Pak. J. Bot., 48(3): 1263-1271, 2016.

PRODUCTION AND CHARACTERIZATION OF TANNASE FROM A NEWLY ISOLATED *BACILLUS SUBTILIS*

MUHAMMAD NAUMAN AFTAB, HAMID MUKHTAR AND IKRAM-UL-HAQ

Institute of Industrial Biotechnology, GC University, Lahore-54000, Pakistan

Abstract

The work describes the production and characterization of tannase from a newly isolated *Bacillus subtilis*. The strain was isolated from the garden soil and was capable of producing tannase at particular temperature (41ºC) and pH (5) in 24 h. Addition of 10 % glucose as a carbon source and 12% tannic acid as an inducer resulted in the improved rate of enzyme production. The enzyme was purified up to 4.86 fold with 96.25% yield. It exhibited optimal temperature and pH tolerance of 45ºC and 5, respectively. However, the enzyme was found to be notably more functional in a broad range of temperature (20-80ºC) and pH (3-10). Furthermore it remained remarkably stable at wide range of pH (3-8) and at a higher salt concentration (~3M). The shelf life of enzyme was also prolonged and remained stable up to a maximum of 8 months.

Key words: Tannase; *Bacillus subtilis*; Characterization; Fermentation; Enzyme; Temperature.

Introduction

Tannin acyl hydrolase (*E.C. 3.1.1.20*) is commonly referred as tannase, an enzyme which catalyses the breakdown of tannins such as tannic acid, methyl gallate, ethyl gallate, *n*-propylgallate and isoamyl gallate (Lekha & Lonsane, 1997). It is an extracellular microbial enzyme and hydrolyses the ester bonds of tannic acid through 2, 3, 4, 6-tetragalloyl glucose and two kinds of monogalloyl glucose (Aguilar *et al*., 2007). The enzyme is being extensively used for the preparation of instant tea, acron wine, soft drinks, clarification of beer and fruit juices, pharmaceutical industry, de-tannification of food and in industrial effluent treatment (Kar & Banerjee, 1997; Mondal & Pati, 2000). It is also used for the production of gallic acid which is mainly used as an important substrate for the synthesis of propyl gallate and trimethoprim widely used as a food antioxidant and antibacterial agent, respectively (Hadi *et al*., 1994; Lekha & Lonsane, 1997). It is also used as photosensitive resin in semiconductor production and as a sensitive analytical probe to determine the structure of naturally occurring gallic acid esters (Hadi *et al*., 1994). Although, tannins are toxic bacteriostatic compounds and have non-reversible reaction to protein (Scalbert, 1991), yet 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. Tannase has been reported as an inducible enzyme produced by a limited number of microorganisms including fungi and bacteria (Maity *et al*., 2009). In particular, the species of *Aspergillus*, *Penicillium*, *Rhizopus*, *Paecilomyces*, *Lactobacillus, Klebsiella pneumoniae* and *Citrobacter freundii* (Kumar *et al*., 1999) have been reported to produce the enzyme through fermentation (Mondal *et al*., 2001). However, some species of *Bacillus* have also been found to produce considerable amounts of tannase in the culture broth such as *B. cereus* and *B. licheniformis* (Mondal *et al*., 2001; Maity *et al*., 2009).

Microbial production of tannase is carried out through solid, liquid surface and submerged fermentation as the enzyme is extracellular and is secreted directly into the medium (Lekha & Lonsane, 1997). Several studies regarding advantages of solid state fermentation (SSF) over the others have been carried out (Pinto 2006); however, production of tannase from a bacterial source usually requires submerged fermentation for the enhanced yield. The optimization of cultural conditions during fermentation results in a considerable increase in the production of tannase by microorganisms. The addition of carbon sources such as glucose, fructose, sucrose, maltose, arabinose (Bradoo *et al*., 1997) and folic or pantothenic acid (Belmares *et al*., 2004) to the culture medium improves tannase production. The addition of different substrates and moisture levels, evaluation of supplementary nitrogen and phosphate sources has been reported to improve enzyme production (Purohit *et al*., 2006). Various tannin-rich substrates like sugarcane baggase, wheat bran, Jamun (*Syzygium cumini*) leaves and *Creosote bush* leaves have also been optimized (Lekha & Lonsane, 1994; Sabu *et al*., 2005; Kumar *et al*., 2006; Trevino-Cueto *et al*., 2007). In this study, we isolated tannase producing *Bacillus subtilis*. Different parameters for the optimal enzyme production were investigated. Later, enzyme characterization was also accomplished. From the available literature, this is perhaps the first ever report of its kind from a novel bacterial culture.

Materials and Methods

Microorganism: A number of tannase producing bacterial strains were isolated by plate dilution technique from different soils of municipal disposal sites (Beijing city) using selective medium consisting of (% w/v); tannic acid, 1; K_2HPO_4, 0.05; KH_2PO_4, 0.05; $MgSO_4$, 0.05; NH_4NO_3, 0.3 and agar, 2 (pH adjusted to 5). One gram of different soil samples were suspended in 100 ml of sterile saline water and subjected to heat shock for 2 min at 70ºC. Approximately, 0.5 ml of a suitably diluted suspension was spread on to the petri plates which were incubated at 37ºC for 24-48 h. Appearance of clear transparent zone around the colony was indicative of tannase production. The selected strains were transferred to the nutrient agar slants for growth and maintenance.

Identification of the isolated strain was carried out on the basis of various morphological, cultural and biochemical characteristics according to Bergey's

Manual of Detreminative Bacteriology (Buchanan and Gibbons, 1974). The species designation was further confirmed through the amplification of one of the 16S rRNA genes using two oligonucleotides: P1 (5'-AAGTCGAGCGGACAGATGG-3') and P2 (5'-CCAGTTTCCAATGACCCTCCCC-3'), annealing to nucleotides 59-79 and 625-646 of the bacterium, respectively (Wattiau *et al*., 2001).

Inoculum preparation: Cells from a newly prepared (48 h old) slant were transferred to a 250 ml Erlenmeyer flask containing 50 ml of pre-culture medium consisting of (g/l) peptone from meat, 5 and yeast extract, 3. The flask was incubated at 37ºC on a rotary shaker (Gallenkamp, UK) at 200 rpm for 24 h. The inoculum so prepared was used to carry out fermentation experiments.

