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Optimization of cellulase production by a versatile *Aspergillus fumigatus* fresenius strain (AMA) capable of efficient deinking and enzymatic hydrolysis of Solka floc and bagasse

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ABSTRACT

This study reports the optimization of cellulase production by a versatile *Aspergillus fumigatus* fresenius (AMA) strain capable of efficient deinking and enzymatic hydrolysis of Solka floc and bagasse. The culture produced maximal levels of cellulase on basal salt medium containing rice straw as carbon and beef extract as nitrogen source at 45 °C. Further optimization of enzyme production was carried out using Box–Behnken design of experiment to study the influence of process variables (beef extract, temperature and tween 80) on cellulase and xylanase production. The model computed for R^2 value ranged between 95.3% and 99.9% indicating that they are appropriate and can be useful to predict the level of beef extract, temperature and tween 80 required to achieve optimal cellulase production by *A. fumigatus* fresenius (AMA). The culture under optimized conditions produced 240.2, 9.73, 470, 15, 2800 (units/g of substrate) of endoglucanase, Fpase, β-glucosidase, cellobiohydrolase (CBH) and xylanase corresponding to 2.45, 2.88, 2.13 and 1.29 folds improvement, respectively, in enzyme activities when compared to those obtained under unoptimized conditions. The treatment of composite paper pulp with enzyme extracts (@ 0.5 U CMCase/g pulp) for 30 min resulted in 53% removal of ink and increased the brightness of the hand sheets by 4.32% ISO. The concentrated culture extracts from *A. fumigatus* fresenius also efficiently hydrolyzed Solka floc SW 40 and bagasse (@ 7%, w/v) resulting in 90 and 87% saccharification, respectively.

1. Introduction

Cellulases are important glycosyl hydrolases that are involved in degrading on and recycling the abundant cellulosic biomass in nature. From biotechnological standpoint, cellulases have a vital role to play in the generation of potentially sustainable energy sources such as glucose, ethanol, hydrogen and methanol (Kamm and Kamm, 2004; Zang and Lynd, 2005). Cellulases are produced by a variety of microorganisms including bacteria, actinomycetes and fungi. Fungi, however, are known to secrete cellulases in large amounts. Cellulase is a complex of enzyme system comprising of endoglucanase (EC 3.2.1.4), exoglucanase (EC 3.2.1.91) and β-glucosidase (EC 3.2.1.21) that act in a synergistic fashion to carry out the complete hydrolysis of cellulose. Endoglucanase acts internally on the chain of cellulose cleaving 1,4-β-linked bonds and liberating oligosaccharides of varying degrees of polymerization. The exoglucanases act processively from reducing and non-reducing ends removing cellobiose in a sequential manner. Finally, β-glucosidase completes the saccharification by splitting cellobiose and small cello-oligossaccharides into glucose molecules (Wither, 2001). Currently, cellulases have gained significant commercial importance due to their potential application in deinking of mixed office waste (MOW), magazines and newspapers, denim stone washing, etc. (Cavaco-Paulo, 1998; Soni et al., 2008; Vyas and Lachke, 2003). In addition, cellulases along with hemicellulases in the enzyme cocktails have the great potential in the saccharification of lignocellulosics to fermentable sugars, and perhaps the most sought after application for its role in bioconversion of cellulosics to ethanol (Elegir et al., 2000).

The application of cellulases in these processes are carried out at relatively higher temperatures (~50–60 °C) and therefore those enzymes are preferred that are capable of withstanding high temperature for a longer period of time. The thermophilic and thermotolerant fungi are known to thrive at high temperature and produce appreciable levels of thermostable enzymes that are suitable for these applications (Maheshwari et al., 2000; Sharma et al., 2008). This paper reports cellulase production by *Aspergillus fumigatus* fresenius (AMA) strain isolated from the degrading paper/polythene composite waste. The strain was found to produce an endoglucanase devoid of cellulose binding domain (CBD)

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which efficiently removed ink particles from printed paper waste (Soni et al., 2008). The cellulase of this culture was also evaluated for saccharification of Solka floc and bagasse. Since the cost of cellulases is one of the most important factors in commercialization of these processes, therefore, approaches for optimization of culture conditions for improving cellulase production is being described here.

2. Materials and methods

2.1. Culture

A thermotolerant fungal strain isolated from degrading paper/polythene composite waste, Amritsar (India), was identified as *A. fumigatus* fresenius (AMA) on the basis of morphological and molecular characterization (Soni et al., 2008). The fungus was grown and maintained on a yeast potato soluble starch (YpSS) agar of the following composition (%; w/v), starch, 1.5; yeast extract, 0.4; KH_2PO_4, 0.2; K_2HPO_4, 0.23; $MgSO_4{\cdot}7H_2O$, 0.05; citric acid, 0.057 and agar, 2.0. The pH of the medium was adjusted to 7.0. The fungus was cultured at 45 °C for 7 days on agar slopes and stored at 4 °C.

