FORMULATION OF BIO BASED TOOTHPASTE

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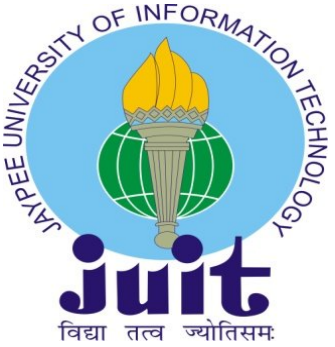

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CERTIFICATE

This is to certify that the work titled "**FORMULATION OF BIO BASED TOOTHPASTE**" submitted by "**NITISH VIKRAM SHAHI**" in partial fulfillment for the award of degree of M. Tech Biotechnology. of Jaypee University of Information Technology, Waknaghat has been carried out under my supervision. This work has not been submitted partially or wholly to any other University or Institute for the award of this or any other degree or diploma.

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DECLARATION

I hereby declare that the work reported in the M.TECH thesis entitled **"FORMULATION OF BIO BASED TOOTHPASTE"** submitted at **Jaypee University of Information Technology, Waknaghat India,** is an authentic record of my work carried out under the supervision of Dr. Vijay Kumar Garlapati. I have not submitted this work elsewhere for any other degree or diploma.

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Nitish Vikram Shahi

Date:

SUMMARY

Biosurfactant was obtained from *Staphylococcus arlettae* and it was used in formulation of toothpaste, replacing Sodium Lauryl Sulphate SDS/SLS which is normally used in commercial toothpaste as a surfactant. This biosurfactant toothpaste was qualitatively analyzed by several tests like Spread ability Test, Foaming ability Test, Abrasiveness Test, and cleaning ability test. The results indicate that biosurfactant are more efficient and less toxic surfactant compare with chemical surfactant, the biosurfactant obtained from *Staphylococcus arlettae* can act as good ingredient in place of chemical surfactant for toothpaste formulation. So with the advantage of environmental compatibility, in future the biosurfactant may be used in other cosmetic formulation like Shampoo formulation, face wash formulation, Soaps etc. as an alternative for the chemical surfactant

The normal role of toothpaste is to remove dental pellicle and plaques which are results of *Actinomyces viscosus* and *Streptococcus sanguis* colonies forming a layer on enamel surface of teeth. The organic constituents of plaque include biomolecules such as polysaccharides, proteins, glycoprotein's and lipid material. Toothpaste with specific enzymes can also remove plaque, stain without affecting the tooth surface and the surrounding soft tissues and thereby improving the gingival health.

Like chemical surfactants, biosurfactant are excellent emulsifiers and maintain wetting and foaming properties, non toxic in natures that are valued in several applications including the cosmetics industry. Unlike chemical surfactant, biosurfactant are readily biodegradable contributing to environmental compatibility.

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(NITISH VIKRAM SHAHI) (Dr. GARLAPATI VIJAY KUMAR)

Date: Date:

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ABBREVIATIONS

°C	Dergee Celsius
%	Percentage
µM	Micromolar
ATCC	American Type Culture Collection
BSA	Bovine Serum Albumin
CM	Carboxymethyl
DOE	Design of Experiment
EC	Enzyme Classification
EDTA	Ethylenediaminetetraacetic acid
h	Hours
KDa	Kilo Dalton
L	Liter
LB	Luria Bertani
Nm	Nano meter
mg	Milligram
mM	Mill molar
MTCC	Microbial Type Culture Collection
O.D	Optical Density
p- NPP	p-Nitrophenyl Palmitate
PHB	Polyhyroxybutyrate
RPM	Revolution Per Minute
SDS/SLS	Sodium Dodecyl/ Lauryl Sulphate
Smf	Submerged Fermentation
SSF	Solid State Fermentation
TLC	Thin Layer Chromatography
U/ml	Unit Per millilitre
Vmax	Maximum Reaction Rate
ST	Surface tension
BS	Bio surfactant
FOR	Formulation
COM	Commercial
Sol.	Solution

Chapter 1

INTRODUCTION

1.1 Enzymes

Enzymes are natural catalysts. They are produced by living organisms to increase the rate of an immense and diverse set of chemical reactions required for life. They are involved in all processes essential for life such as DNA replication and transcription, protein synthesis, metabolism and signal transduction, *etc*. And their ability to perform very specific chemical transformations has made them increasingly useful in industrial processes. Enzymes are among the most important products obtained for human needs through microbial sources. An enzyme works by binding to a given substrate in such a geometrical fashion that the substrate is able to undergo its inherent reaction at a more rapid rate. The enzyme does not actually react with the substrate but merely brings the substrate in to the proper alignment or configuration for it to react spontaneously or in conjunction with other molecules. Enzymes have been used by man throughout the ages. The history of modern enzyme technology really started in the 19th century; Current developments in biotechnology are yielding new applications for enzymes.

1.2 Lipases

Lipases (triacylglycerol acylhydrolases are ubiquitous in nature and produced by various plants, animals and microorganisms. Lipase belongs to a class of serine hydrolases which catalyze the hydrolysis of triglycerides to glycerol and free fatty acids. Lipases represent the most versatile enzymes that display a broad spectrum of substrate specificity. In addition, lipases catalyze the hydrolysis and transesterification of other esters as well as the synthesis of esters and exhibit enantioselective properties. Microbial lipases especially from bacteria and fungi represent the most widely used class of enzymes in biotechnological applications (Gupta et al., 2004).

1.3 Bio surfactants

Bio surfactants are amphiphilic compounds produced on living surfaces, mostly microbial cell surfaces, or excreted extracellularly and contain hydrophobic and hydrophilic moieties that reduce surface tension (ST) and interfacial tensions between individual molecules at the surface and interface, respectively. Since Bio surfactants and bio emulsifiers both exhibit emulsification properties, bio emulsifiers are often categorized with Bio surfactants, although emulsifiers may not lower surface tension. A bio surfactant may have one of the following

structures: mycolic acid, glycolipids, polysaccharide–lipid complex, lipoprotein or lipopeptide, phospholipids, or the microbial cell surface itself.

Considerable attention has been given in the past to the production of surface-active molecules of biological origin because of their potential utilization in food-processing, pharmacology, and oil industry. Although the type and amount of the microbial surfactants produced depend primarily on the producer organism, factors like carbon and nitrogen, trace elements, temperature, and aeration also affect their production by the organism.

Hydrophobic pollutants present in petroleum hydrocarbons, and soil and water environment require solubilisation before being degraded by microbial cells. Mineralization is governed by desorption of hydrocarbons from soil. Surfactants can increase the surface area of hydrophobic materials, such as pesticides in soil and water environment, thereby increasing their water solubility. Hence, the presence of surfactants may increase microbial degradation of pollutants. Use of bio surfactants for degradation of pesticides in soil and water environment has gained importance only recently. The identification and characterization of bio surfactant produced by various microorganisms have been extensively reviewed. Therefore, rather than describing the numerous types of bio surfactants and their properties, this article emphasizes the production of bio surfactants and their role in biodegradation of pesticides.(N. G. K. Karanth et. Al.)

1.4 Global Enzyme Demand

Global enzymes market is estimated to rise 7 percent at a healthy pace to $8.0 billion in 2015. Gains will reflect a continued world economy rebound from the global financial crisis of 2009.

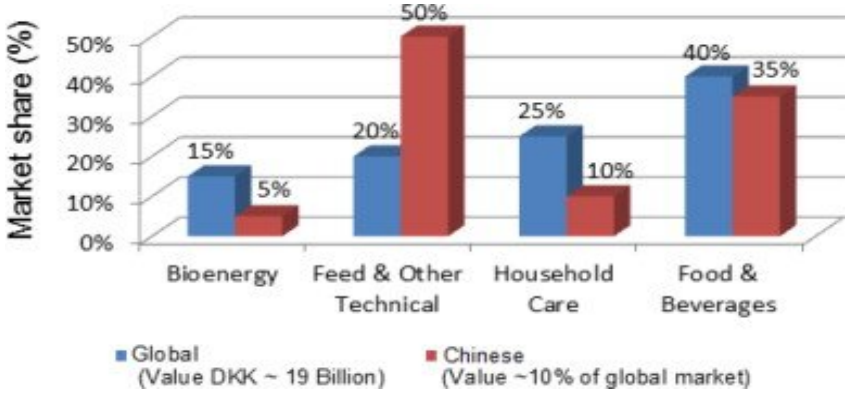


Figure 1. Chinese versus global enzyme market in 2010 (Roughly estimated)

Enzymes are employed in a diverse array of applications in industries and scientific research, ranging from the degradation of various natural substances in the starch processing, detergent and textile industries, to the manipulation of DNA/RNA in biotechnology research. As illustrated in Fig. 1, the global enzyme market was dominated by the food and beverage industry, which benefits from the expansion of the middle class in rapidly developing economies. Growth came mostly from baking enzymes and other smaller applications such as fat and oil processing. However, the growth pace will be moderate as maturity in developed regions North America and Western Europe offsets faster growth in the Asia/Pacific and other developing regions. The fast growth over the past decade has also been seen in a wealth of other industries spanning from organic synthesis in pharmaceutical industry to diagnostics enzyme with expanded access to medical care in developing countries, and the advent of health care reform in the United States. Meantime the detergent industry, once the largest sector in the global enzyme market, experienced a decline due in part to the pricing pressures from the main detergent manufactures after the turn of the century. Demand for cleaning enzymes was accelerated by 2005 as the product lines were reformulated with more-effective new enzymes launched continuously. Bio energy production enzyme demand was limited by the new legislative mandates for grain based ethanol. While the development of the second generation bio fuels derived from cellulosic raw materials will be in favour of demand growth over a long time. (shuang *li et. al.)*

Enzymes are important bio molecules which successfully replace inorganic catalyst owing to their attributes of their specificity, high rate of reaction, eco-friendly and biodegradable nature etc. During the past few years, there is a noticeable sustainable growth in enzyme market which forecasts the further enhancement in a near future. The global market for industrial enzymes is estimated at $3.3 billion in 2010 and is expected to reach $4.4 billion in 2015. India has a marginal share in the global market for industrial enzymes which is estimated to be at about $ 3387. 30 million. On the basis of application, industrial enzymes could be divided into four major categories, i.e. toothpaste enzymes, technical enzymes, food enzymes and feed enzymes. The technical enzymes segment could further be divided into textile enzymes, leather enzymes, pulp and paper enzymes, fuel ethanol enzymes and others. The major enzymes used in industrial enzymes market are amylase, lipase, protease, ligase, phytase, cellulose, xylanase etc. The market segmentation for various areas of application shows that 34 % of market is for food and animal feed followed by toothpaste and cleansers

(29 %). However the year on year growth registered by technical enzymes segment was highest in past 4-5 years due to dramatic growth of the fuel enzymes segment.

