Screening of Important Factors for Xylanase and Cellulase Production from the Fungus *C. cinerea* RM-1 NFCCI-3086 through Plackett-Burman Experimental Design

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A Plackett-Burman design was employed to evaluate the effect of different culture conditions associated with xylanase and cellulase production by the fungus *C. cinerea* RM-1 NFCCI 3086 using agro-residues as substrate. Eight variables were assessed for their significance on xylanase and cellulase production under solid state fermentation. The optimal culture conditions for xylanase production were developed by maintaining the variables of temperature, incubation period, substrate concentration, particle size, inoculum size, and inoculum age at their higher levels, while keeping pH and moisture ratio at their lower levels. For cellulase production, temperature, incubation period, substrate concentration, inoculum size, and inoculum age were fixed at their higher levels, and pH, particle size, and moisture level were kept at their lower levels. Of the eight variables, temperature, incubation period, and pH had significant influence on xylanase and cellulase production. These three variables can be further optimized for increased enzyme production.

Keywords: Plackett-Burman Design; Screening; Xylanase; Cellulase

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INTRODUCTION

Xylanase and cellulase are important enzymes because they have applications in different industries (Subramaniyan and Prema 2002), such as biofuel, pulp and paper, feed, food, textiles, and baking. Xylanase and cellulase are widely spread in nature and produced by a wide variety of bacteria, yeast, actinomycetes, fungi, marine algae, protozoa, snails, insects, and seeds of terrestrial plants (Dekker and Richards 1976; Breen and Singleton 1999). Fungal enzymes are commonly used in industry due to the feasibility of obtaining enzymes at high concentrations by solid state fermentation (Mitchell and Lonsane 1992). The white-rot fungi (Basidiomycetes) are the most efficient producers of xylanase, cellulase, and other extracellular lignocellulolytic enzymes. *C. cinerea* is a multicellular basidiomycete mushroom belongs to the family Psathyrellaceae (McKnight and McKnight 1987). It is a higher fungus (Agaricales) comprised of many cell types and, therefore, provides a window on the development of multicellularity within a single kingdom.

Many studies have been conducted involving the use of several cheap lignocellulosic wastes such as wheat straw, wheat bran, rice straw, corn cob, sugarcane bagasse *etc.* as the substrate for the production of xylanase and cellulase (Jiang *et al.* 2005; Singh *et al*. 2009). Several factors including incubation time, pH, carbon and nitrogen

source, temperature, and substrate concentration influence the production of fungal enzymes significantly during fermentation (Xiong *et al.* 2004). Therefore, screening of important nutritional factors is essential to determine the optimal parameters for efficient production. Plackett-Burman Design (PBD) (Helle *et al.* 1993) is a powerful tool to screen 'n' variables in just 'n+1' experiments, which may reduce the total number of experiments; it has been widely used to optimize fermentation processes (Kachlishvili *et al.* 2006). This technique cannot determine the interaction effect, but it is very useful for the initial step of an optimization procedure. Also, it evaluates the necessity of each factor in relatively few experiments. Therefore, the present study screened various operating physico-chemical parameters and growth conditions for maximum production of xylanase and cellulase enzyme by isolated fungal strains through solid state fermentation, using agro-residues as the substrates.

EXPERIMENTAL

Microorganism and Substrates

The fungus *C. cinerea* RM-1 NFCCI-3086 was isolated from decomposing wood samples and identified by the National Fungal Culture Collection of India, Agharkar Research Institute, Pune, India. The fungal culture was maintained on PDA slants, incubated at 37 °C for 4 days and then kept at 4 °C for storage. Wheat bran, corn cob, and sugarcane bagasse were purchased from the regional market.

