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Full Length Research Paper

Effect of medium composition and ultrasonication on xylanase production by *Trichoderma harzianum* MTCC 4358 on novel substrate

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Sunflower sludge was used as the sole carbon source for xylanase production, an important industrial enzyme used in pulp and paper industry, by the fungi *Trichoderma harzianum* MTCC 4358. Sunflower sludge was subjected to alkaline pretreatment which showed higher enzyme activity than the substrate used without pretreatment. The effect and significance of the medium components were studied using the Plackett-Burman design. The experimental xylanase activity was subjected to statistical analysis using MINITAB 15 software. It was found that peptone, urea, $CaCl_2.2H_2O$, Tween 80, $(NH_4)_2.SO_4$, $MgSO_4.7H_2O$ and $MnSO_4$ significantly influenced xylanase production (8.63 U/g of dry substrate). The enzyme showed maximum activity at pH 5 (8.75 U/g of dry substrate), 45°C (8.66 U/g of dry substrate) and 80% initial moisture content (8.84 U/g of dry substrate) and a period of 7 days incubation (8.57 U/g of dry substrate) was necessary for maximum enzyme production. Under these optimized physical conditions and in optimized medium *T. harzianum* MTCC 4358 was inoculated and subjected to ultrasonication for every 12 h (for a period of 8 days), which resulted in higher enzymatic activity of 9.27 U/g of dry substrate.

Key words: Xylanase, *Trichoderma harzianum*, ultrasonication, sunflower sludge, Plackett-Burman design.

INTRODUCTION

Lignocellulosic wastes are generated from agricultural practices and industrial processes, particularly from agro-allied industries such as breweries, paper-pulp, textile and timber industries throughout the world. These wastes generally accumulate in the environment, thereby causing pollution (Okafor et al., 2007). These wastes are, however, biodegradable and can be converted into valuable products such as biofuels, chemicals and cheap energy sources for fermentation, improved animal feeds and human nutrients (Howard et al., 2003). Ligno-cellulosic wastes refer to plant biomass wastes that are mainly composed of lignin, cellulose and hemicelluloses. They may be grouped into different categories such as wood residues (including sawdust and paper mill discards), waste paper and agricultural residues including straw, stover, nutshells, bagasse, domestic wastes (lignocellulose garbage and sewage), food industry residues and municipal solid wastes (Mtui, 2009) The lignocellulosic biomass, which represents the largest renewable reservoir of potentially fermentable carbo-hydrates on earth (Mtui and Nakamura, 2005), are mostly wasted in the form of pre-harvest and post-harvest agricultural losses and wastes of food processing industries. Due to their abundance and renewability, there has been a great deal of interest in utilizing lingo-cellulosic wastes for the production and recovery of many value-added products (Pandey et al., 2000; Foyle et al., 2007).

Sunflower (*Helianthus annuus*) is one of the most important oil seed crops. It is cultivated worldwide for oil extraction. The by-product rendered by the oil industry is known as sunflower sludge, which is produced in large quantities and remains as waste. The chemical com-position of sunflower sludge was studied by fractionating it into three main components, a lignocellulosic fraction (LCF), a proteinaceous fraction (PF) and a soluble

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fraction (SF), which represent 23.2 to 25.3%, 55.4 to 57.6% and 17.1 to 21.4% of the dry weight, respectively. After the removal of the PF, the remaining sub products (LCF and SF), had a high potential for use as fermentation sources. Sunflower sludge LCF is a suitable fermentation source for solid-state fermentation, as is shown by the growth of different fungi (Bautista et al., 1990).

D-Xylan is the major hemi-cellulose found in woods and accounts for 20 to 35% of the total dry weight of hardwood and perennial plants (Haltrich et al., 1996). The basic structure of xylan is a β- D-(1,4)-linked xylo-pyranosyl residue with a few branch points (Kulkarni et al., 1999). The major backbone carries relatively short side chains of variable lengths. Due to the abundance and the structural heterogeneity of xylan, xylan-degrading enzymes are diverse. Typical xylan-degrading enzymes are endo-β-xylanases (EC 3. 2. 1. 8), which attacks the main chains of xylans, and β-xylosidases (EC 3. 2. 1. 37), which hydrolyze xylo-oligosaccharides into D-xylose. These two enzymes also required for complete hydrolysis of native cellulose and biomass conversion, are produced by many bacteria and fungi. Potential applications of xylanase in biotechnology include bio-pulping of wood, pulp bleaching, processing food to increase clarification, treating animal feed to increase digestibility (Wong et al., 1988) and converting lignocellulosic substances into feed stocks and fuels (Kim et al., 2000). Given the potential usefulness of the enzyme for different applications, development and optimization of enzyme production methods with the ultimate aim of reducing the over-all enzyme production cost could be very important. One alternative is the use of solid-state fermentation.

Solid-state fermentation is defined as the fermentation involving solids in the absence (or near absence) of free water. The substrate, however, must contain sufficient moisture to support the growth and metabolism of the microorganisms (Pandey, 2003). Solid-state fermentation offers advantages over submerged fermentation due to its simplicity and its closeness to the natural way of life for many microorganisms. For example, large amount of wastes are not added to the biological materials, fermented volumes remains small, necessary manipulations become less expensive and the cost of downstream is minimized (Ferreira et al., 1999). A large number of fungi are known to grow well on moist substrates in the absence of free-flowing water, whereas many bacteria are unable to grow under these conditions. As a result, the majority of studies involving solid-state fermentation are conducted using fungi (Shah and Madamwar, 2005).

