Isolation and Purification of Bacterial Psychrophilic Enantioselective Esterase for its potential use in pharmaceutical industry

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Dedication

I dedicate my work to
My beloved Family

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List of Abbreviations

APS	Ammonium persulfate
bp	Base pairs
BSA	Bovine serum albumin
c	Conversion
CAS	Cassette
cm	Centimetre
Da	Dalton
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleoside triphosphate
E	Enantioselectivity
E. coli	*Escherichia coli*
epPCR	Error prone PCR
g	Gram
GC	Gas Chromatography
H	Hour
HPLC	High pressure liquid chromatography
kb	Kilo base
kDa	Kilo Dalton
K_M	Michaelis constant
l	Liter
LB	Laura Bertani
M	Mole per liter

mA	Milliampere
oligo	oligonucleotide
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PFE I	*Pseudomonas fluorescens* esterase I
pNPA	*p*-Nitrophenyl acetate
UV	Ultraviolet
T_M	Melting temperature
sec	Second
SDS-	Sodium dodecyl sulphate
TEMED	N, N, N‘,N‘-Tetramethylethylendiamine
Tris	(hydroxymethyl)- aminomethane
V	Volt
°C	Degree Celsius
µg	Microgram
µl	Microliter
%(w/v)	Mass percent
%(v/v)	Volume percent

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Abstract

Enzymes have been used as biocatalyst since the very beginning of scientific knowledge. With advancement in research techniques and increase in awareness psychrophilic enzymes have attracted the attention of scientists. In this study a recently evolved Pseudomonal species has been isolated from Siachen glacier of Pakistan and its esterase producing capacity has been explored. The enzyme has been found to be active at 4^{o}C, pH 10.5 both on Rhodamine B oil emulsion plates and UV spectroscopy using para nitro phenyl acetate at 410 nm. The concentration of the enzyme was found to be maximum in LB medium substituted with 1% yeast extract at 72 hours. Molecular weight determination through SDS-PAGE followed by MALDI-TOF mass spectrometry revealed the molecular mass 43 kDa. Our work has highlighted an industrially useful enzyme and its activity parameters have been optimized. Further purification will be done to determine the structure of the protein. The applications will be established practically. An alternative approach is to computationally predict the possible substrate of this enzyme and evaluate the outcomes experimentally.

Chapter 1

Introduction:

Esterases as the name indicates are Carboxyl Hydrolases which are involved in the catalysis of ester bonds (Romano, Bonomi et al. 2015). These enzymes participate in formation (Oh, Nguyen et al. 2012), breakdown and transesterification of short chain fatty acid esters. Biochemical analysis of esterase revealed an α/β-hydrolase fold (Oh, Nguyen et al. 2012) as a part of their structure. A consensus motif G-X-S-X-G is also present around the active site, X denotes any amino acid (Fu, Leiros et al. 2013). The centrally located serine in the active site is the reason they are called serine hydrolases (Derewenda and Derewenda 1991). This serine is nucleophilic in nature, aspartate or glutamate with catalytic carboxyl group and a histidine (Derewenda, Derewenda et al. 1994). Another common feature present in all these enzymes is the oxyanion hole which stabilizes the tetrahedral reaction intermediate which is the important transition state (Infanzón, Valenzuela et al. 2014).

Mechanism of esterase action:

The mechanism for ester hydrolysis is similar for esterases and lipases consisting of four main steps. When the enzyme interacts with the substrate its forms a tetrahedral intermediate in which the catalytic histidine and aspartate residues play a stabilizing role. Then an alcohol is released resulting in acyl enzyme complex. Water attacks this complex and forms a tetrahedral intermediate again. In the fourth and final step an acid molecule is released and enzyme is liberated. During transesterification, water substitutes for alcohol and a new ester is formed.

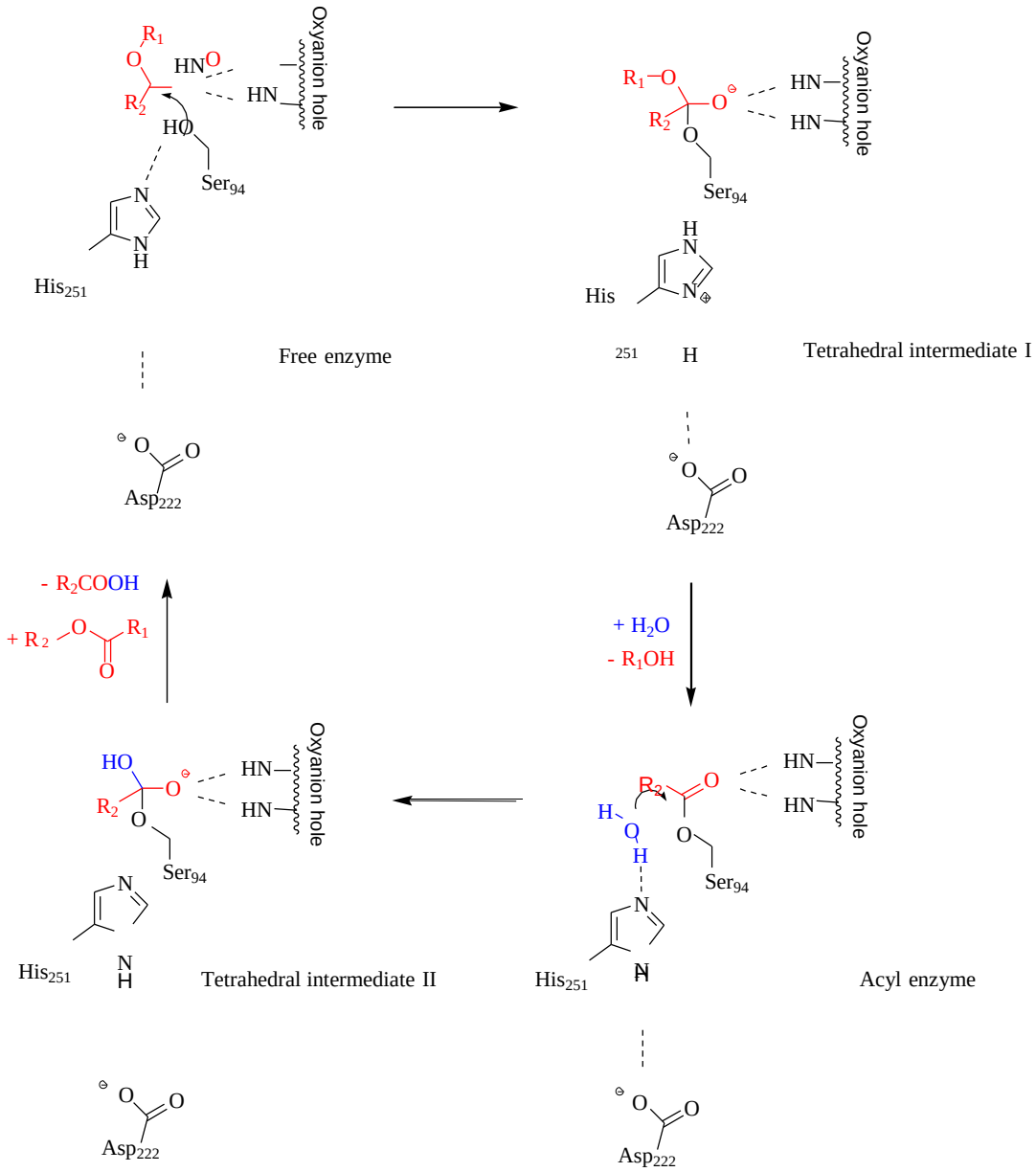


Figure 1.1: **Mechanism of action of esterases:** nucleophilic attack of serine oxygen on substrate, release of alcohol with the formation of acyl-enzyme complex. Nucleophilic attack of another nucleophile i.e. oxygen from water or alcohol, and release of the production liberation the enzyme.

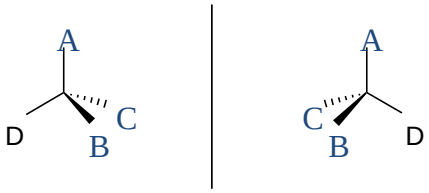
Esterases belong to the group of hydrolases (Bornscheuer and Kazlauskas 2006). Hydrolases are involved in the hydrolytic breakdown of several organic compounds such as carboxylic esters, amides and their formation (van Rantwijk and Sheldon 2004). Additionally, these enzymes hydrolyze nitriles (Banerjee, Sharma et al. 2002) and epoxides (Holmquist 2000) as well. They have been assigned enzyme class 3 (EC3) commission number by the International Union of Biochemistry and Molecular Biology (Webb 1992). Hydrolases have multiple advantages due to which they are extensively used in organic chemistry. Enzymes of this class have broad substrate specificity (Chakraborty, Asgeirsson et al. 2012) therefore, are very lucrative for the synthesis of related compounds (Kim, Song et al. 1997). They do not require cofactors for their activity which are difficult in regeneration thus, are industrially favorable (Steiner, Janssen et al. 2010). EC3 also exhibit tolerance towards organic solvents a key factor in organic compound synthesis (Dandavate, Jinjala et al. 2009). These enzymes can be used to catalyze reversible reactions (Bornscheuer and Kazlauskas 2006) by altering the reaction parameters, such as water is a prerequisite for hydrolysis and on removing water from the reaction mixture, it can be directed towards synthesis (Schoemaker, Mink et al. 2003) (Kourist, Bartsch et al. 2007). Moreover, many of these enzymes are highly enantioselective, making them suitable for synthesis of optically pure molecules for fine chemistry thus, profitable option for pharmaceutical chemistry (Patel 2008). Enzymes display several properties which make them viable alternatives to chemical catalysts;

Enzymes are often regio-, chemo- and stereospecific in their action. As their action is site selective, therefore, there is no need of protecting groups. Thus, reaction steps are saved. Their reaction conditions are greener than the ones of the chemical alternatives (chemical and physical parameters). Protein engineering is an option for improving the properties (for example substrate selectivity, thermostability, pH profile). There is a high demand for selective

catalyst, as with passing time demand for optically pure compounds in the pharmaceutical industry and fine chemistry is increasing (Wong and Whitesides 1994).

Esterases have already established their importance in the industrial sector, as in case of naproxen synthesis, paving way for more (Quax and Broekhuizen 1994). Therefore, the scientists are carrying out extensive research to find and optimize newer enzymes and optimize new processes (Wong and Whitesides 1994). Selection between chemical or biological catalyst is based on many factors like required purity of the product depending upon the nature of use, effect of the catalyst price on the end product, recovery of catalyst from the reaction mixture and down-stream process cost. Product synthesis steps are also affected greatly by the consumer choice as all of them do not prefer biological catalysts in their product some of them prefer their consumable without the interference of gene technology thus option a chemical process instead.

Human body has been observed to respond differently towards the chiral compounds as the active site of the enzymes is 3D in nature and response of the enzyme towards enantiomers is different. It is very important that we monitor the effects of the chemical entities administered in the form of pharmaceuticals in the body. This fact got prominent after the Thalidomide disaster between 1957 and 1961 (McBride 1961). Birth deformities were observed on large scale of population and when the causes were determined this drug was found to be the culprit. Thalidomide was sold under the brand name Contergan® to the pregnant women as remedy for morning sickness and depression. As an optically active drug the R-enantiomer is responsible for the adverse effect and then in-vivo racemization (Eriksson, Björkman et al. 1995) completely ruled out its use in the pregnant ladies. Many other such effects of chiral drugs were observed after this incident and in 1992 the Food and Drug Administration (FDA) issued a statement that all the chiral drugs should be tested with their enantiomers individually before administration to the patient.



Example for a chiral center (A-D are different substituents)

Therefore, synthesis of chemical compound as pure chiral molecules became a prerequisite for many drug therapies (Tomaszewski and Rumore 1994). Many chemical procedures have been devised to purify the chiral compounds from each other increasing the tediousness of the process. Biological catalysts have shown to be extremely useful in this regard, synthesis of Naproxen® (Chang and Tsai 1997) being an example to start with. In addition to the chiral compounds some bulk chemicals are also produced with the help of enzyme catalysts. Production of Acrylamide (Yamada and Kobayashi 1996) with nitrile hydratase is an example.

Habitat of the organism from which enzymes are extracted is very important because it determines the nature of the product. Earlier, the enzymes used were generally from the mesophilic origin, i.e. they were able to perform at temperatures 25-40^{o}C and had certain constraints associated with them. These enzymes were less efficient and less specific, and some reactions needed to be catalyzed at lower temperatures owing to product stability (Joseph, Ramteke et al. 2008). Thus cold adapted catalyst became the subject of interest in such problems. Moreover the rate of reaction of the psychrophilic enzymes was found to be up to one order of magnitude higher than the mesophilic or thermophilic counterparts (D'Amico, Collins et al. 2006). Factors responsible for this change in activity is the adaptability of psychrophilic organisms for which they have certain marked changes in their structural make-up. The proteins have been observed to have decreased number of proline residues, aromatic interactions and ion pairs (Joseph, Ramteke et al. 2008). Furthermore, they possess decreased

hydrophobic packing and their residues are re-distributed to facilitate interactions with the solvent molecules. The stabilizing co-factors have a weaker binding with the enzyme active site (Feller and Gerday 2003).

The aims of this study were to isolate a psychrophilic bacterium from the ice sampled from Siachen glacier of Pakistan and to characterize it via 16S RNA gene sequencing, BLAST analysis followed by phylogenetic study. Then, ferment the purified bacterium, isolate esterase enzyme from it and optimize the activity parameters including pH and wavelength. In addition to these, purification of this enzyme and determination of molecular mass of the enzyme were also the aims for conducting this study.

Chapter 2

Literature review:

2.1: Biocatalysis:

Biocatalysis is the use of enzymes obtained from various sources as biological catalysts (Buchholz, Kasche et al. 2012). These biocatalysts may be crude preparations from their source in the form of whole cells or cellular extracts depending upon the nature of the product or they can be purified lyophilized proteins. Both types of the biocatalysts have their pros on cons i.e. pure enzymes guarantee the purity of the product but they do not have the necessary cofactors with them. The cofactors are mandatory for the activity and an expensive addition to the process (Dordick 2013). The stability may be enhanced by modifying the physical condition of the protein such as immobilizing it through various ways. Immobilization ease away the down streaming and regeneration of the valuable catalyst (Cantone, Ferrario et al. 2013).