Industrial enzymes operate in an oligopolistic market with the presence of three major suppliers, Novozymes, Genencor International Inc., and DSM N.V. Their main market segments are food (e.g., dairy, baking, brewing, beverage), animal feed, and technical applications.

Novozymes is the largest supplier in food, feed and technical segments during 2010-2011, with an estimated market share of 47 % of the industrial enzyme market in 2010. Microbial lipases are of special interest because of their stability in organic solvents, lack of requirement for cofactors, their broad substrate specification and their high enantioselectivity. In 1994, NovoNordisk introduced the first recombinant commercial lipase Lipolase® which originated from the fungus *Thermomyces lanuginosus* and expressed in *Aspergillus oryzae*.

Table 1: Novo Nordisk in commercial Lipase production

Name	Action	Application
Lipopan®	Hydrolysis and Oxygen uptake	Baking Industry
Lipozyme®	Interesterification	Oil and fats industry
Novazym®27007	Hydrolysis	Pasta/noodles
Palatase	Hydrolysis	Dairy industry
Novozyme®871	Emulsification	Pet food industry

In 1995 Genencor International produced two bacterial lipases, Lumafats® from *Pseudomonas mendocina* and Lipomax® from *Pseudomonas alcaligenes*. Lipase produced by microorganisms like bacteria, fungus, and eukarya as well as by plants and animals is studied extensively. However, efforts are made to further to explore the application part of lipase and improve through directed evolution like techniques to enhance their efficiency and efficacy in industrial processes. Further, their commercialization is important which leads to further increase in share in enzyme markets.

1.5 Lipase producing microorganisms

Microbial lipases are more preferred than plants and animal lipases because of higher yield, regular supply irrespective of seasonal changes, easily handling and short generation time. Microbial lipases also work at neutral and alkaline pH optima and are often thermo stable. (Jaeger et al., 1999 and Joseph et al., 2007).The significance of microbial lipases in an industrial arena has already been greatly acknowledged with respective to their vast potential

in numerous industrial applications (Hasan et al., 2006) . Table 2 depicts the major bacterial sources for lipase production. Lipase production has been reported from a variety of bacteria, fungi and actinomycetes. However, the presence of lipases has been observed from 1901 A.D. for *Bacillus pyocyneus*, and *Bacillus fluoresces* which represent some of today's best studied lipase producers, now named, *Serratia marcescens*, *Pseudomonas aeruginosa* and *Pseudomonas fluorescence* respectively. Fungi and yeast like *Rhizopus nigricans, Aspergillus nidulans, Yarrowia lipolytica*, etc (Pignede et al., 2000). have been reported for lipase production. Lipase producers have been isolates from diverse regions range from soil or spoiled food material to extremophilic regions such as hot springs and rock salt mines. Lipases with novel properties have been studied from microorganism isolated from Antarctica Ocean, hot springs, high salt and sugar environment.

Table 2: Lipase producing bacteria and actinomycetes

Bacteria / Actinomycetes	Reference
Achromobacter lipolyticum	Hostacka, 2000
A. calcoaceticus 69-V,	Haferburg and Kleber, 1983
Acenetobactor haemolyticusi TA106	Jagtap et al., 2010
Aeromonas hydrophila MCC-2	Chuang et al., 1997
Alteromonas	Saimoku et al. 1999
Bacillus acidocaldarius,	Manco et al., 1998
B. alcalophilus,	Ghanem et al., 2000
Bacillus subtilis EH 37	Ahmed et al., 2010
P. aeruginosa PseA	Gaur et al., 2008

Lipase producer also isolated from desert soil, thermal station soil, oil mill, etc. However due to applications of lipases in organic synthesis, isolation of microorganisms which survive in the presence of toxic solvent is the current focus of research. The first report on the isolation of solvent-tolerant bacterial strain was put forward by Inoue and Horikoshi in 1989 for *Pseudomonas putida* The isolation of solvent tolerant microbes has been reported from ecological niches such as soil, deep sea and are identified as belonging to genera *Pseudomonas*, *Bacillus*, *Rhodococcus,* and *enterobacter* . In some cases, non-solvent tolerant strains are mutated to obtain tolerant strain such as utilization of spontaneous mutation of *E. coli* K-12 (Aono et al., 1991) to obtain some organic solvent-tolerant mutants. Komatsu et al.,

1994 isolated *Pseudomonas aeruginosa* in n-hexane and p-xylene tolerant strain through repetitive sub culturing. Most of the bacterial isolates for lipase productions grow at various pH and temperature but generally prefer neutral pH. However, there are also reports on alkaliphilic bacteria. On the contrary, fungus prefers acidic conditions for growth and lipase production. Beside these psychrophiles and thermophilic bacterial isolates, the microorganisms having different oxygen demand (Aerobic, microaerophilic and anaerobic), are also reported to produce lipase.

1.6 Microbial lipase production through Smf

Bacterial lipases, like most other types of industrial enzymes, can be produced either by submerged fermentation (SmF) or solid-state fermentation (SSF). In SmF, the nutrients and microorganisms are both submerged in water and nutrients are uniformly distributed. Solid-state fermentation (SSF) is a process that involves solid matrix and occurs in the absence of any fluid in the space between substrate particles. Lipase production through submerged fermentation is dependent upon a number of factors including carbon and nitrogen sources, pH, temperature, aeration and inoculums size. Besides, Aguilar et al., 2004 reported that the expression of proteins may differ in SSF and SmF. Industrially important enzymes have traditionally been obtained from submerged fermentation (SmF) because of the ease of handling and greater control of environmental factors such as temperature and pH. SSF has some limitations, such as the limited choice of microorganisms capable of growth under reduced moisture conditions, as well as the control and monitoring of parameters such as temperature, pH, humidity and air flow.

Bacterial lipases are mostly inducible enzymes, requiring some oil, fatty acids or fatty acid esters for induction. Lipases are mostly secreted extracellularly into the production medium during growth, but there are also reports on intracellular lipase production. Lipase production is organism oriented, but generally enzyme is activated and released during late logarithmic or stationary phase. The fast growing microorganisms tends to secretes biocatalyst within 12-24 hours. Despite of the fact that lipase production is extensively studied, but it still requires a systematic approach to select the best production conditions for fermentation owing to their important effect on final yield. The earlier studies also highlighted the role of various environmental factors such as temperature, pH, nitrogen, carbon and lipid sources, agitation, and incubation period on lipase production.

Lipase production has been studied from various microorganisms using different substrates as inducer and their effect have been studied from time to time. Studied the effect of different oils on lipase production. In their study they use olive oil, corn seed oil and sunflower oil as

inducer and carbon substrate for lipase production for *Fusarium oxysporium*. Soybean oil was demonstrated an effective inducer for the production of fungal enzymes (lipases) that degrade plant seed oils. However, Pogaku reported olive oil as best inducer for lipase production from *Staphylococcus sp*. Lp12 when compared with ground nut oil, coconut, castor oil. The importance of oil in lipase induction was anlaysed by Fadiloglu and Erkmen, 2002. Medium was supplemented with and without olive oil and it has been found that medium containing olive oil yield more lipase (5.5 U/ml). Lipase production has been studied using carbon sources like beef tallow, wool-sour effluent, whey, n-hexadecane. Jansen et al., 1996 describes the production of thermostable lipase from *Bacillus sp.* strain Wai 28A45 in tripalmitin as a carbon source at 70^0 C. A comparative study conducted by Gao and Breuil, 1995 to evaluate the effect of different oils like olive oil, soybean oil, sunflower, sesame, corn, peanut and seed cotton oil on lipase production from fungus *Ophiosstoma piceae,* maximum activity was seen with olive oil as inducer. However, high concentration of oil is inhibitory to enzyme production. However, further increment in concentration decreases the yield of biocatalyst significantly. The production of alkaline thermostable lipase from *Bacillus sterothermophilus* cited by Kim and Oh, 1998 in I L medium containing beef tallow and palm oil. Gerritse et al., 1998 reported alkaline lipase from *Pseudomonas aeruginosa* M-1 in medium supplemented with citric acid and soybean oil and inducer. Thermoalkalophiles which were capable of growing in pH 9 and at temperature 55 ^{0}C isolated by Bayoumi et al., 2007. Bacterial lipases have a neutral or alkaline optimum pH with the exception of lipase from *P. fluorescens* SIK W1 that had an acidic optimum pH 4.8.

However,lipases from *Bacillus stearothermophilus* SB-1, *B. atrophaeus* SB-2 and *B. licheniformis* SB-3 are active over a broad pH range 3-12. Bacterial lipases generally have temperature optima in the range of 30-60 °C. For *B. licheniformis* MTCC-10498 maximum lipase production was observed at pH 7.5 (~0.4 U/ml). However, bacterial lipases with optima in both lower and higher ranges have been reported. Thermal stability data are available only for species of *Bacillus*, *Chromobacterium*, *Pseudomonas* and *Staphylococcus*. Eltaweel et al., 2005 studied the effect of various carbon sources like sorbitol, fructose and arabinose did not improve the lipase activity whereas addition of starch, rhamnose, melibiose and myo-inositole decreased the lipase production on lipase production from *Bacillus sp* stain 42. Sztajer and Maliszewsa, 1998 reported the enhanced lipase production in medium when supplemented with 5 % peptone, corn steep liquor and soybean meal for *Penicillum citrinum.* A thermo stable lipase from *Pseudomonas aeruginosa* Kwi-56 was produced in medium containing 0.2% peptone and 0.1 % yeast extract at 60 ^{0}C after 24 hrs (Izumi et al., 1990).

