ISOLATION AND CHARACTERIZATION OF BACTERIA FOR LIPASE PRODUCTION AND ITS BIOTECHNOLOGICAL APPLICATION

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CERTIFICATE

This is to certify that the research work embodied in this thesis entitled "Isolation and characterization of bacteria for lipase production and its biotechnological application" submitted by Lovely Aktar was carried out under my supervision in the Laboratory of Microbiology, Department of Botany, University of Dhaka. This is further to certify that the research work presented here is original work and suitable for the fulfillment of the Degree of Doctor of Philosophy. To the best of my knowledge this thesis has not been submitted elsewhere.

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DECLARATION

I, Lovely Aktar declare that the research work embodied in this thesis entitled “Isolation and characterization of bacteria for lipase production and its biotechnological application” submitted by me for the Degree of Doctor of Philosophy is the record of work carried out by me during the time from 16 November, 2014 to 15 November, 2018 under the guidance of Dr. Mihir Lal Saha, Professor, Department of Botany, University of Dhaka and has not been submitted elsewhere.

Lovely Aktar

Dedicated

To

My Father

Acknowledgement

All praises belong to the Almighty Allah, who is the most benevolent and merciful, for successful completion of the work.

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-Author

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List of Symbols & Abbreviations and their details

Symbols	Details
&	and
α	alpha
%	Percent
$	Dollar
λ	Gama
ω	Omega
<	Less than
°C	degree Celsius
J.	Journal
U	Unit
p	Page
pp	Pages
M	Molar
N	Normal
d	day
h	Hour
min	Minute
sec	Second
L or l	Litre
g	Gram
mg	Milligram
ml	Millilitre
mm	Millimeter
µ	Micron
µg	Microgram
µl	Microlitre
ng	Nanogram
µm	Micrometer
nm	Nanometer

Symbols	Details
µM	Micromole
kb	Kilobyte
mA	Milliampere
Ed.	Editor
ed.	Edition
et al.	et alibi (with others)
etc.	Etcetra
Fig.	Figure
i.e.	Id est (that is)
M. R.	Methyl Red
No.	Number
NaCl	Sodium Chloride
pH	Negative logarithm of hydrogen ion concentration
sp.	Species (singular)
spp.	Species (plural)
Univ.	University
UV	Ultra Violet
Viz.	Videli (namely)
e.g.	For example
Vol.	Volume
cfu	Colony forming unit
v/v	Volume/volume
w/v	Weight/volume
(-)ve	Negative
(+)ve	Positive
max	Maximum
OD	Optical Density
Co.	Company
Ltd.	Limited
rpm	Rotation Per Minute
abs	Absorbance

Abstract

Lipase is one of the important enzymes can be easily obtained from bacteria and can be used in different important sectors. Water pollution is a big burning issue all over the world. Lipid is an important component of domestic wastes that causes severe environmental pollution. Wastewater with lipid often caused major problems in biological wastewater treatment process. Different microbes producing lipases are used for this wastewater remediation process. The present study was undertaken to isolate potential indigenous lipase producing bacteria from the lipid-rich environment. The isolated bacteria were tested for desired lipase production along with their biotechnological applications. The aerobic heterotrophic bacterial load of the samples ranged in between 4.6×10^3 and 1.37×10^7, 1.65×10^3 and 1.25×10^7, 1.28×10^3 and 1.56×10^7, 8.3×10^2 and 2.64×10^5 cfu/g or cfu/ml in NA, PYG, TBA and LB media, respectively. The highest bacterial count (1.56×10^7 cfu/g) was observed in soil of Dairy Farm from Savar and lowest (8.3×10^2 cfu/ml) in water of The Buriganga River. The highest percentage (94.51%) was found in soil of Edible Oil Mill and the lowest (23.44%) in water of The Buriganga River.

Desired indigenous lipase producing bacteria were isolated from soil and water of lipid-rich environment. On the basis of lipase activity, 30 isolates showed better lipase activity among the total isolates. Result showed that both Gram positive and Gram negative lipase positive bacteria were found to be associated with studied samples. Among them *Bacillus*, *Staphylococcus*, *Micrococcus* and *Planococcus* were Gram positive while *Acinetobacter*, *Acetobacter*, *Pseudomonas*, *Alcaligenes* and *Serratia* were Gram negative bacteria found to be associated in the lipid-rich environments. Qualitatively lipase activity was measured as zone ratio on TBA medium and the zone ratio was found to be ranged in 1.55 – 4.08. Among them 10 better isolates were selected for molecular identification and quantitative lipase production. Among 10 isolates 9 were matched with their conventional identification. Conventionally identified *Acetobacter liquifaciens* was found to be as *Stenotrophomonas maltophilia* e-a22 in case of molecular identification. Among 10 isolates, *Stenotrophomonas maltophilia* e-a22 showed the highest lipase activity (26.89 U/ml) at 72 h of incubation before optimization. Three isolates could produce lipase more than 10 U/ml among the selected isolates. These three isolates *viz*. *Stenotrophomonas*

maltophilia e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B were optimized for lipase production. Before optimization these isolates could produce enzyme 26.89, 10.89 and 13.50 U/ml, respectively. Through optimization, enzyme production increased up to 136.47, 96.33 and 84.30 U/ml by the *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B, respectively. Among the indigenous bacterial isolates *Stenotrophomonas maltophilia* e-a22 found to be potential for lipase production, which could produce a considerable amount of lipase (136.47 U/ml).

Bacterial growth was deterimend by viable cell count using serial dilution technique. After incubation it was found that the highest growth (6.7×10^7 cfu/ml) was obtained at 72 h of incubation in case of *Stenotrophomonas maltophilia* e-a22 and the cell number decreased over this time of cultivation. The highest lipase activity was found in late exponential phase (72 h). *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B showed the highest growth (3.7×10^7 and 6.9×10^7cfu/ml) at 84 h of incubation and cell number decreased over this time. The result clearly reflected that the lipase activities were the highest in exponential growth phase at 36 h of incubation.

These three bacterial isolates and their consortium were used for biotechnological application with special reference to synthetic wastewater treatment. The initial COD value of synthetic wastewater was 1,200 mg/L. Among three indigenous bacterial isolates *Stenotrophomonas maltophilia* e-a22 showed better COD removal performance (74.75%) in case of monoculture. As per speculation consortium of three isolates showed better COD removal than that of monoculture and a maximum of 83.33% COD reduction was achieved by the used consortium. Therefore, it could be concluded that consortium of the isolated three indigenous isolates could be useful for lipid-rich wastewater treatment as a seeding material with special reference to lipid-rich wastewater. Biotechnologically this indigenous consortium of *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B could be useful and play an important role in the water pollution management in Bangladesh.

Chapter-1

Introduction

Introduction

Man has been using enzymes for ages, in different forms, as extracts obtained from vegetables or animal organs or as microbes. The history of modern enzyme technology can be dated from the late 19th century when the Danish chemist Christian Hansen produced rennet by extracting dried calves' stomachs with saline solution (Sangeetha *et al*. 2011). This was followed by extensive research on the resources and applications of enzymes. The industrial biotechnology sector experienced a major breakthrough when it was understood that enzymes could be exploited commercially. A large number of enzymes are being produced and sold for various purposes and the blooming industrial enzyme market is one of the major revenue generators in the life sciences-industry sector. Global Industry Analysts Inc. has reported a comprehensive analysis on worldwide market for industrial enzymes. According to this global strategic business report, the world market for enzymes would exceed $2.9 billion by 2012 and the major product segments include carbohydrases, proteases and lipases (www.reportlinker.com/World-Industrial-Enzymes-Market).

1.1 Enzymes

Enzymes are biological catalysts that make easy the conversion of substrates into products by lowering the activation energy of the reaction. Some enzymes can make their conversion of substrate to product occur many millions of times faster. Enzymes are generally globular proteins, acting alone or in larger complexes. Enzymes must bind their substrates before they can catalyze any chemical reaction. Enzymes are usually very specific as to what substrates they bind and then the chemical reaction catalyzed. Enzymes serve a wide variety of functions inside living organisms. Enzymes are used in the chemical industry and other industrial applications when extremely specific catalysts are required.

The global industrial enzymes market is driven by three leading categories: carbohydrases, proteases and lipases:

– Carbohydrases segment accounts for almost 50% of the industrial enzymes market, up to 70% for the food and beverages application;
– Proteases represent between 25 and 30% of the global market;

– Lipases account for 10% market share.

Other categories include oxidases (e.g. glucose oxidase), phytase, transglutaminase and other enzymes, which are most of the time associated with specific applications and markets combinations (Guerrand 2017).

1.2 Lipases

1.2.1 Introduction to lipase

A lipase is an enzyme that catalyzes the hydrolysis of fats or long chain triglycerides. Lipases are subclass of the esterases. Based on their properties, lipases are widely used at industrial scale with applications as additives in detergents. Pollution generated by residues with fat content such as olive mill wastes is an important problem and novel solutions for their management are needed. Lipase could play a very positive role towards this waste management in relation to fats and oils.

1.2.2 Historical background of lipase

In 1856, Claude Bernard first discovered a lipase in pancreatic juice as an enzyme that hydrolyzed insoluble oil droplets and converted them to soluble products. Lipases have traditionally been obtained from animal pancreas and are used as a digestive aid for human consumption either in crude mixture with other hydrolases (pancreatin) or as a purified grade. Initial interest in microbial lipases was generated because of a shortage of pancreas and difficulties in collecting available material (Sangeetha *et al*. 2011).

The presence of lipases has been observed as early as in 1901 in *Bacillus prodigiosus*, *B. pyocyaneus* and *B. fluorescens* by the microbiologist Sir Eijkman, which represents today's best studied lipase producing bacteria now named *Serratia marcescens*, *Pseudomonas aeruginosa* and *P. fluorescens*, respectively. Enzymes hydrolyzing triglycerides have been studied for well over 300 years and the ability of the lipases to catalyze the hydrolysis and also the synthesis of esters has been recognized nearly 70 years ago (Hossain *et al.* 2010).

Lipases differ greatly both in their origins (which can be bacterial, fungal, mammalian, etc.) and their properties. They can catalyze the hydrolysis or synthesis of a wide range of different carboxylic esters and liberate organic acids and glycerol. They all show highly specific activity towards glyceridic substrates (Hasan *et al.* 2006).

1.2.3 Classification of lipase

Lipase can be classified into two categories:

- **Intracellular lipase**

Intracellular lipase occurs in living cells, where they catalyze and regulate reactions of biochemical pathways essential to the existence of the living system.

- **Extracellular lipase**

Extracellular lipase originally defined as enzymes which are external to the cell wall and in contact with the surrounding medium. Most of the industrial enzymes are extracellular and hydrolytic used for the depolymerization of natural substrates with high molecular mass.

1.2.4 Lipase as biocatalyst

Lipase (triacylglycerol acylhydrolase, EC 3.1.1.3) catalyzes the hydrolysis of the carboxyl ester bonds in triacylglycerols to produce fatty acids and glycerol under aqueous conditions (Kaur *et al*. 2016) (Fig. 1.1).

Lipases are acylhydrolases and water-soluble enzymes that play a key role in fat digestion by cleaving long-chain triglycerides into polar lipids. Because of an opposite polarity between the enzyme (hydrophilic) and their substrates (lipophilic), lipase reaction occurs at the interface between the aqueous and the oil phases (Reis *et al*. 2008). Under the controlled conditions, lipases catalyze a wide range of reactions which are reversible (Joseph *et al*. 2008).

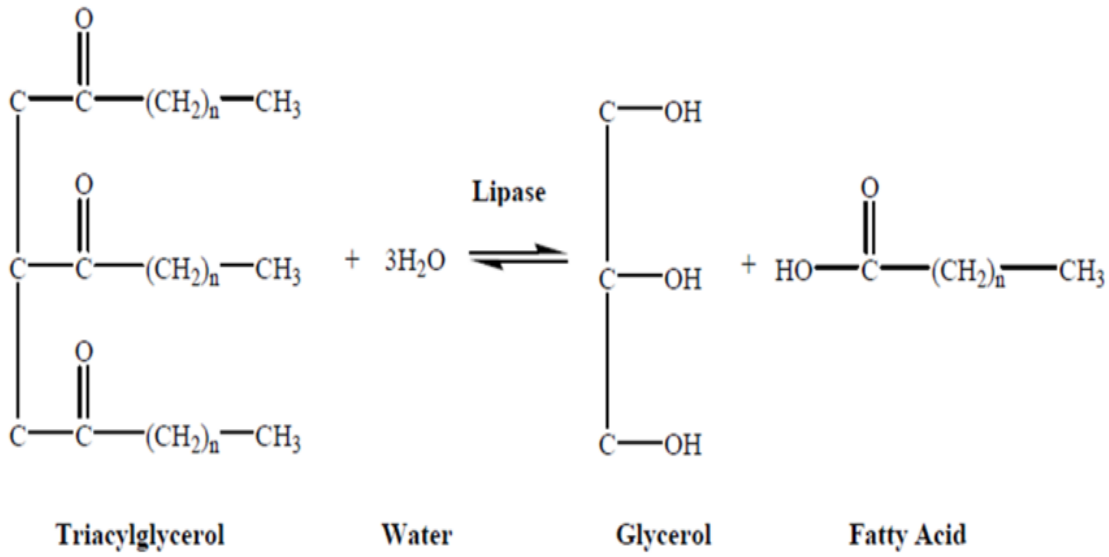


Fig. 1.1: Hydrolysis or synthesis of triacylglycerol substrate catalyzed by lipase.

For the past few decades, lipases have gained much attention due to their versatile activities toward extreme temperature, pH, organic solvents and chemo-, regio- and enantioselectivities (Priji *et al.* 2014*).* Lipases constitute the third most important category of enzymes, next to carbohydrases and proteases (Kavitha 2016). Biotechnological potential of lipases is relying on their ability to catalyze not only the hydrolysis of a given triglyceride, but also its synthesis from glycerol and fatty acids. Lipase catalyzed reactions are widely used in the manufacturing fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture, production of cosmetics, industrial waste management etc. Lipases are also used for accelerate the degradation of fatty wastes and polyurethane (Jager *et al*. 1999).

1.2.5 Properties of lipases

The number of available lipases has increased since the 1980s and used as industrial biocatalysts because of their properties like bio-degradability, high specificity and high catalytic efficiency. Some unique properties of lipases such as their specificity, temperature, pH dependency, activity in organic solvents and nontoxic nature leads to their major contribution in various industries (Anduallema and Gessesse 2012). Lipases from different sources have investigated for their hydrolytic as well as synthetic activity. The most desired characteristics of the lipase are its ability to utilize all mono-, di-, and tri-glycerides as well as the free fatty acids in transesterification,

low product inhibition, high activity in non-aqueous media, low reaction time, resistance to altered temperature, pH, alcohol and reusability of immobilized enzyme (Verma and Kanwar 2008). Additionally, lipases can carry out reactions under mild conditions of pH and temperature and this reduces energy needs to direct reactions at unusual temperatures and pressures. As a result, unstable reactants and products are protected from destruction (Jaeger and Reetz 1998). Generally, bacterial lipases have neutral or alkaline pH value and show activity in a broad pH range (pH 4-11). The thermal stability of lipases ranging from 20º C to 60º C. Bacterial lipases influenced by nutritional and physico-chemical factors; such as temperature, pH, nitrogen and carbon sources, presence of lipids, inorganic salts, agitation and dissolved oxygen concentration (Rosenau and Jaeger 2000). The major factor for the expression of lipase enzyme is carbon source since lipases are inducible enzymes. Lipases generally produced in the presence of lipid source such as oil, triacylglycerols, fatty acids, hydrolyzable esters, tweens and glycerols addition to carbon source. The type of nitrogen source is also influence the production of lipases. Generally, organic nitrogen source is preferred by bacteria, such as peptone and yeast extract (Gupta *et al*. 2004). Growth conditions also affect the synthesis of lipase by microorganisms.

Usually enzymes are not stable in organic solvents and they are tending to denature and lose their activities. But lipases remain stable and active in organic solvents without any stabilizer. Substrates of lipase are often insoluble or partially soluble in water and thus the use of organic solvents or organic-aqueous solutions is in favor of some reactions (Zhao *et al*. 2008) because of its ability to catalyze in organic solvents, many new biotechnological applications of lipases have been identified. One of the applications is the synthesis of important drugs and drug intermediates (Singh and Banerjee 2007). Substrates and products of lipase-catalyzed mechanisms are often insoluble in aqueous solution. Reactions catalyzed by lipases are carried out in organic-aqueous interface. That process is desirable because the separation of enzyme from substrates or products is easy (Rahman *et al*. 2005).

1.3 Microbial lipases

Lipases are currently given much attention with the rapid development of enzyme technology. Lipases are present in microorganisms, plants and animals. Compared to plants and animals, microorganisms have been found to produce high yields of lipases within a short period of time (Mendes *et al*. 2010). Microbial sources of lipase were explored when the industrial potential of lipases enhanced and when the demand for lipases could not be met by the supply from animal sources (Sangeetha *et al*. 2011). This is because their production, commercialization and application at industrial scale is more simple than animal and plant ones (Antczak *et al*. 2009). Microbial lipases have different enzymological properties and substrate specificities (Liu *et al*. 2009). Microbial enzymes are also more stable than their corresponding plant and animal enzymes and their production is more convenient and safer (Hasan *et al.* 2006).

For the past few decades, the world-wide production and consumption of microbial lipases have increased considerably owing to their fascinating industrial applications (Priji *et al*. 2014). Microbial lipases are high in demand due to their specificity of reaction, less energy consumption, hydrolytic and synthetic activities, high yields, ease of genetic manipulation, coupled with exponential growth of the producing microbes in inexpensive media and absence of seasonal fluctuations (Benjamin and Pandey 1996, Kumar *et al*. 2012a). Moreover, most of the microbial lipases do not require cofactors for their activation and they exhibit broad range of substrate specificity and high enantioselectivity (Kirk *et al.* 2002). The potential for the production of lipases enable these microbes to utilize the non-conventional carbon sources such as lipids that cannot directly pass through the cell membrane and have to hydrolyze partially to release free fatty acids prior to the cellular uptake (Najjar *et al.* 2011).

Many microbial lipases have been commercially available in free or immobilized form. Numerous species of bacteria (*Bacillus*, *Pseudomonas*, and *Burkholderia*), yeasts (*Candida rugosa*, *Yarrowia lipolytica* and *Candida antarctica*) and molds (*Aspergillus* and *Trichoderma viride*) produce lipases with different enzymological properties and specificities (Sharma *et al*. 2017). Of all these, bacterial lipases are more economical and stable (Sagar *et al.* 2013). Lipases from microbial origin,

mainly bacterial and fungal are most commonly used in biotechnological applications due to easy extraction and easy cultivation of microbes (Ulker and Karaoglu 2012).

According to substrate specificity microbial lipases divided into three categories; non-specific, regio-specific and fatty acid-specific. Non-specific lipases behave randomly on the triacyglyceride molecule and produce complete breakdown of triacyglyceride to fatty acid and glycerol. Conversely, regio-specific lipases are 1, 3-specific lipases which hydrolyze only primary ester bonds (ester bonds at atoms C1 and C3 of glycerol) and thus hydrolyze triacylglyceride to give fatty acids. Fatty acid-specific lipases display activity in fatty acid presence (Thomson *et al*. 1999, Gupta *et al*. 2004).

1.4 Bacterial lipases

Bacterial lipases are important for applications in various industries because of ecofriendly, non-toxic and no harmful residues. Bacterial lipases were first observed in the year 1901 in the strains of *Serratia marescens* and *Pseudomonas aeruginosa* (Hasan *et al*. 2006). Ever since that lipase production by many different bacterial species has been extensively studied and reported. Among different bacteria, *Bacillus* sp. and *Pseudomonas* sp. constitute a major source of lipase enzyme (Chakraborty and Raj 2008).

Lipases are an important class of enzymes that help microorganisms to derive energy from triacylglycerols. It can be extracellular or intracellular in nature. Extracellular and intracellular nature of lipase varies from organism to organism, however, majority of the bacteria have been found to secrete extracellular lipases (Saxena *et al.* 2003). Cost of lipase production process was considered as a major obstacle in the industries. Therefore, many efforts are being made to use wastes as raw materials for lipase production (Fotouh *et al.* 2016). Lipases from bacterial sources are commercially more significant since they have a lower cost of production, higher stability and availability than other sources and their bulk production is much easier (Kiran *et al*. 2008).

Lipase producing bacteria have been found in diverse habitats such as industrial wastes, oil processing factories, dairies, slaughterhouse, soil contaminated with oil,

decaying foods etc. (Mobarak-Qamsari *et al.* 2011). A variety of extracellular lipases of bacterial origin with different properties and specificities have been described and characterized. Extracellular lipase was isolated from many different bacterial species, including *Bacillus, Pseudomonas, Acinetobacter, Serratia, Burkholderia* etc. (Svendsen 2000). Particular attention is focused on specific classes of enzymes of species *Pseudomonas* that are among the first studied and used in biotechnological. Enzymes of *P. aeruginosa, P. cepacia* and *P. fluorescens* obtained in industrial conditions and are used in organic synthesis, including catalysis of reactions in aqueous solutions (Karadzic *et al*. 2006). A large number of *Pseudomonas* species have been studied for finding attractive and effective lipase application in different fields (Mobarak-Qamsari *et al.* 2011). The majority of the strains of *Pseudomonas* sp. are producers of lipase and phospholipase-C20 (Veerapagu *et al.* 2013).

Many bacterial species produce lipases which hydrolyze esters of glycerol with long-chain fatty acids. They act at the interface generated by a hydrophobic lipid substrate in a hydrophilic aqueous medium. Lipases shows interfacial activation, a sharp increase in lipase activity observed when the substrate starts to form an emulsion. As a consequence, the kinetics of a lipase reaction does not follow the classical Michaelis-Menten model. With only a few exceptions, bacterial lipases are able to completely hydrolyze a triacylglycerol substrate although an ester bonds are more favorable (Jaeger *et al*. 1994).

Bacterial lipases produced form the genera *Burkholderia* and *Pseudomonas* are commercially available. Lipase PS isolated from *Burkholderia cepacia* and Lipase AK isolated from *P. fluorescens* are supplied by Amano and Lipase SL and Lipase TL isolated from *B. cepacia* and *P. stutzeri* are supplied by Meito Sangyo (Japan) (Sangeetha *et al*. 2011).

1.4.1 Estimation of bacterial lipase activity

Several methods have been developed for the estimation of lipase activity in crude or purified lipase preparations.

1.4.1.1 Qualitative assay

A simple and reliable method for detecting lipase activity in bacteria is adding surfactant tween 80 (Fig. 1.2) as a lipidic substrate into the growth medium which was used by Schoofs *et al*. (1997). The formation of opaque zone around the colonies is an indication of lipase producing organisms. Screening of lipase producers on agar plates is frequently done by using tributyrin (Fig. 1.3) as a substrate (Cardenas *et al.* 2001) and clear zones around the colonies indicate production of lipase. Rhodamine B (Fig. 1.4) plates which indicate lipolysis by the formation of fluorescent orange halos are widely used (Kim *et al*. 2001).

1.4.1.2 Quantitative assay

- **Titrimetric method**

Titrimetric method for assaying lipase is popular, the oldest and sensitivity in many applications. A successful measurement of lipase activity by titration requires stable emulsions and absolute control of pH during hydrolysis. Titration methods for the determination of the lipase activity are wide spread by using its tributyrin or olive oil as a substrate. Reaction starts by addition of a lipase solution. Liberated fatty acids are continuously titrated with suitable bases (Sadasivam and Manickam 1996). Titrimetric methods are time consuming process than others.

- **Spectrophotometric method**

Spectrophotometric lipase assay is other important assay. P-nitrophenyl esters with aliphatic acyl chains of various lengths commonly used for investigating lipase activity. Hydrolysis of the substrate by lipase releases p-nitrophenol, which can be measured spectrophotometrically at 410 nm. Enzyme activity is expressed as μmoles of p-nitrophenol released per minute (Thomson *et al*. 1999).

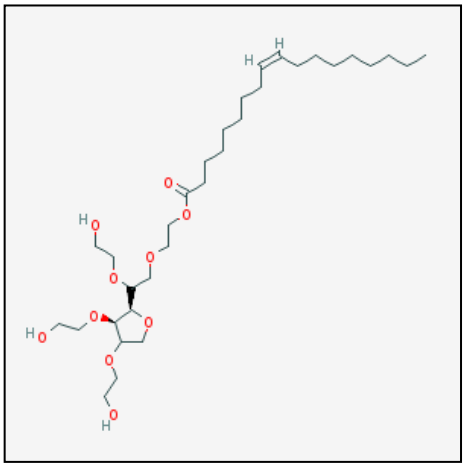


Fig. 1.2: Chemical structure of tween 80.

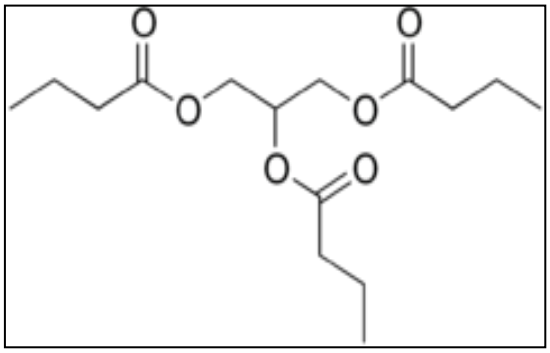


Fig. 1.3: Chemical structure of tributyrin.

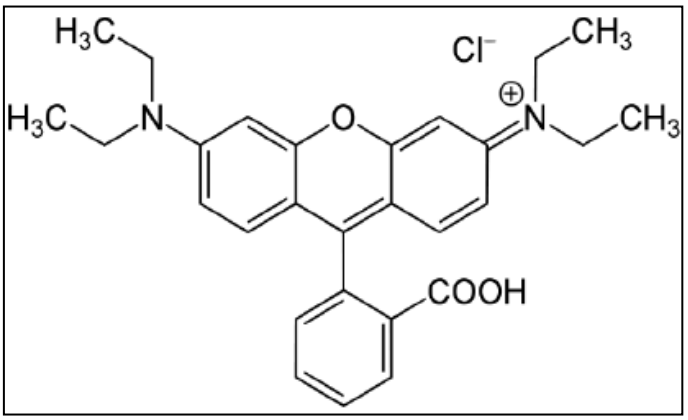


Fig. 1.4: Chemical structure of rhodamine B.

- **Colorimetric method**

The colorimetric methods are based on measuring the complexes formed between the released free fatty acids and a divalent metal ion, usually copper. Fatty acids complex with copper to form cupric salts or soaps that absorb light in the visible range (λmax 715 nm), yielding a blue color. Quantification of fatty acid released by lipase is determined by reference to a standard curve prepared using oleic acid (Marseno *et al*. 1998).

1.4.2 Applications of bacterial lipase

In the industrial and analytical fields biotechnology bacterial lipase is considered as a useful alternative to conventional process technology because of biological systems can accomplish complex chemical conversions with high specificity and efficiency unlike the chemical catalysis. Moreover biological systems help in ingredient substitution, less undesirable products, increased plant capacity, increased product yields and at the same time they are less energy intensive and less polluting. The variety of chemical transformations catalyzed by biocatalysts i.e. enzymes, which are now a prime target of exploitation by the emerging biotechnology based industries. Diverse industrial applications of lipases are overviewed in Table 1.1.

Table 1.1: Overview of industrial applications of bacterial lipases (Patil *et al*. 2011).

Industry	Action	Nature of Application
Dairy food	Hydrolysis of milk, fat, cheese ripening, modification of butter fat	Development of flavoring agent in milk cheese and butter
Bakery food	Flavor improvement	Shelf life prolongation
Beverages	Improved aroma	Alcoholic beverages e.g., sake wine
Food dressing	Quality improvement	Mayonnaise dressing and whippings
Health food	Transesterification	Health foods
Meat and fish	Flavor development	Meat and fish product fat removal
Laundry	Reducing biodegradable strains	Cleaning cloths
Cosmetics	Esterification	Skin and sun-tan creams, bath oil etc.
Surfactants	Replaces phospholipases in the production of lysophospholipids	Polyglycerol and carbohydrates fatty acid esters used as industrial detergents and as emulsifiers in food formulation such as sauces and ice creams
Agrochemicals	Esterification	Herbicides such as phenoxypropionate
Pharmaceuticals	Hydrolysis of exopolyester alcohols	Produce various intermediates used in manufacture of medicine
Fuel industries	Transesterification	Biodiesel production
Pollution Control	Hydrolysis and transesterification of oils and grease	To remove hard stains, and hydrolyze oil and greases

In wake of recent advancements in microbiology and biotechnology, lipases have emerged as key enzyme owing to their multifaceted properties which find use in a wide array of industrial applications (Shaini and Jayasree 2016). Bacterial lipases claim a wide variety of industrial applications due to the ease for mass production and versatile specificities. Bacterial lipases are widely diversified in their enzymatic properties and substrate specificity which make them very attractive for industrial applications (Ray 2012). Biotechnological applications of bacterial lipase enzymes are given below-

- **Hydrolysis of oils**

Hydrolysis of oils is usually performed to concentrate the fatty acids present in the oil. Ramani *et al*. (2010) used an immobilized acidic lipase from *Pseudomonas gessardii* for olive oil hydrolysis. The Polyunsaturated Fatty Acids (PUFAs) are simple lipids with two or more double bonds and play diverse physiological roles which contribute to the normal healthy life of human beings. PUFAs belong to two major families namely n-3 (ω3) and n-6 (ω6). These are currently in high demand as they are formulated in nutraceutical and pharmaceutical products. The demand is met by concentrating these PUFAs from algae, fish oil, fish by-products and edible oil (Chakraborty and Vijayan 2010). Purification and concentration of PUFAs can be the best done using lipases and many bacterial lipases have been studied for their efficiency in enhancing PUFA content (Kumar *et al*. 2005, Chakraborty and Raj 2008).

Byun *et al*. (2007) have used a *Pseudomonas* lipase to hydrolyze sardine oil in the presence of emulsifiers and observed a decrease in the level of saturated fatty acids and increase in the levels of mono and poly unsaturated fatty acids after hydrolysis. Yamauchi *et al*. (2005) have purified arachidonic acid using lipases from *Alcaligenes* and *Bacillus cepacia*.

- **Modification of fat and oil**

The health potential of fats and oils depends largely on the distribution pattern of the fatty acid present in them. Tailored vegetable oils with structured triglycerides popularly known as structured lipids are desirable as they contribute health benefits. Many vegetable oils like sunflower, coconut, olive, corn and rice bran oil are rich in

ω6 fatty acids and fish, linseed oil, walnuts and milk are rich in ω3 fatty acids. The ingested ratio of ω6/ω3 needs to be monitored. This ratio is significant and should be balanced between 1 and 4; a high ω6/ω3 ratio may pose health hazards (Griffin 2008). The best strategy to improve the ω6/ω3 ratio is the enzymatic modification method which uses lipase-catalyzed interesterification (Mitra *et al*. 2010).

- **Synthesis of flavor esters**

Flavor compounds that are extracted from plants are too expensive and hence are replaced by flavor esters synthesized using catalysts. Flavor or fragrance materials which include various aliphatic and aromatic compounds share a major market of food additives throughout the world. Flavor esters are low molecular weight compounds synthesized by the esterification of fatty acids, preferably by microbial lipases. Some of the esters synthesized by esterification reactions catalyzed by bacterial lipases are ethyl acetate, ethyl butyrate, ethyl methyl butyrate, ethyl valerate and ethyl caprylate (Dandavate *et al*. 2009, Ahmed *et al*. 2010).

- **Lipolysed milk fat**

The lipolysed milk fat (LMF) is prepared from condensed milk or butter oil using lipases which release free fatty acids and give it a cheesy aroma. The LMF is used in chocolate coatings, artificial flavor additives, margarine etc. The bacterial lipase used to prepare LMF includes those obtained from *Achromobacter* and *Pseudomonas* (Sangeetha *et al*. 2011).

- **Cheese**

Lipases from *Lactobacillus* play a prominent role in manufacture of bacterial ripened cheese like Parmesan and Grana Padano cheese. Mandrich *et al*. (2006) have investigated the role of lipase/esterase from *Alicyclobacillus acidocaldarius* in milk and cheese models. They observed that the recombinant enzyme was more efficient than the native enzyme and could be used in dairy industry to impart flavor or enhance cheese ripening. Esterification produces new esters like ethyl butanoate, ethyl hexanoate which confer characteristic flavors to cheese (Fenster *et al*. 2003).

- **Bread making**

Emulsifiers are additives required as a bread improver. Bread improvers improve bread volume, texture and dough stability. These emulsifiers are detectable in the final baked and marketed loaf and thus find a place in the label. Enzymes on the other hand get denatured during baking and thus provide bread improving functions without appearing on the label. Lipase from *Bacillus subtilis* has been proved to play a role in bread making in a study by Sanchez *et al*. (2002). Bacterial lipase can completely or partially replace the traditional volume improving agents (Moayedallaie *et al*. 2010).

- **Detergents**

The most noteworthy application of hydrolytic lipases is their use in house-hold and laundry detergents. Lipases were developed as detergent enzymes after the successful introduction of proteases in powder and liquid detergents. Lipases serve as detergent additives due to their stability at alkaline pH, solubility in water, tolerance to detergent proteases and surfactants and low substrate specificity (Quax 2006). Genencor International introduced commercial bacterial lipases Lipomax from *Pseudomonas alcaligenes* and Lumafast from *P. mendocina* and Amano Pharmaceutical Co. Ltd. introduced Lipase P from *P. fluorescens* which could be used as detergent enzymes (Gurung *et al*. 2013).

A detergent stable lipase was isolated from *Bacillus cepacia* by Rathi *et al*. (2001). The enzyme was found to meet all the criteria necessary for a detergent additive and exhibited better stability than commercially available Lipolase (Trade name), a detergent stable lipase marketed by Novo Nordisk, Denmark. Suzuki (2001) has patented an alkaline *Pseudomonas* lipase which remains active at low temperatures and has improved wash performance. Wang *et al*. (2009) and Zhang *et al*. (2009) have isolated lipases from *B. cepacia* and *P. fluorescens* and the lipases proved to be suitable for detergent industry.

Bacterial lipase from *Staphylococcus arlettae* JPBW-1 has been assessed for its use in laundry formulations which exhibited good stability towards surfactants and oxidizing agents, and removed about 62 % of olive oil from cotton fabrics (Chauhan *et al.* 2013). Recently, many cold-active lipases have been reported in bacteria such as *Pseudoalteromonas* sp. NJ 70 (Wang *et al.* 2012), *Bacillus sphaericus* (Joseph and

Ramteke 2013), *Microbacterium luteolum* (Joseph *et al.* 2012), *Pichia lynferdii* (Park *et al*. 2013) etc. Most of them showed good tolerance to salt, synthetic surfactants and oxidizing agents.

- **Leather industry**

The different stages of leather processing are curing, soaking, liming, dehairing, bating, pickling, degreasing and tanning. All these stages use many chemicals and enzymes. Lipases are employed in soaking, bating and degreasing stages along with proteases (Choudary *et al*. 2004). Large quantities of natural fat present in the skin, particularly sheep skin cannot be removed by the liming operation. It is initially performed with lipase alone was not satisfactory and hence a combination of protease and lipase was evaluated. La Forestal Tanica has marketed Forezym WG-L, Forezym DG as degreasing bacterial lipases (Hasan *et al.* 2006).

Lipase specifically degrades fat but do not damage the leather which is proteinaceous in nature, it hydrolyses the fat on the outside of the hides and skins as well as inside the skin structure. Thus, lipase assisted treatment of leather gives the leather with far better quality and finish with uniform color and cleaner appearance as compared to conventional chemical agents. It was found that the lipase produced by *Bacillus subtilis* can be used for the degreasing process, thereby removing all the fat within 8-12 h of incubation by maintaining natural skin color (Saran *et al.* 2013). NovoLime, a protease/lipase blend for enzyme-assisted liming of hides and skins, and NovoCor AD, an acid lipase for degreasing of hides and skins, are some of the commercially available lipases for the leather industry.

- **Textile industry**

Textile industry promises a refined and polished look to the finished fabric. Desizing is such a process and is required to remove the size material which has been impregnated from the fabric prior to weaving. Enzymatic desizing has multiple advantages over the traditional process and uses enzymes like cellulase, amylase, protease and lipase depending on the sizing agent (www.expresstextile.com). Bacterial lipases can be used if the size material is a synthetic sizing agent like polyesters (Sangeetha *et al.* 2011). Lund (2001) has patented a process for combined desizing and stone-washing of denim and lipolytic enzymes from *Pseudomonas*

cepacia, *P. fluoresecens*, *P. fragi* and *P. stutzeri* were evaluated and the lipase of *P. cepacia* was preferred.

- **Biosensors**

Qualitative and quantitative determination of lipids and lipid-binding proteins is possible with the help of biosensors which may be of chemical or biochemical in nature. Bacterial lipases have been used as biosensors and this exploits immobilized lipases. Such biosensors are used to detect triglycerides in food and clinical samples, pollution analysis like pesticide contamination and pharmaceutical industry (Pandey *et al*. 1999). Huang *et al*. (2001) immobilized lipase on a micro-emulsion based gel to fabricate a glass-electrode-based lipase biosensor. Setzu *et al*. (2007) fabricated a potentiometric biosensor using a lipase immobilized on a mesoporous silica matrix.

1.5 Water pollution and bacterial lipase

1.5.1 Importance of water

Living organisms are about 70% water. A human being, for instance, has to consume an average 1.5 liters of water per day to survive. Importance of water could be considered under following heads:

- Water is one of the important gifts to mankind.
- Water covers 70.9% of the Earth's surface, and is vital for all known forms of life.
- Water is a resource that has many uses, including recreational, transportation, agricultural, domestic, industrial and commercial uses.
- Pollution of waters often negates the benefits obtained from the development of these water resources.
- Water pollution is a major problem in the global context.
- Pollution can be caused by wide variety of inorganic, organic compounds and microorganisms.
- Some observers have estimated that by 2025 more than half of the world population will be facing water-based vulnerability.

1.5.2 Water pollution

Water pollution is a major global problem. It has been suggested that it is the leading worldwide cause of deaths and diseases, and that it accounts for the deaths of more than 14,000 people daily. Increasing water pollution from accelerating domestic, industrial & agricultural activities is a major issue for nearly all Asian developing countries. Pollution sources can be divided into two:

- Point sources: Refers to contaminants that enter a waterway from a single, identifiable source such as pipe, ditch, factory, sewage plant.
- Non-point sources: Refers to diffuse contamination that does not originate from a single discrete source. Non-point sources (NPS) pollution is often cumulative effect of small amounts of contaminants gathered from a large area.

1.5.3 Prevention of water pollution

Increasing water pollution from accelerating domestic, industrial and agricultural activities is a major issue for nearly all Asian developing countries. Unless the present perceptions and attitudes change radically, it is likely to be a critical water problem of the future. Therefore, we have to stop water pollution by any means. Following are the basic steps to prevent water pollution:

- **Education**: Education of the public and industry about the effects and consequences of pollution can be prevented water pollution.
- **Promotion**: Promotion of good environmental practices can reduce water pollution.
- **Laws**: Environmental laws can make it tougher for people to pollute and hence pollution can be prevented.
- **Wastewater Treatment**: Environment-friendly wastewater treatment strategies can reduce water pollution from domestic and/or industries wastewater. The volume of water being contaminated and the need to reclaim wastewater are both increasing with growth in population and industrial use of water. Treatment of wastewater is essential to prevent the contamination of drinking water and the entry

of contaminants into the food chain. Wastewater originates from four primary sources:

i. Sewage,
ii. Industrial effluents,
iii. Agricultural runoff,
iv. Storm water and urban runoff.

1.5.4 Bacterial lipase and water pollution management

Treating oily wastewater sources is very broad, as the oil in the oil industry, oil refining, oil storage, transportation and petrochemical industries in the production process generate lot of oily wastewater (Chen and He 2003, Machin-Ramirez *et al*. 2008). Therefore, oily wastewater treatment is urgently needed in today's field of environmental problems.

Bioremediation, the use of microorganisms or microbial process to detoxify and degrade the oil effluents is among the innovative technologies. Different microbes producing lipases are used for the oil effluent remediation process (Bhumibhamon *et al.* 2002, Creencia *et al.* 2014). Enzymatic treatment technique has gained more attention because of stringent environmental regulations and friendly applications (Prasad and Manjunath 2011). Lipolytic strains isolated from industrial effluents show potential utility in biodegradation and bioremediation. The biofilm formed by lipase secreting organisms can be used to degrade fats and oils (Gururaj *et al*. 2016). In the field of biotechnology a wide range of enzymes were commercially synthesized from screened and selected microorganisms. These selected microorganisms have been characterized, optimized and purposely designed in order to produce a high-quality enzymes. These enzymes have wide industrial applications for various biological processes.

