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Improved Production of Endoglucanase Enzyme by *Aspergillus terreus*; Application of Plackett-Burman Design for Optimization of Process Parameters

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Abstract: In this study, bagasse was used as substrate for endoglucanase (carboxymethyl-cellulase; CMCase) production using locally isolated *Aspergillus terreus* and the culture parameters were optimized for enhancing cellulase yield. The fungus showed 0.9 U mL^{-1} endoglucanase (CMCase) activity, during growth on basal salts medium at 35°C, initial pH value of 5.5 and in presence of 5% bagasse as a sole c-source. Preliminary experiments to address the most suitable nitrogen source as well as the optimal substrate (bagasse) treatment revealed that the optimal enzyme activity were 2.1 and 2.43 U mL^{-1} in presence of 3 g yeast extract and 1 N HCl or 1 NaOH, respectively. Statistically based experimental design was applied to optimize the production of endogluconase by *A. terreus*. To evaluate the effect of different culture conditions on the production of CMCase enzyme, Plackett-Burman factorial design was carried out. Twelve variables were examined for their significance on enzyme production. Treated bagasse (T_2), non treated bagasse (NT), K_2HPO_4, $NaNO_3$, trace elements, KCl, temperature and pH were the most significant factors encourage CMCase enzyme production, whereas treated bagasse (T_3), yeast extract and $MgSO_4$, were the most significant factors decreasing CMCase enzyme production. The pre-optimized medium showed approximately 4 folds increase in cellulase enzyme production.

Key words: Carboxymethyl-cellulase (CMCase), experimental design, optimization of enzyme production, *Aspergillus terreus*, bagasse

INTRODUCTION

In recent years, one of the most important biotechnological applications is the conversion of agricultural wastes and all lignocellulosics into products of commercial interest such as ethanol, glucose and single cell protein (Ojumu *et al*., 2003). The key element in bioconversion process is the hydrolytic enzymes mainly cellulases. Cellulase enzyme has been reported by Fan *et al*. (1987), Wu and Lee (1997), Kansoh *et al*. (1999), Ojumu *et al*. (2003) and Immanuel *et al*. (2007) for the bioconversion of lignocellulosics to these useful products. Lignocellulosics are abundant sources of carbohydrate, continually replenished by photosynthetic reduction of carbon dioxide by sunlight energy (Fan *et al*., 1987). Thus, they are the most promising feedstock for the production of energy, food and chemical (Wu and Lee, 1997; Ojumu *et al*., 2003). The bioconversion of cellulosic materials is now a subject of intensive research as a contribution to the development of a large-scale conversion process beneficial to mankind (Kumakura, 1997). Such process would help alleviate shortages of food and animal feeds, solve modern waste disposal problem and diminish man's dependence on fossil fuels by providing a convenient and renewable source of energy in the form of glucose. However, some features of natural cellulosic materials, like the degree of crystallinity, lignification and capillary structure, are known to inhibit their degradation or bioconversion (Solomon *et al*., 1990, 1999). Many physical, chemical and microbial pretreatment methods for enhancing bioconversion of cellulosic materials have been reported by Kumakura (1997), Wu and Lee (1997), Kansoh *et al*. (1999), Depaula *et al*. (1999), Solomon *et al*. (1999) and Ojumu *et al*. (2003). Cellulase production by members of the genus *Aspergillus* using different agricultural wastes has been reported by Gokhale *et al*. (1991), Prasetsan *et al*. (1997), Jecu (2000), Ojumu *et al*. (2003), Milala *et al*. (2005) and Immanuel *et al*. (2007). Furthermore, wild type and mutants of the fungus *Trichoderma reesei* have been reported for celluololytic enzyme activity especially under various fermentation conditions (Gadgil *et al*., 1995;

