

# Microbial Production of Alkaline Proteases by Locally Isolated *Bacillus subtilis* PCSIR-5

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**Abstract.**-Extra-cellular proteases producing bacteria were isolated from local habitats and screened on 1 % skim milk agar plates. Depending upon the zone of hydrolysis strain, 5 was selected for further investigations. Studies regarding the effects of pH, size and age of inoculum revealed that maximum proteolytic activity (107 PU/ml) was indicated after 48 hours by using 10% v/v of 24 h old inoculum in fermentation medium. The enzyme had an optimal pH of 8.5 and exhibited its highest activity at 50°C. The proteases were then tested for collagenolytic activity on leather dust. Results indicate that protease produced by *Bacillus subtilis* PCSIR-5 has greater collagenolytic activity and it can be used for bio-treatment of leather dust for reduction of the environmental pollution.

**Keywords:** Alkaline protease, leather dust, *Bacillus subtilis* PCSIR-5.

## INTRODUCTION

**P**roteases are the single class of enzymes, which occupy a pivotal position with respect to their applications in both physiological and commercial fields. Proteases are degradative enzymes, which catalyze the total hydrolysis of proteins. The worldwide sales of industrial enzymes are \$1 billion. Proteases represent one of the three largest groups of industrial enzymes and account for about 60% of the total worldwide sale of enzymes (Woods *et al.*, 2001). These enzymes are obtained from plants, animals and microbial sources (Puvanakrishanan and Dhar, 1986) and used in dairy, baking, protein hydrolysis, leather, and detergents industries (Adinarayana *et al.*, 2003). Among these, bacterial proteases are the most significant as compared with fungal and animal proteases (Shumi *et al.*, 2004). *Bacillus* species are the specific producers of extra cellular proteases and can grow in pH range of 7-11 (Siezan and Leunissen, 1997). The vast diversity of proteases, in contrast to the specificity of their action, has attracted worldwide attention in attempts to exploit their physiological and biotechnological applications (Hibbs *et al.*, 1985). In leather industries proteases are used as a substitute of toxic chemicals like lime, sodium sulphide, salts and

solvents etc. in pre-tanning process of leather processing. Environmental and public health hazards caused by leather industry can be overcome either by employing proper effluent treatment or controlling pollutions occurring at different stages of leather manufacturing.

The aim of the present study was to produce proteases at optimized conditions by employing a locally isolated strain of *Bacillus subtilis* PCSIR-5 and seek their utilization for the reduction of the leather dust by degrading it into polypeptide chains and amino acids.

## MATERIALS AND METHODS

### *Isolation and identification of microorganism*

Soil samples collected from the drainage of slaughterhouse were diluted in sterile normal saline. The diluted samples were placed at 80°C for 15 minutes to destroy all vegetative microbial cells. The suspension was then plated out on skim milk agar plates containing peptone (0.1%), NaCl (0.5%), agar (1.5%) and skim milk (1.0%). The inoculated plates were incubated at 37°C for 24 hours. Depending upon the zone of clearance, the isolate PCSIR-5 was selected for further experimental studies. To identify, the isolated bacterium was examined for various morphological and biochemical characteristics *viz.* Gram and endospore stainings, motility, oxygen requirement, catalase and oxidase tests etc. in accordance with the methods

recommended in Bergey's Manual of Determinative Bacteriology (Holt *et al.*, 1994). It was grown at 37°C for 24 hours on nutrient agar slants and then maintained at 4°C for further use.

#### *Inoculum preparation*

Inoculum was prepared by transferring a loopful culture from the slants in 250 ml Erlenmeyer flasks containing 50 ml of sterile inoculum medium comprising of glucose (0.2%), peptone (0.5%), soy bean meal (0.5%), NaCl (0.5%), KH<sub>2</sub>PO<sub>4</sub> (0.25%) and MgSO<sub>4</sub>.7H<sub>2</sub>O (0.05%). The flasks were then subsequently incubated at 37°C on a rotary shaker at 120 rpm for 24 hours.

#### *Shake flask experiments*

Different growth conditions like fermentation period (Table I), effect of pH (Table II), effect of age of inoculum (Table III) and size of inoculum (Table IV) were studied in shake flask experiments. The production medium was comprised of glucose (0.5%), peptone (0.5%), soy bean meal (0.5%), NaCl (0.5%), KH<sub>2</sub>PO<sub>4</sub> (0.25%) and MgSO<sub>4</sub>.7H<sub>2</sub>O (0.05%). pH of medium was adjusted at 7.5 before sterilization with 1 N NaOH/ HCl. The inoculated flasks were then placed on shaking incubator at 37±1°C and 120 rpm for 48 hours. The growth medium was then centrifuged at 10,000 rpm for 10 minutes at 4°C. The supernatant was saved and used as an enzyme solution. All the experiments were performed in triplicates.