Shake flask studies: Shake flask studies were carried in 250 ml Erlenmeyer flasks having 50 ml of selective tannic acid-enriched medium consisting of (g/l): tannic acid, 10; NH_4NO_3, 3; KH_2PO_4, 0.5; K_2HPO_4, 0.5; $CaCl_2$, 1 and $MgSO_4$, 0.5 (pH 5). The flasks were cotton plugged and autoclaved at 15 Ib/in^2 for 15 min. The medium was cooled and inoculated with 1 ml of the inoculum as prepared above. The fermentation experiments were carried out at 37ºC in a rotary shaker (Gallenkamp, UK) at a stirring speed of 200 rpm for 48 h. Different fermentation conditions like incubation period, temperature, pH of the medium, glucose and tannic acid concentration, nitrogen source and substrate screening were optimized for the enhanced production of tannase. All experiments were performed in triplicate using standardized lab ware and analytical grade reagents.

Tannase assay: The enzyme activity was determined by the procedure of Deschamps *et al*., (1983). To 4 ml of the reaction mixture, 1 ml of 1 % tannic acid (in 0.5 M citrate-phosphate buffer, pH 5.0), 2 ml of 0.5 M citrate-phosphate buffer (pH 5) and 1 ml of suitably diluted culture filtrate were added. This mixture was incubated at 50ºC for 30 min in a water bath and the reaction was terminated by adding 4 ml of 2 % bovine serum-albumin (BSA) solution. In control, BSA was added into the reaction mixture prior to incubation. The tubes were left for 20 min at room temperature to precipitate the residual tannins and were then centrifuged at 3000×*g* for 20 min. Tannase activity was estimated by diluting 20 µl of the supernatant 500-fold with double distilled water, and the absorbance was read at 260 nm against double distilled water as blank in a UV spectrophotometer (Shimadzu, Japan). The amount of gallic acid produced in the reaction mixture was estimated from the respective standard curve**.**

One unit of tannase is defined as the amount of enzyme required to release 1 mol of gallic acid per millilitre per minute of culture filtrate under the standard assay conditions.

Protein estimation: The protein content in the culture filtrate was estimated according to the procedure of Lowery *et al.,* (1951). The optical density of the mixture was measured at 650 nm wave length and the amount of protein was estimated using standard curve of BSA.

Enzyme concentration: Fermentation broth (about 2 L) containing extra cellular tannase was obtained by compressing polyurethane foam (PUF) in a Buchner funnel. The PUF was washed with 100 mM citrate/phosphate buffer (pH 7). The extract was then centrifuged at 7000×*g* for 15 min at 4°C and concentrated to 100 ml by ultrafiltration on an Amicon membrane with a 50 kDa molecular mass cut-off (Millipore). The extract was dialyzed against water prior to preparative isoelectric focusing.

Results and Discussion

Identification of *Bacillus subtilis* strain: The isolated bacterial strain capable of producing tannase was identified on the basis of morphological and biochemical characters and identified as *Bacillus subtilis*. The strain is a gram positive, spore forming, and motile rod shaped bacterium capable of hydrolyzing starch, casein and urea. It formed white colored colonies with undulating margins on nutrient agar medium (Supplementary file 1). The identified strain was further confirmed by a PCR test based on the 16S rDNA (Wattiau *et al*., 2001). A band size of 595 bp was obtained when PCR product was run on 6 % agarose gel thus confirming the isolated strain (Fig. 1). Although it has been reported that some species of *Bacillus* like *B. pumilus, B. polymixa*, *B. licheniformis* (Mondal & Pati, 2000; Mohapatra *et al*., 2007) and *B. cereus* (Mondal *at al*., 2001) can produce tannase; this is for the first time demonstrated that *Bacillus subtilis* can also produce tannase.

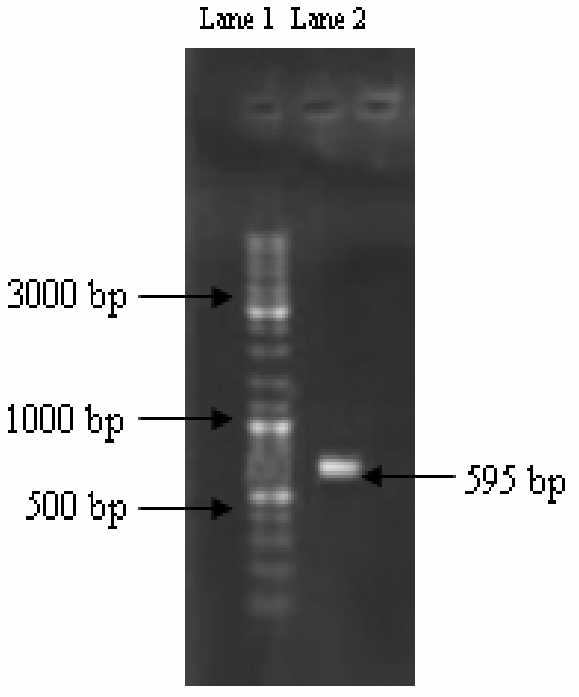

Fig. 1. An amplication of 16S rRNA of *Bacillus subtilis.* The Gene Ruler^{TM} DNA ladder mix bands are in lane 1 and 595 bb band of 16S rRNA gene is in lane 2.

Optimization of tannase production: The effect of incubation period on the production of tannase by *Bacillus subtilis* IBP 120 in a selective tannic acid-enriched medium was studied by carrying out experiments up to 36 h of fermentation. The analysis of the fermented broth after a specific period indicates that maximum enzyme production 0.32 U ml^{-1} was achieved after 24 h of inoculation i.e., during stationary phase of the bacterial growth (Fig. 2A). The results are in compliment with some previous studies in which *Bacillus cereus* KBR9 produced maximum tannase after 24 h of incubation (Mondal *et al*., 2001). It is also reported that *B. licheniformis* has shown maximum production of tannase after 21 h i.e., during exponential phase of growth (Mondal & Pati, 2000) whereas maximum tannase production by some other bacterial strains have been reported just after an incubation period of 6 h i.e., during lag phase of growth (Deschamps *et al*., 1983; Kumar *et al*., 1999).

Tannase production at different incubation temperatures ranging from 0-60ºC showed that bacterium produced maximum titer of tannase at 41ºC. The enzyme production was recorded to be 0.15, 0.28 and 0.30 U ml^{-1} at 25, 30 and 37ºC, respectively and reached maximal (0.34 U ml^{-1}) at 41ºC (Fig. 2 B). Then a decreasing trend in the enzyme production was observed and it was reached to almost negligible at 60ºC. Previous studies indicated the best temperature range for *Paecilomyces variotii, Rhizopus oryzae* and *Aspergillus niger* PKL 104 based on tannase productions and ranged from 28-34ºC (Lekha & Lonsane, 1994). However, the production of tannase by *Aspergillus niger* 3T5B was observed between the temperature range of 32-40ºC (Pinto, 2003).

