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Extracellular β-glucosidase Production by a Marine *Aspergillus sydowii* BTMFS 55 under Solid State Fermentation Using Statistical Experimental Design

K. M. Madhu*, P. S. Beena, and M. Chandrasekaran

Department of Biotechnology, Cochin University of Science and Technology, Cochin 682 022, India

Abstract A potential fungal strain producing extracellular β-glucosidase enzyme was isolated from sea water and identified as *Aspergillus sydowii* BTMFS 55 by a molecular approach based on 28S rDNA sequence homology which showed 93% identity with already reported sequences of *Aspergillus sydowii* in the GenBank. A sequential optimization strategy was used to enhance the production of β-glucosidase under solid state fermentation (SSF) with wheat bran (WB) as the growth medium. The two-level Plackett-Burman (PB) design was implemented to screen medium components that influence β-glucosidase production and among the 11 variables, moisture content, inoculums, and peptone were identified as the most significant factors for β-glucosidase production. The enzyme was purified by $(NH_4)_2SO_4$ precipitation followed by ion exchange chromatography on DEAE sepharose. The enzyme was a monomeric protein with a molecular weight of ~95 kDa as determined by SDS-PAGE. It was optimally active at pH 5.0 and 50°C. It showed high affinity towards *p*NPG and enzyme has a K_m and V_{max} of 0.67 mM and 83.3 U/mL, respectively. The enzyme was tolerant to glucose inhibition with a K_i of 17 mM. Low concentration of alcohols (10%), especially ethanol, could activate the enzyme. A considerable level of ethanol could produce from wheat bran and rice straw after 48 and 24 h, respectively, with the help of *Saccharomyces cerevisiae* in presence of cellulase and the purified β-glucosidase of *Aspergillus sydowii* BTMFS 55. © KSBB

Keywords: Aspergillus sydowii*, β-glucosidase, Plackett-Burman, SSF, wheat bran*

INTRODUCTION

The β-glucosidase has been the focus of recent research because of their important roles in a variety of fundamental biological processes. *Trichoderma reesei* [1], *Aspergillus* sp. [2], and *Penicillium* sp. [3] are the major β-glucosidase producers among the microbial kingdom. The production of ethanol from lignocellulosic residues is considered to be one of the major applications of β-glucosidase, with *Saccharomyces cerevisiae*. Ethanol produced from biomass is today the most widely used biofuel when blended with gasoline. As the carbon-dioxide released by combustion is recycled into biomass, the use of biofuels can significantly reduce the accumulation of greenhouse gas. Biomass conversion to ethanol has been advocated for a long time due to its potential to foster sustainable energy supply, reduce green house gas emissions, boost rural economies, and reduce the country's dependence on foreign oil.

Several industrial enzymes are derived from terrestrial sources. Whereas the marine environment, which encompasses about 71% of the earth's surface; potentially a vast resource for useful enzymes, remains largely unexplored. Marine microorganisms take an active part in the mineralization of complex organic matter and contribute to the secondary production in the sea. Their participation in the degradation of organic compounds and retting of ropes and fibers, testify to their potential as a rich source of hydrolytic enzymes of industrial importance [4]. There are very few reports on β-glucosidase from marine environment on fungi [5-7]. It is only advantageous to have more potential strains that produce enzymes with varied potentials. In this context, in the present study, an attempt was made to isolate a potent β-glucosidase producing fungus from the marine environment and to develop a bioprocess towards industrial produc-

***Corresponding author**
Tel: +91-484-2576267 Fax: +91-484-3922574
e-mail: madhukmani@gmail.com

tion and explore their possible application for bioethanol production.

MATERIALS AND METHODS

Microorganism and Media

The fungal strain was isolated from the seawater of the coastal areas of Kerala, available in the culture collection of the Microbial Technology Laboratory, Department of Biotechnology, Cochin University of Science and Technology, India, was used. The culture was grown at room temperature (28 ± 2°C) in GMY (glucose, malt extract, and yeast extract) fungal medium with the following composition (w/v; %): glucose, 1; malt extract, 1; NH_2NO_3, 0.1; $MgSO_4$, 0.05; yeast extract, 0.1; NaCl, 1; and pH 5.5. Culture was maintained on the slants of the same GMY media at 4°C. Conidial inoculum was prepared from a freshly raised 8 days old GMY agar slant by dispersing the spores in sterile physiological saline containing 0.1% Tween 80. One millilitre of this spore suspension (22×10^8 CFU/mL) was used as inoculum.