Whole cell preparations are particularly useful when both the reactants and the products can pass through the membrane of the cell as down streaming becomes difficult if any part of the product remains inside the cell. Any product or the intermediate should not be toxic for the cell. Moreover other enzymes present inside the cell should not alter the product stability and integrity (Andrade, Freitas et al. 2012).

Crude extracts including the lyophilized preparations possess the advantage of containing the necessary cofactors without the need of products and substrates to pass through the cellular membranes (Andrade, Freitas et al. 2012). Though the undesired enzymes from the host cell may be present in them which lead to side reactions and the catalyst may be lost during product harvesting.

With the advancements in the purification process and increase in knowledge of the researchers way of using biocatalysts has changed a great deal. Earlier wild cells be it bacterial,

fungal or animal plant extracts were used as such. This practice had many constraints and newer processes required increased sophistication. Crude catalysts had lower concentration of enzymes, quantity is not similar between batches making quality control parameters impossible to be achieved. Moreover in food and pharmaceutical industry cultural and ethnic issues need to be taken in consideration. Products from halal animals are permitted in Islamic society therefore on industrial scale particular importance is given while taking such measures (Flickinger 2010).

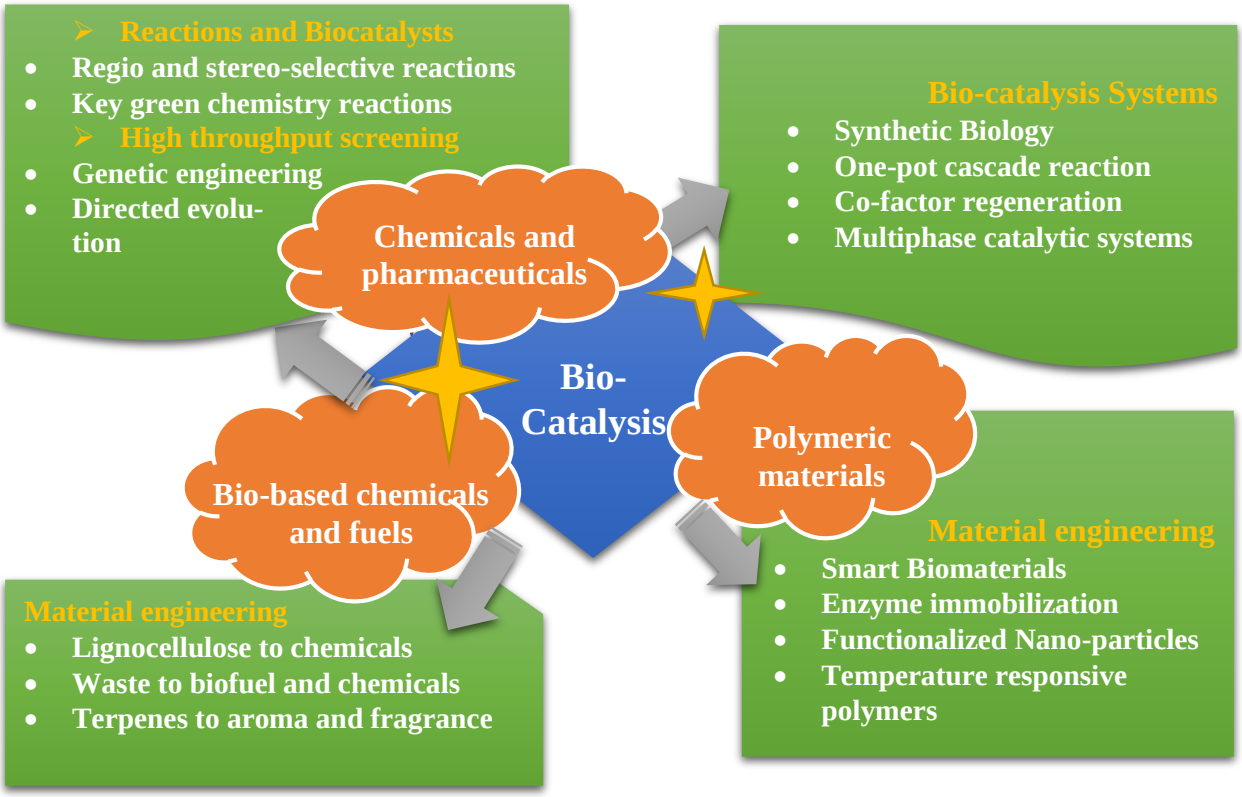


Figure 2.1: General flow chart diagram of bio-catalysis: Biocatalysts are employed in multiple fields of interests. Chemical industry has employed a huge number of enzymes in various reactions. Engineering industry has modified and made the technology increasingly sophisticated by making the processes more reproducible.

2.2. Enantioselectivity:

Most of the biological molecules are organic in nature and have carbon as the main component of their backbone. Carbon atom has a valency of 4 thus, makes four bonds to

complete their octet and achieve stability (Corey and Guzman-Perez 1998). When these bonds are made with four different substituents it gives rise to a property called chirality (Corey and Guzman-Perez 1998). Chiral molecules lack plane of symmetry and their mirror images cannot be superimposed. These molecules rotate the plane of polarized light in opposite directions thus, are optically active. Isomers possessing such properties are called enantiomers (Cahn, Ingold et al. 1966). Enantiomers behave identically in environments where there are no chiral molecules but, in their presence enantiomers behave differently (Jacques, Collet et al. 1981).

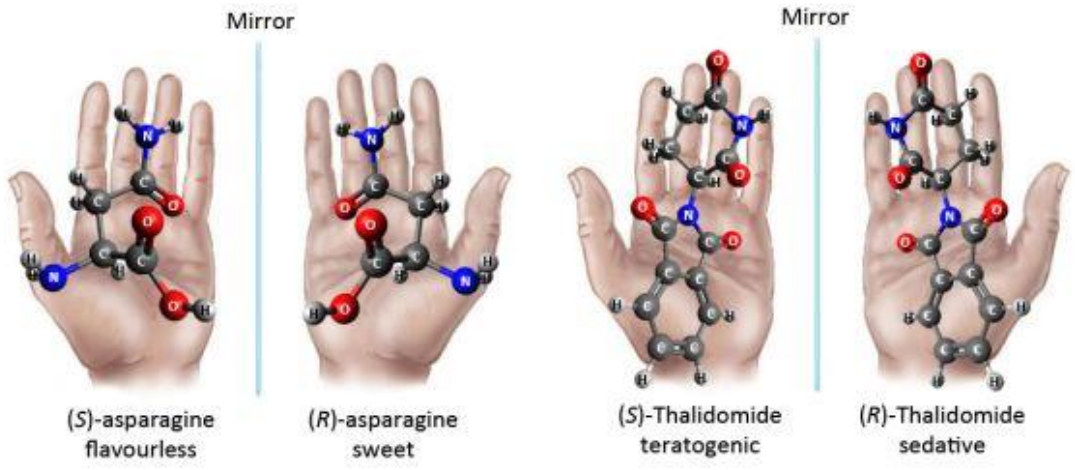


Figure 2.2: Examples of Enantiomeric Compounds: Asparagine and thalidomide in both enantiomeric forms with physical properties. Retrieve from http://doktori.bme.hu/bme_palyazat/2013/honlap/Bagi_Peter_en.htm

Enzymes and receptors have a 3D structure and their active site is in the form of a pocket which is capable of accommodating one of the enantiomers in them (Sonnet 1988). This property makes the biological systems respond differently towards the enantiomers. The taste receptors behave in a similar manner for example, the levo amino acids do not trigger any taste sensation whereas, dextro amino acids are sweet to taste (Meierhenrich 2008). Moreover, the olfactory receptors can clearly distinguish between R- carvone present in spearmint leaves and S-carvone in the caraway seeds (Leitereg, Guadagni et al. 1971). Similar is the case when it

comes to pharmaceutical agents and it can have drastic consequences when one of the enantiomers is toxic in nature (Gotor-Fernández, Brieva et al. 2006).

The Cahn Ingold Prelog rule (Cleaves II 2011) is the rule of thumb for designating enantiomers. The four substituents present on the carbon are assigned precedence based on their atomic number. The substituent with the smallest atomic number is rotated around the chiral center and the sequence of the rest of the substituents is observed accordingly. If it is decreasing in clockwise direction the enantiomer is labelled as *R*, found otherwise the enantiomer is labelled as *S (Cleaves II 2011).*

2.3. Carboxyl Esterase and Lipase Structure:

Like all hydrolases esterases possess the α/β hydrolase fold which is their characteristic feature and highly conserved in this class of enzyme (Hotelier, Renault et al. 2004). There are usually 8 β- strands in the α/β hydrolase fold connected by 6 α-helices as shown in the figure 2.3.

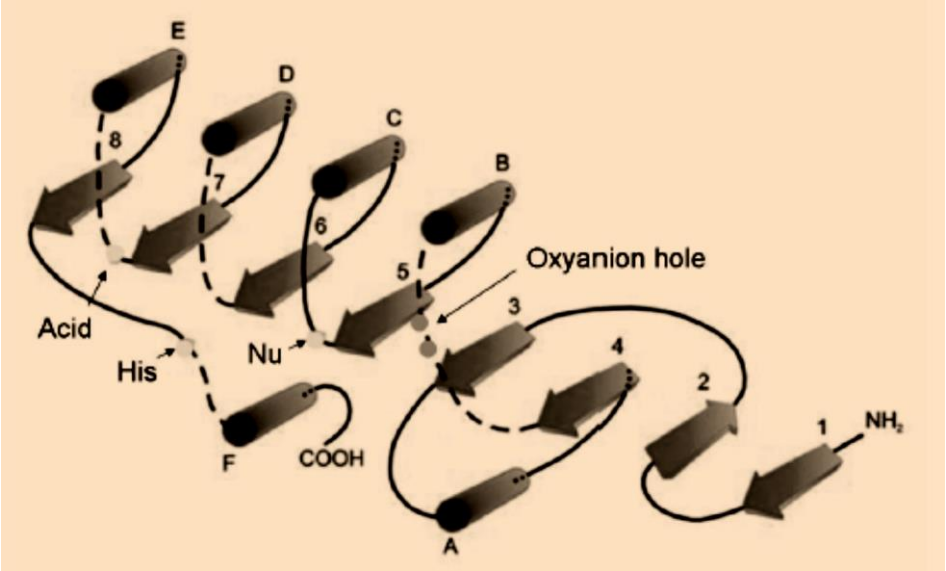


Figure 2.3. Schematic representation of the a/b-hydrolase fold: Helices are shown as pink cylinders, b-strands as blue arrows. Yellow dots: catalytic residues, green dots: oxyanion hole residues. Dashed lines indicate possible insertions. (Ollis, Cheah et al. 1992)

α/β hydrolases also possess a nucleophilic elbow, due the presence of a nucleophilic serine at the sharp γ turn, present in the middle of a β-strand and an α-helix (Nardini and Dijkstra 1999). Then there is a catalytic triad which is composed of a serine, histidine and an aspartate residue. In some lipases there is a Glutamate residue instead of an aspartate. When primary sequence of the protein is observed a Gly-X-Ser-X-Gly pentatpeptide is found, where X denotes any amino acid (Nardini and Dijkstra 1999) (Hotelier, Renault et al. 2004). Additionally when the 3D structure of the enzyme is studied a lid is present which is found covering the active site.

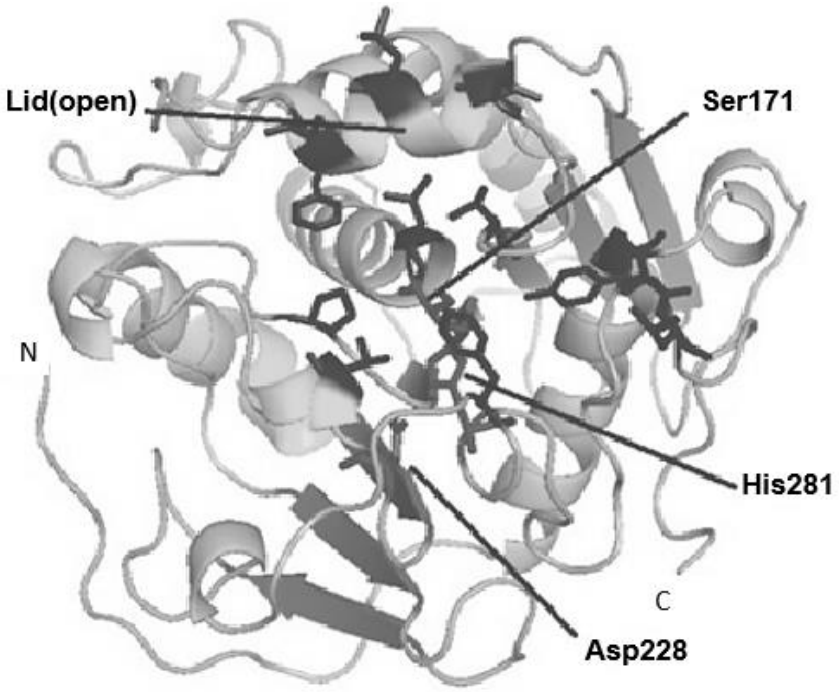


Figure 2.4. Cartoon view of the modeled DAG like lipase from *Malassezia globosa* in the open conformation. The catalytic pocket and the residues constituted the oxyanion hole highlighted in blue, while the catalytic triads were colored red. Taken and modified from (Xu, Lan et al. 2015)

2.4. Secretion pathway of Lipolytic enzymes:

These enzymes are extracellular in nature and there transcription is regulated by the lipase operon *lipA/H* from promotor P1. This promotor requires a sigma factor for its activation and a second promotor P2 300bp upstream of P1. The promotors requiring the sigma factor for their activation also require a cognate transcription activator leading to the proposal for existence of lipase regulator *lipR.* Extracellular enzyme is secreted when the organism enters the stationary phase suggesting a quorum sensing mechanism for the secretion activation.

