FERMENTATIVE PRODUCTION OF LIPASE IN SOLID STATE FERMENTATION BY ISOLATED STRAIN

Thesis Submitted For The Partial Fulfilment Of The Degree Of

Master Of Technology In Food Technology And Biochemical Engineering

Submitted By

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CERTIFICATE OF MERIT

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This is to certify that the work recorded in the report entitled "Fermentative Production of Lipase in Solid State Fermentation by Isolated Strain" submitted by Subhojit Mallick (M.Tech, 2nd Year, Department of Food Technology and Biochemical Engineering, Jadavpur University) is the faithful and bonafide research work carried out under my personal supervision and guidance. The result of investigation recorded in the report has not so far been submitted to any other project. The assistance and help received during the course of the process has been fully acknowledged.

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I hereby declare that this thesis contains literature survey and original research work by me as part of my Master of Technology in Food Technology and Biochemical Engineering studies.

All information in this document have been obtained and presented in accordance with academic rules and ethical conduct.

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Acknowledgement

I would like to express my gratitude to Prof. Dr. Uma Ghosh, my mentor teacher and the former H.O.D., Food Technology and Biochemical Engineering Dept., Jadavpur University, for her constant moral support to carry out the research work. I would like to express my gratitude to Associate Prof. Dr. Dipankar Halder, H.O.D., Food Technology and Biochemical Engineering Dept., Jadavpur University for his enormous support to carry out my project work. I would also like to convey my regards to all other senior faculty members of the Dept. of FTBE specifically Asst. Prof. Dr. Sunita Adhikari (Nee Pramanik), F.T.B.E. Dept., J.U. for guiding me with her valuable knowledge and all the staff members for helping me to complete the research work smoothly within time.

I am happy to oblige all the research scholars of my laboratory for their suggestions and continuous support.

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ABSTRACT

Lipases are group of heterogeneous enzymes which catalyses the hydrolysis of triacylglycerol to free fatty acids and glycerol. Lipase enzymes have applications in a wide range of industries including food, pharmaceutical, leather, cosmetics, textile, detergents etc. In the present study microbial cultures having lipolytic activities were isolated from a petrol-diesel spilled soil sample of a motor vehicle garage. These microbial strains were grown in wheat bran for Solid state fermentation. Maximum lipase production was achieved at a temperature of 37ºC for a fermentation time of 48 hours when 12gm of wheat bran was taken in 250ml Erlenmeyer flask and hydrated at a ratio of 0.92 (weight of wheat bran : volume distilled water & inoculums). Maximum lipase activity was observed when the fermentation was carried out by supplementation of wheat bran with 0.5% maltose and 0.5% beef extract and the lipase activities observed were 2561.7 U/gm and 2933.8 U/gm respectively.

At the optimum environmental conditions of temperature, weight of substrate, hydration ratio and incubation time the enzyme activities observed were 2290.4 U/gm (unit of enzyme activity per gram of wheat bran), 1525.7 U/gm, 2327.7 U/gm and 2234.9 U/gm.

Key Words: Solid State Fermentation, SSF, Lipase, Isolated strain

INTRODUCTION

Lipases are hydrolytic enzymes which are widely distributed in microorganisms, animals and plants. Lipases especially of microbial origin, have great potential in many commercial and analytical applications that act in aqueous-organic interfaces to release di-acylglyceride, mono-acylglyceride, free fatty acid and glycerol. However, in the environments where low water is available, lipases are able to catalyse many reactions such as esterification, interesterification and transesterification reactions. Therefore, lipases can be designated as a very versatile biocatalyst (Sharma et al., 2001; Pandey et al., 1999). Due to the different reactions lipases are able to catalyse and due to their regio and enantio selectivity, lipases find an increasing range of applications, such as in the detergent, food, pharmaceutical, fine chemicals, leather and pulp and paper industries (Freire and Castilho, 2000).

According to functions of enzymes they are classified into six major categories having different EC number (classification based on mechanism of working of enzyme). EC1 Oxidoreductases (catalyze reduction or oxidation reactions), EC2 Transferases (catalyze the shifting of a functional group from one molecule to another), EC3 Hydrolases (undergoes hydrolysis), EC4 Lyases (undergoes formation of double bonds), EC5 Isomerases (catalyze structural alterations within a molecule), EC6 Ligases (undergoes ligation). Among all of these enzymes, the most significantly used enzyme is Lipase. Before the mid-1980s, in most cases lipases were used in laundry applications and in the modification of triglycerides. Advanced research data has proved that they are also very effective biocatalysts to synthesize optically pure compounds for example cyclohexane (Gurung et al., 2013). The hydrolysis of triacylglycerides into fatty acids and glycerol is catalyzed by lipases. Such a process is known as lipolysis (Svendsen, 2000).

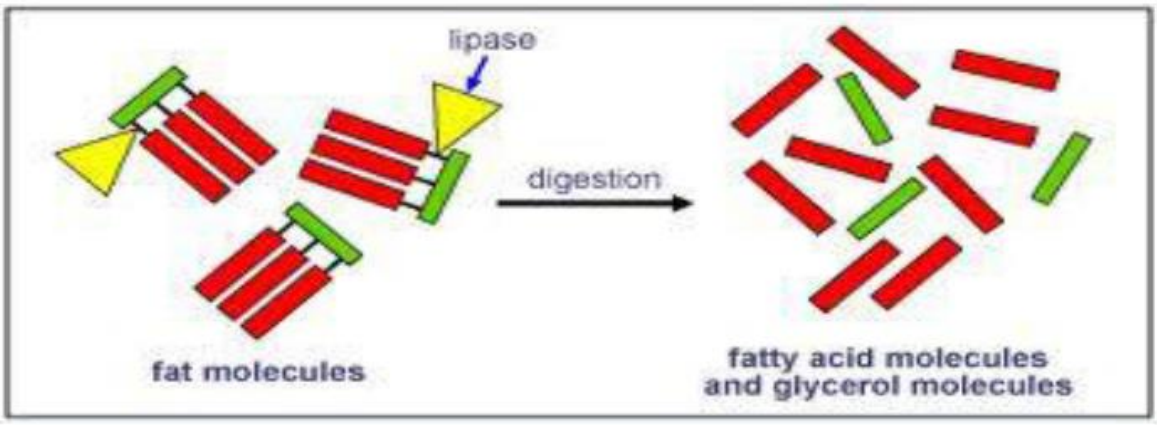


Fig. 1: Working of Lipase

Shumaila Kiran et al., 2016

The use of lipase as an industrial catalyst is increasing day by day as it has many favourable properties like high catalytic efficiency, bio-degradability and high specificity (Arife et al., 2015). The distinctive characteristics of lipase including temperature (Optimum temperature is 55 °C) specificity, non-toxicity towards nature, pH dependency and activity in organic solvents are major factors that are contributing to lead the demand of lipase in food industry. Lipases are investigated for synthetic and hydrolytic having different extraction sources. The utilization of mono-glycerides, di-glycerides, tri-glycerides and free fatty acids during trans-esterification, high yield or activity in non-aqueous medium, resistance to variation in temperature (Lithauer et al., 2002), low product inhibition, less reaction time and activity upto pH 8.0 are most desired features (Kumar et al., 2012). Furthermore, under mild conditions of temperature and pH, lipases can also undergo reaction. This characteristic of lipases helps to reduce energy demand at infrequent pressures and temperatures to direct reactions. Thus, the destruction of reactants and products that remains unstable during reaction can be protected because it changes the kinetics of the reactions (Cambon E et al., 2009). Lipases can act without co-factor with their substrate and they also show stability in organic solvents. These features are main reason for increasing of demand of microbial lipase in biotechnology (Yi-Shu Tai, Kechun Zhang, 2015).

The modification of fats and oil is one of the most important areas (Gupta et al., 2003). The properties of lipids can be altered by lipases when the position of fatty acid is changed in glycerides and also by exchanging one or more than one fatty acids with new ones. Thereby a less valuable and less needed lipid can be converted into greater value fat. By the use of highly selective phospholipases, phospholipids in vegetable oils can be eliminated. This process is latest development and non-toxic to environment (Clausen, 2001).

Detergent making with enzymes is common now and now-a-days. In term of volume and value enzymes are mainly consumed in detergent industries. The detergent becomes eco-friendly with the use of enzymes to avoid the use of harsh strains. Lipase in conjunction with amylase, proteases and cellulases are found in many laundry detergents (Jeon et al., 2009). The detergents which are used in laundry have become more popular due to the wide use in washing machine, resiliency to fabrics, softness producers and anti-staticness. Fabrics quality and texture can be retained with lesser wash temperature which is also an energy saving option in detergent industry (Weerasooriya and Kumarasinghe, 2012).

Lipases have many activities in production of aroma and so has great potential in perfumeries and cosmetics (Metzger and Bornscheuer, 2006). Derivatives of beta-carotene which is vitamin A that is retinoid in pharmaceutical and cosmetics has great applications like the products of skin cares, need explanation for the relation of vitamin A with lipase (Maugard et al., 2002)..

Lovastatin can be produced from lipase isolate from micro-organism *Candida rugosa*, it decreases the serum cholesterol level (Yang F., et al., 1997). Poly unsaturated fatty acids (PUFAs) obtained by using microbial lipases from plant and animal lipids, like oil of menhaden, borage and tuna oil. A variety of pharmaceutical can be produced by using free poly unsaturated fatty acids and their mono and di-acyl glycerides. PUFAs, due to their

metabolic benefits are used remarkably as pharmaceutical, nutraceuticals and food additives. Immobilized lipases are employing for production of nutraceuticals (Abhijit, 2012).

Lipolytic enzymes have popular use in bakery industry. Recently it is suggested that lipases are used to emulsify the supplement or substitute and due to this, lipases break the polar wheat lipid into emulsifying lipids in situ (Collar et al., 2000). Initially the flavour content of the products of bakery was enhanced with the help of lipases through esterification by liberating the short-chain fatty acids. The shelf-life of bakery products can also be enhanced with the help of lipase along with the enhancement of flavours. Through lipase action the softness and texture can also be improved. In baking industry *Aspergillus oryzae* is an artificial lipase producer which was used in processing. Lipase, xylanase, amylase and all other hydrolytic enzymes help in enhancing the specific volume of breads by reducing the initial firmness (Fariha et al., 2006).

Lipase in conjunction with other enzymes acts as desizer in textile industry. It removes the adhesive lubricant from the wrap thread that helps the high absorbency levelness in dyeing. In the system of denim abrasion, the frequency of cracks and streaks is also reduced with the help of lipases. Lipase enzymes and alpha amylase commercially used for desizing of cotton fabrics and denim (Macedo *et al*., 2003).

Lipases are used widely to improve the characteristics of food stuff. Although lipases have many applications in food industry but mostly they are used in flavour development and cheese ripening. The use of lipases is ex situ to obtain the food stuff having high nutrients, modification of structure by Tran- or inter-esterification and to develop flavour (Reetz, 2002). To enhance flavour in food stuff, the production of esters of fatty acids bearing short chain and alcohols is done. These are mostly used flavour compounds. Thus, to maintain the life time of food and to enhance the flavour, lipases are most important and widely used biocatalyst (Macedo et al., 2003).

Lipases also bear some draw backs like high production cost, less commercialization and slow reactivity in some lipase-mediated processes. Due to these factors the use of lipase is restricted to few industries. But in near future these draw backs are going to overcome by new innovative features of lipases that are under study. Many genes of lipase enzymes with unique features are still unknown and need to be explored (Shumaila Kiran et al., 2016).

However, an even greater industrial application of these enzymes would depend on the development of low-cost processes for the production of lipases. In this regard, solid-state fermentation (SSF) appears to be an interesting low-cost alternative technique for the production of biomolecules. In Solid State Fermentation, agro-industrial residues or wastes can be utilized as substrate medium for fermentation. These low-value and easily available raw materials contribute to reduce production costs (Freire and Castilho, 2000). Additionally, SSF, which is characterized by microbial growth on moist solids, has proven to be an efficient way to produce enzymes, especially by filamentous fungi, since it provides the microorganisms with environmental conditions similar to their natural habitat (Pandey et al., 1999; Durand, 2003)**.**

However, industrial scale application of SSF is not yet fully developed. The low moisture contents used in SSF pose limitations to mass and heat transfer, producing heterogeneous environments in full-scale bioreactors, which contribute to overheating and deficient oxygen supply (Ashley et al., 1999; Mitchell et al., 1999)**.** Due to these problems, very few types of SSF bioreactors are found in the literature (Couto et al., 2003). More research is needed in this field to allow successful industrial scale applications of the SSF process to be developed (Ashley et al., 1999).

