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Effect of Fermentation Parameters on Extra Cellular Tannase Production by *Lactobacillus plantarum* MTCC 1407

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Abstract: *Lactobacillus plantarum* MTCC 1407 represents a valuable source of an economically attractive stable long-life tannase with potential for application in various industries. The effect of fermentation parameters such as pH, temperature and agitation speed on the growth of biomass and production of tannase using liquid medium were determined at the end of fermentation period. The optimum values of pH, reaction temperature and agitation speed for tannase activity were 6.0, 30 °C and 125 rpm respectively. The maximum tannase activity was found to be 9.29 U/mL.

Keywords: Tannase, Tannic acid, Agitation speed, Optimum, Fermentation, Temperature.

Introduction

Tannase (tannin acyl hydrolase, E.C.3.1.1.20) is an inducible, extra cellular hydrolase enzyme that catalyzes the breakdown of ester and depside bonds present in hydrolysable tannins or gallic acid esters, liberating glucose and gallic acid (GA). Tannase cleaves the ester linkages between galloyl groups present in various compounds such as epigallocatechin and epigallocatechin gallate that are present in green tea leaves[1]. Tannase find wide speared application in the field of food industries used as antioxidant and is mainly used in the production of gallic acid, instant tea, acron wine, coffee flavored soft drinks and high grade leather tannin. Tannase is also used as a clarifying agent in clarification of beer, fruit juice and various food stuffs. Act as a hydrolyzing agent in cleaning up the highly polluting tannin (polyphenols) from the effluent of leather industry[1].

Tannin rich parts of the plants such as fruits, leaves, branches and barks possess considerable amount of tannase. Plants like penduculate oak (*Quercus rubra*), myrobolano (*Terminalia chebula*) and badul were rich in tannase[2]. Tannase can be extracted from bovine intestine and ruminal mucous. The enzyme produced from microbial sources find immense application in various industries due to its higher stability and availability. Among the various microbial sources for tannase production, filamentous fungi like *Ascochyta, Aspergillus, Chaetomium, Mucor, Myrothecium, Neurospora, Rhizopus, Trichothecium, Fusarium, Trichoderma* and *Penicillium* were studied extensively[1]. Tannase producing yeasts have also been isolated but they were not extensively studied. Bacterial sources such as *Bacillus, Corynebacterium, Klebsiella*[3], *Streptococcus bovis* and *Selenomonas ruminantium*[4] have been studied for tannase production. Lactic acid bacteria play a vital role in hydrolyzing tannins present in food and intestines. High tannase activity was reported in lactic acid bacteria *Lactobacillus plantarum*[5].

Microorganism maintenance and inoculum preparation

Lactobacillus plantarum MTCC 1407 was obtained from the Microbial Type Culture Collection (MTCC) and Gene Bank Centre, Institute of Microbial Technology, Chandigarh, India. The *Lactobacillus plantarum* MTCC 1407 stock culture was maintained in agar slants containing (g/L); beef extract, 10.0, glucose, 20.0, yeast extract, 5.0, peptone, 10.0, Na_2HPO_4, 2.0, sodium acetate, 5.0, triammonium citrate, 2.0, $MgSO_4.7H_2O$, 0.2, $MnSO_4.4H_2O$, 0.2, agar, 2.5 and tween 80, 1 (mL/L). Inoculum was prepared by growing the organism in 100 mL sterile seed medium (composition same as maintenance medium excluding agar) in 250 mL Erlenmeyer flask for 24 h on a temperature controlled rotary shaker at 35 °C and 120 rpm. The medium components and chemicals used in this study were procured from Himedia Ltd, Mumbai, India.

Batch fermentation

The tannase production by *Lactobacillus plantarum* MTCC 1407 was conducted in 250 mL Erlenmeyer flask with 100 mL of the production medium. The production medium was adjusted to the initial pH of 5.5 using 1 M NaOH or 1 N HCl and sterilized (121 °C for 20 min). Growth profile of the organism *Lactobacillus plantarum* MTCC 1407 in the seed medium was shown in Figure 1. The production medium was inoculated with 5% (v/v) of seed culture in the mid exponential phase at 24 h (Figure 1). The flasks were incubated in an orbital shaker at 120 rpm and 30 °C for the fermentation period of 60 h. Aliquot of sample from the fermentation broth was withdrawn at 6 h interval without much change in the culture volume to maintain constant oxygen transfer. The cells were separated from the medium by centrifugation at 10,000 rpm for 15 minutes. The clarified supernatant was used for the analysis of tannase activity, protease activity, total soluble protein and glucose.