It is well known that 30 to 40% of the production cost of industrial enzymes is taken up by the cost of growth medium (Seeta Laxman et al., 2005). Carbon and nitrogen sources together with fermentation time have been reported to play significant roles in the determination of the final morphology of the culture (Papagianni, 2004). Therefore, it is significant to optimize these conditions for low-cost enzyme production using powerful statistical techniques. Plackett-Burman design (PBD) has been used as a successful statistical tool for optimization of medium compositions in a fermentation process for enzyme production and is a popular method that is useful for screening key nutrients, as well as for screening culture conditions (pH, temperature, etc.) (Chauhan et al., 2007; Gennaro et al., 2006). However, few literatures have reported the use of response surface methodology (Senthilkumar et al., 2005; Kim et al., 2007) and the second-order regression for rotation-orthogonal composite design for process optimization (Zhang et al., 2007).

Ultrasonication is a kind of mechanical elastic wave. When traveling in a medium, it has the function of transmission in substance and the phenomena of calefaction and cavitation (Zhu et al., 2001). Recent studies show that ultrasound under the selected condition can be used to increase ethanol production and to improve the de-inking of recycled paper. It also stimulated the secretion of riboflavin produced by *Ecemothecium ashbyii* (Chuanyun et al., 2004). Ultrasound treatment is mainly carried out so as to eliminate the accumulation of xylanase in the cell, hence bringing up the improvement of xylanase production.

The aim of this study was to evaluate, optimize and standardize process parameters on the extracellular xylanase produced by *T. harzianum* MTCC 4358, grown in solid-state fermentation on an industrial waste, sunflower sludge, as the sole substrate for carbon source, thereby minimizing the cost of raw materials.

MATERIALS AND METHODS

Microbial strain

T. harzianum MTCC 4358 obtained from MTCC, Chandigarh, India was used for the production of xylanase. Stock cultures were maintained in the malt extract medium (40 g/L) and stored at 4°C with periodic (15 days) sub-culturing.

Pre-treatment of the substrate

Sunflower sludge was collected from Kaleeshwari refinery, Chennai, Tamil Nadu, India. Briefly, 5 g of the substrate was subjected to alkali pretreatment with 20 ml of 0.1 N NaOH at room temperature and left to soaked in the solution overnight. After treatment, the sunflower sludge was thoroughly washed with distilled water to neutral pH and dried in hot air oven at 80°C. This pretreated substrate was used as sole carbon source for the production of the enzyme.

Production medium

The medium described by Seyis and Aksoz, (2005) was used for growth and enzyme production. This medium contained (in g/L); peptone, 0.5; urea, 0.3; KH_2PO_4, 0.2; $CaCl_2.2H_2O$, 0.3; Tween-80,

Table 1. Actual values of the process variables.

Symbol	Component	g/L	
		L	H
A	Peptone	0.5	1.0
B	Urea	0.1	0.5
C	KH_2PO_4	0.1	0.3
D	$CaCl_2.2H_2O$	0.1	0.5
E	Tween 80	0.1	0.3
F	$(NH_4)_2.SO_4$	1.0	1.8
G	$MgSO_4.7H_2O$	0.1	0.5
H	$FeSO_4$	0.03	0.07
I	$ZnSO_4.7H_2O$	0.0010	0.0018
J	$COCl_2.6H_2O$	0.01	0.03
K	$MnSO_4$	0.013	0.019

The letters 'H' and 'L' represent the two different levels (higher and lower) of the independent variable under investigation

0.2; $(NH_4)_2SO_4$, 1.4; $MgSO_4{\cdot}7H_2O$, 0.3; 1% of birchwood xylan as carbon source and 0.1% of trace element was added. The trace element solution contained (in g/L): $FeSO_4$, 0.05; $ZnSO_4{\cdot}7H_2O$, 0.014; $CoCl_2.6H_2O$, 0.02; $MnSO_4$, 0.016. The medium was sterilized in an autoclave at 121°C for 20 min. After sterilization, 100 ml of the medium was inoculated with 1 ml of spore suspension containing $1{\times}10^6$ spores/ml. Production of xylanase enzyme in solid state fermentation was carried at pH 6, temperature 35°C and moisture content 70% in static condition.

Screening of different nitrogen sources and urea

In order to investigate the effect of different nitrogen sources on Seyis et al. (2005) medium, peptone in the medium was replaced with inorganic nitrogen sources such as NH_4NO_3, $NaNO_3$ and NH_4Cl. On the other-hand, the effect of urea on xylanase production was also investigated by adding urea as a supplementary nitrogen source in the medium. Incubation was done for 7 days after inoculation under pH 6, temperature 35°C and moisture content 70% in static condition.

Effect of different surfactants

In order to investigate the effect of different additional surfactants, Tween 80 in the medium was replaced with Triton X 100 and 0.1% sodium dodecyl sulfate (SDS). Xylanase activity was studied 7 days under pH 6, temperature 35°C and moisture content of 70% in static condition.