Identification of the Fungal Strain

Molecular identification of the fungal strain was done using a primer pair for 28S rDNA, NL1F (5′-GCATATCAATAAGCGGAGGAAAAG-3′), and NL4R (5′-GGTCCGTGTTTCAAGACGG-3′). A portion of the 28S rRNA gene was amplified from the genomic DNA [8]. PCR was carried out using 1X PCR buffer, 1 μLTaq polymerase (Genei, Bangalore), and 1 μL each of the primers. The PCR reaction was carried out for 35 cycles of denaturation at 94°C for 30 sec, annealing at 62°C for 30 sec, and extension at 72°C for 1 min. Products after PCR amplification were subjected to sequencing, followed by homology analysis. Nucleotide sequences were determined by the ABI Prism 310 genetic analyzer using the big dye Terminator kit (Applied Biosystems) at BioServe India Ltd., Hyderabad.

Analytical Methods

β-glucosidase activity was assayed by a reaction mixture containing 1 mL of 0.1% *p*-Nitrophenyl β-D-glucopyranoside (pNPG) in 0.1 M Glycine-NaOH buffer (pH 8.0) and 0.5mL of diluted enzyme solution [9]. The reaction was carried out at 50°C for 15 min and stopped by addition of 2 mL cold 1 M Na_2CO_3. *p*-Nitrophenol (*p*NP) released was quantified at 410 nm. One unit of enzyme activity was defined as the amount of enzyme producing 1 μmol of a *p*NP per mL per minute. Protein content was determined according to the method of Lowry *et al.* [10].

Media Optimisation by Statistical Approach

Plackett-Burman Experimental Design

Plackett-Burman design [11] was applied for the statistical optimization of the production of β-glucosidase under SSF by *A. sydowii* BTMFS 55. Wheat bran (10 g) taken in each flasks was added with each of the nutrients as per the matrix design (Table 3) at the corresponding pH and sterilized at 121°C for 30 min. Inoculated flasks were incubated at the appropriate temperatures and the enzyme was extracted after 72 and 120 h, and the enzyme activity was determined. Statistical significance of the model equation was determined by Fisher's test and the proportion of variance explained by the model was presented by the multiple coefficient of determination, R^2 value. Design Expert (Version 7.1.0; Stat-Ease Inc., USA) was used for the matrix design and interpretations. A validation run was performed to confirm the results after getting the Plackett-Burman results.

Table 1. Levels of factors chosen the Plackett-Burman design for β-glucosidase production by *A. sydowii* BTMFS 55

Variable code	Variable	Levels	
		+1	–1
A	Moisture (%)	70	50
B	Temperature (°C)	30	25
C	pH	9	7
D	Inoculum (%)	20	10
E	Particle size (μ)	600	425
F	CMC (g/L)	10	2
G	Peptone (g/L)	10	1
H	$(NH_4)_2SO_4$ (g/L)	10	1
J	NaCl (g/L)	7.5	2.5
K	Tween 80 (%)	1	0.5
L	Incubation time (h)	120	72

Eleven variables representing ten nutritional components and initial pH of the medium were used. For each nutrient variable, a high (+) and a low (–) concentration were tested. The main effect of each variable upon β-glucosidase activity was estimated as the difference between both averages of measurements made at the high (+) and at the low level (–). A 12 run Plackett-Burman design was used to screen eleven factors and the experimental responses were analyzed by the method of least squares to fit the following first-order model:

$$Y = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_4 X_4 + \beta_5 X_5 + \beta_{11} X_{11}$$

where, Y was the predicted response (β-glucosidase yield), β_0, β_1, β_2 β_{11} were the regression coefficients, and X_1, X_2 X_{11} were the coded levels of the independent variables (Table 1).

Enzyme Purification and Characterization

Ammonium Sulphate Fractionation

Ammonium sulphate precipitation was done according to Englard and Seifter [12], it is required for precipitating the β-glucosidase enzyme and was optimized by addition at varying levels of concentrations to the crude extract. The resulting precipitate was collected by centrifugation at 10,000 rpm for 10 min, dissolved in the smallest possible volume of 0.01 M phosphate buffer (pH 8.0), and then dialyzed overnight

against the same buffer.

Ion Exchange Using DEAE Sepharose Column

The DEAE sepharose column was equilibrated with phosphate buffer of pH 8.0 (0.1 M) for 24 h, and 4 mL of dialyzed sample with protein content of 8.86 mg/mL was applied to the column with height 25 cm, which was pre-equilibrated with the same buffer. The unbound proteins were washed off with the buffer until the OD_{280} reached near zero. Stepwise elution was done at a flow rate of 10mL/h using 0~0.5 M NaCl in the phosphate buffer. 5 mL fractions were collected and protein content was estimated by measuring the absorbance at 280 nm. The active fractions were pooled and dialyzed overnight against phosphate buffer; assayed for β-glucosidase activity, protein content, and specific activity.

Electrophoretic Methods

Ion exchange purified sample was subjected to electrophoresis by Native and SDS-PAGE in a 10% polyacrylamide gel according to the method of Laemmlli [13]. Zymogram was performed by incubating the SDS polyacrylamide gel with 0.01% solution of 4-methylumbelliferyl β-D-glucopyranoside (MUG) in Glycine-NaOH buffer (0.1 M, pH 8.0) at 50°C for 10 min and the β-glucosidase activity was visualized under UV light [14].

