# STUDIES ON UTILIZATION OF WASTE FOR PRODUCTION OF ALCOHOL

THESIS SUBMITTED

by

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# PROFORMA – 1

## "Statement of Originality"

I…**Swati Ray**.…registered on ……**24.05.2016** ….....do hereby declare that this thesis entitled **" Studies on utilization of waste for production of alcohol"** contains a literature survey and original research work done by the undersigned candidate as part of Doctoral studies.

All information in this thesis have been obtained and presented in accordance with existing academic rules and ethical conduct. I declare that, as required by these rules and conduct, I have fully cited and referred all materials and results that are not original to this work.

I also declare that I have checked this thesis as per the "Policy on Anti Plagiarism, Jadavpur University, 2019", and the level of similarity as checked by iThenticate software is ___6___%.

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1. 2.

# PROFORMA - 2

## CERTIFICATE FROM THE SUPERVISOR/S

*This is to certify that the thesis entitled* "**Studies on utilization of waste for production of alcohol**" *submitted by* Smt. **Swati Ray** *who got her name registered on* **24.05.2016** *for the award of* **Ph.D (Engineering)** *degree of Jadavpur University is absolutely based upon her own work under the supervision of* **Dr. Uma Ghosh** *and* **Dr. Avik Mukherjee** *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. ----------

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# CONTENTS

# OBJECTIVE OF THE STUDY

This study was aimed at producing bioethanol, which is an alternative to fossil fuel, by utilizing agro-industrial waste bagasse. Bagasse is a waste generated in sugar processing and juicing of sugarcane, and it is often openly dumped in the environment causing solid waste accumulation and microbial invasion. The dumped bagasse contains some nutrients, which can be reused in some other forms of valuable products. In this current study, at first selection was made among some of the easily available food processing and agriculture wastes. Then the selected waste i.e., bagasse was water-extracted in order to solubilize sugars, and the aqueous extract was used as a substrate for fermentation. Fourier Transform Infrared Spectroscopy was performed on the aqueous extract for its compositional analysis, and a comparison between wet and dry bagasse extracts on various parameters were carried out. The optimization of fermentation conditions like pH, time, temperature, inoculum age and size were also performed. Nutritional supplementations using carbon source, nitrogen source and mineral source were optimized as well. Cumulative effects of all these factors were also studied using statistical approach that was helpful to optimize ethanol yield, which was measured by titrimetric method. Since product recovery and reusability of the fermenting cell was necessary, immobilization of cells were done using suitable matrix. Optimization of immobilized cell fermentation was carried out using the previously optimized conditions.

# PLAN OF WORK

**Chapter 1: Selection of different wastes generated in different food processes or food industries**

Some of the wastes will be selected based on their availability and amount of production and among them the best one will be selected based on reducing sugar content and ethanol production.

**Chapter 2: Treatment of wastes to make them suitable as substrate for fermentation**

The selected waste will be pre-treated by suitable method to ensure increase in ethanol yield after pre-treatment process.

**Chapter 3: Optimization of the fermentation conditions utilizing bagasse extract**

The conditions under which fermentation will be carried out will be optimized on the basis of the following parameters:

- pH
- Fermentation time
- Fermentation temperature
- Inoculum size
- Inoculum age

**Chapter 4: Optimization of the nutritional parameters for the fermentation process**

The conditions under which nutritional supplementation will be carried out will be optimized on the basis of the following parameters:

- Carbon source
- Nitrogen source

- Mineral source

**Chapter 5: Ethanol production from immobilized cells of Saccharomyces cerevisiae MTCC 180**

Cells of the fermenting organism will be immobilized in a suitable matrix and optimization of the conditions under which the immobilized cells will produce ethanol, will be carried out.

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INTRODUCTION

## A. WHAT IS AGRO-INDUSTRIAL WASTE

Population increase in certain parts of the world coupled with tendency towards urban migration has resulted in an increased demands for food and agriculture, which in turn generated large amounts of agricultural wastes at all levels. In many developing and underdeveloped countries, the agricultural produce is transported from rural areas, where they are cultivated and harvested, to cities mostly in unprocessed forms leading to significant losses of these produce. These food processing and agricultural wastes can be both solids and liquids, and are generated from the production, harvesting, transportation, handling, preparation, and/or consumption of food. Wastes are also generated through disposal of already treated waste after its secondary utilization. Due to lack of planning, poor public awareness, lack of sound Government policies, and insufficient utilization of resources, agro-industrial waste accumulation have become one of the major roadblocks to cleaner environment [1-3].

A substantial portion of the global food production, about 1.3 billion tons, i.e., one third of the total food production are lost or wasted annually. Among these, wastages of fruits and vegetables, roots and tubers the highest value that amounts to 520–650 million tons every year. In the European Union alone, 89 million tons of foods are wasted per year, while total agricultural residue production amounts to 367 million tons per year [3]. Additionally, it has been estimated that 147.2 million tons of fibre residues; whereas 709.2 and 673.3 million tons of wheat and rice straw residues, respectively, are generated, as part of agro-industrial wastes. The enriched characteristics of these residues has enabled them to be used as substrate for new product innovation and development. These nutrient rich primal matters are fitting substrates that can be recycled and/or reused through solid state or submerged microbial fermentation to produce various high-value, useful products[2].

## B. SIGNIFICANCE AND EFFECTS OF FOOD WASTE GENERATION ON ENVIRONMENT

The earth is equipped with a natural cycle, by which it maintains environmental equilibrium, and recover from routine environmental degradation in a systematic manner. For example, forest soil is rich and fertile due to biomass recycling of leaves and other plant and animal residues that falls on it. This natural enrichment of soil has resulted in massive deforestation, to avail fertile land for enhancing agricultural production. The process of agricultural production in turn, generates loads of wastes, which have been, and can be recycled into some forms of usable products or substances. For example, rice straw is one of the agricultural wastes, which is used to make roofs of huts, and to feed livestock since ancient times. The twigs and small branches of trees were utilized as fuel for millennium. Agricultural and processing wastes are also composted to produce organic fertilizers, which are profusely used by farmers, as organic foods are gaining market all across the globe. This process has helped many poor farmers to decrease their production cost sustaining their soil fertility [1] [4].The raw material source decides the composition and quantity of agro-industrial wastes along with the nature of the products, manufacturing operations, and processing steps. In general, these wastes have high values of Biochemical Oxygen Demand (BOD), Chemical Oxygen Demand (COD) and suspended solids causing severe water, soil and air pollution problems, if not properly managed or treated prior to disposal.

- *Water Pollution:* It is caused by highly biodegradable effluents having soluble organic compounds that are difficult to remove chemically or by pigments in various disposed wastes that causes water discoloration or by putrescible liquid wastes that cannot be stored for long. Effluent from edible oil processing contains high concentrations of suspended fats, oils, and/or floating grease. They usually have very high $BOD_5$, suspended solids, dispersed organics and dissolved solids. Some of the common

examples are whey from dairy industry, brine from pickle manufacturing, and wash water from livestock, which are rigged with organic, inorganic matter, microorganisms and parasite eggs.

- *Solid waste accumulation***:** Frozen and canned meat or muscle products may form putrescible wastes from peeling and trimming or screening operations. These wastes need to be treated quickly to convert them into by products such as fertilizers and/or feed. Peanut and cocoa industries accumulate large quantities of shells and hulls whereas tea chest, spent tea and coffee grounds are primary by products of tea or coffee manufacturing industries. Processing of poultry, crab, shrimp or fish produce large quantities of shells, entrails, offal, feathers, fins, etc., which are commonly utilized to manufacture animal feed.
- *Air Pollution:* Odours and smoke from processing operations, wastewater treatment, solid waste disposal, visible moisture from steam plumes, entrained particulates, etc. can cause air pollution, if not properly treated, maintained, utilized (where appropriate), and/or disposed. Particulate emissions from handling of beans, food grains, or pomace may discharge occasional toxin(s) or specific allergen(s). Roasting odour from tea, coffee or chocolate industries and odours emanating from cages or burns, as a result of accumulation of livestock wastes, putrefaction of organic matter in manure, animal urine, and/or from redundant feed are some of the routine causes of air pollution from agriculture, and agro-based industries [4] [5].

These organic wastes forms rich biomass that may support or promote growth of microorganisms including human pathogens, if left untreated and/or inadequately treated. These untreated and unutilized wastes are often either dumped in the open or burned in landfills. Burning of these waste releases pollutants like carbon monoxide, nitrous oxide, nitrogen dioxide and carbon particles, accompanied with formation of ozone and nitric acid.

These chemicals gets mixed up in soil and water streams causing acid deposition that may compromise ecological balance. Some of these components leaches from the dumping ground and landfills, and pollute water bodies resulting in invasion of water weeds. [6-8].

## C. TYPES OF WASTES FROM FOOD AND AGRICULTURAL SECTOR

Food and agricultural sector have huge prospects of industrialization / commercialization of waste utilization, including utilization of secondary and tertiary wastes to produce valuable by-products. Although, it may or may not be possible to get rid of hundred percent of the agriculture and food processing wastes even after recycling, but their minimization in the environment is a rather logical and achievable goal. The following table lists some of the major staple and industrial crops with the residues they generate annually (Table 1).

**Table 1.** Agricultural residues for staple crops and industrial crops [5]

| Staple crops | | |
|---|---|---|
| Name of the crop | Crop residue rate (%) | Crop residues (kg) per 1000 tons |
| Rice | 55 | 21,298 |
| Maize | 250 | 11, 328 |
| Sweet Potato | 45 | 595 |
| Cassava | 75 | 7,046 |
| Total | | 40, 269 |
| Industrial Crops | | |
| Sugarcane | 65 | 10,483 |
| Peanuts | 20 | 106 |
| Soybean | 10 | 26 |
| Total | | 10,616 |

Livestock rearing creates solid wastes such as manure and other organic materials, liquid wastes like urine, wash water (for bathing of animals) from cage, barn, and/or slaughter houses. Most of these spontaneously developed farms lack proper treatment facilities for these wastes, and thus cause serious pollution problems.

**Table 2.** Generation rate of solid waste from breeding activities [5]

| Animal | Cow | Buffalo | Pig | Poultry | Goat, sheep | Horse | Deer |
|---|---|---|---|---|---|---|---|
| Average value(kg/head/day) | 18 | 15 | 2 | 0.2 | 1.5 | 4 | 2.5 |

According to the Department of Animal Health (DAH), India, stored manure (25%) is collected and packaged for sale to targeted consumers who will use it as fertilizer or fish food; 20% is recycled for biogas production, 10% is for composting, and remaining 45% is discharged into the environment without any treatment. This waste can generate greenhouse gases, and also cause water pollution. Animal manure may contain various microorganism and parasite eggs, which can survive to harm human and animal health. Additionally, farm wastes may include a considerable amount of redundant food residues, corpses of cattle and poultry, which are sources of invasion by pathogenic microorganisms. Table 2 shows solid waste generation rate due to breeding activity of different animals. The nature of these waste depends upon the feed consumed by the animal, its gender, species, hygienic practices and method of waste treatment followed in the animal husbandry facility [5]. While agriculture mainly deals with raw or unprocessed wastes from plant and animal origin, food industries deal with both raw materials, semi-processed and processed by products, and design their waste treatment accordingly.

Based on the nature of the agro-industrial wastes generated, they may be classified into two broad divisions- agricultural waste and food processing waste. Agricultural wastes may include different wastes generated from various agricultural practices from sowing to harvesting, examples of which include rice straw, corn cob, cassava stem, potato peels, sugarcane bagasse etc. Food processing wastes may include wastes that are generated from

different food processing and treatment operations e.g., oil cakes, fruits pomace and skin, internal organs of animals, etc. The elaborate classification of these wastes is summarized in Figure 1 [2].However, quite understandably, the classified residues often can show redundancy as industrial, agricultural, or food processing wastes. Industries or agricultural farms may adopt different treatments or processes of handling these wastes depending on their choice or final utilization.

## I. Food Industry Wastes

About 20% of the total fruit and vegetable production in India are wasted during the postharvest stage, handling and transportation from production farms to markets. Besides, large quantities of organic wastes and effluents are generated by beverage, edible oil, extruded food, flesh, sweet and savoury delicacies, green harvest processing units, and many others. There has been a remarkable increase in number of food and beverage processing industries in order to meet the increasing demands for semi-processed and processed foods. In edible oil industries, large amount of oil cakes, by-products of oil extraction from the seeds, causes air, water, and soil pollution, as they are rich in greasy pollutants. These food processing wastes have high BOD, COD and TSS value, which make them nutrient-rich media for microbial processing to produce industrially important valuables. Thus, these industrial residues are pre-enriched with desired nutrients, rendering them as low-cost alternative substrates for fermentation, and thus, can be cost effectively utilized as a source [2].

## II. Agricultural wastes

Agriculture wastes are two types-those generated as land residues and processing residues. Land residues are post-harvest wastes composed of green leaves, plant parts etc.; the processing residues belong to post-processing wastes such as molasses, bran, bagasse, other

by products etc. that can be utilized in making forage, improving fertility of topsoil, making up fertilizer etc.

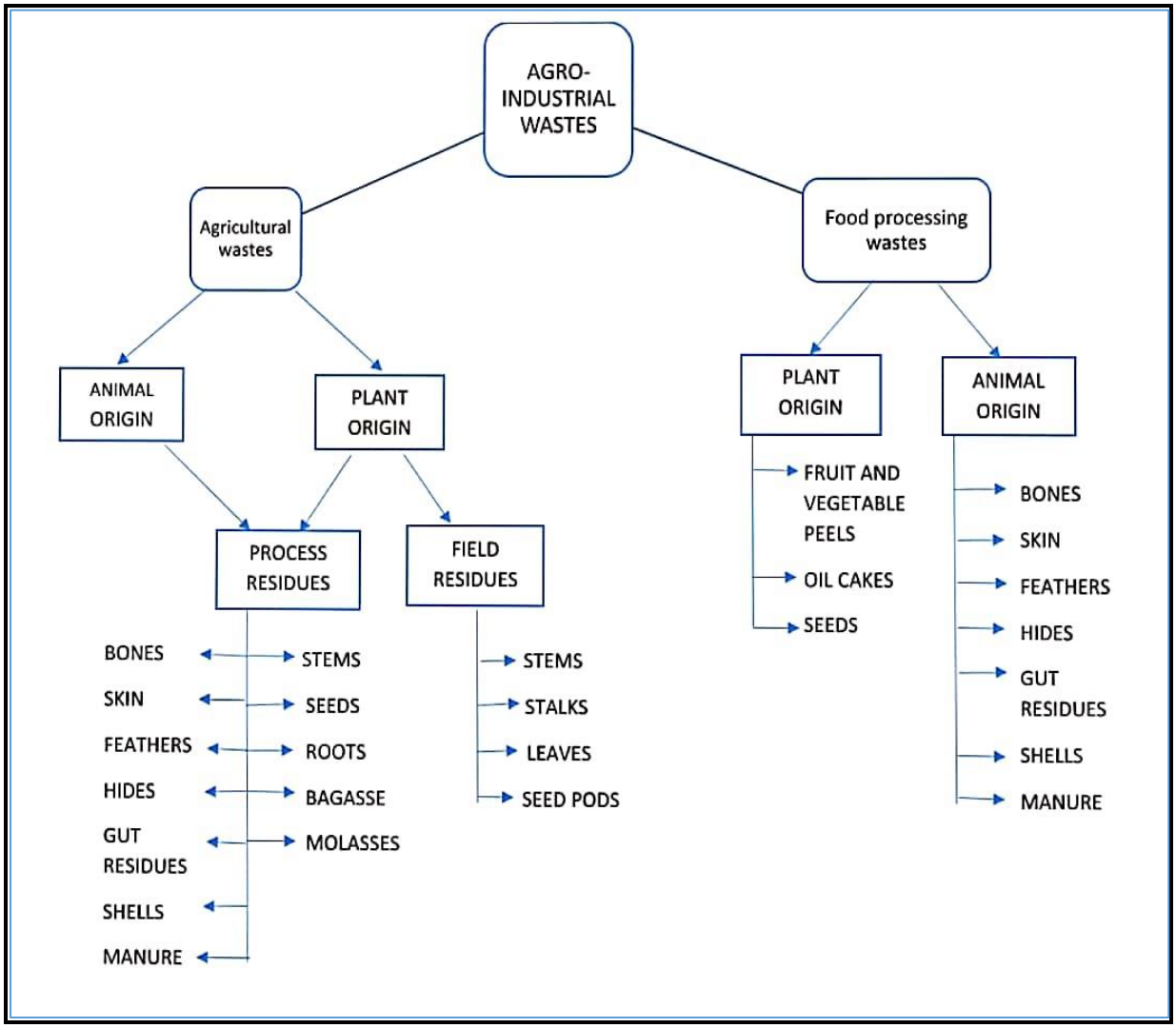


**Figure 1:** Different types of agro-industrial wastes produced from different sources

They can improve the irrigation process and can control soil erosion as well. These phytomass consist of cellulose, hemicellulose and lignin, which are strongly bound by chemical cross-linking, and are also termed as lignocellulose (LCB) accumulation. They are also rich in starch, and both lignocellulose and starch can be converted to biofuels or biopolymers, as important renewable resources. Two types of extracellular enzyme systems to utilize agricultural residues such as sugarcane bagasse, wheat bran, rice bran, corn cob,

wheat straw etc. are hydrolytic (polysaccharide degradation) and ligninolytic (lignin degradation that opens phenyl rings) enzymes, used to produce bio-alcohol [2]. Fermentation is a cost-effective process of utilizing low-value substrates like agricultural wastes, as potential alternatives to the traditional method of acid hydrolysis [9].

## D. REUTILIZATION OF WASTE

Most of the agro industrial wastes are either directly used as a raw material or are pre-treated to a suitable material or ingredient to be processed into different valuable products. Fermentation is one such commonly used process to manufacture industrially important products from these wastes. Figure 2 is a flow-diagram that illustrates the fate of waste products to generate industrially important materials.

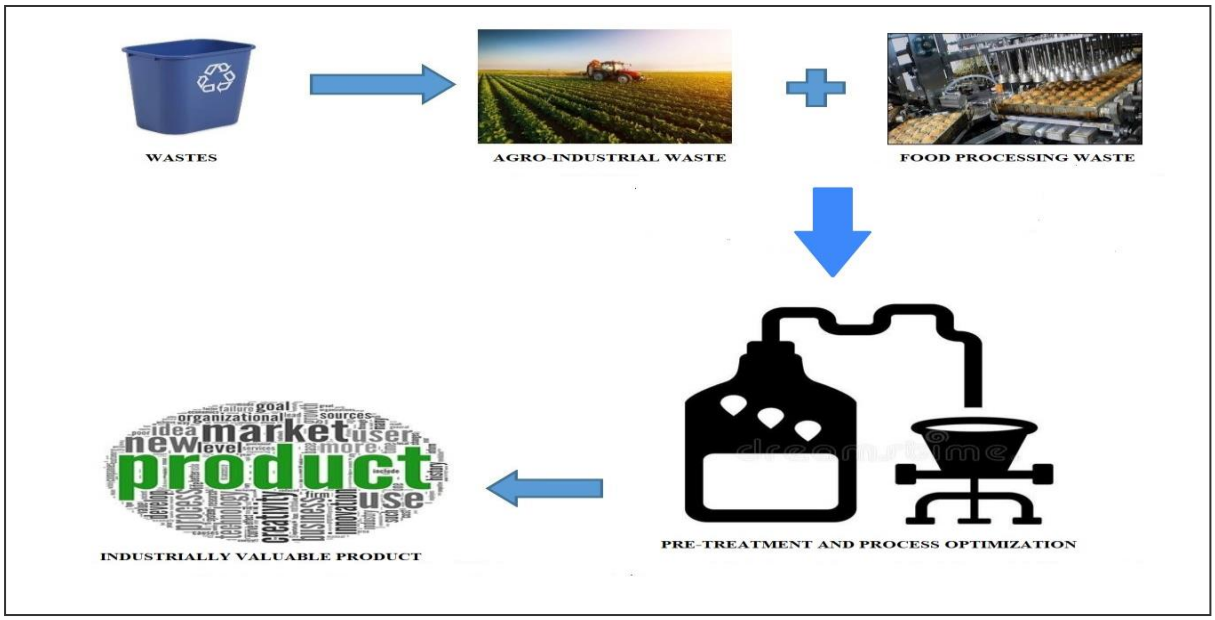


**Figure 2.** Flow diagram of fate of processing of agro-industrial and food processing wastes

The other wastes can be thoroughly pre-treated or minimally treated to either make it suitable for fermentation or can be directly used as animal feed. The pre-treatment may include physical treatments like cutting, grinding, size reduction and steam digestion, or chemical treatments like acid or alkali hydrolysis, enzyme treatment, solvent extraction / treatment etc.

These pre-treatment converts the complex compounds in these wastes into simpler, easily degradable molecules, which are metabolized by microorganisms to generate primary and secondary metabolites.

## E. MANUFACTURE OF INDUSTRIALLY IMPORTANT PRODUCTS FROM WASTE

The valuables that can be generated by processing of these wastes may have therapeutic or medicinal values; may be utilized in various chemical or pharmaceutical industries; may be used to make some regional cuisines or simply utilized to produce solid, liquid or gaseous biofuels (Table 3).

**Table 3.** Production of different industrially important products from agriculture and food wastes

| Name of the waste | Nature of fermentation with microorganism | Product generated | Ref. |
|---|---|---|---|
| **Antimicrobial/ Anticancer/ Antioxidant** | | | |
| Pineapple waste | Solid State Fermentation using *Kluyveromyces marxianus* NRRL Y-8281 | Anticancer and antioxidant property of the methanol extracted waste | [10] |
| Apple and Avocado skin and residue | Extraction | Epicatechin, catechins, anthocyanins, quercitin glycosides, chlorogenic acid, hydroxycinnamates, phloretin glycosides, procyanidins, gallic acid, cyanidin 3-glucoside, homogentisic acid | [11] |
| Banana peel | Extraction | Gallocatechin, anthocyanins, delphindin, cyaniding,catecholamine | [11] |
| **Antibiotics** | | | |
| Corn cob and pomace, sawdust, rice hulls, Groundnut shell, cassava peels and household kitchen waste. | Solid State Fermentation *using Streptomyces rimosus* TM-55, *Streptomyces rimosus* etc. | Antibiotic oxytetracycline | [12-14] |
| Coconut oil cake, groundnut oil cake, | Solid State fermentation using *Amycolatopsis* | Rifamycin B | [15] |

| | | | |
|---|---|---|---|
| ground nut shell and rice husk. | *Mediterranean* MTCC 14 | | |
| Apple pomace, cotton seed meal, soy bean powder and wheat bran | Solid State fermentation using *Streptomyces fradiae* NCIM 2418 | Neomycin | [16] |
| **Regional delicacies** | | | |
| Peanut press-cake | -- | Fermented product Oncom Kacang. | [17-18], |
| Tahoo waste (Curd of soybean) | -- | Fermented product Oncom Tahoo. | [17-18], |
| Hunkwe (starch flour) and *Phaseolus radiata* or Mungbean waste | -- | Fermented product Oncom Ampas Hunkwe. | [19] |
| Soy bean milk wastes | -- | Protein-rich human food like Tempeh or similar kinds. | [20] |
| **Enzymes** | | | |
| Agri wastes | Solid state fermentation using thermophilic fungus strain, i.e., *Thermoascus aurantiacus.* | β- glucosidaseandEndoglucanase | [21] |
| Corn cob | Solid state fermentation coupled with enzymatic treatment using *Sporotrichum thermophile* | Cinnamoyl esterase and xylanase | [22] |
| Wheat bran, green gram bran, black gram bran, corn flour, barley flour, jowar flour, maize bran, rice bran and wheat rawa. | Solid state fermentation using *Aspergillus sp.* | Amylase and Glucoamylase | [23-24], |
| Wheat bran | Solid state fermentation using *Aspergillus awamori* nakazawa MTCC 6652 | Amylase and Protease | [25] |
| Agricultural waste, Papaya Waste, Orange peel, Coconut oil cake, Rice bran, wheat bran, black gram bran, and soybean. | Solid state fermentation using *Aspergillus niger* MTCC 104,<br>*Aspergillus niger, Aspergillus oryzae etc.* | α-amylase | [26-30] |
| Rice bran and Corn bran | Solid state fermentation using *Bacillus* sp PS7 | Thermostable α-Amylase | [31] |
| Candelilla stalks, coconut husks, corn | Solid state fermentation | Ellagitannase | [32] |

| | | | |
|---|---|---|---|
| cobs , sugarcane bagasse | using *Aspergillus niger* GH1 | | |
| Palm kernel oil cakes, Groundnut oil cake and Linseed oil cake. | Solid state fermentation using *Aspergillus ibericus.* MUM 03.49, *Penicillium aeruginosa* and *Candida rugosa*. | Lipase | [33-35] |
| Wheat bran and orange peel | Solid state fermentation using *Penicillium notatum* NCIM 923. | Pectin methyl esterase | [36] |
| Fruits peel waste | Solid state fermentation using *Aspergillus niger* | Invertase | [37] |
| **Organic acids** | | | |
| Outer cover of gallo seeds | Fermentation using *Rhizopus oryzae* | Gallic acid | [38] |
| Tea wastes with sugarcane molasses | Fermentation using *Aspergillus niger* | Gluconic acid | [38] |
| Wheat kernels | Fermentation using *Aspergillus oryzae* | Oxalic acid | [38] |
| Carrot-processing waste, Sweet sorghum, Sugarcane bagasse and Cassava bagasse | Fermentation using *Rhizopus oryzae, Lactobacillus paracasei* and *Lactobacillus delbrueckii* | Lactic acid | [38] |
| Pineapple wastes | Fermentation using *Aspergillus foetidus* | Citric acid | [38] |
| **Organic chemicals** | | | |
| Corn cob | Solid state fermentation coupled with enzymatic treatment using *Sporotrichum thermophile* | Phenolics | [22] |
| Orange peel waste | Stirred tank reactor using *Bacillus subtilis* OK 2 | Poly-3 hydroxybutyrate | [39] |
| Castor oil, coconut oil, corn oil, motor oil, olive oil, olein, rapeseed oil, sunflower oil, barley bran, cassava flour waste, peanut cake, potato waste, rice bran and wheat bran. | *Pseudomonas aeruginosa* PB3A. | Biosurfactant | [40] |
| Waste like potato peel | Submerged Fermentation using *Xanthomonas* | Xanthan | [41] |

| | | | |
|---|---|---|---|
| | *campestries* MTCC 2286, *Xanthomonas oryzae*, *Xanthomonas musacearum*, *Xanthomonas citri* | | |
| **Immobilized matrix** | | | |
| Ten agro wastes which includes creosote bush leaves, variegated Caribbean agave, lemon peel, orange peel, apple pomace, pistachio shell, wheat bran, coconut husk, pecan nutshell, and bean residues. | Solid state fermentation in immobilized form | Immobilized carrier | [42] |
| Salacca wallichiana stem | Immobilization carrier for lipase production | Immobilized matrix | [43] |
| Chicken egg shell membrane powder | Immobilized enzyme carrier of β galactosidase | Immobilization matrix of enzymes | [44] |
| **Biomass** | | | |
| 16 diverse agro-industrial wastes(Rice straw, Cotton waste, Khaya ivorensis, erminalia ivorensis, Androgon sp, Groundnut shell, Melon pericarp, Cassava peels, Banana leaves, Palm wastes, Con cob, Cocoa leaves, Coffee leaves, Paper wastes, Soybean wastes and Poultry manure) | Submerged condition of fermentation | Oyster mushroom species *Pleurotus tuber-regium* | [45] |
| Banana stalks, Bahia grass, Rice and wheat straw. | Solid bed of agro waste | Oyster mushroom pink and grey species and the species *Pleurotus sajor-caju* | [46, 47] |
| Coffee husks | Solid bed of coffee husks | Oyster mushroom | [48] |
| Paddy straw, Sorghum stem, Varagu straw and Sugarcane trash | Solid bed of agro waste | Oyster Mushroom species *Pleurotus eous* and *Pleurotus platypus.* | [49] |
| Cucumber and orange peels | Submerged fermentation using *Sachharomyces cerevisiae* | Single cell protein (SCP) | [50] |

| | | | |
|---|---|---|---|
| **Pigments** | | | |
| Molasses supplemented with sucrose, corn extract, yeast autolysate or extract, zinc sulfate and magnesium sulfate | Fermentation using *Penicillium oxalicum* | Arpink Red | [51] |
| Corn fibre material and the leftovers of ethanol processing like Distiller's dry grains with solubles | Fermentation using genetically modified fungus *Fusarium sporotrichioides* | Lycopene | [51] |
| Orange processing waste | Solid state, Semi-solid state and Submerged fermentation using *Monascus purpureus* ATCC 16365 and *Penicillium purpurogenum* CBS 113139. | Orange pigment | [52] |
| **Flavors** | | | |
| Maize, rice, wheat, and sugar beet pulp by products | Extraction using organic solvents | Ferulic acid ( Precursor of Vanillin production) | [38] |
| Olive mill waste | Microbial fermentation using *Rhizopus oryzae* and *Candida tropicalis* | D-limonene | [53] |
| Cassava bagasse, apple pomace, amaranth and soya bean | Solid state fermentation using *Ceratocystis fimbriata* | Fruits aroma ( mixture of Alcohol, aldehyde, ketones and esters) | [54] |
| **Gas production** | | | |
| Corn straw | Pre-treatment and anaerobic digestion | Biogas | [55] |
| Agriculture residues rice straw, cassava pulp, pineapple peel, decanter cake and empty fruit bunches along with *Eichornia crassipes* and *Typha angustifolia* L. | Pre-digested Solid state fermentation using natural microflora | Biogas methane | [56] |
| Soybean straw, wheat stalk, ground nut shells, black gram straw and red gram straw. | Digestion using concentrated sulphuric acid and catalysts. | Biogas | [57] |

| | | | |
|---|---|---|---|
| | **Solid fuel** | | |
| Grape Marc from winery | hydrothermal carbonization to make hydro-char | Solid biofuel | [58] |
| Palm oil fronds and trunks | hydrothermal treatment | Solid fuel | [59] |
| Mixture of rice husk, corn starch, glycerol, and acetic acid. | Homogenization and oven drying | Solid fuel | [60] |

## I. Manufacture of bioalcohol

Fermentation of these organic wastes into bio-alcohol is an effective strategy to deal with these wastes and their environmental impact [61]. The inflation in oil market brought light to bioalcohol, especially bio-ethanol, bio-diesel, biohydrogen, etc. Bioalcohol can be classified as first and second generation. First generation are manufactured from carbohydrates, lipids and oils or other agro-industrial wastes using conventional methodologies, whereas second generation bioalcohol are derived from lignocellulosic biomass including various plant parts, stalks, stems, wood etc. Some of the second breed of biofuels like biological hydrogen, biological methanol or alcohols in blended form are still in investigation and research. It has been observed that greenhouse gas emission is lesser for second-generation biofuels compared to the first-generation [62, 63].

