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Optimization of alkaline protease production from *Bacillus subtilis* NS isolated from sea water

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The protease producing bacterial strain *Bacillus subtilis* was isolated from sea water and identified by 16S rRNA sequencing. The strain named as *B. subtilis* NS. Optimization of the strain revealed that the most suitable nitrogen source to enhance protease production was beef extract. Among various carbon sources tested, maximum production of protease was registered in medium with added glucose. The effect of metals ions indicated that maximum protease production was observed in medium supplemented with magnesium chloride (MgCl). Investigating the effect of sodium chloride (NaCl) concentration on protease production revealed that 7% yielded higher protease production. The most suitable pH and temperature for maximum protease production revealed that pH 9 and a temperature of 40°C gave optimal protease production.

Key words: Alkaline protease, *Bacillus subtilis*, media optimization, marine bacteria.

INTRODUCTION

Proteolytic enzymes are degradative enzymes which catalyse the cleavage of peptide bonds in other proteins. Alkaline protease, which works optimally in alkaline pH, constitutes 60 to 65% of the global industrial enzyme market (Amoozegara et al., 2004). Proteases are the class of enzymes which occupy key position with respect to their applications in both physiological and commercial fields (Godfrey et al., 1996). Protease derived from microorganisms such as bacteria, fungi and yeast has found wide spread applications in many fields. Among various proteases, bacterial proteases are most significant, compared with animal and fungal proteases (Fujiwara et al., 1991). Alkaline protease of microbial origin possess considerable industrial potential due to their biochemical diversity and wide applications in tannery and food industries, medicinal formulations, silver recovery, detergent, waste water treatment and resolution of amino acid mixtures (Rao et al., 1998). Currently a large proportion of a commercially available alkaline proteases are *Bacillus* strains (Yang et al., 2000) although several fungal sources are being increasingly employed (Banerjee, 1999). Among these, *Bacillus subtilis* is the most important group of bacteria that are involved in the enzyme industries and also *B. subtilis*

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produce a variety of extracellular and intracellular protease (JahirAlam Khan et al., 2011). In view, the present study was undertaken to optimize culture conditions for alkaline protease production by marine water isolate *B. subtilis* NS.

MATERIALS AND METHODS

Screening and isolation of proteolytic bacteria

The protease producing bacterial strain was isolated from sea water of Cuddalore coast, Tamilnadu, India, using Zobell marine agar medium and plates were incubated at 37°C for 5 days (pH 9.0). It was identified by morphological, biochemical identification schemes and confirmed by 16S rRNA gene sequencing. In brief, DNA was isolated by phenol chloroform method (Marmur, 1961). The primer sequences were selected from the conserved regions as previously reported for the bacterial 16S rRNA gene (Saitou et al., 1987). Sequencing was done using forward primer (5′-CAGGCCTAACACATGCAAGTC-3′) and reverse primer (5′-GGGCGGTGTGTACAAGGC-3′). PCR were performed with following conditions: 35 cycles consisting of 95°C for 1 min and 72°C for 5 min, followed by final extension of 5 min at 72°C. The 16S rRNA gene sequences were obtained by an automated DNA Sequencer (Megabace, GE) and homology of the isolated gene with sequences in the Gene Bank database was analyzed.

Enzyme production medium

Production medium contained glucose 0.5 g (W/%), peptone 1 g, $FeSO_4$ 0.1 g, KH_2PO_4 0.5 g, $MgSO_4$ 0.5 g and NaCl 3 g. 10 ml of medium was taken in a 100 ml conical flask. The flasks were sterilized in autoclave at 121°C for 15 min and after cooling, the flask was inoculated with overnight grown bacterial culture. The inoculated medium was incubated at 37°C in shaker incubator for 48 h. At the end of the fermentation period, the culture medium was centrifuged at 5000 rpm for 15 min to obtain the supernatant which was used for further studies.

Protease assay

To 0.25 ml culture supernatant, 1.25 ml Tris buffer (100 mM; pH 9.0) and 0.5 ml 1% aqueous casein solution were added. The mixture was incubated for 30 min at 30°C. Then, 3 ml 5% trichloroacetic acid (TCA) was added to this mixture, whereby it formed a precipitate. The mixture was further incubated at 4°C for 10 min, and then centrifuged at 5,000 rpm for 15 min. Thereafter, 0.5 ml supernatant was taken, to which 2.5 ml 0.5 M sodium carbonate was added, mixed well and incubated for 20 min. To this mixture, 0.5 ml folin phenol reagent was added and the absorbance was read at 660 nm using a UV Spectrophotometer. The amount of protease produced was measured with the help of a tyrosine standard graph (Takami et al., 1989).

