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Statistical optimization of FPase production from water hyacinth using *Rhizopus oryzae* PR 7

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Abstact

Pretreated water hyacinth was used as sole carbon source for the production of cellulase enzyme by *Rhizopus oryzae* PR 7 MTCC 9642 in both submerged fermentation (SmF) and solid state fermentation (SSF) that was measured by the FPase activity. To maximize the FPase production, the critical parameters like substrate concentration, cultivation temperature and pH on enzyme production were optimized using response surface methodology using Central Composite Design (CCD). The SmF was found to be better than SSF for the production of FPase. The best preferred combination for highest FPase activity from SmF was with substrate concentration 1.25%,pH 7.32 and temperature 25.25°C. Estimated optimum conditions for FPase production from SSF was a combination of substrate concentration of 0.5%, pH 6, temperature 18°C. Under the optimized cultivation condition, the strain synthesized 123 U/ml and 48U/ml FPase from SmF and SSF respectively and the highest production was achieved within only 48 hours of cultivation.

Key words: Central composite design, FPase, Water hyacinth, *Rhizopus oryzae*

Introduction

Bioconversion of cellulose to fermentable sugars and bioalcohols could be accomplished by microbial cellulases ,a multi component enzyme complex consisting of 3 types of enzymes namely endoglucanase, cellobiohydrolase and β-glucosidase (Bhat 2000).

A generally accepted practical measure of the cellulose hydrolyzing capacity of microbial cellulase preparations is the filter paper activity (FPA) (Ghose 1987) which measures not individual enzyme activities, but the overall activity of multi component enzyme complexes for cellulose hydrolysis (Urbanszki et al. 2000).Although a number of reports are available on cellulases , precisely on exo and endoglucanases and β-glucosidases, FPase was considered as a side product and very few reports are available exclusively on FP ase production.

Although cellulases have a number of applications (Karmakar and Ray 2011), the high cost of production of these enzymes has hindered the industrial application of cellulose bioconversion (Narasimha *et al*. 2006).Hence a reduction in cellulase production cost, an improvement in cellulase performance and an increase in sugar yields are all vital to reduce the processing cost in biorefineries.(Percival *et al*, 2006).Utilization of plant biomass and agricultural wastes can effectively replace the costly soluble pure substrates with successful solution of garbage disposal problem. Aquatic weed water hyacinth (*Eichornia crassipes*), causing several hazards in pond ecosystem, was evaluated for its potential to be used as feedstock for fermentable sugar production via enzymatic hydrolysis (Mukhopadhyay *et al.* 2008) and could be used as sole carbon source for microbial cellulase production.

For quantitative cellulase assay using water hyacinth, some problems may be encountered as heterogeneity of insoluble cellulose , unclear dynamic interactions between insoluble substrate and cellulase components, and the complex competitive and/or synergistic relationship among cellulase components limit rational design and/or strategies (Percival et al, 2006).Therefore, measurement of total cellulase by FPase assay appears to be more significant than determination of individual component enzymes.

As statistical optimization of factors affecting enzymatic hydrolysis would definitely enhance the enzyme production and response surface methodology was proved to be a more cost and time saving method than classical one-at-a-time or mathematical methods (Siva Kiran *et al.*2010), in the present paper attempts were made to describe the optimization of FP ase production from submerged fermentation (SmF) and solid state fermentation (SSF) of water hyacinth by *Rhizopus oryzae*.

