# STUDIES ON EFFECTS OF PREBIOTICS ON VIABILITY AND ANTIMICROBIAL ACTIVITIES OF MICROENCAPSULATED PROBIOTICS

**Thesis Submitted by**

***Debolina Banerjee***

***Doctor of Philosophy (Engineering)***

***Chemical Engineering Department***
***Faculty Council of Engineering & Technology***
***Jadavpur University***
***Kolkata, India***
***2017***

**JADAVPUR UNIVERSITY**
**KOLKATA- 700 032, INDIA**

INDEX NO. 28/12/E

**1. Title of the thesis:** STUDIES ON EFFECTS OF PREBIOTICS ON VIABILITY AND ANTIMICROBIAL ACTIVITIES OF MICROENCAPSULATED PROBIOTICS

**2. Name, Designation & Institution of the Supervisors:**

1. Dr. Ranjana Chowdhury
Professor
Department of Chemical Engineering
Jadavpur University, Kolkata 700 032

2. Dr.Pinaki Bhattacharya
Professor (Retd.)
Chemical Engineering Department
Jadavpur University, Kolkata 700 032

**3. List of Publications:**

1**. Debolina Banerjee**, Ranjana Chowdhury, Pinaki Bhattacharya**.** *In-vitro* evaluation of targeted release of probiotic *Lactobacillus casei* (2651 1951 RPK) from synbiotic microcapsules in the gastrointestinal (GI) system: Experiments and modeling**.** LWT-Food Science and Technology, 83, 2017, 243-253.

2. **Debolina Banerjee**, Puja Bag, Ranjana Chowdhury, Pinaki Bhattacharya. Sustainability of the probiotic *Lactobacillus casei* in fortified Indian milk cakes under different preservation conditions-effects of co-immobilization of *L. casei* and commercial prebiotic inulin (chicory

based) and millet inulin. International Journal of Pharmacy and Pharmaceutical Sciences, Vol 9, Issue 1, 2017, 152-157.

3. **Debolina Banerjee**, Ranjana Chowdhury, Pinaki Bhattacharya. The prebiotic influence of inulin on growth rate and antibiotic sensitivity of *Lactobacillus casei.* International Journal of Pharmacy and Pharmaceutical Sciences, Vol 8, Issue 4, 2016, 181-184.

4. Sharmistha Samanta (Koruri), **Debolina Banerjee**, Ranjana Chowdhury, Pinaki Bhattacharya. Studies on prebiotic food additive (inulin) in Indian dietary fibre sources - garlic (*Allium sativum),* wheat *(Triticum spp.),* oat *(Avena sativa)* and dalia *(Bulgur)* .International Journal of Pharmacy and Pharmaceutical Sciences, Vol 6, Issue 9, 2014, 278-282.

**4. List of Patents:** None

**5. List of Presentations in National/International/Conferences/Workshops**

1**. Debolina Banerjee**, Monojit Chowdhury, Sauradeep Majumdar, Ranjana Chowdhury, Pinaki Bhattacharya. Parameter optimization of microencapsulation of *Lactobacillus casei* (NCIM) through rice bran oil based w/o/w type internal gelation, Chemcon 2014.

2**. Debolina Banerjee**, Ranjana Chowdhury, Pinaki Bhattacharya. Immobilization of *L. casei* using Entrapment and Microencapsulation using Internal and External Gelation --- A comparative study, Bioprocessing INDIA 2014.

3**. Debolina Banerjee**, Ranjana Chowdhury; Probiotic (*Lactobacillus casei* and *Bifidobacterium spp*)- Prebiotic(Pure and Natural Inulin) Interaction with Reference to Preservation of Tomato Ketchup and Local Milk Based Sweet meat- Experiments and Modelling; EFFOST 2013. Annual Meeting, Italy.

4**. Debolina Banerjee**, Ashwiny Pandey, Ranjana Chowdhury; Effects of Commercial Prebiotic on Suceptibility of *L. casei* against Norfloxacin and Effects of Immobilized Form on Antimicrobial Activity of *L. casei*; Chemcon 2013.

# CERTIFICATE FROM THE SUPERVISORS

This is to certify that the thesis entitled "**STUDIES ON EFFECTS OF PREBIOTICS ON VIABILITY AND ANTIMICROBIAL ACTIVITIES OF MICROENCAPSULATED PROBIOTICS**" submitted by Smt. **Debolina Banerjee**, who got her name registered on 04.07.2012 for the award of **Ph.D. (Engineering)** degree of **Jadavpur University** is absolutely based upon her own work under the supervision of **Prof. Ranjana Chowdhury,** Chemical Engineering Department, Jadavpur University and **Dr. Pinaki Bhattacharya**, Professor (Retd.), Chemical Engineering Department, Jadavpur University and that neither her thesis nor any part of the thesis has been submitted for any degree/diploma or any other academic award anywhere before.

1. _____

Prof. Ranjana Chowdhury
Chemical Engineering Department
Jadavpur University, Kolkata

2. _____

Dr. Pinaki Bhattacharya
Professor (Retd.)
Chemical Engineering Department
Jadavpur University, Kolkata

***Dedicated***

***To***

***My mother***
***&***
***My beloved husband***

# ACKNOWLEDGEMENT

I would like to take this opportunity to convey my sincere gratitude, respect and deep regards to Prof. Ranjana Chowdhury, Chemical Engineering Department, Jadavpur University and Prof. Pinaki Bhattacharya, Ex Prof.& Head, Chemical Engineering Department, Jadavpur University, Kolkata, India), Currently: Professor, Chemical Engineering department, Heritage Institute of Technology, Kolkata for their kind cooperation, able guidance, constant help and encouragement without which it would not have been possible for me to shape this thesis in the present form.

I would like to extend my sincere gratitude to the Council of Scientific and Industrial Research (CSIR), India by providing Senior Research Fellowship (File number: 9/96(0725)2K12-EMRI) for their financial assistance.

I am deeply indebted to my parents and husband for their unconditional love, support and care.

My sincere gratitude goes to my mother-in-law (mamoni), father-in-law (bapi) and to all my in-laws for their continuous support, patience and help during the whole research period.

Above all, I am sincerely thankful to all my family (especially late dida, boromama and mejomashi) members for their support and encouragement all through my life.
Last but not the least, I would also like to thank all of my lab-mates and the Staff Members of Chemical Engineering Department, Jadavpur University for their constant help throughout the course of my work.

*Debolina Banerjee*

# CONTENTS

# List of Figures

# LIST OF TABLES

# Chapter 1

# INTRODUCTION

Development of neutraceuticals using probiotic bacteria is a rapidly expanding area. For the exploitation of full benefits from the bacteria two criteria, namely, viability of the cells and high concentration of cells at the location of its delivery, i.e., the large intestine should be fulfilled. The free cell administration of this bacteria results in loss of viability during the passage through the gastro-intestinal tract particularly through the stomach due to high acidity. Providing a physical barrier for the probiotic microorganisms against adverse environmental conditions is therefore necessary. The technology of microencapsulation of probiotic cells is gaining interest from this perspective. The controlled release of bacteria, immobilized using microcapsules and beads, has been reported to reduce the risk of loss of viability of probiotic cells. There are also scopes for development of new encapsulation technologies to ensure large number of viable probiotic bacterial cells necessary for the use in industry for the development of new probiotic products. Since the synergistic combinations of probiotic bacteria and prebiotic carbohydrates is a new concept in food processing, co-encapsulation of probiotic-prebiotic conjugate may also be developed for the enhancement the shelf life of microencapsulated probiotic bacteria. It is well known that prebiotic bio-molecules enhance the growth rate of probiotics which, in turn, act against pathogenic bacteria through the secretion of bacteriocin [1]. There are scopes for the studies on the effect of prebiotics on the enhancement of sustainability of immobilized probiotic cells. In India many natural food materials have been reported to be rich in prebiotic molecules. However, the extraction processes of prebiotic molecules from only a selective natural source have been investigated [2, 3]. For the commercial viability of production of prebiotics, their extraction from other abundant Indian food sources should be attempted. It is expected that microencapsulated probiotic cells will increasingly be used in food and pharmaceutical industries. Therefore, more focus should be given on the formulation of microcapsules exploring the possibility of incorporation of probiotic-prebiotic conjugates for the enhancement of sustainability of probiotics. The elucidation of the performance of microcapsules in health food and in the human GI system is also necessary from the perspective of delivery of probiotic cells.

## 1.1. Probiotics

A probiotic (derived from the Greek language) is a live microbial feed supplement, which beneficially affects in the host animal by improving its intestinal microbial balance [4, 5]. Probiotics have also been recently defined as "live microbes which transit the gastro-intestinal tract and in doing so benefit the health of the consumer" [6]. The word probiotic is derived from the Greek meaning "for life". Probiotics were first defined by Kollath in 1953 to denote all organic and inorganic food complexes in contrast to harmful antibiotics [7]. Probiotic includes some health benefits: boosting of the immune system, inhibition of the growth of pathogenic microorganisms, prevention of diarrhea from various causes, prevention of cancer, reduction of the risk of inflammatory bowel movements, improvement of digestion of proteins and fats, synthesis of vitamins, and detoxification and protection from toxins [8]. According the FAO/WHO, probiotics are defined as mono or mixed cultures of "live microorganisms which, when administered in adequate amounts confer a health benefit on the host" [9]. They are also called "friendly bacteria" or "good bacteria" and can be used as complementary and alternative medicine, a group of diverse medical and health care systems, practices, and products that are not presently considered to be part of conventional medicine. In other words, they are defined as the microbial food supplement with nearly 20 known species, which beneficially affect the host by improving its intestinal microbial balance.

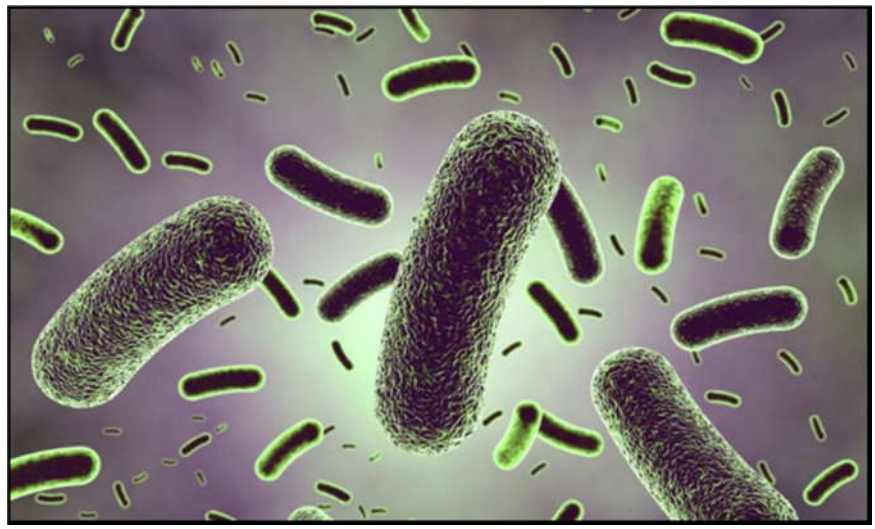


**Figure 1.1: Morphology of Lactic Acid Bacteria [12]**

Lactic acid bacteria and *Bifidobacteria* occurring in foods such as yogurt, cottage cheese, buttermilk and other fermented dairy products are the most familiar probiotics [10]. *Lactobacillus* species [*L acidophilus, L reuteri, L plantarum, L casei, L salivarius, L bulgaricus, L fermentum, L gasseri, L johnsonii, L lactis, L paracasei*]**,** *Bifidobacterium* species [*B bifidum, B infantis, B lactis, B longum, B breve, B adolescentis*]**,** *Saccharomyces* species [*S boulardii*]**,** *Streptococcus* species [*S thermophilus, S salivarius subsp thermophilus*], other bacterium [*Propionibacterium freudenreichii***,** *Entercoccus, Escherichia coli*] are some of the various types of probiotic reported in the literature[11].

### 1.2. Mechanism of Action of Probiotic Bacteria

Different important underlying mechanisms, namely, a) Enhancement of the epithelial barrier, b) Increased adhesion to intestinal mucosa, c) inhibition of pathogen adhesion,

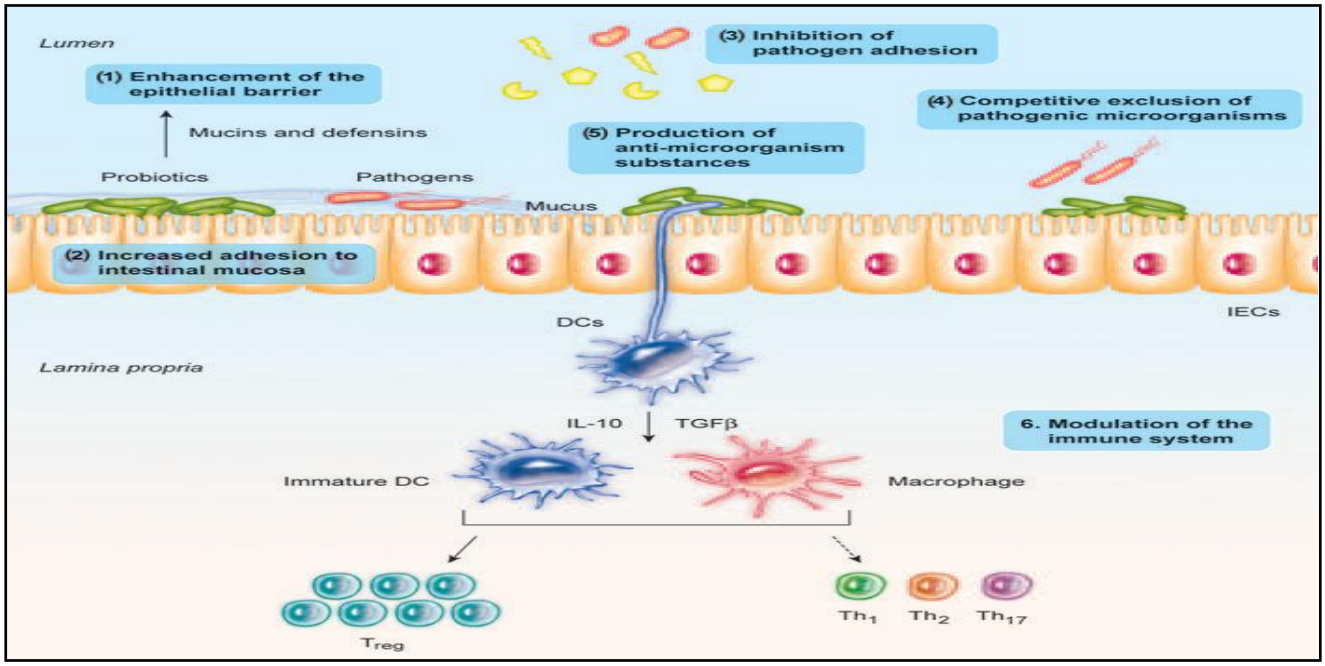



**Figure1.2:Mechanism of Action of Probiotic Bacteria [13]**

d) competitive exclusion of pathogenic microorganism, e) production of anti-pathogenic substances, f) modulation of the immune system, represented in Figure 1.2 are responsible for the antagonistic effects of probiotics against various pathogenic microorganisms [13].

### 1.3. Prebiotics

The term ‘‘prebiotic’’ refers to selectively fermented ingredients which result in specific changes in composition and/or the activity of gastrointestinal flora, with consequent health benefits for the host [14]. According to the FAO/WHO, prebiotics are “nondigestible substances that provide a beneficial physiological effect on the host by selectively stimulating the favourable growth or activity of a limited number of indigenous bacteria”. ‘‘Dietary fiber’’ is a broader term that refers to various carbohydrates and lignin, which resist hydrolysis by human digestive enzymes, but may be fermented by colonic microflora and/or partly excreted in feces. Prebiotics may be added to food preparations containing probiotics to enhance the stability of food matrix and viability of probiotic cultures [15]. Chemically, the prebiotics are mostly oligosaccharides in nature. The various oligosaccharides including fiber gums, fructo-oligosaccharides, inulins, isomalto-oligosaccharides, lactilol, lactosucrose, lactulose, oligofructose, pyrodextrins, oligosaccharides, transgalacto-oligosaccharides, and xylo-oligosaccharides, classified as prebiotics that are added to processed foods and supplements. There is no concern about oligosaccharides being destroyed while in storage or in route to the intestine through the stomach acid and digestive enzymes, as usually faced by the probiotics. Prebiotics are not broken down by gastric enzymes, but pass unaltered into the large intestine, where they are then selectively fermented, producing beneficial effects, such as production of short chain fatty acids [16].

### 1.4. Prebiotic-rich natural food

Usually, food stuffs, rich in dietary fibre, serve as prebiotic sources. The fruits and vegetables as bananas, berries, asparagus, garlic, wheat, oatmeal, barley (and other whole grains), dahlia, millets, psyllium husks, flaxseed, tomatoes, Jerusalem artichoke, onions and chicory, greens (especially dandelion greens but also spinach, collard greens, chard, kale, mustard greens, and others) and legumes (lentils, kidney beans, chickpeas, navy beans, white beans, black beans) have been reported to be naturally rich in prebiotic carbohydrates [17]. Among the natural prebiotics inulin is the most abundant one.

## 1.5. Inulin

Inulin has been defined as a polydisperse carbohydrate material consisting mainly, if not exclusively, of β-(2-1) fructosyl-fructose links [18]. Among different probiotics, inulin, a fructan consisting of glycosidic bonds like fructosyl-fructose with a terminal glucose unit is one of the most popular one [19-22]. A few reports are available, showing the positive effect of inulin on the growth of probiotic organism [15, 23, 24]. Huebner, 2007 reported that inulin enhanced the growth of *Lactocallus casei* [25]. In addition to the inclusion of inulin in yogurt, it is also used as a substitute of fat in low fat emulsions like salad dressings etc [26].

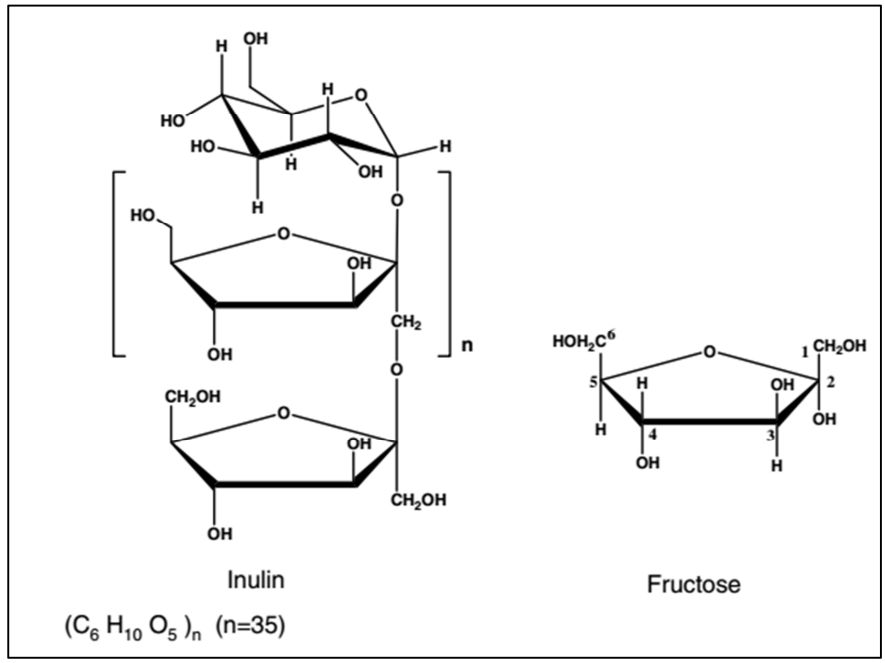



**Figure 1.3: Structure of Inulin [19]**

## 1.6. Inulin-rich Indian food

Inulin producing plant species are found in several monocotyledonous and dicotyledonous families, including Liliaceae, Amaryllidaceae, Gramineae and Compositae [27]. The photographs of some inulin-rich natural vegetables and pulses have been provided in Table 1.1 along with their common and scientific names and inulin content.

**Table 1.1: Inulin-rich Indian food**

| Indian Food source | Photographs |
|---|---|
| **Chicory** (***Cichorium intybus***) |   [28] |
| **Garlic** (***Allium sativum***) |  [29] |
| **Sorghum (Jowar)** (***Sorghum vulgare***) |  [30] |
| **Pearl Millet (Bajra)** (***Pennisetum glaucum***) |  [30] |

| | |
|---|---|
| **Finger Millet (Ragi)** (***Eleusine coracana***) |  [30] |
| **Oat** (***Avena sativa***) |  [31] |
| **Wheat** (***Triticum***) |  [32] |
| **Banana** (***Musa***) |  [33] |

| | |
|---|---|
| **Onion**<br>**(*Allium sepa*)** |  [34] |

From the Table 1.1, it is clear that although the inulin content of several Indian foods has been determined, no quantitative data is available for Indian millets. It has been reported that since these cereals are constitutionally similar to wheat [35], they are expected to contain prebiotic molecules like inulin besides arabinoxylan found in the latter [3]. Although these coarse cereals have long been considered as sources of dietary fibre, no efforts have so far been made to extract prebiotic inulin from them. There is an initiative of Government of India to enhance the production of millets [36] and popularize these coarse cereals in the global market. The extraction of inulin from these sources is expected to boost this initiative.

### 1.7. Synergistic effect of probiotic-prebiotic

Synergistic combinations of probiotic bacteria and prebiotic carbohydrates are new concepts in food processing [37]. Prebiotic bio-molecules enhance the growth rate of probiotics which in turn act against pathogenic bacteria through the secretion of bacteriocin [38]. Thus, prebiotics may be combined with dairy products namely yoghurt etc. which contain an array of probiotic bacteria [39]. Ideally, the prebiotic molecule should not support the growth of pathogens. The main reason for using a synbiotic is that a true probiotic, without its prebiotic food, does not survive well in the digestive system. As prebiotics provide nutrition for the probiotics, the population of these good bacteria is sustained. The synergism of probiotic and prebiotic conjugates is shown in Figure 1.4.

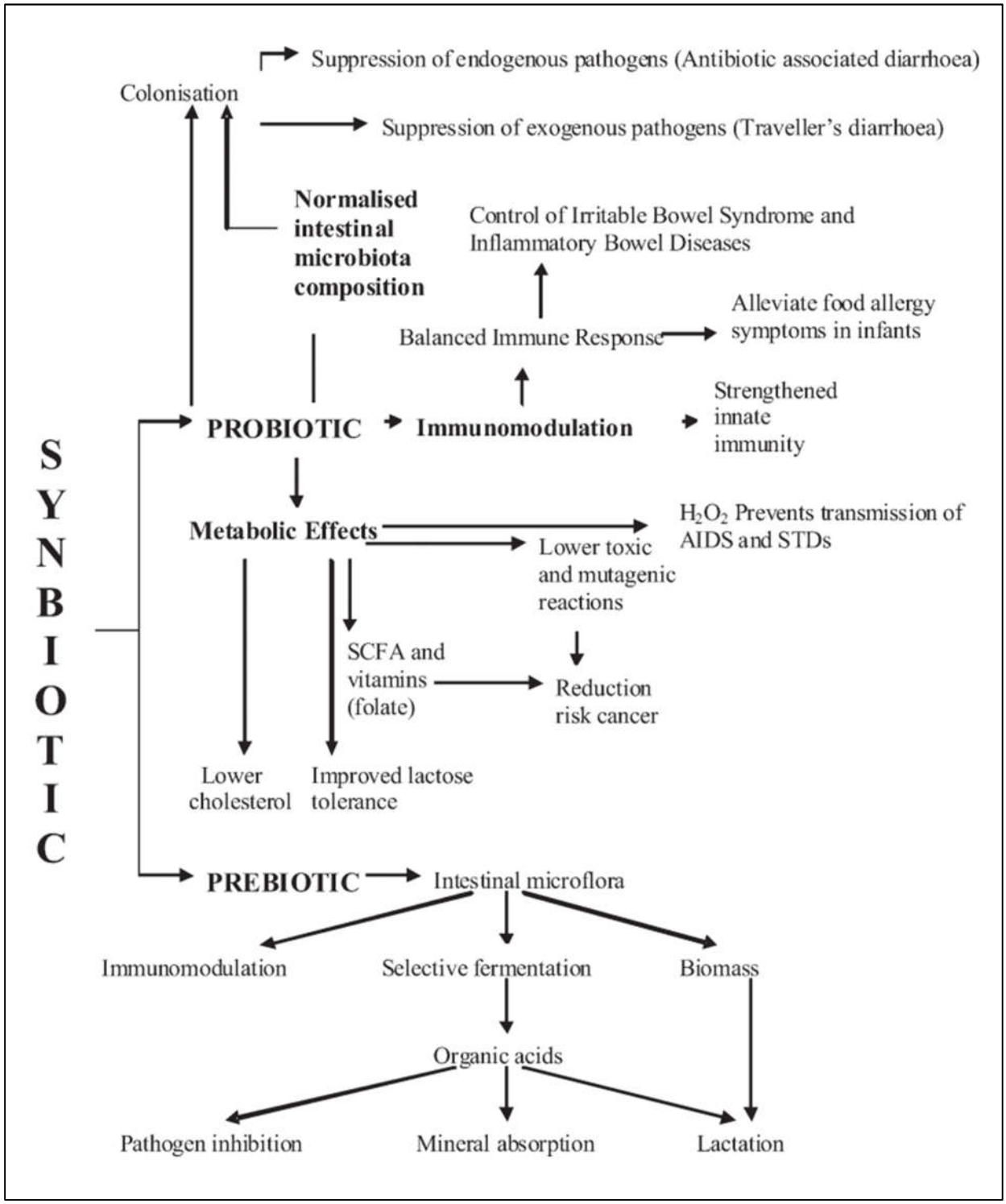



**Figure 1.4: Synergistic effect of probiotic-prebiotic [39]**

### 1.8. Microencapsulation

Microencapsulation is a technique of protection of desired product from detrimental environmental influences and is done either through physical or physicochemical or chemical routes [40]. The most common biomaterial used for microencapsulation or immobilization of probiotic bacteria is alginate which is a linear polymer of heterogeneous structure composed of monosaccharides called α-L glucuronic acid and β- D mannuronic acid linked by β (1→4) glycosidic bonds [41]. Entrapment is one of the techniques of immobilization. The size of beads may vary in the range of 2-3 mm. Spray drying, Spray coating, Extrusion, Emulsification are some basic methods of microencapsulation. External gelation and internal gelation are the two different methods of chemical emulsification. Internal gelation is superior to external one as it ensures the formation of spherically structured microcapsules of sizes ≤ 1000 μm with very narrow size distribution [42, 43]. It is a simple, fast and low cost method of emulsification. One of the important features of immobilized prebiotic-probiotic conjugate in real time operation is the 'release time' of cells from the inert enclosure. Similarly evaluation of *In-vitro* concentration profiles of microcapsules (internal) and released probiotics in the simulated GI system is another important aspect to be considered in immobilized system.

### 1.9. Application of microencapsulated probiotic microorganism in food

The delivery of viable micro encapsulated probiotic bacteria will become important in the near future [44]. Nowadays, a great attention has been given in extending the category of foods carrying probiotics in order to broaden the groups of people that usually have no access to probiotic food [45]. Probiotic products for human diet include neutraceuticals or functional foods, food ingredients and supplements that have an important effect on the intestinal microflora [46, 47]. The intended health benefits of probiotics can only be obtained when the food contains the required minimum viable microorganism count at the time of consumption. US FDA has recommended that minimum probiotic count in the probiotic food should be at the least $10^6$ CFU $ml^{-1}$ [48-50].

### 1.10. Microencapsulated probiotics in Human GI system

After consumption, a microencapsulated probiotic will pass quickly through the esophagus at which the greatest viability loss of bacteria is expected due to high levels of acid [42]. The pH values of the stomach lie within pH 1–2.5. The emptying of stomach contents is usually a result of peristaltic action known as the migrating myoelectric complex. In addition to acid, the stomach also contains pepsin, a proteolytic enzyme, which breaks down proteins. After passage through the stomach, the microcapsules enter the small intestine. The pH of the small intestine lies in the range of 6.80–7.88. After passage through the small intestine the capsules reach the large intestine at which point the pH 7.0. The large intestine, unlike the rest of the GI tract, is home to a large concentration of indigenous bacterial species, which themselves offer an opportunity for targeted delivery as some polysaccharides are biodegraded by the colonic microflora. Development of neutraceuticles using probiotic bacteria is a rapidly expanding area.

## REFERENCES


1. Saad, N., Delattre, C., Urdaci, M., Schmitter, J.M., Bressollier, P. (2013) "An overview of the last advances in probiotic and prebiotic field". LWT-Food Science and Technology, 50, 1-16.
2. Mavumengwana, V.B. (2004) "Isolation Purification and Characterisation of inulin and fructo-oligosaccharides from *Chicoriumintybus* and inulinase from *Aspergillus niger*". Thesis for the degree of M. Sc. Department of Biochemistry, Microbiology and Biotechnology, Rhods University.
3. Samanta (Koruri), S., Banerjee, D., Chowdhury, R., Bhattacharya, P. (2014) "Studies on prebiotic food additive (inulin) in Indian dietary fibre sources garlic (*Allium sativum*), wheat (*Triticum spp*.), oat (*Avena sativa*) and dalia(BULGUR)". International Jornal of Pharmacy and Pharmaceutical Sciences. 6 (9).
4. Fuller, R. (1989) "Probiotics in man and animals". Journal of Applied Bacteriology, 66,365-368.
5. Nithya,V., Halami, P.M. (2013) "Evaluation of the probiotic characteristics of *Bacillus species* isolated from different food sources". Annals of Microbiology, 63, 1, 129–137.
6. Tannock, G. W., Munro, K., Harmsen, H. J. M., Welling, G. W., Smart, J., Gopal, P. K. (2000) "Analysis of the Fecal Microflora of Human Subjects Consuming a Probiotic Product Containing *Lactobacillus rhamnosus* DR20". Applied Environmental Microbiology, 66, 6, 2578-2588.
7. Kotikalapudi, B.L. (2009) "Characterization and encapsulation of probiotic bacteria using a pea-protein alginate matrix". University of Saskatchewan Saskatoon, Saskatchewan, Canada, MSc thesis.
8. Hobbs, C. (2000) "Pro-life therapy with probiotics". Health World Online (Online at: http://www.healthy.net/asp/templates/ article.asp? id =953).
9. Food and Agriculture Organization of the United Nations and World Health Organization Report. http:// www.fao.org/es/ESN/probio/ probio.htm (accessed 15 May, 2007).

10. Collado, M.C. (2008) “Role of probiotics in health and disease”. In: Lee YK, Salminen S, eds. Handbook of Probiotics and Prebiotics. Wiley-Interscience, 2nd ed, Chapter 4, 257-376.
11. Gupta, V., Garg, R. (2009) “Probiotics”. Indian Journal of Medical Microbiology, 27, 202-209.
12. https://www.articleblotter.com/lactobacillus-rhamnosus-a-probiotic-for-gut-health/
13. Bermudez-Brito, M., Plaza-Díaz, J., Muñoz-Quezada,S., Gómez-Llorente, C., Gil, A. (2012) “Probiotic Mechanisms of Action”. Annals of Nutrition and Metabolism ,61,160–174.
14. de Gastroenterología, O.M. Guía Prácticadela Organización Mundial de. (2016) “Gastroenterología: Probióticosyprebióticos”. Guías Mundiales la WGO Probióticos y prebióti-cos. 2011; 29. Available in: http://www.worldgastroenterology.org/UserFiles/file/guidelines/probiotics-spanish-2011.pdf4.
15. Boeni, S., Pourahmad, R. (2012) “Use of inulin and probiotic *Lactobacilli* in synbiotic yogurt production”. Annals of Biological Research, 3(7), 3486-91.
16. Williams, N.T. (2010) “Probiotics”. American Journal of Health System Pharmacy, 67, 49-458.http://www.deoxy.com/index.php?option=com_content &task=view&id=9&Itemid=6.
17. Crittenden, R., Payne, M.J. (2008) “Nutrition News. Facts and functions of prebiotics, probiotics and synbiotics”. pp 1-2. Department of Human Nutrition, K-State Research and Extension, Kansas State University; Prebiotics. In: Lee YK, Salminen S, eds. *Handbook of Probiotics and Prebiotics*. Wiley-Interscience, 2nd ed; 2008: Chapter 4, 535-582.
18. Arihara, R.K., Ota, H.R., Itol, M., Sameshima, Y., Yamanaka, H., Akimoto, M., Kanai, S. (1998) “*Lactobacillus acidophilus* group Lactic-acid bacteria applied to meat fermentation”, Journal of Food Sciences, 63(3), 544-547.
19. Manso, J., Luz, M.M., Ya´n˜ez-Seden˜o, P., Jose´ Pingarro´n, M. (2007) “Bienzymeamperometric biosensor using gold nanoparticle-modified electrodes for the determination of inulin in foods”. Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040, Madrid, Spain.

20. De Souza, O., Perego, P., de Oliveira, M.N., Converti, A. (2011) "Effect of inulin as prebiotic and synbiotic interactions between probiotics to improve fermented milk firmness". Journal of Food Engineering, 107, 36–40.
21. Azza, A., Arab, A., Hala, A.T., Ferial, M., Salem, A. (2011) "Physico-chemical properties of inulin produced from jerusalem artichoke tubers on bench and pilot plant scale". Australian Journal of Basic and Applied Sciences, 5, 1297-1309.
22. Toneli, J.T.C.L., Park, K.J., Ramalho, J.R.P., Murr, F.E.X., Fabbro, I.M.D. (2008) "Rheological characterization of chicory root *(Cichorium intybus*) inulin solution". Brazilian Journal of Chemical Engineering, 25, 461-471.
23. Oliveira, R.P.D.S., Perego, P., Converti, A., Oliveira, M.N.D. (2009) "Effect of inulin on growth and acidification performance of different probiotic bacteria in co-cultures and mixed culture with *Streptococcus thermophilus*". Journal of Food Engineering, 91(1), 133-139.
24. Rodrigues, D., Teresa, A.P., Santos, R., Pereira, C.I., Gomes, A.M., Malcata, F.X. (2011) "The potential effect of FOS and inulin upon probiotic bacterium performance in curdled milk matrices". LWT Food Science and Technology, 44(1), 100-108.
25. Huebner, J., Wehling, R.L., Hutkins, R.W. (2007) "Functional Activity of commercial probiotics". International Dairy Journal, 17, 770-775.
26. Mantzouridou, F., Spanou, A., Kiosseoglou, V. (2012) "An Inulin-based dressing emulsion as a potential probiotic food carrier". Food Research International, 46, 260-269.
27. Parada, J.L., Caron, C.R., Adriane, B.P., Medeiros, S.C.R. (2002) "Bacteriocins from Lactic acid-bacteria: Purification". Brazilian Archives Biology and Technology, 522.
28. http://www.wildcraft.com.au/?product=chicory-root-roasted
29. http://www.care2.com/greenliving/11-proven-health-benefits-of-garlic.html
30. Gangaiah,B. "Agronomy – Kharif Crops, Millets". Senior Scientist, Division of Agronomy Indian Agricultural Research Institute, New Delhi – 110 012.
31. http://www.wisegeek.com/what-is-oat-flour.htm
32. http://aspireexim.com/category/wheat
33. http://saltmarshrunning.com/2014/09/10/why-runners-love-bananas/
34. http://weknowyourdreams.com/onion.html

35. Koehler, p., Wieser, h. (2013) "chemistry of cereal grains". German research center for food chemistry (chapter 2)
36. Passi, S.J., Jain, A. (2014) http://pib.nic.in/newsite/mbErel.asp.
37. Gaggìa, F., Mattarelli, P., Biavati, B. (2010) "Probiotics and prebiotics in animal feeding for safe food production". International Journal of Food Microbiology, 141, S15–28.
38. Kosin, B., Rakshit, S.K. (2006) "Microbial and processing criteria for production of probiotics: a review". Food Technology and Biotechnology, 44, 371–379.
39. Sekhon, B.S. and Jairath, S. (2010) "Prebiotics, probiotics and synbiotics: an overview", Journal of Pharmaceutical and Educational Research, 1(2), 13-36.
40. Champagne, C.P., Lacroix, C., and Sodini-Gallot, I. (1994) "Immobilised cell technologies for the dairy industry". Critical Reviews in Biochemistry and Molecular Biology, 14, 109-134.
41. Ahmed, M.M., El-Rasoul, S.A., Auda, S.H., Ibrahim, M.A. (2013) "Emulsification/internal gelation as a method for preparation of diclofenac sodium–sodium alginate microparticles". Saudi Pharmaceutical Journal, 21, 61–69.
42. Cook, M.T., Tzortzis, G., Charalampopoulos, D., Khutoryanskiy, V.V.(2012) "Microencapsulation of probiotics for gastrointestinal delivery". Journal of Controlled Release, 162, 56–67.
43. Chun, H., Kim, C.H., Cho, Y.H. (2014) "Microencapsulation of *Lactobacillus plantarum* DKL 109 using External Ionic Gelation Method". Korean Journal of Food Science and Technology, 34, 692-699.
44. Kailasapathy, K. (2002) "Microencapsulation of Probiotic Bacteria: Technology and Potential Applications". Current Issues in Intestinal Microbiology, 3, 39-48.
45. Iyer, C., Kailaspathy, K. (2005) "Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under in vitro acidic and bile salt conditions and in yogurt". Journal of Food Sciences, 2005, 70, M18 – 23.
46. Parwal, M., Pareek, R. (2011) "Probiotic: - A Treatment new approach". Asian Journal of Biochemical and Pharmaceutical Research, 3 (1), 448-454.
47. Kawatra, P., Aiyappa, C. (2015) "Probiotics: the undervalued conquerors". Asian Journal of Biochemical and Pharmaceutical Research, 8(4), 97-100.

48. Guarner, F., Khan, A.G., Garisch, J., Eliakim, R., Gangl, A., Thomson, A., Krabshuis, J., Mair, T. (2009) "World gastroenterology organisation practice guidelines: probiotics and prebiotics". Arabian Journal of Gastroenteritis,10, 33 – 42.
49. Tripathi, M.K., Giri, S.K. (2014) "Probiotic functional foods: survival of probiotics during processing and storage". Journal of Functional Foods, 9, 225-41.
50. Prisco, A.D., Mauriello, G. (2016) "Probiotication of foods: a focus on microencapsulation tool". Trends in Food Science and Technology, 48, 27-39.

# Chapter 2

# LITERATURE REVIEW

The reported literatures on the probiotic bacteria, particularly *Lactobacilli*, prebiotic inulin, their synergistic combination, immobilization of probiotics and prebiotic-probiotic conjugates and their performance in functional food and simulated human gastrointestinal systems have been reviewed to assess the state-of-the-art of these research areas.

### 2.1. Probiotics, prebiotics and conjugates

| Observations | References |
|---|---|
| • The development of suitable technology for probiotic production<br>• Overview of probiotic selection studies including new technologies for isolation/identification, adhesion and immune response.<br>• The development of suitable probiotics in food and feed needs good proof of their efficacy and function in order to be accepted as a valuable product. | **Bussarin Kosin et al., 2006 [1]** |
| • Determination the probiotic potential of a large number of autochthonous lactic acid bacteria isolated from fruit and vegetables.<br>• Survival under simulated gastric and intestinal conditions showed that 35% of the strains, mainly belonging to the species *Lactobacillus plantarum* maintained high cell densities.<br>• All strains inhibited enterohemorragic *Escherichia coli* K12 and *Bacillus megaterium* F6 isolated from human sources. The results of this study showed that some autochthonous lactic acid bacteria from raw fruit and vegetables have functional features to be considered as novel probiotic candidates. | **Beatrice Vitali et al., 2011 [2]** |
| • Inulin, a nondigestible carbohydrate, is a fructan molecule. This is found in many plants as a storage carbohydrate and also a part of man's daily diet for several centuries. It is present in many vegetables, fruits and cereals, including leek, onion, garlic, wheat, chicory, artichoke, and banana. Industrially, inulin | **Anne Franck, Leen De Leenheer [3-5]** |

| | |
|---|---|
| is obtained from chicory roots, and is used as a functional food ingredient that offers a unique combination of interesting nutritional properties and important technological benefits.<br>• Rose, a German scientist, first isolated a peculiar substance of plant origin from a boiling water extract of *Inula helenium* in 1804, and the substance was later called inulin by Thomson (1818).<br>• Inulin has been extracted from chicory root by following the protocol described by Mavumengwana. | |
| • Various aspects of potential health benefits of fructo-oligosaccharide and inulin<br>• The origin, history of inulin<br>• Common intakes of inulin<br>• The production and caloric value of dietary fibers | **Linda S. Boeckner et al., 2001[6]** |
| • Use of inulin as bifidogenic agents, stimulating the immune system of the body, decreasing the levels of pathogenic bacteria in the intestine, relieving constipation, decreasing the risk of osteoporosis by increasing mineral absorption, especially of calcium, reducing the risk of atherosclerosis by lowering the synthesis of triglycerides and fatty acids in the liver and decreasing their level in serum.<br>• These fructans modulate the hormonal level of insulin and glucagon, thereby regulating carbohydrate and lipid metabolism by lowering the blood glucose levels<br>• Fructans are also effective in lowering the blood urea and uric acid levels, thereby maintaining the nitrogen balance. The biochemical basis of these beneficial effects of inulin and oligofructose has been discussed. | **Narinder Kaur and Anil K Gupta, 2002 [7]** |
| • The effect of storage temperature of inulin concentrated solution as well as temperature on the rheological behavior of liquid and precipitated phases obtained from a process of phase separation were evaluated.<br>• The precipitated phase of inulin was evaluated under two conditions: pure and formulated with encapsulating agents. | **Toneli et al., 2008 [8]** |

| | |
|---|---|
| • It was observed that a reduction in storage temperature resulted in a higher inulin precipitation | |
| • Lactic acid fermentation of Jerusalem artichoke tuber was performed with strains of *Lactobacillus paracasei* without acidic or enzymatic inulin hydrolysis prior to fermentation.<br>• Some strains of *L. paracasei*, notably KCTC13090 and KCTC13169, could ferment hot-water extract of Jerusalem artichoke tuber more efficiently compared with other *Lactobacillus spp.* such as *L. casei* type strain KCTC3109. The *L. paracasei* strains could utilize almost completely the fructo-oligosaccharides present in Jerusalem artichoke.<br>• Direct lactic fermentation of Jerusalem artichoke tuber extract at 111.6 g/L of sugar content with a supplement of 5 g/L of yeast extract by *L. paracasei* KCTC13169 in a 5 L jar fermentor produced 92.5 ce:hsp sp="0.25"/>g/L of lactic acid with 16.8 g/L fructose equivalent remained unutilized in 72 h. The conversion efficiency of inulin-type sugars to lactic acid was 98% of the theoretical yield. | **Hwa-Young Choi et al., 2012[9]** |
| • Lactic acid producing bacteria was isolated from Whey.<br>• The isolated strain *Lactobacillus fermentum* KN02 was potent producer of bacteriocins. Bacterioicin produced by the isolate KN02 had a large spectrum of inhibition against food spoilage microorganisms (*Escherchia coli, Staphylococcus aureus, Salmonella typhi, Proteus mirabilis, Pseudomonas aeuroginosa, Klebsiella pneumonia, Bacillus cereus*).<br>• The bacterioicin inhibited the growth of pathogens but it showed high activity against *Bacillus*. The bacteriocins were found to be heat stable at 100°C.<br>• The maximum antimicrobial activity was retained within the pH range of 2, and completely affected by proteolytic enzyme (papain). | **Kanagaraj Nithya et al., 2012 [10]** |

| | |
|---|---|
| • Bacteriocins are used to control the frequent development of pathogens and spoiling microorganism in food and feed. Growth of *L. fermentum* KN02 was inhibited only by few antibiotics suggesting that the strain may be used as a probiotic by the individual receiving medical treatment.<br>• The new bacterioicin producing *L. fermentum* KN02 has been selected for identification and application of bacteriocins to food products (Milk and Mushroom) which reduces the growth of pathogens. The bacteriocins producers *L. fermentum* KN02 was identified on the basis of phenotypic analysis and 16S rRNA sequences. | |
| • The health benefits of dietary fiber<br>• Only polysaccharides were included in dietary fiber originally, but more recent definitions have included oligosaccharides as dietary fiber, not based on their chemical measurement as dietary fiber by the accepted total dietary fiber (TDF) method, but on their physiological effects. Inulin has bifidogenic effect. | **Joanne Slavin, 2013[11]** |
| • Millets, an alkaline forming food, have nutraceutical properties in the form of antioxidants which prevent deterioration of human health such as lowering blood pressure, risk of heart disease, prevention of cancer and cardiovascular diseases, diabetes, decreasing tumor cases etc.<br>• Other health benefits are increasing the time span of gastric emptying, provides roughage to gastro intestine.<br>• Millets contain 60-70% carbohydrates, 7-11% proteins, 1.5-5% fat, and 2-7% crude fibre and are also rich in vitamins and minerals.<br>• They are excellent source of vitamin B, magnesium, and antioxidants. Millet is also a good source of other dietary minerals like manganese, phosphorus and iron. Millets are rich in inulin.<br>• Pearl millet are highly effective in inhibiting the growth of all 3 examined phytopathogenic fungi. | **Sarita and Ekta Singh, 2016 [12-14]** |

| | |
|---|---|
| • This study showed that millets are used as "food medicine". Millet is source of antioxidants such as phenolic acids and glycated flavonoids. Millet foods are also characterized to be potential prebiotic and can enhance the viability of probiotics with potential health benefits. | |
| • Inulin is a water soluble storage polysaccharide and belongs to a group of non-digestible carbohydrates called fructans. Inulin has attained the GRAS status in USA and is extensively available in about 36,000 species of plants, amongst, chicory roots are considered as the richest source of inulin.<br>• Inulin is used as a prebiotic, fat replacer, sugar replacer, texture modifier and for the development of functional foods in order to improve health due to its beneficial role in gastric health.<br>• This review provides a deep insight about its production, physicochemical properties, role in combating various kinds of metabolic and diet related diseases and utilization as a functional ingredient in novel product development. | **Muhammad Shoaib et al., 2016 [15-34]** |
| • A detailed description about Sorghum, pearl millet and finger millet. Their availability throughout the India and their detailed composition has been described by Dr. B. Gangaiah. | **Dr. B. Gangaiah [35]** |
| • Synbiotics are nutritional supplements that are combinations of probiotic bacteria and prebiotic food ingredients.<br>• The manipulation of composition of the gut microbiota in infants and adults through dietary supplementation is possible by probiotic/prebiotic/synbiotic therapies.<br>• Probiotics are generally concerned with bacteria in the small intestine, while prebiotics with bacteria in the large intestine and colon. Synbiotics enable to improve the viability of probiotics and to deliver specific health benefits.<br>• Probiotic/prebiotic/synbiotic characteristics include antimicrobial, anticarcinogenic, antidiarrheal and | **Bhupinder Singh Sekhon et al, 2010 [36]** |

| | |
|---|---|
| antiallergenic qualities, osteoporosis prevention, ulcerative colitis, reduction in serum fats and blood sugars, regulation of the immune system and treating liver-related brain dysfunction.<br>• Clinical effects of probiotic/prebiotic/synbiotic therapies include: severe pediatric surgical cases, liver transplantation, biliary cancer, acute pancreatitis, in asthma and atopic dermatitis and patient under emergency care.<br>• Probiotic/prebiotic/synbiotics are strengthening their roles in the reduction of antibiotic associated diarrhea, management of rotavirus diarrhea, and diarrhea due to various causes; exhibiting positive effect in the management of lactose intolerance, irritable bowel syndrome, inflammatory bowel disease, and research is in progress in various nongastrointestinal diseases.<br>• Synbiotics products offer the potential to develop prebiotics targeted at specific probiotic strains to optimize health benefits. | |

**2.2. Immobilization of probiotic bacteria and prebiotic-probiotic Conjugates**

| Observations | References |
|---|---|
| • Immobilization method of *Lactobacillus casei* cells using alginate capsules that possess an interphosic membrane and a liquid core.<br>• The capsules were found to offer more space for cellular growth than gel-core beads, which resulted in 1.5-fold higher cell concentration than in the latter; however, the Ca-alginate structure was unstable during repeated batch fermentations for lactic acid production. | **Ik-Keun Yoo et al., 1996 [37]** |

| | |
|---|---|
| • Ba-alginate capsules were chemically and physically more stable than the Ca-alginate capsules in phosphate and lactate solutions. Attempts were also made to use various hardening agents to stabilize the structure of the Ba-alginate capsules.<br>• It was found that the treatment with a mixture of chitosan and $BaCl_2$, solution gave the best results for hardening. Finally, stable lactic acid production was possible with a productivity of more than 2.7 g /l by *L. casei* cells. | |
| • Encapsulation with alginate does not improve the cell viability upon exposure to low pH solutions, but does improve viability during storage in foods.<br>• This review has provided an overview of progress in this field as well as draw attention to areas where studies have fallen short. | **Hansen et al., 2002 and Sultana et al., 2000 [38, 39]** |
| • The small diameter of microcapsule helps to reduce mass transfer limitations.<br>• The nutrients and metabolites can diffuse through the semi-permeable membrane easily. The membrane serves as a barrier to cell release and minimises contamination.<br>• The encapsulated core material is released by several mechanisms such as mechanical rupture of the cell wall, dissolution of the wall, melting of the wall and diffusion through the wall<br>• The residual oil, emulsifier and surfactant in the encapsulated material can be toxic to live bacterial cells and may interact with sensitive food components<br>• Probiotic bacteria must be able to colonise the gut and therefore survive gastric acidity, bile salts, enzymes, toxic metabolites, bacteriophages, antibiotics and anaerobic conditions. | **Kaila Kailasapathy, 2002 [40-42]** |
| • The tolerance of *Lactobacillus casei* NCDC-298 encapsulated in different alginate concentrations (2%, 3% | **S. Mandal et al.,** |

| | |
|---|---|
| or 4%), to low pH (1.5), high bile salt concentration (1% or 2%) and heat processing (55, 60 or 65ºC for 20 min).<br>• The release of encapsulated cells in simulated aqueous solution of colonic pH was also assessed.<br>• The survival of encapsulated *L. casei* was better at low pH, high bile salt concentration and during heat treatment as compared to free cells.<br>• The survival increased proportionately with increasing alginate concentrations without affecting the release of entrapped cells in solution of colonic pH. | **2006 [43]** |
| • The development of functional foods through the addition of bioactive compounds holds many technological challenges.<br>• Microencapsulation is a useful tool to improve the delivery of bioactive compounds into foods, particularly probiotics, minerals, vitamins, phytosterols, lutein, fatty acids, lycopene and antioxidants. These technologies could promote the successful delivery of bioactive ingredients to the gastrointestinal tract. | **Claude P Champagne et al., 2007 [44]** |
| • *Lactobacillus casei* cells were immobilized on wheat grains and the effect of nine cryoprotectants during freeze-drying was investigated.<br>• Survival and fermentative activity of the freeze-dried immobilized biocatalysts was studied by monitoring pH, lactic acid and lactose content in successive fermentations batches of both synthetic lactose medium and milk.<br>• Freeze-dried *L. casei* cells immobilized on wheat grains without using cryoprotectants resulted in high cell survival and metabolic activity. The same biocatalysts were stored at room temperature for 9 months and at 4 ºC and -18 ºC for 12 months. Reactivation of the stored biocatalysts was carried out in synthetic lactose medium. Storage at room and low temperatures (4º C and -18ºC) | **Loulouda A. Bosnea et al., 2009 [45]** |

| | |
|---|---|
| • The suitability of the immobilized biocatalysts for the production of mild and low pH dairy products. The immobilization of a probiotic microorganism, such as *L. casei*, on boiled wheat which contains prebiotic compounds might provide a potential synbiotic preparation.<br>• Wheat grains can be used for immobilization of *L. casei* cells since the immobilization technique is easy and cheap.<br>• Freeze-drying of *L. casei* cells immobilized on wheat without cryoprotective media lead to low contamination risks and reduction of cost without significant losses in viability and fermentative activity. | |
| • Consumption of probiotics brings health benefits to consumers.<br>• The current microencapsulation studies are focused mainly on probiotic sustainability in the food product with limited experimentation on the probiotics viability during delivery into the body.<br>• Microencapsulation offers significant benefits to food technology and production.<br>• It provides a solution to the low viability of probiotics incorporated in dairy products.<br>• Double microencapsulation provides multiple protection for probiotics, both during storage and during delivery into the body. | **Mohammed Akhiar, 2010 [46]** |
| • The physical properties of lyophilized calcium (Ca)–alginate beads as a carrier material for the stabilization of encapsulated living cells.<br>• Improvements in the sphericity, flowability and mechanical strength of the dried beads were attributed to the filler, which provided structure and reinforcement to the Ca–alginate hydrogel networks, as verified by X-ray microtomography and scanning electron microscopy.<br>• A quantitative analysis of the micro-images revealed the less porous nature of the alginate–starch beads compared to the control. | **Eng-Seng Chan et al., 2011[47]** |

| | |
|---|---|
| • The beads with filler were also found to be less hygroscopic. The results also show that the cells encapsulated within the beads with reduced porosity and hygroscopicity were clearly more stable during lyophilization and storage than the control. | |
| • Pro-and prebiotics-loaded bioadhesive microparticles made of HA and low methyl pectin has successfully been developed for a pharmaceutical use.<br>• The operating conditions allowing the preparation of favorable microparticles for probiotic encapsulation have been determined through an experimental design.<br>• The microparticle obtained exhibited adhesive properties as shown by the rheological measurements and a sustained release of probiotics. | **D. Pliszczak et al., 2011 [48]** |
| • A trial to formulate diclofenac sodium as controlled release microparticles that might be administered once or twice daily. This could be achieved via emulsification/internal gelation technique applying Box-Behnken design to choose these formulae.<br>• Box-Behnken design determined fifteen formulae containing specified amounts of the independent variables.<br>• The prepared microparticles were characterized for their production yield, sizes, shapes and morphology, entrapment efficiency and Diclofenac sodium in vitro release as well.<br>• The results showed that the production yield of the prepared diclofenac sodium microparticles was found to be between 79.55% and 97.41%.<br>• The formulated microparticles exhibited acceptable drug content values that lie in the range 66.20–96.36%.<br>• By increasing surfactant concentration, microspheres' surfaces become smoother and slightly porous. | **Mahmoud M. Ahmed et al., 2013 [49]** |

| | |
|---|---|
| Kinetic treatment of the in vitro release from drug-loaded microparticles indicated that the zero order is the drug release mechanism for the most formulae. | |
| • The alginate–chitosan microcapsules containing probiotics (Yeast, Y235) were prepared by emulsification/ external gelation and emulsification/internal gelation techniques respectively.<br>• The gel beads by external gelation showed asymmetrical structure, but those by internal gelation showed symmetrical structure in morphology.<br>• The cell viability was approximately 80% for these two techniques. However, during cell culture process, emulsification/internal gelation microcapsules showed higher cell growth and lower cell leakage.<br>• It indicated the growth process of cells in microcapsule was important and beneficial to keep enough active probiotics under harmful environment stress. Therefore, the emulsification/internal gelation technique was the preferred method for application in food or biotechnological industries.<br>• In the study, the properties of microencapsulated cells were evaluated including the morphology, size and size distribution, cell viability, cultural characteristics and survival rates in simulated gastrointestinal conditions. | **Huiyi Song et al., 2013[50]** |
| • The two potential probiotic strains namely *Staphylococcus succinus* (MAbB4) and *Enterococcus fecium* (FIdM3) selected from previous probiotic property studies were co-encapsulated with complementary prebiotics.<br>• Two different prebiotics selected by in vitro fermentation namely sugarbeet and chicory were separately encapsulated with both the strains in 2 g/100 mL alginate and were tested for the efficiency in improving the viability compared to free cells under in vitro acidic conditions.<br>• The concept of co-encapsulation offers the potential increased efficacy of survival of the probiotics. In this | **S. Sathyabama et al., 2013[51]** |

| | |
|---|---|
| study the results indicated significant improvement ($P < 0.05$) in survival of co-encapsulated cells when exposed to acidic (pH 2.0-3.0) and bile (0.3, 0.6 and 0.8 g/100 mL) conditions. The encapsulated cells showed about 98.75-88.75% of survivability in simulated gastric environment.<br>• Viability was maintained throughout the storage period and ranges from 8.1 log cfu/mL (Colony Forming Unit) to 7.9 log cfu/mL for about a period of 30 days at 4 °C. | |
| • The probiotic foods provide several health benefits, as they help in maintaining a good balance and composition of intestinal flora, and increase the resistance against invasion of pathogens.<br>• The demand of probiotic functional foods is growing rapidly due to increased awareness of consumers about the impact of food on health.<br>• Development of foods with adequate doses of probiotics at the time of consumption is a challenge, because several factors during processing and storage affect the viability of probiotic organisms.<br>• The presence of probiotics in food products may also adversely affect their quality and sensory properties.<br>• Major emphasis has been given to protect the microorganisms with the help of encapsulation technique.<br>• This contribution provides an overview of probiotic foods, factors responsible for survival of probiotics, and advance technologies used to stabilize their viability during processing and storage. | **M.K. Tripathi et al., 2014 [52]** |

## 2.3. Application of microencapsulated probiotic and/ prebiotic-probiotic conjugates in Functional foods

| Observations | References |
|---|---|
| • A modified method using calcium alginate for the microencapsulation of probiotic bacteria is reported in this study.<br>• Incorporation of Hi-Maize starch (a prebiotic) improved encapsulation of viable bacteria as compared to | **Khalida Sultana et al.[2000] [39]** |

| | |
|---|---|
| when the bacteria were encapsulated without the starch.<br>• Inclusion of glycerol (a cryo-protectant) with alginate mix increased the survival of bacteria when frozen at 22°C.<br>• A preliminary study was carried out in order to monitor the effects of encapsulation on the survival of *Lactobacillus acidophilus* and *Bifidobacterium* spp. in yoghurt over a period of 8 weeks.<br>• This study showed that the survival of encapsulated cultures of *L. acidophilus* and *Bifidobacterium* spp. showed a decline in viable count of about 0.5 log over a period of 8 weeks while there was a decline of about 1 log in cultures which were incorporated as free cells in yoghurt.<br>• The encapsulation method used in this study did not result in uniform bead size, and hence additional experiments need to be designed using uniform bead size in order to assess the role of different encapsulation parameters, such as bead size and alginate concentration, in providing protection to the bacteria.<br>• A recent study indicates that the survival of alginate coated bacteria may be depend on gel concentration and bead size. | |
| • The influence of the addition of *Lactobacillus paracasei* and *Streptococcus thermophilus* on the fructan content at the beginning and at the end of storage at 4 ± 1 °C of a potentially synbiotic fresh cream cheese manufactured with inulin was investigated.<br>• Three cheese-making trials were prepared, all supplemented with a lactic culture of *S. thermophilus* (T1, T2 and T3). *L. paracasei subsp. paracasei* was added in T1 and T2. Inulin was added in T2 and the fructan content was measured after 1 and 21 days of storage. Samples of T2 possessed similar mean concentrations of fructans after 1 and 21 days of storage, 7.32% and 7.27%, respectively, and no | **Fla´via C.A. Buriti et al[2007] [53]** |

| | |
|---|---|
| significant difference was observed.<br>• These results indicated that the metabolism of starter and probiotic bacteria did not degrade the fructans present in those cheeses.<br>• Additionally, synbiotic cheeses possessed a fructan content higher than 7 g per 100 g, sufficient to confer prebiotic potential during the entire storage period of these products.<br>• The cultures employed in this study were not able to degrade fructan and the concentration of this compound remained above 7% during the whole storage of the synbiotic cheese.<br>• This concentration of fructan, allied to the populations of potential probiotic *L. paracasei* above 7 log cfu $g^{-1}$ throughout storage is sufficient to confer synbiotic functional properties to this cheese. | |
| • The work was focused to evaluate the use of sonicated pineapple juice as substrate for producing a probiotic beverage by *Lactobacillus casei* NRRL B442.<br>• Maximal microbial viability was found by cultivating *L. casei* at 31°C and at optimized pH 5.8. After fermentation, samples of sweetened and non-sweetened juice were stored. After 42 days of storage under refrigeration (4°C), the microbial viability was 6.03Log CFU/mL in the non-sweetened sample and 4.77Log CFU/mL in the sweetened sample.<br>• The pH of both samples decreased during storage due to lactic acid production (post acidification). The characteristic colour of the juice was maintained throughout the shelf life and no browning was observed.<br>• Sonicated pineapple juice was shown to be a suitable substrate for *L. casei* cultivation and for the development of an alternative non-dairy probiotic beverage. This study showed that sonication can be applied as a pre-treatment for cultivating the probiotic strain *L. casei* B-442, which was then able ferment sonicated pineapple juice without any nutrient supplementation. | **Mayra Garcia Maia Costa et al.,2013[54]** |

| | |
|---|---|
| • The effect of inulin and/or okara flour on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb- 12 viability in a fermented soy product (FSP) and on probiotic survival under in vitro simulated gastrointestinal conditions were investigated throughout 28 days of storage at 4°C.<br>• Employing a 22 design, four FSP trials were produced from soymilk fermented with ABT-4 culture (La-5, Bb-12, and *Streptococcus thermophilus*).<br>• Probiotic viabilities ranged from 8 to 9 log cfu/g during the 28 days of storage, and inulin and/or okara flour did not affect the viability of La-5 and Bb-12. Bb-12 resistance to the artificial gastrointestinal juices was higher than for La-5, since the Bb-12 and La-5 populations decreased approximately 0.6 log cfu/g and 3.8 log cfu/g, respectively, throughout storage period.<br>• Even though the protective effect of inulin and/or okara flour on probiotic microorganisms was not significant, when compared to a fresh culture, the FSP matrix improved Bb-12 survival on day 1 of storage and may be considered a good vehicle for Bb-12 and could play an important role in probiotic protection against gastrointestinal juices. | **Raquel Bedani et al., 2013 [55]** |
| • Chocolate is consumed by people of all ages for its popularity and it is mainly associated with its potential to arouse sensory pleasure and positive emotions. Increasing awareness of the link between healthy eating and well-being is reflected in the current views of the general consumers.<br>• Consumers perceive functional foods as a member of the specific food category to which they belong.<br>• A key trend at the moment is confectionery products that deliver functional benefits for health and well-being, such as functional chocolate.<br>• In this review, studies related with production of prebiotic, probiotic and synbiotic chocolates as a functional food were investigated and positive and negative aspects of these functional products. | **Nevzat Konar et al., 2016 [56]** |

| | |
|---|---|
| • From this study it could be concluded that the studies associated with chocolate which could play a role in transportation of probiotics and prebiotics might be supported by studies in which bioavailability and bioaccessibility characteristics of them in vivo and in vitro media was determined.<br>• Moreover, in order to improve bioavailability and bioaccessibility properties product quality optimization studies might be required in the future. | |
| • The effects of encapsulation with alginate and chitosan, with or without inulin, on viability of probiotic *Lactobacillus rhamnosus* GG in apple juice during 90 days storage at 4 and 25 °C were evaluated.<br>• The survival rate of free bacteria decreased to 13.6% through the storage. The encapsulated *L. rhamnosus* experienced greater survival rate which was 4.5 times higher than that of free bacteria at day 90.<br>• Encapsulation of *L. rhamnosus* improved bacterial viability since 27.7% of bacterial population survived gastrointestinal transition model.<br>• Furthermore, inulin addition had a significant positive effect ($p < 0.05$) on survival of encapsulated bacteria.<br>• The sensory scores showed that the probiotic encapsulation brought an improvement in all characteristics. The results of this research suggested the encapsulation of probiotic bacteria as an effective method to improve bacterial survival both during apple juice storage and in gastrointestinal condition without any adverse organoleptic effect. | **Hassan Gandomi et al., 2016 [57]** |

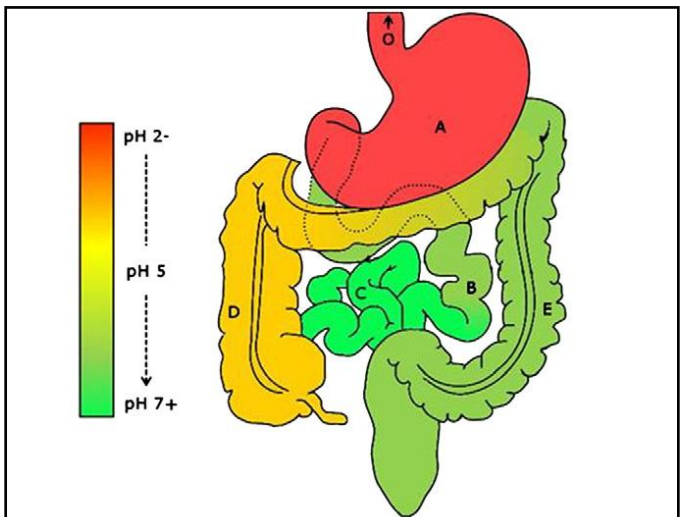
**2.4 Investigation on effects of free and microencapsulated probiotics and prebiotic-probiotic conjugates in simulated human GI system**

| Observations | References |
|---|---|
| • A three-stage compound continuous culture system was used to study the effect of retention time (27.1 and 66.7 h) on the catabolism of organic carbon and nitrogen sources in mixed populations of human colonic bacteria.<br>• In this study the results showed that the majority of carbohydrate breakdown and short-chain fatty acid production Conversely, dissimilatory amino acid metabolism, as evidenced by formation of branched-chain fatty acids and phenolic compounds.<br>• Bacteriological measurements of intestinal contents, in which nine groups of marker organisms were studied, showed that, with the exception of bifidobacteria, no major differences in relative bacterial cell numbers were evident in the proximal and distal colons.<br>• These organisms were also studied in the continuous culture system, where marked reductions in *Escherichia coli.*<br>• Correlations between in vivo chemical and bacteriological measurements and data obtained in vitro demonstrate that the three-stage fermentation system provided a useful model for studying the physiology and ecology of large intestinal microorganisms under different nutritional and environmental conditions.<br>• The three-stage continuous culture system employed in this investigation was designed to reproduce the main nutritional and environmental conditions that affect fermentation reactions in the proximal and distal colons. | **G.T. Macfarlane et al., 1998 [58]** |
| • Fluorescence in situ hybridization was used to quantitate bacteria growing in a three-stage continuous culture system inoculated with human faeces, operated at two system retention times (60 and 20 h). | **Matthew W. Child et al.,2006** |

| | |
|---|---|
| • Twenty-three different 16S rRNA gene oligonucleotide probes of varying specificities were used to detect bacteria.<br>• Their objectives were to compare the population structure of the original faecal inoculum to communities that established in different fermentation vessels and to determine the effect of system retention time, which is analogous to large bowel transit time, on the types of bacteria that colonized the fermentation system, together with their metabolic activities. | **[59]** |
| • The encapsulation and controlled release technologies have enjoyed their fastest growth in the last two decades. This book attempts to illustrate various aspects of encapsulation and controlled release applications in food systems using practical examples. | **Jamileh M. Lakkis, 2007 [60]** |
| • Probiotic Encapsulation Technology (PET) has the potential to protect microorganisms and to deliver them into the gut.<br>• However, there are still many challenges to overcome with respect to the microencapsulation process and the conditions prevailing in the gut.<br>• PET will be of importance in delivering viable strains of probiotic to consumers. | **Gildas K. Gbassi et al., 2012 [61]** |
| • The oral administration of most bacteria results in a large loss of viability associated with passage through the stomach, which is attributed to the high acid and bile salt concentrations present.<br>• This loss of viability effectively lowers the efficacy of the administered supplement. The formulation of these probiotics into microcapsules is an emerging method to reduce cell death during GI passage, as well as an opportunity to control release of these cells across the intestinal tract.<br>• The majority of this technology is based on the immobilization of bacteria into a polymer matrix, which retains it structure in the stomach before degrading and dissolving in the intestine, unlike the diffusion | **Michael T. Cook et al., [2012][62]** |

based unloading of most controlled release devices for small molecules.

- In particular, digestion of an encapsulation matrix by the colonic microflora may be a better method of targeting release to the colon than pH.




| Letter | Region | pH | Transit Time |
|---|---|---|---|
| O | Oesophagus | ~7.0 | 10-14 seconds |
| A | Stomach | 1-2.5 (up to 5 fed) | Half emptying: ~80.5 mins |
| B | Proximal Small Intestine | 6.15-7.35 | 3.2 ±1.6 hrs (combined) |
| C | Distal Small Intestine | 6.80-7.88 | |
| D | Ascending Colon | 5.26-6.72 | Highly variable, dependent on bowel evacuations |
| E | Descending Colon | 5.20-7.02 | |

**Figure 2.1: Characteristics of the GI tract, showing the pH at the different parts**

- The objective was to evaluate the potential of inulin as wall material for encapsulation and controlled release of Oregano essential oil.
- In order to understand both the characteristics of the wall capsules and the way the essential oil is there at retained the use of several accessing methodologies are needed.
- The ability of inulin, a prebiotic material, as encapsulation matrix was explored. IR and Raman analysis

**Sara Beirão-da-Costa et al., 2013 [63]**

| | |
|---|---|
| showed that drying conditions clearly affected the capsules properties probably being related to the phase transitions of inulin.<br>• Encapsulation of oregano essential oil in those matrices conducted also to differentiated releasing profiles that are explained by the dissimilar properties of the wall. Those different patterns may be very useful in finding the most suited formulation and processing condition for a specific end use of encapsulates. | |
| • An updated knowledge was presented about the involvement of gut microbiota in GI tumorigenesis, including its underlying mechanisms.<br>• This study also presented a comprehensive review of the evidence from animal and clinical studies using probiotics and/or prebiotics in the prevention and/or therapy of GI tumours, of GI cancer therapy-related toxicity and of post-operative complications.<br>• It summarized the anticarcinogenic mechanisms of these biotherapeutics from in vitro, animal and clinical interventions.<br>• Well-designed, randomized, double blind, placebo-controlled human studies using probiotics and/or prebiotics, with adequate follow-up are necessary in order to formulate directions for prevention and therapy | **Daniela Elena Serban [64]** |
| • The most frequently used dietary method of influencing the gut flora composition is the consumption of probiotics.<br>• Over the years many species of micro-organisms have been used. They consist mainly of lactic acid bacteria (lactobacilli, streptococci, enterococci, lactococci and bifidobacteria). *Bacillus spp*. may also be used.<br>• The study aimed at comparing the persistence of probiotics within the human gastrointestinal tract. | **Prof G.R. Gibson and Prof. J. Brostoff [65-69]** |

| | |
|---|---|
| Probiotics are marketed as health or functional foods whereby they are ingested for their purported positive advantages in the digestive tract and/or systemic area.<br>• To exert any beneficial properties, probiotics must have robust survival properties in the gut. Given their wide use in the UK, purported health aspect and the fact that they contain living organisms, all new probiotics would be subject to a rigorous pre-market safety evaluation under the terms and conditions of the Novel Foods Regulation (EC) 258/97 before they can be sold in the EU, and all existing probiotic products must comply with the Food Safety Act (1990). | |
| • Investigated the effects of incorporating locust bean (LB) and xanthan XT gums into the high density (HD) capsules of alginate (AGN) coated with chitosan in terms of (i) the capsules' stress tolerance upon freeze drying, heat treatment and acid exposure in simulated gastric juice, and in terms of (ii) their cell release profiles in simulated gastrointestinal fluid.<br>• Similar to the AGN-only capsules, the HD capsules of AGN-LB and AGN-XT were superior to their planktonic counterparts in their freeze drying viability and thermo tolerance, thus the addition of LB and XT gums did not negate the superiority of the HD capsules.<br>• The AGN-LB capsules possessed the optimal cell release profile in which a considerably larger number of cells are released in the simulated intestinal juice than in the gastric juice, unlike the AGN and AGN-XT capsules.<br>• The superior cell released characteristics of the AGN-LB capsules were attributed to their high swelling capacity and stronger interaction between chitosan coating and AGN-LB matrices, resulting in the delay of the onset of bulk capsule dissolution. In this research article, the results thus have demonstrated the importance of the capsule matrix material in the resultant cell release profile and acid tolerance. | **Wean Sin Cheow et al., 2014[70]** |

| | |
|---|---|
| • Designed and assembled a simulated human intestinal system (SHIS) consisting of a stomach and a small intestine.<br>• Each reactor vessel has several ports such as the input and output of medium, a sampler of liquid phase, a pH electrode, a pH control (acid and base), and a thermometer.<br>• There were no difference on the degree of digestion in the alginate capsule and alginate/polyethylene glycol (alginate/PEG) capsule prepared with 0.1-0.3% of PEG in the stomach model.<br>• As a result, the alginate matrix remained in a shrunken state due to conversion of sodium alginate into insoluble alginic acid, which acted as a barrier to chitosan microparticles and it was effective for digesting by intestinal fluid and releasing of the sugar. | **Ae-Jin Choi et al., 2015 [71-75]** |
| • The regenerated cellulose microgels (RCMs) were modified by introducing CaCO3 during the preparation process to obtain the microgels with various porosity and pore structure. Then the RCMs and modified RCMs were used to encapsulate *L. plantarum* cells.<br>• The porosity and pore structure had a great influence on the distribution and release behavior of *L. plantarum* cells<br>• Due to the interpenetrating porous structure, *L. plantarum* cells were not only adhered to the surface but able to penetrate into the core of microgels.<br>• Core-shell structure with pH-responsibility was designed to protect cells in microgels from liberating in gastric fluid before they reached intestine tracts. The composite core-shell gels showed controlled release of *L. plantarum* cells in simulated intestine fluid. The porous cellulose microgels were promising for scaffold in biomedicine area due to its high loading capacity, and the designed composite core-shell gels would further protect the bioactive nutrients with prolonged stability and viability | **Wei Li et al., 2016 [76-81]** |

## REFERENCES


1. Kosin, B. and Rakshit, S.K. (2006) "Criteria for Production of Probiotics, Microbial and Processing Criteria for Production of Probiotics: A Review". Food Technology and Biotechnology, 44 (3), 371–379.
2. Vitali, B., Minervini, G., Rizzello, C.G., Spisni, E., Maccaferri, S., Brigidi, P., Gobbetti, M. and Di Cagno, R. (2012) "Novel probiotic candidates for humans isolated from raw fruits and vegetables". Food Microbiology, 31, 116-125.
3. Franck, A., De Leenheer, L. Orafti, Belgium. Inulin, Chapter 14, page 439-448.
4. Singh, R.S., and Singh, R.P. (2010) "Production of Fructooligosaccharides from Inulin by Endoinulinases and Their Prebiotic Potential". Food Technology and Biotechnology, 48 (4), 435-450.
5. Mavumengwana, V.B. (2004) "Isolation Purification and Characterisation of inulin and fructo-oligosaccharides from *Chicorium intybus* and inulinase from *Aspergillus niger*". Thesis for the degree of M. Sc. Department of Biochemistry, Microbiology and Biotechnology; Rhods University.
6. Boeckner, L.S., Schnepf, M.I., Tungland, B.C. (2001) "Inulin: a review of nutritional and health implications". Advances in food and nutrition research, 43, ISBN: 0-12-016443-4 Copyright Academic Press.
7. Kaur, N. and Gupta, A.K. (2002) "Applications of inulin and oligofructose in health and nutrition". Journal of Biosciences, 27(7), 703–714, © Indian Academy of Sciences.
8. Toneli, J. T. C. L., Park, K. J., Ramalho, J. R. P., Murr, F. E. X. and Fabbro, I. M. D. (2008) "Rheological characterization of chicory root (*Cichorium intybus* l.) Inulin solution". Brazilian Journal of Chemical Engineering, 25 (03), 461 – 471.
9. Choi, H.W., Ryu, H.K., Park, K.M., Lee, E.G., Lee, H., Kim, S.W., and Choi, E.S. (2012) "Direct lactic acid fermentation of Jerusalem artichoke tuber extract using *Lactobacillus paracasei* without acidic or enzymatic inulin hydrolysis". Bioresource Technology, 114, 745–747.
10. Nithya,V., Halami, P.M. (2013) "Evaluation of the probiotic characteristics of Bacillus species isolated from different food sources". Annals of Microbiology, 63, 1, 129–137.