Fermentation Medium

Solid substrate and nutrient salt solution (NSS)

As optimized previously (Poonam 2015), the combination of wheat bran and corn cob (7:3) was used for xylanase; for cellulase production, sugarcane bagasse was used as the solid substrate. Solid substrates were powdered using a laboratory grinder and sieved by using screens of different mesh size, and fractions so obtained were stored in air tight polythene bags at room temperature. NSS was prepared as described (Vishniac and Santer 1957), optimized by a one-variable-at-a-time approach (data not shown), and modified as follows. For xylanase production, it contained: KH_2PO_4, 1.5 g/L; $MgSO_4.7H_2O$, 0.5 g/L; $(NH_4)_2SO_4$, 3.0 g/L; KCl, 0.5 g/L; Tween-80, 0.1 g/L; and yeast extract, 1.0 g/L, in distilled water with trace elements solution (0.04 mL/L) comprising $ZnSO_4.7H_2O$ (180 µg/L), $FeSO_4.7H_2O$ (200 µg/L), and $MnSO_4.7H_2O$ (20 µg/L). For cellulase production, it contained KH_2PO_4, 1.5 g/L; $MgSO_4.7H_2O$, 0.5 g/L; $(NH_4)_2SO_4$, 3.0 g/L; KCl, 0.5 g/L; Tween 80, 0.2 g/L; peptone, 1 g/L, and lactose, 1 g/L, in distilled water with the same trace elements solution.

Solid State Fermentation and Enzyme Extraction

Solid state fermentation was performed in 250 mL Erlenmeyer flasks containing 5 g of substrate and 15 mL of NSS (no free water available) to maintain the moisture ratio of 66.67% (Khandeparkar and Bhosle 2007). The culture medium was autoclaved at high pressure (15 psi) for 15 min, inoculated with 2 discs (5 mm each) of actively growing fungal isolate RM-1 (4 days old culture), and incubated at the desired temperature. The enzyme was harvested by crushing and filtering the flask contents through four-layered cheese cloth. The filtrate was centrifuged at 5000 × *g* at 4 °C temperature for 10 min. The

clear supernatants were used as crude enzyme samples and assayed for xylanase and cellulase activity. Results are expressed as the mean of at least three different cultures.

Analytical Methods

Estimation of xylanase activity

Xylanase activity was estimated by measuring the amount of reducing sugar released after the incubation of diluted culture supernatant (crude enzyme) with 1% birch wood xylan solution (in 0.05 M sodium phosphate buffer) in a ratio of 1:9 at 55 °C (Bailey and Biely 1992). After 15 min, 3 mL of DNS reagent was added to stop the reaction (Miller 1959). The reaction mixture was then boiled for 5 min and cooled under tap water. Blanks were repeated in a similar manner using reagents and enzyme, separately. Xylanase activity was reported in terms of IU. One IU per mL of xylanase was defined as 1 µmol of reducing sugar (xylose) produced in 1 min by 1 mL of enzyme under the assay conditions. The xylose units released in this reaction were estimated at 540 nm using UV-Vis spectrophotometer (Cary 100, Bio Varian, Australia) at 25 °C.

Estimation of cellulase (CMCase) activity

The cellulase activity (CMCase) was determined using CMC as the substrate (Mandels 1975). The reaction mixture, in a total volume of 4 mL, contained 2 mL of 2% (w/v) CMC prepared in 0.05 M citrate buffer and 2 mL of the enzyme preparation in an appropriate dilution. The mixture was incubated at 55 °C for 30 min. Thereafter, the tubes were kept in an ice bath, and 3 mL of DNS reagent (Miller 1959) was added to 1 mL of reaction assay, followed by mixing of the mixture in a boiling water bath for 5 min. The tubes were immediately cooled under tap water. The controls containing reagent and enzyme separately were also treated similarly. The cellulase activity was expressed as IU equivalent to µ moles of glucose units released in 1 min by 1 mL of enzyme at 55 °C. The reducing sugars released were measured at 575 nm with use of a UV-Vis spectrophotometer.

Screening of Factors through PBD

The Plackett-Burman experimental design was employed to investigate the significance of various culture conditions on xylanase and cellulase production. This was a fractional factorial design with certain combinations of the eight factors, *i.e.*, initial pH, incubation time, substrate concentration, temperature, particle size, moisture ratio, inoculum size, and inoculum age (Jiang *et al.* 2005; Singh *et al*. 2009). The eight variables were selected from the literature as possible factors affecting xylanase and cellulase production, and they were tested at two distinct levels, *i.e*., high and low, denoted by "+" and "–" respectively.