Optimization for protease production

Effect of pH on protease production

The optimum pH for protease production was determined by adjusting the production medium to different pH values, for which pre-autoclaved medium was prepared individually at pH 3, 4, 5, 6, 7, 8, 9, 10, 11 and 12, and inoculated with experimental bacterium at 37°C.

Effect of temperature on protease production

Production medium at pH 9 was inoculated with overnight grown selected bacterial strain. The broth was incubated at different temperatures from 20, 30, 40, 50, 60, 70 and 80°C for 48. At the end of incubation period, the cell free culture filtrate is obtained and used as enzyme assay.

Effect of carbon sources on protease production

The effect of various carbon sources such as starch, glucose, maltose, lactose, xylose and fructose was examined in the production medium.

Effect of nitrogen sources on protease production

The different nitrogen sources like yeast extract, beef extract, peptone, urea, ammonium chloride, sodium nitrate and ammonium sulphate were examined for their effect on protease production.

Effect of NaCl concentration on protease production

The basal media were supplied with different concentrations of NaCl (1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%) for the efficiency of maximum protease production.

Effect of metal ions on protease production

Influence of various metal ions on protease production was determined by incubating the medium with different metal ions such as $CaCl_2$, $MnCl_2$, $CuSO_4$, KCl, and $MgCl_2$ at a concentration of 0.2%.

RESULTS AND DISCUSSION

Microorganism

In the present study, a protease producing strain *B. subtilis* was isolated from sea water of Cuddalore coast, Tamil nadu, India. Morphological and biochemical characteristics of the strain revealed that it is a gram-positive, endospore-forming bacillus with catalase enzyme activity 16S rRNA gene sequence analysis confirmed the identity of the strain was submitted to NCBI as *B. subtilis* NS and based on the evolution distance and the phylogenetic tree, this strain was identified as *B. subtilis* and designated *B. subtilis* NS (GenBank accession no KF735656) (Figure 1).

Effect of pH on protease production

Physical factors are important in any fermentation for optimization of biochemical production. The important physical factors that determine the rate of bioprocessing are pH and temperature. In the present study, the effect

