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# Response surface methodology for standardisation of lignocellulosic biomass saccharification efficiency of NSF-2 fungus isolate

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**Abstract**

Lignocellulosic biomass can be used as a low cost substrate for cellulase production. In the present study an attempt was made to optimize physico- chemical condition standardization for simultaneous cellulase production by NSF-2 fungal isolate, using wheat straw as a substrate and enzymatic hydrolysis of wheat straw by cellulase produced *in-vitro* by NSF-2 fungal isolate. Experiments showed maximum saccharification after 5$^{th}$ day of incubation. Optimum pH and temperature for saccharification were 5 and 30˚C respectively. Further optimization was carried out by response surface methodology using Box-Behnken design (BBD). BBD was designed with different combinations of three variables (peptone as nitrogen source, lignocellulosic biomass as substrate and Tween– 80 as surfactant, each at three levels 1 g $l^{-1}$, 2 g $l^{-1}$, 3 g $l^{-1}$, 0.5 g $l^{-1}$, 2.5 g $l^{-1}$, 5 g $l^{-1}$ and (0.05 %, 0.10%, 0.15%) respectively. The model computed for $R^2$ value (99.55%) indicated that this was appropriate and could be useful in predicting the effect of the studied variables. Experimental results showed maximum saccharification at the middle concentration of peptone and substrate *i.e.* 2 g $l^{-1}$ and 2.5 g $l^{-1}$ respectively. Surfactant did not show much significant result.

**Key words**

NSF- 2 fungal isolate, Response surface methodology, Saccharification efficiency

## Introduction

Continuous utilisation of fossil fuels are resulting in depletion of these resources. Burning of fossil fuels such as petroleum oil and coal releases a lot of harmful gases into the environment which are the major cause of global warming. So, idea about sustainable development has become a crux for many countries. Among several options, use of biomass seems to be the best. For energy generation, biomass can be used in three ways. Firstly, it can be burned directly to produce heat energy and electricity; secondly it can be changed to gaseous fuels, like methane, hydrogen and carbon monoxide and thirdly it can be changed in to a liquid fuel. (Demirbas *et al.,* 2010). Liquid fuels, also called bio-fuels, include mainly two forms of alcohol: ethanol and methanol. Because biomass can be changed directly into liquid fuel, it can someday supply much of our transportation fuel need for cars, trucks, buses, airplanes, and trains (Demirbas *et al.,* 2010).

Conversion of abundant lignocellulosic biomass to biofuel presents a viable option for improving energy security and reducing negative impact on environment (Jonsson *et al.,* 2013). Biofuel (bioethanol) production from agricultural waste require 2 steps viz., hydrolysis of pre-treated biomass using enzyme like cellulases and hemicellulases, which coverts cellulosic material to simple sugars, during a process called saccharification, followed by fermentation (Sukumaran *et al.,* 2010).

Saccharification can be either brought about by chemical or biological hydrolysis. Chemical hydrolysis involves the use of harsh chemicals like concentrated acids. Use of concentrated acids poses several threats to user as well as environment, also being corrosive in nature they degrade the container (Sun and Cheng 2002). Because of all these reasons chemical hydrolysis is not a good choice. Another option is biological hydrolysis which is brought about by means of enzymes like cellulases, xylanases and lignases which are capable of degrading biomass. Most

