FORMULATION OF BIOBASED CONTACT LENS SOLUTION

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CERTIFICATE

This is to certify that project report entitled "**Formulation of biobased contact lens solution**.", submitted by Radhika Sharma in partial fulfillment for the award of Degree of Master of Technology in Biotechnology to Jaypee University of Information Technology, Waknaghat, Solan has been carried out under my supervision.

This work has not been submitted partially or fully to any other University or Institute for the award of this or any other degree or diploma.

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SUMMARY

Lipases occur widely in nature, but only microbial lipases are commercially significant. In this study, Lipase produced from bacterial source *Staphylococcus arlettae* through submergerd fermentation. Determined lipase activity of bacterial lipase by spectrophotometric method by using p-nitrophenol palmitate as a substrate and measured protein content in bacterial lipase by lowry methods. 60% ammonium sulphate preciepitation was used to purification of lipase. Determined specific activity of crude and purified lipase. Fold purification was increased after the purification of lipase. Protease assay performed to determine the protein activity in lipase enzyme. Compatibility of purified lipase with surfactant and oxidizing agent present in commercial contact lens solution was checked by lipase assay and relative activity of each contact lens solution was determined on the basis of their compatibility test. Biotrue multipurpose contact lens solution (Bausch and lomb) is most compatible with *S.arlettae* lipase than renu multipurpose solution and purecon on the basis of lipase assay. Prepared different type of contact lens solution and evaluate their effectiveness on the basis of transmittance of contact lens. Visibility of the lens is determine in terms of transmittance. On the basis of their transmittance, it was easily determined removal of protein from coated lens was increased with time when lenses were treated with different type of contact lens solution and lipase based contact lens solution have capability to remove the deposits from contact lens.

(Radhika Sharma)	(Dr. Garlapati Vijay Kumar)
Date	Date

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LIST OF SYMBOLS AND ABBERIVIATION

°C	Degree Celsius
%	Percentage
μM	Micromolar
EDTA	Ethylenediaminetetraacetic acid
h	Hours
KDa	Kilo Dalton
Km	Michaelis Menton Constant
L	Liter
LB	Luria Bertani
mg	Milligram
mM	Mill molar
MTCC	Microbial Type Culture Collection
O.D	Optical Density
p- NPP	p-Nitrophenyl Palmitate
RPM	Revolution Per Minute
Smf	Submerged Fermentation
SSF	Solid State Fermentation
U/ml	Unit Per milliliter
Vmax	Maximum Reaction Rate

CHAPTER 1
INTRODUCTION

1.1 Enzymes

Enzymes are biological catalysts. They increase rate of chemical reaction taking place with in living cells without themselves suffering any overall changes. They reactants of enzyme catlysed reactions are termed substrates and each enzyme is quite specific in character , acting on a particular substrate or substrate to produce a particular product or products. Microbial enzymes are commercially important. They have wide application in various industry. The history of modern enzyme technology really started in the 19th century, while studying the fermentation of sugar to alcohol by yeast. Louis Pasteur came to the conclusion that this fermentation was catalyzed by a vital force contained within the yeast cells called "ferments", which were thought to function only within living organisms (Manchester et al., 1995). In 1877, German physiologist Wilhelm Kuhne was first used the term "*enzyme*", which comes from Greek *ενζυμον* "in leaven", this process. An enzyme's name is often derived from its substrate or the chemical reaction it catalyzes, with the word ending in *–ase*. A large number of industrial processes in the areas of pharmaceutical, environmental and food biotechnology utilize enzymes at some stage or the other.

1.2 Lipases.

Lipases (triacylglycerol acylhydrolases) are industrially significant enzymes and are produced by a variety of microorganisms. Enzyme catalyzes the hydrolysis of triglycerides to fatty acid and glycerol and under certain condition, catalyses the several reaction forming glycerides from glycerols and fatty acids. Lipases can be classified in several groups according to their specificity as non-specific lipases, 1, 3-specific lipases, 2-specific lipases and fatty acyl lipases (Sugihara et al., 1994). Lipases occur widely in nature but only microbial lipases are commercially significant because of their shorter generation time, ease of bulk production, which is further enhanced with advancement in fermentation technologies and ease of manipulation, either genetically or environmentally. Lipase producing microorganisms have been found in diverse habitats such as industrial wastes, vegetable oil processing factories, dairies, soil contaminated with oil etc. Each lipase has a number of unique characteristics such as substrate specificity, region-specificity and chiral selectivity and some enzymes are important for industrial production of free fatty acids, synthesis of

useful esters and peptides. This proves that microbial lipase has vast applications in food industry.

1.3 Lipase producing microorganisms

Lipases occur widely in nature, but only microbial lipases are commercially significant. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the great variety of catalytic activities available, the high yields possible, ease of genetic manipulation, regular supply due to absence of seasonal fluctuations and rapid growth of micro organisms or inexpensive media (Saxena et al., 1991). Microbial lipases are also more stable than their corresponding plant and animal enzymes and their production is more convenient, safer and can be obtained in bulk at low cost. Lipase-producing microorganisms belong to bacteria, fungi, yeasts and actinomyces. The lipases found among these microbial sources are quite diverse and typically vary from one another in physical, chemical and biological properties. Such factors as pH range, tolerance of emulsification and surfactants, temperature tolerance, storage capability and the like are important considerations in the selection and development of a commercially useful producer. Different microorganisms have been used for the production of lipases such as *Rhizopus species, Rhizomucor mehei, Aspergillus niger*, *Penicillium species*, *Bacillus subtilus*. Fungi have also potential to produce lipase in bulk amount.*Achromobacter* sp., *Alcaligenes* sp., *Arthrobacter* sp., *Pseudomonas* sp., *Staphylococcus* sp., and *Chromobacterium* sp. have been exploited for the production of lipases. Fungi as potential candidates that secrete enzyme lipases*, Absidia corymbifera, Aspergillus fumigatus, Aspergillus japonicus, Aspergillus nidulans, Aspergillus terrus, Cunninghamella verticillata, Curvularia pallesoens, fussarium oxysporum, Geotricum candidum , Mucor racemosus, Penicillium citrinum., Penicillium frequentans, Rhizopus stolonifer* and *Trichoderma vinide.* Preliminary screening studies for lipase production by fungi were carried out on agar plates using olive oil or emulsified tributylin by gum Arabic . screened fungi with bromophenol blue dye supplemented agar plates with olive oil as the substrate.(Fadiloglu et al., 1998)

1.4 Microbial lipase production through Submerged fermentation

Microbial lipases are produced mostly by submerged fermentation but solid state fermentation methods can be used also. The Solid State Fermentation (SSF) is an interesting alternative for microbial enzyme production due to the possibility of using residues and by-products of agro-industries as nutrient sources and support for microorganism development. The use of by-products as substrates for lipase production, adds high value and low-cost substrates may reduce the final cost of the enzyme (Rodriguez *et al*., 2006). Optimal culture and nutritional requirement for lipase production in submerged fermentation However, production of lipase through submerged fermentation needs, large space, complex media and also needs complex machinery, equipment and control systems. Moreover, submerged fermentation for production of lipase at large scale demands high energy demand, higher capital and recurring expenditure. Generally, lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature and dissolved oxygen concentration. Lipase production is influenced by the type and concentration of carbon and nitrogen sources, the culture pH, the growth temperature, and the dissolved oxygen concentration. Lipidic carbon sources seem to be generally essential for obtaining a high lipase yield. Production of lipase depend on the fermentation condition like temp., pH, and media. Lipase activity is decreased because of limitation of the nitrogen. Production of extracellular and intracellular lipase enzyme depends on source of the medium. and continuous culture of microbes in carbon sources mixtures which is increased the production of lipases in continuous process.The major factor for the expression of lipase activity has always been carbon, since lipases are by and large inducible and are thus generally produced in the presence of a lipid source such as an oil or any other inducer, such as triacylglycerols, fatty acids, hydrolyzable esters, tweens, bile salts and glycerol. However, their production is significantly influenced by other carbon sources, such as sugars, sugar alcohol, polysaccharides, whey, casamino acids and other complex sources . Certain long-chain fatty acids, such as oleic, linoleic and linolenic acids, are known to support lipase production from various bacteria, such *as Pesudomonas mephitica* . However, lipases from *P. aeruginosa* EF2 (Gilbert et al., 1991) . Besides carbon source, the type of nitrogen source in the medium also influences the lipase titers in production broth (Ghosh et al., 1996). Generally, organic nitrogen is preferred, such as peptone and yeast extract, which have been used as nitrogen source for lipase production by various Bacillus spp (viz. Bacillus strain A30-1, *B. alcalophilus*, B. licheniformis strain H1) and various pseudomonads (viz.

