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Optimization of media and submerged fermentation conditions using central composite design for increased endoglucanase production by *Cladosporium* sp. NCIM 901

[*Cladosporium* sp. NCIM 901 ile artmış endoglukanaz üretiminin merkezi kompozit düzenleme kullanılarak vasat ve dipüstü fermentasyon durumunun optimizasyonu]*

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ABSTRACT

Objective: Present work was focused on utilization of lignocellulosic substrates like bagasse and citrus peel powder for CMCase (endoglucanase) production through submerged fermentation by *Cladosporium* sp. NCIM 901.

Methods: Fungal strain *Cladosporium* sp. was procured from NCIM, Pune. Peracetic acid pretreated bagasse was used in order to reduce the crystallinity of bagasse. Mary Mandels medium was used for enzyme production. Central composite design was evaluated to study the effect of five variables on CMCase production and to optimize the medium composition.

Results: Experimental results showed the optimal medium for higher enzyme production was composed of 10% (w/v) bagasse substrate, 4.5% (w/v) citrus peel powder, 0.17% (w/v) $MgSO_4$, 0.17% (w/v) urea with the initial pH of medium at 5.5. Maximum enzyme yield (15.02 IU/ml) was obtained on by the interaction of optimal level of process involved parameters. There is a good correlation between actual and predicted results. The coefficient of determination (R^2) is 0.9414.

Conclusion: Use of cheap and environment friendly available agricultural and fruit waste substrates for enzyme production eventually reduces the production cost of enzymes. Peracetic acid pretreated bagasse was used with citrus peel powder as fermentation medium constituent to induce the enzyme production. Observed actual enzyme activity was well agreed with the predicted enzymatic activity, shown the model was applicable for enzyme production through submerged fermentation.

Key Words: Sugarcane bagasse, citrus peel, optimization, central composite design, CMCase

Conflict of Interest: The authors have declared that no conflict of interest exists.

ÖZET

Amaç: Bu çalışma, *Cladosporium* sp. NCIM 901 ile dipüstü fermentasyon yoluyla CMCaz (endoglukonaz) üretimi için sellüloz bağı içeren bagas ve turunçgil kabuk tozunun substrat olarak kullanımı üzerine odaklanmıştır.

Yöntem: *Cladosporium* sp. mantar suşları NCIM, Pune'den tedarik edildi. Bagasların kristalliğini azaltmak için perasetik asit ile ön işlem yapıldı. Enzim üretimi için Mary Mandels (MM) vasatları kullanıldı. CMCaz üretimi üzerine ve vasat bileşenlerinin optimizasyonunda beş değişkenin etkisini çalışmak için merkezi kompozit düzenleme değerlendirildi.

Bulgular: Deney sonuçları, en yüksek enzim üretimi için gerekli ideal vasat koşulunun başlangıç pH'ının 5.5 olduğunu, %10 (w/v) bagas substrat, %4.5 (w/v) turunçgil kabuk tozu, %0.17 (w/v) $MgSO_4$ ve %0.17 (w/v) üreden oluştuğunu gösterdi. Maksimum enzim kazancı (15.02 IU/ml) süreçte bulunan parametrelerin optimal düzeyleri ile etkileşimden elde edildi. Gerçek ve öngörülen sonuçlar arasında iyi korelasyon bulundu. Kararlılık katsayısı (R^2) 0.9414'dir.

Sonuç: Enzim üretimi için ucuz ve çevreye dost sağlanabilen, ziraat ve meyve atıkları gibi substratların kullanımı sonuçta enzim üretim maliyetini düşürmektedir. Perasetik asit ile ön işleme giren bagasların turunçgil kabuk tozları ile fermentasyon vasat bileşeni olarak kullanımı enzim üretimini indüklemiştir. Gözlenen gerçek enzim aktivitesi ve öngörülen enzim aktivitesinin biribirini desteklemesi ile dipüstü fermantasyon yoluyla enzim üretiminin kabul edilebilir bir model olduğu gösterilmiştir.

Anahtar Kelimeler: Şekerkamışı posası, turunçgil kabuğu, optimizasyon, merkezi komposit düzenleme, CMCaz

Çıkar Çatışması: Yazarlarin çıkar çatışması bulunmamaktadır.

Introduction

Lignocellulosic biomass represents the largest renewable reservoir of potentially fermentable carbohydrates on earth [1], generally contain up to 75% of cellulose and hemicelluloses which cannot be easily converted to simple monomeric sugars due to their recalcitrant nature. The utilization of cellulosic biomass for ethanol production continues to be a subject of worldwide interest in view of fast depletion of our oil reserves and food shortages [2]. Large quantities of lignocellulosic wastes are generated through forestry, agricultural practices and industrial processes, particularly from agro-allied industries such as breweries, paper pulp, textile and timber industries [3]. Lignocellulosic substrate should be cheap, well processable available in high amounts and its composition should be suited for both hydrolysis and production of cellulolytic enzymes. Production of cellulases on-site instead of using commercial enzymes can improve the economy of the process. Degradation of lignocellulosic materials to monomeric sugars through the concerted action of cellulolytic enzymes has great importance, since sugars can serve as raw materials in a number of biotechnological production processes [4].

The cellulase enzyme complex consists of three types of enzymes that act synergistically in cellulose hydrolysis. Endoglucanases randomly attack cellulose chains and release cello-oligosaccharides, exoglucanases cleave cellobiose units from the end of cellulose chains and β-glucosidase converts the resulting cellobiose to glucose [5]. Cellulase production from various waste cellulosic materials using different cellulolytic microfungi is being vigorously studied for cost reduction strategies. Although a large number of microorganisms (fungi, bacteria and actinomycetes) are capable of degrading cellulose, only a few of them produce significant quantities of cell-free enzyme fractions capable of complete hydrolysis of cellulose *in vitro* [6]. Many fungi capable of degrading cellulose synthesize large quantities of extracellular cellulases that are more efficient in depolymerising the cellulose substrate. Most commonly studied cellulolytic organisms include fungal species: *Trichoderma*, *Humicola, Penicillium* and *Aspergillus* [7]. Cellulase activity has been reported in temperate isolates of *Cladosporium* sp. and considerable research work was carried out for endoglucanase production by *Cladosporium* sp. on native and modified cellulosic substrates [8, 9].

