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OPTIMIZING THE REACTION CONDITIONS FOR EXTRACELLULAR TANNASE FROM ASPERGILLUS TPF-4.

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ABSTRACT

The present research investigation reports an attempt to optimize the reaction conditions for previously purified tannase from *Aspergillus* sp. TPF-4. Buffer system, buffer pH, buffer molarity, reaction temperature & reaction time were the main parameters that were optimized for maximum activity of *Aspergillus* TPF-4 tannase. Furthermore the effect of various metal ions and inhibitors on specific activity of enzyme was also assessed. 0.05M citrate buffer (pH 5.5) was reported as the most prodigious for maximal activity of tannase amongst various buffers tested. The optimum temperature and best reaction time recorded for maximum tannase activity was 45°C & 3 minutes respectively. Sodium azide was reported as the most potent inhibitor of tannase activity amongst various metal ions and inhibitors tested. The prospects of tannin hydrolysis by purified tannase from *Aspergillus* TPF-4 tannase look quite promising. Thus future studies to enhance the tannin hydrolysis rate as well as to assure enhanced process control for increased activity of tannase would be envisaged.

KEYWORDS: Purified tannase, *Aspergillus* TPF-4, optimization, potent, inhibitor, maximal activity.

INTRODUCTION

Tannase or tannin acly hydrolase (E.C.3.1.1.20), is an inducible microbial enzyme that catalyzes the hydrolysis of ester and depside bonds in hydrolysable tannins like gallotannins and gallic acid esters to release gallic acid and glucose.[1] Various sources of hydrolysable tannins principally utilized in industrial sector are chestnut (*Castanea sativa*, Bhat *et al.*, 1998), sumac (*Rhus coriaria)*, myrobalan nuts (*Terminalia chebula)*, chinese gall *(Rhus semialata)*, chestnut gum arabic tree (*Acacia nilotica*, Lal *et al.*, 2012), red gram (*Cajanus*

cajan, Kuppusamy *et al.*, 2015) and Cashew testa (*Anacardium occidentales*, Lokeshwari, 2016).[2,3,4,5] Tannins are the fourth most abundant plant constituents after cellulose, hemicelluloses and lignin. Tannase can be obtained from tannin rich plants and animal tissues, but, microbial sources are preferred for industrial applications on account of their better stability and credibility. Microbial enzymes hold a clear cut upper edge over chemical technologies for various industrial applications since microbial enzymes offer several advantages like better control over various process parameters, environmental safety, process reproducibility, ease of cultivation & reusability of microorganisms etc. Over the last 150 years of tannase research fungal tannase has received a great deal of attention for industrial applications credited to higher activity over a broad range of temperature and pH. From the various research studies involving tannase production from microbial sources, it is quite evident that fungal tannase has been most widely studied and utilized for industrial applications in comparison to bacterial tannase. Amongst various fungal sources of tannase *Aspergillus* sp. has been most profoundly studied and significantly utilized for efficient tannase production. Even though tannase production from microbial sources has been studied worldwide over the years, but it is still considered as one of the costly enzymes loaded with enormous biocatalytic potential for utilization in various industrial applications. Thus in this context it always remains a challenging task to search out potent tannase produces with better activity and stability. So keeping the above facts in view it was considered meaningful to optimize the reaction parameters for previously purified extracellular tannase from *Aspergillus* TPF-4 isolated from soil sample of Gharuan, Punjab.

2. MATERIAL AND METHODS

2.1 Inoculum preparation & enzyme production

Inoculum preparation from fully sporulated culture of *Aspergillus* TPF-4 isolated from soil sample of Gharuan, Punjab was done on Potato Dextrose Agar plates. Inoculation of spore suspension of *Aspergillus* TPF-4 was accomplished in production media (100ml) having Potato Dextrose Broth 2.4g; yeast extract 1.0g and tannic acid (2.5g). Incubation of the culture was done at 30^{o}C for 120 h in an incubator shaker at 100 rpm. Culture contents were centrifuged at 10,000 rpm for 10 min at 4°C. Cell biomass was separated by utilizing Whatman No. 1 filter paper and supernatant (crude enzyme) was assayed for tannase activity and amount of protein content. Crude enzyme was purified thereafter through ammonium sulphate precipitation method and chromatographic techniques.