Pseudomonas sp., P. fragi, P. fluorescens BW 96CC), Staphylococcus haemolyticus while tryptone and yeast extract have been used in the case of S. haemolyticus L62 (Oh et al. 1999). Inorganic nitrogen sources such as ammonium chloride and diammonium hydrogen phosphate have also been reported to be effective in some microbes(Rathi et al., 2001). Divalent cations stimulate or inhibit enzyme production in microorganisms.) observed stimulation in lipase production from Burkholderia sp. in the presence of Ca2+ and Mg2+ (Rathi et al.,2001; Sharma et al.,2001) also reported stimulation in lipase production from *Bacillus sp.* RSJ1 in the presence of calcium chloride. However, most other metal ion salts were inhibitory to lipase production. Iron was found to play a critical role in the production of lipase by *Pseudomonas sp*. G6 (Kanwar et al. 2000,Shabita et al., 1991)). In addition to the various chemical constituents of a production medium, physiological parameters such as pH, temperature, agitation, aeration and incubation period also play an important role in influencing production by different microorganisms. The initial pH of the growth medium is important for lipase production. Largely, bacteria prefer pH around 7.0 for best growth and lipase production, such as in the case of Bacillus sp. , The optimum temperature for lipase production corresponds with the growth temperature of the respective microorganism. For example, the best temperature for growth and lipase production in the case of Bacillus sp. RSJ1 was 50°C (Sharma et al. 2001). It has been observed that, in general, lipases are produced in the temperature range 20–45°C. Incubation periods ranging from few hours to several days have been found to be best suited for maximum lipase production by bacteria. An incubation period of 12 h was optimum for lipase production by A. calcoaceticus and Bacillus sp. RSJ1 (Sharma et al. 2001) While maximum lipase was produced after 72 h and 96 h of incubation, respectively, in the case of the Pseudomonas sppP. fragi and P. fluorescens BW 96CC .Thus, bacterial lipases are generally produced in the presence of oil or any other lipidic substrate (viz. fatty acid esters, fatty acids, glycerol) as carbon in the presence of any complex nitrogen source. The requirement for metal ions varies with the organism. However, physical para meters such as pH, temperature, agitation and aeration influence lipase production via modulating the growth of the bacterium. Lipases are produced throughout bacterial growth, with peak production being obtained by the late log phase. The production period for lipases varies from a few hours to a few days.

1.5 Lipase assay methods

There are numerous methods available for lipase activity estimation and they have been well reviewed in the literature (Beisson et al., 2000). Most of these methods are designed to estimate the products of hydrolytic reactions. Several assays for lipase activity estimation are based on spectroscopic measurements. (Rollof et al.,1987) developed an assay, which involved direct turbidometric estimation of residual lipids, after reaction of lipase with lipid emulsion. A turbidometric assay was developed) for estimation of lipase activity in serum(Robinson et al.,1989). Triacylglycerols are natural substrates of lipases and many spectrophotometric methods use them as substrates .A few spectrophotometric assays are based on methods, which render colour to fatty acids released after hydrolysis of triacylglycerols used rhodamine 6G for complexation with free fatty acids liberated during lipolysis. A pink colour appeared and the absorbance was monitored at 513 nm. The lauric acid released was converted to copper laureate and measured spectrophotometrically at 435 nm. A method developed using immobilized triacylglycerols (Safrik et al, 1991). In this method, the fatty acids released after hydrolysis were extracted with benzene and converted to their corresponding Cu (II) salts, which were measured spectrophotometrically. Another method used in used metachromatic properties of the cationic dye, safranine, to detect a change in the net negative charge at the lipid: water interface, which was monitored by the change in of. safranine. This assay is very sensitive and very low quantities of lipolytic enzyme can be detected using this method. There are enzymatic assays based on estimation of either glycerol or fatty acids released after action of lipase on triacylglycerols. A kinetic colorimetric method for assaying lipase activity in serum by using a natural long-chain fatty acid 1,2-diglyceride. . A kinetic colorimetric method for assaying lipase activity in serum by using a natural long-chain fatty acid 1,2-diglyceride (Fossati et al,1992) In the pres In the presence of co-lipase, deoxycholate and calcium ions, pancreatic lipase hydrolyzed the clear substrate solution to produce a 2-monoglyceride, which in turn, released glycerol by the action of a 2-monoglyceride lipase. Glycerol was then assayed by a sequence of enzymic reactions (glycerol kinase, glycerol phosphate oxidase, and peroxidase) that produced a violet quinone monoimine dye with peak absorption at 550 nm. An enzymatic method described for the determination of the amount of free fatty acids released from triglyceride by lipoprotein lipase (Woollett et al.,1984). The quantity of free fatty acids present in the medium before and after incubation is measured spectrophotometrically by the oxidation of NADH in the final reaction of a series of coupled enzymic reactions.

1.6 Purification and characterization of lipase

Purification of enzymes are necessary for biotechnology purpose like biocatalytic reaction and food, dairy and detergent industry. The purification of lipase normally involves several steps depending on the purity desired In case of extracellular lipases, normally the first step is the removal of cells by centrifugation or filtration. In case of intracellular lipases, an additional step of cell lysis is required. The crude lipase preparation can then be concentrated by ultrafiltration or can be subjected to optional solvent or salt precipitation. 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 enzymes suffer from dilution problem to concentrate the desired extracellular protein precipitation can be attempted .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 (Sharma et al., 2001).. 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 et al., 1994) although with limited purification, and such enzyme preparations are apt for use in contact lens solution formulations. Purification methods consist of ultrafiltration, followed by ammonium sulphate precipitation. The lipase was partially purified through ammonium sulphate resulting in 17.8 fold purification fold and 41.9 U/ml of 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 purification was performed using sequential protocol of precipitation with ammonium sulphate followed by dialysis, DEAE- sepharose anion exchange chromatography and sepharose-6B gel filtration chromatography with 375 purification fold and have 29.35 % final yield. The molecular weight of the protein was 45 KDa. The purified enzyme was further studied for hydrolytic activity which come optimum at temperature 45 ^{o}C and pH 6.5, values of Km and Vmax calculated from the Lineweaver–Burk plot using p-nitrophenyl palmitate (p-NPP) as hydrolysis substrate were 0.02 mM and 38.9 mol min$-^{1}$ mg$-^{1,}$ respectively. Affinity matrices with fatty acids as ligands have been used for purification of microbial lipases. Staphylococcus epidermidis lipase has been

purified using metal-affinity chromatography (Simons et al., 1998). A one-step purification of cloned Bacillus licheniformis lipase (Nthangeni et al.,2001). The lipase was purified using Ni2+- Nitriloacetic acid affinity chromatography, facilitated by 6 histidine residue tag introduced in the C-terminal region of the cloned enzyme. Lipases have also been purified by aqueous-two-phase extraction systems.

1.7 Industrial applications of lipases

Microbial lipases constitute an important group of biotechnologically valuable enzymes, mainly because of the versatility of their applied properties and ease of mass production. Microbial lipases are highly diversified in their enzymatic properties and substrate specificity which make them very attractive for industrial applications. Table 1.1 depicts industrial application of microbial lipase. One the unique characteristics of lipases is that they remain active in organic solvents in field of industrial application.

Table 1.1 Important areas of industrial application of microbial lipase.