Worldwide consumption of cellulase from submerged fermentation is roughly 23,000 tonnes annually. The sales volume of cellulase is around $ 125 million, which represents over 10% of all industrial enzymes sales [10]. In submerged fermentation or deep tank fermentation the fungi are grown in a fully liquid system which has the advantage of control over the process parameters such as temperature, pH, aeration and dispersion for efficient growth and yield of the intensive units [11]. Cellulase production by different organisms in submerged fermentation has received more attention and is found to be cost-prohibitive because of high cost of process engineering [12]. Approximately 90% of all industrial enzymes are produced in submerged fermentation (SmF), frequently using specifically optimized and genetically manipulated microorganisms. In this respect SmF process offers an insurmountable advantage over solid state fermentation (SSF) [13, 14]. For commercial production, optimization of medium composition is one of the essential steps to minimize the amount of unutilized components for a cost-effective yield [15]. It is impractical to optimize all fermentation parameters in conventional methodology to establish the optimum conditions by understanding the interactions of all parameters, as this involves numerous experiments if all possible combinations are to be investigated. Statistically planned experiments effectively reduce the number of experiments by developing a specific design of experiments which also minimizes the error in determining the values for significant parameters [16]. The submerged fermentation medium was optimized based on the change-one-factor-at-a time approach and detailed studies were carried out using response surface methodology (RSM) to optimize the recovery of endoglucanase from optimized medium. The RSM is an empirical statistical technique used to find the optimum conditions of a process response variable when the mechanism underlying the process is either not well understood or is too complicated to allow the exact model to be formulated from theory. It evaluates the relation existing between a group of controlled experimental factors and the observed results of one or more selected variables [17]. Central composite design (CCD) has been successfully utilized to optimize composition of fermentation medium [18-20] for enzyme and ethanol production.

Amongst the various agricultural crop residues, sugarcane bagasse is the most abundant agricultural material in India at an average production rate of 179 metric tons/year, next to Brazil with highest production rate of 672 metric tons/year [21]. Pretreatment was applied to substrate for enhancing bioconversion of cellulosic materials. Pretreatment of cellulose opens the structure and removes secondary interaction between glucose chains [22, 23]. Cellulase production level of 5.6×10^{-2} U/ml using *Aspergillus flavus* on bagasse pretreated with caustic soda was reported by Solomon et al. [24]. Cellulase production depends on the type of substrate, pretreatment and strain of microorganism used [25].

To improve yield and rate of the enzymatic hydrolysis, research has focused on the optimization of the hydrolysis process and enhancement of cellulase activity [26]. The present study was undertaken in order to ascertain to the effect of different fermentation constituents on CMCase production by *Cladosporium* sp. NCIM 901 under submerged conditions through statistical optimization.

Materials and methods

Microorganism

The cellulase producing fungal strain, *Cladosporium* sp. NCIM 901 was procured from NCIM, Pune, India, and maintained on potato-dextrose agar at 4°C. It was sub-cultured onto potato dextrose agar (PDA) in petri dishes at 30°C for 1 week prior to inoculation of submerged fermentation.

Inoculum preparation for SmF

Spores were collected from 5-day old agar-slant cultures by washing with 10 ml of sterile water with 0.1% Tween-80 (v/v), counted in a Neubauer counting chamber [27] and dilute to give 1×10^8 spores/ml. This suspension was used as the inoculum.

Substrate preparation

Substrate sugarcane bagasse preparation and pretreatment was done according to procedure of Mohan et al. [20]. Citrus peel waste was collected and dried in oven at 60-70ºC for 6-8 h. Dried peel was made into powder using mixer grinder.

Culture conditions and enzyme production by SmF

The fungus was grown in Mary Mandels mineral medium (MM medium) [28]. The MM medium contained (in g/l of distilled water) KH_2PO_4, 2.0; $(NH_4)_2SO_4$, 1.4; $CaCl_2{\cdot}2H_2O$, 0.3; Proteose peptone, 0.25; Yeast extract, 0.2 and trace metal solution, 1 ml [$FeSO_4{\cdot}7H_2O$, 5; $MnSO_4{\cdot}7H_2O$, 5.6; $ZnSO_4{\cdot}7H_2O$, 3.34; $CoCl_2{\cdot}2H_2O$, 2 mg/l], Tween-80, 1 ml. The pH of the medium was adjusted according to design model after autoclaving by separately sterilized 1 M Na_2CO_3.

The MM medium supplemented with bagasse and citrus peel powder was inoculated with spore suspension of 5-day-old sporulating slant on PDA. Enzyme production was carried out in 250 ml Erlenmeyer flask containing 100 ml MM medium with bagasse powder as the sole carbon source. The culture was incubated at 35°C on a rotary shaker at 200 rpm. The samples were withdrawn at regular intervals. The mycelium was removed by centrifugation at 7000 rpm at 4°C for 15 min to obtain a clear supernatant. This preparation was used for measurement of enzyme activities. Results given here are the mean of at least triplicate experiments.

Central composite design (CCD)

The experimental design and statistical analysis were performed according to the response surface analysis method using Design-Expert 8.0.6.1 (Stat-Ease, 2010) version software. Analysis of variance (ANOVA) and response surface plots were generated using Design-Expert 8.0.6.1. CCD for five variables and three levels each with four concentric point combinations was used to find the optimized process variables for the CMCase enzyme production (IU/ml).

The overall second order polynomial mathematical relationship of the response Y (CMCase, IU/ml) and the five variables can be approximated by the quadratic Eq. (1).

$$Y = b_0 + b_1X_1 + b_2X_2 + b_3X_3 + b_4X_4 + b_5X_5 + b_{11}X_1^2 + b_{22}X_2^2 + b_{33}X_3^2 + b_{44}X_4^2 + b_{55}X_5^2 + b_{12}X_1X_2 + b_{13}X_1X_3 + b_{14}X_1X_4 + b_{15}X_1X_5 + b_{23}X_2X_3 + b_{24}X_2X_4 + b_{25}X_2X_5 + b_{34}X_3X_4 + b_{35}X_3X_5 + b_{45}X_4X_5 \quad (1)$$

The experimental design for the variables, i.e. substrate (3–8% w/v), citrus peel powder (2–6% w/v), pH (3–8), $MgSO_4$ (5–10% w/v) and urea (2–6% w/v) were taken for measuring the enzyme activity. The design was applied for selection range of each variable (minimum and maximum), total of 50 experiments were designed by the model and performed.

The design consisted of 2^5 CCD factorial points having eight replicates at the central point and ten axial points (α). Optimized values of five independent variables for maximum activities were determined using a numerical optimization package of Design-Expert 8.0.6.1.