Bacillus strain A30-1 (ATCC 53841) was reported for lipase production using 0.1 % yeast extract and 1 % ammonium chloride at pH 9.5 and 60 ^{0}C (Wang et al., 1995). An ammonium dihydrogen phosphate has the best nitrogen source for lipase production from *Pseudomonas fluorescence* NS2W, which was found almost threefold more compared to the basal medium. However, lipase production was very low in medium soybean meal and corn steep liquor (Kulkarni and Gadre, 2002). A tryptone and yeast extract in combined form in 0.6 % and 0.2 % concentration as the best nitrogen source for lipase production from *Bacillus sp* strain 42 reported by Eltaweel et al., 2005. Along with carbon and nitrogen sources metal ions plays an important role in lipase production. Metal ions although required in millimolar concentration for microorganisms but influencing lipase production significantly. Kok et al., 1995 reported enhanced extracellular lipase production when medium was supplemented with $Mg2^+$, $Ca2^+$, Cu2+, Co2+ from *Acinetobacter colcoaceticus* BD413. *Bacillus* species found to produce 7-fold increase in lipase production when growth medium was supplemented with Mg, Fe, and Ca ions (Janseen et al., 1994). The metal ions effected the lipase activity of *Pseudomonas pseudoalcaligenes* KKA-5 which retained activity in $Cu2^+$ and $Mg2^+$ but slightly inhibited by the presence of $Mn2^+$, $Cd2^+$ and $Cu2^+$ in the production medium (Sharon et al., 1999).

1.7 Lipase assay methods

There are a number of assays available for determination of lipase activity reviewed in the literature (Beisson et al., 2000 and Jaeger et al., 1994). Most of the methods are devised to estimate the products of hydrolytic reactions. Table 3 depicting the different assay methods practiced for estimation of lipase activity. More advancement has been made for lipolytic activity determination through spectroscopic methods. Rollof et al., 1984 devised assay methods which involve direct turbidometric estimation of residual lipids, after reaction of lipids with substrates in emulsion. Similarly Robinson et al., 1989 developed a turbidometeric method for lipase activity. Besides these a few methods are developed where the color released after the hydrolysis of triglycerides used as a substrate is measured. Van Autryve et al., 1991 used rhodamine 6G for complexation with free fatty acids liberated during the reaction.

The pink color was monitored at 513 nm. However, Medcova et al., 1981 assayed the monoglyceride lipase activity using tween 20 as substrate. In this case, lauric acid released was converted to copper laureate and measured at 435nm. Safarik, 1991 developed a method where substrate is immobilized and fatty releases were extracted with benzene and converted to their corresponding Cu [II] salt and measured spectrophotometrically. There are enzymatic assays reported based on estimation of either release of glycerol or fatty acids as a

consequence of biocatalysis by lipase on triacylglycerol. Wollett et al., 1984 reported the methods for estimation of released fatty acids. The quantity of fatty acid present in the medium was measured before and after incubation through spectrophotometrically. Fossati et al., 1992 described a colorimetric method for assaying lipase in serum by employing long-chain 1, 2,-diglyceride fatty acid as substrate, where glycerol released was assayed by a sequence of enzymatic reactions. Besides this in some spectrophotometric method, certain synthetic substrate are also used which upon hydrolysis imparts colour. For example β-Naphthyl Capryplate was used for assayed lipase in skim milk. The product β-Naphthol formed after catalysis was reacted with fast blue BB and colored product was then extracted in ethanol and measured spectrophotometrically (McKellar, 1986). Kurooka et al., 1977 described an assay using 2, 3-dimercaptopropan-1-ol tributyrate as substrate and 5, 5' dithiobis (2- nitro-benzoic acid) as chromogenic reagent. Richardson et al., 1989 used substituted arylethene derivatives as substrate and the products of these compounds are coloured, making them suitable precursors for chromogenic enzyme substrates.

Table: 3 Overview of lipase assay

Substrate	**Product**	**Method**	**Final Product**	**Wavelength in nm**	**Ref.**
2,3-dimercaptopropan-1-ol Tributyrate	Glycerol analogue [2over 3 positions]	Reaction with DTNB	TNB	412	Kurooka et al., 1977
T	*p*-nitrophenol	Product is colored		410	Winkler and Stuckmann 1979
Aryleethene Derivatives	**Hydrolysis**	**Products**	**are colored**	Variable	Richardson et al., 1989
Glycerides	Free fattyAcid	Complex Formation	Rhodamine 6G	513	Van Autryve et al, 1991
Glycerides	Free fatty Acid	Complex Formation	Cu [II] salt	715	Safarik 1991
1-2- diglycerides	Glycerol	Enzymatic Conversion	Quinine	550	Fossati et al., 1992

Para- nitrophenylesters of various chain length fatty acids are also used as substrates (Winkler and Stuckmann, 1979). However, these compounds are not suitable for specific

lipase assays because they are cleaved by esterase also (Stuer et al., 1986). Some spectrophotometer assay can be performed in the presence of organic solvent that is quite useful during lipase purification with reverse micelle methods (Aires- Barros and Cabral, 1991). Rogel et al., 1989 employed spectrophotometer property of some natural occurring fatty acids like cis- parinaric for lipase activity estimation.

1.8 Purification and characterization of lipase

Enzyme purification is a series of processes intended to isolate a single type of enzyme from a complex mixture. Wide ranges of techniques are available and the choice depends on factors such as nature of the source i.e., extracellular or intracellular, scale of operation, stability of the enzyme etc. The extracellular products mainly suffer from dilution problem where the level of contaminants are considerably less as the selective secretion of the products takes place through the cell wall barriers. The level of the contamination is more for the intracellular products, where the dilution problem is not predominating because of the other intracellular products being released upon disruption of the cell. Purification methods are employed in order to understand enzyme functions, structure and its relationship with an environment. Purification of lipase normally involves steps that depend upon the purity desired. Many lipases are purified, characterized and crystallized. In a case of extracellular lipases, the first step is the removal of cells by ultracentrifugation or centrifugation, but in case of intracellular lipases an additional step of cell lysis is required. The extracellular enzymes suffer from dilution problem to concentrate the desired extracellular protein precipitation can be attempted. Ammonium sulphate or any other suitable organic solvent can be used as the precipitating agent. After complete precipitation the fraction has to be centrifuged to separate the precipitated protein. Precipitation often gives a high average yield (Aires-Barros et al., 1994) although with limited purification, and such enzyme preparations are apt for use in toothpaste formulations. Borkar et al., 2009 reported the purification of extracellular lipases from *Pseudomonas aeruginosa* SRT 9 using ammonium sulphate (30 % - 90 %) followed by dialysis and then subjected to chromatographic column containing phenyl sepharose CL-4B and mono QHRS/S column. Purification of thermo-alkaliphilic lipases from *Bacillus licheniformis* B42 was reported by Bayoumi et al., 2007. Purification methods followed by ammonium sulphate precipitation. The lipase was partially purified through ammonium sulphate and resulting fold purification and specific activity. Since the importance of lipases from industrial application point of views necessities the critical evaluation of microorganisms isolated from solvent rich conditions. The lipase enzyme was found to be stable at pH range of 6-8.5 and temperature ranges 25-50 ^{0}C. Demir and Tekul, 2010 reported

the purification and characterization of lipase from photosynthetic cyanobacterium *Spirulina platensis*. The purification was performed using sequential protocol of precipitation with ammonium sulphate. The purified enzyme was further used for protein estimation Protein content of cell-free supernatant was determined according to modified Lowry method (Lowry et al.,1951) using BSA as a standard

However, in spite of a growing interest in the use of halophilic enzymes for biotechnological applications, there are relatively few reports in the literature about their production and characterization (Bhatnagar, 2005). Halophilic microorganisms have greater capacity to produce salt and thermo tolerant enzymes like cellulases, amylases, proteases, lipases and xylanases (Govender et al., 2009).

1.9 Catalytic Versatility of lipases

Lipases are very versatile enzyme in terms of biocatalysts. They are serine hydrolases which attracted attention because of their capability to carry out wide range of reactions. They catalyze the hydrolysis of acylglycerol and synthesis of esters from glycerol and long chain fatty acids. Besides this, exchange of acyl group (Inter and transesterification) among different substrates or even among different chemical groups of the same compound is catalyzed by lipases.

1.10 Industrial applications of lipases

Lipases are important hydrolytic enzymes with innumerable applications and industrial potential. Lipases are the most versatile biocatalysts and they bring about a range of bioconversion reactions such as hydrolysis, interesterification, esterification, alcoholysis, acidolysis and aminolysis. These reactions used in food technology applications in fats and oil industry, dairy industry, pharmaceuticals and bakery industry provide a platform for lipase to act as a potential industrial enzyme. The alkaline thermophillic lipases find application in toothpaste industry. Table 4 enumerates a few of the most significant industrial applications of microbial lipases.

1.10.1 Lipase in cosmetic industry

Unichem international (Spain) has launched the production of isopropyl myristate, isopropyl palmitate and 2- ethylhexylpalmitate for use as an emollient in personal care products such as skin and sunscreen lotion (Hasan et al., 2006). Wax ester hace similar application in personal care products and are manufactured by enzymatically (Croda Universal Ltd.) using lipase from *C. cylindracea* in batch bioreactor. Retionoids (vitamin A and derivatives) with great potential in personal care product and prepared by lipase through immobilized lipase (Maugard et al., 2002).

