Development of a media for Fermentative production of lipase by polymeric flocculant producing bacteria

Dissertation submitted in partial fulfilment

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Submitted by

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CANDIDATE'S DECLARATION

I hereby declare that the work which is being presented in the dissertation entitled "Development of a media for Fermentative production of lipase by polymeric flocculant producing bacteria" in the partial fulfilment of the requirements for the award of degree of **Master of Technology in Biotechnology**, Thapar Institute Of Engineering Technologies, Patiala, is an authentic record of the work carried out during a period of twelve months from July 2017 to June 2018, under the supervision of **Prof. Moushumi Ghosh ,TIET- Patiala,** and I have not submitted the matter embodied in this dissertation for the award of any other degree or diploma.

SURBHI SHARMA

Patiala

Date

CERTIFICATE

I hereby certify that the work which is being presented in thesis entitled, **"Development of a media for Fermentative production of lipase by polymeric flocculant producing bacteria"** in partial fulfilment of the requirement for the award of degree of **Master of Technology In Biotechnology,** Thapar Institute Of Engineering And Technology, Patiala, is an authentic record of work carried out under the supervision of **Prof. Moushumi Ghosh (TIET, Patiala).** The matter presented in this thesis has not been submitted for the award of any other degree of this or any other university.

This is certified that the above statement made by the candidate is correct and true to the best of my knowledge.

Moushumi Ghosh

Dr. Moushumi Ghosh
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SURBHI SHARMA

CONTENTS

LIST OF ABBREVIATIONS

LA/LB	Luria agar/broth
FTIR	Fourier transformed infrared spectroscopy
DNSA	Di-Nitrosalicylic acid
SEM	Scanning electron microscopy
RSM	Response Surface Methodology
CRD	Cell regulation and development

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ABSTRACT

The present study investigated the fermentative production of lipase by an extracellular polymer producing strain of bacteria in a media formulated from agro resources. The media which comprised of corn, garlic and onion possessed desirable attributes including optimal C:N ratio and sulphur as constituents. One bacterial strain capable of extracellular polymer production was assessed for lipolytic activity. Under UV light at 350nm light this strain produced strong orange fluorescent colonies on Rhodamine B agar plates upon irradiation under was selected and grown on the developed media. Maximum lipase activity was observed in this growth medium with 0.5% olive oil, 0.5% yeast extract and 1% gum Arabic under shaking conditions at 37°C. The lipase was predominantly extracellular and had optimal pH of 6.5 and temperature of 30°C. RSM was used to validate the optimisation of culture parameters for lipase production. Enhanced polymer yields were observed during lipase production; the purified polymer were subjected to FTIR and SEM to characterize the biopolymer; results indicated no significant ($p<0.05$) difference of its characteristics with that produced by the same culture in reference media. The extracellular polymer demonstrated excellent flocculating activity against a wide range colloidal particles. Overall, the present study indicate that production of both lipase and polymeric flocculant could be achieved by the selected bacterial strain economically in the developed vegetable media. The relevance of the newly developed media for fermentative production and its commercial relevance is suggested.

CHAPTER – 1 INTRODUCTION

Several industrial effluents contain a significant quantity of oil in addition to other fat or fatty acid rich materials; an important example being the palm oil processing industries. Treatment of waste water from such facilities usually require microbial strains for both cost effectiveness and environmental safety. However suitable strains with provision of value addition in terms of both remediation and production of industrially important enzymes or bioproducts are few and are currently intensively explored. In such a context, bacterial strains which have capability to produce lipase and extracellular polymeric flocculants should offer possibilities to current alternatives and expand the horizons of presently used strains of lipase producing microorganisms. While extracellular flocculants produced by bacteria have become popular for remediating a variety of waste water, the potential for such bacteria in simultaneous production of other promising metabolites is an interesting and important proposition. Lipases have important and expanding scope of applications in the chemical industry, environment and pharmaceuticals. This is because they can cause hydrolysis of glycerines and fatty acids under aqueous solutions (Shire *et al*.,2005). Also in the presence of organic solvents they are able to perform reverse reaction or causes the replacement of aryl groups among different alcohols, esters and amines (Rueda., 2005,Jaeger and Eggert.,2005). Lipases produced by microorganisms are most prefrred because of their stability (Gupta *et al*, 2004) and their easy production with provision to enhance the yield in many cases through molecular approaches.

Consequently, an enormous effort has been invested for fermentative production of Lipase from various microorganisms such as bacteria, yeast and 'fungi'. Some applications of which has been in commercial regime. Use of fermentation involves analysis of important aspects such as suitable microorganisms, substrates, optimisation of media components and purification of the product (Basnett *et al*, 2009) and is most preferred for production of bioproducts. Besides, fermentation can be performed using waste raw materials especially agro-wastes which minimize environmental problems, do not generate toxic compounds and reduce the overall cost of production.

An important pre-requisite of developed media to be used for the above cited purpose is the presence of desirable nutrients (Osman *et al*., 2016) and economics. Growth media comprised of vegetable or agro-wastes for fermentative production of lipases could offer an important alternative to currently used media for lipase production, moreover the capability of such bacteria to produce polymeric flocculants could offer an advantage of recovering flocculants during fermentation along with lipase. Such agro-materials are abundantly available and many after primary processing retain a substantial quantity of nutrition which may be utilized for growth and biochemical substance of bacteria and other microorganisms. However, few studies have attempted to produce lipase using bacterial strains with capability of polymeric flocculant production; moreover, media specifically designed using agro materials for production of both lipase and flocculant has not been reported till now.

In view of this, the present study investigated the fermentative potential for lipase production by a strain of bacteria selected from amongst 20 extracellular floccuant producing bacterial strains based on its enhanced lipolytic capability. A vegetable media was formulated and then used for lipase production by optimizing culture variables of the selected bacterial strain. The biopolymeric flocculant produced by this strain was characterized for its constituents and flocculating potential.

Objectives of the study:

The current study was designed with the following objectives;

(1)Designing an appropriate media using easily available and cost effective agro materials (vegetables) and characterization of key chemical constituents.

(2)Selection of effective lipase producing bacteria from amongst polymeric flocculant producing strains.

(3)Optimization of culture components using RSM and experimental validation for fermentative; production of lipase by selected strain of bacteria in the media developed.

(4) Characterization of the polymeric bioflocculant and evaluation of its functionality:.

Gaps in research :

Simultaneous production of lipase and polymeric flocculant by bacterial strains represent a a economical process . Besides the fermentative production using a cost effective media would an advantage for economizing the scale up for these . Though extensive studies have been carried out for the lipase production a high cost of media have failed to offer practical considerations (Kessler *et* al., 2008).Polymeric flocculants on the other hand suffers from high processing costs, a substantial part being incurred upon by the media itself. It is imperative to find solutions which can offer a feasibility in terms of commercial aspects.The aim of our study was therefore to use vegetable media as source of nutrients for the production of lipase as well as polymeric flocculant using a suitable bacterial strain.

Scope of the present study:

Based on the above deliberations, it is clear that cost effective production of lipase and biopolymers need to be addressed. Bacterial lipase production needs to be attempted using media which may be easily and economically obtained.

In a similar fashion, biopolymer production has been largely restricted to laboratory scale on account of the high cost of growth media. In view of these gaps, the present study attempted to evaluate a vegetable media for production of both lipase and polymeric flocculant. The results of this study should enable further detailed explorations in simultaneously scaling up the two important bio-products from the selected microbial strain described in this study and economize their yields.

CHAPTER – 2 LITERATURE REVIEW

2.1 Lipases

Lipases triacylglerols hydrolases are cluster of enzyme that are responsible for the catalysis of acylglycerides and other fatty acids. They causes the hydrolysis under aqueous conditions and synthesis of esters (Shirega *et al*., 2005). These enzymes are very important from biotechnology point of view and have large number of wide applications in various industries like food, dairy, detergent etc. They are highly specific and thus play an important role in industries (Sober on-Chavez and Palmer's, 1994). Lipases are produced by large number of bacteria and each bacteria produce specific type of lipases that play an important role in various commercial applications.

There are several reasons that lipase has become an attractive commercial enzyme. Firstly, they are highly specific. Secondly, they can be easily produced in large quantities by microorganisms. Thirdly, they facilitate design of rational engineering strategies as there structures have been solved. Finally, they don't require cofactors. These properties make lipases as important biocatalysts in many industries (Jaeger and Eggert, 2002).

The biological relevance of lipids has led to the evolution of large number of lipid degrading enzyme, among all of them esterase's is considered the most important biocatalysts due to biotechnological potential (Bornscheuer, 2002). Esterase catalysis the hydrolysis of many ester bonds of lipid compounds, although they can hydrolyze non lipid compounds. Esters have been divided into 84 enzymatic classes by IUBMB (International union of biochemistry and molecular biology) according to the bond they hydrolyze. Carboxylic hydrolases that act on acylglycerols to liberate fatty acids and glycerol are called lipases (Gupta *et al*., 2004).

2.2 Catalytic properties of lipase

Lipase possesses Catalytic versatility such as hydrolysis of esters, phospholipids and acylglycerols. They are also capable of performing catalysis of reversible reactions of ester synthesis in the presence of organic solvents(Schmidt-Dannert, 1998); Gupta *et al*., 2004b; Hedfors *et al*., 2010; Zhang *et al*., 2006). Lipases are regiospecific and enantioselective

(Reetz, 2002; Gotor-Fernández *et al*., 2006). Different reactions mediated by lipases are shown below.

Transesetrification

Acidolysis: R1–COO–R2 + R3 –COOH R3–COOR2 + R1–COOH

Alcoholysis: R1–COO–R2 + R3–OH R1–COO–R3 + R2–OH

Aminolysis: R1–COO–R2 + R3–NH2 R1–CONH–R3 + R2–OH

Ester exchange: R1–COO–R2 + R3–COO-R4 R1–COO–R4 + R3–COO–R2

Hydrolysis:

R1–COO–R2 + H_2O R1–COO–H + R2–OH

Ester synthesis:

R1–COOH + R2–OH R1–COO–R2 + H_2O

Intermolecular esterification:

HO–R–COOH R-COO–R (Lactones)

2.3 Mechanism and Action of lipase

Lipase being a substrate specific does not require any cofactor. The substrate of lipase such as acylglycerols possess very less solubility in water. They cause the hydrolysis of ester bonds at interface between insoluble substrate phase and aqueous phase in which enzyme is dissolved. Lipases are highly specific and this specific property is called enzyme specificity.

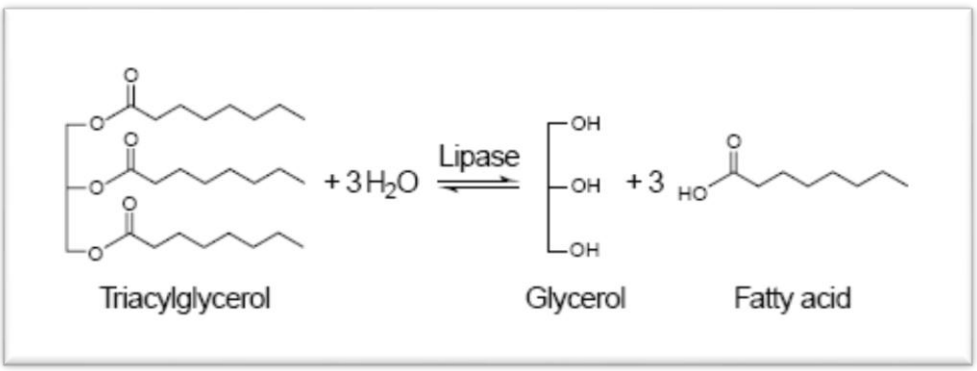

Fig. 2.1 Hydrolytic and Synthetic Actions of Lipase

(Source: Jaeger and Reetz 1998)

When specific lipase acts on different compounds they show different degree of lipolysis such as TAGs, DAGs, or other esters whereas nonspecific act on any random on TAGs which results in the breakdown to glycerol's (Aravindan *et al.*, 2009). They can easily distinguish structural features of acyl such as position, number, configuration bonds, and nature of acyl source PAC1R (Misset *et al*., 1994).

Being stereo and region specific lipase can easily distinguish between enantiomers of racemic mixtures. Property of being reiospecific depends on the factors such as structure of substrate and interactions on the active site (Muralidhar *et al.*, 2002). Steriospecificity is the most important property of lipases in field of biotechnology (Shaoxin *et al*., 2007; Zhao *et al*., 2008a). Lipases from *Pseudomonas* sp. fall into this category (Jaeger and Reetz, 1998).