All over the world, Governments and regulatory bodies are funding and encouraging research aimed at innovation in this area in order to deal with the looming global energy crisis [62]. Bio-alcohol, especially bioethanol has very high blending capacity with petrol, and can be used in lower concentration in vehicles without any modifications. Since 1979, Brazil has significant proportion of its vehicles run purely on bioethanol, and the Brazilian economy is

one of the rare examples across the globe that is completely independent of imported fossil fuel [64].

## II. Generation of bio-alcohol from agro-industrial and food processing waste

Bio-alcohol production and consumption are mostly concentrated to the United States, Europe, and Brazil, Brazil being the largest producer and exporter of sugar cane, and not surprisingly, the second largest producer of bioethanol in the world [3]. Different methods of bio-alcohol generation that are possible from various wastes generated daily are listed in Table 4.

**Table 4:** Bio-alcohol production through fermentation of wastes

| Name of the waste | Nature of fermentation with microorganism | Product | Ref. |
|---|---|---|---|
| Vegetable' waste like potato peel, carrot peel, and onion peel. | Solid state fermentation using *Saccharomyces cerevisiae* with saccharification process by *Penicillium* sp. | Bioethanol | [65] |
| Pseudo banana stem | Co-culture fermentation with pre-treated hydrolysate of *Aspergillus ellipticus* and *Aspergillus fumigatus* and using the organism *Saccharomyces cerevisiae* NCIM 3570. | Bioethanol | [62] |
| Water extract of Bagasse, spent tea liquor and potato wash water | Submerged Fermentation using *Saccharomyces cerevisiae* MTCC 180 | Bioalcohol | [66] |
| Agro-industrial waste and starch industry waste water. | Fermentation using *Clostridium beijerinckii* | Biobutanol | [67] |
| Straw, sawdust and corncobs | Solid state fermentation using *Saccharomyces cerevisiae* NCYC 3451 | Bioethanol | [64] |
| Young coconut husk, custard apple seeds and broiler chicken skin. | Modified Bligh and Dyer method and trans-esterification | Biodiesel | [68] |
| Coconut milk, pineapple juice and tuna juice | Submerged fermentation using *Saccharomyces cerevisiae* strain CDBB 790 | Bioethanol | [69] |
| Sunflower straw and cracked olive stones | Fermentation of thermo-chemically pre-treated and enzymatically saccharified substrate using yeast *Pachysolen tannophilus* | Bioethanol and methane | [70] |
| Carob pod extract and beet molasses | Solid state fermentation using *Saccharomyces cerevisiae* | Bioethanol | [71] |
| Olive pulp and fragmented olive stones | Simultaneous saccharification and solid state fermentation | Bioethanol | [72] |

| | | | |
|---|---|---|---|
| Wheat straw | Pre-treatment and Fermentation using *Clostridium beijerinckii* P260 | Acetone–butanol–ethanol | [73] |
| Rice bran (RB) and de-oiled rice bran | Pretreatment and Fermentation using *Clostridium saccharoperbutylacetonicum* N1-4. | Acetone–butanol–ethanol | [74] |
| Grape and sugar beet pomace | Solid state fermentation using *Saccharomyces cerevisiae* | Bioethanol | [75] |
| Rye bran | Fermentation using *Streptomyces fulvissimus* CKS7 | Bioethanol | [76] |
| Waste animal fat | High temperature treatment using methanol along with catalysts nickel and magnesium. | Biodiesel | [77] |
| Waste fish oils and used cooking oils | Direct utilization or mild heat treatment | Biofuel | [78] |
| Palm shell | Pyrolysis | Biooil | [79] |
| Brunei rice husk | Catalytic pyrolysis using Zeolite-Socony Mobil—5 (ZSM-5) | Biooil | [80] |

## F. WASTE MINIMIZATION AND SUSTAINABLE DEVELOPMENT

Sustainable industrial development, including agro- and food industries, require four-pronged (4-R) strategy that ensure source Reduction, waste Recovery, Recycle and eco-friendly detoxification or neutralization for Reuse. Green productivity can only be achieved through zero discharge, zero emission, zero pollution, cost-effective processing, and application of clean production technology. The regulatory agencies and the food-processing industries can work together to develop new technologies for waste management that is commercially viable and environment friendly [81]. In this context, it is obvious that water pollution is the more serious problem, as solid wastes have better chances of recovery and recycling. Water is used in variety of purposes such as an ingredient of food product, for washing and cleaning purposes, for generating steam, as a coolant, etc., which makes the types and concentration of pollutants widely variable.

The suspended solids in the effluent are either floated in the form of scum or sediment at the bottom, and the latter increases the cost of wastewater treatment. A large reservoir of the treated wastewater is often maintained, as this pigmented water cannot be discharged into the

natural water bodies, despite maintaining Biological Oxygen Demand limits. Approaches of waste management and handling may vary from one country to another, with the principle technologies being the same in most cases. In fact, different countries have proposed different models for effective handling of wastes with the basic steps as follows.

- ✓ **Waste reduction:** Measures like in-plant modification is a major approach, and the methods used in this approach depend on local conditions.
- ✓ **Waste conservation:** The preliminary step for an efficient preventive measure to minimize generation of wastes is waste conservation. It is much effective to control at source i.e. not to produce waste in the first place. Effective use of resources can give both optimum economic benefit and environmental protection.
- ✓ **Waste segregation:** Segregation or categorization of wastes, not only minimizes the burden of solids from wastewater but also lowers the waste load and treatment cost. Wastewater should be separated according to their characteristics; greasy or non–greasy, clear or dirty, chilled, cold or hot, surface water or sanitary wastes, etc. which should be collected and treated separately.
- ✓ **Waste utilization (reuse, recovery / recycling):** Even if waste minimization practices are operational, it is inevitable that some wastes will be generated. The recovery, reuse, or recycling of wastes ensures utilization of raw materials for the production of other valuable products. [4].

Few of the other approaches that are undertaken in different regions and countries are 3R approach [5], Clean Technology [63], community based food management systems [82], 9-stage sustainable management systems etc. [83]. However this particular sector have huge potential and is termed as 'Bio-economy' by the European Union which means spent wastes from one raw material can be used for other processes for generation of useful products [3]. Reutilization of wastes is helping us to control wastage, reduce the use of

natural resources, and to generate newer and smarter products. Practicing recycling also helps the future generations by providing them with a better and greener environment for sustainable development. However, there are many constraints to effective utilization of wastes, as listed below.

- Sourcing of waste residue due to their seasonal and scattered nature bearing high transportation costs
- Variation in the quality of wastes and their deterioration with time
- Lack of adequate technologies or their information
- Absence of incentives and lack of assurance from market for finished goods
- Consumer prejudices and religious habits
- Inadequacy of infrastructure to support this emerging sector
- Lack of funds and manpower for Research & Development work in waste recycle
- Insufficient managerial or administrative initiatives to develop and promote schemes to entrepreneurs in both Government sector and Private organizations [4]

## G. SOCIO ECONOMIC IMPORTANCE

One of the most important needs of any growing population is energy. This growth has resulted in exponential rise in cost of unrefined oil, continuous deforestation, decrease in natural assets, political turbulence, environmental imbalances, and replacement to biomass in order to meet the energy demands of a growing economy like India. India faces an intimidating summon to fulfil energy requirements to cater to its expanding human load and industrialization. Thus, India must invest in renewable energy options to reduce its current imports, which is as much as two-third of its fuel needs. Almost the entire food grains production being used to feed its increasing population, India needs more affordable and renewable sources such as agro- and food industry wastes to produce bioalcohol / bioethanol.

The pre-treatment methods for bioethanol production can be further improved using faster enzymatic hydrolysis of the wastes. Besides, a significant amount of vegetable fibers have prospective use as alternatives to wood for pulp, paper and other applications [62]. Bioethanol production has mitigated the problem of agro- and food industry wastes, created jobs, increased farmers' income, encouraged entrepreneurs, and has helped to partially meet the energy crisis. However, there are certain potential drawbacks e.g. greenhouse gas emission from repeated reuse of wasteland, lack of Government subsidies and other market interventions to make bioethanol competitive with fossil fuels.

### I. Market value and bio-economy

About 181.5 billion tonnes of lignocelluloisic biomass is produced globally in a year, of which 8.2 billion tonnes are currently utilized and the rest remain untreated and degrade as waste. Approximately 7 billion tonnes are produced from by-product of agricultural, forest and grassland activities, of which 1.2 billion tonnes are merely agricultural residues. So, efficient utilization of these wastes are becoming a necessity to expand the prospects of developing bio-economy [84]. These agro-industrial waste biomass is widely available and forms the substrate for the production of biofuel, bioenergy and various value added products. Optimized technologies, life cycle analysis and economic assessment of these processes can contribute in the development of these nature friendly concepts. Various types of pre-treatment methodologies, valorisation techniques, integrated approach and optimization can increase the recycling value of these substances. The processes are not only a step towards the sustainable circular bio-economy but also an art of living life in a zero waste world [85].

## II. Commercialization and business development

Although, various alternative processes are developed for bio-alcohol production from agro-industrial wastes but their high-end costs and wide variety of substrate composition has limited their implementation on a commercial scale. Nevertheless, several integrated bioprocessing techniques is researched increasingly nowadays to produce second generation fuel and it has proven itself to be one of the most promising approach [61].

*Venezuela case study*

Venezuelan company, Etaven CA, which has patented production of "YARETANOL" (a cassava yare bioethanol). They trade to companies producing petrochemical for blending to generate fuel for vehicles. For its suitable positioning, the company can get cheap plant as well as residual plant waste (yare) associated with cassava flour production. After processing this waste, it obtains half yield of ethanol at half of the retail price, for benefit. By using this waste, ETAVEN has good influence on local population and the environment since it has reduced the cyanide pollution due to non-disposal in water bodies, lowered harmful gas evolution, created an alternative fuel source for transportation, created several jobs and improved the livelihood of locals.

- ✓ Achievement indicator on outcome basis as of 2012 by ETAVEN:
- ✓ ***Investment on source****:*2.5 million USD, ***Repairing and prolongation cost:***375,000 USD per year,
- ✓ ***Product yield:*** 30 Tonne /day , ***Period of cost return:***2 years approx,
- ✓ ***Rate of return:*** Less than 50%, ***Gross margin:***99%
- ✓ In spite of huge success of some of the industries, trying to reutilize agro-industrial wastes, most of them fails to get all ends meet. Many experts have tried to suggest

several business models to rectify the limitations, but it needs more research and funding if must be developed to a promising alternative.

*Potential business performance*

This proposed business model, as demonstrated in Figure 3, is one such example. It scores better in innovation for developing improved technologies or operations, and creation of meaningful partnerships on the block, like raw material providers, process makers, management groups and advisories.

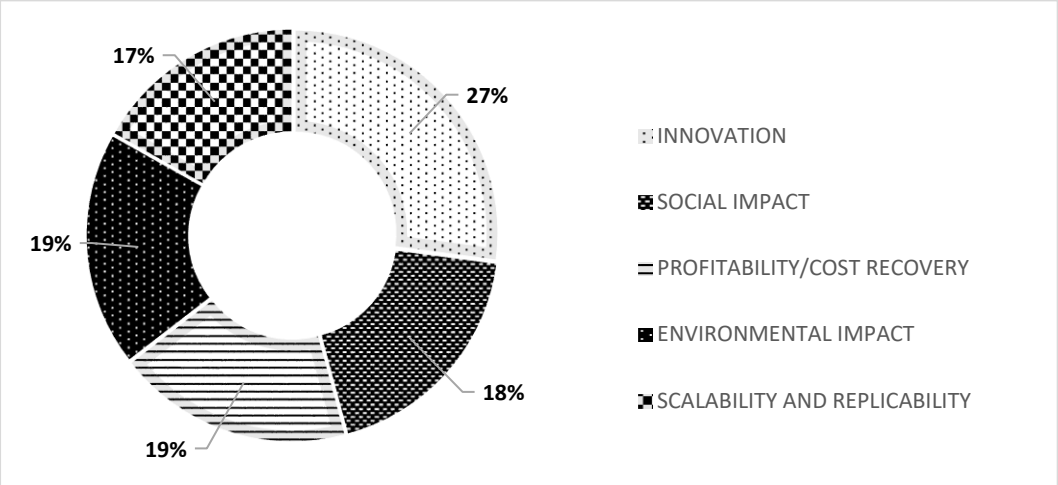


**Figure 3:** Doughnut chart showing an approximated and proposed business model for Bio-alcohol production [86]

However, the model fails to score high on other areas due to the need for personalized application of technologies by each industry. The primary risks associated with adopting it to a business level are:

- ✓ **Market risks:** Uncertainty in successful launching of new product from research and development stage to commercialization.

- ✓ **Technological risks:** Uncertainty of the operations involved in a system after industrial scale up. As it is combination of manifold steps, it may involve dangerous commercial investments.
- ✓ **Political and regulatory risks:** Challenges from unfavourable business environment, possible resistance from Government to obtain permits and lengthy process for obtaining approval for patent.
- ✓ **Safety, environmental and health risks:** Risk of environmental pollution and generation of hazard, if bio-alcohol production from agro-waste does not remove pathogens and pollutants completely, and is discharged in open [86].

## H. PRETREATMENT OF LIGNOCELLULOSIC BIOMASS

The component obtained by directly or indirectly through photosynthesis is called a biomass. They have high moisture content, low energy or bulk density and irregular shape and size. Due to heterogeneous nature, origin, composition and application of lignocelluloisic biomass, their nature of pre-treatment varies widely. Since it is difficult and/or less efficient to use them in their natural form, hence pre-treatment is necessary to convert them for maximum product recovery. There are two pathways by which any biomass can be converted into useable substances – biochemical and thermochemical. Pre-treatments may lead to physical, chemical or structural modifications to the mass that can be easily converted to useful products. Biochemical process involves using microorganisms or enzymes to break down the biomass whereas thermochemical process involves heat treatment to generate energy.

The pre-treatment processes can be subdivided into three broad types – physical, chemical and biological. The classification that are involved in the conversion of lignocelluloisic biomass are shown in Figure 4 [87].

**Physical pre-treatment:**

Mechanical processes that reduce particle size, increase surface area and pore size, reduce crystallinity, decrease polymerization are often used as pre-treatment(s). For example, milling is a vital step to improve enzymatic treatment of biomass. Particle size reduction helps in improving mass and heat transfer processes. Besides milling, chipping, briquetting, drying, densification, microwaving, extrusion, ultra-sonication, palletisation, torrefaction, etc. are all considered as various forms of physical pre-treatments. The limitations of this method include inability to digest lignin, high consumption of energy and cost intensiveness [88] [89].

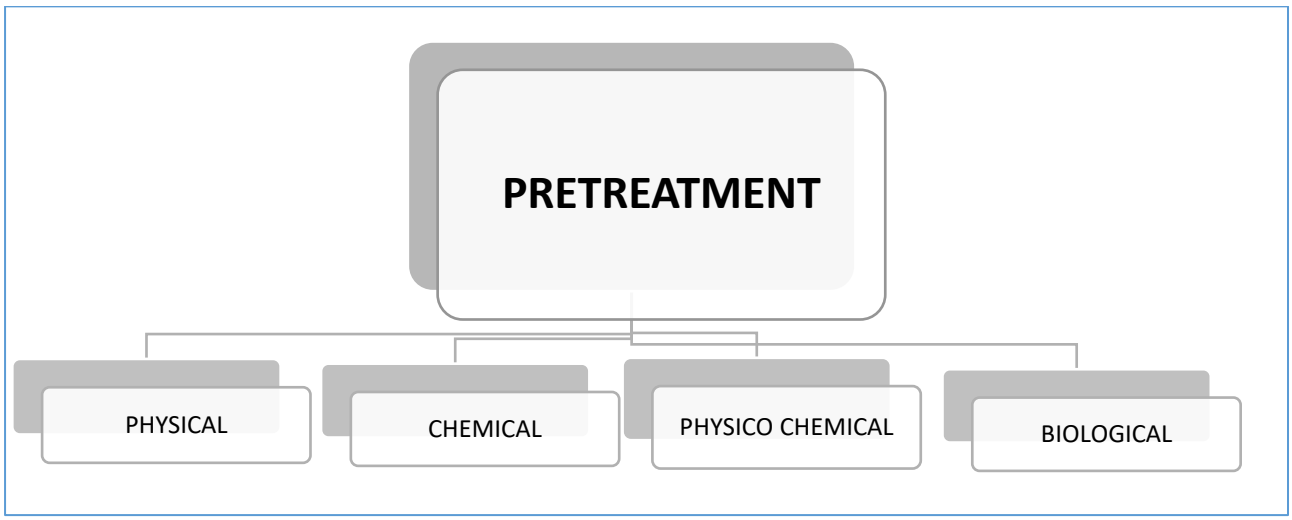


**Figure 4:** Pre-treatment processes involved in saccharification of waste substrates

**Chemical pre-treatment:**

Organic or inorganic chemicals disrupts the structure of biomass to make it usable. Lignocellulose, however, are resistant to chemical breakdown due to their recalcitrant nature, which includes complexity of structure, heterogeneous nature, crystalline cellulose and degree of lignification. Chemical conversion of lignocellulose isolates bio-polymeric

constituents of the material helping them to breakdown into smaller molecules. Acid, alkali, organic solvent, deep eutectic solvent and ionic liquid are the chemicals used for pre-treatment [88] [90-92].

**Physicochemical treatment:**

The process involves the combination of both physical and chemical treatment, in many cases they are found to be more effective than single methods. Steam explosion, carbon-di-oxide explosion, ammonia explosion, liquid hot water treatment, etc. can be considered under this category [93].

**Biological pre-treatment:**

It mostly involves fungal action including degradation, depolymerisation and cleavage of the lignocelluloisic components. Advantages includes zero toxin output, high yield of products at a lower energy input, and high degree of specificity [94] [95]. Biological treatments includes both whole cell and enzymatic pre-treatments having limitation such as higher residence time, need for careful control, space requirement etc. [96]. Brown rot, white rot, soft rot, *Sacccharomyces*, *Actinomycetes*, certain bacterial species are some of the microorganisms involved in this method, which effectively removes lignin and hemicellulose, while having less effect on cellulose [97].

The major bottlenecks of successful pre-treatment processes are high capital cost, inconsistent yield, and lack of rapid and reliable tools to accurately assess biomass components. Since pre-treatment methods involves complicated upstream and downstream processes, chemical recycling and waste treatment techniques, optimization and control becomes difficult in most cases [98].

## I. PROCESS OPTIMIZATION AND ITS IMPORTANCE

Any process optimization involves continuous adaptation of a process in order to improve them while maintaining certain limitations. It may include identification of weakness, strengths, opportunities and threats, and finding appropriate solutions to overcome them. It is also required to achieve desired competitiveness with respect to an existing standard process. .Fermentation process condition and media composition play vital roles as they affect the production, concentration and overall yield of any fermentation end product. Since this is directly related to cost effectiveness of that process, therefore it is important to take into account the overall optimization of the fermentation process. Any optimization process is very laborious, open ended, time consuming and expensive in nature as it involves repeated replicates to be performed for a certain experiment. Besides, generation of new variants and mutants also necessitates periodical optimization of any bioprocess. Experiments with different combinations of factors like process conditions, process sequence, media components etc. must be performed to investigate best growth conditions that produces highest amount of product.

During optimization of fermentation, it is important to decide that whether we should perform the experiments in close ended or in open ended systems. Close ended systems follow simple strategies, where fixed number and type of components are analysed. On the other hand open ended systems relies on analysis of any number and type of components for optimization, which is beneficial, since this system makes no assumption of the best possible parameters to be selected. Therefore, it is always better to start with an open ended system and then analyse with a closed system [99].

During fermentation, composition of the fermenting substrate plays a vital role, which may affect final product concentration and yield along with cost of downstream and recovery

processes. The factors that determine the media composition include type of fermentation, nature of strain, type of primary and secondary metabolites produced and separation process adapted for product recovery. Media composition can be successfully statistically optimized taking into account all empirical and theoretical data, and it is considered as a distinct part that connects upstream and downstream systems of any fermentation process [100] [101].

Optimization techniques relies on various methods starting from borrowing, component swapping, biological mimicry, one-factor-at-a-time, factorial design to Plackett and Burman design, central composite design, response surface methodology, evolutionary operation, evolutionary operation factorial design, artificial neural network, fuzzy logic, genetic algorithms etc. Each of these methods have their own set of advantages and limitations and have to be selected depending on their suitability to the type of experiments carried out [99].

## J. CELL IMMOBILIZATION

Cell immobilization can be defined as the physical confinement or localization of intact cells to a certain region of space; without loss of desired biological activity. Scientists have successfully performed microencapsulation and Nano-encapsulation of cells collectively called bio-encapsulation. The physical and biochemical properties of the immobilizing matrix and the nature of application determines the suitability of the immobilization technique for any specific microorganism. The following are some of the desirable characteristics of any cell immobilization system:

- ✓ High capacity of cell mass loading
- ✓ Easy access to the nutrients of media
- ✓ Simple and nontoxic method
- ✓ Have high surface to volume ratio
- ✓ Ability to do optimum mass transfer
- ✓ Can be easily sterilized and reused

- ✓ Cells and carriers can be easily separated from media
- ✓ Good for cell suspension and anchorage in conventional or non-conventional reactor systems
- ✓ Biocompatibility with animal cell
- ✓ Should be economical

Immobilized cell system that meets all the criteria are found to be advantageous as compared to free cell during fermentation process. Generally the yield is found to be higher for the former along with the convenience of reusing, product purification, recovery and preservation. The only limitation is the biomass loss that may happen sometimes through the immobilization matrix into the solution decreasing the efficiency of the process.

**I. Types of cell immobilization**

Based on physical localization and type of microenvironment, immobilized cells can be classified as follows (Figure 5):

These immobilization techniques utilizes different types of matrices in order to remain embedded with the cells [102].

**II. Cell immobilization matrices**

Cell immobilization matrices are made of different materials that can range from natural, synthetic, inorganic etc. Natural polymers can be prepared from natural materials whereas synthetic and inorganic matrices are artificially generated. Synthetic polymers can be ion exchange resins that provides insoluble supports which can hold and trap cells in them. Inorganic polymers are made of inorganic materials which provides a sturdy support which can hold cells inside its porous structure. Table 5 enlists some of the variations in matrices of each type that are capable of immobilizing some specific cells.

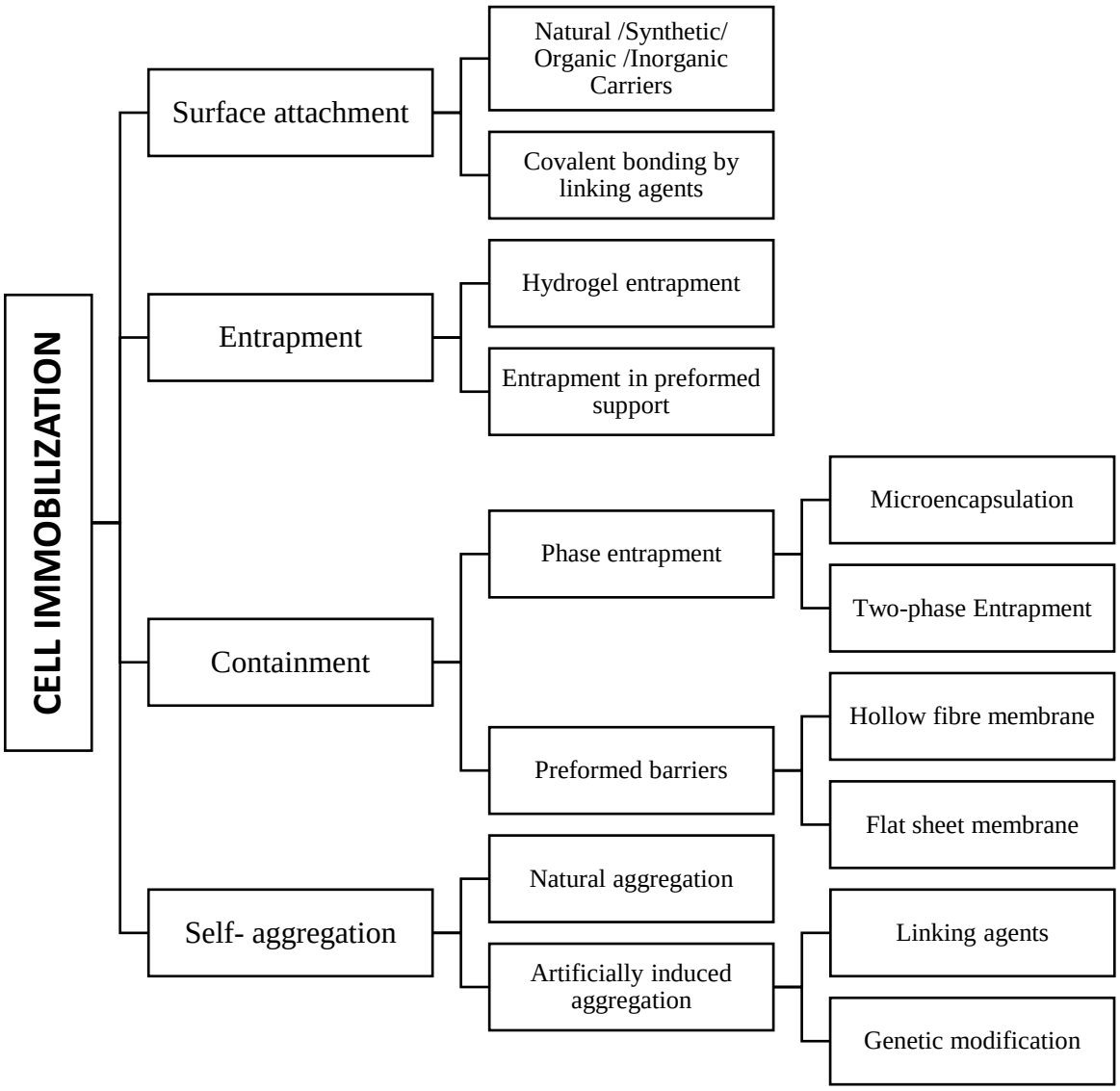


**Figure 5:** Types of cell immobilization process

The table below has enlisted some of the frequently used combinations; however, new and innovation combinations are continuously developing in the area of cell immobilization for performing cost effective sustainable fermentation process.

**Table 5:** Microorganisms and their respective matrices for immobilization

| **Natural polymers** | | |
|---|---|---|
| ***Name of the matrix*** | ***Microorganisms used*** | ***Reference*** |
| Alginate | *Saccharomyces cerevisiae, Bacillus sp, Pseudomonas sp.* | [103, 104, 105, 106] |
| Agar/ Agarose | *Bacillus sp, Escherichia coli, Lycinibacillus sp, Yeast.* | [107, 108, 109, 110] |

| | | |
|---|---|---|
| Chitosan/Chitin | *Bacillus sp, Escherichia coli* | [104, 111] |
| Collagen | *Microbacterium arborescens, Saccharomyces cerevisiae* | [112, 113] |
| Carrageenan | *Zymomonas mobilis, Escherichia coli, Saccharomyces cerevisiae, Bacillus sp.* | [114,115, 116, 117] |
| Gelatin | *Echerichia coli, Streptomyces roseofulus, Saccharomyces cerevisiae, Erwinia* sp. | [118, 119, 120] |
| Cellulose | *Streptomyces griseobrunneus, Bacillus paramycoides, Lactobacillus delbruecki, Yarrowia lipolytica, Echerichia coli.* | [121, 122, 123, 124] |
| Starch | Lactic Acid Bacteria and Yeast | [125] |
| Pectin | *Nocardia tartaricans, Saccharomyces cerevisiae, Candida guilliermondii* | [126,127] |
| | **Synthetic polymers** | |
| Polyvinyl Chloride | Yeast, Ammonia-oxidizing bacteria. | [128, 129] |
| | **Inorganic polymers** | |
| Zeolite | *Aureobasidium pullulans, Aspergillus oryzae.* | [130, 131,137] |
| Ceramics | *Phanerochaete chrysosporium, Saccharomyces cerevisiae* | [132] |
| Diatomaceous Earth | *Alcaligenes denitrificans, Bacillus sphaericus* | [133, 134, 135] |
| Silica Glass | *Escherichia coli, Staphylococcus aureus, Aspergillus oryzae.* | [136] |
| Activated carbon | *Aspergillus oryzae,* Methanogenic organisms | [137, 138,139] |
| Charcoal | *Bacillus firmus, Pseudomonas, Achromobacter, Ochrobactrum y Stenotrophomonas, Bacillus cereus.* | [140,141,142] |

## K. APPLICATION OF RESPONSE SURFACE METHODOLOGY (RSM) IN PROCESS OPTIMIZATION

One of most useful and advanced tool of process optimization especially fermentation can be achieved through response surface methodology or RSM. It helps to identify the best operating conditions for a particular process and how the objective is affected by a set of conditions related to that objective. It minimizes experimental load and saves time considering all possible combinations to simulate the final outcome [143].

RSM operates on different types of models and expertise is needed in order to choose the model suitable for any optimization. The central composite design (CCD) is one such model which is advantageous as it is very accurate and eliminates the necessity of three level factorial experiment for getting a quadratic model. It is also known as "Box-Wilson Central Composite Design". In this model, the centre points are augmented with group of points that

allows estimation of equation. The centre points are aligned with ±1 and ± α values with |α| > 1where the value of α depends on specific properties of the concerned design. CCD contains various factors on which more than two or many star points i.e. higher and lower extremities are applicable. The CCD model can be extended on a two level factor which makes them very suitable for response surface modelling and optimization [144].

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CHAPTER 1

# Chapter title: Selection of wastes for fermentation

## 1.1. INTRODUCTION

Constant disposal of food and agricultural wastes on a daily basis is hazardous to the environment, and contributes to increased carbon footprint of the world. Food and Agricultural Organisation (FAO) pointed out that this practice may also severely affect climate change [1]. Local vendors and large supermarkets are some of the chief contributors to this waste problem [2, 3]. Food waste can categorically affect different elements of ecosystem to create global nuisance and food shortage. [4, 5].

