

Tannase production by *Lactobacillus* sp. ASR-S1 under solid-state fermentation

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Abstract

A tannase yielding bacterial strain was isolated from sheep excreta. It was identified as *Lactobacillus* sp. ASR S1. The bacterial strain produced extracellular tannase under solid-state fermentation (SSF) using tamarind seed powder (TSP), wheat bran (WB), palm kernel cake (PKC) and coffee husk (CH). Among different substrates, coffee husk resulted maximal extra-cellular production of tannase. To optimize the extracellular yield of tannase under SSF various physico-chemical and nutritional parameters were studied. Supplementation of tannic acid was found useful for enzyme synthesis by the bacterial culture selectively depending up on the substrate. Maximum tannase production (0.85 U/gds) was obtained when SSF was carried out using coffee husk, supplemented with 0.6% tannic acid and 50% (w/v) moisture, inoculated with 1 mL cell suspension and incubated at 33 °C for 72 h.

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1. Introduction

Tannin acyl hydrolase (E.C.3.11.20) commonly referred to as tannase is an inducible enzyme, produced mainly by fungi [1–4] but also by yeast [5] bacteria [6–9] and plants [10]. Tannase catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins such as tannic acid, releasing glucose and gallic acid. Tannins are present in nutritionally important forage trees, shrubs legumes cereals and grains. Tannins can impair the digestive process by complexing with secreted enzymes and endogenous proteins. One important adaptation of ruminal metabolism to counter anti-nutritive effects of forage tannins may involve the production of tannases and such enzymes are able to depolymerize tannins in to gallic acid and ellagic acid [11,12]. Gallic acid is then decarboxylated in the rumen to pyrogallol and converted to resorcinol and phloroglucinol. Rumen bacteria involved in this degradative pathway include *Eubacterium oxidoreducens*, *Streptococcus bovis*, *Syntrophococcus sucromutans* and *Coprococcus* sp. [11,13,14].

SSF is defined as any fermentation process occurring in the absence or near-absence of free water, employing a natural substrate or an inert support [15]. SSF is a batch process using natural heterogeneous materials containing complex polymers like Lignin, Pectin and Lignocelluloses. Bacteria, yeasts and fungi can grow on solid substrates and find applications in solid state fermentation process. Some economic applications of solid-state fermentation are bio transformation of crop residues, food additives, bioremediation and biodegradation of hazardous compounds, biological detoxification of agro-industrial wastes, enzyme production, bioactive products, organic acid production, biofuel and other miscellaneous compounds like mycotoxin, gibberellins, alkaloids, antibiotics and hormones [15]. Some advantages of SSF as described by Hesseltine [16], include: fermentation vessels may be small relative to product yield since little water is used and the substrate is concentrated, seed tanks are not necessary and spore inocula may be used, low moisture reduces the problem of contamination, conditions for fungal growth are similar to those in natural habitats, culture agitation inhibits mold sporulation, reducing the risk of laboratory contamination, aeration is facilitated by spaces between substrate particles and particle mixing, product yields may be much higher than those in liquid media and are reproducible, fermented solids may be extracted immediately by the direct addition of solvents

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or maintained in frozen storage before extraction, products may be incorporated directly into animal feeds, it is good for shear-sensitive organisms. Substrates traditionally fermented in the solid state include a variety of agricultural products such as rice, wheat, millet, barley, corn and soybeans. SSF mainly deals with the utilization of agro industrial residues as its substrates. Application of agro industrial residues as substrates for SSF is extremely economical and reduces pollution.

Over the past decade several studies reported that bacterial species such as *Streptococcus galloyticus* and *Lonepinealla koalarum* that were isolated from Koalas, goats or sheep [17,18] showed tannase activity. More recently this enzymatic activity was found in *Lactobacillus* strains isolated from human feces and fermented foods [8] and also from *Lactobacillus plantarum* [19]. *Lactobacillus plantarum*, *L. paraplantarum* and *L. pentosus* have been reported to play an important role in the production of many fermented foods including diary products, silage, pickled vegetables, meat and fish products and this being proposed as a potential probiotic [20]. Further more the tannase activities of these microorganisms in the human alimentary tract have significant effects on pharmacological aspects of dietary tannins that are prevalent in beverage and tea [8]. Naveena et al. [21] reported the use of a number of cheaply available raw substrates for a single step process for the production of lactic acid by *Lactobacillus amylophilus* GV 6 in SSF. Till date there are no reports available in the literature on the production of tannase through SSF by bacterial cultivation. The goal of the present study was to examine the scope of utilization of *Lactobacillus* sp. isolated from the excreta of sheep for the production of tannase in SSF.