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Mekala *et al*., 2008; Latifian *et al*., 2007). Since, the production of cellulase enzyme(s) is a major factor in the hydrolysis of cellulosic materials, it is important to make the process economically feasible. Although, much study has been done on the production of cellulase from lignocellulosics (Solomon *et al*., 1999; Depaula *et al*., 1999; Kansoh *et al*., 1999; Milala *et al*., 2005; Immanuel *et al*., 2007; Alam *et al*., 2008), much emphasis has been placed on bagasse. Reducing the costs of enzyme production by the optimization of the fermentation medium and cultivation condition is the goal of basic research for industrial application. Most of the reports concerning cellulases production are dealt with the purification and characterization of the enzyme, very few reports regarding optimization studies especially using experimental design. These statistical methods, as compared to the common one-factor-at-a-time method, proved to be powerful and useful tools. Interestingly, many reports have been published on the application of statistical experimental methodology for the optimization of xylanase production by members of the genera *Aspergillus* and *Trichoderma* (Prasetsan *et al*., 1997; Li *et al*., 2007; Cao *et al*., 2008). However, few studies on the application of statistical designs for medium optimization in cellulase production by *Trichoderma reesei* have been reported by Latifian *et al*. (2007), Alam *et al*. (2008) and Mekala *et al*. (2008). Therefore, there is growing interest for the application of such methodology in the optimization of cellulase production by members of the genus *Aspergillus*.

The objective of the present research was to study the production of endoglucanase enzyme by local fungal isolate identified as *Aspergillus terreus*. Emphasis was given to the preliminary investigation on the optimal nitrogen source as well as substrate (bagasse) concentration affecting enzyme production. Furthermore, the effect of different pretreatments of bagasse substrate on enzyme production was also conducted. Plackett-Burman experimental design was applied to evaluate the impact of various culture conditions, including nutritional and physical variables, on CMCase enzyme production.

MATERIALS AND METHODS

Lignocellulosic source and pretreatments: The substrates used for this study was bagasse; it is cheap and readily available source of lignocellulose. The bagasse was collected as a waste product of fruit juice substrate and dried at 60°C for 48 h to reduce the moisture content and to make it more susceptible for crushing (Kanosh *et al*., 1999). During cultivation, the milled bagasse material was either used directly without treatment (NT) or subjected to several pretreatment methods using different concentration of HCl (T_1: 0.5, 1 or 2 M), sodium hydroxide solution (T_2: 0.5, 1 or 2 N) or soaked in sodium hydroxide solution (T_3: 2 or 3 N) at 1:10 (substrate : solution) ratio (Gharpuray *et al*., 1983; Solomon *et al*., 1999) for 2 h at room temperature. It was washed free of the chemicals and autoclaved at 121°C (15 psig steam) for 1 h. At the end of each treatment, the pretreated substrate was then filtered and washed successively with distilled water until the wash water was neutral.

Fungus isolation and characterization: The fungus used throughout this study was isolated from garden soil by the use of Czapek-Dox medium containing carboxymethyl-cellulose as a sole carbon source. After several transfers to fresh medium fungal growth sample was subsequently transferred to solid medium. Single pure colonies were screened for CMCase activity. The purified fungus was tested for cellulase production by submerged cultivation on basal salts medium containing 0.3% $NaNO_3$; 0.05% $MgSO_4.7H_2O$; 0.1 K_2HPO_4; 0.05% KCl and supplemented with 0.1 mL $FeSO_5.7H_2O$ as trace element. The medium was adjusted to pH 5.5 and inoculated aseptically by adding spore suspension (approximately 2×10^7) to 50 mL of sterilized medium. The purified isolate was maintained as stock culture in Czapek-Dox agar slants. Stock culture was subcultured at regular intervals of one month and stored under refrigeration. The fungus was characterized and identified by the help of Mycological Center in Assuit University, Egypt. The fungus was closely related to members of the genus *Aspergillus* especially *A. terreus*.

Inoculum: The organism was maintained as direct stock culture from which inocula were prepared. It was grown on Czapek-Dox agar slants at 30°C for 5 days and stored at 4°C with regular subculturing. Fungal inoculum was prepared by inoculation of 50 mL of the basal salts production medium with spore suspension of *A. terreus*. The inoculum was kept in shaker (200 rpm) at 35°C for 24 h before use in fermentation process.