Bagasse is one of the lignocellulosic biomasses generated after sugarcane juice expression, which has been used for the production of bio-alcohol either by direct inoculation [6, 7, 8] or by using immobilized cells to improve yield [9]. Some studies reported genetically engineered xylose fermenting strain used to ferment sugarcane bagasse hydrolysate that may contain some inhibitory substances from food waste [10].Beside *Saccharomyces*, some novel strains having capacity to ferment pentose were also used for bagasse waste utilization [11].

Spent tea is one of the popular solid phase medium for cell mass growth, when enzymatic or acidic saccharification process were adopted at optimized conditions[12,13] for different sources of tea. Spent worm tea shows good potential as a pre-treatment process for second generation bioethanol production [14]. Fungal biomass can be grown on spent tea to be used as inoculum for fermentation process [15].

Hydrolyzed potato wash water contains more sugar than raw potato wash water. The hydrolysis and saccharification can be done both by acidic treatment [16, 17] and by enzymatic reactions of the potato starch [18]. Overnight soaked water [18], potato mash [19], rotten soft potato [20], potato tubers [21] or potato peels [22] are some of the substrates from

potato which may generate acetone, butanol and majorly ethanol. Integrated membrane extraction of the fermented substrate helps in extraction of different components from potato waste [23].

In the current chapter, three wastes; potato wash water, spent tea and bagasse were selected based on their availability, cost, bulk production, ease of processing and handling. Extract of these wastes were preferred than solid state fermentation, since the latter may involve much elaborate processes that will increase the cost due to involvement of more number of unit operations.

## 1.2. MATERIAL AND METHODS

### *1.2.1. Treatment of potato wash water*

The potato wash water was collected from the local potato chips factory where they have soaked potato slices in water for approximately 15 minutes as shown in (Figure 1.1 a).The resulting wash water was subjected to hydrolysis using–i) 5% (v/v) of concentrated hydrochloric acid under condition of 95° - 100°C for 60 minutes [17] and ii) 1% (v/v) of concentrated hydrochloric acid under condition of 121°C for 15 minutes at 15 psi respectively.

### *1.2.2. Water extraction of spent tea*

The spent tea leaves were collected from local tea sellers and air dried for a while. 10 g spent tea sample was mixed with 150 ml water to boil for 2 to 5 minutes. Five different extracts

were prepared following the same procedure every time reusing the spent tea from the previous extract (Figure 1.1b).

a  b 

**Figure 1.1.** *(a) Potato pieces soaked in water to get potato wash water and (b) Spent tea extract kept after extracting 5 times simultaneously (1st to 5th extraction)*

### *1.2.3. Water extraction of bagasse*

Bagasse was collected from local sugarcane juice sellers and was cut into small pieces (Figure 1.2a) of 1 inch in length approximately (Figure 1.2b). Every 5 g batch of bagasse 200 ml water was added to get the extract under boiling condition (Figure1.2c). The water extract was collected for three different time periods 5 minutes, 30 minutes and 60 minutes to check the extent of extraction of sugar.

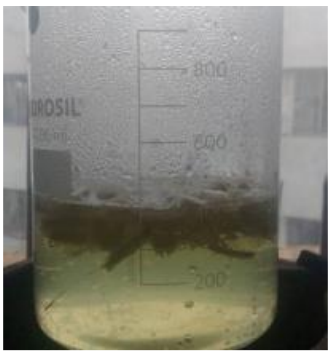
a  b  c 

**Figure 1.2.** *(a) Collected bagasse pieces from local vendor, (b) Bagasse cut pieces each of approximately 1 inch length (c) Extraction of bagasse pieces in water at 90-100 °C*

*1.2.4. Estimation of reducing sugar*

After treatment and neutralization, all the samples were analysed for reducing sugar content by using dinitrosalicylic acid method with respect to a standard glucose solution using an UV-Vis spectrophotometer (Shimadzu, Japan) at 540 nm wavelength [17, 24, 25].

*1.2.5. Inoculum preparation and fermentation of substrate*

The reducing sugar content helped to determine the conditions, under which the waste substrates can be utilized to produce ethanol. 50 ml of each of the three of waste sample was taken, and seeded with 5 % (v/v) of *Saccharomyces cerevisiae* MTCC 180 [26] culture (Figure 1.3a). The inoculum preparation includes culturing the strain in Yeast Extract-Peptone-Dextrose (YEPD) broth media [27] for 48 hours. Fermentation was carried out (Figure 1.3b) in an acidic pH of 3-4 at 30-32°C, and the ethanol content readings were taken at every 24 hours for up to 72 hours.

*1.2.6. Estimation of ethanol content*

The ethanol content was estimated by using dichromate titration method. The sample to be tested was placed in a small beaker above 10 ml acidified dichromate solution at 30 °C overnight. Water and ethanol slowly evaporates and comes in contact with dichromate where it gets dissolved. With time all the ethanol evaporates and react with the excess dichromate and become oxidized to form ethanoic acid. The amount of unreacted dichromate in the solution was estimated by adding potassium iodide solution and titrating with a standard solution of sodium thiosulfate. The colour turns light yellow when starch solution is added

and then on continuation of titration the blue black colour turns colourless marking the end point [28, 29, 30]. Readings were taken every 24, 48 and 72 h during fermentation (Figure 1.3c) to estimate the amount of alcohol generated in the process.

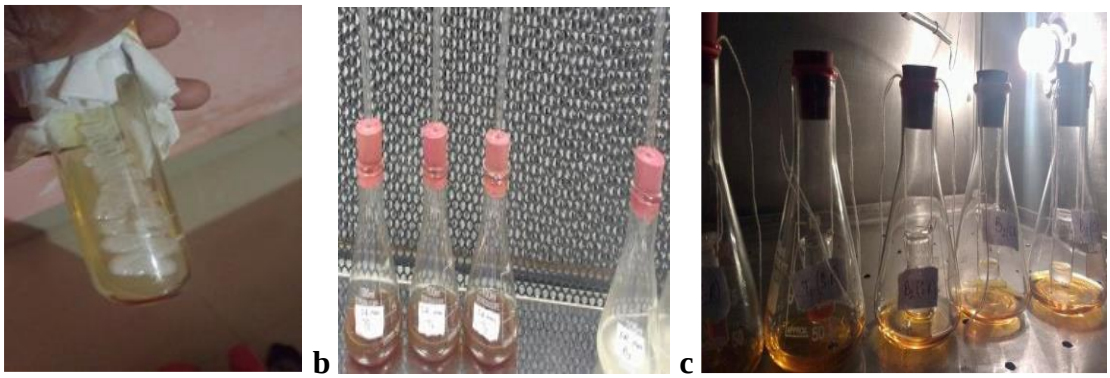


**Figure 1.3.** *(a) Picture showing Saccharomyces cerevisiae MTCC 180 strain preserved in streaked condition in a YEPD slant in the Department of Food Technology, Haldia Institute of Technology, (b) Picture showing batch fermentation of different selected wastes using yeast under anaerobic condition (c) Picture showing overnight hanging and incubation of fermented sample for ethanol estimation using dichromate method.*

### *1.2.7. Statistical Analysis*

Three replicates of experiments were performed for all the values. Statistical values of standard deviation for each finding were analysed using one way ANOVA (MS excel 2013), and the results were analysed using least significant differences ($P < 0.05$).

## 1.3 RESULT AND DISCUSSION

### *1.3.1. Selection based on reducing sugar content of the samples*

The reducing sugar estimation of the wastes i.e., potato wash water, spent tea and bagasse are shown in (Table 1.1) and elaborated in Figure 1.4.

**Table 1.1** Reducing sugar content of three different treated and raw wastes

| Name of the waste | Sample name | Reducing sugar (G/litre) |
|---|---|---|
| Potato wash water | Untreated Potato wash water | 0.13± 0.003[a] |
| | Autoclaved Potato wash water | 0.495± 0.01[b] |
| | Hydrolysed Potato wash water | 0.705± 0.03[b] |
| Spent tea | 1st extraction | 0.053± 0.02[a] |
| | 2nd extraction | 0.012± 0.007[b] |
| | 3rd extraction | 0.0087± 0.00[b] |
| | 4th extraction | 0.0087± 0.007[b] |
| | 5th extraction | 0.0048± 0.001[b] |
| Bagasse | 5 min Extract | 0.524± 0.07[a] |
| | 30 min Extract | 0.764± 0.05[b] |
| | 60 min Extract | 0.679± 0.09[a] |

*Values in the table are Mean ± S.D of three replicates; superscript letters adjacent to each value indicate significant difference ($p<0.05$)*

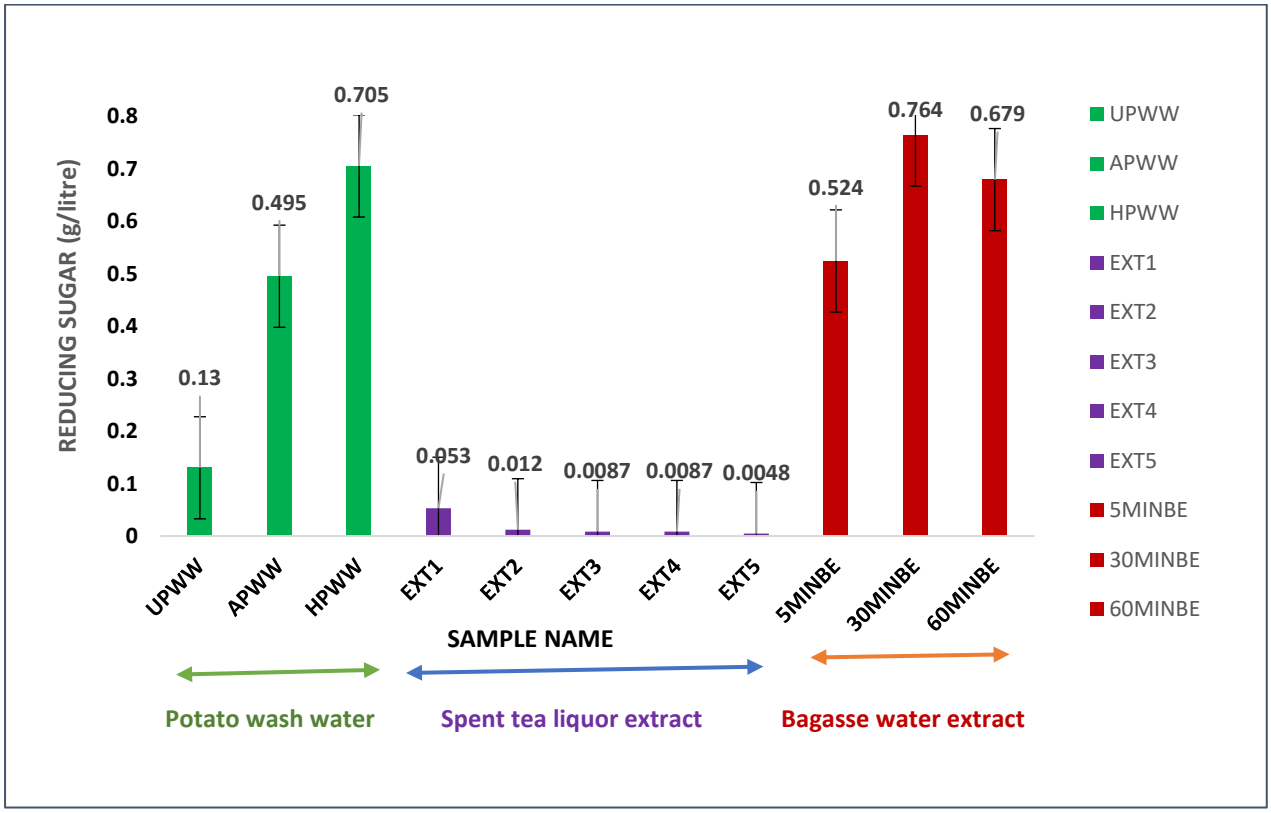



**Figure 1.4.** *Graph showing reducing sugar content of the selected wastes measured using dinitrosalicylic acid method*

The reducing sugar contents in three different waste samples, as estimated by DNS method, were significantly different with the bagasse water extract showing higher value of 0.764± 0.005 g/litre at 30 min extraction. The potato wash water sample yielded lower concentrations of reducing sugar, which improved upon heat treatment and hydrolysis. The higher mean value of 0.705± 0.03 g/litre of reducing sugar from hydrolysed potato wash water was comparable with results obtained from bagasse extract. The spent tea sample showed higher value with the first extraction at 0.053± 0.02 g/litre, and decreased sharply subsequently up to fifth extraction. The best substrate combinations in all the three wastes were selected for further experiments.

This study reveals that hydrochloric acid hydrolysis of potato starch gives higher glucose yield than any other method applied to treat potato wash water, and this was consistent with the results reported by another study [31]. Spent tea aqueous extracts were also used for ethanol production and found to be more effective producing 21% (v/v) of alcohol at temperature of 80°C and pH 8 [32]. However, sugarcane bagasse aqueous extracts showed in bioethanol generation in the world and extensively used as a substrate in various forms [33].

### *1.3.2. Selection based on ethanol content of the samples*

The 30 min bagasse water extract, the hydrolysed potato wash water and the first extraction of spent tea were fermented using *Saccharomyces cerevisiae* MTCC 180, and the ethanol contents are tabulated below (Table 1.2).

**Table 1.2** Ethanol contents of three different selected wastes

| Name of the waste | Ethanol content (g/ 100ml) | | |
|---|---|---|---|
| | **24 h** | **48 h** | **72 h** |
| 30 min bagasse water extract | 0.126±0.00[a] | 0.202±0.03[a] | 0.094±0.00[a] |
| Hydrolysed potato wash water | 0.054±0.00[c] | 0.145±0.00[c] | 0.017±0.01[b] |
| First extraction of spent tea | 0.0207±0.00[b] | 0.073±0.00[b] | 0.047±0.00[c] |

*Values in the table are mean ± SD of three determinations, superscript letters in a column differ significantly ($p<0.05$)*

The ethanol estimation gives higher values with bagasse extract followed by hydrolysed potato wash water and spent tea. The 24 h ethanol yield for bagasse extract started with moderate value of 0.126 g/100 ml, which then showed higher ethanol yield of 0.202±0.03 g/ 100ml at 48 h which gradually decrease at 72 hr. The ethanol yield pattern for hydrolysed potato wash water was more or less same as bagasse extract only with lower ethanol yields. The first extraction of spent tea showed relatively flat curve with lower values of ethanol at all times during fermentation (Figure 1.4).

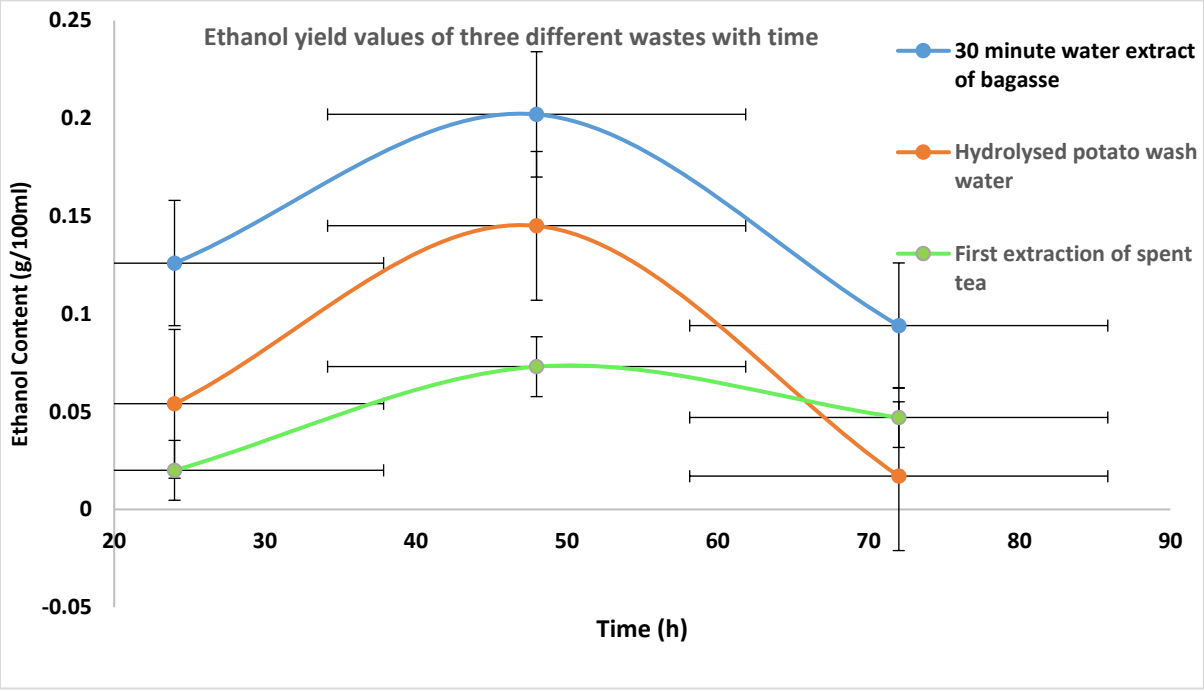



**Figure 1.5.** *Graph showing variations in ethanol content of fermented bagasse extract, spent tea extract and potato wash water sample with change in fermentation time*

Therefore, based on both the reducing sugar content and ethanol production, the 30 min sugarcane bagasse extract was chosen as the best waste substrate for the current study.

Scientific investigation reported water extraction of alkaline bagasse pulp at 160 °C for 30 min to get a low viscosity liquor having lower amount of bleaching residues present [34].

Hot water pre-treatment of bagasse was reported as environmentally beneficial as there is reduced deterioration of polysaccharides, and consequently lower inhibitor generation [35]. Inhibitors like furfural generation is also lowered at reduced temperature and absence of acidic environment [36], which is advantageous in case of bioethanol production.

## 1.4. CONCLUSION

Wastes generated on a daily basis in different food industries due to processing of food creates the problem of waste generation and accumulation in the environment. These wastes can be reutilized to form various important compounds like ethanol, which may help to deal with the worsening fossil fuel crisis as well. In this study, three different waste substrates were selected, and acid and water extracts were prepared from these wastes. Reducing sugar content and ethanol estimation of the aqueous extracts revealed that 30 min extraction of sugarcane bagasse resulted in the best substrate for ethanol production through *S. cerevisiae* MTCC 180. However, further optimization of the fermentation conditions and nutritional supplementation of the chosen substrate are needed to increase ethanol yield.

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CHAPTER 2

# Chapter title: Treatment of wastes to make them suitable as substrate for ethanol fermentation

## 2.1 INTRODUCTION

Municipal solid waste contains about 50%–60% organic wastes including food waste, sometimes referred to as organic fraction of municipal solid waste. Food waste consists of both cooked and uncooked wastes generated from different public places like community kitchens, hotels, households, festivals, canteens as well as food processing industries, and from even from each home kitchen. Treatment or disposal of these wastes may sometimes lead to production of uncontrolled amount of greenhouse gases from the landfill. The diversification of waste in the landfill can be regulated if it is treated at source using techniques like anaerobic digestion, UV treatment, fermentation, composting, etc. Though these biological processes are gaining potential in terms of waste treatment, abundant availability of water needed to perform successful waste treatment operation remains a major challenge. Food wastes having low solid do not require any pre-treatment but if the solid content is more than 15 %, pre-treatment methods can be used [1].

Local sugarcane juice vendors and sugar refineries produces huge amount of bagasse wastes that forms 30 – 40 % of the total waste generated globally. Bagasse is the fibrous solid left after juice extraction from sugarcane that is used as raw material for sugar production. One tonne of sugarcane processing operation produces about 280 kg of bagasse, and tonnes of this wastes are regularly processed worldwide to reduce or convert it into useful products. Compared to other agro industrial wastes, low ash content of bagasse (about 1.9 % by wt.) makes it suitable for valorisation into various value added products, which is an effective solution to the problem associated with environmental accumulation of this waste. Bagasse

primarily contains cellulose (48.3%), hemicellulose (28.6%) and lignin (23.5%) along with some other ingredients in lesser quantities [2].

Pre-treatment methods can be broadly classified as physical, chemical and biological. Treatments such as acid or alkali treatment, drying, organic solvent treatment, steam explosion, hot water treatment or fermentation using fungal biomass have been used for efficient utilization of this waste. [3]. Many studies reported the use of aqueous extract of bagasse, pre-treated or without any treatment, in order to produce industrially valuable products [4, 5, 6].Dry bagasse contains 1.86 % of reducing sugar, which, if extracted in solution, can be fermented into ethanol using microbes such as yeasts as fermenting starter [7]. Thus, bioethanol is one of the major industrial products that can be generated from bagasse [8] along with other applications like energy production through combustion, production of biochar, activated carbon generation, in gasification, pulping to name a few. In the current study, aqueous extracts of wet and dry bagasse were utilized as potential substrates for ethanol production by yeast fermentation.

## 2.2. MATERIAL AND METHODS

### *2.2.1. Fourier Transform Infra-red (FTIR) spectroscopy of aqueous extract of bagasse*

The aqueous extract of bagasse was investigated using mid FTIR range spectroscopy (Thermo-Fisher Scientific, Nicolet 6700) for analysis of the substrate before it was supplemented with the nutrients. Within the mid infrared range, observations were taken between the wave numbers 400 $cm^{-1}$ and 4000 $cm^{-1}$. The results were obtained as absorbance vs. wave number, and the peaks were analysed using the Origin Pro®, Version 2022 b software, Massachusetts.

*2.2.2. Drying of wet bagasse*

Wet bagasse was collected from sugarcane juice seller containing approximately 40-50% moisture, cut into 1 inch pieces, weighed, and put into drying. To dry the bagasse, the tray drier (IIC-INSTIND, Kolkata) was preheated and brought to a temperature of 70°C. The bagasse were put into the tray for drying [9]. The moisture content in bagasse samples measured using the following equation.

$$\text{Moisture \%} = \frac{(\text{Initial weight of Bagasse} - \text{Final weight of Bagasse after drying}) * 100}{\text{Initial weight of Bagasse}}$$

*2.2.3. Preparation of water extract of wet and dry bagasse*

The water extract of both wet and dry bagasse were prepared by taking 5 g wet or dry bagasse mixed with 200 ml water, and boiled at 95°-100°C for 30 min. Then the extract was subjected cooled to room temperature, the suspended solids and fibres were separated using a double layered muslin cloth, and the filtrate was used as the substrate for fermentation [10].

*2.2.4. Determination of quality parameters of wet and dry bagasse extract*

The pH of the wet and dry bagasse extracts were measured by digital pH meter (Systronics μ pH system 361, India) and pH paper simultaneously. The total soluble solid in the aqueous extracts were determined by a hand-held Refractometer (ERMA RBH 62, New Delhi, India). Reducing sugar content was estimated by dinitrosalicylic acid (DNS) method using an UV-Vis spectrophotometer (Shimadzu-UV 1800, United States) using at 540 nm wavelength [11].

The Dextrose and the dinitrosalicylic acid were sourced from SRL chemicals and Himedia laboratories Pvt. Ltd. Respectively.

*2.2.5. Inoculum preparation and substrate fermentation*

50 ml of wet and dry bagasse extracts were autoclaved after pH adjustment, cooled and seeded with 5 % (v/v) of *Saccharomyces cerevisiae* MTCC 180 culture [12]. The inoculum preparation was carried out in YEPD broth for 48 hours [13].Fermentation was carried out in an acidic pH of 3-4 at 30-32°C, and ethanol contents were measured at 24 h intervals. The hydrochloric acid and the sodium hydroxide, yeast extract, peptone and dextrose were sourced from Qualigens fine chemicals Pvt. Ltd. and SRL chemicals respectively.

*2.2.6. Estimation of ethanol*

The process has been described in details in chapter 1 section 1.2.6. of materials and method.

## 2.3. RESULT AND DISCUSSIONS

*2.3.1. Analysis of FTIR peaks*

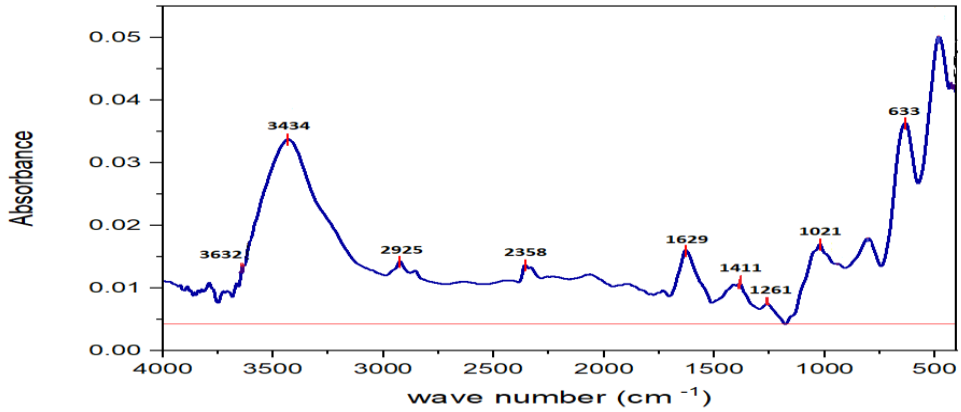


**Figure 2.1** *Figure showing FTIR spectra of aqueous extract of bagasse*

The FTIR plot (Figure 2.1.) clearly shows presence of majority of carbohydrate molecules along with some other contaminants and impurities. The carbohydrates contains both alcohol and ether linkages in their structure. The alcohols can be primary or secondary alcohols depending on the molecule. So, they have distinct C-O and O-H stretching regions with some wagging as well. More hydroxyl groups are indicative of extensive hydrogen bonding, whereas the strong broad O-H stretch in the plot is indicative of the same hydrogen bonding in carbohydrates. The presence of a small peak at 3632 and a strong peak at 3433 are indicative of the presence of very small amount of free alcohol and some substantial amounts of intermolecular bonded alcohol. The region shows striking similarity with IR spectrum of sucrose as described by Smith (2017), who worked with glucose and sucrose solutions individually [14]. This indicates presence of some amounts of sucrose in the bagasse extract, which have remained in the waste, and were transferred into the extract. The wavenumber 1021 $cm^{-1}$ shows association with multiple sugar rings connected by ether linkages present in many carbohydrates. It is also indicative of presence of cellulose molecule that gives peak at 1029 $cm^{-1}$. The small peaks at 1411 may indicate presence of xylan, a major hemicellulose component, which usually gives peak at 1419, while the blob at 1261 may indicate presence of lignin, which usually gives peak at 1266 $cm^{-1}$[15]. The peak at 1629 indicates absorbed water in carbohydrate molecules. It gives a small peak, which indicates atmospheric water absorption by several O-H groups due to their polar and hygroscopic nature. The lone saturated hydrogen molecules present will give a C-H stretch at around 2900. In this sample, due to mixture of carbohydrates and lesser number of lone hydrogen, the peak is comparatively less intense, low in height, and the peak value is 2925 [14, 16]. The peak at the 2358 wavenumber indicates presence of O = C = O bond, which usually gives peak at 2350 [17]. The peak at 633 is indicative of the presence of hydrocarbon structures. C-H bending vibrations of terminal alkynes can be found in the range of 610-700 in the form of a strong

peak that is similar to the results reported by another study [18].Beside these peaks, some insignificant peaks in the diagram may indicate presence of impurities in the extract.

*2.3.2. Drying values for wet bagasse*

The tray drying to get dry bagasse was carried out for 150 min, at 70 ºC which are shown in Figure 2.2.

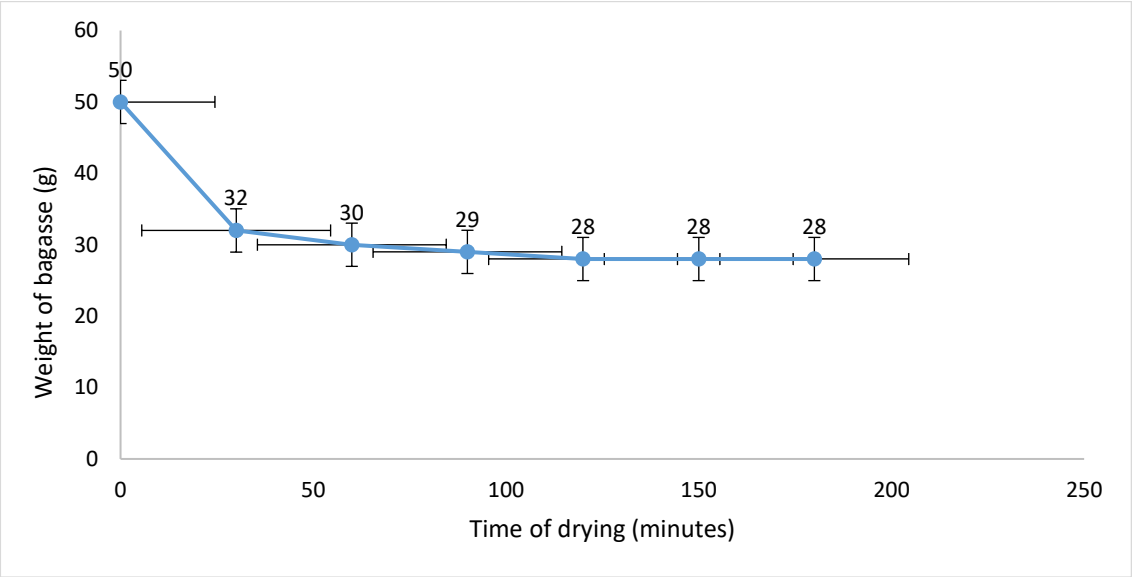


**Figure 2.2.** *Drying parameters of wet bagasse sample in tray drying operation with increase in drying time*

The tray drying is not a very common practice when it comes to drying of bagasse, and traditionally, solar drying or oven drying is commonly used. In this study, 50 g of the collected bagasse sample was dried at 70°C for 90 min in a tray drier.

Hot air oven drying and open air drying are the two most traditional methods of drying bagasse. Both these methods are very time consuming and laborious. Microwave oven heating of bagasse was carried out for drying bagasse to be used as fuel and the process took 20-25 minutes [19] as compared to other methods like solar drying, burning other fuel for drying, counter-current drying, rotary drum drying etc. [20]. Kinetic study of fixed bed tray drying of Caja bagasse was carried out, and the results revealed that drying temperature of 75°C, with dryer inlet air velocity of 6.0 m.s-1 and cake thickness of 0.8 cm resulted in the

most efficient drying [21]. However, nowadays industrial scale bagasse drying is done using rotary drum dryer or pneumatic dryers.

