

## Optimization of Protease Enzyme Production Using *Bacillus* Sp. Isolated from Different Wastes

<sup>1</sup>Udandi Boominadhan, <sup>1</sup>Rajendran Rajakumar,  
<sup>2</sup>Palanivel Karpaga Vinayaga Sivakumaar and <sup>2</sup>Manoharan Melvin Joe

<sup>1</sup>Department of Microbiology, Faculty of Science, Annamalai University, India  
<sup>2</sup>Department of Microbiology, Faculty of Agriculture, Annamalai University, India

**Abstract:** The bacterial isolates including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, *Bacillus megatherium* and *Bacillus licheniformis* isolated from different wastes were screened for protease enzyme production. Among the different sources glucose was found to be best carbon source for all the four *Bacillus* isolates. Yeast extract was found to be the optimum nitrogen source for protease enzyme production by all the test isolates. A temperature of 50°C was found to be optimum for all the isolates for enzyme production. Soy cake found to be the best substrate for enzyme production among different agro based wastes for all the four test isolates grown under submerged fermentation conditions.

**Key words:** *Bacillus* sp % Protease % Process Optimization

### INTRODUCTION

Commercial proteases account for nearly 60% of the total industrial enzyme market. This proteolytic enzyme finds numerous applications in pharmaceutical, food and detergent industries [1]. Proteases of commercial importance are produced from microbial, animal and plant sources [2]. Microbial proteases are produced from high yielding strains including species of *Bacillus* sp., *Alcaligenes faecalis*, *Pseudomonas fluorescens* and *Aeromonas hydrophilia* grown under submerged culture conditions. Among these, *Bacillus* sp is the most important group of bacteria that are involved in the enzyme industry and this bacterium is also known to produce proteolytic enzymes quite effectively. Despite this, only a few studies have been done on proteolytic enzymes from *Bacillus* sp. Furthermore studies have showed that nutritional factors including sources of carbon and nitrogen can influence protease enzyme production. Besides this nutritional factor, physical factors such as inoculum concentration[3], temperature, pH [4] and incubation time [5] can also significantly affect protease production.

The present investigation was aimed at optimization of growth conditions of different *Bacillus* sp. isolated different sources to enhance the protease enzyme production.

### MATERIALS AND METHODS

**Culture and Growth Condition:** The bacterial cultures were isolated from different sources, such as slaughterhouse waste, diary industry effluent, market waste and sewage waste and the individual bacterial colonies were screened for proteolytic enzyme production on Skim agar medium. The inoculated plates were taken inoculated at 37°C for 48 hrs and observed for zones of clearance, which indicate proteolytic activity.

Based on morphology and biochemical studies, the isolates were identified as *Bacillus subtilis* DL-1, *B. licheniformis* SH-2, *B. megatherium* MW-1 and *B. licheniformis* SW2. The bacterial cultures were purified and maintained on nutrient agar slants having pH 8.5 at 35±2°C slants.

**Assay for Protease:** The enzyme was assayed in the reaction mixture containing 2.0 ml of 0.5 per cent casein solution in 0.1 M  $\text{CO}_3\text{-HCO}_3$  buffer (pH 9.5) and 1 ml enzyme solution in a total volume of 3.0 ml. After inoculation at 30°C for five minutes, the reaction was stopped by addition of 3.0 ml of 10 per cent ice cold TCA and centrifuged at 10000 rpm for five minutes. Protein in the supernatant was estimated by the method of Lowry *et al.* [6].

To fifty ml quantities of standard growth broth containing 1 per cent casein, various carbon sources viz., starch, glucose, fructose, maltose, sucrose and cellulobiose at 0.5 per cent levels were added in separate 100 ml Erlenmeyer flasks without adding carbon source. They were sterilized, inoculated and incubated at 30°C for 3 days and at the end of incubation, the cell free filtrate served as the enzyme source.

**Effect of Different Nitrogen Sources:** To fifty ml qualities of standard broth containing one per cent casein, various organic nitrogen sources viz., beef extract, yeast extract, peptone and inorganic nitrogen sources viz., ammonium nitrate, Ammonium carbonate and urea at a concentration of 0.5 per cent was added in separate 100 ml Erlenmeyer flasks without adding nitrogen source. They were sterilized, inoculated and incubated at 30°C for 48 hours and at the end of incubation the cell free extract serve as the enzyme source.

