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Full Length Research Paper

Optimization of growth parameters for lipase production by *Ganoderma lucidum* using response surface methodology

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Lipases comprise a group of hydrolytic enzymes which catalyze reversibly the hydrolysis and synthesis of triacylglycerides in the oil water interface. In this study, a response surface approach was used to study the production of extracellular lipase from *Ganoderma lucidum* which has diverse applications in various fields. Interactions were studied for five different variables (moisture, canola oil cake, olive oil, pH and time of incubation) which were found influential for lipase production. Using the statistical approach (response surface methodology), the maximum yield of lipase (4838 U/gds) by *G. lucidum* was observed under optimum conditions. The optimum values of these parameters were as follows: canola oil cake (12.50 g), moisture level (60%), pH (4.5), olive oil as inducer (2.0%) and incubation period (96 h) at 30°C. Exploration of fungal diversity for improved production of lipases using statistical models makes it a high-profile area for novel discovery with enormous potential of massive returns.

Key words: Lipases, *Ganoderma lucidum*, statistical approach, solid state fermentation, incubation period, canola oil cake.

INTRODUCTION

The art of fat splitting has been completely revolutionized by the entry of lipases (EC.3.1.1.3) into the industrial arena. The conventional physicochemical means of lipolysis is now overshadowed by the biocatalysis of microbial lipases (Benjamin and Pandey, 1997). Lipases (triacylglycerolhydrolases, EC 3.1.1.3) served as versatile biocatalysts, which have been widely used in various industrial applications. These cannot only catalyze hydrolysis reaction in aqueous media (Teng and Xu, 2008), but also catalyze synthesis reactions such as esterification and transesterification in non-conventional media (Wang et al., 2008). Lipases also known as triacylglycerol ester hydrolase (EC 3.1.1.3) belong to the class of serine hydrolases that contain the consensus sequence G-Xl-S-X2-G as the catalytic moiety, where G is glycine, S is serine, Xl is histidine and X2 is glutamic acid or aspartic acid (Saxena et al., 2003). However, in the absence of water or micro-aqueous conditions, they catalyze the reverse reaction of synthesis (Kaushik et al., 2006). Numerous important products such as flavor esters (Larios et al., 2004), monoacylglycerols (Monteiro et al., 2003), optically pure building blocks (Ghanem and Aboul-Enein, 2004) and also biodiesel (Fukuda et al., 2001) have been produced by these reactions. Lipases are drug targets or marker enzymes in the medical sector (Kumar et al., 2010).

Microbial lipases have attracted much attention because of their easy extraction, potential for an unlimited supply (Kumar and Gupta, 2008), relative ease of preparation, broad substrate specificity, including chain length selectivity, position selectivity and sterioselectivity (Rajendran and Thangavelu, 2009). Microbial lipases are widely diversified in their enzymatic properties and substrate specificity, which make them very attractive for industrial applications (Kumar et al., 2010). Lipases from various microorganisms can be induced by lipids or lipid-related substrates. A variety of fatty acids could also play an important role in the lipase production. Besides lipids, other medium components also influence the lipase production. Consequently, the choice of the culture conditions particularly the medium composition, is

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important for the production of lipase with defined and reproducible properties (Wang et al., 2008). Generally, a higher productivity has been achieved by culture medium optimization (Rajendran et al., 2008).

The most difficult task in culture condition optimization is the presence of the interactive effects of medium components and culture condition factors. Medium composition significantly affects the product concentration, yield and productivity. There is a general practice of determining optimal concentration of media components by varying one factor at a time. However, this method does not depict the net effect of total interactions among the various media components. Experimental design techniques present a more balanced alternative to the one-factor-at-a time approach to fermentation improvement (Sifour et al., 2010).

Response surface methodology (RSM) is a collection of statistical techniques applicable to experimental design, model building, evaluating the effects of factors and screening optimum conditions of factors for desirable responses. RSM is advantageous over conventional methods available and it includes less experiment numbers, suitability for multiple factor experiments and search for common relationship between various factors towards finding the most suitable production conditions for the bioprocess and forecast response (Shukla et al., 2007). The use of a good and reliable model is essential to develop better strategies for the optimization of the fermentation processes (Rajendran et al., 2008). To the best of our knowledge, there is no report on the medium optimization for lipase from *Ganoderma lucidum*. Thus, in this study, central composite design was used to evaluate the fermentation medium components for the enhanced production of lipase from *G. lucidum* using response surface methodology.

MATERIALS AND METHODS

Microorganism and inoculum development

G. lucidum employed in the study for lipase production was obtained from the Institute of Horticultural Sciences, University of Agriculture, Faisalabad. It was maintained on potato dextrose agar (PDA) slants at 4°C. The development of inoculum was carried out by transferring spores from 4 to 5 days old slants to 500 ml Erlenmeyer flask containing 150 ml of sterile Vogel's medium with the help of a sterile loop in aseptic conditions (Bhatti et al., 2007). The pH of the medium was adjusted to 5.0 using hydrochloric acid or sodium hydroxide solution. The inoculum flasks were incubated at 30°C (150 rpm) for 72 to 96 h to get homogenous spores suspension (10^7 - 10^8 spores /ml).

