**Journal of**
**Environmental and Applied Bioresearch**
Published online January 23, 2016 (http://www.scienceresearchlibrary.com)

Vol. 04, No. 1, pp. 09 – 15
ISSN 2319 8745

**Research Article** **Open Access**

# OPTIMIZATION OF PRODUCTION AND PARTIAL CHARACTERIZATION OF ALKALINE PROTEASE BY NEWLY ISOLATED *BACILLUS SUBTILIS* STRAIN RS2 FROM DESERT SOIL

**Shine K[1*], Kanimozhi K[1], Panneerselvam A[1], Muthukumar C[2], Thajuddin N[2].**

[1]Department of Botany and Microbiology, AVVM Sri Pushpam College (Autonomous) Poondi 613503, Tamil Nadu, India

[2]Department of Microbiology, School of Life Sciences, Bharathidasan University, Tiruchirappalli 620 024, Tamil Nadu, India

Received: Nov 08, 2015 / Accepted : Dec 11, 2015


## Abstract

A bacterial strain RS2 with high proteolytic activity was isolated from desert soil samples of Riyadh, Saudi Arabia. The strain was identified as *Bacillus subtilis* by morphological and biochemical characteristics as well as by 16S rRNA gene sequence analysis. Process parameters influencing the maximum production of extracellular alkaline protease by *Bacillus subtilis* strain RS2 were optimized using one factor constant at a time method along with its characterization. The maximum level of alkaline protease enzyme production was obtained when the incubation temperature was $40^0$ C in growth medium of pH 9 with 2% inoculum and continuous agitation at 140 rpm for 48 hours. The *Bacillus subtilis* strain RS2 utilized several carbon sources for the production of alkaline proteases, lactose was the best carbon source followed by glucose and sucrose. Among the various organic and inorganic nitrogen sources, casein was found to be the best nitrogen source for the maximum production of alkaline protease. The crude alkaline protease enzyme showed maximum activity at pH 9 and temperature $50^0$ C. These findings suggested that the protease enzyme produced by *Bacillus subtilis* strain RS2 has a potential for industrial applications by its activity at high temperature and high alkaline pH.

**Keywords:** *Bacillus subtilis*, Alkaline protease, Desert soil, Optimization

*Corresponding authors:shine141@gmail.com

## Introduction

Protease constitutes one of the most important groups of industrial enzymes, accounting for more than 65% of total worldwide sales of industrial enzymes (Banik and Prakash 2004). Plants, animals and microbes are the main sources for protease production. Among these, the microorganisms represent an excellent source of enzymes due to their broad biochemical diversity and susceptibility to genetic manipulation. Indeed, microbial proteases account for approximately 40% of the total industrial enzyme market (Rao et al., 1998). Interestingly, bacteria of the genus *Bacillus*, produced the most commercial proteases used today (Rao et al., 1998, Mehrotra et al., 1999). The most dominant species used in industry for alkaline protease production are *Bacillus subtilis*, *Bacillus licheniformis* and *Bacillus pumilus* (Gupta et al., 2002). The performance of alkaline protease in detergent is influenced by several factors such as pH and temperature of washing solution as well as detergent composition. Ideally, proteases used in detergent formulations should have high level of activity over a broad range of pH values and temperatures (Kumar et al., 1999).

Alkaline proteases have diverse applications in a variety of industrial sectors, such as food, detergent, leather, pharmaceutical, textile and silk gumming, silver recovery and production of protein hydrolysates (Banik and Prakash 2004). The new applications will be continued to grow in the present and future as the increased demands for stable biocatalysts capable of withstanding the harsh conditions of the operations (Bruins et al., 2001, Rao et al., 1998, Bryan 2000, Siezen and Leunissen 1997).

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 and 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).

The culture conditions that promote production of enzymes like proteases are significantly different from the culture conditions promoting cell growth (Moon and Parulekar 1991). Optimization of culture medium components which have been predicted to play a significant role in enhancing the production yield of extracellular protease enzymes. It is greatly influenced by media components, especially 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.

In the present investigation, an alkaline protease producing *Bacillus subtilis* strain RS2 was isolated from desert soil of Saudi Arabia and optimized the production conditions for maximum enzyme yield and partial characterization of crude enzyme was also studied.

## 2. Materials and methods

### 2.1. *Isolation and screening of protease producing bacteria*

Soil Samples were collected from five different desert places of Riyadh, Saudi Arabia. Collected sample was serially diluted in sterile normal saline water and the dilutions ($10^{-3}$, $10^{-4}$) were plated in nutrient agar plates and kept for incubation at $37^0$ C. Colonies were picked based on divergence in morphology, size and colour. Proteolytic activity of bacterial strain was screened by inoculating on to skim milk agar plates. Plates were incubated at $37^0$ C for 24 h. A clear zone formation on the media indicates the production of protease by organisms. The isolates showing maximum clear zone was selected for further optimization studies to enhance the protease production.

