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# Production and Characterization of Multi-Polysaccharide Degrading Enzymes from *Aspergillus aculeatus* BCC199 for Saccharification of Agricultural Residues

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Enzymatic hydrolysis of lignocellulosic biomass into fermentable sugars is a key step in the conversion of agricultural by-products to biofuels and value-added chemicals. Utilization of a robust microorganism for on-site production of biomass-degrading enzymes has gained increasing interest as an economical approach for supplying enzymes to biorefinery processes. In this study, production of multi-polysaccharide-degrading enzymes from *Aspergillus aculeatus* BCC199 by solid-state fermentation was improved through the statistical design approach. Among the operational parameters, yeast extract and soybean meal as well as the nonionic surfactant Tween 20 and initial pH were found as key parameters for maximizing production of cellulolytic and hemicellulolytic enzymes. Under the optimized condition, the production of FPase, endoglucanase, β-glucosidase, xylanase, and β-xylosidase was achieved at 23, 663, 88, 1,633, and 90 units/g of dry substrate, respectively. The multi-enzyme extract was highly efficient in the saccharification of alkaline-pretreated rice straw, corn cob, and corn stover. In comparison with commercial cellulase preparations, the BCC199 enzyme mixture was able to produce remarkable yields of glucose and xylose, as it contained higher relative activities of β-glucosidase and core hemicellulases (xylanase and β-xylosidase). These results suggested that the crude enzyme extract from *A. aculeatus* BCC199 possesses balanced cellulolytic and xylanolytic activities required for the efficient saccharification of lignocellulosic biomass feedstocks, and supplementation of external β-glucosidase or xylanase was dispensable. The work thus demonstrates the high potential of *A. aculeatus* BCC199 as a promising producer of lignocellulose-degrading enzymes for the biomass conversion industry.

**Keywords:** Cellulase, hemicellulase, solid-state fermentation, response surface methodology, biomass saccharification, biorefinery

## Introduction

Lignocellulosic feedstock is considered as a promising renewable raw material and carbon source for the production of second-generation biofuel and valorized chemicals. In comparison with conventional fossil resources, the use of various plant biomass such as agricultural and forestry by-products and energy plants can provide environmental benefits and promote the energy security of the country [6]. The core concept of lignocellulose bioconversion constitutes three sequential steps, involving the pretreatment of lignocellulosic materials for lignin deconstruction, enzymatic hydrolysis of holocellulose into sugars, and fermentation of sugars to produce biofuel and biochemicals [20]. The major impediments for the commercialization of a lignocellulose-based sugar production platform are the low hydrolysis rate and the relatively high cost of enzyme used in the enzymatic saccharification process [33]. To overcome these limitations, establishment of an enzyme production platform that can produce a large quantity of enzymes economically,

and development of a powerful enzyme system that can efficiently convert cellulose and hemicellulose into sugars should be addressed.

Enzymatic saccharification of the recalcitrant plant biomass feedstocks generally requires a mixture of cellulases, hemicellulases, and other auxiliary enzymes that cooperate in a synergistic fashion to degrade the substrate [32]. Basically, cellulases consist of three enzymes: endoglucanase (EG; E.C. 3.2.1.4), cellobiohydrolase (CBH; E.C. 3.2.1.91), and β-glucosidase (BGL; E.C. 3.2.1.21), which act cooperatively on cellulose hydrolysis. Hemicellulases contain a variety of enzymes responsible for the degradation of heterogeneous hemicellulose polymers. Complete degradation of xylan, the major component of hemicelluloses, requires the action of endo-1,4-β-xylanase (XYL; E.C. 3.2.1.8), β-xylosidase (BX; E.C. 3.2.1.37), α-glucuronidase (E.C. 3.2.1.139), α-L-arabinofuranosidase (E.C. 3.2.1.55), and acetyl xylan esterase (E.C. 3.1.1.72) [18]. Production of these cellulolytic and hemicellulolytic enzymes can be performed by various filamentous fungal species, including *Trichoderma reesei* that is the most well-known cellulase producer. Although *T. reesei* cellulases contain high cellulolytic activity, the insufficient amount of β-glucosidase in the native fungal system has restricted the hydrolysis yield of the lignocelluloses degradation by the *T. reesei* cellulases [34]. Therefore, it requires supplementation of β-glucosidase and other accessory enzymes to provide more efficient hydrolysis of the lignocellulose substrate [12].

