Acid Xylanase Production from Low Cost Solid State Fermentation Medium

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Abstract;

Characterization of xylanase produced by *Aspergillus niger* was done after extracting the xylanase from wheat bran (WB) which was used as a substrate for solid state fermentation (SSF) process. The extraction parameters for xylanase were optimized by central composite design (CCD). The maximum recovery of xylanase (1460 U/g) was obtained employing water (3.468 ml/g) as extractant at 200 rpm for 2h. Xylanase was found to be active in a vast range of pH from 3.6 to 9 with an optimum at 5. The optimum temperature of this xylanase was 50°C. In the presence of metal ions such as Ca^{2+} and Mn^{2+}, the activity increased while Mg^{2+}, Zn^{2+}, Fe^{2+} and Co^{2+} showed a decreasing trend. Hg^{2+} ions completely inhibited the activity of xylanase. Surfactants caused a negative effect on the activity, when on tested with tween 80 the activity increased. The xylanase showed specific activity towards different xylans of which birchwood xylan yielding the maximum activity.

Keywords: *Aspergillus; Central composite design; Characterization; Extraction; Solid State Fermentation; Xylanase*

Introduction

Xylanases (endo-_-1,4-xylanases, EC 3.2.1.8) are important group of industrial enzymes responsible for hydrolysis of xylan, the major component of hemicelluloses in plant cell walls, to xylo-oligosaccharides and xylose. These end-products has considerable importance in commercial applications are furfural and xylitol [1]. The global market for industrial enzymes was estimated to be about US$ 2.3 billion in 2007 with a projected increase of 4% in annual growth rates [2]. Today the world feed xylanase market is probably worth in excess of US$500m per annum. The commercial applications for xylanase include the chlorine-free bleaching of wood pulp prior to the papermaking process, and the increased digestibility of silage. Xylanases are also used as food additives to poultry, in wheat flour for improving dough handling and quality of baked products, for the extraction of coffee, plant oils, and starch, in the improvement of nutritional properties of agricultural silage and grain feed, and in combination with pectinase and cellulase for clarification of fruit juices and degumming of plant fiber sources such as flax, hemp, jute, and ramie.

In recent years, the biotechnological use of xylanases has grown remarkably [3-6. Currently, xylanase and cellulase, together with pectinases, account for 20% of the world enzyme market. Commercially, xylan can be converted to β-D-xylopyranosyl and its oligosaccharides via acid hydrolysis. However, formation of toxic compounds and corrosion of metallic equipment when contact with acid is major problems raised during acid hydrolysis. To avoid the problems, recently, some industrial companies have shown interest in the development of efficient enzymatic processes. Xylanases are used in animal feed along with glucanases, pectinases, cellulases, proteases, amylases, phytase, galactosidases and lipases. These enzymes break down arabinoxylans in the ingredients of the feed, reducing the viscosity of the raw material [7]. They are produced by fungi, bacteria, marine algae, insects, seeds, etc. but the principal source of commercial xylanases remains to be filamentous fungi because they are capable of producing high levels of extracellular enzymes and can be cultivated very easily. On an industrial scale, xylanases are produced mainly by *Aspergillus* [8] and *Trichoderma* sp. in solid-state- fermentation (SSF) [9]. Xylanase from *Streptomyces* sp.[10], *Aspergillus awamori* [11], *Aspergillus fischeri* [12], *Aspergillus terreus*, *Aspergillus foetidus* [13], *Trichoderma reesei* [14], *Burkholderia* sp [15] and many other species has been reported.

The interest towards xylanase enzyme preparations is basically by their wide applications in various fields of industry. The enzyme hydrolysis of xylan lies in the basis of its utilization as an energy source in different biotechnological processes [16]. The partial enzyme hydrolysis of xylan changes its physical and chemical properties, which concerns the quality of different products of the food and flavor industry [17]. Xylanase finds applications in fruit juices and wines clarifying [6, 18]. In brewing, xylanase is applied in filtering improvement [19, 20]. The utilization of xylanase in bread-making significantly improves the desirable texture, loaf volume and shelf life of bread [17, 21]. Xylanase enzyme preparations are also widely used in bio-bleaching in paper industry. They facilitate the delignification of the plant pulp in the production of high-quality paper [22]. In that way, the chlorine-containing bleaching agents, that are a serious ecological problem, can be reduced [23]. Most of the industrial processes are carried out at higher temperature, where many enzymes are often unstable above certain physiological conditions [24]. There is an ample scope to study the thermostability, pH stability and effects of various reagents that inhibit the activity and it can be extended to industrial process. Maintaining the desired level of enzyme activity over a long period of time and improving its stability are important parameters for the selection and design of xylanase. The knowledge gained in this way will offer new possibilities in improving the potential use of this enzyme in such diverse and broad areas.