Effect of Temperature and pH on Enzyme Activity and Stability

Temperature optimum for enzyme activity was determined by incubating the reaction mixture for 30 min at different temperatures ranging from 30 to 80°C. For temperature stability, the residual activity was estimated at 30 min, 1 h, 12 h, and 24 h of incubation, where the sample kept at 4°C was taken as control. The effect of pH on the activity was studied by performing the enzyme assay at a pH range of 3 to 10. The buffer systems used were, citrate buffer (pH 3~6), phosphate buffer (pH 7~8), and Glycine-NaOH buffer (pH 9~10). Enzyme activity and relative activity were calculated. The pH stability of the enzyme was evaluated by incubating the enzyme with the above said buffers for 24 h at 4°C.

Effect of Organic Solvents on Enzyme Activity

Impact of various organic solvents on enzyme activity was evaluated by adding each solvent into the reaction mixture at the final concentration of 10 to 50% (v/v). After incubating the enzyme with each organic solvent for 30 min, enzyme was assayed for the residual activity.

Effect of Substrate Specificity

The substrate specificity of the purified enzyme was evaluated by conducting enzyme assay with different substrates (5 mM, v/v) such as cellobiose, lactose, maltose, sucrose, trehalose, raffinose, and *p*NPG. Depending on the substrate, the enzyme activity was determined under optimal conditions (50°C, pH 8.0, 30 min) by measuring the release of reducing sugar (by DNS method, A_{540}) or the release of *p*-nitrophenol (A_{410}).

Enzyme Kinetics and Glucose Inhibition

The effect of substrate (*p*NPG) concentration on the activity of the enzyme was determined by using different concentrations of *p*NPG (1~4 mM) in the reaction mixture and estimating the enzyme activity. The kinetic parameters such as the substrate concentration (K_m) at which the reaction velocity is half of maximum and V_{max} were also calculated from the data using double reciprocal plots [15]. The inhibition of glucose on the enzyme activity was measured by adding various concentrations of glucose (50, 100, and 150 mM) into the reaction mixture with *p*NPG (0.5, 1, 2.5, and 3 mM) as substrate. The inhibition constant (K_i) was obtained at the intersection of the line of a Dixon plot [16].

Bioethanol Production-application Studies

Microorganism and Media

Commercially available *S. cerevisiae* (baker's yeast) was used for the fermentation. A 10 mL pre-culture of yeast in MYGP media was transferred to 100 mL same media, incubated for 9 h, and collected from the pellet by centrifugation; re-suspended the pellet in sterilized 0.05 M citrate buffer and 10% of this was used as the inoculum (MYGP media, %: malt extract, 0.3; yeast extract, 0.3; peptone, 0.5; glucose, 1; and pH 5.0 ± 0.1).

Enzymes

A commercial cellulase enzyme (Zytek; 78 FPU/mL) and purified β-glucosidase (1166 U/mL) from *A. sydowii* BTMFS 55 were used.

Substrate and Pretreatment

Wheat bran and rice straw were purchased from local market in Cochin area, and the rice straw cut into small pieces before the pretreatment. Forty grams each of wheat bran and rice straw was taken in Erlenmeyer flask containing 1 L distilled water with 1% H_2SO_4 and subjected to autoclaving (121°C) for 30 min. After cooling the samples, decanted the water, and washed the pretreated substrate with sterile distilled water for 5 more times, to remove the residual glucose and other inhibitors.

Simultaneous Saccharification and Fermentation

Experiments were carried out in 1 L flasks with 250 mL working volume. The pretreated substrate (4%) was added with 250 mL MYGP media (without glucose) with cellulase (6 FPU/g substrate), β-glucosidase (12 FPU/g substrate), and 10% inoculum; incubated at 38°C on an orbital shaker at 150 rpm for a total period of 120 h. Samples were taken aseptically and assayed for ethanol and reducing sugars.

Analytical Techniques

Ethanol was estimated by gas chromatography (Chemito, India) in which a flame ionization detector and stainless steel column packed with Poropak were used. The column oven was operated isothermally at 155°C, and the detector and injection port were kept at 255 and 175°C, respectively. Nitrogen was used as carrier gas at a flow rate of 30 mL/min

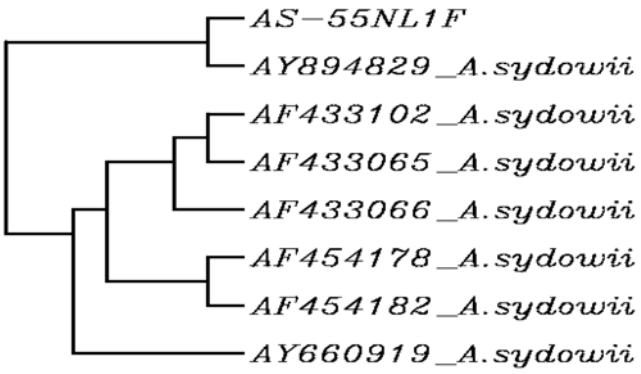

Fig. 1. Dendrogram representing the relationship of *A. sydowii* BTMFS 55 (AS-55 NL1F) with other reported *A. sydowii*.