In a previous study, we had successfully established a ternary enzyme mixture consisting of commercial *T. reesei* cellulase, *Aspergillus aculeatus* multi-enzyme preparation, and *Bacillus subtilis* expansin, which is highly active on degradation of pretreated rice straw compared with the *T. reesei* cellulases alone [31]. It has been revealed that the *A. aculeatus* crude enzyme is capable of acting synergistically with commercial *T. reesei* cellulases and contains not only cellulolytic enzymes, but also a large variety of the hemicellulolytic enzymes endo-1,4-β-xylanase, β-xylosidase, α-glucuronidase, endo-1,4-β-mannanase, α- and β-galactosidase, α-L-arabinofuranosidase, and acetyl hydrolase. *A. aculeatus* has also been reported to produce α-amylase and a number of polysaccharide-degrading enzymes that could be applied for non-thermal saccharification of cassava pulp with no prior pre-gelatinization step of starch at high temperature [29]. Despite the fact that this fungal species has great potential in biotechnological applications, little is known about optimization of the media and conditions for enzyme production from *A. aculeatus*, the characteristics of their multiple plant polysaccharide-degrading enzymes, and their ability on biomass saccharification.

Here, we report the optimization of biomass-degrading enzyme production from *A. aculeatus* BCC199 in solid-state fermentation, which is the important process for economical enzyme production [14]. By using a statistical approach, the important parameters that significantly contribute to the productivity of cellulolytic and hemicellulolytic enzymes were identified by using Plackett-Burman design (PBD). The optimal condition for the simultaneous production of cellulases and hemicellulases at high level was subsequently studied by using response surface methodology (RSM). The crude fungal enzyme was then characterized and used for saccharification of various agricultural residues compared with widely used commercial cellulases. The work demonstrates the potential of *A. aculeatus* BCC199 as a promising candidate for on-site enzyme production in the biorefinery industry.

## Materials and Methods

### Lignocellulosic Biomass and Pretreatment

Various agricultural residues, including corn cob, corn stover, rice straw, wheat bran, rice bran, and cassava pulp, were obtained locally and were physically ground using a disk mill (model FFC-23; Asako) with sieve size of 1.2 mm, before used as substrates for biomass-degrading enzyme production in solid-state fermentation. When necessary, the biomass was pretreated with 1% (w/v) NaOH or 1% (v/v) $H_2SO_4$ (with a liquid/solid ratio of 3/1) at room temperature for 2 h or at 90°C for 90 min. The pretreated biomass was washed with distilled water until the pH became neutral and then dried at 60°C. For enzymatic hydrolysis, the agricultural residues were pretreated with 5% (w/v) NaOH at 90°C for 90 min in an autoclave machine. The pretreated biomass was washed with distilled water until neutral pH was obtained. The biomass was then dried at 60°C.

### Microorganism and Enzymes

The filamentous fungus *A. aculeatus* BCC199 obtained from Thailand's BIOTEC Culture Collection (www.biotec.or.th/bcc) was used in this study. The fungus was grown on potato dextrose agar (PDA) plates with incubation at 30°C for 5 days and stored at 4°C until used. For commercial enzymes, cellulase from *Trichoderma reesei* ATCC 26921 (Celluclast1.5 L) was obtained from Novozymes (Bagsvaerd, Denmark), and two cellulases from genetically modified *T. reesei* (SpezymeCP, Accellerase1500) were obtained from Genencore (Rochester, NY, USA).

