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ARTICLE

Screening and optimization of protease production from a halotolerant *Bacillus licheniformis* isolated from saltern sediments

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KEYWORDS

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Abstract Protease producing halotolerant bacterium was isolated from saltern pond sediment (Tuticorin) and identified as *Bacillus licheniformis* (TD4) by 16S rRNA gene sequencing. Protease production was enhanced by optimizing the culture conditions. The nutritional factors such as carbon and nitrogen sources, NaCl and also physical parameters like incubation time, pH, agitation, inoculum size were optimized for the maximum yield of protease. Studies on the effect of different carbon and nitrogen sources revealed that xylose and urea enhances the enzyme production. Thus, with selected C–N sources along with 1 M NaCl the maximum protease production (141.46 U/mg) was obtained in the period of 24 h incubation at pH 8 under 250 rpm compared to the initial enzyme production (89.87 U/mg).

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1. Introduction

Enzymes are biocatalysts produced by living cells to bring about specific biochemical reactions generally forming parts of the metabolic processes of the cells. Enzymes are commercially exploited in the detergent, food, pharmaceutical, diagnostics, and fine chemical industries. More than 3000 different enzymes have been identified and many of them being used in biotechnological and industrial applications [21]. Even small improvements have been significant for commercial success in biotechnological enzyme production processes [18]. Extremophiles are considered an important source of enzymes [7]. Halophiles inhabit extreme environments and they can be classified into three groups on the basis of their response to NaCl. Slight halophiles (*Shewanella* sp.) which grow optimally at 2–5% NaCl, moderate halophiles (*Halomonas meridian*) show rapid growth at 5–20% NaCl and extreme halophiles (*Salinibacter ruber*) which optimally grow at 20–30% NaCl. Halophilic enzymes have unique enzymatic functions compare to non-halophilic enzymes. It requires high salt concentrations in the range of 1–4 M for higher activity and longer stability [1]. Microbial proteases

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account for approximately 60% of the total enzyme sales in the world [4]. Proteases are one of the most important groups of industrial enzymes with broad applications including meat tenderization, detergents, cheese-making, de-hairing, baking, waste management and silver recovery [8]. Of all proteases, alkaline proteases produced by *Bacillus* species are of significant importance in detergent industry due to their high thermal and pH stability. Isolation and characterization of new promising strains using cheap carbon and nitrogen source is a continuous process of enzyme production for industrial uses [14]. With this in view, we have isolated a halotolerant bacterium (*Bacillus licheniformis* TD4) and characterized its protease production under various chemical and physical conditions.

2. Materials and methods

2.1. Chemicals

Casein enzymatic hydrolysate, Nutrient broth, all carbon and nitrogen sources (99% purity) used in this study was purchased from Hi-Media Laboratories (Mumbai, India).

2.2. Sample collection and isolation of bacterial strain

Samples were collected in a sterile container from the saltern pond of Tuticorin, Tamil Nadu, India. Collected sample was serially diluted in sterile saline water and the dilutions were plated in nutrient agar plates with 5% sodium chloride and kept for incubation at 37 °C. Colonies were picked based on divergence in morphology, size and colour.

2.3. Screening of protease producing bacteria

Proteolytic production of the bacterial strains was screened on agar plates supplemented with 5% NaCl and 1% casein (MNA). The plates were incubated overnight at 37 °C. The protease producing strains were selected based on the zone of clearance.

2.4. Identification of the bacterial strain

The protease producing strain was identified by various biochemical test and 16S rRNA gene sequencing. Genomic DNA was extracted as per the standard protocol [3] and it was amplified by using the following universal 16S rRNA primers: forward primer 5′ GAGTTTGATCCTGGCTCAG3′ (*Escherichia coli* positions 8–27) and reverse primer 5′ACGGCTACCTTGTTACGACTT3′ (*E. coli* positions 1494-1513). The PCR product was amplified and sequenced by Macrogen. (Seoul, Republic of Korea). Phylogenetic tree was constructed with the MEGA v5.04 [13] using neighbor joining method with a bootstrap value of 1000. The 16S rRNA gene sequence was submitted to GenBank under the accession number JF769746.