important of all these are cellulases, which act upon cellulose (the most abundant fraction of biomass) to break it into simple sugars. In India, there are number of companies like Genecor and Novazyme producing cellulases. Commercial cellulases can be used to convert lignocelluloses into reducing sugars (Wen *et al.* 2004), but use of pure enzyme in biomass conversion is uneconomic due to high cost of commercial cellulases (Sukumaran *et al.,* 2005). The current problem can be resolved if cellulases can be produced directly from the biomass to be degraded and then used further to degrade the same. However, the cost of obtaining sugars directly from biomass using microorganisms is still high mainly because of very low yield of cellulase producing microorganisms (Norma *et al*., 2010). The cost of cellulase production can be minimized by optimizing the yield of enzyme production. Optimizing refers to improving the performance of a system, a process or a product in order to obtain maximum benefit from it. Traditionally, optimization is carried out by one-variable-at-a-time approach. But it leads to increase of time and expenses, as well as, consumption of more reagents and materials due to more number of experiments. In order to overcome this problem; optimization can be carried out using multivariate statistic techniques. Response surface methodology (RSM) is one of those multivariate statistical techniques of optimization (Bezerra *et al.,* 2008). In many studies, response surface methodology has been used successfully to optimize a number of production processes viz, lactic acid production (Lima *et al.,* 2010), protease production (Venil and Lakshmana Perumalsamy, 2010) and cellulase production (Muthuvelay udham and Viruthagiri, 2010). Keeping in mind all the challenges optimization of cellulase production process was carried out in the present study with aim of for reducing cost of the production process.

## Materials and Methods

**Screening of cellulase enzyme :** Samples were collected in sterile polythene bags by means of sterilized spatulas from litter of wheat field and then brought to the laboratory on same day and processed further. One g of each sample was shaken with 100 ml sterilized distilled water on rotary shaker for 1 hr of in conical flasks. The extract was filtered and the supernatant was serially diluted ($10^{-1}$, $10^{-2}$, $10^{-3}$, $10^{-4}$ etc.) into separate test tube. Inoculums (100 µl of each) from each dilution were spread onto potato dextrose agar plates with sterile glass rods. The plates were incubated for 3 days at 30°C in incubator. Heterogeneous fungal colonies appeared and well-grown single colonies were picked up, purified using standard technique of sub culturing and the purified strains were maintained on potato dextrose agar slants.

Purified fungi were tested for their ability to produce cellulase using plate assay. Fungus to be screened was inoculated in centre of plate containing 1% carboxymethyl cellulose in a basal salt media. The plates were then incubated for 2 days at 25°C. Thereafter, the plates were flooded with 0.1% Congo red and allowed to react for 30 min, followed by destaining with 1M NaCl for 60 min. The width of fungal growth and the zone of clearing in cellulose medium were measured at three locations along streak line. The index of relative enzyme activity (which compared the width of clearing zone with width of fungal growth) was determined in order to choose a fungus which was best for producing cellulases.

**Cellulase production :** The strain (NSF-2) showing highest index of enzyme activity was used for enzyme production. Fungal strain (NSF-2) was maintained on potato dextrose agar at 28°C. Spore suspension of $1\times10^{6}$ spores $ml^{-1}$ was prepared by harvesting 1-week-old culture and 3 ml spore suspension was used as inoculum for 50 ml medium in a shake flask containing mineral salt medium together with 1% (w/v) of the substrate (wheat straw). The flasks were incubated at 28°C on a shaker at 180 rpm. Samples were drawn at 24, 48, 72, 96, 120 and 140 hrs after inoculation and centrifuged at 10,000 rpm at 4°C to analyse enzyme activities in the supernatant.

**Enzyme assay :** FPase activity (filter paper activity), using a 1 cm × 6 cm strip of Whatman No. 1 filter paper and endoglucanase activity (carboxymethyl cellulase activity), using sodium salt of carboxymethyl cellulose was assayed by the method of Ghosh (1987). Units (IU) of endoglucanase and FPase were defined as micromole of glucose equivalent liberated per minute of culture filtrate under assay conditions. Reducing sugar was estimated as glucose by DNS method (Miller, 1959).

**Optimization of culture conditions for enzyme production :** To determine optimal pH, NSF -2 was cultivated in a 150ml flask containing 50 ml optimized medium with different pH ranges from 3.0 to 8.0.The flasks were kept at 120 rpm at 37 °C for 5 days of cultivation. In order to determine the optimum temperature for cellulase production by the NSF -2, fermentation was carried out at 5 °C intervals in the range of 20º to 45º C. Incubation period is an important parameter for enzyme production by any strain. In this study, fermentation experiment was carried out up to 10 days and production rate was measured every 24 hr intervals.