Mutation studies revealed that the *lipR* is a part of two component system, second being lipQ, both these genes are found to be involved in the encoding of transducing protein. This two component system is also activated by a global regulator gene GacA, overproduction of

this regulator increased the lipase production via *rhlR/I* system. Figure 2.5 summarizes the regulatory process of the lipolytic enzyme production.

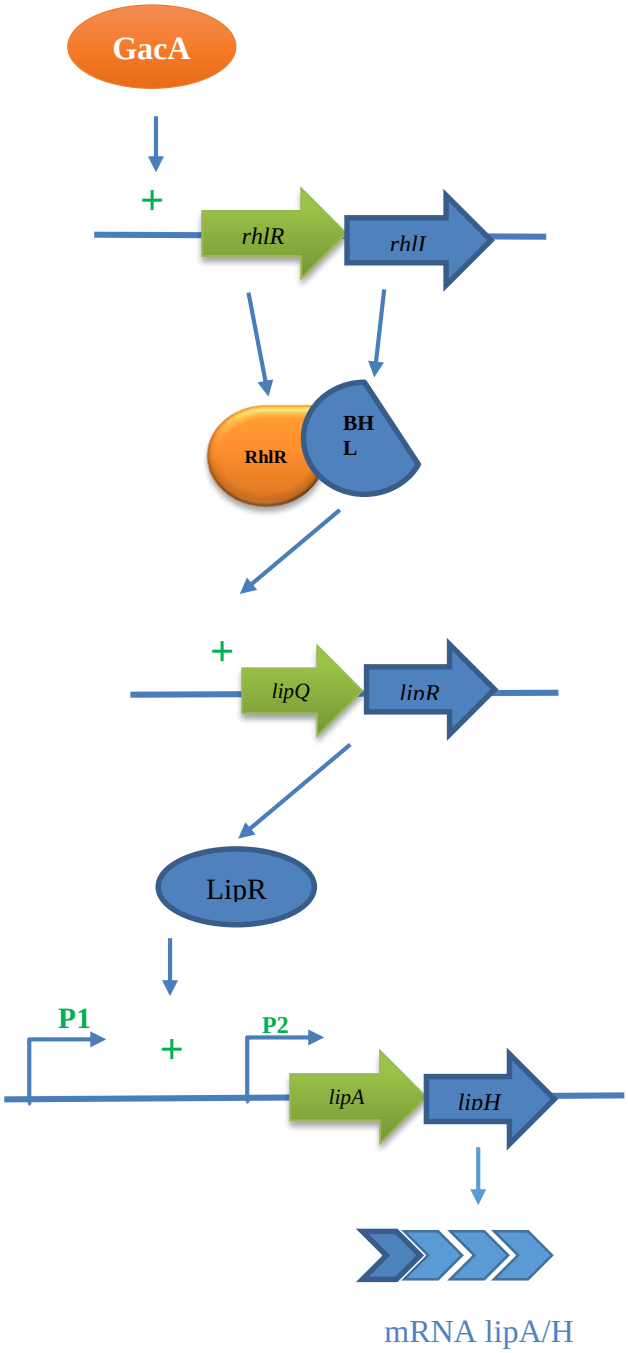


Figure 2.5: Model representing the regulation of the operon *LipA/H* involved in expression of Lipase in *Pseudomonas aeruginosa*.

2.5. Classification of Esterases and Lipases

After discovery of a number of bacterial esterases and lipases there was a need to classify these enzymes. In 1999 Aprigny and Jaeger compared them on the basis of the amino acid sequence, relationship to other enzyme families, presence of the specific foldases required for lipase sand mode of secretion of the enzyme out of the cell. They compared 53 sequences and 8 different families were formed grouping them respectively.

True lipases were classified into Family I (block L in ESTHER database, http://bioweb.ensam.inra.fr/ESTHER) and subsequently classified into 6 sub-families. Subfamilies I.1 and I.2 were similar in respect of having aspartic acid residues for Ca^{2+} binding sites and two cysteine for forming disulfide bridge. They need a Lipase-specific foldase for proper folding. But these families differ in size, family I.1 range in size from 30 to 32 kDa wheras family I.2 are slightly heavier being 33kDa and contain a sequence which forms a double β-strand at the protein surface which is anti-parallel. The subfamily I.3 has molecular mass even higher than the first two families i.e. 50 kDa and 65 kDa reported in *Pseudomonas flourescens* and *Serratia marcescens* respectively. Then the N-terminal signal peptide and the cystiene residues are absent. Next are the lipases from the gram positive organisms, in them the Glycine residue of the pentapeptide is replaced with Alanine Ala-Xaa-Ser-Xaa-Gly. The small molecular weight lipases from *Bacillus subtilis* and *Bacillus pumilus* of 20 kDa have been placed in the 4th sub-family, whereas the higher molecular weight lipases from bacillus species of 45 kDa and staphylococcal species of 75 kDa in the 5th sub-family. The 6th sub-family includes the lipases from *Propionibacterium acnes* and from *Streptomyces cinnamoneus* which are different from other lipases of the true lipase family but show 50% similarity to I.2 and *Bacillus subtilis* lipases.

Family II is called the GDSL family; it does not possess the conventional pentapeptide but a Gly-Asp-Ser-(Leu) motif containing the active site Serine residue. Instead of the catalytic

triad there is a dyad and the acidic side chain stabilizing the positive charge of active site Histidine residue is replaced by the carbonyl located three positions upstream of the histidine. These enzymes show similarity with the platelet activating factor acetylhydrolase. *Streptococcus scabies* esterase belongs to this family.

Third family enzymes have a canonical fold of the α/β hydrolase, contains the typical triad and 20 % similarity with the human platelet activating factor acetylhydrolase. *Streptococcus exfoliatus* enzyme is a member of this family. The Hormone sensitive lipase (HSL) family is the fourth family of the lipolytic enzymes. The bacterial enzymes show a striking resemblance to the mammalian hormone sensitive lipase. Psyhrophillic, mesophillic and thermophillic bacteria are present in this family. *Escherichia coli, Moraxella sp*. And *Archeoglobus fulgidus* enzymes are the examples. The 5th family enzymes also have versatile bacteria producing them from cold moderate and hot environment. They show similarity with the non-lipolytic bacterial enzymes which have α/β-hydrolase fold and a catalytic triad. *Acetobacter pasteurianus* enzyme belongs to this family. The smallest size esterases are member of the 6th family of the lipolytic enzymes. Active form of these enzymes is a dimer. They also have the typical catalytic triad and are the α/β-hydrolases. They are similar in sequence about 40% to the human eukaryotic lysophospholipases. Synechocystis sp. enzymes are member of this family. Esterases of relatively larger size i.e. 55 kDa are found in the 7th lipolytic enzyme family. They show 30% to 40 % sequence similarity with the eukaryotic acetylcholinestrase and the intestinal carboxylesterases. *Bacillus subtilis* esterase is an example of this family enzyme. 8th family of the enzymes has a striking resemblance in sequence to the well-known β-lactamases. The active site has a different sequence i.e. Ser-Xaa-Xaa-Lys than the super family, therefore the catalytic residue is also different. The catalytic mechanism needs to be studied in these enzymes. *Arthrobacter globiformis* stereoselective esterase belongs to this family (Arpigny and Jaeger 1999).

2.6. Psychrophilic enzymes

Sustaining life at low temperature requires a new paraphernalia altogether at all levels of metabolism taking place inside the cell (Feller, Arpigny et al. 1997). The physicochemical features of the cold environment are entirely different from those of mesophilic or thermophilic area, the viscosity of water is different at that temperature leading to lesser diffusion rate along with decrease in biochemical reactions (Margesin, Schinner et al. 2007). Low temperature results in disturbance in weak molecular interactions that are involved in identification of molecules and the interaction between them. Solubility of gases is increased resulting in accumulation of toxic substances inside the cell and the transmembrane material exchange is also reduced (Margesin, Schinner et al. 2007). To adjust to these circumstances psychrophilic organisms have specific features which have been detected in their genome sequences (Methé, Nelson et al. 2005). Whole genome sequences of various psychrophilic bacteria and archea have been obtained and are yet to be analyzed (Casanueva, Tuffin et al. 2010). A eukaryotic microalga has also been sequenced recently (Cadoret, Garnier et al. 2012) with the advancement in the high throughput screening techniques.

To determine the properties of the psychrophilic enzymes the crystal structures are of prime importance, Table 2.1 provides an overview of the crystal structures of the psychrophilic enzymes reported till 2013 (Feller 2013). Until now only one NMR structure of a thiol-disulphide oxidoreductase has been published from an Antarctic bacterium. In comparison to thermophilic enzymes that thrive at higher temperature, the psychrophilic enzymes face more subtle challenges. For instance, the rate of enzyme catalyzed reactions are strongly reduced at lower temperatures, as the molecular motions associated with proteins are slower down too. This temperature dependence with the activity of enzymes can be understood through the relation.

The maximum number of substrate molecules converted to product per active site per unit of time is a measure of enzyme activity and is related as the catalytic constant k_{cat}.

$$k_{cat} = \kappa \frac{k_B T}{h} e^{-\Delta G^{\#}/RT}.$$

κ is the transmission coefficient generally close to 1,

k_B is the Bolzmann constant (1.38×10^{-23} J K^{-1}),

h the Planck constant (6.63×10^{-34} J s),

R the universal gas constant (8.31 J K^{-1} mol^{-1}), and

$\Delta G^{\#}$ the free energy of activation or the variation of the Gibbs energy between the activated enzyme-substrate complex ES^{*} and the ground state ES (Feller 2013).

Table 2.1: Representative psychrophilic enzymes of known crystal structure. (Feller 2013)

Cold-adapted protein	Source	PDB entry
Alpha-amylase	Antarctic bacterium *Pseudoalteromonas haloplanktis*	1AQH
Xylanase	Antarctic bacterium *Pseudoalteromonas haloplanktis*	1H12
Cellulase	Antarctic bacterium *Pseudoalteromonas haloplanktis*	1TVN
Superoxide dismutase	Antarctic bacterium *Pseudoalteromonas haloplanktis*	3LJF
S-Formylglutathione	Antarctic bacterium *Pseudoalteromonas haloplanktis*	3LS2
Ca^{2+} Zn^{2+} protease	Antarctic bacterium *Pseudomonas* sp.	1G9K
Beta-lactamase	Antarctic bacterium *Pseudomonas* sp.	2QZ6
Citrate synthase	Antarctic bacterium *Arthrobacter* sp.	1A59
Beta-galactosidase	Antarctic bacterium *Arthrobacter* sp.	1YQ2
Subtilisine	Antarctic bacterium *Bacillus* sp.	2GKO
Aliphatic amidase	Antarctic bacterium *Nesterenkonia* sp.	3HKX
Alkaline phosphatase	Antarctic bacterium	2IUC
Aminopeptidase	Arctic bacterium *Colwellia psychrerythraea*	3CIA
Phenylalanine hydroxylase	Arctic bacterium *Colwellia psychrerythraea*	2V27
Malate dehydrogenase	Arctic bacterium *Aquaspirillum arcticum*	1B8P
Serine proteinase	Arctic bacterium *Vibrio* sp.	1SH7
Isocitrate dehydrogenase	Arctic bacterium *Desulfotalea psychrophila*	2UXQ
Aspartate	Deep-sea bacterium *Moritella profunda*	2BE7
Adenylate kinase	Bacteria *Bacillus globisporus* and *Marinibacillus marinus*	1S3G, 3FB4
Triose-phosphate isomerase	Marine bacterium *Vibrio marinus*	1AW1
Ca^{2+} Zn^{2+} protease	Marine bacterium *Flavobacterium* sp.	1U1R
Tyrosine phosphatase	Bacterium *Shewanella* sp.	1V73
Catalase	Bacterium *Vibrio salmonicida*	2ISA
Endonuclease I	Bacterium *Vibrio salmonicida*	2PU3
Lipase	Bacterium *Photobacterium lipolytica*	2ORY
Superoxide dismutase	Bacterium *Aliivibrio salmonicida*	2W7W
Alkaline phosphatase	Bacteria *Vibrio* sp. *and Shewanella* sp.	3E2D, 3A52
Alkaline phosphatase	Arctic shrimp *Pandalus borealis*	1K7H
Lactate dehydrogenase	Antarctic ice fish *Champsocephalus gunnari*	2V65
Trypsin	Atlantic salmon *Salmo salar*	2TBS
Elastase	Atlantic salmon *Salmo salar*	1ELT
Pepsin	Atlantic cod *Gadus morhua*	1AM5
Uracil-DNA glycosylase	Atlantic cod *Gadus morhua*	1OKB

Table 2.2: Selected potential biotechnological applications of cold-adapted enzymes.