CMCase (endoglucanase) assay

Approximately 0.5 ml of 1% carboxymethyl cellulose in 0.05 M Na–citrate buffer, pH 4.8 was soaked for 10 min at 50°C. After that 0.5 ml of an appropriately diluted enzyme was added to the tube and incubated at 50°C for 30 min and appropriate controls were also run along with the test. At the end of the incubation period, tubes were removed from the water bath, and the reaction was stopped by addition of 3 ml of 3,5-dinitrosalicylic acid reagent per tube. The tubes were incubated for 5 min in a boiling water bath for color development and were cooled rapidly. The reaction mixture was diluted appropriately and was measured against a reagent blank at 540 nm in a Spectrophotometer. The released reducing sugars were estimated colorimetrically with 3, 5-dinitrosalicylic acid by using glucose as standard [29]. The concentration of glucose released by enzyme was determined by extrapolating with standard curve constructed similarly with known concentrations of glucose. One unit of enzyme activity was defined as the amount of enzyme required for liberating 1 µM of glucose per milliliter per minute.

Results and Discussion

Screening of most important variables and their optimization was attempted to improve the enzymatic yield under SmF on the pretreated bagasse was done. The experimental CCD for the five cultural variables/parameters was studied for measuring the CMCase production. The design was applied for selection of each parameter (maximum and minimum) as shown in Table 1. Total 50 experiments were designed and performed (Table 2).

The experimental results associated with processing set of each independent variable are listed in Table 1. To study the combined effects of these factors/variables, experiments were conducted at different combinations

Table 1. CCD of actual and coded levels of variables for the optimization of medium constituents

Factor	Name	Units	Low level	Middle level	High level	Low coded level	Middle coded level	High coded level
A	Substrate	% w/v	1	6	16	-1	0	1
B	Citrus peel powder	% w/v	1	5	12	-1	0	1
C	pH		3	5	8	-1	0	1
D	$MgSO_4$	% w/v	0.05	0.17	0.3	-1	0	1
E	Urea	% w/v	0.05	0.17	0.3	-1	0	1

*A, B, C, D and E represents the process parameters denoted as X_1, X_2, X_3,X_4 and X_5 respectively, for regression equation

of these parameters using statistically designed experiments. For each run, the experimental response along with predicted response calculated from the regression equation (Eq.2) is shown in Table 2.

A second order polynomial equation (Eq.2) was derived to represent the CMCase production as a function of independent variables tested.

$$Y = 11.54 + 1.54\ X_1 - 0.09\ X_2 - 1.94\ X_3 + 0.07\ X_4 - 0.14\ X_5 - 1.95\ X_1^2 - 1.01\ X_2^2 - 0.72\ X_3^2 - 0.86 X_4^2 - 1.04\ X_5^2 - 0.047\ X_1X_2 - 0.67\ X_1X_3 + 0.13\ X_1X_4 - 0.22\ X_1X_5 + 0.3\ X_2X_3 + 0.087\ X_2X_4 - 0.57\ X_2X_5 + 0.64\ X_3X_4 + 0.3\ X_3X_5 - 0.081 X_4X_5 \quad (2)$$

Where Y = Predicted response (CMCase, IU/ml), X_1,X_2,X_3,X_4 and X_5 are coded values of independent variables such as substrate concentration, citrus peel powder, pH, $MgSO_4$ and urea, respectively. The regression equation was used to calculate the predicted responses given in Table 2. Comparison of the predicted values with the experimentally obtained actual values indicated that these data are in reasonable agreement (Figure 1).

Statistical testing of the model was done by means of Fisher's statistical test for ANOVA using Design-expert software and the obtained results are presented in Table 3. Generally the 'F' value with a low probability 'P' value indicates high significance of the regression model. The quadratic regression model is significant with a computed F value of 20.81 implies the model is significant (P<F lower than 0.05) and there is only a 0.01% chance that a "Model F-Value" this could occur due to noise [30]. The fitness and adequacy of the model was judged by the coefficient of determination (R^2). The R^2 which can be defined as the ratio of the explained variation to the total variation was a measure of the degree of fit. The closer the R^2 value to unity, the better the empirical model fits the actual data. The value of determination of coefficient R^2 is 0.9414, which indicated that model could explain 94.14% of variability and unable to

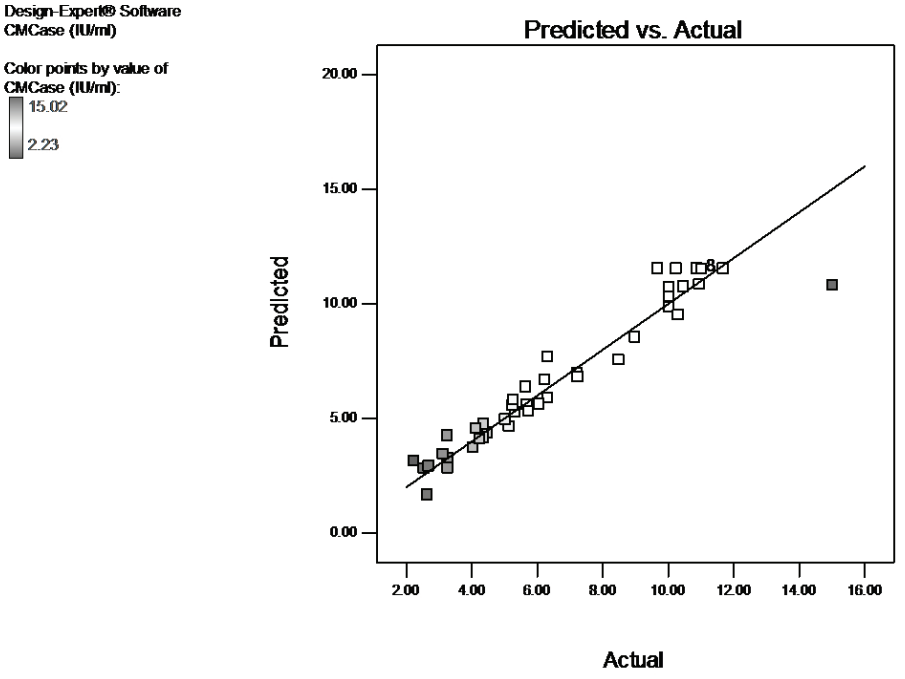

Figure 1. Actual and predicted results.