### 2.2. *Identification of selected bacterial strain*

The selected protease producing strain was identified using morphological and biochemical properties, according to Bergey's Manual of Determinative Bacteriology (Holt et al., 1994) and 16S rRNA sequencing analysis. Genomic DNA was extracted as per the standard protocol (Babu et al., 2009) and it was amplified by using two universal 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 conducted using software package MEGA 5 (Tamura et al., 2011).

### 2.3. *Protease Production*

One ml of overnight grown bacterial culture was inoculated in 49 ml GYP basal production medium (Kumar and Bhatla 2004) (Components: Glucose 1%, Yeast extract 0.5%, Peptone 0.5%, $MgSO_4$ 7.$H_2O$ 0.02%, $K_2HPO_4$ 0.1%) contained in 250 ml Erlenmeyer flasks and incubated at $40^0$ C on incubator shaker for 48 hours at agitation rate 120 rpm. After incubation culture broth was centrifuged at 10,000 rpm at $4^o$ C. The supernatant was used as the crude enzyme for the assay of alkaline protease activity.

### 2.4. *Assay of proteolytic activity*

Protease activity was measured by the sigma's non-specific protease assay method described by Cupp-Enyard (2008). Total protein content was measured by method of Bradford (1976) with bovine serum albumin as the standard. The specific activity of protease enzyme was expressed in U/mg.

### 2.5. *Optimization of Temperature, pH, agitation, inoculum size and incubation period for protease production*

The effect of various physical parameters on protease production was assessed by growing bacterial culture in the GYP medium. The effect of temperature on the production of extracellular proteases was studied by assaying the enzyme after 24 hours of incubation period in the culture medium at varying temperatures (i.e., 30, 35, 40, 45, 50, 55 and $60^o$ 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 bacterial culture at a range of 60–160 rpm with 20.0 unit variation. Effect of varying inoculum percentage from 1% to 7% with 1% variation on protease production was determined. Similarly, for optimal incubation time for protease production, the bacterial culture was inoculated in the GYP medium and incubated for 24, 48, 72, 96 and 120 hours. 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 various carbon sources such as lactose, sucrose, maltose, mannose, xylose, fructose, galactose, mannitol (1% w/v) and nitrogen sources such as urea, tryptone, casein, ammonium nitrate, potassium nitrate, sodium nitrate (1% w/v). After 48 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 considered.

### 2.7. *Effect of temperature and pH on protease activity*

To determine the optimum temperature for crude enzyme, the activity values of protease were measured by incubating the reaction mixture at various temperatures (30, 40, 50, 60, 70 and 80° C) for 10 min during standard enzyme assay.

The effect of pH on protease activity was studied by incubating the reaction mixture at pH values ranging from 5.0 to 12.0, in the following buffer systems 0.1M sodium phosphate (pH 5.0– 7.0), 0.1M Tris–HCl (pH 8.0–10.0), 0.1M glycine–NaOH (pH 10.0–12.0).

## 3. Results and Discussion

### 3.1. *Isolation and screening of protease producing bacteria*

In the present study, a total number of 81 bacterial isolates were isolated from five different desert soils of Riyadh region and screened for protease enzyme production on skim milk agar plates. Among the 81 isolates, 4 strains were identified as protease producers by zone of clearance around the colonies and quantified their alkaline protease activity. In protease assay, strain RS2 showed highest production when compared to other strains (Fig. 1). Hence, the isolate RS2 was used for optimization studies.

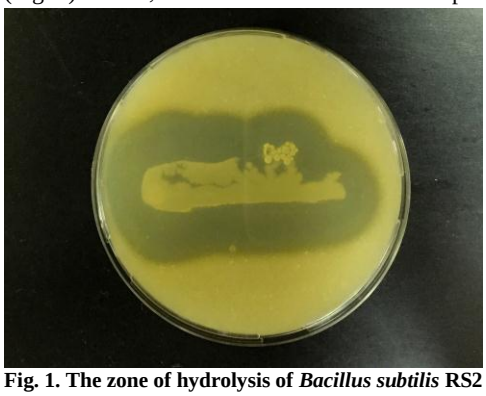

**Fig. 1. The zone of hydrolysis of *Bacillus subtilis* RS2 on skim milk agar.**

### 3.2. *Identification of the selected bacterial isolate*

Various biochemical tests were performed to identify the isolate RS2 according to the methods described in Bergey's Manual of Systematic Bacteriology (Holt et al., 1994). Isolate RS2 is an aerobic, spore forming, Gram-positive rod shaped motile bacteria, which is catalase and oxidase positive. The morphological, physiological and biochemical properties of the

strain RS2 are summarized in Table 1. The results indicated that the isolate RS2 belongs to the genus *Bacillus.*