### *2.3.3. Quality parameters comparative study*

The pH, total soluble solids and reducing sugar content in wet and dry bagasse are enlisted in Table 2.1.

**Table 2.1.** Quality parameters of wet and dry bagasse

| Sample | pH | Total Soluble solid (°B) | Reducing sugar content (mg/ml) |
|---|---|---|---|
| 30 minute water extract of wet bagasse at 95-100 ° C | 6.6 | 0.9 | 0.764 ± 0.05 |
| 30 minute water extract of dried bagasse at 95-100 °C | 6.51 | 1 | 0.778 ± 0.06 |

The comparative quality analysis of wet and dry bagasse reveals that they have similar pH, total soluble solid and reducing sugar contents. The pH values for both samples have shown almost neutral values of approximately 6.5 compared to 3-4 pH of pre-hydrolysed bagasse, as reported in the literature [7]. Yeast strains need lower values of pH for fermenting bagasse to produce ethanol. One study reported a fermentation pH of 5.5 at 32°C utilizing bagasse as the substrate [22]. Another confirmed production of bioethanol at pH 4.5 and 35°C temperature by saccharomyces yeast [23].Though some strains like *Pichia stipites* can produce compounds like xylose at 30 °C and pH 6.0 which saccharomyces is unable to produce at that pH [24].This means that an optimization of pH of the bagasse extract is needed to achieve optimum ethanol yield by *S. cerevisiae* fermentation. The soluble solid content range of

hydrolysed bagasse is 12-23% [25] compared to a lower value range of 0.9-1%, which was quite obvious, as the latter was not hydrolysed. *Saccharomyces* yeast fermenting tchapalo showed a decrease of TSS value from 13.5 to approximately 11 [26]. The requirement of higher range of TSS value showed that the media must be supplemented with nutrient(s) in order to get optimum ethanol yield. The reducing sugar content decreased significantly in the aqueous extracts, as compared to normal, untreated or pre-hydrolysed bagasse [7].Normally yeast has a maximum sugar tolerance of about 40%; some may even be able to tolerate up to 65% sugar [8].

*2.3.4. Alcohol content after fermentation*

After fermentation of both wet and dry bagasse extracts by *S. cerevisiae* MTCC 180, the ethanol contents are tabulated in Table 2.2.

**Table 2.2.** Alcohol content of fermented aqueous extract of wet and dry bagasse

| Sample | Alcohol content (g/100ml) |
|---|---|
| 30 minute water extract of wet bagasse at 95-100 °C | 0.126± 0.009 |
| 30 minute water extract of dried bagasse at 95-100 °C | 0.151 ± 0.023 |

The alcohol content in case of dry bagasse extract was slightly higher as compared to that in case of wet bagasse extract, but the difference was not significant. This signifies that two pronged fermentation strategy may be adopted. Firstly, nutritional supplementation to improve the nutritional content of the fermentation substrate or selecting an yeast strain that is capable of giving good ethanol yield even at lower concentrations of reducing sugar. So, in this study, a secondary fermenting strain of yeast i.e., *S. cerevisiae* MTCC 180 was selected

which was used as an inoculum in kinnow wine preparation and it has high alcohol tolerance as reported in a previous study [27].

## 2.4. CONCLUSION

Agro-industrial wastes like sugarcane bagasse can become an environmental nuisance if it is openly dumped and/or if it ends up in landfill. This waste can typically be used to create a wide range of industrially important products. Bioethanol can be obtained from this waste after simple pre-treatment or nutritional supplementation, followed by fermentation using different yeast strains. Such pre-treatment processes can enhance the available amount of fermentable sugar to be utilized by the fermenting yeast. This study reveals that the composition of aqueous extract chiefly consists of disaccharides along with some absorbed water and various other impurities. Drying has no significant effect on primary quality parameters of bagasse, but it may lead to slightly higher ethanol yield, but the yield was not very significantly different from wet bagasse ethanol yield. Therefore we will not include this operation for the further studies on this substrate for fermentation.

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CHAPTER 3

# Chapter title: Optimization of the ethanol fermentation conditions utilizing bagasse extract

## 3.1. INTRODUCTION

All over the world, people are alarmed by the impact of industrialization and fossil-fuel derived energy consumption on environment. Moreover, conventional fuels, including fossil fuels, are becoming more expensive due to their increasing scarcity [1]. These global concerns have fuelled research in the areas of non-conventional and renewable energy sources. Solid wastes, including food processing / handling wastes are also major environmental concerns in many countries. In this context, renewable, alternative resources such as bio-alcohol, biogas, bio-oils or solid wastes (including food processing / handling wastes) have been gaining popularity and research interests in recent years [2,3,4,5].Biofuels can be produced from discarded agricultural biomass such as post-harvest residues, sugarcane processing waste, or wastewater from food processing industries. The cost effectiveness of the raw materials helps them remain economically feasible in competition with fossil fuels. The added advantage is the environment friendly nature and renewable resources having significant contribution towards reduction of greenhouse gas (GHG) emission [6].About 150 billion tons of lignocellulosic materials are used annually as renewable natural resources for fuel generation worldwide [7].Crop residues like carrot or onion peel [8],straw [9],fruit wastes like date palm [10],olive pulp [11],or food processing wastes like molasses [12],corncobs [13], apple pomace [14], etc. can be used as substrate(s) for *Saccharomyces cerevisiae* fermentation. They can be used to generate industrially important and commercially valuable second generation (2G) bioethanol through fermentation.

Bagasse is a common agro-industrial waste, which is generated not only by the sugar processing industries, but also by local sugarcane juice vendors. About 30-34 tonnes of bagasse is generated after processing 100 tonnes of sugarcane in a factory [15, 16].Small scale industries and local vendors usually dispose these wastes with hardly any treatment, and often in locations, where they may cause harmful impacts on environment e.g. growth and proliferation of pathogenic microorganisms in untreated wastes. *S. cerevisiae* MTCC 180 is a secondary fermentation strain, and can perform better to give good ethanol yield even with a substrate containing low total soluble solids. Many scientific investigations have reported on the efficiency of the strain in alcohol generation [17, 18]. This chapter standardized different fermentation conditions for *S. cerevisiae* MTCC 180 fermentation of aqueous extract of bagasse, and statistically optimized some parameters using central composite design model of response surface methodology.

## 3.2. MATERIALS AND METHODS

### *3.2.1. Preparation of inoculum*

The yeast strain *Saccharomyces cerevisiae* MTCC 180 was procured from the MTCC, preserved at Department of Food Technology, Haldia Institute of Technology, West Bengal, in Yeast Extract Peptone Dextrose (YEPD) media (3 g yeast extract, 10 g peptone, 20 g dextrose, and 15 g agar mixed with 1000 ml distilled water; pH 6.5 ± 0.2) under refrigerated condition (4°C). The strain was sub-cultured in YEPD broth for preparation of fermentation inoculum [19]. All chemicals used were sourced from SRL chemicals.

### *3.2.2. Preparation of aqueous extract of bagasse*

The process has been described in details in chapter 2 section 2.2.2. of materials and method.

### *3.2.3. Optimization of fermentation conditions*

Five different parameters were chosen initially using one variable at a time (OVAT), in order to optimize bioethanol yield based on pre-experiments and literature survey. The effect of pH of the substrate was studied by varying the pH from 3 to 7 using 0.1 (N) sodium hydroxide and/or 0.1 (N) hydrochloric acid. Each time, the pH was measured by using pH paper and by digital pH meter (Systronics μ pH system 361, India) simultaneously. The temperature of fermentation was varied from 25° to 40°C, while the duration of fermentation was varied from 24 to 72 h to study the ethanol yield. . The process of fermentation was carried out using different stages of *S. cerevisiae* MTCC 180 culture growth, starting from 24 h to 168 h culture, and the inoculum size was varied from 5 to 10% (v/v) for optimization of ethanol yield. The fermentation processes were carried out in 100 ml conical flasks, where the bagasse extract (50 ml) was adjusted to the fermentation conditions, and the final substrate was autoclaved. After cooling to ambient temperature, the sterilized substrate was inoculated with measured volume of freshly sub-cultured *S. cerevisiae* MTCC 180 culture. The set up was kept under incubation at the required temperature for a specific period of time, as detailed earlier.

### *3.2.4. Experimental design and statistical data analysis*

Three replicates of each of the experiments were performed in OVAT, and the averages and the standard deviations were analysed using one way ANOVA (MS Excel 2013) at significance level of $P< 0.05$. The Coefficient of variation (CoV), $t_{critical}$ values at α value of 0.05 were also obtained. Fisher's Post Hoc test of least significant difference (LSD) values

were calculated to check the significance of the obtained value in accordance with the given formula in equation 1.

(1) $LSD = t_{crit}\sqrt{MSW\left({}^{1}/_{N_1} + {}^{1}/_{N_2}\right)}$

Where, MSW is the mean square value within groups, $N_1$ and $N_2$ are the respective sample sizes. Since three replicates for each of the values were obtained, so in this case, $N_1 = N_2 = 3$ [20].

The effect of temperature and time were again optimized using RSM. The CCD was utilized for interpreting values that matches with polynomial model of second order having two independent factors time and temperature and one dependent factor ethanol yield. The data was taken in five level having five centre points. The fitness of the model was expressed by the values of coefficient of determination ($R^2$). The three dimensional response surface plots give the relationship between the responses and the factors to maximize ethanol production. The amalgamation of these reformed variables that produced better yield of ethanol was examined for model validation. The absolute residual error was found out from the given formula in equation 2 [21].

(2) $Absolute\ residual\ error\ (\%) = \frac{Experimental\ value - Predicted\ value}{Experimental\ value} \times 100$

Design-Expert Version 7.0.0, was used for analysis for statistical validation 2021, Stat-Ease (Minneapolis, USA).

*3.2.5. Ethanol Estimation*

The process has been described in details in chapter 1 section 1.2.6. of materials and method. The ethanol production rate was calculated by the following equation 3 [22].

(3) $Ethanol\ production\ rate\ (g/L/h) = \frac{Final\ ethanol\ concentration\ (g/L)}{Fermentation\ time\ (h)}$

## 3.3. RESULTS AND DISCUSSION

### *3.3.1. Fermentation condition optimization using OVAT*

The optimization study of fermentation carried out under submerged conditions using *S. cerevisiae* MTCC 180 in bagasse extract resulted in the following ethanol contents, as shown in Table 3.1. and elaborated in Figure 3.1.

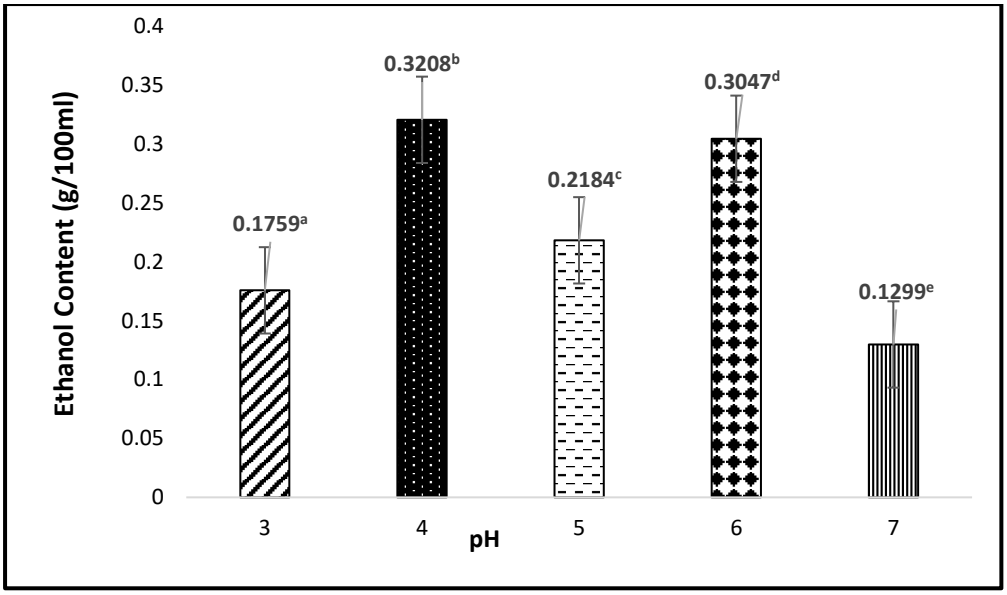
**Table 3.1:** Statistical values for optimization of fermentation conditions using OVAT

| | **Values** | **Ethanol content (g/100ml)** | **CoV** | **$t_{0.05}$** | **$LSD_{0.05}$** |
|---|---|---|---|---|---|
| **pH** | 3 | 0.1759±0.0034[a] | 0.0196 | 2.2281 | 0.009 |
| | 4 | 0.3208± 0.0034[b] | 0.0107 | | |
| | 5 | 0.2184±0.002[c] | 0.0092 | | |
| | 6 | 0.3047±0.05[d] | 0.0173 | | |
| | 7 | 0.1299±0.08[e] | 0.067 | | |
| **Temperature (° C)** | 25 | 0.1847±0.005[a] | 0.0282 | 2.306 | 0.007 |
| | 30 | 0.3162±0.002[b] | 0.0063 | | |
| | 35 | 0.2483±0.003[c] | 0.0138 | | |
| | 40 | 0.2633±0.005[d] | 0.02 | | |
| **Time ( h)** | 24 | 0.3162±0.002[a] | 0.0063 | 2.4469 | 0.01 |
| | 48 | 0.2437±0.007[b] | 0.0294 | | |
| | 72 | 0.2092±0.05[c] | 0.0252 | | |

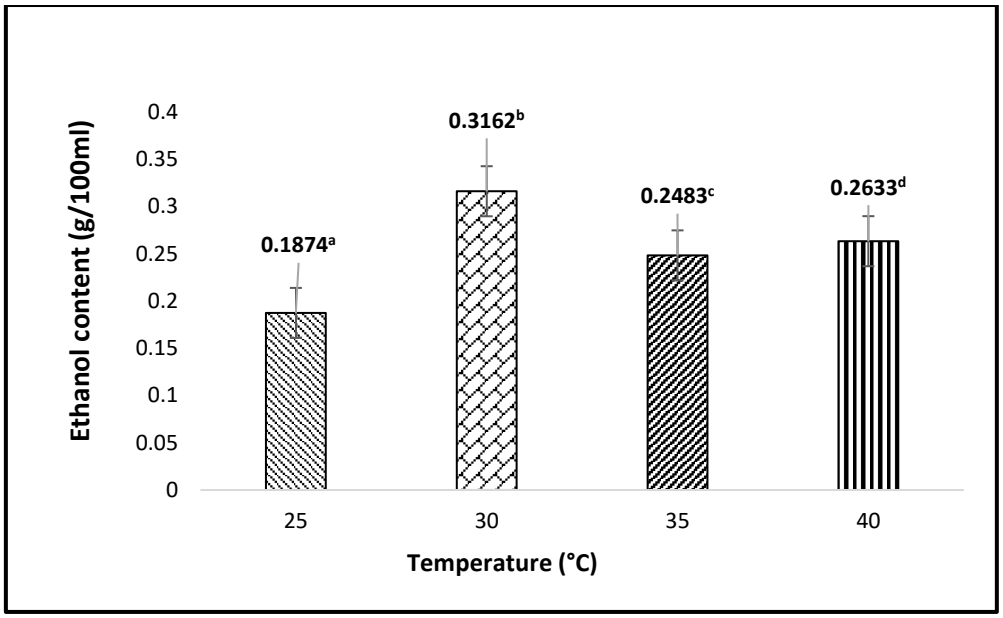
| | | | | | |
|---|---|---|---|---|---|
| **Inoculum age (h)** | 24 | 0.1678± 0.001[a] | 0.0116 | 2.1447 | 0.007 |
| | 48 | 0.3185±0.001[b] | 0.0061 | | |
| | 72 | 0.2093±0.003[c] | 0.019 | | |
| | 96 | 0.1977±0.002[d] | 0.0102 | | |
| | 120 | 0.0908±0.005[e] | 0.0582 | | |
| | 144 | 0.0517±0.003[f] | 0.0666 | | |
| | 168 | 0.0287±0.008[g] | 0.3012 | | |
| **Inoculum size (%)** | 5 | 0.3151±0.003[a] | 0.0126 | 2.1788 | 0.007 |
| | 6 | 0.3265±0.001[b] | 0.006 | | |
| | 7 | 0.1345±0.003[c] | 0.0256 | | |
| | 8 | 0.1437±0.003[d] | 0.2777 | | |
| | 9 | 0.169±0.006[e] | 0.0408 | | |
| | 10 | 0.115±0.003[f] | 0.0346 | | |

Fermentation reaction is sensitive to pH changes, and yeasts generally prefer acidic pH for their growth. The highest ethanol yield of about 0.3208±0.0034 g/100 ml was achieved at pH 4. Similar results were reported when *Aspergillus* spp. S4B2F strain was used on enzymatically hydrolysed sugarcane bagasse [23]. The effect of temperature on yeast activity has been well established by published reports [24]. In distiller's malt wort, it was reported that maximum yeast production occurred at 30°C in non-aerated culture, while it was highest at 35°C in aerated culture.

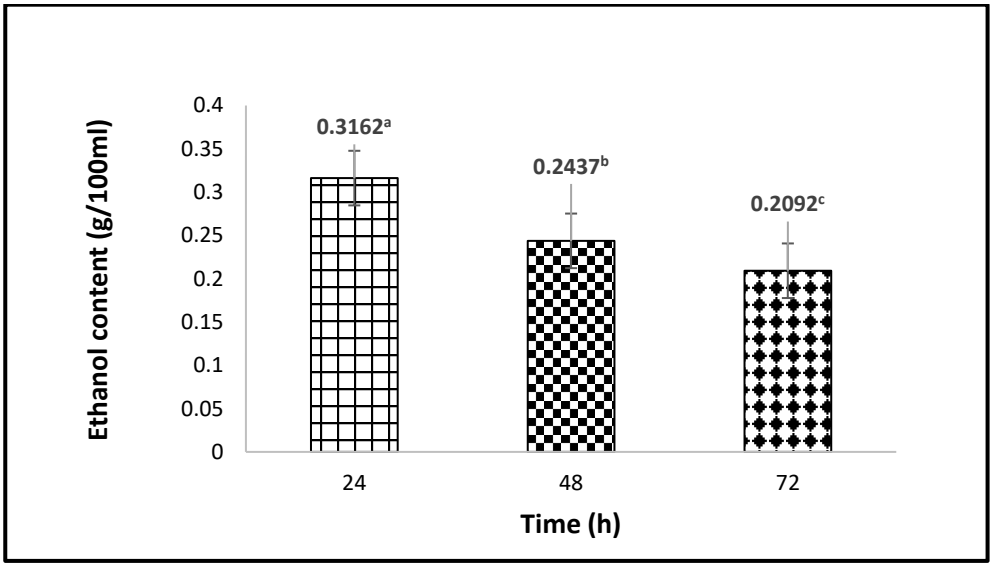
a


Ethanol Content (g/100ml)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.1759a
0.3208b
0.2184c
0.3047d
0.1299e
3
4
5
6
7
pH

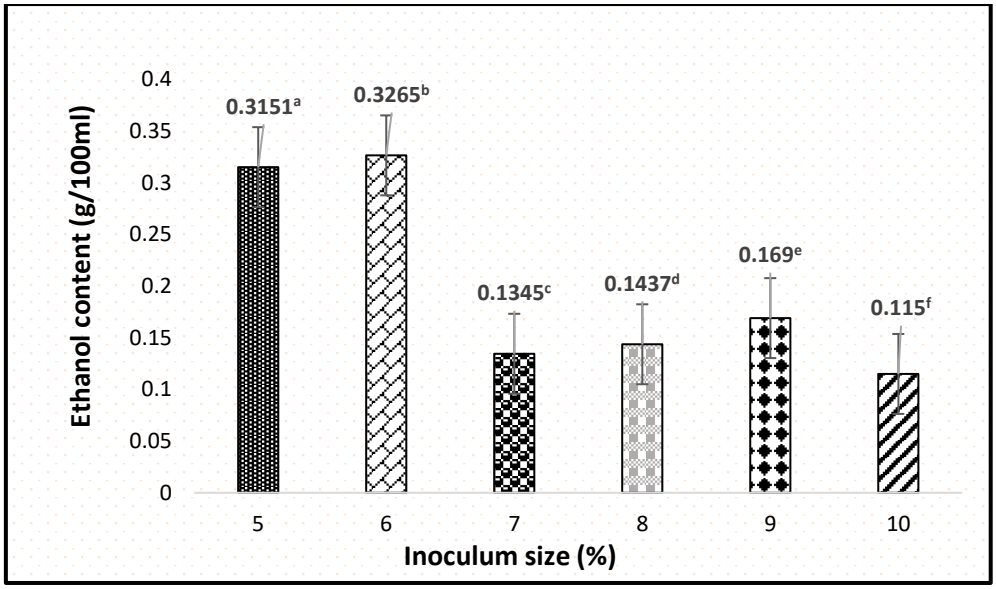
b


Ethanol content (g/100ml)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.1874a
0.3162b
0.2483c
0.2633d
25
30
35
40
Temperature (°C)

c


Ethanol content (g/100ml)
0.4
0.35
0.3
0.25
0.2
0.15
0.1
0.05
0
0.3162a
0.2437b
0.2092c
24
48
72
Time (h)

d




e

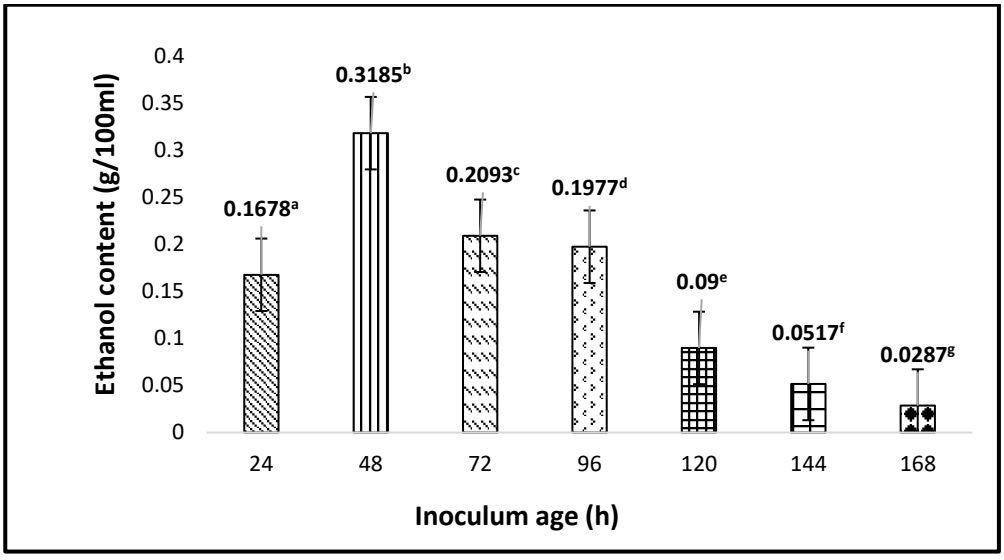



**Figure 3.1.** *Graphical representation of optimization of fermentation conditions- a) pH, b) temperature, c) time, d) inoculum size and e) inoculum age, using OVAT*

In this study, the optimum ethanol yield of 0.3162±0.002 g/100 ml was achieved at 30°C, while the yield decreased at lower and higher temperatures of incubation. Similar result was also reported by another study [25] about optimum ethanol yield at 30°C fermentation. The *S. cerevisiae* MTCC 180 culture showed a gradual decrease in the ethanol content as fermentation time progressed beyond 24 h (0.3162±0.002 g/100 ml). This decrease in ethanol production can be attributed to not only the organism reaching equilibrium growth at around

24 h, but also due to some loss of ethanol due to evaporation and/or due to rapid metabolism by the yeast. Optimum ethanol production may be achieved by much shorter incubation period, if bagasse hydrolysate is used as fermentation substrate, instead of intact bagasse or aqueous extract of bagasse [25]. A wide variation in ethanol production was achieved using different size of inoculum, further increase of which resulted in faster depletion of nutrients leading to decline in ethanol yield. An ethanol yield of 0.3265±0.001g/100 ml was obtained at 6% inoculum size that was found to be significantly higher compared to those obtained using other inoculum sizes. Numerous similar studies, reported optimum ethanol yield at 5% (v/v) *S. cerevisiae* inoculum size in fodder beet [26], bagasse hydrolysate [25], and paper sludge [27].Maximum ethanol yield of 0.3185±0.001 g/100 ml was obtained using a 48 h old culture for fermentation. After 48 h, most cells in the yeast culture were mature and active, and if inoculated at this level of maturity, it produces primary metabolite like ethanol at their rapid growth phase. However, further ageing of yeast cells may lead them into their death phase, rendering the culture incapable of producing optimum ethanol yield. Some reported optimum ethanol production by 36 h old *S. cerevisiae* culture fermenting rice wine cake[28], while a 48 h old *Aspergillus niger* culture was reported to produce optimum ethanol yield from palm fruit fermentation [29].

### *3.3.2. Optimization of Ethanol production using Response Surface Methodology*

The two most influential factors viz., time and temperature were further optimized through response surface methodology. The actual and coded values for the design of the experiment are enlisted in Table 3.2.

**Table 3.2:** The actual and coded values for the experiment using yeast for fermenting aqueous extract of bagasse

| Factor | Name with Unit | Low Actual | High Actual | Low coded | High coded | Mean | Std. dev |
|---|---|---|---|---|---|---|---|
| A | Time (h) | 21.17 | 26.83 | -1.000 | 1.000 | 24.00 | 2.219 |
| B | Temperature (°C) | 28.59 | 31.41 | -1.000 | 1.000 | 30.00 | 1.109 |

The final equation in terms of actual factors is given as follows-

*Ethanol Content = -39.59845 +0.55004* Time +2.23188 * Temp -8.31250E-003*Time * Temp -6.61562E-003* Time$^2$ -0.033837 * Temp$^2$*

The experimental design, shown in Table 3.3., gives the possible combinations of process parameters to maximize ethanol yield.

**Table 3.3:** Experimental design and responses of time-temperature optimization using central composite design

| Run | Time (hours) | Temperature (° C) | Observed Response | Predicted Response |
|---|---|---|---|---|
| 1 | 21.17 | 28.59 | 0.22 | 0.20 |
| 2 | 24.00 | 30.00 | 0.27 | 0.31 |
| 3 | 24.00 | 30.00 | 0.33 | 0.31 |
| 4 | 26.83 | 31.41 | 0.13 | 0.11 |
| 5 | 28.00 | 30.00 | 0.12 | 0.14 |
| 6 | 24.00 | 32.00 | 0.17 | 0.18 |
| 7 | 24.00 | 28.00 | 0.15 | 0.17 |
| 8 | 21.17 | 31.41 | 0.28 | 0.27 |
| 9 | 20.00 | 30.00 | 0.26 | 0.27 |
| 10 | 24.00 | 30.00 | 0.32 | 0.31 |

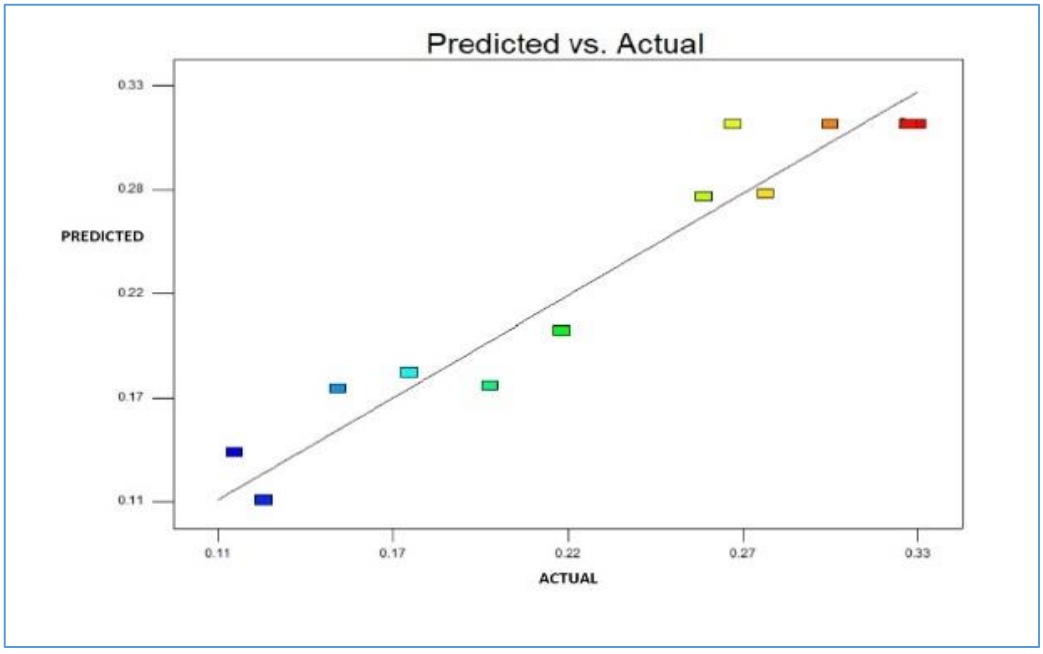
| | | | | |
|---|---|---|---|---|
| 11 | 26.83 | 28.59 | 0.20 | 0.17 |
| 12 | 24.00 | 30.00 | 0.33 | 0.31 |
| 13 | 24.00 | 30.00 | 0.30 | 0.31 |

**Table 3.4:** Analysis of variance (ANOVA) data for optimization of aqueous extract of bagasse fermentation

| **Source** | **Sum of Squares** | **DF** | **F value** | **Prob > F** | |
|---|---|---|---|---|---|
| Model | 0.068 | 5 | 20.74 | 0.0005 | Significant |
| A-Time | 0.018 | 1 | 27.68 | 0.0012 | |
| B-Temp | 7.268E-005 | 1 | 0.11 | 0.7496 | |
| AB | 4.422E-003 | 1 | 6.71 | 0.0359 | |
| $A^2$ | 0.019 | 1 | 29.56 | 0.0010 | |
| $B^2$ | 0.032 | 1 | 48.34 | 0.0002 | |
| Residual | 4.614E-003 | 7 | | | |
| Lack of Fit | 2.140E-003 | 3 | 1.15 | 0.4299 | Not Significant |
| Pure Error | 2.473E-003 | 4 | | | |
| Cor Total | 0.073 | 12 | | | |

| | | | |
|---|---|---|---|
| Std. Dev. | 0.026 | R-Squared | 0.9368 |
| Mean | 0.24 | Adj R-Squared | 0.8916 |
| C.V. % | 10.91 | Pred R-Squared | 0.7385 |
| PRESS | 0.019 | Adeq Precision | 11.386 |

The data shows fitness with a second order polynomial equation, which are considered statistically significant at $P < 0.05$. In this case, the linear as well as quadratic effect of fermentation time (A, $A^2$), interactive effect of time and temperature (AB), and quadratic effect of temperature ($B^2$) are the significant model terms. F-value of model of 20.74 proves the significance of model ($P < 0.05$).Only 0.05% chance that this large F valueis by noise.Lack of Fit value of 1.15 implies the Lack of Fit as not significant with respect to the pure error. Only 42.99% chance that a "Lack of Fit " is by noise. Goodness of fit was checked by values of determination coefficient ($R^2$). As shown in Table 3.4., the $R^2$ value of 0.9368 was in good agreement with the adjusted $R^2$ value of 0.8916 and predicted $R^2$ value of 0.7385 having adequate precision of 11.38. The predicted versus actual curve shows conformation between the predicted value and the actual data (Figure 3.1.a). Lack of trend in the plot shows data with acceptable variance without any outliners of experimentation (Figure 3.1.b).The coefficient of variation (CV) was also as low as 10.91, which is indicative of low deviations between experimental and predicted values. Value of "adequate precision ratio" of 11.38 indicates an ample signal.




