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Optimization of culture conditions using response surface methodology for synergism production of cellulase, xylanase and pectinase by *Rhizopus delemar* F2 under solid state fermentation

Shruti Pathania, Nivedita Sharma and Shweta Handa

Abstract
In the present study Response surface methodology (RSM) was used to investigate the combined effect of relevant process variables to maximize the production of multiple carbohydrase in solid state fermentation by *Rhizopus delemar* F2 using environmental waste i.e. apple pomace a major horticultural waste of Himachal Pradesh. The process variables include temperature (25, 30, and 35°C); moisture (1:2-1:5) and incubation days (4-10). A 20 factorial central composite design (CCD) using response surface methodology (RSM) was employed to obtain interaction between the process variables and optimizing these variables. Total 20 experiments were carried out in shake flask and a three dimensional response surface was generated to determine the effect of process variables on enzymes production. The optimal calculated values of tested variables for maximal production of enzymes were: temperature 30°C, moisture ratio of 1:3:5 and 7 days of incubation with 10.40 U/gds of cellulase, 126.80 U/gds and 31.20 U/gds.

Keywords: Cellulase, Xylanase, Pectinase, *Rhizopus delemar* F2, CCD; Solid state fermentation; Process parameter optimization.

Introduction
Biofuel production from renewable sources is widely considered to be one of the most sustainable alternatives to petroleum sourced fuels and a viable means for environmental and economic sustainability (Farinas *et al*., 2011) [4]. India is a country with a positive outlook towards renewable energy technologies and committed to the use of renewable sources to supplement its energy requirements. The country is one among the few nations to have a separate ministry for renewable energy which address the development of biofuels along with other renewable energy sources.

Production of waste material is an undeniable part of human society. The wastes are produced by several sectors including industries, forestry, agriculture and municipalities. The accumulation of waste and throw-away philosophy result in several environmental problems, issues and safety hazards and prevent sustainable development in terms of resources recovery and recycling of waste materials. A perspective aimed at promoting greater sustainable development and resource recovery has influenced solid waste management practices and gradually implemented through policy at national levels in the number of industrialized and even developing countries.

Biomass-degrading enzymes are one of the most costly inputs affecting the economic viability of conversion of substrate into enzyme. This enzymatic complex is produced by a wide variety of microorganisms (bacteria and fungi) however, the aerobic fungi are known for their higher growth and enzyme production using cheap substrate (Holker *et al*., 2005) [6]. The quality of the enzymatic complex is an important issue, since a cocktail containing cellulases, hemicellulases, pectinases, and other accessory enzymes, acting in synergy in the degradation process, is necessary due to the high recalcitrance of plant biomass (Gabriela *et al*., 2012) [5]. The potential value of the enzymes for diverse applications is noteworthy only through the development and optimization of enzyme production methods with an eventual aim of reducing the overall production cost, use of inexpensive substrates for enzyme production and for purification. However, the relatively high cost of enzyme production has hindered the industrial applications of the enzymatic process (Xia and Cen, 1999) [12].

Solid state fermentation is an attractive process to produce cellulase and xylanase economically due to its lower capital investment and lower operating expresses (Xin and Geng, 2010) [13]. To reduce the cost of enzyme, selection of a cheap and easily available substrate

appears to be essential (Beg *et al*., 2000; Senthilkumar *et al*., 2005) [1, 10]. It is an attractive method for fungal enzymes production because it stimulates the natural growth of microorganisms on a moist insoluble substrate in the absence or near absence of free liquid (Pandey, 2002) [8].

Optimizations of process conditions are the most important factors to reduce the production cost. In preliminary study, optimization of enzyme production was done using response surface methodology (RSM) was used to optimize the media composition and few process variables. RSM is a compilation of numerical and statistical techniques useful for analyzing the effect of several independent variables. The process consists of a low order polynomial equation in a predetermined region of independent variables. These independent variables are later analyzed to locate the optimum values of the independent variables for the best response. It can be used to evaluate the relationship between a set of controllable experimental variables and observed results. The interaction among the possible influencing variables can be evaluated with limited number of experiments. RSM and CCD are proved to be important tools to study the effect of multiple process variables with fewer experimental trials.