Table 2. Experimental design with coded values of variables and experimental and predicted responses of the central composite design (CCD) matrix model

Std	A:Substrate (%, w/v)	B:Citrus peel powder (% w/v)	C:pH	D:$MgSO_4$ (% w/v)	E:Urea (% w/v)	CMCase (IU/ml)
1	-1	-1	-1	-1	-1	6.23 (6.69)
2	1	-1	-1	-1	-1	10.46 (10.76)
3	-1	1	-1	-1	-1	7.22 (6.97)
4	1	1	-1	-1	-1	10.95 (10.86)
5	-1	-1	1	-1	-1	2.63 (1.67)
6	1	-1	1	-1	-1	3.26 (3.08)
7	-1	1	1	-1	-1	2.23 (3.14)
8	1	1	1	-1	-1	4.48 (4.37)
9	-1	-1	-1	1	-1	5.32 (5.27)
10	1	-1	-1	1	-1	10.03 (9.87)
11	-1	1	-1	1	-1	6.32 (5.9)
12	1	1	-1	1	-1	10.02 (10.31)
13	-1	-1	1	1	-1	2.54 (2.82)
14	1	-1	1	1	-1	4.36 (4.75)
15	-1	1	1	1	-1	5.14 (4.64)
16	1	1	1	1	-1	5.64 (6.39)
17	-1	-1	-1	-1	1	8.49 (7.56)
18	1	-1	-1	-1	1	10.03 (10.74)
19	-1	1	-1	-1	1	5.24 (5.56)
20	1	1	-1	-1	1	8.98 (8.56)
21	-1	-1	1	-1	1	4.04 (3.73)
22	1	-1	1	-1	1	3.25 (4.25)
23	-1	1	1	-1	1	2.68 (2.93)
24	1	1	1	-1	1	3.28 (3.26)
25	-1	-1	-1	1	1	5.26 (5.82)
26	1	-1	-1	1	1	10.31 (9.52)
27	-1	1	-1	1	1	4.36 (4.17)
28	1	1	-1	1	1	6.32 (7.68)
29	-1	-1	1	1	1	4.12 (4.56)
30	1	-1	1	1	1	5.68 (5.60)
31	-1	1	1	1	1	4.24 (4.11)
32	1	1	1	1	1	5.02 (4.96)
33	1	0	0	0	0	15.02 (15.82)
34	2.378	0	0	0	0	3.12 (3.44)
35	0	0	0	0	0	9.68 (11.54)
36	0	2.378	0	0	0	6.05 (5.63)
37	0	0	0	0	0	10.88 (11.54)
38	0	0	2.378	0	0	3.26 (2.84)
39	0	0	0	-0.04	0	11.02 (11.53)
40	0	0	0	2.378	0	7.23 (6.81)
41	0	0	0	0	-0.04	10.24 (11.54)
42	0	0	0	0	2.378	5.73 (5.31)
43[a]	0	0	0	0	0	11.68 (11.54)
44[a]	0	0	0	0	0	11.68 (11.54)
45[a]	0	0	0	0	0	11.68 (11.54)
46[a]	0	0	0	0	0	11.68 (11.54)
47[a]	0	0	0	0	0	11.68 (11.54)
48[a]	0	0	0	0	0	11.68 (11.54)
49[a]	0	0	0	0	0	11.68 (11.54)
50[a]	0	0	0	0	0	11.68 (11.54)

Std= Standard run order, [a]Central value

Table 3. Analysis of variance for CMCase production by SmF

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	543.31	20	27.17	23.3	< 0.0001
A-Substrate (% w/v)	50.49	1	50.49	43.29	< 0.0001
B-Citrus peel powder (% w/v)	0.27	1	0.27	0.23	0.6347
C-pH	123.11	1	123.11	105.57	< 0.0001
D-$MgSO_4$ (% w/v)	0.16	1	0.16	0.14	0.7124
E-Urea (% w/v)	0.64	1	0.64	0.55	0.4632
AB	0.069	1	0.069	0.059	0.809
AC	14.19	1	14.19	12.17	0.0016
AD	0.54	1	0.54	0.46	0.5023
AE	1.59	1	1.59	1.36	0.2526
BC	2.85	1	2.85	2.44	0.1288
BD	0.24	1	0.24	0.21	0.6536
BE	10.39	1	10.39	8.91	0.0057
CD	13.2	1	13.2	11.32	0.0022
CE	2.87	1	2.87	2.46	0.1273
DE	0.21	1	0.21	0.18	0.6747
A^2	112.86	1	112.86	96.78	< 0.0001
B^2	30.28	1	30.28	25.96	< 0.0001
C^2	15.64	1	15.64	13.42	0.001
D^2	22.37	1	22.37	19.18	0.0001
E^2	32.45	1	32.45	27.83	< 0.0001
Residual	33.82	29	1.17		
Lack of Fit	29.96	20	1.5	3.5	0.0291
Pure Error	3.86	9	0.43		
Cor Total	577.13	49			

CMCase, IU/ml: R^2=0.9414, Adjusted - R^2=0.9010, CV=14.84

explain only 5.86% of the total variation. The closer the value of R to 1 indicate the better correlation between the observed and predicted values suggesting a good fit for SmF. The adjusted R^2 was a corrected value for R^2 after elimination of the unnecessary model terms. If many non-significant terms have been included in the model, the adjusted R^2 would be remarkably smaller than the R^2. The adjusted R^2 was 0.9010, which is more suitable for comparing models with different numbers of independent variables. The coefficient of variation (CV) is a measure of residual variation of the data relative to the size of the mean; the small values of CV give better reproducibility. A lower value for the CV 14.84% clearly indicate high degree of precision and higher reliability of the experimental values. The significance of individual variables can be evaluated from their P values, the more significant terms having a lower P value. The P values are used to check the significance of each coefficient which also indicates the interaction strength between each independent variable. Table 3 also gives the P values of each of the variables and their quadratic and interaction terms. Values of "Prob>F" less than 0.05 indicate model terms are significant. In this case A, C, AC, BE, CD, A^2, B^2, C^2, D^2, E^2 are significant model terms. Values greater than 0.10 inidicate the model terms are not significant [30]. Besides the relationship between the actual experimental values and predicted values (Figure 1) showed that plotted points cluster around the diagonal line, indicating good fitness of the model.

Response surface curves were plotted to understand the interaction of variables and for identifying the optimal levels of each parameter for attaining maximum enzyme yield. The response surfaces can be used to predict the optimum range of different variables and the major interactions between the tested variables can be identified from the circular or elliptical nature of contours.