**a**

b

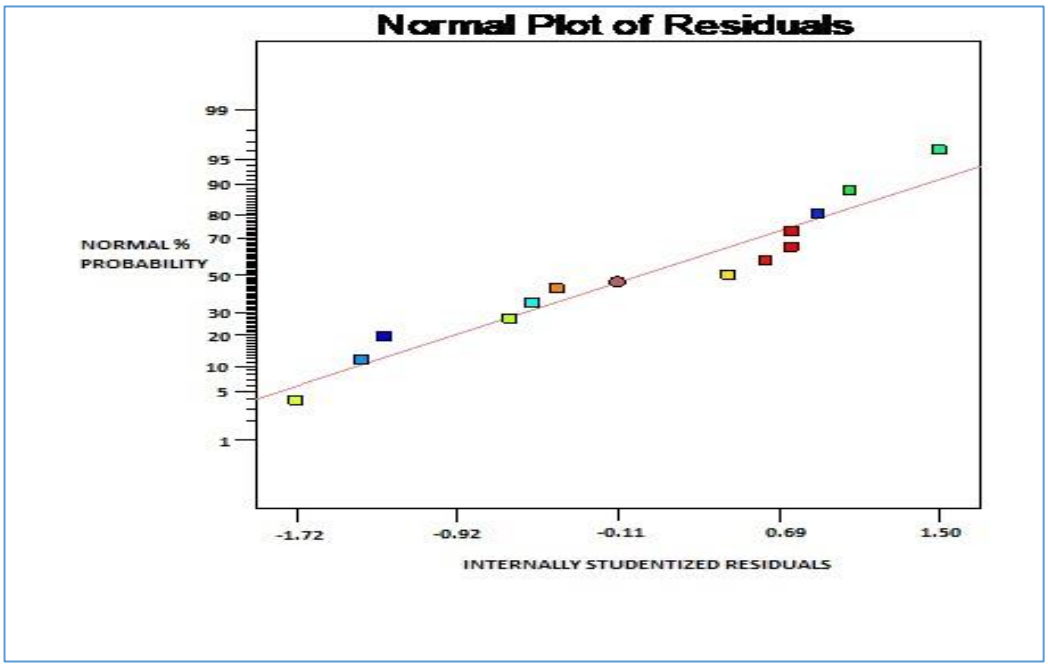
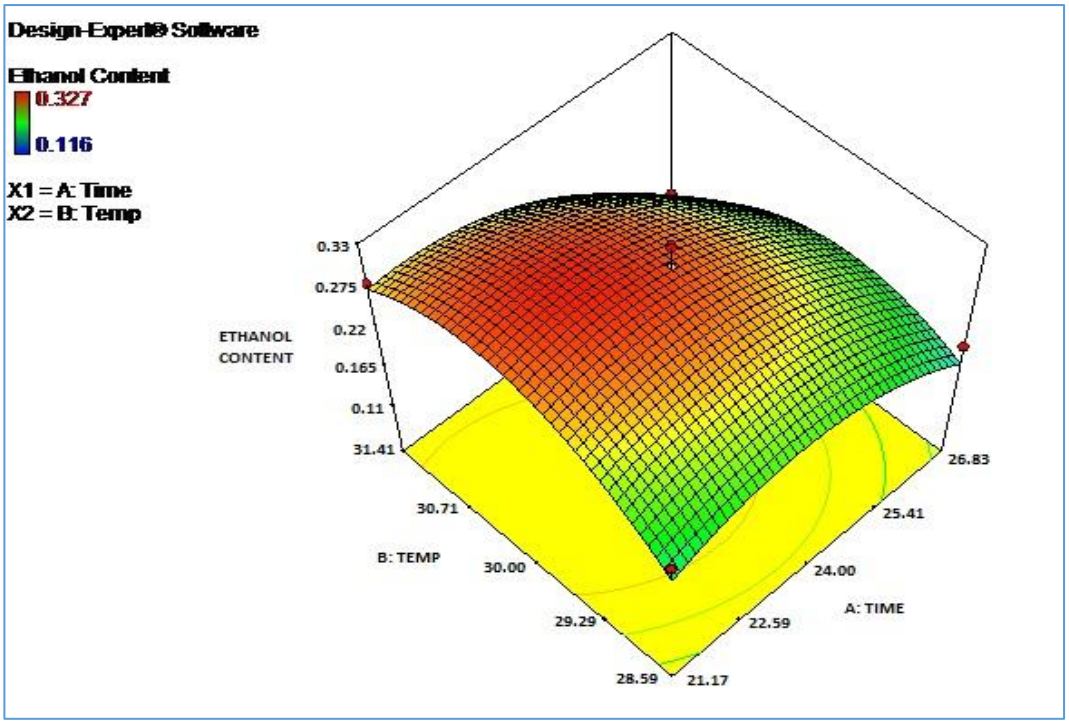



**Figure 3.2.** *Plot of experimental versus predicted values (a) and plot of studentized residual versus predicted values (b) of bagasse extract fermentation by S. cerevisiae.*

The three dimensional and interaction plots help to find out the values for each factor to maximize ethanol production from *Saccharomyces cerevisiae* MTCC 180 which can be shown in Figure 3.2. From the results shown as the graphical interpretation, it is very clear that with increase in time, the ethanol production has slightly increased up to 22.6 h after which there is a gradual decrease.

a




b

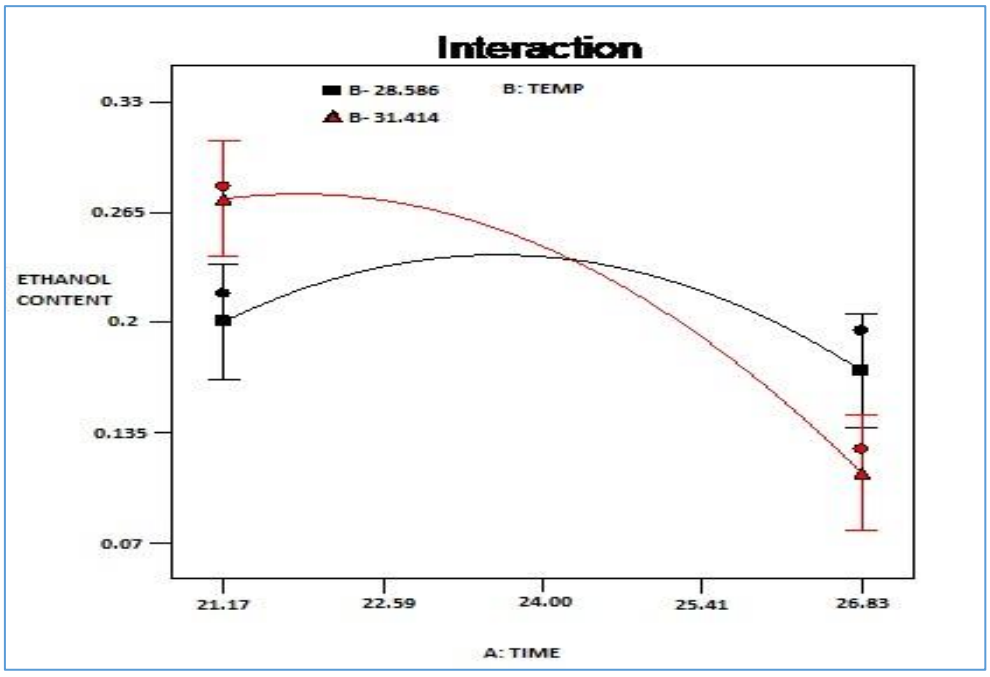



**Figure 3.3.** *Three dimensional (a) and linear plots (b) of the interaction of time and temperature variables with ethanol yield for bagasse extract fermentation by S. cerevisiae*

The ethanol productivity however has been found to increase sharply up to 30°C after which there is a gradual decline. At the lower value of temperature 28.58°C, the ethanol content decreases at a much slower pace; whereas at higher value of temperature 31.41°C, the ethanol

content shows a very sharp decline with time. Overall, though the nature of ethanol production follows almost similar patterns at lower as well as higher values of temperatures; the amount of ethanol produced is more at lower temperature and less fermentation time.

### *3.3.3. Validation of the predicted model*

The predicted time-temperature values best suited for ethanol production is at 22.60 h at30.20°C having ethanol yield of 0.321 gm/100 ml. The desirability function value of 0.975 is indicative of significance of the obtained result. In this condition, validation experiments were conducted to validate the obtained data. The experimental result was around 0.342 gm/100 ml which was conforming to the predicted value of 0.321gm/100 ml, having absolute residual error of 6.14 %. Several studies also found fermentation time and temperature as important parameters in studying optimization of fermentation process using synthetic media or various agro-industrial wastes for efficient generation of bioethanol [30, 31, 32, 33, 34]

## 3.4. CONCLUSION

This study yielded valuable results that may be useful in the utilization of one of the most important lignocelluloisic biomass generated in sugarcane processing. Bagasse, one of the most abundantly available agro-industrial and sugarcane processing wastes, was utilized in this study, and its aqueous extract was easily prepared, and converted into a nutrition-rich fermentable substrate for ethanol production. *S. cerevisiae* MTCC 180 strain was selected as the fermenting yeast strain, as it is capable of fermenting substrates with moderate to low sugar concentration. The optimization of fermentation parameters using single factor led to average ethanol yield of 0.3196 ± 0.004 g/100 ml at the rate of 0.133 g/L/h achieved at a substrate pH of 4, at 30°C fermentation temperature for 24 h using an inoculum size and age

of 6% (v/v) and 48 h, respectively. Rotatable central composite design of response surface methodology was applied for optimization of fermentation time (22.60 h) and temperature (30.20°C) which showed an ethanol yield of 0.321 g /100 ml at the rate of 0.142 g/L/h which is quite consistent with the result obtained initially by OVAT. The un-optimized fermentation media was giving 2.5 times less yield than optimized substrate having an absolute residual error of 6.14 %.Further nutritional supplementation studies using different sugar or nitrogen sources and introducing mineral sources can be evaluated to further improve fermentation efficiency and yield.

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CHAPTER 4

# Chapter title: Optimization of the nutritional parameters for the fermentation process

## 4.1. INTRODUCTION

Sugarcane bagasse is currently one of the world's principle solid wastes generated chiefly from sugar processing. India being one of the leading sugar-producing countries, about 370 million tonnes of sugarcane was produced in 2020 showing an average annual growth rate of 2.78% from 126 million tonnes produced in 1971 [1]. The manufacturing of sugar may generate several types of by-products and/or wastes that includes bagasse, molasses, fly ash, pressed mud, spent wash, etc., bagasse being the most economically important. Bagasse is the fibrous residue after juice is expressed, and it mainly consists of cellulose, hemicellulose and lignin [2]. It finds its use in several ways like fuel, ash for brick making, in paper industry or as fermentation substrate for production of ethanol.

The forms of bagasse used for fermentation substrate varied from simply using the solid residue after some chemical pre-treatment [3], or bagasse hydrolysate after mechanical refining [4] or vacuum concentration [5]. Several studies confirmed the use of various species of *Saccharomyces* yeast for fermenting food or agricultural wastes as well as lignocellulosic biomass like bagasse [6, 7]. *Saccharomyces bayanus* was used on pre-treated solid bagasse [8], while xylose fermenting *S. cerevisiae* strain fermented sugarcane bagasse hydrolysate to yield ethanol [9]. Fermentation conditions optimization and nutritional enrichment of these organic waste substrates can enhance the overall yield of bioethanol [10, 11]. Nutritional enrichment of low-cost media has been proven to be helpful in various studies especially for ethanol production using *Saccharomyces* species [12, 13, 14].

This chapter is focused on optimization of different nutritional supplementations for *S. cerevisiae* MTCC 180fermentation of aqueous extract of bagasse, including statistical optimization of some parameters using one factor at a time and one way ANOVA initially, and later using central composite design model of response surface methodology.

## 4.2. MATERIALS AND METHODS

### *4.2.1. Collection of sample and culture maintenance*

The process has been described in details in chapter 3 section 3.2.1. of materials and method.

### *4.2.2. Aqueous extract of bagasse preparation*

The process has been described in details in chapter 2 section 2.2.2. of materials and method.

### *4.2.3. Selection and optimization of nutritional conditions*

The aqueous extract of bagasse was fermented using the secondary fermenting yeast *S. cerevisiae* MTCC 180 culture. The conditions of fermentation were previously optimized i.e., pH of 4, fermentation duration of 22.06 hours, temperature of 30.20°C, inoculum size of 6%,and48 h old culture, as discussed in detail in chapter 3. Keeping these conditions constant, at first the sugar source, nitrogen source and the mineral source for the said process was selected and restructured using OVAT. Three different sugar sources such as glucose, fructose and sucrose at 1 % (w/v) concentration, and nitrogen sources like ammonium chloride, ammonium sulphate and urea at 1 % (w/v) concentration were tested for selection. To select a suitable mineral source, magnesium sulphate, ferrous sulphate, calcium chloride, potassium chloride and sodium chloride were used at 0.1 mg/ml concentration each.

Quantification of the sugar, nitrogen and mineral source was done after each selection process over a range of values.

*4.2.4. Experimental design and statistical data analysis*

Three replicates of each of the experiments were performed in OVAT, and the averages and the standard deviations were analysed using one way ANOVA (MS Excel 2013) at significance level of $P< 0.05$. The Coefficient of variation (CoV), $t_{critical}$ values at α value 0.05 were also obtained. Fisher's Post Hoc test of least significant difference (LSD) values were calculated to check the significance of the obtained value in accordance with the given formula in equation 1.

$$(1)\ LSD = t_{crit}\sqrt{MSW\left({}^{1}/_{N_1} + {}^{1}/_{N_2}\right)}$$

Where, MSW is the mean square value within groups, $N_1$ and $N_2$ are the respective sample sizes. Since three replicates for each of the values were obtained, so in this case, $N_1= N_2= 3$[15].

The variables were optimized using RSM. The CCD was utilized to interpret data conforms to second order polynomial model having three independent variables sucrose content, Ammonium sulphate content and potassium chloride content, and one dependent factor ethanol yield. The data was taken in five level having six centre points and the fitness of the model was expressed by the values of coefficient of determination ($R^2$). The three-dimensional response surface plots give the relationship between the responses and the factors to maximize ethanol production. The combination of these predicted parameters that gave maximum yield of ethanol were tested to validate the model. Design-Expert Version 7.0.0, was used for analysis for statistical validation 2021, Stat-Ease (Minneapolis, USA).

*4.2.5. Ethanol Estimation*

The process has been described in details in chapter 1 section 1.2.6. and chapter 3 section 3.2.5 of materials and method.

## 4.3. RESULT AND DISCUSSIONS

*4.3.1. Nutritional condition selection and optimization using OVAT*

Enrichment of the fermenting substrate with sugar, nitrogen and mineral sources resulted in optimum ethanol yield. Among the three sugars used for supplementation, sucrose was found to have the highest increment in ethanol production (Fig. 4.1.a) followed by fructose and glucose. Table 4.1.a) and b) gives the detailed values of ethanol contents, standard deviations, coefficient of variation (CV), $t_{critical}$ and least significant difference (LSD), which also complements the graphical data. As the sucrose supplementation was increased, ethanol yield increased, and reached the highest at 4% (w/v) level at 1.771 ± 0.1 g/ 100 ml (Fig. 4.1. b). After this value, there is a slight dip followed by almost a constant ethanol yield. This may be attributed to either increase in hydrolysate concentration that may inhibit yeast activity and/or to deactivation of the yeast enzymes due to increase in ethanol production.

**Table 4.1. a):** Selection of different sugars for fermentation using bagasse extract

| Sugar source | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| Glucose | $0.558\pm 0.01^{a}$ | 0.022 | | |
| Fructose | $0.584\pm 0.005^{b}$ | 0.008 | 2.44 | 0.018 |
| Sucrose | $0.605\pm0.009^{c}$ | 0.015 | | |

**Table 4.1. b):** Sucrose optimization for fermentation using bagasse extract

| Sucrose % | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| 0.5 | 0.520 ± 0.006[a] | 0.012 | | |
| 1 | 0.605 ± 0.009[a] | 0.015 | | |
| 2 | 0.759 ± 0.069[b] | 0.09 | | |
| 3 | 1.38 ± 0.069[c] | 0.05 | 2.11 | 0.135 |
| 4 | 1.771 ± 0.105[d] | 0.059 | | |
| 5 | 1.518 ± 0.119[e] | 0.078 | | |
| 6 | 1.632 ± 0.052[e] | 0.032 | | |
| 7 | 1.633 ± 0.105[e] | 0.064 | | |

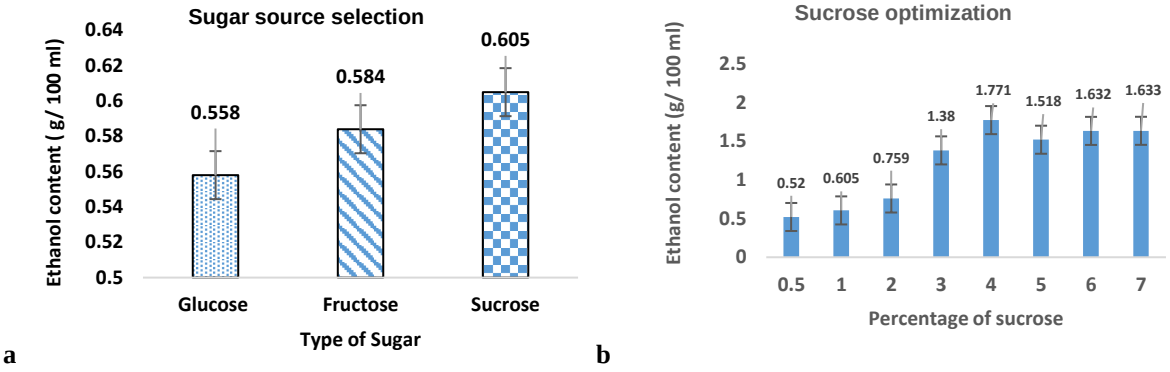



**Fig. 4.1.** *Graphs showing ethanol content after substrate supplementation with a) different sugar sources, b) optimization and quantification of selected sugar (sucrose) supplementation*

Some *S. cerevisiae* species may lack the major hexose transporters (hxt1-hxt7), and (gal2), and prefer sucrose instead of monosaccharide like glucose and fructose [16].The MTCC 180 strain used in this study may also have similar genotypic characteristic. Another study used sucrose as the primary sugar supplement in fermenting cashew apple bagasse for alcohol production by *S. cerevisiae* [17]. Published report also emphasized that fermentation

performance of *Saccharomyces* spp. may be hampered with high sucrose concentration above 3.1 % [18].

Three different sources of nitrogen such as ammonium sulphate, ammonium chloride and urea were used for supplementing the fermentation substrate at a fixed sucrose concentration of 4% (w/v). The values (Table 4.2.a) depicts that among the three nitrogen sources, ammonium sulphate supplementation produced the highest ethanol yield (Fig. 4.2.a). Though the ethanol yield values for ammonium sulphate and ammonium chloride were not significantly different; but based on previously published reports, as well as considering higher mean value of ethanol yield, ammonium sulphate was chosen as more suitable for nitrogen supplementation. Table 4.2.b shows that the highest ethanol content of 1.897±0.03 g/100 ml achieved at 0.5 % (w/v) ammonium sulphate concentration , and any further increase in concentration reduced the ethanol yield (Figure 4.2.b). A similar study used organic sources like yeast extract, peptone, tryptone and urea, and inorganic sources like ammonium sulphate, ammonium nitrate and ammonium chloride, each at 1% concentration, as nitrogen supplement in the fermentation substrate of bagasse hydrolysate to get ethanol yield of 61ml/l [19].

**Table 4.2. a):** Selection of different nitrogen source for fermentation using bagasse extract

| Nitrogen source | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| Ammonium Sulphate | 1.598±0.189[a] | 0.1188 | | |
| Ammonium Chloride | 1.253±0.071[a] | 0.0572 | 2.44 | 0.58 |
| Urea | 0.425± 0.468[b] | 1.101 | | |

**Table 4.2. b):** Ammonium sulphate optimization for fermentation using bagasse extract

| Ammonium sulphate % | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| 1 | 1.598±.189[a] | 0.118 | | |
| 0.5 | 1.897±0.03[b] | 0.018 | | |
| 0.1 | 1.632± 0.207[a] | 0.127 | 2.228 | 0.262 |
| 0.05 | 1.667 ± 0.143[ab] | 0.086 | | |
| 0.01 | 1.437± 0.052[a] | 0.036 | | |

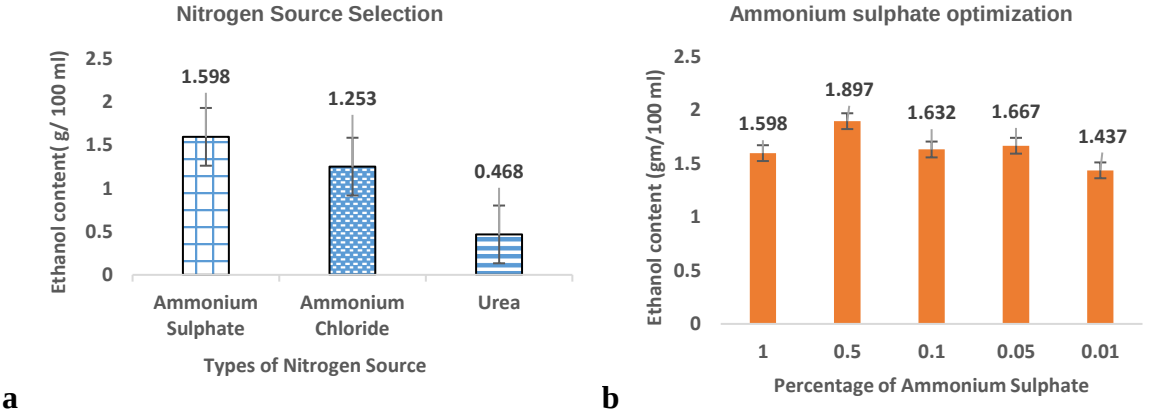



**Fig. 4.2.** *Graphs showing ethanol content for substrate supplementation with a) selected nitrogen sources, b) optimization and quantification of selected nitrogen source (ammonium sulphate) supplementation*

The mineral source selection showed the highest ethanol yield using potassium chloride closely followed by magnesium sulphate (Figure 4.3.a), when sucrose and ammonium sulphate were supplemented at 4% and at 0.5% (w/v) concentrations, respectively (Table 4.3.a). Potassium chloride optimization gives highest ethanol yield (1.598± 0.05 g/ 100 ml) at 0.1 mg/ml concentration (Figure 4.3.b, Table 4.3.b). A study using genetically modified *S. cerevisiae* in co-fermentation of glucose and xylose reported similar results, and suggested that substrate supplementation with salt can be, to a certain extent, inhibitory to the yeast

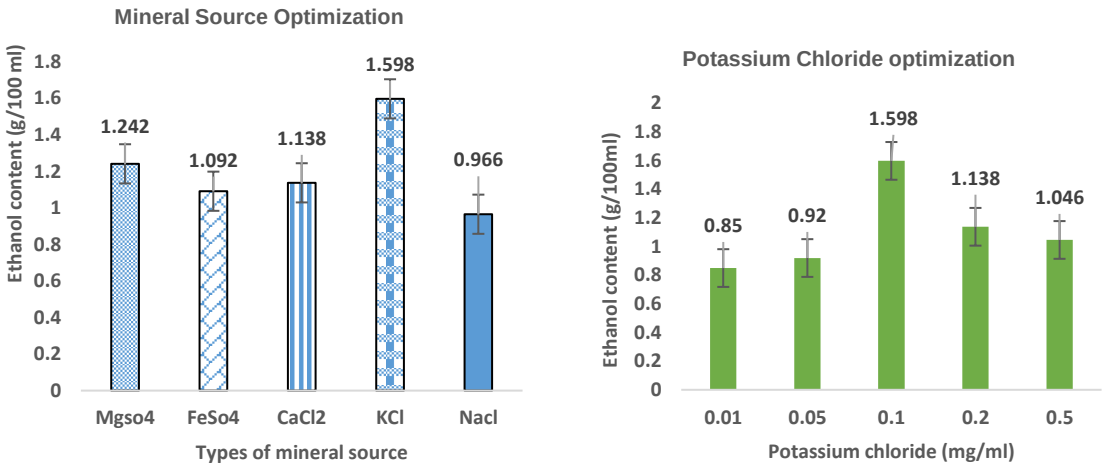
fermentation. However, they also reported that potassium salts are less inhibitory than others [20].

**Table 4.3. a):** Selection of different mineral source for fermentation using bagasse extract

| Mineral source | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| $MgSO_4$ | $1.242\pm 0.03^{a}$ | 0.027 | | |
| $FeSO_4$ | $1.092\pm 0.13^{a}$ | 0.119 | | |
| $CaCl_2$ | $1.138\pm 0.1^{a}$ | 0.09 | 2.228 | 0.176 |
| KCl | $1.598\pm 0.05^{b}$ | 0.033 | | |
| Nacl | $0.966\pm 0.12^{ac}$ | 0.128 | | |

**Table 4.3. b):** Potassium Chloride optimization for fermentation using bagasse extract

| Potassium chloride (mg/ml) | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| 0.01 | $0.85\pm 0.13^{a}$ | 0.153 | | |
| 0.05 | $0.92\pm0.1^{a}$ | 0.114 | | |
| 0.1 | $1.598\pm 0.05^{b}$ | 0.033 | 2.228 | 0.23 |
| 0.2 | $1.138\pm0.15^{ac}$ | 0.139 | | |
| 0.5 | $1.046\pm 0.16^{a}$ | 0.155 | | |




**a** **b**

**Fig. 4.3.** *Graphs showing ethanol content for substrate supplementation with a) selected mineral sources, b) optimization and quantification of selected mineral source (potassium chloride) supplementation*

### *4.3.2. Nutritional optimization using Response Surface Methodology*

The three factors for optimization of nutrients-sugar source, nitrogen source and mineral source were re-optimized through response surface methodology. The actual and coded values of the experimental design can be shown in Table 4.4.

**Table 4.4.:** The actual and coded values for the experiment in response surface methodology

| **Factor** | **Name with Unit** | **Low Actual** | **High Actual** | **Low coded** | **High coded** | **Mean** | **Std. dev** |
|---|---|---|---|---|---|---|---|
| A | Sucrose (%) | 3.41 | 4.59 | -1.000 | 1.000 | 4.000 | 0.491 |
| B | Ammonium Sulphate (%) | 0.44 | 0.56 | -1.000 | 1.000 | 0.500 | 0.049 |
| C | Potassium Chloride (mg/ml) | 0.070 | 0.13 | -1.000 | 1.000 | 0.100 | 0.025 |

The final equation predicted in terms of coded and actual factors are given respectively as follows-

*Ethanol content (coded factor) = +2.39 + 0.081* A+ 0.031* B+ 0.13* C+ 0.016* A * B - 0.025* A * C-0.031* B * C- 0.36* $A^2$- 0.34* $B^2$- 0.18* $C^2$*

*Ethanol content (actual factor) = -41.79523+ 8.15400 * Sucrose+ 97.15373* Ammonium Sulphate+ 59.21060* Potassium Chloride +0.46315 * Sucrose * Ammonium Sulphate - 1.42128 * Sucrose * Potassium Chloride -17.46554* Ammonium Sulphate * Potassium Chloride -1.01332* $Sucrose^2$ -96.73228* $Ammonium\ Sulphate^2$ -201.52913* $Potassium\ Chloride^2$*

The experimental design shown in Table 4.5. gives all possible combinations of process parameters for increasing ethanol yield. The data shows fitness with a second order polynomial equation, which are considered statistically significant at P-value less than 0.05. In this case, sucrose content (A), potassium chloride content (C) and quadratic effect of all the factors ($A^2$, $B^2$, $C^2$) are the significant model terms.