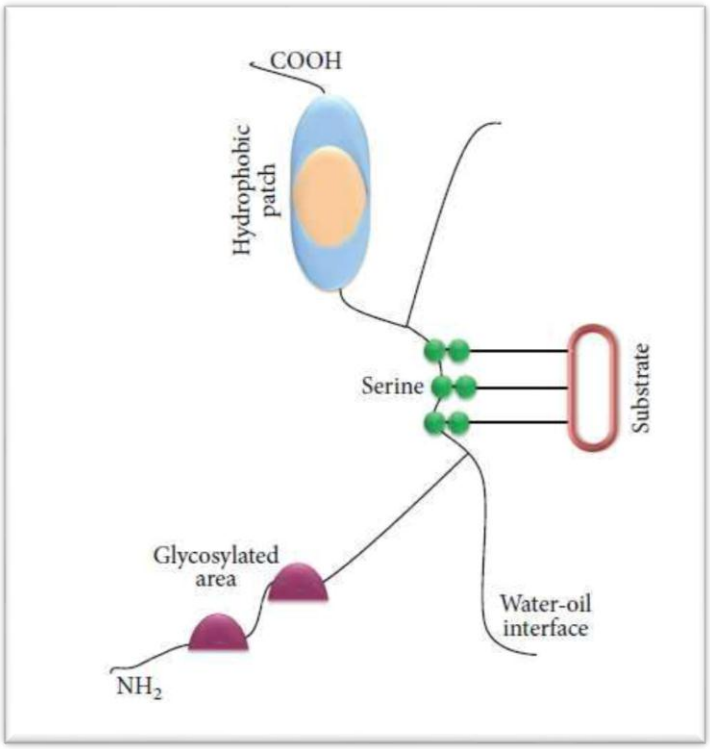

Fig.2.2 Main features of Lipase Molecule shown in Diagrammatic representation

(Ghosh *et al*., 1996)

2.4 Common Sources of Lipases

Lipases occurs in all the prokaryotes which includes bacteria, Achaea and eukaryotes. Eukaryotes includes plants and animals and fungi (Cai-hong, *et al*. 2008). Lipases produced from bacteria are more stable than the lipases produced from animals and plants as they can be easily manipulated and show rapid growth in inexpensive media. Also there are no seasonal fluctuations shown by microorganism so lipase can be produced whole of the year. Productions by microbial lipases are easy and safer to use as industrial applications and hence are used in various industries (Schmidt-Dannert 1998).

Also the fungal enzymes are used but less in comparison to microbial enzymes. Microbial enzymes are preferred as of many reasons like bacterial manipulations can be easily subjected on the bacterial cells, they have short generation time, less nutritional needs and can be easily screened (Hasan, *et al*. 2006). Microbes represent as essential sources of protein and hence can be cultured in huge amount in very less duration of time by fermentation. They can produce large amount of proteins and can secrete into the culture medium. For example large number of proteolytic and amylolytic enzymes produced by *bacilli*. Such protein produced have to go through downstream processing as there are smaller amount of proteins that needs to be separated from the product of interest (Kaul, 1995).

Further, Growth medium also represent significant part in the production of enzymes. Likewise, nutritional requirement of animals are more crucial than plants and microbes as there requirement of oxygen is less and grow very slowly (Wiseman, 2007). Enzymes produced by microorganisms are used extensively in number of applications for example synthesis of various antibiotics, vitamins or biopolymers. Industrial production by microbial enzymes have gained lots of attention because these enzymes are highly selective, are less polluting, require less energy and mild conditions (Cherry and Fidantsef, 2003). Thus the interest has been increased in past years for isolation of enzymes and isolating new enzyme producing strains. Among so many enzymes, lipases, pectinases, amylases, cellulases are considered as important enzymes to be researched on (Cherry and Fidantsef, 2003). Commercially existing microbial lipases along with their application is listed in the table.

Table 2.1 Commercially existing microbial lipases and their applications

Type	source	Application
Bacteria	*Burkholderia epacia*	Organic synthesis
	P alcaligenes	Organic synthesis

	P. mendocina	Detergent additive
Fungi	*C. rugosa*	Organic synthesis
	C.antartica	Organic synthesis
	T.lanuginosus	Detergent additive
	R.meihei	Food processing

Source: Jaeger and Reetz (1998)

2.5 Microbial production of lipase

There are large numbers of organisms that produce lipase but those produced by microorganisms are of more importance. These microorganisms are found in diverse habitats such as soil contaminated by oil, oil producing factories, industrial wastes (Szatejer *et al*.,1988), coal tips, hot springs (Wang *et al*.,1995). Lipase activity can be easily detected and has been mentioned by Sieraa (1957). In this method he has describes the use of Tween 80 to detect the lipolytic activity. Indication of lipase producing bacteria can be detected by the halo zones created around the colonies, tween surfactants in combination with Nile blue can be used as modification in detection. Tributyrin agar as substrate is also used for the screening of lipase producing organisms (Cardenas *et al*., 2001). Chromogenic substrates are frequently used in the detection (Yeoh *et al*., 1986) . Rhodamine agar plates are also used for the confirmation of lipase producing bacteria in which lipase producing colonies are observed to produce orange colored fluorescent halos around the colonies when irradiated with 350nm UV light (Kouker and Jaeger,1987 ;Hou, 1994).

There are three major factors for the detection of lipase producing bacteria which includes growth of organism, culture conditions, sensitive methods to detect lipolytic activity (Shelley, 1987). Nutrients are important modulators for Microbial production of lipase for instance carbon source, nitrogen source, pH, temperature influence the production of lipase (Gupta *et al*., 2004). Lipase being an inducible enzyme, carbon source ha significant influence in its production.Also, production of enzyme is reliant on the presence of lipids, olive oil or any other oil or fatty acids, tweens play an important role in this (Treichel *et al*.,2009). Triolin is the most common substrate used as inducer; alternatively olive oil can also be used since it is economical (Jensen 1983).

2.6 Fermentative production of Lipase

Lipase are known to be easily produced by solid state fermentation however is usually produced by submerged culture (Iso *et al*., 2001). Optimal conditions and nutritional requirement are the basics for a submerged culture and studies have been done to find out the best conditions .lipase production is influenced by lipid carbon sources, nitrogen sources, pH, temperature however many authors have been able to produce lipase even if there is no fats and oils added (Sharma *et al*.,2001).

Microorganism breakdown carbon sources which are converted to amino acids, vitamins, fatty acids and further converted to building blocks proteins, coenzymes, nucleic acids, polysaccharides used for the growth. Lipase production by submerged fermentation is widely used and 90% of microbial enzymes are produced by this technique. Solid state fermentation has also gained attention as it has large number biotechnological benefits linked with it (Holker *et al*., 2004). Immobilized cell culture is also used for the production of lipase (Elibol and Ozer, 2000a). Since submerged fermentation is the widely used technique of fermentation for the production of microbial enzymes it has been applied for the production of lipase. The yield of lipase, usually reported as an extracellular enzyme is dependent on temperature, pH, aeration, agitation etc.

Liquid state condition is required for the bacterial cultivation as more water activity is required by the bacteria. *Pseudomonas* MS 1057 was observed to have highest lipase activity with 750U/mL yield (Kiran *et al*., 2008). In *Bacillus stearothermophilis* an activity of 1585U/mLwas reported under optimum submerged conditions (Abada *et al*., 2008). 104 U/mL of activity was reported in *Trichosporon asahii* MSR-54 (Gupta *et al.,* 2007), while in *Rhizopus chinensis* using liquid state fermentation ,14 U/mL yield at optimized experimental conditions (Teng and Xu , 2008) was documented. Maximum lipase yields, 16 U/mL, 41 U/mL, 12.7U/mL and 17 U/mL by *Fusarium oxysporum*, *Aspergillus niger* NCIM 1207 and *Rhizopus oryzae* and *Geotrichum* sp. were observed under optimized liquid state fermentation conditions (Burket *et al*.,2004; Shukla *et al*., 2007; Rifaat *et al*., 2010).

Bacteria are mostly grown under submerged fermentation conditions but many reports have been seen as there growth in solid state fermentation. 1084 u/g of yield was produced by *Pseudomonas aeruginosa* PseA (Mahanta *et al.* (2008). Alkan *et al*. (2007) and Fernandas *et al*. (2007) reported highest lipase activities of 149 and 108 U/g by *Bacillus coagulans* and *Bacillus cepacia*. Diaz *et al*. (2006) reported the lipase production in a 50-g packed-bed bioreactor. It had temperature 40°C and 50 mL min−1 of air was supplied . A maximum

lipase activity of 1,500 U/ g was seen after 12 hr of fermentation. The lipase production in a 1-kg tray bioreactor was reported by Mala *et al*. (2007) .The production was very less when it was compared to the yield that was obtained by 100-g tray bioreactor.

2.7 Modulation of lipase production by carbon source

Type and amount of carbon and nitrogen source used both effect the production of lipase. Various carbon sources have been used as growth substrates for the production of many enzymes and antibiotics such as corn,coconut oil, starch, glucose, sucrose, soybean oil. Growth sometimes become restricted due to the opposite effect exerted by carbon source. A regulatory mechanism called CCR (Carbon catabolite regulation) is performed in many microbial systems to make sure that best carbon source is utilized when more than one carbon source is present. Cell catabolises the best carbon source that is available in the medium and the synthesis of other substrate is suppressed until primary substrate isn't utilized (Saxena *et al*., 2003).

Lipase production in Bacillus sp. was studied by Sugihara *et al*., (1991). . Although enzyme activity occurred in the lack of olive oil also after long cultivation. The lipase production with 1% olive oil as an inducer was observed by the author. Palm oil and fructose were observed to be the best carbon source for lipase production by *Rhodotorula*s sp. However on comparison between two sources palm oil is considered to be more productive at concentration of 1% (Papaparaskevas *et al*., 1992).

2.8 Effect of nitrogen source on Lipase production

A large numbers of nitrogen sources can be used as growth medium for the production of lipase. However there are only some sources of nitrogen that supports the growth Good sources of nitrogen include ammonia, glutamine where as urea is considered as poor source of nitrogen. In order to utilize the best source for growth organisms follow CRD (Cell Regulation And Development) mechanism and hence utilizes the best and repress the other source (Saxena *et al*., 2003)

Peptone is reported as the best nitrogen source in comparison to soybean meal and steep corn liquor. Urea inhibited the lipase synthesis and ammonium sulphate (Szatejar and Malisszewaska, 1989). *Pseudomonas* sp. produced lipase under a medium which was having peptone (2%) and yeast extract (0.1%) (Izumi *et al*., 1990). The maximum lipase activity that

was reported was with olive oil as carbon source and yeast extract and protease and peptone as nitrogen source (Fadiloglu and Erkmen, 2002).

2.9 Effect of inducer on production of Lipase

Upon exposure of microorganisms to varying environment certain variations are produced through changing patterns of structural proteins, toxins, enzymes etc so that they can adapt to prevailing conditions. The amount of enzyme produced is a function of the source used for its production; this source is called "inducer" which turns on the production in a way that enzyme or metabolite are synthesized only when needed (Saxena *et al.,* 2003). Higher yield of lipase was observed when oil was used as inducer which enhanced the production by many folds although production was observed in the absence of inducer but failed to enhance the production in the absence of oil (Ifithakar and Husain, 2002).

2.10 Effect of temperature and pH

The optimal temperature and pH varies from organism to organism for growth and lipase production. Different species and strains of bacteria have variable needs of temperature and pH for their growth. In a study performed by Ertuğrul *et al* (2007) it was observed that pH 7 was best suited for the lipase production. At pH 7.5 highest lipase activity was seen which produced 0.73g/l of total protein while no growth of streptomycin was seen at pH 7.5. Also the growth decreased at pH from 9.0-11.0 (Chaiyaso, 2007).

Growth temperature is another factor that influences growth and lipase production. *Burkholderia* strain of bacteria however many can even grow at 37°C and 40°C but usually grows at 30°C and produce lipase at all temperatures (Brenner *et al*., 2005). *Bacillus* sp. produce lipase at the temperature of 70°C and maximum lipase production was seen at 50° C (Lee *et al*., 1999).

2.11 Strategies for purification of Lipase

Purification is considered to be the important part in enzyme production as it leads to efficient and successful usage of enzyme. Also, the three dimensional structure of a protein can be easily determined if an enzyme is purified. To determine whether lipase is pure X-ray studies can be important (Ghosh *et al.,* 1996). In many industries lipase in purified form is used for the production of large number of chemicals. It is also used for the production of pharmaceuticals and cosmetics. The major problem in traditional purification process is the

time and lower yield, however new method of purification has emerged such as gel filtrations; immune purification etc. Industries use such kind of purification methods which are less expensive (Saxena, 2003). Purification by gel filtrations and acetone precipitation was reported in 1990 (Izumi *et al),* the purification factor that was achieved was 13.9 with 2.9% of overall recovery. Molecular weight of enzyme was estimated 33 kDa by SDS-PAGE. Centrifugation is an age old trusted method for purifying extracellular enzymes.Lipase being predominantly an extracellular enzyme, it is important that cells from the culture broth should be removed by filtrations or centrifugation. Cell free culture broth is concentrated by ultra filtrations or extraction is done with organic solvents (Saxena *et al*., 2003). Most of the bacterial cells are centrifuged by applying centrifugal force of 5000g for 10-15minute. Higher centrifugal force is required to remove large cell debris. Force of 10000g for 10-15minute is applied. Although centrifugation is most widely used method for the removal of cell debris but alternate methods has also been developed which are cost effective.