11. Slavin, J. (2013) “Fiber and Prebiotics: Mechanisms and Health Benefits”. Nutrients, 5, 1417-1435, doi: 10.3390/nu5041417.
12. Sarita, Singh, E. (2016) “Potential of Millets: Nutrients Composition and Health Benefits”. Journal of Scientific and Innovative Research, 5(2), 46-50.
13. Liu, R.H. (2007) “Whole Grain Phytochemicals and Health”. Journal of Cereal Sciences, 46, 207–219.
14. Radhajeyalakshmi, R., Yamunarani, K., Seetharaman, K. and Velazhahan, R. (2003) “Existence of Thaumatin-Like Proteins (Tlps) in Seeds of Cereals”. Acta Phytopathologica et Entomologica Hungarica, 38(3–4), 251–257.
15. Shoaib, M., Shehzad, A., Omar, M., Rakha, A., Raza, H., Sharif, H.R. Shakeel, A., Ansari, A., Niazi, S. (2016) “Inulin: Properties, health benefits and food applications”. Carbohydrate Polymers, 147, 444–454.
16. Aidoo, R. P., Afoakwa, E. O., and Dewettinck, K. (2014) “Optimization of inulin and polydextrose mixtures as sucrose replacers during sugar-free chocolate manufacture −rheological, microstructure and physical quality characteristics”. Journal of Food Engineering, 126(1), 35–42.
17. Anderson, J. W., Baird, P., Davis, R. H., Jr., Ferreri, S., Knudtson, M., Koraym, A., et al. (2009) “Health benefits of dietary fiber”. Nutrition Reviews, 67(4), 188–205.
18. Apolinario, A. C., de Lima Damasceno, B. P., de Macedo Beltrao, N. E., Pessoa, A., Converti, A., and da Silva, J. A. (2014) “Inulin-type fructans: a review on different aspects of biochemical and pharmaceutical technology”. Carbohydrate Polymer, 101, 368–378.
19. Biedrzycka, E., and Bielecka, M. (2004) “Prebiotic effectiveness of fructans of different degrees of polymerization”. Trends in Food Science & Technology, 15(3–4), 170–175.
20. Boeckner, L. S., Schnepf, M. I., and Tungland, B. C. (2001) “Inulin: a review of nutritional and health implications”. Advances in Food and Nutrition Research, 43, 1–63.
21. Bonnema, A. L., Kolberg, L. W., Thomas, W., and Slavin, J. L. (2010) “Gastrointestinal tolerance of chicory inulin products”. Journal of the American Dietetic Association, 110(6), 865–868.

22. Bornet, F. R. J. (2008) “Fructo-oligosaccharides and other fructans: chemistry, structure and nutritional effects”. Advanced Dietary Fibre Technology, Blackwell Science Ltd. 480–493.
23. Bosscher, D., Van Loo, J., and Franck, A. (2006) “Inulin and oligofructose as prebiotics in the prevention of intestinal infections and diseases”. Nutrition Research Reviews, 19(2), 216–226.
24. Cherbut, C. (2002) “Inulin and oligofructose in the dietary fibre concept”. British Journal of Nutrition, 87, S159–S162.
25. Chi, Z. M., Zhang, T., Cao, T. S., Liu, X. Y., Cui, W., and Zhao, C. H. (2011) “Biotechnological potential of inulin for bioprocesses”. Bioresource Technology, 102(6), 4295–4303.
26. Cho, S. S., and Samuel, P. (2009) “Fiber ingredients: food applications and health benefits”. pp. 41–55. Boca Raton, FL, USA, CRC Press.
27. Flamm, G., Glinsmann, W., Kritchevsky, D., Prosky, L., and Roberfroid, M. (2001) “Inulin and oligofructose as dietary fiber: a review of the evidence”. Critical Reviews in Food Science and Nutrition, 41(5), 353–362.
28. Guarner, F. (2005) “Inulin and oligofructose: impact on intestinal diseases and disorders”. British Journal of Nutrition, 93, S61–S65.
29. Judprasong, K., Tanjor, S., Puwastien, P., & Sungpuag, P. (2011) “Investigation of Thai plants for potential sources of inulin-type fructans”. Journal of Food Composition and Analysis, 24(4–5), 642–649
30. Kalyani Nair, K., K., Kharb, S., and Thompkinson, D. K. (2010) “Inulin dietary fiber with functional and health attributes—a review”. Food Reviews International, 26(2), 189–203.
31. Kelly, G. (2008) “Inulin-Type Prebiotics—a review” part 1. Alternative Medicine Review, 13(4), 315–329.
32. Krasaekoopt, W., and Watcharapoka, S. (2014) “Effect of addition of inulin and galacto-oligosaccharide on the survival of microencapsulated probiotics in alginate beads coated with chitosan in simulated digestive system, yogurt and fruit juice”. Lwt-Food Science and Technology, 57(2), 761–766.
33. Van Loo, J. A. (2004) “Prebiotics promote good health: the basis, the potential: and the emerging evidence”. Journal of Clinical Gastroenterology, 38, S70–S75.

34. Rubel, I. A., Pérez, E. E., Manrique, G. D., and Genovese, D. B. (2015) "Fibre enrichment of wheat bread with Jerusalem artichoke inulin: effect on dough rheology and bread quality". Food Structure, 3, 21–29.
35. Gangaiah, B., "Agronomy – Kharif Crops". Senior Scientist, Division of Agronomy, Indian Agricultural Research Institute, New Delhi.
36. Sekhon, B.S. and Jairath, S. (2010) "Prebiotics, probiotics and synbiotics: an overview", Journal of Pharmaceutical and Educational Research, 1(2), 13-36.
37. Yoo, I.K., Seong, G.H., Chang, H.N. and Park, J.K. (1996) "Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production". Enzyme Microbiology and Technology, 19, 426-433.
38. Hansen, L.T., Allan-Wojtas, P.M., Jin, Y.L., Paulson, A.T. (2002) "Survival of Ca-alginate microencapsulated *Bifidobacterium spp.* in milk and simulated gastrointestinal conditions". Food Microbiology, 19, 35–45.
39. Sultana, K., Godward, G., Reynolds, N., Arumugaswamy, R., Peiris, P. (2000) "Encapsulation of probiotic bacteria with alginate–starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt". International Journal of Food Microbiology, 62, 47–55.
40. Kailasapathy, K. (2002) "Microencapsulation of Probiotic Bacteria: Technology and Potential Applications". Current Issues Intestinal Microbiology, 3, 39-48.
41. Franjione, J. and Vasishtha, N. (1995) "The Art and Science of microencapsulation". Technology Today.
42. Godward, G.N. (2000) "Studies on enhancing the viability and survival of probiotic bacteria in dairy foods through strain selection and micro encapsulation". University of Western Sydney, Master of Hons. Thesis.
43. Mandal, S., Puniya, A.K., Singh, K. (2006) "Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298". International Dairy Journal, 1190–1195.
44. Champagne C.P. and Patrick Fustier, P. (2007) "Microencapsulation for the improved delivery of bioactive compounds into foods". Current Opinion in Biotechnology, 18, 184–190.

45. Bosnea, L.A., Kourkoutas Y., Albantaki, N., Tzia, C., Koutinas, A.A., and Kanellaki, M. (2009) "Functionality of freeze-dried *L. casei* cells immobilized on wheat grains", LWT - Food Science and Technology, 42, 1696–1702.
46. Mohammed, Akhia, N.S.A. (2010) "Enhancement of probiotics survival by microencapsulation with alginate and prebiotics". MMG 445 Basic Biotechnology, 6, 13-18.
47. Chan, E.S., Wong, S.L., Lee, P.P., Lee, J.S., Ti, T.B., Zhang, Z., Poncelet, D., Ravindra, P., Phan, S.H., Yim, Z.H.( 2011) "Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells". Carbohydrate Polymers. 83, 225–232.
48. Pliszczak, D., Bourgeois, S., Bordes, C., Valour, J.P., Mazoyer, M.A., Orecchioni, A.M., Nakache, E., Lantéri, P. (2011) "Improvement of an encapsulation process for the preparation of pro- and prebiotics-loaded bioadhesive microparticles by using experimental design". European Journal of Pharmaceutical Sciences, 44, 83–92.
49. Ahmed, M.M., El-Rasoul, S.A., Auda, S.H., Ibrahim, M.A. (2013) "Emulsification/internal gelation as a method for preparation of diclofenac sodium–sodium alginate microparticles". Saudi Pharmaceutical Journal, 21, 61–69.
50. Song, H., Yu, W., Gao, M., Liu, X., Ma, X. (2013) "Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process". Carbohydrate Polymers, 96, 181– 189.
51. Sathyabama, S., Kumar, M.R., Bruntha devi, P., Vijayabharathi, R., Brindha priyadharisini, V. (2014) "Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in simulated gastric environment". LWT - Food Science and Technology, 57, 419-425.
52. Tripathi, M.K., Giri. S.K. (2014) "Probiotic functional foods: Survival of probiotics during processing and storage". Journal of functional foods, 9, 225–241.
53. Fla´via, C.A., Buriti, Haı´ssa, R., Cardarelli, T., Filisetti, M.C.C., Saad, S.M.I. (2007) "Synbiotic potential of fresh cream cheese supplemented with inulin and *Lactobacillus paracasei* in co-culture with *Streptococcus thermophilus*". Food Chemistry, 104, 1605–1610.

54. Beirão-da-Costa, S., Duarte, C., Bourbon, A.I., Pinheiro, A.C., Isabel, M., Januário, N., Vicente, A.A., Beirão-da-Costa M.L., Delgadillo, I. (2013) "Inulin potential for encapsulation and controlled delivery of Oregano essential oil". Food Hydrocolloids, 33, 199-206.
55. Bedani, R., Rossi, E.A., Saad, S.M.I. (2013) "Impact of inulin and okara on *Lactobacillus acidophilus* La-5 and *Bifidobacterium animalis* Bb-12 viability in a fermented soy product and probiotic survival under in vitro simulated gastrointestinal conditions". Food Microbiology, 34, 382-389.
56. Konar, N., Toker, O.S., Oba, S., Sagdic, O. (2016) "Improving functionality of chocolate: A review on probiotic, prebiotic, and/or synbiotic characteristics". Trends in Food Science & Technology, 49, 35-44.
57. Gandomi, H., Abbaszadeh, S., Misaghi, A., Bokaie, S., Noori, N. (2016) "Effect of chitosan-alginate encapsulation with inulin on survival of *Lactobacillus rhamnosus GG* during apple juice storage and under simulated gastrointestinal conditions". LWT - Food Science and Technology, 69,365-371.
58. Macfarlane, G.T., Macfarlane, S., Gibson, G.R. (1998) "Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon". Microbial Ecology, 35, 180–187.
59. Child, M.W., Kennedy, A., Walker, A.W., Bahrami, B., Macfarlane, S., Macfarlane, G.T. (2006). "Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques". FEMS Microbiology Ecology, 55, 299–310.
60. Lakkis, J.M**.** (2007) "Encapsulation and Controlled Release Technologies in Food Systems". Blackwell Publishing.
61. Gbassi, G.K. and Vandamme, T. (2012) "Probiotic Encapsulation Technology: From Microencapsulation to Release into the Gut". Pharmaceutics, 4, 149-163, doi:10.3390/pharmaceutics4010149.
62. Cook, M.T., Tzortzis, G., Charalampopoulos, D., Khutoryanskiy, V.V.(2012) "Microencapsulation of probiotics for gastrointestinal delivery". Journal of Controlled Release, 162, 56–67.

63. Costa, M.G.M., Fonteles, T.V., de Jesus, A.L.T., Rodrigues, S. (2013) "Sonicated pineapple juice as substrate for *L. casei* cultivation for probiotic beverage development: Process optimisation and product stability". Food Chemistry, 139, 261–266.
64. Serban, D.E. (2014) "Gastrointestinal cancers: Influence of gut microbiota, probiotics and Prebiotics". Cancer Letters, 345, 258–270.
65. Gibson, G.R., Rouzaud, G. Food and Microbial Sciences Unit, University of Reading, Brostoff, J., Rayment, N. King's College London. "An evaluation of probiotic effects in the human gut microbial aspects". Final Technical report for FSA project ref G01022.
66. Brigidi, P., Swennen, E., Vitali, B., Rossi, M., Matteuzzi, D. (2003) "PCR detection of Bifidobacterium strains and *Streptococcus thermophilus* in feces of humans subjects after oral bacteriotherapy and yogurt consumption". International Journal of Food Microbiology, 81, 203-209.
67. Casas, I. A. and Dobrogosz, W. J. (2000) "Validation of the probiotic concept: *Lactobacillus reuteri* confers broad-spectrum protection against disease in humans and animals". Microbial Ecology in Health and Disease, 12, 247-285.
68. Charteris, W.P., Kelly, P.M., Morelli, L., Collins, J.K. (1998) "Development and application of *in vitro* methodology to determine the transit tolerance of potentially probiotic *Lactobacillus* and *Bifidobacterium* species in the upper human gastrointestinal tract". Journal of Applied Microbiology, 84, 759-768.
69. Yuki, N., Watanabe, K., Mike, A., Tagami, Y., Tanaka, R., Ohwaki, M., Morotomi, M. (1999) "Survival of a probiotic, *Lactobacillus casei* strain *Shirota*, in the gastrointestinal tract: selective isolation from faeces and identification using monoclonal antibodies". International Journal of Food Microbiology, 48, 51-57.
70. Cheow, W.S., Kiew, T.Y., Hadinoto, K. (2014) "Controlled release of *Lactobacillus rhamnosus* biofilm probiotics from alginate-locust bean gum microcapsules". Carbohydrate Polymers, 103, 587– 595.
71. Choi, A.J., Buisson, N., Kim, C.T. (2015) "Digestion characteristics and kinetic analysis of biomolecules in a simulated human intestinal system". Integrative Food Nutrition Metabolism, 2(3), 189-192.
72. Marteau, P., Minekus, M., Havenaar, R., Huis in't Veld, J.H. (1997) "Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: validation and the

effects of bile". Journal of Dairy Sciences, 80, 1031-1037.

73. Minekus, M., Marteau, P., Havenaar, R., Huisin´t Veld, J.H.J. (1995) "A multicompartmental dynamic computer controlled model simulating the stomach and small intestine". American Theoretical Library Association, 23, 197-209.
74. Huguet, M.L., Dellacherie, E. (1996) "Calcium alginate beads coated with chitosan: effect of the structure of encapsulated materials on their release". Process Biochemistry, 31, 745-751.
75. Lentle, R.G., Janssen, P.W.M. "The Physical Processes of Digestion". Springer New York Dordrecht Heidelberg London. ISBN 978-1-4419-9448-6. DOI 10.1007/978-1-4419-9449-3.
76. Li, W., Zhu, Y., Feng, Y., Li, B., Luo, X., Liu, S. (2016) "Probiotics in cellulose houses: Enhanced viability and targeted delivery of *Lactobacillus plantarum*". Food Hydrocolloids, doi:10.1016/j.foodhyd.2016.07.019.
77. Bron, P. A., van Baarlen, P., Kleerebezem, M. (2012) "Emerging molecular insights into the interaction between probiotics and the host intestinal mucosa". Nature Reviews Microbiology, 10(1), 66-90.
78. Cook, M. T., Saratoon, T., Tzortzis, G., Edwards, A., Charalampopoulos, D., Khutoryanskiy, V. V. (2013) "CLSM Method for the Dynamic Observation of pH Change within Polymer Matrices for Oral Delivery". Biomacromolecules, 14(2), 387-393.
79. Cook, M. T., Tzortzis, G., Charalampopoulos, D., Khutoryanskiy, V. V. (2011) "Production and Evaluation of Dry Alginate-Chitosan Microcapsules as an Enteric Delivery Vehicle for Probiotic Bacteria". Biomacromolecules, 12(7), 337, 2834-2840.
80. Cotter, P. D., Ross, R. P., Hill, C. (2013) "Bacteriocins - a viable alternative to antibiotics?". Nature Reviews Microbiology, 11(2), 95-105.
81. Huq, T., Khan, A., Khan, R. A., Riedl, B., Lacroix, M. (2013) "Encapsulation of Probiotic Bacteria in Biopolymeric System". Critical Reviews in Food Science and Nutrition, 53(9), 909-916.

# Chapter 3

# AIMS & OBJECTIVES

From the foregoing discussion it is evident that application of prebiotic-probiotic mixture (as netraceuticles) in conventional nutritional food will grow rapidly in near future. Moreover, the synbiotic effect of prebiotic-probiotic mixtures may also pave a new avenue in the area of food preservation. Literature survey shows that while extensive research are now being conducted in these areas, these studies are limited only to a few selected sources of prebiotics. It is now well established that inulin, a fructo-oligosaccharide is possibly the best choice as prebiotic. However, its application is constrained due to the prohibitory high cost of the material. This scenario demands searching of new sources of raw materials, rich in fructo-oligosaccharide inulin and their subsequent isolation following a cheaper route. On the other hand, the utilization of probiotic organism in health food is restricted by the adverse effect of acidic gastric juice and alkaline small intestinal juice against the free cells. The protection of probiotic cells in the food substances under preservation is also necessary. Thus probiotic microencapsulation technology has been developed to protect the cells. The selection of the proper microencapsulation method and study of the behavior of probiotic cells in microencapsulated vector are necessary. The effect of prebiotics on the microencapsulated probiotic should also be investigated. From the literature review it is clear that the studies on immobilized prebiotic-probiotic conjugates are still in its infancy. In the present investigation it is proposed to study immobilized conjugate for its antimicrobial activity as well as to explore the possibility of using the immobilized whole cells as a potential source of food preservative. With these intentions, the present investigation will focus on the following aims:

1. Extraction and characterization of prebiotic biomolecule, namely inulin from garlic and abundant Indian pulses, namely the Indian millets.
2. Bioprocess studies of the growth of *Lactobacillus casei* (2651 1951 RPK) on carbohydrate sources, namely lactose and inulin from different sources in free cell system
3. Immobilization of *Lactobacillus casei* (2651 1951 RPK) and the prebiotic-probiotic conjugate through entrapment and microencapsulation techniques along with optimization and determination of antimicrobial activity.
4. Mathematical Modeling for prediction of system performance.

5. Application of probiotic and prebiotic-probiotic beads and microcapsules in Indian foods and assessment of enhancement of the survival of probiotic bacteria using preservation time and temperature as parameters.
6. Cost analysis of extraction process of millet prebiotic, identified as the best growth enhancer of the probiotic cell under study.

The objectives for the fulfillment of the above aims are follows:

**Objectives under aim-1**

- The solvent extraction technique, followed by vacuum evaporation and lyophilizing will be used for the isolation of inulin from garlic and Indian millet sources, namely, pearl millet, great millet and finger millet.
- Characterization of isolated inulin will be done with the help of Thin layer Liquid Chromatography (TLC) and Fourier transform infrared spectroscopy (FTIR). Presence of inulin in the isolated samples will be authenticated by comparing the result with the commercially available inulin. Once identified, concentration of inulin in each source will be determined quantitatively by the standard 3-5 Di-nitro salicylic acid (DNS) method.
- The most effective millet inulin with respect to *Lactobacillus casei* will be identified by comparing the 24 h growth of the probiotic microorganism on different millet inulins extracted under the present study.
- The process parameters for the extraction of the most effective millet inulin will be optimized by conducting experiments at pre-set conditions using central composite design. The conditions corresponding to maximum yield of inulin will be identified using statistical modeling.

**Objectives under aim-2**

- Optimization of the growth medium of a selected probiotic microorganism, namely *Lactobacillus casei* with respect to the concentrations of the carbohydrates, namely, lactose and inulin.

The first step towards achieving significant cell growth is the selection of composition of growth medium. Thus in the present investigation a statistical optimization of the cell growth medium will be carried out with concentration of lactose and concentration of inulin as dependent variables with an objective to get maximum specific cell growth rate response. It is proposed to use Response Surface Methodology (RSM) as the optimization tool.

- Determination of growth dynamics of *Lactobacillus casei* on inulin as carbohydrate sources.

Effect of any prebiotic as probiotic cell growth enhancer is an important parameter for its usefulness. Quantitatively this can be expressed in terms of cell growth kinetics. In the present investigation a set of programmed batch mode experiments will be carried out to generate concentration time histories of both cell and the substrate. Initial concentration of substrate as well as the selected prebiotic inulin will be considered as operating variables. A comprehensive theoretical analysis will be carried out in predicting the cell growth dynamics of *Lactobacillus casei* in absence and in presence of the prebiotic inulin. A comparison will also be made in order to establish the usefulness of selected prebiotic source. Fulfillment of this objective will thus lead to the understanding of complete cell dynamics of the prebiotic-probiotic conjugate along with the quantitative knowledge of intrinsic kinetic parameters.

- Determination of Antimicrobial activity and antibiotic sensitivity of inulin- *Lactobacillus casei* conjugate.

In order to achieve the objective, the quantitative ranking method suggested by Hubner et al will be utilized [1]. A programmed set of experiments on antimicrobial activity will be carried out with two well performing prebiotic-probiotic conjugates in presence of the pathogen, *Escherichia coli*. In each case the prebiotic score will be evaluated which will help to establish the preferential effectiveness of the prebiotic under investigation. A separate set of experiments will also be conducted to evaluate the zone of inhibition provided by the selected conjugate in presence of a broad spectrum antibiotic, Norfloxacin.

**Objectives under aim-3**

- Three different techniques of immobilization, viz., entrapment in bead and microencapsulated through external and internal gelation will be used with an intention to determine the most effective method of immobilization.
- The process parameters for the best immobilization method, under study, will be optimized using response surface methodology to maximize the efficiency of encapsulation.
- Antimicrobial activity of immobilized conjugates towards a specific pathogen, *Escherichia coli,* will be studied in detail in terms of zone of inhibition.

**Objectives under aim-4**

- Mathematical modeling for the prediction of lactose and biomass concentration profiles in probiotic microcapsules and beads suspended in lactose rich MMRS medium and green coconut water.

Prediction of internal concentration of lactose and biomass with progress of time is one of the most important requirements for long term use of immobilized probiotic whole cell in food preservation. In the present investigation, another mathematical model will be developed incorporating both the diffusion of lactose across the outer surface of microcapsules and beads and the growth of cells immobilized within the internal core. The partial and ordinary differential mass balance equations for substrate and cells will be solved using MATLAB 7. The release time of cells from the probiotic beads and microcapsules either suspended in MMRS medium or lactose rich liquid food will also be predicted using this model. The model will be validated by the comparison with the experimental data.

- Mathematical modeling for the determination of "release time" of probiotic cells from the entrapment beads and microcapsules suspended in simulated gastrointestinal (GI) juices.

The bursting time of prebiotic-probiotic microcapsules will be predicted using Malthusian population balance equation for cell growth [2]. Experimental data will be used to validate the proposed model equations.

- Mathematical modeling of a simulated ***in-vitro*** GI system to predict the concentration-time histories of microcapsules and released probiotic cells.

The human GI system will be simulated by using a series of three continuous stirred tank bioreactors (CSTBRs) representing the "stomach", "small intestine" and the "large intestine". In order to predict concentration time history of microcapsules and probiotic cells *Lactobacillus casei* in the simulated GI tract a set of ordinary differential equations correlating the mass and number balance equations for the microcapsules and probiotic cells around the CSTBRs under transient condition will be developed using simplified and logical assumptions. The concentration time histories of microcapsules and probiotic cells in the "stomach", "small" and the "large" intestines will be simulated by solving the differential equations along with the boundary conditions using MATLAB 7, ode 45 software. The simulated profiles will be compared with the experimental ones to validate the mathematical model.

**Objectives under aim-5**

Application of these immobilized samples as potential sources of food preservative will also be studied at two different temperature levels and preservation times on polysaccharide enriched food materials viz., Indian milk cake and jaggery.

**Objectives under aim-6**

The cost analysis of the extraction of the most effective millet inulin will be done for a small production plant considering the recurring and non-recurring expenditure into account. The cost of extracted millet inulin will be compared with that of commercially available inulin to assess the competitiveness.

## REFERENCES

1. Huebner, J., Wehling, R.L. and Hutkins, R.W. (2007) "Functional Activity of commercial probiotics". International Dairy Journal, 17, 770-775.
2. Clark, H., Blanch, W. (1997). Biochemical Engineering, Edition 2, CRC Press.

# Chapter 4

# MATERIALS & METHODS

## 4.1. Chemicals

4.1.1. Beef extract (Merck, India)

4.1.2. Yeast extract (Himedia, India)

4.1.3. Peptone (Himedia, India)

4.1.4. Sodium acetate (LobaChemie, India)

4.1.5. di-potassium hydrogen phosphate (Himedia, India)

4.1.6. Tri-ammonium citrate (LobaChemie, India)

4.1.7. Magnesium sulphate (Himedia, India)

4.1.8. Manganese sulphate (Himedia, India)

4.1.9. Lactose (Merck, India)

4.1.10. Food grade inulin (Himedia, India)

4.1.11. Benzene (Ranbaxy, India)

4.1.12. Acetic acid (Merck, India)

4.1.13. Methanol (Merck, India)

4.1.14. Resorcinol (Merck, India)

4.1.15. Ethanol (Merck, India)

4.1.16. Sulphuric acid (Merck, India)

4.1.17. HCL (Merck, India)

4.1.18. Calcium hydroxide (Merck, India)

4.1.19. n-butanol (Merck, India)

4.1.20. Oxalic acid (Merck, India)

4.1.21. Orcinol (Merck, India)

4.1.22. Glucose (LobaChemie Laboratory reagents and fine chemicals, India)

4.1.23. Calcium chloride (Himedia, India)

4.1.24. Sodium alginate (Merck, India)

4.1.25. Rice bran oil (vegetable oil) (Local market)

4.1.26. Tween 80 (Merck, India)

4.1.27. Glycerol (Merck, India)

4.1.28. Sodium Dodecyl Sulphate (SDS) (Merck, India)

4.1.29. Paraffine oil (Merck, India)

4.1.30. Acetonitrile

**4.2. Materials**

4.2.1. TLC Silica gel G 60 Aluminium sheets 20 x 20 cm (Merck HX 816976, Germany)

**4.3. Simulated reagents**

4.3.1. Saline water

4.3.2. Phosphate buffer

4.3.3. Peptone water

4.3.4. Gastric juice

4.3.5. Saliva solution

4.3.6. Simulated Small and Large intestinal juices

**4.4. Antibiotic**

4.4.1. Norfloxacin (Local medical shop)

**4.5. Vegetables and cereals**

4.5.1. Garlic (A*llium sativum*) (Local Market)

4.5.2. Jowar (Great Millet) (*Sorghum vulgare*) (Andheri market, Mumbai)

4.5.3. Bajra (Pearl millet) (*Pennisetum glaucum*) (Andheri market, Mumbai)

4.5.4. Ragi (Finger Millet) (*Eleusine coracana*) (Andheri market, Mumbai)

**Garlic cloves [1]**

**Jowar, Bajra, Ragi seeds (clockwise)**

### 4.6. Food materials

4.6.1. Jaggery (Local Market)

4.6.2. Home- made sweet (milk-cake)

4.6.3. Milk (Amul Taza) (Local Market)

4.6.4. Coconut water (Local Market)

### 4.7. Microorganisms

The bacterial strains used under the present research study are as follows:

4.7.1. *Lactobacillus casei* (2651 1951 RPK) culture was purchased from National Collection of Industrial Microorganisms, Pune, India.

4.7.2. *Escherichia coli* (2065ATCC 8739) culture was purchased from National Collection of Industrial Microorganisms, Pune, India.

### 4.8. Microbial culture media

#### 4.8.1. Culture Media for *Lactobacillus casei* (2651 1951 RPK)

Modified de-Mann Rogosa Sharp (MMRS) medium:

**Table 4.1: Composition of Modified de-Mann Rogosa Sharp (MMRS) medium**

| Serial No. | Components | Quantity |
|---|---|---|
| 1. | Beef extract | 10g |
| 2. | Yeast extract | 5g |
| 3. | Peptone | 10g |
| 4. | Sodium acetate | 5g |
| 5. | di-potassium hydrogen phosphate | 2g |
| 6. | Tri-ammonium citrate | 2g |
| 7. | Magnesium sulphate | 0.05g |
| 8. | Manganese sulphate | 0.05g |
| 9. | Lactose | 20g |
| 10. | Distilled water | 1 L |

### 4.8.2. Culture Media for *Escherichia coli* (2065ATCC 8739)

Luria Bertani (LB) medium:

**Table 4.2: Composition of Luria Bertani medium**

| Serial No. | Components | Quantity |
|---|---|---|
| 1. | Tryptone | 1g |
| 2. | Yeast extract | 0.5g |
| 3. | Sodium chloride | 1g |
| 4. | Distilled water | 100 mL |

**Table 4.3: Composition of Phosphate buffer**

| Serial No. | Components | Quantity |
|---|---|---|
| 1. | Potassium di-hydrogen phosphate | 1.361 g |
| 2. | Distilled water | 100 mL |

The pH was adjusted using 35 g/L solution of disodium hydrogen phosphate.

**Table 4.4: Composition of Peptone water**

| Serial No. | Components | Quantity |
|---|---|---|
| 1. | Peptone digest | 10 g |
| 2. | Sodium chloride | 100 mL |
| 3. | Distilled water | 1L |

**Table 4.5: Composition of Simulated gastric juice**

| Serial No. | Components | Quantity |
|---|---|---|
| 1. | Sodium Chloride | 0.9g |
| 2. | Pepsin | 0.3g |
| 3. | Distilled water | 100mL |

The pH was adjusted to 2.0 with HCl.

**Table 4.6: Composition of Simulated saliva solution** [2]

| Serial No. | Components | Quantity %(w/v) |
|---|---|---|
| 1. | Sodium Chloride | 0.62 |
| 2. | Potassium Chloride | 0.22 |
| 3. | Calcium Chloride | 0.02 |
| 4. | $NaHCO_3$ | 0.12 |

**Table 4.7: Composition of Green Coconut Water** [3]

| Serial No. | Components | Quantity %(w/v) |
|---|---|---|
| 1. | Total solids | 4.83 |
| 2. | Citric acid percent | 1.35 |
| 3. | Total reducing sugar | 3.94 |

The normal pH of green coconut water is 4.68.

### 4.9. Preparation of lactose rich green coconut water

Lactose was added to fresh green coconut water to maintain a concentration of 20g/L and the pH was adjusted to pH 7 by the addition of phosphate buffer solution of pH 8.

### 4.10. Simulated Small and large intestinal juices

As reported in the literature, the gut juice can be simulated using phosphate buffer saline (PBS) (NaCl: 8 g/L; $Na_2HPO_4$: 1.44 g/L and $KH_2PO_4$: 0.24 g/L) with pH adjusted in the range of 6.5 to 8 [4]. Under the present investigation PBS having pH of 8.0 and 7.0 was used to simulate small and large intestinal gut juices respectively. Since bile and pancreatin have no role in the growth of *Lactobacillus casei* (2651 1951 RPK), these enzymes were not used.

## 4.11. Equipments

The following equipments were used under the present research study

4.11.1. BOD-Shaker incubator (G.B. Enterprises, Kolkata, India)

4.11.2. Hot Air Oven (G.B. Enterprises, Kolkata, India)

4.11.3. Autoclave (G.B. Enterprises, Kolkata, India)

4.11.4. Laminar Air Flow (G.B. Enterprises, Kolkata, India)

4.11.5. Heating Mantle (G.B. Enterprises, Kolkata, India)

4.11.6. Refrigerator (Whirlpool; Corona Deluxe, India)

4.11.7. Freezer -20ºC (Blue Star, India)

4.11.8. Weighing machine (Sartorius BS124S, Germany)

4.11.9. Magnetic Stirrer (Remi; 5MLH, India)

4.11.10. Freeze dryer (Lyophilization Systems, Inc. India)

4.11.11. Cooling centrifuge (Plasto Crafts, Model-Superspin R-V/$F_M$)

4.11.12. Constant temperature bath (S.C Dey & Co., India)

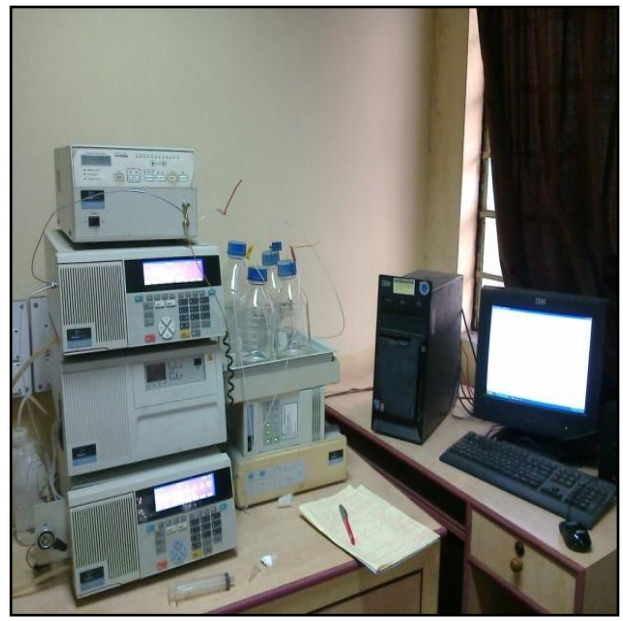
**Optical Microscope**

**FTIR**

**Laminar airflow chamber**

**Cooling centrifuge**

**Rotary evaporator**



**HPLC**

**Constant Temperature bath**

## 4.12. Analytical Instruments

The following analytical instruments were used in the present research work

4.12.1. Fourier Transform Infrared Spectrometer (FT-IR) (Schimadzu Company; VERTEX 70)
4.12.2. UV-visible spectro-photometer (Varian, Cary 50 Bio USA)
4.12.3. Field Emission Scanning Electron Microscope (FESEM) (JSM 6700A, Jeol Ltd., Japan)
4.12.4. Rotary evaporator (Buchi Labor Technik AG R3, Switzerland)
4.12.5. Optical microscope (OPTIKA, Italy)

## 4.13. TLC (Thin layer Liquid Chromatography)

The standard inulin solutions of different concentrations (5g/L, 10g/L, and 20g/L) were hydrolyzed using 5% oxalic acid and heated in boiling water bath for 30 minutes [5]. 1 mL of each inulin solution was hydrolyzed with 4 ml oxalic acid. Filtrate obtained from suspensions of aqueous test solutions of raw natural prebiotics at concentration of 20g/L each were hydrolyzed using the same protocol. Approximately 10 µl of each hydrolyzed sample was applied to spot on the aluminium sheet (TLC Silica gel G 60 Aluminium sheets) by a micropipette (make:

Biohitproline 0.5 to 10 µl) and was developed for ~ 3 h in the n-butanol: acetic acid: methanol solvent (4:2:2) mixture in a closed chamber [6]. The mobile phase (solvent) was at the bottom of the tank. The mobile phase gradually moved upward carrying the spotted samples. Once the solvent front moved ~ 2/3 of the sheet, the sheet was removed; the solvent front was marked immediately and dried with a portable dryer. The sheet was sprayed with spraying reagent which had been prepared by mixing 0.2% orcinol in sulphuric acid and was incubated in hot air oven for 15 minutes at 70˚C. Dark brown spots appeared for different samples. $R_f$ values (Relative Fronts of the solute and the solvent) were calculated using the following equation.

$R_f$ = distance moved by the solute/ distance moved by the solvent (4.1)

From the Thin Layer Chromatogram of each sample, the spot, obtained at the same position as inulin, was scraped out and collected in a sample vial containing 10 mL distilled water. OD values of supernatants were determined with the spectrophotometer at 540 nm, to quantify the concentration of inulin. Standard curve generated using OD values of TLC spots corresponding to pure inulin solutions of different concentrations have been used to determine inulin concentration in the natural prebiotic sources.

### 4.14. Extraction of Inulin

The prebiotic native sources, namely, garlic, pearl, finger and great millets were washed properly with distilled water. In case of garlic, peeling of outer skin was done manually and the core portion was taken for the preparation of paste. 100 g natural samples were taken and with addition of 150 mL distilled water was blended with Mixer-Grinder. The protocol for the extraction of inulin from chicory, as suggested by Mavumengwana, was followed with some modifications [7]. Same protocol was also used by Samanta (Koruri) [8]. The flow sheet showing different operations in the protocol of inulin extraction is shown in Figure 4.1.

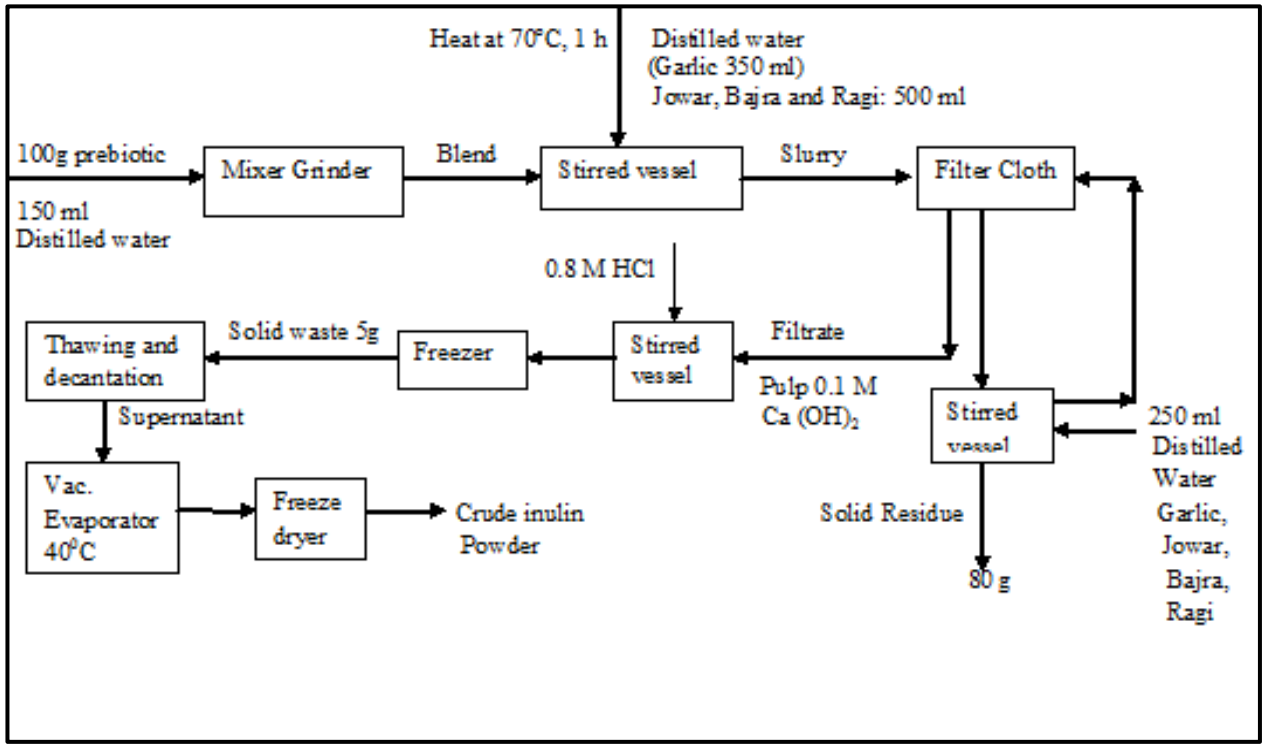



**Figure 4.1: Process of inulin extraction from natural prebiotic sources**

According to the protocol, suspensions of the aqueous pastes of different samples were prepared. The volume of distilled water used for the preparation of suspension was 750 mL for all samples. The suspension was heated up to 70°C and was subsequently screened. Heating up to 70°C had been recommended by previous workers, to de-activate the inulinase, which might have otherwise led to the conversion of inulin to fructose [7, 9]. The solid cake from the filter cloth was re-suspended several times in distilled water and was recycled to the filtration unit. Finally the filtrate was treated with calcium hydroxide to raise the pH up to 8.0. The pH was readjusted to 7.0 by using 0.8 M HCl. After the adjustment of pH, filtrate was frozen at -22°C for 3h and was thawed. The supernatant was evaporated under vacuum to remove water and to obtain crude inulin powder and all the inulin samples were characterized.

### 4.15. Optimization of the parameters for most desirable millet inulin yield

A statistical model correlating the response variable, namely, yield of inulin defined by g inulin extracted/g millet, was developed using response surface methodology (RSM). For this purpose, a set of 20 experiments was conducted at different combinations of independent variables, pre-

set by central composite design. Independent variables, namely, temperature, concentration of HCl and heating period were varied in the range of 24-70˚C, 0.5-2 M and 20 - 180 min respectively. The development of model equation using RSM and the optimization of inulin yield were carried out using Design-Expert 8.1 (Stat-Ease, Inc., Minneapolis, USA) software.

### 4.16. Characterization of Extracted Inulin Powder

The presence of inulin in different natural prebiotic samples (selected for this study) was qualitatively and quantitatively determined using Thin layer Liquid Chromatography (TLC), DNS method and Fourier transform infrared spectroscopy (FTIR).

#### 4.16.1. TLC (Thin layer Liquid Chromatography)

The same protocol of TLC, performed for the qualitative and quantitative determination of inulin in raw natural prebiotics, was followed for the inulin extract powder derived from them.

#### 4.16.2. DNS (3-5 Di-nitro salicylic acid) method

1g of natural inulin powder of each prebiotic sample was dissolved in 5 ml distilled water. 5 ml 4:1 acetonitrile: water solution mixture was added to the solution. The resulting mixture was allowed to precipitate. The clear solvent layer was decanted and the precipitate was washed repeatedly with distilled water and the final suspension of 4 mL was divided into two parts. One part was taken directly for fructose determination using DNS (3, 5- Di-nitro salicylic Acid) method and another part was hydrolyzed with 5% oxalic acid following the same protocol used in case of TLC analysis and was subsequently analyzed using DNS method to determine the total content of reducing sugar[10]. The inherent fructose content of the extract powder was evaluated by the DNS analysis of the first part. On the other hand the DNS analysis of the second part would determine the summation of the fructose/glucose produced through hydrolysis of inulin and the inherent fructose content. The quantity of fructose produced from hydrolysis of inulin was determined by subtracting the inherent fructose content from the total value of the content of reducing sugar, obtained through the DNS analysis of second part.The concentration of pure inulin in the extract inulin powder was obtained by the simple molar ratio relationship given by Mavumengwana [7].

$$[I] = 162 \times [F] / 180 \quad (4.2)$$

Where [I] = concentration of inulin, g/L and [F] = concentration of fructose obtained from inulin hydrolysis, g/L.

### 4.16.3. FTIR (Fourier Transformed Infrared Spectroscopy) Analysis

The results of all were compared with that of the pure food grade inulin.

## 4.17. Determination of Degree of Polymerization

The degree of polymerization, or DP, is usually defined as the average number of base units per molecule if the molecules are composed of regularly repeating units, or as the average number of monomeric units (mers) per molecule [11]. The extracted inulin samples were hydrolyzed. DNS method was followed to determine the glucose concentration in the hydrolysate. On the other hand, inulin content of the extract was determined using the TLC method. The DP has been calculated using the following procedure:

According to Irene A. Rubel et al, 2014 and Simonovska, 2000, the degree of polymerization, n, may be correlated to contents of glucose and fructose in inulin as follows [12, 13]:

$$n = \frac{F_i}{G_i} + 1 \quad (4.3)$$

if the general formula of Inulin be $GF_{n-1}$

Where,

G= glucose, F= fructose,

$F_i$= Content of fructose originating from inulin sample

$G_i$= Content of glucose originating from inulin sample

Again, according to Stefano Curcio et al, 2014 [14]

$$i = 0.9 \times F_i + G_i$$

(4.4)

Therefore, by dividing both sides of Equation (4.4) by $G_i$,

$$\frac{i}{G_i} = 0.9 * \frac{F_i}{G_i} + 1 \qquad (4.5)$$

Therefore, by using Equation (4.3) and (4.5)

$$i = 0.1 * G_i + 0.9 * n * G_i$$

(4.6)

Thus, by determining the content of inulin, i and that of glucose, obtained on hydrolysis, the value of DP may be calculated from Equation (4.6).

### 4.18. Pre-adaptation of *Lactobacillus casei* (2651 1951 RPK) to different carbohydrate sources

The probiotic bacteria, namely, *Lactobacillus casei* was pre-adapted to different carbohydrate sources, namely, lactose, commercial inulin and three millet inulins. For lactose, sterile modified de-Man Rogosa Sharpe (MMRS) medium was prepared by using 50 g/L lactose as the carbohydrate source. Four other stock cultures were also prepared using MMRS media in which the food grade commercial inulin and three millet inulins at a concentration of 20 g/L were separately used as the carbohydrate source. For pre-adaptation, each medium was inoculated with 1% (v/v) of *Lactobacillus casei* in a 50 mL flask and was incubated for 18 h at 37$^{o}$C based on sufficient growth ($5x10^{10}$cfu/ml). Since the purity of each inulin extract is dependent on the natural source, different quantity of inulin extracts were required to prepare the inulin solution of equal strength.

### 4.19. Pre-adaptation of *Escherichia coli* (2065ATCC 8739)

*Escherichia coli* (2065ATCC 8739) grown in Luria Bertani (LB) medium was pre-adapted to sterile MRS and modified de-Man Rogosa Sharpe (MMRS) media containing 20g/L glucose and

20 g/L inulin as the carbohydrate sources respectively. For pre-adaptation, each medium was inoculated with 1% (v/v) of *Escherichia coli* (2065ATCC 8739) in 50 mL flask and was incubated for 18 h at 37$^{o}$C until sufficient growth ($5x10^{10}$cfu/ml) was detected.

### 4.20. Scanning Electron Microscopy (SEM) of *Lactobacillus casei* (2651 1951 RPK)

SEM analysis of 24 h batch culture of *Lactobacillus casei* using two types of MMRS media, one containing 20 g/L lactose and the other containing 20 g/L lactose and 20 g/L commercial inulin, were used to observe the morphological changes of bacterial cell.

### 4.21. Optimization of specific growth rate of *Lactobacillus casei* (2651 1951 RPK)

The multivariate functionality of $\mu_{ave}$ (average specific growth rate) on two independent variables, namely concentrations of lactose and inulin was determined by designing the experiment using Central Composite Design (CCD) technique. 13 batch mode experiments were conducted in Erlenmeyer flasks to determine the specific growth rate of *Lactobacillus casei* by varying the initial concentrations of lactose and inulin in the ranges of 10-30 g/L and 0.164-0.624 g/L respectively. Response Surface Methodology has been used to develop the model equation to correlate the specific growth rate with the above mentioned independent variables. Design-Expert 8.1 (Stat-Ease, Inc., Minneapolis, USA) software has been used for the purpose of the development of statistical model as well as for the identification of the optimum values of the independent variables corresponding to maximum specific cell growth rate.

### 4.22. Batch studies for the determination of growth kinetic of *Lactobacillus casei* (2651 1951 RPK) on lactose, inulin and mixture of lactose and inulin

Batch growth of *Lactobacillus casei* was investigated using lactose as the carbon source and the initial concentration was varied in the range of 10 - 30 g/L. Similarly, another set of experiments was conducted using inulin as the sole carbon source. The initial concentration of inulin was maintained in the range of 0.035-0.624 g/L. A separate set of batch experiments was performed using a mixture of lactose and inulin as the carbon source. At each value of initial concentration of lactose of 10, 20, 30 and 40 g/L, the initial concentration of inulin was varied in the range of 0.164-0.624 g/L. Separate set of experiments was conducted for commercial inulin and the most

effective millet inulin. For each experiment, incubation was done for 24 h at pH and temperature of 7 and 37˚C respectively. The cultures were withdrawn at 1 h interval and the concentration of biomass was determined using UV-VIS spectrophotometer. Each experiment was repeated thrice.

### 4.23. Determination of the effectiveness of millet inulins for the growth of *Lactobacillus casei* (2651 1951 RPK)

Batch studies were conducted in three 250 mL conical flasks using 100 mL modified MRS medium containing lactose and different millet inulins. The concentration of millet inulin and lactose in the MMRS medium in each conical flask were maintained at the optimum levels, as determined under section 6.B.1. The pearl millet inulin, finger millet inulin and great millet inulin were respectively used in the first, second and third flasks. Each flask was seeded with 10% (v/v) stock culture of *Lactobacillus casei*. The flasks were kept in an incubator for 24h at 37°C. The cell concentration in each flask was measured using spectrophotometric method determining the optical density of the growth medium at 600 nm. The cell concentrations of growth medium in three flasks at 24h were compared to identify the most effective millet inulin for the growth of *Lactobacillus casei*.

### 4.24. Antibiotic Sensitivity

The sensitivity of the selected microorganisms, *Lactobacillus casei* towards a common antibiotic namely, norfloxacin was tested using well diffusion method. MRS agar plate containing 20 g/L glucose and MMRS agar plate containing optimum concentrations of glucose and inulin corresponding to maximum specific growth rate and the zone of inhibition was measured in each plate. In each plate 0.1 g norfloxacin was applied in the well. The *Lactobacillus casei* cell culture was spread with a glass spreader. The plates were kept in an incubator at 37ºC for 24 h.

### 4.25. Prebiotic activity score

Both the bacterial strains namely, probiotic, *Lactobacillus casei* and *Escherichia coli* were grown in MRS and MMRS media containing 20 g/L glucose and 20 g/L inulin as carbohydrate source

respectively. The inocula (0.1%) of probiotic bacteria and *Escherichia coli* were obtained from the pre-adapted stock cultures of the respective strains. The growth cultures were incubated for 24 h at 37°C. The cell concentrations at the initial condition and after 24 h were determined for each growth medium using spectro-photometric method. Separate sets of experiments were conducted using commercial inulin and the most effective millet inulin as the carbohydrate source in the MMRS medium. The prebiotic activity score for both commercial and millet inulin were determined by using the Equation (5.2) (Theoretical analysis section) [15].

### 4.26. Co-entrapment and entrapment of *Lactobacillus casei* (2651 1951 RPK) with and without inulin

The protocols described by Chan et al, 2011 and Yoo et al, 1996 was principally followed [16, 17] for the entrapment and co-entrapment. *Lactobacillus casei* cultures, pre-adapted to lactose and commercial inulin were respectively spiked up to 20 g/L lactose and 0.3225 g/L commercial inulin and were individually mixed with 2% sterile sodium alginate solution in the volumetric ratio of 1:4. Each mixture was then added drop wise into a 1% $CaCl_2$ solution at room temperature and stirred continuously. The beads containing entrapped *Lactobacillus casei* and co-entrapped *Lactobacillus casei* and inulin were allowed to harden and were washed with saline water and stored at 4 $^{o}$C. The size of the beads was determined using optical microscope.

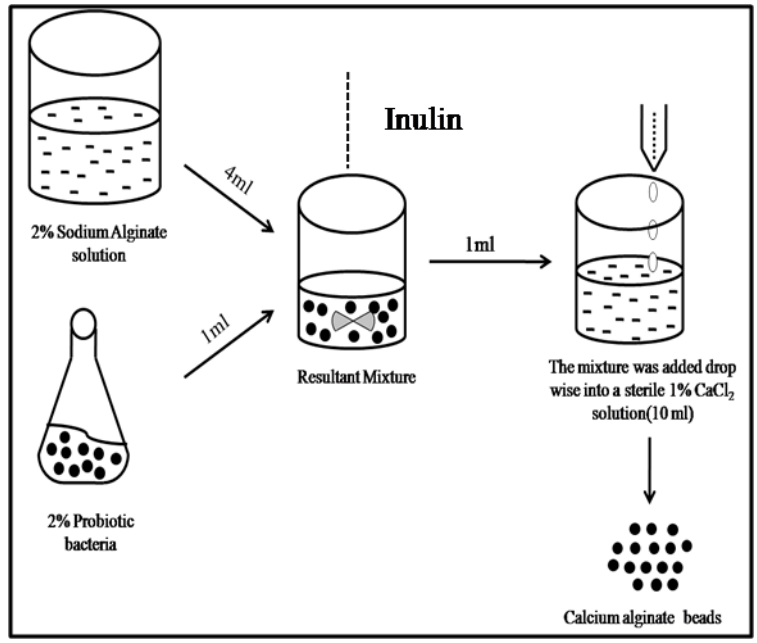



**Figure 4.2: Schematic representation of co-entrapment and entrapment of *Lactobacillus casei* (2651 1951 RPK) with and without inulin**

### 4.27. Co-encapsulation and encapsulation of *Lactobacillus casei* (2651 1951 RPK) with and without inulin through external gelation

For the co-encapsulation and encapsulation of *Lactobacillus casei* with and without inulin, via external gelation method, the protocol suggested by Song et al, 2013 have been principally followed [18]. *Lactobacillus casei* cultures, pre-adapted to lactose and commercial inulin were spiked with the respective carbohydrates up to the concentration levels as used for pre-adaptation, i.e., 20 g/L and 0.3225g/L for lactose and inulin respectively. Each culture was mixed with sodium alginate solution in the volumetric ratio of 1:4. 1 mL of the mixture was stirred with 5 mL of vegetable oil (rice bran oil). Tween 80 (emulsifier) and SDS were added to the resulting solution to maintain their concentrations at 0.2 % and 0.25 % respectively. This mixture was then stirred at 200 rpm for 30 minutes. $CaCl_2$ was added quickly but gently down the side of a beaker, in which the mixture was taken. The formed micro-beads were kept undisturbed for 30 minutes. They were then filtered with muslin cloth. Finally, the microspheres were washed with 0.9% saline water containing 0.05% glycerol. The washed micro-beads were stored at 4˚C. The size of the microcapsules was determined through microscopic analysis.

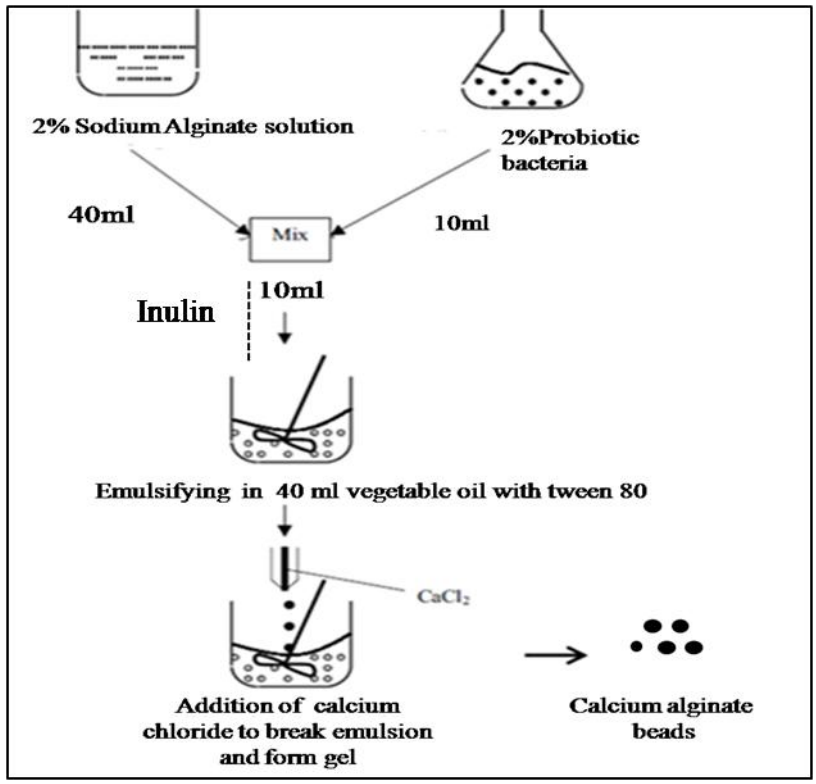
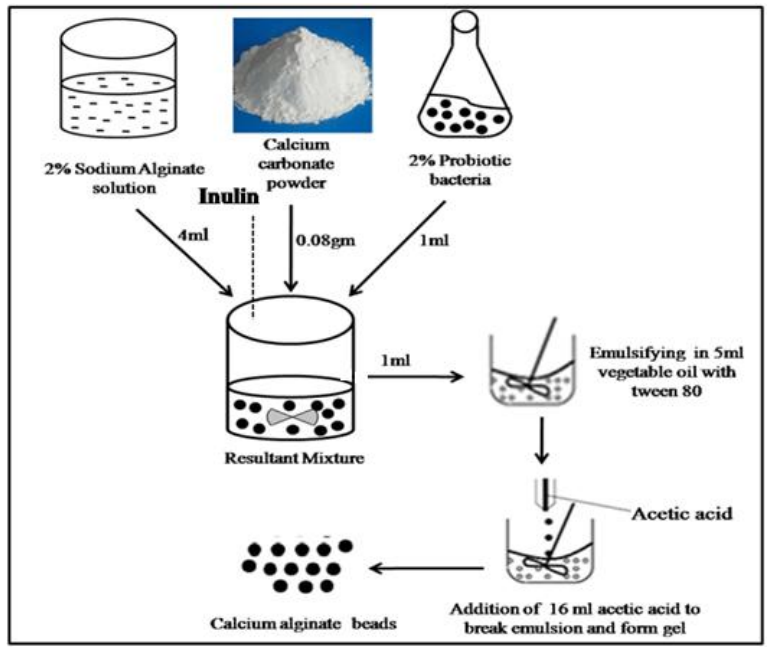



**Figure 4.3: Schematic representation of co-encapsulation and encapsulation of *Lactobacillus casei* (2651 1951 RPK) with and without inulin through external gelation**

### 4.28. Co-encapsulation and encapsulation of *Lactobacillus casei* (2651 1951 RPK) with and without inulin through internal gelation

The protocol suggested by Song et al, 2013 and Ribeiro et al, 2005 was principally followed with some modifications for the encapsulation of cells through internal gelation method [17, 18]. *Lactobacillus casei* cultures pre-adapted to lactose, commercial inulin and the millet inulin, most effective for the growth of the probiotic microorganism, were at first spiked with the respective carbohydrate sources as described in case of entrapment and external gelation i.e., up to 20 g/L and 0.3225 g/L for lactose and inulin samples respectively. Each culture was then individually mixed with 2 % sodium alginate solution in the volumetric ratio of 1:4. $CaCO_3$ powder and Tween-80 were added to each mixture up to a concentration level of 0.8 g/L and 0.5 % (v/v). This mixture was then combined with vegetable oil (rice bran oil) in the volumetric ratio of 1:5 and stirred at 300 rpm for 30 minutes. The temperature of the mixture was maintained at 25 $^{o}$C by circulating water from a constant temperature bath and acetic acid was next added drop wise

to the solution to release the $Ca^{2+}$ from the insoluble $CaCO_3$ powder. Microcapsules were formed and were filtered with muslin cloth and washed with 1% (v/v) aqueous tween 80 solution and distilled water. The microcapsules were then stored at 4 $^{o}$C. The size of the microcapsules was determined through microscopic analysis.



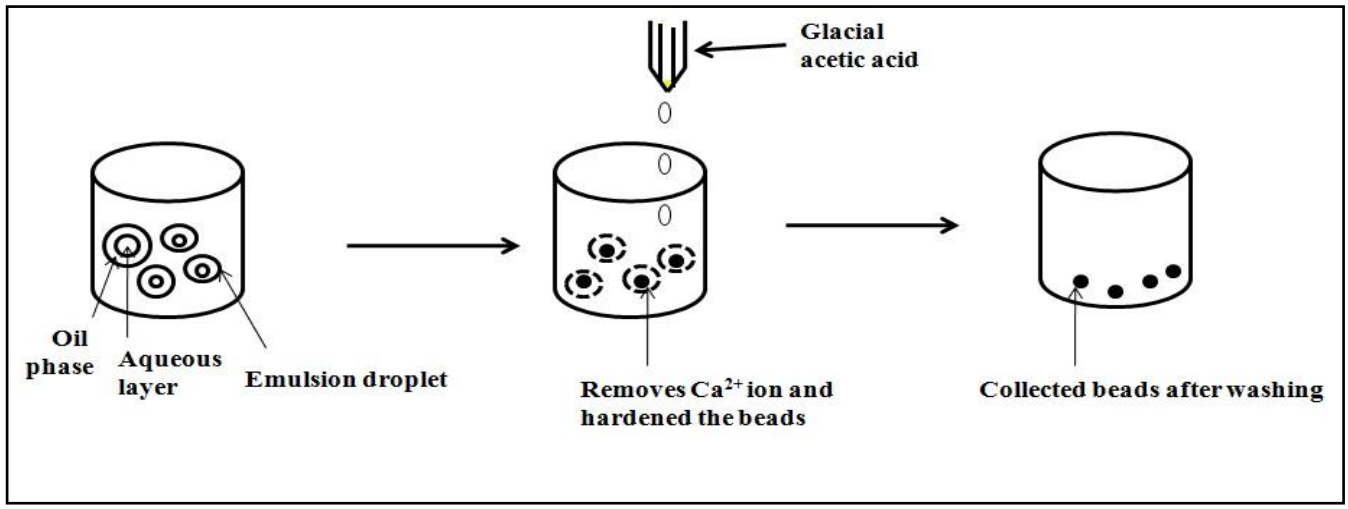




**Figure 4.4: Schematic representation of co-encapsulation and encapsulation of *Lactobacillus casei* (2651 1951 RPK) with and without inulin through internal gelation**

### 4.29. Size Analysis of Beads and Microcapsules

Samples of 10 beads and microcapsules were chosen randomly from the respective total population and the mean size was determined using the optical microscope. The mean of three samples chosen from each population of microcapsules and beads was determined along with the standard deviation.

### 4.30. Determination of Entrapment and Encapsulation efficiencies ($E_e$)

Microcapsules, weighing 0.1 g, were put in phosphate buffer (pH-7).These were rotated at 15000 rpm using a high speed homogenizer for 60 seconds.1mL of the homogenate was then 10 fold diluted with sterile peptone water and 0.1 mL of the final solution was spread plated on MMRS agar. The plates were incubated at 37˚C for 24 hours. The numbers of viable bacteria, released in the homogenate, were determined by counting the bacterial colonies. The initial bacterial count in the culture used for entrapment or encapsulation was also determined by spread plate method followed by colony counting. The entrapment and encapsulation efficiency were determined using the original cell counts and the viable cell counts in the homogenate. The entrapment and encapsulation efficiencies were calculated by using Equation (5.8) of Theoretical analysis section.

### 4.31. Optimization of the efficiency of Microencapsulation through Internal Gelation

A statistical model correlating the response variable, encapsulation efficiency ($E_e$) of internal gelation process with the independent factors, namely, alginate concentration (1–2%), emulsifier concentration (Tween 80) (0.5-1.5%) and post cooling temperature (15-25°C) was developed using response surface methodology (RSM). For this purpose, a set of 20 experiments was conducted at different combinations of independent variables, pre-set by central composite design. The development of model equation using RSM and the optimization of inulin yield were carried out using Design Expert software 7.0.

### 4.32. Antimicrobial activity

The antimicrobial activity of *Lactobacillus casei* -inulin entrapped or encapsulated in calcium alginate bead or microcapsules was determined against *Escherichia coli* (2065ATCC 8739).

Different concentrations of *Lactobacillus casei* -inulin conjugate beads or microcapsules, were applied to the well of MRS agar plate (containing 20 g/L glucose). The plates were kept in an incubator at 37 ºC for 24 h. The antimicrobial activity was determined by measuring zone of inhibition against selected pathogen, *Escherichia coli*.

### 4.33. Determination of "Release time" of *Lactobacillus casei* (2651 1951 RPK) from the beads or microcapsules suspended in lactose rich green coconut water and MMRS medium

3g beads and capsules, synthesized via the aforementioned methods, were suspended in 30 mL of MMRS and lactose rich (20g/L) green coconut water in 50 mL conical flasks. The mixture was incubated under anaerobic condition at 37$^{o}$C for 60h. The content of the conical flask was observed periodically so as to obtain the times at which the capsules are burst and there by the turbidity increases abruptly due to the release of the entrapped cells. Each experiment was repeated thrice.

### 4.34. Determination of "Release time" of *Lactobacillus casei* (2651 1951 RPK) from prebiotic-probiotic microcapsules (Internal gelation) in simulated saliva and GI juices

Microcapsules containing a mixture of *Lactobacillus casei* and commercial inulin were used for this assay. For obtaining the "release time" of probiotic cells from the capsules in the simulated saliva solution, "gastric juice", and small and large intestinal juices, the microcapsules weighing 3 g were suspended in 30 mL of each medium in a 50 mL conical flask and the mixture was incubated under anaerobic condition at 37 $^{o}$C for 60 h. The content of the conical flask was observed periodically so as to obtain the times at which the capsules burst and thereby the turbidity increases abruptly due to the release of the entrapped cells. Each experiment was repeated thrice.

### 4.35. Operation of Three stage bioreactors simulating the human GI tract

Three continuous stirred tank bioreactors representing the "stomach", the "small intestine" and the "large intestine" of volumes 300 mL, 1 L and 5 L respectively were connected in series. As per literature data the passage time of microcapsule in

the stomach, small intestine and large intestine are 80.5 mins, 3.2± 1.6 h and 6-32 h [2]. Initially the reactors representing stomach, large and small intestine were filled up respectively with gastric, small intestinal and large intestinal juices and were sparged with nitrogen to maintain anaerobic condition. The "stomach" bioreactor was fed with 30g probiotic-prebiotic (*Lactobacillus casei* (2651 1951 RPK)-inulin) microcapsules, already soaked in saliva solution for 30 minutes, so that the concentration of microcapsules in gastric solution was 0.1g/L [20].The stomach was fed and emptied at a constant volumetric flow rate of PBS solution of 3.72 mL/min using peristaltic pumps and the effluent was fed to the next bioreactor, i.e., the "small intestine". The effluent from this reactor was fed to the bioreactor representing "large intestine" from which the outlet was withdrawn at the same volumetric flow rate. The reservoir for PBS was also sparged with nitrogen to maintain anaerobic condition. The pH of each reactor (pH=2 for "Stomach"; pH=8 for "small intestine" and pH=7 for "Large intestine") was adjusted continuously by 0.1 M HCl and 0.1 M NaOH. The values of hydraulic retention time of liquid in the reactors, namely stomach, small intestine and large intestine are 80.6 min, 4.48 h and 22.4 h respectively. Therefore, the total retention time in the simulated GI system was 1694min. The effluent from the large intestine was collected in a reservoir. Fluid samples were withdrawn from each reactor at 50 min interval and the release of probiotics was monitored by measuring the optical density using a spectro-photometer at 600nm. The concentration of microcapsules in each reactor was measured after 2000 minutes, which was much greater than the total retention time in the simulated GI system.

### 4.36. Application of beads and Microcapsules in Indian food

#### 4.36.1. Preparation of Indian cottage cheese for milk cakes

Indian cottage cheese (chhena) was prepared from 1L milk. Firstly the milk was allowed to boil, followed by addition of lemon juice into it stirring continuously. When the milk curdled well, cottage cheese was strained using muslin cloth and washed with water. After removing the excess water, 200 g chhena was obtained. The chhena was added to a pan and was mixed well with sugar and cardamom powder on a low flame until a mixture of thick consistency was obtained. The mixture was then cooled and kneaded properly and was autoclaved at 120 $^{o}$C.

### 4.36.2. Preparation of milk cakes with and without fortification with probiotic or probiotic--prebiotic microcapsules

The sterile 200 g chhena, as described above, was divided into eight parts—first part was mixed with probiotic Ca-alginate beads spiked by lactose; second part was mixed with Ca-alginate beads spiked by commercial inulin; third part was mixed with probiotic microcapsules (external gelation) spiked by lactose; fourth part was mixed with probiotic microcapsules (external gelation) spiked by commercial inulin; fifth part was mixed with probiotic microcapsules (internal gelation) spiked by lactose; sixth part was mixed with probiotic microcapsules (internal gelation) spiked by commercial inulin; seventh part was mixed with probiotic microcapsules spiked by millet inulin, most effective for the growth of the probiotic microorganism and the eighth part was kept unfortified. From each part, one milk cake weighing 25 g was prepared using sterile molds. All operations for the preparation of milk cake were conducted in an UV-sterilized hood. During the fortification 10 % (w/w) beads or microcapsules were used. The milk cakes are shown in Figure.4.5. All milk cakes were wrapped with sterile aluminium foils (Figure.4.5) before preservation.


**Figure 4.5: Indian sweet (milk cakes) samples (a) wrapped by aluminium foil (b) original sample**

### 4.36.3. Preparation of jaggeries with and without fortification with probiotic or probiotic--prebiotic microcapsules

The sterile 40 mL jaggery, was divided into eight parts—first part was mixed with probiotic Ca-alginate beads spiked by lactose; second part was mixed with Ca-alginate beads spiked by commercial inulin; third part was mixed with probiotic microcapsules (external gelation) spiked by lactose; fourth part was mixed with probiotic microcapsules (external gelation) spiked by commercial inulin; fifth part was mixed with probiotic microcapsules (internal gelation) spiked by lactose; sixth part was mixed with probiotic microcapsules (internal gelation) spiked by commercial inulin; seventh part was mixed with probiotic microcapsules spiked by millet inulin, most effective for the growth of the probiotic microorganism and the eighth part was kept unfortified. From each part, one jaggery weighing 5 mL was prepared using sterile molds. All operations for the preparation of jaggery were conducted in an UV-sterilized hood. During the fortification 10 % (w/w) beads or microcapsules were used. All jaggeries were distributed in sterile vials (Figure.4.6) before preservation.

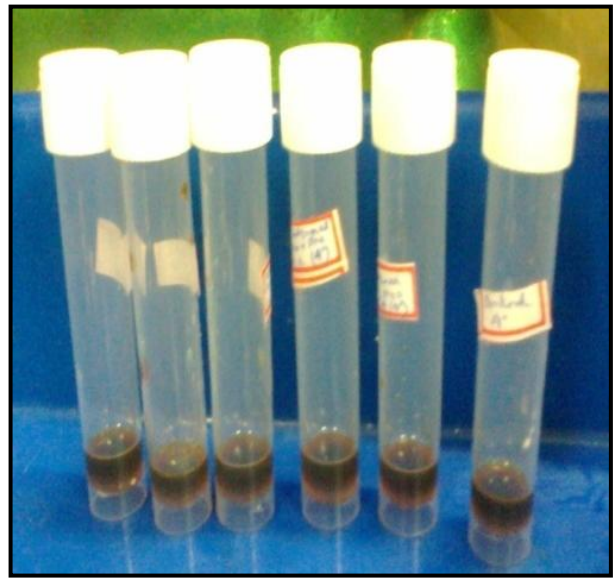


**Figure 4.6: Indian jaggeries distributed in sterile vials before preservation**

### 4.36.4. Viability assay of the probiotic cells in food products

The fortified Indian milk cakes were stored at 4 $^{o}$C and -20 $^{o}$C over a period of 4 weeks. Unfortified samples were used as controls. Viability assay was performed every one week interval as per the following protocol: in an UV-sterilized hood, 5mL of phosphate buffer was first mixed with 1 g of food samples and then 1ml from this mixture was serially diluted with peptone water by 10 fold for six times. 0.1 ml of the diluted mixture was plated on MRS agar using spread plate technique. The agar plates were incubated under anaerobic condition for 24 h at 37 $^{o}$C and the colonies on each plate were counted. According to this technique the concentration of cells per milliliter of the original phosphate buffer solution of the food sample $10^7$ times the count obtained from the spread plate method [21]. Since 1 g of food sample was originally present in 5mL phosphate buffer, the concentration of cells in the food sample is $5*10^7$ times that obtained from spread plate method.

All experiments were carried out in triplicate and the averages of three replicate experimental results have been reported.

## REFERENCES


1. http://garlicmatters.com/how-to-grow-garlic/garlic-plant-structure/
2. Cook, M. T., Tzortzis, G., Charalampopoulos, D., and Khutoryanskiy, V. V. (2011) "Production and Evaluation of Dry Alginate-Chitosan Microcapsules as an Enteric Delivery Vehicle for Probiotic Bacteria". Biomacromolecules, 12(7), 2834-2840.
3. Rojas, M.L., Trevilin, J.H., Funcia, E.d.S., Gut, J.A.W., Augusto, P.E.D. (2017) "Using ultrasound technology for the inactivation and thermal sensitization of peroxidase in green coconut water". Ultrasonics Sonochemistry, 36, 173–181.
4. Gbassi, G.K. and Vandamme, T. (2012) "Probiotic Encapsulation Technology: From Microencapsulation to Release into the Gut". Pharmaceutics, 4, 149-163, doi:10.3390/pharmaceutics4010149.
5. Teris, A.V.B., Paul, M., Bonnie, M.K., Edith, L., Alphons, G.J., Voragen, A.D.G. (1990) "Bitter sesquiterpene lactones from chicory roots". Journal of Agricutural Food Chemistry, 38, 1035-1038.
6. Druart, N., De Roover, J., Van den Ende, W., Goupil, P., Van Laere, A., Rambour, S. (2001) "Sucrose assimilation during early developmental stages of chicory (*Chicorium intybus L*.) plants". Planta , 212, 436-43.
7. Mavumengwana, V.B. (2004) "Isolation Purification and Characterisation of inulin and fructo-oligosaccharides from *Chicorium intybus* and inulinase from *Aspergillus niger*". Thesis for the degree of M. Sc. Department of Biochemistry, Microbiology and Biotechnology, Rhods University.
8. Samanta (Koruri), S., Banerjee, D., Chowdhury, R., Bhattacharya, P. (2014) "Studies on prebiotic food additive (inulin) in Indian dietary fibre sources garlic (*allium sativum*), wheat (*triticum spp*.),oat (*avena sativa*) and dalia (BULGUR)". International Journal of Pharmacy and Pharmaceutical Sciences, 6 (9).
9. Singh, M.K., Sharma, S.L. (2008) "Quantitative estimation of some metabolites and enzymes in insect induced leaf galls of *Mangifera indica*". Asian Journal Experimental Sciences, 22(3), 343-346.