Tannase activity assay

Tannase activity was determined spectrophotometrically by the method of Libuchi *et al*[6]. 0.5 mL of culture supernatant (crude enzyme) was added to 2.0 mL of 0.35% (w/v) tannic acid solution in 0.05 M citrate buffer (pH 5.5) in a test tube. 20 µL of the reaction mixture was taken out and 2.0 mL of 95% ethanol was added to the reaction mixture to stop the enzyme reaction. The absorbance at 310 nm was noted immediately (t_1). The test tube was then incubated in a water bath at 37 °C for 10 min (t_2), after which ethanol was added to the reaction mixture (20 µL) to stop the enzyme reaction. The absorbance was measured at 310 nm. One unit (U) of tannase activity is defined as the amount of enzyme required to hydrolyze 1 µ mol of ester in 1 min and is expressed as unit/mL min.

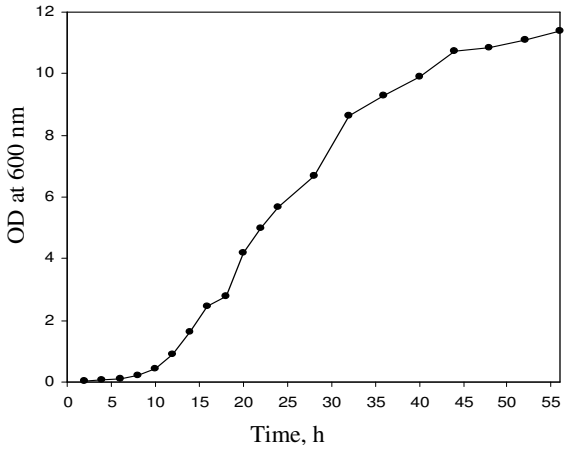

Figure 1. Growth profile of *Lactobacillus plantarum* MTCC 1407 in the seed medium (g L^{-1}): beef extract- 10.0, glucose - 20.0, yeast extract - 5.0, peptone - 10.0, Na_2HPO_4 - 2.0, sodium acetate - 5.0, triammonium citrate - 2.0, $MgSO_4.7H_2O$ - 0.2, $MnSO_4.4H_2O$ - 0.2 and tween 80 - 1 (mL L^{-1}).

Results and Discussion

The objective of the present study is to optimize the fermentation parameters for tannase production by *Lactobacillus plantarum* MTCC 1407. Various reports on microbial production of tannase revealed that the fermentation parameters namely culture condition, culture composition, and substrate concentration had significant effect on production of tannase and biomas[7].

Effect of fermentation parameters on tannase production

Effect of pH on tannase production

The fermentation medium containing (g/L): tannic acid -13.16, glucose -1.5, NH_4Cl -1.0, $CaCl_2$ -1.0, K_2HPO_4 -0.5, KH_2PO_4 -0.5, MgSO4.7H2O -0.5 and $MnSO_4.7H_2O$ -0.03 was obtained the maximum tannase production. The optimized fermentative culture medium selected for further optimization of physicochemical parameters, such as pH, temperature and agitation speed. To study the effect of pH on tannase production, the pH ranges from 4-8 was selected and other fermentation parameters kept constant level. The optimum pH for the maximum tannase activity was found to be 6 (Figure 2). Upon varying the pH from (4.0–8.0), the tannase enzyme was active at acidic pH and activity decreased as the pH approached to the alkaline pH range. From the result of optimized pH value, it could be concluded that tannase enzyme need an acidic environment to be active in the case of produced by *Lactobacillus plantarum* strain. This result shows the similarity with earlier published reports by Batra and Saxena[8]. Naturally any changes in pH may affect the protein structure and a decline in enzyme activity beyond the optimum pH could be due to enzyme inactivation or its instability nature. Tannase has been reported to be an acidic protein, with an optimum[1] pH around 5.5. There are reports describing of the optimum pH as 5.5 of tannase obtained from *A. oryzae* and in the case of *P. chrysogenum* and *A. niger* using as microbial source can be obtained maximum[9] tannase activity at 6.0. Normally the effect of pH on the enzyme activity is determined by the nature of the amino acid at the active site, which undergoes protonation and deprotonation and by the conformational changes induced by the ionization of the amino acids.