The effect of initial pH of the medium on the production of tannase by *B. subtilis* was studied and it was found that the bacterium was capable of producing enzyme at a wider range of medium pH between 3-6.5 but maximum production for tannase was achieved at pH 5 (Fig. 3A). Above this pH value, the enzyme production was gradually decreased and there was no enzyme production above pH 8. The result was in agreement to the previous reports for the production of tannase by both bacteria and fungi (Barthomeuf *et al*., 1994; Hadi *et al*., 1994; Kumar *et al*., 1999; Mondal & Pati, 2000). The possible explanation for the production in acidic medium might be due to the fact that the strain was isolated from municipal disposal site that was acidic in nature. It was also observed that the pH of the broth decreased sharply as the fermentation was initiated and reached around pH 2 after 24 h, the phase of the growth at which bacterium produced maximum tannase (Fig. 3B). After 24 h of incubation, the pH of the medium was increased slowly. This was due to the assimilation of glucose and production of gallic acid in the medium. These findings are also comparable with earlier studies (Vermeire & Vandamme, 1988; Mondal & Pati, 2000).

The effect of different concentrations of glucose (2-20 %) in the fermentation medium on the production of the tannase was observed. It was found that the addition of glucose in the culture medium enhanced the tannase production and maximum residual production i.e., 125 % (0.35 U ml^{-1}) was achieved with the addition of 10 % glucose in comparison with the medium without glucose i.e., control (Fig. 4 A). A decrease in the production was observed when glucose concentration exceeded upto 10 % in the medium. It seems that glucose acted as catabolic inducer for tannase production. Similar results were demonstrated by Hadi *et al*., (1994) for the production of fungal tannase.

The supplementation of the culture medium with different levels (2-20 %) of tannic acid was carried out to study its effect on the production of tannase by *B. subtilis* IBP20. The addition of tannic acid to the culture medium resulted in an increase in tannase production, but the values greater than 12 % resulted in a decreased level (Fig. 4B). So tannic acid is found to be an inducer for tannase production by *B. subtilis* as reported earlier (Mondal & Pati, 2000). It is being degraded by tannase into gallic acid and glucose, which are ultimately utilized by the organism for growth (Lekha & Lonsane, 1997). It was also noted that an increment in the amount of inducer results in an increase in enzyme synthesis, however, this increase may not necessarily cause an equivalent increase in enzyme synthesis. The solubility; toxicity level and saturation of the inducer might affect the optimal production (Pinto, 2003). Previous studies showed that *A. niger* (Lekha & Lonsane, 1994), *Aspergilus oryzae* (Lekha & Lonsane, 1997) and *Paecilomyces variotii* (Battestin & Macedo, 2007) required the addition of 4-12 % of tannic acid into the culture medium for maximal enzyme synthesis.

Tannase production depends on the availability of nitrogen source in the medium which has regulatory effects on enzyme synthesis (Patel *et al*., 2005). The effect of supplementation of three different nitrogen sources viz. ammonium nitrate (NH_4NO_3), ammonium chloride (NH_4Cl) and ammonium phosphate (NH_4PO_4), at the concentration of 0.2-2.0 % on the tannase production was studied (Fig. 4 C). Among all nitrogen sources tested, NH_4NO_3 at a level of 1 % was found to be the most suitable as it resulted in the highest residual activity of 123 % (0.38 U ml^{-1}) with 23 % increase in production when compared to control (medium without nitrogen source). The ammonium nitrate (ions) stimulates the synthesis of proteins and is a source of readily utilizable nitrogen (Djekrif-Dakhmouche *et al*., 2006). Among other nitrogen sources, ammonium phosphate has some effect on the production but ammonium nitrate has negligible effect on the production of tannase. Concentrations more than 1 % resulted into a declined rate of production of tannase in all the cases. On the basis of present results, we concluded that microorganisms require a low level of nitrogen in order to produce enzymes because nitrogen may be a limiting factor (Djekrif-Dakhmouche *et al*., 2006). Previous study showed that 0.8 % of NH_4NO_3 as a nitrogen source is required for optimum production of tannase in *Paecilomyces variotii* (Battestin & Macedo, 2007).

Different substrates such as gallocatechin gallate, gallic acid methylester, tannic acid, epigallocacatechin gallate, epicatechin gallate and catechin gallate were screened for the production of tannase by *Bacillus subtilis* (Fig. 5). The results showed that tannase production from *B. subtilis* can be induced by a variety of substrates. However, maximum activity (0.36 U ml^{-1}) was observed when tannic acid was used as a substrate in the culture medium. However, the reasonable amount of enzyme production was also observed in case of gallocatechin gallate (0.32 U ml^{-1}). Some amount of tannase was also produced when gallic acid methyl ester (0.24 ml^{-1}) and epigallocatechin gallate (0.19 U ml^{-1}) were used as substrates.

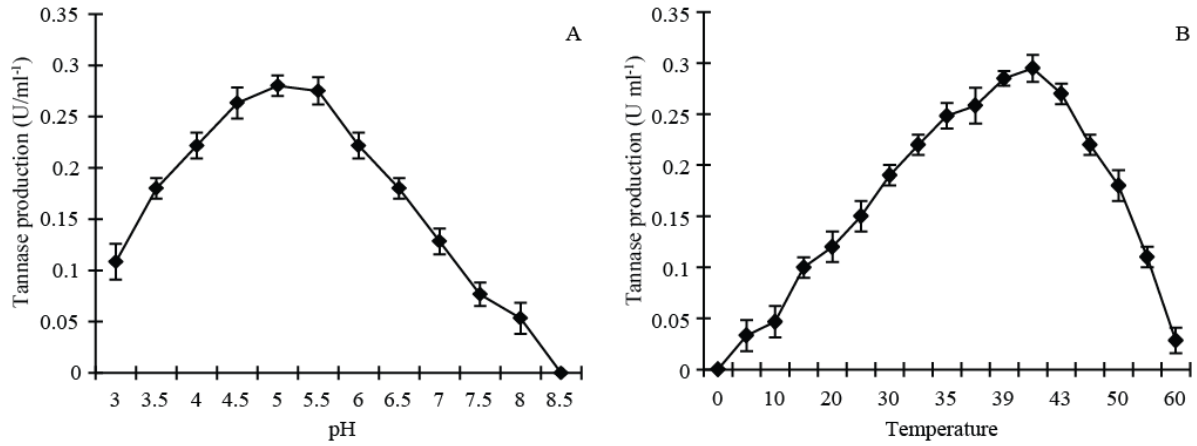

Fig. 2. (A) Effect of time period on the production of tannase. Maximum production was achieved after 24 h of incubation. (B) Effect of temperature on the production of tannase. Maximum production was obtained at 41ºC.

