



Evaluation of selected agri-industrial residues as potential substrates for enhanced tannase production via solid-state fermentation

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ABSTRACT

Tannase is an important enzyme widely used in the production of food, feed, pharmaceuticals, beverages, leather and cosmetics, but its industrial application is still limited because of its high production cost. Agri-industrial residues are generally rich in tannins and can be used as low-cost substrates for tannase production. In this study, selected agri-industrial residues were evaluated as potential substrates for enhanced tannase production by a locally isolated fungal strain via solid-state fermentation. Among all four agri-industrial residues tested, rice bran, a nutrient-rich substrate with a low tannin level, showed the highest tannase activity during solid-state fermentation, followed by brewer's rice, spent coffee ground and desiccated coconut residue. Spent coffee ground contained the highest tannin content (155 mg tannin/g substrate), but exhibited poor fungal growth, possibly because of the high tannin content in spent coffee, which might have inhibited fungal growth. However, the incorporation of small amounts of spent coffee ground (0.5% tannic acid equivalent, TAE) into rice bran enhanced tannase activity by 1.8-fold and 3-fold in comparison to rice bran used as single substrate or with the addition of tannic acid, respectively, with 260.39 U/g tannase being produced. The use of nutrient- and tannin-rich agri-industrial residues, such as rice bran and spent coffee ground, enhances tannase activity, suggesting that such low-cost substrates can be used in industrial tannase production.

1. Introduction

Tannin acyl hydrolase (EC 3.1.1.20), also known as tannase, catalyses the breakdown of ester bonds from hydrolysable tannins such as gallotannins, producing gallic acid and glucose (Kumar et al., 2016; Aguilar et al., 2007). Tannase is an inducible extracellular enzyme and produced by a variety of plants, animals and microbial sources. Microbes, predominantly filamentous fungi, are considered as the most important commercial tannase producers because microbial tannases are more stable than those produced by other sources. Filamentous fungi, especially species of the genera *Aspergillus* and *Penicillium*, are widely studied as they can tolerate up to 20% of tannin (Lekha and Loshane, 1997; Belmares et al., 2004).

Tannins are naturally occurring water-soluble polyphenolic secondary metabolites in plants and considered as the fourth most abundant plant constituents after cellulose, hemicellulose and lignin

(Lokeswari and Kumar, 2013). They are widely distributed not only among different families of higher plants, but also found in tea, cashew nuts, hazelnuts, walnuts, grapes, mangoes and strawberries. One of the major characteristics of tannins is their ability to form strong complexes with macromolecules such as carbohydrates and proteins (Malgiredy and Nimma, 2015). High-molecular tannins have stronger anti-nutritional effects and lower biological activities due to their ability to form complexes with proteins, carbohydrates and digestive enzymes such as lipases, amylase, protease, pectinase and cellulase; resulting in reduced nutritional values as well as lower feed intake and protein bioavailability in ruminants (Combs, 2016).

Tannase is an important enzyme with a wide range of industrial applications in the production of food, feed, beverages, cosmetic products, pharmaceutical and chemical substances and leather (Aguilar and Gutierrez-Sanchez, 2001; Aguilar et al., 2007). Due to its potential application in various industrial sectors, its commercial usage has

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accelerated in recent years (Govindarajan et al., 2016). For example, in the beverage industry, it is used as clarifying agent in wines, fruit juices, coffee-flavoured refreshing drinks and in the production of instant tea. The high concentration of tannins present in fruit juices, iced tea, beer, wine and coffee-flavoured beverages results in the formation of precipitates due to their interaction with other molecules present in these beverages, and these undesirable effects of tannins can be reduced or eliminated by enzymatic treatment. Tannase reduces the concentration of tannin via hydrolysis and produces glucose and gallic acid. The latter is the major hydrolytic product of tannic acid and used in food, cosmetics and adhesives in the synthesis of a potent antioxidant, propyl gallate (Viswanath et al., 2016).

