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ORIGINAL RESEARCH

Optimization of process parameters for β-glucosidase production by *Byssochlamys fulva* in slurry state fermentation

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ABSTRACT
Byssochlamys fulva showing glucose tolerant β-glucosidase production was used in the present study. The production of BGL and GBGL in slurry state fermentation using this strain was optimized using statistical methodology. To screen factors affecting BGL and GBGL production, an 'Optimal Factorial Design' was created. Of the four factors selected, wheat bran concentration and incubation days showed significant positive effect on both BGL and GBGL production. Maximum BGL production of 1300.98 U/ml was noticed with incubation days 8, pH 8, agitation-100 rpm and wheat bran concentration 30 % w/v. Wheat bran concentration and incubation days were selected for further optimization using Response Surface Methodology (Central Composite design). After Response Surface optimization, there was a 2.52 % increase in BGL production (from 1300.9 U/ml to 3281 U/ml) and 1.53 % increase in GBGL production (from 231.6 U/ml to 355.1 U/ml).

KEY WORDS: *Slurry state fermentation, β-glucosidase, Byssochlamys fulva, optimal factorial design, central composite design, response surface.*

Introduction

Cellulose is the most abundant and renewable non fossil carbon source on earth. Lignocelluloses are generally composed of 30-56 % cellulose, 10-27 % hemicellulose and 3-30 % lignin (Emtiazi and Nahvi, 2000). Cellulose is a linear polysaccharide of glucose residues connected by β-1,4 linkages. The beta linkage between the glucose units make cellulose rigid and thus are less prone to disintegration compared to alpha linked counterparts. The use of lignocellulose for saccharification and subsequent fermentation to bioethanol is gaining momentum particularly with declining productivity of ageing fossil reserves and the rising price of fossil fuels. The hydrolyzing enzymes that can act on cellulose are collectively called as cellulase and consist of three different enzymes- endoglucanase, exoglucanase and β-glucosidase.

Endoglucanase (EG) acts on cellulose chains and cut them at random in to short chains. Thus it increases the reducing ends in the cellulose chains by cleaving them in to a number of small units. Exoglucanase or Cellobiohydolase (CBH) acts on these short chains at their reducing ends releasing cellobiose. Beta-glucosidase (BGL) acts on the cellobiose and cleaves it in to two glucose units. In *T. reesei* and many other fungi the BGL is produced in lowest quantities compared to the other two classes of enzymes and it is slow acting, making it the rate limiting component in the hydrolysis of cellulose. Glucose is a strong repressor of cellulases but at the pace which it is generated and consumed by the organism under natural conditions, there is no over accumulation of glucose. However, this economy practiced

by the fungus is not desired when it has to be used as an industrial source of cellulase.

Thus BGL forms the major limiting factors in cellulose hydrolysis, which affect the overall rate of enzyme hydrolysis. Most fungi including cellulase hyper producing mutants of *Trichoderma reesei* are deficient in BGL (Duff *et al.*, 1986). Moreover, most of the reported BGLs are subject to product inhibition which means that the enzyme gets inhibited when glucose concentration reaches a certain threshold. This in turn results in the accumulation of cellobiose which causes the inhibition of endoglucanases and exoglucanases, as well as a repression of the synthesis of these enzymes (Lymar *et al*, 1995; Riou *et al*, 1998; Kaur *et al*, 2007) in addition to β-glucosidase itself (Woodward and Wiseman, 1982; Schmid and Wandrey, 1987). Hence to enhance cellulose hydrolysis, cellulases of *Trichoderma reesei* may be supplemented with glucose tolerant β-glucosidases from other sources (Mathew *et al*., 2008).

Statistical methods of the design of experiments (DOE) are widely employed in the optimization of process variables with considerable amount of success. This involves screening of factors that have significant effect on response through factorial design and study of interactions of factors by Response Surface Methodology. In the present study an attempt is made to screen out factors that show influence in BGL production in slurry state fermentation using *Byssochlamys fulva* and to study effect of interaction of factors in BGL production.

Materials and Methods

Fungal strain

Byssochlamys fulva showing glucose tolerant β-glucosidase production was used in the study. The fungus was grown in Potato Dextrose Agar (PDA) slants and the sporulated slant was used as the primary stock. Secondary working stocks were prepared by sub culturing from the primary stock.

