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A N   I N T E R N A T I O N A L   J O U R N A L

Multivariable Parameter Optimization for the Endoglucanase Production by *Trichoderma reesei* Rut C30 from *Ocimum gratissimum* seed

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ABSTRACT

The aim of this study was to evaluate the interaction effects of the physico-chemical parameters on the endoglucanase (CMCase) production by Trichoderma reesei Rut C30 *on a cellulosic agro-residue by the solid-state fermentation (SSF) and to determine their optimum values by the EVOP factorial design technique. The best combination of physical parameters for the maximum production of the endoglucanase (CMCase) was 28^oC temperature, 79% relative humidity and 4.8 pH of the medium. The best combination of the chemical parameters was (mg/L) nicotinic acid 15, naphthalene acetic acid 7, ferric chloride 5 and Tween-80 6. With the application of this technique, the yield of the CMCase increased by ~ 2.3 fold.*

Key words: Enzyme, endoglucanase, carboxymethylcellulase (CMCase), EVOP factorial design technique

INTRODUCTION

Cellulases have attracted much interest because of the diversity of their application. Since the cost of production is the major constraint in the cellulase production, much attention is drawn on the availability of low cost carbon source and on effective fermentation system. Fungal cellulases are produced in large amounts, which include all the components of multi enzyme system acting in a synergism for the complete hydrolysis of the cellulose. *Trichoderma reesei* Rut C30 is known to be one of the best cellulolytic fungal strain, which makes it an ideal test organism for the cellulase production. The organism produces CMCase in major level than the other two enzymes, exo-β-glucanase and β-glucosidase (Chahal et al*.,* 1982; Juhász et al*.,* 2004). Both the liquid and solid - state fermentations have been tried for the cellulase production by *Trichoderma* sp*.* (Esterbauer et al*.,* 1991; Kubicek, 1992; Montenecourt, 1983). Wheat bran has been extensively used in SSF (Jabbar and Ilahi, 1981). Reports on the production of the cellulase by *Trichoderma* sp. in SSF (Chahal, 1985; Chahal, 1986; Chahal, 1991; Kim et al*.,* 1985; Sim et al*.,* 1990) are numerous because different strains and mutants, culture conditions and substrates have been used under different studies. In the present study, the fungus was grown on a novel substrate (mixture of sawdust and seeds of *Ocimum gratissimum,*) and through optimization of the physicochemical parameters of the solid-state fermentation process, enhanced yield of CMCase was expected.

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In the traditional single variable search technique, the probable interaction among the factors gets ignored, and thus, the true optimum condition may not be determined. The EVOP factorial design technique has been reported as useful technique to study bioprocesses. (Banerjee and Bhattacharyya, 1993; Tunga et al., 1999).

In the EVOP, the tool for optimizing the multivariable parameters, the decision-making procedure is easy and comprehensive. Moreover, it predicts the changes of variables towards the objective maximum and minimum values. The aim of the present investigation was to study the impact of three physical and four chemical factors on maximizing the CMCase yield.

MATERIALS AND METHODS

Microorganism

Trichoderma reesei Rut C30 was maintained on PDA slants at 28^oC and subcultured once in two weeks.

Raw Materials and Chemicals

Sawdust from Sal wood (*Shorea robusta* L.*)* and dry intact seeds of *Ocimum gratissimum* L. were processed locally. All chemicals used were of analytical grade and were obtained from the s.d. fine chemicals, Merk, SRL, India.

Medium and Culture Conditions

The medium used for the growth of the fungal culture of the organism contained (g/L) KH_2PO_4 2, $(NH_4)_2SO_4$ 1.4, $MgSO_4.7H_2O$ 0.3, $CO(NH_2)_2$ 0.2, peptone 0.2, yeast extract 0.2, lactose 1 and $NaNO_3$ 1. The sawdust (1g) and seed (2g) were mixed with the liquid medium in 1:3 ratio in 250ml Erlenmeyer flasks and autoclaved at 121^oC for 20 min. The mixed substrate was inoculated with 2ml spore (conidia) suspension of the fungal culture containing ~ 3.6×10^6 spores/ml.

Fermentation was carried out for six days. Extraction of enzyme from the fermented biomass was carried out by soaking 3g of biomass with 3ml of 5% glycerol for 2h at room temperature.

CMCase assay was done using 0.5ml of 1% Na-CMC (carboxymethyl cellulose) as substrate and 0.25ml of 0.4M Glycine-HCl (pH 4) as buffer. Activity was measured by adding 0.25ml of diluted enzyme incubated for 10min at 50°C.

