



Production and optimization of alkaline protease by *Bacillus cereus* RS3 isolated from desert soil

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Abstract

A bacterial isolate RS3 with significant proteolytic activity was isolated from desert soil of Riyadh, Saudi Arabia. The isolate was identified as *Bacillus cereus* based on morphological, biochemical and molecular characterization. Factors influencing the maximum production of extracellular alkaline protease by *Bacillus cereus* RS3 were optimized via the use of the one-factor-at-a-time method. The maximum alkaline protease enzyme yield was achieved when the incubation temperature was 45 C in growth medium with a pH of 9 and 4% inoculum, and continuous agitation at 170 rpm for 24 hours. *Bacillus cereus* RS3 exploited multiple sources of carbon for its alkaline proteases' production; fructose was the best source of carbon, whereas sucrose and galactose followed. Among the various organic and inorganic nitrogen sources tested, yeast extract was found to be the best nitrogen source for the maximum production of alkaline protease.

Keywords: Alkaline protease, *Bacillus cereus*, Optimization, Desert soil.

1. Introduction

Proteases are a large group of hydrolytic enzymes that catalyze protein hydrolysis by cleaving the intra-amino acid peptide bonds in protein molecules (Shankar *et al.*, 2011). Proteases are of great importance in the industrial sector, comprising over 65 percent of the total industrial enzyme market (Banik and Prakash, 2004) and are used in food processing, detergents, feather processes, silk gumming, pharmaceuticals, biosynthesis, bioremediation and biotransformation (Bhaskar *et al.*, 2007, Jellouli *et al.*, 2009, Wang *et al.*, 2008). Recently, the synthesis of oligopeptides via the use of proteases was explored as an alternative to the chemical approach (Ma *et al.*, 2007, Wang *et al.*, 2009). The new applications are expected to grow now and in the future, because of the increase in demand for biocatalysts of high stability,

capable of enduring the rigid conditions of the various operations (Bruins *et al.*, 2001, Bryan, 2000, Rao *et al.*, 1998, Siezen and Leunissen, 1997).

Commercial proteases are mostly obtained from several bacteria; It has been reported that almost 35 percent of the enzymes utilized in the detergent industry, are bacterial proteases (Ferrero *et al.*, 1996). Among bacteria, *Bacillus* sp. are specific producers of extracellular proteases (Priest, 1977) and can grow over extreme pH and temperature conditions to create products that are, in turn, stable in a large variety of rigid environments (Han and Damodaran, 1997). In addition, many members of the *Bacillus* sp. synthesize proteases in excess than those needed for their physiological activities (Bhosale *et al.*, 1995). Hot and

cold deserts were shown to host peculiar microbial arrays ready to adapt with threatening environment and/or to quickly adapt to changing conditions. This adaptation is inferred to particular community structure behavior and specific metabolic capacities allowing cells to overcome water stress, fluctuating temperature, and high salinity (Cherif *et al.*, 2015). Hence, exploring these habitats will provide access to novel bacteria and their robust enzymes that can act under multiple extreme conditions (Singh *et al.*, 2010, Singh *et al.*, 2012). Isolation and screening of alkaline proteases producing *Bacillus* spp. from different ecological environments can result in isolation of novel alkaline proteases with unique physiochemical characteristics (Shumi *et al.*, 2004, Singh *et al.*, 1999).

Culture conditions that favor the production of enzymes like proteases differ significantly from those that promote the growth of cells (Moon and Parulekar, 1991). A number of components in culture media that play an important role in enhancing the production yield of extracellular protease enzymes have been found. The optimization of the production yield is a process that is heavily influenced by these components and especially the carbon and nitrogen sources (Kaur *et al.*, 2001, Oberoi *et al.*, 2001). Protease synthesis is also greatly affected by physical factors such as temperature, pH, incubation time and dissolved oxygen (Hameed *et al.*, 1999, Puri *et al.*, 2002). Therefore optimization of media components and physical conditions is imperative for high yield of extracellular protease production. The present investigation mainly focused on the isolation and screening of protease producing bacteria from desert soil collected from Riyadh province of Saudi Arabia. The conditions were optimized for the maximal protease enzyme production in *Bacillus cereus* RS3.