Minimal Media

Mandel's and Weber Media (1969) were used as the minimal media in the optimization studies. The medium contained (In g/l) KH_2PO_4 -2.0, $CaCl_2.2H_2O$- 0.3, $MgSO_4.7H_2O$-0.3, $FeSO_4.7H_2O$- 0.005, $MnSO_4.H_2O$-0.016, $ZnSO_4.7H_2O$-0.014, $CoCl_2.6H_2O$-0.002, Peptone-0.75 and Yeast extract- 0.25. pH was adjusted to 7.4.

Inoculum and Inoculum preparation

Potato dextrose agar slants were used as the sporulating slant. Loop full of fungal spore was streaked on to PDA slants and was kept for incubation at 30° C for 6 days. The spores so formed were used as the inoculum. Four ml of sterile tween 80 (0.01%) was added on to the sporulated slant. The spores were dispersed in the tween by pipetting the sterile tween up and down using sterile tips. This was added on to 50 ml sterile tween in 100 ml conical flask and 1 ml of this spore solution containing 5.0 X 10^6 spores/ml (determined using hemocytometer) was used as the inoculum. The spore count was maintained the same throughout the experiment.

Media composition and culture conditions

In the present study, statistical optimization in Slurry state fermentation was carried out to determine the effect of each factor on the response. Also the effect of interaction of factors on the response can be studied. 'Factorial Design' was used to screen out unimportant factors that had no or minimal effect on response and 'Response Surface Design' was used to study interaction between factors.

The solid substrate used in the present study was wheat bran. Wheat bran purchased locally was dried in oven at 70° C for 2 days. 250 ml conical flasks were used as culturing vessels. 50 ml of minimal media were poured in to each flask and the required amount of wheat bran was weighed and added in to these flasks as per the experiment. The pH of the media was adjusted as per the experiment model and the flasks were autoclaved at 121° C for 15 minutes.

To each flask, 1 ml of spore suspension was added aseptically in a Laminar Air Flow, mixed well and was incubated at room temperature (30° ± 3 °C) with or without agitation as per the experiment for 4 or 8 days.

Optimization of enzyme production

Factorial design – 'Optimal Design'

Factorial design for assessing BGL production, glucose tolerant BGL production and glucose tolerance was created. 'Optimal Design' was selected as the factorial design. Four factors at two levels were selected to study their effect

Table 1. Optimal design with responses

	Factor 1	Factor 2	Factor 3	Factor 4	Response 1	Response 2
Run	A:Incubation (days)	B:pH	C:Agitation (rpm)	D:Wheat bran (% w/v)	BGL (U/ml)	GBGL (U/ml)
1	8	8	0	15	615.7	92.9
2	4	8	0	30	879.7	155.5
3	8	8	100	15	477.0	114.9
4	8	4	100	30	1147.0	152.1
5	4	8	0	15	504.0	81.0
6	8	4	0	30	1086.1	123.3
7	4	4	100	30	1028.6	148.7
8	4	4	0	15	411.0	57.3
9	4	8	100	30	883.1	184.2
10	8	4	0	15	637.7	121.6
11	8	8	0	30	1143.62	179.15
12	4	8	100	15	270.53	43.79
13	4	4	100	15	303.24	26.87
14	8	8	100	30	1300.98	231.61
15	4	4	0	30	207.92	40.41
16	8	4	100	15	397.43	141.93

Table 2. Central Composite Design with responses

	Factor 1	Factor 2	Response 1	Response 2
Run	A:Wheat bran (% w/v)	B:Incubation (days)	BGL production (U/ml)	GBGL production (U/ml)
1	20	6	737.53	125.01
2	25	4	801.83	114.86
3	15	8	1028.56	236.68
4	27.07	6	2163.79	355.13
5	20	6	1588.49	290.83
6	20	6	1960.74	344.98
7	15	4	493.88	50.56
8	20	6	1028.56	162.23
9	20	3.17	300.98	20.10
10	20	8.83	1926.90	243.45
11	12.93	6	737.53	84.40
12	20	6	879.66	94.55
13	25	8	3280.54	331.44

on production and tolerance. The factors selected were: Wheat bran concentration at 15 % and 30 % w/v, pH at 4 and 8, stationary and agitated (100 rpm) and incubation at 4 and 8 days. The Design was produced using Design Expert (Version 8.0.5, Stat-Ease, Inc., Minneapolis, USA) software. 16 runs were performed, each in duplicates. The experimental model is shown in Table 1.

