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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

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Abstract

Palm kernel cake (PKC), the residue obtained after extraction of palm oil from oil palm seeds and tamarind seed powder (TSP) obtained after removing the fruit pulp from tamarind fruit pod were tested for the production of tannase under solid-state fermentation (SSF) using *Aspergillus niger* ATCC 16620. The fungal strain was grown on the substrates without any pretreatment. In PKC medium, a maximum enzyme yield of 13.03 IU/g dry substrate (gds) was obtained when SSF was carried out at 30 °C, 53.5% initial substrate moisture, 33×10^9 spores/5 g substrate inoculum size and 5% tannic acid as additional carbon source after 96 h of fermentation. In TSP medium, maximum tannase yield of 6.44 IU/gds was obtained at 30 °C, 65.75% initial substrate moisture, 11×10^9 spores/5 g substrate inoculum, 1% glycerol as additional carbon source and 1% potassium nitrate as additional nitrogen source after 120 h of fermentation. Results from the study are promising for the economic utilization and value addition of these important agro residues, which are abundantly available in many tropical and subtropical countries.

Keywords: Palm kernel cake; Tamarind seed powder; Tannase; Solid state fermentation; Agro residue

1. Introduction

In nature, solid organic substrates such as animal and plant residues, wood, crop residues and fruits, undergo complex microbial degradation and transformation by various microbiological processes. In the industrial sector, this natural process may be utilized in a controlled form and pure culture may be employed if a specific end product is desired. Natural SSF is carried out mostly by mixed cultures. Modern biotechnological industries are highly selective and they generally utilize the potential of a single microorganism under well defined conditions for developing a bioprocess (Pandey et al., 1999). Two types of substrates are used in SSF—one in which the solid substrate itself is used by the microorganisms as the carbon and energy source and the other in which substrate acts only as support. SSF mainly deals with the utilization of agro industrial residues as its substrates. Application of agro industrial residues as substrates is certainly economical and it also reduces environmental pollution. Several naturally occurring agricultural byproducts such as wheat bran, coconut oil cake, groundnut oil cake, rice bran, wheat and paddy straw, sugar beet pulp, fruit pulps and peels, corn cobs, saw dust, maize bran, rice husk, soy hull, sago hampas, grape marc, coconut coir pith, banana

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waste, tea waste, cassava waste, aspen pulp, sweet sorghum pulp, apple pomace, peanut meal, cassava flour, wheat flour, corn flour, steamed rice, steam pre-treated willow, starch etc. could be used in one or the other industrial bioprocess for the production of value added products through SSF (Pandey et al., 2001).

Hydrolysable tannins are present in most of the residues from higher plants and are polyphenolic compounds formed by the association of sugars and gallic and ellagic acids via ester linkages (Kumar and Singh, 1984). Tannins are able to form complexes with proteins and are able to inhibit the growth of microorganisms. By virtue of their chelating property, they precipitate proteins and are known enzyme inhibitors. These characters are highly anti-nutritional and it prevents the utilization of tannin rich plants as a source of animal feed. One important adaptation of ruminal metabolism to counter anti-nutritive effects of forage tannins may involve the microbial degradation of these compounds (Skene and Brooker, 1995; Selinger et al., 1996).

Palm kernel cake (PKC) is the byproduct of oil palm industry and is generated after the kernel is crushed to squeeze the oil. It is a useful source of protein and energy which contains (% w/w) dry matter (90); crude protein (16.1); ether extract (0.8); crude fiber (15.2); Ash (4); N-free extract (63); calcium (0.29); phosphorous (0.71) and metabolized energy—N7/kg (6.2). Tamarind seed powder (TSP) is obtained after removing the fruit pulp from tamarind fruit pod, which is commercially used as a feed material. The chemical constituents of tamarind seed powder include (% w/w) carbohydrate (73.68); protein (14.38 + 0.21); Ash (3.28 + 0.26) and moisture (8.67 + 0.05) (Marathe et al., 2002).

Tannase (tannin-acyl-hydrolase, E.C. 3.1.1.20) catalyzes the hydrolysis of tannic acid by breaking its ester and depside bonds releasing glucose and gallic acid. The fundamental application of tannase in nature is litter degradation and as a defensive mechanism against the attack of phytopathogens. Tannases are used widely in the food and pharmaceutical industry. It is used in processing of tea and also for production of pharmaceutically important compounds like gallic acid. Gallic acid, a tannin product is the substrate for chemical synthesis of propyl gallate and trimethoprim, which are important in the food and pharmaceutical industries. Tannase is also used in the treatment of tannery effluents, for the stabilization of malt polyphenols, clarification of beer and fruit juices, for the prevention of phenol-induced madeirization in wine and fruit juices and for the reduction of antinutritional effects of tannins in animal feed (Adachi et al., 1968; Aguilar and Sanchez, 2001). In the present report, the potential of two agro residues viz. palm kernel cake and tamarind seed powder were examined to see if these residues could be used as substrates for SSF using the fungal strain *Aspergillus niger* ATCC 16620 for the production of the high value industrial enzyme—tannase.