Table 2. The estimated effects of individual parameters during SSF for β-glucosidase production by *A. sydowii* BTMFS 55

Code	Parameter	Estimated effects
A	Moisture	224.1
B	Temperature	70.5
C	pH	17.3
D	Inoculum	274.7
E	Particle size	-18.6
F	CMC	78.5
G	Peptone	387.3
H	$(NH_4)_2SO_4$	-35.5
J	NaCl	-40.8
K	Tween 80	0.4
L	Time	102.5

and the combustion gas was a mixture of hydrogen and air. The glucose was estimated according to the DNS method [17].

RESULTS AND DISCUSSION

Marine environments are unique by virtue of their salinity, wide range of mineral content, and well-knitted ecosystem when compared to terrestrial environments, which is constantly disturbed by human activities. The marine fungi have not been experimentally tried for their potential in many of the human endeavours in which they would have a major role to play as a biocatalyst, through their enzymes or whole cell systems. *A. sydowii* BTMFS 55 isolated from seawater was found as a potential strain that produced extracellular β-glucosidase under SSF.

Molecular Identification of the Fungal Strain

The PCR amplification of rDNA sequences using taxon-specific primers which were derived from sequence data and were checked for cross reaction with related fungi is thought to be the most powerful molecular tool for fungal diagnosis [18]. A portion of the 28S rRNA gene (~600 bp) was amplified from the genomic DNA using a primer pair for 28S rDNA (NL1F and NL4R) and the partial nucleotide sequence obtained after sequencing was of 494 bp. The identity of the sequence was determined by BLAST software and the resultant sequence showed 93% identity with the already available sequences of *A. sydowii* in the GenBank. The partial sequence of the 28S rRNA gene was submitted to GenBank (EF 570064) through BankIt programme at NCBI (http://www.ncbi.nlm.nih.gov/BankIt). Phylogenetic tree was constructed using the dendrogram method implemented in CLUSTAL W (http://align.genome.jp) (Fig.1). Tree was constructed using nucleotide evolutionary model based on synonymous and non-synonymous nucleotide substitutions. *A. sydowii* BTMFS 55 shared a close affinity with most of the terrestrial species of *Aspergillus* suggesting that *A. sydowii* BTMFS 55 could have migrated to marine sediments through surface drain from terrestrial environments and could have adapted to marine environments in due course of time. It was also identified as *A. sydowii* by MTCC, IMTECH, India, by morphological analysis.

Media Optimisation by Statistical Analysis under SSF

Medium optimization by the one-factor-at-a-time method involves changing one independent variable while fixing others at certain levels. This method is not only time-consuming, but also often leads to an incomplete understanding of the behavior of the system, resulting in confusion and a lack of predictive ability [19]. Hence statistical approach was attempted towards optimization of bioprocess variables for β-glucosidase production, which not only allows quick screening of a large experimental domain, but also reflects the role of each of the components.

In the present study, the Plackett-Burman experimental design was used to optimize the solid state culture conditions for the production of β-glucosidase by *A. sydowii* BTMFS 55. The estimated effects of individual parameters and the predicted and experimental responses are presented in the Tables 2 and 3. The results indicated that although most of the parameters had a positive influence, the moisture content (*A*), inoculum (*D*), and peptone (*G*) had significant influence on β-glucosidase under SSF. A graphical representation of the positive (orange bars) and negative (blue bars) effects of the variables tested was presented as a Pareto chart in Fig. 2. The significant ones were labelled by respective codes and ranked based on their estimated effects. On the basis of the experimental values, statistical testing was carried out using Fisher's test for analysis of variance (ANOVA) (Table 4). The results were used to fit a first order polynomial equation and the model equation for β-glucosidase yield could be written as:

$$Y = 834.18 + 112.04\,A + 137.34\,D + 193.69\,G$$

where, *Y* is yield of β-glucosidase, *A* is moisture content (%), *D* is inoculum concentration (%), and *G* is peptone concen-

Table 3. Result of the Plackett-Burman experimental design for optimization of process variables for β-glucosidase production by *A. sydowii* BTMFS 55 under SSF