Industry	Effect	Product
Bakery	Flavour improvement and shelf life prolongation	Bakery products
Beverages	Improved aroma	Beverages
Chemical	Enantioselectivity	Chiral building blocls
Cleaning	Synthesis hydrolysis	Chemicals, Removal of cleaning agent like surfactant
Cosmetics	Synthesis	Emulsifiers, miosturising
Diary	Hydrolysis of milk ,fat, cheese ripening	Flavour agents
Fats and oils	Transestrification Hydolysis	Cocoa butter, margarine Fatty acids, glycerol, mono and diglycerides
Food dressing	Quality improvement	Mayonnaise, dressing and whippings
Health food	Transestrification	Health food
Leather	Hydrolysis	Leather products
Meat and fish	Flavour development and fat removal	Meat and fish products
Paper	Hydrolysis	Paper products
Pharmaceuticals	Transestrification Hydrolysis	Specially lipids Digestive aids

Lipases are widely used in the processing of fats and oils, detergents and degreasing formulations, food processing, the synthesis of fine chemicals and pharmaceuticals, paper manufacture and production of cosmetics.(Jaeger et al 2002)

1.8 Microbial enzyme based contact lens solution

In normal course of wearing contact lenses, tear films and proteinaceous molecules have a tendency to deposit on lens surface which affects the optical clarity of the lenses. This surface deposit also increases the possibility of pathogenic bacterial adhesion such as *Psedomonas aeroginosa*. Mainly, contact lens cleaning solutions are prepared by using plant (papain) or animal (trypsin and chymotrypsin) drived enzyme. In most of the cases these gives a unpleasant odour after few hours of use cause irritation and turns allergic, while microbial enzyme do not produce any of these drawbacks and are gaining importance. Microbial enzymes are often more useful than enzymes derived from plants or animals because of the abundant variety in catalytic activities, their high yield, ease of genetic manipulation, independent of seasonal fluctuations which gives regular supplies and rapid growth on inexpensive media. These enzymes are also more stable than the plant and animal enzymes and their production is more convenient and safer.(Ogunbiyi et al., 1985)

CHAPTER 2
REVIEW OF LITERATURE

2.1 Formulation considerations of contact lens products

Although contact lens products may not contain therapeutic agents, various factors must be considered during their formulation. These factors include pH, viscosity, isotonicity, stability, sterility and preservation. Eye tissues can tolerate formulations having a range of pH from 6.6 to 7.8 without any discomfort; a solution that has a pH value beyond this range may lead to tearing, burning and stringy mucus formation .The pH values of contact lens solutions vary from 4.2 to 8.6. Generally, highly buffered solutions are avoided as they may cause irritation or possible ocular damage. In order to maintain normal corneal thickness and functional integrity, contact lens solutions should be made isotonic with tears.. When a hydrogel lens is soaked in hypor hypertonic solutions, then placed on the eye, it adheres to the cornea. The strength of such adhesion is dependent on the difference in tonicity between the contact lens and cornea. Ideally, contact lens solutions should be adjusted to a tonicity of 0.9 percent to 1 percent equivalent of NaCl; but actual tonicity values of contact lens solutions may vary from as low as 0.14 percent to as high as 1.4 percent. A major concern with contact lens solutions is their potential for bacterial contamination, so lens products must contain a preservative that is effective against common contaminants, in addition to maintaining efficacy over the shelf life, being nonirritating and nontoxic to the ocular tissues following their daily use. The commonly used preservatives, such as benzalkonium chloride (BAK), chlorobutanol and thimerosal, may trigger toxic or allergic reactions, depending upon the concentration. Polyquad, a quaternary ammonium compound, has been shown to be less sensitizing than BAK or thimerosal.(Zhu et al., 2007)The preservatives used in contact lens products. Symptoms of reactions to preservatives include stinging, burning and red eyes. The routine care for soft lenses is different from that for hard lenses.

2.2 Contact lens solution for soft contact lenses

Care, cleaning and disinfection of soft lenses poses some unique problems. Since hydrophilic soft lenses absorb water and water soluble substances, any chemicals in the solutions used in routine care may also be absorbed into the lens; if any of the absorbed substances prove toxic, ocular damage may result when the lenses are worn and the substance is released onto the

cornea .The water content of the lens may also be a factor in the care of the lens, lenses with water content of 60 percent or higher being more susceptible to deterioration from heating(Fowler et al., 1985). These lenses may also absorb or adsorb more foreign material, such as preservatives or enzymes, when treated with lens care solutions (Zhuh et al., 2007) . Foreign substances as diverse as oils, creams and cosmetics can contaminate lenses; hydrophilic soft lenses have a greater tendency to accumulate surface deposits than hydrophobic lenses(Botempo et al., 1994, Castillo et al., 1986). Such deposits generally consist of proteins and lipids, which can also act as a growth medium for microbial organisms (Rasika et al., 2006; Maiscca et al 2000); daily cleaning is necessary for removal. Buildup of these deposits can create an irregular surface on the lens, may irritate the lids and corneal epithelium, and may even lead to infection (Hart et al., 1986). Decreased visual acuity and lens wear time are likely consequences of a cloudy lens or allergic reactions. Soft contact lenses may be cleaned using a surfactant cleaner or an enzymatic cleaner(Stanley et al., 1987). Cleaning with a surfactant cleaner must be done daily, or even each time the lenses are removed in the case of extended wear lenses; enzymatic cleaning should be performed weekly or as recommended by the manufacturer of the lens.

Enzymatic cleaners contain a proteolytic enzyme (papain, pancreatin or subtilisin) that helps in removing tenacious protein debris, which may not be removed with the surface active cleaners. These enzymes hydrolyze polypeptide bonds of protein and dissolve the protein deposits. As a result, they increase wearing time and comfort. The enzyme cleaners are provided in tablet form and are dissolved in preserved saline or sterile nonpreserved saline. The soiled lens is soaked in the solution for a period of time, depending upon the type of lens. It is usually sufficient to use enzyme cleaning as a weekly supplement to daily cleaning with surfactant cleaners. In order to reduce the cleaning steps and to increase patient compliance, combination products are designed which typically contain an enzymatic cleaner and a disinfecting agent. Both methods are reliable for most ocular pathogens, but the latter method has become more popular with the introduction of hydrogen peroxide as a chemical disinfectant (U.S. patent, US4670178A). The main advantage of disinfecting lenses by thermal means is the avoidance of any preservatives to cause toxic or allergic reactions. Thermal disinfecting involves placing the cleaned lenses into separate compartments of a storage case containing saline. The saline used contains either no preservatives or only a sufficient preservative to prevent contamination of the solution while in the bottle. The case is then placed in a heating unit, and the temperature is increased to a specific level for a

prescribed time, it may weaken the lens and shorten its useful life. Solutions containing newer disinfecting preservatives such as sorbic acid, Polyquad and Dymed tend to be less toxic or allergenic (Stanley et al., 1987) but may also be less effective against fungi and protozoans. Solutions containing hydrogen peroxide as the antimicrobial agent have received wider acceptance, as it is a highly reactive oxidizing agent. The disinfecting mechanism is thought to entail reaction of "free radicals" with the bacterial cell wall. The effectiveness of hydrogen peroxide is dependent upon its concentration and time of exposure to the organism. Typically, hydrogen peroxide systems require about 20 minutes to 30 minutes to complete; then the peroxide is neutralized by different types of neutralizing agents. When neutralization is incomplete, trace amounts of hydrogen peroxide remaining on the lens may cause pain, photophobia, redness and even corneal epithelial damage. Saline solutions. Saline solution can be an effective medium for microbial growth and may serve as a potential source for infections of the eye. The hydrophilic soft contactlens must be maintained in a constant state of hydration; normal saline, either preserved or preservativefree, is the basic solution used for rinsing, thermally disinfecting and storing soft contact lenses. Saline preserved with sorbic acid Polyquad and Dymed are also available for patients who are sensitive to other preservatives. Preservativefree buffered saline is available in unitofuse containers and in several sizes of multiuse bottles, and nonpreserved saline is also available as an aerosol spray. Some nonpreserved saline products contain ethylenediamine tetraacetate. EDTA is a chelating agent and prevents deposits of calcium and other divalent ions on the surface of the lens and inhibits growth of certain bacteria, thus extending the shelf life of the product(Puker et al., 2010)

2.3 Contact lens solution for hard contact lenses

Hard lens care involves three steps: cleaning, soaking and wetting. For optimal lens care, all three steps should be performed each time the lenses are removed from the eye. The appropriate use of lens care products helps the wearer to avoid potential problems by offering a degree of safety as well as providing comfort. Proteins, mucus and lipids from the tear film, as well as other foreign material such as eye makeup can contaminate the lens surface (Berry et al., 2003, Rathi et al, 2002). The hydrophobic substances found in normal tears may adhere to the surface of a hard lens during normal daily wear and can harden to form tenacious deposits that can produce an irregular lens surface if not properly removed. Typical cleaning solutions contain surfactants that lower surface tension, emulsify oils and aid in solubilizing

other debris (U,S,patent, 4670178A).Proteins and lipids are soluble in highly alkaline media, but a high pH may cause lens decomposition(Castillo et al, 1986;Brennan et al.,2000). Consequently, weak alkaline solutions are useful in dislodging deposits from the lens when combined with the surfactants. Mild abrasive compounds may also be included in the formulation.

