ISSN: 0975-8585

Research Journal of Pharmaceutical, Biological and Chemical Sciences

Isolation, Purification and Application of Enzymes from *Bacillus subtilis*

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ABSTRACT

The present study was focused on the partial 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 specific activity of purified lipase and protease obtained from ammonium sulfate precipitation were found to be 10.00 U/mg and 1.02 U/mg respectively and the specific activity after dialysis were found to be 10.86 U/mg and 1.62 U/mg respectively. The lipase was substituted for a chemical surfactant (SDS) in the formulation of the toothpaste and further protease also tested for its efficiency as a contact lens cleaner.

Keywords: *Bacillus subtilis*, Lipase, Protease, Toothpaste formulation, Contact lens cleaner

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ISSN: 0975-8585

INTRODUCTION

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 [1].

Lipases are the subclass of esterase's those hydrolyse the triacylglycerides to free fatty acids and glycerol. They have a wide range of properties depending on their sources with respect to positional specificity, fatty acid specificity, thermo-stability, pH optimum, etc [2]. Lipases are involved in diverse biological processes ranging from normal metabolism of dietary triglycerides to cell signalling [3] and inflammation [4]. Thus, some lipase activities are confined to specific compartments within cells while others work in extracellular spaces.

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 [5]. In higher organisms, they perform wide range of physiological reactions from simple digestion of food proteins to highly regulated cascades. Bacterial proteases are important to the carbon and nitrogen cycles in the recycling of proteins [6].

Lipase are used in industries for the hydrolysis of fats, production of fatty acids and food additives, synthesis of esters and peptides, cosmetic and biodiesel industries, resolution of racemic mixtures. Lipases such as phospholipases have industrial applications in egg yolk treatment for the production of mayonnaise and other emulsifiers, in lecithin modification, and for the oil-degumming step in there fining of vegetable oils. They are used in food industries, pharmaceutical industries, pulp and paper industries [7]. While proteases are used in meat tenderization, silk industries [8], protein recovery and solubilisation, industrial waste treatment, paper industry [9], silver recovery from X-ray [10]. In general lipases and proteases are used in detergents [11], pharmaceuticals, food industries, leather industries.

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 [12, 13].

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 [14]. This surface deposit also increases the possibility of pathogenic bacterial adhesion such as *Psedomonas aeroginosa* [15]. 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 [14]. Hence, the present study focused on the production of lipase and protease from bacteria and their application in the formulation of toothpaste and as contact lens cleaner respectively.

MATERIALS AND METHODS

Chemicals

Media and chemicals were purchased from Hi Media Private limited, Mumbai, India and Sisco Research Laboratories Pvt. Ltd. and Merck specialities Private Limited, Mumbai, India respectively.

Sample collection

The rhizosphere soil samples were collected from the Vellore district (12.92°N, 79.13°E), Tamil Nadu, India during the month of February 2013. The samples were collected from 5-6cm depth using sterile spatula and transferred in sterile plastic bags which were then transferred to the laboratory and stored at 4°C until further use.

Isolation of lipolytic and proteolytic bacteria

The isolation was performed by serial dilution and followed by spread plate technique. The soil sample was diluted to ten-folds and 0.1 ml from 10^{-4} dilution was plated directly on tributyrin agar and the plates were incubated at 37°C for 24 hrs.

The lipolytic bacterial isolates obtained were inoculated on gelatin agar media and the plates were incubated at 37°C for 24 hrs. After incubation, a mixture of $HgCl_2$, concentrated HCl and distilled water was flooded onto the plate to detect the proteolytic activity of the bacterial isolates.

Identification of bacteria

The potential isolate was identified based on colony characters on differential media, morphological characters (Gram staining, endospore staining and motility) and biochemical tests [16].

Production media

- Lipase production media contained (g/L) peptone (5), yeast extract (5), NaCl (0.5), $CaCl_2$ (0.05) and olive oil 5 ml and the pH was adjusted to 8.0 was prepared and incubated on rotary shaking incubator for 24 hrs at room temperature. The supernatant was collected by centrifugation at 8,000 rpm for 10 mins at 4°C.