10. Chapin, K.C., Lauderdale, T.L. (2003) "Reagents, Stains and Media: Bacteriology". In Manual of Clinical Microbiology, 8th edition, American Society for Microbiology: Washington, DC, USA, pp. 15–35.
11. Wiley, J. and Sons, Inc. (2011) "Degree of Polymerization". Encyclopedia Of Polymeric Sciences and Technology, DOI: 10.1002/0471440264.pst444 Copyright © 2002.
12. Irene, A., Rubel Ethel, E. P., Diego, B. G., Guillermo, D. M. (2014) "In vitro prebiotic activity of inulin-rich carbohydrates extracted from Jerusalem artichoke (*Helianthus tuberosus L.*) tubers at different storage times by *Lactobacillus paracasei*". Food Research International, 62, 59–65.
13. Simonovska, B. (2000) "Determination of Inulin in Foods". Journal of AOAC International, 83, 3.
14. Curcio, S., Ricca, E., Calabrò, V. and Iorio, G.(2014) "Effect of the Degree of Polymerization of Inulin on the Rate of Hydrolysis Using Immobilized Inulinase". Food Technology and Biotechnology, 52 (3).
15. Huebner, J., Wehling, R.L., Hutkins, R.W. (2007) "Functional Activity of commercial probiotics". International Dairy Journal, 17,770-775.
16. Chan, E.S., Wong, S.L., Lee, P.P., Lee, J.S., Ti, T.B., Zhang, Z. (2011) "Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells". Carbohydrate Polymer, 83, 225–232.
17. Yoo, I.K., Seong, G.H., Chang, H.N. and Park, J.K. (1996) "Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production". Enzyme and Microbial Technology, 19,426-433.
18. Song, H., Yua, W., Gaoa, M., Liub, X., Maa, X. (2013) "Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process". Carbohydrate Polymer, 96,181–189.
19. Ribeiro, A.J., Silva, C., Ferreira, D., Veiga, F. (2005) "Chitosan-reinforced alginate microspheres obtained through the emulsification/internal gelation technique". European Journal of Pharmaceutical Sciences, 25, 31–40.
20. Sathyabama, S., Ranjithkumar, M., Brunthadevi, P., Vijayabharathi, R., Brindhapriyadharisini, V. (2014) "Co-encapsulation of probiotics with prebiotics on

alginate matrix and its effect on viability in simulated gastric environment". LWT - Food Science and Technology. 57. 419-425.

21. Doran, P.M. (1995). Bioprocess Engineering principles, Elsevier Science & Technology Publishers.

# Chapter 5

# THEORETICAL ANALYSIS

## 5.1. Prebiotic activity score

As described by Huebner et al. [1], the Prebiotic Activity Score (PAS), a quantitative measure of the effectiveness of any prebiotic material, is defined as follows:

$$PAS = \left( \frac{\log \frac{N_{24}}{N_O}\Big|_{prebiotic}}{\log \frac{N_{24}}{N_0}\Big|_{glucose}} \right)_{probiotic} - \left( \frac{\log \frac{N_{24}}{N_0}\Big|_{prebiotic}}{\log \frac{N_{24}}{N_0}\Big|_{glucose}} \right)_{E.coli} \quad (5.1)$$

Where $N_{24}$= Number of cells of probiotic bacteria or *E. coli* in 24 h growth culture in medium containing either a prebiotic compound or glucose as the carbohydrate source.
$N_0$= Number of cells of probiotic bacteria or *E. coli* in the initial growth culture.

Under the present study, concentrations of cells have been used instead of number of cells. Therefore, the definition of PAS reduces to the following:

$$PAS = \left( \frac{\log \frac{C_{24}}{C_O}\Big|_{prebiotic}}{\log \frac{C_{24}}{C_0}\Big|_{glucose}} \right)_{probiotic} - \left( \frac{\log \frac{C_{24}}{C_0}\Big|_{prebiotic}}{\log \frac{C_{24}}{C_0}\Big|_{glucose}} \right)_{E.coli} \quad (5.2)$$

$C_{24}$ = Concentrations of cells of probiotic bacteria or *E. coli* in 24 h growth culture using in medium containing either a prebiotic compound or glucose as the carbohydrate source.
$C_0$= Concentrations of cells of probiotic bacteria or *E. coli.*

## 5.2. Growth Kinetics of *Lactobacillus casei* (2651 1951 RPK) on single carbon source

In order to regress the cell growth dynamics of *L. casei* on single carbon source, the classical Monod type substrate uninhibited unstructured model has been attempted first. The equation is as follows:

$$\mu = \frac{\mu_m C_s}{K_s + C_s} \quad (5.3)$$

This on rearrangement becomes

$$\frac{1}{\mu} = \frac{K_s}{\mu_m}\frac{1}{C_s} + \frac{1}{\mu_m} \quad (5.3a)$$

where, $\mu$ is specific growth rate defined as follows:

$$\mu = \frac{1}{C_X}\frac{dC_X}{dt} \tag{5.4}$$

$\mu_{m=}$ Maximum specific cell growth rate, $Time^{-1}$

$K_s$= Substrate saturation constant, mass/volume

$C_s$= Concentration of lactose, mass/volume

$C_X$= Concentration of cell, mass/volume

A double reciprocal plot has been made using inverse of initial values of μ and $C_s$ as ordinate and abscissa respectively. If the double reciprocal plot is linear, the values of intrinsic kinetic parameters, $\mu_{max}$ and $K_s$ have been determined from the intercept and the slope.

Haldane type model has been attempted if Monod model is not valid and substrate inhibition is present. Haldane equation is as follows:

$$\mu = \frac{\mu_{max} C_S}{K_S + C_S + \frac{C_S^2}{K_I}} \tag{5.5}$$

Where, $K_I$= Inhibition constant, mass/volume

At the initial concentration of substrate $C_{s_{max}}$ corresponding to the maximum value of μ [2],

$$C_{S_{max}} = (K_S K_I)^{0.5} \tag{5.6}$$

The validity of Haldane model is verified by the analysis of the pattern of the plot of μ against the corresponding initial substrate concentration. A bell-shaped plot is obtained if the Haldane model is valid. After verifying the validity of Haldane model, the values of kinetic constants, $\mu_{max}$ and $K_S$ have been determined by making the double reciprocal plot using the values of inverse of μ and $C_S$ in the uninhibited growth region, i.e, by using the specific growth rates corresponding to initial substrate concentrations below $C_{s_{max}}$. The value of $K_I$ has been determined using the Equation 5.6.

### 5.3. Growth Kinetics of *Lactobacillus casei* (2651 1951 RPK) on two carbon sources (Inulin and Lactose)

Under the present study, the growth kinetics of *L. casei* in a medium containing two carbohydrate sources, namely, inulin and lactose have been determined by attempting summative model [3]. The summative type kinetic equation for the growth of *L. casei* is as follows:

$$\mu = \mu_L + \mu_I \tag{5.7}$$

Where,

$\mu_L$= Specific growth on lactose

$\mu_I$= Specific growth on inulin

The validity of the summative model is verified by the comparison of the experimental and simulated values of specific growth rates at different combinations of values of lactose and inulin.

### 5.4. Encapsulation efficiency

Encapsulation or entrapment efficiency ($E_e$) is calculated using the following correlation [4, 5]:

$$E_e = \frac{N_f}{N_i} \times 100 \tag{5.8}$$

Where, $E_e$ represents the encapsulation/entrapment efficiency, $N_f$ is the number of viable bacteria released in the homogenate of microcapsules/beads and $N_i$ is the number of bacteria used for encapsulation/entrapment.

### 5.5. Optimization using Response Surface Methodology (RSM)

Statistical analysis has been done to correlate different response parameters with the influencing factors using Response surface methodology. Design-Expert 8.1(Stat-Ease, Inc., Minneapolis, USA) software has been used for this purpose. Composite Design of experiments has been done by using central composite design. In order to investigate the effects of individual parameters as well as their interactive effects on the response variable, a general second order polynomial response surface model was selected and is expressed by Equation (5.9):

$$Y_k = b_{k0} + \sum_{i=1} b_{ki} X_i + \sum_{i=1} b_{kii} X_i^{\,2} + \sum_{i=1} \sum_{j=1} b_{kij} X_i X_j \tag{5.9}$$

Where, $Y_k$ is response variable, $b_{k0}$ is a constant intercept; $b_{ki}$, $b_{kii}$, and $b_{kij}$ are the linear, quadratic and interaction regression coefficients, respectively. $X_i$ and $X_j$ represent the coded values of the process variables (factors). The regression Equation (5.9) was considered for simultaneous multiple optimizations in order to maximize $Y_k$ using the numerical optimization program of the same Design Expert software 8.1.

The adequacy of the fit and the significance of the terms in the have been performed through Fischer-F test and Student-t test respectively. The results have been provided in ANOVA tables.

### 5.5.1. Optimization of the parameters for pearl millet inulin yield

The yield of inulin extracted from pearl millet has been maximized with respect to operating variables, namely, temperature, concentration of HCl and heating period using RSM.

The face centered central composite design (FCCD) was used to design the experimental runs to optimize i.e., g inulin extracted/ g natural resource used, the yield of inulin with respect to temperature, HCl concentration and heating period in terms of ±1 levels created by entering the factors. To ascertain reproducibility of the data, each experimental run was conducted in triplicate. Face-centered CCD has been used for the optimization study of extraction process of inulin where extraction temperature was varied from 24-70˚C, HCL concentration was varied from 0.5-2 M and heating period was varied from 20 - 180 min. In a typical experimental run, all the operating variables were pre-set at predetermined design values and the corresponding value of yield of inulin was determined. The experiments were conducted randomly to avoid systematic biasness. A model equation correlating the inulin yield and the above mentioned independent factors was developed using RSM and the extraction conditions was optimized using Design Expert software 8.1.

### 5.5.2. Optimization of growth of *Lactobacillus casei* (2651 1951 RPK) with respect to Concentrations of inulin and lactose

The specific growth rate of *Lactobacillus casei* has been correlated to the concentrations of inulin and lactose and subsequently optimized using response surface methodology.

### 5.5.3. Optimization of Encapsulation efficiency

The efficiency of microencapsulation ($E_e$) of *L. casei* through internal gelation method has been correlated with three important factors, namely, alginate concentration (1–2%), emulsifier concentration (Tween 80) (0.5-1.5%) and post cooling temperature (15-25°C) using RSM technique. The response variable, i.e., $E_e$ has been maximized with respect to the independent variables.

## 5.6. Mathematical Model for the Prediction of "Release time" of microorganisms from Immobilized Prebiotic/probiotic Beads and Microcapsules in simulated large intestinal fluid

The cells are released to the "gut" environment as soon as the volume of biotic phase, i.e., the cells exceed the internal volume of the microcapsules. Thus, it is proposed that "burst release" mechanism [6], characterized by pulse release of the encapsulated active, is followed. This is shown in Figure 5.1. This is justified by the fact that due to large dimensions with respect to the pores of microcapsules, the trans-boundary diffusion of cells driven by concentration gradient is not possible. Thus, the cells are released through the rupture of the capsules as soon as the internal core volume of the capsules is exceeded by the biotic cell volume. The burst release of active molecules has already been reported for hydrogel systems [6].

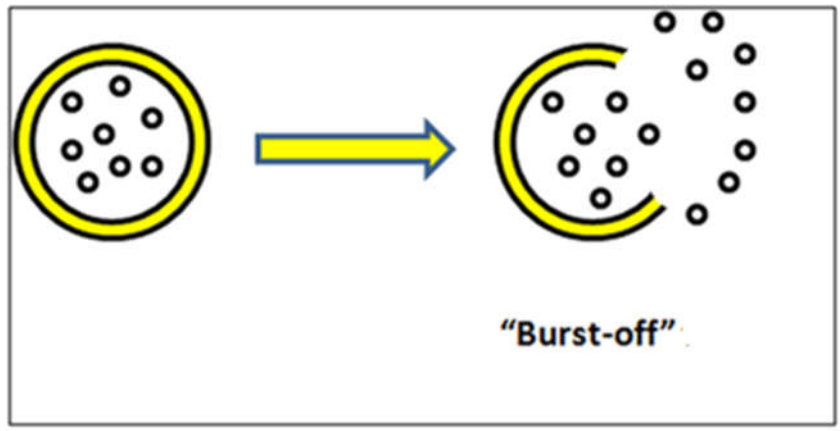



**Figure 5.1: Bursting of microcapsules or beads/Release of Probiotic Cells**

It is assumed that the microcapsules and beads suspended in simulated large intestinal fluid of pH=7 behave like batch type bioreactors. As suggested by Doran, the specific growth rate, μ, in

the batch culture remains almost constant and is equal to $\mu_{max}$ [7]. Thus, for the present case, the specific growth rate of *L. casei* on lactose and inulin may be represented as

$$\mu = \mu_{max\,L} + \mu_{max\,I} \tag{5.10}$$

The decaying rate of the probiotic cells within the microcapsules is negligible.

Mathematically, the number of cells encapsulated within a particular bead can be predicted using the assumption of balanced growth during exponential growth in a batch reactor. Under balanced growth condition, the cell number specific growth rate $v = \frac{1}{N}\frac{dN}{dt}$ is equal to the specific growth rate, $\mu = \frac{1}{C_X}\frac{dC_X}{dt}$, calculated on the basis of cell biomass[8]. Therefore, by expressing the dependence of rate of increase of cell number on the total cell number by Malthusian model [8],

$$\frac{dN}{dt} = \mu N \tag{5.11}$$

The initial condition is

$$\text{At } t = 0,\ N = N_0 \tag{5.12}$$

Integrating, Equation 5.12, we get

$$\int_{N_0}^{N} \frac{dN}{N} = \mu \int_{0}^{t} dt \tag{5.13}$$

Or,

$$N = N_0 e^{\mu t} \tag{5.14}$$

Incorporating the encapsulating efficiency, $E_e$ in the Equation 5.13,

$$N_0 = N_i E_e \tag{5.15}$$

Where, $N_i$ is concentration of cells (number basis) in the stock culture from which the beads or capsules were formed. Hence, Equation 5.14 reduces to the following form:

$$N = N_i E_e e^{\mu t} \tag{5.16}$$

Using assumption 1, μ is replaced by $\mu_{max\,L} + \mu_{max\,I}$

Therefore, Equation 5.16 reduces to the following form:

$$N = N_i E_e e^{(\mu_{max\,L} + \mu_{max\,I})t} \tag{5.17}$$

When $N \geq N_r$; i.e, the cell number exceeds a certain critical value, the capsules burst, releasing entrapped *L. casei*. To predict $N_r$, we assume that bursting of the beads occurs when the entire inner core of the capsule is completely filled with *L. casei* cells. Therefore, mathematically:

$$N_r = \frac{V_{capsule}}{V_{cell}} \tag{5.18}$$

Where; $V_{capsule}$: Volume of the inner core, i.e., the aqueous part of the capsule.

$V_{cell}$: Volume of an individual *L. casei* cell.

Therefore,

$$N_r = N_i E_e e^{(\mu_{maxL} + \mu_{maxI})t_r} \tag{5.19}$$

Where, $t_r$ is the release or bursting time.

$$t_r = \frac{\ln(\frac{N_r}{N_i E_e})}{\mu_{max\,L} + \mu_{max\,I}} \tag{5.20}$$

The value of $t_r$ may be determined using Equation 5.20 for beads and microcapsules produced by internal and external gelation.

## 5.7. Mathematical model for the evaluation of *In-vitro* concentration profiles of microcapsules (internal) and released probiotics in the simulated GI system

The mathematical model for the GI system has been developed using the concepts of combination of reactors, as used by pioneering researchers [9-11]. Although from the literature it is apparent that the activity of the stomach during the digestion of slurries containing polymeric solids is periodic in nature, the stomach, the small intestine and the large intestine have been considered as continuous stirred stank bio-reactors under transient condition.

The route followed by the microcapsule within the human gastro-intestinal tract may be schematically represented as follows:

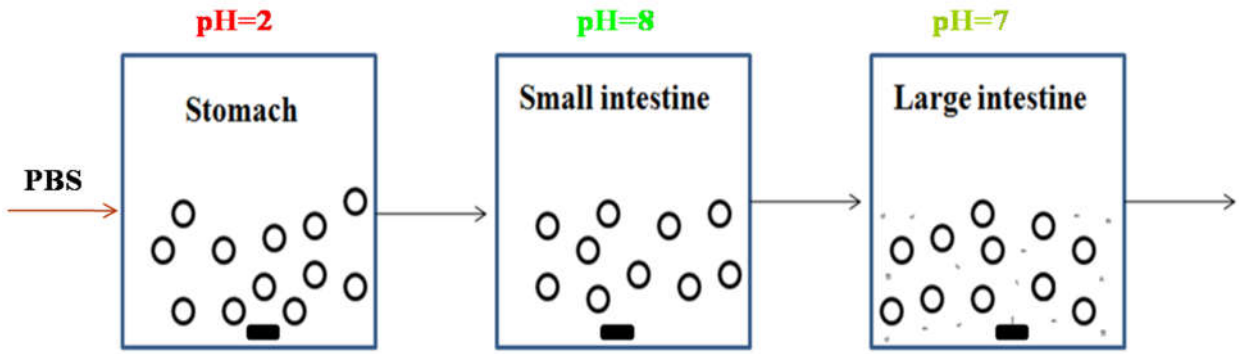



**Figure 5.2: Schematic representation of simulated human gastro-intestinal tract**

**○: Microcapsule, The dots represents probiotic cells released from microcapsules,**

**▬: Magnetic stirrer**

The mathematical model has been developed using the following assumptions:

1. The probiotic cells does not grow within the microcapsules during their passage through stomach (pH= 2.0) and small intestine (pH = 8). This has been justified by the experimental verification using simulated gastric and small-intestinal juices.
2. The change of structure of microcapsules in stomach and hence the "mechanical destructive force for size reduction" is neglected. This may be justified by the fact that the size of food ingredients >1mm is reduced in the stomach [12, 13].
3. Enzymatic dissolution and subsequent absorption in the small intestine, which usually occur in case of digestion of food materials, have been neglected for microcapsules. This has been verified by experimental study.
4. The release of cells from the microcapsules in the "large intestine" reactor follows the same behavior as described in section 5.6. The release of cells from the microcapsule may be represented by the following equation:

$$Mc \rightarrow pX \qquad (5.21)$$

Where, Mc and X stand for microcapsule and probiotic cells respectively, "p" represents the number of probiotic cells released at the time of bursting of microcapsules.

At any time greater than release time of cell from microcapsules, i.e., $t_r$, there will be two types of microcapsules, namely, ruptured and un-burst ones. Therefore, for $t>t_r$

$$C_{MC_T} = C_{MC_{RUP}} + C_{MC_{UNB}} \tag{5.22}$$

5. The stomach, small intestine and large intestine represented by CSTBRs are under dynamic conditions and never attain steady state.
6. Although the GI fluids are non-Newtonian in nature [13], Newtonian behavior has been assumed for the simplification of the model.
7. The exit of probiotic microorganisms from the large intestine is negligible. This may be justified by the fact that the probiotic bacteria in the large intestine adhere to the mucus for further action [10].

The balance equations for the concentration of microcapsules and probiotic cells in the reactors representing "stomach", "small intestine" and "large intestine" are provided in Table 5.1.

**Table 5.1: Balance equations for the concentration of microcapsules and probiotic cells in human gut**

| Reactor | Microcapsules | Probiotic cells |
|---|---|---|
| Stomach (St) | $\frac{dC_{(MC)\,St}}{dt} = -D_{St} C_{(MC)\,St}$ (5.23) | $\frac{dC_{X\,St}}{dt} = -D_{St} C_{X\,St}$ (5.24) |
| Small Intestine (SI) | $\frac{dC_{(MC)_{SI}}}{dt} = D\left(C_{MCst} - C_{MC_{SI}}\right)$ (5.25) | $\frac{dC_{X_{SI}}}{dt} = D\left(C_{X_{St}} - C_{X_{SI}}\right)$ (5.26) |
| Large Intestine (LI) | For $t<t_r$<br>$\frac{dC_{MC_{T\,LI}}}{dt} = D_{LI}\left(C_{MC_{SI}} - C_{MC_{T_{Li}}}\right)$ (5.27)<br>$C_{MC_{RUP\,LI}} = 0$ (5.28)<br>For $t>t_r$,<br>$\frac{dC_{MC_{T\,LI}}}{dt} = D_{LI}\left(C_{MC_{SI}} - C_{MC_{T\,LI}}\right)$ (5.30)<br>$C_{MC_{RUPLI}}(n+1) = C_{MC_{T_{LI}}}(n+1) - C_{MC_{T_{LI}}}(n)$ (5.31)<br>$C_{MC_{UNB\,LI}}(n) = C_{MC_{T_{LI}}}(n) - C_{MC_{RUP\,LI}}(n)$ (5.32) | For $t<t_r$<br>$\frac{dC_{X_{LI}}}{dt} = D_{LI} C_{X_{SI}}$ (5.29)<br>For $t> t_r$<br>$C_{X_{LI}}(n+1) = C_{X_{LI}}(n) + p * C_{MC_{RUP\,LI}}(n)$ (5.33) |

In Equations 5.30, 5.31, 5.32 and 5.33, (n+1) and ((n) represent the conditions at $n^{th}$ and $(n+1)^{th}$ time grids when the time domain is divided into equal intervals equivalent to the experimental value of the release time $t_r$, of the probiotic cells. In Equation 5.33, "p" represents the number of cells available from 1 g of microcapsule produced through internal gelation and is equal to 150.41E9. The Equations 5.23 to 5.33 of Table 5.1 have been solved using ode45 of MATLAB 7.The initial conditions and the values of the constants have been provided in Table 5.1.

## 5.8. Mathematical Model Probiotic Microcapsules and Beads suspended in lactose-rich MMRS medium and green coconut water: Prediction of internal concentration profiles and release time

The bead or microcapsule has been schematically represented in Figure 5.3.

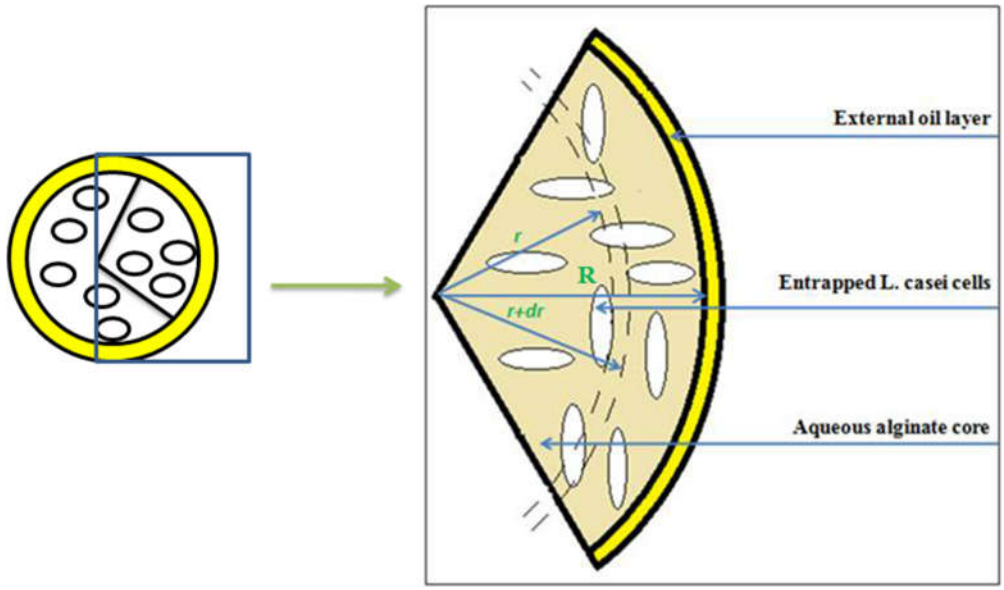



**Figure 5.3: Schematic representation of a microcapsule**

### 5.8.1. Schematic Representation of Microcapsules/Beads

Although thorough washing of oil has been done, the external layer of oil is present in case of microcapsules obtained via internal/ external gelation, not in case of beads synthesized via direct entrapment.

As shown in Figure 5.3, a differential volume element, enclosed within r and r + Δr, has been considered. The following assumptions have been made:

1. The diffusion of lactose occurs from the bulk, into the interior of the capsule and bead.
2. Lactose is the only limiting carbohydrate responsible for the growth of immobilized *L. casei* suspended in lactose-rich green coconut water and MMRS.
3. The mass transfer resistance due to oil layer is negligible for the transport of lactose from the exterior to the internal core of the microcapsules. This may be justified by the fact that the oil layer is thoroughly washed and hence its thickness is almost negligible.
4. The bulk concentration of lactose in the MMRS medium and the lactose-rich green coconut water remains constant over the whole period of incubation. This is justified due to low diffusion and consumption rates of lactose with respect to its total quantity of originally present in the system.

Therefore, the mathematical formulations of the concentration profiles for beads and beads are same. By performing the lactose balance over the differential volume element for time Δt:

$$4\pi r^2 \Delta r C_l\Big|_{t+\Delta t} - 4\pi r^2 \Delta r C_l\Big|_{t} = \left(\left( - D \; 4\pi r^2 \frac{\partial C_l}{\partial r} \right)\Big|_{r} + \left(D \; 4\pi r^2 \frac{\partial C_l}{\partial r} \right)\Big|_{r+\Delta r} + 4\pi r^2 \Delta r \eta r_l \right)\Delta t \tag{5.34}$$

Where, $C_l\big|_{t+\Delta t}$ and $C_l\big|_{t}$ are the concentration of lactose at time t+Δt and t; $C_l\big|_{r+\Delta r}$ and $C_l\big|_{r}$ are respectively the values of lactose concentration at radius r+Δr and r; D is the diffusivity of glucose in Ca-alginate, $r_l$ is intrinsic reaction rate and η is the effectiveness factor [14].

Dividing both sides of Equation 5.34 by $4\pi \; \Delta r \; \Delta t$ and limiting $\Delta r \to 0$ and $\Delta t \to 0$;

$$\frac{\partial}{\partial t}\left(r^2 C_L\right) = \frac{\partial}{\partial r}\left( D \; r^2 \frac{\partial C_L}{\partial r} \right) + r^2 \eta r_L \tag{5.35}$$

Or, by dividing both sides of Equation 5.35 by $r^2$,

$$\frac{\partial C_L}{\partial t} = D\left( \frac{2}{r}\frac{\partial C_L}{\partial r} + \frac{\partial^2 C_L}{\partial r^2} \right) + \eta r_L \tag{5.36}$$

Where,

$$r_l = -\frac{1}{Y_{X/l}}\left(\frac{dC_X}{dt}\right) \tag{5.37}$$

Where, $Y_{X/l}$ is the yield coefficient of biomass growth with respect to lactose

Since the growth of *L. casei* simultaneously on lactose follows Monod model,

$$\frac{dC_X}{dt} = \frac{\mu_{max\,L} C_L}{K_{SL} + C_L} \tag{5.38}$$

Thus, Equation 5.38 reduces to,

$$\frac{\partial C_L}{\partial t} = D\left(\frac{2}{r}\frac{\partial C_L}{\partial r} + \frac{\partial^2 C_L}{\partial r^2}\right) - \frac{\eta}{Y_{X/l}}\left(\frac{\mu_{max\,L} C_L}{K_{SL} + C_L}\right) \tag{5.39}$$

The initial and boundary conditions are as follows:

- IC( Initial Condition): i) At t=0; $C_L$ =0; 0<r<R

  ii) At t=0; $C_x$ = 2.17 g/L; 0<r<R (5.40)

- B.C ( Boundary Condition): i) At r=0; $\frac{dC_s}{dr} = 0; t \geq 0$

  ii) At r=R; $C_s = C_b = 20 g/L; t \geq 0$ (5.41)

The above set of partial differential equations, coupled with their B.C's and I.C's; have been solved using MATLAB R2010a to predict the concentration profiles of biomass and lactose within the microcapsules (internal and external) and beads. The "Release time" corresponding to the maximum concentration of biomass in the immobilized matrix, as described under section 6.E.3, has been determined for all cases.

### 5.9. Cost analysis

Cost analysis of a small process plant for the production of pearl millet inulin under optimum condition has been performed. The small plant is constituted of the units detailed in Table 5.2.

**Table 5.2: Units of small plant**

| Unit | Quantity | Capacity | Power Rating (PR) (in W) | Cost (1$ = Rs. 67.93) | | Write-off Period (Years) |
|---|---|---|---|---|---|---|
| | | | | (INR) | ($) | |
| Mixer-Grinder | 5 | 1.5 L | 750 | 10000 | 147.20 | 2 |
| Heating Mantles | 10 | 1.0L | 100 | 3000 | 44.16 | 1 |
| Magnetically stirred vessel | 20 | 2.0L | 500 | 10000 | 147.20 | 1 |
| Refrigerator | 2 | 190L | 400 | 20000 | 294.40 | 3 |
| Rotary vacuum evaporator | 1 | 5L | 60 | 1000000 | 147198.45 | 3 |
| Freeze dryer | 1 | 2.3 kg over 24 h | 350 | 100000 | 14719.845 | 3 |

The schedule of plant operation is as follows

**Table 5.3: Schedule of plant operation**

| | |
|---|---|
| Working hours for the plant ($n_W$) | 8h |
| Working days per year($n_{dY}$) | 280 |

Referring to the extraction scheme of inulin shown in Figure 4.1, each stirred vessel placed on a heating mantle handles 100 g of prebiotic, pearl millet suspended in slurry of 500 ml in 1 h. Since, 10 heating mantles are in operation, total quantity of pearl millet which can be processed annually ($M_{PA}$) by this small plant is calculated as follows:

$$M_{PA}(kg) = \frac{10*100*n_W*n_{dy}}{1000} \quad (5.42)$$

If the fractional extraction and the purity of inulin obtained under optimum condition be denoted by $Q_{opt}$ (0.4) and $P_{opt}$ (0.473) the annual production capacity of pure inulin by the small process plant $Q_{iy}$ is given by following correlation

$$Q_{IA}(kg) = M_{PA} * Q_{opt} * P_{opt} \quad (5.43)$$

From the extraction scheme of inulin from pearl millet it is also clear that approximately 1 L of water is needed to process 100 g of millet inulin. Therefore the total annual consumption of water ($W_Y$) for the process of inulin extraction is as follows:

$$W_Y(L) = M_{pA} * \frac{0.75*1000}{100} \quad (5.44)$$

The quantity of HCl needed for 100 g of pearl millet under processing is approximately 1mL. Therefore total annual consumption of HCl ($H_Y$) is calculated as follows:

$$H_Y(L) = M_{pA} * \frac{0.001*1000}{100} \quad (5.45)$$

The recurring cost for the plant has been calculated using the following data

**Table 5.4: Recurring cost for the plant**

| | |
|---|---|
| Grid electricity tariff (Calcutta electricity supply corporation) ($E_C$) | Rs.6.77/kWh |
| Annual Maintenance cost ($MC_A$) of Freeze dryer and rotary vacuum evaporator | Rs.50,000 |
| Manpower (2 operators) ($MP_A$) | Rs.9,00,000 |
| Cost of water (Rs/L) | $W_C$ (Rs.6/L) |
| Cost of HCl (Rs/L) | $H_C$ (Rs.1000/L) |
| Cost of pearl millet(Rs/kg) | $P_C$ (Rs.8/kg) |

| | |
|---|---|
| Cost of other Chemicals and filter cloth (Rs) | Co (Rs.100000) |

Total annual energy consumption ($E_Y$) of the plant has been calculated considering the plant schedule and the power rating (PR) of each unit, i.e., mixer-grinder ($PR_{MG}$), heating mantle ($PR_{HM}$), Rotary vacuum evaporator ($PR_{RVE}$), Freeze dryer ($PR_{FD}$), magnetic stirred vessel ($PR_{MGST}$), refrigerator ($PR_{RF}$).

Therefore,

$$E_y(kWh) = \frac{n_w * n_{dY} * (PR_{MG} + PR_{HM} + PR_{RVE} + PR_{FD} + PR_{MGST} + PR_{RF}}{1000} \quad (5.46)$$

Total energy cost ($E_T$) is calculated as follows:

$$E_T = E_Y * E_c \quad (5.47)$$

Total material cost ($M_Y$) is contributed from pearl, millet, water and HCl and cost of other chemicals, filters etc ($C_{Ot}$), Therefore,

$$M_Y = M_{PA} * P_C + W_Y * W_C + H_Y * H_C + C_{Ot} \quad (5.48)$$

The total annual recurring cost ($R_{cY}$) is the summation of energy cost ($E_Y$), Material cost ($M_Y$), manpower cost ($M_{PY}$) and the annual maintenance cost ($M_{CY}$)

$$R_{CY} = E_Y + M_{PY} + M_{CY} + M_Y \quad (5.49)$$

Total annual fixed cost ($FC_y$) has been incurred by the investment cost for the equipment, namely, mixer-grinder ($FC_{MG}$), heating mantle ($FC_{HM}$), Rotary vacuum evaporator ($FC_{RVE}$), Freeze dryer ($FC_{FD}$), magnetic stirred vessel ($FC_{MGST}$), refrigerator ($FC_{RF}$), distributed manually over the write-off period.

Therefore,

$$FC_y = FC_{MG} + FC_{HM} + FC_{RVE} + FC_{FD} + FC_{MGST} + FC_{RF} \quad (5.50)$$

$$FC_y = \frac{Co_{MG}}{n_{w_{MG}}} + \frac{Co_{HM}}{n_{w_{HM}}} + \frac{Co_{RVE}}{n_{w_{RVE}}} + \frac{Co_L}{n_{w_{FD}}} + \frac{Co_{MGST}}{n_{w_{MGST}}} + \frac{Co_{RF}}{n_{w_{RF}}} \quad (5.51)$$

Where, Co = cost of equipment * number of equipment

$n_{wr}$ = write-off period

Total annual cost (CT) is a summation of recurring ($R_{CY}$) and fixed cost ($FC_Y$)

Therefore,

$$CT = R_{CY} + FC_Y \quad (5.52)$$

The extraction cost ($E_{XT}$) per unit kg of pure inulin may be calculated using the following correlation.

$$E_{X_T} = CT / Q_{IA} \quad (5.53)$$

## REFERENCES


1. Huebner, J., Wehling, R.L. and Hutkins, R.W. (2007). "Functional Activity of commercial probiotics". International Dairy Journal, 17, 770-775.
2. Shuler, M.L. and Kargi, F. (2002). Bioprocess Engineering: Basic Concepts, Edition 2, Pearson, USA.
3. Douglas, S., Clark, H., Blanch, W. (1997). Biochemical Engineering, Edition 2, CRC Press.
4. Zou, Q., Liu, X., Zhao, J., Tian, F., Zhang, H., Zhang, H. (2012). "Microencapsulation *Lactobacillus delbrueckii* of *Bifidobacterium bifidum F-35* in whey protein-based microcapsules by transglutaminase-induced gelation". Journal of Food Science, 77(5), M270–M277.
5. Rosas-Flores, W., Ramos-Ramirez, E.G., Salazar-Montoya, J.A. (2013) "Microencapsulation of *Lactobacillus helveticus* and using alginate and gellan gum". Carbohydrate Polymers,98, 1011– 1017.
6. Lakkis, J.M. (2007) "Encapsulation and Controlled Release Technologies in Food Systems". Blackwell Publishing.
7. Doran, P.M. (1995). Bioprocess Engineering principles, Elsevier Science & Technology Publishers.
8. Clark, H., Blanch, W. (1997). Biochemical Engineering, Edition 2, CRC Press.
9. Cook M.T., Tzortzis, G., Charalampopoulos D., Khutoryanskiy, V.V. (2012) "Microencapsulation of probiotics for gastrointestinal delivery". Journal of Controlled Release, 162, 56–67.
10. Macfarlane, G.T., Macfarlane, S., Gibson, G.R.(1998) "Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon". Microbial Ecology, 35,180–187.
11. Child, M.W., Kennedy, A., Walker, A.W., Bahrami, B., Macfarlane, S., and Macfarlane, G.T. (2006) "Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques". FEMS Microbiological Ecology, 55, 299–310.

12. Roger, G., Janssen, P.W.M. “The Physical Processes of Digestion”. ISBN 978-1-4419-9448-6. DOI 10.1007/978-1-4419-9449-3. Springer, New York, Dordrecht Heidelberg, London.
13. Kong, F. and Sing, R.P. (2008) “Disintegration of Solid Foods in Human Stomach”. Journal of food science (Concise Reviews and Hypotheses in Food Science), 73, 5.
14. Levenspiel O., Chemical Reaction Engineering, John Wiley & Sons, Edition 3, New York, Chichester, Weinheim Brisbane, Singapore Toronto.

# Chapter 6

# RESULTS AND DISCUSSION

## 6.A. Characterization of inulin isolated from different sources

In the present investigation the prebiotic inulin a fructo-oligosaccharide has been isolated from three different Indian millets, viz., pearl millet (*Pennisetum glaucum*), great millet (*Sorghum bicolor*) and finger millet (*Eleusine coracana*). Inulin has also been isolated from the most common natural source garlic (*Allium sativum).* In order to ascertain the effectiveness of isolated desired product, an elaborate comparison has been made between the isolated inulin and the commercially available inulin (taken as standard sample) by different instrumental analysis. The results are given below:

### 6.A.1. Thin layer Liquid Chromatography (TLC) Analysis

Thin layer Liquid Chromatography has been used to analyze inulin isolated from the natural food stuffs. Commercial inulin and the samples derived from the natural sources (garlic, great millet, pearl millet and finger millet) have been used for this purpose. In order to standardize the TLC procedure different quantity of inulin was subjected to TLC analysis with an intention to identify the sample with the best spot. This is shown in Figure 6.A.1. It is observed from the figure that 0.2 g of pure inulin gives the best result for comparison. It may be mentioned that TLC picture of inulin has been confirmed from the published literature [1].

In order to confirm the presence of inulin isolated from natural food stocks TLC analysis has been carried out following the same procedure as in the case of pure inulin. Results are shown for garlic and millet extracts in Figures 6.A.1 and 6.A.2 respectively. The results obtained from the diagram clearly indicate the presence of inulin in garlic, great millet (jowar), pearl millet (bajra) and finger millet (ragi) taken for the present investigation. The Relative Fronts, ($R_f$) values of the extracts from garlic and millet sources have been compared with that obtained for commercial inulin. Results are shown in Table 6.A.1.The closeness of $R_f$ values of different inulin extract with commercial sample confirms the presence of inulin.

**Table 6.A.1: $R_f$ values of prebiotic food samples and commercial inulin**

| Samples | $R_f$ Values |
|---|---|
| Commercial inulin | 0.80 |
| Garlic | 0.80 |
| Great millet (Jowar) | 0.80 |
| Pearl millet (Bajra) | 0.80 |
| Finger millet (Ragi) | 0.75 |

In order to determine the concentration of inulin present in the isolated samples spectro-photometric analysis of aqueous solutions of colored spots extracted from TLC plate has been carried out. Noting the optical density of each sample solution and comparing it with the pure commercially available sample the concentration of the each isolated sample has been computed. Results are shown in Table 6.A.2. It is observed that pearl millet contains maximum concentration (47 %) of inulin.

**Table 6.A.2: Concentrations of inulin in different prebiotic Indian food materials**

| Natural prebiotics | Quantity of Inulin Extract (powders)(%)(w/w) |
|---|---|
| Garlic | 16.60 |
| Great millet | 30 |
| Pearl millet | 47 |
| Finger millet | 32 |

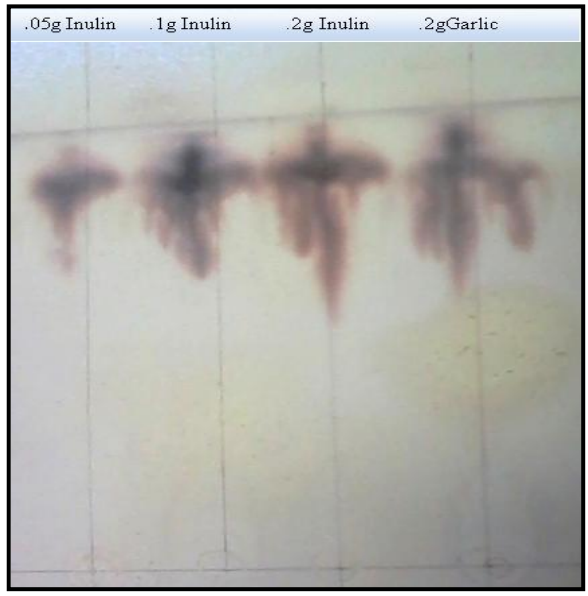



**Figure 6.A.1: Silica gel G plate for TLC analysis for different concentrations of hydrolyzed products of prebiotics by 5% oxalic acid**

[First three lanes are respectively 0.05 g (10%), 0.1 g (20%) and 0.2 g (40%) hydrolyzed commercial inulin and fourth lane is 0.2 g hydrolyzed garlic solution]

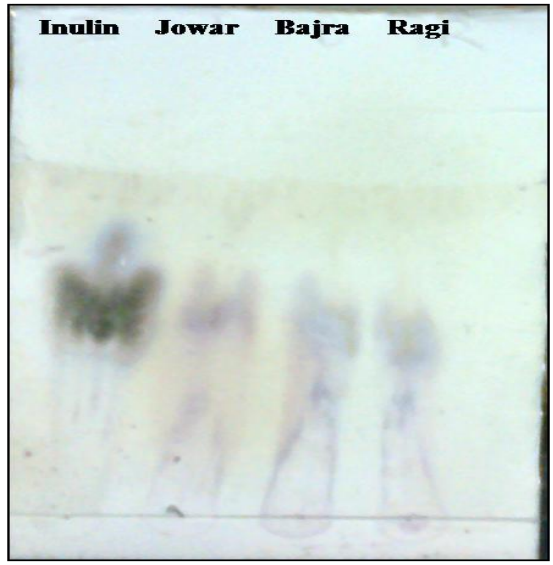



**Figure 6.A.2: Silica gel G plate for TLC analysis for (0.2 g or 40%) of hydrolyzed products of prebiotics by oxalic acid**

[First lane is hydrolyzed inulin, second lane is hydrolyzed Great millet (jowar), third lane is hydrolyzed Pearl millet (bajra), and fourth lane is hydrolyzed Finger millet (ragi) solution]

### 6.A.2. Estimation of inulin in extract powder by DNS (3-5 Di-nitro salicylic acid) method

DNS method has been applied to determine the quantity of inulin content in inulin extract powder obtained from different natural prebiotic samples. The results obtained from these experiments are shown in Table 6.A.3. From the table it also appears that the purity of inulin obtained from garlic is the maximum compared with the other prebiotic samples. This is due to very low content of other carbohydrates present in garlic.

**Table 6.A.3: Inulin content (%w/w) in the Natural Extract Powder**

| Natural prebiotic sample | Fructose content, g/L | Inulin content, g/L | Purity of inulin in extract powder(% w/w) |
|---|---|---|---|
| Garlic | 276.30 | 248.67 | 99.46 |
| Great millet | 83.95 | 75.55 | 30.22 |
| Pearl millet | 131.31 | 118.18 | 47.27 |
| Finger millet | 89.31 | 80.38 | 32.15 |

### 6.A.3. Characterization of chemical bonds through FTIR (Fourier Transformed Infrared) spectroscopy

TLC analysis of the prebiotic samples used in the present investigation has confirmed the presence of inulin in them. To compare the chemical bonds of natural inulin with those of commercial sample, FTIR analysis has been carried out. The FTIR chromatogram of the commercial inulin sample (used for comparison purpose) and four other samples are shown in Figures 6.A.3 through 6.A.7. By comparing the chromatograms the distributions of chemical bonds in the standard sample and the samples under study have been evaluated and the results are shown in Table 6.A.4. On closer examination it is revealed that in each of the candidate food materials inulin is present. The chromatograms of natural extracts from garlic, great millet, pearl millet and finger millet are similar to that of standard inulin. A close comparison of

chromatogram (Figure 6.A.3 and Table 6.A.4) evidently indicates that the centroid of the main maximum of pure inulin sample and the hydrolyzed samples of natural inulin extracts fall on the wavelength between 3240 $cm^{-1}$ to 3433 $cm^{-1}$. Although the centroids of commercial inulin and garlic inulin falls on the same wavelength between 3240 $cm^{-1}$to 3290 $cm^{-1}$ those of jowar, bajra and ragi shift towards higher wavelength. The centroids of great millet, pearl millet and finger millet are obtained at a higher wavelength of 3433 $cm^{-1}$, 3422 $cm^{-1}$ and 3414 $cm^{-1}$ respectively. This may be due to the presence of many carbohydrates other than inulin in these food samples [2].

**Table 6.A.4: Wavelength numbers of four natural prebiotic samples**

| **FTIR wavelength numbers** | | | | | **Range of functional groups** |
|---|---|---|---|---|---|
| **Commercial Inulin** | **Garlic** | **Great millet** | **Pearl millet** | **Finger millet** | |
| 1365 | ---- | 1380 | 1383 | 1408 | 1365 and 1390 $cm^{-1}$ is often due to a t-Bu-group |
| 1450 | ---- | 1458 | 1457 | 1547 | A strong peak around 1450 $cm^{-1}$ indicates the presence of methylene groups ($CH_2$) [$CH_2$, $CH_3$ (bend)] |
| 1633.71 | 1633.71 | 1651 | 1650 | 1653 | (1620-1680) –C=C- Alkene **[**3]**;**(1638.84)C=O [4] |
| 1965.46 | 1965.46 | ---- | ---- | ----- | (1908-1960) O-H stretch first overtone, C=O stretch second overtone, O-H stretch/O-H bend combination |
| 1988.61 | 1988.61 | ---- | ---- | ---- | (1980) Asym N-H stretch/N-H in plane bend; C-N stretch combination [5] |
| 2015.61 | 2015.61 | ---- | ---- | ---- | |
| 2038.76 | 2036.83 | ---- | ---- | ---- | |
| 2061.90 | 2061.90 | ---- | ---- | ---- | |
| 2086.98 | 2088.91 | ---- | ---- | ---- | |

| | | | | | |
|---|---|---|---|---|---|
| 2104.34 | 2100.48 | 2140 | 2149 | 2134 | |
| 2351.23 | 2353.16 | ---- | ----- | ---- | (2100-2260)-C≡C- stretch in alkynes, -C≡N- stretch in Nitrile, OH bond, C-O stretch [6] |
| 2389.80 | ---- | ---- | ---- | ---- | |
| 2931 | ---- | 2925 | 2928 | 2928 | |
| 3240.41 | 3240.41 | ---- | ---- | ----- | 2931 (C-H aliphatic) |
| 3290.56 | 3290.56 | 3433 | 3432 | 3414 | (3200-3500) O-H bond[7] |

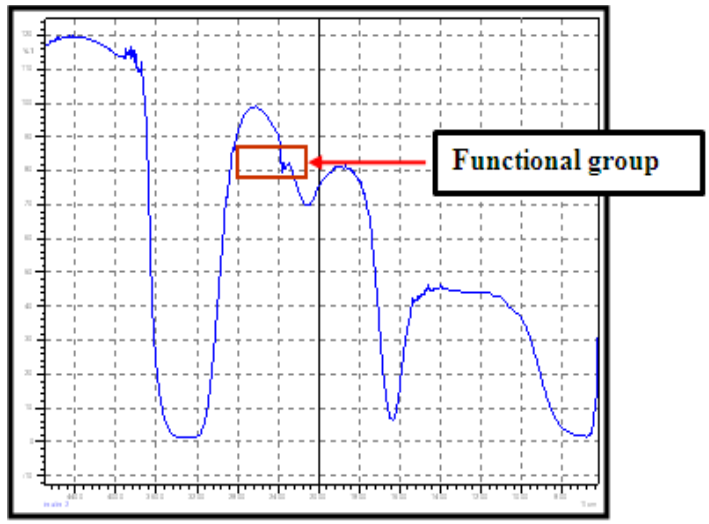



**Figure 6.A.3: FTIR analysis of Commercial Inulin**

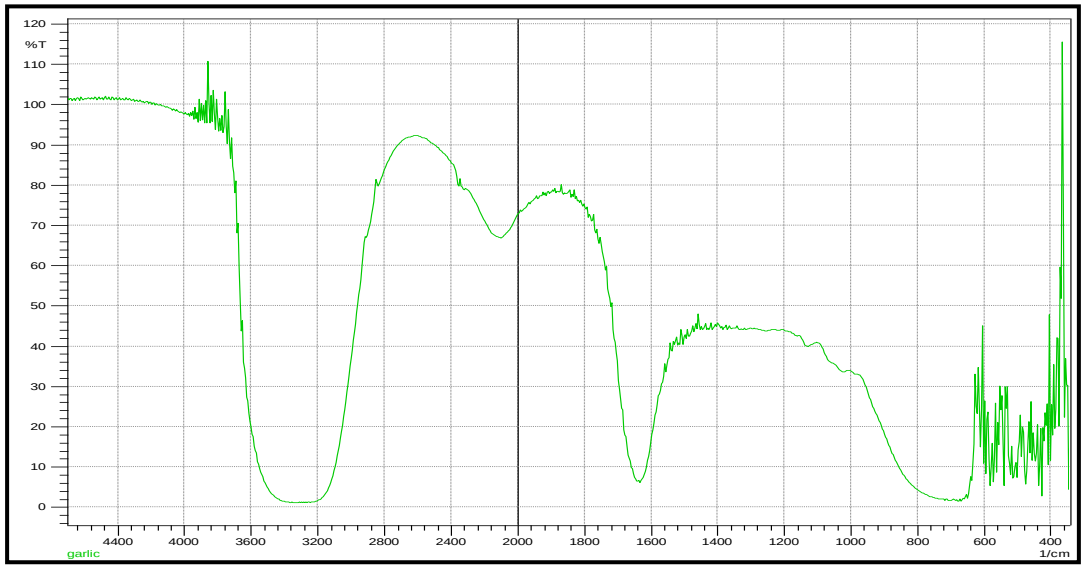



**Figure 6.A.4: FTIR chromatogram of garlic**

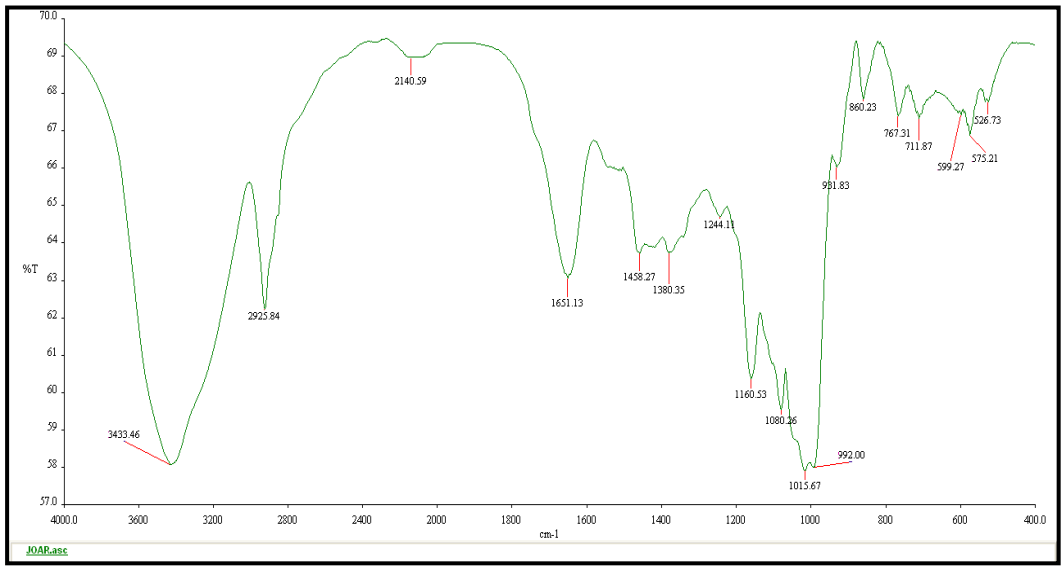



**Figure 6.A.5: FTIR analysis of Great millet**

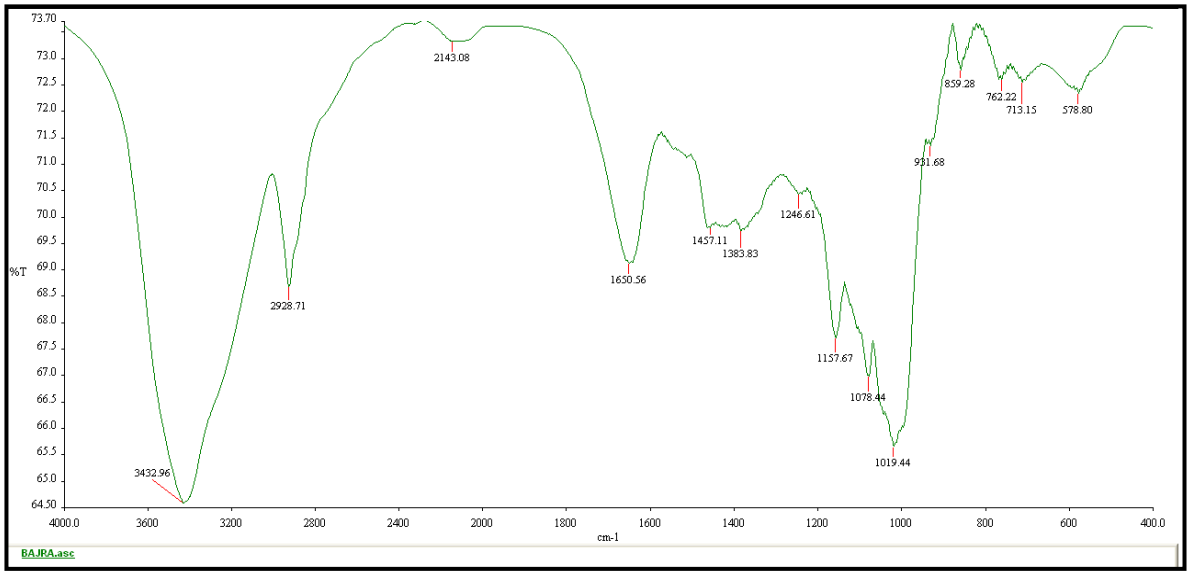



**Figure 6.A.6: FTIR analysis of Pearl millet**

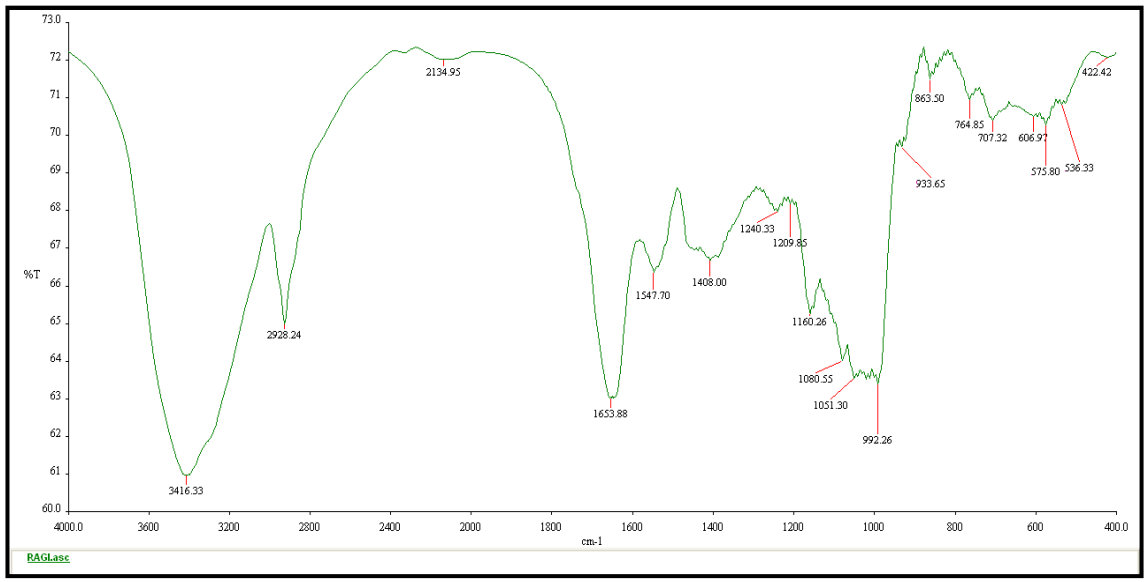
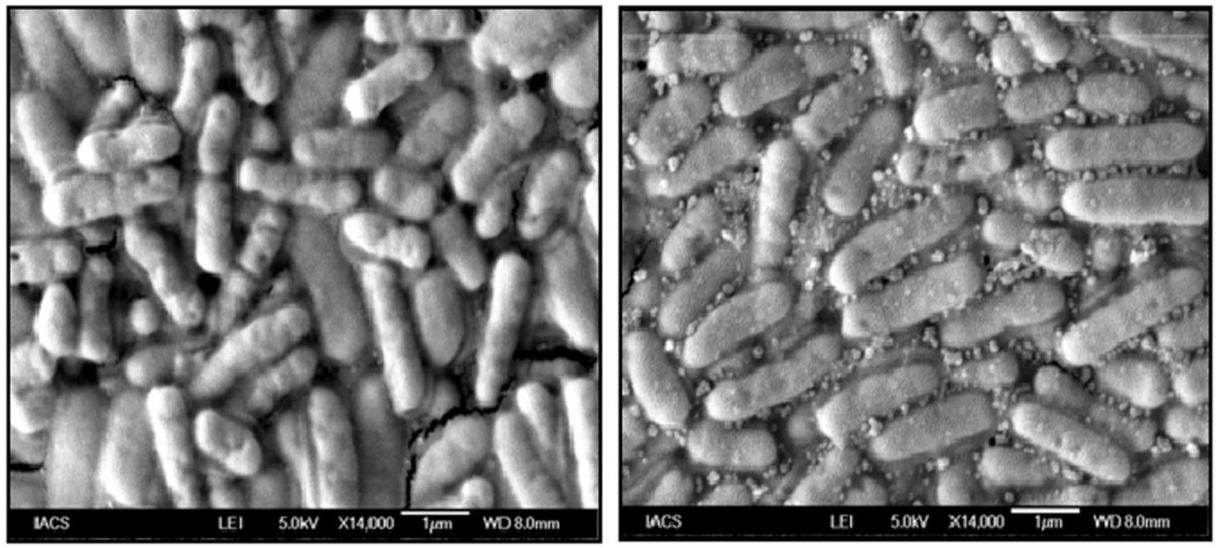



**Figure 6.A.7: FTIR analysis of Finger millet**

### 6.A.4. Field Emission Scanning Electron Microscopy images of *Lactobacillus casei* (2651 1951 RPK)

Figure 6.A.8 (a) and (b) depict the FESEM image of *Lactobacillus casei* grown with and without inulin.




**(a)** **(b)**

**Figures 6.A.8: (a) SEM image of *Lactobacillus casei* cell grown without inulin, (b) with inulin**

It is evident from above figures that the prebiotic inulin plays an important role in the metabolic growth of *Lactobacillus casei*. Figure 6.A.8 (b) reveals the presence of a layer of inulin over the cell wall. This may be due to the fact that inulin, being a large molecule cannot be transported inside the cells and they are decomposed enzymatically to glucose and fructose which are in turn assimilated by *Lactobacillus casei* [8]. A close inspection also reveals that the *Lactobacillus casei* cells treated with inulin has been elongated in size compared to its non-inulin treated counterpart. This observation directly indicates that the prebiotic inulin induces a metabolic reorientation of *Lactobacillus casei* cells resulting in formation of the desired enzymes in greater quantity.

## 6.B. Optimization of growth medium, yield of inulin from pearl millet and efficiency of encapsulation during immobilization using Response Surface Methodology

It is now well understood that initial concentrations of inulin and lactose have profound effect on the cell growth of *Lactobacillus casei*. While both lactose and inulin are electron donors for the

candidate microorganism under investigation experimental data have clearly shown that the specific cell growth rate does not monotonically increase with increase in inulin concentration. More importantly when lactose and inulin are used simultaneously as carbon sources, their mutual interaction also affects the cell growth. Such observation evidently indicates the necessity of detailed optimization study of these two parameters to achieve the optimum cell growth rate.

While there are several optimization techniques available for use, in the present situation the most convenient and widely accepted technique Response surface methodology (RSM) has been the most favored choice by the investigators in recent time because of its simplicity, user-friendly software and high degree of confidence. Thus in the present investigation response surface methodology has been used in all the three situations stated above.

### 6.B.1. Optimization of concentrations of lactose & inulin for achieving maximum specific cell growth rate of *Lactobacillus casei* (2651 1951 RPK) using Response surface methodology technique

In standard MRS medium composition carbohydrate concentration is specified as 20 g/L. Thus in the present investigation the carbohydrate source lactose was taken within the concentration range 10-30 g/L. The concentration range of another independent variable inulin has been selected from the previous knowledge that inulin inhibits the specific cell growth rate at higher concentration level. Thus, in the present optimization study inulin concentration kept in a lower region of concentration (0.164-0.624 g/L). The experimental design matrix was prepared with the help of Design-Expert 8.1 and the experimental results of the response variable μ for 13 set of experiments are given in Table 6.B.1.

**Table 6.B.1: Experimental design matrix***

| Run | Concentration of Lactose ($C_L$ ,g/L) | Concentration of inulin( $C_I$ ,g/L) | Coded value of $C_L$ , g/L | Coded value of $C_I$ , g/L | Response (μ) ($h^{-1}$) |
|---|---|---|---|---|---|
| 1 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 2 | 30.00 | 0.62 | +1 | +1 | 0.0920 |
| 3 | 10.00 | 0.62 | -1 | +1 | 0.1823 |
| 4 | 20.00 | 0.75 | 0 | +1 | 0.1510 |

| | | | | | |
|---|---|---|---|---|---|
| 5 | 20.00 | 0.13 | 0 | -1 | 0.2173 |
| 6 | 5.90 | 0.32 | -1 | 0 | 0.1200 |
| 7 | 20.00 | 0.31 | 0 | 0 | 0.8285 |
| 8 | 30.00 | 0.00 | +1 | -1 | 0.2830 |
| 9 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 10 | 10.00 | 0.00 | -1 | -1 | 0.1787 |
| 11 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 12 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 13 | 30.41 | 0.31 | +1 | 0 | 0.4143 |

*****Here, $\mu$ = Specific growth rate ($h^{-1}$).

The Design Expert 8.1 software also provides the quantitative relationship of two interacting independent variables $C_L$ and $C_I$ with the objective function $\mu$. The relationship as follows

$$\mu = 0.83 + 0.054 * C_L - 0.035 * C_I - 0.049 * C_L * C_I - 0.29 * C_L^2 - 0.33 * C_I^2 \quad (6.B.1)$$

In order to understand the relative significance of each coded independent variable as well as their combination "analysis of variance" (ANOVA) of the proposed quadratic model of response has been made and results are given in Table 6.B.2.

**Table 6.B.2: Analysis of Variance (ANOVA) for Response Surface Quadratic Model***

| **Source** | **Sum of squars** | **df** | **Mean square** | **F value** | **p-value Prob>F** |
|---|---|---|---|---|---|
| **Model** | 1.25 | 5 | 0.25 | 70.39 | <0.001(significant) |
| **$C_L$** | 0.023 | 1 | 0.023 | 6.53 | 0.0378 |
| **$C_I$** | 9.882E-003 | 1 | 9.882E-003 | 2.79 | 0.1387 |
| **$C_L C_I$** | 9.467E-003 | 1 | 9.467E-003 | 2.67 | 0.1460 |
| **$C_L^2$** | 0.59 | 1 | 0.59 | 166.47 | <0.0001 |
| **$C_I^2$** | 0.77 | 1 | 0.77 | 217.31 | <0.0001 |
| **Residual** | 0.025 | 7 | 3.541E-003 | | |

| | | | | | |
|---|---|---|---|---|---|
| **Lack of Fit** | 0.027 | 3 | 8.262E-003 | | |
| **Pure Error** | 0.000 | 4 | 0.000 | | |
| **Cor total** | 1.27 | 12 | | | |

*****Here, F value= Fischer test value, p-value= lack of fit value.

The F value (Fischer test value) (70.39) and p-value (<0.001) clearly indicate that in the present system $C_L$, $C_I$, $C_L*C_I$, $C_L^2$ and $C_I^2$ are all significant model terms.

In order to verify the usefulness of the model equation (Eq. 6.B.1) a comparison has been made by plotting the predicted response against the experimentally obtained response. This is shown in Figure 6.B.1.

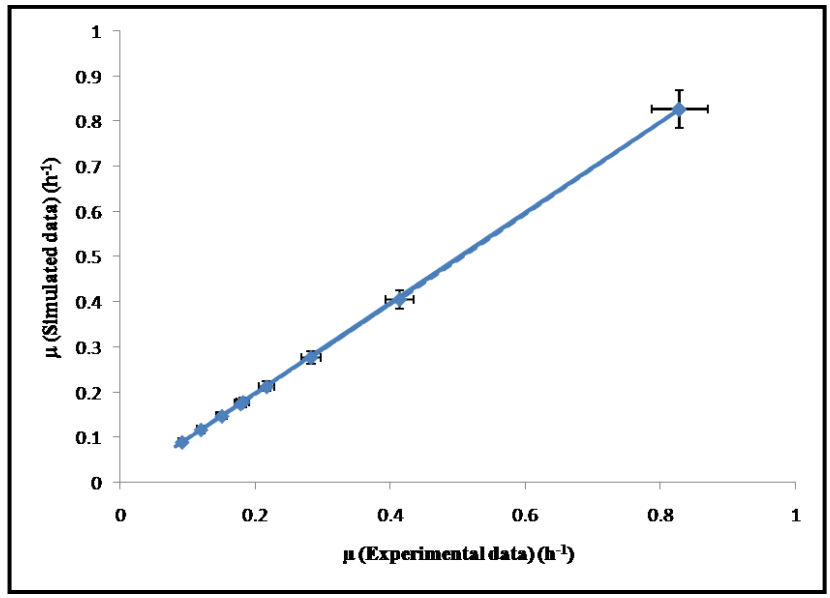



**Figure 6.B.1: Comparison of experimental and simulated values of specific growth rate (μ)**

A statistical summary on the model fit between the actual and predicted responses is shown in Table 6.B.3.

**Table 6.B.3: Model Fit summary statistics for final specific growth rate***

| Std. Dev. | Mean | C.V.% | PRESS | R-Squared | AdjR Squared | PredR-Squared |
|---|---|---|---|---|---|---|
| 0.060 | 0.44 | 13.38 | 0.18 | 0.9805 | 0.9666 | 17.684 |

***Here, C.V. = Coefficient of variance

In order to understand the effect of lactose concentration and inulin concentration on specific cell growth rate, the result obtained through response surface methodology technique have been presented in three dimensional plot shown in Figure 6.B.2. It is observed that the maximum specific growth rate of *Lactobacillus casei* is obtained at 20 g/L of lactose concentration and 0.32 g/L of inulin concentration. The contour plot shown in Figure 6.B.3 clearly confirms the optimum values of the response variables. Solutions for optimal conditions of specific growth rate are shown in Table 6.B.4.

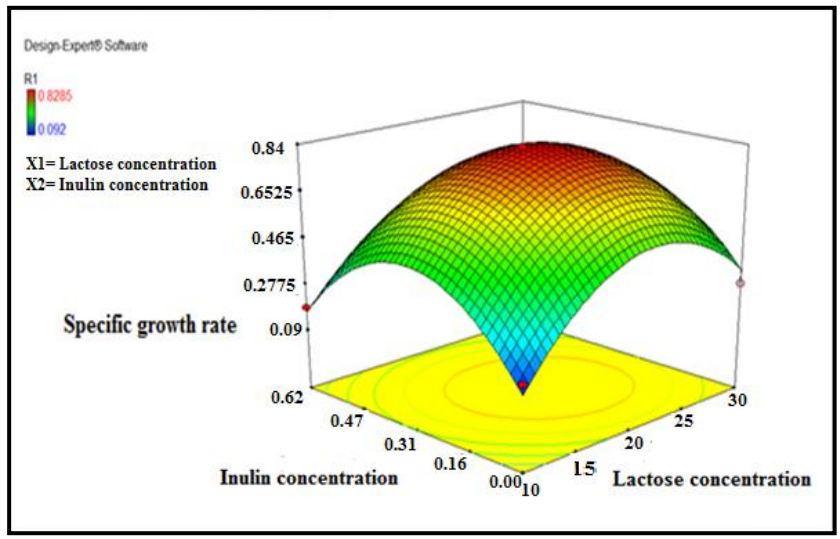



**Figure 6.B.2: Three dimensional plot for optimization of specific growth rate of *Lactobacillus casei* (2651 1951 RPK). Here R1= Response (μ or specific growth rate)**

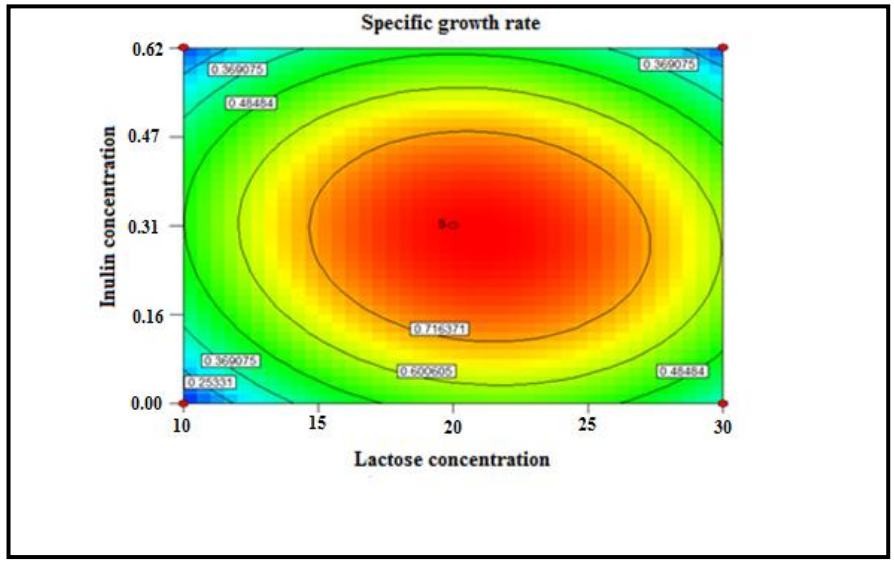



**Figure 6.B.3. Contour plot for optimization of specific growth rate of *Lactobacillus casei* (2651 1951 RPK)**

**Table 6.B.4: Solutions for optimal conditions of specific growth rate**

| Number | Lactose concentration ($C_{L)}$ | Inulin concentration ($C_I$) | Desirability |
|---|---|---|---|
| 1 | 20.00 | 0.32 | 1.0 (selected) |
| 2 | 10.00 | 0.62 | 0.909 |
| 3 | 10.00 | 0.00 | 0.909 |

## 6.B.2. Optimization of Temperature, Concentration of HCl and Heating period for achieving maximum pearl millet inulin yield using Response surface methodology technique

In the present investigation the temperature, concentration of HCl and heating period were taken within the range 24 to 70°C, 0.5 to 2 M and 20 to 180 min. For obtaining maximum inulin yield (with respect to purity) the temperature, concentration of HCl and heating period are specified as 70 °C, 0.8 M and 60 min. The experimental design matrix was prepared with the help of Design-Expert 8.1 and the experimental results of the response variable inulin yield (obtained at different

values of Temperature, HCl concentration and heating period) for 20 set of experiments are given in Table 6.B.6.

The model equation predicted using the values of response variables corresponding to the preset experimental conditions using the CCD table has been used for optimization of pearl millet inulin yield with respect to temperature, HCl concentration and heating period in terms of ±1 levels created by entering the factors (Table 6.B.5).

**Table 6.B.5: Experimental ranges and levels of the factors (process variables) for response surface study**

| Uncoded factor | Coded factor | Unit | Uncoded value | Coded value |
|---|---|---|---|---|
| Temperature | A | ˚C | 24 | -1 |
| | | | 70 | +1 |
| HCL concentration | B | M | 0.5 | -1 |
| | | | 2 | +1 |
| Heating period | C | min | 20 | -1 |
| | | | 180 | +1 |

**Table 6.B.6: Experimental design matrix**

| Run | Temperature, ˚C | HCl concentration, M | Heating period, Min | Inulin yield(%) |
|---|---|---|---|---|
| 1 | 65.00 | 1.25 | 80.00 | 70.2 |
| 2 | 47.00 | 1.25 | 100.00 | 45.0 |
| 3 | 24.00 | 2.00 | 20.00 | 16.0 |
| 4 | 70.00 | 0.80 | 60.00 | 90.0 |
| 5 | 47.00 | 0.01 | 100.00 | 27.0 |
| 6 | 47.00 | 1.25 | 100.00 | 45.0 |
| 7 | 24.00 | 0.50 | 180.00 | 28.8 |
| 8 | 70.00 | 0.50 | 180.00 | 45.0 |

| | | | | |
|---|---|---|---|---|
| 9 | 47.00 | 1.25 | 234.54 | 32.0 |
| 10 | 24.00 | 2.00 | 20.00 | 16.0 |
| 11 | 24.00 | 2.00 | 180.00 | 28.8 |
| 12 | 47.00 | 1.25 | 100.00 | 45.0 |
| 13 | 70.00 | 0.80 | 60.00 | 90.0 |
| 14 | 47.00 | 2.51 | 100.00 | 29.0 |
| 15 | 47.00 | 1.25 | 100.00 | 45.0 |
| 16 | 8.32 | 1.25 | 100.00 | 16.0 |
| 17 | 47.00 | 1.25 | 100.00 | 45.0 |
| 18 | 70.00 | 2.00 | 20.00 | 18.0 |
| 19 | 47.00 | 1.25 | 34.54 | 18.0 |
| 20 | 47.00 | 1.25 | 100.00 | 45.0 |

The Design Expert 8.1 software also provides the quantitative relationship of three interacting independent variables temperature, HCl concentration and heating period with the objective function inulin yield from pearl millet. The relationship as follows

$$\text{Inulin yield} = 47.96 + 15.59*A - 5.79*B + 0.93*C - 18.31*A*B - 13.68*A*C + 0.57*B*C - 1.00*A^2 - 7.80*B^2 - 8.98*C^2 \quad \ldots \quad (6.B.2)$$

Where, A,B and C are the temperature, concentration of HCL and heating period.