Fermentation experiment: The cultures were incubated aerobically in 250 mL Erlenmeyer flasks under submerged conditions at 35°C for 5 days. At the end of incubation period, culture were centrifuged at 4000 x g for 15 min and extracellular protein and CMCase activities were measured in the culture supernatant. All experiments and analysis were carried out in duplicate.

Endoglucanase (carboxymethyl cellulase; CMCase) activity: Endoglucanase activity was measured as

previously described by Ghose (1987) by determination of reducing sugar release from carboxymethyl cellulose (CMC). 0.1 mL of the culture supernatant was incubated with 1 mL 2% CMC in 0.05 M sodium acetate buffer, pH 4.8 at 50°C for 10 min. The reducing sugar produced was assayed by dinitrosalicyclic acid (DNS) method (Miller, 1959) using glucose as standard. Controls for carbohydrate produced from substrate and enzyme preparation were included. One unit (1U) of endogluconase activity was defined as the amount of enzyme which produced 1U mole of glucose equivalents per minute under assay conditions.

Protein determination: The protein content of cell free supernatant was determined by Lowry *et al.* (1951) method with bovine serum albumin as standard.

Growth and production conditions: The fungus was grown in 50 mL aliquot of basal salts medium dispensed in 250 mL Erlenmeyer flask and incubated at 35°C for 24 h at 200 rpm. One percent inoculum of the overnight culture was used to inoculate the basal salt production medium of the following composition (g L^{-1}): 3 $NaNO_3$, 3; $MgSO_4.7H_2O$, 0.5; K_2HPO_4, 1; KCl, 0.5 and supplemented with 1 mL $FeSO_5.7H_2O$ as trace element. The medium was adjusted to pH 5.5 and inoculated aseptically by adding spore suspension (approximately 2×10^7) to 50 mL of sterilized medium. During fermentation, bagasse substrate was added at a concentration of 5% (w/v) and was used as a sole carbon source. To assess the optimal nitrogen source, six different nitrogen sources were tested (on equal nitrogen bases) namely; $NaNO_3$, $(NH_4)_2SO_4$, peptone, yeast extract, NH_4Cl and urea. The 50 mL medium was inoculated with 500 μL of the preculture. Cellulase enzyme activity and protein content were determined in culture supernatants after clarifying cultures by centrifugation.

Fractional factorial design: For screening purpose, various medium components and culture parameters have been evaluated based on the Plackett-Burman factorial design. Plackett-Burman experimental design (Plackett and Burman, 1946) was applied to investigate the significance of various medium components on cellulase production. Twelve culture variables were tested in two levels: -1 for low and +1 for high level based on Plackett-Burman matrix design, which is a fraction of two level factorial design and allows the investigation of n-1 variables in at least n-experiments. Table 3 represents the lower and higher levels of each variable. In this study the independent variables were screened in 22 combinations according to the matrix shown in Table 4. The main effect of each variable was calculated simply as the difference between the average of measurements made at high setting (+1) and the average of measurements observed at low setting (-1) of that factor.

Plackett-Burman experimental design is based on the first order model Eq. 1:

$$Y=\beta_0+\Sigma\ \beta_i x_i \quad (1)$$

where, Y is the predicted response (specific activity of cellulase enzyme), β_0, β_i are constant coefficients and x_i is the coded independent variables estimates or factors.

Analysis of data: The data on the specific activity of cellulase enzyme were statistically analyzed. Essential experimental design free software (Steppan *et al.*, 1999) was used for data analysis, determination of coefficients, as well as polynomial model reduction. Factors having highest t-value and confidence level over 95% were considered to be highly significant on cellulase enzyme production.

