

Optimization conditions of alkaline protease production by *Streptomyces* sp.H1 isolated from red sea coastal region in submerged culture

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Abstract

A potent alkaline protease producing strain characterized and identified as *Streptomyces* sp. H1 was isolated from soil around red sea shore. The enzyme was produced extracellularly in submerged culture revealed maximum level during early stationary phase. Alkaline protease showed the highest activity at incubation time, pH and inoculum size of 3 days, 9 and 8% respectively. Among different carbon sources beet molasses gave a maximum production followed by starch, sucrose and fructose. High yield of protease production was noticed with casein followed by peptone, yeast extract and ammonium sulphate. Furthermore, it was optimized with 7g/l NaCl resulted in higher level of protease. Optimization of the process parameters resulted in about 3.4 fold increase in the alkaline protease. Partial purification of the crude enzyme was achieved by fractional precipitation using ammonium sulfate at 50% saturation. Due to the maximum production of protease in the presence of cheaper substrate as beet molasses, stability at alkaline pH 9 and temperature up to 70 oC besides salt tolerance make the strain and its enzyme useful in different industrial applications.

Keywords: 16S Rdna; Alkaline Protease; Optimization; *Streptomyces* SP; Submerged Culture.

1. Introduction

Besides the production of drugs, microorganisms serve as an excellent source for the synthesis of various useful industrial enzymes. Proteases are the most important group has wide applications as additive to detergents in several industrial processes (Suthindhiran, et. al., 2014). They catalyze the hydrolysis of protein into amino-acid especially alkaline type is increasingly finding potential future applications in pharmaceutical industry too (Singhal, et. al., 2012). Different kinds of microorganisms; bacteria, fungi, yeast, actinomycetes, in addition plants and mammalian tissues produced alkaline proteases (Mukesh, et. al., 2012 and El-Shafei, et. al., 2010). Moreover, microbial process may meet more easily the current market demand for industrial enzymes due to the rapid doubling time of microbes compared with plants or animals. The majority of microbial enzymes produced on a commercial scale are extracellular, and are potentially employed in industries because they exhibit stability to chemical and physical changes in the medium (Ramesh, et. al., 2009). Actinomycetes particularly *Streptomyces* are known to secrete multiple (greater) proteases in culture medium, which is generally regarded as safe with food and drug administration. *Streptomyces* spp., that have been reported to produce proteases include *S. clavuligerus*, *S. griseus*, *S. rimonus*, *S. thermoviolaceus* and *S. thermovulgaris* (Okpukpara and George-Okafor, 2016). As the composition of culture medium strongly influences enzyme production (Jayasree, et. al., 2009 and Arifuzzaman, et. al., 2010), therefore, this study was aimed in selecting novel alkaline protease-producing actinomycete and optimizing different fermentation parameters for obtaining high alkaline protease yield.

2. Materials and methods

2.1. Isolation and screening of microorganisms

Samples were collected from soil around red sea shore. For isolation of actinomycetes, starch casein agar medium was used and its composition was as follow: starch 15 g; casein 5 g; peptone 5.0 g; beef extract 3.0 g; NaCl 5.0 g; Nalidixic acid 10 µl/ml; nystatin 25 µl/ml, cyclohexamide 10 µl/ml; agar 15 g, distilled water 1000 ml [8]. All the isolated actinomycetes were screened for protease production by streaking separately on 1% casein and gelatin agar and the plates were incubated at 32°C temperature. After 5 days, the plates were observed for clear zones caused by hydrolysis of casein and gelatin around the colonies. Among 20 actinomycetes, an isolate was selected as it produced highest proteolytic activity.

2.2. Identification of the potent isolate

2.2.1. Phenotypic identification

The strain was identified by morphological, cultural, physiological, and biochemical characteristics according to the methods described in International *Streptomyces* Project (ISP) (Shirling and Gottlieb, 1966). Color of aerial mycelium was determined from mature, sporulating aerial mycelia of the actinomycetes colonies on starch-casein agar media after 14 days at 30 °C (Pridham, 1964). Morphological observations of spore and mycelia were studied by light microscope through the oil immersion (1000×, Olympus) microscope done by cover slip culture method (Kawato and Shinobu, 1959) as well as scanning electron microscope (JEOL JSM 5300, JEOL

Techniques Ltd., Japan). The observed structure was compared with Bergey's manual of Determinative Bacteriology (Bergey's, 2000) and the organism was identified.

