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The Suitability of Natural Tannins from Food and Agricultural Residues (FAR) for Producing Industrially Important Tannase and Gallic Acid through Microbial Fermentation

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Abstract

In India more than 40% of solid waste generated annually is from organic and agricultural sources. There is a growing concern for these accumulating wastes as they are either being dumped in landfills, burnt, or left to rot in the open, leading to severe environmental pollution. A sustainable solution would be to utilize these solid wastes as an 'economical' alternative to costly raw materials and produce industrially important products of practical utility. Tannase and the associated byproduct gallic acid have many potential applications in food, pharmaceutical and chemical industries. Many plant species have naturally occurring tannins in their biomass. The present study focuses on utilization of these solid food and agricultural residues (FAR) as a source of tannic acid for producing tannase and gallic acid through microbial fermentation.

Native micro-organisms were isolated from FAR and soil from FAR dump sites in Delhi NCR region. Screening and selection of tannase producing isolates was made on the basis of zone of clearance obtained of synthetic tannic acid supplemented media. From a total of twenty one isolates studied, one fungal (Aspergillus sp. - F1) and two bacterial isolates (B 2.2 and B 2.7) proved to be comparatively superior. Various FAR tested for the suitability of tannase and gallic acid production were pomegranate peel, spent tea powder, tamarind seed powder, coconut coir, Banana peel, corn husk, keekar and jamun leaf powders. These substrates were used individually as well as in a variety of combinations in a ratio of 1:1 based on the nutrient

availability of the substrates. Our study revealed that the production of tannase and gallic acid was dependent not only on the type of substrate, but also on the specific ratio in which the substrates were combined and the microbial isolate applied for fermenting the substrate. While certain substrate combinations and isolates have shown very poor tannase activity (only 1.97 U/g) and gallic acid (that is as little as 0.77 mg/g), the Aspergillus sp. F1 has shown 10 times more tannase activity. Amongst various substrate combination tested, pomegranate peel with spent tea has shown maximum tannase activity of 19.02 U/g and gallic acid content of 5.31 mg/g of substrate. The study reveals that these tannin-rich FAR could be an economical alternative to the commercially available costlier raw material for industrial production of tannase and gallic acid.

Keywords: Tannase; Gallic acid; Food and Agriculture Residues (FAR); SSF.

1. Introduction

Rapid industrialization and increasing population in India have contributed to an increased generation of variety of solid waste. Currently in India, about 960 million tones of solid waste is being generated annually as by-products during industrial, mining, municipal, agricultural and other processes. More than 40% of the waste will be from agricultural and food sources. There is a growing concern for these wastes, which are mostly being dumped to the landfills and are left to rot in the open, leading to land and water pollution or being burnt thereby contributing considerably to global warming (Pappu et al, 2007; Sharholy et al, 2008). The present day trend involves utilization of these solid wastes as economical raw material for the production of industrially important products of great practical significance. This boosts up high economic returns in many industries. Besides, restricting the quantum of organic wastes reaching the landfills, it also helps to solve pollution problem or otherwise their disposal would be minimized. With the advent of biotechnology, attempts have been made globally to utilize FAR for production of value added products such as enzymes, organic acids, bioactive secondary metabolites etc. There is a huge demand for tannase and gallic acid due to their potential applications in food, pharmaceutical and chemical industries (Aguilar et al, 2007; Paranthaman et al, 2009).

Tannase (tannin acyl hydrolase, E.C. 3.1.1.20) is an inducible, hydrolytic enzyme. It catalyzes the hydrolysis of the ester bonds present in the hydrolysable tannins into tannic acid and gallic acid. Currently tannase is produced on industrial scale by employing microorganisms to metabolize pure (synthetic) tannic acid acting as both inducer as well as available carbon source. Pure tannic acid used as substrate for tannase production is expensive which adds on to the cost of the final product (Cruz-Hernández et al, 2006; Sivashanmugam and Jayaraman, 2011). Apart from this it was observed that few microorganisms utilize tannins as substrates for growth, to produce

