Isolation and Bioprocess Optimization of Halophilic and Alkaline Protease From Marine Streptomyces and Its Use As Contact Lens Cleaner

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Abstract

In the present study, an alkaline protease was isolated from marine *Streptomyces* sp. GS – 3. The strain was isolated from the backwaters of Puthuvypeen, Kerala, and identified as *Streptomyces* sp. by polyphasic taxonomy. The media for protease production was optimized by response surface methodology (RSM) using the Box-Behnken model. The maximum yield (357 U/ml) was observed using wheat bran as substrate (5.5%), pH 5.5, and the incubation period of 9 days at 28 °C. The optimum temperature and pH conditions for the maximum enzyme activity were 45 °C, and 9 respectively. The K_m and V_{max} values of the enzyme were determined as 5.88%, and 38.46 $\mu\text{mol l}^{-1} \text{min}^{-1} \text{mg}^{-1}$, respectively, using 1% casein as substrate. The enzyme was isolated by solvent extraction with acetone, and the crude enzyme was characterized by Sodium Dodecyl Sulphate Poly-Acrylamide Gel Electrophoresis (SDS-PAGE) and Circular Dichroism (CD) studies. The enzyme sustained at high temperature (50 °C for 6 h), and alkaline pH (10) conditions, and was active in the presence of metal ions, and organic solvents. The purified enzyme was inhibited by phenylmethylsulphonyl fluoride (PMSF) suggesting it belong to serine protease. Further to exploit its application, contact lenses were incubated with the enzyme solution, and the protein debris on it was found to be scrubbed at an optimum time of 60 minutes. Therefore, it increases the transmittance of the contact lens indicating its use as a potential contact lens cleansing agent.

1. Introduction

Contact lenses are often affected by the tear film, which is a complex fluid composed mainly of water, lipids, proteins, sugars, mucin, and carbohydrates (Nichols and Sinnott 2006). These components are natural deposits on the contact lenses which are formed by the interaction of natural tears, and contact lenses (Dutta et al. 2012). Adhesion and colonization by microorganisms, particularly bacteria, on contact lenses are responsible for several adverse events including microbial keratitis, contact lens-related acute red eye, contact lens peripheral ulcer, and infiltrative keratitis (Szcotka-Flynn et al. 2010; Dong et al. 2017). Thus, a periodical cleansing of contact lenses is highly advisable. Proteases have been used for preparing contact lens cleaning solutions; mainly plant (papain), and animal (trypsin, chymotrypsin, and pancreatin) proteases. Proteases are one of the most industrially important enzymes that have been extensively explored during these recent years (Sarrouh 2014). Proteases (EC 3.4) are a group of enzymes found in plants, animals, and microorganisms (Ellaiah et al. 2002). Microorganisms are the most preferred source of commercial protease because of their fast growth rate, strain improvement by genetic mutations, and enzyme overproduction (Almas et al. 2009).

In previous reports, some microbial proteases from *Bacillus* sp, and *Aspergillus* sp. were proved as promising agents for contact lens cleaning (Pawar et al. 2009). However, in many cases, these enzymes possess an unpleasant odor in the cleaning solution, and other contaminations that limit its utility. Proteases obtained from marine actinobacterial sources are gaining importance to overcome these disadvantages, and to render a safe cleaning composition viz. odorless, non-allergic response or not causing eye irritation. Alkaline protease plays an important role in the manufacture of different cleansers,

and hence can also be used as contact lenses cleanser (Vishalakshi et al. 2009). The present study describes the isolation, screening, and identification of an alkaline protease producing marine actinomycetes, *Streptomyces* sp. GS-3 from the backwaters of Puthuvypeen, and Valapad, Kerala. The growth conditions were optimized, and protease was obtained in partially purified form. This enzyme was characterized, and its application as a potent contact lens cleansing agent was evaluated.

2. Materials, And Methods

2.1. Isolation of strain

The marine sediment sample was collected from the backwaters of Puthuvypeen (9.9950° N, 76.2247° E), Kerala, India. The sample was pretreated with CaCO₃ and kept at 45 °C for 24 hours to minimize the growth of other contaminants. Serially diluted samples (10⁻⁴ and 10⁻⁵) were used for the isolation using three different media viz. starch casein agar, actinomycetes isolation agar, and ISP agar – 2. The media were supplemented with 2M NaCl concentration, and alkaline pH (pH – 8) to selectively isolate halophilic, and alkaliphilic strains. Antibiotics viz cycloheximide (25 µg/ml), and nalidixic acid (25 µg/ml) were added to the growth medium to prevent Gram-negative bacterial, and fungal contamination. The selected strains were enriched with 1–5% skim milk solution, and subsequently plated on skim milk agar. The protease producing isolates were screened, and cultured in skim milk broth for 9–12 days for the enzyme production (Mehtani et al. 2017) The broth was centrifuged, and the supernatant was used for protease estimation according to (Lowry et al. 1951). The effective strain GS-3 was selected for morphological, and taxonomical characterization using conventional methods as described earlier (Cappuccino and Sherman 2014). The obtained results were compared with Bergey's Manual of Systematic Bacteriology (D H Bergey; et al. 2012).