The present work investigates the effects of operational conditions on the production of biomass-secreting multienzyme carbohydrase (containing FPase, endoglucanase, and xylanase) by a selected strain of *Rhizopus delemar* F2 cultivated under SSF using apple pomace using Response surface methodology.

Material and methods

Microorganism

The strain of *Rhizopus delemar* F2 was isolated from the decaying wood in cookes red bengal medium and the culture was maintained and sub-cultured every two weeks. The culture sequences was submitted in NCBI and provided with an accession number of KX512312.

Inoculum preparation and Production of enzymes under solid state fermentation

Added 10% of four days old fungus culture scratch with 10ml of autoclaved distilled water and fix their inoculum size 1×10^7 spores/ml. The fungus was preferred in 250ml of Erlenmeyer flask containing 10g of untreated and pretreated biomass. The moistening agent used was Basal salt medium of pH 5.5. The flask were cooled down at room temperature and then inoculated with 1×10^6 spores and incubated for 5-6 days at temperature 30 ^{0}C.

Optimization of multiple carbohydrase production

Response surface methodology was used to optimize the Solid state fermentation process and enhance the cellulose, xylanase and pectinase production. Design expert software (version 7.0) was used for the statistically design of experiments and data analysis. Four experimental factors (temperature, moisture and incubation days) were found to have significant effect on enzymes production as determined. The range and centre point values of the three independent variables are presented in Table 1. The full experimental design with respect to the real value of the independent variables and attained values for the response (cellulose, xylanase and pectinase) is presented in Table 2. RSM using a three level central composite design was applied to optimize the response of three variables. A 20 factorial design was used in order to study the effect of ion. Incubation time for enzymes production was 7 days. Therefore enzyme activity after 7 days of production was measured as responses. The statistical analysis of the results was performed using Design Expert ver. 6.0.9 statistical software (Stat-Ease Inc, Minneapolis, MN). Different enzyme activity was analyzed using the analysis of variance (ANOVA) combined with the Fischer test to evaluate if a given term has a significant effect ($p\leq 0.05$). The optimum levels of the variables were obtained by graphical and numerical analysis using Design Expert program.

Table 1: Levels of factor chosen for the experimental design

Factor	Unit	Symbols	Actual levels of coded factors	
			-1	1
Temperature	^{O}C	A	20	40
Moisture	%	B	2	5
Incubation days	Days	C	4	10

Table 2: Central composite designs for the experimental design and predicted results for enzyme activity (U/gds).

Run	Temperature	Moisture	Incubation days	Cellulase (U/gds)	Xylanase (U/gds)	Pectinase (U/gds)
1	30	3.50	7.00	1.1	45.5	9.8
2	30	6.02	7.00	9.8	122.4	28.8
3	30	3.50	7.00	5.9	66.6	12.3
4	30	3.50	7.00	1.1	42.2	1.2
5	30	3.50	1.95	4.4	5.4	21.1
6	20	2.00	10	9.9	128.2	29.8
7	30	46.62	7.00	10.1	129.9	29.4
8	40	2.00	4.00	1.9	54.8	5.3
9	20	5.00	4.00	2	5	11.9
10	30	3.50	7.00	10.3	128.7	25.9
11	30	3.50	7.00	9.9	127.9	30.0
12	40	5.00	10.00	5.6	63.3	21.1
13	40	5.00	4.00	0.9	0.12	10.0
14	30	3.50	7.00	10.4	126.8	31.2
15	30	3.50	12.05	3.7	21.2	30.0
16	20	2.00	4.00	1.7	53.2	7.8
17	13	3.50	7.00	3.4	55.5	8.3
18	20	2.00	10.00	4.4	5.4	12.3
19	30	0.58	7.00	6.6	63.9	9.8
20	20	5.00	10.00	5.5	58.1	10.9

Extraction of extracellular repeated extraction method
To 10g of pretreated biomass, 50ml of phosphate buffer (0.1M, pH 6.9) with 0.1% Tween -80 was added in 250ml Erlenmeyer flask. The contents were kept in the shaker for 1h at 120 rpm and then filtered through muslin cloth. The process was repeated twice with 25 ml of phosphate buffer. After filtration, contents were centrifuged at 5,400 rpm for 10 min at 4^0C. The supernatant was collected for further studies (Bollg and Edelestein, 1993) [2].