**Table 4.5.:** Experimental design, observed and predicted responses of nutritional optimization using central composite design

| Run | Sucrose (%) | Ammonium Sulphate (%) | Potassium Chloride (mg/ml) | Observed Response | Predicted Response |
|---|---|---|---|---|---|
| 1 | 3.41 | 0.56 | 0.07 | 1.26 | 1.32 |
| 2 | 4.00 | 0.50 | 0.05 | 1.67 | 1.66 |
| 3 | 4.00 | 0.50 | 0.10 | 2.48 | 2.39 |
| 4 | 4.59 | 0.44 | 0.13 | 1.69 | 1.69 |

| | | | | | |
|---|---|---|---|---|---|
| 5 | 4.59 | 0.56 | 0.13 | 1.67 | 1.72 |
| 6 | 4.00 | 0.50 | 0.10 | 2.30 | 2.39 |
| 7 | 4.00 | 0.40 | 0.10 | 1.44 | 1.37 |
| 8 | 4.00 | 0.50 | 0.10 | 2.42 | 2.39 |
| 9 | 4.00 | 0.50 | 0.10 | 2.45 | 2.39 |
| 10 | 4.00 | 0.50 | 0.10 | 2.20 | 2.39 |
| 11 | 3.41 | 0.44 | 0.07 | 1.23 | 1.23 |
| 12 | 3.41 | 0.56 | 0.13 | 1.58 | 1.57 |
| 13 | 4.00 | 0.60 | 0.10 | 1.48 | 1.48 |
| 14 | 5.00 | 0.50 | 0.10 | 1.52 | 1.52 |
| 15 | 4.00 | 0.50 | 0.10 | 2.50 | 2.39 |
| 16 | 4.00 | 0.50 | 0.15 | 2.18 | 2.11 |
| 17 | 4.59 | 0.56 | 0.07 | 1.62 | 1.57 |
| 18 | 3.41 | 0.44 | 0.13 | 1.50 | 1.61 |
| 19 | 4.59 | 0.44 | 0.07 | 1.36 | 1.41 |
| 20 | 3.00 | 0.50 | 0.10 | 1.31 | 1.24 |

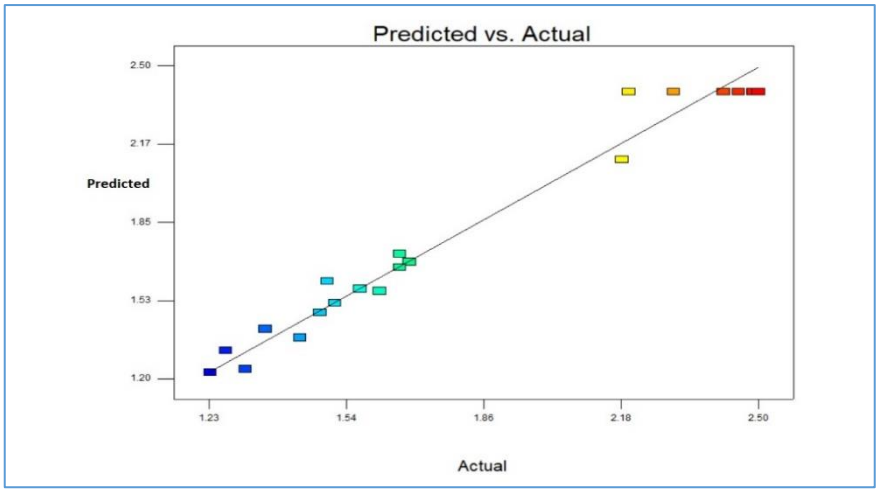
F-value of model 39.38 implies that the model is significant having P-value < 0.0001. There is only a 0.01% chance that a Model F-Value" is by noise. Lack of Fit F-value of 0.52 implies the Lack of Fit is not significant relative to the pure error. There is a 75.30 % chance that a "Lack of Fit F-value" this large could occur due to noise. The goodness of fit of the model was checked by the values of determination coefficient ($R^2$). As shown in Table 4.6., the $R^2$ value of 0.9726 was in confirmation with the adjusted $R^2$ value of 0.9479 having adequate precision of 15.95. The predicted $R^2$ value of 0.8972 is in reasonable conformation withadjusted $R^2$ value of 0.9479. The CV was also as low as 5.76 that is indicative of low deviations between experimental and predicted values predicts accuracy and model

redundancy. Adequate precision ratio of 15.95 indicates an ample signal which measures the signal to noise ratio. The predicted versus actual curve shows very less deviations between the values. (Figure 4.4.a).

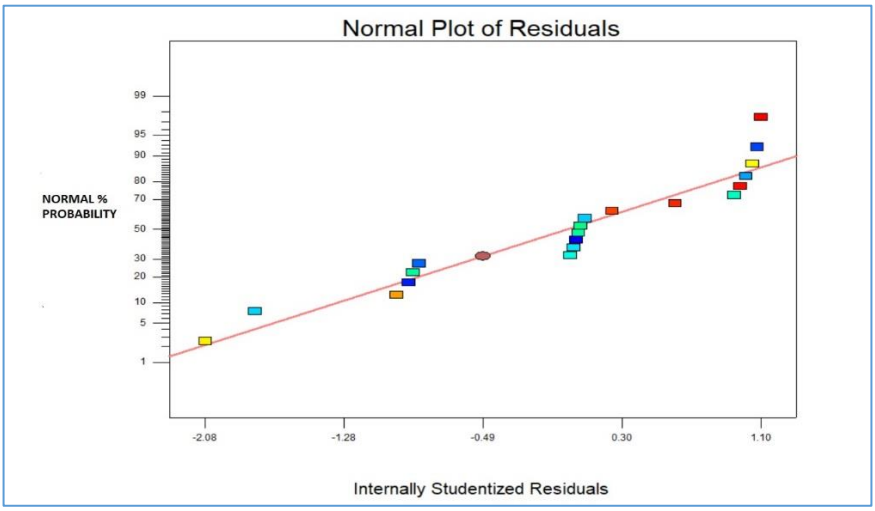
**Table 4.6.:** Analysis of variance (ANOVA) data for nutritional optimization

| Source | Sum of Squares | DF | Mean Square | F value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 3.77 | 9 | 0.42 | 39.38 | < 0.0001 | Significant |
| A-Sucrose | 0.090 | 1 | 0.090 | 8.50 | 0.0154 | |
| B-Ammonium Sulphate | 0.013 | 1 | 0.013 | 1.26 | 0.2874 | |
| C-Potassium Chloride | 0.24 | 1 | 0.24 | 22.83 | 0.0007 | |
| AB | 2.145E-003 | 1 | 2.145E-003 | 0.20 | 0.6630 | |
| AC | 5.050E-003 | 1 | 5.050E-003 | 0.47 | 0.5066 | |
| BC | 7.626E-003 | 1 | 7.626E-003 | 0.72 | 0.4171 | |
| $A^2$ | 1.85 | 1 | 1.85 | 173.80 | < 0.0001 | |
| $B^2$ | 1.69 | 1 | 1.69 | 158.38 | < 0.0001 | |
| $C^2$ | 0.46 | 1 | 0.46 | 42.96 | <0.0001 | |
| Residual | 0.11 | 10 | 0.011 | | | |
| Lack of Fit | 0.037 | 5 | 7.310E-003 | 0.52 | 0.7530 | not significant |
| Pure Error | 0.070 | 5 | 0.014 | | | |
| Cor Total | 3.88 | 19 | | | | |
| Std. Dev. | 0.10 | | R-Squared | 0.9726 | | |
| Mean | 1.79 | | Adj R-Squared | 0.9479 | | |
| C.V. % | 5.76 | | Pred R-Squared | 0.8972 | | |
| PRESS | 0.40 | | Adeq Precision | 15.959 | | |

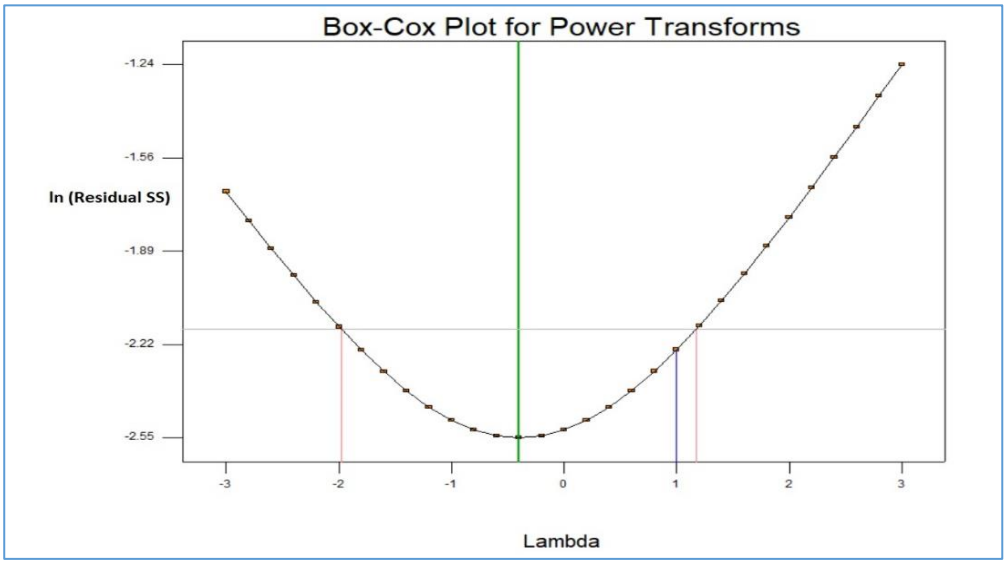
**a**




**b**




**c**



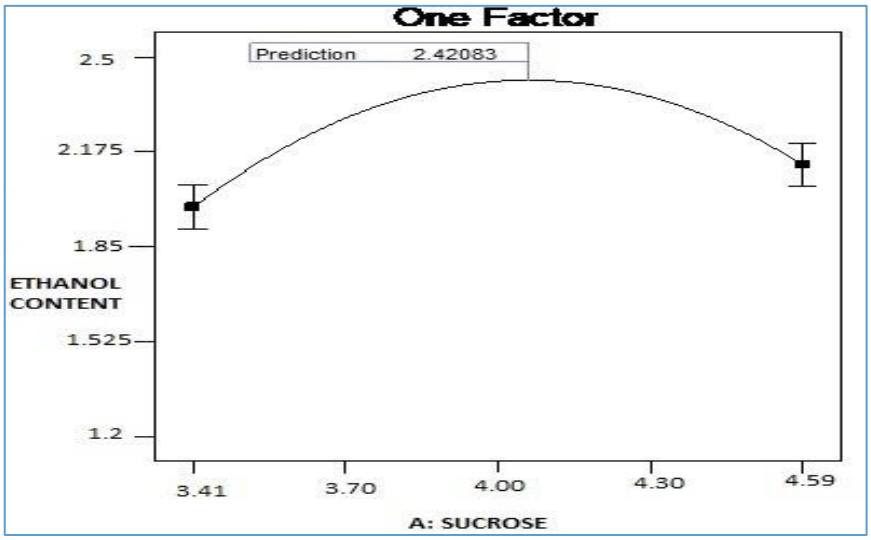

Lambda; Current =1, Best= -0.4, Low C.I. = -1.98, High C.I. = 1.17

Recommended Transform: None (Lambda =1)

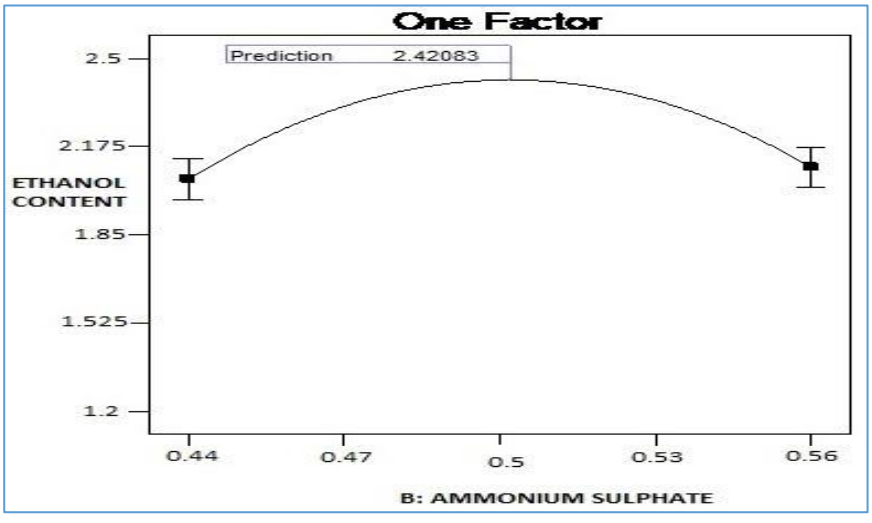
**Fig 4.4.** *Plot of predicted versus actual (a), Normal Plot of residuals (b), and Box–Cox plot of model transformation (c) of nutritional optimization of aqueous extract of bagasse used as a substrate for optimizing fermentation by S. cerevisiae MTCC 180*

Lack of any trend in the plot of studentized residual versus the values predicted by the model shows that the variances in the data are acceptable, without outliers. (Figure 4.4.b). The Box–Cox plot of model transformation (Figure 4.4.c) shows that the optimum value 1of lambda present between the two red lines interpreting no requirement of data transformation. The predicted best value of lambda is shown at -0.4, which signifies that the current lambda value of 1 is more towards the higher lambda value limit.

**a**




**b**



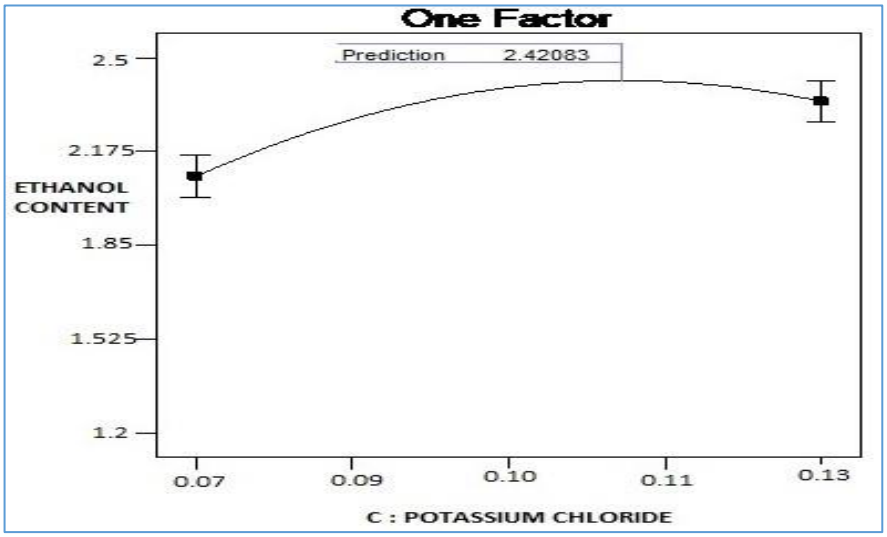
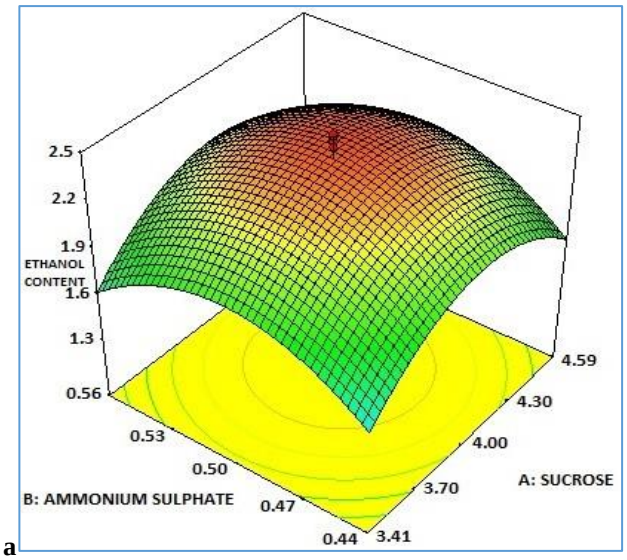
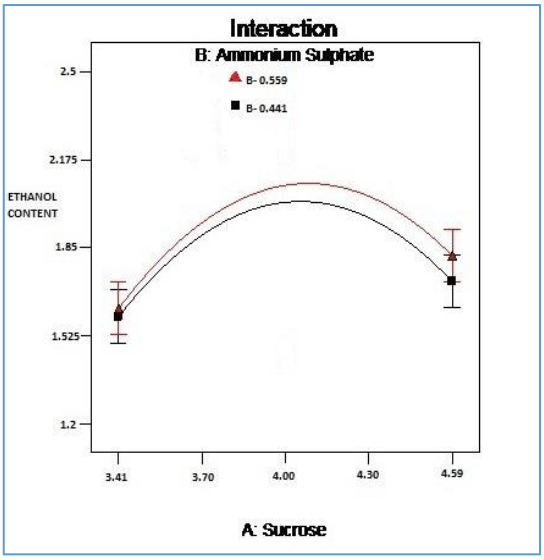
c 


**Fig. 4.5.** *Interaction curves showing ethanol production with a) Sucrose optimization, b) Ammonium sulphate optimization, c) Potassium chloride optimization for bagasse extract fermentation by S. cerevisiae MTCC 180*

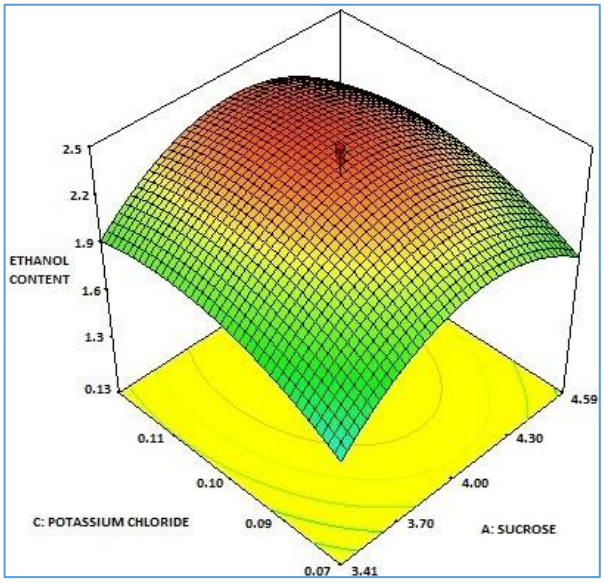
The linear plots show the interaction between the independent variables, and helps to find out the optimum value for each factor to maximize ethanol production, as shown in Figure 4.5. It is evident that initially at around with 3.4 % sucrose supplementation, the ethanol yield was less, the yield gradually increased until 4% supplementation, and subsequently it decreased considerably (Figure 4.5.a). Ammonium sulphate shows a rather symmetrical rise in ethanol yield at the mean value of 0.5%, and the yield decreases in the same manner at higher levels of supplementation (Figure 4.5.b). Potassium chloride resulted in a very flat rise in the ethanol yield curve to a maximum at 0.11 mg/ml supplementation, followed by insignificant but discernible decrease beyond that value (Figure 4.5.c).


2.5
2.2
1.9
ETHANOL CONTENT
1.6
1.3
0.56
0.53
0.50
0.47
0.44
B: AMMONIUM SULPHATE
3.41
3.70
4.00
4.30
4.59
A: SUCROSE

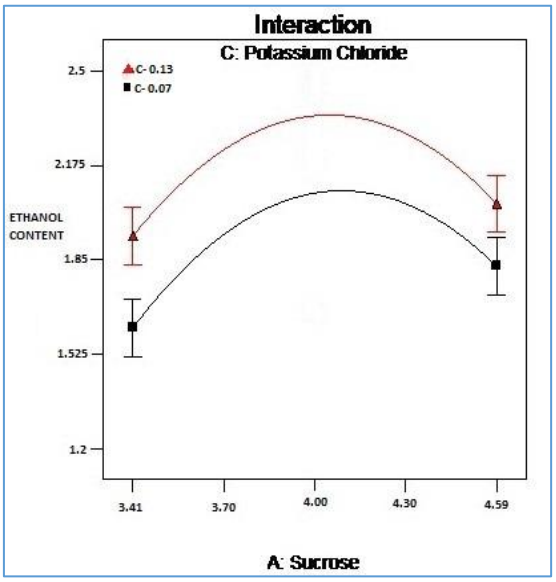
a


Interaction
B: Ammonium Sulphate
B- 0.559
B- 0.441
2.5
2.175
ETHANOL CONTENT
1.85
1.525
1.2
3.41
3.70
4.00
4.30
4.59
A: Sucrose

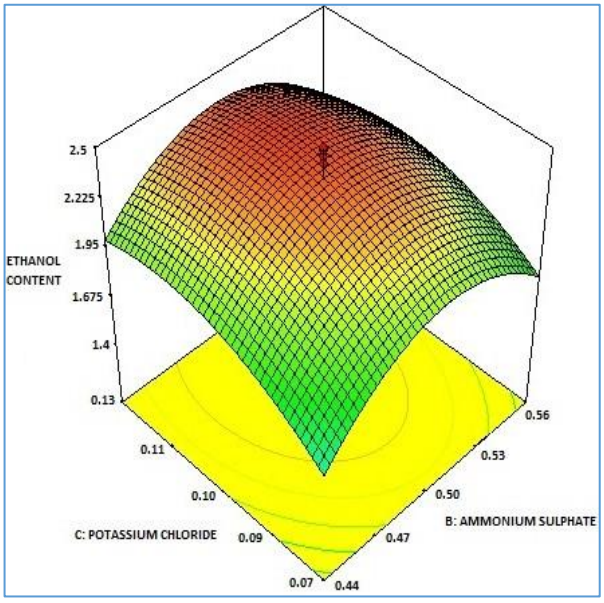
b


2.5
2.2
1.9
ETHANOL CONTENT
1.6
1.3
0.13
0.11
0.10
0.09
0.07
C: POTASSIUM CHLORIDE
3.41
3.70
4.00
4.30
4.59
A: SUCROSE

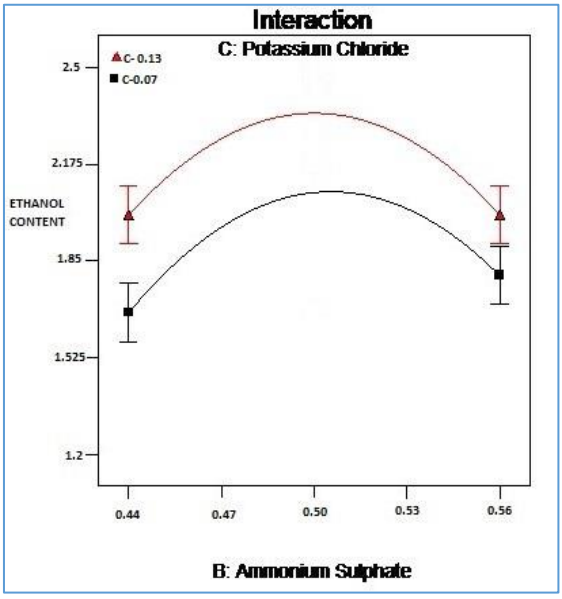
c


Interaction
C: Potassium Chloride
C- 0.13
C- 0.07
2.5
2.175
ETHANOL CONTENT
1.85
1.525
1.2
3.41
3.70
4.00
4.30
4.59
A: Sucrose

d


2.5
2.225
1.95
ETHANOL CONTENT
1.675
1.4
0.13
0.11
0.10
0.09
0.07
C: POTASSIUM CHLORIDE
0.44
0.47
0.50
0.53
0.56
B: AMMONIUM SULPHATE

e


Interaction
C: Potassium Chloride
C- 0.13
C-0.07
2.5
2.175
ETHANOL CONTENT
1.85
1.525
1.2
0.44
0.47
0.50
0.53
0.56
B: Ammonium Sulphate

f

**Fig. 4.6.** *Three dimensional and linear interaction plots for (a and b) A – sucrose supplementation and B- Ammonium sulphate supplementation, (c and d) A – sucrose supplementation and C – Potassium chloride supplementation (e and f) and B – Ammonium sulphate supplementation and C – Potassium chloride supplementation (Red dots within the response curves represent experimental design points obtained during the CCD-RSM while contour lines show prediction outputs calculated by the mathematical model.)*

The three dimensional plots of dual factor interaction among the independent variables show that the basic nature of the curve is convex (Figure 4.6). That means it starts with low initial values, rises in beteween at its optimal value, and decreases further upon increasing the nutritional supplementation. Keeping potassium chloride content of 0.1mg/ml, the first curve (Figure 4.6a,b) depicts that at 0.44 % ammonium sulphate, the ethanol yield rises and falls at 0.559 % upon increasing the amount of sucrose in the substrate. The overall ethanol productionis higher at 0.559 % ammonium sulphate and with increased sucrose supplementtion in the feremntation substrate. Though the initial yield was almost same, the ethanol content slightly increases afterwards. The second part of the curve (Figure 4.6c, d) shows that at potasssium chloride content of 0.07 or 0.13 mg/ml, the nature of the curve is exactly same starting from lower to higher value of sucrose supplementation, when ammonium sulphate value is kept constant at 0.5 %. The net ethanol production is significntly higher in case of potassium content of 0.13 mg/ml. In the third part of the curve (Figure 4.6e, f), sucrose supplementation was kept at a constant level of 4%, both lower and higher values of potassium chloride between the range of 0.07 or 0.13 mg/ml gives the same graphical pattern. The net ethanol content is more in case of potassium chloride content of 0.13 mg/ml. From these graphs we can say that sucrose content as well as the mineral content of the substrate quite significantly affects the overall ethanol content.

### *4.3.3. Confirmation of the predicted model*

The study of the plots gives the best predicted values of nutritional enrichment at 4.06 % sucrose, 0.5% ammonium sulphate and 0.11 mg/ml potassium chloride having an ethanol yield of 2.42 gm/100 ml with desirability function value of 0.941 which is indicative of good significance of the obtained result. Using such conditions, replicate experiments were conducted again. The experimental result was around 2.345 gm/100 ml which was largely in agreement with the predicted value of 2.42 gm /100 ml of ethanol content. The experimental value was96.9 % of the predicted value of the modified quadratic model (Table 4.7). So, the observed value was in good agreement with the predicted value that confirms to the sufficiency and significance of model.

**Table 4.7:** Validation data for the predicted model for fermenting bagasse aqueous extract using saccharomyces cerevisiae MTCC 180

| **Response** | **Software prediction value (g /100 ml)** | **Validity experiment value (g /100 ml)** | **95% CI low** | **95% CI high** |
|---|---|---|---|---|
| Ethanol yield | 2.42 | 2.345 | 2.33 | 2.51 |

## 4.4. CONCLUSIONS

India is among the world's leading generator of bagasse wastes, which is abundantly available, and can be put to various industrial uses including production of ethanol as a biofuel through fermentation. The aqueous extract of bagasse was easily prepared, and converted into a nutrition-rich fermentable substrate for ethanol production by supplementing

nutrients. When the factors were optimized using Design expert® software, the ethanol yield of 2.42 g/100 ml at the rate of 1.07 g/L/h was obtained. The validation of nutritional parameters gave maximum ethanol yield of 2.345 g/100 ml at the rate of 1.037 g/L/h achieved at 4.06% sucrose, 0.5 % ammonium sulphate and 0.11 mg/ml potassium chloride. The results were consistent with the results of 4% sucrose, 0.5 % ammonium sulphate and 0.1 mg/ml potassium chloride as obtained from the studies of one variable at a time. The non-supplemented medium, even under optimized fermentation conditions, gave 7.5 times lesser yield. The study reveals that sucrose, ammonium sulphate and potassium chloride significantly improved ethanol yield, and the modified quadratic model suggested by the software was adequate ($p < 0.0001$), as the $R^2$, adjusted $R^2$ and CV values showed acceptability and accuracy of the model.

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CHAPTER 5

# Chapter title: Ethanol production from immobilized cells of Saccharomyces cerevisiae MTCC 180

## 5.1. INTRODUCTION

Chemical synthesis of industrially valuable products like fuels, chemicals and various other materials gives high yield but are damaging to the environment due to production of huge amount of toxic by-products. The trend is towards the development of sustainable biocatalytic processes which not only are environment friendly but also gives substantial amount of yield [1]. Biocatalysis using whole cells allow production either through multistep reactions, or by cofactor regeneration with high regio- and stereo selectivity but under mild operational conditions [2]. These advantages are magnified if these whole cells are immobilized in a matrix to perform the process. The definition of immobilization of whole cells states that "It is the physical confinement or localization of intact cells to a certain region of space; without loss of desired biological activity" [3]. Immobilization of cells have been found to be much more efficient than its freely suspended counterparts as it allows easy separation of biomass or product recovery.

Second generation ethanol production is the latest trend, which could be developed successfully by utilizing some wastes with low-cost yeast immobilization. Effective sugar utilization and resistance to inhibitors are more evident in immobilized cells. Most common types of yeast immobilization uses calcium alginate as the carrier along with agar-agar, alginate, chitosan, biochar, carrageenan, luffa sponge etc.; multispecies biofilm membrane being the latest one [4]. Agar immobilization of yeast was performed by some researchers that included production of ethanol in a tubular reactor using agar immobilized *Saccharomyces cerevisiae* [5] orco-immobilizing yeast using agar-alginate combinations [6].

*Saccharomyces cerevisiae* MTCC 174 was immobilized in agar cubes produced bioethanol of 9.4 g/L which gave much better yield than free cells [7].

Food processing and agro-industrial wastes are a global problem that needs to be eradicated by conversion techniques that will fetch useful products. This biomass can be converted to fuel like bioethanol, and can be utilized as a potential renewable source of energy or can be used as laboratory solvent. About 10 -14 % of global energy crisis is now being solved by the use of lignocelluloisic biomass as a substrate for production of bioethanol. Among various organisms involved in this biocatalysis, *Saccharomyces cerevisiae* is one of the most popular that can be used for fermenting the waste to generate bioethanol [8].

This chapter deals with bioethanol production from immobilized *S. cerevisiae* MTCC 180 under conditions of optimization. The entrapment of the species was done using agar-agar matrix in the form of cubes prepared to perform the fermentation of aqueous extract of bagasse. The optimization was performed initially using one variable at a time, and then using central composite design of response surface methodology.

## 5.2. MATERIALS AND METHODS

### *5.2.1. Fermentation substrate preparation*

The process has been described in details in chapter 2 section 2.2.2. of materials and method.

### *5.2.2. Maintenance and preparation of inoculum*

The process has been described in details in chapter 3 section 3.2.1. of materials and method.

### *5.2.3. Measurement of cell count*

Cell count gives us an idea of the growth rate of cells in a given population. The 48 h old culture, grown in 50 ml YEPD broth, was plated in YEPD agar media to count the

approximate number of cells present using spread plate technique [9]. The temperature of incubation was kept at 30°C and a dilution of $10^7$ was performed using buffered peptone water. After 48 h incubation, the plates were counted using digital colony counter.

*5.2.4. Immobilization of culture, preservation and reuse*

In accordance with a previously optimized fermentation conditions as described in chapter 3, the 48 h old inoculum was considered for further experiments. Agar containing 0.9% sodium chloride were mixed in a solution of distilled water and autoclaved at 15 psi for 15 min at 121°C. The sterile agar is then cooled to 30°C and mixed with the prepared inoculum. The mixture was poured in Petriplates and kept for solidification of the agar. On solidification, they were cut into pieces of small cubes and preserved in 0.1 (M) phosphate buffer solution of pH 5.5 in refrigerated conditions for 1 h. For reusing the immobilized culture, it must be taken out of refrigeration to achieve room temperature and washed with cold sterile water 2-3 times before they are used as inoculum [10].