Experimental design

The Plackett-Burman design for eleven variables, including peptone (A), urea (B), KH_2PO_4 (C), $CaCl_2.2H_2O$ (D), Tween 80 (E), $(NH_4)_2.SO_4$ (F), $MgSO_4.7H_2O$ (G), $FeSO_4$ (H), $ZnSO_4.7H_2O$ (I), $COCl_2.6H_2O$ (J) and $MnSO_4$ (K) at two levels (H and L) (Table 1) (Plackett and Burman, 1946) was used for screening. The signs 'H' and 'L' represent the two different levels (higher and lower) of the independent variable under investigation. The total number of experiments performed was n + 1 according to PB design, where n is the number of variables. In the experimental design, each row represented an experiment, and each column represented an independent variable (Table 2). The statistical significance was determined by F-value and T-value.

Effect of incubation time

Erlenmeyer flasks containing sunflower sludge media with the addition of trace elements, surfactants and nitrogen sources were inoculated with a solution of spores of *T. harzianum* MTCC 4358 at a concentration of $1{\times}10^6$ spores/ml, and incubated at 35°C for 24, 48, 72, 96, 120, 144, 168 and 192 h.

Effect of pH

To evaluate the effect of pH on xylanase production, the production media was adjusted to different pH ranging from 4 to 9 using 1 N HCl and NaOH. The effect of pH on xylanase production was monitored throughout the 7 days of incubation time in static condition.

Effect of temperature

The effect of temperature on the xylanase production by *T. harzianum* MTCC 4358 was determined by incubating the inoculated flask at different temperatures, ranging from 20 to 40°C for 7 days under optimized pH in static condition.

Effect of moisture content

The effect of moisture content on xylanase production was tested by varying the moisture content in the range of 65 to 80%. All the liquids added to the flask were taken into consideration in calculating the moisture content. The effect of the moisture content on xylanase production was studied by incubating the flasks at 35°C for 7 days under optimized pH and temperature in static condition.

Effect of ultrasonication

Under the optimized fermentation conditions (pH 5, temperature 45°C and moisture content 80%), the optimized media was subjected to low ultrasonic waves of frequency 24 KHz for 30 min

Table 2. Plackett-Burman design and enzyme activity (U/g of dry substrate).

Trial number	A	B	C	D	E	F	G	H	I	J	K	Enzyme activity (U/g of dry substrate)
1	0.75	0.3	0.2	0.3	0.2	1.4	0.3	0.05	0.0014	0.02	0.016	7.53
2	0.50	0.5	0.3	0.1	0.3	1.0	0.1	0.03	0.0018	0.03	0.019	7.09
3	1.00	0.5	0.1	0.5	0.1	1.0	0.1	0.07	0.0018	0.03	0.013	7.76
4	1.00	0.1	0.3	0.1	0.1	1.0	0.5	0.07	0.0018	0.01	0.019	7.28
5	0.75	0.3	0.2	0.3	0.2	1.4	0.3	0.05	0.0014	0.02	0.016	7.53
6	1.00	0.1	0.1	0.1	0.3	1.8	0.5	0.03	0.0018	0.03	0.013	7.65
7	1.00	0.5	0.3	0.1	0.3	1.8	0.1	0.07	0.0010	0.01	0.013	8.27
8	1.00	0.5	0.1	0.5	0.3	1.0	0.5	0.03	0.0010	0.01	0.019	8.33
9	0.50	0.5	0.3	0.5	0.1	1.8	0.5	0.03	0.0018	0.01	0.013	7.18
10	1.00	0.1	0.3	0.5	0.1	1.8	0.1	0.03	0.0010	0.03	0.019	7.34
11	0.50	0.1	0.3	0.5	0.3	1.0	0.5	0.07	0.0010	0.03	0.013	6.57
12	0.50	0.5	0.1	0.1	0.1	1.8	0.5	0.07	0.0010	0.03	0.019	6.99
13	0.50	0.1	0.1	0.5	0.3	1.8	0.1	0.07	0.0018	0.01	0.019	6.63
14	0.75	0.3	0.2	0.3	0.2	1.4	0.3	0.05	0.0014	0.02	0.016	7.53
15	0.50	0.1	0.1	0.1	0.1	1.0	0.1	0.03	0.0010	0.01	0.013	6.42

with 30 s working time and 30 s resting time in turns. The fermentation media was stimulated once every 12 h after inoculation. The distance between Erlenmeyer flask and the ultrasonic probe was approximately 2 mm. The treatment device used was based on Chuanyun et al. (2004), which consist of two parts, the ultrasonic treatment and fermentation device. The distance between ultrasonic probe end and the flask was approximately 1 mm.

Enzyme extraction

After suitable periods of growth time in each experiment, xylanase was extracted from the fermented medium by adding 25 ml of sodium acetate buffer (pH 5) and gently shaking in room temperature for 60 min. The suspended materials and fungal biomass was separated by centrifugation (5000 rpm for 10 min) and the clarified supernatant was used as the crude enzyme.

Enzyme assay

Assay for xylanase was performed using 0.5 % soluble birchwood xylan (sigma) in 50 mM sodium phosphate buffer, pH 7. The reaction mixture was composed of 1.5 ml substrate and 0.5 ml crude enzyme. The mixture was incubated in water bath at 45°C for 15 min. The released reducing sugar was measured by the dinitrosalicylic acid (DNS) method (Miller 1959). The absorbance was measured at 540 nm with xylose as the standard. The amount of reducing sugar was calculated from the standard curve based on the equivalent xylose units. One unit of xylanase activity was described as the amount of enzyme producing 1 µmole of reducing sugar per ml medium per min under standard test conditions.

