A study on psychrotrophic bacteria from Arctic region with special emphasis on cold active lipase production, optimization and characterization

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By

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A study on psychrotrophic bacteria from Arctic region with special emphasis on cold active lipase production, optimization and characterization

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Certificate

*This is to certify that the thesis entitled "**A study on psychrotrophic bacteria from Arctic region with special emphasis on cold active lipase production, optimization and characterization**" is an authentic record of research work carried out by Ms. Neethu C.S. under my supervision and guidance in the Department of Marine Biology, Microbiology and Biochemistry, School of Marine Sciences, Cochin University of Science and Technology, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Microbiology and no part thereof has been presented before for the award of any degree, diploma, or associateship in any University.*

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*I hereby declare that the thesis entitled "**A study on psychrotrophic bacteria from Arctic region with special emphasis on cold active lipase production, optimization and characterization**" is a genuine record of research work done by me under the supervision and guidance of Dr. A.V. Saramma and Dr. A. A. Mohamed Hatha, Professors, Department of Marine Biology, Microbiology and Biochemistry, School of Marine sciences, Cochin University of Science and Technology and that no part of this work has been presented for the award of any degree, diploma, associateship in any University or Institution earlier.*

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This is to certify that all the relevant corrections and modifications suggested by the audience during the pre-synopsis seminar and recommended by the doctoral committee of Mrs. Neethu C.S. has been incorporated in the thesis entitled ***"A study on psychrotrophic bacteria from Arctic region with special emphasis on cold active lipase production, optimization and characterization"***

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Neethu C.S.

Dedicated to……

Almighty God and my family

Abbreviations

˚C	Degree Celsius
%	Percentage
A	Amplicillin
AM	Amoxicillin
ANOVA	Analysis of variance
Bp	Base pair
BLAST	Basic Local Alignment Search Tool
BSA	Bovine serum albumin
CB	Carbenicillin
CEP	Cefpodoxime
CA	Ceftazidime
CH	Cephalothin
C	Chloramphenicol
CF	Ciprofloxacin
CL	Colistin
CCD	Central Composite Design
Da	Dalton
DEAE	Diethyl amino ethyl
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DOE	Design of Experiments
EDTA	Ethylene- diamine tetra acetic acid
FCCCD	Face Centered Central Composite Design
G	Gentamicin

G	Gram
Hrs	Hours
IAA	Iodo acetic acid
KDa	Kilo Dalton
L	Litre
M	Molar
Mg	Milligram
Ml	Milli litre
Mm	MilliMolar
mol/L	Moles per litre
N	Normal
NA	Nalidixic acid
nm	Nanometer
O. D	Optical density
PAGE	Poly Acrylamide Gel Electrophoresis
PBD	Plackett- Burman Design
PCR	Polymerase chain reaction
PMSF	Phenylmethylsulphonyl fluoride
Ppm	parts per million
pNP	Para nitrophenol
pNPP	Para nitrophenyl palmitate
RSM	Response surface methodology
SDS	Sodium dodecyl sulphate
Sec	seconds
S	Streptomycin
SM	Sulphamethoxazole
T	Tetracycline

TR	Trimethoprim
U	Lipase Unit
µg	microgram
µl	microlitre

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Chapter 1
General Introduction

The Arctic is a polar region that lies north of Arctic Circle or region higher than latitude 66° 33' 44" north of the equator. The Arctic region consists approximately 14.5 million square kilometers dominated by Arctic Ocean covering 14,056,000 square kilometers and land mass from parts of eight countries - Canada, Greenland, Finland, Alaska (United States), Iceland, Norway, Sweden and Russia. The Polar Regions are the coldest part on the Earth. Because of Earth's axial tilt the polar region never receives direct sunlight. Sunlight reaches at an oblique angle and is subjected to scattering, absorption and reflection while travelling through Earth's atmosphere for long distance. This is mainly responsible for extreme cold and harsh climate of polar region. The precipitation in the Arctic is mainly in the form of snow and annual precipitation is low, mostly less than 50 cm. The Arctic is turned away from sun during the winter which causes 24 hours darkness during winter months and in summer the Earth is tilted towards the sun which causes 24 hours of sunlight in summer months.

Average winter temperatures could be as low as -40 °C and coldest temperature reported is -68 °C (near Siberia). The size and shape of the Arctic Ocean basins are similar to the Antarctic continent and 1.5 times the size of continental US. The Arctic Ocean is considered to be the shallowest of the five major ocean divisions with an average depth of 1038 m. The deepest point is found at Litke Deep in the Eurasian basin at 5450m. The Arctic Ocean is partly covered in sea ice throughout the year and completely covered in the winter which is a distinctive feature of polar Oceans. When this ice melts or freezes, it causes seasonal variations in surface temperature and salinity. Large freshwater bodies drain into the Arctic Ocean. The major rivers that drain into the Arctic are Lena, Ob, Yenisey, Pechora, Kolima, Rivers in Northern Canada and Yukon River systems. In large parts of the Arctic Ocean, the top layer remains to be lower in salinity and temperature than the rest. It is required that the upper 100 to 150m of Ocean remain in freezing temperature of -1.8 °C for sea ice to form. Some of the land parts of the Arctic such as Greenland are covered with ice sheets while others such as Alaska have tundra.

1.1 Microbial life in the Arctic

Studies and developments in microbial ecology and evolution in the past few decades have proven that the microbial life, especially prokaryotic life, can survive and thrive in harsh environments previously thought uninhabitable on Earth. Despite presenting adverse conditions for life such as freezing temperatures, low nutrient availability, reduced membrane fluidity and high water viscosity; permanently cold environments such as the Arctic is successfully colonized by all three domains of life (Steven *et al.*, 2007; Carrasco *et al.*, 2012). Most biologists tend to neglect the fact that the dominant environment of the biosphere is cold. The Polar Regions and oceans cover 14 and 71 % of earth's surface respectively and more than 90 % of the ocean (by volume) is 5 °C or lower. These environments provide natural habitat for cold adapted psychrophilic and psychrotrophic bacteria.

Mortia (1975) defined psychrophiles as bacteria or archaea having an optimal temperature of growth at 15 °C or lower and maximal temperature of growth at about 20 °C and psychrotrophs to denote microbes that have the ability to grow at low temperature and maximal growth at 20 °C. Gonout (1986) defined psychrophiles and psychrotrophs as microorganisms that have the ability to grow at 0 °C. Psychrotrophs are organisms having maximum temperature of growth

above 20 °C and is widespread in natural environment while psychrophiles have a maximum temperature for growth at 20 °C or below and confined to permanently cold habitats.

The thermal sensitivity of psychrophilic bacteria indicates the adaptation of cold adapted bacteria to their environment. The most important functional adaptation is found to be the role of dihydrouridine in the maintenance of conformational flexibility of RNA. Dihydrouridine rich RNA in cold adapted organisms helps to retain their membrane fluidity at low temperatures which is otherwise known as homeophasic adaptation. This appears to be the primary adaptation in cold temperature since it allows the nutrient transport to take place (Mayer and Morita, 2007). Antifreeze protein and cryoprotectants reduce the ice crystal formation inside cells and these proteins have been successfully isolated from polar bacteria (Gilbert *et al*., 2004; Gilbert *et al*., 2005). Exopolysaccharide production is also found to play an important role in cryo-protection in psychrophile and psychrotrophs (Krembs *et al*., 2002; Nicholas *et al.,* 2005). Apart from these modifications, the enzymes of psychrophilic and psychrotrophic bacteria are adapted to catalyze all metabolic processes at the low temperatures. Crystallographic analysis and gene homology studies revealed that different enzymes use multiple strategies such as decreased inter subunit interactions; reduction in number of ionic pairs, hydrogen bonds and hydrophobic interactions; increased interaction with solvent; higher accessibility to active site; increased exposure to nonpolar residues in the solvent etc. to achieve the conformational flexibility at low temperature (Mayer and Morita, 2007).

Various distinct microbial habitats have been reported in the Arctic region including glacial ice, sea ice, tundra wet lands, permafrost, oceanic water, subglacial soil, periglacial soil and tundra soil (Reddy *et al.*, 2009). International Polar Year (IPY) program in 2008 reported the climate change impacts on microbial diversity and vulnerability of polar ecosystems to global climate change. These reports reiterate the significance of microbial diversity and ecosystem studies in the polar region. Marine sediment represents one of the most complex habitats on Earth, and microbes thriving in these habitats apart from contributing to biomass, play very significant role in remineralization of organic matter (Ravenschlag *et al*., 2000; Tian *et al*., 2009). Accumulation of organic matter in the northern environments immobilizes nutrients and thus the Arctic is considered to be a carbon sink (Vincent *et al.*, 2009). The organic matter concentration of marine

sediments is 10^4 to 10^5 fold higher when compared to sea water. This serves as a rich nutrient source for growth and abundance of microorganisms (Zeng *et al.*, 2011).

Arctic and Antarctic ice packs with its vast extension are proved to have high biological productivity and constitute one of the most important polar ecosystems. Arctic Ocean, in contrast to the Southern Ocean is influenced by warm Atlantic waters and high terrestrial inputs (Amon *et al.*, 2001; Brinkmayer *et al.*, 2003). Crump *et al.* (2012) suggested that patterns of microbial diversity in Arctic waters are structured by initial inoculation from soil reservoirs of microbes which is followed by species sorting process during down slope dispersal of microbes. Permafrost represents 26 percentage of terrestrial soil ecosystem and is defined as soil that remains at or below 0 °C for at least two consecutive years (Willian and Smith, 1989). Permafrost harbour many bacterial communities with diverse metabolic groups, many researchers (Boyd and Boyd, 1964; Gilichinsky *et al*., 2002; Bakermans *et al*., 2003; Vishnivetskaya *et al*., 2003) reported the existence of methanogens, sulfur-reducing bacteria, aerobic and anaerobic bacteria from Siberian, Canadian and Alaskan permafrost. Microbes isolated from permafrost survive geological time scales at subzero temperatures and it is interesting to note that majority of them are psychrotrophic bacteria rather than true psychrophlies.

1.2 Antibiotic resistance in the Arctic bacteria

Even though Antibiotic resistant pathogens are profoundly important to human health, the environmental reservoirs of resistance determinants are poorly understood (Allen *et al*., 2010). The prevalence of horizontal gene transfer through presence of antibiotic resistance genes on plasmids, transposons and integrons are the major mechanisms by which antibiotic resistance is disseminated to non resistant bacteria (Thomas, 2005). There are lots of evidences of transfer of resistance factors to known human commensal bacteria and pathogens more over gene transfer is shown to be extensive in the human intestinal micrbiome (Witte, 1998; Aarestrup *et al*., 2005). The information regarding the sources and reservoirs of these transferable genes are lacking. The circumstances that lead to dissemination of antibiotic resistance genes in pathogens will be understood only with the detailed examination of origin and role of resistance in natural environments.

As in the case of hospital environment, the agricultural use of antibiotics selects for antibiotic resistance. It is argued that in the agricultural fields, dissemination is higher owing to the wide administration of prophylactic antibiotics in the feed and water in the farms (Allen *et al*., 2010). Antibiotics from both urban and agricultural sources pollute and persist in the soil and aquatic environments (Thiele-Bruhn *et al*., 2003; Segura *et al*., 2009). The selection pressure applied by such antibiotics in clinical and agricultural fields has promoted the evolution and dissemination of resistance genes regardless of their origin. The use or misuse of antibiotics is not the only selection pressure of antibiotics; compounds and conditions that present in the natural microbial communities provide an additional selection pressure. Most of the antibiotic producing bacteria also carry genes for resistance to the antibiotics they produce. Besides, antibiotics are mostly produced by bacteria and fungi that occur in natural environment.

Antibiotics have multiple functions in a microbial community and evidences show that antibiotic resistance genes are common in natural environments and existed even on plasmids (Allen *et al*., 2010). Prevalence of bla_{TEM} genes, the resistant determinants for β-lactam antibiotics was reported from the Arctic environments by Brusetti *et al.* (2008). During this study, it was found that a high proportion of bacteria with extracellular *β*-lactamase activity exist in the Arctic environment. Functional metagenomic studies on resistosome of bacterial communities from different layers of the Canadian permafrost have shown that microbial communities harboured diverse resistance mechanisms at least 5000 years ago. Eight genes were isolated conferring clinical levels of resistance against aminoglycoside, tetracycline and β-lactam antibiotics. Four of the genes among resistance genes obtained conferred resistance against a modern semi synthetic antibiotic amikacin (Perron *et al*., 2015). These evidences indicate that antibiotic resistance genes probably were functional and even diverse much before the human use of antibiotics, contributing to the evolution of natural reservoirs of resistance genes.

Antimicrobial drug resistance in *Escherichia coli* from fecal samples of Arctic bird was studied by Sjolund *et al*. (2008). Among the 17 antibiotic tested, *E. coli* isolates from the Arctic bird showed resistance to 14 antibiotics. Antimicrobial drug resistance in normal flora of Arctic bird could be explained by mechanisms such as spontaneous mutation, horizontal gene transfer from other microbes, bacteria with resistance could be imported into the region by migratory birds or through human refuse. Nevertheless, the study proved that multiple antibiotic resistance exists in

one the most remote place on Earth also warrants further investigations in drug resistance in the Arctic.

Fecal indicator organisms were isolated from migratory Canada geese and their antibiotic resistance pattern was detected and it was found that migratory water fowl might serve as reservoir of antibiotic resistance. Out of the 10 antibiotics tested, 95 % of *E. coli* was resistant to a number of antibiotics such as penicillin, ampicillin, cephalothin and sulfathiazole. *Enterococci* were found to be resistant to streptomycin, sulfathiazole, cephalothin and gentamycin (Middleton and Ambrose, 2005). Although *Enterococci* are rarely pathogenic, they are now listed under most common causes of nosocomial infections (Jeljazewicz *et al*., 2000). Polar bears are the major predators in the Arctic marine ecosystems and are closely associated with sea ice. Gut microbial diversity and antibiotic resistance study of Polar bear showed that even though bacterial diversity in faeces of polar bear is low, they do harbor bacteria with bla_{TEM} alleles (Glad *et al*., 2010). These studies reinforce the necessity of detailed study of Antibiotic resistance from pristine environments of the Arctic.

1.3 Enzyme profiling of the Arctic bacteria

Prokaryotic organisms do not have temperature regulation and their internal temperature will be always close or identical to the surrounding external environment. Despite the pressure of low temperature on the biochemical reactions, they carry out their metabolic processes at a rate similar to that of organisms living in the temperate environments. Enzymes are the target for adaptations to make life possible in the extreme cold environments in the cold adapted microorganisms. Cold adapted enzymes have a high specificity at low and moderate temperatures but they have low thermal stability compared to their mesophilic counterparts (Gerday *et al*., 2000). The available data on mechanisms of cold adaptation in the enzymes indicates that the increased flexibility is the main structural characteristic of cold adapted enzymes and balance between stability, activity and flexibility is the key to the enzyme catalysis at low temperatures.

Cold adapted enzymes produced by psychrophilic and psychrotrophic bacteria have the ability to catalyze reactions at low or moderate temperatures and are considered to be a versatile tool for development and improvement of biotechnological processes (Gomes and Steiner, 2004). Cold

adapted enzymes occupy a significant position in bioprospecting of extremozymes mainly due to their specific stability under extreme conditions, improved raw material usage and low waste generation (Margesin *et al*., 2007; Cavicchioli *et al*., 2011; Kumar *et al*., 2012). Various cold adapted enzymes including proteases, lipases, amylases, cellulases, invertases and inulinases have high demand in the global market for their application in food, biofuel and detergent industries (Margesin, 2010).

Even though microbes offer enormous metabolic diversity, only 2 percentage of world's microbial diversity is tested for their ability to produce enzymes. Compared to plant and animal enzymes microbial enzymes are more sought after for their industrial and biotechnological applications because of the ease of genetic manipulation, absence of seasonal fluctuations, cost effective high production and lesser waste generation. The growth in the volume of industrial enzymes is assessed to be 4-5 % AAGR (average annual growth rate) in 2003. This growth is accompanied by decreasing prices due to increasing number of smaller companies competing in the market. In general industrial enzyme market is classified as food enzymes, technical enzymes and animal feed enzymes. Under the technical enzymes category, enzymes used in the detergent, pulp and paper industries have the major share of 52 %**.** The use of cold active lipase in detergents for cold washing would greatly reduce the energy consumption and tear of textile fibers. Cold active proteases and keratinases are used for dehairing of hides and skins at low temperature, which is advantageous because of low energy consumption and reduction of toxic chemicals used for dehairing. Other potential applications of cold active enzymes include lactose hydrolysis in milk using β-galactosidases, use of cellulases in stone washing and biopolishing of textile products, tenderization and flavour enhancement of refrigerated meet using lipases and proteases, clarification of fruit juices and beverages using pectinases and xylanases etc.

1.4 Cold active lipase

Lipases (Triacylglycerol acylhydrolases, EC3.1.1.3) catalyze reactions such as partial or complete hydrolysis of triacylglycerol and lipid esterification, transesterification and interesterification (Colla *et al.*, 2010). These varied catalytic properties generated interests in large scale production of lipases in various industries for their applications as additives in food industry, fine chemical synthesis, pharma, detergents, cosmetics, leather processing, biodiesel

production, biomedical assays and waste water treatments (Elibol and Ozer 2000; Kamini *et al.*, 2000; Burkert *et al.,* 2004; Colla *et al.*, 2010).

Despite cold active lipase producing bacteria and yeast are reported from around the globe, production of cold active lipases is under explored (Joseph *et al.*, 2007). The ability of cold active enzymes to catalyze reactions at low or moderate temperature provides industrial and biotechnological potential (Gomes and Steiner, 2004). Cold active enzymes have the additional advantage that they can be inactivated by treatment at low temperature for short time periods (Margesin *et al.*, 2002) reducing energy consumption and cost. Some of the best studied lipase producing bacteria includes *Serratia marcescens, Pseudomonas aeruginosa, Bacillus* sp.*, Burkholderia* sp. and *Pseudomonas fluorescens.* The regional and enantiospecific nature of lipases have caused a lot of interest recently among scientists and industrialists (Saxena, 2005). Following carbohydrases and proteases, lipases are the largest group of enzymes used in the industry.

Cold adapted lipases are structurally modified to increase the flexibility of polypeptide chain for easy accommodation of the substrates at low temperatures. The major modifications of amino acid sequences include a low proportion of arginine residues when compared to lysine residues, a small amount of proline residues, a reduced number of bonding such as salt bridges and of aromatic- aromatic interactions and a small hydrophobic core. In 2006, Ryu *et al*. deduced the catalytic core of cold active lipase to be oxyanion hole consisting of three amino acid sequences (Ser 174, Asp 236 and His 312) at active site and RG residues (Arg236 and Gly91) through a study conducted on Sequence alignment of cold active lipase from *Pseudomonas lipolyticus.* Alquati *et al*. (2002) reported that a large number of charged residues are exposed at the protein surface of cold active lipases from *Pseudomonas fragi*. Substrate selectivity and thermostability of cold active lipases from *P. fragi* was improved by a mutation in the lid region of catalytic triad (Ser 174, Asp 236 and His 312) and it is believed that introduction of polar residues to the lid region might be responsible for improved protein flexibility and substrate specificity (Santarossa *et al*., 2005).

1.4.1 Characterization and purification

A number cold active lipases are purified and characterized from different microbial sources. The degree of purification of lipase depends upon the kind of commercial application intended. Most of the industrial applications do not demand the need for homogenous preparation of

enzymes. However, certain degree of purity is necessary in industries such as fine chemicals, cosmetics and pharmaceuticals (Taipa *et al.*, 1992; Saxena *et al.*, 2003). Industrial purification processes are designed in such way that they are economically viable, rapid, high yielding and amenable to large scale operations (Saxena *et al.*, 2003). Traditional processes of lipase purification include various chromatographic techniques namely anion exchange chromatography (DEAE group), cation exchange chromatography (Carboxymethyl group), strong ion exchangers such as triethylaminoethyl groups and Q-sepharose groups, gel filtration chromatography and affinity chromatograpy. New purification methods are gaining momentum in lipase purification processes including immunopurification techniques, membrane processes, aqueous two-phase extraction systems and hydrophobic interaction chromatography.

Bacterial lipases from species of *Bacillus, Pseudomonas, Staphylococcus, Acinetobatcer* and *Serratia* have been studied with respect to a number of properties such as pH, temperature, stability in organic solvents, substrate specificity and effect of inhibitors and activators. Lipases that are stable in organic solvents, surfactants and metal ions are sought after by many industries such as fine chemical synthesis, cosmetics and detergents.

1.4.2 Optimization and applications

Besides using a potent strain, fermentation conditions and media composition play a significant role in enhancing lipase production (Kim *et al*., 2006). Production of lipase depends on various process parameters such as carbon source, nitrogen source, pH, temperature, substrate concentration, inducer sources and its concentration and inoculum level (Lin *et al*., 2006). In addition to media, other factors including agitation and parameters related to fermentor designs such as impeller configuration, impeller spacing and diameter are also thought to play a key role in large scale fermentation reactions (Puthli *et al*., 2008). Thus improvement of lipase production could be achieved through manipulation of nutritional and physical parameters.

There are several strategies used for modelling and optimization of fermentation parameters using conventional and statistical experimental designs. Conventionally, medium optimization using ‘one-at-a-time’ approach was used for enhancing lipase production. This method is considered to be cumbersome, time consuming and limiting considering the interaction effects of media components. During the fermentation process, the operating conditions interact and its

influence is reflected in the yield of enzymes. Thus advanced statistical methods such as response surface methodology (RSM) which analyze the interaction effects and evaluate set of process conditions are considered to be superior for media optimization (Salihu *et al*., 2011). RSM is a collection of statistical techniques that are used for modelling and analysis, wherein a response of interest is influenced by several variables and the objective is to optimize this response (Myers and Montgomery, 2002).

Microbial lipases are highly diversified in the substrate specificities and enzymatic properties which make these enzymes a highly sought after enzyme in the industries. Because of the ease of mass production large amount of purified lipase have become available for varied commercial applications. Lipases find promising applications in detergent formulations, synthesis of biosurfactants, oleochemical industry, organic chemical synthesis, nutrition, cosmetics, pharmaceuticals, leather, textile and food processing industries. Cold active lipases which act under mild conditions have greater rapidity and better specificity for production of fine chemicals. In the food industries, reactions carried out at higher temperatures are undesirable since it diminishes the nutrient value and flavour of the product and thus cold active enzymes have become an integral part of modern food industry. Enantioselective interesterification and traesterification have great significance in pharmaceutical industries for selective aceylation and deaceylation

One of the main areas in lipase research now involves the use of different microbes, supplements and substrates to obtain the best combinations for high value lipases by operational conditions that offer low production cost at industrial scale (Rigo *et al.*, 2010). Agro industrial waste and their complex organic contents constitute a significant source of residual nutrients which serve as a rich media for microbial growth and production of the enzymes (Alonso *et al.,* 2005). Even though agricultural residues are produced in large quantities in developing countries, they are mainly utilized as animal feed and landfills (Salihu *et al.*, 2012). India is world's second largest fruit and vegetable producer and encounters waste close to 25 % worth of produce (NIAM, 2012). In recent times, agricultural wastes have been made to use in biotechnological processes such as production of value added compounds and substrates for microbial isolation. These help in disposal problems which otherwise lead to pollution (Pandey *et al.*, 2000; Graminha *et al.*, 2008; Mussatto, 2009). Oil cakes from different oil extraction industries have been utilized for

fermentative lipase production mainly because; the residual oil content serves as inducer for lipase production (Singhania *et al.*, 2008; Salihu *et al.*, 2012).

In the present study, psychrotrophic bacteria isolated during the second Arctic Expedition were subjected to detailed studies.

1.5 Objectives of the study:

- To identify and characterize psychrotrophic bacteria isolated from the Arctic sediment and water by biochemical and molecular methods.
- To evaluate the prevalence of antibiotic resistance and heavy metal tolerance among these heterotrophic bacteria.
- To find out the ability of these bacteria to produce extracellular enzymes.
- To optimize the production of cold active lipase using the most potent lipase producer, purification and characterization of the enzyme and to study it's possible applications.

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Chapter 2
Characterization of heterotrophic bacterial diversity of the Arctic sediment and water by phenotypic and molecular methods

Contents:

Kongsfjord is a high latitude region (79°N) situated at the west coast of Norwegian Svalbard Archipelago, Arctic. It presents a unique site for marine ecosystem and distribution studies in the European Arctic. Kongsfjord is an ideal research spot since it provides infrastructure, accessibility, arctic climate and association with the Barents Sea. Kongsfjord is comparatively a small fjord which opens to the Sea through Kongsfjordrenna, Kongsfjord is divided into outer

part and inner part by a sill in the middle. The outer part of Kongsfjord is under the influence of west Spitsbergen current and the inner part is influenced by glaciers such as Conwaybreen, Blomstrandbreen, Kronebreen, and Kongsvegan (Svendsen, 2002).

The water column of kongsfjord is in connection with the Arctic and Atlantic water masses while fresh water is dislodged at the head of fjord by tidal glaciers which strongly influence the stratification in salinity and temperature. This unique environment creates a comparatively stable ecosystem at the entrance of the fjord and becomes unstable towards inner fjord which is brackish in nature (Weslawski *et al.*, 2000). Even though many researchers have reported hydrography, mesozooplankton and higher trophic level studies of Kongsfjord, reports regarding microbial distribution and abundance are scarce (Hop *et al.*, 2002). Some of the previous studies on microbes and their processes from this area were reported by Jankowska *et al.* (2005), Wiktor and Wojciechowska (2005), Thingstad *et al.* (2008), Piwosz *et al.* (2009), Wang *et al.* (2009) and Topper (2012). Ecosystems in Svalbard, Barrent Sea and other part of Arctic are not only stressed by climate change but also by increased anthropogenic activities such as tourism, shipping and mining.

Heterotrophic bacteria are essential components of marine ecosystem and studies on their distribution, abundance, production and involvement in nutrient cycling will throw more light on understanding the marine environment. Heterotrophic bacteria are responsible for recycling of a large fraction of sinking organic matter. Interaction between heterotrophic bacteria and sinking organic matter such as fecal pellets and post bloom aggregates often constitute the bulk of carbon biomass (Cho and Azam, 1990; Ducklow, 2001) in pelagic ecosystem and it is considered to be important in maintaining life below euphotic zone. The three major heterotrophic activities observed in marine systems include cycling of essential elements, microbial loop and utilization of dissolved organic matter.

2.1 Review of literature

2.1.1 Heterotrophic bacteria in the Arctic

For over more than 100 years, it has been established that bacteria can grow and carry out all metabolic reactions at or below 0 °C. Since more than 70 % of the oceans in the world has a temperature of 5 °C or below, marine scientists have shown keen interest in the physiology, distribution, and *in situ* activities of organisms growing at low temperatures (Wiebe *et al.*, 1992).

In the past few decades, researchers around the globe have shown great interest in polar research and reported bacterial biomass, diversity and heterotrophic activities from various Arctic environments. Arctic being one of the most vulnerable areas affected by climate change, many researchers have shown increased interest in biological studies in Arctic.

Kongfjord represents a border area between the biogeographic zones of Arctic and Atlantic. The biodiversity of Kongfjord is thus strongly influenced by both the ends. An increased influx of Atlantic water would change the species composition towards the boreal species while inner part of the fjord receives glacial waters making it more Arctic (Hop *et al*., 2002). Arctic water masses now encounter climate induced changes and might influence the marine ecosystems and biogeochemical pathways (Arctic climate impact assessment, 2004). Basic understanding on structure and function of ecosystem is thus important in predicting future changes in the Arctic ecosystems (Iversen and Seuthe, 2011). Since high-latitude seas are heterogenous in nature, the community structure and function of marine ecosystems of Arctic region may differ from each geographical area (Carmack, 2008). The Arctic region offer several distinct habitats such as glacial ice, Sea ice, tundra wetlands, permafrost, oceanic water, periglacial soil, subglacial soil, and tundra soil for growth and reproduction of various microbial populations (Reddy *et al.*, 2009).

2.1.1.1Heterotrophs in Arctic Ocean and sediment

Microbial ecology is often defined as the relationship of organisms to each other and with environment. A number of biotic and abiotic factors interact and influence the bacterial community composition and diversity. Major abiotic factors include availability of light, nutrients, pH and temperature. Major biotic factors are the structure of microbial food web and its interaction within itself which create individual niches for various autotrophic and heterotrophic bacteria (Field *et al.*, 1998, Topper *et al.*, 2012). Although heterotrophs and autotrophs utilize different carbon sequestering mechanisms, they compete for mineral nutrients and nitrogen sources. In the Arctic Ocean, these limiting nutrients are represented in a concentration of nanomolar to micromolar levels which plays a significant role in the determination of bacterial community structure (Thingstad *et al*., 1998; Vadstein, 2000; Havskum *et al*., 2003; Thingstad *et al*., 2008; Seuthe *et al*., 2011; Vadstein 2011).

Availability of dissolved organic carbon (DOC) is a major factor determining the distribution and diversity of heterotrophs in the marine environment. Apart from DOC from the primary production various other biological sources such as aquatic animal excreta and viral lysis act as provider of DOC to heterotrophic bacteria (Ducklow *et al.*, 2000; Nagata *et al.*, 2000; Williams, 2000). Vadstein *et al.* (2012) reported that the heterotrophic bacteria itself often contribute to the DOC pool. It is believed that efficiency of nutrient uptake is higher in bacteria (higher surface area to volume ratio) when compared to phytoplanktons. In the Arctic Ocean, autotrophic bacteria are subjected to nitrogen limitation and heterotrophic bacteria are limited by phosphorus availability (Topper, 2012; Vadstein, 2012). Because of scarcity of studies, factors governing microbial growth and diversity in the Arctic are not very clear. However, some studies indicate a direct correlation between viral lysis and heterotrophic distribution. Moreover, abiotic factors like nature of the substrate, its availability, depth of the water column and closeness to the coast are also found to be relevant in determining the diversity (Galand *et al.*, 2010; Pommier *et al.*, 2010; Thingstad *et al*., 2008).

A very large amount of particulate and dissolved organic matter from several large rivers enter into the strongly stratified, comparatively small Arctic Ocean (Anderson 2002, Dittmar and Kattner 2003; Benner *et al.*, 2005). The fate of this terrestrial organic matter, whether they are immediately remineralized or preserved for longer period of time will have significant impact on global carbon cycle. Many biogeochemical studies indicate low amount of biological and photochemical remineralization of dissolved organic matter in the shelf waters due to a large amount of export of this organic matter to the North Atlantic through East Greenland current (Benner *et al*., 2005).

Higher latitudes are proved to contain lesser bacterial richness when compared to lower latitudes and similarly, it was found that bacterial richness of Arctic Ocean is less (except a report of lower richness in Mediterranean Sea, Pommier *et al.,* 2010) (Fuhrman *et al.*, 2008; Topper, 2012). World Oceans are dominated by bacteria in the order of *Alphaproteobacteria*, *Gammaproteobacteria* and *bacteroides*. Even though Arctic Ocean is found to have a similar trend, there is gaining evidence of domination of *Gammaproteobacteria* in some studies (Bano & Hollibaugh, 2002; Pommier *et al*., 2007; Arnosti *et al.*, 2008; Galand, 2009; Kirchman *et al*., 2010; Teske *et al.*, 2011; Topper, 2012). It is also suggested that *Flavobacteria* may predominate in the Arctic Ocean in future due to increased nutrient load and CO_2 resulting from global

warming (Topper *et al*., 2010; Seuthe *et al*., 2011). Due to the global warming, sea ice melts and results in an increased light penetration to the upper layer which in turn supports the primary production by phytoplankton. An increase in phytoplankton production will influence the diversity of heterotrophic bacteria and previous studies suggest a trend towards C*ytophaga-Flavobacteria-Bacteroidetes* group (Horner-Devine, 2003; Sakshaug, 2004). However these studies are rarely conclusive and demand further studies in distribution of heterotrophs in Arctic region.

In some PCR based diversity analysis, bacterial communities of Arctic Ocean showed a high sequence similarity among the isolates. Ecological significance of such closely clustered isolates is so far unknown and warrant more close study on Arctic bacterial assemblage. One of the proposed methods for analyzing the ecological role is the investigation of the abundance and distribution of specific micro-diverse clusters using Fluorescence *in situ* hybridization (FISH). It is also suggested that FISH analysis might provide information about the underrepresented bacterial isolates (Amann, 1995; Malstorm *et al*., 2007). However, it is suggested that some microbes might be present in dormant states such as spores and cysts which might not be represented in diversity analysis by molecular techniques.

In the Arctic Ocean, the incidence of photochemically active irradiance is low and transmission of this irradiance through ice cover round the year is also minimal. This phenomenon results in the photochemical reactions that are not efficient for sinking significant fractions of the organic matter (Bélanger *et al*., 2006). These relatively weak photochemical reactions may restrict the formation of biologically labile dissolved organic matter which in turn reduces microbial population in Arctic. However, it is proven that the psychrophilic and psychrotolerant bacteria occurring in the Arcric Ocean actively grow even at sub-zero water temperatures (Moran *et al.,* 2000; Yager *et al.,* 2001; Miller *et al.,* 2002; Connelly *et al.*, 2006).

When compared with sea water, marine sediments contain 10^4-10^5 fold concentration of organic matter which is used as substrates and energy sources by microbes (Zeng *et al.*, 2011). Marine sediment represents one of the most complicated microbial habitats on Earth and bacteria in these sediments are a major reservoir of genetic variability similar to soil systems (Xuezheng *et al.*, 2014). The bacterial diversity studies on Arctic sediment are less compared to the other habitats although culture dependent and culture independent bacterial diversity is reported to be high. In the highly diverse bacterial population of Arctic sediments, γ- proteobacteria is found to

be dominant by most of the studies (Li *et al.*, 2009; Tian *et al.*, 2009; Zeng *et al.*, 2011). A significant fraction of retrieved sequences in the marine sediments by culture independent methods did not belong to any known taxonomic division indicating the presence of novel species and reiterate the importance of biodiversity analysis (Gongtang *et al.*, 2007; Xuezheng *et al.*, 2014).

Pomeroy and Weibe (2001) suggested that the complex interaction between low temperature effects and substrate limitation are the key to the understanding of relatively low growth rate observed in polar waters. Sherr *et al.* (2003) demonstrated the synchronous relationship that exists between autotrophic and heterotrophic biomass and abundance. Furthermore, this study also revealed dependence of bacterial growth on autochthonous substrates in the central Arctic Ocean. The fundamental differences in physical and geochemical parameters such as nature and type of organic carbon substrates, surface area, extend of mixing and available electron acceptors in the water and sediment provide different challenges to the survival of heterotrophic bacteria in these environments. Teske *et al.* (2011) demonstrated that the bacterial communities isolated from Svalbard water column and sediment at the same site was compositionally different. The laboratory experiments have significantly contributed to our knowledge about bacterial growth in cold waters. However, the complex and intricate relationships between substrate availability, bacterial growth at molecular scale, nutrient regeneration, grazing effects and viral lysis are difficult to reproduce under laboratory conditions (Belzile *et al.*, 2008).

2.1.1.2 Heterotrophs in Permafrost

Permafrost represents one of the most important habitats in northern hemisphere consisting of 24 % of the total landmass and compared to other Arctic habitats, microbiological properties of this habitat is well documented. Recently reported microbial diversity from Arctic permafrost includes the Siberian and Alaskan permafrost containing aerobic and anaerobic heterotrophic communities apart from sulfur reducing bacteria and methanogens (Vishnivetskaya, *et al.*, 2000; Gilichinsky, 2002; Bakermans *et al.*, 2003). Another interesting study was reported by Rivkina *et al.* (2004), they observed that majority of the microbes isolated from permafrost were psychrotrophs and not true psychrophiles, and these permafrost microbes survive geological time scale below freezing temperatures. Investigations on survival mechanisms of permafrost bacteria

showed altered cellular structures such as thick capsular layer around the cells and intracellular aggregates.

2.1.1.3 Heterotrophs in Tundra

Microorganisms play critical role in the tundra or boreal environment which cover 22 % of terrestrial landmass of the planet and is highly sensitive to climate change. In tundra soil, species diversity is assumed to be poor and this may be a probable reason for fewer studies in this area (Heal, 1999; Hodkinson and Wookey, 1999). However, molecular studies carried out in the Arctic tundra contradict this assumption and DNA hybridization analysis from various regions of high Arctic tundra proved that genetic diversity of these regions is similar to that of temperate regions (Neufeld and Mohn, 2005). Furthermore, Restriction fragment length polymorphism (RFLP) analysis of 43 clones from Siberian tundra showed maximum possible diversity with unique patterns for all isolates (Zhou *et al.*, 1997).

2.1.1.4 Heterotrophs in Coastal ecosystem

North Pole is the focus of many studies on climate change and its impacts in the past few decades and coastal ecosystems especially are found to be vulnerable to the impacts (Garneau *et al.*, 2006, Serreze *et al.*, 2000). Garneau *et al*. (2009) showed that there is a 52 % inter group similarity in the distribution of prokaryotes in the global ocean. The major factors influencing the coastal water ecosystems are large river water inflows, increased delivery of organic matter to the inshore Arctic sea and melting of permafrost in Arctic and subarctic region (Peterson *et al.*, 2002; Payette *et al.*, 2004). The Arctic Ocean receives annual discharge of 3299 to 4280 km^3 fresh water and it has extended shelf area coastal zones. This unique nature of the Sea is thought to play significant role in biological processes. However, prokaryotic diversity in this wide habitat is under explored (Amon, 2004). Particle associated bacterial heterotrohy and its importance in the Arctic coastal ecosystem was reported by Garneau *et al.* (2009) and concluded

that under warm climatic conditions, heterotrophs might become part of major share of biogeochemical processes and carbon fluxes.

The conditions existing in the coastal waters are characterized by permanently cold temperatures, extensive coverage of ice and short summer. However, primary production of Arctic waters is proved to be similar to that of temperate regions. During the winter period organic sedimentation is minimal and in the short summer period, ice breaks and this process results in high sedimentation (Hebbeln and Wefer, 1991). The respiration rates in the Arctic sediment are not affected by low temperature but by influx of organic matter and similar to temperate sediments, mineralization in the Arctic is found to be high (Glud *et al.*, 1998).

A large amount of organic matter is drained into coastal Arctic Ocean by Mackenzie River; out of this organic matter 30 % is young and derived from plant material. The greater part of the organic matter is land derived, mostly consisting of highly degraded almost recalcitrant molecules (Goñi *et al*., 2000; Goñi *et al.*, 2005). Due to the climate change, Arctic climate is warming up and permafrost is melting which results in an increased input of aged organic compounds into the coastal Arctic Ocean. Meanwhile, northward movement of tree line will bring more young organic carbon into the coastal Arctic Ocean. This is proposed to have a consequence in the ocean chemistry such amount of particulate material and hence will influence particle associated bacterial communities (Serreze *et al*., 2000; Garneau *et al*., 2009).

2.1.1.5 Heterotrophs in sea ice

Sea ice represents coldest environment on Earth for marine ecosystem, still an abundant microbial community develop in this coldest highly variable environment (Garrison *et al*., 1986; Horner *et al*., 1988; Palmisano, 1993). Microbial loop and analysis of bacterial production proved that major share of organisms in sea ice is represented by heterotrophic bacteria (Sullvian, 1984; Grossmann and Dieckmann, 1994; Helmke, 1995). Culturable diversity analysis recently revealed several new species belonging to α- proteobacteria and γ – proteobacteria. Some gram positive bacteria and *Cytophaga – Flexibacter- bacteroides* were also recognized from sea ice (Bowman *et al*., 1998; Gosnik *et al*., 1998).

2.1.2 Climate change, carbon load and Arctic microbial diversity

It is well established that atmospheric CO_2 levels have increased drastically due to natural and anthropogenic activities such as fossil fuel consumption and are estimated to rise further. There is an equilibrium existing between surface seawater and atmospheric carbon dioxide, as the atmospheric CO_2 increases, the in dissolved organic carbon in the sea also increases (Hedges, 2002; Riebesell *et al.*, 2007). A direct effect of global warming in the Arctic is the melting of permafrost and increased river inflow into the Arctic Ocean. These waters are accompanied by high nutrient influx into the Arctic Ocean which, in turn, influences the unique Arctic ecosystem. Apart from the increased nutrient flux, reduction in transport of inorganic nutrients is also noticed. It was also observed that the light availability to the ecosystem is increased by surface ice melting (Vadstein, 2011)

Ocean could be divided into net- phototrophic and net-heterotrophic depending on whether the organic matter is produced by phototrophs or consumed by heterotrophs. Heterotrophic bacteria consume most of the dissolved organic matter and subsequently pass it on to higher trophic levels. Under the increased dissolved organic matter and nutrient availability due to climate change, dominance and distribution of heterotrophic bacteria might shift and thus a through and continuous study is required to understand carbon dynamics and microbial distribution of the Arctic Ocean (delGiorgio, 1997; Seuthe *et al.*, 2011). It is interesting to note the contradictory effects of temperature on organic carbon utilization by heterotrophs exist. An increase in the organic carbon requirement at low temperature was revealed by Wiebe *et al.* (1992) and reduction in the degradation of organic matter was reported by Pomeroy and Wiebe in 2001. However, Thingstad *et al.* (2008) concluded in their studies that rather than temperature, availability of nutrients such as organic carbon and minerals determine the dominance and distribution of bacteria. Furthermore, a shift in the bacterial composition with respect to change in nutrient availability was also noted (Topper *et al.*, 2012).

An important consequence of increased CO_2 concentration is decrease in pH of the Ocean hence acidification (Caldeira and Wickett, 2003). It is assumed that ocean acidification would affect the heterotrophic bacterial community in various ways; Witt *et al.* (2011) reported a shift in the community composition as a result of ocean acidification. Ocean acidification might also cause changes in ocean chemistry for example an increase in the production of particulate organic

matter such as transparent exopolymer particulates (TEP) (Engel *et al.*, 2004). TEP contain large surfaces and act as an ideal medium for bacterial attachment, which could possibly enhance the abundance of attached bacteria (Allgaier *et al.*, 2008).

2.1.3 Identification of Microbial diversity

Microbial diversity is studied as culturable diversity and culture independent diversity depending on the isolation method. The culturable diversity depends upon the identification and characterization of culturable cells which represent only 1% of the total microbial community. However, characterization of microbial diversity is essential to understand the functional dynamics and survival of communities in the ecosystem. In culture independent diversity analysis, the total nucleic acid from an environment is extracted and analyzed to include all the microbial population in the sample. Culture independent methods using molecular technologies are gaining momentum in the diversity studies and have revolutionized our understanding about microbial diversity. However, culturable organisms from Arctic may provide novel compound or enzymes with exceptional properties which may find application in industries and thus making the study of retrievable heterotrophs and its characterization more relevant.

Biochemical and physiological characterization help in the tentative identification of bacterial isolates. It also helps in the differentiation of biochemical types of genetically similar groups, 15 strains of nitrogen fixing bacteria isolated from Canadian high Arctic were turned out to be *Pseudomonas* but biochemical characterization proved them to be physiologically distinct strains (Lifshitz *et al.*, 1986). Denner *et al.* (2002) carried out 16S rDNA analysis for previously identified marine Antarctic isolate *Sphingomonas paucimobilis*, and molecular identification showed 97.4 % similarity to *Psychrobacter glacincola.* Phenotypic and chemotaxonomic characterization of this isolate was coincided with the genus *Psychrobacter* confirming the identity and thus it can be concluded that phenotypic and chemotaxonomic identification aid in the confirmation of species.

In the past few decades, bacterial phylogeny has advanced to peak due to the growing information on sequencing of chronometers such as 16S rRNA and 18S rRNA genes. Phylogenetic identification of microorganisms using 16S rRNA often has proved the inadequacy

of classical methods in identification of microbes. The 16S rRNA is about 1500bp in length and consists of highly conserved sequences which may reveal deep branching into classes and phyla. It also contain variable regions within the sequence which aid in the discrimination of species within the same genus (Wiik *et al.*, 1995). Introduction of 16S rRNA gene cloning and sequencing in microbial diversity studies enable the researchers to carry out detailed investigation of biodiversity in aquatic microbial communities (Dorigo *et al.*, 2005). This molecular technique permits the efficient analysis of community composition and diversity of complex habitats.

In the last two decades, >90 % of the diversity and distribution studies in the Arctic environment was based on 16S rRNA whether it was retrievable or non retrievable diversity analysis. Groudieva *et al*. (2004) used 16S rRNA sequencing to evaluate bacterial communities associated with Svalbard sea ice, their findings grouped bacterial isolates into five phylogenetic groups and was predominated by *alpha* and *gamma proteobacteria*. However, sediment analysis of bacterial isolates showed a different trend which was predominated by *beta, gamma* and *delta proteobacteria* (Ravenschlag, 2001).

2.2 Materials and Methods

2.2.1 Sampling and Isolation

Samples were collected from four different predetermined stations at Kongsfjord, Arctic (79 N, 12 E). Sediment and water samples were collected using Van Veen Grab and Niskin bottles respectively deployed from Research Vessel Teisten. Soon after hauling up the sample on board, water and sediment temperature was checked by centigrade digital thermometer. Salinity was determined by refractometer (Atago, Japan) and pH of the samples was determined using a pH meter. Water and sediment samples were collected aseptically in sterile Duran Schott glass bottle and polythene bags respectively. Samples were then transported to the laboratory on shore within four hours of collection and were then serially diluted and plated on to ZoBells Marine Agar (ZMA), Thiosulfate citrate Bile salts Agar (TCBS agar) and Eosine Methylene Blue Agar (EMB) and were incubated at 10 ˚C for 1-2 weeks for isolation of heterotrophic bacteria.

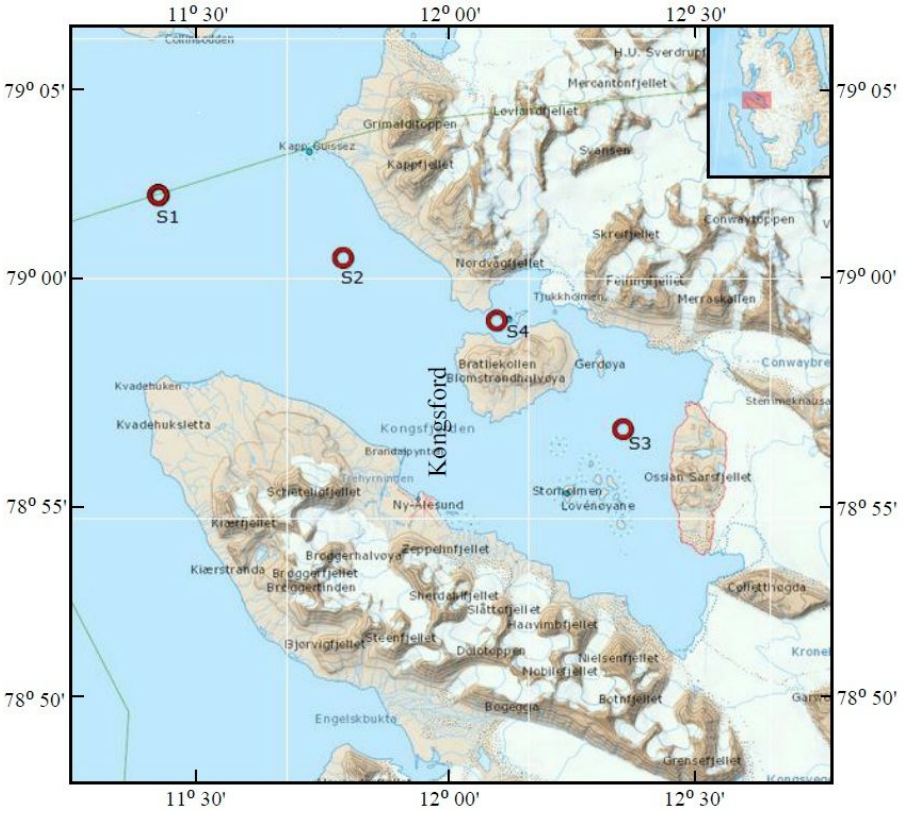


Figure 2.1: Location of sampling sites in Kongsfjorden, Ny Ålesund, Arctic

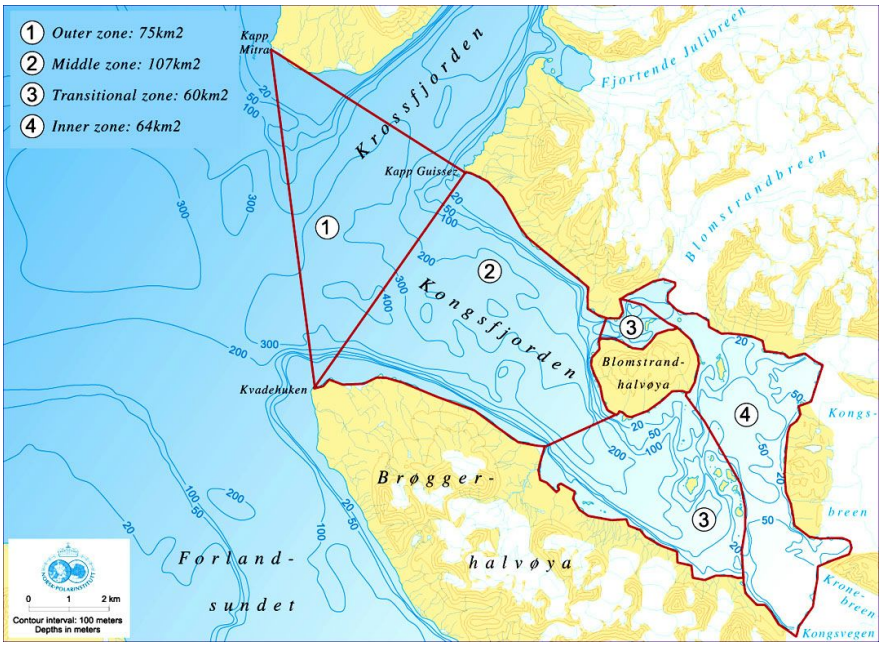


Figure 2.2: Differentiation of Arctic water into different zones

2.2.2 Phenotypic Identification

The phenotypic identification was carried out as per standard scheme based on Bergey's Manual of Systematic Bacteriology (2005).

Gram staining

Smears were prepared from young cultures and allowed to air dry and heat fixed. Primary stain (Crystal violet) was added to these smears and allowed to react for 1 min. Slides were then washed with distilled water. Gram's iodine was added to smears and again allowed to react for 1 min. Smears were rinsed with distilled water and decolourizing agent (acetone) was added drop by drop until crystal violet fails to wash off from the smear. The smears were then washed with distilled water and counter stained with Safranin for 30 sec. Slides were washed again with distilled water and air dried. After drying, slides were observed under oil immersion objective of light microscope.

Negative staining

Negative staining is performed to visualize accurate picture of true size and shape of the cell. Nigrosin stain was placed at one end of the slide and a loopful of inoculum was mixed with nigrosin stain. Another slide was placed at 45° angle to the slide with nigrosin suspension and pushed away such that it created a dark area at one end and a grayish lighter area at another end. Smear was allowed to air dry and observed under oil immersion objective lens of light microscope.

Spore staining

Gram positive bacilli selected from gram staining were subjected to spore staining. Smears were prepared, air dried and heat fixed. Smears were then flooded with malachite green. Slides were heated by passing the slides over a Bunsen burner flame to drive the stain into the spore coat. Care was taken to keep the slide wet by adding malachite green periodically. The slides were removed and rinsed gently with distilled water and counter stained with safranin for 30 sec.

Smears were then rinsed with distilled water, blot dried and observed under oil immersion objective of the light microscope.

Motility assay

a) Soft agar method

Motility of the bacteria was tested in the soft agar medium. Cultures isolated at their log phase were stab inoculated into the medium using a straight wire loop and then incubated at 15 °C for 48hr. Growth in rhizoidal pattern from the line of inoculation towards the peripheral area of the test tube was considered as the sign of motility.

b) Hanging drop method

Petroleum jelly was placed at the four corners of a clean cover slip. A loop of the bacteria grown in ZoBell's Marine Broth (ZMB) at 15 °C for 48 hr was placed at the centre of the cover slip. The cavity slide was placed with the concave facing down over the cover slip so that the depression covers the drop of the culture. Slide was gently pressed to form a seal between the slide and the cover slip. This preparation was inverted quickly so that the drop of culture was seen hanging from the cover slip. The slide was placed under the objective lens and observed for actual movement of the cells that could very well be differentiated from Brownian movement.

Cytochrome oxidase test

The organisms were freshly grown on ZoBell's Marine Agar (ZMA). A wooden applicator was used to pick colony and a compact smear was made on a filter paper moistened with 2-3 drops of 1 % solution of tetramethyl –p-phenylene diamine dihydrochloride (TPDD). A positive reaction was indicated by the development of intense deep purple colour within ten seconds. Negative reaction was indicated by the absence of characteristic colour within 10 seconds

Marine oxidation-fermentation reaction

This experiment was devised to evaluate the ability of organisms to utilize the sugar glucose. Marine oxidation fermentation media (HiMedia, Mumbai) was employed for this test. This reaction was determined by inoculating the organisms into basal media supplemented with 1%

glucose in the culture tubes. The tubes were stabbed and streaked and incubated at 15 °C. The results were recorded as follows

O – Oxidative (Yellow colouration in the slope)

F- Fermentative (Yellow colouration in both butt and slant)

FG- Fermentative with gas production

Alk/ N- alkaline reaction (Pink or purple colouration in the slant and no reaction in the butt)

Hydrogen sulfide production

Triple sugar iron agar (TSI) was used for this test. The culture was inoculated by stabbing and streaking the TSI slant. The slant was incubated at 15°C for 48 hrs and checked for H_2S production. Blackening of media was positive for H_2S production

Utilization of Amino acids

The amino acid decaboxylase test demonstrates the bacterial decarboxylation of lysine, arginine and ornithine, and these tests are of particular use in identifying members of *Enterobacteriaceae.* Decarboxylase basal broth (HiMedi, Mumbai) incorporated with 1 % of the L-amino acid (lysine dihydrochloride, ornithine dihydrochloride, and arginine dihydrochloride) was used for this test

The test organism from a solid medium was inoculated into the specific broth with a straight inoculating wire through paraffin overlay. Suitable controls were placed. Tubes were incubated and examined daily for 4 days. As a result of the bacterial fermentation of the glucose in the medium, the colour of the indicator changed to yellow. The control tube without the amino acid remained yellow; but a subsequent change to violet or purple in the tests indicated that alkaline degradation products were produced in the course of decarboxylation of the particular amino acid. A colour change in medium from purple to yellow and back to purple was positive for this test.

Production of Indole

Indole test was performed to check the ability of the organisms to convert tryptophan into Indole. Test organism was grown in Tryptone broth for 24 hrs. Two hundred microlitres of Kovac's reagent was added to 5 ml of culture medium. Formation of a cherry red ring is the positive result for indole production

Methyl Red and Voges-Proskauer test

These tests were carried out with cultures grown in glucose phosphate peptone water.

Methyl red test

Methyl red test was performed to identify the mixed acid fermentation of glucose. A few drops of methyl red indicator were added to the culture both and a resultant definite red colour was considered positive.

Voges- Proskauer test

VP test was used to detect acetoin production in the culture media by organisms. Barritt's reagent A and B were added in the ratio 3:1 to culture broth. A positive reaction was indicated by development of a pink colour in 2-5 minutes, becoming crimson in 30 minutes. The tube was shaken at intervals to ensure maximum aeration.

Citrate Utilization

This test was used to demonstrate the ability of the microbes to utilize citrate as a sole source of carbon. Simmon's citrate agar slants were streaked with a loopful of culture and incubated. The colour of bromothymol blue indicator changed from green to blue upon utilization of citrate.

Growth at different salinity

Growth of the organisms at different salinity was tested by preparing tryptone broth at different concentrations of sodium chloride (2, 5, 8, 10 and 13 % w/v). A loopful of culture was inoculated into this broth and incubated at 15 °C. Growth was detected visually by observing turbidity.

Catalase test

The ability to produce the enzyme catalase was checked in this test. The test organisms were grown on the slope of a nutrient agar slant. A thick smear of the organism was made from a 24 hrs culture on a clean slide and a drop of hydrogen peroxide was placed on it. Immediate development of effervescence was considered as positive result.

Growth on Mac-Conkey agar

Mac-Conkey agar is a differential medium for enteric group of organisms based on lactose utilization and pigmentation. Test culture was streaked onto Mac-Conkey agar plates and incubated at 15 °C for 48 hrs. Culture that utilizes lactose in the medium turns pink. Non lactose fermenting bacteria produce white/ cream colonies. Some organisms of genera *Klebsiella* and *Enterobacter* produce mucoid glassy colonies.

Utilization of various sugars

The ability of bacteria to utilize various sugars was tested using purple HiVeg broth (HiMedia, Mumbai). One percentage of the sugar was added to the media and a loopful of culture was inoculated. After the incubation period yellow colouration of the broth was considered as positive result.

2.2.3 Molecular Characterization

Biochemically typed strains were further characterized by 16S rDNA amplification; the total genomic DNA was extracted using the bacterial genomic DNA (prep) kit (Chromus Biotech, India). DNA extracts were verified by gel electrophoresis and 16S rDNA genes were amplified by using the polymerase chain reaction (PCR) with universal primer (Bossard et al, 2000) 27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492r (5'- GGTTACCTTGTTACGACTT-3').

The PCR reaction was set up using 100 ng of genomic DNA, 1.5 µL of 10 pmol of both forward and reverse primers, 5 µL of standard 1X Taq buffer, 3 µl of 1.5 mM $MgCl_2$, 0.4 µl of 200 µM dNTP mix and 0.3 µl of 1.5 U Taq DNA polymerase (Banglore Genei Pvt. Ltd., Bangalore, India) in 50 µl reaction volume. The PCR amplification was carried out in PCR tubes using Biorad CFX96 (USA), for 30 cycles after an initial denaturation at 94 C for 5 min. Each PCR

cycle comprised three steps: 30s at 95 C (denaturation), 30s at 45 C (annealing) and 120s at 72 C (extension). A final extension of 10 min was given at 72 C. PCR products were checked by gel electrophoresis and purified using the PCR Clean-Up Kit (Chromous Biotech, India) and sequenced using an ABi 3730 XL Genetic Analyser (Applied Biosystems, USA).

In order to identify the nearest taxa, 16S rRNA gene sequences (1418-1542 bases) obtained was subjected to BLAST sequence similarity search (http://blast.ncbi.nlm.nih.gov/BLAST). 16S rRNA gene sequences were aligned using the CLUSTAL W and phylogenetic trees were constructed using two tree making algorithms, maximum likelihood (ML) and Neighbour-joining (NJ) methods using MEGA version 6. All 16S rDNA sequences of the strains selected from biochemically typed groups were deposited in GenBank with accession numbers.

2.3 Results

2.3.1 Phenotypic Identification

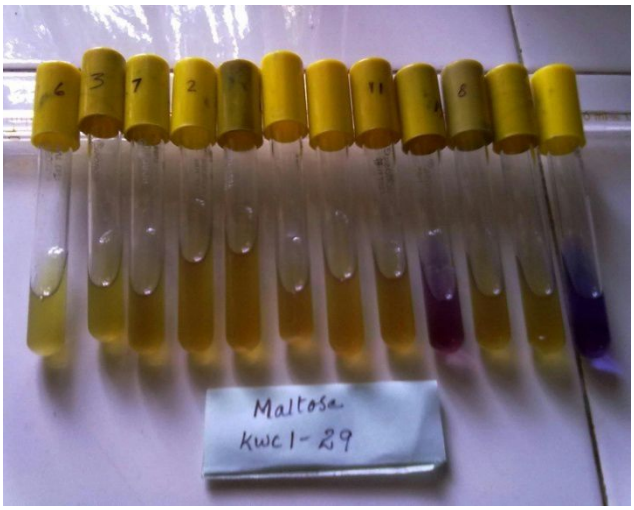
The predetermined sampling stations ranged between the coordinates 78°57'29" - 79°02'36" N and 11°19'14"-12°19'27" E. Bottom depth of different stations were 320, 185, 45 and 110 m in station 1, 2, 3 and 4 respectively. The surface water temperature varied from 3.6 to 6. 1 °C while bottom water temperature of all stations was around 1°C. Salinity at station 3 and 4 was found to be 33.1 PSU which was lower than station 1 (33.69) and station 2 (33. 56). Variation in the colour of sediment and water at different stations were also noted.