For molecular characterization, the genomic DNA of GS–3 was extracted according to (Kutchma et al. 1998) with minor modifications. PCR amplification (Sambrook and Russel, D 2000) of the 16S rRNA gene was performed using actinomycetes specific primers 243F, and A3R (Monciardini et al. 2002). The amplified products were sequenced using Sanger sequencing methods (Veda Scientific, Bangalore), and compared with other reported sequences using BLAST (NCBI). The sequences were aligned using CLUSTAL W (Larkin et al. 2007) followed by the construction of the phylogenetic tree by MEGA, version 6 (Tamura et al. 2013). Bootstrap values were calculated for 1000 replicates.

2.2. Media Optimization

GS-3 was inoculated in production media containing various substrates viz. skim milk, casein, wheat bran, and rice bran (1% w/v), and the respective protease production was determined (Sarkar and Suthindhiran 2020). All these four substrates were used with 10 different basal media (BM1–BM10). Media compositions (g/l) are presented as follows: BM1 - Peptone – 10, Sucrose – 10, NaCl – 90, K₂HPO₄ – 0.5, MgSO₄.7H₂O – 0.5, CaCl₂ – 0.5; BM2 - Glucose – 0.5, Peptone – 10, KNO₃ – 0.6, NaCl – 90, K₂HPO₄ – 0.5, MgSO₄.7H₂O – 0.5, CaCl₂ – 1, ZnSO₄. 5H₂O – 0.5; BM3 - Peptone – 5, Yeast Extract –

3, Glucose – 10, NaCl – 90, Beef Extract – 3, CaCO₃ – 3; BM4 - Solution A (800 ml) - KH₂PO₄ – 3, Na₂HPO₄ – 3, NH₄Cl – 2, NaCl – 90, Solution B (200 ml) - Glucose – 8, MgSO₄ – 1; BM5 - Starch – 20, Yeast Extract – 3, K₂HPO₄ – 1, CaCO₃ – 3, FeSO₄ – 0.01, NaCl – 90; BM6 - Sucrose – 30, KCl – 0.5, FeSO₄ – 0.01, MgSO₄ – 0.5, K₂HPO₄ – 1, KNO₃ – 3, NaCl – 90; BM7 - Starch – 2, K₂HPO₄ – 1.2, MgSO₄ – 0.5, NaCl – 90; BM8 - Glucose – 1, Peptone – 0.5, Yeast Extract – 0.5, NaCl – 90, CaCl₂ – 0.2; BM9 - (NH₄)NO₃ – 5, KH₂PO₄ – 3, NaCl – 90, MgSO₄ – 1; BM10 - Maltose – 10, Yeast Extract – 2, Beef Extract – 1, NaCl – 90.

2.3. Response Surface Methodology using Box-Behnken Method

Three factorial Box-Behnken model was studied to determine the optimum growth conditions and parameters for maximum protease yield. The basal media supplemented with wheat bran as substrate giving the highest yield was chosen for the study. Substrate concentration (1–10%), pH (5–10%), and incubation period (5–14 days) were used as variables.

2.4. Enzyme production, and purification

GS-3 was incubated in the optimized media for 10 days at a temperature of 45 °C at 150 rpm. After incubation, the culture was centrifuged for 10 minutes at 10,000 rpm, and the supernatant was obtained. The supernatant has been further analyzed for the estimation of protein. The protein present in the supernatant was precipitated using acetone at different concentrations (50%, 75%, and 100%). The precipitated protein was further characterized as described earlier (Suthindhiran et al. 2014).

2.5. Enzyme Characterization

2.5.1. SDS –PAGE

SDS-PAGE was performed to determine the molecular weight of the purified enzyme (Laemmli 1970). The bands of the desired protein were visualized in blue background gel in a gel documentation system (Azure Biosciences, Canada). The desired b, and was selected, and trypsin digested. The isolated protein was retrieved, and used for further analysis.