(Siddiqui and Cavicchioli 2006)

Applications	Enzymes
Food and Feed Industry:	
Animal feed for the improvement of digestibility and assimilation	Lipase, protease, phytase, glucanase, xylanase
Removal of hemicellulosic material from feed	Protease
Meat tenderizing	Chitinase
Single-cell protein from shellfish waste	α-amylase, glucoamylase
Starch hydrolysis	Pectinase, xylanase
Clarification of fruit, vegetable juices and wine	Pectate lyase, pectinase
Cheese ripening	α-amylase, xylanase
Dough fermentation, bakery products	β-galactosidase
Removal of lactose from milk, conversion of lactose in whey into glucose and galactose in dairy industry	Laccase
Wine and beverage stabilization	Feruloyl esterase
Detergent and cleaning industry:	
Additive to detergents for washing at room temperature	Lipase, protease
Fine chemical synthesis by reverse hydrolysis in organic solvents:	
Flavor modification, optically active esters	Lipase, protease
Asymmetric chemical synthesis	Dehydrogenase
Peptides, oligosaccharides	Protease, feruloyl esterase
Epoxide	Epoxide hydrolase
Organic compounds	Peroxidases
Environmental Biotechnology:	
Bioremediation, degradation and removal of xenobiotics and toxic	Lipase, protease, hydrocarbon degrading enzyme, peroxidase
Tanning and hide industry	Collagenase (deseasin)
Biobleaching in paper and pulp industry	Xylanase
Biofuels and energy production:	
Biodiesel production by trans-esterification of oils and alcohols	Lipase
Conversion of chitin and ethanol	Chitinase and yeast
Conversion of cellulose to ethanol	Cellulose β-glucosidase complex
Bioethanol production from dairy waste	β-galactosidase
Pharmaceutical, medical and domestic industry:	
Hydrolysis of chitin to chitosan, chitooligosaccarides, glucosamine	chitinase
Anti-fungal drug and additive for antifungal creams and lotions	Chitinase
Mosquito control at larval stage	Endochitinase and lipase
Synthesis of citroneliol laurate	Lipase
Cosmetics	Lipase, laccase
Antibacterial agent	Lysozyme
Anti-microbial, antioxidant, photiprotectant (ferulic acid)	Feruloyl esterase
Antibiotic degradation	β-lactamase
Chiral resolution of drugs to increase potency and spectrum	Esterase
Chiral resolution and synthesis of chemicals	Peroxidase
Manufacture of anticancer drugs	Laccase
Preparation of precursors of antibiotics	Imidase
Textile industry:	
Stone washing	Cellulose
Desizing denim jeans	α-amylase
Retting of flax, jute ramie, hemp etc	Xylanase

2.7. Applications of Carboxyl Esterases and Lipases

Synthesis and hydrolysis of ester bonds is carried out by hydrolytic enzymes comprising a large group called lipases and esterases (Bornscheuer and Kazlauskas 2006). These enzymes are widely used by pharmaceutical companies, in food processing, for detergent treatment and kinetic resolution. There is particular interest in these enzymes because these enzymes have novel characteristic including require no dependency on cofactors, ease in synthesis, have stability, show activity in organic solvents and possess high region and stereospecificity (Dordick 2013).

2.7.1. Applications in the Detergent Industry

The primary additives utilized in house hold detergents and industrial laundries are lipases and esterases owing to due to their ability to hydrolyze fats. For example, Novo Nordisk introduced the first lipase for commercial use i.e. In 1994, isolated from *Thermomyces lanuginosus*, a fungus and expressed via recombinant technology within *Aspergillus oryzae*. Additionaly, in 1995 two bacterial lipases namely LipomaxTM from *Pseudomonas alcaligenes* and LumafastTM from *Pseudomonas mendocina* were introduced by Genencor International for use as detergent additives. Suitable enzyme properties for this particular application comprise low specificity for the substrate and high tolerance to temperature, pH shifts and solvents. These requirements are all resolved by either enhancing enzyme properties via protein engineering or screening enzymes that are naturally stable (Arpigny and Jaeger 1999).

2.7.2. Applications in the Pharmaceutical Industry

A wide array of regio-, stereo- and chemo-specific transformations are catalyzed by esterases and lipases, thus providing a great variety in organic syntheses. For instance we can consider the synthesis of optically pure (R)- and/or (S)-ketoprofen [2-(3-benzoylphenyl) propionic acid] using esterase from *Trichosporon brassicae*. This compound is particularly

utilized in the pain relief and reduction of inflammation that occur due to sunburn, arthritis, fever and menstruation. Naproxen, which is another anti-inflammatory drug, is synthesized by carboxyl esterase from bacteria *Bacillus subtilis* called NP.

Ninety-Nine Percent enantiomerically pure (S)-naproxen is generated in this, and yield for this reaction is usually in excess of 95 %. Acetylcholine esterase inhibitors, taxol synthesis, throumboxane-A2-antagonist, anti-infective drugs , anti-cholesterol drugs, Ca2+ channel blocker drugs, anti-arrhythmic agents antiviral agents and K channel blocking drugs are some other examples of stereospecific conversions in the synthesis of pharmaceutical intermediates.

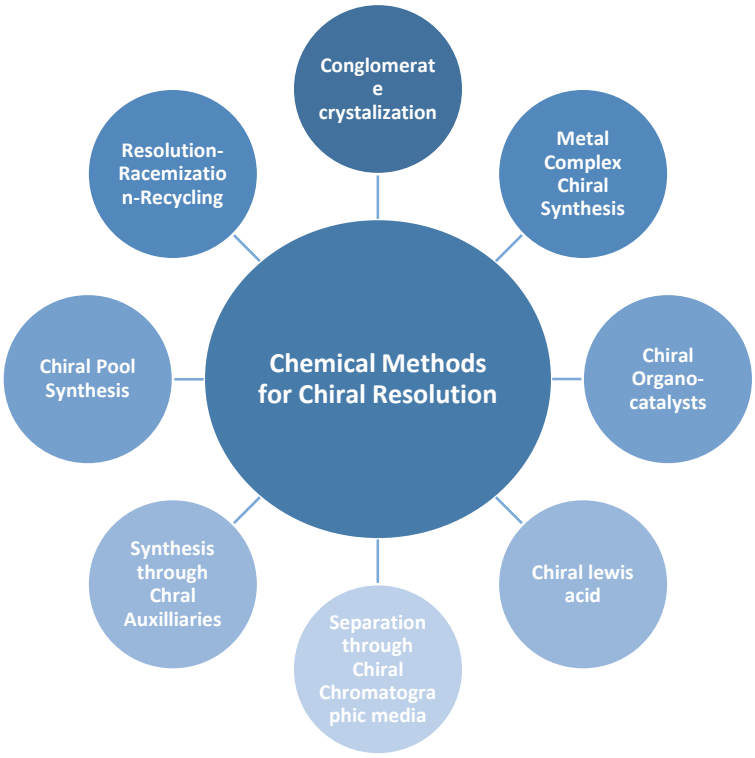


Figure 2.6: chemical methods of chiral resolution. Conglomerate crystallization (Zbaida, Lahav et al. 2000), Metal Complex Chiral Synthesis (Trost and Crawley 2003), Chiral Organo-catalysts (Bartók 2009), Chiral lewis acid (Evans, Murry et al. 1995), Separation through Chiral Chromatographic media (Bjørnsdottir, HonoréHansen et al. 1996), Synthesis through Chiral Auxiliaries (Seyden-Penne and Curran 1995), Chiral Pool Synthesis (Nugent, RajanBabu et al. 1993), and Resolution- Racemization-Recycling (Gladiali and Alberico 2006).

2.7.3. Applications in the Food Industry

The nutritional value and physical properties of triglycerides is highly effected by the chain length, degree of unsaturation and degree of the fatty acids. As oils and fats are vital components of food, esterases and lipases can be utilized for synthesizing higher value fats a relatively economical way. For example, the position of fatty-acid chains can be altered by these enzymes in the glyceride substrate and substitute either one or more of fatty acids with ones that are more desirable.

Upgradation of palm oil to attain the properties of coca butter for chocolate production is a typical example of the use of these enzymes in food industry. Undesired palmitic acids with selected stearic acids have been substituted by using lipases from *Rhizomucor miehei*. Some other examples involve lipase-catalyzed exclusion of fat from meats as well as extracting polyunsaturated fatty acids from animals and plants for use as nutraceuticals.

2.7.4. Other Industrial Applications

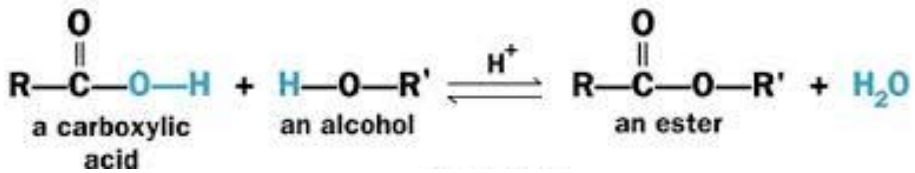
Esterases and lipases, in addition to the above-mentioned applications are utilized in many other industries including pulp and paper processing, perfume industries as well as in degradation of synthetic materials. Arylesterases and feruloyl esterases, which form a sub-group of esterases, are better and suitable alternatives for the production of anti-oxidant additives and flavors from lignin-derived phenolic acids. For instance, feruloyl esterases on combination with xylanases release ferulic acid from xylan, which has a potential of conversion to vanillin. Feruloyl esterases are members of carbohydrate esterase family 1, and have been derived from various fiber-active microorganisms such as *Fusarium oxysporum* and *Aspergillus niger*. Additionally, synthetic pollutants including plastics, polyesters and polyurethane have been degraded using polyurethenase and cholesterol esterases. Isolation of these enzymes was done from *Pseudomonas aeruginosa* and *Pseudomonas chlororophis*.

2.8. Esterase and Lipase Catalyzed Transesterification

Esterases and lipases have demonstrated ability in organic chemical synthesis, catalyzing a wide range of chemo-, regio-, and stereo-selective transformations. Corresponding (trans-) esterification reactions are favored in the absence of water, whereby an ester bond is synthesized between an alcohol and a carboxylic acid (or carboxylic ester) by synthesizing the acid or exchanging the organic group (R3) of the ester with the organic group (R2) of the alcohol. For instance, the commercial lipase, PS "Amano" SD, originated from *Burkholderia cepacia*, catalyzes a variety of transesterification reactions and successfully resolves many racemic mixtures.

2.9. Schematic representation of esterification and transesterification reactions.

During esterification, an ester linkage is formed between an alcohol and carboxylic acid by release of a water molecule.



While, a new ester is formed by exchanging the organic group (R3) of an ester with the organic group (R2) of the alcohol in a transesterification.

As described earlier, the water content determines the equilibrium of reaction i.e. hydrolysis vs synthesis, thermo-inactivation of biocatalysts as well as the distribution of products in the reaction vessel. It has been shown by the researchers that elevation of

temperatures yield in higher stability of enzymes relatively hydrophobic environment in comparison to hydrophilic environments (Ebbing and Gammon 2007). Therefore, organic solvents are being used as reaction media as they not only favor (trans-) esterification, but also have the ability to increase the solubility of organic substrates and resulting in higher reaction rates.

Hence, in transesterification reactions, the only water existent in the system is the water that is attached to the enzyme itself; this water is vital in maintianing the structural integrity of the enzyme. According to Klibanov *et al* enzymes can function in principally non-aqueous environment or an environment with low water providing the essential water layer sorrounding them is not exposed off (Klibanov 1989) (Ebbing and Gammon 2007).

The activity of an enzyme can be associated with the hydrophobicity of an organic solvent. Log *Poct/w* is defined as the logarithm of the partition coefficient of a solvent with respect to the octanol/water two-phase system, and shows good correlation with enzyme activity. Usually, organic solvents that have log $P \leq 2$ are not considered favorable for reaction media because polar solvents may destroy the essential water layer surrounding the biocatalyst. Relatively, organic solvents, which possess log $P \geq 4$, are considered more suitable. The behavior of organic solvents possessing log *P* between 2 and 4 is determined by the nature of the biocatalyst. Pancreatic lipase is an exception, which functions in all tested organic solvents having log *P* that ranges from -1.3 to 13.7. Nonetheless, this enzyme still performs better in hydrophobic organic solvents in comparison with hydrophilic organic solvents. According to Lanne *et al.* (1987), the outer part of the pancreatic lipase is relatively more hydrophobic than other lipases, thus binding its essential water more tightly.

2.10. Esterase enzyme assay methods

Plate assay containing olive oil or tributyrin emulsified with triolein in the growth medium is the most widely used petri plate assay for detection of the esterase producers.(Jaeger,

Dijkstra et al. 1999). The Enzyme activity is indicated by the presence of clear halos around the active colonies in the petri dishes containing differential media (Collaboration 1996). The detection of the colonies can be enhanced by adding Rhodamine-B to the above medium, after the enzyme activity the free fatty acids produced in the medium complex with the fluorescent dye and produce orange fluorescence under Ultra Violet light 340 nm. (Kouker and Jaeger 1987). The activity of esterase produced in the medium can be quantified by using chromogen substances like p-nitrophenyl esters (Schmidt and Bornscheuer 2005) (Vorderwülbecke, Kieslich et al. 1992) which upon hydrolysis produce yellow coloured compound p-nitrophenol measured at 410 nm (Jaeger, Dijkstra et al. 1999). The Carboxyl hydrolases have two main subtypes, lipases and esterases which can be distinguished from each other by using the substrates *p*-nitrophenyl palmitate (cleaved by lipases) vs. *p*-nitrophenyl butyrate. Lipases cleave the long chain ester i.e. p-nitrophenyl palmitate whereas, esterases cannot. (Jaeger, Dijkstra et al. 1999). The limitation in this assay is the lack of sensitivity and stability of the commonly employed substrate such as p-nitrophenyl acetate (Gutfreund and Sturtevant 1956) for which immense care has to be taken to minimize the background signals such as the Blank solution and reducing the light in the environment where assay is being carried out. A solution to this problem was found by substituting nitrophenyl esters with –cyano- containing esters derived from the pyrethroid class of compounds (Shan and Hammock 2001) upon hydrolysis it produces an acid and a cyanohydrin. This cyanohydrin is an intermediate compound and rearranges to meta-phenoxybenzaldehyde spontaneously in the reaction mixture(Shono, Ohsawa et al. 1979). The aldehyde molecule has a stronger capacity to absorb the UV light than former compound and its alcohol derivative. Thus, it provides us with a more sensitive assay than the nitrophenyl esters (Shono, Ohsawa et al. 1979). The scientist reinforced this idea by synthesizing more pyrethroid like compounds and subjected them to hydrolysis by the esterase resulting in the production of strongly fluorescent molecules.

Chapter 3:

Materials and Methods:

3.1. Sample Collection:

Ice samples were collected from the Siachen Glacier of Pakistan. Sterile axe, tongs and vacuum vessel was used for the purpose. Sterile cold chain was maintained till the samples were received in the laboratory. In the lab the ice pellets were then transferred to sterilized plastic bottles and kept till further tests were performed.

3.2. Isolation of bacteria from ice:

Isolation of the desired bacterium from the sample was carried out in two steps.

1. Promoting growth of all the organisms in the sample
2. Isolation using selective medium

3.2.1. General growth:

Ice pellets were ethanol treated and inoculated in nutrient broth (Oxoid™ Musa ji Traders). 1.2% of nutrient broth was prepared and sterilized through moist heat autoclaving. After inoculation of the ice pellets in the broth, incubation was done at 4^{o}C for three days.