2.2 Optimization of physicochemical reaction parameters

Optimization of the best reaction conditions for maximal tannase activity of purified tannase was executed by stepwise optimizations of various parameters.

2.2.1 Buffer system and pH optimization

Most suitable buffer system and optimum pH for maximum tannase activity were optimized by using different buffers viz. citrate phosphate buffer (pH 3.5-6.0), citrate buffer (pH 4.5-6.5), potassium phosphate buffer (pH 6.5-8.5), and sodium phosphate buffer (pH 7.0-8.5) at 0.05M concentration in the reaction mixture. Tannase activity was assayed using the standard assay method as described by Sharma *et al*., (2000) and optimized buffer system & pH were used for further studies.[6]

2.2.2 Optimization of buffer molarity

Optimization of the buffer molarity for maximum tannase activity was accomplished by using different molar concentrations (25mM to 200mM) of citrate buffer (pH 5.5) in the reaction mixture. Tannase activity was assayed using the standard assay method as described by Sharma *et al*., (2000) and optimized buffer molarity used for further studies.

2.2.3 Optimization of reaction temperature

Optimization of the most appropriate reaction temperature for maximum tannase activity was accomplished by incubating the reaction mixture in the temperature range 30 °C to 60 °C. Tannase activity was assayed using the standard assay method as described by Sharma *et al*., (2000) and optimized reaction temperature was used for further studies.

2.2.4 Optimization of reaction time

Optimization of the most appropriate reaction time for maximum tannase activity was accomplished by incubating the reaction mixture at 45 °C for different time intervals ranging from 2 min to 7 min. Tannase activity was assayed using the standard assay method as described by Sharma *et al*., (2000) and the optimized reaction time was used for further studies.

2.2.5 Effect of metal ions & inhibitors

The effect of metal ions on tannase activity was investigated by using different metal ions at 1mM concentration in the production medium individually. The enzyme activity was assayed using using the standard assay method as described by Sharma *et al*., (2000).

3. RESULTS AND DISCUSSION

3.1 Enzyme production

Tannase production from *Aspergillus* TPF-4 was accomplished in production medium (pH-5.5) containing PDB- 2.4 g, yeast extract- 1.0 g and substrate tannic acid- 2.5 g. Purification of crude enzyme was accomplished thereafter through ammonium sulphate precipitation method and chromatographic techniques. Briefly when crude extracellular tannase was purified through ammonium sulphate precipitation & anion exchange chromatography; purification folds of 8.5 & 28.55 were recorded respectively in comparison to crude tannase.

3.2 Buffer system and pH optimization

Fungal tannases are generally acidic enzymes. [7] Citrate buffer (pH 5.5) (Fig 3.2(a) proved to be the most significant for maximum activity (456.20 U/mg) of purified tannase amongst various buffers assesed. Comparable enzyme activity was also observed in Citrate phosphate buffer. Least tannase activity was observed in sodium phosphate buffer. Maximum tannase activity activity (519.85 U/mg) was obtained with 0.0 75 M citrate buffer (pH 5.5) (Fig 3.2(b)) while least activity (262.24 U/mg) was observed in 0.15M citrate buffer.

Chhokar et al., 2010 recorded highest tannase activity at pH of 5.5 for the tannase from *Aspergillus heteromorphus* MTCC 8818. [8] Renovato *et al.*, 2011 reported optimum enzyme activity at pH 6.0 for tannase from *Aspergillus niger*.[9] Costa *et al.*, 2012 reported highest enzyme activity for TAH I tannase from *A. tamarii* at pH 5.0-6.0.[7]