Multienzyme activity assay
Cellulase activity (Reese and Mendel, 1963) [9]
The reaction mixture contained 0.5ml of 1% of CMC in citrate buffer (0.055M, pH-5) and 0.5ml of diluted enzyme (supernatant). Reaction mixture was incubated at 50°C for 30 min. After incubation 3ml of DNSA reagent was added. Tubes were immersed in boiling water bath and removed after 15 min when color development was complete. Control was run with all the components except the enzyme. Tubes were cooled at room temperature and O.D was read at 540nm in spectrophotometer against the reagent blank i.e. 1ml of distilled water and 3ml of DNSA reagent. The standard curve was made from the stock solution of glucose (0.4 mg/ml). The enzyme activity was expressed in terms of International Unit (IU) and specific activity (SA).

Xylanase assay (Miller, 1959) [7]
Selected isolates were quantitatively assayed by growing them in Reese medium (HIMEDIA, Mumbai) at 50ºC at 120 rpm. Xylanase activity in the culture broth was assayed in triplicates. 0.2 ml of crude enzyme was mixed with 0.3 ml of citrate buffer (pH 5.0) and 0.5 ml of xylan solution (kept overnight at 37ºC in citrate buffer pH 4.0, centrifuged and clear suspension was used) and incubated at 45ºC for 10 min and then reaction was terminated by adding 3 ml of Dinitrosalicylic acid (Miller, 1959) [7]. The absorbance (Thermo electron spectrometer) was measured against the control at 540 nm, using xylose as a standard.

Pectinase activity (Miller *et al*., 1959) [7]
To 1ml of pectin was dissolved in 0.1 ml of citrate buffer (0.6M, pH-6) and 1 ml of culture supernatant was added. Reaction mixture was incubated at 35°C for 30 min. After incubation 3ml of DNSA reagent was added. Tubes were immersed in boiling water bath and removed after 15 min when colour development was complete. Control was run with all the components except the enzyme. Tubes were cooled at room temperature and O.D was read at 540nm in spectrophotometer against a reagent blank i.e. 1ml of distilled water and 3ml of DNSA reagent. The standard curve was prepared from the stock solution of glucose (0.4mg/ml). The enzyme activity was expressed in terms of International Unit (IU) and specific activity (SA).

Results and Discussion
Optimization of culture conditions by central composite design
Temperature, moisture and incubation days were chosen as the process parameters to optimize the conditions for maximum enzyme production by a statistical design (CCD and RSM). A CCD with three coded levels for all the three factors moisture (A), temperature (B) and incubation day (C). The maximum and minimum value of these parameters were depicted in Table 1. A total of 20 experiments were performed following the experimental design. The approach was chosen to preserve the significance one at a time while keeping the other variables constant.

Optimization of Cellulase production
In case of cellulase, of all the screened parameters i.e. moisture, temperature and incubation day found to have positive effect. The model for cellulase showed an F value corresponding to 29.77 indicating the significance of the model indicating the significance of the model with p-value < 0.05 (Table 3). A lower value of CV corresponding to 0.77 indicated a greater reliability of the experiment performed. The analysis showed that the form of the model chosen to explain the relationship between the factors and the response is correct. The Adj R-squared value of 0.931 was found to be very close to Pre R-squared value of 0.734 and R^2 value corresponding to 0.964 indicated that the model was appropriate and can be used for the quantitative predicated value of cellulase production. The final model equation for cellulase in terms of coded factor may be written as:
Cellulase = 0.81+0.036*A-0.012*B+0.10*C-0.26*A2-0.070*B2-0.064*C2-0.019*C2-
0.019*A*B+0.039*A*C+0.066*B*C
Where A, B and C are moisture, temperature and incubation time respectively. The respective effect of various variable is also explained by the values of coefficient estimates which is depicted in Table 3. The t test value of the significant variables indicated the greatest positive effect of incubation time.
To validate the optimum concentrations an experiment with the above specified conditions was performed and the result yield was 10.40U/gds which is quite close to the predicted value, hence validating that the model chosen for optimization was correct.

