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Production and characterization of tannase from *Bacillus cereus* KBR9

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A tannase-producing soil bacteria has been isolated and identified as *Bacillus cereus.* It can degrade tannic acid and produce maximum tannase (0.22 U/ml) at stationary phases of growth (24 h). Maximum growth and enzyme production occurred with initial medium pH of 4.5–5.0. Partial purified tannase showed optimum activity at pH 4.5 and 40°C. It remains stable up to 30°C and pH 4.5 to 5.0. The enzyme is salt tolerant, stable up to 2 M of NaCl and retains 82% original activity in 3 M.

Key Words——*Bacillus cereus*; tannase

Introduction

Tannic acid, a heteropolymer of glucose and gallic acid (1 : 9), is one of the most abundant reserve materials of plants (Bhat et al., 1998). Industrial bioconversion of tannic acid is generally accomplished by enzyme tannase for the production of gallic acid (3,4,5-trihydroxy benzoic acid). Gallic acid is mostly utilized in the pharmaceutical industry for manufacture of trimethoxy benzaldehyde, which is used in the production of a broad-spectrum antibiotic, trimethoprim (Bajpai and Patil, 1996). Besides gallic acid production, tannase is extensively used for the preparation of instant tea, acron wine, coffee-flavored soft drinks, clarification of beer and fruit juices, detannification of food and in industrial effluent treatment (Kar and Banerjee, 2000; Lekha and Lonsane, 1997; Mondal and Pati, 2000; Suseela and Nandy, 1985).

Tannic acid is a polyphenolic compound, because of which it is generally considered an antinutrient and antimicrobial agent (Lewis and Starkey, 1969). But a large number of fungi have been reported to degrade tannins by producing tannase (Bhat et al., 1998; Lekha and Lonsane, 1997). In this regard some literature is available on tannase from bacterial origin. Deschamps et al. (1983) first reported that *Bacillus polymyxa*, *B. pumilus*, *Corynebacterium* sp. and *Klebsiella pneumoniae* are able to produce tannase in a liquid culture containing chestnut tannin. Kumar et al. (1999) isolated tannase-producing *Citrobacter freundii* from tannery effluent. In our previous reports, the occurrence and production of tannase by *B. licheniformis* was mentioned (Mondal et al., 2000; Mondal and Pati, 2000).

The present paper deals with the newly isolated *Bacillus cereus* that produces tannase and degrades tannic acid in broth culture. Some properties of the enzyme have also been considered.

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Materials and Methods

Isolation and identification bacteria. Tannase-producing bacteria were isolated from lateritic forest soil of Midnapore district, West Bengal, India on selective tannic acid-agar media (Bradoo et al., 1996; Mondal et al., 2001b). This organism was identified in our laboratory following Bergey's Manual of Systematic Bacteriology (1986) and it is also confirmed from Microbial Type Culture Collection and Gene Bank (MTCC), Institute of Microbial Technology, India.

Production of enzyme. An inoculum was first prepared for tannase production by growing the organism at 30°C for 24 h in selective medium (pH 5.0) with the following composition (g L^{-1}): tannic acid, 10; K_2HPO_4, 0.5; KH_2PO_4, 0.5; $MgSO_4$, 0.5; NH_4Cl, 1; $CaCl_2$, 0.01 and glucose, 0.5. Then the broth was centrifuged (5,000×*g*×5 min) and the pellet washed twice in sterilized distilled water and used as inoculum.

For enzyme production, 1% (v/v) inoculum was transferred to fresh culture medium having identical composition with the medium used for inoculum preparation except glucose. Growth of the organism was carried out in a 250 ml Erlenmeyer flask containing 50 ml liquid medium and incubated at 30±2°C in a rotary shaker (200 rpm) for 48 h. The culture supernatant obtained by centrifugation was assayed periodically for enzymatic activity. The effect of medium pH on growth and enzyme production were studied by adjusting it at various level (pH 3.0–7.0) with 1 N HCl or 1 M NaOH. Growth of the organism was estimated on the basis of biomass dry weight (mg ml^{-1}). The remaining tannin content in the fermented broth was estimated by the modified protein precipitation method of Hagerman and Butler (1978).

Assay of tannase. A new colorimetric method has been used here to determine the tannase activity, based on measuring the residual tannic acid content after enzymatic reaction (Mondal et al., 2001a). The reaction mixture consisted of substrate tannic acid 0.3 ml (0.5% (w/v), in buffer) and 0.05 ml crude enzyme (cell free supernatant). The enzymatic reaction was stopped by the addition of bovine serum albumin (1 mg ml^{-1}), which also leads to the precipitation of the remaining tannic acid. In the same way a reference tube was prepared with heat-denatured enzyme. The tubes were then centrifuged (5,000×*g*, 20 min) and the precipitate dissolved in SDS-triethanolamine (1% (w/v) of SDS in 5% (v/v) triethanolamine) solution and the absorbance was measured with $FeCl_3$ at 530 nm. One unit of the tannase was defined as the amount of enzyme that can hydrolyze 1 μmol of ester linkage of tannic acid in 1 min under specific conditions.

