Optimization of Xylanase Production from *Penicillium citrinum* in Solid-State Fermentation

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Solid-state fermentation of sugarcane bagasse by *Penicillium citrinum* MTCC 2553 was optimized to maximize the yield of xylanase. Preliminary experiments carried out with various lignocellulosic materials revealed sugarcane bagasse to be the most suitable substrate for producing xylanase. Response surface methodology was used in the optimization. Xylanase activity was maximized in a 5-day batch fermentation carried out under the following conditions: a substrate-to-moisture ratio of 1:5 by mass, an initial pH of 7.0 and an incubation temperature of 30 °C. Under the optimal conditions, the final activity of xylanase was 1645 U g^{-1} of dry substrate. Xylanase was recovered from an extract of the fermented solids by ammonium sulfate precipitation. The crude enzyme was further purified by dialysis. The activity of the enzyme was enhanced in the presence of Na^{+}, Mg^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Co^{2+} and Tween 80. The enzyme was inhibited by Hg^{2+}, Ca^{2+} and the chelating agent ethylene diamine tetra acetic acid (EDTA).

Key words:
Xylanase, hemicellulose, sugarcane bagasse, solid-state fermentation, *Penicillium citrinum*

Introduction

The enzyme xylanase (EC 3.2.1.8) catalyzes the hydrolysis of the linear polysaccharide β-1,4-xylan to xylose. Xylan is the major constituent of hemicellulose and therefore xylanases together with other hydrolytic enzymes are used in breaking down hemicellulose. Xylanases are potentially useful in processing of food, feed, pulp and paper.[1–3] Many microorganisms are known to produce extracellular xylanases. Certain filamentous fungi are particularly good producers.[4,5]

Fungal xylanases can be produced both by submerged fermentation and solid-state fermentation (SSF).[6] Production of extracellular enzymes by solid-state fermentation is generally less expensive compared to production by submerged fermentation. The fermented solids can be extracted with buffers to provide a preparation of crude xylanases[7] that can be used directly without further processing. Low-cost agro-industrial residues such as wheat bran, rice bran, wheat straw, rice husk, sugarcane bagasse and corncobs have been effectively used as substrates for producing enzyme by SSF.[8] Fungal xylanases have been previously reviewed.[3] Substantial information exists on molecular structures and mode of action of certain xylanases.[4,9]

This work discusses the SSF production of xylanase by *Penicillium citrinum*. The fermentation process is optimized by a response surface method[10] to maximize the activity of the enzyme. Optimization of substrate type was done by one factor at a time approach. The other parameters of the fermentation process were optimized with respect to the following factors: the initial pH of the fermentation; the initial moisture content in the substrate; the duration of the batch fermentation; and the incubation temperature using response surface methodology. As these factors influence the final enzyme activity interactively, the conventional optimization method of measuring the response (i.e. enzyme activity) in experiments involving variation of a single factor at a time is not a satisfactory strategy for identifying the conditions that maximize enzyme production. Statistical design of experiments in combination with the response surface method (RSM) has been successfully used in optimizing many fermentation processes.[11–21]

Although RSM has been used to optimize production of microbial xylanases[15,22,23] less work has been reported on xylanase production by SSF using *Penicillium citrinum*. The enzyme produced by SSF

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was partially purified. The temperature and pH for optimal activity of this enzyme were determined. The influence of the various metal ions on the enzyme activity was established.

Materials and methods

Materials

All chemicals used were of analytical grade. Potato dextrose agar (PDA) was obtained from Hi-media (India). Birchwood xylan was purchased from Sigma-Aldrich (Germany). Wheat straw, wheat bran, rice straw, rice bran, sawdust, corncobs, and sugarcane bagasse were purchased from the local market.

Microorganism and culture condition

The filamentous fungus *Penicillium citrinum* MTCC 2553 (procured from Institute of Microbial Technology, Chandigarh, India) was used throughout. The microorganism was maintained on PDA slants and stored at 4 °C.

