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ORIGINAL ARTICLE

# High-level tannase production by *Penicillium atramentosum* KM using agro residues under submerged fermentation

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**Abstract** A tannase-yielding fungal strain identified as *Penicillium atramentosum* KM was isolated from tannery effluent collected from a small-scale tannery. The fungal strain produced extracellular tannase under submerged fermentation (SmF) using amla (*Phyllanthus emblica*), ber (*Zyzyphus mauritiana*), jamoa (*Eugenia cuspidate*), jamun (*Syzygium cumini*) and keekar (*Acacia nilotica*) powdered leaves. Among the different substrates, amla and keekar leaves resulted in maximal extracellular production of tannase. Various process parameters were studied to optimize the extracellular yield of tannase under SmF. Maximum yield of tannase i.e., 32.8 and 34.7 U/ml was obtained with amla leaves (2% w/v) and keekar leaves (3% w/v), respectively, in selective mineral salt and production media, inoculated with 3 x $10^6$ spores/ml cell suspension by incubating at 30°C for 72 h. The production of enzyme was induced strongly by the presence of maltose (0.2% w/v) as carbon source. Crude enzyme was optimally active at pH 5.5 and pH 7.5 using amla and keekar leaves, respectively. The fungal strain produced yields of tannase about twice as high as the highest reported yield of tannase. Our findings suggest that agro residues in the form of amla and keekar leaves can be one of the best and most cost effective alternatives to the costly pure tannic acid for industrial production of microbial tannase. The novelty in the present investigation is that is that *P. atramentosum* KM is the first fungal isolate found to produce tannase at high pH (i.e. 7.5) using keekar leaves.

**Keywords** Tannin · Tannin acyl hydrolase · *Penicillium atramentosum* · Keekar leaves · Amla leaves · Submerged fermentation

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## Introduction

Tannin acyl hydrolase (EC 3.1.1.20), commonly called tannase, catalyzes the hydrolysis of ester bonds in hydrolysable tannins such as tannic acid, thereby releasing glucose and gallic acid. Tannins are widespread in the plant kingdom, are found in leaves, fruits, bark and wood, and are often considered nutritionally undesirable because they form complexes with protein, starch and digestive enzymes and cause a reduction in the nutritional value of food (Aguilar et al. 2007). Tannase finds widespread application in the food, feed, and pharmaceutical industries, as well as in leather and beverage processing. At the moment, most commercial applications of tannase are in the manufacturing of instant tea, where it is used to eliminate the water-insoluble precipitates, and in wine, beer and coffee-flavored soft drinks. Another important application of tannase in the food industry is its use as a substrate for the chemical synthesis of pyrogallol or ester gallates, which are used as preservatives (Aguilar et al. 2007). Gallic acid is also used in the enzymatic synthesis of propyl gallate, which is used mainly as an antioxidant in fats and oils, as well as in beverages (Banerjee et al. 2005).

Although tannase is present in plants, animals and microorganisms, it is produced mainly by microorganisms. Filamentous fungi of the *Aspergillus* genus have been used

widely for tannase production (Aguilar et al. 2007). The production of tannase by *Aspergillus* sp. can occur in the absence of tannic acid, but these fungi tolerate tannic acid concentrations as high as 20% without exhibiting any deleterious effect on either growth or enzyme production. The use of the submerged fermentation (SmF) is advantageous because of the ease of sterilization, and process control is easier to engineer in such systems. Depending on the strain and the culture conditions, the enzyme can be constitutive or inducible, showing different production patterns. Although *Penicillium* sp. grows well in tan liquors and is known to produce tannase, little information is available on the isolation and production of tannase obtained from this source. There are few reports on tannase production by *Penicillium* sp. under SmF (Batra and Saxena 2005; Rajkumar and Nandy 1983; Van de Lagemaat and Pyle, 2005). Rajkumar and Nandy (1983) reported tannase production using Czapeck's Dox medium supplemented with costly pure 2% tannic acid as carbon and tannin source, whereas Batra and Saxena (2005) reported the use of 1% pure tannic acid in the same medium under SmF. Van de Lagemaat and Pyle (2005) reported the development of a mathematical growth model for tannase production by *Penicillium glabrum* under solid-state fermentation using polyurethane foam cubes, which served only as an inert support for the organism, impregnated with a liquid medium containing tannic acid as the main carbon source. In the present investigation, we report tannase production by *Penicillium atramentosum* KM under SmF using cheap and locally available agro residues like jamun and keekar leaves as an alternative to tannic acid, which is a costly substrate.

## Materials and methods

### Raw materials

Amla (*Phyllanthus emblica*), ber (*Zyzyphus mauritiana*), jamun (*Syzygium cumini*), jamoa (*Eugenia cuspidate*) and keekar (*Acacia nilotica*) leaves were collected from local orchards. The leaves were first dried at 60°C in an oven and then finely ground to powdered form in a grinder mixer. The powder was stored in a dry place at room temperature and used as source of tannins in SmF. The tannin content was estimated by using the protein precipitation method (Haggerman and Butler 1978). Dried leaves were ground to a fine powder in 70% methanol and kept overnight at 4°C. One milliliter of the extract was placed in a test tube, and 3 ml bovine serum albumin (BSA) solution (1 mg/ml BSA with 0.17 M NaCl in 0.2 M sodium acetate buffer of pH 5.0) was added. The reaction mixture was kept for 15 min at room temperature. Then, the mixture was centrifuged (5,000 *g*, 10 min), and the precipitate was dissolved in 3 ml SDS-triethanolamine solution. Absorbance was measured at 530 nm after addition of 1 ml $FeCl_3$ reagent.