2. Methods

2.1. Solid substrates

Palm kernel cake was a gift from the Malaysian Agricultural Research and Development Institute, Kuala Lumpur, Malaysia. Tamarind seed powder was collected from the local market in Trivandrum, India. These substrates were used without any pre-treatment.

2.2. Microorganism and inoculum preparation

A fungal strain of *A. niger* ATCC 16620, purchased from the American Type Culture Collection (ATCC), Manassas, Virginia was used throughout this study. The strain was grown and maintained on Potato Dextrose Agar (PDA. Hi-Media, India) slants by culturing at 30 °C. Cultures were preserved at 4 °C for short-term storage. To a fully sporulated 1-week old agar slant culture, 10 ml of sterile distilled water with 0.1% Tween-80 was added. The spores were scraped using an inoculation needle under strict aseptic conditions. The spore suspension thus obtained was used as the inoculum. Viable spores in the spore suspension were determined by plate count technique.

2.3. Moistening medium

A salt solution with 0.5% w/v NH_4NO_3, 0.1% w/v $MgSO_4 \cdot 7H_2O$ and 0.1% w/v NaCl was used as the moistening medium for SSF. Final pH of the medium was adjusted to five.

2.4. Preparation of SSF medium for inoculation

Five grams of PKC and TSP were separately added to 250 ml Erlenmeyer flasks, moistened with 5 ml of salt solution, autoclaved at 121 °C for 20 min, cooled to room temperature and inoculated with 1 ml of the fungal spore inoculum (11×10^9 spores). The contents were mixed thoroughly and incubated at 30 °C in an incubator for the desired period of time.

2.5. Enzyme extraction

The fermented substrates were mixed thoroughly by keeping the flasks on a rotary shaker (Scigenics, India) at 150 rpm for 10 min after adding 50 ml of distilled water with 0.01% Tween 80. Crude enzyme from the fermented matter was extracted by direct filtration using Whatman #1 filter paper. The filtrate was collected in vials and preserved at 4 °C for further analysis.

2.6. Enzyme assay

Tannase activity was estimated by the method of Sharma et al. (2000). The method is based on the for-

mation of a chromogen between gallic acid (released by the action of tannase on methyl gallate) and rhodanine (2-thio-4-ketothiazolidine). The pink color developed was read at 520 nm using a spectrophotometer (Shimadzu UV-160A, Japan). Tannase activity was expressed in international units. One unit of tannase activity was defined as the amount of enzyme required to liberate one micromole of gallic acid per minute under defined reaction conditions. Enzyme yield was expressed as units/gram dry substrate (U/gds)/min.

2.7. Determination of soluble protein and fungal biomass

The soluble protein was determined by the method of Lowry et al. (1951) and was expressed in mg/ml. Glucosamine present in the cell wall of culture was estimated to determine the fungal biomass in fermented solid substrate and was expressed in mg/gds. A known quantity of the fermented matter was taken in a test tube and hydrolysed with concentrated sulphuric acid for 24 h. This mixture was then diluted and autoclaved at 121 °C for 1 h. After cooling the mixture was filtered and the filtrate was neutralized to a pH of 7 by adding 1 N NaOH. The glucosamine thus liberated was then estimated by the method of Sakurai et al. (1977).

2.8. Optimization of process parameters

The SSF medium containing the solid substrates and moistening media described in the previous section were taken as a basal medium and the process parameters under study were varied. Incubation time (0–168 h), incubation temperature (25–45 °C), initial moisture content (47–75%), inoculum concentration (spore concentration ranging from 2.75×10^9–66×10^9 spores), supplementation of additional carbon sources (glucose, starch, sucrose, maltose, glycerol, tannic acid, methyl gallate and gallic acid at 1%, w/v), supplementation with different organic nitrogen sources (peptone, yeast extract, malt extract and corn steep liquor), and inorganic nitrogen sources (ammonium nitrate, ammonium chloride, sodium nitrate and potassium nitrate, at 1% w/v—ammonium nitrate was excluded in the SSF moistening media during this study) and finally a time course experiment (0–168 h) was conducted incorporating all the optimized parameters where the specific enzyme activity and the biomass content (in terms of glucosamine) were also determined. The procedure adopted for optimization of various process parameters influencing tannase production was to evaluate the effect of individual parameters (keeping all other parameters as constant) and to incorporate it at the optimized level in the experiment before optimizing the next parameter.