**Table 1. Morphological and Biochemical Characterization of *Bacillus subtilis* RS2**

| Characterization | Isolate RS2 |
|---|---|
| Gram staining | + |
| Morphology | Rod shape |
| Spore staining | + |
| Motility | + |
| Nitrate reduction | + |
| $H_2S$ Production | - |
| Oxidase | + |
| Catalase | + |
| Methyl red test | + |
| Vogues Proskaeur | + |
| Indole production | - |
| Citrate Utilization | + |
| Hydrolysis of starch | + |
| Hydrolysis of gelatin | + |
| Hydrolysis of casein | + |

**+ Positive reaction, - Negative reaction**

To confirm the identity of the isolate, 16S rRNA gene sequence analysis was carried out. The 16S ribosomal RNA gene was sequenced and submitted to NCBI GenBank and the accession number is KT962235. The blast result of the isolate was found 100% sequence similarity with *Bacillus subtilis*. The phylogenetic tree constructed by the neighbour-joining method indicated that the isolate RS2 was formed separate cluster along with *B. sublilis* (Fig. 2). Overall, physiological, biochemical and molecular characterization, the isolate was identified as *Bacillus subtilis* strain RS2.

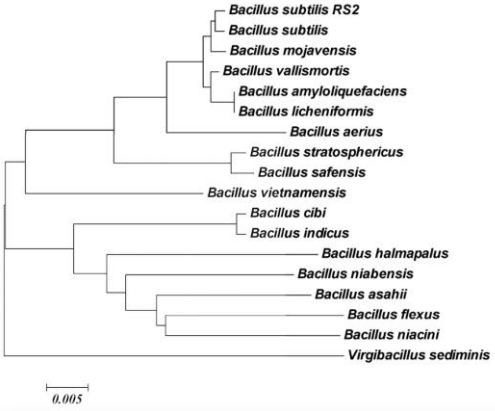


**Fig. 2. Phylogenic tree showing the relationship of protease enzyme producing *Bacillus subtilis* RS2**

### 3.3. *The effect of Temperature, pH, agitation, inoculum level and incubation period for protease production*

The effect of different incubation temperatures on protease production was evaluated. It is known that temperature is one of the most critical parameters that have to be controlled in bioprocess and it varies from organism to organism (Chi and Zhao, 2003). Temperature influences secretion of extracellular enzymes by changing the physical properties of the cell membrane. The results (Fig. 3) revealed that the *B. subtilis* strain RS2 produce extracellular enzyme over a wide range of temperatures ($30^0$ to $60^0$ C), the maximum protease enzyme specific activity 91 U/mg was found to be at $45^0$ C, hence the optimum incubation temperature for protease production by *B. subtilis* strain RS2 is $45^0$ C. Temperatures at $45^0$ C have been reported to be optimum for the production of protease by *B. subtilis* (Pant et al., 2015).

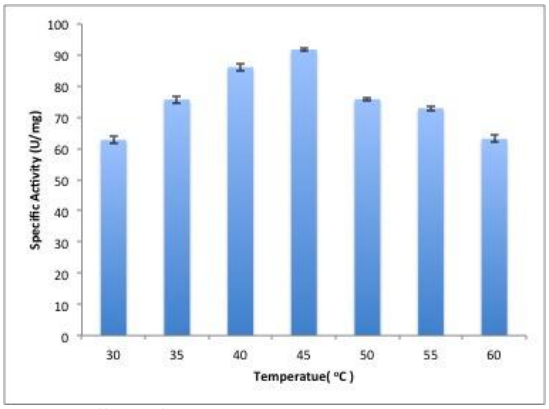


**Fig. 1. Effect of temperature on protease production by *Bacillus subtilis* RS2. The bars indicate the standard deviation of three replicates analyzed**

It has been noted that one of the important characteristic of most microorganisms is their strong dependence on the extracellular pH for cell growth and enzyme production (Kumar and Tagaki, 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 RS2 has produced reasonable amount of protease under neutral and alkaline conditions and the highest specific activity 93.7 U/mg was observed at pH9 (Fig. 4). Hence, in the following experiments, the pH of the growth medium was kept at pH 9. Most of the *Bacillus* spp. have been reported the optimum protease production at pH 7.0 - 11.0 (Joo and Chang 2005, Shivanand and Jayaraman 2009).