2.4 Microbial enzyme based contact lens solution

In normal course of wearing contact lenses, tear films and proteinaceous molecules have a tendency to deposit on lens surface which affects the optical clarity of the lenses. This surface deposit also increases the possibility of pathogenic bacterial adhesion such as *Pseudomonas aeroginosa*(Lorentz et al., 2010, Keith et al., 2001). Mainly, contact lens cleaning solutions are prepared by using plant (papain) or animal (trypsin and chymotrypsin) protease. In most of the cases these gives a unpleasant odour after few hours of use cause irritation and turns allergic, while bacterial protease do not produce any of these drawbacks and are gaining importance . Hence, the present study focused on the production of protease from bacteria and their application in the contact lens cleaner by replacing PBS respectively(Rasika et al., 2009). Protease can be used over chemical cleansing solution with a little more incubation time. Even the enzymatic solution was observed to be odourless (Rasika et al., 2009). Since these enzymes are obtained from bacterial source it is easier to produce in large scale and in short period of time as compared to animal or plant sources. Enzyme could be effectively used to remove protein deposits from contact lens indicating its potential to increase in transmittance of lenses (Sayali et al., 2013).The present studies shows proteinaceous tear films and debris are removed from contact lenses with aqueous solutions of bacterial proteolytic and carbolytic enzymes, principally protease and amylase with or without lipase. The solutions are substantially odor-free, non-allergenic, require no activator/stabilizer and are completely water soluble (European patent, EP014066941).An enzymatic contact lens cleaner containing an effective, non-toxic amount of a protease derived from a *Bacillus, Streptomyces or Aspergillus* microorganism, such that when dissolved in an aqueous solution will effectively remove at least protein and carbohydrate films and debris from contact lens surfaces. The enzyme cleaners may contain protease alone derived from the above genera of bacteria or mold. The enzyme(s) will preferably be comprised of a mixture predominantly of protease and amylase, and optionally, a minor amount of lipase. The bacterial protease enzyme can be used along with chemical disinfectants to clean the contact lenses .The

microbial protease-containing lens cleaning solutions are especially effective in digesting and removing denatured protein and carbohydrate films and debris from contact lenses without enzyme activators, and therefore, present fewer manufacturing and packaging problems in formulating the various cleaning preparations contemplated herein. The enzymatic contact lens cleaners are especially effective in removing contact lens film and debris in one hour or less by high temperature cleaning methods. In addition, the bacterial enzyme cleaners may perform with little or no residual binding or concentrating onto lens surfaces, and therefore, eye tissue sensitivity normally manifested as stinging and inflammation are virtually eliminated. Enzyme cleaners are generally viewed as being efficacious, safe and capable of removing the principal component of contact lens film and debris, namely protein. Some also have the ability to remove carbohydrate and lipid deposits from contact lenses. Heretofore, the supply of proteolytic, carbolytic and lipolytic enzymes e.g.....proteases, amylases and lipases for use in contact lens cleaning solutions was restricted to plant and animal sources..Contact lens cleaning solutions prepared with plant and animal derived proteases like papain, chymopapain, pancreatin, trypsin, chymotrypsin, pepsin, ficin, carboxypeptidase, aminopeptidase, and bromelin are described in several patent publications e.g..U.S. Patent 3,910,296. In addition to the patent citations, enzymatic lens cleaners prepared with proteases from pork, namely.pancreatin have been commercially available from Alcon Laboratories. Enzymatic contact lens cleaners prepared with plant proteases i.e. papain have also been available from Allergan Pharmaceuticals under the registered trademark Soflens Enzymatic Cleaning Tablets. Although these preparations are generally effective in cleaning contact lenses, In some cases, plant and animal proteases and amylases will discolor lenses. they have shortcomings problems That is, besides the propensity for unpleasant odors and potential for discoloring lenses, cleaners containing proteases like pancreatin from pork or beef can induce an allergic response among some users. In addition, solutions containing pancreatin have a tendency to become cloudy and turbid.Plant proteases for example papain, normally require lengthy cleaning cycles ranging from 4 to 12 hours in order to remove film and debris from lenses. Such lengthy cycles can be an inconvenience to the user. In addition, cleaning solutions prepared with plant and animal proteases require the application of heat e.g. 80°C which is needed not only to disinfect the lenses, but also to inactivate the enzyme. Contact lens cleaners containing enzymes also require stabilizers/activators. For example, papain requires cysteine. Pancreatin requires calcium salts. Without the use of an activator papain and other similar plant enzymes will remain dormant. Activators like cysteine are hygroscopic and have a tendency to pick-up moisture creating manufacturing difficulties.

Such enzyme products can only be manufactured and packaged under stringent standards to eliminate any moisture from entering the packaging otherwise it will autoreact and shorten the shelf life of the cleaner.. The tablets and solutions of this patent employ a neutral protease referred to as a metallo-enzyme having an optimum activity at a pH of 6 to 8. Because metals are an integral part of the enzyme, its activity is inhibited by the presence of chelating agents which are customarily employed in contact lens cleaning preparations to bind calcium and other unwanted metals from reacting with proteins and depositing on lenses. Consequently, enzymes which are inhibited by chelating agents are generally unsatisfactory for use with contact lenses. An enzyme containing cleaning liquid for soft contact lenses consists of a solution containing a lipolytic enzyme (mainly for reducing the lipids) and, optionally, a proteolytic enzyme, such as papain or bromelain, (for reducing the Albumin deposits) and, additionally, a buffering agent, such as a phosphate. Such cleaning liquid is preferably hypertonic to its nature, i.e. its osmotic pressure exceeds that of a physiological solution, so that in treatment with the solution some dewatering of the lens takes place, which seems to be beneficial to the cleaning effect. During after-treatment with an isotonic solution, for example a saline solution, the lens reversibly again takes up water to revert to its original state.A method of cleaning a soft contact lens in accordance with the invention to remove deposits on the surface of the lens by enzymatic action comprises the steps of placing at least one drop of a solution containing Papain or Bromelain and in addition a Lipolytic Enzyme, on the contact lens which is to be cleaned to reduce both Albumin and Lipids present to water soluble peptones, fatty acids and esters, and subsequently removing the resulting products by rinsing and boiling in a physiological saline solution. Preferably the enzyme activity in the cleaning solution is of the order of 100 tyrosine units per ug of protein.When a proteolytic enzyme and a lipolytic enzyme are used in combination it is preferred in order to avoid undue interaction between the enzymes to include in the solution a so-called "aqueous polymer complex", which is conventional in the art and have for a purpose to bind the lipolytic enzymes so that it will not be unduly destroyed by the proteolytic enzyme. The polymers used in the manufacturing of soft contact lenses at the present time, PMMA, HEMA and PvP all have a common factor, that is, they are lipid and protein retensive. New materials have been introduced such as silicone, even in this material there is lipid retention. Chemically the deposits are composed of phospholipids, probably in the form of lecitin, forming together with the protein a lecitoprotein, (lecitin on exposure to heat and light tends to autocxidise or decompose into yellowish substances) or cholesterol and fat esters which are white in colour.Plaques or what one might call lesions also appear on the lenses after a period of time.