In order to understand the relative significance of each coded independent variable as well as their combination “analysis of variance” (ANOVA) of the proposed quadratic model of response has been made and results are given in Table 6.B.7.

**Table 6.B.7: ANOVA for Response Surface Model**

| Source | Sum of squars | df | Mean square | F value | p-value Prob>F |
|---|---|---|---|---|---|
| **Model** | 7913.07 | 9 | 879.23 | 5.97 | 0.0050(significant) |
| **A-** | 1814.60 | 1 | 1814.60 | 12.31 | 0.0056 |

| | | | | | |
|---|---|---|---|---|---|
| **Temperature** | | | | | |
| **B-HCl concn** | 324.80 | 1 | 324.80 | 2.20 | 0.1685 |
| **AB** | 662.22 | 1 | 662.22 | 4.49 | 0.0600 |
| **AC** | 323.03 | 1 | 323.03 | 2.19 | 0.1695 |
| **BC** | 0.66 | 1 | 0.66 | 4.458E-003 | 0.9481 |
| **$A^2$** | 5.18 | 1 | 5.18 | 0.035 | 0.8550 |
| **$B^2$** | 755.41 | 1 | 755.41 | 5.13 | 0.0470 |
| **$C^2$** | 1002.95 | 1 | 1002.95 | 6.81 | 0.0261 |
| **Residual** | 1473.50 | 10 | 147.35 | | |
| **Lack of Fit** | 1473.50 | 3 | 491.17 | | |
| **Pure Error** | 0.000 | 7 | 0.000 | | |
| **Cor total** | 9386.57 | 19 | | | |

The F value (Fischer test value) (5.97) and p-value (0.005) clearly indicate that in the present system A, B, A*B, A*C, B*C, $A^2$, $B^2$ and $C^2$are all significant model terms.

In order to verify the usefulness of the model equation (Eq. 6.B.2) a comparison has been made by plotting the predicted response against the experimentally obtained response. This is shown in Figure 6.B.4.

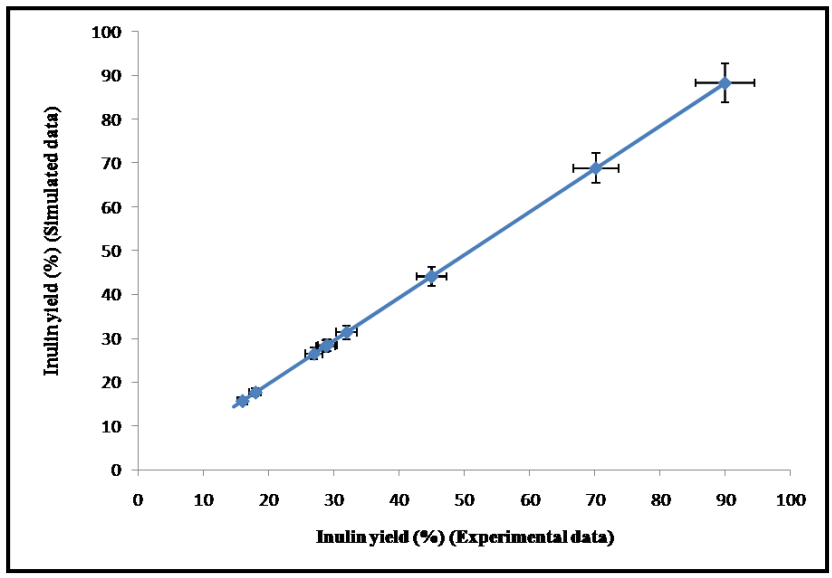



**Figure 6.B.4: Comparison of experimental and simulated values of maximum inulin yield obtained from pearl millet**

A statistical summary on the model fit between the actual and predicted responses is shown in Table 6.B.8.

**Table 6.B.8: Model Fit summary statistics for inulin yield**

| Source | Std. Dev. | $R^2$ | Adj $R^2$ | Pred $R^2$ | PRESS |
|---|---|---|---|---|---|
| Linear | 17.27 | 0.4914 | 0.3960 | 0.0918 | 8525.01 |
| 2FI | 15.22 | 0.6793 | 0.5313 | -1.3717 | 22262.09 |
| Quadratic | 12.14 | 0.8430 | 0.7017 | -5.7179 | 63057.75 |

In order to understand the effects of temperature, HCL concentration and heating period on inulin yield, presented three dimensional plots and contour plots have been shown in Figure 6.B.5(a)-(f). It is observed that the maximum inulin yield is obtained at 70˚C temperature, 0.8 M HCl concentration and 60 minutes of heating period with desirability of unity.

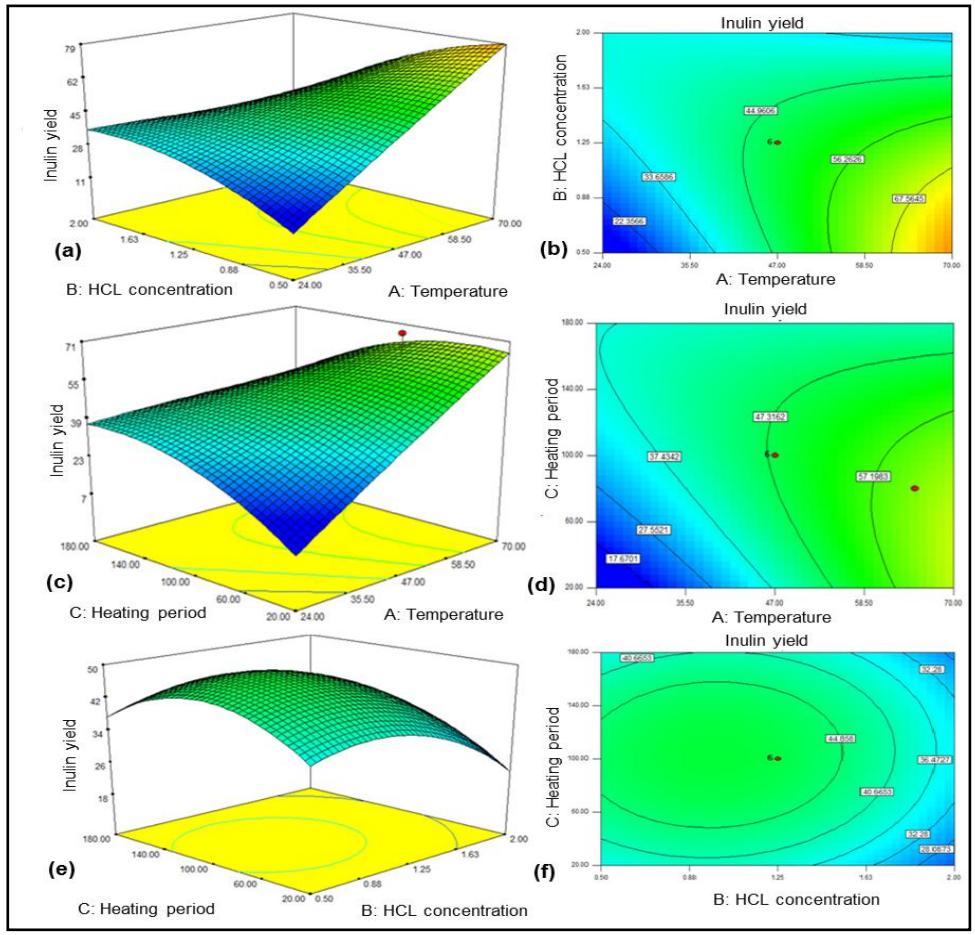



**Figure 6.B.5(a)-(f): Three dimensional plots and corresponding contour plots for optimization of pearl millet inulin yield depending on Temperature (A), HCl concentration (B) and Heating period (C)**

### 6.B.3. Optimization of concentrations of sodium alginate, surfactant and post cooling temperature for achieving maximum Encapsulation efficiency ($E_e$) of alginate/ *Lactobacillus casei* (2651 1951 RPK) microcapsules (synthesized via internal gelation) using Response surface methodology (RSM) technique

In the present investigation the concentrations of sodium alginate, surfactant (Tween 80) and post-cooling temperature were taken within the range 1 to 3%, 0.5 to 2 M and 20 to 180 min. For obtaining maximum encapsulation efficiency the concentrations of sodium alginate, surfactant (Tween 80) and post-cooling temperature are specified as 2%, 0.5% and 25°C respectively. The

experimental design matrix was prepared with the help of Design-Expert 8.1 and the experimental results of the response variable "Encapsulation efficiency"($E_e$) for 20 set of experiments are given in Table 6.B.9.

**Table 6.B.9: Experimental matrix for analysis via CCD**

| Run | Alginate concentration (%) | Surfactant concentration (%) | Post-cooloing temperature (°C) | $E_e$(%) |
|---|---|---|---|---|
| 1 | 1.50 | 1.00 | 20.00 | 85 |
| 2 | 1.50 | 1.00 | 20.00 | 85 |
| 3 | 1.50 | 1.00 | 20.00 | 85 |
| 4 | 2.00 | 0.50 | 15.00 | 90 |
| 5 | 2.00 | 1.50 | 15.00 | 72 |
| 6 | 1.00 | 1.50 | 15.00 | 82 |
| 7 | 1.00 | 0.50 | 15.00 | 91 |
| 8 | 1.00 | 1.50 | 25.00 | 92 |
| 9 | 1.50 | 1.00 | 15.00 | 90 |
| 10 | 2.00 | 1.50 | 25.00 | 94 |
| 11 | 1.00 | 0.50 | 25.00 | 87 |
| 12 | 1.50 | 1.00 | 20.00 | 85 |
| 13 | 1.50 | 1.00 | 20.00 | 85 |
| 14 | 1.50 | 1.50 | 20.00 | 82 |
| 15 | 1.00 | 1.00 | 20.00 | 82 |
| 16 | 2.00 | 1.00 | 20.00 | 94 |
| 17 | 1.50 | 1.00 | 25.00 | 83 |
| 18 | 1.50 | 1.00 | 20.00 | 85 |
| 19 | 2.00 | 0.50 | 25.00 | 98 |
| 20 | 1.50 | 0.50 | 20.00 | 89 |

Generally the dependency of independent variables on the output is given by:

$$y^{\lambda} = f(x_1, x_2, x_3.....) + e \quad (6.B.3)$$

Where, y is the output, $\lambda$ is the power of the output, "f" is the function of input variables $x_1$, $x_2$, $x_3$ and e is the error. To find the correct transformation, Box-Cox plot (Figure 6.B.6) was used where the ln (residual Sum of Squares) was plotted against $\lambda$. $\lambda$ was chosen such that the value of the residual was minimum, which is shown by green line. Since the $\lambda$ value did not fall in 95% confidence level, so the $\lambda$ was recommended by the software; which is 1.

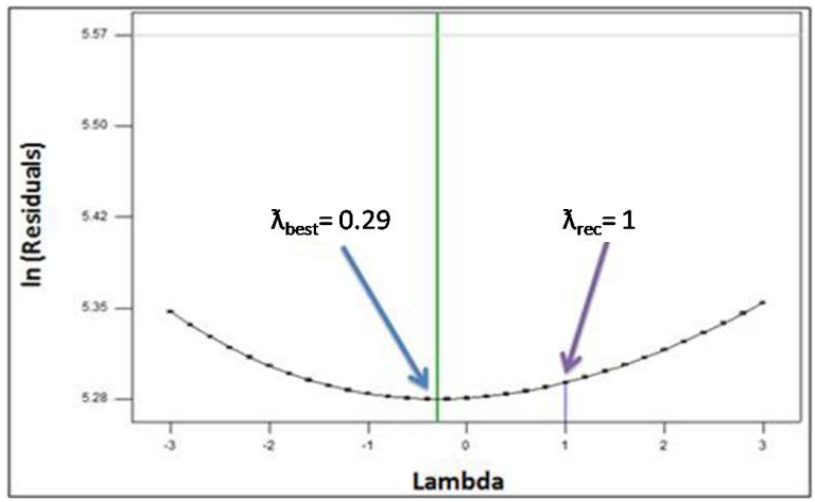



**Figure 6.B.6: Box-Cox plot for power transforms**

The polynomial quality was determined by the determination coefficients, $R^2$, $R_{adj}^2$ and predicted $R^2$. The equations for calculating $R^2$ and $R_{adj}^2$ are given in Equations (6.B.4) and (6.B.5).

$$R^2 = 1 - \frac{SS_{residual}}{SS_{model} - SS_{residual}} \quad (6.B.4)$$

$$R_{adj}^2 = 1 - \frac{SS_{residual}/DF_{residual}}{(SS_{model} + SS_{residual})/(DF_{model} + DF_{residual})} \quad (6.B.5)$$

In these equations, DF is the degree of freedom and SS is the sum of squares. The experimental data were fitted using multiple regression to the quadratic equation which is given by

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + e \quad (6.B.6)$$

where $X_1$, $X_2$ and $X_3$ are the independent variables, Y is the output variable, $a_0$ and $a_i$ are intercept and linear constant coefficients, $a_{ij}$ is regression 2-factor interaction between $X_i$ and $X_j$ and e is the residual part.

The final equation correlating $E_e$ with the independent variables is as follows:

$$E_e = 86.80 + 1.40*A - 3.30*B + 2.90*C - 2.25*A*B + 3.00*A*C + 3.50*B*C \quad (6.B.7)$$

In this equation, the terms containing B, C, AC and BC are significant. In Figure 6.B.8, 3-D surface plots illustrate the variation of $E_e$ with the interaction factors. In all these figures, the third variable (i.e, the variable that has not been plotted) has been held constant at its mean value within the given range, like, in the first plot; C, i.e; temperature has been maintained at 20°C.

To analyze the effects and interactions, analysis of variance (ANOVA) was done which is shown in Table 6.B.10. In ANOVA, any factor with p-value less than 0.05 has significant contribution to response with 95% confidence level.

**Table 6.B.10: ANOVA for response surface 2FI (2 factor interaction) model**

| Source | Sum of squars | df | Mean square | F value | p-value Prob>F |
|---|---|---|---|---|---|
| **Model** | 423.1 | 6 | 70.51667 | 4.627545 | 0.0099(significant) |
| **A-Alginate concentration** | 19.6 | 1 | 19.6 | 1.286219 | 0.2772 |
| **B-Surfactant concentration** | 108.9 | 1 | 108.9 | 7.146391 | 0.0191 |
| **C-Temperature** | 84.1 | 1 | 84.1 | 5.51893 | 0.0353 |
| **AB** | 40.5 | 1 | 40.5 | 2.657749 | 0.1270 |
| **AC** | 72 | 1 | 72 | 4.724886 | 0.0488 |
| **BC** | 98 | 1 | 98 | 6.431095 | 0.0248 |
| **Residual** | 198.1 | 13 | 15.23846 | | |
| **Lack of Fit** | 198.1 | 8 | 24.7625 | | |

| | | | | | |
|---|---|---|---|---|---|
| **Pure Error** | 0.0 | 5 | 0.0 | | |
| **Cor total** | 621.2 | 19 | | | |

The model F-value of 4.627545 and p-value of 0.0099 implies that the model is significant. Also the linear terms like B and C (A: Sodium alginate concentration; B: Surfactant concentration and C: Post-cooling temperature) and the 2FI terms – BC and AC, have significant contribution to encapsulation efficiency ($E_e$). But A and AB and are insignificant since their p-value is greater than 0.05.

In order to verify the usefulness of the model equation (Eq. 6.B.7) a comparison has been made by plotting the predicted response against the experimentally obtained response. This is shown in Figure 6.B.7.

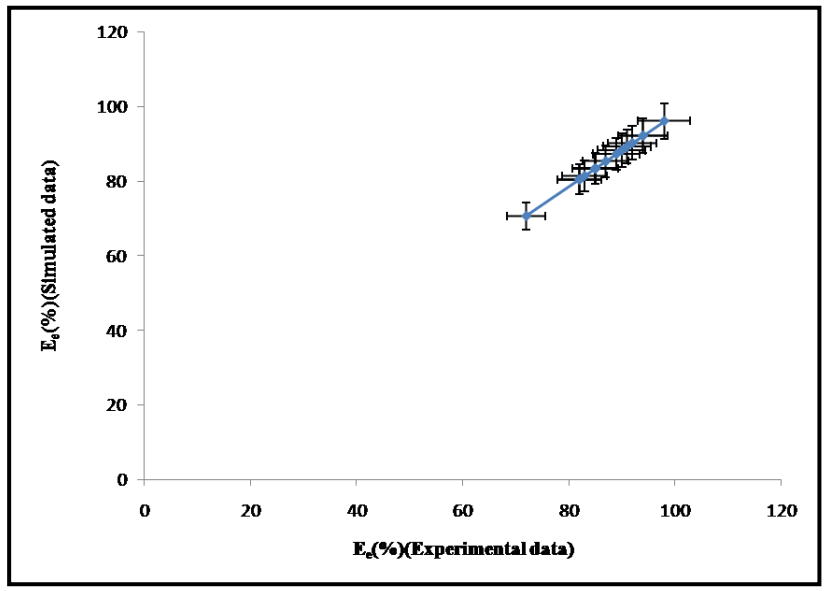



**Figure 6.B.7: Comparison of experimental and simulated values of Encapsulation efficiency ($E_e$)**

The values of $R^2$ and $R_{adj}^2$ are presented in Table 6.B.11 and since we aim at maximizing the sum of the values of $R_{adj}^2$ and predicted $R^2$, it clearly indicates that the 2 factor interaction (2FI) equation provides the best fit since the sum of $R_{adj}^2 = 0.5339$ , is maximum.

**Table 6.B.11: Model Summary Statistics**

| Source | Std. Dev. | R-Squared | Adjusted R-Squared | Predicted R-Squared | Comment |
|---|---|---|---|---|---|
| Linear | 5.05 | 0.3422 | 0.2189 | -0.3106 | |
| 2FI | 3.90 | 0.6811 | 0.5339 | -0.3456 | Suggested |
| Quadratic | 3.96 | 0.7477 | 0.5206 | -0.9509 | |
| Cubic | 0.47 | 0.9978 | 0.9932 | -1.6433 | Aliased |

In order to understand the effect of sodium alginate concentration, surfactant concentration and temperature on encapsulation efficiency, the result obtained through response surface methodology technique have been presented in three dimensional plots and corresponding contour plots shown in Figure 6.B.8.(a)-(f).

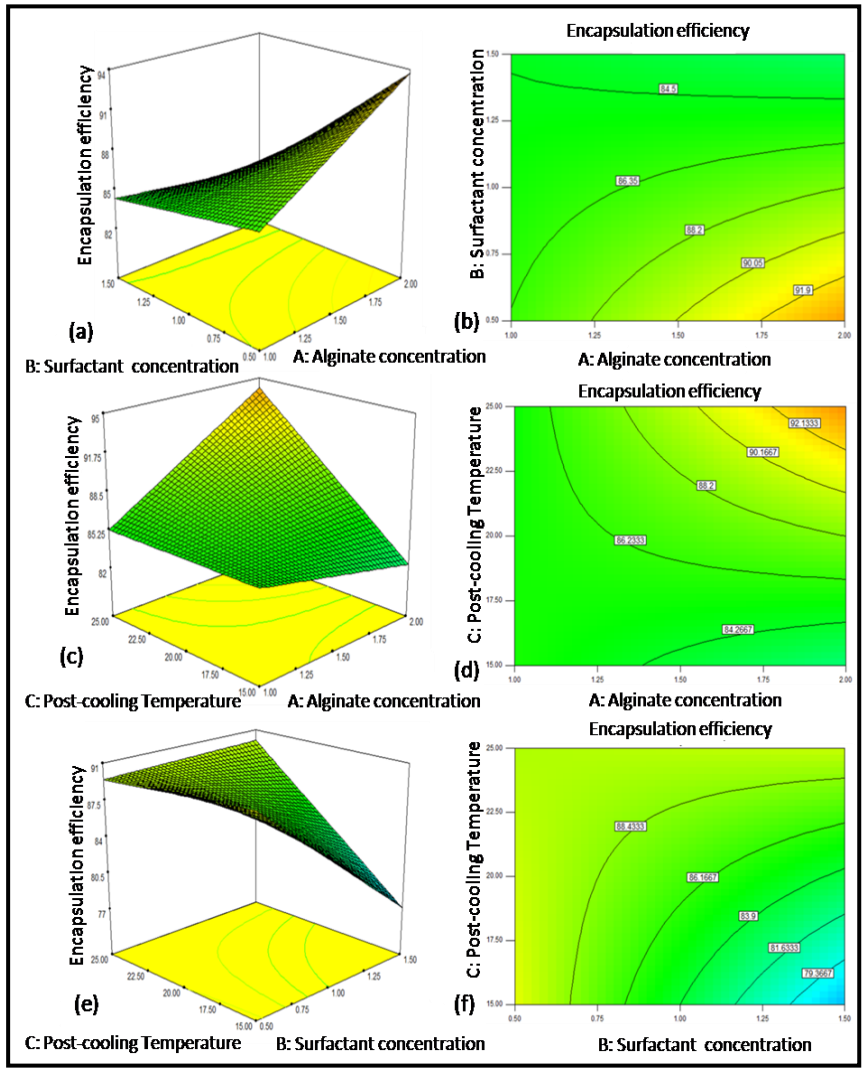



**Figure 6.B.8(a)-(f): 3-D surface and contour plots depicting the variation of encapsulaton efficiency with A: Alginate concentration, B: Surfactant concentration and C: Post cooling temperature**

From the above section, we summarize that Encapsulation efficiency ($E_e$) is a strong function of alginate concentration, surfactant concentration and post-cooling temperature, within the given ranges of the respective variables. However, $E_e$ decreases with the increase in surfactant concentration. This may be due to the fact that increased surfactant molecules may lead to excessive perturbation, disrupting the formation, the above plots suggest that $E_e$ increases as the alginate concentration and post-cooling temperature are increased. Higher temperature and alginate may lead to better bonding within the alginate core.

For the maximization of Encapsulation efficiency with the input parameters within the specified ranges, Design Expert 8.1 suggested 18 solutions, of which the solution having a maximum desirability of 0.929; was selected. These were obtained with alginate concentration of 2%; surfactant concentration of 0.5% at a post-cooling temperature of 25°C, which corresponded to an encapsulation efficiency of 98%.

## 6.C. Studies on cell growth dynamics of *Lactobacillus casei* (2651 1951 RPK)

Cell growth dynamics of *Lactobacillus casei* have been studied in detail in order to understand the effect of prebiotic inulin on the selected probiotic, *Lactobacillus casei*. Two different approaches, viz., cell growth study in complete absence of inulin and in presence of both lactose and inulin have been investigated in order to get a quantitative idea on the synbiotic effects of prebiotic-probiotic interaction.

### 6.C.1. Studies of cell growth dynamics in presence of lactose as carbon source

Cell growth dynamics of *Lactobacillus casei*, a probiotic under investigation has been carried out by following the cell growth in presence of lactose as sole electron donor. This study in complete absence of the prebiotic inulin has provided a sound comparison basis with the cells grown in presence of both inulin and lactose.

Experimental data of *Lactobacills casei* cell concentration time histories with initial lactose concentration as parameter are given in Table 6.C.1.

**Table 6.C.1: Cell growth dynamics of *Lactobacillus casei* in presence of lactose as carbon source**

| Time (h) | Initial lactose concentration(g/L) | | |
|---|---|---|---|
| | 10 | 20 | 30 |
| | Cell concentration (g/L) | | |
| 0 | 0.0030 | 0.0009 | 0.0007 |
| 1 | 0.0056 | 0.0018 | 0.0014 |
| 2 | 0.0106 | 0.0036 | 0.0030 |

| | | | |
|---|---|---|---|
| 3 | 0.0198 | 0.0073 | 0.0061 |
| 4 | 0.0371 | 0.0147 | 0.0127 |
| 5 | 0.0694 | 0.0295 | 0.0261 |
| 6 | 0.1294 | 0.0591 | 0.0539 |
| 7 | 0.2401 | 0.1187 | 0.1111 |
| 8 | 0.4400 | 0.2376 | 0.2288 |
| 9 | 0.7865 | 0.4741 | 0.4705 |
| 10 | 1.9378 | 1.3378 | 1.4333 |
| 11 | 1.8493 | 1.8174 | 1.9555 |
| 12 | 1.9942 | 3.2521 | 3.8478 |
| 13 | 2.0026 | 3.9935 | 5.9550 |
| 14 | 2.0030 | 4.0009 | 6.0007 |
| 22 | 2.0030 | 4.0009 | 6.0007 |
| 23 | 2.0030 | 4.0009 | 6.0007 |
| 24 | 2.0030 | 4.0009 | 6.0007 |

In order to understand the nature of cell growth dynamics qualitatively, a plot of cell concentration against time with three different initial lactose concentrations as a parameter has been made and is shown in Figure 6.C.1.

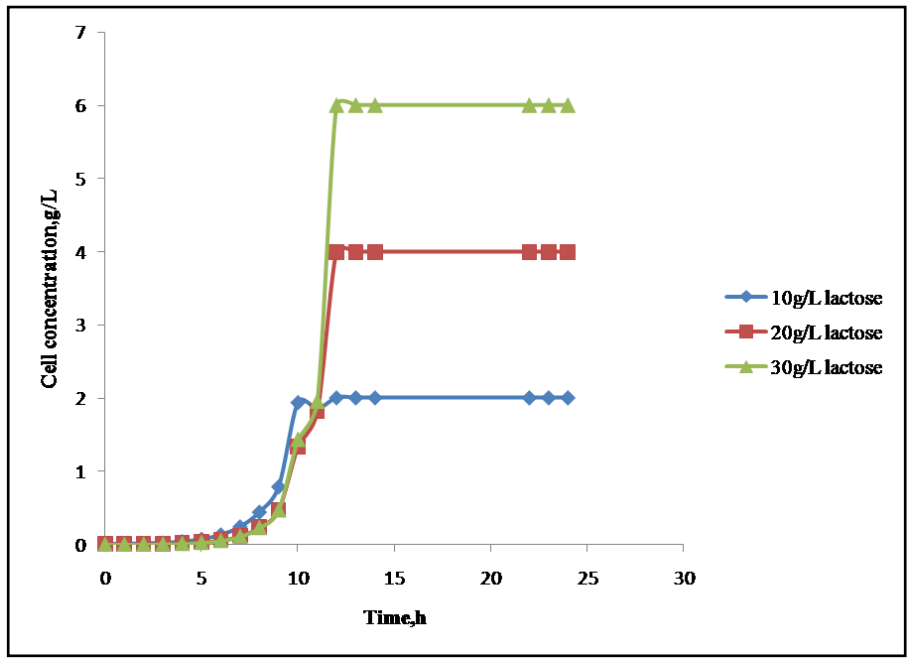



**Figure 6.C.1: *Lactobacillus casei* (2651 1951 RPK) cell concentration time history plot in presence of lactose as sole carbon source**

It is observed from the figure that there are distinct appearance of lag phase, exponential phase, stationary phase and death phase in the cell growth history. Such type of cell dynamics are qualitative indication of the validity of Monods classical form of substrate uninhibited unstructured model. It is also observed that with increase in initial lactose concentration the cell growth increases and the rate of cell growth is also higher with increase in substrate concentration. This is an expected behavior since with the availability of more food (lactose in the present case) generation of cell will be more with a higher rate.

In order to predict cell growth dynamics a priori quantitatively, the Monod substrate uninhibited unstructured model equation has been selected in its classical form:

$$\mu = \frac{\mu_m C_s}{K_s + C_s} \qquad (6.C.1)$$

$$\mu = \frac{1}{C_x}\frac{dC_x}{dt} \qquad (6.C.2)$$

Where,

$\mu$= Specific cell growth rate, $h^{-1}$

$\mu_{m=}$ Maximum specific cell growth rate, $h^{-1}$

$K_s$= Substrate saturation constant, g/L

$C_s$= Concentration of lactose, g/L

X= concentration of cell, g/L

Equation 6.C.1 contains two intrinsic kinetic parameters $\mu_m$ and $K_s$. In order to verify the validity of Monods equation in the present case and the subsequent evaluation of $\mu_m$ and $K_s$, Equation 6.C.1 has been rearranged in double reciprocal form as follows:

$$\frac{1}{\mu} = \frac{K_s}{\mu_{max}} \frac{1}{C_s} + \frac{1}{\mu_{max}} \qquad (6.C.3)$$

Thus, a plot of $1/\mu$ versus $1/C_s$ will provide a straight line if Monod equation is valid.
In the present investigation the specific cell growth rate ($\mu$) has been computed from the slope of cell growth curves shown in Figure 6.C.1 in the exponential phase region and subsequently dividing it by the average cell concentration within this selected range. The computed data are shown in Table 6.C.2.

**Table 6.C.2. Specific cell growth rate as a function of initial lactose concentration**

| **Substrate concentration, g/L** | **Specific cell growth rate,$h^{-1}$** | **$1/C_S$, L/g** | **$1/\mu$, h** |
|---|---|---|---|
| 10 | 0.6299 | 0.100 | 1.587554 |
| 20 | 0.6984 | 0.050 | 1.431844 |
| 30 | 0.7247 | 0.033 | 1.379881 |

A plot of $1/\mu$ versus $1/C_S$ has been made using the data shown in Table 6.C.2. This is shown in Figure 6.C.2.

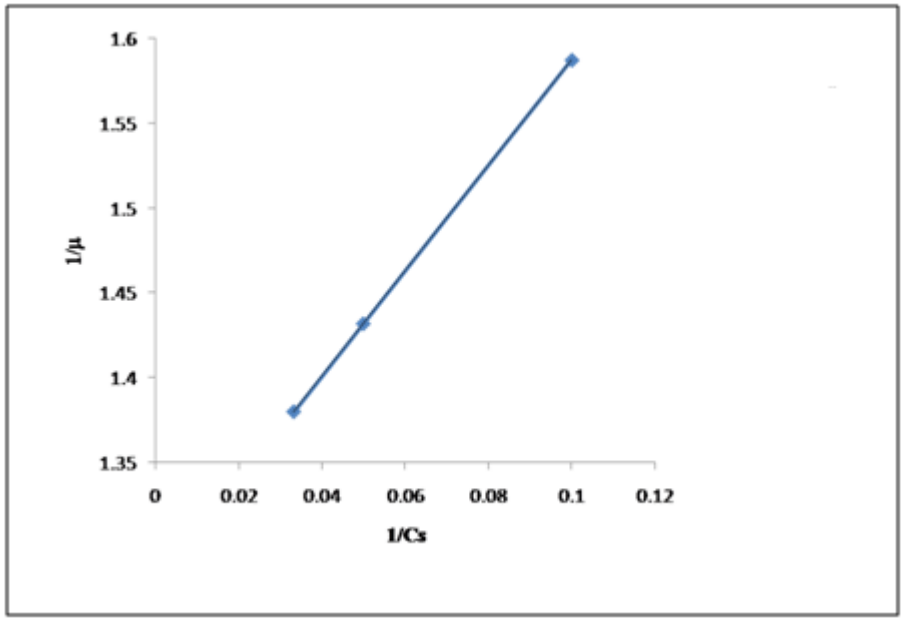



**Figure 6.C.2: Double reciprocal plot of specific cell growth rate and substrate concentration**

It is evident from the figure that a reasonably good plot has been obtained showing the validity of Monod equation in predicting the cell growth dynamics in the present case. From the linear plot the slope and intercept have been computed as 3.114 and 1.276 respectively. Thus, $K_s/\mu_m$=3.114 and 1/ $\mu_m$= 1.276. The intrinsic kinetic parameters $K_s$ and $\mu_m$ are therefore

$K_s$= 2.44g/L, $\mu_m$= 0.7836 $h^{-1}$

The *Lactobacillus casei* growth rate equation in presence of lactose as only carbon source can now be written as

$$r_x = \frac{0.7836 h^{-1} C_s C_x}{2.44 + C_s} \frac{g cell form ed}{L.h} \qquad (6.C.4)$$

### 6.C.2. Studies of cell growth dynamics in presence of commercial inulin as only carbon source

In order to determine the sole effect of prebiotic inulin on the probiotic *Lactobacillus casei* under investigation, the results obtained from a programmed experiment on time history of cell growth are shown in Table 6.C.3.

**Table 6.C.3: Concentration time history data of *Lactobacillus casei* (2651 1951 RPK) in presence of inulin**

| Time (h) | Initial inulin concentration(g/L) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **0.035** | **0.05** | **0.095** | **0.164** | **0.175** | **0.322** | **0.487** | **0.624** |
| | **Cell concentration (g/L)** | | | | | | | |
| **0** | 0.0700 | 0.0700 | 0.0700 | 0.0700 | 0.0700 | 0.0700 | 0.0700 | 0.0700 |
| **1** | 0.0705 | 0.0707 | 0.0711 | 0.0714 | 0.0714 | 0.0716 | 0.0715 | 0.0714 |
| **2** | 0.0710 | 0.0714 | 0.0721 | 0.0728 | 0.0729 | 0.0732 | 0.0731 | 0.0728 |
| **3** | 0.0715 | 0.0720 | 0.0732 | 0.0742 | 0.0743 | 0.0748 | 0.0746 | 0.0743 |
| **4** | 0.072 | 0.0727 | 0.0742 | 0.0756 | 0.0758 | 0.0765 | 0.0763 | 0.0758 |
| **5** | 0.0724 | 0.0733 | 0.0753 | 0.0771 | 0.0773 | 0.0783 | 0.0779 | 0.0773 |
| **6** | 0.0729 | 0.0739 | 0.0763 | 0.0785 | 0.0788 | 0.0800 | 0.0796 | 0.0789 |
| **7** | 0.0733 | 0.0745 | 0.0774 | 0.0800 | 0.0803 | 0.0818 | 0.0814 | 0.0805 |
| **8** | 0.0737 | 0.0751 | 0.0784 | 0.0815 | 0.0818 | 0.0837 | 0.0831 | 0.0821 |
| **9** | 0.0741 | 0.0757 | 0.0794 | 0.0829 | 0.0834 | 0.0855 | 0.0850 | 0.0838 |
| **10** | 0.0745 | 0.0762 | 0.0804 | 0.0844 | 0.0850 | 0.0875 | 0.0868 | 0.0855 |
| **11** | 0.0749 | 0.0768 | 0.0814 | 0.0859 | 0.0865 | 0.0894 | 0.0887 | 0.0873 |
| **12** | 0.0753 | 0.0773 | 0.0824 | 0.0874 | 0.0881 | 0.0914 | 0.0907 | 0.0891 |
| **13** | 0.0757 | 0.0778 | 0.0833 | 0.0888 | 0.0897 | 0.0935 | 0.0927 | 0.0909 |
| **14** | 0.0760 | 0.0783 | 0.0843 | 0.0903 | 0.0913 | 0.0956 | 0.0947 | 0.0928 |
| **24** | 0.0789 | 0.0825 | 0.0927 | 0.1045 | 0.1069 | 0.1186 | 0.1182 | 0.1142 |
| **25** | 0.0791 | 0.0829 | 0.0934 | 0.1058 | 0.1084 | 0.1211 | 0.1208 | 0.1166 |
| **26** | 0.0794 | 0.0832 | 0.0941 | 0.1070 | 0.1098 | 0.1236 | 0.1235 | 0.1191 |
| **27** | 0.0796 | 0.0835 | 0.0948 | 0.1083 | 0.1113 | 0.1262 | 0.1263 | 0.1217 |
| **28** | 0.0798 | 0.0838 | 0.0955 | 0.1095 | 0.1127 | 0.1288 | 0.1292 | 0.1243 |
| **29** | 0.0800 | 0.0841 | 0.0961 | 0.1107 | 0.1140 | 0.1314 | 0.1321 | 0.1269 |
| **30** | 0.0802 | 0.0844 | 0.0967 | 0.1118 | 0.1154 | 0.1340 | 0.1351 | 0.1297 |
| **31** | 0.0803 | 0.0847 | 0.0973 | 0.1129 | 0.1167 | 0.1367 | 0.1381 | 0.1325 |
| **32** | 0.0805 | 0.0849 | 0.0978 | 0.1140 | 0.1179 | 0.1393 | 0.1412 | 0.1354 |
| **33** | 0.0807 | 0.0852 | 0.0984 | 0.1150 | 0.1192 | 0.142 | 0.1444 | 0.1383 |
| **34** | 0.0808 | 0.0854 | 0.0989 | 0.1160 | 0.1203 | 0.1446 | 0.1477 | 0.1414 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **35** | 0.0810 | 0.0857 | 0.0994 | 0.1170 | 0.1215 | 0.1473 | 0.151 | 0.1445 |
| **36** | 0.0811 | 0.0859 | 0.0999 | 0.1179 | 0.1226 | 0.1499 | 0.1544 | 0.1476 |
| **37** | 0.0813 | 0.0861 | 0.1003 | 0.1188 | 0.1237 | 0.1525 | 0.1578 | 0.1509 |
| **38** | 0.0814 | 0.0863 | 0.1007 | 0.1196 | 0.1247 | 0.1551 | 0.1613 | 0.1543 |
| **39** | 0.0815 | 0.0865 | 0.1011 | 0.1204 | 0.1256 | 0.1576 | 0.1649 | 0.1577 |
| **40** | 0.0816 | 0.0866 | 0.1015 | 0.1212 | 0.1266 | 0.1601 | 0.1685 | 0.1612 |
| **41** | 0.0818 | 0.0868 | 0.1019 | 0.1219 | 0.1274 | 0.1625 | 0.1722 | 0.1648 |
| **42** | 0.0819 | 0.0870 | 0.1023 | 0.1226 | 0.1283 | 0.1649 | 0.1759 | 0.1685 |
| **43** | 0.0820 | 0.0871 | 0.1026 | 0.1232 | 0.1291 | 0.1672 | 0.1797 | 0.1723 |
| **44** | 0.0821 | 0.0873 | 0.1029 | 0.1238 | 0.1298 | 0.1695 | 0.1835 | 0.1761 |
| **45** | 0.0822 | 0.0874 | 0.1032 | 0.1244 | 0.1305 | 0.1716 | 0.1874 | 0.1801 |
| **46** | 0.0822 | 0.0875 | 0.1035 | 0.1249 | 0.1312 | 0.1737 | 0.1913 | 0.1842 |
| **47** | 0.0823 | 0.0877 | 0.1038 | 0.1255 | 0.1318 | 0.1757 | 0.1951 | 0.1883 |
| **48** | 0.0824 | 0.0878 | 0.104 | 0.1259 | 0.1324 | 0.1776 | 0.199 | 0.1926 |

A wide range of initial inulin concentration, viz., 0.035 to 0.624 g/L were employed for this purpose. The cell growth dynamics as a function of initial inulin concentration are shown in Figure 6.C.3.

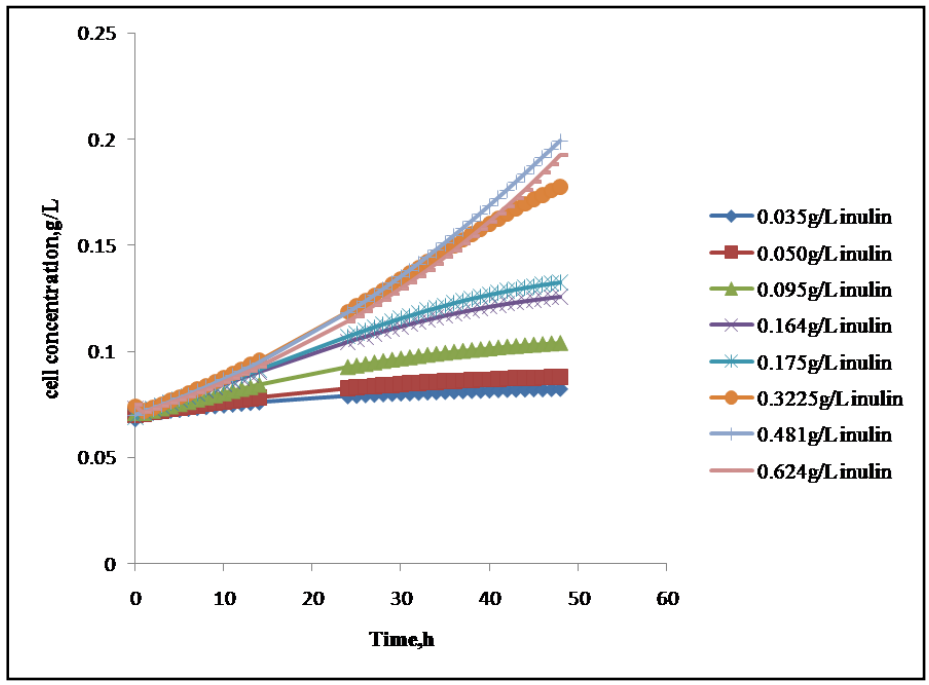



**Figure 6.C.3: Concentration time history of *Lactobacillus casei* (2651 1951 RPK) in presence of inulin as sole carbon source with initial inulin concentration as parameter**

It is observed from the figure that in each case presence of lag phase and exponential phase are predominantly visible. Interestingly it is also observed that cell concentrations rapidly increases with increase in inulin concentration upto 0.175g/L, a reverse trend is observed above inulin concentration 0.175g/L. Qualitatively such behavior indicates that cell growth dynamics is a function of initial inulin concentration and follow a different route beyond a critical inulin concentration.

In order to understand the cell growth behavior in a quantitative manner specific cell growth rates (μ) for different initial inulin concentration have been computed from Equation 6.C.2 and the results are shown in Table 6.C.4.

**Table 6.C.4: Specific cell growth rate of *Lactobacillus casei* (2651 1951 RPK) as a function of initial inulin concentration**

| Substrate (inulin) concentration ($C_I$), g/L | Specific cell growth rate (μ),$h^{-1}$ | $1/C_I$, L/g | 1/μ, h |
|---|---|---|---|
| 0.035 | 0.006 | 28.57143 | 166.6667 |
| 0.050 | 0.009 | 20.00000 | 111.1111 |
| 0.095 | 0.014 | 10.52632 | 71.4285 |
| 0.164 | 0.019 | 6.097561 | 52.6315 |
| 0.175 | 0.019 | 5.714286 | 52.6315 |
| 0.322 | 0.022 | 3.100775 | 45.4545 |
| 0.481 | 0.021 | 2.079002 | 47.6190 |
| 0.624 | 0.020 | 1.602564 | 50.0000 |

In order to understand the effect of initial inulin concentration on specific cell growth rate of *Lactobacillus casei* a plot of specific cell growth rate (μ) and initial inulin concentration ($C_I$) has been made and is shown in Figure 6.C.4.

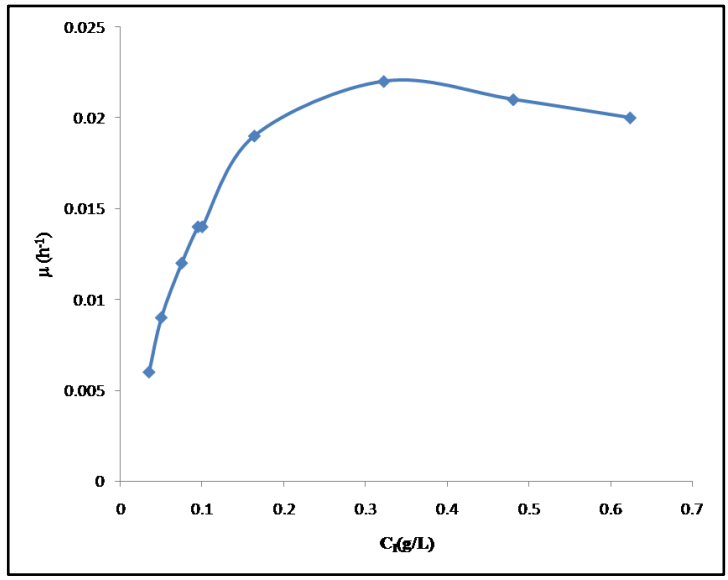



**Figure 6.C.4: Specific cell growth rate (μ) versus initial inulin concentration ($C_I$)**

It is observed that specific cell growth rate increases monotonically upto 0.3225 g/L of inulin above which the rate declines rapidly indicating the inhibitory effect of inulin on cell growth above the critical inulin concentration 0.3225g/L. It therefore follows that the cell growth dynamics should be explained in terms of a suitable model equation associated with non-competitive substrate inhibition. Since, the most celebrated non-competitive substrate inhibited model equation proposed by Haldane is widely used in such cases, in the present investigation the Haldane equation given by its simplified form given by

$$\mu_I = \frac{\mu_{max_I} C_I}{K_{S_I} + C_I + \frac{C_I^2}{K_I}} \qquad (6.C.5)$$

Where,

$\mu_I$ = Specific growth rate of *Lctobacillus casei* in presence of inulin, $h^{-1}$

$\mu_{max_I}$ = Maximum specific cell growth rate in presence of inulin, $h^{-1}$

$K_{S_I}$ = Substrate saturation constant for inulin, g/L

$C_I$ = Concentration of inulin, g/L

has been selected to test its validity towards predicting the cell growth dynamics of *Lactobacillus casei*. This equation contains two intrinsic kinetic parameters, viz., $\mu_m$ and $K_s$ and one thermodynamic constant $K_I$. Numerical analysis has been carried out by solving the non linear Equation 6.C.5 and Equation 5.6 to evaluate the values of $\mu_m$, $K_s$ and $K_I$. A selected range of experimental data ranging from specific cell growth rate 0.014 $h^{-1}$ to 0.02 $h^{-1}$and their corresponding inulin concentration have been selected from Table 6.C.4 for evaluating the parameters. The results are given in Table 6.C.5.

**Table 6.C.5: Simulation constants present in Haldane equation**

| **Kinetic Parameters** | | |
|---|---|---|
| **$\mu_{max}$($h^{-1}$)** | **$K_s$(g/L)** | **$K_I$(g/L)** |
| 0.05 | 0.2 | 0.52 |

The Haldane equation can therefore be written as

$$\mu_I = \frac{0.05h^{-1}C_I}{0.2 + C_I + \frac{C_I^2}{0.52}}, h^{-1} \quad (6.C.6)$$

The proposed equation has now been tested for its ability to predict real time data with the help of an extended range of experimental data. Table 6.C.6 shows the predicted and experimental values of specific cell growth rate for five different initial inulin concentrations not used for evaluating kinetic parameters.

**Table 6.C.6: Comparison of simulated and experimental data of specific cell growth rate at five different initial inulin concentrations**

| Substrate (inulin) concentration, g/L | Specific cell growth rate,$h^{-1}$(Experimental) | Specific cell growth rate,$h^{-1}$(Predicted) |
|---|---|---|
| 0.035 | 0.0059 | 0.006 |
| 0.050 | 0.0089 | 0.009 |
| 0.075 | 0.0119 | 0.012 |
| 0.164 | 0.019 | 0.018 |
| 0.25 | 0.020 | 0.021 |
| 0.481 | 0.0209 | 0.021 |

In order to find out the deviation between the experimental and simulated value from the Haldane equation a comparative plot of experimental and predicted values of μ at different $C_I$ has been made and is shown in Figure 6.C.5. It is evident from the figure that the predicted values from the Haldane equation are well within the confidence level. It may therefore be concluded that Haldane equation can be taken as the model equation for predicting cell growth rate in presence of synbiotic effect of inulin on *Lactobacillus casei*.

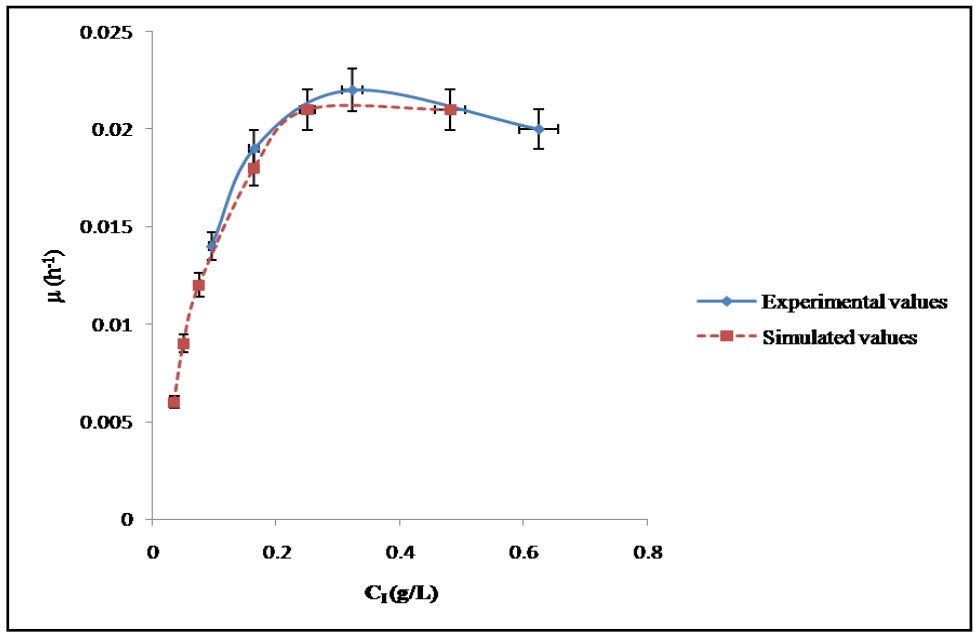



**Figure 6.C.5: Comparative plot of experimental and predicted values of specific cell growth rate**

### 6.C.3. Studies on combined effect of commercial inulin and lactose as electron donors on the cell growth of *Lactobacillus casei* (2651 1951 RPK)

The previous two sections show the investigation results on the effect of single electron donor, viz., lactose and inulin on the cell growth kinetics of *Lactobacillus casei*. It is, however, understood that while maintenance of cell growth of *Lactobacillus casei* requires the presence of lactose, inulin strongly enhances the cell growth rate. Thus studies on the combination of two electron donors, viz., lactose and inulin are essential to understand the synbiotic effect of prebiotic and probiotic. Experimental results on the cell growth of *Lactobacillus casei* in presence of different initial inulin concentrations ranging from 0.164-0.624 g/L at three different initial concentrations of lactose are given in Tables 6.C.7.(a) through 6.C.7.(c).

**Table 6.C.7.(a): Concentration time history of *Lactobacillus casei* in presence of inulin at 10g/L initial lactose concentration**

| Time (h) | Initial inulin concentration(g/L) | | |
|---|---|---|---|
| | **0.164** | **0.3225** | **0.624** |
| | **Cell concentration (g/L)** | | |
| 0 | 0.0030 | 0.0032 | 0.0034 |
| 1 | 0.0056 | 0.0068 | 0.0078 |
| 2 | 0.0106 | 0.0306 | 0.0806 |
| 3 | 0.0598 | 0.0798 | 0.1998 |
| 4 | 0.0971 | 0.1571 | 0.3671 |
| 5 | 0.1694 | 0.2694 | 0.6691 |
| 6 | 0.3294 | 0.3994 | 0.9995 |
| 7 | 0.4401 | 0.5401 | 1.2401 |
| 8 | 0.7400 | 0.8300 | 1.5400 |
| 9 | 0.9865 | 1.2865 | 1.7865 |
| 10 | 1.9600 | 1.9800 | 2.0100 |
| 11 | 4.8493 | 6.8493 | 8.5493 |
| 12 | 8.9710 | 9.3400 | 9.1600 |
| 13 | 8.5226 | 9.1260 | 9.0760 |
| 14 | 8.2630 | 9.0600 | 9.0400 |
| 22 | 8.0500 | 9.0130 | 9.0050 |
| 23 | 8.0300 | 9.0050 | 9.0030 |
| 24 | 8.0030 | 9.0009 | 9.0007 |

**Table 6.C.7.(b): Concentration time history of *Lactobacillus casei* in presence of inulin at 20g/L initial lactose concentration**

| Time | Initial inulin concentration(g/L) | | |
|---|---|---|---|
| | **0.164** | **0.3225** | **0.624** |
| | **Cell concentration (g/L)** | | |
| 0 | 0.0009 | 0.00092 | 0.00094 |
| 1 | 0.0017 | 0.0051 | 0.0050 |
| 2 | 0.0050 | 0.0080 | 0.0160 |
| 3 | 0.0730 | 0.0760 | 0.0634 |
| 4 | 0.1247 | 0.1697 | 0.1450 |
| 5 | 0.2595 | 0.2995 | 0.3495 |
| 6 | 0.4791 | 0.5671 | 0.7691 |
| 7 | 0.6187 | 0.8987 | 0.9787 |
| 8 | 0.9376 | 1.1376 | 1.0976 |
| 9 | 1.1741 | 1.2541 | 1.1741 |
| 10 | 1.3600 | 1.3900 | 1.4200 |
| 11 | 4.8174 | 5.8374 | 1.8174 |
| 12 | 8.2100 | 8.5070 | 8.3930 |
| 13 | 7.9935 | 8.2435 | 8.1945 |
| 14 | 7.3409 | 8.1109 | 8.0601 |
| 22 | 7.0509 | 8.0709 | 8.0097 |
| 23 | 7.0309 | 8.0059 | 7.0900 |
| 24 | 7.0009 | 8.0010 | 7.0060 |

**Table 6.C.7.(c): Concentration time history of *Lactobacillus casei* in presence of inulin at 30g/L initial lactose concentration**

| Time | Initial inulin concentration(g/L) | | |
|---|---|---|---|
| | **0.164** | **0.3225** | **0.624** |
| | **Cell concentration (g/L)** | | |
| 0 | 0.0007 | 0.00072 | 0.00075 |
| 1 | 0.0014 | 0.0025 | 0.0022 |
| 2 | 0.0030 | 0.0070 | 0.0060 |
| 3 | 0.0051 | 0.0061 | 0.0091 |
| 4 | 0.0727 | 0.0127 | 0.0526 |
| 5 | 0.1561 | 0.0541 | 0.0983 |
| 6 | 0.3539 | 0.0939 | 1.0159 |
| 7 | 0.9831 | 1.0611 | 1.0831 |
| 8 | 1.0988 | 1.1288 | 1.1235 |
| 9 | 1.1905 | 1.2705 | 1.2300 |
| 10 | 1.4600 | 1.4900 | 1.5200 |
| 11 | 5.9555 | 6.4500 | 5.5255 |
| 12 | 9.8975 | 10.1500 | 9.9924 |
| 13 | 9.5000 | 10.0650 | 9.4550 |
| 14 | 9.2107 | 10.0217 | 9.2107 |
| 22 | 9.0707 | 10.0090 | 9.0507 |
| 23 | 9.0023 | 10.0010 | 9.0097 |
| 24 | 9.0011 | 10.0007 | 9.0005 |

In order to understand the effect of initial inulin concentration in presence of lactose the experimental data of cell concentration time history have been plotted with initial inulin concentration as parameter at three different initial lactose concentrations viz.,10,20,30 g/L. These are shown in Figures 6.C.6(a) through 6.C.6(c).

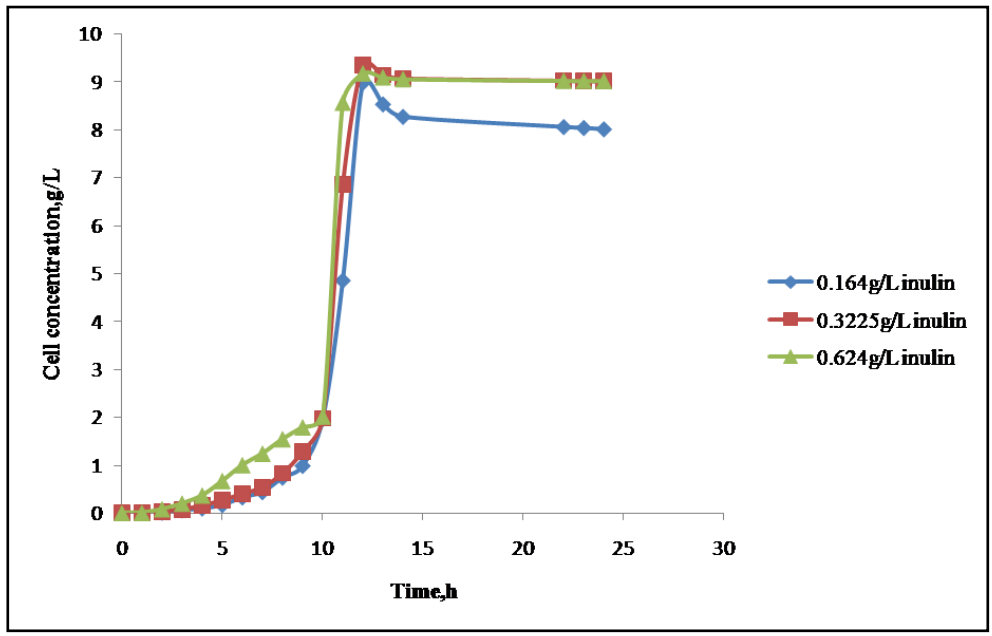



**Figure 6.C.6(a): Experimentally obtained cell response at initial lactose concentration 10g/L**

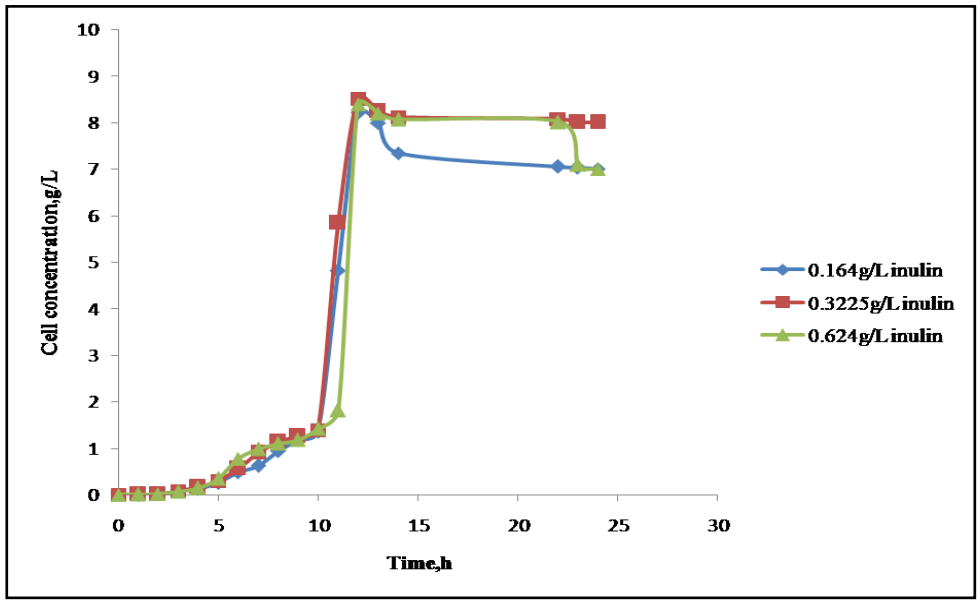



**Figure 6.C.6(b): Experimentally obtained cell response at initial lactose concentration 20g/L**

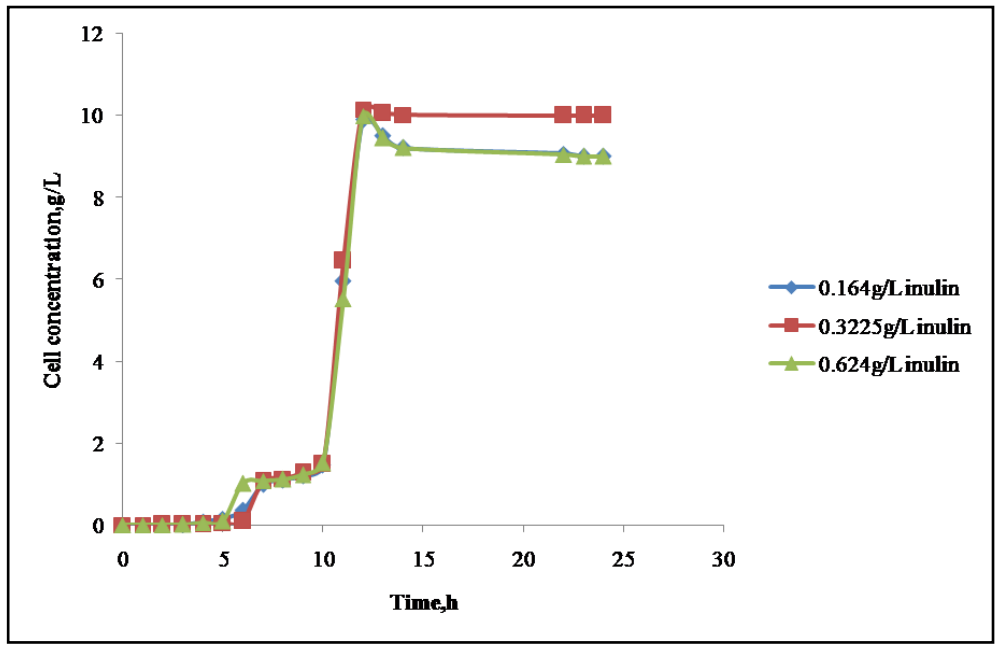



**Figure 6.C.6(c): Experimentally obtained cell response at initial lactose concentration 30g/L**

It is evident from the figures that within the range of inulin concentration studied, increase of inulin concentration increases the cell growth at all levels of initial lactose concentration. This observation is in sharp contrast with the cell growth of *Lactobacillus casei* in presence of single substrate inulin, where the cell growth is maximum at 0.175g/L above which cell growth declines. It may therefore be concluded that in the present two substrate system lactose plays a dominating role in the cell growth of *Lactobacillus casei*. It is also evident from the figures that three distinct growth regions viz., lag phase, exponential phase and stationary phase are visibly present.

In order to model the cell growth rate theoretically for the present system a summative type rate equation combining Monod equation and Haldane equation as suggested by Blunch & Clerk has been tested here[9]. The summative type cell growth rate equation is given by

$$\mu = \frac{\mu_{m_1} C_s}{K_s + C_s} + \frac{\mu_{m_2} C_I}{K_{s_I} + C_I + \frac{C_I^2}{K_I}} \frac{\text{gcellforme d}}{\text{L.h}} \qquad (6.C.7)$$

Where,

$\mu_{m_1}$ = Specific growth rate of Monod equation, 0.7836 $h^{-1}$

$\mu_{m_2}$ = Specific growth rate of Haldane equation, 0.05 $h^{-1}$

$K_{s_I}$ = Substrate saturation constant for inulin, 0.2 g/L

$K_I$ = Substrate inhibition constant, 0.52 g/L

$K_s$ = Lactose saturation constant, 2.44 g/L

The proposed cell response equation is

$$r_x = \frac{dC_x}{dt} = \frac{0.7836h\ ^1C_sC_x}{2.44 + C_s} + \frac{0.05h\ ^1C_IC_x}{0.2 + C_I + \frac{C_I^{\ 2}}{0.52}} \quad (6.C.8)$$

The specific cell growth rate is given by

$$\mu = \mu_1 + \mu_2 = \frac{0.7836h\ ^1C_s}{2.44 + C_s} + \frac{0.05h\ ^1C_I}{0.2 + C_I + \frac{C_I^{\ 2}}{0.52}} \quad (6.C.9)$$

In order to verify the validity of Equation 6.C.9 simulated data of the specific cell growth rate at different levels of inulin and lactose concentrations are given in Table 6.C.8.

**Table 6.C.8: Simulated values of specific cell growth rate at different concentrations of lactose and inulin**

| $C_s$ (g/L) | $C_I$(g/L) | $\mu$ ($h^{-1}$) |
|---|---|---|
| 10 | 0.1640 | 0.6487 |
| 10 | 0.3225 | 0.6570 |
| 10 | 0.6240 | 0.6480 |
| 20 | 0.1640 | 0.7177 |
| 20 | 0.3225 | 0.7265 |
| 20 | 0.6240 | 0.7178 |
| 30 | 0.1640 | 0.7440 |
| 30 | 0.3225 | 0.7525 |
| 30 | 0.6240 | 0.7438 |

It is evident from the figures that in each level of lactose concentration a brief lag phase followed by exponential phase and satationary phase are predominantly present. The specific cell growth rate (μ) have been computed in each case using Equation 6.C.2 and the results are shown in Table 6.C.9.

**Table 6.C.9: Experimentally obtained specific cell growth rate in presence of lactose and inulin**

| $C_s$ (g/L) | $C_I$(g/L) | $\mu_{expt}$ ($h^{-1}$) |
|---|---|---|
| 10 | 0.1640 | 0.6422 |
| 10 | 0.3225 | 0.6504 |
| 10 | 0.6240 | 0.6410 |
| 20 | 0.1640 | 0.7162 |
| 20 | 0.3225 | 0.7192 |
| 20 | 0.6240 | 0.7106 |
| 30 | 0.1640 | 0.7365 |
| 30 | 0.3225 | 0.7440 |
| 30 | 0.6240 | 0.7360 |

In order to verify the validity of Equation 6.C.9 both experimental and simulated values of specific cell growth rate have been plotted against concentration of inulin with concentration of lactose as parameter. This is shown in Figure 6.C.7.

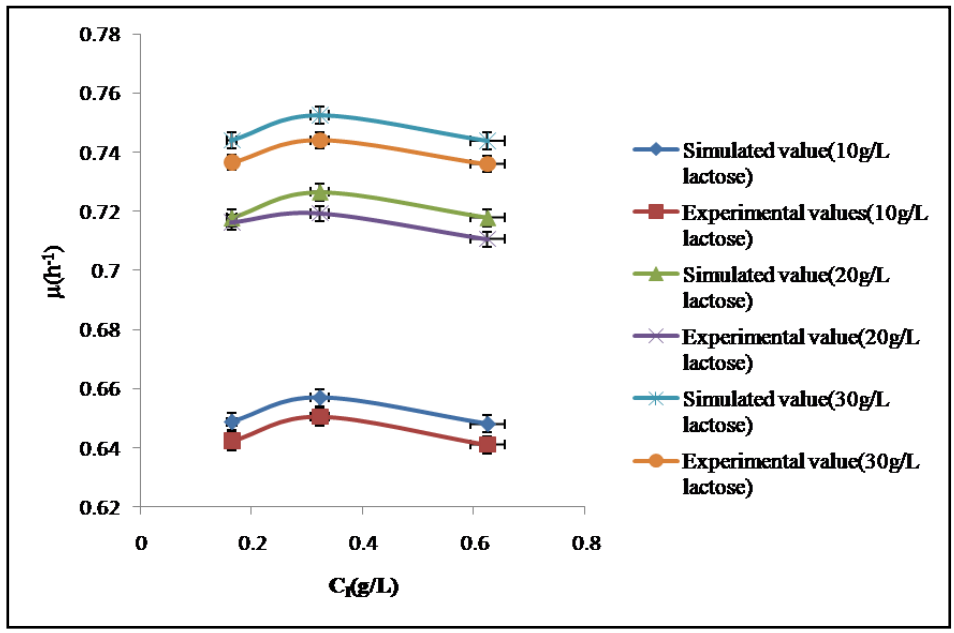



**Figure 6.C.7: Comparison of simulated and experimentally obtained cell growth rate**

It is observed that in all the cases experimentally obtained specific cell growth rate fit well with the simulated values with appreciable confidence level. It may therefore be concluded that *ab initio* prediction of the cell response of *Lactobacillus casei* in presence of two electron donors lactose and inulin can be done with appreciable confidence level by Equation 6.C.9.

## 6.C.4. Studies on effectiveness of inulin as prebiotic isolated from three different millet sources and garlic

In the present investigation an attempt has been made to study the effectiveness of inulin as prebiotic on the specific probiotic *Lactobacillus casei*. After establishing the synbiotic effect of commercially available inulin and *Lactobacillus casei* a detailed investigation has been carried out on the synbiotic effect of inulin isolated in-house from four different sources. Cell response in each case with constant lactose concentration 20 g/L as maintenance carbon source has been studied by following the cell concentration time histories.

### 6.C.4.1. Prebiotic effect of inulin isolated from garlic

Experimental data on cell concentration time history are given in Table 6.C.10 (a).

**Table 6.C.10 (a): Concentration time history data of *Lactobacillus casei* in presence of garlic inulin at 20g/L initial lactose concentration**

| **Time (h)** | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell concentration (g/L)** | 0.00091 | 0.00021 | 0.00026 | 0.00051 | 0.00103 | 0.00254 | 0.00330 | 0.00950 | 0.01950 | 0.04910 | 1.05100 | 1.15000 | 1.29150 | 1.41007 | 1.53526 | 1.74772 | 1.85513 | 1.85004 |

### 6.C.4.2. Prebiotic effect of inulin isolated from great millet

Experimental data on cell concentration time history are given in Table 6.C.10 (b).

**Table 6.C.10 (b): Concentration time history data of *Lactobacillus casei* in presence of great millet inulin at 20g/L initial lactose concentration**

| **Time** | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell concentration (g/L)** | 0.00091 | 0.00509 | 0.01212 | 0.02938 | 0.05289 | 0.07589 | 0.09134 | 1.14600 | 1.51000 | 2.13014 | 2.14667 | 2.20888 | 2.31773 | 2.41442 | 2.56021 | 2.73747 | 2.73789 | 2.68003 |

### 6.C.4.3. Prebiotic effect of inulin isolated from pearl millet

Experimental data on cell concentration time history are given in Table 6.C.10(c).

**Table 6.C.10 (c): Concentration time history data of *Lactobacillus casei* in presence of pearl millet inulin at 20g/L initial lactose concentration**

| **Time** | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Cell concentration (g/L)** | 0.00091 | 0.00913 | 0.02536 | 0.04576 | 0.07520 | 0.09204 | 1.25060 | 1.59080 | 2.57030 | 2.76053 | 3.22001 | 3.48004 | 3.51008 | 3.54038 | 3.60041 | 3.64022 | 3.53086 | 3.4422 |

### 6.C.4.4. Prebiotic effect of inulin isolated from Finger millet

Experimental data on cell concentration time history are given in Table 6.C.10 (d).

**Table 6.C.10 (d): Concentration time history data of *Lactobacillus casei* in presence of finger millet inulin at 20g/L initial lactose concentration**

| Time | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 | 13 | 14 | 22 | 23 | 24 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Cell concentration (g/L) | 0.00091 | 0.00036 | 0.00051 | 0.00071 | 0.00151 | 0.00454 | 0.00730 | 0.01340 | 0.02050 | 0.06310 | 0.12510 | 0.24160 | 0.45915 | 0.62007 | 0.73926 | 0.92272 | 0.95213 | 1.9304 |

Cell concentration time history data in each of the four cases have been plotted simultaneously on a single figure. Data obtained for commercially available inulin have also been superimposed on the same figure for the purpose of comparison. This is shown in Figure 6.C.8.

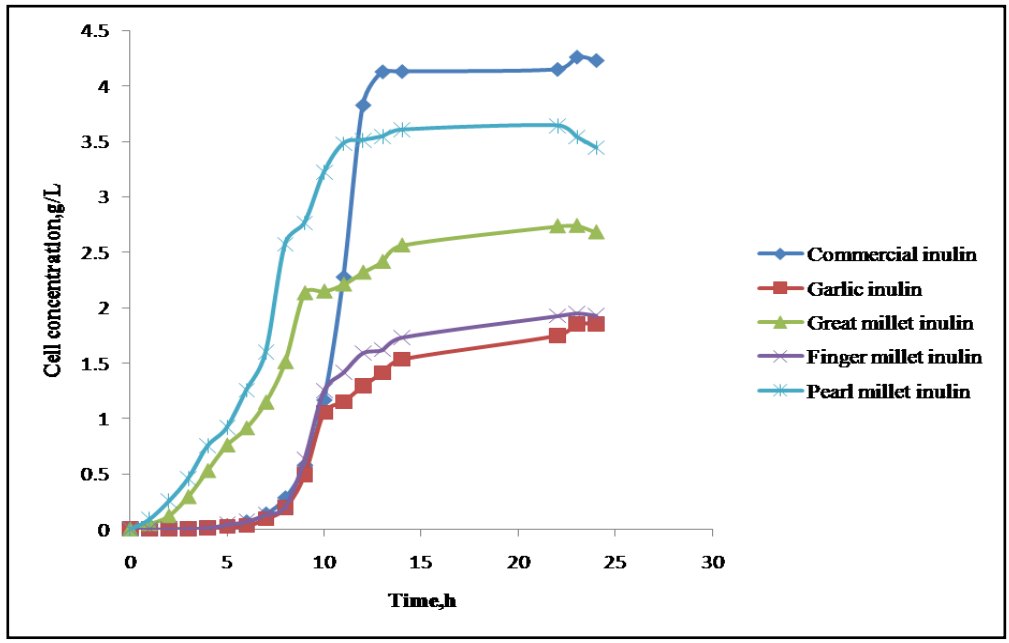



**Figure 6.C.8: The cell concentration time histories of *Lactobacillus casei* in presence of inulin extracted from different natural sources**

It may be noted that in each case the initial concentration of cell (0.00091 g/L) and the initial concentration of inulin (0.3225g/L) were kept constant. It is evident from the figure that inulin extracted from pearl millet (Indian name: bajra) shows the highest effectiveness as prebiotic amongst the four in-house sample. The reason behind this unique and interesting finding may be due to the variation of degree of polymerization (DP) of inulin obtained from different sources [10]. As reported by Ricca et al., [8] inulin is hydrolyzed to glucose and fructose by fructo-

enzymes. As per the present investigation, the pearl millet inulin has the highest DP (=39) compared to that obtained from the great millet (=27) and finger millet (=23) inulin and hence there is maximum availability of assailable monomer on enzymatic hydrolysis. This is possibly the justification for the observation of maximum growth of *Lactobacillus casei* on pearl millet inulin among the all varieties studied. It may, however, be noted that the commercially available inulin gives the better performance because of its greater purity compared with the laboratory samples.

From experimental results obtained in the present investigation, the effectiveness of inulin isolated from four different natural sources as prebiotic may be arranged as follows:
Commercial inulin (chicory) > Pearl millet inulin > Great millet inulin > Finger millet inulin > Garlic inulin.

### 6.C.5. Growth Dynamics of *Lactobacillus casei* (2651 1951 RPK) in presence of Pearl millet inulin

In order to study the cell response in presence of inulin derived from pearl millet as only electron donor experiments were performed to record the cell concentration time history with four initial inulin concentrations as parameter. The results are given in Table 6.C.11.