2. Materials and Methods

2.1. Isolation and screening of alkaline protease producing bacteria

The soil samples were collected aseptically from seven desert places of Riyadh, Saudi Arabia. To isolate the alkalophilic bacteria, the collected soil sample was serially diluted up to 10^{-6} dilutions in normal saline and plated on to Horikoshi alkaline medium (pH 10.) contained glucose (10 g/L), peptone (5 g/L), yeast extract (5 g/L), K_2HPO_4 (1 g/L), $Mg_2SO_4 \times 7H_2O$ (0.2 g/L), NaCl (50 g/L), Na_2CO_3 (10 g/L), and bacteriological agar (15 g/L). After 24 hours of incubation, morphologically distinguished colonies were picked and purified in nutrient agar medium.

Purified isolates were screened for extracellular protease production by streaking onto skim milk agar medium contained peptone (2 g/L), sodium chloride (5 g/L), yeast extract (2 g/L), beef extract (1 g/L), bacteriological agar (15 g/L), skim milk powder (10 g/L). After incubation, clear zone formation on the media indicates the production of protease by the bacterial isolates. The isolate RS3 showed maximum clear zone, hence the isolate was selected for further optimization studies to enhance the protease production.

2.2. Identification of selected bacterial isolate

The selected protease producing isolate RS3 was identified using morphological and biochemical characteristics, according to Bergey's Manual of Determinative Bacteriology (Holt JG, 1994) and also 16S rRNA sequencing analysis. Genomic DNA was extracted as per the standard protocol (Babu *et al.*, 2009) and it was amplified by using universal bacterial primers (27F: 5'-AGA GTT TGA TCM TGG CTC AG-3', 1492R: 5'-TAC GGY TAC CTT GTT ACG ACT T-3'). The purified PCR products of approximately 1400 bp were sequenced using Applied Biosystems model 3730 XL automated DNA sequencing system (Applied BioSystems, USA). The sequence alignments and the phylogenetic tree construction were analyzed using MEGA 5 (Tamura *et al.*, 2011).

2.3. Protease Production

For the production of alkaline protease, the overnight grown bacterial culture was inoculated in 50 ml production medium contains glucose (10 g/L), peptone (5 g/L), yeast extract (5 g/L), K_2HPO_4 (1 g/L), $MgSO_4 \cdot 7H_2O$ (0.2 g/L), Na_2CO_3 (10 g/L), NaCl (5 g/L) in 250 ml Erlenmeyer flasks and incubated at 40°C on incubator shaker for 24 hours at agitation rate of 120 rpm. After incubation the culture broth was centrifuged at 10,000 rpm at 4°C and the supernatant was used as the crude enzyme for the estimation of proteolytic activity.

2.4. Assay of proteolytic activity

Protease activity was determined by a slight modification of the method described by Kembhavi *et al.* (1993) with casein as a substrate. Briefly, a 1 mL crude enzyme was mixed with 1 mL of 100 mM Tris-HCl buffer (pH 9.0) containing 1% (w/v) casein and incubated for 30 min at 37°C. The reaction was then stopped by the addition of 3 mL of trichloro acetic

acid (5% TCA), after which it was allowed to stand at room temperature for 15 min. Then the precipitate was removed by centrifugation at 12,000 rpm for 15 min. Finally, the supernatant was measured spectrophotometrically at 280 nm. One unit of the protease activity was defined as the amount of enzyme required to liberate 1 μ g of tyrosine in 1 min. Total protein content was measured by the method of Bradford (Bradford, 1976) with bovine serum albumin as the standard.