2.3. Molecular identification

2.3.1. DNA isolation and manipulation

Two milliliters of the potent isolate spore suspension were inoculated into the starch–nitrate broth and incubated for 3 days on a shaker incubator at 200 rpm and 30°C to form a pellet of vegetative cells (pre-sporulation). Then the culture was centrifuged for 10 min at 5000 rpm and the supernatant was discarded and the preparation of total genomic DNA was carried out (Sambrook, et. al., 1989).

2.3.2. Amplification and sequencing of the 16S rDNA gene

The polymerase chain reaction (PCR) using primers designed to amplify 1500 bp fragment of the 16S rDNA gene of the local actinomycete strain was performed. A total volume of 100 µl, which contained 1 µl DNA, 10 µl of 250 mM deoxyribonucleotide 5'-triphosphate (dNTP's); 10 µl PCR buffer, 3.5l 25 mM MgCl₂ and 0.5µl Taq polymerase, 4 µl of 10 pmol (each) with forward 16S rRNA primer 27f (5'-AGATTTGATCMTGCCTCAG-3') and reverse 16S rRNA primer 1492r (5'-TACGGYTACCTGTAC-GACTT-3') and water was added up to 100 µl. Amplification was conducted for 30 cycles of 1 min at 94°C, 1 min of annealing at 53°C, and 2 min of extension at 72°C. The PCR reaction mixture was then analyzed via agarose gel electrophoresis, and the remaining mixture was purified using QIA quick PCR purification reagents (Qiagen, USA). The purified PCR product was sequenced by using two primers, 518F; 5'-CCA GCA GCC GCG GTA ATA CG-3' and 800R; 5'-TAC CAG GGT ATC TAA TCC-3' using a Terminator Cycle Sequencing kit (ABI Prism 310 Genetic Analyzer, Applied Biosystems, USA). Sequencing product was resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

2.3.3. Sequence similarities and phylogenetic analysis

The BLAST program (www.ncbi.nlm.nih.gov/blst) was employed in order to assess the degree of DNA similarity. Multiple sequence alignment and molecular phylogeny were evaluated using BioEdit software (Hall, 1999). The phylogenetic tree was displayed using the TREE VIEW program.

2.4. Medium for protease production

The entire study on nutritional factors affecting protease production was carried out in Erlenmeyer flasks (250 ml) containing 50 ml of the production fermentation medium: glucose 5 g/l; peptone, 10 g/l; NaCl, 5g/l; K₂HPO₄, 0.5 g/l; MgSO₄.7 H₂O, 0.5 g/l). The pH of the media was adjusted at [7]. For inoculation, sterile medium containing yeast extract 4 g/l, malt extract 10 g/l, dextrose 4 g/l was prepared and inoculated with a fully sporulated slant of the producer isolate. Inoculated flask was shaken in an incubator at 200 rpm and 32°C. After about 48h (at logarithmic phase), 1.5 ml of the seed culture was transferred into 50 ml of sterile production media and incubated at 32 °C for 6 days on a rotary shaker at 200 rpm. After incubation period, the cells were removed by centrifugation at 5000 rpm for 10 min., and then the mycelium free supernatant was used as a crude enzyme for estimation of protease activity. The experiments were carried out in triplicate and the average values are presented.

2.5. Optimization of culture conditions

Various parameters were studied for maximum alkaline protease production in submerged culture include investigation of incubation time (1-6days), pH (7-11) and inoculum size (1-5 percent). The cultures were incubated at 32°C and analyzed for protease activity. 2.6.

Effect of different carbon and nitrogen sources on protease production

To test the effect of different carbon sources on protease production, glucose in the basal medium was substituted with (0.5%w/v) of fructose, starch, sucrose, beet molasses and glycerol which were separately added as a sole carbon. To test the effect of different nitrogen sources, peptone in the basal medium was substituted with 1% w/v of yeast extract, casein, ammonium sulphate, ammonium nitrate, sodium nitrate and urea. Whereas, a control represented by the production basal medium was performed at the same time. Media were inoculated and incubated at 32 °C for 6 days with shaking at 200 rpm.