### Chemicals

All media ingredients and reagent chemicals were of analytical grade and were procured from E-Merck, Hi-Media, SRL, Genex health bio, Qualigens Chemicals (India), and Sigma Aldrich (St. Louis, MO).

### Microorganism and inoculum preparation

The fungal strain used in the present investigation was isolated from tannery effluent using routine mycological procedures and screened for tannase enzyme production using tannase screening medium comprising 0.5% tannic acid as the substrate through enrichment technique. The isolated fungal strain was identified as *Penicillium atramentosum* KM. The fungus was identified by A. Aggarwal, Mycologist, Department of Botany, Kurukshetra University, on the basis of morphological characteristics. The identity of the isolate was confirmed by genetic characterization using ITS4 and ITS5 primers that specifically identify *Penicillium* by amplifying 600-bp fragment at the genus level, and the species-level specificity of the fungal strain was tested using primer sets ITS4 and ITS5 and PgrisF1-1, PatraR1 (Sugita et al. 2004, Oliveri et al. 2007). A product of approximately 625 bp was amplified by PCR from the tested fungal strain. The fungal culture was maintained on Czapeck's Dox agar slants at 4°C. For preparation of inoculum, 10 ml sterilized distilled water supplemented with 0.1% Tween-80 was added to 1-week-old fully sporulated agar slant culture.

### Production of extracellular tannase by *P. atramentosum* KM in submerged fermentation

A 100 ml Erlenmeyer flask containing 25 ml production medium (MS I; pH 6.0) containing (g/l) $(NH_4)_2HPO_4$ (3.0); magnesium sulphate (1.0); $K_2HPO_4$ (0.5); calcium chloride (0.3) was used for production of tannase. After optimization of all process parameters we found that a 100-ml Erlenmeyer flask containing 25 ml selective mineral salt medium (g/l) supplemented with $KH_2PO_4$ (0.5); $(NH_4)_2HPO_4$ (3.0); $ZnSO_4$ / copper sulfate (2.0); maltose (0.2% w/v) was used with keekar leaves (3% w/v), which served as a sole tannin source for the fermentation process. On the other hand, selective production medium containing (g/l) $KH_2PO_4$ (0.5); $NH_4Cl$ (2.0); magnesium sulfate (2.0) was used with amla leaves (2% w/v) that served as sole carbon and tannin source. The culture medium was

maintained at pH 5.5 for amla and pH 7.5 for keekar leaves, and was autoclaved at 120°C for 20 min. After cooling, the flasks were inoculated with 0.5 ml *P. atramentosum* KM (3 x $10^6$ spores/ml) and incubated in an orbital shaker (150 rpm) at 30°C for 72 h. The biomass was separated by filtration through Whatman No. 1 filter paper. The cell-free culture broth was assayed for extracellular tannase activity. All experiments were carried out in triplicate and mean values with standard deviation are reported.

### Tannase assay

The qualitative assay of tannase enzyme activity was carried out by culturing the microorganism on Czapeck's Dox agar plates containing tannic acid (0.3% w/v). A clear hydrolyzing zone around the colonies indicated tannase activity.

Tannase activity was estimated by the colorimetric method of Mondal et al. (2001). This method is based on changes in optical density of the substrate tannic acid after enzymatic reaction at 530 nm. The reaction mixture contained 0.3 ml substrate tannic acid (0.5% w/v in 0.2 M sodium acetate buffer, pH 5.5) and 0.1 ml enzyme. This reaction mixture was incubated at 30°C for 1 h. The enzymatic reaction was terminated by addition of 3 ml BSA solution (1 mg/ml), which also precipitated the residual tannic acid. A control was prepared in parallel using heat-denatured enzyme. The tubes were then centrifuged (5,000 *g*, 10 min) and the precipitate were dissolved in 3 ml SDS-triethanolamine (1% w/v SDS in 5% v/v triethanolamine) solution. One milliliter of $FeCl_3$ reagent (0.01 M $FeCl_3$ in 0.01 *N* HCl) was added to the tube, which was then kept for 15 min at room temperature for stabilization of the color. Absorbance was read at 530 nm against the blank (i.e., without tannic acid).

The specific extinction co-efficient of tannic acid at 530 nm was found to be 0.577 (Mondal et al. 2001). Using this co-efficient, one unit of tannase activity is defined as the amount of enzyme required to hydrolyze 1 mM tannic acid in 1 min under assay conditions.

## Results and discussion

### Effect of substrate concentration

Tannase is an inducible enzyme that is produced only in the presence of tannins. The concentration of tannic acid is a crucial factor for growth and tannase induction. Our findings demonstrate that amla and keekar leaves as substrates were the best sole carbon and tannin sources in the case of *P. atramentosum* KM, yielding 15.9 U/ml and 18.5 U/ml tannase, respectively (Table 1). The organism produced maximum tannase in production medium (MS I; pH 6.0). These results may be due to differences in the nitrogen and metal ion composition of the two media used here. The constituents of MS I favored tannase synthesis by both organisms. The same medium composition was also reported for maximum tannase production by *Aureobasidium pullulans* DBS66 (Banerjee and Pati 2007) and *A. aculeatus* DBF9 (Banerjee et al. 2007a). However, negligible growth and enzyme production was observed even after 7 days of incubation when ber, jamun and jamoa leaves were used as sole carbon and tannin source. Hence, amla and keekar were chosen for further experiments. The effect of different concentrations of the selected substrates (1–5%) on enzyme production was also studied. The results revealed that maximum tannase activity was obtained by using 2% (w/v) amla leaves and 3% (w/v) keekar leaves, yielding about 24.4 and 26.5 U/ml tannase, respectively (Fig. 1). The high production of tannase in the case of keekar and amla leaves may be due to some inducing factors, like the presence of sufficient tannins, and carbon sources that accelerate synthesis of the enzyme. The results obtained are in agreement with those of other workers who also reported maximum tannase production with 2% (w/v)