RESULTS AND DISCUSSION

Monitoring of cellulase enzyme production during growth of *A. terreus* on bagasse: In this experiment, the time course of cellulase enzyme production, in presence of 2 levels of bagasse (2 and 5%), was closely investigated. The enzyme activity and extracellular protein content versus the time course of fermentation are shown in Fig. 1. It was clear that the pattern of enzyme activity was nearly similar. Two clear peaks of activities were recognized; the first after 30 and 75 h and the second after 160 and 220 h, for the 2 and 5% bagasse, respectively. The depression in cellulase activity between those two main activity peaks may be due to cumulative effect of

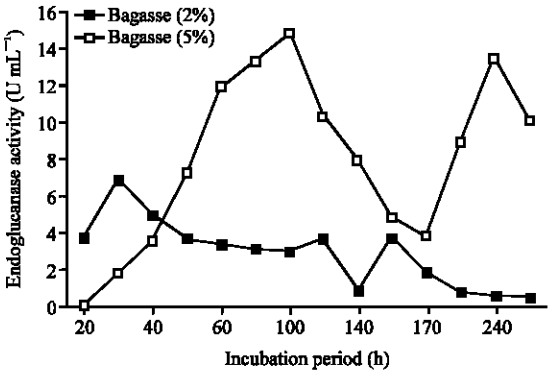

Fig. 1: Production of endoglucanase (CMCase) enzyme by *Aspergillus terreus* in presence of different bagasse concentrations

Table 1: Effect of different bagasse and yeast extract concentrations on endoglucanase enzyme activity of *Aspergillus terreus*

Concentration	Endoglucanase activity (U mL^{-1})	Protein content (mg mL^{-1})	Specific activity (U mg^{-1} protein)
Bagasse (%)			
1	0.32	0.90	0.35
2	0.47	1.83	0.25
3	0.65	2.50	0.26
4	0.88	3.16	0.28
5	0.96	3.30	0.29
6	0.73	1.88	0.39
Yeast extract (g L^{-1})			
2.0	1.40	2.17	0.65
2.5	1.90	1.82	1.04
3.0	2.47	2.04	1.21
3.5	1.99	2.70	0.74
4.0	1.67	2.68	0.63
4.5	1.51	3.10	0.48
5.0	1.35	3.76	0.35
5.5	1.18	3.20	0.37
6.0	0.74	2.52	0.29

cellobiose, a dimer of glucose which is known to inhibit endogluconase activity (Ojumu *et al*., 2003). Hatakka (1983) also suggested that delignification produces aromatic water-soluble products which can repress the celluolytic action of the enzyme. Generally, there was an obvious increase in cellulase enzyme activity in presence of 5% bagasse, recording approximately 2 fold increase as compared to 2% bagasse concentration.

Influence of different bagasse levels: In a trail to reduce the costs for enzyme production, bagasse raw material was used as substrate. In this experiment, the influence of different bagasse concentrations on cellulase enzyme production by *A. terreus* was investigated. Results shown in Table 1 represent the maximal values of protein and the specific activity expressed as CMCase activity per mg of protein at different bagasse levels. As it can be observed, the values of endoglucanase activity and extracellular protein (0.9 U mL^{-1}, 3.30 mg mL^{-1}) were higher for a 5% bagasse concentration. However, any decrease in bagasse concentration led to simultaneous decline in CMCase activity.

Influence of different bagasse pretreatment: For enhancing conversion of cellulosic material by making it more accessible and susceptible for cellulolytic enzyme activity, bagasse substrate was subjected to different pretreatment processes. Results shown in Table 2 indicated that treatment methods have a similar effect on endogluconase activity. However, maximum specific activity was recorded during growth on bagasse substrate pretreated with (0.5 or 1 N HCl) and 1 N NaOH, that was 2.26, 2.38 and 2.64, for each of the tested substrate, respectively. As compared with nontreated substrate, 2.2-fold increase in specific cellulase activity was recorded

Table 2: Effect of different bagasse pretreatments on endoglucanase enzyme activity of *Aspergillus terreus*