#### *Enzyme assay*

Proteolytic activity was determined by the method of Kunitz (1965). One proteolytic unit (PU) was defined as amount of the enzyme that released one microgram of tyrosine under assay conditions.

#### *Protein assay*

Protein was estimated according to the method described by Lowry *et al.* (1951) using bovine serum albumin (BSA) as standard.

#### *Bio-treatment of leather dust*

Maximum enzyme production was carried out at the optimized conditions. The enzyme which has proteolytic unit 107 (PU)/ml was used for the bio-treatment of leather dust. Different parameters, like effect of time period with 0.1 g of sample at 3210

PU up to 192 hours and effects of employing different proteolytic units *i.e.* 2140, 3210, 4280, 5350, 6420 and 7490 PU, and varying amounts of leather dust (0.05g, 0.1g, 0.15g and 0.2 g) were studied for the bio-treatment of the pollutant. Hydroxyproline released as a result of bio-treatment of leather dust was measured by the method of Leach (1951). All the experiments concerning the biotreatment of leather dust were performed at 37±1°C.

## RESULTS AND DISCUSSION

It is evident from the results that production of the enzyme is affected significantly by fermentation period, initial pH, age and size of inoculum. Proteases production throughout this study was determined at 37±1°C temperature and 120 rpm agitation speed in shake flask cultures by using the locally isolated strain of *Bacillus subtilis*-PCSIR5. The isolated strain was Gram positive, coccobaccilli, spore forming, motile, aerobic, catalase and oxidase positive.

Maximum production of the enzyme was obtained after 48 hours of fermentation period (Table I). The results are in good agreement with those reported by Adinarayana *et al.* (2003). These workers also observed maximum enzyme production after 48 hours of incubation with *Bacillus subtilis* PE-11. Similarly, in an earlier study Qadeer *et al.* (1990) also showed that cell free filtrate of 48 hours incubated culture of another strain of *Bacillus subtilis* possessed maximum enzyme yield. The decrease in enzyme's yield after 48 hours might be due to depletion of nitrogen source in the medium. Another reason may be the increase in pH values accompanying the bacterial growth. This is suggestive for incorporation of some buffer to the fermentation media for prolonged production of the enzyme. pH of the growth media affected the production of proteolytic enzymes and maximum production was observed at initial pH 7.5 (Table II). Increase in initial pH (7.5-9.0) caused decrease in enzyme's yield. These results are in accordance with the observation of Takii *et al.* (1990). They got maximum enzyme production at pH 7.6 at 37°C by *Bacillus alcalophilus sub spp. halodurans* KP 1239. While, Huang *et al.* (2003)

**Table I.-** Effect of fermentation period on the production of extra-cellular proteases by *Bacillus subtilis* PCSIR-5 in the production medium at initial pH 7.5, temperature 37 °C and agitation speed of 120 rpm in shake flask cultures.

Incubation Period (hours)	Final pH	Proteolytic Activity (PU/ml)	Total protein (mg/ml)	Specific Activity (PU/mg)
12	6.20±0.10 <sup>a</sup>	39.33±1.20	0.81±0.01	48.34±1.53
24	6.93±0.15	78.00±2.34	0.79±0.02	99.67±2.08
36	7.77±0.13	94.67±1.50	0.74±0.01	127.34±1.15
48	8.33±0.11	106.33±1.00	0.68±0.02	156.53±1.52
60	9.13±0.10	83.34±1.06	0.67±0.03	124.33±1.73
72	9.30±0.14	62.66±1.57	0.66±0.01	94.54±1.00

a: Values are means of three replicates±S.D.

**Table II.-** Effect of initial pH on the production of extra-cellular proteases by *Bacillus subtilis* PCSIR-5 in the production medium at temperature 37°C and agitation speed of 120 rpm after 48 hours of incubation in shake flask cultures.

Initial pH	Final pH	Proteolytic Activity (PU/ml)	Total protein (mg/ml)	Specific Activity (PU/mg)
7.0	8.00±0.10 <sup>a</sup>	82.33±1.15	0.62±0.01	132.33±1.53
7.5	8.46±0.15	107.00±1.00	0.67±0.02	160.34±1.52
8.0	9.13±0.13	88.00±2.00	0.64±0.01	138.66±1.64
8.5	9.23±0.18	79.34±0.58	0.59±0.01	134.00±1.00
9.0	9.30±0.10	69.66±1.52	0.57±0.02	121.67±1.44

a: Values are means of three replicates±S.D.