Solid-state fermentation (SSF) has been defined as the fermentation process which involves solid matrix and is carried out in absence or near absence of free water; however, the substrate must possess enough moisture to support growth and metabolism of the microorganism. The solid matrix could be either the source of nutrients or simply a support impregnated by the proper nutrients that allows the development of the microorganisms [1, 2]. SSF has experienced renewed interest due to many potential advantages of this bioprocess in comparison with submerged fermentation (SmF). These advantages include smaller bioreactor volumes, reduced downstream processing costs, superior productivity, simpler techniques, reduced energy requirements, low wastewater output [3, 4]. For the majority of filamentous fungal species, solid-state media are the natural life media. Growth can occur on the surface or within the whole substrate, depending on the porosity. Industrial SSFs have been developed largely in traditional food industries such as cheese, oriental fermentations, fermented vegetables, meat, and other products, and in biotechnological industries such as antibiotics and enzymes [5].

The experimental design is powerful tool for studying the individual and synergistic effects of different operating parameters concurrently. Especially, Response Surface Methodology (RSM) is utilizes quantitative data based on adequate experimental planning in order to determine and simultaneously resolve multi-variate equations. This method is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors, and analyzing optimum conditions of factors for desirable responses [25]. RSM has been extensively employed for to optimize many enzyme producing processes, such as pectinases. Much information can be obtained using experimental designs based on statistical principles, performing a minimum number of experiments. Different statistical designs for medium optimization regarding xylanase production have been reported; among which factorial experiments and RSM are included [12, 26, 27]. In this study, we describe recovery of xylanase from *Aspergillus niger* in SSF. The ultimate objective of extraction is to achieve high enzyme activity. Like other techniques, the extraction can be affected by factors including the natural properties of the solid substrate and extraction conditions. To obtain high yield, it is important to understand the relationship between these two goals and the extraction conditions and to optimize conditions accordingly. The extraction of xylanase is influenced by factors such as volume of extractant, time and agitation speed. The effects of operating parameters of extraction were optimized using RSM. Based on experience and economic feasibility, a (Central composite Design) CCD was employed. Biochemical characteristic of the enzyme and inhibition of its activity on action of various reagents were also studied.

Materials and Methods

Microorganism and Solid state fermentation

Universal primer used in this study, NL1 (5'GCATATCAATAAGCGGAGGAAAAG) and NL4 (5'GGTCCGTGTTTCAAGACGG). The pure culture of *A. niger* MTCC 1344 procured from microbial type culture collection (MTCC), Chandigarh, India was maintained in Czapeck Dox Agar medium in petriplates and stored at 4 ◦C.

Screening of Xylanase producing organism

The cultures were carried out in 250 ml Erlenmeyer flasks incubated in a tray chamber (50x50x70 cm) with temperature and humidity automatically controlled. Production studies of xylanase on wheat bran (WB) from *A.niger* were carried out. The appropriate quantity of solids that absorbs 10mL of the nutrient solution without drainage was determined previously. To avoid any nutrient limitations when using WB these substrates were moistened with the same nutrient solution; 10 g solid was moistened with 10mL solution. WB was moistened with mineral salt solution (1.0:1.0 w/v) containing lactose 10; soyaflour toasted 5; NH_4SO_4 0.4; Tween 80 0.01%; KH_2PO_4 1.0. The best optimum conditions were 120 h incubation time, pH of 5.5 at 40°C, 72h old inoculum with 15% inoculum size.

Extraction of xylanase

After 120h of fermentation, the contents of each flask were extracted with 100 ml of cold 0.1 M acetate buffer (pH 5.0). A spatula was used to break the solid culture into smaller particles. The flasks were subsequently transferred to an incubator shaker at 4 ^oC and 200 rpm for approximately 120 min. After shaking, the contents were centrifuged at 13, 500 rpm for 20 min. The supernatant was filtered through Whatman No. 1 filter paper and the crude culture filtrate containing fungal enzymes were stored at 20 ^oC for 24 h prior to xylanase assays.