2.5.2. Kinetics studies

The kinetics of enzyme production was measured with different substrate concentration (1–10% casein) (Vishwanatha et al. 2010). The enzyme production was analyzed for 14 days followed by enzyme quantification. The graph was plotted for the substrate and velocity of the reaction. The K_m and V_{max} values were calculated from the slope of the graph, and derived from the following equation:

$$v = \frac{v_{\max}[s]}{k_m + [s]}$$

2.5.3. Determination of optimum pH, and temperature for enzyme stability

The optimum pH for the purified enzyme was determined by incubating the enzyme with buffers of various pH (2–10). The buffers used were 0.1 M glycine-HCl buffer (pH 2–5), 0.1 M acetate buffer (pH 5–7), and 0.1 M glycine-NaOH (pH 7–10). The enzyme was incubated in the same buffer range to determine the pH stability (pH 2–10) for 24 hours at 28 °C. For determining the optimum temperature, the enzymatic assay was performed with temperature ranges of 35–70 °C. Casein (1%) in 0.2 M Tris-HCl buffer (pH – 8.5) was used as a substrate. The enzyme was incubated in a temperature range of 20–50 °C for 24 hours to check the enzyme stability. The assays were conducted after every 6 hours to check the enzymatic activity (Vishwanatha et al. 2010).

2.5.4. Effect of protease inhibitors, metal ions, and solvents on the enzyme

The enzyme solution was incubated at room temperature for 30 minutes with protease inhibitors such as EDTA (2.5–10 mM), and PMSF (2.5–10 mM). Similarly, the effect of metal ions (Mg^{2+} , Ca^{2+} , Pb^{2+} , Mn^{2+} , Cu^{2+} , Zn^{2+} , and Hg^{2+}) on the enzymatic activity were examined. Metal ions (1 mM) were incubated with the enzyme solution for 30 minutes, and subsequently, the activity was determined by enzymatic assay. The stability of the enzymes in different organic solvents (1 ml) was also determined by incubating the enzyme solution (3 ml) at 37 °C for 30 minutes at 150 rpm. The organic solvents used were aniline, acetone, acetonitrile, benzene, chloroform, DMSO, ethanol, ethyl acetate, hexane, isopropanol, pyridine, and toluene. The enzyme activity was measured by protease assay, and the enzyme solution without solvent was taken as control (100%). The residual activity was calculated based on control (Bhunja et al. 2013).

2.6. Assay for lens cleansing

The enzyme was used for cleansing protein-coated contact lenses. Protein removal was assayed by the method used by Jadhav et al. with minor modifications (Jadhav et al. 2014). The contact lenses were coated with an artificial tear solution. The artificial tear solution was prepared with 0.2% lysozyme in electrolyte solution consisting of 0.22 g Na_2CO_3 , and 0.7 g NaCl (pH – 7.5). The solution was incubated at 50°C for 20 minutes for the proper denaturing of protein. The solution was filter-sterilized and used for the coating of the lenses. The light transmission reading was taken for all the contact lenses (Lenskart, Vellore, India) before initiating the experiment using a spectrophotometer (Hallmark Optomechatronics Pvt. Ltd.) at 500 nm. The contact lenses were incubated in 3 ml of artificial tear solution for 20 min at 30 °C. The readings for the coated lenses were recorded followed by the enzyme treatment for 10, 30, 60, and 90 min. Readings were taken post enzyme treatment. A set of control lenses were treated with phosphate buffer (pH – 7), and readings were recorded at similar intervals (Jadhav et al. 2014)(Jadhav et al. 2014)(Jadhav et al. 2014)(Jadhav et al. 2014)(Jadhav et al., 2014)(Jadhav et al., 2014).

3. Results

3.1. Isolation and screening of the potent strain

In the present study, 12 morphologically distinct halophilic strains were isolated from the samples of Puthuvypeen. These strains were further screened for protease production out of which, 4 strains (P1, P6, P11, and V4) showed significant results by forming halo zones on skim milk agar. Among these strains, P11 showed the maximum zone formation. Hence it was further selected for the study, and designated as GS-3.

The strain GS-3 produced white powdery colonies on starch casein agar and was gram-positive. The long mycelia were made up of chains of elongated spores (Fig. 1). The morphological and biochemical characteristics of GS-3 were also studied. The strain was found to be positive for catalase, and oxidase but was unable to utilize citrate. Nitrate reduction and H₂S production were also positive. The strain was able to utilize different forms of sugars such as glucose, galactose, sucrose, maltose, and starch. GS-3 also showed significant growth at alkaline pH (8–10), and a wide temperature range (28–50 °C). The strain also showed proper growth until 3M NaCl concentration while moderate growth was observed for 4M NaCl concentration. The BLAST analysis was performed with the 16S-rRNA gene sequence of GS-3. The search results showed 99% homology with different strains belonging to the genus *Streptomyces*. Phylogenetic tree construction, accomplished by the neighbor-joining method showed that the isolate was closely related to other *Streptomyces* strains. Based on the results obtained, the strain was identified as *Streptomyces* sp. GS-3 and the sequence were submitted to NCBI GenBank with accession number MN435583.