RESULTS AND DISCUSSION

Pretreatment of substrate

Pretreatment of sunflower sludge with 0.1 N NaOH showed a 2-fold increase in xylanase production over the control (Figure 1) since the cellulose and hemicellulose are cemented together by lignin. Lignin is responsible for integrity, structural rigidity and prevention of swelling of lignocelluloses. Thus, lignin content and distribution constitute the most recognized factor which is responsible for recalcitrance of lignocellulosic materials to enzymatic degradation by limiting the enzyme accessibility. Therefore, pretreatment techniques lead to a change in physical nature of lignin, increase in the available surface area, increase in pore sizes, partial depolymerization of hemicellulose, decrystallization of cellulose and deacetylation of hemicellulose, which enhance availability of the substrate (Shah and Madamwar, 2005). Sunflower sludge contains a good amount of lignin, which protects the xylan from attack by hydrolytic enzymes. To make components of lingocellulosics more accessible, alkali pretreatment was done. Thus, there is mostly increase in enzyme activity after subjecting lignocellulosics for pretreatment which is also supported by Goyal et al. (2008).

Screening of different nitrogen sources and urea

The nitrogen source used in the production medium is one of the major factors affecting enzyme production. Peptone (organic nitrogen source) gave better results (8.66 U/g of dry substrate) as shown in Figure 2 because of its amino-acid content and other nitrogenous compounds. These results were in agreement with those reported in the literature where a fungus was found to produce high xylanase activity on organic nitrogen sources than with inorganic sources (Lemos et al., 2001; Bakri et al., 2003). The best nitrogen source obtained

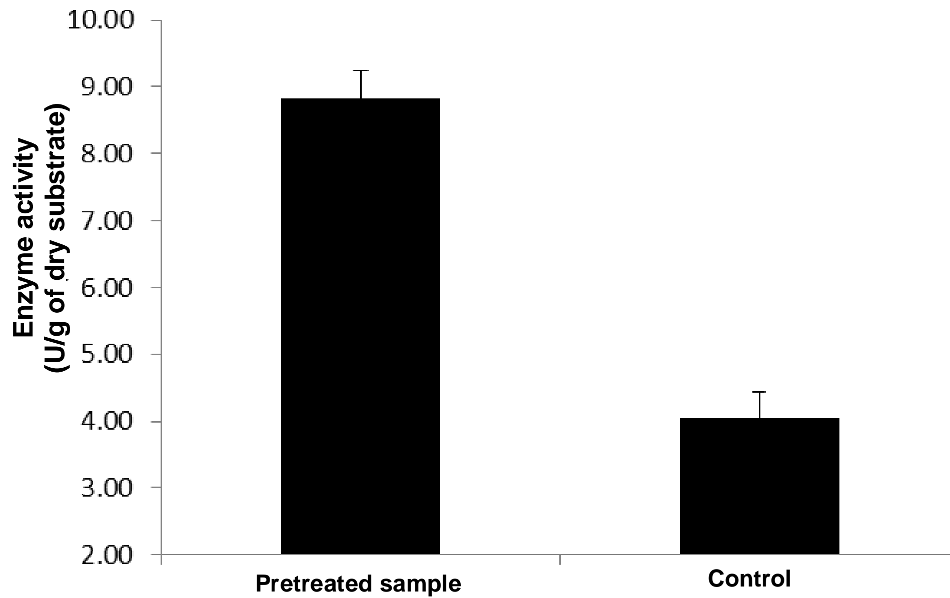

Figure 1. Effect of pretreatment on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

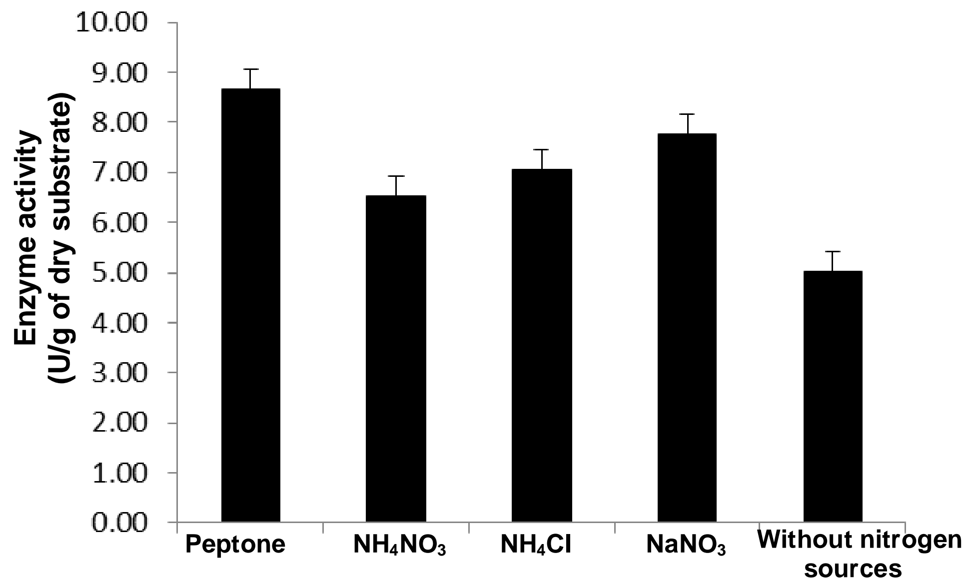

Figure 2. Effect of different nitrogen sources on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

from this was taken into account for medium optimization using PB design.