Enzyme activities (IU) were expressed as 1μmole of glucose equivalents released per milliliter per minute. The reducing sugar was measured with the dinitrosalicylic acid reagent (Miller, 1959). Total protein was estimated using bovine serum albumin as described by (Lowry *et.al*, 1951).

Optimization of variables by the EVOP based on factorial technique

The proposed methodology combines the advantage of factorial technique for designing experiments with three and four parameters separately and that of EVOP methodology for the systematic analysis of experimental results, and facilitates the selection of optimum conditions.

Apart from the control, the total number of new experiments to be conducted is 2^3 for the study of 3 variable systems and 2^4 for the study of 4 variable systems. The parameters for the above experiments are kept in both the higher level (+) and lower level (-) compared to the search level (control) region (0). The total number of experiments in 3 variable parameters is represented in a matrix of $[n \times (2^n +2)]$ i.e., 30 and the total number of experiments in a 4 variable parameters is represented in a matrix of 72. Matrices are divided into two blocks (block I and block II). Each of them is having one set of control experiment (search level). Each new experiment in block I has an odd number of lower level (-) parameter (s), such as 1, 3, 5 and block II should have an even number of lower level (-) parameter (s), such as 2, 4. When block I exhibits lower level (-) responses, block II exhibits higher level (+) responses compared to those of control experiments.

All the experiments were conducted two times (cycle I and cycle II) to minimize the standard deviation and error limits.

The 'n' variable system can have $^nc_1 = n$ numbers zero order interaction, $^nc_2 = n (n-1)/2!$ numbers first order interaction, $^nc_3 = n (n-1)(n-2)/3!$ numbers second order interaction, $^nc_4 = n (n-1)(n-2)(n-3)/4!$ numbers third order interaction and so on.

So the total number of effects can be expressed as $[^nc_1 + {}^nc_2 + {}^nc_3 + \ldots\ldots {}^nc_n]$ + the change in mean effect.

$$= \sum_{i=1}^{n} {}^nC_1 + \text{the change in mean effect}$$

The calculation of the effect can be made based on the well-known theory of fractional factorials (Adler et al., 1975; Davis, 1954).

All possible interaction level for 'n' variable system was derived by the multiplication of the

individual level. Hence, any effect can be estimated from the generalized form as shown in the equation-1

$$E_1 = 1/n´ \left[\sum_{i=1}^{n´} \text{(Higher interaction level effects)} - \left(\sum \text{Lower interaction level effects} \right) \right] \quad i = 1$$

and the change in mean effects of all the experimental conditions can be expressed in generalized form as shown in equation ---2

$$E_2 = 1/2 \left[1/m \left(\sum_{i=2}^{m} a_i - ma_1 \right) + 1/m \left(\sum_{j=m+2}^{2m} a_j - ma_{m+1} \right) \right]$$

Where,
E_1 --- effect
E_2 --- change in mean effects
$n´$ --- No. of higher or lower interaction level effects
m --- No. of experiments in Block – I or Block- II

The standard deviation and error limits based on a 95% confidence level are estimated from the differences according to the relationship given in the literature (Box *et.al*, 1959; Pearson et.al, 1962) and are calculated as given below:
σ (standard deviation) $= 1/2 (\sigma_1 + \sigma_2)$,
where, $\sigma_1 = R_1 f$ and $\sigma_2 = R_2 f$;
R_1 = (largest difference – smallest difference) in Block – I
R_2 = (largest difference – smallest difference) in Block – II

f (statistical constant)$= 0.3$ for number of cycles (n") = 2 and number of new experiments per cycle (k) = 32.

Error limits:
For average: $\pm (2/\sqrt{n"}) \sigma$,

For effects: $\pm (0.71 \times 2/\sqrt{n"}) \sigma$,

For change in mean: $\pm (0.63 \times 2/\sqrt{n"}) \sigma$.

After the calculation of change in mean effects and error limits, it is necessary to examine whether any change in the control (search level) experimental condition will help to improve the objective function (i.e., response) and if so, which is the desired direction of change (Banerjee and Bhattacharyya, 1993). For that the magnitudes of the effects are compared with that of the error limits. If all or any of the effects are larger than the error limits, the change in the experimental conditions may yield better results. The decision on the desired direction of change of a variable has been described in the literature (Tunga et al., 1999).

In the present work, the control experimental conditions were selected based on the results of the effect of the individual parameters on the cellulase production by the SSF. Then, experimental conditions were selected with the lower and higher levels of the parameters compared to the control or search level. The fermentation was carried out at these levels of parameters and all the experiments were repeated for two cycles. CMCase yield was estimated following the procedure described earlier. Differences in the cellulase yield between the cycles I, and II and the average cellulase yield were calculated to estimate the effects and error limits. The magnitude of the effects, error limits and change in mean effect was examined as per the decision-making procedure to arrive at the optimum.