### Culture Conditions

Solid-state fermentation was conducted in 250 ml Erlenmeyer flasks containing 5.0 g of each agricultural residue as a carbon source and 2.1 g of soybean meal as a nitrogen source. Soybean

meal (7.94% N as determined by the Kjeldahl method) was purchased locally for used in all experiments. The solid medium was moistened by addition of 6.4 ml of basal medium containing 15.6 g/l yeast extract, 15.6 g/l peptone, 7.8 g/l $KH_2PO_4$, 17.6 g/l $Na_2HPO_4$, 1.2 g/l KCl, 0.1 g/l $ZnSO_4 \cdot 7H_2O$, 0.1 g/l $FeSO_4 \cdot 7H_2O$, 0.02 g/l $CuSO_4 \cdot 5H_2O$, and 0.004 g/l $NiCl_2 \cdot H_2O$. The medium was then mixed and sterilized. The spore suspension was prepared in 0.1% (v/v) Tween 20 solution and inoculated into the culture medium at $1 \times 10^7$ spore/g biomass to give the moisture level of 60%. The SSF culture was incubated at 30°C for 5 days under static condition. For harvesting, 50 ml of 50 mM sodium acetate buffer (pH 5.0) supplemented with 0.1% (v/v) Tween 20 was added and the flask was shaken at 150 rpm for 1 h. The cells and residual substrates were removed by centrifugation at 12,857 ×*g* for 10 min, twice. The supernatant was used for enzyme activity assay. For the hydrolysis experiment, the selected crude enzyme extracts were concentrated by ultrafiltration on a Minimate tangential flow filtration (TFF) system using a Minimate TFF capsule with a 10-kDa MWCO membrane (Pall Corp, Ann Arbor, MI, USA). The enzyme solutions were kept at 4°C until used.

**Enzyme Assays**

The cellulolytic and hemicellulolytic enzyme activities were measured as previously described [33]. In brief, filter paper activity (FPase) was determined in the 1 ml reaction mixture containing a piece of 1 × 1.5 cm Whatman No. 1 filter paper in 50 mM sodium acetate buffer (pH 5.0) and the reactions were incubated at 50°C for 60 min. For endoglucanase and xylanase activities, the assay was conducted in the reaction mixture comprising 50 mM sodium acetate buffer (pH 5.0) and 1% (w/v) carboxymethyl cellulose (CMC) or 1 (w/v) % beechwood xylan as substrate for endoglucanase or xylanase, respectively. The amount of reducing sugars that were liberated was measured by the 3,5-dinitrosalicylic acid (DNS) method [21]. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 μmol of glucose or xylose per minute under the assay conditions. Activities of β-glucosidase and β-xylosidase were measured with 40 mM *p*-nitrophenyl-β-D-glucopyranoside (*p*NPG; Sigma) or *p*-nitrophenyl-β-D-xylopyranoside (*p*NPX; Sigma) as a substrate, respectively. The assays were carried out in a 96-well plate containing 10 mM sodium acetate buffer (pH 5.0) and incubated at 50°C for 10 min. Then, a final concentration of 100 mM $Na_2CO_3$ was added into the well in order to terminate the enzyme reaction. The quantity of *p*-nitrophenolate was measured spectrophotometrically at 405 nm. One unit of enzyme refers to the amount of enzyme that produces 1 μmol *p*-nitrophenol per minute under the assay conditions. U/gds represents an enzyme unit per g of dry substrate. In the case of the hydrolysis experiment, the standard measurement of total cellulase activity as recommended by the International Union of Pure and Applied Chemistry (IUPAC) was performed in a reaction mixture containing 1 × 6 cm Whatman No. 1 filter paper and 50 mM citrate buffer (pH 4.8) [11]. The total protein concentrations of the crude enzyme extracts were determined using Bradford's method with the Bio-Rad protein assay reagent (Bio-Rad, Hercules, CA, USA) and bovine serum albumin (BSA) as the standard protein.