Table:4 Industrial application of Lipase

Source	Applications/ properties	Referances
A. calcoaceticus	Waste management -Heating oil/furnace oil, removal of fats, oils and greases	Mrin et al., 1995
Bacillus subtilis	Biomedical applications-Cephalosporin derivative	Usher et al.,1995
Bacillus sphaericuc 206Y	Produces solvent tolerant lipase	Hun et al.,2003
Sphinobacterium Mizutae	Mineralization of a high concentration of isopropanol	Mohammad et al., 2006
Cnadida antarctica lipase B	Biomedical application: alcoholytic resolution of [R,S]-flurbiprofenyl azolides fo	Ciou et al., 2011
Pseudomonas aeruginosa PseA	Produces solvent-tolerant lipase	Gaur et al.,2005; Gupta et al., 2008
Bacillus sp	Food and Processing industry	Ahmed et al., 2010
Mucor racemosus	Biomedical application: treatment of cellulite	Mohemed et al., 2011

1.10.2 Lipases in toothpastes

Lipases were generally added to the toothpastes primarily in combination with proteases and cellulases. However, other enzymes such as amylases, peroxidases and oxidases are also reported to be added in toothpaste preparations (Kottwitz et al., 1994) Removal of oil/fatty deposits by lipase are attractive owing to its suitability under milder washing conditions. To be a suitable additive in toothpastes, lipases should be both thermo stable as well as alkalophilic and capable of functioning in the presence of the various components of washing powder formulations (Jaeger et al., 1994). *Pseudomonas* lipase preparations have been used for preparation of washing powder formulations. *Pseudomonas medocina* (Lumafast) and *Pseudomonas alcaligenes* (Lipomax) lipases have been manufactured by Genencor international USA, as toothpaste additive (Jaeger et al., 1994 Jaeger and Reetz , 1998). The Novo group has reported a highly alkaline, positionally non-specific lipase, from a strain of

Streptomyces sp. that was useful in laundry and dish-washing toothpastes as well as industrial cleaners (Pandey et al., 1999). Several lipase-producing organisms and their manufacturing processes are patented for preparation of toothpaste lipases (Lawler and Smith, 2000).

1.10.2.1 Disadvantages of chemical toothpastes

- **Surfactants** studies have shown that toothpastes which produce a lot of lather due to the presence of the toxic chemical (detergent surfactant) **sodium lauryl sulfate** (SLS, or sodium dodecyl sulfate or SDS) are actually bad for the gums.
- **Pyrophosphate** compounds are ingredients in many tartar control tooth pastes. Many people with sensitive skin have experienced burning, itching, or red cracked skin around the mouth.

1.10.2.2 Advantages of Bio- based toothpaste

- Non toxicity,
- Higher biodegradability
- Better environmental compatibility
- Higher foaming
- High selectivity and
- Lowering production cost

Chapter 2

REVIEW OF LITERATURE

2.1 Harmful effect of SDS or SLS

2.1.1 The Truth About SDS

(SDS; also known as *sodium lauryl sulfate*, or SLS): Is a synthetic detergent obtained by treating lauryl alcohol with sulfur trioxide or other sulfur compounds. SDS is a coarse powder that is often used as a foaming agent or detergent in soaps. Commercial SDS is often a mixture of other alkyl sulfates, dodecyl sulfate being the main component. The structure of SDS is somewhat similar to that of sodium laurate, which is the salt of a natural fatty acid obtained by saponification of fats and a component of most sops and shaving creams. The hydrocarbon tail of both SDS and sodium laurate is, in fact, identical. A sulfate group replaces the carboxylic acid group in SDS, this replacement makes SDS a more powerful detergent than sodium laurate. Sulfonate cleaners like SDS do not form insoluble precipitates in hard water and for this reason, they have become a popular choice for cosmetics, household cleaning and personal care products. SDS can be found in a variety of products including, laundry detergents, liquid soap, all-purpose degreasers, shampoos, and toothpaste. In medicine, sodium lauryl sulfate is used as a laxative in enemas, and as an excipient in some dissolvable caplets. SDS can also be found in candy.

2.1.2 What does SDS do?

Like all detergents, SDS removes oils from the skin and can cause eye and skin irritation, and due to its versatility at dissolving oils, it has a wide variety of industrial applications: the same properties that make SDS useful for cleaning your hair make it also useful for cleaning your clothes or a garage floor. Obviously, different applications demand changes in concentrations or strength and that explains the versatility of SDS. In addition, these same properties make SDS a potentially effective topical microbicide that can also inhibit, and possibly prevent, infection by various viruses.

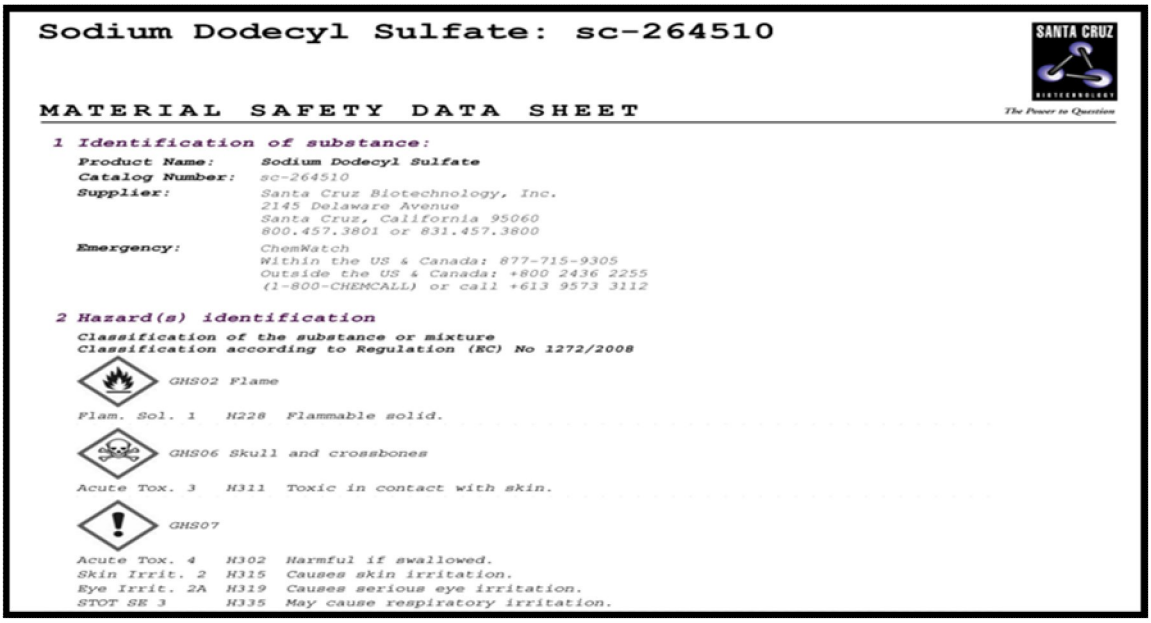
2.1.3 How bad can SDS/ SLS be on your skin?

SDS has been shown to irritate facial skin after prolonged and constant exposure (more than an hour) in young adults. SDS may worsen pre-existing skin conditions in individuals with chronic skin hypersensitivity. According to a report published in the Journal of The American College of Toxicology in 1983, concentrations as low as 0.5% could cause irritation and concentrations of 10-30% caused skin corrosion and severe irritation. The report concludes

that “Tests show permanent eye damage in young animals from skin contact in non-eye areas. Studies indicated sodium lauryl sulfate kept young eyes from developing properly by possibly denaturing the proteins and not allowing for proper structural formation. This damage was permanent.”

Did you know that over more than 50% of main stream cleaning products on the market contain some type of evidence of Sodium Dodecyl Sulfate. Although used as an abrasive cleaning ingredient, in literature studies it was shown to have a negative effect on the skin and eye. Researchers found that many concentrations of SDS caused irritation and redness in most skin samples, proving it to be harmful towards the epidermis. SDS was also tested on rabbit corneas, resulting in impaired vision as well as even blindness in some cases. This led to mimic the skin and eyes by testing the skin and eye cells. Serratia marcescens is a biofilm that forms on most shower heads around the country. Thriving in the damp, moist environment, the bacteria attaches to the shower head so that when the water is turned on, it travels with water, hitting the body. This bacteria is shown in some cases to be pathogenic to the human body. The effect of Sodium Dodecyl Sulfate on Eye Cells, Skin Cells, and Serratia marcescens. Sodium Dodecyl Sulfate (SDS) is an ingredient found in many shampoos and soaps that cause them to have a lathering effect. This widely used ingredient has a detrimental effect on the human body

Santa cruz biotechnology clearly stated the harmful effect of SDS in its supplier safety sheet.



Sodium Dodecyl Sulfate: sc-264510

SANTA CRUZ BIOTECHNOLOGY
The Power to Question

MATERIAL SAFETY DATA SHEET

1 Identification of substance:

Product Name: Sodium Dodecyl Sulfate
Catalog Number: sc-264510
Supplier: Santa Cruz Biotechnology, Inc.
2145 Delaware Avenue
Santa Cruz, California 95060
800.457.3801 or 831.457.3800

Emergency: ChemWatch
Within the US & Canada: 877-715-9305
Outside the US & Canada: +800 2436 2255
(1-800-CHEMCALL) or call +613 9573 3112

2 Hazard(s) identification

Classification of the substance or mixture
Classification according to Regulation (EC) No 1272/2008

GHS02 Flame

Flam. Sol. 1 H228 Flammable solid.

GHS06 Skull and crossbones

Acute Tox. 3 H311 Toxic in contact with skin.

GHS07

Acute Tox. 4 H302 Harmful if swallowed.
Skin Irrit. 2 H315 Causes skin irritation.
Eye Irrit. 2A H319 Causes serious eye irritation.
STOT SE 3 H335 May cause respiratory irritation.

IDENTIFICATION AND USE: Sodium lauryl sulfate is a white or cream-colored crystal, flake, or powder with a faint odor.Sodium dodecyl sulfate is used in general as a detergent, dispersant, and surfactant. Pure sodium dodecyl sulfate is used mainly in dentifrice products (a powder, paste, or liquid for cleaning the teeth), in hair shampoos, and in emulsion polymerization. The rest is either used in special cosmetic formulations, e.g. for bubble baths and hair bleaches, or as a fine chemical, e.g. as denaturing agent in gel electrophoresis. Besides pure sodium dodecyl sulfate detergent, manufacturers usually produce "technical grade" sodium dodecyl sulfate, a product that consists of approximately 70 % sodium dodecyl sulfate and 30 % sodium tetradecyl sulfate. This product is generally called sodium lauryl sulfate. Technical grade sodium dodecyl sulfate is used as a detergent in dish-washing products (main use), as additive for plastics and latices, and in paints and lacquers. Sodium lauryl sulfate is used as a flea and tick repellant in one registered pesticide product--a flea and tick shampoo for cats and dogs. Sodium lauryl sulfate also is a widely used component of many nonpesticidal consumer products currently marketed in the United States, including shampoos and fruit juices. It is also used in hydraulic fracturing to prevent the formation of emulsions in the fracture fluid.