3.2. Media Optimization

Streptomyces sp. GS-3 was inoculated in different basal media along with different substrates, and its growth and protease production were examined (Table 1). The protease yield of *Streptomyces* sp. GS-3 was recorded maximum with wheat bran (357 U/ml) as substrate followed by casein (315 U/ml), skim milk (307 U/ml), and rice bran (287 U/ml) respectively. Among the 10 different basal media, BM3 containing wheat bran produced the highest yield i.e. 357 U/ml. Rice bran showed the lowest yield (213 U/ml) as a substrate. BM3 supplemented with wheat bran was hence used for further optimization studies.

Table 1
Protease extracted from *Streptomyces sp* GS-3 in different basal media combined with different substrates

	SKIM MILK (U/ml)	CASEIN (U/ml)	WHEAT BRAN (U/ml)	RICE BRAN (U/ml)
BM 1	291	295	325	282
BM 2	239	275	340	247
BM 3	241	286	357	231
BM 4	239	277	248	284
BM 5	246	315	279	268
BM 6	244	302	341	275
BM 7	301	249	335	246
BM 8	285	298	324	274
BM 9	307	269	326	287
BM 10	294	304	315	235

3.3. Response Surface Methodology

The experimental design with 17 different setups is explained in Table 2 along with the predicted, and experimental values. Each run was replicated, and the mean value of the obtained yield was calculated. The predicted yield was calculated by applying the multiple regression analysis methods according to the following equation:

Table 2

The Box-Behnken design matrix for the variables along with actual, and predicted responses

Order	Substrate (%) (A)	pH (B)	Incubation (Days) (C)	Yield (U/ml)	Predicted yield
1	10	1	0	254	262.881795
2	10	0	-1	54	73.002035
3	5.5	0	0	359	369.806645
4	5.5	0	0	357	369.806645
5	10	-1	0	72	78.88172
6	5.5	1	-1	43	37.1269425
7	5.5	-1	-1	34	-27.8731075
8	1	0	-1	20	50.751785
9	1	0	1	55	26.01425
10	5.5	0	0	365	369.806645
11	5.5	-1	1	109	57.8894225
12	10	0	1	301	284.26448
13	1	1	0	39	11.131455
14	5.5	1	1	123	137.8893225
15	5.5	0	0	354	369.806645
16	1	-1	0	30	50.13158
17	5.5	0	0	253	369.806645

$$\text{Yield} = -1861.81914 + 8.26358 * A + 375.63778 * B + 137.64383 * C + 4.95556 * A * B + 2.91358 * A * C + 0.3333 * B * C - 5.22963 * A^2 - 26.104 * B^2 - 7.674 * C^2$$

where substrate concentration, pH, and incubation days were represented as A, B, and C respectively. The model was proved significant by the ANOVA (Table 3), and F-test with a low probability value [(P-value > F) = 0.0002]. The value of the adjusted R^2 (0.9242) showed that 92% of the enzyme yield in the proposed model. The closer the value of R^2 (0.9668) to 1, the greater the correlation between the expected, and experimental values, indicating a good relation. The non-significant value of lack of fit (0.1351) revealed that the model is statistically significant for the maximum yield. The three-dimensional response surface plots the graphical representations of the equation (Fig. 3). After analyzing the plots, the optimum parameters were calculated as a substrate concentration of 5.5%, pH 9.5, and incubation of 9.5 days. The total yield was calculated as 357 U/ml under these optimum growth conditions.

Table 3
ANOVA for Response Surface Quadratic Model of *Streptomyces* sp. GS – 3

Source	Sum of Squares	df	Mean Square	F Value	p-value > F	
Model	228188	9	25354.22	22.68327	0.0002	significant
A-Substrate	36046.13	1	36046.13	32.24883	0.0008	
B-pH	5724.5	1	5724.5	5.121449	0.0581	
C-Incubation Time	23871.13	1	23871.13	21.35641	0.0024	
AB	7482.25	1	7482.25	6.694028	0.0361	
AC	11236	1	11236	10.05234	0.0157	
BC	6.25	1	6.25	0.005592	0.9425	
A^2	29006.32	1	29006.32	25.95063	0.0014	
B^2	54002.37	1	54002.37	48.31346	0.0002	
C^2	45980	1	45980	41.13621	0.0004	
Residual	7824.25	7	1117.75			
Lack of Fit	5610.25	3	1870.083	3.378651	0.1351	not significant
Pure Error	2214	4	553.5			
Cor Total	236012.2	16				

3.4. Enzyme purification

The protein was precipitated with different concentrations of acetone (50, 75, and 100%). 50% acetone successfully precipitated 57% of the protein from the crude extract. By increasing the acetone concentration to 75%, 68% protein gets precipitated. 89% of the protein was precipitated using 100% acetone. The precipitated protein was again purified using a dialysis membrane (Sigma – Aldrich), and the final obtained protein estimated was 297.35 mg/ml.