Despite the varied application of tannase, its industrial application is still limited, mainly because of the high enzyme production costs, caused by increasing costs of media components and processing (Meena et al., 2013). In commercial tannase production, generally, pure tannic acid is used, which serves both as an inducer as well as a carbon source. Pure tannic acid is, however, relatively costly and not suitable for large-scale enzyme production (Lokeswari and Kumar, 2013). Recently, the interest in tannase production from agricultural residues has remarkably increased because of its low cost and wide application. The use of different agri-industrial by-products, such as tamarind seed powder and palm kernel cake (Sabu et al., 2005), sugarcane bagasse and rice straw powder (Paranthaman et al., 2010), tea stalks (Xiao et al., 2015; Wang et al., 2013) and cashew bagasse (Liu et al., 2016), employing different *Aspergillus* strains, enhances the tannase production through solid-state fermentation (SSF). This approach could reduce the high enzyme production costs (Kumar et al., 2016; Viswanath et al., 2016). The above-mentioned agri-industrial residues are rich in carbohydrates and other nutrients but often under utilized or being used as low-value niche market applications such as animal feed or bio-fertilisers (Chutmanop et al., 2008). A number of agri-industrial residues are also rich in tannins (Pandey et al., 1999) and can be considered as suitable substrates to substitute pure tannic acid as inducer to enhance tannase production. Therefore, tannin-rich agri-industrial residues as natural tannin resources can be good sources of low-cost substrates for tannase production. Among the most important agri-industrial residues available in Malaysia are rice by-products such as rice bran and brewer's rice from the rice milling industry.

Solid-state fermentation (SSF), using agri-industrial residues as substrate, is often favoured over submerged fermentation (SmF) because it offers numerous advantages. Among the main advantages of SSF are the higher economic viability, the use of cheaper substrates such as agri-industrial residues, lower production costs, higher enzyme yields and less energy consumption (Pandey, 2003). However, studies on the tannase production profile through SSF and its correlation with other biochemical changes during fermentation are rare. In this context, we employed a local tannase-producing fungal strain, *Aspergillus niger* PN1, isolated from banana peel, for tannase production through solid-state fermentation. Several types of agro-based residues were evaluated for their feasibility as substrates for tannase production through solid-state fermentation, and the tannase production profiles of the selected substrates were investigated.

2. Materials and methods

2.1. Chemicals

All chemicals used in this study were of analytical grade and produced by Himedia, India.

2.2. Microorganism maintenance

A potential tannase-producing fungal strain used in this study was isolated from banana peels and identified as *Aspergillus niger* PN1 (GeneBank Accession Number KY931504.1), based on microscopic

observation and ITS gene sequencing, performed in our previous study at our laboratory (Mansor et al., 2018). The fungal isolate was kept in the Collection of Functional Food Culture (CFFC, MARDI Serdang, Malaysia) and maintained in potato dextrose agar (PDA) slants at 4 °C for working cultures and at -20 °C in potato dextrose broth (PDB) containing 16% glycerol for long-term storage.

2.3. Inoculum preparation

A volume 10 mL of 0.01% Tween 80 was added to 7-day-old fully sporulated fungi. The spores were scrapped from the surface to prepare the spore suspension, which was then shaken at 200 rpm, 30 °C, for 2 h to produce a homogeneous spore suspension. The spore count was calculated using a haemocytometer to obtain a spore count of 1×10^8 spores/mL, which was used as inoculum (Batra and Saxena, 2005).

2.4. Substrate preparation

Different agri-industrial residues, such as desiccated coconut residue and spent coffee ground, were collected from a local market and Starbucks restaurant, Serdang, respectively. Rice bran and brewer's rice were obtained from the rice milling factory Padiberas Nasional Berhad (BERNAS), Selangor, Malaysia. All samples were oven-dried at 50 °C for 16 h until a moisture content of less than 5% was reached, packed in vacuum packs and stored at 4 °C until use. The solid substrates used in the screening process had different particles sizes, i.e. 150–425 µm (rice bran and spent coffee ground), 0.425–1.77 mm (brewer's rice) and 1.4–1.6 mm (desiccated coconut residue).

2.5. Solid-state fermentation

Solid-state fermentation was conducted in 100 mL Erlenmeyer flasks containing 30 g of solid substrate, sterilized at 121 °C for 20 min at 15 atm. After cooling down at room temperature, the substrates were added with sterilized distilled water to obtain a moisture content of approximately 50%, inoculated with 1 mL of spore suspension and incubated at 32 °C with sampling at every 3 days intervals for 18 days.

2.6. Estimation of moisture content

The moisture content of the substrates was estimated by drying 1 g of substrate to constant weight at 80 °C, using a moisture analyser (A&D MX-50, Japan).