2.2. Solid substrate culturing for enzyme production

Solid-state fermentation was carried out in Erlenmeyer flasks (250 ml) that contained ground rice straw to act as a carbon source (5 g) and basal medium BM (15 ml) of following composition (%; w/v) KH_2PO_4, 0.4, CH_3COONH_4, 0.45, and $(NH_4)_2SO_4$, 1.3. Prior to sterilization, the initial pH and moisture content of the medium was adjusted to 7.0 and 75%, respectively. The culture medium was then inoculated with a mycelial suspension (2 ml) of 24 h old culture grown on glucose pre-cultured medium (%; w/v); (glucose, 1.5; yeast extract, 0.4; K_2HPO_4, 0.2; $MgSO_4{\cdot}7H_2O$, 0.1; pH 7.0) and incubated in a water saturated atmosphere at 45 °C for 5 days in an incubator. Thereafter, the enzyme was harvested by adding 50 ml of sodium citrate buffer (50 mM pH 6.0) to the flasks and kept at 45 °C for 1 h under mild shaking. The resultant slurry was filtered through a muslin cloth and centrifuged at 8800 × *g* for 10 min and finally the extracts were used for enzyme assay.

2.3. Optimization of parameters for cellulase and xylanase production by classical approach

Optimization of production parameters was carried out using the approach of one variable at a time. The effect of different basal media types, i.e., carbon and nitrogen sources, pH, temperature, inoculum age and level and additives on cellulase production were studied.

2.4. Box–Behnken design

Response surface methodology (RSM) using the Box–Behnken design (Box and Behnken, 1960) of experiments was carried out to develop a mathematical correlation among three process variables, tween 80 level (X_1), temperature (X_2) and beef extract level (X_3) on production of endoglucanase (EG), β-glucosidase, FPase, cellobiohydrolase (CBH) and xylanase. The variables were studied at low, middle and high concentration levels and were designated as −1, 0 and +1 (coded values), respectively (Tables 1 and 2).

The behavior of the system was explained by the following quadratic model equation.

$$G = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3$$

Table 1
The level of variable chosen for the trials.

Tween 80 (%, v/v)	Temperature (°C)	Beef extract (%, w/v)
0.10 (−1)	30 (−1)	0.10 (−1)
0.25 (0)	40 (0)	0.25 (0)
0.4 (+1)	50 (+1)	0.4 (+1)

where G is the predicted response, β_0 intercept, β_1, β_2 and β_3 linear coefficient, β_{11}, β_{22} and β_{33} square coefficient, and β_{12}, β_{13} and β_{23} interaction coefficients. A total of 17 experiments were necessary to study the 10 coefficients of the model. The MINITAB statistical (MINI-TAB 15, USA) was used to compute the results and generate response surface graphs.

2.5. Enzyme assay

Endoglucanase and xylanase activities were determined using 1% (w/v) CM-cellulose and 1% (w/v) birch wood xylan, prepared in sodium citrate buffer (50 mM, pH 6.0), respectively. The reaction mixture, containing equal amounts of suitably diluted enzyme (0.500 ml) and substrate (0.500 ml), was incubated at 50 °C for 10 min and 5 min, respectively. Total cellulase activity (FPase) was measured by using Whatman No. 1 filter paper (1 cm × 6 cm) strip as substrate (Wood and Bhat, 1988). For FPase, a rolled 1 cm × 6 cm strip (50 mg) of the filter paper was dipped into 0.500 ml of sodium citrate buffer (50 mM, pH 6.0) and incubated with 0.500 ml of diluted enzyme at 50 °C for 1 h. The reaction was stopped by addition of DNS followed by boiling (Miller, 1959); the colour developed was read at 540 nm using Novaspec II spectrophotometer (Pharmacia). The amounts of released reducing sugars were determined using DNS, and also quantified using glucose and xylose as standards. The enzyme activities (units/ml) were expressed as amount of enzyme required to release 1 μmol of reducing sugar per minute under assay conditions. The enzyme activities were expressed as units/gram dry weight substrate.

β-Glucosidase and cellobiohydrolase was assayed using p-nitro phenyl-β-D-glucopyranoside (pNPG) and p-nitro phenyl-β-D-lactopyranoside (pNPL) respectively, in a micro titre plate based method (Parry et al., 2001). Appropriately diluted enzyme (25 μl) was mixed with 50 μl of sodium acetate buffer (50 mM, pH 5.0) and the reaction was started by adding 25 μl of pNPG (10 mM)/pNPL (3 mM) and incubated at 50 °C for 30 min. The reaction was terminated by adding 100 μl of NaOH–glycine buffer (0.4 M, pH 10.8) and the developed yellow color was read at 405 nm using ELISA Reader (MULTISKAN; Lab system). One unit of β-glucosidase/cellobiohydrolase activity was expressed as the

Table 2
The Box–Behnken design for three independent variables.

Trial no.	Tween 80	Temperature	Beef extract
1	−1	−1	0
2	1	−1	0
3	−1	1	0
4	1	1	0
5	−1	0	−1
6	1	0	−1
7	−1	0	1
8	1	0	1
9	0	−1	−1
10	0	−1	−1
11	0	−1	1
12	0	1	1
13	0	0	0
14	0	0	0
15	0	0	0
16	0	0	0
17	0	0	0