Enumeration of viable heterotrophic bacteria was performed with four different media and revealed a bacterial load of 1.2 to 1.7 x 10^3 cfu/ ml in water and 1.4 x 10^5 to 8.5 x 10^6 cfu/ g in sediment. Based on the colony morphology 272 isolates were selected for biochemical characterization. Figure 2.3 represents images of various biochemical tests performed during the study. Biochemically typed strains belonging to 33 groups were then characterized using 16S rDNA sequencing. Gram negative bacteria predominated in all stations compared to Gram positive bacteria. Predominance of short rods were also noted from both sediment and water samples. All gram negative bacteria turned out to be proteobacteria belonging to a single class

Gammaproteobacteria. Negative staining revealed 3 distinct morphologies such as rods, short rods and cocci of which the dominance of short rods was confirmed

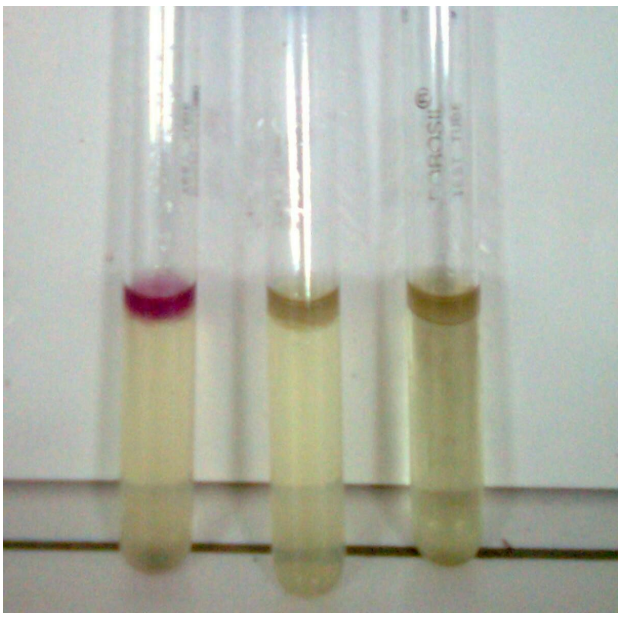
2.3.2 Molecular Identification

The thirty three bacterial strains, each representative of the groups were identified using 16S rDNA sequencing. Following BLAST analysis of the sequences, 12 distinct species belonging to *Enterobacter ludwigii, Stenotrophomonas maltophilia, Brachybacterium paraconglomeratum, Micrococcus luteus, Bacillus flexus*, *Staphylococcus cohinii, Enterobacter cancerogenus, Pseudomonas fragi* , *Pseudomonas koreensis* , *Bacillus thuringiensis, Pseudomonas sabulinigri* and *Halomonas boleviensis* were identified. Figure 2.4 and 2.5 show phylogenetic relationship of sediment and water isolates with its nearest neighbors. The sequences showed a homology ranging between 97.7 to 100 % to the closest neighbors.



Carbohydrate utilization test showing positive and negative reactions

Citrate utilization test showing positive and negative reactions



Indole test showing positive and negative reactions

Triple sugar iron test showing positive and negative reactions

Figure 2.3: Images of various biochemical reactions used for identification

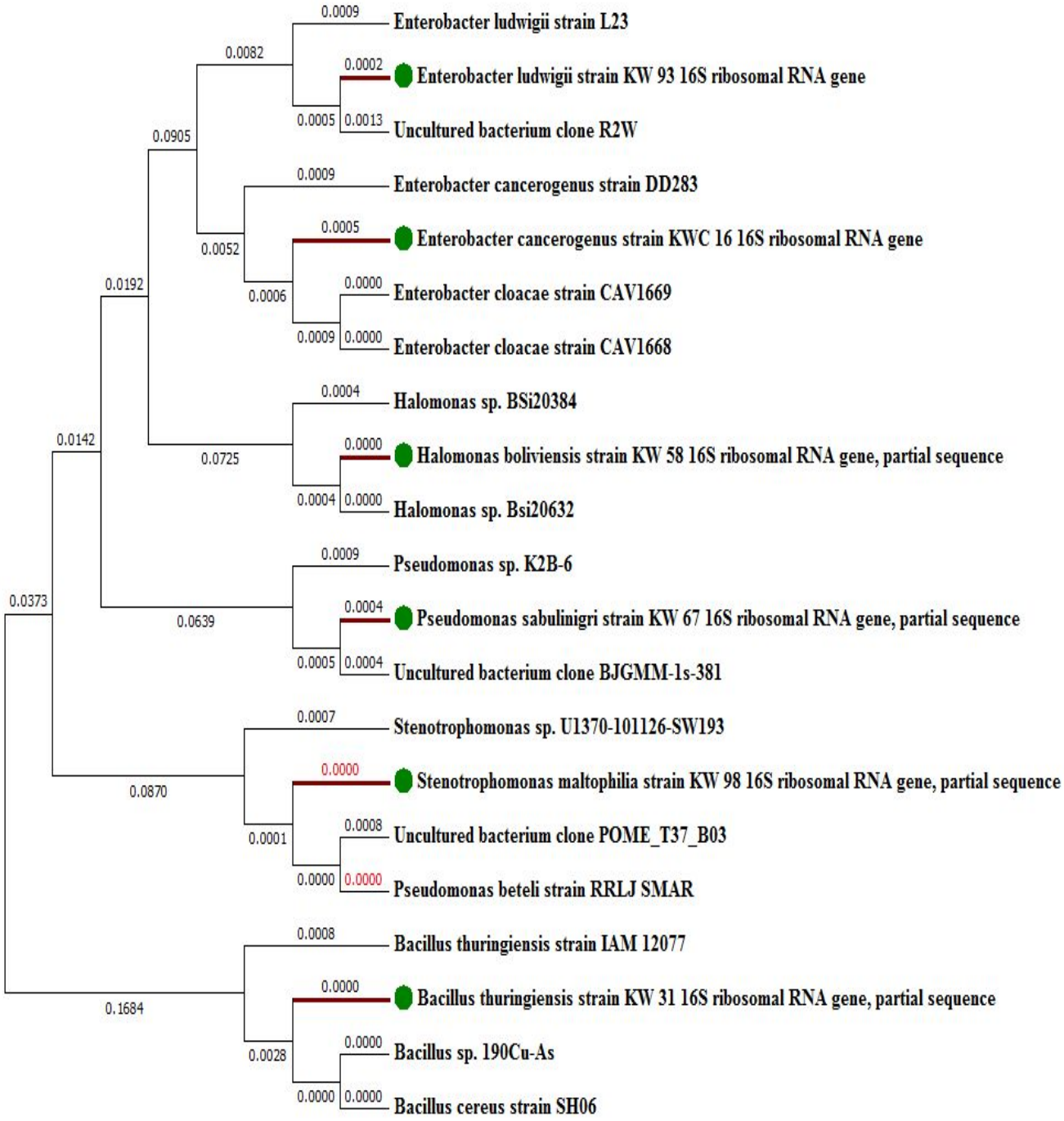


Figure 2.4: Phylogenetic tree of bacterial isolates from Kongsfjord water showing the relationship to closest neighbours

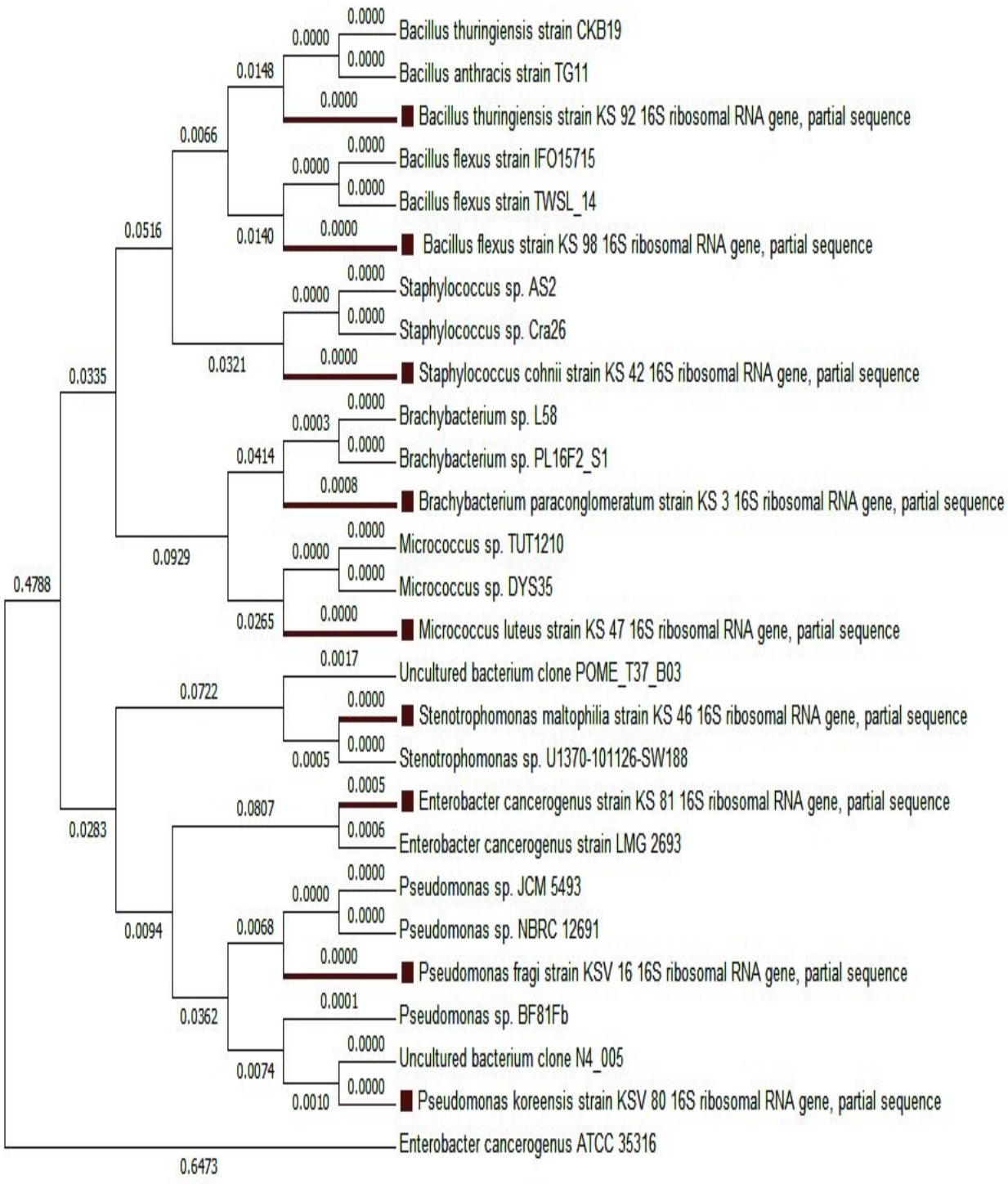


Figure 2.5: Phylogenetic tree of bacterial isolates from Kongsfjord sediment showing the relationship to closest neighbours

Three phyla namely Proteobacteria, Firmicutes and Actinobacteria were identified from sediment and water samples. From the sediment three classes of bacteria were identified namely *Gammaproteobacteria, Actinobacteria and Bacilli* (Figure 2.6) and from the water *Gammaproteobacteria* and *Bacilli* were detected (Figure 2.7). Proteobacteria was restricted to *Gammaproteobacteria* and represented major share in sediment and water. In sediment 66 % of the total isolates were *Gammaproteobacteria* and in water they were represented by 91 %. *Actinobacteria* was found only in the sediment and represented 20 % of the total isolates. In the phylum firmicutes, all isolates belonged to the class *Bacilli* and in water they were represented by 9 % and in the sediment by 14 %.

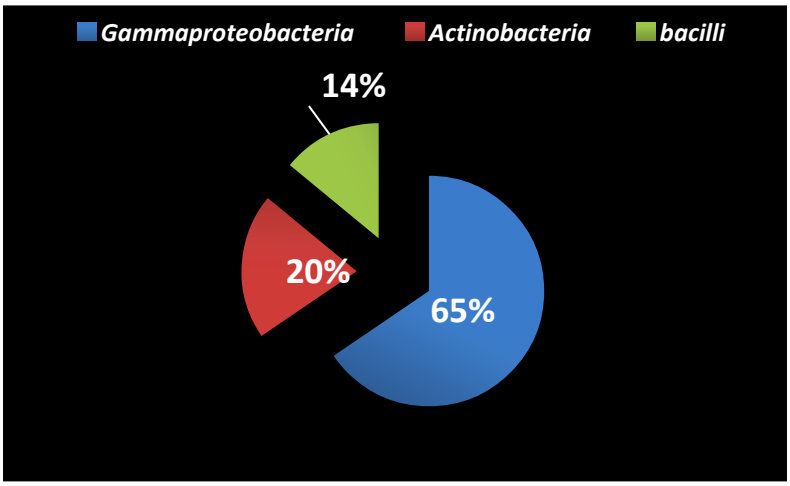


Figure 2.6: Percentage distribution of different classes of bacteria isolated from sediment samples

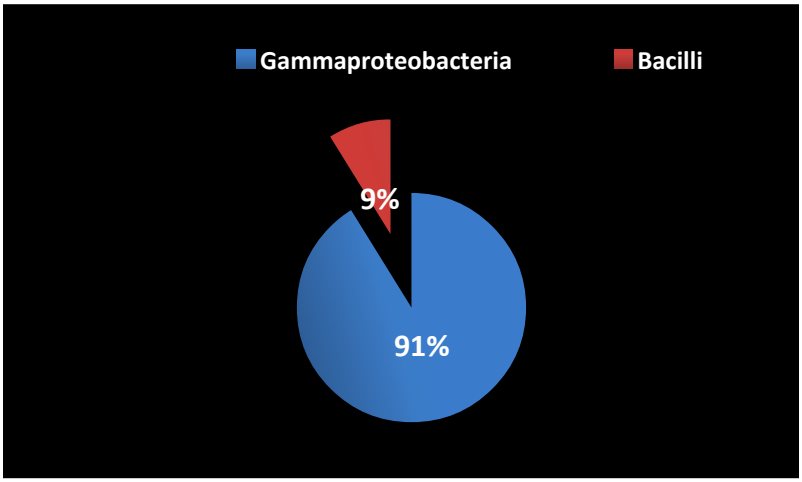


Figure 2.7: Percentage distribution of different classes of bacteria isolated from water samples

Among the sediment isolates, maximum species diversity was observed for *Gammaproteobacteria* with five distinct species including *Stenotrophomonas maltophilia, Enterobacter ludwigii, Enterobacter cancerogenes, Pseudomonas fragi* and *Pseudomonas koreensis*. Even though *Actinobacteria* ranked second with number of isolates, species diversity was restricted to two species including *Micrococcus luteus* and *Brachybactetrium paraconglomeratum*. Bacilli represented 11 % of the sediment isolates and included *Bacillus flexus, Bacillus thuringiensis* and *Staphylococcus cohnii* ssp. *urealyticus*. Figure 2.8 shows the percentage distribution of different species from the sediment sample. The figure clearly indicates dominance of species such as *Enterobacter ludwigii, Stenotrophomonas maltophilia* and *Brachybactetrium paraconglomeratum.*

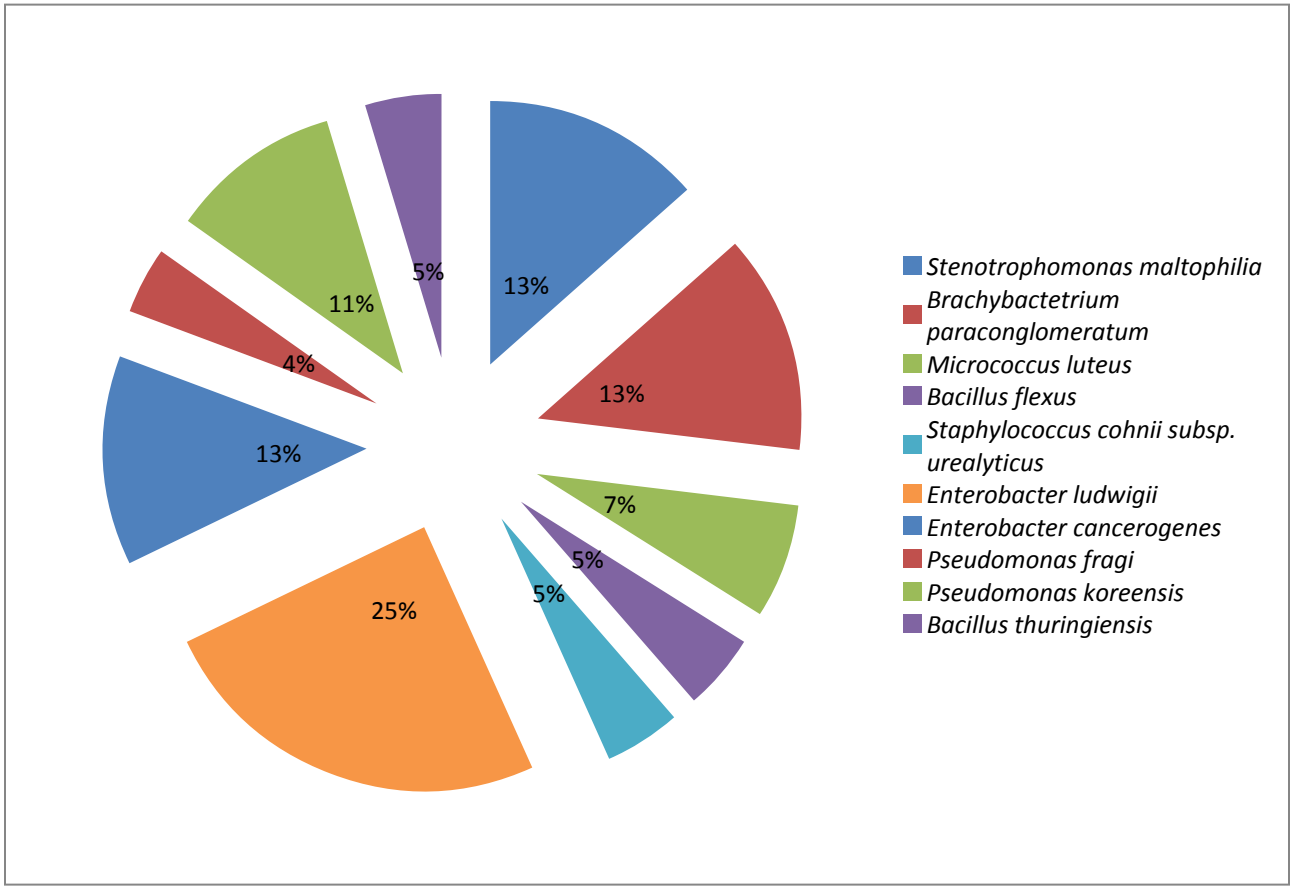


Figure 2.8: Percentage distribution of different species isolated from the sediment samples

Water samples represented seven distinct species. Similar to sediment isolates, water isolates were also dominated by *Gammaproteobacteria* consisting of six species such as *Enterobacter ludwigii, Stenotrophomonas maltophilia, Halomonas boliviensis, Pseudomonas sabulinigri, Enterobacter cancerogenus,* and *Pseudomonas fragi.* A single species of bacilli (*Bacillus thuringiensis*) represented 9 % of total water isolates. *Enterobacter ludwigii, Stenotrophomonas maltophilia, Bacillus thuringiensis, Enterobacter cancerogenus* and *Pseudomonas fragi* were found in both sediment and water samples. In the sediment samples, species such as *Stenotrophomonas maltophilia* and *Brachybactetrium paraconglomeratum* represented a high fraction followed by *Enterobacter ludwigii*. Among the water isolates was *Stenotrophomonas maltophilia* was followed by *Enterobacter ludwigii* and *Enterobacter cancerogenus* (Figure 2.9).

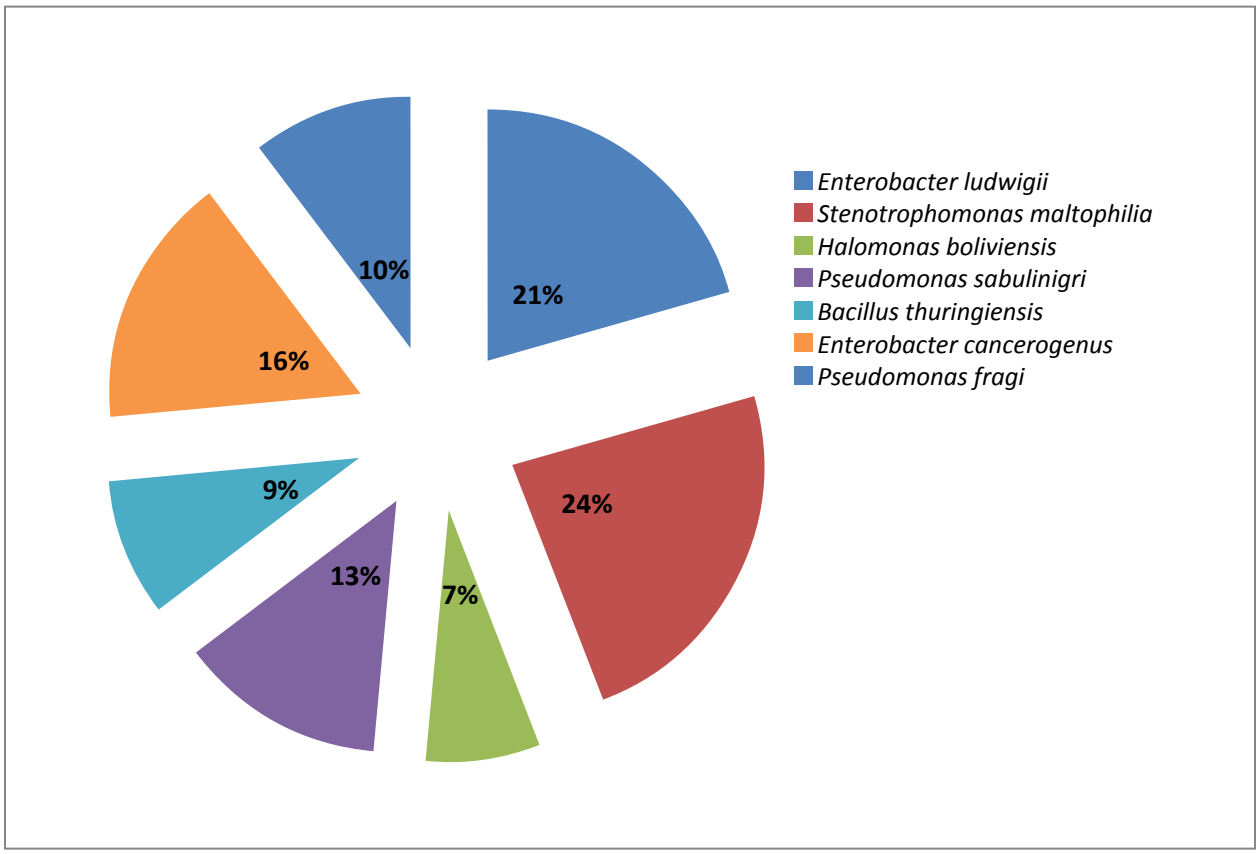


Figure 2.9: Percentage distribution of different species isolated from the water samples

2.4 Discussion

Arctic ecosystem is a highly dynamic ecosystem influenced by seasonal and climatological fluctuations. The temperature of the surface water ranged from 3.6 to 6.1 ^{o}C. This could be explained by the summer sampling and suggests a free exchange of water masses between the open sea and fjords. Bottom water temperature of all stations was around 1 ^{o}C. Salinity was found to be ranging between 33.11 to 33.69 PSU and stratification was noted from surface to bottom water in the stations that was influenced by glacial run off. The colour of the sediments varied between stations from light brown to chocolate brown. This is probably due to the difference in organic content of the sediments (Jorgensen *et al.*,1997). Waters of the inner basins were found to be comparatively turbid and towards the mouth of the fjord water was observed to be clearer.

Retrievable heterotrophic bacterial load in the water and sediment samples ranged from 1.2 to 1.7 x 10^{3} cfu/ ml and 1.4 x 10^{5} to 8.5 x 10^{6} cfu/g respectively and was comparable to previous reports (Steven *et al*., 2006; Perreault *et al*., 2008). Sediment sample from station four represented the highest number of isolates. Station four is situated between the outer zone and inner zone of the sea commonly known as transitional zone (Figure 2.2). It is influenced by glacial and terrestrial run off due to its close proximity to Blomstrand –halvoya and Bloomstrandbreen. Similar to previous studies, more heterotrophic bacteria were retrieved from sediments than those from water samples. When compared to seawater, organic matter composition of sediment is higher to a magnitude of 10^{4} - 10^{5} folds (Zeng *et al.*, 2011). Bacteria in sediment could be considered as a major reservoir of genetic diversity (Torsvik, 2002).

Depending on the various colony characteristics such as size, elevation, pigmentation and margin 272 isolated colonies were selected for further characterization. The size of various colonies ranged from very small, small, medium and large; the pigmentation exhibited includes white, creamy, yellow and orange colonies. Morphological characteristics of the isolates were carried out using Gram staining, spore staining and capsular staining. The shape of the isolates detected using negative staining clearly indicated dominance of short rods and coccobacilli over long rods and cocci. Bacterial shapes contribute to aid in many basic mechanisms of life including accessing nutrients, partitioning materials to progeny, attachment and dispersion (Young *et al.*, 2006). In general, marine bacteria are found to be smaller and motile in nature which helps them

escape predation more frequently than comparatively larger and slower cells (Young *et al.*, 2006). Gram staining revealed that Gram negative bacteria were highest in number in both sediment and water samples. Similar to the present study, Gram negative bacteria are reported larger in number than Gram positive bacteria from Polar Regions and glacial samples (Skidmore *et al.*, 2005; Amato *et al.*, 2006; Tropeano *et al.*, 2012). Spore staining revealed two different kinds of spores; one category with central large spore and another category with smaller sub-terminal spore. It has been known that several physiological characteristics such as spore formation and pigmentation help in the survival at subzero temperatures (Fong *et al.*, 2001; Mueller *et al.*, 2005).

Biochemical characterization of 272 isolates yielded 33 groups which were then subjected to molecular characterization by 16S rDNA sequencing. Following BLAST analysis of the sequences, 12 distinct species were identified belonging to Proteobacteria, Firmicutes and Actinobacteria. The bacterial communities of Kongsfjord sediment and water were diversified in composition. Proteobacteria was the major phyla in both sediment and water samples belonging to a single class of *Gammaproteobacteria*. In the sediment sample, *Gammaproteobacteria* represented 66 % of the total isolates.

Presence and dominance of *Gammaproteobacteria* in Svalbard sediments were reported by Teske *et al.* (2011). In their study, 49 % of the total sediment isolates turned out to be *Gammaproteobacteria* and 9 % of total isolates in deep sediment samples. Culturable diversity and extra cellular enzyme production study conducted in Anarctic sediment, soil and some other habitats showed predominance of *Gammaproteobacteria* (Loperena *et al.*, 2012). Arnosti *et al*. (2008) reported dominance of *Delta* and *Gammaproteobacteria* from Smeerenburgfjord and in Hornsundfjord in Arctic sediment. Thus it is evident from these studies that the dominance of *Gammaproteobacteria* exists in some Arctic sites. Actinobacteria from sediment samples represented 20 % of the total isolates. Xuezheng *et al.* (2013) also detected the presence of Actinobacteria in Arctic sediment. Actinobacteria in the snow cover of Arctic was reported from Spitzberg in Svalbard by Amato *et al.* (2006). Microbial diversity study conducted on permafrost of high Arctic contained 38 % of the clone library of Actinobaceria (Steven *et al.*, 2005). Firmicutes/ Bacilli represented 14 % of the sediment isolates. Several reports suggest the

presence of firmicutes in different locations of Arctic (Neufeld *et al.*, 2005; Amato *et al.*, 2006; Steven *et al.*, 2006; Perreault *et al.*, 2008; Schostag *et al.*, 2015).

Gammaproteobacteria represented 91 % of total heterotrophic bacteria from water samples. Dominance of proteobacteria in Arctic surface and bottom waters has been reported (Christner *et al*., 2008; Simon *et al*., 2002; Zeng *et al*., 2002; Miteva and Brenchley, 2005). However; it is suggested that limitation of retrievable bacteria by culture dependent methods often present dominance of particular class of organisms. Wind and fresh water are the major factors that influence the water mass in upper water columns (Harmes *et al.*, 2007). Three major groups of bacterial community *Alphaproteobacteria*, *Gammaproteobacteria* and *Bacteroidetes* were found to contain 63.8 % of surface water and 71.5 % of bottom water in Kongsfjorden in a study carried out by Zeng *et al.* (2009). Another study conducted in Arctic Ocean reported that 77 % of bacterial communities represented *Alphaproteobacteria*, *Gammaproteobacteria* and *cytophaga/ flavobacteria* group (Bano and Hollibaugh, 2002). It was suggested that 70 % of isolates from fjords of Spitsbergen was affiliated to the *Gammaproteobacteria* however; this could possibly due to the limitations of culture technique (Groudieva *et al.*, 2004).

Malmstorm *et al.* (2007) carried out molecular diversity analysis of bacterial communites of western Arctic Ocean and reported dominance of *Gammaproteobacteria* over *Alphaproteobacteria* and *Bacteroidetes.* Thus the above studies prove the existence and dominance of *Gammaproteobacteria* by both culture- dependent and culture- independent methods. Among the water isolates Phylum Firmicutes were represented by a single class - *Bacilli*. Presence of *Bacilli* in Arctic waters has been reported but never as dominant organisms. Similarly, in this study only 9 % of water isolates was confirmed to belong to class *Bacilli*. Study of bacterial characterization of snow cover by Amato *et al.* (2006) reported 2 genera from phylum Firmicutes including *Paenibacillus* and *Bacillus* sp. Steven *et al.* (2006) reported three genera from Firmicutes representing only 4 % of the total isolates. Firmicutes were also reported from cold springs of High Arctic containing 18 % of the total bacteria (Perreault *et al.*, 2008).

Diversity of species from water and sediment samples was comparatively less as expected and a similar number of species was reported from previous works. A total of 12 species were successfully isolated and identified from sediment and water which include *Enterobacter ludwigii, Stenotrphomonas maltophilia, Halomonas boliviensis, Pseudomonas sabulinigri,*

Enterobacter cancerogenus, Pseudomonas fragi, Pseudomonas koreensis, Bacillus flexus, Bacillus thuringiensis, Staphylococcus cohnii subsp. *urealyticus*, *Micrococcus luteus* and *Brachybactetrium paraconglomeratum*. Sediment samples showed higher diversity with ten species compared to water samples with seven species. This finding is in agreement with most of the previous studies supporting dominance of sediment diversity over water (Arnosti *et al.*, 2005; Arnosti *et al.*, 2006; Perreault, 2008; Teske *et al.*, 2011).

It was interesting to note that some species such as *Enterobacter ludwigii, Stenotrophomonas maltophilia, Bacillus thuringiensis, Enterobacter cancerogenus* and *Pseudomonas fragi* were isolated from both sediment and water samples. Potential source of these organisms to the water column would be re-suspension of bottom sediments caused by wind - induced mixing or coastal upwelling (Garneau *et al.*, 2006). Sediment re-suspension and transport due to hydrodynamic processes may cause short term variations in bacterial communities (Garneau *et al.*, 2009). However, studies of microbial communities representing the water and sediment from same sampling sites are scarce (Teske *et al.*, 2011),

Enterobacter ludwigii was found to represent in the highest numbers in the sediment samples. It was the first report of the species *Enterobacter ludwigii* from Arctic environment. However; other members of *Enterobacteriaceae* was reported from Arctic environment (Glad *et al.*, 2007) and also from Arctic animal sources such as Polar bear and Arctic birds (Sjolund *et al.*, 2008; Glad *et al.*, 2010). Survival of *Enterobacter ludwigii* in Arctic has not been previously investigated although psychrotrophic bacterium, *Enterobacter ludwigii* isolated from Indian Himalayan soil successfully expressed cold shock protein Csp E. Julian *et al.* (2009) isolated *Enterobacter ludwigii* from root nodules of *Medicago* plants growing near nuclear power plant and all strains produced thick acidic exopolysaccharides (EPS) and thus arguably play a role in survival in the extreme environments. *Enterobacter ludwigii* had been isolated from clinical specimens and reported to cause nosocomial infections (Khajuria *et al.*, 2013).

Sediment and water habitats were also found to harbour another species of the genus *Enterobacter – Enetrobacter cancerogenus*. *Enterobacter cancerogenus* is usually recovered from environmental sources and is widely distributed in nature. *Enterobacter cancerogenus* is recognized as phytopathogen although some severe infections of bones and joints after crush

injuries have been reported in humans (Abbott and Janda, 1997; Garazzino *et al.*, 2005; Kazaks, 2012).

Stenotrophomonas maltophilia was also isolated from sediment and water samples and there are no previous reports of this bacterium from any Arctic environment. *Stenotrophomonas maltophilia* is ubiquitous in aquatic environment, soils and plants. Furushita *et al.* (2005) reported *Stenotrophomonas maltophilia* from marine fish *Seriola quinqueradiata. Stenotrophomonas maltophilia* was originally classified as *Pseudomonas maltophilia* and DNA hybridization analysis led to the reclassification into *Stenotrophomonas maltophilia.* In the present study phylogenetic analysis of *Stenotrophomonas maltophilia* revealed this close relationship to *Pseudomonas* by showing 99 % similarity to *Pseudomonas beteli* and *Pseudomonas* sp R5.

Three species of *Pseudomonas* were identified from water and sediment and *Pseudomonas fragi* was isolated from both the samples. *Pseudomonas fragi* is a known psychrophilic organism and is not known to produce siderophores indicating iron availability in the environment. Different species of *Pseudomonas* have been isolated from various Arctic habitats (Steven *et al.*, 2006). *Pseudomonas koreensis* was isolated from sediment alone and *Pseudomonas sabulinigri* was unique to water sample. *Pseudomonas koreensis* was previously reported from environmental samples such as soil and it was reported to grow at 4 °C but not 37 °C (Tvrzova *et al.*, 2006). *Pseudomonas sabulinigri* was recently reported from black sand from Jeju Island Korea and found to grow at temperature between 4- 37 °C (Kim *et al.*, 2009).

Bacillus, *Micrococcus* and *Staphylococcus* were the three genera identified from phylum Firmicutes from sediment and water. *Micrococcus luteus* and *Staphylococcus cohnii* subsp. *urealyticus* was detected in the sediment samples. Strains of *Micrococcus luteus* was detected from Arctic permafrost and snow cover by Steven *et al.* (2006) and Amato *et al.* (2006) respectively by culture- dependent method. Wilhem *et al.* (2011) reported permafrost sample containing *Staphylococcus* sp from the permafrost of High Arctic. There are several reports of genus *Bacillus* from various habitats of Arctic such as snow cover in Spitzberg and permafrost from different regions (Amato, 2006; Steven *et al.*, 2006; Wilhelm *et al.*, 2011). It is interesting to note that the entire *Bacillus* sp reported from Arctic was isolated via culture dependent techniques. It is suggested that some microbial populations may be rare or present in the form of

spores and thus might not be detected using molecular tools such as FISH or T-RFLP (Finlay, 2002; Baldwin *et al.*, 2005). Thus some major communities of bacteria might be present in Arctic environment as spores and only germinate under favorable conditions such as spring or summer.

Halomonas boliviensis was a unique bacterium isolated from water sample and it was not detected in sediments. There is only one previous report on genus *Halomonas* from marine Arctic sediment by Xuezheng *et al.* (2013). *Halomonas* is a typical halophilic organism growing over a wide range of salinity. Poli *et al.* (2013) reported a *Halomonas* species from Antarctica which grows optimally at 30 °C, 10 % NaCl and at pH 9. Another halophilic organism obtained in the present study belongs to the genus *Brachybactetrium* isolated from sediment. *Brachybactetrium paraconglomeratum* species was not previously reported from Arctic sediment although a single report exists of *Brachybactetrium* from Arctic permafrost by Steven *et al.* (2006). *Brachybactetrium paraconglomeratum* represented 13 % of the total water isolates and was the major fraction of *Actinobacteria*.

Detailed analysis of phylogenetically identified species revealed several biochemical types within many species. *Pseudomonas fragi* species showed two distinct biochemical types differing in utilization of sorbitol, inositol, urea hydrolysis, and gelatin liquefaction. *Enterobacter ludwigii* was found to have four biochemical types distinct from each other in the utilization of urea, voges proskuer test, ornithine hydrolysis, utilization of sugars and ONPG test. *Stenotrophomonas maltophilia* strains showed difference in growth at different temperatures, salinity requirement, and production of gas during fermentation reaction. Thus it is clear that biochemical characterization help in the detection of strains with different physiological capabilities. This might aid not only in better understanding of survival in different environments but also their possible applications in biotechnological industries.

Culture-independent methods are widely used nowadays for the microbial ecology analysis and theoretically culture-independent organisms represent the entire microbial population from the environmental samples (Spiegelman *et al.*, 2005). However, by comparing several reports on microbial diversity from various Arctic sites it could be inferred that retrievable bacteria differ from non-cultivable bacteria. Steven *et al.* (2006) characterized microbial diversity of permafrost from High Arctic and they used culture dependent and culture independent methods to analyse

the diversity of bacterial population. In their study it was observed that culturable diversity was dominated by firmicutes and molecular diversity indicated dominance of *Actinobacteria* although all phyla detected by culturable method were found in culture independent method. Moreover 5 species of *Bacillus,* 6 species of *Paenibacillus and* 2 species of *Sporosarcina* were detected in culturable method while culture -independent method represented only 2 isolates belonging to the genus *Bacillus*.

Bacterial diversity analysis of Arctic marine sediment was carried out in another study by culture -dependent and culture-independent methods and similar observations could be made. (Xuezheng *et al.,* 2014). In their study, culture-dependant methods identified 3 phyla *Gammaproteobacteria*, *Actinobacteria* and *Bacteriodetes* which were also found in culture independent analysis however; the composition of the phyla differed in both the methods. Genera such as *Shewanella, Halomonas, Acinetobacter, Psychrobacter, Arthrobacter,* and *Microbacterium* were detected in culture dependant method but not in culture-independent method.

Culturable diversity detected in the current study represented many species which are considered to be rare. However, most of the Genera identified including *Bacillus*, *Halomonas, Brachybactetrium, Micrococcus* and *staphylococcus,* were previously reported from different regions of the Arctic. It was interesting to note that these genera were observed in previously reported culturable diversity studies only, except the genus *Pseudomonas*. Genus *Pseudomonas* was detected in both culture-dependant and culture-independent methods from the Arctic. Thus it is evident that culturable diversity is significant in the determination of species diversity even though culture- independent methods are superior in detection of microbial diversity. It should be noted that with the advancement of molecular detection methods in the past two decades, diversity studies were concentrated on culture- independent methods with a few reports on culturable diversity making the comparative analysis difficult. Moreover, diversity analysis by culture independent methods was often restricted to analysis of different classes rather than to species richness (Neufeld *et al.*, 2005; Garneau *et al.*, 2006; Malmstorm *et al.*, 2007; Arnosti, 2008). Furthermore, microbial species diversity analysis from same sites of sediment and water sources is rarer (Teske *et al.*, 2011).

Analysis and comparison of phylogenetically identified species revealed several distinct strains within the species. Strains of the species with novel biochemical properties might be used as a tool in industrial processes. Many isolates detected during the study posses unique properties such as tolerance of wide range of pH, salinity and growth over wide range of temperature. Some of the species such as *Pseudomonas fragi, Enterobacter ludwigii, Stenotrophomonas maltophilia,* and *Bacillus thuringiensis,* identified during the study are known for their biotechnological potential. *Pseudomonas fragi* have been reported for the production of esters, improvement of cheese flavour, aroma and lipase production (Raymond *et al*., 1991; Alquati *et al.*, 2002). *Enterobacter ludwigii* have been recently recognized for their ability to promote plant growth (Shoebitz *et al.*, 2009), hydrocarbon degradation (Yousaf *et al.*, 2011) and production of tannase (Singh *et al.*, 2012). A strain of *Stenotrophomonas maltophilia* was found to degrade RDX (Binks *et al.,* 1995) and polycyclic aromatic hydrocarbon degradation was reported by Boonchan *et al.* (1998). *Bacillus thuringiensis* has been recognized for a long time in protecting plants by acting as a biocontrol agent (Betz *et al.*, 2000).

To conclude, bacterial diversity analysis of sediment and water from Kongsfjord detected some species such as *E. ludwigii, S. maltophilia, E. cancerogenus, P. koreensis,* and *P. sabulinigri* that were not previously reported from Arctic although most of the genera were previously reported from the Arctic environments. Bacterial load was comparable to previous studies based on culture-dependent analysis. *Gammaproteobacteria* was found to be most abundant in both sediment and water samples and support the suggestion that 70 % of the total Kongsfjord bacteria belong to *Gammaproteobacteria* (Groudieva *et al*., 2004). Comparison of genera with previously reported diversity from the Arctic revealed that the generic diversity is only comparable with previous studies carried out via culture dependent methods. Only the genus *Pseudomonas* was reported by both culture-dependent and culture-independent methods. It is evident that microbial diversity analysis of sediment and water samples are scarce and present study demands further investigations into microbial diversity for a thorough understanding of microbial ecology of Arctic sediment and water. Biochemical characterization of phylogenetically analyzed strains revealed many biochemical types within the species. The current study also recognized species which are already known for their biotechnological

potential reiterating the significance of identifying novel organisms from wild sources such as the Arctic environments.

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Chapter 3
Antibiotic resistance profiling of heterotrophic bacteria from Arctic sediment and water

Contents:

Microorganisms represent the most established life form on earth surviving in extremes of temperature, pH, salinity, metal polluted and ionizing radiation polluted habitats. This great flexibility demonstrated by microbes to different challenges is a result of their genomic plasticity, high frequency of mutation and large population size enabling them to mutate and adapt. Similarly, capacity of microbes to respond and adapt to antibiotic stress is not a novel phenomenon but a coping mechanism that exist to establish bacterial life in the stressed habitat whether it is the wild or urban environments. However, decades of study on antibiotic resistance has established the role of anthropogenic activities that accelerated dissemination of multidrug resistant microbes and especially pathogens across the globe.

The Arctic is a region located at the northern-most part of the earth which is considered as a pristine environment with minimum impacts of anthropogenic activity on the ecology. Thus Arctic region provides a wild habitat to investigate the origin, spread and evolution of antibiotic

resistant genes. The ecosystem in Svalbard, the Barents Sea and other parts of Arctic is subject to increasing pressure from climate change and resultant change in nature. In addition to this, stress due to increased anthropogenic activity such as cruise tourism, shipping and extraction of natural resources also exist in Arctic region. Discoveries of oil, mineral, diamonds and development of research and ecotourism are bringing many non-indigenous populations to Arctic, which may enhance the spread of antibiotic resistance to this wild environment. Although the Arctic is far away, events elsewhere in the world have consequences here and the changes in the Arctic can impact our everyday life.

3.1 Review of literature

3.1.1 Antibiotic resistance - The global scenario

First reports of drug resistance appeared soon after the wide spread use of antibiotics and it was naturally from hospitals where it was used most frequently. *Streptococcus pyogenes* resistant to sulfonamides was reported from military hospital in 1930s. Following the discovery of penicillin, London civilian hospitals reported penicillin resistant *Staphylococcus aureus* in 1940s. Interestingly, *Mycobacterium tuberculosis* with streptomycin resistance was also reported from pulmonary tuberculosis in 1948 (Barber, 1948; Crofton and Mitchison, 1948). Today the cost of treating infectious organisms which are resistant to many antibiotics is very high and many reports have evaluated the cost of antibiotic resistance. In brief, antibiotic resistant infections would cost double the duration of hospital stay, high mortality and number of recurring infections. It was observed that difference of cost in treating methicillin resistant *Staphylococcus aureus* infections (MRSA) to that of methicillin sensitive *Staphylococcus aureus* (MSSA) infection is 22 % higher (Rubin *et al.,* 1999).

Recent reports suggest an increasing number of multiple drug resistance (MDR) infections, many patients succumb to infections, and this phenomenon is reported especially in developing countries (www.who.int/mediacentre/factsheet/fs194/en/). These reports also indicate that in developing countries MDR infections are predominated by organisms such as *Salmonella enteritidis*, *Shigella flexneri* and *Vibrio cholerae* causing enteric infections. Forty to sixty percentage of nosocomial infections of methicillin resistant *Staphylococcus aureus* are caused by multiple drug resistant strains in United States and United Kingdom (David and Daum, 2010).

Some strains of *E. coli*, which are common cause of urinary tract infections and enteric infection, are found to be resistant to up to six drugs (Ahmad *et al.*, 2015). Differences in drug resistance in

different parts of the world has also been noted; in southeast Asia and China 60-70% of *E. coli* has been reported to be resistant to fluoroquinolones while in United States and industrialized countries the resistance was found to be 10 % (Zervos, 2000; Karlowsky *et al.,* 2002).

Different antibiotics have different mechanisms to fight or kill bacteria, beta-lactam antibiotics such as penicillins, cephalosporins, carbapenems and glycopeptide – vancomycin inhibit bacterial cell wall synthesis, quinolones and rifampicin affect nucleic acid metabolism, aminoglycosides, chloramphenicol, tetracycline and macrolids affect protein synthesis and trimethoprim and sulfonamides inhibit important metabolisms or metabolites of the cell. Bacteria possess intrinsic resistance to some of these antibiotics and such resistance is not affected by the use or misuse of antibiotics. It is best exemplified by membrane of gram-negative bacteria, outer membrane of these organisms are impermeable to hydrophobic compounds such as macrolides. Bacteria also use enzymes such as β-lactamase to cleave antibiotics or efflux pump to block or reduce the uptake of antibiotic into the cell. Mechanisms such as efflux pump render the bacterium with multiple drug resistance (Bennet *et al*., 2008). Mulvey and Simer (2009) showed that resistance of *Mycobacterium tuberculosis* towards isoniazid compounds are non transferable. However, antibiotic resistance is mainly transferred through genetic material by mechanisms such as horizontal gene transfer.

Resistance in bacteria is often induced by the presence of antibiotics, resistant bacteria become predominant when the antibiotic destroys all the susceptible bacteria in the environment. Hence antibiotics do not bring about resistance by itself instead act as a selection pressure. McMahon *et al*. (2007) proved that apart from antibiotics, detergents and solvents were also able to induce antibiotic resistance. These compounds acts as environmental stress to induce efflux pump such as arcAB efflux pump which render bacteria with multiple drug resistance.

Considering the ecological distribution of resistance, the amount of antibiotics used is directly proportional to resistance selection and its effect. Continuous selection of resistance can be observed, possibly due to persistence of antimicrobials in natural environments. Kummerer and

Henninger (2003) reported that waste water contains antimicrobials and their amount is increasing which also was responsible for environmental selection of antibiotic resistant bacteria.

Whatever may be the sources of resistance it is essential to preserve the efficiency of antibiotics to treat bacterial infections. The wise and careful use of antibiotics has been advised by scientists for decades now (Allen *et al.,* 2011). In agricultural field, antibiotics such as amoxicillin and erythromycin are used without any discrimination as prophylactic measure and in animal feed. Similar to hospital environment, agricultural use and misuse also lead to selection of antibiotic resistance. Estimates of antimicrobials used in livestock showed that more than 24 million pounds of antibiotics are used in feed and water per year in United States (Landers *et al*., 2012) and these antibiotics are often poorly metabolized by the animals. Chee-Sanford *et al*. (2001) showed that 75 % of tetracycline administered on swine was excreted unaltered. Thus, antibiotics from both agricultural and urban sources cause persistence of antibiotic molecules in the environment leading to specific resistance against antibiotics. Another field of wide spread use of antibiotics is fish and fishery products and reports suggest that resistant bacteria from such sources can transfer genes to human pathogens (Cabello, 2006).

The age before use of antibiotics or before introduction of sulfonamide lies before 1930s and this age is considered to be pre-antibiotic era. Historical analysis of resistant studies revealed that resistant gene**s** were present in bacteria before wide spread use of antibiotics. Some *E. coli* isolates lyophilized before 1950s were found to have multiple antibiotic resistance and resistant elements were able to conjugate into *E. coli* (Smith, 1967). Some enteric bacterial strains (433) isolated between the time period of 1917 and 1952 were tested for plasmid production and 24% of the total isolates were able to transfer plasmids of which 11 strains were resistant to ampicillin or tetracycline. These studies indicate the existence of antibiotic resistance in pre-antibiotic era and which would have spread to pathogens via horizontal gene transfer (Allen *et al.,* 2011).

The production of antibiotics and antibiotic resistance has various functions in microbial communities and natural environment. Many soil bacteria such as *Pseudomonas fluorescens, Burkholderia cepacia, Streptomyces* sp., *Flavobacterium* and some unidentified cultures were shown to have ability to use antibiotic as a sole source of carbon (Pramer and Starkey, 1951; Kameda *et al*., 1961, Abd-El-Malek *et al*., 1961., Malik and Vining, 1970; Johnsen, 1977; Beckman and Lessie, 1979). Role of resistance gene in environment is not absolutely clear but

believed to have some primary roles in the environment. Certain classes of efflux pumps offer general mechanisms of resistance since they pump various toxins including heavy metals and other toxic molecules out of cells (Nies, 2003; Poole, 2005).

Spread of antibiotics via wind and watershed are considered to be an important factor in dissemination of antibiotic resistance. Wild environment are also known to harbour antibiotic resistant genes which serve as ideal environment to study biological mechanisms of spread of antibiotic resistance. Gilliver *et al*. (1999) isolated bacteria from mice from rural area of London and 90 % of them turned out to be resistant to beta-lactam antibiotics. In a similar study carried out in Finland on enteric bacteria from wild elk showed almost no resistance (Osterblad *et al*., 2001). The possible explanation given for this scenario is that a lower population density exists in most parts of Finland when compared to England. A comparative study of *E. coli* isolates from Australia and Mexico from wild animals showed the isolates from Mexico were more antibiotic resistant (Souza *et al.,* 1999). The reasons suggested for the differences were widespread settlement and increased use of antibiotics in Mexico compared to Australia.

Many studies have revealed the role of migratory birds and waterfowls in dissemination of drug resistant bacteria into the wild environments. It was found that gulls and geese nesting near waste and agricultural waste harbour more antibiotic resistant *E. coli* when compared to birds associated with unpolluted water (Dolejska *et al.,* 2007; Cole *et al.,* 2009). Many migratory birds such as *Branta canadensis* travel to northern most parts such as Arctic. These migratory birds were found to harbour *E. coli* and *Enterococcus* with multiple drug resistance (Middleton and Ambrose, 2005; Sjolund *et al.,* 2008).

In general, the reservoirs of antibiotic resistance genes are present in the environment as a combination of natural, animal and human wastes, and selective effects of pollutants (Wellington *et al*., 2015). Resistance genes are commonly associated with mobilome (mobile genetic elements) including integrons and transposons and are easily transferred to unrelated bacteria. Mobile genes usually do not carry genes essential for cellular functions. There are many transponsable elements associated with antibiotic resistance although some are closely associated. Karim *et al.* (2001) showed association of plasmid mediated extended spectrum beta-lactamase genes in Insertion sequences. Sequence comparison of several plasmids showed that genes of resistance were found in transposable elements and integrons (Wellington *et al*., 2015).

For many integrative and conjugative elements such as plasmids is often associated with the stress response such as presence of antibiotics and pollutants in the environment.

Antibiotic resistance genes (ARG) reach the environment or human beings through several ways such as crops contaminated with sludge or waste, live stock with bacteria containing drug resistance gene, fish and fishery products and ground water contaminated pharma residue. Many antibiotics could not be degraded naturally and synthetic antibiotics especially persist in the soils or landfills at high concentrations for long periods of time (Kummerer, 2004; Jjemba and Robertson, 2005). Kolpin *et al*. (2001) and Monterio *et al*. (2010) analyzed the persistence of pharmaceutical compounds in water and established the presence of tetracyclines, fluoroquinolones, sulfonamides and macrolides. The third generation cephalosporins are used in veterinary medicine including ceftiofur which is licensed for use in cattles and pigs. In Japan strains of *E. coli* was found to have CTX M2 gene and it was isolated from cattle faeces (Shiraki *et al*., 2009). Extended spectrum antibiotic genes such as SHIV 12, CTX M 1, CTX M 9 and CTX M 14 were found in *E. coli* strains isolated from pig slurry (Escudero *et al*., 2010).

3.1.2 Mechanisms of antibiotic resistance development

Several antibiotics are known to influence mutation rate such as SOS response, nucleotide-pool unbalancing and general stress response (Rodriguez-Rojas *et al.,* 2013). How reactive oxygen species (ROS) influence the mode of action of antibiotics was studied in detail by Dwyer *et al*. (2009). This study explored the formation of ROS in response to antibiotic and characterized a novel pathway for oxidatively damaged induced cell death. The common pathway follows an increased respiration rate, permanent oxidation of iron sulfur clusters and depletion of NADH causing the formation of highly reactive hydroxyl radicals which ultimately culminate in cell death (Kohanski *et al.,* 2007; Dwyer *et al.,* 2009).

It is well established that ROS can cause damage to proteins, lipids and DNA. If these DNA damages are not repaired, it will lead to accumulation of mutations. Kohanski *et al.* (2010) showed that such mutations can happen as a result of exposure to antibiotic at sub-lethal concentrations. Another consequence of ROS increase is activation of SOS response which in turn activates specialized DNA polymerase which help to bypass DNA lesions. Many antibiotics including quinolones and ciprofloxacin are known to cause such effects and increased mutation

in bacteria. In *Streptococcus pneumonia*e ciprofloxacin was reported to cause fivefold increase in rifampicin resistance (Henderson-Begg *et al.,* 2006). *Pseudomonas aeruginosa* was also found to have same effects on ciprofloxacin exposure (Tanimoto *et al.,* 2008).

Antibiotics such as penicillins and cephalosporins are also found to generate SOS response. These antibiotics cause obstruction of cell division which in turn triggers a response effector system – *dpiBA* operon. The effector molecule DpiA binds at the origin of replication thereby inhibiting the replication of DNA which ultimately activates the SOS response. Antibiotics such as trimethoprim follow another pathway of influencing SOS response wherein nucleotide pool imbalance results in reduced accuracy of DNA replication. It is also postulated that sub-inhibitory levels of antibiotics bring about recombination effects. This creates an ideal environment such as human body to act as recombinant hot spot which ultimately result in phenotypic variations which help in emergence, maintenance and dissemination of antibiotic resistance (Lopez *et al.,* 2007; Lopez-Blazquez, 2009; Rodriguez-Rojas *et al.,* 2013).

Apart from mutations, major mechanisms that operate in bacteria that influence antibiotic resistance include horizontal gene transfer and intra-chromosomal recombination. It is believed that most of the antibiotic resistance genes are acquired through horizontal gene transfer (Binnewies *et al.,* 2006). During horizontal gene transfer a portion of foreign DNA is assimilated by other organism. This mechanism is thought to play a significant role in bacterial evolution, evasion of immune response, distribution of virulence and dissemination of antibiotic resistance (Guttmann and Dykhuizen, 1994; Lawrence and Ruth, 1996; Lawrence and Ochman, 1998; De la Cruz and Davies, 2000). Horizontal gene transfer mainly operates via conjugation, transduction and transformation. Furthermore, such gene transfer occurs among diverse bacteria easily and genes are maintained even in the absences of selection pressure.

Beta-lactamases (EC 3. 5. 2. 6) are produced by many microbes that catalyze hydrolysis of β-lactam ring structure of antibiotics such as penicillins, carbapenems and cephamycins. These antibiotics have a common molecular structure of β-lactam ring and hydrolysis of the ring structure hampers the antibacterial activity. Generally, most of the gram-negative bacteria express β-lactamase constitutively while in gram-positives β-lactamase production is induced (Bennet and Chopra, 1993). It is known for decades that some bacterial populations survive in presence of antibiotic without being resistant by genetic mechanisms. This phenomenon, called

persistence, enables bacteria to survive without making a hereditary change and conserve the genetic pool. Levin and Rozen (2006) reviewed different mechanisms of un- inherited resistance in bacteria. Dorr *et al.* (2009) reported that most of the resistance exhibited by such organisms towards quinolones and ciprofloxacin are in SOS-dependant manner.

Resistance of bacteria towards β-lactam antibiotics, quinolones, aminoglycosides, trimethoprim and chloramphenicol are mostly enzyme dependent and usually encoded by multi-resistant plasmids. However, other mechanisms such as modification in membrane permeability leading reduced uptake and decreased drug accumulation have also been reported. Many strains of bacteria such as *Enterobacter, Pseudomonas, Serratia* and *Salmonella* are reported to have such one-step multidrug resistance mechanisms (Dang *et al.,* 1988).

3.1.3 Antibiotic resistance in Arctic region

Antibiotic resistance genes are common in natural environment and existed even on plasmids, before the use of antibiotics (Allen, 2010). However, environmental reservoirs of antibiotic resistance genes from Arctic are not clearly known. Production of an antibiotic is associated with the presence of genes encoding one or more self protection processes. Many of these genes retain their original essential functions for survival in the environment. Many bacteria which produce the antibiotic also have predisposition of genes for its resistance. Antibiotic resistance is introduced in microbial communities through mechanisms of different dimensions.

The unique ability of the bacteria to develop resistance mechanism to antimicrobial agents is resulting ineffective treatment of many infectious diseases and multi drug resistant pathogens that pose formidable challenge to the scientific community. The prevalence of horizontal gene transfer among bacteria by which they acquire genes from environment has made it easy for pathogens to exchange the antibiotic resistance elements among them. Though it is widely assumed that the selection pressure offered by polluted environments are key to the spread of the drug resistant strains, strains from pristine environment also show drug resistance (Allen *et al*., 2010).

The emergence of multidrug resistance has been reported from distant and wild habitats including extreme cold environments. Sudha *et al*. (2013) reported that 32 % of total bacteria from Arctic sediment and water were resistant to multiple drugs. De Souza *et al.* (2006) studied

antibiotic and metal resistance of bacteria from Antarctic region and reported 100 % resistance to ampicillin at a concentration of 50 ppm. The study also reported 63 % of resistance towards chloramphenicol and streptomycin at concentrations of 25 ppm and 250 ppm respectively. Bacterial isolates from Siberian permafrost were found to be resistant to some aminoglycosides such as gentamycin, kanamycin and streptomycin in addition to chloramphenicol and tetracycline (Mindlin *et al*., 2007). In the same study, antibiotic resistance genes such as *strA*, *strB*, and *aadA* were also detected. Perron *et al.* (2015) used metagenomic approach to study antibiotic resistance genes from Arctic soil. They could isolate eight genes which would provide resistance against aminoglycoside, beta-lactam and tetracycline antibiotics. Among the eight antibiotic resistance genes, four genes were able to provide resistance against synthetic antibiotic, amikacin.

Many wild animals and birds were reported to harbor bacteria with multi drug resistance and Arctic environment is no exception to it. Sjolund *et al.* (2008) carried out investigation on *E coli* from Arctic birds to study antibiotic resistance. The antibiotics such as ampicillin, cefadroxil, cefuroxime, cefpodoxime, chloramphenicol, ciprofloxacin, fosfomycin, trometamol, gentamicin, imipenem, mecillinam, nalidixic acid, nitrofurantoin, streptomycin, sulfamethoxazole, tetracycline, trimethoprim, and tigecycline were tested and resistance was found against 14 of them.

Faeces from polar bear were analyzed for bacterial diversity and antibiotic resistance index was calculated for the isolated strains (Glad *et al*., 2010). This study reported ampicillin resistance up to 44% and the strains were positive for beta-lactamase production. Middleton and Ambrose (2005) studied the antibiotic resistance of bacterial isolates from feacal matter of Canada geese. Feacal indicator organisms such as *E coli* and *Entercoccus* were screened for sensitivity to 10 antibiotics and resistance was found against penicillin G, ampicillin, cephalothin, streptomycin, sulphamethaxizole and chloramphenicol.