Table 3: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental designs (Cellulase)

Source of model	Sum of square	DF	Mean square	F value	Prob>F	
	2.28	9	0.25	29.77	< 0.0001	
A	7.34 E=003	1	7.349E-003	0.86	0.3744	
B	9.48 E-003	1	9.489E-003	1.12	0.374	
C	0.27	1	0.27	31.99	0.315	
A2	1.02	1	1.20	119.59	0.002	
B2	0.33	1	0.33	38.50	<0.0001	**Significant**
C2	0.98	1	0.98E-003	114.87	0.001	
AB	9.113E-003	1	7.813E-003	1.07	<0.0001	
AC	7.813E-003	1	2.812E-003	0.92	0.324	
BC	2.812E-003	1	0.035	0.33	0.360	

Std: 0.092; R^2= 0.964; Mean= 0.54; Adj R-squared= 0.931; C.V. = 0.79; Pred R-squared- 0.934; Press= 0.63; Adeq Precision= 14.933

Interactions among the factors
The graphical representative of the regression equation is presented in the form of contour plots and the 3D surface graph indicating the interactions between two factors for the

optimization of conditions for cellulase production. The contour plot and 3D graph obtained as a function of moisture and temperature concentration indicate that increase in the concentration of both induced the cellulase production. The mutual interactive effect of both the parameters predicted a maximum cellulase yield of 10.1 U/g (Figure 1 a, b, c and d).

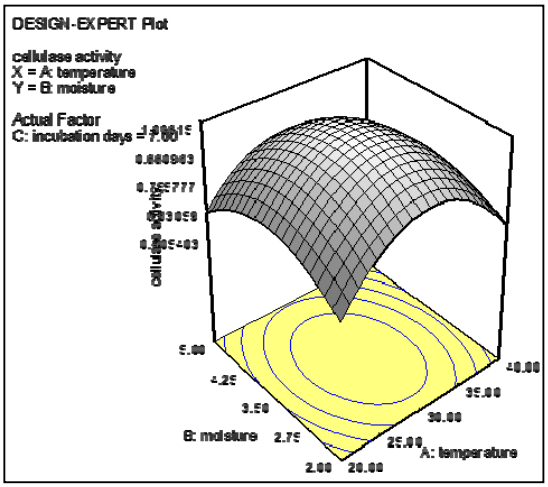

Fig 1: Effect of temperature and moisture on cellulase production by *Rhizopus* sp.

Xylanase production

The model analyzed the quadratic regression model was found to be significant. The xylanase model showed an F value corresponding to 21.93 indicating the significance of the model with p-value < 0.05 (Table 4). The lowest value of CV corresponding to 0.69 indicated a greater reliability of the experiment performed. The analysis showed that the form of the model chosen to explain the relationship between the factors and the response is correct. Further the Adj R-squared value of 0.908 was found to be very close to Pre-squared value of 0.939 and the corresponding R^2 value of 0.951 indicated that the value was appropriate and can be used for the quantitative predication of xylanase production.

Xylanase=+2.45+0.059*A+0.12*B+0.22*C-0.60*A2-
0.34*B2-0.25*C2-0.16*A*B
+0.038*A*C-0.020*B*C

Where A, B and C are moisture, temperature and incubation time. The respective effect of various variables is also explained by the value of coefficient estimates which is predicated in Table 4 When the pretreated apple pomace was microwave pretreated and incubated at variable moisture 1:3.5, temperature 30^0C and incubation time of 7 days yielded a maximum 126.80 U/gds, the value is quite close to the predicted values, hence validating that the model chosen for optimization was correct (Figure 2 a, b, c and d).