**Statistical Optimization of Enzyme Production**

An experimental design approach was applied for determination of the optimal condition for cellulase and hemicellulase production by *A. aculeatus* BCC199. At first, Plackett-Burman design (PBD), which is a 2-level factorial design, was carried out for screening of significant factors that affected the production level of cellulases (FPase, endoglucanase, β-glucosidase) and hemicellulases (xylanase and β-xylosidase) without consideration of interaction among variables. In this study, 10 independent factors were tested at low (-1) and high (1) concentrations and 12 different experimental conditions were generated using Minitab 16.0 software (Minitab Inc., State College, PA, USA). These experiments were conducted in quadruplicate. After analysis of the experiment, the most significant factors that play critical roles in cellulase and hemicellulase production were selected for the following optimization by the response surface methodology (RSM).

The optimum condition for enzyme production was studied by the RSM approach. In this study, the Box-Behnken design (BBD), with the experimental point consisting of the middle points of the edges and center point, was applied in order to avoid the extreme value of variables. The initial pH and concentration of soybean meal, yeast extract, and Tween20 were varied at three levels (high, middle, and low). A set of 24 experimental points plus three replicates at the center points were generated and tested in quadruplicate. In this experiment, all chemical compositions in basal medium except the yeast extract were kept constant, initial moisture was fixed at 60%, and the number of spores was always applied at $1 \times 10^7$ spore/g. Production levels of FPase, endoglucanase, β-glucosidase, xylanase, and β-xylosidase served as response variables ($Y_1$-$Y_5$) for the multiple regression analysis and were fitted to a second-order polynomial equation according to this following equation:

$$Y = \beta_0 + \sum_{i=1}^{4} \beta_i x_i + \sum_{i=1}^{4} \beta_{ii} x_i^2 + \sum_{i=1}^{3} \sum_{j=i+1}^{4} \beta_{ij} x_i x_j \quad (1)$$

where Y is a predicted response, $\beta_0$ is a constant, $\beta_i$ is a linear coefficient, $\beta_{ii}$ is a quadratic coefficient, $\beta_{ij}$ is an interaction coefficient, and x is a coded level of the independent variable [24].

Then, the statistical data and iso-response contour plots were generated. The predicted optimal condition for maximum production of cellulases and hemicellulases was verified by performing the experiment at the prediction point.

**Multi-Enzyme Characterization**

The effects of temperature and pH on the activities of the composite enzyme in the crude enzyme preparation were studied. The activities of FPase, endoglucanase, β-glucosidase, xylanase, and β-xylosidase were measured at the same condition described above (pH 5.0), except that temperature was changed from 30°C

to 70°C. For the influence of pH, the enzyme activities were determined at constant temperature (50°C) with different pH ranging from 3 to 10 by using different buffer systems; namely, 50 mM glycine-HCl (pH 3.0–3.5), 50 mM sodium acetate (pH 4.0–6.0), 50 mM citrate phosphate (pH 6.5–7.0), 50 mM phosphate (pH 7.5–8.0), and 50 mM Tris-HCl (pH 8.5–10.0).

**Zymogram Analysis**

An amount of crude BCC199 enzyme (5 mg) was separated by 10% non-denaturing polyacrylamide gel electrophoresis (PAGE) at 90 V for approximately 3 h at 4°C. After electrophoresis, zymograms for endoglucanase and xylanase were examined by using a previously described procedure [7] with some modifications. The PAGE gel containing crude BCC199 enzyme was incubated in 50 mM sodium acetate buffer (pH 5.0) for 15 min at room temperature and then overlaid with a polyacrylamide gel containing 2% (w/v) CMC or 0.5% (w/v) beechwood xylan for 2 h at 50°C. After that, the overlaid gel was discarded and the PAGE gel containing crude BCC199 was stained with 0.2% (w/v) Congo red solution for 40 min. Destaining was carried out by immersing the gel in 1 M NaCl and then 10% (v/v) acetic acid. Clear bands corresponding to endoglucanase or xylanase isozymes appeared against the dark background. For activity staining of β-glucosidase, the assay was performed using the method of Kwon *et al*. [19] in which 0.1% (w/v) esculin was used as the substrate. Esculetin, a product by action of β-glucosidase, was then reacted with ferric ion to form a black precipitate, revealing the enzyme bands. All assays were repeated at least two times.