2.5. Optimization of temperature, pH, agitation, incubation period and inoculum size for protease production

The effect of different physical parameters on alkaline protease production was optimized by growing bacterial isolate RS3 in the protease production medium. The effect of temperature on the production of alkaline proteases was evaluated by assaying the enzyme after 24 hours of incubation in the culture medium at varying temperatures (i.e., 30, 35, 40, 45, 50, 55 and 60 $^{\circ}$ C). For optimizing pH, the medium was prepared by varying the pH from 5.0 to 11.0 at 1.0 unit interval. Agitation was determined by incubating the production medium with the isolate RS3 in different range of 70–200 rpm with 30.0 unit variation. Similarly, for optimal incubation time for protease production, the bacterial isolate RS3 was inoculated in the protease production medium and incubated for 24, 48, 72, 96 and 120 hours. Effect of varying inoculum percentage from 1% to 7% at 1% variation on protease production was determined. Specific activity of crude protease enzyme was determined.

2.6. Optimization of carbon and nitrogen sources on protease production

The effect of different chemical parameters on protease production was studied by using various carbon sources such as galactose, fructose, sucrose, starch, maltose, lactose, mannitol (1% w/v) and nitrogen sources such as urea, tryptone, casein, ammonium nitrate, potassium nitrate, sodium nitrate (1% w/v). After 24 hours incubation, the cell free supernatants were used to detect specific activity of protease enzyme. All the experiments were conducted in triplicate and then the mean values were calculated.

3. Results and Discussion

3.1. Isolation and screening of protease producing bacteria

Totally twenty four bacterial isolates were obtained from seven soil samples and screened for proteolytic

activity by inoculating on to skim milk agar plates. Out of these isolates, six isolates showed zone of hydrolysis indicating the extracellular protease production. The isolate RS3 showing maximum clear zone was selected for further experimental studies in order to optimize the production of protease.

3.2. Identification of the selected bacterial isolate

Morphological and physiological characteristics of the organism were investigated according to the methods described in Bergey's Manual of Systematic Bacteriology (Holt JG, 1994). Results of phenotypic and biochemical characterization of the isolate RS3 showed that aerobic, spore forming, Gram-positive rod shaped bacteria. The morphological, physiological and biochemical properties of the isolate RS3 was presented in Table 1. Based on the morphological, physiological and biochemical characterization the isolate RS3 belongs to the genus *Bacillus*. The organism was further identified by 16S rRNA gene sequencing analysis, the genomic DNA of the isolate RS3 was amplified and obtained the product length of 1449 bp nucleotides. The 16S ribosomal RNA gene was sequenced and submitted to NCBI GenBank and the accession number is KT962236. The blast result of the isolate was found 100% sequence similarity with *Bacillus cereus*. The phylogenetic tree constructed by the neighbour-joining method indicated that the isolate RS3 was formed separate cluster along with *B. cereus* (Fig. 1). Overall, physiological, biochemical and molecular characterization, the isolate was identified as *Bacillus cereus* RS3.

3.3. Optimization of temperature, pH, agitation, incubation period and inoculum level for protease production

The effect of various incubation temperatures on alkaline protease production was studied. It has been observed that temperature is one of the most crucial factor that have to be controlled in bioprocess and it varies from organism to organism (Chi and Zhao, 2003). Production of extracellular enzymes could be influenced by altering the physical properties of the culture media. The results (Fig. 2) revealed that the *B. cereus* isolate RS3 producing alkaline protease over a wide range of temperatures (30 $^{\circ}$ to 60 $^{\circ}$ C). The maximum alkaline protease production was found to be at 45 $^{\circ}$ C with specific activity 118 U/mg. While the incubation temperature was increased up to 60 $^{\circ}$ C and decreased the yield to 104 U/mg. The results of the present study also coincided with previous reports. Temperatures at 45 $^{\circ}$ C have been reported to be optimum for the production of protease by *B. subtilis*

(Pant *et al.*, 2015) and also *Bacillus* spp. (Atalo and Gashe, 1993, Sen and Satyanarayana, 1993). It is well known that pH is one of the important characteristic of most of microorganisms for their cell growth and enzyme production (Kumar and Takagi, 1999). The production medium was adjusted at different initial pH values (5-11) were used to study their effect on the

protease production. The isolate RS3 was produced reasonable amount of protease under neutral and alkaline conditions and also the highest specific activity 128 U/mg was observed at pH 9 (Fig. 3). Most of the *Bacillus* spp. have been previously reported the optimum protease production at pH 7.0 - 11.0 (Joo and Chang, 2005, Shivanand and Jayaraman, 2009).