Sagawa *et al.* (2013) isolated antibiotic genes using PCR techniques from glaciers and snow field from around the world including Arctic, Antarctic, Central Asia, North and South America and Africa. Their study showed that most wide spread antibiotic resistance genes were from samples of Central Asia, Greenland, Africa and North and South America. They also suggested that the most probable mode of dissemination of antibiotic genes could be through migrating birds and

air borne bacteria. Allen *et al*. (2009) carried out gene hunt for beta-lactamase production in bacteria from remote Alaskan soil using metagenomic approach. They reported several kinds of beta-lactamases and some of the enzymes were functional in *E. coli*. They also suggested that resistance genes in remote Alaskan soil indicate that even in the absence of selection pressure microbial communities can harbor antibiotic resistance genes. Perron et al. (2015) to study the resistosome of bacteria from Arctic ancient soil used functional metagenomics. Canadian Arctic permafrost of at least 5000 years old was studied and they could isolate eight resistant genes from it. In another study different soil samples were collected from places such as Mainland Norway, New Zealand, Svalbard and Italy which was then analyzed to check the prevalence of bla_{TEM} gene (Brusetti *et al*., 2008). Their study showed that bacteria with bla_{TEM} carrying genes account for only < 1 per 1000 or 100000 bacteria tested.

3.1.4 Heavy metal resistance

There are many reports on association of antibiotic resistance with heavy metal resistance. The pollution of the environment with toxic heavy metals is spreading throughout the world along with industrial progress (Raja *et al.*, 2009). Increased use of metals and chemicals in the process industries has resulted in generation of large quantities of effluents containing toxic heavy metals and these effluents pose environmental disposal problems due to their non-degradable and persistent nature (Ahluwalia *et al.*, 2006). Microorganisms living in such environments adopt different mechanisms to adapt to these heavy metal stresses. Among various adaptation mechanisms, metal sorption, mineralization, uptake and accumulation, extracellular precipitation, enzymatic oxidation or reduction to a less toxic form and efflux of heavy metals from the cells have been reported (Nies, 2000; Shoheb, 2006; Rathnayake, 2009). Heavy metal resistance of bacteria from environment such as waste water, sewage and polluted soils are studied extensively but studies from pristine environment such as Arctic are scarce.

Heavy metal contamination of water bodies and air is a growing concern all over the world. Arctic is under long term surveillance program such as gaseous elemental mercury measurements (Berg *et al*, 2013) and Arctic monitoring and assessment program (AMAP, 2009) for heavy metal contamination. Sources of these heavy metals in Arctic can be from air and water. Heavy metals are carried through air in various ways such as combustion of fuel to produce heat and electricity, industrial processes including mineral mining and smelting of ores, sources relating to tourism development and waste disposal including waste incineration.

Similarly, heavy metals in water also find various sources, of which river systems plays a commendable role. Major river systems draining into Arctic Ocean that may carry dissolved metals and suspended solids are Lena, Ob, Yenisey, Pechora, Kolima, rivers in northern Canada and Yukon River systems. Ice transport and Ocean currents are two other sources of heavy metal contamination (AMAP, 2009; Berg *et al.*, 2013).

Arctic Monitoring and Assessment Program (AMAP, 2011) reported that Arctic organisms are impacted by multiple stressors and the effects observed in them will typically be the result of combined stressors (For example interaction between Se and Hg). Based on their studies, observed levels of Cd^{2+} and Hg in some marine birds and animals are high enough to be of concern. But there are no reports of microbial interaction with these heavy metals or its effect in Arctic. Although available information on sub-lethal biological effects in Arctic organisms is limited, the use of metal resistant bacteria as bioindicators of polluted environment might through some light on sub lethal toxicity (Blaise *et al.,* 1985; Trevers *et al.,* 1985; AMAP, 2011). In integrated ecotoxicological approach a combination of bioassays (fish, algae, and bacteria) is highly recommended to gain better insight into levels of toxicity. It is also proposed that metal tolerance of bacteria can be considered as an indicator of potential toxicity to all other forms of life.

Mercury (Hg) is a major pollutant in the Arctic environment, and there is evidence of increasing concentrations in some marine ecosystems over time. Atmospheric Hg is recognized as one of the main sources to the environment and particular attention has been paid to understand the importance of atmospheric mercury depletion events (AMDE) as a means of delivering Hg to polar ecosystems (AMAP, 2011; Berg *et al.*, 2013). Similarly, heavy metals such as mercury, cadmium, lead, arsenic and selenium are believed to produce biological effects in Arctic (AMAP, 2011). Thus in the current study bacteria with high antibiotic resistance were also tested for heavy metal resistance.

3.2 Materials and Methods

Microorganisms used

A total of 272 heterotrophic bacterial isolates from sediment and water of Kongsfjord were used for the Antibiotic resistance study. The isolates were inoculated into ZoBell's Marine Broth (ZMB) and incubated for 48 hr at 20 °C for obtaining cultures having an optical density value 1.0 at 620 nm. These enriched cultures were used for antibiotic susceptibility studies.

3.2.1 Antibiotic Sensitivity Test

Antibiotic susceptibility test was performed for all the isolates on Mueller-Hinton Agar (HiMedia, Mumbai) by Kirby-Bauer method (Bauer *et al.,* 1966). The enriched cultures were aseptically swabbed on to sterile, surface dried Mueller Hinton Agar plates using sterile cotton swabs. After 30 min. of pre-diffusion time antibiotic impregnated discs were placed on the surface of inoculated plates and incubated at 20 °C for 24 hr. After incubation, the diameter of inhibition zone was measured and was interpreted based on recommendations of Clinical laboratory Standards Institute (CLSI, 2011) and classified the isolates as sensitive, intermediate and resistant. The antibiotics used for the antibiotic sensitivity testing were amplicillin (A, 10 µg), amoxicillin (AM, 30 µg), carbenicillin (CB, 100 µg), cefpodoxime (CEP, 10 µg), ceftazidime (CA, 30 µg), cephalothin (CH, 30 µg), chloramphenicol (C, 10 µg), ciprofloxacin (CF, 5 µg), colistin (CL, 25 µg), gentamycin (G, 10 µg), nalidixic acid (NA, 30 µg), streptomycin (S, 10 µg), sulphamethaxizole (SM, 300 µg), tetracycline (T, 30 µg) and trimethoprim (TR, 5 µg). The antibiotics belonged to 7 classes according to their chemical structure beta-lactams (amplicillin, amoxicillin, carbenicillin, cefpodoxime, ceftazidime, cephalothin, and chloramphenicol), quinolones (ciprofloxacin, nalidixic acid), aminoglycosides (gentamycin, streptomycin), tetracyclins (tetracycline), sulfadrugs (sulphamethaxizole), polypeptides (colistin) and others (trimethoprim).

3.2.2 MAR Indexing of the isolates

MAR indices and MAR profiles were determined for each isolate from water and sediment. The MAR index, when applied to a single isolate is defined as *a/b*, where *a* represents the number of antibiotics to which the isolate is resistant, and *b* represents the number of antibiotics to which the isolate was exposed.

Resistance pattern for the isolates are designated by the abbreviation of the antibiotics to which the isolate is resistant. For example an isolate which is resistant to ampicillin, amoxicillin and cephalothin is represented as A, AM CH. Data was further processed to calculate the percentage resistance of each antibiotic in sediment and water by calculating the percentage of organisms resistant to the antibiotic to the total number of isolates tested.

3.2.3 Plasmid Isolation

The antibiotic resistant isolates were subjected to plasmid extraction using alkali lysis process as described by Brimboim and Doly (1979). The cultures were inoculated into Luria Bertani broth and incubated at 20 °C for 24 hr at a shaking speed of 150 rpm. These cultures (1.5 ml) were then centrifuged at 6000 rpm for 5 min and supernatant was discarded. Lysis buffer (10 % SDS, 0.5 M EDTA, 10 N NaOH) was added to the pellets and placed in a boiling water bath for 10 min and then 50 µl of 1mM $MgCl_2$ was added. Tubes were kept on ice for 2 min and then centrifuged again at 12000 rpm for 2 min. To this mix, 50 µl of 3 M potassium acetate was added followed by centrifugation at 12000 rpm for 2 min. The supernatant was transferred to a fresh tube and 600 µl of isopropanol was added to it and kept on ice for 10 min. Tubes were then subjected to centrifugation at 10000 rpm for 10 min. Supernatant was discarded and pellet was washed with 70 % ethanol at 5000 rpm for 5 min, air dried followed by addition of 30 µl distilled water and kept at 4 °C overnight for dissolving. Electrophoresis was performed on a 0.8% agarose gel (Agarose, Origin, India) 1% (w/v) in 1X TBE buffer containing 0.5 µg/ml of ethidium bromide. Electrophoretic separation was carried out at 75 V for 1 h and molecular weight marker (supercoiled DNA ladder, HiMedia) was included.

3.2.4 Assessment of metal toxicity

Metal toxicity assessment was carried out on multiple antibiotic resistant strains which included selenium, cadmium, mercury, zinc, lead and copper in their salts forms as cadmium chloride ($CdCl_2$), mercuric chloride ($HgCl_2$, pH was adjusted to 6.5), zinc chloride ($ZnCl_2$), lead acetate ($Pb(CH_3COO)_2$), and copper sulphate ($CuSO_4.5H_2O$). These metals were prepared in different concentrations 20 µg/l, 50 µg/l, 100 µg/l, 200 µg/l, 500 µg/l, 1000 µg/l, 2000 µg/l, 3000 µg/l and 4000 µg/l. The stock solutions were prepared in double distilled water, sterilized at 120 ˚C for 15 min and were kept at 4 ˚C for no longer than 1 month. All glassware used during the study were

washed in 2 N HNO_3 and rinsed several times with distilled water to avoid any metal contamination. During the toxicity test, a different media instead of isolation media was used, since isolation media contain large amount of complex metal ions which might possibly interfere with microbe-metal interaction. Thus Nutrient Agar (NA) which supported good growth and lesser in trace metal content than the isolation media was used for the tolerance test. A preliminary non quantitative assessment was carried out by incorporating heavy metals into nutrient agar before proceeding to quantitative assays by incorporating heavy metals into nutrient agar. These metal containing plates were then spot inoculated with cultures enriched in nutrient broth for 24 h. Development of colonies on plates indicated resistance against the heavy metal. Quantitative assessment of heavy metal effects were carried out by plate diffusion and tube dilution method

Plate diffusion method

To each of nutrient agar plates, actively growing bacterial cultures (final OD value 1.0 at 620nm) were inoculated by swabbing. When inoculums were completely absorbed (20-30min), salts of heavy metal solutions were introduced to the plates as follows. Plates were divided into 4 quadrants and wells of 10mm diameter and 4mm depth were cut into each agar quadrant with a sterile gel borer. Into each well, 25µl of heavy metal solution of appropriate concentrations such as 20 µg/l, 50 µg/l, 100 µg/l and 500 µg/l were added except a well bored in the centre, which was kept as a control well (25µl deionized water). The tests were done in triplicates and the plates were incubated at 20˚C for 48hrs. The diameter of inhibition zone was measured in millimeters and was used in toxicity assessment.

Tube dilution method

Nutrient broths with appropriate heavy metal concentrations were prepared in 25 ml test tubes with a final volume of 10 ml. In to each tube, 200 µl of actively growing cultures were added (OD value 1.0 at 620 nm). A tube without metal and another tube without the culture served as positive and negative controls respectively. Tubes were then incubated at 20 ˚C for four days. All tests were conducted in triplicates and average of three readings was taken for computation of toxicity. Tube with lowest concentration of metal that prevented growth was considered as minimum inhibitory concentration of the metal.

Statistical analysis

Statistical analysis was carried by using IBM SPSS Statistics 20 software and ANOVA was used to test the null hypothesis that the concentration of heavy metals did not have any effect on resistance and growth of the bacteria. Significance of difference between the samples were tested with one way ANOVA at $P = 0.05$.

3.3 RESULTS

3.3.1 Antibiotic sensitivity of heterotrophic bacteria

Analysis of antibiotic resistance from bacteria isolated from sediment and water was carried out by agar diffusion method. The results revealed that sediment isolates were clearly more resistant than the water isolates. Resistance was found against all antibiotic tested in the sediment isolates whereas resistance was found only against 12 antibiotics in the isolates from water samples. None of the isolates from water sample has shown resistance towards chloramphenicol, ciprofloxacin and trimethoprim.

Table 3.1 represents the percentage of isolates from sediment and water that were resistant against different antibiotics tested. Bacterial isolates from the sediment showed maximum resistance against ampicillin (67.5 %) followed by amoxicillin (59.65 %) and cephalothin (48.65 %). The order of resistance showed by the sediment isolates were ampicillin > amoxicillin > cephalothin > cefpodoxime > tetracycline > sulphamethaxizole > carbenicillin > ciprofloxacin > ceftazidime = gentamycin > colistin > streptomycin = chloramphenicol > nalidixic acid > trimethoprim.

Isolates from water samples showed lower resistance compared to isolates from the sediment samples. Maximum resistance was shown towards cephalothin (58.5 %) followed by carbenicillin (41.16 %) and cefpodoxime (36 %). The percentage resistance of water isolates were in the order cephalothin > carbenicillin > Cefpodoxime > ampicillin > amoxicillin > colistin > gentamycin = sulphamethaxizole > tetracycline > nalidixic acid > streptomycin > ceftazidime.

Table 3.1: Percentage resistance of bacteria against different antibiotics.

Sl No:	Antibiotic	Sediment isolates (% resistance)	Water isolates (%resistance)
1	Ampicillin	67.5	33.33
2	Amoxicillin	59.65	10.83
3	Cephalothin	48.65	58.5
4	Chloramphenicol	7.5	0
5	Ciprofloxacin	15.5	0
6	Carbenicillin	15.9	41.16
7	Colistin	9.6	8.3
8	Ceftazidime	11	1.02
9	Cefpodoxime	42.31	36
10	Gentamycin	11	6.5
11	Nalidixic acid	5.6	2.5
12	Streptomycin	7.5	1.5
13	Sulphamethaxizole	25.69	6.5
14	Tetracycline	31.89	3
15	Trimethoprim	4.7	0

1	0.2	A,AM,CH	7.4	10.83
		A,CEP,CH	2.1	58.5
		A,AM,CB	1.3	0

3.3.2 MAR Index and resistance pattern

A total of 27 resistant patterns were detected from the heterotrophic bacteria from sediment and water. MAR index was calculated as described in materials and methods (Section 3.2) for each isolate. Most frequently observed MAR index belonged to 0.2, 0.266, 0.33, 0.4, and 0.466 (Table 3.2). Different patterns were seen under each MAR index such as 8 patterns for 0.2, 7 patterns 0.266, 4 patterns for 0.33 and 2 patterns for 0.466. As depicted in Table no. 3. 3, most frequently encountered resistance pattern among heterotrophic bacteria was A, AM, CH, CEP with a frequency of 31.96% followed by A, AM, CH with a frequency of 7.4%. The least encountered pattern was A, AM, CEP under MAR Index 0.2 with a frequency of 0.30%.

Table 3.2: MAR Index and Resistance pattern of bacteria from Arctic sediment and water

		CH,CB,AM	1.59
		CH,CB,TR	0.63
		AM,CH,CEP	0.933
		A,AM,CEP	0.301
		CEP,CH,G	0.606
2	0.266	A,AM,CH,CEP	31.96
		A,AM,CH,T	1.87
		A,CH,CEP,SM	0.933
		A,AM,CH,SM	1.87
		A,CH,CEP,T	0.63
		A,AM,CH,CB	9.09
		S,CB,T,NA	0.303
3	0.33	A,AM,CH,CEP,TR	0.933
		A,AM,CH,CEP,SM	2.7
		A,AM,CH,CB,CEP	3.46
		CA,CB,CL,CEP,TR	0.933
4	0.4	A,AM,CH,CEP,NA,T	0.933
		A,AM,CH,CEP,CA,SM	0.933
		A,AM,CH,NA,T,SM	1.89
		A,AM,CH,CEP,T,SM	1.56
		A,AM,CEP,T,NA,G	0.303
		A,AM,CEP,CB,T,SM	1.56
5	0.466	A,AM.CH,CEP.NA,T,SM	1.871
		A,AM,CA,CH,CB,CL,CE P	1.265

Figure 3.1 (a-l) shows the antibiotic resistance of bacteria isolated from the Arctic sediment and water. Ninety two percentage of *Stenotrophomonas maltophilia* was resistant to ampicillin and high resistance was also found against amoxicillin and cephalothin (75.12%). Figure 3.1 (b) shows resistance of *Brachybacterium paraconglomeratum* against different antibiotics. The strains of *Brachybacterium paraconglomeratum* showed 45.45% resistance against ampicillin and 31.81% resistance against cephalothin..

Figure 3.1 (a): Antibiotic resistance of *Stenotrophomonas maltophilia* isolated from Kongsfjord

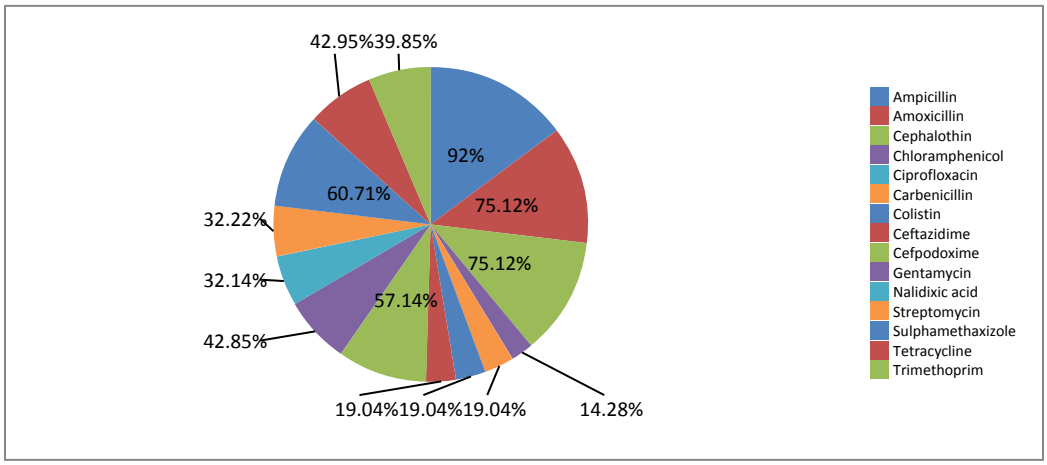
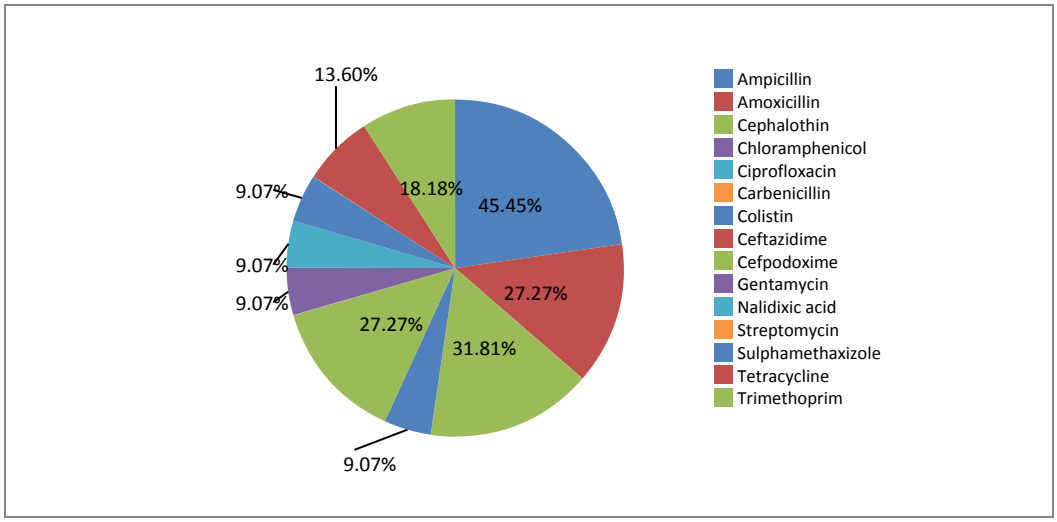


Figure 3.1 (b): Antibiotic resistance of *Brachybacterium paraconglomeratum* isolated from Kongsfjord



Almost 63% of the strains of *Bacillus flexus* were resistant against ampicillin and 38 % of strains were resistant against cephalothin and amoxicillin (Figure. 3.1(c)). Resistance of the strains of *Micrococcus luteus* is represented in the Figure 3.1(d) which shows 42 % resistance against ampicillin, amoxicillin and cefpodoxime.

Figure 3.1 (c): Antibiotic resistance of *Bacillus flexus* isolated from Kongsfjord

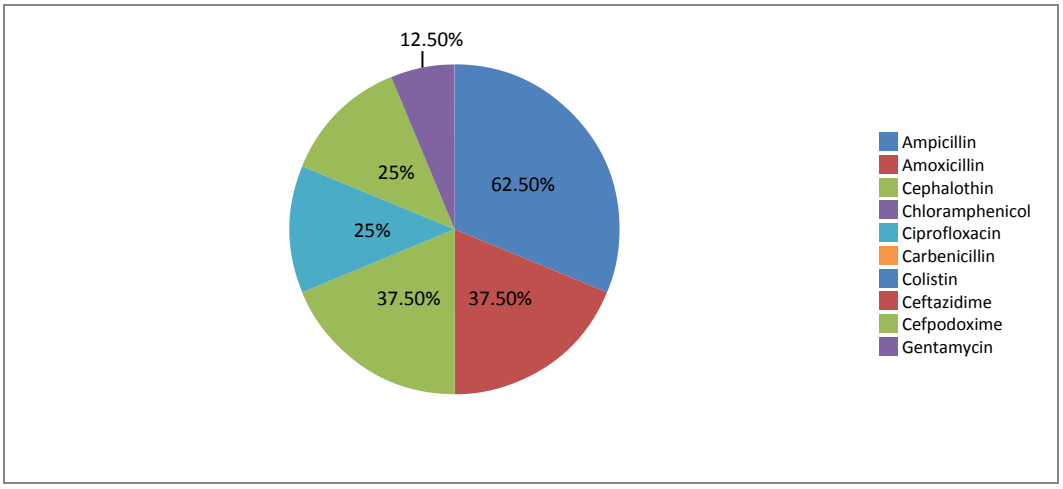
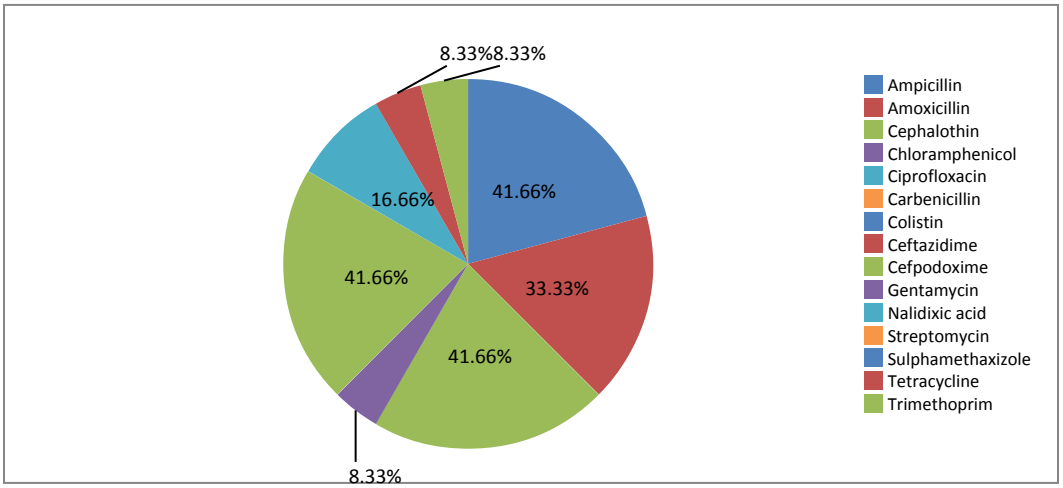


Figure 3.1 (d): Antibiotic resistance of *Micrococcus luteus* isolated from Kongsfjord



Resistance of strains of *Staphylococcus cohinii* towards caphalothin was found to be 75 % followed by ampicillin (38 %) and is represented in the figure 3.1(e). Similar resistance percentage was found against the antibiotic tetracycline, sulphamethaxizole and gentamycin (25 %). Strains of *Enterobacter ludwigii* showed resistance against 11 antibiotics and 98 % of the strains were resistant against cephalothin followed by resistance against ampicillin (97 %) and amoxicillin (92 %).

Figure 3.1 (e): Antibiotic resistance of *Staphylococcus cohinii* isolated from Kongsfjord

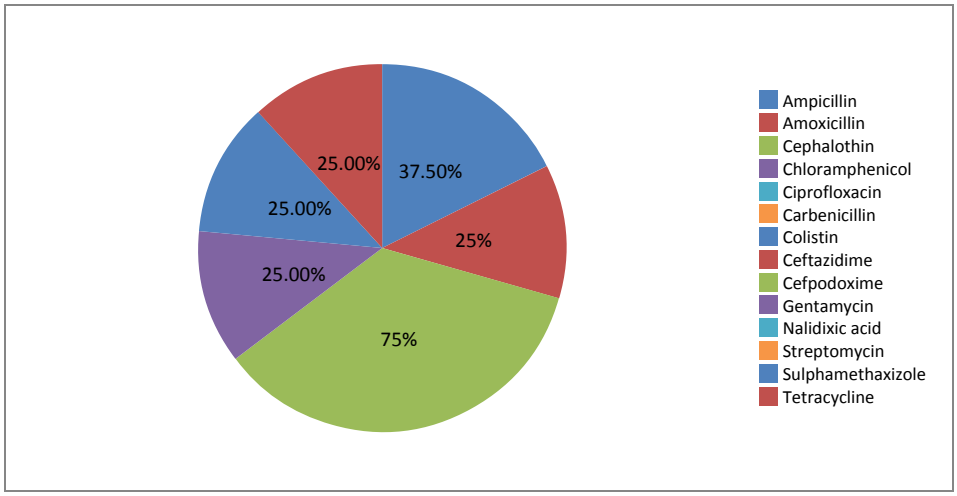


Figure 3.1 (f): Antibiotic resistance of *Enterobacter ludwigii* isolated from Kongsfjord

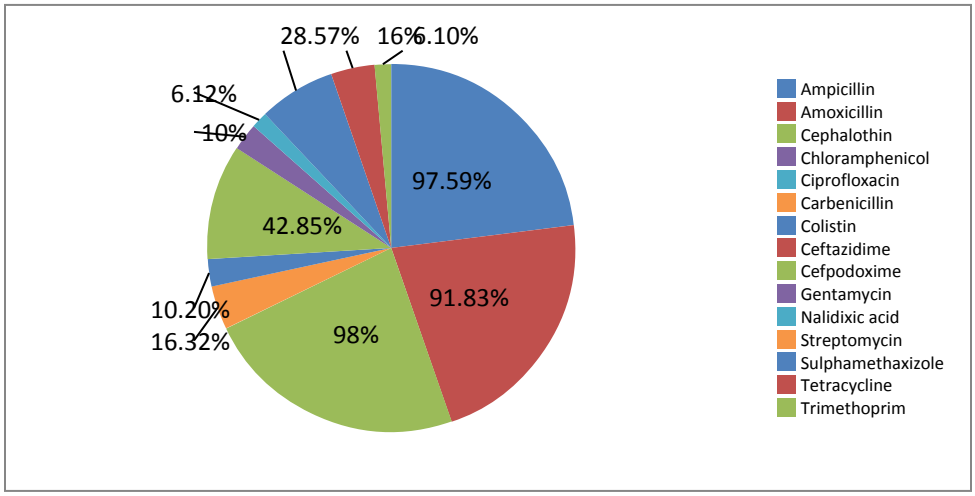


Figure 3.1(g) shows resistance of *Bacillus thuringiensis* from Kongsfjord sediment and water against antibiotics. Strains of *Bacillus thuringiensis* showed resistance against 7 antibiotics and 37.5% resistance was found against 4 antibiotics such as cephalothin, ampicillin, cefpodoxime, and gentamycin. As depicted in Figure 3.1 (h), strains of *Pseudomonas koreensis* showed resistance against 9 antibiotics and 99% resistance was found against ampicillin and cephalothin. Strains of *Pseudomonas koreensis* showed low resistance towards tetracycline, streptomycin and ceftazidime (<3.5%)

Figure 3.1 (g): Antibiotic resistance of *Bacillus thuringiensis* isolated from Kongsfjord

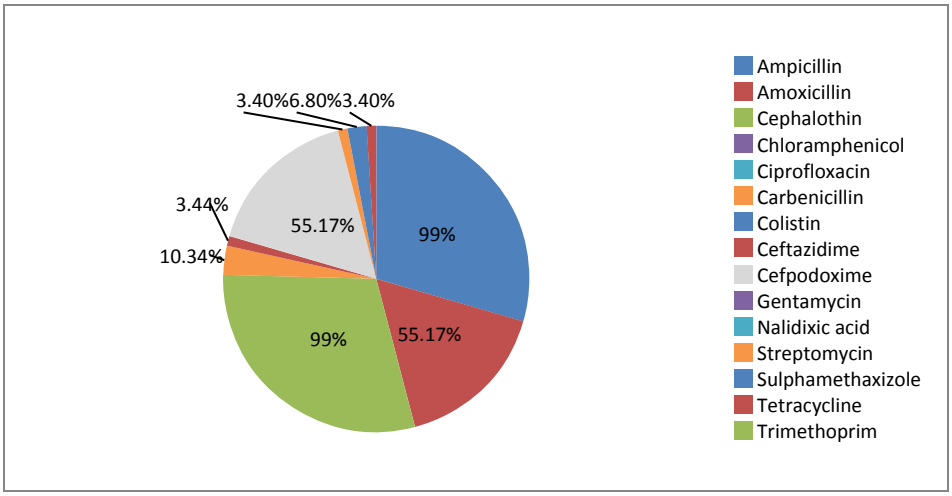


Figure 3.1 (h): Antibiotic resistance of *Pseudomonas koreensis* isolated from Kongsfjord

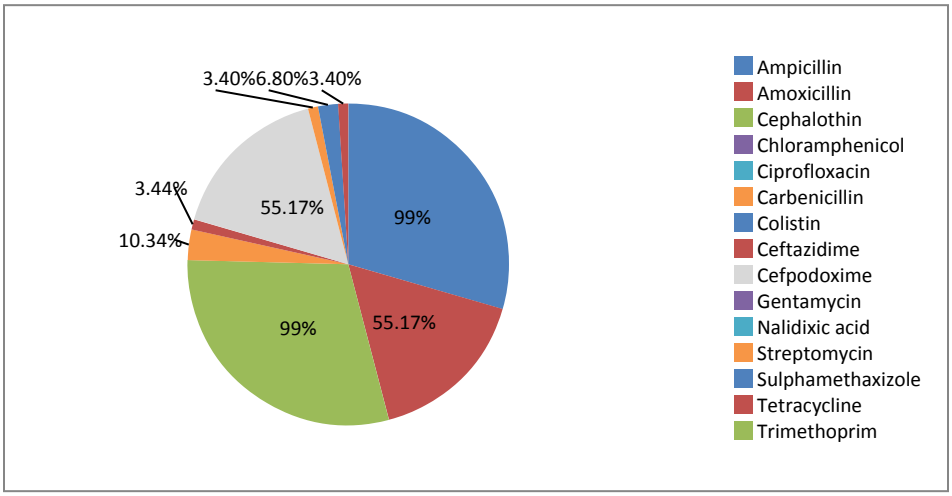


Figure 3.1 (i) show the resistance of strains of *Enterobacter cancerogenus* against different antibiotics. *Enterobacter cancerogenus* strains also showed resistance against 11 antibiotics and highest resistance was found against cephalothin (98 %). Strains of *Pseudomonas fragi* showed resistance against 7 antibiotics and resistance to ampicillin and cephalothin was found to be 99 % as represented in the Figure 3.1 (j).

Figure 3.1 (i): Antibiotic resistance of *Enterobacter cancerogenus* isolated from Kongsfjord

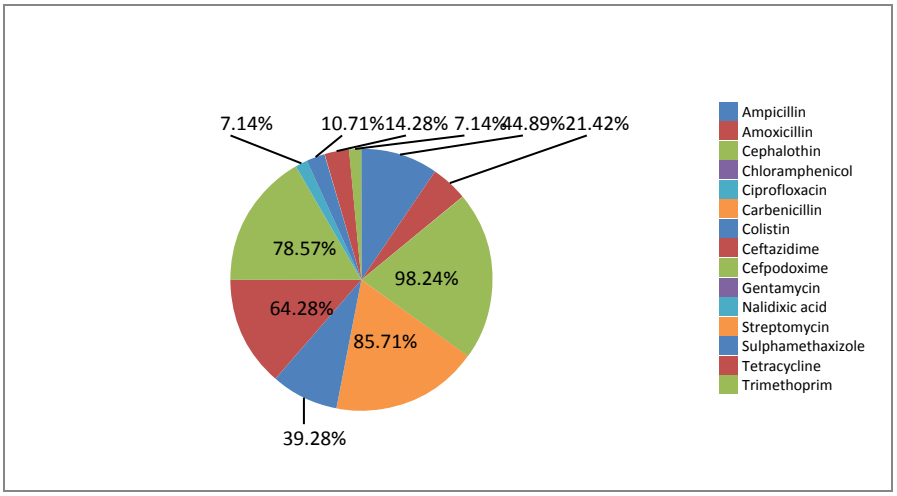
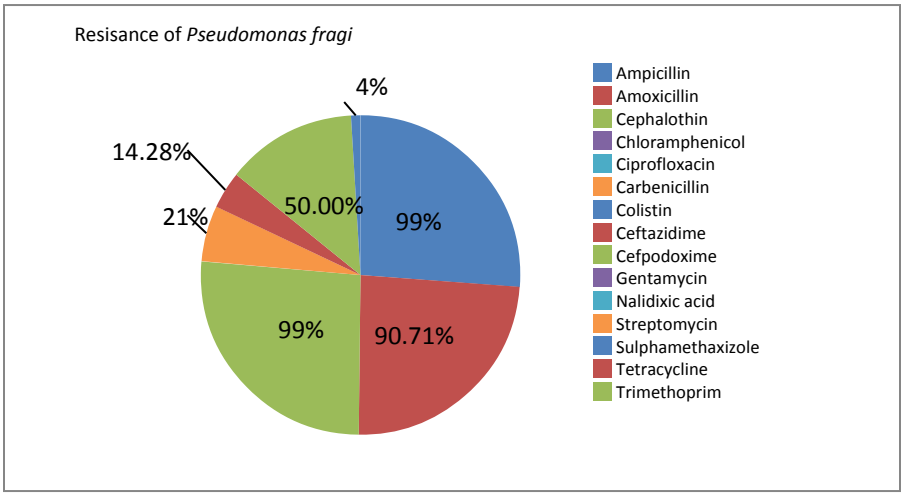


Figure 3.1 (j): Antibiotic resistance of *Pseudomonas fragi* isolated from Kongsfjord



As shown in Figure 3.1(k), strains of *Pseudomonas sublingiri* showed resistance against only 5 antibiotics such as ampicillin, cephalothin, carbenicillin, cefpodoxime and streptomycin. Strains of *Halomonas boliviensis* showed resistance against only 4 antibiotics including ampicillin, amoxicillin, cephalothin and gentamycin (Figure 3.1(l)).

Figure 3.1 (k): Antibiotic resistance of *Pseudomonas sublingiri* isolated from Kongsfjord

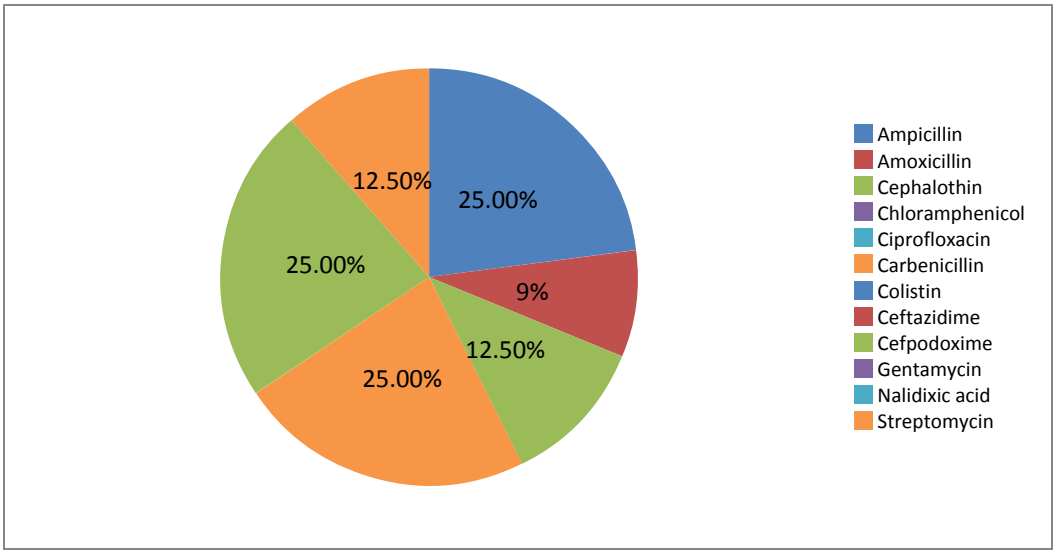


Figure 3.1 (l): Antibiotic resistance of *Halomonas boliviensis* isolated from Kongsfjord

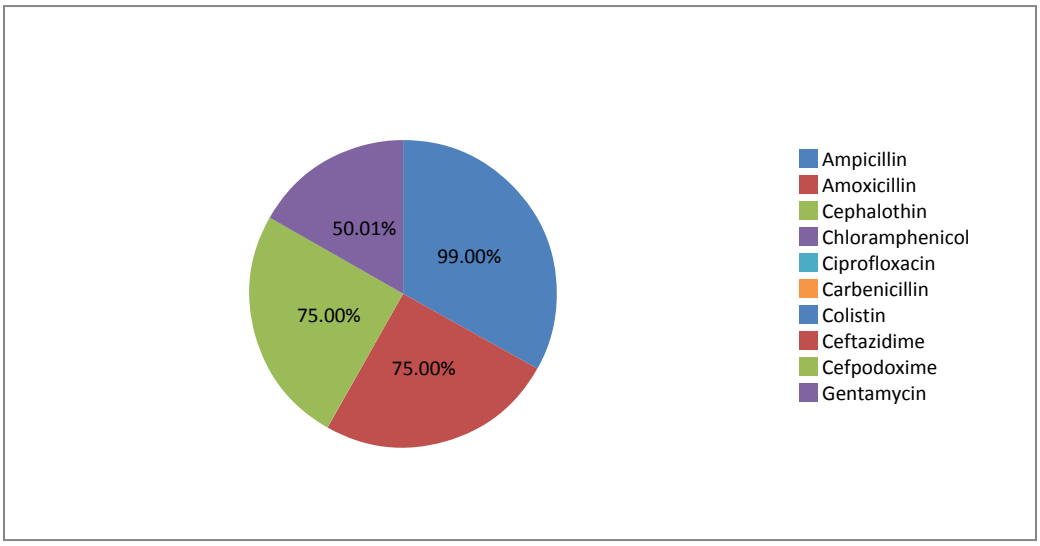
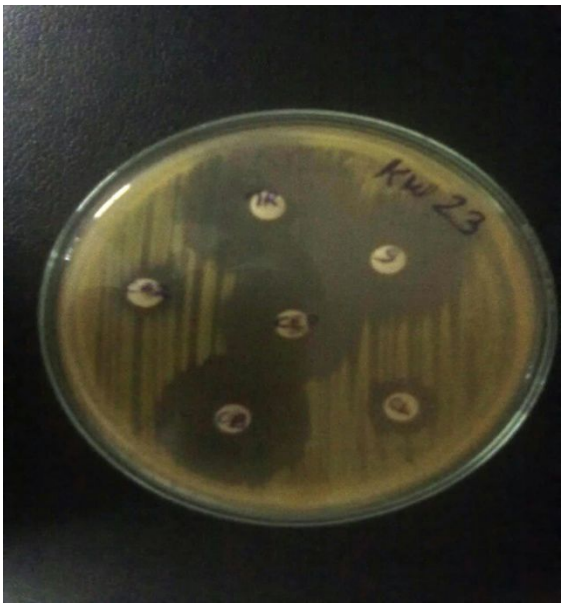
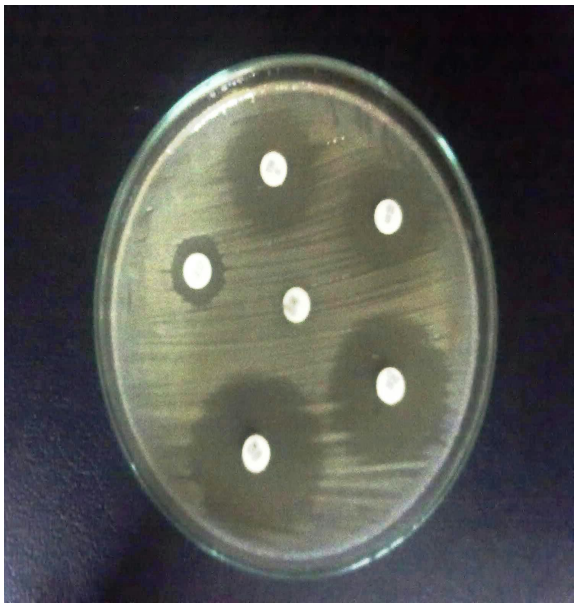
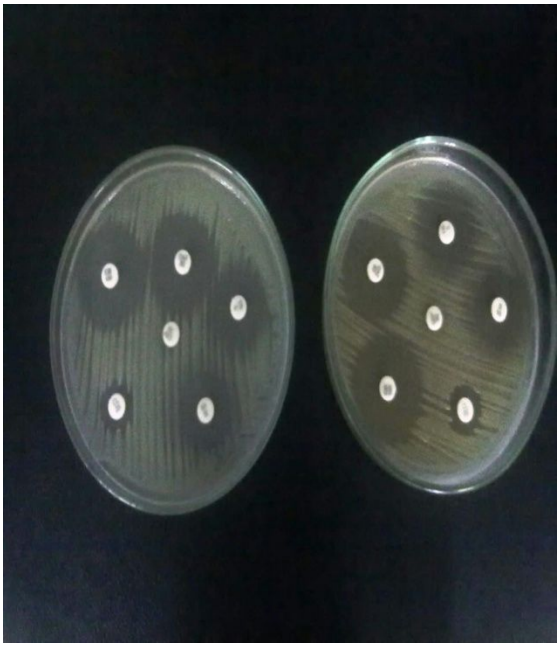
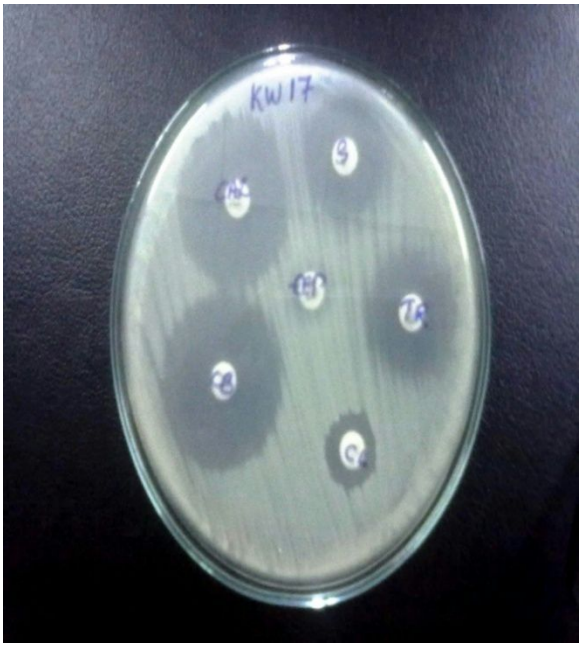


Figure 3.2: Images showing antibiotic resistance of different heterotrophic bacteria









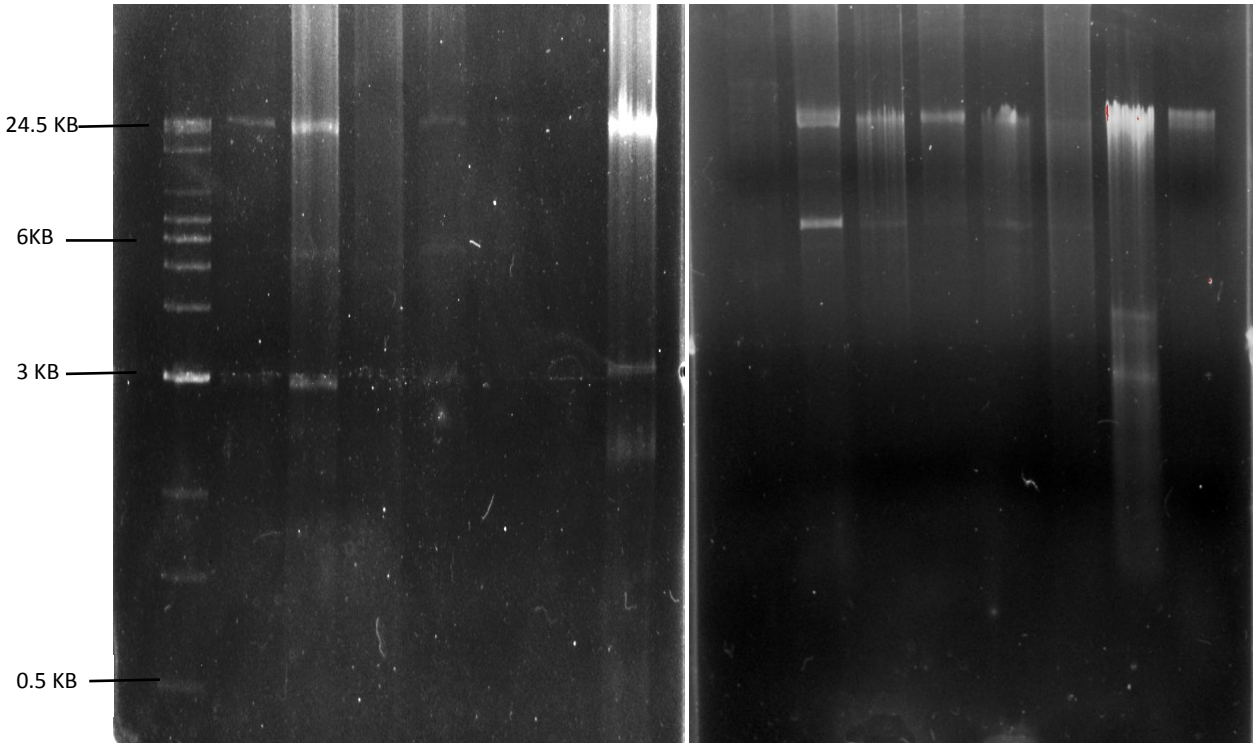
3.2.2 Plasmid profiling

All water and sediment isolates that showed multiple antibiotic resistances were subjected to plasmid extraction. From the multiple antibiotic resistant strains, plasmids were isolated from 81 strains. These isolates showed different pattern of plasmid ranging from 1 band to 4 bands in some isolates. Maximum number of isolates were found to have single plasmid of size ~24.5 Kb. As can be observed from table: 3.3 seven patterns of plasmids carried a band approximately at 1.8 Kb. Agarose gel electrophoresis of plasmids showing the bands of different molecular weight is represented in the figure 3.3

Table 3.3: Pattern of plasmids and size of detected plasmids from heterotrophic bacteria

Sl. No.	Plasmid pattern (Kb)	No. of isolates
1	~1.8	3
2	~3.0	5
3	~24.5	53
4	~1.3, ~3.0	1
5	~1.8, ~ 6.0	2
6	~6.0, ~24.5	3
7	~1.8, ~ 24.5	4
8	~1.8, ~3.0, ~9.0	3
9	~ 1.8, ~ 3.0, ~ 6.0	2
10	~ 1.8, ~ 3.0, ~ 24.5	2
11	~1.8, ~9.0, ~ 24.5	3

Figure 3.3: Agarose gel electrophoresis showing plasmids of various sizes isolated from antibiotic resistant isolates from sediment and water.



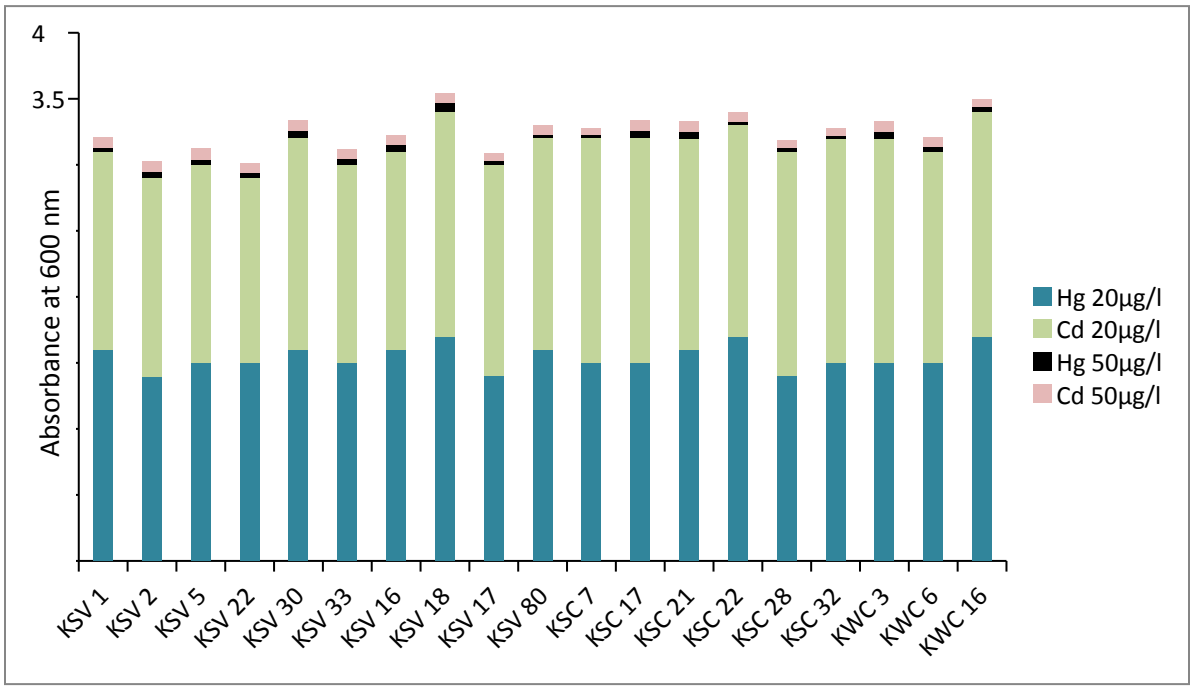
Lane 1: Marker, Lane 2-13 multiple antibiotics resistant isolates showing plasmids

3.3.3.Heavy metal resistance of Arctic bacteria

Mercury was found to be the most toxic of all heavy metals tested by both the methods. The range of tolerance was heterogeneous among different strains. As can be envisaged from Figure 3.4, the 20 µg/l concentration of mercury was well tolerated by most of the isolates but increasing the concentration to 50 µg/l was toxic to all bacteria. Thus 50 µg/l mercury has significantly higher toxicity (P = < 0.0001, F = 291.08). Even though tube dilution method also expressed the same MIC, at 20 µg/l concentrations, growth was seen only after prolonged incubation and many isolates showed very scanty growth compared to positive control. Cadmium had shown a minimum inhibitory value of 50µg/l but the size of inhibition zone was smaller than that of mercury and growth in tube dilution method was significantly higher in presence of cadmium than mercury confirming higher toxicity of mercury over cadmium.

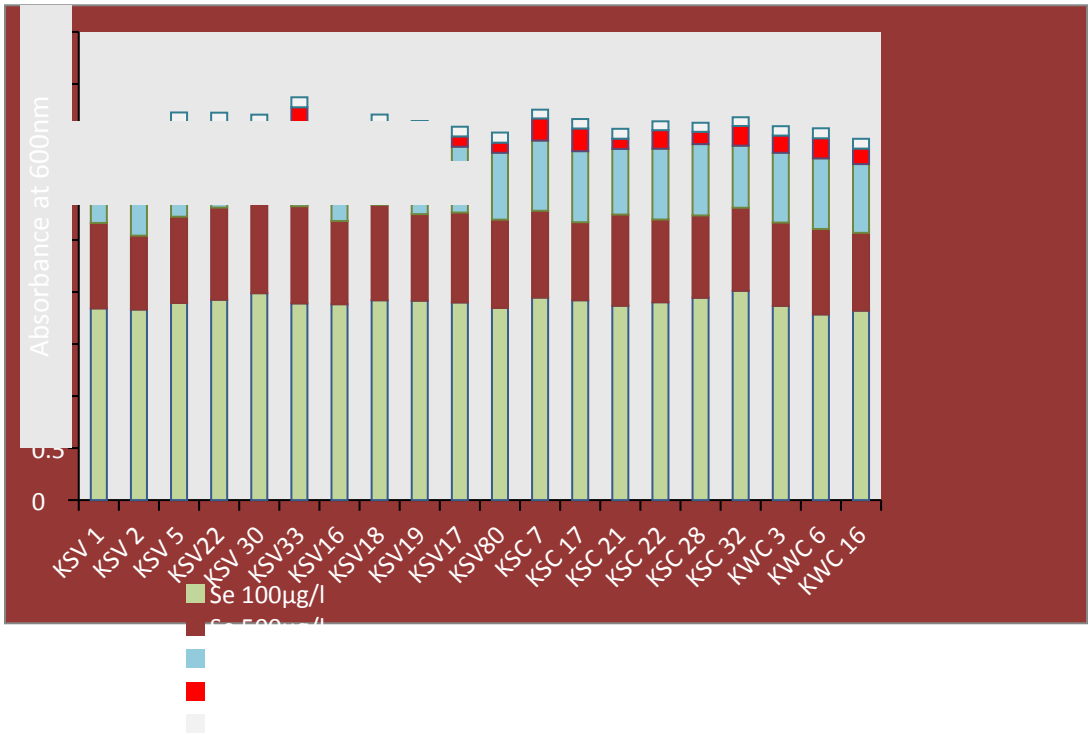
Depending on the percentage of isolates showing metal resistance, the order of toxicity was found to be Hg > Cd > Cu > Zn > Pb > Se.

Figure 3.4: Mercury and Cadmium resistance of selected isolates from Arctic sediment and water by tube dilution method



Selenium was the most tolerated of all heavy metals tested and no toxic effects to growth were observed up to 1000 µg/l concentration. While increasing the concentration to 2000 µg/l, 62 % of the strains were resistant and no growth was observed at 3000µg/l. Thus at a concentration of 3000 µg/l the growth was significantly less (P = <0.0001, F = 75.6) compared to concentrations of 100 µg/l, 500 µg/l, 1000 µg/l, and 2000 µg/l.

Figure 3.5: Resistance of Arctic bacterial isolates against different concentrations of selenium by tube dilution method



Copper and Zinc were moderately tolerated by the bacteria and minimum inhibitory concentration turned out to be 2000 µg/l. 2000 µg/l copper has shown significantly higher toxicity (P = <0.0001, F = 59.0) compared to 100 µg/l, 500 µg/l, and 1000 µg/l. MIC of Zinc was found to be 2000 µg/l and toxicity was significantly higher (P = <0.0001, F = 48.78) compared to lower concentrations. Even though copper and zinc have the same MIC; copper could be more toxic than zinc since at a concentration of 1000 µg/l, 78% of the isolates were resistant to copper and 85.05 % were resistant to zinc.

Figure 3.6. Resistance of Arctic bacterial isolates against different concentrations of Zinc by tube dilution method

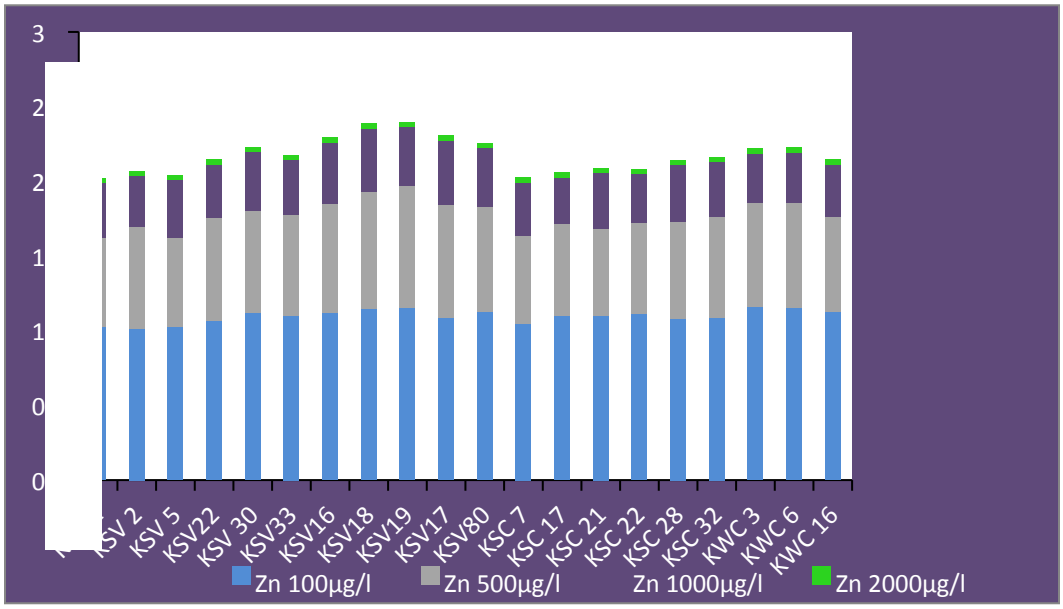
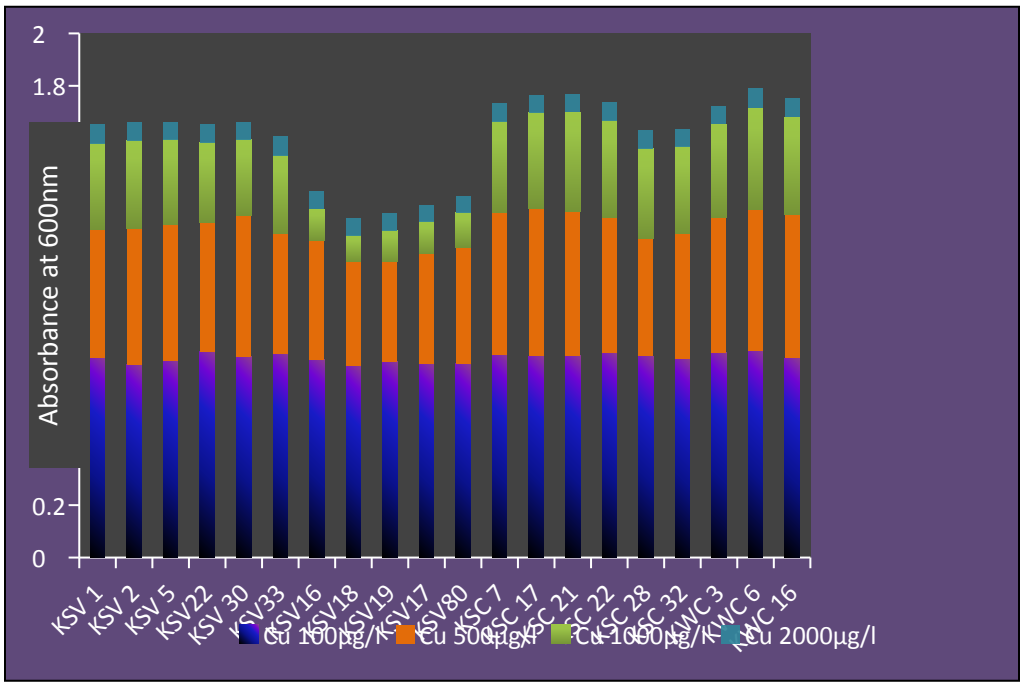


Figure 3.7: Resistance of Arctic bacterial isolates against different concentrations of Copper by tube dilution method



All the isolates showed tolerance to Lead up to 1000 µg/l at a concentration of 2000 µg/l, 49 % of isolates was found to show resistance. At a concentration of 3000 µg/l significantly lesser growth was observed (P = < 0.0001, F = 51.79) and MIC was determined to be 3000 µg/l.