2.7. Enzyme assay

Protease activity in the culture supernatant was determined using casein as a substrate [15]. To 3 ml of 0.6% casein solution (pH 9.0, prepared in 10 mM Tris-HCl buffer), 0.5 ml of diluted enzyme solution was added and the reaction mixture was incubated at 40°C for 10 min. The reaction was terminated by the addition of 3.2 ml of a mixture of 0.11 M Trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid and the reaction mixture was allowed to stand for 15 min followed by centrifugation to separate the non-reacted casein at 5000 rpm for 20 min. The supernatant was mixed with 2.5 ml of 0.4 mol/l Na₂CO₃ and 1 ml of 3-fold diluted Folin-Ciocalteu phenol reagent was added. The resulting solution was incubated at room temperature in the dark for 30 min and the absorbance of the blue color developed was measured 660 nm (Tsuchida, et. al., 1986 and Lowry, et. al., 1951) against a reagent blank using a tyrosine standard. One unit of protease activity is expressed as the amount of enzyme which converts 1.0 mg of protein per 10 min at 40°C.

2.8. Determination of protein content

To determine the protein content, a standard curve of protein concentration was generated using Coomassie Brilliant Blue (CBB) and bovine serum albumin (BSA) [17]. The absorbance was plotted against the protein content. The protein content of the unknown sample was calculated from the standard curve.

2.9. Partial purification of alkaline protease from culture filtrate

The ammonium sulphate was added slowly to the supernatant containing the crude enzyme with stirring until the required saturation of ammonium sulphate was reached ranging from 35-80% saturation. The saturated enzyme solution was kept overnight at 4°C. The precipitated protein obtained from each saturation was collected by centrifugation at 5000 rpm for 30 min at 4°C, dissolved in deionised water and dialysed for 2 days against phosphate buffer 0.1M pH 7.0. Then the enzyme was assayed for its activity.

2.10. Effect of temperature on enzyme activity and stability

The partially purified enzyme was stored at different temperatures ranging from 30 to 70° C. The time of incubation of the samples varied from 30 to 60 minutes at pH 9. After incubation, the samples were submitted to determination of protease activity.

3. Results and discussion

3.1. Screening for protease activity

Results showed that almost all actinomycete isolates were capable of hydrolyzing protein in agar media. A difference in protease activity of the tested isolates was observed, this is might be due to taxonomic differences between strains living in the same zone of isolation (Bradford, 1976 and Strzelczyk & Szpotański, 1989). Out of the 20 colonies of actinomycetes screened, one isolate showed prominent proteolytic activity with maximum zone of clearance on

casein and gelatin agar. The result obtained from the proteolytic assay revealed that actinomycetes isolate was found to have the highest potential for protease production. The result was similar to proteolytic activity obtained from *Streptomyces pulveraceus* (Jayasree, et. al., 2009). Actinomycetes, are known to be good protease producers particularly *Streptomyces* sp. (Al-Askar, et. al., 2015a). It was discovered that aerial mycelium formation and sporulation are related to the production of extracellular proteases (Kitadokoro, et. al., 1994).

3.2. Identification of the isolate

Based on colonial appearances, the powdery whitish gray colored isolate with highest proteolytic activity was a Gram positive (Korkare, et. al., 2004), filamentous organism showed no distinctive or diffusible pigments, produced mycelium, aerial hyphae and non-motile spores with filamentous colony margin. The isolate showed reflexible spore chain, spores appear abundantly, white in color, oval to cylindrical in shape, and smooth in surface (Fig.1). The cell wall hydrolysate contains LL-diaminopimelic acid with no characteristic sugars. Other characteristics of the selected strain were observed on the basis of morphological, physiological and biochemical tests are summarized in Table 1. Based on results of growth characterization, the isolate was identified as a species belonging to the genus *Streptomyces* (Holt, et. al., 1994).

The ability of the actinomycetes species mostly reported by *Streptomyces* which produce whitish gray colored colony with its mycelia, which has good hydrolytic properties (Jayasree, et. al., 2009).