2.9. Data analysis

All the experiments were carried out in duplicate. Results obtained were analyzed using the Microsoft Excel programme to determine the standard deviations.

3. Results and discussion

3.1. Incubation time

Studies on the optimization of incubation time required for the production of tannase by *A. niger* ATCC 16620 indicated that an incubation time of 144 h was optimum for maximal production of tannase in case of PKC, where an enzyme activity of 3.36 U/gds was obtained. In TSP medium, maximal enzyme production (0.64 U/gds) was obtained after 24 h of incubation and as the incubation period increased the production decreased. In PKC medium also there was decrease in enzyme yield with further increase in incubation time. This could be due to the reduced nutrient level of medium, affecting the enzyme synthesis. Decreased enzyme yield on prolonged incubation could also be due to inhibition and denaturation of the enzyme (Gautam et al., 2002). It has been reported before that tannase was produced during the primary phase of growth and thereafter the activity decreases either due to the decrease in production or due to enzyme degradation (Suseela and Nandy, 1985).

3.2. Incubation temperature

Experiments on the optimization of incubation temperature for the utilization of PKC and TSP for the production of tannase under SSF revealed that there was increase in enzyme yield till 30 °C where maximal production (3.36 U/gds for PKC and 0.64 U/gds for TSP) was recorded. With further increase in temperatures above 30 °C there was a sharp decrease in enzyme activity. Although nearly nondetectable enzyme was present for PKC at 40 and 45 °C the fungal culture could grow and produce 0.25 U/gds enzyme on TSP medium at 40 °C. There are reports that described optimum temperature for tannase from 30 to 35 °C (Lekha and Lonsane, 1997). In the case of *Aspergillus japonicus*, the enzyme production was similar at 30 and 37 °C where biomass was also maximum (Bradoo et al., 1996).

3.3. Initial moisture content

Optimum level of initial moisture content is required for maximum substrate utilization and enzyme production in SSF. Results obtained (Table 1) from the experiment to optimise the initial moisture content of the substrate revealed that in PKC medium, maximum

Table 1
Impact of initial moisture content of the media on tannase production

Initial moisture content in palm kernel cake (%)	Tannase activity (U/gds)	Initial moisture content in tamarind seed powder (%)	Tannase activity (U/gds)
45.2	1.67 ± 0.015	47.58	0.02 ± 0.009
50.6	1.57 ± 0.009	56.53	0.18 ± 0.006
53.5	3.36 ± 0.019	60.25	0.64 ± 0.009
57.1	1.09 ± 0.014	63.89	0.51 ± 0.009
60.1	0.95 ± 0.008	65.75	0.72 ± 0.004
63.7	1.01 ± 0.01	69.88	0.13 ± 0.007
66.4	0.45 ± 0.021	71.32	0.06 ± 0.004
69.7	0.57 ± 0.01	75.12	0.00 ± 0.001

enzyme production was recorded at 53.5% moisture content, i.e. 3.37 U/gds. TSP medium resulted maximal production (0.72 U/gds) of tannase at 65.8% moisture level. Initial moisture content is a critical factor for growth and enzyme production. For both substrates, increase in moisture level beyond a particular level was found to inhibit the production of enzyme. With increasing water content and constant substrate volume, the air content of the substrate (air occupied within the interparticle space) decreases. At the lowest and the highest water content the decomposition rate of the total organic matter was also found to decrease and this in turn affects the enzyme production (Pandey, 1994). Initial moisture content is a critical factor for growth and enzyme production (Pandey, 1992a). Also moisture is a factor that is intimately related to the definition of SSF because it is necessary for new cell synthesis (Pandey, 1992a,b).