Run	Coded variable level											Y_1^a (U/g IDS)	Y_2^b (U/g IDS)
	Moisture (%)	Temperature (°C)	PH	Inoculum (%)	Particle size (μ)	CMC (g/L)	Peptone (g/L)	$(NH_4)_2SO_4$ (g/L)	NaCl (g/L)	Tween 80 (%)	Incubation time (h)		
1	50	25	9	10	600	10	1	10	7.5	1	72	305.247	391.111
2	50	30	7	20	600	2	10	10	7.5	0.5	72	941.568	1053.17
3	70	25	9	20	425	10	10	10	2.5	0.5	72	1250.41	1277.26
4	50	25	7	10	425	2	1	1	2.5	0.5	72	303.916	391.111
5	50	30	9	10	600	10	10	1	2.5	0.5	120	941.568	778.494
6	50	25	7	20	425	10	10	1	7.5	1	120	1106.64	1053.17
7	70	30	7	10	425	10	1	10	7.5	0.5	120	703.28	615.198
8	70	25	9	20	600	2	1	1	7.5	0.5	120	863.026	889.872
9	70	25	7	10	600	2	10	10	2.5	1	120	964.198	1002.58
10	50	30	9	20	425	2	1	10	2.5	1	120	733.898	665.785
11	70	30	7	20	600	10	1	1	2.5	1	72	933.58	889.872
12	70	30	9	10	425	2	10	1	7.5	1	72	962.867	947.336

[a]experimental responses, [b]predicted values calculated from the fitted first-order model.

Table 4. Analysis of variance for the factorial model on β -glucosidase production in SSF by *A. sydowii* BTMFS 55

Source	Sum of squares	DF[a]	Mean square	*F* Value	*p*-Value Prob > F[b]
Model	8.272E + 005	3	2.757E + 005	29.15	0.0001
A-Moisture	1.506E + 005	1	1.506E + 005	15.92	0.0040
D-Inoculum	2.263E + 005	1	2.263E + 005	23.92	0.0012
G-Peptone	4.502E + 005	1	4.502E + 005	47.59	0.0001
Residual[c]	75682.39	8	9460.30		
Corrected total[d]	9.029E + 005	11			

[a]Degree of freedom, [b]Significant at "Prob > F" less than 0.05, [c]Difference between experimental and predicted points, [d]Total of all information corrected for the mean. R_2 = 0.9162, Adjusted R_2 = 0.8847, Predicted R_2 = 0.8114, Adequate Precision = 15.780.

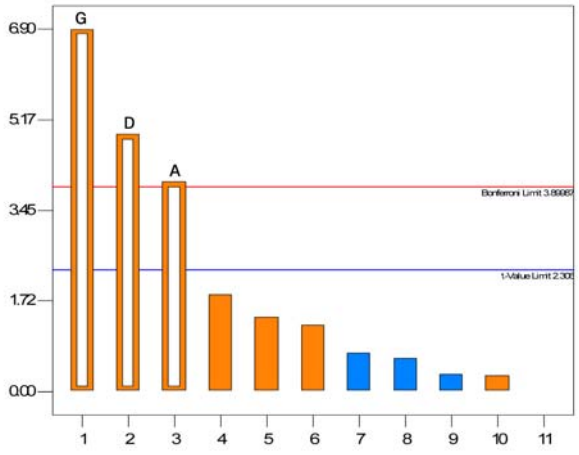

Fig. 2. Pareto chart of standardized effects of the selected parameters during SSF by *A. sydowii* BTMFS 55 (*G*-concentration of peptone, *D*-concentration of inoculum, and *A*-moisture content).

tration (g/L).

The model *F*-value of 29.15 and values of prob > *F* (< 0.05) indicated that the model terms are significant. The test model was statistically significant with a confidence levels above 95%. The quality of fit of the polynomial model equation was expressed by the coefficient of determination (R^2), which equalled 0.9162, indicating that 91.62% of the variability in the response could be explained by the model. The R^2 value is always between 0 and 1. The closer the R^2 is to 1.0, the stronger the model and the better it predicts the response [20]. The purpose of statistical analysis is to determine the experimental factors, which generate signals that are large in comparison to the noise. Adequate precision measures signal to noise ratio. An adequate precision of 15.78 for β-glucosidase production was recorded. The predicted R^2 of 0.8114 was in reasonable agreement with the adjusted R^2 of 0.8847. This indicated a good agreement between the experimental and predicted values for β-glucosidase production. The value of the adjusted determination coefficient (0.8847) was also very high to advocate for a high significance of the model. These results indicated that the response equation provided a suitable model for the

Table 5. Purification of β-glucosidase from *A. sydowii* BTMFS 55

Purification step	Total protein (mg)	Total activity (U/mL)	Specific activity (U/mg)	Yield of protein (%)	Yield of activity (%)	Fold of purification
Crude extract	1560	134160	86	100	100	1
$(NH_4)_2SO_4$	35	5830	167	2	4	2
DEAE sepharose	2	1270	635	0.1	0.9	7

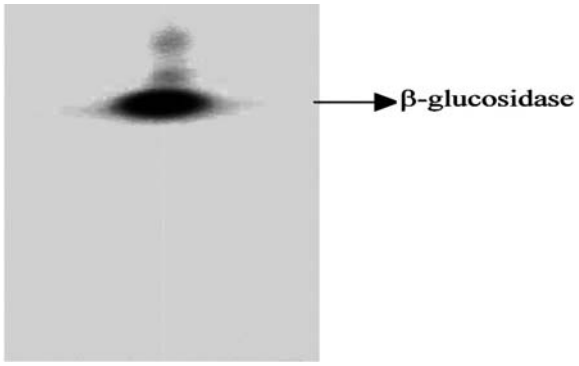

Fig. 3. Native PAGE analysis of *A. sydowii* BTMFS 55 β-glucosidase.