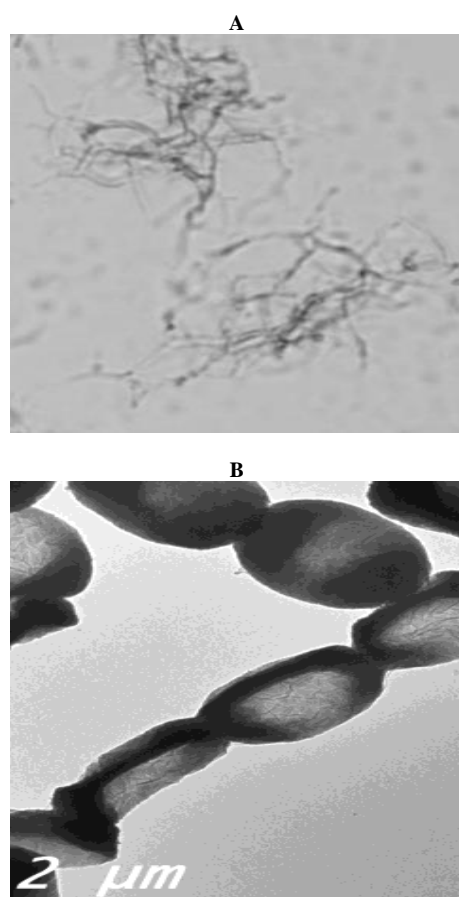


Fig. 1: A) Light Microscope 100X Magnification Showing Substrate and Aerial Hyphae and B) TEM Micrograph for Spores (Smooth) of the Potent *Streptomyces* Isolate Grown in Optimized Medium at 32 °C for 7days.

Table 1: Growth Characteristics of the *Streptomyces* Isolate

Morphological characteristic	Result
Grams stain	+
Cell morphology	Mycelial
Spore chains	rectiflexible
Spore surface	smooth
Colony color	White/ grey
Diffusible pigment	-
Motility	-
Cell wall hydrolysate	
Diaminopimelic acid (DAP)	+
Physiological and biochemical properties	
Hydrolysis of	
Starch	+
Protein	+
Casein	+
Citrate utilization test	+
Catalase	+
Gelatin Liquefaction	+
H ₂ S production	-
Indole production	-
Nitrate reduction	+
Degradation of	
D - glucose	+
L - rhamnose	+
D - mannitol	+
Mannose	+
Dextrine	+
D - xylose	+
D - galactose	+
D - fructose	+
Lactose	+
Raffinose	+
L- inositol	+
Cellulose	+
Sucrose	+

After genomic DNA separation and purification, the sequence of the 16S rRNA gene was determined in order to achieve the phylogeny. Search for similar sequences in the GenBank database and subsequent alignment of the retrieved sequences indicated that the DNA sequence showed high similarity (99–100%) to the known 16S rRNA gene sequences from *Streptomyces*. Based on molecular taxonomy and phylogenetic analysis for precise genera identification, the strain was identified as *Streptomyces* sp and designated as *Streptomyces* sp. H1. A neighbor-joining tree based on 16S rRNA gene sequence revealed that the strain H1 belongs to the new species as it occupies a distinct phylogenetic position and different from other strains of *Streptomyces* (Fig. 3).

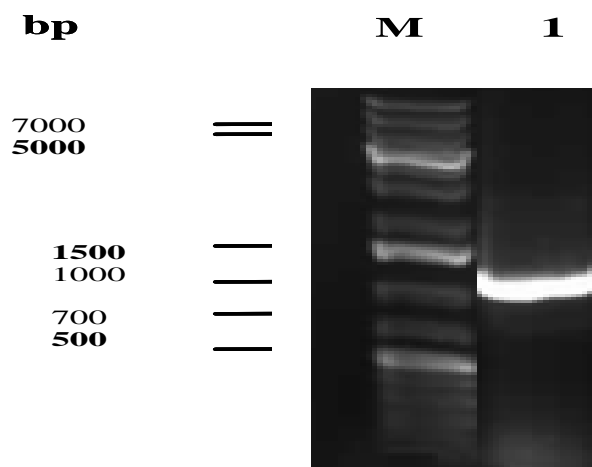


Fig. 2: M: Agarose Gel Electrophoresis for PCR Product: Kb-Standard, Lane 1: the Amplified Fragment Using *Streptomyces*'s Primer for the Selected Isolate.

