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Production of Tannase through Submerged Fermentation of Tannin-containing Plant Extracts by *Bacillus licheniformis* KBR6

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A b s t r a c t

Tannins are water-soluble polyphenolic compounds found in plants as secondary metabolites. The presence of these substances in the barks of eight different plants was initially examined and their crude extracts were used separately as a substrate for production of tannase through submerged fermentation by *Bacillus licheniformis* KBR6. Tannase production as well as biodegradation of the substrate reached a maximum within 15 to 18 h against crude tannin extract obtained from *Anacardium occidentale*. Among different concentrations of the crude tannin tested, 0.5% (w/v) induced maximum synthesis of enzyme. Tannase production was higher by almost two-fold in the presence of crude tannin compared to pure tannic acid used as a substrate. It seems that industrial production of tannase, using bark extract of *A. occidentale* can be a very simple and suitable alternative to presently used procedures.

K e y w o r d s: *Bacillus licheniformis*, plant tannins, submerged fermentation

Introduction

Tannins are the fourth most abundant plant constituent after cellulose, hemicellulose and lignin (Swain, 1965). Generally tannins are accumulated as secondary metabolites in the bark and heartwood of plants. Although these substances have negligible value for growth, they play a great role in the immunity of plants. Tannin protects the vulnerable parts of the plants from microbial attack by inactivating viruses and invasive microbial extracellular enzymes (Field and Lettinga, 1992). In spite of its antimicrobial effect, some organisms use this compound as a nutrient for growth, utilizing tannase-hydrolyzing enzyme (Lewis and Starkey, 1969).

Tannase (tannin-acyl-hydrolase, E.C: 3.1.1.20) has been known to hydrolyze the ester and depside linkages of hydrolysable tannins into glucose and gallic acid. Nowadays, the enzyme has wide applications in food, beverage, brewing, cosmetics and chemical industries (Lekha and Lonsane, 1997). It is used mainly for the preparation of gallic acid, instant tea, acron wine, coffee flavoured soft drinks, high-grade leather tannin, clarification of beer and fruit juice, detannification of food and to clean-up highly polluting tannin from the effluent of leather industry (Lekha and Lonsane, 1997). Gallic acid, a hydrolytic product of tannin, has different uses like preparation of trimethoprim, pyrogallol, propyl gallate, dyes, *etc.* (Hadi *et al*., 1994; Mukherjee and Banerjee, 2003).

Most of the reported tannase-producing organisms are fungi (Aoki *et al*., 1976; Bhat *et al*., 1998; Mondal *et al*., 2001c; Ramirez-Coronel *et al*., 2003) and only a few are bacteria (Deschamps *et al*., 1983; Mondal and Pati, 2000; Mondal *et al*., 2001a). Many authors studied tannase production by these organisms in the medium containing pure tannic acid acting as both inducer as well as available carbon source. Pure tannic acid is a very costly substrate and is not suitable for large-scale production of the enzyme. In this respect crude tannin could be cost effective and suitable for the commercial production of the enzyme. Agro-residues and forest products are generally considered the best source of tannin-rich substrate (Pandey *et al*., 1999).

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Production of tannase by *Rhizopus oryzae* and *Aspergillus foetidus* from the powdered fruits of *Terminalia chebula* and *Caesalpinia digyna* has been reported (Mukherjee and Banerjee, 2004). In this regard there are no reports on bacterial tannase production using tannin containing any agro-based substrates.

In the present publication we are reporting for the first time the production of tannase by *Bacillus licheniformis* KBR6 through submerged fermentation of crude tannin extracted from the barks of various forest plants.

Experimental

Materials and Methods

Microorganism. The non-pathogenic tannase-producing soil bacterium, *Bacillus licheniformis* KBR 6 (IMI: 379224) described earlier (Mondal and Pati, 2000) was used in the present study.