2. Materials and methods

2.1. Microorganism and culture maintenance

Lactobacillus sp. ASR-S1 used in the present study is a microaerophile and was isolated from sheep excreta. One gram of excreta collected from domestic sheep which usually feed on green leaves of wild plants was dissolved in 10 mL sterile distilled water. One millilitre from this was inoculated to 10 mL of MRS broth containing 0.2% tannic acid. Aliquots from this was plated to MRS agar containing 0.2% tannic acid and incubated at 30 °C for 72 h. Bacterial colonies which are able to form a clearing zone around the colony due to the hydrolysis of tannic acid were selected and purified. Selected strains were then identified. The culture was maintained on MRS broth (Hi Media, India).

2.2. Preparation of inoculum

The *Lactobacillus* strain was cultured in 250 mL conical flasks containing 45 mL of MRS broth inoculated with 5 mL of an 18 h culture. The flasks were then incubated at 30 °C for 18–20 h and the inoculum thus obtained was used for the inoculation of the solid substrate medium.

Total viable cell count of the selected strain in MRS broth was determined by Colony Count Technique. One millilitre of the cell suspension was serially diluted and 0.1 mL of the same was taken from each diluted sample and was poured on to sterile Petri plates containing MRS agar medium and then spread uniformly. The plates were then incubated at 30 °C for 48 h and the colonies were counted using a colony counter. It was found that there was a cell density of 8×10^8 cells/mL.

2.3. Solid substrates

Wheat bran (WB), palm kernel cake (PKC), tamarind seed powder (TSP) and coffee husk (CH) were used as the solid substrates for the present study.

Table 1

Chemical composition of solid substrates used for tannase production

Component	WB (%) [22]	PKC (%) [23]	CH (%) [24]	TSP (%) [25]
Pentosans	25	–	–	–
Protein	15.7	16	6.8	14.38
Fat	3.77	–	1.5	–
Fiber	10.4	15.2	31.8	–
Ash	4.0	4.0	6.03	3.28
Moisture	9.67	–	11.98	8.67
Tannin	–	–	–	9.3
Carbohydrate	–	–	26.5	73

Wheat bran and tamarind seed powder were obtained from the local market in Trivandrum. Palm kernel cake was a gift from Malaysian Agricultural Research and Development Institute, Kuala Lumpur, Malaysia. Coffee husk was collected from a coffee processing unit located at Idukki district of Kerala, India. Chemical Composition of the different substrates are given in Table 1.

2.4. Moistening media

A salt solution containing (% w/v) NH_4NO_3 (0.5), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (0.1) and NaCl (0.1) having a pH of 5 was used as the moistening media for solid state fermentation.

2.5. Solid-state fermentation

Each substrate (5 gms) was taken in 250 mL conical flasks. The moistening media was added as per the required level for various substrates. The flasks were autoclaved at 121 °C at 15 lbs for 20 min. After cooling the sterilized solid substrates were inoculated with 1 mL cell suspension (18–20 h old) under aseptic conditions. These flasks were then incubated at 30 °C for 48 h.

2.6. Enzyme extraction

After 48 h of fermentation, crude enzyme was extracted from the fermented matter by adding 50 mL of 0.05 M citrate buffer (pH 5). The flasks were then kept in a rotatory shaker (Scigenics, India) for 10 min at 180 rpm and the crude enzyme was separated by filtration over Whatman No.1 filter paper. The filtrate was collected in vials and preserved for further analysis.