Typically the plaque consists of a central core of lipid lying free on the polymer and protruding into the material matric causing a sand grain sensation when the lens lies in the eye.Unfortunately, we have only theories to explain how fatty substances in the tear fluid are transformed into obstructive plaques. However, these plaques start from the same observation - an excess of lipids - and in particular cholesterol and lecithin.Based on these observations it is therefore quite apparent that a method for cleaning soft contact lenses presently and in the future must be one that can remove the lipid and protein deposits formed in the soft contact lens material during wearing. Due to the fact that new materials are being investigated it is necessary that the cleaning method must be compatible with these materials. An enzymatic method whereby a lipase is used is without doubt the most gentle method and probably the most efficient for removing fatty deposits from soft contact lenses.It is also evident that the greater the water content of the polymer the greater the binding of protein and lipids, this binding tends to be normally a surface adsorption but in those polymers that are combined with copolymers of certain types there is a possibility that a covalent binding can occur.(Zhu et al, 2007) This type of binding is naturally more difficult to separate than an ordinary surface adsorption. It is, however, possible with the use of lipase in combination with a tenside; the tenside increases the water/oil interphase and allows the enzyme to react upon the lipids.A preferred variety is lipase derived from cand. cylindracae, suitably prepared by lyophilization. As a proteolytic enzyme any protein-digesting enzyme is useful, preferred examples being bromelain and papain. When using in combination both a lipolytic enzyme and a proteolytic enzyme, the latter being papain, it will be noted that the beneficial effect of free sulfhydryl groups on the activity of papain will be satisfied by the presence of the lipase containing sulfhydryl groups. Thus, such combination of enzymes is particularly preferred, especially when used in solutions of a hypertonic character.(Ogunbiyi et al, 1985)

Objectives

The aim of the present research is intended to replace PBS with Lipase and protease from contact lens solution formulations. Hence, the objectives of the present work has been framed as follows:

- Production of lipase by *Staphylococcus arlettae* JPBW-1 through SmF.
- Partial Purification of *Staphylococcus arlettae* lipase.
- Compatability of lipase with contact lens formulation adjuncts
- Formulation of Bio-based contact lens solutions and its evaluation

CHAPTER 3
MATERIALS AND METHODS

3.1 Materials

3.1.1 Microorganism

Staphylococcus arlettae JPBW-1 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 .

Systemic position

Kingdom : Bacteria

Phylum : Firmicutes

Class: Bacilli

Order: Bacillales

Family: Staphylococcaceae

Species: ***S. arlettae***

3.1.2 Raw material

Luria Bertani media were utilized for lipase production from *S. arlettae*.

3.1.3 Chemicals

All chemicals used were of AR grade from Merck, SRL, Sdfine, Qaligens and Himedia Indis. All standards were prepared from Sigma Co., USA. The chemicals include:

Ammonium sulphate, boric acid, bovine serum albumin (BSA),calcium carbonate, calcium chloride, casein copper sulphate, ,ethanol, ethyldiamine tetraacetic acid, Folin- ciocalteu reagent, gelatine, glycine, gum acia, hydrochloric acid, Luria bertani agar, p- nitrophenol palmitate, potassium chloride, potassium sodium tartarate, propanol, proyelene glycol sodium carbonate, sodium hydroxide, tetraacetic acid, terasodium phosphate, tris buffer, triton –X.

Biotrue multipurpose contact lens solution, renu multipurpose contact lens solution and purecon contact lens solution obtained from local market.

3.1.4 Instruments and apparatus

The instruments used in the present research work are listed below:

Autoclave : Used for sterilization of media and glassware.

Centifuge: For separation of insoluble protein content.

Digital balance: For weighing chemicals in mg and g level.

Distilled water plant (Ion Exchang, India Ltd).:For distilling the water used for media and reagent preparation.

Humidity controlled chamber (Remi, India) : To maintain desired humidity during lab fermentation.

Incubator(Sambros , India) : For growth of microorganism at constant temperature.

Laminar air flow cabinet: For inculation under sterile condition.

pH meter (Elico, India): Used for pH measurement.

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

Shaker (Instrumentation India)**:**Employed for imparting shaking condition to the reaction mixture.

Spectrophotometer (Shimadzu double beam UV-Vis(260)),Japan: For routine optical measurements.

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

Weighing balance (Asiatic Machinery Corpn(P) Ltd): Used for weighing chemicals and substrates.

3.2 Methods

3.2.1 Media

3.2.1.1 Luria Bertani Agar medium

LB agar 2% (w/v).Required weight of LB agar was mixed thoroughly in distilled water, heated with frequent agitation and boiled for 1 min to completely dissolve the powder. It was then autoclaved at 121 $^{\circ}$C for 15 min, cooled it and 5-7 ml of it per test tube was poured subsequently to make slant.

3.2.1.2 Medium for lipase production from S.arlettae

Luria Bertani broth (LB broth) meadia was used for lipase production. The medium composed following components.

Casein enzymic hydrolase	10 g/L
Yeast extract	5 g/L
NaCl	5g/L

3.2.2 Buffer: All solution were made in distilled water

3.2.2.1 0.1 M Tris- HCL buffer (pH 8)

a) Tris 6.05 g/l

b) 1M HCL 36.46 ml/l

The solution (a) was adjusted with solution (b) to pH 8.

3.2.2.2 1M Potassium Phosphate Buffer (pH 7.5)

a)KH_2PO_4 6.8 g/L

b) K_2HPO_4 11.4 g/L

The solution (b) was adjusted with solution (a) to pH 8.0

3.2.2.3 0.2 M Glycine NaOH buffer(pH 8.0)

a) Glycine (0.2 M) 15 g/L

b) NaOH(0.2 M) 40 g/L

The solution (a) was adjusted with solution (b) to pH 8.0

3.2.3 Reagents: All solution were made in distilled water.

3.2.3.1 For lipase assay

a) **Solution A:** 40 mg pNPP in 12ml propanol.

b) **Solution B:** 0.1M Tris-HCL buffer (pH 8), 40µl Triton X-100 and 0.1 g gum acia.

3.2.3.2 For protein estimation (Lowry et al., 1991)

a) **Solution A:** 2% sodium carbonate in 0.1N NaOH
b) **Solution** B: 5%$CuSo_4$ in 1% sodium potassium tartrate
c) **Solution** C: Mix 50ml of solution A with 1ml of solution B
d) **Follins- Ciocalteu reagent**: Dilute the commercial reagent (2N) with an equal volume of distilled water.
e) **Stock standard solution**- 5 mg of Bovine serum albumin(BSA)/5ml of water

3.2.3.3 For protease assay

a) **Solution A**: 2% Casein solution in Phosphate Buffer
b) **Solution B**: Glycine NaOH Buffer(pH 8.0)
c) **Solution C**: 5% TCA solution
d) **Follins- Ciocalteu reagent**: Dilute the commercial reagent (2N) with an equal volume of distilled water.
e) **Stock standard solution**- 5 mg of Bovine serum albumin(BSA)/5ml of water

3.2.4 Strain maintenance inoculums preparation

The isolated culture were maintained on luria agar slants at 4 ^{o}C, subcultured after 15 days to maintain its viability.

3.2.5 Lipase production

Bacterial lipase was produced through submerged fermentation by cultivating 100ml 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 rotar shaker (125 rpm) at 37 °C. After 3 h, the fermented broth was centrifuged at 5367 g for 15 min at 4 °C and the cell free supernatant was used for estimation of lipase activity.

3.2.6 Lipase assay.

Lipase activity is determined by using p-nitrophenyl palmitate standard curve. The lipase activity was evaluated spectrophotometrically by measuring *p*-nitrophenol produced by hydrolysis of *p*-nitrophenyl palmitate at 410nm(Pancreac et al., 1996). Substrate solution which was mixture of propanol, pNPP, Tris-HCL.buffer, Triton X-100 and gum acia was prepared. 9 ml of solution B was taken in beaker and 1ml of solution A is added drop by drop in beaker which is kept in the water bath at tempreture>50 $^{\circ}$ C. 1ml of this solution was taken in test tube and incubated at 37°C for 10min then the enzyme was added to the substrate solution, 2ml distilled water was added to stop the reaction and take absorbance at 410nm. Unknown concentration of p-nitrophenol released was determined from standard curve of p-nitrophenol. Following formula is used to determine enzyme activity

$$E = \frac{(S)\times(A)}{T\times V\times 12}$$

Where, E=Enzyme activity(U/ml)

S= Slope^{-1}(from p-NPP standard curve)

A= Absorbance at 410nm

T= Incubation time

V= Volume of enzyme taken.

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.