**Enzymatic Hydrolysis of Biomass**

The enzymatic hydrolysis of alkaline-pretreated biomass was conducted in 1.5 ml tubes with a total reaction volume of 1 ml containing 5% (w/v) pretreated biomass, 50 mM sodium acetate buffer (pH 5.0), 1 mM sodium azide, and 7.5 FPU/g enzyme loading or 15 FPU/g enzyme only for Napier grass. The hydrolysis reaction was incubated at 50°C with 200 rpm shaking for 48 h and was performed in quadruplicate. The total reducing sugar product was analyzed using the DNS method. The monomeric sugar yields were analyzed on the Water 2960 high-performance liquid chromatography system (Waters, Milford, MA, USA) equipped with a refractive index detector using an Aminex HPX-87H column (Bio-Rad, Hercules, CA, USA). The column temperature was set at 65°C. The samples were eluted at a flow rate of 0.6 ml/min with 5 mM $H_2SO_4$ in doubly deionised water.

## Results and Discussion

### Effects of Carbon and Nitrogen Sources and Pretreatment Methods

In solid-state fermentation, carbon and nitrogen sources could be considered as the most critical factors, since they

**Table 1.** Effects of carbon sources, nitrogen sources, and pretreatment methods on enzyme production.

| Treatment | Enzyme activity (U/gds) | | | | |
|---|---|---|---|---|---|
| | FPase | EG | XYL | BGL | BX |
| C source | | | | | |
| Cassava pulp | 8.23 | 390.74 | 252.34 | 15.72 | 25.56 |
| Rice straw | 9.66 | 445.85 | 598.92 | 24.00 | 41.67 |
| Wheat bran | 3.77 | 331.30 | 274.85 | 20.75 | 21.01 |
| Rice bran | 6.96 | 458.33 | 494.37 | 14.85 | 29.41 |
| Corn cob | 13.86 | 565.42 | 1049.70 | 41.36 | 61.40 |
| Corn stover | 11.38 | 514.39 | 860.29 | 42.75 | 30.22 |
| N source | | | | | |
| Soybean meal | 12.84 | 556.74 | 1002.48 | 36.90 | 52.65 |
| $NH_4NO_3$ | 10.76 | 359.46 | 605.19 | 19.62 | 50.91 |
| $(NH_4)_2SO_4$ | 10.00 | 385.79 | 985.12 | 19.92 | 36.71 |
| $NH_4Cl$ | 5.34 | 225.92 | 498.56 | 8.65 | 32.52 |
| Urea | 3.33 | 0.00 | 14.96 | 1.37 | 3.97 |
| Pretreatment | | | | | |
| Untreated | 14.74 | 599.80 | 1109.53 | 36.13 | 61.37 |
| Alkaline (RT, 2 h) | 16.03 | 589.86 | 1237.46 | 33.43 | 91.97 |
| Alkaline (90°C, 90 min) | 13.14 | 480.72 | 811.49 | 20.48 | 100.47 |
| Acid (RT, 2 h) | 16.36 | 623.46 | 1472.27 | 37.69 | 76.09 |
| Acid (90°C, 90 min) | 19.80 | 714.45 | 1700.44 | 30.03 | 77.41 |

not only procure the nutrients for microbial growth, but also provide the surface area for cell anchorage [25]. In this study, various kinds of carbon and nitrogen sources were initially tested for cellulase and hemicellulase production by *A. aculeatus* BCC199 strain (Table 1). Among the tested agricultural residues, the most appropriate carbon source for *A. aculeatus* BCC199 was corn cob, which provided the maximum cellulase and hemicellulase production, and corn stover was the second most effective carbon source. The composition of corn cob used in this study is 33.3% cellulose, 38.3% hemicelluloses, and 14.8% lignin. Similar to other reports, the production of endoglucanase, FPase, and β-glucosidase by *A. terreus* and *A. niger* were usually induced in corn stover and wheat bran rather than other substrates [3, 10]. Then, *A. aculeatus* BCC199 was grown in SSF medium containing various organic and inorganic nitrogen sources. It was found that the biosynthesis level of each composite enzyme varied considerably, and soybean meal was the best nitrogen source tested. This result is similar to previous reports that fungi grow more favorably on organic nitrogen source with a high amount of enzyme accumulation [10, 16].