Bagasse treatment	Endoglucanase activity (U mL^{-1})	Protein content (mg mL^{-1})	specific activity (U mg^{-1} protein)
Non-treated	2.47	2.04	1.21
0.5 N HCl	2.44	1.08	2.26
1 N HCl	2.09	0.88	2.38
2 N HCl	1.50	1.68	0.89
0.5 N NaOH	2.38	1.21	1.97
1 N NaOH	2.43	0.92	2.64
2 N NaOH	2.36	1.83	1.29
Auto* (2 N NaOH)	2.47	1.89	1.31
Auto* (3 N NaOH)	1.93	2.55	0.76

*Auto: Autoclaved

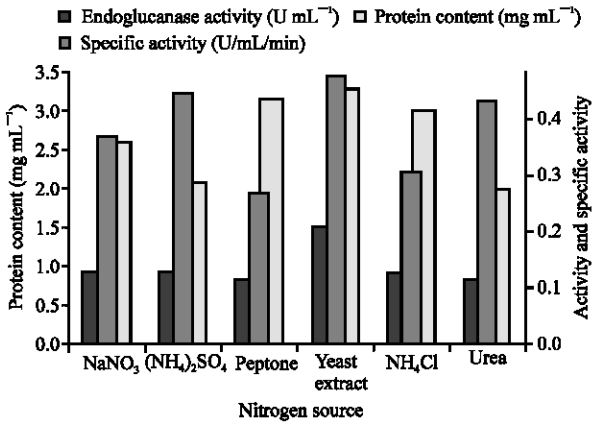

Fig. 2: Production of endoglucanase enzyme by *Aspergillus terreus* in presence of different nitrogen sources

when bagasse was treated with 1N NaOH. Interestingly, many physical, chemical and microbial pretreatment methods for enhancing bioconversion of cellulosic materials have been reported by Kumakura (1997), Wu and Lee (1997), Kansoh *et al*. (1999), Depaula *et al*. (1999), Solomon *et al*. (1999) and Ojumu *et al*. (2003) in order to make cellulosic material more accessible and susceptible for cellulolytic activities.

Influence of different nitrogen sources on endoglucanase production: In an attempt to maintain low fermentation costs during endoglucanase production, relatively inexpensive organic nitrogen sources (peptone and yeast extract) and inorganic nitrogen sources (sodium nitrate, ammonium sulfate, ammonium chloride andurea) were used. Endoglucanase activity by *A. terreus* grown on different nitrogen sources is shown in Fig. 2. The effectiveness of nitrogen source in supporting endoglucanase production along with growth and secretion of extracellular protein by *A. terreus* decreased in the following order; yeast extract, $NaNO_3$, NH_4Cl, $(NH_4)SO_4$, urea, peptone. Yeast extract was the

most preferable nitrogen source yielding maximal endoglucanase CMCase activity, as well as highest extracellular protein also noticed (1.46 U mL^{-1}, 3.10 mg mL^{-1}, respectively). The preference of the fungus for yeast extract as a sole N-source was justified independently. Different levels of yeast extract ranging from 2 to 6% were individually supplemented to the cultures, highest activity (2.47 U mL^{-1}) was obtained when using 3 g L^{-1} yeast extract concentration below or above this level showed an adverse effect on the metabolic activities of the test organism (Table 1). In the contrary, growth of *Trichoderma reesei* on cellulase production medium without nitrogen source increased cellulase enzyme production (Turker and Mavi, 1987). Narasimha *et al.* (2006) indicated that urea is the optimal N-source for cellulose production by *Aspergillus niger*. Furthermore, 2% urea were the optimal N-source during production of xylanase and cellulase by *Aspergillus niger* ATTC 6275 during growth on palm mill wastes (Prasetsan *et al*., 1997).