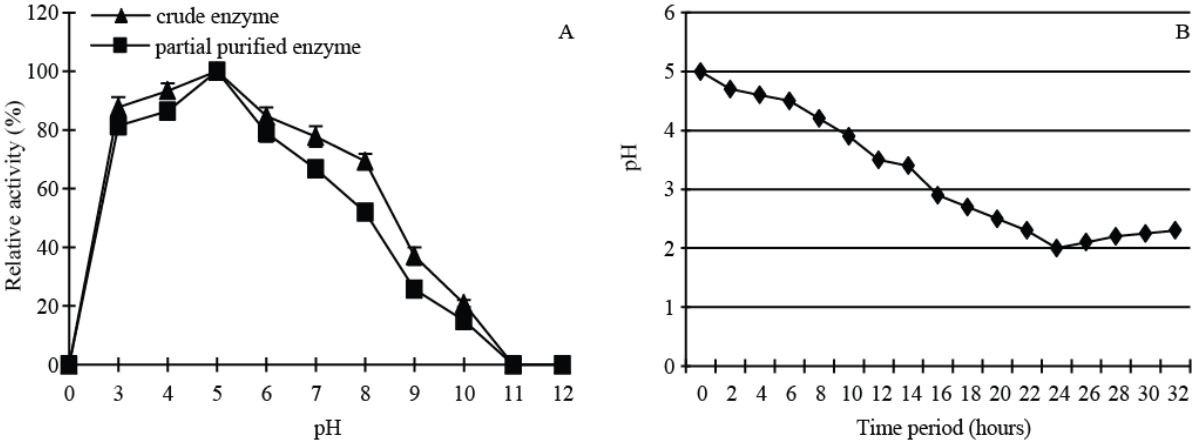

Fig 3. (A). Effect of pH of the medium on the production of tannase. Optimum pH for the production of tannase is 5.0. (B). Periodical measurement of pH after equal interval of time during production of tannase.

Partial purification and characterization of tannase: A single step partial purification of tannase was carried out through ultra filtration. The culture filtrate containing the enzyme was concentrated to one-tenth the original volume by ultra-filtration using 100 kDa membrane cartridge, resulting in 96.25 % yield and 4.86-fold purification (Table 1). Both crude and partial purified tannase was used for characterization. Suitable dilutions of crude and purified enzymes were made in 0.5 M citrate-phosphate buffer while immobilized enzyme (100 mg) was suspended in 0.5 ml citrate-phosphate buffer (0.01 M, pH 5).

Table 1. Comparison of different parameters before and after partial purification of tannase. Partial purification of tannase 120 was carried out by ultra-filtration using 100 kDa membrane cartridge.

	Before purification	After purification
Volume (ml)	1000	195
Total tannase (U)	350	337
Total proteins (mg)	99	20
Specific activity (U/mg)	3.53	16.85
Yield (%)	100	96.28
Fold purification	1.0	4.86

The effect of temperature on the activity of crude, purified and immobilized tannase was studied by incubating the reaction mixtures at different temperatures ranging from 25-100ºC for 30 min. Tannase produced by *B. subtilis* was found active from 20-80ºC (Fig. 6 A), however, the maximum activity was observed at 45ºC which shows that enzyme was quite thermostable. At 20ºC, crude and partially purified enzyme showed 71 and 66 % relative activity, respectively. At 70ºC, more than 5 and 45 % relative activities were retained by the crude and partially purified enzymes, respectively. At 80ºC, both crude and partially purified enzymes exhibited sharp decrease in activity with 24 and 18 % relative activities, respectively and with almost no activity at 90ºC. Tannase from *A. niger* van *Tieghem* (Sharma *et al*., 1999) and *Bacillus cereus* KBR 9 (Mondal *et al*., 2001) was reported to have an optimal temperature range from 45 to 60ºC but most of the fungal tannases have optimum temperatures ranged between 30-40ºC (Farias *et al*., 1994; Yu *et al*., 2004; Sabu *et al.,* 2005).