3.2.2. Isolation of the enzyme producing bacterium:

In the second step the growth from the above mentioned medium (1.2% nutrient broth) was innoculated on selective medium reported earlier (Kouker and Jaeger 1987). i.e. the medium which helped us identify the enzyme producers. The selective media was composed of 1% olive oil Sasso™, 2.8% Nutrient Agar (Oxoid™ Musa Ji Traders), 0.1% Rhodamine B (**R6626 Sigma-Aldrich™**) dye. These components were autoclaved and then stirred

vigorously to make a uniform emulsion. This emulsion was poured in sterile petri dishes while hot and left to settle.

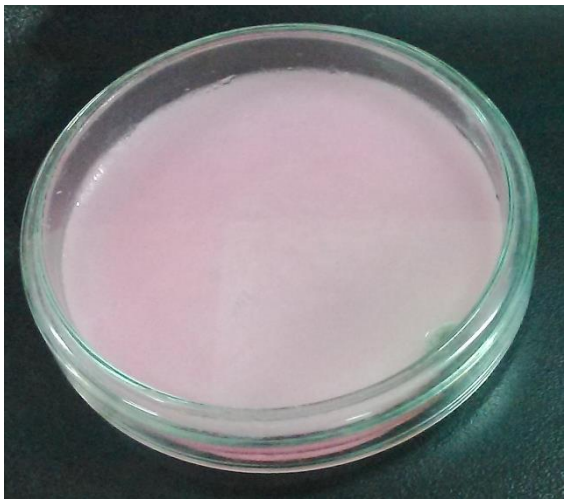


Figure 3.1: Rhodamine B olive oil emulsion plate for the isolation of esterase producer. Olive oil is the substrate for esterase and Rhodamine B aids in detecting the presence of hydrolysis by complexing with the free fatty acids.

After inoculation by spread plate method, the petri plate was observed after three days of incubation at 4^{o}C when growth was visible under UV light to check the presence of the enzyme producer. The active colonies were having orange florescence. The colony which was producing florescence in UV-light was picked with sterile loop and streaked on a fresh sterile selective medium plate and again incubated for three days. The whole procedure provided us with pure enzyme producing bacterium. 30% Glycerol stocks in nutrient broth and nutrient agar slants were made for future use.

3.3. Characterization of the Isolated Enzyme Producer

3.3.1. DNA Extraction:

Bacterial template DNA was extracted from the streaked plates. The colony was picked with the help of sterile pipette tip and suspended in 100uL of NF water in 0.5 mL micro-

centrifuge tube (Eppendorf ™) and mixed thoroughly to form a turbid solution. The tube was then given a heat shock for 20 minutes at 85^{o}C. Then the micro-centrifuge tube was centrifuged at 4000 G for 3-4 minutes until the pellet was formed. The supernatant was then collected in a separate sterile tube and the pellet was discarded. The supernatant contained the bacterial chromosomal and plasmid DNA.

3.3.2. Determination of DNA concentration:

Concentration of DNA was obtained by measuring the optical density (OD) at 260 nm using 1/100 equals 50ug/mL DNA dilution in quartz cuvette (Barbas, Burton et al. 2007). The purity of the nucleic acid was indicated by the ratio of two readings i.e. one reading taken at 260 nm and the other at 280nm wavelength. The measurement of DNA quality is that the absorbance at 260 nm is twice than that at 280 nm, if the solution contains pure DNA. In Case of any contamination like protein, there is some additional OD which decreases the absorbance ratio between 260 and 280 nm respectively. Pure nucleic acid samples would have an A_{260}/A_{280} ratio of 1.8.

3.3.3. Primer Designing:

Primers for ribotyping were obtained as reported in the literature (Akram, Shafaat et al. 2014). The nucleotide sequence of the primers is given in the table below.

Table 3.1: Primers used for PCR amplification of 16S RNA gene.

Target Gene	Primer Code	Sequence 5’ 3’	Product size
Ribotyping	RS1	AAACTCAAATGAATTGACGG	445
	RS3	ACGGGCGGTGTGTAC	

3.3.4 PCR Amplification:

The DNA samples were subjected to PCR amplification using the above primers and profile conditions were optimized. Total reaction mixture volume of 25uL was used containing 2mM DNTPs, 10X PCR buffer, 50mM $MgCl_2$, 50 pmol of each primer, 5uL DNA sample 1 unit of thermostable Taq DNA polymerase (Fermentas, USA) and the final volume was adjusted using nuclease free water. The reaction mixture was centrifuged for thorough mixing using microcentrifuge (Edison, New Jersey USA) then PCR was performed in Swift™ MaxPro Thermal cycler (Applied Biosystem, Foster City, USA).

3.3.5. PCR profile for Ribotyping:

Initial denaturation was given at 95°C for 5 minutes followed by 35 cycles of denaturation at 95°C 45 seconds, annealing at 51°C for 45 seconds and extension at 72°C for 1 minute. The reaction was completed with a final extension of 5 minutes at 72°C and kept on hold at 4°C until tubes were taken out of the cycler.

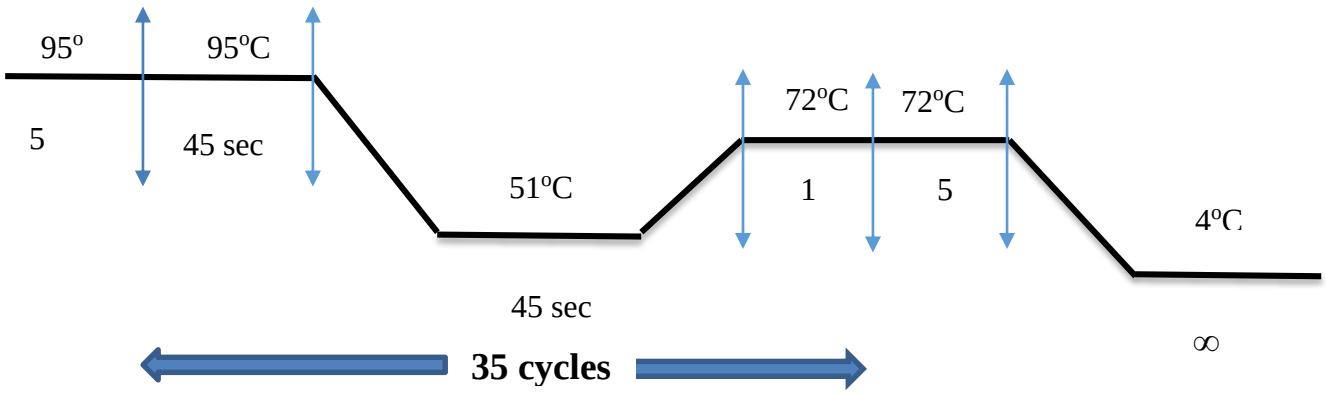


Figure 3.2: Polymerase chain reaction (PCR) thermo cyclic program for amplification of 16S RNA gene

3.3.6. Agarose Gel Electrophoresis:

In order to analyze the PCR products, 2% (w/v) agarose gel was prepared in 1x TAE (In 990mL distilled water, 10 mL 1M Tris HCl: pH 8.0 and 400uL of 0.5M EDTA). The gel was prepared by dissolving 0.8 g agarose was dissolved in 40 mL of 1x TAE by in heating in

microwave oven. The gel mixture was cooled to ~60 ^{o}C and upon cooling 0.05% of ethidium bromide was added to stain the gel. For analysis 7uL of the PCR product was run on the gel with 1x loading dye (0.05% ethidium bromide). The gel was subjected to electrophoresis (Wealtec, Sparks USA) run at constant current 60mA for half an hour to resolve the DNA under electric field. After half an hour the voltage was increased up to 80 volts for complete resolution of the product. The gel was visualized under UV transilluminator (Biometra, Goettingen, Germany) and photographed by Gel Documentation System (Wealtec Dolphin Sparks, USA) (S/NN470883). The PCR product was stored at -20^{o}C for further use.

3.3.7. Sequencing:

Sequencing service was acquired from Macrogen™ Korea. The results from sequencing were analyzed using NCBI (National Center for Biotechnology Information) BLAST (Basic Local Alignment Tool).

3.3.8. Phylogenetic Analysis:

Phylogenetic analysis was done using the software Mega6. Phylogenetic trees were constructed using neighbor joining method and minimum evolution tree.

3.4 Time course studies:

Determination of the relationship between the life cycle and enzyme production time was done, for which time course studies were conducted. Fermentation was done in nutrient broth (Oxoid™ Musa Ji traders) supplemented with 1% yeast extract (Oxoid™ Musa Ji traders). 1 mL of the fermentation medium was withdrawn every subsequent day. Serial dilutions were made in normal saline and 500uL was spread on nutrient agar plates in triplicates. These plates were incubated at 4^{o}C for three days and colony forming units were counted on the 4th day of incubation. Simultaneously enzyme assay was done following the above procedure for each day using phosphate buffer of strength 10mM and pH 10.5.

Colony forming units and absorbance values were plotted against the day of sampling to obtain the time of the life cycle when enzyme production is maximum.

3.5. Enzyme assay:

For determining the activity of the enzyme, physical and chemical parameters were optimized, by keeping two of them constant i.e. Temperature and incubation time.

Para nitro phenyl acetate (Merck™) was used as the substrate for the enzymatic reaction. As a result of enzyme activity the substrate is hydrolyzed yielding acetic acid and para nitro phenol (a yellow colored compound). The activity of the enzyme is directly proportional to the amount of the colored compound produced. Therefore the absorbance of the reaction mixture measured by UV-Vis Spectrophotometer (Biotechnology Medical Services, Canada) was taken as the measure for the enzyme activity. (Huggins and Lapides 1947).

3.5.1. Wavelength Optimization:

An 8mM solution of hydrolyzed Para Nitro Phenyl Acetate PNPA (Merck™) was used to optimize the wavelength for absorbance. Absorbance was measured at various wavelengths from 405 to 425nm. Graph was plotted for absorbance versus wavelength to obtain maximum absorbance value.

3.5.2. pH optimization:

3.5.2.1. Sample preparation:

1 mL of the fermentation medium was collected in a sterile environment. It was centrifuged at 4000rpm for 10 minutes at 4^{o}C. Supernatant was collected and the pellet was discarded.

3.5.2.2. Substrate preparation:

50 mL 8mM of PNPA (Para nitro phenyl Acetate (Merck™) was prepared in isopropanol and stored in an airtight opaque jar. The jar was stored at 4^{o}C for future use.

3.5.2.3. Buffer preparation:

Phosphate buffer was made with varying pH values using mono sodium hydrogen phosphate $NaHPO_4$ (Merck ™) and Di sodium hydrogen phosphate Na_2HPO_4 (Merck™). The quantities of the respective salts were calculated using Javascript Phosphate Buffer Calculator (http://clymer.altervista.org/buffers/phos2.htmL). Buffers ranging from 7 to 11.5 were prepared to test the enzyme activity against every pH value.

3.5.2.3. Assay procedure:

2.9mL of the buffer, 50uL of the sample and 50uL of substrate were incubated at 4^{o}C for 10minutes. The absorbance of this mixture was measured using UV Spectrophotometer at 410nm. To nullify the effect of medium sample blank was used i.e. 1.2% nutrient broth substituted with 1% yeast extract. The absorbance of sample blank was subtracted from the absorbance of the sample. These absorbance values obtained were plotted against the pH values in Microsoft Excel to acquire a graph showing maximum absorbance against a specific pH value.

3.6. Molecular weight determination of Enzyme:

Molecular weight of the enzyme was determined in two steps. To approximate the molecular weight SDS-PAGE was done then to confirm the exact molecular weight MALDI_TOF was conducted.

3.6.1. SDS-PAGE:

3.6.1.1. Sample preparation:

Freshly fermented bacterial batch medium was used for the extraction of protein. 3 mL of the medium during the stationary phase was harvested and centrifuged at 4000rpm to separate cells, followed by ammonium sulfate precipitation and again pelleted down at 13000rpm to obtain total protein content from the medium. The supernatant was discarded and pellet was re-suspended in phosphate buffer and stored in ice-bath. 15uL of the protein solution and 5uL of solubilization buffer (1M Tris pH 6.8: 1 mL, 1M DTT: 2 mL, SDS: 0.4 gm, Bromophenol blue: 0.02 gm, 100% glycerol: 2 mL, final volume was adjusted to 20 mL) were mixed and the sample was denatured in heat block for 10 minutes.

3.6.1.2. Gels preparation:

12 % SDS PAGE gel, as described by Laemmli (Laemmli 1970), was used to analyze the protein expression using BIO-RAD system (Singapore). In order to make 10% resolving gel 3 mL of distilled water, 2.5mL of 30% degassed Acrylamide (Acrylamide: 29 gm, N'N'-bis-methylene acrylamide: 1 gm. Both of these reagents were dissolved in 100 mL distilled water and solution stored at 4ºC). 1.9 mL 1.5 M Tris-HCL gel buffer (Tris base: 18.15gm dissolved in 80 mL distilled water and pH was adjusted at 8.8. final volume as adjusted to 98 mL. Then 2 mL of 20% Sodium Dodecyl Sulfate (SDS) was added along with 50uL 10% Ammonium per sulfate (APS). 50 uL of N,N,N',N', tetraethylmethylendiamin (TEMED) (Research Organics,Cat#3009T) was mixed to it and poured in between the gel plates leaving some top area for stacking gel. Now distilled water was poured over the gel to prevent oxidation and removal of bubbles. Gel was left for 20-30 minutes for polymerization. Now the water was removed carefully with 5cc syringe and 6% stacking gel was poured which was prepared by mixing 1.95 mL of distilled water, 0.625 mL of 30% Acrylamide/BIS, 0.95mL 0.5M Tris-HCl gel buffer (Tris base: 6 gm, dissolved in 60 mL distilled water and adjusted pH to 6.8 and make

final volume to 98 mL add 2 mL of 20 % SDS), 100 µL 10% APS and 50 µL TEMED. Comb was placed in the stacking gel immediately after pouring and left it until it polymerized. After polymerization, the gel plates were fixed in electrophoresis apparatus and placed in the gel tank (BIO-RAD,Singapore). Tank was filled with gel running buffer (1X Tris-Glycine) up to the marked area for 2 gels. Sample was loaded in the well and the gel was run at 20 amperes till loading dye entered the resolving gel. Later the current was increased to 25 amperes.