**Response surface model :** In the present study Box-Behnken design (Box and Behnken, 1960) was selected for optimization of production of FPase and endoglucanase (EG), by NSF-2, and to develop a mathematical correlation between three independent variables on endoglucanase (EG) and FPase production. Three independent variables were peptone concentration (g $l^{-1}$), wheat straw (g $l^{-1}$) and surfactant (%). Low, middle and high concentration level of each were designated as 1, 0 and +1 respectively. For response surface methodology based on the Box–Behnken design, 17 experimental runs with different combinations of three variables were carried out. This design was applied using Design Expert 6.0 with three variables at three levels. Three different variables; peptone concentration (0.5, 1.75, 3 g $l^{-1}$), wheat straw (0.5, 2.75, 5 g $l^{-1}$) and surfactant (0.05,

0.125, 0.2 %) were chosen as critical variables and designated as A, B and C, respectively. The behaviour of the system was explained by the following quadratic model equation:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC$$

Where Y is the predicted response; $\beta_0$ is a constant; $\beta_1$, $\beta_2$, $\beta_3$ are linear coefficients; $\beta_{12}$, $\beta_{23}$, $\beta_{13}$ are cross-coefficients; $\beta_{11}$, $\beta_{22}$, $\beta_{33}$ are quadratic coefficients. Data were analysed using Design Expert 6.0 program including ANOVA to find out interaction between variables and response. The quality of fit of this model was expressed by coefficient of determination ($R^2$) in the same programme.

## Results and Discussion

After screening 70 different strains of fungi, NSF-2 was selected for further study on the basis of highest zone of hydrolysis obtained around the colony. There existed a strong influence of initial pH of medium on enzymatic saccharification. Very low and high pH is not suitable for saccharification (Fig. 1a). Saccharification efficiency gradually increased with increasing pH up to optimum point (pH 5). After that further increase in pH results in a decrease in saccharification efficiency. Similar results were also reported by Ahmed *et al.* (2012) on of saccharification sugarcane baggase at pH 5.

Incubation temperature and period are also important factors for enzyme production. The results showed that optimal temperature for enzyme production was 30 °C (Fig 1b) and maximum activity was attained on the 5$^{th}$ day of incubation period (Fig 1c).

**Table 1 :** Box-Behnken Design and observed values for three independent variables.

| Trial No | Peptone (g l$^{-1}$) | wheat straw (g l$^{-1}$) | Surfactant (%) | Cellulase activity | |
|---|---|---|---|---|---|
| | | | | FPA (U ml$^{-1}$) | CMCase (U ml$^{-1}$) |
| 1 | -1 | -1 | 0 | 0.99 | 34.7 |
| 2 | 1 | -1 | 0 | 0.79 | 35 |
| 3 | -1 | 1 | 0 | 1.2 | 34.7 |
| 4 | 1 | 1 | 0 | 1.25 | 36.6 |
| 5 | -1 | 0 | -1 | 0.69 | 34.4 |
| 6 | 1 | 0 | -1 | 0.57 | 31.1 |
| 7 | 1 | 0 | 1 | 0.47 | 31.1 |
| 8 | 1 | 0 | 1 | 0.33 | 31.1 |
| 9 | 0 | —1 | -1 | 0.78 | 31.2 |
| 10 | 0 | 1 | -1 | 0.78 | 32.5 |
| 11 | 0 | -1 | 1 | 0.39 | 34.7 |
| 12 | 0 | 1 | 1 | 1.2 | 30.6 |
| 13 | 0 | 0 | 0 | 0.62 | 31.1 |
| 14 | 0 | 0 | 0 | 1.19 | 31.1 |
| 15 | 0 | 0 | 0 | 1.28 | 31.2 |
| 16 | 0 | 0 | 0 | 0.75 | 32.5 |
| 17 | 0 | 0 | 0 | 0.51 | 36.9 |