tannase and other products which are industrially important. One such industrially important product is gallic acid. Gallic acid is a substrate for the chemical or enzymatic synthesis of propyl gallate, a potent antioxidant used in the food industry and is an important intermediary compound in the synthesis of antibacterial drugs, trimethroprim, used in the pharmaceutical industry. The worldwide annual demand of gallic acid is about 8,000 tons. Conventionally gallic acid is produced by acid hydrolysis of tannins, but this process releases a large amount of toxic effluent that causes environmental hazard. It also involves high production cost, low yield and purity (Aguilar et al, 2001; Paranthaman et al, 2009; Reddy and Kumar, 2011). FAR reported to have hydrolysable tannins are pomegranate peels, banana peels, spent tea leaves, keekar leaves, jamun leaves, coconut coir and tamarind seeds etc. Therefore these FARs, containing natural tannins can be considered as an alternative source of tannic acid for producing tannase. Besides that, it can be proved to be a potential source for production of gallic acid, beneficial for the economic tannase production and alternative method to convert FAR into useful products (Sabu et al, 2006; Battestin and Macedo, 2007).

Hence the present study was focused on (i) Isolation and screening of native micro-organisms for their ability to produce tannse and gallic acid and (ii) Utilization of FAR for the production of tannase and gallic acid (alternative to synthetic tannic acid) through solid state fermentation

2. Material and Methods

2.1 Isolation and enrichment of tannase producing microbes upon selective media

Native Microbes having tannase producing ability were isolated from FAR and soil from FAR dump sites of Delhi NCR region. Fungal cultures as well as bacterial cultures were isolated and enrichment of bacterial/fungal cultures was done using spread plate method on respective enrichment media containing synthetic tannic acid (2%,filter sterilized) as sole carbon source. After obtaining the pure cultures, screening of the pure cultures having better tannase producing ability was carried out as per the procedure demonstrated by Kumar et al, (2010). From a total of twenty one isolates based on their zone of clearance one fungal culture of genus *Aspergillus* (F1) and two bacterial cultures (B 2.2 and B 2.7) were selected. The cultures thus screened were maintained on agar plates containing 2% tannic acid and stock cultures were stored at 4 °C.

2.2 Preparation of FAR used for the production of tannase and gallic acid

Pomegranate peel (PP), spent tea leaves powder (STP), tamarind seed powder (TSP), keekar leaves powder(KL), Jamun leaves powder (JL), coconut coir (CL)and banana peel (BP) were collected from local sources and dried at 60 °C. The dried material was powdered in a grinder and stored in airtight containers for further use. Selection of these FAR and formulation of their combination was based on estimation of two main nutrient supplements i.e. tannin and reducing sugar content of the substrates. There is

published literature on the presence of tannins in these substrates and also information is available on physico-chemical characterization, expressed as total carbohydrate content. In order to design an optimal SSF substrate, it is important to know the actual concentration of tannins and also available sugars in terms of glucose. Moreover the specific nutrient composition of plant/ agro residues could vary from plant variety to variety and also on habit and habitat of the source plant. Hence the actual tannin and reducing sugar content of these FAR was estimated and tabulated in Table 1 to facilitate design of SSF substrate combinations.

Table 1: Reported and estimated tannin and reducing sugar content in FAR chosen for the study.

Raw material	Reported Percentage		Estimated Percentage	
	Tannin content	Carbohydrate content	Tannin content	Reducing Sugar content
Pomegranate peel (PP)	8.00	9.3	18.89	18.6
Spent tea powder(STP)	13 to 17	NA	12.29	1.4
Banana peel (BP)	30	59	10.5	4.0
Tamarind seed powder (TSP)	20	73.68	12.91	4.4
Coconut coir powder	29	NA	10.27	NDL
Keekar leaves powder	4	3.62	0.9	0.98
Jamun leaves powder	3.52	5.72	1.3	1.98

* NDL: not in detectable level; NA data not available.

Tannin content in the FAR was extracted from dried substrate powders using 70% (v/v) aqueous acetone solution as per the protocol discussed in Barman, 2004. The filtrate obtained after extraction was used for the total phenol and non-tannin phenol estimation using Folin-Ciocalteu reagent and absorbance was read at 725nm as per the procedure discussed by Makkar et al, (1993). Tannin content was calculated by subtracting non-tannin phenol from total phenol and expressed on dry mass basis i.e. mg/g substrate.

