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KINETIC AND THERMODYNAMIC CHARACTERIZATION OF *PLEUROTUS ERYNGII* MTCC 1798 XYLANASE AND *FUSARIUM OXYSPORUM* MTCC3300 XYLANASE: A COMPARISION

K. R. SUGUMARAN, S. N. SRIVASTAVA and V. PONNUSAMI*

School of Chemical and Biotechnology, SASTRA University, Tirumalaisamudram,
THANJAVUR – 613401 (T.N.) INDIA

ABSTRACT

This study investigates the potentials of *Pleurotus eryngii* and *Fusarium oxysporum* fungal species to produce xylanase, an industrially important enzyme. The xylanase was produced in a batch fermenter using starch as carbon source at pH 6.5 and pH 5.5, respectively for *P. eryngii* and *F. oxysporum*. Maximum enzyme production was achieved on fifth day and third day for *P. eryngii* and *F. oxysporum*, respectively. Effect of pH, substrate concentration, and temperature on enzyme activity was studied. Batch enzyme kinetics was studied using Michalis-Menton model. The values of K_m and V_m were found to be 1.45 U/mL, 7.21 mg/mL respectively for P. eryngii xylanase and 0.4 U/mL, 1.43 mg/mL, respectively for *F. oxysporum* xylanase by line-weaver plot. Optimum pH for xylanase reaction was found to be 6.5 for both *Pleurotus eryngii* and *Fusarium oxysporum*. Optimum temperatures for xylanase from *Pleurotus sp.,* and *Fusarium sp.,* are found to be 60°C and 50°C, respectively. Thermodynamic parameters such as activation energy for xylanase activity were determined to be 28.093 and 37.155 KJ/mol from *P. eryngii* and *F. oxysporum*. Thermodynamic parameters such as enthalpy change of activation (ΔH^*), entropy change of activation ΔS^* were also determined. Half life was obtained as 30.13 min for xylanase by *P. eryngi* and 26.65 min for *F. oxysporum* at 90°C. This shows that among these two enzyme produced *P. eryngi* xylanase had shown better thermal stability.

Key words: Xylanase, Submerged fermentation, Enzyme kinetics and Thermodynamics.

INTRODUCTION

Since ancient times, several enzymes have been used in many manufacturing processes, such as manufacture of wine, cheese, bread, and starch modification process etc. Optimum xylanase activity was found at the temperature below 50°C and acidic or neutral

*Author for correspondence; E-mail: vponnu@chem.sastra.edu

pH[1,2]. Xylanes are polysaccharides containing β-1, 4 linked to D-xylosyl residue on back born[3]. Xylanases (E.C.3.2.1.8) plays major role in breaking up of xylan. Corn, wheat stem, barley shells and similar material contain xylan[4-7]. Xylanase production had gained growing attention owing to its numerous biotechnological applications[8]. Among many micro-organisms such as bacteria, yeast, actinomycetes and filamentous fungi producing xylanase[7]. Filamentous fungi such as *Aspergillus sp.,* and *Trichoderma sp*., can produce high concentrations of xylanase than yeast and bacteria. Xylanase was produced by *Plueurotus eryngii* and *Flamulina velutipes* under submerged fermentation[9]. *Plueurotus sp.,* is a fungi with important medicinal properties in biotechnological applications. It is capable of producing hydrolytic and oxidative enzymes[9,10]. Xylanase was produced by *Fusarium oxysporum* and partial characterization of xylanase work was done[11]. However, previous reports on production of xylanase by *F. oxysporum* and *P. eryngii* are restricted to optimization of fermentation medium condition and effect of process variables on xylanase activity. But reports on thermodynamic parameters on xylanase activity are very limited in literature.

Due to greatest applications of xylanase, it is essential to characterize the kinetic parameters such as Vm and Km and thermodynamic parameters. The knowledge of above parameters are important to design the enzymatic reactors.

The purpose of this report was to compare and investigate the potential of *F. oxysporum* and *P. eryngii* xylanase production and to study the effect of process variables namely initial pH, substrate concentration and temperature on xylanase activity and to optimize the conditions for enzyme activity. In addition, the thermodynamic parameters have been described using transition state theory. Also thermo stability study on xylanase activity was performed.