Lipid-rich wastewater treatment processes remove lipidic residues by air-floatation and discard it in sanitary landfill dumping yard. These pose a threat to the waterbeds and ground water by decreasing the oxygen transfer rate and hence bioremediation is adopted to control pollution (Mongkolthanaruk and Dharmsthiti 2002). Oil spills in the soil and water during rigging and refining can be handled using lipases (Pandey *et al*. 1999). The most common method of treating wastewater is by cultivating pure

cultures which produce lipase and mixed cultures which produce lipase and other enzymes. Effluents emanating from food processing, dairy, tannery, automobile industries, oil mill, restaurant and fast-food outlets generates strong wastewaters characterized by high biological oxygen demand (BOD) and chemical oxygen demand (COD) concentrations representing their high organic content. These effluents consist of wide range of proteins, fats and possibly other additives and can be treated by cultivating lipase producing bacteria (Nelson and Rawson 2010). The commonly used bacterial genera for these wastewater treatment are *Pseudomonas, Bacillus* and *Acinetobacter* (Mongkolthanaruk and Dharmsthiti 2002).

Lipases are utilized in activated sludge and other aerobic waste processes, where thin layers of fats must be continuously removed from the surface of aerated tanks to permit oxygen transport (to maintain living conditions for the biomass). This skimmed fat-rich liquid is digested with lipases (Momsia and Momsia 2013). Biodegradation of petroleum hydrocarbons is a result of indigenous cold-adapted microorganisms able to degrade these contaminants. Some bacteria such as *Pseudomonas putida*, *Acinetobacter* sp. and *Mycobacterium* sp. involve in the degradation of alkanes, aromatic hydrocarbons and polycyclic aromatic hydrocarbons (Gopinath *et al.* 1998). Wang and Zhang (2009) have found that soil microbial lipase activity is a valuable indicator of diesel oil biodegradation.

1.8 Lipid associated problems

Lipid, characterized by fats, oils and grease (FOG) and long chain fatty acids (LCFA) are one of the major organic components of wastewater. Lipid in domestic wastes that causes severe environmental pollution. Lipid-rich wastewater produced from edible oil refinery, slaughter house, wool scouring and dairy products industry contains a high concentration (> 100 mg/l) of lipids (Dimirel *et al*. 2005). Wastewater with lipid often cause major problems in biological wastewater treatment process because it can form oil film on water surface, preventing the diffusion of oxygen from air into water and leading to the death of many forms of aquatic life (Cammarota and Freire 2009). Aggregates formed by the oil droplets and other particles present in wastewater can also block water drainage lines. In addition, fats may solidify at lower temperatures causing operational damage such as clogging, and may develop unpleasant odors

(Masse *et al.* 2001). It causes high COD in wastewater. Lipase showed potential applications in degrading the oil and fats in the wastewater (Mendes *et al*. 2010). Wastewaters are treated using physico-chemical and biological treatment methods. However, since the reagent costs are high and the soluble COD removal is poor in physico-chemical treatment processes, biological processes are usually preferred to other processes (Vidal *et al*. 2000).

Wastewater treatment in developing countries is a major concern and solution has become challenging for various unfavorable conditions. Bangladesh is also facing several water related problems both in the urban and rural region. The condition of water contamination is quite different in urban and rural areas. In urban areas water scarcity is a major concern and mainly surface water is contaminated by the illegal effluent discharge into water bodies. In rural areas relatively more people have accessibility to water sources. However, the condition has been changed by some significant approaches to mitigate this problem.

Insufficiency in wastewater treatment facility is making effluent water harmful for the environment. Most of the untreated effluents discharge to the nearest water bodies. Now a day's water scarcity is very common problem in Bangladesh. According to the department of public health and engineering of Bangladesh, most of the treatment facilities include filtration, flocculation, sedimentation and disinfection. Some also include ion exchange and filtration depending on the quality of collected water.

Ground water depletion is increasing with time. The country requires some urgent solution to eradicate problems regarding wastewater management. Wastewater treatment can make a remarkable change in the wastewater issues in Bangladesh. A number of local and international organizations are trying to mitigate the water related issues (Knappett *et al*. 2012). Discharge of untreated effluent in the surface water is results in pollution. In addition, contamination of the environment with crude oil causing severe pollution. Wide scale production, transport use and disposal of oil globally have made it a major contamination in both prevalence and quantity in the environment. The oil gets mixed with the river or marine water by many ways as accidental spills or discharge of refineries in river or other water bodies. Biological treatment is one of the best options to remove organic material from such wastewater.

Aerobic processes such as activated sludge, rotating biological contactor, trickling filter, and lagoons are suitable for the removal of organic compounds (Leal *et al*. 2006). Biological wastewater treatment would be impossible without microorganisms. Wastewater treatment plants are huge microbiological powerhouses, where microorganisms are responsible for the conversion of compounds and degradation of pollutants. Therefore, using of microorganisms for treatment and bioremediation purposes affords a very efficient tool for purifying contaminated effluents and natural water (Glazer & Nikaido 1995). However, due to economical instability centralized solutions seem hard to implement. Small scale solution could play an active role for developing countries like Bangladesh. As small scale solutions are comparatively economical and less skill required than centralized system. The major problems lie in the establishment of the most suitable microbial population for waste to be treated (Yacob *et al*. 2006). Multiple initiatives have been developed to resolve the problem of wastewater pollution.

1.9 Aims and objectives of the research

The enzyme lipase has wide range of significance, therefore lipases remain a subject of intensive study. Lipases from a large number of bacterial, fungal, plant and animal sources have been purified. In this context indigenous bacteria of lipid-rich environment could be a good reservoir of lipases. Industrial organic toxic waste causes ecology damages for aquatic organisms, plant, animal, and equally, mutagenic and carcinogenic for human being (Lan *et al.* 2009, Islam *et al.* 2013). They discharge from different sources to form a layer on water surface that decreases dissolved oxygen. The FOG layer reduces biological activity of treatment process where oil film formation around microbes in suspended matter and water. This lead to decrease dissolved oxygen levels in the water. Then oxygen molecules are difficulty to be oxidative for microbial on hydrocarbon molecules and cause ecology damages to water bodies (Alade *et al.* 2011, Facchin *et al.* 2013).

As industrial wastes are rich in various components which support the luxurious growth of different bacteria for this reason, lipid-rich environment has chosen for isolation of lipase producing bacteria. Considering the importance and applications of biotechnologically and industrially valuable bacterial enzymes the present study was

aimed to isolate and characterize indigenous lipase producing bacteria from lipid-rich environment for biotechnological application.

Followings were the aims and objectives of the present study:

1. Enumeration and isolation of lipase producing bacteria from different lipid-rich environment in and around Dhaka Metropolitan City.
2. Screening and characterization of lipase positive isolated bacterial isolates.
3. Provisional and molecular identification of selected bacterial isolates.
4. Determination of phylogenetic relationship.
5. Lipase production and estimation of lipase activity of the selected bacterial isolates.
6. Optimization of different physico-chemical parameters on lipase production.
7. Laboratory scale wastewater treatment by the isolated potential bacterial isolates.

Chapter-2

Materials and Methods

Materials and Methods

2.1 Location of sampling

Dhaka is the capital and the largest city of Bangladesh. It is one of the major megacities of South Asia. It is located on the bank of the river Buriganga. Dhaka, along with its Metropolitan area, has an estimated population of over 17 million (2015), making it the largest city in Bangladesh (Wikipedia).

To get a fair idea about the bacterial load in the soil and water of lipid-rich environments in and around Dhaka Metropolitan City, samples were collected from ten different habitats *viz*. The Turag River, Dairy Farm, Kitchen Waste, Tannery Industry, Bus Depot, The Buriganga River, Navana Car Workshop, Navy Dockyard, Edible Oil Mill and Kohinoor Chemicals Co. Ltd.

2.2 Collection of samples

During collection of samples, plastic bags, marker, pen, field notebook were taken to the sampling sites. Lipid-rich soil samples were collected aseptically in the sterilized plastic bags and water samples were collected in the sterilized plastic bottles. A total of 10 soil and water samples were collected. After collection, the samples were labeled properly and brought into the laboratory as soon as possible. Date of collection, sampling sites and types of sample are shown in Table 2.1.

Table 2.1: Date of collection, sampling sites and sample types.

Sample No.	Date	Sampling sites	Sample types
1	08.02.15	The Turag River, Gabtoli, Dhaka	Water
2	16.02.15	Dairy Farm, Savar, Dhaka	Soil
3	22.02.15	Kitchen Waste, Mirpur, Dhaka	Soil
4	09.03.15	Tannery Industry, Hazaribagh, Dhaka	Water
5	31.03.15	Bus Depot, Kalyanpur, Dhaka	Water
6	12.04.15	The Buriganga River, Dhaka	Water
7	30.04.15	Navana Car Workshop, Tejgaon, Dhaka	Water
8	11.05.15	Navy Dockyard, Narayanganj	Water
9	25.05.15	Edible Oil Mill, Elephant road, Dhaka	Soil
10	17.06.15	Kohinoor Chemicals Co. Ltd., Tejgaon, Dhaka	Soil

2.3 Preservation of the samples

Collected samples were preserved in a refrigerator at 4 °C before and after the analysis.

2.4 Culture media and techniques used for the enumeration and isolation of bacteria

2.4.1 Culture media for enumeration and isolation of bacteria

Nutrient Agar (NA), Peptone Yeast Extract Glucose Agar (PYG), Tributyrin Agar (TBA) and Luria Bartani (LB) media were used for the enumeration and isolation of aerobic heterotrophic bacteria present in soil and water samples. Tributyrin Agar (Cardenas *et al.* 2001) and Tween Agar (Schoofs *et al*. 1997) media were two lipid based media to detect lipase producing isolates associated with the collected samples. Tryptone Soya Broth (TSB) (Anbu *et al*. 2011) was used for *in vitro* lipase production. The pH of the media was adjusted to 7.0 before the addition of agar and sterilization.

2.4.2 Techniques employed for enumeration and isolation of bacteria

Serial dilution technique (Clesceri *et al*. 1998) was used for the isolation of bacteria. One gram of soil sample or one ml of water sample was diluted (1:100 or 1:99) with required amount of sterile water in a sterile conical flask and shaken well. This suspension was transferred to 9 ml of sterile water in a test tube for ten-fold (1:10) dilution and further diluted up to 10^5 for the plating in four different media *viz*. NA, PYG, TBA and LB.

One ml of each of the diluted samples was taken in a sterilized petri plate. Then molten agar medium was poured and mixed thoroughly by rotating the petri plate, first in one direction and then in the opposite direction. Plating in duplicated plates was made for each diluted samples. After setting the medium the plates were placed invertedly and incubated at 37 °C for 24 h in an incubator (Memmert GmbH + Co Kg 8540 Sehwabach).

2.5 Enumeration of bacteria

After 48 h of incubation the plates having well discrete colonies were selected for counting. The selected plates placed on colony counter (Digital colony counter, DC-8 OSK 100086, Kayagaki, Japan) and the colonies were counted. Lipase positive bacteria grown in TBA plates were also counted separately.

2.6 Isolation of bacteria

Well discrete desired aerobic heterotrophic bacterial colonies were selected after counting. The selected colonies were isolated on Nutrient Agar slants for further studies.

2.7 Preliminary selection of the isolated bacterial isolates

Preliminary selection of the isolated possible lipase producing bacteria was made on the basis of their distinctive colony morphology.

2.8 Screening of lipase producing bacterial isolates

Tributyrin Agar (TBA) and Tween Agar (TA) media were used to identify and the potentiality of the isolates to produce lipase enzyme.

Bacteria capable of hydrolyzing lipid to form fatty acids and glycerol possess the enzyme lipase. As an extracellular enzyme, lipase diffuses outward from the bacterial cells and break down lipid. This test reveals the presence or absence of the enzyme lipase in the organisms.

For this test, TBA and TA plates were inoculated with the isolated bacteria and the plates were incubated at 37 °C for 72 h.

Formation of a clear zone around the colony in TBA plates and opaque zone around the colony in TA plates indicated lipase positive.

The degree of lipase activity was determined by zone ratio on TBA plates. The isolates forming a clear zone around the colonies were determined by mm scale. The following formula was used to determine the zone ratio.

$$\text{Zone ratio} = \frac{\text{Zone diameter (mm)}}{\text{Colony diameter (mm)}}$$

The lipase activities of isolates were observed by measuring zone ratio and intensity of clear zone in TBA plates and intensity of opaque zone in tween agar plates around the colonies (Bueno *et al*. 2014). The selected bacterial isolates were considered for purification towards further morphological and biochemical tests.

2.9 Purification of the bacterial isolates

Although a few bacteria are so morphologically remarkable as to make them identifiable without isolation, pure cultures are nearly always a necessity before one can attempt identification of an organism. It is important to realize that the single selection of a colony from a petri plate does not assure purity. Testing of pure cultures is essential to the success of any microbial identification system.

After preliminary selection, the selected lipase producing bacterial isolates were purified by streak plate method. When a plate yielded only one type of colony, the organism was considered to be pure.

2.10 Colonial morphology of the bacterial isolates

Bacteria grow on solid media as colonies. A colony is defined as a visible mass of microorganisms all originating from a single mother cell. Key features of these bacterial colonies serve as important criteria for their identification.

The isolated lipase producing bacterial colonies on plating medium were morphologically studied as their shape/form, pigmentation, surface elevation, margin, surface and opacity (Bryan 1950, Eklund and Lankford 1967).

2.11 Maintenance and preservation of bacterial isolates

The purified isolates were then transferred on Nutrient Agar slants. The slants were kept in polythene bags and preserved as stock cultures in refrigerator at 4 °C for further studies. Periodical transfers of isolates on agar slants were done for maintaining viability of the organisms.

2.12 Morphological observation of bacterial isolates

For the identification of selected isolates, following morphological characters were studied and recorded.

2.12.1 Microscopic examination of bacterial isolates

Bacteria are microscopic single-celled prokaryotic organisms. Microscope is an essential tool for bacterial cell observation. Bacterial cell suspension made by using fresh culture with physiological saline. The prepared suspensions were used to make smear on glass slides. A good quality glass slide was used for this purpose. Thin smear was prepared on the clean and oil free slide. The smear was allowed to dry in air and get fixed. Two different staining methods *viz.* (i) Simple staining and (ii) Differential staining were employed to stain the fixed smears.

2.12.1.1 Simple staining

Manual of Microbiological Methods (SAB 1957) was followed for simple staining. Basic dyes *viz.* safranin was used. The fixed smear was flooded with safranin solution for one minute. The flooded smear was washed off with slow stream of tap water and dried in air.

2.12.1.2 Differential staining

Staining procedures that make visible differences between microbial cells or parts of cells termed as differential staining (Pelczar *et al.* 1993). In differential staining process, a combination of dyes is used that take advantage of chemical differences among cells (Claus 1995). The differential staining methods most frequently used are the Gram stain, Acid-fast stain and Spore stain (Tortora *et al*. 1998). For this purpose, fixed smear is exposed to more than one dye solution to differentiate cell and or its parts. In this study, two differential techniques were used *viz.* (i) Gram staining and (ii) Spore staining.

2.12.1.2.1 Gram staining

Gram staining is one of the most important and widely used differential staining techniques which is considered as one of the important steps in identifying an

unknown bacterium. For Gram staining, method described by Claus (1995) was followed. For this purpose, smear was made from 18-24 h old bacterial culture.

Fixed smear was treated with the following solutions and after application of each solution, the slide was gently washed off with slow stream of tap water.

Crystal violet solution 60 sec., Lugol's iodine solution 60 sec., 95% Ethyl alcohol 15 sec., Mercurochrome solution 60 sec. After staining and washing the slide was blotted dry and observed under advanced research microscope (Nikon Microphot, UFX-IIA, Japan).

The results were recorded as Gram positive (blue-violet) and Gram negative (light red).

2.12.1.2.2 Spore staining

The method described by Claus (1995) was applied in spore staining. For this purpose, smear was made from 36-48 h old bacterial culture. The fixed smear was flooded with 5% aqueous solution of malachite green and heated over a brass plate for about 15 minutes taking care that the dye must not be dried off. Excess dye was then washed gently and safranin used as a counter stain for 1 minute. The slide was washed gently, dried and examined under microscope. The shape and position of the spores within sporangia were observed. The swelling nature of the sporangium was also observed and properly recorded.

Spores were stained with green color of malachite green and vegetative cells or sporangia were stained with red color of safranin.

2.12.2 Microscopic observation of the stained bacteria

The size and shape of vegetative cells of selected bacterial isolates were observed. The arrangement of cells whether single or in chains or clusters were carefully recorded. Gram reaction and spore formation of the isolates were also studied and recorded. Photomicrographs of the observed cells were taken using advanced research microscope (Nikon Microphot, UFX-IIA, Japan).

2.13 Physiological and biochemical studies of the bacterial isolates

Few Physiological and biochemical characteristics are used for identification of bacteria e.g. carbohydrate utilization, amino acid degradation, enzyme production (catalase, amylase, protease, nitrate reductase, oxidasae etc.), motility etc. Following Bergey's Manual (Krieg and Holt 1984, Sneath *et al.* 1986), Manual of Microbiological Methods (SAB 1957), Microbiological Methods (Collins and Lyne 1984) and Understanding Microbes (Claus 1995) the major important physiological and biochemical tests in relation to identification of the isolated bacteria were carried out.

2.13.1 Acid production from carbohydrate (Sneath *et al.* 1986)

The selected bacterial isolates were tested for their acid production from different major carbohydrates.

For this purpose, inorganic nitrogen based agar medium was used. Bromocresol purple as an indicator and 1% carbon source (w/v) were added to this medium. D-glucose, D-Xylose, L-arabinose, D-mannitol, D-galactose, D-mannose, L-rhamnose (monosaccharides), sucrose, D-trehalose, D-cellobiose (disaccharides) and D-raffinose (oligosaccharides) were used as carbon sources. All these were sterilized. The medium was poured into the sterilized petri plates and allowed to solidify. Inoculation was done by point inoculation method and the plates were incubated for 48 h at 37 °C.

Acid production from carbohydrate was determined by yellow color formation around the colony.

2.13.2 Gas production from glucose (SAB 1957)

Gas production during fermentation test from glucose is of considerable significance in the identification and classification of bacteria.

Fermentation tubes with glucose were made using bromothymol blue as an indicator. One Durham's tube was introduced in each of the test tubes. Then the tubes were inoculated in duplicates with 24 h old culture suspension with the help of sterilized pipette and incubated at 37 °C for 48 h.

The change of color of the indicator from green to yellow indicated the production of acid. Presence of gas in the Durham's tube indicated the positive result. No gas indicates negative result.

2.13.3 Potassium hydroxide solubility test (Schaad 1988)

Potassium hydroxide solubility test was done with 3% potassium hydroxide (KOH) solution. One to two drops of 3% KOH solution was placed on a clean and dry glass slide. A loop full of 24 h old culture of bacterial cells were transferred and mixed thoroughly with the inoculation loop and the slide was kept for 10 sec.

The organism was considered positive when KOH solution became viscous and showed a slimy thread and it was negative when there was no slime. The organisms showing positive reaction to KOH are supposed to be Gram negative bacteria.

2.13.4 Catalase test (Claus 1995)

Catalase is a common enzyme found in nearly all living organisms exposed to oxygen. It catalyzes the decomposition of hydrogen peroxide to water and oxygen. It is a very important enzyme in protecting the cell from oxidative damage by reactive oxygen species (ROS).

$$2H_2O_2 \longrightarrow 2H_2O + O_2\uparrow$$

To demonstrate catalase activity, a loop full of test organisms were taken by a sterilized loop on a glass slide and a drop of hydrogen peroxide was added to it.

The evolution of bubbles indicated the positive result *i.e.* the organism having the enzyme catalase.

2.13.5 Oxidase test (Claus 1995)

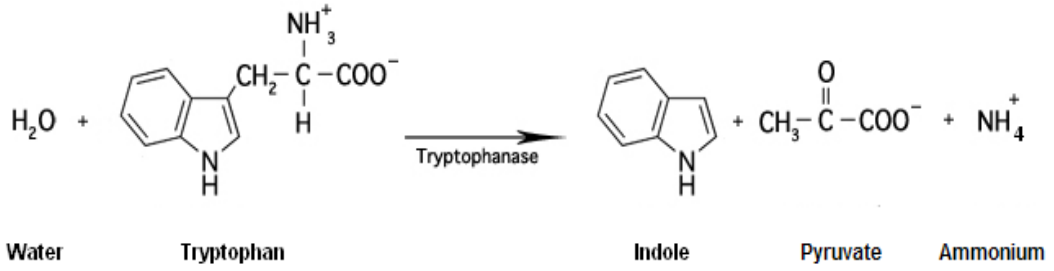
The enzyme oxidase, present in certain bacteria catalyzes the transport of electron from donor bacteria to the redox dye tetra-methyl-para-phenylene-diamine dihydrochloride. The dye in the reduced state has a deep purple color.

To perform this test, filter papers were soaked in 1% aqueous tetramethyl-phenylene-diamine dihydrochloride. Fresh young cultures were then rubbed on the filter paper with a clean glass rod. Results were recorded within 10 seconds.

Blue color indicated positive result.

2.13.6 Production of indole (Atlas 1997)

Indole is generated by reductive deamination from tryptophan via the intermediate molecule indole pyruvic acid. Tryptophanase catalyzes the deamination reaction, during which the amine (-NH_2) group of the tryptophan molecule is removed. Final products of the reaction are indole, pyruvic acid, ammonia (NH_3) and energy.



For this test, Kovac's modification method was followed. In this method 1% tryptone broth medium was used. The inoculated tubes were incubated at 37 °C for 72 h. After incubation 2 ml of the test reagent (Kovac's reagent) was added.

A rose pink color indicated formation of indole.

2.13.7 Nitrate reduction test (SAB 1957)

Nitrate reduction is evident by complete or partial disappearance of nitrate accompanied by appearance of nitrite, ammonia or free nitrogen. This test performed to observe the organisms' capability on the reduction of nitrate to nitrite. The formation of nitrite indicated the presence of the enzyme nitrate reductase in the organisms.

The following three reagents were required for this test:

Reagent A: Sulfanilic acid – acetic acid solution:

Sulfanilic acid	-	8.0 g
5N acetic acid	-	1000 ml

(1 part chemically pure acetic acid and 2.5 parts distilled water).

Sulfanilic acid was dissolved in acetic acid and stored in brown glass bottle.

Reagent B: Dimethyl-α-napthalamine solution:

Dimethyl-α-napthalamine	-	6.0 ml
5N acetic acid	-	1000 ml

(Stored in brown glass bottle)

Reagent C: Zinc dusts

The tubes of nitrate broth in duplicates were inoculated with test organisms and then incubated at 37 °C for 72 h. After incubation, 1 ml of **reagent A** was added to the incubated tube and shaken. Then 1 ml of **reagent B** was also added to each tube and shaken well.

Formation of a distinct red or pink color indicated the reduction of nitrate to nitrite. Absence of nitrite may be due to complete conversion of nitrate as well as no reduction at all. A pinch of zinc dust then should be added to the tube showing absence of nitrite and it was allowed to stand for a few minutes. Any remaining nitrate (in case) would be reduced to nitrite by zinc and the characteristic pink or red color would appear and no color indicated complete reduction.

2.13.8 Production of hydrogen sulfide (SAB 1957)

To detect hydrogen sulfide (H_2S) production, peptone-iron agar deeps were inoculated with test bacteria by stabbing each bacterium with a straight inoculating needle. After incubation at 37 °C for 48 h, culture tubes were examined for H_2S production.

Any blackening of the medium due to the formation of ferrous sulfide (FeS), shows positive reaction while the absence of black color demonstrates negative result.

2.13.9 Motility test (Eklund and Lankford 1967)

Motile bacteria can move through semisolid, soft motility agar and their growth clouds the medium. Non-motile organisms will remain still and only grow near the site of inoculation.

A chemical 2,3,5-triphenyltetrazolium chloride, which is reduced from the colorless form to the insoluble red formazan by growing bacteria, was added to the medium at 0.001% (w/v). The color is developed only in that part of medium in which bacteria are present.

Color formation in the medium apart from inoculation site revealed the motility of bacteria.

2.13.10 Voges-Proskauer Test (Sneath *et al.* 1986)

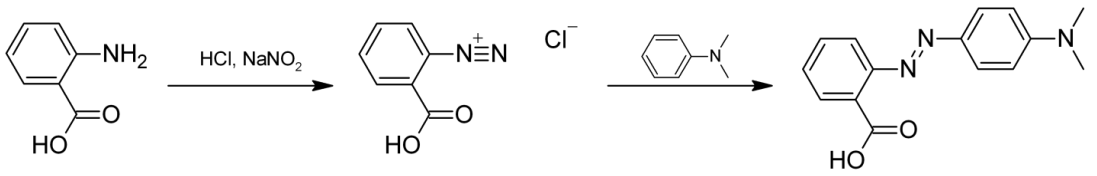
Voges-Proskauer (VP) test is a color reaction test for the production of a neutral product during glucose fermentation by microorganisms. Acetoin or acetyl-methyl carbinol oxidised to diacetyl, which reacts with creatine and forms a red complex.

For this test, VP broth tubes were inoculated with the test bacteria and incubated for 72 h at 37 °C. When sufficient bacterial growth was observed, 3 ml of 5% alcoholic α-napthol solution added to each tube followed by 1 ml of 40% potassium hydroxide and 0.3% creatine solution. The tubes were then shaken vigorously and allowed to stand.

Development of crimson to ruby red color indicates a positive reaction that is the production of acetyl-methyl carbinol.

2.13.11 Methyl Red test (Sneath *et al.* 1986)

Methyl Red (MR) test is the test for mixed acid fermentation of glucose by microorganisms. Excreted acid contains large amount of formic, acetic, lactic and succinic acid and causes a major decrease in pH that can be detected by Methyl Red indicator.



For this test, tubes with VP broth was inoculated with the test bacteria and incubated at 37 °C for 72 h.

After incubation 5 drops of methyl red indicator added to the culture broth. Red color throughout the broth indicated positive reaction whereas yellow or any yellowish red indicated negative reaction.

2.13.12 Hydrolysis of casein (Collins and Lyne 1984)

Casein is a milk protein. Casein comprises about 85% of the total protein in milk. Many microorganisms have the capacity to hydrolyze casein. This test demonstrates the ability of microbes to degrade casein into soluble peptides and amino acids by the enzyme caseiase.

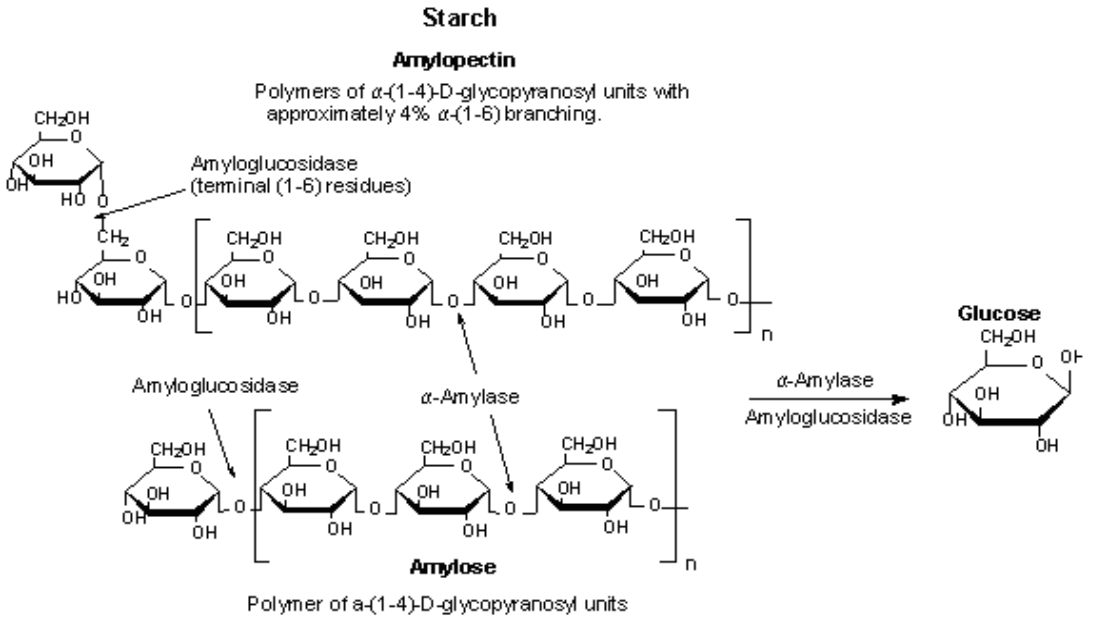
One ml of sterilized milk was taken in a sterilized petri-plate and then melted agar medium was poured and mixed thoroughly. After solidifying, the plates were inoculated with fresh cultures and incubated at 37 °C for 24 h.

Formation of a clear, transparent zone around the bacterial growth indicated hydrolysis of casein.

2.13.13 Hydrolysis of starch (Claus 1995)

Hydrolysis of starch test was used to identify and the potentiality of amylase production of the isolates.

Organisms capable of hydrolyzing starch to form monosaccharide or disaccharide possess the enzyme amylase. As an extracellular enzyme, amylase diffuses outward from the bacterial cells and breakdown starch. This test reveals the presence or absence of the enzyme amylase in the organisms.



For this test, starch-agar plates were inoculated with test bacteria and the plates were incubated at 37 °C for 48 h. After incubation, the surface of the plates was flooded with iodine solution.

Iodine reacts with starch and form starch iodide and gives the color deep blue. Development of a clear zone around the bacterial growth indicated starch hydrolysis and the presence of amylase.

2.13.14 Deep glucose agar test (SAB 1957)

Microorganisms vary widely in their requirements for oxygen. The nature of microbial growth in agar deeps reflects the cells' relative need for oxygen or an oxygen free environment. In relation to free oxygen, organisms are generally classified as strict aerobes, microaerophiles, facultative anaerobes and strict anaerobes.

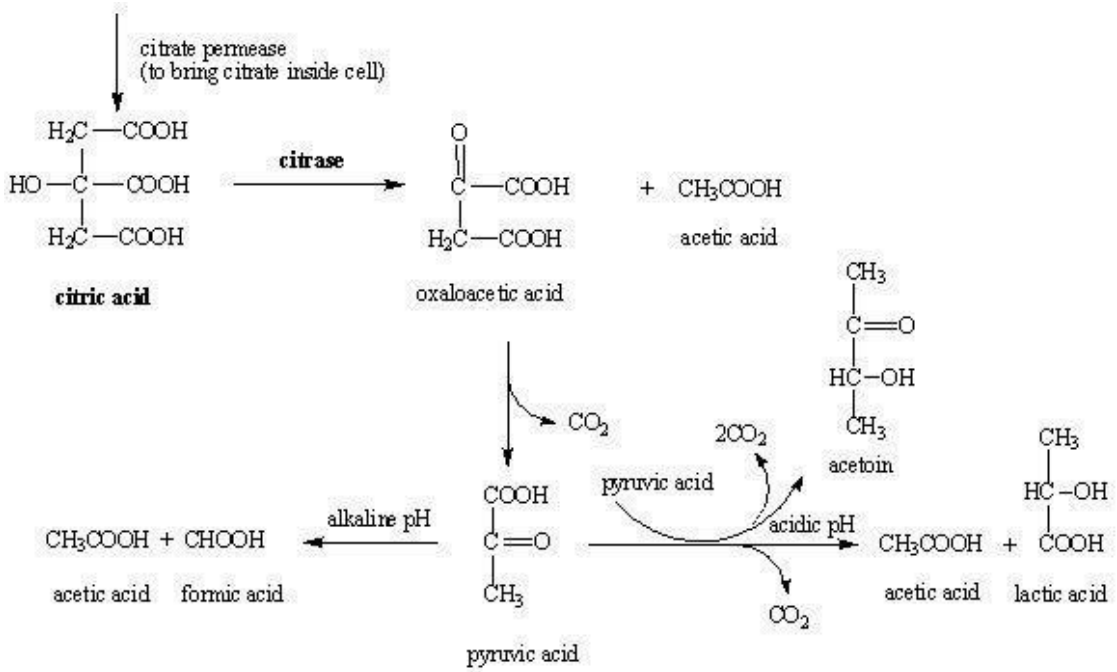
A tube of deep glucose agar medium was inoculated with the test bacteria in fluid condition approximately at 45° C. The tube was rotated to mix the inoculums with the medium and allowed to solidify. Inoculated test tubes were incubated at 37 °C for 72 h.

Observation was made after incubation at 37 °C for 72 h to find out whether the bacteria grew on the surface and in the upper layer of the medium (strict aerobes), or the organisms grew just a few millimeters below the surface

(microaerophiles), or the organisms grew throughout the medium (facultative anaerobes), or the organisms grew deeper in the medium (strict anaerobes).

2.13.15 Utilization of citrate (Atlas 1997)

This test demonstrates the ability or inability of test organisms to use sodium citrate as sole source of carbon for metabolism and growth. The citrase enzyme hydrolyses the citrate to form oxaloacetic acid and acetic acid.



Tubes containing Simmon's citrate agar were inoculated with the test bacteria and incubated at 37 °C for 48 h.

Utilization of citrate was determined by change of the color from green to blue.

2.13.16 Utilization of propionate (Sneath *et al.* 1986)

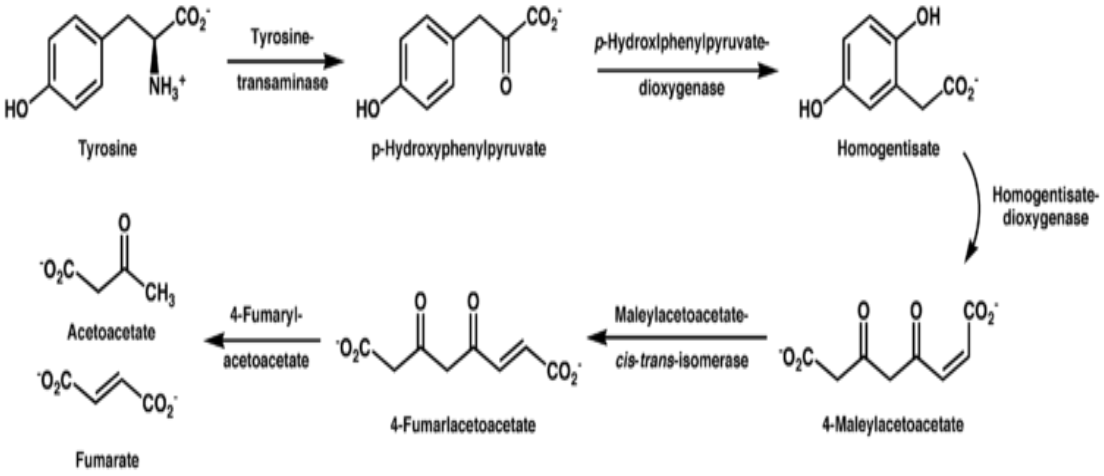
Propionate is a short chain fatty acid and utilization of propionate is one of the key characters of bacterial idedtification.

Propionate agar slants were inoculated with 24 h old bacterial culture and incubated at 37 °C for 48 h.

Production of a pink color indicated the utilization of propionate by the isolates.

2.13.17 Degradation of tyrosine (Sneath *et al.* 1986)

Tyrosine is one of the 20 common amino acids found in all biological systems. It is catabolized as part of normal intermediary metabolism and in the breakdown of external proteins by microorganisms.



Tyrosine agar plates were inoculated with the test bacteria by point inoculation method and incubated for 72 h at 37 °C.

Clearing of tyrosine crystals around and below the bacterial growth revealed degradation of tyrosine.

2.13.18 Egg-yolk lecithinase test (SAB 1957)

Bacterial lecithinases are of special interest because of the possible role of these enzymes in pathogenicity. Lecithinases or phospholipases are enzymes released by bacteria that have the ability to destroy animal tissues. Phospholipid complexes are usually emulsifying agents occurring in tissues, serum and egg yolk. Lecithin is a normal component of the egg yolk. Bacterial lecithinases break down this lecithin to an insoluble diglycerides resulting in an opaque halo, surrounding the colony when grown on the egg yolk agar medium.

After autoclaving the medium, 1.5 ml egg-yolk aspirated aseptically was added to 100 ml of basal medium. After solidifying, the plates were inoculated with fresh bacterial cultures and incubated at 37 °C for 48 h.

Bacteria that produce lecithinase appear as colonies surrounded by a zone of insoluble precipitate. Bacteria that produce lipase appear colonies with a pearly iridescent sheen.

2.13.19 Levan test (Schaad 1988)

Levan is a poly-fructose which is an extracellular capsular substance. Levan formation is detected on nutrient agar plate to which 5% sucrose (w/v) was added. Inoculated plates were incubated at 37 °C for 72 h.

After 72 h incubation the presence of convex white mucoid bacterial colonies indicate levan formation.

2.13.20 Arginine dihydrolysis test (Schaad 1988)

The arginine dihydrolase catalyzes the hydrolysis of arginine into ornithine, NH_3 and CO_2 with a gain of ATP. Arginine dihydrolase is detected in routine laboratory tests by the anaerobic tube method of Thornley (1960).

The test medium of Thornley was stab inoculated with the test bacteria at the base of the medium and each tube instantly sealed with 3 ml of the molten (3%) agar and incubated at 37 °C for 24 h.

A positive alkaline reaction was indicated by the development of deep red color.

2.14 Identification of the bacterial isolates

2.14.1 Provisional identification of the bacterial isolates

Provisional identification of selected bacterial isolates isolated from lipid-rich environment was carried out according to their observed morphological and biochemical characteristics. Gram negative bacteria were provisionally identified following Bergey's Manual of Systematic Bacteriology Vol.1 (Krieg and Holt 1984) and Gram positive bacteria were provisionally identified following Bergey's Manual of Systematic Bacteriology Vol. 2 (Sneath *et al.* 1986).

2.14.2 Molecular identification of the bacterial isolates

Potential isolates were studied for molecular identification. Selection was done based on lipolytic zone ratio and intensity of zone in TBA and TA medium. Diversity of species was also considered for the selection of the bacterial isolates.

2.14.2.1 PCR amplification

In order to identify the selected bacterial isolates based on sequence comparison, partial amplification of 16S rRNA was necessary. For the partial amplification of 16S rRNA gene the following primer pair was used-

CC(F) 16S rRNA: 5'-CCAGACTCCTACGGGAGGCAGC-3'

CD(R) 16S rRNA: 5'-CTTGTGCGGGCCCCCGTCAATTC-3'

- **Preparation of Primer**

Primers were dissolved in sterile MiliQ water following the manufacture's instruction to obtain 100μM stock concentration of each primer. To make working concentration each primer was diluted 10 fold further.

- **Preparation of Template**

The bacterial isolates were cultured as single colony and one colony for each isolate was suspended in 10 μl sterile water and subject to heat lysis by heating (95 °C) in a PCR machine for 10 minutes. The lysed cell suspension was centrifuged for 5 minutes at 3,000 rpm in a microcentrifuge (Mikro 200R) and then the supernatants were used as template DNA for PCR amplification of 16S rRNA.

The following components were used to prepare PCR cocktail. The total volume of PCR cocktail was 500 μl for 20 samples.

Table 2.2: Components of PCR cocktail (for 20 reactions).

Sl. No.	Reagents	Amount for 1 sample	Amount for 20 samples
1	Sterile de-ionized distilled water	17.95 µl	359.0 µl
2	Taq Buffer B 10X	2.5 µl	50.0 µl
3	Primer Forward	1.0 µl	20.0 µl
4	Primer Reverse	1.0 µl	20.0 µl
5	dNTPs 10 mM	0.5 µl	10.0 µl
6	Taq DNA Polymerase 5U/µl	0.05 µl	1.0 µl
7	Template DNA 25 ng/µl	2.0 µl	40.0 µl
	Total	25.0 µl	500.0 µl

During the experiment, PCR buffer, dNTPs, Primers and DNA sample solution were thawed in ice from frozen stocks. PCR master mix was prepared for each primer to be tested by adding the components of PCR in the following order (mentioned above in Table 2.2): ddH_2O, buffer, primer, $dNTP_S$ and taq DNA polymerase, mixed thoroughly and kept in ice. In the meantime the PCR tubes were marked compatibly with the thermocycler and the master mix was aliquoted to individual tubes marked for each DNA samples. Template DNA (25 ng/µl) were pipetted (2.0 µl) into PCR tubes containing PCR mix and mixed by tapering the tube following short spin of the tubes to recollect the total mixture. The tubes were then sealed and placed in a thermocycler. PCR amplification was carried out in an oil-free thermo cycler (UNO II, Biometra).