HUMAN EXPOSURE AND TOXICITY: Among 242 patients suffering from eczematous dermatitis, the percentage of allergic reactions reached 54.6%. A great number of allergic reactions to sodium lauryl sulfate (6.4%) was observed. The study was conducted to compare the effects of sodium lauryl sulfate (SLS)-free and SLS-containing dentifrice (a powder, paste, or liquid for cleaning the teeth) in patients with recurrent aphthous stomatitis (RAS). Although SLS-free products did not reduce the number of ulcers and episodes, it affected the ulcer-healing process and reduces pain in daily lives in patients with RAS.

ANIMAL STUDIES: The repeated dose toxicity of sodium dodecyl sulphate was studied extensively. Tests range from sub-acute (28 days) to chronic (2 years in rat) studies. Further, the substance was tested in two different species (rat and dog) and by means of two different routes of administration (diet and gavage). The primary effect of sodium dodecyl sulphate in animals isa local irritation of the gastro-intestinal tract. Developmental toxicity/teratogenicity of sodium dodecyl sulphate was investigated in three different species. Mice and rabbits are the most sensitive species. Sodium dodecyl sulphate was extensively tested for genetic toxicity. Neither the bacterial tests nor the various tests in mammalian systems have shown any indication of genotoxicity with or without metabolic activation. Published reports suggest that sodium lauryl sulfate has low acute mammalian toxicity and no known chronic effects.

ECOTOXICITY STUDIES: Uptake, tissue distribution, and elimination of lauryl sulfate were investigated in carp. The chemical concentrated in hepatopancreas and gallbladder. Maximum whole-body levels were reached during 24-72 hr. Survival time decreased with increased water hardness. Sodium lauryl sulfate has been tested as a repellent against several species of sharks. It did not provoke a repellency response at a low enough concentration to function effectively as a classical, surrounding-cloud type, repellent. The range of potency, however, does allow it to be used as a directional repellent.

2.2 Lipase in toothpaste

The purification of lipase and protease enzymes from *Bacillus* sp and their application in the formulation of tooth paste and as a contact lens cleaner. The rhizosphere soil sample was collected and screened for lipase and protease producing bacteria on tributyrin agar and gelatine agar respectively. The potential isolate SS-7 was selected and tested for various morphological and biochemical tests which confirmed SS-7 as *Bacillus subtilis*. The crude lipase and protease were obtained, partially purified with ammonium sulfate precipitation and dialysis. The lipase was substituted for a chemical surfactant (SDS) in the formulation of the toothpaste.

Many microorganisms like Actinobacteria are able to produce a wide range of amphipathic compounds which is normally known as bio surfactant. Due to their amphipathic nature, bio surfactant can partition at the interface between difference fluid phases such as oil, water or air interfaces. There are many types (e.g. glycolipids, rhamnolipids, sophorolipids etc) of bio surfactants each produced by specific microorganisms. Glycolipids and lipopeptides are low molecular weight bio surfactants that effectively lower surface and interfacial tensions. High molecular weight compounds include extracellular polysaccharides, lipopolysaccharides, proteins, and lipoproteins that have a high affinity for surface binding Bio surfactant have special advantage over the chemical surfactants (e.g. Sodium Lauryl Sulphate), such as non toxicity, higher biodegradability, better environmental compatibility, higher foaming, high selectivity and specific activity at extreme temperature, pH, and salinity. Hence, biosurfactant provide highly valued surface properties, they have a higher degree of biodegradability and environmental compatibility which synthetic surfactants lack. Even with potential for several applications, production cost must be lowered, and high yield mutant strains need to be developed for these compounds to be profitable on a commercial scale. Exhibiting unique characteristics including antiradical properties, stimulation of dermal fibroblast metabolism, and hygroscopic properties to support healthy skin physiology, future prospects of bio

surfactant based products include several types of facial cosmetics, lotions, beauty washes and hair products. Bio surfactant production and analysis is currently a wide and active field of study and bio surfactant easily can be used in cosmetic formulation replacing the chemical surfactant. Bio surfactant also has enzyme activity and it's known as bacterial lipases. Bacterial lipases are ubiquitous enzymes of considerable physiological significance and industrial potential. Lipases are widely used in cosmetic industry and it was used as an emulsifiers and moisturizers. The market for enzyme used in cosmetic is set to grow 5% per year up to 2015 driven by technological progress and consumer awareness of seemingly potent power of enzymes. Normally toothpastes are complex mixtures of abrasives, surfactants, anticaries agents, tartar control ingredients, pH buffers, humectants and binders to provide consistency and shape. Toothpastes are the essential cosmetics of daily uses. The primary function of toothpaste is the cleaning of accessible surfaces of the teeth. Following aspects of toothpaste were planned for the formulation and carried out to the application of bio surfactant in cosmetic formulation instead of using chemical surfactant.

2.3 ANHYDROUS TOOTHPASTE FORMULATION


United States Patent Office 3,574,824 Patented Apr. 13, 1971

This is anhydrous toothpaste formulation patent from USPO from this formulation we have to replace SLS with lipase.

This invention relates to a substantially anhydrous toothpaste formulation which may be used as a vehicle for Water incompatible enzymes, bleaching agents, fluorides and other therapeutic dentifrice additives suitable for use in the oral cavity. Commercially acceptable products are obtained. This invention provides a substantially anhydrous toothpaste base, suitable for use in the oral cavity, which when formulated, has a consistency, foam ability and taste equivalent to the commercially available hydrous dentifrices. Further, it provides a substantially anhydrous toothpaste which may contain 30% to 70% of an abrasive. Additionally, water incompatible dentifrice additives such as enzymes, bleaching agents, fluorides and the like, when added to this substantially anhydrous toothpaste base or formulation, remain stable throughout the normal shelf life of the toothpaste product.

Standard prior art toothpastes generally contain synthetic detergents, abrasives, formulating liquids (such as glycerin, propylene glycol, sorbitol solution, water and a ‘ alcohol), and thickeners which may include starch, tragacanth, alginate, carrageenan and cellulose derivatives. Non-carbohydrate sweetening agents may also be present. Typical abrasives used in these toothpaste formulations are: calcium carbonate, the calcium phosphates, calcium sulphate, insoluble sodium metaphosphate, hydrated aluminium oxide, magnesium carbonates and phosphates, sodium bicarbonate, and sodium chloride. Representative hydrous toothpaste contains the following ingredients:

CMC 120 H, Glycerin, Propylene glycol, Purifed water, Methyl paraben, Saccharin sodium, solution 50% , Peppermint oil, Mineral oil, Sodium lauryl sulphate, Dicalcium phosphate, in very fine powder.

OBJECTIVES

The aim of the research is to replace chemical surfactant from toothpaste and to add biological surfactant, the work has been categorized under the following headlines

- Production of lipase by *Staphylococcus arlettae* JPBW-1 through SmF.
- Purification of *Staphylococcus arlettae* lipase.
- Evaluation of partially purified *s.arlettae* lipase by its addition in toothpaste formulation
- Formulation of Bio-based toothpaste with partially purified lipase

Chapter 3

MATERIALS AND METHODS

3.1 Materials

The materials such as microorganism, raw materials, chemicals, instrument and apparatus used in the present research work are briefly presented in this section.

3.1.1 Microorganism

Staphylococcus arlettae was used for the lipase production, which was isolated from the one and only rock salt mine of India, Darang, HP and deposited in MTCC, Chandigarh as *Staphylococcus arlettae* JPBW-1 MTCC5589, maintained on Luria agar slants at 4 ^{o}C , Provided by JUIT.

3.1.2 Chemicals and reagents

Triton X and *P*nitrophenyl palmitate (*p*-NPP) was procured from Sigma, USA. Acacia, acetone, agar, ammonium nitrate, ammonium sulphate, calcium carbonate, calcium chloride, carboxy methyl cellulose, ethanol, Folin- Ciocalteau reagent, tris-buffer, Glycerin, Propylene glycol, Purifed water, Methyl paraben, Saccharin sodium, solution 50% , Peppermint oil, Mineral oil, Sodium lauryl sulphate, Dicalcium phosphate, in very fine powder. Commercial toothpaste of Indian market namely Closeup, sensodyne, colgate salt, colgate total, colgate herbal , colgate regular and Pepsodent..

3.1.3 Instruments and apparatus

Glasswares like Testubes, Petri plates, Beaker, Conical flask. BOD Bottles were used of borossil, JSBW.

Distilled water plant (Ion Exchange, India Ltd.): For distilling the water used for media

Autoclave (Testing Instruments, Mfg. Co. India): Used for sterilization of the media.

Heater: Used for heating solutions.

Hot air oven: (SISCO India): Used for drying glasswares.

Incubator (Sambros, India): For growth of micro-organism at constant temperature.

Water bath (Thermostat) (Hijli co-operative stores Ltd., India): Used for maintaining constant temperature.

Refrigerator (Whirlpool, India), **Deep fridge** (Godrej, India): For storage of heatsensitive materials.

3.2 Methods

3.2.1 Lipase production

Production of lipase from bacterial strain *Staphylococcus arlettae was conducted by* Sub culturing of *Staphylococcus arlettae* Bacterial lipase was produced through submerged fermentation by cultivating 100 ml inoculum (48 h old) in a shaking flask (250 ml) with 100 ml of the LB broth medium. The culture was incubated for 3 h on a rotary shaker (125 rpm) at 37 °C. After 3 h, the fermented broth was centrifuged at 7000 rpm for 15 min at 4 °C and the cell-free supernatant was collected and used for estimation of lipase activity.

3.2.2 Lipase assay

Lipase activity was determined using *p*-NPP as substrate (Garlapati and Banerjee, 2010). One unit (U) of lipase activity was expressed as the amount of enzyme that liberates one micromole of *p*-nitrophenol released per minute under the assay conditions. Two solutions were prepared soln1 & soln2

Sol 1: 40mg PNP in 12ml Propanol.

Sol 2: 90% Tris cl pH 8 + 40µl Triton X + 1mg Gum acacia.