3.4. Inoculum concentration

The size of inoculum plays a significant role in the production of metabolites under SSF (Pandey, 1994). In the present study, fungal spores were used as the inoculum and different inoculum sizes were put under study to enhance the utilization of the solid substrate and thereby improving tannase activity. Results show the effect of inoculum size on tannase production under SSF using PKC and TSP as substrate. In PKC medium, the yield of tannase increased with the increase in inoculum size and maximal tannase activity (3.94 U/gds) was recorded with an inoculum size of 33×10^9 spores. In TSP medium, there was an increase in tannase production with increasing inoculum size up to a spore concentration of 11×10^9 where a yield of 0.72 U/gds was obtained. With further increase in inoculum size, the enzyme production decreased. Lower level of inoculum may not be sufficient for initiating growth and enzyme synthesis on different substrates. An increase in the number of spores however, ensures a rapid proliferation of biomass and enzyme synthesis (Kashyap et al., 2002). After a certain limit, the enzyme production could decrease because of the depletion of nutrients, which result in decrease in metabolic activity. A balance between the proliferating biomass and available material would yield maximum enzyme (Pandey et al., 2000; Kashyap et al., 2002).

3.5. Supplementation of carbon and nitrogen sources

3.5.1. Carbon sources

In PKC medium, addition of tannic acid, methyl gallate and gallic acid significantly affected the enzyme production and maximum activity was obtained with tannic acid (7.65 U/gds) (Fig. 1). Methyl gallate and gallic acid stimulated slightly less tannase. Other carbon sources were found inhibitory to tannase production.

In view of the effectiveness of tannic acid, it was thought desirable to evaluate the response to different concentrations in PKC medium in tannase production. Accordingly tannic acid was supplemented in the SSF medium at 0.5–10% concentration. There was further increase in the enzyme production and maximum tannase (13.03 U/gds) was obtained with 5% supplementation. Beyond this level, the enzyme synthesis remained constant (data not shown).

The impact of supplementation of these compounds to TSP medium for tannase production was quite different from that of PKC medium. As is evident from Fig. 1, although here also all the tested compounds resulted in increase in enzyme production by the fungal culture,

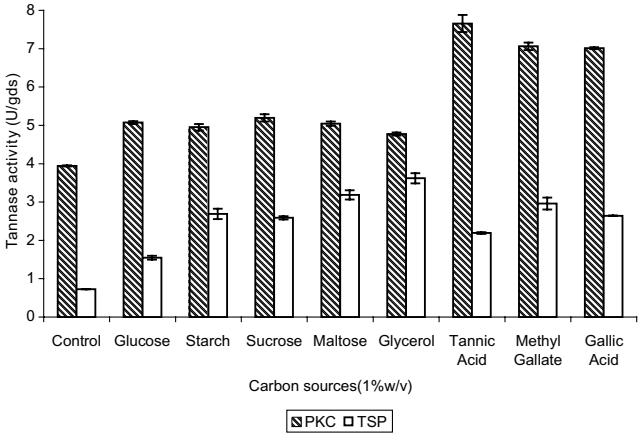

Fig. 1. Impact of additional carbon sources on tannase production.

glycerol stimulated tannase production about 5.7 fold. Methyl gallate and gallic acid was better than tannic acid in enhancing tannase production. Among the carbohydrates, maltose, sucrose and starch also gave comparable yield. In view of the effectiveness of glycerol, different concentrations (0.5–5%) were tested. It was found that 1% (w/v) was most suitable, resulting in 3.62 U/gds tannase. Tannase production was also enhanced by the addition of glucose, sucrose, maltose, starch and glycerol and the order of effectiveness was glucose > sucrose > maltose > starch > glycerol (Fig. 1).

Available reports on the role of carbon sources on the extracellular secretion of tannase are contradictory (Aguilar et al., 2000). As per these reports tannase could be induced only when the microorganism is grown in the presence of tannic acid (Nishira and Mugibayashi, 1953) and is contradictory to the reports of Seiji et al. (1973) who mentioned that tannase activity is expressed when the organism is grown only on glucose. In the present report, in PKC tannic acid induced enhanced synthesis and secretion of tannase. The sugars stimulated tannase production less but they never inhibited tannase production, hence the chances of any feed back inhibition could be ruled out.

3.5.2. Nitrogen sources

The effect of supplementation of different inorganic and organic nitrogen sources on tannase production was evaluated and results are shown in Fig. 2. Since the moistening media contained ammonium nitrate, it was excluded from that media for the present study in order to avoid any repetition. Evidently all the tested compounds exerted harmful impact on tannase production by the fungal culture in comparison to the control in PKC. This showed that PKC had enough nitrogen required for the activity of the culture and supplementation of the additional nitrogen sources resulted in imbalanced C/N ratio, affecting the cultures activity harmfully. However, in case of TSP medium it was the opposite observation. All the tested compounds irrespective of their organic or inorganic nature resulted in marginal increase in tannase activity. Potassium nitrate was the most effective among all, which gave 5.59 U/gds tannase (Fig. 2). In this case also, as with previous compounds, it was decided to evaluate different concentrations of potassium nitrate to determine the optimal level for tannase production. Accordingly TSP medium was supplemented with 0.5–3.0% potassium nitrate. Maximum enzyme yield (5.59 U/gds) however, was obtained with 1% concentration. Nitrogen can be an important limiting factor in the microbial production of enzymes. Probably the presence of additional nitrogen sources along with nitrogenous compounds present in the substrate promoted enhanced growth and consequent enzyme production (Chandrasekaran et al., 1991).