The reaction conditions for PCR are given below-

	Initial denaturation	95 ºC	For	5 min
30 cycles	Denaturation at	94 ºC	For	45 sec
	Annealing at	65 ºC	For	30 sec
	Extension at	72 ºC	For	40 sec
	Final extension at	72 ºC	For	5 min

After completion of cycling program, the reactions were held at 4 ºC.

2.14.2.2 Electrophoresis of the amplified products and documentation

The amplified products were separated electrophoretically on 1% agarose gel. The gel was prepared using 1.0 g agarose powder and 100 ml 1xTAE buffer. Ethidium bromide was added later. Gel was run at 80 Volts for 1.5 h. 100 bp DNA ladder was electrophoresed alongside the amplified sample DNA. DNA bands were observed on UV-transilluminator and photographed by a Gel Documentation system.

2.14.2.3 Automated sequencing

The bacterial isolates can be identified based on alignment of partial sequence of 16S rRNA gene with the existing sequences available in the database. In the present experiment, 10 different samples were used to amplify their 16S rRNA gene and PCR amplified DNA of the bacterial isolates were gel purified using phenol freeze method and sent for automated sequencing.

2.14.2.4 Molecular identification

The sequence generated from automated sequencing of PCR amplified DNA was analyzed through NCBI-BLAST database (http://blast.ncbi.nlm.nih.gov/) and rRNA BLAST (http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi) programs to find out possible similar organism through alignment of homologous sequences.

2.14.3 Phylogenetic analysis of the bacterial isolates

Phylogenetic tree was constructed by taking the sequence obtained in BLAST search. Sequence obtained from BLASTN (nucleotide BLAST) was obtained in FASTA format and relation between each sequence could be known by multiple sequence alignment using a software CLUSTAL OMEGA algorithm. The tree was generated using neighbor joining (NJ) a distance- based algorithm of phylogenetic analysis.

2.15 Estimation of lipase activity of the selected bacterial isolates

2.15.1 Preparation of inoculum

For this experiment, inoculum prepared in Nutrient Broth medium and incubated at 37 ºC on a rotary shaker (DAIHAN-LABTECH) at 150 rpm for 24 h. In order to keep the inoculum potential constant for each isolate, inoculum was prepared to obtain an initial cell density to adjust the turbidity of 0.5 McFarland standard (1.5×10^8 cfu/ml).

2.15.2 Preparation of 0.5 McFarland standards

McFarland turbidity standards are used to standardize the approximate number of bacteria in a liquid suspension by comparing the turbidity of a test suspension. Standard was prepared by adding barium chloride ($BaCl_2$) to H_2SO_4 to obtain a barium precipitate. 85 ml of 1% H_2SO_4 added to a 100 ml volumetric flask. 0.5 ml of 1.175% $BaCl_2$ added drop wise to the 1% H_2SO_4 while constantly swirling the flask. The volume was adjusted to 100 ml with 1% H_2SO_4. Then it was stirred for 5 min until the solution appears homogeneous and free of clumps. Optical density was measured at 625 nm wavelength.

2.15.3 Lipase production by submerged fermentation process

Lipase production was carried out by submerged fermentation process (Anbu *et al*. 2011). The Tryptone Soya Broth (TSB) with 1% olive oil as substrate was used for lipase production. Olive oil used as substrate because it is good and inexpensive substrate for lipase production. The experiment was carried out in 100 ml plugged Erlenmeyer flask containing 50 ml of TSB medium inoculated with 1% inoculum. The inoculated flasks were then incubated at 37 ºC for 96 h with constant shaking at 150 rpm in a rotary shaker (DAIHAN-LABTECH). The experiment was performed in triplicates.

2.15.4 Separation of crude enzyme

After incubation in submerged fermentation cell free supernatant was recovered from the fermentation broth by centrifugation at 24 h intervals at 9,000 rpm for 20 min at 4 ºC in a centrifuge (Thermo Scientific Sorvall ST 8R, Germany). The clear supernatant was collected as source of crude enzyme for determination of lipolytic activity.

2.15.5 Preparation of oleic acid standard curve

A standard curve of oleic acid was prepared before the estimation of lipase activity of the bacterial isolates. As olive oil used as substrate for lipase production and it releases oleic acid as free fatty acid so first oleic acid standard curve was prepared according to the method of Marseno *et al*. (1998). A series of 2 – 10 (2, 4, 6, 8 and 10) µmoles of oleic acid (fatty acid) were prepared by dissolving in 2 ml iso-octane. Then 0.4 ml of 5% cupric acetate pyridine was added and was mixed vigorously for

90 sec by hand. The mixture was vortexed for 5 sec and allowed to stand for 10 min. Then oleic acid content in the iso-octane fraction was measured spectrophotometrically at 715 nm. Using the data standard curve was made.

2.15.6 Estimation of lipase activity of the bacterial isolates

Marseno *et al.* (1998) method was followed for the estimation of lipase activity of the bacterial isolates. For this purpose**,** 5% (w/v) cupric acetate pyridine pH 6.0 was prepared. Five grams of cupric acetate was dissolved in 80 ml of distilled water and the pH was adjusted to 6.0 using pyridine. Then the volume was made to 100 ml using distilled water.

Lipase activity was estimated in a screw cap vial containing 2 ml of reaction mixture containing 60% (v/v) olive oil in iso-octane. The 60% (v/v) olive oil in iso-octane was prepared by mixing 60 ml of olive oil with 40 ml of iso-octane. The reaction was started by adding of 20 µl of crude enzyme solution at 30 ºC at 150 rpm for 30 min. Then the reaction was stopped by placing the reaction mixture in an ice bath for 10 min. About 200 µl of the aliquots was added to the mixture containing 1800 µl of iso-octane and 400 µl of cupric acetate pyridine at pH 6.0. The upper layer (iso-octane fraction) was pipetted and the amount of free fatty acid that dissolved in iso-octane layer was determined spectrophotometrically by measuring the optical density at 715 nm (Shimadzu, UV-120-02, Japan). Lipase activity was estimated by quantification of oleic acid released as free fatty acid from olive oil by bacterial lipase which is determined following oleic acid standard curve. Released oleic acid was estimated up to 96 h and results recorded every 24 h intervals. Oleic acid liberated during hydrolysis of olive oil substrate by bacterial lipase was determined colorimetrically using cupric acetate pyridine reagent. Fatty acid complex with copper to form cupric salts or soaps that absorb light in the visible range (715 nm), yielding a blue color.

$$\text{Lipase activity (U/ml)} = \frac{X \times \text{Dilution factor} \times 1000}{\text{Time (min)} \times \text{Sample volume (µl)}}$$

X = Value obtained from standard curve

Dilution factor = 10

Sample = 20 µl

Time = 30 min

Lipase unit

One unit of lipase activity was defined as the amount of enzyme that produced one µmole fatty acid per min per ml.

2.16 Optimization of lipase production of the bacterial isolates

Optimization of different parameters in lipase production was carried out for better enzyme activity showing bacterial isolates. To perform this experiment lipase production was carried out in 100 ml Erlenmeyer flask containing 50 ml of liquid medium on a rotary shaker (DAIHAN-LABTECH) with 150 rpm and incubated at 37 °C for 48 h and the enzyme activity was estimated. All experiments were carried out in triplicate.

2.17 Optimization of different physico-chemical parameters

Optimization of different physic-chemical parameters such as pH, temperature, NaCl, carbon source, organic and inorganic nitrogen source, inoculum concentration, substrate and medium were carried out. 1% (v/v) olive oil was also used as an inducer for lipase production (Kumar *et al*. 2012c, Veerapagu *et al.* 2013).

2.17.2 Effects of pH on lipase production

Effects of pH on lipase production was performed at varying pH (5-9) of the medium at temperature 37 °C. The bacterial isolates were inoculated in the lipase production medium and incubated at 37 °C for 48 h.

2.17.3 Effects of temperature on lipase production

To test the effect of temperature on lipase production, five different temperatures such as 20, 30, 37, 40 and 50° C were selected at pH 7. The bacterial isolates were inoculated in the lipase production medium and incubated at selected different temperature for 48 h.

2.17.4 Effects of NaCl on lipase production

Effects of NaCl on lipase production was carried out by adding six different NaCl concentrations (0.5, 1.0, 1.5, 2.0 and 2.5%) to the lipase production medium by

keeping all other parameters same. The bacterial isolates were inoculated to the medium and incubated at 37 °C for 48 h.

2.17.5 Effects of carbon sources on lipase production

Effects of carbon sources on the lipase production was analyzed by adding different carbon sources such as glucose, sucrose, starch, arabinose, xylose and mannitol at a concentration of 1% (w/v) in the production medium. The inoculated lipase production medium was incubated at 37 ^{o}C for 48 h.

2.17.6 Effects of organic nitrogen sources on lipase production

Effects of organic nitrogen sources on the lipase production was studied. The nitrogen sources *viz*. peptone, tryptone, yeast extract and beef extract at a concentration of 1% (w/v) was added to the lipase production medium and incubated at 37 ºC for 48 h.

2.17.7 Effects of inorganic nitrogen sources on lipase production

Effects of inorganic nitrogen sources on lipase production was studied by adding three different nitrogen sources such as ammonium chloride (NH_4Cl), ammonium nitrate (NH_4NO_3) and ammonium ortho phosphate ($NH_4H_2PO_4$) at a concentration of 1% (w/v) to the medium. The inoculated lipase production medium was incubated at 37 ºC for 48 h.

2.17.8 Optimization of better carbon and nitrogen sources on lipase production

Better carbon and nitrogen sources which support maximum lipase production were added at 0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% (w/v) in the production medium. For optimization the inoculated lipase production medium was incubated at 37 ºC for 48 h.

2.17.9 Effects of inoculum concentration on lipase production

To evaluate the effect of inoculum concentration on lipase production varied cell concentrations 1.0 to 7.0% were added and the inoculated medium was incubated at 37 ºC for 48 h.

2.17.10 Effects of olive oil concentration on lipase production

Effects of olive oil concentration on lipase activity was determined using different concentrations of olive oil (0.5, 1.0, 1.5, 2.0, 2.5 and 3.0% v/v) to the medium. The inoculated lipase production medium was incubated at 37 ºC for 48 h.

2.17.11 Effects of media on lipase production

The following media were tested on lipase production:

Nutrient Broth (NB)
Luria Bartani (LB)
Tributyrin Broth (TBB)
Tween Broth (TB) and
Tryptone Soya Broth (TSB)

2.18 Lipase production using all optimum conditions

Considering all parameters for optimization of lipase production the best condition was employed. For this purpose, 5% inoculum was added to 50 ml production medium in 100 ml Erlenmeyer flask. Inoculated flasks were incubated at 37 °C for 96 h with 150 rpm on a rotary shaker (DAIHAN-LABTECH). Sampling for lipase estimation was carried out periodically at 12 h intervals.

2.19 Determination of bacterial growth

Bacterial growth was determined by counting bacterial colony. Bacterial count was recorded as colony forming unit (cfu/ml) using the dilution plate technique. Serially diluted 1 ml culture mixed with molten Nutrient Agar. The inoculated plates were incubated at 37 °C for 24 h. After incubation, the total bacterial colonies developed in the inoculated plates were counted using a colony counter.

2.20 Laboratory scale wastewater treatment

Laboratory scale wastewater treatment was carried out by using synthetic wastewater. Synthetic wastewater was prepared in the laboratory with the following compositions as described by Chen *et al*. (2009). Prepared synthetic medium was diluted 100 times (1 ml stock + 99 ml distilled water). One percent olive oil was added in the diluted medium. The pH was adjusted to 7 and the media was autoclaved at 121 °C for 15 min.

Seed culture was prepared in nutrient broth. Inoculated Erlenmeyer flasks (250 ml) containing 100 ml synthetic wastewater with 5% (5 ml) seed culture at OD value with 600 nm =1 were then incubated in a shaking incubator (DAIHAN-LABTECH) at 37 °C

at 150 rpm for 96 h. COD analysis was done periodically at 24 h intervals. Control test carried out without inoculation.

COD is the oxygen required by the organic substances in water to oxidize them by a strong chemical oxidant. This shows the oxygen equivalent of the organic substances in water that can be oxidized by a strong chemical oxidant such as potassium dichromate in acidic solution. Potassium dichromate ($K_2Cr_2O_7$) in the presence of sulphuric acid is generally used as an oxidizing agent in the determination of COD. The sample is treated with potassium dichromate and sulphuric acid and titrated against ferrous ammonium sulphate (FAS) using ferroin as an indicator. The amount of $K_2Cr_2O_7$ used is proportional to the oxidizable organic matter present in the sample.

COD was calculated by following this formula-

$$\text{COD (mg/L)} = \frac{(\text{A-B}) \times \text{N} \times 8000}{\text{ml sample}}$$

A = ml used for blank

B = ml used for control

N = normality of Mohr's salt

2.21 Statistical analysis

Statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) v.20.0 for windows (SPSS, SAS Institute Inc. Cary, USA). The data were analyzed to determine the descriptive statistics such as Mean and Standard deviation. Evaluation on differences between groups was based on the oneway ANOVA test at 95% confidence interval with Duncan's Multiple Range Test. Value of $p < 0.05$ was regarded as statistically significant.

Chapter-3

Results

Results

Water and soil samples were collected aseptically in the sterilized polythene bags or in the sterilized plastic bottles from some selected area of lipid-rich environment in and around Dhaka Metropolitan City. Collected samples were used for isolation of indigenous bacteria having biotechnological potential with special reference to lipase production. The enzyme lipase is very important in different sectors *viz.* food, detergent, textile, leather, wastewater management, etc. In case of wastewater management bacterial lipase has very important role in removing lipidic or oily substances from wastewater.

3.1 Sampling sites and sample types

Considering potential indigenous bacteria ten different sampling sites were selected for the enumeration and isolation of bacteria. Sampling sites were mainly industrial area and river receiving untreated industrial effluents. Six water and four soil samples were collected from ten different possible lipid-rich environments in and around Dhaka Metropolitan City. The map of the Dhaka Metropolitan City and some of the sampling sites are shown in Fig. 3.1 and 3.2.

3.2 Aerobic heterotrophic bacteria and lipase positive bacterial load of the collected samples

A good number of aerobic heterotrophic bacteria were found to be associated with the collected samples (Table 3.1). Bacterial load of the collected samples ranged in between 4.6×10^3 and 1.37×10^7, 1.65×10^3 and 1.25×10^7, 1.28×10^3 and 1.56×10^7, 8.3×10^2 and 2.64×10^5 cfu/g or cfu/ml in NA, PYG, TBA and LB media, respectively. The maximum bacterial count (1.56×10^7 cfu/g) was observed in TBA medium and in the soil sample collected from Dairy Farm soil of Savar region. On the other hand the lowest bacterial load (8.3×10^2 cfu/ml) was observed in LB medium and in the water sample collected from The Buriganga River.

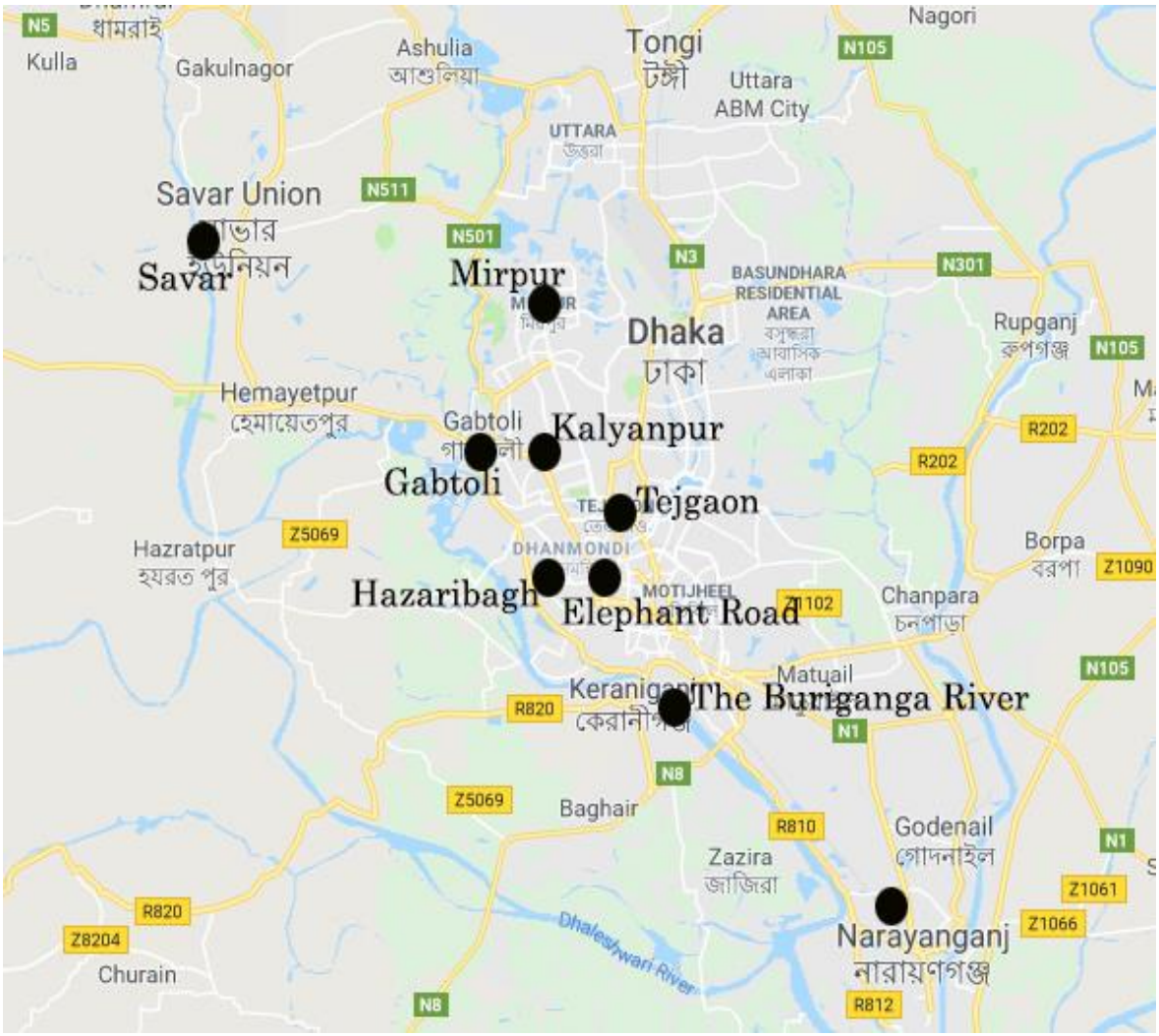


Fig. 3.1: Photograph showing the map of Dhaka Metropolitan City. The black mark shows the location of sampling sites.

Fig 3.2 (A-D): Photographs showing some of the sampling sites.

A. The Turag River, Gabtoli, Dhaka B. Bus Depot, Kalyanpur, Dhaka

C. Navy Dockyard, Narayangonj and D. The Buriganga River, Dhaka.

Table 3.1: Aerobic heterotrophic bacterial load of collected samples.

Sample No.	Sampling sites	Samples	Bacterial load (cfu/ml or cfu/g)			
			NA	PYG	TBA	LB
1	The Turag River, Gabtoli, Dhaka	Water	3.25×10^4	4.51×10^4	1.07×10^7	ND
2	**Dairy Farm, Savar, Dhaka**	**Soil**	1.37×10^7	1.25×10^7	**1.56×10^7**	ND
3	Kitchen Waste, Mirpur, Dhaka	Soil	6.8×10^5	6.9×10^6	3.1×10^6	ND
4	Tannery Industry, Hazaribagh, Dhaka	Water	8.3×10^5	6.8×10^6	1.36×10^6	ND
5	Bus Depot, Kalyanpur, Dhaka	Water	1.39×10^5	1.15×10^5	7.3×10^4	4.4×10^4
6	**The Buriganga River, Dhaka**	**Water**	4.6×10^3	1.65×10^3	1.28×10^3	**8.3×10^2**
7	Navana Car Workshop, Tejgaon, Dhaka	Water	1.8×10^6	2.1×10^6	2.91×10^5	2.64×10^5
8	Navy Dockyard, Narayangang	Water	1.21×10^4	2.41×10^4	7.6×10^3	6.8×10^3
9	Edible Oil Mill, Elephant Road, Dhaka	Soil	1.18×10^4	1.11×10^4	9.1×10^3	1.18×10^4
10	Kohinoor Chemicals Company Ltd., Tejgaon, Dhaka	Soil	3.93×10^5	3.7×10^5	2.4×10^5	3.87×10^4

NB: NA=Nutrient Agar, PYG=Peptone Yeast Extract Glucose, TBA=Tributyrin Agar, LB=Luria Bartani, ND=Not done, cfu/ml, in case of water and cfu/g, in case of soil.

Lipase positive bacterial load and their percentage were observed in TBA medium (Table 3.2). Lipase positive bacterial load in TBA medium was ranged in 3×10^2 cfu/ml to 1.17×10^7 cfu/g in the water sample of The Buriganga River and the soil sample of Dairy Farm, respectively. The highest percentage (94.51%) was found in Edible Oil Mill soil and the lowest (23.44%) was in The Buriganga River water. The results revealed that lipase producing bacteria are widely distributed in lipid-rich environment.

3.3 Isolation and selection of bacteria

During this study, a total of 273 aerobic heterotrophic bacterial colonies were primarily selected from different collected samples based on different colony morphology. Fig. 3.3 showed bacterial colonies developed in different steps of isolation. Bacteria with different colony morphology along with pigment formation were observed.

3.4. Purification of the selected bacteria

Purification was done by streak plate method. Well discrete single colony was considered as pure culture. Fig. 3.4 showed purification of some isolates as purified on Nutrient Agar plates. The selected pure cultures were studied for screening of lipase positive isolates.

Table 3.2: Lipase positive bacterial load and their percentage.

Sample No.	Sampling sites	Samples	Lipase positive bacterial load in TBA medium (cfu/ml or cfu/g)	% of lipase positive bacteria
1	The Turag River, Gabtoli, Dhaka	Water	6.8×10^{6}	63.55
2	**Dairy Farm, Savar, Dhaka**	**Soil**	**1.17×10^{7}**	75
3	Kitchen Waste, Mirpur, Dhaka	Soil	2.92×10^{6}	94.19
4	Tennary Industry, Hazaribagh, Dhaka	Water	5.35×10^{5}	39.34
5	Bus depot, Kalyanpur, Dhaka	Water	3.5×10^{4}	47.95
6	**The Buriganga River, Dhaka**	**Water**	**3×10^{2}**	**23.44**
7	Navana Car Workshop, Tejgaon, Dhaka	Water	1.16×10^{5}	39.86
8	Navy Dockyard, Narayangang	Water	6.8×10^{3}	89.47
9	Edible Oil Mill, Elephant Road, Dhaka	**Soil**	8.6×10^{3}	**94.51**
10	Kohinoor Chemicals Company Ltd., Tejgaon, Dhaka	Soil	1.9×10^{5}	79.17

NB: cfu/ml, in case of water and cfu/g, in case of soil.

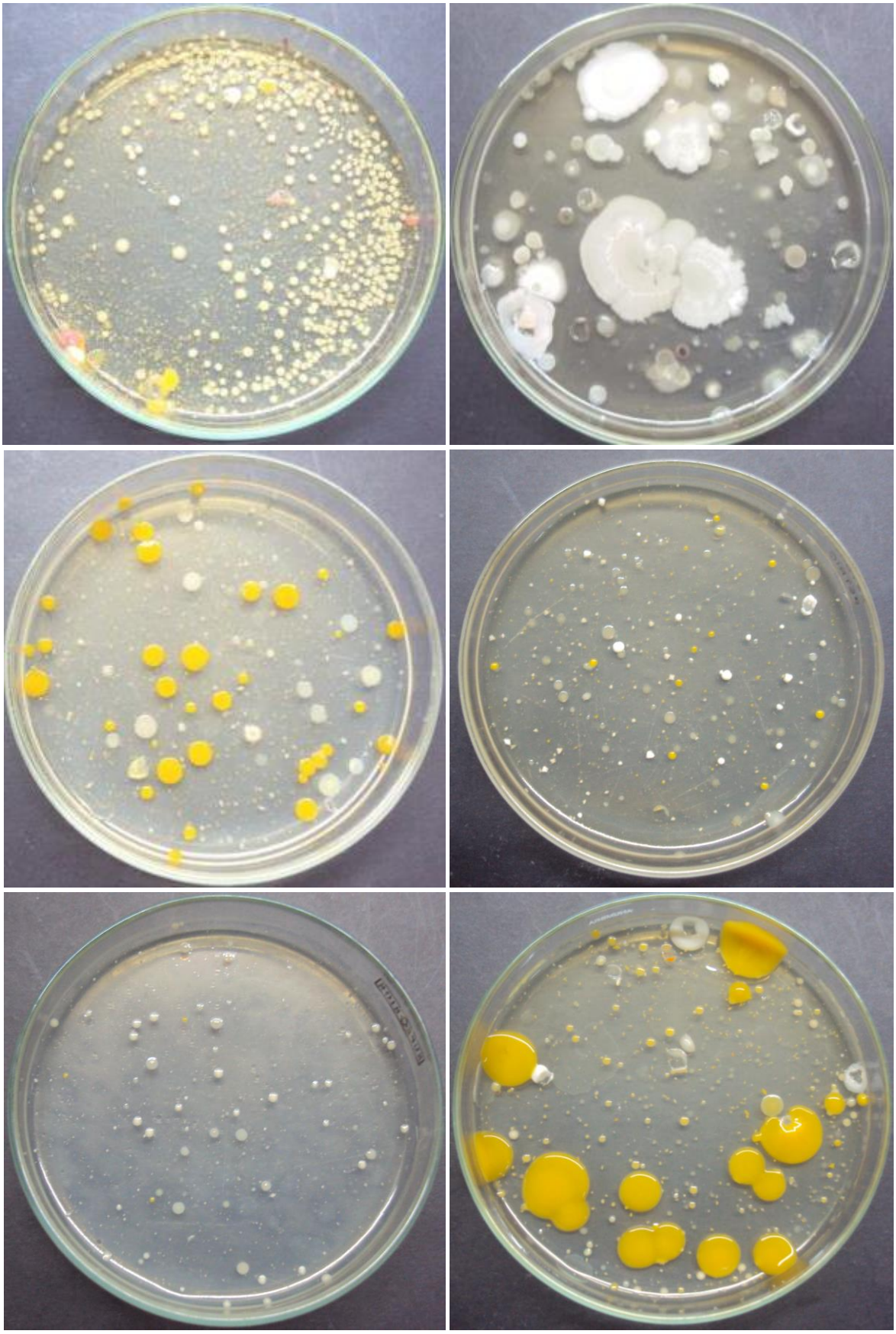


Fig. 3.3: Aerobic heterotrophic bacterial colonies developed during isolation.

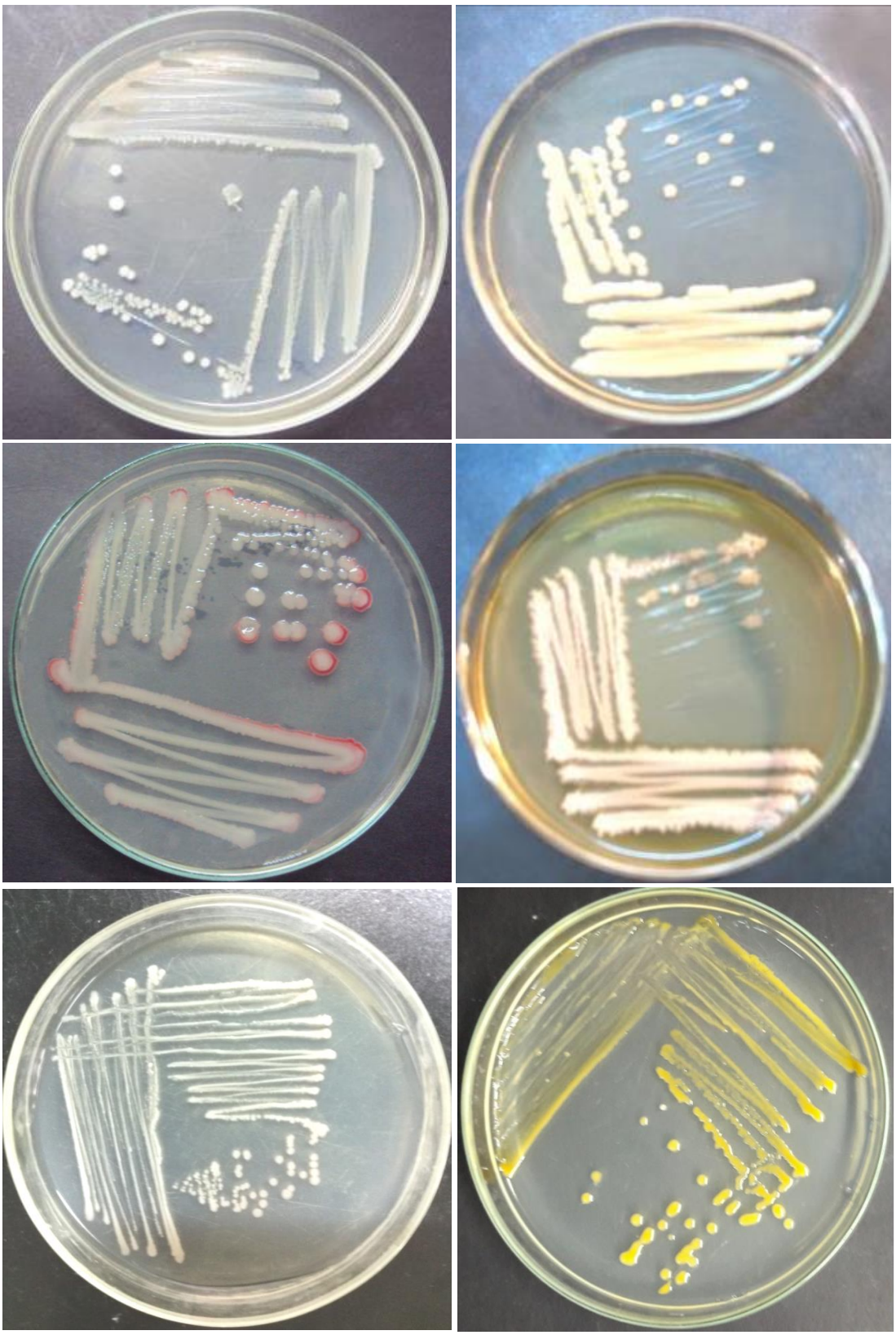


Fig. 3.4: Photographs showing streak plate method for purification on Nutrient Agar medium.

3.5 Screening of lipase producing bacteria

The preliminary selected bacterial colonies were subjected to qualitative screening for lipase producing isolates. Selected 273 isolates were point inoculated in two lipid based media (TBA and TA) to screen out the lipase producing bacteria through the formation of clear zone on TBA medium due to hydrolysis of tributyrin and opaque zone on TA medium due to hydrolysis of tween 80. There were about 186 isolates were found to be lipase positive. Among them 30 showed better lipase activity. These 30 isolates were selected for detailed study towards the morphological, physiological and biochemical characteristics for identification. Lipase positive and negative bacterial colonies developed on TBA plate as shown in Fig. 3.5.

The percentage of lipase activity showing isolates is shown in Fig. 3.6. Among the total 273 isolates, 68% showed lipase positive. Considering hydrolysis of tributyrin and clear zone formation, top 30 selected better lipolytic bacterial isolates are shown in Fig. 3.7.

For analysis of the degree of lipolysis by the selected isolates, zone ratio of 30 selected isolates was calculated from TBA plates and is shown in Table 3.3. Zone ratio was calculated after 72 h of incubation. Among the isolates, the highest lipolytic activity was shown by the isolate S_4P-4 and the measured zone ratio was 4.08 ± 0.47. The lowest activity showing isolate was S_5L-2 and the measured zone ratio was 1.55 ± 0.18.

Growth on TA medium after 72 h of incubation is shown in Fig. 3.8. Hydrolysis of tween 80 and formation of opaque zone around the inoculated bacterial colony was observed due to precipitation of Ca.

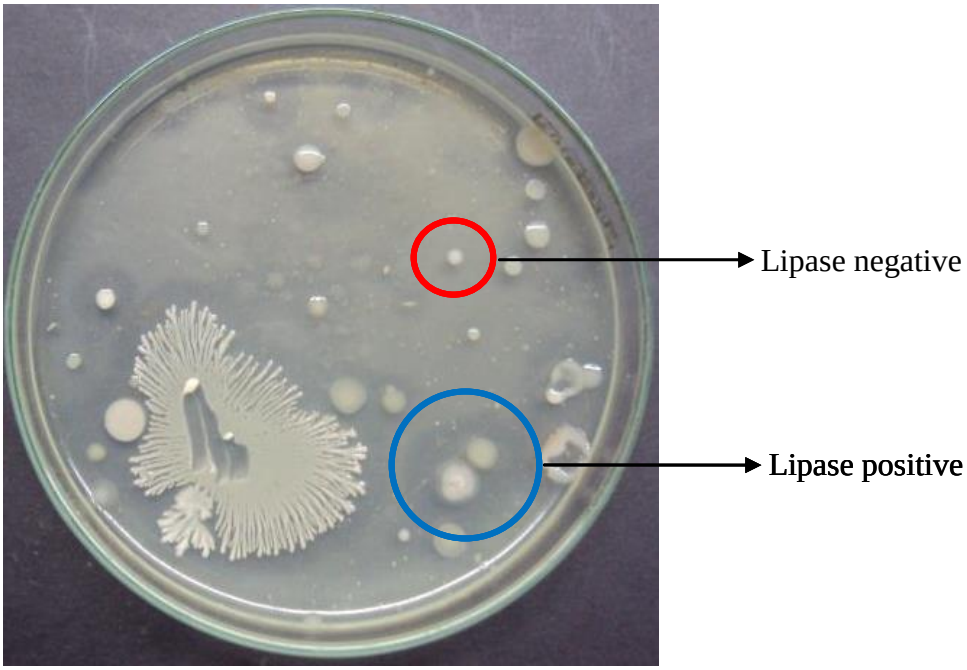


Fig. 3.5: Photograph showing lipase producing bacterial colonies developed on TBA plate.

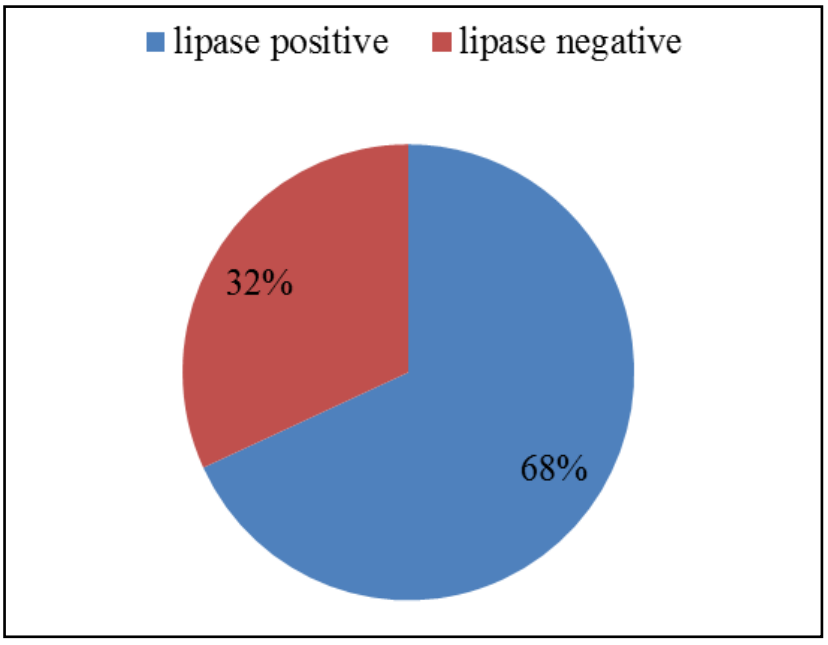


Fig. 3.6: Percentage of lipase activity showing isolates.

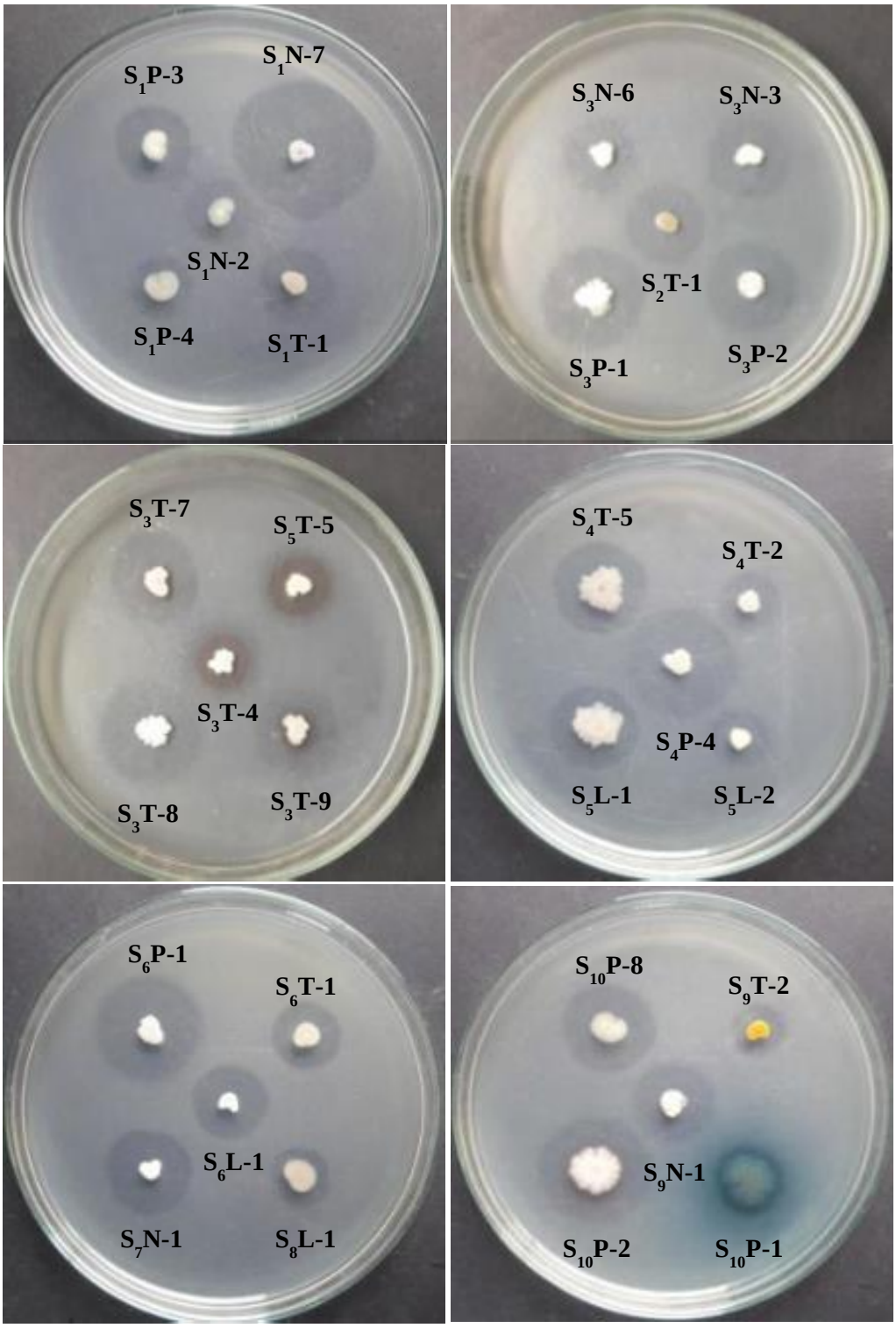


Fig. 3.7: Clear zone formation by selected 30 bacterial colonies on TBA medium.

Table 3.3: Zone ratio on TBA plate of the selected 30 bacterial isolates.