2.5. Assay of proteolytic activity

Overnight culture of protease producing strain (1%) inoculum was added in the nutrient broth with 5% NaCl and 1% casein, pH 7.0 and kept for 24 h incubation at 37 °C under shaking condition of 150 rpm. After incubation the culture was centrifuged at 10,000 rpm for 15 min at 4 °C. The cell free supernatant was used for protease assay by Anson method [9]. The reaction mixture contains 1 ml of enzyme was added to 1 ml of casein (1% w/v in 50 mM potassium phosphate buffer, pH 7.5) and the mixture was incubated for 10 min at 37 °C. The reaction was terminated by adding 2 ml of 10% trichloroacetic acid reagent, kept for 30 min incubation at room temperature and then centrifuged for 15 min at 10,000 rpm. Then 2 ml of filtrate was mixed with 3 ml of 500 mM sodium carbonate solution and absorbance was measured at 280 nm. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of tyrosine per min under the defined assay conditions. The range of concentration 50–250 μg of tyrosine was used as standard. The potential producer was taken for further optimization studies to enhancement the protease production.

2.6. Optimization of pH, agitation, inoculum, incubation period for protease production

The effect of various physical parameters on protease production was assessed by growing bacterial culture in the nutrient broth with 1% casein, pH 7.0 (MNB). For optimizing pH, the medium was prepared by varying the pH from 2.0 to 10.0 at 1.0 unit interval. Agitation was determined by incubating the bacterial culture at a range of 50–300 rpm with 50.0 unit variation. Effect of varying inoculum percentage from 0.2% to 1% with 0.2% variation on protease production was determined. Similarly, for the investigation of optimal incubation time for protease production, the bacterial culture was inoculated in the nutrient broth and kept for 48 h at 37 °C. Samples were withdrawn aseptically every 6 h interval and protease activity was determined by Anson method.

2.6.1. 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 0 to 2 M with 0.5 M variation in MNB. MNB was supplemented with various carbon sources such as xylose, dextrose, fructose, sucrose, galactose, mannose and lactose (1% w/v) and nitrogen sources such as potassium nitrate, urea, sodium nitrate, ammonium chloride, ammonium nitrate and ammonium carbonate (1% w/v). After 24 h incubation, the cell free supernatants were quantified for protease production.

3. Results and discussion

3.1. Isolation and screening of protease producing bacteria

In the present study, a total of 15 halotolerant bacteria from saltern pond have been screened for the presence of protease production on MNA plates. Six strains were identified as protease producers by zone of hydrolysis around the colonies (Fig. 1) and quantified their activity. In protease assay, TD4 showed highest production (89.87 U/mg) compared to other strains and used for protease optimization studies.

3.2. Characteristics of the potential strain

TD4 was found to be Gram-positive rod shaped bacterium and showed NaCl tolerance up to 2 M. Their morphological and

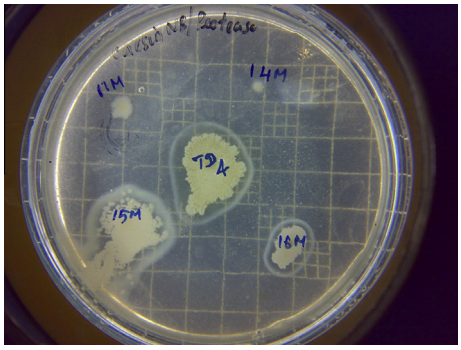

Figure 1 TD4 strain showing protease production (zone of clearance) around the colonies on Caesin agar plates.

Table 1 Morphological and biochemical characterization of TD4 strain isolated from saltern sediments.

Characteristic feature	TD4 strain
Colony morphology	Large, round, irregular, mucoid
Gram staining	Gram positive, rod
Spore staining	Spore former
Hanging drop	Motile
Indole	Positive
Methyl red	Negative
Voges-proskauer test	Positive
Citrate	Positive
Catalase	Positive
Oxidase	Negative
Nitrate test	Positive
H_2S production	Negative
Starch	Positive
Glucose	Positive
Sucrose	Positive
Lactose	Negative

biochemical characteristics were listed in Table 1. The results showed an emblematic characteristic of *Bacillus* sp. Most of the *Bacillus*sp. were reported as 3.0–5.2 μm in length and 0.4–0.7 μm in width [16]. Based on 16S rRNA gene analysis, the strain was phylogenetically characterized and identified the closest relative using BLAST (NCBI) search. Thus identified, the strain belonged to *Bacillaceae* family (Fig. 2). TD4 displayed 97% sequence similarity with its closest relative *B. licheniformis* (CP000002), an industrial bacterium [20].

