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Characterization of Extracellular Tannin Acyl Hydrolase and Gallic Acid Produced on Pomegranate Rind (*Punica granatum*) under Submerged Fermentation by an *Aspergillus niger* Isolate

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An isolated *Aspergillus niger* strain (ITCC 6514.07) was found to utilize pomegranate rind (PR) as substrate in submerged fermentation (SmF) most efficiently compared to other tannin rich agro residues tested for the production of extracellular tannin acyl hydrolase (E.C.3.1.1.20) and gallic acid. Maximum enzyme production took place at 37°C, pH 5.0 with 4% (w/v) PR and mineral salt solution (MSS) after 72 h. The enzyme production was stimulated 3.3 fold by the addition of 1% tannic acid. The optimum temperature and pH of the partially purified enzyme were found to be 35°C and 6.2 respectively. The K_m value of the enzyme produced on PR as substrate were found to be 0.012 mM. The nature of the experimental data permitted excellent polynomial fit on the basis of which, a master equation corresponding to the isolated strain was derived for estimation of enzyme activity for any set of values of incubation time, substrate concentration, inoculum size, temperature and pH within the indicated range.

Key words: Extracellular, Tannase, Gallic acid, Pomegranate rind, *Aspergillus niger*, Submerged fermentation.

Tannins are widely distributed in nature and occur at high levels in various plants[1]. Tannin acyl hydrolase (E.C.3.1.1.20) commonly known as tannase, catalyses the hydrolysis of the ester and depside bonds in hydrolysable tannins such as tannic acid producing glucose and gallic acid. The enzyme has been used in the prevention of phenol induced mediarization in wine, manufacture of coffee flavoured soft drinks, instant tea, clarification of beer and fruit juices. Gallic acid is used in dyemaking, food, pharmaceutical, leather and chemical industries[2,3]. Because of abundant presence of tannins in various agro residues they can serve as substrates for the production of tannase and gallic acid by appropriate microorganism. Thus agro residues such as *Caesalpinia spinosa* and *Rhus coriaria*[4], *Terminalia chebula* and *Caesalpinia digyna*[5], *Gobernadora*[6], tamarind seed powder and palm kernel cake[7] have been used as a substrate for tannase production by different microorganisms under solid state fermentation (SSF). Conventionally solid insoluble agriculture residues are chosen as the natural substrates for solid state fermentation. Soluble substrates, on the other hand are preferred over insoluble ones for submerged fermentation (SmF) as is evident from

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the published literature. Submerged fermentation is advantageous because of its ease of sterilization and easier process controls. It can be made more attractive economically by using solid agricultural residues as substrate. Bulk of the existing literature on tannase production by SmF report the use of pure tannic acid as substrate and fungal strains of *Aspergillus*[8,9,10], *Penicillium*[11] producing the enzyme at temperature lower than the present strain and *Candida* species[12].

The objective of this work was to establish the potential of the tannin rich agroresidue pomegranate rind (PR), not reported before as a substrate for tannase and gallic acid production by SmF, using an *A.niger* strain, identify the optimum fermentation conditions, characterize the kinetic parameters of the enzyme and examine the effect of tannic acid on the fermentation process. Also a master equation for estimation of enzyme activities for any set of values of these parameters is presented.

MATERIAL AND METHODS

Microorganism

The strain in this work was isolated on Czapek Dox medium containing 1% (w/v) tannic acid on the basis of zone of lysis produced by the strain[13]. The strain was identified as *Aspergillus niger* by the Indian Type Culture Collection New Delhi, and deposited in their culture collection unit (ID no. ITCC 6514.07).

Inoculum preparation

The culture was maintained on tannic acid agar slants stored at 4^0C and subcultured routinely after every three-four weeks. For inoculum preparation the culture was grown at 37°C for 7 days and the spores (5.0×10^7) were scraped into 5 ml of sterile Tween 80 solution which was used to inoculate 50 ml of fermentation medium.