**Effect of pH:** Erlenmeyer flasks containing 50 ml quantities of modified standard growth broth containing the optimum casein concentration and carbon sources were taken and the pH of the broth was adjusted to 7.0, 8.0, 9.0, 10.0 and 11.0 in different flask using pH meter with 1 N HCl or 1 N KOH and sterilized. The bacterial cultures were inoculated and incubated at 30°C for 3 days. At the end of incubation period the cell free culture filtrate was obtained and used as enzyme source.

**Effect of Incubation Temperature:** Fifty ml quantities of modified standard growth broth were dispensed in 100 ml Erlenmeyer flasks. The pH adjusted to 10 by adding 0.1 N NaOH. The flasks were sterilized, inoculated and incubated at different temperatures viz., 35, 40, 45, 50, 55 and 60°C for 3 days. At the end of inoculation period, the cell free culture filtrate was obtained and used for the enzyme assay.

### Influence of Different Agro Based Waste as Substrates

**on the Protease Production:** To find out the suitability of agro-based waste as substrate for protease production, different substrates viz., groundnut cake, coconut cake, brewery spent grain, soy cake and wheat bran (Agro based industrial wastes) were taken by replacing casein in the growth media under submerged condition (pH 10). The enzyme activity was measured at 3 days interval for protease production.

## RESULTS AND DISCUSSION

Proteases, represent about 60% of total enzymes market sales [7]. At present, the largest part of the hydrolytic enzyme market is occupied by the alkali proteases.

In the present study, glucose was found to be the optimum carbon source for protease activity by all the four *Bacillus* isolates followed by sucrose, fructose, maltose, starch and cellulose. Glucose was the best carbon source for protease production by *Bacillus subtilis* [8], starch was found to be best carbon source for protease production by *B. licheniformis*. This results are in conformity with our present findings.

In the present study, Beef extract was found to be the best organic nitrogen source for augmenting the protease enzyme production for the *Bacillus* sp. Among the different inorganic nitrogen studied ammonium carbonate was found to be the best for the *Bacillus* isolates. The results of our present study are in line with the findings of [9]; beef extract among the different organic nitrogen sources and ammonium carbonate among the different inorganic nitrogen sources lead to a high proteolytic activity by *Bacillus* sp., at 48 hrs of incubation period.

In the present study, among the different pH levels tested, pH 8 was found optimum for all the test isolates. Manjeet Kaur *et al.* [3] reported that even though pH initially decreased, it reached alkalinity when secondary

Table 1: Effect of different carbon source on the proteolytic activity of the *Bacillus* sp.

Sl. No.	Casein concentration in per cent	Enzyme activity (U/mL)			
		<i>B. subtilis</i>	<i>B. amyloliquefaciens</i>	<i>B. megaterium</i>	<i>B. licheniformis</i>
1.	Glucose	590.24 ± 1.89 <sup>a</sup>	531.14 ± 1.14 <sup>b</sup>	512.24 ± 2.42 <sup>b</sup>	570.12 ± 1.17 <sup>a</sup>
2.	Fructose	540.12 ± 2.43 <sup>c</sup>	560.12 ± 2.22 <sup>a</sup>	540.12 ± 1.74 <sup>a</sup>	514.72 ± 0.90 <sup>c</sup>
3.	Sucrose	564.12 ± 1.22 <sup>b</sup>	470.12 ± 0.42 <sup>c</sup>	440.12 ± 1.20 <sup>c</sup>	542.12 ± 1.28 <sup>b</sup>
4.	Maltose	300.14 ± 0.46 <sup>d</sup>	300.14 ± 0.72 <sup>d</sup>	274.12 ± 1.34 <sup>d</sup>	270.12 ± 0.56 <sup>d</sup>
5.	Starch	224.12 ± 2.02 <sup>e</sup>	214.12 ± 1.34 <sup>e</sup>	191.12 ± 1.12 <sup>e</sup>	194.12 ± 1.48 <sup>e</sup>
6.	Cellulose	154.12 ± 2.13 <sup>f</sup>	194.12 ± 0.74 <sup>f</sup>	170.12 ± 2.20 <sup>f</sup>	101.67 ± 1.58 <sup>f</sup>

One unit of enzyme activity is the amount of enzyme that released 1 µg of tyrosine/mL/min

Values are a mean of three-replication ± SD.