Solid state fermentation uses culture substrates where water levels are low, thereby reduced water activity, which is appropriate for mold. This method used to grow filamentous fungi using SSF which allow the best reproduction of their natural environment. The medium is saturated with water but should not be free flowing. The solid medium provides both the substrate and the solid support over which the fermentation takes place. The most commonly used substrates are composed of vegetable by products like wheat bran or beet pulp.

SSF has been used traditionally in Asian countries to produce Koji using rice to manufacture alcoholic beverages such as Sake or Koji with soybean seeds. The latter, produces sauces such as soy sauce or other foods. Traditional manufacturing process of many foods in Western countries uses solid state fermentation. Examples of such include fermented bakery products like bread or the manufacture of cheese. To prepare raw materials of chocolate and coffee, SSF is widely used typically cacao bean fermentation and coffee bean skin removal are SSF process carried out under natural tropical conditions.

Enzymes and enzymatic complexes are able to break down macromolecules which are difficult to transform such as cellulose, hemicelluloses, pectin and proteins. SSF is very much recommended for the production of various enzymatic complexes composed of multiple enzymes. Enzymatic compounds generated by SSF find outlets in all sectors where digestibility, solubility or viscosity is needed.

Growing Environmental awareness and economic changes generates new perspectives for solid state fermentation. SSF adds value to insoluble agricultural by products thanks to its higher energy efficiency and reduced water consumption.

Solid state fermentations could be used for the production of various enzymes utilizing various agro-industrial wastes. Lipase production in SSF under variable process conditions, with different types of microorganisms and substrates has been reported (Godoy et al., 2009; Hernandez-Rodriguez et al., 2009, Sun et al., 2009). However, most of the studies were done using a few grams of substrate, mesophilic temperatures and pure cultures of known microorganisms. There are only few studies have been carried out at pilot plant or industrial scales (Kumar et al., 2009; Edwinoliver et al., 2010). SSF compensates the problems related to mass and heat transfer phenomena associated with solid substrates (Pandey et al., 2008). The use of natural solid substrates can hinder downstream processes (Rodriguez-Couto and Sanroman, 2006), specifically at the time of extraction of lipophilic enzymes like lipases (Mala et al., 2007). Fermented solids have been used as naturally immobilized biocatalysts for synthesis reactions in lyophilized (Hernandez-Rodriguez et al., 2009) or dried form

(Hellner et al., 2010). This attempt leads to lower cost of enzyme preparations since no extraction and purification step has to be carried out. The study on scalable solid state fermentations with vegetable oil refining industry wastes has shown that these kinds of wastes can be a good source of substrate for lipolytic enzymes production with many promising properties. Use of fermented solid substrates as biocatalysts is also promising in terms of low-cost production process with high yield potential because of reduction of loss during extraction process. Further research could explore the application of the obtained lipases in novel synthetic routes and their identification. There is another point which needs attention is the reproducibility of the source microorganisms used since sludge and, in general organic solid wastes are inherently variable in chemical composition and in the characterization of the existing microbial communities.

CHAPTER ONE

Isolation & identification of lipase producing strain

1.1 Isolation and identification of strain

In microbiological aspect, the term isolation is defined as separation of a strain from a natural, mixed population of living microbes, as present in the environment, for example in water or soil flora, or from living beings with skin flora, oral flora or gut flora, in order to identify the microorganisms of interest. Historically, in the bacteriological and parasitological field the laboratory techniques were first developed during the 19th century, after that in the 20th century it was developed in the field of virology. Thereafter, methods of microbial isolation have drastically changed over the past 50 years, from a labour perspective with increasing mechanization, and in regard to the technology involved, and hence speed and accuracy.

In order to isolate a microbial strain from a natural, mixed population of living microbes, as present in the environment, for example in water or soil flora, or from living beings with skin flora, oral flora or gut flora, the first assignment is to separate it from the mix.

In traditional methods microbes have been cultured in order to identify the microbe(s) of interest based on its growth characteristics. Depending on the expected density and viability of microorganisms present in a liquid sample, physical methods to increase the gradient as for example serial dilution or centrifugation may be chosen. In order to isolate organisms in materials with high microbial content, such as sewage, soil or stool, serial dilutions will increase the chance of separating a mixture.

Identification of the selected strain was carried out according to Bergey's manual of determinative bacteriology (Holt et al 1994). For this purpose, different media required for different biochemical tests were prepared.

Then morphological, physiological and biochemical characteristics of the selected strain were studied in detail.

1.2 Materials and Methods

The lipase producing bacterial strain was isolated from a petrol and diesel spilled soil sample collected from a motor vehicle garage, located at Chandrakona village of West Midnapore, West Bengal. A definite weight of Soil sample was first suspended in sterilized distilled water with the help of a magnetic stirrer (Remi Make). Soil suspended water then serially diluted in sterilized isotonic solution containing 0.9% NaCl. Serially diluted soil samples then plated in Tributyrin agar medium (TBA medium) containing 1% (v/v) Glycerol Tributyrate, 0.5% Peptone, 0.3% Yeast Extract and 3% Agar. pH of the medium was maintained 7.5 and temperature of incubation maintained at 37 °C for 24 hours.

Then the isolated strains were used for SSF using wheat bran as fermentation medium for secondary screening of potent lipase producing strain. The wheat bran was purchased from local market for the study. 12 gm of wheat bran was taken in three different 250ml Erlenmeyer flasks, sterilized in autoclave at 15psig, for 15 min. Nutrient broth medium (1.5% Peptone, 0.3% Yeast extract, 0.1% Dextrose, 0.6% NaCl, pH 6.8 – 7.0) was prepared as inoculum**s** medium and sterilized in autoclave. The isolated strains were inoculated in different 250ml Erlenmeyer flasks containing sterilized nutrient broth medium incubated at 37 °C for 24 hours at 130 rpm.

Each of 10ml of 24 hours grown culture and 1ml of sterilized distilled water was transferred to three different Erlenmeyer flasks containing previously sterilized 12gm wheat bran using sterilized pipette. Then the contents of each flask mixed thoroughly with a sterilized glass rod. Then all the flasks incubated at 37 °C for 48 hours at inclined position to maximize surface area.

After 48 hours of fermentation 120ml of distilled water (Wheat bran: Distilled water = 1:10) was added to each Erlenmeyer flasks and kept in refrigerator for soaking overnight to extract enzyme.

The content of each flask was then filtered through clean cloth and squeezed to extract maximum volume of aqueous extract. Volume of the filtrate was measured then and a portion of the filtrate then transferred to centrifuge tubes & centrifuged at 4000 rpm for 5 min. The clear supernatant was used as crude enzyme. The clear crude enzyme preparation then decanted test tubes for lipase activity determination.

Extracellular Lipase activity was measured using polyoxyethylene sorbitan ester (Tween 80) as substrate by the method described by Tirunarayanan and Lundbeck (Tirunarayanan and Lundbeck, 1968) with slight modifications. The reaction mixture contains 0.2ml of 10% Tween 80 in 50mM Tris hydrochloride buffer (pH 7.6), 1.0ml of concentrated culture supernatant as a source of enzyme, 0.2ml of 1M $CaCl_2$ in Tris buffer, and 4.6ml of Tris buffer (pH 7.6). Reaction mixture with 1.0ml of deionized water instead of supernatant was considered as a blank. Then the reaction mixtures were incubated for 2 hours at 37 °C in an incubator. In this spectrophotometric assay technique (Vivek S. Vishwe et al. 2015), Tween which is the ester of oleic acid, was cleaved to produce fatty acid and alcohol. Presence of calcium in the reaction mixture leads to the formation of an insoluble fatty acid salt, giving a

precipitate which can be measured spectrophotometrically at 400nm. One unit of Lipase activity was defined as the amount of enzyme resulted in an increase of optical density at 400nm (OD at 400) of 0.01 after 2 hours under the assay conditions. Lipase activity of all isolated lipolytic microorganisms were determined and expressed in U/gm of Wheat bran.

After determining the strain which produces the maximum unit activity of lipase, we tried to identify the strain by morphological, physical and biochemical tests.

Morphology

The size and shape of the single strain was measured by the microscopic view of the strain after staining with crystal violet (0.5% aqueous solution**)** (Holt et al., 1994). The isolated strain was first smeared on a clean glass slide. The slide containing the organism then stained with crystal violet, observed through optical microscope and with the help of ocular and stage micrometer the size of the selected strain was measured.

Gram Characteristics

A smear was prepared from a 24hr nutrient agar slant culture of the isolated strain. It was dried and fixed on slide. The smear was covered by sufficient Crystal Violet stain (0.5% aqueous solution) and allowed to remain for 1 min. Excess stain was poured off and the slide was washed in tap water. Gram's Iodine solution (Iodine – 1.0 g, Potassium Iodide – 2.0 g, Distilled Water – 300 ml) was applied to the slide and allowed to remain for 1 min. The slide was washed using tap water and air dried. The smear was covered with 95 % alcohol. The slide was washed in tap water and dried. The smear was counter stained with Safranine for ½ min. Again, the slide was washed in tap water, drained and air dried. The slide was examined under oil immersion lens (Holt et al., 1994).

Spore staining

Heavy suspension of the organism in a few drops of distilled water in a test tube was prepared and then equal quantity of Carbolfuchsin solution was added and the mixture was boiled for 20 min. Carbolfuchsin solution was prepared by dissolving 0.3 gm of Basic fuchsin in 10 ml of alcohol, separately 5 gm of phenol dissolved in 100 ml D.W. and then the two solution was mixed then. Next one Loop full of the stained preparation with one loop full of Nigrosin solution (prepared by dissolving 10 gm of Nigrosin in 100ml D.W. then it was boiled for 30 min and 0.5ml of 40% formaldehyde was added as preservative, after that the medium was filtered through double filter paper) was mixed. The smear was prepared as thin as possible and dried rapidly. Then the slide was examined under oil immersion objectives lens of the microscope (Holt et al., 1994).

Motility test

A loopful of culture was taken on a clean glass slide. A drop of distilled water was added over it and it was covered with a cover slip. The slide was observed under microscope at total magnification of 1000X (Holt et al., 1994).

Growth in liquid medium at stationary and shaking conditions

50ml of the prepared nutrient broth medium was taken in two different 250ml conical flask, autoclaved to sterilize and then a loopful of culture was inoculated to the sterilized nutrient broth medium aseptically and incubated for 24 hour and 48 hours at 37 ºC (one flask at stationary condition and other one at 130 rpm. After incubation period the flasks were observed for microbial growth (Holt et al., 1994).

Growth in solid medium of strain

The nutrient agar medium (peptone 0.5%, beef extract 0.3%, agar 3%, D.W. 100 ml, pH – 7.2) was prepared and then sterilized in autoclave. A portion of the sterilized medium was then added to a sterilized petri dish and allowed to solidify. When it got solidified, it is inoculated with the microbial strain with an inoculating needle and then the plate was incubated at 37 °C for 24 hours. After incubation is over, the plate was examined by visual observation (Holt et al., 1994).

Spectrophotometric method of Growth Study

Sterile nutrient broth media was prepared that will be used for inoculation. Inoculate the sterile nutrient broth medium with the isolated strain and incubated at 37ºC. After 24 hours of incubation optical density (O.D.) of the culture medium was recorded at 600nm wavelength in spectrophotometer using sterile uninoculated nutrient broth medium as blank. Similarly, after 48 hours of incubation the O.D. was recorded again. Similar measurement was done again after 72 hours of incubation (Holt et al., 1994).

Physiochemical characteristics study

Rhodamine Test

The isolated culture in Nutrient agar slant was inoculated in Rhodamine-Tributyrin Agar medium plates containing 1% (v/v) Glycerol tributyrate, 0.5% Peptone, 0.3% Yeast Extract, 0.00001% (w/v) Rhodamine B and 3% Agar using streaking technique. pH of the medium was maintained 7.5, temperature and time of incubation were 37 °C and 24 hours respectively (Rajesh.B et al., 2013).

Nitrate reduction

50 ml solution containing 2 % (W/V) peptone and 1 % (W/V) potassium nitrate was prepared. Then 10 ml of this medium was distributed in different test tubes with an inverted Durham tube in each test tube and then test tubes were cotton plugged and sterilized at 15 psig pressure at 121 ^{0}C for 15 minutes.