**Table 1** Efficiency of agro-residues as substrates for tannase production

| Agro residue (0.2% w/v) | Tannase activity (U/ml) | |
|---|---|---|
| | MS I | MS II |
| *Eugenia cuspidate* | 0.526±0.43 | 0.58±0.435 |
| *Syzygium cumini* | 1.376±0.34 | 1.204±0.564 |
| *Acacia nilotica* | 18.553±0.342 | 2.511±0.543 |
| *Zyzyphus mauritiana* | 0.224±0.401 | 0.205±0.351 |
| *Phyllanthus emblica* | 15.972±0.543 | 1.85±0.761 |

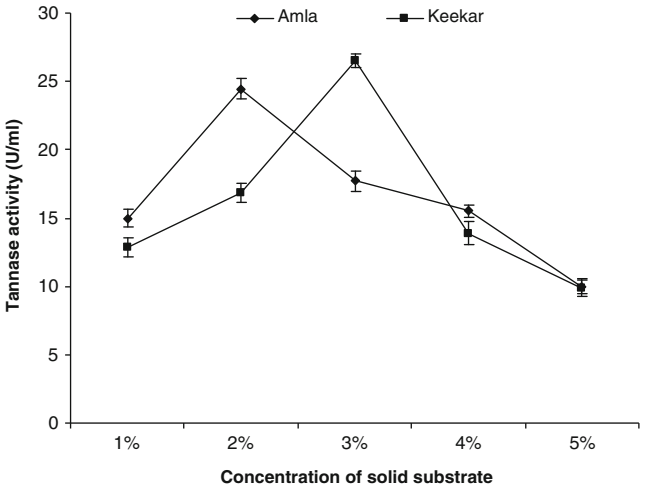


**Fig. 1** Effect of concentration of selected solid substrate on tannase production by *Penicillium atramentosum* KM

**Table 2** Effect of incubation time, temperature and pH on tannase activity

| | Tannase activity (U/ml) | |
|---|---|---|
| | Amla leaves | Keekar leaves |
| Time (h) | | |
| 24 | 9.551±0.561 | 9.223±0.476 |
| 48 | 15.015±0.578 | 17.462±0.723 |
| 72 | 24.024±0.342 | 27.54±0.551 |
| 96 | 16.65±0.492 | 13.42±0.615 |
| 120 | 8.71±0.782 | 6.85±0.429 |
| Incubation temperature (°C) | | |
| 20 | 10.62±0.546 | 12.52±0.563 |
| 25 | 13.23±0.436 | 18.523±0.435 |
| 30 | 28.42±0.567 | 30.142±0.437 |
| 35 | 19.76±0.546 | 21.274±0.589 |
| 40 | 6.7±0.716 | 5.67±0.527 |
| 45 | 2.455±0.654 | 2.65±0.824 |
| pH | | |
| 5 | 22.7±0.662 | 16.4±0.456 |
| 5.5 | 28.56±0.456 | 20.15±0.527 |
| 6 | 20.43±0.428 | 21.58±0.432 |
| 6.5 | 15.77±0.675 | 24.3±0.783 |
| 7 | 10.15±0.768 | 25.65±0.832 |
| 7.5 | 7.431±0.412 | 30.75±0.671 |
| 8 | 4.34±0.582 | 21.27±0.835 |

*Caesalpinia digyna* powder (Kar and Banerjee 2000), amla and keekar leaves (Selwal et al. 2010) as sole source of carbon and tannin. Banerjee et al. (2007b) also reported maximum tannase production with 3% (w/v) raw tannins obtained from *Cassia siamea* plants. Srivastava and Kar (2009) reported optimum tannase production with 4% (w/v) pomegranate rind powder under SmF conditions. The actual mode of tannase induction at a particular concentration of tannin has not yet been explained clearly.

## Effect of incubation period

The incubation period for harvesting tannase from production medium was optimized. In our study, the time course for enzyme production was monitored up to 120 h. Enzyme production on amla and keekar was started from early growth and reached the highest levels (24.0 and 27.5 U/ml, respectively) after 72 h, thereafter starting to decrease (Table 2). The incubation time generally depends on the micro-organism and medium used. Some authors also reported an incubation period of 72 h for maximum tannase production by different fungi such as *Rhizopus oryzae*, *Aspergillus foetidus* (Kar and Banerjee 2000; Mukherjee and Banerjee 2006), *Aspergillus aculaetus* DBF9 (Banerjee et al. 2007a) and *Aspergillus niger* ITCC 6514.07 (Srivastava and Kar 2009). Other workers also reported an incubation period of 96 h to be the best for higher tannase production using palm kernel cake (Sabu et al. 2005). The variations in optimal incubation period might be due to differences in fungal strain and medium composition used for tannase production.