Figure 3 shows the importance of urea in the production media. When urea was used as a supplementary nitrogen source in the medium, it increased xylanase activity slightly (6.97 U/g of dry substrate without and 8.39 U/g of dry substrate with urea). It was therefore concluded that urea should be present in the production medium, if maximizing xylanase activity is the major objective. xylanase production.

Effect of surfactants

The effect of different surfactants (Tween-80, Triton X-100 and SDS) on the xylanase production by *T. harzianum* MTCC 4358 was investigated. Among these materials, only Tween-80 exerted a marked effect on the

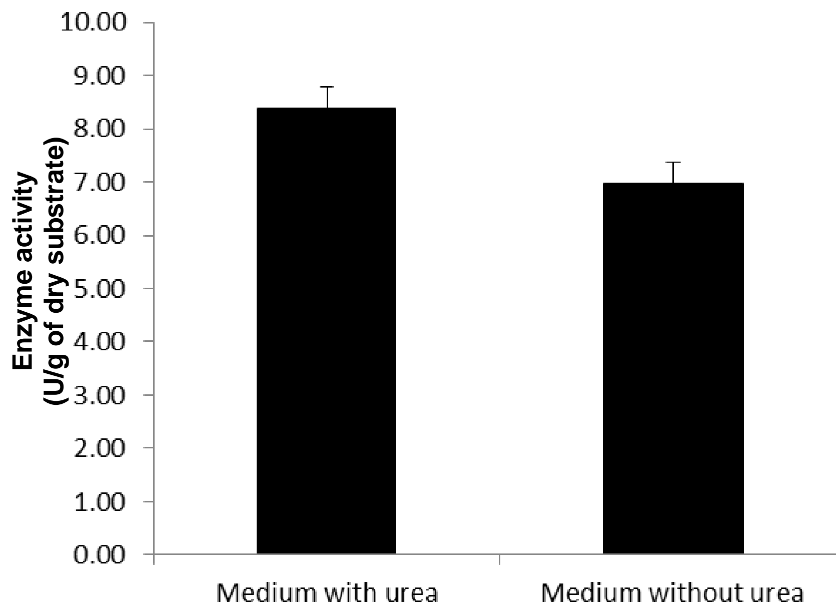

Figure 3. Effect of urea on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

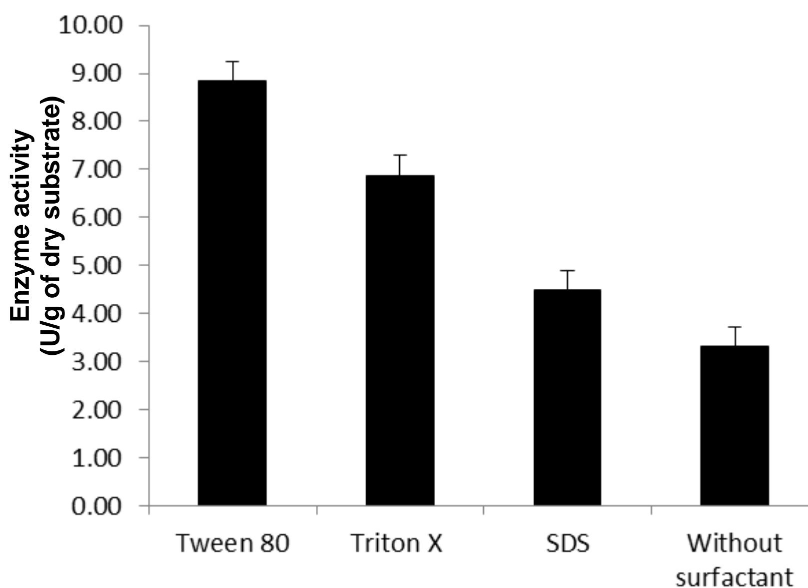

Figure 4. Effect of surfactants on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

Figure 4 indicates the effect of surfactants on the xylanase activity. The mechanism for the enhanced enzyme production by Tween-80 may be related to the increased permeability of the cell membrane, allowing a more rapid secretion of the enzyme, leading to greater enzyme synthesis (Eriksson et al., 2002). Another possible explanation is that Tween-80 has an influence on the level of glycosylation and thus on protein stability (Kruszewska et al., 1990). Due to the large amount of enzyme production, the xylanase activity was also increased. The results obtained were found to be similar to that of Jiang et al. (2005).

Plackett-Burman (PB) design

Medium optimization was done using MINITAB 15 software. The data in Table 2 were obtained by taking the

Table 3. Statistical analysis of Plackett-Burman design on enzyme activity (U/g of dry substrate).