3.6.1.3. Commassie staining & destaining

After completion of electrophoresis, gel was stained in commassie brilliant blue R-250 solution (50mg dye in methanol: acetic acid: H2O (45:9:45) by placing it carefully in a petri dish with the staining solution for 20 minutes. It was then destained with a destaining solution (5% methanol and 7% acetic acid in water) for 2 hours. After complete destaining of gel, it was stored in destaining solution and photographed.

3.6.2. MALDI_TOF mass Spectrometry:

Freshly fermented bacterial batch medium was used for the extraction of protein. 3 mL of the medium during the stationary phase was harvested and centrifuged at 4000rpm to separate cells, followed by ammonium sulfate precipitation and again pelleted down at 13000rpm to obtain total protein content from the medium. The supernatant was discarded and pellet was re-suspended in phosphate buffer and stored in ice-bath. The protein sample was mixed with 2, 5-Dihydroxyactetophenone. 2, 5-DHAP is a MALDI matrix used for preparations of proteins with a mass of 8–100 kDa.

Chapter: 04

Results

4.1: Sampling of ice from the Glacier:

Ice sample was collected from the Siachen Glacier of Pakistan, located in the eastern Karakoram Range in the Himalaya Mountains at about 35.421226°N 77.109540°E, just east of the Line of Control between India-Pakistan. At 70 km (43 mi) long, it is the longest glacier in the Karakoram and second-longest in the world's non-polar areas. It falls from an altitude of 5,753 m (18,875 ft) above sea level at its head at Indira Col on the China border down to 3,620 m (11,875 ft) at its terminus. The vessel was ethanol treated to make it sterile. This sample was brought to the lab enclosed so that there is no chance of contamination.

4.2: Isolation of bacteria from the ice samples:

Coring of the ice pellets was done through ethanol treatment and surface melting. The treated ice pellets were inoculated in nutrient broth and incubated at 4^{o}C for three days to ensure growth of all the microbes present in the sample. Figure 4.1 shows the growth in nutrient broth.

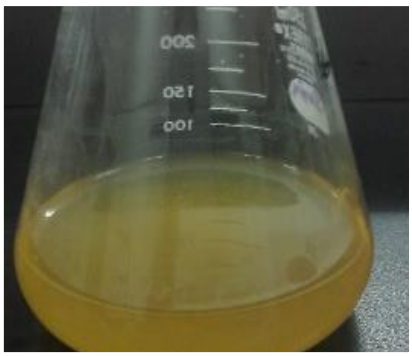


Figure 4.1: Fermentation in nutrient broth: Ice pellets inoculated in nutrient broth and grown to high turbidity ensuring the growth of all the microbes present.

The enzyme producer was identified with spread plate technique on the selective medium.

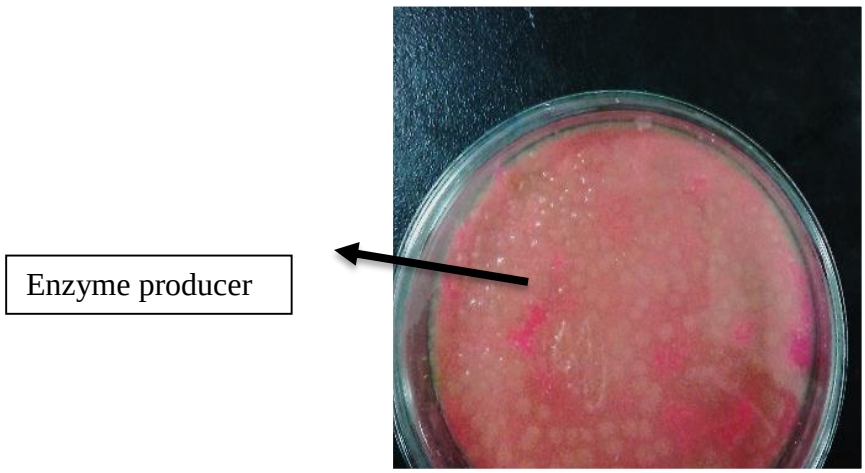


Figure 4.2: Isolated colonies using spread plate technique. Bacterial cell emulsion made in normal saline and 0.5 uL of the suspension spread on the differential medium. Incubated at 4oC for 3 days.

These isolated colonies were re-streaked on the selective medium and pure enzyme producer was obtained. Orange florescence was observed on the plate when viewed under UV-Light. This is due to the enzyme activity. As the free fatty acid produced from the oil in the medium forms complex with the florescent dye Rhodamine B present in the medium as well. This complex when exposed to UV light produces orange coloured florescence. Figure 4.3 shows the growth of isolated colonies on differential medium.

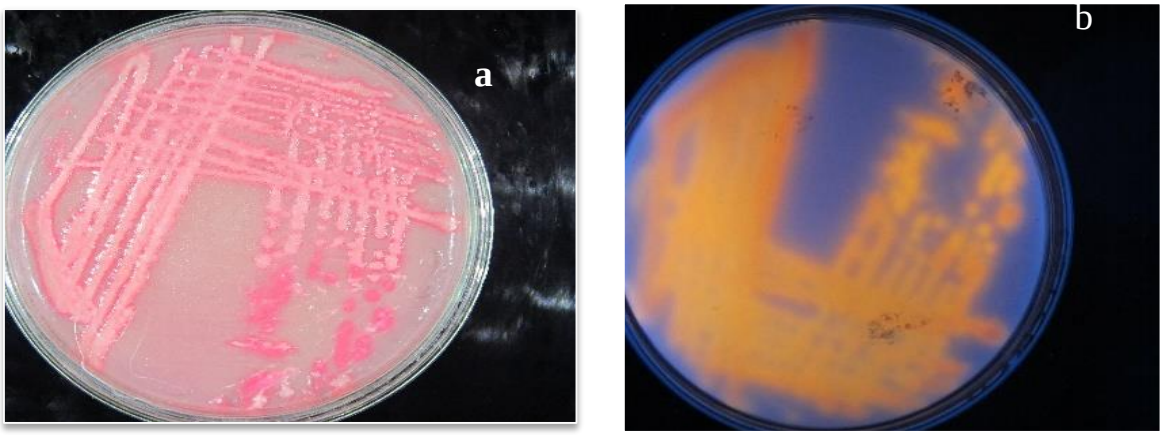


Figure 4.3: Purified bacterial colonies on the selective medium (Rhodamine B oil emulsion medium): isolated colony from the spread plate streaked over Rhodamine oil emulsion plate. a) under visible light b)under UV light.

4.3: Characterization of the isolated enzyme producer:

Determination of strain characteristics DNA analysis was done through 16s RNA sequencing. For this colony PCR was conducted using primers mentioned in section 3.3.3. Table 3.1. Then DNA was separated through agarose gel electrophoresis.

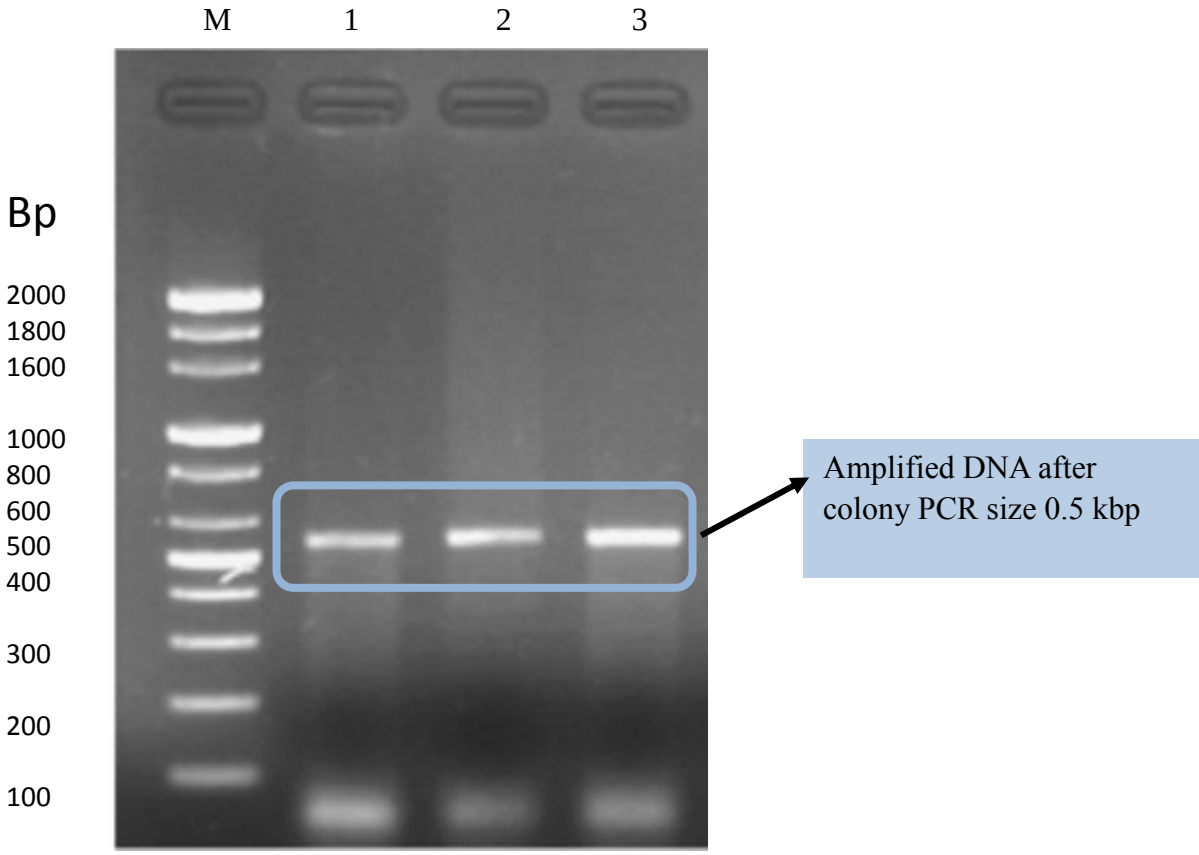


Figure 4.4: Agarose gel electrophoresis: Picture of agarose gel under UV light showing the 0.5 kb length of DNA as a result of colony PCR. Lane M shows the DNA ladder for comparison of size of the DNA fragment. Lane 1,2 and 3 shows the DNA extracted from the bacterium.

16S r RNA analysis of the isolate was done by Macrogen Korea™ using reading 675 base pairs in the forward direction and 848 base pairs in the reverse direction. A contiguous fragment was formed by Macrogen sequencing service and the sequence was matched with the already reported bacterial strains using BLAST (Basic Local Alignment Search Tool). 99% sequence homology was found with already reported strains collected from various regions of the world.

4.4: Phylogenetic analysis:

4.4.1: Phylogenetic analysis with respect to closely related taxonomic units:

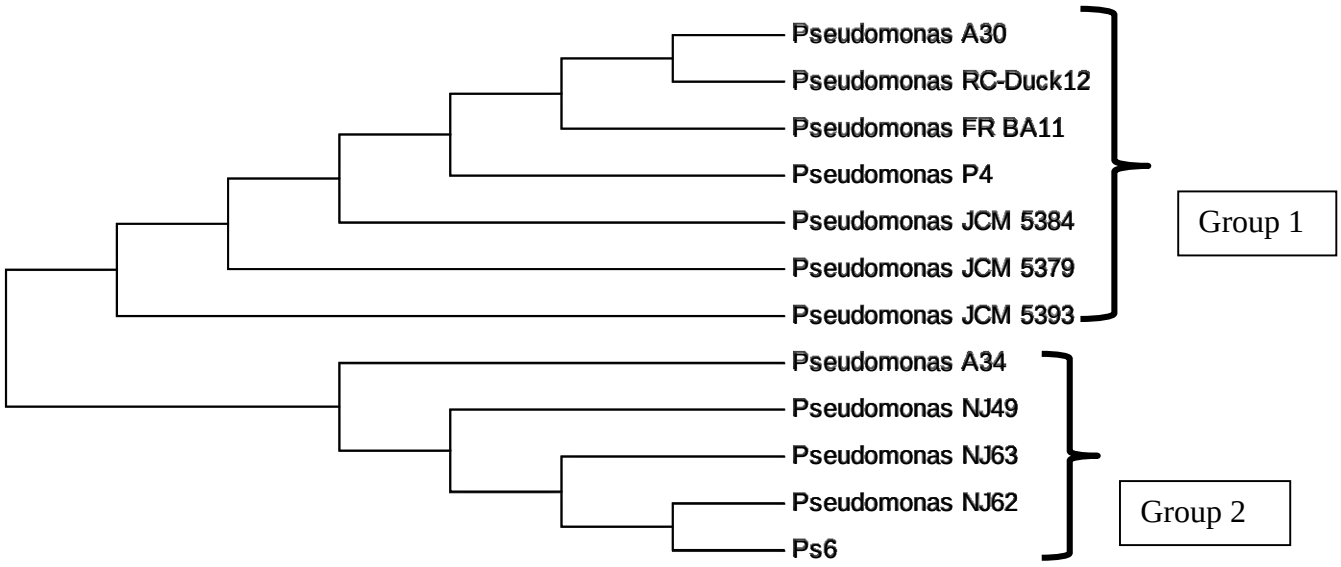


Figure 4.5: Phylogenetic Tree with respect to ancestors of the species: Ps6 is closely related to Pseudomonas NJ62. It belongs to group 2 consisting of 5 members which have evolved from the same ancestor. Neighbor joining method has been used to construct this tree with a bootstrap value 100

This phylogenetic tree has been constructed to determine the type of species and the relationship of Ps6 with the already discovered strains. Sequencing results obtained were analyzed using MEGA6. The nucleotide sequence of different species were aligned using CLUSTALW to figure out similarities and differences in them. The alignment results were then used to construct trees using two algorithms, i.e. neighbor joining method and minimum evolution.