2.7. Enzyme assay

Tannase activity was estimated using the method of Sharma et al. [26]. The method is based on the formation of chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2-thio-4-ketothiazolidine). Tannase assay procedure includes the addition of 0.2 mL of crude enzyme to 0.55 mL of citrate buffer (0.05 M, pH 5). This was followed by the addition of 0.25 mL methyl gallate (0.01 M), 0.3 mL methanolic rhodanine (0.067% w/v) and 0.2 mL of potassium hydroxide (0.5 M) solution with incubation at 30 °C for 5 min after each addition. Dilute the reaction mixture with 4 mL of glass-distilled water and again incubated at 30 °C for 10 min. The pink color developed was read at 520 nm using a Spectrophotometer (Shimadzu UV-160 A). A set of blanks and controls were used simultaneously. One unit of tannase activity was defined as the amount of enzyme required to liberate 1 μmol of gallic acid/min under defined reaction conditions.

2.8. Optimization of process parameters

Optimum physico-chemical and nutrient parameters required for maximum tannase production by *Lactobacillus* sp. under SSF using the four different substrates were determined for initial moisture content (37–58% w/w), incubation temperature (25–40 °C), inoculum size (0.5–5 mL), supplementation of carbon sources (lactose, starch, glucose, maltose and sucrose) at 1% w/v and addition of tannic acid (0.1–1% w/v). The protocol adopted for optimization of

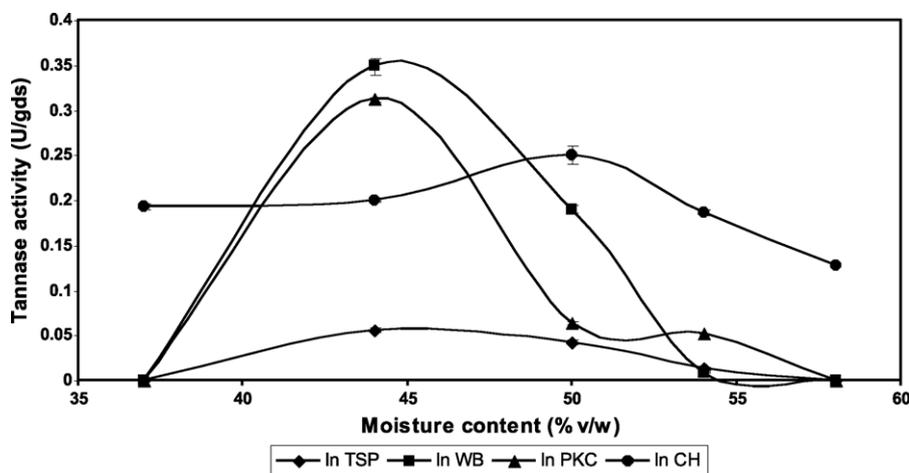


Fig. 1. Optimization of initial moisture content for the production of tannase under solid state fermentation.

various process parameters was to evaluate the effect of an individual parameter and to incorporate it at the optimized level in the experiment before optimizing the next parameter. After optimizing various parameters, a time course experiment was conducted incorporating all the optimized parameters. All experiments were carried out in triplicate and the mean values were reported with standard deviation.

3. Results and discussions

Lactic acid bacteria may play an important role during the digestive process and there are reports that *Lactobacilli* produce tannase [8]. Tannase production is induced and generated during the first steps of microbial growth [27]. Extracellular production of tannase by *Lactobacillus* sp. ASR-S1 under SSF using TSP, WB, PKC, and CH as substrates was evaluated. Results obtained from the various experiments conducted are described here with relevant discussion.

Fig. 1 shows the results on effect of substrate moisture on tannase production using different substrates. In case of WB, PKC and TSP, there was gradual increase in enzyme production with increase in the moisture in the substrate by *Lactobacillus* sp. ASR-S1 and maximum tannase was obtained at 44% moisture level on all these substrates. In case of CH, maximum tannase

was obtained at 50% substrate moisture. Wheat bran produced maximum yield of 0.35 U/gds (Fig. 1). In coffee husk, the production was not much affected from 37–54% moisture level.