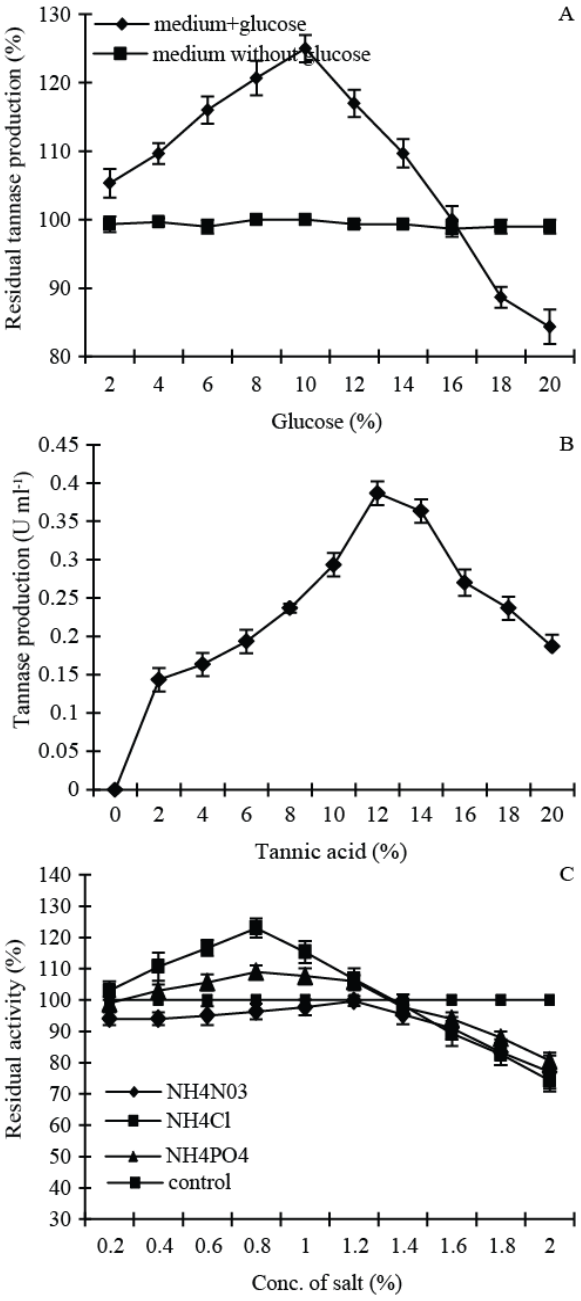

Fig. 4. (A) Residual tannase production of tannase with and without glucose in the medium. Maximum production was obtained with the addition of 10 % glucose in the medium with increase of approximately 25 %. (B) Effect of addition of tannic acid in the medium on the production of tannase. Maximum production was obtained when 12 % tannic was added in the medium. (C) Effect of addition of different nitrogen sources in the medium on the tannase production. Approximately 25 % increased production was achieved with the addition of 1 % ammonium chloride (NH_4Cl) as compared with control (medium without nitrogen source).

The thermostability of all the three forms of tannase was determined by incubating the enzyme in 0.5 M citrate phosphate buffer at temperatures ranging from 25 to 100ºC for different time intervals (0.5, 1, 6, 12 and 24 h). After the desired incubation periods, enzyme aliquots were taken and assayed at optimal assay conditions to determine the residual tannase activities. It was found that both the forms of tannase showed stability in the range of 20-60ºC for 24 h retaining more than 70 % residual activity at 20ºC, around 85 % at 45ºC, about 78 % at 60ºC (Table 2). At 70ºC, after 6 h of incubation, residual activity for both crude and partially purified enzyme was dropped sharply and was observed at approximately 17 and 12 %, respectively. At 80ºC, the residual activity was retained for up to only 3 h at approximately 22 and 13 % for crude and partially purified enzyme, respectively.

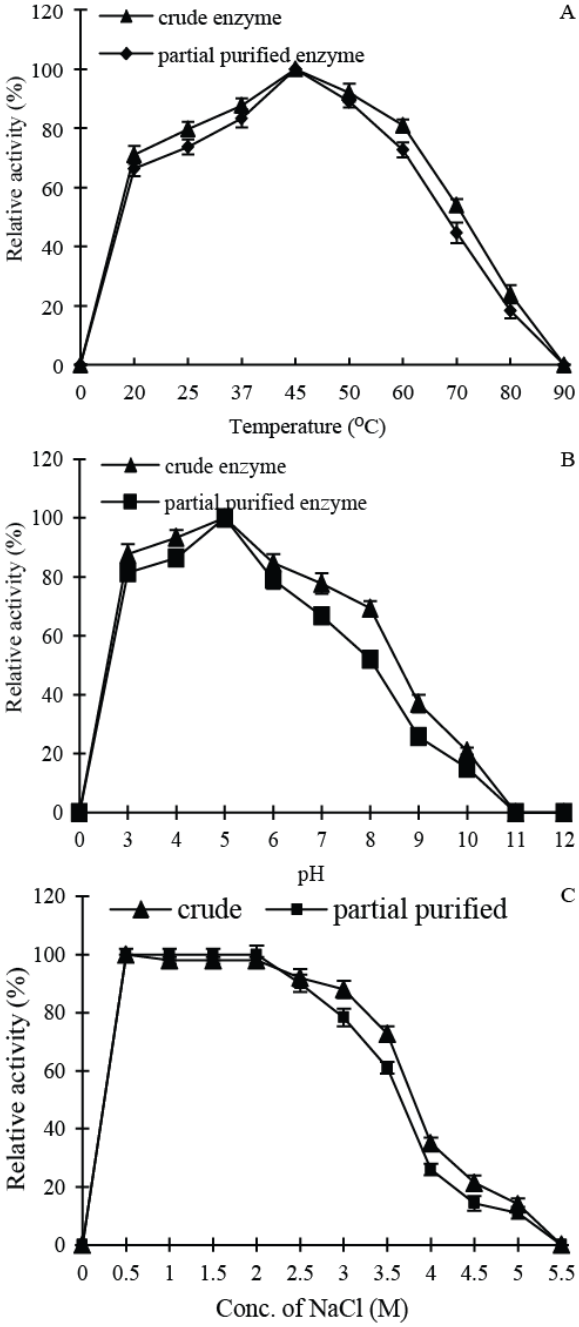

Fig. 6. (A) Temperature tolerance of tannase 120. Maximum tolerance of the enzyme is observed at 45ºC. (B) pH tolerance of tannase 120 of *B. subtilis* IBP 120. (C) Salt tolerance of *B. subtilis* IBP 120 tannase 120.

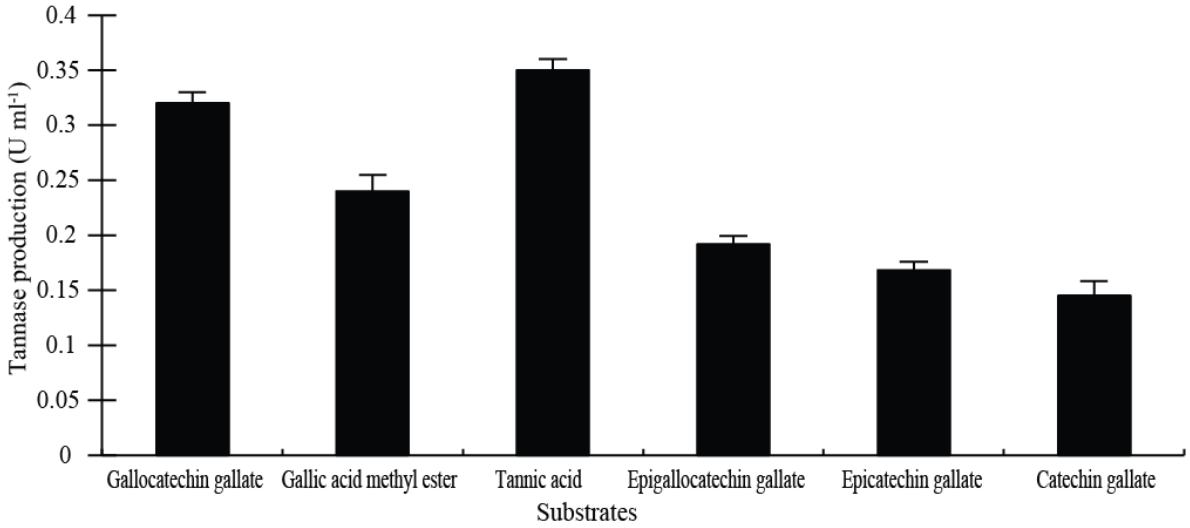

Fig. 5. Effect of the addition of different tannase producing substrates in the medium on the production of tannase after 24 hours.