The maximum enzymatic activity was obtained at higher substrate concentration with the medium pH at their middle level. The Figure 2 represents the interaction of substrate and pH on enzyme production. The interactive effect of the variables on the production of CMCase was significant. Any change in substrate concentration does not affect the enzyme production. The highest enzymatic activity 15.02 IU/ml was well agreed with predicted enzymatic activity of 15.82 IU/ml.

The response surface curves in Figure 3 indicate the significant interaction of citrus peel powder (4.5%) and urea (0.17%) on enzyme production. The highest enz-

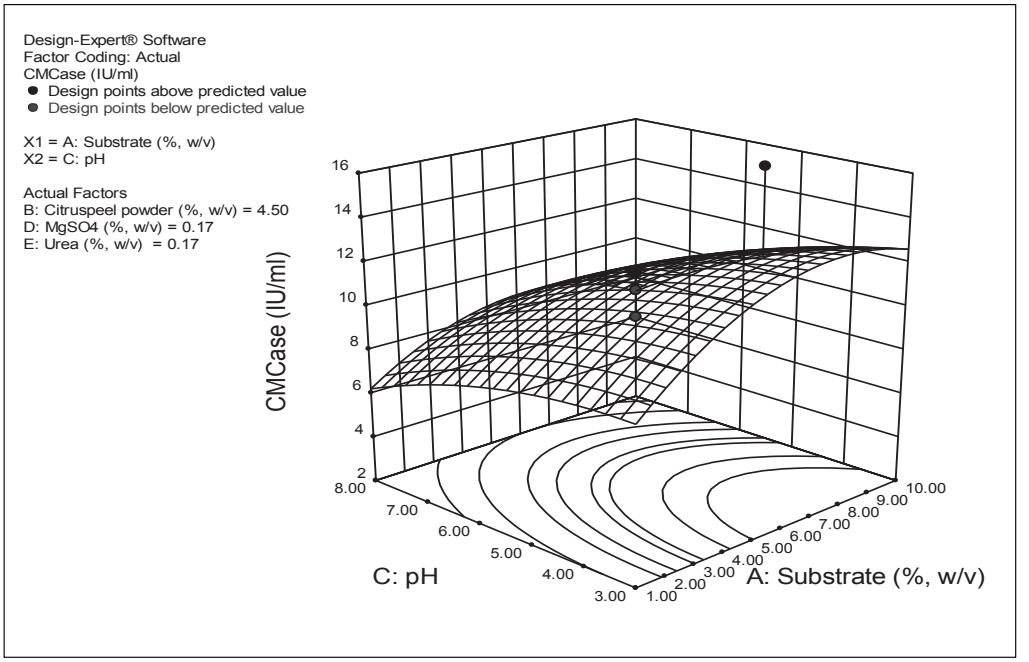

Figure 2. Response surface plots showing the interaction of substrate and pH on enzyme production

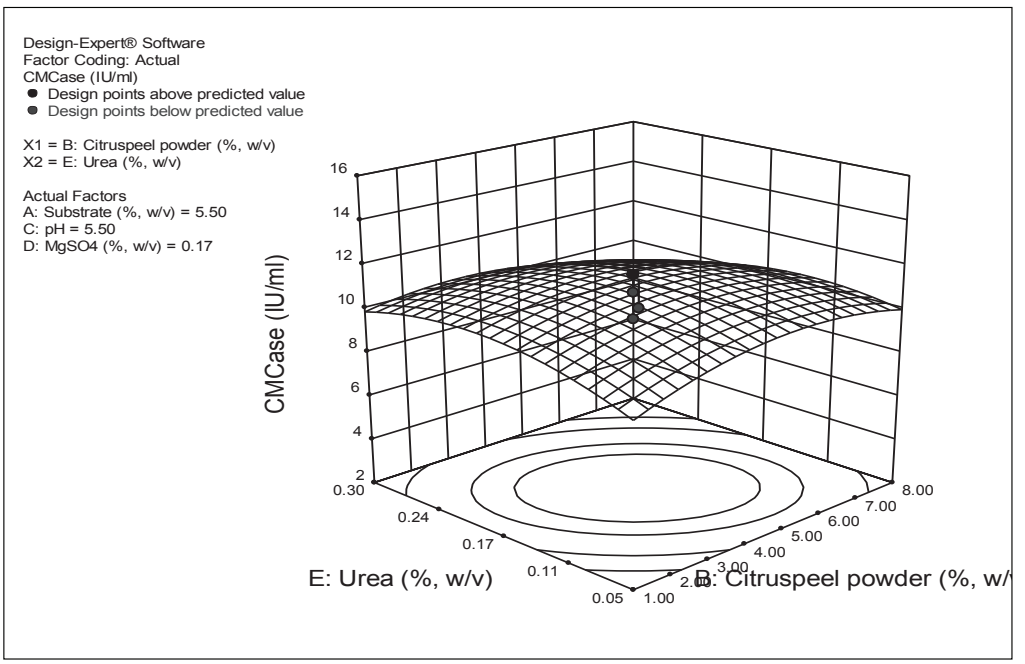

Figure 3. Response surface plots showing the interaction of citrus peel powder and urea on enzyme production

ymatic activity (15.02 IU/ml) was obtained when both of these process parameters were at their middle level of concentration. Increased or decreased concentration of these variables eventually decrease the enzyme activity.

The significant interaction effect of pH (5.5) and $MgSO_4$ (0.17%) on highest enzymatic activity is presented in Figure 4. The optimal level of both of the process variables leads to increase the enzymatic activity, any changes in both of these variables affect the enzymatic activity. The highest enzymatic activity was predicted 15.82 IU/ml on the center point with the optimal level of process variables.