The highest total cellulase activity (1.28 U ml$^{-1}$) was observed in trial run 15 (Table 1). The final response equation that represented a suitable model for total cellulase production is given below:

$$1.22+0.18*A+0.043*\ B+0.030*C0.34*A^2-0.2*B^2-0.28*C^2+0.15*A\ B-0.018*AC-0.083*BC$$

In order to build a second-order model which can describe the enzymatic activity (dependent variable) concerning peptone, wheat straw and surfactant, ANOVA was employed for determination of significant parameters. $R^2$ represents the proportion of variation in the response data that can be explained by the fitted model. The regression equation indicated that coefficient of determination ($R^2$) was 0.9955 (Table 2) (a value of

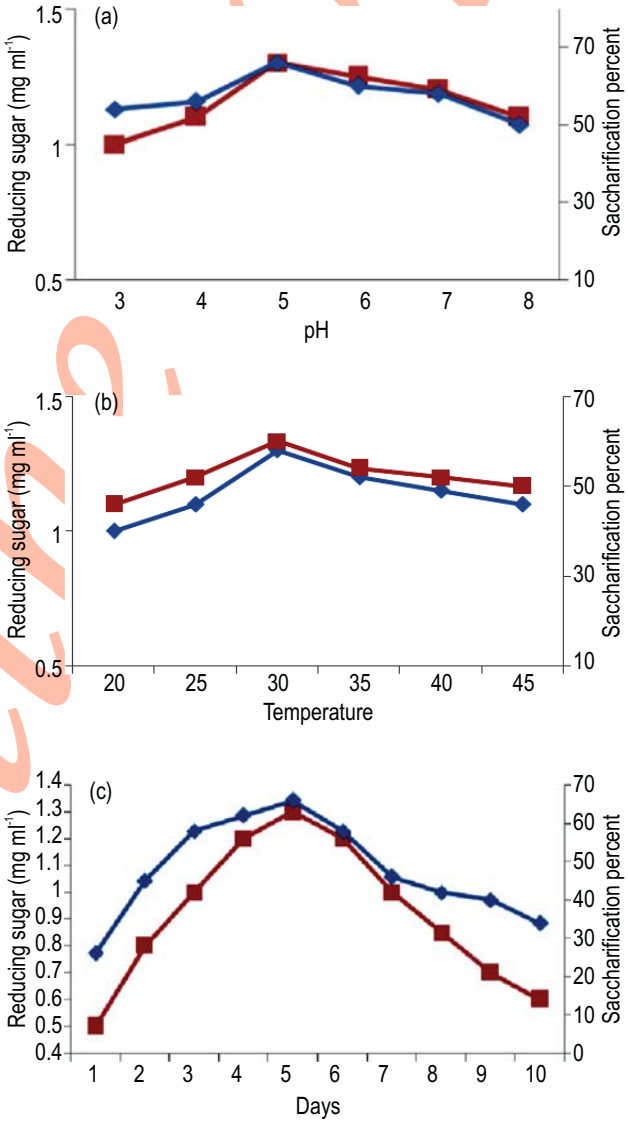


**Fig. 1 :** Effect of pH (a), Temperature (b) and Incubation time (c) on % saccharification (■) of wheat straw and released sugars (♦) (mg ml$^{-1}$)

$R^2$ > 0.75 indicates excellence of model for total cellulase production, thus model could explain more than 99.55 % of variability in response. Moreover, $R^2$ is in reasonable agreement with adjusted $R^2$ of 0.9896. The adjusted $R^2$ corrects the $R^2$ value for the sample size and 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 predicted $R^2$. The "Pred R-Squared" of 0.9818 is in reasonable agreement with the "Adj R-Squared" of 0.9896."Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. The ratio of 35.522 indicates an adequate signal. The value of $R^2$ and Adj-$R^2$ close to 1.0 implies that there is strong correlation between observed values and predicted values. The coefficient of variation (CV) indicates the degree of precision with which the treatments were compared. Usually, the higher the value of CV, the lower is the reliability of experiment. Here, a lower value of CV (4.00%) indicated better precision and reliability of the experiments. The lack-of-fit term in the residual indicates the variation due to model inadequacy. The computed F-value (170.37), which is the ratio of mean square due to regression to the mean square due to error, indicates the influence (significant or not) of each controlled factor on tested the model. The low probability p-value (<0.05) indicated that model terms are significant. For total cellulase production in this case A, B, C, $A^2$, $B^2$, $C^2$, AB, BC were significant model terms. The concentration of peptone, varyed between 0.5 to 1.75 g $l^{-1}$ increased enzymatic activity but when concentration increased up to 3g $l^{-1}$, enzymatic activity decreases. So the result showed that maximal cellulase was produced at the middle level of peptone concentration (coded value 1). Similarly middle concentration of wheat straw shows maximal results (coded value 1) (Table 1). Surfactant does not show much significant result.