Table 1. Physiological and biochemical characteristics of *B. cereus* RS3

Characterization	Isolate RS3
Gram staining	+
Morphology	Rod
Spore formation	+
Motility	+
Nitrate reduction	+
Catalase	-
Methyl red test	+
Vogues Proskauer	+
Indole production	-
Citrate Utilization	+
Hydrolysis of starch	+
Hydrolysis of gelatin	+
Hydrolysis of casein	+

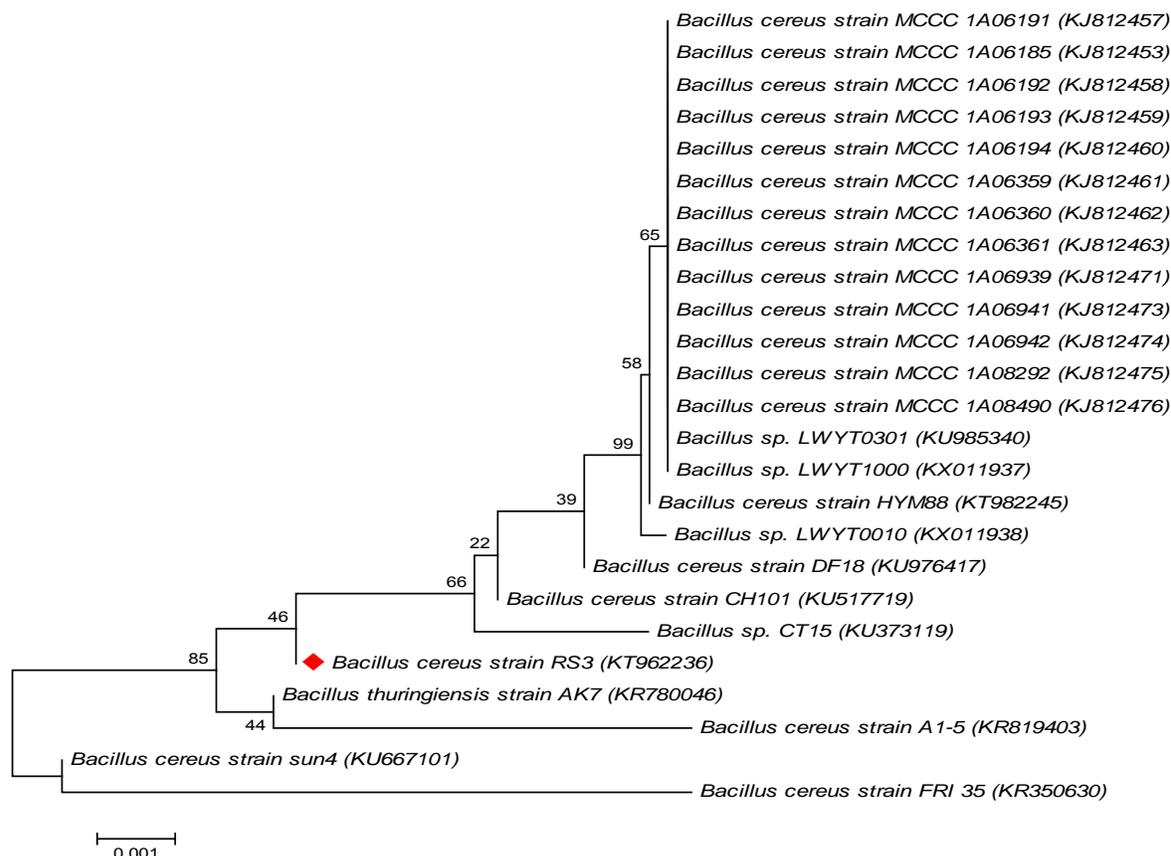


Fig. 1. Phylogenetic tree constructed using 16S rRNA gene sequences by neighbour-joining method for *Bacillus cereus* RS3 and other closely related gene sequences. Numbers in the parentheses are GenBank accession number.