## Effect of incubation temperature

Incubation temperature affects various metabolic processes such as protein denaturation, enzymatic inhibition, promotion or inhibition on production of a particular metabolite, cell death, etc. Thus, the effect of temperature ranging from 20 to 45°C on tannase production was studied. In the

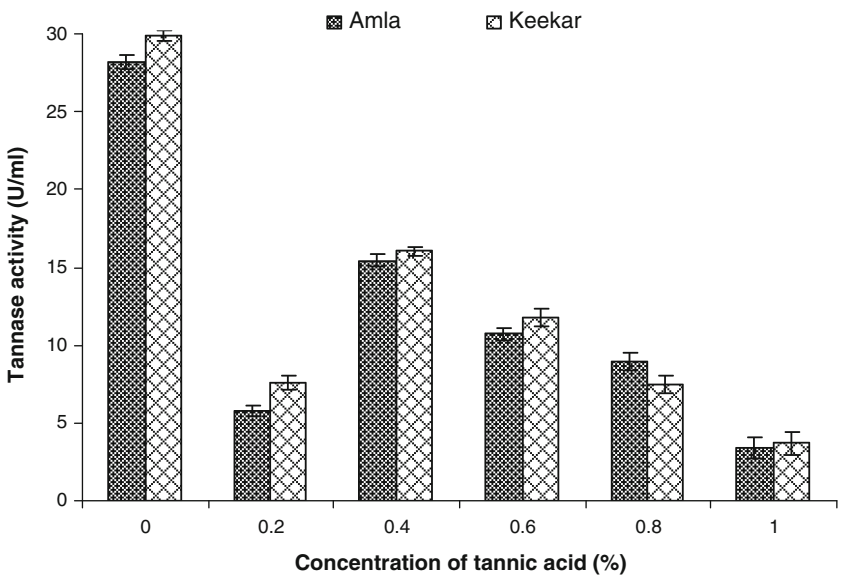


**Fig. 2** Effect of various concentrations of tannic acid on tannase production by *P. atramentosum* KM

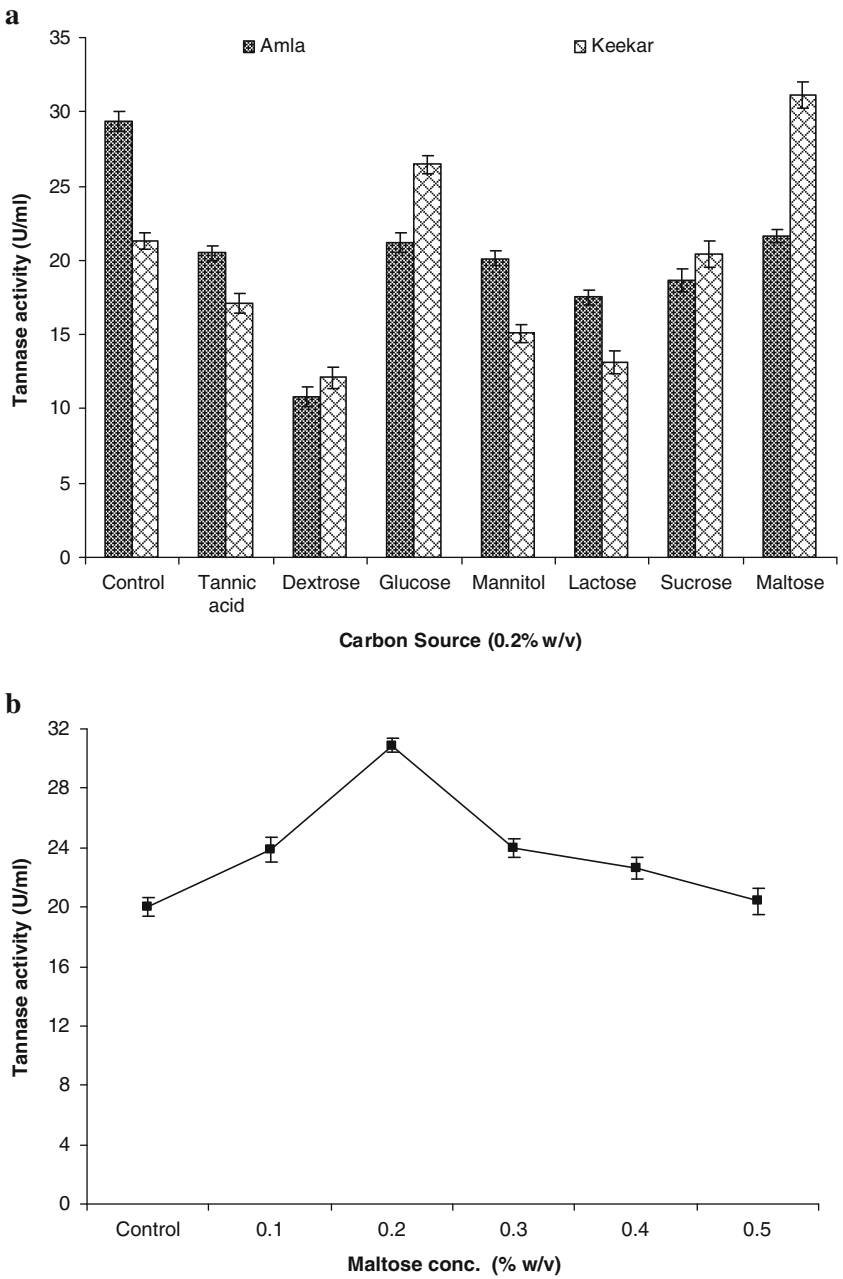


**Fig. 3** **a** Effect of various carbon sources on tannase production. **b** Effect of maltose concentration on tannase production