- Protease production media contained (g/L) gelatin (1), peptone (10), $CaCl_2$ (0.1), $MnSO_4$ (0.1), K_2HPO_4 (0.5) and yeast extract (0.2) and the pH was adjusted to 7.0

was prepared and incubated on rotary shaking incubator for 24 hrs at room temperature. The supernatant was collected by centrifugation at 8,000 rpm for 10 mins at 4°C.

Protein estimation

The total protein content was estimated by Lowry's method (1951) using Bovine Serine Albumins (BSA) as standard [17].

Enzymatic assay

Lipase assay

One ml of culture free supernatant was added to assay substrate, containing 10 ml of 10% (v/v) homogenized olive oil in 10% (w/v) gum acacia, 2.0 ml of 0.6% $CaCl_2$ solution and 5 ml of phosphate buffer (pH 7.0). The enzyme substrate mixture was incubated on rotary shaker with 150 rpm at 30°C for one hour. 20 ml of alcohol: acetone (1:1) mixture was added to the reaction mixture. Liberated fatty acids were titrated with 0.1N NaOH using phenolphthalein as an indicator.

Protease assay

The crude enzyme (1 ml) was mixed with 1 ml of gelatin solution in borate NaOH buffer (10 mM, pH 8.0) incubated at 40°C for 10 mins. One ml of 10% TCA (Trichloroacetic acid) solution was added immediately and incubated for 20 mins and it was centrifuged at 10,000 rpm for 5 mins. The Tyrosine content in the supernatant was determined spectroscopically at 280 nm using standard tyrosine curve. One unit of enzyme activity was defined as the amount of enzyme that liberated one microgram of tyrosine from substrate (gelatin) per minute under assay conditions.

Partial purification

Ammonium Sulfate Precipitation

The Lipase cell free extract (50 ml) was precipitated with ammonium sulfate up to 70% 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 for further purification.

The Protease cell free extract (50 ml) was precipitated with ammonium sulfate up to 70% 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 7.0 for further purification.

Dialysis

Desalting of both the precipitates was achieved by dialysis. About 1.5 ml of enzyme precipitate was placed in the dialysis bags (tightly closed from both the side) and it was

suspended in a beaker containing 1 mM Tris HCl buffer. This beaker was then placed on a magnetic stirrer for 24 hrs at 4°C.

Application

Lipases

Lipase was used for formulation of the toothpaste, substituting the SDS (surfactant). It was prepared and kept at room temperature for 10 days. The compatibility (uniformity, spreading ability, pH, abrasiveness, foaming ability, cleaning ability) was checked and compared with another formulation prepared with SDS.

Formulation material

Sodium bicarbonate (baking soda), Sodium chloride (common salt), Calcium Carbonate, Glycerine and sodium lauryl sulphate (SDS)[24].

Proteases

Artificial tear solution was prepared and modified by replacing lysozyme with gelatine in original process given by Rasika et al [14]. This solution was heated for 15 min at 50°C for denaturation of gelatine protein and used for contact lens coating. Light transmission was noted at 500 nm. These lenses were kept in artificial tear for 10 mins for coating of protein and the transmittance was recorded in visible length. Further these lenses were treated with protease enzyme (0.660 U/mg) for 30 mins and 60 mins. The transmittance was checked in the same range as earlier. Similarly, the another set of lens (control) was treated with phosphate buffer (pH 7) and transmission was correlated.

RESULTS AND DISCUSSION

Isolation and Identification of lipolytic and proteolytic bacteria

After 24 hrs of incubation, 8 colonies showing maximum zone of clearance on tributyrin agar plates were observed and designated as SS-1 to SS-8. The SS-7 exhibited highest lipolytic activity with a clear zone of 14 mm and proteolytic activity (on gelatin agar) with a clear zone of 12 mm (Table 1 and Fig 1). In controversy, Vijay et al (2012) and Folasade and Joshua (2005) reported *Bacillus subtilis* showed higher lipolytic activity and lesser proteolytic activity respectively [18,19]. This might be because of variation in the geographical condition.