EXPERIMENTAL

Materials and methods

Microbial culture

Fungal strains *P. eryngii* (MTCC 1798) and *F. oxysporum* (MTCC 3300) were purchased from MTCC, Chandigarh, India. Stock cultures were maintained on potato sucrose agar slants at 4^{o}C. Potato agar composition was potato smash 200 g/L, sucrose 20 g/L Agar 20 g/L. The potato pulp was taken and sucrose, agar agar were added to it until it get dissolved. pH was maintained at 6.5 and then media was sterilized. The strains were streaked (Quadrant streaking) on separate plates and they were kept undisturbed in the incubator.

Shake flask studies

Fungal cultures obtained from MTCC were cultivated in growth medium. (200 g/L potato, sucrose 20 g/L). 5% (V/V) of inoculum was introduced into 150 mL sterilized production medium (glucose 6 g/L, yeast extract 0.2 g/L, peptone 0.5 g/L potassium di-hydrogen phosphate 1 g/L, magnesium sulfate 0.5 g/L) in 250 mL Erlenmeyer flask, which is kept in a rotary orbital shaker at 150 rpm and 30^{o}C.

Various carbon sources such as starch, sucrose and maltose were also tried in place of glucose to screen the best carbon source. Maximum dry cell weight concentration and soluble protein concentration were observed when starch was used as a carbon source and same was used as a carbon source for further analysis. Similarly effect of initial pH on cell concentration and xylanase concentration was studied in shake flask. Optimum pH was found to be 6.5.

Batch fermenter studies

Batch fermenter (Bioflow110, New Brunswick Scientific, USA) study was carried out with a working volume of 1.5 L at room temperature. 5% (v/v) inoculum was introduced in to production medium under aseptic conditions. Sterile air was supplied at the rate of 1.5 vvm under 150 rpm, respectively. Cell concentration was estimated at regular time interval. Harvested broth was taken and subjected to centrifugation at 12000 × g for 20 min at 4^{o}C. Supernatant obtained from centrifugation was analyzed for enzyme concentration as the total soluble protein concentration by Lowry's method[12]. Dissolved oxygen concentration in the fermentation broth was checked at regular time intervals using dissolved oxygen probe.

Enzyme assay

Enzyme centrifugation was performed at a temperature below 4^{o}C. The centrifugal supernatant was taken as the enzyme source for the enzyme analysis. Xylanase activity was measured using 1% (W/V) birchwood xylan (4-O-methylglucuronoxylan) solution as a substrate[13]. The removal of reducing sugars in 10 min at 50^{o}C, pH 5.3 (0.05 M citrate buffer) was measured as xylose equivalents using the dinitrosalisylic acid method[14].

Enzyme kinetics

Effect of pH (2, 4, 6 and 8), effect of substrate concentration (0.5-2 wt. %) on enzymatic reaction and effect of temperature (30^{o}C to 90^{o}C) on enzyme activity were

studied. Thermo stability of enzyme was studied by exposing the enzyme to 90°C for varying heating time durations prior to enzyme reaction.

Kinetics and thermodynamics

The Michealis-Menten[15] and transition state theory[16] are represented by the following equations respectively

$$v = \frac{v_m S}{K_m + S} \quad \ldots(1)$$

$$\ln\left(\frac{v}{T}\right) = \ln\left(\frac{K_B}{h}\right) + \frac{\Delta S^*}{R} - \frac{\Delta H^*}{RT} \quad \ldots(2)$$

Where T, K_B, h, R, ΔH* and ΔS* are absolute temperature (K), Boltzmann constant (1.38×10^{-23} J/K), Planck's constant (6.626×10^{-34} Js), Gas constant (8.314 J/ molK), enthalpy change of activation and entropy change of activation, respectively.

The kinetic constants (v_{max}, Km) were obtained from Michealis-Menten plot using Eq. (1). Using the eqn. (2), ΔH* was calculated. Free energy change of activation is given by the following eqn. (3) at constant temperature T in K.

$$\Delta G^* = \Delta H^* - T\Delta S^* \quad \ldots(3)$$

Where ΔG* is change in free energy change of activation at constant temperature.