Optimization of lipase production media by response surface methodology (RSM)

Canola oil cake was used as substrate for the production of lipase by the fungus *G. lucidum* under solid state fermentation. Fermentation was carried out in 250 ml Erlenmeyer flasks containing media prepared according to the design (RSM). Media optimization for enhanced lipase production was carried out following the statistical method. The fundamental factors studied, influencing the lipases production were: moisture (%), substrate concentration (g), inducer effect (olive oil %), pH and time of incubation (h). These factors were subjected to RSM to obtain maximum concentration and level of individual factors. A 2^n factorial central composite design (CCD) developed by the design expert software was used to optimize the concentration of above five significant factors yielding a set of 50 experiments. The medium pH was adjusted to the corresponding values using 1 M HCl and NaOH. After sterilization, flasks were inoculated with the 2 ml inoculum having 1×10^8 spores /ml under aseptic conditions. Inoculum was sprinkled on the bed of the substrate (carbon source) with the injector to obtain the homogenous growth. The incubation was done at 30 ± 1°C for a specific period of time set according to the design expert software. The fermented mash was harvested after incubation for a specified period of time. The filtrate was used for lipase assay and activity was recorded as response at the end of the experiments. These experiments were performed in triplicate. The response data obtained after CCD were then fed and analyzed by the software which generated 3D contour plots and standard analysis of variance (ANOVA) indicating the optimum concentrations and interaction among these factors.

Lipase assay

The lipase activity in the culture supernatant was determined using *p*-nitrophenyl palmitate (PNPP) as substrate. Appropriate diluted enzyme (100 µl) was mixed with 0.9 ml (900 µl) of the solution containing: 3 mg PNPP dissolved in 1 ml of propane-2-ol diluted in 9 ml of the 50 mM Tris-HCl (pH=8) buffer containing 40 mg Triton ×-100 and 10 mg of gum Arabic. The stated mixture was incubated at 37°C for 30 min. The liberated *p*-nitrophenol was immediately recorded at 410 nm (Gupta et al., 2002; Yagiz et al., 2007). One unit of lipase activity was defined as the amount of enzyme required to produce 1 µmol of the *p*-nitrophenol per ml per minute under the earlier stated assay conditions.

Protein estimation

Total protein was estimated by using Bradford method (Bradford, 1976). Bovine serum albumin (BSA) was used as standard.

RESULTS AND DISCUSSION

RSM is a successive exploratory approach which allows the establishment of the relationship between multiple variables with obtained responses more efficiently than traditional design. Five factors; pH, moisture, incubation time, inducer concentration (olive oil %) and substrate concentration (g) were selected and central composite design (CCD) was used to determine their optimum concentrations. A total of 50 experiments with different combinations of factors were generated by RSM. These experiments were performed in triplicate and responses obtained were fed to the design of expert software, which gave the following quadratic regression equation,

$$\text{Lipase activity} = 1477.34+38.985A+80.751-B+14.0575C+401.618D-19.645E-37.596AB-57.280AC-27.433AD-36.062AE-26.610BC-123.253BD+7.249BE-4.315CD+8.448CE-5.959DE+9.660A^2+13.571B^2-$$

Table 1. ANOVA for response surface quadratic model.

Source of variation	Sum of squares	df	Mean square	F-value	p-value	prob > F
Model	18444830	20	922241.5	257.655	< 0.000	significant
A-Moisture	65830.43	1	65830.43	18.392	0.000	significant
B-Substrate	282433.6	1	282433.6	78.906	< 0.000	significant
C-pH	8559.34	1	8559.34	2.391	0.133	
D-Time of incubation	6893963	1	6893963	1926.026	< 0.000	significant
E-Inducer effect	16716.05	1	16716.05	4.670	0.039	significant
AB	45232.05	1	45232.05	12.637	0.001	significant
AC	104990.8	1	104990.8	29.332	< 0.000	significant
AD	23650.41	1	23650.41	6.607	0.016	significant
AE	41615.4	1	41615.4	11.626	0.002	significant
BC	22658.42	1	22658.42	6.330	0.018	significant
BD	477418.4	1	477418.4	133.380	< 0.000	significant
BE	1681.565	1	1681.565	0.470	0.498	
CD	585.247	1	585.247	0.163	0.689	
CE	2284.035	1	2284.035	0.638	0.431	
DE	1115.927	1	1115.927	0.312	0.581	
A^2	5182.848	1	5182.848	1.448	0.239	
B^2	10229.22	1	10229.22	2.858	0.102	
C^2	8056.098	1	8056.098	2.251	0.144	
D^2	9979587	1	9979587	2788.084	< 0.000	significant
E^2	3722.394	1	3722.394	1.040	0.316	
Residual	103801.8	29	3579.372			
Lack of fit	94685.08	22	4303.867	3.305	0.055	not significant
Pure error	9116.696	7	1302.38			
Cor total	1854862	49				

Values of "Prob > F" less than 0.050 indicate that model terms were significant. In this case A, B, D, E, AB, AC, AD, AE, BC, BD, D^2 were significant model terms. Values greater than 0.100 indicate that the model terms were not significant. Standard deviation, 59.828; Mean,1857.128; C.V. %, 3.221; R-Squared, 0.994; adjusted R-squared, 0.990; Pred R-Squared, 0.981; Adeq Precision, 96.319.