The reducing sugar concentration in FAR was estimated by DNSA method using dinitro-salicylic acid (DNSA) reagent. The absorbance was recorded at 575 nm against blank and a control set was maintained for all the experiments. The reducing sugar was estimated on the basis of standard curve of glucose and expressed as mg/g of substrate.

2.3 Experimental design for SSF

The FAR used in this study were reported to have tannin content. Some of these substrate were used individually for the production of tannase and gallic acid by different research groups (Sabu et al, 2005; Sabu et al, 2006; Srivastava and Kar, 2009; Jana et al, 2012). The amount of FAR to be utilized in our study was decided by comparing the estimated levels of tannins and available sugars. The requirement of mineral was met by adding 0.5X modified Czapek-Dox media (MCD) devoid of sugars. Care has been taken that in all the combinations tested a minimum of

approximately 2% tannin was present. Based on these estimated values twelve different combinations of FAR were made (Table 2). The studied experimental design thus contained 12 substrate combination X 3 microbial isolates inoculated individually X 3 replicates i.e. 36 treatments with 3 replicates each.

Table 2: Experimental design for the production of tannase and gallic acid.

Combination of FAR	PP	STP	TSP	BP	CC	JL	KL
PP	*	*	*	*	*		
STP	*	*					
TSP	*		*				
BP	*			*			
CC	*				*		
JL						*	*
KL						*	*

Substrate combination were prepared by mixing representative FAR in 1:1 ratio by volume.

2.4 Solid state fermentation for tannase and gallic acid production

Two volumes of different agro and food residue powders were taken in jam bottles (250 ml capacity) as per the experimental design mentioned in the Table. 2. Five volume of 0.5 X concentration of modified Czapek-Dox media (MCD) devoid of sucrose content was used as the wetting medium. Media was autoclaved at 121 °C for 20 min. The sterilized media was cooled to room temperature. All the twelve combinations were inoculated with three organisms separately. The inoculum of fungal culture was prepared by growing the fungal culture on tannic acid agar media for 5 days at 30 °C. Two stubs of 5 mm cut from the growing tip of mycelium of 5 day old culture were used for inoculating the jam bottle containing SSF media. The bacterial culture used was grown overnight in a medium containing 2% tannic acid. Jam bottles were inoculated with 1 ml of overnight grown bacterial culture. The incubation period for fungal culture was for 72 hr and in case of bacterial cultures it is 48 h at 30 °C as per the optimized incubation time in preliminary studies. All the experiments were performed in triplicate.

2.5 Estimation of Tannase activity

Tannase activity was determined using spectrophotometric method based on the formation of chromogen between rhodanine and gallic acid (that is released by the action of enzyme-tannase on substrate i.e. methyl gallate). 100 µl of culture supernatant was used for the estimation of enzyme and absorbance was recorded against blank at 520 nm using Shimadzu UV-1800 spectrophotometer (Sharma et al, 2000). Tannase activity was calculated based on the standard graph of gallic acid and expressed in terms of U/g of substrate.

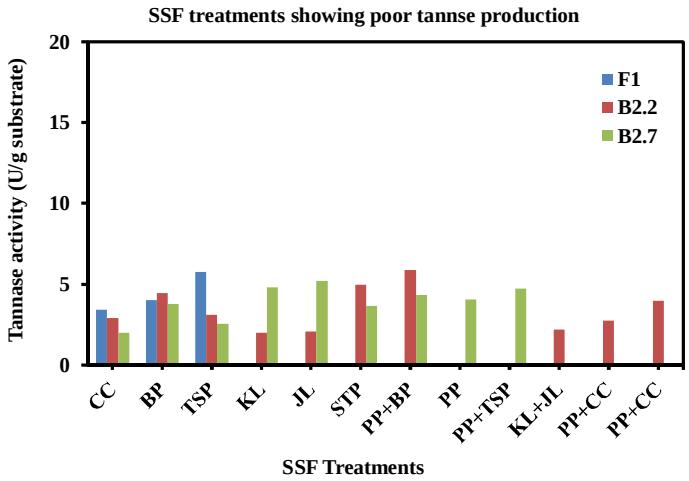
2.6 Gallic acid estimation

Gallic acid was estimated by spectrophotometric method using methanolic rhodanine at 520 nm using spectrophotometer (Shimadzu UV-1800) as per the procedure Sharma *et al,* (2000). Gallic acid content was calculated on the basis of standard curve of gallic acid. Gallic acid is expressed as mg/g of substrate.