Table 4: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental designs (Xylanase)

Source of square	Sum of square	DF	Mean square	F value	Prob>F	
	363.58	9	40.40	21.93	< 0.0001	
A	0.013	1	0.013	7.173E-003	0.934	
B	8.36	1	8.36	4.54	0.059	
C	0.023	1	0.023	0.012	0.913	
A2	94.97	1	94.97	51.56	< 0.0001	
B2	135.75	1	135.75	73.70	< 0.0001	**Significant**
C2	139.85	1	139.85	75.92	< 0.0001	
AB	14.44	1	14.44	7.84	0.0188	
AC	16.44	1	16.44	8.92	0.0136	
BC	14.39	1	14.39	7.81	0.0190	

Std Dev= 1.36; R-squared= 0.9518; Mean= 6.76; Adj R squared= 0.908; CV= 0.912; Pre R squared= 0.9397;

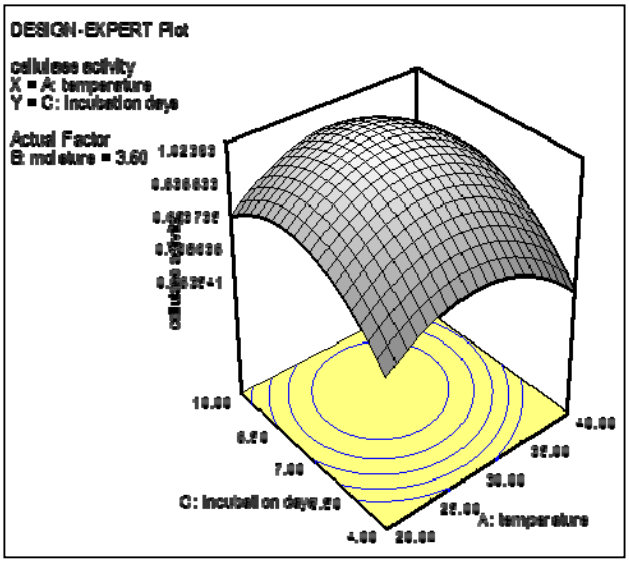

Fig 2: Effect of temperature and incubation day on cellulase production by *Rhizopus* sp.

Pectinase production

In case of pectinase production, of all the parameters moisture and incubation time exerted a positive effect. Of all the variables promoting moisture exerted the most significant effect. The pectinase model showed F value corresponding to 20.31 indicating the significance of the model with p value of <0.05 (Table 5). The lower value of CV corresponding to 0.890 indicated a greater reliability of the experiment performed. The analysis showed that the form of the model choosen to explain the relationship between the factors and the response is correct. Further the Adj R-squared value of 0.998 was found to be very close to pre R-squared value of 0.948 and the corresponding R^2 value of 0.948 indicated that the model was appropriate and can be used for the quantitative of pectinase production.

Pectinase=+12.46-.097*A-0.61*B+1.28*C-2.46*A2-
2.83*B2-0.61*C2 1.11*A*B+1.39*A*C+1.72*B*C

The respective effect of various variables is also explained by the values of the coefficient estimates which is depicted in Table 5. The t test values of the significant variables indicated the greatest positive effect. The moisture (A), temperature (B) and incubation time (C) in 450 watt microwave pretreated apple pomace supplemented with moistening agent 1:3.5 incubation for 7 days pH 5.5 incoluated with hyphae and spore indicated an increase in pectinase production with 31.20 U/gds (Fig 3a,b,c and d).