RESULTS AND DISCUSSION

Fermenter studies

Fermentation was carried out in batch mode using starch as a substrate at pH 6.5. Cell concentration in dry weight and protein concentration was monitored. Maximum protein concentration was obtained on the fourth day for *P. eryngii* and on third day for *F. oxysporum.* Maximum xylanase production of 0.62 g/L was achieved by *F. oxysporum* on third day. Meanwhile maximum xylanase production of 0.59 g/L was obtained using *P. eryngii* on fourth day of fermentation. The volumetric mass transfer co-efficient for *P. eryngii* and *F. oxysporum* were found to be 0.039 and 0.021 sec^{-1}. Xylanase production was achieved by *Penicillium canescens* 10-10c in 5 l bioreactor and mass transfer co-efficient was studied in submerged fermentation[17]. The comparison of xylanase produced by these two species is shown in Fig. 1.

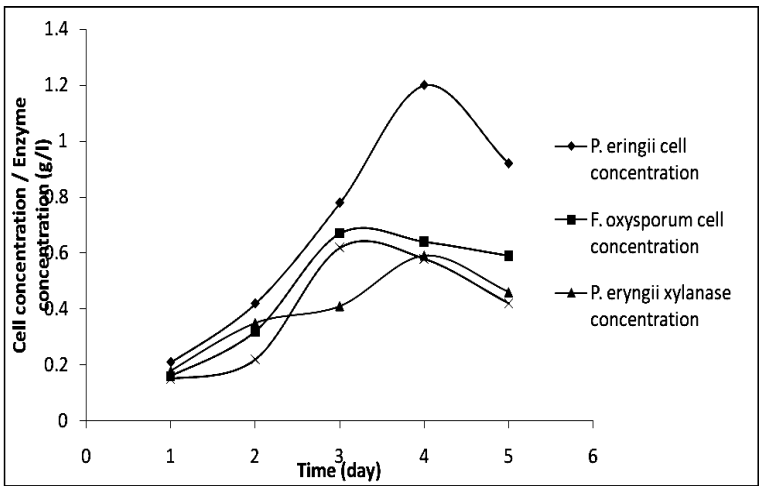

Fig. 1: Comparison of cell concentration and xylanase concentration

Effect of pH on xylanase activity

In this work, Fig. 2 explains that optimum pH for xylanase reaction was found to be 6.5 for both *P. eryngii* and *F. oxysporum* at room temperature, which is coincide with the findings of Turkiewicz et al., Xylanase activity was maximum around pH 5[18]. Xylanase from *Aspergillus spp.,* was showed a optimum pH range of 4.0 and 6.0 and a optimum temperature of 50°C[19].

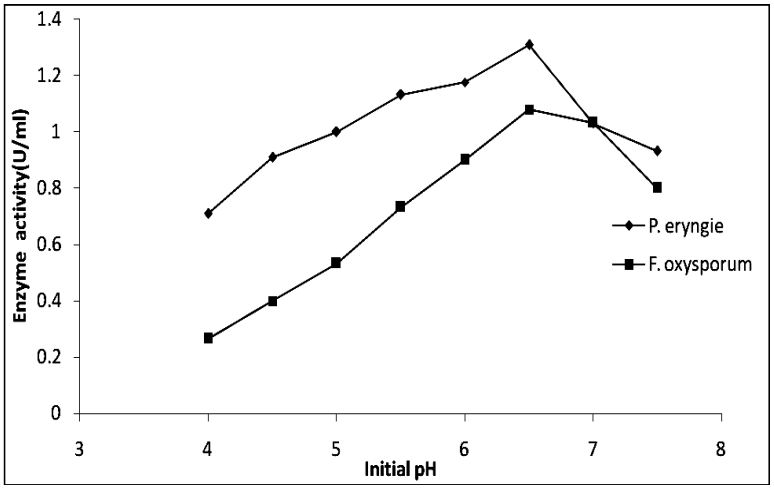

Fig. 2: Effect of initial pH on enzyme activity of *P. eryngii* xylanase and *F. oxysporum* xylanase