present investigation, maximal tannase production was observed on both substrates at 30°C, i.e., 28.4 U/ml in amla and 30.1 U/ml in keekar leaves (Table 2). Further, an increase in temperature beyond the optimum might have caused denaturation of enzyme and made it inactive. Different organisms require different temperatures for enzyme synthesis and utilization of substrate. As in our results, many other workers also reported an optimum temperature of 30°C for maximal tannase production from various fungi such as *R. oryzae* and *A. foetidus* (Batra and Saxena 2005; Mukherjee and Banerjee 2006), *A. niger* (Cruz-Hernández et al. 2006; Sabu et al. 2006; Treviño-Cueto et al. 2007; Cruz-Aldaco et al. 2009), *A. aculeatus* DBF9 (Banerjee et al. 2007a), *Aureobasidium pullulans* DBS66 (Banerjee and Pati 2007), *A. tamarii* (Costa et al. 2008) and *Paecilomyces variotii* (Battestin and Macedo 2007). Some workers obtained maximum tannase activity at 25–28°C from *Aspergillus fumigatus* MA (Manjit et al. 2008), and *A. niger* (Huang et al. 2005) while Kasieczka-Burnecka et al. (2007) reported an optimum temperature of 16°C for maximum tannase production by *Verticillium* sp. P9.

## Effect of pH

The pH of the production medium plays a significant role in the production of metabolites. In the present study, various

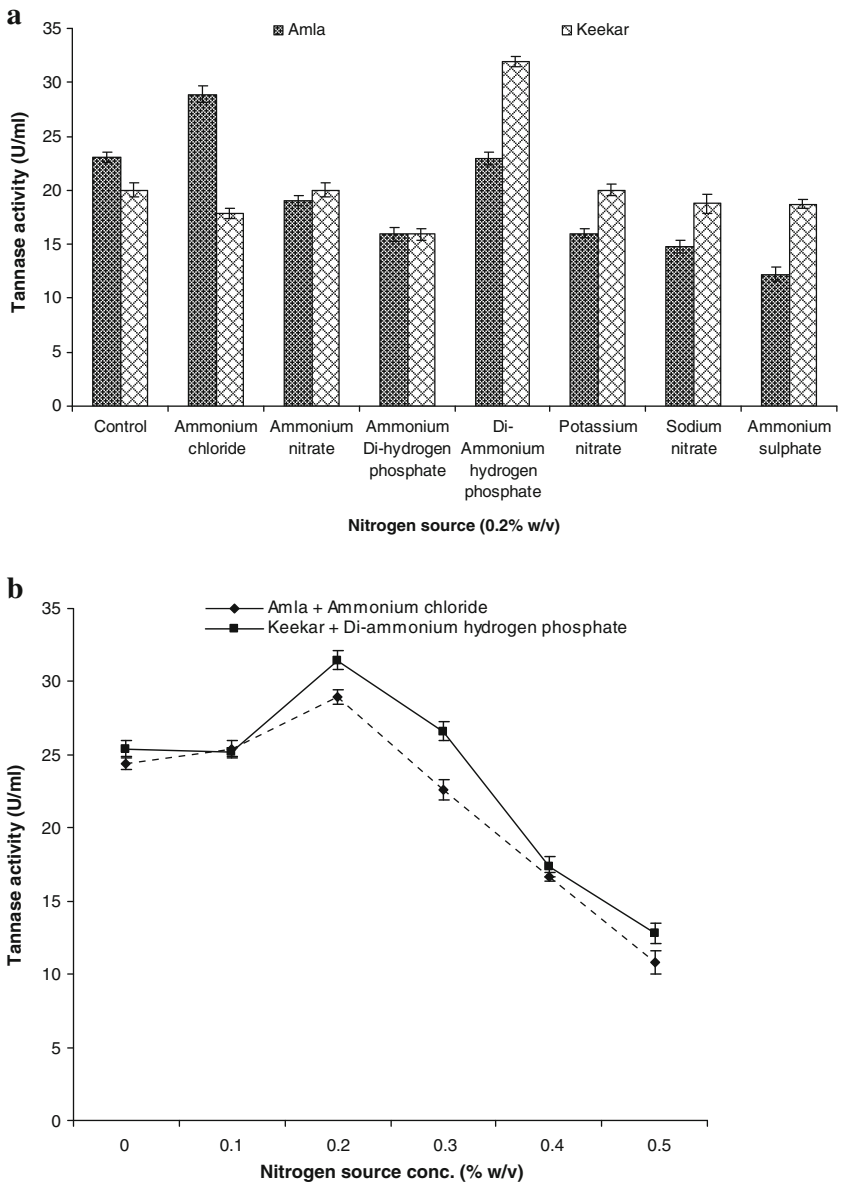


**Fig. 4** **a** Effect of various nitrogen sources on tannase production. **b** Effect of selected nitrogen source concentration on tannase production

ranges of pH (5–8) were tested in order to enhance tannase yield. The results revealed that *P. atramentosum* KM gave maximum tannase production of 28.6 U/ml in amla leaves at pH 5.5 and 30.7 U/ml in keekar leaves at pH 7.5 (Table 2). It may be concluded from the results that tannase from the isolate needs an acidic-to-neutral environment to be active. Similar to our observations, maximum tannase production was observed at pH 7.0 by *A. niger* in a 20-l fermenter (Pourrat et al. 1982). Recently, we reported maximum tannase production by *Pseudomonas aeruginosa* IIIB 8914 using amla leaves at pH 7.0 and keekar leaves at pH 5.5 (Selwal et al. 2010). Tannase production from *A. niger* was higher at pH 5.5 (Cruz-Aldaco et al. 2009; Treviño-Cueto et al. 2007).