Set of four experiments were performed to verify the optimization result to validate the present model. Medium was composed with the parameters of the independant variables is shown in Table 4. Highest enzymatic activity of 15.46 IU/ml, was obtained in the optimum condition, which is slightly higher than the predicted value

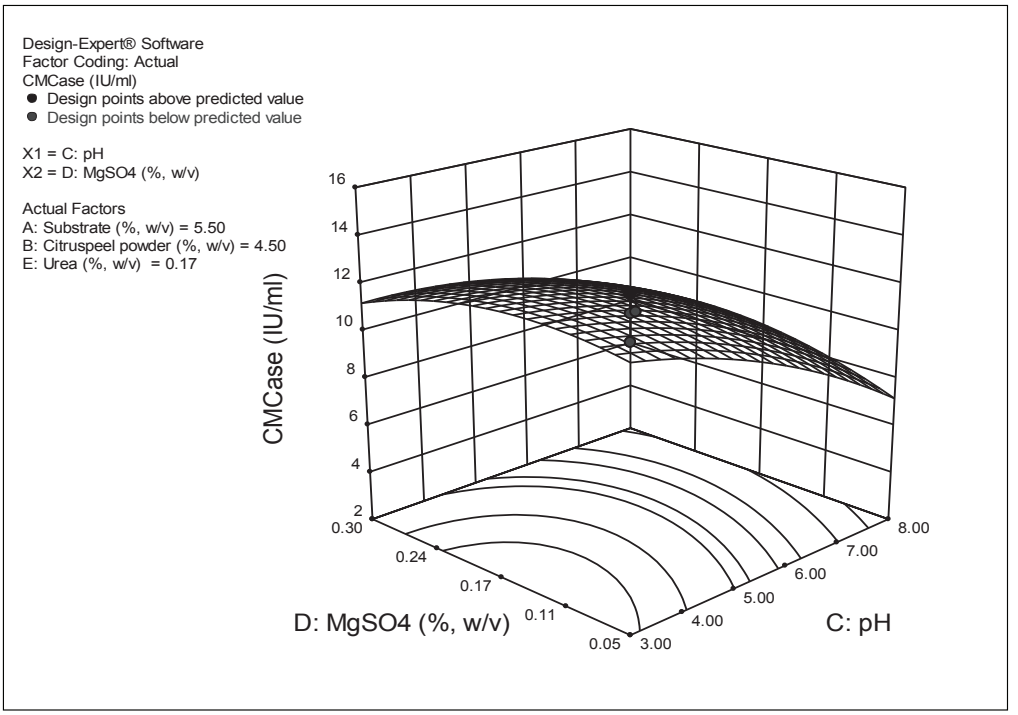

Figure 4. Response surface plots showing the interaction of pH and $MgSO_4$ on enzyme production

Table 4. Validation of model

Std	A:Substrate (%, w/v)	B:Citrus peel powder (%, w/v)	C:pH	D:$MgSO_4$ (%, w/v)	E:Urea (%, w/v)	CMCase (IU/ml)
1	1	8	8	0.3	0.05	5.14 (4.64)
2	1	1	3	0.3	0.05	5.32 (5.27)
3	1	8	3	0.3	0.3	4.36 (4.17)
4	10	4.5	5.5	0.17	0.17	15.46 (15.26)

*Values are mean of three replicates.

15.26 IU/ml. Validated results conforms the accuracy of the present model.

Statistical optimization experiments like CCD was used to optimize the fermentation process variables for increased CMCase activity under SmF conditions. Enzymatic degradation of cellulosic waste by the fungal enzymes has been suggested as feasible alternative for the conversion of lignocellulosics into fermentable sugars and ethanol [31]. According to studies of Muthuvelayudham and Viruthagiri [32] pretreatment of sugarcane bagasse and rice straw offers very digestible cellulose and potentially less inhibition. In the present study, bagasse substrate was pretreated by the lignin selective degrading chemical like peracetic acid and citrus peel was made powdered through physical pretreatment such as grinding for decreasing cellulose crystallinity and both substrates were used for endoglucanase production by fungi. Reduction of substrate particle size makes it readily usable for the mold because of decreasing crystallinity and degree of polymerization, and increasing the surface area and the bulk density of the raw materials. As a result, more cellulase enzyme produced to break down the cellulosic compounds [33]. Cellulase production was enhanced by multiple carbon sources because of diauxic pattern of utilization of substrates. Increase in enzyme production with additional carbon sources have been demonstrated by Solis-Pereira et al. [34] in both the SmF and SSF systems as a result of good growth.

In the present study the maximum enzyme activity was obtained with the medium having 10% (w/v) of bagasse substrate along with 4.5% (w/v) citrus peel powder after which enzyme activity is started to decrease significantly. The results were compared to the work of Shanmugam et al. [35] reported that the maximum CMcase

production (1.59 U/ml) and β-glucosidase production (1.82 U/ml) were increased by fungus *Tricothecium roseum* in PDYE media amended with citric acid at 37ºC and demonstrated that citric acid is a good inducer for extracellular cellulolytic enzyme production by the fungus. The present study clearly shows that the citrus peel powder has the ability to induce cellulolytic enzyme production by *Cladosporium* sp.

Mixed substrates in the form of carbon sources were used in the present study for increased CMCase production. Present study was compared with results obtained by Oberoi et al. [36] used combination of kinnow pulp and wheat bran for increasing the cellulase activity, reported by Singhania et al. [37] using wheat bran alone as a substrate employing *T. reesei* Rut C-30. Xue-Cai Hao et al. [38] employed statistical design of experiments using wheat bran, avicel, soybean cake flour, corn steep flour along with other fermentation parameters for cellulase production. The present results were compared with the results of Rashid et al. [39] obtained CMCase activity of 18.53 IU/ml using palm oil mill effluent waste in liquid state bioconversion. The different results for concentration of carbon source may be due to different nutrient composition, concentration, different nature of the substrate (particle size and consistency) and the process physiological conditions.

Some environmental factors also influence the growth of organisms as well as maximum production of enzymes will be at certain optimum temperature, pH and salt concentration. [40]. Among the physical parameters, pH of the growth medium plays an important role by inducing morphological changes in microbes and in enzyme secretion. The pH change observed during the growth of microbes also affects product stability in the medium [41]. The production of the cellulase was slightly more sensitive to the change in pH than the substrate concentration. The highest enzymatic activity was obtained in the present study was at the initial pH of the medium at 5.5. Similar findings were observed by Xue-Cai Hao et al. [38] reported that the maximum cellulase activity by mutated *T. reesei* was obtained at the medium initial pH of about 5.4 and RSM was applied for optimizing the medium constituents. The highest enzymatic activity (15.02 IU/ml) in the present study was compared with the results obtained by Muthuvelayudham and Viruthagiri [32]. Gokhale et al. [42] reported that the optimum pH for cellulase production by *A. niger* NCIM 1207 was 3.0–5.5. The cellulase activity has a broad pH range between 3.0 and 9.0. Akiba et al. [43] found the optimal pH in between 6.0 and 7.0 for cellulase from *A. niger*.