3.5. Characterization of the enzyme

3.5.1. SDS –PAGE

The purified enzyme was analyzed in 12.5% SDS-PAGE and visualized after CBB. 25kD b, and was detected for the isolated protease. The molecular weight of the isolated enzyme was compared with the standard (Protease from *Streptomyces griseus*). Gelatin zymography showed white bands in blue background.

3.5.2. Kinetics studies

K_m and V_{max} values were determined to be 5.88%, and 38.46 $\mu\text{M}/\text{min}/\text{mg}$ respectively, using 1% casein as substrate. These parameters were calculated from experiments in which the rates of reactions were determined at different substrate concentrations.

3.5.3. Determination of optimum pH, and temperature for enzyme

The enzyme showed 50% stability at a wide range of temperatures (20–50 °C) until 6 hours (Fig. 4a). After 2 hours, the enzyme showed the maximum activity. After 6 hours, the enzyme activity decreases, and till the 10th hour, the enzyme could only retain 20% of its activity. The enzyme activity was best stable at 50 °C till the 10th hour. The optimum temperature of the enzyme was recorded as 45 °C in which it recorded its maximum activity. Simultaneously, the optimum pH for the enzyme activity was determined to be 9 (Fig. 4b). The enzyme sustained its activity even at alkaline pH (10). In the acidic pH (2–5), the enzyme showed more than 60% of its activity. The increase in the activity of the enzyme towards alkaline pH shows its potential as an alkaline protease.

3.5.4. Effects of different inhibitors, metals, and solvents on the isolated enzyme

The effect of different protease inhibitors is explained in Fig. 4c. PMSF inhibited enzymatic activity to up to 40% at a minimum concentration of 2.5 mmol l^{-1} . With the increase in the concentration of PMSF, the enzyme activity reduces to less than 20%. At the highest PMSF concentration (10 mmol l^{-1}), the enzyme exhibited only 10% of its activity. The enzyme showed less inhibition in the presence of minimum concentration (2.5 mmol l^{-1}) of EDTA but showed inhibition of up to 60% when the concentration was increased to 10 mmol l^{-1} , thus, indicating that the enzyme is not a metalloprotease. The enzyme activity gets affected by up to 50% when incubated with most of the metal ions (Cu^{2+} , Hg^{2+} , Mg^{2+} , Pb^{2+} , Zn^{2+} , and Ca^{2+}) whereas Mn^{2+} recorded the highest enzyme activity with slight inhibition (Fig. 4d). This result indicates that Mn^{2+} has the least effect on the enzyme reaction. The enzyme showed maximum stability in acetone, and the lowest stability in aniline, followed by acetonitrile (Fig. 4e). Besides, it showed approximately 80% stability in other water-miscible solvents, such as ethanol, ethyl acetate, DMSO, chloroform, hexane, isopropanol, benzene, pyridine, and toluene.

3.6. Application of protease in contact lens cleansing

To study the efficiency of the enzyme in removing proteinaceous wastes deposited over the lenses, the enzyme was used as a cleansing agent. The lenses were coated with artificial tear solution before enzymatic treatment, and the % transmittance was recorded (Table 4). Before treatment, the uncoated, and coated lenses showed 97%, and 68% transmittance respectively. After the enzyme treatment, the transmittance of the coated lenses started increasing from 68% until the final transmittance (95%). The

final transmittance was recorded after 60 minutes of incubation. However, the coated lens treated with phosphate buffer recorded 70% transmittance. Thus, the enzyme can be further used as a potent contact lens cleansing agent.