RESULTS AND DISCUSSION

Optimization of Physical Parameters

Table 1 shows the experimental conditions for the first set of optimization of three physical parameters and the corresponding cellulase yields. Calculation of the effects resulted with temperature (T, -4.82), pH (P, -2.94), relative humidity (R, +2.66), (TP, -1.00), (TR, +2.22) and (PR, -0.23). Analysis of the results showed that most of the effects were smaller than the error limits, for the averages (± 4.45), for the effects (± 3.17) and for the change in the mean (± 2.80); the change in the mean effect was negative and large (-7.16), which was a confirmation of achieving optimum condition. Moreover, the effect of the temperature (T, -4.82), which was higher, was not much large in comparison to the error limits. Hence, as per the decision making rule, maximum CMCase yield was achieved with the search region A_1 and A_6, which was the optimum physical condition for the maximum yield of the CMCase.

Optimization of Chemical Parameters Table 2 shows the experimental conditions for the first set of optimization and the corresponding CMCase yields. Results given in Tables 4 and 5 showed that half of the effects were smaller than the error limits and the change in the mean effect was positive and but not large in comparison to the error limits. Magnitudes of most of the effects were still higher and the change in the mean effect was positive but not large. Moreover, the CMCase yield in set A_{18} was highest, which contained all the parameters at their higher level (+). Thus, it didn't ensure that the condition at the search region A_1 and A_{10} of set-I was the real optimum condition and a second set of experiments was required with the higher search level of A_{18} as control level.

The experimental conditions and results of set-II are presented in Table 3. The results given in Table 4 and 5, showed that the change in mean effect (- 32.37) was large and negative and most of the effects were also less than the error limits, for the averages (± 10.77), for the effects (± 7.66) and for change in the mean (± 6.80). The four variable interaction effects were very small compared to error limits.

Table 1 - Results of optimization of three physical parameters

Experimental Set up	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}
Temperature ^{0}C	28	26	30	30	26	28	30	26	26	30
pH	4.8	4.5	5.1	4.5	5.1	4.8	5.1	4.5	5.1	4.5
Relative Humidity %	78	76	76	80	80	78	80	80	76	76
CMCase Activity (IU/gds) Cycle-I	74.86	66.6	57.22	67.79	67	74.6	66.86	67.92	60.79	68.71
CMCase Activity (IU/gds) Cycle-II	70.5	64.55	63.36	61.58	70.04	76.6	60.78	70.23	63.69	65.94
Difference in Activity	+4.36	+2.05	-6.14	+6.21	-3.04	-2	+6.08	-2.31	-2.9	+2.77
Average	72.68	65.57	60.29	64.68	68.52	75.6	63.82	69.07	62.24	67.32

[gds- gram dry substrate]   Block- I   Block- II

Table 2 - Results of optimization of four chemical parameters Set-I

Experimental Parameters	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}
FeCl$_3$ (mg/l)	3	5	1	1	1	5	5	1	5	3
Tween-80 (ml/l)	4	2	6	2	2	6	2	6	6	4
Nicotinic acid (mg/l)	10	5	5	15	5	15	15	15	5	10
Napthalene acetic acid (mg/l)	5	3	3	3	7	3	7	7	7	5
CMCase Activity (IU/gds) Cycle-I	112.32	105.72	93.83	118.93	126.86	122.89	105.72	132.15	118.93	113.65
CMCase Activity (IU/gds) Cycle-II	108.36	103.74	105.06	121.57	130.16	118.93	100.43	126.86	122.89	105.72
Difference in Activity	+3.96	+1.98	-11.23	-2.64	-3.3	+3.96	+5.28	+5.28	-3.96	+7.93
Average	109.68	105.72	99.11	120.25	128.84	120.91	103.07	129.50	120.91	109.68

Con. Table 2

Experimental Parameters	A_{11}	A_{12}	A_{13}	A_{14}	A_{15}	A_{16}	A_{17}	A_{18}
FeCl$_3$ (mg/l)	1	5	5	5	1	1	1	5
Tween-80 (ml/l)	2	6	2	2	6	6	2	6
Nicotinic acid (mg/l)	5	5	15	5	15	5	15	15
Napthalene acetic acid (mg/l)	3	3	3	7	3	7	7	7
CMCase Activity (IU/gds) Cycle-I	97.13	104.39	131.49	123.56	114.31	129.5	116.29	152.63
CMCase Activity (IU/gds) Cycle-II	94.48	107.04	122.24	118.27	123.55	124.22	120.91	156.59
Difference in Activity	+3.3	-2.64	+9.25	+5.28	+9.25	+5.28	-4.62	-3.96
Average	95.8	105.72	126.86	120.91	118.93	126.86	118.27	154.65