Interactive effects of extraction parameters on xylanase recovery

Optimum condition for extraction of xylanase was determined by response surface methodology (RSM) and the important class of second-order design called central composite design (CCD) was used for the analysis. Optimization studies were carried out by considering the effect of three variables such as, extraction time (h), agitation (rpm) and volume of extractant (ml/g dry substrate) at five levels (preliminary experiments were carried out to determine the parameters range) was used to study their interactive influence on xylanase recovery from optimized solid-state medium. The result of CCD with six replicates at the center point was used to fit the second order response surface. The independent variables chosen in this study were coded according to the equation given below:

$$x_i = \frac{X_i - X_0}{\Delta X_i} \qquad (1)$$

Where, x_i is the dimensionless coded value of the i^{th} independent variable; X_i the natural value of the i^{th} independent variable; X_0 the natural value of the i^{th} independent variable at the center point and ΔX_i the step change value. Once the experiments were performed, the experimental results were fitted with a 2nd order polynomial function:

$$Y = b_0 + b_1 x_1 + b_2 x_2 + b_3 x_3 + b_{11} x_1^2 + b_{22} x_2^2 + b_{33} x_3^2 + b_{12} x_1 x_2 + b_{13} x_1 x_3 + b_{23} x_2 x_3 \quad (2)$$

Where, Y is the predicted response; b_0 the intercept; b_1, b_2, b_3 the linear co-efficient; b_{11}, b_{22}, b_{33} the squared co-efficient and b_{12}, b_{13}, b_{23} the interaction co-efficients

Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA). This analysis included the fisher's F-test (overall model significance), its associated probability P (F), determination coefficient R^2 which measures the goodness of fit at regression models. For each variable, the quadratic model was represented as contour plots were generated using Minitab 15.

Xylanase assay

Xylanase activity was assayed by the method of Khanna and Gauri [28] and the release of reducing sugar from soluble xylan using 3,5-dinitrosalicylic acid (DNS) method as described by Miller [29]. The standard assay mixture contained 0.1 ml of appropriately diluted enzyme and 0.9 ml of McIlvaine buffer (pH 5.5) containing 1% (w/v) oat spelt xylan. After incubation at 70 ◦C for 10 min, the reaction was terminated by adding 1.5 ml of DNS reagent. The mixture was then boiled for 5min and cooled to room temperature, and the absorption at 540nm measured. Each reaction and its control were run in triplicate

Effect of pH on activity and stability

The relative xylanase activity using 1% (w/v) xylan was determined at various pH. For the optimum pH determination, 0.1M buffer solutions ranging from pH 3.6 to 9.0 were used at the optimal temperature previously determined. Acetate buffer was used for pH 3.6–5.6, phosphate buffer for pH 5.6–7.5 and Tris–HCl buffer for pH 7.5–9.0. For pH stability studies, purified enzyme was pre-incubated in 0.1M appropriate buffers for 2 h at 40 ◦C. The relative activity was measured at standard assay conditions.

Effect of temperature on activity and stability

The optimal temperature for xylanase was obtained by assaying the enzyme activity at different temperatures (30–70°C). The thermostability of xylanase was monitored by incubating the enzyme solution at a fixed temperature, in the range of 30–60 °C and measuring the activity at 60 min interval for 9 h.

Effect of metal ions

The effect of metal ions ($HgCl_2$, $ZnCl_2$, $CuSO_4$, $CaCl_2$, $MnSO_4$, $MgSO_4$, $FeCl_2$ and $CoCl_2$), on relative activity of xylanase was determined by adding the metal ions to xylanase at various concentrations and incubated at 50°C for 30 min. Relative activity was expressed as the percentage of the activity observed in the absence of any compound.

Effect of surfactants

To determine the effects of non-ionic (Tween-80 and Triton X 100) anionic surfactants (SDS) on xylanase, the surfactants were added to xylanase and incubating the enzyme at varying concentrations of surfactants and oxidizing agent at 50°C for 30min.

Substrate specificity

Substrate specificity of the enzyme was determined using different cellulose and hemicellulose substrates. Birchwood, Beechwood and Oat- spelt xylan were tested for its activity. The reaction was carried out in 50mM citrate phosphate (pH 5.0) containing 2.0 mg/ml of each substrate at 50 ◦C for 10 min. For each assay, three different substrate concentrations were prepared in 50mM citrate phosphate (pH 5.0), and incubated with the purified enzyme at 50 ◦C for 30min.