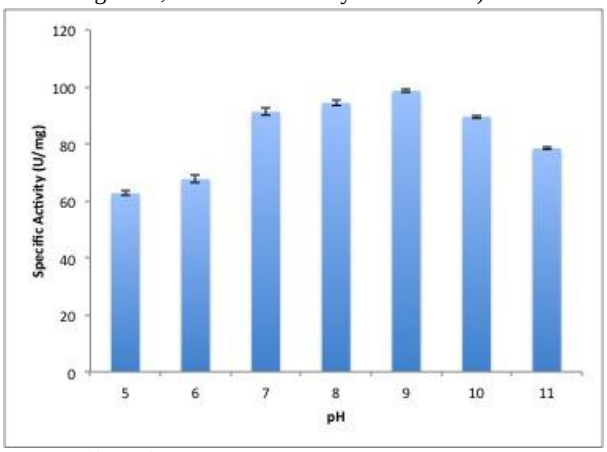


**Fig. 2. Effect of pH on protease production by *Bacillus subtilis* RS2. The bars indicate the standard deviation of three replicates analyzed**

The effect of different agitation rates on alkaline protease production is shown in Fig. 5. The isolate RS2 showed an increase in protease production with the increase in the agitation rate up to 140 rpm with a maximum specific activity of 105.2 U/mg. Further increase in agitation rates, decreased the alkaline protease production. The various agitation speeds have been found to influence the extent of mixing in the shake flasks or bioreactors and will also affect the nutrient availability during the enzyme production (Nascimento and Martins, 2004).

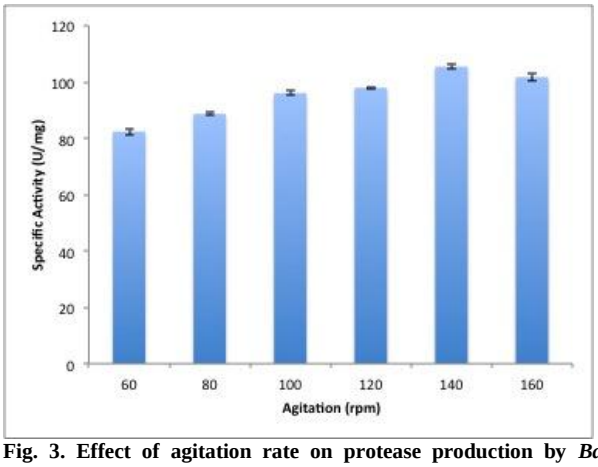


**Fig. 3. Effect of agitation rate on protease production by *Bacillus subtilis* RS2. The bars indicate the standard deviation of three replicates analyzed**

The level of inoculum is also an important factor for the production of alkaline protease. Various inoculum levels (1-7 %) were used to study their effect on protease production. A higher level of inoculum (7 %) was reducing the protease production than if the lower size of inoculum 1% (v/v) was used. The results (Fig. 6) showed that the highest production of alkaline protease by the isolate RS2 was 110 U/mg at the level of 3 %. Therefore, high level of inoculum size might not necessarily give higher protease production. The increase in protease yield using lower size inoculum was recommended due to the higher surface area to volume ratio ensuing in increased protease production (Rahman, 2005). There was a reduction in protease production when inoculum size was reduced and these may be due to insufficient number of bacteria which would have led to a reduced amount of enzyme secretion (Shafee et al., 2005). Higher level of inoculum in fermentation medium may cause reduced dissolved oxygen and increased competition towards nutrients (Smita et al., 2012).

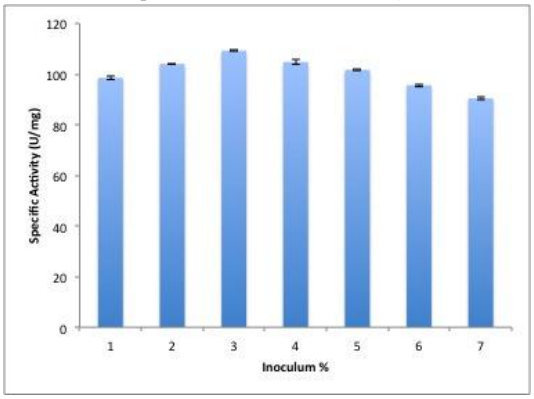


**Fig. 4. Effect of inoculum size on protease production by *Bacillus subtilis* RS2. The bars indicate the standard deviation of three replicates analyzed**

The appropriate incubation time for achieving maximum enzyme yield is governed by characteristics of the culture and enzyme production. The results of different incubation time on protease production by *B. subtilis* strain RS2 was shown in Fig. 7. The maximum production of protease (117.32 U/mg) was recorded after 48 h of incubation. The effect of incubation periods on biosynthesis of alkaline protease was found to vary with different microorganisms (Morimura et al., 1994, Battaglino et al., 1991, Nehra et al., 2002).