2.7. Sampling and extraction

The fermented mass was mixed with distilled water at the ratio of 1:4. The obtained slurry was thoroughly mixed on an orbital shaker at 200 rpm, 30 °C, for 2 h and subsequently filtered through a muslin cloth, followed by centrifugation at 10,000 g at 4 °C for 10 min to obtain the crude enzyme. The enzyme extract was stored at -20 °C for further analysis, and the fermented biomass was dried at 80 °C to measure the glucosamine content.

2.8. Tannase assay

Tannase activity was determined using the colorimetric method described in Mondal et al. (2001). One unit of the enzyme tannase was defined as the amount of enzyme able to hydrolyse 1 mM of ester bond of tannic acid per minute under specific conditions, expressed as U/g (units per gram of dry substrate).

2.9. Determination of fungal biomass

Fungal growth was determined by estimating the glucosamine amount present in the fungal cell wall, expressed in milligrams per

gram of fermented substrate (mg/g). A known quantity of the whole dried fermented sample was placed in a screw-capped test tube, hydrolysed with 10 M HCl and heated at 100 °C for 16 h. The pH of the filtrate was neutralised to pH 7, and the glucosamine content was estimated using the method of Sakurai et al. (1977).

2.10. Determination of tannin content

The tannin extraction method used was as described in Shilpa (2010), with modifications. Fat was removed by incubating 1 g of sample in 20 mL of petroleum ether for 4 h at room temperature and subsequently discarded the petroleum ether. A volume of 20 mL of 70% methanolic acid (pH 3) was added and stored for 16 h at 4 °C. Then, 1 mL of tannin extract was added to 3 mL of bovine serum albumin, BSA, and the mixture was centrifuged at 10,000 rpm, 10 °C, for 7 min. The supernatant was discarded prior to the addition of 3 mL of sodium dodecyl sulphate, SDS. Finally, 1 mL of FeCl₃ was added and the absorbance of the sample was read at 530 nm after a resting period of 15 min. The standard tannic acid solution was prepared at different concentrations of 0, 5, 10, 15 and 20 mg/mL tannic acid.

2.11. Determination of total reducing sugars

Total reducing sugars were determined according to Miller (1959). Briefly, the reaction mixture containing 0.5 mL of crude extract and 0.5 mL dinitro salicylic acid reagent was heated in a boiling water bath for 10 min. After cooling to room temperature, 9 mL of distilled water was added and the absorbance was measured at 540 nm. A calibration curve was prepared using glucose as standard.

2.12. Statistical analysis

Mean values and standard deviations were calculated from the values of three replicates. To determine the significance of the data, one-way analysis of variance (ANOVA) was conducted using the statistical software package Minitab (Version 14). Differences between means with a p-value of < 0.05 were considered statistically significant.

3. Results and discussion

3.1. Screening and selection of agri-industrial residues as substrates for solid-state fermentation of tannase

Fig. 1 depicts the screening of four different agri-industrial residues by a local fungal strain isolated from banana peels and identified as *Aspergillus niger* PN1 for the production of tannase through solid-state fermentation (SSF). Four types of agri-industrial residues, namely rice bran (RB), brewer's rice (BR), desiccated coconut residues (CN) and

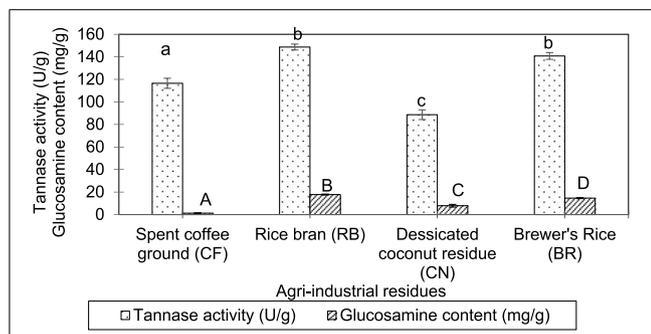


Fig. 1. Highest tannase activity in four different types of agri-industrial residues at day 12 for CF and day 15 for RB, CN, and BR, where maximum tannase production was attained. Means with different letters are significantly different (Tukey's HSD, $p < 0.05$).