$12.044C^2+424.663D^2+8.187E^2$

The result obtained after CCD were then analyzed by standard ANOVA. ANOVA showed that the factor A (moisture %), B (substrate concentration, gram), E (inducer effect %) and D (incubation period, h) were significant factors and AB, AC, AD, AE, BC, BD and D^2 were significant model terms. The analysis of variance results are summarized in Table 1. The p-value serves as a tool for checking the significance of each of the coefficients. The responses taken from Table 1 reveal that linear coefficient of A, B, D and E ($p < 0.05$) and quadratic coefficients of AB, AC, AD, AE, BC, BD and D^2 ($p < 0.05$) had remarkable effects on the lipase production.

The quality of fit of the model was checked by the coefficient of determination (R^2). The R^2 value (0.994) for lipase production, pointed to the accuracy of the model. The ANOVA for the responses indicated that the model was significant. The R^2 value provides a measure of how much variability in the observed response values can be explained by the experimental factors and their interactions. The R^2 value is always between 0 and 1. The closer the R^2 value is to 1.00, the stronger the model is and the better it predicts the response. When expressed as a percentage, R^2 is interpreted as the percent variability in the response explained by the statistical model. This implied that the sample variation of 99.44% for lipase production was attributed to the independent variables and only 0.56% of the total variation was not explained by the model. This ensured a satisfactory adjustment of the quadratic model to the experimental data. The purpose of statistical analysis was to determine which experimental factors generated signals, which were large in comparison to the noise. Adequate precision measures signal-to-noise ratio; a ratio greater than 4 is desirable. An adequate precision of 96.319 for lipase activity indicated an adequate signal. So, this model can be used to navigate the design space. The "Pred R-Squared" of 0.981 was in reasonable agreement with the "Adj R-Squared" of 0.990. The 'Pred R^2 of 0.981 indicated that there was a good agreement between the experimental and predicted values for lipase production. The adjusted R^2 corrected the R^2 values for

Table 2. The actual values of factors in the central composite design (CCD).

Factor	Name	Unit	Low coded	Low actual	Central value	High coded	High actual
A	Moisture (v/w)	%	-1	52	60	+1	68
B	Substrate concentration	Gram	-1	9.35	12.50	+1	15.65
C	pH	-	-1	3.87	4.50	+1	5.13
D	Time of incubation	Hours	-1	45	60	+1	75
E	Inducer effect (v/w)	%	-1	1.16	2	+1	2.84

the sample size and for the number of terms in the model. If there are many terms in the model and the sample size is not very large, the adjusted R^2 may be noticeably smaller than the R^2. In this case, the adjusted R^2 value is very close to the R^2 value. The coefficients of regression equation were calculated using design of expert software. The model F-value of 257.65 implied that the model was significant. There was only a 0.01% chance in the "model F-value," this could occur due to noise. The value of CV (3.22%) was very less due to the low residue between the actual and predicted values of the enzyme activity.

Values of 'Pred > F' less than 0.050 indicated that the model terms were significant; values greater than 0.100 indicated that the model terms were not significant. The "lack of fit F-value" of 3.30 implied that there was a 5.48% chance that a "lack of fit F- value" which was large could occur due to noise. The 'lack of fit value' of 0.055 for lipase activity implied that the lack of fit was non-significant and the model was adequate. The optimum concentrations of the five factors for maximum lipase production was found by studying the responses in detail for all possible combinations (Table 2) by keeping 3 factors constant at a time using point prediction feature of the software. Maximum lipase yield/activity was obtained with moisture contents of 60%, substrate concentration of 12.50 g, pH of 4.50, time of incubation of 96 h and olive oil as inducer at 2.0%. Under these conditions, the experimental yield of lipase was 4838 U/gds. Sifour et al. (2010) reported that the enzyme activity of 578 U/ml was reached under the following conditions of glycerol of 2.24% (v/v), Tween 80 of 0.76% (v/v), glucose of 0.76% (w/v) and K_2HPO_4 of 0.38% (w/v).