In preliminary work, the aforementioned solid substrates were individually evaluated for production of xylanase by SSF. Thus, each substrate was ground to a particle size of ≤0.5 mm. The ground substrate (5 g) was mixed with a moistening medium in a 250 mL Erlenmeyer flask such that the mass ratio of the substrate-to-medium was 1:5. The aqueous moistening medium contained the following (g L^{-1}): sucrose 30, yeast extract 5, K_2HPO_4 1, $NaNO_3$ 30, KCl 5, $MgSO_4$ 5 and $FeSO_4$ 0.1. The pH of the moistening medium was adjusted to 7.

The flasks containing the moistened substrate were then sterilized at 121 °C, 15 min. The flasks were cooled to 30 °C and inoculated with a 1 mL suspension of fungal spores in sterile water. The spore suspension had been standardized to provide 10^8 spores g^{-1} of dry substrate. The inoculated substrate was incubated at 30 °C, for 5 days, in flasks plugged with sterile cotton wool. The initial pH was 7.0.

The substrate that gave the maximum final xylanase activity in the above study, was used for all future work. In some cases, the selected substrate was washed with distilled water, dried and pretreated prior to use in fermentations. The following pretreatments were tested: (1) alkali treatment using 1 mol L^{-1} NaOH followed by neutralization with 1 mol L^{-1} HCl and repeated washing with distilled water; (2) acid treatment with 1 mol L^{-1} HCl followed by neutralization with 1 mol L^{-1} NaOH and washing with distilled water; and (3) steam treatment of dry substrate at 121 °C for 2 h. In pretreatments (1) and (2), 100 g of dry substrate was completely immersed in about 500 mL of acid or alkali for overnight. The pretreated substrate was dried at 50 °C to constant mass, ground to a particle size of ≤0.5 mm, and stored in polyethylene bags until use. The untreated substrate was used as control. All fermentations used the aforementioned standardized inoculum.

Experimental design and data analysis

Optimum operating conditions were determined using the response surface method (RSM) with 2^4 factorial design (Table 1) for the process variables (pH, temperature, substrate-to-moisture mass ratio, incubation time) with three levels each.

Enzyme activity (U g^{-1} of dry substrate) after desired period of incubation was measured as the response. The response was modeled using the following polynomial:

$$P = b_0 + \sum b_i X_i + b_{ii} X_i^2 + \sum b_{ij} X_i X_j \quad (1)$$

where P is predicted response (xylanase activity); b_0 is regression coefficient at the center of the regression model; and b_i, b_{ii}, b_{ij}, are coefficients estimated by the model for the linear, quadratic, and interactive effects of the coded variables (A, B, C, D). In eq. 1 X_i and X_j are the process variables.

Table 1 – *Factor levels used in the experimental design*

Factor	Symbol	Actual levels of coded factors		
		–1	0	+1
pH	*A*	6.5	7.0	7.5
Temperature (°C)	*B*	25	30	35
Substrate-to-moisture mass ratio	*C*	1:4	1:5	1:6
Time (d)	*D*	4	5	6

Design Expert ver. 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN, USA) was used for the statistical analyses. Data was analyzed to estimate whether a given term had a significant effect ($p < 0.05$) on enzyme activity using the analysis of variance (ANOVA) in combination with the Fischer test. Graphical and mathematical analyses were performed using the Design Expert program to determine the optimum intensity level of the variables. The overall second order polynomial relationship between P and the variables could be represented by the following quadratic equation:

$$P = b_0 + b_1 A + b_2 B + b_3 C + b_4 D + \\ + b_{11} A^2 + b_{22} B^2 + b_{33} C^2 + b_{44} D^2 + \\ + b_{12} AB + b_{13} AC + b_{14} AD + b_{23} BC + b_{24} BD + b_{34} CD \quad (2)$$

where, A, B, C, D are the coded variables for pH, temperature (°C), substrate-to-moisture mass ratio, and incubation time (d). In eq. 2, b_0, b_1, b_2, b_3, b_4, b_{11}, b_{22}, b_{33}, b_{44}, b_{12}, b_{13}, b_{14}, b_{23}, b_{24}, b_{34} are the coefficients for the various effects.

Enzyme extraction

The crude enzyme from each fermentation flask was extracted with 50 mL of 0.05 mol L^{-1} citrate buffer at pH 6.0. The suspended solids were removed by filtering the slurry through a sterile muslin cloth. The solids remaining in the filtrate were removed by centrifugation (4 °C, 10,000 g, 20 min). The resulting cell free supernatant was used as the crude enzyme preparation in subsequent measurements.