Table 5: Analysis of variance (ANOVA) for response surface quadratic model obtained from experimental designs. (Amylase)

Source of model	Sum of Square	Df	Mean Square	F value	Prob>F	
	18.15	9	2.02	20.31	< 0.0001	**Significant**
A	0.028	1	0.028	0.28	0.609	
B	0.91	1	0.91	9.14	0.012	
C	2.26	1	2.26	22.77	00.008	
A2	11.26	1	11.26	113.43	<0.0001	
B2	3.69	1	3.69	37.12	0.0001	
C2	1.75	1	1.75	17.62	0.0018	
AB	0.15	1	0.15	1.47	0.2335	
AC	0.27	1	0.27	2.68	0.1324	
BC	2.450E-003	1	2.450E-003	0.025	0.8783	

Std Dev = 0.32; R-squared= 0.948; Mean= 1.73; Adj R-squared= 0.901; CV=0.890; Pre R-squared= 0.948; Adeq Precision= 12.490

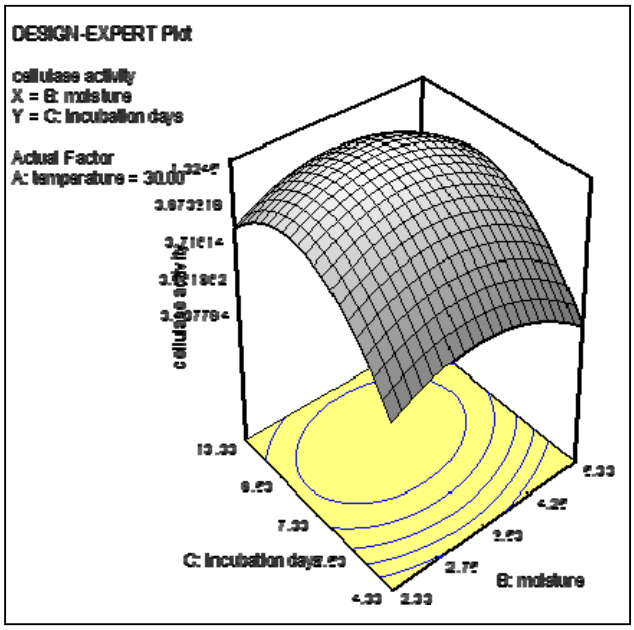

Fig 3: Effect of moisture and incubation day on cellulase production by *Rhizopus* sp.

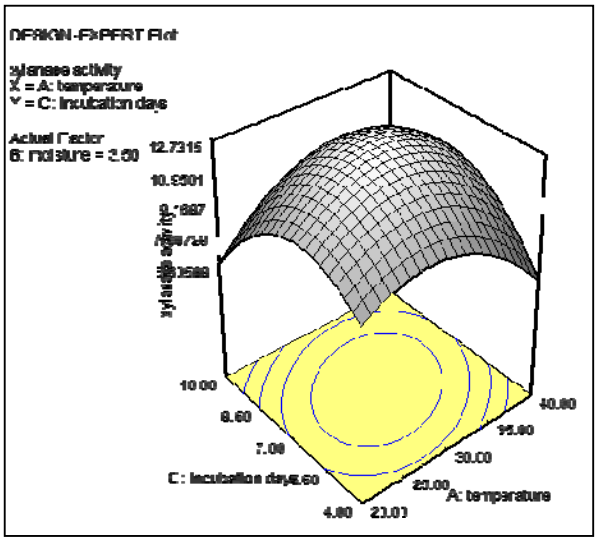

Fig 4: Effect of temperature and incubation day on xylanase production by *Rhizopus* sp.