3.3. Optimization of culture conditions for protease production

3.3.1. Effect of NaCl, carbon and nitrogen on protease production

The effect of salt on protease production was shown in (Fig. 3). Maximum protease production was observed in the medium

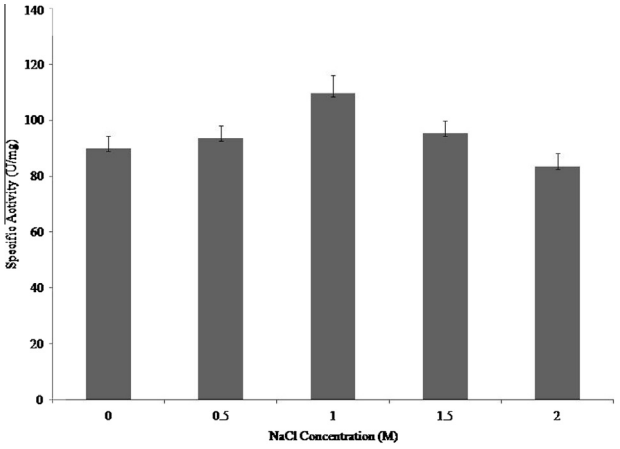

Figure 3 Effect of various concentration of NaCl on protease production in *Bacillus licheniformis* (TD4) isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

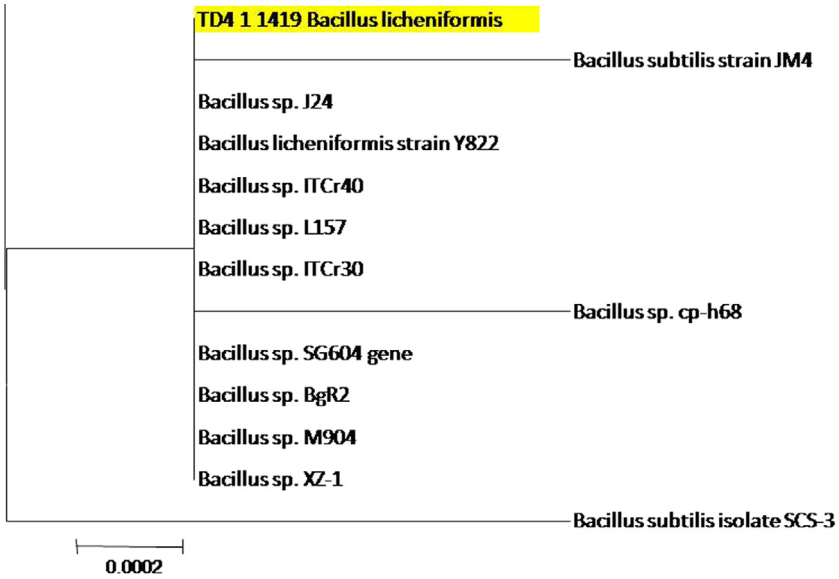

Figure 2 Phylogenetic relationship of strain TD4 (Underlined) isolated from saltern sediments. The tree is constructed using 16S rRNA gene sequence using the Neighbour-joining method.

containing 1 M NaCl (109.52 U/mg) after 24 h of incubation. The growth and production of protease was gradually reduced when salt concentration increases above 1.5 M NaCl. An increased salt concentration creates change in the lipid composition of cell membrane. So, the growth rate decreases along with enzyme production. Mostly, gram positive moderate halophiles are often reported in the reduction of enzyme production at high salt concentration [26]. In a previous report a similar NaCl concentration was found to be optimum for the production of protease [22].

Among various carbon sources used, protease production was highest in the medium containing xylose (94.77 U/mg) followed by fructose (94.26 U/mg) shown in (Fig. 4). Less production of enzyme was recorded in the medium containing starch (87.13 U/mg). Xylose can be easily utilized by our strain TD4 compared to other saccharides. An increased yield of enzyme production from various carbon sources such as lactose [11] maltose [17] and sucrose [25] have been reported by other researchers.