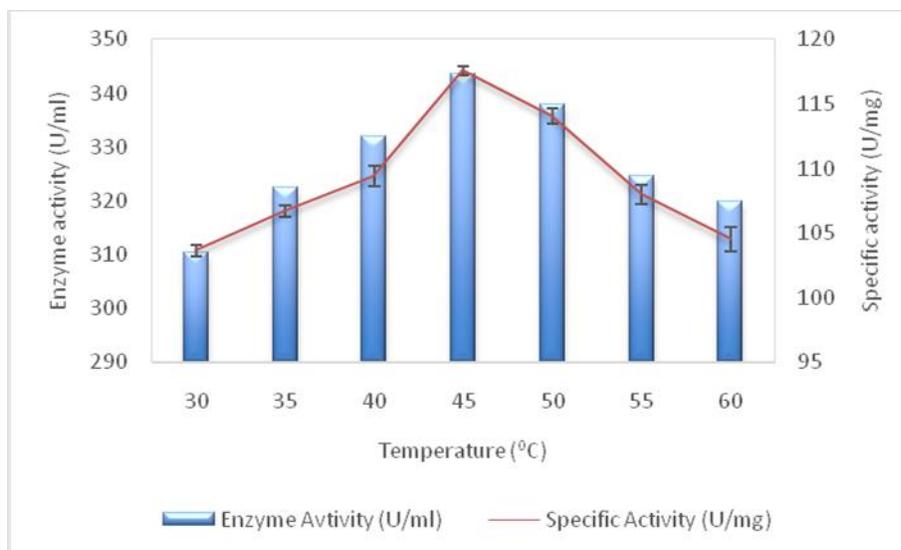


Fig. 2. Effect of different incubation temperature on protease production by *Bacillus cereus* RS3

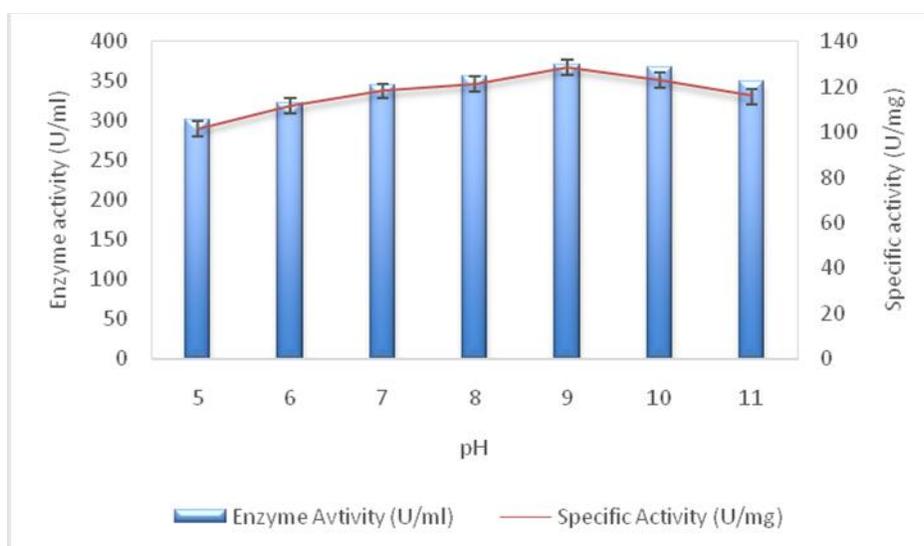


Fig. 3. Effect of different pH on protease production by *Bacillus cereus* RS3

The effect of different agitation rates on alkaline protease production was studied and the results are presented in Fig. 4. The isolate RS3 showed an increase in protease production with the increase in the agitation rate up to 170 rpm with a maximum specific activity of 144 U/mg. Further increase in agitation rates, the alkaline protease production yield started to decrease. The various agitation speeds have been found to effect the extent of mixing in the shake flasks or bioreactors and will also affect the availability of nutrients during the enzyme production (Nascimento and Martins, 2004). The incubation period affects the extracellular protease yield and the results were showed in Fig. 5. The significant rate of protease