Response Surface Design – Central Composite Design

To study the effect of interaction between selected factors, a Response Surface Design was created. Here a 'Central Composite Design' was chosen with each factor at five levels. In the present design, two factors that had significant effect on BGL production were taken -wheat bran concentration and incubation days. The Design was produced using Design Expert (Version 8.0.5, Stat-Ease, Inc., Minneapolis, USA) software. 13 runs were performed. The experimental model is shown in Table 2.

Enzyme extraction

After the required incubation time, the enzyme is extracted out by squeezing the fermented substrate. The crude extract was then centrifuged at 3000 rpm for 10 minutes and was stored at 4 °C.

Enzyme assay

Beta-glucosidase activity was assayed using p-nitrophenyl- β D-glucopyranoside (pNPG) as substrate. The reaction mixture consisting of 0.5 ml of citrate buffer (0.05 M, pH 4.8), 0.25 ml of culture extract and 0.25 ml of 10 mM pNPG was incubated at 50 °C for 15 minutes. The reaction was terminated by adding 1 ml of 0.2 M Na_2CO_3. The absorbance of p-nitrophenol released was measured at 410 nm. (Ghose & Bisaria, 1987). For estimating glucose tolerance, the assay was performed in presence of 1M. One unit of enzyme activity was defined as the amount of enzyme required for releasing 1µM of p-nitrophenol per minute and was expressed as U/ml. Glucose tolerance was defined as the % activity retention when assayed in presence of 1M glucose. .

Results and Discussion

Among a large number of non-pathogenic microorganisms capable of producing useful enzymes, filamentous fungi are particularly interesting due to their easy cultivation, and high production of extracellular enzymes of large industrial potential (Guimarães *et al*., 2006). Since majority of fungi are decomposers that depends on plant material as carbon source, they can be good sources of cellulase and hence β-glucosidase. The fungi used in the present study was found to secrete glucose tolerant BGL and hence an attempt was made to enhance enzyme production in slurry state fermentation using statistical methodology.

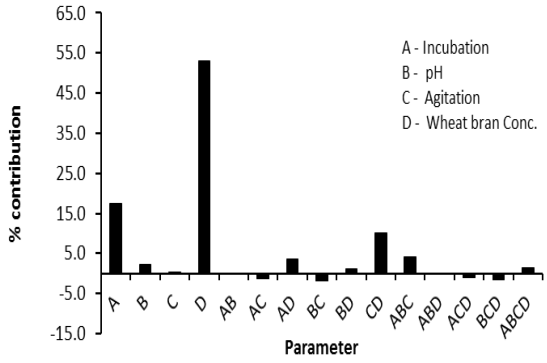

Figure 1. Contribution of factors to Response 1 (BGL production)

Factorial Design – Optimal Design

The fungus showed BGL production in all runs. Maximum BGL production (1300.98 U/ml) was noticed in Run No. 14 with incubation days 8, pH 8, agitation-100 rpm and wheat bran concentration 30 % w/v (Table no 1). For glucose tolerant BGL production, conditions in flask No 14 was optimal.For BGL production, ANOVA indicated the model to be significant with a p value 0.0004 and a model F Value of 15.52 indicating only 0.04% chances of noise The Final Equation in Terms of Coded Factors: BGL = +705.88+ 144.85 x A +253.77 x D.