Then 1 ml of 24 hr inoculum of the strain was added to each test tube and the test tubes were incubated at 37 ^{0}C for 24 hours along with a control test tube (without inoculum).

Meanwhile Griess – Hesvay's reagent was prepared which was

(a) 8 g sulphanilic acid in 1000 ml of 5 (N) acetic acid.

(b) 5 g α - Nepthylamine in 1000 ml of 5 (N) acetic acid.

After the incubation of the test tubes, 1 ml of the reagent (a) and reagent (b) was added to each test tube, including the control (Holt et al., 1994).

Catalase reaction

At first one loop full of the isolated strain was taken on a clean glass slide and spread with the inoculating needle. Then few drops of H_2O_2 (Hydrogen Peroxide) was poured over the strain and then observed for effervescence (Holt et al., 1994).

Starch hydrolysis

For this test, nutrient agar medium (peptone 0.5%, beef extract 0.3%, agar 3%, D.W. 100 ml, pH – 7.2) was prepared. The medium was taken in some test tubes, taking 10 ml in each test tube and then cotton plugged, wrapped with brown paper and sterilized at 15 psig pressure, 121 ºC temperature for 15 minutes.

Again starch – agar solution was prepared by heating the nutrient agar with 1 % starch on a hot water bath. The medium was taken in some test tubes taking 5 ml in each test tube and the test tubes were cotton plugged and sterilized at 15 psig pressure, 121 °C temperature for 15 minutes.

After sterilization, 10 ml of hot nutrient agar was poured in each sterile petri dish and after solidification of the medium 5 ml of hot starch-agar solution was poured on the solid masses, and cooled.

Then the solution was taken forma slant culture with a wire loop and streaked on petri dishes with present medium and then the petri dishes were incubated at 37 ºC for 24 hours.

After incubation, about 10 ml of Gram's Iodine solution was added to the sterile petri dish and then it was observed whether any clear zone was formed around the streaking line (with indicates hydrolysis of starch) or any reddish – brown zone around the streaking line (which indicates partial hydrolysis of starch) or the whole plate turns blue due to unhydrolyzed starch (Holt et al., 1994).

Production of ammonia from urea (Urease test)

For this test Christensen's Urea agar medium was prepared. Composition of the medium was as follows, Peptone 0.5 g, NaCl 0.5 g, KH_2PO_4 0.2 g, D – glucose 0.1 g, Agar 3.0 g, Phenol red 0.2 g, Distilled Water 100 ml, pH - 7.0. About 16 ml of medium was distributed in each of a few test tubes and then test tubes were cotton plugged and sterilized at 15 psig pressure, 121 ºC temperature for 15 minutes. Then the test tubes were cooled down to 50 °C. 20% Urea solution which was previously sterilized was added to the basal medium in sufficient amount so that final concentration of urea in the basal medium was 2 %. Then the medium was allowed to set.

Hot molten medium was aseptically added to each sterile petri dish from each test tube and after cooling the medium a loop full of the strain was aseptically taken from a slant culture and aseptically streaked on the solid medium with the same loop. Then the petri dishes were incubated at 33 ºC for 7 days (Holt et al., 1994). After incubation it was observed whether any colour change from yellow to pink or red occurred by the production of ammonia (Urease hydrolyses urea into ammonia).

1.3 Results and Discussions

In the primary isolation technique lipase producing microbial strain was isolated from a petrol and diesel spilled oil sample by on tributyrin agar medium where formation of clear halo zones around the grown colonies indicate that those strain are lipolytic in nature and are able to produce lipase enzymes. Tributyrin agar is a differential medium that tests the ability of an organism to produce an exoenzyme, called lipase. Tributyrin oil forms an opaque suspension in the agar. When an organism produces lipase and breaks down the tributyrin, a clear halo surrounds the areas where the lipase-producing organism has grown.

Among the all incubated plates three numbers of colonies were observed as producers of clear halo zones surrounding the growth areas. Those particular colonies were then transferred to Nutrient agar slants and kept preserved at refrigerator and marked them as ‘B1’, ‘B2’ and ‘B3’. The approximate D-d values [Diameter of the total clear hydrolytic halo including colony (D) – Diameter of the colony (d)] of the clear halo zones formed on the TBA medium was 16 – 20 mm (refer to Fig. 1.1) compared to D-d value reported from a study 20 – 29 mm, where bacterial strains isolated from sun dried spoiled coconut (Rajesh.B et al., 2013)**.**

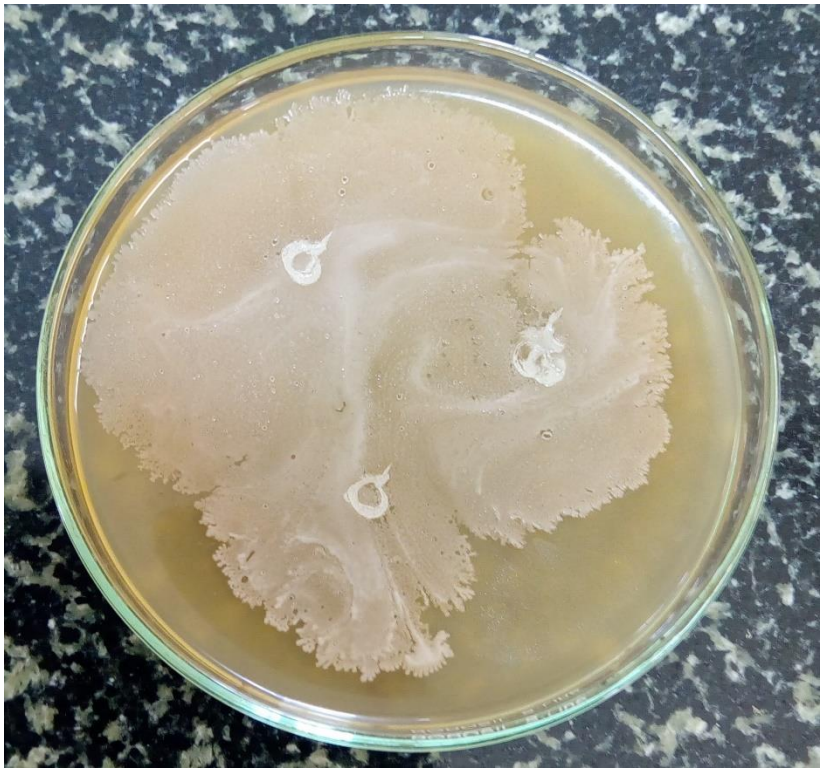
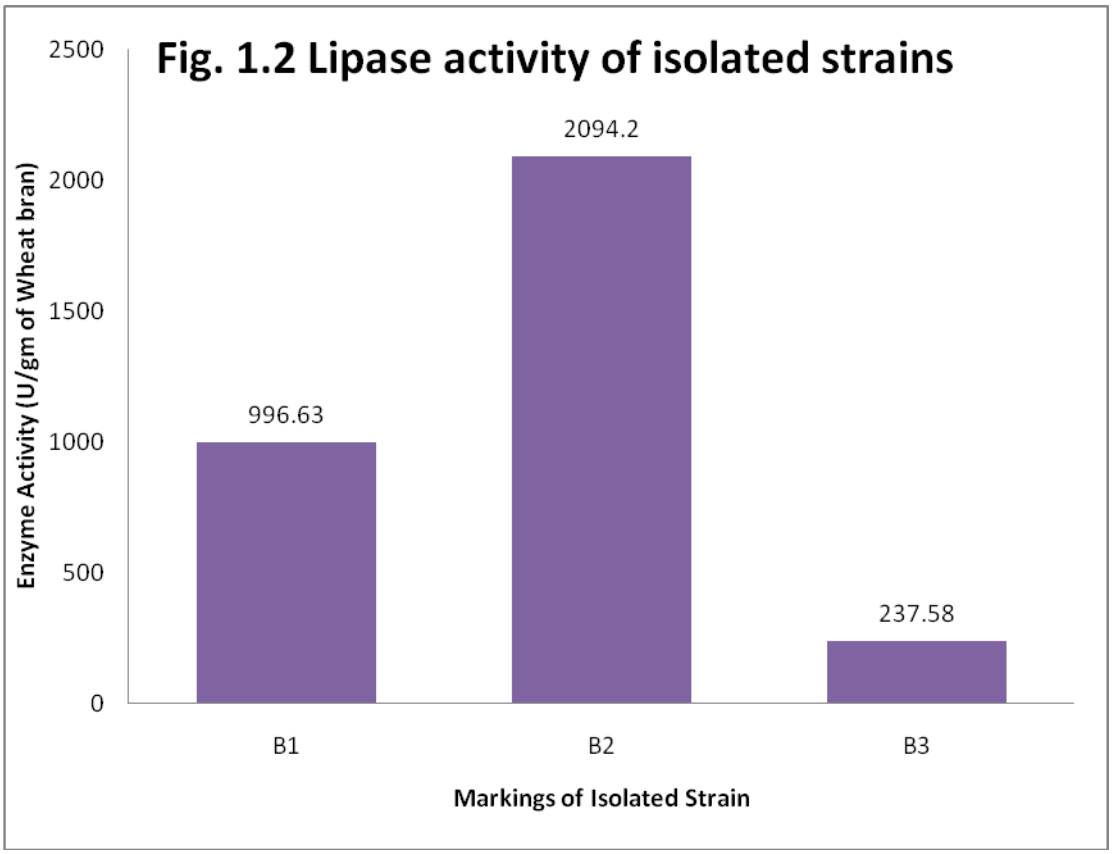


Fig. 1.1 Tributyrin agar medium showing halo zones around growth areas of the isolated strain

From the results obtained from the experimental methods to identify the best lipase producing microbial strain among the three isolated strain from petrol and diesel spilled soil sample and partial identification of the best isolated strain it is found that among the three different isolated strains B1, B2 and B3 produces 996.63, 2094.20 and 237.58 Units of enzyme activity per gram of dry wheat bran (Table 1.1 and Fig. 1.2). Therefore, the strain marked 'B2' has been isolated as the best productive strain among the three isolated strains. The results thus obtained is comparable to lipase activity of 26.4 U/g, (Elisa d'Avila Costa Cavalcanti et al. 2005) using packed bed bioreactor by *Penicillium simplicissimum* strain used in this work was isolated from the solid residues generated during the industrial processing of babassu seeds (Freire et al., 1997), lipolytic activity reached of 120,000 UA/g of dry matter (Angélica Santis-Navarro et al.2011) where lipases were produced by a microbial consortium derived from a mixture of wastewater sludges in a medium containing solid industrial wastes rich in fats, under thermophilic conditions (temperature higher than 45 ºC for 20 days) in 4.5-L reactors. The lipases were extracted from the solid medium using 100 mM Tris–HCl, pH 8.0 and a cationic surfactant agent (cetyltrimethylammonium chloride).

Table 1.1 Enzyme activities of isolated microbial strains		
Markings of Isolated Strain	**Wt. Of wheat bran (gm)**	**Enzyme Activity (U/gm of Wheat bran)**
B1	12	996.63 ± 6.96
B2	12	2094.20 ± 27.88
B3	12	237.58 ± 7.13



Identification of the isolated microbial strain was only done partially. For complete identification more, biochemical tests and molecular characterization test to be performed.

From the morphological study (refer Fig. 1.3) as seen after gram staining of the prepared smear of the organism under the microscope, it was found that the isolated organism appeared oval in shape, violet in colour. Therefore it can be concluded that the organism is gram-positive and coccus shaped (refer Table 1.2). The size of the strain was determined by calibrating stage micrometer scale with the ocular micrometer scale at total magnification of 1000X (1 ocular division = 1 micrometer).

Staining of the isolated strain with nigrosin solution shows that the organism is non-spore former.

A loopful of the isolated culture in a drop of water over a glass slide when examined under the microscope, little movement of the cells was observed. Therefore it can be said that the isolated B2 strain is motile.

After 24 hours incubation at stationary condition at 37ºC in 50ml nutrient broth medium kept in 250ml Erlenmeyer flask, very little growth of the organism was observed with little sedimentation at the bottom. Whereas in shaking condition of incubation for similar time and temperature fair growth was observed with no sedimentation (see Table 1.3).

Smooth, opaque, off-white coloured colonies of diameter 1mm to 3mm were observed after 24 hours of incubation at 37ºC in nutrient agar medium in petri dishes.