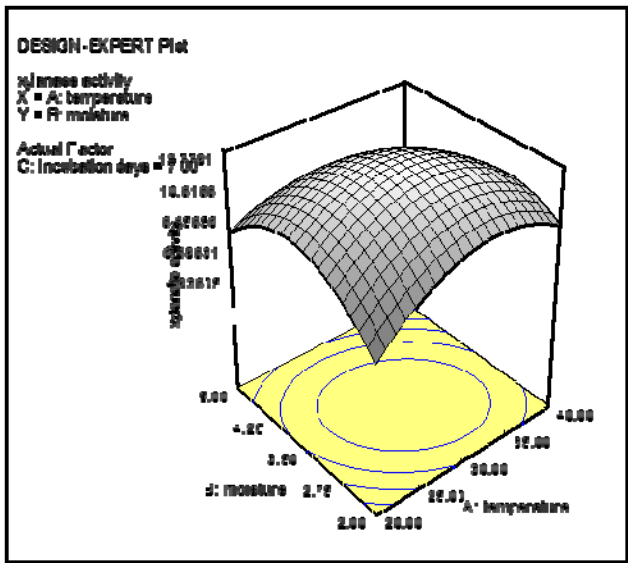

Fig 5: Effect of temperature and moisture on xylanase production by *Rhizopus* sp.

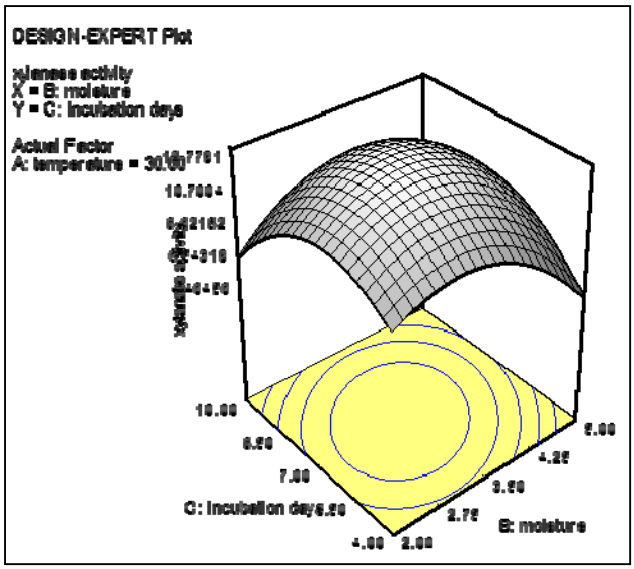

Fig 6: Effect of moisture and incubation day on xylanase production by *Rhizopus* sp.

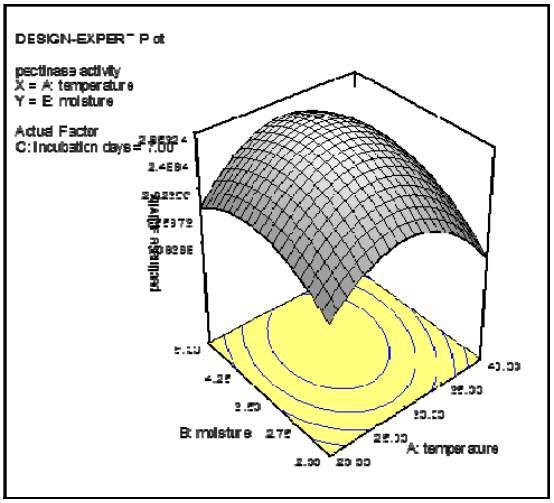

Fig 7: Effect of temperature and moisture on pectinase production by *Rhizopus* sp.

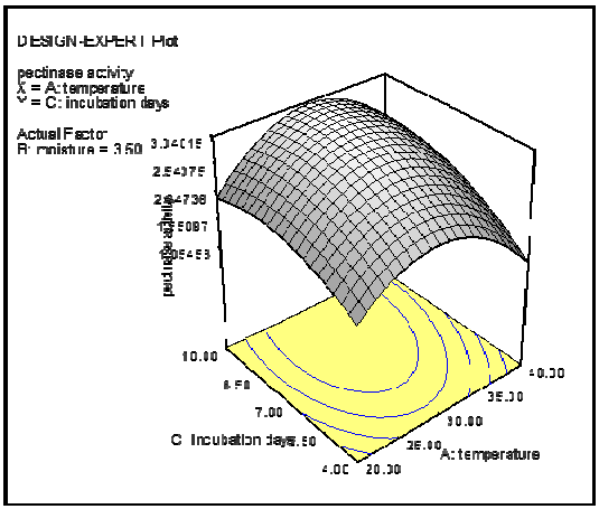

Fig 8: Effect of temperature and incubation day on pectinase production by *Rhizopus* sp.