Total protein and enzyme assays

The total protein in the crude enzyme extract was measured using the Lowry method[24] with bovine serum albumin as the standard protein solution.

The activity of xylanase was measured at 50 °C using a 10 g L^{-1} solution of Birchwood xylan as the substrate in 0.05 mol L^{-1} citrate buffer, pH 6.0. The enzyme solution (0.2 mL) was incubated with the substrate (1.8 mL) for 5 min. The reaction was then terminated by adding 3 mL of dinitrosalicylic acid solution and boiling for 5 min.[25] Absorbance of the resulting mixture was read at 540 nm. One unit of enzyme activity was defined as the quantity of enzyme liberating 1 μmol of xylose per min.[26] Xylanase activity was expressed as U g^{-1} of dry substrate.

As cellulase is commonly produced together with xylanase, the activity of cellulase in the crude enzyme extract was also measured. Thus 0.5 mL of the crude extract was incubated with the substrate (50 mg of Whatman no.1 filter paper suspended in 0.5 mL of 0.05 mol L^{-1} phosphate buffer at pH 6.0) for 1 h at 50 °C. The reaction was stopped by adding 2 mL of dinitrosalicylic acid solution followed by boiling for 5 min. Absorbance of the resulting solution was measured at 540 nm. One unit of cellulase activity was defined as the quantity of enzyme that released 1 μmol of glucose min^{-1}.[27] Cellulase activity was expressed in U g^{-1} of dry substrate. All measurements were done in triplicate.

Partial purification of xylanase

The extracted crude enzyme was partially purified by ammonium sulfate precipitation (60 %) followed by dialysis overnight in 0.05 mol L^{-1} citrate buffer for desalting and used for characterization studies.

Determination of pH optimum, temperature optimum, pH stability and thermostability of xylanase

For determining the optimum pH of xylanase, the activity was measured at 50 °C in buffers of pH values ranging from 3 to 11. The following buffers were used: citrate buffer for pH 3 to 6; phosphate buffer for pH 6.5 to 8; and glycine NaOH buffer for pH 8.5 to 11. The pH stability of the enzyme was determined by incubating the enzyme in the above specified buffers at 30±2 °C for 24 h. The activity was then measured and compared to the initial activity.

The optimum temperature was determined by measuring the xylanase activity for 5 min at incubation temperatures ranging from 25 to 90 °C at pH 6 (citrate buffer). The temperature stability was determined at 50 °C by measuring the remaining activity after incubation of the enzyme at the specified temperature for 24 h, pH 6 (citrate buffer), in the absence of the substrate. All measurements were done in triplicate.

Effect of additives on xylanase activity

Effect of various additives on xylanase activity was determined by incubating the xylanase solution (pH 6.0) with 10 mmol L^{-1} of the specified additive at 30±2 °C. Samples were withdrawn at 15 min intervals and the xylanase activity was measured. The additives were: Na^+, Mg^{2+}, Ca^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Co^{2+}, Hg^{2+}, Tween 80 and EDTA. The measured activity was compared to the activity of the control preparation (xylanase solution incubated as above but with no additive). All measurements were done in triplicate.

Results and discussion

Effect of substrates on xylanase yield

In preliminary batch fermentations (5-days, 30 °C, initial pH of 7), the highest yield of xylanase (151.7 U g^{-1} of dry substrate) was obtained on sugarcane bagasse and only a negligible amount of cellulase (0.06 U g^{-1} of dry substrate) was produced. In comparison, the xylanase yields on sawdust, corncobs, wheat bran, xylan, rice bran, rice straw, and wheat straw were 45.5, 44.6, 141.5, 128.5, 76.5, 20.5 and 12.2 U g^{-1} of dry substrate, respectively. In view of the high yield, sugarcane bagasse was used in all future fermentations. The high xylanase yield on bagasse was likely due to a relatively high level of hemicellulose in this substrate.[28]

As shown in Table 2, the acid, alkali and steam pretreatments of sugarcane bagasse actually re-