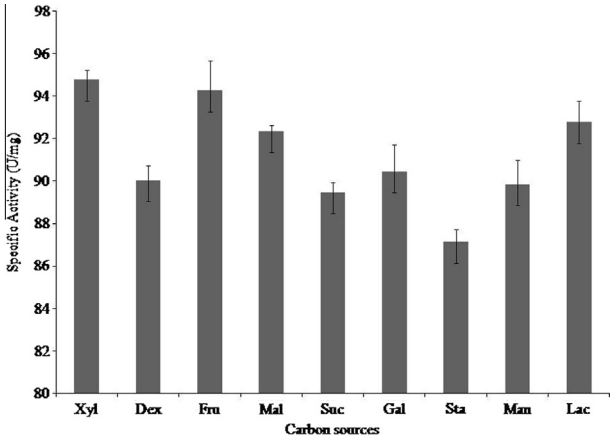

Figure 4 Effect of different carbon sources on protease production in *Bacillus licheniformis* (TD4) isolated from saltern sediments. (Xyl-Xylose, Dex-Dextrose, Fru-Fructose, Suc-Sucrose, Gal-Galactose, Man-Mannose, Lac-Lactose). The bars indicate the standard deviation of three replicates analyzed.

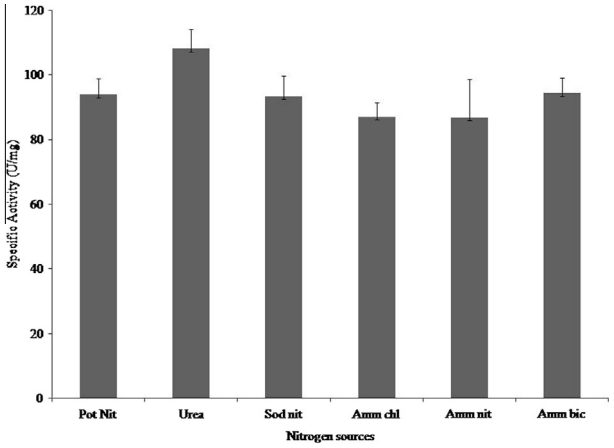

Figure 5 Effect of different nitrogen sources on protease production in *Bacillus licheniformis* (TD4) isolated from saltern sediments. (Pot Nit-Potassium nitrate, Ure-Urea, Sod Nit-Sodium nitrate, Amm Chl-Ammonium chloride, Amm Nit-Ammonium nitrate, Amm Car-Ammonium carbonate). The bars indicate the standard deviation of three replicates analyzed.

Various nitrogen sources were investigated for protease production. High yield of protease production was observed (Fig. 5) in urea (108.13 U/mg) and ammonium bicarbonate (94.34 U/mg). In earlier reports soybean meal, casamino acids, and peptone were found effective ingredients for the protease production [12,10,15]. Both organic and inorganic nitrogen compounds were utilized by TD4 strain. Its shows the versatility of the bacteria utilizing a range of compounds.

3.3.2. Effect of pH, agitation, inoculum, incubation period on protease production

B. licheniformis TD4 could grow and produce protease over a wide range of pH (2.0–10.0). Maximum protease production was observed at pH 8 (135.50 U/mg) (Fig. 6). The production

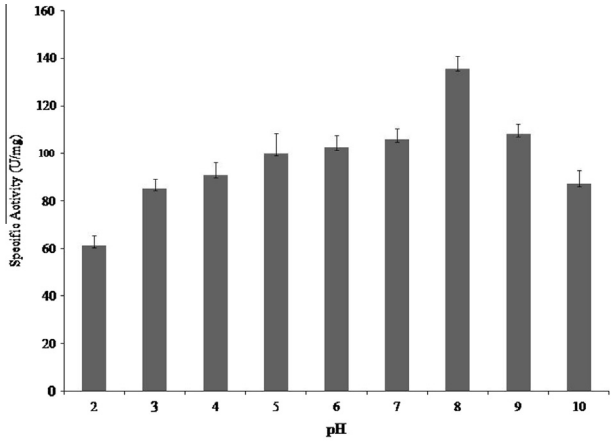

Figure 6 Effect of various incubation period on protease production in *Bacillus licheniformis* (TD4) isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

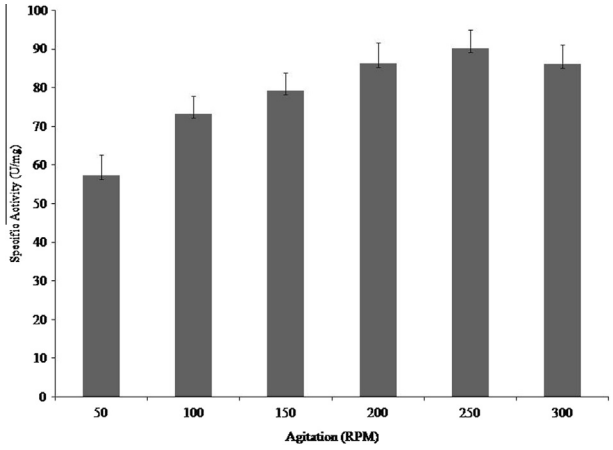

Figure 7 Effect of various pH on protease production in *Bacillus licheniformis* (TD4) isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

at pH 7 and 8 was relatively comparable. Most of the *Bacillus* sp. reported have optimum pH from 7.0 to 11.0 [10,22] for the production of protease.