production was achieved in early stationary phase at 145 U/mg and decreased the enzyme production after 24 hrs of incubation period. The inoculum size also influenced the protease production, in the present study, maximum protease production was observed at 4% inoculum and the specific activity 158 U/mg (Fig. 6). There was a reduction in protease production when inoculum size was reduced (1 %), these may be due to insufficient size of the bacterial population, leading to low enzyme biosynthesis. Moreover, an inoculum size of more than 4% could result reduced the enzyme production which is due to lower concentration of dissolved oxygen and higher competition for nutrients (Smita *et al.*, 2012).

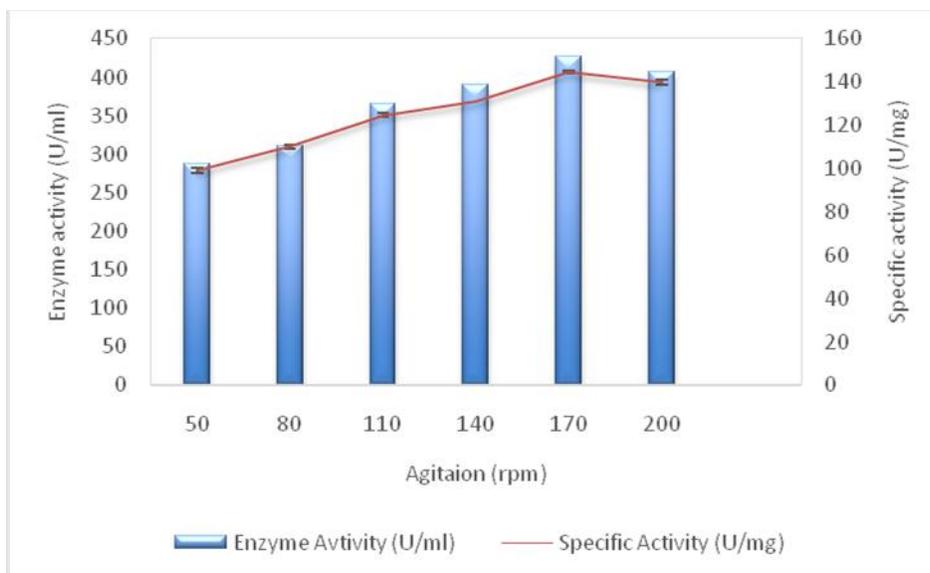


Fig. 4. Effect of different agitation speed on protease production by *Bacillus cereus* RS3

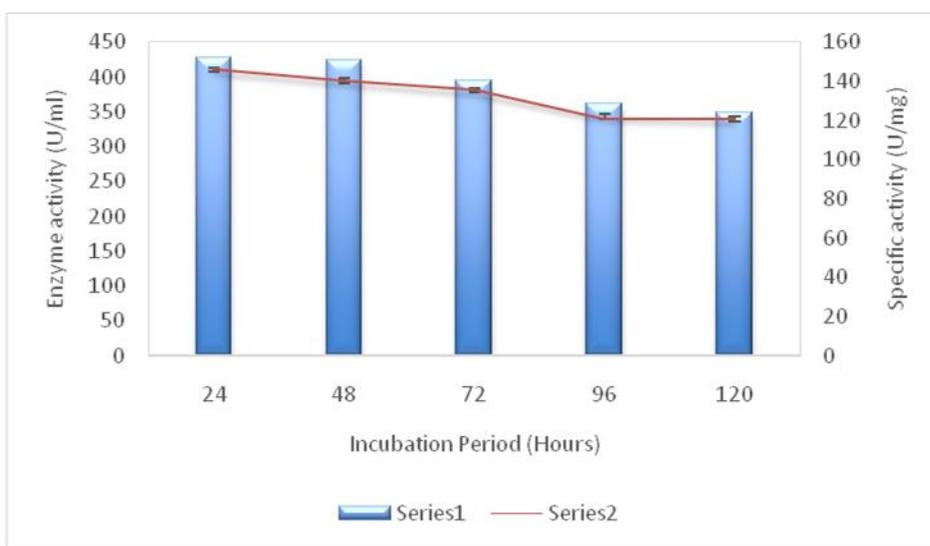


Fig. 5. Effect of different incubation period on protease production by *Bacillus cereus* RS3