## Effect of tannic acid supplementation

The effect of tannic acid on tannase yield was studied by adding different concentrations of this substrate (0.2–1% w/v) to the production medium. No inductive effect of different concentrations of tannic acid was observed in either substrate (Fig. 2). One of the most striking observations in this study is that the highest enzyme production by *P. atramentosum* KM was obtained in medium containing amla (28.2 U/ml) or keekar leaves (29.9 U/ml) as tannin rich substrate as compared to medium supplemented with pure, i.e., 0.4%, tannic acid (15.4 U/ml and 16.0 U/ml). This observation reveals amla and keekar leaves to be superior, as well as cheap, substrates as an alternative to the costly pure tannic

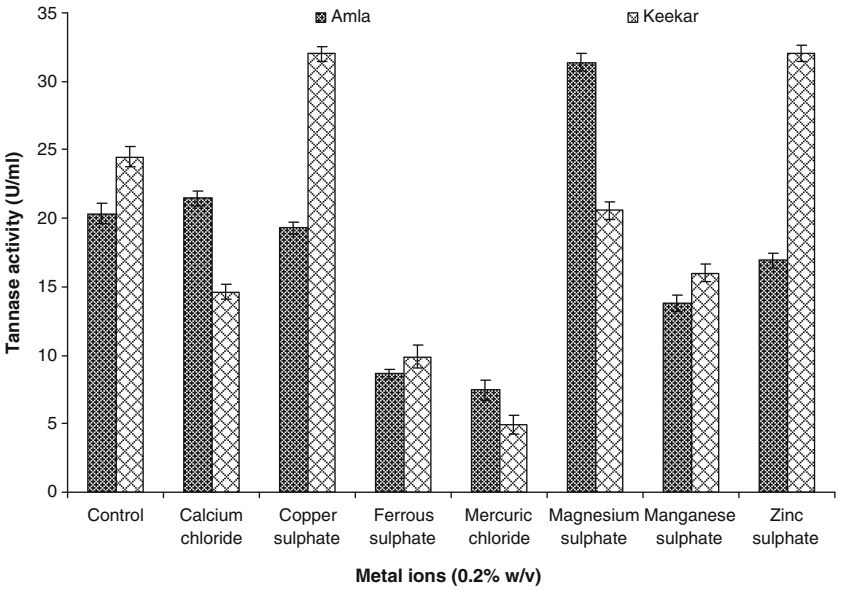


**Fig. 5** Effect of various metal ions on tannase production by *P. atramentosum* KM

acid for industrial production of microbial tannase. This may be due to the fact that higher concentrations of tannin lead to the formation of non-reversible bonds with surface proteins and impair the metabolism as well as the growth of the organism (Selwal et al. 2010). Similarly, some workers also observed an inhibitory effect of tannic acid on tannase synthesis by *Lactobacillus* sp ASR-S1 (Sabu et al. 2006) and *P. aeruginosa* IIIB 8914 (Selwal et al. 2010) using agro residues like wheat bran, palm kernel cake, amla and keekar leaves. A concentration of 1% tannic acid was found to be optimal for production of maximum tannase in *A. pullulans* (Banerjee and Pati 2007), while 2% (w/v) tannic acid was reported to yield maximal enzyme production in *R. oryzae* and *A. japonicus* (Bradoo et al. 1997). Some workers reported maximum tannase production with 1% tannic acid from the genera *Aspergillus* and *Penicillium* (Banerjee et al. 2007a, 2007b; Batra and Saxena 2005; Costa et al. 2008; Sabu et al. 2005).

## Effect of carbon source supplementation

Carbon is an energy source that is essential for the growth of microorganisms. The effect of supplementation of different carbon sources (0.2% w/v) viz. tannic acid, dextrose, glucose, glycerol, maltose, mannitol, lactose and sucrose, on the production of tannase was evaluated. The results showed the stimulatory effect of glucose and maltose on tannase production as carbon source in keekar leaves whereas all the carbon sources inhibited tannase production with amla leaves. The strain *P. atramentosum*