3. Result and Discussion

3.1 Suitability of FAR for the production of tannase and gallic acid

Suitability of FAR for producing tannase and gallic acid through solid state fermentation was evaluated on 12 different combinations of FAR, each inoculated by 3 different organisms individually, thus making a total of 36 treatments (12 combinations X 3 microbes) in totality. The three organisms used in this study are: F1, B2.2 and B2.7. The range of tannase production varied between 1.98 U/g to 19.02 U/g and gallic acid from 0.51 mg/g to 5.31 mg/g. Based on the tannase and gallic acid production by different organism on different substrate combinations, results were divided into three groups i.e. poor, moderate and highest producers of enzyme/byproducts. Range for poor, moderate and highest tannase production was 0 - 6 U/g, 6 to12 U/g and 12 to20 U/g substrate, respectively. Similarly the range of gallic acid content for poor, moderate and highest production was 0 to 1.5 mg/g, 1.5 to 2.5 mg/g and 2.5 to 6 mg/g, respectively. Among 36 treatments tested for tannase production, 22 treatments belonged to poor, 10 to moderate and 4 were highest producers (Fig. 1). In case of gallic acid production 23 treatments were poor, 9 were moderate and 4 were highest producers (Fig. 2). The maximum tannase activity of 19.02 U/g was produced by the native isolate of genus *Aspergillllus* F1 with pomegranate peel and spent tea powder in the ratio of 1:1. Same substrate combination and organism were found to produce highest gallic acid content of 5.32 mg/g. The study reveals that the production of tannase and gallic acid is not only dependent on the organism where as it is also dependent on the combination of the substrate and the availability of nutrients particularly sugars in different combinations.



The lower enzyme production in case of other treatments except PP and STP may be attributed to their higher tannin concentration as compared to PP and STP. Concentration of tannin is a very important determining factor for tannase biosynthesis. It was reported that higher concentration of tannin lead to formation of non-reversible bonds with surface proteins of the organisms and impair the metabolism as well as growth of the organism which would finally result in decreased enzyme production (Bradoo et al., 1997; Mondal et al., 2000).