The boundary limits of each variable were chosen as per the literature (Jiang *et al.* 2005; Singh *et al*. 2009). This design did not describe the interaction among the factors. It was used to screen and evaluate the important factors that influenced the response (xylanase or cellulase activity) and to rank the factors according to their importance. Minitab-16 software (Minitab, Inc., State College, PA, USA) was used to generate the design table and analyze data. Variables with the highest t-value and confidence level over 95% were considered highly significant.

RESULTS AND DISCUSSION

Analysis of PBD

Table 1 illustrates the maximum and minimum levels of eight variables chosen for trials in PBD. The design of 12 trials with two levels for each variable with the resultant enzyme activities are shown in Table 2. Plackett-Burman experiments showed a wide variation in xylanase (from 302 IU/mL to 705 IU/mL) as well as in cellulase activity (from 0.96 IU/mL to 2.25 IU/mL). This variation indicated towards the importance of optimization for attaining higher productivity.

Table 1. Variables and their Levels Employed in PBD for Screening of Culture Conditions Affecting Xylanase and Cellulase Production

SI. No.	Variable		Level	
	Variable code	Variable name	-1	+1
1	A	Initial pH	6	8
2	B	Incubation time (days)	4	7
3	C	Substrate concentration (g/flask)	3	5
4	D	Incubation temperature (°C)	30	40
5	E	Particle size (μm)	300	600
6	F	Moisture ratio (%)	66.67	80
7	G	Inoculum size (No. of disc, mm)	1	3
8	H	Inoculum age (days)	3	5

Table 2. PBD for Xylanase and Cellulase Production

Run No.	A	B	C	D	E	F	G	H	Xylanase activity (IU/mL)	Cellulase activity (IU/mL)
1	-1	-1	1	1	1	-1	1	1	705.61	2.04
2	-1	1	1	1	-1	1	1	-1	670.36	2.25
3	-1	1	-1	-1	-1	1	1	1	579.34	1.65
4	1	-1	1	-1	-1	-1	1	1	401.21	1.34
5	1	-1	1	1	-1	1	-1	-1	385.56	1.17
6	1	1	-1	1	-1	-1	-1	1	582.19	1.90
7	1	1	1	-1	1	1	-1	1	595.35	1.45
8	1	1	-1	1	1	-1	1	-1	634.28	1.96
9	-1	-1	-1	-1	-1	-1	-1	-1	396.35	1.40
10	-1	1	1	-1	1	-1	-1	-1	652.21	1.75
11	1	-1	-1	-1	1	1	1	-1	302.12	0.96
12	-1	-1	-1	1	1	1	-1	1	479.61	1.52
Solid substrate: For xylanase = WB+CC, 7:3 For cellulase = SB			Assay conditions: Xylanase: 55 °C, pH = 6.4, incubation time = 15 min Cellulase: 55 °C, pH = 4.8, incubation time = 30 min							

The significance of each variable was indicated by a larger Student's t-test value and smaller *p*-value (Table 3; Khuri and Cornell 1987). The pH (A), incubation period (B), and temperature (D) showed significant influence on xylanase and cellulase production ($p < 0.05$, significant at 95% confidence level). The main effects of all variables were also calculated. When the main effect value of a tested variable is positive, its influence on response variables is greater at high level; when it is negative, its influence is greater at low level. For xylanase production, the initial pH (A) and moisture ratio (F) displayed negative effects, while all other variables had positive effects. For cellulase production,

initial pH (A), particle size (E), and moisture ratio (F) displayed negative effects, while all other variables had positive effects. pH was significant but with a negative coefficient, as most fungi grow well on slightly acidic media (Reid 1989). pH regulates the production of enzymes by inactivating of the enzyme system if fermentation is carried out at above or below the optimum range of pH.

Table 3. Analysis of PBD for Xylanase and Cellulase Production

Sl. No.	Variable		Xylanase			Cellulase		
	Variable code	Variable name	Effect	t-ratio	p-value	Effect	t-ratio	p-value
1	A	Initial pH	-97.13	-4.16	0.025	-0.3050	-4.17	0.025
2	B	Incubation time (days)	173.8	7.45	0.005	0.4217	5.77	0.010
3	C	Substrate concentration (g/flask)	72.73	3.12	0.063	0.1017	1.39	0.258
4	D	Incubation temperature (°C)	88.51	3.79	0.032	0.3817	5.22	0.014
5	E	Particle size (μm)	59.03	2.53	0.085	-0.0050	-0.07	0.950
6	F	Moisture ratio (%)	-59.92	-2.57	0.083	-0.2317	–3.17	0.054
7	G	Inoculum size (No. of disc, mm)	33.61	1.44	0.245	0.1683	2.30	0.105
8	H	Inoculum age (days)	50.40	2.16	0.120	0.0683	0.93	0.419
1	A	Initial pH	-97.13	-4.16	0.025	-0.3050	-4.17	0.025

