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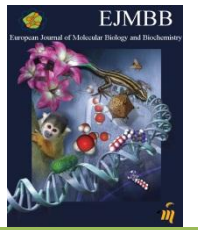

PRODUCTION AND OPTIMIZATION OF PROTEASE FROM *BACILLUS SUBTILIS* ISOLATED FROM AGRICULTURAL SOIL SAMPLE

R. Seetha and K. Padmalochana*

Department of Biochemistry, Sri Akilandeswari Womens College, Wandiwash – 604408, Tamilnadu, India.

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ABSTRACT

Microbial protease enzyme has vast applications in different industries and environmental bioremediation. The objective of this study was to isolate, characterize and optimize the protease producing bacteria. Protease producing bacteria was isolated from garden soil by serial dilution and identified as *Bacillus subtilis*. Protease production was enhanced by optimizing the fermentation culture conditions like carbon sources and nitrogen sources, NaCl, incubation time, pH, and agitation speed. The Optimum conditions for maximum protease production were achieved by using fructose as carbon Source and urea as nitrogen Source. Maximum protease production was attained at 1 M NaCl concentration. The optimum pH, incubation time and agitation speed for proteolytic activity were 8, 24 h and 250 rpm, respectively. Therefore, this reports showed excellent proteolytic activity of isolated and screened bacteria *Bacillus subtilis* under optimized physical and chemical conditions.

INTRODUCTION

Proteases are proteolytic enzymes that involved in the biological conversion ofproteins into amino acids by broke down of peptide bond. Mainly protease enzymes are important in digestive tract for the digestion of many foods [1,2]. Protease enzymes are most important and largest group of enzymes [3]. Protease enzymes were used in dry cleaning, detergents, meat processing, cheese making, photographic, and pharmaceutical industry [4-6]. Protease enzymes are classified into three categories based on their acid base behaviour are acid protease, alkaline protease and neutral protease.

Using microorganisms in synthesis of protease enzyme have replaced chemical hydrolysis of starch in starch processing industry. Microbes are considered as a bio factory and most suitable sources for production of protease enzymes.

Corresponding Author

K. Padmalochana
Email: - kpadmalochana@gmail.com

The enzyme production by an organisms are strongly influenced by physic chemical factors such as pH, temperature incubation time agitation, and medium components like carbon, nitrogen and NaCl sources [7]. The optimization of these parameters is the main study in an enzyme production process [8]. Microbial production of enzymes is easy and more convenient process because of their rapid growth, the limited space required for their cultivation and the ease to generate new enzymes with desirable applications either environmentally or genetically with altered characters [9].

Even though a wide variety of microorganisms like fungi (*Aspergillus flavus, Aspergillus miller*, *Aspergillus niger* and *Penicillium griseofulvin*, Mucor and Rhizopus) and bacteria (*Clostridiumsp, Pseudomonas fluorescens, Bacillus subtilus, E.coli and Serratiamarscens*) [10], yeast and molds. *Bacillus* produces a large proportion of extracellular enzymes, including proteases especially alkaline proteases [11, 12].

Several Bacillus species involved in protease production are e.g. *B. cereus*, *B. sterothermophilus*, *B. mojavensis*, *B. megaterium* and *Bacillus licheniformis* [13-15].In the study, *Bacillus subtilis*was isolated from garden soil and screened for proteolytic activity. Identified bacteria was subjected to highest protease production under some fermentations conditions like carbon, and nitrogen sources, pH, NaCl, incubation, agitation etc. the optimized medium was considered for highest production od protease.

MATERIALS AND METHODS

Sample Collection and isolation of bacteria

Agricultural soil from Around Sri Agilandeswari women college, Wandiwash was collected in a sterile container and it was brought to the laboratory for further processing. The collected sample was serially diluted up to 10^{-7} dilution using sterile saline as a blank and the diluted samples were plated using spread plate method into the sterile Nutrient agar (NA).