Bacterial isolates	Zone diameter (mm) (Mean±SD)	Colony diameter (mm) (Mean±SD)	Zone ratio (Mean±SD)
S_1N-2	17.17 ± 1.89	7.83 ± 0.29	2.19 ± 0.28
S_1N-7	23.17 ± 1.89	6.33 ± 0.58	3.66 ± 0.29
S_1P-3	17.33 ± 2.93	6.17 ± 0.29	2.81 ± 0.41
S_1P-4	15.00 ± 1.32	8.5 ± 0.00	1.76 ± 0.15
S_1T-1	16.33 ± 1.89	7.83 ± 0.29	2.09 ± 0.22
S_2T-1	15.50 ± 1.50	7.00 ± 0.50	2.21 ± 0.38
S_3N-3	19.33 ± 2.08	7.17 ± 0.58	2.70 ± 0.09
S_3N-6	17.33 ± 2.36	6.83 ± 0.29	2.54 ± 0.47
S_3P-1	19.50 ± 4.77	7.00 ± 0.50	2.79 ± 0.62
S_3P-2	16.83 ± 3.33	7.17 ± 0.76	2.35 ± 0.67
S_3T-4	22.67 ± 2.02	9.17 ± 1.26	2.47 ± 0.27
S_3T-5	22.83 ± 1.53	9.67 ± 0.58	2.36 ± 0.10
S_3T-7	17.33 ± 2.75	6.33 ± 0.58	2.74 ± 0.18
S_3T-8	23.33 ± 4.91	7.17 ± 1.15	3.25 ± 0.15
S_3T-9	25.00 ± 4.09	9.50 ± 2.18	2.63 ± 0.46
S_4P-4	24.50 ± 0.87	6.00 ± 0.50	**4.08 ± 0.47**
S_4T-2	12.67 ± 2.47	7.67 ± 0.29	1.65 ± 0.36
S_4T-5	18.00 ± 0.60	6.33 ± 0.29	2.84 ± 0.17
S_5L-1	17.33 ± 0.29	9.17 ± 1.26	1.89 ± 0.29
S_5L-2	18.83 ± 0.29	12.17 ± 1.53	**1.55 ± 0.18**
S_6P-1	16.50 ± 2.18	5.33 ± 0.76	3.10 ± 0.55
S_6T-1	14.50 ± 2.00	6.00 ± 0.00	2.42 ± 0.34
S_6L-1	14.67 ± 1.59	8.00 ± 0.50	1.83 ± 0.23
S_7N-1	16.83 ± 4.48	5.67 ± 0.76	2.97 ± 0.90
S_8L-1	23.17 ± 1.76	7.17 ± 0.29	3.23 ± 0.29
S_9N-1	17.33 ± 1.53	5.33 ± 0.29	3.25 ± 0.18
S_9T-1	10.67 ± 1.04	5.17 ± 0.58	2.06 ± 0.36
S_{10}P-1	23.50 ± 3.00	14.69 ± 1.76	1.60 ± 0.13
S_{10}P-2	29.83 ± 2.75	15.33 ± 1.15	1.95 ± 0.15
S_{10}T-8	21.5 ± 1.76	8.33 ± 0.76	2.58 ± 0.24

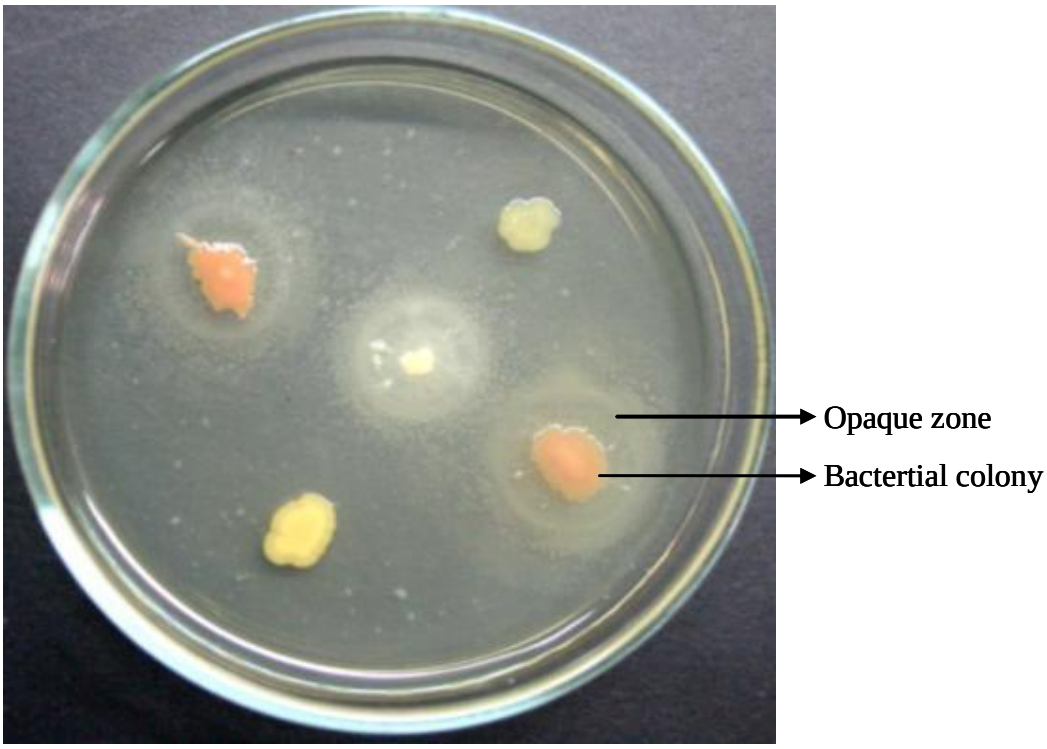


Fig. 3.8: Photograph showing growth and opaque zone on TA medium.

3.6 Colony morphology of the selected bacterial isolates

Colony morphology of the selected isolates was recorded on the basis of their shape, elevation, margin, surface, optical characteristics and pigmentation (Table 3.4). Most of the colonies of the selected isolates were found to be circular, flat, entire, smooth, moist, opaque and off-white. Fig. 3.9 showed the percentage of colony morphology of the selected isolates with special reference to colony shape. Circular type of colony was found to be dominant (67%) over the irregular (23%) and punctiform (10%).

3.7 Microscopic observation of the selected bacterial isolates

Staining properties and microscopic observation of the selected bacterial isolates revealed that 13 isolates were Gram positive rod shaped, 3 were Gram positive coccus and 14 were Gram negative short rod shaped (Table 3.5). Among 13 spore former bacteria most of the spores were ellipsoidal and showed not swollen type of sporangia. Photomicrographs of some of the selected isolates are shown in Fig. 3.10. Rod shaped bacteria were found to be single, in pair and in chain. While round shaped bacteria were found to be as single, in pair and in cluster.

Table 3.4: Colony morphology of the selected bacterial isolates.

Bacterial isolates	Shape	Elevation	Margin	Surface	Dryness	Optical characteristics	Color
S_1N-2	Circular	Raised	Undulate	Smooth	Moist	Transluscent	Cream
S_1N-7	Circular	Convex	Entire	Smooth	Moist	Opaque	White
S_1P-3	Irregular	Flat	Undulate	Smooth	Moist	Transluscent	Colorless
S_1P-4	Punctiform	Raised	Entire	Smooth	Moist	Transluscent	Colorless
S_1T-1	Circular	Raised	Undulate	Smooth	Moist	Transluscent	Off white
S_2T-1	Circular	Raised	Entire	Smooth	Moist	Transluscent	Cream
S_3N-3	Irregular	Effuse	Undulate	Smooth	Moist	Transluscent	Off white
S_3N-6	Punctiform	Flat	Entire	Smooth	Moist	Opaque	Off white
S_3P-1	Circular	Flat	Undulate	Smooth	Moist	Opaque	Off white
S_3P-2	Circular	Flat	Undulate	Smooth	Moist	Opaque	Off white
S_3T-4	Circular	Flat	Undulate	Smooth	Moist	Opaque	White
S_3T-5	Circular	Flat	Undulate	Smooth	Moist	Opaque	Off white
S_3T-7	Irregular	Flat	Erose	Contoured	Dry	Opaque	Light pink
S_3T-8	Circular	Flat	Undulate	Smooth	Moist	Opaque	Off white
S_3T-9	Irregular	Flat	Erose	Contoured	Dry	Opaque	Off white
S_4P-4	Circular	Flat	Entire	Smooth	Moist	Opaque	White
S_4T-2	Circular	Convex	Entire	Smooth	Moist	Opaque	Orange
S_4T-5	Punctiform	Flat	Entire	Smooth	Moist	Transluscent	Cream
S_5L-1	Circular	Flat	Entire	Smooth	Moist	Transluscent	Cream
S_5L-2	Circular	Raised	Entire	Smooth	Moist	Transluscent	Off white
S_6P-1	Circular	Raised	Entire	Smooth	Moist	Opaque	White
S_6T-1	Circular	Flat	Entire	Smooth	Moist	Transluscent	Off white
S_6L-1	Circular	Flat	Entire	Smooth	Moist	Transluscent	Cream
S_7N-1	Circular	Flat	Entire	Smooth	Moist	Opaque	White
S_8L-1	Circular	Flat	Undulate	Smooth	Moist	Opaque	Off white
S_9N-1	Circular	Flat	Entire	Smooth	Moist	Opaque	White
S_9T-1	Circular	Convex	Entire	Smooth	Moist	Opaque	Orange
S_{10}P-1	Irregular	Effuse	Undulate	Smooth	Moist	Transluscent	Cream
S_{10}P-2	Irregular	Flat	Undulate	Smooth	Moist	Opaque	Off white
S_{10}T-8	Irregular	Flat	Undulate	Smooth	Moist	Transluscent	cream

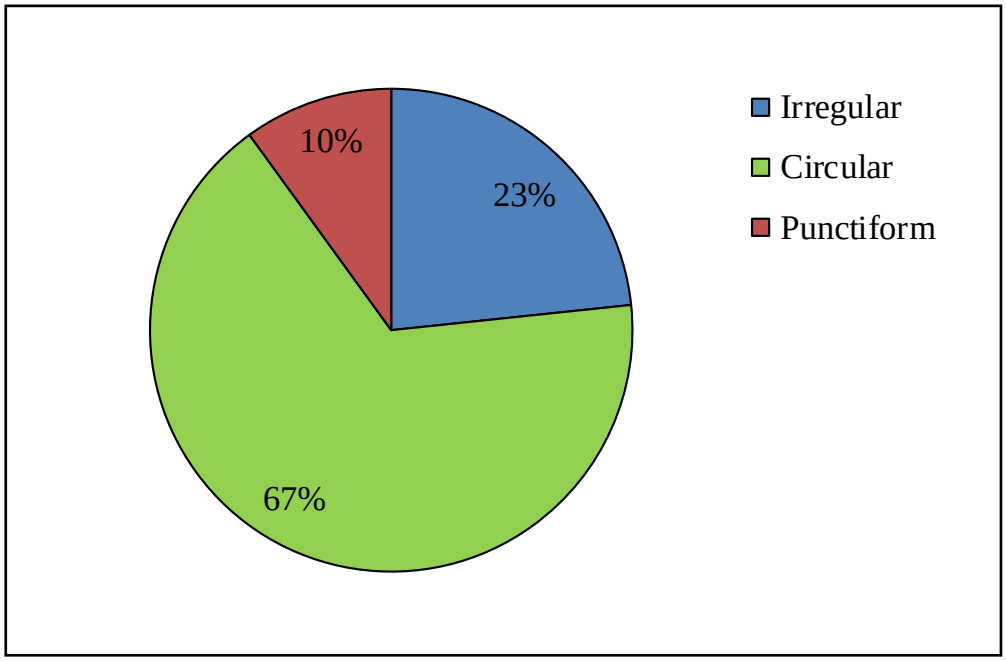


Fig. 3.9: Colony morphology with special reference to shape of colony of the isolated bacteria.

Table 3.5: Microscopic observation of the selected bacterial isolates.

Bacterial isolates	Vegetative cell	Spore	Sporangia	Gram reaction
S_3N-3	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_3N-6	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_3P-1	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_3P-2	Rod, occur singly, pair and in chain	Ellipsoidal	Not Swollen	+
S_3T-4	Rod, occur singly and in pair	Ellipsoidal	Swollen	+
S_3T-5	Rod, occur singly, pair and in chain	Ellipsoidal	Swollen	+
S_3T-7	Rod, occur singly	Ellipsoidal	Not Swollen	+
S_3T-8	Rod, occur singly	Ellipsoidal	Not Swollen	+
S_3T-9	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_4P-4	Rod, occur singly and in pair	Ellipsoidal	Swollen	+
S_8L-1	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_9N-1	Rod, occur singly and in pair	Ellipsoidal	Not Swollen	+
S_{10}P-2	Rod, occur singly	Ellipsoidal	Not Swollen	+
S_6P-1	Coccus, occur single, pair and in cluster	Non spore former		+
S_7N-1	Coccus, occur in cluster	Non spore former		+
S_9T-1	Coccus, occur single, pair and in cluster	Non spore former		+
S_1N-2	Short rod, occur singly and in pair	Non spore former		-
S_1N-7	Short rod, occur singly and in pair	Non spore former		-
S_1P-3	Short rod, occur singly and in pair	Non spore former		-
S_1P-4	Short rod, occur singly and in pair	Non spore former		-
S_1T-1	Short rod, occur singly and in pair	Non spore former		-
S_2T-1	Short rod, occur singly and in pair	Non spore former		-
S_4T-2	Short rod, occur singly and in pair	Non spore former		-
S_4T-5	Short rod, occur singly and in pair	Non spore former		-
S_5L-1	Short rod, occur singly and in pair	Non spore former		-
S_5L-2	Short rod, occur singly	Non spore former		-
S_6T-1	Short rod, occur singly and in pair	Non spore former		-
S_6L-1	Short rod, occur singly and in pair	Non spore former		-
S_{10}P-1	Short rod, occur singly and in pair	Non spore former		-
S_{10}T-8	Short rod, occur singly and in pair	Non spore former		-

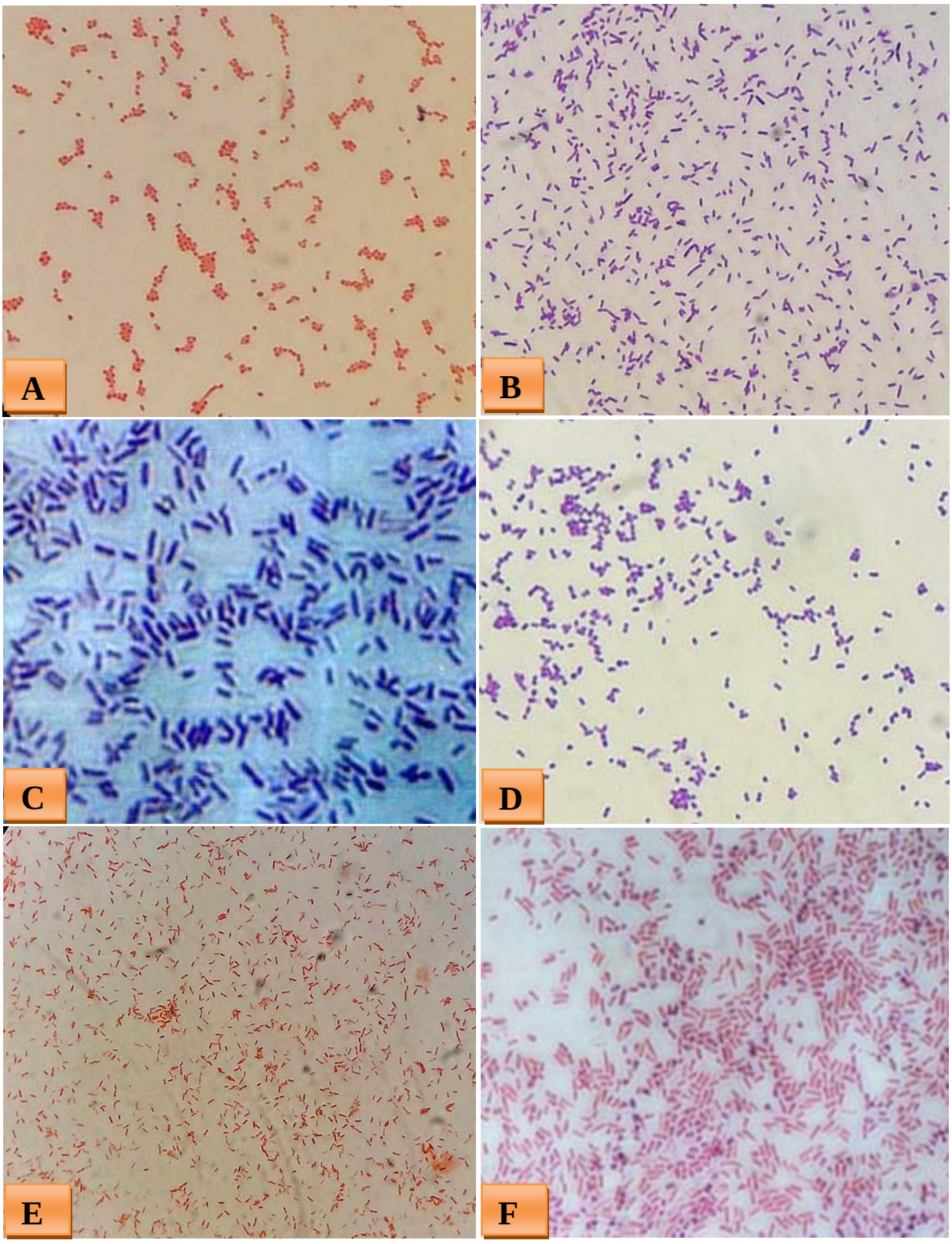


Fig. 3.10 (A-F): Photomicrographs of some isolates.

Simple staining (A. S_6P-1), Gram positive (B. S_4P-4, C. S_3T-9 and D. S_3N-6) and Gram negative (E. S_1P-4 and F. S_5L-1).

3.8 Physiological and biochemical characteristics of the selected bacterial isolates

The selected 30 bacterial isolates were studied for their major and important physiological and biochemical characteristics. The results of the fermentation tests with selected carbohydrates are shown in Table 3.6 and Fig. 3.11. Among the 30 isolates, ten isolates *viz.* S_1N-7, S_1T-1, S_2T-1, S_3T-4, S_3T-5, S_3T-8, S_3T-9, S_4P-4, S_8L-1 and S_{10}P-1 could ferment all the tested carbohydrates. But the isolates S_1P-3 and S_4T-5 could ferment none of the tested carbohydrates. Interestingly none of the selected isolates could produce gas during glucose fermentation.

The results of some major physiological and biochemical tests are shown in Table 3.7-3.9. Among 30 isolates, 14 showed positive in KOH solubility test. All the tested isolates were catalase positive and were negative in case of indole formation. There are 7 isolates showed positive in oxidase and 16 were positive in nitrate reduction. All isolates showed negative in H_2S production except S_{10}P-1. Motility test showed that 24 isolates were motile and 6 were non motile.

Table 3.8 showed VP, MR, hydrolysis of casein and starch, and deep glucose agar test. Among 30 bacterial isolates, 17 isolates showed positive result in VP and 15 isolates showed positive result in MR test. Out of 30 isolates, 8 were found to be starch hydrolysis positive and 20 isolates were positive in casein hydrolysis. Most of the isolates showed growth only at the surface of the deep glucose agar medium and thus considered as aerobes. Only two isolates (S_4P-4 and S_6T-1) were facultative anaerobes.

Some of the major physiological and biochemical characteristics of the selected isolates are shown in the Table 3.9. Among 30 bacterial isolates 8 showed positive in both citrate and propionate utilization test. Eight isolates showed positive results in tyrosine degradation, 27 positive in lecithinase production and 5 in levan. Among 14 Gram negative isolates, 4 isolates (S_1N-2, S_1N-7, S_4T-5 and S_{10}P-1) showed positive results in arginine dihydrolysis. Fig. 3.12 and 3.13 showed photographs of different physiological and biochemical test of the isolated bacteria.

Table 3.6: Fermentation test of the selected carbohydrates.

Bacterial Isolates	D-Glucose	L-Arabinose	D-Xylose	D-Mannitol
S_1N-2	A	-	-	-
S_1N-7	A	+	+	+
S_1P-3	-	-	-	-
S_1P-4	A	+	+	-
S_1T-1	A	+	+	+
S_2T-1	A	+	+	+
S_3N-3	A	-	-	-
S_3N-6	-	+	+	-
S_3P-1	A	+	+	-
S_3P-2	-	+	+	-
S_3T-4	A	+	+	+
S_3T-5	A	+	+	+
S_3T-7	A	+	+	-
S_3T-8	A	+	+	+
S_3T-9	A	+	+	+
S_4P-4	A	+	+	+
S_4T-2	A	+	+	-
S_4T-5	-	-	-	-
S_5L-1	A	-	+	-
S_5L-2	A	-	+	-
S_6P-1	A	-	-	-
S_6T-1	-	-	+	-
S_6L-1	A	+	+	-
S_7N-1	A	-	-	-
S_8L-1	A	+	+	+
S_9N-1	A	+	+	-
S_9T-1	A	-	+	-
S_{10}P-1	A	+	+	+
S_{10}P-2	A	+	-	-
S_{10}T-8	A	-	-	-

'+'= positive result, '-' = negative result and A=Acid

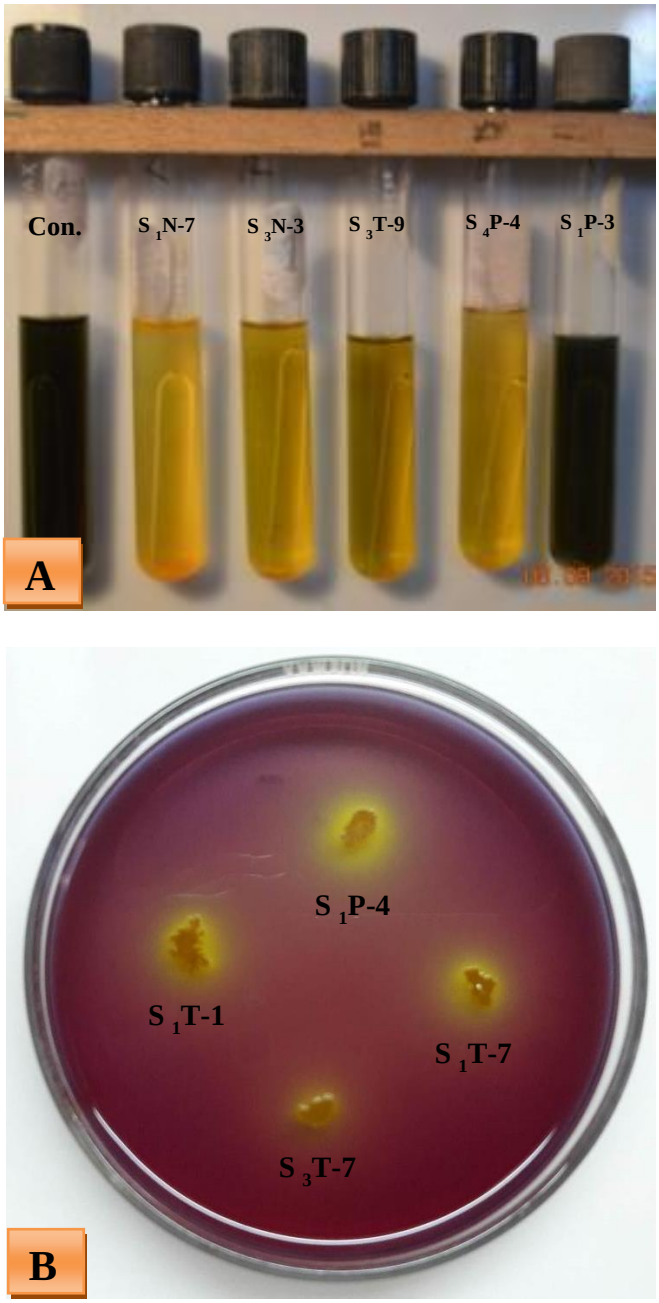


Fig 3.11: Photographs showing the fermentation test of the carbohydrates. (A) D-glucose and (B) D-xylose.

Table 3.7: Some major physiological and biochemical characteristics of the selected 30 bacterial isolates.

Bacterial isolates	KOH test	Catalase	Oxidase	Indole	Nitrate reduction	H_2S production	Motility
S_1N-2	+	+	-	-	-	-	Motile
S_1N-7	+	+	-	-	+	-	Motile
S_1P-3	+	+	-	-	-	-	Motile
S_1P-4	+	+	-	-	+	-	Non motile
S_1T-1	+	+	-	-	-	-	Non motile
S_2T-1	+	+	-	-	-	-	Non motile
S_3N-3	-	+	-	-	-	-	Motile
S_3N-6	-	+	-	-	-	-	Motile
S_3P-1	-	+	-	-	-	-	Motile
S_3P-2	-	+	-	-	-	-	Motile
S_3T-4	-	+	-	-	+	-	Motile
S_3T-5	-	+	-	-	+	-	Motile
S_3T-7	-	+	-	-	+	-	Motile
S_3T-8	-	+	-	-	+	-	Motile
S_3T-9	-	+	-	-	+	-	Motile
S_4P-4	-	+	-	-	+	-	Motile
S_4T-2	+	+	+	-	-	-	Motile
S_4T-5	+	+	+	-	+	-	Non motile
S_5L-1	+	+	+	-	+	-	Motile
S_5L-2	+	+	+	-	+	-	Motile
S_6P-1	-	+	-	-	+	-	Non motile
S_6T-1	+	+	+	-	-	-	Motile
S_6L-1	+	+	+	-	+	-	Motile
S_7N-1	-	+	-	-	+	-	Motile
S_8L-1	-	+	-	-	+	-	Motile
S_9N-1	-	+	-	-	-	-	Motile
S_9T-1	-	+	-	-	+	-	Motile
S_{10}P-1	+	+	+	-	-	+	Motile
S_{10}P-2	-	+	-	-	-	-	Motile
S_{10}T-8	+	+	-	-	-	-	Non motile

'+'= positive result and '-' = negative result

Table 3.8: Some major physiological and biochemical characteristics of the selected 30 bacterial isolates.

Bacterial isolates	VP test	MR test	Hydrolysis of casein	Hydrolysis of starch	Deep glucose agar test
S_1N-2	-	-	+	-	Strict aerobes
S_1N-7	+	+	+	-	Strictly aerobe
S_1P-3	+	+	-	-	Strictly aerobe
S_1P-4	-	-	-	-	Strictly aerobe
S_1T-1	-	-	-	-	Strictly aerobe
S_2T-1	-	-	-	-	Strictly aerobe
S_3N-3	+	+	+	-	Strictly aerobe
S_3N-6	+	+	+	-	Strictly aerobe
S_3P-1	+	+	+	-	Strictly aerobe
S_3P-2	+	+	+	-	Strictly aerobe
S_3T-4	+	+	+	+	Strictly aerobe
S_3T-5	+	+	+	+	Strictly aerobe
S_3T-7	+	+	+	+	Strictly aerobe
S_3T-8	+	+	+	+	Strictly aerobe
S_3T-9	+	-	+	+	Strictly aerobe
S_4P-4	+	+	+	+	Facultative anaerobe
S_4T-2	-	-	-	-	Strictly aerobe
S_4T-5	+	-	+	-	Strictly aerobe
S_5L-1	-	-	-	-	Strictly aerobe
S_5L-2	-	-	-	-	Strictly aerobe
S_6P-1	+	+	+	-	Strictly aerobe
S_6T-1	-	-	-	-	Facultative anaerobe
S_6L-1	-	-	-	-	Strictly aerobe
S_7N-1	-	+	+	-	Strictly aerobe
S_8L-1	+	-	+	+	Strictly aerobe
S_9N-1	-	-	+	-	Strictly aerobe
S_9T-1	+	+	+	-	Strictly aerobe
S_{10}P-1	-	-	+	-	Strictly aerobe
S_{10}P-2	+	+	+	+	Strictly aerobe
S_{10}T-8	-	-	-	-	Strictly aerobe

'+'= positive result and '-' = negative result

Table 3.9: Some major physiological and biochemical characteristics of the selected 30 bacterial isolates.

Bacterial isolates	Utilization of citrate	Utilization of Propionate	Degradation of tyrosine	Production of lecithinase	Levan test	Arginine dihydrolysis
S_1N-2	-	-	-	+	-	+
S_1N-7	+	-	-	+	+	+
S_1P-3	-	-	-	+	-	-
S_1P-4	+	+	+	+	-	-
S_1T-1	+	+	+	+	-	-
S_2T-1	+	+	+	+	-	-
S_3N-3	-	-	-	+	-	ND
S_3N-6	-	-	-	+	-	ND
S_3P-1	-	-	-	+	-	ND
S_3P-2	-	-	-	+	-	ND
S_3T-4	-	-	-	+		ND
S_3T-5	-	-	-	+	-	ND
S_3T-7	-	-	-	+	-	ND
S_3T-8	-	-	-	+	-	ND
S_3T-9	-	-	-	+	-	ND
S_4P-4	-	-	-	+	-	ND
S_4T-2	-	-	-	-	-	-
S_4T-5	-	-	-	+	-	+
S_5L-1	+	-	+	+	+	-
S_5L-2	+	-	+	+	+	-
S_6P-1	-	-	-	-	-	ND
S_6T-1	-	+	-	+	-	-
S_6L-1	+	+	+	+	-	-
S_7N-1	-	-	-	+	-	ND
S_8L-1	-	-	-	+	+	ND
S_9N-1	-	-	-	+	-	ND
S_9T-1	-	+	-	-	-	ND
S_{10}P-1	+	+	+	+	+	+
S_{10}P-2	-	-	-	+	-	ND
S_{10}T-8	-	+	+	+	-	-

'+'= positive result, '-' = negative result and ND=not done

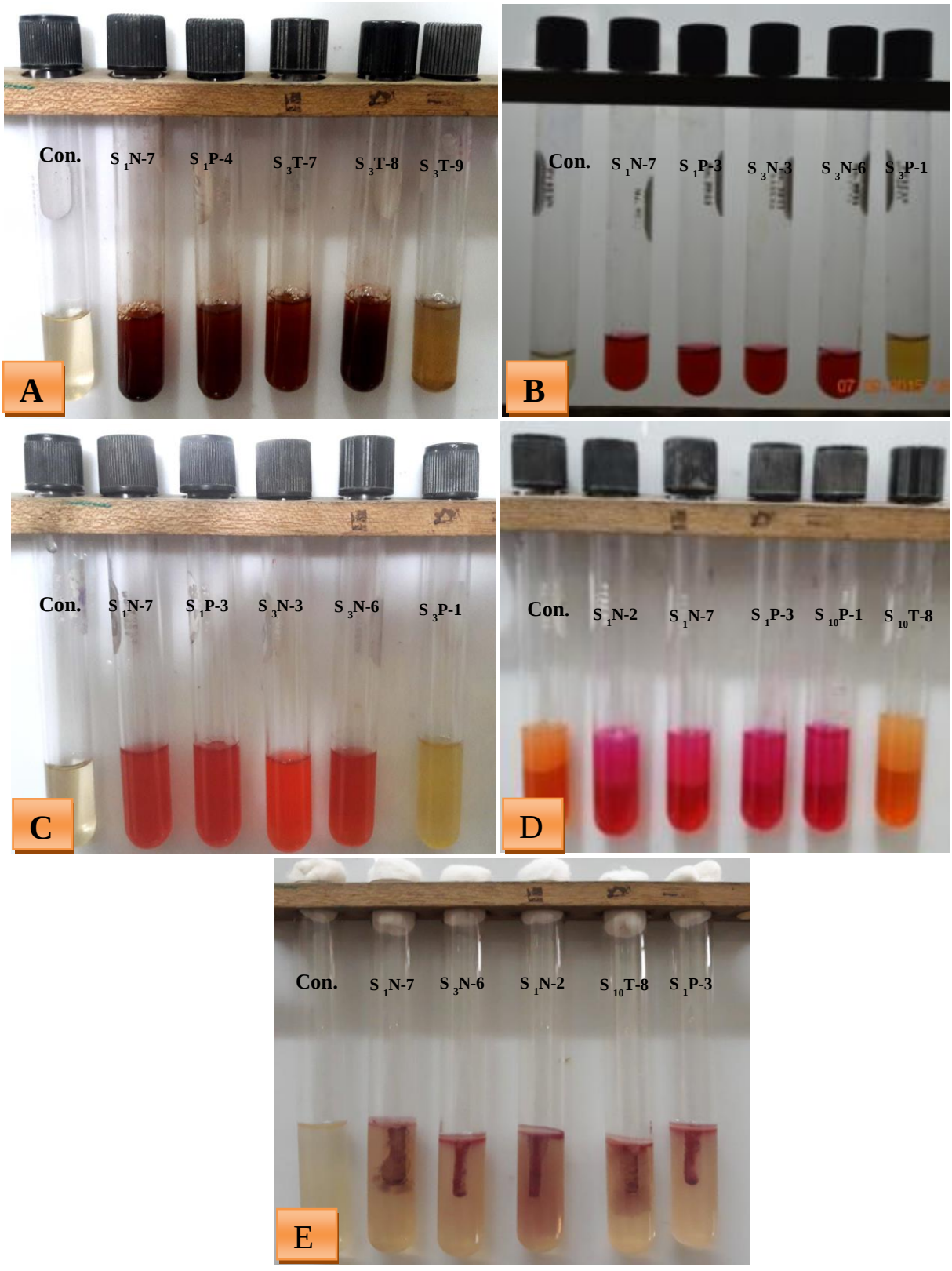


Fig. 3.12 (A-E): Photographs showing some physiological and biochemical tests. (A) Nitrate Reduction test, (B) VP test. (C) MR test (D) Arginine dihydrolysis and (E) Motility test.

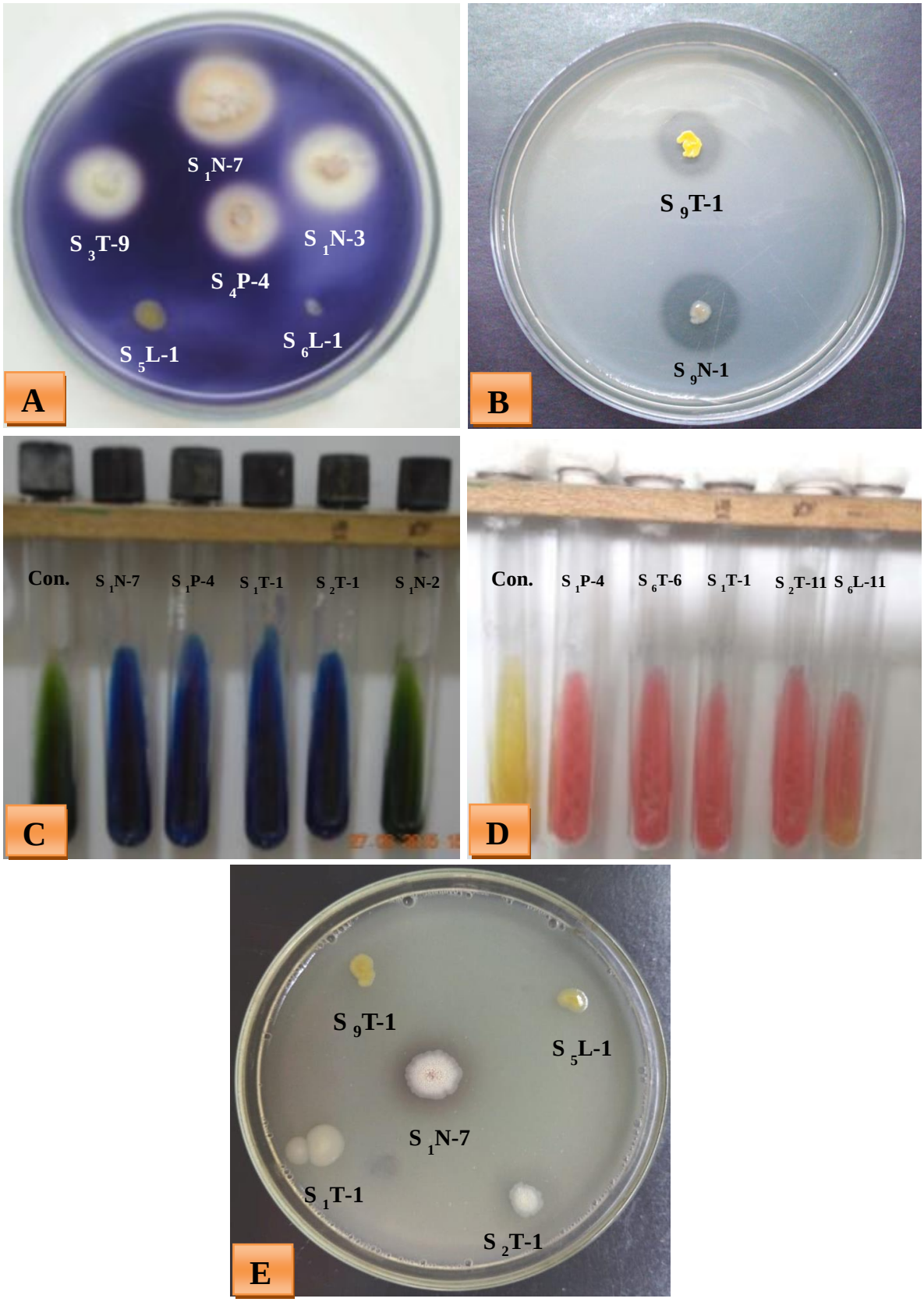


Fig. 3.13 (A-E): Photographs showing some physiological and biochemical tests. (A) Hydrolysis of starch, (B) Hydrolysis of Casein, (C) Citrate (D) Propionate and (E) Egg yolk lecithinase test.

For identification, 14 Gram negative isolates were tested for utilization of some carbohydrates. Results are shown in the Table 3.10. All the isolates showed positive result in utilization of sucrose and negative in case of sorbitol. Except the isolates S_4T-5 and S_1N-2, all were positive in case of utilization of mannose and raffinose. They were negative in rhamnose and inositol except S_1N-7 and S_4T-2. Among 14 isolates, 7 showed positive result in trehalose, 12 in galactose and 11 in cellobiose. The result clearly reflected that the tested bacteria showed different patterns of utilization of carbohydrates. The bacterial isolates S_1N-7 and S_4T-2 were found to be maximum (seven) carbohydrate utilizers. On the other hand, the isolate S_1P-3, S_4T-5 and S_5L-1 could utilize only four carbohydrates.

3.9 Identification of the isolates

After thorough characterization isolated bacterial isolates considered for both provisional and molecular identification.

3.9.1 Provisional identification of the selected bacterial isolates

Consulting all observed morphological, biochemical and physiological characters of the isolates, provisional identification was done with the help of Bergey's Manual of Systematic Bacteriology Vol.-1 (Krieg and Holt 1984) and Bergey's Manual of Systematic Bacteriology Vol.-2 (Sneath *et al*. 1986). Provisionally identified Gram positive bacteria are shown in the Table 3.11. Gram positive bacteria were the members of *Bacillus, Staphylococcus, Micrococcus* and *Planococcus*. Among them *Bacillus* was the dominating genus. The genus *Bacillus* comprises of various species matched with the standard species described in the Bergey's Manual of Systematic Bacteriology and the selected isolates were provisionally identified on the basis of resemblance to that of standard strains. *Bacillus subtilis* (5) were found to be dominating over *B. pumilus* (4), *B. polymyxa* (3) and *B. lentus* (1).

Table 3.12 shows the provisionally identified Gram negative bacterial isolates. There were five genera *viz*. *Acinetobacter, Acetobecter, Pseudomonas, Alcaligenes* and *Serratia.* Among Gram negative bacteria *Acinetobacter* was the dominating genus. *Serratia rubidaea* was the single species among the Gram negative bacterial isolates. Fig. 3.14 shows the percentage frequency of occurrence of the identified bacterial isolates of the lipid-rich environment.

Table 3.10: Utilization of some carbohydrates of the 14 selected Gram negative bacterial isolates.

Bacterial isolates	Mannose	Rhamnose	Raffinose	Trehalose	Galactose	Cellobiose	Inositol	Sorbitol	Sucrose	Number of positive results
S_1N-2	+	-	-	-	+	+	-	-	+	4
S_1N-7	+	-	+	+	+	+	+	-	+	7
S_1P-3	+	-	+	-	-	+	-	-	+	4
S_1P-4	+	-	+	-	+	+	-	-	+	5
S_1T-1	+	-	+	+	+	+	-	-	+	6
S_2T-1	+	-	+	+	+	+	-	-	+	6
S_4T-2	+	+	+	+	+	+	-	-	+	7
S_4T-5	-	-	+	+	-	+	-	-	+	4
S_5L-1	+	-	+	-	+	-	-	-	+	4
S_5L-2	+	-	+	+	+	-	-	-	+	5
S_6T-1	+	-	+	-	+	+	-	-	+	5
S_6L-1	+	-	+	-	+	+	-	-	+	5
S_{10}P-1	+	-	+	+	+	-	-	-	+	5
S_{10}T-8	+	-	+	-	+	+	-	-	+	5

‘+’= positive result and ‘-’ = negative result

Table 3.11: Provisional identification of 16 Gram positive bacterial isolates.