Table 2 – *Effect of different pretreatments of sugarcane bagasse on xylanase activity*

Pretreatment of substrate	Xylanase activity (U g^{-1} ds)
Untreated	151.7
Alkali treatment (1 mol L^{-1} NaOH)	115.8
Acid treatment (1 mol L^{-1} HCl)	96.1
Steam treatment (121 °C, 2 h)	108.5

duced the enzyme yield relative to the untreated substrate. This may have resulted from inhibitory compounds being produced in some of the treatments and/or leaching of nutrients from the substrate as a consequence of the treatment process. Alternatively, pretreatments may have altered the porosity of the substrate[29] to make it less suitable for solid-state fermentation. In view of the results (Table 2), all subsequent fermentations used untreated bagasse as the substrate.

Effect of process variables on xylanase activity

Data on the final activity of xylanase produced under different combinations of the initial pH (pH 6.5–7.5), temperature (25–35 °C), substrate-to--moisture mass ratio (1:4 to 1:6), and incubation time (4–6 d), are reported in Table 3. The combined effects of the process variables on the final xylanase activity could be expressed in the form of eq. 2, using the coded variables (A–D, Table 1); thus:

$$P = 1309.01 - 130.81A - 98.11B + 82.64C - \\ - 98.11D - 124.08A^2 - 178.89B^2 - 51.61C^2 - \\ - 94.03D^2 - 35.61AB + 25.0AC + 6.89AD - \\ - 35.50BC - 143.31BD - 140.0CD \quad (3)$$

Table 3 – *Experimental design and results of the central composite design for xylanase activity*

Run number	Block	Factors				Xylanase activity (U g^{-1} ds)	
		A	*B*	*C*	*D*	observed	predicted
1	Block 1	7.5	25.0	1:6	6	1120	1114.8
2	Block 1	6.5	35.0	1:6	6	720	684.5
3	Block 1	7.5	25.0	1:4	6	990	984.8
4	Block 1	7.0	30.0	1:5	5	900	864.5
5	Block 1	7.5	35.0	1:4	4	1020	984.5
6	Block 1	7.0	30.0	1:5	5	1320	1314.8
7	Block 1	6.5	25.0	1:4	4	640	634.8
8	Block 1	7.0	30.0	1:5	5	890	854.5
9	Block 1	6.5	35.0	1:4	6	1330	1378.2
10	Block 1	7.5	35.0	1:6	4	1340	1378.2
11	Block 1	6.5	25.0	1:6	4	1330	1378.2
12	Block 1	7.0	30.0	1:5	5	1350	1378.2
13	Block 2	6.16	30.0	1:5	5	1080	1108.8
14	Block 2	7.0	30.0	1:5	5	640	668.8
15	Block 2	7.0	30.0	1:3.32	5	870	898.8
16	Block 2	7.84	30.0	1:5	5	540	568.8
17	Block 2	7.0	21.59	1:5	5	890	954.8
18	Block 2	7.0	30.0	1:6.68	5	1240	1232.8
19	Block 2	7.0	30.0	1:5	6.68	1110	1138.8
20	Block 2	7.0	30.0	1:5	3.32	780	808.8
21	Block 2	7.0	30.0	1:5	5	1350	1239.8
22	Block 2	7.0	38.41	1:5	5	1360	1239.8

Fisher's test for analysis of variance (ANOVA) was used for statistical testing of the model (eq. 3) and the results are shown in Table 4. The ANOVA revealed that the response surface quadratic model (eq. 3) was significant because the *p*-value for the model was less than 0.05 while the corresponding *F*-value was 12.75 (Table 4). A insignificant value for the lack of fit indicated that the quadratic model was valid for the present study.[13] The R^2 value for the model was 0.967, indicating general good agreement of the model and the data. The small value of the coefficient of variation of 8.62 suggested a high level of precision in the data (Table 4).

The various *p*-values in Table 4 indicate the significance of the coefficients of the corresponding terms in eq. 3. The *p*-value of less than 0.05 for *A*, *B*, *C*, *D*, A^2, B^2, D^2, *BD* and *CD* revealed these model terms to be significant. The negative quadratic coefficient values (eq. 3) for all the variables implied the existence of a peak point xylanase activity with respect to the individual variables and an inhibitory effect of the variable at other than the peak point.