Preparation of inoculum. Inoculum was prepared by growing a loopful amount of stock culture of the bacterium in 50 ml sterile tannic acid medium (pH 5.0) at 35°C for 20 h. Composition of the medium was (g%, w/v): tannic acid, 1; NH_4Cl, 0.3; KH_2PO_4, 0.05; K_2HPO_4, 0.05 and $MgSO_4$, 0.05.

Extraction of crude tannins. Collected barks of different forest plants from south West Bengal, India, were cut into small pieces and dried in hot air-oven at 60°C for 24 h. The barks (50 g) were then mixed with distilled water (200 ml) and kept at room temperature overnight. After soaking, the mixture was boiled for 10 min. The filtered solutions were used as source of crude tannin (Schanderi, 1970).

Detection of tannin by paper chromatography. Presence of tannin in the plant extract was confirmed through paper chromatographic analysis. A descending mode of solvent system containing n-butanol, acetic acid and water (4:1:5) was used for the study. Detection of the spot was made by $FeCl_3$ (0.1 g % in 30% methanol) as colouring spray reagent (Mondal and Pati, 2000) and confirmed after comparing it with standard tannic acid.

Measurement of tannin biodegradation. The tannin content of the crude plant extracts was measured before and after fermentation by Folin-Denis method (Schanderi, 1970). The crude extract (0.2 ml) was initially diluted with 8.3 ml of distilled water and then mixed with 0.5 ml of Folin-Denis reagent. After proper mixing, 1 ml of 15% (w/v) Na_2CO_3 was added to it and kept in the dark for 30 min at room temperature. The absorbency of tannin was measured at 700 nm (SL 171 MINI SPEC, ELICO, INDIA) and its concentration was calculated using pure tannic acid as standard.

Mode of fermentation. Tannase production by *B. licheniformis* KBR6 was achieved through submerged fermentation of crude tannin at 35°C in a rotary shaker (200 rpm). Different concentrations of tannin were prepared by diluting the measured crude tannin with distilled water. The pH of the medium was adjusted to 5.0 after sterilization. Fermentations were carried out separately in individual 250 ml Erlenmeyer flasks containing 50 ml medium with 1% (v/v) fresh inoculum. The cell-free fermented broth was used as the source of the enzyme. The growth of the organism in culture media was monitored by measuring dry weight of the biomass (mg/ml). All experiments were done in triplicate and data presented as mean ± SE.

Assay of tannase. Tannase activity in the fermented medium was determined by the colorimetric method of Mondal *et al*. (2001b). For assay, 0.1 ml of enzyme was incubated with 0.3 ml of substrate tannic acid (1.0% w/v in 0.2 M citrate buffer, pH 5.0) at 50°C for 30 min. The reaction was terminated by the addition of 3 ml BSA solution (1 mg/ml), which also precipitates the residual tannic acid. A control reaction was done side by side using heat-denatured enzyme. The tubes were then centrifuged (5000 × g, 10 min) and precipitate was dissolved in 2 ml of SDS-triethanolamine (1% w/v, SDS in 5% v/v, triethanolamine) solution. Absorbency was measured at 530 nm after addition of 1 ml of $FeCl_3$ (0.13 M).

The specific extinction co-efficient of tannic acid at 530 nm was 0.577 (Mondal *et al.*, 2001b). Using this co-efficient, one unit of tannase activity is defined as the amount of enzyme required to hydrolyze 1 mM substrate (tannic acid) in 1min at 50°C and pH 5.0.

Results and Discussion

The selection of a substrate for large-scale enzyme production by fermentation depends upon its availability and cost. In this regard several low cost agro-forest residues were used as substrates for obtaining the desired fermented product (Pandey *et al*., 1999). Different substrates (tannin containing plant extracts) were tested in this experiment for production of tannase through submerged fermentation by *B. licheniformis* KBR6.

Tannin contents of the bark of some commonly available plants were initially examined by paper chromatography and quantified by colorimetric method (Table I). Among the eight plant species tested, the maximum amount of tannin was found in the extract of the bark of *Acacia auriculiformis.* On the basis of tannin content, the studied plants can be arranged in the following order: *Acacia auriculiformis* > *Casuarina equisetifolia* > *Psidium guazava* > *Anacardium occidentale* > *Delonix regia* > *Eucalyptus tereticornis* > *Cassia fistula* > *Ficus benghalensis* (Table I).