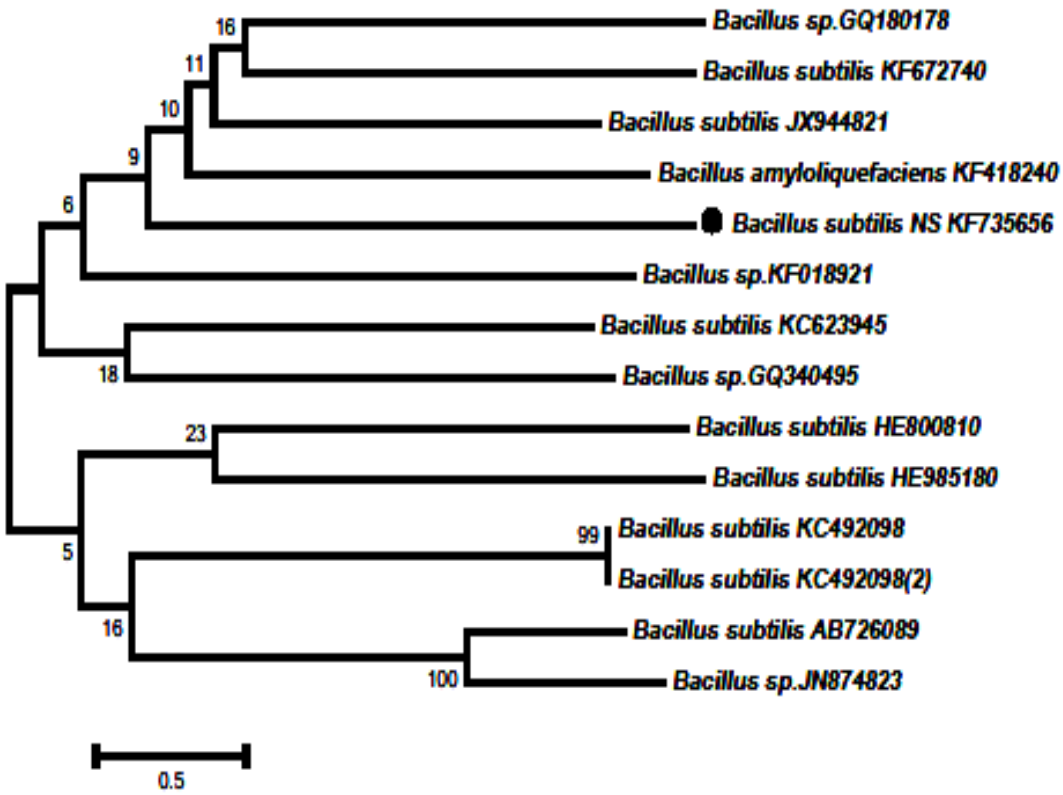

Figure 1. Phylogenetic tree of *Bacillus subtilis* NS strain 16S rRNA gene sequence with other *Bacillus* species.

of pH on protease production by *B. subtilis* NS revealed that pH 9 was optimal (123.5 U/ml) and enzyme production declined at the higher pH tested (Figure 2). This may be attributed to growth reduction and enzyme inactivation at higher pH (Tsujibo et al*.,* 1990; Mukesh Kumar et al*.,* 2012).

Effect of temperature on protease production

The effect of initial temperature on protease production showed that the higher protease production found at 40°C (117.4 U/ml) and minimum production (23.4 U/ml) was obtained in 80°C (Figure 3). The temperature influence enzyme production by changing the physical properties of the cell membrane. Usharani and Muthuraj (2010) were reported that protease production by *Bacillus laterosporous* was best at 37°C which indicates the same trend.

Effect of carbon source on protease activity

In the present study five different carbon sources were used for protease production. Since carbon is considered as the primary nutrient for the bacteria, different carbon source like sucrose, maltose, glucose, lactose, starch, fructose were analysed for the protease production. Maximum production of protease (199.01 U/ml) was observed in glucose when compare to other carbon sources (Figure 4). Maximum protease productions were obtained in xylose and maltose supplied medium by *Bacillus* sp. (Prakasham et al., 2006). Samarntarn et al. (1999) reported that protease production was high in the presence of supplementary carbohydrate carbon sources, especially lactose for microbes.

Effect of nitrogen source on protease production

The nitrogen source is important in fermentation media supplying a suitable nitrogen source favors higher level enzyme or metabolite production. In the present study, supplementary nitrogen sources accelerated protease production. Furthermore, this experiment showed that the complex organic nitrogen sources gave higher protease production than inorganic nitrogen sources, with production being highest in beef extract (118.42 U/ml)

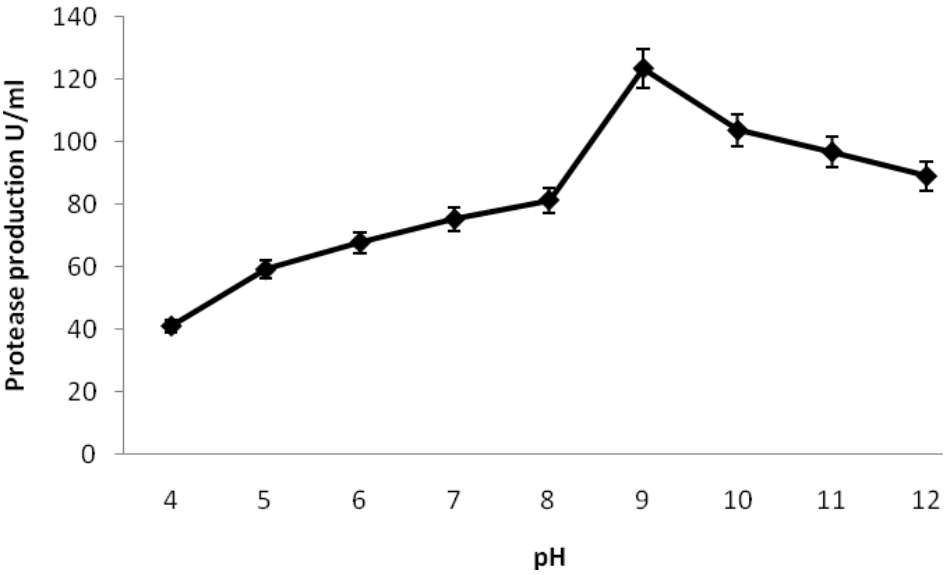

Figure 2. Effect of pH on protease production from *Bacillus subtilis* NS.