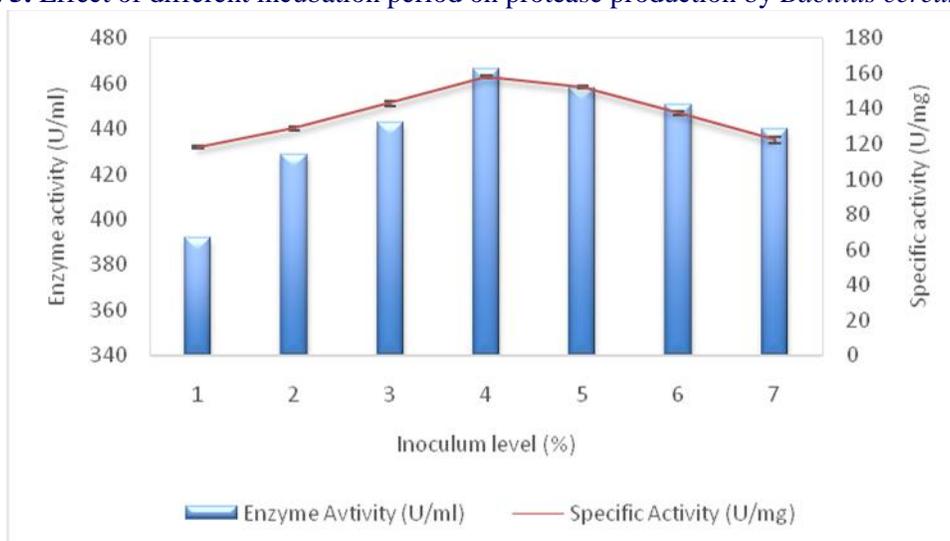


Fig. 6. Effect of different level of inoculum on protease production by *Bacillus cereus* RS3

3.4. Optimization of carbon and nitrogen sources on protease production

The carbon and nitrogen sources in the production medium have play an important role in increasing the production of alkaline proteases.(Gupta *et al.*, 2002) *Bacillus cereus* RS3 was able to utilize different sources of carbon, however, protease production varied with each carbon source (Fig. 7). The result showed that fructose was the most suitable source of carbon for the production of alkaline proteases by *B.*

cereus RS3 and yielded 173U/mg, followed by sucrose (163.5 U/mg) and galactose (153 U/mg). The combination of lactose and maltose provided a moderate to good degree of protease activity. This finding agrees with previous research on supplemented with fructose as a carbon source and increased the enzyme production in *Bacillus* species (Kumar *et al.*, 2002, Sen and Satyanarayana, 1993). However, the different carbon sources have different effects on biosynthesis of extracellular enzyme by different strains (Chi and Zhao, 2003).