In 3D contour plots, the responses were studied taking two factors at a time while keeping others at a fixed level. From the stated experiments, the main effects of moisture, substrate concentration and time of incubation had significant effect on the lipase production. The level of moisture contents in the fermentation medium is a crucial factor in SSF, which often determines the success of a process. An increase or decrease in the moisture content will significantly affect the biosynthesis of the enzymes. Higher moisture levels decrease substrate porosity, alter substrate particle structure, promote development of stickiness, reduce gas volume and exchange leading to poor oxygen transfer and diffusion and enhance formation of aerial mycelium, while lower moisture contents reduce the solubility of nutrients contained in solid substrate, resulting in improper swelling and a higher water tension (Bhatti et al., 2007; Shaheen et al., 2008; Sun and Xu, 2008). Low diffusion of nutrients and metabolites takes place in lower water activity conditions whereas compaction of substrate occurs at higher water activity. Hence, maintenance of adequate moisture level in the solid matrix along with suitable water activity is an essential element for SSF processes. The optimum moisture was essential for a better release of nutrients which were substrate-bound and their release occurred during autoclaving. If the initial moisture is at a supra-optimum, which would block the inter-particular air spaces in the substrate and eventually retard the growth of the organism, then growth would not be promising (Benjamin and Pandey, 1998). Results obtained by design of expert software indicated that 60% of the moisture content gave the highest yield of lipase (4838 U/gds). Sun and Xu (2008) reported that 70% moisture contents gave higher yields of lipase.

As the substrate concentration increases, the enzyme production will go up at a directly proportional rate until the media becomes saturated with the substrate. When this saturation point is reached, then addition of extra substrate will make no difference. Solid substrates should have generally large surface area per unit volume (in the range of 10^3 to 10^6 m^2/cm^3 for the ready growth on the solid/gas interface). Smaller substrate particles provide larger surface area for microbial attack, but pose difficulty in aeration/respiration due to limitation in inter-particle space availability. Larger particles provide better aeration/ respiration opportunities but provide lesser surface area. Results obtained by DOE software indicated that 12.50 g of the substrate gave the optimum response.

Different incubation times (24 to 96 h) were employed to study the effect on lipase production. The maximum enzyme activity was observed after 96 h of fermentation. These results indicate that lipase gene activation for the production of lipase occurs at the senescence phase of growth. Induction or switching on of the lipase gene (activation) is a significant step in its expression as a

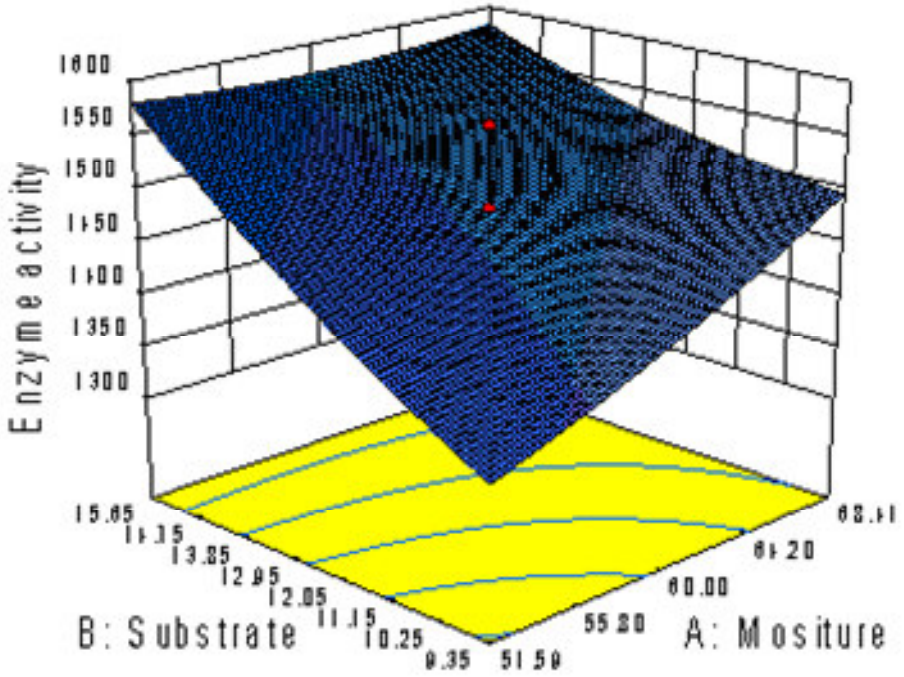

Figure 1. Contour plot of lipase activity as a function of substrate concentration and moisture.

lipase protein (Bejamin and Pandey, 1997).

pH is another important factor which affects the growth and enzyme production during solid state fermentation (SSF). Each microorganism possesses a unique optimum pH and pH range for its growth and activity (Amin et al., 2008; Bhatti and Nawaz, 2009). Filamentous fungi are supposed to thrive over a broad range of pH under solid state culture, because the solid substrate holds a better buffering capacity (Sun and Xu, 2008). The data in Table 1 (ANOVA) indicated that, lipase production was not significantly affected by pH variations and *G. lucidum* was capable of producing lipase in the pH range of 3 and 7 with the optimum been 4.50.