Tannase production by *B. licheniformis* KBR6 was studied using different crude tannins as submerged fermentation media. It has been found that extract of *A. occidentale* was the best for induction of tannase (0.62 ± 0.04 U/ml) and as much as 73% of tannin in the culture media was degraded by it (Table I). Enzyme production by the organism was found to reach a maximum within 15–18 h of growth in all the tannin

Table I
Measurement of tannin content in crude plant extract, tannin biodegradation and tannase production by *B. licheniformis* KBR6

Plant source	Tannin content in the crude extract (g% w/v)	Biodegradation (%) of tannins through fermentation	Tannase production (U/ml)
Acacia auriculiformis	1.33 ± 0.18	27 ± 3.60	0.32 ± 0.09
Anacardium occidentale	0.65 ± 0.10	73 ± 4.65	0.62 ± 0.04
Casuarina equisetifolia	0.85 ± 0.09	34.5 ± 5.33	0.06 ± 0.10
Cassia fistula	0.43 ± 0.16	64.4 ± 1.88	0.52 ± 0.01
Delonix regia	0.54 ± 0.09	21 ± 3.38	0.12 ± 0.07
Eucalyptus tereticornis	0.45 ± 0.12	58 ± 6.10	0.17 ± 0.02
Ficus benghalensis	0.39 ± 0.20	53 ± 1.58	0.40 ± 0.11
Psidium guazava	0.73 ± 0.03	0.08 ± 0.01	0.32 ± 0.06

The organism was grown for 18 h at specified conditions, which are described in Materials and Methods. The amount of tannase production is significantly correlated with the biodegradation of crude tannins (correlation coefficient, r = + 0.54).

extracts except for *Eucalyptus*, (Fig. 1). A similar type of time related enzyme production by the same organism was also reported with pure tannic acid as substrate (Mondal and Pati, 2000). Tannase production by the organism was found to be maximal in the extract of *A. occidentale* compared to other plant extracts (Fig. 1). It is not clear to us why the production of enzyme in the extract of *A. occidentale* is high, but we assume that there may be some inducing factors that accelerate enzyme synthesis.

Enzyme production was also studied at different concentrations (0.5–2.0 %, w/v) of crude tannins. It was observed that a specific concentration of crude tannin from a plant influenced enzyme production the strongest (Table II). In our experiments a maximal amount of enzyme was produced in medium containing 0.5% (w/v) of crude tannin of *A. occidentale*, but the highest enzyme production (Mondal *et al*., 2000) was observed when 1.5% (w/v) of pure tannic acid was used as substrate in the medium The concentration of tannin is thus a very important determining factor for tannase biosynthesis for most fungi and bacteria (Lekha and Lonsane, 1997; Mondal *et al*., 2000; Banerjee *et al*., 2001). The actual mode of tannase induction in a particular concentration of tannin has not been properly explained until now. Lewis and Starkey (1969) mentioned that higher concentrations of tannin lead to non-reversible bonds with surface proteins and impair the metabolism as well as growth of the organism. One of the most striking observations in this experiment is that enzyme production was increased about four-fold in the medium containing basal salt with crude tannin (0.5%) compared to medium containing crude tannin extract (0.5%) of *A. occidentale* alone (Table III). This result revealed that some specific microelements (salts and ions) are probably essential