A positive value of the linear coefficient for *C* (eq. 3) indicated that the xylanase activity increased with substrate-to-moisture ratio, a factor that is known to profoundly affect many SSF processes.[29] Too much moisture leads to water logging of the substrate and poor diffusion of oxygen. Too little water means a water activity that may be too low to solubilize the substrate and support fungal growth.[29]

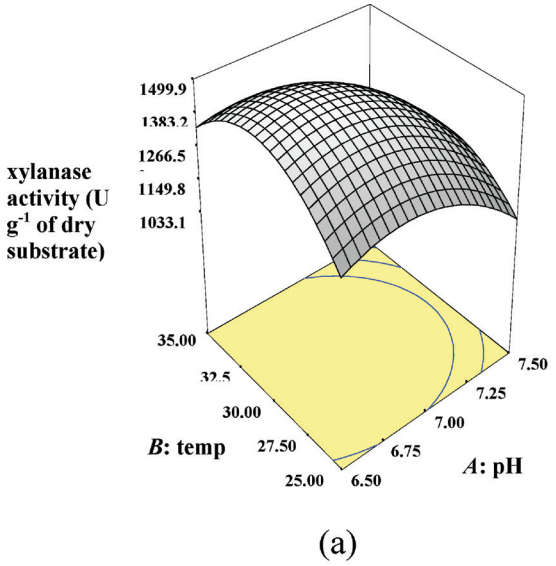
Response surface plots were generated to identify the optimal levels of the process variables for maximizing the activity of xylanase (Figs. 1–2). The response surfaces were generated for the variation of two independent variables while keeping other variables at fixed *B* levels. The response plots in Figs. 1–2 take into account the possible combinations of independent variables.

Table 4 – *Analysis of variance for the response surface model (eq. 3)*

Source	Sum of squares	DF	Mean of squares	*F*-value	*p*-value	
Block	47345.61		47345.61			
Model	$1.425 \cdot 10^6$	14	$1.425 \cdot 10^6$	12.75	0.003[a]	Significant
A	96800.0	1	96800.0	12.12	0.013[a]	
B	54450.00	1	54450.00	06.82	0.040[a]	
C	93271.85	1	93271.85	11.68	0.014[a]	
D	54450.0	1	54450.0	06.82	0.040[a]	
A^2	$2.372 \cdot 10^5$	1	$2.372 \cdot 10^5$	29.71	0.002[a]	
B^2	$4.93 \cdot 10^5$	1	$4.93 \cdot 10^5$	61.75	0.000[a]	
C^2	41033.09	1	41033.09	05.14	0.064	
D^2	$1.362 \cdot 10^5$	1	$1.362 \cdot 10^5$	17.06	0.006[a]	
AB	4201.92	1	4201.92	0.53	0.496	
AC	5000.00	1	5000.00	0.63	0.459	
AD	157.33	1	157.33	0.02	0.893	
BC	8450.00	1	8450.00	01.06	0.343	
BD	68058.78	1	68058.78	08.52	0.027[a]	
CD	$1.568 \cdot 10^5$	1	$1.568 \cdot 10^5$	19.64	0.004[a]	
Residual	47905.29	6	7984.21			
Lack of fit	47580.29	2	23790.14	292.80	<0.0001	Insignificant
Pure error	325.00	4	81.25			
Cor. total	$1.52 \cdot 10^6$	21				

R^2 = 0.967, adjusted R^2 = 0.896, C.V. = 8.62, mean = 1036.82

[a]Significant model terms, C.V.: coefficient of variance, DF degree of freedom



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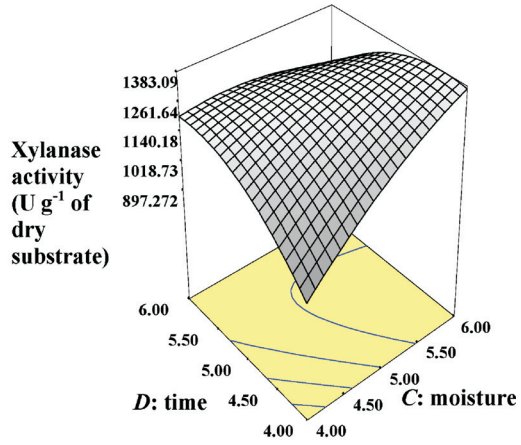

