SCREENING, OPTIMIZATION OF PRODUCTION AND PARTIAL CHARACTERIZATION OF ALKALINE PROTEASE FROM HALOALKALIPHILIC BACILLUS SP

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Abstract

Bacillus strains isolated from the salteren pond (Kakinada) were screened and identified for high alkaline protease activity. The isolates which were positive on skim milk agar (1%) were selected as protease producing strains. Of the ten bacterial isolates screened, isolate S-8 was observed as a potential haloalkaline protease producer and it was identified as Bacillus cereus strain S8 (MTCC NO: 11901) by 16S rRNA gene sequencing, phylogenetic tree analysis and by different biochemical tests. Protease production was enhanced by optimizing the culture conditions. The nutritional factors such as carbon and nitrogen sources, NaCl and also physical parameters like temperature, incubation time, pH, inoculum size were optimized for the maximum yield of protease. Studies on the effect of different carbon and nitrogen sources revealed that maximum protease production was obtained in the medium supplemented with Molasses,1%(w/v); Potassium nitrate, 0.75%(w/v); salt solution- 5%(v/v) {$MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4, 0.5%(w/v)}; $FeSO_4.7H_2O$, 0.01%(w/v) and $CaCO_3$, 0.5% respectively. Thus, with selected carbon and nitrogen sources along with 1 % NaCl and 2% inoculum the maximum protease production (205.0 U/ml) was obtained in the period of 72 h incubation at pH-12.0 under 160 rpm when compared to the initial enzyme production (165.0 U/ml). The crude enzyme extract of this strain was also characterized with respect to temperature, pH, incubation period and different concentrations of casein which was used as enzyme substrate. This study shows that the enzyme has wide range of pH stability from 8 to 11 with optimum activity at pH-10.0. It is thermostable with optimum activity at 70°C (392U/ml) with 1h incubation of enzyme with 1% casein as its substrate. From the above investigations it was concluded that the protease production by these microorganisms at wide temperatures and pH ranges could be explored for varied industrial applications.

***Keywords:** Bacillus cereus strain S8, Alkaline protease, Optimization, Protease production, 16S rRNA gene sequencing, phylogenetic tree analysis, Enzyme characterization.*

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1. INTRODUCTION

Proteases are one of the most important classes of enzymes, occupying a major share of 60% of total enzyme market [1]. Proteolytic enzymes are ubiquitous in occurrence, being found in all living organisms, are essential for cell growth and differentiation. Proteases represent one of the three largest groups of industrially important enzymes [2]. Alkaline proteases are of great interest because of their high proteolytic activity and stability under alkaline conditions [3, 4]. These enzymes find applications in detergents, feather processing, food processing, silk gumming, pharmaceuticals, bioremediation, biosynthesis, biotransformation, silver recovery from photographic film, production of digestive and certain medical treatments of inflammation and virulent wounds [5, 6, 7, 8, 9].The majority of commercial alkaline proteases are produced by bacteria, especially *Bacillus* sps. [9]. Since the first alkaline protease Carlsberg from *Bacillus licheniformis* was commercialized as an additive in detergents in 1960s [4], a number of *Bacillus* derived alkaline proteases have been purified and characterized because of their significant proteolytic activity, stability, broad substrate specificity, short period of fermentation, simple downstream purification and low cost [3, 10].

Multiple applications of these enzymes stimulated interest to discover them with novel properties and considerable advancement of basic research into these enzymes. Microbial proteases also play a crucial role in numerous pathogenic processes mainly responsible for degradation of elastin, collagen, proteoglycans and also proteins that function in vivo host defence. Identification and characterization of microbial protease are prerequisite for understanding their role in pathogenesis [11]. Furthermore studies have shown that nutritional factors including sources of carbon and nitrogen can influence protease enzyme production [12]. Besides these nutritional factors, physical factors such as inoculum concentration [13], temperature, pH [14] and incubation time [15] can also significantly affect protease production and its

activity. The present investigation was aimed to isolate and characterize new promising strains using cheap carbon and nitrogen sources for enzyme production of different industrial uses [16] and to optimize the growth conditions of isolated Bacillus sps to enhance the protease production. With this in view, a halotolerant bacterium (Bacillus cereus strain S8) was isolated from salteren soil and characterized its protease production under various chemical and physical conditions, which could be a potential candidate for industrial use.