Figure 3.8: Resistance of Arctic bacterial isolates against different concentrations of lead by tube dilution method

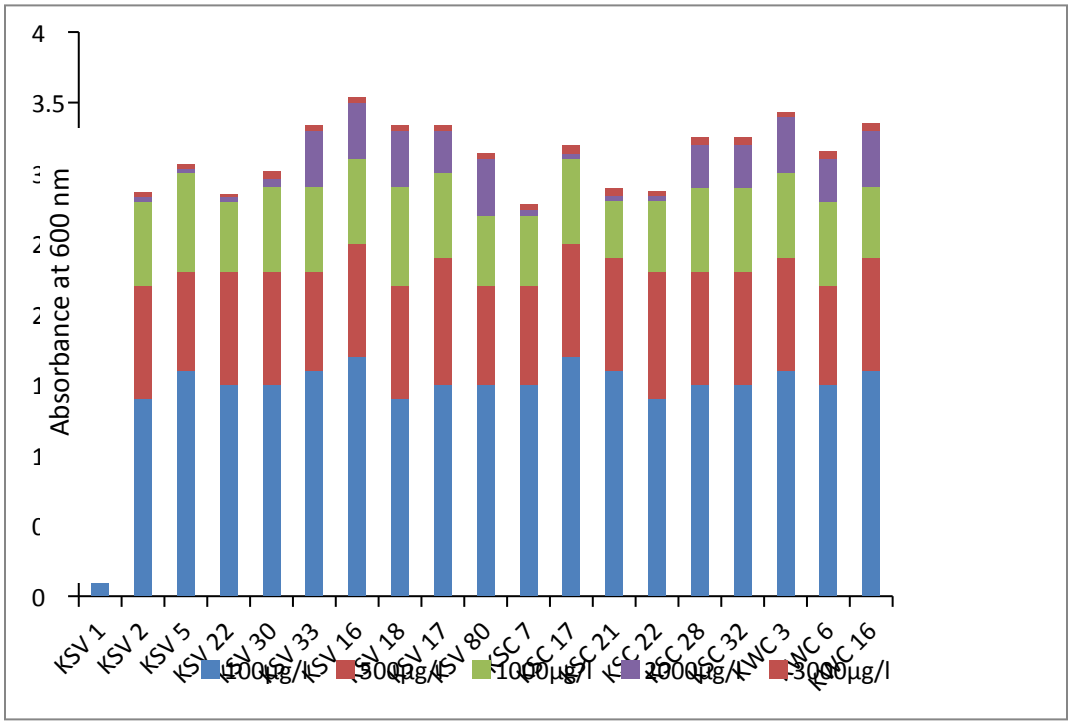
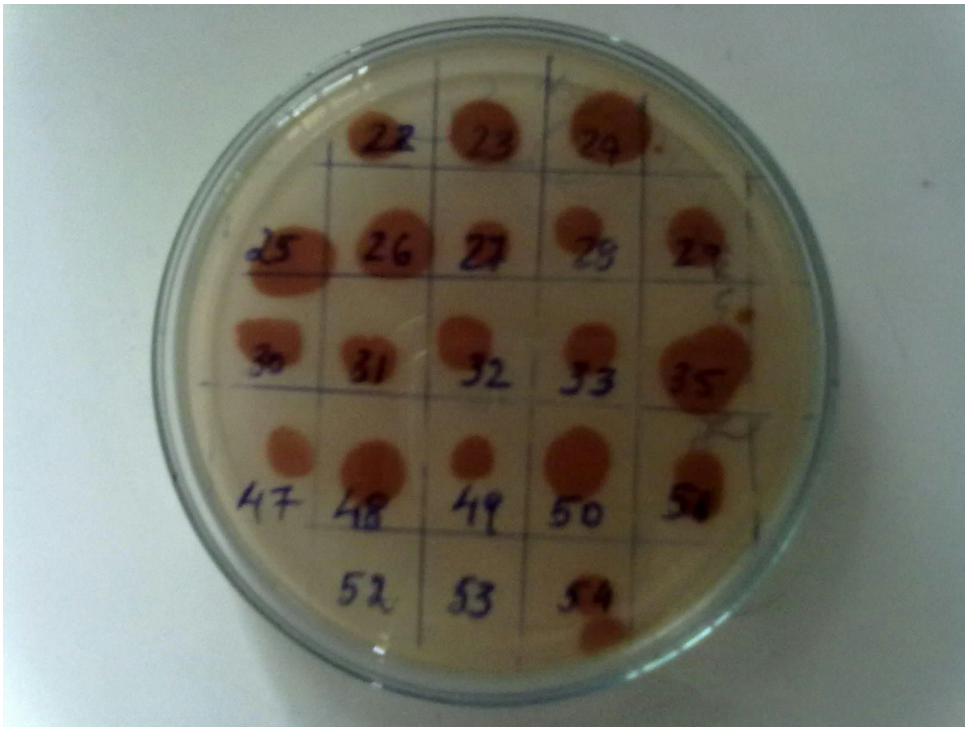


Figure 3.9: Images showing selenium resistant and copper resistant isolates from Arctic sediment and water



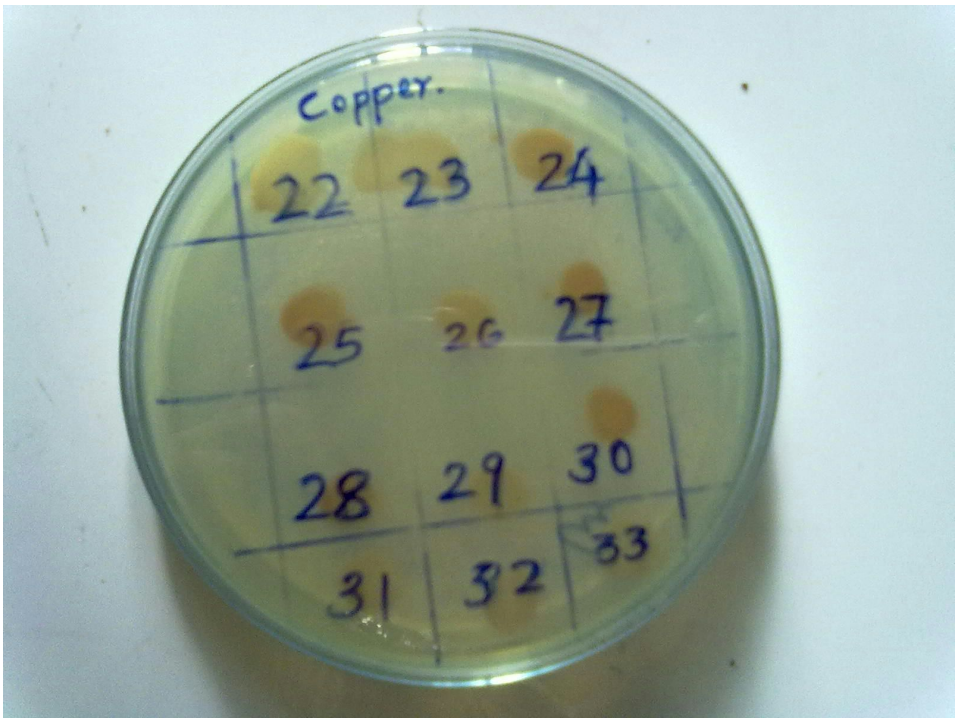
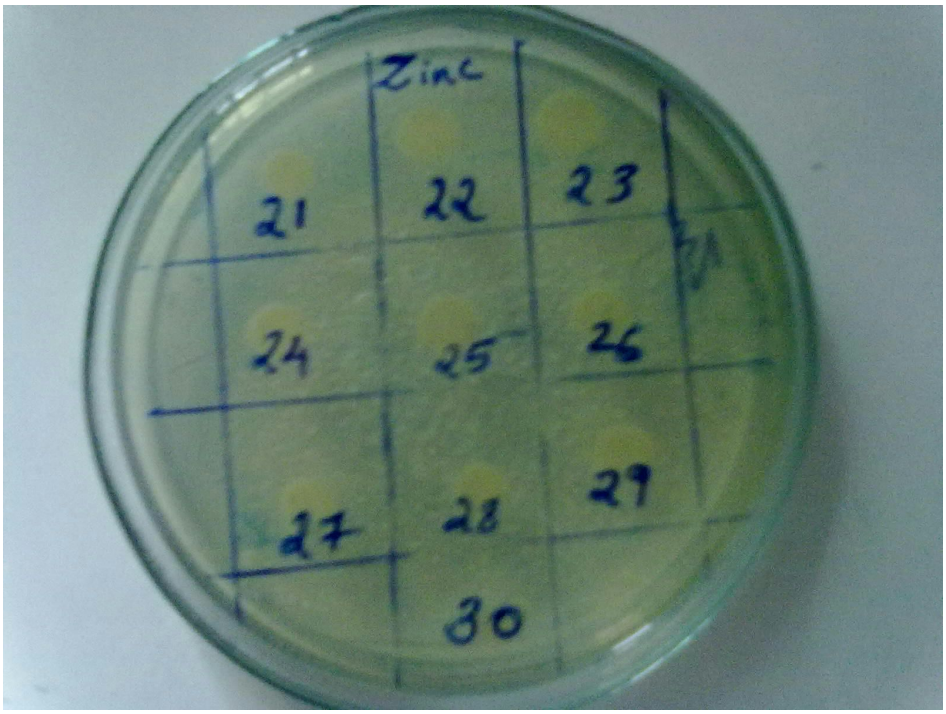
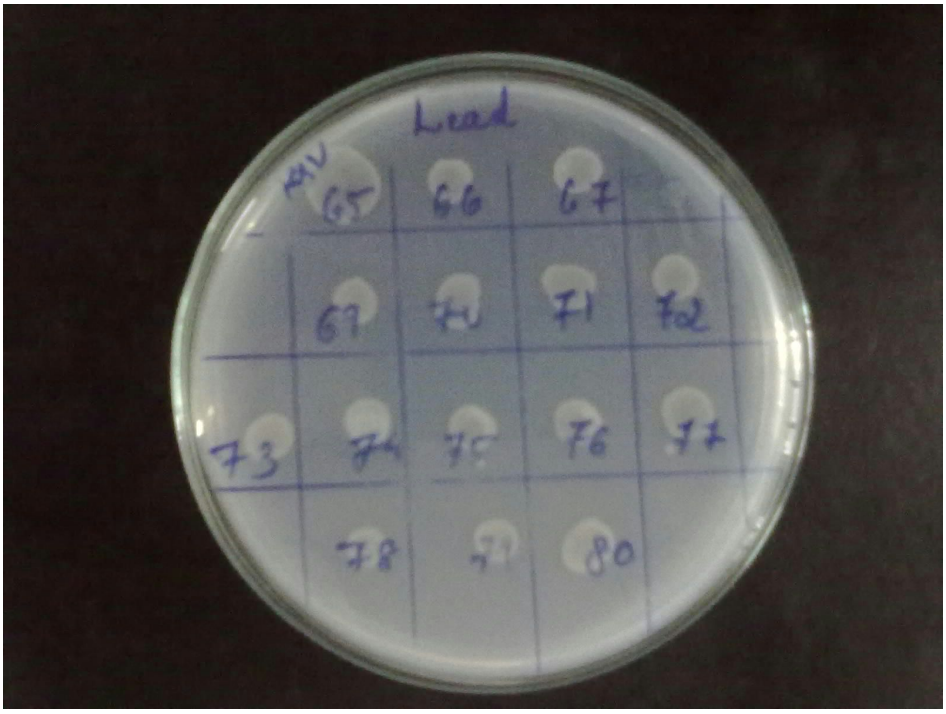


Figure 3.10: Images showing zinc resistant and lead resistant isolates from Arctic sediment and water





3.4 Discussion

Antimicrobial drug resistance among the bacteria has been reported from Arctic region (Goodman *et al*., 2008; Sjolund *et al*., 2008; Glad *et al*., 2010; Sudha *et al*., 2013). Most of the studies are confined to animal fecal indicator bacteria; there are not many studies exploring antibiotic resistance of microbes of natural habitats such as water, snow and sediment. A total of fifteen antibiotics belonging to class beta- lactam, extended spectrum beta- lactam, amino-glycosides, polypeptides, sulfonamides and quinolones were tested against 272 sediment and water bacterial isolates from the Arctic. Beta- lactam and extended spectrum beta- lactam antibiotic resistance are most frequently reported from wild environments (Glad *et al*., 2008; Sudha *et al*., 2013).

In the present study, sediment isolates were found to show more resistance towards antibiotics when compared to water isolates. Resistance was observed against all 15 antibiotics by sediment isolates. Ampicillin and amoxicillin resistance was observed to be highest with percentage resistance values of 67.5 % and 59.65 %. Ampicillin and amoxicillin are penicillins with beta-lactam rings. Cephalothin and cefpodoxime resistance were found to be high as well with a percentage resistance of 48.65 % and 42.31 %. Both cephalothin and cefpodoxime are first generation cephalosporins which are, in turn, the fourth generation beta-lactam antibiotics. Thus, it can be clearly concluded that sediment isolates showed high resistance to beta-lactam antibiotics. Similar observations were made by Sudha *et al*. (2015) from sediment and water isolates collected from the Arctic. In their study, the order of resistance was cefixime > cephalexin > ampicillin; all are beta-lactam antibiotic. Cefixime is third generation cephalosporin while cephalexin is first generation cephalosporin.

The most common mechanism of beta-lactam resistance among bacteria is via induction of beta-lactamase production. A distinct feature of beta-lactamase is that genes encoding them have high ability to adapt and evolve (Henriques *et al*., 2006). A study conducted to characterize the beta-lactamase genes of isolates from natural environment such as water revealed three families of beta-lactamases and are found to be similar to clinincal isolates (Henriques *et al*., 2006). The ability of Arctic bacteria to exhibit resistance towards different classes of beta-lactam antibiotics could be due to this high rate of adaptability of genes. Allen *et al.* (2008) carried out metagenomic study to detect genes for beta-lactamase production from Alaskan permafrost of

over 5000 years old. This study revealed several classes of beta-lactamase genes from the ancient soil and indicate that even without selection pressure, bacteria from pristine environment can harbor many resistance genes. An interesting observation during the current study was that ceftazidime, which is a third generation cephalosporin showed lower resistance than other cephalosporins tested (11%). Similarly, carbenicillin which is a carboxy-penicillin showed lower resistance than the other penicillins tested. Takeuchi *et al*. (2013) carried out a wide-spread study on resistance genes of different glacial environments around the globe. This study reported that beta-lactamase genes were present in Asian and African isolates while they could not find any beta-lactamase genes from Greenland and Alaskan glaciers.

The pattern of resistance in the sediment isolates were found in the order ampicillin > amoxicillin > cephalothin > cefpodoxime > tetracycline > sulphamethaxizole > carbenicillin > ciprofloxacin > ceftazidime = gentamycin > colistin > streptomycin = chloramphenicol > nalidixic acid > trimethoprim. These patterns reiterate the predominance of beta-lactam resistance in sediment isolates. It was also noted that low resistance was shown against nalidixic acid (quinolone), trimethoprim (dihydrofolate reductase inhibitor), colistin (polymyxin), ciprofloxacin (quinolone) and chloramphenicol. Quinolones are essentially synthetic antibiotics. Mode of action of quinolones is by preventing the DNA replication by blocking DNA from unwinding and duplicating. Two antibiotics tested in the current study were quinolones; nalidixic acid belongs to first generation quinolones and ciprofloxacin belongs to second generation quinolones. The first acquired antibiotic resistance gene against quinolones for which distant analogues exist in the wild was recently discovered in clinical isolates (Perron *et al*., 2015). Although quinolone resistance was low in the Arctic region it might serve as a reservoir of resistance in the pristine environment.

Tetracycline and sulfonamide resistance was found to be 31.89 % and 25.89 % respectively. Mode of action of tetracycline is by inhibiting protein synthesis by preventing the attachment of aminoacyl-tRNA to the ribosomal acceptor site. Tetracyclines are increasingly used in animal feed in many countries such as The United States of America (Chopra and Roberts, 2001). These wide spread use of tetracycline in agricultural and animal feed might deliver commensal and pathogenic bacteria from different ecosystems. This might increase the momentum of tetracycin-resistant bacteria in different ecological niches (Andersson and Hughes, 2012). Rahman *et al.*

(2008) reported tetracycline genes in intestinal flora such as enteric bacteria in endemic penguins (*Pygoscelis adeliae*) of Antarctica.

Sulfonamides are structural analogs to para-aminobenzoic acid (PABA) and act as a competitive antagonist. They inhibit utilization of PABA for synthesis of folic acid, an important metabolite during DNA synthesis. Middleton and Ambrose (2005) reported sulfonamide resistant *E. coli* from fecal matter of migratory Canada geese. Migratory birds are known to carry antibiotic resistant gene as reported by Sjolund *et al.* (2008).

Water isolates were found to have lower resistance than sediment isolates. Antibiotic resistance observed in water isolates could be expressed as three classes, first class contains antibiotics in which isolates showed high resistance including cephalothin (58.5 %), carbenicillin (41.16 %), cefpodoxime (36 %), ampicillin (33.33 %) and amoxicillin (10.83 %). The second class contains antibiotics such as colistin, ceftazidime, nalidixic acid, streptomycin, sulfamethaxizole and tetracycline which showed less than 10 % antibiotic resistance. The third class of antibiotics contains antibiotics against which no resistance was shown (chloramphenicol, ciprofloxacin and trimethoprim).

Cephalothin is a first generation cephalosporin antibiotic and resistance to this antibiotic is associated with beta-lactamase production. Workman and Farrar (1970) analyzed the correlation between beta-lactamase productions associated with cephalothin resistance in *Enterobacter*. This showed the relationship between combined resistance shown against cephalothin and oxacillin; moreover, similar to the present study, cephalosporins were hydrolyzed at a faster rate than penicillins. The current study contains many strains of *Enterobacter* which is a probable reason for showing similar pattern of resistance.

Resistance to first class antibiotics also reiterates the dominance of beta-lactam antibiotic resistance. Similar to sediment isolates low or no resistance was found towards nalidixic acid, ciprofloxacin, trimethoprim and chloramphenicol in water isolates. However, major difference in resistance was observed for sulfamethaxizole, tetracycline and ceftazidime. Only very low percentage of water isolates showed resistance against sulfamethaxizole (6.5 %), tetracycline (3.0 %) and ceftazidime (1.02 %) compared to high percentage resistance of sediment isolates against sulfamethaxizole (25.69 %), tetracycline (31.89 %) and ceftazidime (11 %). Resistance

pattern observed in water isolates were in the order cephalothin > carbenicillin > Cefpodoxime > ampicillin > amoxicillin > colistin > gentamycin = sulphamethaxizole > tetracycline > nalidixic acid > streptomycin > ceftazidime. Out of 15 antibiotics tested water isolates showed resistance against 12 antibiotics. Calculation of MAR Index of the heterotrophic bacteria revealed a total of 5 MAR index which revealed the diversity of resistance patterns within each MAR Index. It was interesting to note that the indices such as 0.2 and 0.266 had a diverse pattern within them showing upto 8 and 7 patterns respectively.

Antibiotic resistance was observed in different species of heterotrophic bacteria from Kongsfjord sediment and water. Species such as *Brachybacterium paraconglomeratum*, *Stenotrophomonas maltophilia*, *Enterobacter ludwigii* and *Enterobacter cancerogenus* showed resistance against 10 or more antibiotics. Strains of *Pseudomonas fragi* and *Pseudomonas koreensis* showed resistance against 7 antibiotics and 9 antibiotics respectively. Strains of *Micrococcus luteus* showed resistance against 8 antibiotics and *Bacillus thuringiensis* strains showed resistance against 7 antibiotics. Strains of species such as *Pseudomonas sublingiri, Bacillus flexus, staphylococcus cohinii* and *Halomonas boliviensis* showed relatively low resistance, only against 4-6 antibiotics.

Plasmid profiling results showed the following bands after gel electrophoresis ~1.3 Kb, ~1.8 Kb, ~3.0 Kb, ~6.0 Kb, ~9.0 Kb and ~24.5 Kb. Maximum of the isolates (62) was found to have plasmid of size ~24.5 and 53 isolates among them had a single plasmid of size ~24.5. Many high molecular weight plasmids are reported to harbor antibiotic resistance. Tsuchida *et al.* (2010) reported plasmids approximately 24 Kb which was found to contain antibiotic resistance genes. Watanabe and Sato (2002) reported *Enterobacter* sp. that has 100 Kb plasmids for resistance against Streptomycin, kanamycin, ampicillin, tetracycline and chloramphenicol. Antibiotic resistance and plasmid profiling of *Vibrio* sp. was carried out and showed plasmids of size between 9 to 123 Kb and 15 out of 51 isolates were found to have 1- 4 plasmids (Jun Li., 1999). Sudha *et al.* (2015) demonstrated various plasmids from *Pseudomonas* sp. isolated from Arctic sediment and water; these plasmids ranged between 5.2 Kb to 9.5 Kb. In the current study, a total of 11 patterns of plasmids were detected from all isolates tested. Among these patterns, ten isolates were found to have three plasmids and the patterns observed were (1.8Kb, 3.0 Kb, 6.0 Kb), (1.8 Kb, 3.0 Kb, 24.5 Kb), (1.8 Kb, 3.0 Kb, 9.0 Kb) and (1.8 Kb, 9.0 Kb 24.5 Kb)

Antibiotic resistance is often associated with heavy metal resistance and many comparative studies have proved this association (De Souza *et al*., 2006). Thus multiple antibiotic resistant strains were tested for heavy metal resistance. Out of all heavy metals tested mercury was found to be the most toxic. The range of tolerance was heterogeneous among different strains. 20 µg/l concentration of mercury was well tolerated by most of the isolates but increasing the concentration to 50µg/l was however toxic to all bacteria. Even though tube dilution method also expressed the same MIC, at 20 µg/l concentrations, growth was seen only after prolonged incubation and many isolates showed very scanty growth compared to positive control. Chelation, sorption, and complexation might be responsible for the higher resistance against the metal in solid medium. Mercury is a major pollutant in Arctic environment, and there is evidence of increasing concentrations of it in marine ecosystem over time (AMAP, 2011). This study also indicates that even though no increasing trend of atmospheric mercury was seen in recent years (Berg *et al.*, 2013), chelation and complex formation by mercury might result in biomagnification of metals in ecosystem.

Cadmium is monitored as the second most important heavy metal in Arctic ecosystem (AMAP, 2009). Cadmium had shown a minimum inhibitory value of 50µg/l but the size of inhibition zone was smaller for mercury and growth in tube dilution was significantly higher for cadmium than mercury confirming higher toxicity of mercury over cadmium. Even though cadmium is considered as a minor micronutrient; toxicity in higher amount, prolonged half life and deposition in live tissue have raised concern (Mehler *et al.,* 1987; Tang *et al.*, 2006; Chong *et al*., 2012). A very wide range of tolerance to cadmium (0.2 to 400mg/l) was reported for different strains of *Pseudomonas* (Mehler *et al.,* 1989, Hassen *et al.,* 1998, Raja *et al.,* 2006)

Heavy metals copper, zinc and selenium did not show significant difference in the MIC values in liquid media compared to solid media but the percentage of strains showing resistance was slightly lower in liquid media. No significant difference was observed for lead. Depending on the percentage of isolates showing metal resistance, the order of toxicity was found to be Hg > Cd > Cu > Zn > Pb > Se.

Selenium was the most tolerated of all heavy metals tested and no toxic effects to growth were observed up to 1000 µg/l concentration. While increasing the concentration to 2000 µg/l, 62 % of the strains were resistant and no growth was observed at 3000µg/l. An interesting observation

was noted during the non quantitative plate assay method, most isolates turned brick red color in selenium plates. This might be due to complex formation by selenium inside the cells and the study proposes the scope of using selenium resistant strains in remediation of metal polluted environment. There was no particular pattern observed among isolates towards selenium tolerance but interaction of selenium with mercury, forming mercuric selenides is of concern. Selenium monitoring for lichens and mosses are thus investigated thoroughly in Arctic because of this synergy between mercury and selenium (AMAP, 2009).

Copper and Zinc were moderately tolerated by the bacteria and minimum inhibitory concentration turned out to be 2000 µg/l. Many heavy metals are essential part of cellular metabolism and retaining suitable concentrations of metals such as copper while rejecting toxic metals like lead and cadmium was probably one of the toughest challenges of the living cells (Gatti *et al.*, 2000). Some bacterial isolates exhibit metal resistance to other metals (such as copper, lead etc.) associated with zinc resistance (Tada and Inoue, 2000; Xie *et al.*, 2010; Ceylan and Ugur, 2012).

Zinc is one the most abundant divalent cation found in the environment and also serves as a cofactor in microbial metabolism. Zinc tolerance results from immobilization, accumulation and detoxification assisted by zinc binding protein (CZC) (Mago *et al.,* 1994, Nies 2000). Even though copper and zinc had the same MIC; copper could be more toxic than zinc since at a concentration of 1000 µg/l, 78 % of the isolates were resistant to copper and 85.05 % were resistant to zinc. All isolates showed tolerance up to 1000 µg/l for lead. At a concentration of 2000 µg/l, 49 % of isolates was found to show resistance. Thus lead was better tolerated than zinc and copper, and similar to selenium there was no pattern observed in strains towards lead tolerance.

During the present study, tube dilution technique was found to be superior to plate diffusion technique for determination of MIC. There are similar reports on the superiority of tube dilution method for calculation of MIC (Jonas, 1989; Hassen *et al.*, 1998). Some metals such as mercury and cadmium as observed in the study form complexes, and cause chelation effect in the solid media, thus reducing the availability of the metals.

Spatial pattern and form of existence are critical in determining the nature and extend of impact of heavy metals. Spatial pattern exist for different heavy metals found within individual species

and tissues. Metals such as cadmium and mercury exist in particulate or complexes in Arctic (Moline *et al.*, 2008; AMAP, 2009; Berg *et al.,* 2009; AMAP, 2013). Even though atmospheric mercury concentration is not of concern, particulate forms might lead to toxic effects in ecosystems. Mercury appears to be converted to a particulate form, possibly in association with bromine oxide and is deposited onto the snow pack. The present study, poses some concerns in this scenario; a high selenium resistance and moderate mercury resistance were shown by most of the isolates. The particulate mercury thus formed might gain entry into the food chain through these resistant isolates. The possibility of Hg storage as nontoxic mercury selenides is already under investigation in whales (AMAP, 2009). Role of microbes in chemical weathering, organic and inorganic cycling is well established in Arctic (Skidmore *et al.*, 2005; Jiao *et al.*, 2010; Piontek *et al.*, 2012) thus the possibility of metal complexes moving up in the increasing food chain might lead to bioaccumulation (Rajbanshi, 2008). Levels of selenide mercury in some fishes (Like trout, Arctic beluga) are above action levels and health advisories have been issued (AMAP, 2009).

Most antibiotic resistant isolates tested for heavy metal resistance have shown multiple resistance to heavy metals as well. Some strains of *E. ludwigii* (KSV 20, KSC 34, KSC 38) showed resistance to heavy metals and up to six antibiotic. Strains of *Pseudomonas fragi* (KSV 16, KSC 18) also showed high resistance to antibiotics. Antibiotic resistance of the isolates tested positively correlated with metal resistance. Association of antibiotic resistance with metal tolerance was reported by many workers (Dhakephalkar *et al.*, 1994; Rosen, 1996, Verma *et al.*, 2001, Raja *et al.*, 2006). Bacterial resistance may be due to the presence of R-plasmid containing genes for both antibiotics and heavy metal resistance. (Timoney *et al.*, 1978; Calomiris *et al.*, 1984, Saikia *et al.*, 2008).

A strain of *E. ludwigi* KSV 30 showed resistance to 6 antibiotics tested and a general resistance tendency was shown towards β-lactam antibiotics by most isolates. Metal tolerance may also be related to efflux pumps and production of capsular polysaccharides usually by *Enterobacter* group of organisms, which can combine with metals to protect themselves from the toxicity of metals but more often it is related to plasmids (Bitton and Freihofer, 1978, Saikia *et al.*, 2008). A positive correlation of antibiotic and metal resistance is also observed during the present study. Thus the possibility of developing assays by incorporating tolerance studies using bacteria in

combination with higher organisms may serve as a better model to study ecological effects of heavy metal pollution and antibiotic resistance in wild habitats.

It can be concluded that antibiotic resistance exists in pristine and wild environments of sediment and water. Resistance of sediment isolates was found to be much higher when compared to water isolates. In both sediment and water isolates, resistance against beta-lactam antibiotics was dominant. Thus the study reiterates the existence of drug resistance in wild environments without much selection pressure. Resistance towards quinolones and aminoglycosides was found to be very low or nil among the heterotrophic bacteria. Most of the multiple antibiotic resistant isolates tested were found to be positive for heavy metal tolerance. Mercury was found to be the most toxic of all heavy metals tested followed by cadmium. The study calls for continuous monitoring of antibiotic and metal resistance in the Arctic region. Investigations on molecular mechanisms of resistance will shed more light on our understanding of origin of resistance and its role in wild ecology.

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Chapter 4
Enzyme profiling of heterotrophic bacteria from Arctic sediment and water

Contents:

Seawater and sediment present a wide variety of carbon, nitrogen and various different energy sources that could support microbial life. These nutrients and energy sources are present in various complex forms in the natural environment causing their accession difficult to the microbial population. Heterotrophic bacteria produce and exude extracellular enzymes to access these polymeric compounds. The complex structures are degraded to form simpler molecules of monomers and oligomers which are recognized by cell wall receptors. The size of molecules that can be transported across cell membrane into the cytoplasm is approximately 700 Da; making extracellular enzymes an essential component for the survival. The ability of heterotrophic bacteria to produce extracellular enzymes varies within the community and often responsible for microbial diversity and structure.

Microbial enzymes are a significant part of many industries and play a key role in metabolic catalysis. Microbial enzyme market has shot up in the past few decades and over 500 industrial products are made using microbial enzymes alone. Global demand for development for green

technologies has expanded the arena of enzyme research. Enzyme production using microbes are superior to using other organisms due to many advantages such as production in bulk, fast growth rate, low consumption of area, waste management etc. Prior to the enzyme era, most of the catalytic reactions were carried out in harsh conditions such as high temperature, pH, strong organic solvents and high pressure. Most of the industrially used enzyme catalyzed reactions are carried out in mild conditions compared to non enzymatic reactions. Use of cold active enzymes for industrial productions has proved to reduce the energy consumption further. In addition to this, enzyme catalyzed reactions could be modified to increase its efficiency by genetic and chemical modifications.

4.1 Review of literature

4.1.1Functional differences in microbial communities

Heterotrophic microorganisms produce extracellular enzymes that act up on the organic matter in the marine environment to acquire carbon, nitrogen and other mineral nutrients. The fate of organic matter in sediment and water is largely determined by the heterotrophic degradation. Extracellular enzyme activity is in turn depends on a number of physical and chemical parameters such as availability of nitrogen, C/N ratio, temperature, pH and oxygen availability (Fabiano and Danovaro, 1998; Schnecker *et al.*, 2014). Degradation of organic matter depends upon the composition of microbial communities which in turn depend on their enzymes. Thus, a change in microbial community composition due to climate change would possibly affect the biodegradation of organic matter, hence the global carbon cycling (Topper, 2012).

Determination of microbial processes and enzyme activities in their natural environment is difficult due to interactions within the microbial communities and interactions with different factors (Schnecker *et al.*, 2014). However, indirect measurement of microbial activity could be performed by substrate utilization assays. Detection of organic matter degradation by monomer-fluorophore bond method is gaining interest in which change in substrate molecular weight is measured directly (Teske *et al*., 2011).

Another approach for determination of activity is by using genomic investigations to identify functional genes for enzyme production. This method is still in the budding stage and currently presents some limitations. These limitations include gene sequences in the data base represent

insufficient microbial diversity of natural environment, correct assignment of gene function is difficult and expression of identified sequences often yield different results (Cottrell *et al.*, 2005; Hardemn and Sjoling, 2007).

Organic matter from sediment and water differ in physical and geochemical parameters and hence the microbial populations that act up on them also differ. Similarly, significant difference in extracellular enzyme production in sediment and water is also reported. Heterotrophs in the organic rich sediment produce broader and higher range of enzymes compared to heterotrophs in water column. It was reported that compared to temperate region polar waters contain low extracellular enzyme activity and higher sedimentation rates. This phenomenon results in sinking of organic matter forming nutrient rich sediments (Vetter and Deming, 1994; Cattaneo-Vietti and Fabiano, 1998; Fabiano, 1998). A contrasting observation was made by Arnosti *et al*. (2009) wherein a shallow water column showed similar enzyme activities compared to underlying highly permeable sandy sediment.

Dissolved organic carbon (DOC) from primary production consists of sugar and amino acids which are easily accessible. Although this DOC makes only less than 1% of the total organic matter, it has a high turnover in the marine ecosystems (Skoog *et al*., 2005; Nagata *et al*., 2008). Dissolved organic matter find its' way into Arctic Ocean in various ways and mainly influenced by terrestrial run off which accounts for 150 µM of average DOC.

In addition to organic matter degradation, heterotrophic bacteria play a significant role in cycling of minerals such as phosphorus. Phosphorus is considered as a major limiting nutrient for growth of organisms since they are usually found in inaccessible bound form in sediment**s** as absorbed to silt and clay. Different forms of phosphorus are solubilised by acid hydrolysis or enzymatic hydrolysis. It was reported that extracellular alkaline phosphatase degrade these inaccessible form of phosphorus into orthophosphate in sediment and seawater (Paytan, 2007).

4.2.2 Molecular mechanism of action of cold active enzymes

Permanently cold environments exert various challenges that an organism must overcome to grow and reproduce in near zero or sub zero temperatures. The physiochemical pressures include reduced rate of biochemical reactions, decrease in molecular diffusion rate, increased water viscosity, reduced fluidity of membranes, ice crystal formation inside the cytoplasm, persistence

of toxic metabolites and increased solubility of gases. The organisms thriving in cold habitats have developed many adaptive measures to circumvent such adverse effects. Ranging from membrane structure, transport of solutes, to nucleic acid and protein structure all cellular components and functions are subjected to cold adaption and the enzymes are the major target for such adaptation.

The most important adaptation found in all cold adapted enzymes is the flexibility of the active site of the enzymes and heat labile nature. A number of modification lead to flexibility of active sites including reduction in inter and intra specific interactions, reduction in proline and arginine residues, increased glycine residues and a hydrophobic core. Advancements in proteomics and transcriptomics led to better understanding of protein synthesis and folding of cold adapted enzymes and revealed that the rate limiting step for growth in cold environment is protein synthesis. To cope with this limitation, cellular machinery has adapted in different ways such as stimulation of synthesis of ribosomal proteins and RNA chaperons at low temperature, high number of rRNA and tRNA, over expression of RNA helicase, over expression of ribosome binding trigger factor (TF), under expression of some heat shock proteins and reduced proline isomerization during folding of proteins (Feller and Gerday, 2003; Lee *et al*., 2004; Médigue *et al*., 2005; Methé *et al*., 2005; Rodrigues *et al*., 2008; Feller, 2010; Ting *et al.*, 2009).

Various cold active enzymes have been studied in the past two decades to analyze the 3D crystalline structure of these enzymes. Investigation on structure of α-amylase, protease, triose phosphate isomerase, citrate synthase and malate dehydrogenase showed that adaptations are unique to each enzyme. For example cold adaptation of an Antarctic α-amylase showed that mutations at four sites bring about changes in melting temperature of the enzyme. Most striking similarity of cold active enzymes is that all side chains involved in catalytic mechanism are strictly conserved. Comparison of cold active enzymes with its mesophilic counterparts revealed several structural modifications at the catalytic site.

Jung *et al* .(2008) reported that catalytic cleft opening of α-amylase in psychrophilic enzymes are wider and achieved by replacing bulky side chains with short side chains and specific orientation of loops to achieve broadness. Several deletions at the loop region in cold active citrate synthase were also reported (Russell *et al*., 1998). These conformational changes result in better accessibility, accommodation of substrates at lower energy cost, and reduction in activation

energy to form enzyme substrate complex (Raeder *et al*., 2010; Feller, 2013). Differences in electrostatic potential around active site were also studied and compared with mesophilic homologues. All the Cold active enzymes were found to have discrete distribution of charged residues which help in better enzyme substrate contact and orientation (Garfe *et al*., 2000; Papaleo *et al*., 2007; Olufsen *et al*., 2008; Raeder *et al*., 2010).

It is generally known that low temperature slow down the reaction rate, molecular movement and hence the protein functions. K_{cat} is the catalytic constant defined as the rate at which maximum number of substrate molecules converted to products per unit time per active site. K_{cat} is exponentially depended on temperature at which the reaction is carried out and related by the following equation

κ = transmission coefficient, K_B= Bolzmann constant (1.38 × 10^{-23} J/ K), h = planks constant (6.63 × 10−34 J s), R= universal gas constant (8.31 J K−1 mol−1), and = free energy of activation

Thus it is clear that biochemical reactions catalyzed by enzymes are directly proportional to temperature and in case of mesophilic enzyme catalyzed reactions, a drop in temperature from 37 °C to 0 °C will reduce the activity to 20 to 80 times of the original activity. Psychrophiles have enzymes that have high *specific activity* (tenfold higher) at low temperature to circumvent slow activity rates at low temperature. It also causes a shift in the optimal activity of cold active enzymes to low or ambient temperature which is reflected in their moderate heat inactivation compared to mesophilic enzymes.

Comparative protein unfolding studies of psychrophilic, mesophilic and thermophilic enzymes showed that active site of psychrophilic enzymes are extremely sensitive to heat inactivation. Psychrophilic enzymes were found to have lost the activity even before unfolding of the entire protein structure takes place while mesophilic and thermophilic enzymes have lost their activity after unfolding of 3D structure. Multi subunit cold adapted enzymes were found to contain heat labile catalytic subunit and stable non catalytic domain similar to mesophilic enzymes (Qian *et al*., 1994; Fields and Somero, 1998; Lonhienne *et al*., 2000; Suzuki *et al.,* 2005; Chiuri *et al*., 2009).

Cold adapted enzymes have evolved in such a way that free energy of activation is low reducing the height of energy barrier. For all enzyme catalyzed reactions, enzyme and substrate interact with each other to form enzyme-substrate complex (ES). In their activated state ES* eventually break down into enzyme and product. The difference between ground state ES and activated ES* is defined as free energy of activation. When the free energy of actvation is low the rate at which reaction proceeds becomes higher. Cold adapted enzymes have a lower affinity towards their substrates when compared to mesophilic enzymes and this result in low energy requirement for ES* formation (Feller *et al*., 2013). Another strategy used by cold active enzymes to reduce could be explained by enthalpy and entropy contributions from the equation

ΔH^* and ΔS^* represent change in enthalpy (enthalpy of activation) and change in entropy during the reaction and clearly influence $K_{cat.}$ When enthalpy of activation is reduced, the reaction proceeds at a faster rate. Many studies on cold active enzymes have proved to have a low enthalpy of activation (Thomas and cavichiioli, 2000; Collins *et al*., 2003; Georlette *et al*., 2003; Altermark *et al.*, 2007). Some studies suggest that $T\Delta S^*$ of cold adapted enzymes are large and negative contributing to increased reaction rate. Increased active site flexibility of cold adapted organisms corresponds to broader distribution of conformational states of ES. Reduction in disorder between ground state with loose conformation and compact transition state ultimately result in large and negative entropy (Lonhienne *et al*., 2000; Feller, 2013).

4.1.3 Prospecting of cold active enzymes

Apart from making essential contributions to nutrient cycling and organic matter decomposition cold active enzymes are widely used for various industrial applications. The potential use of cold adapted enzymes are mainly attributed to 1) High specific activity at low concentrations of substrate thereby reducing the production cost 2) activity at low or ambient temperature making heating process unnecessary for domestic or industrial applications 3) low thermal stability at elevated temperatures corresponding to effective heat inactivation at moderate heat application 4) selective inactivation in reaction mixture by lower heat application. Cold adapted enzymes also have some additional advantages such as they are environmental friendly due to

biodegradability, improved use of raw material and reduced waste products (Margesin *et al*., 2005; Loperna *et al*., 2011).

Proteases

Proteases are essential enzymes used by all organisms to cut long sequences of amino acids into short fragments and regulate physiological processes (Sheng, 2011). The quality and quantity of proteases in application fields depend upon the kind of industries they are used. Proteases used in detergent and food industries are used in crude form and in bulk whereas in pharmaceuticals they are used in small quantities and in highly purified form. Out of all industrial enzymes used in the market, proteases have the largest production share of 60 % (Dastager *et al*., 2008). Proteases are considered as one of the most important enzyme in the industry since it is a convenient tool to remove protein whenever required (Rao *et al*., 1998). Cold adapted enzymes with low temperature optimum are specifically beneficial for some industries such as food processing, detergent and biotransformation of chemicals (Margesin and Schinner, 1994; Kuddus and Ramteke, 2008).

Cold active proteases have been reported from cold environments belonging to various genera such as *Alcaligenes, Azospirillum, Bacillus, Candida, Flavobacterium, Pseudomonas, Serretia, Stenotrophomonas* and *Vibrio* (Ray *et al*., 1992; Kristjansson *et al*., 1999; Kaur *et al*., 2001; Chessa *et al*., 2004; Vazquez *et al*., 2004; Kobayashi *et al*., 2007; Patil *et al*., 2011; Tariq *et al*., 2011; Zhang *et al*., 2011) Maximum number of cold active proteases was reported from *Pseudomonas* and *Bacillus*.

Arctic habitats represent a potential source for isolation of cold active protease producing microorganisms. Kim *et al.* (2011) isolated 48 strains of cold adapted protease belonging to *Pseudoalteromonas*, *Flavobacterium, Pseudomonas* and *Arthrobacter* from Arctic marine and terrestrial habitats. Protease producing bacteria isolated from biofilm of a floating tire from Arctic water showed optimum activity at 10°C and represented three species *Pseudoalteromonas elykovii, Exiguobacterium oxidotolerum and Pseudomonas jessenii* (Lee *et al*., 2005). Compared to other permanently cold habitats such as Antarctic and Alpine region, Arctic region is under explored for studies related to cold adapted protease producers.

α-Amylase

Amylases belong to the class hydrolases that cleaves the 1, 4-α-D- glucosidic linkages between adjacent glucose subunits. The specificity of the bond that is cleaved by *α*-amylase depends upon the source and kind of enzyme. Cold adapted amylase from *Alteromonas haloplanctis* was studied in detail and its 3D structure was deduced (Feller *et al*., 1992; Aghajari *et al*., 1996). Most of the structural features of the enzyme were found to be similar to mesophilic amylase. However, three distinct domains and characteristic ion binding sites were found in cold adapted amylase. Most of the cold adapted *α-amylase* is produced by submerged fermentation and was found to be influenced by several nutritional and physiochemical factors.

Reddy *et al.* (2009) reported *α-amylase* from *Bacillus* sp strains from Arctic glacier, Midtre Lovenbreen. Some of the unidentified *Gammaproteobacteria* isolated from Arctic Ocean were also found to have *α-amylase* activity (Tatiana *et al*., 2004). The enzyme distribution study of several culturable bacteria from Arctic Svalbard also showed *amylase* producers (Srinivas *et al*., 2009).

Similar to protease, Antarctic region is reported to have higher number of studies on *α-amylase* compared to Arctic region. Cold adapted *α-amylases* are most widely used in detergent industry, textile industry, bioremediation and for production of maltose. α-amylases have promising applications in textile industry for desizing grey fabric, strengthening of fabric and texture improvement. α-amylases are used in the food industries for the preparation of maltosugar syrup from sweet potato, potato and cassava. Addition of maltose lowers the freezing point of water and is used as a replacement for sucrose in the syrups (Aiyer, 2005).

*Lipase*s

Lipases (Triacylglycerol acylhydorlases, EC3.1.1.3) catalyze reactions such as partial or complete hydrolysis of triacylglycerol and lipid esterification, transesterification and interesterification (Colla *et al.*, 2010). These varied catalytic properties generated interests in high production of lipases in various industries for their applications as additives in food industry, fine chemical synthesis, pharma, detergents, cosmetics, leather processing, biodiesel production, biomedical assays and waste water treatments (Elibol and Ozer 2000; Kamini *et al.*,

2000; Burkert *et al.,* 2004; Colla *et al.*, 2010). It was suggested that production of industrially useful chemicals made from fats and oils could be replaced by enzymatic processes at milder conditions and will be energetically viable.

Despite cold active lipase producing bacteria and yeast are reported from around the globe, production of cold active lipases is under explored (Joseph *et al.*, 2007). The ability of cold active enzymes to catalyze reactions at low or moderate temperature represents industrial and biotechnological potential (Gomes and Steiner, 2004). Cold active enzymes have additional advantage that they can be inactivated by treatment at low temperature for short time periods (Margesin *et al.*, 2002) reducing energy consumption and cost.

Cold active lipases are the most important enzyme in pharmaceutical industries for their application in the preparation of enantioselective compounds, digesive aids and emulsifiers (Margesin *et al*., 2002). *Candida antarctica* and *Pseudomonas* sp. have been reported for the preparation of N-actylamines which is an important component in pharma preparations and pesticides (Smidt *et al*., 1996). Cold adapted lipase is widely used in food industries for flavour improvement, synthesis of lipophilic antioxidants, meat tenderization and lean meat preparation (Tan *et al*., 1996; Joseph *et al*., 2007). In leather and tanning industry lipases are used for removal of fats during the dehairing of hides and skins. Detergents and dish washers used for domestic application contain cold active lipases for the removal of fats and oils. Lipases that are active in low or moderate temperature hydrolyze fatty stains in tap water effectively and thus reducing the energy requirement. In addition to this, colour and texture of the fabric is protected from harsh treatments of heat and chemicals (Feller and Gerday, 2003; Jeseph *et al*., 2007). Environmental applications of cold active lipases are not utilized effectively, it is suggested that lipases that are active at low temperature could be used for bioremediation of fat contaminated cold environment and waste waters (Buchon *et al*., 2000).

β-galactosidase

β –D-galactoside galactohydrolase (EC 3.2.1.23) commonly known as *β*-galactosidase hydrolyses lactose into glucose and galactose by cleaving β 1, 4–D- galactosidic linkage. Cold active β-galactosidase producing organisms have been isolated from various environments including polar region. Hoyoux *et al.* (2000) identified an Antarctic marine bacterium,

Pseudoalteromonas haloplanktis producing β-galactosidase which was found to be active at 4 °C. An Arctic bacterium *Alkalilactibacillus ikkense* was reported to have 60 % activity at 0 °C and optimum activity was found between 20-30 °C at pH 8 (Schmidt and Stougaard, 2010).

Lactose intolerance is a common medical condition affecting approximately 65 % of world population. Thus, β-galactosidase finds their maximum utilization in dairy industry for the reduction of lactose in the milk. Lactose crystallizes faster and tends to absorbs odours and flavours while refrigerated, thus reduction of lactose in milk improves the quality of milk and milk products during storage. β-galactosidase is also used in dairy industry for various other applications such as improvement of cheese ripening, enhanced quality of dairy products during shipment, and hydrolysis of whey with β-galactosidase to produce a sweet syrup which is used in baking and soft drink industries.

Chitinase

Chitin is the second most abundant polysaccharide in the world and the enzyme chitinase play a significant role in cycling of this carbon source. Bacterial extracellular chitinase hydrolyze β-1,4 linkage in chitin between N-Acetylglucosamine residues. Two strains of bacteria isolated from Arctic Ocean was found to have chitinase gene that were found to be similar to ChiA and ChiB gene of *Arthrobacter* isolated from Antarctic. The similarity in heat labile nature of the enzymes indicates their functional similarity in these biogeographical locations (LeCleir *et al*., 2004).

Chitinase found its application in various fields such as food industries, production of fine chemicals and medicine. Ramli *et al.* (2011) suggested that chitooligosaccharide produced by cold active chitinase could be used as biocontrol agents of microbial spoilage of refrigerated food and as a mycocide of phytopathogenic fungi in cold environments.

Alkaline Phosphatase

Alkaline phosphatase is a hydrolytic enzyme which removes phosphate groups by dephosphorylation of a variety of molecules. Phosphate is a limiting nutrient in many environments since phosphorus lack free existing state and often depends on the microbial release of phosphates. Thomas and Dieckmann (2002) reported existence of alkaline phosphatase producing bacteria in sea ice from Antarctic. Some other reports also suggested the presence of

cold active alkaline phosphatase producing bacteria from polar region indicating the wide distribution of this enzyme (Kobori *et al*., 1984; Koutsioulis *et al*., 2008).

Increasing evidences of use of alkaline phosphatase in molecular biology indicate the possible uses of alkaline phosphatase in industries. Alkaline phosphatase is currently used in molecular techniques for dephosphorylation and rephosphorylation of DNA vectors. Cold active alkaline phosphatase that could be deactivated with mild heat present an alternative to minimize nucleic acid loses during the subsequent steps. New England Biolabs (USA) has already proposed the market of cold active alkaline phosphatase from Antarctic strains under the name Antarctic phosphatase. However, further research is necessary for the optimization and development of bacterial cold active alkaline phosphatase to be used as a tool in molecular biology techniques.

Xylanase

Xylan is a component of hemicelluloase and Xylanase catalyzes the hydrolysis of β-1,4- D glycosidic bond. Most of the catalytic action of the enzyme Xylanase on Xylan yields xylotriose and xylotetrose. Xylanase production is reported from many marine habitats since rhodophytes and chlorophytes were found to be rich sources of xylan (Arnosti *et al*., 1998). Xylanase activity was reported from polar region by Humphry *et al.* (2001) from a marine Antarctic bacterium *Flavobacterium frigidarium*. Another polar bacterium *Pseudoalteromonas haloplanktis* was found to show optimum xylanase activity at 25 °C (Collins *et al*., 2002).

It is proposed that hydrolysis of lignocelluloses by cold active xylanase could be useful in manufacture of liquid fuel, single cell proteins, solvents and other chemicals (Wong *et al*., 1988). Xylanase is used in food and soft drink industries for clarification of juice and juice products. Other industries using xylanase enzyme for lignocellulose degradation include paper and pulp industry, bread making and animal feed (Polizeli *et al*., 2005; Collins *et al*., 2006).

Cellulase

Cellulose is the most abundant biomass on Earth and the principal component of cell wall of the plants. Cellulase enzyme cleaves β-1, 4-linkage and break down the carbohydrate to glucose subunits. Cold active cellulases are reported from permanently cold environments such as Polar water, sediments, sea ice and tundra. Nineteen strains of cold active cellulase producing

Pseudoalteromonas sp. were isolated from Arctic sea ice (Yu *et al*., 2009). Violot *et al* (2005) identified strains of *Pseudoalteromonas haloplanktis* from Antarctic marine environment and its structure was deduced by X-ray diffraction and scattering studies.

Cellulases are currently used in detergent formulations, extraction of fruits and vegetable juices, starch processing, textile for fiber finishing and denim finishing (Cammasola and Dillon, 2007). In pulp and paper industries, cellulases are used for fiber improvement and drainage clearance (Cherry and Fidant, 2003; Sethi *et al*., 2013). Further investigations on enzymes from cold adapted microbes will expand the arena of applications of cold active enzymes.

4.2 Materials and Methods

4.2.1 Enrichment of heterotrophic bacteria

Heterotrophic bacteria isolated from Arctic sediment and water samples were purified on nutrient agar plates. Pure cultures were selected and inoculated into ZoBells Marine broth (ZMB). These tubes were incubated at 20 °C for three days and were used as fresh cultures for enzyme assays.

4.2.2 Detection of enzyme activity

Protease assay (Vijayaraghavan and Vincent, 2012)

Heterotrophic bacterial isolates were spot inoculated onto nutrient agar plates containing 1% gelatin as protein source. Plates were incubated at 20 °C for three days. Following the incubation period, plates containing the cultures were flooded with 20 % mercuric chloride solution. Positive isolates will produce a clearance zone around the colonies.

Amylase assay (Fossi and Tavea, 2013)

Cultures were spot inoculated onto nutrient agar incorporated with 1% starch. These plates were incubated at 20 °C for three days. After the incubation period, plates were flooded with Grams' iodine solution. The plate turns purple in color as iodine binds to starch and positive reaction for amylase production is indicated by lack of purple color around the colonies.

Lipase assay (Yuan *et al*., 2011)

Bacterial isolates were spot inoculated onto nutrient agar with 1% tributyrin and Tween 80. Plates were incubated at 20 °C for five days. Positive reaction was indicated by change in the opacity (clearance) of the medium around the colonies.

β – galactosidase

β – galactosidase production was tested (as per instructions of HiMedia, Mumbai) using a synthetic compound O-nitrophenyl β-D galactoside (ONPG). β – galactosidase cleaves this artificial substrate to liberate galactose and O-nitrophenol which is yellow in color. ONPG discs were placed in sterile micro-centrifuge tube, and added 100 µl of 0.85 % sodium chloride. A loopful of inoculum was added to this micro-centrifuge tube and incubated at 20 °C and within 6hr of incubation change in the color of the solution was noted. Late lactose fermenters were detected after 24hr of incubation.

Alkaline phosphatase

Two sets of nutrient agar plates were prepared by incorporating 1 % solution of phenolphthalein diphosphate at pH 7 and pH 9. Heterotrophic isolates were spot inoculated and incubated at 20 °C for three days. The plates were then inverted over 10 % ammonia solution. The colonies producing phosphatase turn pink in color which was taken as positive reaction.

Xylanase

Xylanase agar was prepared and heterotrophic bacteria were spot inoculated onto the plates and incubated for five days at 20 °C. The plates were flooded with 1 % aqueous congo red solution for 1 hr followed by de-staining with 1M NaCl. The positive isolates produced yellow zone around the colonies.

Cellulase

Nutrient agar was prepared and 1 % carboxymethyl cellulose was incorporated into the media. Bacterial strains were spot inoculated onto the plates and incubated for five days at 20 °C. The

plates were flooded with 1 % aqueous congo red solution for 1 hr followed by de-staining with 1M NaCl. The positive isolates produced yellow zone around the colonies.

Chitinase

Nutrient agar was prepared and 1.5 % of colloidal chitin was incorporated. Heterotrophic bacteria were spot inoculated onto the plates and incubated for five days at 20 °C. Positive reaction was indicated by change in the opacity (clearance) of the medium around the colonies.

Pectinase

Nutrient agar medium was prepared and 1 % pectin was added to the media. Pure cultures were spot inoculated onto the media and incubated at 20 °C for five days. After the incubation period, potassium iodide solution was added onto the plates. Clearance zone around the colonies were taken as positive reaction for pectinase production.

Urease

Urease media was prepared by adding 2 % urea and phenol red as indicator. Cultures were inoculated onto the agar and incubated at 20 °C for 24 hrs. Positive result was indicated by a change in color from pink to yellow. Urea gets hydrolyzed to ammonia and carbon dioxide which causes a change in pH, hence the color change.

4.3 Results

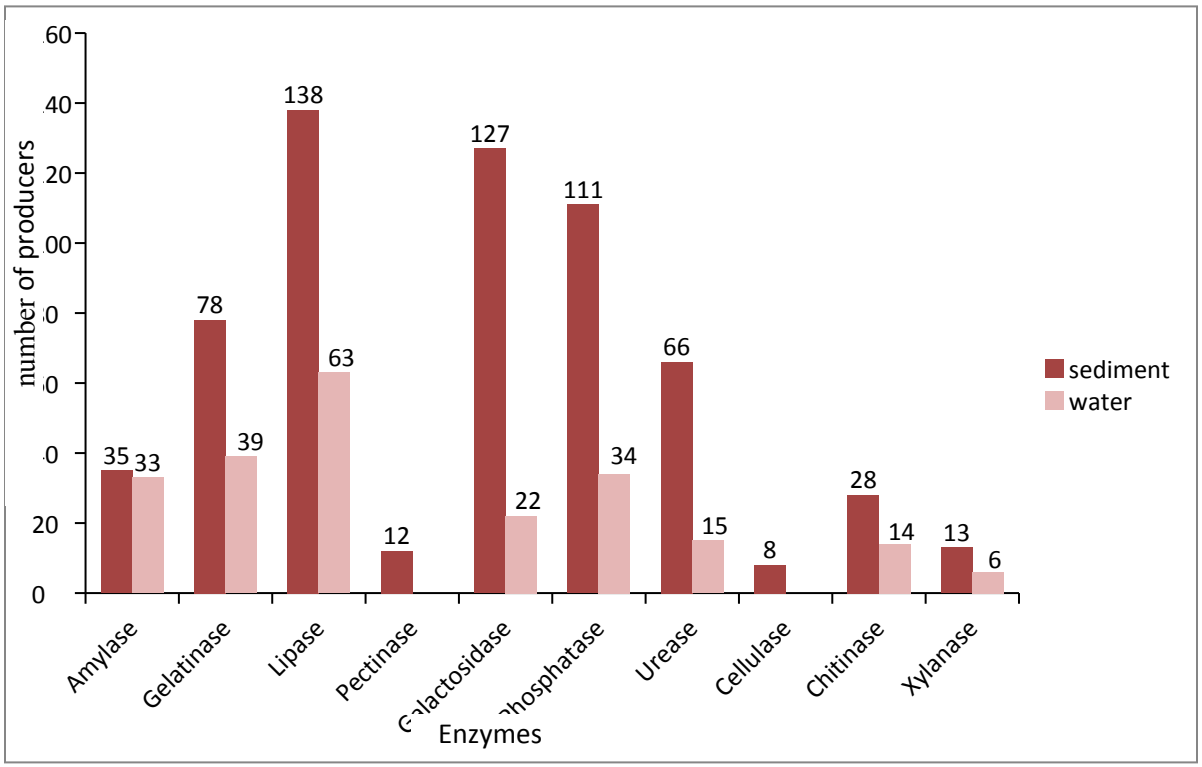
4.3.1Distribution of enzyme producing bacteria in sediment and water

In the current study, hydrolytic enzyme producing bacteria were found to be more in Arctic sediment than in water. Amylase, lipase, protease, β-galactosidase and phosphatase producers were found in substantial numbers in both sediment and water whereas cellulase and pectinase producers were present only in the sediment.

Figure 4.1 shows the distribution of various enzymes producing heterotrophic bacteria in sediment and water of Kongsfjord. Sediment isolates showed higher production of enzymes that water isolates. This difference is more predominant for enzymes such as lipase, β-galactosidase,

gelatinase, phosphatase and urease. Pectinase and cellulase producting bacteria were found only among the sediment isolates. Station wise analysis of number of enzyme producing bacteria revealed that station 4 contains highest number of enzyme producers.

Figure 4.1: Distribution of various enzyme producing heterotrophic bacteria in sediment and water of Kongsfjord.