The final response equation that represented a suitable model for endoglucanase production is given below:

$$60.45+9.11*A+3.15*B+1.03*C-14.34*A^2-11.56*B^2-13.19*C^2+8.90*AB+0.26*AC-4.05*BC$$

Production of CMCase follows the similar pattern as observed for FPase. The highest endoglucanase production (36.9 U $ml^{-1}$) was observed at the middle level of peptone and wheat straw concentration (coded value 1) in run 17 (Table 1). The independent variables were fitted into the second order model equation and examined for the goodness of fit. Several indicators were used to evaluate the adequacy of the fitted model and results are shown in Table 2. The value of coefficient of determination ($R^2$) was 0.9953; adjusted $R^2$ was 0.9893, "Pred R-Squared" of 0.9379, Adeq Precision of 34.467. The low probability p-value (<0.05) indicated that model terms were significant. For endoglucanase production, in this case A, B, $A^2$, $B^2$, $C^2$, AB, BC are significant model terms. Again a lower value of CV (3.67 %) indicated better precision and reliability of the experiments.

It means that both the substrate (in range of 0.5 – 2.75 g $l^{-1}$) and peptone (in the range of 0.5 – 1.75g $l^{-1}$) showed a positive effect on both FPase and endoglucanase production. Substrate concentration is an important factor which shows very significant effects on yield and initial hydrolysis of cellulose. At low substrate concentration, increase in substrate concentration normally results in an increase in the yield and reaction rate of hydrolysis. However, high substrate concentration lowers enzyme production. The observed response at high concentration may be due to substrate inhibition (Sun and Cheng, 2002). A similar positive contribution of peptone concentration to production of cellulase in the range between 0.5- 1.0 % (w/v) was also observed by Rashid *et al.* (2009) and Gautam *et al.* (2011).

The three dimensional response surface graphs were plotted to study the interaction among the various factors selected and to determine the optimum concentration for attaining maximum FPase (Fig. 2a, b, c) and endoglucanase production (Fig. 3a, b, c). These plots are graphical representation of the regression equation. The plots were generated by plotting response using the z-axis against two independent variables while keeping the other independent variables at their O-level.

**Table 2 :** ANOVA for response surface quadratic model for FPase and endoglucanases

| Source | Sum of Squares | | Mean square | | F Value | | P value | |
|---|---|---|---|---|---|---|---|---|
| | Fpase | Endoglucanase | Fpase | Endoglucanase | Fpase | Endoglucanase | Fpase | Endoglucanase |
| Model | 1.61 | 3546.66 | 0.18 | 394.07 | 170.37 | 165.78 | <0.0001 | <0.0001 |
| Residual | 7.370E-003 | 16.64 | 1.053E-003 | 2.38 | | | | |
| Lack of Fit | 1.250E-003 | 13.52 | 4.167E-004 | 4.51 | 0.27 | 5.78 | 0.8431 | 0.0616 |
| Pure Error | 6.120E-003 | 3.12 | 1.530E-003 | 0.78 | | | | |
| Cor Total | 1.62 | 3563.30 | | | | | | |
| $R^2$ | 0.9955 | 0.9953 | | | | | | |
| Adj $R^2$ | 0.9896 | 0.9893 | | | | | | |
| Pred $R^2$ | 0.9818 | 0.9379 | | | | | | |
| Adeq Precision | | 35.522 | 34.467 | | | | | |
| C.V. | 4.00 | 3.67 | | | | | | |
| PRESS | 0.030 | 221.22 | | | | | | |