Nitrogen sources in the fermentation medium for the production of cellulolytic enzymes vary from organism to organism and influence the enzyme production. In the present study urea at middle level (0.17% w/v) induces the enzyme production on by interacts with other fermentation parameters. These results are in agreement with the Brown et al. [44] reported that urea and corn-steep liquor were the best nitrogen sources for the production of cellulase by *Penicillium pinophillum*. Similarly, Narasimha et al. [45] reported that at 0.03% urea, peptone and $NaNO_3$ used as nitrogen source, the activity of cellulase obtained were 0.824, 0.421 and 0.401 IU/mL, respectively. Effect of minerals on enzyme production is dependant on it's concentration in the bioconversion medium [46]. In the present study cellulase activities of the *Cladosporium* sp. were increased with the middle level of (0.17% w/v) $MgSO_4$ on by interaction with other fermentation parameters. Lower or higher concentration of $MgSO_4$ decreases the enzyme production, magnesium is needed for cellulase production, but it is also inhibitory at high concentrations [47]. Similarly, Shahriarinour et al. [48] reported that the highest growth and cellulase activities of *A. terreus* were increased with the presence of magnesium (5 mM) in the culture medium than with low (0 mM) or higher magnesium (10 mM) levels. Youssef and Berekaa [49] studied the production of endoglucanase by *Aspergillus terreus* by applying the Plackett-Burman design for optimization of process parameters and this study agreed with our results in that, both KH_2SO_4 and $MgSO_4$ positively affected CMCase production. High levels of KH_2SO_4 and low levels of $MgSO_4$ maximize enzyme production. The present results indicate the suitability of cheaply available lignocellulosic substrate and fruit waste for enzyme production through SmF on by optimizing the medium constituents. Using natural substrates as inducers for enzyme production will eventually reduce the production cost in fermentation process.

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References

[1] Ahmadi AR, Ghoorchian H, Hajihosaini R, Khanifar J. Determination of the amount of protein and amino acids extracted from the microbial protein (SCP) of lignocellulosic wastes. Pak J Biol Sci 2010; 13: 355–361.

[2] Singh A, Singh N, Bishnoi NR. Production of cellulases by *Aspergillus heteromorphus* from wheat straw under submerged fermentation. International Journal of Civil and Environmental Engineering 2009; 1: 23–26.

[3] Abu EA, Onyenekwe PC, Ameh DA, Agbaji AS, Ado SA. Cellulase (E.C.3.2.1.3) production from sorghum bran by *Aspergillus niger* SL 1: An assessment of pretreatment methods. Proceedings of the International Conference on Biotechnology: Commercialization and Food security, Abuja, Nigeria. 2000; 153–159.

[4] Juhasz T, Szengyel Z, Reczey K, Siika-Aho M, Viikari L. Characterization of cellulases and hemicellulases produced by *Trichoderma reesei* on various carbon sources. Process Biochem 2005; 40:3519–3525.

[5] Bhat MK, Bhat S. Cellulose degrading enzymes and their potential industrial applications. Biotechnol Adv 1997; 15:583–620.

[6] Jayanth M, Rashmi J, Shailendra M, Deepesh Y. Production of cellulase by different co-culture of *Aspergillus niger* and *Penicillium chrysogenum* from waste paper, cotton waste and bagasse. Journal of Yeast and Fungal Research 2011; 2 (2): 24–27.

[7] Gautam SP, Bundela PS, Pandey AK, Awasthi MK, Sarsaiya S. (2009) Prevalence of fungi in Municipal solid waste of Jabalpur city (M.P). J Basic Appl Mycol 2009; 8: 80–81.

[8] Abrha B, Gashe BA. Cellulase production and activity in a species of *Cladosporium*. World J Microbiol Biotechnol 1992; 8: 164–166.

[9] Duncan SM, Farrell RL, Thwaites JM, Held BW, Arenz BE, Jurgens JA, Blanchette RA. Endoglucanase-producing fungi isolated from Cape Evans historic expedition hut on Ross Island, Antarctica. Environ Microbiol 2006; 8(7):1212–1219.

[10] Tolan JS, Foody B. Cellulase from Submerged Fermentation. In: Advances in Biochemical Engineering/Biotechnology, Scheper, Third. (Ed.). Springer,Berlin/ Heidelberg. 1999; 65: 41–67.

[11] Rajeev KU, Mukerji KG, Chamola BP. Biocontrol potential and its exploitation in sustainable agriculture. Springer, Berlin / Heidelberg. 2001; 2: 30.

[12] Anita Singh, Namita Singh, Bishnoi NR. Production of cellulases by *Aspergillus Heteromorphus* from wheat straw under submerged fermentation. International Journal of Civil and Environmental Engineering 2009; 1:1-4.

[13] Filer K. The newest old way to make enzymes. Feed Mix 2001; 9:27–29.

[14] Pandey A, Soccol CR, Rodriguez-Leon JA, Nigam P. Solid state fermentation in biotechnology: fundamentals and applications. Asiatech, New Delhi, 2001.

[15] Subba Rao Ch, Sathish T, Maha Laxmi M, Suvarna Laxmi G, Sreenivas Rao R, Prakasham RS. Modeling and optimization of fermentation factors for enhancement of alkaline protease production by isolated *Bacillus circulans* using feed-forward neural network and genetic algorithm. J Appl Microbiol 2008; 104: 889–898.

[16] Suvarna Lakshmi G, Subba Rao Ch, Sreenivas Rao R, Hobbs PJ, Prakasham RS. Enhanced production of xylanase by a newly isolated *Aspergillus terreus* under solid state fermentation using palm industrial waste: A statistical optimization. Biochem Eng J 2009; 48: 51–57.

[17] Park Y, Kang S, Lee J, Hong S, Kim S. Xylanase production in solid-state fermentation by *Aspergillus niger* mutant using statistical experimental designs. Appl Microbiol Biotechnol 2002; 58: 761–766.

[18] Muthuvelayudham R, Viruthagiri T. Application of central composite design based response surface methodology in parameter optimization and on cellulase production using agricultural waste. International Journal of Chemical and Biological Engineering 2010; 3: 97–104.

[19] Sivakumar T, Shankar T, Vijayabaskar P, Ramasubramanian V. Statistical optimization of keratinase production by *Bacillus cereus*. Global Journal of Biotechnology and Biochemistry 2011; 6 (4): 197–202.

[20] Mohan PR, Ramesh B and OVS Reddy. Production and optimization of ethanol from pretreated sugarcane bagasse using *Saccharomyces bayanus* in simultaneous saccarification and fermentation. Microbiology Journal 2012; 2: 52–63.