The NA plates were incubated at 37°C for 24 hours. The isolated colonies were further screened and purified by streak plate method using sterile media plates. The pure cultures were inoculated into sterile nutrient agar slants and nutrient broth for further characterization studies. The morphological and physiological characterization of the isolate was performed according to the methods described in Bergey's Manual of determinative bacteriology [16].

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.

Enzyme production

Preparation of Inoculum for bacteria

The inoculum for further production of enzyme and other studies was prepared using Luria broth (LB). The pure culture was inoculated into sterile inoculum broth and was incubated at 37°C in a rotary shaker for overnight. The fresh overnight culture was used as an inoculum for production of Enzyme.

Production

The enzyme production was carried out by shake flask fermentation using production mediumwhich comprising of Glucose as a carbon source and amended with Peptone as a proteinacious substrate with pH 7. Five hundred ml of sterile Production broth was prepared in one-litre conical flask and 5% inoculum was transferred aseptically in to the production medium. The inoculated medium was incubated at 37°C for 48 hours. The medium was agitated at 200 rpm for better aeration and growth of the organism.

Assay of proteolytic activity

Overnight culture of protease producing strain (1%) inoculums 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. 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 lmol of tyrosine per min under the defined assay conditions. The range of concentration 50–250 g of tyrosine was used as standard. The potential producer was taken for further optimization studies to enhancement the protease production.

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 2M 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.

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.

RESULTS AND DISCUSSION

Identification of proteolytic bacterial culture

The isolated microorganisms were examined using various cultural and biochemical test for identification. The cultural characteristics of microorganisms as a help in identifying and classifying organisms into taxonomic groups. Based on biochemical characters the isolated culture is identified as *Bacillus subtilis* (Table 1).The proteolytic activities of *Bacillus subtilis* was assayed using casein medium; it showed high proteolytic activity.

Optimization of culture conditions for protease production

The effect of salt on protease production was shown in (Figure. 1).Maximum protease production was observed in the medium 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. In a previous report a similar NaCl concentration was found to be optimum for the production of protease. 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 (Figure2). Less production of enzyme was recorded in the medium containing starch (87.13 U/mg). Similar results of fructose was the best carbon source for protease production was reported by Sangeetha et al and Mabrouk et al [17, 18]. Xylose can be easily utilized by our strain *B. subtilis* compared to other saccharides. An increased yield of enzyme production from various carbon sources such as lactose,maltoseand sucrose have been reported by other researchers. Various nitrogen sources were investigated for protease production. High yield of protease production was observed (Figure3) 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 [19]. Both organic and inorganic nitrogen compounds were utilized by bacterial strain. Its shows the versatility of the bacteria utilizing a range of compounds. The effect of incubation period on the protease production was shown in (Figure 4). *B. subtilis* 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. and *Halobacterium sp*. have been reported that maximum protease production was achieved at 48 and 96 h incubation period respectively. When comparing the earlier reports our strain *B. subtilis* has shorter period of incubation for the production of protease. B. *subtilis* 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) (Figure 5). The production at pH 7 and 8 was relatively comparable. Most of the *Bacillus* sp. reported has optimum pH from 7.0 to 11.0 for the production of protease.

The effect of various agitation rates was investigated for protease production; optimum production of protease (Figure6) 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 and B. *subtilis* showed optimum yields of alkaline protease production under the condition of 200 rpm. In the present study, maximum protease production was observed (Figure 7) 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. Pseudomonas sp. has been reported that 1.5% inoculums showed maximum enzyme production.

Table 1. Cultural and biochemical characters of identified bacteria *Bacillus subtilis*

S.No	Biochemical Tests	*Bacillus subtilis*
1	Gram staining	Gram +ve, Rod
2	Agar slant	Abundant, white, waxy
3	Motility	-ve
4	Spore staining	Positive
5	Indole	Positive
6	Methyl red	positive
7	VogesProskauer	positive
8	Citrate Utilization	positive
9	Starch hydrolysis	Positive
10	Oxidase	Positive
11	Catalase	-ve
12	Urease	Negative
13	H_2S Production	Negative
14	Mannitol salt agar	negative
15	Triple sugar iron agar test	Acid and alkaline

Figure 1. Effect of various concentration of NaCl on protease production in *Bacillus subtilis* isolated from garden soil. The bars indicate the standard deviation of three replicates analysed.