2.12 Industrial applications of Lipases: Lipases represent 4% of the enzyme market and is considered to be the important enzyme from the biotechnological prospective (Hasan, *et al*., 2006). Lipase possesses such properties which are more favorable than chemical catalysts and are used as biocatalysts. Being substrate specific lipase catalyzed reactions are highly specific while the chemical process is non-specific. Due to specificity there is no waste products formed by them. Moreover, the use of enzymes reduce side reactions and because of this they can be isolated easily (Pandey, *et al*., 1999), Hasan, *et al*., 2006). This makes this enzyme environmental friendly. Lipase reactions are carried out at mild conditions hence reduced the energy cost and products are protected from getting destroyed. Microbial lipases are highly stable and do not require any cofactors which makes there biotechnological potential more effective (Jaeger and Reetz 1998). By growing lipase producing bacteria in suitable substrate, lipase can be used in different industries for various applications (Pandey, *et al*. 1999). Lipases are used in food industries for the production of variety of products like fruit juice, baked foods, cheese, and butter. By synthesis of fatty acids and alcohols lipase is used for giving special flavor and taste to food (Gandhi 1997). Lipases are used in milk industry for the hydrolysis of milk fats. It's been used in meat industry to remove excess of fats, fat is removed by adding lipases to the meat and this process is named as biolipolysis (Sharma *et al.,* 2001; Seitz 1974). Lipases are also used in detergent industry as additives commonly in household and industrial industry. Lipases are added to detergents along with protease and cellulases (Pandey, *et al*. 1999) which in turn leads to the hydrolysis of fatty

stains into hydrophilic parts which can be easily detached (Joseph, *et al*. 2007). In addition to their possible use in laundry industry they are extensively used in many other applications also such as dish washing, contact lens cleaning etc (Hasan, *et al*. 2006). Lipases in leather industry are used to remove of subcutaneous fats and dehairing. Using surfactants to remove fats an cause damage to environment as dangerous compounds are formed as side product so lipases are employed for removal (Hasan, *et al*. 2006). Lipases are used in paper and pupl industry to remove pitch (trigylerides and waxes) which causes problem in the production of paper and pulp industry. Pitch control system have been found to remove most of the wood triglycerides by industry called Nippon Paper Industries. This system uses lipase produced from *Candida rugosa* (Jaeger and Reetz 1998). Furthermore lipases are used as biosensors and in pharmacy.

2.13 Microbial polymers: overview

Polymers are natural and synthetic compounds which consist of monomers as repeating units. Due to their high weight they are classified as macromolecules. These polymers are classified into:Natural polymers such as proteins,Synthetic polymers such as PVC and polyethyleneBiopolymers such as PHA.

Natural polymers produced by living organisms includes chitin, proteins, peptides, DNA etc. biopolymer is the term used in place of bioplastics but have two different definitions, first is the source of raw material and second is the biodegradability. Biopolymers can be differentiated into three types: Bio-based polymers that are made up of renewable raw materials and are biodegradable, Bio-based polymers that are made up of renewable raw materials and are non-biodegradable and polymers made up of fossils and are biodegradable Biopolymers can be also divided into categories namely biodegradable or non-biodegradable. Those which respond to heat and are classified as thermoplastics, thermo sets (Raquez *et al*., 2010).

Based on the origin biopolymers can be divided into three main categories:

Polymers derived from biomass e.g. starch and cellulose.
Polymers by chemical synthesis using bio based monomers e.g. PLA
Polymers produced by microorganisms. This category, includes PHA, PHB and PHBV.
Microbial polymers are produced by microorganisms and common ones are PHA, PHB, PHBV etc.

1) PHA are the natural aliphatic polysters which are synthesized by the process of fermentation of sugars, alkenes, alkanes with the help of certain bacteria such as *Bacillus, Pseudomonas, Azotobacter* sp. and many other strains (Sukan, 2015).

2) PHB are natural macromolecules extracted from *Bacillus megatarium* having semi crystal line behaviour and high melting point with brittle properties. This polymer shows thermally unstable behavior and hence is very expensive. During processing the viscosity decreases gradually. This polymer is synthesized by bacteria as a storage material. It is fully biodegradable polyster with very fine barrier properties. This biopolymer is used in wide range if applications such as tablet packaging, food industry for example bottles, foils and many more (Katarzayna Leja, 2009)

3) PHBV is a copolymer of PHB which is produced by the fermentation for improving the properties for industrial applications. The application of this biopolymer is mainly in production of disposal materials, packaging, medicines etc (Brunel, 2014).

There are certain biopolymers which are widely used:

Starch thermoplastic is considered to be the most widely used bioplastic. It constitutes 50% of the bioplastic market. Addition of additives such as plasticizer can modify the property of the material which is called as thermo plastic starch.

Cellulosic bioplastics which are made from cellulosic derivatives.

Corn starch, PLA is produced from corn.

PHAs produced by microorganism by fermentation by the use of waste materials.They are produced as homopolymers or co polymers.

In addition, biopolymers have been exploited for virtually every application and sector including electronics, healthcare and diagnostics and environment. The application of biopolymers for flocculation in waste water treatment has been an important achievement wherein a large segment of waste water originating from variety of industries have been trialled. Bacterial extracellular polymers with capability to flocculate is an important addition to the repertoire of microbial polymers and represent a promising tool for water treatment , reclamation in view of their safety and sustainaibility.

CHAPTER – 3 MATERIALS AND METHODS

3.1 Materials

1) Chemicals: *p*-Nitrophenly-laurate(p-NPL) used was purchased from Sigma (Mo,USA). All other reagents that were used were of analytical grade and were purchased from Merch (E.Merck, Darmstadt, Germany). Branded Oils used, were commercially available and purchased from local supermarket.

Media: Three different types of media that were used were FIB media, Rhodamine B agar media and enrichment media. Media were purchased from Himedia,Mumbai. Enrichment media was vegetable waste media such as corn powder (0.5g), onion powder (1g) and garlic powder (0.5g) with 1% gum Arabic, olive oil (2.5%3%) and Rhodamine B solution (1mg/ml), 2g agar**.**

3.2 Methods

Measurement of lipolytic activity

Lipase activity in all cases in supernatant was determined by spectrophotometric assay using *p*- nitrophenly-lauarte (p-NPL) as substrate. The reaction mixture consists of 0.1ml enzyme solution, 0.8ml 50mM Tris- HCL buffer (pH 8.0) and 0.1ml 10mM pNPL dissolved in ethanol. The hydrolytic reaction is carried out at 65°C for 30 minute. Following the incubation 0.25ml of 0.1 M Na_2CO_3 is added to stop the reaction. The mixture was centrifuged (10000 * g for 15 min) and the absorbance at 410nm is determined (Ertugrul *et al*., 2007)spectrophotometrically.

Estimation of Total Organic carbon, Nitrogen, total proteins, total organic carbon and reducing sugars in formulated media:

1)Folin Lowry's method (Lowry *et al*.., 1951) was used to determine the protein content in formulated media. In this method bovines serum albumin (BSA) was used as standard. Different conc. of BSA and sample was prepared, mix the 0.1ml of protein with 2ml of alkaline copper phosphate reagent, and mix it well. Solution was

incubated at room temperature for 10 minutes. 0.2ml folins reagent was added and each tube and incubated for 30 minutes. Absorbance at 660nm was taken. The standard curve between concentration and optical density was plotted.

2)DNSA method was used to analyze the sugar reducing content in the formulated media using glucose as standard. Different concentration of glucose (0.1 to 0.8 mg/ml) and sample containing media was prepared (0.9mg/ml). 3ml of DNSA (3,5- Dinitrosalicylic acid) was added to each test tube. The solution was incubated for 5 minute at 80°C. Absorbance was taken at 540nm. The standard curve of glucose was used to determine sugar content in the media.

3)TOC method was performed for the estimation of carbon content in the vegetable media . 1ml of sample was mixed with 10ml of potassium dichromate and 20ml of sulphuric acid. Sample was diluted with 70ml of water and 10ml of orthophosphoric acid and 0.5gm sodium fluoride was added and titrated with ferrous ammonium sulphate. 1ml od diphenyl amine is added as indicator. End point is taken till green color is achieved. TOC was calculated by the following formula

Organic carbon(%)= 10(B-S)-B*[0.003*100/wt. of sample]

4)Kjeldahl's method is performed for the estimation of nitrogen content. Digestion, distillation and titration was done by the specified procedure and nitrogen content was calculated by the following formula.

% of nitrogen in the sample= 1.4*Normality of HCl * Vol of HCl used/mass of the compound taken (Kjeldahl's.,1883)

3.3 Experimental methods

The experimental method was used to validate the results of RSM (Research Surface Methodology) for the optimization of different media parameters. The statistical analysis was carried out using RSM software.

Detection of lipase producing bacteria

Lipase producing bacteria was characterized from amongst 20 pre-existing bacterial cultures with ability to produce polymeric flocculants. Purity was checked by gram

staining and microscopy and glycerol stocks were made for storing cultures for further investigations. *In situ* detection of lipase production was performed in two stages:

Bacterial isolates were first investigated for their lipolytic activity by Tween 80 agar method. LB agar medium was supplemented with 0.01% $CaCl_2$.H2O and Tween 80, which were separately autoclaved for 15min at 120°C. Tween 80 was added to the molten agar medium at 45°C to provide a final concentration of 1%. The medium was agitated until Tween 80 had dissolved completely and solution is poured into Petri dishes. As an indication of positive outcome for the test, an opaque halo occurred around the colonies.

For final confirmation of Lipase production, the Rhodamine B-olive agar plate method was used. LB agar medium was supplemented with Arabic gum (to yield a final concentration of 1%) and olive oil which were autoclaved at 121°C for 15 minute. The medium was cooled to about 60°C and 10ml Rhodamine B solution (1mg/ml) and olive oil (to yield a final concentration of 2.5%) were added and media was stirred vigorously and emulsified by mixing for 1min in a homogenizer. Aliquots of 20ml were poured into Petri dish and left to solidify. Stock cultures were streaked into Rhodamine B olive agar plates and were incubated at 65°C for 24-48 hours. The lipase producing bacteria were indicated by the presence of orange fluorescent halos around the colonies when the plates were irradiated with 350nm UV light.Both methods were used as well on solid vegetable media

3.3.1 Lipase production in formulated vegetable media

Lipase production in vegetable media was analyzed by dissolving corn powder (0.5g), onion powder (1g), garlic powder (0.5g) in 100ml distilled water. After complete dissolution for 30minutes it was filtered and 0.005g yeast extract was added and autoclaved. Solid media was made by adding 2g agar.; 1% gum Arabic, 2.5% olive oil and 10ml of Rhodamine B solution was added after cooling to 55-60°C. Solid media plates were streaked three successive times for the detection of lipase producing bacteria by presence of fluorescent halos around the colonies when plates were irradiated with 350nm UV light.

3.4 Kinetics for lipase production

Kinetics of lipase production was important to gain insights of enzyme affinity to substrates. Substrate concentration was kept constant for the kinetics studies. The effect of substrate concentration was measured at different concentration levels from 0.05mM to 0.7mM using culture supernatants.

3.5 Optimization of media components for lipase production

1)The effect of carbon source : effect of lipase production of strain was studied using LB containing different carbon sources (olive oil, glycerol, glucose, fructose, sucrose, almond oil) at concentration of 0.1, 0.5 and 1%. 0.5% gum Arabic was added to the LB medium. Suitable carbon source with highest lipase activity was selected for advance study.

2)The effect of nitrogen source: effect on lipase production of the strain was studied using medium containing 0.5% olive oil, 0.5% gum Arabic, 1% NaCl and different nitrogen sources (yeast extract, peptone, meat extract, tryptone and ammonium sulphate) at concentration of 0.5%. Suitable nitrogen source with highest lipase activity was chosen for advance study.

3)The effect of pH: effect on lipase production was investigated with the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum Arabic at different pH values (6.0, 6.5, 7.0, and 7.5). Suitable pH showing highest lipase activity was selected for advance study.

4)The effect of temperature: effect on lipase production is investigated with the optimized medium containing 0.5% olive oil, 0.5% peptone, 1% NaCl and 0.5% gum Arabic at different temperatures (30, 35, 40, 45, 50°C) at pH 6.5. Suitable temperature showing highest lipase activity was selected for advance study.

3.5.1 Location of enzyme

Cell mass was washed, sonicated (10min approx under ice) and centrifuged and supernatant was used for assaying. Enzyme activity from one milligram protein in supernatant was compared with cell mass.

3.6 Response Surface Methodology

Response surface methodology was applied with CCD (Central Composite Design) to optimize the media components according to the results that were obtained by screening experiments. The design expert trial package was used for the experimental design and

regression analysis of data. The media components were studied at three levels (-), (0), (+), for low intermediate and high concentrations respectively. For statistical calculation, coding of factors was done by the equation.

Lipase production at 12 hours: 0.0035+0.0025 A+ 0.0036 B-0.0025 AB-0.0006A2-0.0033B2

Lipase production at 36 hours: 0.0031+0.0.0014A+0.0028B-0.0019AB-0.000A2-0.0026B2

Lipase production at 60 hours: 0.0029+0.0029A+0.0034B+-0.0024AB-0.0010A2-0.0028B2

Lipase production at 80 hours: 0.0033+0.0020A+0.0031B-0.0021AB-0.0006A2-0.0027B2

Lipase production at 96 hours: 0.0028+0.0027A+0.0043B-0.0037AB-0.0009A2-0.0030B2

Where, A: Olive Oil; B: Yeast Extract

Table 3.1 Experimental design matrix showing different levels.