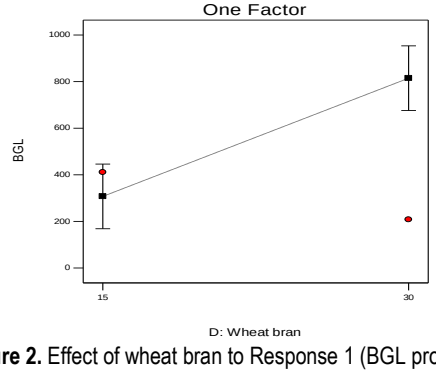

Figure 2. Effect of wheat bran to Response 1 (BGL production)

Of the four factors selected in the present design, two factors were found to show significant effect in BGL production- Wheat bran concentration (D) and incubation period (A). Both showed positive effect i.e., increase in the factor value caused increase in production (Figure 1). Wheat bran concentration showed 53.2 % effect while incubation period showed 17.3 % effect on the response.

With increase in wheat bran concentration from 15 % to 30 % W/V, there was a sharp increase BGL in production from 320 to 800 U/ml (Figure 2.). Similarly with increase in incubation days from 4 to 8, there was an increase in response from 340 to 590 U/ml (Figure 3).

In *Aspergillus niger*, wheat bran concentration and agitation had significant positive effect in BGL production under submerged fermentation conditions (Hu *et al*., 2008).

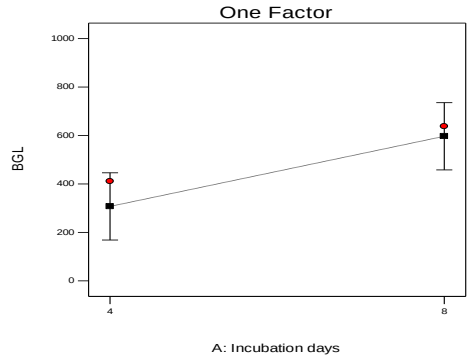

Figure 3. Effect of incubation days to Response 1 (BGL production)

Increasing wheat bran concentration increases the available carbon and nitrogen content that help the organism in increasing biomass. The same is the case with increasing incubation time as spores formed during incubation can act as second level inoculum thus enhancing biomass. Since enzyme production is a primary metabolism, product yield will be directly proportional to biomass. Generally pH has significant effect on enzyme production. According to Manzur *et al*., 2003, variations in medium pH can affect the expression and secretion of different isoforms of an enzyme. However, in this case pH showed less significant effect. This may be because of the buffering capacity of the wheat bran (Fadel, 1992).

For GBGL production, the ANOVA indicated the model as significant with a p value 0.0042 and a model F Value of 8.61 indicating only 0.42% chances of noise.

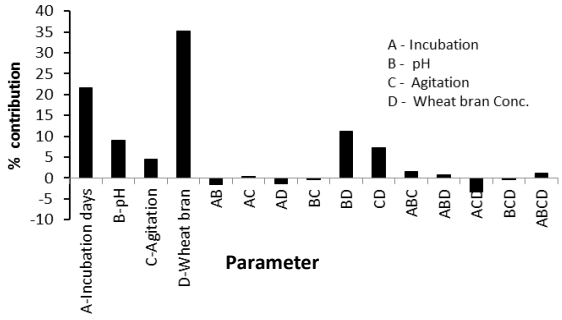

Figure 4. Contribution of factors to Response 2 (GBGL production)

The Final Equation in Terms of Coded Factors: GBGL = +118.45 + 26.23 x A + 33.42 x D. Here also wheat bran concentration (D) and incubation period (A) were found to show significant effect in GBGL production. Both showed positive effect on the response (Figure 4). Wheat bran concentration showed 35.1 % effect while incubation period showed 22.5 % effect on the response.

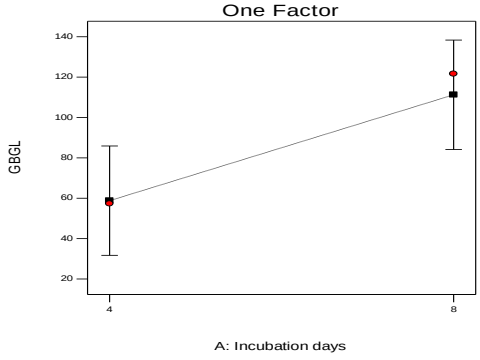

Figure 5. Effect of incubation days to response 2 (GBGL production)