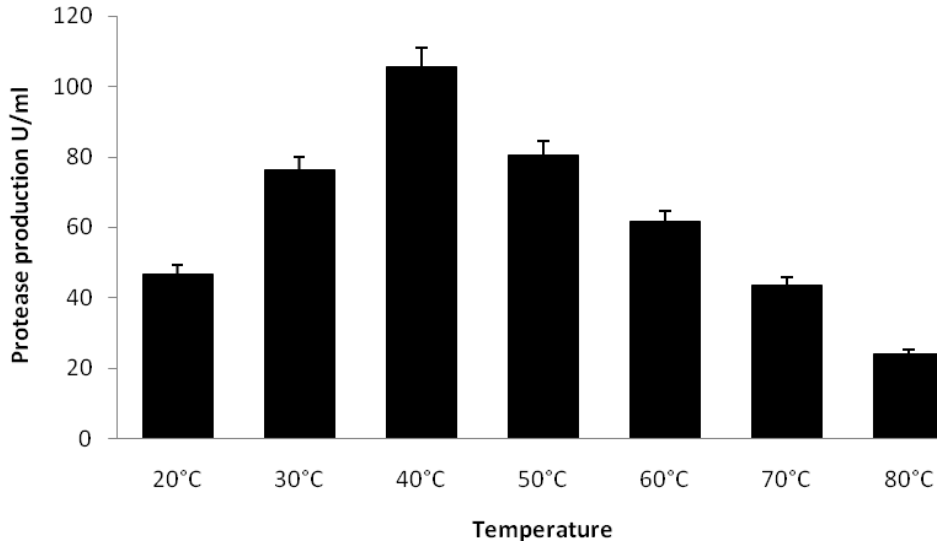

Figure 3. Effect of temperature on protease production from *Bacillus subtilis* NS.

supplied medium. Other organic nitrogen sources also support high protease production (Figure 5). It has been reported that organic nitrogen source like peptone, casein, yeast extract, favoured maximum protease production by *B. subtilis*. Next to that, inorganic nitrogen sources like ammonium carbonate followed by ammonium chloride, ammonium citrate and potassium nitrate were used as good nitrogen sources. Gupta et al. (2007) reported that the optimization of protease production in *Pseudomonas aeruginosa* PseA by using complex nitrogen sources. Our results also comply with the complex nitrogen sources induced protease production in *Aspergillus tamari* (Anandan et al., 2007).

Effect of metal ions on the protease production

The effect of various metal ions on protease production

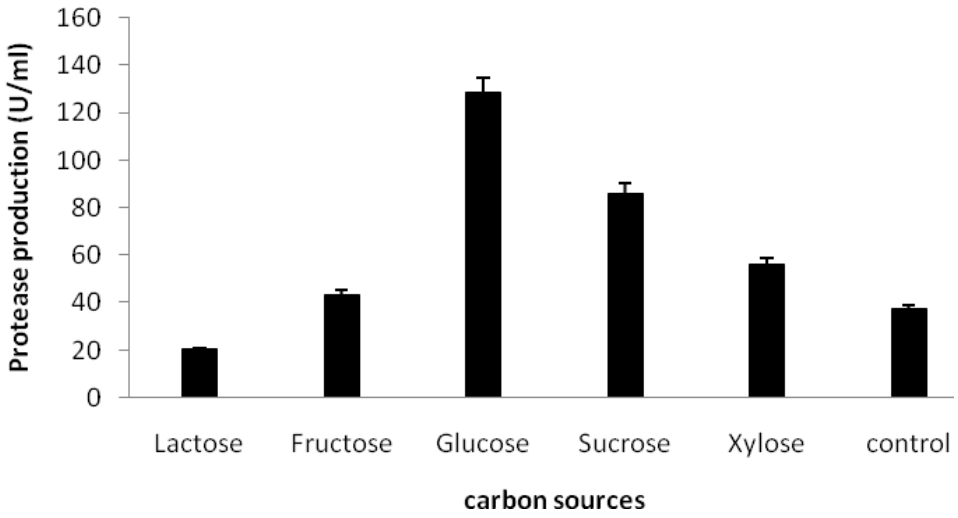

Figure 4. Effect of Carbon sources on the activity of protease enzyme from *Bacillus subtilis* NS.