In the spectrophotometric study of microbial growth as soon as the O.D. observations show values above 1.0, it indicates that stage of growth of the organism is either in logarithmic phase or stationary phase. But when it shows a value below 1.0 it indicates the declining growth phase/ death phase. The O.D. readings after 24 hours and 48 hours of growth in nutrient broth medium were above 1.0 but after 72 hours it was reduced to 0.732. Therefore, it was clear from the observations that the organism maintains logarithmic or stationary growth phase upto 48 hours of incubation at 37ºC.

Rhodamine test was perforemed with the isolated B2 strain using streak plate method. Use of fluorescence dye such as Rhodamine B for isolating lipolytic organisms by using tributyrin oil as substrate is widely used. The extracellular lipase produced by the microorganism acts upon tributyrin oil present in the medium and liberates free fatty acids, which form complexes with the Rhodamine dye and produces orange fluorescence while non-lipase producers make pink colour. After 24 hours of incubation the plate was examined in a UV Transilluminator under the exposure of UV rays. Development of orange colour in the bacterial growth regions (see Fig. 1.4) was observed which clearly indicate that the organism has produced lipase. Development of orange colour in the growth areas confirms the ability of the following strain for lipase production (Rajesh.B et al., 2013).

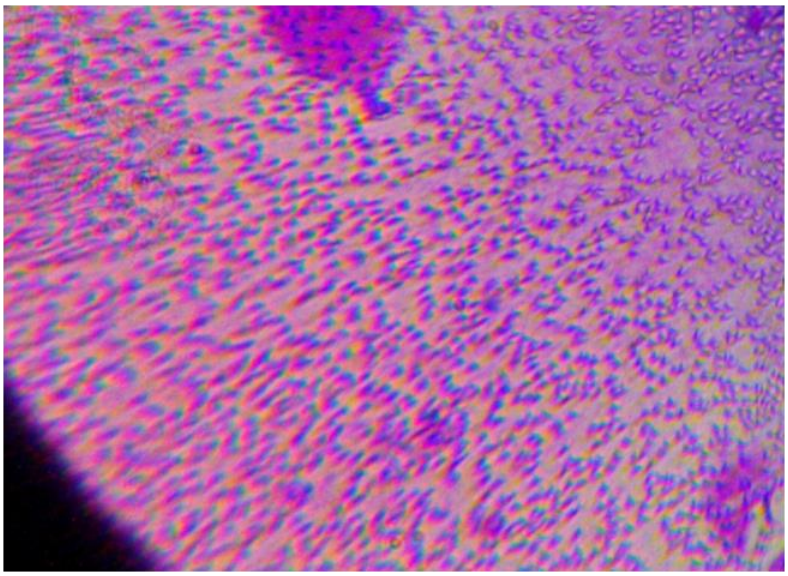


Fig. 1.3 Image of Gram Staining

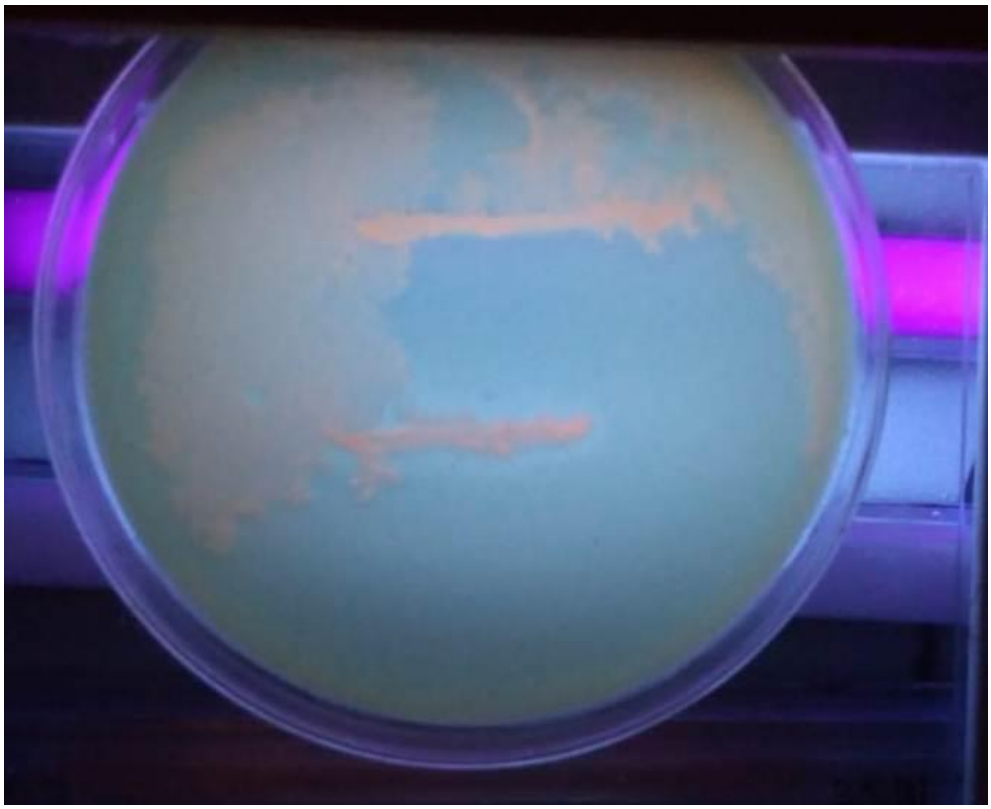


Fig. 1.4 Image of Rhodamine test shows development of orange colour in streaked growth zones

The nitrate reduction test is based on the detection of nitrite and its ability to form a red compound when it reacts with sulfanilic acid to form a complex (nitrite-sulfanilic acid) which then reacts with a α-naphthylamine to give a red precipitate (prontosil), which is a water-soluble azo dye. However, only when nitrate is present in the medium, red color will be produced. If there's no red color in the medium after addition of sulfanilic acid and α-naphthylamine, it means only that nitrite is not present in the medium. After 24 hours of incubation when sulfanilic acid solution and α-naphthylamine solution was added to the contro test tube and test tube containing B2 strain no red colour formation was observed even no sign of gas formation observed in the inverted durham tubes. It confirms the absence of nitrite in the tubes. On addition of a small quantity of zinc dust to the tubes formation red colour was observed which indicate the presence of nitrate. It proves the inability of the strain to reduce nitrate to nitrite (see Table 1.4).

The catalase reaction test demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide (H_2O_2). The presence of the enzyme in a bacterial isolate is evident when a small inoculum is introduced into hydrogen peroxide, and the rapid elaboration of oxygen bubbles occurs. The lack of catalase is evident by a lack of or weak bubble production. When few drops of hydrogen peroxide was added over a loopful culture of the isolated strain on a glass slide, rapid formation of effervescence was observed. It clearly indicates that the strain is catalase positive (see Table 1.4).

In the starch hydrolysis test, the test organisms are grown on agar plates containing starch. If the bacteria have the ability to hydrolyze starch, it does so in the medium, particularly in the areas surrounding their growth while the rest of the area of the plate still contain non-hydrolysed starch. Since no colour change occurs in the medium when organisms hydrolyze starch, iodine solution is added as an indicator to the plate after incubation. While the non-hydrolysed starch forms dark blue colour with iodine, its hydrolyzed end products do not acquire such dark blue colour with iodine. Consequently, transparent clear zones are formed around the colonies that hydrolyze starch while the rest of the plate show a dark blue coloration as iodine forms the coloured complex with starch. In the test, no blue colour was observed around the colonies of the isolated B2 strain on addition of grams iodine solution to the incubated plates. It clearly indicates that the isolated strain is capable of hydrolysing starch (see Table 1.4).

Urea is the product of decarboxylation of amino acids. Hydrolysis of urea produces ammonia and CO_2. The formation of ammonia alkalinizes the medium, and the pH shift is detected by the colour change of phenol red from light orange at pH 6.8 to magenta (pink) at pH 8.1. After 24 hours of incubation of the petri plates no colour change was observed which indicates the organism's inability to produce urease (see Table 1.4).

Table 1.2 Morphological Characteristics

Experiment	Observation
i. Morphology of vegetative cells	Cell shape – oval (cocci) Cell size – **1.75 ± 0.5 μm (Dia)**
ii. Arrangement	Single, double
iii. Spore staining	Negative
iv. Motility	Positive
v. Gram characteristics	Gram positive

Table 1.3 Cellular Characteristics

Experiment	Observation
i. Growth in 50 ml medium taken in 250 ml Erlenmeyer flask	A. Stationary condition (a) After 24 hr: Poor growth, no ring formation, no pellicle formation, sedimentation at the bottom, upper portion of the broth was clear. (b) After 48 hr: Same as after 24 hr but the growth was fair.

	B. Shaking condition. (a) After 24 hr: Fair growth, turbid, no sedimentation, no pellicle formation, no pigmentation, no ring formation. (b) After 48 hr: Same as after 24 hr.
ii) Growth in solid medium	A. Nutrient agar medium in petri plate Colony characteristics: 1mm – 3mm (diameter) Opacity: opaque Surface growth: Smooth Colour: Off-white Pigmentation: Nil
iii) Spectrophotometric method of growth study	**O.D. at 600nm** After 24 hours: 1.082 After 48 hours: 1.154 After 72 hours: 0.732

Table 1.4 Biochemical Test

Parameters	**Characteristics**
Rhodamine test	Lipase Positive
Nitrate reduction	Negative
Catalase reaction	Positive
Starch hydrolysis	Positive
Urease test	Negative

CHAPTER TWO

Optimization of Environmental parameters for Production of Lipase in SSF

2.1 Environmental Parameters Optimization

To increase the production and the productivity of the lipase enzyme, effect of different physiological parameters like temperature, weight of wheat bran, extent of hydration and incubation time have been studied. The optimization of lipase production was carried on the variation of physical parameters.

In the classical medium optimization technique, one-factor-at-a-time (OFAT) experiments, only one factor variable can be varied at a time while keeping all other variables constant. Because of its ease and convenience, the OFAT has been the most preferred choice among the researchers for designing the medium composition and used in the initial stages in diverse fields (Gonzalez et al., 1995). This technique is still in use even today, during the initial stages of medium formulation for the production of new metabolite or known compound from a new source. Based upon the approach applied OFAT has further sub-groups:

Removal experiments where all the medium components are removed from the production medium one-by-one and after proper incubation period, their effects on the production of secondary metabolite or interested product is observed in terms of suitable parameters.

Supplementation experiments are normally performed for evaluation of the effects of different carbon and nitrogen supplements on metabolite production.

Replacement experiments performed for medium formulation, carbon or nitrogen sources showing enhanced metabolite production in supplementation experiments are tried to use as a whole carbon or nitrogen source.

In addition to chemical and biological variables, many researchers used OFAT (One Factor At a Time) experiments to standardize the physical parameters like temperature, pH, agitation, aeration requirements etc. of fermentation process (Niwas et al., 2013).

To meet the increasing demand of the industries, low-cost medium is required for the production of lipase enzyme. Though, both solid state fermentation (SSF) and submerged fermentation (SmF) could be the choice for the production of lipase, although these have been obtained traditionally from submerged cultures because of the ease of handling of media and greater control of environmental factors such as pH and temperature. Mostly synthetic media have been used for the production of bacterial lipase but the raw materials are very much expensive and there is a solubility problem of oily substrates in the liquid medium. Therefore, those expensive raw materials could be replaced by cheaper agricultural by-products for the reduction of cost of the medium. SSF utilizes natural microbiological processes such as composting and ensiling, which is then can be utilized in controlled way to produce a desired product. SSF has been utilized from long ago to convert moist agricultural polymeric substrates to such products including industrial enzymes. SSF defined as growth of microorganisms on moist substrates with negligible free water. The substrate used in solid state fermentations not only supports the microorganisms but also provides nutrition. Solid state fermentation constitutes an interesting alternative since the metabolites those are

produced are concentrated and therefore the less costly purification processes (Pandey et al., 2000; Pandey 1992; Nigam and Singh, 1995).