Term	Main effect	Coefficient	t-value	p-value
Constant	-	72.68	21803.50	0.00
PEPTONE	9.12	4.56	1367.50	0.00
UREA	5.76	2.88	863.50	0.00
KH_2PO_4	-0.38	-0.19	-57.50	0.00
$CaCl_2.2H_2O$	0.53	0.27	80.00	0.00
TWEEN 80	2.13	1.07	320.00	0.00
$(NH_4)_2SO_4$	0.67	0.33	100.00	0.00
$MgSO_4.7H_2O$	1.20	0.60	180.00	0.00
$FeSO_4$	-1.20	-0.60	-180.00	0.00
$ZnSO_4.7H_2O$	-0.03	-0.02	-5.00	0.04
$COCl_2.6H_2O$	-0.68	-0.34	-102.50	0.00
$MnSO_4$	0.01	0.01	1.50	0.27

Table 4. Analysis of variance for enzyme activity (U/g of dry substrate) (coded units).

Source	Degrees of freedom (DF)	Sum of squares (SS)	Mean square (MS)	F-value	P-value
Main effects	11	375.08	34.10	255738.93	0.00
Curvature	1	15.71	15.71	117811.25	0.00
Residual error	2	0.00	0.00		
Total	14	390.79			

number of center points per replicate as three in this software. The data on xylanase activity using PB experiments showed a wide variation from 6.42 to 8.33 U/g of dry substrate. From the analysis of regression coefficients and t-value of the 11 factors, peptone (A), urea (B), KH_2PO_4 (C), $CaCl_2.2H_2O$ (D), tween 80 (E), $(NH_4)_2.SO_4$ (F), $MgSO_4.7H_2O$ (G) and $MnSO_4$ (K) showed a positive effect (Table 3) on xylanase production thus enhancing the enzyme activity, whereas other factors showed a negative effect on the enzyme production and were considered less significant when compared to those variables showing a positive effect. P values of 0.00 indicate a confidence level greater than 95% and those variables were considered as significant. Table 4 shows the analysis of variance (ANOVA) for linear model on effect of independent variables on xylanase production. From the software, the following model equation was proposed for xylanase activity:

$$\text{Enzyme activity} = 72.68 + 4.56A + 2.88B + 0.96C + 0.92D + 1.07E + 0.33F + 0.60G + 0.01K \quad (1)$$

Where, A, B, C, D, E, G and K are the concentration (g/l) of peptone, urea, KH_2PO_4, $CaCl_2.2H_2O$, Tween 80, $MgSO_4.7H_2O$, $MnSO_4$, respectively. The predicted response using the MINITAB 15 for xylanase activity was found to be 8.63 U/g of dry substrate and the following medium composition (with negative coefficients removed) was generated by the software for the maximum xylanase activity. The medium composition is as follows in (g/L): peptone, 1; urea, 0.5; KH_2PO_4, 0.1; $CaCl_2.2H_2O$, 0.5; Tween-80, 0.3; $(NH_4)_2.SO_4$, 1.8; $MgSO_4.7H_2O$, 0.5; and $MnSO_4$, 0.019. Xylanase activity of 8.63 U/g of dry substrate was obtained using the above-mentioned medium. This medium was used for the further studies.

Effect of incubation time

The present research demonstrated that culturing time is a determining factor in the process of xylanase production by *T. harzianum* 5348 because production reaches the maximum in a gradual manner and then falls abruptly. Activity in the first 24 h was 0.12 U/g of dry substrate, reaching a maximum activity at 168 h culturing (8.57 U/g of dry substrate), and falling to 6.26 U/g of dry substrate during the following 24 h (Figure 5). Production effectively started on the second day, attaining its maximum on the seventh day, and suffered a rapid decline on day 8. As previously reported, the optimum culturing time varies for the production of xylanase by this genus. The optimum production time obtained with *T. harzianum* was on day 8 (Sater et al., 2001), day 7 with the *T. harzianum* (Kheng et al., 2005) and with *Trichoderma viridae*, the maximum activity was found on day 6 (Simoes et al., 2009). The rapid decline after attaining the maximum might be due to the depletion of macro-and micronutrients in the fermentation medium with the lapse in time, which stressed the fungal physiology resulting in the inactivation of secretary machinery

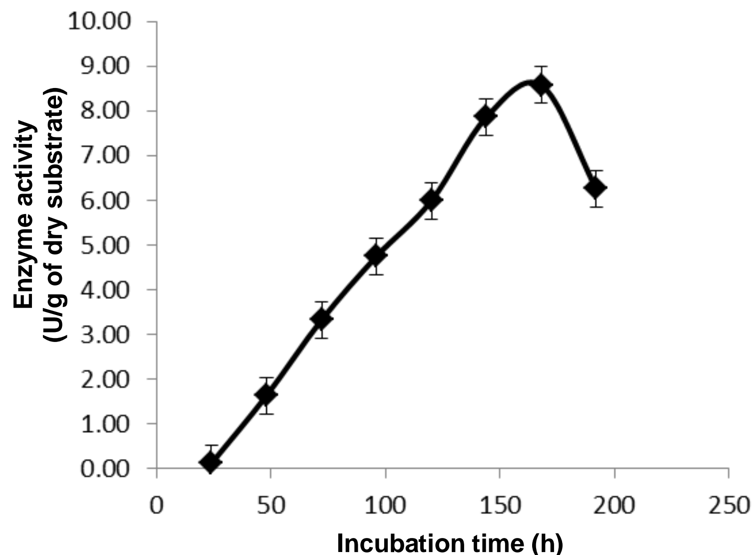

Figure 5. Effect of incubation time on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