Neighbor joining method is used to find the pairs of operational taxonomic units. The above phylogenetic tree represents the relation of Ps6 with the related strains. All these strains have descended from two ancestors. Thus forming two groups. Group 1 of 7 strains from one

ancestor and the second group of 5 strains from the other. The isolated new strains belongs to the second group. In the second group the sister strain of Ps6 is Pseudomonas NJ62.

4.4.2: Phylogenetic analysis with respect to evolution:

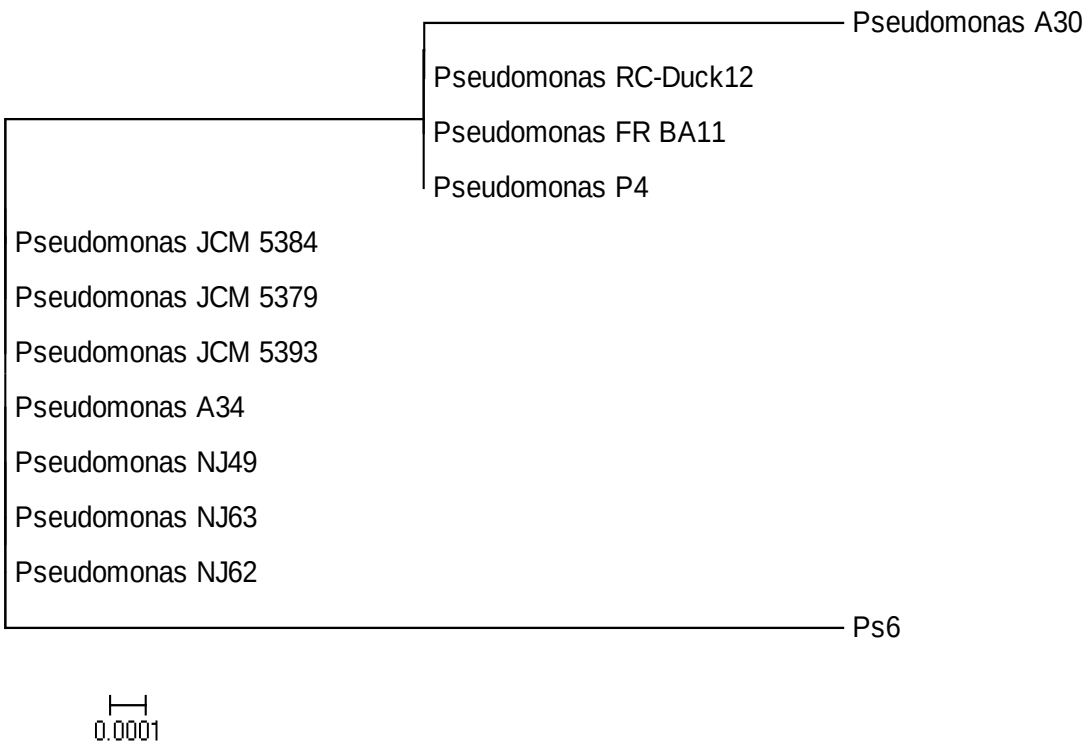


Figure 4.6: Phylogenetic tree with respect to Evolution: This tree has been constructed using minimum evolution algorithm. The branch lengths of the taxonomic units correspond to the time of evolution in comparison to the other members of the tree. Ps6 has evolved later than the other strains and close to Pseudomonas A30.

The above phylogenetic tree is the minimum evolutionary tree. This tree does the comparative analysis of the time of evolution of the organisms. The branch lengths of strains correspond to the evolution time of that strain. Pseudomonas A30 and Ps6 are the most recent strains as their branch lengths are longer than the other members of the tree. Phylogenetic analysis has confirmed the strain to be from genus pseudomonas. Additionally the time of evolution of the strain has also been revealed. Phylogenetic trees have been shown in Figure 4.5 and 4.6 respectively.

4.5. Time Course studies:

To measure the time when the organism starts producing the enzyme, time course studies were conducted. Colony forming units were counted each day and enzyme assay was done simultaneously. According to the activity values the enzyme production starts at late log phase and is maximum absorbance is observed during stationary phase. The graph has been shown in figure 4.7.

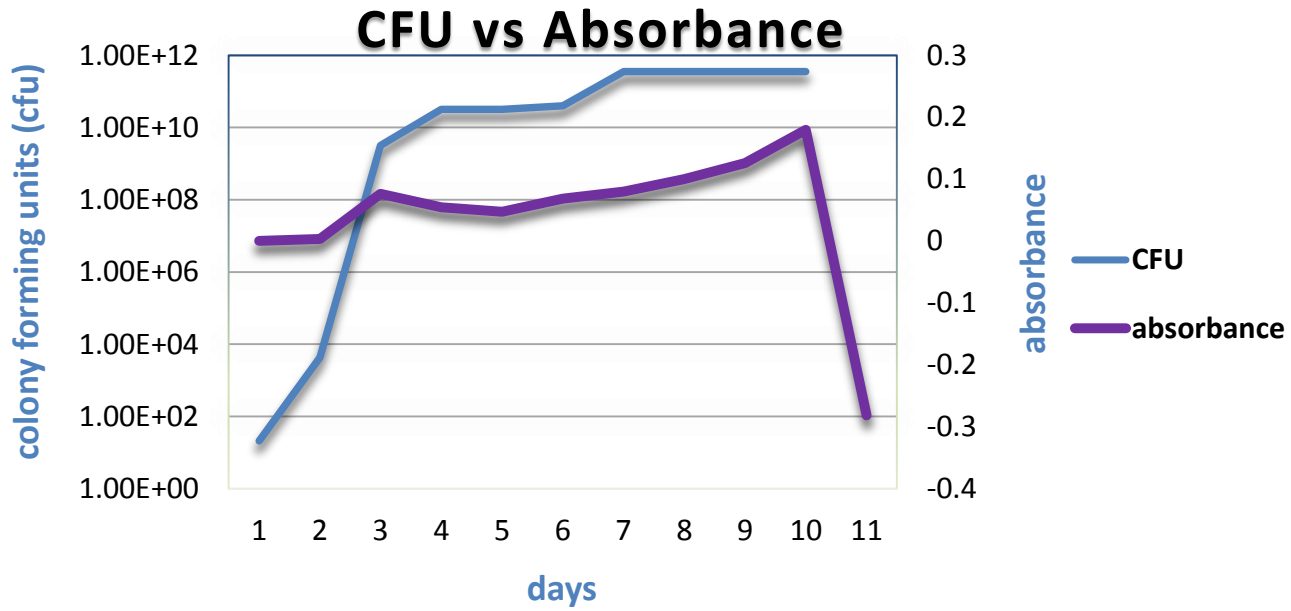


Figure 4.7: Graph showing relationship between life cycle of bacterium and enzyme production. Fermentation in LB medium substituted with 1% yeast extract, colony forming units counting and enzyme activity test done simultaneously. x-axis shows the time passed in days, on y axis on the left side of the graph number of colony forming units is present and y-axis on the right hand side is depicting the absorbance by the chromogen after hydrolysis as a result of enzyme activity. The enzyme is mainly being produced in the stationary phase, i.e. at the end of log phase. For fermentation the optimum time for harvesting is found to be approximately 3 days.

4.6: Enzyme Assay:

The activity of the enzyme is quantified via a substrate, which produces a yellow colored compound upon hydrolysis. i.e. para nitro phenol. More the activity of the enzyme more will be the intensity of the color. Thus absorbance was taken as the measure for enzyme activity.

Two parameters were kept constant. Temperature at 4^0C and incubation time of 10 minutes.

4.6.1: Wavelength optimization:

The substrate used to quantify the activity of the enzyme is a chromogen. Optimization of the wavelength for absorption is a prerequisite for the enzyme assay. Absorbance value was determined at various wavelengths. Maximum value was observed at 410 nm. Graph is shown in Figure 4.7.

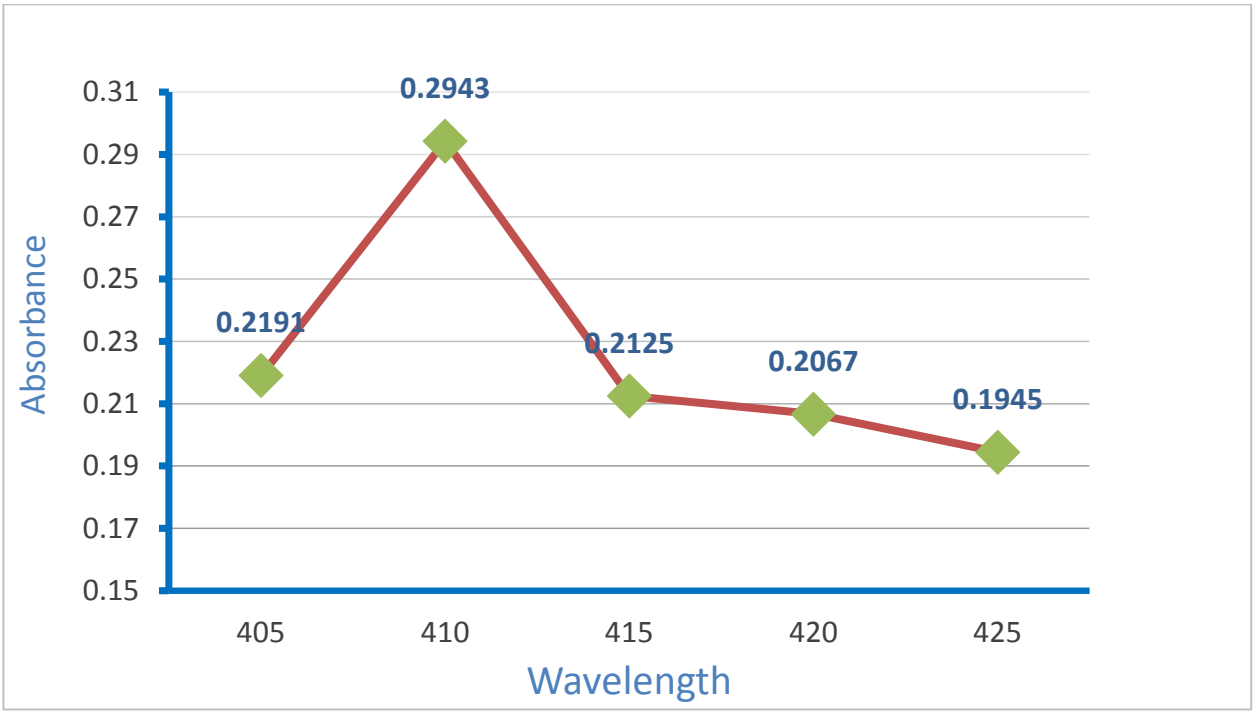


Figure 4.8: Graph showing absorbance at different wavelengths. Enzyme activity using pNPA as substrate determined at wavelengths from 405nm to 425 nm. X-axis shows the wavelength and at y-

axis absorbance by the chromophore is present. Maximum value obtained at 410nm. The optimum wavelength for wavelength determination has been obtained.

4.6.2: pH optimization:

The enzymes require a certain pH to work. To maintain the pH buffers were made of pH range from 7 to 11.5. Corresponding to these pH values absorbance was measured. The maximum value was measured against the pH value 10.5. Thus trend line shows that the optimum pH for the enzyme activity is 10.5. The graph showing the optimum pH value has been shown in figure 4.8.

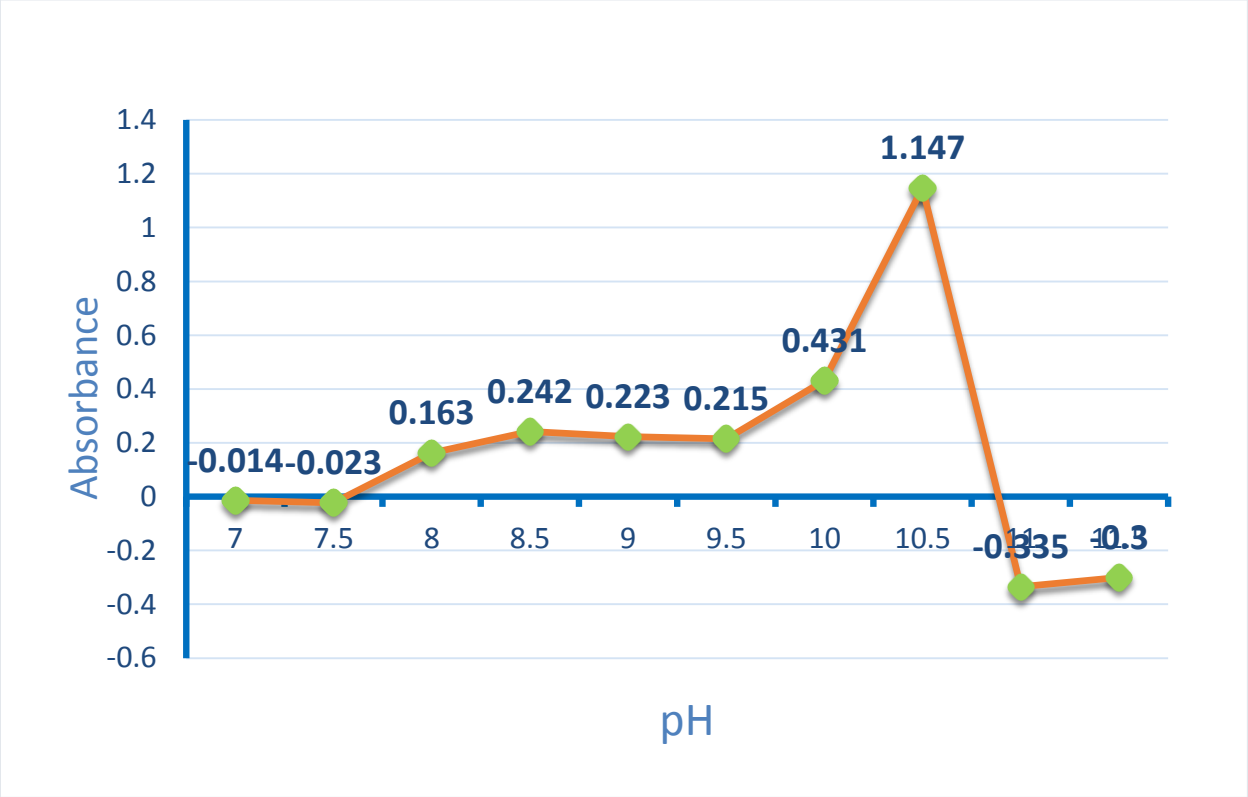


Figure 4.9: Graph showing absorbance at various pH values. Enzyme activity using pNPA as substrate observed at pH values 7 to pH 11.5. X-axis depicts the pH values and y-axis shows the absorbance by the chromogen after hydrolysis. Maximum activity was observed at pH value 10.5. Optimum pH value for the enzyme activity is 10.5.