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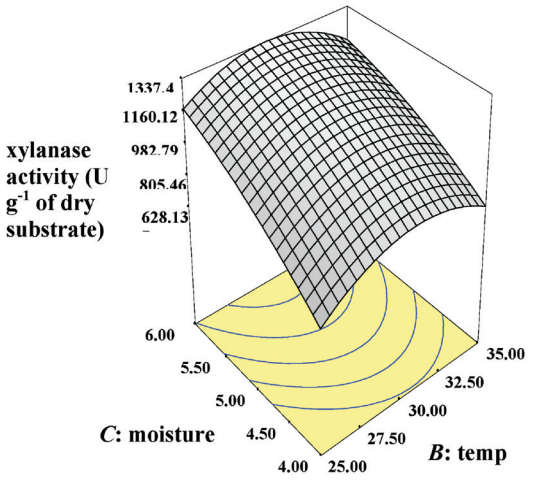

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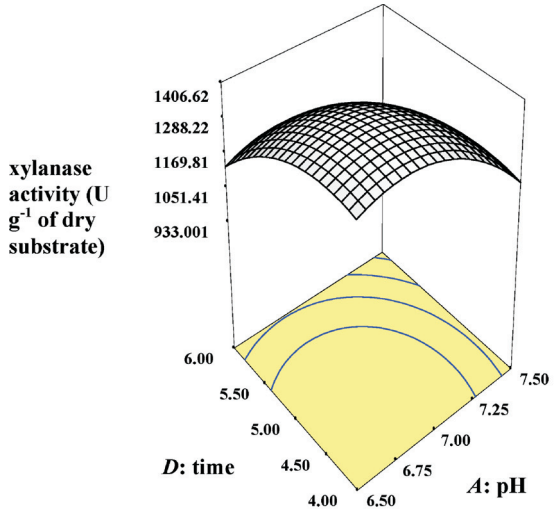

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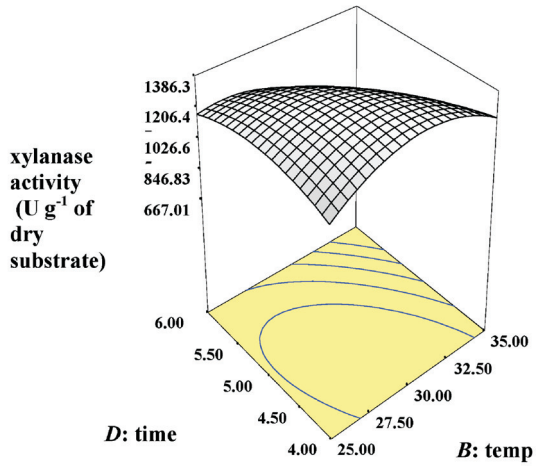

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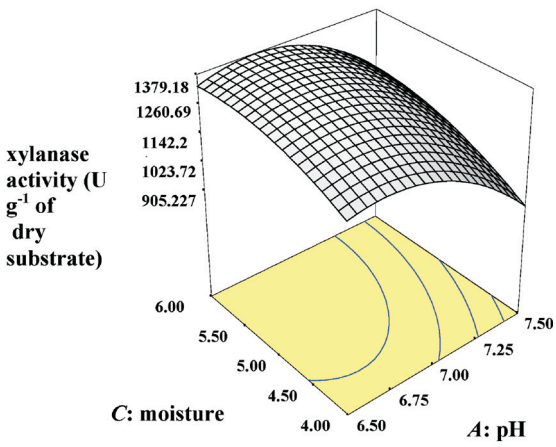

(c)

F i g . 1 – *Combined effect of (a) media pH and incubation temperature (b) incubation time and substrate to moisture ratio (c) substrate to moisture ratio and temperature on xylanase activity*

F i g . 2 – *Combined effect of (a) incubation time and pH (b) incubation time and temperature (c) substrate to moisture ratio and pH of the fermentation media on xylanase activity*