3.2.7 Protein estimation

Protein content of cell-free supernatant was determined according to modified lowry method using BSA as a standard (Lowry et al.,1951). The protein concentration exhibited by a color change of the sample solution in proportion to protein concentration which can be measured by using colorimetric technique. Under alkaline conditions the divalent copper ion forms a complex with peptide bonds in which it is reduced to a monovalent ion and Folin Ciocaltaeu reagent which contains phosphomolybdic complex which is a mixture of sodium tungstate, sodium molybdate and phosphate, along with copper sulphate solution and the protein, a blue purple colour is produced which can be assessed by measuring the absorbance at 700 nm. Three solutions were prepared solution A solution B and solution C. Solution for protein estimation.50µl cell free supernatant fluid is taken in test tube and added 450µl water is

added and add solution C and incubate at 37 °C for 10 min and added 500µl of Follin reagent is added and similarly blank is prepared without sample and absorbance was taken at 750nm. Protein content of cell free supernatant determined by BSA standard curve

Determination of specific activity

The specific activity(U/mg) was calculated by using the following formula:

$$\text{Specfic activity (U/mg)} = \frac{E}{P}$$

Where, E(U/ml) = activity per unti volume of culture extract and

P(mg/ml)= protein concentration of crude extract

3.2.8 Partial purification (ammonium sulphate precipitation)

The Lipase cell free extract was precipitated with ammonium sulfate up to 60% of saturation.100 ml of supernatant containing lipase was taken in beaker while constant stirring, ammonium sulphate was added slowly to the supernatant.Throughout the reaction, maintained the beaker temperature to be 4° C. After addition of ammonium sulphate, beaker was stored at 4° C for 2 hr. 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. Formula used for obtaining various salt concentrations is:

$$\text{Amount of ammonium sulphate added} = \frac{533 \times (S2 - S1)}{100 - 0.3 \times S2}$$

where S1and S2 are intial and final ammonium sulphate concentrations respectively.

After partial purification again Lipase assay is done to check the stability of enzyme after purification. Relative activity test or we can say compatibility of Lipase with contact lens solution for commercial contact lens solution and the procedures for that is as following. We got 3 commercial contact lens solution from market i.e namely renu multipurpose solution, biotrue multipurpose solution, and purecon contact lens solution..

3.2.9 Protease assay

In this assay, casein acts as a substrate. When the enzyme are testing digests casein, the amino acid tyrosine is liberated along with other amino acids and peptide fragments. Folin &

Ciocalteus Phenol, or Folin's reagent primarily reacts with free tyrosine to produce a blue colored chromophore, which is quantifiable and measured as an absorbance value on the spectrophotometer.(Han et al.,2002). The more tyrosine that is released from casein, the more the chromophores are generated and the stronger the activity of the protease. Absorbance values generated by the activity of the protease are compared to a standard curve, which is generated by reacting known quantities of tyrosine with the F-C reagent to correlate changes in absorbance with the amount of tyrosine in micromoles. From the standard curve the activity of enzyme samples can be determined in terms of Units. Substrate solution which was mixture of casein and phosphate buffer. 0.65 ml of solution B, 2ml of Solution C ml was taken in a test tube and add 0.05(V2) lipase enzyme and incubate 37 °C for 20 min. 0.2ml of 1N HCL was added to stop the reaction and the unhydrolysed casein was precipitated with 5 ml of Solution D. After precipitation the clear solution (hydrolyzed solution) was separated by centrifugation at 5000 rpm for 20 min. From this 0.5 ml (V_1) was taken and the peptide fragment were measured by the Lowry method as for protein estimation (Lowry et al., 1951). The activity (Y) of protein was calculated according to the following formula,

$$Y = \frac{V \times \Delta c}{V1 \times V3}$$

Where, Y= protein activity(U/ml)

V= total volume of solution(ml)

Δc=amount of peptie fragment(mg)

V1= volume of sample(ml)

V2 = amount of enzyme(ml)

One unit of enzyme is defined as the amount of enzyme that liberates peptide fragments equivalent to 1 mg of BSA under the assay condition.

3.2.10 Compatibility of lipase with surfactant and oxidizing agent present in commercial contact lens solution

To investigate the lipase compatibility with various commercial contact lens solution, respective contact lens solution were added to the reaction mixture at a concentration of 7mg/mL and assayed under standard conditions and expressed in terms of percent relative activity. Contact lens solution were inactivated by heating for 1 h at 65 ° C prior to the

addition of enzyme preparation. To determine the stability, an aliquot of enzyme sample was incubated with equal volume of contact lens solution (7mg/ml of respective contact lens solution) in Tris-HCl buffer (0.1 M, pH 8.0) for 1 h at 30 °C. The relative activity (%) of each sample was determined and compared with the control without contact lens solution. The relative activity of control was defined as the enzyme activity without contact lens solution, incubated under the similar conditions, and was taken as 100%.

3.2.11 Formulation of contact lens solution

3.2.11.1 Formulation 1

Commercial available Biotrue multipurpose solution(Bausch and lomb)

3.2.11.2 Formulation 2

Addition of lipase in Biotrue multipurpose solution (Bausch and lomb)

3.2.11.3 Formulation 3

Replacing PBS(component of PBS i.e sodium phosphate(monobasic and diabasic anhydrous), sodium chloride replaced) with lipase enzyme. PBS replaced by protease enzyme (Rasika et al, 2009).

Table 3.1 Composition of contact lens solution

Ingredients	% W/W
Boric acid	0.830
Sodium phosphate(dibasic anhydrous)	0.310
Sodium phosphate(monobasic anhydrous)	0.155
Sodium chloride	0.375
Poloxamine	1.000
Tetrasodium phosphate	0.100
Polyhexamethyelene biguanide HCL	0.0008
Alexidine	0.0002
Sodium carbonate	0.100
Sodium hydroxide, 1N and/or Hydrochloric acid	As required for PH adjustment
Purified water	Balance to 100

3.2.11.4 Preparation of own contact lens solution

This solution was prepared by weighing out the necessary amount of the ingredients, including sodium carbonate, the tetrasodium salt of l-hydroxyethylidene-1,1- diphosphonic acid into a glass beaker. The solution is prepared by gradually heating 80 percent of the water to 80°C while dissolving the tetrasodium phosphate and the buffer substances. Then addition of surfactant poloxamine in the solution. After the solution is cooled to room temperature,alexidine, polyhexamethyelene biguainde HCL,and carbonate in solution are added through a sterile filter and add lipase enzyme.The pH of the resulting solution was between about 7.3 to 7.5. (If necessary, the pH of the solution may be adjusted by use of an appropriate amount of hydrochloric acid or sodium hydroxide, as indicated in Table 3.1)

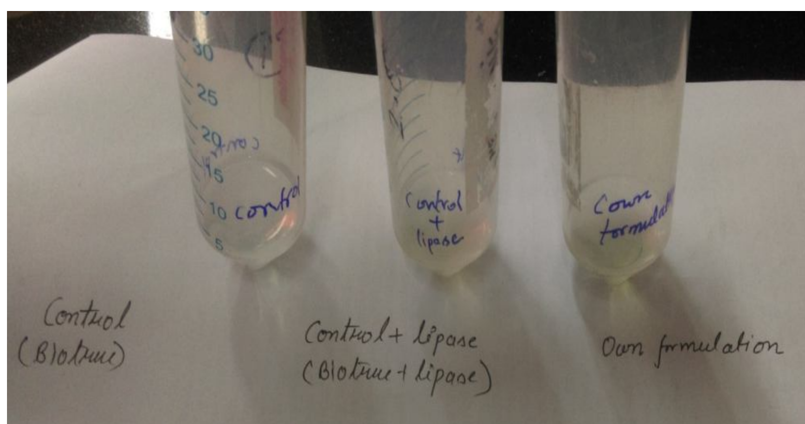

Fig: 3.1 Formulation of contact lens solution

3.2.12 Evaluation of effectiveness of contact lens solution

3.2.12.1 Preparation of artificial tear solution

Fig:3.2 Lenses coated with 0.2 % gelatine

Artificial tear solution is used to develop adddtional tear film. They adsorb the protein, lipid, carbohydrate and mucus onto the lens surface and decrease optical clarity of contact lens. Contact lens solution is used to overcome this problem. Contact lens solution effectively remove these component from contact lens to make clear optical clear visibility. A filter sterilized artificial tear solution was prepared with 0.2% gelatin in electrolyte solution (0.22g Na2CO3 ,0.7 g NaCl, CaCl 0.0005 and KCl 0.15 pH 8). This solution was heated at 50 °C to denature gelatin protein and used for coating of the lenses.