Properties of enzyme. Partially purified enzyme was used to determine the stability and optimum activity of tannase. After 24 h of incubation the culture broth was centrifuged (5,000×*g*, 15 min), and the supernatant was then treated with solid ammonium sulfate (80% saturation) and allowed to stand overnight at 4°C. The precipitate was collected by centrifugation (15,000×*g*, 30 min), dissolved in acetate buffer (0.2 M, pH 5.0) and dialyzed against the same buffer for 2 days. The dialysate was used as the source of partially purified enzyme. The optimum pH and temperature of enzyme activity were measured at different pH values from 3.0 to 8.0 and temperature 20 to 70°C.

The pH stability was determined by incubation of enzyme at various pH values for 24 h at 4°C followed by measurement of enzyme activity. The thermal stability was estimated by incubating the enzyme at different temperature for 1 h at pH 5.0.

Salt tolerance test. The enzyme was incubated in 0.02 M acetate buffer (pH 5.0) containing various concentration of NaCl (1 to 5 M) for 24 h at 4°C, and each case activity of the enzyme was measured.

Results and Discussion

A bacterial strain capable of producing tannase was isolated from lateritic forest soil that designated as KBR9. According to morphological and biochemical properties the strain was identified as *B. cereus* (Table 1).

In tannic acid liquid medium the organism attained maximum growth at 21 h after 3 h of initial lag period (Fig. 1). The enzyme production was started from its early growth and reaches highest level at 24 h, after which it decreases. The tannic acid content in fermented broth decreases sharply and is completely utilized within 21 h of growth (Fig. 1).

Generally tannins are toxic as well as bacteriostatic compounds and have non-reversible reaction to protein (Scalbert, 1991). Nevertheless, some microorganisms degrade this compound by producing tannase and play an active role in the soil for nutrient recycling through decomposition of tannin-containing plant materials (Lewis and Starkey, 1969). Though a few bacterial strains have been reported to produce tannase

Table 1. Characterization of *Bacillus cereus* KBR 9.

Properties studied	Result
Gram's staining	+
Size	rods
Color (pigment)	white
Spore	+
Fluorescence (UV)	−
Motility	+
Growth at	
i. pH	3–9
ii. Temperature	20–50°C
iii. Salt (NaCl)	10%
iv. Lysozyme (0.001%)	−
Growth on MacConkey agar	−
Starch hydrolysis	+
Casein hydrolysis	+
Citrate utilization	+
Urea hydrolysis	−
Indole production	−
VP test	−
Anaerobic growth	−
Nitrate reduction	+
Gelatin liquefaction	+
Catalase	+
Oxidase	+
Utilization of carbohydrates (acid production)	
i. Glucose	±
ii. Fructose	−
iii. Arabinose	−
iv. Galactose	−
v. Xylose	−
vi. Inositol	−
vii. Mannitol	−
viii. Salicin	−
ix. Raffinose	−
x. Sucrose	−

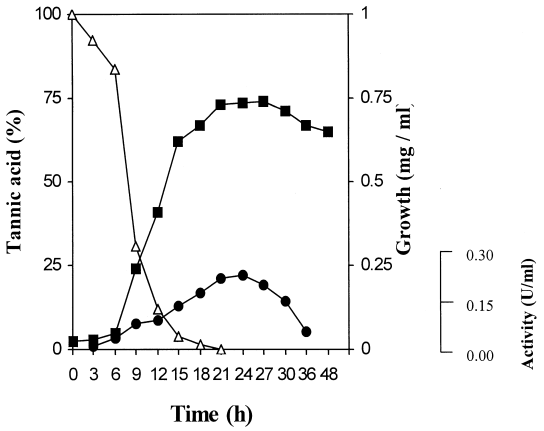


Fig. 1. Time course of growth (■), tannase production (●) and tannic acid degradation (△) by *B. cereus* KBR9.

Growth condition: basal medium containing 1.0% (w/v) tannic acid considered as 100%, pH 5.0, incubation temperature 30±2°C.

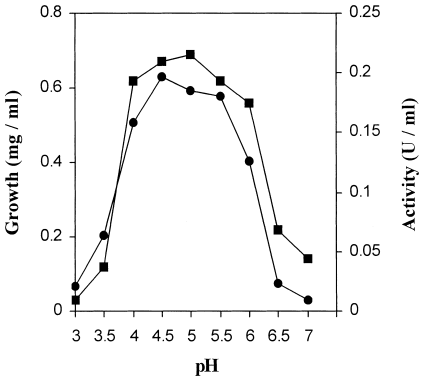


Fig. 2. Influence of initial medium pH on growth (■) and tannase production (●) by *B. cereus.*

pH of the medium was adjusted after sterilization. Growth and enzyme production were measured after 24 h of incubation at 30±2°C.