Results and Discussion

Production of xylanase

Excellent growth of *Aspergillus niger* was noted on nutrient-supplemented WB. The first sign of fungal mycelial growth on WB during SSF was seen 2nd day after inoculation. WB was completely colonized by fungal mycelia within 6 days. Different incubation time (1- 7 days) was employed to study the effect on xylanase production using WB as substrate. The fermentation was carried out keeping all other conditions at above optimum levels. The enzyme production increased with time of incubation reaching a maximum after 120h (Fig.1). The enzyme activity was maximum 891 U/g of WB after 120h and minimum 513 U/g of WB after 168h of incubation. With extending incubation time the yield of xylanase had decreasing trend. This result confirmed that highest activity of enzyme would not occur until a sufficient biomass was formed, and that further extending incubation time had no effect on improving the enzyme yield. The difference in incubation period may be due to the substrates, difference of organisms, growth rate of microorganism and its enzyme production pattern [30, 31].

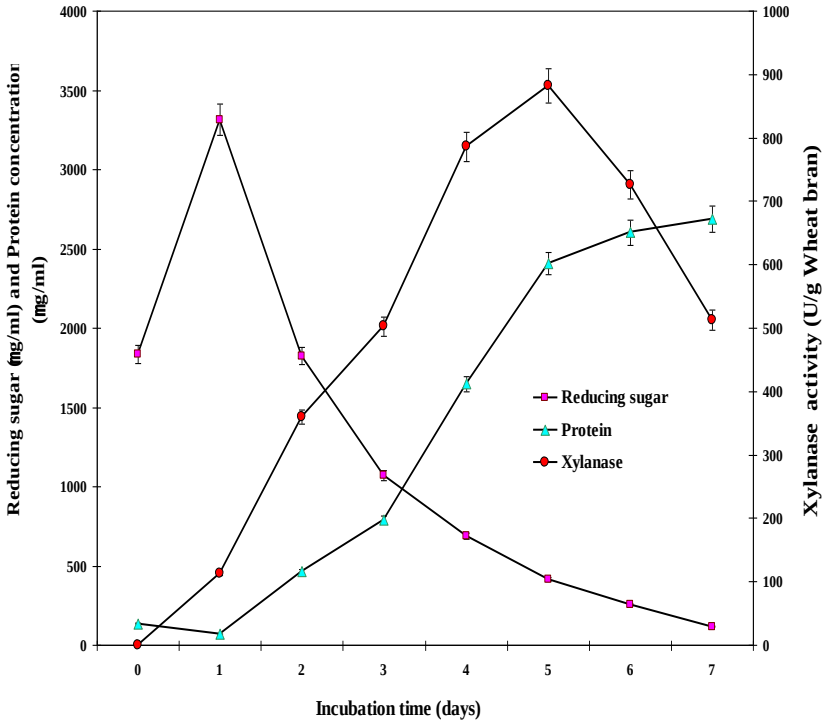

Fig. 1: Production of Xylanase from *Aspergillus niger*

The soluble protein assay used to assess fungal biomass indirectly showed a rapid increase after day 4 (Fig.1). This suggested that the increase in protein content in the culture was partly due to the secretion of enzymes such as xylanase and other extracellular enzymes responsible for the degradation of WB. The soluble protein content of WB increased at an almost linear rate until reaching a maximum after 7 days of SSF with an average value of 2.76 mg/g.

Enzyme extraction and properties of the crude extract

The first step for obtaining the xylanase from the fermented system is leaching, a process devoted to the recovery of the enzyme from solids in order to obtain a crude extract by using cold 0.1M acetate buffer (pH 5.0). To employ a statistical approach for optimization of xylanase extraction, it was necessary to first gain information about extraction conditions and the nature of the experiments. Xylanase extraction was optimized by the traditional technique of varying one variable at a time to achieve optimal recovery. Therefore, preliminary extraction was performed with the cold 0.1M acetate buffer (pH 5.0). Various experiments at different agitation time (1 – 3 h), agitation speed (100-300 rpm) and volume of extractant (6 – 14 ml/g) were performed (data not shown). As it is seen all these three parameters have major influence on the extraction of xylanase. Results showed that, 840U/ml of xylanase were obtained in the crude extract. The preliminary extraction provided important information regarding each parameter and this information was used in RSM experiments.

At this stage, xylanase were not completely obtained for satisfactory recovery and further fine tuning of optimization of accurate extraction conditions was needed.