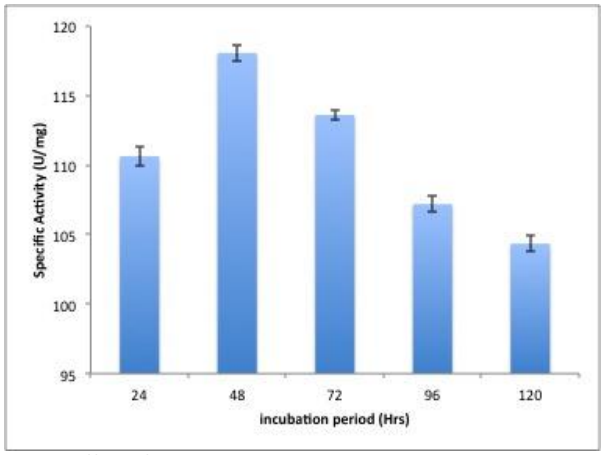


**Fig. 5. Effect of incubation period on protease production by *Bacillus subtilis* RS2. The bars indicate the standard deviation of three replicates analyzed**

### 3.4. *The effect of carbon and nitrogen sources on protease production*

The result showed that the suitable carbon source for production of protease enzyme by *B. subtilis* strain RS2 was lactose (Fig. 8). Less yield of protease enzyme was observed in the medium containing mannitol. Similar result was also reported by El-Safey and Abdul-Raouf (2004) who found that lactose as a best carbon source that induced the biosynthesis of protease by *B. subtilis.* However, increased yields of alkaline proteases were also reported by several researchers in the presence of different sugars such as glucose (Gessesse. 1997), maltose (Tsuchiya et al., 1991), sucrose (Phadatare et al., 1993) and fructose (Sen and Satyanarayana 1993). The different carbon sources have different effects on biosynthesis of extracellular enzyme by different strains (Chi and Zhao 2003).

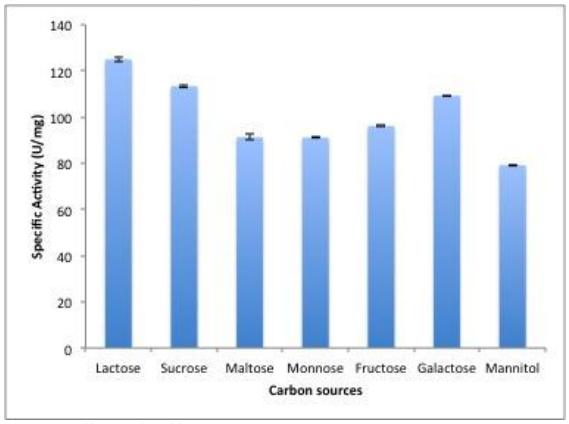


**Fig. 6. Effect of different carbon sources on protease production by *Bacillus subtilis* RS2. The bars indicate the standard deviation of three replicates analyzed**

The alkaline protease comprises 15.6% nitrogen and its biosynthesis is dependent on the presence of both nitrogen and carbon sources in the production medium (Kole et al., 1988). Various organic and inorganic nitrogen sources were used in relation to biosynthesis of protease enzyme by *B. subtilis* strain RS2 (Fig. 9). The optimum nitrogen source for maximum protease production was found as casein. The requirement for a specific nitrogen source for protease production differs from organism to

organism.

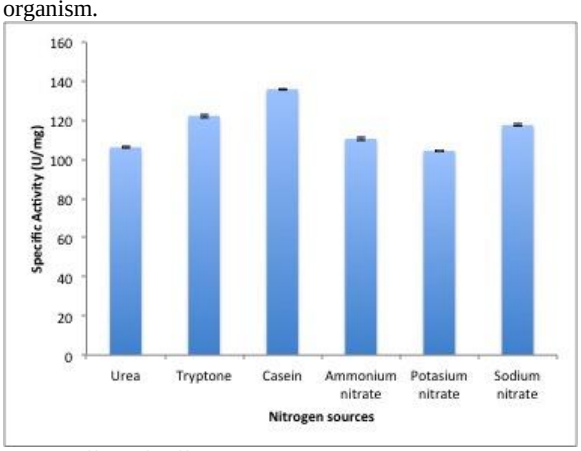


**Fig. 7. Effect of different nitrogen sources on protease production by *Bacillus subtilis* RS2. The bars indicate the standard deviation of three replicates analyzed**

### 3.5 *Effect of temperature and pH on protease activity*

The crude protease obtained from *B. subtilis* strain RS2, was further subjected to preliminary characterization study. To determine the effect of temperature and pH on the catalytic activity was studied by using casein as a substrate under the standard assay conditions. The maximum enzyme activities were taken as 100 % of relative activity. The relative protease activities, measured at various temperatures, were shown in Fig. 10. The optimum temperature for activity of protease from *B. subtilis* strain RS2 was 50º C and showed quite good activity over a temperature range of 40–60º C. Some earlier studies reported that optimum temperature for *Bacillus pumilus* (Aoyama et al., 2000) and *B. subtilis* (Uchida et al., 2004) was 50º C, which coincides with the present investigation. However, a higher optimum temperature of 60º C was observed in *Bacillus* sp. SMIA-2 (Nascimento and Martins, 2004) and in another *Bacillus* strain (Horikoshi, 1990, Banerjee et al., 1999).