Table 4
Comparison of transmittance (%), and removal of protein debris from contact lenses

Lenses in different enzyme solutions	Transmittance (%)			
	0 min	15 min	30 min	60 min
Untreated lenses	68	68	67	67
Lenses in phosphate buffer	68	68	69	70
Lenses in an enzymatic solution	69	87	93	95

4. Discussion

Enzymes are regarded as environmentally friendly chemicals that help to replace or reduce the use of dangerous chemicals in industrial processes, thereby encouraging sustainable production, and manufacturing. Among different industrial enzymes, microbial proteases dominate the world enzyme market due to their potential application in various industries like food (de Souza et al. 2015), pharmaceutical (Monciardini et al. 2014), textile (Jani et al. 2012), photographic (Parpalliwar et al. 2015), leather (Mamun et al. 2016), and detergent (Sarrouh 2014). However, the proteases must be robust enough to meet the process conditions that are usually hostile for successful industrial applications (Olajuyigbe and Falade 2014). Proteases for industrial applications must have activity, and stability over a wide range of temperatures, and pH extremes for prolonged periods, and even in the presence of various potential enzyme inhibitors (Singh et al. 2016). Contact lenses bear tear films, and protein residues on their surface, which affect the optical clarity of the lenses. Mostly, contact lens cleansing solutions have been prepared using plant, and animal proteases, while microbial proteases are also gaining importance (Pawar et al. 2009).

In the present study, protease producing actinomycetes were isolated from the backwaters of Puthuvypeen, Kerala. These sampling sites have high salinity, and alkaline conditions, and are known to be a rich source for various extremophilic isolates (Ballav et al. 2012). A total of 12 halophilic strains were isolated, out of which 4 strains showed protease production. GS-3 exhibited the best results for protease production among the 4 strains. The phenotypic characteristics reported that the strain belongs to the genus *Streptomyces*. The BLAST result confirmed that the isolate GS-3 belongs to the genus *Streptomyces*, and hence designated as *Streptomyces* sp. GS-3. The isolate GS-3 was considered for the maximum protease production at extreme conditions (high salt concentration, and alkaline pH). Thus, the enzymes obtained from this strain were expected to be highly stable under extreme conditions. *Streptomyces* sp. GS-3 also showed a higher yield of protease (371 U/ml) than previously reported strains, and, hence, might have a bigger role in a variety of industrial practices. Other actinomycetes

strains such as *S. pseudogrisiolus* NRC-15 produce approximately 200 U ml^{-1} of protease (El-Sayed et al. 2012), whereas *S. griseus* SJ_UOM-07-09, *Streptosporangium roseum* SJ_UOM-18-09, and *Streptomyces* sp. LD48 exhibits significantly lower enzyme production of 87, 82, and 16 U ml^{-1} , respectively (Jogaiah et al. 2016; Mehtani et al. 2017).

The growth media for *Streptomyces* sp. GS-3 was optimized, and wheat bran was found to be the best substrate used for protease production followed by casein, skim milk, and rice bran respectively. Wheat bran was previously proved to be an important substrate to stimulate protease production in different microorganisms (Agrawal et al. 2005; Kalaiarasi and Sunitha 2009). Wheat bran also gave the best yield for protease production by different actinomycetes like *Thermoactinomyces thalpophilus* PEE 14 (Divakar et al. 2006). The media contained peptone, and yeast extract as the nitrogen source, and glucose as carbon source. These nutrients play an important role in microbial growth, and enzyme production (Al-Askar et al. 2015). RSM by Box–Behnken design was used for further optimization (Fig. 3). The model was used to monitor the optimum values for each variable in an efficient way to maximize the yield. The enzyme production increased when the substrate concentration increased from 1–5.5%. Further increase in substrate concentration decreases the overall production (Fig. 3a). The excess substrate caused growth inhibition, and hindrance in metabolism resulting in low enzyme production (Ait Barka et al. 2016). A sharp decline in enzyme production was noted as the pH was increased beyond 9.5 (Fig. 3b). Though *Streptomyces* sp. GS-3 can survive in an alkaline environment and sustain enzyme production, excess alkalinity (> 9.5) restricts its growth. Similar findings were earlier observed with the other strains of *Streptomyces* sp. E-99-1333 (Kontro et al. 2005). After 9.5 d of inoculation, the yield decreases (Fig. 3c) the bacterium entered the death phase due to the lack of nutrients in media, causing a notable decrease in enzyme production after the 10th day. The degradation of the existing enzyme in the media is also responsible for the lowered enzyme yield (Sharma et al. 2009).