In addition, the effect of acid or alkaline pretreatment methods under ambient temperature and high temperature (90°C) on enzyme production was investigated. From Table 1, corn cob pretreated with acid at 90°C showed approximately 19.1–53.3% increase in production level for FPase, endoglucanase, and xylanase compared with the non-pretreated corn cob, whereas production of β-glucosidase was not improved in any pretreatment conditions. The production level of β-xylosidase was improved up to 49.9–63.7% using alkaline-pretreated corn cob. However, this result differs from the study of *A. niger*, in which cellulase production improved more than 10-fold by using alkaline-pretreated substrates [3]. Owing to little improvement obtained by using these pretreatment methods, untreated corn cob was applied in all subsequent experiments.

**Statistical Optimization of Simultaneous Cellulase and Hemicellulase Production**

In this study, PBD was applied for the screening of critical factors from both culture medium compositions and growth parameters that affected cellulase and hemicellulase production. Ten different parameters, including medium components (soybean, yeast extract, and peptone), culture conditions (initial moisture, initial pH, and inoculum), surfactants (Triton X-100 and Tween 20), and pure celluloses (filter paper and Avicel), were screened for the effects on cellulase and hemicellulase production levels. In total, 12 experimental runs, where each factor was tested at high and low levels, were examined by incubation at 30°C for 5 days. It was found that yeast extract, which provided vitamins, amino acids, and nitrogen to the organism, had a significant positive effect on the production of all enzymes (***$p < 0.001$) with high magnitude of effect values (Table S1). The initial pH had a substantial effect on FPase and xylanase productivities. The Tween 20 and soybean meal were also selected for further optimization based on their relatively high effects on the production level of endoglucanase and β-glucosidase, respectively. However, peptone and filter paper were omitted from the medium owing to their insignificant and negative effects for each enzyme in PBD analysis.

Then, the optimum production condition and interaction between the variables for co-production of cellulases (endoglucanase, FPase, and β-glucosidase) and hemicellulases (xylanase and β-xylosidase) were determined by the BBD method. In the BBD experiment with four independent variables (Table S2), a set of 24 experimental runs were performed with three additional runs at the center point. Production levels of each composite enzyme were found to vary greatly under the different conditions tested (Table S3). Multiple regression analysis revealed the response model for each enzyme production level with the coefficient of determination ($R^2$) ranked from 90.1–98.1% and insignificant "lack of fit" tests. These results suggested that the estimated second-order polynomial models for all five enzyme activities were fitted effectively to the experimental data and could be adequately applied for response prediction.

From Table 2, the production of all five enzymes was significantly affected by yeast extract and soybean in both linear and square terms with prominent coefficients (***$p < 0.001$). This result confirmed the crucial role of yeast extract (supplying nitrogen and vitamin sources) and soybean meal (supplying nitrogen source) on fungal growth and enzyme production. In addition, initial pH had significant effects (***$p < 0.001$) in both linear and square terms on all enzyme production except endoglucanase, and Tween 20 had significant effects (***$p < 0.001$) in both linear and square terms on xylanase and β-glucosidase production. Moreover, the interaction between yeast extract and pH was significant (***$p < 0.001$) for all enzymes.

The iso-response contour plots (Figs. 1A–1C) depicted the effects of yeast extract, soybean, initial pH, and Tween 20 on the production yield of FPase, endoglucanase, and β-glucosidase. Interestingly, the optimal production of FPase (20 U/g of dry substrate (gds)), endoglucanase (660 U/gds),