Certain quantity of water is essential for new cell synthesis. Initial moisture content of the solid substrate is an important factor which dictates the growth of the organism and enzyme production. In the case of fungi a wider moisture range (20–70%) supports better growth and metabolic activities but for bacteria only a higher moisture content of the solid matrix can yield better performance. At the lowest and the highest water content the decomposition rate of the total organic matter was found to decrease and this in turn affects the enzyme production [15,28]. So in SSF a particular moisture range, which is optimum, is maintained.

As shown in Fig. 2, optimum temperature for tannase production varied from one substrate to the other. In general enzyme yield was comparatively low in TSP at all the tested temperatures. PKC gave maximal yield (0.51 U/gds) between 33 and 36 °C. This could be an added advantage while going for scale up. In the case of CH there was increase in enzyme yield from 25 to 36 °C and further increase in temperature resulted in a decrease in enzyme yield.

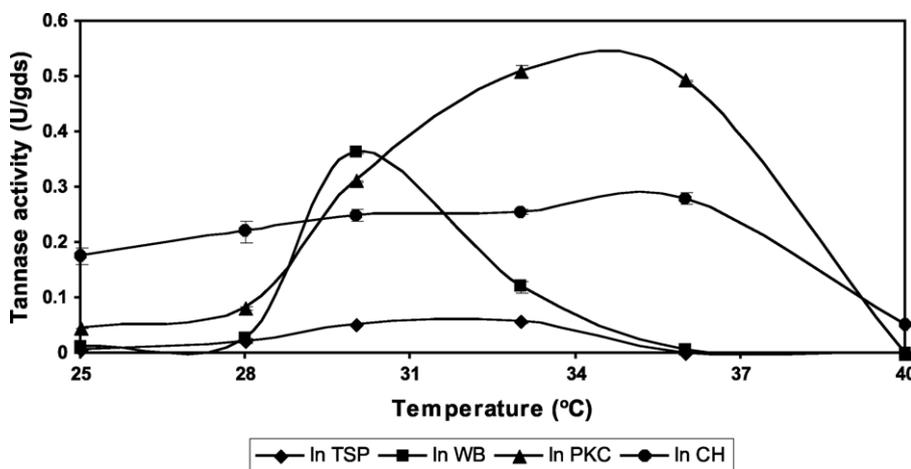


Fig. 2. Optimization of incubation temperature for the production of tannase under solid state fermentation.

Temperature determines effects as important as protein denaturation, enzymatic inhibition, promotion or inhibition on production of a particular metabolite, cell death, etc. Biological processes are characterized by the fact that they develop in relatively narrow range of temperature. Lactobacilli are mesophilic as well as thermophilic microorganisms. It is

common to have maximal growth around 36 °C. In the case of tannase production by fungi there are reports that the optimum temperature ranges from 30–35 °C [4].

The size of inoculum plays a significant role in the production of metabolites under SSF. In the present study, bacterial cells were used as inoculum and different inoculum

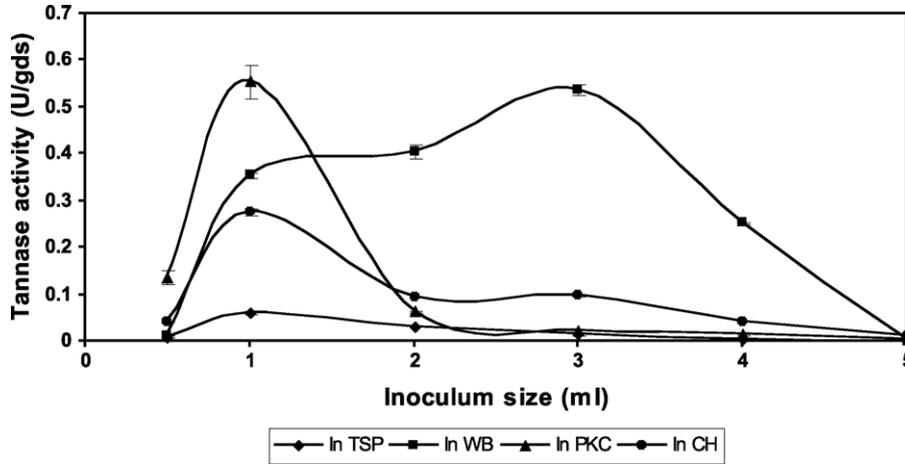


Fig. 3. Effect of inoculum size for tannase production under solid state fermentation.