Add 9ml soln2 + 1ml soln1 drop by drop and this addition must be done in water bath maintaining temperature $\leq 50^0$ c , with continuous shaking now this composition is taken 1ml in each of 5 Test tubes 25µl enzyme in 4 tubes ,where as 1 keep as control out of these 5 tubes, incubation for 10 min at 37^0c. After completion of incubation 2ml distilled water is added in each tube then at last O.D. at 410nm

3.2.3 Protein estimation

Protein content of cell-free supernatant was determined according to modified Lowry method (Lowry et al.,1951) using BSA as a standard. Three solutions were prepared soln1 soln2 and soln3. Preparation for protein estimation is as follows

Sol 1: 2% sodium carbonate in .1N NaOH

Sol 2: .5%$CuSo_4$ in 1% sodium potassium tartrate

Sol 3 :50ml of soln1 + 1ml of soln2

Follins reagent - 1:1 ratio nof follin : distilled water.

50 µl of supernatant is allowed to be taken in 4 test tubes and then 450 µl of distilled water is added to it where as for control 500 µl of distilled water in a single test tube, 5ml soln3 is added in each of the tubes including control, Incubatation for 10 min at room temperature, 500 µl of follin reagent is added in each tube, Incubatation at room temperature for 30 mins optical density is expected to be taken at 750nm because the color which is expected to turn blue from yellow.

3.2.4 Partial purification (ammonium sulphate precipitation)

The Lipase cell free extract was precipitated with ammonium sulfate up to 60% of saturation. Precipitate (pellets) was obtained by centrifugation at 10,000 rpm at 4° C for 15 mins and dissolved in 1 mM Tris HCl buffer of pH 8.0

In 100ml of supernatant add 60% ammonium sulphate , while addition constant stirring is done for proper mixing and temperature maintained at 4^0c, incubation for 2 hrs at 4^0c, then centrifugation at 7000 rpm for 15 min, supernatant is discarded and finally add Tris cl to dissolve palate us min amount of buffer. After partial purification again Lipase assay is Performed to check the stability of enzyme after purification.

3.2.5: Compatibility of lipase with surfactants and commercial toothpastes

Relative activity test or we can say compatibility of Lipase with toothpaste for commercial toothpaste and the procedures for that is as following. We got 7 commercial toothpastes from market i.e namely Closeup, sensodyne, colgate salt, colgate total, colgate herbal , colgate regular and Pepsodent.

To investigate the compatibility of lipase in various surfactants and commercial toothpastes, respective surfactants and toothpastes were added to the reaction mixture at a concentration of 7 mg/ml and assayed under standard assay conditions and expressed as percent relative activity. The endogenous lipases contained in these toothpastes were inactivated by heating the diluted toothpastes for 1 h at 65 ^{0}C prior to the addition of the enzyme preparation. To determine the stability, an aliquot of enzyme sample (50 U/ml) was incubated with equal volume of toothpaste solution (7 mg/ml of respective toothpaste) in Tris- HCl buffer (0.1 M, pH 8.0) for 1 h at 30 ^{0}C. The relative activity (%) of each sample was determined and compared with the control without toothpaste. The relative activity of control was defined as

the enzyme activity without toothpaste, incubated under the similar conditions and was taken as 100 %.

3.2.6 Enzymatic toothpaste formulation

Three formulations were there FOR1, FOR2 and FOR3

FOR1 --- Commercial toothpaste

FOR2 --- Commercial + Lipase

FOR3 --- Only Lipase

Formulation 1 : Commercial toothpaste available in market is used after checking the relative activity of different toothpaste available then selecting the toothpaste showing higher activity.

Formulation 2 : this formulation is done by simply mixing bio surfactant with formulation 1.

Formulation 3 : own formulation using Lipase. The following ingredients are been used in our formulation base [-1]:

- CMC 120 H _____ __ 0.9gm
- Glycerin _____ __ 1.0
- Propylene glycol _____ __ 18.0
- Purifed water _____ __. 13.5
- Methyl paraben _____ __ 0.1
- Saccharin sodium solution 50% _____ __ 0.1
- Peppermint oil _____ __ 0.3
- Mineral oil _____ __ 1.0
- Lipase---------- 2.5
- Dicalcium phosphate, in very fine powder _____ 54.0

Formulation procedure :

All these ingredients were added in a beaker and after that stir it continuously until it gains the consistency of normal commercial toothpaste.

3.2.7 Efficiency tests and comparison

3.2.7.1 Abrasiveness Test:

Pea sized amount of commercial toothpaste, formulation2 toothpaste and formulation 3 toothpaste were placed on three different clean slides and one drop of distilled water was added on each toothpaste sample. With the help of cotton swab each toothpaste sample was rubbed in a back and forth motion for 25 times using short 1cm stroke. Then carefully the slides were rinsed off and dried with soft tissue paper. Each slide was examined under a dissecting microscope and the amounts of scratches are determined on the surface of the slide

3.2.7.2 Determination of Spread ability

One gm of commercial toothpaste, for2, for3 was placed on the centre of the glass plate and another glass plate is placed over the sample. 1kg weight on top of the glass plates was placed. After 10 min, the weight is removed and the diameter of the paste is measured in centimetre. The experiment was carried out in triplicate.

3.2.7.3 Determination of pH

In 250 mL beaker 2.0 gm commercial toothpaste was taken and 80 mL of distilled water was added and the solution is stir well. After 30 mins, the pH of the solution is measured with the help of pH meter. This test was repeatedly done for formulation2 and formulation3 measurement.

3.2.7.4 Determination of foaming ability

In a test tube 5 mL of distilled water was taken and followed by 0.5 gm of commercial toothpaste was added. The top of the test tube was covered with cork and the test tube is shaken properly. The nature and stability of the foam thus formed were studied and the height of the foam above the water is measured in centimetre. This test was repeated for formulation2 and formulation3 toothpaste.

3.2.7.5 Cleaning ability Test

The original human tooth available from dentist i.e kapoor dental clinic, shimla was having blood stains on it was used for the research and human teeth were brushed with the wet tooth brush for 5 to 10 strokes (back and forth motion). After that small amount of commercial toothpaste placed on tooth brush and human teeth were brushed by 5 to 10 strokes. Whole test is repeated for formulation2 and formulation3 toothpaste to check their cleaning ability. The results interrupted as follows '+++' 95% cleaning ability, '++' 85-95% cleaning ability, '>85%' cleaning ability.

Chapter 4

RESULTS AND DISCUSSION

4.1 Partial Purification of Lipase

Lipase assay and protein estimation were measured for crude and purified separately and other parameters were calculated by these two results as shown in table different formulas were used to calculate fold , yield, specific activity and those formulas are stated below. BSA standard curve used for Protein estimation and for lipase assay p-NP curve was used. The unknown conc. of p- NP released was determined from standard curve of p-NP. Lipase assay found in crude lipase is 0.0069 U/ml. Protein estimation result can be validated by BSA standard plot.

Table 5: purification of lipase from *Staphylococcus arlettae*

Purification	**Total protein (mg/ml)**	**Total activity (U/ml)**	**Specific activity (U/mg)**	**Fold purification**	**Yield(%)**
Crude	0.146	0.0069	0.048	1.00	100 %
Ammonium sulphate	0.052	0.0044	0.084	1.75	63.7 %

4.2 Compatibility of lipase with surfactants and commercial toothpastes

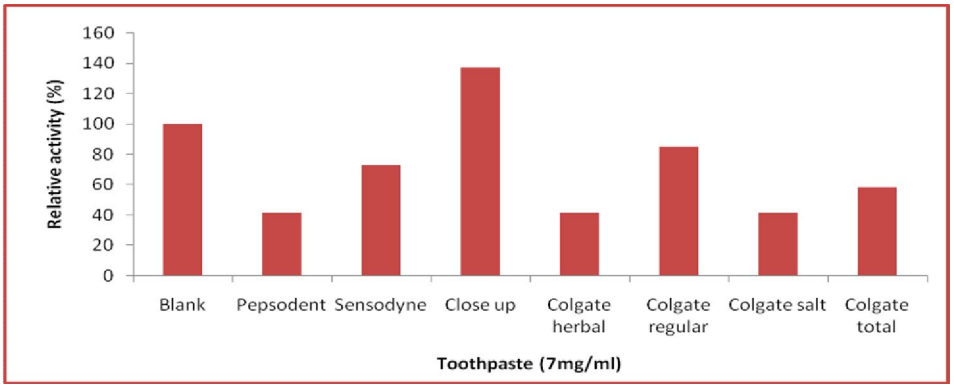


Fig.2: Compatibility of *S. arlettae* lipase with Toothpaste. For the control, lipase was incubated with buffer alone without Toothpaste and its activity was taken as 100%.

Therefore, after doing this experiment we found closeup is relatively more compatible then other commercial toothpastes, so closeup is considered for further use.

4.3 Enzymatic toothpaste formulation

- Formulation 1 (FOR 1)= commercial i.e. close up….WITH SDS
- Formulation 2 (FOR 2)= close up + Lipase
- Formulation 3 (FOR 3)= own formulation …. Only Lipase

4.4 Efficiency tests and comparison

4.4.1 Efficacy Test

Table 6: Efficacy test

Experiments	Commercial (FOR1)	Formulation 2	Formulation 3
Abrasiveness Test(scratch)	+++	+	++
Spread ability Test	+	++	+++
Foaming ability	++	+++	+
Cleaning ability	+	++	+++
pH Test	+	++	+++

This table is showing the results of our formulation in comparison with other commercial one and is showing that formulation3 is more effective as compared to others in all the parameters. As shown below each of the parameters of efficacy test. The effect of the efficacy test performed, showed positive effect in formulation consistinglipase in comparison with the commercial one consisting SDS.

Results having high pH means the toothpaste is more basic and less acidic which is good for gums and rashes in the inner portion of mouth can be avoided due to its basic nature. Less abrasive means that toothpaste is smooth as compared to commercial one. In the results we found that toothpaste consisting lipase is high in spread ability means the smoothness of the toothpaste the foaming ability is less in comparision to commercial one because that consists SDS which is a chemical foaming agent whereas, lipases is a biological surfactant and giving

less foam then SDS but sufficient amount of foam is seen in the test, lipase is giving foam but not as much as SDS.

4.4.2 pH test result

The pH value of toothpaste also plays a crucial role. The pH value gives an indication of inorganic constituents in toothpaste. Acidic pH encourages the growth of oral bacteria that cause dental carries. The pH test result showed that formulation1 and formulation2 toothpaste are more basic in nature compare to commercial toothpaste.