Selection of substrate

Tannins are widely distributed in various plants. Tannin content was estimated in agro residues such as brewed tea, pigeon pea coat, almond shell, pomegranate rind and bark of trees like *Eucalyptus* and *Terminalia* arjuna. Among all the substrates tested PR was found to have the highest tannin content of 17% (w/v) and was selected as a substrate for the present work.

Preparation of substrate

Pomegranate rind was spreaded on trays and oven dried at 70°C for 24 h. The dried rind was ground and sieved to obtain particle size of 425 mm and stored in polyethylene bags at room temperature (30 ± 5°C).

Medium composition and growth conditions

Fifty milliliter of Czapek dox medium supplemented with 4% (w/v) PR as the sole carbon source containing (g/l): $NaNO_3$ 6.0; KCl 0.52; $MgSO_4.7H_2O$ 0.52; KH_2PO_4 1.52; $Cu(NO_3)_2.3H_2O$ traces; $ZnSO_4.7H_2O$ traces; $FeSO_4$ traces pH 4.0in 250 ml Erlenmeyer flask was inoculated with 5.0×10^7 spores and incubated at 37°C in an orbital shaker at 220 rpm for 96 h. Extracellular tannase and gallic acid were estimated at an interval of 24 h, from the fermented broth. All the experiments were carried out in triplicates and analyses were done in duplicates. Mean values are shown in the table and figures.

Tannase assay

Tannase was estimated by the method based on chromogen formation between gallic acid and rhodanine (14). The reaction mixture containing 0.25 ml of 0.01 M methyl gallate in 0.05 M citrate buffer and 0.25 ml of extracellular enzyme was incubated at 30°C for 10 min and 0.3 ml of methanolic rhodanine (0.667% w/v) was then added. After 5 min. 0.2 ml of 0.5 M KOH was added. A control was run where enzyme was added after the addition of KOH. Finally the reaction mixture was diluted by 4.0 ml distilled water and incubated at 30°C for 10 min and absorbance was recorded at 520 nm. One unit of tannase is the amount of enzyme which liberated 1ìmol of gallic acid in one minute.

Gallic acid estimation

Gallic acid was estimated in the fermented broth. To 0.5 ml of broth, 0.3 ml of methanolic rhodanine was added followed by 0.5 M KOH and gallic acid content was estimated by the method described above using a calibration graph using (10mg- 50mg) of gallic acid.

Optimization of process parameters

Optimum tannase and gallic acid production were determined for incubation period (24 h-96 h), substrate concentration (1%-5% w/v), temperature (30-40°C), pH (3.0-7.0) and inoculum size (5% - 15% v/v). Effect of tannic acid (0.2% - 1.5% w/v) on tannase and gallic acid

Combining the above equation (1 to 5), one obtains the following universal equation (6) for the enzyme activity which can be used to obtained the value of the enzyme activity in units/ml for any set of values of t (Incubation time between 24 and 96 h), S (substrate concentration between 1 to 5%), I (Inoculation size between 5 to 15%), T (temperature between 30°C and 40°C), pH (between 3.0 and 7.0).

Universal Equation

$$EA(t, S, T, p, I) = [EA(t)] \times [EA(S)] \times [EA(T)] \times [EA(p)] \times [EA(I)] / A \times B \times C \times D \quad (6)$$

where A = 29.78, B = 28.72 , C = 28.72, D = 28.72, A,B,C,D being the normalization constants.

CONCLUSIONS

The tannin rich agroresidue, pomegranate rind has been used in this study for the first time as a substrate for tannase and gallic acid production under SmF by an isolated *A.niger* species, at 37°C rarely reported so far in the literature. The optimum fermentation conditions and kinetic parameters of the enzyme are also being reported. A master equation has been provided that permits the estimation of the enzyme for the isolated strain for any set of values of incubation time, substrate concentration, inoculum size, temperature and pH within the indicated range.

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