2. MATERIALS AND METHODS

2.1. Chemicals

All chemicals (99% purity) used in this study were purchased from Hi-Media Laboratories Merck (Mumbai, India) and Sigma (U.S.A).

2.2. Isolation and Screening of Protease Producing Bacterial Strain

Soil samples were collected in a sterile container from the Saltern Lake of Kakinada, East Godavari District, Andhra Pradesh, India. Collected sample was serially diluted in sterile saline water and the dilutions were placed on skim milk agar medium plates containing skim milk powder 1.0%, peptone 0.5%, sodium chloride 5% and agar 2.5%. The pH of the medium was adjusted to 9.0 with 1N HCl/1N NaOH before sterilization at 121^0C for 15 minutes. The inoculated plates were then incubated at 37^0C for 24hrs and observed for zones of clearance which indicates proteolytic activity. The proteolytic activity of these isolates was assayed by well boring method. In this method a well was created with the help of borer in the centre of the plate containing skim milk agar and 100µl of broth culture (isolated organisms) was added in the centre of the well and plates were incubated at 37^0C for 24 hours. Hydrolysis is expressed as diameter of clear zone in mm. The bacterial isolates with prominent zones of clearance were further processed.

2.3. Characterization and Identification of the Bacterial Strain

The protease producing strain S8 with highest diameter of proteolytic zone was characterized and further processed for the determination of morphology, gram's characteristics, motility, oxidase, citrate utilization, catalase, indole, vogues prosaukeur, casein, gelatin, starch, acid production from different sugars and was subjected to 16S rRNA gene sequencing and Phylogenetic tree analysis for its confirmation. The chromosomal DNA of strain S8 was isolated according to Rainey [17]. The 16S rRNA gene was amplified with primers 8-27f (5'-AGAGTTTGATCCTGGCTCAG-3') and 1500r (5'-AGAAAGGAGGTGATCCAGCCA-3'). The amplified DNA fragment was separated on 1 % agarose gel, eluted from the gel and purified using a QIAquick gel extraction kit (Qiagen). The purified PCR product was sequenced with four forward and three reverse primers, namely 8-27f, 357f (5'-CTCCTACGGGAGGCAGCAG-3'), 704f (5'-TAGCGGTGAAATGCGTAGA-3'), 1114f (5'-GCAACGAGCGCAACC-3'), 685r (5'-TCTACGCATTTCACCGCTAC-3'), 1110r (5'-GGGTTGCGCTCGTTG-3') and 1500r (*Escherichia coli* numbering system). Sequencing of the amplified product was done by dideoxy chain terminator method using the Big Dye terminator kit followed by capillary electrophoresis on an ABI 310 genetic analyzer (Applied Biosystems, USA). The identification of phylogenetic neighbours and the calculation of pairwise 16S rRNA gene sequence similarities were achieved using the EzTaxon server [18]. The 16S rRNA gene sequence of S-8 and the members of the closely related genera were retrieved from EzTaxon server and aligned using the MEGA software version 5.0 [19].Phylogenetic trees were constructed using the neighbour-joining as well as maximum parsimony algorithms. Bootstrap analysis was performed to assess the confidence limits of the branching [20].

2.4 Preparation of Inoculum

A slant culture of strain S8 was inoculated into 50ml of sterilized media of glucose, 0.5%(w/v); peptone, 0.75%(w/v); salt solution- 5% (v/v){($MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4, 0.5% (w/v)}; and $FeSO_4.7H_2O$, 0.01%(w/v) in 250 ml Erlenmeyer conical flask and incubated at 37^0C for 24 h in a shaker incubator.

2.5 Production of Enzyme

Protease enzyme production was carried out using standard media glucose, 0.5%(w/v); peptone, 0.75%(w/v); salt solution-5%(v/v) {($MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4 0.5%(w/v)}; and $FeSO_4.7H_2O$, 0.01%(w/v) at 160rpm. The culture medium was harvested and was subjected to centrifugation at 10,000 rpm for 20 min to obtain crude extract, which was used as enzyme source. The potential producer strain S8 was taken for further optimization studies to enhance the protease production.

2.6 Protein Estimation

Protein estimation was determined according to the method of Lowry [21], using crystalline bovine serum albumin as standard.