Interactive effect of extraction parameters on recovery of xylanase

A quadratic regression model was developed using coded values from the estimation of data. A full factorial CCD was applied to study the interactive effect of extraction time (min), agitation speed (rpm) and volume of extractant (ml/g) on the recovery of xylanase and to derive a statistical model for their effects. To make the regression model accurate, the center point was repeated six times. Twenty experiments were performed using different combinations of the variables, as per the CCD. The experimental design and results of the CCD are shown in Table 1. The time of agitation varied from 1 – 3h, while agitation speed between100 – 300 rpm and volume of extractant diverges from 6 - 14 (ml/g). Maximum enzyme activity from the recovery parameters was observed at run 18 (1460 (U/g)). In this run, a very low volume of the extractant was used for the enzyme recovery. On comparison with run 7, wherein, 10 ml/g was used, showed a relatively low activity of 886 (U/g). This is due to the fact that, when higher volumes of extractant are used, extraction decreases due to dilution [32]. The model was modified based on the insignificancy of some model terms. The model equations based on coded values for the enzyme activity is represented by Eq. (3)

Table 1 Experimental design with observed and predicted value of Enzyme activity

Run order	Time (h)	Agitation (rpm)	Volume of extractant (ml/g)	Enzyme activity (observed(U/g))	Enzyme activity (predicted(U/g)
1	3	300	6	840	936.46
2	2	36.7	10	472	461.79
3	2	200	10	777	810.68
4	3	100	6	1093	1096.06
5	2	363.3	10	412	460.81
6	2	200	10	816	810.68
7	2	200	10	886	810.68
8	3	100	14	540	589.74
9	3	300	14	502	410.14
10	1	100	6	712	778.11
11	1	300	14	532	503.20
12	2	200	10	803	810.68
13	2	200	16.532	503	609.03
14	1	300	6	1032	956.51
15	3.633	200	10	819	771.61
16	2	200	10	782	810.68
17	0.367	200	10	502	587.98
18	2	200	3.468	1460	1392.57
19	1	100	14	467	344.79
20	2	200	10	813	810.68

$$Enzyme\,activity(U/g) = 810.689 + 91.814x_1 - 0.487x_2 - 391.769x_3 - 130.885x_1^2 - 349.385x_2^2 + 190.115x_3^2 - 225.335x_1x_2 - 48.667x_1x_3 - 13.333x_2x_3 \quad (3)$$

Where x_1 = time; x_2 = Agitation; x_3 = Volume of extractant

The coefficients of determination (R^2=0.9367) was also reasonably good which suggested that 93% of the effect on the enzyme activity was explained by the variation in the process variables. The R^2 value provides a measure of variability in the observed response values that can be explained by the experimental factors and their interactions. The closer the R^2 value to 1, the stronger the model is and better it predicts the response [33]. Therefore, equation (3) can be used for predicting the response at any combination of three predicted variables in and around their experimental range. The enzyme activity (response) at specified combination of the three variables can be predicted by substituting the corresponding coded values in the equation. ANOVA results of these quadratic models are presented in Table 2, indicating that these quadratic models can be used to navigate the design space.

Table 2 Analysis of Variance for Enzyme activity Observed

Source	DF	Seq SS	Adj SS	Adj MS	F	P
Regression	9	1209714	1209714	134413	16.45	0.000
Linear	3	809563	809563	269854	33.03	0.000
Square	3	340165	340165	113388	13.88	0.000
Intraction	3	59987	59987	19996	2.45	0.124
Residual Error	10	81696	81696	8170		
Lack-of-Fit	5	74001	74001	14800	9.62	0.013
Pure Error	5	7695	7695	1539		
Total	19	1291411				

Apart from the linear effect of the variables on the extraction process, the second order RSM also gives insights into their quadratic and interaction effects. The significance of each co-efficient was determined by Student's t-test (Table 3). The smaller the p- and larger the t-value, the more significant is the corresponding co-efficient [34]. The second order terms of agitation and volume of extractant were also significant. The interaction terms were insignificant. All the linear co-efficients were highly significant ($p \leq 0.01$). The high significance denotes that they can act as limiting factors and a small variation in their values will affect xylanase recovery to an extent [35]. The sign and magnitude of the co-efficients indicate the effect of the variable on the response.

Table 3 Co-efficients of regression equation

Terms	Co-efficient	t-value	p- value
Constant	810.689	22.055	0.000
Time	91.814	2.271	0.046
Agitation	-0.487	-0.012	0.991
Volume of extractant	-391.769	-9.692	0.000
$Time^2$	-130.885	-1.973	0.077
$Agitation^2$	-349.385	-5.268	0.000
Volume of $extractant^2$	190.115	2.866	0.017
Time * Agitation	-225.335	-2.644	0.025
Time *Volume of extractant	-48.667	-0.571	0.581
Agitation* Volume of extractant	-13.333	-0.156	0.879