Bacterial isolates	Source	Sample	Provisionally identified names
S_3T-7	Kitchen Waste, Mirpur, Dhaka	Soil	*Bacillus subtilis*
S_3T-8	Kitchen Waste, Mirpur, Dhaka	Soil	*B. subtilis*
S_3T-9	Kitchen Waste, Mirpur, Dhaka	Soil	*B. subtilis*
S_8L-1	Navy Dockyard, Narayangang	Water	*B. subtilis*
S_9N-1	Edible Oil Mill, Elephant Road, Dhaka	Soil	*B. subtilis*
S_3N-3	Kitchen Waste, Mirpur, Dhaka	Soil	*B. pumilus*
S_3N-6	Kitchen Waste, Mirpur, Dhaka	Soil	*B. pumilus*
S_3P-1	Kitchen Waste, Mirpur, Dhaka	Soil	*B. pumilus*
S_3P-2	Kitchen Waste, Mirpur, Dhaka	Soil	*B. pumilus*
S_3T-4	Kitchen Waste, Mirpur, Dhaka	Soil	*B. polymyxa*
S_3T-5	Kitchen Waste, Mirpur, Dhaka	Soil	*B. polymyxa*
S_4 P-4	Tannery Industry, Hazaribagh, Dhaka	Water	*B. polymyxa*
S_{10}P-2	Kohinoor Chemicals Company Ltd., Tejgaon, Dhaka	Soil	*B. lentus*
S_7N-1	Navana Car Workshop, Tejgaon, Dhaka	Water	*Staphylococcus intermedius*
S_6P-1	The Buriganga River, Dhaka	Water	*Micrococcus lylae*
S_9T-1	Edible Oil Mill, Elephant Road, Dhaka	Soil	*Planococcus citreus*

Table 3.12: Provisional identification of 14 Gram negative bacterial isolates.

Bacterial isolates	Source	Sample	Provisionally identified names
S_1P-4	The Turag River, Gabtoli, Dhaka	Water	*Acinetobacter calcoacetius*
S_2T-1	Dairy Farm, Savar, Dhaka	Soil	*A. calcoacetius*
S_1T-1	The Turag River, Gabtoli, Dhaka	Water	*A. baumanni*
S_4T-5	Tannery Industry, Hazaribagh, Dhaka	Water	*A. lwoffi*
S_{10}T-8	Kohinoor Chemicals Company Ltd., Tejgaon, Dhaka	Soil	*A. johnsonii*
S_1N-2	The Turag River, Gabtoli, Dhaka	Water	*Acetobacter liquifaciens*
S_1P-3	The Turag River, Gabtoli, Dhaka	Water	*A. pasteurianus*
S_6L-1	The Buriganga River, Dhaka	Water	*A. aceti*
S_5L-1	Bus Depot, Kalyanpur, Dhaka	Water	*Pseudomonas pseudoalcaligenes*
S_5L-2	Bus Depot, Kalyanpur, Dhaka	Water	*P. pseudoalcaligenes*
S_{10}P-1	Kohinoor Chemicals Company Ltd., Tejgaon, Dhaka	Soil	*P. aeruginosa*
S_4T-2	Tannery Industry, Hazaribagh, Dhaka	Water	*Alcaligenes paradoxus*
S_6T-1	The Buriganga River, Dhaka	Water	*A. faecalis*
S_1N-7	The Turag River, Gabtoli, Dhaka	Water	*Serratia rubidaea*

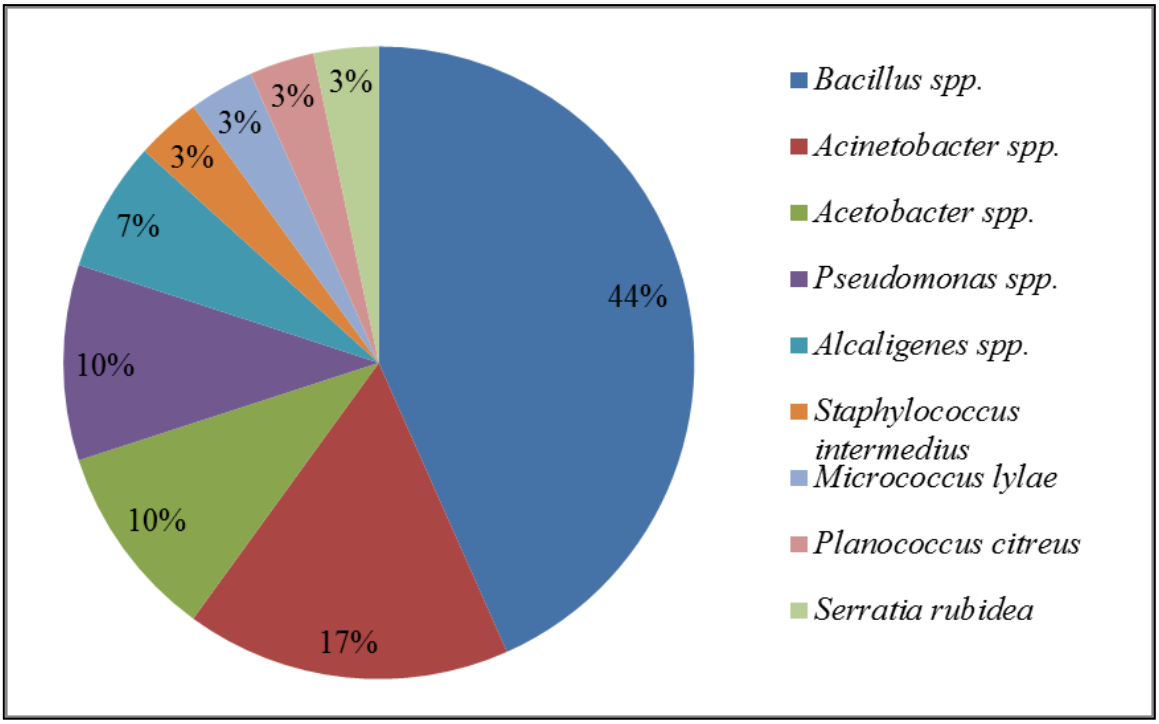


Fig. 3.14: Percentage frequency of occurrence of the identified bacterial isolates.

3.9.2 Molecular identification of the selected bacterial isolates

Among 30 bacterial isolates, potential 10 lipase producers were selected for molecular identification and enzyme production. Selection was done based on zone ratio and intensity of clear zone in TBA medium. Species diversity was also considered for final selection.

Using a pair of bacterial universal primer the 16S rRNA gene was amplified from 10 better lipase activity showing bacterial isolates. The PCR amplified DNA of the 10 isolates (S_1N-2 = lane 1, S_1N-7= lane 2, S_3P-1= lane 3, S_3T-5= lane 4, S_3T-9= Lane 5, S_4P-4= lane 6, S_7N-1= lane 7, S_{10}P-1= lane 8, S_{10}P-2= lane 9 and S_{10}T-8= Lane 10) and 100 bp ladder (lane M) were gel purified and automatically sequenced (Fig. 3.15). The sequences obtained were used for BLAST and rRNA BLAST search to find out possible correct match present in the data base using the following links: http://blast.ncbi.nlm.nih.gov/
and http://bioinformatics.psb.ugent.be/cgi-bin/rRNA/blastform.cgi, respectively (Fig. 3.16-3.25).

Followed by genomic DNA extraction from ten isolates, primers CC(F) and CD(R) were used to amplify 16S rDNA sequences from bacterial genomic DNA. PCR products were sequenced and BLAST (NCBI) searches were conducted on the DNA sequence to compare the different 16S rDNA sequences and determine the most closely related species. Molecular identification of 10 bacterial isolates is shown in Table 3.13.

3.10 Comparison between provisional and molecular identification

A comparative analysis was done in between provisional and molecular identification of 10 isolates as shown in Table 3.14. Among 10 isolates, 9 genera were matched with their provisional identification. Molecularly identified *Stenotrophomonas maltophilia* differ with its provisional identification. It was provisionally identified as *Acetobacter liquifaciens.* The results revealed that the provisional identification based on morphological, physiological and biochemical characters were found to be valid to some extent.

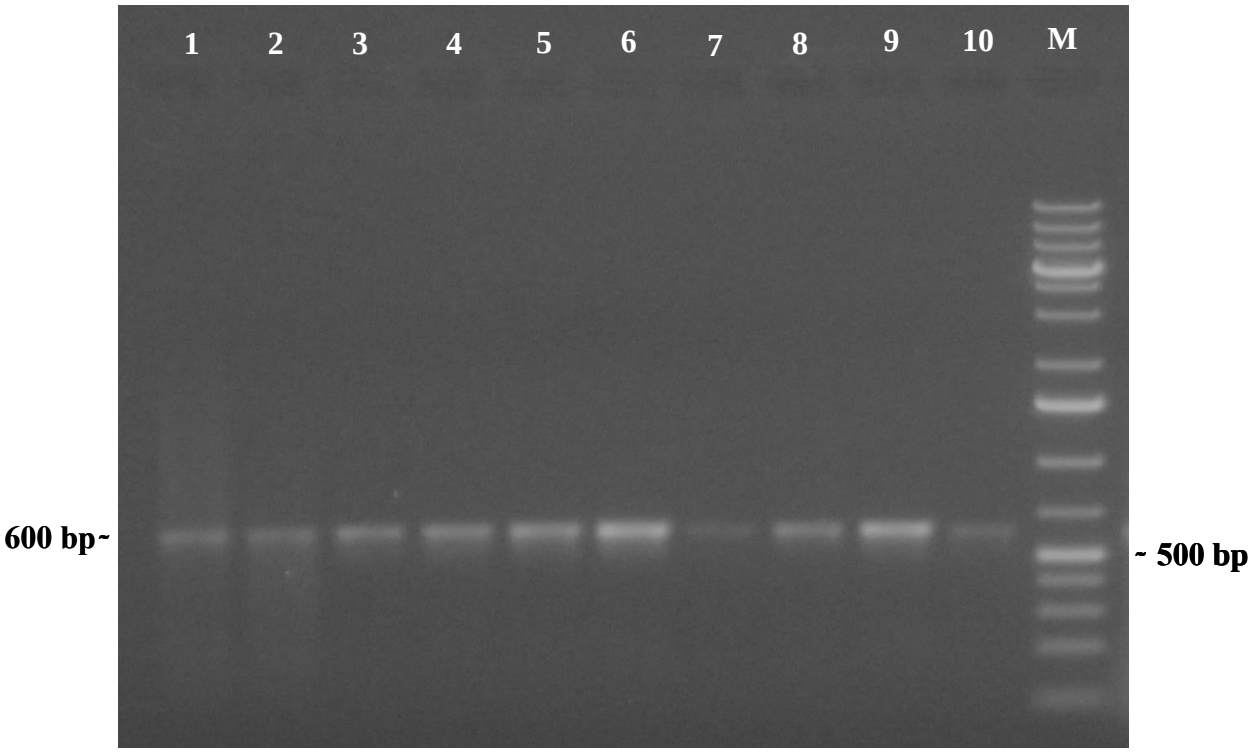


Fig. 3.15: PCR amplification of part of the 16S rRNA gene.

Lanes 1-10 are representing 10 different bacterial isolates *viz*. S_1N-2, S_1N-7, S_3P-1, S_3T-5, S_3T-9, S_4P-4, S_7N-1, S_{10}P-1, S_{10}P-2, S_{10}T-8 and M=100 bp ladder. In the gel size of the amplified DNA bands were approximately 600bp.

TTCCAGACTCCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG
GCGCAAGCCTGATCCAGCCATACCGCGTGGGTGAAGAAGGCCTTTCGG
GTGTAAAGCCCTTTTGTTGGGAAAGAAATCCCAGCTGGCTAATACCCG
GTTGGGATGACGGTACCCAAAGAATAAGCACCGGCTAACTTCGTGCCA
GCAGCCGCGGTAATACGAAGGGTGCAAGCGTTACTCGGAATTACTGG
GCGTAAAGCGTGCGTAGGTGGTCGTTTAAGTCCGTTGTGAAAGCCCTG
GGCTCAACCTGGGAACTGCAGTGGATACTGGGCGACTAGAGTGTGGT
AGAGGGTAGCGGAATTCCTGGTGTAGCAGTGAAATGCGTAGAGATCA
GGAGGAACATCCATGGCGAAGGCAGCTACCTGGACCAACACTGACAC
TGAGGCACGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAG
TCCACGCCCTAAACGATGCGAACTGGATGTTTTGTGCAATTTGGCACG
CAGTATCGAAGCTAACGCGTTAAGTCGCCGCCTGGGAGTACGTCGCAA
GACAGTTAGG

Fig. 3.16: 16S rRNA partial sequence of isolate S_1N-2.

TCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGC
GCAAGCCTGATGCAGCCATGCCGCGTGTGTGAAGAAGGCATTCGGGTT
GTAAAGCACTTTCAGCGAGGAGGAAGGTGGTGAACTTAATACGTTCAT
CAATTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCA
GCCGCGGTAATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCG
TAAAGCGCACGCAGGCGGTTTGTTAAGTCAGATGTGAAATCCCCGAGC
TTAACTTGGGAACTGCATTTGAAACTGGCAAGCTAGAGTCTCGTAGAG
GGGGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAG
GAATACCGGTGGCGAAGGCGGCCCCCTGGACGAAGACTGACGCTCAG
GTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGTCGATTTGGTGTTTCCCTTGAGGCGTGGCTTCCG
GAGCTAACGCGTAAATCGACCGCCTGGGATACGCCGCAAGTAGCT

Fig. 3.17: 16S rRNA partial sequence of isolate S_1N-7.

TCCAGACCTCCCTCGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGAC
GAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGAT
CGTAAAGCTCTGTTGTTAGGGAAGAACAAGTGCGAGAGTAACTGCTCG
CACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCG
TAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGC
TCAACCGGGGAGGGTCATTGGAAACTGGGAAACTTGAGTGCAGAAGA
GGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGA
GGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAG
GAGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCA
CGCCGTAAACGATGAGTGCTAAGTGTTTTTGGGTTTCCGCCCCTTAGTG
CTGCAGCTAACGCATAAGCACTCCGCCTGGGATACGTCGCAGACGTAC
G

Fig. 3.18: 16S rRNA partial sequence of isolate S_3P-1.

CCAGACTCCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGGACG
AAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCGGATCG
TAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAGGGCGGT
ACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGCA
GCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATTATTGGGCGT
AAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAAGCCCCCGGCT
CAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGAGTGCAGAAGAG
GAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGTAGAGATGTGGAG
GAACACCAGTGGCGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGG
AGCGAAAGCGTGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCAC
GCCGTAAACGATGAGTGCTAAGTGTTTTGGGGTTTCCGCCCCTTAGTGC
TGCAGCTAACGCATAAGCACTCCGCCTGGGAGTACGTCGCAGACATGC
G

Fig. 3.19: 16S rRNA partial sequence of isolate S_3T-5.

GTTTGTTTTTCGGCATGGAGTAGTCTGACGGAGCACGCCGCGTGAGT
GATGAAGGTTTTCGGATCGTAAAGCTCTGAAAAGGGAAGAACAAGT
ACCGTTCGAATAGGGCGGTACCAAGACGGTACCTAACCAGAAAGCC
ACGGCTAACTACGTGCCAGCAGCCGCGGTAATACGTAGGTGGCAAG
CGTTTTTTTCGACTTATTGAGGGCGTAAAGGGCTCGCAGGCCGGTTT
CTTAAGTCTGATGTTGAAAGCCCCCGGGCTCAACCGGGGGAGGGTC
ATTTGAAACTGGGGAACTTGAGTGGCGGAAGAAGGAGAATGGAAAT
CCCCCGTGAGCGGTGAAAGGCTAAAAGATGTGGAAGGAAACCAGTG
GCGAAGGCACTCTTTGGTCTGGTACTGGACCTTAAGAACCAAACGGG
GGGGGGGAACAGAATAGATACCCTGTAGTCCCGCGTAACGATGTGG
TTAGTGTTAAGGGGTTCCCCTTTATGCTGCGCTACGTATAGCATCCGC
TGAGAATCCGTCGAGATGAATCCAGGATTACGGGCCGCTCATAGAA

Fig. 3.20: 16S rRNA partial sequence of isolate S_3T-9.

TTTCAACAGTAGGGATTCTTCCCCAAGGCAGAAATCTGTAGGGACCA
CCGCCCCGGTATTATGAAGTTTTCGGTTGGAAAGCTCCTTTTTTGGGA
AAAACAAGTACCGTTCGAATTGGGCGGTACCTTGACGGTACCTTACC
AGAAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGG
TGGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGGGCTCGCAGGCG
GTTTCTTAAGTCTGATGTGAAAGCCCCCGGCTCAACCGGGGAGGGTC
ATTGGAAACTGGGGAACTTGAGTGCAGAAGAGGAGAGTGGAATTCC
ACGTGTAGCGGTGAGGATACATAGAGATGTGGAGGAACACCAGTGG
CGAAGGCGACTCTCTGGTCTGTAACTGACGCTGAGGAGCGAAAGCG
TGGGGAGCGAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTTGCTAAGTGTTAGGGGGTTCCGCCCCTTAGTGCTGCAGCTAA
CGCATAAGCATCCGCCTGGCCCACTTCGCAAGGGGCTTC

Fig. 3.21: 16S rRNA partial sequence of isolate S_4P-4.

TTCCAGCTTCCTACGGAGGCACCAGTAGGAATCTCCGCCAAGGGCGA
AAGCCTGACGGAGCAACGCCCGTGAGTATGAAGGTCTCGATCGTAA
ACTTCTGTATTAGGGAGGAACATACGTGTAAGTAACTATGCACGTCT
TGACGGTACCTAATCAGAAAGCCACGGCTAACTACGTGCCAGCAGC
CGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGA
AAGCGCGCGTAGGCGGTTTTTTAAGTCTGATGTGAAAGCCCACGGCT
CAACCGTGGAGGGTCATTGGAAACTGGAAAACTTGAGTGCAGAAGA
GGAAAGTGGAATTCCATGTGTAGCGGTGAAATGCGCAGAGATATGG
AGGAACACCGGGAAAAGAAGGCGACTTTCTGGTCTGTAACTGACGC
TGATGTGCGAAAGCGTGGGGATCAAACAGGATTAGATCCCCTGGTA
GTCCACGCCGTAAACGATGAGTGCTAATTGTTTGTGGTTTCCGCCCC
TTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGTAGTACTTTTT
TTAACGTTTTTTTTT

Fig. 3.22: 16S rRNA partial sequence of isolate S_7N-1.

TTCCCAATTCCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATG
GGGCGAAAGCTTGATCCAGCCATGCCGCGTGTGTGAAGAAAGGTCT
TCGGATTGTAAAAGCACTTTAAGTTGGGAGGAAGGGCAGTAAGTTA
ATACCTTGCTGTTTTGACGTTACCAACAGAATAAGCACCGGCTAACT
TCGTGCCAGCAGCCGCGGTAATACGAAGGGTGCAAGCGTTAATCGG
AATTACTGGGCGTAAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGT
GAAATCCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCT
AGAGTACGGTAGAGGGTGGTGGAATTTCCTGTGTAGCGGTGAAATG
CGTAGATATAGGAAGGAACACCAGTGGAGAAGGCGACCACCTGGAC
TGATACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTA
GATACCCTGGTAGTCCACGCCGTAAACGATGTCGACTAGCCGTTTGA
TCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTG
GGATACGCCGCAAAGTATGCT

Fig. 3.23: 16S rRNA partial sequence of isolate S_{10}P-1.

TCCAGACTTCCCTACGGGAGGCAGCAGTAGGGAATCTTCCGCAATGG
ACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGATGAAGGTTTTCG
GATCGTAAAGCTCTGTTGTTAGGGAAGAACAAGTACCGTTCGAATAG
GGCGGTACCTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGTGGCAAGCGTTGTCCGGAATT
ATTGGGCGTAAAGGGCTCGCAGGCGGTTTCTTAAGTCTGATGTGAAA
GCCCCCGGCTCAACCGGGGAGGGTCATTGGAAACTGGGGAACTTGA
GTGCAGAAGAGGAGAGTGGAATTCCACGTGTAGCGGTGAAATGCGT
AGAGATGTGGAGGAACACCAGTGGCGAAGGCGACTCTCTGGTCTGT
AACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATTAGAT
ACCCTGGTAGTCCACGCCGTAAACGATGAGTGCTAAGTGTTTGGGGG
TTCCGCCCCTTAGTGCTGCAGCTAACGCATTAAGCACTCCGCCTGGG
ATACGGCGCAGACGTTAGG

Fig. 3.24: 16S rRNA partial sequence of isolate S_{10}P-2.

TCCAGACTCCCTACGGGAGGCAGCAGTGGGGAATATTGGACAATGG
GCGGAAGCCTGATCCAGCCATGCCGCGTGTGTGAAGAAGGCCTTTTG
GTTGTAAAGCACTTTAAGCGAGGAGGAGGCTACTAGTACTAATACTA
CTGGATAGTGGACGTTACTCGCAGAATAAGCACCGGCTAACTCTGTG
CCAGCAGCCGCGGTAATACAGAGGGTGCGAGCGTTAATCGGATTTA
CTGGGCGTAAAGCGTGCGTAGGCGGCTGATTAAGTCGGATGTGAAA
TCCCTGAGCTTAACTTAGGAATTGCATTCGATACTGGTCAGCTAGAG
TATGGGAGAGGATGGTAGAATTCCAGGTGTAGCGGTGAAATGCGTA
GAGATCTGGAGGAATACCGATGGCGAAGGCAGCCATCTGGCCTAAT
ACTGACGCTGAGGTACGAAAGCATGGGGAGCAAACAGGATTAGATA
CCCTGGTAGTCCATGCCGTAAACGATGTCTACTAGCCGTTGGGGCCT
TGAGGCTTTAGTGGCGCAGCTAACGCGATAAGTAGACCGCCTGGGA
GTAGCGTCGCAAGTAATTCCT

Fig. 3.25: 16S rRNA partial sequence of isolate S_{10}T-8.

Table 3.13: Molecular identification of 10 bacterial isolates.

Bacterial isolates	Molecular identification			
	Scientific name	Strain	Max score	Identity match (%)
S_1N-2	*Stenotrophomonas maltophilia*	e-a22	1013	98
S_1N-7	*Serratia rubidaea*	9B	672	99
S_3P-1	*Bacillus pumilus*	Ja02	996	98
S_3T-5	*Bacillus* sp.	BTMASC2	1024	99
S_3T-9	*Bacillus subtilis*	20B	619	89
S_4P-4	*Bacillus subtilis*	HRBS-10TDI13	784	95
S_7N-1	*Staphylococcus epidermidis*	6E02	869	95
S_{10}P-1	*Pseudomonas aeruginosa*	12	982	98
S_{10}P-2	*Bacillus subtilis*	CI1	1016	99
S_{10}T-8	*Acinetobacter johnsonii*	372	1024	99

Table 3.14: Comparison between provisional and molecular identification of 10 bacterial isolates.

Bacterial isolates	Provisional identification	Molecular identification
S_1N-2	*Acetobacter liquifaciens*	*Stenotrophomonas maltophilia*
S_1N-7	*Serratia rubidaea*	*Serratia rubidaea*
S_3P-1	*Bacillus pumilus*	*Bacillus pumilus*
S_3T-5	*Bacillus polymyxa*	*Bacillus* sp.
S_3T-9	*Bacillus subtilis*	*Bacillus subtilis*
S_4P-4	*Bacillus polymyxa*	*Bacillus subtilis*
S_7N-1	*Staphylococcus intermedius*	*Staphylococcus epidermidis*
S_{10}P-1	*Pseudomonas aeruginosa*	*Pseudomonas aeruginosa*
S_{10}P-2	*Bacillus lentus*	*Bacillus subtilis*
S_{10}T-8	*Acinetobacter johnsonii*	*Acinetobacter johnsonii*

3.11 Phylogenetic tree

Phylogenetic tree of three isolates (*Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B) were generated using neighbor joining (NJ) a distance based algorithm of phylogenetic analysis based on 16S rRNA sequences previously acquired (Fig. 3.26-3.28). In the phylogenetic tree it could be seen that the closely related bacterial strains were grouped together while strains having distant relationships were placed separately.

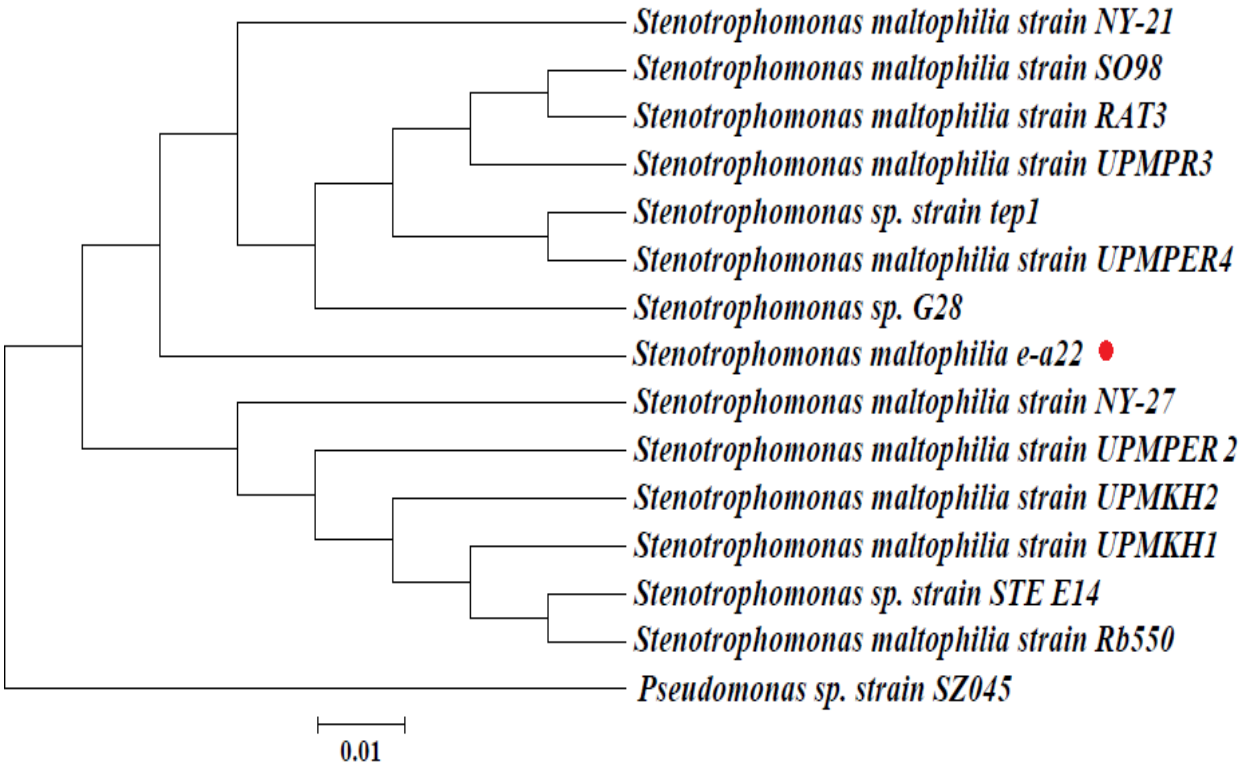


Fig. 3.26: A neighbor-joining phylogenetic tree of *Stenotrophomonas maltophilia*.

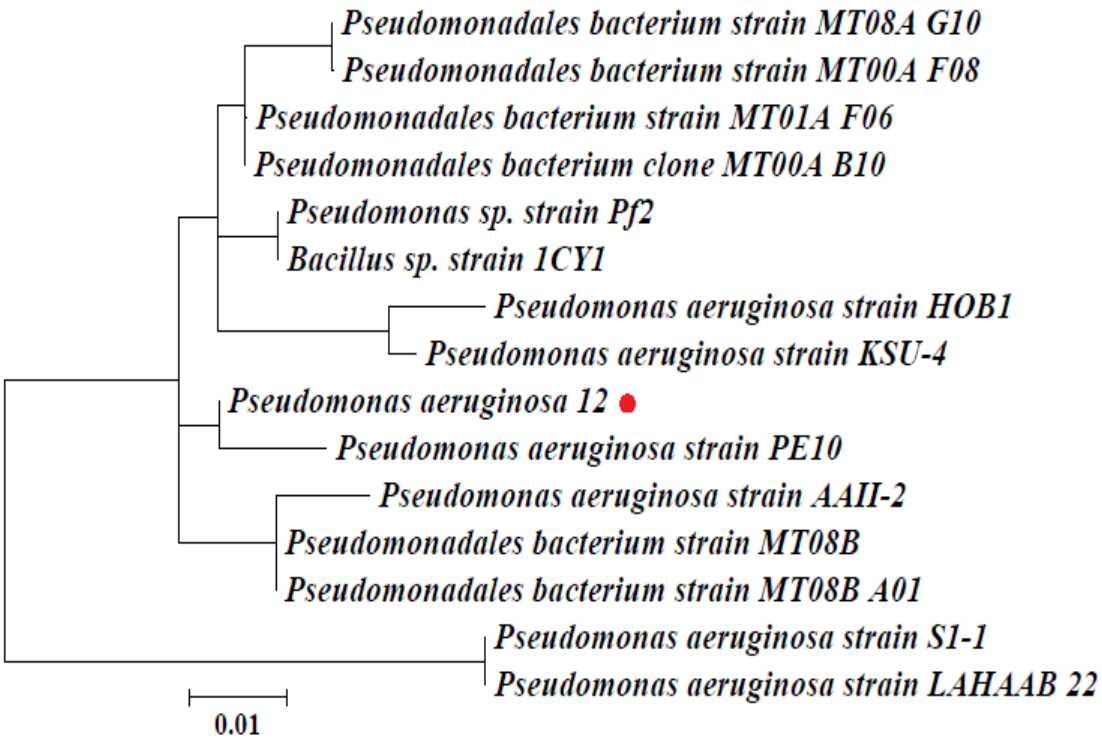


Fig. 3.27: A neighbor-joining phylogenetic tree of *Pseudomonas aeruginosa*.

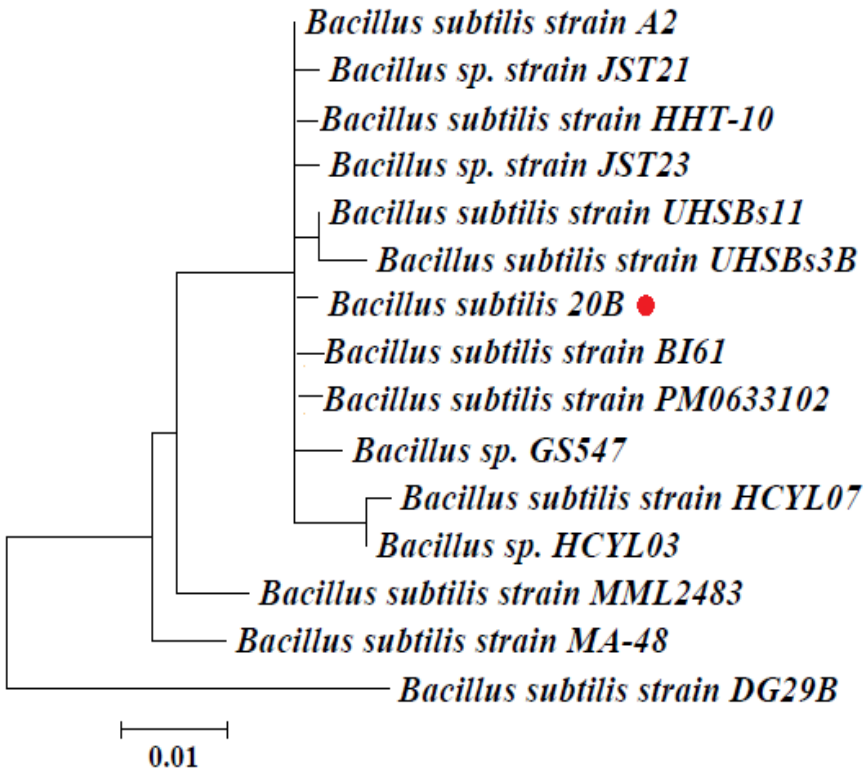


Fig. 3.28: A neighbor-joining phylogenetic tree of *Bacillus subtilis*.

3.12 Lipase production and estimation of lipase activity

Isolated indigenous bacteria from lipid-rich environment were tested for lipase production of commercial interest as well as lipid-rich wastewater management. Lipase production test was carried out in Erlenmeyer flask. Inoculated flasks were incubated at 37 °C for 96 h and samples were analyzed in every 24 h intervals. In this test olive oil used as inducer for lipase production and it releases oleic acid as free fatty acid. For estimation of lipase activity oleic acid standard curve was prepared.

Oleic acid standard curve was prepared by using a series of oleic acid concentration (2-10 µmole). Standard curve was carried out by plotting the concentration of oleic acid in X axis against the absorbance in Y axis (Fig. 3.29). Absorbance was determined at 715 nm. Result showed that an increase in absorbance with the increase of the concentration, giving a linear line. From this curve it could be found a linear line equation is $y=0.1179x + 0.0682$.

The comparative study of the isolates for lipase production was carried out in TSB medium at 37 °C. Lipase production and estimation of lipase activity are shown in Fig. 3.30. The potentiality of 10 better lipolytic activity showing bacterial isolates (6 Gram positive and 4 Gram negative) was examined for lipase producing test. The enzyme producing capabilities of Gram positive isolates ranged in between 0.89±0.63 and 13.50±0.14 U/ml and in case of Gram negative isolates it was in between 1.28±0.25 and 26.89±2.68 U/ml. The Gram negative *Stenotrophomonas maltophilia* strain e-a22 was found to be the most potent isolate for lipase production which could produce lipase up to 26.89±2.68 U/ml at 72 h of incubation and the isolated Gram positive *Bacillus pumilus* JaO2 was the lowest potent isolate with lipase activity 0.89±0.63 U/ml at 96 h of incubation.

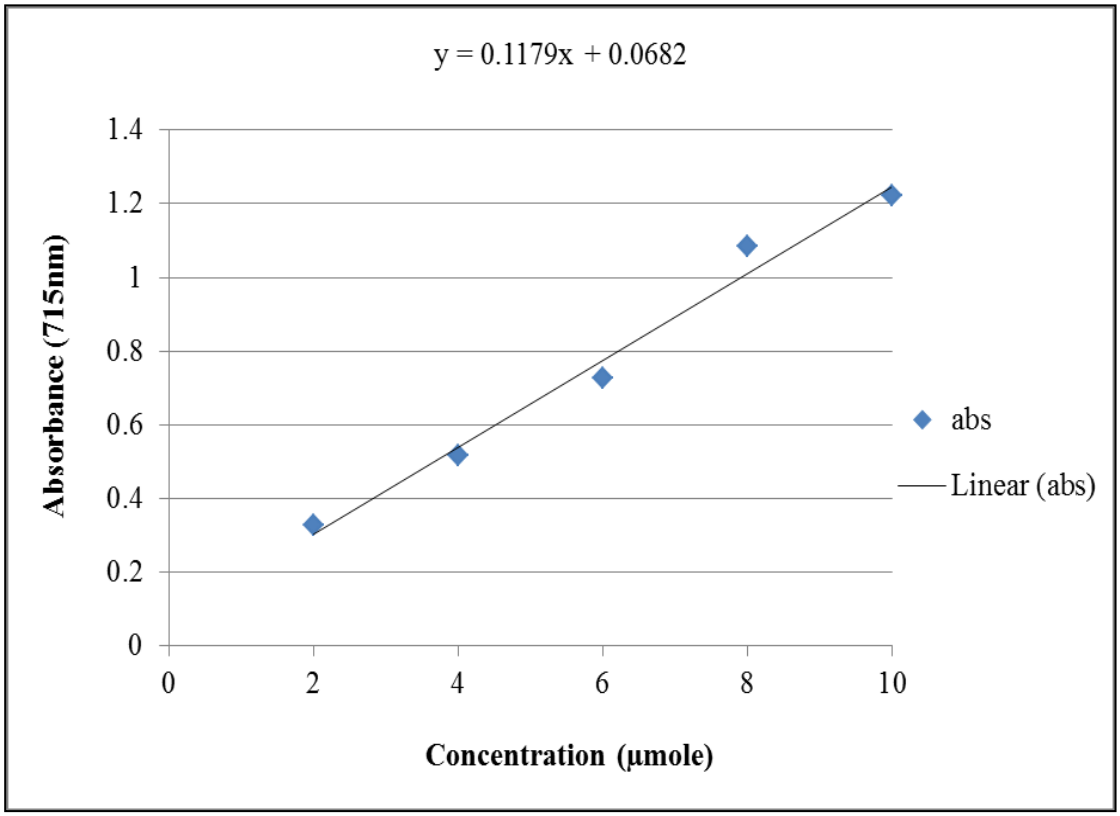
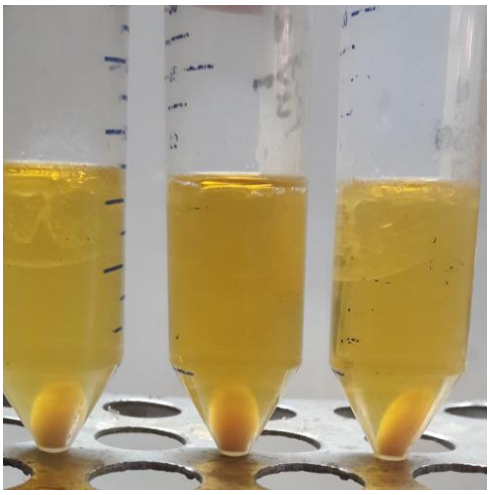
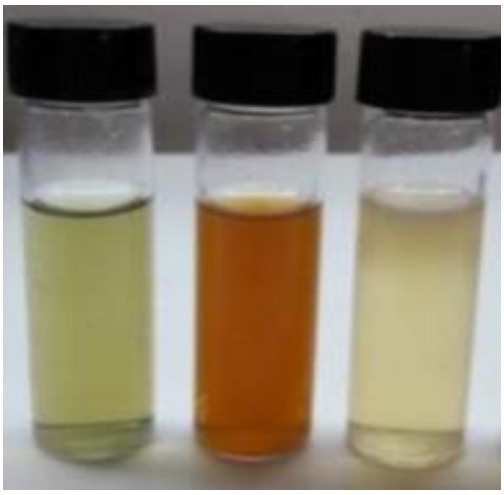


Fig. 3.29: Standard curve of oleic acid.

Control **(A) Production of enzyme**



(B) After centrifugation



(C) Crude enzyme (Supernatant)

(D) Estimation of enzyme activity

Fig. 3.30 (A-D): Enzyme production and estimation of enzyme activity.

Lipase activity of 6 Gram positive bacterial isolates is shown in Fig. 3.31. *Bacillus subtilis* 20B, *Bacillus pumilus* JaO2 and *Staphylococcus epidermidis* 6EO2 showed maximum lipase activity at 24 h of incubation and then lipase activity gradually decreases. The result clearly indicated increased amount of lipase production was observed by the two isolates (*Bacillus subtilis* CI1 and *Bacillus subtilis* HRBS-10TDI13) with the period of incubation up to 48 h. *Bacillus* sp. BTMASC2 showed maximum lipase activity at 72 h of incubation and then lipase activity decreased.

Lipase activity of 4 Gram negative bacterial isolates is shown in Fig. 3.32. *Pseudomonas aeruginosa* 12 and *Acinetobacter johnsonii* 372 showed the highest lipase production at 24 h of incubation. *Serratia rubidaea* 9B showed maximum lipase activity at 48 h of incubation and then decreased lipase activity was found. The result clearly indicated increased amount of lipase production was observed by the isolate *Stenotrophomonas maltophilia* e-a22 with the period of incubation up to 72 h.

Among the 10 bacterial isolates, 3 isolates *viz. Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B showed enzyme activity higher than 10 U/ml and were chosen for further study towards optimization of lipase production and synthetic wastewater treatment in the laboratory.