*5.2.5. Examination of immobilized pieces with and without cell using Scanning Electron Microscopy (SEM)*

The topology of immobilized agar pieces was observed with and without yeast cells using Scanning Electron Microscope (Merlin, Zeiss) at varied magnification. The samples were mounted on the stub, and then coated with gold for observation under microscope.

*5.2.6. Optimization of immobilized conditions using OVAT*

The bagasse extract was fermented using *S. cerevisiae* MTCC 180 culture in immobilized form. The conditions of fermentation and nutritional supplementation were previously

optimized and those optimized conditions were used keeping the fermentation pH at 4, time 22.06 hours, temperature 30.20°C, using inoculum size of 6% with 48 h old culture of inoculum, containing 4% sucrose, 0.5 % ammonium sulphate and 0.1 mg/ml potassium chloride, as discussed in chapter 3 and 4. Keeping these conditions constant, four different parameters- fermentation time, agar concentration, gel formation time and cell concentration were selected and optimized using OVAT method. The fermentation time was varied from 20 to 28 h, agar concentration from 3 to 5 %, gel formation time from 10 to 30 minutes and cell concentration from 5 to 12 %. The optimized values were selected to carry out further experiments.

*5.2.7. Experimental design and statistical data analysis*

Each of the experiments were performed in three replicates in OVAT where the mean value and the standard deviations were analysed using one way ANOVA (MS Excel 2013) at significance level of $P< 0.05$. The Coefficient of variation (CV), $t_{critical}$ values at α value 0.05 were also obtained. Fisher's Post Hoc test of least significant difference (LSD) values were calculated to check the significance of the obtained value in accordance with the given formula in equation 1.

(1) $$LSD = t_{crit}\sqrt{MSW\left({}^{1}/_{N_1} + {}^{1}/_{N_2}\right)}$$

Where, MSW is the mean square value within groups, $N_1$ and $N_2$ are the respective sample sizes. Since three replicates for each of the values were obtained, so in this case, $N_1= N_2= 3$ [11].

Three different independent variables were further optimized using rotatable central composite design (RCCD) of response surface methodology (RSM). The data fits on a second order polynomial model having three independent factors fermentation time (h), agar

concentration (%) and cell concentration (%), and one dependent factor ethanol yield (g/100 ml). The data was taken in five level having six centre points and the fitness of the model was determined by the values of coefficient of determination ($R^2$). The three dimensional response surface plots gives the relationship between the responses and the factors to maximize ethanol production. The combination of these predicted parameters that gave maximum yield of ethanol were tested to validate the model. Statistical evaluation was performed using Design-Expert Version 7.0.0, 2021, Stat-Ease (Minneapolis, USA).

### *5.2.8. Ethanol Estimation*

The process has been described in details in chapter 1 section 1.2.6. and chapter 3 section 3.2.5 of materials and method.

## 5.3. RESULT AND DISCUSSIONS

### *5.3.1. Cell count value of inoculum*

The incubation of the cells showed growth on the agar plate that can be calculated using digital colony counter. The average readings on three successive replicate plates was found to be 209x $10^7$ CFU/ml. The approximated CFU per beads ranges from 17.41x $10^7$ - 41.8 x $10^7$

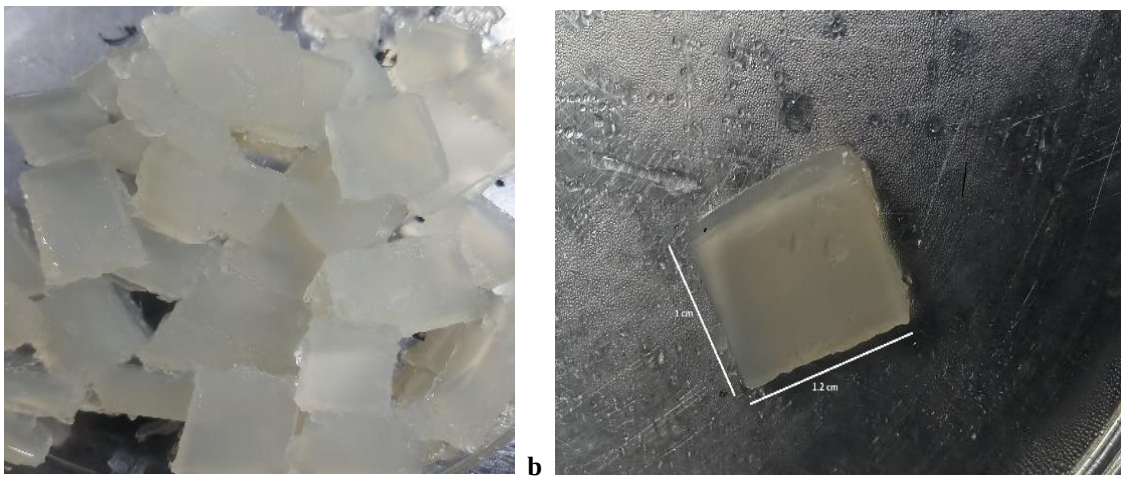
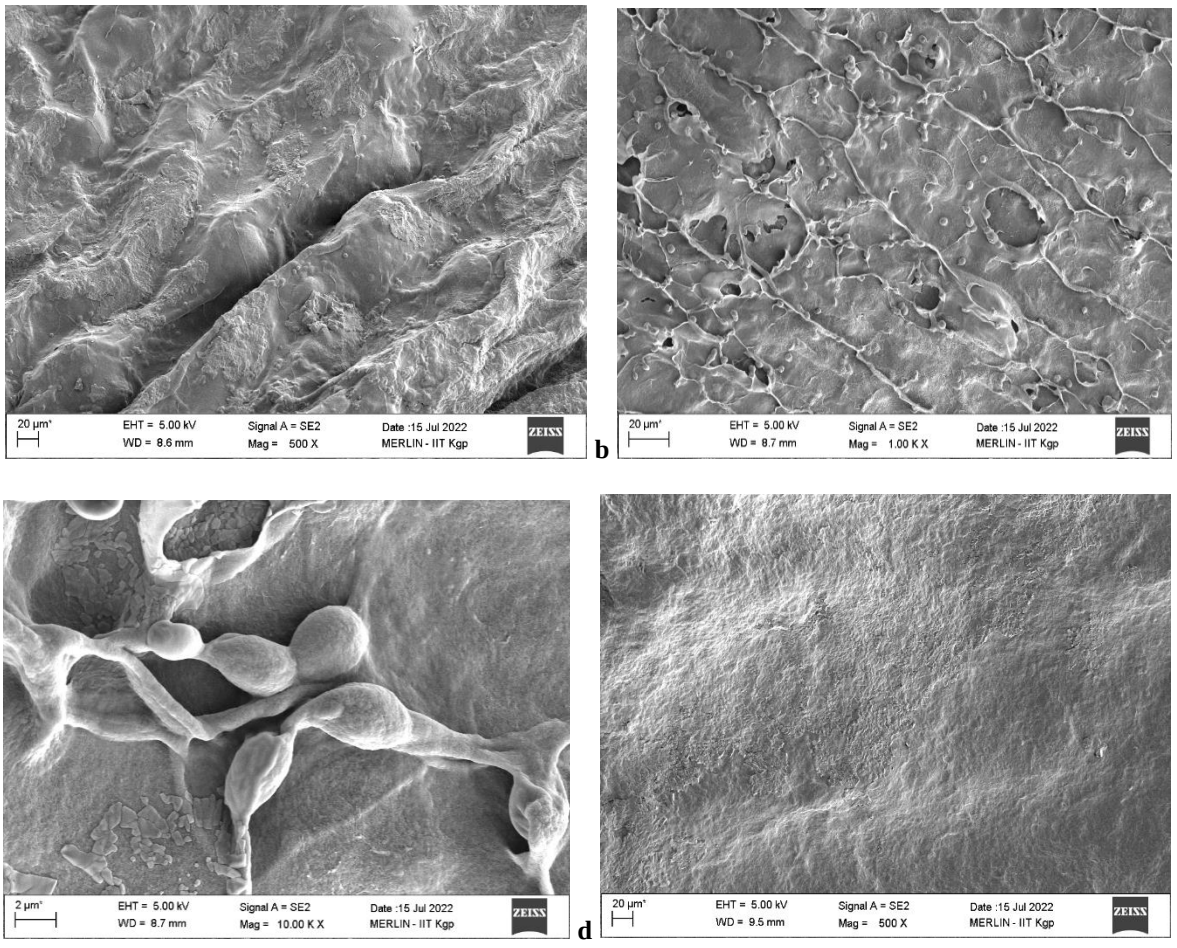



**Fig. 5.1.** *(a) Yeast cells immobilized in agar blocks (b) Approximate size of each block 1.2x1 cm*

depending on the cell concentration used for immobilization. Yeast immobilized on polyurethane foam used for bioremediation of phenol showed a cell concentration of 2.5 x$10^6$ CFU/ cm at 24 h which is suggestive of biofilm formation [12] whereas when *Saccharomyces cerevisiae var boulardii* was immobilized on apple pieces, it maintained a viable count of 6.7–6.9 log CFU/g for all contact times starting from the time of immobilization [13].

*5.3.2. SEM view of immobilized cells*

The agar pieces with immobilized yeast cells are made into blocks of approximate size 1.2 x1 cm (Figure 5.1).The Scanning electron microscope view of the agar blocks with and without cells can be shown in Figure 5.2.




**a** **b**

**c** **d**

**Fig. 5.2.** *Distribution and topology of Saccharomyces cerevisiae MTCC 180 cells immobilized in agar matrices in varied magnification 500X, 1000Xand 10,000X (a, b and c) and agar matrix without cells at 500X magnification (d).*

The microscopic view at 500 times magnification (Figure 5.2 a) of the immobilized cells reveals irregular surface scaffolds of agar where almost even distribution of yeast cells can be seen. At higher magnification of 1000 and 10000 times (Figure 5.2 b and c) it was found that the cells are evenly distributed in the matrix, and structural similarities reveal the presence of *S. cerevisiae* MTCC 180 culture. The view of agar matrix with cell only reveals wavy surface of agar along with presence of some geometrically shaped salt crystals (Figure 5.2 d). Beads of porous agar matrix used for ethanol production from cane molasses showed similar structural patterns [14]. Clustered yeast cell and biofilm formation was evident as reported by researchers when they tried to immobilized yeast using alginate beads or any other fibrous matrices [15, 16].

### *5.3.3. Optimization of parameters of immobilization using OVAT*

Several different types of factors influence the immobilization efficiency and in turn affects the fermentation ability and yield. The fermentation time under immobilized conditions was found to give better results of 1.978 ± 0.1 g/100 ml ethanol yield at 20 h followed by gradual decrease in yield (Table 5.1.a).Agar concentration of 4 % gives ethanol content of 2.293± 0.34 g/100ml, when the fermentation time was kept at 20 h (Table 5.1.b).The upper or lower value of agar at 3% or 5% are not very significantly different. At constant value of fermentation time and agar concentration of 20 h and 4% respectively, the gel formation time gives best result at 20 minutes (Table 5.1.c); the upper and lower values were found to be

insignificant. Cell concentration is one of the most important factors in case of cell immobilization, since it takes higher cell concentration value in case of immobilized cell than free cell.

**Table 5.1.** Ethanol content, CV, $t_{critical}$ and LSD values in varied fermentation time (a), agar concentration (b), gel formation time (c) and cell concentration (d) of immobilized cell fermentation using *Saccharomyces cerevisiae* MTCC 180 utilizing bagasse water extract

| Fermentation Time (h) | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| 20 | 1.978 ± 0.1[a] | 0.053 | | |
| 24 | 1.506± 0.22[b] | 0.147 | 2.446 | 0.339 |
| 28 | 1.368± 0.16[b] | 0.118 | | |

**a**

| Agar Concentration (%) | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| 3 | 1.747± 0.13[a] | 0.079 | | |
| 4 | 2.293± 0.34[b] | 0.149 | 2.446 | 0.496 |
| 5 | 1.70± 0.21[a] | 0.128 | | |

**b**

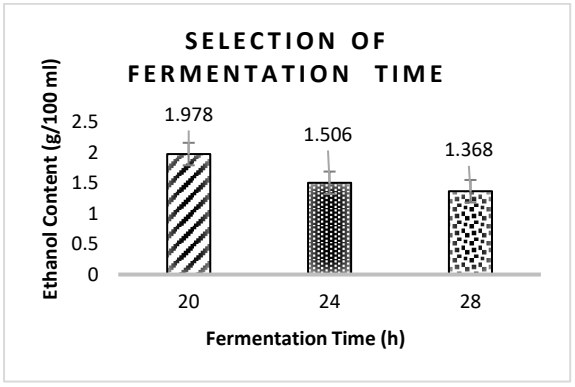
| Gel formation time (minutes) | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| 10 | 1.678± 0.07[a] | 0.042 | | |
| 20 | 1.977± 0.05[b] | 0.026 | 2.446 | 0.131 |
| 30 | 1.609± 0.07[a] | 0.044 | | |

**c**

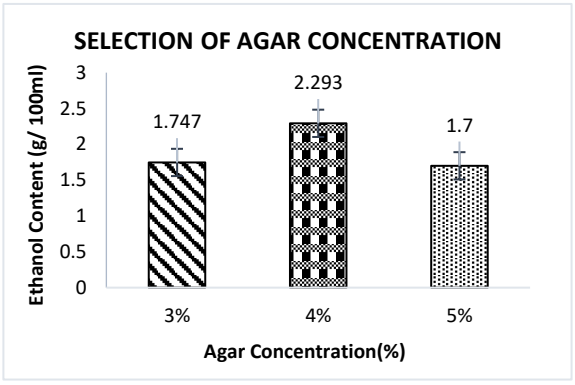
| Cell concentration (%) | Ethanol content (g/100ml) | CV | $t_{0.05}$ | $LSD_{0.05}$ |
|---|---|---|---|---|
| 5 | 1.782± 0.05[a] | 0.029 | | |
| 6 | 1.759± 0.13[a] | 0.078 | | |
| 7 | 1.943± 0.26[a] | 0.137 | 2.178 | 0.259 |
| 8 | 2.254± 0.17[b] | 0.077 | | |
| 10 | 1.874± 0.05[a] | 0.028 | | |
| 12 | 1.724± 0.03[a] | 0.02 | | |

**d**

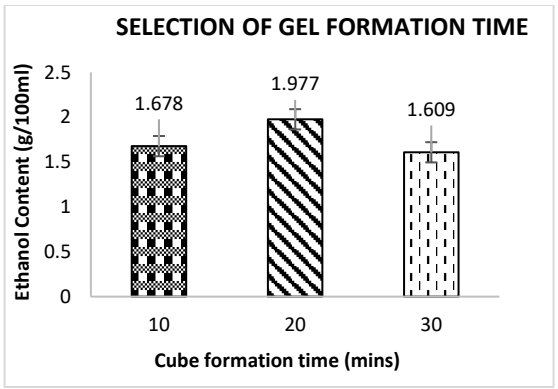
The significantly higher ethanol content was found at 8% cell concentration, when the other three factors were kept at a constant at previously optimized values(Table 5.1.d).The coefficient of variation (CV), $t_{critical}$ and LSD values also helped to understand the correlation between these factors and are in confirmation with the graphical representation of the data (Figure 5.3.). So, the optimized values using OVAT were found to be 20 h, 4%, 20 minutes and 8% of fermentation time, agar concentration, gel formation time and cell concentration respectively. Very limited number of studies reports immobilization of yeast cells in agar matrices. Beads of agar were produced with suspended yeast cells at 3% agar concentration using chilled water and vegetable oil mixture as reported by a few studies where they fermented cane molasses using immobilized yeast cells [5][14]. Recently some researchers reported the use of agar matrix along with other matrices to form combination matrix for yeast cell immobilization [6] while others tried different organism like *Lycinibacillus fusiformis* for immobilizing in agar matrices [12].



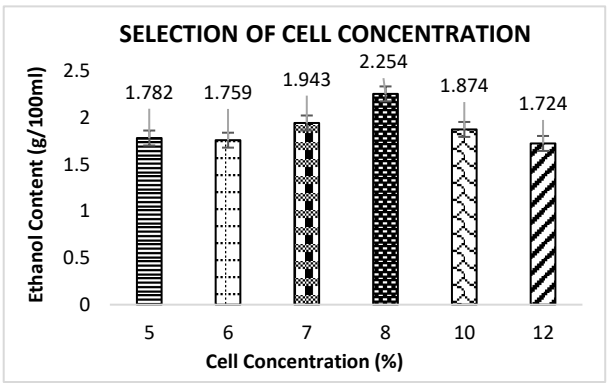

a




b




c




d

**Fig. 5.3.** *Graphical representation of selection of fermentation time (a), agar concentration (b), gel formation time (c) and cell concentration (d) in immobilized fermentation of Saccharomyces cerevisiae MTCC 180 using bagasse water extract*

### *5.3.4. Optimization of yeast immobilization parameters using Response Surface Methodology*

The three factors for optimization of yeast immobilization-agar concentration, fermentation time and cell concentration were re-optimized through response surface methodology. The actual and coded values of the experimental design are shown in Table 5.2.

**Table 5.2.** The actual and coded values for the experiment in response surface methodology

| Factor | Name with Unit | Low Actual | High Actual | Low coded | High coded | Mean | Std. dev |
|---|---|---|---|---|---|---|---|
| A | Fermentation time (h) | 18.81 | 21.19 | -1.000 | 1.000 | 20.000 | 0.983 |
| B | Agar concentration (%) | 3.41 | 4.59 | -1.000 | 1.000 | 4.000 | 0.491 |
| C | Cell concentration (%) | 6.81 | 9.19 | -1.000 | 1.000 | 8.000 | 0.983 |

The final equation predicted in terms of coded and actual factors are given respectively as follows-

*Ethanol content (Coded Factors) =+2.16+1.934E-003*A-0.065*B-0.017*C-0.018*A* B+0.032*A* C-0.035* B * C-0.26 * $A^2$-0.10 * $B^2$-0.17* $C^2$*

*Ethanol content ( Actual Factors)=-83.09233+7.26468* Fermentation time+3.13726* Agar conc+1.64756*Cell conc-0.025102*Fermentation time * Agar conc+0.022627**

*Fermentation time * Cell conc-0.049144* Agar conc * Cell conc-0.18359* Fermentation time$^2$-0.29387* Agarconc$^2$-0.11984*Cellconc$^2$*

The experimental design shown in Table 5.4. gave all possible combinations of process parameters for increasing ethanol yield. The data shows fitness with a second order polynomial equation, which are considered statistically significant at P-value less than 0.05. In this case, Agar concentration(B), interactive effect of fermentation time and cell concentration (AC), interactive effect of agar concentration and cell concentration (BC), and quadratic effect of all the factors ($A^2$, $B^2$, $C^2$) are the significant model terms.

**Table 5.3.** Experimental design, observed and predicted responses of immobilization optimization for yeast using central composite design

| Run | Fermentation time (h) | Agar concentration (%) | Cell concentration (%) | Observed Response | Predicted Response |
|---|---|---|---|---|---|
| 1 | 21.19 | 4.59 | 9.19 | 1.50 | 1.53 |
| 2 | 18.81 | 4.59 | 6.81 | 1.68 | 1.66 |
| 3 | 21.19 | 3.41 | 9.19 | 1.72 | 1.76 |
| 4 | 20.00 | 4.00 | 10.00 | 1.70 | 1.65 |
| 5 | 20.00 | 4.00 | 8.00 | 2.13 | 2.16 |
| 6 | 20.00 | 3.00 | 8.00 | 2.02 | 1.98 |
| 7 | 20.00 | 4.00 | 8.00 | 2.15 | 2.16 |
| 8 | 21.19 | 4.59 | 6.81 | 1.57 | 1.57 |
| 9 | 18.81 | 4.59 | 9.19 | 1.50 | 1.50 |
| 10 | 20.00 | 4.00 | 8.00 | 2.19 | 2.16 |
| 11 | 20.00 | 4.00 | 8.00 | 2.16 | 2.16 |
| 12 | 21.19 | 3.41 | 6.81 | 1.64 | 1.66 |

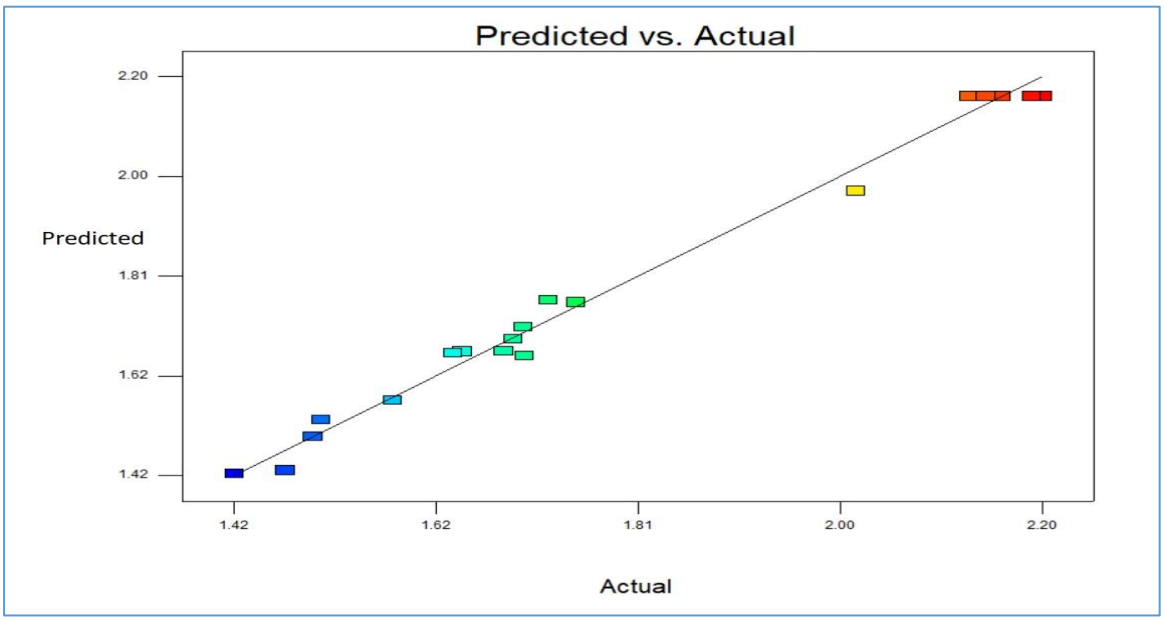
| | | | | | |
|---|---|---|---|---|---|
| 13 | 22.00 | 4.00 | 8.00 | 1.47 | 1.43 |
| 14 | 20.00 | 4.00 | 6.00 | 1.70 | 1.71 |
| 15 | 18.00 | 4.00 | 8.00 | 1.42 | 1.42 |
| 16 | 20.00 | 5.00 | 8.00 | 1.75 | 1.76 |
| 17 | 18.81 | 3.41 | 9.19 | 1.63 | 1.66 |
| 18 | 18.81 | 3.41 | 6.81 | 1.69 | 1.69 |
| 19 | 20.00 | 4.00 | 8.00 | 2.20 | 2.16 |
| 20 | 20.00 | 4.00 | 8.00 | 2.14 | 2.16 |

The Model F-value of 116.04 implies that the model is significant ($P < 0.0001$). The "Lack of Fit F-value" of 2.40 implies the Lack of Fit is not significant relative to the pure error. There is a 17.96 % chance that a "Lack of Fit F-value" this large could occur due to noise. The goodness of fit of the model was checked by the values of determination coefficient ($R^2$). As shown in Table 5.3., the $R^2$ of 0.9905 was in adequate to adjusted $R^2$ of 0.9820 having adequate precision of 28.33. The predicted $R^2$ value of 0.9451 is in reasonable confirmation withadjusted $R^2$ of 0.9905. The CV was very low as 2.05 that is indicative of low deviations between experimental and predicted values, which predicts accuracy and model redundancy. Adequate precision ratio of 28.33 indicates adequate signal. The predicted versus actual curve shows very less deviations between the value predicted by the model and the actual data (Figure 5.4.a).

**Table 5.4.:** Analysis of variance (ANOVA) data for immobilized yeast fermentation optimization

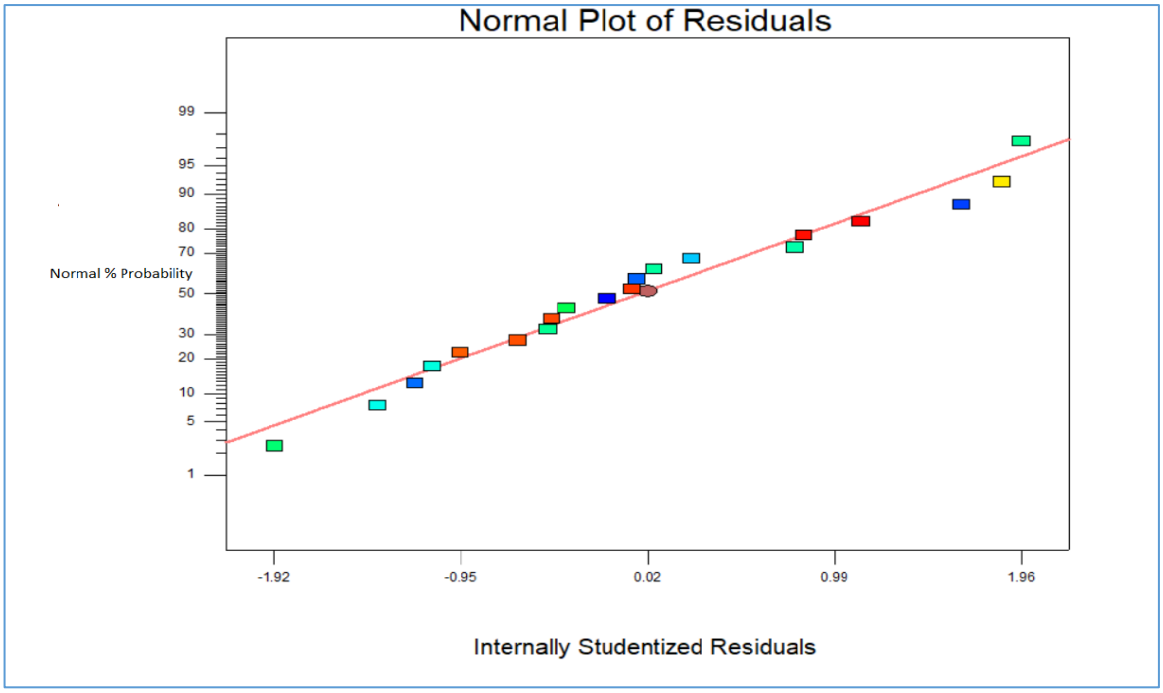
| Source | Sum of Squares | DF | Mean Square | F value | Prob > F | |
|---|---|---|---|---|---|---|
| Model | 1.42 | 9 | 0.16 | 116.04 | < 0.0001 | Significant |
| A- Fermentation time | 5.106E-005 | 1 | 5.106E-005 | 0.038 | 0.8500 | |
| B- Agar concentration | 0.057 | 1 | 0.057 | 42.22 | < 0.0001 | |

| | | | | | | |
|---|---|---|---|---|---|---|
| C-Cell concentration | 3.750E-003 | 1 | 3.750E-003 | 2.77 | 0.1272 | |
| AB | 2.520E-003 | 1 | 2.520E-003 | 1.86 | 0.2026 | |
| AC | 8.192E-003 | 1 | 8.192E-003 | 6.04 | 0.0338 | |
| BC | 9.660E-003 | 1 | 9.660E-003 | 7.13 | 0.0235 | |
| $A^2$ | 0.97 | 1 | 0.97 | 716.63 | < 0.0001 | |
| $B^2$ | 0.16 | 1 | 0.16 | 114.75 | < 0.0001 | |
| $C^2$ | 0.41 | 1 | 0.41 | 305.35 | < 0.0001 | |
| Residual | 0.014 | 10 | 1.356E-003 | | | |
| Lack of Fit | 9.566E-003 | 5 | 1.913E-003 | 2.40 | 0.1796 | not significant |
| Pure Error | 3.990E-003 | 5 | 7.980E-004 | | | |
| Cor Total | 1.43 | 19 | | | | |
| Std. Dev. | 0.037 | | R-Squared | 0.9905 | | |
| Mean | 1.80 | | Adj R-Squared | 0.9820 | | |
| C.V. % | 2.05 | | Pred R-Squared | 0.9451 | | |
| PRESS | 0.078 | | Adeq Precision | 28.332 | | |