2.7 Assay of Alkaline Protease Enzyme Activity

The enzyme activity was determined by using Mc Donald & Chen method [22]. One ml of enzyme was added to 2 ml of casein (1% w/v in 0.1N Glycine – NaOH buffer pH 10) and the mixture was incubated for 15 min at 60^0C. The reaction was terminated by adding 3 ml of 10% trichloroacetic acid reagent and then centrifuged for 15 min at 10,000 rpm. Then 1 ml of filtrate was mixed with 5ml of alkaline copper reagent and after 15 min 0.5ml of Folin-ciocalteau reagent was added, up on

standing for 30 min the absorbance was read at 700nm. Similarly blank was carried out by replacing enzyme with distilled water. One unit enzyme activity is defined as the amount of enzyme that releases 1µg of tyrosine per ml per min under the assay conditions. The range of concentration 50–250 µg of tyrosine was used as standard.

2.8. Optimization of pH, Inoculum Size, Incubation Period and Temperature for Protease Production

The effect of various physical parameters on protease production was assessed by growing bacterial culture in the standard growth media. For optimizing pH, the medium was prepared by varying the pH from 2.0 to 12.0. Effect of varying inoculum percentage from 0.5% to 5% with 0.5% variation on protease production was determined. Similarly, for the investigation of optimal incubation time for protease production, the bacterial culture was inoculated in the growth media and optimized for different incubation periods up to 120h at 160rpm. Samples were withdrawn aseptically for every 6 h intervals and protease activity was determined. Different temperatures (20^0C to 70^0C) were also tested for optimizing the temperature.

2.9. Optimization of NaCl, Carbon and Nitrogen Sources on Protease Production

The effect of various chemical parameters on protease production was studied by varying the salt concentrations from 1% to 3% variation in standard growth media. Standard media was supplemented with various carbon sources such as glucose, mannose, maltose, soybean meal, wheat flour, sugarcane bagasse, rice bran, rice husk and molasses. It was also optimized with different inorganic and organic nitrogen sources such as potassium nitrate, sodium nitrate, ammonium chloride, ammonium sulphate and peptone, casein, skim milk powder, beef extract and yeast extract. Effects of various substrate concentrations (1%-5%) were also studied. After incubation with 2% inoculum at 160rpm, the cell free supernatants obtained by centrifugation at 10,000g were quantified for protease production. Addition of $CaCO_3$ to the optimized medium also showed significant increase in enzyme activity.

3. CHARACTERIZATION OF CRUDE PROTEASE ENZYME

The crude protease obtained from the strain S8, which showed the highest potential for proteolytic activity, was further subjected to preliminary characterization study. Therefore, the effects of pH, incubation period, temperature and substrate concentration on enzyme activity were studied. The procedures are outlined in detail below.

3.1. Effect of pH on Activity of Protease

The effect of pH on the proteolytic activity of crude alkaline protease from strain S8 was determined by assaying the enzyme activity at different pH values ranging from 2.0 to 12.0 using the following buffer systems: KCl-HCl (pH 2.0), Citrate (pH 3.0 to 6.0), phosphate (pH 7.0), Tris–HCl (pH 8.0 to 9.0), Glycine–NaOH (pH 10 to12).

3.2. Effect of Incubation Period on Activity of Protease

The effect of incubation period was determined by incubating the reaction mixture with enzyme at pH 10.0 at different incubation periods ranging from 5 minutes to 70 minutes with interval of about 5 minutes.

3.3. Effect of Temperature on Activity of Protease

The effect of different temperatures ranging from 20^0C to 100^0C with an interval of 5^0C was carried out by incubating the reaction mixture with enzyme at pH 10.0 for 1hr.

3.4. Effect of Substrate Concentration on Activity of Protease

Casein was used as substrate for enzyme assay. Different concentrations of casein (1% to 5%) in Glycine-NaOH buffer pH 10.0 was used as enzyme substrate with the above mentioned parameters to determine optimum concentration of substrate.

4. RESULTS

4.1 Isolation and Screening Of Protease Producing Bacterial Strain

In the present study, a total of 17 halotolerant bacteria from the soil of saltern pond have been screened for the presence of protease production on skim milk agar plates. Ten strains were identified as protease producers by zone of hydrolysis around the colonies were quantified their activity. Out of all the strains, strain S8 showed highest diameter of zone of hydrolysis (4.6cm) (Fig. 1) and production (165.0 U/ml) with respect to other strains and it was used for further protease optimization studies.