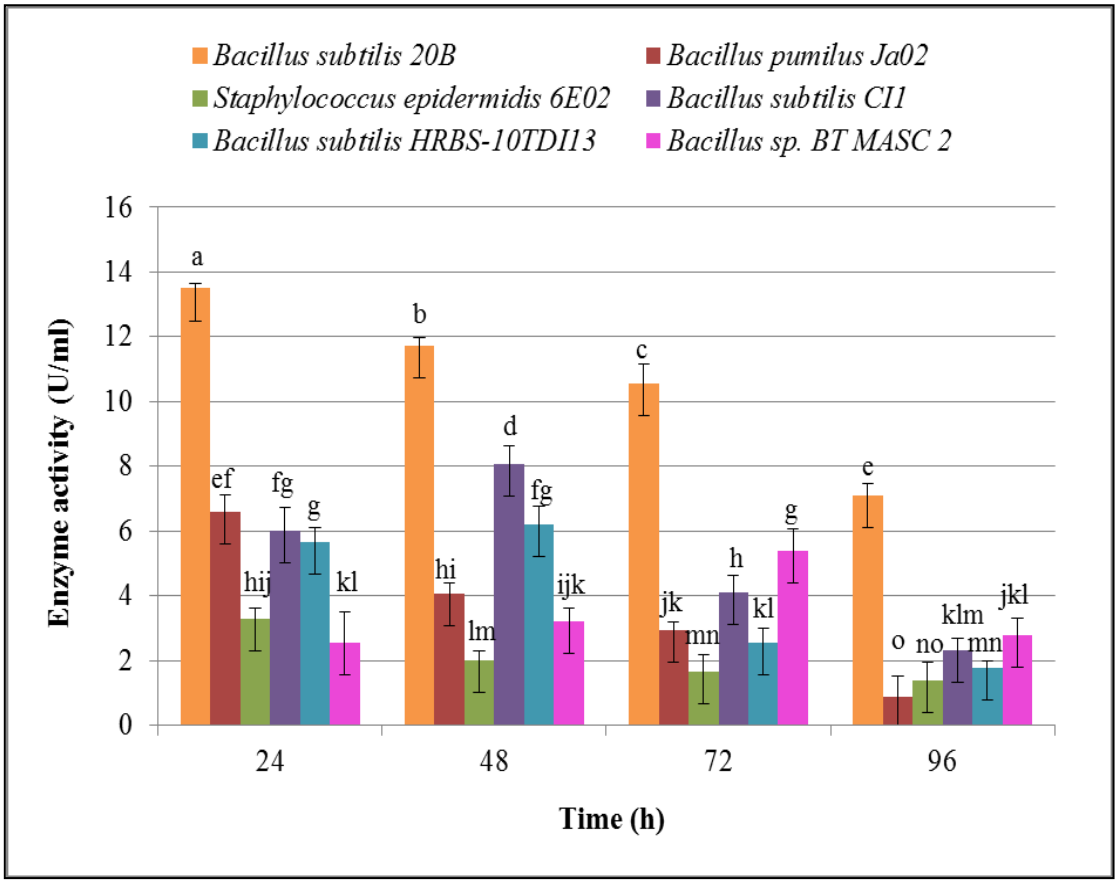


Fig. 3.31: Enzyme activity of 6 Gram positive bacterial isolates.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

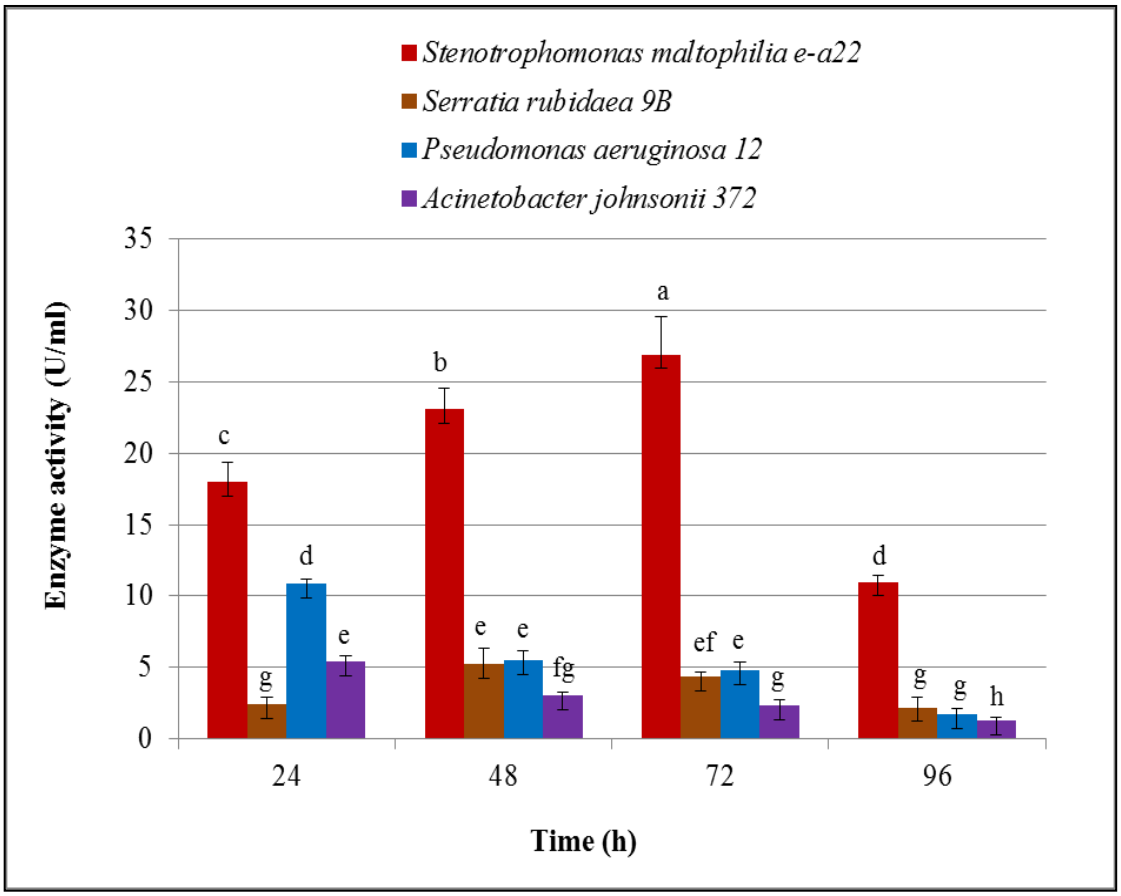


Fig. 3.32: Enzyme activity of 4 Gram negative bacterial isolates.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

3.13 Optimization of lipase production

Optimization of lipase production was carried out at 48 h of incubation using different parameters (pH, temperature, NaCl, carbon, nitrogen source, inoculum concentration, olive oil concentration and media). Three isolates *viz*. *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B were selected for optimization.

3.13.1 Effects of pH on lipase production

Initial pH of the production medium is one of the most critical parameters affecting both growth and lipase production. Lipase production was tested against different pH ranging from 5 to 9. The result clearly indicated that all three isolates showed the highest activity at pH 7 (Fig. 3.33).

Maximum lipase activity was 23.11±1.42, 5.50±0.72 and 11.72±0.25 U/ml by *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa and Bacillus subtilis,* respectively at pH 7 while minimum activity 2.33±0.34, 1.72±0.25 and 1.78±0.42 U/ml obtained at pH 5. Therefore, it could be concluded that the pH 7 would be optimum for the lipase production for the isolated bacteria.

3.13.2 Effects of temperature on lipase production

Temperature would be one of the important parameters for better lipase production. The effect of temperature was carried out against different temperatures (20, 30, 37, 40 and 50 °C). Fig. 3.34 shows the effect of temperature on lipase production. Results revealed that all three isolates gave maximum production of lipase at temperature of 37 °C.

The incubation temperature revealed that increasing the incubation temperature up to 37 °C resulted an increasing enzyme activity up to 23.11±1.42, 5.50±0.72 and 11.72±0.25 U/ml, respectively. *Stenotrophomonas maltophilia* and *Bacillus subtilis* showed the lowest activity (3.11±0.51 and 1.28±0.35 U/ml) at 20 °C whereas *Pseudomonas aeruginosa* showed the lowest activity (1.89±0.26 U/ml) at 50 °C. The activity drops rapidly above 40 °C in all three isolates.

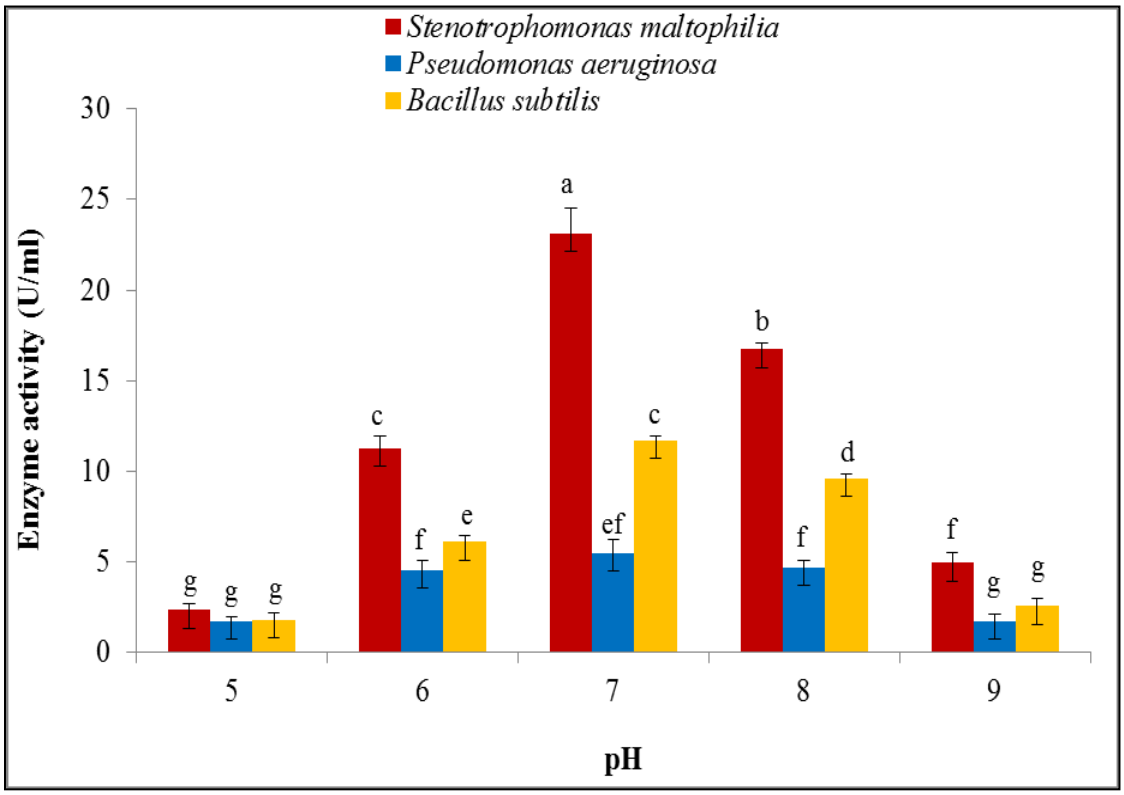


Fig. 3.33: Effects of pH on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

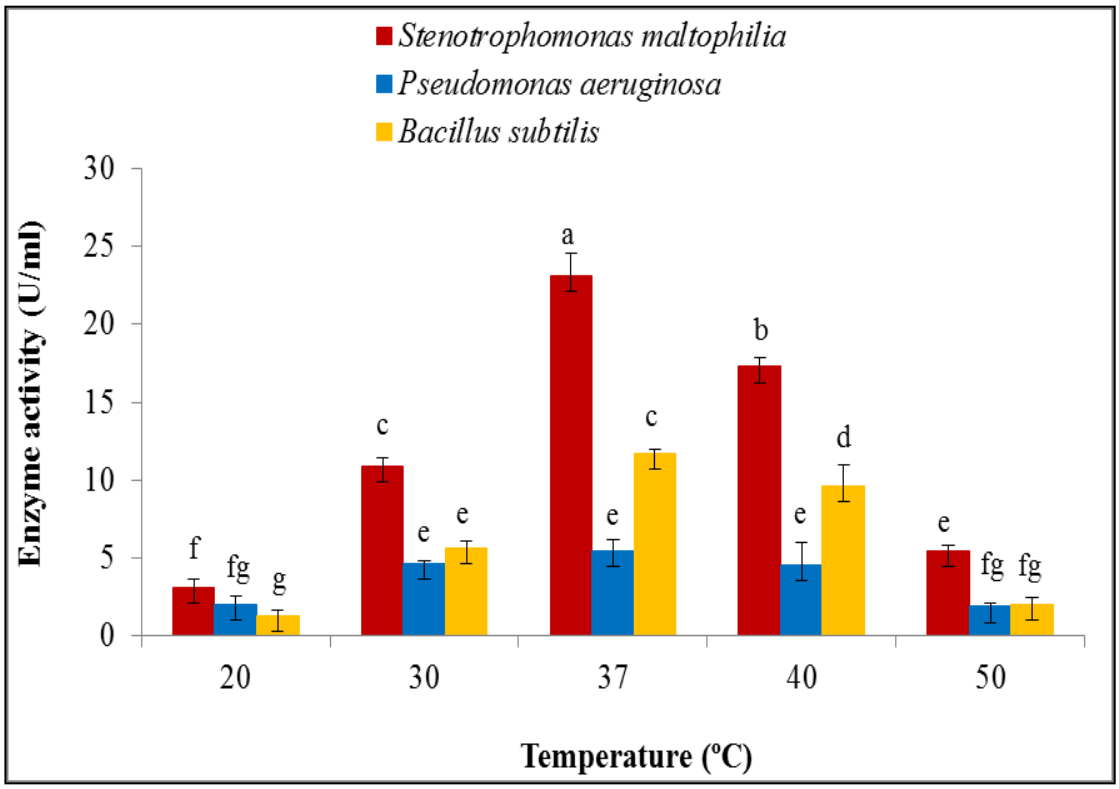


Fig. 3.34: Effects of temperature on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

3.13.3 Effects of NaCl concentration on lipase production

To test the effect of NaCl concentration, different concentrations of NaCl (0.5, 1.0, 1.5, 2.0%) were added in the production medium. Fig. 3.35 shows the effect of NaCl concentration on lipase production. Results showed that high salt concentration affected lipase production. Decrease amount of lipase production was observed against higher NaCl concentration. The highest enzyme production was recorded at 0.5% of NaCl while lowest enzyme units were obtained at 2% of NaCl.

In this test, three isolates showed maximum lipase activity *viz*. 28.33±1.00, 14.44±0.59, 17.28±0.75 U/ml at 0.5% concentration of NaCl and the minimum activity 6.50±1.17, 2.39±0.59, 3.39±0.42 U/ml at 2% concentration of NaCl by *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis,* respectively.

3.13.4 Effects of carbon source on lipase production

In this study, various carbon sources at 1% concentration were added to the production medium and their effects on lipase production were evaluated. Glucose, sucrose, starch, arabinose, xylose and mannitol were used as major carbon sources for identifying the most suitable carbon source for lipase production.

Effects of carbon source is shown in the Fig. 3.36. In case of carbon source *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* showed positive response with supplementation of glucose in the culture medium as the carbon source. Before adding any carbon source *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Bacillus subtilis* could produce lipase 23.11±1.42, 5.50±0.72 and 11.72±0.25 U/ml, repectively. The maximum enzyme activity of 47.33±0.50 and 32.00±0.50 U/ml were achieved after 48 h of incubation by *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* when medium provided with glucose. The addition of glucose enhanced lipase production up to 2.05 and 5.82 fold higher than without adding glucose. In case of starch the highest lipase activity attained (28.39±1.19 U/ml) by the isolate of *Bacillus subtilis* which is 2.42 fold higher than without adding starch. Mannitol showed minimum response for lipase activity (24.56±0.42, 22.78±0.51 and 15.45±0.63 U/ml) in these three isolates.

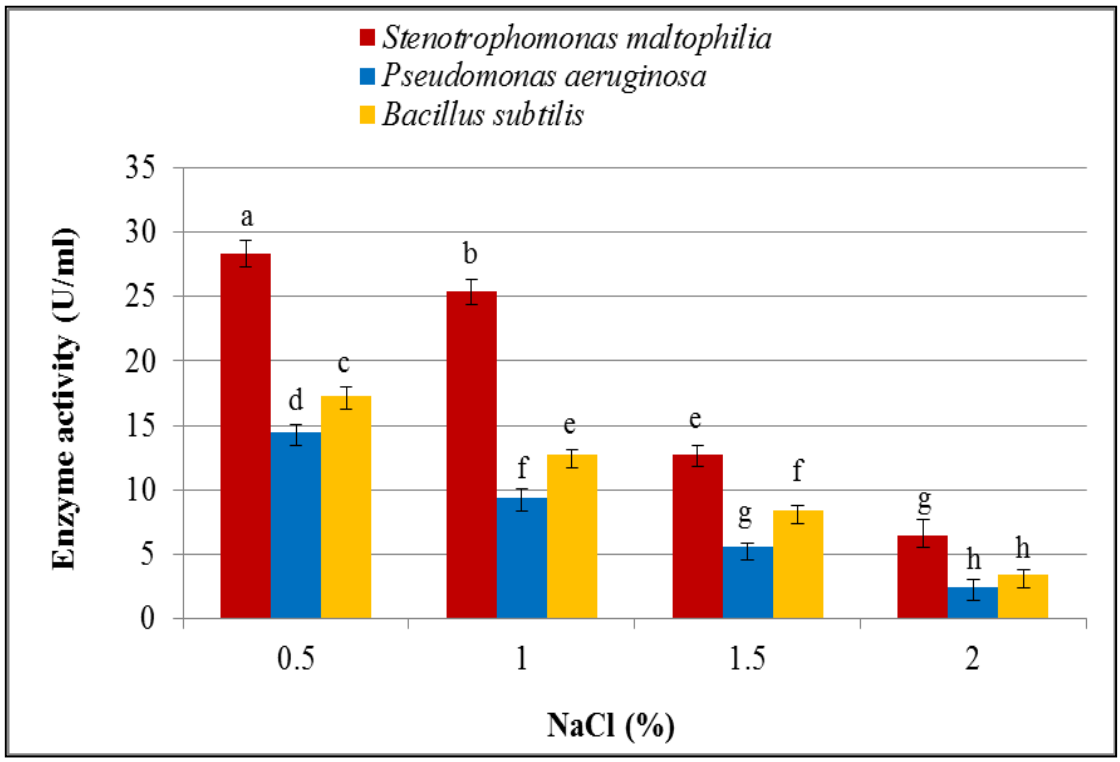


Fig. 3.35: Effects of NaCl concentration on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

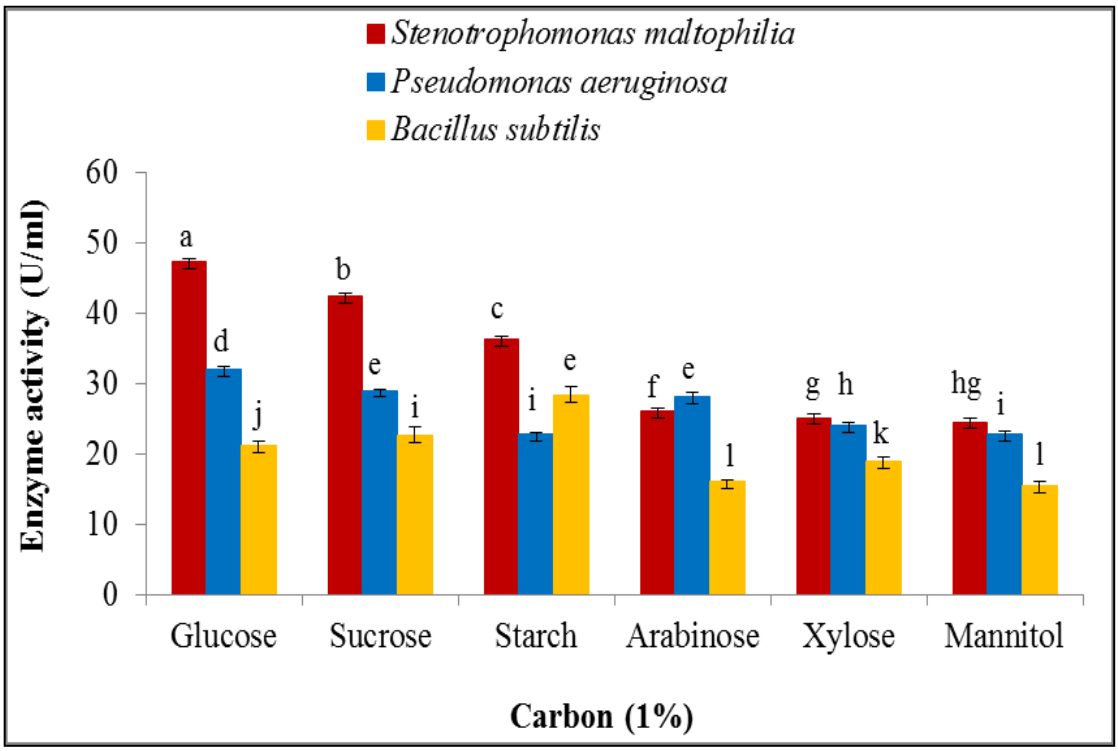


Fig. 3.36: Effects of carbon source on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

3.13.5 Effects of better carbon source concentration on lipase production

After screening the carbon sources, varying concentrations of better carbon source were added to the production medium and incubated at 37 ºC and enzyme activity was estimated after 48 h of incubation.

Various concentrations of glucose (0.5 – 3.0%, w/v) were supplemented in the medium to investigate the effect on lipase production of *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*. Effects of glucose concentration on *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* are shown in the Fig. 3.37 and 3.38, respectively. Results showed that there was an increase amount of lipase production in these two isolates with increase in the concentration of glucose up to 2.0% (w/v) and thereafter, a decrease concentration was observed. The lipase activities showed at this concentration of glucose were 57.33±0.44 and 43.28±0.51 U/ml by *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, respectively. Lipase production was found to be the lowest by *Stenotrophomonas maltophilia* (37.33±0.67 U/ml) at 3% and *Pseudomonas aeruginosa* (21.28±0.58 U/ml) at 0.5% of glucose.

Like glucose concentration for *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, various concentrations of starch (0.5 – 3.0%, w/v) were supplemented in the medium to investigate the effect on lipase production by the isolate of *Bacillus subtilis* since starch was found to be suitable for lipase production. The highest enzyme activity (37.39±0.67 U/ml) showed at 1.5% and the lowest (20.28±0.51 U/ml) at 0.5% of starch (Fig. 3.39).

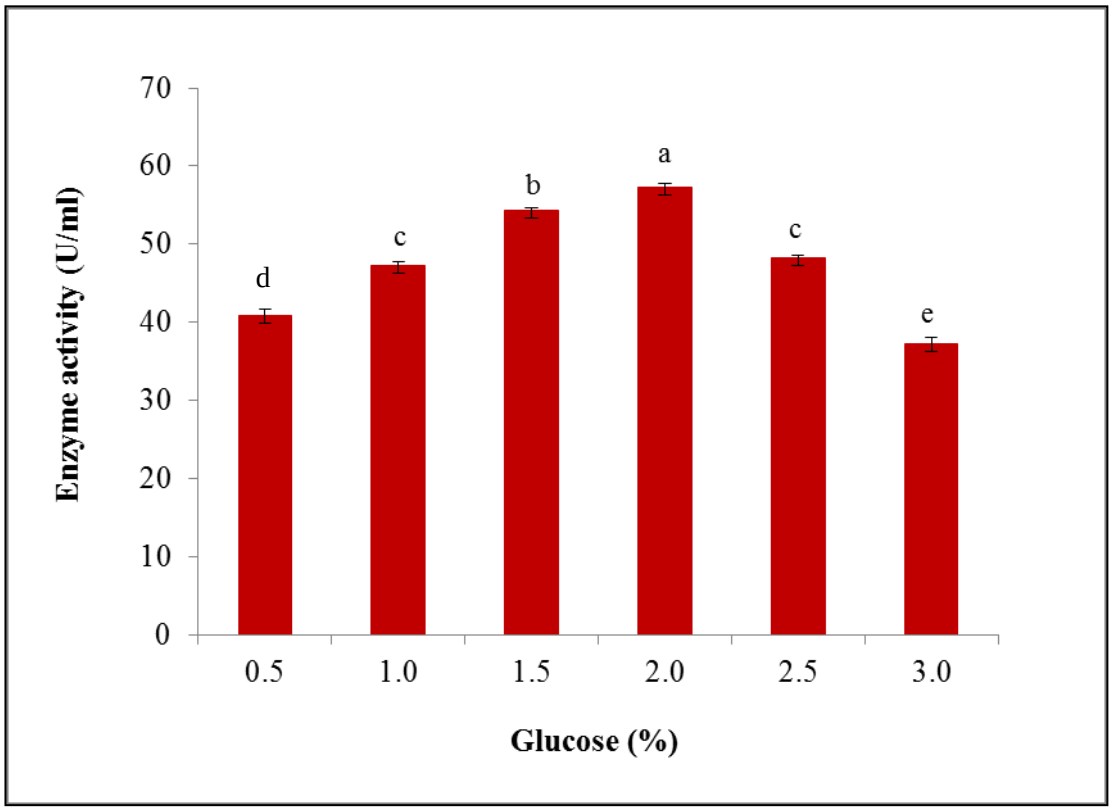


Fig. 3.37: Effects of glucose concentration on lipase production in *Stenotrophomonas maltophilia.*

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

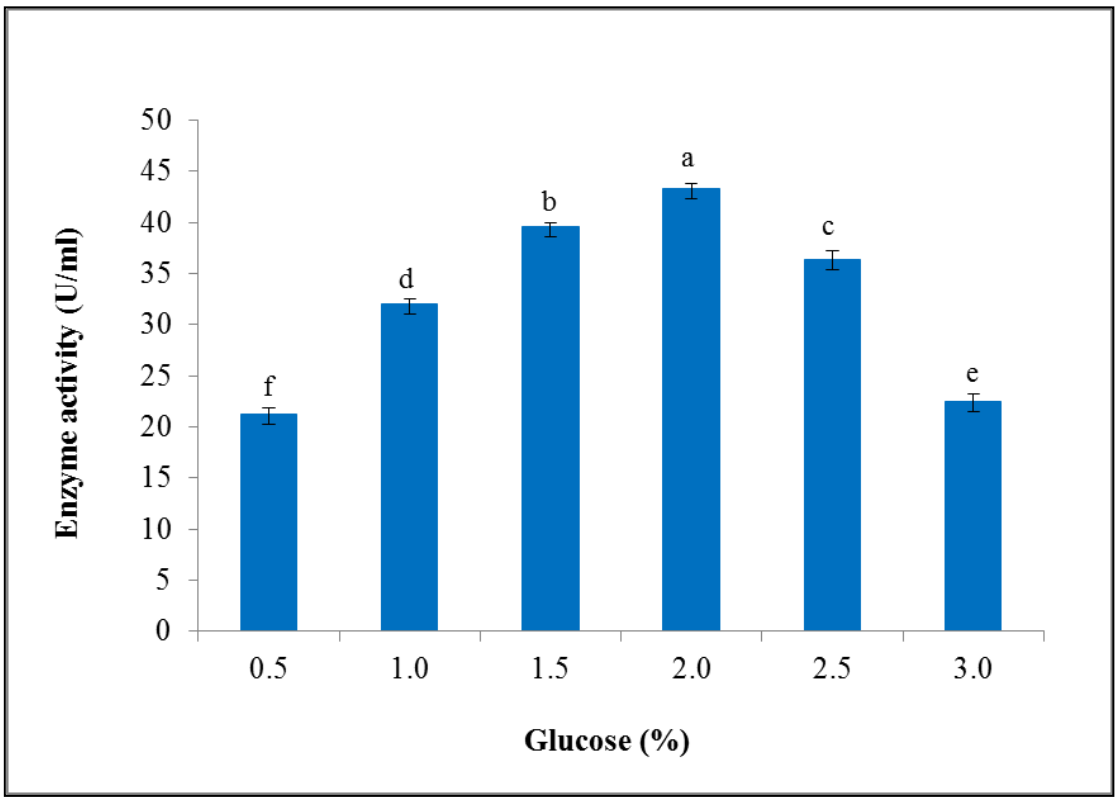


Fig. 3.38: Effects of glucose concentration on lipase production in *Pseudomonas aeruginosa*.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by the Duncan's Multiple Range Test (n=3).

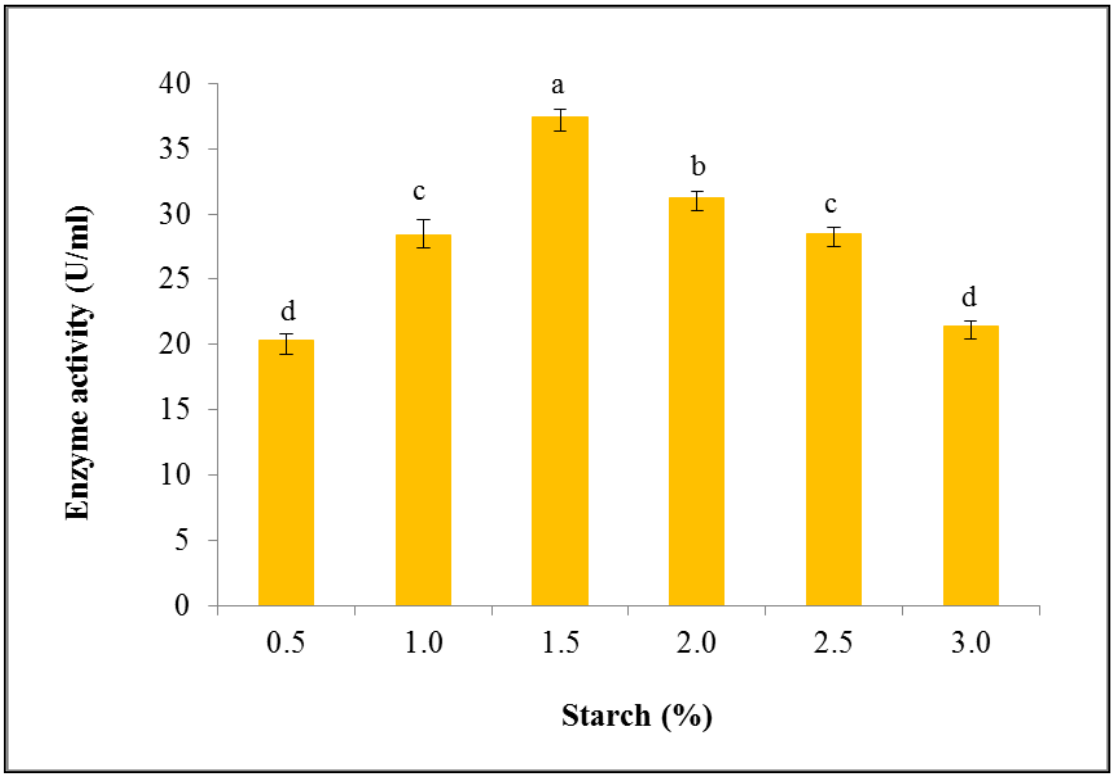


Fig. 3.39: Effects of starch concentration on lipase production in *Bacillus subtilis*.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

3.13.6 Effects of organic nitrogen source on lipase production

Like carbon source, the effect of nitrogen sources on the lipase production was tested by supplementing production medium with different organic nitrogen sources (1% w/v) *viz*. peptone, tryptone, yeast extract and beef extract and enzyme activity was estimated.

Among different organic nitrogen sources, the highest activity of lipase by *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* were obtained in case of peptone which attained the enzyme activity up to 42.17±0.84 and 21.45±0.75 U/ml and in case of yeast extract it was found to be 21.61±0.75 U/ml by *Bacillus subtilis* (Fig. 3.40). Lipase production was minimum when tryptone was used and the production amount was 27.22±0.51 U/ml by the *Stenotrophomonas maltophilia,* 14.28±0.75 U/ml in case of yeast extract by the *Pseudomonas aeruginosa* and 15.78±1.00 U/ml in case of beef extract by the *Bacillus subtilis.*

3.13.7 Effects of better organic nitrogen source concentration on lipase production

For maximum lipase production the best organic nitrogen source was added in different concentration to the production medium. Peptone was found to be better for the isolates *Stenotrophomonas maltophilia* and *Pseudomona aeruginosa*. Therefore, various concentrations of peptone (0.5 – 3.0%, w/v) were supplemented in the medium for *Stenotrophomonas maltophilia* and *Pseudomona aeruginosa*. Results showed that there was an increase in lipase production with the increase in the concentration of peptone up to 1.5% (w/v) (Fig.3.41 and 3.42). The enzyme activity showed at 1.5% peptone concentration was 48.11±0.84 and 27.33±0.34 U/ml by *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*, respectively. Lipase activity (32.22±0.51 and 19.28±0.51 U/ml) was low at 3% concentration in both the isolates.

Yeast extract was found to be better for *Bacillus subtilis* so that various concentrations of yeast extract (0.5 – 3.0%, w/v) were supplemented in the medium to investigate the effect on lipase production of *Bacillus subtilis*. The highest amount of lipase production (25.17±0.67 U/ml) showed at 1.5% and the lowest (16.11±0.75 U/ml) at 0.5% concentration of yeast extract (Fig. 3.43).

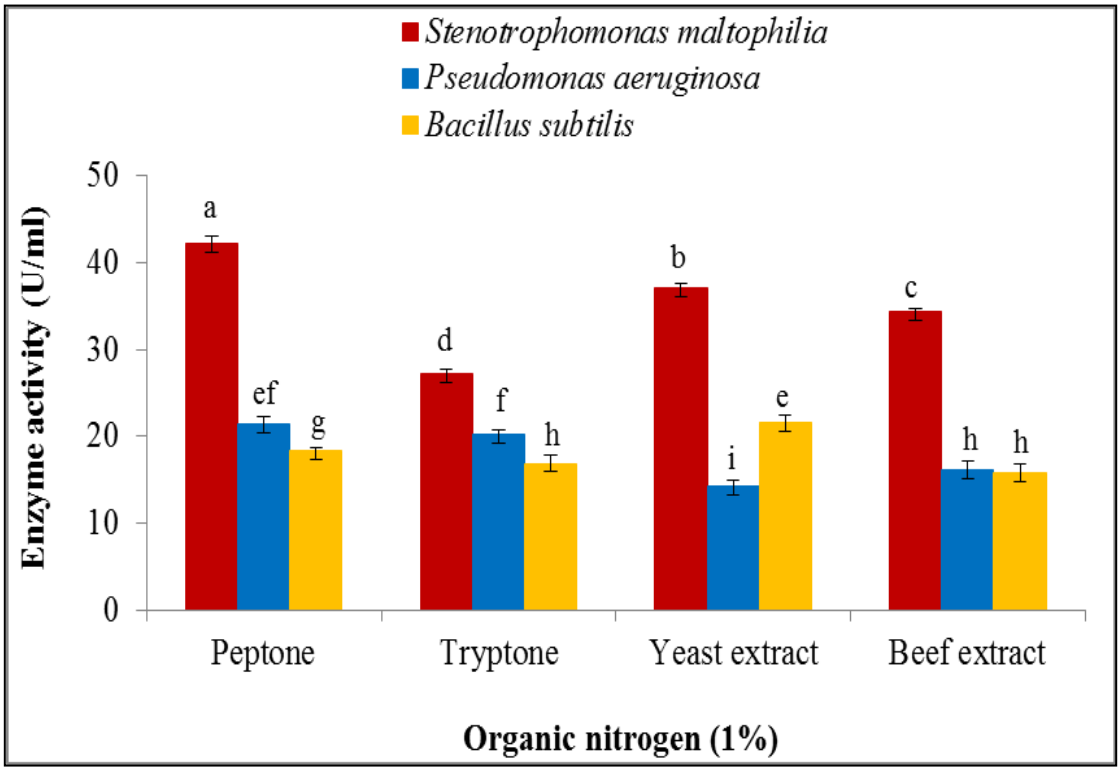


Fig. 3.40: Effects of organic nitrogen source on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

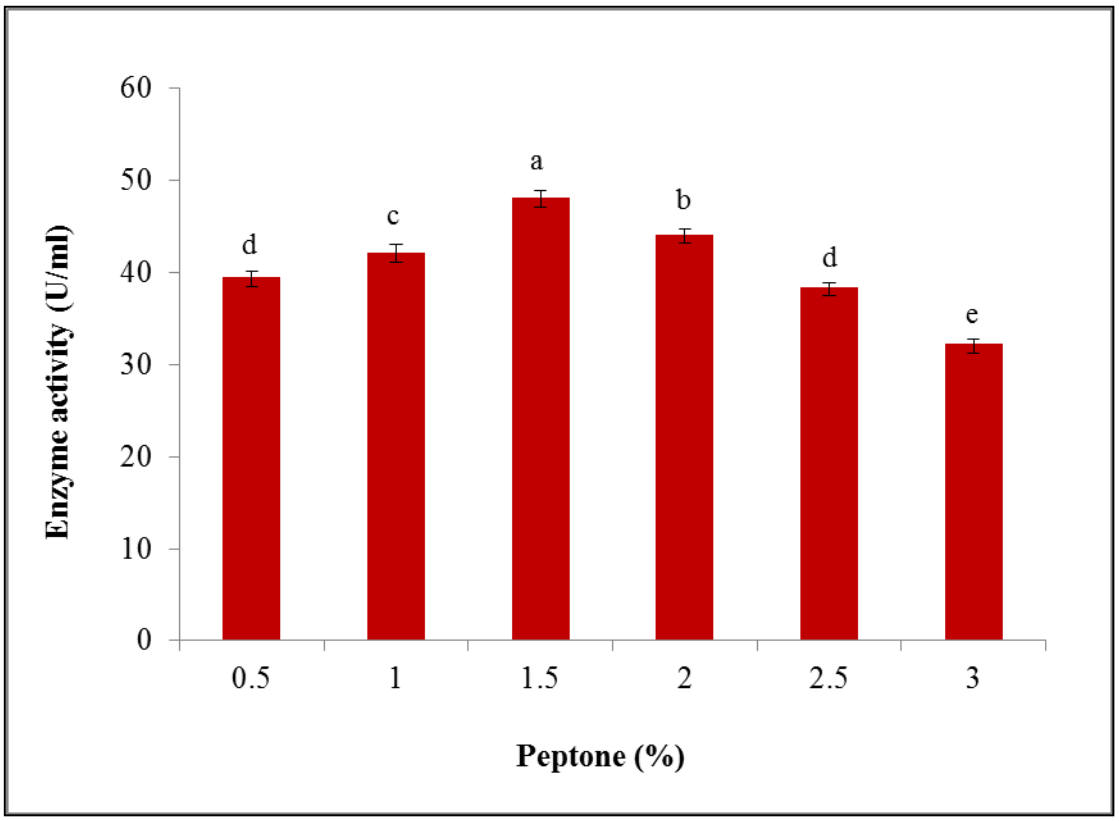


Fig. 3.41: Effects of peptone concentration on lipase production in *Stenotrophomonas maltophilia.*

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

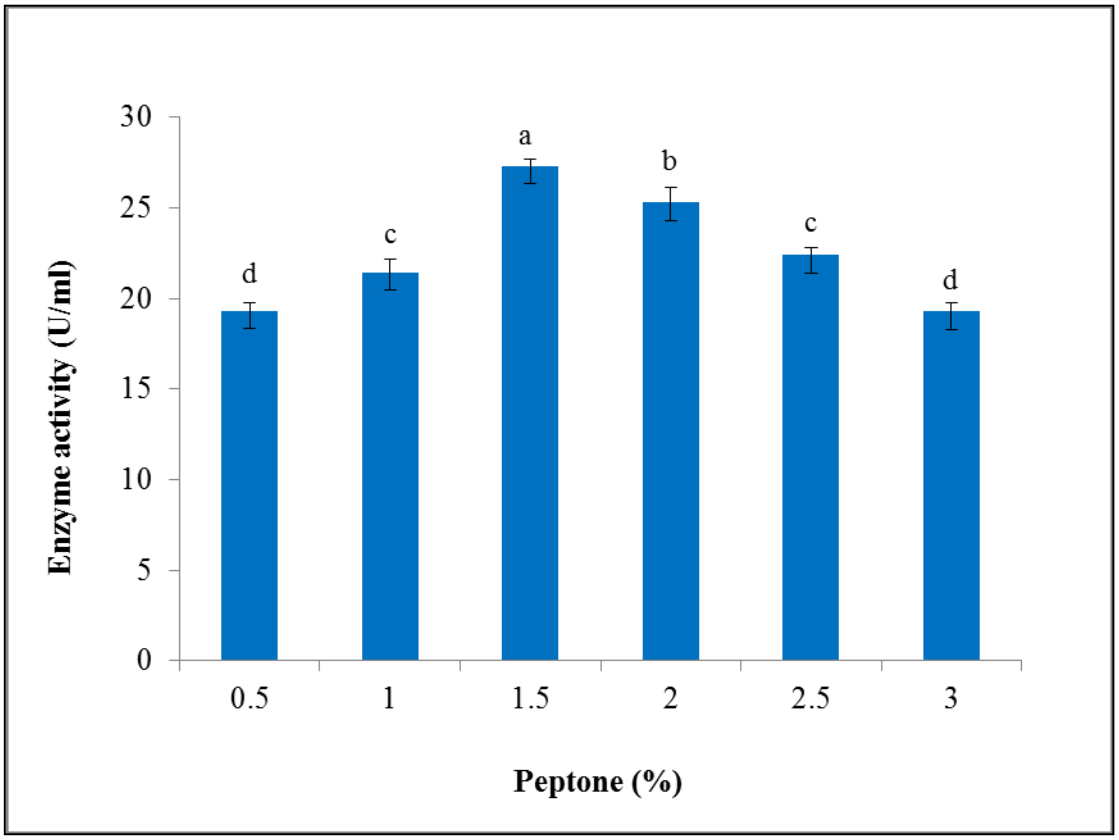


Fig. 3.42: Effects of peptone concentration on lipase activity in *Pseudomonas aeruginosa*.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

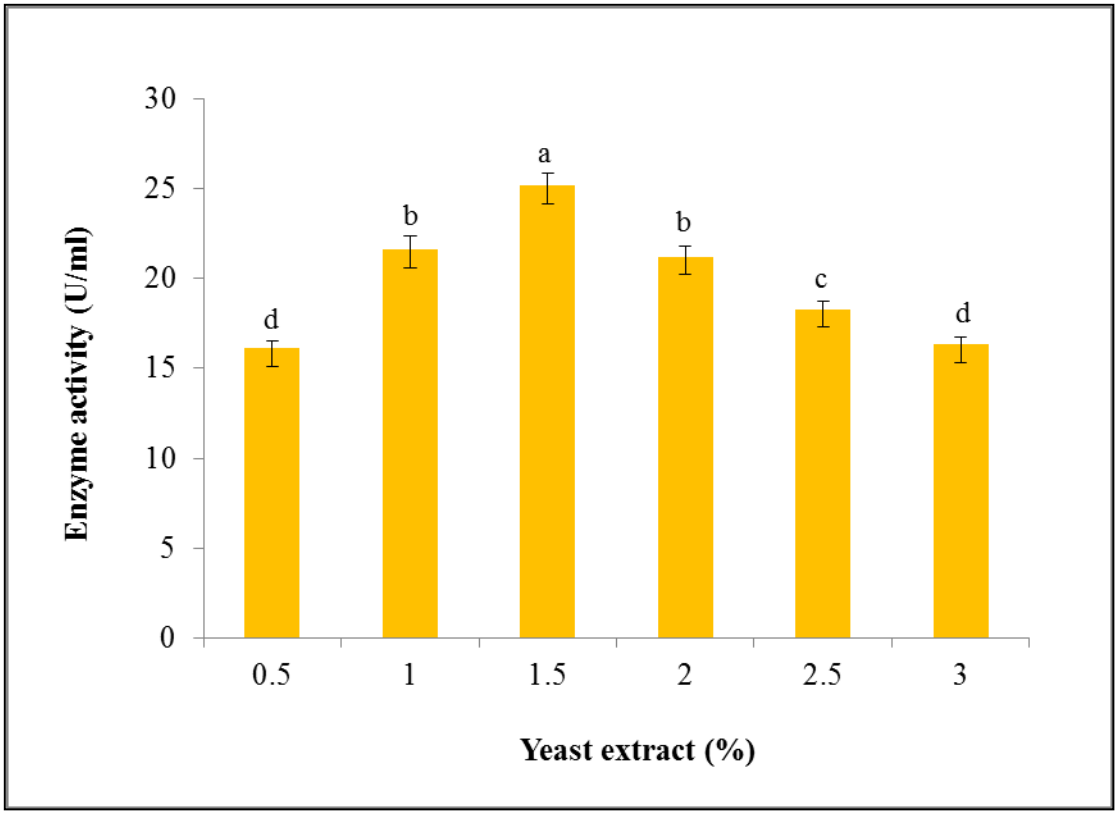


Fig. 3.43: Effects of yeast extract concentration on lipase production in *Bacillus subtilis*.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

3.13.8 Effects of inorganic nitrogen source on lipase production

Effects of inorganic nitrogen source on lipase production was carried out by using different inorganic nitrogen sources such as ammonium chloride, ammonium nitrate and ammonium orthophosphate at 1% (w/v) concentration to the medium. Enzyme activity was estimated after 48 h of incubation. In the present investigation ammonium chloride was found to be the best inorganic nitrogen source for lipase production by the *Stenotrophomonas maltophilia* (27.83±0.50 U/ml) and *Bacillus subtilis* (17.06±0.42 U/ml) and ammonium nitrate for *Pseudomonas aeruginosa* (16.06±0.51 U/ml) (Fig. 3.44).