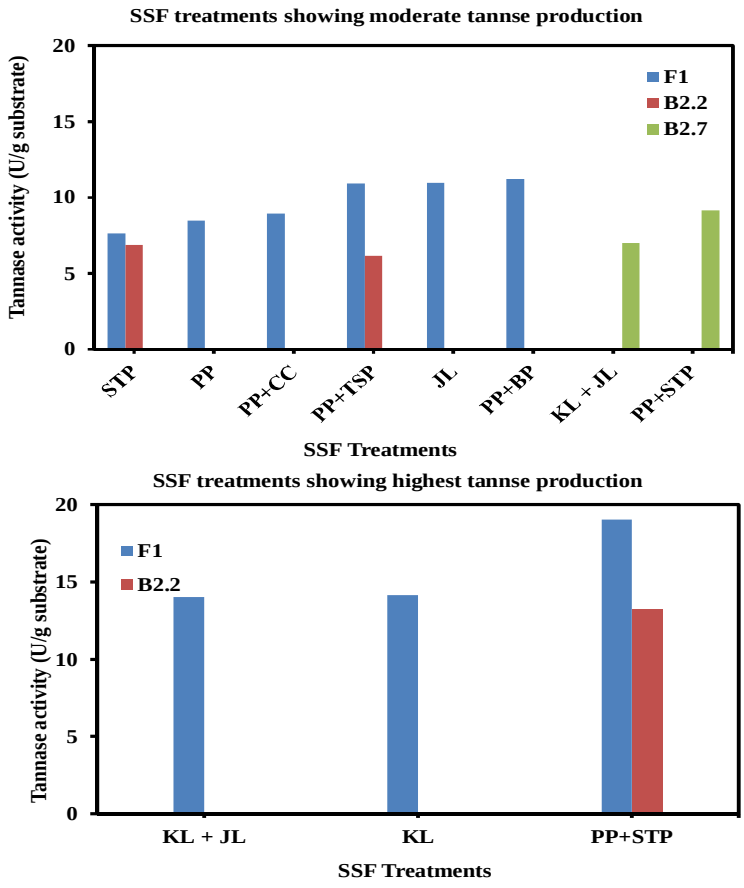
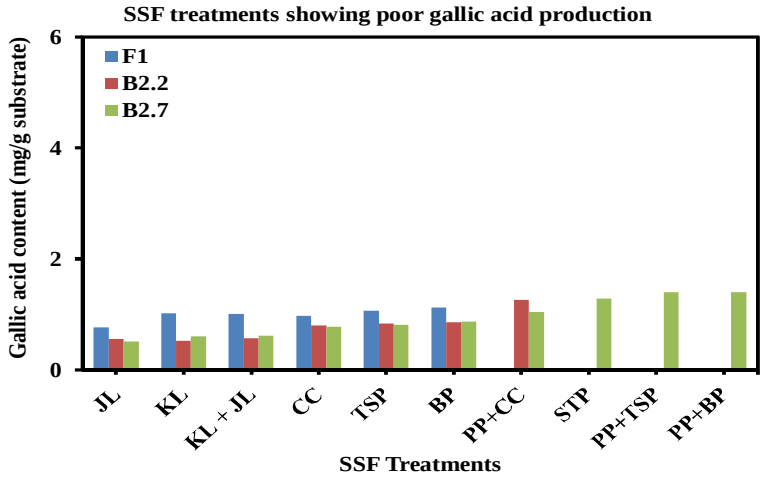
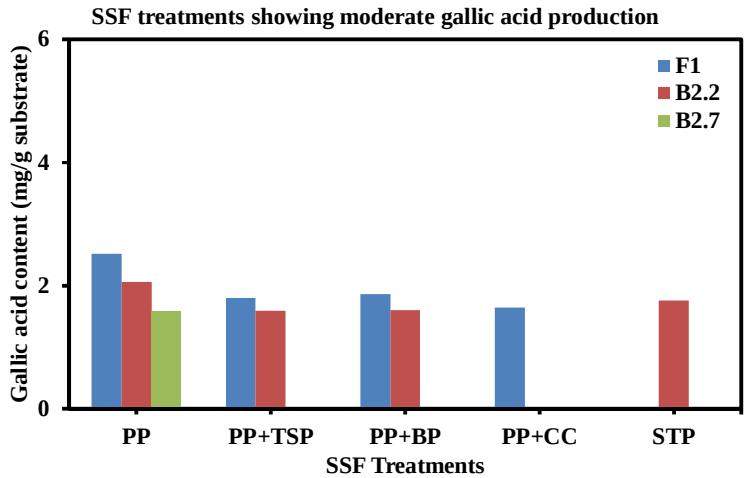


Figure 1: Tannase production on different SSF treatments.

Results from the reducing sugar utilization assay of different treatments have given very conclusive evidence about the metabolic preference of respective microbes to specific substrate concentrations. Reduction in reducing sugar was found to be higher in those treatments that have yielded maximum tannase and gallic acid in SSF. These results clearly indicate that where ever the organism was able to utilize available sugars at a faster pace, the metabolism of tannin substrate was also faster and hence the enzyme and byproduct produced were also higher in such substrates. It was observed that maximum utilization of sugars was up to 70% by the most preferable treatment *viz.*, PP+STP with Fungal F1 (Fig 3). This was closely followed by the next best