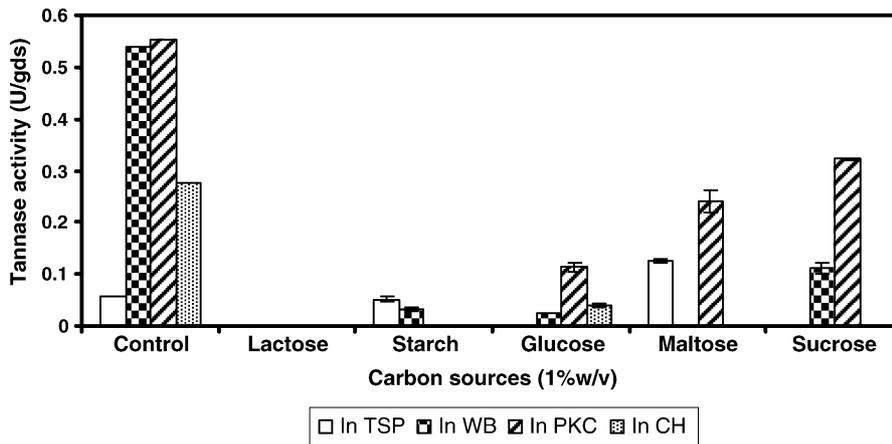


Fig. 4. Effect of various carbon sources on tannase production under solid state fermentation.

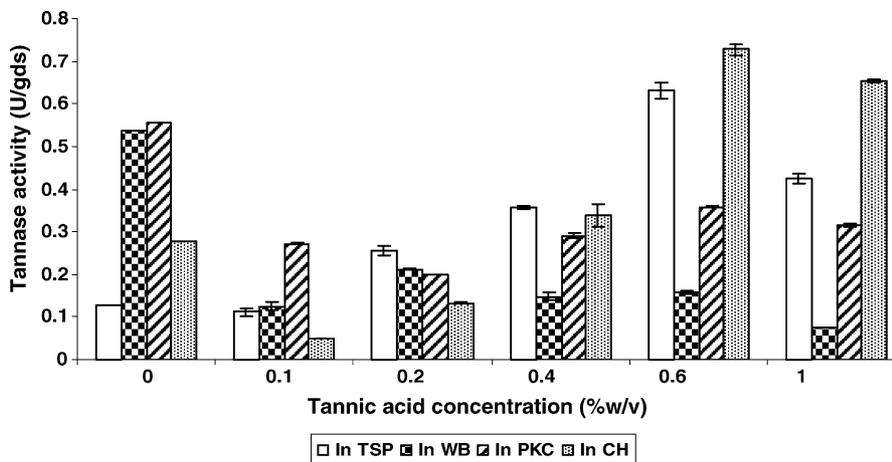


Fig. 5. Inductive effect of tannic acid on *Lactobacillus* sp. for tannase production under solid state fermentation.

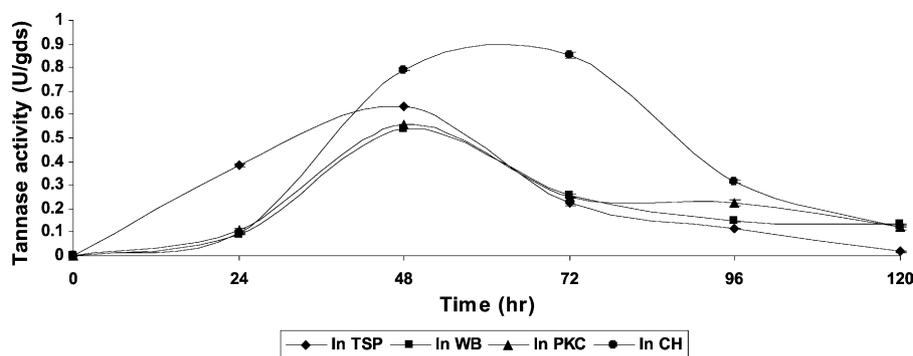


Fig. 6. Time course of tannase production under solid state fermentation.

