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Enhanced tannase production by *Bacillus subtilis* PAB2 with concomitant antioxidant production

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ABSTRACT

The present study was aimed at finding the optimal conditions for tannin biodegradation by *Bacillus subtilis* PAB2, a newly isolated soil bacterium, and to study its fermentative byproducts. Tannase production by *B. subtilis* PAB2 in optimum level was studied by one variable at a time (OVAT) approach followed by Box–Behnken response surface methodology (RSM) using six important variables. The maximum tannase production (10.69 U/ml) was achieved in the presence of 0.47% (w/v) tannic acid, 0.23% (w/v) ammonium chloride (NH_4Cl), 0.1% (w/v) potassium dihydrogen phosphate (KH_2PO_4) and 0.046% (w/v) magnesium sulphate ($MgSO_4$) with the initial medium pH of 5.9 and incubation at 34.1 °C for 36 h under shaking condition (120 rpm). An overall 2.06 fold increase in tannase production was achieved after RSM global formulation. One of the major end product of tannin degradation i.e. gallic acid, was accumulated in the highest level (6.45 mg/ml) in 36 h of fermentation. Liquid chromatography and mass spectroscopy results indicated the presence of both gallic acid and pyrogallol in the fermentative end product. Purification of gallic acid and pyrogallol was achieved through high pressure liquid chromatography followed by crystallization and confirmed by Fourier transform infrared spectroscopy. Associated production of tannase as well as gallic acid and pyrogallol by *B. subtilis* PAB2 makes it obvious in different biotechnological interest.

1. Introduction

Tannins are water-soluble polyphenolic secondary metabolites of plants and believe as the fourth most profuse constituents after cellulose, hemicelluloses and lignin. They are found in a large array of herbaceous and woody plants (Scalbert, 1991). Two groups of tannins are eminent according to their structures: hydrolyzable and condensed ones (Regerat et al.,1989). Tannins play an important role in the immunity of the plants by protecting the vulnerable parts of the plants from microbial attack by inactivating viruses and invasive microbial extracellular enzymes (Field and Lettinga, 1992). Inspite of some beneficial effect, tannin also causes various processing and nutritional problems like protein indigestibility, inhibition of enzymatic reactions and essential microbial processes for beer brewing (Chavan et al., 1979; Watson, 1975). Thus high concentrations of tannins depress voluntary feed intake, digestive efficiency, animal productivity and develop some forms of cancer (Reed, 1995). Hydrolysable tannins are readily hydrolyzed chemically by acidification or biologically by an enzyme known as tannase (tannin acyl hydrolase, E.C. 3.1.1.20).

Tannase is an industrially important inducible enzyme that hydrolyzes ester and depside linkages in hydrolysable tannin to gallic acid and glucose. Tannase is used pervasively in different industrial sectors like in manufacturing of instant tea, acron wine, coffee-flavoured soft drinks, clarification of beer and fruit juices (Das Mohapatra et al., 2007; Lekha and Lonsane, 1997) etc. Gallic acid, the hydrolytic product of tannic acid, is also used in the synthesis of propyl gallate (a potent antioxidant), trimethoprim (an antibacterial agent) and as photosensitive resin in semiconductor production (Das Mohapatra et al., 2006; Hadi et al., 1994). It can be used in the manufacture of ordinary writing inks and dyes, as photographic developer, in the tannery industry for homogenization of tannins, for the production of pyrogallol and gallic acid esters among other compounds. Pyrogallol (metabolic end product of gallic acid) also have several industrial importance for staining of leathers, fur, colouring of hair, photographic plate developing agent, anti-lung cancer drug, anti-tumor drug etc. (Zeida et al., 1998). Microbial production of tannase is most preferable in pharmaceutical and food industries due to maximum production in the shortest period of cultivation (Ayed and Hamdi, 2002). Fungi, bacteria and yeast are the major tannase producing

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microbes, few of them have been reported as potent industrial strains (Belmares et al., 2004; Belur et al., 2010; Beniwal and Chhokar, 2010). Production optimization and physico-chemical characterization of fungal tannase has been extensively studied in a number of scientific publications (Rodriguez et al., 2011), but information about bacterial tannase is very sparse. That is why, the researcher are continuously searching for a potent bacterial strain which could be exploited industrially and meet the high rise global demand of tannase.

During pilot scale production of secondary metabolites, the physico-chemical environments of microbes are fully controlled externally. The optimization of fermentation conditions is an important issue in the development of economically feasible bioprocesses. Designing an appropriate fermentation condition is of significant importance because it is an important corner stone which greatly influence product concentration, yield, and volumetric productivity (Haaland, 1989). The conventional, one variable at a time (OVAT) method has several inadequacies towards complete optimization. Being single dimensional, this laborious and time-consuming method often does not guarantee for determination of optimal conditions (Box and Behnken, 1960). Statistical optimization not only allows quick screening of large experimental domain but also reflects the role of each of the factors and their interactions.

In the present investigation, a newly isolated bacterium *Bacillus subtilis* PAB2 has been used to evaluate its tannase production ability to convert tannin into gallic acid and pyrogallol by optimizing tannase production through submerged fermentation. The changes in tannin content during fermentation, the accumulation of antioxidants (gallic acid and pyrogallol) and the enzymes produced in fermentation media of were also estimated.