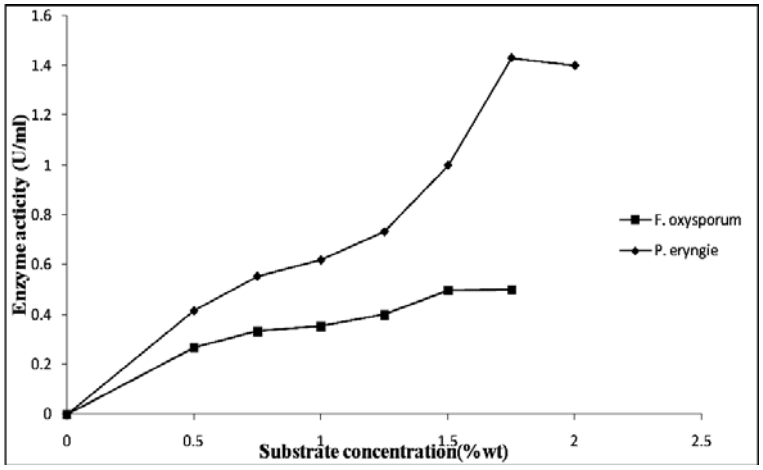

Fig. 3: Effect of sustrate concentration on enzyme activity of *P. eryngii* xylanase and *F. oxysporum* xylanase

Effect of substrate concentration on xylanase activity

In this study, Vm and Km values are found to be 1.45 U/mL, 12 mg/mL, respectively for *P. eryngii* and 0.5 U/mL, 2.5 mg/mL, respectively for *F. oxysporum* in Fig. 3. Optimum substrate concentration was 1.75 wt% for *P. eryngii* xylanase and 1.5 wt% for *F. oxysporum* xylanase. High values of Km were obtained as 5.6 and 7.0 mg/mL using oat spelt xylan from partially purified xylanase which were isolated from two *Aspergillus sp*.,[20]. Two purified xylanase from *A. tamarii* had higher Km values which were reported viz. 5.8 and 8.4 mg/mL using birchwood xylan as the substrate and maximum velocity of reaction were observed to be 694 and 1700 micro mol/mg/min[21].

Effect of temperature on xylanase activity

From Fig. 4 optimum temperatures for xylanase from *Pleurotus sp.,* and *Fusarium sp.,* are found to be 60^{o}C and 50^{o}C, respectively. The optimum temperature for xylanase activity was 60^{o}C from *T. harzianum 1073 D3*[18]. The optimum temperature of xylanase was varied between 45 and 60^{o}C by *Trichoderma sp*.,[4,22]. Optimum temperature for fungal xylanase was obtained as 60^{o}C[23,24].

According to the optimum temperature, enzymes can be classified as mesophilic 40–60^{o}C, thermophilic 50–80^{o}C and hyperthermophilic above 80^{o}C[25]. Now, thermostable nature of xylanase could be obtained from fungal sources like *Thermoascus aurantiacus*[26], *F. oxysporum* F3[11] and *Penicillium capsulatum*[1]. These strains were well active at temperature in the range of 50^{o}C and 80^{o}C.

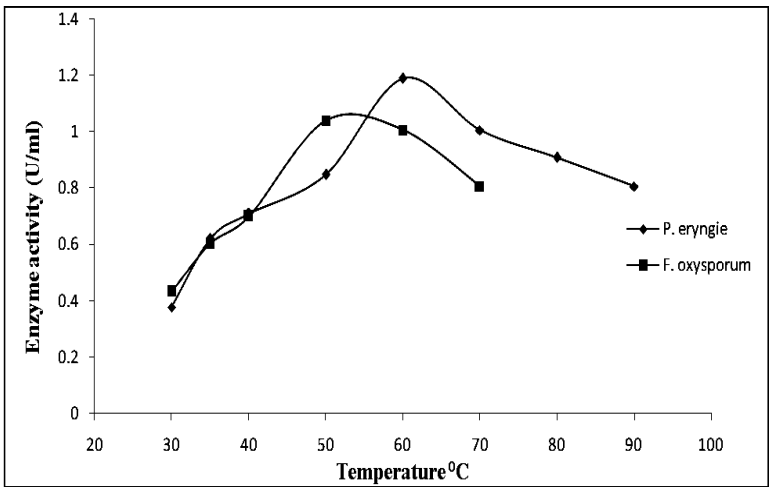

Fig 4: Effect of reaction temperature on enzyme activity of *P. eryngii* xylanase and *F. oxysporum* xylanase