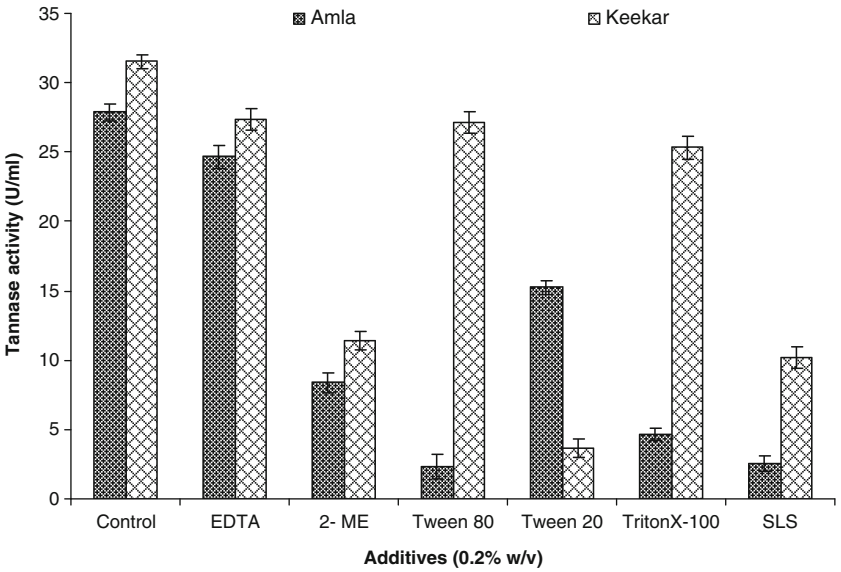


**Fig. 6** Effect of various additives on tannase production by *P. atramentosum* KM

**Table 3** Comparison of tannase production under unoptimized and optimized conditions

| Organism | Tannase activity of unoptimized culture filtrate (U/ml) | Tannase activity of optimized culture filtrate (U/ml) | Fold increase |
|---|---|---|---|
| Amla leaves | 15.972 | 32.78 | 2.05 |
| Keekar leaves | 18.553 | 34.75 | 1.88 |

KM produced maximum tannase of 29.4 U/ml in the case of amla leaves, while 31.1 U/ml tannase was produced in the case of keekar leaves supplemented with 0.2% (w/v) maltose as a carbon source (Fig. 3a). This may be due to the fact that amla leaves as a substrate are already rich enough to supply the carbon required for fungal growth and tannase production. Furthermore, when the effect of various concentrations of maltose in keekar leaves was evaluated, an increase in activity was obtained with 0.2% (w/v) of maltose, i.e., 30.9 U/ml (Fig. 3b). This might be due to the fact that the higher concentration of carbon source interrupted sporulation of the fungal isolate by affecting metabolic activities. Similar to our results, Sabu et al. (2006) reported that tannase yield from *Lactobacillus* sp. was slightly improved by the addition of 1% (w/v) maltose as carbon source in the case of tamarind seed powder. Bradoo et al. (1997) observed that 0.2% (w/v) glucose favored both growth and tannase production, whereas a higher concentration of glucose created an osmotic stress that depressed enzyme synthesis in *A. japonicus*. A stimulatory effect of a low concentration of glucose on tannase production from *P. glabrum* (Van de Lagemaat and Pyle 2005) and *A. pullulans* DBS66 (Banerjee and Pati, 2007) was also reported. Tannase production was also enhanced by the addition of glucose, sucrose, maltose, starch and glycerol (1% w/v each) by *A. niger* ATCC 16620 using tamarind seed powder (Sabu et al. 2005)

## Effect of nitrogen sources

A suitable nitrogen source is an essential component for growth and enzyme production by microorganisms. Nitrogen sources like ammonium tartrate, oxalate, sulfate, nitrate, chloride, sodium nitrate, urea, peptones, and amino acids stimulate conidia formation. The specificity for tannase production of different nitrogen sources (ammonium chloride, ammonium nitrate, ammonium di-hydrogen orthophosphate, di-hydrogen ammonium phosphate, ammonium sulphate, sodium nitrate and potassium nitrate) at 0.2% (w/v) was evaluated. The results showed maximum tannase production by *P. atramentosum* KM was obtained with ammonium chloride, i.e., 28.9 U/ml using amla and di-ammonium hydrogen phosphate gave 31.9 U/ml using keekar leaves (Fig. 4a). When the effect of concentration of the two nitrogen sources was evaluated, the results showed that 0.2% (w/v) of both nitrogen sources was effective for maximal tannase production with both substrates, i.e., 28.9 U/ml in amla leaves and 31.4 U/ml in keekar leaves (Fig. 4b). In both cases, higher concentration of nitrogen sources might have inhibited the formation of fungal conidia and mycelia growth. Similar to our results, Banerjee and Pati (2005) also observed the highest growth and tannase production by *A. pullulans* in the presence 0.3% (w/v) di-ammonium hydrogen phosphate [$(NH_4)_2HPO_4$]. Other workers reported maximum production of tannase in the presence ammonium chloride (Kar et al. 2003), 0.2% (w/v) ammonium sulphate (Manjit et al. 2008; Podrigues et al. 2007), and ammonium nitrate (Selwal et al. 2010). Sabu et al. (2005) reported that 1% (w/v) potassium nitrate was the most effective nitrogen source for maximum production of tannase by *A. niger* ATCC 16620 using tamarind seed powder.

**Table 4** Effect of laboratory scale transfer on tannase production through submerged fermentation (SmF)

| Erlenmeyer's flask (ml) | Production medium (ml) | Tannase activity (U/ml) | |
|---|---|---|---|
| | | Amla leaves | Keekar leaves |
| 250 | 50 | 33.8±0.58 | 35.7±0.67 |
| 500 | 100 | 23.78±0.65 | 23.78±0.45 |
| 1,000 | 200 | 12.78±0.88 | 13.78±0.48 |

## Effect of metal ions

Tannase requires the presence of metal ions to express its full catalytic activity. Hence, it is important to know the type of ions and the concentration required to achieve maximal reaction efficiency (Aguilar et al. 2007). Therefore, the effect on tannase production of different di-valent cations (0.2% w/v) such as chlorides of $Ca^{+2}$, $Cu^{+2}$, $Hg^{+2}$ and sulfates of $Fe^{+2}$, $Zn^{+2}$, $Mn^{+2}$ and $Mg^{+2}$ was assessed. *P. atramentosum* KM gave higher tannase production with $MgSO_4 \cdot 7H_2O$ in case of amla (31.4 U/ml) and copper sulfate / zinc sulfate in case of keekar leaves, i.e., 32.0 U/ml with copper sulfate and 32.0 U/ml with zinc sulfate (Fig. 5). This activation by metal ions might be due to changes in the equilibrium constant of the enzyme reaction or by causing a change in the surface charge of the enzyme protein. Ionic interactions between an enzyme-bound metal and the substrate can help orientate the substrate to facilitate the reaction. The presence of the divalent ion $Mg^{+2}$ at low concentration might have caused precipitation of the enzyme in the reaction mixture, thus enhancing tannase activity (Aguilar et al. 2007; Kar et al. 2003). Similar to our observations, traces of $Fe^{+2}$, $Zn^{+2}$ and $Cu^{+2}$ have been reported to be stimulatory for the production of tannase by