Table 2. Themostability of tannase produced by *B. subtilis IBP* 120

Temp	30 min		1 h		3 h		6 h		12 h		18 h		24 h	
(ºC)	CT	PPT	CT	PPT	CT	PPT	CT	PPT	CT	PPT	CT	PPT	CT	PPT
20 TA	0.31	0.32	0.30	0.29	0.31	0.28	0.28	0.27	0.26	0.26	0.26	0.25	0.25	0.25
TRA	91.25	91.48	87.91	88.88	86.25	87.40	80.41	82.96	77.08	79.25	74.58	77.77	72.08	76.66
25 TA	0.32	0.31	0.32	0.30	0.31	0.29	0.3	0.29	0.28	0.27	0.27	0.26	0.27	0.26
TRA	91.66	94.44	91.25	91.85	89.58	90.00	86.66	88.51	82.08	82.96	79.16	81.11	77.50	78.88
37 TA	0.33	0.32	0.33	0.31	0.33	0.31	0.33	0.30	0.32	0.30	0.32	0.29	0.31	0.28
TRA	95.83	96.25	95.00	95.18	94.58	94.44	93.75	91.85	92.08	89.62	90.83	87.77	89.16	85.55
45 TA	0.35	0.33	0.35	0.33	0.35	0.33	0.33	0.31	0.33	0.31	0.32	0.29	0.31	0.28
TRA	100	100	100	100	100	100	96.66	96.29	95.41	94.81	92.91	90.74	90.00	87.77
50 TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.33	0.30	0.32	0.30	0.31	0.29
TRA	100	100	100	100	100	100	100	100	95.00	92.96	92.50	91.48	90.41	89.25
60 TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.31	0.29	0.30	0.27	0.29	0.25
TRA	100	100	100	100	100	100	100	100	90.41	88.51	87.50	82.59	84.58	78.14
70 TA	0.31	0.29	0.29	0.27	0.18	0.14	0.15	0.11	0.16	0.04	–	–	–	–
TRA	88.75	88.51	83.75	82.22	51.66	44.07	42.91	36.29	17.5	12.22	–	–	–	–
80 TA	0.18	0.14	0.16	0.09	0.07	0.04	–	–	–	–	–	–	–	–
TRA	51.66	43.33	45.83	27.77	22.08	13.70	–	–	–	–	–	–	–	–
90 TA	–	–	–	–	–	–	–	–	–	–	–	–	–	–
TRA	–	–	–	–	–	–	–	–	–	–	–	–	–	–

CT: crude tannase; PPT: partial purified tannase; TA: tannase activity (U/ml); TRA: 100 % tannase residual activity (at 0 min) corresponds to 0.35 U^{-1} for CT and 0.33 U ml^{-1} for PPT.

Table 3. pH stability of *B. subtilis* IBP 120 tannase.

pH	30 min		1 h		3 h		6 h		12 h		18 h		24 h	
	CT	PPT	CT	PPT	CT	PPT	CT	PPT	CT	PPT	CT	PPT	CT	PPT
3 TA	0.30	0.27	0.28	0.26	0.27	0.25	0.24	0.24	0.26	0.21	0.21	0.19	0.23	0.18
TRA	87.5	81.85	80.41	78.88	77.08	76.66	68.33	73.33	74.16	63.70	70.83	59.62	66.66	55.55
4 TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
5 TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
6 TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
7 TA	0.29	0.26	0.28	0.25	0.26	0.24	0.24	0.23	0.23	0.20	0.21	0.19	0.19	0.17
TRA	85.0	79.62	78.75	77.03	73.75	72.96	67.91	68.88	64.58	62.96	62.08	58.51	56.66	51.48
8 TA	0.28	0.25	0.26	0.24	0.25	0.23	0.23	0.21	0.22	0.2	0.21	0.18	0.19	0.15
TRA	79.58	76.66	75.41	74.44	72.08	70.0	66.25	65.55	62.08	60.74	59.16	55.15	53.75	46.66
9 TA	0.19	0.19	0.18	0.15	0.16	0.14	0.15	0.13	0.13	0.11	0.10	0.09	0.09	0.07
TRA	55.41	58.14	50.41	45.92	47.08	43.33	42.50	38.88	37.08	34.07	31.25	28.88	26.66	21.85
10TA	0.16	0.14	0.14	0.13	0.13	0.10	0.11	0.09	0.09	0.07	0.07	0.05	0.06	0.04
TRA	45.41	43.33	40.83	40.37	36.25	31.48	32.08	26.29	26.66	21.85	21.25	16.29	16.25	12.96

CT: crude tannase; PPT: partial purified tannase; TA: tannase activity (U/ml); TRA: 100 % tannase residual activity (at 0 min) corresponds to 0.35 U^{-1} for CT and 0.33 U ml^{-1} for PPT.

The pH activity profile of all the three forms of tannase i.e., crude, partially purified and immobilized was studied by incubating the enzyme-substrate mixture in buffers of different pH values ranging from 3 to 11 at optimal temperature (50ºC). The buffers used included 0.5 M citrate-phosphate buffer (pH 3-5), 0.5 M phosphate buffer (pH 6-7), 0.5 M Tris buffer (pH 8-9) and 0.5 M glycine-NaOH buffer (pH 10-11). The results showed that tannase from B. subtilis IBP 120 have its functionality in a broad range of pH i.e., 3-10 (Fig. 6B) however; maximum activities for both crude and partially purified enzyme were observed at pH 5. At pH 8 both types of enzymes showed more than 50 % relative activity while the residual activity dropped very rapidly at pH 9 and 10. Previous studies reported optimum pH in the range of 3-6 (Banerjee *et al*., 2001; Ramirez-Coronel *et al*., 2003; Yu *et al*., 2004; Sabu *et al*., 2005).

The pH stability of all enzyme forms was also studied by incubating the enzyme in different buffers of pH values ranging from 3 to 10 for 0.5, 1, 6, 12 and 24 h at 37ºC. After the desired incubation period, the residual tannase activity was estimated. Tannase showed stability in pH range of 3-8 for 24 h (Table 3). At pH 3, crude and partially purified enzyme showed approximately 66 and 55 % residual activities, respectively. At pH 4-6, there was almost 100 % residual activity exhibited by the enzyme, whereas at pH 9 and 10, the stability of crude and partially purified tannase was dropped quickly and only 26 and 21 % at pH 9 and 16 and 12 % residual activities at pH 10 were observed. Previous studies showed that tannase remained stable only between pH range of 4.5-5.5 (Lekha *et al*., 1994).