DF = degree of freedom; CV= Coefficient of variation; PRESS= Predicted sum of squares

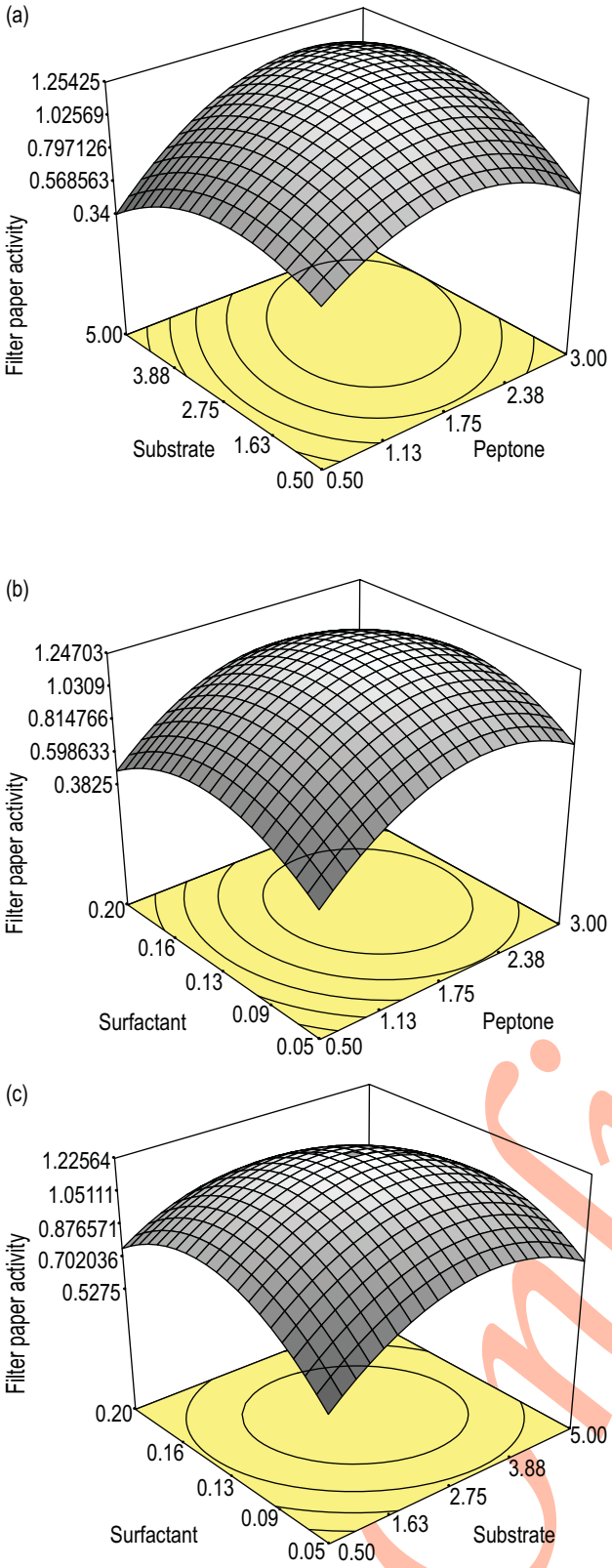


**Fig. 2 :** Response surface plot showing effect of substrate and peptone (a), surfactant and peptone (b), substrate and surfactant (c) on FPase activity