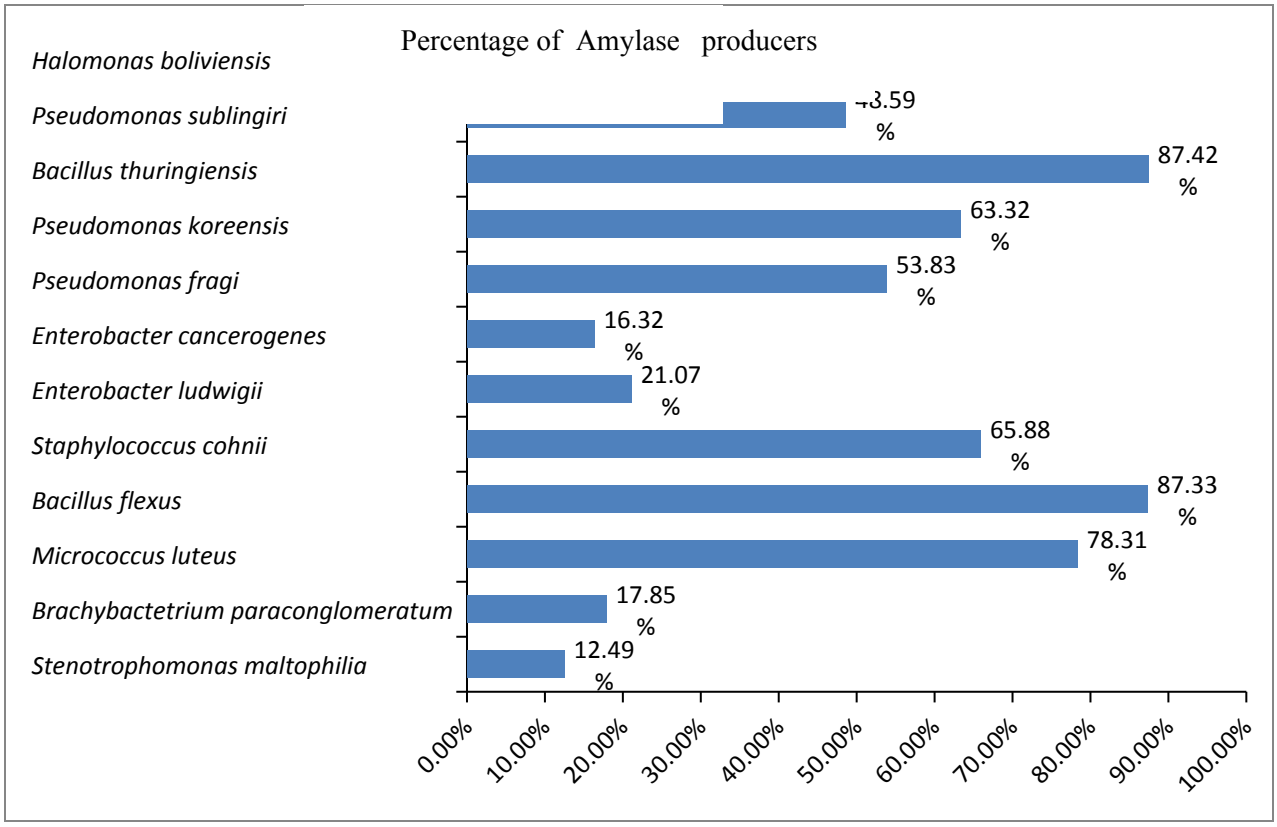


4.3.2 Percentage distribution of enzyme producers in different genera

Distribution of amylase producing bacteria was found to be similar in water and sediment sample. In sediment the number of amylase producers was observed to be 35 and in water it was found to be 33. Gelatin was found to be degraded by 78 sediment isolates and 39 water isolates, clearly showing the dominance of sediment isolates in protease production. Lipase enzyme was produced by 138 isolates from sediment and 63 isolates from water and it was found to be the most widely produced enzyme among all the enzymes screened. Amylase, protease and lipase had similar distribution in sediment and water.

Figure 4.2 represent the percentage distribution of amylase producing bacteria from sediment and water. Strains of *Bacillus thuringiensis, Bacillus flexus* and *Micrococcus luteus* showed comparatively high production of amylase since more than 75 % of the strains produced amylase. Percentage of strains producing amylase was found to less than 20 % for species such as *Halomonas boliviensis, Enterobacter cancerogenus, Brachybacterium paraconglomeratum* and *Stenotrophomonas maltophilia.*

Figure 4.2 Percentage of amylase producing strains of heterotrophic bacteria from Kongfjord sediment and water



Gelatinase production by strains of different species is depicted in the Figure 4.3. More than 75% of the strains of 4 species namely, *Bacillus flexus*, *Bacillus thuringiensis, Staphylococcus cohinii* and *Micrococcus luteus* was found to produce gelatinase. Strains of species such as *Pseudomonas koreensis, Enterobacter cancerogenus* and *Pseudomonas fragi* showed comparatively low gelatinase production (<20 %).

Figure 4.3: Percentage of gelatinase producing strains of heterotrophic bacteria from Kongfjord sediment and water

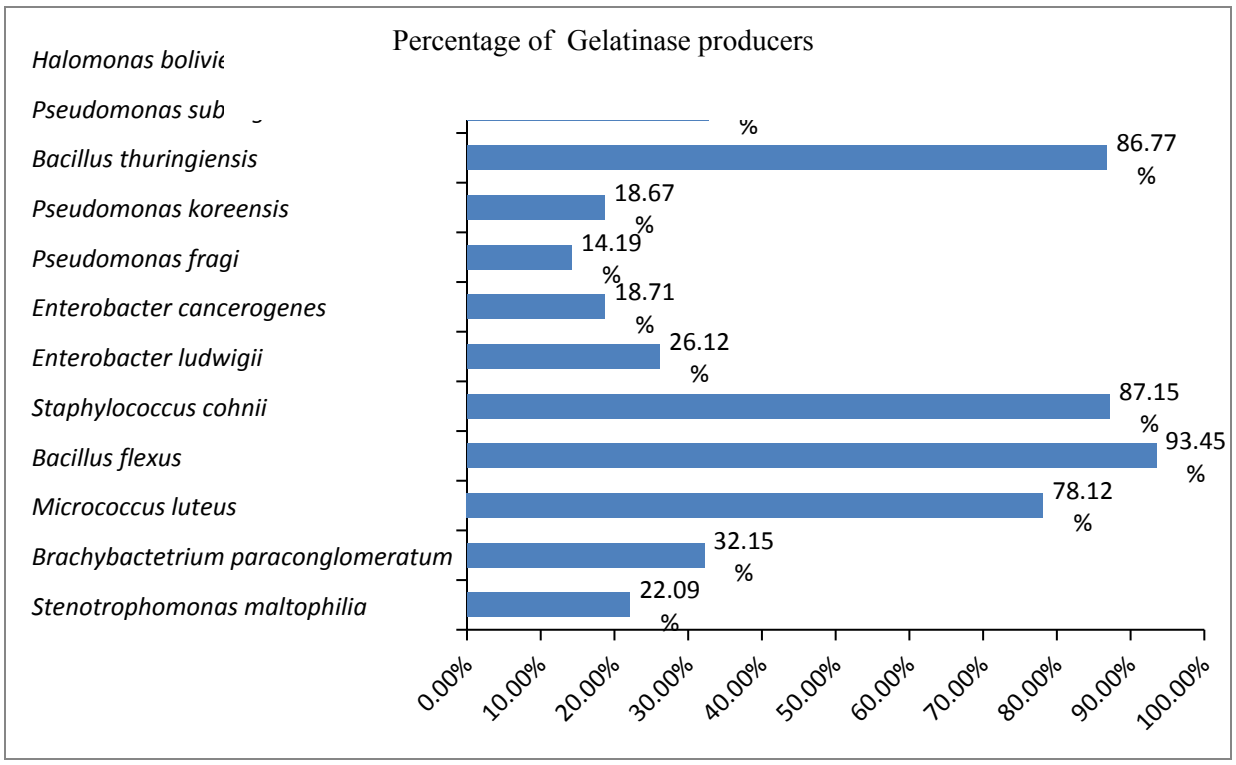


Figure 4.4 shows percentage of lipase producing strains from Kongsfjord sediment and water. It was noted that more than 30 % of the strains of all species produced lipase enzyme and strains of species such as *Halomonas boliviensis, Pseudomonas koreensis, Pseudomonas fragi, Enterobacter cancerogenus, Enterobacter ludwigii, Micrococcus luteus, Brachybactetrium paraconglomeratum* and *Stenotrophomonas maltophilia* were found to have >70 % lipase producers. Strains of *Enterobacter cancerogenus* and *Enterobacter ludwigii* showed >90 % lipase producers.

Figure 4.4: Percentage of lipase producing strains of heterotrophic bacteria from Kongfjord sediment and water

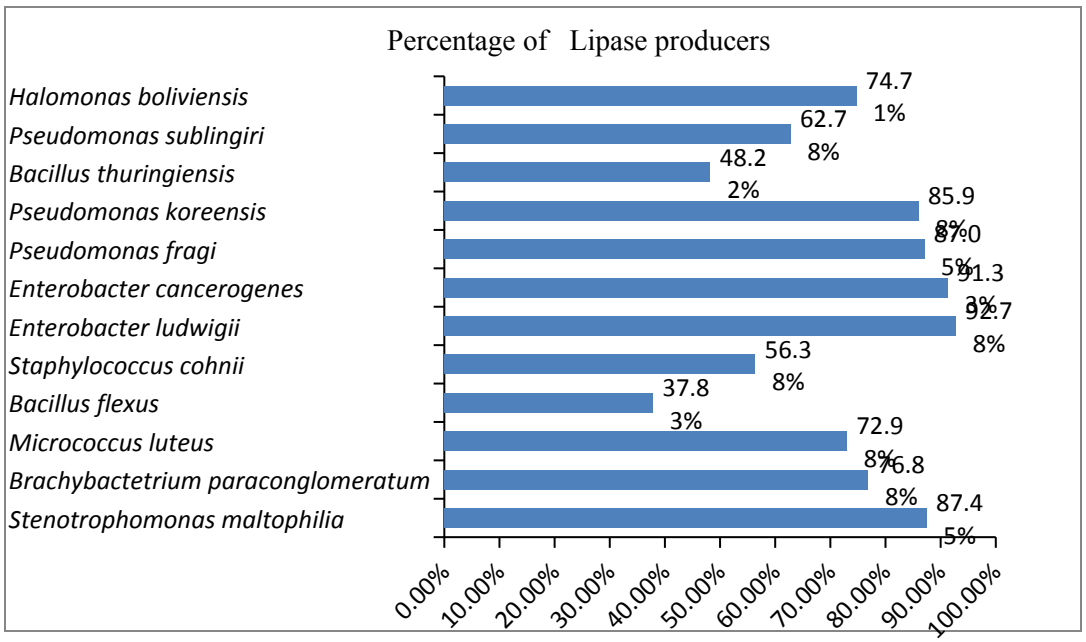
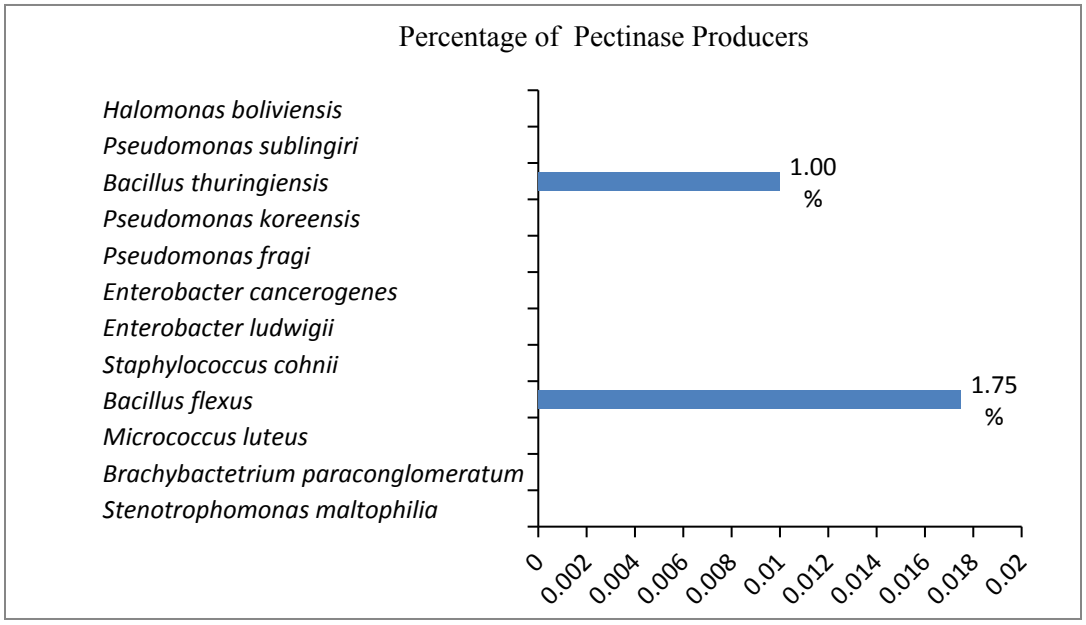


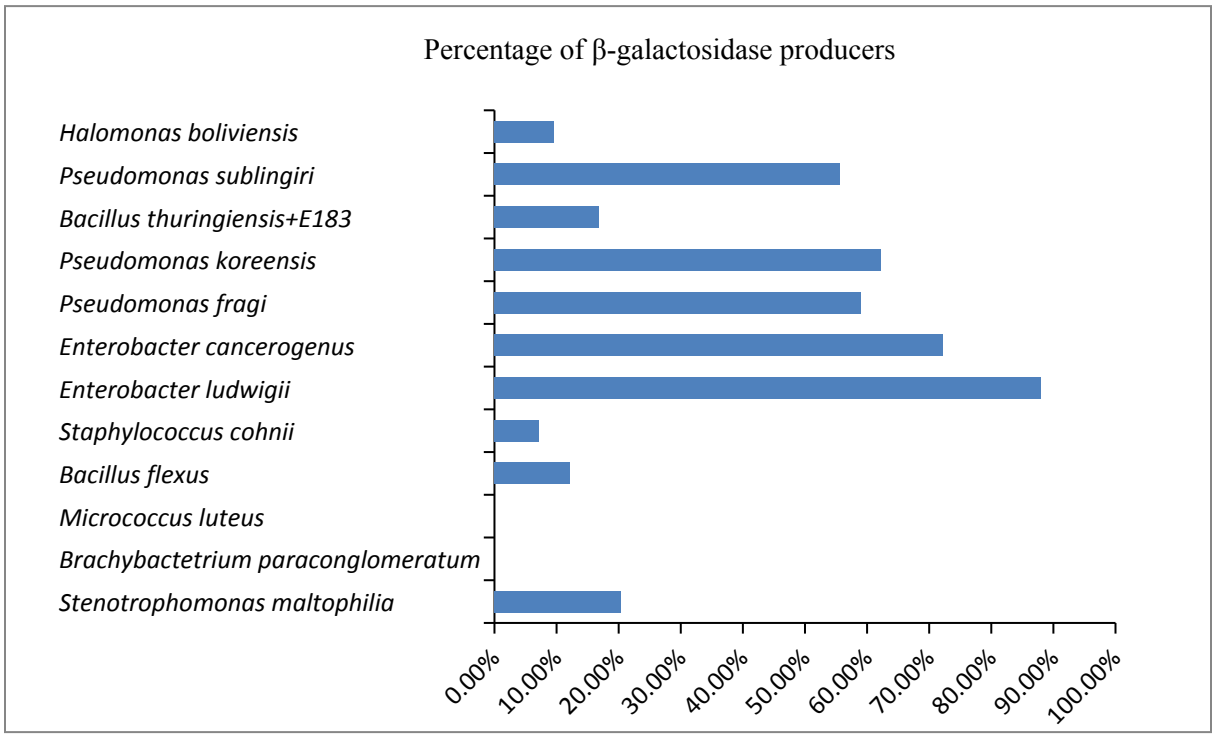
Figure 4.5: Percentage of pectinase producing strains of heterotrophic bacteria from Kongfjord sediment and water



Pectin was found to be degraded by a few of the sediment isolates and no isolates from water degraded this carbon source. Twelve isolates from sediment showed pectinase production. Only

a few strains of *Bacillus thuringiensis* and *Bacillus flexus were capable of producing* the enzyme pectinase (Figure 4.5)

Figure 4.6: Percentage of β-galactosidase producing strains of heterotrophic bacteria from Kongfjord sediment and water



β-galactosidase was found to be produced by 128 sediment isolates and 22 water isolates. A marked difference in production of β-galactosidase from sediment and water isolates was noted. Strains of only 2 species were found have > 70 % β-galactosidase producers such as *Enterobacter ludwigii* and *Enterobacter cancerogenus*. Strains of *Halomonas boliviensis, Staphylococcus cohnii, Bacillus flexus* and *Bacillus thuringiensis* showed <20 % β-galactosidase production (Figure 4.6).

Figure 4.7 shows percentage of cellulase producers from Kongsfjord sediment and water. The strains of *Pseudomonas fragi, Pseudomonas koreensis* and *Bacillus flexus* from sediment were found to produce the enzyme cellulase.

Figure 4.7: Percentage of cellulase producing strains of heterotrophic bacteria from Kongfjord sediment and water

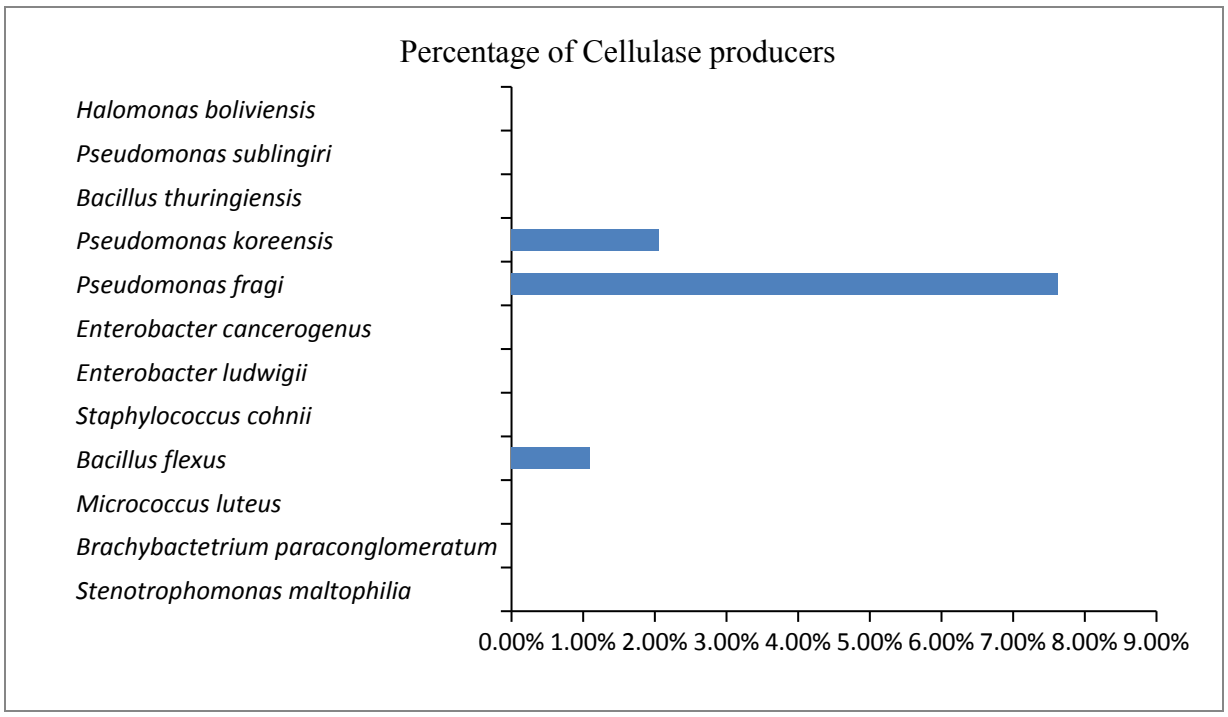
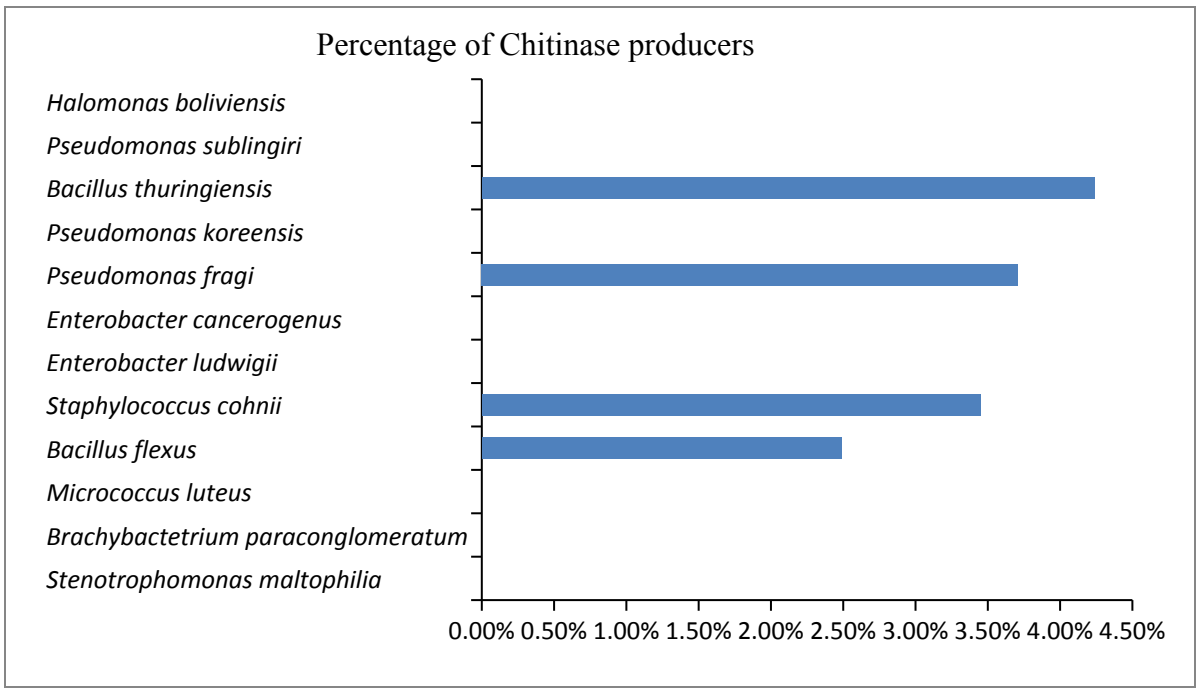
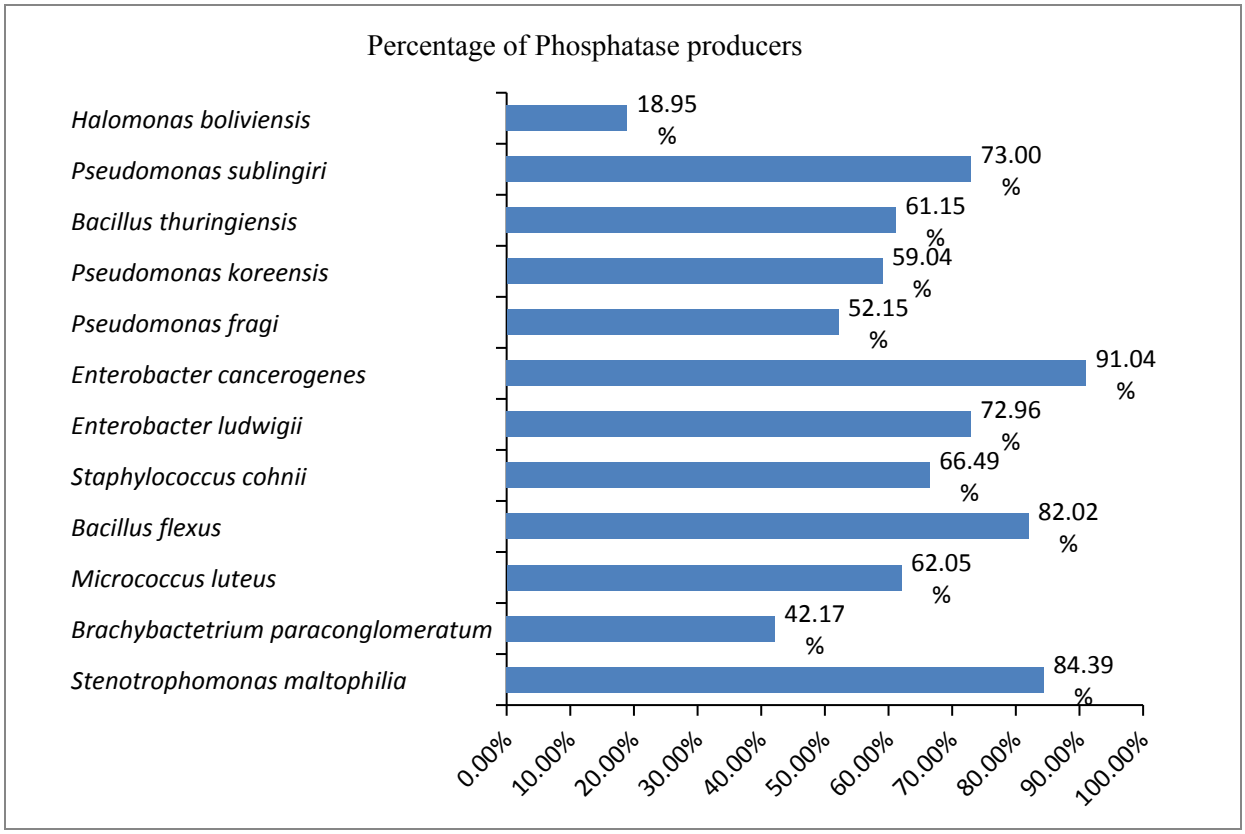


Figure 4.8: Percentage of chitinase producing strains of heterotrophic bacteria from Kongfjord sediment and water



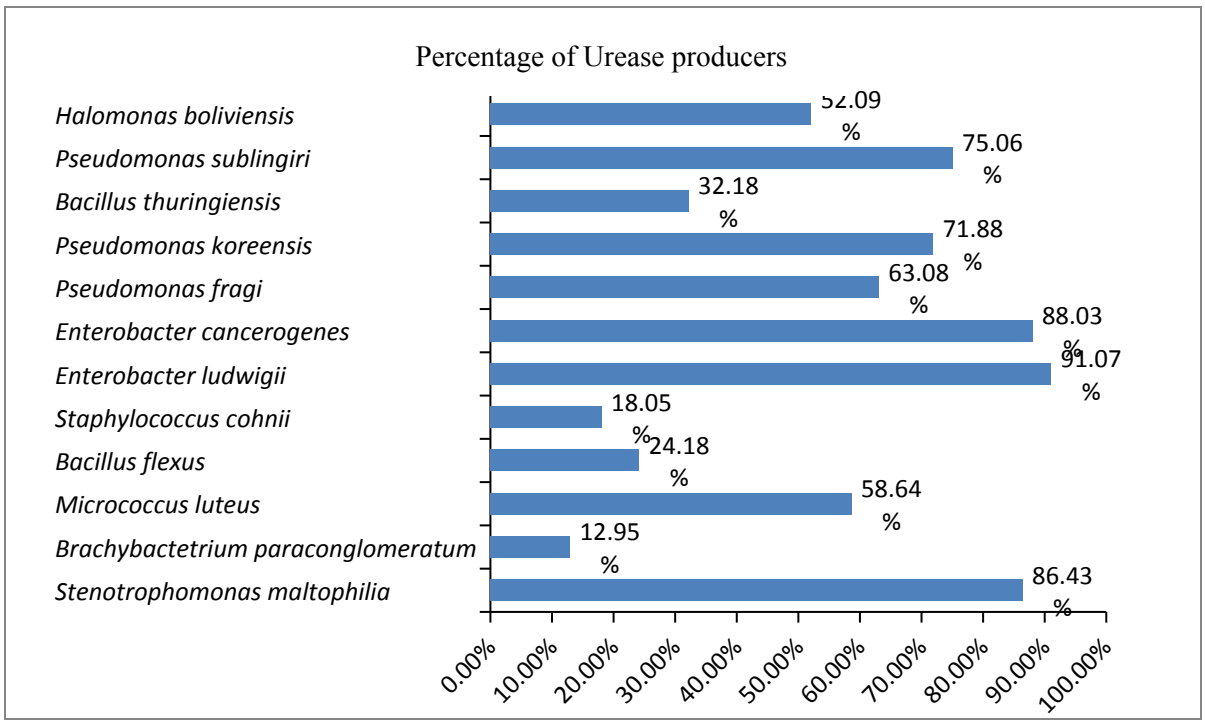
Sediment samples were found to have 28 chitinase producers and water samples were shown to have 14 chitinase producers. Figure 4.8 shows chitinase producing strains of heterotrophic bacteria. Strains of *Bacillus thuringiensis, Pseudomonas fragi,* and *Staphylococcus cohnii* were found to produce the enzyme chitinase.

Figure 4.9: Percentage of phosphatase producing strains of heterotrophic bacteria from Kongfjord sediment and water



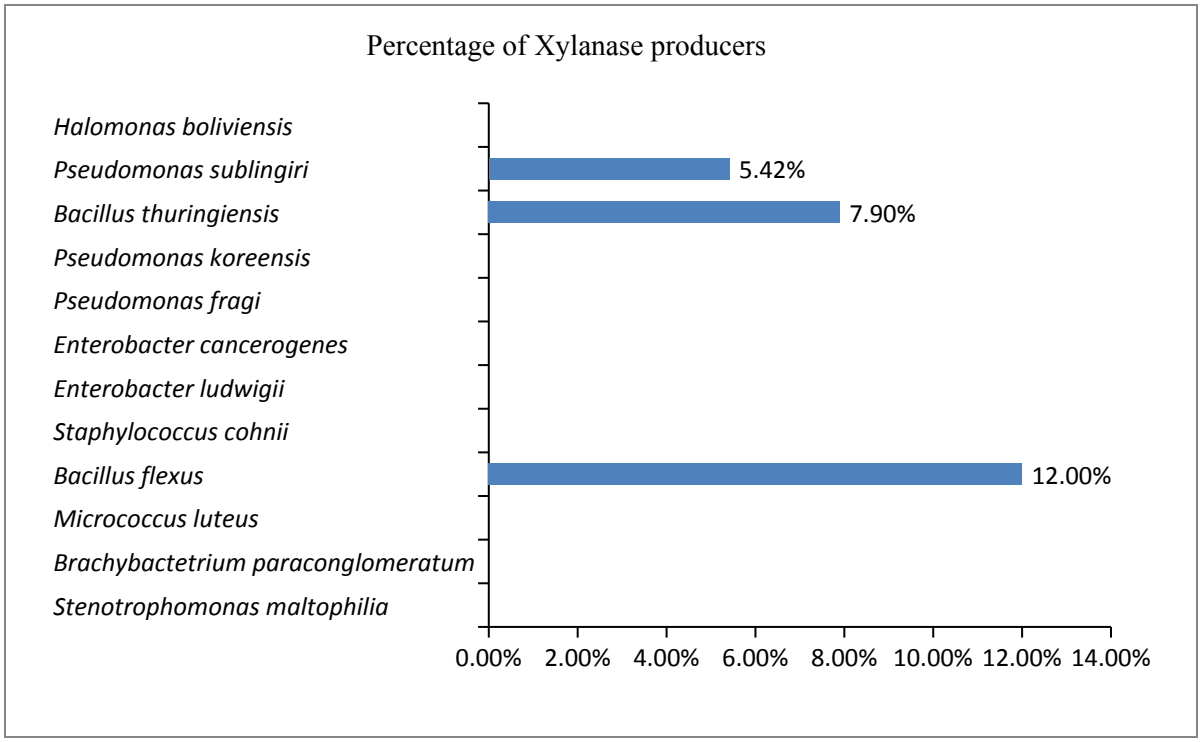
Phophatase was produced by 111 isolates from sediment and 34 isolates from water. Figure 4.9 shows the percentage of phosphatase producing strains of heterotrophic bacteria from Kongfjord. More than 70 % Strains of *Pseudomonas sublingiri, Enterobacter cancerogenus, Enterobacter ludwigii, Bacillus flexus* and *Stenotrophomonas maltophilia* have shown the ability to produce phosphatase. Most of the species were found to have strains with >60 % phosphatase producers.

Figure 4.10: Percentage of Urease producing strains of heterotrophic bacteria from Kongfjord sediment and water



Urea was found to be degraded by 66 sediment isolates and 15 water isolates. For both phosphatase and urease station 4 was found have maximum producers in sediment and water. Strains of *Pseudomonas sublingiri, Pseudomonas koreensis, Enterobacter cancerogenus, Enterobacter ludwigii* and *Stenotrophomonas maltophilia* were found to have >70 % urease producers. Twenty percentage strains of *Brachybactetrium paraconglomeratum* and *Staphylococcus cohnii* showed urease production (Figure 4.10).

Figure 4.11: Percentage of xylanase producing strains of heterotrophic bacteria from Kongfjord sediment and water



Xylanase producers were found to be less in Arctic marine habitat with 13 and 6 producers from sediment and water respectively. Xylase producing strains were found in *Pseudomonas sublingiri* (5.42 %), *Bacillus thuringiensis* (7.90 %) and *Bacillus flexus* (12 %). However, all species were found to have only <20 % strains producing the enzyme xylanase.

Figure 4.12: Image of plate showing gelatinase producing heterotrophic bacteria from Kongsfjord sediment and water

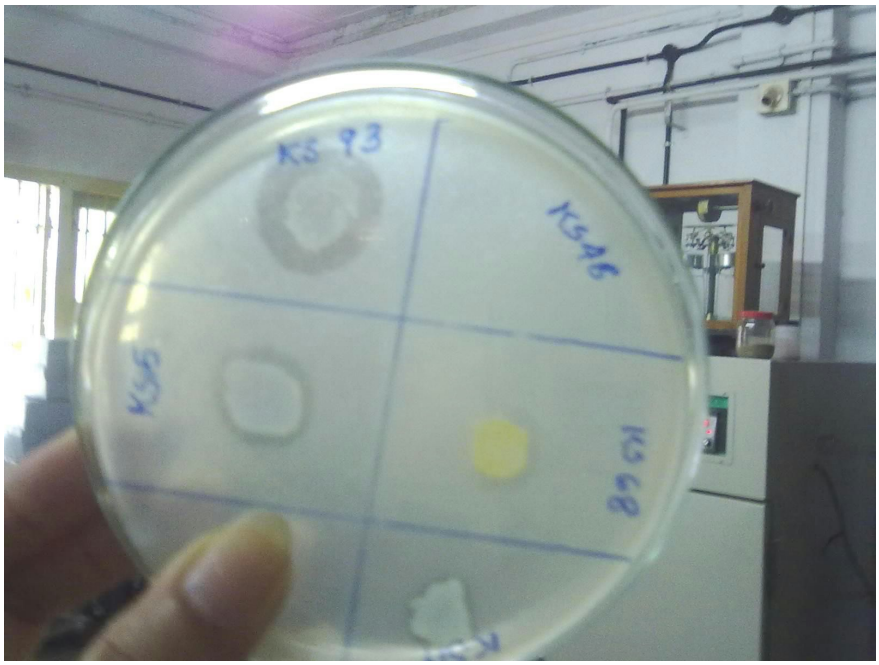


Figure 4.13: Image of plate showing lipase producing heterotrophic bacteria from Kongsfjord sediment and water

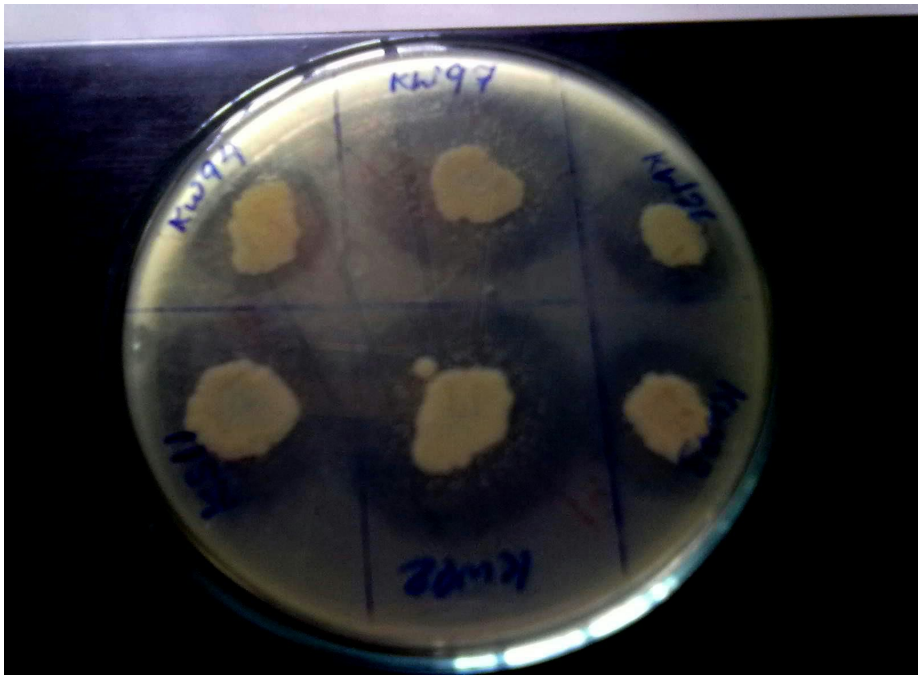
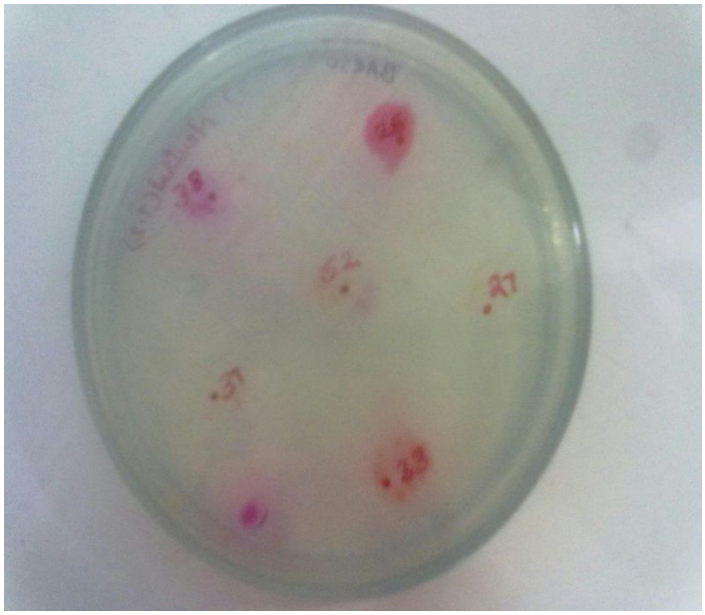


Figure 4.14: Image of plate showing amylase producing heterotrophic bacteria from Kongsfjord sediment and water

Figure 4.15: Image of plate showing phosphatase producing heterotrophic bacteria from Kongsfjord sediment and water



4.4 Discussion

Cold adapted enzymes produced by microorganisms are largely responsible for nutrient recycling and organic matter degradation (Gerday *et al.*, 2000). Abundance, bacterial count and presence of particulate extracellular polymeric substances have been studied by Collins *et al* (2008) to investigate the spatial heterogeneity in Arctic winter ice but studies on spatial variability of extracellular enzyme activities on sediment and water are very scarce. The substrate specificity of different enzymes either as a measure of enzyme diversity or as a way to differentiate reaction mechanisms could possibly resolve enzyme activities that attack specific detritus components in a major nutrient pool. Within each of these nutrient pools, there are specific compounds or substrates against which major classes of enzymes are active (Caldwell, 2005). Many extracellular enzyme producing bacteria belonging to phylogenetic groups - α, β, γ, δ- proteobacteria, Cytophaga, Flavobacteria, and Bacteroides have been isolated and identified from Arctic sediment and water (Teske *et al*., 2011; Karan *et al*., 2012; Vester *et al*., 2014).

The heterotrophic bacteria isolated from water and sediment of Kongsfjord was tested for their ability to produce hydrolytic enzymes. The simple plate assay method was followed for the detection of enzyme production. Several investigations have proved the sensitivity of this semi-quantitative method and it has been successfully used for the identification of enzymes (Leon *et al*., 2007; Li *et al*., 2009; Loperna *et al*., 2011). Plate assay of enzymes showed that heterotrophic bacteria from sediment were dominant in enzyme production and showed wider spectrum of enzymes. The result was not unexpected since diversity and number of heterotrophic bacteria from sediment was found to be higher than those from the water. Several studies have proved the superiority of enzyme production in sediment when compared to seawater (Arnosti, 2000; 2004; Arnosti *et al*., 2005; Arnosti *et al*., 2008; Teske *et al*., 2011). According to Teske *et al.* (2011) the differences in the ability of microbial population to degrade organic matter are independent of the number of microbial cells present in sediment and water. Furthermore, their study proved that the differences in the enzyme activities were the result of distinction between sediment and water community composition.

Zeng *et al.* (2011) showed that organic matter composition of sediment is 10^4 - 10^5 folds higher than seawater. The ability to degrade this organic matter is unevenly distributed among microbes because of the differences in extracellular enzyme production and a substrate is found to be labile only in the presence of appropriate enzyme (Arnosti, 2011). Out of the ten enzymes tested

during the study, eight were present in sediment and water isolates. Extracellular enzymes are produced by either free living or particle associated bacteria in the Ocean. The suspended particles may aggregate and sink to the bottom carrying the microbes along with it and these microbes colonize in the sediments. Enzymes could be attached to the cell surface, released into the surroundings freely or by viral lysis (Arnosti, 2013). This could be the possible reasons for elevated number of enzyme producers in the sediment samples.

A number of studies showed the superiority of enzyme production by *Gammaproteobacteria* in cold environment (Teske *et al*., 2011; Arnosti *et al*., 2008; Arnosti *et al*., 2011; Loperna *et al*., 2011). In the current study, sediment and water communities represented a majority of *Gammaproteobacteria*. However, marked differences in the quantity of enzyme producers in sediment and water samples were noted. It is also known that the entire microbial communities not the individual organism exhibit distinct substrate specificity (Arnosti *et al*., 2013).

Figures 4.2 to 4.11 show the distribution of various enzyme producers among the heterotrophic bacteria isolated from sediment and water. Hydrolytic enzymes producers are found distributed widely in the arctic environment. Seventy percentage strains of *Bacillus thuringiensis, Bacillus flexus* and *Micrococcus luteus* were found to have the ability to produce amylase. Amylase producers were found to have comparatively similar distribution in the sediment and water. Cold active nature of amylase activity was observed at 20 °C and various psychrotrophic amylase producers have been reported from Arctic and Antarctic (Feller *et al*., 1992; Chessa *et al*., 1999; Mihaela *et al*., 2009; Chattopadhyay *et al*., 2013).

Loperna *et al.* (2012) reported maximum number of amylase producers among the heterotrophic bacteria isolated from Antarctic at 20 °C. Amylases are one of most sought after enzyme in industry having 25-30 % share of 2.7 billion US$ enzyme market (Azad *et al*., 2009; Deb *et al*., 2013). Major applications of amylase include manufacture of maltose, preparation of high fructose containing syrups and maltotriose containing syrups, synthesis of high molecular weight branched dextrans, de-sizing in textile industry and direct fermentation of starch to ethanol (Kuddus and Roohi, 2010). Many species of *Bacillus* have been recognized as potent producers of protease and many researchers have reported the production and optimization of protease from this genus (Calik *et al*., 2000; Beg *et al*., 2003; Chauhan *et al*., 2004). In the present study also more than 80% strains of *Bacillus thuringiensis* and *Bacillus flexus* showed protease activity

Strains of *Staphylococcus cohinii* was also found to have potent protease producers (>80 %). The global demand for protease enzyme is very high and leading market is controlled by the U.S.A and China. Consumption of protease enzyme in China is growing by 6 % and expected to reach $272.6 million by 2019. This high demand of protease enzyme is mainly attributed to its applications in advanced laundry detergents to high quality food ingredients (Rohan, www.marketsandmarkets.com).

The current study shows that most of the heterotrophic bacteria isolated from the Arctic are capable of producing lipase (138 sediment and 63 water isolates). All species tested during the study were found to have some strains that could produce lipase. Highest number of lipase producing strains (>90 %) belonged to species *Enterobacter ludwigii* and *Enterobacter cancerogenus*. Strains of *Pseudomonas fragi*, *Pseudomonas koreensis* and *Stenotrophomonas maltophilia* were also found to have high number of lipase producers (>80 %).

Lipases from the Arctic environment was previously reported by Srinivas *et al* (2009) and 26 isolates were lipase producers at 5 °C and 20 °C. Another psychrotrophic lipase producer was found to be *Pseudomonas* sp. from Arctic soil by Dey *et al.* (2014). The high proportion of cold active enzyme producers from the current study indicates the possibility of development of potent lipase producers that could be used for industrial applications. Cold active lipases are used for many biotechnological applications such as pharmaceuticals for the preparation of enantio-pure chemicals, food industries, bakery and cheese manufacture, textile industries, detergent industry, chemical synthesis and environmental bioremediation (Joseph *et al*., 2007; Ramteke *et al*., 2005; Gerday *et al*., 2000; Margesin *et al*., 2002; Ganapati and Piyush, 2005).

Pectinolytic bacteria were found to be very few in the Arctic. Only the strains of *Bacillus thuringiensis* and *Bacillus flexus* were able to produce the enzyme pectinase. Pectinase has been used in food industries for degradation of pectin to improve texture and flavour. Pectinase is used for liquefaction, clarification and juice extraction (Adapa *et al*., 2014). Use of cold active pectinase in the preparation of wine under cold condition has been proposed by Merin and Ambrosini (2015). The cellulase producing bacteria from Kongsfjord sediment and water include strains of *Bacillus flexus, Pseudomonas fragi* and *Pseudomonas koreensis*. Pectinase and cellulase production was found only in sediment samples and production was found to be less. This result is in agreement with previous reports of pectinase and cellulase producing bacteria

from Polar Regions. Yu *et al.* (2009) could isolate 11 strains of cellulase producing *Pseudoalteromonas* from Arctic sea ice. Loperna *et al.* (2011) isolated 15 bacteria producing pectinase enzyme and 2 bacteria producing cellulase from marine environment of Antarctic region. Carrasco *et al.* (2012) reported pectinase and cellulase producing yeast from soil and water from Antarctic region.

Cellulase has been used in various industries such as agricultural, chemical, food and paper industries. In agricultural industries cellulases are used for control of fungal infections, to improve soil quality and enhance germination of seeds. Cellulase is used for conversion of cellulose to various chemicals and solvents (ethanol, organic acids, and single cell proteins). Brewing industries apply cellulase for processing of malt and mashing, improving aroma and viscosity of wine, increasing filterability of wart and improving primary fermentation. Paper and pulp industries use cellulase for pulp bleaching, improvement of draining, improved paper brightness and production of biodegradable cardboard (Kuhad *et al*., 2011).

β-galactosidase and phosphatase production by sediment and water isolates were found to be high compared to urease, chitinase, cellulase, pectinase and amylase. However, there was difference in the percentage of strains producing these enzymes from sediment and water samples. Many species of heterotrophic bacteria were found to produce enzyme β-galactosidase and strains of *Enterobacter ludwigii* and *Enterobacter cancerogenus* were found to have >70 % producers. Strains of species *Bacillus flexus* and *Staphylococcus cohinii* were found to have <20 % producers.

β-galactosidase producers from polar region have been characterized by many workers and the important genera include *Pseudoalteromonas*, *Arthrobacter*, and *Cytophaga,*(Oikawa *et al*., 2001; Hoyoux *et al*., 2001; Coker *et al*., 2003; Karasova-Lipovova *et al*., 2003). Unlike the other enzymes tested, β-galactosidase is an intracellular enzyme and has many applications in dairy industries and pharmaceuticals. β-galactosidase is mainly used in dairy industry for the production of milk with reduced lactose content to circumvent lactose intolerance problem for the affected population. Lactase is also added to reduce lactose crystallization thereby increasing the refrigeration quality of milk and milk associated products. β-galactosidase is used in pharma for preparation of sweet syrups and in food industries for ripening of cheese (Panesar *et al*., 2010).

Phosphatase activityhas been shown by several members, 128 isolates from sediment and 34 isolates from water were able to produce phosphatase. Sevaral strains of *Stenotrophomonas maltophilia*, *Enterobacter ludwigii, Enterobacter cancerogenus, Bacillus flexus* and *Pseudomonas sublingiri* were found to have the ability to produce phosphatases (>70 %). Enzyme phosphatase represents a major enzyme that plays a key role in phosphate mineralization in marine habitats. It was reported that presence of organic phosphorus is 5-10 folds higher than inorganic phosphorus in the surface waters of Oceans. Phosphatase production was previously reported from permanently cold habitats such as Antarctic region (Thomas and Dieckmann, 2000; Koutsioulis *et al*., 2008; Kobori *et al*., 1984). However, reports from Arctic region are very rare and thus the comparison becomes difficult. Research in the fieid of production and characterization of alkaline phosphatase is gaining momentum because of its recognition as a biotechnologically potential enzyme. Cold active alkaline phosphatase is currently used in molecular biotechnology for dephosphorylation of DNA vectors. Heat labile alkaline phosphatase from Arctic shrimp is marketed by Biotec Pharmacon, ASA (Tromso, Norway) and GE healthcare life sciences (Little Chalfont, UK) (Feller, 2013).

Chitinase production has been detected only in four genera isolated from the Arctic. Strains of *Bacillus thuringiensis, Pseudomonas fragi, Staphylococcus cohnii* and *Bacillus flexus* were shown to produce chitinase enzyme. However, production was found to be less when compared to other enzymes. Chitinase producers were found to be 28 in the sediment and 14 in the water. Whereas, more than 70 % isolates of *Pseudomonas sublingiri, Pseudomonas koreensis, Enterobacter cancerogenus, Enterobacter ludwigii* and *Stenotrophomonas maltophilia* were found to produce urease enzyme.

Urease producers in the sediment were found to be 66 and in the water only 15. Several urease and chitinase producers have been reported from permanently cold environment. Recently, urease isolated from jack bean (DOS47) has exhibited anticancer properties and has raised new interest in urease research. Another plant urease from *Cucumis melo* has been used in pharma preparation of diuretic medicines. Urease is used in brewing industry for the removal of urea from wine since urea and ethanol combine to form ethyl carbamate which is a carcinogen (Sujoy and Aparna, 2013). Chitinase has been identified as an inhibitor and biopesticide for control of fungal infections and used in agricultural industries. Chitinase is used for the production of

Single cell protein (SCP) for animal and aquaculture feed. Chitinase produced by *Serratia marcescens* is widely used for the conversion of chitin to aminosugars (Matsumoto *et al*., 2006).

Xylanase producing strains were found to be distributed in different species, such as *Pseudomonas sublingiri, Bacillus thuringiensis* and *Bacillus flexus*. However, Xylanase producing isolates were found to be less when compared to other enzymes tested. This could be possibly due to indirect measurement using plate assay since xylanase detection using direct methods have identified substantial amount of xylanase producers from Arctic. Most of the advanced studies used the direct measurement of xylanase enzyme in water using fluorescently labeled substrates and change in the molecular weight of the substrate is measured (Arnosti and Steen, 2013). Xylan is a major part of planktonic cells and hence ocean organic matter. Bacterial degradation of xylan is significant in recycling of this carbon source. Arnosti (2008) investigated high molecular weight organic carbon degradation including xylan derived from algae. This study showed similar result wherein xylanase producers were found to be more in sediment than in water. Difference in xylanase production in Arctic tundra during different seasons was demonstrated using fluorescently labeled substrates by Wallenstein *et al.* (2009). Arnosti and Steen (2013) reported high production of bacterial xylanase (ranking second) while studying different carbohydrate utilization from surface and pelagic communities of Arctic water.

Many countries including Japan, USA, Finland and Germany produce xylanase in commercial scale and share 20 % of enzyme market along with pectinase. Xylanases are used in the preparation of animal feed for the degradation of arabinoxylan. Xylanase is used in the food and baking industries along with other enzymes such as α-amylase, glucose oxidase and protease to improve flavour and texture. Addition of this enzyme in the baking preparations delays the crumb formation and improves the dough quality (Twomey *et al*., 2003). Xylanase along with cellulase is used in pulp industry to improve the digestion of wood and wood shavings (Polizeli *et al*., 2005).

Microbial enzymes hold a fair share in global market with a turnover of 3.3 billion US$ and is regarded as a very competitive field with Novozymes as the largest player followed by DSM (Koninkiijke DSM). The main areas of competition are product quality, performance, intellectual property rights and development of innovative methods (Adrio and Demain, 2014). Thus expansion of search for novel organisms or organisms with novel properties becomes a necessity

and pristine environments with extreme conditions such as the Arctic might harbour organisms with great potential. Nature presents a wide spectrum of microbial enzymes to be tapped for biotechnological applications. Proper utilization of these immense sources depends of many factors such as screening and exploring extremophilic diversity (Adrio and Demain, 2014).

The study shows that extracellular enzyme producing bacteria are widely distributed in the Arctic sediment and water. These bacteria might play an important role in mineralisation of complex organic matter. Further, the extracellular enzyme production potential of these strains could be exploited for production of commercially important enzymes such as cold active lipases.

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Chapter 5
Production of cold active lipase and Optimization of Culture conditions

Contents:

Lipases are hydrolases (triacylglycerol acylhydrolases; EC 3.1.1.3) that catalyze hydrolysis of carboxyl ester bonds present in acylglycerols to liberate fatty acids and glycerols. The enzyme is also known to catalyze esterification, interesterification, acidolysis, alcohololysis and aminolysis. Lipids constitute a major part in the global biomass and lipases play a significant role in the cycling of organic carbon. Lipases are either produced intracellularly for mobilization of lipidic substances within the cell or extracellularly for absorption of nutrients from the surrounding environment. Lipid molecules are usually composed of hydrogen and carbon but often oxygen, nitrogen and phosphorus are also present giving the molecule a polar head structure with a non polar tail. Lipases act up on these molecules at lipid – water interface releasing glycerol and fatty acids.

Lipases are usually divided into true lipases and carboxylesterases, true lipases display specificity for long chain acylglycerols and carboxylesterases act up on the short chain acylglycerols. However, clear definition of long chain and short chain does not exists although esters with an acyl chain length of ≥ 10 carbon atoms are considered to be long chains and <10 carbon atoms are considered to be short chains. Another important criterion to distinguish between lipases and carboxylesterases is the interfacial activation. Only true lipases have the ability for interfacial activation (Dheeman, 2011)

There are a considerable number of reports by authors such as Macra and Hammon (1985) regarding the industrial break down of lipids using physical and chemical processes. However, they are capital intensive and are high on energy requirement. In such a context, the search for alternative processes or mechanisms for breaking down of lipids assume significance. Lipases of microbial origin are considered to be better than their industrial counterparts since they have been found to be less draining not only on capital and energy requirement but also on quality and recovery of end product (Kaur *et al*., 1993). Because of the ability to catalyze various reactions, the lipases are used widely in production of medicines, developing flavor in vegetable, meat, fruit and dairy products, detergent and pharmaceutical industries (Aravindan *et al*., 2007 and Li *et al*., 2005). Thus, considering the multifarious advantages that microbial lipases offer, there are not too many studies that have been conducted to study the factors controlling production of microbial lipase.

Cold active lipases have attracted a lot of interest in recent years due to its various applications in different industries. Their ability to catalyze reactions at low or moderate temperature could be useful in industries such as pharmaceuticals, food and detergents. Cold active lipases are also known for their low heat of inactivation which helps in the protection of frail compounds and saving energy. Cold adapted enzymes are currently used in detergent industries, food and brewing industries, cheese manufacture, bakery, lean meat preparation, wine making and in environmental bioremediations of oils, xenobiotics and composting. However, compared to thermophilic enzyme, cold active lipases are less explored for their use in biotechnological fields and future researches in cold active lipases might open new fields for its potential use.

5.1 Review of literature

5.1.1 Structural properties and Catalytical function of lipases

Structural analysis of enzyme will aid in better understanding of catalytic function and help in improvement of its activity through structural modification. The structure of an archeal lipase isolated from *Archaeoglobus fulgidus* has been studied in detail. The lipase consisted of an N-terminal region which has α/β hydrolase fold domain, a lid domain (smaller) and C-terminal with β-barrel domain. The catalytic domain which was a part of N-terminal domain has six β-sheets and seven α-helices. Lipid binding region of the protein was C-terminal domain containing 14 strands of β-sheets and it also has a substrate covering motif on top of the hydrophobic substrate binding site.

Generally all lipases have similar structure of α/β- hydrolase fold; it has a canonical structure with eight strands of β-sheets at the centre (Jaeger, 1999). Lipases are found to have a lid structure located close to the active site and found to have both hydrophilic and hydrophobic domains. Interfacial activation mechanism of true lipases was studied by Angkawidjaja *et al*. (2007) on *Pseudomonas* sp. using x-ray crystallographic studies and molecular dynamics simulation. Their study showed two lid structures, lid 1 remains open and represent the active confirmation. Confirmation of lid 2 (open or close) depend up on the presence or absence of substrate and the study also proved the role of Ca^{2+} in the stabilization of open confirmation of active site. Ca^{2+} is essential to maintain the open confirmation of the lid structure and hence the active state of the enzyme.

All the enzymes were found to have a catalytic triad in which the loops of the structure contain highly conserved elements. Among the aminoacids, histidine was found to be most conserved in the nucleophilic-histidine-acid catalytic triad. However, nucleophile and acid loops could accommodate several amino acids. The three dimensional structure of bacterial lipases were further analyzed by Jaeger (1999) who revealed a catalytic triad consisting of a nucleophilic serine located in the highly conserved pentapeptide of Gly-X-Ser-X-Gly. Furthermore, an aspartate or glutamate residue, hydrogen bonded to a histidine was also noted. This study also revealed the substrate binding sites of lipases as an oxy-anion hole and three pockets accommodating the fatty acids recognized as *sn-1, sn-2,* and *sn-3*. The enantio-specific nature of

lipases is determined by the nature of these pockets depending on whether they are hydrophilic or hydrophobic elements.

Crystallographic analysis of inhibitor-lipase complexes has revealed the details of mechanisms of lipase catalysis. According to Jaeger (1999) it follows 4 stages, in the first stage lipidic substrate bind to the nucleophilic serine residue and transferring its proton to histidine residue. Following the activation, oxygen atom of serine hydroxyl group reacts with the lipid ester bond at the carbonyl carbon region. On the second stage of catalysis, an unstable tetrahedral intermediate structure is formed consisting of oxygen atom at the centre and surrounded by carbon atoms of the carbonyl atoms. The additional proton of histidine is then donated to ester oxygen of the susceptible carbonyl bond followed by its cleavage. This would result in the esterification of the acid component of the substrate while the alcoholic component of the substrate gets diffused away. Third stage is the deacetylation step wherein water molecules hydrolyse the acyl-enzyme and the acid component of the substrates gets esterified. Proton from the water molecules are removed by catalytic histidine active sites. This results in the formation of OH^- ion that reacts with carbon of the acyl group that is attached to serine residue. In the last stage, proton from histidine is transferred to oxygen atom of serine. This results in breakage of ester bond between serine and acyl group followed by release of acyl component.

5.1.2 Detection of lipase activity

Many authors have reported that detection of lipase activity is difficult due to the nature of lipase enzyme. Lipases are soluble enzymes which act on water insoluble substrates (Kanchana *et al*., 2011; Redondo *et al*., 1995; Verger, 1997). There are different methods used for detection of lipase activity depending on parameters such as activity markers, substrate solubilisation methods and detection system. Most of the detection method widely used includes chromogenic and flourogenic substrates which are easiest for testing large number of samples (Wahler and Reymond, 2001).

Plate assay

Plate assays are semi-quantitative method and are widely used for preliminary screening of lipase production. In the plate assay method substrates such as tributyrin, triolein etc. are incorporated in the media. Detection is usually by appearance of clearance zone around the lipase producing colonies. Liberated fatty acids can also be detected by addition of pH indicators or by adding fluorophores like rhodamine B. and fluorophores form complex with fatty acids which produce an orange-pink color under UV irradiation.

Spectrophotometric assay

Spectrophotometric assays are the most widely used quantitative method for detection of lipase. Direct and indirect methods of detection are used for spectrophotometric methods. Triacylglycerols contain α-eleosteric acid which is a chromophore released from the hydrolysis and can be analyzed by spectrophotometery. Acylglycerols (AGs) such as p-nitrophenyl esters of fatty acids (pNPPs) when hydrolyzed release p-nitrophenol which is a yellow colored compound and read at 400-410 nm. One disadvantage of using pNPs is that short chain pNPs could be hydrolyzed by other enzymes or basic solutions (Beisson *et al*., 2000).

Titrimetric method

Titrimetric method is a widely used quantitative method since it is cheap and simple although they have low sensitivity. Lipase activity is measured on a mechanically stirred emulsion of natural or synthetic triacylglycerols either by manual titration of released fatty acids with standardized NaOH or by pH-stat equipment, neutralizing the fatty acids released by adding standardized NaOH in order to maintain the pH at a constant end point value (Beisson *et al*., 2000)

Substrate specificity of lipases

Lipases from different sources show variations in properties especially with respect to carbohydrate content, regional specificity and fatty acid specificity (Gupta *et al*., 2004). Better spectrum of substrate utilization coupled with enantioselectivity and enhanced efficiency in non aqueous medium is always desirable in new lipases (Doukyu and Ogino, 2010). Different substrate specificities among lipases are attributed to differences in geometry and size of their active sites (Aloulou *et al*., 2006).