**Table 6.C.11: Concentration time history data of *Lactobacillus casei* in presence of different initial pearl millet inulin concentration**

| Time(h) | Initial pearl millet inulin concentration(g/L) | | | |
|---|---|---|---|---|
| | 0.164 | 0.322 | 0.481 | 0.624 |
| | Cell concentration (g/L) | | | |
| 0 | 0.0600 | 0.0600 | 0.0600 | 0.0600 |
| 1 | 0.0614 | 0.0616 | 0.0615 | 0.0614 |
| 2 | 0.0624 | 0.0632 | 0.0631 | 0.0628 |
| 3 | 0.0642 | 0.0648 | 0.0646 | 0.0643 |
| 4 | 0.0657 | 0.0665 | 0.0663 | 0.0658 |
| 5 | 0.0673 | 0.0683 | 0.0679 | 0.0673 |
| 6 | 0.0687 | 0.0650 | 0.0696 | 0.0689 |
| 7 | 0.0705 | 0.0718 | 0.0714 | 0.0705 |

| | | | | |
|---|---|---|---|---|
| **8** | 0.0713 | 0.0737 | 0.0731 | 0.0721 |
| **9** | 0.0728 | 0.0755 | 0.0754 | 0.0738 |
| **10** | 0.0750 | 0.0850 | 0.0790 | 0.0750 |
| **11** | 0.0757 | 0.0864 | 0.0787 | 0.0773 |
| **12** | 0.0772 | 0.0880 | 0.0817 | 0.0774 |
| **13** | 0.0788 | 0.0835 | 0.0827 | 0.0809 |
| **14** | 0.0803 | 0.0856 | 0.0847 | 0.0828 |
| **24** | 0.0945 | 0.1086 | 0.1082 | 0.1042 |
| **25** | 0. 0958 | 0.1113 | 0.1108 | 0.1066 |
| **26** | 0. 0970 | 0.1136 | 0.1135 | 0.1091 |
| **27** | 0. 0983 | 0.1162 | 0.1163 | 0.1118 |
| **28** | 0. 0995 | 0.1188 | 0.1192 | 0.1143 |
| **29** | 0.1007 | 0.1214 | 0.1221 | 0.1168 |
| **30** | 0.1018 | 0.1240 | 0.1251 | 0.1197 |
| **31** | 0.1029 | 0.1265 | 0.1281 | 0.1224 |
| **32** | 0.1040 | 0.1293 | 0.1312 | 0.1257 |
| **33** | 0.1050 | 0.1321 | 0.1344 | 0.1283 |
| **34** | 0.1062 | 0.1346 | 0.1377 | 0.1314 |
| **35** | 0.1071 | 0.1373 | 0.1420 | 0.1345 |
| **36** | 0.1076 | 0.1397 | 0.1441 | 0.1376 |
| **37** | 0.1088 | 0.1425 | 0.1480 | 0.1409 |
| **38** | 0.1095 | 0.1453 | 0.1514 | 0.1443 |
| **39** | 0.1104 | 0.1475 | 0.1547 | 0.1477 |
| **40** | 0.1112 | 0.1501 | 0.1583 | 0.1513 |
| **41** | 0.1119 | 0.1524 | 0.1625 | 0.1548 |
| **42** | 0.1126 | 0.1549 | 0.1659 | 0.1583 |
| **43** | 0.1135 | 0.1560 | 0.1694 | 0.1623 |
| **44** | 0.1138 | 0.1595 | 0.1733 | 0.1661 |
| **45** | 0.1144 | 0.1616 | 0.1774 | 0.1701 |
| **46** | 0.1149 | 0.1635 | 0.1813 | 0.1741 |
| **47** | 0.1155 | 0.1657 | 0.1850 | 0.1783 |
| **48** | 0.1158 | 0.1674 | 0.1891 | 0.1824 |

Using the experimental data given under Table 6.C.11, the cell growth dynamics curves for *Lactobacillus casei* have been plotted in Figure 6.C.9.

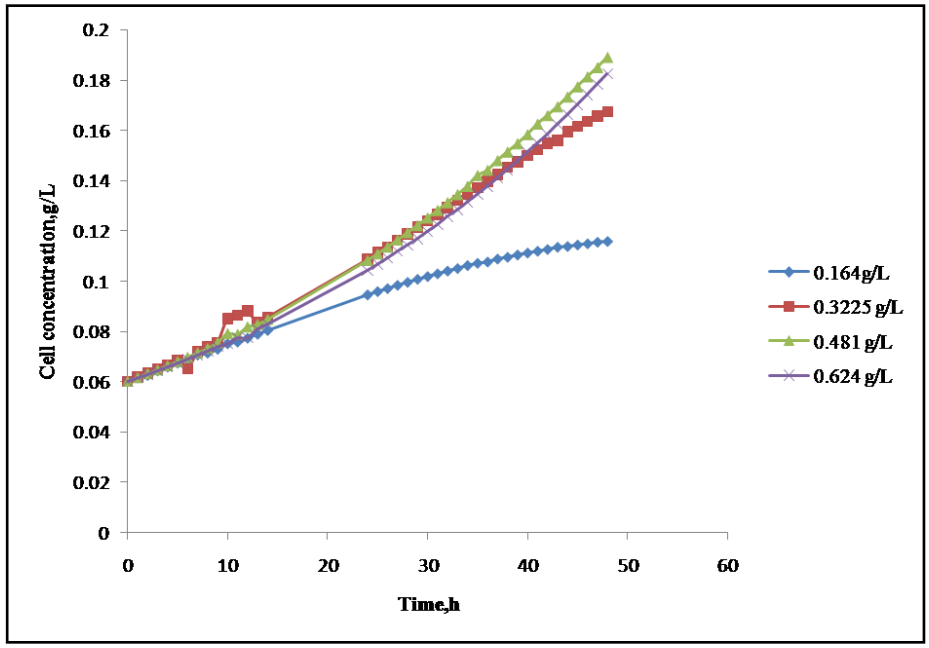



**Figure 6.C.9: Concentration time history of *Lactobacillus casei* in presence of pearl millet inulin as sole carbon source with initial inulin concentration as parameter**

It is noted from the nature of cell dynamics curves of Figure 6.C.9 that similar to the case of pure commercial inulin, here also an inhibitory effect of inulin on the cell growth is present. It is evident from the figure that the cell growth is enhanced upto the initial inulin concentration 0.3225 g/L and above this value the cell growth declines. Although similar behavior has been observed in case of pure commercial inulin, in the present situation maximum cell concentration (0.1664 g/L) is less compare to pure commercial inulin (0.1776 g/L).

The specific cell growth rates for each of the above four initial inulin concentrations have been computed from the progress curve Figure 6.C.9 and using Equation 6.C.2 and Equation 5.5 (Theoretical section). The results are given in Table 6.C.12.

**Table 6.C.12: Specific cell growth rate of *Lactobacillus casei* as a function of initial pearl millet inulin concentration**

| Substrate (pearl millet inulin) concentration ($C_I$), g/L | Specific cell growth rate (μ), $h^{-1}$ |
|---|---|
| 0.164 | 0.0148 |
| 0.322 | 0.0178 |
| 0.481 | 0.0173 |
| 0.624 | 0.0162 |

In order to understand the effect of initial pearl millet derived inulin on the specific cell growth rate, a plot of μ versus $C_I$ have been made and it is shown in Figure 6.C.10.

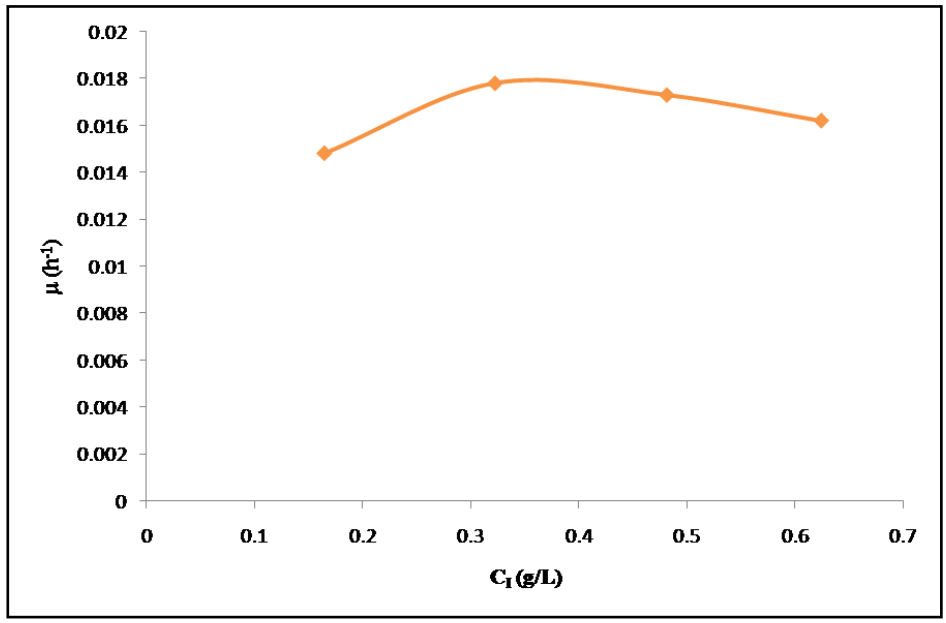



**Figure 6.C.10. Specific cell growth rate versus initial pearl millet inulin concentration**

The bell shaped curve so obtained evidently indicates the inhibitory effect of pearl millet inulin on *Lactobacillus casei* cell growth rate.

In order to model the specific cell growth rate as a function of pearl millet inulin an attempt has been made to taste the validity of Haldane equation in the present case. The Haldane equation in its general form is given by Equation 6.C.5.

$$\mu_I = \frac{\mu_{max_I} C_I}{K_{S_I} + C_I + \frac{C_I^2}{K_I}} \qquad (6.C.5)$$

The simulation constants $\mu_{max_I}$, $K_{S_I}$ and $K_I$ have been evaluated with three experimental data, viz., 0.3225 g/L, 0.481 g/L and 0.624 g/L by solving the non linear Equation 6.C.5 with the help of Matlab 7.0 and the results are shown in Table 6.C.13.

**Table 6.C.13: Simulation constants present in Haldane equation**

| **Kinetic Parameters** | | |
|---|---|---|
| **$\mu_{maxI}$ ($h^{-1}$)** | **$K_{S_I}$ (g/L)** | **$K_I$(g/L)** |
| 0.045 | 0.271 | 0.47 |

The Haldane equation can therefore be written as

$$\mu_I = \frac{0.045 h^{-1} C_I}{0.271 + C_I + \frac{C_I^2}{0.47}}, h^{-1} \qquad (6.C.10)$$

In order to validate the applicability of Equation 6.C.10 in the present case the predicted values of specific cell growth rate with five more initial pearl millet inulin concentrations, viz., 0.164 g/L, 0.175 g/L, 0.250 g/L, 0.500 g/L,0.782 g/L have been compared with the experimental value. This is shown in Table 6.C.14.

**Table 6.C.14: Comparison of simulated and experimental data of specific cell growth rate at three different initial pearl millet inulin concentrations**

| Substrate (pearl millet inulin) concentration, g/L | Specific cell growth rate,$h^{-1}$(Experimental) | Specific cell growth rate,$h^{-1}$(Predicted) |
|---|---|---|
| 0.164 | 0.0146 | 0.0148 |
| 0.175 | 0.0152 | 0.0154 |
| 0.250 | 0.0171 | 0.0172 |
| 0.500 | 0.0172 | 0.0173 |
| 0.782 | 0.0147 | 0.0149 |

In order to arrive at a conclusive decision regarding the validity of Haldane equation in the present case a plot of μ versus $C_I$ has been made with simulated data taken from Table 6.C.14. This is shown in Figure 6.C.11. Experimental data have been superimposed on the same plot.

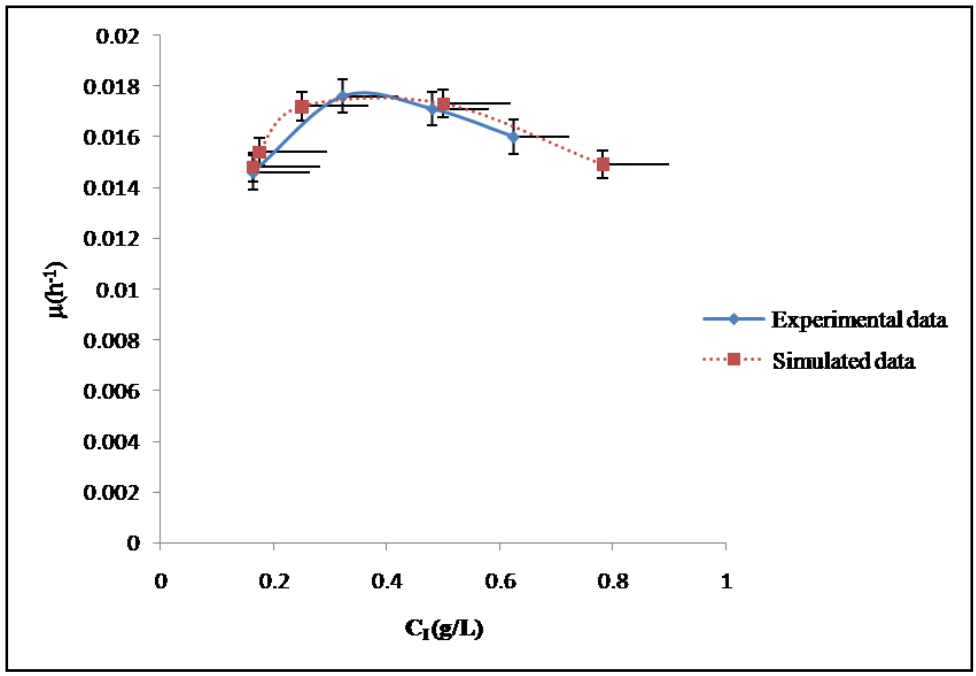



**Figure 6.C.11. Comparative plot of experimental and predicted values of specific cell growth rate**

It is evident from the figure that the experimental data fit well with the simulated data confirming the validity of Equation 6.C.10.

### 6.C.6. Growth Dynamics of *Lactobacillus casei* (2651 1951 RPK) in presence of both pearl millet inulin and lactose

In order to compare the performance of inulin derived from pearl millet as prebiotic with the commercially available inulin in presence of lactose a programmed investigation has been carried out on the cell growth dynamics. The results for four different initial inulin concentrations ( viz., 0.164, 0.3225, 0.481 and 0.624 g/L) and four different initial lactose concentrations ( viz., 10, 20, 30 and 40 g/L) are given in Table 6.C.15(a) through 6.C.15 (d).

**Table 6.C.15 (a): Concentration time history of *Lactobacillus casei* in presence of pearl millet inulin at 10g/L initial lactose concentration**

| Time (h) | Initial inulin concentration(g/L) | | | |
|---|---|---|---|---|
| | 0.164 | 0.3225 | 0.481 | 0.624 |
| | Cell concentration (g/L) | | | |
| 0 | 0.0020 | 0.0020 | 0.0021 | 0.0023 |
| 1 | 0.0035 | 0.0036 | 0.0035 | 0.0037 |
| 2 | 0.0072 | 0.0073 | 0.0073 | 0.0074 |
| 3 | 0.0150 | 0.0139 | 0.0139 | 0.0130 |
| 4 | 0.0260 | 0.0560 | 0.0265 | 0.0350 |
| 5 | 0.0700 | 0.0900 | 0.0500 | 0.0500 |
| 6 | 0.1100 | 0.1560 | 0.0960 | 0.0950 |
| 7 | 0.2100 | 0.2500 | 0.1820 | 0.2100 |
| 8 | 0.3300 | 0.4270 | 0.3410 | 0.3580 |
| 9 | 0.9800 | 0.7320 | 0.6300 | 0.6440 |
| 10 | 1.2100 | 1.3500 | 1.2900 | 1.2500 |
| 11 | 2.6900 | 3.7400 | 4.5400 | 3.7280 |
| 12 | 5.5500 | 6.2600 | 5.9700 | 5.7700 |

| | | | | |
|---|---|---|---|---|
| 13 | 5.0600 | 5.5060 | 5.6270 | 5.6270 |
| 14 | 4.9600 | 5.1190 | 5.4560 | 5.5500 |
| 22 | 4.5600 | 4.9310 | 5.3440 | 5.4100 |
| 23 | 3.9700 | 4.5100 | 5.1940 | 5.2510 |
| 24 | 3.6000 | 4.1310 | 5.0440 | 5.0910 |

**Table 6.C.15 (b): Concentration time history of *Lactobacillus casei* in presence of pearl millet inulin at 20g/L initial lactose concentration**

| **Time** | **Initial inulin concentration(g/L)** | | | |
|---|---|---|---|---|
| | **0.164** | **0.3225** | **0.481** | **0.624** |
| | **Cell concentration (g/L)** | | | |
| 0 | 0.0008 | 0.00081 | 0.00084 | 0.00083 |
| 1 | 0.0018 | 0.0018 | 0.0018 | 0.0018 |
| 2 | 0.0037 | 0.0038 | 0.0038 | 0.0038 |
| 3 | 0.0076 | 0.0077 | 0.0077 | 0.0077 |
| 4 | 0.0360 | 0.0157 | 0.0157 | 0.0157 |
| 5 | 0.0617 | 0.0922 | 0.0721 | 0.0320 |
| 6 | 0.1140 | 0.2180 | 0.1860 | 0.1520 |
| 7 | 0.2310 | 0.4340 | 0.3400 | 0.2300 |
| 8 | 0.5670 | 0.6730 | 0.4720 | 0.4160 |
| 9 | 0.7400 | 0.8550 | 0.7530 | 0.6280 |
| 10 | 0.9800 | 1.1000 | 1.0500 | 1.0200 |
| 11 | 3.1020 | 4.1750 | 3.1620 | 2.1490 |
| 12 | 5.8220 | 6.6000 | 6.2800 | 6.0800 |
| 13 | 5.5620 | 6.4200 | 6.1610 | 4.1741 |
| 14 | 5.0660 | 6.2820 | 5.9650 | 4.2266 |
| 22 | 4.9660 | 5.9900 | 5.5330 | 4.1505 |
| 23 | 4.4560 | 5.4290 | 5.1933 | 3.9505 |
| 24 | 4.2650 | 5.2190 | 4.9330 | 3.2505 |

**Table 6.C.15 (c): Concentration time history of *Lactobacillus casei* in presence of pearl millet inulin at 30g/L initial lactose concentration**

| Time | Initial inulin concentration(g/L) | | | |
|---|---|---|---|---|
| | **0.164** | **0.3225** | **0.481** | **0.624** |
| | **Cell concentration (g/L)** | | | |
| 0 | 0.0006 | 0.00061 | 0.00063 | 0.0006 |
| 1 | 0.0013 | 0.0913 | 0.0013 | 0.0013 |
| 2 | 0.0026 | 0.0126 | 0.0026 | 0.0026 |
| 3 | 0.0055 | 0.0556 | 0.0045 | 0.0055 |
| 4 | 0.0115 | 0.1170 | 0.0156 | 0.0116 |
| 5 | 0.0241 | 0.2245 | 0.0244 | 0.0143 |
| 6 | 0.0505 | 0.3514 | 0.0513 | 0.0309 |
| 7 | 0.1257 | 0.5079 | 0.1076 | 0.0967 |
| 8 | 0.2210 | 0.9261 | 0.2254 | 0.1535 |
| 9 | 0.4610 | 1.1733 | 0.4718 | 0.3672 |
| 10 | 0.9000 | 1.2500 | 1.1500 | 1.0700 |
| 11 | 3.9656 | 2.0360 | 2.0313 | 2.0083 |
| 12 | 5.9500 | 8.3760 | 7.6840 | 7.1190 |
| 13 | 5.7228 | 8.1991 | 7.4570 | 6.9599 |
| 14 | 5.5661 | 8.0910 | 7.1908 | 6.8417 |
| 22 | 5.2662 | 7.9296 | 6.9300 | 6.5502 |
| 23 | 5.0662 | 7.5296 | 6.4300 | 6.2602 |
| 24 | 4.9662 | 7.1296 | 6.2930 | 6.1502 |

**Table 6.C.15 (d): Concentration time history of *Lactobacillus casei* in presence of pearl millet inulin at 40g/L initial lactose concentration**

| Time | Initial inulin concentration(g/L) | | | |
|---|---|---|---|---|
| | **0.164** | **0.3225** | **0.481** | **0.624** |
| | **Cell concentration (g/L)** | | | |
| 0 | 0.0005 | 0.00052 | 0.00053 | 0.00051 |
| 1 | 0.0011 | 0.0012 | 0.0013 | 0.0010 |
| 2 | 0.0023 | 0.0025 | 0.0023 | 0.0023 |
| 3 | 0.0038 | 0.0058 | 0.0048 | 0.0046 |
| 4 | 0.0102 | 0.1303 | 0.0153 | 0.0102 |
| 5 | 0.0616 | 0.2219 | 0.0718 | 0.0517 |
| 6 | 0.1458 | 0.3466 | 0.1464 | 0.0861 |
| 7 | 0.2971 | 0.4991 | 0.2488 | 0.1181 |
| 8 | 0.4060 | 0.6108 | 0.3102 | 0.2083 |
| 9 | 0.6364 | 0.7481 | 0.5466 | 0.4422 |
| 10 | 0.8500 | 0.9500 | 0.9000 | 0.8800 |
| 11 | 1.9343 | 2.0038 | 1.9988 | 1.9758 |
| 12 | 5.9900 | 6.7920 | 6.4190 | 6.2450 |
| 13 | 5.4828 | 7.7070 | 6.2606 | 6.1406 |
| 14 | 5.2660 | 8.1293 | 6.1921 | 6.0680 |
| 22 | 5.1461 | 8.1295 | 5.9290 | 5.9001 |
| 23 | 5.0661 | 8.1295 | 5.7929 | 5.8501 |
| 24 | 4.9661 | 8.1295 | 5.7129 | 5.7901 |

The experimentally obtained cell concentration time histories with initial inulin concentration as parameter for four different initial lactose concentrations are shown in Figures 6.C.12(a) through 6.C.12(d).

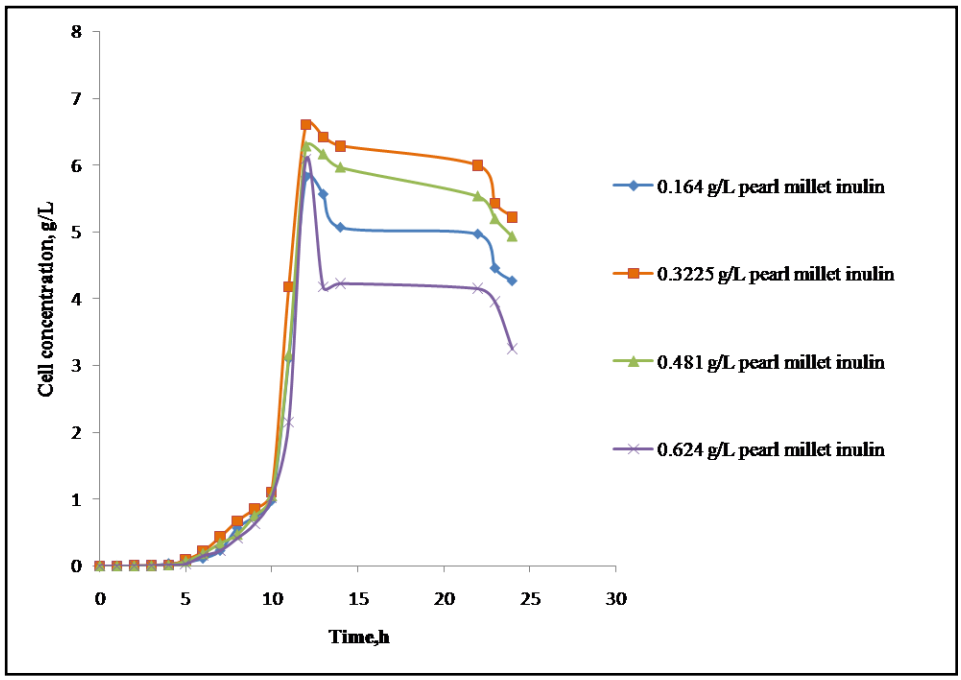



**Figure 6.C.12(a): Experimentally obtained cell response at initial lactose concentration 10g/L**

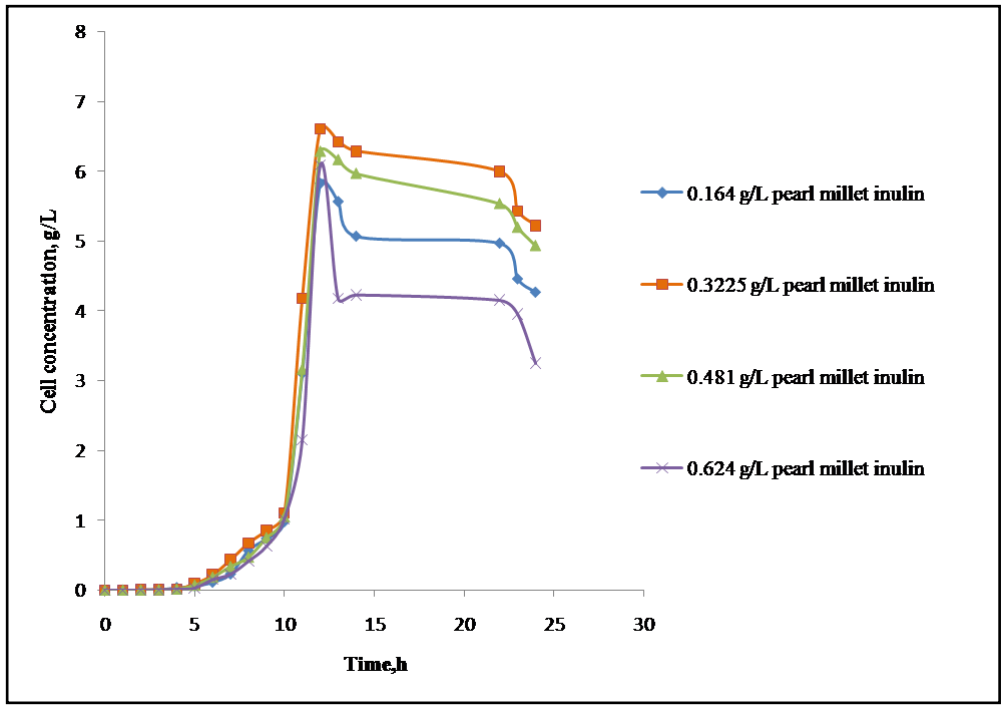



**Figure 6.C.12(b): Experimentally obtained cell response at initial lactose concentration 20g/L**

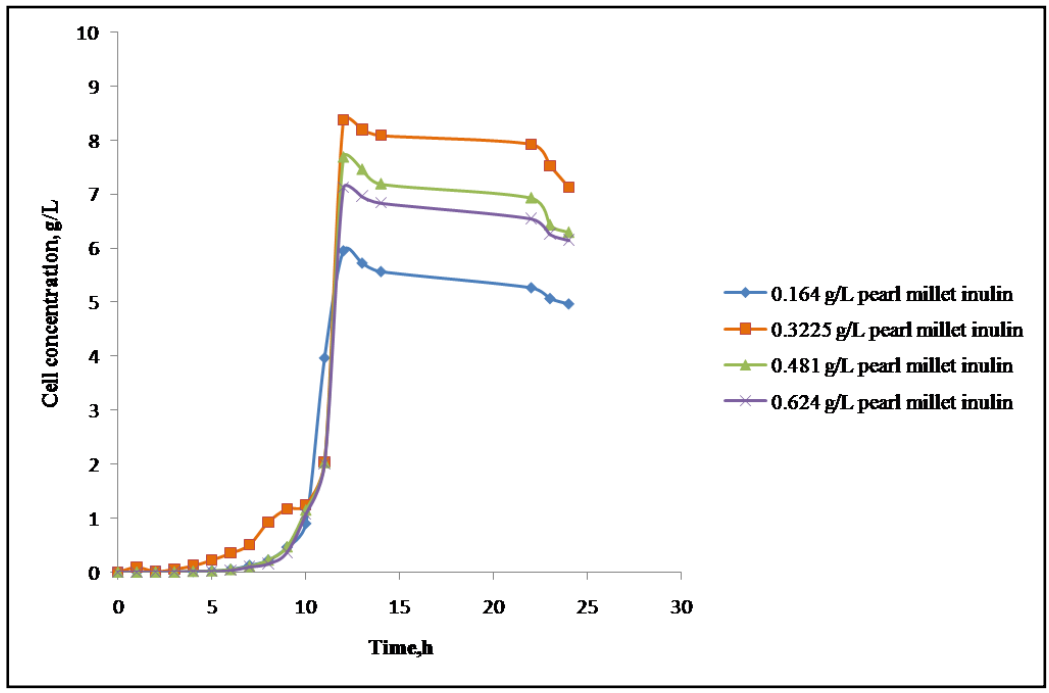



**Figure 6.C.12(c): Experimentally obtained cell response at initial lactose concentration 30g/L**

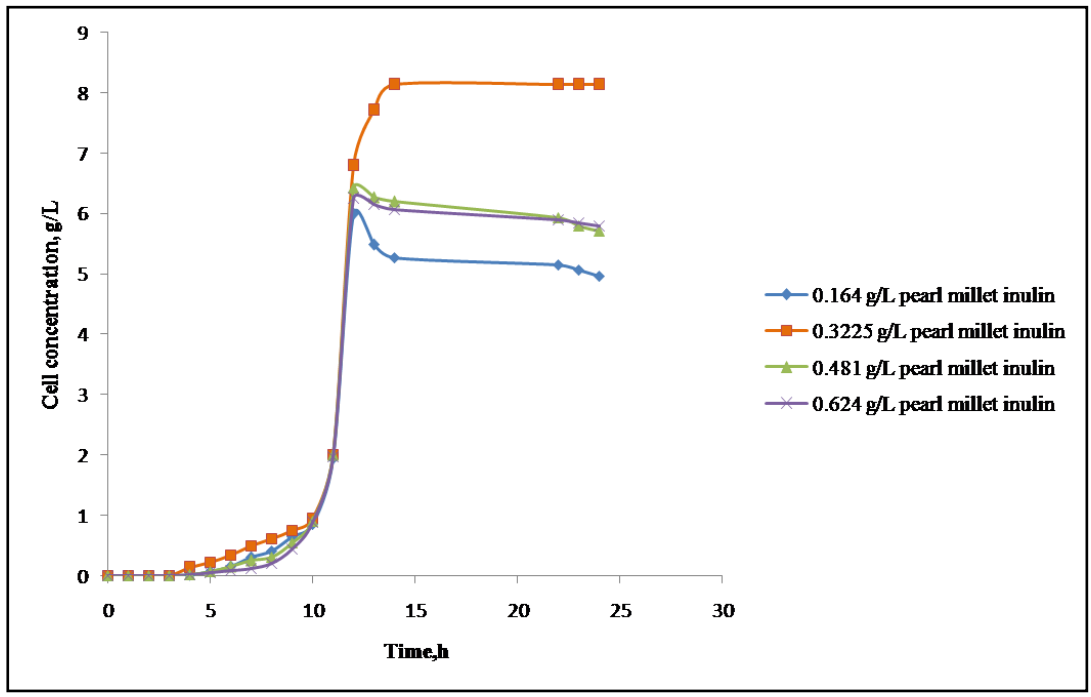



**Figure 6. C.12(d): Experimentally obtained cell response at initial lactose concentration 40g/L**

Comparing these figures with those obtained in the case of commercially available inulin it is observed that although similar trends in cell response exist in all the cases, the maximum cell concentration in the stationary phase of growth is less in case of inulin derived from pearl millet. This is because of the fact that the purity of commercially available inulin is appreciably higher compare to the inulin derived from pearl millet.

Since the cell response curves are similar in nature to those of commercially available inulin system it is expected that a summative type rate equation (as in the case of commercially available inulin, Equation 6.C.7 should be able to predict a real time situation. The specific cell growth rate is thus related as

$$\mu = \frac{\mu_{m_1} C_s}{K_s + C_s} + \frac{\mu_{m_2} C_I}{K_{s_I} + C_I + \frac{C_I^2}{K_I}} \frac{\text{gcellforme d}}{\text{L.h}} \qquad (6.C.7)$$

The simulation constants $\mu_{m_2}$, $K_{s_I}$ and $K_I$ have been evaluated by solving the non linear equation using numerical analysis. Matlab7.0 software has been used for computation purpose. Four different initial pearl millet inulin concentrations, viz., 0.164, 0.3225, 0.481 and 0.624 g/L have been studied to facilitate evaluation of intrinsic kinetic parameters.

Experimentally obtained specific cell growth rate in presence of lactose and pearl millet inulin have been computed for four different sets of inulin concentrations as well as four different initial concentration of lactose. The results are shown in Table 6.C.16.

**Table 6.C.16: Experimentally obtained specific cell growth rate in presence of lactose and pearl millet inulin**

| $C_s$ (g/L) | $C_I$(g/L) | $\mu_{expt}$($h^{-1}$) |
|---|---|---|
| 10 | 0.1640 | 0.6425 |
| 10 | 0.3225 | 0.6455 |
| 10 | 0.4810 | 0.6450 |
| 10 | 0.6240 | 0.6439 |
| 20 | 0.1640 | 0.7113 |

| | | |
|---|---|---|
| 20 | 0.3225 | 0.7143 |
| 20 | 0.4810 | 0.7138 |
| 20 | 0.6240 | 0.7127 |
| 30 | 0.1640 | 0.7373 |
| 30 | 0.3225 | 0.7403 |
| 30 | 0.4810 | 0.7398 |
| 30 | 0.6240 | 0.7387 |
| 40 | 0.1640 | 0.7516 |
| 40 | 0.3225 | 0.7546 |
| 40 | 0.4810 | 0.7541 |
| 40 | 0.6240 | 0.7530 |

The above sets of values have been used for finding out the simulation constants.

The numerical values of the intrinsic kinetic parameters, $\mu_{m2}$ and $K_{S_2}$ as well as the inhibition constants $K_I$ are shown in Table 6.C.17.

**Table 6.C.17: Simulation constants present in Haldane equation**

| **Kinetic Parameters** | | |
|---|---|---|
| **$\mu_{m2}$ ($h^{-1}$)** | **$K_{S_2}$ (g/L)** | **$K_I$(g/L)** |
| 0.045 | 0.271 | 0.47 |

The proposed cell response equation can therefore be written as

$$r_x = \frac{dC_x}{dt} = \frac{0.7836h^{-1}C_sC_x}{2.44 + C_s} + \frac{0.045h^{-1}C_IC_x}{0.271 + C_I + \frac{C_I^2}{0.47}} \qquad (6.C.11)$$

and

The specific cell growth rate is given by

$$\mu = \mu_1 + \mu_2 = \frac{0.7836h\ {}^{1}C_s}{2.44 + C_s} + \frac{0.045h\ {}^{1}C_I}{0.271 + C_I + \frac{C_I{}^2}{0.47}} \qquad (6.C.12)$$

Effects of two different independent variables viz., initial concentration of inulin and initial concentration of lactose on specific cell growth rate are shown in the three dimensional diagram Figure 6.C.13 to understand the mutual distribution of initial inulin concentration and initial lactose concentration.

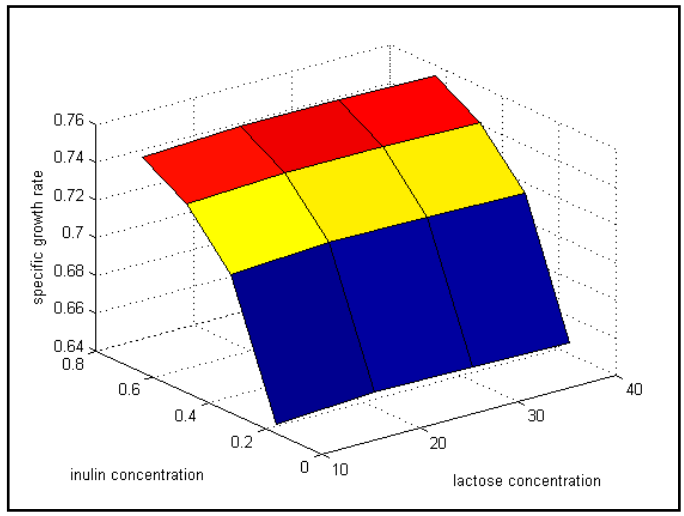



**Figure 6.C.13: 3D plot with experimental data of specific growth rate as a function of lactose and pearl millet inulin concentrations.**

All concentrations are in g/L and specific cell growth rate (μ) is in $h^{-1}$

## 6.D. Studies on prebiotic activity score and antimicrobial activity of *Lactobacillus casei* (2651 1951 RPK)

### 6.D.1. Prebiotic activity score

Prebiotic activity score is a quantitative measure of the effectiveness of any prebiotic material, like inulin. The values of prebiotic activity score against the selected pathogen *Escherichia coli* (2065ATCC 8739) for three different inulin samples are given in Table 6.D.1.

**Table 6.D.1: Prebiotic score of inulin against *Escherichia coli***

| Source of Inulin | Commercial | Garlic | Pearl millet |
|---|---|---|---|
| **Prebiotic activity score** | 1.0 | 0.7 | 3.2 |

The quantitative representation of prebiotic score is shown in terms of bar diagram (Figure 6.D.1)

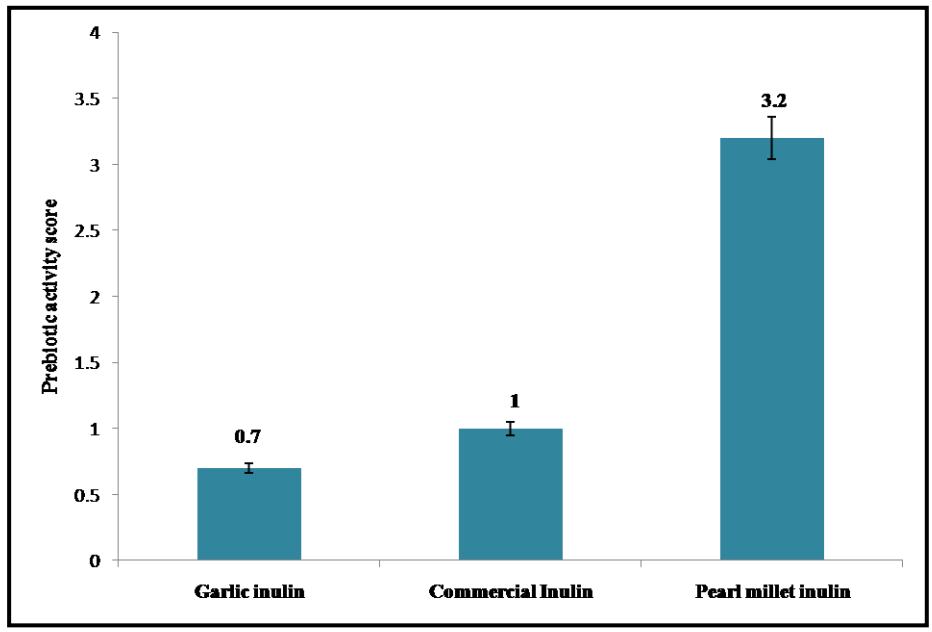



**Figure 6.D.1: Comparison of prebiotic score of inulin against the pathogen *Escherichia coli***

It immediately reveals that pearl millet inulin has much better score indicating that this variety of the inulin molecule is superior to chicory based inulin from the perspective of prebiotic activity on *L. casei*. This result is also in agreement with the largest biomass concentration at any batch time using pearl millet inulin. Higher prebiotic score inulin derived from pearl millet in comparison to the other two samples is possibly due to presence of higher oligosaccharide concentration which intern results in formation of greater amount of inhibitory enzymes within the cells. These inhibitory enzymes reduce the activity of pathogen resulting in higher prebiotic score.

### 6.D.2. Studies on synbiotic effect of inulin – *Lactobacillus casei* (2651 1951 RPK) conjugate on antibiotic sensitivity

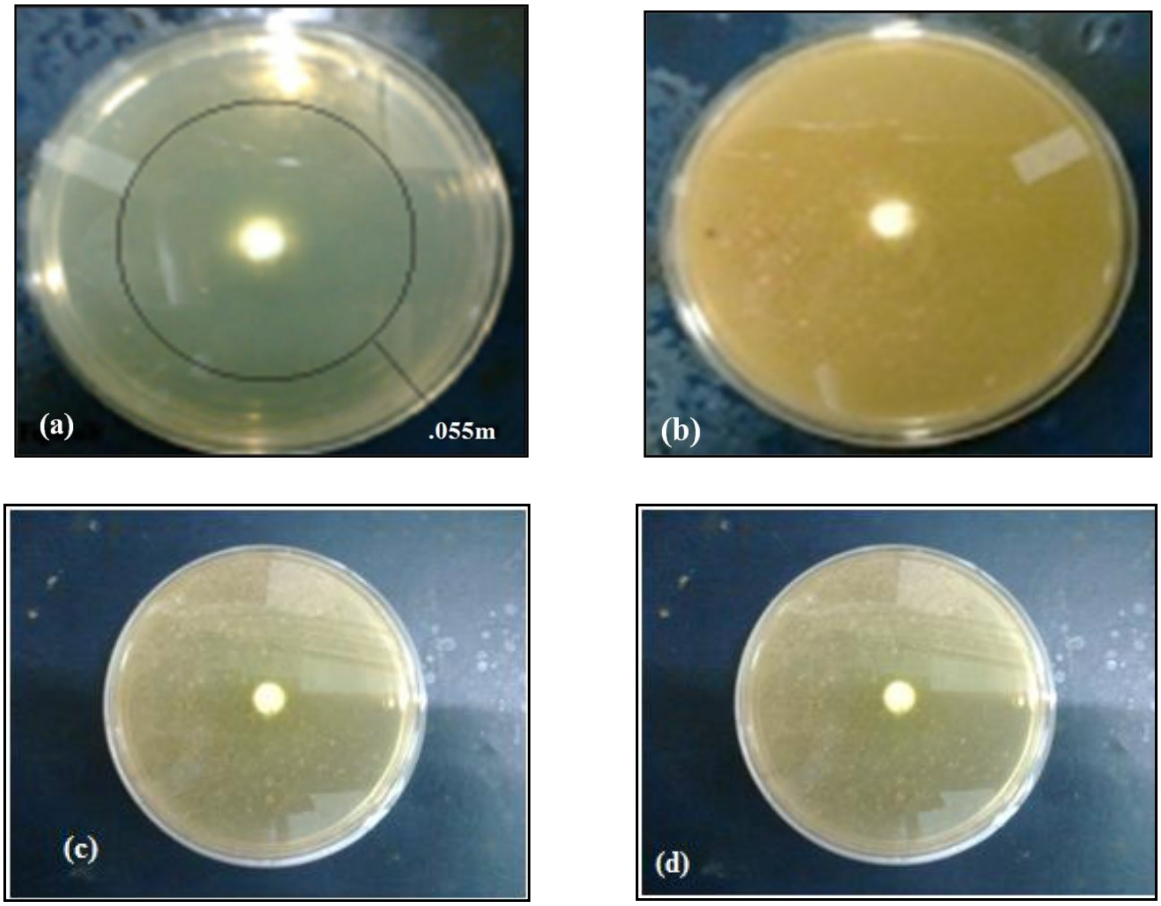
One of the main intensions of the present investigation is to study antibiotic sensitivity on the selected prebiotic- probiotic conjugate, viz., inulin treated *Lactobacillus casei*. A standard experiment in this regard is to perform well diffusion experiment with and without the presence of prebiotic and measuring the zone of inhibition. In the present investigation the photographs of petri-plates of *Lactobacillus casei*, used for the determination of antibiotic (norfloxacin) sensitivity through well diffusion technique with and without inulin are shown in Figures 6.D.2 (a)(b)(c)(d) and (e). The zone of inhibition measured in terms of linear distance in the five different cases is given in Table 6.D.2.

**Table 6.D.2: Measurement of zone of inhibition in presence of norfloxacin**

| System | Zone of inhibition(mm) |
|---|---|
| ***Lactobacillus casei* -in absence of inulin** | 55 |
| ***Lactobacillus casei* -in presence of commercial grade inulin** | 0 |
| ***Lactobacillus casei* -in presence of great millet inulin** | 0 |
| ***Lactobacillus casei* -in presence of pearl millet inulin** | 0 |
| ***Lactobacillus casei* -in presence of finger millet inulin** | 0 |

This experimental observation reveals that while the candidate bacterium *Lactobacillus casei* inherently susceptible towards norfloxacin, a broad spectrum antibiotic, it has developed the said antibiotic resistance in presence of prebiotic inulin. The antibacterial effect of norfloxacin, a fluoroquinolone, may be through the inhibitory action on the enzyme DNA gyrase, essential for replication, repair and transcription of DNA and topoisomerase essential for entangling DNA of the bacteria under attack [11]. The development of resistance in presence of inulin may be due to

the chemical bonding of inulin molecule with fluoroquinolone group thus eliminating the possibility of extinction of the probiotic cell. The formation of a layer of coating of inulin around the cell wall (referring Figure 6.A.8) may also impedes the transport of fluoroquinolone group from bulk phase to inside the cell. Effect of Norfloxacin on *L. casei* in absence and in presence of prebiotic inulin is shown in Figures 6.D.2 (a),(b),(c) and (d) respectively.



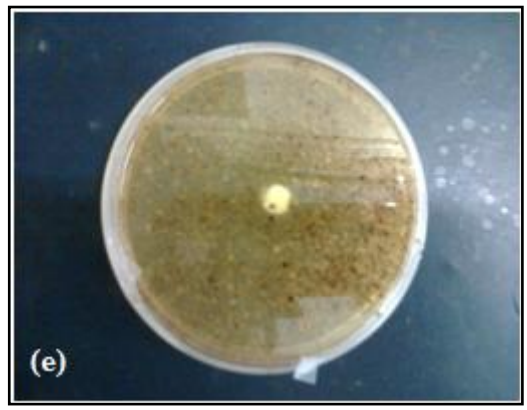



**Figure 6.D.2 (a): Effect of norfloxacin on *Lactobacillus casei* in de-Mann Rogosa Sharp (MRS) agar plate. The marked area indicates the zone of inhibition (0.055 m), (b)(c)(d)(e): Effect of norfloxacin on *Lactobacillus casei* in Modified de-Mann Rogosa Sharp (MMRS) agar plate in presence of glucose and commercial, great millet, pearl millet and finger millet inulin respectively.**

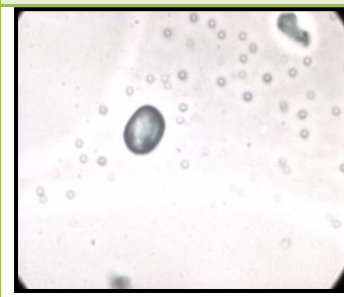
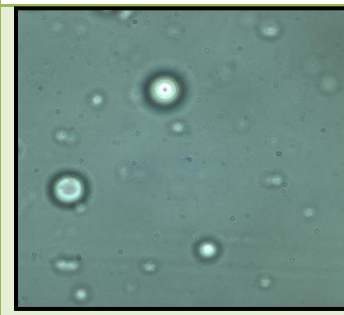
It is evident from the figures that while the inulin treated *Lactobacillus casei* have grown profusely in presence of the antibiotic, Norfloxacin, native *Lactobacillus casei* could not grow in the identical environment showing the effectiveness of inulin as prebiotic compound.

## 6.E. Studies on Immobilized *Lactobacillus casei* (2651 1951 RPK)

### 6.E.1. Physical nature of the whole cell-matrix conjugate

It has already been noted from literature that immobilization of whole cell in an inert carrier increases the shelf life of cells. Reusuability of immobilized cells reduces the cost of target product biomolecule. As stated under material and method section three different techniques of immobilization of *L.casei* have been carried out using calcium alginate as carrier material. The physical nature of the immobilize particle and the average particle size in each case are given in Table 6.E.1.

**Table 6.E.1: Microscopic size analysis of Beads and Microcapsules***

| Microencapsulation Process | Bead/microcapsules Diameter | Physical nature of the product |
|---|---|---|
| **Entrapment method** | 2.98333mm<br>(± 0.028868mm) |  |
| **Emulsification/external gelation** | 298.333 µm<br>(±2.886751 µm) |  |
| **Emulsification/internal gelation** | 23.9333µm<br>(± 0.11547 µm) |  |

* Values are mean (n=3) ±standard deviation

### 6.E.2. Encapsulation efficiency

Bacterial cell loading within the matrix is different for three different immobilization methods. Encapsulation efficiencies for entrapment, external gelation and internal gelation have been computed from Equation 5.8 the results in the present case are given in Table 6.E.2.

**Table 6.E.2: Quantitative representation of cell loading for three different immobilization techniques**

| Immobilization processes | Entrapment | External gelation | Internal gelation |
|---|---|---|---|
| Encapsulation efficiency (%) | 62 | 76 | 98 |

### 6.E.3. *Lactobacillus casei* (2651 1951 RPK) cell concentration in immobilized Systems

Concentrations of cells in the internal aqueous core of Ca-alginate beads and microcapsules (internal and external) just after preparation and at the "release time" have been calculated as follows:

- **Internal gelation**

By using 10 mL of cell solution containing 8mg/mL biomass, 1g of $16.302*10^6$ microcapsules was obtained.

Approximately, $10^9$ cells weigh 1mg [12]

Encapsulation efficiency =98%

Therefore initial number of cells/microcapsule (internal gelation) = $\frac{80*10^9*0.98}{16.302*10^6} = 4.809*10^3$

Diameter microcapsule (internal) =24μm

Since the microcapsule is prepared using 1:1 oil to aqueous phase ratio, therefore, the volume of aqueous phase in each microcapsule = $\left(\frac{1}{2}*\frac{\pi}{6}*\left(24*10^{-6}\right)^3\right) = 3.617*10^{-15}$

Therefore, initial concentration of cells per unit aqueous volume of microcapsules = $\frac{4.809*10^3}{3.617*10^{-15}} = 1.329*10^{18}/\mathrm{m}^3$

Size of the bacterial strain (*L. casei*):

Length = 2μm

Diameter = 0.5 μm

Therefore, cell volume = $\left(\pi/4 * (0.5*10^{-6})^2 * 2*10^{6}\right)$

$=3.926*10^{-19}\ m^3$

Maximum number of cells that can be accommodated in each microcapsules = $\frac{3.617*10^{-15}}{3.92*10^{-19}}$ = $9.227*10^3=9227$

The number of cells per unit volume of microcapsules = $\frac{9227}{3.617*10^{-15}}$ = $2551*10^{15}/m^3$

The maximum biomass concentration in the aqueous core= $2551*10^{15}*10^{-9} mg/m^3$

$=2551*10^6 mg/m^3$

$=2.551 g/mL$

- **External gelation**

  By using 10 mL of cell solution containing 8mg/mL biomass, 1g of 8360 microcapsules was obtained.

  Encapsulation efficiency =76%

  Therefore, applying the same principle used in case of microcapsules obtained through internal gelation, initial number of cells/microcapsule (external gelation) = $\frac{80*10^9*0.76}{8360}$ = $7.27*10^6$

  Diameter of microcapsule=300μm

  The volume of aqueous core of alginate microcapsule = $0.5*\frac{4}{3}\pi(150*10^{-6})^3$

  $= 0.705*10^{-11} m^3$

  Therefore, initial concentration of cells per unit aqueous volume of microcapsules = $\frac{7.27*10^6}{0.705*10^{-11}}$ $= 10.312*10^{17}/m^3$

  Following the principle used in case of microcapsules obtained through internal gelation, maximum number of cells that can be accommodated in each microcapsules(external) = $\frac{0.705*10^{-11}}{3.92*10^{-19}}$ $= 17.98*10^6$

The number of cells per unit volume of microcapsules (external)= $\frac{17.98*10^{6}}{0.705*10^{-11}} = 2.55*10^{18}/\mathrm{m}^3$

The maximum biomass concentration in the aqueous core= 2.55g/mL

- **Direct entrapment**

By using 10 mL of cell solution containing 8mg/mL biomass, 1g of 8.36 beads were obtained.

Encapsulation efficiency =62%

Therefore initial number of cells/bead (Ca-entrapment) = $\frac{80*10^{9}*0.62}{8.36} = 5.933*10^{9}$

Diameter of bead=3mm

The volume of alginate bead = $\frac{4}{3}\pi(1.5*10^{-3})^3 = 1.41*10^{-8}\mathrm{m}^3$

Therefore, initial concentration of cells per unit aqueous volume of beads = $\frac{5.933*10^{9}}{1.41*10^{-8}} = 4.207*10^{17}/\mathrm{m}^3$

Maximum number of cells that can be accommodated in each microcapsules = $\frac{1.41*10^{-8}}{3.92*10^{-19}} = 3.596*10^{10}$

The number of cells per unit volume of microcapsules = $\frac{3.596*10^{10}}{1.41*10^{-8}} = 2.55*10^{18}/\mathrm{m}^3$

The maximum biomass concentration in the aqueous core= 2.55g/mL

### 6.E.4. Antimicrobial activity of immobilized *Lactobacillus casei* (2651 1951 RPK)

The antimicrobial activity of *L. casei* entrapped in calcium alginate beads and microcapsules is shown in Figures 6.E.1(a) through 6.E.1(c). For the purpose of comparison petri-plate containing free cell is also shown in Figure 6.E.1(d).

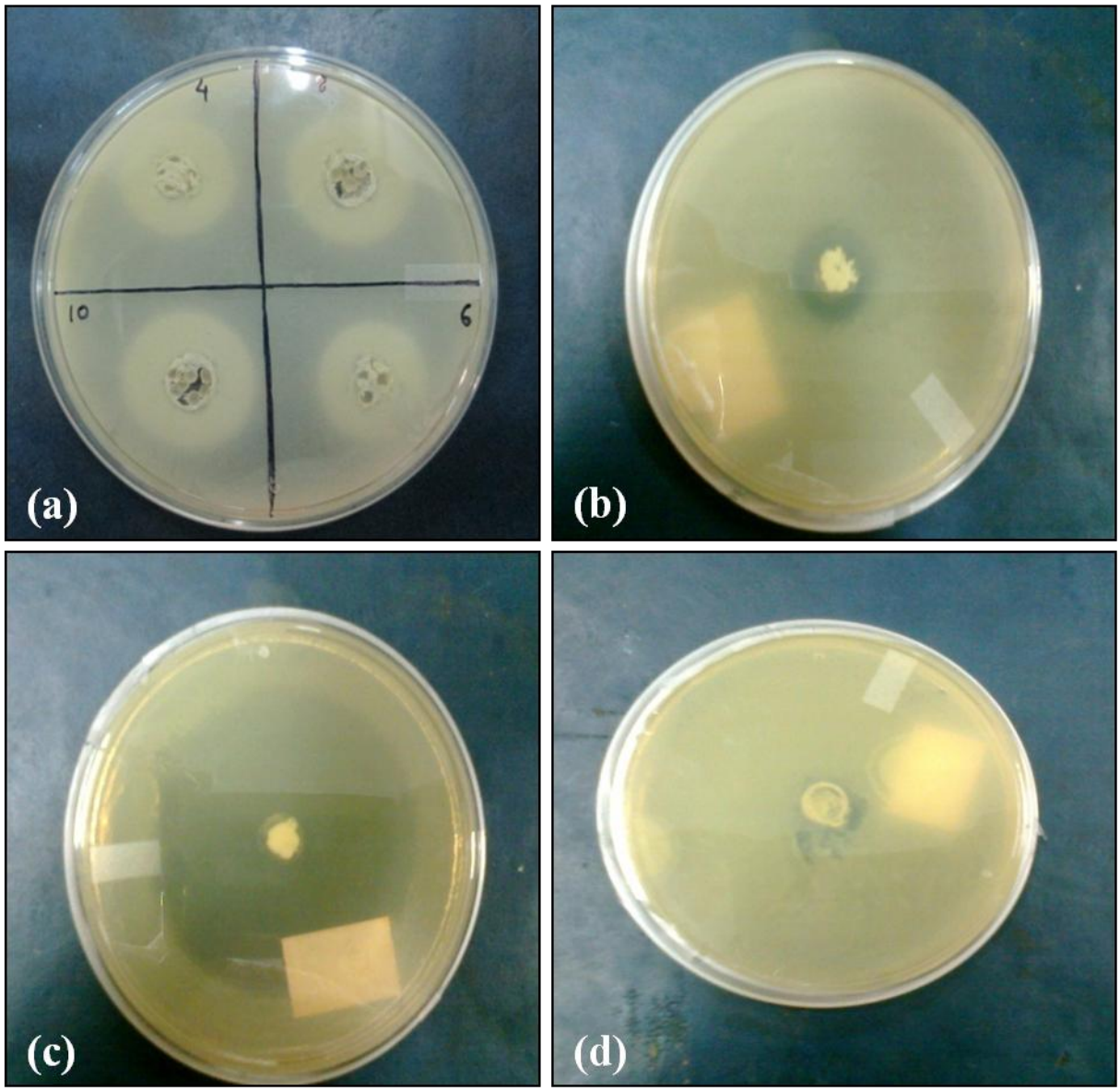



**Figure 6.E.1(a),(b),(c),(d): Antimicrobial activity of *Lactobacillus casei* entrapped in calcium beads (variation in number of beads), encapsulated through external and internal gelation tachniques against *Escherichia coli***

In Figure 6.E.1(a), the agar plate has been divided into four quadrants each containing different number of *Lactobacillus casei* entrapped beads. The figures clearly shows that in each case the growth rate of the selected pathogen, *Escherichia coli* has been considerably reduced due to the synbiotic effect of prebiotic-probiotic conjugate. It has also been observed that the zone of inhibition is more for the quadrant containing more number of immobilized beads. The specific

advantage in the immobilized system is that the probiotic *Lactobacillus casei* remains intact within the matrix but extending its action in the immediate vicinity of the immobilized cell beads. From Figure 6.E.1 (b), it is apperent that immobilized *Lactobacillus casei* by external gelation renders marginally less antimicrobial activity compare to immobilized cells obtained by entrapment method. On the other hand, Figure 6.E.1(c) which contains cells immobilized by internal gelation technique shows greater antimicrobial activity compare to external gelation technique although its performance is marginally lower than the case of cell system obtained by entrapment technique. Figure 6.E.1(d) clearly indicates that antimicrobial activity of cells under all three different immobilized condition are much superior to that of the free cells. The quantitative results on antimicrobial activity for four different cases are given in Table 6.E.1(a) and 6.E.1 (b).

**Table 6.E.3(a): Antimicrobial activity of entrapped *Lactobacillus casei* cell treated with inulin**

| Number of entrapped beads | **4** | **6** | **8** | **10** |
|---|---|---|---|---|
| Zone of inhibition (mm) | 21 | 22.5 | 24 | 25 |

For free probiotic (*L. casei*) cell (zone of inhibition) =10mm

For,

Ca-entrapment, 1 g of bead=8 beads

External gelation, 1g of microcapsules= 8360 microcapsules

Internal gelation, 1g microcapsules= $16.302*10^6$ microcapsules

**Table 6.E.3(b): Antimicrobial activity of immobilized *Lactobacillus casei* cell treated with inulin**

| Amount of microcapsules | Immobilization methods | Zone of inhibition (mm) |
|---|---|---|
| **1g** | External gelation | 22 |
| **1g** | Internal gelation | 37 |

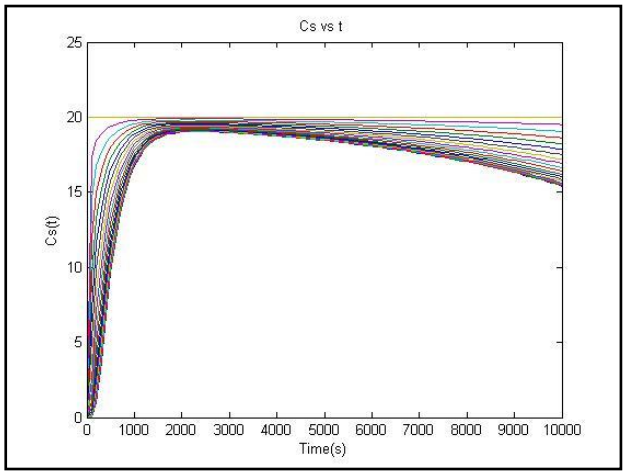
## 6.F. Mathematical modeling for the prediction of lactose and biomass concentration profiles in probiotic microcapsules and beads suspended in lactose rich MMRS medium and green coconut water

### 6.F.1. Simulated Profiles

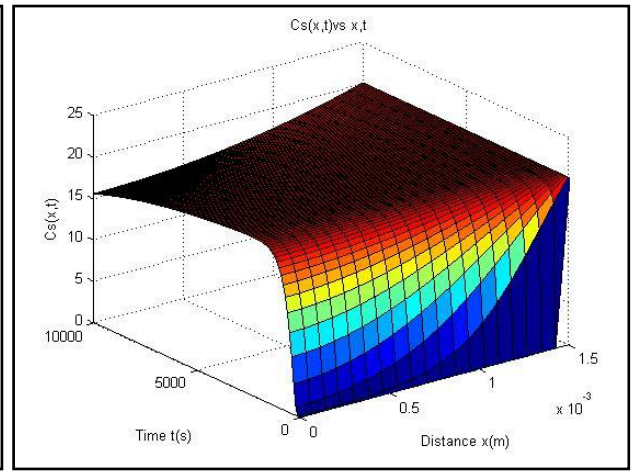
The simulated concentration profiles of substrate and biomass considering their evolution over different values of incubation time (t=10000s and t=600min) have shown in Figures 6.F.1 (a)-(g) and 6.F.2 (a)-(g) for Ca-alginate beads and microcapsules obtained through internal and external gelation techniques respectively.

#### 6.F.1.1. Substrate and biomass concentration profile for three immobilization processes at t=10,000 seconds

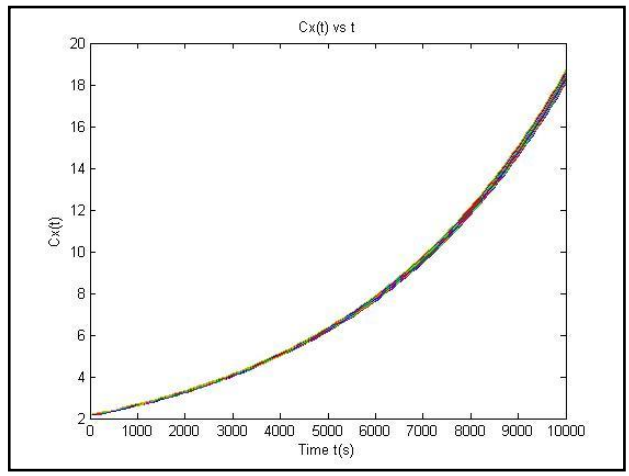
- **For Ca-entrapped beads**




**(a)**

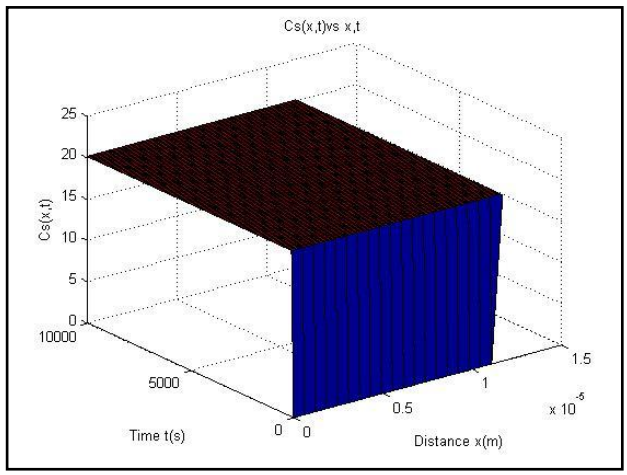



**(b)**

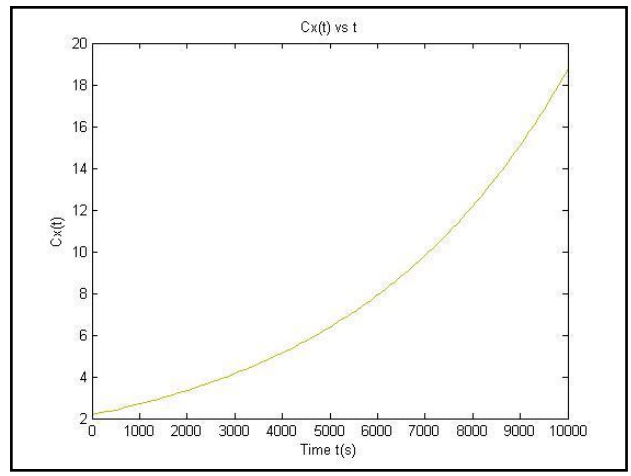



**(c)**

❖ **For microcapsules immobilized through External gelation**

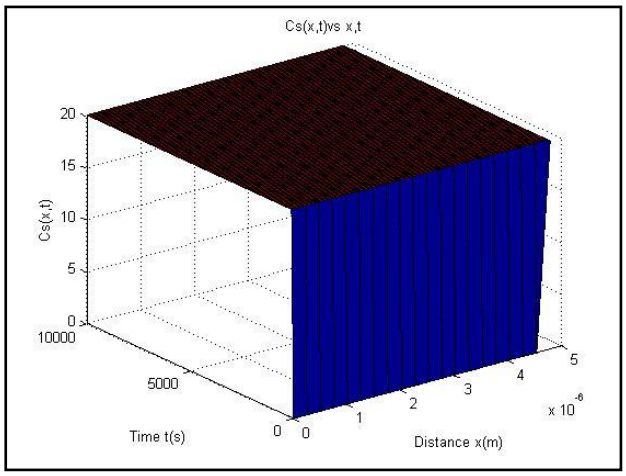


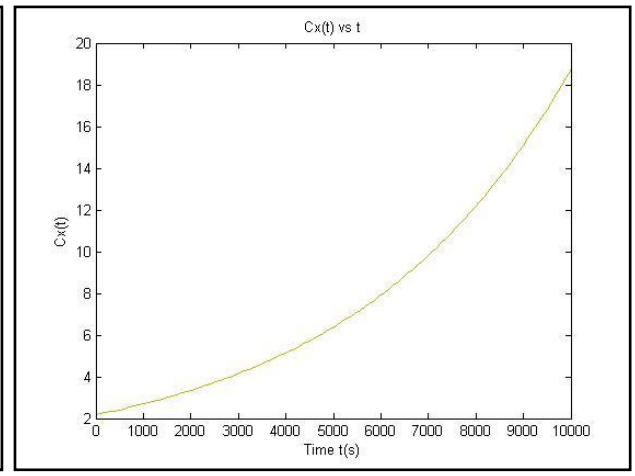

**(d)**




**(e)**

❖ **For microcapsules immobilized through Internal gelation**



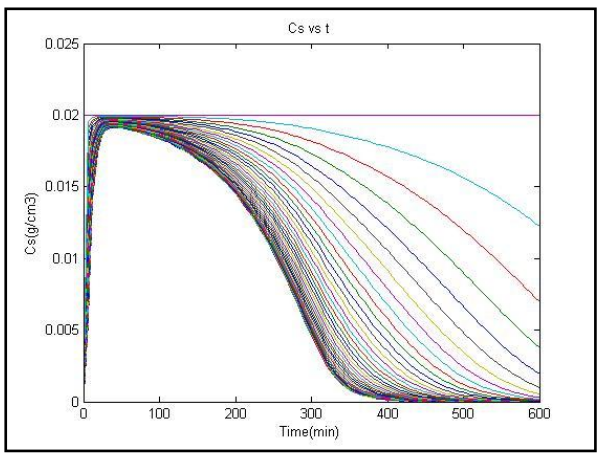



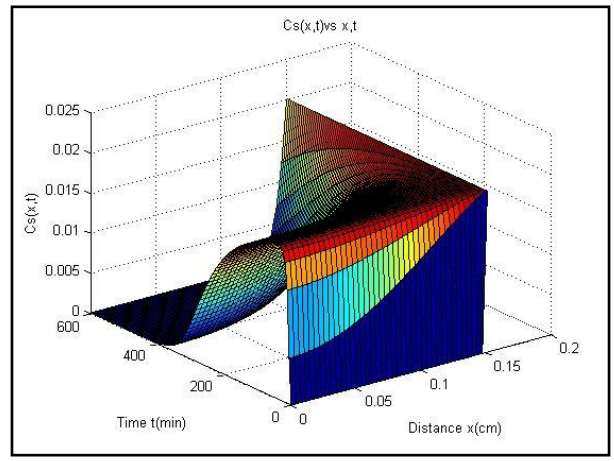

**(f)** **(g)**

**Figure 6.F.1(a)-(g): Substrate and biomass concentration profile for three immobilization processes at t=10,000 seconds**

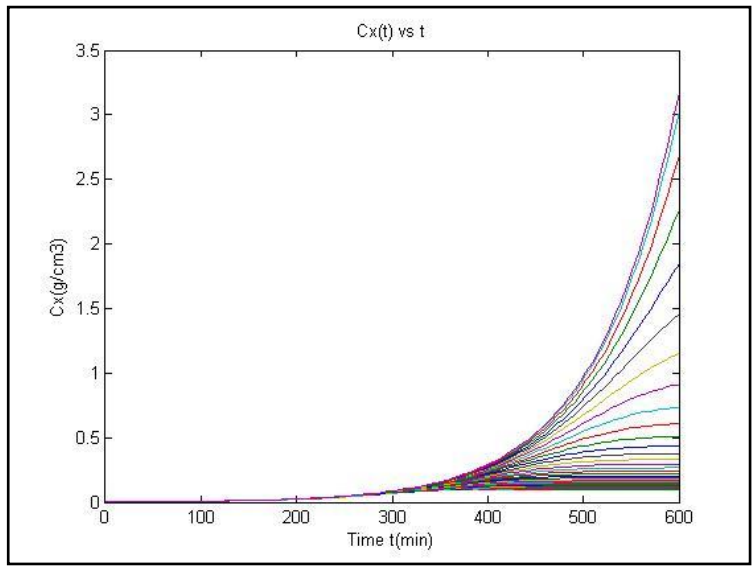
**6.F.1.2. Substrate and biomass concentration profile for three immobilization processes at t=600min**

❖ **For Ca-entrapped beads**



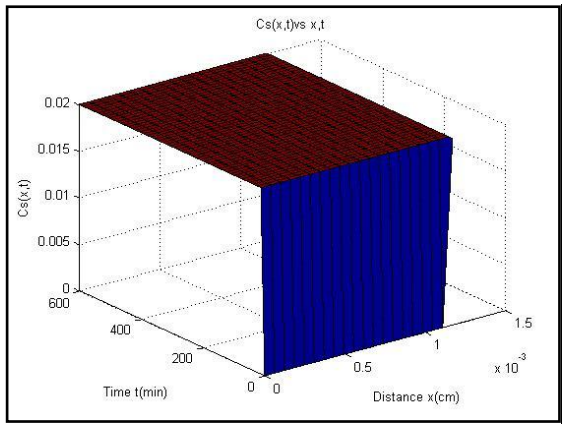




**(a)** **(b)**

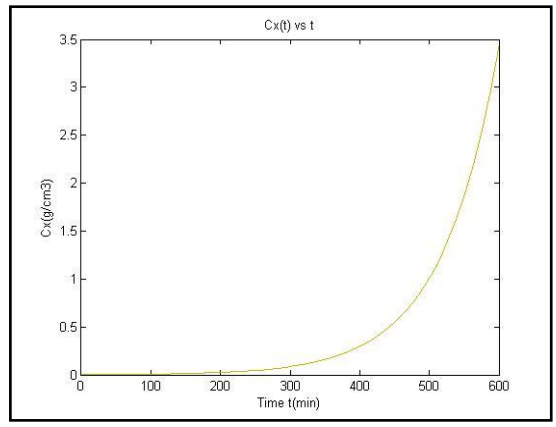



**(c)**

- **For microcapsules immobilized through External gelation**

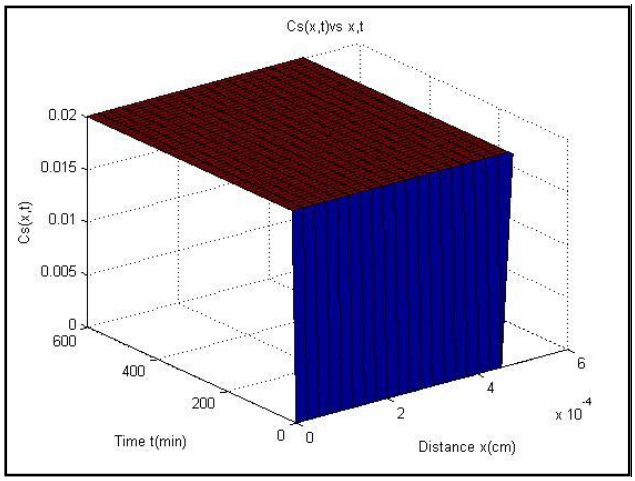


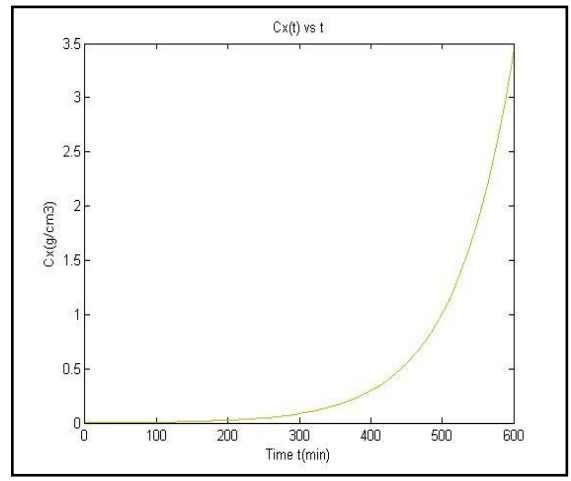

**(d)**




**(e)**

❖ **For microcapsules immobilized through Internal gelation**



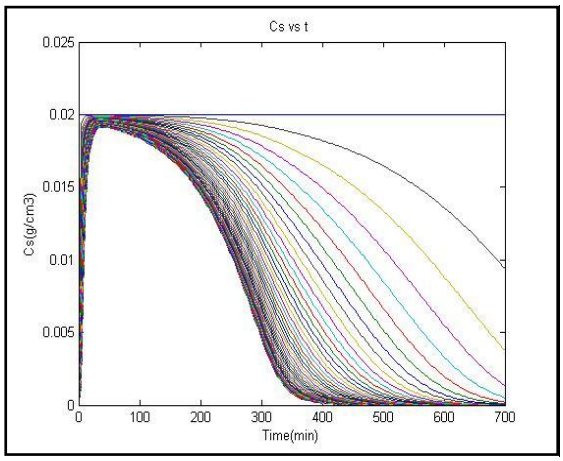



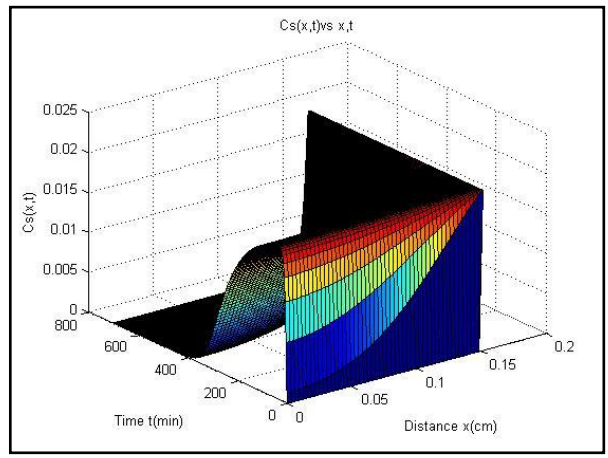

**(f)** **(g)**

**Figure 6.F.2(a)-(g): Substrate and biomass concentration profile for three immobilization processes at t=600min**

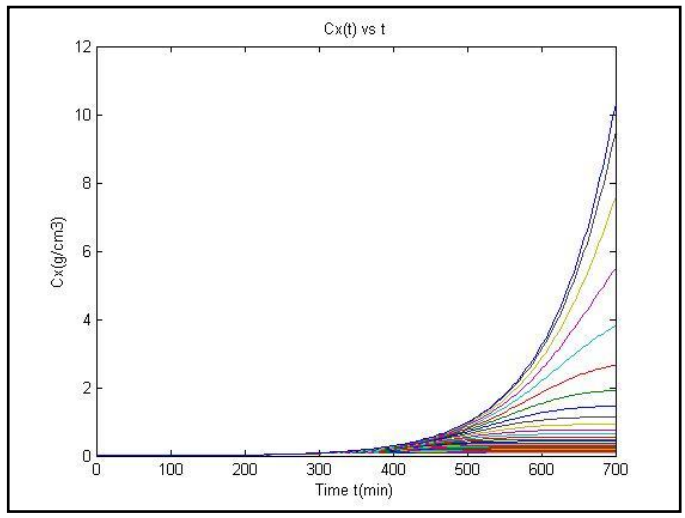
### 6.F.1.3. Substrate and biomass concentration profile for Ca- alginate entrapment; t=700min.