Values followed by different letters are significantly differed at a P value of 0.05 according to DMRT

Table 2: Effect of organic and inorganic nitrogen source on the proteolytic activity of the *Bacillus* sp

Sl. No.	Casein concentration in per cent	Enzyme activity (U/mL)			
		<i>B. subtilis</i>	<i>B. megaterium</i>	<i>B. amyloliquefaciens</i>	<i>B. licheniformis</i>
<b>Organic N source</b>					
1.	Beef extract	612.12 ± 1.04 <sup>a</sup>	580.12 ± 3.04 <sup>a</sup>	621.14 ± 0.94 <sup>a</sup>	591.12 ± 1.12 <sup>a</sup>
2.	Yeast extract	540.12 ± 1.72 <sup>c</sup>	560.14 ± 3.84 <sup>b</sup>	570.13 ± 1.04 <sup>b</sup>	540.12 ± 1.03 <sup>b</sup>
3.	Peptone	400.12 ± 2.04 <sup>e</sup>	372.14 ± 0.95 <sup>e</sup>	460.12 ± 1.96 <sup>d</sup>	424.12 ± 0.84 <sup>e</sup>
<b>Inorganic N source</b>					
4.	Ammonium nitrate	540.12 ± 1.12 <sup>c</sup>	510.14 ± 1.47 <sup>c</sup>	570.12 ± 0.98 <sup>b</sup>	540.12 ± 1.96 <sup>b</sup>
5.	Ammonium carbonate	598.64 ± 2.44 <sup>b</sup>	582.12 ± 3.40 <sup>a</sup>	560.17 ± 0.81 <sup>c</sup>	521.14 ± 0.98 <sup>c</sup>
6.	Urea	502.42 ± 2.42 <sup>d</sup>	469.76 ± 3.24 <sup>d</sup>	540.17 ± 0.98 <sup>e</sup>	491.71 ± 1.12 <sup>d</sup>

Protease activity was determined by the procedure.

One unit of enzyme activity is the amount of enzyme that released 1 µg of tyrosine/mL/min

Values are a mean of three-replication ± SD.

Values followed by different letters are significantly differed at a P value of 0.05 according to DMRT

Table 3: Process optimization of *Bacillus* sp. for protease production using different protein rich substrates

Sl. No.	Substrates	Enzyme activity (U/mL)			
		<i>B. subtilis</i>	<i>B. megaterium</i>	<i>B. amyloliquefaciens</i>	<i>B. licheniformis</i>
1.	Soy cake	594.12 ± 0.91 <sup>a</sup>	571.78 ± 1.28 <sup>a</sup>	554.12 ± 2.75 <sup>a</sup>	520.12 ± 1.18 <sup>a</sup>
2.	Coconut cake	434.12 ± 1.18 <sup>c</sup>	401.12 ± 0.88 <sup>c</sup>	390.12 ± 1.20 <sup>c</sup>	340.12 ± 2.63 <sup>c</sup>
3.	Wheat bran	560.12 ± 1.68 <sup>b</sup>	544.12 ± 0.91 <sup>b</sup>	494.12 ± 1.01 <sup>c</sup>	462.12 ± 1.87 <sup>c</sup>
4.	Groundnut cake	494.12 ± 1.52 <sup>c</sup>	464.12 ± 1.00 <sup>d</sup>	454.12 ± 0.677 <sup>d</sup>	467.45 ± 2.25 <sup>c</sup>
5.	Brewery spent grain	486.80 ± 1.68 <sup>d</sup>	502.14 ± 0.80 <sup>c</sup>	528.12 ± 1.38 <sup>b</sup>	500.14 ± 1.23 <sup>b</sup>

One unit of enzyme activity is the amount of enzyme that released 1 µg of tyrosine/mL/min

Values are a mean of three-replication ± SD.