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Materials and Methods

The microorganism used in the present study was *Rhizopus oryzae* PR7 MTCC 9642 (Karmakar and Ray 2010), which was isolated from the decaying vegetation enriched soil of West Bengal, India. The strain was deposited to Microbial Type Culture Collection, India. Water hyacinth *(Eichornia crassipes)* was used as the sole carbon source in the cultivation media. Its foliage was collected, oven dried at 60°C for 12 hours , pulverized and pretreated with steam explosion by double autoclaving before using in the culture media. The strain, for submerged fermentation was cultivated in 100 ml Erlenmeyer flasks each containing 20 ml Basal Medium (BM) composed of (gl^{-1}): peptone 0.9; $(NH_4)_2HPO_4$ 0.4; KCl 0.1;$MgSO_4.H_2O$ 0.1 and pretreated water hyacinth of variable concentration, at variable pH and at variable temperature for 48 hrs in static condition. For solid state fermentation, the strain was cultured similarly with totally dried water hyacinth dust and salts (based on 10 ml medium) moistened with 0.5 ml distilled water. The grown culture was centrifuged at 10,000 rpm for 5 min at 4°C and the supernatant was used as the crude enzyme. To measure the activity of FPase, the assay mixture (1ml) containing equal amount of enzyme diluted with 0.1(M) phosphate buffer (pH-6) and 1 %(w/v) Whatman No1 filter paper strips was incubated at 33°C for 30 minutes. The reducing sugar released was measured by the dinitrosalicylic acid method (Bernfeld 1955) taking glucose as standard. Blanks were prepared with inactivated enzymes. One unit of FPase was defined as that amount of enzyme that liberated 1µ mole of glucose per ml per minute of reaction. An evaluation copy of the statistical software, Design-Expert version 7.1.6, from Stat-Ease, Inc., Minneapolis, USA was used for analysis of experimental data and to plot response surface. ANOVA was used to estimate the statistical parameters. The response surface methodology (RSM) is used to determine the optimum operational conditions for the process. A regression model containing 3 linear ($\beta_1, \beta_2, \beta_3$), 3 quadratic ($\beta_{11}, \beta_{22}, \beta_{33}$), 3 interaction ($\beta_{12}, \beta_{23}, \beta_{13}$) and β_0, intercept term is used. The overall second order polynomial mathematical relationship of the response Y and the three variables, i.e. A denoted as substrate concentration (% w/v), B was the pH and C was the temperature (°C) could be approximated by the quadratic Eq. (1).

$$Y=\beta_0+\beta_1A+\beta_2B+\beta_3C+\beta_{11}A^2+\beta_{22}B^2+\beta_{33}C^2+\beta_{12}AB+\beta_{23}BC+\beta_{13}AC \quad \text{Eqn 1}$$

Each set of experiment was performed in triplicate and their values averaged.

Results and Discussion

Detection of the substrate concentration, effects of temp and pH for optimum enzyme production by RSM

To detect the effect of three major key factors responsible for enzyme production, each factor in the design was studied at five different levels ($-\alpha, -1, 0, +1, +\alpha$) (Table 1) by central composite design. To detect the effect of three major key factors responsible for enzyme production, each factor in the design was studied at five different levels ($-\alpha, -1, 0, +1, +\alpha$) (Table 1) by central composite design. Total twenty experiments were designed by the model and performed (Table 2). Each of the flasks were triplicated and average value is taken.

Table 1. The central composite design for optimizing the parameters in FP ase production by *Rhizopus oryzae*

Independent variables	Symbols	Code levels			
		-1	+1	- α	+ α
Substrate conc (%)	A	0.5	1.25	0.244328	1.50567
pH	B	6	9	4.97731	10.0227
Temp(°C)	C	18	40	10.5003	47.4997

Optimization of parameters in case of submerged fermentation

On the basis of quadratic polynomial equation of response surface model (Eq. (1)), the present model and data analysis allowed not only to define optimum culture conditions in submerged fermentation process for FPase activity but also showed combined effect of independent variables such as substrate concentration, pH and temperature on the FPase activity in Eq. (2).

FPase production from SmF (X1) = +88.69+33.31*A-7.82*B-1.54*C+5.38*A*B-4.11*A*C+3.48*B*C+6.24*A^2-15.24*B^2-8.86*C^2 Eqn (2)

where A,B and C denoted substrate concentration(w/v), pH,and the temperature (° C) respectively.