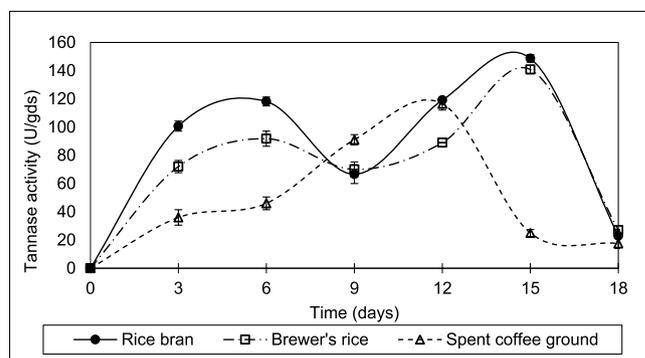


Fig. 2. Profiles of tannase activity in different types of agri-industrial residues.

spent coffee ground (CF), were screened in terms of their suitability as solid-state fermentation substrate for tannase production. These substrates were selected because they are relatively cheap, easily and widely available and can be readily used without further processing steps. Among the four substrates tested, rice bran had the highest tannase activity with 148.7 U/g, followed by brewer's rice, spent coffee ground and desiccated coconut residue with 140.8, 116.52 and 88.64 U/g, respectively (Fig. 1). Fungal growth, as indicated by the glucosamine content, was also higher in rice bran and brewer's rice compared to the other substrates, with 17.8 and 14.6 mg/g dry substrate, respectively.

Further, three substrates, namely spent coffee ground (CF), rice bran (RB) and brewer's rice (BR) were selected and investigated in terms of the tannase production profile and other biochemical changes. Fig. 2 shows a similar trend of an increase in tannase activity in both RB and BR up to day 6, with a slight reduction afterwards. A subsequent increase in enzyme activity was observed after day 9, with the highest activity at day 15, i.e. 148.7 and 140.8 U/g in RB and BR, respectively (Fig. 2). On the other hand, a gradual increase of tannase activity was observed in CF, and the highest activity was attained at day 12, reaching 116.52 U/g.

Similarly, fungal growth was higher in RB and BR, as shown by the amount of glucosamine content in these substrates, compared to CF. The maximum glucosamine levels in both RB and BR were 17.86 and 14.6 mg/g substrate, respectively, at day 12 of the fermentation (Fig. 3). Despite the high tannase activity in CF, fungal growth was poor, probably due to the high tannin content in this substrate. According to a previous study, high tannin contents inhibit fungal growth due to the formation of strong complexes with proteins and carbohydrates (Malgireddy and Nimma, 2015). In addition, the poor fungal growth was probably due to the low amounts of nutrients available in CF, based on the low level of reducing sugars (Fig. 4).

A high concentration of reducing sugars was found for both RB and BR, produced from starch hydrolysis during fermentation of both

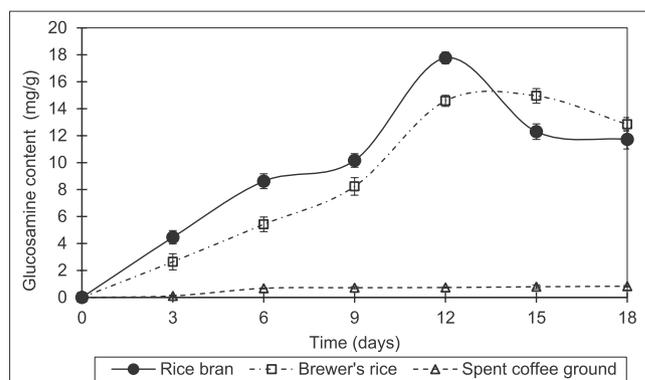


Fig. 3. Glucosamine contents in different types of agri-industrial residues.

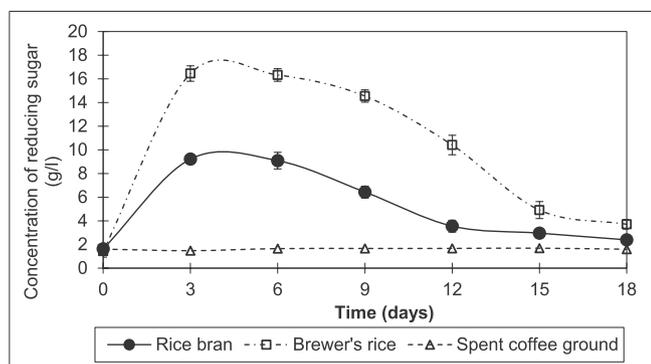


Fig. 4. Concentrations of reducing sugars in different types of agri-industrial residues.

substrates that contain high levels of carbohydrates. Among the three substrates; CF, RB and BR, the latter has the highest carbohydrate content, resulting in the highest level of reducing sugars (Fig. 4). A drastic increase in reducing sugars was observed in the early stage of fermentation at day 3, with maximum glucose concentrations of 16.32 and 9 g/L in BR and RB, respectively. After day 3, reducing sugar levels gradually decreased and were depleted at days 12 and 15, respectively, indicating glucose consumption for both fungal growth and enzyme production.