**For r = 0.9R; Cx = 2.5 g/cm3**




**(a)** **(b)**




**(c)**

**Figure 6.F.3(a)-(c): Substrate and biomass concentration profile for Ca-entrapment method processes at t=700 min**

In case of encapsulation via direct entrapment, the generation of radial concentration profile of both substrate and biomass at each value of incubation time has been observed. No such radial profile of concentration of substrate is obtained for any of the microcapsules. This may be due to relatively larger diameter of the beads compared to that of microcapsules. From the analysis of the Figure 6.F.1 (a), it is evident that the saturation level of substrate concentration of 20g/L at the outermost radial position is attained at 10000s for Ca-alginate beads. In case of microcapsules the substrate concentration reaches the saturation value of 20g/L at each radial position (Figure 6.F.1 (d) and 6.F.1 (f)). On the other hand, an ever increasing trend in the time-concentration plot of biomass has been observed for all encapsulated system (Figure 6.F.1(c), 6.F.1 (e), 6.F.1(g)) . However, unlike microcapsules, spatial gradient of biomass is also observed for the Ca-alginate beads. The Figures 6.F.2(a)-(g) show the dependence of concentration of substrate and biomass on the incubation time up to 600 min and radial position for Ca-alginate beads and microcapsules respectively. The time concentration profile of substrate for beads, depicted in Figure 6.F.2 (a), clearly indicates a decreasing trend after reaching a maximum value at a particular incubation time of 50 min at each radial position. This can be due to higher assimilation rate of substrate by the probiotic cells in comparison to its rate of diffusion from the abiotic external medium. The biomass concentration, however, shows an increasing pattern over

the entire time span. In case of microcapsules, while the substrate concentration remains saturated, increasing pattern of time-concentration plot of biomass is sustained. This indicates that the rate of diffusion of substrate is almost offset by the rate of consumption by the biomass (Figure 6.F.2.(d)-(g)). As evident from the previous calculation, the maximum attainable biomass concentration in all types of immobilized system is 2.55 g/mL. This value is achieved at 574 and 587 minutes for the microcapsules produced through internal and external gelation respectively. Figures 6.F.3 (a)-(c) represent the time-concentration profiles for substrate and biomass within Ca-alginate beads up to 700 minutes as a function of radial position. The profiles for both substrate and biomass are very similar to those obtained at 600 min. Further analysis of the Figure 6.F.3 (b) indicates that the biomass concentration of 2.55 g/mL is attained at 668minutes. These values have been compared with the experimental ones obtained for lactose rich MMRS medium and green coconut water sample containing initial lactose concentration of 20g/L in Figure 6.F.4 and good agreement has been established indicating the validity of the model. This mathematical model is expected to be applicable for the prediction of the behavior of encapsulated probiotics used for the preservation of food. The delayed release of probiotics due to the immobilization clearly enhances their availability for prolonged preservation period [13]. Since the bacteriocin, caseicin in case of *Lactobacillus casei* having antimicrobial activity against pathogenic bacteria can diffuse out of the encapsulated environment due to their small size the shelf life of the preserved food is enhanced even when the probiotics are in immobilized form. After the release of probiotics into the food matrix their growth is sustained till the supply of nutrients is ensured. The survival of encapsulated probiotic organisms in another food matrix, viz carrot juice has been reported by Nazzaro et al and Ying et al during the studies on preservation at 4°C [14, 15]. The mathematical model developed under this study is the first one for immobilized probiotics suspended in food matrix and it is expected to be successfully implemented for the further designing of probiotic food. In a recent literature, Kailasapathy pointed out that the probiotic micocapsules will play a determining role in food processing industry and future food regulations will make it mandatory to mention the viability of cells in the "probiotic food matrix"[16]. This mathematical model will be particularly useful for this purpose.

### 6.F.2. Experimental "Release time" of microorganisms from entrapped systems of probiotics exposed to MMRS medium and lactose rich green coconut water

The values of "release time" of probiotic cells from the Ca-alginate beads and different microcapsules exposed to MMRS medium and lactose-rich green coconut water are provided in Table 6.F.1. From the analysis of the table, it is evident that the bursting of Ca-alginate beads require the longest time, while the microcapsules produced through internal gelation are ruptured first. From of the comparison of release time of probiotics from the immobilized systems in MMRS medium and lactose rich green coconut water it is clear that the values obtained for MMRS medium are always lower than those obtained from green coconut water. This is due to the fact that although the probiotics are supplied with same concentration of lactose (20 g/L) for both the media, the essential micronutrients are present in MMRS are not available in green coconut water. Therefore, the growth rate of cells within the immobilized system suspended in MMRS medium is expected to be higher than that obtained in green coconut water resulting in early bursting of immobilization matrices in the former.

**Table 6.F.1: "Release time" of immobilized probiotic cells in lactose rich MMRS medium and green coconut water**

| Immobilization methods | "Release time" (Minutes) | |
|---|---|---|
| | MMRS | Green coconut water |
| Internal gelation | 570 | 572 |
| External gelation | 580 | 585 |
| Ca-entrapment | 664 | 670 |

The Table 6.F.1 provides the matrix of the experimental values of burst-off times for the beads or microcapsules; prepared via different methods of encapsulation when suspended in MMRS medium. "Burst-off" time for beads and capsules varies with the methods through which they are formed. As per expectation, the maximum "burst-off" time is required for Ca-alginate beads.

This is due to the presence of high diffusional resistance of substrate i.e. lactose and lowest surface area to volume ratio.

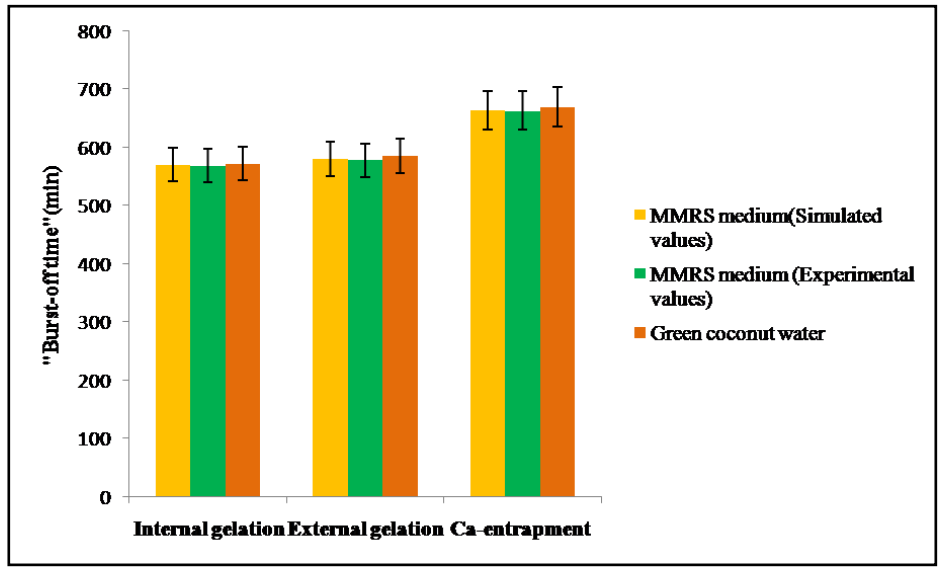



**Figure 6.F.4: Comparison of experimental and simulated burst off time for beads and microcapsules in lactose-rich MMRS medium and Green coconut water**

## 6.G. Mathematical modeling for the determination of "Release time" of probiotic cells from the entrapment beads and microcapsules suspended in simulated gastrointestinal (GI) juices

### 6.G.1. Experimental "Release time" of microorganisms from Prebiotic-Probiotic entrapped systems exposed to simulated GI juices

It is experimentally observed that while the simulated large intestinal fluid medium becomes turbid after a certain time interval the other media remain clear for entire 24 h. The time at which the turbidity suddenly appears in the simulated large intestinal medium corresponds to the release time of microcapsules from the respective immobilized systems. In Table 6.G.1, the values of burst off time of *Lactobacillus casei* from beads and different microcapsules in various simulated media have been provided.

**Table 6.G.1: "Release time" of *Lactobacillus casei* from prebiotic-probiotic beads and microcapsules in various simulated media**

| Simulated media | Immobilization methods | "Release time" (min) |
|---|---|---|
| Saliva juice | Internal gelation | --- |
| | External gelation | --- |
| | Ca-alginate entrapment | --- |
| Gastric juice | Internal gelation | --- |
| | External gelation | --- |
| | Ca-alginate entrapment | --- |
| Small intestinal juice | Internal gelation | --- |
| | External gelation | --- |
| | Ca-alginate entrapment | --- |
| Large Intestinal juice | Internal gelation | 50 |
| | External gelation | 63 |
| | Ca-alginate entrapment | 908 |

From the analysis of the table it can be inferred that the microorganisms cannot release from the entrapped systems in simulated saliva juice, gastric juice and small intestinal juice. On the other hand in the simulated large intestinal fluid medium the microorganisms are suddenly released after different exposure time. The value of released time from the microcapsules produced through internal gelation is the least among those of all three varieties of entrapped systems under consideration. On the other hand the release time for the Ca-alginate bead is the longest among all. This observation may be justified by the fact that the capacity of holding of the maximum number of cells is minimum in case of internal microcapsules and the maximum in case of Ca-alginate beads. The reason behind the incapability of release of probiotics in saliva, gastric and small intestinal juice environment is due to unfavorable pH of these media with respect to the growth of *Lactobacillus casei* in the internal core.

### 6.G.2. "Release time" of microorganisms from Prebiotic/probiotic Beads and Microcapsules in simulated large intestinal fluid: comparison between experimental and simulated values

Using the Equation 5.20 developed under section 5.6, the release time of the probiotic *Lactobacillus casei* from Ca-alginate beads and microcapsules produced through internal and external gelation in large intestinal fluid environment have been evaluated. The detailed calculation has been provided in the Appendix.

**Determination of Burst-off time**

**Internal gelation**

$2551*10^{15}= 1.329*10^{18}*e^{0.8338*t_{cr}}$

$t_r$=0.7815 h= 46.89 min

**Direct entrapment**

$2.55*10^{18}= 4.207*10^{17}*e^{0.8338*t_{cr}}$

$t_r$=15.16 h

**External gelation**

$2.55*10^{18}= 10.312*10^{17}*e^{0.8338*t_{cr}}$

$t_r$= 1.08 h

The simulated values have been compared with the experimental one in Figure 6.G.1.

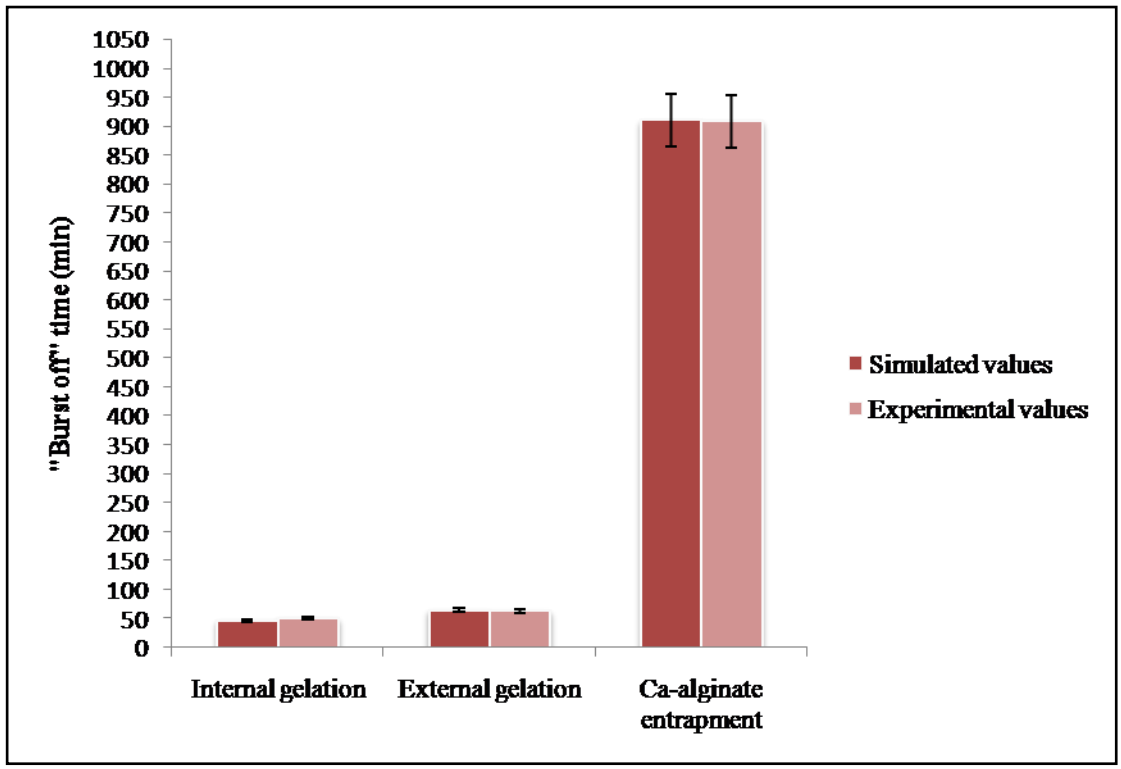



**Figure 6.G.1: Comparison of experimental and simulated burst off time for beads and microcapsules in simulated large intestinal fluid**

From the figure it is clearly evident that the computed data are in good agreement with the experimental ones. Therefore, it may be inferred that the proposition of "burst-release" of probiotics from the beads and microcapsules is valid. However this should be further verified with the data obtained through *In-vivo* experiments using real human systems.

## 6.H. Mathematical modeling of a simulated *in-vitro* GI system to predict the concentration- time histories of prebiotic/probiotic microcapsules (internal) microcapsules and released probiotic cells in the simulated GI system

In Figure 6.H.1 (a) and (b) the time histories of unburst (UNB) microcapsules and the probiotic cells have been depicted. The mass balance equations provided in Table have been used for the simulation. The *in-vitro* experimental values of concentration of probiotic cells have been superimposed on the same figure. The concentration of microcapsules after total pumping time

of PBS of 2000 min have been measured and have been plotted on the same figure. The agreement between the experimental and simulated values of microcapsule concentration at 2000 min and that of patterns of time histories of probiotic cells in the reactors representing the stomach and the small intestine validates the mathematical model. From the close analysis of the simulated time histories it is clear that the concentrations of microcapsules fall exponentially with time in the stomach. On the other hand the absence of probiotics in the stomach is also established from the figure. The pattern of time histories of the microcapsules in the gastric (stomach) environment can be justified by the fact that once fed into the stomach the microcapsules are swept away from this reactor at a constant rate. No prebiotics are detected in the stomach since there is no rupture of microcapsules in this acidic environment. From the analysis of Figure 6.H.1 (b) it appears that the time history of the microcapsules passes through a maximum in the small intestine. This is justified by the fact that the microcapsules are both fed and removed from the small intestine and there is initially an apparent increase in microcapsules due to the disparity between the hydrodynamic residence time in the stomach (80.5min) and small intestine (268.8min). Declining pattern is observed as soon as the concentration of microcapsules in the stomach becomes zero and hence the outlet PBS stream from the stomach is free from microcapsules. Similar to the gastric environment, the immobilized probiotics are not released in the small intestine due to unfavorable pH.

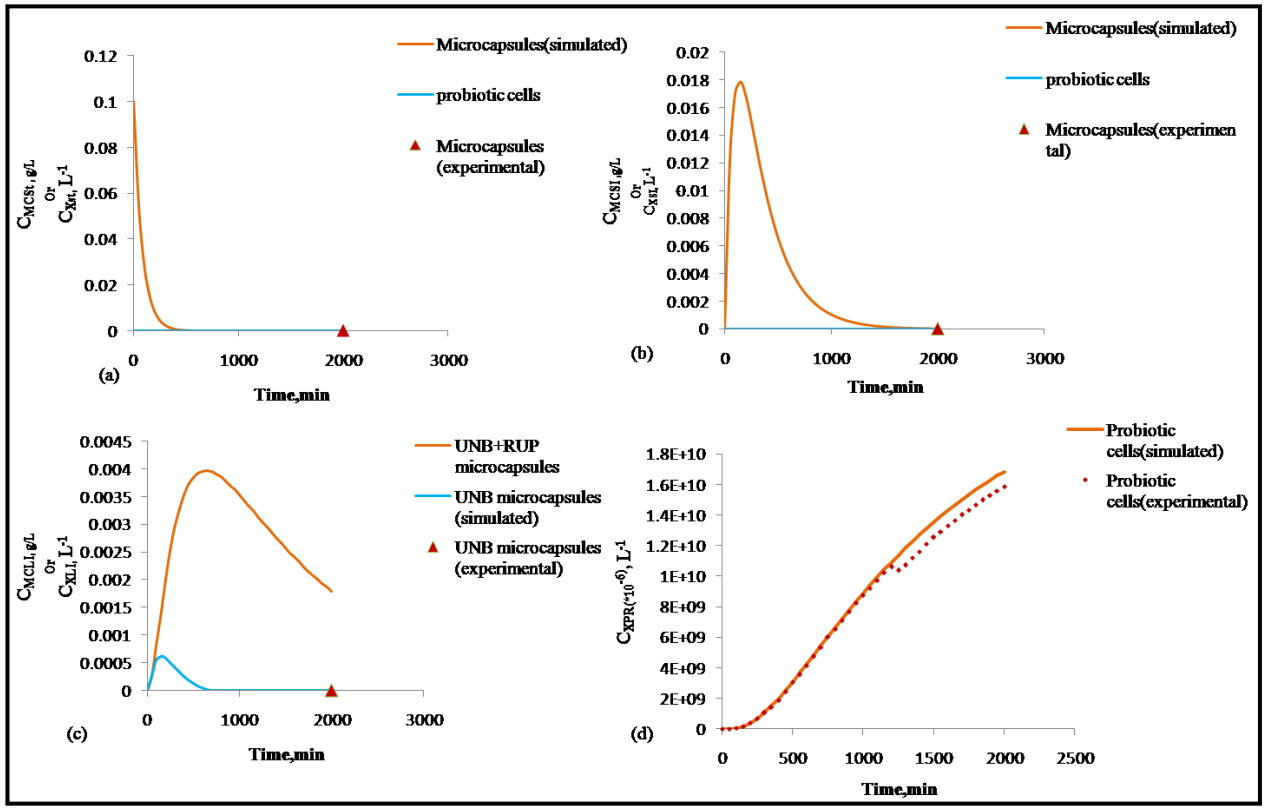



**Figure 6.H.1: Concentration profiles for human gut system**

$C_{MCSt}$: Concentration of microcapsules in "stomach"; $C_{MCSI}$: Concentration of microcapsules in "small intestine"; $C_{MCLI}$: Concentration of microcapsules in "large intestine"; $C_{XPR}$: Concentration of probiotic cells

Figure 6.H.1 (c) depicts the simulated time-concentration plots of unburst microcapsules as well as the total number of microcapsules that would have been present if there were no bursting of cells. The *in vitro* experimental value of the concentration of unburst microcapsule at 2000 minutes of pumping time of PBS solution has also been graphed in the same figure. From the patterns of the simulated plots it is clearly evident that both the concentrations of whole microcapsules and the summation of concentrations of unburst and ruptured microcapsules pass through maxima. The maximum in the time concentration plot of unburst microcapsules is obtained much earlier than that on the time-concentration plot of total microcapsules (unburst + ruptured). The pattern of variation of concentration of unburst microcapsules with time may be explained by the fact that while the concentration increases due to the feeding of PBS stream from the small intestine containing microcapsules it diminishes as a result of rupture of

microcapsules (RUP) after each 50 min of residence time and due to the outlet. On the other hand the pattern of time history of total microcapsule shows the pattern if there were no burst releases of probiotics and hence is guided by the hydrodynamics. The simulated value of concentration of unburst microcapsule at 2000 minutes is in agreement with its experimental counterpart. Figure 6.H.1 (c) depicts the simulated time concentration profile for the probiotic cells in the reactor representing large intestine. The experimental values of concentration of cells have also been superimposed on the Figure 6.H.1 (d). Expectedly, the time history of the probiotic cell shows an increasing trend. The agreement between the experimental and simulated values validates the mathematical model for the prediction of behavior of prebiotic-probiotic microcapsules in the *in vitro* simulated GI system. The predictions of the model should be verified by comparing with the results of *in-vivo* experiments on real human system. From the *in-vitro* experiment and from the prediction of the mathematical model, it is clear that after the intake of a dose of prebiotic/probiotic microcapsules, there will be sustained release of probiotic cells up to 2000 min and later. Since the prebiotic biomolecules are also released and other carbohydrates are available in the real large intestinal environment, their growth after release will also be supported. However, it is understandable that as different types of food substances are simultaneously up-taken by human beings in reality, complex mathematical models incorporating the interaction of microcapsules with other food molecules may be necessary.

Kailashpathy has identified that co-encapsulation of prebiotic biomolecules and probiotic bacteria within a single capsule will serve as an useful tool for the controlled release of the later through the enhance mental growth due to synbiotic effects [16]. This is the first mathematical model to predict the release time for the probiotics in the GI environment. Although many pioneering mathematical models have been developed for the growth behavior of probiotic cells in the GI environment [17-21]. However the present model is a “proof of concept” type one and needs further modification by incorporating the real behavior of the GI environment, viz., consideration of non-Newtonian behavior of fluids, effects of interaction between other food molecules and the microcapsule and so on. Ultimately the validity of the model should be checked by the comparison with the *in-vivo* results.

# 6.I. Application of immobilized probiotic *Lactobacillus casei*(2651 1951 RPK) in food material

## 6.I.1. Application of immobilized probiotic in Indian milk cake

One of the major factors of immobilized prebiotic-probiotic conjugate system is their shelf lives when stored in different temperatures. In order to get quantitative idea of cell viability of such conjugate system, in the present investigation a set of programmed experiments has been carried out. Milk cake a very common sweet material of Asian subcontinent people has been selected as the candidate food material as the principle source of support material. All the three immobilized samples obtained after following three different immobilization techniques, viz., microencapsulation (both external and internal gelation) and gel entrapment have been studied separately. In each case immobilized native *Lactobacillus casei* has been used for comparison purpose. Viability of probiotic *Lactobacillus casei* cell in fortified food product after preservation has been noted at two different temperatures, viz., 4 $^{o}$C and -20 $^{o}$C respectively. Results are shown in Table 6.I.1. In the present investigation viability of cell has been reported in terms of cell count of *Lactobacillus casei*.

**Table 6.I.1: Results of viability assay (cell concentrations) for fortified milk cakes***

| Weeks | Microcapsules (External gelation) ($x10^{-7}$)/mL | | | | Beads (Entrapment) ($x10^{-7}$)/mL | | | | Microcapsules (Internal gelation) ($x10^{-7}$)/mL | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PRO | | PRO+CI | | PRO | | PRO+CI | | PRO | | PRO+CI | | PRO+PMI | |
| | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| 1 | 22.0 ± 0.000 | 69.6 ± 0.577 | 51.6 ± 0.577 | 72± 0.000 | 13.6 ± 0.577 | 25.6 ± 0.577 | 24.6 ± 0.577 | 34.6 ± 0.577 | 40± 0.000 | 71.6± 0.577 | 60± 0.000 | 88.6 ± 0.577 | 84.6± 0.577 | 94.6± 0.577 |

| | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 2 | 12.7 ± 0.577 | 35.6 ± 0.577 | 17.6 ± 0.577 | 62.6 ± 0.577 | 9± 0.000 | 10.6 ± 0.577 | 13± 0.000 | 19.6 ± 0.577 | 28.6± 0.577 | 43± 0.000 | 41.6± 0.577 | 62.6 ± 0.577 | 42.6± 0.577 | 64± 0.000 |
| 3 | 6.7 ± 0.577 | 13± 0.000 | 8.6± 0.577 | 35.6 ± 0.577 | 4.6± 0.577 | 9± 0.000 | 9.6± 0.577 | 12.6 ± 0.577 | 14.6± 0.577 | 34.6± 0.577 | 21.6± 0.577 | 46± 0.000 | 23.6± 0.577 | 37.6± 0.577 |
| 4 | 3.3± 0.577 | 7± 0.000 | 5± 0.000 | 14.6 ± 0.577 | 3.6± 0.577 | 6.6± 0.577 | 7.6± 0.577 | 9± 0.000 | 6.6± 0.577 | 16.6± 0.577 | 11.6± 0.577 | 24.6 ± 0.577 | 11± 0.000 | 24.6± 0.577 |

*****For Table 6.I.1, PRO: fortified with only probiotic; PRO+CI: fortified with probiotics and commercial inulin; PRO+PMI: fortified with probiotics and pearl millet inulin; A: preservation at $4^{o}$C; B: $-20^{o}$C.

*Values are mean (n=3) ±standard deviation

### 6.I.2. Viability of the probiotic cells in fortified food products after preservation

In Figure 6.I.1 (a) and 6.I.1 (b) the photographs of the spread plates obtained for unfortified food samples and that fortified with encapsulated *L. casei* are provided. It clearly indicates that while the control is fully contaminated, no contamination is observed in case of the fortified one. This establishes the antimicrobial activity of the immobilized probiotic culture against contaminating microorganisms and the elongation of shelf life. Due to unavailability of literature data, the results cannot be compared.

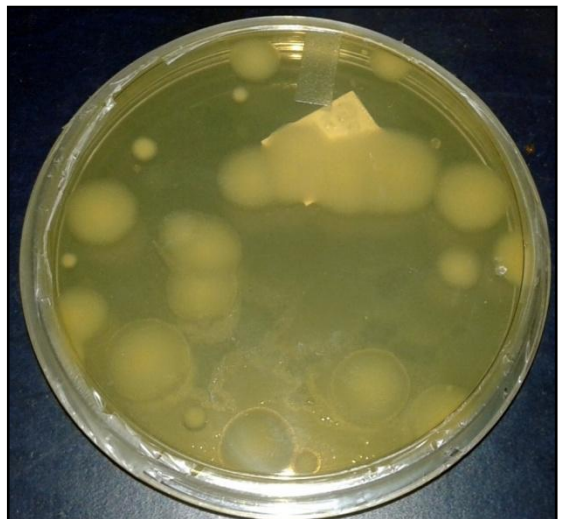


**Figure 6.I.1(a): Plate for control sample of milkcake (without probiotics) on 4$^{th}$ week after preservation at 4 $^{o}$C**

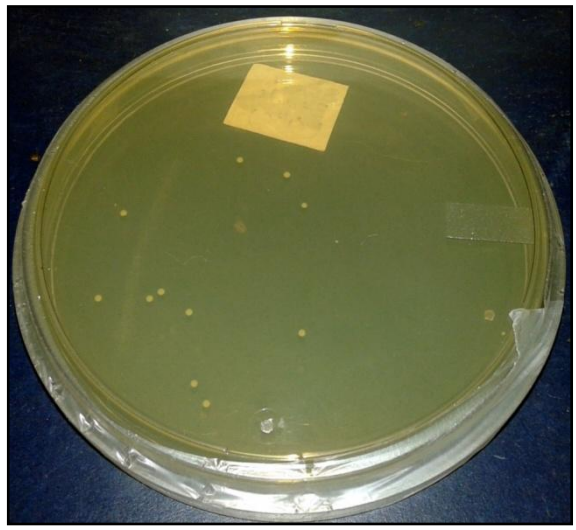


**Figure 6.I.1(b): Plate for milk cake sample fortified with encapsulated probiotics on 4$^{th}$ week after preservation at 4 $^{o}$C**

The bar plots (shown in Figures 6.I.2. (a), through 6.I.2.(d)) depict the concentrations of viable *Lactobacillus casei* cells in fortified milk cake samples. Fortification has been done by gel entrapped beads and microcapsules containing either native probiotics or mixture of probiotics and prebiotic commercial inulin (derived from chicory). Results of samples preserved for four weeks at an interval of one week each have been reported.

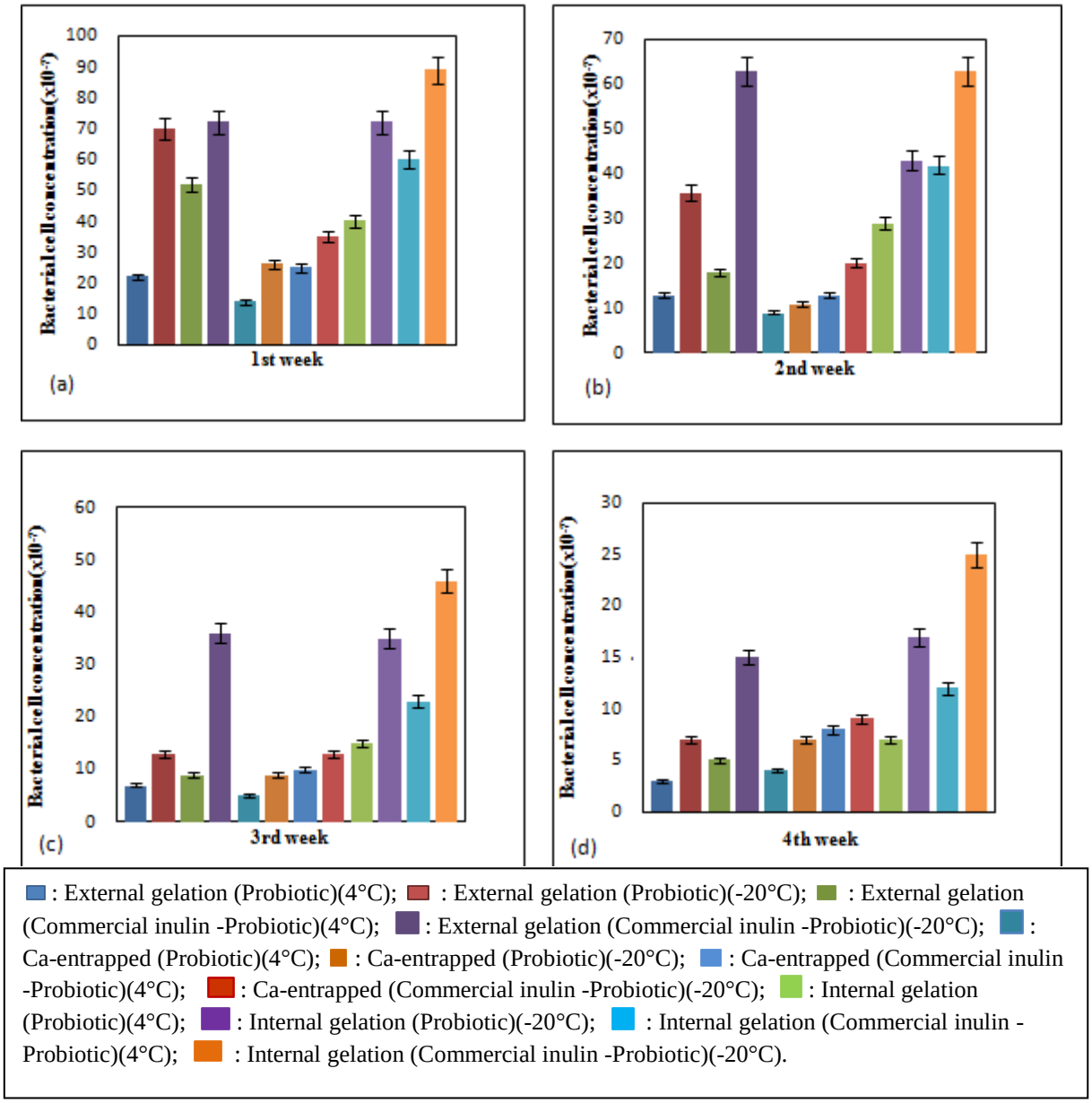



**Figure 6.I.2(a)-(d): Comparison of effects of immobilization methods and fortification vectors (only Probiotic or Commercial inulin -Probiotic) on viability of probiotics in fortified milk cakes preserved at 4$^{o}$C and -20$^{o}$C.**

It is clearly indicated in the Figure 6.I.2 (a) through (d) that irrespective of the methods of immobilization, fortification vectors and period of preservation, the viability of the cells is more for preservation condition of -20 $^{o}$C in comparison to that exhibited at 4$^{o}$C. This is due to better preservation of cells at -20 $^{o}$C compared to that at 4 $^{o}$C. Similar observations were obtained by

Martin et al during their studies on preservation of immobilized *L. fermentum* at -20 $^{o}$C and 4 $^{o}$C for 30 days [22]. Irrespective of immobilization method preservation temperature and fortification vector, it is observed that the viability of cells decreases even at -20 $^{o}$C with the increase of preservation period. This may be due to the fact that on prolonged preservation at sub-zero temperature cells age, and lysis takes place. While analyzing the effect of immobilization technique on the viability of cells, it is revealed that whatever might be the fortification vector and the preservation time and condition, the best viability of cells was shown by the microcapsules produced through internal gelation method. This is justified by the fact that due to small size of microcapsules the surface area available for the transport of substrates present in the extracellular food materials into the cells was increased resulting in more sustainability of cells. This is also due to the low leakage of cells from the microcapsules in case of internal gelation beads as compared to the external beads and the Ca-alginate entrapped beads [22,23]. Better viability of preserved probiotic cells immobilized through internal gelation in comparison to those immobilized with entrapment and external gelation techniques has also been observed by other researchers [22]. It is clearly indicated from the comparison of performance of fortification vectors that irrespective of immobilization method and preservation period, the best viability of cells is exhibited by the co-immobilized combination of probiotic cells and commercial inulin. This is due to the fact that even under immobilized condition, the prebiotic inulin serves as a suitable substrate for the sustainability of the probiotic cells. As no reported data are so far available on the effect of co-immobilization of *L. casei* and a prebiotic compound, the comparison could be made with the literature data. It is observed that the variation of the probiotic cell count (CFU) in the 0.2 g/mL solution of food sample in phosphate buffer, as described under experimental section, is in the range of 3-95 x$10^{7}$ /mL and is much higher than the recommended lower limit of cell count of $10^{6}$ CFU/mL in probiotic food. Thus all the milk cakes fortified by different vectors are suitable as probiotic food. Among all samples, combination of probiotic cells with prebiotic inulin, immobilized through internal gelation, is the most effective one. As expected, the prebiotic inulin is effective in enhancement and sustainability of probiotic cells even in co-immobilized condition. All the food samples are suitable for human intake even after 4 weeks of preservation.

The viability of probiotic cells in milk cakes fortified by microcapsules produced through internal gelation containing combinations of *L. casei* and either commercial inulin or pearl millet inulin is indicated in the Figure 6.I.3.

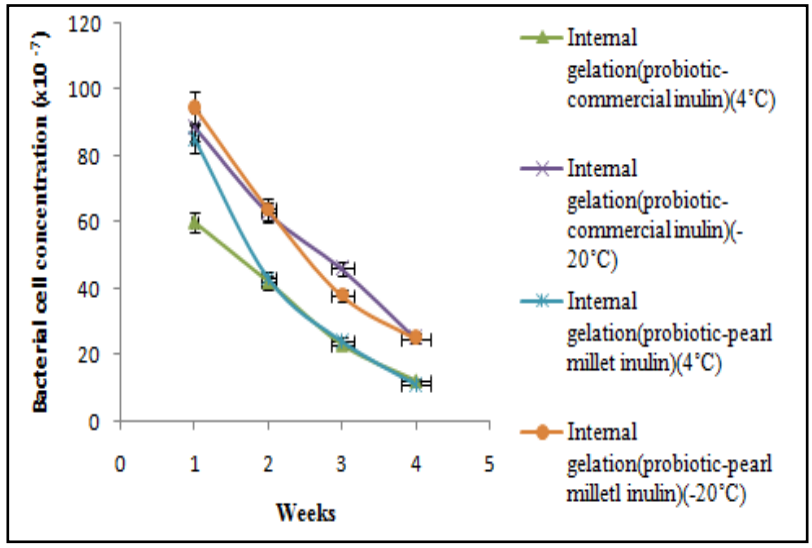



**Figure 6.I.3: Comparison of effect of microcapsules obtained by internal gelation method for both the combination probiotic-commercial inulin and probiotic-pearl millet inulin at $4^{o}$C and $-20^{o}$C in case of milk cake.**

(Results are average of those obtained from three replicate observations. Standard deviation varies from 0 to 0.57735)

A close analysis of the figure clearly reveals that the effectiveness of pearl millet inulin in enhancement of sustainability of probiotic cells is marginally better than that of commercial inulin. This may be due the difference in degree of polymerization of inulin derived from chicory and pearl millet inulin. However, more experiments have to be conducted to reveal the exact fact. Substitution of commercial inulin by derived from pearl millet seems to be promising on commercial scale in near future.

### 6.I.3. Application of immobilized probiotic in Indian jaggery

The promising results obtained on sweet milk cake paves the way of diversification in other perishable food materials. Thus in the present investigation a programmed experiment has been

carried out on the viability of cell count for immobilized prebiotic-probiotic fortified jaggery. Experimental procedure similar to that followed in case of Indian milk cake. The results are shown in Table 6.I.2.

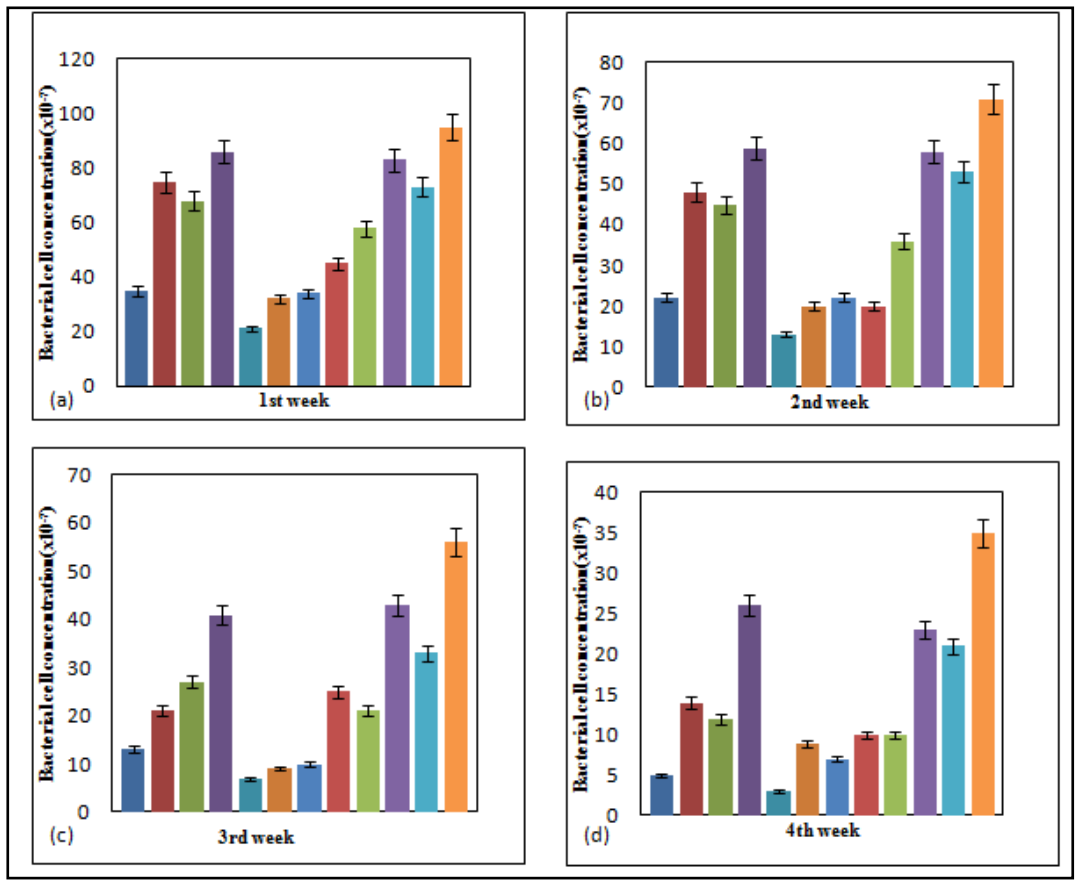
**Table 6.I.2: Results of viability assay (cell concentrations) for fortified jaggery***

| Weeks | Microcapsules (External gelation) ($x10^{-7}$)/mL | | | | Beads (Entrapment) ($x10^{-7}$)/mL | | | | Microcapsules (Internal gelation) ($x10^{-7}$)/mL | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PRO | | PRO+CI | | PRO | | PRO+CI | | PRO | | PRO+CI | | PRO+PMI | |
| | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| 1 | 34.6 ± 0.577 | 75± 0.000 | 67.6 ± 0.577 | 85.6 ± 0.577 | 20.6 ± 0.577 | 32± 0.000 | 33.6 ± 0.577 | 44.6± 0.577 | 57.6± 0.577 | 82.6± 0.577 | 73± 0.000 | 94.6 ± 0.577 | 97.6± 0.577 | 104.6± 0.577 |
| 2 | 22 ± 0.000 | 47.6 ± 0.577 | 44.6 ± 0.577 | 59± 0.000 | 13± 0.000 | 19.6 ± 0.577 | 21.6 ± 0.577 | 30.6± 0.577 | 35.6± 0.577 | 58± 0.000 | 52.6± 0.577 | 70.6 ± 0.577 | 75± 0.000 | 84.6± 0.577 |
| 3 | 12.6 ± 0.577 | 20.6 ± 0.577 | 27± 0.000 | 41± 0.577 | 6.6± 0.577 | 10.6 ± 0.577 | 14.6 ± 0.577 | 25± 0.000 | 20.6± 0.577 | 42.6± 0.577 | 32.6± 0.577 | 56± 0.000 | 35.6± 0.577 | 68.6± 0.577 |
| 4 | 4.6± 0.577 | 13.6 ± 0.577 | 11.6 ± 0.577 | 26± 0.577 | 2.6± 0.577 | 8.6± 0.577 | 7± 0.000 | 9.6± 0.577 | 10± 0.000 | 22.6± 0.577 | 20.6± 0.577 | 34.6 ± 0.577 | 17.6± 0.577 | 28± 0.000 |

*****For Table 6.I.2, PRO: fortified with only probiotic; PRO+CI: fortified with probiotics and commercial inulin; PRO+PMI: fortified with probiotics and pearl millet inulin; A: preservation at $4^{o}C$; B: $-20^{o}C$.

*Values are mean (n=3) ±standard deviation

The results clearly indicate that performance of prebiotic-probiotic conjugate is much better in case of jaggery compare to the Indian milk cake system. This is possibly due to abundant availability of glucose present in jaggery enables the probiotic to grow in a larger rate compare to the Indian milk cake system. Thus the performance of immobilized *L. casei* in association with the prebiotic either commercial inulin or pearl millet inulin( isolated in-house) is much better in case of jaggery compare to the Indian milk cake. A close inspection of the experimental data also reveals that the performance of immobilized *L. casei* in association with pearl millet inulin marginally better compare to that of its commercial inulin counterpart.




: External gelation (Probiotic)(4°C); : External gelation (Probiotic)(-20°C); : External gelation (Commercial inulin -Probiotic)(4°C); : External gelation (Commercial inulin -Probiotic)(-20°C); : Ca-entrapped (Probiotic)(4°C); : Ca-entrapped (Probiotic)(-20°C); : Ca-entrapped (Commercial inulin -Probiotic)(4°C); : Ca-entrapped (Commercial inulin -Probiotic)(-20°C); : Internal gelation (Probiotic)(4°C); : Internal gelation (Probiotic)(-20°C); : Internal gelation (Commercial inulin -Probiotic)(4°C); : Internal gelation (Commercial inulin -Probiotic)(-20°C).

**Figure 6.I.4: Comparison of effects of immobilization methods and fortification vectors (only probiotic or Commercial inulin- Probiotic) on viability of probiotics in fortified jaggery preserved at 4°C and -20°C.**

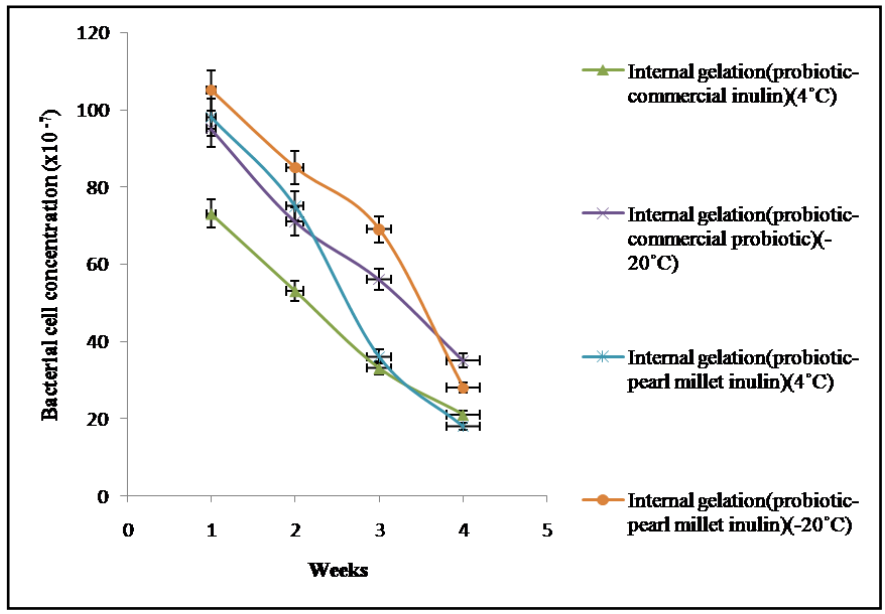



**Figure 6.I.5: Comparison of effect of microcapsules obtained by internal gelation method for both the combination probiotic-commercial inulin and probiotic-pearl millet inulin at 4°C and -20°C in case of jaggery.**

## 6.J. Cost analysis of pearl millet inulin

The commercialization of any product depends on its production cost to attain a competitive position with respect to similar products in the market. A cost analysis of production of inulin extract from pearl millet (bajra) has been done based on the optimum condition determined through RSM using laboratory scale experimental data.

On the annual basis the following results were obtained using the correlations derived in section 5.9 (Theoretical analysis).

**Table 6.J.1 Cost analysis of extraction of inulin from pearl millet (bajra) in small scale**

| Parameter | Significance | Value |
|---|---|---|
| $M_{PA}$ | Quantity of pearl millet processed | 2240 kg |
| $Q_{IA}$ | Quantity of pure inulin extracted | 423.808 kg |
| $W_Y$ | Volume of water used, mass of pearl millet processed | 16800L |
| $H_Y$ | Volume of HCl used, mass of pearl millet processed | 22.4 L |
| $E_y$ | Energy consumption | 4838.4 kWh |
| $E_T$ | Total energy cost | Rs. 32756 |
| $M_Y$ | Total material cost ( Pearl millet+ water+ HCl+ Other chemicals+ Filter cloths etc.) | Rs. 2,41,120 |
| $R_{CY}$ | Total annual recurring cost | Rs. 12,23,876 |
| $FC_y$ | Total annual fixed cost | Rs. 9,35,000 |
| $C_T$ | Total annual cost | Rs. 2158876 |
| $Ex_T$ | Extraction cost of inulin | Rs.5094.091/kg |

The cost of 25g commercial inulin is Rs. 2000. According to the lab scale experimental data the same quantity of pearl millet inulin may be produced by the investment of Rs. 127.35. Although, it is understandable that more purification of the pearl millet inulin is required before its full commercialization, it is expected that pearl millet inulin will also be a close and cost effective competitor of commercial inulin presently available in Indian market.

## REFERENCES


1. Chesini, M., Neila, L.P., de la Parra, D.F., Rojas, N.L., Esquivel, J.C.C., Cavalitto, S.F., Ghiringhelli, P.D. and Hours, R.A.( 2013) "*Aspergillus kawachii* produces an inulinase in cultures with yacon (*Smallanthus sonchifolius*) as substrate". Electronic Journal of Biotechnology, ISSN: 0717-3458, 16, 3.
2. Milorad, D., Cakic Goran, S.N. and Ljubomir, A. I. (2002) "FTIR Spectra of Iron (III) complexes with dextran, pullulan and inulin oligomers". Bulletin of the Chemists and Technologists of Macedonia, 21(2), 135-146.
3. www.senseair.se/senseair/gases-applications/hydrocarbons.
4. Cardenas, G.T. and Salinas, S. (2004) "TGA-FTIR of PdSn Fine solid supported on MgO cyclohexene reduction". Journal of Chilean Chemical Society, 49(3), 267-271.
5. Burn, D.A. and Qurczak, E.W. (Eds). (1992) Handbook of Near infrared Analysis. Practical Spectroscopy series, Marcel Dekker, Inc, NewYork, 13, 393-395.
6. wikis.lawrence.edu/display/chem.
7. Dinixchemy.Jumatdinintahyontahdakblogspot.in/2012/12/v, 2012.
8. Ricca, E., Calabrò, V., Curcio, S. and Iorio, G. (2009) "Fructose production by chicory inulin enzyme hydrolysis: Kinetic study and reaction mechanism". Process Biochemistry, 44, 466–471.
9. Douglas, S., Clark, H. and Blanch, W. (1997) Biochemical Engineering. Second Edition, CRC Press.
10. Li, W., Zhang, J., Yu, C., Li, Q., Dong, F., Wang, G., Gu. G. and Guo, Z. (2015) "Extraction, degree of polymerization determination and prebiotic effect evaluation of inulin from Jerusalem artichoke". Carbohydrate Polymers, 121, 315–319.
11. Nelson, D.L. and Cox, M.M. (2004) Lehninger principles of biochemistry, 4th edition, New York, WH Freeman.
12. Van Dan-mieras, M.C.E., James, J.W., Leach, C.K. , de Jew, W.H., de varies, J.(1992) "IN VITRO CULTIVATION OF MICRO-ORGANISMS". Published on behalf of open universiteit & Thans polytechnic (Netherland, UK),Butterwarth & Hanimann(publisher).
13. Inoue, S.K. (1997) "A Moving Boundary Model of Calcium Alginate Gel Formation and the Estimation of Diffusion and Mass Transfer Coefficients". Oregon State University.

14. Nazzaro, F., Fratiannia, F., Coppol, R., Sada, A., Orlando, P. (2009) "Fermentative ability of alginate-prebiotic encapsulated *Lactobacillus acidophilus* and survival under simulated gastrointestinal conditions". Functional Foods, doi:10.1016/j.jff.2009.02.001.
15. Ying, D.Y., Schwander, S., Weerakkody, R., Sanguansri, L., Gantenbein-Demarchic, C., Augustina, M.A. (2012) "Microencapsulated Lactobacillus rhamnosus GG in whey protein and resistant starch matrices: Probiotic survival in fruit juice". Journal of Functional Foods, http://dx.doi.org/10.1016/j.jff.2012.08.009.
16. Kailasapathy, K. (2002) "Microencapsulation of Probiotic Bacteria: Technology and Potential Applications". Current Issues Intestinal Microbiology, 3, 39-48.
17. Gildas, K., Gbassi, G.K. and Vandamme, T. (2012) "Probiotic Encapsulation Technology: From Microencapsulation to Release into the Gut". Pharmaceutics, 4, 149-163; doi: 10.3390/pharmaceutics4010149.
18. Macfarlane, G.T., Macfarlane, S. and Gibson, G.R. (1998) "Validation of a Three-Stage Compound Continuous Culture System for Investigating the Effect of Retention Time on the Ecology and Metabolism of Bacteria in the Human Colon". Microbial Ecology, 35,180–187.
19. Sathyabama, S., Ranjith kumar, M., Bruntha devi, P., Vijayabharathi, R. and Brindha priyadharisini,V. (2014) "Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in simulated gastric environment". LWT - Food Science and Technology, 57, 419-425.
20. Cook, M.T., Tzortzis, G., Charalampopoulos, D., Khutoryanskiy, V.V.(2012) "Microencapsulation of probiotics for gastrointestinal delivery". Journal of Controlled Release, 162, 56–67.
21. Child, M.W., Kennedy, A., Walker, A.W., Bahram Bahrami, B., Macfarlane, S., and Macfarlane, G.T. (2006) "Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques". FEMS Microbiology Ecology, 55, 299–310.
22. Martin, M.J., Villoslada, F.L., Ruiz, M.A., Morales, M.E. (2013) "Effect of unmodified starch on viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716". LWT - Food Science and Technology, 53, 480-486.

23. Chan, E.S., Wong, S.L., Lee, P.P., Lee, J.S., Ti, T.B. and Zhang, Z. (2011) “Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells”. Carbohydrate Polymer, 83, 225–232.

# Chapter 7

# CONCLUSION

In the present investigation comprehensive experiments have been carried out to understand the synbiotic effect of prebiotic on probiotic in free and immobilized form. Inulin, a compound in fructo-oligosaccharide group has been used as prebiotic agent while *Lactobacillus casei*, a member of Lactic Acid Bacteria (LAB) group has been selected as the candidate probiotic. Initially inulin has been isolated from a selected range of abundant natural sources. This includes sources like garlic and Indian millets, viz., pearl millet, great millet and finger millet. An improvised method of solvent extraction technique has been used for isolation of inulin from the raw material. Chemical characterization of isolated inulins from different sources has been carried out with the help of Thin layer Liquid Chromatography (TLC), Fourier Transform Infra-Red spectroscopy (FTIR) and DNS (3-5 Di-nitro salicylic acid) methods. Similar experiments have been conducted with commercial inulin for the purpose of comparison.

Investigation on cell dynamics is initiated with the optimization of cell growth media. With concentrations of lactose and inulin as independent variables the specific cell growth response has been optimized. Response surface methodology technique has been used as the optimization tool and a dedicated Design expert 8.1 has been used for this purpose.

Programmed and systematic experiments have been conducted on the cell growth response of *Lactobacillus casei* (2651 1951 RPK) (*L. casei*) in absence and in presence of prebiotic inulin isolated from different sources. Similar experiments have been performed with the commercially available inulin. A rigorous mathematical modeling exercise has been carried out to understand the reaction engineering behavior of the prebiotic inulin on the probiotic, *L. casei* and validation of these equations has been made with the help of experimental data. It is observed that inulin free system using lactose as the carbohydrate source follows classical substrate uninhibited Monod equation. Interestingly, system in presence of inulin as the only carbon source follows Haldane equation. The growth of *L. casei* on inulin and lactose as dual carbohydrate sources follows summative model equation. It is observed that in these cases beyond a inulin concentration 0.3225 g/L inhibition sets in. A comparative study on the cell growth dynamics using inulin from different prebiotic sources clearly indicates that the effectiveness of inulin isolated from pearl millet is much superior to other systems under investigation. However, when compared with effectiveness of commercially available inulin it is observed that the effectiveness of inulin isolated from pearl millet is marginally lower than the former one.

In order to compare the effectiveness of inulin isolated from pearl millet and garlic with that obtained from commercial source, experiments on antimicrobial activity of these three prebiotics have been conducted. The results have been expressed in terms of prebiotic scores as suggested by Huebner et al [1]. It is observed that the performance of prebiotic inulin isolated from pearl millet source is significantly superior than that of commercially available inulin. Performance of inulin isolated from garlic ranks lowest in this series. It may therefore be concluded that inulin isolated from pearl millet has a great future market potential. Effectiveness of prebiotic can also be expressed in terms of its providing resistance to antibiotic. In the present investigation

experimental results show that the resistivity of prebiotic inulin - *L. casei* conjugate towards the selected broad spectrum antibiotic, Norfloxacin is appreciably high. An attempt has also been made to explore the possibility of introducing immobilization technique in prebiotic-probiotic conjugate system for finding newer applications of the conjugate. It is observed that inulin-*L. casei* conjugates, immobilized through internal gelation, possesses significantly high resistance to the candidate pathogen *E. coli*.

Three mathematical models have been developed for the prediction of release time of *L. casei* from microcapsules formulated through internal gelation technique under optimum condition for different systems. The concept of "burst release" mechanism of probiotic cells from the microcapsules has been proposed under the present study. One model has been able to predict the 'release time' of probiotic cells and the concentration profiles of substrate lactose and the *L. casei* cells in probiotic microcapsules suspended in lactose rich MMRS medium and green coconut water. The second model has been developed to predict the release time of *L. casei* from prebiotic-probiotic microcapsules suspended in simulated large intestinal juice. Both the models have been validated by the successful comparison with the experimental data. In the third model the human GI system has been represented by three continuous stirred tank bioreactors, namely, "stomach", "small intestine" and "large intestine" in series. The concentration time histories of microcapsules and *L. casei* in the model "stomach", "small intestine" and "large intestine", expected to be obtained after the intake of a quantum of prebiotic-probiotic microcapsules by human body, have been simulated. The model has been validated by the comparison with the experimental data of *in-vitro* GI system.

In order to find the newer applications of immobilized system a detailed study has been conducted on two polysaccharide rich food products, viz., Indian milk cake and jaggery for improving preservation time. It is observed that the immobilized conjugate obtained by internal gelation technique is the most efficient compared to other two systems obtained by following gel entrapped and external gelation techniques. It is also observed that the performance of inulin obtained from pearl millet performs even better than commercial inulin as food preservatives for these two selected Indian foods. It may, therefore, be concluded that immobilized prebiotic-probiotic conjugate may find wide application as food preservative containing highly useful probiotics.

The cost analysis of the extraction process of pearl millet inulin indicates the economic viability of the process and its competitiveness with the commercial inulin.

## References

[1] Huebner, J., Wehling, R.L. and Hutkins, R.W. (2007). "Functional Activity of commercial probiotics". International Dairy Journal, 17, 770-775.

# Chapter 8

# SCOPE OF FUTURE WORK

The observations obtained in the present study evidently demand a more comprehensive and systematic investigation for the commercial implementation of the process. It is proposed to standardize the isolation method of inulin followed in laboratory scale in the present investigation on a bench-scale size. This may later be extended to the pilot plant study to evolve technology of prebiotic isolation suitable for adaption on commercial scale. Scale up exercise may be done following chemical similitude.

Once the method of isolation of prebiotic is standardized, application of such isolated prebiotics as probiotic cell growth enhancer may be carried out in different type of bioreactors so as to identify the most suitable contacting device for getting favorable product distribution, yield of the probiotic *Lactobacillus casei* in the present case.

In the present investigation antimicrobial activity of the prebiotic-probiotic conjugate have been studied with respect to a single pathogen *Escherichia coli*. It is felt that this antimicrobial activity investigation should be extended against several other common pathogens present in human gut. This includes the antimicrobial activity of *L. casei*-inulin conjugate against *Salmonella*, *Enterococcus fecalis*, *S. aureus*. Studies on sensitivity of prebiotic-probiotic mixture when exposed to antibiotics are highly important since this information provides the effectiveness of prebiotic. In the present investigation a single broad spectrum antibiotic Norfloxacin has been tested. However it is felt that this investigation should be extended against other antibiotics namely ciprofloxacin, levofloxacin, erythromycin and others.

Immobilized prebiotic-probiotic conjugate has been proved to be significantly effective for their high antimicrobial activity. In the present investigation, a humble effort has been made to use this property in the preservation of polysachharide rich food products. It is suggested that future work should be initiated on more sensitive food products in general and infant and elderly food products in particular.

In the present investigation cell growth associated immobilization techniques have been adopted. It is proposed to extend the same investigation using immobilized cell prepared under non-growth associated condition. Above two studies will be highly useful in selecting suitable bioreactors. The mathematical models developed under the present study are very general in nature. Therefore, these models may be further used for other probiotic-prebiotic pairs. Moreover, the further validation of the models can be done by comparing with the data of *in-vivo* GI systems of a large population samples.

# Appendix

**Determination of Release time of Probiotic Cells, $t_r$**

The release time has been determined using Equation 5.20. The values of $N_i$ and $N_{cr}$ have been determined as follows:

By using 10 mL of cell solution containing 8mg/mL biomass, 1g of $16.302*10^6$ microcapsules was obtained.

Approximately, $10^9$ cells weigh 1mg [1]

Encapsulation efficiency =98%

Therefore initial number of cells/microcapsule (internal gelation) = $\frac{80*10^9*0.98}{16.302*10^6}$ = $4.809*10^3$

Diameter microcapsule (internal) =24µm

Since the microcapsule is prepared using 1:1 oil to aqueous phase ratio, therefore, the volume of aqueous phase in each microcapsule = $\left(\frac{1}{2}*\frac{\pi}{6}*\left(24*10^{-6}\right)^3\right)$= $3.617*10^{-15}$

Therefore, initial concentration of cells per unit aqueous volume of microcapsules = $\frac{4.809*10^3}{3.617*10^{-15}} = 1.329*10^{18}/\mathrm{m}^3$

$$N_i = \frac{1.329*10^{18}}{0.98} = 1.356*10^{18}/m^3$$

Size of the bacterial strain (*L. casei*):

Length = 2 $\mu$m

Diameter = 0.5 $\mu$m

Therefore, cell volume = $\left(\frac{\pi}{4}*\left(0.5*10^{-6}\right)^2*2*10^{\ 6}\right)$

$=3.926*10^{-19}\ \mathrm{m}^3$

Maximum number of cells that can be accommodated in each microcapsules = $\frac{3.617*10^{-15}}{3.92*10^{-19}}$ = $9.227*10^{3}$=9227

The number of cells per unit volume of microcapsules = $N_{cr}=\left(\frac{9227}{3.617*10^{-15}}\right)$= $2551*10^{15}/m^{3}$

Therefore, according to Equation 16,

$2551*10^{15}= 1.356*10^{18}*0.98*e^{0.8338*t_r}$

$t_r$=0.7815 h= 46.89 min

## REFERENCES

1. Van Dan-mieras, M.C.E., James, J.W., Leach, C.K. , de Jew, W.H., de varies, J.(1992) "IN VITRO CULTIVATION OF MICRO-ORGANISMS". Published on behalf of open universiteit & Thans polytechnic (Netherland, UK),Butterwarth & Hanimann(publisher).

## Response to Examiners' Queries

**Reviewer #1:**

**Query1**. Letter ‘c’ should be capital-front page 3.1, 3.3, 5.2
**Response:** Changes have been done accordingly in the final copy of the thesis.

**Query 2.** Some title of the paper/ presentation is given in uppercase (3.1, 3.2, 3.3, 5.1), others in sentence case (5.2, 5.3, 5.3)
**Response:** Changes have been done accordingly in the final copy of the thesis.

**Query 3.** Page (vii) ‘T’ to be deleted in ANNEXTURE
**Response:** Correction has been made in the final copy of the thesis.

**Query 4.** Page 2, line 3 “that’ to be deleted
**Response:** Correction has been made in the final copy of the thesis.

**Query 5.** Page 4, line 11 “of’ to be deleted
**Response:** Correction has been made in the final copy of the thesis.

**Query 6.** Page 13, ref.7, Msc-‘s’ to be capital
**Response:** Correction has been made in the final copy of the thesis.

**Query 7.** Page 130, last line “of the’ to be deleted
**Response:** Correction has been made in the final copy of the thesis.

**Query 8**. Reference list
Inclusion of ‘pp’-ref. 5 page13; ref.8, 10 page 41; ref. 22 page42; ref.10, 17 page 14:
Page no. missing- ref.39, 46, 47 page 16; ref. 36 page 44:
Incomplete- ref.4 page 41; ref. 37 page 44; ref. 21 page 84
**Response:** Correction has been made in the final copy of the thesis.

**Query 9.** Cell concentration has been given in g/L unit, but the concentration of cell biomass has been determined by Spectrophotometric method by observing optical density which generally represents the number of cells.
**Response:** Under the present investigation, the biomass concentration on the basis of dry cell weight has been correlated to absorbance. Although in spectrophotometric method, the CFU is

often correlated to the absorbance, the research studies using the correlation between dry cell concentration with absorbance are also reported. The second method is justified by the fact that the Beer-Lambert Law, originally designed for molar concentrations, can be also applied for mass concentrations (dry cell weight per volume) since the relationship between the dry cell weight and the number of moles of dry cells are related by a molecular weight,

The Beer-Lambert law states that "the absorbance is linearly proportional to the measurement path length and the concentration of a species in mol/L with a wavelength dependent coefficient 'a'".

It is, however, expected that the Beer-Lambert law experiences deviations in absorptivity coefficient 'a' at high concentrations above >0.01mol/L, which may cause a nonlinear relationship between absorption and concentration. Since the molecular weights of our samples are unknown, it is impossible to predict at what dry cell weights the deviation from Beer-Lambert relationship would start. In the present case, the spectrophotometric method has been used in the linear region of correlations between the absorbance and dry cell weight concentration with $R^2 \geq .0.95$ ( Ref: Lawrence Lai,Omar Shkeir, Lun Guo, Photobioreactor Analysis Methods Characterization, Brown Industries Lab (2012); Martin Silberberg, Chemistry: The Molecular Nature of Matter & Change, Fifth Edition, Publisher: McGraw-Hill Higher Education).

**Query 10.** The release of cells in functional food namely Milk cake and Jaggery has not been discussed

**Response:** The viability of probiotic bacteria has been determined in functional food namely Milk cake and Jaggery by bacterial colony count. .However the bursting time of encapsulated probiotic bacteria in functional foods can also be evaluated in future studies, as done in case of *in-vitro* GI system.

# Annexure

LWT - Food Science and Technology 83 (2017) 243–253

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

# *In-vitro* evaluation of targeted release of probiotic *Lactobacillus casei* (2651 1951 RPK) from synbiotic microcapsules in the gastrointestinal (GI) system: Experiments and modeling

Debolina Banerjee [a], Ranjana Chowdhury, Ph.D. [a, *], Pinaki Bhattacharya [b]

[a] Chemical Engineering Department, Jadavpur University, Jadavpur, 188 Raja S C Mullik Road, Kolkata, India
[b] Chemical Engineering Department, Heritage Institute of Technology, Kolkata, 700107, India

ARTICLE INFO

*Article history:*
Received 30 December 2016
Received in revised form
16 April 2017
Accepted 10 May 2017
Available online 13 May 2017

*Keywords:*
Mathematical model
"Burst release" mechanism
Synbiotic microcapsules
Simulated GI system

ABSTRACT

The microencapsulation processes serve potentially to protect probiotic cells against adversity in the GI tract. The article focuses on the behavior and release characteristics of *Lactobacillus casei* from synbiotic microcapsules. Monod ($\mu_{max} = 0.78\ h^{-1}$, Ks = 2.44 g/L) Haldane ($\mu_{max} = 0.05\ h^{-1}$, Ks = 0.2 g/L, $K_I = 0.52$ g/L) and summative models are followed by *L. casei* during its growth on lactose, inulin and the combination of these carbohydrates respectively. The internal micro-encapsulation efficiency (98%) for synbiotic microcapsules (24 μm) has been optimized with respect to concentrations of sodium alginate (2%), tween-80 and post-cooling temperature (25 °C) using Response Surface Methodology. In agreement with the prediction of proposed "burst release" mechanism, probiotic cells are released after 50 min if 1 g synbiotic microcapsules are initially suspended in 10 mL simulated large intestinal juice. A mathematical model has been developed to predict the concentration profiles of microcapsules and released probiotic cells in the GI system and has been validated through the successful comparison of simulated data with the results of *in-vitro* experiments. The concentration of released probiotic cells rises to 1.7E10/L in the large intestine. Interestingly, this is the first study on the prediction of release of *L. casei* from synbiotic microcapsules and is expected to be useful for similar systems.



## 1. Introduction

Development of nutraceuticals using probiotic bacteria is a rapidly expanding area of pharmaceutical and food industries. This is because of the anti-microbial property of these microorganisms against pathogens. These nutraceuticals are also intended to replenish naturally occurring gut bacteria, adversely affected due to rampant use of antibiotics, chemotherapy against carcinoma etc. The administration of these bacteria in free form results in loss of viability during the passage through the gastro-intestinal tract, particularly through the stomach. The immobilization of probiotic cells in microcapsules and beads has been reported to ensure controlled release of bacteria and reduce the risk of loss of viability (Lakkis, 2007). Thus, the microencapsulation technology of probiotic cells has emerged to develop nutraceuticals. External and internal gelations are the two different methods of micro-emulsification. Internal gelation is superior to external one as it ensures the formation of spherically structured microcapsules of sizes ≤1000 μm with very narrow size distribution (Ahmed, El-Rasoul, Auda, & Ibrahim, 2013; Chun, Kim, & Cho, 2014). The most common biomaterial used for microencapsulation of probiotic bacteria is alginate which is reported to show resistance against acidity of gastric juice which ultimately protect the probiotic cells against destruction in the GI tract (Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2012; Chourasia and Jain, 2003). The microcapsules serve as reservoirs or micro containers for probiotics that ultimately release them at the targeted site i.e. the large intestine. Kailasapathy, 2002 identified that co-encapsulation of prebiotic biomolecules and probiotic bacteria within a single capsule will serve as a useful tool for the controlled release of the latter due to synbiotic effects. A few pioneering studies have also been reported on co-immobilization of prebiotics and probiotics (Pliszczak et al., 2011; Sathyabama, Ranjithkumar, Brunthadevi, Vijayabharathi, & Brindhapriyadharisini, 2014). Sathyabama et al. selected *Staphylococcus succinus* (MAbB4) and *Enterococcus fecium* (FIdM3) as the probiotics and oligosaccharides,

* Corresponding author.
*E-mail address:* ranjana.juchem@gmail.com (R. Chowdhury).

http://dx.doi.org/10.1016/j.lwt.2017.05.011
0023-6438/

namely, sugarbeet and chicory as the prebiotics for immobilization through emulsification and observed that the incorporation of prebiotics increased the viability of co-encapsulated probiotics and the resistance of microcapsules against the acidity of gastric juice and bile salt. They suggested for the requirement of more research studies on the efficacy of co-encapsulation in the human gut environment through modeling. Plizzack et al. reported preparation of bio-adhesive microparticles through emulsification/gelation method using the probiotics, namely, *Lactobacillus rhamnosus, L. salivarius, L. brevisandL. L. plantarum* and prebiotics, namely, FOS Actilight_ 950S and 950P. They observed complete release of probiotics in simulated vaginal fluid at the end of 16 h. They identified the necessity for the study of kinetics of probiotic delivery at the target site. The present research group has also reported the elongation of shelf-life of food in presence of synbiotic microcapsules (Banerjee, BAG, Chowdhury, & Bhattacharya, 2017). However, no attempt has been made to study the dynamics of release of probiotics in the food matrix from the microcapsules. From the literature review, it is clear that although the popularity of probiotic health products is increasing rapidly, a gap still remains regarding the unwinding of the mechanism of release of cells at the gut through modeling and prediction of release pattern of probiotics from the synbiotic microcapsules (Gbassi & Vandamme, 2012; Kailasapathy, 2002; Pliszczak et al., 2011; Sathyabama et al., 2014). The release time is predominantly guided by the growth pattern of probiotic cells within the capsules and the mechanism of release. The prediction of the release time of cells from the probiotic neutraceuticals at the large intestine is possible through the development of mathematical model. From the reported literature it is clear that a few pioneering *in-vitro* models have been developed predicting the viability of free probiotic cells in gastrointestinal environment (Macfarlane, Macfarlane, & Gibson, 1998; Child et al., 2006). However, no studies have been reported on modeling the release of co-encapsulated probiotic cells from probiotic or synbiotic microcapsules in the gut environment. Under the present research study the growth kinetics of *L. casei* individually on lactose and prebiotic inulin and in presence of combination of lactose and inulin have been determined. The resistance of the microcapsules co-encapsulating probiotic *L. casei* and prebiotic chicory inulin against simulated gastric (pH 2) and small intestinal (pH 8) juice has been evaluated experimentally. The human GI tract has been simulated by three continuous stirred tank bio-reactors, representing the “stomach”, “small intestine” and “large intestine”. A mathematical model has been developed to predict the characteristics of release of probiotic cells from synbiotic microcapsules during the passage through the simulated GI system. The model-predicted concentration profiles of microcapsules and released probiotic cells have been compared with those obtained from the simulated *in-vitro* experimental set-up.

## 2. Materials and methods

### 2.1. Chemicals

Calcium chloride, sodium alginate, tween 80, acetic acid, glycerol, Sodium Dodecyl Sulphate (SDS), beef extract, calcium carbonate, lactose, purchased from Merck, India were used. Yeast extract, peptone, sodium acetate, di-potassium hydrogen phosphate, tri-ammonium citrate, magnesium sulphate, manganese sulphate, sodium chloride, di-sodium hydrogen phosphate, potassium di-hydrogen phosphate, glucose, purchased from Himedia, India, pancreatin (SIGMA-ALDRICH), bile salt (cholic acid sodium salt, ~50%; deoxycholic acid sodium salt, ~50%) (SIGMA-ALDRICH) were used for this study. Rice bran oil, purchased from local market was used.

### 2.2. Microorganism

*Lactobacillus casei* (2651 1951 RPK) was purchased from NCIM, Pune. The SEM image of the cells is shown in Fig. 1. The length and diameter of the microorganism are 2 μm and 0.5 μm respectively.

### 2.3. Modified de-Mann Rogosa Sharpe (MMRS) medium

Beef extract: 10 g/L; Yeast extract: 5 g/L; Peptone: 10 g/L; Sodium acetate: 5 g/L; di-potassium hydrogen phosphate: 2 g/L; Tri-ammonium citrate: 2 g/L; Magnesium sulphate: 0.05 g/L; Manganese sulphate: 0.05 g/Land Lactose: 20 g/L; pH 7.