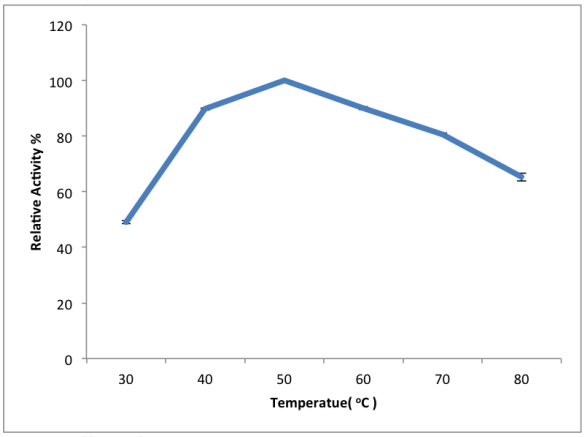


**Fig. 8. Effect of temperature on protease activity**

The relative protease activity at different pH values ranging from 5.0 to 12.0 were recorded (Fig. 11). The crude protease of *B. subtilis* strain RS2 was active over wide ranges of pH between 5 and 12 and optimum at pH 9.0. Generally, commercial proteases from microorganisms have maximum activity in the alkaline pH range of 8-12 (Durham et al., 1987, Rao et al., 1998, Kumar et al., 1999, Gupta et al., 2002). The proteases with an optimal pH of 9–12 are desirable for applications in detergents and tanning industries (Haddar et al., 2009).

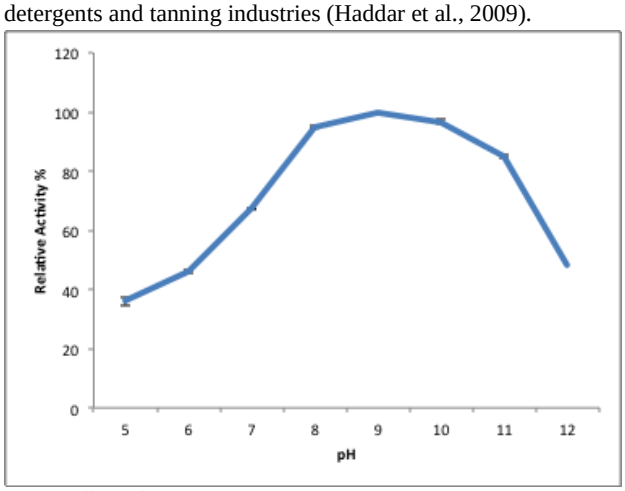


**Fig. 9. Effect of pH on protease activity**

## 4. Conclusion

In the present study, alkaline protease producing bacterial strain was isolated from desert soil samples and identified as *B. subtilis* by morphological, biochemical and molecular characterization including 16S rRNA gene sequencing. The optimal media components and conditions for the highest protease production were recorded. The maximum production of alkaline protease was observed at pH 9 and a temperature of 45º C with lactose and casein as the carbon and nitrogen sources respectively. The crude protease obtained from *B. subtilis* strain RS2, was further subjected to preliminary characterization study and determined the optimum pH 9 and optimum temperature 50º C for catalytic activity of the enzyme. *B. subtilis* strain RS2 can be used for large-scale production of alkaline protease to meet present-day needs in the industrial sector.

## References

Aleksieva P, Djerova A, Tchorbanov B, Girarov J. (1981). Submerged cultivation of a strain of *Humicola lutea* 72 producing acid protease. European Journal of Applied Microbiology and Biotechnology 13, 165-169.

Aoyama M, Yasuda M, Nakachi K, Kobamoto N, Oku H, Kato F. (2000). Soybean-milk- coagulating activity of *Bacillus Pumilus* derives from a serine proteinase. Applied Microbiology and Biotechnology, 53, 390-395.

Babu TG, Nithyanand P, Babu NC, Pandian SK. (2009). Evaluation of cetyltrimethylammonium bromide as a potential short-term preservative agent for stripped goat skin. World Journal of Microbiology and Biotechnology, *25*, 901-907.

Banerjee UC, Sani RK, Azmi W, Sani R. (1999). Thermostable Alkaline Protease from *Bacillus brevis* and Its Characterization as a Laundry Detergent Additive. Process Biochemistry, 35, 213-219.

Banik RM, Prakash M. (2004). Laundry detergent compatibility of the alkaline protease from *Bacillus cereus*. Microbiological Research, 159, 135–140.