3.6. Time course studies of tannase production

After optimizing the various process parameters, a time course study was conducted to see the cumulative effect of various physical parameters and nutrient sources. The experiment was conducted incorporating all the optimized parameters and samples were taken after every 24 h. The result of time course study on TSP is shown in Fig. 3, where a maximum activity (6.44 U/gds) was obtained after 24 h of incubation and there was a decline in enzyme yield after 24 h. Analysis of the specific activity of the enzyme indicated that maximal activity occurred at 24 h and further incubation resulted in accumulation of soluble protein and a decrease in specific enzyme activity (Fig. 3). The time course experiment with PKC showed that maximal synthesis of extra cellular tannase was obtained after 96 h of incubation where a maximum yield of 13.03 U/gds was obtained. Here the specific activity of tannase was maximum after 48 h of incubation and further incubation resulted in a decrease in specific activity.

To monitor the growth of *A. niger* on both substrates, biomass of the fungi was estimated through the analysis of glucosamine. Results obtained showed that in TSP maximal enzyme production and biomass increase

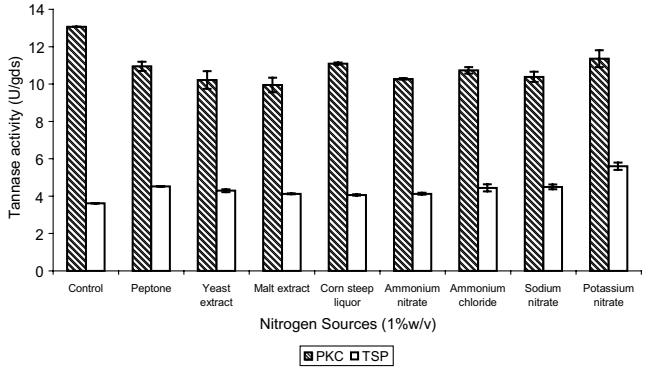

Fig. 2. Impact of addition of nitrogen sources to the SSF media on tannase production.

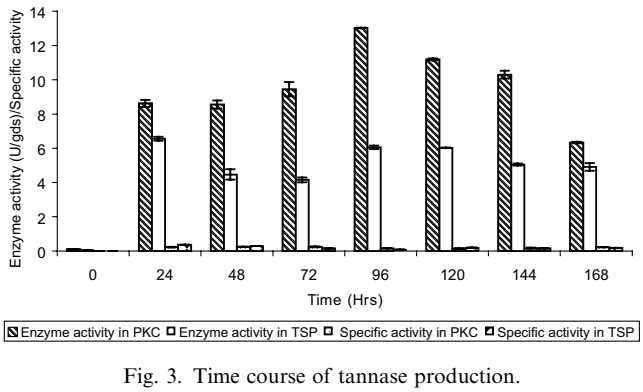

Fig. 3. Time course of tannase production.

(35.69 mg/gds) occurred after 24 h of incubation and further increase in incubation time resulted in a decline in growth rate of the organism, but in PKC, glucosamine content was found increasing with time till 144 h (60.92 mg/gds). These results are in agreement with the earlier reports on enzyme production through SSF using fungal strains, which indicated that tannase yield was growth associated (Sabu et al., 2000).

4. Conclusions

In most of the countries where the economy is largely based on agriculture and where the farming practice is very intensive, accumulation of agricultural residues is a serious problem. Presence of tannins and its derivatives in agro residues is a major hurdle in their utilization as feed material. Solid state fermentation technology using non pathogenic microorganisms which can produce hydrolytic enzymes such as tannase will be advantageous for the proper utilization of these residues. Along with the production of tannase under SSF, tannins present in the residues will undergo hydrolysis and thus it improves the nutrient quality of the fermented matter as a feed material. Utilization of agricultural residues through biotechnology is becoming more and more significant with the dual goal of waste disposal and value addition. Since microbial activity especially fungal activity is the key aspect in this area, there is enormous opportunity for the cost effective production of tannase, which is an important enzyme in the food and pharmaceutical industry.

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