Fig: 1. Isolate- S8 showing zone of hydrolysis by well boring method

4.2. Morphological Characterization and Identification of the Bacterial Strain

Strain S8 was found to be Gram-positive rod shaped bacterium and showed NaCl tolerance up to 2%. Their morphological and biochemical characteristics were listed in Table 1. The results showed characteristic feature of *Bacillus sp.* Based on 16S rRNA gene analysis (Fig.2), the strain was phylogenetically characterized (Fig.3) and identified as the closest relative of *Bacillus cereus*, thus identified the strain belongs to Bacillaceae family and it was confirmed as new protease producing *Bacillus cereus strain S8* (MTCC NO: 11901).

Table.1 Details of parameters used for identification of isolated strain S8.

Biochemical Tests	Isolated strain S8
Morphology	Rods
Gram staining	Gram Positive
Endospore staining	Positive
Oxidase	Positive
Motility	Motile
Vogues prosakauer	Negative
Casein hydrolysis	Positive
Gelatin hydrolysis	Positive
Starch hydrolysis	Positive
Citrate utilization	Negative
Indole	Negative
H_2S production	Negative
Urease	Negative
Methyl red	Positive
Nitrate broth reactions	Positive
Acid production from	
Mannose	Positive
Glucose	Negative
Lactose	Negative
Galactose	Positive
Maltose	Negative

16S rRNA gene sequence

GATGAACGCTGGCGGCGTGCCTAATACATGCAAGTCGAGCGAATGGATTAAGAGCT
TGCTCTTATGAAGTTAGCGGCGGACGGGTGAGTAACACGTGGGTAACCTGCCCATA
AGACTGGGATAACTCCGGGAAACCGGGGCTAATACCGGATAACATTTTGAACCGCA
TGGTTCGAAATTGAAAGGCGGCTTCGGCTGTCACTTATGGATGGACCCGCGTCGCAT
TAGCTAGTTGGTGAGGTAACGGCTCACCAAGGCAACGATGCGTAGCCGACCTGAGA
GGGTGATCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCA
GTAGGGAATCTTCCGCAATGGACGAAAGTCTGACGGAGCAACGCCGCGTGAGTGAT
GAAGGCTTTCGGGTCGTAAAACTCTGTTGTTAGGGAAGAAACAAGTGCTAGTTGAAT
AAGCTGGCACCTTTGACGGTACCTAACCAGAAAGCCACGGCTAACTACGTGCCAGC
AGCCGCGGTAATACGTAGGTGGCAAGCGTTATCCGGAATTATTGGGCGTAAAGCGC
GCGCAGGTGGTTTCTTAAGTCTGATGTGAAAGCCACGGCTCAACCGTGGAGGGTCAT
TGGAAACTGGGAGACTTGAGTGCAGAAGAGGAAAGTGGAATTCCATGTGTAGCGGT
GAAATGCGTAGAGATATGGAGGAACACCAGTGGCGAAGGCGACTTTCTGGTCTGTA
ACTGACACTGAGGCGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGT
CCACGCCGTAAACGATGAGTGCTAAGTGTTAGAGGGTTTCCGCCCTTTAGTGCTGAA
GTTAACGCATTAAGCACTCCGCCTGGGGAGTACGGCCGCAAGGCTGAAACTCAAAG
GAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTAATTCGAAGCAACGC
GAAGAACCTTACCAGGTCTTGACATCCTCTGAAAACCCTAGAGATAGGGCTTCTCCT
TCGGGAGCAGAGTGACAGGTGGTGCATGGTTGTCGTCAGCTCGTGTCGTGAGATGTT
GGGTTAAGTCCCGCAACGAGCGCAACCCTTGATCTTAGTTGCCATCATTAAGTTGGG
CACTCTAAGGTGACTGCCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAATC
ATCATGCCCCTTATGACCTGGGCTACACACGTGCTACAATGGACGGTACAAAGAGCT
GCAAGACCGCGAGGTGGAGCTAATCTCATAAAACCGTTCTCAGTTCGGATTGTAGGC
TGCAACTCGCCTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGCATGCCGCG
GTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCACGAGAGTTTGTAAC
ACCCGAAGTCGGTGGGGTAACCTTTTTGGAGCCAGCCGCCTAAGGTGGGACAGATG
ATTGGGGTG

Fig.2: 16S rRNA sequence of isolated strain S8 done by dideoxy chain terminator method.