In our study we used wheat bran as the substrate for the fermentative production of lipase. Though other substrates such as sunflower meal, olive cakes, rice husk, cottonseed meal, soybean meal and rice bran have also been utilized for lipase production through SSF. Though SSF is mostly utilized for fermentative production of various metabolites by fungi, however successful bacterial growth in SSF is known in much natural fermentation (Lonsane and Ramesh, 1990; Ramesh and Lonsane, 1991). The production of lipolytic enzymes by SSF is limited to the genus *Acinetobacter radioresistens* (Zhao et al., 2013), *Pseudomonas sp.* (Latip et al., 2016), *Pseudomonas aeruginosa* (Bose et al., 2013), *Staphylococcus caseolyticus* (Sharma et al., 2014), *Bacillus sterothermophillus* (Ekinci et al., 2015). The production of bacterial lipase using the SSF technique requires less fermentation time which leads to considerable reduction in the capital and recurring expenditure. Research on the selection of suitable substrates for SSF has mainly been centered on agro-industrial residues due to their potential advantages for lipase producing bacteria, which are capable of penetrating into the hardest of these solid substrates.

Now-a-days fermentation technology is widely used for the production of various economically important compounds which have applications in the energy production, pharmaceutical, chemical and food industry. Though, fermentation processes are used from generations, the need for sustainable production of products, meet the market requirements in a cost-effective manner has put forward a challenging demand. For any fermentation-based product, the most important thing is the availability of fermented product equal to that of market demand. According to many reports, various microorganisms produce an array of primary and secondary metabolites, but in a very low quantity. So, to meet the market demand, several high yielding techniques have been discovered in the past, and successfully implemented in various processes, like production of primary or secondary metabolites, biotransformation, oil extraction etc. (Dubey et al., 2008, 2011; Singh et al., 2009).

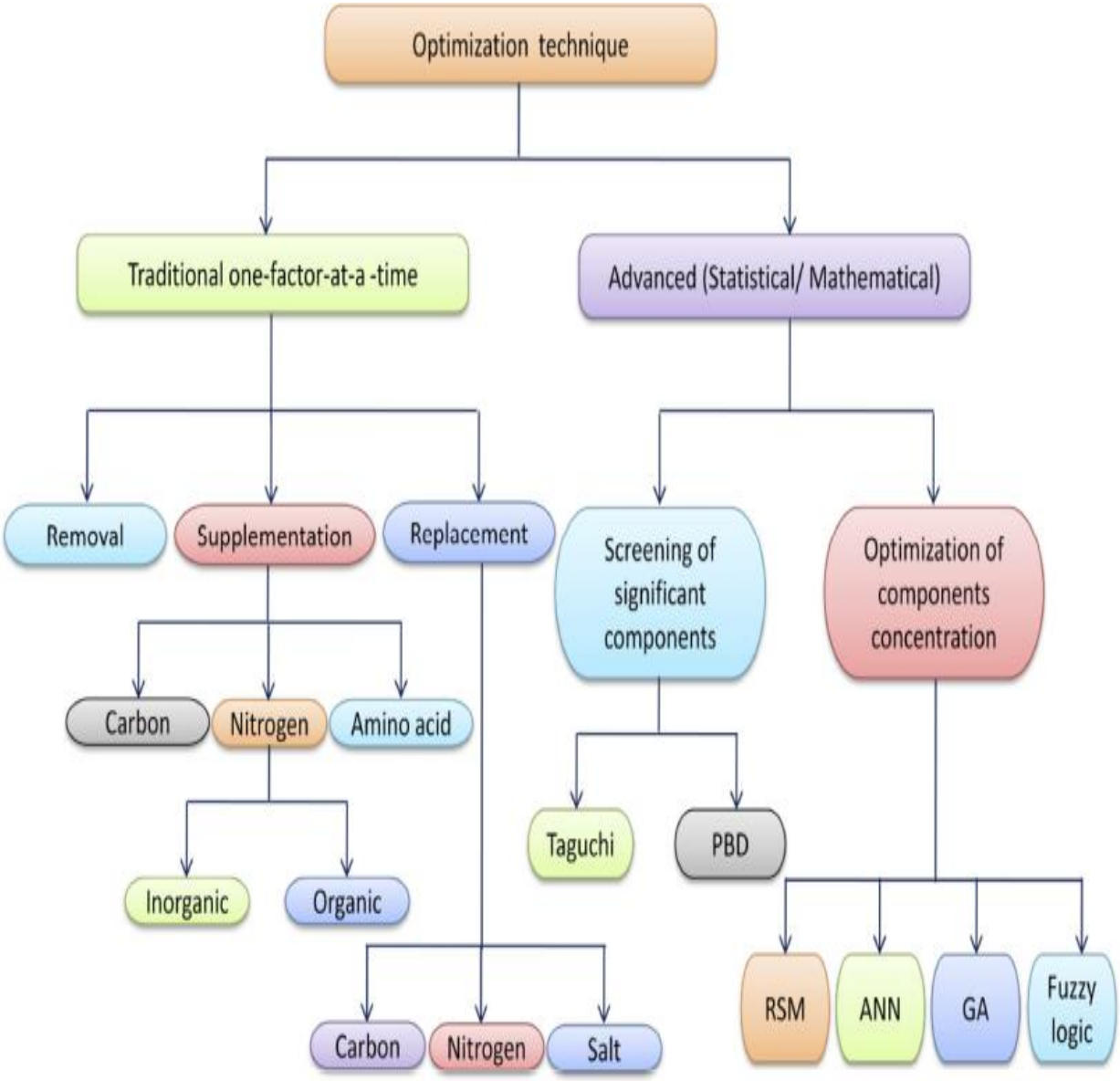


Fig. 2.1 (A)

Schematic diagram of optimization technique
Vineeta Singh et al., 2017

2.2 Materials & Methods

Effect of Temperature on Enzyme Production

To find out the optimum temperature required for maximum lipase production, the lipase producing solid state media was inoculated with 24 hours grown cultures in nutrient broth medium and incubated at different temperatures (28, 37 and 45 °C) for 48 hours.

For this study 12 gm of wheat bran was taken in three different 250 ml Erlenmeyer flask, sterilized in autoclave at 121 °C, 15 psig for 15 min. Then all the flasks were inoculated with 10 ml of inoculums and 1 ml of sterile distilled water was added to each flask for the purpose of hydration. The fermentation media were inoculated with the isolated best lipase producing strain (marked as 'B2') and then incubated at different temperature such as 37°C, 45 °C and 28°C.

Substrate Weight Optimization

For substrate weight optimization study at first 8gm, 12gm and 16gm of wheat bran was taken in 3 separate 250ml Erlenmeyer flasks. Then 1ml of D.W. and 10 ml of 24 hours inoculums were added to each flask and flasks were incubated at 37 °C for 48 hours.

Effect of Hydration on Enzyme production

For this study 12gm of wheat bran was taken in three separate 250ml Erlenmeyer flasks and sterilized in autoclave. Then 1ml, 3ml and 5ml of sterile D.W. was added to 3 individual flasks and then 10 ml of 24 hour grown inoculums were added to each individual flasks and flasks were incubated at 37 °C for 48 hours.

Effect of Fermentation Time on Enzyme Production

For this study 12gm of wheat bran was taken in 5 different 250ml Erlenmeyer flasks and sterilized in autoclave. After sterilization 3ml of sterilized D.W. was added to each flask. Then 10ml of 24 hours grown inoculums was added to each flask. All the flasks were incubated at 37 ºC for varying periods of time (16, 24, 48, 72 and 96 hours).

2.3 Results & Discussion

From the result of the optimization study it has been observed that isolated culture from the petrol and diesel spilled soil sample has the capability of sustain in an environment where mineral oil level in quiet moderate. The isolated organism gives maximum productivity of lipase when it is incubated at 37ºC (see Table 2.1 and Fig. 2.1(B)). In a study of lipase production from by SSF using vegetable oil refining waste under thermophilic conditions (temperature higher than 45ºC for 20 days) it was reported the lipolytic activity reached up to 120,000 UA/g of dry matter (Angélica Santis-Navarro et al., 2011).

Table 2.1 Effect of temperature on enzyme production

Temperature of Incubation [°C]	**Enzyme Activity (U/gm of Wheat bran)**
28	2059.0 ± 22
37	2290.4 ± 18.85
45	1369.2 ± 38.1

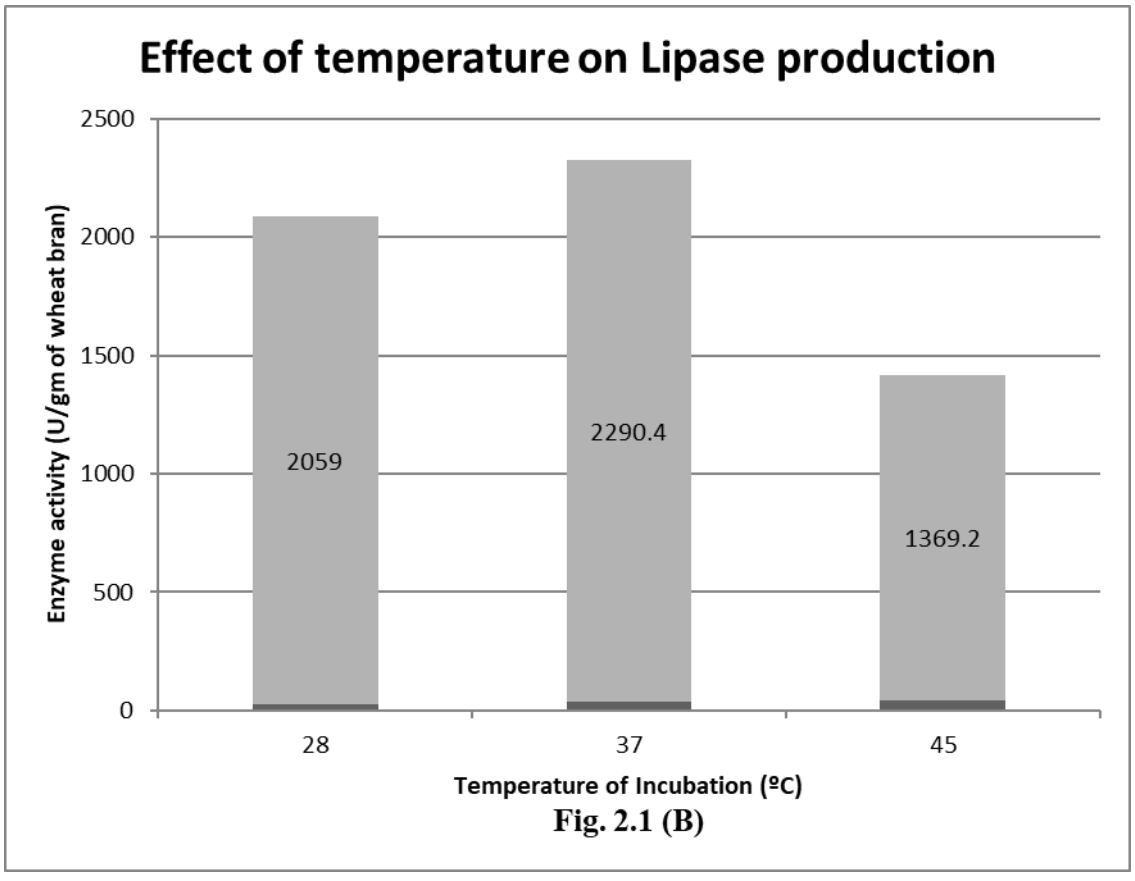


Fig. 2.1 (B)

From the study of optimum weight of substrate to be used in a 250 ml Erlenmeyer flask to obtain maximum productivity it was observed that 12gm of wheat bran as substrate gives the best result, compared to 8gm and 16gm wheat bran (see Table 2.2 and Fig. 2.2). In a reported study fermentations were carried out in a lab scale with 5 g of the dry substrate [(wheat bran with castor oil (WBC) and wheat bran with olive oil (WBO)] and 9.0 mL of Mineral Growth Medium using *Aspergillus flavus.* The lipase activity reported 107.78 U/gm of dry substrate for WBO and 103.13 U/gm of dry substrate for WBC after 120 hours of incubation (Lydia Toscano et al., 2013).