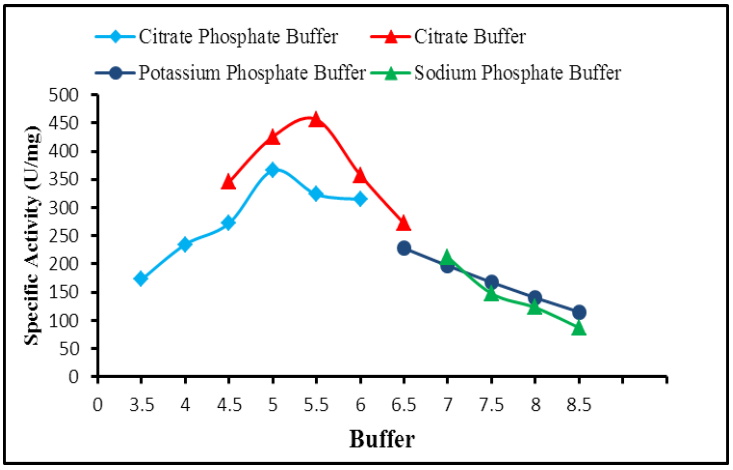

Fig. 3.2(a) Optimization of buffer system & pH.

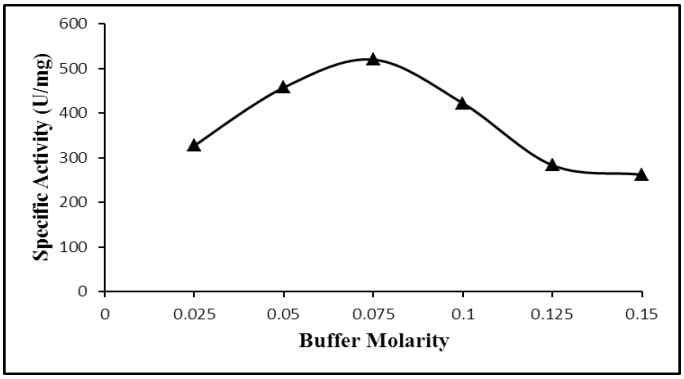

Fig 3.2(b) Optimization of buffer molarity.

3.3 Optimization of reaction temperature

The optimum temperature for tannase typically ranges between 30-50ºC (Mahendran *et al.*, 2006; Kasieczka-Burnecka *et al*., 2007).[10] [11] Maximum specific activity (562.41 U/mg) was recorded at 45 °C and least activity (355.76 U/mg) was recorded at 60 °C (Fig. 5.3). With further increase in temperature tannase activity was found to decrease. Increase in temperature increases the rate of denaturation of enzyme, with loss of secondary and tertiary structure. Similar thermal stabilities were documented for tannases from several other fungal species. Sharma *et al*. (2008), reported tannase from *Penicillium variable* showed maximum activity at 50°C.[12] Lokeswari *et al*., (2010), reported tannase from *Tricoderma viride* (MTCC 167) exhibited optimum activity at 45°C.[13] The optimal temperature for the activity of TAH I tannase from *Aspergillus tamarii* was in the range between 30 and 35°C.[7]

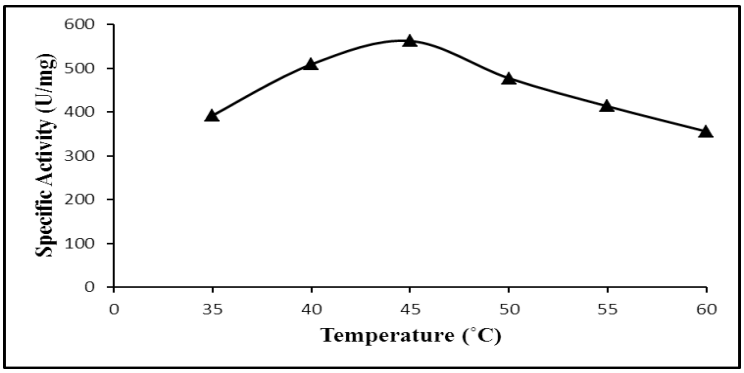

Fig. 3.3 Optimization of reaction temperature for the activity of purified tannase from *Aspergillus* TPF-4.