**a**

b

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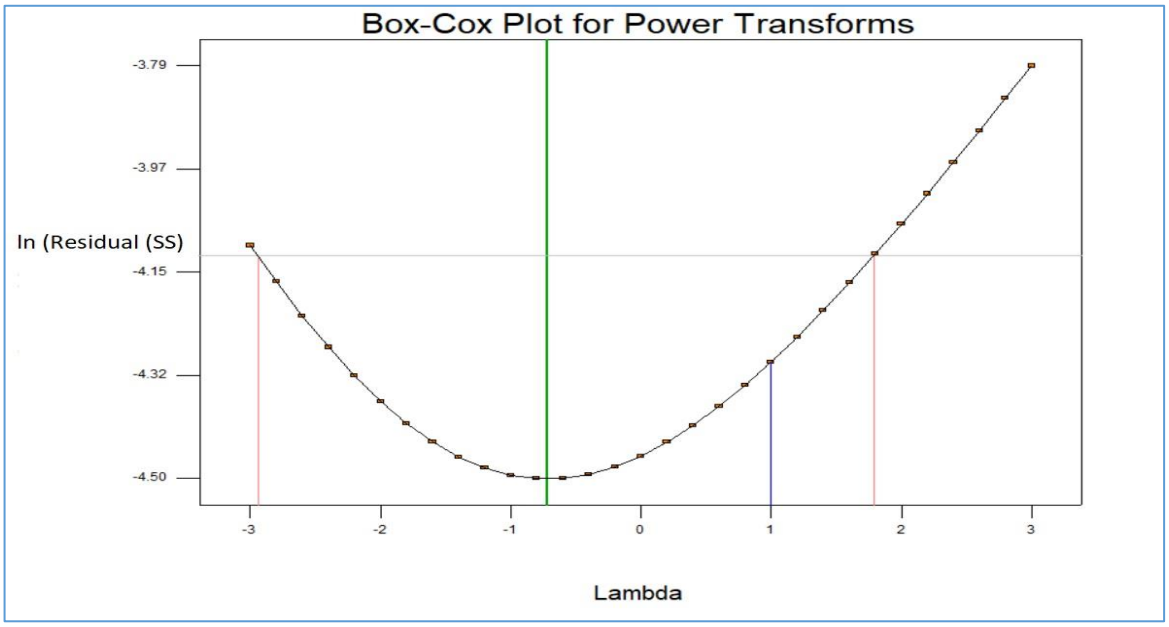
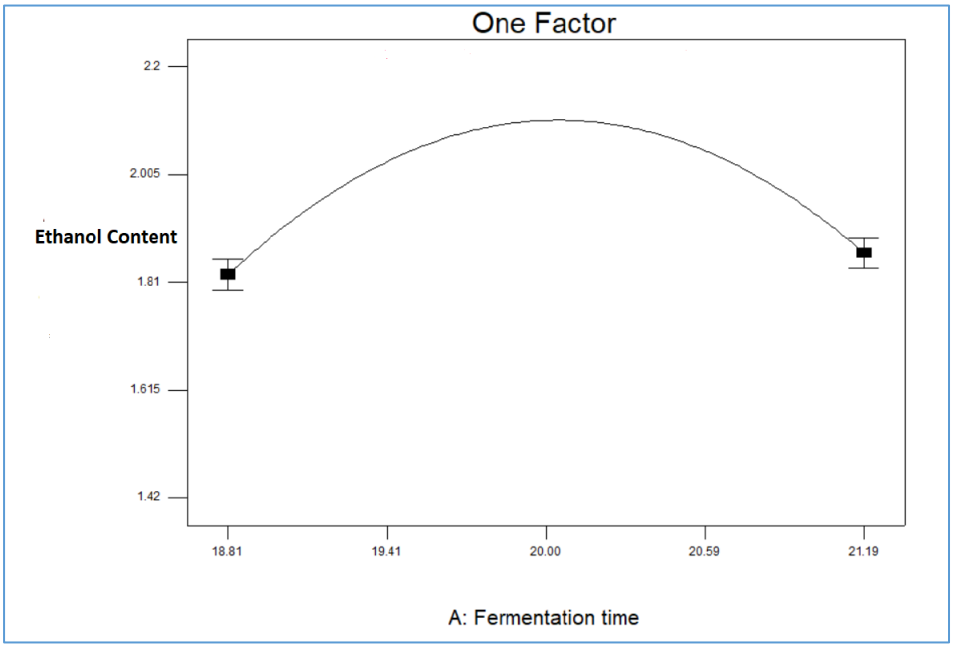



Lambda; Current =1, Best= -0.72, Low C.I. = -2.94, High C.I. = 1.79

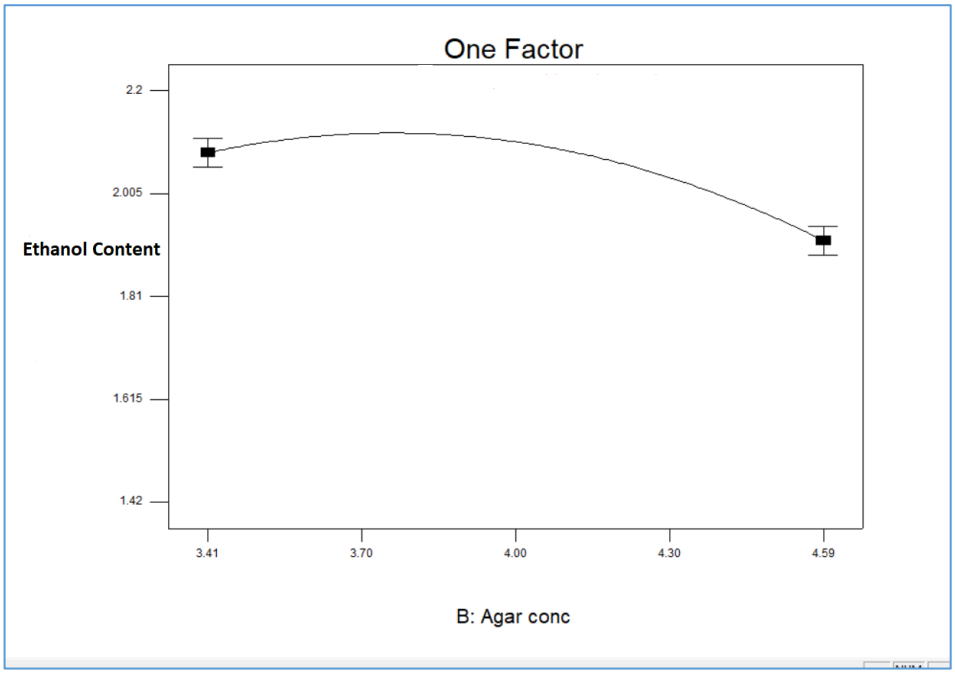
Recommended Transform: None (Lambda =1)

**Fig 5.4.** *Plot of predicted versus actual (a), Normal Plot of residuals (b), and Box–Cox plot of model transformation (c) of immobilized yeast fermentation optimization of aqueous extract of bagasse used as a substrate for optimizing fermentation by S. cerevisiae MTCC 180*

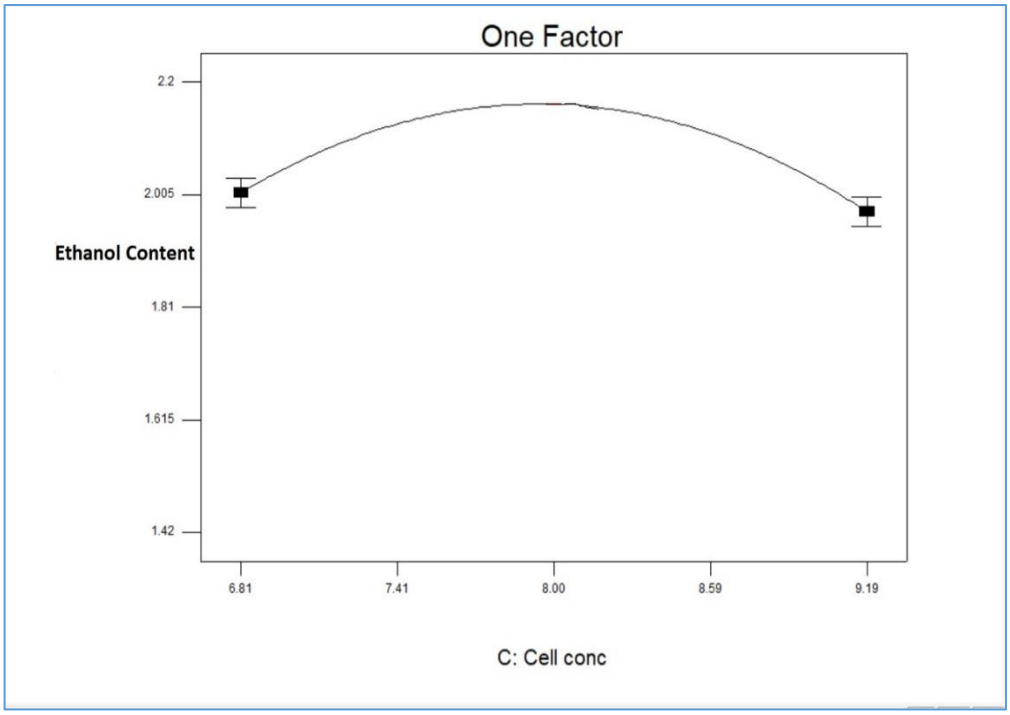
No formation of trends in plot shows acceptable variances and no outliers (Figure 5.4.b). The Box–Cox plot of model transformation (Figure 5.4.c) shows that the optimal value of Lambda (λ) of 1 lies between the two vertical red lines interpreting no requirement of data transformation. The predicted best value of lambda is shown at -0.72, which signifies that the current lambda value of 1 is more towards the higher lambda value limit.




**a**




**b**

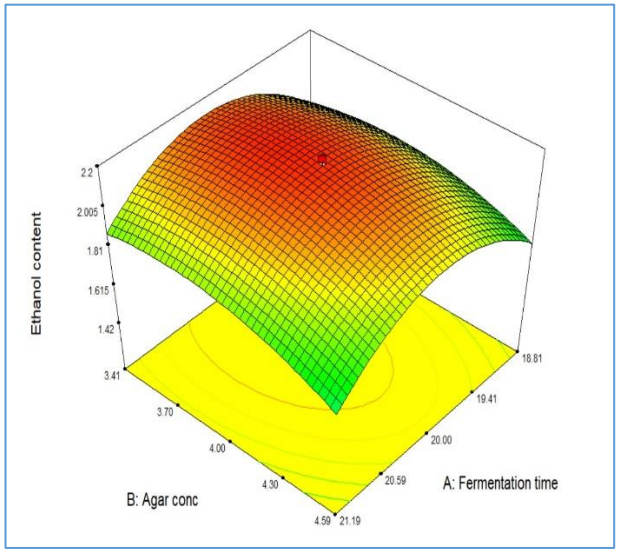



**c**

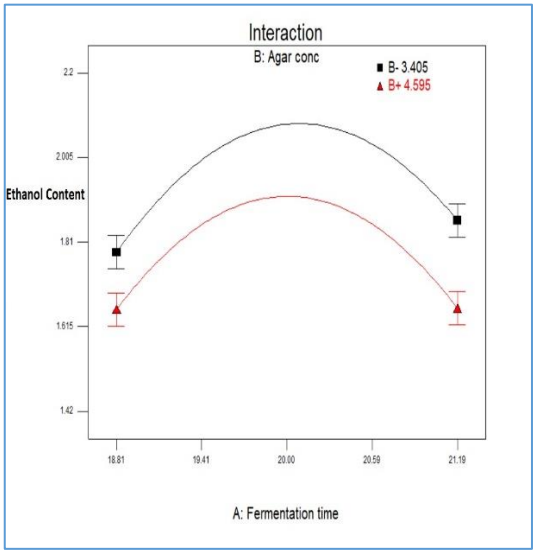
**Fig. 5.5.** *Single factor interaction curves showing production of ethanol with varying a) time of Fermentation, b) Agar concentration and c) cell concentration optimized with immobilized Saccharomyces cerevisiae MTCC 180 fermentation of bagasse extract.*

The single factor plots shows individual effects of each of the independent variable and helps to get an estimate of the optimum value for each factor to maximize ethanol production as shown in Figure 5.5. The factor fermentation time gives lower ethanol yield in lower value like 18.81 h, gradually increases at around 20 h and then decreases symmetrically at higher values of 21.19 h giving a perfect convex pattern. The overall ethanol yield was less at lowest value than the highest value of fermentation time taken. (Figure 5.5. a). Higher ethanol yield can be observed at lower value of agar concentration at 3.41 % which remained almost steady just below 4 % and decrease slightly in higher value of 4.59 %. Compared to the higher value of agar concentration, the lower value gave significantly higher ethanol (Figure 5.5. b). At lower value of yeast cell concentration of 6.81 %, the ethanol yield was low, increases to its maximum value at around 8% and then decreases at higher value of 9.19 %. The lowest value

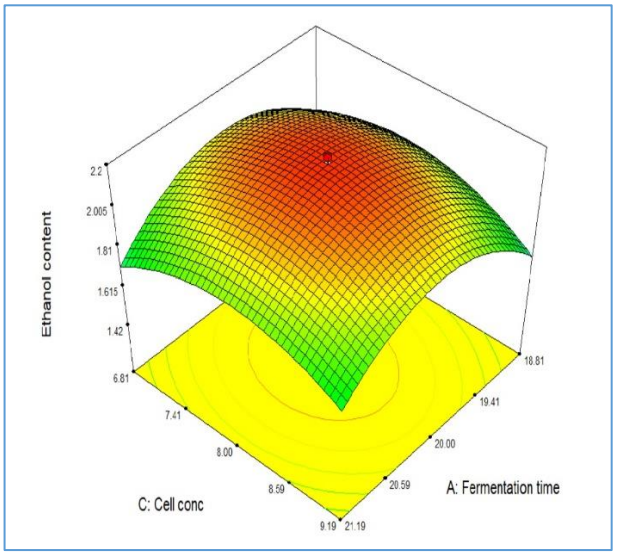
of cell concentration taken gave slightly higher ethanol yield than the highest value (Figure 5.5. c).



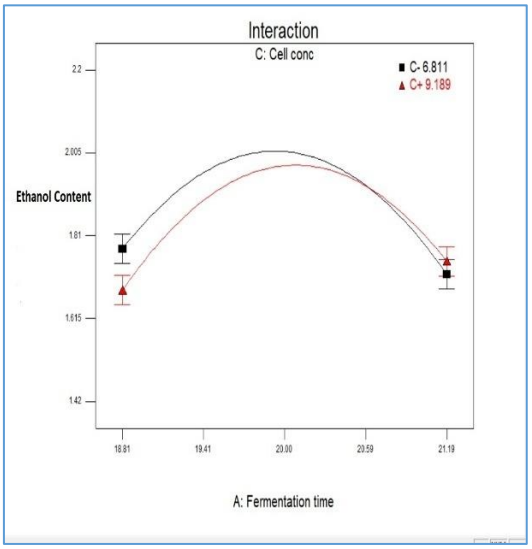

a



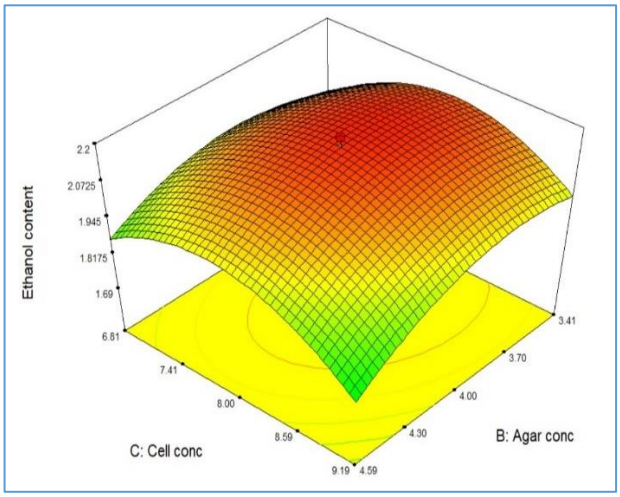

b



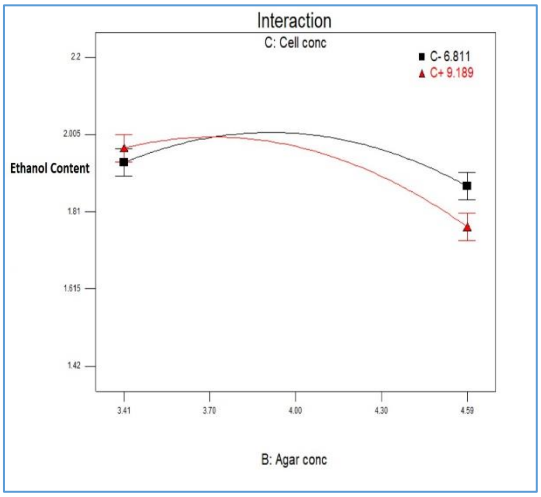

c




d




e




f

**Fig. 5.6.** *Three dimensional and linear interaction plots for (a and b) A – fermentation time and B- agar concentration , (c and d) A – fermentation time and C – cell concentration (e and f) and B – agar concentration and C – cell concentration (Red dots within the response curves represent experimental design points obtained during the CCD-RSM while contour lines show prediction outputs calculated by the mathematical model.)*

The three dimensional plots of two factor interaction among the independent variables with respect to ethanol yield gave variety of interpretations (Figure 5.6.). At constant cell concentration of 8%, the first interaction curve (Figure 5.6. a, b) shows that at 3.405 % agar concentration, the ethanol yield gives less value at 18.81 h and 21.19 h fermentation time with a peak in between at 20 h. Similar pattern can be seen when agar concentration of 4.595 % was used at same cell concentration. The overall ethanol content was more with lower value of agar concentration of 3.405 % than 4.595 % and 20 h fermentation time. The second interaction curve (Figure 5.6. c, d) keeps agar concentration constant at 4%. At 6.811 % cell concentration, the ethanol content was less at 18.81 h that increases slowly near 20 h and finally falls again at 21.19 h of fermentation time. Whereas the higher value of cell concentration at 9.189 %, the ethanol was less at lower value of fermentation time 18.81 h, increase around 20 h and then takes a slight dip at 21.19 h. So, the lower value of cell concentration gave better ethanol yield than higher value starting at initial to 20 h fermentation time. At the end of the curve, the higher value of cell concentration gave slightly better ethanol yield than lower one. In the third part of the curve (Figure 5.6. e, f), fermentation time was kept at a constant value of 20 h. The cell concentration of 6.811 % gave less yield at 3.41 % agar concentration, increases in a flat manner around 4% and again slightly decrease at 4.59 %. When cell concentration was 9.189 %, at 3.41% agar concentration, the ethanol content was more which took a slight peak around 3.7% and then decreases gradually to a much less value at 4.59 %.Therefore, initially the ethanol yield was

less at lower value of cell concentration compare to higher values but near the end of the curve, the ethanol content was much greater at lower value than the higher value of cell concentration. From these graphs we can say that the interaction of factors fermentation time-cell concentration (AC) and agar concentration- cell concentration (BC) significanty affect overall ethanol yield.

*5.3.5. Confirmation of the predicted model*

The study of the plots gives the best predicted values of fermentation by immobilized yeast at 20.05 h fermentation time, 3.41 % agar concentration and 8.07 % cell concentration ( approx. 27.87 $x10^7$ CFU/bead) having an ethanol yield of 2.123 g/100 ml with desirability function value of 0.95which is indicative of good significance of the obtained result. Under these conditions, repeating experiments were conducted in replicates. The experimental result was around 2.056 g/100 ml which was matching with the predicted value of 2.123 g /100 ml of ethanol content. The experimental value was96.8%of the predicted value of the modified quadratic model (Table 5.6.). So, the observed value was in conformation with the predicted value that confirms to the acceptability of the model.

**Table 5.5:** Validation data for the predicted model for fermenting bagasse aqueous extract using immobilized saccharomyces cerevisiae MTCC 180

| Response | Software prediction value (g /100 ml) | Validity experiment value (g /100 ml) | 95% CI low | 95% CI high |
|---|---|---|---|---|
| Ethanol yield | 2.123 | 2.056 | 2.09 | 2.16 |

## 5.4. CONCLUSION

Immobilization of microbial cell brings about certain advantages in fermentation process over free cell. Yeast immobilization is a very popular practice among brewers who tries to yield ethanol by reutilizing cells and want to recover the final product without any hassle. The yeast cells could be successfully immobilized in agar matrices having even distribution as revealed in scanning electron microscopic view. The bagasse water extract, which was used as the fermentation media, was optimized and supplemented for ethanol production, and fermented using immobilized *S. cerevisiae* MTCC 180 gave successful results. When the immobilization factors were optimized using Design expert® software, the ethanol yield of 2.123gm/100 ml at the rate of 1.05 g/L/h was obtained. The model validation of immobilization parameters gave maximum ethanol yield of 2.056 g/100 ml at the rate of 1.025 g/L/h achieved at 20.05 h fermentation time, 3.41 % agar concentration and 8.07 % cell concentration. The study reveals that the factors fermentation time, agar concentration and cell concentration sucrose significantly affected ethanol yield, and the modified quadratic model suggested by the software was adequate ($p < 0.0001$), as the $R^2$, adjusted $R^2$ and CV values showed acceptability and accuracy of the model.

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# SUMMARY

This study attempts to investigate the potential of aqueous extract of sugarcane bagasse using a secondary fermenting strain *Saccharomyces cerevisiae* MTCC 180. Some types of food wastes which is generated after any processing step was collected. They include- Potato wash water that is obtained after washing the peeled potatoes during chips manufacturing, spent tea that is obtained after making tea and bagasse which is obtained after expression of sugarcane juice. All these wastes have a very high BOD value and usually thrown or dumped into the environment that may cause serious environmental pollution. These wastes were reutilized to obtain substrate for fermentation of bioethanol. The potato wash water that was soaked for at least 15 minutes approximately, was treated in two different ways: in one method it was subjected to hydrolysis at 1% v/v of concentrated hydrochloric acid autoclaving at 121 ºC, 15 psi pressure for 15 minutes and in the other method hydrolysis was performed using 5% v/v of concentrated hydrochloric acid at 90-100 ºC for 60 minutes. The spent tea (10 g) was water extracted (150 ml) taking each extract after 2-5 minutes up to $5^{th}$ extraction and mixing the extracts together one at a time to get the fermentation broth. Bagasse was taken (5g) and cut into small pieces of approximately 1 inch length and extracted using water (200ml) boiling at 95-100 ºC. The three different potato wash water samples; Untreated, treated and autoclaved and hydrolysed were taken. Spent tea liquor consisted of samples; 1st extraction, 2nd extraction, 3rd extraction, 4th extraction, 5th extraction, (1st+2nd) extraction, (1st+2nd+ 3rd) extraction, (1st=2nd +3rd+4th) extraction and (1st+ 2nd+ 3rd+4th+ 5th) extraction. Bagasse aqueous extract includes all the 12 extracts starting from 5 minutes to 60 minutes at an interval of 5 minutes each. All these extract were prepared and tested for reducing sugar using Dinitrosalicylic acid method with respect to standard glucose solution. This helped us to get an estimate of the amount of fermentable monosaccharide present that may be useful in

the fermentation initiation. Then the selection was made among the three waste variants based on higher values of reducing sugar and it was found that the hydrolysed potato wash water, first extraction of spent tea and 30 minutes extraction of bagasse scored higher than the others. The final substrate selection was made based on fermentation of these substrate using *Saccharomyces cerevisiae* MTCC 180 to get ethanol at an interval of 24 h up to 72 h. Ethanol content was estimated by titration using dichromate technique and 30 minutes aqueous extract of bagasse gave the highest value of ethanol. Therefore, this substrate was used for further experiments in fermentation.

Bagasse itself can be treated with various pre-treatments before fermentation which may increase ethanol yield. Estimation of our substrate here revealed that it contains very minimum amount of approximately 0.0032g/L of furfural which may increase if any acidic or alkaline treatment was performed. For secondary utilization of bagasse, it needs to be dried and stored. Drying operation on the bagasse was performed using tray dryer which was preheated and brought to a temperature of 70°C. The drying operation was carried out for 150 minutes to get dry bagasse. 5 g dried bagasse was extracted in water and pH, total soluble solid and reducing sugar were analysed. Alcohol estimation was also done using dried bagasse water extract. Results showed almost similar values for both dried and normal bagasse in terms of the above mentioned quality parameters. So, drying was found to have insignificant effect on fermentation of bagasse aqueous extract.

Optimization of fermentation conditions and nutritional supplementation was done on the substrate using statistical methods. The fermentation conditions like pH, temperature, time, inoculum size and inoculum age were optimized at first using one factor at a time and then time and temperature were analysed using response surface methodology where the upper and lower limits were selected based on one factor optimized values. The best conditions for fermentation was found to be at pH 4, fermentation time 22.60 h, temperature 30.20°C,

inoculum size 6% (v/v) and inoculum age of 48 which gave 2.5 times more yield than un-optimized substrate having an absolute residual error of 6.14 %. The nutritional conditions like carbon, nitrogen and mineral sources were selected and optimized. At first they were selected and optimized using one factor at a time. The values from OFAT were used to select the upper and lower limit in response surface method to consider the all possible combinations for the experiments. Sucrose, ammonium sulphate and potassium chloride was selected that gave better ethanol yield at 4.06%, 0.5 % and 0.11 mg/ml respectively. The non-supplemented medium, even under optimized fermentation conditions, gave 7.5 times lesser yield.

The fermentation was further carried out using immobilized *Saccharomyces cerevisiae* MTCC 180 cells in 0.9% sodium chloride mixed agar matrix. On mixing and solidification, they were cut into pieces of small cubes and preserved in 0.1 (M) phosphate buffer solution of pH 5.5 in refrigerated conditions for 1 hour. For reusing the immobilized culture, it was taken out of refrigeration to achieve room temperature and washed with cold sterile water 2-3 times before they were used as inoculum for fermentation. The conditions of immobilization were optimized varying fermentation time, agar concentration, gel formation time and cell concentration with eth help of response surface methodology. The model validation of immobilization parameters gave maximum ethanol yield of 2.056 g/100 ml at the rate of 1.025 g/L/h achieved at 20.05 h fermentation time, 3.41 % agar concentration and 8.07 % cell concentration.

# CONCLUSION

The above study was based on evaluation of the potential of aqueous extract of sugarcane bagasse using a unconventional strain *Saccharomyces cerevisiae* MTCC 180. Food wastes like Potato wash water, spent tea and Bagasse were collected and sample variations were made. Various possible variations of extracts from these wastes were prepared and tested for reducing sugar and ethanol content. The test reveal that 30 minutes aqueous extract of bagasse gave the highest value of reducing sugar and ethanol. Therefore, this substrate was used for further experiments in fermentation.

The FTIR analysis of the aqueous extract of bagasse reveals majority of presence of disaccharides, very small amount of other polysaccharides, absorbed water and other impurities. Tray drying of the bagasse to get dry bagasse was performed to check its capacity to produce ethanol and found that it was not significantly different from all aspects with respect to wet bagasse. So, this insignificant unit operation was not included for further experiments.

Optimization of fermentation conditions was done on the substrate using statistical methods. The best conditions for fermentation was found to be at pH 4, fermentation time 22.60 h, temperature 30.20°C, inoculum size 6% (v/v) and inoculum age of 48 which gave having an ethanol yield of 0.342 g /100 ml produced at the rate of 0.142 g/L/h. It generated2.5 times more yield than un-optimized substrate having an absolute residual error of 6.14 %. The nutritional conditions like carbon, nitrogen and mineral sources were selected and optimized. Sucrose, ammonium sulphate and potassium chloride was selected that gave better ethanol yield at 4.06%, 0.5 % and 0.11 mg/ml respectively of 2.345 g/100 ml produced at the rate of 1.037 g/l/h. The non-supplemented medium, even under optimized fermentation conditions, gave 7.5 times lesser yield.

The fermentation was further carried out using immobilized *Saccharomyces cerevisiae* MTCC 180 cells in 0.9% sodium chloride mixed agar matrix. The conditions of immobilization were optimized varying fermentation time, agar concentration, gel formation time and cell concentration which gave maximum ethanol yield of 2.056 g/100 ml at the rate of 1.025 g/L/h achieved at 20.05 h fermentation time, 3.41 % agar concentration and 8.07 % cell concentration.

## FUTURE PROSPECTS

The investigation presented here is clearly problem problem-specific approach to mitigate some of the environmental pollutants produced daily as agriculture or food processing waste. An insightful conclusion of this is that an unconventional strain like Saccharomyces cerevisiae MTCC 180 can be utilized for fermenting a novel substrate like an aqueous extract of bagasse without any physical or chemical pre-treatment. The research can further be extended with a pilot scale study to check the yield at scale-up conditions. Different types of matrices can be tried other than agar to check their stability and compatibility for cell immobilization. The metabolic study of the microorganism and immobilized cell reuse can be studied in more detail. The bioethanol produced can further be studied in terms of its different parameters and efficiency as a biofuel in crude as well as blended form. Other potential applications of generated bioethanol can also be investigated. Embracing technologies such as this can be a sustainable approach towards effective waste utilization serving the objective of narrowing down the pollution burden on Earth, and making it a better habitat for our descendants.

# LIST OF CHEMICALS

| Name of the Chemical | Supplier company |
|---|---|
| Agar | SRL Chemicals. |
| Ammonium chloride | SRL Chemicals. |
| Ammonium sulphate | SRL Chemicals. |
| Calcium chloride | SRL Chemicals. |
| Dextrose | SRL Chemicals. |
| Dinitrosalicylic acid reagent (DNS) | HiMedia Laboratories Pvt.Ltd. |
| Disodium hydrogen phosphate | Merck |
| Ethanol | HiMedia Laboratories Pvt.Ltd. |
| Ferrous sulphate | SRL Chemicals. |
| Fructose | SRL Chemicals. |
| Hydrochloric acid | Qualigens Fine Chemicals Pvt. Ltd. |
| Magnesium sulphate | SRL Chemicals. |
| Peptone | SRL Chemicals. |
| Potassium chloride | SRL Chemicals. |
| Potassium dichromate | SRL Chemicals. |
| Potassium di-hydrogen phosphate | Merck |
| Potassium iodide | SRL Chemicals. |
| Rectified spirit | Bengal Chemicals & Pharmaceuticals Limited. |
| Sodium chloride | SRL Chemicals. |
| Sodium hydroxide | SRL Chemicals. |
| Sodium thiosulphate | SRL Chemicals. |
| Starch soluble | SRL Chemicals. |
| Sucrose | HiMedia Laboratories Pvt.Ltd. |
| Sulphuric acid | Merck |

| | |
|---|---|
| Tween 20 | BRM Chemicals. |
| Urea | SRL Chemicals. |
| Yeast extract | SRL Chemicals. |

## LIST OF INSTRUMENTS

| **Name of the Instrument** | **Company /Model** |
|---|---|
| Autoclave | M/s Instrumentation India, Kolkata. |
| BOD Cooling Incubator Shaker | M/s Instrumentation India, Kolkata. |
| Cooling centrifuge | Remi, 412 LAG. |
| Digital colony counter | Optics Technology Pvt. Ltd. |
| Digital pH meter | Systronics, µ pH system 361. |
| Fourier-transform infrared spectroscopy (FTIR) | Thermo Fisher Scientific, NICOLET 6700 FT-IR |
| Halogen Moisture Balance | Wenser, HMB50H. |
| Horizontal Tray dryer | M/s Instrumentation India, Kolkata. |
| Hot air oven | M/s Instrumentation India, Kolkata. |
| Incubator Shaker | M/s Instrumentation India, Kolkata. |
| Laminar air flow chamber | M/s Instrumentation India, Kolkata. |
| Refractometer | ERMA RBH 62 |
| Refrigerator | Whirlpool, DC 200 E-2019. |
| Rough weighing balance | Wenser, TTB 20 HH. |
| Scanning Electron Microscope | Merlin, Zeiss 6105 |
| Sensitive weighing balance | Wenser, PGB 220. |
| Temperature controlled hot plate | Ajay Pvt. Ltd. |
| Temperature Controlled Water bath | M/s Instrumentation India, Kolkata. |
| UV-Vis Spectrophotometer | Shimadzu, UV-1800. |

# LIST OF SOFTWARE

| Name of the Software | Company /Version |
|---|---|
| One way ANOVA | Microsoft office Excel, 2013 |
| Design-Expert | Stat-Ease, Version 7.0.0, 2021. |
| Origin-Pro | Origin-Pro 2022 b |