It has been reported that, both extra- and intra-cellular lipase biosynthesis can be increased by the addition of triglyceride or fatty acid to the media, as well as olive oil and oleic acid. Olive oil is the commonly used substrate in lipases fermentation; it serves not only as inducer for lipase production, but also as the carbon resource for microorganism growth (Teng and Xu, 2008). In this study, the addition of olive oil in the fermentation medium resulted in remarkable increase in the lipase production and 2% (v/w) olive oil gave the optimum response of 4838 U/gds. These results are in agreement with those described by Teng and Xu (2008). Kumar et al. (2010) showed that olive oil was found to be the most suitable carbon source to induce lipase production with 98.88% confidence level; 1% (v/v) olive oil produced enzyme activity of 5.59 IU/ml. It had been proposed that the fatty acids had important effects on lipase production.

Therefore, the idea represented that the effect of oil on the lipase production was caused by the fatty acids from the metabolism of olive oil and better lipase production appeared to be correlated with a higher content of oleic acid in oil; oleic acid is the main fatty acid of olive oil (>70%) (Wang et al., 2008).

The optimal value of each variable was clearly represented in the three dimension surface plots. Figure 1 show the function of moisture and substrate concentration on the enzyme activity, when other variables were kept at their central points. Figure 2 represents the enzyme activity as a function of pH and moisture (by keeping pH and moisture at central point). The middle region of the graph shows the point of maximum enzyme activity. According to the plot, the optimal value lied toward the middle range of the pH and moisture. Figure 3 represents the enzyme activity as function of moisture and time of incubation. The maximum activity was found towards higher region than the center point of time of incubation and middle region in the case of moisture. Figure 4 represents the enzyme activity as a function of variable inducer concentration and moisture. According to the plot, the optimal value lied toward the middle range of the inducer concentration and moisture. Figure 5 shows the activity of lipase as a function of pH and substrate concentration at central points. Figure 6 represents the activity of lipase as a function of substrate concentration and the time of incubation. The maximum activity of lipase was found towards higher region than the center point of time of incubation and middle region in the case

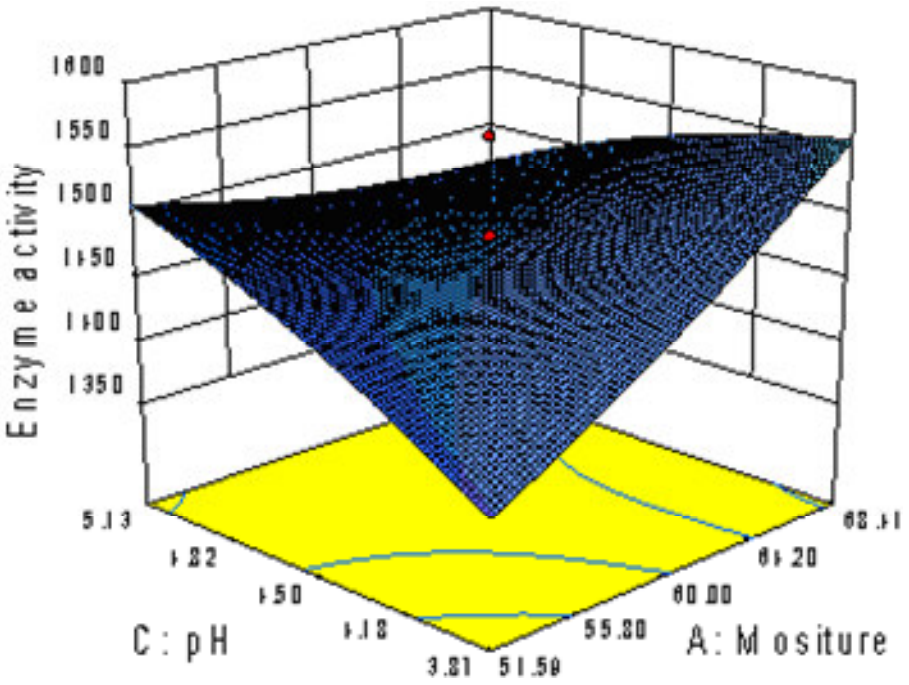

Figure 2. Contour plot of lipase activity as a function of moisture and pH.