Evaluation of different process parameters affecting endoglucanase (CMCase) production: Screening is indicated when the investigator is faced with a large number of factors and is unsure which settings are likely to produce optimal or nearly optimal responses. Identifying the key response(s) and identifying all possible process factors are crucial steps in experimental design methodology. Factor level selection can be a difficult part of the experimental process. Experience, prior experimentation and the literature can be valuable resources for choosing factor settings (Strobel and Sullivan, 1999). In order to evaluate factors affecting cellulase enzyme production by *A. terreus*, Plackett-Burman statistical design was employed. Settings of the examined twelve independent variables are shown in Table 3. The experiments were carried out according to the experimental matrix presented in Table 4, where endoglucanase enzyme activity and the calculated specific activity were the measured responses. A wide variation in specific activity of endoglucanase enzyme (0.2-6.52 U mg^{-1}) was recorded, which reflects the importance of medium optimization to attain high yield of the interested product. Furthermore, the pre-optimized medium showed approximately 4 folds increase in cellulose enzyme production. The main effect of examined

Table 3: Variables and their levels employed in Plackett-Burman design for screening of culture conditions affecting on endoglucanase production by *Aspergillus terreus*

Variable code	Variables	Levels -1	Levels +1
X_1	Bagasse nontreated (NT) (g L^{-1})	10.0	50.0
X_2	Bagasse treated (T_1) (g L^{-1})	10.0	50.0
X_3	Bagasse treated (T_2) (g L^{-1})	10.0	50.0
X_4	Bagasse treated (T_3) (g L^{-1})	10.0	50.0
X_5	Yeast extract (g L^{-1})	2.0	6.0
X_6	$NaNO_3$ (g L^{-1})	2.0	5.0
X_7	KCl (g L^{-1})	0.5	2.0
X_8	KH_2PO_4 (g L^{-1})	1.0	3.0
X_9	$MgSO_4$ (g L^{-1})	0.5	2.0
X_{10}	Trace elements (mL L^{-1})	0.5	2.0
X_{11}	Temperature	20.0	35.0
X_{12}	pH	4.0	6.5

*Bagasse treatments: T_1: Hydrolysis with 0.5 M HCl, T_2: Hydrolysis with 1 N NaOH, T_3: Autoclaved and hydrolyzed with 2 N NaOH

Table 4: Plackett-Burman experimental design for evaluation of factors affecting endoglucanase enzyme production by *Aspergillus terreus*

Trail	X_1	X_2	X_3	X_4	X_5	X_6	X_7	X_8	X_9	X_{10}	X_{11}	X_{12}	Specific activity (U mg^{-1} protein)
1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	1	0.135
2	-1	1	1	-1	1	1	-1	-1	-1	-1	1	-1	2.03
3	1	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1.42
4	-1	-1	1	1	-1	1	1	-1	-1	-1	-1	1	1.44
5	1	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1.83
6	1	-1	1	1	1	1	-1	-1	1	1	-1	1	2.68
7	1	1	-1	-1	1	1	-1	1	1	-1	-1	-1	1.30
8	0	0	0	0	0	0	0	0	0	0	0	0	1.52
9	1	1	1	1	-1	-1	1	1	-1	1	1	-1	4.79
10	1	1	1	-1	-1	1	1	-1	1	1	-1	-1	5.39
11	-1	1	-1	1	-1	1	1	1	1	-1	-1	1	1.62
12	-1	1	-1	1	1	1	1	-1	-1	1	1	-1	1.71
13	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1	4.50
14	1	-1	-1	-1	-1	1	-1	1	-1	1	1	1	5.24
15	1	-1	1	-1	1	1	1	1	-1	-1	1	1	6.52
16	-1	-1	-1	1	-1	1	-1	1	1	1	1	-1	1.45
17	0	0	0	0	0	0	0	0	0	0	0	0	1.50
18	-1	1	1	1	1	-1	-1	1	1	-1	1	1	1.19
19	1	-1	1	1	-1	-1	-1	-1	1	-1	1	-1	1.11
20	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	-1	1.45
21	-1	-1	1	-1	1	-1	1	1	1	1	-1	-1	3.33
22	1	1	-1	1	1	-1	-1	-1	-1	1	-1	1	0.20

X_1: Bagasse (NT), X_2: Bagasse (T_1), X_3: Bagasse (T_2), X_4: Bagasse (T_3), X_5: Yeast extract, X_6: $NaNO_3$, X_7: KCl, X_8: KH_2PO_4, X_9: $MgSO_4$: X_{10}: Trace elements, X_{11}: Temperature, X_{12}: pH