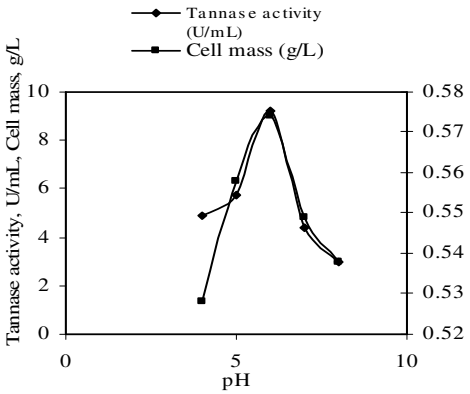

Figure 2. Effect of initial pH on tannase production.

Effect of temperature on tannase activity

The optimized medium was selected to study the effect of temperature on tannase production by changing the temperature and kept all other fermentation conditions were constant. The optimum temperature was found to be 30 ºC when the effect of temperature (20–40 ºC) on enzyme activity was studied. There was an increase in tannase activity with an increase in temperature up to 30 ºC, and a subsequent decrease. The optimum temperature for tannase production was 30 ºC shown in Figure 3. The higher temperature range is preferred for the industrially useful enzymes. But in the case of tannase enzyme the activity did not increase continuously along with the temperature nature. When the temperature increases, the kinetic energy of the substrate and enzyme molecules also increase which affects the reaction rate. The number of collisions per unit time of tannase activity and its substrate, tannic acid increases, resulting in a higher activity with the continuous increases in the temperature level. When the optimum level of temperature obtained, the energy of the molecules increased thorough out the process, but the chemical potential energy increases enough, some of the weak bonds determining the three-dimensional shape of the active proteins break leading to thermal denaturation of the tannase protein causing its inactivation.

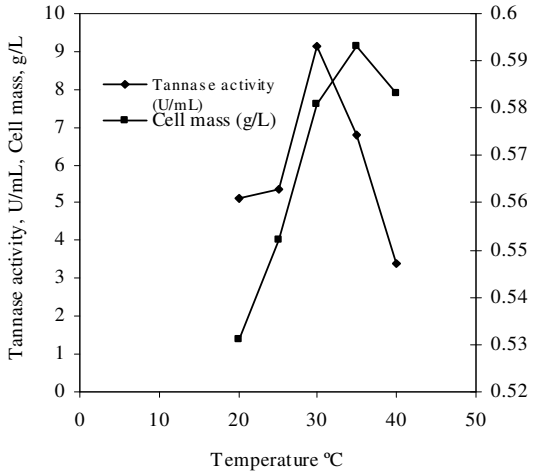

Figure 3. Effect of temperature on tannase production.

Thus, an increase in temperature beyond the optimum value caused reduces in the catalytic rate of tannase as either the enzyme or substrate became denatured and inactive. Here the temperatures above the optimum (30 ºC) level also affect the protein active state, which thus resulted in a reduction in enzyme activity. The optimum temperature for fungal tannase in most of the cases has been found to be near about 30 ºC. Yamada *et al*[9]. reported an optimum of 30 ºC for tannase production by *Aspergillus flavus* and Rajakumar and Nandy[10] found 28 ºC as the optimum for tannase production by submerged fermentation using *Penicillum chrysogenum* NCIM 722.

Effect of agitation speed on tannase activity

The above mentioned medium was selected to study the effect of agitation speed on tannase production by subjective the different agitation speed and the other conditions temperature of the fermentation was maintained at 30 ºC, initial pH 6.0 and the inoculum size of 5% v/v. The optimum agitation speed for the maximum tannase activity was found to be 125 rpm (Figure 4) upon varying the agitation speed (50-200 rpm). The increase in agitation rate beyond 200 rpm resulted in a drastic fall in specific enzyme activity. The agitation speed below 150 rpm resulted in inadequate mixing of the broth towards the later stages of growth.