Phylogenetic tree

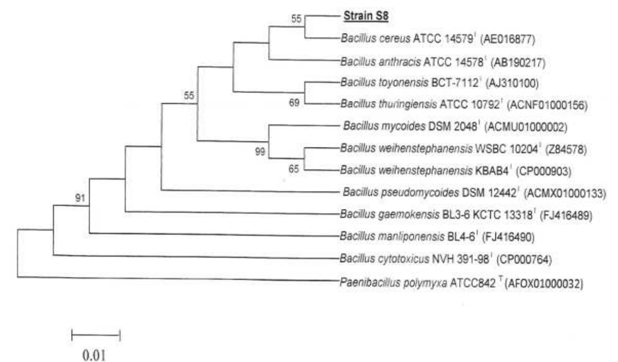


Fig 3: Phylogenetic relationship of strain S8 (Underlined) isolated from saltern sediments. The tree is constructed using 16S rRNA by neighbour joining and parsimony method.

4.3. Optimization of Culture Conditions for Protease Production

4.3.1. Effect of pH, Inoculum Size and Incubation Period and Temperature on Protease Production

B. cereus strain S8 could grow and produce protease over a wide range of pH (2.0–12.0). Maximum protease production was observed at pH 12 (160.50 U/ml) (Fig 4). The production at pH 10 and 11 was relatively comparable. In the present study, maximum protease production was observed at 2%

inoculum (170.00 U/ml) (Fig 5). The effect of incubation period on the protease production was shown in Fig 6. It shows that *B. cereus strain S8* has optimum protease production (174.30 U/ml) after 72h of incubation. The *B. cereus strain S8* also shows growth stability up to 60^0C with optimum growth rate at 37^0C (Fig 7).

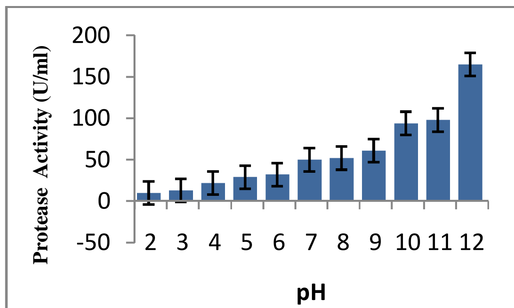


Fig 4: Effect of pH on protease production in *Bacillus cereus strain S8* isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

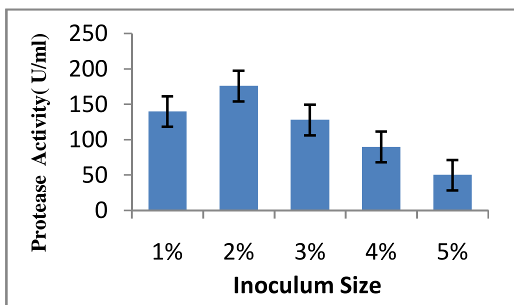


Fig 5 Effect of different inoculum concentrations on protease production in *Bacillus cereus strain S8* isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

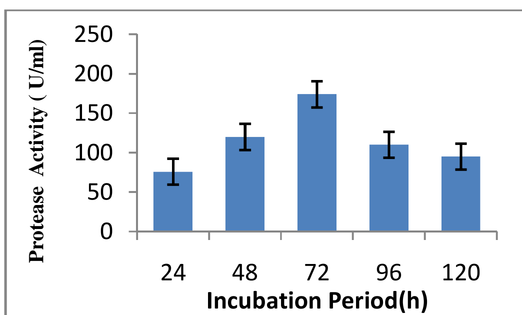


Fig 6: Effect of different incubation periods on protease production in *Bacillus cereus strain S8* isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

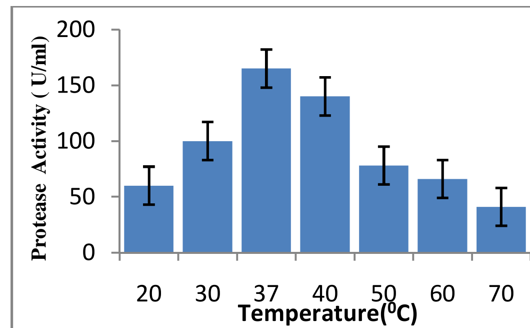


Fig 7 Effect of different temperatures on protease production in *Bacillus cereus strain S8* isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