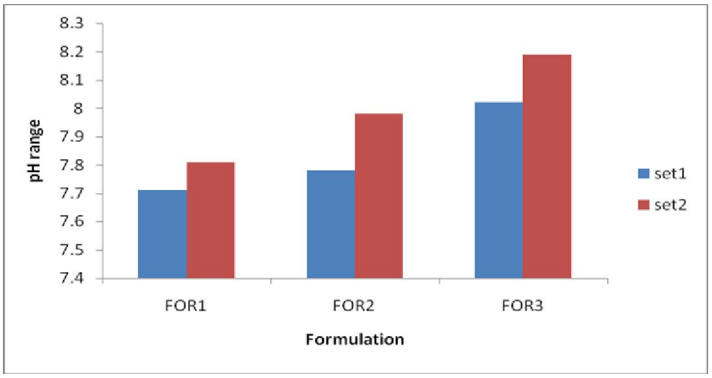


Fig 3: pH test

4.4.3 Spreadability test result

Spreadability was another characteristic of toothpaste. It showed the consistency of paste. Basically the toothpastes are homogenous in nature and it should not separate into liquid and solid ingredients. The large spread area has shown the good consistency of toothpaste. In Spreadability test formulation1 toothpaste showed more consistency (3 cm) compared to formulation2 (4 cm) and commercial toothpaste (5.2 cm).

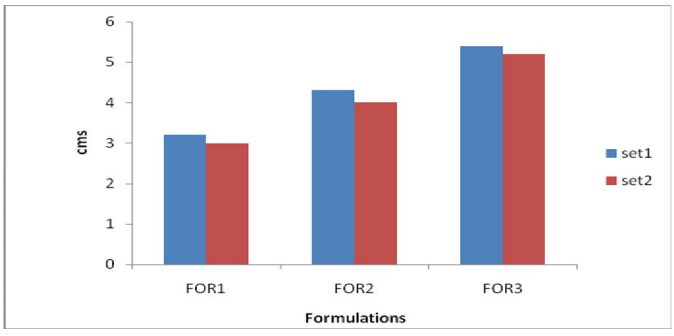


Fig 4: Spreadability test

4.4.4 Foaming test results

All toothpaste usually has foaming property. The foaming ability test of toothpastes showed that the efficiency and detergency of a particular toothpaste. Here commercial toothpaste and formulation1 showed almost same result in foaming ability test. So, this result indicates that biosurfactant are efficient greater than equal to chemical surfactant and biosurfactant also act as a good detergent in toothpaste

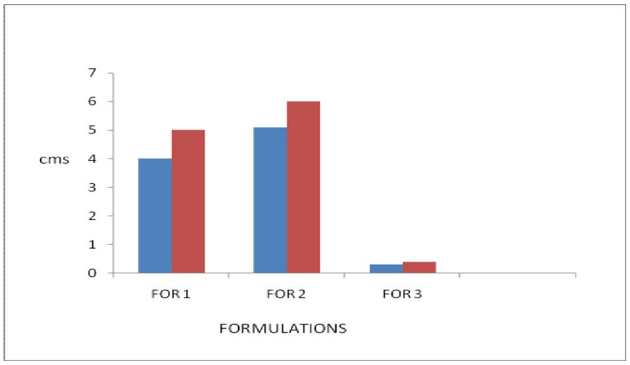


Fig 5: Foaming test

4.6.4 Abrasiveness test result

Each slide was examined under a dissecting microscope and the amounts of scratches are determined on the surface of the slide. Here in case of abrasiveness test the result showed that commercial tooth paste has more abrasive particle because of that reason the scratches are coming more compare to other two formulations. In case of formulation1 and formulation2 less scratch are observed because Dicalcium phosphate

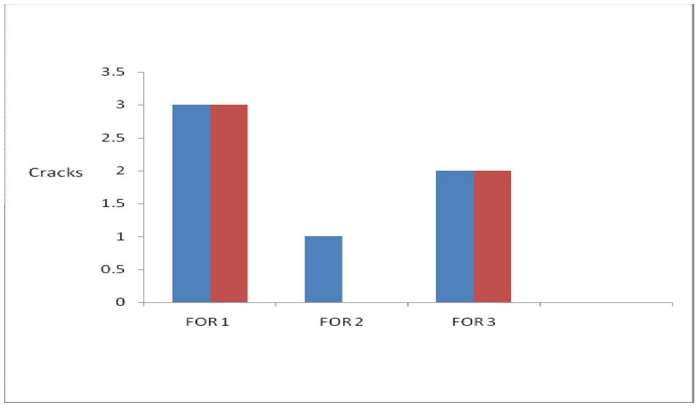
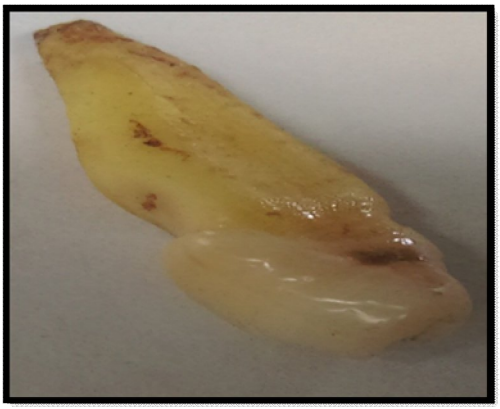
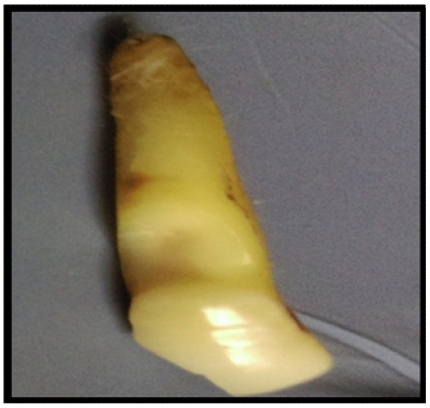


Fig 6. Abrasiveness test

4.4.6 CLEANING TEST



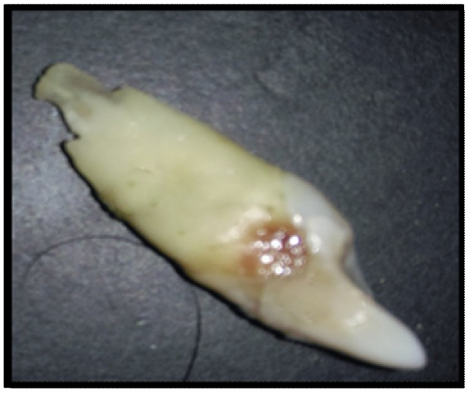
Before brushing



After brushing

(a)

Before brushing



after brushing

(b)

Before brushing

after brushing

(c)

Fig 7: Cleaning ability test of (a) Formulation 1 (b) Formulation 2 (c) Formulation 3, before brushing and after brushing

Collected 3 teeths from kapoor dental clinic , shimla in very dirty condition blood stains were there on the teeth yellowness was fond on teeth can be seen easily in pictures after brushing tooth really showing positive change in colour .Another important characteristic of toothpaste is cleaning ability. In case of human, when organic pellicle which is secreted by the saliva and pellicle reacts with colouring agent such as cigarette smoke, beverages, coloured fruits, chocolates etc and those things stained the teeth. Removing spots and stains faces serious problems. Stained pellicle sticks firmly to the teeth and resist against removing. Those stained pellicle can be removed by toothpastes. In commercial toothpaste chemical surfactant are responsible ingredients for removing the stain from teeth. The cleaning ability test results reported that biosurfactant had equal ability to remove the stain from teeth

Chapter 5
CONCLUSIONS

Following conclusion have been drawn from the above work

- Production of lipase from *Staphylococcus arlettae* by submerged fermentation is done lipase assay is done to check the stability of enzyme partial purification is done to make it ready to use in toothpaste by 60% ammonium sulphate purification. Formulation of toothpaste by using this enzyme as a biological surfactant in it, replacing SDS which is a chemical surfactant and is found harming skin, which was the purpose of this research.

- Like chemical surfactants, bio surfactant are excellent emulsifiers and maintain wetting and foaming properties, non toxic in natures that are valued in several applications including the cosmetics industry. Unlike chemical surfactant, bio surfactant are readily biodegradable contributing to environmental compatibility. Overall it may conclude that bio surfactant can act as a replacement candidate for chemical surfactant in toothpaste formulation.

- On comparison with substituting SDS with lipase enzyme in formulation and it was observed that formulation with enzyme showed maximum spreading, incomplete uniformity, high pH and less abrasiveness. A major difference was observed in foaming ability and cleaning ability. This result indicates that lipase can be altered to some extent for SDS reducing its concentration in the formulation just too impact foaming ability.

REFERENCES

Ahmed, E. H., T. Raghavendra and D. Madamwar. (2010) An alkaline lipase from organic solvent tolerant Acinetobacter sp. EH28: Application for ethyl caprylate synthesis .Science direct 101(10): 3628-3634

Abdel-Naby, MA; Ismail, AMS; Abdel-Fattah, AM; Abdel-Fattah, AF (1997) Production and immobilization of alkaline protease from Bacillus mycoides. Biores tech. 64: 205-210.

Aires-Barros M.R. and Cabral, J.M.S. (1991) selective separation and purificationof two lipases from Chromobacterium viscosum using AOT reversed micelles. Biotechnol. Bioeng. 38, 1302-1307.

Banerjee, R. K. Sani, W. Azmi, and R. Soni (1999) Thermostable alkaline protease from Bacillus brevis and its characterization as a laundry detergent additive. Proc Biochem. 35: 213-219.

Benjamin, S, Ashok Pandey (1995) Optimization of liquid media for lipase production by Candida rugosa. Bioresource technol. 55:167-170.

Bergy (1984). Systematic bacteriology. Lippincott William and Wilkins, Baltimore, USA. 1:1-4

Borkar, P. S., R. G. Bodade, S. R. Rao, and C. N. Khobragade. (2009) Purification and characterization of extracellular lipase from a new strain-Pseudomonas aeruginosa SRT 9. Journal of Microbiology. 40:358–366.