The effect of moisture level on enzyme production may be because it alters the physical properties of a solid substrate. The free excess liquid reduces porosity and oxygen transfer and increases the swelling of the solid substrate, which results in a gummy texture, while low moisture level reduces the swelling of the solid substrate and decreases the solubility of nutrients (Raimbault and Alazard 1980). Singh *et al*. (2009) obtained maximum xylanase production by different white rot fungi at substrate to moisture ratio of 1:3 under SSF using wheat bran as the substrate. Latifian *et al*. (2007) obtained maximum cellulase activity by *T. reesei* QM9414 at 70% moisture content under SSF using rice bran as the substrate.

The particle size of the solid substrates affects the surface/volume ratio and accessibility of the nutrients for the microorganism (Assamoi *et al.* 2008). Hence, substrate particles of an appropriate size are required for optimum microorganism growth and production of enzymes. Sugarcane bagasse particles of 300 μm may possibly provide sufficient surface area and good aeration for growth of *C. cinerea* RM-1 under SSF, resulting in increased cellulase production. Lower xylanase production obtained on smaller particles of wheat bran and corn cob may be due to increased thickness of fungal mycelia around the solid substrate particles, which reduces the porosity of the substrate and diffusivity of oxygen in the bed (Rajagopalan and Modak 1995). Jiang *et al.* (2005) obtained maximum xylanase activity for strain *Thermomyces lanuginosus* CAU44 on corn cob particles of 0.300 μm to 450 μm.

Pareto graphs were used to rank the variables according to their importance and to show the effect of all variables on xylanase and cellulase production (Fig. 1). The Pareto graph represents the magnitude of each variable, and it is an easy way to view the results of a PB design (Strobel and Sullivan 1999). The Pareto graphs confirmed the significance of the variables of initial pH (A), incubation period (B), and temperature (D) for xylanase and cellulase production.

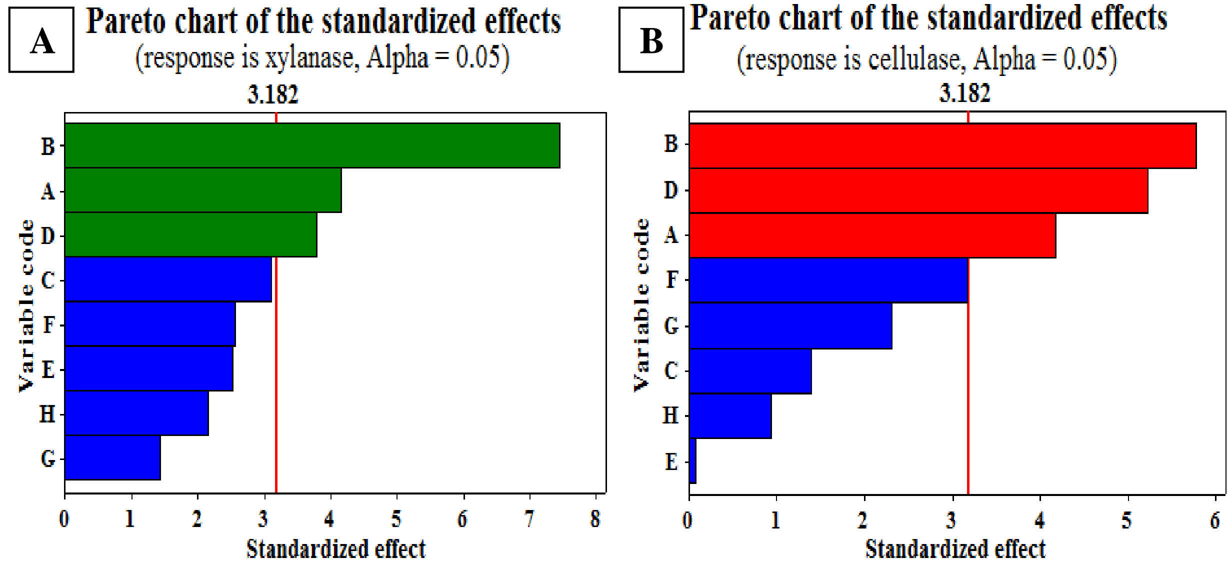

Fig. 1. Effects Pareto plots for xylanase (A) and cellulase (B) production
* Green and red color represent the significant factors for xylanase and cellulase, respectively while blue color represents the factors having insignificant effect.