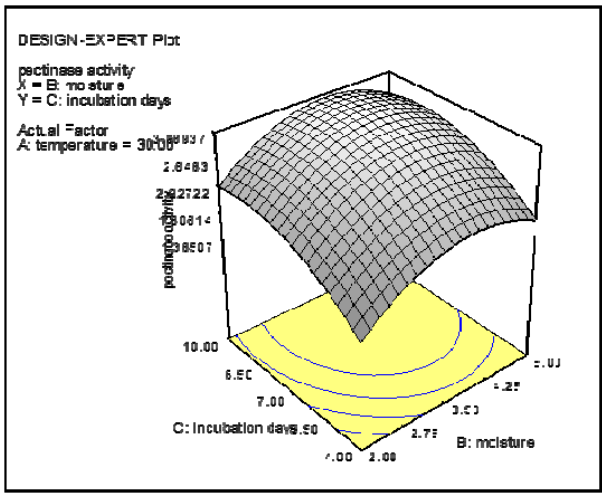

Fig 9: Effect of moisture and incubation day on pectinase production by *Rhizopus* sp.

Validation of the Experimental Model

Validation of the predicted results was accomplished by performing additional experiments in triplicate with the parameters suggested by the numerical modeling. These three sets of experiments yielded an average enzyme production of 1.0 IU/ml cellulase, 3.0 IU/ml pectinase, 13.0 IU/ml xylanase. Good agreement between the predicted and experimental results confirmed the experimental adequacy of the model and the existence of the optimal point. The response surface describing the quadratic effect of temperature. Incubation day and moisture ratio on enzymes production i.e. cellulase, xylanase and pectinase by *Rhizopus sp*. Maximum production of enzyme obtained 30°C, moisture ratio of 1:3.5 after 7 days of incubation. The effect of temperature, moisture and incubation day on enzymes production.

Chen *et al*. (2013) [3] studied the RSM to optimize the co-production of a mixture of crude cellulosic and hemicellulosic by A.terreus K1 with maximum endoglucanase, mannose, xylanase (17.37, 41.24 and 265.57 U/g respectively using palm kernel expeller (PKE) with 62.7% moisture, 6% inoculums, pH 5.8. *Serratia marcescens* SB08 used the response surface methodology were pH 6.0, agitation 100rpm, incubation time 51.0h and yeast extract 3.0g/l with maximum production of 281.23 U/ml (predicted response 275.66 U/ml) by Venil and Lakshmanaperumalsamy (2009) [11].

Acknowledgment

The funds received from DBT, New Delhi, India to carry out this work is acknowledged with thanks.

Conclusion

A mixture of celluloytic, hemicelluloytic and pectinolytic enzymes could be produced by *Rhizopus delemar* F2 in solid state fermentation using pretreated apple pomace as a substrate. The enzyme cocktail could efficiency hydrolyze waste rich in cellulose, hemicelluloses and pectin into efficient enzymes. *Rhizopus delemar* F2 is a promising organism for production of different consortium of enzymes and statistical design like RSM can be used for optimization of process parameters and their interactions. RSM and CCD permitted studying and exploring fermentation conditions for the production of enzymes in just 20 experimental runs with overall increase in enzymes production. The optimum fermentation conditions for the production of enzymes were established and the corresponding values of variables were: temperature 30ºC, moisture ratio 1:3.5 and incubation day of 7 days. The potentials of using fungi as biotechnological sources of industrially relevant enzymes has stimulated interest in the exploration of extracellular enzyme activity. The utilization of implicates an easy, already available and cheap biomass thus can be done for production of enzymes production. Therefore utilizing these unutilized waste lying resulting in deterioration of environment and loss of potentially valuable resources.

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