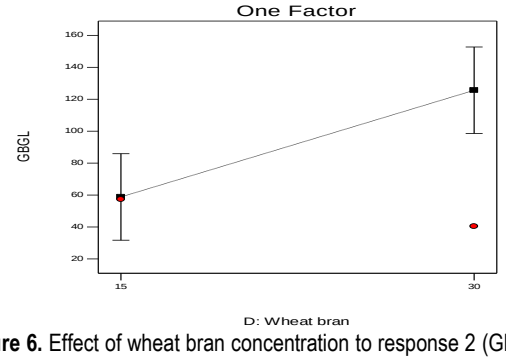

Figure 6. Effect of wheat bran concentration to response 2 (GBGL production)

With increase in wheat bran concentration from 15 % to 30 % W/V, there was a sharp increase BGL in production from 58 to 120U/ml (Figure 5.). Similarly with increase in incubation days from 4 to 8, there was an increase in response from 59.5 to 110 U/ml (Figure 6).

Response Surface Design – Central Composite Design

The two factors – wheat bran concentration and incubation days that had significant effect on BGL and GBGL production were further optimized using response surface design. Each factor was taken at 5 levels and the result of the design so created is provided in Table 2.

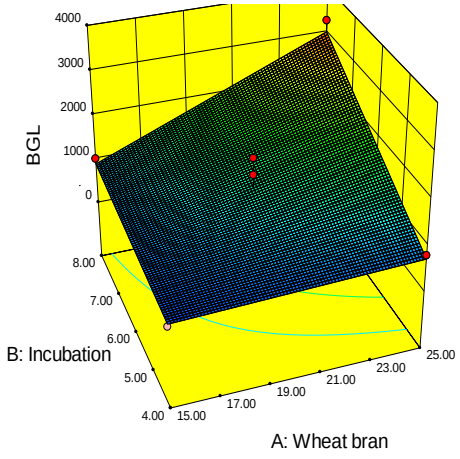

Figure 7. Response Surface for BGL production

For BGL production, ANOVA indicated the model to be significant with a p value 0.0006 and a model F Value of 15.85 indicating only 0.06% chances of noise Final equation in terms of coded factors: BGL = + 1302.23 + 572.12 x A + 664.10 x B + 486.01 x A x B. Incubation days and wheat bran concentration showed interactions. At 4 days of incubation, as wheat bran concentration was increased, there was a slight increase in response. But at incubation days of 8, increase in wheat bran concentration had significant increase in response (Figure 7.) Similarly at 25 % w/v wheat bran, increase in incubation day had significant enhancement in the response. Maximum response was noted to the extremes of the two parameters

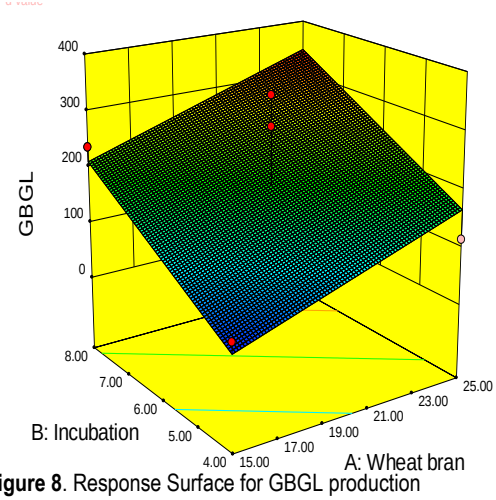

Figure 8. Response Surface for GBGL production

For GBGL production, the model was significant with a p value 0.0089 and a model F Value of 7.86 indicating only 0.89% chances of noise. Final equation in terms of coded factors: GBGL = +188.79 + 67.74 x A + 89.82 x B. Incubation days and wheat bran concentration showed similar interactions. At 4 days of incubation, with increase in the wheat bran concentration, there was a slight increase in response. This was the same with 8 days of incubation. Similarly at 25 % w/v wheat bran, increase in incubation day showed enhancement in the response. Again a similar response was noticed with 15 % w/v wheat bran (Figure 8.)

The study was thus successful in optimizing the parameters for BGL and GBGL production by *Byssochlamys fulva* in slurry state fermentation. After Response Surface optimization, their was a 2.52 % increase in BGL production (from 1300.9 U/ml to 3281 U/ml) and 1.53 % increase in GBGL production (from 231.6 U/ml to 355.1 U/ml).

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