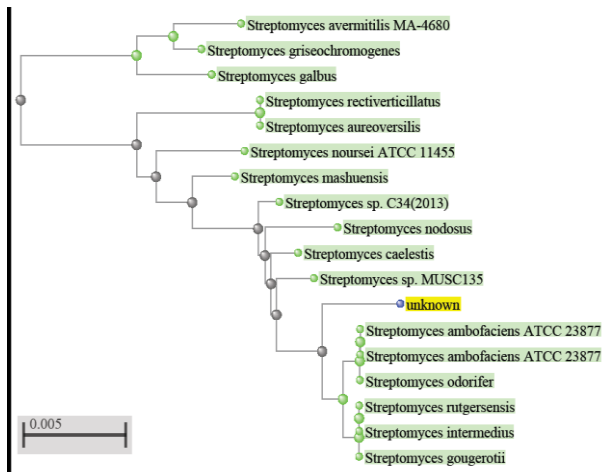


Fig. 3: Neighbour Joining Phylogenetic Tree of the Streptomyces Sp.H1 (The Unknown) Based on 16S Rrna Gene Sequences Shoing H1 Strain and the Nearest Related Taxa.

Description	Ident	Accession
Streptomyces ambofaciens ATCC 23877 complete genome	99%	CP12382.1
Streptomyces ambofaciens ATCC 23877 left chromosomal arm	99%	4023893.1
Streptomyces rubeus strain DSM 43077 16S ribosomal RNA partial sequence	99%	NR_119348.1
Streptomyces intermedius strain DSM 43074 16S ribosomal RNA partial sequence	99%	NR_119347.1
Streptomyces rubeus strain DSM 43024 16S ribosomal RNA partial sequence	99%	NR_119346.1
Streptomyces odorifer strain DSM 43047 16S ribosomal RNA gene partial sequence	99%	NR_326535.1
Streptomyces caelestis strain 1895_2419 16S ribosomal RNA partial sequence	99%	NR_326268.1
Streptomyces nodosus strain ATCC 14959 genome	98%	CP098313.1
Streptomyces galbus strain MUSC 135 complete genome	99%	CP151089.1
Streptomyces leucostictus genome assembly strC4 chromosome chromosome	99%	LN61730.1
Streptomyces mashuensis strain DSM 43071 16S ribosomal RNA partial sequence	98%	NR_326174.1
Streptomyces rectiverticillatus strain 1895_13079 16S ribosomal RNA gene partial sequence	98%	NR_119284.1
Streptomyces aureoveris strain 1895_13021 16S ribosomal RNA gene partial sequence	98%	NR_119283.1
Streptomyces noursei ATCC 11455 complete genome	97%	CP151533.1
Streptomyces parvulus 1895_14993 16S ribosomal RNA complete genome	96%	RA000039.4
Streptomyces galbus strain DSM 43088 16S ribosomal RNA partial sequence	96%	NR_326173.1
Streptomyces griseochromogenes strain ATCC 14571 complete genome	96%	CP162079.1

Fig. 4: Similarity of 16S RRNA Gene Sequences in the Genbank.

3.3. Optimization of alkaline protease production

3.3.1. Effect of incubation period

The study was carried out from 1-7 days incubation time and maximum enzyme formation (230 u/ml) was recorded with 3 days of incubation period (Fig.5). The results revealed that there was an induction phase of proteolytic activity in the first 24 h. During which considerable alkaline protease was synthesized. It was indicated by an activity of 22 u/ml and 58.36 u/ml in the first and second days respectively. The specific activity sharply increased to a maximum value in the third day of incubation as well. Following this phase, Both enzyme activity and specific activity decreased gradually from the 4th day to the 7th day. These results confirmed the observation reported previously which described an increase of protease production by Streptomyces cyanens and S. rimosus during log phase. This indicates that high level of alkaline protease production was observed during production of active biomass (Jayasree, et. al., 2009). This result was somewhat different from the other organism Streptomyces clavuligerus which starts synthesis of protease in the post-exponential phase of growth (Moreira, et. al., 2001).

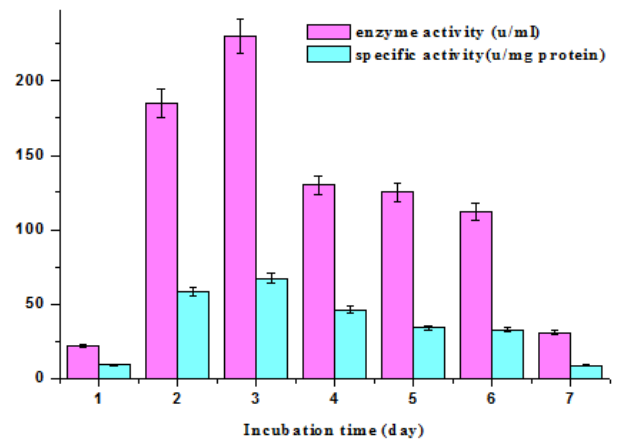


Fig. 5: Effect of Various Incubation Period on Protease Production by the Selected Strain Streptomyces Sp.H1. the Bars Indicate the Standard Deviation of Three Analyzed Replicates.