Run	A: Olive Oil	B: Yeast Extract	O.D (550nm)(12 h)	36h	60 h	84 h	96h
1	0.000	1.000	0.004	0.003	0.004	0.004	0.004
2	0.000	1.414	0.002	0.002	0.002	0.002	0.003
3	0.000	0.000	0.004	0.004	0.004	0.004	0.004
4	1.000	1.000	0.003	0.003	0.003	0.003	0.002
5	0.000	0.000	0.003	0.003	0.003	0.003	0.002
6	-1.000	1.000	0.003	0.004	0.002	0.003	0.004
7	1.414	0.000	0.006	0.005	0.005	0.005	0.005
8	0.000	0.000	0.004	0.003	0.003	0.003	0.002
9	-0.600	0.000	0.002	0.002	0.001	0.002	0.001
10	1.000	-1.000	0.001	0.001	0.001	0.001	0.001
11	-1.000	-1.000					
12	0.000	0.000	0.003	0.003	0.002	0.003	0.003
13	0.000	0.000	0.003	0.003	0.002	0.003	0.003

The design expert trial package was used for the statistical and regression analysis. Statistical analysis of model was verified by applying analysis of variance (ANOVA). Overall model significance was tested by Fischer's F test and its associated probability P (F'). Coefficient of determination (R^2) and adjusted R^2 was used to judge the quality of polynomial model equation. Three dimensional contour plots were further used to illustrate the relation between responses and experimental levels of each independent variable.

3.7 Production of bacterial extracellular polymer

One litre of media containing corn powder (5gm), onion powder (10gm) and garlic powder (5gm) was taken and inoculated with 5% bacterial cells grown overnight (with cell density (3 * 10^6 cells/ml). It was incubated at 37°C for 48hr and culture was centrifuged at 10000 rpm for 10minute. Supernatant was collected and concentrated by lyophilizing it to 1/10 of original volume. Equal volume of ethanol was added to supernatant and was left overnight at 4°C. Next day, precipitates were collected and centrifuged at 12000 rpm for 20minute at 4°C. Deionized water was added to pellet and contents were transferred to beaker and put on stirrer for 2hrs; 2% CPC treatment was given and mixture allowed to stand for precipitation at room temperature.Therefatrer, 2 mL of 0.1N NaCl was added to precipitate and again centrifuged at 10000 rpm at 4°C for 5minute. Pellet was collected and washed thrice with 100% ethanol and centrifuged again, pellet was collected and washed with milli Q water thrice. Dialysis was performed for 4-5 day, lyophilized and polymer powder collected and stored (Ghosh *et al*., 1996)for the characterization.

3.7.1 Structural and functional characterization

FTIR and SEM were used for their characterization of biopolymer. FTIR techniques have less solvent nature and requires less time for the analysis therefore, reduces the exposure to dangerous chemicals while providing faster output. FTIR technique is also suitable for routine monitoring of the biopolymer production. Interactions between biopolymer were recorded by FTIR (Fourier transform infrared spectrophotometer). Wave number range was recorded to reduce the spectrum of biopolymer. Surface properties of a biopolymer were analyzed by SEM. Sample of biopolymer were analyzed by SEM at 20.0kV.

Biopolymer powder produced after lyophilization was stored for structural and functional characterization by FTIR and SEM.

3.7.2 Determination of flocculating activity of the extracellular polymer:

The polymer produced extracelluarly by the bacterial strain was evaluated for its ability to flocculate suspended solids of varied size.A standard solution of suspended solids of 2000 NTU was made by suspending active carbon, silica, magnesium hydroxide, cellulose and yeast in 100mL water. A suspension of desired turbidity was prepared by diluting the suspension form stock to desired turbidity with a turbidimeter (Cyber Scan TBDIR1000 Meter, Eutech, Netherlands). The assay was carried out by adding 10mL $CaCl_2$ (5mM),

0.5mL biopolymer (2mg/l) and 9.5mL of distilled water to 80mL of these solutions. The pH was adjusted to 7±0.2 and the solutions were allowed to stand at room temperature for 5min. 20mL of aliquots were withdrawn from the upper phase and its turbidity was measured. The flocculating activity was calculated by recording its optical density with a spectrophotometer at 550nm against distilled water as control as described by Kurane et al., (1986). Activity (%) was defined and calculated as (B–A)/ B × 100. The activity was expressed as the mean value from duplicate determinations.

CHAPTER – 4 RESULTS AND DISCUSSION

4.1 Detection of lipase producing bacteria:

Bacterial cultures from amongst a repository of 20preexisting extracellular polymer producing producing isolates were spot inoculated on Tween 80 Agar to presumptively check lipolytic activity. Out of these only one strain which showed strong lipolytic activity was designated as A1 and used for further analysis. The positive outcome of Tween 80 screen assay lies on production of opaque halo around the colonies of bacteria (Kouker and Jaeger, 1987).The positive strain wss further verified for orange fluorescent colonies umde UV light in Rhodamine B agar medium (Fig 4.2)

It has been suggested that Rhodamine B assay for the aerobic culture conditions produce dimers of Rhodamine B which was considered as the probable mechanism for the detection of fluorescence observed with lipolytic activity. However recent modification of the explanation is that development of Rhodamine B-LCFA (long chain fatty acid) conjugate (Jarvis and Thiele, 1997) is not responsible for fluoroscence. This fact was supported by Rueda (2005) who postulated that Rhodamine B solution when it forms complexes with fatty acids develop orange-pink fluorescent under UV light radiation. The orange fluorescent color can be used as a confirmation for the detection of bacteria that produce lipase.

In present study, Rhodamine B agar method was selected for the isolation and screening of lipase producing bacteria because of several advantages in comparison to other methods. Rhodamine B agar plate is not sensitive to pH changes, it causes no inhibition in the growth of microorganism and changes in physiological properties, and is highly specific and distinguish lipase producing bacteria easily (Kouker and Jaeger, 1987).

Fig 4.1 Lipolytic activity of extracellular polymer producing bacterial isolates on Tween 80 media. Opaque zones are indicative of positive lipolysis

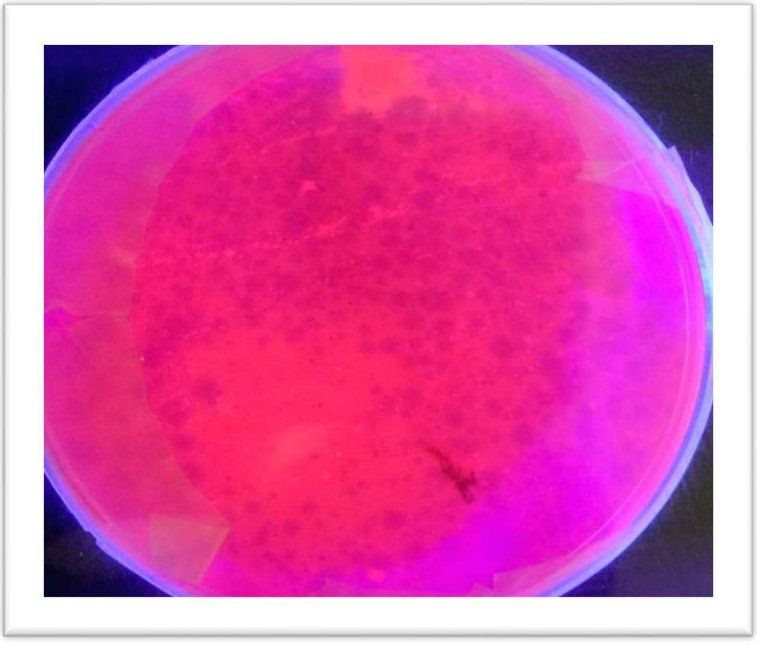

Fig 4.2 Detection of Lipase production by extracellular polymer producing bacterial isolate on Rhodamine agar plates. Lipase production is visualized as orange fluorescence in colonies exposed under UV light.

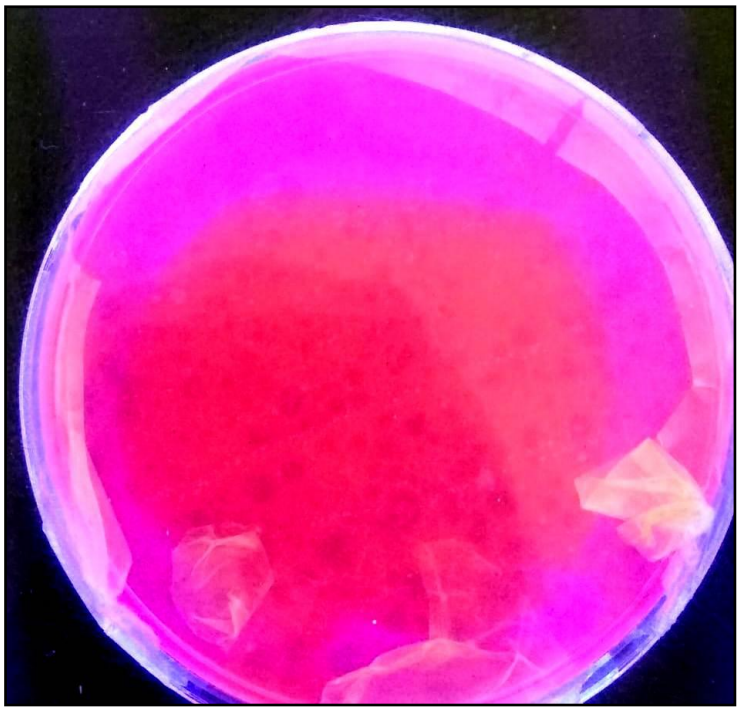

Fig 4.3 Confirmation of Lipase production by selected bacterial strain on Rhodamine agar plates.

4.2 Characteristics of the formulated vegetable media

The concept of formulating media from vegetable utilizes the fact that the component vegetables considered, could be obtained at very low cost. Discarded potatoes, onions from potato snack processing units, garlic and corn from superstores after grading. The chief advantage is consumer unacceptable/rejected ones can be used for preparation the media. Therefore the method also provides an alternative of valorizing agri-wastes. All materials were further cleaned, ground to powder and sun dried completely. Finally they were ensured to be free from microbial contaminants by surface plating (APC < 200 CFU/g).

The color, consistency and solubility of the media formulated using powders of vegetables were satisfactory. The gelling property, coloration upon addition of agar–agar was also not affected indicating applicability of the media for preparing solid plates. The formulated media was stored under hygroscopic condition in dark at 28^0C. Although, very critical, a study on extended shelf life could not be conducted, the media remained intact and was able to support bacterial growth excellently after 2 months of storage. This indicated the stability

of media components and feasibility for large scale production of this possibly for commercial purposes. The Protein content and reducing sugar in the media was estimated by Lowry's method and DNSA(3,5-Dinitrosalicylic acid). The media contained 20mg/ml of protein, whereas the reducing sugar content was found to be 60mg/ml.

Both Protein and Sugar has been suggested important as media constituents and govern cellular pathways for production of exopolysaccharides. The media carbohydrate content should be high and protein content should be low. The C:N ratio is an important consideration for triggerring lipase and biopolymer production (Wang and Lee, 1997). The total organic carbon was 5.66%and total nitrogen determined was 0.044% respectively implying satisfactory ratio suggested for extracellular polymer production. Nitrogen source in media is used for the synthesis of proteins, amino acids, DNA and RNA and carbon source is used for the growth of the bacteria (Ward 1991; Stanbury *et al*. 1995). Too high and low C: N ratio causes decline in the formation of lipase and biopolymer. The components – garlic contains several amino acids, thioslufinates etc. Corn has been used (cornsteep liquor) in commercial fermentations and is a good source for carbohydrate while onions contain flavonoids and polyphenols in addition to sulphur containing amino acids. Together, the ingredients provide a complete nutritional profile enabling adequate biochemical processes nad growth for bacterial growth. However, prior to commercial exploitation a detailed analysis of the developed would be necessary.

Table 4.1 Key constituents of the formulated vegetable media.

Method	**Content**
Total protein	20mg/ml
Reducing sugar	60mg/ml
Total organic carbon	5.66%
Total Nitrogen(Kjeldahl)	0.0448%

4.3 Kinetics for lipase production

The effect of *p*-nitrophenyl laurate was measured at different substrate concentrations. The crude lipase showed a variable specificity towards different concentration of triacylglycerols.

The spectrophotometric assay was used for each concentration with respect to time (Fig. 4.4).