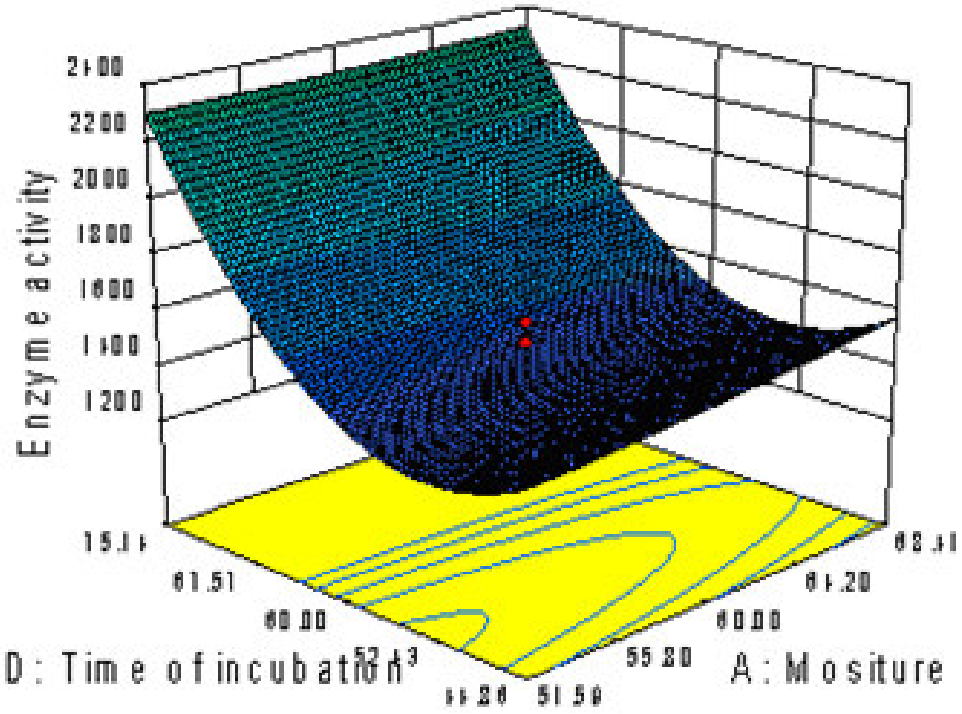

Figure 3. Contour plot of lipase activity as a function of moisture and time of incubation.

of substrate concentration. The residual differences were not large when compared with the actual and predicted one. This was due to the presence of less noise during the experimentation. Figures 7 and 8 represent the differences between the actual and predicted responses. Figure 7 show that, neither the response transformation was needed nor there was any apparent problem with normality. Kaushik et al. (2006) used response surface

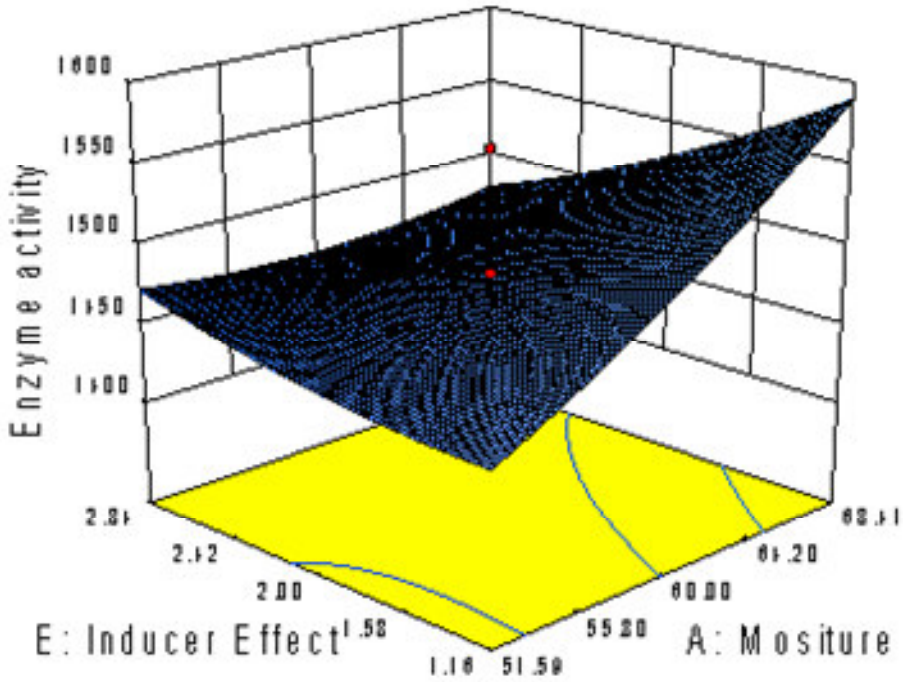

Figure 4. Contour plot of lipase activity as a function of moisture and inducer effect.