(Deschamps et al., 1983; Kumar et al., 1999; Mondal and Pati, 2000), this may be the first observation that *B. cereus* is also a tannase producer. The bacterium is able to grow and degrade tannic acid by producing extracellular tannase in liquid culture. The kinetics of tannase production by *B. cereus* is linear and maximum synthesis occurs at stationary phase of growth. Actually tannase is an inducible enzyme in bacteria and produced only in the presence of tannic acid (Mondal and Pati, 2000). The enzyme degrades the tannic acid into gallic acid and glucose, which are ultimately utilized by the organism for growth (Lekha and Lonsane, 1997). In comparison to this report, *B. licheniformis* (Mondal et al., 2000) produce maximum tannase at exponential phase (21 h), whereas other reported bacteria produce it at the initial phase (6 h) of growth (Deschamps et al., 1983; Kumar et al., 1999).

This bacterium is able to grow in tannic acid medium with an initial pH of 3.0–7.0 (Fig. 2). The maximum enzyme production is observed at pH 4.5, whereas highest growth occurs at pH 5.0. This result is comparable to findings of other reported organisms including fungi (Barthomeuf et al., 1994; Hadi et al., 1994; Mondal et al., 2001b) and bacteria (Kumar et al., 1999; Mondal and Pati, 2000). Actually most of the microbial extracellular enzymes are produced in greatest yield at a growth pH somewhere near the pH for maximum en-

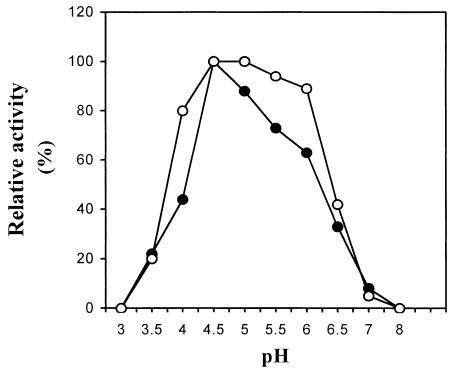
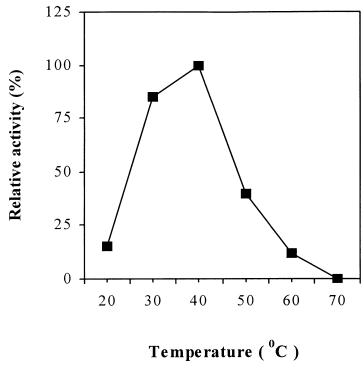
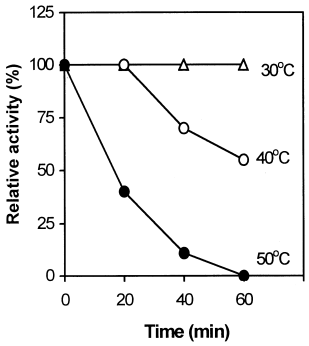


Fig. 3. Effect of pH on activity (●) and stability (○) of tannase.

pH of the substrate was adjusted at different values with 0.2 M of acetate and phosphate buffer. The relative activity, 100%, is 0.9 U/ml of enzyme.



(a)



(b)

Fig. 4. (a) Effect of temperature on the activity of tannase (■).

The relative activity, 100%, is 0.85 U/ml of enzyme.

(b) Thermostability of tannase from *B. cereus* KBR9.

Enzyme (0.83 U/ml) was incubated at different temperatures for 1 h and after then residual activities was measured at optimum pH and temperature.

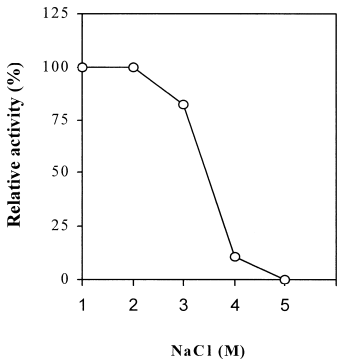


Fig. 5. Activity of tannase at various concentrations of NaCl.

The relative activity, 100%, is 0.92 U/ml of enzyme.

zyme activity (Volesky and Luong, 1985).

The partially purified tannase remained active at pH 3.5–7.0. It showed optimum activity at pH 4.5 and was completely stable within the range of pH 4.5–5.0 (Fig. 3). It lost only 20% and 10% of original activity at pH 4.0 and 6.0 respectively. Earlier Lekha and Lonsane (1997) mentioned that tannase is an acidic protein with an optimum pH around 5.5.

Tannase from *B. cereus* showed optimum activity at 40°C (Fig. 4a). It was stable up to 30°C for 1 h and for 20 min at 40°C (Fig. 4b). Tannase produced by most of the potent strains like *Aspergillus oryzae*, *Penicillium chrysogenum* and *Aspergillus niger* also showed temperature optima at 30°C (Lekha and Lonsane, 1997). So far, no report is available on the temperature stability of bacterial tannase.

It has been found that this enzyme is stable up to 2 M and retains 82% activity at 3 M of NaCl (Fig. 5). This is the first observation that tannase is also a salt-tolerant enzyme. This salt-tolerant property of the enzyme will be very useful in the treatment of hard and slightly acidic effluent containing tannin residues in pollution control mechanisms.

This newly isolated *B. cereus* is very suitable for tannase production because it can grow easily and produce a huge amount of tannase within a short period. The novelty of this enzyme is that it is salt-tolerant as well as stable over a wide pH range. These features make the strain promising for industrial exploitation in various fields.

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