The effect of different concentrations of NaCl on the activity of *B. subtilis* based tannase was studies (Fig. 6C). It was found that the enzyme remained ~100 % active when treated with up to 3 M NaCl for 24 h and retained 67 and 62% residual activities at 4 M salt concentration for crude and partially purified enzymes, respectively (Table 4). However, at 4.5 M NaCl concentration, the enzyme lost its activity quickly and reached almost zero at 5 M salt concentration. Previous report also showed the stability of tannase at 3 M salt concentration (Mondal *et al*., 2001).

Shelf-life: The shelf-life studies on tannase in crude and partially purified form at 4 and 37ºC were carried out for period of eight months. The results showed that more than 90 % residual activities were retained till 15 days in crude and partially purified enzyme forms both at 4 and 37ºC (Table 5). Approximately 70 % residual activity was retained after 5 months and more than 50 % residual activity was exhibited by tannase even after 8 months of storage at 4 and 37ºC, respectively.

Table 4. Salt stability study of tannase produced by *B. subtilis* IBP 120.

NaCl Concen.	30 min		1 h		3 h		6 h		12 h		18 h		24 h	
	CT	**PPT**	**CT**	**PPT**	**CT**	**PPT**	**CT**	**PPT**	**CT**	**PPT**	**CT**	**PPT**	**CT**	**PPT**
0.5M TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1M TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
1.5M TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2M TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
2.5M TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33
TRA	100	100	100	100	100	100	100	100	100	100	100	100	100	100
3M TA	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.35	0.33	0.33	0.31
TRA	100	100	100	100	100	100	100	100	100	100	100	100	95.83	94.44
3.5M TA	0.35	2.7	0.35	2.7	0.35	2.7	0.32	0.30	0.29	0.29	0.28	0.27	0.27	0.24
TRA	100	100	100	100	100	100	91.6	92.59	83.33	88.88	80.36	81.48	77.27	74.07
4M TA	0.34	0.31	0.33	0.30	0.32	0.28	0.30	0.27	0.28	0.25	0.26	0.24	0.23	0.20
TRA	97.91	95.55	96.25	91.48	91.66	85.55	85.41	81.48	81.25	74.81	72.91	71.48	67.5	62.59
4.5M TA	0.31	0.30	0.29	0.28	0.27	0.26	0.24	0.23	0.21	0.20	0.18	0.16	0.15	0.14
TRA	89.58	91.48	84.16	85.55	77.03	80.0	67.5	70.0	60.0	61.11	52.51	48.88	44.16	42.96
5M TA	0.25	0.25	0.23	0.25	0.19	0.22	0.15	0.17	0.12	0.15	0.10	0.12	0.09	0.08
TRA	71.24	78.14	65.0	74.81	52.91	67.03	42.91	52.59	34.16	45.92	30.83	38.88	24.58	23.70
5.5M TA	0.02	0.01	–	–	–	–	–	–	–	–	–	–	–	–
TRA	5.0	4.07	–	–	–	–	–	–	–	–	–	–	–	–

CT: crude tannase; PPT: partial purified tannase; TA: tannase activity (U/ml); TRA: 100 % tannase residual activity (at 0 min) corresponds to 0.35 U^{-1} for CT and 0.33 U ml^{-1} for PPT

Table 5. Shelf-life of crude and partial purified tannase at 4ºC and 37ºC.

Number of days	% Residual activity at 4ºC		% Residual activity at 37ºC	
	Crude enzyme	Partially purified enzyme	Crude enzyme	Partially purified enzyme
0	100	100	100	100
7	99	99	99	99
15	96	94	95	93
30	88	94	93	94
45	83	90	91	92
60	80	86	87	86
90	76	82	81	79
120	71	79	75	76
150	67	75	70	74
180	62	72	67	69
210	58	66	62	66
240	55	61	59	64

Conclusion

Bacillus subtilis that can produce tannase is reported for the first time whose tannase producing ability is higher than the previously reported tannase producing bacterial species. The optimum temperature for the production of tannase by newly isolated *Bacillus subtilis* has also an edge on other tannase producing bacteria at least in the regions with higher average temperatures. The enzyme is novel as it possesses properties such as stability at extreme conditions of pH and temperature and good shelf-life permits its biotechnological potential to be exploited in food, feed, beverage and brewing, pharmaceutical and chemical industries. Due to its tolerance against high concentrations of salts, tannase could also be efficiently used in the treatment of effluents containing tannins deposits in pollution control systems.

References

Aguilar, C.N., R. Rodriguez, G. Gutierrez-Sanchez, C. Augur, E. Favela-Torres, L. A. Prado-Barragan, A. Ramirez-Coronel and J. C. Contreras-Esquivel. 2007. Microbial tannases: advances and perspectives. *Appl. Microbiol. Biotechnol*, 76: 47-59.

Banerjee, D., K.C. Mondal and B.R. Pati. 2001. Production and characterization of extracellular and intracellular tannase from newly isolated *Aspergillus aculeatus* DBF 9. *J. Basic Microbiol*., 41: 313-8.

Barthomeuf, C., F. Rogerat and H. Pourrat. 1994. Production, purification and characterization of a tannase from *Aspergillus niger* LCF 8. *J. Ferment. Bioeng*., 77: 320-323.

Battestin, V. and G. A. Macedo. 2007. Tannase production by *Paecilomyces variotii*. *Bioresour. Technol*., 98: 1832-7.

Belmares, R., J. C. Contreras-Esquivel, R. Rodríguez-Herrera, A. Ramírez, R. Rodriguez-Herrera, C. R. Ascension and N. A. Cristobal. 2004. Microbial production of tannase: an enzyme with potential use in food industry. *Lebensm Wiss Technol*., 37: 857-864.

Bradoo, S., R. Gupta and R.K. Saxena. 1997. Parametric optimization and biochemical regulation of extracellular tannase from *Aspergillus japonicus. Proc. Biochem*., 32: 135-139.

Buchanan, R.E. and N.E. Gibbons. 1974. Bergey's Manual of Determinative Bacteriology, 8th Ed., Williams and Wilkins Co., Baltimore, Md.