[21] Kapoor RK, Chandel AK, Kuhar S, Gupta R, Kuhad RC. Bioethanol from crop residues, production forecasting and economics: An Indian perspective R.C. Kuhad, A. Singh (Eds.), Lignocellulose Biotechnology: Current and Future Prospects, I.K. International, New Delhi, pp. 2006; 32–45.

[22] Fan LT, Gharpuray MM, Lee YH. Cellulose hydrolysis. Berlin, Germany: Springer-Verlag. 1987; 3: 1–68.

[23] Tang LG, Hon DNS, Pan SH, Zhu YQ, Wang Z, Wang ZZ. Evaluation of microcrystalline cellulose changes in ultrastructural characteristics during preliminary acid hydrolysis. J Appl Polym Sci 1996; 59: 483–488.

[24] Solomon BO, Amigun B, Betiku E, Ojumu TV, Layokun SK. Optimization of cellulase production by *Aspergillus flavus* Linn isolate NSPR 101 grown on bagasse. JNSChE 1999; 16: 61–68.

[25] Ali S, Sayed A, Sarker RT, Alam R. Factors affecting cellulase production by *Aspergillus terreus*. World J Microb Biot 1991; 7: 62–66.

[26] Sun Y, Cheng J. (2002) Hydrolysis of lignocellulosic materials for ethanol production: a review. Bioresource Technol 2002; 83: 1–11.

[27] Raimbault M, Alazard D. Culture method to study fungal growth in solid fermentation. European J Appl Microbiol 1980; 9: 199–209.

[28] Reese ET, Mandels M. Enzymatic hydrolysis of cellulose and its derivatives. In: Whistler L Editor. Methods in Carbohydrate Chemistry, Academic Press New York, London: 1963; 139–143.

[29] Miller GL. Use of dinitrosalicylic acid reagent for determination of reducing sugars. Anal Chem 1959; 31: 426–428.

[30] Design-Expert® Software. 2010 Version 8 user's guide.

[31] Szengyel Z, Zacchi G, Varga A, Reczey K. Cellulase production of *Trichoderma reesei* RUT C30 using steam- pretreated spruce. Hydrolytic potential of cellulases on different substrate. Appl Biochem Biotech 2000; 84-86:679–691.

[32] Muthuvelayudham R, Viruthagiri T. Fermentative production of kinetics of cellulase protein on *Trichoderma reesei* using sugarcane bagasse and rice straw. Afr J Biotechnol 2006; 5(20): 1873–1881.

[33] Awafo VA, Chahal DS, Simpson BK. Evaluation of combination treatments of sodium hydroxide and steam explosion for the production of cellulase-systems by two *Trichoderma reesei* mutants under solid-state fermentation conditions. Bioresource Technol 2000; 73: 235–245.

[34] Solis-Pereira S, Favela-Torres E, Viniegra-Gonzalez G. Effects of different carbon sources on the synthesis of pectinase by *Aspergillus niger* in submerged and solid state fermentation. Appl Microbiol Technol 1993; 39 (1): 36–41.

[35] Shanmugam P, Madhumathi M, Narayanasamy M. Biosynthesis of cellulolytic enzymes by *Tricothecium roseum* with citric acid mediated induction, Afr J Biotechnol 2008; 7 (21): 3917–3921.

[36] Oberoi HS, Chavan Y, Bansal S, Dhillon GS. Production of cellulases through solid state fermentation using kinnow pulp as a major substrate. Food Bioprocess Technol 2010; 3:528–536.

[37] Singhania RR, Sukumaran RK, Pandey A. Improved cellulase production by *Trichoderma reesei* RUT C30 under SSF through process optimization. Appl Biochem Biotech 2007; 142: 60–70.

[38] Xue-Cai Hao, Xiao-Bin Yu, Zhong-Li Yan. Optimization of the medium for the production of cellulase by the mutant *Trichoderma reesei* WX-112 using response surface methodology. Food Technol Biotech 2006; 44 (1): 89–94.

[39] Rashid SS, Alam MZ, Karim MIA, Salleh MH. Optimization of the nutrient supplements for cellulase production with the basal medium palm oil mill effluent. World Academy of Science, Engineering and Technology 2009; 60: 809–814.

[40] Immanuel G, Dhanusa R, Prema P, Palavesam A. Effect of different growth parameters on endoglucanase enzyme activity by bacterial isolated from coir retting effluents of estuarine environment. Int J Environ Sci Tech 2006; 3(1): 25–34.

[41] Gupta R, Gigras P, Harapriya M, Vineet Kumar G, Bhavna C.

Microbial α-amylases: a biotechnological perspective. Process Biochem 2003; 38: 1599–1616.

[42] Gokhale DV, Patil SG, Bastawde KB. Optimization of cellulase production by *Aspergillus niger* NCIM 1207. Appl Biochem Biotech 1991; 30: 99–109.

[43] Akiba S, Kimura Y, Yamamoto K, Kumagai H. Purification and characterization of a protease-resistant cellulase from *Aspergillus niger*. J Ferment Bioeng 1995; 79: 125–130.

[44] Brown JA, Collin SA, Wood TM. Enhanced enzyme production by the cellulolytic fungus *Penicillium pinophilum*, mutant strain NTG III/6. Enzyme Microb Tech 1987; 9:176–180.

[45] Narasimha G, Sridevi A, Buddolla V, Subhosh CM, Rajsekhar RB. Nutrient effect on production of cellulolytic enzymes by *Aspergillus niger*. Afr J Biotechnol 2006; 5(5): 472–476.

[46] Lee YJ, Kim BK, Lee BH, Jo KI, Lee NK, Chung CH, Lee YC, Lee JW. Purification and characterization of cellulase produced by *Bacillus amyloliquefaciens* DL-3 utilizing rice hull. Bioresource Technol 2008; 99:378–386.

[47] Mandels M, Reese ET. Fungal cellulases and the microbial decomposition of cellulosic fabric. J Ind Microbiol Biotechnol 1999; 22: 225–240.

[48] Shahriarinour M, Abdul Wahab MN, Mohamad R, Mustafa S, Ariff AB. Effect of medium composition and cultural condition on cellulase production by *Aspergillus terreus*. Afr J Biotechnol 2011; 10(38): 7459–7467.

[49] Youssef GA, Berekaa MM. Improved production of endoglucanase enzyme by *Aspergillus terreus*: application of Plackett-Burman design for optimization of process parameters. J Biotechnol 2009; 8(2): 212–219.