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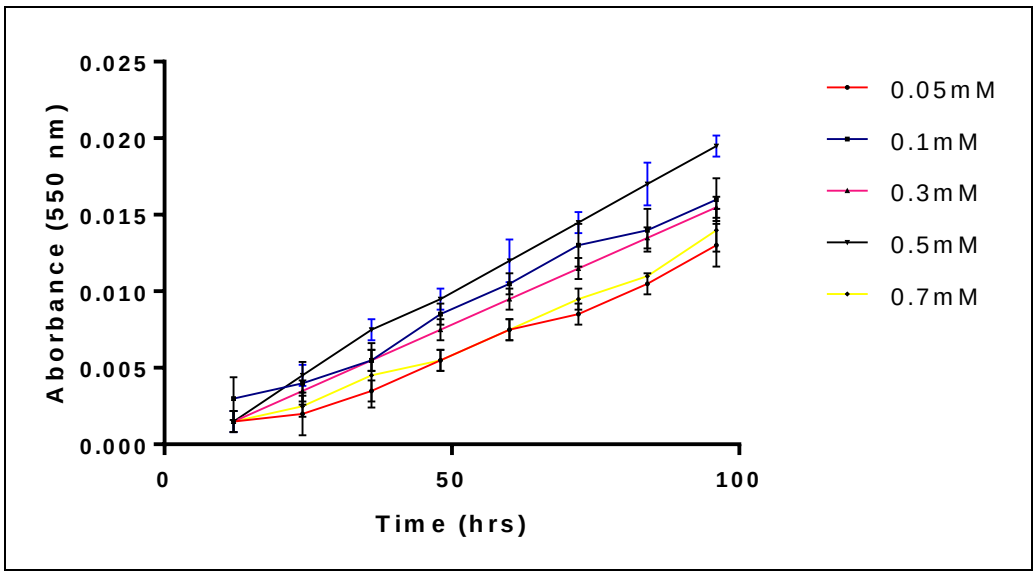
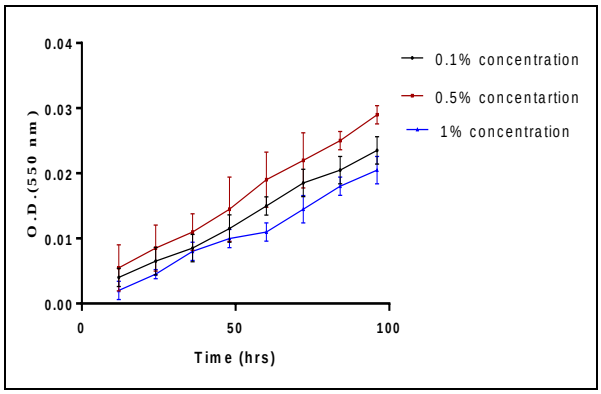


Fig. 4.4 Kinetics of lipase production shown by bacterial strain A1

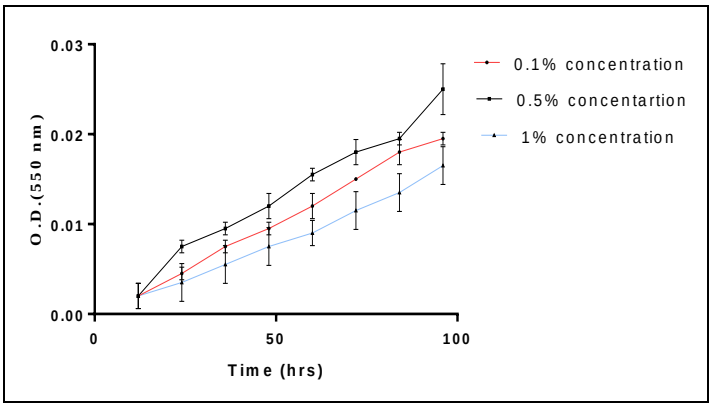
4.4 Optimization of lipase production

4.4.1 Effect of carbon sources on lipase production

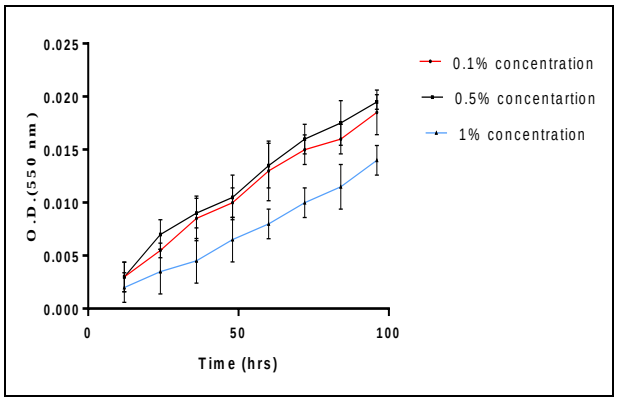
Although many reports indicate the production of lipase in the absence of oil (Handlesman and Shoharn, 1994; Ifithkhar and Husain, 2002), it has been suggested that production of lipase is also stimulated by the action of oil (Sharma *et al*., 2001) that contains long fatty acids (LCFA) (Taqac and Marul, 2008). Therefore different oils have been selectetd to determine their effect as well as other carbon sources (sucrose, fructose, glucose, glycerol) on lipase production by the selected bacterial strain. The results indicated(Fig 4.5) the production of lipase by different oils and carbon sources at different concentration of 0.1%, 0.5% and 1%.



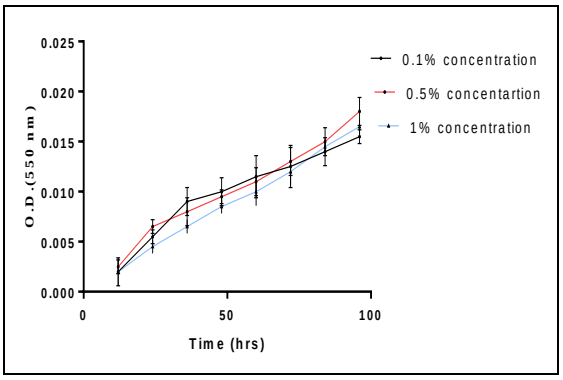
A (olive oil)



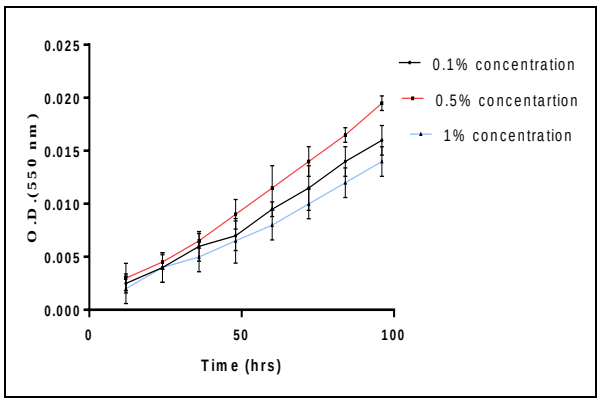
B (corn oil)



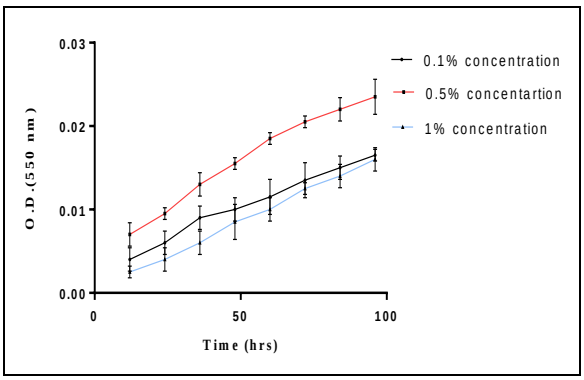
C (Glycerol)



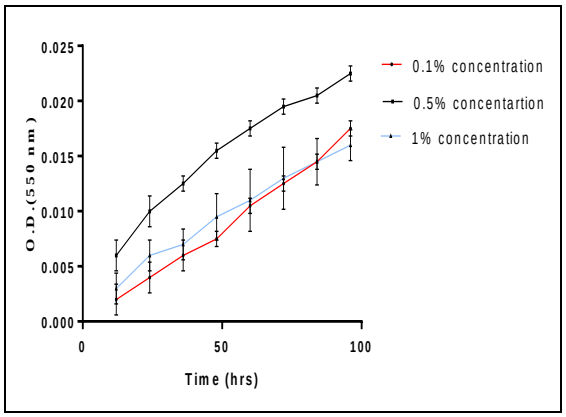
D (Glucose)



E (Fructose)



F (Sucrose)



Graph G (Almond oil)

Fig. 4.5 Graphs (A), (B), (C), (D), (E), (F) and (G) -the effect of different carbon sources at various concentrations after every 12hrs.

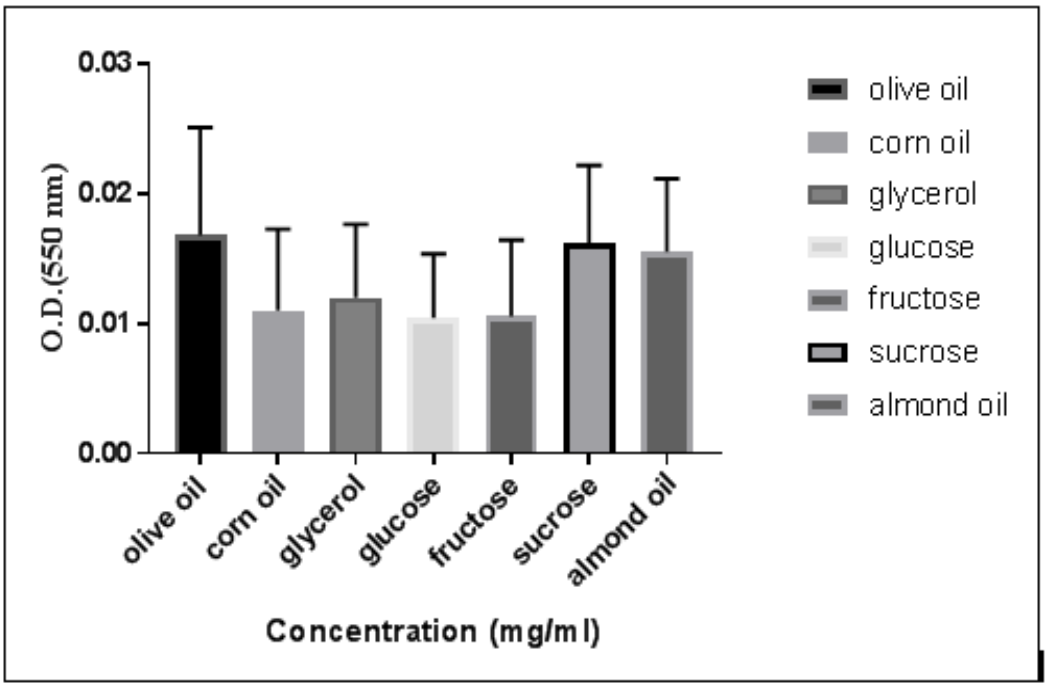


Fig. 4.6 Lipase activity in olive oil at 0.5% , corn oil, glycerol, glycerol, glucose, fructose, sucrose and almond oil after 96hrs at 1%NaCl, 0.5% gum Arabic and 0.5% of different carbon sources.

The result of different oils on production of lipase by bacterial strain was studied for 96 hrs at 37°C carried out in growth medium. The oils included olive oil, corn oil, glycerol, glucose, fructose, sucrose and almond oil which had three different concentrations 0.1%, 0.5%,1%. After every 12hrs optical density was checked at 550nm. The highest lipase activity was obtained at 0.5% by olive oil, corn oil, glycerol, glucose, fructose, sucrose and almond oil. At 0.5% concentration of each oil highest optical density at 550nm was obtained. Out of the different oils, olive oil showed highest lipase activity with highest optical density increasing after every 12hr. After olive oil, corn oil, glycerol, glucose ,fructose ,sucrose ,almond oil showed lipase activity but lesser than olive oil. Corn oil also showed good lipase activity as an inducer for lipase production. The total activity of lipase production using corn oil was not much different with lipase produced from olive oil. However olive oil was selected for the further studies since it had highest lipase activity. The other oils such as almond oil and corn oil were also good inducers beside other carbon sources such as glycerol, glucose ,fructose and sucrose.

4.4.2 Effect of nitrogen source on lipase production

The effect of various nitrogen sources on lipase production by bacterial strain were examined by growing it in a vegetable medium which contained 0.5% olive oil, 0.5% gum Arabic, 1%NaCl and different nitrogen sources such as yeast extract, peptone, tryptone, meat extract and ammonium sulphate at 0.5% concentration. Lipase activity and bacterial growth was detected very low with growth medium containing non organic nitrogen source. Lipase production was improved by using organic nitrogen source.