*A. niger* (Yamada et al. 1968). Likewise, a stimulatory effect of $Mg^{+2}$ on tannase production was reported in *Verticillium* sp. P9 (Kasieczka et al. 2007). Mukherjee and Banerjee, (2006) reported that $Mg^{+2}$ ions enhanced tannase activity produced by a co-culture of *R. oryzae* and *A. foetidus*. Kar et al. (2003) reported that $Mg^{+2}$ or $Hg^{+}$ ions stimulated tannase activity produced by *A. foetidus* and *R. oryzae*. Chhokar et al. (2010) reported that metal ions $Mg^{+2}$, $Mn^{+2}$, $Ca^{+2}$, $Na^{+}$, and $K^{+}$ stimulated the tannase activity produced from *A. awamori* MTCC 9299, while $Cu^{+2}$, $Fe^{+3}$, and $Co^{+2}$ acted as inhibitors of the enzyme. Recently, Selwal et al. (2010) reported maximum tannase production by *P. aeruginosa* IIIB 8914 in the presence of $MgSO_4 \cdot 7H_2O$ with amla leaves and $HgCl_2$ with keekar leaves.

### Effects of additives

Surfactants play a vital role in enzyme production (Kim et al. 1995). We evaluated the effect of different surfactants (0.2% w/v) on tannase production, and observed that all the surfactants tested inhibited tannase production by *P. atramentosum* KM using amla (27.9 U/ml) and keekar (31.5 U/ml) leaves as compared to the control (Fig. 6). This inhibition may be due to a combination of factors such as a reduction in the hydrophobic interactions that play a crucial role in holding together the tertiary protein structure, and direct interactions with the protein molecule (Kar et al. 2003). Earlier reports on the effect of surfactants showed that all the surfactants and chelators inhibited tannase activity in *A. foetidus* and *R. oryzae* (Kar et al. 2003), while Tween 80 enhanced tannase activity. Tween 80 and Tween 20 caused a decrease in tannase activity in *P. variotii* (Battestin and Macedo 2007) and *Verticillium* sp. (Kasieczka et al. 2007). Srivastava and Kar (2009) also reported inhibitory effect of surfactants on tannase production.

Using optimized production medium, we are able to achieve enzyme production of 32.7 U/ml using amla leaves and 34.7 U/ml using keekar leaves using *P. atramentosum* KM. We found an increase in tannase activity of approximately 2.0- to 2.5-fold after optimization of certain physical and nutritional parameters during SmF (Table 3). Our isolates produced a high tannase yield as compared to other fungal and bacterial tannase producers under SmF reported in the literature. Rajkumar and Nandy (1983) reported 10.8 U/ml tannase from *P. chrysogenum* NCIM-722. Batra and Saxena (2005) reported various Penicillii producing approximately 4.8 U/ml tannase from *P. charlesii* (4.8 U/ml), *P. variable* (4.7 U/ml), *P. crustosum* (4.7 U/ml) and *P. restrictum* (4.4 U/ml). In our previous report (Selwal et al. 2010), we reported maximum yields of tannase, i.e., 13.6 and 12.9 U/ml by *P. aeruginosa* IIIB 8914 under SmF using 2% (w/v) amla and keekar leaves, respectively. Costa et al. (2008) reported ca. 20.6 U/ml tannase by *A. tamarii*. Selwal et al. (2011) also reported maximal tannase production of 170. 7 U/g dry solid and 165.5 U/g dry solid by *P. atramentosum* KM under solid state fermentation using jamun and keekar leaves, respectively.

### Laboratory scale-transfer of tannase production under SmF conditions

During the scale transfer of tannase production in Erlenmeyer flasks, using keekar and amla leaves, the tannase yield was found to decrease. An enzyme titer of 35.7 U/ml by *P. atramentosum* KM using keekar leaves and 33.8 U/ml using amla leaves were obtained when the fungal strain was grown in 50 ml selective mineral salt medium and selective production medium, respectively (Table 4). Further increase of the culture medium volume to 100 ml and 200 ml decreased enzyme yield. Reasons for the decrease in enzyme production during the scale transfer could be due to the non-maintenance of nutritional and fermentation conditions required in the scale-up experiments.

## Conclusion

The present investigation suggests that agro residues such as jamun and keekar leaves can be one of the best and most cost-effective alternatives to costly pure tannic acid for industrial production of microbial tannase. The enzyme produced by *P. atramentosum* KM has interesting characteristics and this fact encourages further studies including its production at industrial scale. The novelty in the present investigation is that this is the first fungal isolate reported to produce tannase at the high pH of 7.5 using keekar leaves. The production of tannase by this isolate should be attempted at higher levels. Optimization of growth parameters in a bioreactor is needed to determine its commercial viability. As the range of applications of this enzyme is very wide there is always a scope for novel tannase with better characteristics, which may be suited to diverse fields of application.

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