3.3.2. Effect of initial pH

The effect of initial pH of the fermentation medium on alkaline protease production from the selected strain Streptomyces sp.H1 was studied. It was indicated that maximum enzyme activity (244 u/ml) and specific activity (72.83 u/mg protein) occurred when the selected strain Streptomyces sp.H1 was cultured in a medium having pH 9 (Fig.6). These results are in accordance other observations reported when Str. clavuligerus grows optimally at pH 9.0. Similarly, it was reported that alkaliphilic Streptomyces sp. grows at an optimum pH 8-9 with scant growth at pH 7.0 (El-Sayed, et. al., 2012). Therefore, optimized pH 9 was applied in all he following experiments.

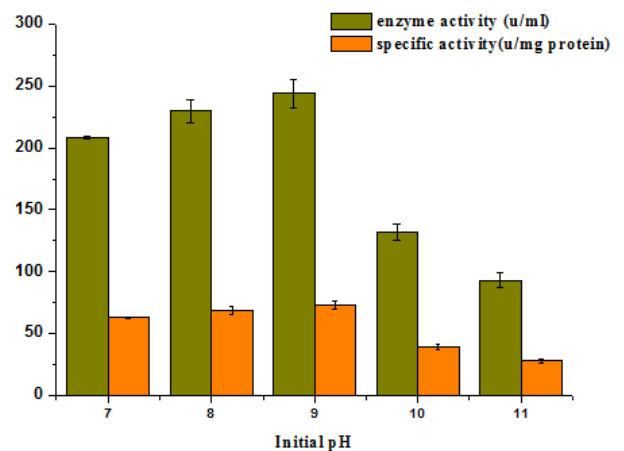


Fig. 6: Effect of Various Ph on Protease Production by the Selected Strain Streptomyces Sp.H1. the Bars Indicate The Standard Deviation of Three Analyzed Replicates.

3.3.3. Effect of inoculum sizes

The result revealed that, protease production and the specific activity were optimum when 8.0% (v/v) of inoculum size was used recording 505.42 u/ml and 196.5 u/mg protein respectively. Higher inoculum sizes 10% decreased the protease production by 7% (Fig.7). Therefore, high cell densities in inoculum size over 8% might not necessarily give higher alkaline protease, it could result in nutrient depletion and the lack of oxygen and in the culture media and yield (Haritha, et. al., 2012).

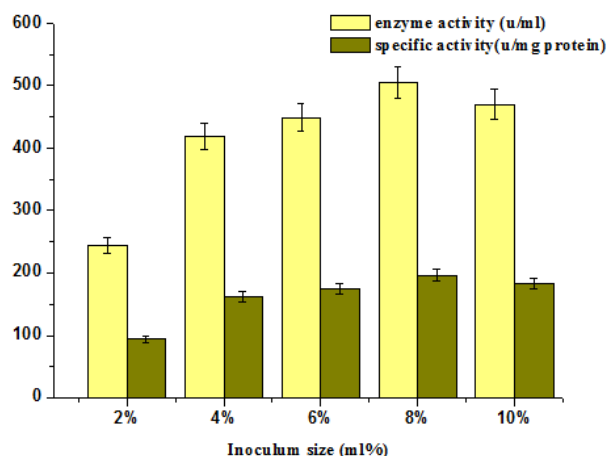


Fig. 7: Effect of Different Inoculum Percentages on Alkaline Protease Production by the Selected Strain *Streptomyces* Sp.H1. the Bars Indicate the Standard Deviation of Three Analyzed Replicates.

3.3.4. Effect of different carbon sources

Among various carbon sources used, beet molasses enhanced alkaline protease production (Fig. 8). It showed the highest activity of 720 u/ml corresponding to specific activity of 220 u/mg protein. Starch and sucrose were the second carbons that showed better activity than recorded in the control basal medium with optimized pH 9 and inoculum size 8% containing glucose as a carbon source. Sugars like fructose and glucose showed a moderate enzyme activity while glycerol showed the least activity (Fig.8). Similarly, results reported by Chi and Zhao (2003) revealed that different carbon sources have different influence on extracellular enzyme production by different strains. Sugar beet molasses is a well-known multifunctional nutrient contains sucrose which support slower growth than glucose but a higher rate of specific productivity. In addition, it contains many of the essential minerals and trace elements that may act as macro or micronutrient (Lotfy, 2007). According to these results, beet molasses will substitute glucose in the basal medium in the following experiments.