4.3.2. Effect of NaCl, Carbon and Nitrogen Sources on Protease Production

The effect of salt on protease production showed optimum protease production in the medium containing 1% NaCl (109.52 U/ml) after 72 h of incubation with growth tolerance up to 3% NaCl. The growth and production of protease was reduced when salt concentration increases above 1% NaCl (Chart.7). Among various carbon sources used, protease production was highest in the medium containing molasses (205.00 U/ml) followed by maltose, rice bran and wheat flour as shown in (Chart. 8). Molasses can be easily utilized by *B. cereus strain S8* when compared to other carbon sources. Various nitrogen sources were investigated for protease production. High yield of protease production was observed in potassium nitrate (198.00 U/ml) (Chart. 9) followed by peptone and skim milk powder as nitrogen sources. Effects of different concentrations of carbon and nitrogen sources were carried out. It shows that maximum production was achieved at a concentration of Molasses (1%) and Potassium nitrate (0.75%).

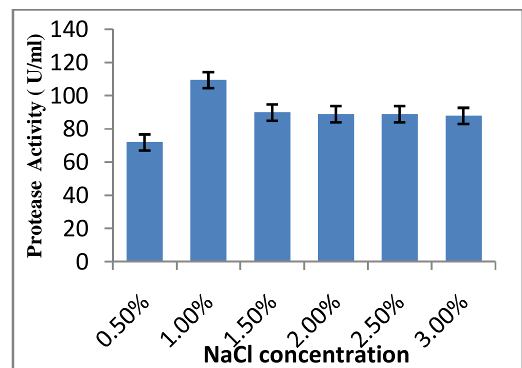
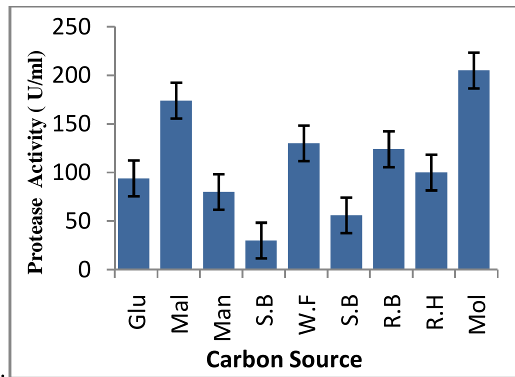
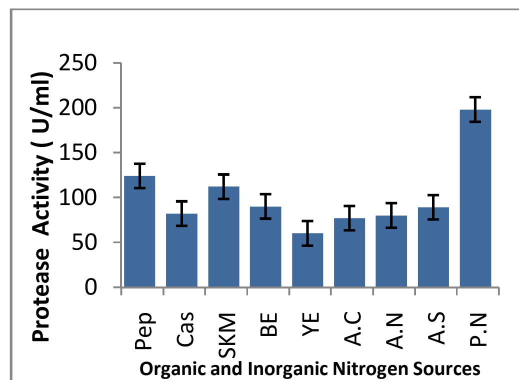


Fig 8. Effect of different NaCl concentrations on protease production in *Bacillus cereus strain S8* isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.



Glu: Glucose, Mal: Maltose, Man: Mannose, S.B: Sugarcane Bagasse, R.B: Rice bran, R.H: Rice husk, Mol: Molasses

Fig 9 Effect of different Carbon sources on protease production in *Bacillus cereus strain S8* isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.



Pep: Peptone, Cas: Casein, SKM: Skim milk powder, BE: Beef extract, YE: Yeast extract, A.C: Ammonium chloride, A.N: Ammonium nitrate, A.S: Ammonium sulphate, P.N: Potassium nitrate.

Fig 10 Effect of different Organic and Inorganic nitrogen sources on protease production in *Bacillus cereus strain S8* isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

3.8. Characterization of the Crude Enzyme obtained from Strain S8

Bacillus cereus Strain S8 demonstrated a higher potential for alkaline protease activity. Therefore, a preliminary study on the characterization of this enzyme was carried out. According to this study, it was observed that, this enzyme was thermostable with an optimum temperature of 70^0C (Fig 11) at a pH 10.0 (Fig 12) with 1h incubation (Fig 13) of enzyme reaction mixture. Casein was used as substrate for this enzyme and among different concentrations of casein used, 1% casein (Fig 14) showed optimal enzyme activity.