Table 2.2 Effect of substrate weight on enzyme production

Weight of Substrate (gm)	Enzyme Activity (U/gm of Wheat bran)
8	1310.63 ± 22.6
12	1525.7 ± 14.2
16	1441.83 ± 21.5

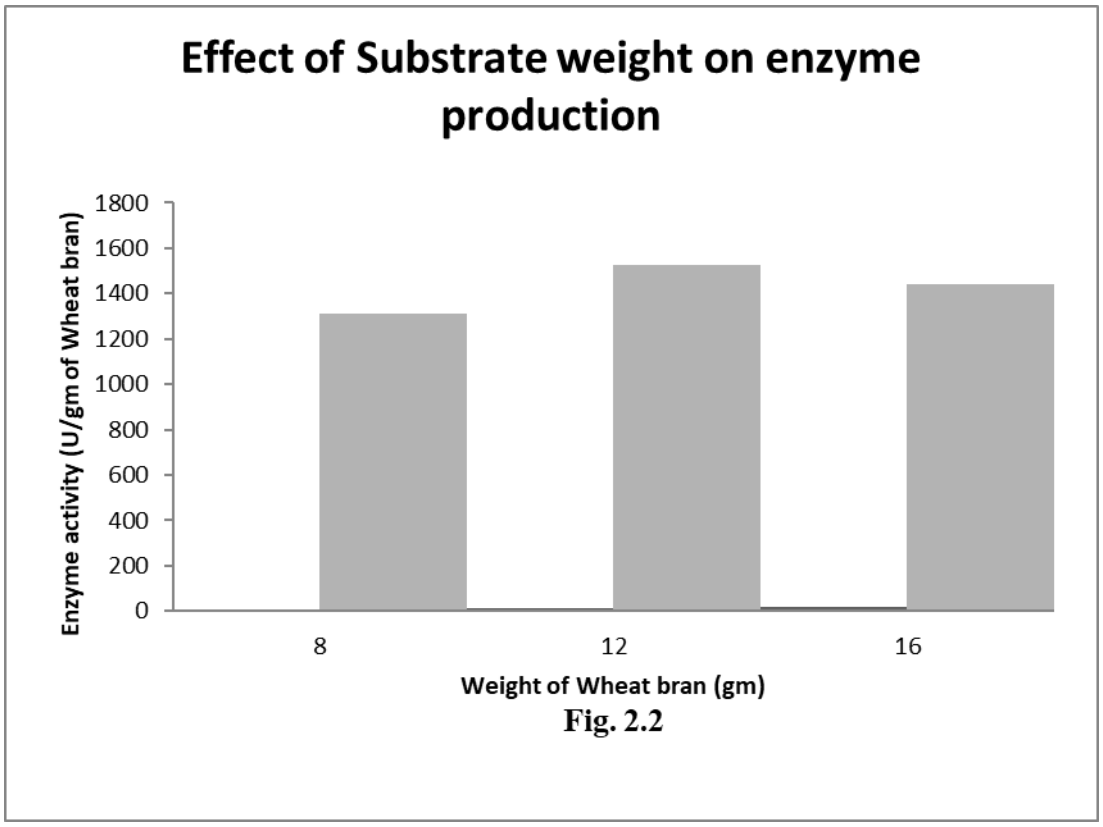


Fig. 2.2

Hydration is one of most important controlling parameter for SSF. There should not be free flowing water but the moisture content should be such a level that it will enhance the secondary metabolite production. From the results obtained from the study it has been observed that a ratio of 12:3:10 (weight of wheat bran: volume of D.W.: volume of inoculums) gives the better productivity (see Table 2.3 and Fig. 2.3) compared to other ratios of 12:1:10 and 12:5:10 which were used for the study. As per a study for fermentative production of sorbitol in SSF by Lactobacillus plantarum from pretreated Meranti wood sawdust. The highest product yield was obtained at 50% moisture content, at 10 hours of fermentation time and 35℃ of incubation temperature where the concentration of sorbitol was 25.68 g/L (Zuriana S. A. and Mimi S. A. M., 2017).

Table 2.3 Effect of hydration on enzyme production

Wt. Of wheat bran	**volume of D.W. Added for Hydration (ml)**	**enzyme activity (U/gm of wheat bran)**
12	1	2295.2 ± 5.73
12	3	2327.7 ± 13.6
12	5	2248.7 ± 9.2

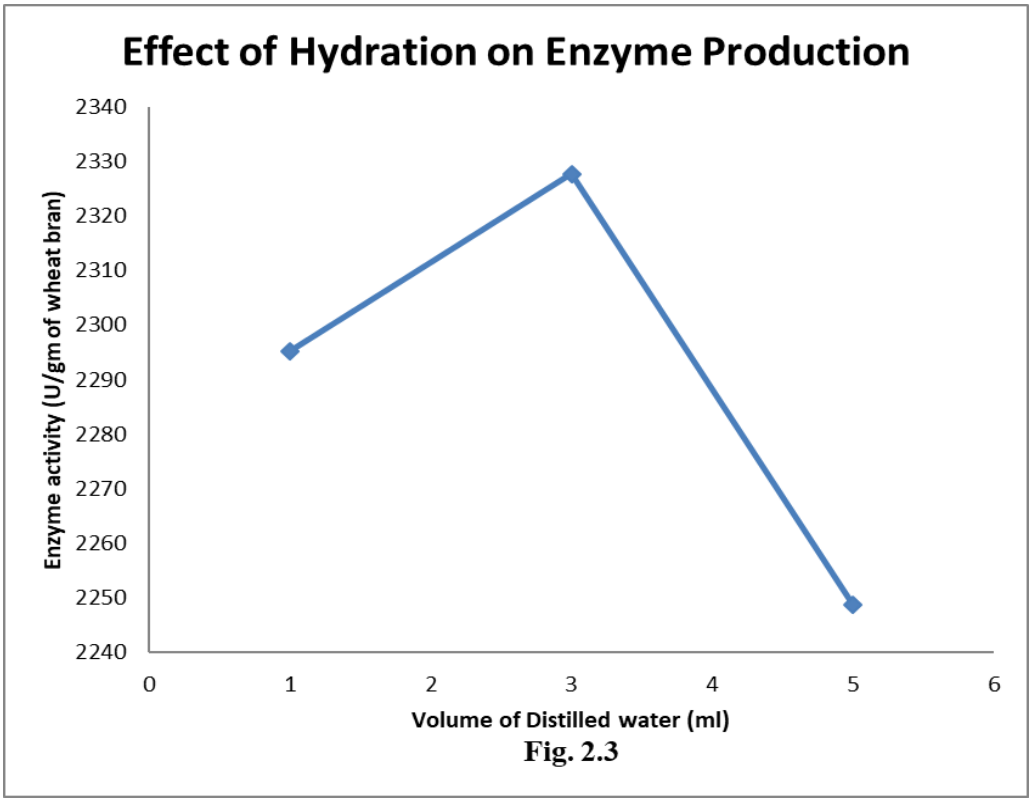


Fig. 2.3

Study of optimum incubation period is another important parameter to enhance the productivity as both above and below the proper incubation period the productivity of a product may reduce significantly. From the results obtained from the study of optimization of fermentation time, it has been observed that 48 hours of incubation period gives the better productivity (see Table 2.4 and Fig 2.4). Both above and below that optimum time a decrease in productivity of lipase has been observed. In a reported study fermentations were carried out in a lab scale with 5 g of the dry substrate [(wheat bran with castor oil (WBC) and wheat bran with olive oil (WBO)] and 9.0 mL of Mineral Growth Medium using *Aspergillus flavus.* The lipase activity reported 107.78 U/gm of dry substrate for WBO and 103.13 U/gm of dry substrate for WBC after 120 hours of incubation (Lydia Toscano et al., 2013).

Table 2.4 Effect of fermentation time on enzyme production

Duration of Fermentation (Hours)	Enzyme Activity (U/gm of wheat bran)
16	1130.9 ± 10.2
24	1760.5 ± 10.2
48	2234.9 ± 9.2
72	1819.3 ± 15.5
96	1821.8 ± 12

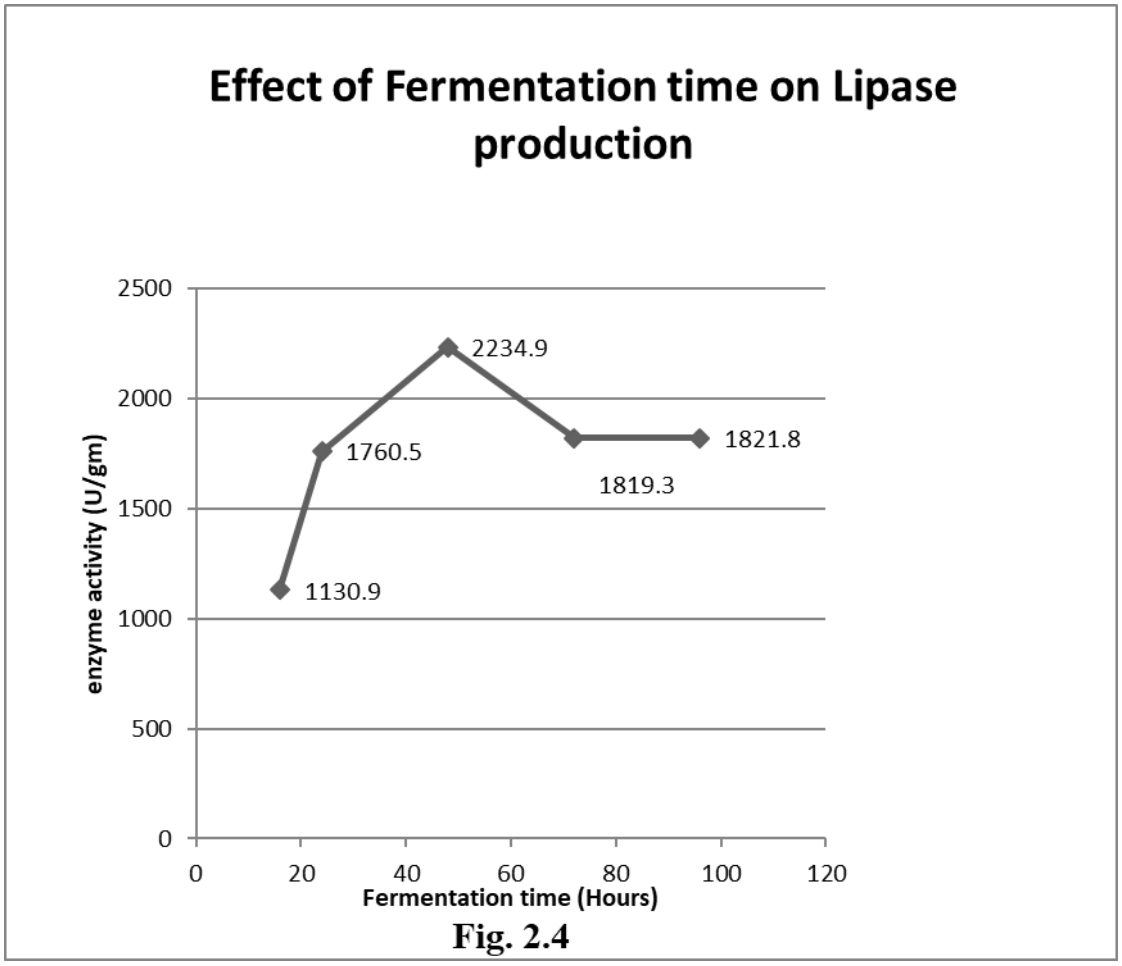


Fig. 2.4

CHAPTER THREE

Optimization of Nutritional parameters for Production of Lipase in SSF

3.1 Nutritional Parameters Optimization

Optimization study of fermentation medium is a very important job to maximize metabolite yield. This can be achieved by using different techniques ranging from classical "one-factor-at-a-time" to modern statistical and mathematical techniques, viz. Artificial neural network (ANN), genetic algorithm (GA) etc. Every individual technique comes with its own advantages and disadvantages, and because of the drawbacks some techniques are applied to obtain best results.

Fermentation medium optimization is still one of the most critically investigated phenomenon that is carried out before any large-scale metabolite production, and it possess many challenges. Before 1970s, media optimization was carried out by using classical methods, which were expensive, time consuming, involving plenty of experiments with compromised accuracy. Nevertheless, with the advent of modern mathematical/statistical techniques, media optimization has become more vibrant, effective, efficient, economical and robust in giving the results. For designing a production medium, the most suitable fermentation conditions (e.g., pH, temperature, agitation speed, etc.) and the appropriate medium components (e.g., carbon, nitrogen, etc.) must be identified and optimized accordingly. Further, by optimizing the above said parameters, maximum product concentration could be achieved (Gupte and Kulkarni, 2003; Franco-Lara et al., 2006; Wang et al., 2011).