The effect of various agitation rates was investigated for protease production; optimum production of protease (Fig. 7) was under the condition of 250 rpm (90.15 U/mg). The production of protease from 150 to 250 rpm was quite comparable. *Bacillus subtilis* ATCC 14416 [5] and *B. licheniformis* [23] showed optimum yields of alkaline protease production under the condition of 200 rpm.

In the present study, maximum protease production was observed (Fig. 8) at 1% inoculum (104.14 U/mg). There was a reduction in protease production when inoculum size was reduced (0.2%), these may be due to insufficient number of bacteria, which would lead to reduced amount of enzyme production. Higher inoculum size may have resulted reduced dissolved oxygen and increased competition towards nutrients [24]. *Pseudomonas* sp. [6] has been reported that 1.5% inoculum showed maximum enzyme production.

The effect of incubation period on the protease production was shown in (Fig. 9). TD4 has ability to produce maximum protease in the period of 24–30 h. Optimum production of protease (101.30 U/mg) was observed in the period of 24 h incubation. *Bacillus* sp. [19] and *Halobacterium* sp. [2] have been reported that maximum protease production was achieved at 48 and 96 h incubation period respectively. When comparing the earlier reports our strain TD4 has shorter period of incubation for the production of protease.

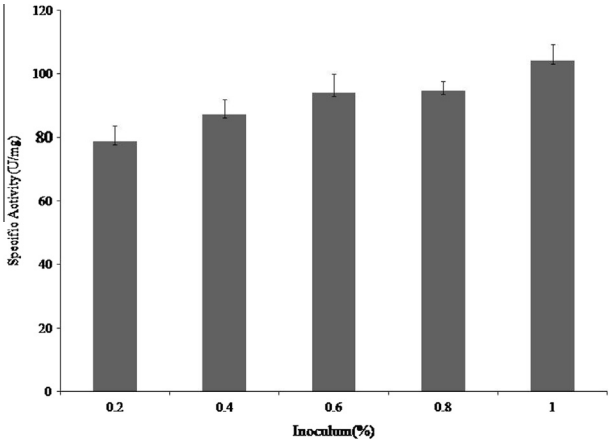

Figure 8 Effect of different agitation speed on protease production in *Bacillus licheniformis* (TD4) isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

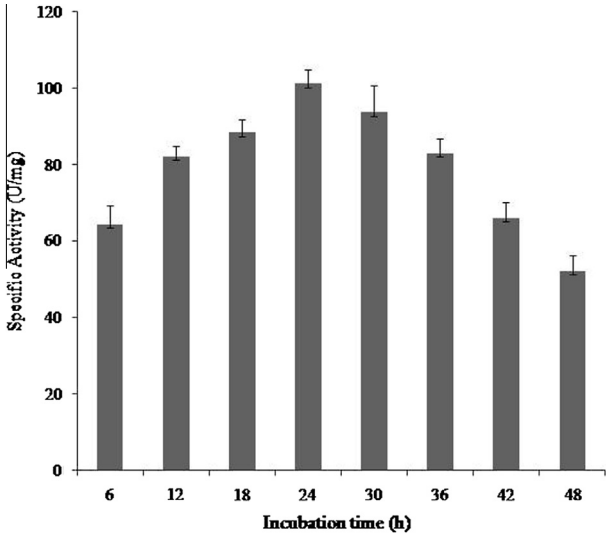

Figure 9 Effect of different inoculums percentage on protease production in *Bacillus licheniformis* (TD4) isolated from saltern sediments. The bars indicate the standard deviation of three replicates analyzed.

4. Conclusion

In the present study, we have isolated *B. licheniformis* TD4 from saltern pond sediments (Tuticorin) and investigated the optimal media components for highest protease production. The optimum salt concentration, incubation time, pH, agitation, inoculum size, carbon and nitrogen sources for protease production were determined as 1 M, 24 h, 8, 250, 1%, xylose and urea, respectively. With the optimized parameters protease production was enhanced to 141.46 U/mg. Further studies are required to find out the potential applications of the protease obtained from this study.

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