5.1.3 Lipases from microorganisms

Lipase productions have been widely reported from many microbial species such as bacteria and yeasts. Various microbial sources of lipase are *Bacillus subtilis*, *B. liqueniformis, B. stearothermophilus*, *B.catenulatus* (Lee *et al*., 1991), *Candida rugosa* (Benjamin and Pandey, 1995), and *Rhizopus delemar* (Acikel *et al*., 2011).

Lipase production is dependent on various factors such as temperature, pH, carbon source, nitrogen source, inoculum size, inducers, inhibitors, substrate concentration and their interaction with each other (Lin *et al.*, 2006; Acikel *et al.*, 2011). It is well documented that carbon and nitrogen play important role in growth and lipase production. (Corzo and Revah, 1999; Fickers *et al.*, 2004). C/N ratio and nitrogen concentration are critical factors in the determination of lipase production (Adinarayana, 2002). There is an increased interest in optimization of carbon source for lipase production and also to investigate cheaper alternative substrates for large scale production of lipase (Abdelmoez *et al.*, 2013; Kanmani *et al.*, 2015; Salihu *et al.,* 2012).

Over the years, many studies have been conducted to assess the role played by the media components in lipase production. Sekhon *et al.* (2006) studied the ability of *Bacillus megaterium* AKG -1 to produce extracellular lipase in a submerged fermentation and found that using 0.2 % (w/v) mannitol as the primary carbon source gives the highest production of lipase (848 U/ ml). Kumar *et al.* (2010) used the Plackett-Burman statistical package to determine the optimum production of lipase from *Bacillus pumillus* RK 31. The study 'proved that olive oil, Tween 80 and KH_2PO_4 played significant role in lipase production. Many reports suggest that lipase production is in abundance when nitrogen used in the media is in the form of organic or organic and inorganic combination rather than inorganic nitrogen alone (Fickers *et al.*, 2003; Lima *et al*., 2003).

In general, presence of triglycerides has shown to improve the lipase production in the media. Many researchers have reported enhancement of lipase production by adding extra carbon sources such as glucose, sucrose and maltose (Acikel *et al*., 2011; Chang *et al*., 1994; Gilbert *et al*., 1991; Burkert *et al*., 2003; Marcin *et al*., 1993; Chartrain *et al*., 1993). However, there are also reports of suppression of lipase production by carbon sources such as glucose (Valero *et al*., 1991) and thus it is suggested that expression of lipase gene is governed by multiple mechanisms

(Lotti *et al.*, 1998). It has also been noticed that, carbon from lipid sources generally lead to a greater production of lipase (Boonchaidung *et al*., 2013).

Fattah (2002) evaluated lipase production from a novel thermophilic *Bacillus* sp. using the Placket-Burman and concluded that Tween 80, temperature, olive oil, aeration, beef extract and age of inoculum had the greatest affirmative impact while pH and calcium chloride had the most significant negative impact on lipase production. Shariff *et al.* (2007) evaluated the impact of nutritional and physical factors on *Bacillus* sp. strain L2 and observed that Ca^{2+}, Tween 60 and casamino acid had positive correlation with increased lipase production.

Deive *et al.* (2012) studied the extracellular thermostable lipase production by *Bacillus thermoamylovorans* which was found to produce maximum amount of the enzyme between 50-55 °C and at pH 7.0. Agitation and aeration at 300rpm and 0.33vvm were also found to have positive correlation with lipase production.

In the recent years considerable research activities have centered on cold adaptive lipases since they work very well at low temperatures – their optimum temperatures being substantially low. Cold adaptive lipases are being increasingly used for production of chemically sensitive compounds (Joseph *et al*., 2008). Cold adaptive lipases which are isolated from different regions vary from each other in their active sites, optimum temperature, pH, nutrient factors etc. (Joseph *et al*., 2008). Tutino *et al.* (2009) isolated cold active lipase from *Pseudoalteromonas haloplanktis* TAC 125.

Pascale *et al.* (2008) studied the genes of *Pseudoalteromonas haloplanktis* TAC 125 isolated from Antarctica for production of esterase. An esterase named PhTAC125 Lip 1 was found to possess unique biochemical features such as conserved active site, cytoplasmic location and absence of canonical lid and calcium binding pockets. Sirisha *et al.* (2010) had studied the role of different carbon sources, nitrogen sources, pH, and temperature and incubation period on lipase activity and maximum lipase production was observed in the media containing palm oil and peptone as carbon and nitrogen sources.

Fickers *et al.* (2003) studied the role of carbon and nitrogen sources in lipase production by *Yarrowia lipolytica* mutant. Tryptone N1 and Oleic acid were found to be most suitable for lipase production by this organism. Lima *et al.* (2003) studied the effects of nitrogen and carbon

sources on lipase production by a wild fungus identified as *Penicillium aurantiogriseum* and maximum yields were obtained with ammonium sulphate as nitrogen source and olive oil as carbon source. Dalmau *et al.* (2000) studied the impact of carbon sources on lipase production by the yeast *Candida rugosa* and found that carbohydrates and acids not related to fats did not play any significant role to induce lipase production.

Sekhon *et al.* (2005) studied the influence of media components, pH and oil on lipase production from *Bacillus megaterium* AKG -1. Mannitol was found to be the best suited carbon source at concentration of 0.2 % (w/v) whereas, ammonium nitrate was found to be the best nitrogen source for lipase production. Addition of oils as soyabean oil, tween 80 and bile salts induced enhanced production of lipase. Just as the addition of seed cakes like cotton and neem, wheat bran were found to increase lipase production. There are other reports of 0.1 % rice bran enhancing lipase production in *Bacillus* sp. THL 027 (Dharmsthiti and Luchai, 1999). However, for *Lactobacillus delbrueckii* subsp. *bulgaricus*, wheat bran was found to be a suitable nitrogen source (El-Sawah *et al*., 1995).

Thus, it may be inferred that different organisms have different requirements and have different tolerance level to any particular factor. Increased levels of mannitol (>0.2 %), glucose, galactose, xylose and sucrose lead to an increased production of biomass but led to decline in enzyme production (Mahadik *et al.,* 2002).

The most commonly used approach for optimization of different parameters is by 'one-variable-one-at a time'. In this method, one parameter is changed while keeping the other parameters at constant level. Many researchers have used this method for successful optimization of various enzyme productions. However, this method has some disadvantages such as it does not include interactive effects among the variables and it is time consuming. Optimization using statistical approach such as response surface methodology has become popular method among researchers in past two decades.

5.1.4 Optimization of lipase production by response surface methodology

Response surface methodology (RSM) was designed by Imperial chemical Industries (1951). The graphical perspective of mathematical model has led to the term RSM (Bas and Boyaci, 2007). RSM is widely used by researchers in different fields for optimization studies. Statistical

approach has an upper hand over non statistical approach in industrial processes (Haaland, 1989; Gupta *et al*., 2004; Beg *et al*., 2003). RSM has important application in the design, development and formulation of new products as well as in the improvement of existing product design. It defines the effect of the independent variables, alone or in combination on the process. In this method, a mathematical model is generated which explains chemical and biochemical processes (Myers and Montgomery, 1995). The relationship between the response and the input is given in Eq:

$$\eta = f(x_1, x_2 \ldots\ldots\ldots x_n) + \varepsilon$$

Where η is the response, f is the unknown function of response, $x_1, x_2 \ldots\ldots\ldots x_n$ represents the independent variables, also called natural variables, 'n' denotes the number of independent variables and ε is the statistical errors that represent other sources of variability not accounted for by f. These sources include the effects such as the measurement error. It is generally assumed that ε has a normal distribution with mean zero and variance.

Optimization using RSM can be divided into three stages. The first stage is the preliminary steps which determine the independent parameters and their levels are calculated. The first stage of RSM has major effects on the biochemical processes and preliminary screening is used to identify the independent parameters (Bas and Boyaci, 2007).

The second stage involves the selection of experimental design and prediction and verification of model equation. These designs are different from one another with respect to their selection of experimental points, number of runs and blocks. Once the design is selected, the model equation can be defined and coefficients of the model equation are predicted. The model generally used in RSM is a full quadratic equation or the diminished form of this equation. The second order model can be written as follows:

$$Y = \beta_0 + \sum \beta_i X_i + \sum \beta_{ii} X_i^2 + \sum\sum \beta_{ij} X_i X_j$$

Where Y is the predicted response, β_0, β_i, β_{ii}, and β_{ij} are regression coefficients for intercept, linear, quadratic and interaction co-efficient respectively. X_i and X_j are independent variables. The system of equations given above is solved using the method of least squares (MLS). Once the

regression coefficient is calculated, the estimated response can be calculated from the model equation (Bas and Boyaci, 2007).

The central composite design (CCD) is the most frequently and extensively used RSM design. CCD is a well established widely used statistical technique for determining the key factors from a large number of medium components by a small number of experiments. The CCD has three groups of design points *Two level factorial or fractional factorial design points*: All possible combinations of the +1 and -1 levels of factors (2^K) *the axial points* (Sometimes called 'star' points): All of the factors are set to '0' the midpoint except one factor, which has the value +/- alpha. In face centered central composite design (FCCCD) all of the factors are set to 0 (midpoint), except one factor, which is set at the +1/-1 value and *center point:* Points with all levels set to coded level 0 which is the midpoint.

The third stage in RSM is the graphical presentation of the model equation and determination of optimal operating conditions. The visualization of the predicted model equation can be obtained by the response surface plot and contour plot. The response surface plot is the theoretical three-dimensional plot showing the relationship between response and the independent variables. The contour plot helps to visualize the shape of a response surface.

When the contour plot displays ellipses and circles, the center of the system refers to a point of maximum or minimum response. Sometimes, contour shows hyperbolic or parabolic systems of contours. For such structures the stationary point is called a saddle point and it is considered as neither a maximum nor a minimum point. Even though these plots provide useful information about the fitted model, they might not represent the true nature of the system (Myers and Montgomery, 1995). A flat response surface and circular contour of dependent variables indicate that the response can tolerate variations in the processing of variables without the responses being seriously affected. A very pointed surface and elliptical or saddle nature of contour indicate that the responses would be sensitive to the processing variable (Montgomery, 2000).

Lipase optimization has also been reported using response surface methodology. Wu *et al*., (1999) optimized process parameter for ethyl ester production by lipase from the bacteria *Pseudomonas cepacia*. Ruchi *et al*. (2008) isolated organic solvent tolerant *Pseudomonas aeruginosa* and lipase production by this strain was optimized using response surface

methodology. In this study, parameters such as tryptone and gum arabic concentrations were optimized using response surface methodology. Box-Behnken experimental design was used by Acikel *et al*. (2011) and five fermentation parameters were optimized by response surface methodology to obtain maximum lipase production. Ebrahimpour *et al*. (2008) optimized thermostable lipase from *Geobacillus* sp. and 4.7 fold increase was obtained after the optimization. Kumari *et al*. (2009) isolated lipase from *Enterobacter aerogenes* and optimization was carried out for physiochemical parameters including temperature, oil concentration, inoculum volume, pH and incubation period.

Demirkol *et al.* (2006) used lipase from *Rhizomucor miehei* for methanolysis of soyabean oil and used response surface methodology for optimization. Ognjanovic *et al.* (2009) used sunflower oil as a substrate for biodiesel production using lipase from *Candida antarctica* and process parameters were optimized by RSM. Sharma *et al*. (2009) optimize physical parameters for lipase production from *Arthrobacter* using RSM. Teng and Xu (2008) used response surface methodology for whole cell lipase production by submerged fermentation. Burkert *et al.* (2003) optimized extracellular lipase production by *Geotrichum* sp. by statistical approach. Ten fold of increase in the lipase production was yielded after optimization of several parameters such as concentration of corn steep liquor, ammonium nitrate and soy oil.

Lo *et al.* (2012) used response surface methodology to optimize the lipase production from *Burkhoderia* sp. In their study, the optimized medium included olive oil (0.65 %), tryptone (2.42 %) and tween 80 (0.15 %). The R^2 value was found to be 0.9866 for the regression model and 3 fold increases in lipase production achieved. Sifour *et al*. (2010) studied lipase production by thermophilic *Geobacillus stearothermophilus* using response surface methodology. The lipase production was enhanced to five fold after the optimization.

In the current study, various heterotrophic bacterial isolates were tested for lipase production. The isolate which showed the highest production was identified and further characterization and optimization was restricted to isolate showing highest activity.

5.2 Materials and Methods

Organisms used

Lipase positive heterotrophic bacteria isolated from Arctic sediment and water were used for the study. The lipase positive isolates were subjected to quantitative spectrophotometric assay for confirmation of lipase production. The isolate with highest lipase production is identified as *Stenotrophomonas maltophila* and it was used for further study.

5.2.1 Spectrophotometric assay of lipase

The lipase activity of cell free supernatant or cell extracts was quantitatively assayed using p-nitrophenol palmitate as substrate. Para nitrophenyl palmitate ester was freshly prepared in isopropanol (solution A) at a concentration of 0.3 % (w/v). This solution was emulsified for 3 min with continuous pulse at 135 watts. Solution B was prepared using 0.1 % (w/v) gum acacia and 0.4 % (v/v) triton X 100 in distilled water. Substrate stock was prepared by adding 0.5 ml of solution A to 9.5 ml of solution B. The reaction mixture contained 900 µL of substrate emulsion, 50 µL of tris buffer (pH-7) and 50 µL of lipase enzyme. The assay was run for 15 min at the optimum temperature and the activity was terminated by adding 2.0 ml of Na_2CO_3. The liberated *p*-nitrophenol was determined by measurement of absorbance at 410 nm ($\varepsilon_{410\ nm}$ = 0.017 µ/M/cm) using a UV-Visible spectrophotometer. One international unit (IU) of lipase activity was defined as the amount of enzyme needed to liberate 1 µmol of *p*-nitrophenol per minute under standard conditions.

Selection of most potent strains

Based on the screening studies, the most potent lipase producing isolate was found to be KS 46. Isolate KS 46 was a gram negative bacterium, non sporing, and motile isolate and molecular identification (chapter 2, section 2.2) revealed the isolate to be *Stenotrophomonas maltophilia*.

5.2.2 Lipase production using different natural oils.

The ability of the isolates to grow and produce lipase using different natural oils was tested. The basal medium (nutrient agar) was supplemented with various lipid sources such as sunflower oil,

coconut oil, gingelly oil, groundnut oil, olive oil and palm oil. The activity was determined semi-quantitatively by plate assay method and tributyrin was used as a control substrate.

Relative activity

It is the percentage enzyme activity of the sample with respect to the sample for which maximum activity is obtained

Relative activity =

Residual activity

It is the percentage enzyme activity of the sample with respect to activity of the control (untreated sample)

Residual activity =

Specific activity

Specific activity of the sample was calculated by dividing the enzyme units (U) with the protein content

Specific activity =

The activity of the sample is expressed in units (U) and wherever necessary as specific activity (U/mg).

Estimation of protein content

Concentration of protein was estimated using Bradford protein assay method. Bovine serum albumin (BSA) solutions at concentrations ranging from 0 to 50 µL were used as standards.

Optimization of Media

Different media were tested to identify the media which support good growth of the organism and maximum lipase production. Various media tested were

Media 1 (Joshi *et al*., 2006)

$NaNO_3$	3.0 %
K_2HPO_4	0.5 %
$MgSO_4.7\ H_2O$	0.5 %
KCl	0.05 %
$FeSO_4.\ 7H_2O$	0.01 %
Sucrose	1 %
Olive oil	1 %

Media 2 (Kiran *et al*., 2008)	
Yeast extract	3.0 g
Malt extract	3.0 g
Tryptone	5.0 g
KH_2PO_4	1.0 g
$MgSO_4.\ 7H_2O$	1.0 g
Thiamine	1.0 g
Glucose	5.0 g
Tributyrin	50 ml
Distilled water	1000 ml

Media 3 (Domínguez *et al*., 2003)	
$(NH_4)_2SO_4$	0.1 %
$K`_2HPO_4$	0.09 %
KH_2PO_4	0.06 %
$MgSO_4.7H_2O$	0.02 %
yeast extract	0.01 %
Olive oils	1.0 %

Media 4 (Ruchi *et al*., 2008)	
Peptone	5.0 g
Yeast extract	3.0 g

Olive oil	40 ml
Distilled water	1000 ml

Media 5 (Chen, 2005)	
Olive oil	1 %
$(N`H_4)_2SO_4$	0.5 %
Peptone	2.0 %
K_2HPO_4	0.5 %
$MgSO_4.\ 7H_2O$	0.1 %

Media 6 (Yuan *et al*., 2008)	
Glucose	20 g/L
Yeast extract	5 g/L
Peptone	10 g/L
$MgSO_4$	0.1 g/L
$MnSO_4$	0.1 g/L
Olive oil	10 ml/L

5.2.3 Optimization of process parameters using - one factor at a time

Optimization of different process parameters was carried out by changing one parameter at a time and keeping all other parameters constant. Parameters tested using this approach include incubation period, temperature, agitation, pH, carbon source and nitrogen source. All

experiments were carried out in triplicates and statistical analysis was performed using one – way ANOVA (SPSS statistics 20, IBM). Significance of difference between the samples were tested with one way ANOVA at $P = 0.05$.

5.2.4 Optimization of process parameters using RSM

The process parameters such as pH, agitation and nitrogen concentration were optimized statistically using the full factorial Face Centered Central Composite Design (FCCCD) of the RSM. The software Design expert (Version 9, Stat-Ease Inc, Minnepolis, USA) was used for experimental design, data analysis and the quadratic model building. The effect of these process parameters pH, agitation and nitrogen concentration on lipase production by *Stenotrophomonas maltophilia* was tested by RSM. The optimal levels of the variables were obtained by solving the regression equation and also by analyzing the response surface contour plots using the same software.

A 2^3 factorial (CCD) with six central points and 14 non central points giving a total of 20 experiments were performed for optimization of the chosen independent variables. The lower and higher limits of these parameters were taken as -1 and +1 respectively in face centered central composite design of RSM. The minimum and maximum value with full experimental plan is depicted in table 7.1. Experiments were conducted in 250 ml screw cap conical flasks containing 100 ml of the media and incubated at 25 °C and incubated for 5 days. After the completion of incubation period enzyme was extracted and lipase activity was estimated.

To evaluate the validity of chosen quadratic model, the predicted points of the design expert software were conducted in triplicates. The validation was carried out at optimum values as determined by the model and media was prepared at pH 6.8 with nitrogen concentration of 1.342 g/ml and incubated in a shaker incubator with an agitation of 147.3. After the completion of incubation period lipase activity was estimated and compared with the predicted values. The quality of the fit of the polynomial model equation was expressed by the coefficient of determination – R^2 and adjusted R^2. The fitted polynomial equation was analyzed by software which generated 3D contour plots indicating optimum concentrations and interactions among these factors.

Table 5.1: Levels of variables tested

Independent variables	-1 (low)	0 (Mid)	+1 (High)
Initial pH	6.00	7.00	8.00
Nitrogen concentration	0.5	1.00	1.50
Agitation	100	135	170

Table 5.2: Face centered composite design matrix of three factors

Sl No	Factor 1- pH	Factor 2: Nitrogen concentration (g/100ml)	Agitation (RPM)
1	6	0.5	100
2	8	1.5	100
3	7	1	135
4	8	1.5	170
5	7	1	135
6	7	1	135
7	8	0.5	100
8	6	1.5	100

9	6	1.5	170
10	7	1	135
11	6	0.5	170
12	6	0.5	170
13	7	1	135
14	7	1	135
15	7	1	193.863
16	5.31821	1	135
17	7	0.159104	135
18	7	1.8409	135
19	8.68179	1	135
20	7	1	76.1373

5.3 Results

5.3.1 Selection of the most potent lipase producer

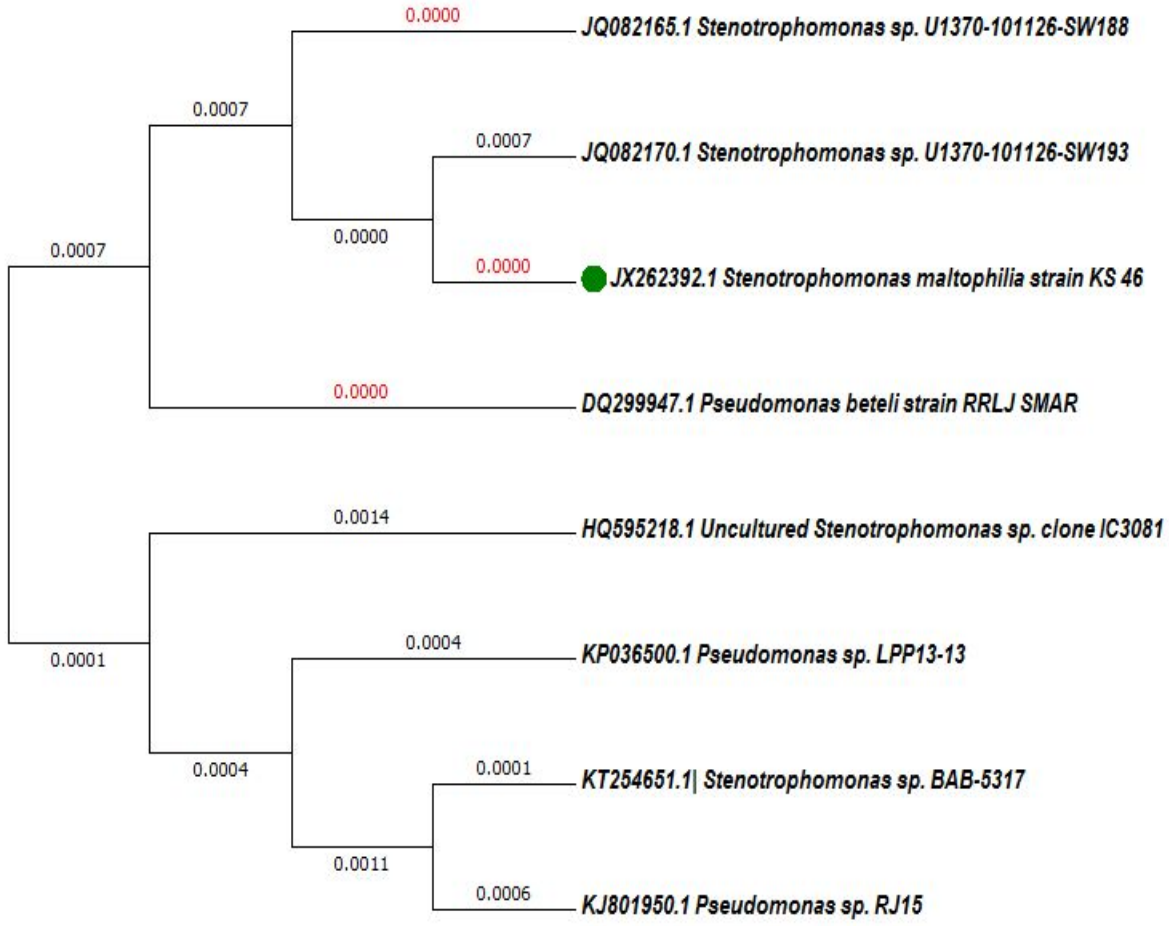
Among the lipase producing isolates detected using plate assay method, 21 isolates showed very good lipase activity by spectrophotometric method. Table 5.3 depicts the lipase activity of different strains and the strain KS 46 showed highest activity with 493.52 U/ml.Other potent lipase producers included KW 42 (*Enterobacter ludwigii)*, KW 41 (*Enterobacter ludwigii)*, KW 97 (*Stenotrophomonas maltophilia)*, KS 97 (*Bacillus thuringiensis*) and KS 96 (*Pseudomonas koreensis)*. Molecular identification of highest lipase producing strain KS 46 by 16S rDNA analysis and sequencing revealed it to be *Stentrophomonas maltophilia* and phylogenetic tree of the isolate is represented in the Figure 5.2.

Table 5.3: Lipase activity of potent isolates using spectrophotometric method

Sl No	Isolate	Lipase activity (U/ml)
1	KS 6	233.34
2	KS 97	338.23
3	KS 27	235.59
4	KS 24	235.29
5	KS 46	493.52

6	KS 7	257.08
7	KS 29	302.64
8	KS 42	300.00
9	KS 92	270.88
10	KS 71	222.94
11	KS 95	235.44
12	KS 25	320.59
13	KS 43	192.64
14	KS 8	302.94
15	KS 90	280.00
16	KS 96	310.30
17	KW 41	388.24
18	KW 42	470.03
19	KW 97	340.88
20	KW 46	302.74
21	KW 30	257.06

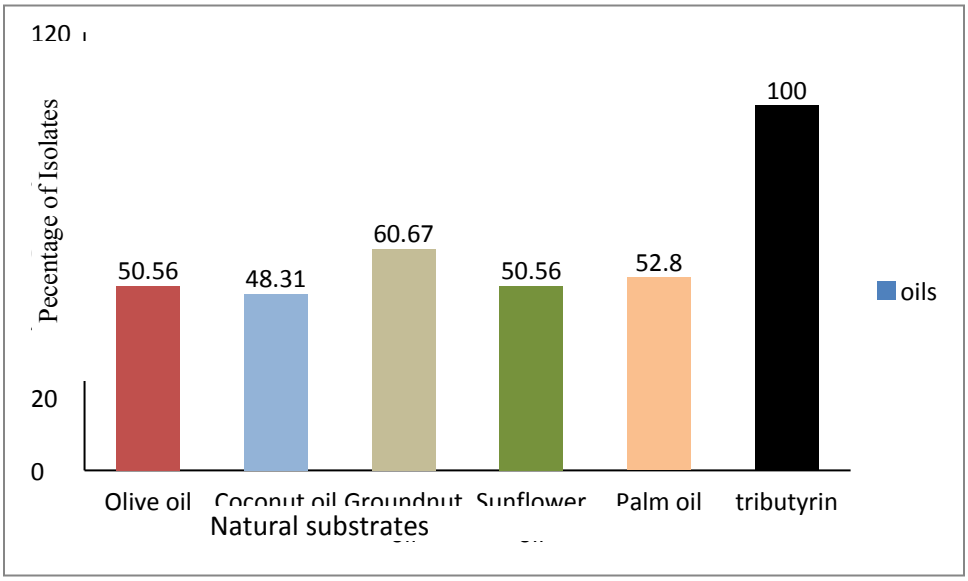
Figure 5.1: Phylogenetic tree of *Stenotrophomonas maltophilia* showing nearest neighbours



5.3.2 Natural substrate utilization of lipase positive Isolates

Natural substrate utilization of all lipase positive isolates that showed activity on tributyrin agar was tested on different substrates. Figure 5.1 shows the percentage of isolates that showed lipase production using different substrates. It can be envisaged from the figure that 60.67 % of isolates were able to degrade groundnut oil followed by palm oil with 52.8 % isolates. More than 50 % of isolates were able to degrade olive oil and palm oil.

Figure 5.2: Percentage of isolates showing production of lipase on natural oils



5.3.3Growth optimization of Stentrophomonas maltophilia

Growth optimization studies carried out for *Stentrophomonas maltophilia* are represented in the figures 5.3 to 5.5. Experiments on incubation period suggested that maximum growth was on 3rd day of incubation at 20 °C. Temperature that supported best growth of *Stentrophomonas maltophilia* was found to be 23 °C- 25 °C. The pH that supported maximum growth was found to be pH 6 and the organism is` tolerant to alkaline conditions till pH 11

Figure 5.3: Time course of growth of *Stenotrophomonas maltophilia*

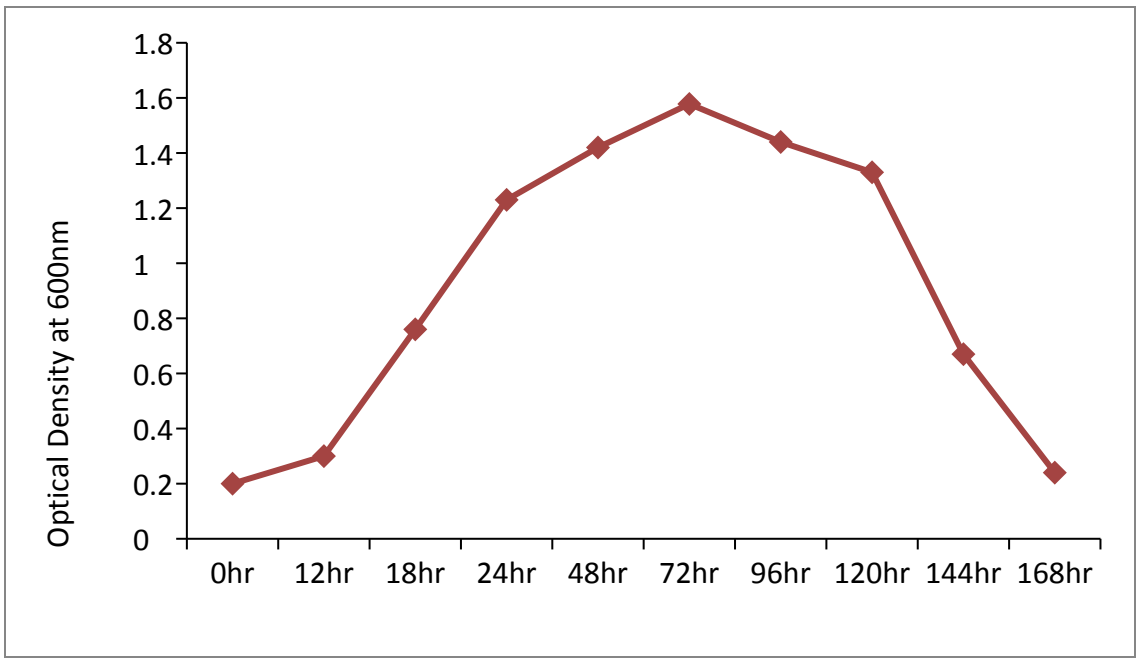


Figure 5.4: Growth of *Stenotrophomonas maltophilia* under different pH

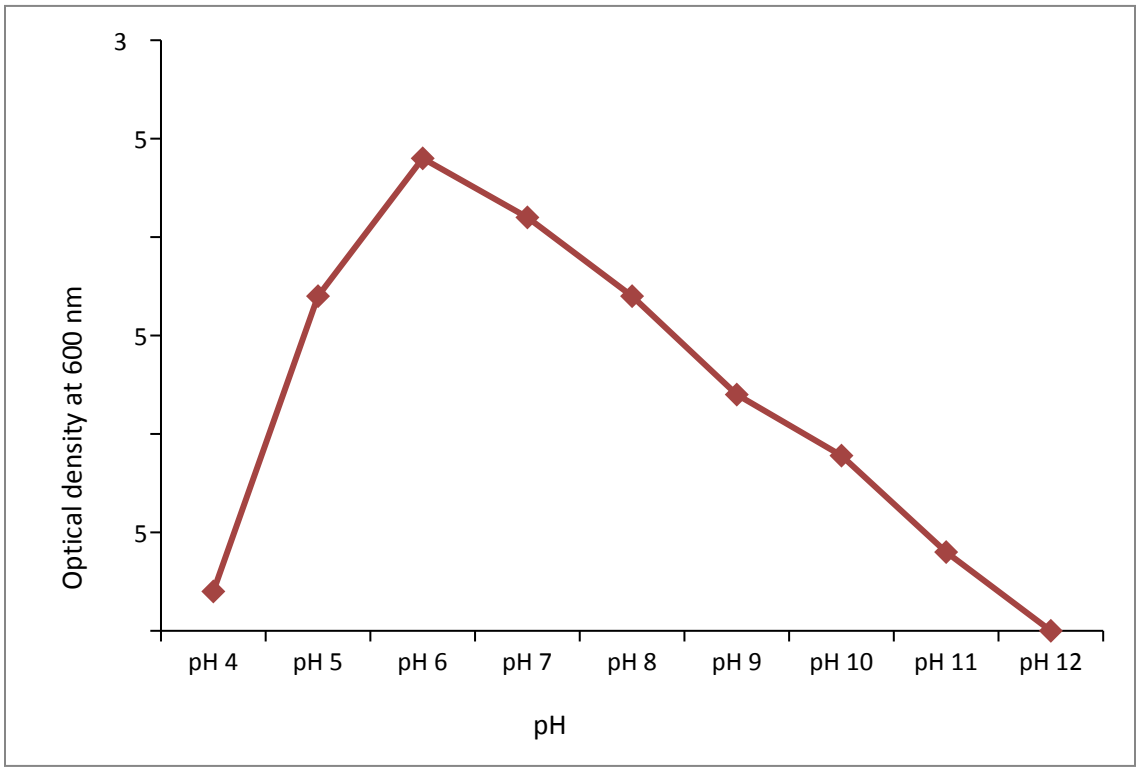
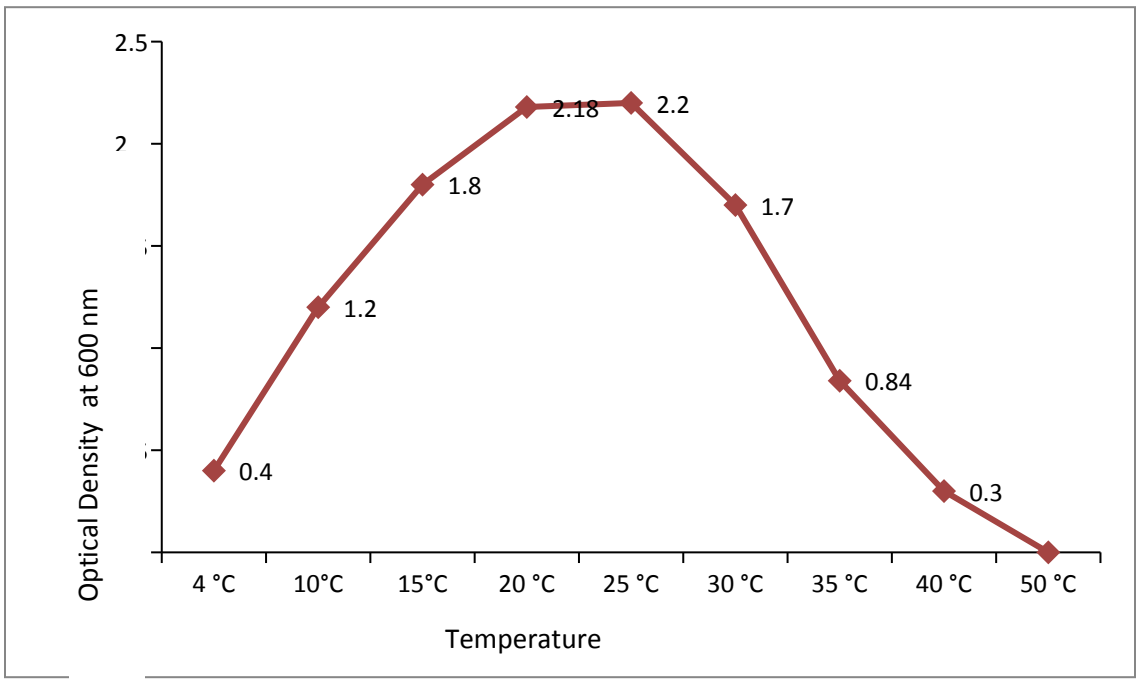


Figure.5.5: Growth of *Stenotrophomonas maltophilia* under different temperature



5.3.4 Optimization of lipase production by one variable at a time

Effect of different parameters on lipase production was standardized by varying one factor at a time. The optimum temperature for lipase production was 25 °C followed by 20 °C which retained more than 95 % activity (Figure 5.6). The maximum production of lipase was found on 5th day followed by a decline after this period. Effect of pH was studied by preparing the media at different pH and results indicated that maximum lipase production occurred in the range pH 6-7. Different carbon sources were found to influence the lipase production and maximum lipase production was found when glucose was used as a carbon source along with 1 % olive oil.

The influence of number of organic and inorganic nitrogen sources on lipase production was tested and results indicated that organic nitrogen sources supported more lipase production when compared to inorganic nitrogen sources (Figure 5.10). A combination of nitrogen sources peptone and yeast extract (2:1) was found to give maximum lipase yield. Agitation at 150rpm was found to support the maximum lipase production and agitation of more than 180 rpm had an adverse effect on lipase production (Figure 5.11).

Figure 5.6: Optimization of temperature for lipase production by *Stenotrophomonas maltophilia*

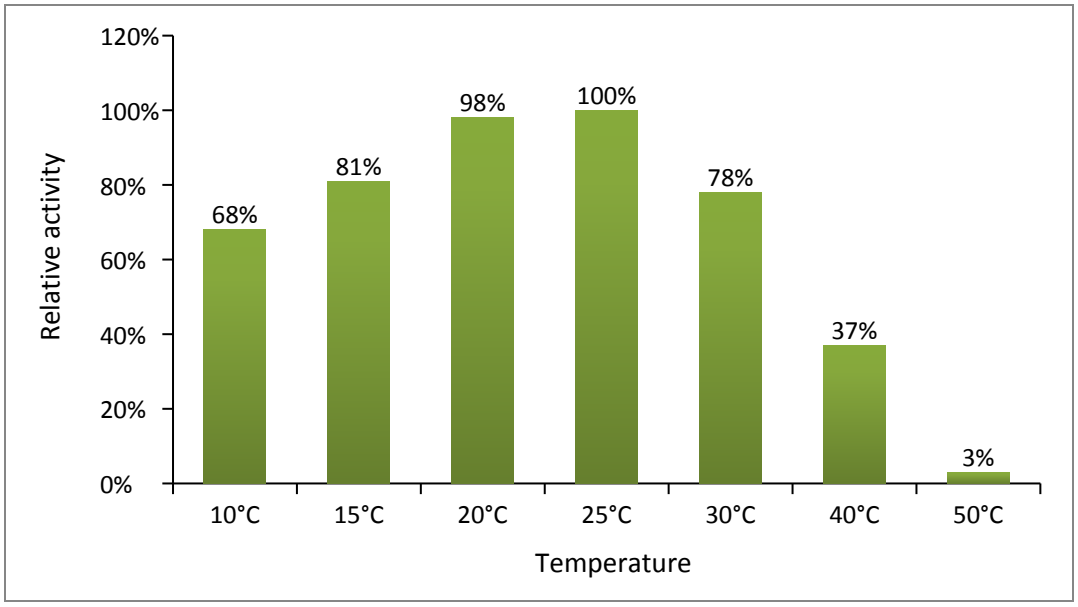


Figure 5.7: Optimization of incubation period for lipase production by *Stenotrophomonas maltophilia*

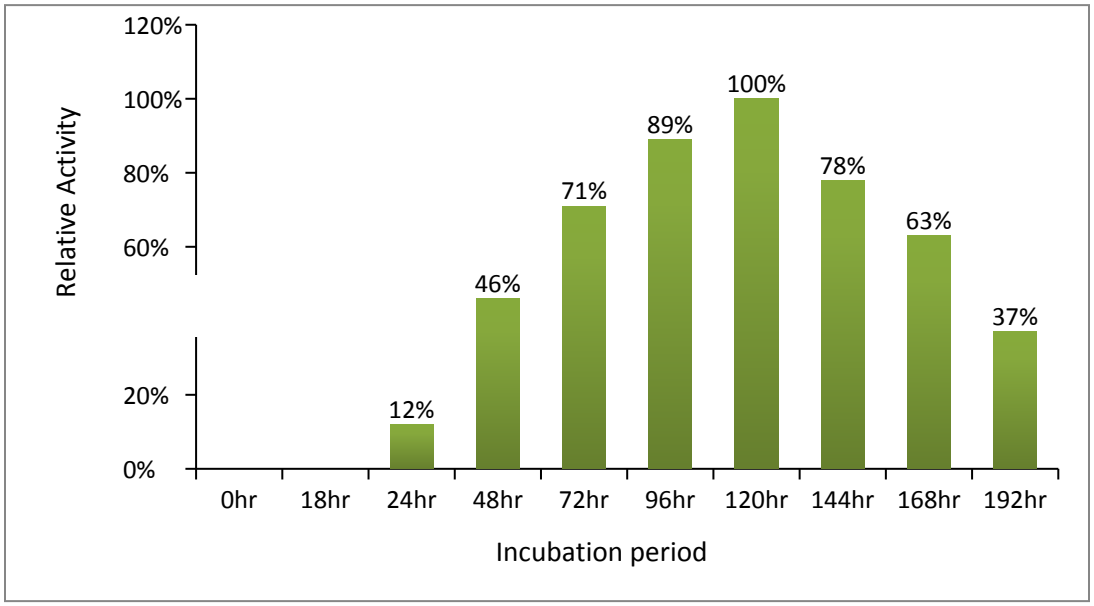


Figure 5.8 Optimization of pH for lipase production by *Stenotrophomonas maltophilia*

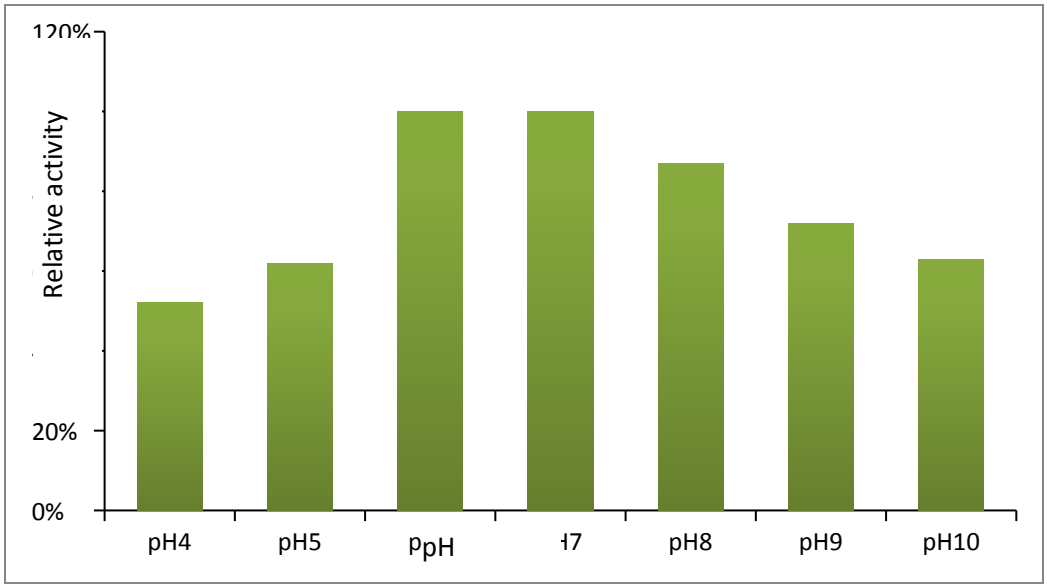
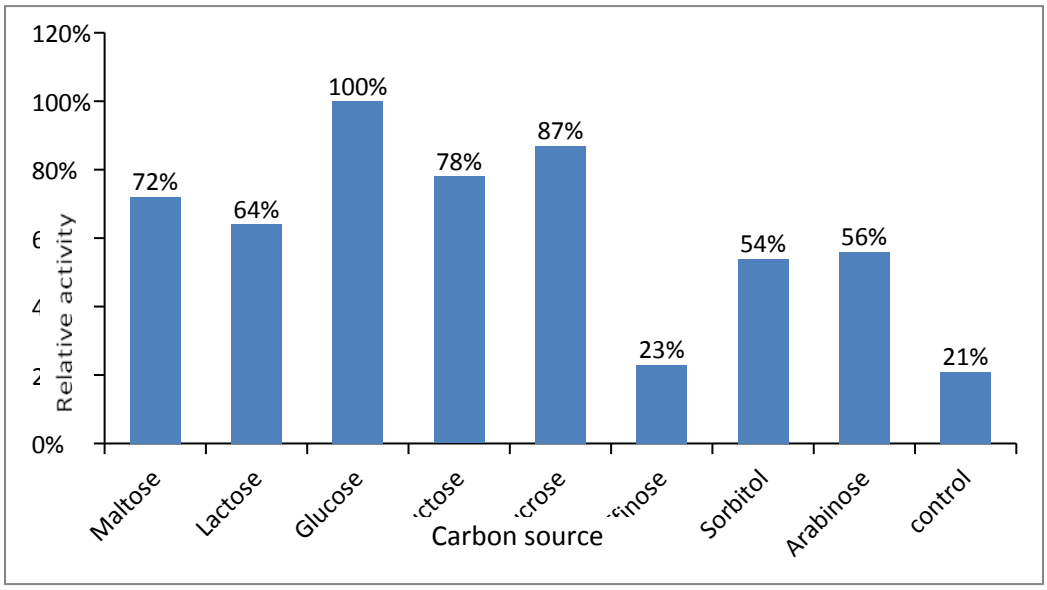


Figure 5.9: Optimization of carbon source for lipase production by *Stenotrophomonas maltophilia*



The influence of number of organic and inorganic nitrogen sources on lipase production was tested and results (Figure 5.10) indicated that organic nitrogen sources supported more lipase production when compared to inorganic nitrogen sources. A combination of nitrogen sources peptone and yeast extract (2:1) was found to give maximum lipase yield. Agitation at 150rpm

was found to support the maximum lipase production and agitation of more than 180 rpm had an adverse effect on lipase production (Figure 5.11).

Figure 5.10: Optimization of nitrogen source for lipase production by *Stenotrophomonas maltophilia*

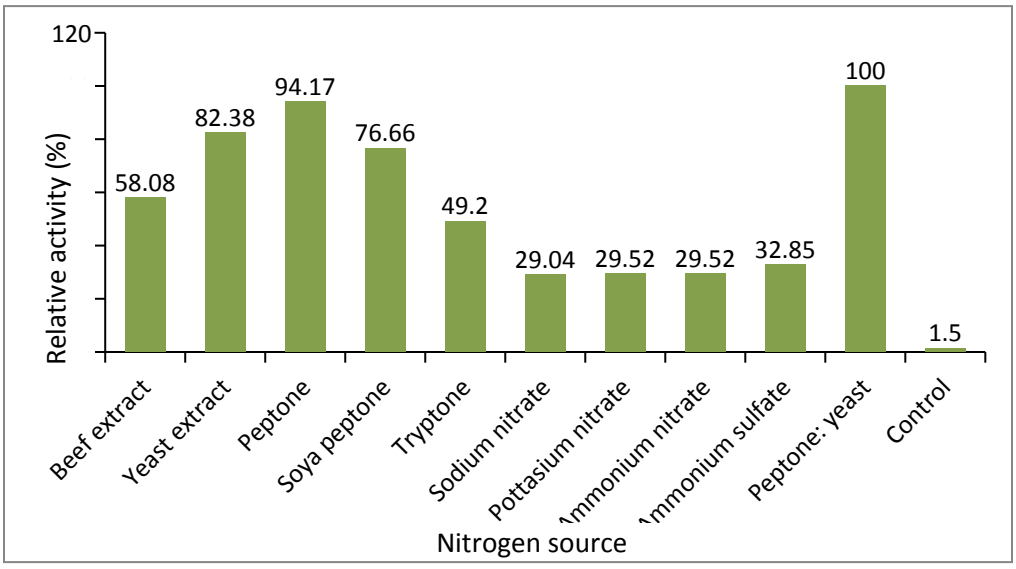
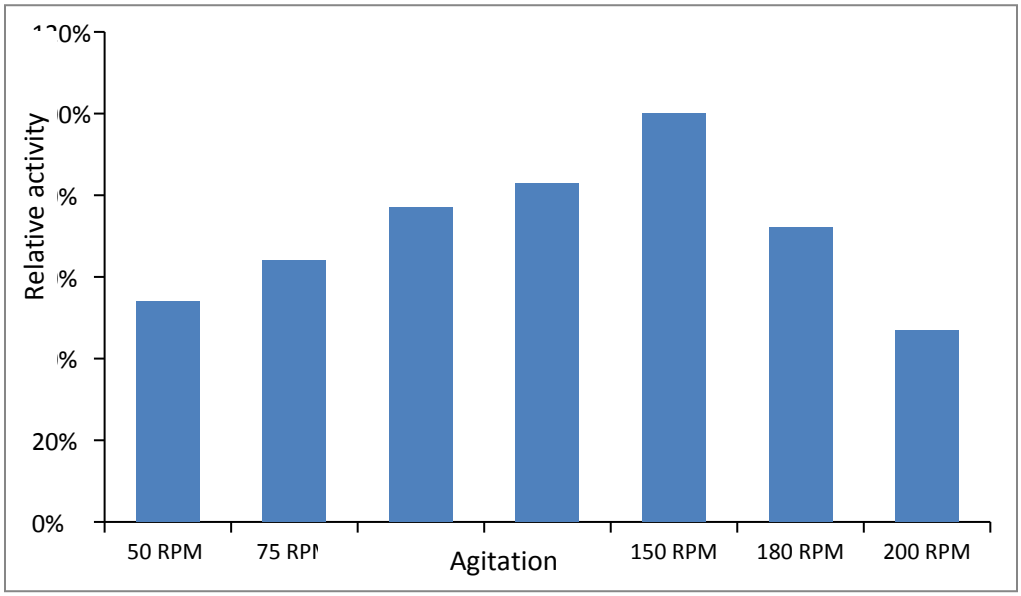


Figure 5.11: Optimization of shaking speed for lipase production by *Stenotrophomonas maltophilia*



5.4.4Optimization by RSM

The response surface methodology was used to investigate the effects of independent variables separately and also interactive effects. The variables and the range were selected on the basis of the 'one- variable at a time strategy'. The study showed that the conditions that supported maximum lipase production were different from the conditions that supported the growth of *Stenotrophomonas maltophilia*. Table 5.4 depicts the lipase production of the experiment (FCCCD) for each individual run with the result and the predicted response. All the experiments were performed in triplicates and average was taken.

ANOVA for the model was performed and is summarized in table 5.6. The 'F-value' for the model was found to be 134.10. High model was also assisted with a low probability value of [(prob > F) less than 0.0001] reiterate the significance of the model. The quadratic terms A^2, B^2 and C^2 were found to be significant since probability was less than 0.0001. The value of multiple correlation was found to be R^2 was found to be 0.9926 which suggested that the model can explain 99.26 % variation in the response and predicted R^2 value was 0.9414. The adjusted R^2 value was found to be 0.9852. The adequate precision value for the model was 25.730 and indicates that the model can be used to navigate the design space. Table 5.7 express the following values such as the coefficient of variation (CV), standard deviation, mean and predicted residual sum of squares (PRESS). Figure 5.12 and 5.13 represents normal plot of predicted and studentized residual values which also conform the precision of the experiment. Relative effects of the independent variables were expressed in 2D contour and 3D response surface plots. The interactive effects of factors such as nitrogen and agitation (BC), pH and agitation (AB), and pH and nitrogen (AC) are expressed in figure 5.14, figure 5.15 and figure 5.16.

Validity of the model was checked experimentally and the value was found to be 821.14 which was closer to the predicted value of 832.63

Table 5.4: Face centered central composite design of the three independent factors along with observed and predicted values of lipase production

Run order	Factor1	Factor 2	Factor 3	Predicted Activity	Actual Activity
1	6	0.5	100	387.27	371.23
2	8	1.5	100	419.59	412.34
3	7	1.0	135	843.59	834.44
4	8	1.5	170	415.75	434.78
5	7	1.0	135	843.59	854.12
6	7	1.0	135	843.59	812.67
7	8	0.5	100	403.48	411.70
8	6	1.5	100	482.38	512.76
9	6	1.5	170	473.04	467.80
10	7	1.0	135	843.59	861.17
11	6	0.5	170	424.74	434.98
12	8	0.5	170	446.45	419.06
13	7	1.0	135	821.69	837.23
14	7	1.0	135	821.69	818.32
15	7	1.0	193	448.74	452.18
16	5.3	1.0	135	433.82	423.76
17	7	0.1	135	421.52	437.81
18	7	1.8	135	475.68	455.17
19	8.8	1.0	135	399.28	405.12
20	7	1.0	76.1	420.45	412.78

Table 5.5: Solution for the model

Solution	Agitation	pH	Nitrogen	Predicted value
1	147.29	6.7	1.3	832.637

Table 5.6: Analysis of variance (ANOVA) for the fitted quadratic polynomial model of lipase from *Stenotrophomonas maltophilia*

Sl no	Source	Sum of squares	DF	Mean square	F value	Prob > F
1	Model	6.902E+005	9	76687.30	134.10	<0.0001

2	A	1440.07	1	1440.07	2.52	0.1470
3	B	3540.98	1	3540.98	6.19	*0.0345*
4	C	965.90	1	965.90	1.69	*0.2260*
6	AB	3120.11	1	3120.11	5.46	*0.0443*
7	AC	15.15	1	15.15	0.026	*0.8743*
8	BC	1095.82	1	1095.82	1.92	*0.1996*
9	A^2	2.954E+005	1	2.954E+005	516.64	*< 0.0001*
10	B^2	2.505E+005	1	2.505E+005	438.13	*< 0.0001*
11	C^2	2.697E+005	1	2.697E+005	471.65	*< 0.0001*
12	Residual	5146.68	9			
13	Lack of fit	3553.58	5		1.78	0.2974
14	Pure error	1593.30	4			
15	Cor total	7025E+005	19			

Table 5.7: Important parameters of ANOVA for lipase production from *Stenotrophomonas maltophilia*

Std Deviation	23.91	R^2 square	0.9926
Mean	553.50	Adj. R^2 square	0.9852
C. V %	4.32	Pred. R^2 square	0.9414
Press	40771.26	Adeq precisior	25.730

Figure 5.12: Normal plot of extremely studentized residues and percentage of normal probability

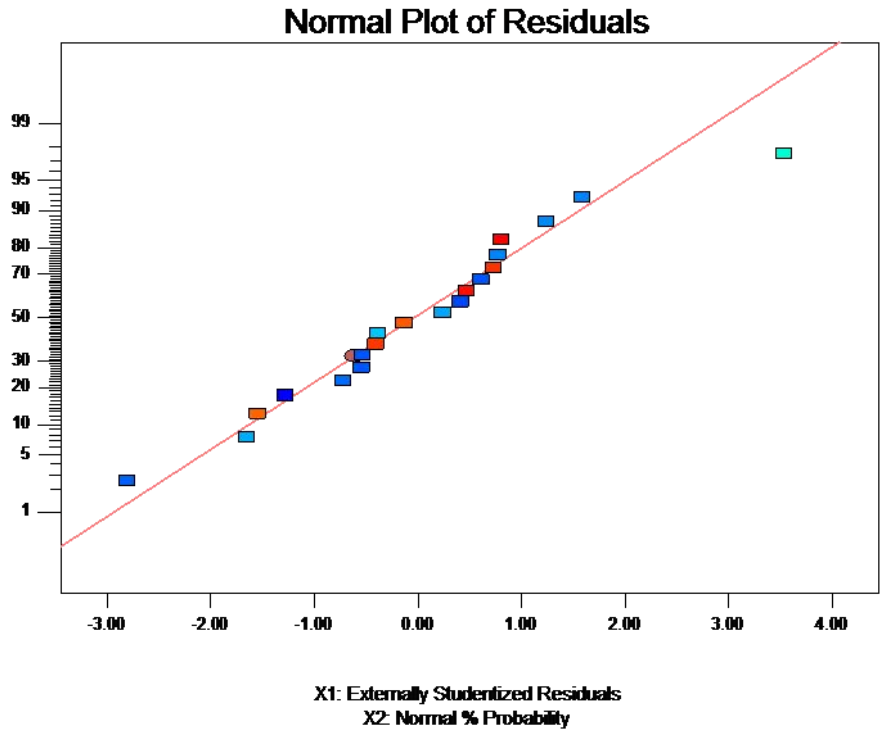


Figure 5.13: Plot of predicted Vs studentized residual

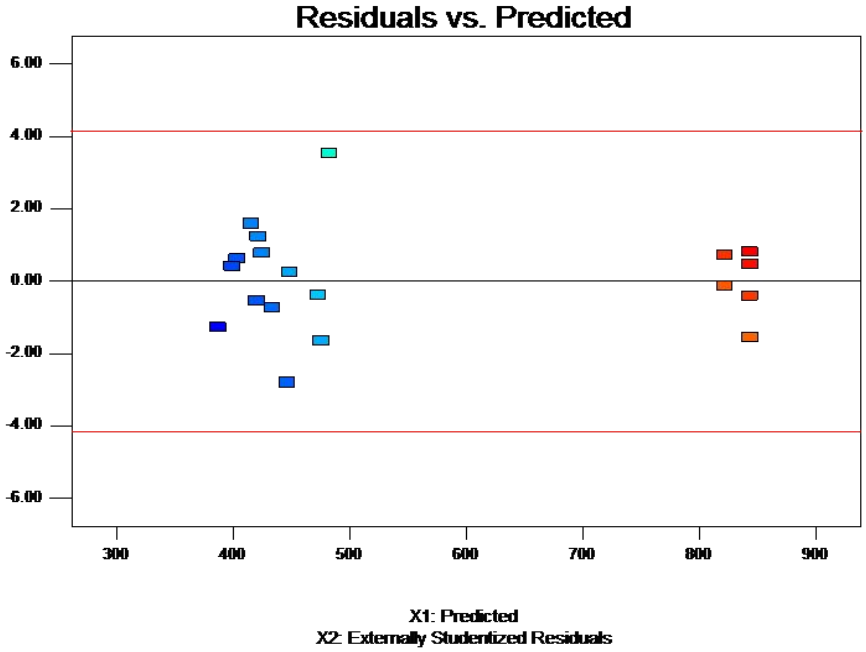
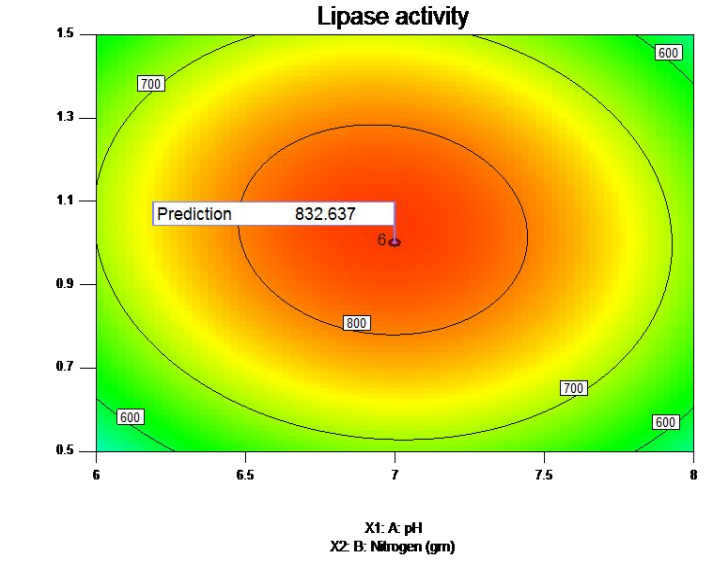


Figure 5.14: 2D contour plot and response surface plot of relative effects of pH and nitrogen concentration. The other variable agitation was kept constant



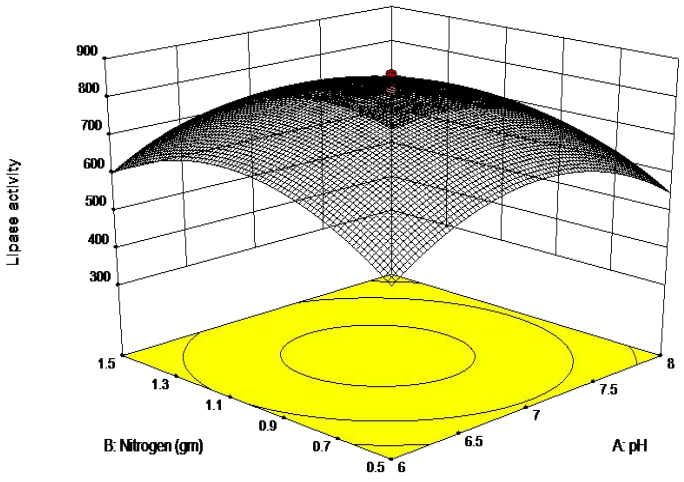
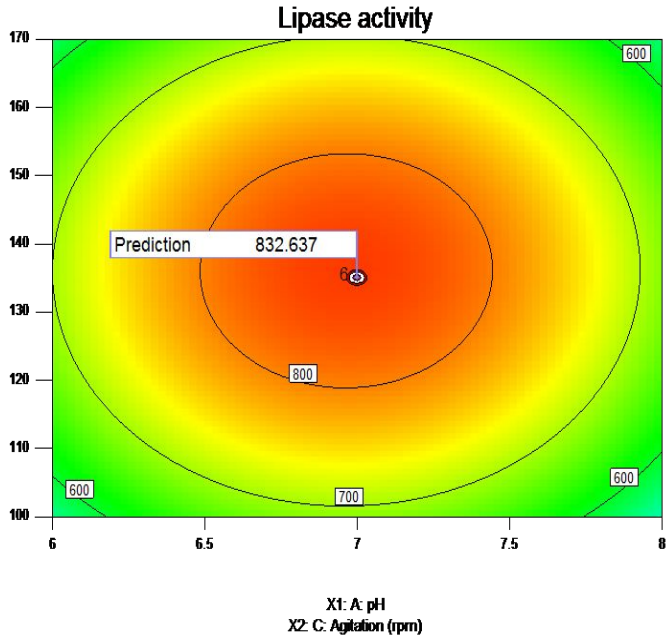


Figure 5.15: 2D contour plot and response surface plot of relative effects of pH and agitation. The other variable nitrogen concentration was kept constant.