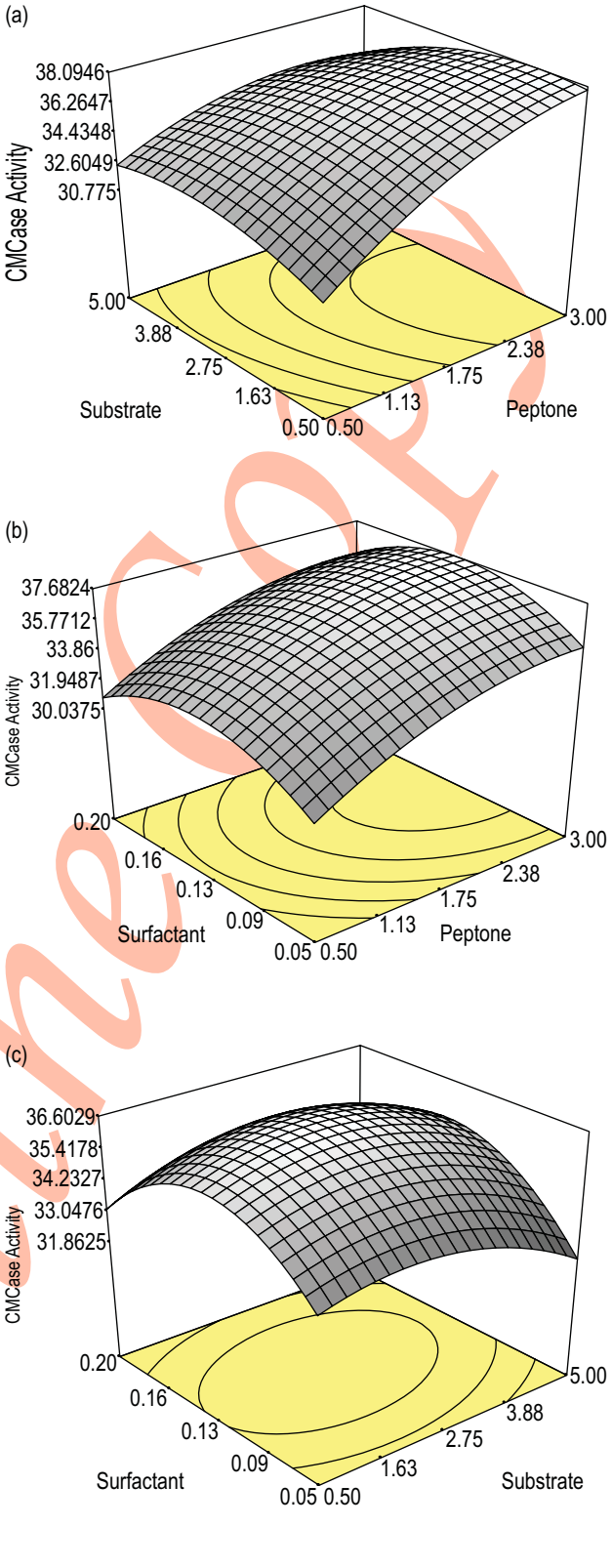


**Fig. 3 :** Response surface plot showing effect of substrate and peptone (a), surfactant and peptone (b), substrate and surfactant (c) on endoglucanase activity

The coordinates of central point within the highest contour levels in each of the figures correspond to the optimum concentrations of the respective components. Three dimensional contour plots for Fpase (Fig. 2a) and endoglucanase activity (Fig. 3a) with substrate and peptone concentration had an elliptical nature and a clear elongated running diagonal indicating a significant interactive effect on response between the two independent variables. On the other hand, the contour plot of Fpase (Fig. 2b, c) and endoglucanase activity (Fig. 3b, c) with substrate and surfactant concentration and peptone and surfactant concentration respectively had a circular nature, suggesting that the interactive effects of above said factors were not significant and these factors were independent or slightly interdependent. Each contour plot had clear maxima which implied that maximum enzyme yield could be obtained inside the design boundaries (Ghosh and Hallenbeck, 2010).

Response surface model proved to be a useful and accurate method to optimize the enzymatic activity and is a significant concern in developing a suitable bioprocess for cellulase production. Thus, enhancing cellulase production in submerged state on cheap substrate could go a long way in bringing down the cost of cellulases which would eventually help to develop economical progress for biofuel production.

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