A proper choice of level combination of variables is desirable for maximizing the enzyme extraction, which can be made from contour plots of individual response (Fig.2). Fig 2a elucidates the enzyme activity with respect to agitation (x_2) and volume of extractant (x_3) while keeping the other parameter (time) unchanged. Fig 2b depicts the change in activity of xylanases owing to the simultaneous change in time (x_1) and agitation (x_2) at constant volume of extractant. Fig 2c explains the concurrent change in time(x_1) and volume of extractant(x_3). The interaction between extraction time and volume of the extractant is statistically significant and the contour curve indicates that xylanase recovery is affected dominantly and positively by volume of extractant without being much affected by the time. It also reveals that maximum activity can be obtained when low volume of extractant is used. At higher agitation rates and time, a decreased activity of the enzyme was observed. This is because; at higher and prolonged agitation the enzyme denatures which leads to loss in enzyme activity [36]. The plots infer that xylanase recovery was sensitive to change in all the variables.

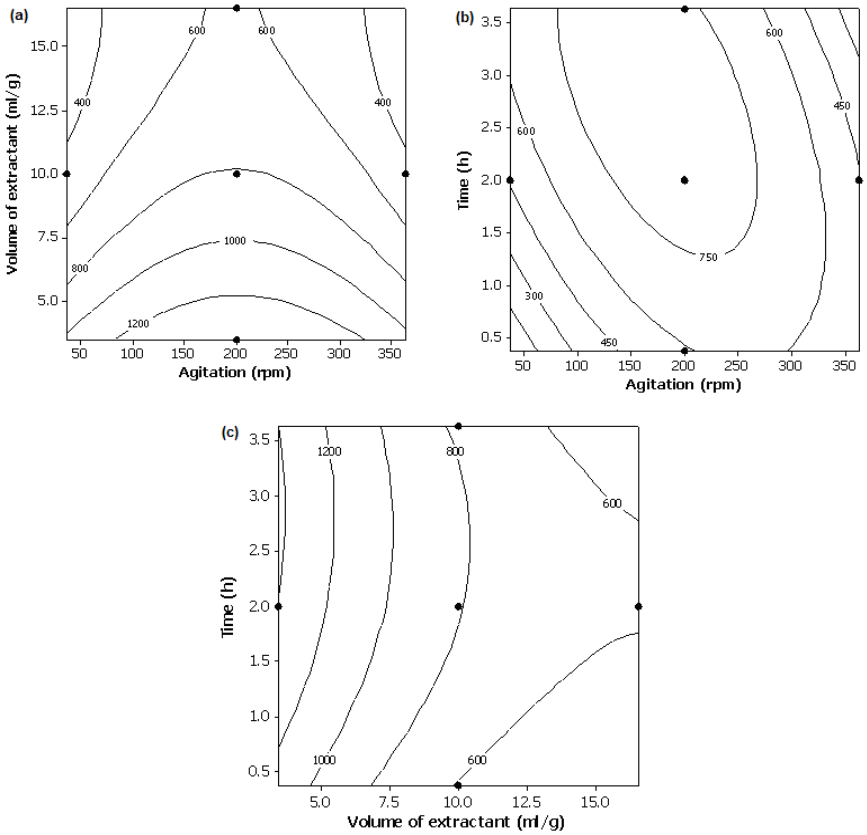

Fig 2: Two dimensional contour graph showing the effects of volume of extractant, time and agitation speed on enzyme activity.

Effect of pH on Xylanase activity and stability

pH affects the enzyme activity to a considerable extent. This is because substrate binding and catalysis are often dependent on charge distribution on both, substrate and particularly enzyme

molecules [13, 15]. The enzyme was highly active in a wide range of pH, showing more than 50% of the maximum activity in the pH range of 3.6 to 9. The optimum pH for xylanase activity of *A.niger* was at 5 (fig 3c) suggesting that it may be of great value in fruit juice processing where the acidic pH is favored. A pH stability study is an essential part of any enzyme characterization before it can be exploited commercially. [9]. The optimum pH was approximately 5 in the similar experiments on some fungal xylanases [37, 38]. A remarkable drop in the enzyme activity was observed below pH 5 and above pH 5.6. The pH optima of bacterial xylanases in general, are slightly higher than fungal xylanases. Xylanase was also studied for its pH stability and it was found to be stable in the pH range 4–7. The activity was studied for 7 hours incubation time (fig 3d). The activity seemed to be high at lower pH range and less at higher pH.