3.2.12.3 Spectrophotometric assay

Before initiating the coating and cleansing process, light transmission reading for all soft contact lenses used in the study was rrecorded using spectroscope at 500 nm. Contact lenses were placed in lens cases and soaked in the 3ml filter sterilized artificial tear solution for 10 min at 30°C to coat the lens with gelatin protein and light transmission readings were recorded. Lenses employed for enzyme treatment were then transferred to 3ml enzymatic based contact lens solution and placed in lens cases. Enzyme treatment was done for 30, 60, 90 min at 37°C. Light transmission readings were recorded post enzyme treatment. Similarly, a control set of lenses was soaked in tear solution but treated with formulation 1(commercial available Biotrue multipurpose solution (Bausch and lomb) contact lens solution and buffer) instead of enzymatic based contact lens solution and another one treated with formulation 2(addition of lipase with commercial available Biotrue multipurpose contact lens solution(Bausch and lomb) and light transimission readings were recorded in similar way as a mentioned above. Protein removal was spectrophotometrically assayed in visible range according to method described by with same modification (Harris et al, 2000,)

CHAPTER 4
RESULTS AND DISCUSSION

4.1 Role of lipase and protease in contact lens solution

Proteases are the protein digesting enzymes which occur naturally in all the organisms. They can hydrolyse the long chain polypeptides into shorter polypeptides and individual amino acids. There are different types of proteases those differ from each other in the properties such as substrate specificity, active site and mechanism of action. The bacterial protease enzyme can be used along with chemical disinfectants to clean the contact lenses (Karageozian et al, 1996) .Tear film proteins, particularly lysozyme ,accumulate on the surface of hydrogel lenses over time. These proteins become loosely attached to the lens surface on insertion and bind to surface over time either as films or discrete deposits(Keith et al., 2001). Bound protein is not removed from the lens surface by most surfactant cleaners. The microbial protease-containing lens cleaning solutions are especially effective in digesting and removing denatured protein and carbohydrate films and debris from contact lenses without enzyme activators .The enzymatic contact lens cleaners are especially effective in removing contact lens film and debris in one hour or less by high temperature cleaning methods. In addition, the bacterial enzyme cleaners may perform with little or no residual binding or concentrating onto lens surfaces, and therefore, eye tissue sensitivity normally manifested as stinging and inflammation are virtually eliminated. Enzyme cleaners are generally viewed as being efficacious, safe and capable of removing the principal component of contact lens film and debris, namely protein. Protease has potential to remove protein, carbohydrate and lipid deposits from contact lenses with addition of lipase. A lipase is used is without doubt the most gentle method and probably the most efficient for removing fatty deposits from soft contact lenses.

4.2 Lipase assay

Standard curve of p-nitrophenol palmitate

The concentration of *p*-Nitrophenol determined by comparing to a standard curve of known concentrations of *p*-Nitrophenol.To determine the activity of lipase, p-nitrophenyl palmitate was used as substrate, it is worth mentioning that lipase catalyzed reaction cannot be

performed at acidic medium (pH 4-6) or highly alkaline medium (pH 10-12), it is best at neutral or mildly alkaline. However, based on the standard curve prepared lipase activity was 0.0072 U/ml .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.

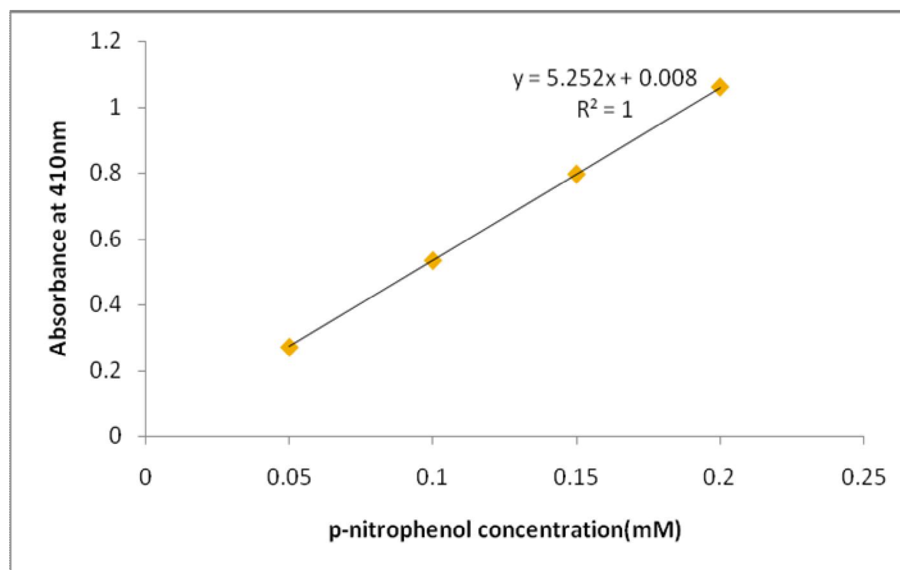

Fig 4.1 : Standard curve of p-nitro phenol

4.3 Protein estimation

Standard curve of BSA

Protein concentration of enzyme was determined by BSA standard curve. Based on lowry method, the protein concentration of the crude enzyme mixture 0.144mg/ml, it was observed that the color of the assay was stable blue and in gradient based on the concentration of BSA in preparation of standard curve and the sample was dark blue indicating the presence of active protein.

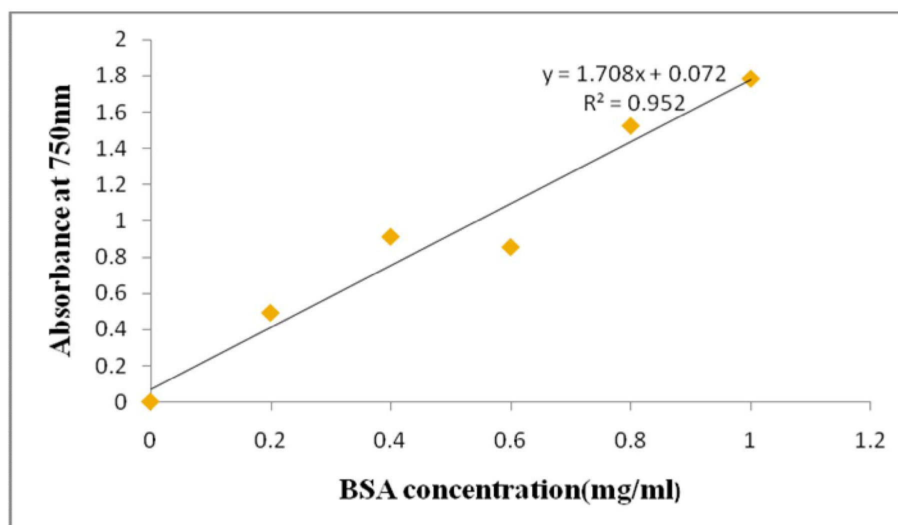

Fig.4.2 : Standard curve of BSA

Protein content in cell free supernatent was determined by BSA standard curve.

4.4 Partial purification

The lipases enzyme was purified to 1.6 fold and showed a specific activity of 0.08 U/mg after ammonium sulfate precipitation (60%) respectively (Table 4.1). Specific activity of crude enzyme and purified enzyme was 0.05 U/mg and 0.08U/mg.

Table 4.1. Specific activity of crude enzyme and purified enzyme

Purification	Total activity (U/ml)	Total protein mg/ml)	Specific activity (U/mg)	Fold purification	Yield(%)
Crude	0.0072	0.144	0.05	1.00	100
Ammonium sulphate precipitation (60%)	0.0044	0.052	0.08	1.6	61

4.5 Protease assay

Based on assay, the protein concentration of the crude enzyme mixture 0.059 mg/ml=59µg/ml, it was observed that the color of the assay was stable blue and in gradient based on the concentration of BSA in preparation of standard curve (shown in Figure 4.2), and the sample was dark blue indicating the presence of active proteins and that the reaction occurred between the protein and the dye. The mixture is incubated at 35°C to give the enzyme a best shape of active side to the attachment with the substrate for suitable time, then the reaction was terminated by trichloroacetic acid (TCA). Dark blue color formed when testing the sample that indicates the reaction of lipase in the sample with the F-C reagent and show the protein activity in sample...

4.6 Compatibility of lipase with surfactants and oxidizing agent present in commercial contact lens solution

For effective use under cosmetic industry condition, lipolytic enzyme must be compatible and stable with all commonly used contact lens solution compounds such as surfactants which mainly present in any contact lens solution formulation. The *S. arlettae* lipase was tested for its potential as an additive in contact lens solution.