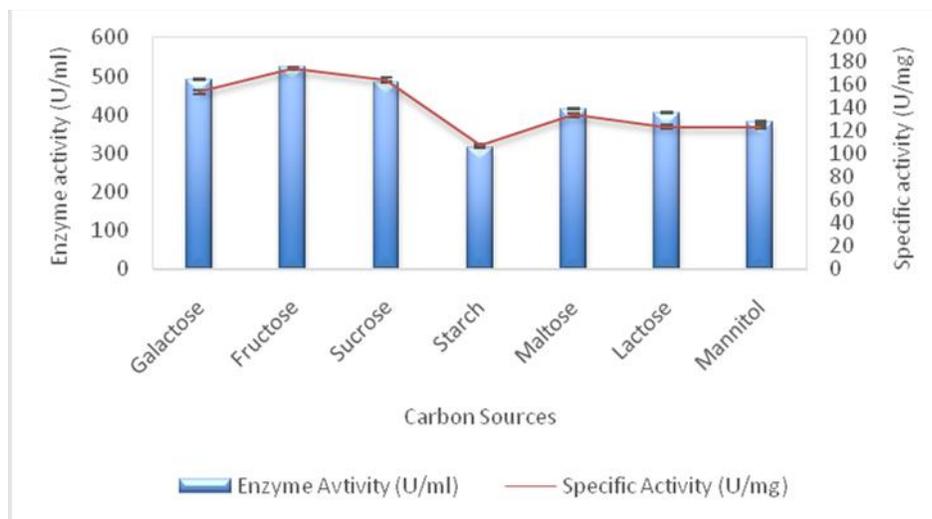


Fig. 7. Effect of various carbon sources on protease production by *Bacillus cereus* RS3

Various organic and inorganic nitrogen sources were used in relation to biosynthesis of protease enzyme by *B. cereus* RS3 (Fig. 8). The optimum nitrogen source for maximum protease production was found to be as yeast extract. The requirement for a specific nitrogen

source for protease production differs from organism to organism and also the alkaline protease biosynthesis is depends up on the presence of both nitrogen and carbon sources in the production medium (Kole *et al.*, 1988).

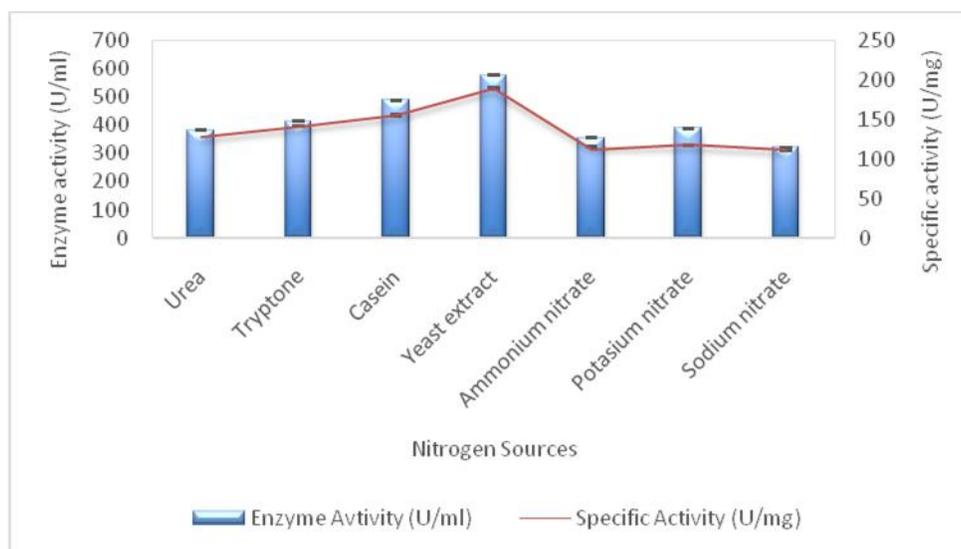


Fig. 8. Effect of various nitrogen sources on protease production by *Bacillus cereus* RS3

4. Conclusion

In this study, an extracellular alkaline protease producing bacterial isolate RS3 was isolated from desert soil samples and identified as *B. cereus* RS3 by morphological, biochemical and molecular characterization including 16S rRNA gene sequencing. The optimal media components and conditions for the highest protease production were optimized. The maximum production of alkaline protease was observed at pH 9 and a temperature of 45°C with fructose and yeast extract as the carbon and nitrogen sources respectively. *B. cereus* RS3 can be used for large-scale production of alkaline protease to meet present-day needs in the industrial sector.

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How to cite this article:

Shine K, Kanimozhi K, Panneerselvam A, Muthukumar C, Thajuddin N. (2016). Production and optimization of alkaline protease by *Bacillus cereus* RS3 isolated from desert soil. *Int. J. Adv. Res. Biol. Sci.* 3(7): 193-202.