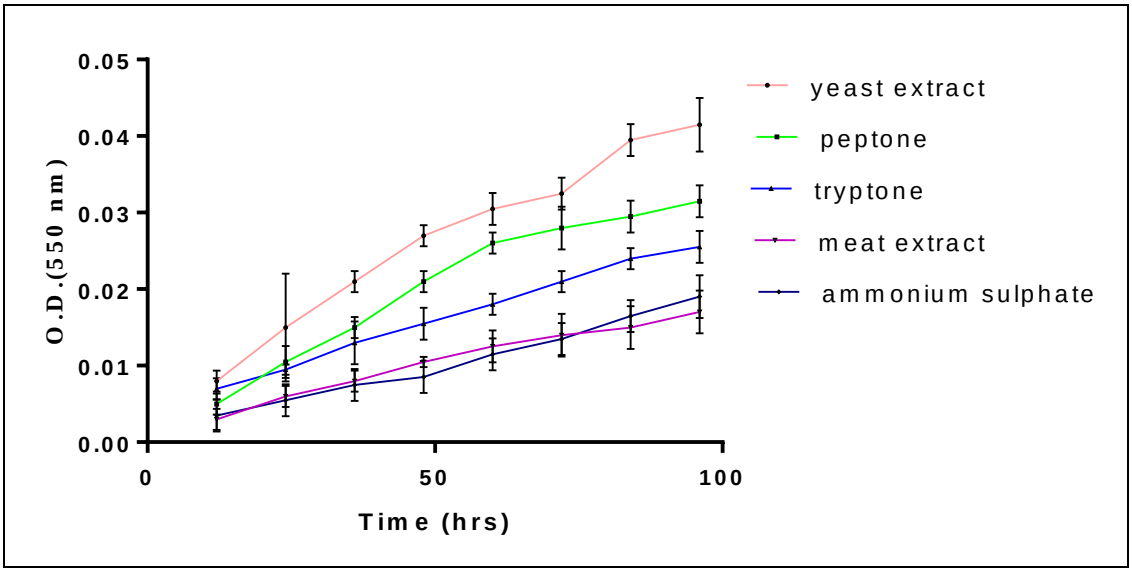


Fig 4.7 Effect of nitrogen source on lipase production by the bacterial srain

Among numerous nitrogen sources that were experimented, yeast extract was observed to be the best organic source for the production of lipase by bacterial strain. Lipase production by using peptone and tryptone was not much different when compared with production of lipase using yeast extract. However yeast extract was selected for the next factor determination for the lipase production while meat extract and ammonium sulphate did not show good yields for the lipase production by bacterial strain.

A comparable result was observed that reported yeast extract was the best nitrogen source for lipase production by bacterial strain as substitution of ammonium sulphate with yeast extract led to 3 fold increase in production of lipase (Gupta *et al*., 2007). Similar type of results was reported by Chaiyaso (2007) who observed that production of lipase by *Burkholderia multivoras* in a medium containing tryptone was improved by 17 fold when compared with production of lipase without organic nitrogen source.

Lipase synthesis by *Candida rugosa* was also found to increase in presence of olive oil and organic nitrogen source (Fadiloglu and Erkmen, 2002). Organic nitrogen sources affect the production of enzyme as it provides amino acids and several growth factor. These factors are necessary for protein synthesis (Ifithkar *et al.*, 2008) .

4.4.3 The effect of pH on the lipase production

The effect of initial pH on production of bacterial strain was examined for various pH values from 6.0-7.5 throughout 96 hr of cell cultivation which was carried out in growth medium

at temperature of 37°C. (Fig 4.8) show highest level of lipase production that was obtained at pH 6.5. These showed bacteria preferred a pH of 6.5 for lipase production. The results also showed that bacteria grew at 6.0, 7.0 and 7.5 where it produced highest lipase activity with no major difference in lipase production. No lipase activity was observed at pH 8.0 and 8.5. A pH of 6.5 pH was the best suited pH for the lipase production and was chosen for further parameter determination.

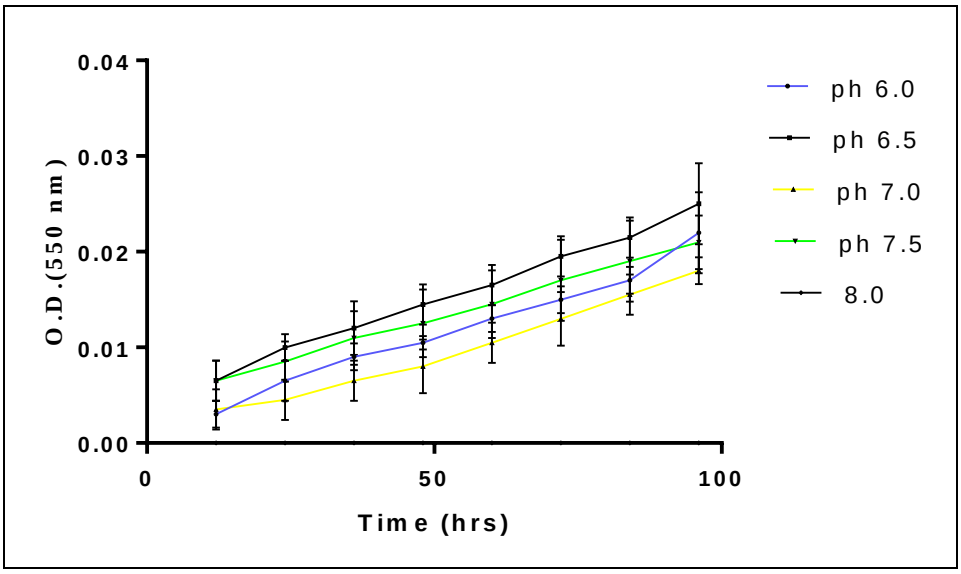


Fig. 4.8 Effect of initial pH on lipase production by bacterial strain in vegetable medium

According to Gupta *et al* (2004) it was reported that pH affects the production of lipase. More detail was explained by Stocker *et al* (2006) that specific intracellular pH values are required to reach equilibrium in enzyme catalyzed reactions. Therefore it was reported that intracellular pH can change extracellular pH of bacterial cells and can weaken enzyme synthesis. additional report was done by Etrugal *et al* (2007) to examine the best suited pH for the lipase production in *bacillus* sp. for 96hr at 37°C. The results showed highest lipase activity was achieved on 6.0 and 6.5 pH.

4.4.4 Effect of temperature on lipase production

The effect of temperature on lipase activity was evaluated at different temperatures from 30°C to 50°C .It was seen that lipase activity was more than twice at 35°C than compared to lipase activity at room temperature (25°C). Lipase activity decreased at temperature of 50°C. Highest lipase activity was determined at 30°c while it dramatically declined at 40, 45 and 50°C. (Fig 4.9).

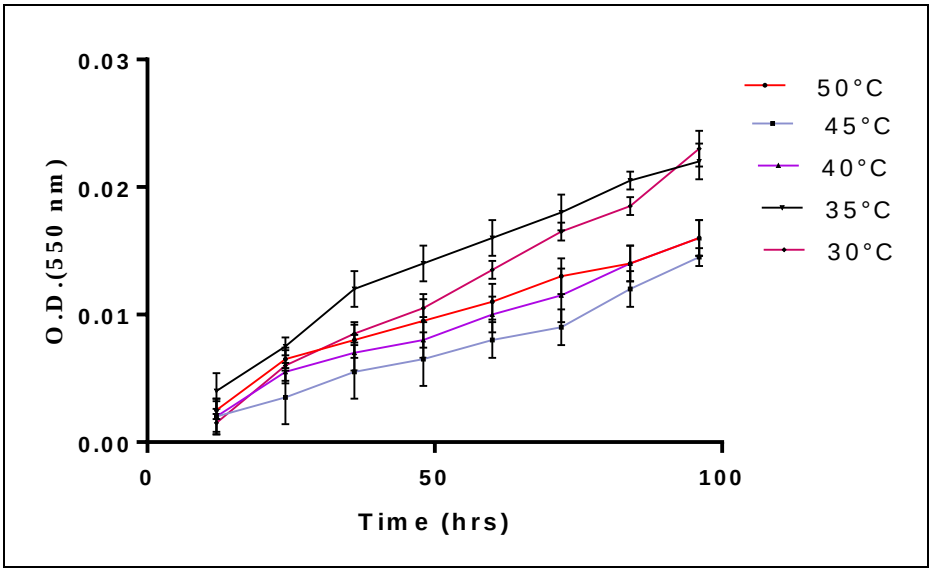


Fig.4.9 Effect of temperature on lipase production by bacterial strain in growth medium.

4.5 Location of enzyme

Location of enzyme was determined by disrupting the cell (cell free extract) and supernatant. Enzyme activity from 1mg protein in supernatant and cell free extract was checked by assaying for lipase activity at 410nm. The supernatant exhibited maximum enzyme activity

of 0.062mg/ml in comparison to cell free extract(0.002mg/ml). This result suggested that predominantly extracellular location of lipase.

Table 4.2 Location of Lipsae in the polymeric flocculant producing bacterial strain

Location of enzyme	Lipase activity (mg/ml)
Supernatant	0.062
Cell free extract	0.002

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4.6 Response surface methodology for the optimization of parameters

Based on the results obtained by screening of optimization parameters, media components such as carbon source olive oil at 0.5% concentration and nitrogen source ,yeast extract at 1% concentration were selected as most influencing variables and subjected to further

optimization using RSM. In this method, batch runs were conducted and response along with runs is shown in table. The regression coefficients were calculated and data was fitted into second polynomial equation.

ANNOVA was performed to verify the significance of second order response surface model and results are given below in table. ANNOVA showed that model was highly significant as it was evident from low p-value (<0.0001).The Model F-value of 6.02 implies the model is significant. There is only a 2.46% chance that an F-value this large could occur due to noise.

P-values less than 0.0500 indicate model terms are significant. In this case A, B, AB, B^2 are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. If there are many insignificant model terms (not counting those required to support hierarchy), model reduction may improve model.

The Lack of Fit F-value of 0.10 implies the Lack of Fit is not significant relative to the pure error. There is a 91.04% chance that a Lack of Fit F-value this large could occur due to noise. Non-significant lack of fit is good -- we want the model to fit. Since the lack of fit is not significant therefore the model is significant.

Table 4.3 ANNOVA of quadratic polynomial model and significant test.

	12h			36h			60h			84 h			96h		
Source	Df	F-Value	P-value	df	F-value	P-value	df	F-value	P-Value	df	F-value	P-Value	df	F-value	P-value
Model	5	13.63	0.0032	5	11.99	0.0044	5	5.20	0.0346	5	12.84	0.0037	5	6.02	0.0246
A	1	24.35	0.0026	1	10.07	0.0192	1	14.42	0.0090	1	21.93	0.0034	1	13.87	0.0098
B	1	40.02	0.0007	1	32.55	0.0013	1	16.82	0.0064	1	41.16	0.0007	1	27.30	0.0020
AB	1	16.48	0.0067	1	12.47	0.0123	1	6.86	0.0396	1	15.30	0.0079	1	17.38	0.0059
A2	1	2.51	0.1642	1	0.0025	0.9618	1	3.18	0.1249	1	3.28	0.1200	1	2.98	0.1353
B2	1	52.15	0.0004	1	41.78	0.0007	1	16.90	0.0063	1	49.76	0.0004	1	21.19	0.0037

At 12hrs, the Predicted R^2 of 0.6664 showed reasonable agreement with the Adjusted R^2 of 0.8517; i.e. the difference is less than 0.2.Adequate

At 36hrs, the Predicted R^2 of 0.6163 is not so close to the Adjusted R^2 of 0.8332 as one might normally expect; i.e. the difference is more than 0.2.

At 60hrs, the Predicted R^2 of 0.4817 is in reasonable agreement with the Adjusted R^2 of 0.6560; i.e. the difference is less than 0.2. Adequate Precision measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 8.209 indicates an adequate signal. This model can be used to navigate the design space.

At 80hrs, the Predicted R^2 of 0.6325 is different slightly close to the Adjusted R^2 of 0.8433 as one might normally expect; i.e. the difference is more than 0.2.

At 96hrs, the Predicted R^2 of 0.4373 is not as close to the Adjusted R^2 of 0.6955 as one might normally expect; i.e. the difference is more than 0.2.

Table 4.4 Statistical analysis showing ANNOVA for lipase production.

	12hrs	36hrs	60hrs	84hrs	96hrs
Std Dev.	0.0005	0.0004	0.0007	0.0004	0.0007
Mean	0.0032	0.0030	0.0027	0.0030	0.0028
C.V.%	15.41	14.22	27.07	13.78	24.68
PRESS					
R-Squared	0.9191	0.9090	0.8124	0.9145	0.8339
Adj R-Squared	0.8517	0.8832	0.6560	0.8433	0.6955
Pred R-Squared	0.6664	0.6163	0.4817	0.6325	0.4373
Adequate Precision	14.0494	13.5311	8.2095	13.8441	8.0930

4.6.1 Final equation in terms of coded equation

In 12hrs, the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor..

In 36hrs, the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

In 60hrs, the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor.