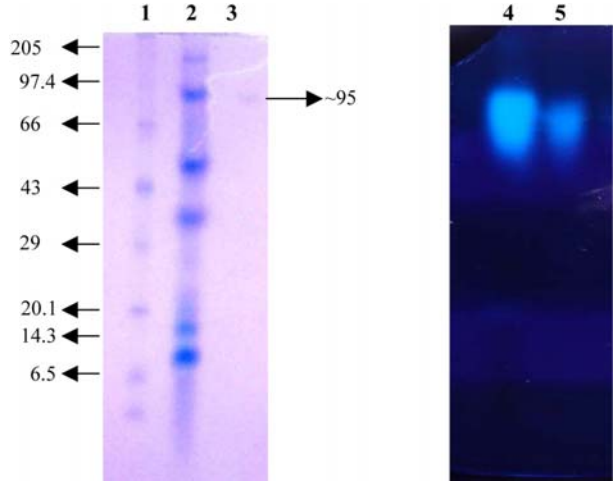

Fig. 4. SDS-PAGE analysis of β-glucosidase showing (A) Coomassie blue staining and (B) Zymogram analysis (Lane 1, Protein Marker (kDa); lanes 2 and 4, $(NH_4)_2SO_4$ precipitated fraction; lanes 3 and 5, Ion exchange purified sample).

Plackett-Burman design experiment.

The adequacy of the model was further examined at additional independent conditions that were not employed to generate the model. It was observed that the experimental and predicted values of β-glucosidase production showed good correlation. The optimum conditions predicted for the production of 1257.09 U/g IDS β-glucosidase were as follows: moisture (X_1) - 68.6 (%), inoculum (X_2) - 19.93 (%), and peptone (X_3) - 9.94 (g/L). The actual experimental value obtained at these predicted conditions was 1335.61 U/g IDS, which was in good agreement with the predicted value.

Enzyme Purification and Characterization

β-glucosidase could be precipitated with 60% ammonium sulphate saturation. Table 5 summarizes the data of the purification steps of the extracellular β-glucosidase. During ion exchange chromatography on DEAE sepharose, the β-glucosidase was eluted as a single active peak.

Native and SDS-PAGE

The β-glucosidase enzyme showed a single polypeptide band on both native and SDS-PAGE analysis, having a mass of ~95 kDa (Figs. 3 and 4). The zymographic analysis of the corresponding band exhibited β-glucosidase activity as fluorescence under UV light, after staining with MUG. It should be noted that the molecular mass of most fungal β-glucosidases are often greater than 80 kDa [21]. Extracellular β-glucosidases which has molecular masses of 96 kDa [22], 100 kDa [23], and 105 kDa [24] has been purified from three different strains of *A. niger*.

Effect of Temperature on β-glucosidase Activity and Stability

The temperature optimum for maximal β-glucosidase activity was 50°C and it demonstrated significant activity (up to 40% of the maximal activity) over a temperature range of 40 to 70°C. The thermal stability of the β-glucosidase was investigated by measuring the residual activity after 30 min, 1 h, 12 h, and 24 h of incubation at temperatures ranging from 30 to 80°C. The enzyme showed 10% increase in residual activity at 50°C, compared to control (4°C). It could withstand the temperatures of 40 to 60°C even after 24 h of incubation. The enzyme was not stable at 80°C, whereas it showed a weaker stability pattern at 30 and 70°C. The optimum temperature of the purified enzyme is similar to that of *A. oryzae* [2], *A. nidulans* [25], *Candida peltata* [26], *Stachybotrys* sp. [27], *Volvarialla volvacea* [28], and *T. reesei* [29].

Effects of pH Activity and Stability

The β-glucosidase enzyme displayed a pH optimum of 5.0 at 50°C. The enzyme was able to retain 65% activity at pH 6.0 followed by 45% at 4.0, 27% at 7.0, 18% at 8.0, and 13% at 9.0 and was completely inactivated at pH 10.0. The enzyme was stable at pH 4.0 to 6.0. After 24 h, 86% of the activity remained at pH 5.0 where 50% remained at pH 6.0. Maximal enzyme stability was displayed at pH 4.0 and the optimal pH of the purified enzyme was seemed to be acidic, ranging from 4.0 to 7.0. It is similar to that of *A. oryzae* [30], *Neurospora*

crassa [31], *A. fumigatus* [32], and *Stachybotrys* sp. [27].