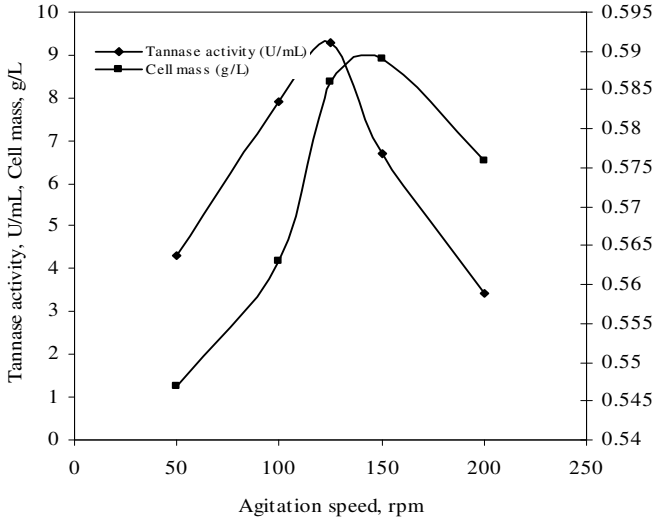

Figure 4. Effect of agitation speed on tannase production.

From the present work tannase production by *Lactobacillus plantarum* MTCC 1407 was found to be growth associated in all the fermentation runs conducted, as the tannase production increased with the cell mass. Mondal and Pati[7], reported that the tannase production was directly proportional to the growth of *Bacillus licheniformis* KBR6 and the extracellular enzyme accumulation increased with the number of cells.

Kinetics of tannase production

The kinetics of tannase fermentation using *L. plantarum* was studied using various culture medium at 30 ºC, initial pH of 5.5 and agitation speed at 120 rpm. The maximum tannase production was observed in the medium contains the following composition (g/L): tannic acid - 10, glucose - 1, NH_4Cl- 3, $MgSO_4.7H_2O$ - 2, KH_2PO_4 - 0.5, K_2HPO_4 - 0.5 and $CaCl_2$ - l. The kinetic profile of tannase activity, cell mass concentration, substrate utilization, and pH is given in Figure 5.

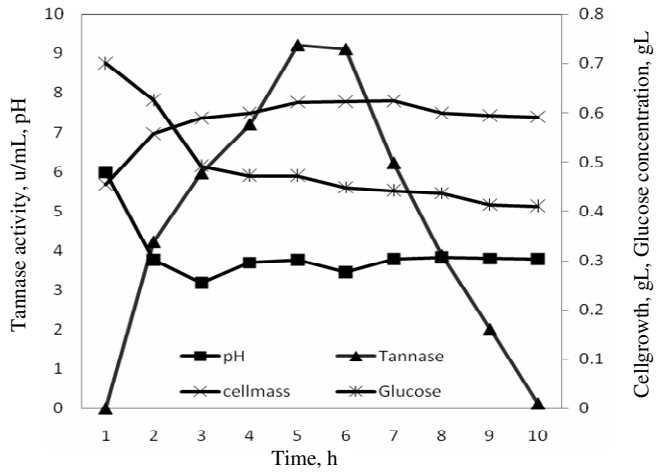

Figure 5. Profile of tannase activity (▲), glucose concentration (∗), pH (■), and cell mass concentration (×) in batch tannase fermentation by *Lactobacillus plantarum.*

The tannase production was found to increase gradually from 8 h of the fermentation period when the growth of the microorganism reaches the early exponential phase which shows that the tannase production is growth associated product. The maximum tannase activity was found in the mid exponential phase and early stationary growth phase of *L. plantarum* as reported by Ayed and Hamdi[5].

Conclusion

This is the first report of bacterial tannase production by *Lactobacillus plantarum,* describing the effect of fermentation parameters to the best of our knowledge. The *Lactobacillus plantarum* strain has the potential to produce tannase in the medium containing both higher amount of tannic acid and lower amount of glucose as carbon source. And also the advantage of bacterial strain is that it can produce maximum product yield within a short period of cultivation time. From the results obtained the best favorable condition for the production of tannase by *Lactobacillus plantarum* were: temperature 30 °C, pH 6.0 and agitation speed 125 rpm.

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