The lowest amount of lipase was produced with ammonium orthophosphate which gave 23.22±0.35, 5.00±0.33 and 13.28±0.42 U/ml by *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively. The result showed that the supplementation of the inorganic nitrogen source enhanced lipase production by the three bacterial isolates.

3.13.9 Effects of better inorganic nitrogen concentration on lipase production

Various concentrations (0.5 – 3.0%, w/v) of better inorganic nitrogen source were added in the medium to investigate the effect on lipase production. Ammonium chloride was found to be better inorganic nitrogen source for *Stenotrophomonas maltophilia* and *Bacillus subtilis*. Therefore, different concentrations of ammonium chloride were added to the medium for *Stenotrophomonas maltophilia* and *Bacillus subtilis*. It was observed that lipase production was increased with increase concentration of ammonium chloride up to 1.5% and thereafter, lipase production was declined (Fig. 3.45 and 3.46). At this concentration ammonium chloride attained lipase activity 30.28±0.51 and 17.22±0.51 U/ml by *Stenotrophomonas maltophilia* and *Bacillus subtilis*, respectively.

Ammonium nitrate was found to be better inorganic nitrogen for *Pseudomonas aeruginosa.* Therefore, different concentrations of ammonium nitrate (0.5 – 3.0%, w/v) were supplemented in the medium for *Pseudomonas aeruginosa*. The highest lipase production (16.06±0.51 U/ml) showed at 1.0% concentration of ammonium nitrate (Fig. 3.47). After that lipase production decreased.

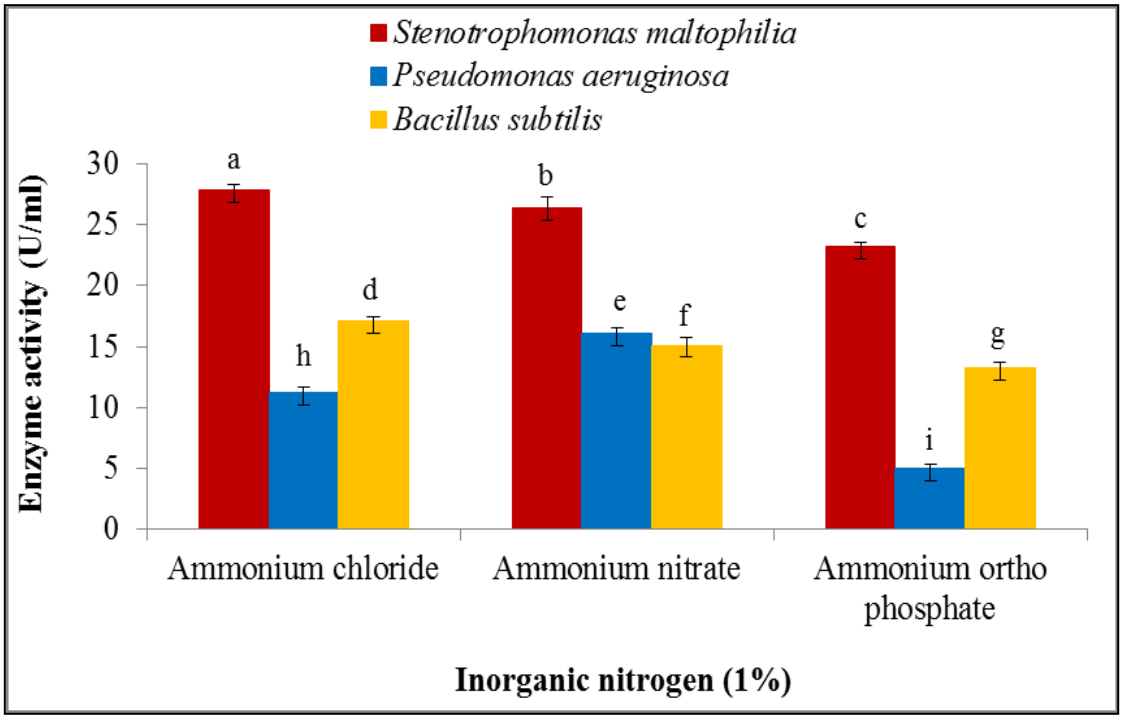


Fig. 3.44: Effects of inorganic nitrogen sources on lipase activity.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

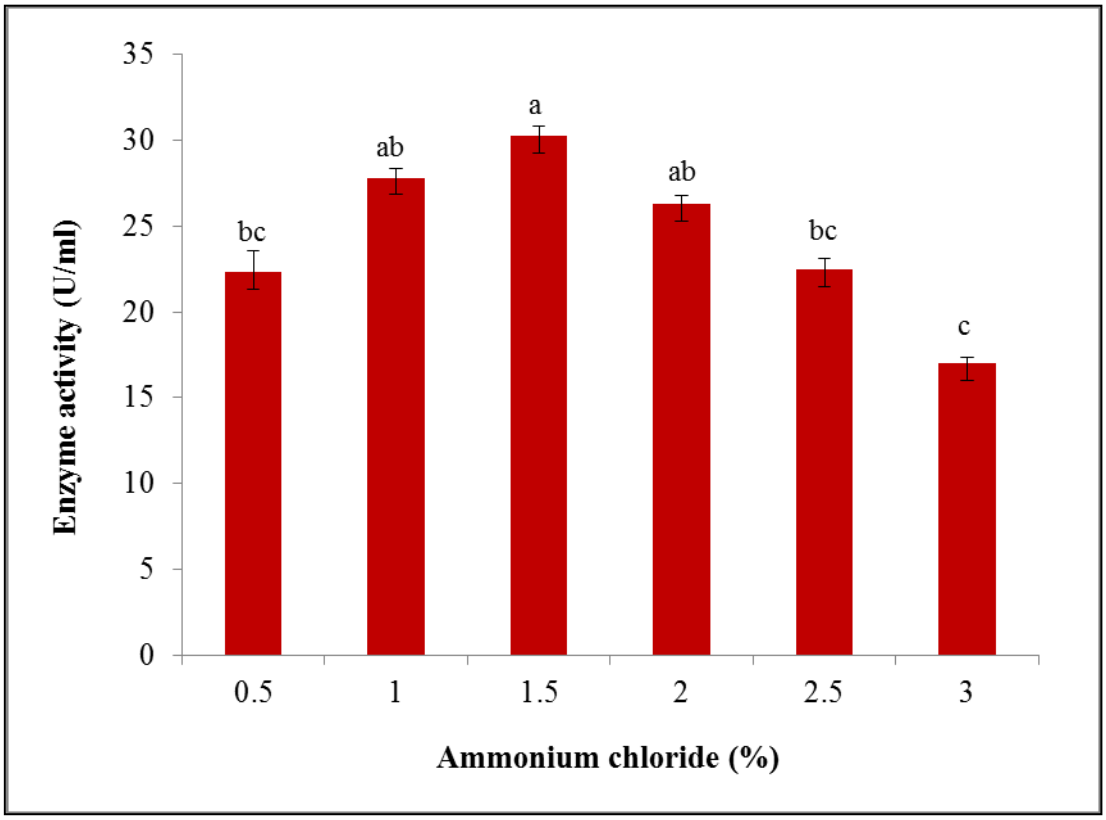


Fig. 3.45: Effects of ammonium chloride concentration on lipase production in *Stenotrophomonas maltophilia*.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

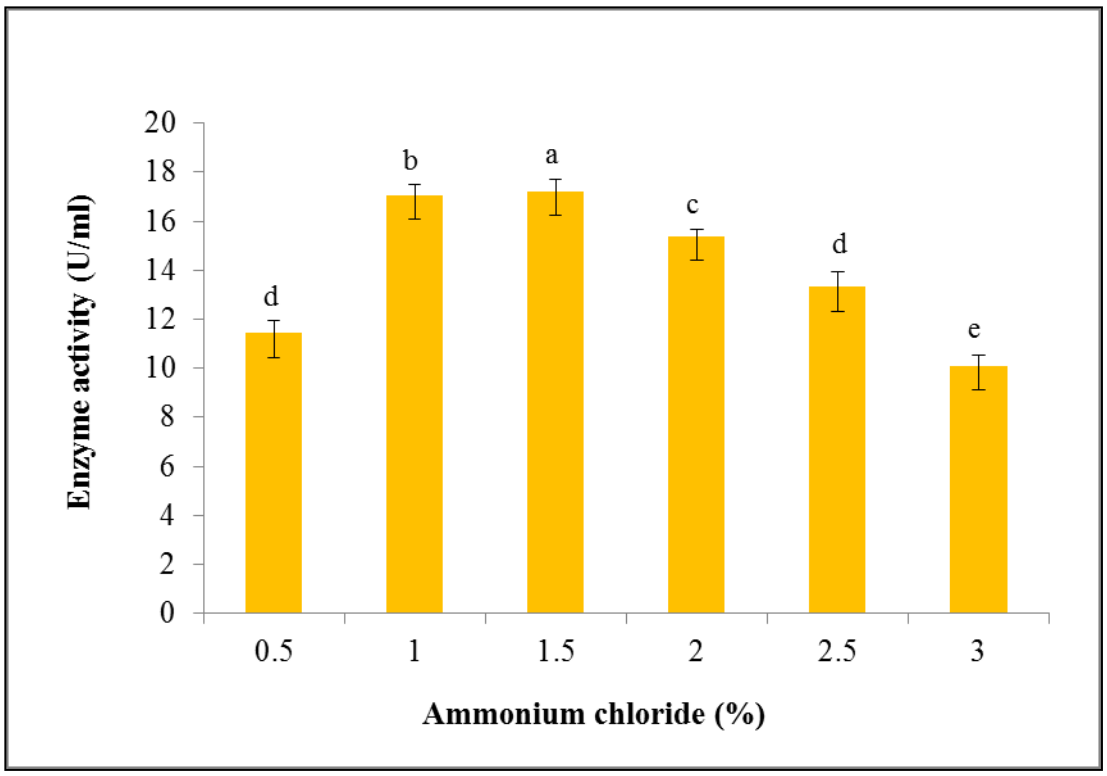


Fig. 3.46: Effects of ammonium chloride concentration on lipase production in *Bacillus subtilis*.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

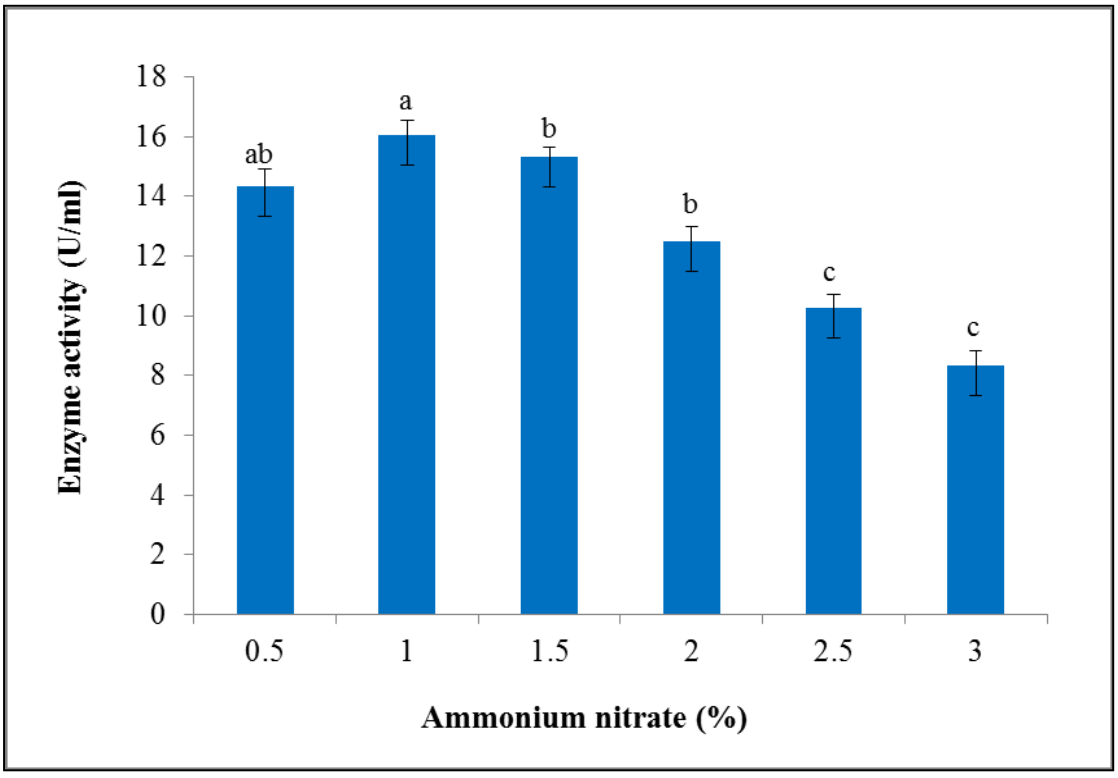


Fig. 3.47: Effects of ammonium nitrate concentration on lipase activity in *Pseudomonas aeruginosa*.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

3.13.10 Effects of inoculum concentration on lipase production

Effects of inoculum concentration was tested for lipase production. For this purpose, 1-7% fresh inoculum was used for the lipase production. Maximum enzyme activity was obtained at 5% inoculum concentration in all three bacterial isolates (Fig. 3.48). Lipase production in *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis* were 73.50±0.44, 37.05±0.75 and 51.17±0.50 U/ml, respectively which were found to be 3.18, 6.74 and 4.37 fold higher than before optimization (23.11±1.42, 5.50±0.72 and 11.72±0.25 U/ml).

This study also revealed that increase of inoculum concentration up to 5% in production media there was a considerable increase in the lipase production. Decrease lipase production was noticed when more than 5% inoculum concentration was added into the medium.

3.13.11 Effects of olive oil concentration on lipase production

The substrate specially lipid concentration of the fermentation medium had a major influence on lipase production. The effect of lipid as substrate on lipase production was studied by using different concentrations of olive oil (0.5 - 3%, v/v) in the medium.

Interestingly, there was no significant effect of olive oil concentration on lipase production. All three isolates showed the optimum activity at 1% concentration of olive oil (Fig. 3.49). The three isolates *viz*. *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis* showed the maximum lipase activity of 23.11±1.42, 5.50±0.72 and 11.72±0.25 U/ml, respectively.

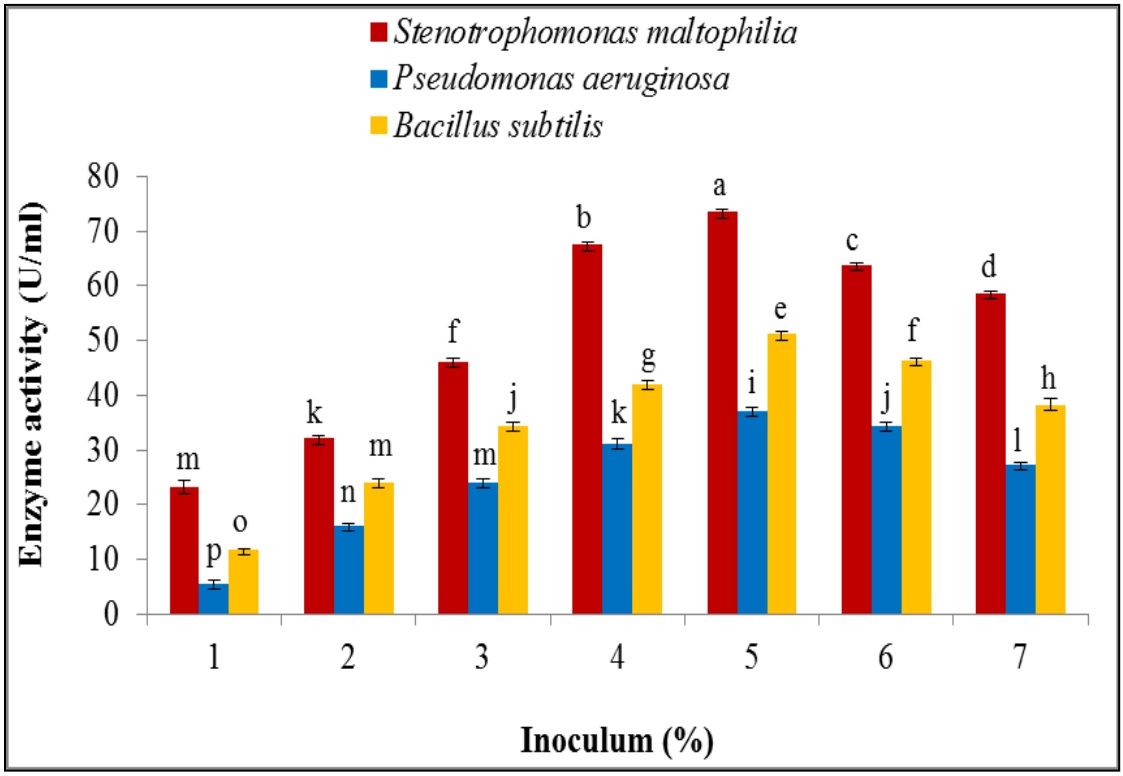


Fig. 3.48: Effects of inoculum concentration on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

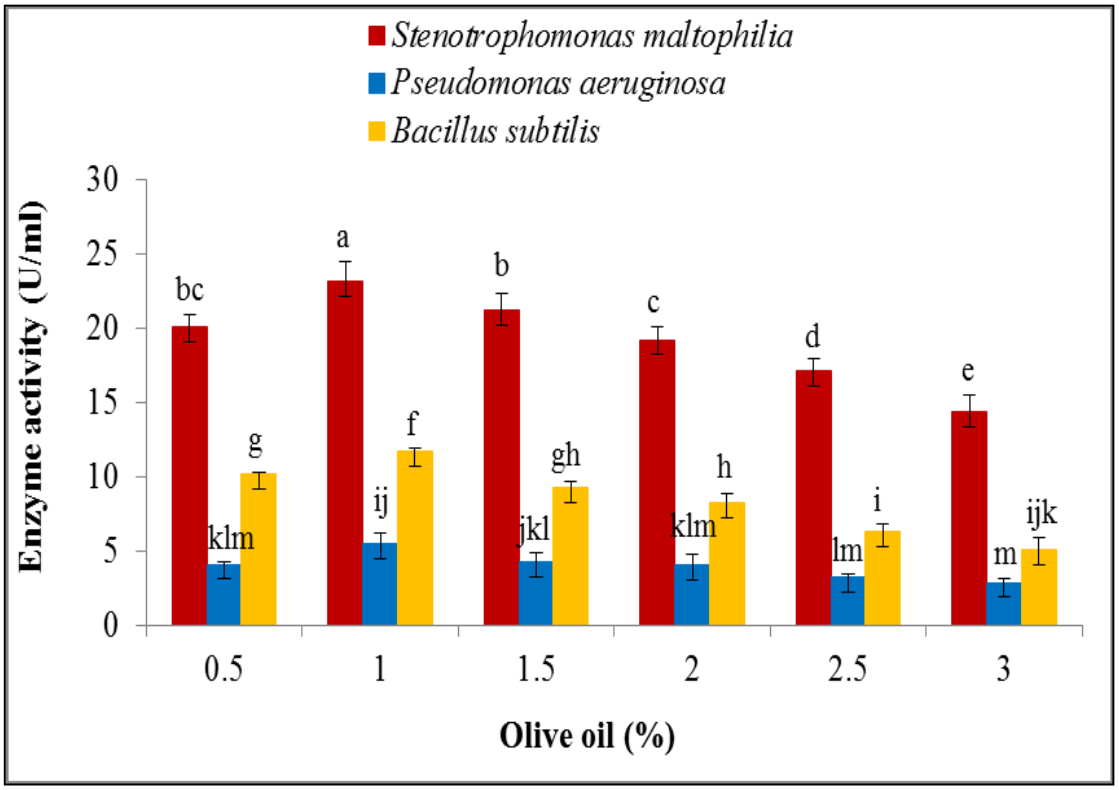


Fig. 3.49: Effects of olive oil concentration on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

3.13.12 Effects of five basal media on lipase production

Getting all possible information in relation to lipase production five conventional basal media were tested for better lipase production. In this experiment Nutrient Broth (NB) medium was found to be better basal medium for lipase production.

Maximum lipase activity *viz*. 27.50±0.60, 13.83±1.17 and 16.28±0.42 U/ml were shown by *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively in NB medium with olive oil as lipid substrate (Fig.3.50). After 48 h of incubation *Stenotrophomonas maltophilia* and *Bacillus subtilis* showed decreased activity in LB medium (21.72±0.92 and 9.28±0.42 U/ml). *Pseudomonas aeruginosa* showed minimum activity in TSB medium (5.50±0.72 U/ml).

3.14: Lipase production in all studied optimum conditions

After optimization of all studied parameters, the best conditions were set for the maximum lipase production by the isolated indigenous bacterial isolates. Inoculated flasks were incubated at 37 ºC for 96 h and samples were analyzed in every 12 h intervals. The result clearly reflected an enhanced production of lipase in the possible optimum condition (Fig. 3.51).

The results showed that the maximum lipase production by *Stenotrophomonas maltophilia* occurred at 72 h of incubation where lipase activity was achieved to 136.47±1.63 U/ml. This was 5.08 fold higher than that of before optimization. *Pseudomonas aeruginosa* and *Bacillus subtilis* showed the highest activity at 36 h of incubation and lipase activities were 96.33±0.21 and 84.30±0.17 U/ml, respectively. On the other hand *Pseudomonas aeruginosa and Bacillus subtilis* showed 8.86 and 6.24 fold higher than before optimization. All three isolates showed minimum activity at 12 h of incubation. Comparative analysis of lipase production is shown in Table 3.15. All the three bacterial isolates showed considerably better performances after optimization.

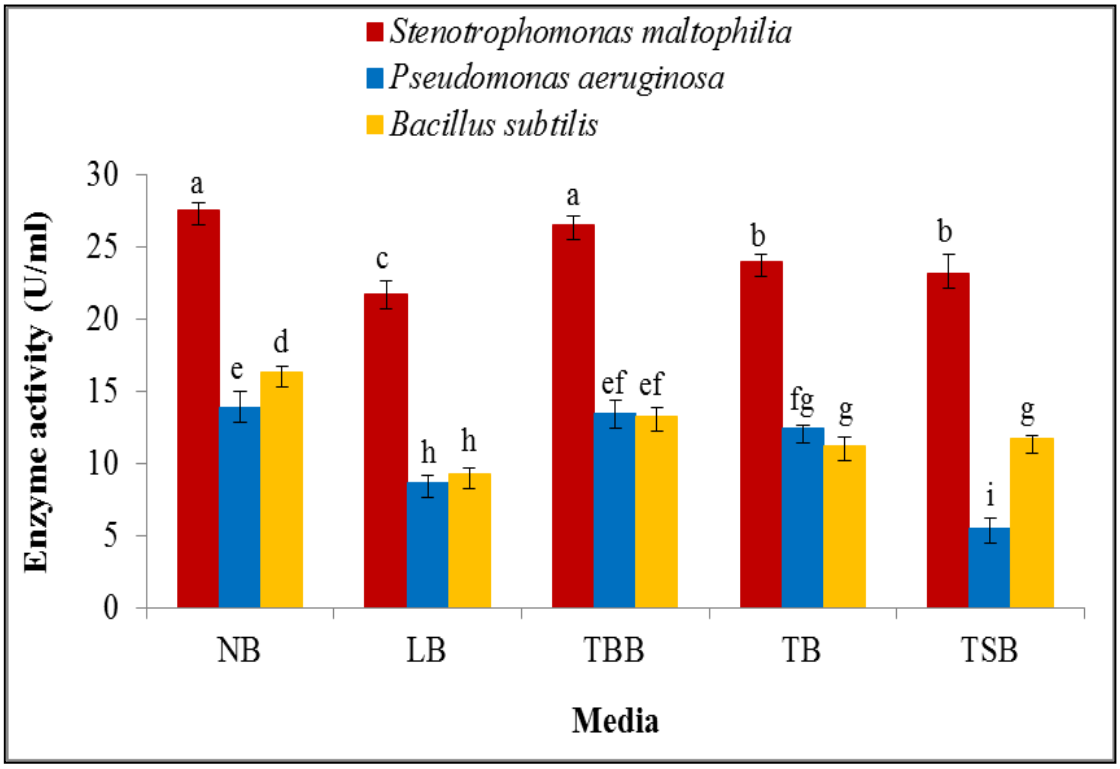


Fig. 3.50: Effects of basal media on lipase production.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=3).

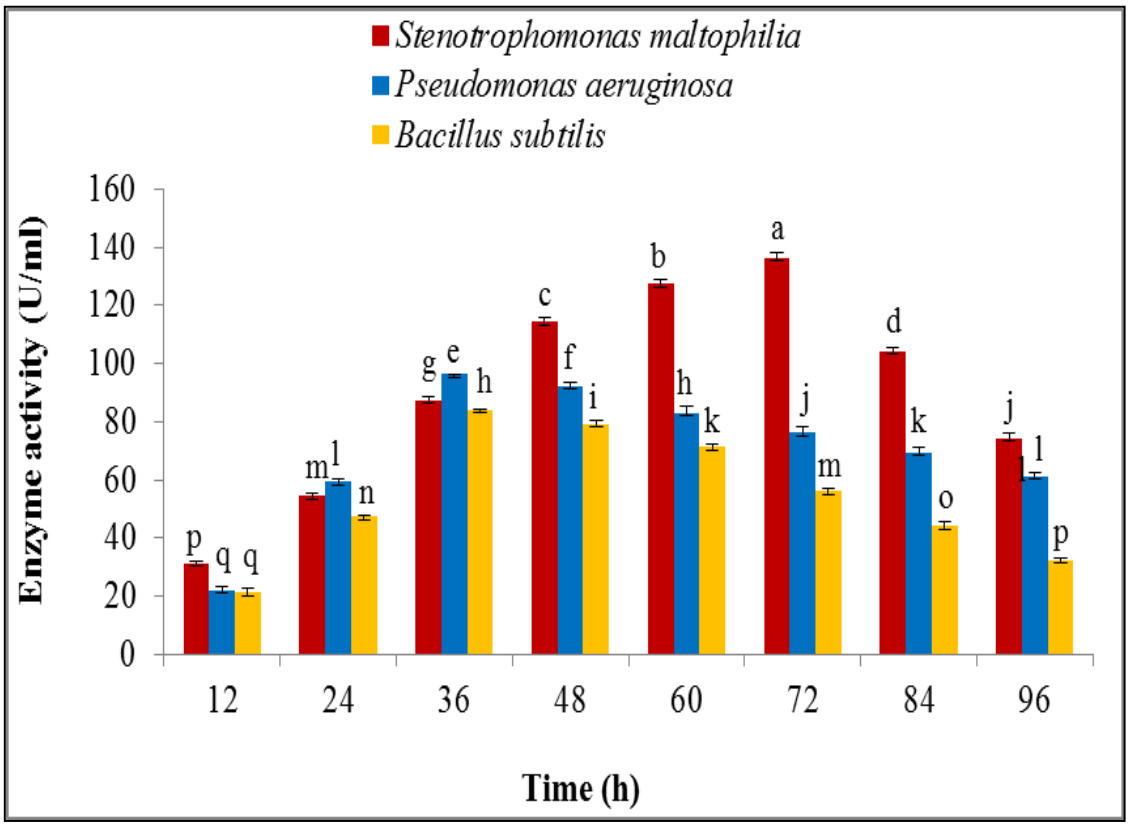


Fig. 3.51: Lipase production in all studied optimum conditions.

Error bar representing the experimental error of Standard deviation. Means with different letters designations within the column are significantly different at $p<0.05$ by Duncan's Multiple Range Test (n=5).

Table 3.15: Comparative analysis of lipase production by the selected three isolates.

Bacterial isolates	Enzyme activity (U/ml)	
	Before optimization	After optimization
Stenotrophomonas maltophilia e-a22	26.89±2.68	136.47±1.63
Pseudomonas aeruginosa 12	10.89±0.30	96.33±0.21
Bacillus subtilis 20B	13.50±0.14	84.30±0.17

3.15 Estimation of bacterial growth of the three bacterial isolates

Bacterial growth was measured by the total viable count. Total viable count of the inoculated bacteria was carried out by serial dilution plate technique at 12 h intervals up to 96 h. Fig. 3.52 shows the bacterial growth pattern by the three bacterial isolates during lipase production using tested all optimum conditions. Initial cell densities of three isolates were 1.5×10^3, 1.6×10^3 and 1.56×10^3 cfu/ml of *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively.

Increasing the incubation time showed that lipase production had been initiated slowly after 12 h and at 48 h the bacteria had obtained logarithmic growth phase. The viable count result showed that there was an increase in the number of cells from time 12 h to 72 h in *Stenotrophomonas maltophilia* and 84 h in *Pseudomonas aeruginosa* and *Bacillus subtilis* and then decreased as the time progressed. The effect of incubation time and growth on lipase activity determined that the highest growth (6.7×10^7 cfu/ml) was obtained at 72 h of incubation in case of *Stenotrophomonas maltophilia* and the highest lipase production was found in late exponential phase. The cell number decreased over 72 h of cultivation. *Pseudomonas aeruginosa* and *Bacillus subtilis* showed highest growth (3.7×10^7 and 6.9×10^7cfu/ml) at 84 h of incubation and cell number decreased over this time. The lipase productions were the highest in exponential growth phase at 36 h of incubation by *Pseudomonas aeruginosa* and *Bacillus subtilis*.

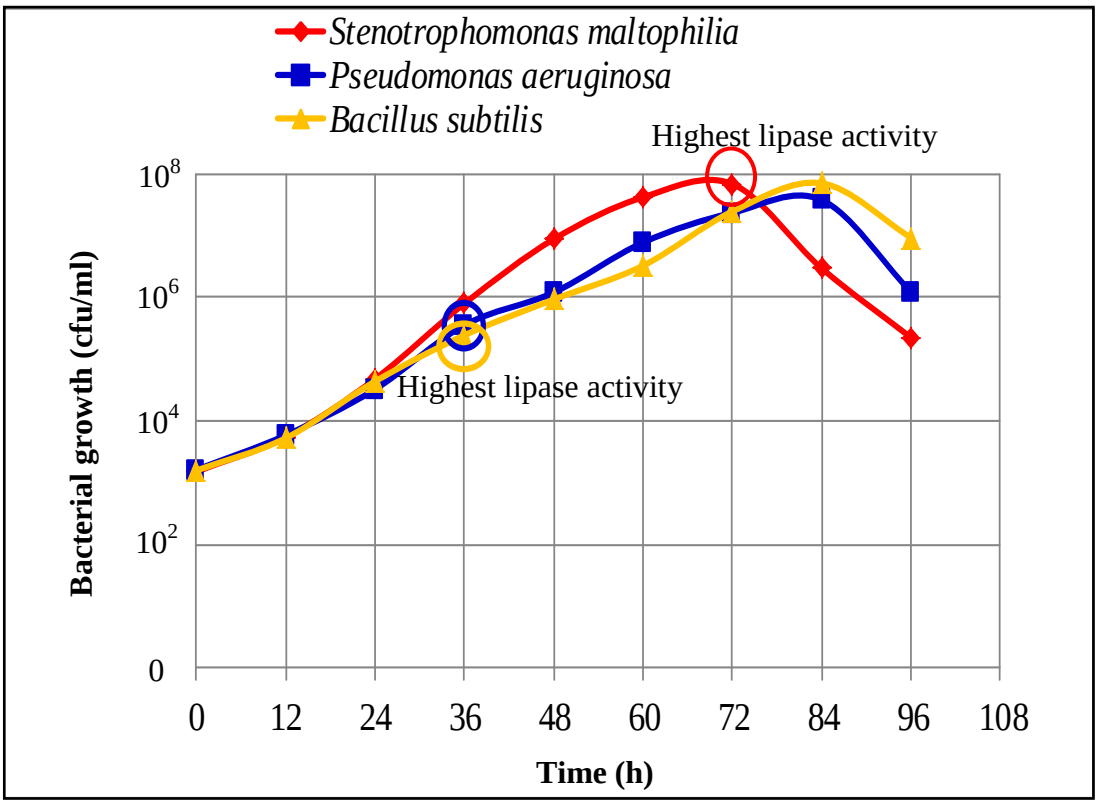


Fig. 3.52: Bacterial growth pattern of three bacterial isolates.

3.16 Laboratory scale wastewater treatment

In the laboratory synthetic wastewater treatment was carried out by two different ways. Firstly, the wastewater treatment was done by monoculture using three individual bacterial isolates *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B. Secondly, wastewater treatment was carried out by using consortium or mix culture of all three bacterial isolates. Fig. 3.53 shows lipase production experiment during wastewater treatment by *Pseudomonas aeruginosa*.

COD of wastewater sample analysis was carried out within 96 h of treatment under aerobic conditions. The initial COD concentration of synthetic wastewater was 1200 mg/L. When the synthetic wastewater was treated with the bacteria individually and as a consortium, there was a reduction in COD level. The COD value of wastewater reduced from 1200 mg/L to 303, 320 and 400 mg/L after 96 h of treatment by the three bacterial isolates (*Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively) when treated with monoculture.

In wastewater treatment, consortium of three bacterial isolates was found to be better than that of monoculture. There was a considerable decrease in COD on the fourth day when consortium was used for the treatment. The COD finally reduced to 200 mg/L at the end of the treatment by consortium. In case of monoculture, the strain *Stenotrophomonas maltophilia* was found to be better candidate where the COD removal rate was 74.75% at 96 h of incubation followed by *Pseudomonas aeruginosa* (73.33%) and *Bacillus subtilis* (66.67%). Overall, consortium showed better result than that of monoculture, where maximum COD removal was attained to 83.33% (Fig. 3.54).

Generally, oil degradation efficiency gradually increased with increasing culture incubation time. At 24 h of incubation, the ability to reduce COD concentration by the consortium was at its lowest efficiency (50%). Progress of the treatment time up to the 96 h resulted to the highest removal rate of 83.33% which is found to be better for COD removal from synthetic lipid-rich wastewater by the consortium.

(a) Inoculated flask **(b) After 96 h incubation**

Fig. 3.53: Lipase production experiment during wastewater treatment by *Pseudomonas aeruginosa*.

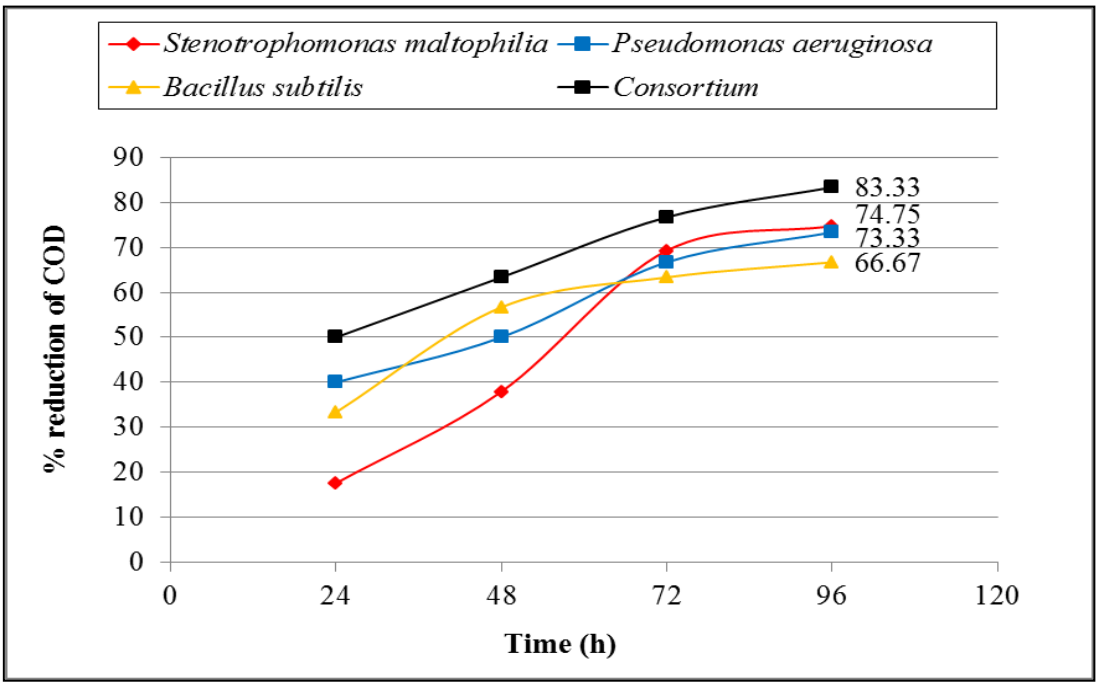


Fig. 3.54: Wastewater treatment using monoculture and consortium.