Chauhan M and Garlapati VK (2013) Production and charecterisation of a halo solvent thermo tolerant alkaline lipase by staphylococcus arlettae JPBW 1 isolated from rock salt mine App. Biochen Biotechnol 171:1429-1443

Chauhan M, Chauhan RS, Garlapati VK (2013) Evaluation of new Lipase from staphylococcus sp. For detergent additive capability Biomed Res. Int 2013:374967

SD, Lee KS, Lo YC, Chen WM, Wu JF, Lin CY and Chang JS (2005) Production of Acinetobacter radioresistens lipase with repeated fed-batch culture. Biochem eng J. 25:195 – 199.

Dharmsthiti S, Sudaporn Luchai.. (1999) Production, purification and characterization of thermophilic lipase from Bacillus sp. THL027. FEMS Microbiol lett. 17:, 241-246

El Taweel, M. A., Rahman, R. N. Z. R. A., Salleh, A. B., and Basri, M., (2005). An organic solvent-stable lipase from Bacillus sp. strain 42. Annals Microbiology 55(3) :187-192.

Piero Fossati, Maurlzlo Ponti, Paolo Paris, Giovanni Berti, (1992) Kinetic colorimetric assay of lipase in serum. Clinical chemistry 2-

Fikret U, Zubeyde B (2004). Production and optimization of process parameters for alkaline protease production by a newly isolated Bacillus species under solid state fermentation. Process Biochemistry 39: 1893-1898.

Fikret Uyar, Zubeyde Baysal. (2004) Production and optimization of process parameters for alkaline protease production by a newly isolated Bacillus species under solid state fermentation. Proc Biochem. 39: 1893-1898.

Garlapati VK, Banerjee R (2010) Optimisation of Lipase production using differential evolution Biotechnol. Bioprocess Eng.15:254-260

Gaur R, Gupta A, Khare SK (2008). Purification and characterization of lipase from solvent tolerant Pseudomonas aeruginosa PseA Process Biochemistry 43(10): 1040-1046

L. Govender, L. Naidoo, and M.E. Setati (2009) Isolation of hydrolase producing bacteria from Sua pan solar salterns and the production of endo-1,4-β-xylanase from a newly isolated haloalkaliphilic Nesterenkonia sp. African Journal of Biotechnology. 2009(8) 5458-5466.

Genckal, H., Tari, C. ,(2006) Alkaline protease production from alkalophilic Bacillus sp. isolated from natural habitats. Enzyme and microbiology tech 39:703 – 710

Gerritse b, Lydia Dankmeyer b, Wim J. Quax b,c (1998) Development of lipase fermentation process that uses a recombinant Pseudomonas alcaligenes strain. J. Appl Environ Microbiol. 64: 2644-2651.

HASAN, F.; SHAH, A. A.; HAMEED, A (2006). Industrial applications of microbial lipases. Enzyme and Microbial Technology 39(2): 235-251.

Haferburg D (1983) Lipase in bacteria. Biotechnological future prospects 2:371–374, .
Hostacká A (2000) Lipase activity of Acinetobacter baumannii after treatment with meropenem. Arzneimittelforschung. 50(12):1134-7

Iizumi T, Nakamura K, Fukase T (1990). Purification and characterization of a thermostable lipase from newly isolated Pseudomonas sp. KWI-56. Agricultural Biology Chemistry 54:1253-1258.

Ismail (1999.) Optimization of alkaline protease productivity by Bacillus licheniformis ATCC 21415. Bioresearch Technology 69:155-159.

James Echeandia, Richmond, Va., Yashvant Kapadia, Lafayette (1971) Anhydrous toothpaste formulation US 3574824 A

Jaeger et al., 1999 and Joseph et al., 2007 Polyhydroxyalkanoates from Palm Oil: Biodegradable Plastics.Current Opinion Struc Biology 15:447–452

Jaeger K.E., Eggert T.(2002) Lipases for biotechnology Current. Opinions in Biotechnology. 13:390–397.

Janssen, P. H., Monk, C. R. and Morgan, H. W (1994). A thermophilic, lipolytic Bacillus sp., and continuous assay of its p-nitrophenyl-palmitate esterase activity. FEMS Microbiology Letters 120(2):195-200.

Jansen, Jongejan, H., Kooijmau, H,(1996). Substrate specificity and kinetics of Candida rugosa lipase in organic media. Enzyme Microbial Technology 18: 340-346.

Karthik L, K Gaurav, KV Bhaskara Rao.,(2013) Antioxidant activity of newly discovered lineage of marine actinobacteria. Asian pacific journal of tropical biomedicine 6(4): 325-332.

Kulkarni, N. and. Gadre RV (1999) A novel alkaline, thermostable, protease free lipase from Pseudomonas sp. Biotechnology. Lett. 21: 897-899.

Kulkarni, N.,(2002). Studies on lipase enzyme from Pseudomonas fluorescens NS2W. Ph.D Thesis. University of Pune, pp: 221.

Karanth NGK (1989) Bacterial lipases: an overview of production, purification and biochemical properties. researcg gate 64(6):763-81

Kazlauskas, R.J., Bornscheuer, U.T.,. (1998) Biotransformations with lipases.Biotechnology New York VCH. 3:37-192.

Kordel M , Wang Y, Fu Z, Huang H, Zhang H, Yao B.,(1991) Extracellular lipase of Pseudomonas sp. Strain ATCC 21808: purification, characterization, crystallization and preliminary X-ray diffraction data. Journal of Bacteriology. 1991;173:4836–4841. [PMC free article] [PubMed]

Liebeton, K., A. Zacharias, and K.-E. Jaeger.., (2001) Disulfide bond in Pseudomonas aeruginosa lipase stabilizes the structure but is not required for interaction with its foldase. Journal of Bacteriology. 2001;183:597–603. [PMC free article] [PubMed]

Lowry O.H., Rosebrough NJ, Farr AL, Randall RJ (1951) Protein measurement with the folin phenol reagent. Journal of Biohemistry. 1951;193:265–275.

Limpon B, Kalita MC (2007)Production and Optimization of Thermostable lipase from a Thermophilic Bacillus sp LBN 4. The Internet Journal of Microbiology 4

Lechevalier MP, Lechevalier, H. (1970) Chemical composition as a criterion in the classification of aerobic actinomycetes. Int. J. Syst. Evol. Microbiol 20: 435-443.

Maia MMD, Heasley A, Camargo de Morais MM., (2000) Effect of culture conditions on lipase production by Fusarium solani in batch fermentation. Bioresearch technology 76: 23-27.

Mohammed A, (2005) An organic solvent-stable lipase from Bacillus sp. strain 42. Annals of microbiology 55:187-192

Maugard T. Laboratoire de Génie Protéique et Cellulaire., (2002) Synthesis of water soluble derivatives by enzymatic method. Biotechnol Prog. 18: 424-428.

Mckellar & Cholette .(1986) Determination of the extracellular lipases of Pseudomonas fluorescens spp. in skim milk with the β-naphthyl caprylate assay 53:301-312

Pignede , Dolezalova L. Musil P. Novak M.., (2002) Characterization of an extracellular lipase encoded by Lip2 in Yarrowia lipolytica. Journal of Bacteriology vol. 182(10): 2802-2810.

Rathi P., Saxena R.K., Gupta R, (2001) A novel alkaline lipase from Burkholdria cepacia for detergent formulation. Proc Biochem 37: 187 – 192.

Sharma A, Bardhan D, Patel R, (2009) Optimization of physical parameters for lipase production from Arthrobacter sp. BGCC#490. Indian J Biochem Biophys vol 46:178–183. [PubMed]

Sharon C, Furugoh S, Yamakido T ,(1999) Purification and characterization of a lipase from Pseudomonas aeruginosa KKA-5 and its role in castor oil hydrolysis. J Ind Microbiol Biotechnol 20:304–307.

Safarik I.(1991) spectrophotometric assay for lipase activity utilizing immobilized triacylglycerols 23:249–253

Shuang li, xiaofeng yang, shui yang Muzi Zhu. (2012) Technology prospecting on enzymes application Marketing and Engineering.Computational and Structural Biotechnology Journal 3:1–11

Shaukat Ali, Zhen Huang & Shun X. Ren.,(2009) Production and extraction of extracellular lipase from the entomopathogenic fungus Isaria fumosoroseus. Biocontrol Science and Technology 19(1): 81-89

Stuer W J.Bacteriol.; Jaeger, K.E.; Winkler, U.K. (1986) Purification of extracellular lipase from *Pseudomonas aeruginosa*. *J. Bacteriol.* 168:1070-1074.

Van A, Wim H. M. Saris (1991).. Determination of total free fatty acids was performed according to wiley online library

Wang SL Wang SL, Lin YT, Liang TW,., (2009) Purification and characterization of extracellular lipase from Pseudomonas monteilii tku009 by the use of soybeans as the substrate. J Ind Microbiol Biotechnol 36:65–73.

Woollett L. A., Beitz D. C., Hood R. L. and Aprahamian S. (1984) An enzymatic assay for activity of lipoprotein lipase. Anal. Biochem. 143:

Formulae used :

- Specific activity = $\frac{\text{Lipase activity}}{\text{Protein content}}$

- Enzymatic activity = $\frac{\text{slope}^{-1} \times \text{absorbance}}{\text{Vol. of enzyme} \times \text{incubation} \times 12}$

- Amount of ammonia (60%) = $\frac{533\ (S_2 - S_1)}{100 - 0.3 \times S_2}$
  - Online calculator also available at ENCOR Biotechnology website

http://www.encorbio.com/protocols/AM-SO4.htm

- Fold Purification = $\frac{\text{specific activity of purified}}{\text{specific activy of crude}}$

- Relative activity = $\frac{\text{sample activity}}{\text{Blank activity}}$

RESUME

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- Chhama Pandey, **Nitish Vikram Shahi** and BH Pawar. Synergistic effect of chitosan and *Trichodermaviride* against *C.paradoxa*, the causal agent of pineapple disease in sugarcane. *The Journal of Rural and Agricultural Research, Vol. 14 No. 2, 70-74 (2014)*

ACADEMIC PROJECTS

- Production of Biodiesel from *jetropha curcas - Jul10*
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CERTIFICATIONS

- Production of Biodiesel from *jetropha curcas - Jul10*
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- IMMUNOTECHNOLOGY
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DECLARATION

I hereby declare that the above information is true to the best of my knowledge.

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