The normal effect plot is a useful resource for visualizing the significance of variables. A significant effect strays farther from the line, but an insignificant effect drops alongside a line (Fig. 2). The effects of incubation period (B) and temperature (D) were placed farther from the line on the right side, which illustrated that their higher levels had a significant effect. The effect of initial pH (A) was on the left side of the line, indicating that its lower level contributed to a higher value of response variables.

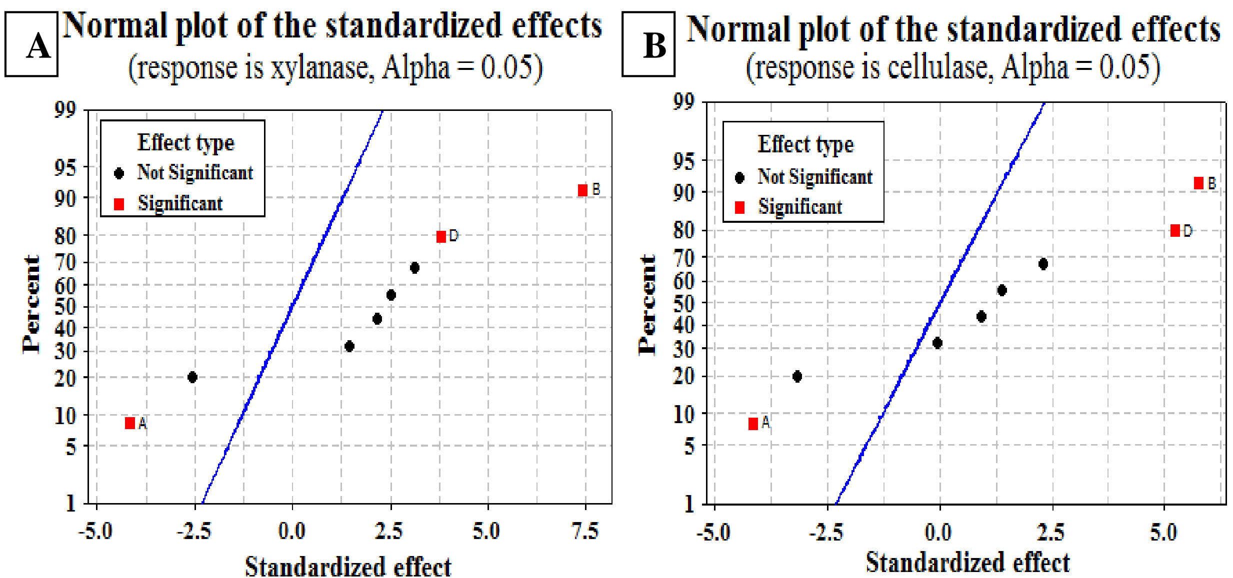

Fig. 2. Normal effect plots for xylanase (A) and cellulase (B) production

CONCLUSIONS

1. Out of the eight variables studied, temperature, incubation period, and pH had significant influence on xylanase and cellulase production.
2. For xylanase production, the optimum values of substrate concentration, particle size, inoculum size, inoculum age, and moisture ratio were 5 g/flask, 600 µm, 3 disks/flask, 5 days, and 66.67%, respectively.
3. For cellulase production, the optimum values of substrate concentration, particle size, inoculum size, inoculum age and moisture ratio were 5 g/flask, 300 µm, 3 disks/flask, 5 days, and 66.67%, respectively.
4. Xylanase and cellulase have great potential in different industries such as biofuel, pulp and paper, feed, food, textile and baking. *C. cinerea* RM-1 produced quite high activity of these enzymes and therefore may be of great interest for these industries.

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