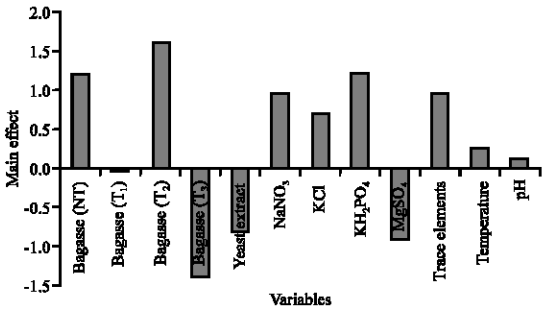

Fig. 3: Effect of environmental and nutritional factors on endoglucanase enzyme production by *Aspergillus terreus* based on Plackett-Burman design

Table 5: Statistical analysis of Plackett-Burman design showing coefficient values, t-stat and p-values for each variable

	Specific CMCase enzyme activity (U mg^{-1} protein)			
Variables	Coefficient	t-stat	p-value	Confidence level (%)
Intercept	2.38	-	-	
X_1	0.58	2.94	0.016	98.4
X_2	-0.01	-0.05	0.960	04.0
X_3	0.83	4.20	0.002	99.8
X_4	-0.71	-3.57	0.006	99.4
X_5	-0.42	-2.10	0.065	93.5
X_6	0.47	2.38	0.040	96.0
X_7	0.35	1.78	0.110	89.0
X_8	0.67	3.39	0.008	99.2
X_9	-0.46	-2.34	0.044	95.6
X_{10}	0.48	2.41	0.039	96.1
X_{11}	0.13	0.68	0.516	48.4
X_{12}	0.07	0.35	0.740	26.0

X_1: Bagasse (NT), X_2: Bagasse (T_1), X_3: Bagasse (T_2), X_4: Bagasse (T_3), X_5: Yeast extract, X_6: $NaNO_3$, X_7: KCl, X_8: KH_2PO_4, X_9: $MgSO_4$: X_{10}: Trace elements, X_{11}: Temperature, X_{12}: pH

factors on endoglucanase (CMCase) enzyme activity was calculated and presented graphically in Fig. 3. On analysis of regression coefficients and t-value of 12 ingredients (Table 5), treated bagasse (T_2), non treated bagasse (NT), K_2HPO_4, $NaNO_3$, trace elements and KCl were the most significant factors increasing endoglucanase enzyme production, whereas treated bagasse (T_3), yeast extract and $MgSO_4$, were the most significant factors decreasing endoglucanase enzyme production, where any increase in their concentration on the production medium will negatively influence enzyme production.

In many fungi, the production and secretion of the celluolytic system are known to be induced by cellulosic substrates (e.g., bagasse) and repressed by easily metabolized compounds such as glucose, however it is difficult to generalize about the response of fungi to inducers and repressors. The generally accepted mechanism of induction of cellulase secretion by insoluble cellulose is that low constitutive levels of cellulases interact with the polymer to induce soluble compounds and some of them act as the real inducer after assimilation by the fungal cell (Magnelli and Forchiassin, 1999).

Interestingly, some features of natural cellulosic materials (crystallinity and lignification) are known to limit or inhibit their degradation/bioconversion (Fan *et al*., 1987; Solomon *et al*., 1990, 1999). Therefore, pretreatment of cellulose opens up the structure and removes secondary interaction between glucose chains (Tang *et al*., 1996; Fan *et al*., 1987). In concordance with the results obtained in this study, Solomon *et al*. (1999) produced cellulase of 0.056 IU mL^{-1} from the growth of *Aspergillus flavus* on bagasse pre-treated with using ballmilling and caustic soda. While, the pretreatment of palm cake gave no improvement in cellulase and xylanase enzyme production by *Aspergillus niger* ATTC 6275 (Prasetsan *et al*., 1997).