**Table III.-** Effect of age of inoculum on the production of extra-cellular proteases by *Bacillus subtilis* PCSIR-5 in the production medium at initial pH 7.5, temperature 37°C and agitation speed of 120 rpm after 48 hours of incubation in shake flask cultures.

Age of Inoculum (hours)	Final pH	Proteolytic Activity (PU/ml)	Total protein (mg/ml)	Specific Activity (PU/mg)
12	7.96±0.12 <sup>a</sup>	69.33±1.53	0.61±0.02	115.67±1.43
18	8.23±0.15	88.67±1.52	0.63±0.01	140.00±2.00
24	8.75±0.11	106.00±1.00	0.67±0.01	158.34±1.75
30	9.13±0.12	82.34±0.57	0.72±0.02	134.66±1.152
36	9.10±0.10	78.00±2.00	0.75±0.01	104.33±1.67

a: Values are means of three replicates±S.D.

**Table IV.-** Effect of size of inoculum on the production of extra-cellular proteases by *Bacillus subtilis* PCSIR-5 in the production medium at initial pH 7.5, temperature 37°C and agitation speed of 120 rpm after 48 hours of incubation in shake flask cultures.

Size of Inoculum (%)	Final pH (Mean±SD)	Proteolytic Activity (PU/ml)	Total protein (mg/ml)	Specific Activity (PU/mg)
5.0	8.03±0.13 <sup>a</sup>	68.67±1.54	0.64±0.01	106.33±2.02
7.5	8.45±0.11	81.34±1.47	0.65±0.02	125.34±1.36
10	8.73±0.21	107.00±1.00	0.68±0.01	157.00±1.00
15	9.28±0.15	90.66±1.63	0.71±0.01	127.00±1.50
20	9.17±0.17	76.33±1.52	0.70±0.02	109.67±1.34

a: Values are means of three replicates±S.D.

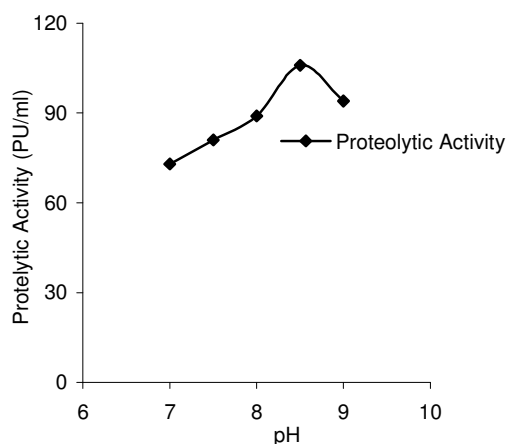


Fig. 1. Effect of pH on activity of alkaline proteases.

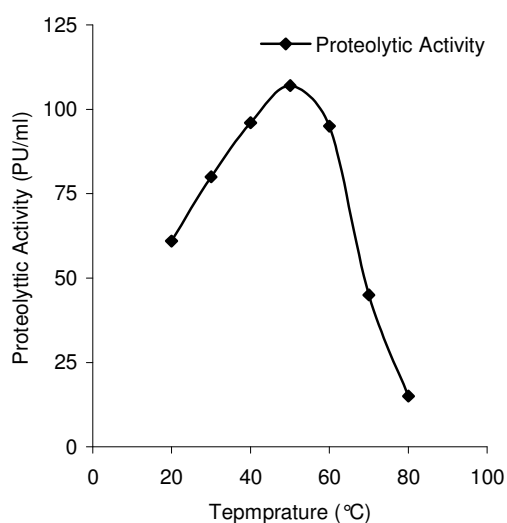


Fig. 2. Effect of temperature on activity of alkaline proteases,

reported maximum protease's yield of de-hairing enzymes from *Bacillus pumilus* at pH 10. Thus the different species and strains of protease producers need different pH levels for highest yield of the enzymes. This metabolic diversity has potential for biotechnological applications in different situations. Regarding the age and size of inoculum, 24 hours old and 10% inoculum gave the height enzyme yield (Tables III and IV). Maximum enzyme production

was obtained with 36 hours old and 5% inoculum size by Chu *et al.* (1992). While, for alkaline proteases from a strain of *B. subtilis* Loperena *et al.* (1994) observed maximum production with 12 hours age of inoculum.