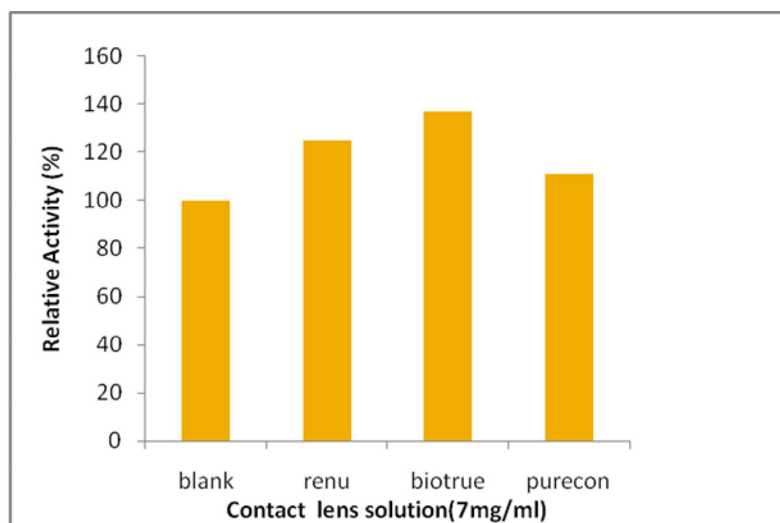

Fig.4.3: Compatibility of *S. arlettae* lipase with with surfactants and oxidizing agent present in commercial contact lens solution. For the control, lipase was incubated with buffer alone without Contact lens solution and its activity was taken as 100%.

The enzyme showed increased stability in presence of renu multipurpose solution, Biotrue multipurpose contact lens solution (Bausch and lomb) showed a maximum 37% increase in activity, while renu multipurpose solution exhibited an increase of approximately 25% and purecon 11% in activity over control (Fig 4.3.).

4.6.1 Relative activity of contact lens solution

Relative activity is the ratio of sample activity and blank activity. Absorbance as recorded at 410nm for different contact lens solution.

Table 4.2 Absorbance of contact lens solution

Sample	Absorbance
Blank	0.3012
Renu multipurpose solution	0.3803
Biotrue	0.4151
Purecon	0.3359

4.6.1 Enzyme activity

Determine enzyme activity of contact lens solution by using formula

$$E = \frac{(S)\times(A)}{T\times V\times 12}$$

Where, E=Enzyme activity(U/ml)

S= Slope-1(from p-NPP standard curve)

A= Absorbance at 410nm

T= Incubation time

V= Volume of enzyme taken.

Table 4.3 Enzyme activity of contact lens solution

Sample	Enzyme activity(U/ml)
1.Blank	1.906
2.Renu multipurpose solution	2.400
3.Biotrue	2.627
4. Purecon	2.126

4.6.2 Relative activity

Determine relative activity of contact lens solution by using formula

$$\text{Relative activity}(\%) = \frac{\text{sample activity}}{\text{blank activity}} \times 100$$

Table 4.4 Relative activity of contact lens solution

Sample	Relative activity(%)
1.Renu multipurpose solution	125
2.Biotrue	137
3. Purecon	111

Therefore, after doing this experiment we found Biotrue multipurpose solution is relatively more compatible then other commercial contact lens solution, so Biotrue multipurpose solution is considered for further use.

4.7 Evalution of effectiveness of contact lens solution

Spectrophotometric assay

In order to study the effectiveness of *S.arlattae lipase* in removing proteinaceous deposits and debris from contact lenses, artificial tear solution was prepared. It was treated with Formualtion 1(commercial available biotrue multipurpose solution, formulation 2(addition of

lipase in biotrue multipurpose solution) and formulation 3(replacing PBS component with lipase enzyme) separately.

Table 4.5 Transmittance (%) of removal of protein from contact lens

Treatments	**Trasmittance%(Biotrue+ buffer)**	**Transmittance% (Biotrue+lipase)**	**Transmittance% (Lipase based enzymatic contact lens cleaner)**
Intial transmittance of leans at 500 nm	`100	100	100
After coating with gelatin for 10 min	85	87.5	86.5
Gelatin coated lens+Biotrue+buffer /Biotrue+lipase/lipase contact lens solution(30 min incubation)	96.2	94.2	94.7
Gelatin coated lens+ Control / Contol+lipase/lipase based contact lens solution(60 min incubation)	97.1	95.0	96.7
Gelatin coated lens + Control / Contol+lipase/lipase based contact lens solution(90 min incubation)	98.5	98.6	96.9

The spectrophotometric analysis indicated that before treatment % transmittance were 85,87.5 and 86.5**%** and after treatment with different formulation of contact lens solution it was 98.5, 98.6 and 96.9% respectively (Table 4.5).The increased transmittance indicated that the enzyme had potential in the removal of protein deposits from contact lens. The optimal time for the degradation was 30,60 and 90 min.

CONCLUSION

Lipase produced from *Staphylococcus arlattae* is most compatible with commercial available biotrue multipurpose contact lens solution than the renu multipurpose solution and purecon. In addition a Lipolytic Enzyme in contact lens solution on the contact lens which is to be cleaned to reduce both Albumin and Lipids present to water soluble peptones, fatty acids and esters. Removal of protein from coated lens was increased with time when lens treated with biotrue solution by the addition of enzyme in it and coated lens treated with lipase based enzymatic contact lens solution, they also showed the effective removal of protein from coated lens. So it conclude that *S. arlattae* lipase based contact lens solution have capability to remove the tear film and protein content from contact lens.

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APPENDICES

1. **Luria bertani agar medium for Maintainence of *staphylococcus arlettae.***

Media composition	Gms/Litre
Casein enzymic hydolysate	10.000
Yeast extract	5.000
Sodium chloride	5.000
Agar	10.000
Final pH (at 25° C)	7.5±0.2

2. **Luria bertani broth medium for maintainence of *staphylococcus arlettae.***

Media composition	Gms/Litre
Casein enzymic hydolysate	10.000
Yeast extract	5.000
Sodium chloride	5.000
Final pH (at 25° C)	7.5±0.2

3. **1 M Phosphate buffer (pH- 7.5)**

Components	Gms/Litre
NaCl	8.0
KCl	0.2
Na_2HPO_4	1.44
KH_2PO_4	0.24

RESUME

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Career Objective

To become a successful professional in the field of Biotechnology by working in an innovative and competitive work environment with committed and dedicated people.

Educational Qualification

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Academics	Name of Institute	University/ Board	Year of Passing	Percentage Marks/GPA
M.Tech	Jaypee Uniersity of Information Technology, Wakhnaghat, Solan (H.P.)	Jaypee University	2015	6.9(3rd sem)
B. Tech	Banasthali University, Jaipur	Banasthali university	2013	68.6%
Senior Secondary School Examination (12th)	Saraswati Shishu Mandir,Morena(M.P.)	M.P.Board	2009	77.6%
Secondary School Examination(10th)	Saraswati Shishu Mandir,Morena(M.P.)	M.P.Board	2007	93.6%

Projects

TITLE 1 : M.Tech project on "**formulation of biobased contact lens cleasing solution"**.(August 2014- May 2015)

Description: Determine the efficacy of contact lens solution by spectrophotometric assay. Effective removal of depostis from contact lens by using commercial available contact lens solution and lipase based solution. Find out s.arlattae lipase has capability to remove the deposits from contact lens..

TITLE 2: Six month training project done at FRI, Deemed University, Dehradun.This training is based on"**Invitro studies on propogation of Elaeocarpus *Sphaericus.*"**. June 2012- December 2012)

Description: Determination of effective medium for shoot and root induction of Elaeocarpus sphaericus.

TITLE 3: 4 week Training at DR&DE Gwalior . This training based on **"Immunosensing studies for the detection of (IgG) based on screen printed electrode."June 2011- July 2011.**

Desription: Comparison of different carbon ink based screen-printed electrodes towards amperometric immunosensing. Amperometric immunosensors made by different carbon inks were compared with standard ELISA in terms of total assay time, amount of biological materials used and sensitivity of detection. A model system containing rabbit anti-mouse immunoglobulin G (RαMIgG) as the capturing antibody, mouse I (MIgG) as antigen and alkaline phosphatase conjugated RαMIgG as revealing antibody was used. In these studies, 1-naphthyl phosphate was used as substrate.

Extra Curricular Activities and Achievements

- First runer up of BIOSCOPE in Banasthali University.
- I have participated in "CLOSING CEREMONY" in Banasthali University.
- I have participitated in the quiz conducted by school at state level.

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Techniques learned-

- ELISA Test, Double diffusion and immune electrophoresis
- Salt precipitation method of enzyme purification
- Sterilization techniques
- Embryo culture
- Tissue culture media preparation.

Personal Profile

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