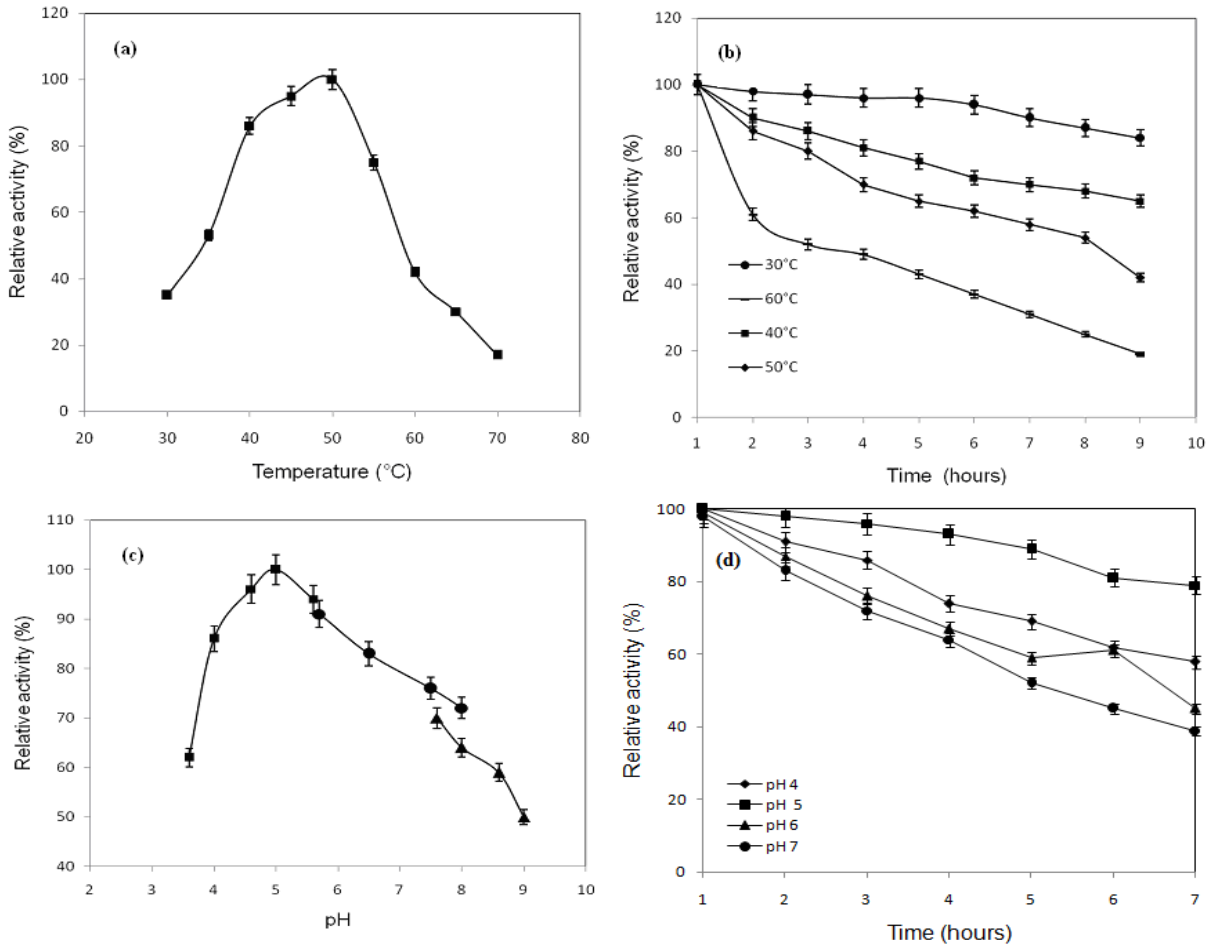

Figure 3 Influence of temperature (a) thermostability (b), influence of pH (c) and pH stability (d) on xylanase activity from *A.niger*. Data are average of three replicates

Effect of temperature on xylanase activity and stability

The temperature profile of xylanase activity from *Aspergillus niger* strain is presented on Fig. 3a. The optimum temperature was observed at 50°C. The enzyme activity significantly increased with increase in temperature from 30 to 50°C and further increase in temperature adversely affected the

enzyme activity. Xylanases from *Fusarium proliferatum* showed a high stability of 55°C, losing its stability at 60°C [39] and *Aspergillus foetidus* showing an optimum of 50°C [13]. Utilization of enzymes in industrial applications often encounters its thermal inactivation. Thermal stability studies were carried out in the temperature range of 30–60 °C. At the end of 9th hour, xylanase showed a decrease in activity of 15%when the enzyme was incubated at 30°C. 35% decrease in the activity was observed on incubating the enzyme at 40°C. At 50 °C, the enzyme seemed to be very sensitive by losing 30% of its activity at 4th hour and 58% of its activity at the end of 9th hour. Xylanase lost almost 50% of its activity at 4th hour and at the end of 9th hour it retained only 19 % of its activity. Fig 3b gives a clear indication of temperature range for industrial usage of xylanase from *A.niger*. Similar results were observed on thermostability studies on xylanase produced by *Jonesia denitrificans* [40].