Maximum alkaline protease activity was found at pH 8.5. However, the enzyme remained active within the range of 7-9 pH (Fig. 1). A decrease in proteolytic activity by increasing pH has been observed by Malathi and Chakraborty (1991). Our findings are in agreement with those reported by Sampath *et al.* (1997). These authors have reported optimum pH range of 7-8.4 for maximum proteases activity by *Streptomyces* sp.

The activity of crude enzyme was determined on different temperatures ranging from 20°C to 80°C. The optimum temperature for *Bacillus subtilis*-PCSIR-5 protease was found to be 50°C (Fig. 2). The enzymes activity gradually decreased at temperature beyond 60°C. Adinarayana *et al.* (2003) have observed maximum proteolytic activity of enzymes from *Bacillus subtilis* at 60°C. While, Thangem and Rajkumar (2002) found maximum enzyme activity of alkaline proteases at 55°C by *A. faecacalis*.

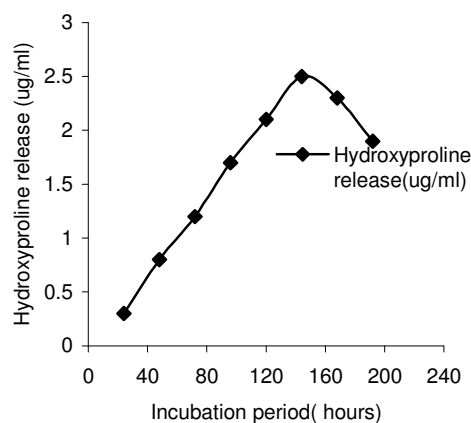


Fig. 3. Effect of time period on the degradation of 0.1 g of leather dust treated with 3210 PU of enzyme at 37°C

Figure 3 shows that maximum hydroxyproline (2.5 µg/ml) was obtained after 144 hours when the substrate was treated with 3210 PU at 37±1°C. For the remaining sampling periods, 0.3, 0.8, 1.2, 1.7,

2.1, 2.3, and 1.9  $\mu\text{g}$  of hydroxyproline /ml were released after 24, 48, 72, 96, 120, 168, and 192 hours, respectively.

Optimum degradation of leather dust (0.1g) was obtained with 5350 PU as shown in Fig. 4. Further increases in proteolytic units (PU) did not affect the degradation. This might be due to the depletion of degradable collagen present in the leather dust or due to the feed back inhibition of the enzymes by the proteolytic products.

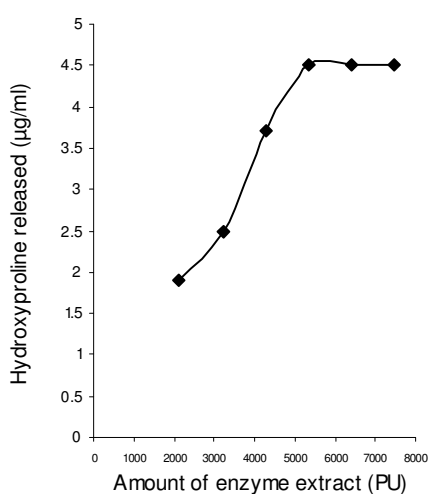


Fig 4. Effect of number of proteolytic units on the degradation of 0.1 g leather dust after 144 hours at 37°C.

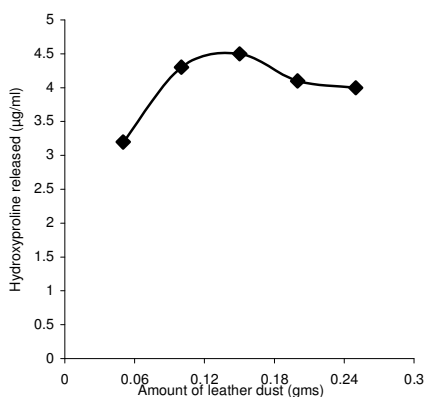


Fig. 5. Effect of amount of leather dust on its degradation, treated with 5350 PU of enzyme after 144 hour at 37°C.

When the effect of different amount of leather dusts was observed (Fig. 5), it appeared that maximum degradation occurred when 0.15 g of the sample was allowed to react with 5350 PU at  $37\pm 1^\circ\text{C}$  for 144 hours. Petushkova *et al.* (1985) observed that *Bacillus subtilis*, *Bacillus lichniformis*, *Bacillus megaterium*, and *Bacillus pumilis* released 1.3, 1.7, 1.5, and 1.4  $\mu\text{g/ml}$  of hydroxyproline, respectively when cultured on leather. Experiments involving continuous culturing of the bacterium and the enzyme immobilization will be more informative for biotechnological applications of proteases described in this study.

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