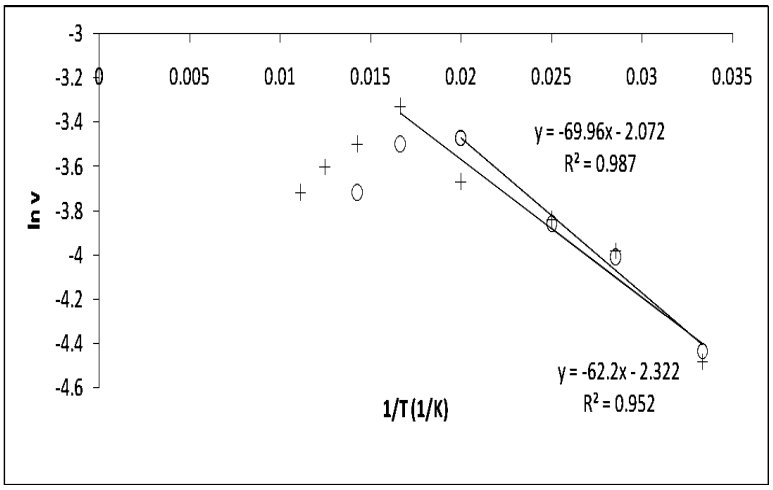

Fig. 5: Arrhenius plot for *P. eryngii* xylanase and *F. oxysporum* xylanase

Thermodynamic Approach of enzymatic reaction

Legends: + *P. eryngii*; O - *F. oxysporum*

From Fig. 5 Activation energy for xylanase activity were determined to be 28.093 and 37.155 KJ/mol from *Pleurotus sp.,* and *Fusarium sp.,* lower the enthalpy values of enzyme, high efficient are the development of transition state between the enzyme-substrate[27]. Activation energy for starch hydrolysis using α-amylase enzyme by *B. licheniformis* EMS-6

was found to contain 25.14 KJ/mol[28]. Activation energy was about 363.7 KJ/mol at 70°C for *B. licheniformis* α-amylase and 317.9 KJ/mol for *Aspergillus oryzae* α-amylase[29]. Thermodynamic parameters for xylanase from *P. eryngii* and *F. oxysporum* are determined using transition state theory. The plot is shown in Fig. 6 and the calculated values of thermodynamic parameters are summarized in Table 1.

Table 1: Optimum temperature, activation energy, enthalpy change of activation, entropy change of activation, free energy change of activation for *P. eryngii* and *F. oxysporum*

	Optimum temp. (°C)	Activation Energy (KJ/mol)	ΔH^* (KJ/mol)	ΔS^* (KJ/Kmol K)	ΔG^* (KJ/mol)
Pleutorus eryngii	60	28.093	25.041	-193.04	89.710
Fussarium oxysporum	50	37.155	33.455	-170.35	88.478

Xylanase obtained from *P. eryngii* has lower activation energy and enthalpy change of activation than from *F. oxysporum.* Lower the enthalpy values of enzyme having more efficient are the formation of transition state between the enzyme-substrate[27]. Gibbs free energy change under isothermal condition (ΔG^*), enthalpy change of activation (ΔH^*) and entropy change of activation (ΔS^*) for binding of α-amylase from *B. licheniformis* EMS-6 were 36968 J/mol, 22.53 KJ/mol and -110.95J/mol/ K, respectively[28]. ΔH^* and ΔS^* of *B. amyloliquefaciens* for α-amylase 29.3 KJ/mol and - 82.6 J/mol/ K, respectively[30].