Table 2.Experimental design of central composite design for three factors and experimental values for production of FP ase in both liquid and solid state fermentations of water hyacinth by *R.oryzae*

Runs	Substrate Conc. (A)	pH (B)	Temp (C)	FP ase (IU/ml)	
				SmF (X1)	SSF (X2)
1	0.5	6	18	48.108	50
2	1.25	6	18	113.94	25
3	0.5	9	18	18.99	33.32
4	1.25	9	18	101.28	20.82
5	0.5	6	40	50.64	41.65
6	1.25	6	40	94.95	33.32
7	0.5	9	40	30.384	29.15
8	1.25	9	40	101.28	29.15
9	0.24	7.5	29	50.64	41.65
10	1.5	7.5	29	164.58	25
11	0.87	4.9	29	62.034	41.65
12	0.87	10	29	31.65	16.66
13	0.87	7.5	10.5	69.63	29.15
14	0.87	7.5	47.49	60.135	29.15
15	0.87	7.5	29	88.62	25
16	0.87	7.5	29	88.62	25
17	0.87	7.5	29	88.62	25
18	0.87	7.5	29	88.62	25
19	0.87	7.5	29	88.62	25
20	0.87	7.5	29	88.62	25

The high value 33.31 for linear coefficient of substrate concentration (Eqn 2) illustrates the positive effect of the variable on the FPase production. Linear positive coefficient indicates the increase in enzyme activity with the increase of substrate concentration. The negative linear coefficient of pH and temperature indicates an increase in FPase activity with the rise of these variables initially in a ranged value. In order to determine the optimum levels of each of the variables (substrate conc., pH, temperature) for maximum FPase production.Contour plots were constructed by plotting the response. The response surface plots were constructed with varying the two independent variables keeping the third variable fixed.

Table 3: Analysis of variance (ANOVA) for quadratic model for FPase activity in case of submerged fermentation.

Source	Sum of Squares	Df	Mean Square	F Value	p-value Prob > F
Model	21661.41	9	2406.824	389.7046	< 0.0001
A	15155.82	1	15155.82	2453.978	< 0.0001
B	835.2588	1	835.2588	135.2422	< 0.0001
C	32.39188	1	32.39188	5.244782	0.0450
AB	231.5982	1	231.5982	37.49959	0.0001
AC	135.4329	1	135.4329	21.92883	0.0009
BC	96.96674	1	96.96674	15.70052	0.0027
A^2	561.9702	1	561.9702	90.99228	< 0.0001
B^2	3347.186	1	3347.186	541.9649	< 0.0001
C^2	1131.752	1	1131.752	183.2493	< 0.0001
Residual	61.76021	10	6.176021		
Lack of Fit	61.76021	5	12.35204		
Pure Error	0	5	0		
Cor Total	21723.17	19			

R2 =0.9972, Adj R2 =0.9946, Pred R2 =0.9775, C.V. =3.25%.

Fisher's statistical test has been done for analysis of variance (ANOVA).The results of ANOVA are given in Table3. The model F-value is as high as 389.70 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise.Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, B, C, AB, AC, BC, A^2, B^2, C^2 are significant model terms. The value of correlation coefficient is 0.9972 (closer the R2 value to 1.0 the better model fitness to experimental values) which indicated that the model can explain 99.9 % of variability and was unable to explain only 0.01% .A very small value of coefficient of variation 3.25% clearly indicated a very high degree of precision and a good reliability of the experimental values.

When certain pH and temperature are applied on the enzyme production, it is necessary to consider both pH, thermal stability. The 0.0001 significant value (Prob > F) for main, interaction and quadratic effects pointed out the strong effect of these variables, and have the most influence on the FPase production in submerged condition. The contour diagrams clearly indicated that the variables have optimal setting (Fig 1,2,3).