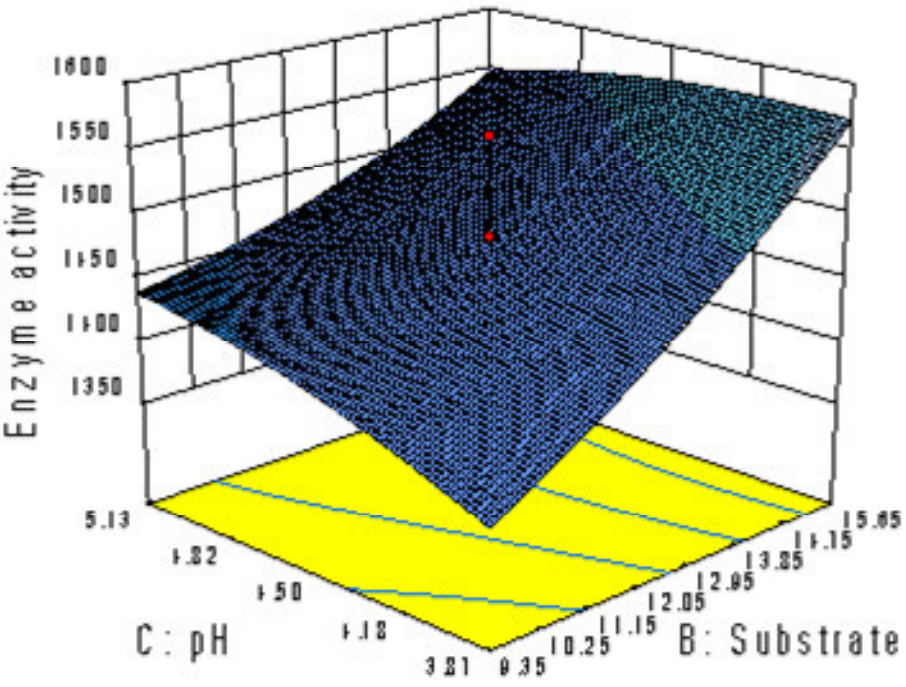

Figure 5. Contour plot of lipase activity as a function of substrate conc. and pH.

approach to find the optimum influential parameters of sunflower oil and incubation period at 1% and 96 h, respectively. Wang et al. (2008) suggested that, olive oil having high contents of oleic acid could be an important substrate for lipase production. The optimized media for lipase activity yields contain 22.99 g/l at pH 6 with other components as well (Wang et al., 2008). Teng and Xu (2008) reported an optimum inducer level of olive oil

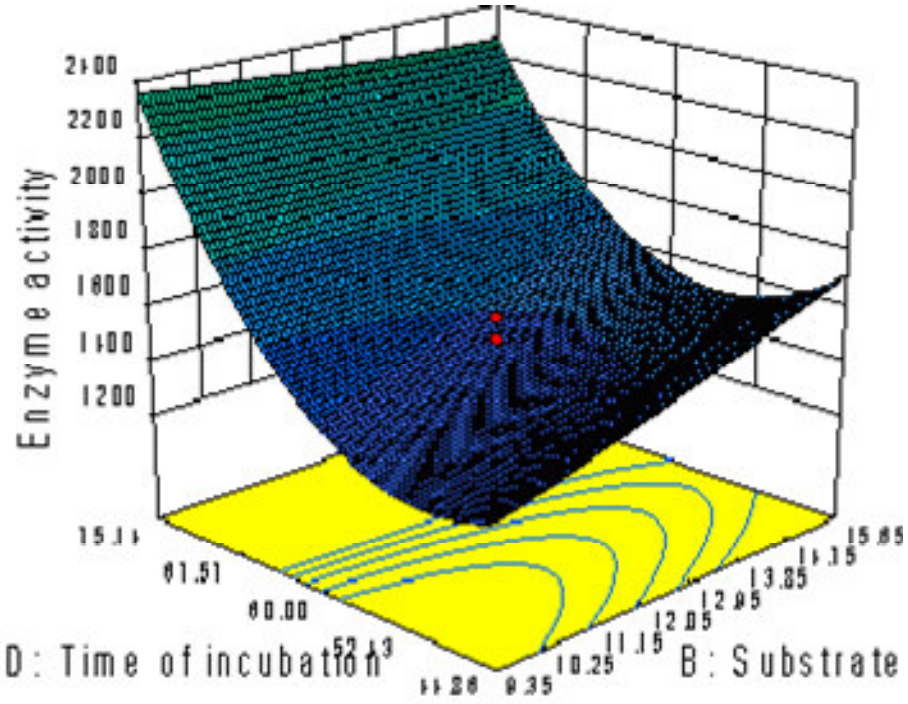

Figure 6. Contour plot of lipase activity as a function of time of incubation and substrate amount.