substrate combinations in terms of enzyme production i.e., PP where utilization was 62% and KL+JL with 55% utilization. Ready utilization of available sugars from particular substrates supports active growth, metabolism and proliferation of the organism in the substrate and might be the contributing factor for better utilization of the complex tannins present and associated enzyme (tannase) release. Though the treatments KL and KL + JL along with F1 were amongst the four highest tannase producing treatments, gallic acid production was observed to be very low in these substrates. Hence two treatments i.e. PP + SPT with F1 and B2.2 were considered as the most preferable SSF systems for production of tannase and gallic acid. Further optimization studies are in progress in the laboratory to enhance the production of tannase and gallic acid. Optimization of parameters will be carried out with respect to physical parameters such as pH, temperature, moisture content etc. and biological parameters like inoculum size, incubation period etc. Apart from these studies in order to maximize production supplementation of media with synthetic tannic acid and co-culturing of fungal with bacterial culture will also be attempted.





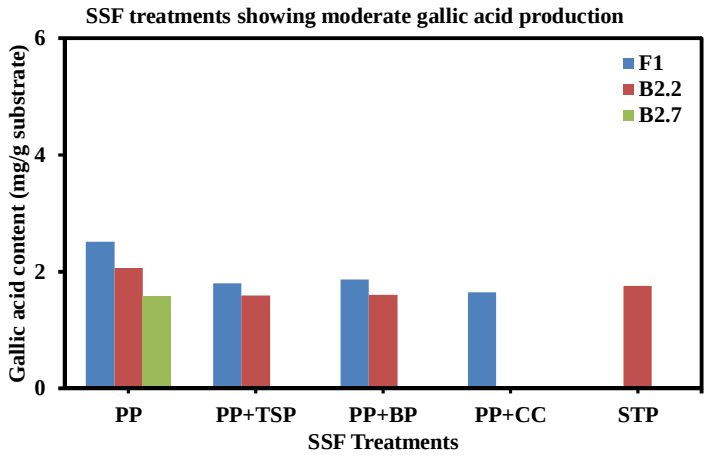


Figure 2: Gallic acid production on different SSF treatments.

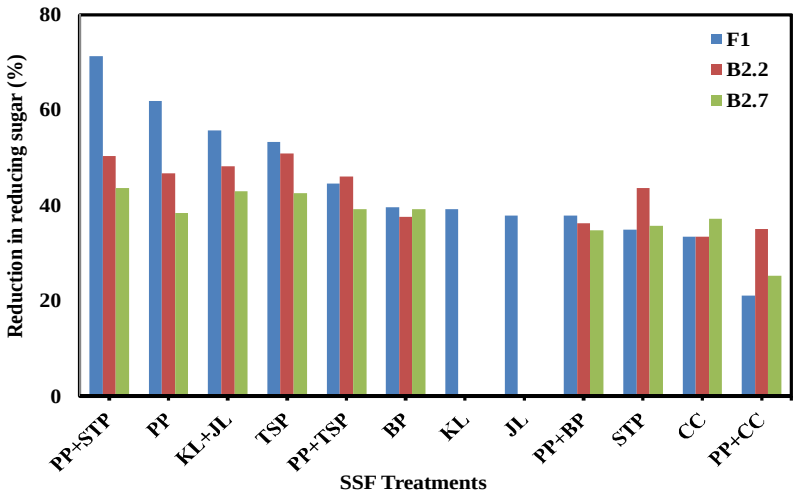


Figure 3: Reducing sugar utilization by different SSF treatments.

4. Conclusions

FAR could be utilized as potential alternatives of synthetic tannic acid for the production of tannase and gallic acid. Thirty six treatments were made using twelve different treatments of substrate with three organisms. Among these 36 combinations, four treatments have been proved to be the best producer of tannase and gallic acid. A 10 fold increase in tannase production was achieved with the best producing combination as compared to the lowest producing combination. The native isolate of the genus *Aspergillus* F1 and bacterial species *Bacilli* B2.2 with PP and STP produced maximum activity of 19.02 U/g and 13.21 U/g respectively. Maximum gallic acid production observed with the same substrate by F1 was 5.32 mg/g and B2.2 was 3.51 mg/g. Further optimization of fermentation conditions with regard to physicochemical conditions can lead to better tannase production.

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