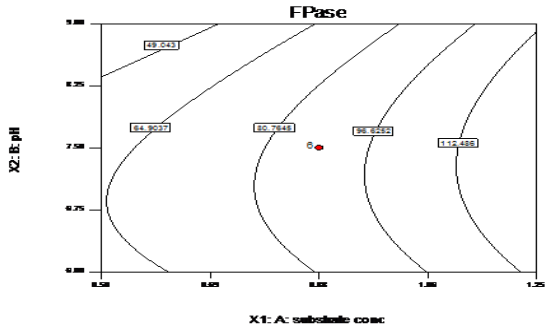

Fig 1. Contour plots showing the effect of substrate concentration and pH on FP ase production in submerged fermentation with other variable constant at middle level

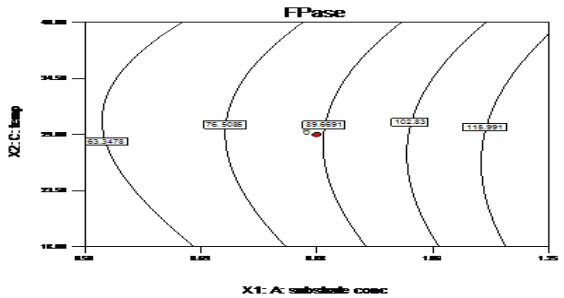

Fig 2: Contour plots showing the effect of temperature and substrate concentration on FP ase production in submerged fermentation with other variable constant at middle level

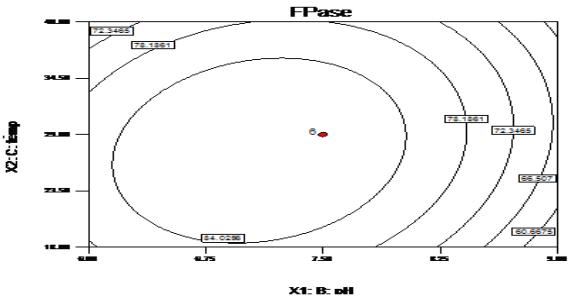

Fig 3: Contour plots showing the effect of temperature and pH on FP ase production in submerged fermentation with other variable constant at middle level

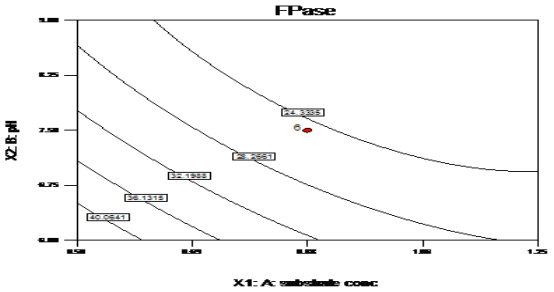

Fig 4: Contour plots showing the effect of substrate concentration and pH on FP ase production in SSF with other variable constant at middle level

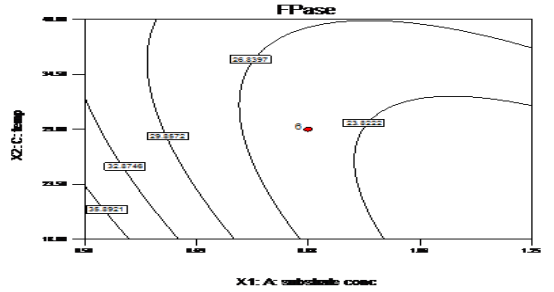

Fig 5: Contour plots showing the effect of temperature and substrate concentration on FP ase production in SSF with other variable constant at middle level

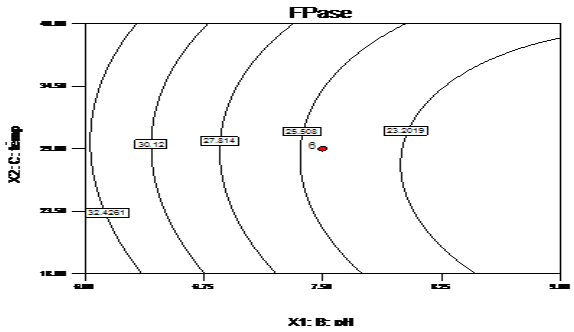

Fig 6: Contour plots showing the effect of temperature and pH on FP ase production in SSF with other variable

Optimization of parameters in case of solid state fermentation by CCD (mathematical analysis):

The response (FPase in SSF) of the CCD design were fitted with a quadratic equation.