An increase in productivity reduces the overall cost of the product, as well as the production cost; therefore, it is one of the important topics for the research. Usually, enhanced productivity can be achieved either by strain improvement or by optimizing the process parameters. But strain improvement and optimization are "Catch-22" situation. One cannot choose a lead strain until you have the best medium and you cannot propose a finest medium until you have the lead strain. Usually, the researchers around the world solve this predicament by sticking to one component at a time. However, both strategies cannot guarantee that one of the preferred strains if another medium is used. With this drawback and Catch-22 situation, various new methods have been suggested and investigated, where both the medium design and strain improvement can be carried out simultaneously.

In this review we have restricted our scope and discussed about the media formulation and media optimization techniques in terms of their utility, application and feasibility to maximize the enzyme yield produced by the solid state fermentation process.

3.2 Materials & Methods

Effect of different Carbon sources on Enzyme production

Carbon is the most important medium component, as it is largest energy source for the microorganisms and plays an important role in the cell growth as well as in the production of various primary and secondary metabolites. Carbon acts as the main energy source for an organism and sometimes controls the productivity of enzymes. Selection of right source of carbon is therefore very important for maximum production of any biochemical products. The rate at which the carbon source is metabolized can often influence the formation of biomass and/or the production of primary or secondary metabolites. While studying antibiotics production from marine bacteria Marwick et al. (1999), noticed that the gradually assimilating carbon sources, like, galactose generally enhances the production of secondary metabolites (antibiotics). A classic example of this is, penicillin production, where glucose is found to have repression effect. After many days, it was found that lactose is a slowly assimilating carbon source and helped in the production of secondary metabolites (i.e., penicillin). Hence, in order to overcome the carbon catabolite repression phenomenon, the production process was established using lactose fermentation.

For this study glucose, fructose, xylose, sucrose and maltose monohydrate has been chosen as the carbon sources. Then 1% each of the carbon sources was added to 5 different 250ml Erlenmeyer flasks containing 12gm of wheat bran medium. 0.12gm each of glucose, fructose, xylose and sucrose was added to four different flasks and 0.126gm of maltose monohydrate was added to the fifth flask because particularly this carbon source contains one molecule of water as water for crystallization. Then all the flasks were sterilized in autoclave at 121ºC, 15 psig for 15 min. After sterilization 3ml of sterilized D.W. was added to each flask. Then 10ml of 24 hours grown inoculum was added to each flask and all the flasks were incubated at 37 ºC for 48 hours.

After the carbon source has been optimized, concentration of the optimized carbon source has been optimized next. As the maltose has been found to be the optimized carbon source concentration of maltose was varied then. Maltose or otherwise called maltobiose or malt sugar, is a disaccharide formed from two units of glucose joined with an alpha 1,4 glycoside bond. In the isomer isomaltose, the two glucose molecules are joined with an alpha 1,6 glycoside bond. Maltose is the two unit member of the amylase homlogus series, the key structure moiety of starch. When beta-amylase breaks down starch, it removes two glucose units at a time, producing maltose. An example of this reaction is found in germinating seeds, which is why it was named after malt.

To study the effect of maltose concentration on lipase production in SSF four different concentrations of maltose (0.5%, 1%, 2% and 3% of weight of wheat bran) were maintained in four different 250ml Erlenmeyer flask containing 12 gm of wheat bran and sterilized in autoclave. After sterilization 10 ml of 24 hours grown inoculums in nutrient broth medium and 3 ml of sterile distilled water added in each of the flasks and properly mixed with a sterile

glass rod. Then all the flasks were incubated in stationary condition at 37 ºC for 48 hours in an incubator.

After fermentation the fermented mediums were extracted by soaking in distilled water, filter through muslin cloth, centrifuged and assayed for lipase activity determination.

Effect of Complex Nitrogen sources on Enzyme production

Unlike carbon source, the selection of nitrogen source and its concentration in the fermentation medium is also play vital role in primary and secondary metabolite production. The microorganisms are able to utilize both inorganic and organic sources of nitrogen. But use of specific amino acids can increase the productivity of enzymes in some cases. Conversely, unsuitable amino acids may inhibit the synthesis of some secondary metabolites (Marwick et al., 1999, Singh et al., 2009) during the optimization of actinomycin V production by *Streptomyces triostinicus* found that biosynthesis of actinomycin V involves tryptophan pathway and addition of amino acid tryptophan to the fermentation medium enhances the production. On the other hand, the same amino acid shows inhibitory effect in the production of candicidin from *Streptomyces griseus* (Sanchez and Demain, 2002). Nevertheless, it is confirmed that nitrogen molecules have some inhibitory effect on the metabolite production in some cases, whereas some enhancing effects of nitrogen have also been reported.

For this study peptone, yeast extract, beef extract and urea has been chosen as complex nitrogen sources to be enriched in the fermentation medium. 12 gm of wheat bran was taken in four different 250 ml conical flask. Then 0.06 gm maltose (0.5%) was added to each of the conical flasks. Then 0.06 gm (0.5%) each of peptone, yeast extract, beef extract and urea were taken in the four different flasks. All the flasks were sterilized in autoclave and then inoculated with 10 ml of 24 hour grown inoculums and hydrated with 3 ml of sterile distilled water, mixed with glass rod and incubated at 37 ºC for 48 hours.

After fermentation the fermented mediums were extracted by soaking in distilled water, filter through muslin cloth, centrifuged and assayed for lipase activity determination by the method cited in chapter one.

Determination of right source of complex nitrogen not only sufficient to obtain maximum productivity of a metabolite but also it is necessary to find out exact concentration of the nitrogen source that must be added to the medium to maximize the productivity. For this study, 12 gm of wheat bran was taken in four different 250 ml conical flask, 0.5% of maltose was added to every flask. Then 0.5%, 1%, 2% and 3% of beef extract was added to four different conical flasks containing wheat bran and maltose. All the flasks were then sterilized in autoclave and then inoculated with 10 ml of 24 hours grown inoculums and hydrated with 3 ml of sterile distilled water, mixed with glass rod and incubated at 37 ºC for 48 hours.

After fermentation the fermented mediums were extracted by soaking in distilled water, filter through muslin cloth, centrifuged and assayed for lipase activity determination by the method cited in chapter one.

3.3 Results & Discussion

Selection of exact carbon source for production of secondary metabolite is very important to improve productivity. From the results obtained from the study where 1% each of glucose, fructose, sucrose, xylose and maltose were used as different carbon sources, it has been found that 1% maltose gives the maximum productivity of lipase (see Table 3.1 and Fig 3.1)

Table 3.1 Effect of different carbon sources on enzyme production

Carbon source	% of carbon source	Enzyme activity (U/gm of wheat bran)
Maltose	1%	2206.9 ± 8
Xylose	1%	2148.9 ± 6.5
Sucrose	1%	2181.1 ± 10
Fructose	1%	2086.1 ± 5.6
Glucose	1%	2189.9 ± 15.3

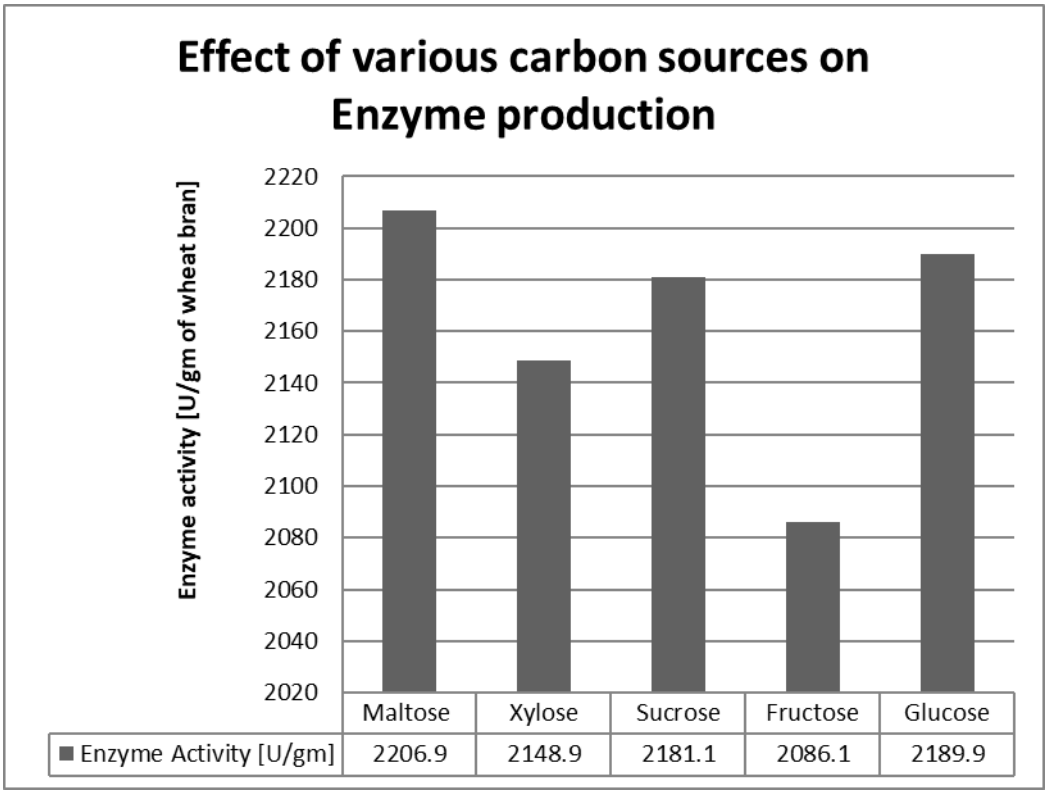


Fig. 3.1

Not only the selection of exact carbon source improves the productivity of a fermentative product but also its exact concentration may improve productivity of the particular metabolite. From the results obtained from the study, among the four different concentrations (0.5%, 1%, 2% and 3%) of maltose used it has been found that 0.5% maltose as carbon source gives best productivity of lipase (see Table 3.2 and Fig. 3.2). Moderate enzyme activity (9.14 IU/g dss) was obtained in SSF using wheat bran, which to the best of the author's knowledge are amongst the highest extracellular lipase activities reported in the literature concerning fungal sources using 1.5% glucose as the optimum concentration of carbon source (Gwen Falony et al., 2006).

Table 3.2 Effect of maltose concentration on enzyme production	
Maltose concentration (%) (w/w)	**Enzyme activity (U/gm of wheat bran)**
0.5	2561.7 ± 9.8
1.0	2289.3 ± 10.9
2.0	2265.9 ± 9
3.0	2233.0 ± 4.6

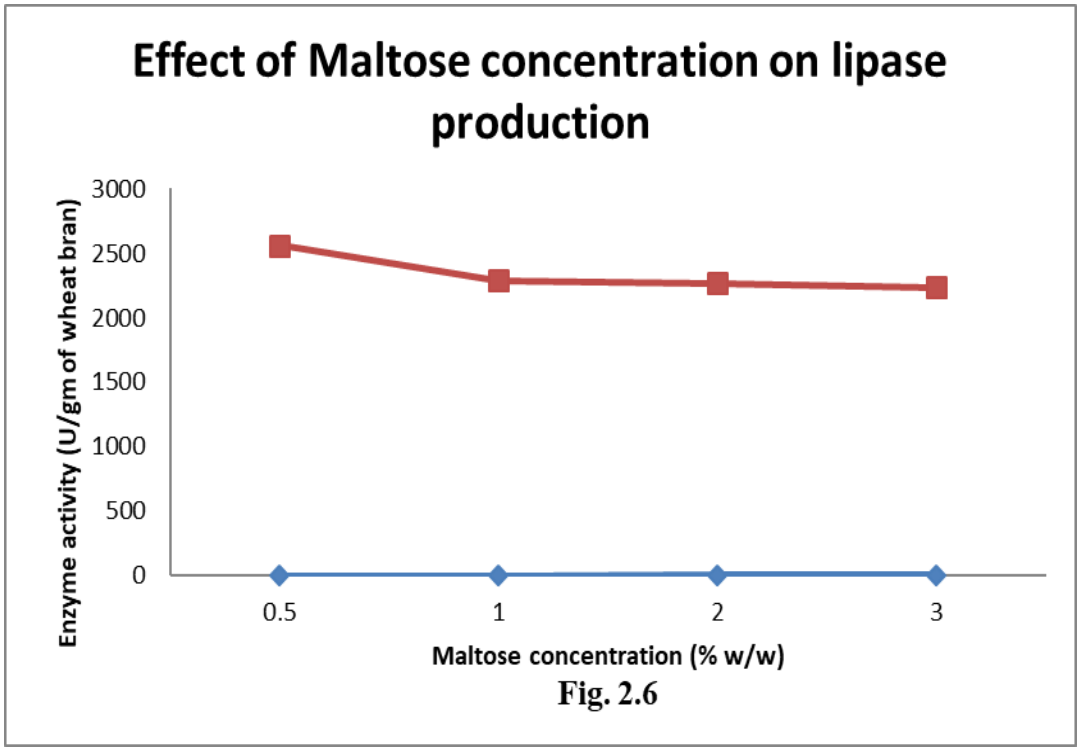


Fig. 2.6

Selection of exact complex nitrogen source for production of secondary metabolite is very important to improve productivity. From the results obtained from the study where 1% each of peptone, yeast extract, beef extract and urea were used as different complex nitrogen sources, it has been found that 1% beef extract gives the maximum productivity of lipase (see Table 2.7 and Fig 2.7)