There was an increase in tannase activity at the early state of fungal growth (day 6), mainly because of the complex substrates used in the solid-state fermentation. Extracellular enzymes such as tannase are produced and secreted to hydrolyse solid substrate into simpler sugars, which can then be absorbed by the fungus. When the glucose concentration was increased, as shown in Fig. 4, enzyme production was slowed down, resulting in the reduction of tannase, as detected at day 6. However, the accumulated simple sugars were immediately used by the actively growing fungi and were rapidly depleted. This, in turn, caused the fungi to produce more hydrolytic enzymes (here, tannase), as shown by the increase in enzyme activity after day 9. Chutmanop et al. (2008) have reported a similar profile, with a slight increase followed by a decrease in protease activity by *Aspergillus oryzae* at day 3 of rice bran fermentation.

Both RB and BR served as good substrates for SSF of tannase, with the highest activity in fermented rice bran. Recently, rice by-products such as BR and RB have received increased attention because of their high amounts of carbohydrates, protein, vitamins, minerals and fibre (Esa et al., 2013). The application of RB, which is rich in nutrients, could provide the required carbon, nitrogen, sugar and protein amounts for fungal growth, leading to protease production (Sumantha et al., 2006). This rice by-product is not only a cheap agri-industrial residue, but also easily available in large quantities and ready to use without further processing.

3.2. Tannin content in agri-industrial residues

The tannin contents of selected agri-industrial residues used in the screening of the best substrates for tannase production are shown in Table 1. Among all four agri-industrial residues tested, the highest

Table 1
Tannin contents in four different agri-industrial residues.

Agri-industrial by-products	Tannin content (mg TAE/g substrate)
Rice bran (RB)	12.60 ± 0.236 ^a
Brewer's rice (BR)	2.16 ± 0.330 ^b
Spent coffee ground (CF)	155.61 ± 0.275 ^c
Desiccated coconut residue (CN)	3.49 ± 0.060 ^b

Means with different letters are significantly different (Tukey's HSD, $p < 0.05$).

tannin content was detected in CF, with 155.6 mg tannic acid equivalent (TAE)/g substrate. The tannin content in agri-industrial residues generally acts as inducer for tannase production, and tannin-rich plants or agricultural residues as potential substrates for tannase production have been used in searching for low-cost substrates (Natarajan and Rajendran, 2012; Viswanath et al., 2016). Some researchers have used natural tannin, obtained from a number of tannin-rich plants or agro-residues, as cost-effective natural substrate for tannase production. In this regard, natural substrates such as jamun leaves, amla leaves (Kumar et al., 2007; Selwal et al., 2011), tamarind seed powder (Sabu et al., 2005), bagasse, ground nut oil cake, wheat bran and rice bran (Natarajan and Rajendran, 2012; Viswanath et al., 2016), coffee pulp and tea residue (Wang et al., 2013) have been used as substrates. These tannin-rich agri-industrial residues were generally added as substrate and/or inducer to substitute pure tannic acid and enhanced tannase production (Lokeswari and Kumar, 2013). In this study, the enhanced fungal growth that resulted in high tannase production was probably due to the high nutrient content, despite the small tannin content in RB.

3.3. Supplementation of spent coffee ground as inducer for tannase production

Microbial tannase is an inducible enzyme, and generally, tannin or tannic acid was used to enhance tannase production. However, high tannin content inhibit fungal growth as indicated by the low glucosamine content in CF. Further investigation on the effect of supplementation of CF as inducer in the fermentation of RB has revealed that 3% of CF gave the highest tannase activity with 260 U/g dry substrate, in comparison to RB as single substrate or with the addition of tannic acid (Fig. 5). The addition of 3% of CF at 0.5% tannic acid equivalent (TAE)/g substrate has resulted in an increase in tannase activity by 1.8-fold, indicating that spent coffee ground can be used to substitute pure tannic acid in the production of tannase via SSF. In other studies, tannin-rich agri-industrial residues such as cashew testa (Viswanath et al., 2016), Indian gooseberry leaves (*Phyllanthus emblica*), Black plum leaves (*Syzygium cumini*), Eucalyptus leaves (*Eucalyptus globus*) and Babul leaves (*Acacia nilotica*) (Kumar et al., 2016) have been used to enhance tannase production.