Effect of metal ions

The activity of xylanase was checked in the presence and absence of metal ions(Table 4). On increasing the concentration of Mg^{2+}, Zn^{2+}, Fe^{2+} and Co^{2+} from 1 to 10 mM, reduced the enzyme activity by 0.68, 0.27, 0.53 and 0.47 fold, respectively. Similarly, increasing the concentration of Mn^{2+} and Ca^{2+} to 10 mM, resulted in 1.55 and 1.17 fold of increased in activity. Hg^{2+} showed a thorough inhibitory effect on the activity of the xylanase enzyme, suggesting that there is an important cysteine residue in or close to the active site of the enzyme [41]. A mediocre inhibition was observed on presence of Zn^{2+}, Co^{2+}, Fe^{2+} and Mg^{2+} whereas Ca^{2+} and Mn^{2+} ions increased the xylanase activity at all concentrations (table 3). Increase in the activity in the presence of Ca^{2+} may be due to stabilization of enzyme in its active conformation rather than it being involved in the catalytic reaction. It probably acts as a salt or ion bridge via a cluster of carboxylic groups [42]. Most of the xylanases were found to be inhibited by Mn^{2+} [39]. But the xylanases of *Aspergillus versicolor* [43] are stimulated by Mn^{2+} ions. It may be interpreted that, Mn^{2+} ion exerts its effect by interacting with some amino acid residues involved in the active site, which causes a change in conformation leading to higher activity, but also higher susceptibility to denaturation at higher temperatures [40].

Table 4 Effect of various metal ions on activity of xylanase produced by *A.niger*
[a] = Relative activity is expressed as a percentage of control (100% of enzyme activity).

Metal ions	Concentration (mM)	Relative activity [a] (%)
Zn^{2+}	1	95
	5	62
	10	26
Hg^{2+}	1	12
	5	0
	10	0
Mn^{2+}	1	112
	5	148
	10	174
Mg^{2+}	1	98
	5	75
	10	62
Ca^{2+}	1	104
	5	108
	10	122
Fe^{2+}	1	95
	5	74
	10	51
Co^{2+}	1	89
	5	63
	10	42
Control	-	100

Effect of surfactants

The effect of surfactants on activity of xylanase produced by *A. niger* has been summarized in table 5. On addition of anionic agents, viz., SDS the activity of xylanase showed a inhibition effect with concentration. Tween – 80 increased the xylanase activity at lower concentrations, while higher concentration of these detergents showed a lesser activity than that in lower concentrations. Therefore, tweenn 80 can be recommended for preservation of xylanase enzyme during storage. The xylanase produced in this study showed a improved stability in the presence of SDS when compared to xylanase from microbial community EMSD5 [44] and relatively lower stability with SDS on xylanase produced by *S. matensis* [45].

Table 5 Effect of surfactants on activity of xylanase produced by *A.niger*

Reagents	Concentration(mM)	Relative activity (%)
SDS	1	92
	5	84
	10	69
Tween – 80	1	126
	5	117
	10	106
Triton X 100	1	103
	5	94
	10	88
Control	-	100

Substrate specificity

The activity of xylanase on artificial substrates was tested (table 6). Birchwood xylan showed the best activity (124%) among the substrates tested, while beechwood (114%) and oat spelt xylan (100%) gave a relatively less activity. Similar result with brichwood xylan giving the maximum activity was reported for *T.reesei* and its recombinant [14].

Substrates	**Relative activity (U/ml)**
Birchwood xylan	124
Beechwood xylan	114
Oat – spelt xylan	100

Conclusions

Xylanase production could be considerably improved by optimizing the extraction parameters for maximum recovery (1460 U/g) at 200rpm, 2h of incubation time and 3.6 ml/g of extractant. Xylanase produced extends its activity in wide range of pH and temperature which is attributing to saccharify lignocellulosic waste as well as industrial pulp demonstrates its potential application in paper and pulp biotechnological sector. The study also shows that RSM was an appropriate [1-7] method to optimize the extraction parameters to obtain maximum enzyme activity. The experimental and the predicted values were very close which reflected the accuracy and applicability of RSM.

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