Bataglino RA, Huergo M, Pilosot, AM, Bartholomai AB. (1991). Culture requirements for the production of protease by *Aspergillus oryzae* in solid state fermentation. Applied Microbiology and Biotechnology, 35, 292-295.

Bradford MM. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72, 248-254.

Bruins ME, Janssen AE, Boom RM. (2001). Thermozymes and their applications: a review of recent literature and patents. Applied Biochemistry and Biotechnology, 90, 155–186.

Bryan PN. (2000). Protein engineering of subtilisin. Biochimica et Biophysica Acta (BBA)-Protein Structure and Molecular Enzymology, 1543, 203-222.

Cherif A, Tsiamis G, Compant S, Borin S. (2015). Biodesert: Exploring and exploiting the microbial resource of hot and cold deserts. BioMed research international.

Chi Z, Zhao S. (2003). Optimization of medium and cultivation conditions for pullulan production by a new pullulan-producing yeast. Enzyme and Microbial Technology, 33, 206–221.

Chi Z, Zhao S. (2003). Optimization of medium and cultivation conditions for pullulan production by a new pullulan-producing yeast. Enzyme and Microbial Technology 33, 206–221.

Cupp-Enyard C. (2008). Sigma's non-specific protease activity assay-casein as a substrate. *Journal of visualized experiments: JoVE*, (19).

Durham DR, Stewart DB, Stellwag EJ. (1987). Novel alkaline and heat- stable serine proteases from alkalophilic *Bacillus* sp. strain GX6638. Journal of Bacteriology, 169, 2762-2768.

El-Safey E.M. and Abdul-Raouf I.M. (2004). Production, purification and characterization of protease enzyme from *Bacillus subtilis.* In International Conference for Development and the Environment in the Arab World, Assiut Univ, Egypt, 14.1.

Gessesse A. (1997). The use of nug meal as a low-cost substrate for the production of alkaline protease by the alkaliphilic *Bacillus sp.* AR-009 and some properties of the enzyme. Bioresource Technology, 62, 59–61.

Gupta R, Beg QK, Lorenz P. (2002). Bacterial alkaline proteases: molecular approaches and industrial applications. Applied Microbiology and Biotechnology, 59, 15-32.

Haddar A, Agrebi R, Bougatef A, Hmidet N, Sellami-Kamoun A, Nasri M. (2009).Two detergent stable alkaline serine-proteases from *Bacillus mojavensis* A21: purification, characterization and potential application as a laundry detergent additive. Bioresource Technology, 100, 3366–73.

Hameed A, Keshavarz T, Evans CS. (1999). Effect of dissolved oxygen tension and pH on the production of extracellular protease from a new isolate of *Bacillus subtilis* K2, for use in leather processing. Journal of chemical technology and biotechnology, 74, 5-8.

Holt JG, Krieg NR, Sneath PHA, Staley JT, Williams ST. Bergey's Manual of Determinative Bacteriology. 9th ed. Baltimore: Williams and Wilkins; (1994).

Horikoshi, K. Enzymes of Alkalophiles. In: MO. St. Louis, editor. Microbial enzyme and biotechnology. 2nd ed. Amsterdam: Elsevier; (1990), pp. 275–294.

Joo H, Chung SC. (2005). Production of protease from a new alkalophilic *Bacillus sp.* I-312 grown on soybean meal: optimization and some properties. Process Biochemistry 40, 1263-1270.

Kaur S, Vohra RM, Kapoor M, Beg QK, Hoondal GS. (2001). Enhanced Production and characterization of a highly thermostable alkaline protease from *Bacillus sp*. P-2. World Journal of Microbiology and Biotechnology, 17, 125-129.

Kole MM, Draper I, Gerson DF. (1988). Production of protease by *Bacillus subtilis* using simultaneous control of glucose and ammonium concentrations. Journal of Chemical Technology and Biotechnology, 41, 197–206.

Kumar CG, Tagaki H. (1999). Microbial alkaline protease: from bioindustrial view- point, Biotechnology Advances, 17, 561–594.

Kumar CG, Tiwari MP, Jany KD. (1999). Novel alkaline serine proteases from alkalophilic *Bacillus* spp.: purification and some properties. Process Biochemistry, 34, 441-449.

Kumar D and Bhatla, T.C. (2004). Purification and characterization of a small size protease form *Bacillus sp.* APR-4, Indian journal of experimental biology, 42, 515-521.

Mehrotra S, Pandey P, Gaur R, Darmwal N. (1999). The production of alkaline protease by a *Bacillus species* isolate. Bioresource Technology, 67, 201-203.

Moon SH, Parulekar SJ. (1991). A parametric study ot protease production in batch and fed-batch cultures of *Bacillus firmus*. Biotechnology and Bioengineering, 37, 467-483.

Morimura S, Kida K, Sonoda Y. (1994). Production of protease using waste water from the manifacture of soochu. Journal of Fermentation and Bioengineering, 77, 183-187.