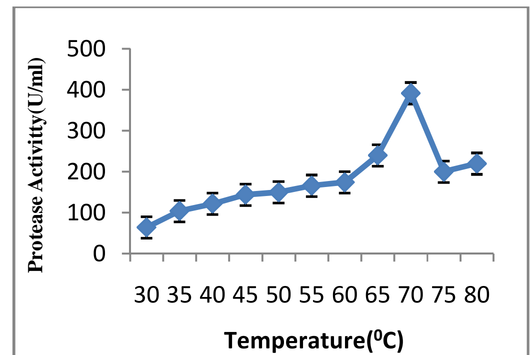


Fig 11: Activity of protease from *Bacillus cereus strain S8* at different temperatures.

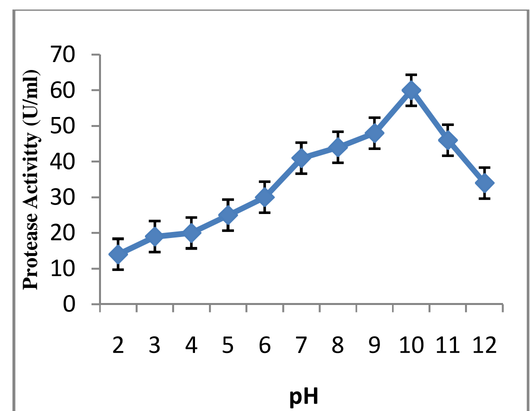


Fig 12: Activity of protease from *Bacillus cereus strain S8* different pH.

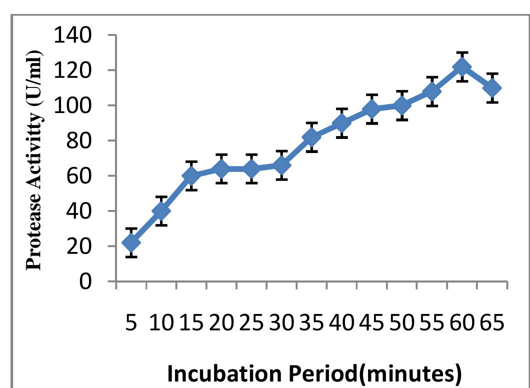


Fig 13: Activity of protease from *Bacillus cereus strain S8* at different Incubation Periods of Enzyme mixture.

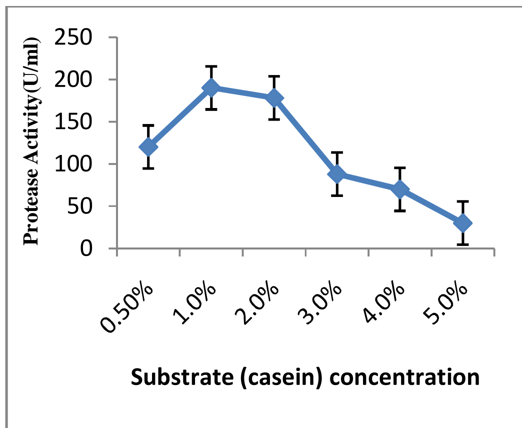


Fig 14: Activity of protease from *Bacillus cereus strain S8* at different concentrations of substrate (casein)

5. DISCUSSION

One of the main concerns of this study was to isolate and identify alkalophilic *Bacillus* sp. having a vital tendency to secrete extra-cellular proteolytic enzyme. Accordingly, 17 bacterial strains were isolated from saltern pond soil sample suspension processed on nutrient agar medium. Out of these isolates, isolate S8 represents genus *Bacillus* and exhibited vivid zone of clearance (4.6 cm) on skim milk agar medium at pH 10. The use of alkaline skim milk agar for the isolation of alkaline protease producing bacteria has earlier been reported by some workers [23, 24, 25]. In fact these bacteria are known for their abilities to secrete large amounts of alkaline proteases having significant proteolytic activity and stability at considerably high pH and temperatures [26, 27]. In the present study, the proteolytic activities of the isolate S8 were tested under extreme alkaline conditions and it was confirmed as *Bacillus cereus isolate S8* by 16S r RNA gene sequencing.