Deschamps, A.M., G. Otuk and J.M. Lebeault. 1983. Production of tannase and degradation of chestnut tannin by bacteria. *J. Ferment. Technol*., 61: 55-59.

Djekrif-Dakhmouche, S., Z. Gheribi-Aoulmi, Z. Meraihi and L. Bennamoun. 2006. Application of a statistical design to the optimization of culture medium for a-amylase production by *Aspergillus niger* ATCC 16,404 grown on orange waste powder. *J. Food Eng*., 73: 190-197.

Farias, G.M., J. R. Gorbea, J.R. Elkins and G.J. Griffin. 1994. Purification, characterization and substrate relationships of the tannase from *Cryphonectria parasitica. Physiol. Mol. Plant Pathol*., 44: 51-63.

Hadi, T.A., R. Banerjee and B.C. Bhattacharya. 1994. Optimization tannase biosynthesis by a newly isolated *Rhizopus oryzae*. *Bioprocess Eng*., 11: 239-243.

Kar, B. and R. Banerjee. 2000. Biosynthesis of tannin acyl hydrolase from tannin- rich forest residue under different fermentation conditions. *J. Ind. Microbiol. Biotechnol*., 25: 29-38.

Kumar, R., J. Sharma and R. Singh. 2006. Production of tannase from Aspergillus ruber under solid-state fermentation using jamun (*Syzygium cumini*) leaves. *Microbiol. Res*.,

Kumar, R.A., P. Gunasekaran and M. Lakshmanan. 1999. Biodegradation of tannic acid by *Citrobacter freundii* isolated from a tannery effluent. *J. Basic Microbiol*., 39: 161-8.

Lekha, P. and B. Lonsane. 1994. Comparative titres, location and properties of tannin acyl hydrolase produced by *Aspergillus niger* PKL 104 in solid state, liquid surface and submerged fermentations. *Proc. Biochem.* 29: 479-503.

Lekha, P.K. and B.K. Lonsane. 1997. Production and application of tannin acyl hydrolase: state of the art. *Adv. Appl. Microbiol*., 44: 215-60.

Lowery, O.H., N.J. Rosebrough, A.L. Farr and R.J. Randall. 1951. Proteins measurement with Folin phenol reagent. *J. Biol. Chem*., 193: 265-275.

Maity, C., P. K. Das Mohapatra, B.R. Pati and K.C. Mondal. 2009. A simple gel detection method of microbial tannin acyl hydrolase (EC 3.1.1.20). *World J. Microbiol. Biotechnol*., 25: 733-735.

Mohapatra, P. K., K.C. Mondal and B.R. Pati. 2007. Production of tannase by the immobilized cells of *Bacillus licheniformis* KBR6 in Ca-alginate beads. *J. Appl. Microbiol*., 102: 1462-7.

Mondal, K.C. and B.R. Pati. 2000. Studies on the extracellular tannase from newly isolated *Bacillus licheniformis* KBR 6. *J. Basic Microbiol*., 40: 223-32.

Mondal, K.C., D. Banerjee, R. Banerjee and B.R. Pati. 2001. Production and characterization of tannase from *Bacillus cereus* KBR9. *J. Gen. Appl. Microbiol*., 47: 263-267.

Patel, R., M. Dodia and S.P. Singh. 2005. Extracellular alkaline protease from a newly isolated haloalkaliphilic *Bacillus* sp.: production and optimization. *Process Biochem*., 40: 3569-3575.

Pinto, G., L. Bruno, M. Hamacher, S. Tarzi and S. Couri. 2003. Increase of tannase production in solid state fermentation by *Aspergillus niger* 3T5B8. 25th Symposium on biotechnology for fuels and chemicals. *Poster presentation, Breckenridge, CO, USA*: 3-68.

Pinto, G., S. Couri and E. Goncalves. 2006. Replacement of methanol by ethanol on gallic acid determination by rhodanine and its impacts on tassae assay. *EJEAFCHe* 5: 5.

Purohit, J.S., J.R. Dutta, R.K. Nanda and R. Banerjee. 2006. Strain improvement for tannase production from coculture of *Aspergillus foetidus* and *Rhizopus oryzae*. *Bioresour.*

Ramirez-Coronel, M.A., G. Viniegra-Gonzalez, A. Darvill and C. Augur. 2003. A novel tannase from *Aspergillus niger* with beta-glucosidase activity. *Microbiology*, 149: 2941-6.

Sabu, A., A. Pandey, M. Jaafar Daud and G. Szakacs. 2005. Tamarind seed powder and palm kernel cake: two novel agro residues for the production of tannase under solid state fermentation by *Aspergillus niger* ATCC 16620. *Bioresour. Technol*., 96: 1223-1228.

Sabu, A., S. Kiran and A. Pandey. 2005. Purification and characterization of tannin acyl hydrolase from *Aspergillus niger* ATCC 16620. *Food Technol. Biotechnol*., 43: 133-138.

Scalbert, A. 1991. Endospore-forming Gram-positive rods and cocci. *In* Antimicrobial properties of tannins. *Phytochemistry*, 30: 3875-3883.

Sharma, S., T.K. Bhat and R.K. Dawra. 1999. Isolation, purification and properties of tannase from A. niger van Tieghem. *World J. Microbiol. Biotechnol*., 15: 673-677.

Tieghem, P. 1867. Sur la fermentation gallique. *CR Acad Sci (Paris)* 65: 1091-1094. *Technol*., 97: 795-801.

Trevino-Cueto, B., M. Luis, J.C. Contreras-Esquivel, R. Rodriguez, A. Aguilera and C.N. Aguilar. 2007. Gallic acid and tannase accumulation during fungal solid state culture of a tannin-rich desert plant (*Larrea tridentata* Cov.). *Bioresour Technol*., 98: 721-4.

Vermeire, A. and E. Vandamme. 1988. Fungal production of tannin acyl hydrolase. *Med. Fac. Landbouw. Rijksuniv. Gent.* 53: 2047-2056.

Wattiau, P., M.E. Renard, P. Ledent, V. Debois,G. Blackman and S.N. Agathos. 2001. A PCR test to identify *Bacillus subtilis* and closely related species and its application to the monitoring of wastewater biotreatment. *Appl. Microbiol. Biotechnol.* 56: 816-9.

Yu, X., Y. Li, C. Wang and D. Wu. 2004. Immobilization of Aspergillus niger tannase by microencapsulation and its kinetic characteristics. *Biotechnol. Appl. Biochem*., 40: 151-5.

(Received for publication 25 March 2015)