In this study, the use of $NaNO_3$ let to significant increase in endoglucanase production. Interestingly, inorganic nitrogen sources namely; ammonium sulphate and ammonium nitrate led to optimal production of cellulase enzyme by *Aspergillus niger* and *Trichoderma reesei* (Gokhale *et al*., 1991; Gadgil *et al*., 1995; Prasetsan *et al*., 1997). On the other hand, statistical analysis indicated that temperature and pH were insignificant for cellulase production by *A. terreus*. In contrast, several scientists found out that temperature and pH have crucial effect on cellulase production in many fungi (Gokhale *et al*., 1991; Prasetsan *et al*., 1997; Jecu, 2000; Immanuel *et al*., 2007; Alam *et al*., 2008). As assayed by DNS method, Immanuel *et al*. (2007) recorded pH optima of 5 and 6 for cellulose production during growth of *A. niger* and *A. fumigatus* on coir waste and sawdust; respectively. Interestingly, Milala *et al*. (2005) reported optimal enzyme production by *A. niger* at pH 3 and substrate concentration of 5% (w/v). Narasimha *et al*. (2006) recorded an optimal pH with a value of 5 for cellulose production by *A. niger*. Romero *et al*. (1991) recorded that pH with a value of 6.5 was optimal for cellulase production by *Neurospora crassa* during growth on wheat straw. In the contrary, the positive significance of temperature on cellulase enzyme production by several *Aspergilli* was recorded, with temperature optima between 28-35°C (Gokhale *et al*., 1991; Romero *et al*., 1991; Prasetsan *et al*., 1997; Jecu, 2000; Alam *et al*., 2008). Interestingly, Immanuel *et al*. (2007) reported that *A. niger* and *A. fumigatus* were capable of producing cellulase enzyme optimally at 40 and 50°C during growth on coir waste and sawdust, respectively.

The t-test for any individual effect allows an evaluation of the probability of finding the observed effect purely by chance. Some investigators find that

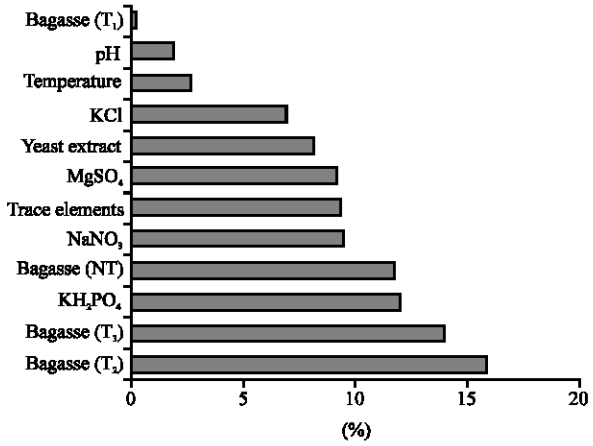

Fig. 4: Pareto plot for Plackett-Burman parameter estimates of endoglucanase enzyme activity of *Aspergillus terreus*

confidence levels greater than 70% are acceptable (Stowe and Mayer, 1966). In these experiments, variables with confidence levels greater than 95% were considered as significant. The quality of fit of the polynomial model equation was expressed by the coefficient of determination R^2. The determination coefficient R^2 of the full model for specific cellulase activity was 0.85.

One of the advantages of the Plackett-Burman design is to rank the effect of different variables on the measured response independent on its nature (either nutritional or physical factor) or sign (whether contributes positively or negatively). Figure 4 shows the ranking of factor estimates in a Pareto chart. The Pareto chart displays the magnitude of each factor estimate and is a convenient way to view the results of Plackett-Burman design (Strobel and Sullivan, 1999).

CONCLUSION

The results of this study collectively helped to optimize endoglucanase (CMCase) enzyme production by locally isolated *Aspergillus terreus* strain through improvement of substrate (bagasse) pretreatment, chemical as well as environmental parameters affecting growth and enzyme production by application of fractional experimental design. The design succeeded to rank factors from different categories to enable better understanding of the substrate as well as medium effect. It is worthwhile to further optimize the significant variables determined in the present study to attain maximum CMCase enzyme production by applying of other suitable designs.

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