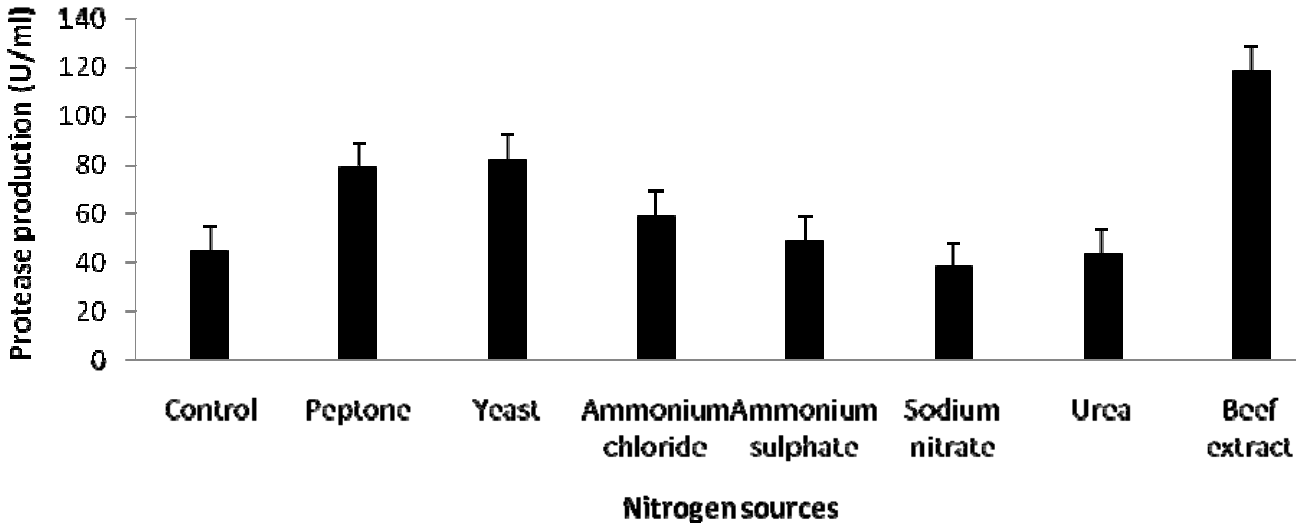

Figure 5. Effect of nitrogen sources on protease production from *Bacillus subtilis* NS.

was evaluated. Among these ions magnesium chloride was found to increase protease production of 149.29 U/ml (Figure 6). The enzyme activity was slightly enhanced by supplementation of K^+, Na^+ and Ca^+ ions compared to control. It was reported that supplementation of Mg^+, Ca_2^+ and K^+ salts to the culture medium exhibited slightly better production of protease. Rahman et al. (2005) observed that protease production was higher by *P. aeruginosa* in metal ions mediated culture. The present observation is in agreement with the earlier study reported by Krishnaveni et al. (2012) where the magnesium sulphate and manganese sulphate enriched medium enhanced the protease production in *B. subtilis.*

Effect of sodium chloride on the protease production

Regarding NaCl concentration, protease production was increased with increasing concentrations from 0 to 10% and reached its maximum at 7% (W/V) (Figure 7). There was a significant reduction in enzyme production found in the absence of NaCl (0%). The strain *B. subtilis* NS used in this study was isolated from marine water and that