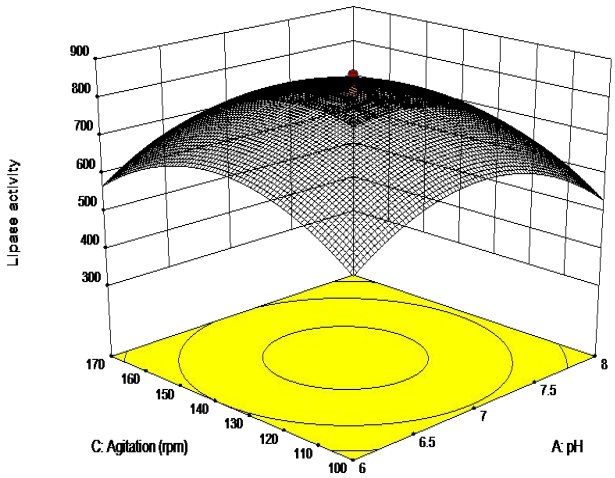
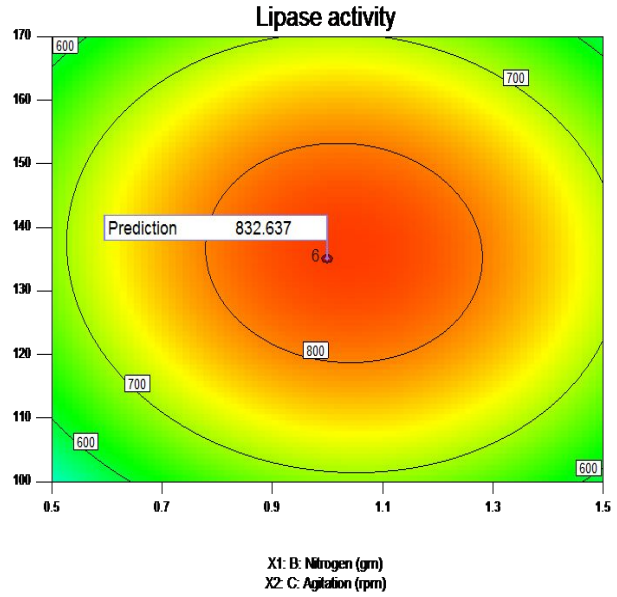
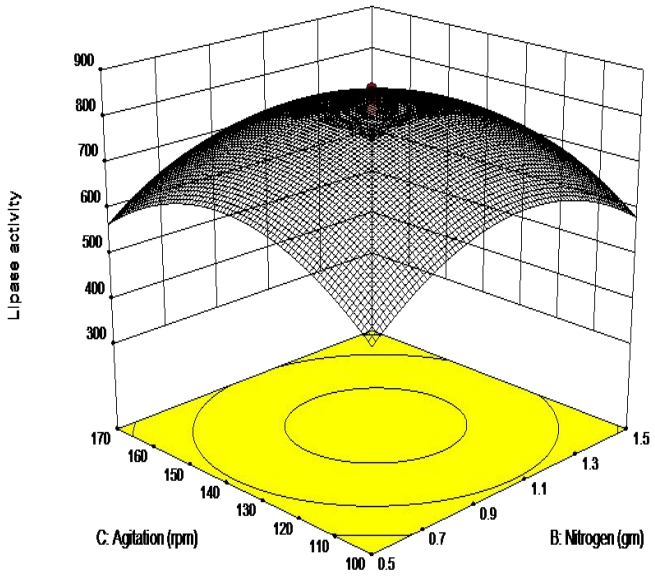


Figure 5.16: 2D contour plot and response surface plot of relative effects of agitation and nitrogen concentration. The other variable pH was kept constant





5.4 Discussion

Lipase production by heterotrophic isolates from Arctic was tested using plate assay method and further confirmed using pNP assay or spectrophotometric assay. p-NP analysis revealed 16 sediment isolates and 5 water isolates showing very high lipase activity compared to other lipase producers. Isolate KS46 showed highest activity of 493.52 U/ml and isolates was identified as *Stenotrophomonas maltophilia.* Beikdashti *et al.* (2012) reported *Stenotrophomonas maltophilia* with lipase activity of 500 U/ml which was very close to the activity reported in the present study. All isolates that tested positive for lipase were checked for their ability to degrade natural lipids such as palm oil, olive oil, groundnut oil, coconut oil and sunflower oil. Most of the isolates were able to degrade groundnut oil (60.67 %) followed by palm oil (52.8 %), olive oil (50.56 %), sunflower oil (50.56 %) and coconut oil (48.31 %).

Optimization studies using one variable at a time have been used by many researchers for optimization of media components and physical parameters. In the present study, various parameters such as carbon source, nitrogen source, pH, agitation, temperature and incubation period was optimized using this approach. Different carbon sources used for the optimization include maltose, glucose, lactose, fructose, sucrose, raffinose, sorbitol and arabinose. Maximal activity was found when the media was supplemented with glucose. Lipase production was supported well by sucrose (87 %) followed by maltose (72 %). Olive oil (1 %) was supplemented in the media as a substrate which acted as an additional carbon source. Optimization of different carbon sources for lipase production by *Candida rugosa* was carried out using 'one variable at a time' approach by Dalman *et al.* (2000) and similar to the present study, glucose was found to be the best carbon source. Ability of glucose to enhance lipase production was reported by Boonchaidung and Papone (2013) in their study to optimize carbon and nitrogen source for lipase production from *Candida* sp.

Different nitrogen sources such as beef extract, yeast extract, peptone, soya peptone, tryptone, sodium nitrate, potassium nitrate, ammonium nitrate, ammonium sulfate and peptone-yeast combination were tested for lipase production. Maximal activity was found when a combination of nitrogen sources, yeast extract-peptone was used. Compared to organic nitrogen sources, inorganic nitrogen sources supported very less lipase production. Superiority of organic nitrogen over inorganic nitrogen was reported by many researchers (Acikel *et al*., 2010; Sirisha *et al*.,

2010; Qamsari *et al*., 2011; Boonchaidung and Papone, 2013; Veerapagu *et al.,* 2013). In the present study, maximum lipase production was observed at the neutral pH values. Veerapagu *et al.* (2013) optimized bacterial lipase production by *Pseudomonas gessardii* isolated from oil spilled soil using 'one variable at a time' method. Pera *et al.* (2006) reported lipase from *Aspergillus niger* which was found to have optimal activity at pH 6.5.

Agitation of the media was also found to enhance the lipase production and agitation of 150 rpm supported maximum lipase production. However agitation of more than 180 rpm had an adverse effect and reduced lipase production. Agitation helps in proper dispersal of nutrients and improves the aeration in the media which would be responsible for enhancing lipase production. Qamsari *et al.* (2011) reported lipase production from *Pseudomonas aeroginosa* KM110 and during their study, an agitation of 150 rpm was found to support maximum lipase production. Salihu *et al*. (2011) showed that optimum agitation for lipase production by *Candida cylindracea* as 150 rpm. Maximum lipase production was observed on 5^{th} day of incubation followed by reduction in lipase production to 78 % on 6^{th} day. Lipase activity reduced to 37 % on 8^{th} day. Maximum growth of the organism was observed on 3^{rd} day and growth started reducing on 6^{th} day and drastically dropped on 7^{th} day. Maximum lipase production was observed at 25 °C and there was very good enzyme production in the temperature range 20-30 °C. Very good lipase production was also noted at low temperatures such as 15 °C and 10 °C. However higher temperatures such as 35 °C and 40 °C adversely affected lipase production. It was noted that growth of the organism was also adversely affected above temperature of 35 °C.

In the present study, RSM was used for optimization of three parameters, concentration of nitrogen source, agitation and pH based on 'one variable at a time' method. In general, it can be concluded that the interaction of tested parameters did not significantly influence the lipase production although nitrogen concentration and pH were found to have slight interactive effects. The effects of different parameters on lipase production were best expressed by the ANOVA table. The analysis revealed the best model to be quadratic regression model. Multiple correlation coefficient or determination coefficient or R^2 value explains the goodness of fit of the model. The R^2 value actually expresses the measure of how much variability in the observed response can be explained by the physical factors used for the study. The R^2 value for the present study was found to be 0.9926 which translates that 99.26 % variation can be explained. The R^2

value of more than 0.9 indicates high correlation and a model value between 0.7 – 0.9 is considered having a good relation (Haaland, 1989). The significance of the model is expressed by Fisher's value or commonly known as F-value. In the present study F value for the quadratic regression model was found to be 134.10 and very low probability value of <0.0001 was observed.

Another factor which explains the goodness of fit is 'adequate precision value'. The adequate precision value' is an index of the signal to noise; signals are controllable factor and noise is the uncontrollable factor. A value of > 4 is considered to be necessary for a model to be a good fit. In the current study, adequate precision value of 25.73 indicated adequate signal. Similarly a low coefficient of variation (CV) indicates precision and reliability of the experiment. Coefficient of variation (CV) for the present model was 4.3 % which indicate high precision and reliability of the experiment.

How well the model fits the data is expressed as a measure of 'Lack of fit'. If the ANOVA table express a significant 'Lack of fit' then it is considered as not a good predictor of the response and should not be used. In the current study 'Lack of fit' for the model was detected as 0.2974 and was found to be non significant. According to the ANOVA table (Table 5.6), the model terms B, AB, B^2, A^2 and C^2 values were considered significant since values of prob>F less than 0.05. However B^2, A^2 and C^2 values were most significant since prob>F values were < 0.0001.

The 2D contour plots and 3D response surface plots generated by the software express the interactive effects of factors tested in the study. The response surfaces are plotted in such a way that one variable is kept constant while other two varied within their experimental range. Figure 5.14 represent the interactive effects of nitrogen and pH, as can be observed from this figure, the 2D contour plot was circular in nature with a slight tilt and 3D response surface was convex. This suggested that there was slight interaction between pH and nitrogen but it was not significant enough to affect lipase production, and there is definite optimum values for the factors. Figure 5. 15 show the interactive effects of the factors pH and agitation. The 2D contour plot was found to be fairly circular and 3D response surface plot was a raised convex. These results indicated the existence of definite optimum values for the factors but interactive effects were not significant enough to influence lipase production. Figure 5.16 represents the interactive

effects between nitrogen and pH; no significant interactive effects were observed for the factors and definite optimum values exist for the factors.

The solution for the model (Table 5.5) suggested that optimum pH that supports maximum lipase production to be 6.7. Similar observation was made by Papagora *et al.* (2013), the optimization of lipase production by yeast *Dabaryomyces hansenii* using RSM showed the optimum pH value to be 6.4. Lipase production by *Pseudomonas aeruginosa* was optimized by Ruchi *et al.* (2007) using response surface methodology and optimum pH was noted as 6.5. Most of the studies reported that optimum pH value for lipase production is 6 to 7 or slightly alkaline pH. Solution for the model suggested that optimum concentration of nitrogen source to be 1.3 g. In the present study combination nitrogen source, peptone – yeast extract was tested in different concentrations using response surface methodology. Nitrogen concentration of 13.4 g/L was found to be optimum for lipase production by *Stenotrophomonas maltophilia*. Acikel *et al.* (2011) optimized media components using response surface methodology for production of lipase from *Rhizopus delemar.* In their study, yeast extract was used as the nitrogen source and concentration was optimized to be 0.53 g/L. Salihu *et al*. (2010) used face centered central composite design (FCCCD) for optimization of media components for lipase production from *Candida cylindracea*. One of the significant components that influence the lipase production was found to be nitrogen source. Teng and Xu (2007) used statistical approach to optimize the parameters for lipase production by *Rhizopus chinensis* and nitrogen source peptone, at a concentration of 4.06 % (w/v) was reported to be optimum for lipase production.

Solution of the model suggested that optimum speed of agitation for maximum lipase production to be 147.3. Agitation is known to enhance the lipase production (Salihu *et al*., 2011). The optimal level of agitation caused an increase in aeration of the media and resulted in increase in nutrient uptake. This is the possible reason for increase in lipase production at optimum agitation (Beg *et al*., 2003). Similar to the present study, speed of agitation for lipase production was previously reported as 150 rpm by Qamsari *et al.* (2011) and Salihu *et al.* (2011). Teng and Xu used statistical approach to optimize the lipase production and agitation of 200 rpm supported maximum lipase production.

In the current study, optimization using RSM yielded 1.7 fold increase in lipase production by *Stenotrophomonas maltophilia.* After optimization of several components Papagora *et al.* (2013)

reported 2.28 fold increase in lipase production from *Dabaryomyces hansenii* using the RSM approach. Edrahimpour *et al.* (2008) used response surface methodology for optimization of a thermostable lipase from *Geobacillus* sp. and obtained 4.7 fold increase in lipase production. 1.4 fold increase in production of lipase was obtained from *Enterobacter aerogenes* using RSM by Kumari *et al.* (2009) was obtained. Enhanced production of lipases was also reported from many microorganisms using RSM optimization such as *Pseudomonas cepacia* (Wu *et al*., 1999), *Pseudomonas aeruginosa* (Gilbert *et al*., 1991), *Rhizomucor miehei* (Demirkol *et al*., 2015), *Candida antarctica* (Ognjanovic *et al.*, 2009) and *Arthrobacter* sp. (Sharma *et al*., 2009).

A total of 21 potent lipase producing isolates were detected in the study using p-NP assay and natural substrate specificity of these isolates revealed a high proportion of groundnut oil degrading organisms. The most potent lipase producing isolate was identified as *Stenotrophomonas maltophilia* with 492.52 U/ml activities. Optimization of culture conditions for maximum lipase production by this strain was carried out primarily by 'one variable at a time' method followed by optimization using RSM for 3 factors- pH, nitrogen and agitation. No significant interactive effects were observed for pH, nitrogen and agitation using RSM although definite optimum values were detected. Experimental validation of the model was found to be 821.14, which was close to the predicted response of 832.63. Using RSM method, 1.7 fold increase in lipase production was achieved.

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Chapter 6
Lipase purification, characterization and applications

Contents:

Lipases are serine hydrolases containing characteristic catalytic region with the consensus sequence G-X_1-S-X_2-G representing amino acids, glycine (G), Histidine (X_1), Serine (S), and glutamic acid/ aspartic acid (X_2). Lipases are used in various industries and purity requirement of the enzyme differ from industry to industry. Stages in purification of lipases follow the same path as most of the proteins which involve extraction or recovery of proteins, concentration, primary purification followed by chromatography. Improved purification techniques such as immunopurification, two phase aqueous extraction system and membrane filtrations are also used for bacterial lipase purification. In all standard procedures, at each stage of purification activity of the enzyme and protein content is evaluated (Palmer and Bonner, 2008).

Purified enzymes are often subjected to characterization studies including molecular mass analysis, identification of lipase gene and sequencing, determination of amino acid composition and deduction of three dimensional structures. Characterization lipase is necessary for proper application of enzymes in industries and stability of enzymes is often revealed by characterization analysis.

Microbial lipases are used in industries such as detergent, food processing, enantiopure chemicals in pharmaceutical industries, processing of fats and oils and bioremediation. Lipases are the most widely used enzymes in organic synthesis and more than 20 % biotransformations are performed with lipases. Cold adapted lipases probably are structurally modified by an increasing flexibility of the polypeptide chain enabling an easier accommodation of substrates at low temperature. Cold adapted lipases are largely distributed in microorganisms existing at low temperatures nearly 5 °C. Although a number of lipase producing microbes are used for industrial productions, only a few bacteria and yeast were exploited for the production of cold adapted lipases (Joseph, 2006). Thus, studies on lipase production from psychrophiles or psychrotrophs and its characterization would help in the development of potent strains capable of producing cold active lipase for industrial applications.

6.1 Review of literature

6.1.1Purification of cold active lipase

Purification of lipase is not considered to be necessary for commercial applications by majority of the industries. However, industries such as pharmaceuticals, cosmetics and fine chemical synthesis use lipase in the purified form. Purification of the enzyme is also required for successful determination of primary amino acid sequence and elucidation of three dimensional structures. Purification is an important stage in X-ray analysis of lipases to establish the interplay between structure and function. Purified lipase is also used in kinetic studies to understand the catalytic mechanisms, synthesis and group exchange of esters. Large scale industries demand purification strategies employed to be cost effective, high yielding, less waste generating and amenable to large scale operations (Gupta *et al*., 2004).

Purification process starts with the extraction of the enzyme from the microbial sources and recovery process varies with intracellular and extracellular enzymes. Most, if not all, of the microbial lipases produced in industries are extracellular in nature which is usually recovered from the fermentation media. Cell separation is usually carried out by centrifugation and at times by filtration for sensitive enzymes. Enzymes present in the fermentation media are in highly diluted form and hence one or more concentration steps are carried out prior to purification. Concentration of culture broth is carried out by filtration, precipitation with ammonium sulphate

or organic solvents. Ultra filtration was introduced as an alternative to concentration by evaporation method and used in many industries. Filtration using dialysis is also followed for removal of salt and changing salt concentrations (Saxena *et al.*, 2003).

The most widely used method for concentration of lipases is precipitation. Ammonium sulphate is a desirable choice for precipitation of proteins because it's inexpensive, highly soluble, and stability of the proteins is maintained. Acetone precipitation is also found to be highly popular for concentration of culture supernatant and is used in various concentrations such as 1: 2 or 1: 3. Acetone, ethanol and ammonium sulphate follow a similar mechanism of lowering the solubility of the desired protein. Precipitation gives a high average yield of enzymes and it is often considered as a kind of limited purification process; such enzyme preparations are used for detergent formulations (Saxena *et al*., 2003).

Proper purification of the protein demands use of chromatographic techniques such as ion exchange chromatography, affinity chromatography and gel filtration chromatography. Lipases show distinct hydrophobic nature and this property of lipase is applied for its advanced purification using aqueous two-phase systems, reversed micellar systems and hydrophobic interaction chromatography. (Saxena *et al*., 2003; Gupta *et al*., 2004).

Diethylaminoethyl (DEAE) anion exchanger is used for most of the lipase purification procedures (Shah and Bhatt, 2011). DEAE-cellulose resin has been widely used by many researchers for the purification of lipase (Isobe *et al*., 1988; Sztajer *et al*., 1992; Choo *et al*., 1997; Kim *et al*., 1997; Abdou *et al*., 2003; Ghori *et al*., 2011; Shah and Bhatt, 2011). DEAE-cellulose is an attractive choice since it is inexpensive and found to have satisfactory levels of purification. DEAE – Sepharose has been alternatively used for ion exchange chromatographic purification of lipase (Lee and Rhee, 1993; Kim *et al*., 2000; Lee *et al*., 2001; Wang *et al*., 2009). Q-sepharose has also attracted many researchers attention because of high levels of purity, however it is found to be expensive compared to other resins used. Hydrophobic interaction chromatography using Butyl toyopearl and butyl speharose for lipase purification has been reported by Sugihara *et al.* (1991), Kim *et al.* (1994) and Kojima *et al.* (1994). Affinity adsorbents usually used for enzyme purification include N-benzoyloxycarbonyl phenylalanine agarose, aprotinin – agarose, and benzamidine-sepharose (Petinate *et al*., 1999; Joo *et al*., 2001).

In general, chromatographic techniques are found to be expensive and new techniques using liquid –liquid extraction with reverse micellar system and aqueous two phase has been proposed as less expensive alternative (Gupta *et al*., 2004). Extraction using two phase system has been suceessfully applied for lipase purification from *Pseudomonas luteola* (Terstappen *et al*., 1992) and *Pseudomonas cepacia* (Litthauer *et al*., 2002). Aqueous two phase uses two incompatible polymers for separation proteins by partitioning it depending on the physiochemical properties (Gupta and Rathi, 2004). Reverse micellar system consist of water droplets dispersed in organic solvents and stabilized by surfactant monolayer. Vicente *et al.* (1990) could separate and purify lipase from *Candida viscosum* using reverse micelles with benzene. Skagerlind *et al.* (1992) and Yamada *et al.* (1993) have reported high efficiency of micellar systems for lipase reactions, purification. Immunopurification technique is considered as the most efficient method for selective purification. A cutinase lipase produced by *Escherichia coli* was purified using IgG-affinity chromatography (Bandmann *et al*., 2000). However high cost involved in immunopurification has made this procedure a less popular choice among researchers and industries (Gupta *et al*., 2004).

6.1.2 Characterization of cold active lipase

Most of the cold active lipases have optimal activity at 20 or near 20 °C and they are stable over a wide range of temperatures (Joseph *et al*., 2007). Suzuki *et al.* (2001) identified cold active lipase producing *Acinetobacter* sp which was found to have optimal activity at 20 °C. A strain of *Bacillus sphaericus* was reported to have optimal activity at 15 °C at pH 8 (Joesph, 2006). Psychrotrophic bacterial isolates *Pseudoalteromonas* and *Psychrobacter* were shown to have wide range of optimal activity between 20 – 30 °C and interestingly at pH 7-8 (Zeng *et al*., 2004; Zeng *et al*., 2004 b; Zeng *et al*., 2007). Most of the cold active lipases were found to show minimal activity at 50 °C and inactivated at 65 °C.

Characterization of some lipases from psychrotrophic bacteria was found to show interesting results; even though the optimal activity was at above 30 °C, they showed good activity at low temperature, even at 5 °C. *Corynebacterium paurometabolum*, isolated from Uttaranchal, India was found to have optimal activity at 25 °C and retained 60 % activity at 20 °C and optimum pH was pH 8.5 (Joshi *et al*., 2006). Alkaline lipase isolated from *Acinetobacter calcoaceticus* had optimal activity at 40 °C and reported to have a relative activity of 85 % at 20 °C and its optimal

pH was 9. Alkaline lipase produced by psychrophilic *Pseudomonas* sp. showed optimal activity around 37 °C but retained 80 % activity at 20 °C and showed high activity between pH 7-9 (Kiran *et al*., 2008). Psychrotrophic bacteria *Serratia marcescens* had an optimal activity at 37 °C while 90 % activity was observed at 5 °C and pH ranged between 7 to 9 (Abdou, 2003).

Cold active lipases were found to respond to the presence of metal ions and organic solvents. Studies have shown that some of the divalent cations such as Ca^{2+}, Mg^{2+}, Zn^{2+} and Mn^{2+} were found to have enhanced the lipase activity (Dheeman, 2011). It is suggested that these cations protect the enzymes from thermal denaturation and play an important role in maintaining the active conformation of enzyme at higher temperatures (Kumar and Takagi, 1999). Inhibitory effect of cations such as Cu^{2+}, zn^{2+}, and Cr^{3+} was also reported (Lee *et al*., 2001; Ulker *et al*., 2010; Tripathi *et al*., 2014). Many researchers have shown inhibitory effects of ethylene – diamine tetraacetic acid (EDTA), methanol, ethanol and isopropyl alcohol (Lee *et al*., 2003; Kiran *et al*., 2008) on lipases.

Stability of lipases in organic solvent is a desirable quality in many industries since polymerization and transesterification process often demands harsh conditions of addition of organic solvents (Sharma and Kumar, 2014). Gang *et al*. (2011) reported that technological quality of lipase can be improved by using organic solvents instead of aqueous media. The improved quality of the enzyme is attributed to enhanced activity and stability, regiospecificity, better solubility of the substrate and ease of product recovery. Kumar and Kanwar (2011) showed the use of organic solvent stable lipase on immobilized kieselguhr. Similarly, surfactant stable enzymes find their use in many industries such as detergents and dish washers. Surfactants are often found to enhance the activity of lipase possibly due to the role of surfactants in reducing surface tension, preventing aggregation of enzyme and increasing the accessibility of substrates to enzymes (Balan *et al.*, 2013)

Molecular mass of bacterial lipases were found to range between19 KDa to 85 KDa and most of them range between 25 to 50 KDa (Gupta *et al*., 2004). Molecular weight of cold active lipase of *Aeromonas* was found to be 50 KDa. Cold active lipase from *Serratia marcescens* was found to have a molecular weight of 52 KDa. Joseph *et al.* (2006) reported that molecular weight of a bacterial lipase active at 15 °C as 40 KDa. Lipase produced by a strain of *Pseudoalteromonas* active at 4 °C, had a molecular mass of 85 KDa (Zeng *et al*., 2004). Cold active lipase from

Pseudomonas sp. reported by Rashid *et al.* (2001) was found to have a molecular weight of 49.9 KDa.

Lipase genes are widely distributed among bacteria and have been isolated from different habitats. There is a steady increase in the detection of novel microbial lipase genes in the last two decades and its expression in suitable hosts may help to produce lipases with desirable qualities. Studies on lipase gene, gene expression, and its regulation may throw more light on functional characteristics of the enzyme. Bacterial lipase gene studies have a significant share in enzyme research due to the possibility of lipases acting as virulence factors in pathogenic bacteria and it is one of the most widely used industrial enzymes. Depending on the amino acid homology many different kinds of lipases have been identified and grouped into six families (Arpigny and Jaeger, 1999).

Industrially used lipases are extracellular in nature and the journey of lipase from cell to the media requires concerted efforts of many cellular processes from transcription of structural genes to translocation and secretion. It is thus evident that regulation of gene expression affects each stage of the process and ultimately the quality and quantity of the enzyme secreted into the media. Several researches indicate the versatility in the regulation of gene expression. Quorum sensing kind of lipase gene regulation was reported from *Xenorhabdus nematophilus*, an insect pathogen and its lipase synthesis was found to be stimulated by N-β- hydroxybutanoyl homoserine lactone (HBHL) (Dunphy, 1997). One of the most widely distributed lipase gene is *lipA* gene; *Acinetobacter calcoaceticus* was shown to produce several lipolytic enzymes coded by *lipA* gene (Kok *et al*., 1995). Detailed studies were carried out on this gene by fusion with *lacZ* gene and integrated into *A. calcoaceticus* chromosome. It was also noted that fatty acids produced by hydrolysis of lipids heavily repress the expression of *lipA* gene (Kok *et al*., 1996).

Pseudomonas sp. has been thoroughly studied for regulation of lipase gene expression. *Pseudomonas aeruginosa* contains lipase operon *lip/lif* and detailed analysis of this operon revealed that transcription of the operon from P1 requires an additional sigma factor δ^{54} (Jaeger *et al*., 1996). Existence of additional lipase regulator called *lipR* was also recognized since δ^{54} dependent promoters require the presence of cognate transcriptional activator (Shingler, 1996). The global regulator GaC A is known to activate transcription of Rh1R which is a quorum sensing activator. Once activated Rh1R activate transcription of *lipQ/R* two component regulator

system of lipase gene. It was also reported that the sensor kinase lipQ could be activated by unknown environmental stimuli or by periplasmic signals such as unfolded or non-secreted enzyme. LipR is a transcriptional activator that binds to upstream activating sequences proceeding the σ^{54} depended promoter P1 of lipase promoter (Rosenau and Jaegar, 2000).

Isolation of novel lipase genes are difficult due to many reasons such as toxicity of expression to heterologous hosts and necessity of additional helper protein required to obtain functional lipase expression. Besides, a very low homology was observed between the lipase genes which makes the PCR amplification and cloning difficult. One of the established methods for obtaining new lipase genes is to sequence the N-terminal amino acid of lipase and design complimentary degenerate oligonucleotide probe (Bills *et al*., 2002). It is also suggested that construction of DNA library from lipase producers that can be screened using specific oligonocleotide probes for lipase gene. Alternatively, these probes could be used as a tool for construction of mini-library enriched for the target lipase (Oh *et al*., 1999; Bills *et al*., 2002).

Na –na *et al*. (2010) isolated lipase gene and partial gene sequencing was performed from *Staphylococcus hominis*. The genus *Staphylococcus* is well studied for lipase productionand lipase is biosynthesized as prolipase in cytoplasm of *Staphylococcus*. Characterization of lipase gene from *Antrodia cinnamomea* was found to have lipase consensus sequences VTVVGHSLGA which encodes a polypeptide of 303 aminoacids (Chu *et al*., 2008). Cai-Hong *et al.* (2008) isolated a novel lipase gene from *Pseudomonas* sp. and sequence analysis was carried out; the study revealed the lipase consensus sequence GXSXG coding 566 amino acid polypeptide. Lee and Kim (2010) carried out an interesting study in which *Burkholderia capacia* and *Pseudomonas aeruginosa* were isolated from activated sludge soil and these strains were found to harbour lipase gene *lipA* coding for Gly-His-Ser-Gln-Gly. Lipase gene from *Pseudomonas alcaligenes* was isolated and details of its promoter and upstream activating sequence was revealed by systematic mutational analysis (Cox *et al*., 2001). Zuo *et al.* (2010) isolated novel lipase gene from oil contaminated soil and expressed it in *Escherichia coli*. Yuhong *et al.* (2009) studied diversity of lipase in glacier soil by analysis of metagenomic DNA fragments.

6.1.3 Applications of lipases

The two important properties of lipases that are most useful for industrial applications include high catalytic activity at low or moderate temperature and thermolability. Cold active lipases are used in the fat and oleochemical industries for preparation of value added fats. Lipases could be used for transesterification catalysis to convert cheap oils such as palm oil to cocoa butter (Sharma *et al.,* 2001). Lipases are being used for manufacturing of polyunsaturated fatty acids (PUFA) which are often used for pharmaceutical purposes (Jaeger *et al*., 1998; Nakajima *et al*., 2000). Butter and margarine production is being supported by lipases using interesterification catalysis by some industries (Tanaka *et al*., 1981). The use of cold active lipase to carry out industrial hydrolysis of tallow has been proposed. Lipases have been used for production of biodegradable polymers. Butanol and oleic acid are directly esterified to produce 1-Butyl to decrease the viscosity of biodiesel in winter use. Some esters such as trimethylpropane are also biosynthesized to use as lubricants. Linko *et al.* (1998) also suggested lipases for Aromatic polymer synthesis.

Textile industry use lipases for many purposes such as removal of lubricants, to improve absorbency of fabric, desizing of denims and cotton fabrics, to reduce streaks and cracks in denim, and to increase levelness of dyeing. Synthetic fibers such as polyesters are modified with lipases for the use in yarn production, fabrics, rugs and other consumer items. Enzymatic degradation of polyesteramide by treatment with enzyme mixtures of esterase, lipase and protease also improve the quality of textile fiber.

Detergents industry use lipase for hydrolysis of fats, in industrial laundry and household detergents. Reports suggest that 1000 ton of lipases are used every year in detergent industry (Godfrey and West, 1996). Feller and Gerday (2003) reported that use of cold active lipase in detergent industries is advantageous due to possible reduction in energy consumption and wear and tear of fabrics. Cold active lipases are also found to be used in preparations of dish wash, clearing of drains clogged by lipids and domestic waste water treatment system (Bailey and Ollis, 1986)

Food ingredients and beverages undergo undesirable side reactions in high temperature and quality of the food is often comes down. Thus, cold active lipases are used for carrying out many

catalytic reactions in food industry. Cold active lipase from *Pseudomonas* is widely used in the industry for the synthesis of n-heptane. Lipases are also used for gelling of fish, improving food texture, flavor modification and production of several fatty acids and inter-esterification of fats (Cavicchioli and Siddique, 2004).

Bioremediation of agricultural wastes

Cold active lipases have great potential to be used for environmental bioremediation processes. Utilization of lipase to remove oil spills in the cold environments such as temperate environments are also recommended. Waste water treatment of fat contaminated cold environment has been suggetsted by (Buchon *et al*., 2000). Another important area of utilization of lipase for environmental remediation is in agro-industrial field. Agriculture related industries such as oil mills and grain processing industries generate large volume of waste material. These waste materials are rich in nutrients thus often used for preparation of animal feed such as peanut meal and coconut meal. However majority of this waste is disposed off or used as landfill, hence utilization of such lipid rich agro-industrial wastes could be exploited as a substrate for lipase production.

Although all agree that enzymatic processes are favorable to the environment than traditional processes, the economic viability of replacing chemical methods with enzymatic processes still poses doubts (Abdelmoez, 2013; Jayannathan and Nielsen, 2013). Thus, exploitation of agricultural residues for enzyme production and other value added products would be of greater significance in future biotechnologies mainly due to their low cost, accessibility and nutrient content (Salihu *et al.,* 2012). One of the main area in lipase research now involve the use of different microbes, supplements and substrates to obtain the best combinations for production of high value lipases by operational conditions that offer low production cost at industrial scale (Rigo *et al.*, 2010).

Agro industrial waste and their complex organic contents constitute a significant source of residual nutrients which serve as a rich media for microbial growth and production of the enzymes (Alonso *et al.,* 2005). Solid state fermentation had been successfully used for bacterial lipase production using agricultural and oil industries waste (Kapoor *et al.*, 2000; Kashyap *et al.*, 2003; Virupakshi *et al.*, 2005; Sabu *et al.*, 2006). Various reports suggest that apart from the

nature of solid substrate (water activity and particle size), physical parameters (temperature, pH, and heat conductivity) also greatly influence the production of lipase on agricultural waste (Echevarria, 1991; Barrios- Gonzalez *et al.*, 1993; Pandey *et al.,* 1994; Liu and Tzeng, 1999).

Even though agricultural residues are produced in large quantities in developing countries, they are mainly utilized as animal feed and landfills (Salihu *et al.*, 2012). India is world's second largest fruit and vegetable producer and encounters waste close to 25 % worth of produce. It is also significant that 40 % of loses occur at post harvest and processing levels while in industrialized countries loses happen only at retail and consumer levels (NIAM, 2012). In recent times, agricultural wastes have been made to use in biotechnological processes such as production of value added compounds and substrates for microbial isolation. These help in disposal problems which otherwise lead to environmental pollution (Pandey *et al.*, 2000; Graminha *et al.*, 2008; Mussatto, 2009). Oil cakes from different oil extraction industries have been utilized for fermentative lipase production mainly because the residual oil content serves as inducer for lipase production (Singhania *et al*, 2008; Salihu *et al.*, 2012). During the present study - groundnut cake, rice bran, wheat bran, neem cake, and coconut cake were tested for fermentative lipase production. Further, the optimal physical conditions for maximum production of enzyme were also determined.

Coconut oil is rich in saturated fatty acids with low content of polyunsaturated and monounsaturated fats hence the waste generated from coconut oil processing is also found to be rich in lipids. Solid waste content produced from coconut milk extraction contains up to 24 wt % oil content (Sulaiman *et al*., 2013). However majority of the waste generated is either used for animal feed preparation or disposed off. Utilization of coconut mill waste for the production of bacterial lipase has been reported (Kanmani *et al.*, 2015).

Azadirachta indica commonly known as neem is indigenous to Indian subcontinent. Neem oil is extracted from the seeds which are used for many purposes such as insecticide, medicine and for rice cultivation. Residues from neem oil extraction industry are currently used as a fertilizer and land fill. Ananthi *et al*. (2014) used neem cake as a substrate for the production of lipase from *Bacillus cereus*. Thus neem cake could be used as an alternative media for lipase production

Groundnut is known to be the most important oil and protein producing crop in the world and subsequently generate large amount of agricultural waste. Usual practice of disposal of this waste which is rich in fibers, proteins and polyphenolics is by landfill. Majority of the groundnut is utilized for oil production, preparation of peanut butter and other food products. These processes generate large amount of waste which is often used either for by product preparations such as pea nut meal or treated and disposed off (Zhao *et al*., 2012). Groundnut has 49 g of fat/ 100 g and is rich in monounsaturated fats (24 g), poly unsaturated fat (16 g) and low in saturated fats (7 g). Thus waste from groundnut processing industries has great potential to be used as a substrate for lipase production.

Rice bran is a byproduct of the rice milling process and rich in nutrients and antioxidants. Rice bran is rich in oils constituting 12-13 % of the total content. Rice bran oil has 20 g of saturated fats, 35 g of polyunsaturated fats, and 39 g of monounsaturated fats, thus has a high potential to be used as a lipid substrate. Ananthi *et al*. (2014) reported use of rice bran for lipase production from Bacillus cereus. Faisal *et al*. (2014) reported lipase production by *Pseudomonas* sp using different bran including rice bran.

Similar to rice bran, wheat bran is a byproduct of wheat milling process and is used in various industries such as food, animal feed, medicine and fermentation. Wheat bran is rich source of nutrients including fibers, proteins and fats. There are many reports of wheat bran being used as a substrate for lipase production. Falony *et al.* (2006) used wheat bran for production of lipase from fungus *Aspergillus niger*. Sekhon *et al.* (2004) used *Bacillus megatarium* for lipase production using wheat bran.

6.2 Materials and Methods

6.2.1 Production of Lipase

Organism used

The selected strain of high lipase producing *Stenotrophomonas maltophilia* was used for the study

Medium used

The medium used for lipase production was glucose yeast peptone medium (GYP medium, Yuan *et al*., 2007) which was supplemented with 1% olive oil and minerals. Modified glucose yeast peptone broth (200ml) was prepared in 500 ml screw capped flasks, pH adjusted to 7, and sterilized at 121 °C for 20 min in an autoclave.

Composition of modified glucose yeast peptone media

Ingredients	Concentration
Peptone	10g
Glucose	20g
Yeast extract	5g
$MnSO_4$	1g
$MgSO_4$	5g
Distilled water	1000 ml
pH	7
Olive oil	10 ml

Inoculum preparation

Stenotrophomonas maltophilia was inoculated onto nutrient agar slant and incubated at 20 °C for 3 days. Following the incubation period, a loop full of culture was inoculated into 10 ml of

Zobell's marine broth and incubated at 20 °C for 48 hr which was then used as a seed culture to inoculate GYP medium.

Inoculation and lipase production

The optical density of the seed culture was read at 600 nm in a UV- Visible spectrophotometer. An adequate volume of seed culture was added to obtain an absorbance of 0.5 at 600 nm for the total volume of production media

Inoculated production media were then incubated at 20 °C for five days in a cooling rotary shaker at 160 rpm. After completion of the incubation period, medium was centrifuged in a cooling centrifuge (eppendorf, Germany) at 10000 rpm for 15 min at 4 °C. Lipase activity and specific activity was checked to confirm lipase production. The cell free supernatant was used as the crude enzyme.

6.2.2 Purification of lipase

The cell free culture supernatant was subjected to purification which involved various steps such as filtration, precipitation by organic solvent and ion exchange chromatography.

Filtration

Crude extract of enzyme was pooled and filtration was carried out by using cellulose nitrate membrane filter of pore size 0.2 µ under vacuum. The filtrate was collected and used for the next stages of purification

Cold acetone precipitation

For concentration of the sample to 1 ml of the filtered sample 2 ml of (two fold volume) acetone stored at -20 °C was added. This mixture was vortexed and then stored at -20 °C for 2 hr. The sample was then centrifuged at 14000 rpm at 4 °C for 30 min, supernatant was discarded and the pellets were dissolved in Tris buffer (pH-6) and subjected to DEAE- Cellulose ion exchange chromatography

DEAE- Cellulose ion exchange chromatography

DEAE- Cellulose resin was purchased from sigma (U.S.A) and activated as per manufacturer's instructions. The resin was packed into the column (1.5 × 20 cm) and care was taken in such a way that air bubbles were not trapped. All the buffers were filtered before each run and the column was pre-equilibrated with 20 mM Tris buffer, pH 7.

Precipitated enzyme (1.5 ml) was loaded into the pre-equilibrated column. Following this, column was washed with the same buffer (20 mM Tris buffer, pH -7) to remove the unbound proteins until the absorbance was read zero at 410 nm. The bound protein was eluted by applying linear gradient of NaCl (0 to 1.0 M) at a flow rate of 0.5 ml/ min and absorbance of the elute was monitored at 410 nm. Eluted fractions (1.0 ml) were collected in microcentrifuge tubes and analyzed for lipase activity.

6.2.3 Determination of molecular weight

Determination of molecular weight of purified lipase was carried out using one dimensional SDS-PAGE. Stacking gel of 4 % was prepared with 0.5 M Tris HCl at pH 6.8 and resolving gel of 12 % was resolving was prepared with 1.5 M Tris HCl at pH 8.8 (Laemmli, 1970). Enzyme samples collected from each stage such as crude enzyme extract, acetone precipitated sample and purified samples were mixed with sampling buffer and subjected to SDS-PAGE.

Sampling buffer

Distilled water	3.55 ml
0.5 M Tris HCl (pH 8)	1.25 ml
Glycerol	2.5 ml
10% SDS	2.0 ml
0.5% bromophenol blue	0.2 ml
β-mercaptoethanol	50 µl

Electrophoresis was carried out at a constant current (150V) using vertical electrophoresis running unit (BioRad, USA). A molecular weight marker – medium size (Merck, U.S.A) was also added. Run was allowed to continue until dye front reached at the bottom of the gel. After

the completion of electrophoresis is completed, glass plates were carefully removed from the unit. Gel was then scooped carefully from the glass plates and stained with 0.025 % Coomassie brilliant blue R250 (methanol 40 %, acetic acid 10 %, Coomassie brilliant blue 0.025 %). After the staining procedure, decoloration was performed with destaining solution (methanol 40 %, distilled water 50 %, 10 % acetic acid). The gel was then visualized under the UV transilluminator (GelDoc EZ imager, BioRad).

Zymogram analysis

Activity of the monomeric band obtained in the SDS-PAGE analysis was checked using zymogram analysis. The single major band was cut and scooped out of the gel carefully and this band is dissolved in Tris buffer (pH 7). Buffer containing the band was kept at 4 °C overnight following which lipase assay was carried out using pNPP assay.

6.2.4 Characterization of enzyme

Effect of pH on enzyme activity and stability

The effect of pH on lipase activity was determined over a pH range of 5 to 12. Stability was checked using different buffers such as acetate buffer (pH 5-6), sodium phosphate (pH-7), Tris Cl (pH 8-9) and Glycine NaOH (pH 11-12) in the reaction mixture. The activity of the enzyme was expressed in terms of relative activity.

The stability of the enzyme at a pH range of 5-12 was tested by pre-incubating the enzyme in the respective buffers for 1 hr and the residual activity (%) was measured. The percentage residual activity was calculated by comparing the activity of the treated enzyme with that of the untreated enzyme which serves as a control and the value was taken as 100 %.

Effect of temperature on the enzyme activity and stability

The effect of temperature on enzyme activity was assessed by carrying out the lipase assay at different temperatures from 10 to 50 °C. The difference in activity was calculated as percentage relative activity.

The temperature stability of the enzyme was assayed by pre-incubating the enzyme at different temperature from 10 to 50 °C for 1 hr followed by assay of the residual activity under standard assay conditions. The activity of the untreated enzyme was taken as 100 %.

Effect of metal ions on enzyme activity

The influence of different metal ions on the lipase activity was tested as follows. Different metal ions in their salt form were prepared at a final concentration of 5mM and purified enzyme was added to the metal ion solution (Dheeman., 2011). This mixture was incubated for 30 minutes at 20 °C. The percentage relative activity was calculated by considering the lipase activity in the absence of metal ions at 20 °C and pH 7 as 100%.

Effect of surfactants on enzyme activity

To determine the stability of enzyme in the presence of surfactants, the purified enzyme was preincubated with the surfactants. The surfactants such as SDS, Triton X- 100, Tween 80, Tween 60 and Tween 20 were prepared in different concentration of 0.1, 0.5 and 1 % and the purified lipase was incubated for 30 min at 20 °C. After the incubation period, activity was calculated in terms of residual activity

Effect of enzyme inhibitors

To study the effect of different inhibitors on purified enzyme, aliquots of enzymes were preincubated in the following known inhibitors such as ethylene-diamine tetra acetic acid (EDTA), iodo acetic acid (IAA), urea, and phenylmethylsulphonyl fluride (PMSF). The reaction was allowed to take place for 30 min at 20 °C. Residual activity was calculated and enzyme preparation without inhibitor was taken as control.

Stability of enzyme in organic solvents

The stability of lipase in different organic solvents such as Dimethyl sulfoxide (DMSO), pyridine, Benzene, Acetone, butanol, methanol, heptane, ethanol, ethyl acetate and 1-propanol were checked by preincubating the purified enzyme in the solvents for 30 min. Residual activity was calculated and purified enzyme without organic solvent was kept as a control of the experiment.

6.2.5 Lipase production using agro-industrial wastes by solid state fermentation

Substrate preparation

Agro-wastes such as groundnut cakes, coconut cakes, wheat bran, rice bran and neem cake were collected from markets and mills, dried in hot air oven at 55°C for overnight, ground in a blender and then sieved. Fermentation media consisted of 10g of each substrate supplemented with 1% peptone (w/w), 0.5 % yeast extract (w/w), 1 % olive oil (v/v) and initially moistened with 50 % distilled water (Mahanta *et al.,* 2008). Media containing different substrates were then sterilized at 15 lbs/inch at 120 °C for 20 min followed by inoculation with 1% (v/v) of inoculum and incubated at 20 °C to maintain psychrotrophic conditions for 5 days.

Extraction and estimation of lipase

After 5 days of incubation, the enzyme was extracted from the SSF media according to the method of Alva *et al.* (2007). Fifty ml of cold phosphate buffer (0.1 M) was added to the fermentation flasks and agitated at 100 rpm at orbital shaker at 25°C for 2 hr. Contents of the flasks were then filtered with autoclaved cheese cloth and centrifuged at 4000 rpm for 10 min. Supernatant was collected and filtered. The filtrate was then centrifuged at 10000 rpm at 4°C (Eppendorf, Germany) for 20 min to obtain crude enzyme extract. Lipase activity was determined according to the method described by Yagiz *et al*. (2007), using Paranitrophenylpalmitate as substrate. One unit of enzyme activity is defined as the amount of enzyme releasing 1 µmol of paranitrophenol/ ml/ min under standard assay conditions

Optimization of different process parameters

The important physical factors influencing lipase production were studied as effect of incubation temperature (15, 20, 25, 30, 35, 40, 45 and 50 °C), initial pH (4, 5, 6, 7, 8, and 9) and moisture content (30, 40, 50, 60, 70 and 80 %). Extraction and estimation of lipase was performed to detect the activity of the enzyme.

6.2.5 Statistical analysis

All experiments of characterization and agro-industrial media optimization were conducted in triplicates. Statistical analysis (ANOVA) was carried out by using IBM SPSS statistics 20 software to test the null hypothesis that physical parameters or chemical parameters did not have significant effects on lipase production. Significance of difference of each physical parameter was tested with one way ANOVA at $P = 0.05$.

6.3 RESULTS

6.3.1 Purification of lipase

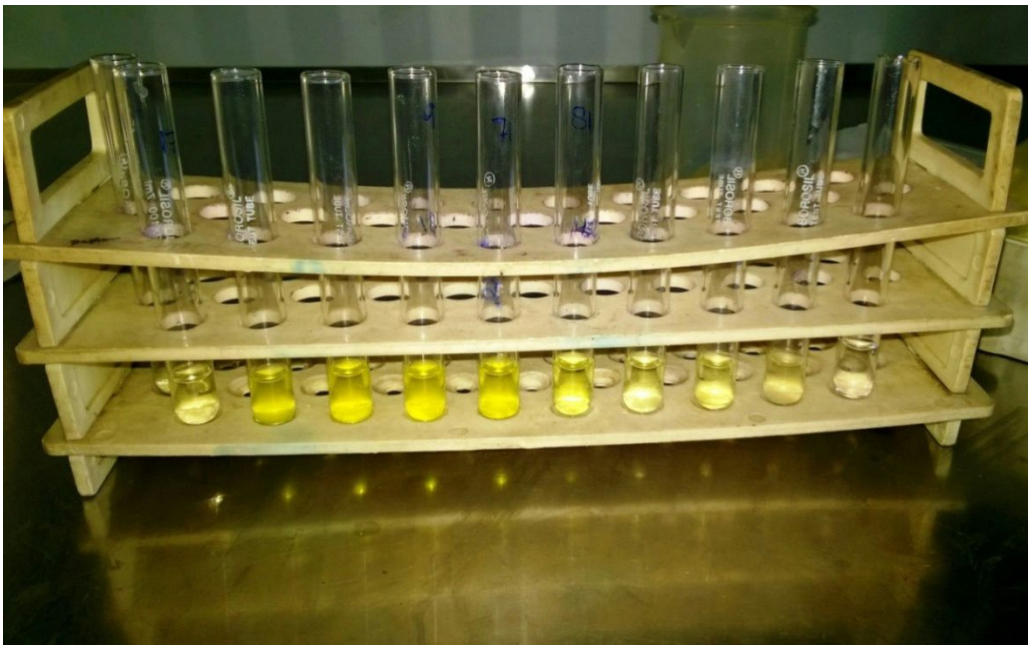
The cell free supernatant was collected by centrifugation of production media and considered as crude enzyme extract. Purification of lipase was carried out by filtration, concentration of the crude extract by precipitation and DEAE anion exchange chromatography. The results of the purification procedure are summarized in table 6.1. Acetone precipitation (2:1) exhibited an activity of 47.22 U/mg and DEAE-Cellulose showed an activity of 176.16 U/mg. Protein precipitated from crude enzyme by acetone precipitation was applied to DEAE- Cellulose anion exchange column, and the fractions eluted were subjected to assay by pNPP. Comparatively high fold precipitation (7.123) was expressed by ion exchange purification.

Figure 6.1 show the different active fractions of eluted samples, active fractions 16, 17, 18 and 19 showed highest lipase activities corresponding to a single peak. These active fractions were pooled and used for the further study.

Table 6.1: Summary of the purification of lipase

Sample	**Protein(mg)**	**Activity (U/ml)**	**Specificactivity (U/mg)**	**Yield (%)**	**Purification fold**
Crude extract	16.4	412.62	25.15	100	1
Acetone precipitation	3.2	146.71	47.22	35.43	1.877
DEAE-Cellulose	0.48	86.17	179.16	20.88	7.123

Figure. 6.1: Image showing activity of eluted fractions of DEAE cellulose chromatography

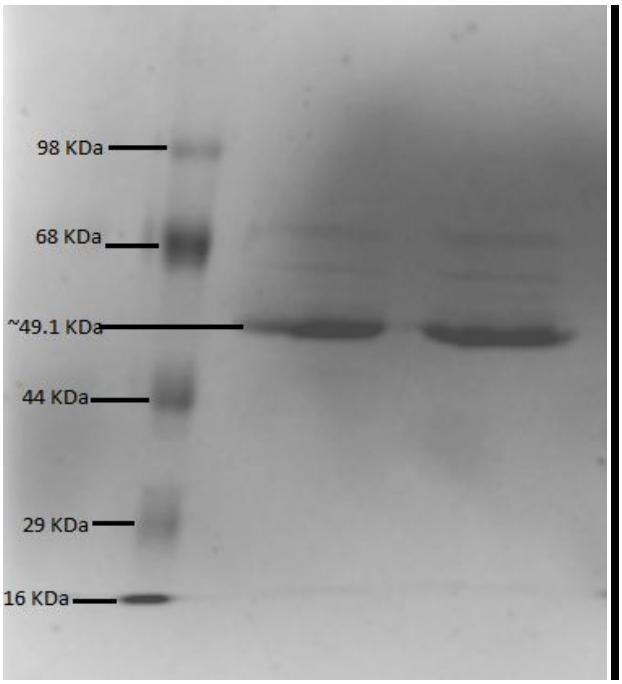


6.3.2 Molecular weight detection

Protein purification was successfully achieved to homogeneity as evident by single sharp band observed at 49.1 KDa on SDS PAGE (Figure 6.2). Figure 6.3 represents SDS PAGE analysis of the chromatographic sample, acetone precipitated sample and crude extract.

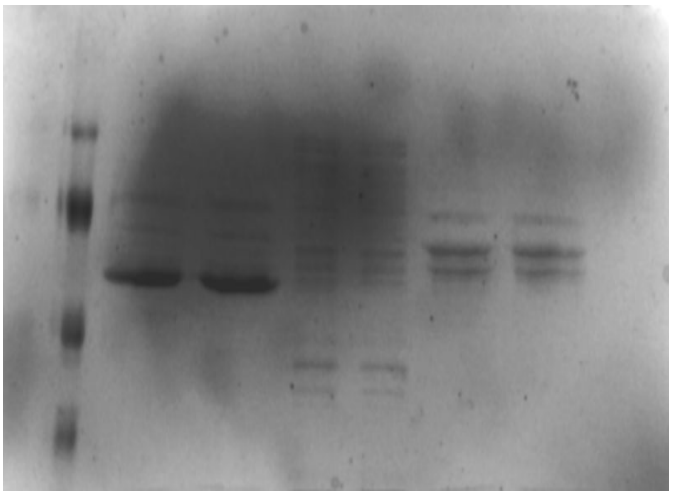
Zymogram analysis was performed by scooping the suspected lipase band from the gel and the enzyme was dissolved in 50 µl of Tris buffer (pH 7), incubated overnight at 4 °C. Figure 6.4 shows the result of lipase assay (Yellow coloration of buffer due to liberation of p-nitrophenol by the lipase activity and lack of color development in the control.

Figure 6.2: SDS- PAGE showing DEAE cellulose purified sample with molecular marker.



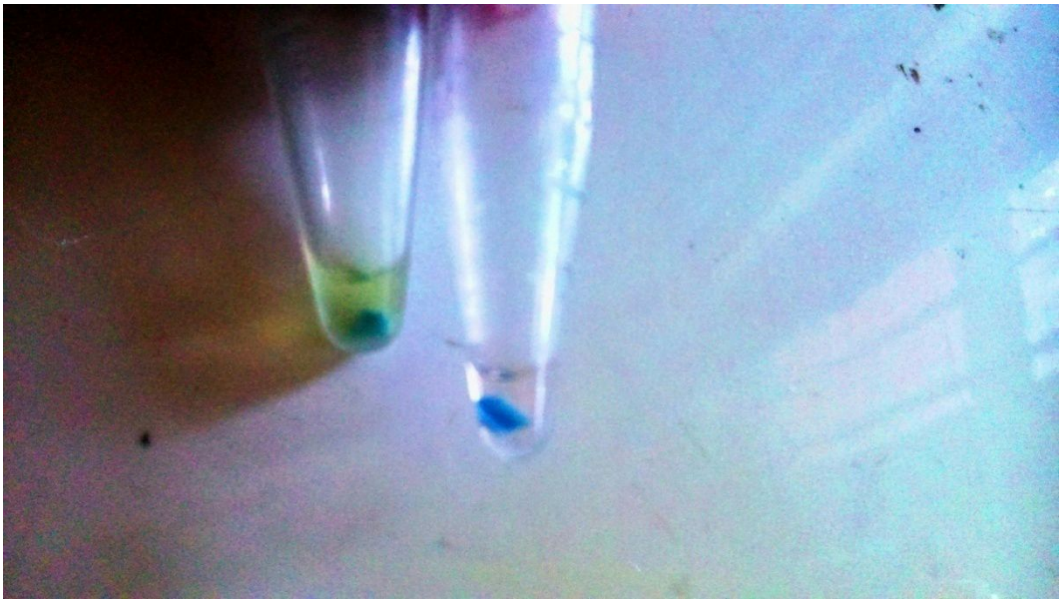
Lane 1: marker; Lane 2: 15 µl of enzyme Lane 3: 20 µl of enzyme

Figure 6.3: PAGE showing crude, precipitated, and DEAE cellulose purified enzyme



Lane 1-marker, Lane 2, 3 - DEAE cellulose purified sample, Lane 4, 5 - cell free supernatant, Lane 6,7 – acetone precipitated sample

Figure 6.4: Image showing zymogram analysis of 49.9 KDa band (yellow) and control band (colourless)



6.3.3 Characterization of enzyme

Effecet of pH on enzyme activity

The effect of pH on lipase activity of purified lipase was determined by using different buffers having different pH values. Results revealed that the enzyme was active at a pH range of 6 to 10 (Figure 6.5). The optimum pH of the enzyme was pH 7 and there was a decrease in activity when pH was increased. Enzyme activity at pH 8 was found to be 91% and at pH 9 it was found to be 72 %. From pH 10 lipase activity decreased drastically and falls to 32 % at pH 12.

Effect of pH on stability of the enzyme

Enzyme activity was found to be highly stable at pH range between pH 6 to pH 9 (Figure 6.6) Highest residual activity was observed at pH 7 followed by pH 6 and then pH 8.

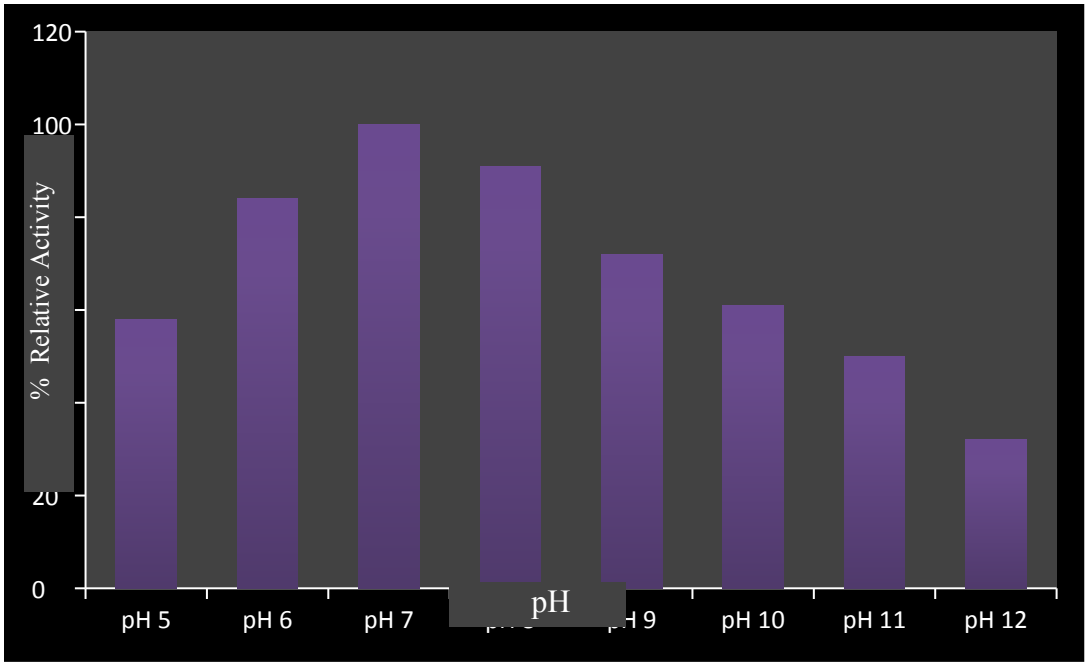


Figure 6.5: Effect of pH on activity of lipase from *S. maltophilia*

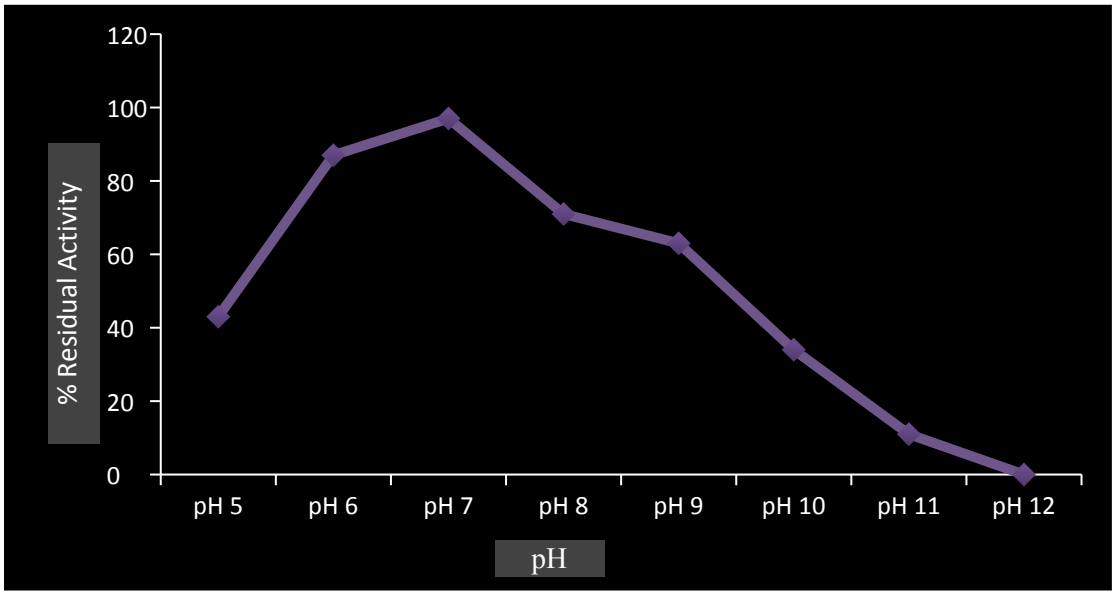


Figure 6.6: Effect of pH on the stability of lipase from *S. maltophilia*

Effect of temperature on lipase activity

Experiments on effect of temperature on enzyme activity revealed the cold active nature of the enzyme. The enzyme showed maximal activity at 25 °C followed by 20 °C exhibiting 94 % activity and 15 °C showing 82 % activity (Figure 6.7). Most of the reported cold active enzymes have maximal activity between 20-25 °C and minimal activity beyond 50 °C. Sharp decline in the activity at 40 °C was indicated by relative activity of 51 % and almost negligible relative activity of 12 % was noted at 50 °C.