4.7: Molecular weight determination of Esterase:

To determine the molecular weight of the enzyme following two procedures were performed

4.7.1; SDS-PAGE:

Sodium Dodecyl sulfate Polyacrylamide Gel Electrophoresis was performed as the first step for molecular weight and protein expression determination of the bacterium.

4.7.1.1. SDS-PAGE results:

Sample preparation was done as 20 ul of the above sample was loaded in 10% SDS-PAGE gel with solubilization buffer and stained and de-stained afterwards. Following figure shows the results obtained. Esterase band appeared between 25kDa and 55kDa, and is darker than the other bands as esterase production was induced with yeast extract. The gel is in shown in figure 4.10.

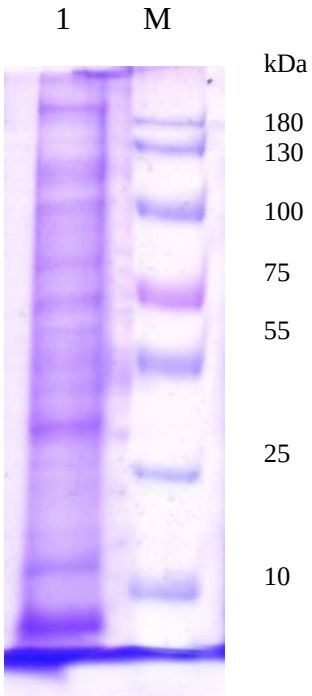


Figure 4.10: Protein expression of bacterium. Column 2 shows the protein ladder used for the reference and column 1 shows the proteins produced by the bacterium. Esterase band is darker as its production was induced by yeast extract.

4.7.1: MALDI-TOF analysis:

Mass Spectrometry is used to determine the size of proteins. Matrix Assisted Laser Desorption Ionization-Time of Flight is the detector used specifically for the purpose. The spectrum is shown in Figure 4.11.

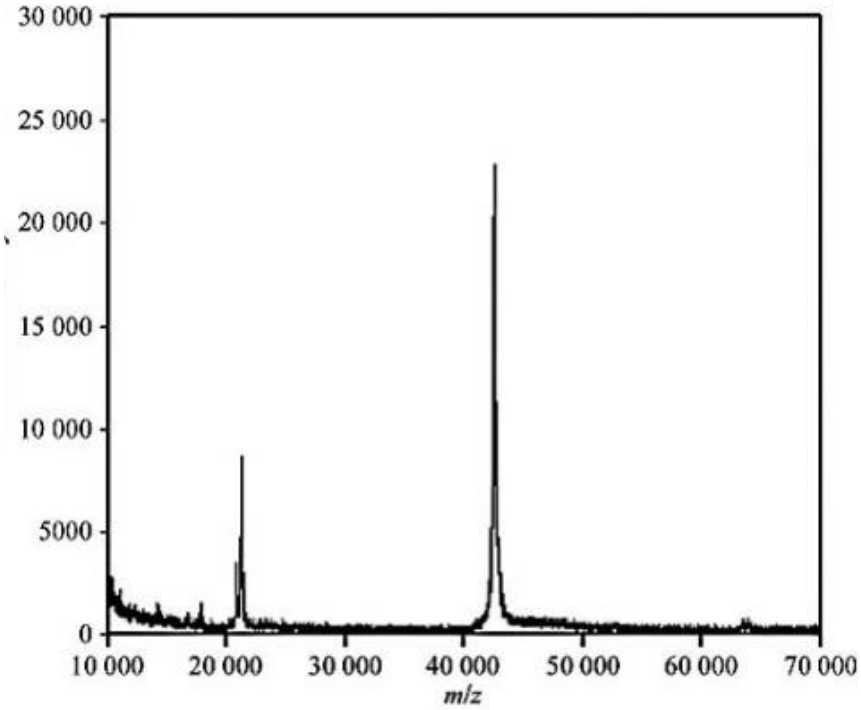


Figure 4.11: Spectrum of the protein sample analyzed through MALDI-TOF mass spectrophotometer. Peak at 43,000 m/z corresponding to the molecular weight of the protein sample. Showing the weight is 43kDa.

MALDI-TOF spectrum obtained from Hussain Ebrahim Jamal Research Institute of Chemistry, University of Karachi, Pakistan. Sample for the analysis was prepared following procedure in section 3.5.2 of chapter 3. Results were analyzed using MASCOT online service confirming the type of protein.

Chapter 5

Discussion:

It has been forecasted by the Global industry analyst that the chiral technology will reach $5.1 billion, primarily due to the demand for enantiopure compounds, moreover advances in technology with respect to efficacy, amount, automation and increased product yield have also affected the need for the chiral product requirement in the years to come. A major factor in driving the demand for chiral intermediates in Pharmaceutical industry is the extension of the patent protection rights in the drug compounds manufactured from enantiomers. (Jose *et al*. 2015).

There are numerous ways through which chiral multiplication can be achieved in organic synthesis. Metal complex catalysts and biocatalysts (enzymes) for asymmetric synthesis are of particular importance to achieve the industrial and economical demands (Gawroński 2005). Of these biocatalysts hydrolases are the most useful ones. Drug synthesis has contributed mainly in the advancement of methodology of synthesis and has greatly benefited from the synthetic chemistry as well. Biocatalyst in the form of whole cell cultures or isolated enzymes has gained importance over the past forty years reaching from laboratory to vast industry (Patrick 2013). These enzymes can cope with the rigorous environmental limitations, other reagents can be added along with them to help in the catalysis and they can also be modified to increase their rate of activity and improve substrate specificity.

Such biocatalysts can be obtained from organisms from different habitats. The cold inhabitants; the psychrophiles, moderate temperature inhabitants; the mesophiles and high temperature inhabitants; the thermophiles (Goldstein 2007). Of these three we are interested in the psychrophiles as at low temperature many undesirable effects can be minimized which can

occur at high tempratures (Jeon, Kim et al. 2009). The three characteristics that are mainly useful of the psychrophiles are, their high activity a low concentration of the catalyst thus reducing the demand for the expensive catalyst production. The process can be conducted at low temperature, thus reducing the energy demand and being thermo-labile they can be selectively inactivated by slightly heating the reaction mixture.

These enzymes have very characteristic properties due to which they have become the area of interest for many researchers. Scientists get the opportunity to investigate their stability and flexibility in relation to their activity. Cold environment has many constraints when the kinetic properties of the existing systems are considered, thus the organisms have evolved certain features which circumvent these limitations (Goldstein 2007). The enzymes of these organisms are flexible in nature which allow them to be active at low kinetic energy. Activation enthalpy value of these enzymes is less and even lesser/ negative values for activation entropy when they are compared with their thermophilic and mesophilic homologues (Siddiqui and Cavicchioli 2006).

The flexibility obtained is due to the presence of following structural properties; decreased core hydrophobicity, increased surface hydrophobicity, lower arginine/lysine ratio, weaker inter-domain and inter-subunit interactions, more and longer loops, decreased secondary structure content, more glycine residues and less proline residues in loops, more proline residues in a-helices, less and weaker metal-binding sites, a reduced number of disulfide bridges, fewer electrostatic interactions (H-bonds, saltbridges, cation–pi interactions, aromatic–aromatic interactions), reduced oligomerization and an increase in conformational entropy of the unfolded state.

When the genomes of the psychrophiles were compared with those of the thermophiles prominent amino acid biases were observed thus temperature adaption achieved (Saunders, Thomas et al. 2003) (Siddiqui and Cavicchioli 2006)

With the above point of view this bacterium was isolated from Siachen glacier with the assessment that the cold inhabitant has the metabolic makeup which works at lower temperature. Then it was inoculated on selective medium for determining the esterase producing capacity. The selective medium was composed of 1% olive oil, 0.5% Rhodamine B Dye and 2.8% nutrient agar. As the bacterium multiplies it produces esterase which hydrolyses the oil in the medium producing free fatty acids. These free fatty acids complex with the florescent dye and produces florescence under UV-light. This florescence is visible surrounding the enzyme producers in the petri plate.

Bacterium characterization is a necessary preamble to get to know the organism with the evolutionary perspective. The bioinformatic report revealed that this strain of pseudomonas has evolved in the recent years and has similarity to those present in other colder regions of the world.

Esterase production was yet to be established with the help of an enzyme assay as plate assay is a general test for lipases and esterases. It cannot distinguish between the two. Para nitro phenyl acetate was used as a substrate as it has a two carbon acetyl group, decisive factor for an esterase (Lopes, Fraga et al. 2011).

Physical and chemical parameters were optimized including wavelength and pH, temperature and incubation time were kept constant. Then time course studies were carried out to determine the time when the enzyme production starts, so, when to harvest. The enzyme was found to be active at low temperature and at a wide range of pH, 10.5 being the optimum. This step confirmed the presence of the enzyme and its activity.

Furthermore this bacterium was fermented to obtain sufficient amount of the enzyme protein for additional analysis. Fermentation was done using nutrient broth supplemented with 1% yeast extract, as yeast extract is a known esterase/lipase inducer (Fuciños, Atanes et al. 2014). Following the time course studies protein harvesting was done on the third day maintaining the

cold chain. As the enzyme is extracellular therefore the medium was centrifuged to separate the cellular mass protein was precipitated from the medium (Kumar, Kumar et al. 2012).

The protein characterization requires protein purification, which in turn is dependent on the molecular weight determination of the protein. Thus SDS-PAGE (Sodium Dodecyl Sulfate-Poly Acrylamide Gel Electrophoresis) was done following the BIO-RAD™ protocol to approximate the molecular weight of the protein. The band appeared between 25kDa and 55kDa corresponding to molecular weight ladder. Then confirmation was done using MALDI-TOF mass spectroscopy (matrix assisted laser desorption ionization-time of flight) analysis.

Our work has highlighted an industrially useful enzyme and its activity parameters have been optimized. Further purification will be done to determine the structure of the protein. Then there is a need for establishing the applications practically. An alternative approach is to computationally predict the possible substrate of this enzyme and then justify them practically. It can reduce the discrepancy between practical results and save time.

CONCLUSION

Psychrophilic esterases are of extreme importance industrially and this area needs to be addressed extensively by the researchers. This study has enabled us to discover a new psychrophilic enzyme which can prove to be a useful entity in chemical sector. The esterase obtained is active over a wide range of pH and maximal activity is observed at 10.5. At low temperature and incubation time of just 10 min has provided with an observable activity experimentally, thus revealing its high catalytic efficiency. Moreover psychrophilic enzyme features are being studied biochemically, and with every addition to the pool study become more reliable. This esterase is being worked upon further so as to achieve the desired outcome.

RECOMMENDATIONS

The Siachen Glacier of Pakistan is rich in natural microflora which needs to be explored further. We can obtain such other useful microbes from that region. Moreover the genome of the obtained microbe should be explored to find such other enzymes in it. Simultaneously these useful entities need to be taken to the market so that our biotechnological sector flourishes and becomes an active part of our economy.

Appendix

Details of the Sequencing product

Name	Read Length (Normal)	Read Length (Q16)	Read Length (Q20)	GC Content
Ps6_contig_1	**1468**	**1384**	**1379**	**53.201634877384194**
Ps6_F	675	666	663	54.074074074074076
Ps6_R	848	846	840	52.358490566037744

Appendix II

BLAST (Basic Local Alignment Search Tool) results

Query		Subject					Score			Identities			
Start	**End**	**Description**	**AC**	**Length**	**Start**	**End**	**Bit**	**Raw**	**EV**	**Match**	**Total**	**Pct.(%)**	**Strand**
1	1468	Uncultured bacterium clone A34 16S ribosomal RNA gene, partial sequence	JN867380.1	1519	39	1505	2699	1461	0.0	1466	1468	99	Plus/Plus
1	1468	Pseudomonas sp. P4(2010) 16S ribosomal RNA gene, partial sequence	HM196356.1	1504	22	1488	2699	1461	0.0	1466	1468	99	Plus/Plus
1	1468	Uncultured bacterium clone FR_B_A11 16S ribosomal RNA gene, partial sequence	GQ443090.1	1502	19	1485	2693	1458	0.0	1465	1468	99	Plus/Plus
1	1468	Uncultured bacterium clone A30 16S ribosomal RNA gene, partial sequence	GQ422762.1	1500	19	1485	2693	1458	0.0	1465	1468	99	Plus/Plus
1	1468	Pseudomonas sp. NJ-49 16S rRNA gene, strain NJ-49	AM421981.1	1498	19	1485	2693	1458	0.0	1465	1468	99	Plus/Plus
1	1468	Pseudomonas sp. Nj-63 16S rRNA gene, strain Nj-63	AM491466.2	1498	19	1485	2693	1458	0.0	1465	1468	99	Plus/Plus
1	1468	Pseudomonas sp. Nj-62 16S rRNA gene, strain Nj-62	AM491465.2	1498	19	1485	2693	1458	0.0	1465	1468	99	Plus/Plus
1	1468	Pseudomonas sp. NJ-62 16S rRNA gene, strain NJ-62	AM396914.1	1500	19	1485	2693	1458	0.0	1465	1468	99	Plus/Plus
3	1462	Pseudomonas fragi gene for 16S ribosomal RNA, partial sequence, strain: JCM 5393	AB685606.1	1459	1	1459	2689	1456	0.0	1459	1460	99	Plus/Plus
3	1462	Pseudomonas fragi gene for 16S ribosomal RNA, partial sequence, strain: JCM 5384	AB685597.1	1459	1	1459	2689	1456	0.0	1459	1460	99	Plus/Plus

Chapter 6

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