The potential strain was identified as *Bacillus subtilis* based on the colony characters, morphological characters and biochemical tests (Table 2).The results were compared and found to be similar to those of results earlier reported by Logan and Berkeleyr (1984), Folasade and Joshua (2005) and Gitishree and Prasad (2010), except casein hydrolysis which was reported as positive by Gitishree and Prasad (2010) while in present study it was observed as negative [19,20,21].

ISSN: 0975-8585

Colony	Shape	Elevation	Colour	Consistency	Grams reaction
SS-1	Irregular	Flat	Off white	Sticky	G+ve cocci
SS-2	Circular	Flat	Off white	Sticky	G+ve rod
SS-3	Circular	Raised	Off white	Sticky	G +ve rod
SS-4	Irregular	Flat	Off white	Sticky	G +ve rod
SS-5	Circular	Flat	Off white	Sticky	G +ve rod
SS-6	Circular	Flat	Off white	Sticky	G+ve cocci
SS-7	Circular	Raised	Off white	Sticky	G +ve rod
SS-8	Irregular	Flat	Off white	Sticky	G +ve rod

Table 1. Colony morphology characteristics of SS-1 to SS-8 on Tributyrin agar

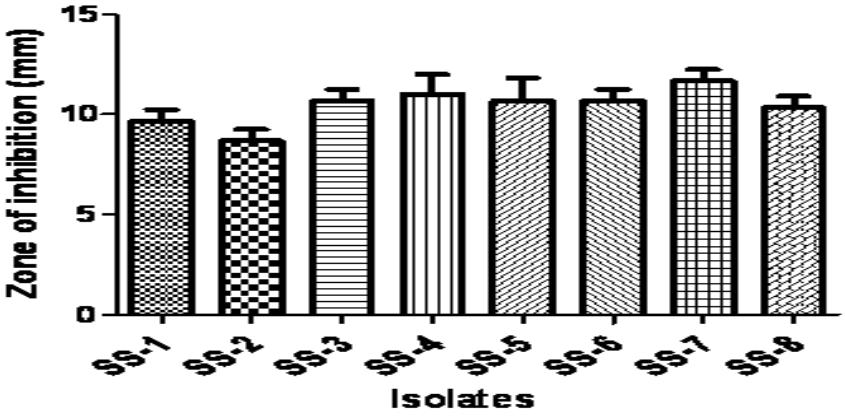

Fig 1. Lipolytic activity of isolated SS-1 to SS-8

Table 2. Morphological and Biochemical characters of isolate SS-7

	Characterization of Bacteria	Results	Earlier results[19,20,21]
Culture characteristics	Colony morphology		
	Trybutyrin agar base	Circular, raised, entire margin, off white, sticky colony.	Matched-Positive
	Bacillus differential media	Circular, raised, creamy colony.	-
Microscopic characteristics	Grams staining	Gram positive rods	Matched-positive
	Endospore staining	Terminal endospore	Matched-Positive
	Motility	Motile	Motile
Biochemical characteristics	Indole	Negative	Negative
	Methyl red	Negative	Negative
	Voges Proskauer	Positive	Positive
	Oxidase	Positive	Positive
	Catalase	Positive	Positive
	Starch hydrolysis	Positive	Positive
	Casein hydrolysis	Negative	Positive
	Gelatin hydrolysis	Positive	Positive

ISSN: 0975-8585

Partial Purification of lipase and protease

The lipases enzyme was purified to 2.40 fold and showed a specific activity of 10.00 U/mg after ammonium sulfate precipitation (Table 3). The fold purification increased to 2.61 with a specific activity of 10.86 U/mg after dialysis. Compared to other reports, good yield and purification was achieved. Similarly, Hassan et al. (2010) reported 10.70 U/mg specific activities with 4.7 purification fold of lipase [22].