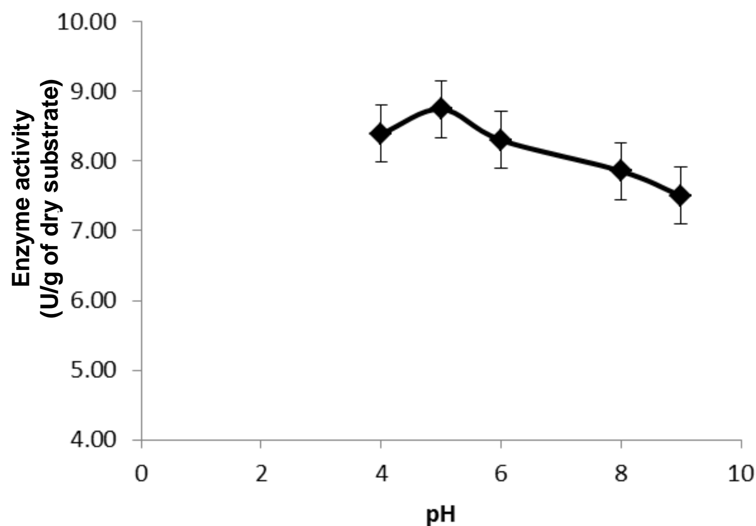

Figure 6. Effect of pH on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

of the enzymes.

Effect of pH

To optimize the xylanase production further, the production media was manipulated by growing the fungus on media with initial pH ranging from 4 to 9. The initial pH showed a profound influence on xylanase production. The fungus showed maximum activity for xylanase (8.75 U/g of dry substrate) when the initial pH was adjusted to acidic pH 5.0 using acid. During fermentation, pH of the medium was monitored and it was observed that the pH changes, which may be due to the production of enzyme from substrate. It was also found that the xylanase activity decreased with increase in pH (Figure 6). The results obtained are similar to those of Silveira et al. (1999).

Effect of temperature

From the Figure 7, it is inferred that maximum xylanase activity occurred at 35°C. Xylanase was produced with

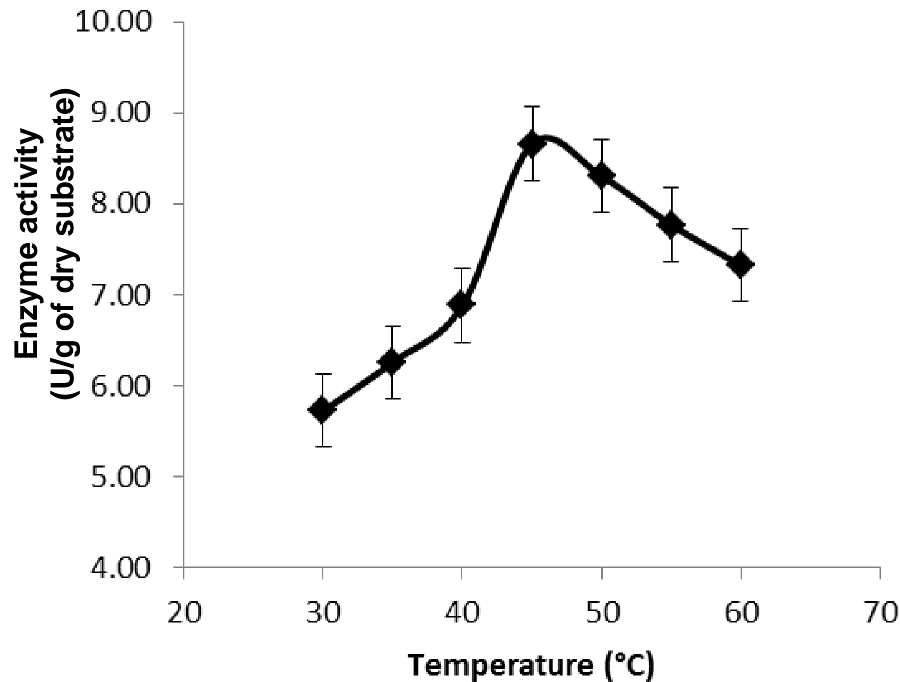

Figure 7. Effect of temperature on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

distilled water as the moistening agent in the temperature range of 25 to 40°C, showing maximum activity (8.66 U/g of dry substrate) after the 7th day of incubation at 45°C. A further increase in temperature above 50°C, not only inhibited fungal growth but also the production of xylanase, indicating that 45°C is the optimum temperature for the maximum xylanase activity. Moreover, the production of xylanase is closely related to the growth of fungus as the optimum temperature for xylanase production is as same as the optimum temperature for the growth of fungus. Similarly, the highest xylanase titers in fungal systems have been reported to occur generally at temperatures that are optimal for growth of cultures in solid-state fermentation (Sanghvi et al., 2010).