Effect of Organic Solvents

It was observed that organic solvents had a stimulating effect on β-glucosidase activity especially alcohols at lower concentrations. Among the solvents tested, ethanol was the most effective one, which could enhance the activity 41% at a concentration of 10% (v/v) followed by methanol (30%) and butanol (21%). Propanol did not inhibit the enzyme activity but it reduced all the concentrations tested. Methanol could enhance the enzyme activity at lower concentrations of 10 and 20% whereas all the others, butanol and ethanol led to increase in the activity only at 10% level. The alcohol tolerant enzymes were reported to have some transglucosylation activities [24]. It is inferred that the purified β-glucosidase from *A. sydowii* BTMFS 55 may have transglucosylation activity. Activation of enzyme by ethanol was also reported from *A. tubingensis* [33], *A. oryzae* [2], *Candida peltata* [26], and *Fusarium oxysporum* [34].

Substrate Specificity

Though the enzyme showed high reactivity towards *p*NPG (control, 100%), it hydrolyzed natural oligosaccharides having (1-4)-β-glycosidic linkages, such as cellobiose and lactose, having a relative initial rate of hydrolysis of 0.56 and 2.14%, respectively. The enzyme could also hydrolyze maltose, which have (1-4)-α-glycosidic linkage with a relative hydrolysis rate of 11.66%, but it was totally inactive on sucrose, trehalose, and raffinose. The enzyme showed high affinity towards the *p*NPG than the cellobiose and other disaccharides tested. Plant *et al.* [35] suggested that the preference of β-glucosidases for aryl glycosides is due to the high electrophilicity of the aglycone moiety, which enhances the stability of the *ortho* or *para* nitrophenoxide anion generated during the first step of catalysis. The β-glucosidase from thermophilic fungus, *Talaromyces thermophilus* showed an affinity of lactose and maltose than cellobiose with relative activities of 75, 61, and 6%, respectively [36]. The β-glucosidase of *A. sydowii* BTMFS 55 also showed such a result that it hydrolyses maltose and lactose more efficiently than cellobiose. β-glucosidases with very broad specificity have been isolated from many fungi [5,37,38].

Enzyme Kinetics

The reaction kinetics of the purified enzyme was determined from Lineweaver-Burk plots with *p*NPG under optimal conditions (30 min, pH 8.0, 50°C). The enzyme had an apparent K_m value of 0.67 mM and a V_{max} value of 83.3 U/mL for the hydrolysis of *p*NPG. The specificity constant (V_{max}/K_m) for the *p*NPG hydrolysis was also determined as 123.37. The molar activity (k_{cat}) and catalytic efficiency (k_{cat}/K_m) of the enzyme were calculated to be 2.14×10^{-2} s^{-1} and 3.19×10^{-2} $mM^{-1}s^{-1}$, respectively. The K_m value of *A. sydowii* BTMFS 55 β-glucosidase supported with that of *A. oryzae* and *A. phoenicis* which have a lower K_m of 0.55 mM and 0.58 mM, respectively [2]. *Thermomyces lanuginosus*, *A. fumigatus* (0.075 mM each) and *Phanerochaete chrysosporium* (0.096 mM) were also exhibited a much lower K_m [32,39,40].

Effects of Glucose on pNPG Hydrolysis

The inhibition constant (K_i) is an indication of how potent an inhibitor is and it is the concentration required to produce half maximum inhibition. The plotting of 1/V against concentration of inhibitor (glucose) at each concentration of substrate (*p*NPG) yielded a family of intersecting lines and the convergence of lines above the X-axis showed the glucose was a competitive inhibitor. The K_i value of glucose was obtained as 17 mM from the intersection of the line of the Dixon plot. Competitive inhibition by glucose is a common characteristic of fungal β-glucosidases and most microbial enzymes show inhibition constants of 0.6 to 8 mM for glucose [41]. The K_i values of *Aspergillus* sp. have been reported to range from 3 to 14 mM [24]. The availability of β-glucosidase insensitive to glucose have a significant impact on the enzymatic conversion of cellulosic biomass to glucose for the subsequent production of fuel ethanol. Glucose tolerant fungal β-glucosidases were reported from *A. oryzae* (136 mM), *A. niger* (543 mM), *Candida peltata* (140 mM), *Pyrococcus furiosus* (300 mM), and *A. tubingensis* (470 and 600 mM) by various authors [2, 24,26,33,42].