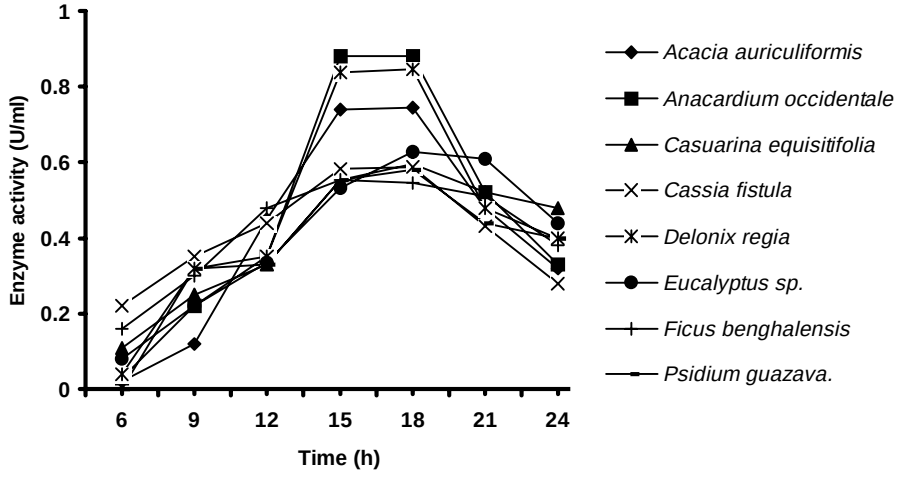

Fig. 1. Time course of tannase production by *Bacillus licheniformis* KBR6 using different plant extracts (crude tannin) as substrate

Table II
Effect of different concentrations of crude tannin
from different plants on tannase production (mean ± SD)

Plant source	Concentration of tannin (%)	Tannase (U/ml)
Acacia auriculiformis	0.5	0.33 ± 0.09
	1	0.42 ± 0.11
	2	0.35 ± 0.19
Anacardium occidentale	0.5	1.23 ± 0.29
	1	0.86 ± 0.08
	2	0.83 ± 0.23
Casuarina equisetifolia	0.5	1.01 ± 0.11
	1	1.12 ± 0.15
	2	1.02 ± 0.14
Cassia fistula	0.5	0.55 ± 0.25
	1	0.83 ± 0.14
	2	0.84 ± 0.30
Delonix regia	0.5	0.36 ± 0.02
	1	0.89 ± 0.42
	2	0.10 ± 0.32
Eucalyptus tereticornis	0.5	0.20 ± 0.15
	1	0.10 ± 0.01
	2	0.210 ± 0.22
Ficus benghalensis	0.5	0.50 ± 0.31
	1	0.83 ± 0.20
	2	0.85 ± 0.08
Psidium guazava	0.5	0.30 ± 0.25
	1	0.86 ± 0.05
	2	0.78 ± 0.16

Table III
Comparative study of tannase production in pure tannic acid and crude tannin extract as
substrates by *Bacillus licheniformis* KBR6

Substrates in fermentation medium	Growth (mg/ml)	Tannase (U/ml)
Crude tannin (0.5 g/100 ml)	0.82 ± 0.18	0.17 ± 0.06
Crude tannin (0.5 g/100 ml) + Basal salts*	1.12 ± 0.22	0.66 ± 0.12
Tannic acid (1.5 g/100 ml) containing enriched medium**	0.58 ± 0.12	0.31 ± 0.13

* Composition (g%, w/v): NH_4Cl, 0.3; KH_2PO_4, 0.05; K_2HPO_4, 0.05 and $MgSO_4$, 0.05.
** Composition (g%, w/v): NH_4Cl, 0.3; KH_2PO_4, 0.05; K_2HPO_4, 0.05; $MgSO_4$, 0.05; glucose, 0.01%; alanine, 0.01%; pyridoxine, 0.003%.
Fermentation was carried out at specified condition for 18 h.

for growth as well as enzyme synthesis by *B. licheniformis* KBR6. Both growth of the organism and enzyme production increased two-fold when it was grown in salt containing crude tannin extract rather than enriched pure tannic acid medium. All these beneficial effects of the plant extract of *A. occidentale* make it promising as one of the best as well as cheaper substrates for the large scale production of microbial tannase.

In conclusion, tannase has now been extensively used in different biochemical industries. The selected bacterium used in this study is able to synthesize high amounts of tannase through fermentation of crude tannin of *A. occidentale*. Exploitation of these plant extracts could be a source of cheaper substrate for industrial production of microbial tannase.

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