### 2.4. Simulated juices

Gastric juice was simulated by using sodium chloride (9 g/L) and pepsin (0.3 g/100 ml). The pH was adjusted to 2.0 with HCl. Saliva solution was simulated by mixing 0.62% (w/v) NaCl, 0.22% (w/v) KCl, 0.02% (w/v) $CaCl_2$ and 0.12% (w/v) $NaHCO_3$(Cook et al., 2012); , The gut juice was simulated using phosphate buffer saline (PBS) (NaCl: 8 g/L; Na2HPO4: 1.44 g/L and KH2PO4: 0.24 g/L) with pH adjusted in the range of 6.5–8 (Chapin & Lauderdale, 2003; Gbassi & Vandamme, 2012). PBS containing 0.09% (w/v) pancreatin and 0.6% (w/v) bile salt with pH adjusted to 8.0 was used to simulate small intestinal juice (Sathyabama et al., 2014).

### 2.5. Equipments and analytical instruments

Magnetic stirrer (Remi, India), Constant temperature bath (S.C Dey& Co., India) and High speed homogenizer (Plasto Crafts (Model: Superspin R-V/$F_M$), India), Optical microscope (OPTIKA, Italy), Spectrophotometer (Varian, Cary 50 Bio USA) and FESEM (JSM 6700A, Jeol Ltd., Japan) were used.

### 2.6. Experimental procedure

#### 2.6.1. Pre-adaptation of Lactobacillus casei (2651 1951 RPK) to different carbohydrate sources

The probiotic bacteria, namely, *Lactobacillus casei* was pre-adapted to different carbohydrate sources, namely, lactose and commercial inulin. For pre-adaptation to lactose and inulin, sterile MMRS medium was prepared by using 50 g/L and 20 g/L lactose and inulin respectively. Each medium was inoculated with 1% (v/v) of *L. casei* and was incubated for 24 h at 37 °C. For pre-adaptation of *L. casei* to combination of both carbohydrates, the concentrations of lactose and inulin were maintained at 50 g/L and 20 g/L simultaneously.

#### 2.6.2. Batch studies for the determination of growth of Lactobacillus casei (2651 1951 RPK) on lactose, inulin and mixture of lactose and inulin

Batch growth of *L. casei* was investigated using lactose as the carbon source and the initial concentration was varied in the range of 10–30 g/L. Similarly, another set of experiments was conducted using inulin as the sole carbon source. The initial concentration of inulin was maintained in the range of 0.035–0.624 g/L. A separate set of batch experiments was performed using a mixture of lactose and inulin as the carbon source. At each value of initial concentration of lactose of 10, 20, 30 and 40 g/L, the initial concentration of inulin was varied in the range of 0.164–0.624 g/L. For each

experiment, incubation was done for 24 h at pH and temperature of 7 and 37 °C respectively. The cultures were withdrawn at 1 h interval and the concentration of biomass was determined using UV-VIS spectrophotometer. Each experiment was repeated thrice. Batch studies for the determination of growth behavior of *L. rhamnosus* have been reported by Berry et al. (1999).

### 2.6.3. Preparation of synbiotic microspheres: design of experiment for optimization of microencapsulation efficiency

For the co-encapsulation of *L. casei* with inulin via internal gelation method, the protocol suggested by Song, Yu, Gao, Liu, & Ma, 2013 has been principally followed. The *L. casei* culture, pre-adapted to combination of lactose and inulin was spiked with lactose and inulin up to the respective optimum concentration levels of 20 g/L and 0.3225 g/L respectively (Banerjee, Chowdhury, & Bhattacharya, 2016). 1 mL of the culture was mixed with sodium alginate solution in the volumetric ratio of 1:4. $CaCO_3$ powder up to a concentration level of 0.8 g/L and Tween-80 were added to each mixture. This mixture was then combined with vegetable (rice bran) oil in the volumetric ratio of 1:5 and stirred at 300 rpm for 30 min. Vegetable oil is used to avoid aggregation of microcapsules during gelation step. Since the viscosity of rice bran oil is moderate (0.0398 Pa s) the microcapsules are expected to have small particle size since it has been reported by Plizzack et al. that microcapsules of large size are obtained using oil having low viscosity (0.01 Pa s). Moreover, the richness of rice bran oil in antioxidants mainly oryzanol etc. is among other reasons behind its choice. The mixture was post-cooled by circulating water from a constant temperature bath and acetic acid was next added drop wise to the solution to release the $Ca^{2+}$ from the insoluble $CaCO_3$powder. The microcapsules were formed and were filtered with muslin cloth and washed with 1% (v/v) aqueous tween 80 solution and distilled water. The microcapsules were then stored at 4 °C.

Design of experiments was done using central composite design and a statistical model correlating the response variable, encapsulation efficiency ($E_e$) with the independent factors, namely, alginate concentration (1–2%), emulsifier concentration (Tween 80) (0.5–1.5%) and post cooling temperature (15–25 °C) was developed using response surface methodology (RSM). For this purpose, a set of 20 experiments at pre-set conditions. The development of statistical model and identification of optimimum conditions for the maximization of the microencapsulation efficiency was performed using Design Expert software 8.1.

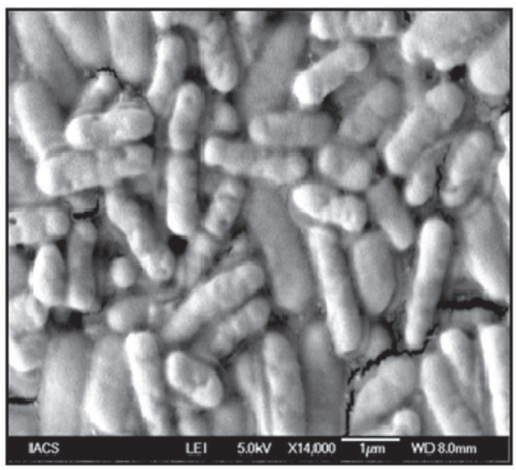



**Fig. 1.** SEM image of *Lactobacillus casei* (2651 1951 RPK).

### 2.6.4. Size analysis of microcapsules

Samples of 10 microcapsules were chosen randomly from the respective total population and the configuration and mean size was determined using the optical microscope. The mean of three samples chosen from each population of microcapsules was determined along with the standard deviation.

### 2.6.5. Determination of "Release time" of Lactobacillus casei (2651 1951 RPK) from synbiotic microcapsules in GI fluids

Microcapsules containing a mixture of *L. casei* and commercial inulin were used for this assay. For obtaining the "burst-off" time of the capsules in the simulated saliva solution, "gastric juice", and small and large intestinal juices, microcapsules weighing 3 g were suspended in 30 mL of each medium in a 50 mL conical flask and the mixture was incubated under anaerobic condition at 37 °C for 60 h. The content of the conical flask was observed periodically so as to obtain the times at which the capsules are burst and there by the turbidity increases abruptly due to the release of the entrapped cells. On release of cells, the medium becomes turbid due to the formation of probiotic suspension. It is well known that the concentration of bacterial suspension can be conveniently determined through the measurement of turbidity. Since a single culture of *L. casei* is involved in the study, a standard plot has been generated using the dry cell concentration and the corresponding value of optical density of the bacterial suspension at 600 nm (Okkers, Dicks, Silvester, Joubert, & Odendaal, 1999). Later, the bacterial concentration has been determined by using the optical density of the suspension formed as a result of release of cells from synbiotic microcapsules. Each experiment was repeated thrice.

### 2.6.6. Operation of three stage bioreactors simulating the human GI tract

Three continuous stirred tank bioreactors representing the stomach, the small intestine and the large intestine of volumes 300 mL, 1 L and 5 L respectively were connected in series. As per literature data the passage time of microcapsule in the stomach, small intestine and large intestine are 80.5 mins, 3.2 ± 1.6 h and 6–32 h (Cook et al., 2012). Initially the reactors representing stomach, large and small intestine were filled up respectively with gastric, small intestinal and large intestinal juices and were sparged with nitrogen to maintain anaerobic condition. The "stomach" bioreactor was fed with 30 g synbiotic (*L. casei*-inulin) microcapsules, already soaked in saliva solution for 30 min, so that the concentration of microcapsules in gastric solution was 0.1 g/L (Sathyabama et al., 2014). The stomach was emptied at a constant volumetric flow rate of PBS solution of 3.72 mL/min using a peristaltic pump and the effluent was fed to the next bioreactor, i.e., the "small intestine". The effluent from this reactor was fed to the bioreactor representing "large intestine" from which the outlet was withdrawn at the same volumetric flow rate. The reservoir for PBS was also sparged with nitrogen to maintain anaerobic condition. The pH of each reactor (pH = 2 for "Stomach"; pH = 8 for "small intestine" and pH = 7 for "Large intestine") was adjusted continuously by 0.1 M HCl and 0.1 M NaOH. The values of retention time of liquid in the reactors, namely stomach, small intestine and large intestine are 80.6 min, 4.48 h and 22.4 h respectively. The effluent from the large intestine was collected in a reservoir. Fluid samples were withdrawn from each reactor at 50min interval and the release of probiotics was monitored by measuring the optical density using spectrophotometric method described in Section 2.6.5. The concentration of microcapsules in each reactor was measured after 2000 min.

## 3. Theoretical analysis

### 3.1. Growth kinetics of Lactobacillus casei (2651 1951 RPK) on single carbon source

In order to regress the cell growth dynamics of *L. casei* on single carbon source, the classical Monod type substrate uninhibited unstructured model has been attempted first (Shuler & Kargi, 2002). The equation is as follows:

$$\mu = \frac{\mu_m C_s}{K_s + C_s} \quad (1)$$

Where, $\mu$ is specific growth rate defined as follows:

$$\mu = \frac{1}{C_X}\frac{dC_X}{dt} \quad (2)$$

$\mu_{m=}$ Maximumspecific cell growth rate, $h^{-1}$
$K_s$ = Substrate saturation constant, g/L
$C_s$= Concentration of lactose, g/L
$C_X$ = concentration of cell, g/L

A double reciprocal plot has been made using inverse of initial values of $\mu$ and $C_s$ as ordinate and abscissa respectively. If the double reciprocal plot is linear, the values of intrinsic kinetic parameters, $\mu_{max}$ and $K_s$ have been determined from the intercept and the slope. The plot has been provided in the supplementary section.

Haldane type model has been attempted if Monod model is not valid and substrate inhibition is present. Haldane equation is as follows (Clark & Blanch, 1997):

$$\mu = \frac{\mu_{max} C_S}{K_S + C_S + \frac{C_S^2}{K_I}} \quad (3)$$

where, $K_I$ = Inhibition constant.

The correlation between the initial concentration of substrate, $C_{s_{max}}$ ,corresponding to the maximum value of specific growth rate and the constants, $K_s$ and $K_I$ are as follows (Shuler & Kargi, 2002).:

$$C_{S_{max}} = (K_S K_I)^{0.5} \quad (4)$$

#### 3.1.1. Method of validation of Haldane model

The validity of Haldane model is verified by the analysis of the pattern of the plot of $\mu$ against the corresponding initial substrate concentration. A bell-shaped plot is obtained if the Haldane model is valid. After verifying the validity of Haldane model, the values of kinetic constants, $\mu_{max}$ and $K_S$ have been determined by making the double reciprocal plot using the values of inverse of $\mu$ and $C_S$ in the uninhibited growth region, i. e, by using the specific growth rates corresponding to initial substrate concentrations below $C_{s_{max}}$. The value of $K_I$ has been determined using Eq. (4).

### 3.2. Growth kinetics of Lactobacillus casei (2651 1951 RPK) on two carbon sources

Summative type model (Clark &Blanch, 1997) has been attempted to correlate the specific growth rate of *L. casei* using lactose and inulin simultaneously as the carbon sources. The summative type kinetic equation for the growth of *L. casei* is as follows:

$$\mu = \mu_L + \mu_I \quad (5)$$

Where,

$\mu_L$ = specific growth rate on lactose
$\mu_I$ = specific growth rate on inulin

The validity of the summative model is verified by the comparison of the experimental and simulated values of specific growth rates at different combinations of values of lactose and inulin.

### 3.3. Encapsulation efficiency

Encapsulation efficiency ($E_e$) is calculated using the following correlation (Rosas-Flores, Ramos-Ramirez, & Salazar-Montoya, 2013; Zou et al., 2012):

$$E_e = \frac{N_f}{N_i} \times 100 \quad (6)$$

where, $E_e$ represents the encapsulation/entrapment efficiency, $N_f$ is the number of viable bacteria released in the homogenate of microcapsules/beads and $N_i$ is the number of bacteria used for encapsulation/entrapment.

### 3.4. Statistical modeling using RSM and optimization

In the present investigation the concentrations of sodium alginate, surfactant (Tween 80) and post-cooling temperature were taken within the range 1–3%, 0.5–2 M and 20–180 min. For obtaining maximum encapsulation efficiency the concentrations of sodium alginate, surfactant (Tween 80) and post-cooling temperature are specified as 2%, 0.5% and 25 °C respectively. The experimental design matrix was prepared with the help of Design-Expert 8.1.

Generally the dependency of independent variables on the output is given by:

$$y^{\lambda} = f(x_1, x_2, x_3 .....) + e \quad (7)$$

where, y is the output, $\lambda$ is the power of the output, "f" is the function of input variables $x_1$, $x_2$, $x_3$ and e is the error.

$$R^2 = 1 - \frac{SS_{residual}}{SS_{model}\ SS_{residual}} \quad (8)$$

$$R_{adj}{}^2 = 1 - \frac{SS_{residual}/DF_{residual}}{(SS_{model}+SS_{residual})/(DF_{model}+DF_{residual})} \quad (9)$$

The polynomial quality was determined by the determination coefficients, $R^2$, $R^2_{adj}$ and predicted $R^2$. The equations for calculating $R^2$ and $R^2_{adj}$ are given in Eqs. (8) and (9).

In these equations, DF is the degree of freedom and SS is the sum of squares. The experimental data were fitted using multiple regression to the quadratic equation which is given by

$$Y = a_0 + a_1X_1 + a_2X_2 + a_3X_3 + a_{12}X_1X_2 + a_{13}X_1X_3 + a_{23}X_2X_3 + e \quad (10)$$

where $X_1$, $X_2$ and $X_3$ are the independent variables, Y is the output variable, $a_0$ and $a_i$ are intercept and linear constant coefficients, $a_{ij}$ is regression 2-factor interaction between $X_i$ and $X_j$ and e is the residual part.

### 3.5. Prediction of "Release time" of microorganisms from synbiotic beads in simulated large intestinal fluid

The cells are released to the "gut" environment as soon as the volume of biotic phase, i.e., the cells exceed the internal volume of the microcapsules. Thus, it is proposed that "burst release" mechanism (Lakkis, 2007), characterized by pulse release of the encapsulated active, is followed. This is shown in Fig. 2. This is justified by the fact that due to large dimensions with respect to the pores of microcapsules, the trans-boundary diffusion of cells driven by concentration gradient is not possible. Thus, the cells are released through the rupture of the capsules as soon as the internal core volume of the capsules is exceeded by the biotic cell volume. The burst release of active molecules has already been reported for hydrogel systems (Lakkis, 2007).

It is assumed that the microcapsules and beads suspended in simulated large intestinal fluid of pH = 7 behave like batch type bioreactors. As suggested by Doran, 1995, the specific growth rate, $\mu$, in the batch culture remains almost constant and is equal to $\mu_{max}$. Thus, for the present case, the specific growth rate of *L. casei* on lactose and inulin may be represented as

$$\mu = \mu_{max\ L} + \mu_{max\ I} \tag{11}$$

The decaying rate of the probiotic cells within the microcapsules is negligible.

Mathematically, the number of cells encapsulated within a particular bead can be predicted using the assumption of balanced growth during exponential growth in a batch reactor. Under balanced growth condition, the cell number specific growth rate $v = \frac{1}{N}\frac{dN}{dt}$ is equal to the specific growth rate, $\mu = \frac{1}{C_X}\frac{dC_X}{dt}$, calculated on the basis of cell biomass (Clark & Blanch, 1997). Therefore, by expressing the dependence of rate of increase of cell number on the total cell number by Malthusian model (Clark & Blanch, 1997),

$$\frac{dN}{dt} = \mu N \tag{12}$$

At $t = 0$,

$$N = N_0 \tag{13}$$

$$N_0 = N_i E_e \tag{14}$$

where, $N_i$ is concentration of cells (number basis) in the stock culture from which the beads or capsules were formed.

Integrating Eq. (12), and using Eqs. (11) and (14),

$$N = N_i E_e e^{(\mu_{max\ L} + \mu_{max\ I})t} \tag{15}$$

When $N \geq N_{cr}$, the capsules rupture, releasing entrapped *L. casei*. $N_{cr}$, is the total number of cells whose volume is equal to the internal core volume of the microcapsules. Therefore, mathematically,

$$N_{cr} = \frac{V_{capsule}}{V_{cell}} \tag{16}$$

Where, $V_{capsule}$: Volume of the inner core, i.e., the aqueous part of the capsule.

$V_{cell}$: Volume of an individual *L. casei* cell.

Therefore,

$$N_{cr} = N_i E_e e^{\left(\mu_{max_L} + \mu_{max_I}\right) t_r} \tag{17}$$

Where, $t_r$ is the release or bursting time.

$$t_r = \frac{\ln\left(\frac{N_{cr}}{N_i E_e}\right)}{\mu_{max\ L} + \mu_{max\ I}} \tag{18}$$

The value of $t_r$ has been determined using Eq. (18). The detailed calculation of the release time of probiotic cells from the microcapsules, formulated under the present study, has been provided in the "Supplementary material".

### 3.6. Mathematical model for the evaluation of in-vitro concentration profiles of microcapsules (internal) and released probiotics in the simulated GI system

The mathematical model for the GI system has been developed using the concepts of combination of reactors, as used by pioneering researchers (Cook et al., 2012; Kailasapathy, 2002; Macfarlane et al., 1998). Although from the literature it is apparent that the activity of the stomach during the digestion of slurries containing polymeric solids is periodic in nature, the stomach, the small intestine and the large intestine have been considered as continuous stirred stank bio-reactors. The path followed by the microcapsule within the human gastro-intestinal tract may be schematically represented in Fig. 3.

The mathematical model has been developed using the following assumptions:

1 The probiotic cells do not grow within the microcapsules during their passage through stomach (pH = 2.0) and small intestine (pH = 8). This has been justified by the experimental verification using simulated gastric and small-intestinal juices.
2 The change of structure of microcapsules in stomach and hence the "mechanical destructive force for size reduction" is neglected. This may be justified by the fact that only the size of food ingredients >1 mm is reduced in the stomach (Kong and Singh, 2008; Lentle and Janssen, 2011).
3 Enzymatic solubilization of microcapsules and subsequent absorption in the small intestine has been neglected. This has been verified by experimental study.
4 The probiotic cells are released from the prebiotic-probiotic microcapsules in the large intestinal fluid following bursting mechanism described in section 3.3.

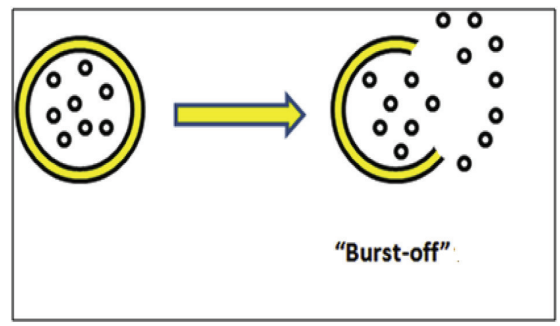



**Fig. 2.** Bursting off microcapsules.

5 The stomach, small intestine and large intestine represented by CSTBRs are under dynamic conditions and never attain steady state.
6 Although the GI fluids are non-Newtonian in nature (Kong and Singh, 2008), Newtonian behavior has been assumed for the simplification of the model.
7 The exit of probiotic microorganisms from the large intestine is negligible. This may be justified by the fact that the probiotic bacteria in the large intestine adhere to the mucus for further action (Yoo, Seong, Chang, & Park, 1996).

The release of cells from the microcapsulein the large intestine may be represented by the following equation:

$$Mc \rightarrow nX \tag{19}$$

where, Mc and X stand for microcapsule and probiotic cells respectively. At any time greater than "release time" of cell from microcapsules, i.e., $t_r$, there will be two types of microcapsules, namely, ruptured (rup) and un-burst (unb) ones. Therefore, for $t > t_r$

$$C_{MC_{LI_T}} = C_{MC_{LI_{rup}}} + C_{MC_{LI_{unb}}} \tag{20}$$

The mass balance equations for the concentration of microcapsules and probiotic cells in the reactors representing "stomach", "small intestine" and "large intestine" are provided in Table 1. All derivations have been provided in the Supplementary material. The concentrations $C_{MC_{St}}$, $C_{MC_{SI}}$ *and* $C_{MC_{unb_{LI}}}$ of microcapsules in the bioreactors representing stomach, small intestine and large intestine have been determined experimentally by filtering the samples withdrawn from reactors and subsequent determination of number of microcapsules per unit volume of sample by microscopic method. The concentrations $C_{X_{St}}$, $C_{X_{SI}}$ *and* $C_{X_{LI}}$ of released probiotic cells in the same bioreactors have been determined by analyzing the samples withdrawn from reactors using the spectrophotometer at 600 nm. The value of dilution rates, $D_{ST}$, $D_{SI}$ and $D_{LI}$ have been calculated by dividing the volume of respective bioreactor, i.e., stomach small intestine and large intestine by the flow rate of the PBS stream used in the in-vitro GI experiment. "n" is the number of time release cycle of probiotics from the synbiotic microcapsules. Since the "burst release" mechanism is followed also in the in-vitro continuous GI system, cells are released from each microcapsules after its residence time of $t_r$ (release time) in the large intestine. The value of the release time has been experimentally determined in Section 2.6.5 and has been provided in Table 2. In Eqs. (21)–(24), (n+1) and (n) represent the conditions at nth and $(n+1)^{th}$ time grids when the time domain is divided into equal intervals of $t_r$. In Eq. (24), p represents the number of cells available from 1 g of microcapsules. The detailed calculation for the evaluation of "p" has been provided in the "Supplementary material". The initial conditions are as follows:

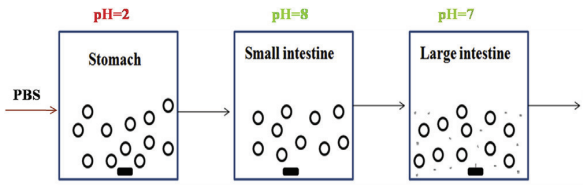



**Fig. 3.** Schematic representation of simulated human gastro-intestinal tract. ○: Microcapsule, The dots represents probiotic cells released from microcapsules, ▬: Magnetic stirrer.

At $t = 0$

$C_{Mc_{st}} = 0.1 g/mL$
$C_{Mc_{SI}} = 0$
$C_{MC_{Tp_{LI}}} = 0$
$C_{MC_{rup_{LI}}} = 0$
$C_{MC_{unb_{LI}}} = 0$
$C_{Mc_{LI}} = 0$
$C_{Mc_{LI}} = 0$
$C_{X_{ST}} = 0$
$C_{X_{SI}} = 0$
$C_{X_{LI}} = 0$

The values of the constants have been provided in Table 3. Eqs. (21)–(31) of Table 1 have been solved using ode45 of MATLAB 7.

## 4. Results and discussion

### 4.1. Growth kinetics of Lactobacillus casei (2651 1951 RPK)

The Monod and Haldane models have been proved to represent the growth kinetics of *L. casei* grown on lactose and inulin as sole carbohydrate sources respectively. The kinetic constants have been provided in Table 4. The detailed discussion has been provided in the Supplementary material.

#### 4.1.1. Growth kinetics of L. casei on combinations of lactose and inulin

In Fig. 4, the experimental values of specific growth rate of *L. casei* have been plotted against the concentration of inulin using the concentration of lactose as a parameter. The values of the specific growth rate, simulated using the summative model, have been superimposed on the same plot. The simulated values are in good agreement with the experimental ones and hence the summative model is validated for the prediction of specific growth rate of *L. casei* simultaneously on lactose and inulin.

### 4.2. Morphology of beads and microspheres

The mean diameter of the microcapsules was 24 μm.The morphology of beads has been shown in Table 5.

### 4.3. Statistical modeling using RSM and optimization

The final equation correlating $E_e$ with the independent variables is as follows:

$$E_e = 86.80 + 1.40*A - 3.30*B + 2.90*C - 2.25*A*B + 3.00*A*C + 3.50*B*C \tag{32}$$

In this equation, the terms containing B, C, AC and BC are significant. In Fig. 5, 3-D surface plots illustrate the variation of $E_e$ with the interaction factors (sodium alginate concentration, surfactant concentration and temperature). To analyze the effects and interactions, analysis of variance (ANOVA) was done which is shown in Table 6. In ANOVA, any factor with p-value less than 0.05 has significant contribution to response with 95% confidence level. The model F-value of 4.627545 and p-value of 0.0099 implies that the model is significant. From the above section, we summarize that Encapsulation efficiency ($E_e$) is a strong function of alginate concentration, surfactant concentration and post-cooling temperature, within the given ranges of the respective variables. However, $E_e$ decreases with the increase in surfactant concentration. This may be due to the fact that increased surfactant molecules may lead to

**Table 1**
Balance equations for the concentration of microcapsules and probiotic cells in human gut.

| Reactor | Microcapsules | | Probiotic cells | |
|---|---|---|---|---|
| Stomach (st) | $\frac{dC_{(MC)_{st}}}{dt} = -D_{St}C_{(MC)_{st}}$ | (21) | $\frac{dC_{X_{St}}}{dt} = -D_{St}C_{X_{St}}$ | (22) |
| Small Intestine (SI) | $\frac{dC_{(MC)_{SI}}}{dt} = D_{SI}\left(C_{MC_{St}} - C_{MC_{SI}}\right)$ | (23) | $\frac{dC_{X_{SI}}}{dt} = D_{SI}\left(C_{X_{st}} - C_{X_{SI}}\right)$ | (24) |
| Large Intestine (LI) | For $t < t_r$<br>$\frac{dC_{MC_{T_{LI}}}}{dt} = D_{LI}\left(C_{MC_{SI}} - C_{MC_{T_{LI}}}\right)$<br>$C_{MC_{rup_{LI}}} = 0$ | (25)<br>(26) | For $t < t_r$<br>$\frac{dC_{X_{LI}}}{dt} = D_{LI}C_{X_{SI}}$<br>For $t > t_r$<br>$C_{X_{LI}}(n+1) = CX_{LI}(n) + p * C_{MC_{rup_{LI}}}(n)$ | (27)<br>(29) |
| | For $t > t_r$<br>$\frac{dC_{MC_{T_{LI}}}}{dt} = D_{LI}\left(C_{MC_{SI}} - C_{MC_{T_{LI}}}\right)$<br>$C_{MC_{rup_{LI}}}(n+1) = C_{MC_{T_{LI}}}(n+1) - C_{MC_{T_{LI}}}(n)$ | (28)<br>(30) | | |
| | $C_{MC_{unb_{LI}}}(n) = C_{MC_{T_{LI}}}(n) - C_{MC_{rup_{LI}}}(n)$ | (31) | | |

**Table 2**
"Release time" of *L. casei* from synbiotic beads and microcapsules in various simulated media.

| | |
|---|---|
| Saliva juice | Not released |
| Gastric juice | Not released |
| Small intestinal juice | Not released |
| Large Intestinal juice | 50 min |

excessive perturbation, disrupting the formation. Higher temperature and alginate may lead to better bonding within the of the alginate core.

### 4.4. Experimental "Release time" of microorganisms from synbiotic microcapsules suspended in simulated GI fluids—verification of burst release mechanism

It is experimentally observed that while the simulated large intestinal fluid medium becomes turbid after a certain time interval the other media remain clear for entire 24 h. The time at which the turbidity suddenly appears in the simulated large intestinal medium corresponds to the release time of microcapsules. In Table 2, the values of release time of probiotic cells in various simulated media have been provided. The reason behind the incapability of release of probiotics in saliva, gastric and small intestinal juice environment is due to unfavorable pH of these media with respect to the growth of *L. casei* in the internal core. The resistance to gastric and small intestinal juice is also established. The resistance of the alginate against thelow and high pH of stomach and small intestine environments respectively is also established (Sathyabama et al., 2014; Cook et al., 2012&Chourasia and Jain, 2003). The simulated (48.89 min) and experimental (50 min) values of release time of proibiotic cells in the large intestinal fluid are in good and hence the "burst release" mechanism is validated.

### 4.5. In-vitro concentration profiles of probiotic/prebiotic microcapsules (internal) and released probiotics in the simulated GI system

In Fig. 6 (a) and (b) the simulated time histories of un-burst (unb) microcapsules and the probiotic cells using Eqs. (21)–(24) in the stomach and small intestine respectively have been depicted. The *in-vitro* experimental values of concentration of probiotic cells have been superimposed on the same figure. The concentration of microcapsules after total pumping time of PBS of 2000 min have been measured and has been plotted on the same figure. The agreement between the experimental and simulated values of microcapsule concentration at 2000 min and that of patterns of time histories of probiotic cells in the reactors validates the mathematical model. From the close analysis of the simulated time histories, it is clear that the concentrations of microcapsules fall exponentially with time in the stomach. The pattern of time histories of the microcapsules in the gastric (stomach) environment can be justified by the fact that once fed into the stomach the microcapsules are swept away from this reactor at a constant rate. No probiotics are detected in the stomach since there is no rupture of

**Table 3**
Values of different constants.

| $D_{St}$ ($min^{-1}$) | $D_{SI}$ ($min^{-1}$) | $D_{LI}$ ($min^{-1}$) | P No./g of microcapsules | $t_r$ (min) |
|---|---|---|---|---|
| 0.012 | 0.00372 | 0.000744 | 150.41E9 | 50 |

D: Diffusion rate, St: Stomach, SI: Small intestine, LI: Large intestine, P: Number of probiotic cells, $t_r$: Release time.

**Table 4**
Values of different kinetic parameters.

| Kinetic Parameters | | | | |
|---|---|---|---|---|
| | $\mu_{max}$ ($h^{-1}$) | $K_s$(g/L) | $K_I$(g/L) | $R^2$ |
| Lactose | 0.7836 | 2.44 | – | 1 |
| Inulin | 0.05 | 0.2 | 0.52 | 1 |

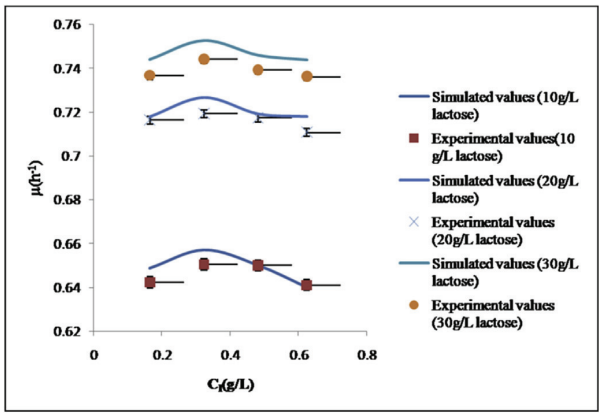
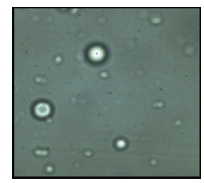



**Fig. 4.** Growth Kinetics of *L. casei* on combinations of lactose and inulin.

microcapsules in this acidic environment. From the analysis of Fig. 6 (b) it appears that the time history of the microcapsules passes through a maximum in the small intestine. This is justified by the fact that the microcapsules are both fed and removed from the small intestine and there is initially an apparent increase in microcapsules due to the disparity between the hydraulic residence time in the stomach (80.5min) and small intestine (268.8min). Declining pattern is observed as soon as the concentration of microcapsules in the stomach becomes zero and hence the outlet PBS stream from the stomach is free from microcapsules. Similar to the gastric environment, the immobilized probiotics are not released in the small intestine due to unfavorable pH. Fig. 6 (c) depicts the simulated time-concentration plots of unburst microcapsules as well as the total number of microcapsules that would have been present if there were no bursting of cells using Eqs. (25) and (31). During this simulation, the experimental value of $t_r$, i. e the release time of probiotics in the large intestinal juice, provided in Table 2 has been used. The *in vitro* experimental value of the concentration of unburst microcapsule at 2000 min of pumping time of PBS solution has also been graphed in the same figure. From the patterns of the simulated plots it is clearly evident that both the concentrations of whole microcapsules and the summation of concentrations of whole and ruptured microcapsules pass through maxima. The maximum in the time concentration plot of whole microcapsules is obtained much earlier than that on the time-concentration plot of total microcapsules (whole + ruptured). The pattern of variation of concentration of whole microcapsules with time may be explained by the fact that while the concentration increases due to the feeding of microcapsules through the PBS stream from the small intestine, it diminishes as a result of rupture of microcapsules after each 50 min of residence time and due to the outlet. On the other hand the pattern of time history of total microcapsule represents a hypothetical condition if there were no burst releases of probiotics and hence is guided only by the hydrodynamics. The simulated value of concentration of whole microcapsule at 2000 min is in agreement with its experimental counterpart. Fig. 6 (d) depicts the simulated time concentration profile for the probiotic cells in the reactor representing large intestine. The experimental values of concentration of cells have also been superimposed on the Fig. 6 (d). Expectedly, the time history of the probiotic cell shows an increasing trend. The agreement between the experimental and simulated values validates the mathematical model for the prediction of behavior of synbiotic microcapsules in the *in vitro* simulated GI system. From the analysis of the results depicted in Fig. 6(a)–(d) it is clear that the synbiotic microcapsules formulated under the present study are resistant to acidic gastric juice and small intestinal juice containing pancreatin and bile salt and are capable of releasing probiotic cells in the large intestine in a sustainable way. The retention of released probiotics in the large intestine, as assumed in the model, is validated by the agreement of simulated and experimental results. This supports the mucosal adhesion of the probiotic cells for their further proliferation in the gut. From the *in-vitro* experiment and from the prediction of the mathematical model, it is clear that after the intake of a dose of synbiotic microcapsules, there will be sustained release of probiotic cells in the large intestine up to 2000 min and later. Since the prebiotic biomolecules are also released and other carbohydrates are available in the real large intestinal environment, their growth after release will also be supported. This type of profile is very interesting from the perspective of the synbiotic microcapsules for GI application which demands controlled release and sustainable proliferation of probiotics. Although pioneering *in-vitro* studies on the release of probiotics from probiotic microcapsules have been reported (Mandal, Puniya, & Singh, 2006; Ding and Shah, 2009; Chandramouli, Kailasapathy, Peiris, & Jones, 2004; Graff, Hussain, Chaumeil, & Charrueaul, 2008; Cook, Tzortzis, Charalampopoulos, & Khutoryanskiy, 2011; Albertini et al., 2010; Sabikhi, Babu, Thompkinson, & Kapila, 2010; Muthukumarasamy, Allan-Wojtas and Holley et al., 2006; Marteau, Minekus, Havenaar, & Huisin't Veld, 1997) the study on release of probiotics from synbiotic microcapsules in *in-vitro* GI system is being reported for the first time. In absence of literature data on such experiments on synbiotic microcapsules, the comparison with the findings of other investigators is not possible. It is understandable that as different types of food substances are simultaneously digested by real human GI tract, complex mathematical models incorporating the interaction of microcapsules with other food molecules may be necessary. Although the initial evaluation of the performance of the synbiotic microcapsules has been possible through the *in-vitro* experiments and the mathematical model, in a future study *in-vivo* model will be investigated.

**Table 5**
Structure of microcapsules.

| Microencapsulation Process | Bead/microcapsules Diameter | Physical nature of the product |
|---|---|---|
| Emulsification through internal gelation | 23.9333 μm (±0.11547 μm) |  |

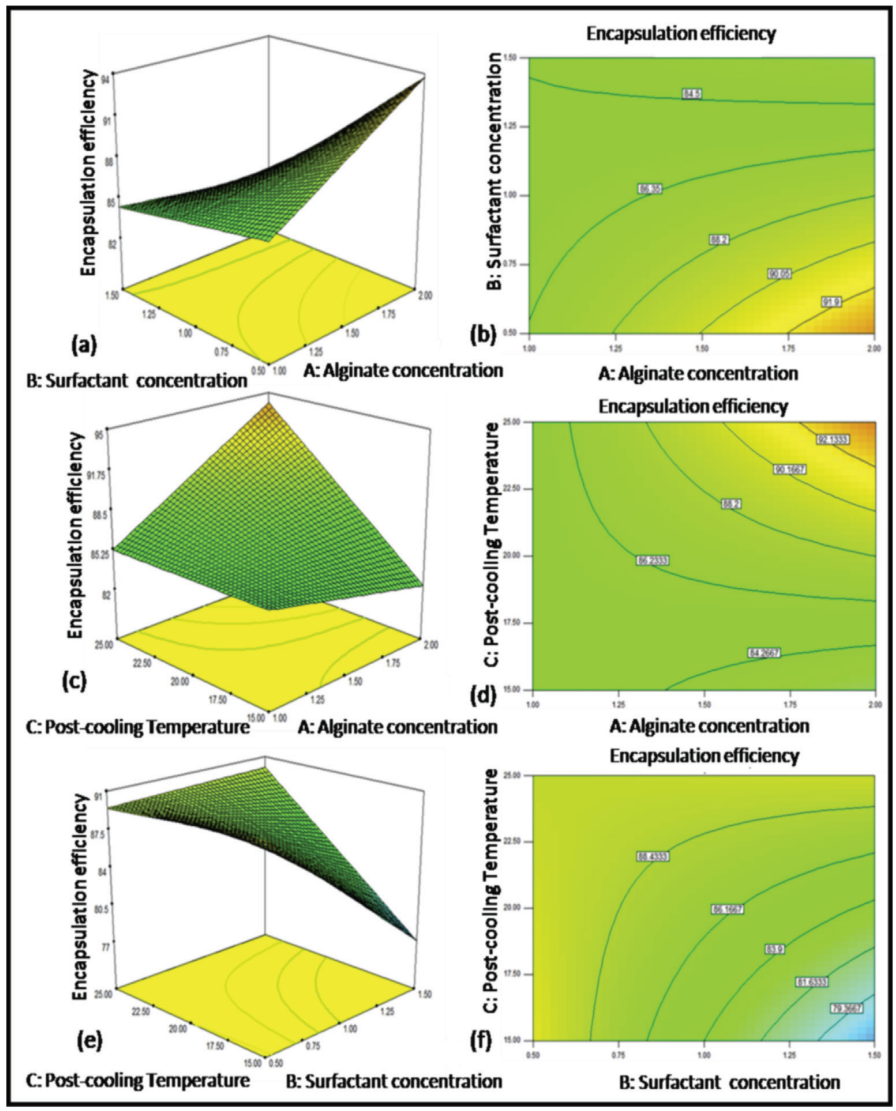



**Fig. 5.** (a)–(f): 3-D surface and contour plots depicting the variation of encapsulaton efficiency with A: Alginate concentration, B: Surfactant concentration and C: Post cooling temperature.

**Table 6**
ANOVA for response surface 2FI (2 factor interaction) model.

| Source | Sum of squars | df | Mean square | F value | p-value Prob > F |
|---|---|---|---|---|---|
| Model | 423.1 | 6 | 70.51667 | 4.627545 | 0.0099 (significant) |
| A-Alginate concentration | 19.6 | 1 | 19.6 | 1.286219 | 0.2772 |
| B-Surfactant concentration | 108.9 | 1 | 108.9 | 7.146391 | 0.0191 |
| C-Temperature | 84.1 | 1 | 84.1 | 5.51893 | 0.0353 |
| AB | 40.5 | 1 | 40.5 | 2.657749 | 0.1270 |
| AC | 72 | 1 | 72 | 4.724886 | 0.0488 |
| BC | 98 | 1 | 98 | 6.431095 | 0.0248 |
| Residual | 198.1 | 13 | 15.23846 | | |
| Lack of Fit | 198.1 | 8 | 24.7625 | | |
| Pure Error | 0.0 | 5 | 0.0 | | |
| Cor total | 621.2 | 19 | | | |

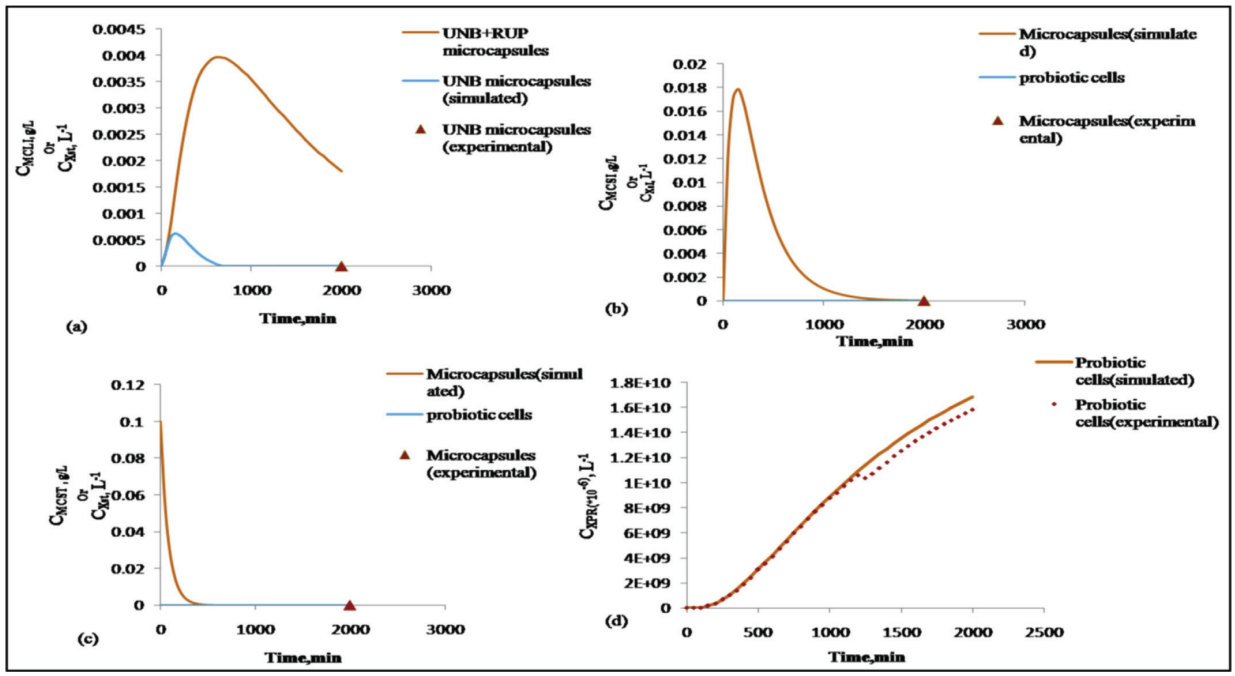



**Fig. 6.** Concentration profiles for simulated human gut system. $C_{MCST}$: Concentration of microcapsules in "stomach"; $C_{MCSI}$: Concentration of microcapsules in "small intestine"; $C_{MCLI}$: Concentration of microcapsules in "large intestine"; $C_{XPR}$: Concentration of probiotic cells.

## 5. Conclusions

The present study focused on the co-encapsulation of probiotic *L. casei* and the prebiotic inulin through internal gelation technique under optimum conditions and subsequently on the performance of the synbiotic microcapsules in *in-vitro* GI environment in batch and continuous mode. Resistance of the synergistic microcapsule to simulated acidic gastric juice and small intestinal juice containing pancreatin and bile salt was observed. The "burst release", proposed under the study for the first time, could predict the release time probiotic cells from the synbiotic microcapsules in the simulated large intestinal juice. The deterministic mathematical model developed under the present study could predict the performance of the *in-vivo* GI system containing three continuous stirred tank bioreactors in series to represent stomach, small intestine and large intestine. This study will be useful for commercialization of synbiotic microcapsules containing this pair of probiotic and prebiotic and other similar one. However, further studies are to be conducted using human and animal models.

## Acknowledgement

The first author (Debolina Banerjee) acknowledges the financial support offered by Council of Scientific and Industrial Research (CSIR), India by providing Senior Research Fellowship (File number: 9/96(0725)2K12-EMRI).

## Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.lwt.2017.05.011

## References

Ahmed, M. M., El-Rasoul, S. A., Auda, S. H., & Ibrahim, M. A. (2013). Emulsification/internal gelation as a method for preparation of diclofenac sodium–sodium alginate microparticles. *Saudi Pharmaceutical Journal, 21*, 61–69.

Albertini, B., Vitali, B., Passerini, N., Cruciani, F., DiSabatino, M., Rodriguez, L., et al. (2010). Development of microparticulate systems for intestinal delivery of *Lactobacillus acidophilus* and Bifidobacteriumlactis. *European Journal of Pharmaceutical Sciences, 40*, 359–366.

Banerjee, D., BAG, P., Chowdhury, R., & Bhattacharya, P. (2017). Sustainability of the probiotic *Lactobacillus casei* in fortified Indian milk cakes under different preservation conditions-effects of co-immobilization of *L. casei* and commercial prebiotic inulin (chicory based) and millet inulin. *International Journal of Pharmacy and Pharmaceutical Sciences, 9*(1).

Banerjee, D., Chowdhury, R., & Bhattacharya, P. (2016). The prebiotic influence of inulin on growth rate and antibiotic sensitivity of *Lactobacillus casei*. *International Journal of Pharmacy & Pharmaceutical Sciences, 8*.

Berry, A. R., Franco, C. M. M., Zang, W., & Middelberg, A. P. J. (1999). Growth and lactic acid production in batch culture of Lactobacillus rhamnosus in a defined medium. *Biotechnology Letters, 21*(2), 163–167.

Chandramouli, V., Kailasapathy, K., Peiris, P., & Jones, M. (2004). An improved method of microecapsulation and its evaluation to protect *Lactobacillus* spp. in simulatedgastric conditions. *Journal of Microbiology Methods, 56*, 27–35.

Chapin, K. C., & Lauderdale, T. L. (2003). Reagents, stains and Media: Bacteriology. In *Manual of clinical microbiology* (8th ed., pp. 15–30). Washington, DC, USA: American Society for Microbiology.

Child, M. W., Kennedy, A., Walker, A. W., Bahrami, B., Macfarlane, S., & Macfarlane, G. T. (2006). Studies on the effect of system retention time on bacterial populations colonizing a three-stage continuous culture model of the human large gut using FISH techniques. *FEMS Microbiology Ecology, 55*, 299–310.

Chourasia, M. K., & Jain, S. K. (2003). Pharmaceutical approaches to colon targeted drug delivery systems. *Journal of Pharmacy and Pharmaceutical Sciences, 6*(1), 33–66.

Chun, H., Kim, C. H., & Cho, Y. H. (2014). Microencapsulation of *Lactobacillus plantarum* DKL 109 using external ionic gelation method. *Korean Journal for Food Science and Animal Resources, 34*, 692–699.

Clark, H., & Blanch, W. (1997). *Biochemical engineering* (Edition 2). CRC Press.

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2011). Production and evaluation of dry alginate–chitosan microcapsules as an enteric delivery vehicle for probiotic bacteria. *Biomacromolecules, 12*, 2834–2840.

Cook, M. T., Tzortzis, G., Charalampopoulos, D., & Khutoryanskiy, V. V. (2012).

Microencapsulation of probiotics for gastrointestinal delivery. *Journal of Controlled Release, 162*, 56–67.
Ding, W. K., & Shah, N. P. (2009). An improved method of microencapsulation of probioticbacteria for their stability in acidic and bile conditions during storage. *Journal of FoodSciences, 74*, M53–M61.
Doran, P. M. (1995). *Bioprocess engineering principles*. Elsevier Science &TechnologyPublishers.
Gbassi, G. K., & Vandamme, T. (2012). Probiotic encapsulation Technology: From microencapsulation to release into the gut. *Pharmaceutics, 4*, 149–163. http://dx.doi.org/10.3390/pharmaceutics4010149.
Graff, S., Hussain, S., Chaumeil, J. C., & Charrueaul, C. (2008). Increased intestinal delivery of viable Saccharomyces boulardii by encapsulation in microspheres. *Pharmaceutical Research, 25*, 1290–1296.
Kailasapathy, K. (2002). Microencapsulation of probiotic Bacteria: Technology and potential applications. *Current Issues Intestinal Microbiolgy, 3*, 39–48.
Kong, F., & Singh, R. P. (2008). Disintegration of solid foods in human stomach. *Journal of Food Science, 73*(5).
Lakkis, J. M. (2007). *Encapsulation and controlled release technologies in food systems*. USA: Blackwell Publishing.
Lentle, R. G., & Janssen, P. W. M. (2011). *The physical processes of digestion*. http://dx.doi.org/10.1007/978-1-4419-9449-3. ISBN 978-1-4419-9448, e-ISBN 978-1-4419-9449-3.
Macfarlane, G. T., Macfarlane, S., & Gibson, G. R. (1998). Validation of a three-stage compound continuous culture system for investigating the effect of retention time on the ecology and metabolism of bacteria in the human colon. *Microbial Ecology, 35*, 180–187.
Mandal, S., Puniya, A. K., & Singh, K. (2006). Effect of alginate concentrations on survival of microencapsulated *Lactobacillus casei* NCDC-298. *International Dairy Journal, 16*, 1190–1195.
Marteau, P., Minekus, M., Havenaar, R., & Huisin't Veld, J. H. (1997). Survival of lactic acid bacteria in a dynamic model of the stomach and small intestine: Validation and the effects of bile. *Journal of Dairy Science, 80*, 1031–1037.
Muthukumarasamy, P., Allan-Wojtas, P., & Holley, R. A. (2006). Stability of *Lactobacillus reuteri* in different types of microcapsules. *Journal of Food Science, 71*, M20–M24.
Okkers, D. J., Dicks, L. M. T., Silvester, M., Joubert, J. J., & Odendaal, H. J. (1999). Characterization of pentocin TV35b, a bacteriocin-like peptide isolated from *Lactobacillus pentosus* with a fungistatic effect on Candida albicans. *Journal of Applied Microbiology, 87*, 726–734.
Pliszczak, D., Bourgeois, S., Bordes, C., Valour, J. P., Mazoyer, M. A., Orecchionid, A. M., et al. (2011). Improvement of an encapsulation process for the preparation of pro- and prebiotics-loaded bioadhesivemicroparticles by using experimental design. *European Journal of Pharmaceutical Sciences, 44*, 83–92.
Rosas-Flores, W., Ramos-Ramirez, E. G., & Salazar-Montoya, J. A. (2013). Microencapsulation of *Lactobacillus helveticus* and using alginate and gellan gum. *Carbohydrate Polymers, 98*, 1011–1017.
Sabikhi, L., Babu, R., Thompkinson, D. K., & Kapila, S. (2010). Resistance of microencapsulated *Lactobacillus acidophilus* LA1 to processing treatments and simulated gut conditions. *Food Bioprocess Technology, 3*, 586–593.
Sathyabama, S., Ranjithkumar, M., Brunthadevi, P., Vijayabharathi, R., & Brindhapriyadharisini, V. (2014). Co-encapsulation of probiotics with prebiotics on alginate matrix and its effect on viability in simulated gastric environment. *LWT - Food Science and Technology, 57*, 419–425.
Shuler, M. L., & Kargi, F. (2002). *Bioprocess Engineering: Basic concepts* (Edition 2). USA: Pearson.
Song, H., Yu, W., Gao, M., Liu, X., & Ma, X. (2013). Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process. *Carbohydrate Polymer, 96*, 181–189.
Yoo, I. K., Seong, G. H., Chang, H. N., & Park, J. K. (1996). Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production. *Enzyme &Microbial Technology, 19*, 426–433.
Zou, Q., Liu, X., Zhao, J., Tian, F., Zhang, H., & Zhang, H. (2012). Microencapsulation *Lactobacillus delbrueckii* of *Bifidobacteriumbifidum F-35* in whey protein-based microcapsules by transglutaminase-induced gelation. *Journal of Food Science, 77*(5), M270–M277.

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 9, Issue 1, 2017

Original Article

# SUSTAINABILITY OF THE PROBIOTIC *LACTOBACILLUS CASEI* IN FORTIFIED INDIAN MILK CAKES UNDER DIFFERENT PRESERVATION CONDITIONS-EFFECTS OF CO-IMMOBILIZATION OF *L. CASEI* AND COMMERCIAL PREBIOTIC INULIN (CHICORY BASED) AND MILLET INULIN

**DEBOLINA BANERJEE[1], PUJA BAG[1], RANJANA CHOWDHURY[1*], PINAKI BHATTACHARYA[2]**

**[1]Chemical Engineering Department, Jadavpur University, Kolkata 700032, India, [2]Chemical Engineering Department, Heritage Institute of Technology, Kolkata 700107, India**
**Email: ranjana.juchem@gmail.com**

*Received: 21 Sep 2016 Revised and Accepted: 17 Nov 2016*

**ABSTRACT**

**Objective:** The objective of the present article is to identify the most suitable Indian millet inulin for the growth of probiotic *Lactobacillus casei* and to evaluate the effects of the fortification vectors (probiotics and probiotic-prebiotic combination in immobilized conditions) and immobilization methods on the sustainability of *L. casei* in a fortified Indian sweet (milk cake) preserved under different conditions.

**Methods:** Inulin was extracted from pearl, finger and great millets. The concentrations of *L. casei*, grown on three millet inulins, were compared in 24 h batch culture. The *L. casei* and probiotic-prebiotic combinations namely *L. casei*-commercial inulin and *L. casei*-pearl millet inulin were immobilized using entrapment, external and internal microencapsulation methods. The Indian milk cake samples were fortified with the immobilized probiotic cells, co-immobilized probiotic-prebiotic combinations. The fortified samples were preserved at different conditions (temperature: 4 °C and-20 °C; Time: 1-4 w). The sustainability of *L. casei* in the preserved samples was determined using spread plate method and the cell concentrations were compared among all fortified samples.

**Results:** Pearl millet inulin is determined to be the most suitable millet inulin for the growth of *L. casei*. The synergistic combination of *L. casei*–pearl millet inulin, co-immobilized with internal gelation technique is the best fortification vector for the viability of *L. casei* in preserved food samples.

**Conclusion:** The *L. casei*, co-immobilized with pearl millet inulin through internal gelation technique, can be utilized as an effective fortification vector for the sustainability of probiotic cells in preserved Indian milk cakes and similar food samples.

**Keywords:** Probiotic, *Lactobacillus casei* (*L. casei*), Prebiotic, Inulin, Pearl millet, Immobilization, Viability, Indian Milk cakes


DOI: http://dx.doi.org/10.22159/ijpps.2017v9i1.15305

## INTRODUCTION

In recent past, concepts of functional foods have moved progressively towards the development of dietary supplementation which plays an important role in maintaining a healthy gut microbial composition and activities. The consumption of probiotic products is constantly gaining popularity among the population throughout the globe. Nowadays, great attention has been given in extending the category of foods carrying probiotics in order to broaden the groups of people that usually have no access to probiotic food [1]. Probiotic products for human diet include nutraceuticals or functional foods, food ingredients and supplements that have an important effect on the intestinal microflora [2, 3]. A wide range of foods including fermented and non-fermented dairy products have been evaluated as possible probiotics carrier. Milk derivatives are considered an ideal vehicle for carrying living probiotic cells because of its high fat content and buffering properties that help the bacteria to overcome the stress factors in the gastrointestinal conditions. With the view of improving the survival of probiotics [4], the effectiveness of prebiotics are well approved. The prebiotics are complex carbohydrates which are available in non-digestible food ingredients and support the growth of probiotics [5]. The most common prebiotics, namely inulin is naturally found in many foods such as wheat, pearl millet (bajra), onions, garlic, leeks, asparagus and chicory roots [6].

Although inulin extracted from chicory roots is available commercially, extraction of inulin from other natural sources should also be conducted. Only a few studies have been conducted on the extraction of inulin from oat, wheat, daliah and garlic [7, 15]. The effect of inulin extracted from other sources on the growth and viability of probiotic cells should also be emphasised. The Indian millets, namely great millet (*Sorghum bicolor*; Indian name: jowar), pearl millet (*Pennisetum glaucum*; Indian name: bajra) and finger millet (*Eleusine coracana*; Indian name: ragi) are reported to be rich in inulin. However, no work is available in the literature on extraction and characterization of millet-based inulin.

The intended health benefits of probiotics can only be obtained when the food contains the required minimum viable microorganism count at the time of consumption. US FDA has recommended that minimum probiotic count in the probiotic food should be at the least $10^6$CFU $ml^{-1}$[8-10]. It is, therefore, important to ensure that probiotics must survive and retain their functional features during the entire food processing operations as well as over the product shelf life in order to maintain consumer confidence in probiotic products. In this regard, a number of efforts have been made to improve the robustness and the viability of the probiotics in the variety of functional foods. Many factors were found to influence the viability of probiotic cells in food products during processing and storage. In this context, microencapsulation is found to be the most potential approach for enhancing the survival of the probiotic microorganisms in the food products from processing to storage until consumption [11-14]. The present article is focused on the systematic studies on the following topics: a) extraction of inulin from Indian millets namely great millet (jowar), pearl millet (bajra) and finger millet (ragi) along with the assessment of their effectiveness as the substrate for the probiotic cells, namely, *L. casei*; selection of the most effective millet inulin thereof, b) assessment of the effects of co-immobilization of *L. casei* and inulin with respect to the enhancement of viability of the former in a traditional Indian food product called milk cake (an Indian cottage cheese or chhena based dairy product); comparison of the effectiveness of co-immobilized combination of probiotic *L. casei* and commercial inulin with that of *L. casei* and millet inulin, c) comparison of the performance of co-immobilized combination of *L. casei* and inulin prepared using different immobilization techniques. Although it is

understandable that the sustainability of probiotic cells is expected to increase by co-immobilization of prebiotics along with probiotics, no such research studies is available in the literature. To the best of our knowledge, this is the first study addressing the above topics and the research findings will open up the possibility of utilisation of prebiotic inulin derived from abundant sources of Indian millet and development of food products fortified with co-immobilized probiotics and millet based prebiotics.

## MATERIALS AND METHODS

### Microorganisms

*Lactobacillus casei* (2651 1951 RPK) culture purchased from NCIM, Pune were used.

### Materials

Food grade inulin, peptone, yeast extract, sodium acetate, dipotassium hydrogen phosphate, tri-ammonium citrate, magnesium sulphate, manganese sulphate, sodium chloride, disodium hydrogen phosphate, potassium dihydrogen phosphate and sodium chloride purchased from Himedia, India, were used. Sodium alginate, acetic acid, tween80, potassium dihydrogen phosphate, disodium hydrogen phosphate, calcium chloride, glycerol, sodium dodecyl sulphate (SDS), beef extract, calcium carbonate, lactose and ethanol purchased from Merck, India, were used.

Milk (Amul India pvt. Ltd.), seeds of pearl millet, great millet and finger millet (Andheri, Mumbai) and rice-bran oil purchased from the local market were used.

### Growth medium and other reagents

### MRS medium

### Composition

Beef extract: 10 g/l; yeast extract: 5 g/l; peptone: 10 g/l; sodium acetate: 5 g/l; di-potassium hydrogen phosphate: 2 g/l; tri-ammonium citrate: 2 g/l; magnesium sulphate: 0.05 g/l; manganese sulphate: 0.05 g/l and lactose: 20 g/l; pH 7.

### Phosphate buffer (0.1M)

### Composition

Potassium di-hydrogen phosphate: 1.361 g/l; di-sodium hydrogen phosphate: 35 g/l (to adjust the pH at 7).

### Peptone water

### Composition

Peptone digest: 10 g/l; sodium chloride: 5 g/l.

### Equipments

Magnetic stirrer (Remi, India), constant temperature bath (S. C Dey and co., India), digital weighing machine (Sartorius), autoclave (Gurpreet engineering works), hot air oven (G. B. Enterprises, Kolkata, India), B. O. D incubator (G. B. Enterprises, Kolkata, India), laminar airflow (G. B. Enterprises, Kolkata, India), mixer-grinder (Crompton Greaves, Kolkata, India) and high speed homogenizer (Plasto Crafts (Model: Superspin R-V/$F_M$), India), refrigerator (Whirlpool; Corona Deluxe, India), freezer-20 °C (Blue Star, India)were used.

### Analytical instrument

Optical microscope (Optika, Italy) was used.

### Extraction of inulin from Indian millets

The prebiotic native sources, namely, pearl (bajra), finger (ragi) and great (jowar) millets were washed properly with distilled water. The same protocol followed for extraction of inulin from wheat, oat, daliah etc. has been used [15].

### Sterilization procedure

MMRS medium, 2 % sodium alginate solution and home-made sweet were autoclaved at 121 °C for 15 min.

### Pre-adaptation of *Lactobacillus casei* to different carbohydrate sources

The probiotic bacteria, namely, *Lactobacillus casei* was pre-adapted to different carbohydrate sources, namely, lactose, commercial inulin and three millet inulins. For lactose, sterile modified de-Man Rogosa Sharpe (MMRS) medium was prepared by using 20 g/l lactose as the carbohydrate source. Four other stock cultures were also prepared using MMRS media in which the food grade commercial inulin and three millet inulins at a concentration of 0.3225 g/l were separately used as the carbohydrate source. Each medium was inoculated with 1 % (v/v) of *L. casei* and was incubated for 24h at 37 °C. The concentration of 0.3225 g/l was chosen for each inulin sample because the maximum growth of *L. casei* on commercial inulin had been observed by the present researchers at this level [15]. Since the purity of each inulin extract is dependent on the natural source, different quantity of inulin extracts was required to prepare the inulin solution of equal strength.

### Determination of the effectiveness of millet inulins for the growth of *L. casei*

Batch studies were conducted in three 250 ml conical flasks using 100 ml modified MRS medium containing lactose and different millet inulins. The concentration of millet inulin and lactose in the MMRS medium in each conical flask were 0.3225 g/l and 20 g/l respectively. The pearl millet inulin, finger millet inulin and great millet inulin were respectively used in the first, second and third flasks. Each flask was seeded with 10 % (v/v) stock culture of *L. casei*. The flasks were kept in an incubator for 24 h at 37 °C. The cell concentration in each flask was measured using spectrophotometric method determining the optical density of the growth medium at 600 nm. The cell concentrations of growth medium in three flasks at 24 h were compared to identify the most effective millet inulin for the growth of *L. casei*.

### Characteristics of millet extracts

FTIR analysis of the extracts of three millet sample has been carried out to determine the presence of inulin qualitatively. The quantity of inulin in each extract has been determined spectrophotometrically following the protocols described by Samanta Koruri *et al.* [16].

### Co-entrapment and entrapment of *Lactobacillus casei* with and without inulin

The protocols described by Chan *et al.*, 2011 and Yoo *et al.*, 1996 have been principally followed [17, 18] for the entrapment and co-entrapment. *L. casei* cultures, pre-adapted to lactose and commercial inulin were respectively spiked up to 20 g/l lactose and 0.3225 g/l commercial inulin and were individually mixed with 2% sterile sodium alginate solution in the volumetric ratio of 1:4. Each mixture was then added dropwise into a 1% $CaCl_2$ solution at room temperature and stirred continuously. The beads containing entrapped *L. casei* and co-entrapped *L. casei* and inulin were allowed to harden and were washed with saline water and stored at 4 °C.

### Co-encapsulation and encapsulation of *L. casei* with and without inulin through external gelation

For the co-encapsulation and encapsulation of *L. casei* with and without inulin, via external gelation method, the protocol suggested by Song *et al.*, 2013 have been principally followed [19]. *L. casei* cultures, pre-adapted to lactose and commercial inulin were spiked with the respective carbohydrates up to the concentration levels as used for pre-adaptation, i.e., 20 g/l and 0.3225g/l for lactose and inulin respectively. Each culture was mixed with sodium alginate solution in the volumetric ratio of 1:4. 1 ml of the mixture was stirred with 5 ml of vegetable oil. Tween 80 (emulsifier) and SDS were added to the resulting solution to maintain their concentrations at 0.2 % and 0.25 % respectively. This mixture was then stirred at 200 rpm for 30 min. $CaCl_2$ was added quickly but gently down the side of a beaker, in which the mixture was taken. The formed microbeads were kept undisturbed for 30 min. They were then filtered with muslin cloth. Finally, the microspheres were washed with 0.9% saline water containing 0.05% glycerol. The washed microbeads were stored at 4 °C.

**Co-encapsulation and encapsulation of *L. casei* with and without inulin through internal gelation**

The protocol suggested by Song *et al.*, 2013 and Sultana *et al.*, 2000 was principally followed with some modifications for the encapsulation of cells through internal gelation method [19, 20]. *L. casei* cultures pre-adapted to lactose, commercial inulin and the millet inulin, most effective for the growth of the probiotic microorganism, were at first spiked with the respective carbohydrate sources as described in the case of entrapment and external gelation i. e up to 20 g/l and 0.3225 g/l for lactose and inulin samples respectively. Each culture was then individually mixed with 2 % sodium alginate solution in the volumetric ratio of 1:4. $CaCO_3$ powder and Tween-80 were added to each mixture up to a concentration level of 0.8 g/l and 0.5 % (v/v). This mixture was then combined with vegetable oil in the volumetric ratio of 1:5 and stirred at 300 rpm for 30 min. The temperature of the mixture was maintained at 25 °C by circulating water from a constant temperature bath and acetic acid was next added dropwise to the solution to release the $Ca^{2+}$from the insoluble $CaCO_3$ powder. Microcapsules were formed and were filtered with a muslin cloth and washed with 1% (v/v) aqueous tween 80 solution and distilled water. The microcapsules were then stored at 4 °C.

**Microscopic characterization of beads and microcapsules**

The size analysis of the beads and microcapsules were done microscopically.

**Preparation of Indian cottage cheese for milk cakes**

Indian cottage cheese (chhena) was prepared from 1L milk. Firstly the milk was allowed to boil, followed by addition of lemon juice into it stirring continuously. When the milk curdled well, cottage cheese was strained using a muslin cloth and washed with water. After removing the excess water, 200 g chhena was obtained. The chhena was added to a pan and was mixed well with sugar and cardamom powder on a low flame until a mixture of thick consistency was obtained. The mixture was then cooled and kneaded properly and was autoclaved at 120 °C.

**Preparation of milk cakes with and without fortification with probiotic or probiotic--prebiotic microcapsules**

The sterile 200 g chhena, as described above, was divided into eight parts—first part was mixed with probiotic Ca-alginate beads spiked by lactose; second part was mixed with Ca-alginate beads spiked by commercial inulin; third part was mixed with probiotic microcapsules (external gelation) spiked by lactose; fourth part was mixed with probiotic microcapsules (external gelation) spiked by commercial inulin; fifth part was mixed with probiotic microcapsules (internal gelation) spiked by lactose; sixth part was mixed with probiotic microcapsules (internal gelation) spiked by commercial inulin; seventh part was mixed with probiotic microcapsules spiked by millet inulin, most effective for the growth of the probiotic microorganism and the eighth part was kept unfortified.

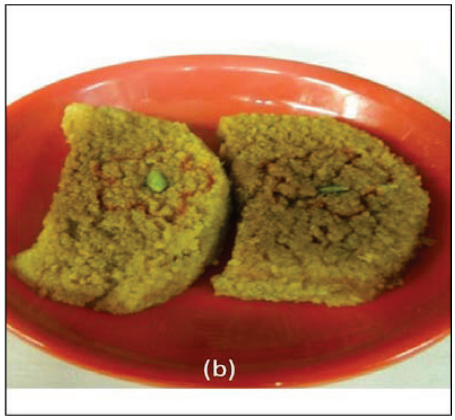



**Fig. 1: Indian sweet (milk cakes) samples (a) wrapped by aluminium foil (b) original sample**

From each part, one milk cake weighing 25 g was prepared using sterile molds. All operations for the preparation of milk cake were conducted in a UV-sterilized hood. During the fortification, 10 % (w/w) beads or microcapsules were used. The milk cakes are shown in fig. 1. All milk cakes were wrapped with sterile aluminium foils (fig. 1) before preservation.

**Viability assay of the probiotic cells in food products**

The fortified Indian milk cakes were stored at 4 °C and-20 °C over a period of 4 w. Unfortified samples were used as controls. Viability assay was performed every one-week interval as per the following protocol: in a UV-sterilized hood, 5 ml of phosphate buffer was first mixed with 1 g of food samples, and then 1 ml from this mixture was serially diluted with peptone water by 10 fold for six times. 0.1 ml of the diluted mixture was plated on MRS agar using spread plate technique. The agar plates were incubated under anaerobic condition for 24 h at 37 °C and the colonies on each plate were counted. According to this technique the concentration of cells per millilitre of the original phosphate buffer solution of the food sample, $10^7$ times the count obtained from the spread plate method. Since 1g of food sample was originally present in 5 ml phosphate buffer, the concentration of cells in the food sample is $5*10^7$ times that obtained from spread plate method.

All experiments were carried out thrice, and the averages of three replicate experimental results have been reported.

**RESULTS AND DISCUSSION**

**Characteristics of millet extracts**

The presence of functional groups of inulin in the three millet samples was clearly indicated through the FTIR results (not shown). The % (w/w) of inulin in great, pearl and finger are provided in table 1. From the analysis of the table 1, it is clear that the inulin content of pearl millet extract is the highest among all millet extracts. The results could not be compared with the literature data due to unavailability.

**Table 1: Content of inulin in millet extracts***

| Millet type | Inulin in extract % (w/w) |
|---|---|
| Great | 30.147±0.127000 |
| Pearl | 47.27±0.155885 |
| Finger | 32.0833±0.076376 |

*Values are mean (n=3)±standard deviation (Standard deviation for sample population of three replicate observations corresponding to each mean result was calculated)

**Effect of naturally extracted inulin on the growth of *Lactobacillus casei***

The concentrations of *L. casei* obtained using the three different millet inulins, following the procedure described in the experimental section, were compared in fig. 2. From the analysis of the fig., it is clear that the pearl millet inulin is the most effective among all millet inulins with respect to the growth of *L. casei*. The effectiveness of pearl millet inulin was followed by that of great millet inulin and finger millet inulin. Better assimilation of the pearl millet inulin by *L. casei* in comparison to inulins derived from other millet sources was the underlying fact. This might be due to the difference in the degree of polymerization of inulin derived from different millet sources. This could be elucidated by the determination of the degree of polymerization defined by a number of monomer units (fructose) per molecule of prebiotic polymer, i.e., inulin derived from great, pearl and finger millets.

**Characterization of entrapped beads and microcapsules**

Table 2 shows the results of microscopic size analysis of beads and microcapsules obtained by entrapment and external/internal micro-emulsification techniques. It is clear that the size of beads is much larger than those of microcapsules. On the other hand, the

microcapsules obtained through internal gelation method are much smaller than those obtained through external gelation. The results are in agreement with those reported by previous researchers [17, 20, 21].

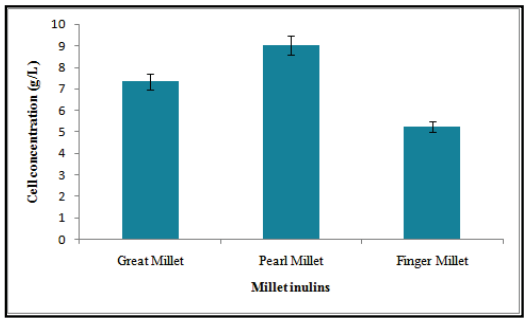



**Fig. 2: Bacterial growth for three different millet inulins**

**Table 2: Microscopic size analysis of beads and microcapsules***

| Microencapsulation processes | Bead/microcapsule diameter |
|---|---|
| Entrapment method | 2.98333 mm (±0.028868 mm) |
| Emulsification/external gelation | 298.333 µm (±2.886751 µm) |
| Emulsification/internal gelation | 23.9333 µm (±0.11547 µm) |

* Values are mean (n=3)±standard deviation

**Viability of the probiotic cells in fortified food products after preservation**

In fig. 4a and 4b the photographs of the spread plates obtained for unfortified food samples and that fortified with encapsulated *L. casei* are provided. It was clearly indicated that while the control was fully contaminated, no contamination was present in the case of the fortified one. This establishes the anti-microbial activity of the immobilized probiotic culture against contaminating micro-organisms and the elongation of shelf life. Due to unavailability of literature data, the results could not be compared.

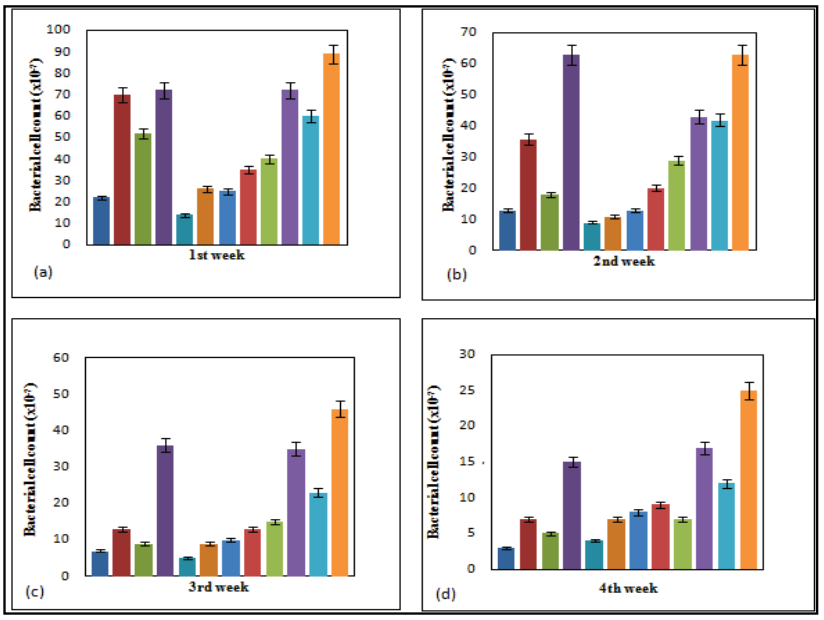



**Fig. 3: Comparison of effects of immobilization methods and fortification vectors (only probiotic or Probiotic-commercial inulin) on viability of probiotics in fortified milk cakes preserved at 4 °C and-20 °C, : External gelation (Probiotic)(4°C); : External gelation (Probiotic)(-20°C); : External gelation (Probiotic-commercial inulin)(4°C); : External gelation (Probiotic-commercial inulin)(-20°C); : Ca-entrapped (Probiotic)(4°C); : Ca-entrapped (Probiotic)(-20°C); : Ca-entrapped (Probiotic-commercial inulin)(4°C); : Ca-entrapped (Probiotic-commercial inulin)(-20°C); : Internal gelation (Probiotic)(4°C); : Internal gelation (Probiotic)(-20°C); : Internal gelation (Probiotic-commercial inulin)(4°C); : Internal gelation (Probiotic-commercial inulin)(-20°C).**

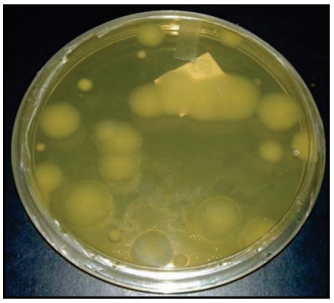


**Fig. 4a: Plate for control sample of milkcake (without probiotics) on 4th week after preservation at 4 °C**

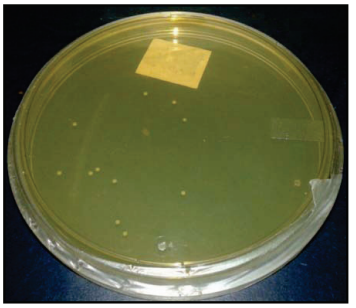


**Fig. 4b: Plate for milk cake sample fortified with encapsulated probiotics on 4th week after preservation at 4 °C**

In table 3, the concentrations of probiotic cells in the fortified Indian milk cakes have been provided.