Interactive effects of variables on the xylanase activity

The p-value of >0.05 for AB (Table 4) indicated that temperature and pH had not significantly interacted in influencing the xylanase titer, but had individually affected the xylanase titer. The negative values of coefficients for A, B, A^2, B^2, AB (eq. 3) indicated that an increase or decrease in pH and temperature from the optimal point reduced xylanase titer. Fig. 1a shows the response surface for the effect of pH and temperature on xylanase activity at constant values of the variables C (= 1:5) and D (= 5 d). From Fig. 1a, the maximum response occurs in the vicinity of 30 °C and pH 7.

In view of p-value of <0.05 for CD (Table 4), the variables of substrate-to-moisture ratio (C) and incubation time (D) had a significant interactive effect on xylanase titer. In the range of interest, the xylanase titer increased with an increased incubation time and an increased level of moisture in the substrate (Fig. 1b). This is generally consistent with other similar observations for production of xylanases by fungi.[22]

Temperature (B) and the substrate-to-moisture ratio (C) did not have a significant interactive influence on the enzyme titer ($p > 0.05$, Table 4). Both these factors individually affected the enzyme titer. As shown in Fig. 1c, the maximum response occurred in the temperature range of 29–30 °C and at a substrate-to-moisture ratio of 1:6.

The interactive effect of pH (A) and incubation time (D) on the enzyme titer was insignificant as p-value for AD was above 0.05 (Table 4). A pH of around 7 in combination of an incubation time of around 5 days maximized the xylanase titer (Fig. 2a) at fixed values of B (30 °C) and C (substrate-to-moisture ratio of 1:5).

The interactive effect of temperature (B) and incubation time (D) on xylanase titer was significant ($p < 0.05$ for BD in Table 4). Fig. 2b indicates that the region of interaction of temperature with incubation time was in the temperature range of 27.5 to 32.5 °C and a substrate-to-moisture ratio range of 1:4 to 1:6.

The pH (A) and the substrate-to-moisture ratio (C) had no significant interactive effect ($p > 0.05$ for AC in Table 4), but both these variable individually affected the xylanase titer (Fig. 2c). Fig. 2c is for a constant incubation temperature of 30 °C and an incubation time of 5 days.

Validation of the experimental model

An additional fermentation was carried out in triplicate to validate the optimal predictions of the response surface model. The values of the variables A–D in this confirmation experiment were set to levels suggested by the Design Expert statistical software and shown in Table 5. The three replicate fermentations yielded an average enzyme production of 1645.3 U g^{-1} of dry substrate, a value within 20 % of the predicted value of 1369.6 U g^{-1} of dry substrate (Table 5). Therefore, the response surface model and its optimum point were verified. Statistical optimization of this fermentation increased the final activity from 151.7 U g^{-1} of dry substrate for unoptimized conditions to 1645.3 U g^{-1} of dry substrate for the optimized case. The, optimized conditions enhanced the final titer of xylanase by nearly 11-fold.

Table 5 – *Predicted and measured values of maximum xylanase activity*

Variable	Value	Xylanase activity (U g^{-1} ds)	
		predicted	measured
pH	7.0	1369.6	1645.3
Temperature (°C)	30		
Substrate-to-moisture mass ratio	1:5		
Incubation time (d)	5.0		

It has been reported in literature that xylanase yield was lesser (approximately 20–100 U g^{-1} solid substrate) in SSF with *Penicillium citrinum* as the producing organism.[30–31] While, with *Aspergillus niger*, the xylanase yield was comparable with our results.[29]

Partial purification of xylanase

The enzyme could be easily concentrated by ammonium sulfate precipitation from the clarified extract of the fermented solids. Nearly half (51.1 %) of the xylanase activity was recovered in the precipitate after the crude extract was supplemented with ammonium sulfate to 40–60 % of the saturation level. The precipitate was redissolved in 0.05 mol L^{-1} citrate buffer and dialyzed overnight against the same buffer for desalting prior to activity measurements. Precipitation concentrated the enzyme activity by 3.5-fold (Table 6). Similar purification levels and concentration factors have been previously reported for recovery of xylanases by ammonium sulfate fractionation from crude extracts and clarified broths of *Aspergillus ochraceus*[32] and *Aspergillus foetidus*.[29]