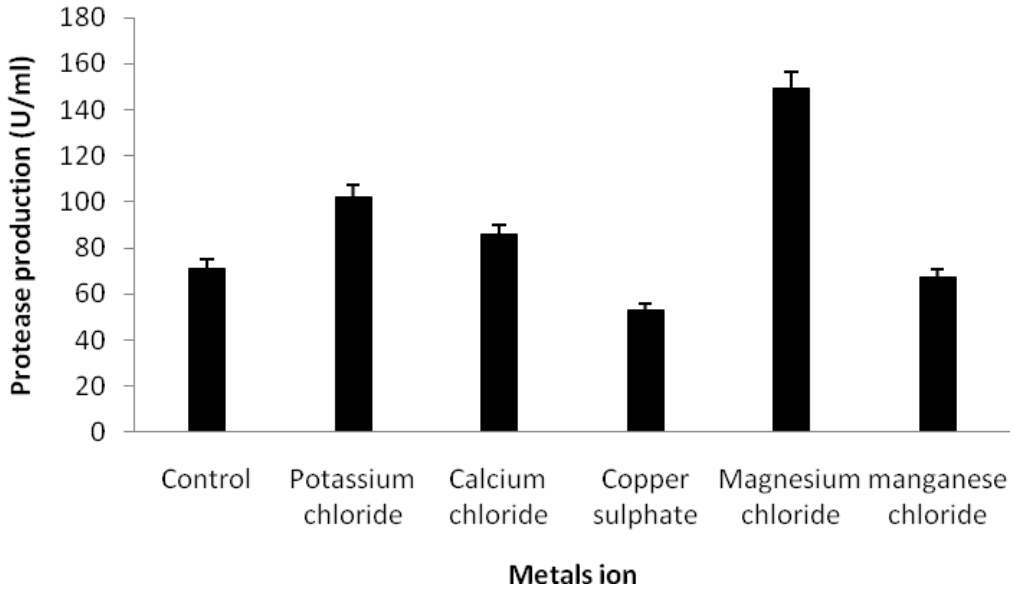

Figure 6. Effect of metal ion on the activity of protease enzyme from *Bacillus subtilis* NS.

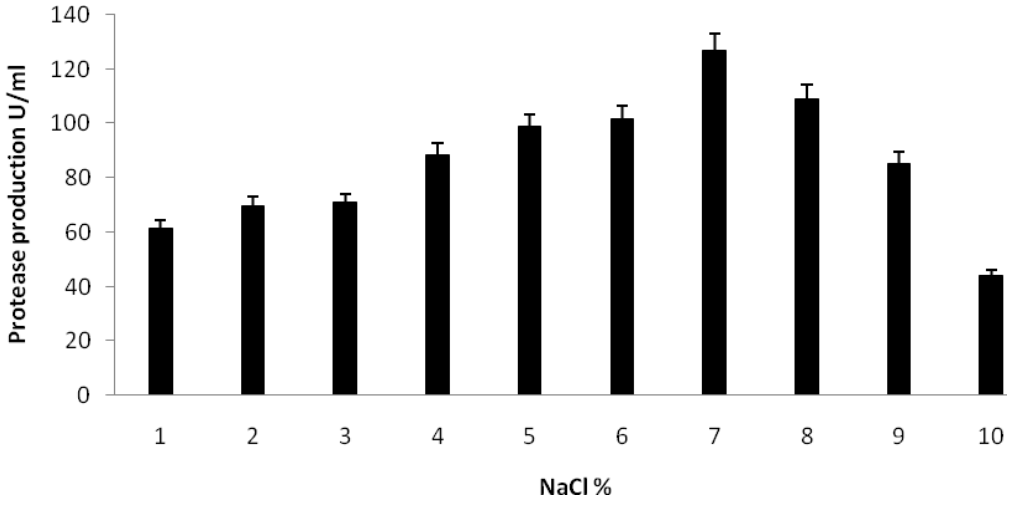

Figure 7. Effect of NaCl on protease production by *Bacillus subtilis* NS.

might be the reason for the higher protease production found at 30% and lesser at 0% NaCl. Similar results were observed in 1 M NaCl concentration by *Bacillus* sp. VITP4 isolated from Indian coastal area (Pooja and Jayaraman, 2009).

Conclusion

Proteases are industrially important enzymes with many applications, especially in the detergents industry. The enzyme from halophilic bacteria is an unexploited bio resource for enzyme production. The present study reports the production of protease by marine water isolate *B. subtilis* NS. Successfully, optimized environmental factors (pH and temperature) and nutrient (carbon, nitrogen, trace elements and sodium chloride) conditions yielded maximum protease production. This proteolytic bacterium could be used effectively for industrial purpose.

Conflict of interests

The author(s) have not declared any conflict of interests.

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