**Table 3: Results of viability assay (cell concentrations) for fortified milk cakes***

| Weeks | Microcapsules (External gelation) ($x10^{-7}$)/ml | | | | Beads (Entrapment) ($x10^{-7}$)/ml | | | | Microcapsules (Internal gelation) ($x10^{-7}$)/ml | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | PRO | | PRO+CI | | PRO | | PRO+CI | | PRO | | PRO+CI | | PRO+PMI | |
| | A | B | A | B | A | B | A | B | A | B | A | B | A | B |
| 1 | 22.0± 0.000 | 69.6± 0.577 | 51.6± 0.577 | 72± 0.000 | 13.6± 0.577 | 25.6± 0.577 | 24.6± 0.577 | 34.6± 0.577 | 40± 0.000 | 71.6± 0.577 | 60± 0.000 | 88.6± 0.577 | 84.6± 0.577 | 94.6± 0.577 |
| 2 | 12.7± 0.577 | 35.6± 0.577 | 17.6± 0.577 | 62.6± 0.577 | 9± 0.000 | 10.6± 0.577 | 13± 0.000 | 19.6± 0.577 | 28.6± 0.577 | 43± 0.000 | 41.6± 0.577 | 62.6± 0.577 | 42.6± 0.577 | 64± 0.000 |
| 3 | 6.7± 0.577 | 13± 0.000 | 8.6± 0.577 | 35.6± 0.577 | 4.6± 0.577 | 9± 0.000 | 9.6± 0.577 | 12.6± 0.577 | 14.6± 0.577 | 34.6± 0.577 | 21.6± 0.577 | 46± 0.000 | 23.6± 0.577 | 37.6± 0.577 |
| 4 | 3.3± 0.577 | 7± 0.000 | 5± 0.000 | 14.6± 0.577 | 3.6± 0.577 | 6.6± 0.577 | 7.6± 0.577 | 9± 0.000 | 6.6± 0.577 | 16.6± 0.577 | 11.6± 0.577 | 24.6± 0.577 | 11± 0.000 | 24.6± 0.577 |

For table 3, PRO: fortified with only probiotic; PRO+CI: fortified with probiotics and commercial inulin; PRO+PMI: fortified with probiotics and pearl millet inulin; A: preservation at 4 °C; B: -20 °C, *Values are mean (n=3)±standard deviation

The concentrations of viable *L. casei* cells in milk cake samples fortified by either beads or microcapsules containing either only probiotics or mixture of probiotics and prebiotic commercial inulin derived from chicory and preserved for 1, 2, 3 and 4 w respectively were depicted in the bar plots in fig. 3a, 3b, 3c and 3d.

It was clearly indicated in the fig. 3a-3d that irrespective of the methods of immobilization, fortification vectors and period of preservation, the viability of the cells was more for preservation condition of-20 °C in comparison to that exhibited at 4 °C. This is due to better preservation of cells at-20 °C compared to that at 4 °C. Similar observations were obtained by Martin *et al.* during their studies on the preservation of immobilized *L. fermentum* at-20 °C and 4 °C for 30 d. [22] Irrespective of immobilization method preservation temperature and fortification vector, it was observed that the viability of cells decreased even at-20 °C with the increase of preservation period. This might be due to the formation of ice crystals on prolonged preservation causing the destruction of probiotic cells. When the effect of immobilization method on the viability of cells was analysed, it was revealed that whatever might be the fortification vector and the preservation time and condition, the best viability of cells was shown by the microcapsules produced through internal gelation method. This was justified by the fact that due to the small size of microcapsules the surface area available for the transport of substrates present in the extracellular food materials into the cells was increased resulting in more sustainability of cells. This was also due to the low leakage of cells from the microcapsules in the case of internal gelation beads as compared to the external beads, and the Ca-alginate entrapped beads [17, 22]. The better viability of preserved probiotic cells immobilized through internal gelation in comparison to those immobilized with entrapment and external gelation techniques had also been observed by other researchers [17]. It was clearly indicated from the comparison of the performance of fortification vectors that irrespective of immobilization method and preservation period, the best viability of cells was exhibited by the co-immobilized combination of probiotic cells and commercial inulin. This was due to the fact that even under the immobilized condition, the prebiotic inulin served as a suitable substrate for the sustainability of the probiotic cells. As no reported data was so far available on the effect of co-immobilization of *L. casei* and a prebiotic compound, the comparison could be made with the literature data. It was observed that the variation of the probiotic cell count (CFU) in the 0.2 g/ml solution of food sample in phosphate buffer, as described under experimental section, was in the range of 3-95 $x10^7$/ml and was much higher than the recommended lower limit of cell count of $10^6$CFU/ml in probiotic food. Thus all the milk cakes fortified by different vectors were suitable as probiotic food. Among all samples, a combination of probiotic cells with prebiotic inulin, immobilized through internal gelation, were the most effective one. As per expectation, the prebiotic inulin was effective in enhancement and sustainability of probiotic cells even in co-immobilized condition. All the food samples were suitable for human intake even after 4 w of preservation. The viability of probiotic cells in milk cakes fortified by microcapsules produced through internal gelation containing combinations of *L. casei* and either commercial inulin or pearl millet inulin was indicated by the fig. 5.

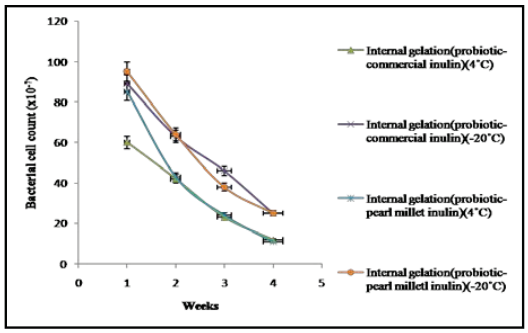



**Fig. 5: Comparison of effect of microcapsules obtained by internal gelation method for both the combination probiotic-commercial inulin and probiotic-pearl millet inulin at 4 °C and-20 °C (Results are average of those obtained from three replicate observations. Standard deviation varies from 0 to 0.57735)**

It was clearly revealed from the close analysis of the fig. that the effectiveness of pearl millet inulin in the enhancement of sustainability of probiotic cells was marginally better than that of commercial inulin. This might be due to the difference in the degree of polymerization of inulin derived from chicory and pearl millet inulin. However, more experiments have to be conducted to reveal the exact fact. Therefore, the substitution of commercial inulin by this nonconventional variety of inulin should be attempted in commercial scale.

## CONCLUSION

Under this study effects of different immobilization methods–Ca-alginate entrapment; external and internal microencapsulation, the fortification vectors, namely the probiotic cells, combination of probiotic cells and commercial inulin/pearl millet inulin, preservation temperature, 4 °C and-20 °C and period of 1-4 w on the sustainability of the probiotic microorganism, *L. casei* in fortified Indian milk cake has been investigated systematically. The pearl millet inulin has been selected based on its best performance among three millet inulins (great, pearl and finger millet) with reference to the growth of the probiotic cells, namely, *L. casei* in free (not immobilized) culture. To the best of our knowledge, this is the first investigation of this type. This study indicates that microencapsulation techniques including internal gelation, external gelation and direct calcium-alginate entrapment methods can significantly support the survival of the probiotic microorganisms in the fortified food product, namely Indian milk cake over a preservation period of up to 4 w at both 4 °C and-20

°C. However, on the basis of the viability of probiotic cells, the internal gelation technique is found to be superior to the other two immobilization techniques. The results show that the contribution of different immobilization methods in increasing the viability of probiotic bacteria in preserved and fortified milk cake follows the order: internal gelation>external gelation>Ca-entrapped. Co-immobilized probiotics with prebiotics, namely, commercial inulin/pearl millet inulin markedly increase the viability of probiotic cells over that obtained through fortification by immobilized probiotics. Thus, the use of both commercial food grade inulin and naturally derived pearl millet inulin has positive effects on the survival of the microencapsulated probiotic cells in the food products during storage. Results of the study suggest that pearl millet inulin offer marginally more potential as prebiotic than the commercial food grade inulin. Overall, the fortification results in elongation of the shelf life of the preserved due to the sustenance of growth of probiotic microorganisms which ultimately inhibit the growth of pathogenic microorganisms in the food products into which they are added. It is expected that the outcome of the present research study can be utilized for the application of pearl millet and the conventional chicory inulin (commercial) for the production of fortified food products using synergistic probiotic-prebiotic combinations co-immobilized through internal gelation technique.

**ACKNOWLEDGEMENT**

The first author (Debolina Banerjee) acknowledges the financial support offered by Council of Scientific and Industrial Research (CSIR), India by providing Senior Research Fellowship (File number: 9/96(0725)2K12-EMRI).

**CONFLICT OF INTERESTS**

Declared none

**REFERENCES**

1. Iyer C, Kailaspathy K. Effect of co-encapsulation of probiotics with prebiotics on increasing the viability of encapsulated bacteria under *in vitro* acidic and bile salt conditions and in yogurt. J Food Sci 2005;70:M18–23.
2. Parwal M, Pareek R. Probiotic:-a treatment new approach. Asian J Biochem Pharm Res 2011;3:448-54.
3. Kawatra P, Aiyappa C. Probiotics: the undervalued conquerors. Asian J Biochem Pharm Res 2015;8:97-100.
4. Solanki HK, Pawar DD, Shah DA, Prajapati VD, Jani GK, Mulla AM, *et al*. Development of microencapsulation delivery system for long-term preservation of probiotics as biotherapeutics agent. BioMed Res Int 2013;620-719. http://dx.doi.org/10.1155/2013/620719
5. Sekhon BS, Jairath S. Prebiotics, probiotics and synbiotics: an overview. J Pharm Educ Res 2010;1:2.
6. Ziemer CJ, Gibson GR. An overview of probiotics, prebiotics and synbiotics in the functional food concept: perspectives and future strategies. Int Dairy J 1998;8:473-9.
7. Gibson GR. Fibre and effects on probiotics (the prebiotic concept). Clin Nutr Suppl 2004;1:25–31.
8. Guarner F, Khan AG, Garisch J, Eliakim R, Gangl A, Thomson A, *et al*. World gastroenterology organisation practice guidelines: probiotics and prebiotics. Arab J Gastroent 2009;10:33–42.
9. Tripathi MK, Giri SK. Probiotic functional foods: survival of probiotics during processing and storage. J Func Foods 2014;9:225-41.
10. Prisco AD, Mauriello G. Probiotic action of foods: a focus on microencapsulation tool. Trends Food Sci Technol 2016;48:27-39.
11. Kailasapathy K. Microencapsulation of probiotic bacteria: technology and potential applications. Curr Issues Intest Microbiol 2002;3:39-48.
12. Krasaekoopt W, Bhandari B, Deeth H. Evaluation of encapsulation techniques of probiotics for yogurt. Int Dairy J 2003;13:3-13.
13. Anal AK, Singh H. Recent advances in microencapsulation of probiotics for industrial applications and targeted delivery. Trends Food Sci Technol 2007;18:240-51.
14. Chavarri M, Maranon I, Villaran MC. Encapsulation technology to protect probiotic bacteria. Bioprocess and Preserv Area 2012;501-29. Doi:10.5772/50046.
15. Banerjee D, Chowdhury R, Bhattacharya P. The prebiotic influence of inulin on growth rate and antibiotic sensitivity of *Lactobacillus casei*. Int J Pharm Pharm Sci 2016;8:181-4.
16. Samanta (Koruri) S, Banerjee D, Chowdhury R, Bhattacharya P. Studies on prebiotic food additive (inulin) in Indian dietary fibre sources garlic (*Allium sativum*), wheat(*Triticum spp.*),oat(*Avena sativa*) and dalia(*Bulgur*). Int J Pharm Pharm Sci 2014;6:278-82.
17. Chan ES, Wong SL, Lee PP, Lee JS, Ti TB, Zhang Z, *et al.* Effects of starch filler on the physical properties of lyophilized calcium–alginate beads and the viability of encapsulated cells. Carbohydr Polym 2011;83:225–32.
18. Yoo IK, Seong GH, Chang HN, Park JK. Encapsulation of *Lactobacillus casei* cells in liquid-core alginate capsules for lactic acid production. Enzyme Microbiol Technol 1996; 19:426-33.
19. Song H, Yua W, Gaoa M, Liub X, Maa X. Microencapsulated probiotics using emulsification technique coupled with internal or external gelation process. Carbohydr Polym 2013;96:181–9.
20. Ribeiro AJ, Silva C, Ferreira D, Veiga F. Chitosan-reinforced alginate microspheres obtained through the emulsification/ internal gelation technique. Eur J Pharm Sci 2005;25:31–40.
21. Sultana K, Godward G, Reynolds N, Arumugaswamy R, Peiris P, Kailasapathy K. Encapsulation of probiotic bacteria with alginate–starch and evaluation of survival in simulated gastrointestinal conditions and in yoghurt. Int J Food Microbiol 2000;62:47–55.
22. Martin MJ, Villoslada FL, Ruiz MA, Morales ME. Effect of unmodified starch on the viability of alginate-encapsulated *Lactobacillus fermentum* CECT5716. LWT-Food Sci Technol 2013;53:480-6.

**How to cite this article**

- Debolina Banerjee, Puja Bag, Ranjana Chowdhury, Pinaki Bhattacharya. Sustainability of the probiotic *Lactobacillus casei* in fortified Indian milk cakes under different preservation conditions-effects of co-immobilization of *l. Casei* and commercial prebiotic inulin (chicory based) and millet inulin. Int J Pharm Pharm Sci 2017;9(1):152-157.

International Journal of Pharmacy and Pharmaceutical Sciences

ISSN- 0975-1491 Vol 8, Issue 4, 2016

Original Article

# THE PREBIOTIC INFLUENCE OF INULIN ON GROWTH RATE AND ANTIBIOTIC SENSITIVITY OF *LACTOBACILLUS CASEI*

**DEBOLINA BANERJEE[1], RANJANA CHOWDHURY[1*], PINAKI BHATTACHARYA[2]**

**[1]Chemical Engineering Department, Jadavpur University, Kolkata 700032, India, [2]Chemical Engineering Department, Heritage Institute of Technology, Kolkata 700107, India**
**Email: ranjana.juchem@gmail.com**

***Received: 04 Dec 2015 Revised and Accepted: 18 Feb 2016***

**ABSTRACT**

**Objective:** This research study is focused on the prebiotic effect of inulin on the antibiotic sensitivity of *Lactobacillus casei* and on the determination of functionality of specific growth rate (μ) of the probiotic bacteria on the concentrations of lactose ($C_L$ = 10-30 g/l) and inulin ($C_I$ = 0.164-0.625 g/l) along with the optimization of growth condition through Response Surface Methodology (RSM).

**Methods:** The sensitivity of *Lactobacillus casei* towards norfloxacin was determined using well diffusion method. Using the initial values of μ ($h^{-1}$) of *Lactobacillus casei* at different values of $C_L$ (g/l) and $C_I$ (g/l), the functionality of μ on the concentrations of the carbon sources have been derived, and the optimum condition has been identified.

**Results:** Although *Lactobacillus casei* is sensitive to norfloxacin, resistance is developed in the presence of inulin. Quadratic model equation $\mu = 0.83+0.054*C_L-0.035*C_I-0.049*C_L*C_I-0.29*C_L^2-0.33*C_I^2$ is valid and the optimum value of specific growth rate is 0.8285 $h^{-1}$ at $C_L$ = 20 g/l and $C_I$ = 0.32 g/l.

**Conclusion:** The interesting observation of the development of antibiotic resistance of *Lactobacillus casei* in the presence of inulin suggests that the intake of probiotic *Lactobacillus casei*, may be done along with prebiotic inulin when a patient is treated with antibiotics like norfloxacin. Moreover, the model equation correlating the functionality of growth rate of *Lactobacillus casei* on lactose and inulin will be helpful in fortifying the probiotic milk products and drugs with prebiotics like inulin.

**Keywords:** *Lactobacillus casei*, Prebiotic, Inulin, Antibiotic sensitivity, Statistical growth model, Optimization of specific growth rate, Response Surface Methodology



## INTRODUCTION

Synergistic combinations of probiotic bacteria and prebiotic carbohydrates are new concepts in food processing [1]. Prebiotic bio-molecules enhance the growth rate of probiotics which in turn act against pathogenic bacteria through the secretion of bacteriocin [2]. Thus, prebiotics may be combined with dairy products namely yoghurt etc. which contain an array of probiotic bacteria [3]. Among different probiotics, inulin, a fructan consisting of glycosidic bonds like fructosyl-fructose with a terminal glucose unit is one of the most popular one [4-7]. Ideally, the prebiotic molecule should not support the growth of pathogens. Although the synbiotic combinations of suitable probiotic-prebiotic pairs are expected to be beneficial for human health, the assessment should be made about the sensitivity of the combinations of broad spectrum antibiotics. The genus *Lactobacillus* (probiotic bacteria) is inherently resistant to tetracycline, vancomycin, erythromycin, streptomycin, clindamycin, gentamicin and oxacillin, but, there may exist some broad spectrum antibiotics against which probiotic strains are susceptible [8].

The combination with prebiotic may also influence the sensitivity of probiotics. No such study elucidating this fact is reported in the literature. Therefore, addressing all these issues regarding the application of synbiotics in food processing, the following novel objectives have been set in the present research by selecting the combination of *Lactobacillus casei (L. casei)* and inulin as the probiotic bacteria and prebiotic biomolecule respectively. These are determination of the sensitivity of *Lactobacillus casei* against a broad spectrum antibiotic, namely, norfloxacin with and without inulin; determination of a statistical growth model; optimization of the growth of *Lactobacillus casei* against the concentration of inulin and lactose using Response Surface Methodology. The functionality of specific growth rate of *Lactobacillus casei* on the concentration of carbohydrate sources, determined for the first time in this report, is very important for the overall production of the microorganism as well as for the metabolic products generated during growth.

## MATERIALS AND METHODS

### Chemicals

Beef extract (10 g/l) (Merck, India), Yeast extract (5 g/l), Peptone (10 g/l) (Himedia, India), Sodium acetate (5 g/l) (Himedia, India), dipotassium hydrogen phosphate (2 g/l) (Himedia, India), Tri-ammonium citrate (2 g/l) (Himedia, India), Magnesium sulphate (0.05 g/l) (Himedia, India), Manganese sulphate (0.05 g/l) (Himedia, India) and Lactose (20 g/l) (Merck, India) were used in the present research study. These are the composition of Modified de-Man Rogosa Sharp (MMRS) medium. Glucose is used as carbon source for standard de-Man Rogosa Sharp (MRS) medium.

### Microorganisms

*Lactobacillus casei* (2651 1951 RPK) culture purchased from NCIM, Pune were used.

### Prebiotic

Food grade commercial inulin purchased from Himedia, India.

### Pre-adaptation of culture

Adaptation of the strain to Modified de-Man Rogosa Sharp (MMRS) medium containing a high concentration of lactose (50 g/l) and inulin (50 g/l) was performed by repetitive subculturing for three times. The pre-culture process was conducted in an incubator at 37 °C using 250 mL Erlenmeyer flasks for 18 h, based on sufficient growth ($5x10^{10}$cfu/ml).

The cell from the last adaptation experiment was stored at 4°C for use in further experiments. The pre-adaptation is needed to activate the culture at the high level of concentrations of lactose and inulin exceeding the upper limits of present concentration ranges for the two carbohydrate sources and to maintain the culture in the exponential phase.

**Kinetic study**

Batch experiments were conducted to determine the kinetics of growth of *Lactobacillus casei* using both carbohydrate sources, namely inulin and lactose simultaneously by varying the initial concentration of lactose, i.e., 10 g/l, 20 g/l, 30 g/l at each initial inulin concentration of 0.00 g/l, 0.164 g/l, 0.32 g/l and 0.63 g/l.

**Optimization study**

The multivariate functionality of $\mu_{ave}$ (average specific growth rate) on two independent variables has been determined by conducting 13 batch mode experiments using the Central Composite Design (CCD) technique to set the experimental conditions.

**Experimental design and optimization**

The Face-centered Central Composite Design (FCCD) was created by entering the factors viz. the concentration of lactose and concentration of inulin in terms of ±1 levels to perform RSM using Design-Expert 8.1 (Stat-Ease, Inc., Minneapolis, USA). The experiments were conducted randomly to avoid systematic biasness. Accordingly, a design layout was created using 13 experimental runs with six center points. In a typical experimental run, all the operating variables were pre-set at a predetermined design value as per the experimental design layout shown in table 1. In order to investigate the effects of individual parameters as well as their interactive effects on the response variable, a general second order polynomial response surface model was selected and is expressed by Eq. (1):

$$Y_k = b_{k0} + \sum_{i=1} b_{ki}X_i + \sum_{i=1} b_{kii}X_i^2 + \sum_{i=1}\sum_{j=1} b_{kij}X_iX_j \text{---------- (1)}$$

Where, $Y_k$ is response variable, $b_{k0}$ is a constant intercept, $b_{ki}$, $b_{kii}$ and $b_{ij}$ are the linear, quadratic and interaction regression coefficients respectively. $X_i$ and $X_j$ represent the coded values of the process variables (factors). The regression Eq. (2) was considered for multiple simultaneous optimizations in order to maximize $Y_k$ using the numerical optimization program of the same Design Expert software.

**Antibiotic sensitivity**

The sensitivity of the selected microorganisms, *Lactobacillus casei* towards a common antibiotic namely, norfloxacin was also tested using well diffusion method. MRS agar plate containing 20 g/l glucose and MMRS agar plate containing 20 g/l glucose and commercial inulin each was prepared. In each plate 0.1 g norfloxacin was applied in the well. The *Lactobacillus casei* cell culture was spread with a glass spreader. The plates were kept in an incubator at 37 °C for 24 h.

**Scanning electron microscopy (SEM)**

SEM analysis of 24 h batch culture of *Lactobacillus casei* using two types of MMRS media, one containing 20 g/l lactose and the other containing 20 g/l lactose and 20 g/l commercial inulin, were used to observe the morphological changes of the bacterial cell.

**RESULTS AND DISCUSSION**

**Growth pattern of *Lactobacillus casei* in presence of inulin**

Time histories of cell concentration have been plotted in fig.1 with inulin (0-0.624 g/l) as a parameter at initial lactose concentration of 20 g/l. From the analysis of the figure, it is evident that the cell concentration at any reaction time increase with the increase of inulin concentration and passes through the maximum at the inulin concentration of 0.32 g/l. Same trends have also been obtained for other concentrations of lactose (not shown).

From fig.1 it is evident that at 0.624 g/l inulin concentration specific growth rate of *Lactobacillus casei* is lower than at 0.32 g/l inulin concentration due to substrate inhibition. As inulin is a heavier molecule, even the enzymatic conversion to its monomers is slow and hence there is an accumulation of inulin on outer cell wall at high concentration levels. Due to the formation of a thick layer, a mass transfer limitation may creep into the system.

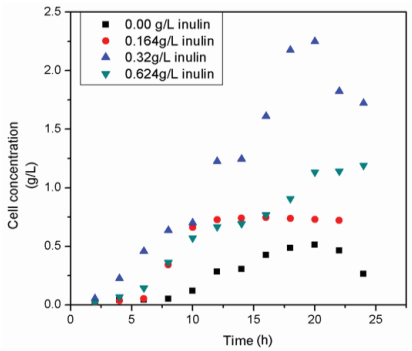



**Fig. 1: Growth curves of *Lactobacillus casei* for 20 g/l lactose concentration using concentrations of inulin as a parameter. ■: without inulin, ●:0.164 g/l inulin, ▲: 0.32 g/l inulin ▼ :0.625 g/l inulin**

**Dependence of specific growth rate (μ) on concentrations of lactose and inulin**

According to the standard MRS medium composition for lactic acid bacteria, carbohydrate concentration is used 20 g/l. Under this study lactose was used as the carbohydrate source. In order to carry out the optimization of specific growth rate (μ) through Response Surface Methodology, the range of concentration of lactose ($C_L$= 10-30 g/l) was chosen around the standard carbohydrate concentration value (20 g/l) of MRS medium. From preliminary observation, it was seen that inulin has inhibitory effect at high concentrations and hence a low concentration range was chosen for inulin under the present study. The values of μ obtained at different values of concentration of lactose ($C_{L)}$ and concentration of inulin ($C_I$) have been shown in table 1.

In accordance with the statistical analysis model fit summary (table 2), a quadratic model was selected as the best fitted with lower standard deviation (0.060) and lower PRESS value (0.18), predicted residuals sum of squares (1.27), higher adjusted $R^2$ (0.9666), regression coefficient (0.9108), predicted $R^2$ regression coefficient (0.8613) and adequate precision in comparison to linear and 2FI, i.e., Two-Factor Interaction model. The data obtained from ANOVA, "analysis of variance" (table 3), for quadratic model shows an insignificant lack of fit (sum of squares = 1.27>0.05), larger F value (Fischer test value) (70.39), high $R^2$value (0.9805) and low C. V., i. e, "coefficient of variance" value (0.89). The model equation showing the dependence of specific growth rate simultaneously on initial concentration of lactose and inulin is as follows:

$$\mu = 0.83+0.054*C_L-0.035*C_I.0.049*C_L*C_I-0.29*C_L2-0.33*C_I^2\text{----------} \quad (2)$$

Where $C_L$ and $C_I$ are the concentrations of lactose and inulin and μ (R1) is specific growth rate. From Eq. 2, it is evident that the factors $C_L$, $C_I$ and $C_L^2$ affected the change in weight of the sample positively.

**Optimization**

In order to understand the effect of lactose concentration and inulin concentration on specific cell growth rate, the result obtained by response surface methodology technique have been presented in three-dimensional plots shown in fig.2a. It is observed that the maximum specific growth rate of *Lactobacillus casei* is obtained at 20 g/l of lactose concentration and 0.32 g/l of inulin concentration. The contour plot shown in fig.2b clearly confirms the optimum values of the response variables. Solutions for optimal conditions of specific growth rate are shown in table 4.

**Effect of inulin on antibiotic sensitivity**

The photographs of petri-plates of *Lactobacillus casei*, used for the determination of antibiotic (norfloxacin) sensitivity through well diffusion technique with and without inulin are shown in fig.3a and fig.3b. By the measurement of zone of inhibition it is clear that while it measures 0.055 m in absence of inulin, no inhibition zone is observed in

the presence of inulin. Thus, it is revealed that while the strain of *Lactobacillus casei* used in the present investigation is inherently susceptible towards norfloxacin, resistance to the antibiotic is developed in the presence of inulin. The results signify that norfloxacin act against the *Lactobacillus casei* strain used under the present investigation. The antibacterial effect of norfloxacin, a fluoroquinolone, may be through the inhibitory action on the enzyme DNA gyrase, essential for replication, repair and transcription of DNA and topoisomerase essential for entangling DNA of the bacteria under attack [9].

However, the exact mechanism may be determined by further investigation. The development of resistance in the presence of inulin may be due to the formation of a coating of inulin around the cell wall as observed (fig. 3b) in the case of growth of the same microorganism on lactose and inulin.

**Table 1: Experimental design matrix**

| S. No. | $C_L$ g/l | $C_I$ g/l | $C_L$ g/l | $C_I$ g/l | Response (μ) ($h^{-1}$) |
|---|---|---|---|---|---|
| 1 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 2 | 30.00 | 0.62 | +1 | +1 | 0.092 |
| 3 | 10.00 | 0.62 | -1 | +1 | 0.1823 |
| 4 | 20.00 | 0.75 | 0 | +1 | 0.151 |
| 5 | 20.00 | 0.13 | 0 | -1 | 0.2173 |
| 6 | 5.9 | 0.32 | -1 | 0 | 0.12 |
| 7 | 20.00 | 0.31 | 0 | 0 | 0.8285 |
| 8 | 30.00 | 0.00 | +1 | -1 | 0.283 |
| 9 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 10 | 10.00 | 0.00 | -1 | -1 | 0.1787 |
| 11 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 12 | 20.00 | 0.32 | 0 | 0 | 0.8285 |
| 13 | 30.41 | 0.32 | +1 | 0 | 0.4143 |

Here, $C_L$= Concentration of lactose, $C_I$= Concentration of inulin, μ = Specific growth rate.

**Table 2: Model Fit summary statistics for final specific growth rate**

| | |
|---|---|
| Std. Dev. | 0.060 |
| Mean | 0.44 |
| C. V.% | 13.38 |
| PRESS | 0.18 |
| R-Squared | 0.9805 |
| AdjR Squared | 0.9666 |
| PredR-Squared | 17.684 |

Here, C. V. = Coefficient of variance, The Analysis of Variance (ANOVA) table is shown in table 3.

**Table 3: Analysis of variance (ANOVA) for response surface quadratic model**

| Source | Sum of squars | df | Mean square | F value | p-value Prob>F |
|---|---|---|---|---|---|
| Model | 1.25 | 5 | 0.25 | 70.39 | <0.001(significant) |
| $C_L$ | 0.023 | 1 | 0.023 | 6.53 | 0.0378 |
| $C_I$ | 9.882E-003 | 1 | 9.882E-003 | 2.79 | 0.1387 |
| $C_LC_I$ | 9.467E-003 | 1 | 9.467E-003 | 2.67 | 0.1460 |
| *$C_L2$* | *0.59* | 1 | 0.59 | 166.47 | <0.0001 |
| *$C_I2$* | *0.77* | 1 | 0.77 | 217.31 | <0.0001 |
| Residual | 0.025 | 7 | 3.541E-003 | | |
| *Lack of Fit* | 0.025 | 3 | 8.262E-003 | | |
| Pure Error | 0.000 | 4 | 0.000 | | |
| Cor total | 1.27 | 12 | | | |

Here, F value= Fischer test value, p-value= lack of fit value

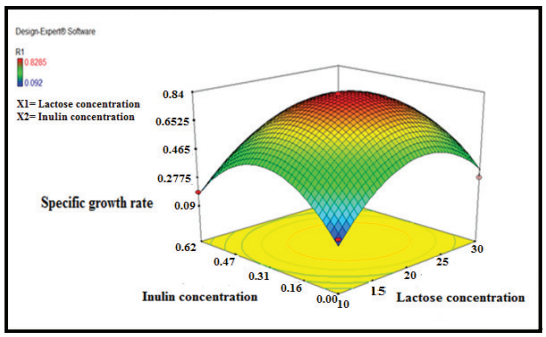



**Fig. 2a: Three-dimensional plots for optimization of specific growth rate of *Lactobacillus casei*. Here R1= Response (μ or specific growth rate)**

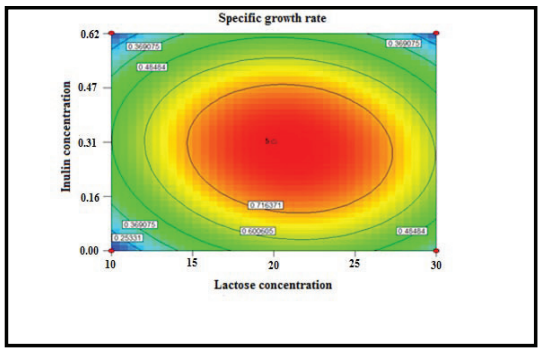



**Fig. 2b: Contour plot for optimization of specific growth rate of *Lactobacillus casei***

**Table 4: Solutions for optimal conditions of specific growth rate**

| Number | Lactose concentration ($C_L$) | Inulin concentration ($C_I$) | Desirability |
|---|---|---|---|
| 1 | 20.00 | 0.32 | 1.0 (selected) |
| 2 | 10.00 | 0.62 | 0.909 |
| 3 | 10.00 | 0.00 | 0.909 |

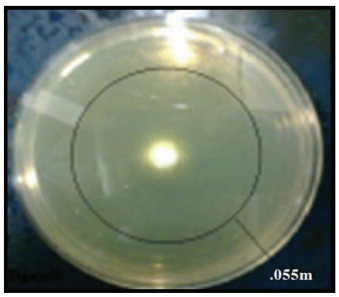



**Fig. 3a: Effect of norfloxacin on *Lactobacillus casei* in de-Man Rogosa Sharp (MRS) agar plate. The marked area indicates the zone of inhibition (.055 m)**

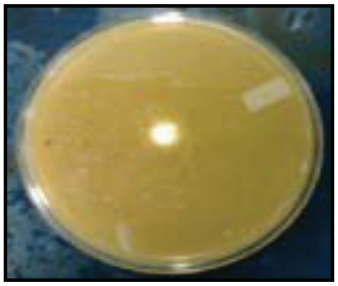


**Fig. 3b: Effect of norfloxacin on *Lactobacillus casei* in Modified de-Man Rogosa Sharp (MMRS) agar plate in presence of inulin**

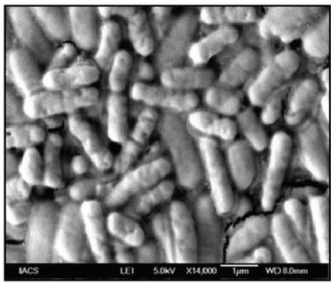


**Fig. 4a: SEM image of *Lactobacillus casei* cell without inulin**

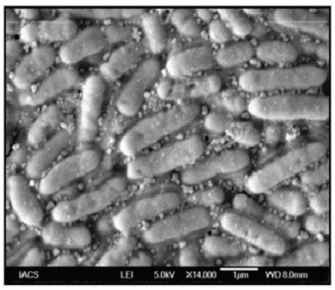


**Fig. 4b: SEM image of *Lactobacillus casei* cell in presence of inulin**

**Scanning electron microscopy image**

The SEM images (fig. 4a and 4b) of *Lactobacillus casei* grown on lactose and on lactose-inulin mixture respectively reveal that in the presence of inulin, there is a formation of a distinct layer of inulin over the cell wall. This may be due to the fact that inulin, being a large molecule cannot be transported inside the cells, and they are decomposed enzymatically to glucose and fructose which are in turn assimilated by *Lactobacillus casei* [10].

**CONCLUSION**

From the experimental findings, it is ascertained that the presence of inulin imparts resistance in *Lactobacillus casei* against norfloxacin, a broad spectrum antibiotic. Thus, inulin may be mixed with dairy products using *Lactobacillus casei* to avoid the destruction of probiotic cells in the human body by the antibiotic. From response surface methodology, it is observed that the specific growth rate is maximum (0.8285 $h^{-1}$) at concentrations of lactose and inulin of 20 g/l and 0.32 g/l respectively.

**ACKNOWLEDGEMENT**

The first author (Debolina Banerjee) acknowledges the financial support offered by Council of Scientific and Industrial Research (CSIR), India by providing Senior Research Fellowship (File number: 9/96(0725)2K12-EMRI).

**CONFLICT OF INTERESTS**

Declare none

**REFERENCES**

1. Gaggìa F, Mattarelli P, Biavati B. Probiotics and prebiotics in animal feeding for safe food production. Int J Food Microbiol 2010;141: S15–28.
2. Saad N, Delattre C, Urdaci M, Schmitter JM, Bressollier P. An overview of the last advances in probiotic and prebiotic field. LWT-Food Sci Technol 2013;50:1-16.
3. Kosin B, Rakshit SK. Microbial and processing criteria for production of probiotics: a review. Food Technol Biotechnol 2006;44:371–9.
4. Manso J, Luz MM, Ya´n˜ez-Seden˜o P, Jose´ Pingarro´n M. Bienzymeamperometric biosensor using gold nanoparticle-modified electrodes for the determination of inulin in foods. Department of Analytical Chemistry, Faculty of Chemistry, University Complutense of Madrid, 28040 Madrid, Spain; 2007.
5. De Souza O, Perego P, de Oliveira MN, Converti A. Effect of inulin as prebiotic and synbiotic interactions between probiotics to improve fermented milk firmness. J Food Eng 2011;107:36–40.
6. Azza A, Abou-Arab, Hala AT, Ferial M, Salem A. Physico-chemical properties of inulin produced from jerusalem artichoke tubers on bench and pilot plant scale. Aust J Basic Appl Sci 2011;5:1297-309.
7. Toneli JTCL, Park KJ, Ramalho JRP, Murr FEX, Fabbro IMD. Rheological characterization of chicory root *(Cichorium intybus*l.) inulin solution. Brazilian J Chem Eng 2008;25:461-71.
8. Ashraf R, Shah NP. Antibiotic resistance of probiotic organisms and safety of probiotic dairy products. Int Food Res J 2011;18:837-53.
9. Nelson DL, Cox MM. Lehninger principles of biochemistry. 4th ed. New York: WH Freeman; 2004.
10. Ricca E, Calabrò V, Curcio S, Iorio G. Fructose production by chicory inulin enzyme hydrolysis: Kinetic study and reaction mechanism. Process Biochem 2009;44:466–71.

Innovare Academic Sciences

International Journal of Pharmacy and Pharmaceutical Sciences
ISSN- 0975-1491 Vol 6, Issue 9, 2014

Original Article

# STUDIES ON PREBIOTIC FOOD ADDITIVE (INULIN) IN INDIAN DIETARY FIBRE SOURCES - GARLIC (*ALLIUM SATIVUM*), WHEAT (*TRITICUM SPP.*), OAT (*AVENA SATIVA*) AND DALIA (BULGUR)

**SHARMISTHA SAMANTA (KORURI)[1], DEBOLINA BANERJEE[1], RANJANA CHOWDHURY[1*], PINAKI BHATTACHARYA[2]**

**Chemical Engineering Department, Jadavpur University, Kolkata 700032, India.**
**Email: ranjana.juchem@gmail.com**

***Received: 15 Jul 2014 Revised and Accepted: 01 Sep 2014***

**ABSTRACT**

**Objective:** In the present investigation inulin has been extracted from dietary fibre rich Indian food stuffs, namely, garlic, wheat, oat and dalia. Inulin in the raw food stuff and in the extract has been assessed qualitatively and quantitatively.

**Methods:** Inulin has been extracted from each food source using a combination of lab-scale chemical processes and unit operations. Qualitative assessment of inulin in different food samples and their extracts has been done using FTIR and TLC and quantitative assessment has been done using high performance liquid chromatography (HPLC) and also through combination of TLC and spectro-photometry.

**Results:** The concentration (on dry weight basis) of inulin in natural prebiotic sources has been determined to be 16.60%, 13.07%, 8.94%, 14.95% for garlic, wheat, oat and dalia respectively. The extraction of inulin from garlic, wheat, oat and dalia was possible up to the extent of 99.46%, 77.94%, 53.31% and 89.15% respectively.

**Conclusion:** It may be concluded that all the food samples, investigated under the study, may serve as potential sources for extraction of prebiotic inulin. The present extraction procedure may be escalated to commercial scale for the production of inulin particularly from garlic for which the efficiency is as high as 99.46%.

**Keywords:** Dietary fibre**,** Prebiotic, Inulin, FTIR, TLC, HPLC.

## INTRODUCTION

Studies on prebiotics and probiotics are recently attracting the attention of scientists and technologists due to immense potential of these two closely related bio-systems. Prebiotics are basically any non-digestible food ingredients that can benefit the human body by stimulating the growth and activity of one or more species of probiotics in the large intestine and produce short chain fatty acids (SCFAs) resulting in a decrease of the colon pH [1]. Prebiotics may be added to food preparations containing probiotics to enhance the stability of food matrix and viability of probiotic cultures [2].

Prebiotics are mostly oligosaccharides in chemical nature. Full-spectrum prebiotics, e. g., oligofructose-enriched inulin (OEI) provide the full range of molecular link lengths from 2-64 links per molecule and nourish bacteria throughout the colon. Inulin has been defined as a polydisperse carbohydrate material consisting mainly, if not exclusively, of β-(2-1) fructosyl-fructose links [3]. A few reports are available, showing the positive effect of inulin on the growth of probiotic organism [4, 5, 2]. Huebner, 2007 reported that inulin enhanced the growth of *L. Casei* [6]. In addition to the inclusion of inulin in yogurt, it is also used as a substitute of fat in low fat emulsions like salad dressings etc [7].

For the fortification of food products with prebiotic, like inulin, the possibility of extraction of the latter from all available natural food grade resources should be investigated. Inulin producing plant species are found in several monocotyledonous and dicotyledonous families, including Liliaceae, Amaryllidaceae, Gramineae, and Compositae [8]. Usually, food stuffs, rich in dietary fibre, serve as prebiotic sources.

In spite of several resources of inulin, only one inulin containing plant species namely Chicory (*Chichorium intybus*) has been used to produce inulin commercially so far, possibly because of technical snags for exploring other resources. It is now established that inulin is naturally available in several fruits and vegetables. Among them garlic, onion, wheat, oat, dalia, banana etc. are some of the common Indian vegetables and fruits which have been reported to be rich in inulin by earlier researchers [9, 10]. It may be noted that no systematic and programmed investigation is available on qualitative and quantitative assessment of inulin in these Indian vegetable stuffs. Although a few scattered information have been reported in the literature review articles [11, 12] on the content of inulin in several Indian vegetables but these are only on piece meal basis and are not suitable for commercial exploitation.

Considering the importance of isolation and purification of inulin from its native source, in the present investigation an attempt has been made to isolate and characterise inulin quantitatively and qualitatively from four candidate materials namely- garlic, oat, wheat, and dalia. Qualitative identification of inulin has been done using Fourier transformed infrared (FTIR) while quantitative determination has been carried out using thin layer chromatography (TLC) followed by spectrophotometry and high performance liquid chromatography (HPLC).

A pure food grade sample of inulin has been used as standard for the purpose of comparison. This investigation presents a programmed study on isolation and characterisation of inulin from four abundantly available food materials.

## MATERIALS AND METHODS

### Materials

Garlic **(*Allium sativum*)**, wheat **(*Triticum spp)*** oat **(*Avena sativa*)** and dalia **(Bulgur)** were purchased from local market. Moisture contents of all prebiotic sources are provided in Table 1. Food grade inulin purchased from Himedia, India, was used. TLC Silica gel G 60 Aluminium sheets 20 x 20 cm (Merck HX 816976, Germany), benzene (Ranbaxy, India), acetic acid(Merck, India), methanol(Merck, India), resorcinol(Merck, India), ethanol (Merck, India), sulphuric acid(Merck, India), HCL (Merck, India), HPLC water (Merck, Germany), Acetonitrile (Merck, Germany), Column: C-18 Phenomenex (250 x 4.6 mm x 5µ) were used.

**Analytical Instruments**

Shimazdu FTIR Spectroscope 8400, Varian UV-visible spectro photometer Cary 50 Bio, Shimadzu Corporation Reverse Phase with RI detector HPLC (Model no: CBM-Ro A) were used.

**Determination of Inulin in Natural Prebiotics**

Inulin in different natural prebiotics was qualitatively and quantitatively determined using thin layer chromatographs (TLC).

**TLC (Thin Layer Chromatography)**

The standard Inulin solutions of different concentrations (5g/L, 10g/L, and 20g/L) were hydrolysed using 5% oxalic acid and heated in boiling water bath for 30 minutes [13]. 1 mL of each inulin solution was hydrolysed with 4 ml oxalic acid. Filtrate obtained from suspensions of aqueous test solutions of raw natural prebiotics at concentration of 20g/L each were hydrolysed using the same protocol. Approximately 10 µl of each hydrolysed sample was applied to spot on the aluminium sheet (TLC Silica gel G 60 Aluminium sheets) by a micropipette (make: Biohit proline 0.5 to 10 µl) and was developed for ~ 3 h in the n-butanol: acetic acid: methanol solvent (4:2:2) [14] mixture in a closed chamber. The mobile phase (solvent) was at the bottom of the tank. The mobile phase gradually moved upward carrying the spotted samples. Once the solvent front moved ~ 2/3 of the sheet, the sheet was removed; the solvent front was marked immediately and dried with a portable dryer. The sheet was sprayed with spraying reagent which had been prepared by mixing 0.2% orcinol in sulphuric acid and was incubated in hot air oven for 15 minutes at 70°C. Dark brown spots appeared for different samples. $R_f$ values (Relative Fronts of the solute and the solvent) were calculated using the following equation.

$$R_f = \frac{\text{Distance moved by the solute}}{\text{Distance moved by solvent}} \quad \ldots\ldots\ldots (1)$$

From the TLC of each sample spot obtained at the same position as inulin, was scraped out and collected in a sample vial containing 10 mL distilled water each and OD values of supernatants were determined with the spectrophotometer at 540 nm, to quantify the concentration of inulin. Standard curve (not shown) generated using OD values of TLC spots corresponding to pure inulin solutions of different concentrations have been used to determine inulin concentration in the natural prebiotic extracts.

**Extraction of Inulin**

The prebiotic native sources, namely, garlic, wheat, oat, and dalia were washed properly with distilled water. In case of garlic, peeling of outer skin was done manually and the core portion was taken for the preparation of paste. 100 g garlic cloves were cut into small pieces and with addition of 150 mL distilled water was blended with Mixer-Grinder. The protocol for chicory reported by Mavumengwana was followed with some modification to extract inulin from garlic, wheat, dalia and oat. The flow sheet showing different operations in the protocol is shown in figure 1.

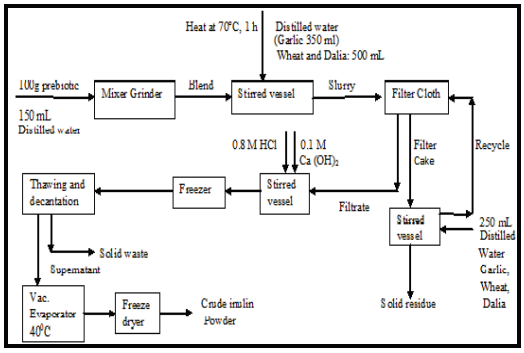



**Fig. 1: Process of inulin extraction from natural prebiotic sources**

According to the protocol, suspensions of the aqueous pastes of different samples were prepared. The volume of distilled water used for the preparation of suspension was 750 mL for all samples except wheat, for which 900 ml water was required. The suspension was heated up to 70°C and was subsequently screened. Heating up to 70$^0$C had been recommended by previous workers [15, 16], to de-activate the inulinase, which might have otherwise led to the conversion of inulin to fructose. The solid cake from the filter cloth was resuspended several times in distilled water and was recycled to the filtration unit. Finally the filtrate was treated with calcium hydroxide to raise the pH up to 8.0. The pH was readjusted to 7.0 by using 0.8 M HCl. After the adjustment of pH, filtrate was frozen at -22°C for 3h and was thawed. The supernatant was evaporated under vacuum to evaporate water and to obtain crude inulin powder. The crude inulin powder obtained from garlic, wheat, dalia and oat were analysed quantitatively and qualitatively using HPLC. Quantity of inulin in the extracts of prebiotic samples was also determined using DNS method. Chemical bonds present in native inulin powder were assayed using FTIR spectroscopy.

**Characterization of Extracted Inulin Powder**

**HPLC (High Performance Liquid Chromatography)**

The extracted inulin powder derived from each natural prebiotic was analysed using HPLC to determine the concentration of inulin. Solutions (1g/ml) of standard inulin and extracted inulin powder of each natural prebiotic sample were prepared in distilled water. Similarly, solutions of pure samples were also made maintaining concentration of 0.2g/mL. The sample was subsequently freeze dried to obtain the solution of 1g/mL. 20 µl of each sample solution was injected through the column using water: acetonitrile (80:20) solvent system with a flow rate of 1 mL/min at column temperature of 25.8°C.

**DNS (3-5 Di-nitro salicylic Acid method)**

1g of natural inulin powder of each prebiotic sample was taken in 5 ml distilled water and 5 ml solution of 4:1 acetonitrile: water was added. This solution was allowed to precipitate. The solvent was decanted and after repeated washing with distilled water the final suspension of 4 mL was divided into two parts. One part was taken directly for fructose determination using DNS (3, 5- Di-nitro salicylic Acid) method and another part was hydrolysed with 5% oxalic acid following the same protocol used in case of TLC analysis was further analysed using DNS [17]. Since extracted inulin powder contents inherently associated sugar molecules, DNS analysis of extracted inulin powder will provide the concentration of sugars associated with inulin powder. On the other hand after hydrolysis of inulin when the sample is subjected to DNS analysis, concentration of sugar obtained from the breakdown of inulin structure will be achieved. The difference of the sugar content between the hydrolysed product and the parent inulin powder will be the fructose content in the inulin. The concentration of pure inulin in the extract inulin powder is obtained by the simple molar ratio relationship given by Mavumengwana.

$$[I] = 162 \times [F]/180 \quad \ldots\ldots\ldots (2)$$

Where [I] = concentration of inulin, g/L and [F] = concentration of fructose obtained from inulin, g/L.

**FTIR (Fourier Transformed Infrared Spectroscopy) Analysis**

In order to verify the presence of inulin in the four selected food materials, under study, FTIR analysis has been carried out to arrive at a conclusive decision. For all the test cases comparison has been done with that of the pure food grade inulin.

**RESULTS AND DISCUSSIONS**

**TLC Analysis**

Results obtained from thin layer chromatogram (Figure not shown) indicate the presence of inulin in each of the four candidate samples taken for study. From TLC experiment $R_f$ values of inulin have been calculated. These values have been compared with the $R_f$ value obtained for standard food grade inulin. Results are shown in Table 1.

**Table 1: $R_f$ values of prebiotic food samples and inulin**

| Samples | $R_f$ Values |
|---|---|
| Inulin | 0.8 |
| Garlic | 0.8 |
| Wheat | 0.9 |
| Oat | 0.92 |
| Daliah | 0.9 |

A quantitative approach has been made using TLC data to find out the content of inulin (weight % dry basis) in each sample. The results are shown in Table 2.

**Table 2: Concentrations of inulin in different prebiotic**

| Natural prebiotics | Quantity of Inulin Extract (powders)(%)(w/w) |
|---|---|
| Garlic | 16.6 |
| Wheat | 13.07 |
| Oat | 8.94 |
| Dalia | 14.95 |

**Indian food materials**

It is evident from the Table that garlic contents the maximum amount of inulin whereas oat content least amount of inulin. Such results are, however, expected. Garlic being fibrous vegetable and containing high fructose should possess high concentration of inulin. On the other hand, oat, a digestible cereal containing the least amount of fructose in it out of the four samples investigated possesses least quantity of inulin.

The OD values obtained by spectrophotometric analysis of aqueous solution of coloured spots on TLC plates have been used for the calculation of percentage of inulin present in the above mentioned natural prebiotic materials. The concentration of inulin in each natural prebiotic source, calculated on dry weight basis, has been provided in Table 2. It is evident that Garlic contains maximum concentration (16.6%) of inulin. Concentration of inulin in garlic is also determined to be 15.69% using HPLC analysis.

**Estimation of Inulin in extract powder by DNS method**

In order to find out the quantity of inulin content (weight % dry basis) in inulin extract powder obtained from different natural prebiotic samples DNS method has been applied. The results obtained from these experiments are shown in Table 3.

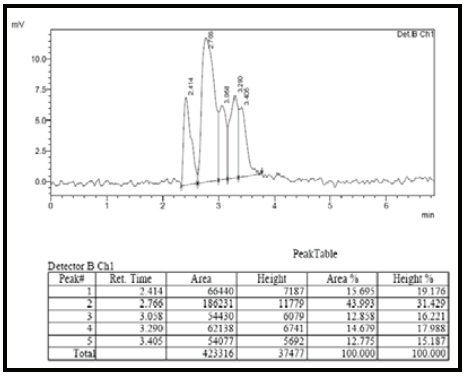
**Table 3: Percentage of inulin content in the inulin extracts powder**

| Natural prebiotic sample | Fructose content, g/L | Inulin content, g/L (from Equation 2) | Percentage inulin in extract powder |
|---|---|---|---|
| Garlic | 276.3 | 248.67 | 99.46 |
| Wheat | 216.5 | 194.85 | 77.94 |
| Oat | 148.08 | 133.28 | 53.31 |
| Dalia | 247.64 | 222.88 | 89.15 |

From the table it also appears that the purity of inulin obtained from garlic is the maximum compared with the other prebiotic samples. Since extracted inulin is a mixture of pure inulin and associated sugars, it may appear that the quantity of extracted inulin is greater than the quantity of pure inulin present in the native source. The difference in quantity of extracted inulin and inulin actually present is minimum in case of garlic because of very low content of other carbohydrate present in it. However, for other samples studied in the present investigation an appreciable difference is noticed in this regard.

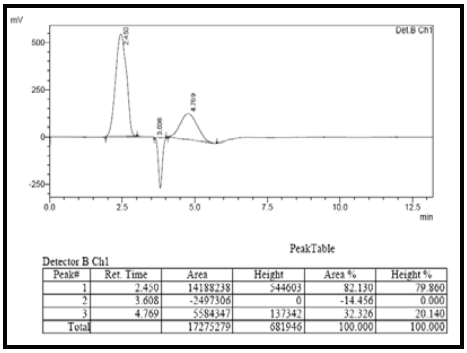
**HPLC**

In order to ascertain the presence of inulin in garlic a qualitative experiment has been performed using HPLC technique. The chromatogram attached (Figure 2 and 3) for raw garlic sample and the standard inulin powder (obtained from market) clearly indicates the peak corresponding to retention time (RT) 2.45 minutes for standard inulin and RT 2.414 minutes for garlic sample. Evidently this close resemblance of RT is a qualitative indication of the presence of inulin. The chromatogram data when processed shows a quantitative content of inulin of 15.7% by weight dry basis in the garlic sample while for standard inulin powder this value is 82.13 %. Similar observations were made for other prebiotic samples, viz., wheat, oat and dalia used for the present investigation. It may be noted that the result obtained from HPLC is comparable to that from TLC.




| Peak# | Ret. Time | Area | Height | Area % | Height % |
|---|---|---|---|---|---|
| 1 | 2.414 | 66440 | 7187 | 15.695 | 19.176 |
| 2 | 2.766 | 186231 | 11779 | 43.993 | 31.429 |
| 3 | 3.038 | 54430 | 6079 | 12.858 | 16.221 |
| 4 | 3.290 | 62138 | 6741 | 14.679 | 17.988 |
| 5 | 3.405 | 54077 | 5692 | 12.775 | 15.187 |
| Total | | 423316 | 37477 | 100.000 | 100.000 |

**Fig. 2: HPLC chromatogram of garlic**




| Peak# | Ret. Time | Area | Height | Area % | Height % |
|---|---|---|---|---|---|
| 1 | 2.450 | 14188238 | 544603 | 82.130 | 79.869 |
| 2 | 3.608 | -2497306 | 0 | -14.456 | 0.000 |
| 3 | 4.769 | 5584347 | 137342 | 32.326 | 20.140 |
| Total | | 17275279 | 681946 | 100.000 | 100.000 |

**Fig. 3: HPLC chromatogram of inulin**

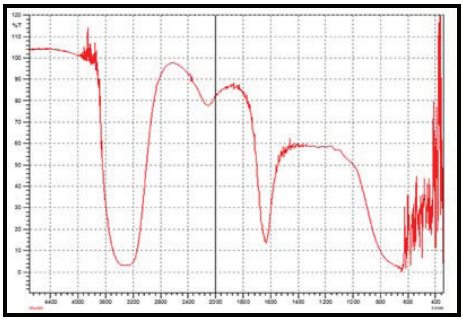


**Fig. 4: FTIR chromatogram of inulin**

**Qualitative analysis of prebiotic samples for their inulin content using FTIR**

Although TLC, HPLC and DNS analyses of the prebiotic samples used in the present investigation have confirmed the presence of inulin in

them, in order to take a conclusive decision FTIR analysis of the samples has been carried out. The FTIR chromatogram of the standard food grade inulin sample (used for comparison purpose) and four other samples are shown in figures 4 and 5 respectively.

By comparing above two figures the distributions of chemical bonds in the standard sample and the sample under study have been evaluated and the results are shown in Table 4.

On closure examination it reveals that in each of the candidate food materials inulin is present. A careful qualitative comparison also shows that chemical composition of garlic extract is almost similar to the standard inulin throughout the wavelength scanned (Table 4 and figure 5). This indicates that the garlic is enriched with inulin and is a potential source of the same. On the other hand chromatograms of wheat, oat and dalia have shown the similarity to the inulin less than that of garlic. A close comparison of chromatograms (figures 4 and 5 and table 4) evidently indicates that the centroid of the main maximum of pure inulin sample and the hydrolysed garlic sample fall on the same wavelength between 3240.41 to 3348.42 cm-1. Therefore it may be stated that garlic is highly enriched in inulin. The centroid of the wheat lies higher than that in the spectrum of inulin about 40 $cm^{-1}$, whereas the centroid of oat and dalia shift to about 31 $cm^{-1}$ higher than that of inulin in each individual case (figures 4 and 5 and table 4). This may be due to the influence of different interfering ions present in these food stuffs [23]. The mass fractions of inulin in wheat, oat and dalia are also appreciable.

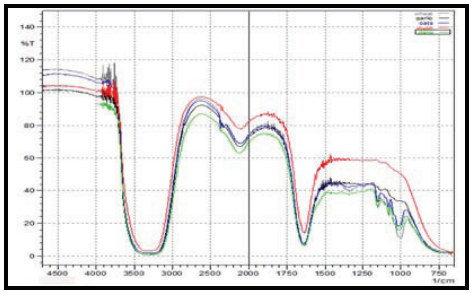



**Fig. 5: FTIR chromatogram comparison between inulin, garlic, wheat, oat and dalia**

**Table 4: Wavelength numbers of prebiotic samples**

| FTIR wavelength numbers | | | | | Range of functional groups |
|---|---|---|---|---|---|
| **Inulin** | **Garlic** | **Wheat** | **Oat** | **Dalia** | |
| 1633.71 | 1633.71 | 1633.71 | 1635.64 | 1633.71 | (1620-1680) –C=C- Alkenes [18] (1638.84) C=O [19] |
| 1965.46 | 1965.46 | 1963.53 | 1905.67 | 1869.02 | (1908-1960) O-H stretch first overtone, C=O stretch second overtone, O-H stretch/O-H bend combination |
| 1988.61 | 1988.61 | 1988.61 | 1940.39 | 1909.53 | (1980) Asym N-H stretch/N-H in plane bend; C-N stretch combination [20] |
| 2015.61 | 2015.61 | 2015.61 | ---- | 1944.25 | |
| 2038.76 | 2036.83 | 2038.76 | ---- | 1967.39 | |
| 2061.90 | 2061.90 | 2061.90 | ---- | 1990.54 | |
| 2086.98 | 2088.91 | 2086.98 | ---- | 2115.91 | (2100-2260) -C≡C- stretch in alkynes, -C≡N- stretch in Nitrile, OH bond, C-O stretch [21] |
| 2104.34 | 2100.48 | 2100.48 | 2104.34 | ---- | |
| 2351.23 | 2353.16 | 2358.94 | 2358.94 | 2358.94 | |
| 2389.80 | ---- | 2328.08 | 2330.01 | ---- | |
| 3240.41 | 3240.41 | 3278.99 | 3271.27 | 3271.27 | (3200-3500) O-H [22] |
| 3290.56 | 3290.56 | --- | --- | --- | |

## CONCLUSION

The present investigation on isolation of prebiotic from different natural sources reveals that the Indian food samples, namely garlic, wheat, oat and dalia contain the prebiotic inulin. The concentration of inulin in each food component has been determined using the combination of TLC supported by spectrophotometric methods and HPLC. The extraction of inulin was also performed for all prebiotic food samples under study. The inulin in extract was analysed qualitatively and quantitatively using HPLC and DNS methods. The purity of inulin extract is 99.46 % in case of garlic and this is the maximum value obtained from the samples studied in the present investigation. The powder was characterised regarding the distribution of chemical bonds using FTIR and was satisfactorily compared with standard inulin. The flow chart used for the purpose of extraction of inulin is a commercially viable process. The evidence of high concentration of inulin in these food stuffs also supports the daily intake of them for the maintenance of good health.

## ACKNOWLEDGMENTS

The first author acknowledges the financial support offered by the University Grant Commission of India for UGC-BSR fellowship.

The second author acknowledges the financial support offered by Council of Scientific and Industrial Research for providing SRF.

## REFERENCES

1. Vankerckhoven VV, Autgaerden TV, Huys G, Vancanneyt M, Swings J, *et al*. Establishment of the Prosafe Collection of Probiotic and Human Lactic acid Bacteria. Microb Ecol Infect Dis 2004;16:131-6.
2. Boeni S, Pourahmad R. Use of inulin and probiotic Lactobacilli in synbiotic yogurt production. Ann Biol Res 2012;3(7):3486-91.
3. Arihara RK, Ota HR, Itol M, Sameshima Y, Yamanaka H, Akimoto M, Kanai S, et al. Lactobacillus acidophilus group Lactic-acid bacteria applied to meat fermentation. J Food Sci 1998;63(3): 544-7.
4. Oliveira RP DS, Perego P, Converti A, Oliveira MND. Effect of inulin on growth and acidification performance of different probiotic bacteria in co-cultures and mixed culture with Streptococcus thermophilus. J Food Engineering 2009;91(1):133-9.
5. Rodrigues D, Teresa AP, Santos R, Pereira CI, Gomes AM, Malcata FX, *et al*. The potential effect of FOS and inulin upon probiotic bacterium performance in curdled milk matrices. LWT Food Sci Technology 2011;44(1):100-8.
6. Huebner J, Wehling RL, Hutkins RW. Functional Activity of commercial probiotics. Int Dairy J 2007;17:770-5.
7. Mantzouridou F, Spanou A, Kiosseoglou V. An Inulin-based dressing emulsion as a potential probiotic food carrier. Food Res Int 2012;46:260-9.
8. Parada JL, Caron CR, Adriane BP, Medeiros, Soccol CR. Bacteriocins from Lactic acid-bacteria: Purification. Brazilian Archives Biology and Technology 2002;522.
9. Roberfroid MB. Inulin-type fructans: functional food ingredients. J Nut 2007;137Suppl 11:2493S-502S.
10. Abrams S, Griffin I, Hawthome K, Liang L, Gunn S, Darlington G, *et al*. A combination of prebiotic short-and long-chain inulin-type fructans enhances calcium absorption and bone

mineralization in young adolescents. Am J Clin Nutr 2005;82(2):471–6.
11. Kaur N, Gupta AK. Applications of inulin and oligofructose in health and nutrition: review. J Bio Sci 2002;27:703–14.
12. Tungland BC. Inulin: a comprehensive scientificreview. http: //members.shaw.ca/duncancrow/inulinreview.html. 2000.
13. Simonovska B. Determination of Inulin in Foods. J AOAC Int 2000;83:3.
14. Teris A VB, Paul M, Bonnie MK, Edith L, Alphons GJ, Voragen, Aede DG. Bitter sesquiterpene lactones from chicory roots. J Agric Food Chem 1990;38:1035-8.
15. Druart N, De Roover J, Van den Ende W, Goupil P, Van Laere A, Rambour S. Sucrose assimilation during early developmental stages of chicory (Chicorium intybus L.) plants. Planta 2001;212:436-43.
16. Mavumengwana VB. Isolation Purification and Characterisation of inulin and fructo-oligosaccharides from Chicorium intybus and inulinase from Aspergillus niger. Thesis for the degree of M. Sc. Department of Biochemistry, Microbiology and Biotechnology; Rhods University: 2004.
17. Singh Marmit K, Sharma SL. Quantitative estimation of some metabolites and enzymes in insect induced leaf galls of Mangifera indica. Asian J Exp Sci 2008;22(3):343-6.
18. www.senseair.se/senseair/gases-applications/hydrocarbons.
19. Cardenas GT, Salinas S. TGA-FTIR of PdSn Fine solid supported on MgO. cyclohexene reduction. J Chilean Chem Soc 2004;49(3):267-71.
20. Burn DA, Qurczak EW (Eds). Handbook of Near infrared Analysis. Practical Spectroscopy series, Marcel Dekker, Inc, NewYork, 1992;13:393-5.
21. wikis.lawrence.edu/display/chem.
22. Dinixchemy.Jumatdinintahyontahdakblogspot.in/2012/12/v, 2012.
23. Milorad D, Cakic, Goran SN, Ljubomir A I. FTIR Spectra of Iron (III) complexes with dextran, pullulan and inulin oligomers. Bull Chem Technol Maced 2002;21(2):135-46.

# Bioprocessing INDIA 2014

**Jointly Organized by**
**Institute of Chemical Technology, Mumbai**
**&**
**Indian Institute of Technology Bombay, Mumbai**
December 17-20, 2014
Mumbai, India


# *Certificate of Participation*

*This is to certify that*

*Dr./Mr./Ms.* **Debolina Banerjee**
*has presented a* ***research paper*** *titled*

**'Immobilization of *L. casei* using Entrapment and Microencapsulation using Internal and External Gelation --- A comparative study'**

*in 'Bioprocessing INDIA 2014' held at*
*Institute of Chemical Technology, Mumbai-400019.*

| **Prof. A. M. Lali** | **Prof. K. V. Venkatesh** | **Dr. S. B. Kale** |
|---|---|---|
| Conference Chair | Conference Chair | Chair and Convener |

INDIAN CHEMICAL ENGINEERING CONGRESS

# CHEMCON 2014

**67th Annual Session of Indian Institute of Chemical Engineers (IIChE)**

***International Conference on***

**CHEMICAL ENGINEERING - EMERGING DIMENSIONS AND CHALLENGES AHEAD**

**&**

***Indo Japanese Symposium on***

**SEPARATION TECHNOLOGY FOR GREEN ENVIRONMENT**

December 27 - 30, 2014

*Organized by*

**Chandigarh Regional Centre,**
**Indian Institute of Chemical Engineers**

*In Collaboration with*

**Dr. SSB University Institute of Chemical Engineering & Technology, Panjab University, Chandigarh**

# Certificate of Participation

This Certificate is being awarded to Prof./Mr/Ms *Debolina Banerjee*

of **Jadavpur University, Jadavpur, Kolkata** for participation/paper presentation (Oral/Poster) in **"CHEMCON 2014"** held on December 27-30, 2014 at Dr S. S. Bhatnagar University Institute of Chemical Engineering & Technology, Panjab University, Chandigarh.

Organising Secretary

Organising Chair

id 11.17 % chlorophyll whereas Anabaena sp. showed presence of 41.51 %protein, 35.58 % carbohydrate, 12.58 % lipid and .63 % chlorophyll 16.3 mg of lipid is extracted from 300 mg of dryChlorella pyrenoidosa cells and converted to fatty acid ıethyl esters (FAME) by the method of acid basedtransesterification to yield 4.4 mg FAME. Similarly, from 300 mg dried .nabaena cells, 39 mg of lipid is extracted to yield13.5 mg of FAME. These results indicate possibility of using both the algae or bio-diesel application; Anabaena sp.showed better growth and lipid production over Chlorella sp.

*eywords:* Bio energy, lipids, FAME

## P106 (BIF003)

## MULTIVARIATE STATISTICAL ANALYSIS OF PHYSICO-CHEMICAL PROPERTIES OFBIOMARKER FOR MUSCULOSKELETAL CANCER/SARCOMA

**S. Soma Sekhara Reddy, C. Sumana***
Chemical Engineering Division, Indian Institute of Chemical Technology, Hyderabad
E-mail: sumana.chenna@gmail.com

*bstract:* Present study is focused on the multivariate analysis of physico-chemical properties of sN-CAD protein data which is a iomarker for Sarcoma. If any protein has to enter a cell or it needs to be expressed in an organism, it has to be in an active soluble ›rm to be functional. The solubility of a protein depends on the physico-chemical properties of the protein. These properties re also useful in preventing the aggregation of a protein during its folding. Analysis of physico-chemical properties is helpful ı predicting the nature of the protein, as well as in optimising expression of the protein. Overall 133 amino acid sequences of N-CAD, mainly belonging to vertebrates are considered for this study. The physico-chemical properties of these sequences are valuated and the data is further subjected to principal component analysis (PCA), a powerful multivariate statistical technique, hich transforms the correlated high dimensional data into uncorrelated lower dimensions. The contribution chart evaluated y PCA has identified some of the important physico-chemical properties of sN-CAD biomarker for musculoskeletal sarcoma. hese studies may be helpful in improving the diagnostic ability of biomarker and also in developing appropriate drug delivery ystems.

*eywords:* principal component analysis, biomarker, physico- chemical properties

## P110 (BPH002)

## EFFECTS OF COMMERCIAL F:.EBIOTIC ON SUCEPTIBILITY OF L.CASEI AGAINSTNORFLOXACIN AND EFFECTS OF IMMOBILIZED FORM ON ANTIMICROBIAL ACTIVITY OFL.CASEI

**Debolina Banerjee[1], Ashwiny Pandey[2], Ranjana Chowdhury[1]***
Department of Chemical Engineering, Jadavpur University, Jadavpur, Kolkata-700032
E-mail: d.banerjee2009@gmail.com1, ashwiny06.pandey@gmail.com2, ranjana.juchem@gmail.com1*

*bstract:* Due to heavy use of antibiotics over the past few decades, microorganisms by their natural ability have adapted emselves to resist many of the antibiotics[4]. So researchers are becoming interested in finding out other alternatives of those ıtibiotics, which do not have much side effects. Probiotic organisms lactic acid bacteria used in yoghurt can be a very good lution of this persisting problem, because they have many health benefits effects due to antimicrobial effects towards many .thogens[5]. The effects of these probiotic organisms can be increased to several degrees with the incorporation of some tural growth inducers, called prebiotics[1]. The current research study is focused on the enhancement of antimicrobial effects probiotic lactic acid bacteria, namely Lactobacillus casei in presence of commercial prebiotic, inulin. The growth kinetics selected probiotic organism, L.casei has been determined both with and without commercial prebiotic supplement (inulin). ebiotic score have been determined for all prebiotics. For both L.casei and E.coli the number of viable cells (N24) determined er 24 h batch growth in modifird MRS medium containing 1%glucose and 1% prebiotic separately as carbohydrate. The sceptibility of the selected microorganisms towards a common antibiotic namely, norfloxacin has also been tested. It has en observed that although L.casei is sensitive towards norfloxacin in absence of inulin, resistance is developed when inulin is esent. This has been observed by measuring the zone of inhibition as a function of inulin concentration. Zones of inhibition e to the application of L.casei in free and immobilized form in E.coli culture have been measured.

*ywords:* Probiotics, Prebiotics, Immobilization, Antimicrobial activity, Inulin

Gmail **Debolina Banerjee <d.banerjee2009@gmail.com>**

# Fwd: EFFoST Annual Meeting 2013: Poster Acceptance

1 message

**Ranjana Chowdhury** <ranjana.juchem@gmail.com> Mon, Jul 1, 2013 at 11:54 AM
To: d.banerjee2009@gmail.com

---------- Forwarded message ----------
From: **Content-EFFoST2013 (ELS-CHN)** <Content-EFFoST2013@elsevier.com>
Date: Thu, Jun 27, 2013 at 7:09 PM
Subject: EFFoST Annual Meeting 2013: Poster Acceptance
To: "ranjana.juchem@gmail.com" <ranjana.juchem@gmail.com>


## Poster Acceptance Letter

**REF NO: EFFO2013_0303 (Please quote in all correspondence)**

27 June 2013

Email: ranjana.juchem@gmail.com

Dear Prof. R. Chowdhury,

Thank you for submitting an abstract to present at 2013 EFFoST Annual Meeting. On behalf of the Organising Committee I am delighted to inform you that your abstract entitled “Probiotic (Lactobacillus casei And Bifidobacterium sp.)- Prebiotic (Pure And Natural Inulin) Interaction With Reference To Preservation Of Tomato Ketcup And Local Milk Based Sweet meat—Experiments and Modelling" has been accepted for **poster presentation** at the Conference.

| | |
|---|---|
| **Title:** | **Probiotic (Lactobacillus casei And Bifidobacterium sp.)- Prebiotic (Pure And Natural Inulin) Interaction With Reference To Preservation Of Tomato Ketcup And Local Milk Based Sweet meat—Experiments and Modelling** |
| | |

| | |
|---|---|
| **Authors:** | **D. Banerjee, R. Chowdhury** |
| **Presenting Author:** | **R. Chowdhury** |

Please check the above details of your presentation carefully, as all conference material will be printed with this information. If there are any corrections please inform us as soon as possible by email to: Content-EFFoST2013@elsevier.com

It is a condition of abstract acceptance that you or a nominated presenting co-author registers for the conference by the **author registration deadline of 20 September 2013. The abstracts of all unregistered presenters will be removed from the poster programme after this date.** Should the addressee above not be the nominated presenter, please inform me of the name and email address of the presenter immediately: Content-EFFoST2013@elsevier.com

**Please register online to attend the conference by following this personal link**: http://conferences.elsevier.com/EFFO2013?email=ranjana.juchem@gmail.com&abstracts=0303

Registration is available online using a credit card. Registration rates are as follows:

| | |
|---|---|
| Early bird student | €300 |
| Standard rate student | €350 |
| Early bird academic | €750 |
| Standard rate academic | €825 |
| Standard rate exhibitor | €1650 |
| Conference Buffet Dinner | €40 |

All authors will be responsible for the payment of their own registration fees, travel and accommodation expenses. Unfortunately the conference organisers do not have funds available to support the attendance of individual delegates.

**Poster Presentations**

Following under separate cover is an information sheet for poster presenters. Please read this carefully for details on production of your poster, paying particular attention to panel size and appropriate fixings. These guidelines are also available on the conference website.

You will be informed of your session date, time and poster number in due course.

**Accommodation**

Rooms have been reserved at the conference hotel at specially negotiated rates for use by delegates.

For further information and to book please access the accommodation page on the website: http://www.effostconference.com/conference-accommodation.html

**Conference Publications**

Upon arrival at the conference all delegates will also receive a programme booklet and prior to the conference all delegates will be sent a link to a secure online system with access to the programme and abstracts.

Please do not hesitate to contact me if you have any queries and I look forward to receiving you completed registration.

Yours sincerely,

Irene Cyrilraj

Content-EFFoST2013@elsevier.com