2. Materials and methods

2.1. Chemicals

All the chemicals used in this study were of analytical grade and procured from Himedia, India and Merck, India.

2.2. Isolation and identification of potent tannase producer

A number of tannase producing bacterial strains were screened by serial dilution technique from the soil of Gurguripal sal (*Sorea robusta* Gaertn.f.) forest, Midnapore, West Bengal, India using selective tannic acid agar medium containing (g/l): tannic acid, 5.0; NH_4Cl, 3.0; KH_2PO_4, 0.5; $MgSO_4$, 0.5; glucose, 0.1 and agar, 30.0. The medium pH was adjusted to pH-5.0 $\pm$ 0.2 and then filter sterilized. The medium was inoculated with the serially diluted soil sample and incubated at 35 °C for 24 h. Appearance of clear transparent zone around the colony was selected as tannase producing strain. The bacterial strain with the highest clear zone to colony size ratio (*cz*/*cs*) was selected and designated as PAB2. The strain was preserved at 4 °C and subcultured at a regular interval on tannic acid agar slant.

The selected bacterium (PAB2) was identified on the basis of its 16S rDNA sequence. DNA from the bacterial cells was isolated using QIAamp DNA Purification Kit (Qiagen, Japan) and electrophoresed in agarose gel. Fragment of 16S rDNA gene was amplified by PCR upto 30 cycles (using the following profile: initial denaturation, 95 °C for 2 min; final denaturation, 94 °C for 30 s; annealing, 52 °C for 30 s; extension, 72 °C for 90 s; final extension, 72 °C for 10 min). Amplified PCR product was purified using Qiagen Mini elute gel extraction kit (Qiagen, Japan). Forward and reverse DNA sequencing reaction of PCR amplicon was carried out with 8–27F (5′AGAGTTTGATCCTGGCTCAG3′) and 1492R (5′ACGGCTACCTTGTTACGACTT3′) primers using BDT v3.1 Cycle sequencing kit on (ABI 3730xl) Genetic Analyzer (Maity et al., 2011). A single discrete PCR amplicon band of 1500 bp was observed when resolved on 1.2% agarose gel. The 1432-bp assembled 16S rDNA sequence was compared with the nr database of NCBI Genbank using MEGABLAST algorithm. Based on maximum identity score, first 10 sequences were selected and aligned using multiple sequence alignment software program ClustalX2. The evolutionary relatedness was inferred using the Neighbor-Joining method (Saitou and Nei, 1987) and distance matrix was generated using RDP database and the phylogenetic tree was constructed using Phylip-3.69 software (at 100 bootstrap).

2.3. Enzyme assay

Tannase activity in the fermented medium was determined by the colorimetric method of Mondal et al. (2001b). One unit (U) of tannase was defined as the amount of enzyme, which is able to hydrolyze 1 μ mole of the ester linkage of tannic acid per minute.

2.4. Estimation of gallic acid

Gallic acid in the culture filtrate was estimated by the method of Bajpai and Patil (1996). Filtrate was diluted to 100-fold in 0.2 M acetate buffer at pH 5.0. The absorbence was recorded at two selective wavelengths of 254.6 and 293.8 nm. The concentration of gallic acid was measured using specific extinction coefficient by the following equation:

Concentration of gallic acid (mg/ml) = 21.77 ($A_{254.6}$) − 17.17 ($A_{293.8}$).

2.5. Optimization of tannase production using OVAT approach

The effect of several physico-chemical parameters on tannase production was evaluated and optimized. The effect of physical parameters like, e.g. incubation period (12–120 h), temperature (25–45 °C), pH (5.0–7.0), inoculum volume (0.5–5% v/v) and chemical parameters like tannic acid concentrations, various nitrogen sources, phosphate sources, metal salts and secondary carbon sources were evaluated for tannase production. The whole optimization process was carried out using freshly grown PAB2 strain (24 h incubation and A_{620}~1.28) grown in tannic acid broth (composition stated in Section 2) at 35 °C and 120 rpm.

2.6. Optimization of tannase production using RSM

Tannase production was further optimized by RSM, using Minitab (Minitab Inc., USA) statistical and process management software. The randomized factorial design of the whole experiment consisted of six variable influencing factors, i.e. incubation temperature (°C) [30(−1), 35(0), 40(+1)], pH [5.0 (−1), 6.0(0), 7.0 (+1)], tannic acid concentration (%) [0.3(−1), 0.5(0), 0.7(+1)], ammonium chloride concentration (%) [0.1(−1), 0.3(0), 0.5(+1)], potassium dihydrogen phosphate (%) [0.05(−1), 0.1(0), 0.15(+1)] and magnesium sulphate (%) [0.03(−1), 0.05(0), 0.07(+1)] were studied at the optimum condition of enzyme catalysis. Following this design total of 54 experiments were conducted (Table 1). The response was tannase activity (U/ml).

The RSM obtained data were subjected to the analysis of variance (ANOVA). The statistical significance of the model equation was determined by Fisher's test value, and the proportion of variance explained by the model was given by the multiple coefficient of determination, *R* squared (R^2) value. The three dimensional graphs were generated to elucidate the effect of selected variables individually and in combination to determine their optimum level for maximal production of tannase. Finally a time course study was conducted keeping all unoptimized and optimized parameters.