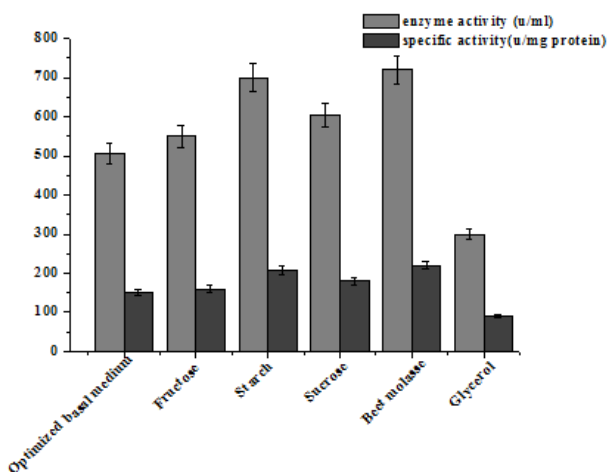


Fig. 8: Effect of Different Carbon Sources on Alkaline Protease Production by the Selected Strain *Streptomyces* Sp.H1. the Bars Indicate the Standard Deviation of Three Analyzed Replicates.

3.3.5. Effect of different nitrogen sources

The extracellular protease production could considerably be affected by the protein substrate and the composition of the medium (Al-Askar, et. al., 2015a). From the results, alkaline protease formation was enhanced by the organic nitrogen sources more than the tested inorganic nitrogen sources (Fig. 9). Among the organic nitrogen sources, Casein was found to enhance maximum enzyme production yield, enzyme activity of (745 u/ml) and specific activity of (208.9 u/mg protein) which was about 3.4% higher than peptone in the control basal medium. It was observed that, peptone followed

by yeast extract, were found to have moderate enzyme production showed activity of 720 and 668u/ml, and specific activity of 201.7 and 187.25 u/mg protein respectively. Nitrogen source has been observed as another factor that influences protease production. Effect of the nitrogen source on extracellular protease produced by *S. clavuligerus* was reported depend on the nature of the nitrogen source, and a transient decrease in protease titers was also observed (De Azeredo, et. al., 2006).

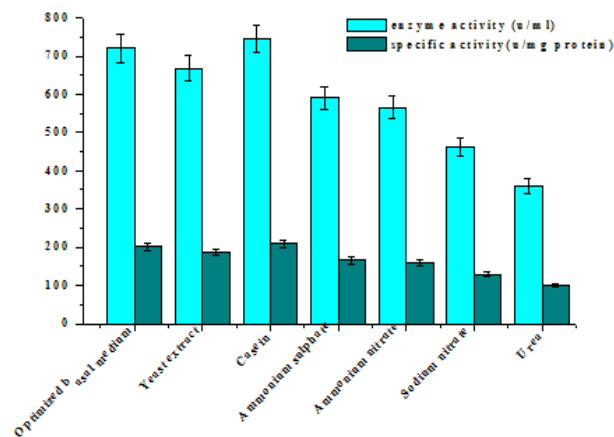


Fig. 9: Effect of Different Nitrogen Sources on Alkaline Protease Production by the Selected Strain *Streptomyces* Sp.H1. the Bars Indicate the Standard Deviation of Three Analyzed Replicates.

3.3.6. Effect of NaCl concentration the effect of salt on alkaline protease production was shown in

Fig. 10. Maximum protease production was observed in the medium containing 7 g/l NaCl (772.6 u/ml) at 72 h of incubation above this value alkaline protease was gradually reduced until 11g/l salt concentration. Salinity has pronounced effect on growth and enzyme production. The salt requirement of a truly halophilic *Streptomonospora Alba* sp. nov., was much more higher than that supported by the selected isolate under study. Similarly, although its presence was not essential for growth, *Kocuria marina* sp. nov., a novel actinomycete, tolerated up to 15% NaCl in growth media (Thumar and Singh, 2007).