Ethanol Production from Wheat Bran and Rice Straw

The production of ethanol from wheat bran and rice straw with help of *S. cerevisiae* in the presence cellulase enzyme and purified β-glucosidase from *A. sydowii* BTMFS 55 showed a considerable yield of ethanol production in range of 24 to 48 h of incubation (Table 6). A time course study of wheat bran fermentation resulted in a yield of 11.22 g/L ethanol, after 48 h, whereas in the case of rice straw, the production was maximal at 24 h (7.15 g/L) (Figs. 5 and 6). It was reported that the ethanol production rate was much higher at the initial stage of the fermentation such as 22 h in the case of wheat straw by a recombinant *E. coli* strain [43]. An incubation period of 24 h has been found to be optimum for production of ethanol by *S. cerevisiae* from acid and enzymatic hydrolysate of agricultural residues [44]. The fermentation of pretreated poplar wood and wheat straw by *Kluyveromyces marxianus* yielded 19 and 18 g/L ethanol in 72 and 82 h, respectively [45]. Ethanol has been produced from a variety of substrates such as sunflower hull [46], raw corn flour [47], lignocellulose from a weedy creeper and sugar cane leaves [48], barley husk [49], and water hyacinth [50].

CONCLUSION

β-Glucosidase is an industrial enzyme known for its applications in saccharification of cellulose, degradation of the anthocyanins into sugars and anthocyanidins, processing of juices and wines, flavour enhancement, and production of ethanol from lignocellulosic biomass. There is a need for better and newer source of this enzyme for industrial applications as the sources are limited. In the present study *A. sydowii* BTMFS 55, producing copious amount of β-glucosidase enzyme, was isolated from the marine environ-

Table 6. Ethanol production from pretreated wheat bran and rice straw by *S. cerevisiae*

Substrate (4%)	Fermentation time (h)	Sugar (g/L)	Ethanol (g/L)	Ethanol (g/g)
Wheat bran	24	36.4	9.98	0.25
	48	31.6	11.22	0.28
	72	13.9	6.83	0.17
	96	13.9	5.96	0.15
	120	13.2	5.35	0.13
Rice straw	24	15.5	7.15	0.18
	48	7.4	3.57	0.09
	72	6.9	3.49	0.09
	96	4.6	1.65	0.04
	120	4.1	1.37	0.03

Substrate concentration, 4% (w/v). Enzyme: cellulose, 6 FPU/g; β-glucosidase, 12 FPU/g. Ethanol (g/g), g ethanol/g cellulose (expressed as potential glucose) in pretreated raw materials.

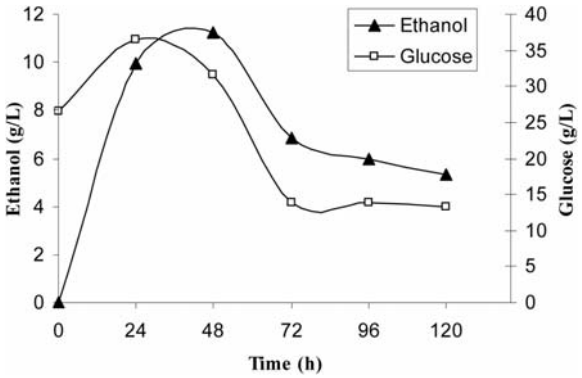

Fig. 5. Ethanol and glucose concentration profiles during simultaneous saccharification and fermentation of wheat bran by *S. cerevisiae*. Substrate, 4%; Cellulase, 6 FPU/g; β-glucosidase, 12 FPU/g; and incubation at 38°C.

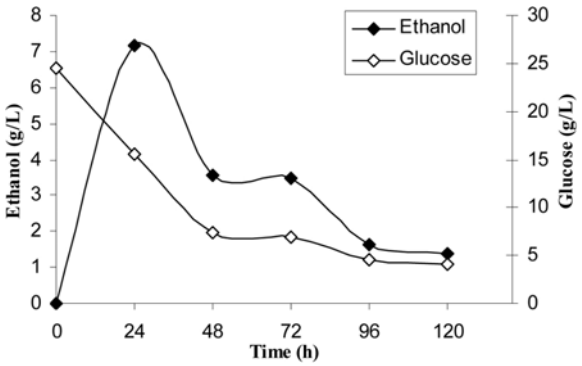

Fig. 6. Ethanol and glucose concentration profiles during simultaneous saccharification and fermentation of rice straw by *S. cerevisiae*. Substrate, 4%; Cellulase, 6 FPU/g; β-glucosidase, 12 FPU/g; and incubation at 38°C.

ment. The critical medium components for attaining high yields of β-glucosidase under SSF were optimised by using statistical experimental design. It offers a practicable approach to the implementation of medium optimization. From an industrial point of view, the optimum medium for β-glucosidase production needs only peptone for the maximal production with appropriate moisture level and inoculum concentration. A high similarity was observed between the predicted and experimental results, which reflected the accuracy and applicability and further, a good coverage of interactions could be explained. Hence, the statistically based experimental design is an efficient tool in this optimization process in a short term experiment. The purified enzyme showed low pH activity and stability, glucose tolerance, and activation by ethanol. It could produce ethanol from wheat bran and rice straw by simultaneous saccharification and fermentation with yeast. There is immense scope for the application of this marine fungus in the biofuel production besides in other industries provided further studies are pursued in exploiting this enzyme and the organism particularly scale up studies with respect to application.

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