Table 6 – *Xylanase activity and yield during various steps of crude extract purification*

Purification step	Volume (mL)	Total xylanase (U)	Total protein (mg)	Specific activity (U mg^{-1})	Yield* (%)	Purification factor
Crude extract	50	4230.6	135	31.3	100	1.00
Ammonium sulfate fraction	5.0	2160.6	19.9	108.8	51.1	3.5
Dialysis	9.5	2136.5	18.9	113.0	50.5	3.6

*Based on the total activity in the crude extract.

pH and temperature optima, pH and temperature stability

Xylanase of *P. citrinum* was stable in the pH range of 3 to 8. Above a pH value of 8, the activity reduced to 10 % of the initial value after 24 h of incubation at 30 °C. The optimum pH for activity was found to be 6. The optimum temperature for activity was 50 °C. At 70 °C, the activity was reduced to 20 % of the initial value after 24 h. Thus, the enzyme could be viewed as relatively thermostable. Often, enzymes are not stable for long at temperatures of >40 °C.[33] In terms of pH stability, optimal pH, optimal temperature and thermostability, the enzyme was generally comparable to the xylanases of *Bacillus pumillus*,[7] *Rhizopus oryzae*[34] and *Streptomyces* sp.[35]

Effect of metal ions on xylanase activity

Relative to control (no additive), the activity of xylanase was enhanced or reduced depending on the additive used (Table 7). EDTA, Hg^{2+} and Ca^{2+} had strong activity reducing effects (Table 7). EDTA is a strong chelator of metal ions. Reduced activity in the presence of EDTA suggests a requirement for a metal ion for the action of xylanase, although xylanases are not metaloproteins.[36] The required ion is obviously not Hg^{2+} or Ca^{2+} as these ions reduced activity (Table 7). Several other multivalent metal ions enhanced activity (Mg^{2+}, Mn^{2+}, Fe^{3+}, Zn^{2+}, Cu^{2+}, Co^{2+}) as did also the monovalent Na^{+} (Table 7). Many xylanases are known to be inhibited by Hg^{2+},[37,38] possibly because the catalytic site of the enzyme contains histidine[36,39] which appears to complex with Hg(II).[40] Compared to other metal ions, Hg(II) has in general a greater binding power for nitrogen ligands[40] such as the ones found in histidine residues. The activity stimulating effect of some of the other metal ions may be linked to their weaker interactions with the active site ligands, although no direct evidence for this appears to exist.

Table 7 – *Effect of additives on xylanase activity relative to control*

Additives	Activity (%)
None (control)	100
Co^{2+}	189
Cu^{2+}	165
Zn^{2+}	142
Fe^{3+}	132
Mn^{2+}	124
Na^{+}	123
Mg^{2+}	113
Tween 80	108
Ca^{2+}	56
Hg^{2+}	21
EDTA	14

Conclusions

Of the substrates tested, sugarcane bagasse proved to be the best for producing xylanase by solid-state fermentation using the microfungus *Penicillium citrinum* MTCC 2553. The optimal conditions for producing the enzyme in 5-day batch fermentation were: substrate-to-moisture mass ratio of 1:5, initial pH of 7 and incubation temperature of 30 °C. Under these conditions, the final activity of xylanase was 1645 U g^{-1} of dry substrate. During hydrolysis of Birchwood xylan, the partly purified xylanase was shown to have temperature and pH optima of 50 °C and 6, respectively. The enzyme was inhibited by 10 mmol L^{-1} Ca^{2+}, Hg^{2+} and EDTA, but was activated by 10 mmol L^{-1} Co^{2+}, Cu^{2+}, Zn^{2+}, Fe^{3+}, Mn^{2+}, Na^{+} and Mg^{2+}.

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List of symbols

P – predicted response (xylanase activity); U g^{-1} of dry substrate
b_0 – coefficient of interception
b_i – coefficients of linear effects
b_{ii} – coefficients of quadratic effects
b_{ij} – coefficients of interactive effects ($i < j$)
X_i and X_j – process variables
R^2 – coefficient of determination

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