Effect of temperature on stability of the enzyme

Results of temperature stability experiments reinforced the cold active nature of the enzyme. Highest residual activity was observed at temperatures 20 °C and 25 °C showing 100 % activity and a great deal of activity was observed (>60 %) from 10 °C to 30 °C. However, stability dropped drastically from 30 °C (73 % activity) to 40 °C with a residual activity of only 22 % (Figure 6.8) and no residual activity was observed at 50 °C.

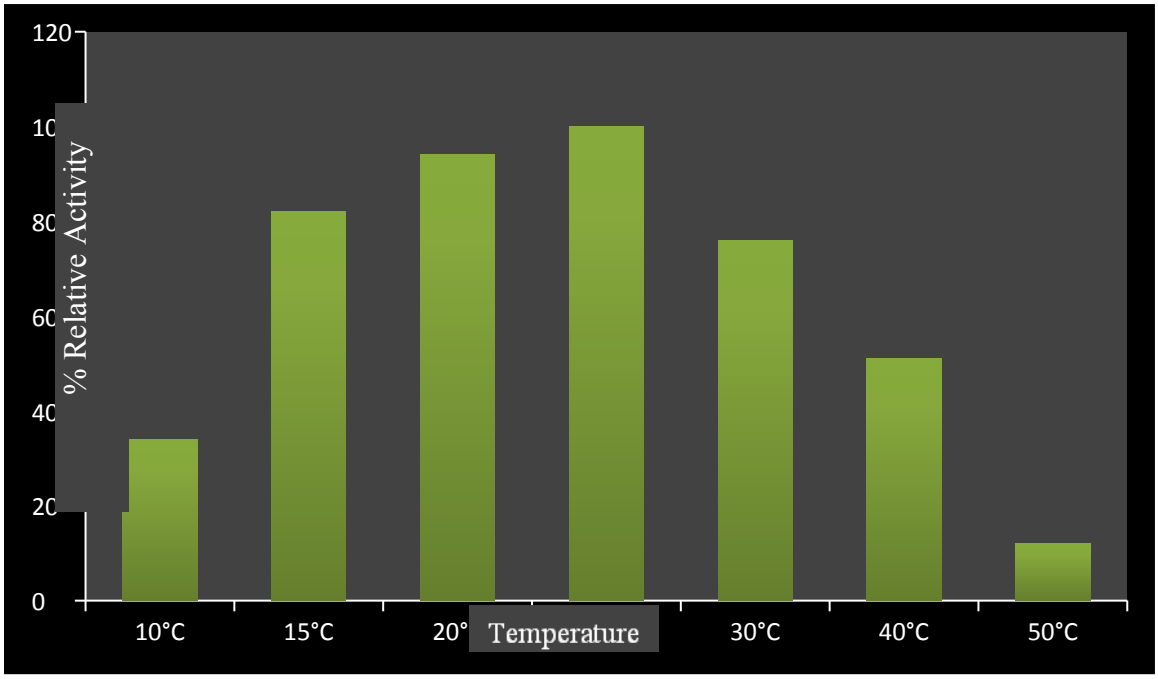


Figure 6.7: Effect of temperature on the activity of lipase from *S. maltophilia*

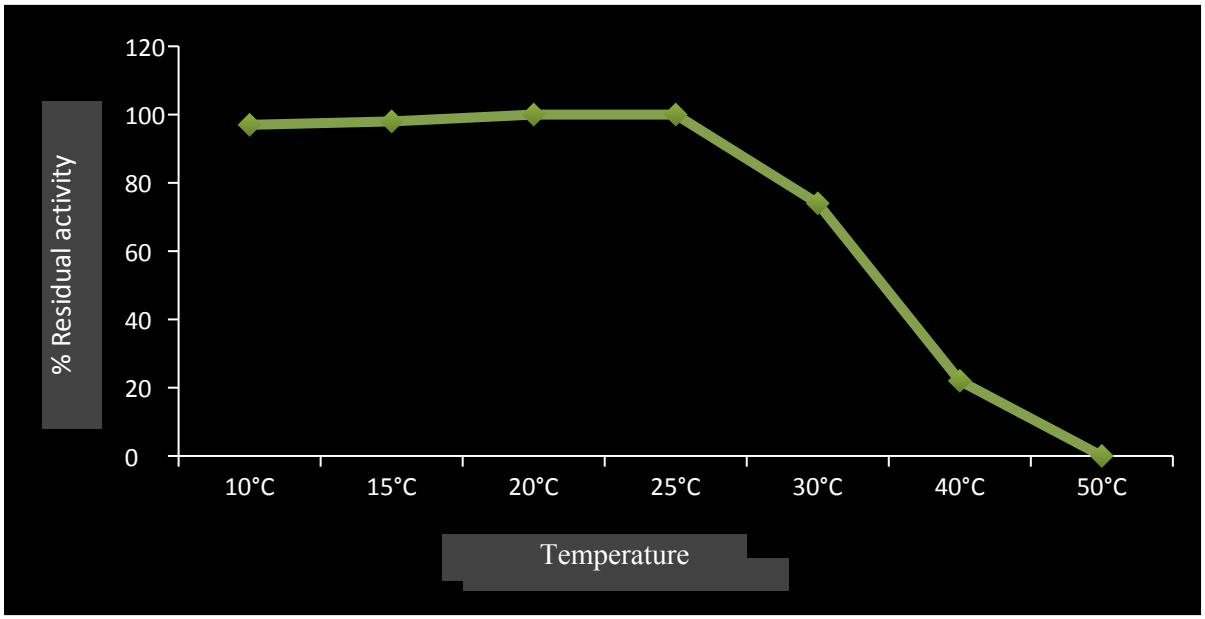


Figure 6.8: Effect of temperature on the stability of lipase from *S. maltophilia*

Effect of organic solvents on lipase activity

Figure 6.9 shows the effect of various organic solvents on the lipase activity. It can be easily understood from the graph that the enzyme was highly stable in most of the organic solvents. The enzyme showed > 60 % activity in the presence of the solvents such as benzene, heptane, acetone, 1-propanol, methanol and ethanol. However, low residual activity of less than 60 % was seen in the presence of butanol (53.33 %) and ethyl acetate (38 %). In organic solvents such as pyridine, benzene, acetone, methanol, and heptane residual lipase activity was observed to be greater than 80% indicating high stability of the enzyme in these organic solvents.

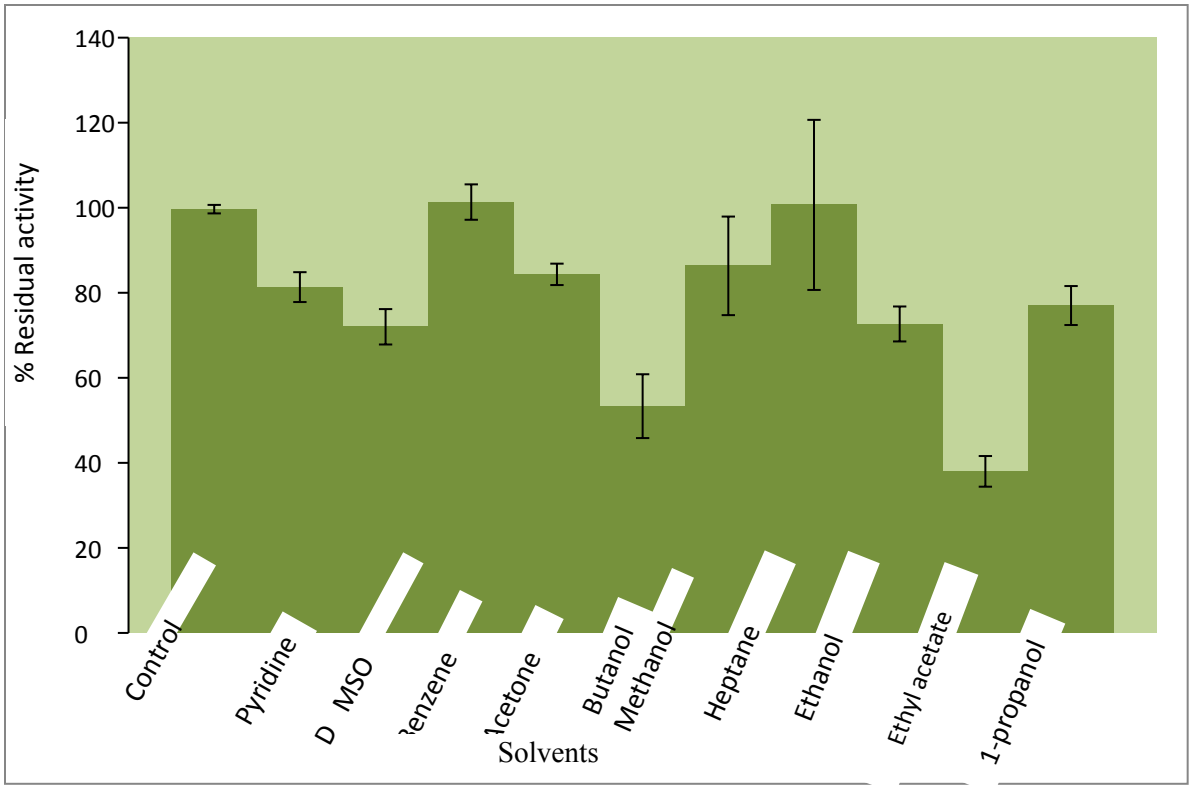


Figure 6.9: Effect of various organic solvents on lipase activity from *S. maltophilia*

Effect of metal ions on lipase activity

The result of the effect of metal ions on lipase activity is represented in the figure 6.10. The metal ions such as calcium, copper, iron, magnesium, lead and zinc did not have any adverse effect on lipase activity and a relative activity of greater than 70 % was exhibited in presence these metals. The metal ions such as cobalt, mercury and lithium significantly reduced the lipase activity by less than 50 %. Mercury was found to be the most inhibitory by reducing the activity to 16 % of the original activity.

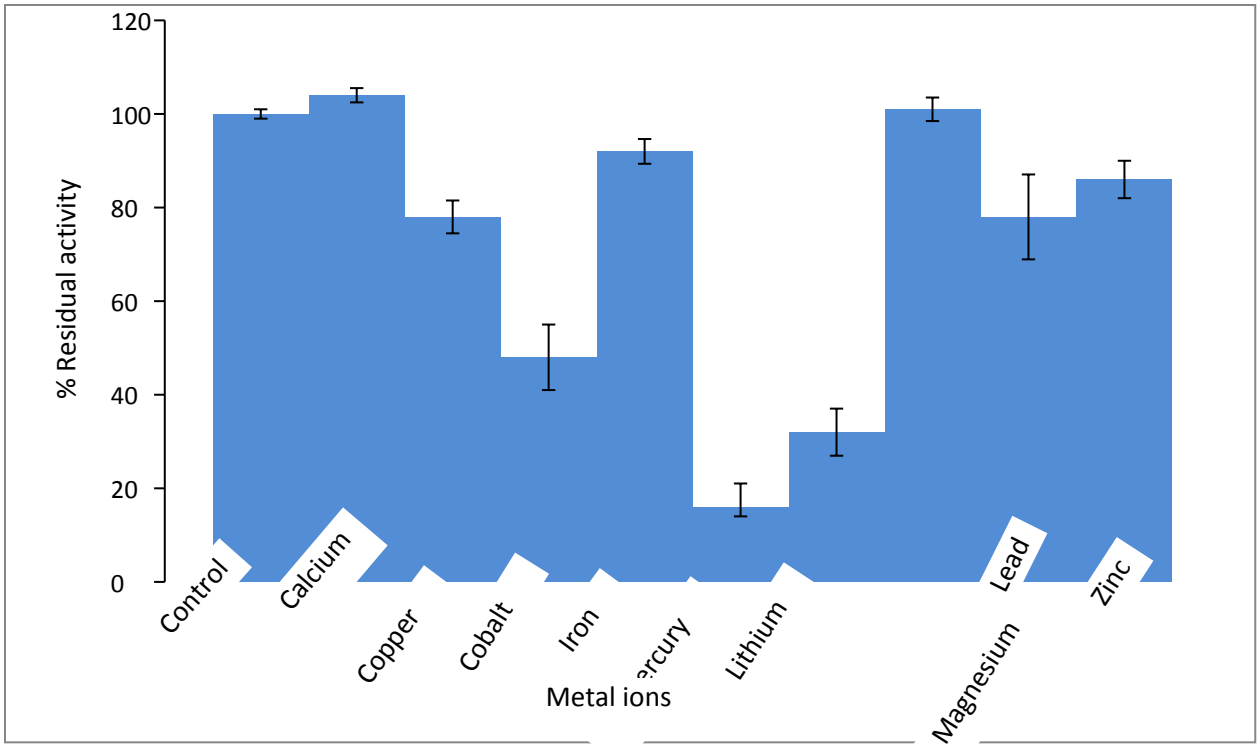


Figure 6.10: Effect of various metal ions on lipase activity of *S. maltophilia*

Effect of inhibitors and surfactants on lipase activity

The inhibitors such as EDTA and IAA at low concentration did not have inhibitory action on lipase activity but PMSF was inhibitory to the enzyme (Figure 6.11). The serine protease inhibitor PMSF was found to have a residual activity of 53 % and 92 % of residual activity was observed for EDTA followed by 87 % activity in IAA. Surfactants such as SDS, Triton X 100, Tween 80, Tween 60 and Tween 20 were checked for their effect on lipase activity. SDS, Triton X 100, Tween 80 and Tween 60 had no significant impact on activity since residual lipase activity of > 60 % was noted. However, Tween 20 was found to be inhibitory and the residual activity was reduced to 57 %.

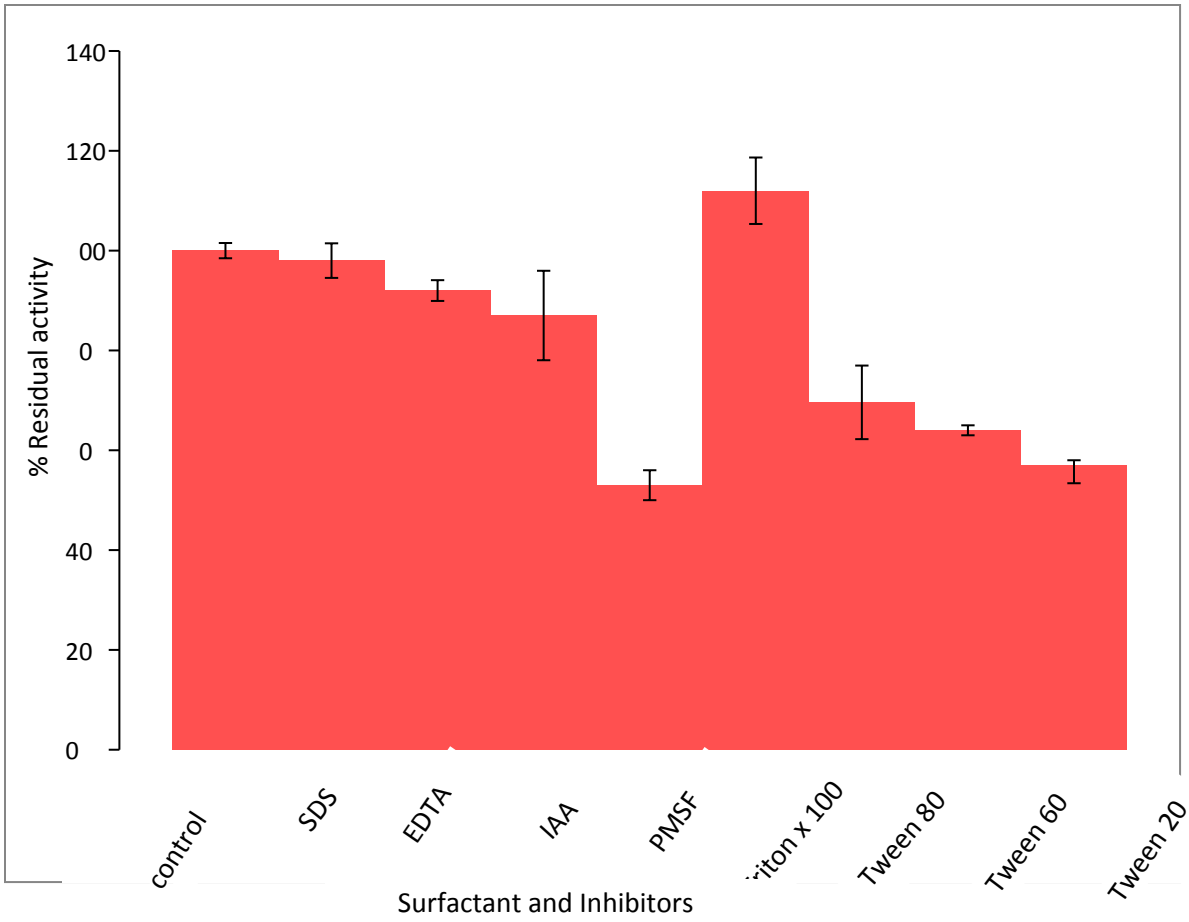


Figure 6.11: Effect of surfactants and inhibitors on lipase activity on *S. maltophilia*

6.3.4 Agro-industrial waste as substrate for enzyme production

During the present study, five agro-industrial waste materials were used as substrates and the most prominent lipase activity was achieved in the media containing the substrate, groundnut cake. Figure 6.12 shows relative activity of the enzyme in different media with respect to groundnut cake (74.11 U/ml). Neem cake (58.73 U/ml) and coconut cake (61.91 U/ml) supported good growth and shown similar lipase production of 80 and 83 % respectively. Wheat bran showed 66 % activity while rice bran was found to have 38 % of activity. . Effect of pH of different substrates on the enzyme production (Figure 6. 13) revealed that at pH 6, significantly high production ($p = < 0.001$) was observed in media containing groundnut, neem and coconut cakes while enzyme production in wheat bran and rice bran was found to maximum at ($p = < 0.001$) pH 7. The enzyme activity at different temperatures in all substrates was found to be similar, increasing gradually from 15 °C and maximum production at 28 °C ($p = < 0.001$)

thereafter dropping at 37 °C (Figure 6.14). The enzyme production reduced drastically at 45 °C indicating thermal denaturation at higher temperatures. To check the influence of moisture content on lipase production, all substrates were moistened with distilled water (30 to 80 %) prior to fermentation. Figure 6.15 shows that moisture content of 60 % was optimal for enzyme production containing groundnut cake, coconut cake, wheat bran and rice bran and lipase production was significantly higher (P = < 0.001) when compared to 30, 40, 50, 70 and 80 %. When neem cake was used as a substrate for lipase production, significantly high activity (P = < 0.001) was observed at a moisture content of 70 %.

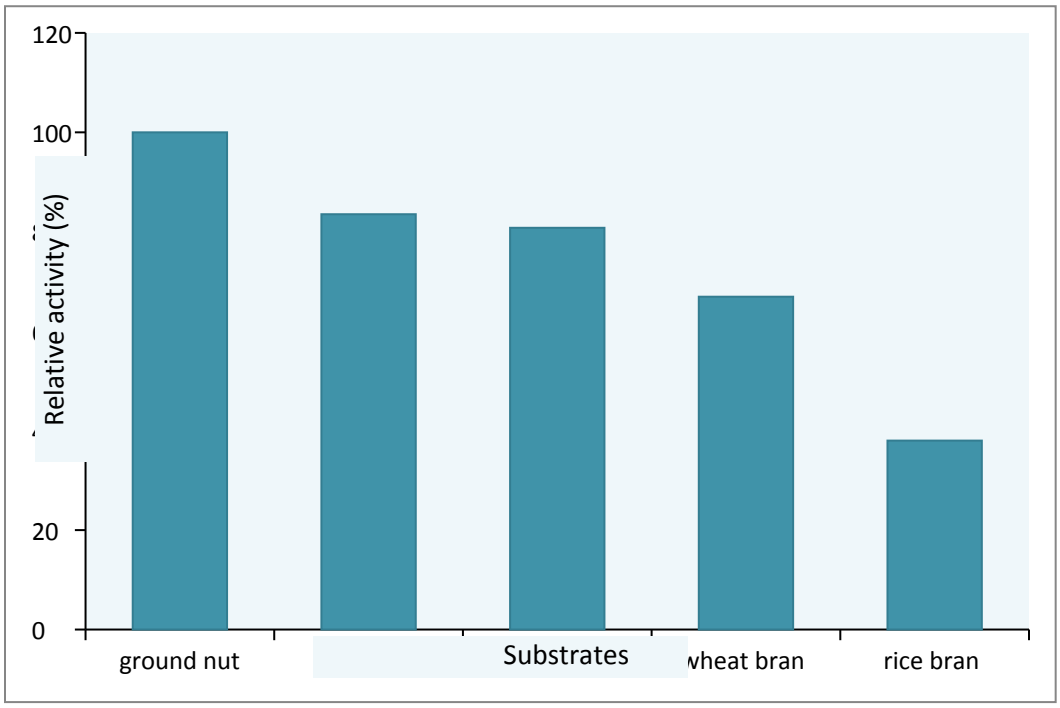


Figure 6.12: Lipase production in various agro-industrial wastes

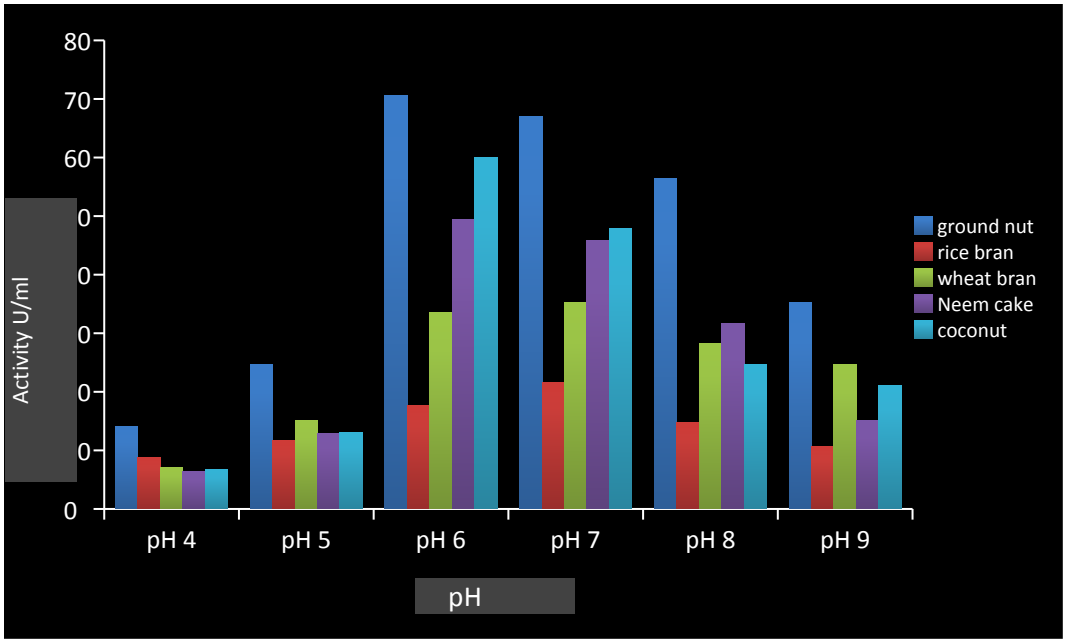


Figure 6.13: Effect of pH on lipase production in various agro-industrial waste media

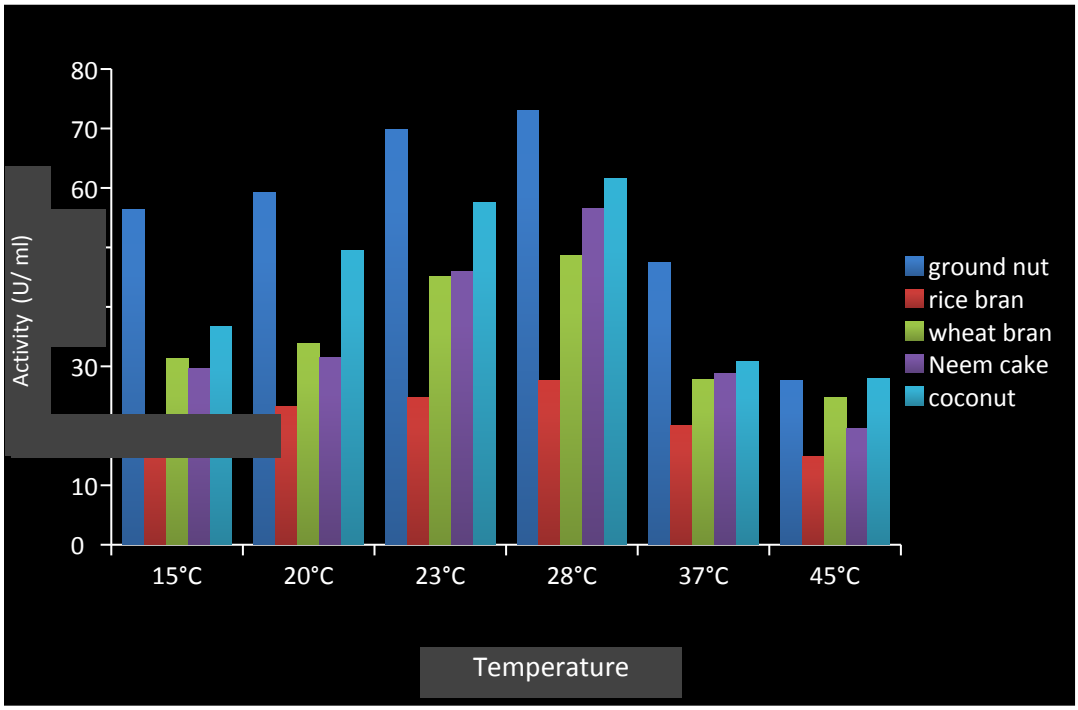


Figure 6.14: Effect of temperature on lipase production in various agro-industrial waste media

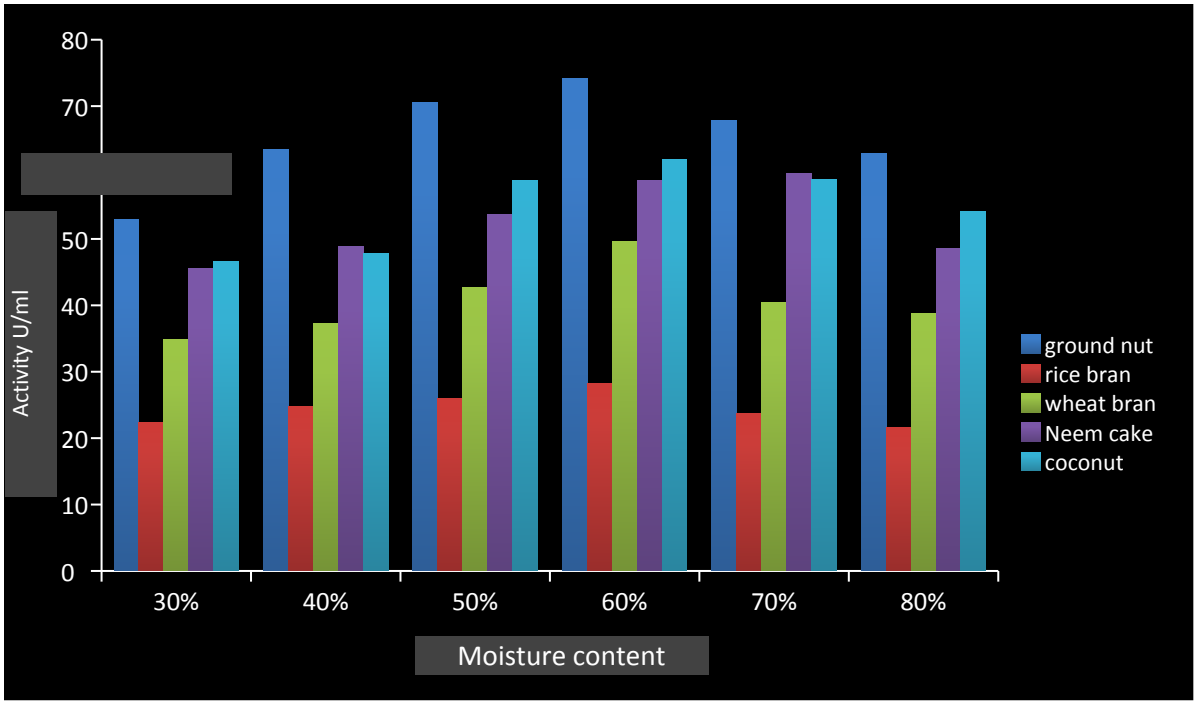


Figure 6.15: Effect of moisture content on lipase production in various agro-industrial waste media

6.4 Discussion

Cold active lipase from *Stenotrophomonas maltophilia* was purified by a two-step process. Purification fold of 7.1 was achieved after DEAE-cellulose ion exchange chromatography. In a similar study, after DEAE-cellulose chromatography for lipase purification, 11.5 fold purification of lipase *Bacillus thermoleovorans* was achieved (Lee *et al.*, 2001). Psychrotrophic *Pseudomonas* lipase was observed to have 8.6 folds purification following the DEAE cellulose chromatography (Choo *et al*., 1997). Purification index reported for cold active lipase from *Serratia marcescens* was to be 3.06 after subjecting to CM-cellulose ion exchange chromatography. An interesting observation noted during the experiment was the high specific activity of the lipase. In the current study, after performing the chromatographic purification, specific activity of cold active lipase was found to be 179.16 U/mg protein. High specific activity at low temperature and high thermolability is a typical characteristic of cold active enzyme (Joseph *et al*., 2008; Sharma *et al*., 2001).

Molecular weight of the lipase produced by *S. maltophilia* was found to be approximately 49.1 KDa which was similar to previously reported molecular weight of lipases. Molecular weight of most of the reported lipase was found to be between 30 to 60 KDa. Lipase produced by the genus *Pseudomonas* was found to have similar molecular weight with lipase from *S. maltophilia* and both the genera have close phylogenetic relationship. Sztajer *et al.* (1992) reported lipase protein with molecular weight of 45 KDa from *Pseudomonas fluorescens*. Lipase protein of 45 KDa was reported from *Pseudomonas putida* by Lee and Rhee (1993). Kim *et al.* (1997) reported lipase protein from Pseudomonas sp with molecular weight of 38 KDa.

Bacterial lipases are generally found to have maximal activity at neutral pH (Dharmshtiti *et al*., 1998; Lee *et al*., 1999) or slightly alkaline pH (Sidhu *et al*., 1998; Sunna *et al*., 2002). The current study also agrees with this general observation as the lipase was having maximal activity at pH 7 and a great deal of activity of >60% was observed at pH 8 and 9.

Characterization of several bacterial lipases showed the neutral nature of lipase. Lipase characterized from *Acinetobacter* showed the maximal activity at neutral pH (Dharmsthiti *et al*., 1998; Pratuangdejkul and Dharmsthiti, 2000). Sugihara *et al.* (1991) showed maximum activity between pH 5.6- 6.2 for *Bacillus* sp. lipase. Another *Bacillus* sp characterized by Kim *et al* (1994) was found to have a pH range of 5.0-9.5. The genus *Pseudomonas* was found to have a narrower range of pH for activity with maximal activity (5.5- 7.0) as reported by Brune and Gotz in 1991; another strain of *Pseudomonas* was found to have maximal activity in the range of pH of 7 to 9 (Bradoo *et al*., 2002).

Effect of temperature on lipase activity was studied and good activity of lipase was found between 20 to 30 °C, which is characteristic of cold active enzyme. Maximal activity was observed at 25 °C followed by activity at 20 °C (98% relative activity). Until 30 °C great deal of activity (>70%) was seen but activity dropped drastically from 40 °C. Cold active nature of lipase in the present study was compared with the previous reports of cold adapted enzymes from different organisms. Zeng *et al.* (2007) characterized lipase from *Pseudoalteromonas* sp. Wp27 which was found to have maximal activity at temperature between 20-30 °C. *Psychrobacter* sp. was found to have maximal activity at a range of 20-30 °C and retained 60 % activity at 4 °C (Zeng *et al*., 2004 and 2007). Suzuki *et al*. (2001) and Joseph *et al*. (2007) reported two bacteria

Acinetobacter sp. strain 6 and *Microbacterium phyllosphaerae* respectively that were found to have optimal activity at 20 °C.

Thermal stability profile of lipase confirmed the cold active nature of the enzyme. The enzyme was found to be highly stable at low or moderate temperature since enzyme showed >70 % residual activity at temperature ranging from 10 to 30 °C. Maximal residual activity of 100 % was observed at 20 °C and 25 °C. High residual activity of >97 % was observed at 10 °C and 15 °C indicating stability of lipase at low temperature. However, increasing the temperature to 40°C caused a drop in stability (22 % residual activity) and no noticeable activity was seen at 50 °C.

Low stability profile of enzymes at high temperature is an adaptive mechanism of cold adapted microorganisms. Compared to mesophilic microorganisms' cold adapted microbes have high activity at low temperature (Joseph *et al*., 2006) at the cost of thermolability at high temperature. This was achieved by cold adapted microbes by showing a high specific activity at low temperature. Similar scenario was also observed in the current study, the enzyme being thermo-labile, showed high activity at low temperatures and high specific activity. These properties of the lipase are beneficial in many industries such as food and pharmaceuticals wherein high catalytic activity is required at low temperature. High temperature treatments are known to cause damage to the quality of the food and beverages and instability of the frail compounds used in pharma preparations (Joseph *et al*., 2007; Sharma *et al*., 2001). Another area of possible application of cold active lipase is in detergent industry where low temperature helps in maintaining fabric quality and reducing the energy demand (Maurer, 2004)

Most of the metal ions tested showed a low inhibitory action on lipase. All metals were tested at a concentration of 5mM and inhibitory actions were shown by cobalt, mercury and lithium. High residual activity of >75 % was found in the presence of cadmium, copper, iron, magnesium, lead and zinc. Cofactors are usually not necessary for the activity of lipase although divalent cations such as calcium are found to stimulate lipase activity (Dheeman, 2011). In the current study, calcium and magnesium was found to stimulate the activity of lipase. Similar findings were reported for divalent cations. Lesuisse *et al.* (1993) reported the stimulatory effect of calcium for lipase produced by *Bacilus subtilis*. *Acinetobacter* lipase was found to have stimulatory activity in the presence of calcium Snellman *et al*. (2002).

Stability of enzyme in organic solvents was checked and the results are summarized in the figure 6.9. The enzyme showed high stability in organic solvents. Benzene and heptane was found to have maximum residual activity and were stimulatory in nature. The enzyme showed > 80% residual activity in the presence of pyridine, methanol and acetone indicating the enzyme's high stability in these solvents. DMSO and ethanol was tolerated well by the enzyme with a residual activity of 72 %. However, presence of butanol and ethyl acetate was shown to adversely affect the enzyme since enzyme activity dropped to <60 % of residual activity.

Stability of enzyme in organic solvents is considered to be significant in industries such as fine chemical synthesis. Enzymes that are stable in organic solvents are used for catalysis of reactions in non- aqueous milieu and optical resolution of chiral compounds (Gargouri *et al*., 1984). In the present study, polar solvents such as ethanol, methanol and 1-propanol were tolerated well by the enzyme and the results are in agreement with previous reports (Kanmani *et al*., 2015; Dheeman *et al*., 2011). However, ethyl acetate strongly inhibited the enzyme activity by showing only 38% residual activity. Non- polar solvents such as benzene and heptane was found to be stimulatory and similar finding were reported by Dheeman *et al*. (2011).

Effect of inhibitors such as EDTA, IAA and PMSF on lipase activity was checked and PMSF was found to inhibit the activity significantly. Kanwar *et al.* (2005) reported complete inhibition of lipase by *Bacillus coagulans* at a concentration of 15 mM. PMSF is usually regarded as serine hydrolase inhibitor and inhibition of lipase was reported by previous studies also (Dheeman *et al*., 2011). IAA and EDTA were not found to be inhibitory at a concentration of 5 mM since >80 % of activity was retained by the enzyme. Surfactants such as SDS, Triton X 100, Tween 80, 60 and 20 were checked for its effects on lipase enzyme. Stimulatory effect of Triton X 100 was noted and similar reports exist on positive effects of this compound. Tripathi *et al.* (2014) reported lipase from *Microbacterium*, the activity of the enzyme was found to be positively enhanced by the addition of Triton X 100. Cold active lipase produced by psychrotrophic *Aeromonas* was also found to be stimulated by the addition of Triton X 100 (Lee *et al*., 2003).

Characterization of cold active lipase from *S. maltophilia* showed that the enzyme was comparatively stable in organic solvents, metal ions and surfactants. Organic solvent stable lipases have many uses in industries since transesterification and polymerization are carried out in the presence of harsh conditions such as organic solvents (Sharma and Kumar, 2014).

Enhanced activity, better stability and ease of product recovery was noted for lipases that are stable in organic solvent (Kumar and Kanwar, 2011). Surfactant stable enzymes are used in industries such as detergents and dish washers. Lipases that are stable in metal ions find their applications in pharmaceuticals, fine chemical synthesis and cosmetics (Joseph *et al*., 2007).

Solid state fermentation is gaining momentum in industrial enzyme production due to easy availability of substrates and low cost. During the present study, five agro-industrial waste materials were used and maximum lipase activity was achieved in the media containing the substrate, groundnut cake. Many researchers have previously reported the superiority of groundnut cake as a substrate for lipase production (Faisal *et al.*, 2012; Rekha *et al.*, 2012; Mary-Suji *et al.*, 2014). Groundnut provides all nutrients for the growth and maximal production of lipase and reports suggest that C/N and nitrogen concentration is considered to be a critical factor in the determination of lipase production. Groundnut cake contain 40- 50 % of crude protein which might significantly enhance the lipase production in the media (Adinarayana *et al*, 2003, Petrovic *et al.*, 1990).

Neem cake (58.73 U/ml) and coconut cake (61.91 U/ml) also supported good growth and lipase production. Neem cake and coconut were successfully used for microbial lipase production by many researchers (Kanmani *et al.,* 2015; Mary-Suji *et al.*, 2014; Marimuthu K, 2013; Selva Mohan *et al.*, 2008). Even though wheat bran provides high nutritional source for bacterial fermentation, pretreatment or processing is often required for release of nutrients from the complex substrate to more accessible form (Kumar *et al.*, 2012). This could be responsible for comparatively less lipase production in wheat bran (49.6 U/ ml) medium which showed only 66 % relative activity and similarly in rice bran where lipase production was found to be only 28.16 U/ml with 38 % relative activity.

It is well established that the moisture content of the substrate is the most important factor for microbial enzyme production in SSF. To check the influence of moisture on lipase production, all substrates were moistened with distilled water (30 to 80 % saturation) prior to fermentation. Moisture content of 60 % was found to be optimal for lipase production in the media containing groundnut cake, coconut cake, wheat bran and rice bran and lipase production was significantly higher ($P = < 0.001$) when compared to moisture content of 30, 40, 50, 70 and 80 %. When neem cake was used as a substrate for lipase production, significantly high activity ($P = < 0.001$) was

observed at moisture content of 70 %. A change in the moisture content, affect SSF due to its impacts on the physical properties of the substrate (Pokerny *et al.*, 1997; Mahanta *et al.*, 2007). An increase in moisture content, reduces porosity which in turn reduces the gas exchange. Similarly, low moisture content do not support optimal growth and lower substrate swelling resulting in reduced enzyme production (Mahadik *et al.*, 2002).

Lipase production at different temperatures in all the media was found to have a similar pattern, increasing gradually from 15 °C and peak activity at 28 °C ($p = < 0.001$) thereafter dropping at 37 °C. The enzyme production reduced drastically at 45 °C indicating that thermal denaturation may proceed at higher temperatures above 45 °C. These results suggest that the lipase is cold active in nature but show good activity at ambient temperatures. Thus while scaling up the process, energy consumption for temperature build up in the reactor will be less as the organism could tolerate a rise in temperature up to 10 °C from the optimum (28 °C) which is an added advantage in reducing the cost of production. Cold or moderate temperature maintenance for enzyme catalyzed reactions are critical to some industries such as in production of fatty acids and in pharmaceuticals. High temperature and pressure used in these industries for removal of coloring materials formed during production causes significant loss of fatty acids and glycerol (Abdelmoez *et al.*, 2013) .Thermal stability of lipase is desirable in many industries which could be achieved by chemical modification of the enzyme such as introduction of salt bridges as reported by Wu *et al.* (2015) but demands further studies in this area.

The initial pH of the media was found to be critical for growth and enzyme production since the metabolic activities of microorganisms are very sensitive to pH change (Rekha *et al.,* 2012). Effect of pH on different substrates revealed that at pH 6, significantly high production ($p = < 0.001$) was observed in media containing groundnut, neem and coconut cakes while wheat bran and rice bran media were found to show the optimal activity ($p = < 0.001$) at pH 7. The lipase production at low pH was found to be less and the result is in agreement with other reports (Adinarayana *et al.*, 2003; Kanmani *et al.*, 2015; Mahanta *et al.*, 2008; Santose *et al.*, 2013). All substrates showed good lipase production from pH 6 to 9 attributing to the tolerance of *S. maltophilia* to wide range of pH. Stability in pH is an important factor in many industries such as detergent and textile. The discrepancy in optimum pH in different media is due to the nature of

the substrate used and the fact that the enzyme may possibly interact with other components in the media (Santos *et al.*, 2013).

Thus, the study shows that agro-industrial waste could be used as an alternative substrate for production of cold active lipase providing double benefits, production of a commercially important enzyme and bioremediation of agricultural wastes.

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Chapter 7

Summary

The thesis presents different characteristics of heterotrophic bacteria isolated from the Arctic region and emphasis was given to lipase production, optimization and characterization. The study is divided into two parts; the first part includes characterization of heterotrophic bacteria from sediment and water samples, antibiotic resistance analysis of isolates and enzyme profiling. The predominant enzyme produced by these strains was found to be lipase. The second part focuses on lipase production. Lipase positive isolates identified by plate assay method were subjected to secondary screening using spectrophotometric method and the ability of isolates to degrade various natural substrates were investigated. The sediment isolate KS 46 with maximum lipase production was selected for detailed study and the strain was identified as *Stenotrophomonas maltophilia*. The optimization of lipase production by *Stenotrophomonas maltophilia* was performed using response surface methodology for parameters such as pH, nitrogen concentration and agitation. The purification of lipase was carried out by DEAE anion exchange chromatography and molecular weight was determined by SDS-PAGE. The scope of using different agro-industrial wastes for lipase production, stability of lipase in organic solvent and surfactants for application in various industries was also studied.

The important finding of the study can be summarized as follows:

- A total of 272 heterotrophic bacteria isolated from sediment and water samples from the Arctic region were subjected to phenotypic characterization and biochemically typed strains belonging to 33 groups were selected for molecular identification using 16S rDNA sequencing.
- The sequencing of 16S rDNA gene from representative strains from 33 groups was carried out by BLAST analysis and the sequence homology ranged between 97.7 to 100 % to the closest neighbors.

- BLAST analysis revealed 12 distinct species belonging to three phyla – Proteobacteria, Firmicutes and Actinobacteria. Diversity of bacteria in sediment samples was higher compared to water samples.
- Proteobacteria represented the major phyla in culturable bacteria in sediment and water and all of these belonged to class *Gammaproteobacteria*.
- In sediment, *Gammaproteobacteria* was represented by 66% followed by *Actinobacteria* (20%) and *Bacilli* (14%). The species detected from sediment includes *Stenotrophomonas maltophilia,Brachybacterium paraconglomeratum, Micrococcus luteus, Bacillus flexus, Staphylococcus cohnii, Enterobacter ludwigii, Enterobacter cancerogenus, Pseudomonas fragi, Pseudomonas koreensis* and *Bacillus thuringiensis.*
- In water, 91% of bacteria were represented by *Gammaproteobacteria* and 9% was represented by *Bacilli* (9%).The species identified from water samples were *Enterobacter ludwigii, Stenotrophomonas maltophilia, Halomonas boliviensis, Pseudomomas sabulinigri, Bacillus thuringiensis, Enterobacter cancerogenus* and *Pseudomonas fragi.*
- Analysis of antibiotic resistance in heterotrophic bacteria was carried out by agar diffusion method against 15 antibiotics. The results revealed that the sediment isolates were clearly more resistant than water isolates.
- In the sediment samples, highest percentage resistance was found against ampicillin (65%). The order of percentage resistance was found to be ampicillin > amoxicillin > cephalothin > cefpodoxime > tetracycline > sulphamethaxizole > carbenicillin > ciprofloxacin > ceftazidime = gentamycin > colistin > streptomycin = chloramphenicol > nalidixic acid > trimethoprim.
- In the water samples, highest percentage resistance was found against cephalothin (58.5%). The percentage resistance of water isolates were in the order cephalothin > carbenicillin > Cefpodoxime > ampicillin > amoxicillin > colistin > gentamycin = sulphamethizole > tetracycline > nalidixic acid > streptomycin > ceftazidime.
- In all, 27 different patterns were detected from sediment and water isolates. Most frequently observed MAR indices were 0.2, 0.266, 0.33, 0.4, and 0.466. Different patterns were observed under each MAR index which included eight patterns in 0.2, seven patterns in 0.266, four patterns in 0.33 and two patterns in 0.466.

- The resistance pattern that was observed in the highest number was A, AM, CH, CEP with a frequency of 31.96%. The least encountered pattern was A, AM,CEP with a frequency of 0.2% .
- All multiple antibiotic resistant isolates were subjected to plasmid extraction and plasmids were extracted from 81 isolates. Different patterns were observed from bacteria ranging from 1 to 4.
- Highest number of isolates was found to have a single plasmid with a size of 24.5 Kb and band of approximately 1.8 Kb was seen in seven patterns.
- Multiple antibiotic resistant isolates were investigated for their resistance against heavy metals by plate assay and tube dilution methods to ascertain the correlation between antibiotic and heavy metal resistance.
- Among the six heavy metals tested, mercury was found to be most toxic of all the metals at a concentration of 50 μg/ L and selenium was tolerated well by most of the isolates up to a concentration of 2000 μg/ L.
- Depending on the percentage of isolates showing metal resistance the order of toxicity was found to be Hg > Cd > Cu > Zn > Pb > Se.
- Potential of heterotrophic bacteria to produce hydrolytic enzymes was tested for enzymes such as amylase, protease, lipase, β-galactosidase, cellulase, pectinase and phosphatase and sediment isolates showed higher potential to degrade different substrates.
- More than 75% strains of *Bacillus thuringiensis, Bacillus flexus* and *Micrococcus luteus* showed amylase production.
- Protease was produced by all species reported in the study and more than 85% strains of *Bacillus thuringiensis, Bacillus flexus* and *Staphylococcus cohinii* showed protease production.
- Lipase was the most widely distributed enzyme in sediment and water, highest number of strains producing lipase belonged to *Enterobacter ludwigii, Enterobacter cancerogenus* and *Stenotrophomonas maltophilia.*
- Pectin was found to be degraded by 12 sediment isolates only and none of the water isolates produced pectinase enzyme.

- β-galactosidase was found to be distributed in 10 species and >70% strains of *Enterobacter ludwigii* and *Enterobacter cancerogenus* were found to produce β-galactosidase.
- Cellulase production was found only in a few species such as *Pseudomonas fragi, Pseudomonas koreensis* and *Bacillus flexus.*
- Both phophatase and urease production was found in all the 12 species identified from sediment and water.
- Chitanse and xylanase producers were very less among culturable bacteria from Arctic. Forty two chitinase and 19 xylanase producing isolates were detected in the species such as *Bacillus thuringiensis*, *Pseudomonas fragi, Staphylococcus cohinii, B. flexus* and *P. sublingri.*
- Spectrophotometric assay of 53 high lipase producing isolates identified from plate assay method revealed 21 potent isolates and highest producer was phylogenetically identified as *Stenotrophomonas maltophilia.*
- Ability of 53 isolates to degrade natural substrates such as sunflower oil, olive oil, coconut oil, groundnut oil and palm oil were investigated by plate assay method. Groundnut oil was degraded by 60.67% isolates followed by palm oil (52.8%), olive oil (50.56%) sunflower oil (50.56%), and coconut oil (48.31%)
- Optimization of lipase production by 'one variable at a time' method was performed and the optimum conditions for lipase production were found to be temperature (25-30 °C), pH(6-7), agitation (150 rpm), carbon source (glucose), nitrogen source (yeast-peptone combination) and incubation period (5^{th} day).
- The parameters such as agitation, pH and nitrogen concentration were optimized using response surface methodology by software Design expert (Version 9, Stat-Ease. U.S.A.).
- Central composite design was employed for designing 20 experiments with 6 central points and 14 non central points as suggested by the software for optimization.
- Model suggested by the software was quadratic regression model and ANOVA of the model was found to be significant with an F value of 134.10. The multiple correlation coefficient of lipase production R^2 was 0.9926 which suggested that the model can explain 99.26% variation in response.

- The adequate precision value for the model was 25.73 and indicates the precision as well as reliability of the experiment. The 'Lack of fit' for the model was found to be 0.2974 which was not significant and it also indicated the prosperity of the model.
- The ANOVA table also indicated that the quadratic terms A^2 (pH), B^2 (nitrogen concentration) and C^2 (agitation) were significant, however, interactions between the factors were found to be insignificant.
- The optimized conditions for lipase production were identified using the model as agitation 147.29, pH 6.7 and at nitrogen concentration 13 g/L. Validation of the model was carried out by experimental analysis. Experimental value was 821.14 which was close to the predicted value of 832.63.
- Purification of lipase was done by acetone precipitation followed by DEAE-cellulose ion exchange chromatography and a purification fold of 7.1 was achieved.
- Determination of molecular weight of lipase was performed using SDS-PAGE analysis which was found to be approximately 49.1 KDa.
- Temperature stability of the enzyme revealed the cold active nature of the enzyme. The enzyme was found to be active at temperature range of 10 to 40 °C and optimum was detected at 25°C. Stability of the enzyme reduced drastically from 30 °C to 40 °C.
- The cold active lipase was found to be active at a wide range of pH ranging from 5-12 and optimum activity was detected at pH 7.0. However stability of the enzyme was found to reduce drastically as pH increased from pH 10 to pH 12.
- Cold active lipases from *Stenotrophomonas maltophilia* was found to be highly stable in presence of solvents such as pyridine, DMSO, benzene, acetone, butanol, methanol, heptane, ethanol, ethyl acetate and 1-propanol and only two organic solvents such as ethyl acetate and butanol reduced the activity to >50%.
- Out of the nine different metal ions tested, mercury, lithium and cobalt were found to have inhibitory effect on lipase activity Calcium and magnesium was noted to have stimulatory effect on lipase activity.
- The enzyme was stable in the presence of surfactants such as SDS, Triton X-100, Tween 80, Tween 60 and Tween 20 and the enzyme inhibitor, PMSF was found to have inhibitory effect on lipase activity.

- Agro-industial waste materials such as groundnut cake, coconut cake, neem cake, wheat bran and rice bran were investigated for production of lipase. Groundnut cake was found to the best suited for lipase production followed by coconut cake and neem cake.
- Moisture content of 60% was found to optimal for lipase production using groundnut cake, coconut cake, wheat bran and rice bran while moisture content of 70% was optimal for neem cake. For all the substrates optimal temperature for enzyme production was detected as 25 °C to 28°C.

The current investigation revealed the different characteristics of heterotrophic bacteria isolated from sediment and water of Kongsfjord (Arctic). The study showed the existence of multiple antibiotic resistance along with multiple metal resistance among these bacteria. Antibiotic resistance is a growing concern around the globe and resistance studies from pristine environment such as the Arctic might shed more light on the mechanism of antibiotic resistance and its dissemination.

Enzyme profiling of heterotrophic bacteria revealed their potential to produce various commercially valuable enzymes. The demand for enzymes is increasing every year and developed countries are gaining momentum in enzyme market which involves billions of dollars. Novel sources of enzymes with unique properties from extreme environments such as wild Arctic may provide unexplored potential enzyme producers and thus warrant further studies in this area. Studies on cold active lipase from *Stenotrophomonas maltophilia* indicated its possible applications in agro-industrial waste management, in detergents, in cosmetics and in fine chemical synthesis. However, a detailed study on the feasibility of large scale production of the enzyme in fermentors is essential for considering its industrial application.

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Appendix

Appendix 1

Compositoin of Growth Media

Table 1.1

ZoBell's Marine Broth

Ingredients	**g/ L**
Peptone digest of animal tissue	5.0
Yeast extract	1.0
Ferric citrate	0.1
Sodium chloride	19.45
Magnesium chloride	8.8
Sodium sulphate	3.2
Calcium chloride	1.8
Pottasium chloride	0.5

Sodium bicarbonate	0.16
Pottasium bromide	0.08
Strontium chloride	0.034
Boric acid	0.022
Sodium silicate	0.004
Ammonium nitrate	0.0016
Disodium phosphate	0.008
Sodium fluorate	0.0024
Final pH	7.6

Table 1.2

Glucose Yeast Peptone Broth

Ingredients	**g/ L**
Peptic digest of animal tissue	10.0
Yeast extract	5.0
Glucose	10.0
pH	7.0

Table 1.3

Luria Bertani Broth

Ingredients	**g/ L**
Casein enzymic hydrolysate	10.0
Yeast extract	5.0
Sodium chloride	10.0
pH	7.5

Table 1.4

Nutrient Broth

Ingredients	g/ L
Peptone	10.0
Beef extract	5.0
Sodium chloride	5.0

pH	7.3

Table 1.5

Mueller Hinton Agar

Ingredients	**g/ L**
Casein acid hydrolysate	17.5
Beef heart infusion	2.0
Starch	1.5
Agar	17.0
pH	7.3

Appendix 2

Table 2.1(a)

Effect of pH on the activity of lipase

pH	**% Relative activity**
5	57.86
6	84.00
7	100
8	91
9	72.15 ± 2.1
10	61.34 ± 1.4
11	49.88 ± 0.7
12	31.73 ± 0.4

Table 2.1 (b)

ANOVA for the effect of pH on lipase activity

	Sum of squares	Df	Mean square	F	Sig.
Between groups	1218.762	7	2863.32	1473.174	0.00
Within groups	18.31	12	1.8		
Total	1237.072	19			

Table 2.2(a)

Effect of pH on the stability of lipase

pH	**% Residual activity**
5	43.18
6	87.05
7	97.14
8	71.43
9	63.14±0.5
10	34.18±0.43
11	10.88±0.84
12	0.12±0.54

Table 2.2 (b)

ANOVA for the effect of pH on lipase stability

	Sum of squares	Df	Mean square	F	Sig.
Between groups	7623.17	7	8643.97	5618	0.00
Within groups	24.157	12	1.5		
Total	7647.17	19			

Table 2.3 (a)

Effect of temperature on activity of lipase

Temperature (°C)	% Relative activity
10	34.12
15	82.37
20	94.54
25	100.11
30	76.05±1.1
40	51.28±0.8
50	11.92±0.5

Table 2.3 (b)

ANOVA for the effect of temperature on lipase activity

	Sum of squares	**Df**	**Mean square**	**F**	**Sig.**
Between groups	3875.16	4	4138.14	4073.96	0.00
Within groups	17.9	13	1.9		
Total	3893.06	17			

Table 2.4 (a)

Effect of temperature on stability of lipase

Temperature (°C)	**% Residual activity**
10	97.17
15	98.56
20	100.06
25	100.21
30	73.97 ± 0.6
40	22.18 ± 0.7
50	0.84 ± 0.2

Table 2.4 (b)

ANOVA for the effect of temperature on lipase stability

	Sum of squares	Df	Mean square	F	Sig.
Between groups	1649.12	3	2901.23	2109.63	0.00
Within groups	15.4	14	1.7		
Total	1664.52	17			

Table 2.5 (a)

Effect of metal ions on the activity of lipase

Metal ions	% Residual activity
Calcium	104.03
Copper	78.52
Cobalt	48.32
Iron	92.43
Mercury	16.04±2.6
Lithium	32.48±0.4
Magnesium	101.514±1.6
Lead	77.94±1.4
Zinc	86.19±1.1

Table 2.5 (b)

ANOVA for effect of metal ions on the activity of lipase

	Sum of squares	**Df**	**Mean square**	**F**	**Sig.**
Between groups	2189.52	9	3195.62	3106.09	0.00
Within groups	18	16	5.7		
Total	2207.52	25			

Table 2.6 (a)

Effect of organic solvents on the activity of lipase

Solvents	**% Residual activity**
Pyridine	81.33
DMSO	72.13
Benzene	101.33
Acetone	84.33
Butanol	53.33 ± 1.4
Methanol	86.33 ± 0.7
Heptane	100.66 ± 1.3
Ethanol	72.66 ± 1.4
Ethylacetate	38.43 ± 1.7

1-propanol	77.06±0.3

Table 2.6 (b)

ANOVA for effect of organic solvents on the activity of lipase

	Sum of squares	Df	Mean square	F	Sig.
Between groups	9874.76	2	11097.6	10876.13	0.00
Within groups	31.7	15	2.9		
Total	9906.46	17			

Articles Published

- *C.S. Neethu, K.M. Mujeeb Rahiman, A.V. Saramma, A.A. Mohamed Hatha (2015) Heavy metal resistance of Gram negative bacteria isolated from Kongsfjord, Arctic.* ***Canadian journal of microbiology.*** *61: 429-435.*
- *C.S. Neethu, K. M. Mujeeb Rahiman, Emilda Rosmine, A. V. Saramma, A. A. Mohamed Hatha (2015) Utilization of agro-industrial wastes for the production of lipase from Stenotrophomonas maltophilia isolated from Arctic and Optimization of physical parameters.* ***Biocatalysis and agricultural biotechnology****. In press*
- *C. S. Neethu, K.M. Mujeeb Rahiman, A.V. Saramma, A.A. Mohamed Hatha (2012) Substrate specificity of cold active lipase of psychrotrophic bacteria from Kongsfjord and its partial characterization.* ***Advanced Biotechnology*** *12: 28-31.*
- *A.A.Mohamed Hatha, C.S. Neethu, S.M. Nikhil, K.M. Mujeeb Rahiman, K.P. Krishnan[b], A.V. Saramma, Relatively high antibiotic resistance among heterotrophic*

bacteria from arctic fjord sediments than water – evidence towards better selection pressure in the fjord sediments. ***Polar Research****. In Press*

- *Shubhankar Ghosh, D. G. Selvam, C. S. Neethu, A. V. Saramma and Mohamed Hatha (2014) Diversity of and antimicrobial activity of Lactic Acid Bacteria from the gut of marine fish Rastrelliger kanagurta against fish, shrimp and human pathogens.* ***Journal of Marine biological assossiation of India****. 55 (2): 22-27*