Effect of moisture content

Low moisture content is known to decrease the metabolic and enzymatic activity probably due to reduced solubility of nutrients from the solid substrate, low substrate swelling and higher water tension (Simoes et al., 2009). Therefore, to study the effect of moisture level, the substrate was moistened with different volumes of nutrient solution. It was taken into consideration that the concentration of medium ingredients was not changed. Maximal activity (8.84 U/g of dry substrate) was attained in the medium with 80% initial moisture content (sunflower sludge: nutrient solution ratio 1:3, v/v, Figure 8). Maximum enzyme activity also occurred at 75% moisture content (Ghanem et al., 2000). This could be attributed to faster growth of the organism at higher moisture content and the subsequent early initiation of the enzyme production. As previously reported, high moisture enhanced fungal growth and xylanases production when lignocellulosic substrates were the carbon sources in solid-state fermentation (Bakri et al., 2003; Narang et al., 2001). Many researchers have reported the similar effect of moisture content on xylanases production (Ferreira et al., 1999; Bakri et al., 2003; Yang et al., 2006).

Effect of ultrasonication

Low-intensity ultrasonic waves under proper conditions could promote the growth of the organism in lag phase and in exponential phase (Lanchun et al., 2003). Xylanase is a growth-associated product and hence ultrasonication increased the yield of xylanase. The experimental results showed that low frequency waves accelerated the enzyme production and a maximum activity of 9.27 U/g of dry substrate (Figure 9) was achieved, which was higher than the production carried out under the above optimized conditions without ultrasonic treatment. These low-intensity ultrasonic waves could promote the activity of enzyme, stimulate the growth of cell, speed up the transfer of substances and improve the penetrability of the cell membrane (Sinisterra, 1992). These results were also in accordance with Lanchun et al. (2003).

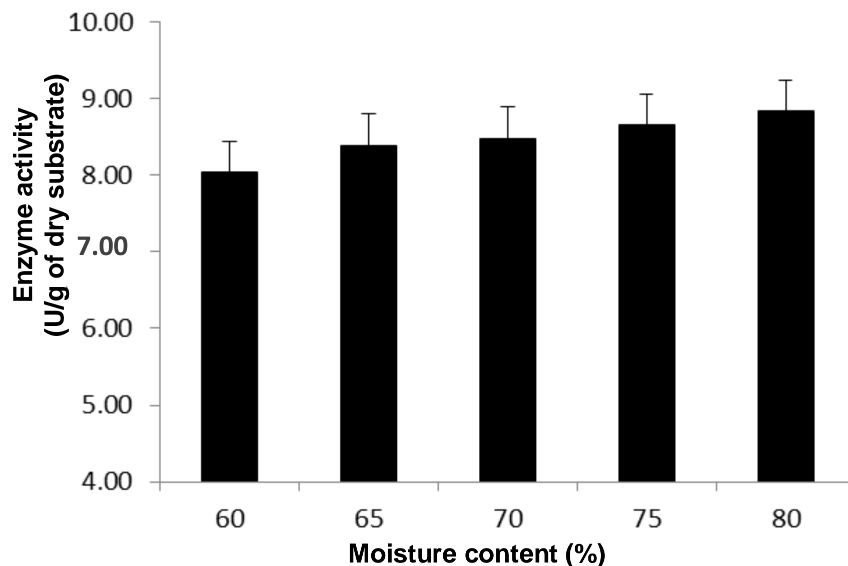

Figure 8. Effect of moisture content on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

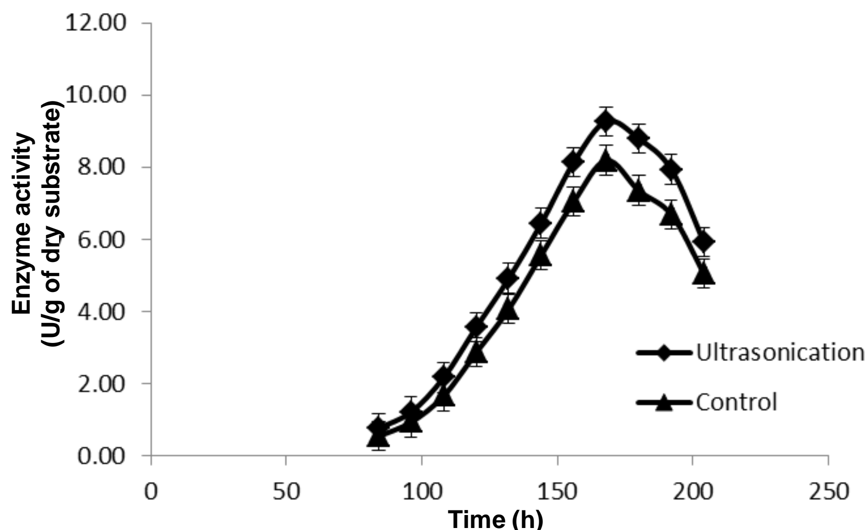

Figure 9. Effect of ultrasonication on xylanase production by *T. harzianum* MTCC 4358 in solid-state fermentation.

Conclusion

It can be concluded that sunflower sludge as a waste, is a promising potential substrate for the production of xylanase. Waste materials constitute renewable resource and can serve as an abundant and inexpensive carbon source. It was also observed that the combined effect of peptone and urea showed a good result for the maximum enzyme production. This observation is interesting due to the low cost of these nitrogen sources. The Plackett-Burman design was employed for optimizing culture media for maximum xylanase production. As a result, the use of the above-mentioned waste in the production of xylanase would decrease the cost of production in an environmentally sound manner. Moreover, the implementation of ultrasonication at a low frequency during fermentation increases the cell membrane permeability, thereby increasing the secretion of xylanase to a higher level.

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