The enzyme extracted from *Streptomyces* sp. GS-3 was purified, and the molecular weight was calculated to be 25 kD. Previous reports show that low molecular weight proteases (18–35 kD) from other actinobacteria belong to the class of serine proteases (Kim et al. 2006). These low molecular weight proteases have significant applications in various industries, such as food, detergent, textile, and leather industries (Li et al. 2013). The K_m and V_{max} values of the enzyme were determined to be 5.88%, and $38.46 \mu\text{mol l}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$, respectively, using 1% casein as substrate. The K_m value depends on the substrate, and other associated conditions such as temperature, and pH. This value represents the binding affinity of the substrate towards the enzyme. V_{max} values represent the number of substrate molecules being catalyzed per minute. The optimum temperature for the maximum enzyme activity was found to be 45°C , which implies that the enzyme is also thermostable (Briki et al. 2016). Such thermostable proteases are important in biotechnological, and industrial applications due to their stability against denaturing agents, and other chemicals (Barzkar et al. 2018). The enzyme shows maximum activity at pH 9, and hence can be classified as an alkaline protease. Similarly, a novel, thermostable alkaline serine protease with an optimum pH, and temperature of 9, and 60°C , respectively were obtained from a newly recognized strain, *Aeribacillus pallidus* C10 (Yildirim et al. 2017). Such enzymes are robust

and have a huge impact on different industries like leather, detergent, and food. Enzyme activity was inhibited by PMSF even at the minimum concentration (2.5 mmol l^{-1}) as it strongly blocks the active site of the enzyme, which leads to complete loss of enzyme activity (Rao et al. 1998). As PMSF is a known inhibitor of serine proteases (Geng et al. 2016), it indicates that the isolated enzyme could be a serine-type protease. The enzyme was mostly stable in the presence of metal ions such as Cu^{2+} , Hg^{2+} , Zn^{2+} , Ca^{2+} , and Mg^{2+} ions. Reports also support the fact that metal ions do not affect protease activity (El-Sayed et al. 2012; Jani et al. 2012). The enzyme was stable in the presence of different organic solvents. However, these solvents act as a medium for enzymatic reactions but can also be responsible for deactivating these enzymes by changing the structural flexibility of the enzyme (Yildirim et al. 2017). This was consistent with the results observed for the protease produced from *Streptomyces* sp. AB-1, which was also stable in different organic solvents (Jaouadi et al. 2010). Proteases that are stable in the presence of organic solvents are very useful for synthetic reactions.

Mostly, contact lenses were cleaned with three different types of solutions such as surfactant, oxidative, and enzymes. Surfactants are non-toxic to lenses but do not remove the protein deposits efficiently (Szczotka-Flynn et al. 2010). Oxidative cleaners are effective in removing non-protein deposits from contact lenses, however, are harmful to the lenses (Jung and Rapp 1993). Enzyme cleaners are safe to lenses and efficient in removing the main component of contact lens debris, namely lysozyme. Bacterial protease degrading lysozyme was known for successfully removing the protein debris, and acting as a cleansing agent (Greene et al. 1996). To study the efficiency of the enzyme as a cleansing agent, the lenses were previously coated with an artificial tear solution. The spectroscopic analysis of the contact lens indicated that before coating the contact lenses with lysozyme the percent transmittance was 97%, and by the earlier studies (Jadhav et al. 2014), after deposition of protein, it was reduced to 68%. After enzymatic treatment, the transmittance was again increased to 95% (Table 5). Thus the increased transmittance indicated that enzyme has potential in the removal of protein deposits from the contact lens. Similarly, a post-treatment transmittance of control using phosphate buffer was 70%, indicating no protein removal. Effects of treatment of lenses with enzyme, and phosphate buffer are statistically significant.

5. Conclusion

In the current study, marine halophilic, and alkaliphilic strain *Streptomyces* sp. GS-3 was isolated as a potential source for alkaline protease. The growth media and the culturing conditions were optimized for *Streptomyces* sp. GS-3. Wheat bran was used as a substrate while peptone and yeast extract served as a nitrogen source. The enzyme was precipitated using 100% acetone and further purified by dialysis method. The activity of extracted protease was 341 U/ml and has maximum activity at 45 °C, and pH 9. The K_m and V_{max} values of the enzyme were determined to be 5.88%, and $38.46 \mu\text{mol l}^{-1} \text{ min}^{-1} \text{ mg}^{-1}$, respectively, using 1% casein as substrate. The enzyme was sustained at high temperature (50 °C for 6 h), alkaline conditions (pH – 10), and the presence of metal ions, and organic solvents. The protein solution was degraded by the purified enzyme in 60 min. Furthermore, the activity of protease on artificial tear

solution was demonstrated. Thus, the protease enzyme can be used along with other chemical disinfectants to clean the contact lenses and has potential application in contact lens cleansing as a non-hazardous, and bio alternative solution.

Abbreviations

RSM

Response Surface Methodology

SDS-PAGE

Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis

CD

Circular Dichroism

PMSF

Phenylmethanesulfonyl fluoride

ISP

International Streptomyces Project

DNA

Deoxyribonucleic acid

PCR

Polymerase Chain Reaction

BLAST

Basic Local Alignment Search Tool

MEGA

Molecular Evolutionary Genetics Analysis

BM

Basal media

EDTA

Ethylenediamine tetraacetic acid

Declarations

Ethics approval and consent to participate –

Not applicable

Consent for publication –

Not applicable

Availability of data and materials –

Not applicable

Competing interests -

The authors declare that they have no competing interests.