Values followed by different letters are significantly differed at a P value of 0.05 according to DMRT

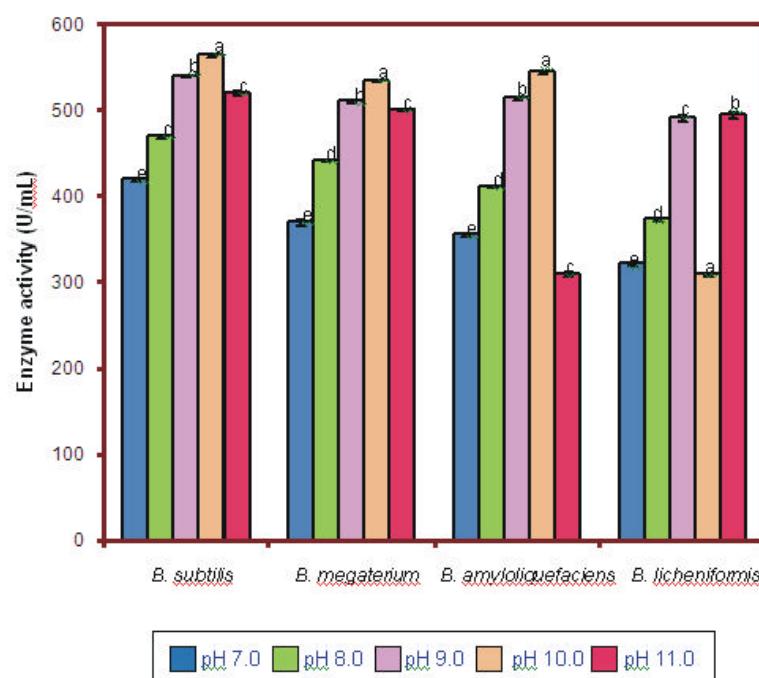


Fig. 1: Effect of pH on the proteolytic activity of the *Bacillus* sp.

Values are a mean of three-replication ± SD.

Values followed by different letters are significantly differed at a P value of 0.05 according to DMRT

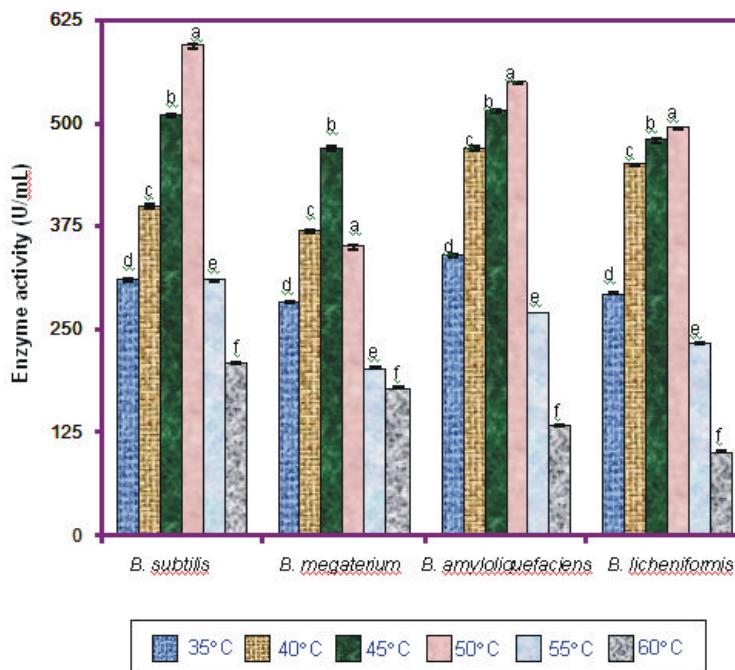


Fig. 2: Effect of temperature on the proteolytic activity of the *Bacillus* sp.  
Values are a mean of three-replication  $\pm$  SD.  
Values followed by different letters are significantly differed at a P value of 0.05 according to DMRT

metabolites were released and become constant. Further studies by *B. licheniformis* S-40 was able to grow in a pH range of 7-12 with better protease production in alkaline range [10].

The influence of incubation temperature on protease production by bacteria was studied by several workers [11-13]. Among the different incubation temperatures tested for protease production 50°C was found to be optimum for all test isolates. The optimum temperature for protease production for *B. licheniformis* was 45°C [10] and for *B. subtilis* 50°C. But inactive at 55-90°C, they further reported that the optimum temperature for protease activity was 50°C.

The effect of agro based byproducts as alternative substrate on bacterial protease production under submerged fermentation was studied by several workers[3]. In the present study, soy cake was found to be the best inducer of protease enzyme production by all the four *Bacillus* isolates.

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