[gds- gram dry substrate]   ⟶   Block- I   ⟵   ⟶   Block- II

Table 3 - Results of optimization of four chemical parameters Set-II

Experimental Parameters	A_1	A_2	A_3	A_4	A_5	A_6	A_7	A_8	A_9	A_{10}
FeCl$_3$ (mg/l)	5	7	3	3	3	7	7	3	7	5
Tween-80 (ml/l)	6	4	8	4	4	8	4	8	8	6
Nicotinic acid (mg/l)	15	10	10	20	10	20	20	20	10	15
Napthalene acetic acid (mg/l)	7	5	5	5	9	5	9	9	9	7
CMCase Activity (IU/gds) Cycle-I	175.09	149.33	146.69	147.34	152.63	144.7	148.66	136.77	128.84	176.42
CMCase Activity (IU/gds) Cycle-II	173.77	154.61	151.97	145.36	140.74	136.11	141.4	125.54	130.16	175.76
Difference in Activity	+1.32	-5.28	-5.28	+1.98	+11.89	+8.59	+7.26	+11.23	-1.32	-0.66
Average	174.43	151.97	149.33	146.02	146.69	140.08	144.7	130.82	129.5	175.76

Con. Table 3

Experimental Parameters	A_{11}	A_{12}	A_{13}	A_{14}	A_{15}	A_{16}	A_{17}	A_{18}
FeCl$_3$ (mg/l)	3	7	7	7	3	3	3	7
Tween-80 (ml/l)	4	8	4	4	8	8	4	8
Nicotinic acid (mg/l)	10	10	20	10	20	10	20	20
Napthalene acetic acid (mg/l)	5	5	5	9	5	9	9	9
CMCase Activity (IU/gds) Cycle-I	146.68	128.19	141.89	146.68	145.36	147.35	146.02	145.36
CMCase Activity (IU/gds) Cycle-II	155.93	132.15	130.16	134.79	146.68	153.29	139.42	135.45
Difference in Activity	-9.25	-3.96	+11.23	+11.89	-1.32	-5.94	+6.60	+9.91
Average	151.31	130.16	135.45	140.74	146.02	149.99	142.72	140.08

[gds- gram dry substrate]   ⟶   Block- I   ⟵   ⟶   Block- II   ⟵

Table 4 - Calculation of effects of four inducer system: Metal ion, $FeCl_3$ (M); Surfactant, Tween-80 (S); Vitamin, Nicotinic acid (V); Hormone, Napthalene acetic acid (H)

Parameters	Set-I	Set-II
M	+2.643	-6.211
S	+7.268	-5.352
V	+11.232	-2.709
H	+13.215	-3.039
MS	+3.964	-2.841
VH	-3.964	-31.98
MV	+1.982	+4.955
MH	-3.303	-29.93
SV	+16.518	+2.378
SH	+7.929	-0.528
MSV	+4.625	+5.814
MSH	+5.946	+0.858
SVH	+9.250	-4.559
VHM	+3.964	+4.493
MSVH	+4.625	-0.330

Table 5 - Calculation of standard deviation and error limits

		Set-I	Set-II
Standard deviation		7.07	7.66
Change in mean effect		+ 8.59	- 32.37
Error limits	Average	± 9.91	± 10.77
	Effects	± 7.07	± 7.66
	Change in mean	± 6.21	± 6.80

Hence, as per the decision making rule, maximum CMCase yield was achieved with the search region A_1 and A_{10} of set-II, was the optimum condition for the same through SSF by *Trichoderma reesei* Rut C30.

Judicious designing of the experiment and the manipulation of the levels to increase the CMCase yield with the critical analysis of the combinatorial impact of the three physical and four chemical parameters resulted ~ 2.3 folds increase in yield with the maximum activity of 175.76 IU/gds in A_{10} of set-II, (Table 5).

CONCLUSION

The EVOP technique is a powerful tool for the optimization of the multiple parameters simultaneously of any complex system such as the solid-state-fermentation. From the laboratory experiments, initial operating and design

conditions of a large- scale production unit can be obtained. The optimum conditions of the temperature, pH and relative humidity were 28 ^{o}C, 4.5 and 78% respectively, while the optimum levels of additives were nicotinic acid (as vitamin source) 15mg/l, napthanlene acetic acid (as hormone) 7mg/l, ferric chloride (as metal ion) 5mg/l and Tween-80 (as surfactant) 6ml/l.

With the application of this technique the cellulase yield increased by ~ 2.3 fold with maximum activity of 175.8 IU/gds and titre value of 21 IU/ml.

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