Table 3.3 Effect of different complex nitrogen sources on enzyme production	
Complex Nitrogen Source	**Enzyme activity (U/gm of wheat bran)**
Peptone	2316.1 ± 16.6
Yeast extract	2304.9 ± 6
Beef extract	2474.7 ± 3.3
Urea	2060.9 ± 3

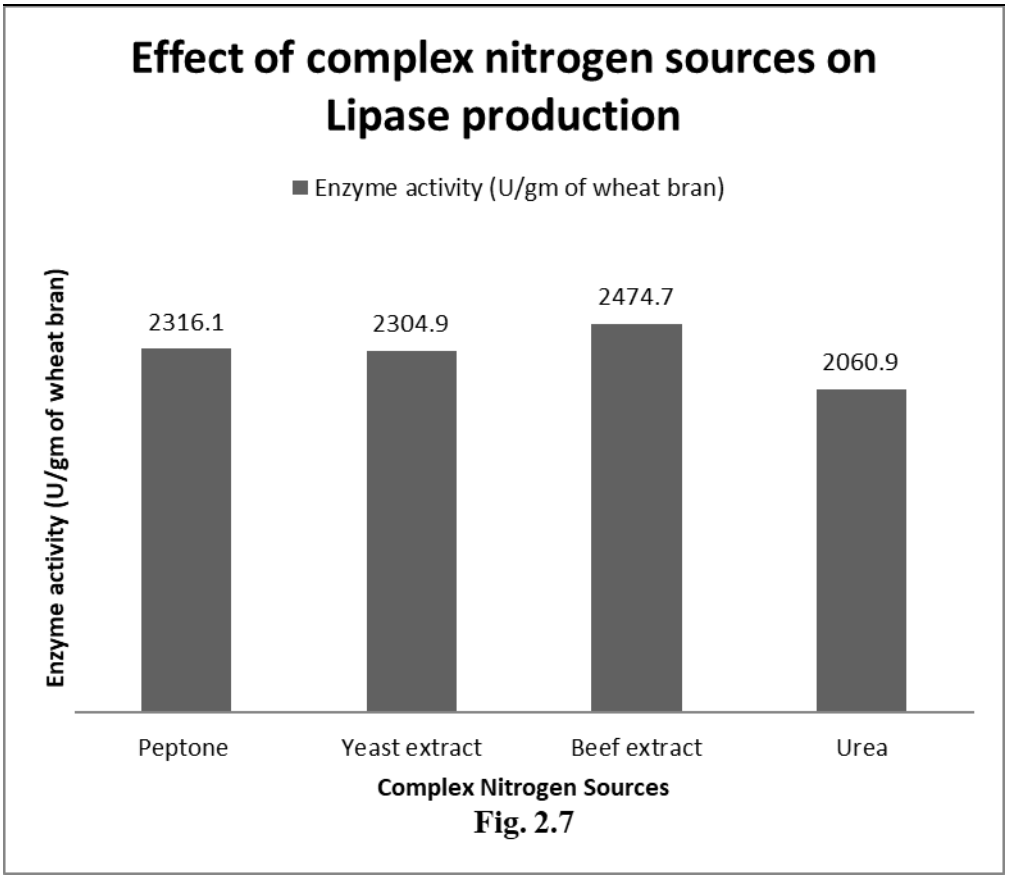


Fig. 2.7

Not only the selection of exact complex nitrogen source improves the productivity of a secondary metabolite but also its exact concentration may improve productivity of the particular metabolite. From the results obtained from the study, among the four different concentrations (0.5%, 1%, 2% and 3%) of beef extract used it has been observed that 0.5% beef extract as complex nitrogen source gives best productivity of lipase (see Table 3.4 and Fig. 3.4). From a recent study maximum lipase activity of 500 unit per gram of solid state substrate was reported using 1% NH_4Cl as nitrogen source (Felisbela Oliveiraa et al., 2016).

Table 3.4 Effect of concentration of beef extract on enzyme production	
Percentage of Beef extract (w/w)	**Enzyme activity (U/gm of wheat bran)**
0.5	2933.8 ± 20
1.0	2367.3 ± 20.3
2.0	2013.4 ± 10.2
3.0	1766.7 ± 22.5

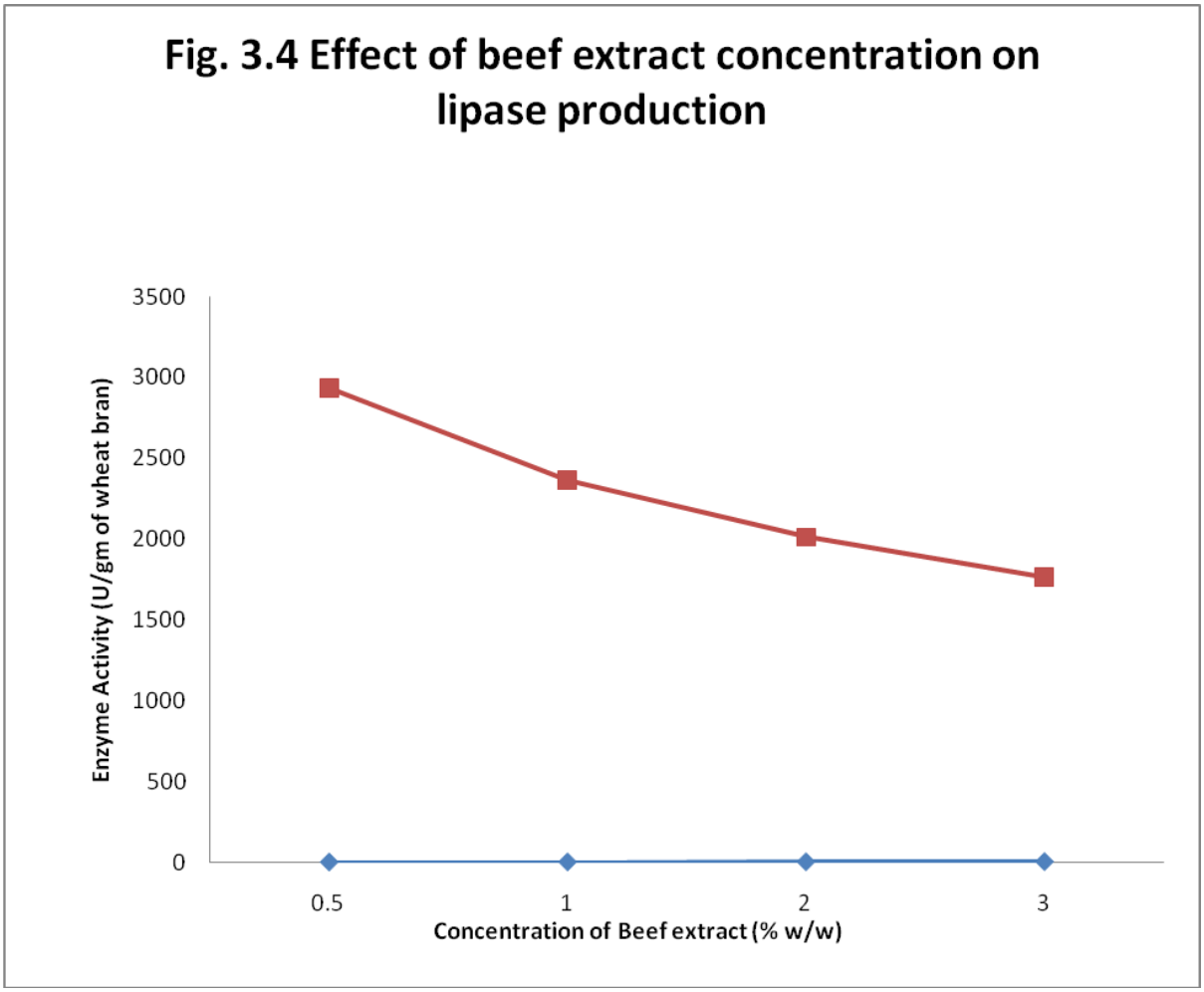


Table 3.5 Optimized Environmental conditions			
Optimum temperature	**Optimum weight of substrate**	**Optimum ratio of hydration (solid:liquid)**	**Optimum incubation period**
37 ºC	12 gm	≈ 0.92	48 hours

Table 3.6 Optimized Nutitional conditions		
Nutrient	**Name of source**	**Concentration (%)**
Carbon source	Maltose	0.5
Complex nitrogen source	Beef extract	0.5

In our study under the optimum physical and nutritional conditions lipase activity found to be 2933.8 U/gm of wheat bran. The results we obtained is quiet comparable to a reported study published in journal where lipolytic activity upto 120000 UA/gm of dry substrate was found (Angelica Santis et al., 2011). But further purification of the enzyme supernatant is required when it comes to application of the enzymes in specific areas like food and pharmaceutical. We have extracted the fermented solids by soaking it overnight in D.W. at refrigerated condition that is one of the low cost extraction techniques for extraction of water soluble lipase. Whereas in most of the published studies, it has been observed, that extraction is done with buffer solutions. A few other study findings are lipase production in SSF in tray type bioreactor of 33 litre scale by *Aspergillus niger* in 96 hours was 1934 U/gm (Adinarayana K et al., 2004), lipase production in packed bed bioreactor in 50 litre scale for 13 hours using *Rhizopus homothallicus* was 1500 U/gm (Diaz JC et al., 2006).

The study on scalable SSF with vegetable oil refining industry waste has shown that this waste is a good source for lipolytic enzymes production with promising properties. The use of fermented solids as biocatalysts is also promising in terms of low-cost production process with high yield potential. Further research should explore the application of the obtained lipases in novel synthetic routes and their identification. Another point that needs attention is the reproducibility of the source of microorganism used since sludge and, in general, organic solid wastes are inherently variable in chemical composition and in the characterization of the existing microbial communities (Angelica Santis et al., 2011).

Summery

Table 4 Summary of optimized conditions with productivity of lipase

Parameters	Optimized condition	Enzyme activity (U/gm of wheat bran)
Optimum temperature	**37 °C**	2290.4 ± 18.85
Optimum weight of substrate	**12 gm**	1525.7 ± 14.2
Optimum ratio of hydration (gm of wheat bran : ml of D.W. & inoculums)	**≈ 0.92**	2327.7 ± 13.6
Optimum incubation period	**48 hours**	2234.9 ± 9.2
Carbon source	**Maltose**	2206.9 ± 8
Concentration of maltose	**0.5% (w/w)**	2561.7 ± 9.8
Complex nitrogen source	**Beef extract**	2474.7 ± 3.3
Concentration of beef extract	**0.5% (w/w)**	2933.8 ± 20

From the comparative study it can be said that a significant amount of improvement in enzyme activity per gram of wheat bran has been observed when several environmental and nutritional factors has been optimized. Further enrichment of medium with trace elements may further improve the productivity lipase.

Conclusion

In our study we have used wheat bran as the solid substrate to carry out the fermentative production of lipase. We have isolated the suitable bacterial strain from soil sample of a motor vehicle garage allowed it produce lipase by providing its suitable environmental and nutritional factors. Environmental factors we optimized are temperature and time of incubation, extent of hydration and weight of wheat bran. Nutritional factors we optimized are suitable source and concentration carbon and complex nitrogen source to maximize yield of enzyme. From the summary of results it can be concluded that an approximate enhancement of 900 Unit of enzyme activity per gram of wheat bran can be achieved with only environmental, carbon source and complex nitrogen source optimization using the isolated strain.

Future Prospects

- **Scaling up of lipase production by SSF in pilot plant**
- **Application of free enzyme in waste treatment**
- **Application of immobilized enzyme in waste treatment**
- **Purification of the enzyme and application in other field**
- **Characterization of the enzyme**
- **Complete identification of the isolated strain.**

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