sizes were tested in order to enhance tannase yield. Fig. 3 shows the effect of inoculum size on tannase production under SSF by the selected strain. Maximal tannase production was observed when 1 mL inoculum of *Lactobacillus* was grown on all solid substrates except WB. In the presence of WB a range of inocula (1–4 mL) resulted in significant tannase production (Fig. 3). This may be due to the simple nature of the substrate which gives an easy access for the microbe for hydrolytic action

Lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis on different substrates. An increase in the number of cells however, ensures a rapid proliferation of biomass and enzyme synthesis. After a certain limit, enzyme production could decrease because of depletion of nutrients due to the enhanced biomass, which would result in a decrease in metabolic activity [29]. A balance between the proliferating bacterial biomass and available substrate material would yield maximum enzyme [30].

The effect of additional carbon sources added to the four different substrates on the production of tannase was evaluated. In general, it was found that tannase production was inhibited by the incorporation of any of the carbon sources to the different substrates except TSP (Fig. 4).

Results revealed that in PKC, WB and CH tannase yield was suppressed due to the presence of additional carbon sources, but in TSP tannase synthesis was slightly improved by the addition of carbon sources. Available reports on the role of carbon sources on the extracellular secretion of tannase are contradictory [31]. Lagemaat and Pyle [1] reported that the glucose if present in the media will be exhausted rapidly and this may lead to the partial induction of tannase.

Different concentrations of tannic acid were added to the fermentation media after filter sterilization, to study the inductive effect of this compound on tannase production. Fig. 5 shows that *Lactobacillus* sp. ASR-S1 grown in TSP or CH gave maximal tannase yield at 0.6% tannic acid concentration. However, in WB or PKC there was no visible inductive effect on tannase synthesis. Recent data from our laboratory show the positive effect of tannic acid towards tannase induction [2].

Finally, after optimizing the various process parameters, a time course study was conducted to see the cumulative effect of various parameters. The experiment was conducted incorporating all the optimized parameters and samples were taken

every 24 h. Fig. 6 shows the result of time course of tannase production by *Lactobacillus* sp. ASR-S1 grown on various substrates. All other substrates except CH reported maximal enzyme yield after 48 h of incubation. In coffee husk maximal yield was after 72 h of incubation with an enzyme activity of 0.85 U/gds. Out of the four substrates CH reported maximal tannase yield (0.85 U/gds) followed by TSP (0.63 U/gds). WB and PKC gave almost similar tannase activity (around 0.5 U/gds). Since the level of tannase activity decreased after 48 h in three of the tested substrates further in depth study is needed to monitor the stability of the enzyme and of any inhibitory agents.

4. Conclusions

The most important source to obtain any enzyme is by microbial way, because the produced enzymes are more stable and the production will be consistent than similar ones obtained from other sources. Microorganisms can undergo new techniques such as genetic manipulation, resulting in an increase in the tannin acyl hydrolase activity. Solid state fermentation (SSF) is re-emerging as a promising tool in biotechnology for the production of microbial metabolites through inexpensive means and it is the most appropriate process for developing countries. Based on the present study it is concluded that *Lactobacillus* sp. ASR-S1 offers scope for the production of tannase under SSF and this is going to be the first report on the application of SSF technology for tannase production. TSP, WB, PKC and CH can be exploited for the production of this industrially important enzyme. Out of these four substrates CH which is accumulating in large quantities at places of bulk coffee production yielded maximal tannase under optimized conditions. After optimization of the various process parameters there was 3.5 fold increase in tannase activity in CH. Tannase yield reported here is comparable to those already reported from *Lactobacillus* sp. under liquid cultures. Since, information on bacterial tannase is scarce results obtained here demand further in-depth study to exploit the isolated strains of *Lactobacilli* for the industrial production of tannase at reduced costs. Research out put in that line will make tannase more attractive and feasible for the food and pharmaceutical industry. Therefore, it is necessary to achieve complete understanding about the regulation, catalytic

capacity, specificity and other aspects of optimization of its production at industrial scale.

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