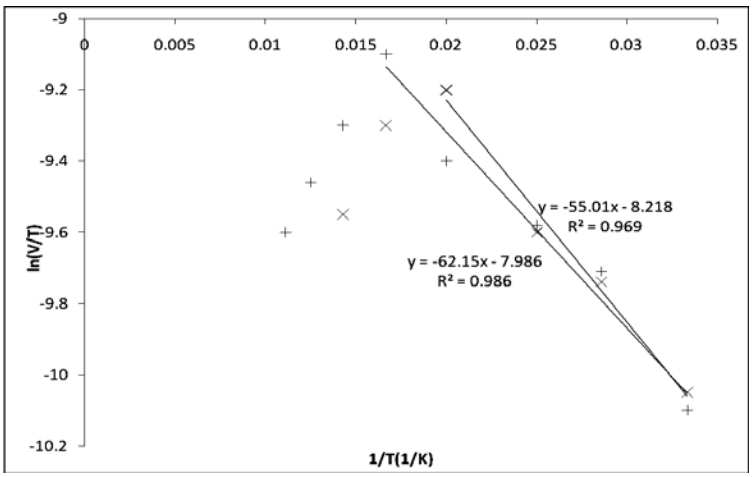

Fig. 6: Transition state theory plot for *P. eryngii* xylanase and *F. oxysporum* xylanase.

+ P. eryngii, x F. oxysporum

Thermal stability study

From Fig. 7, denaturation constant K_d value was found to be 0.023 min^{-1} for xylanase produced by *P. eryngii* and 0.026 min^{-1} for *F. oxysporum*. Half life was obtained as 30.13 min for xylanase by *P. eryngii* and 26.65 min for *F. oxysporum* at 90°C. Half life period of Xylanase from two *Aspergillus sp.,* were found to be 240 and 260 min at 50°C [20].

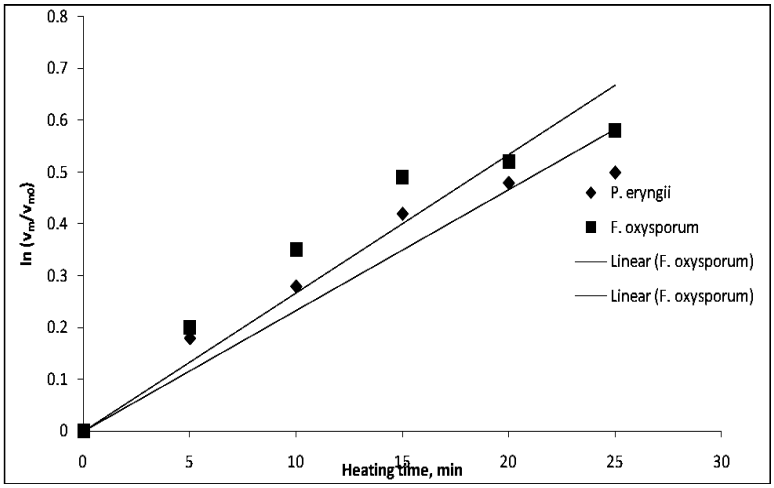

Fig. 7: Thermal stability for *P. eryngii* xylanase and *F. oxysporum* xylanase at 90°C

CONCLUSION

In this work, *P. eryngii* and *F. oxysporum* were cultivated in shake flask and effects of carbon source and pH on cell concentration and protein concentration were studied. Maximum xylanase production was achieved when starch was used as carbon source and optimum pH was found to be 6.5 and 5.5 for *P. eryngii* and *F. oxysporum*, respectively. Thus, xylanase production was investigated in batch fermenter at pH 6.5 using starch as a substrate. Enzyme solution was separated from crude culture by centrifugation. Effects of pH, substrate concentration, temperature on enzyme activity were studied. In this study, Vm and Km values are found to be 1.45 U/mL, 7.211 mg/mL, respectively for *P.eryngi* xylanase and 0.4 U/mL, 1.43 mg/mL, respectively for *F.oxysporum* xylanase by line-weaver plot. Optimum pH for xylanase reaction was found to be 6.5 for both *Pleurotus eryngii* and *Fusarium oxysporum*. Optimum temperatures for xylanase from *pleurotus sp.,* and *Fusarium sp*., are found to be 60°C and 50°C, respectively. Thermodynamic parameters such as activation energy for xylanase activity were determined to be 28.093 and 37.155 KJ/mol

from *P. eryngi* and *F. oxysporum.* Thermodynamic parameters such as enthalpy change of activation (ΔH^*), entropy change of activation ΔS^* were also determined. Half life was obtained as 30.13 min for xylanase by *P. eryngi* and 26.65 min for *F. oxysporum* at 90^oC. This shows that *P. eryngi* xylanase had shown better thermal stability.

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