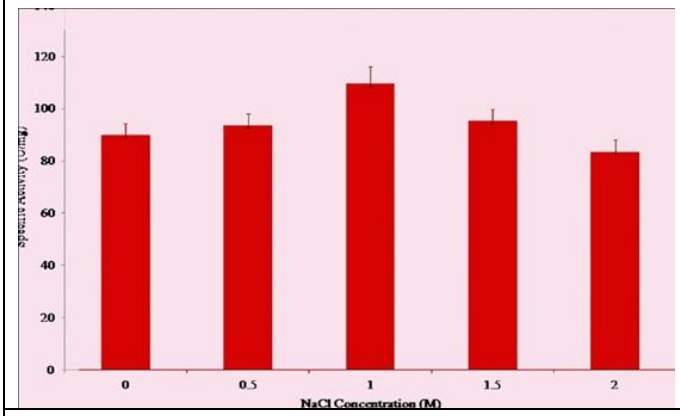


Figure 2. Effect of different carbon sources on protease production in *Bacillus subtilis* isolated from garden soil. (Xyl-Xylose, Dex-Dextrose, Fru-Fructose, Suc-Sucrose, Gal-Galactose, Man-Mannose, Lac-Lactose). The bars indicate the standard deviation of three replicates analysed

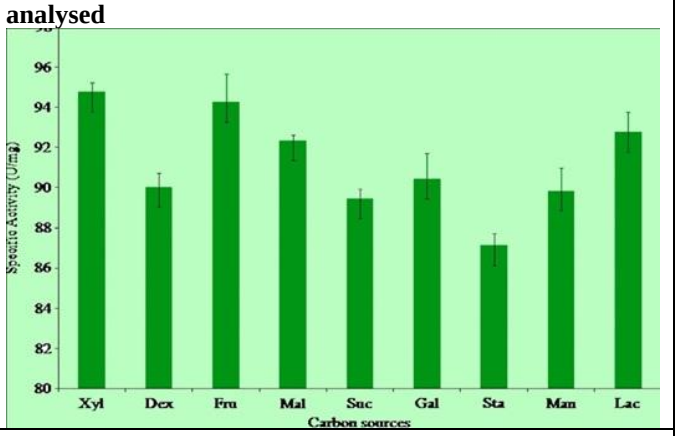


Figure 3. Effect of different nitrogen sources on protease production in *Bacillussubtilis* isolated from garden soil. (Pot Nit-Potassium nitrate, Ure-Urea, Sod Nit-Sodiumnitrate, AmmChl-Ammonium chloride, Amm Nit-Ammonium nitrate, Amm Car-Ammonium carbonate). The bars indicate the standard deviation of three replicates analysed

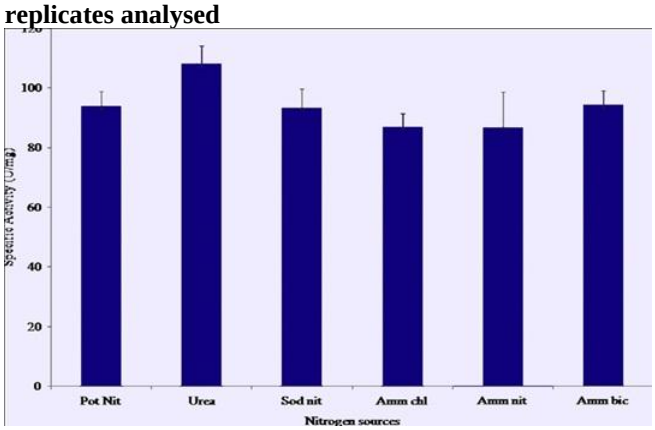


Figure 4. Effect of various incubation period on protease production in *Bacillussubtilis* isolated from garden soil. The bars indicate the standard deviation of three replicates analysed