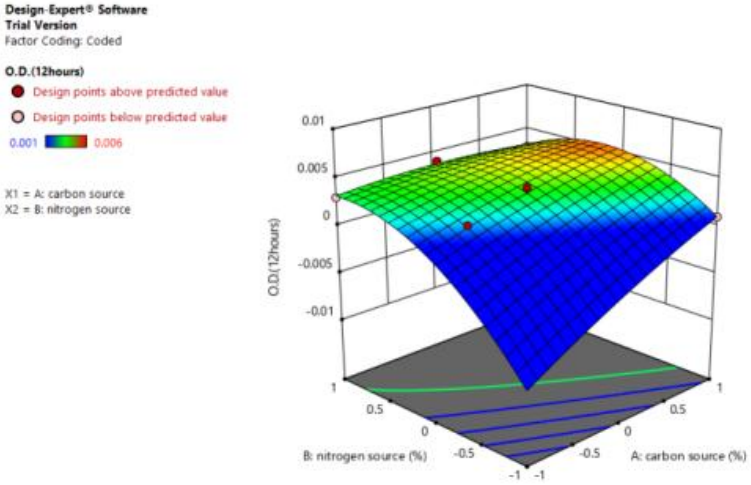
In 80hrs, the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as +1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

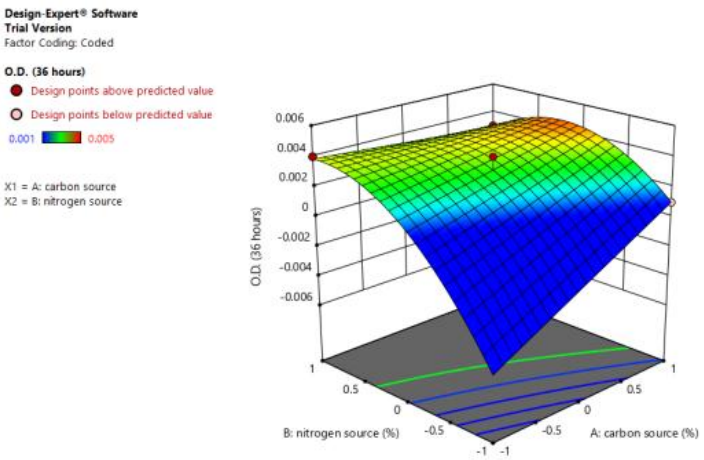
In 96hrs, the equation in terms of coded factors can be used to make predictions about the response for given levels of each factor. By default, the high levels of the factors are coded as

+1 and the low levels are coded as -1. The coded equation is useful for identifying the relative impact of the factors by comparing the factor coefficients.

Table 4.5 Experimental variables for lipase production in terms of coded values.

	12 hrs	36	60	84	96
	+0.0035	+0.0031	+0.0029	+0.0033	+0.0028
*A	+0.0025	+0.0014	+0.0029	+0.0020	+0.0027
*B	+0.0036	+0.0028	+0.0034	+0.0031	+0.0043
*AB	-0.0025	-0.0019	-0.0024	-0.0021	-0.0037
$*A^2$	-0.0006	-0.0000	-0.0010	-0.0006	-0.0009
$*B^2$	-0.0033	-0.0026	-0.0028	-0.0027	-0.0030





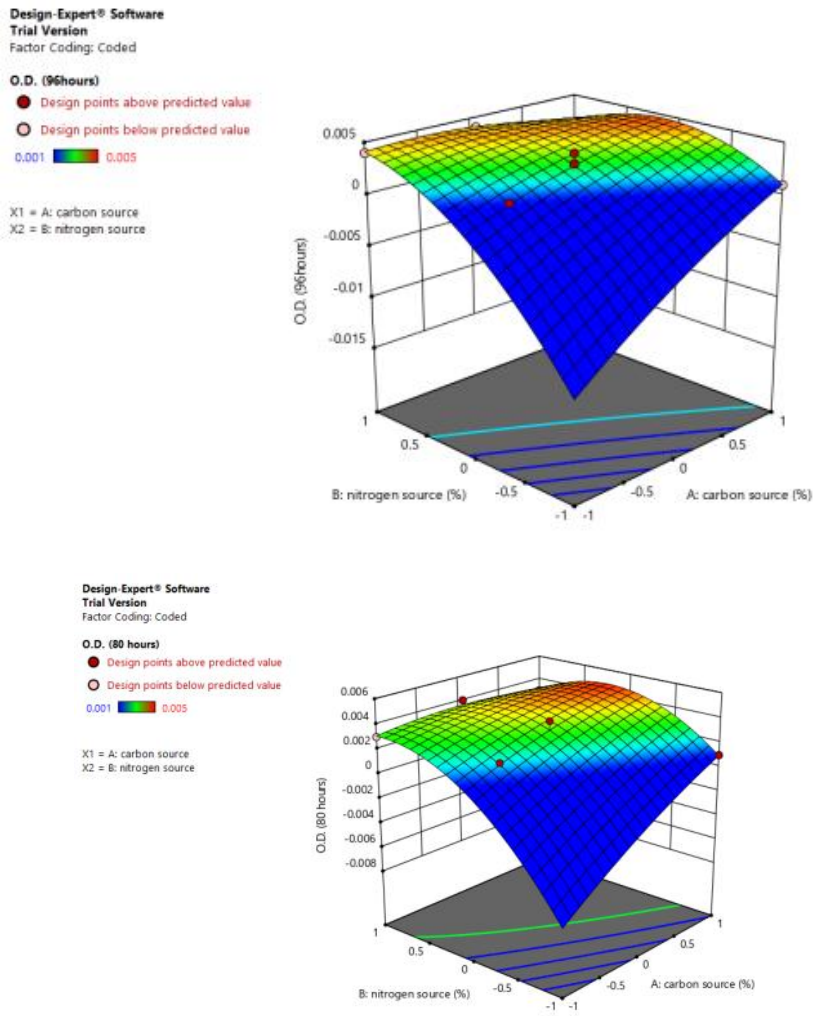


Fig. 4.10 3D representations highlighting interaction between two variables.

From the results, it can be concluded that 1% and 0.4% concentrations of carbon and nitrogen source are optimal for the lipase production.

Table 4.6 Predicted responses of lipase production at different time intervals:

O.D. (Time intervals in hrs)	Predicted response
12	0.00360913
36	0.00378957
60	0.00280422
80	0.00346649
96	0.00413962

4.7 Production of polymeric flocculant

Biopolymer production in general media (FIB) and vegetable media was compared by their corresponding yields. Biopolymer production protocol protocol was carried out as described by Ghosh *et al., (2014).* It was observed that amount of biopolymer produced in the vegetable composed media was 214mg/L and in standard media was 197mg/L (table 4.7). The polymeric flocculant produced in the formulated media was lyophilized stored for characterization by FTIR and SEM.

Table 4.7 (a) Comparative yield of biopolymer produced by the strain in two different media (mg/L)

Media used	Yield (mg/L)of polymeric flocculant
Reference	197mg/L
Vegetable	214mg/L

4.8. Flocculating potential of the extracellular polymer:

Table 4.7(b) Flocculating efficacy of the extracellular bacterial polymer in removal of colloidal particles in solution. The corresponding size of the particles are following: ~4-7µ (Kaolin, yeast cell suspension, cellulose), ~15-20µ (silica), ~100µ (Activated carbon)]. The polymer was used at a concentration from 2-10ppm.

Colloidal particles	**Residual turbidity(NTU)**	**Flocculating activity**
Kaolin clay	168	20 %
Yeast	198	60%
Cellulose	250	80%
Silica	150	30%
Activated carbon	148	38%
Mixed suspension	250	80%

Results revealed that the polymer aggregated a wide range of colloidal particles, over concentrations of 2-10ppm. Table 4.7(b) depicts that most effective flocculation was achieved in suspensions comprising very low and low sized particles at even low

concentration of the biopolymer. Whereas the efficacy of flocculation of suspension of medium sized particles ranged from 30-40% that too at relatively higher concentration of 8-10ppm of the biopolymer. With suspensions of large sized particles 50-65% flocculant activity was observed, at the same concentration range that flocculated in the range of 70-90% in case of low and very low sized particle suspension. This observation may be attributed to non-uniform distribution of pore size on the biopolymer surface; besides, the number of sites available for binding low and very low sized particles might be more than that of binding medium and large sized particles. However, large variations in the degree of flocculation were observed, where the kaolin particles (size~2μ) showed highest flocculating activity (90%). Overall the extracellular polymer was found to have good flocculating activity in line with earlier reports of Khaira etal, (2014).

4.7.1 Economics of the formulated media

A tentative economics were charted for fermentation carried out with 1L of both reference and formulated vegetable media. Table 4.8 depicts the break up of each media components with the cost . It must be noted that down stream processes have an add on cost for ultimately recovering the target bioproducts.

Table 4.8 Economics of media usage for biopolymer and lipase production by the bacterial strain in 1L vegetable media

Vegetable media components (gm/L)	Cost (Rs)
Corn powder -1gm	23
Garlic powder - 1gm	10
Onion powder - 2gm	20
Yeast extract - 0.10gm	12
Olive oil - 5ml	70
Total	135

Table 4.9 Economics of biopolymer and lipase production by the bacterial strain in 1L reference media

Standard media components(gm/L)	Cost (Rs.)
$(NH_4)_2SO4$ - 2gm	106
KH_2PO_4 -2gm	14
Na_2HPO_4 -0.6gm	33
$MgSO4.7H_2O$ - 0.2gm	32
$CaCl_2$ -20mg	38
yeast extract- 0.1gm	49
Fructose - 40gm	994
Total	1266

A comparison of Table 4.8 and 4.9 justifies clearly the cost involved upon using same quantities of two media . The vegetable media proved to be more efficient at Rs. 135 /L in comparison to the reference media (Rs. 1266/L) .Moreover, the ingredients of the vegetable media can be obtained abundantly .

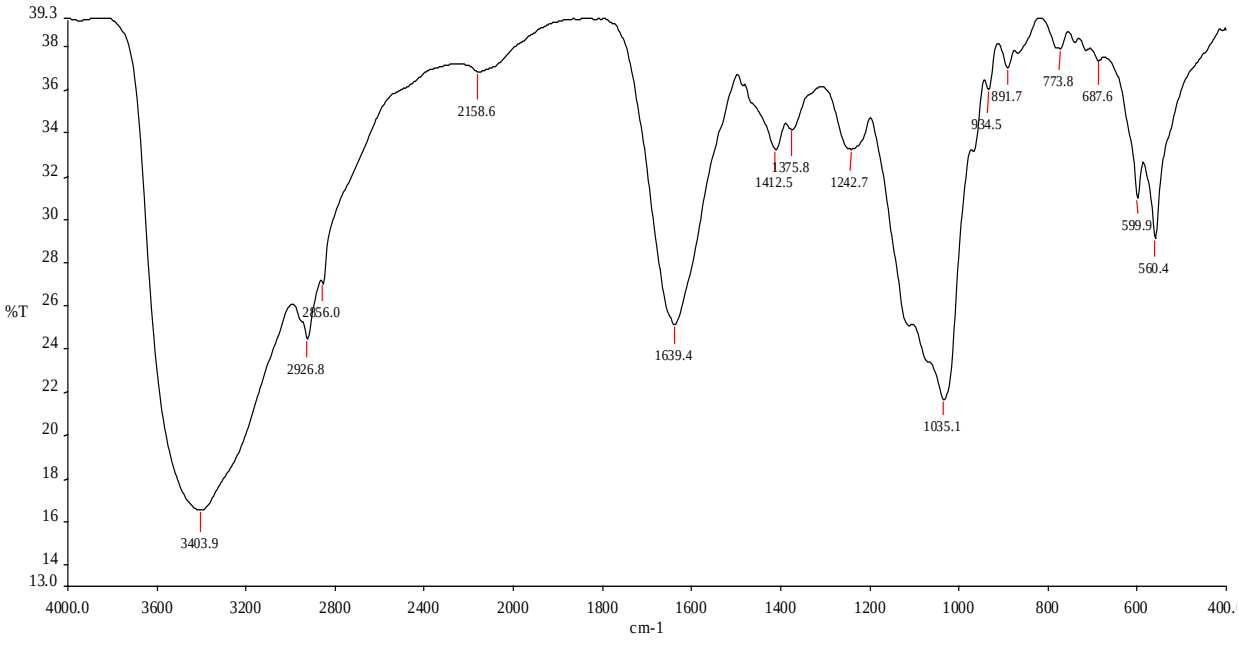


Fig 4.11 FTIR profile of purified polymeric flocculant produced by the bacterial strain

As depicted in Fig. 4.10, FTIR analysis of the polymeric flocculant indicated medium peak at 3403.9 cm^{-1} which is the characteristics of presence of alkyl halides. Strong peak at 2158.6 cm^{-1} indicates the presence of C-H stretch of aromatic compound, medium peak at 1639.4

cm^{-1} indicates the presence of C-N stretch of aliphatic amines. 1412.5 cm^{-1} and 1375.8 cm^{-1} medium peaks indicate the C-H stretch of aromatic compounds. Medium stretch at 1035.1 cm indicates the presence of alkenes. Strong and broad peak at 599.9 cm^{-1} and 560.4 cm^{-1} indicates the presence of O-H stretch and hydrogen bonded alcohols and phenols.