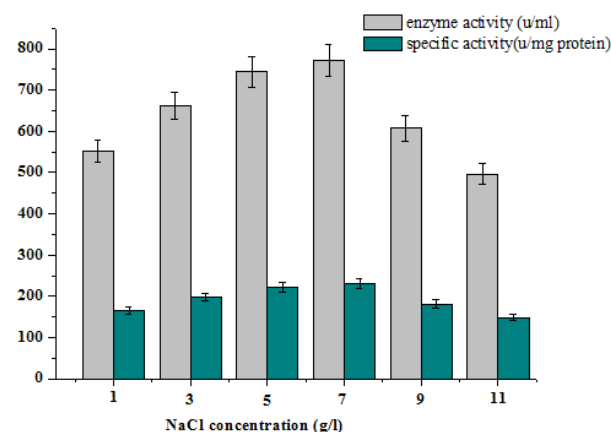


Fig. 10: Effect of Various Concentration of NaCl on Alkaline Protease Production by the Selected Strain *Streptomyces* Sp.H1. the Bars Indicate the Standard Deviation of Three Analyzed Replicates.

3.3.7. Partial purification of alkaline protease

The alkaline protease was partially purified from the culture filtrate of the selected strain *Streptomyces* sp.H1 using $(\text{NH}_4)_2\text{SO}_4$ precipitation (35-80%). The enzyme fraction salted out at 50% ammonium sulphate saturation was the most active fraction exhibited 14 fold purification (Fig.11).

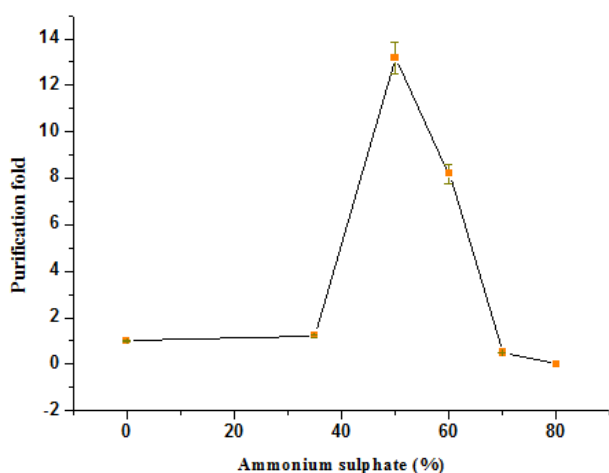


Fig. 11: Partial Purification of Alkaline Protease with Ammonium Sulphate.

3.3.8. Thermal stability of alkaline protease

The stability of the alkaline protease produced by the selected strain *Streptomyces* sp.H1 was tested at temperatures 60 °C and 70°C for 120 min. the results showed that the enzyme was thermostable for at least 1 hour retaining about 30% and loose 70% of its activity after heating at 70°C (Fig.12). Hence it is found that the alkaline protease form *Streptomyces* sp. H1 is thermostable and may be useful to various biotechnological processes.

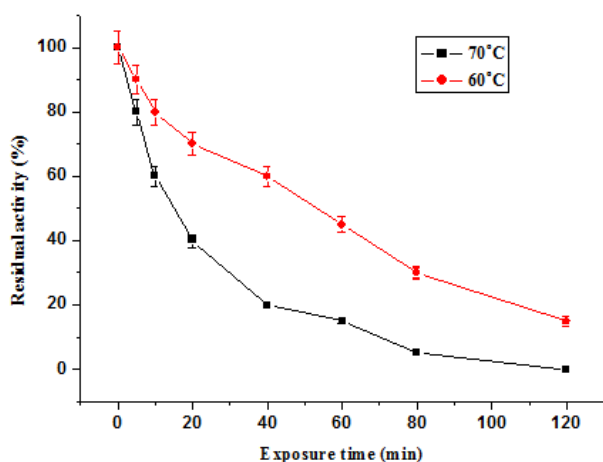


Fig. 12: Thermal Stability of Alkaline Protease at Different Incubation Temperatures 60°C and 70°C.

4. Conclusion

In the present study, an actinomycete isolated from soil around red sea coastal region having higher protease activity was selected for characterization and identification. The organism was identified as *Streptomyces* sp.H1. The use of low-cost carbon source beet molasses in the growth medium for production of this alkaline protease would significantly reduce the cost of enzyme production. The alkaline protease produced by the selected strain is thermostable can retain its activity at 70°C for 1 hour. These properties have a relevant economical and environmental impact in biotechnological applications.

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