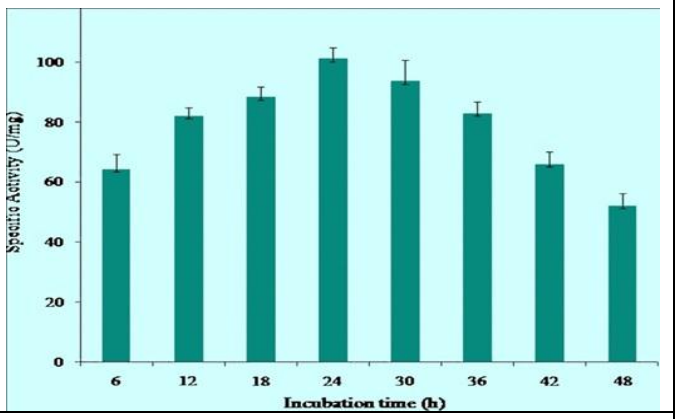


Figure 5. Effect of various pH on protease production in *Bacillussubtilis* isolated from garden soil. The bars indicate the standard deviation of three replicates analysed.

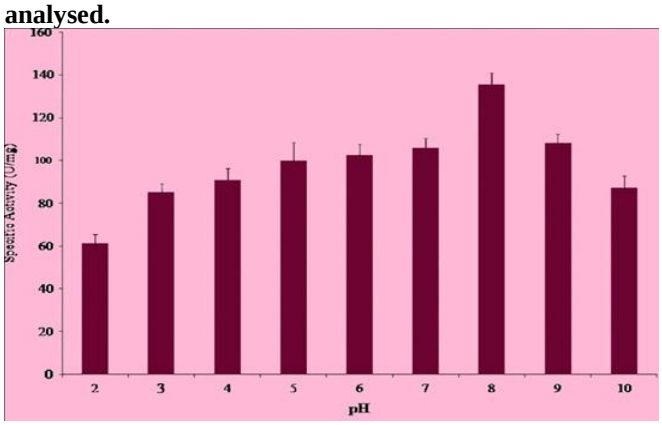
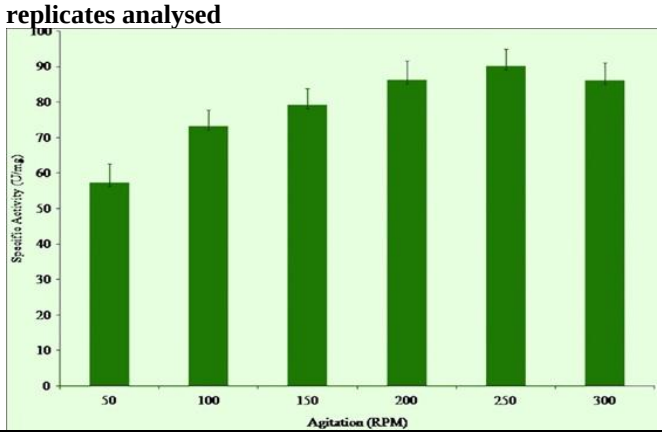


Figure 6. Effect of different agitation speed on protease production in *Bacillussubtilis* isolated from garden soil. The bars indicate the standard deviation of three replicates analysed



CONCLUSION

In this study demonstrated that physical and chemical parameters play an important role in the protease production by microorganisms. Among the various carbon sources fructose and xylose shows maximum activity on protease. The inorganic nitrogen source and urea display enhanced proteolytic activity. The optimum NaCl concentration, pH, incubation time and agitation speed for proteolytic activity were 1 M, 8, 24 h and 250 rpm, respectively. This study concluded that the physical and chemical conditions can affect the proteolytic activity and growth of microorganisms. Therefore, isolated bacteria *B. subtilis* have good proteolytic activity and suitable for industrial applications.

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CONFLICT OF INTEREST: NIL

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