The protease enzyme was purified to 1.54 fold and showed a specific activity of 1.02 U/mg after ammonium sulfate precipitation (Table 4). The fold purification increased to 2.45 with a specific activity of 1.62 U/mg after dialysis. Contrastingly, Soundra et al (2012**)** found that a high level of protease activity from *Bacillus* sp [23].

Table 3. Purification of lipase from *Bacillus subtilis*

purification	Total protein (µg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	1257	5.00	4.16	1.00	100
Ammonium sulphate	450	4.50	10.00	2.40	91
Dialysis	236.4	2.50	10.86	2.61	50

Table4. Purification of protease from *Bacillus subtilis*

Purification	Total protein (µg/ml)	Total activity (U/ml)	Specific activity (U/mg)	Fold purification	Yield (%)
Crude	742	0.49	0.66	1.00	100
Ammonium sulphate	450	0.46	1.02	1.54	93
Dialysis	228	0.37	1.62	2.45	75

Application

Lipases (Toothpaste)

On comparison with substituting of 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 (Table 5 and Fig 2). This result indicates that lipase can be altered to some extent for SDS reducing its concentration in the formulation just too impact foaming ability.

Protease (Contact Lens Cleaner)

There was clarity obtained after treating contact lens with protease. On incubation with crude protease and phosphate buffer (chemical solution) 0.129 and 0.064 (60 min) of transmittance was read which indicated that protease can be used over chemical cleaning solutions with a little more incubation time (Table 6). Even the enzymatic solution was observed to be odourless. There was similarity in results reported by Rasika et al (2009)

[14]. 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.

Table 5. Properties of formulations with lipase

Test	Formulation-1 (lipase)	Formulation-2 (SDS)
Uniformity	Incomplete	Incomplete
Spreading ability	2.4 cm	2 cm
pH	9.79	8.39
Abrasiveness	One scratch	3 scratches
Foaming ability	0.3 cm	3 cm
Cleaning ability	Less clarity than F2	More clarity than F1

Table 6. Removal of protein deposition from contact lens depending on transmittance

Treatments	Transmittance (Protease treatment)	Transmittance (Phosphate buffer treatment)
Intial transmittance of lens at 500nm	0.000	0.000
After coating with gelatine for 20 mins	0.579	0.579
Gelatin coated lens + Protease/phosphate buffer (30 mins incubation)	0.291	0.119
Gelatin coated lens + Protease/ phosphate buffer (60 mins incubation)	0.129	0.064

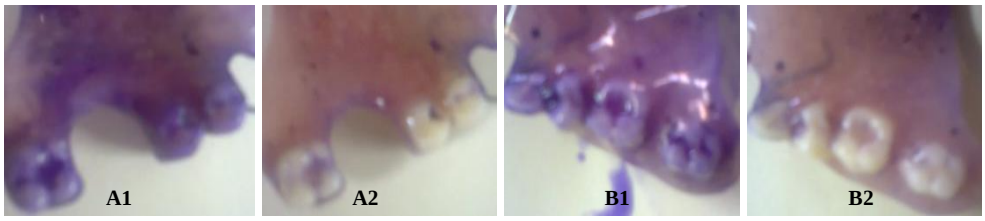

Fig 2. A1 and B1 was the tooth stained with methylene blue. A2 and B2 was the tooth treated with formulation 1 and 2 respectively

CONCLUSION

The results obtained in the present study reveals that, there is promising possibility for substitution of SDS with Lipase with further modification in the formulation which might have foaming ability. Protease can be used in lens cleaning in less concentration instead of higher content of chemical cleaning agents. In future, it can be used for toothpaste and lens cleaner industries.

ACKNOWLEDGEMENT

We would like to thank VIT University, Vellore, Tamil Nadu, India for supporting this work.

ISSN: 0975-8585

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