Nascimento WC, Martins ML. (2004). Production and properties of an extracellular protease from thermophilic *Bacillus* sp. Brazilian Journal of Microbiology, 35, 91–96.

Nehra KS, Dhillon S, Kamala C, Randhin S. (2002). Production of alkaline protease by *Aspergillus sp* under submerged and solid state fermentation. Indian Journal of Microbiology, 42, 43-47.

Oberoi R, Beg QK, Puri S, Saxena RK, Gupta R. (2001). Characterization and wash performance analysis of an SDS-stable alkaline protease from a *Bacillus sp.* World Journal of Microbiology and Biotechnology, 17, 493-497.

Pant G, Anil P, Pavani JV, Sayantan B, Deviram GV, Ajay K, Mitali P, Ravi GP. (2015). Production, optimization and partial purification of protease from *Bacillus subtilis*. Journal of Taibah University for Science, *9*, 50-55.

Phadatare SU, Deshpande VV, Srinivasan MC. (1993). High activity alkaline protease from *Conidiobolus coronatus* (NCL 86.8.20): Enzyme production and compatibility with commercial detergents. Enzyme and Microbial Technology, 15, 72-76.

Puri S, Beg QK, Gupta R. (2002). Optimization of alkaline protease production from *Bacillus sp.* by response surface methodology. Current microbiology, 44, 286-290.

Rahman RN, Geok LP, Basri M, Salleh AB. (2005). Physical factors affecting the production of organic solvent-tolerant protease by *Pseudomonas aeruginosa* strain K. Bioresource Technology, 96, 429-436.

Rao MB, Tanksale AM, Ghatge MS, Deshpande VV. (1998). Molecular and biotechnological aspects of microbial proteases. Microbiology and Molecular Biology reviews, 62, 597-635.

Sen S, Satyanarayana T. (1993). Optimization of alkaline protease production by thermophilic *Bacillus licheniformis* S-40. Indian Journal of Microbiology, 33, 43-47.

Shafee N, Aris SN, Rahman R, Basri M, Salleh AB. (2005). Optimization of environmental and nutritional conditions for the production of alkaline protease by a newly isolated bacterium *Bacillus cereus* strain 146. Journal of Applied Sciences Research, 1, 1-8.

Shivanand P, Jayaraman G. (2009). Production of extracellular protease from halotolerant bacterium, *Bacillus aquimaris* strain VITP4 isolated from Kumta coast. Process Biochemistry, 44, 1088-1094.

Shumi WM, Hossain T, Anwar MN. (2004). Proteolytic Activity of a Bacterial Isolate *Bacillus fastidiosus* den Dooren de Jong. Journal of Biological Sciences, 4, 370-374.

Siezen RJ, Leunissen JA. (1997). Subtilases: the superfamily of subtilisin-like serine proteases. Protein Science : A Publication of the Protein Society, *6*, 501–523.

Singh J, Vohra RM, Sahoo DK. (1999). Alkaline protease from a new obligate alkalophilic isolate of *Bacillus sphaericus.* Biotechnology letters. 21, 921-924.

Singh SP, Purohit MK, Raval VH, Pandey S, Akbari VG, Rawal CM. Capturing the potential of haloalkaliphilic bacteria from the saline habitats through culture dependent and metagenomic approaches. In: Mendez-Vilas A, editor. Current research technology and education topics in applied microbiology and microbial biotechnology. Spain: Formatex Publishers; (2010), pp. 81–7.

Singh SP, Raval VH, Purohit MK, Pandey S, Thumar JT, Gohel SD, Sandip P, Viral G A, Chirantan M. Haloalkaliphilc bacteria and actinobacteria from the saline habitats: new opportunities for Biocatalysis and Bioremediation. In:

Satyanarayana T, Johri BN, Prakash A, editors. Microorganisms in environmental management: microbes and environment. New York: Springer Science Business Media; (2012), pp. 415–29.

Smita GS, Ray P, Mohapatra S. (2012). Quantification and optimization of bacterial isolates for production of alkaline protease. Asian Journal of Experimental Biological Sciences, 3, 180–186.

Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S, (2011). MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. Molecular Biology and Evolution, 28, 2731–2739.

Tsuchiya K, Sakashita H, Nakamura Y, Tetsu K. (1991). Production of thermostable alkaline protease by alkalophilic *Thermoactinomyces sp*. HS682. Agricultural and biological chemistry, 55, 3125– 3127.

Uchida H, Kondo D, Yamashita S, Tanaka T, Tran HL, Nagano H, Uwajima T. (2004). Purification and properties of a protease produced by *Bacillus subtilis* CN2 isolated from a Vietnamese fish sauce. World Journal of Microbiology and Biotechnology, 20, 579-582.