Chapter-4

Discussion

Discussion

Lipase is one of the most important industrial enzymes can be obtained from microorganisms of different habitats. The industrial demand for new lipase sources with different enzymatic characteristics and production at a lower cost has led to the isolation of new lipolytic microorganism from a variety sources (Colen *et al*. 2006). Due to industrial importance of lipase, there is an ongoing interest in the isolation of new bacterial strains producing lipase suitable for applications in wastewater management (Mc Tigue *et al.* 1995). Many attempts have been made to isolate lipase producing bacteria since this enzyme is being used in numerous biotechnological processes (Fatima *et al*. 2014). The oily environment with organic matter may provide a good environment for isolation of lipase producing bacteria (Mobarak-Qamsari *et al.* 2011).

Considering importance and applications of the enzyme, six water and four soil samples were collected from ten different lipid-rich habitats in and around Dhaka Metropolitan City. Nutrient Agar (NA), Peptone Yeast Extract Glucose (PYG), Tributyrin Agar (TBA) and Luria Bartani (LB) media were exploited for the enumeration and isolation of aerobic heterotrophic bacteria associated with the lipid-rich environment. Interestingly, a good number of aerobic heterotrophic bacteria were found to be associated with the collected samples. The bacterial load of the collected samples ranged in between 4.6×10^3 and 1.37×10^7, 1.65×10^3 and 1.25×10^7, 1.28×10^3 and 1.56×10^7, 8.3×10^2 and 2.64×10^5 cfu/g or cfu/ml in NA, PYG, TBA and LB media, respectively. The maximum bacterial count (1.56×10^7 cfu/g) was observed in the soil sample of Savar Dairy Farm soil in TBA medium while the lowest bacterial load (8.3×10^2 cfu/ml) was observed in the water sample collected from the Buriganga River in LB medium (Table 3.1). Lipase positive bacterial load in TBA medium was ranged in 3×10^2 cfu/ml to 1.17×10^7 cfu/g (Table 3.2). The highest percentage (94.51%) of lipase positive bacteria was found in the soil sample of Edible Oil Mill and the lowest (23.44%) was in the water sample of the Buriganga River.

On the basis of colony diversity, primarily a total of 273 aerobic heterotrophic bacteria were isolated. Two different media *viz*. TBA and TA were used for the screening of lipase producing ability by the bacterial isolates. Among them 186

isolates (68%) showed lipase positive (Fig. 3.6). Considering better lipase producer, 30 isolates were selected for further detail study. Clear zone formation on the TBA medium is an indicator of lipase positive isolate. Zone ratio was found to be in the range of 1.55 - 4.08 (Table 3.3). In a work Rathi *et al*. (2001) reported similar zone ratio in case of *Burkholderia* sp. In the present study, Isolate S_4P-4 showed the highest zone ratio (4.08) in TBA medium.

For provisional identification, the morphologically, physiologically and biochemically characterized 30 isolates were compared with the standard descriptions of the Bergey's Manual of Systematic Bacteriology Vol.-1 (Krieg and Holt 1984) and Bergey's Manual of Systematic Bacteriology Vol.-2 (Sneath *et al*. 1986). Among them, 16 were Gram-positive under four genera *viz*. *Bacillus, Staphylococcus, Micrococcus, Planoococcus* and 14 were Gram negative isolates and were the members of *Acinetobacter, Acetobacter, Pseudomonas, Alcaligenes* and *Serrratia*, respectively. Under the genus *Bacillus*, there were four distinct species such as *Bacillus subtilis, B. polymyxa, B. pumilus* and *B. lentus*. Other Gram positive species were *Staphylococcus intermedius, Micrococcus lylae* and *Planococcus citreus.* Under the Gram negative genus *Acinetobacter* there were four distinct species *viz. Acinetobacter calcoacetius, A. baumanni, A. lwoffi* and *A. johnsonii*. Under the genus *Acetobacter* there were three species *viz*. *Acetobacter liquifaciens, A. pasteurianus* and *A. aceti*. In case of the genus *Pseudomonas* there were two distinct species *viz. Pseudomonas pseudoalcaligenes* and *P. aeruginosa*. All other Gram negative isolates were *Alcaligenes paradoxus, A. faecalis* and *Serratia rubidaea.* The findings clearly demonstrated that the lipid-rich environments were the good sources of diversified bacteria. In this research different species of *Bacillus* were found to more predominant than other species. Cipinyte *et al.* (2009) reported more or less similar result where *Bacillus* was the dominating genus among 724 bacterial strains isolated from soil rich in organic matter could degrade lipid. *Acetobacter, Acinetobacter, Pseudomonas, Serratia, Burkholderia* and *Staphylococcus* were known to have the ability to degrade lipid as well as *Bacillus* (Veerapagu *et al*. 2013).

Molecular identification on the basis of the 16S rRNA gene sequence analysis was important for more accurate identification of microbial species than morphological, physiological and biochemical characterization due to the process being cumbersome

and time consuming (Poorani *et al.* 2009). In the present study for authentication, 10 better lipase producer isolates were selected for molecular identification. In molecular identification the bacterial isolate S_1N-2 was identified as *Stenotrophomonas maltophilia* e-a22 having 98% identity match and maximum coverage score was 1013. The isolate S_1N-7 was identified as *Serratia rubidaea* 9B having 99% identity match and maximum coverage score was 672. The isolate S_3P-1 was identified as *Bacillus pumilus* Ja02 having 98% identity match and maximum coverage score was 996. The isolate S_3T-5 was identified as *Bacillus* sp. BTMASC2 having 99% identity match and maximum coverage score was 1024. The isolate S_3T-9 was identified as *Bacillus subtilis* 20B having 89% identity match and maximum coverage score was 619. The isolate S_4P-4 was identified as *Bacillus subtilis* HRBS-10TDI13 having 95% identity match and maximum coverage score was 784. The isolate S_7N-1 was identified as *Staphylococcus epidermidis* 6E02 having 95% identity match and maximum coverage score was 869. The isolate S_{10}P-1 was identified as *Pseudomonas aeruginosa* 12 having 98% identity match and maximum coverage score was 982. The isolate S_{10}P-2 was identified as *Bacillus subtilis* CI1 having 99% identity match and maximum coverage score was 1016. The isolate S_{10}T-8 was identified as *Acinetobacter johnsonii* 372 having 99% identity match and maximum coverage score was 1024.

A comparative analysis was done in between provisional and molecular identification of 10 bacterial isolates (Table 3.14). Among 10 isolates, 9 genera were matched with their conventional provisional identification. Molecularly identified *Stenotrophomonas maltophilia* e-a22 differs with its provisional identification. It was provisionally identified as *Acetobacter liquifaciens.* On this point it could be mentioned that conventional identification based on morphological, physiological and biochemical characters were still found to be valid with minor exceptions. Phylogenetic tree was constructed of three better isolates using the neighbor-joining model. Multiple sequence comparison and phylogenetic tree showed that the strain No. S_1N-2, S_{10}P-1 and S_3T-9 were belonged to *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B, respectively. In the phylogenetic tree the closely related bacterial strains were grouped together while strains having distant relationships were placed separately (Fig. 3.26-3.28).

Based on the zone ratio and intensity of lipase producing ability 10 isolates were chosen for quantitative analysis of lipase activity among 30 isolates. The enzyme producing capabilities of the isolated indigenous Gram positive isolates ranged in between 0.89±0.63 and 13.50±0.14 U/ml and Gram negative isolates were in between 1.28±0.25 and 26.89±2.68 U/ml. Isolated *Stenotrophomonas maltophilia* strain e-a22 was found to be the most potent isolate for lipase production (26.89±2.68 U/ml) at 72 h of incubation. In this study, lipase activities of isolated *Bacillus* sp. BTMASC2, *Staphylococcus epidermidis* 6EO2 and *Acinetobacter johnsonii* 372 were 5.39, 3.28 and 5.39 U/ml, respectively which were almost similar with the findings of Musa and Tayo (2012). They found lipase activities of *Bacillus* sp., *Staphylococcus* sp. and *Acinetobacter calcoaceticus* as 3.8, 2.3 and 4.7 U/ml, respectively.

In this study, most of the isolates *viz. Bacillus pumilus* JaO2, *Bacillus subtilis* 20B, *Staphylococcus epidermidis* 6EO2, *Pseudomonas aeruginosa* 12 and *Acinetobacter johnsonii* 372 showed decreased lipase production after 24 h of incubation with the increase of the incubation period. This is in agreement with work of Rajesh *et al*. (2010) where they mentioned that the lipase production increases as the incubation time increases but later falls with the increase in incubation time. Decrease in the lipase activity may be explained by the substrate depletion, the inhibitory effect of the metabolites and proteolytic degradation of enzymatic system (Musa and Tayo 2012). During this study three isolates *viz*. *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B showed lipase activity higher than 10 U/ml and were selected for detail study for lipase production.

Bacterial lipase is influenced by physicochemical factors and nutritional conditions such as pH, temperature, NaCl, carbon and nitrogen source, substrate, inoculum concentration, media, etc. which were studied earlier by Aires-Barros *et al*. (1994). Among them the pH of fermentation medium and the temperature at which fermentation process were carried out had a major influence on the enzymatic activity. Optimization of lipase production was carried out at 48 h of incubation by three better lipase producing isolates *viz*. *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B.

Initial pH of the culture broth is one of the most critical parameters affecting both growth and lipase production. In this study pH 5-9 were set to investigate the optimum pH by the isolates towards enzyme production. The optimum pH of the enzyme production was found to be at pH 7 by the isolated three bacteria (*Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis*) and showed lipase activity 23.11, 5.50 and 11.72 U/ml, respectively at 48 h of incubation (Fig. 3.33).

Generally, bacteria prefer pH around 7 for the best growth and lipase production in the case of *Bacillus* sp. (Sugihara *et al*. 1991), *Acinetobacter* sp. (Barbaro *et al*. 2001), *Burkholderia* sp. (Rathi *et al*. 2001), *Bacillus* sp. ITP-001(Carvalho 2013) and *Pseudomonas aeruginosa* SRT 9 (Prita *et al*. 2009). Medium pH more than 7 was found to be better in some cases as mentioned by some workers in case of *Pseudomonas* sp. (Dong *et al*. 1999, Kanwar *et al*. 2002).

The pH of the culture medium influences the amount of lipase production. The present findings showed that the bacteria were capable of producing lipase in the range in pH 5 and 9. Similar reports were also mentioned by Sekhon *et al*. (2010) and reported that good bacterial growth occurred at pH 5-9 whereas lipase production was maximum at pH 7 and decreased above or below this pH level. Zouaoui *et al.* (2012) observed that *Pseudomonas aeruginosa* grew best in the pH range of 6-8. Yuzo and Shimizu (2003) also reported that maximum lipase activity (39 U/ml) from *Pseudomonas fluorescens* HU 380 was at pH 7. Considering all available information it could be concluded that maximum lipase activity would be at pH 7.

Temperature would be another important parameter to influence enzyme production. For this reason, 20, 30, 37, 40 and 50 °C temperature were set for the optimization of lipase production. The optimum temperature of the lipase production was found to be at 37 °C in all three isolates. *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis* showed maximum lipase activity of 23.11, 5.50 and 11.72 U/ml, respectively at 37 °C (Fig. 3.34). The enzyme activity was found to decrease below and above 37 °C where lipase may inactivate or lower activity. In a study, Zouaoui and Bouziane (2011) reported similar type observation. In another research, *Bacillus subtilis* isolated from oil-contaminated wastewater had its maximum lipase activity (9.33 U/ml) at 37 °C (Iqbal and Rehman, 2015). Similarly,

Padhiar *et al*. (2016) reported lipase activity by *Pseudomonas aeruginosa* as 8.32 U/ml.

The enzyme activity of the present study drops rapidly above 40 °C in all three isolates. Kiran *et al*. (2008) observed similar type of findings in their study by *Pseudomonas* sp. Mohan *et al.* (2008) found that optimum temperature for lipase production by *Bacillus* sp. was 37 °C. In another research, *Bacillus subtilis* isolated from oil-contaminated wastewater had its maximum lipase activity at 37 °C (Iqbal and Rehman 2015). Noman *et al*. (2010) also obtained the maximum lipase activity from *Pseudomonas aeruginosa* BN-1 at 37 °C and pH at 6.5.

Kumar *et al*. (2012b) mentioned that lipase activity increased with the increase of temperature up to 37 °C and thereafter it decreased. The rate of enzymatic reactions in the cells increases with the increase in temperature till optimum temperature and enzyme inactivation due to protein denaturation beyond this optimum temperature causing the slow metabolism of cells, which ultimately affects the cell growth and productivity. The gelling of plasma membrane at low temperatures also results in slowing down of transport processes in the microbial cells and therefore the enzyme activity (Sahu and Martin 2011, Shah and Bhatt 2011).

For optimization of NaCl concentration in lipase production, various NaCl concentrations (0.5, 1.0, 1.5 and 2.0%) were added in the production medium. The result suggests that high salt concentration tend to inhibit lipase production while low salt concentration does not aid in lipase production. During this work, maximum lipase activities showed by *Stenotrophomonas maltophilia, Pseudomonas aeruginosa and Bacillus subtilis* with 28.33, 14.44 and 17.28 U/ml, respectively at the concentration of 0.5% NaCl (Fig. 3.35). Similar results were also obtained by Sangeetha *et al*. (2014) at 0.5% salt concentration to obtain high lipase yield in *Bacillus pumilus* SG2 with 14.98 U/ml. Kathiravan *et al*. (2012) showed the highest lipase activity by *Pseudomonas aeruginosa* with 4.3 U/ml at 0.5% NaCl. The present result and all available information showed that NaCl is important and optimum concentration was 0.5%.

The major content of lipase production media is the carbon source that also acts as an inducer for lipase production (Lotti *et al*. 1998). In this study glucose, sucrose, starch,

arabinose, xylose and mannitol were used as carbon source (1%, w/v) to investigate the better carbon source in lipase production by the three isolates. *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* showed the highest activity (47.33 and 32.00 U/ml) when production medium containing glucose as carbon source (Fig. 3.36). Kathiravan *et al.* (2012) reported that glucose showed the optimum enzymatic activity by *Pseudomonas aeruginosa* (50.50 U/ml) at 1% (w/v) carbon source. Maximum enzyme production with glucose suggests that glucose could be the best inducer for lipase than other carbon sources used. *Bacillus subtilis* showed the highest activity (28.39 U/ml) when medium containing starch. This result also supported by Joseph *et al*. (2012) in *Bacillus sphaericus* MTCC 7526 (23.67 U/ml) when they used starch for lipase production. Rohit *et al.* (2001) reported that the lipase production was found to be better when olive oil was used as the carbon source. Like others, olive oil was used as inducer in this study. Novototskaya-Vlasova *et al.* (2013) reported that olive oil in combination with glucose increases lipase activity. In the present study the presence of olive oil along with carbon source in growth medium greatly enhanced the lipase activity of three isolates.

After screening the carbon sources, optimization of better carbon source were carried out for the production of lipase. Various concentrations of beter carbon source, glucose were supplemented in the medium with 0.5-3.0%, w/v to investigate the effect on lipase production by the selected better lipase producer *viz. Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*. The highest lipase activities showed at 2% concentration of glucose by *Stenotrophomonas maltophilia* (57.33 U/ml) and *Pseudomonas aeruginosa* (43.28 U/ml) (Fig. 3.37 and 3.38). Various concentrations of starch (0.5 – 3.0%, w/v) were supplemented in the medium to investigate the effect on lipase production by the isolate of *Bacillus subtilis* since starch was found to be suitable for lipase production. The highest enzyme activity (37.39 U/ml) showed at 1.5% concentration of starch (Fig. 3.39).

Nitrogen sources and other components of the media helped in the regulation of the growth of the producer organism and therefore the fermentation process (Rathi *et al.* 2002). To observe the effects of nitrogen source in lipase production, various organic nitrogen sources such as peptone, tryptone, yeast extract and beef extract were added to the production medium. Results using different nitrogen sources showed that

peptone and yeast extract were found to be better for lipase production than other nitrogen sources used. In the present study *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa* showed the highest activity (42.17 and 21.45 U/ml) when peptone was added to the medium as organic nitrogen source whereas *Bacillus subtilis* showed the highest activity (21.61 U/ml) when yeast extract was added in the medium (Fig. 3.40). Yeast extract gave similar results (20 U/ml) in case of *Bacillus* sp. reported by Bora and Bora (2012). Sharma *et al*. (2002) and Bhattacharya *et al*. (2016) showed that when organic nitrogen sources such as peptone and yeast extract were used, the bacteria, especially various thermophilic *Bacillus* spp. and *Pseudomonas* spp. were able to produce higher levels of lipase. Fadiloglu and Erkmen (2002) mentioned better lipase production when the medium contains olive oil as the carbon source along with the presence of nitrogen sources. Ghosh *et al*. (1996) reported that organic nitrogen such as peptone and yeast extract are preferred for lipase activity by various pseudomonads *viz*. *Pseudomonas aeruginosa, P. fragi* and *P. fluorescens* BW 96CC. Noormohamadi *et al*. (2013) reported that olive oil in combination with other nitrogen sources enhanced the lipase production. The organic nitrogen sources were found to be better than inorganic nitrogen sources. This finding was in agreement with the reports on lipase production from *Pseudomonas* sp. (Gupta *et al*. 2004).

The best organic nitrogen source was added in different concentration for maximum lipase production. Peptone was found to be better for the isolates *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa.* Therefore, various concentrations (0.5 – 3.0%, w/v) of peptone were supplemented in the medium for *Stenotrophomonas maltophilia* and *Pseudomonas aeruginosa*. The enzyme activity showed the highest at 1.5% concentration of peptone by *Stenotrophomonas maltophilia* (48.11 U/ml) and *Pseudomonas aeruginosa* (27.33 U/ml) (Fig. 3.41 and 3.42). Yeast extract was found to be better for *Bacillus subtilis* so that various concentrations (0.5 – 3.0%, w/v) of yeast extract were supplemented in the medium to investigate the effect on lipase production of *Bacillus subtilis*. The highest amount of lipase production was shown by *Bacillus subtilis* (25.17 U/ml) at 1.5% of yeast extract (Fig. 3.43). The results reflected that sources and concentration of organic nitrogen varied case by case.

Besides organic nitrogen sources, various inorganic nitrogen sources *viz*. ammonium chloride, ammonium nitrate and ammonium ortho phosphate were also tested to the lipase production medium. In this study ammonium chloride was the best inorganic nitrogen source for lipase production of *Stenotrophomonas maltophilia* (27.83 U/ml) and *Bacillus subtilis* (17.06 U/ml) and ammonium nitrate was the best for lipase production of *Pseudomonas aeruginosa* (16.06 U/ml) (Fig. 3.44). Inorganic nitrogen source such as ammonium chloride has been also reported to be effective in some bacteria (Dong 1999, Rathi 2001).

Various concentrations (0.5 – 3.0%, w/v) of better inorganic nitrogen source were added to the medium for lipase production. Ammonium chloride was found to be better inorganic nitrogen source for *Stenotrophomonas maltophilia* and *Bacillus subtilis*. Therefore, ammonium chloride with different concentration was added in the medium to investigate the effect on lipase production of *Stenotrophomonas maltophilia* and *Bacillus subtilis*. At 1.5% concentration ammonium chloride attained maximum lipase activity 30.28 and 17.22 U/ml were observed by *Stenotrophomonas maltophilia* and *Bacillus subtilis*, respectively (Fig. 3.45 and 3.46). Different concentrations of ammonium nitrate (0.5 – 3.0%, w/v) were supplemented in the medium to investigate the effect on lipase production by *Pseudomonas aeruginosa*. The highest lipase production (16.06 U/ml) showed at 1.0% concentration of ammonium nitrate (Fig. 3.47).

For the increase of lipase production, inoculum concentration was also increased from 1 to 7%. All three isolates showed the highest activity at 5% inoculum (Fig. 3.48). After optimization of inoculum concentration, enhanced lipase production was observed in *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Bacillus subtilis* were 73.50, 37.05 and 51.17 U/ml, respectively.

In this study various concentrations (0.5. 1.0, 1.5 and 2.0%) of olive oil were used as substrate for lipase production. All three isolates showed maximum lipase activity at 1% of olive oil (Fig. 3.49). Increasing concentration of olive oil showed an increase in lipase activity up to 1% and then a gradual decrease with the increase in oil concentration. It could be due to the certain ability of the organism to use it as a substrate and high concentration of such substrate could become toxic for the organism (Iqbal and Rehman 2015). Olive oil as lipid substrate has been found to be

generally important for obtaining a high lipolytic enzyme (Lim *et al*. 2008). Among different oils used as substrate, olive oil had the highest effect on lipase activity (12.5 U/ml) by *Pseudomonas* sp. BWS-5 (Sooch and Kauldhar 2013). Most of the researches revealed that lipid carbon sources stimulate lipase production (Abdel-Fattah 2002, Kaushik 2006). High levels of lipase production were reported from various thermophilic *Bacillus* spp. in the presence of olive oil as carbon source in the culture medium (Eltaweel 2005). Muralidhar *et al*. (2001) mentioned that olive oil was better carbon source for lipase production compared to glucose as a carbon source.

Various media were used to investigate the best production medium for lipase production by the isolates. For this test five different media *viz*. NB, LB, TBB, TB and TSB were used. It has been found that bacteria in NB medium showed their maximum lipase production (27.50, 13.83 and 16.28 U/ml by *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively) (Fig. 3.50). Habibollahi and Salehzadeh (2018) mentioned in their study that lipase production greatly affected by the composition of the medium.

In the present study lipase production with different parameters were studied to maximize the production of lipase. All optimized conditions were provided for maximum lipase production. *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis* showed enzyme activity 136.47, 96.33 and 84.30 U/ml, respectively after optimization of different parameters (Fig. 3.51). The time of incubation showed sharp decrease in lipase activity after 72 h incubation in *Stenotrophomonas maltophilia* and 36 h in *Pseudomonas aeruginosa* and *Bacillus subtilis*. After these periods, the enzyme tends to decrease suggesting that enzyme may have been either degraded or become non-functional due to decrease in lipidic substrate or increase in acidity after consumption of medium. Such a trend has also been reported with *Pseudomonas aeruginosa* by Kashmiri *et al*. (2006) and Kathiravan *et al*. (2012).

In the present study lipase production of isolated *Bacillus subtilis* (84.30 U/ml) is in almost similar with the findings of Kumar *et al*. (2012c) who reported the lipase production of *Bacillus* sp. MPTK912 with 75 U/ml. Kamaladevi *et al*. (2014) showed that lipase production of *Bacillus sp*. LBN4 was maximum 69 U/ml at 30 h of incubation. Therefore, the present result clearly showed better performances than that

of Kamaladevi (2014). Isolated *Stenotrophomonas maltophilia* of this study which was molecularly similar to *Pseudomonas* sp. showed lipase activity 136.47 U/ml which was higher than 114 U/ml produced by *Pseudomonas gessardii* reported by Veerapagu *et al*. (2013). Similar observation was also noticed by Tembhurkar *et al*. (2012) in case of *Pseudomonas* sp. and the optimum lipase activity was 66.26 U/ml obtained after optimization of different fermentation parameters. In a study, Sooch and Kauldhar (2013) reported 81.83 U/ml by *Pseudomonas* sp. BWS-5. In the present study it was 84.30 U/ml by the isolated *Pseudomonas aeruginosa*. Their result showed that there was an increase in enzyme production up to 36 h of incubation and thereafter, it decreased which is similar to this present work. They inferred that a decline in enzyme activity after 36 h of fermentation could be either due to decrease in nutrient availability in the medium or catabolite repression of enzyme. However, some workers found maximum lipase production 79.83 U/ml after 72 h incubation from *Pseudomonas fragi* (Pabai *et al*. 1996). In this study, interestingly, before optimization *Bacillus subtilis* was better than *Pseudomonas aeruginosa* but after optimization *Pseudomonas aeruginosa* performed better than *Bacillus subtilis*. Both medium characteristics and the rate of bacterial growth have an influence on the incubation time for enzyme production. It may be concluded from the present study that three indigenous isolates characterized as *Stenotrophomonas maltophilia, Pseudomonas aeruginosa* and *Bacillus subtilis* can be used as a potential bacterial source of lipase.

A time course of lipase production the strain *Stenotrophomonas maltophilia* showed that after optimization the maximum lipase activity was 136.49 U/ml and was observed at 72 h of incubation at the late exponential phase of bacterial growth curve. *Pseudomonas aeruginosa* and *Bacillus subtilis* showed the highest lipase activity at 36 h of incubation at exponential phase of growth curve. Similar result was obtained by Kanlayakrit and Boonpan (2007) who showed lipase activity in *Pseudomonas aeruginosa* (90.12 U/ml) was detected at 48 h of incubation at exponential phase.

The results of the present study provide useful information for the optimization of culture conditions such as pH, temperature, NaCl, carbon and nitrogen source, substrate, inoculum concentration and media for the best lipase production by the isolates. The present study revealed that a wide range of bacteria were found to have

lipolytic activity. Since the selected organisms have been isolated from nature, there is a scope of increasing their potentialities by genetic manipulation. Therefore, the present investigation and findings can definitely offer impacts for further studies. The optimized lipase production developed in this study can be used for large scale in industrial purpose.

To investigate the biotechnological importance of isolated bacterial lipase with special reference to lipid-rich wastewater treatment, synthetic wastewater treatment was carried out in the Laboratory using three potential isolates *viz*. *Stenotrophomonas maltophilia* e-a22*, Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B and their consortium. For this experiment COD performance was carried out. Wastewater sample was prepared in the Laboratory and after inoculation analyzed for COD in various time intervals to find out the performance. COD removal was observed after each 24 h of incubation up to 96 h. In this study the COD was reduced from initial 1200 to 303, 320, 400 mg/L at the end of the treatment by *Stenotrophomonas maltophilia, Pseudomonas aeruginosa,* and *Bacillus subtilis,* respectively. Consortia of three strains reduced the COD to 200 mg/L. During monoculture the COD removals were 74.75%, 73.33%, 66.67% by *Stenotrophomonas maltophilia*, *Pseudomonas aeruginosa* and *Bacillus subtilis*, respectively after 96 h of treatment. *Stenotrophomonas maltophilia* showed the best result among single isolates. Consortium showed the highest COD removal (83.33%) (Fig. 3.54). There were many reports in relation with the use of lipolytic enzymes in wastewater treatment (Chen *et al*. 2009, Hesnawi *et al*. 2014). Like present study similar work had been carried out with other lipase-producing strain *Pseudomonas aeruginosa* SL-72 (Verma *et al*. 2012). The crude lipase of *Pseudomonas aeruginosa* SL-72 was added to wastewater contaminated with crude oil, resulting 86.39% reduction of COD after 7 days of treatment. Orapinand and Kriangkrai (2010) reported that single culture of *Pseudomonas* sp. was highly efficient and showed COD removal of 95.81% within 8 days of treatment. Bhumibhaman (2002) reported COD reduction 57.2% of kitchen wastewater by *Pseudomonas* sp.

Since consortia gave better result than single isolates so the results indicate that combined strains could reduce COD efficiently from synthetic wastewater. Other investigators have also reported oil and grease removal which is comparable to the

present study. Serikovna *et al*. (2013) reported fat, oil and grease (FOG) removal by *Pseudomonas aeruginosa* G23 with the degradation rate of 62%-66%. Bhumibhamon *et al*. (2002) reported 88.8 and 81.6% organic matter removal by *Acinetobacter* sp. KUL8 and *Bacillus* sp. KUL39 from palm oil and bakery wastewater. Hassan *et al*. (1997) has also reported the treatment of palm oil by *Rhodobacter sphaeroides* with 74.2% organic matter removal. Other reports are *Acinetobacter* sp. (60-65%) by Wakelin and Forster (1997) and *Rhodobacter shaeroide* S (74.2%) by Takeno *et al*. (2005).

In a study by Mongkolthanaruk and Dharmsthiti (2002), a mixed culture composed of *Pseudomonas aeruginosa* LP602, *Acinetobacter calcoaceticus* LP009 and *Bacillus* sp. B304 had been effective in lowering the COD and lipid content of a wastewater within 12 d of treatment under aerobic conditions. Many researcher have reported that introduction of indigenous strains of bacteria can enhance the degradation of organic pollutants in wastewater. Hu *et al.* (2018) had found that *Pseudomonas* sp. was very effective in removing organic matter from industrial wastewater. Riaz *et al*. (2010) found *Bacillus* spp. have high ability in this case. Chen *et al*. (2009) investigated the treatment of municipal wastewater with single strain of bacteria and bacterial consortium.

Obire (1988) reported that biodegradation of oil polluted environment can be enhanced by inoculation with microbial species that will degrade the oil waste more efficiently. The single culture, however, it took longer time to reach their maximum removal than consortia. This study suggests wastewater treatment with highly specialized strains can improve the treatment. In this study the results of degradation of organic matter between mixed cultures and single culture in two types of experiments indicated that the different degradation efficiency might be due to the different reaction system of lipase from each culture. Lipase present not only catalyzed hydrolysis reaction but also catalyzed interesterification reaction, depending on the source of lipase and reaction condition (Macrae 1983). A mixture of three bacterial strains expressed potential to reduce of COD under neutral pH of the wastewater. Neutral pH favored lipase production during wastewater treatment in *Bacillus* spp. reported by Mohan *et al*. (2008).

The difference in percentage degradation rate by different isolates in various studies by researchers could be due to the difference in waste characteristics where each type of oily wastewater has its own characteristic composition (Ainon *et al*. 2010). Oily wastewater produced in oil factories provides various environmental problems, because of having different pollutant compounds. Recently the biological process is the main treatment system in wastewater plant. In biological methods, the microorganisms with high lipase activities can degrade oil. In this study three indigenous bacterial isolates could be used as seeding inocula in the oily wastewater treatment. The performance of a biological process is often enhanced through bioaugmentation of one or more species of specialized microorganisms (Karamalidis *et al*. 2010, Erdogan *et al*. 2011, Semrany *et al*. 2012).

Industrial effluents from different industries may have been creating serious problems. These problems might be ecofriendly solved by the indigenous isolates. It will not be amazing if lipases acquire the peak position in the area of enzyme application and global enzyme market in the upcoming future. As the world oil demand is increasing generation of oil is also increasing. There is a need of appropriate waste minimization or recycling technology which should be easy to operate and cost effective using the microbial resources. The use of lipase in industries is enormous and increasing. The new areas of application are constantly being added (Veerapagu *et al*. 2013). Screening of new lipase producing bacteria will open new, simple routes to solve environmental pollution with lipid. Therefore, it may be concluded that bacteria with lipase activity can be used as a potent candidate for bioremediation of lipid pollution. However further investigations are required to understand the suitability of using these organisms in the treatment of lipid-rich wastewater on a large scale.

Chapter-5

Conclusion

Conclusion

A good number of heterotrophic bacteria were found to be associated with the lipid-rich environment. During this study a total of 176 lipolytic bacterial isolates were isolated from 10 lipid rich water and soil samples from different areas in and around Dhaka Metropolitan City. Isolated bacterial isolates were screened out for lipase producing ability. Among them 68% isolates showed lipase positive. Among them 30 lipolytic isolates were provisionally identified. Molecularly identified 10 better lipolytic isolates were studied in detail with special reference to biotechnological point of view.

The results clearly demonstrated that lipase producing bacteria are widely distributed in environment. The present research revealed that different species of *Bacillus* were more predominant than other species. Three isolates showed lipase activity above 10 U/ml. *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B were better lipase producers.

The cultural conditions had a considerable influence on enzyme production especially carbon, nitrogen and inoculum concentration played important role in enzyme production. Optimum conditions of the parameters investigated gave a baseline for further study involving large scale and cost-effective production of lipase by three isolates. The final optimized medium resulted higher lipase production in compare to initial lipase production. The maximum lipase production was 136.47 U/ml by the *Stenotrophomonas maltophilia* e-a22 which was found to be good for the indigenous bacteria isolated from Bangladesh.

The COD removal test of lipid-rich synthetic wastewater using the three isolated bacteria individually and as a consortium was investigated. After 96 h of treatment, the COD decreased by 74.75, 73.33 and 66.67% by *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B, respectively. The maximum COD reduction was 83.33% by the consortium could be considered good for COD removal from lipid-rich wastewater. These results suggested that they were effective for organic matter removal in wastewater treatment.

In conclusion, it could be mentioned that for the first time *Stenotrophomonas maltophilia* e-a22 isolated from water of The Turag River which may be considered a promising indigenous lipase producing strain aptly suitable for bioremediation. However, as per speculation consortium is found to be better for wastewater treatment than the monoculture. Therefore, consortium of *Stenotrophomonas maltophilia* e-a22, *Pseudomonas aeruginosa* 12 and *Bacillus subtilis* 20B could be considered as potent seeding material for lipid-rich wastewater treatment.

Water pollution is one of the burning issues of Bangladesh and the indigenous potent lipase producing bacterial isolates could play a vital role towards the water pollution management. It was observed considerable COD decrease indicated the potentiality of the isolated indigenous bacteria towards its biotechnological application. The mixed culture or the consortium showed the best performance and it could be applied in lipid-rich wastewater treatment in commercial scale. From this research, it may be suggested that bacteria with better lipolytic activity can be used as industrial candidate as seeding material in the lipid-rich wastewater treatment.

Chapter-6

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Chapter-7

Appendices

Appendices

Composition of the media and reagents used in this study are as follows. In each case, pH was adjusted before sterilization.

1. Ammonium oxalate crystal violet solution (Claus 1995)

Solution A

Crystal violet (85% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Solution **A** and **B** were mixed.

2. Basal medium for fermentation (Gordon 1966)

Beef extract	3.0 g
Ammonium dihydrogen phosphate	1.0 g
Magnesium sulphate	0.2 g
Potassium chloride	0.3 g
Carbohydrate	10.0 g
Bromothymol blue	2.0 g
Distilled water	1000 ml
pH	6.8

3. Cetrimide agar medium (ATCC 1992)

Peptocomplex	20.0 g
$MgCl_2$	1.4 g
K_2SO_4	10.0 g
Cetrimine	14.0 g

Glycerol	10.0 ml
Distilled water	1000 ml
pH	7.2

4. Deep glucose agar medium (SAB 1957)

Beef extract	3.0 g
Peptone	5.0 g
Glucose	10.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

5. Egg-yolk lecithinase medium (SAB 1957)

Peptone	40.0 g
Na_2HPO_4	5.0 g
KH_2PO_4	1.0 g
NaCl	2.1 g
$MgSo_4$	0.1 g
Glucose	2.0 g
Agar	25.0 g
Distilled water	1000 ml
pH	7.6

6. Iodine Solulion (Claus 1995)

Iodine	1.0 g
Potassium Iodide	2.0 g
Distilled water	300 ml

7. Indole Nitrate broth (Biolife Manual 1991)

Peptone	20.0 g
NaH_2PO_4	2.0 g
Glucose	1.0 g

KNO_3	1.0 g
Agar	1.0 g
Distilled Water	1000 ml
pH	7.2

8. Kovac's reagent (SAB 1957)

Paradimethyl amino-benzaldehyde	5.0 g
Butyl alcohol	5.0 ml
HCl (Conc.)	25.0 ml

9. Levan medium (Schaad 1988)

Beef extract	3.0 g
Peptone	5.0 g
Sucrose	50.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

10. Luria Bartani agar medium (Sambrook 1989)

Peptone	10.0 g
Yeast extract	5.0 g
NaCl	10.0 g
Distilled water	1000 ml
pH	7.0

11. Malachite green solution (Claus 1995)

Malachite green	5.0 g
Distilled water	100 ml

12. Mercurochrome solution (SAB 1957)

Mercurochrome	0.5 g
Distilled water	100 ml

13. Methyl Red / Voges-Proskauer broth medium (Bryan 1950)

Peptone	7.0 g
Glucose	5.0 g
Dipotassium phosphate	5.0 g
Distilled water	1000 ml
pH	7.0

14. Methyl red solution (Bryan 1950)

Methyl red	0.1 g
Ethyl alcohol (95%)	300 ml
Distilled water	200 ml

15. Motility medium (Eklund and Lankford 1967)

Nutrient Broth	100 ml
Agar	0.5 g
TTC	0.001 g
pH	7.0

16. Nutrient agar medium (Bryan 1950)

Beef extract	3.0 g
Peptone	5.0 g
Nacl	5.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

17. Nitrate broth medium (SAB 1957)

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	1.0 g
Distilled water	1000 ml

pH	7.2

18. α-Napthol solution (Bryan 1950)

α –Napthol	5.0 g
Ethyl alcohol (95%)	100 ml

19. Oxidase test reagent (Collins and Lyne 1984)

Tetramethyl-p-phenylene-diamine dihydro-chloride	1.0 g
Distilled water	100 ml

20. Peptone broth

Peptone	1.0 g
Water	100 ml

21. Peptone iron agar medium (SAB 1957)

Peptone	15.0 g
Proteose peptone	5.0 g
Ferric ammonium citrate	0.5 g
Sodium glycerophosphate	1.0 g
Sodium thiosulphate	0.08 g
Agar	15.0 g
Distilled water	1000 ml
pH	6.7

22. Peptone Yeast Extract Glucose agar (PYG medium)

Glucose	10 g
Peptone	5 g
Yeast extract	5 g
Agar	15 g
Distilled water	1000 ml
pH	7.2

23. Physiological saline

Sodium chloride	0.85 g
Distilled water	100 ml

24. Propionate agar medium (Sneath *et al.* 1986)

Sodium propionate	2.0 g
$MgS0_4.7H_20$	1.2 g
NH_4HP0_4	0.5 g
KCI	1.0 g
Trace element solution	40.0 ml
Agar	15.0 g
Distilled water	920 ml
Phenol red (0.04% w/v)	20.0 ml
pH	6.8

25. Safranin solution (SAB 1957)

Safranin	0.5 g
Distilled water	100 ml

26. Simmon's citrate agar (Claus 1995)

$MgSO_4$	0.2 g
Mono-ammonium phosphate	1.0 g
Di-potasssium phosphate	1.0 g
Na-citrate	2.0 g
NaCI	5.0 g
Bromo-thymol-blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

27. Starch agar medium (Claus 1995)

Beef extract	3.0 g
Peptone	5.0 g
Soluble starch	10.0 g
Agar	15.0 g
Distilled water	1000 ml
pH	7.2

28. Skim milk agar (SMA) medium (Collins and Lyne 1984)

Skim Milk	6.6 ml
Nutrient agar	100 ml
pH	7.2

29. Synthetic wastewater medium (Chen *et al.* 2009)

Glucose	7.5 g/L
NH_4Cl	1.15 g/L
KH_2PO_4	0.2 g/L
$MgSO_4.7H_2O$	0.2 g/L
$CaCl_2$	0.25 g/L
Trace element	0.5 ml

Trace element solution

H_3BO_3	50 mg/L
$ZnCl_2$	50 mg/L
$CuCl_2$	30 mg/L
$MgSO_4$	500 mg/L
$CoCl_2.H_2O$	30 mg/L
$(NH_4)_6$ $6MoO_2.4H_2O$	50 mg/L
pH	7.0
Autoclaved	15 min

30. Thorney's medium (Schaad 1988)

Bacto peptone	1.0 g
NaCl	5.0 g
K_2HPO_4	0.3 g
L-arginine HCl	10.0 g
Phenol red	0.01 g
Agar	3.0 g
Distilled water	1000 ml
pH	7.2

31. Tributyrin Agar (Cardenas *et al.* 2001)

Peptone	5 g
Yeast extract	3 g
Tributyrin	10 ml
Agar	15 g
pH	7.0

32. Tryptone Soya Broth (Anbu *et al*. 2011)

Pancreatic casein	17 g
Enzymatic digest soybean	3 g
NaCl	5 g
Di-potassium phosphate	2.5 g
Glucose	2.5 g
Distilled water	1000 ml
pH	7.5

33. Tyrosine agar medium (Sneath *et al*. 1986)

L-tyrosine	0.5 g
Distilled water	10.0 ml
Nutrient agar	100 ml
pH	7.2

Tyrosine was sterilized separately and was added to the medium before inoculation.

34. Tween Agar medium (Schoofs *et al*. 1997)

Peptone	10 g
NaCl	5 g
$CaCl_2$	0.1 g
Tween 80	10 ml
Distilled water	1000 ml
Agar	15 g

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