Funding –

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Authors' contributions –

SK gave the concept and designed the work. GS analyzed and interpreted the data. GS drafted the manuscript and SK have done the revision and final correction of the manuscript.

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Tables

Table 1: Protease extracted from Streptomyces sp GS-3 in different basal media combined with different substrates

	SKIM MILK (U/ml)	CASEIN (U/ml)	WHEAT BRAN (U/ml)	RICE BRAN (U/ml)
BM 1	291	295	325	282
BM 2	239	275	340	247
BM 3	241	286	357	231
BM 4	239	277	248	284
BM 5	246	315	279	268
BM 6	244	302	341	275
BM 7	301	249	335	246
BM 8	285	298	324	274
BM 9	307	269	326	287
BM 10	294	304	315	235

Table 2: The Box-Behnken design matrix for the variables along with actual, and predicted responses

Order	Substrate (%) (A)	pH (B)	Incubation (Days) (C)	Yield (U/ml)	Predicted yield
1	10	1	0	254	262.881795
2	10	0	-1	54	73.002035
3	5.5	0	0	359	369.806645
4	5.5	0	0	357	369.806645
5	10	-1	0	72	78.88172
6	5.5	1	-1	43	37.1269425
7	5.5	-1	-1	34	-27.8731075
8	1	0	-1	20	50.751785
9	1	0	1	55	26.01425
10	5.5	0	0	365	369.806645
11	5.5	-1	1	109	57.8894225
12	10	0	1	301	284.26448
13	1	1	0	39	11.131455
14	5.5	1	1	123	137.8893225
15	5.5	0	0	354	369.806645
16	1	-1	0	30	50.13158
17	5.5	0	0	253	369.806645

Table 3: ANOVA for Response Surface Quadratic Model of *Streptomyces* sp. GS – 3

Source	Sum of Squares	df	Mean Square	F Value	p-value > F	
Model	228188	9	25354.22	22.68327	0.0002	significant
A-Substrate	36046.13	1	36046.13	32.24883	0.0008	
B-pH	5724.5	1	5724.5	5.121449	0.0581	
C-Incubation Time	23871.13	1	23871.13	21.35641	0.0024	
AB	7482.25	1	7482.25	6.694028	0.0361	
AC	11236	1	11236	10.05234	0.0157	
BC	6.25	1	6.25	0.005592	0.9425	
A^2	29006.32	1	29006.32	25.95063	0.0014	
B^2	54002.37	1	54002.37	48.31346	0.0002	
C^2	45980	1	45980	41.13621	0.0004	
Residual	7824.25	7	1117.75			
Lack of Fit	5610.25	3	1870.083	3.378651	0.1351	not significant
Pure Error	2214	4	553.5			
Cor Total	236012.2	16				

Table 4: Comparison of transmittance (%), and removal of protein debris from contact lenses

Lenses in different enzyme solutions	Transmittance (%)			
	0 min	15 min	30 min	60min
Untreated lenses	68	68	67	67
Lenses in phosphate buffer	68	68	69	70
Lenses in an enzymatic solution	69	87	93	95

Figures

Figure 1

(a) White powdery colonies of GS-3 on starch casein agar; (b) 40X magnification of the spores joining to form mycelium; (c) SEM image of GS-3 showing the mycelial chains with elongated spores; (d) Phylogenetic tree based on the neighbour-joining method with 1000 bootstrap value. The phylogram shows the position of GS-3 along with the other *Streptomyces* strains.

Figure 2

Three-dimensional response surface plot for the different parameters responsible for protease yield by GS-3; (a) substrate concentration, and pH; (b) Substrate concentration, and incubation time; (c) pH, and incubation time.

Figure 3

(a) SDS-PAGE for the sample using CBB Staining; (b) HPLC chromatogram of the isolated protease showing the retention time of 2.5 minutes; (c) The UV spectra (190 – 320 nm) of the purified enzyme, 1mg/ml of protein in 0.5M Tris - HCl buffer, pH – 8.5;

Figure 4

(a) Effect of different temperature (20 – 50°C) on the protease activity till 10 hours; (b) Effect of different pH (2 – 10) on the protease enzyme activity; (c-e) Residual activity is given in (%); (c) Effect of protease inhibitor on protease activity; (d) Effect on different metal ions (1mM) on protease activity; (e) Effect of organic solvents on protease activity.

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