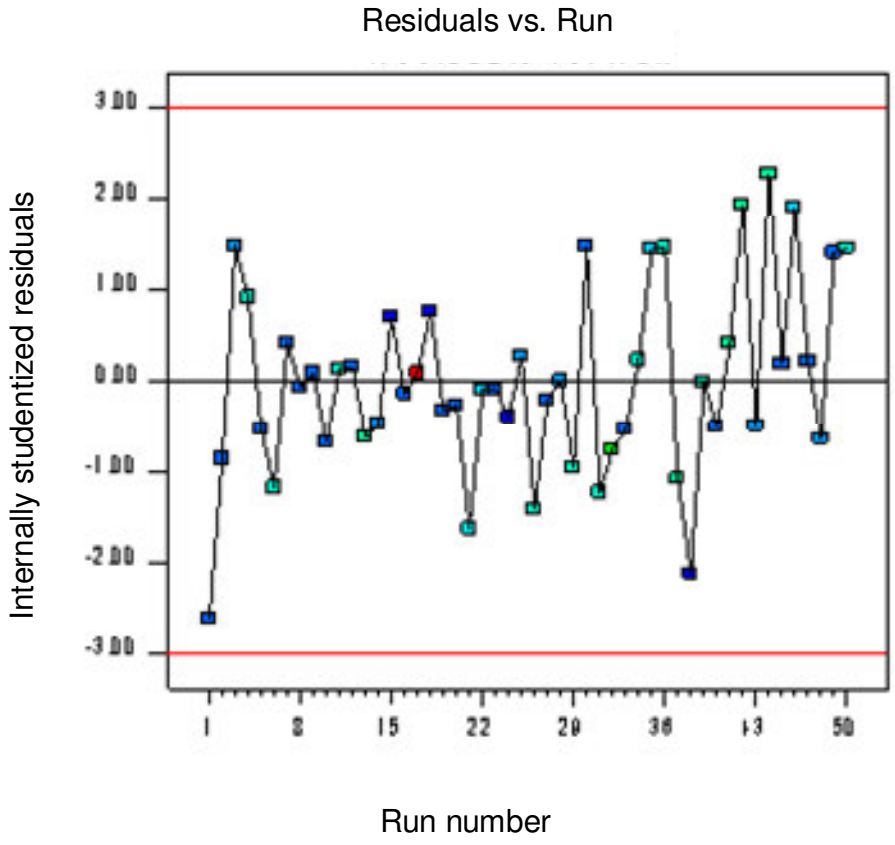

Figure 7. Representation of residual differences between predicted and actual responses of experimental runs under CC design.

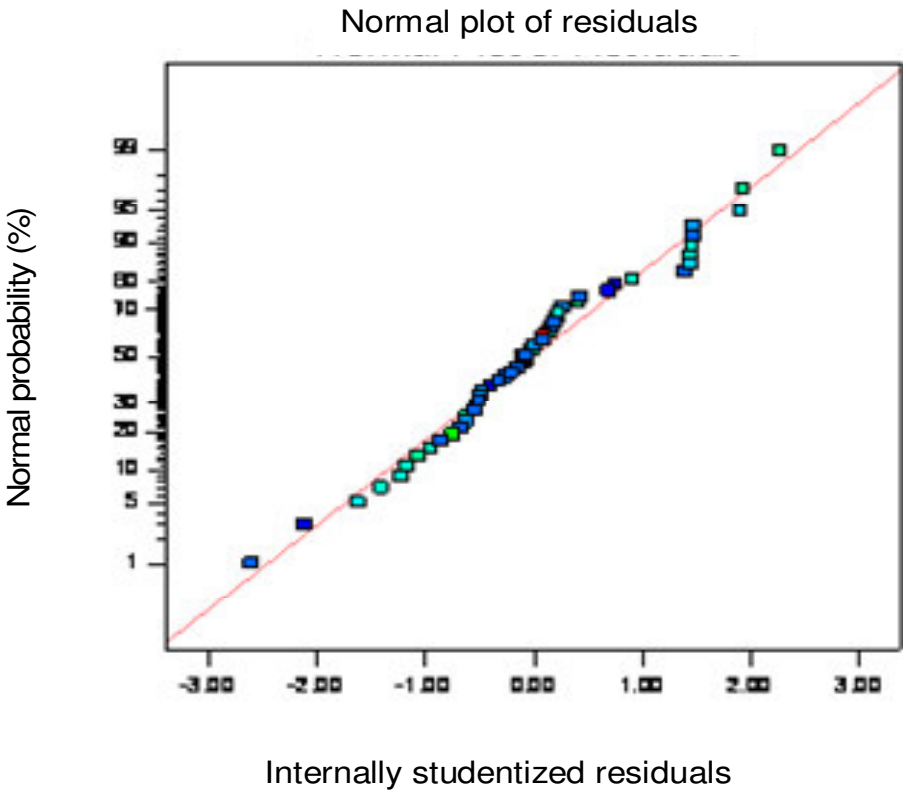

Figure 8. Representation of predicted versus actual response of experimental runs under CC design.

(2.367%) (w/v). Sun and Xu (2008) also reported that, olive oil gave good result at lipase activity of 24,447 U/kg and 70% moisture contents gave higher yields of whole cell synthetic lipase (Sun and Xu, 2008).

Conclusion

Response surface analysis was used to study and optimize the conditions for the enhanced production of lipase by *G. lucidum*. The optimum conditions as stated by further numerical analysis of the responses using the design expert software, revealed that the maximum enzyme activity was 4838 U at moisture concentration of 60%, pH 4.50, substrate concentration of 12.50 g, inducer concentration (olive oil) of 2.0% and time of incubation of 96 h. Exploration of fungal diversity for improved production of lipases using statistical models by employing these approaches for novel bioprocess development makes it a high-profile area for novel discovery with enormous potential of massive returns.

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