In the present work, the self constructed medium of pH-12.0 supported maximum enzyme production (205.00 U/ml) after 72h of incubation. Similar results were also reported in *Bacillus odyssey* with 72 h incubation period [28]. The medium provided primarily adequate amount of nutrients required for the production of alkaline protease. Carbon and nitrogen sources, inorganic salts and other growth factors are important variables that affect the growth and products of microbes [29, 30].

Requirement for specific carbon and nitrogen source differs from organism to organism, or even among the same species isolated from different sources [26]. Among various carbon and nitrogen sources used molasses (1%) and potassium nitrate (0.75%) supported maximum enzyme production. Both organic and inorganic nitrogen compounds were utilized by this strain. A decrease in enzyme production was observed at lower and higher concentrations. The results indicated that proper concentration level of molasses and potassium nitrate played a significant role in enhancing the production of alkaline protease and growth of the *B.cereus isolate S8*. Repressed growth and enzyme production at higher concentration of the substrates might be due the catabolic repression, or substrate inhibition, a traditional property of batch fermentation processes.

Presence of phosphate ions in the medium increases enzyme production. These results were supported by Calik [31] who found 1.55 fold higher enzyme activities in the presence of phosphate ions by *Bacillus subtilis*. While, Rahman [32] observed 39 % increase in enzyme production in the presence of 2mM phosphate ions in the growth medium by *Pseudomonas aeruginosa*. Presence of calcium carbonate in the medium increased the production of protease. These results were in agreement with the earlier findings which showed enhancement of protease activity in the presence of metal ions and it was suggested that these metal ions increased stability of proteases [33].

The enhancing effect of sodium on bacterial alkaline protease has been reported rarely. Chandrasekaran and Dhar [34] observed the beneficial effect of sodium chloride on alkaline protease production by *Streptomyces moderatus* NRRL 3150. An increased salt concentration creates change in the lipid composition of cell membrane. Hence, the growth rate decreases causing reduced enzyme production. Prolonged incubation period has been documented to lead auto digestion of the proteases and proteolytic attack by other proteases resulting in decrease in enzyme activity [35, 36]. It has been noted that microorganisms are dependent on the extra cellular pH for their cell growth and enzyme production [26]. Regarding the effect of inoculum size, maximum enzyme production (170.00 U/ml) was observed with 2.0 % (v/v) inoculum size and thereafter, increase of inoculum size decreased alkaline protease production. Increased protease production with small inoculum size has been suggested to be due to the higher surface area to volume ratio [32]. At higher concentration of inoculum, the nutrient might be consumed rapidly and resulted into a lesser growth as well as lesser alkaline protease production. These findings are in good agreement with the study of Kanekar [37] who have reported maximum enzyme activity (167.28 U/ml) of *Bacillus alcalophilus* with 2.0 % inoculum of 24 h old.

Partial characterisation studies of the crude enzyme showed that, this enzyme was thermostable with an optimum temperature of 70^0C at a pH 10.0 with 1h incubation of enzyme reaction mixture. Similar result was reported by Sandhya and Tambekar [28] in *Bacillus pseudofirmus* and *Bacillus odyssey*. There is a large demand of proteases, which can withstand at high temperature as well as at high pH. The isolation and production of protease enzyme with these characteristics is most important to fulfill the industrial demand [38]. These novel properties exhibited by the protease of *B.cereus strain S8* to meet the present industrial demand.

6. CONCLUSIONS

Alkaline protease producing strain Isolate S8 from the soil of saltern pond was isolated and identified as *Bacillus cereus strain S8* by 16s rRNA gene sequencing and phylogenetic tree analysis. Optimization of the fermentation conditions (temperature, inoculum concentration, carbon and nitrogen sources, incubation time and initial media pH) were optimized. Based on the results, maximum protease production was obtained in the medium supplemented with Molasses,1%(w/v); Potassium nitrate, 0.75%(w/v); salt solution- 5%(v/v) {$MgSo_4.7H_2O$, 0.5%(w/v); KH_2PO_4, 0.5%(w/v)}; $FeSO_4.7H_2O$, 0.01%(w/v) and $CaCO_3$, 0.5% at 37^0C after 72h incubation. Production of protease was achieved with cheap carbon sources like molasses in a cost effective manner and showed halo tolerance. Characterization studies of the crude enzyme showed that the enzyme was thermostable, showing activity at wide pH ranges. These promising features of the isolated *Bacillus cereus strain S8* might increase the scope of the strain to meet industrial demands of various sectors.

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