3.4 Optimization of reaction time

Maximum specific activity (643.32 U/mg) was recorded at 3 minutes of incubation and least activity was recorded at 7 min of incubation (Fig. 5.4). Crude tannase from *Aspergillus niger*

AUMC 4301 showed maximum activity at 20 min.[14] EL-Tanash et al. (2012) reported highest tannase activity at 30.4 min of incubation.[15]

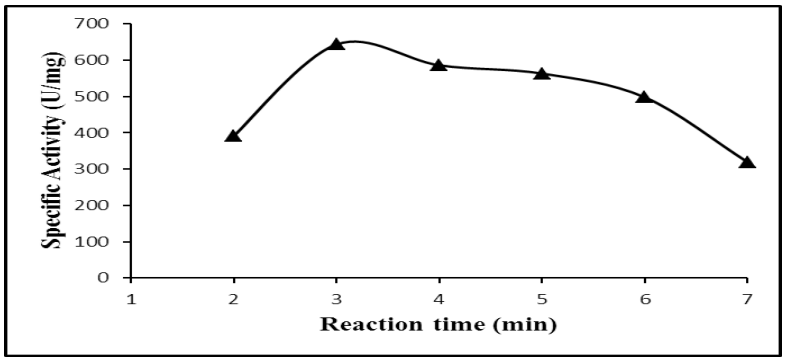

Fig. 3.4 Optimization of incubation time for the activity of purified tannase from *Aspergillus* TPF.

3.5 Effect of metal ions & inhibitors

Several enzymes require metal ions as activators with an objective to attain full catalytic efficiency. On the contrary, many enzymes are strongly inhibited by metal ions and various other organic compounds.[7] The effect of metal ions on tannase activity was investigated by using different metal ions at 1mM concentration. Metal ions Mg^{2+}, Cu^{2+}, Fe^{2+} and Hg^{2+} showed moderate inhibition of tannase activity with 93%, 90%, 81% and 70% residual activity, respectively. None of the metal ions assessed showed increase in maximum activity. From our study it becomes quite evident these metal ions are not necessary for the catalytic activity of tannase. Tannase activity was considerably inhibited by Sodium azide with only 42% residual activity. The decline in tannase activity in the presence of sodium azide could be due to nonspecific binding or aggregation of the enzyme. Chhokar *et al*., 2010 reported marginal inhibition of activity of purified tannase from *Aspergillus heteromorphus* MTCC 8818 by $Hg^{2+,}$ Ag^{+}, Fe^{2+} and Cu^{2+}. [8] Costa *et al*., 2012 reported inhibition of activity of *Aspergillus awamori* TAH I tannase by Hg^{2+}, Cu^{2+}, Zn^{2+}, Fe^{2+}, Ca^{2+} and Co^{2+}.[7]

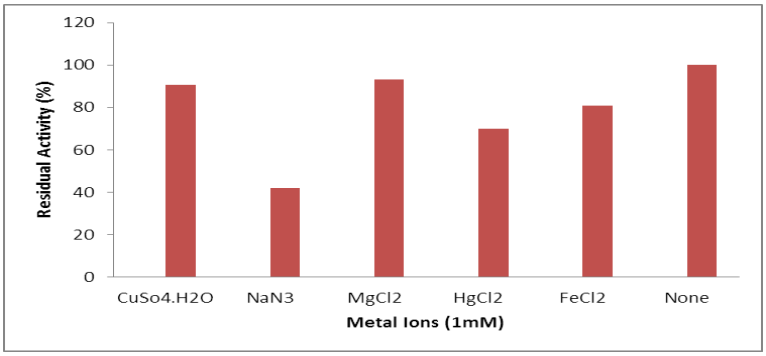

Fig. 3.5 Effect of metals ions and inhibitors on tannase activity of *Aspergillus* sp. TPF-4.

CONCLUSION

The present research study was taken up with a view of optimizing the reaction conditions for maximum activity of previously purified tannase from *Aspergillus* TPF-4. From our study we concluded that the purified enzyme showed considerable rise in activity under optimized reaction conditions. In conclusion, the purified *Aspergillus* TPF-4 tannase presents some qualities under optimized conditions that are desirable for industrial applications. Since the enzyme tannase has a plethora of applications; thus there is always an opportunity for a tannase with better and improved characteristics which may find its utility in various applications in the commercial sector.

CONFLICTS OF INTEREST

The authors declare that there are no conflicts of interest in relation to this research article.

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