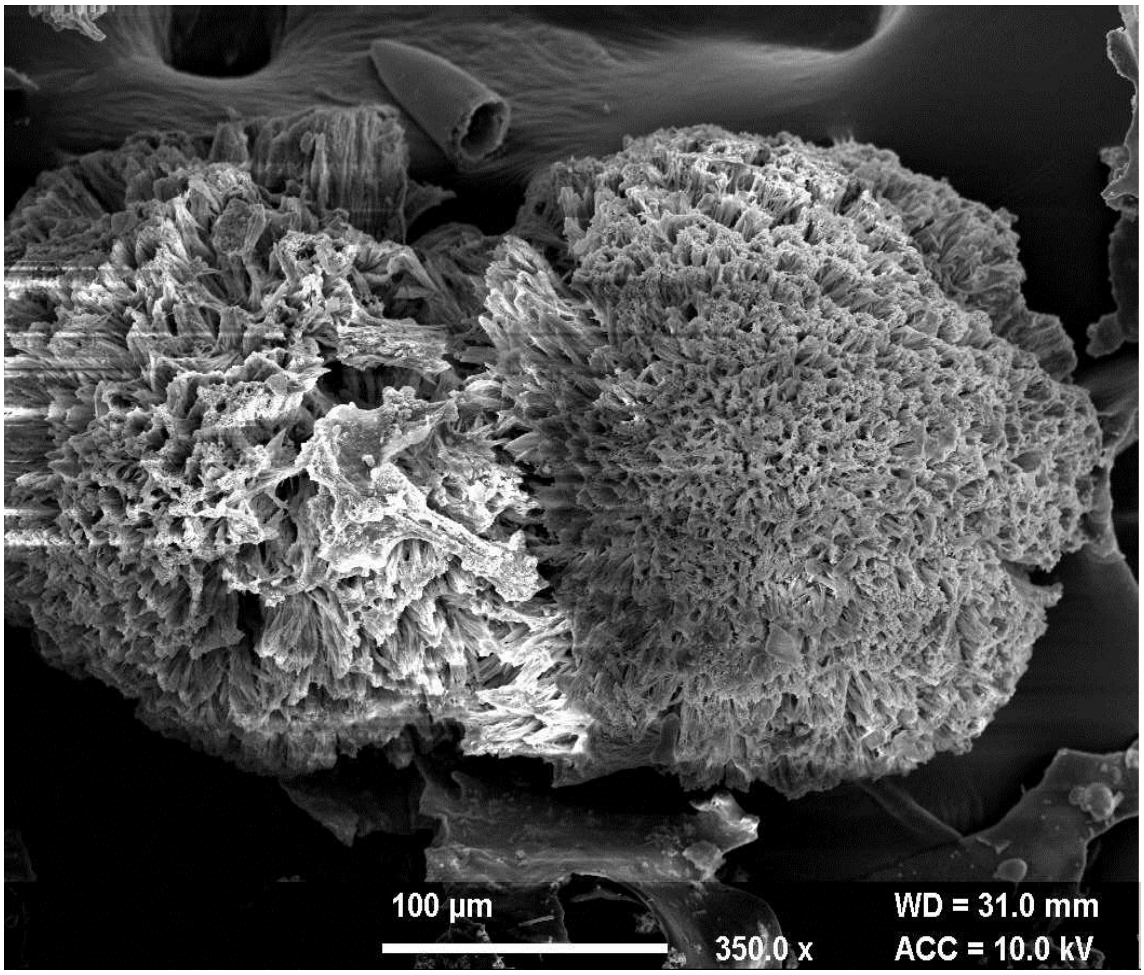


Fig 4.12 Scanning Electron Micrograph of the polymeric flocculant produced in vegetable media by the bacterial culture:

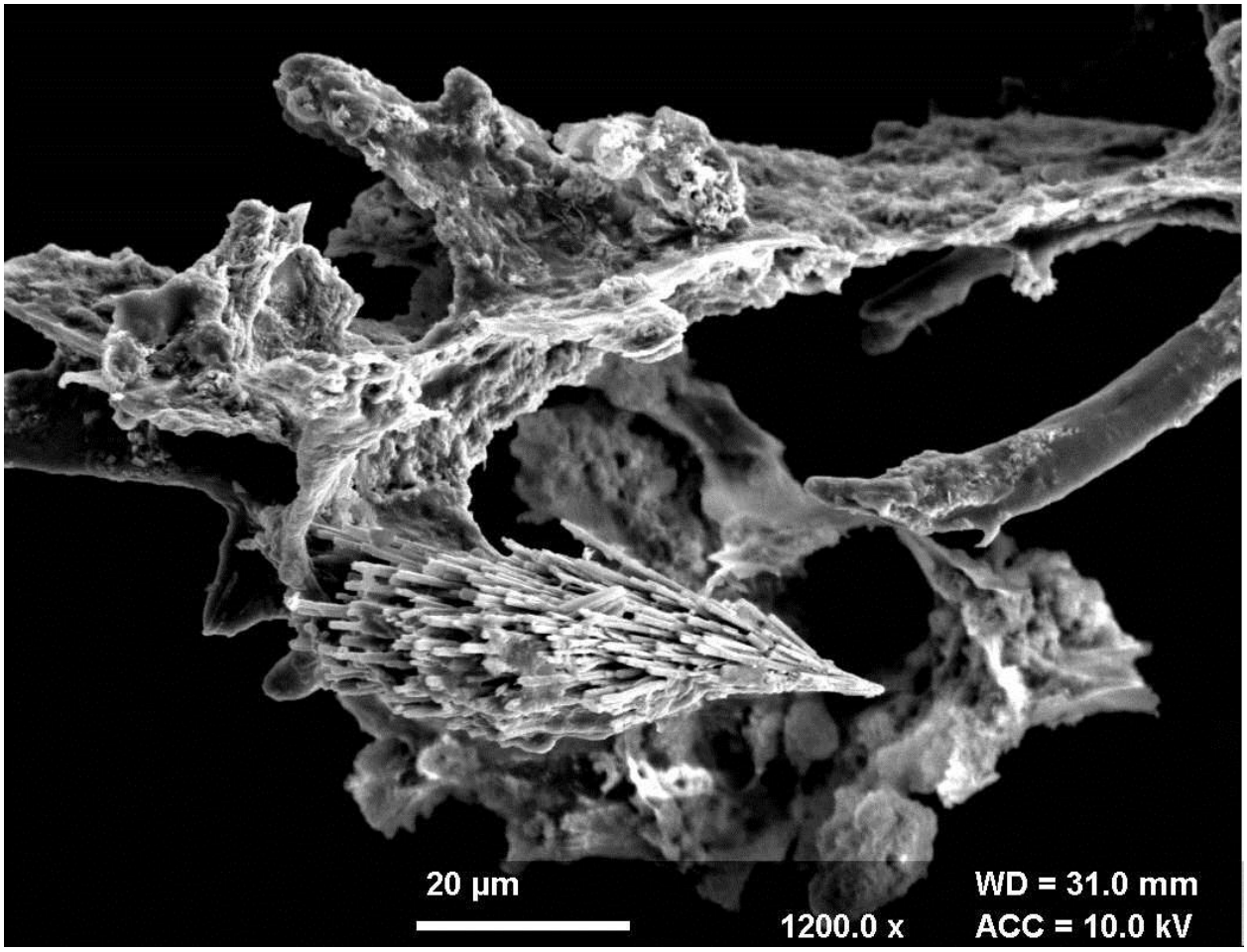


Fig 4.13 Scanning Electron Micrograph of the purified polymeric flocculant produced in reference media by the bacterial strain

The Scanning Electron Micrograph indicated rod shaped structure of the polymeric flocculant, spherical and long fibers like particles were also observed. Single spheres of diameter less than 0.2 µm were present on the surface along with needle shaped structures. Both SEM and FTIR data suggested very close resemblance of the biopolymer produced in vegetable media with that produced by the same culture in reference media, suggesting the applicability of the formulated vegetable media for both lipase and biopolymer production by the selected bacterial strain.

CHAPTER 5
CONCLUSION

The present study explicitly demonstrated an approach for fermentative production of lipase from a extracellular polymer producing bacterial strain in a media formulated from cheap agro resources. The basis of selection for the bacterial strain was its ability to produce high lipolytic activity from amongst 20 bacterial strains from culture repository with properties mentioned.Lipase production was predominantly extracellular and bore strong resemblance to other microbial lipases in terms of pH,temperature optimas.A thorough Optimisation of the media components revealed the best suited carbon source was olive oil, nitrogen source was yeast extract, pH of 6.5 and 30°C temperature for maximum the production of lipase. To rationalize the optimisation, RSM was used, results revealed the model as significant by enabling best lipase activity at concentrations of 0.4% of carbon source and 1% of nitrogen source. These parameters were duely validated experimentally and the parameters were also instrumental in an enhanced yield of the biopolymeric flocculant simultaneously. Typical yields of 214mg/L was obtained in comparison to 197mg/L observed in reference media. The polymeric flocculant, possessed desirable structural and functional attributes as evident from results of FTIR and SEM.

The presence of carboxyl, hydroxyl and other functional groups, amorphous nature and porous structure of the polymer are important considerations for biopolymeric flocculants to retain and bind water. Flocculation activity was notable over a range of colloid particles. The vegetable media formulated from onion, corn and garlic, was found to be satisfactory in terms of characteristics and storage with desirable C:N ratio, crucial for supporting optimal biochemical activities. A cost wise analysis indicated substantial economic benefits of the formulated media compared to fermentative production in reference media. Although detailed

investigations of the lipase produced including novel functionalities, if any, could not be ascertained in this study, further analysis of the lipase should yield important insights on its applicability especially in treating waste water effluents from oil producing facilities. Moreover the polymeric flocculant could be also used for such treatment processes. An important finding of this study was development of an economical method for simultaneously producing two bioproducts, both of considerable value in environmental and other applications.

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APPENDIX 1

1. Basal medium

- NaCl 2 g
- NH_4SO_4 5 g
- $CaCl_2.2H_2O$ 0.5 g
- Yeast extract 0.005 g
- Gum arabic 3 g
- Olive oil 3 ml
- Distilled water 100 ml

2. Nutrient broth

- Peptone 5g
- Distilled water 100ml

3) Vegetable medium

- Corn powder 1g
- Garlic powder 1g
- Onion powder 2g
- Distilled water 1litre
- pH 6.5

APPENDIX 2

Determination of total cell protein by modified Lowry's method

1) Lowry's reagent

Solution A - 20g in Na_2CO_3 in 260ml H_2O

- 0.4g $CuSO_4.5H_2O$ in 20ml H_2O
- 0.2g sodium potassium tartrate in 20ml H_2O

Solution B - 10g SDS in 100ml H_2O

Solution C - 1N NaOH solution

Note: Lowrys reagent is prepared by mixing solution A,B and C

2) Folin's reagent

Folins reagent is freshly prepared before analysis by dluting with distilled water in the ratio of 10:1 (0.2N).

3) Standard curve of BSA protein

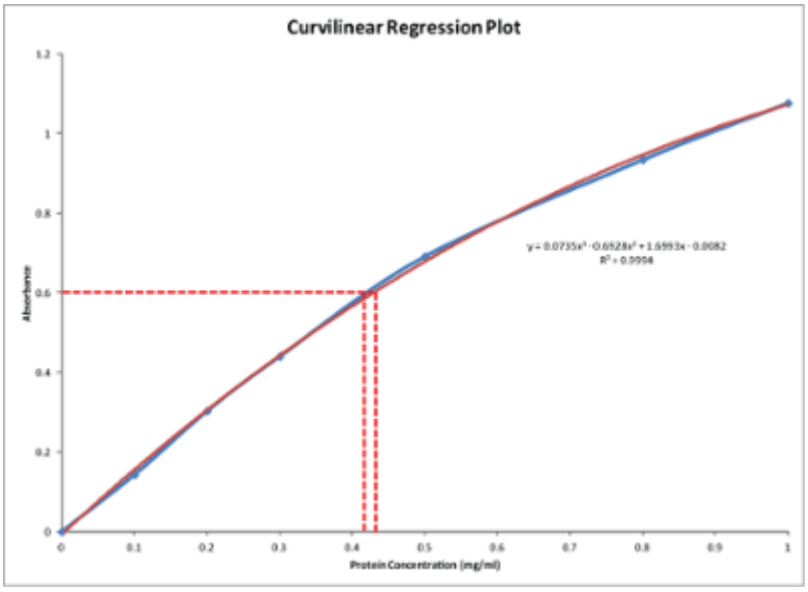

Fig 4.14 standard curve for protein estimation

3) DNSA method for sugar estimation

- Distilled water - 10ml
- DNSA - 3ml
- Standard Glucose solution
- Sodium potassium tartarate - 30 g
- Sodium hydroxide 2N NaOH - 20ml

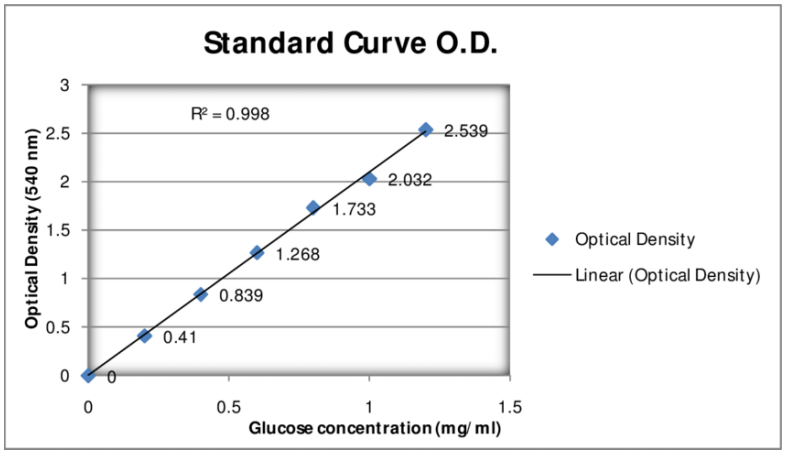

Fig 4.15 standard curve for sugar estimation