FPase production from SSF (X2) = +24.94-5.41*A -5.83*B +0.30*C+2.60*A*B+3.65*A*C+0.52*B*C+3.35*A2+1.87*B2+1.87*C2 …….Eqn(3)

where A,B and C denoted substrate concentration(w/v), pH,and the temperature (° C) respectively.

Table 4 :Analysis of variance (ANOVA) for quadratic model for FPase activity in case of solid state fermentation.

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	1251.686	9	139.0762	33.34583	< 0.0001
A	399.1506	1	399.1506	95.70302	< 0.0001
B	463.4651	1	463.4651	111.1235	< 0.0001
C	1.248963	1	1.248963	0.29946	0.5962
AB	54.23611	1	54.23611	13.00401	0.0048
AC	106.3611	1	106.3611	25.50185	0.0005
BC	2.194513	1	2.194513	0.526171	0.4848
A^2	161.6195	1	161.6195	38.75097	< 0.0001
B^2	50.63931	1	50.63931	12.14162	0.0059
C^2	50.54384	1	50.54384	12.11873	0.0059
Residual	41.70721	10	4.170721		
Lack of Fit	41.70721	5	8.341443		
Pure Error	0	5	0		
Cor Total	1293.393	19			

R2 =0.9678, Adj R2 =0.9387, Pred R2 =0.7528, C.V. =6.86%.

The second-order model was hypothesized and statistically evaluated by analysis of variance (ANOVA). ANOVA result showed that the values of R^2 for the response were in reasonable agreement with the adjusted R^2. Normally, a regression model having an R2 value higher than 0.9 is considered to have a very high correlation .The closer the value of R (correlation coefficient) to 1, the better the correlation between the experimental and predicted values. Here, the value of R^2 (0.967) for Eqn.(3) indicates a close agreement between the experimental results and the theoretical values predicted by the model equation This ensured a satisfactory adjustment of a quadratic model to the experimental data. Values of ''prob > F" obtained was <0.001, indicating that the mathematical model terms A, B, AB, AC, A2, B2, C2 generated were highly significant. The Model F-value of 33.35 implies the model is significant. There is only a 0.01% chance that a "Model F-Value" this large could occur due to noise. The mathematical models' signal to noise ratio was well in control, as assessed by the values of adequate precision (21.331), which were quite higher than the standard value of 4 (Design expert guide). Therefore, this model can be used to navigate the design space. 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 the reliability of experiment is. Here, a lower value of CV (6.86%) indicated a better precision and reliability of the experiments.

Validation of the model (biological experiments)

The highest predicted FPase production from submerged condition was 129.363 U/ml at substrate concentration 1.25%, pH 7.32 and at temperature 25.25° C. Similarly in SSF, the highest FPase production of 49.73 U/ml was achieved at substrate concentration 0.5%,pH 6 and temperature 18°C.

A repeat fermentation of water hyacinth for highest production of FPase by Rhizopus oryzae MTCC 9642 under optimized conditions was carried out for verification, which was found to be 123 U/ml in submerged fermentation, about 4.6% less than the predicted value. In case of SSF, maximum FPase production was 48 U/ml which was only 3.4% less than the predicted value. These discrepancies were probably due to the slight variation in experimental conditions. After optimization the production of FPase was increased 6.4 fold and 1.44 fold from SmF and SSF respectively as compared with the lowest cocktail of enzyme production at run 3 in Central composite design (Table 3).

Conclusion

The strain was found to prefer submerged fermentation than solid state fermentation which might appear unusual, as in most of the reports available, SSF was proved to be a better producer of enzyme than SmF. But, in the present case lower productivity in SSF was presumed to be a result of relatively smaller inoculum size, limitation in aeration and immiscibility with consequent non uniform availability of carbon source, i.e. water hyacinth. Similar observation was found by Karmakar and Ray, 2010, Vintilla, 2008. However the present work gave an overall comparative account on the production of FPase both from SSF and SmF under certain fixed conditions and after optimization, the strain was found to be a better FPase producing one than already reported T.reesei and P.chrysosporium (Deshpande et al 2008) growing on water hyacinth rich cultivation media.

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