On the other hand, the addition of tannic acid to rice bran did not improve tannase production, in fact, a lower tannase activity was observed compared to the use of rice bran alone (Fig. 5). In this study, fungal growth was inhibited as shown by the significant reduction in the glucosamine content in RB supplemented with tannic acid. Similar result has been reported by Sabu et al. (2006), who observed that incorporation of tannic acid into wheat bran has significantly reduced tannase activity and the highest tannase production was obtained in wheat bran without the addition of tannic acid. This might be due to the characteristics of tannic acid that easily forms complexes with

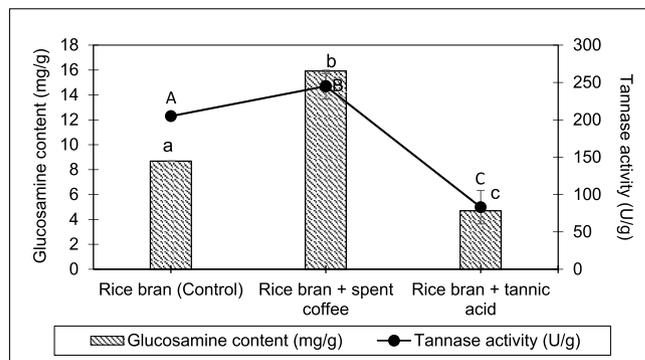


Fig. 5. Comparison of tannase activity and glucosamine content in rice bran used singly or supplemented with inducers. Means with different letters are significantly different (Tukey's HSD, $p < 0.05$).

macromolecules such as carbohydrates, proteins and digestive enzymes, causing a reduction in fungal growth and subsequently, inhibited tannase production (Sabu et al., 2006; Malgiredy and Nimma, 2015). Higher fungal growth was also observed in RB supplemented with CF when compared to RB alone or with tannic acid. This is probably due to the presence of carbon and nitrogen in spent coffee ground (data not shown), which further enhanced tannase production in RB supplemented with CF. The suitability of RB for tannase production could be attributed to its carbon, nitrogen and tannin levels. Although the high tannin content in CF inhibited fungal growth, the supplementation of spent coffee at a low concentration (0.5% TAE) favored enzyme production. The addition of CF, a tannin-rich source, induced tannase production, indicating that it is a viable substitute for pure tannic acid.

The tannase activity found in our study are comparable with those reported by other researchers using other agro wastes under solid-state fermentation. Similar amounts of tannase production by *Aspergillus fumigatus* MA with a maximum yield of 174.32 U/g at 25 °C after 96 h of incubation at pH 5.0 under solid-state fermentation (SSF) of jamun leaves (Manjit et al., 2008). A maximum tannase activity ranging from 97.32 to 301.70 U/gds was obtained by *Aspergillus niger* CEPC 11 (MTCC 5898) under optimised SSF of cashew testa (Viswanath et al., 2016). On the other hand, a slightly lower tannase activity of 87.26 U/gds was attained using *Aspergillus tubingensis* CICC 2651 under solid-state fermentation of tea stalks (Xiao et al., 2015). Another author has also reported considerably lower tannase activity values, with a maximum enzyme yield of 13.03 U/g dry substrate (gds) by *Aspergillus niger* ATCC 16620, using palm kernel cake (PKC) under SSF (Sabu et al., 2005).

4. Conclusions

Agri-industrial residues are, in general, highly nutritious and contain high levels of carbon and nitrogen, facilitating fungal growth and consequently, enhancing enzyme production. The use of such residues as potential cost-effective substrates for enzyme production is therefore, a viable approach. Some agri-industrial residues are high in tannin and can be used as low-cost alternative substrates for pure tannic acid, which is used as substrate in tannase production. Among all agri-industrial by-products tested, highest tannase activity was obtained using rice bran. The incorporation of spent coffee ground (0.5% TAE) into rice bran enhanced tannase production by 1.8-fold and 3-fold in comparison to rice bran alone or rice bran incorporated with tannic acid, respectively. Therefore, solid-state fermentation using nutrient-rich and tannin-rich agri-industrial residues such as rice bran and spent-coffee ground is a promising approach in industrial tannase production. The investigation on other aspects such as the optimisation of fermentation parameters and medium composition could further enhance tannase production by *A.niger* PN1, thereby contributing to the development of the SSF process using agri-industrial residues.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.bcab.2019.101216>.

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