**Table 2.** Multiple regression analysis (ANOVA) results.

| Terms | FPase | EG | BGL | XYL | BX |
|---|---|---|---|---|---|
| Constant | 19.70** | 651.92** | 86.06** | 1306.45** | 91.06** |
| $x_1$ (yeast extract) | -0.90** | -24.05** | 9.07** | 64.03** | 10.49** |
| $x_2$ (Tween 20) | -0.04 | 6.87** | 3.39** | 20.29* | 3.13** |
| $x_3$ (pH) | 0.46** | -13.44** | 2.21** | 40.15** | 5.61** |
| $x_4$ (soybean) | 0.76** | 48.33** | 4.35** | 118.68** | -1.83** |
| $x_1^2$ | -0.30** | -30.20** | -17.86** | -33.77** | -13.36** |
| $x_2^2$ | -0.77** | 4.03 | -2.95** | 67.52** | 0.27 |
| $x_3^2$ | -0.32** | -3.74 | 2.34** | 45.65** | -5.99** |
| $x_4^2$ | -0.96** | -24.75** | -8.33** | -68.37** | -6.69** |
| $x_1x_2$ | -0.84** | -6.84* | 1.58** | -48.69** | 0.24 |
| $x_1x_3$ | 0.28** | 23.15** | 13.54** | 34.77** | 3.54** |
| $x_1x_4$ | -0.11 | -6.28* | -13.53** | -94.81** | -4.86** |
| $x_2x_3$ | 0.54** | 21.01** | 5.82** | 66.31** | 0.27 |
| $x_2x_4$ | 0.48** | -25.76** | 0.88 | -32.84** | -2.17* |
| $x_3x_4$ | 0.03 | -9.98** | -8.87** | -7.33 | 3.16** |
| $R^2$ | 91.04% | 93.61% | 98.12% | 90.15% | 91.06% |
| Analysis of variance | | | | | |
| *F* value | 67.47 | 97.32 | 345.96 | 60.83 | 67.68 |
| Regression | ** | ** | ** | ** | ** |
| Lack of fit | Insignificant | Insignificant | Insignificant | Insignificant | Insignificant |

*$p < 0.05$; **$p < 0.001$.

and β-glucosidase (85 U/gds) could be achieved at similar conditions, where approximately 0.1 g/gds yeast extract, 0.85–1.05 g/gds soybean, pH 5.5–7, and 0.55–1% Tween 20 were applied. Furthermore, the optimal production for xylanase (1,550 U/gds) and β-xylosidase (98 U/gds) production was obtained at the identical initial pH and concentration of Tween 20 as for the cellulase production (Figs. 1D–1E). However, 0.15–0.18 g/gds yeast extract and 0.6–0.8 g/gds soybean were preferable for xylanase and β-xylosidase production. In this study, it was found that addition of the nonionic surfactant Tween 20 had a positive effect on secreted enzyme yield. This nonionic surfactant was assumed to increase the permeability of the cell membrane as well as promote the release of cell-bound enzymes [26]. Similar results were found in studies of *A. fumigatus* and *Melanocarpus* sp., that cellulase secretion was improved with the addition of surfactants [15, 30].

For simultaneous production of all enzymes, the optimum culture condition was estimated by Minitab software. At the optimum culture condition, the predicted production level of FPase, endoglucanase, and β-glucosidase were at 20, 661, and 88 U/gds, respectively. The validation experiment at the optimum condition revealed that the actual production yield of FPase, endoglucanase, and β-glucosidase were in agreement with the predicted amounts and corresponded to 23, 663, and 88 U/gds, respectively. These evidence suggested that the model of FPase, endoglucanase, and β-glucosidase was effective. The predicted production levels of xylanase and β-xylosidase were at 1,508 U/gds, and 91 U/gds, respectively, and the validated production levels were 1,633 U/gds of xylanase and 90 U/gds of β-xylosidase. The optimized condition offers high-level production of all five enzymes, and using statistical response surface methodology, the enzyme production levels were improved significantly, ranging from 1.2–2.1 times, especially for β-glucosidase where the production was increased 2.1 times of that before optimization. This is in accordance with previous observation in *A. fumigatus*, that the levels of endoglucanase, FPase, and β-glucosidase were improved approximately 1.6 times by using RSM optimization [30]. Moreover, a 1.5-time increase in FPase production was achieved by RSM optimization in *A. terreus* [22]. The highly

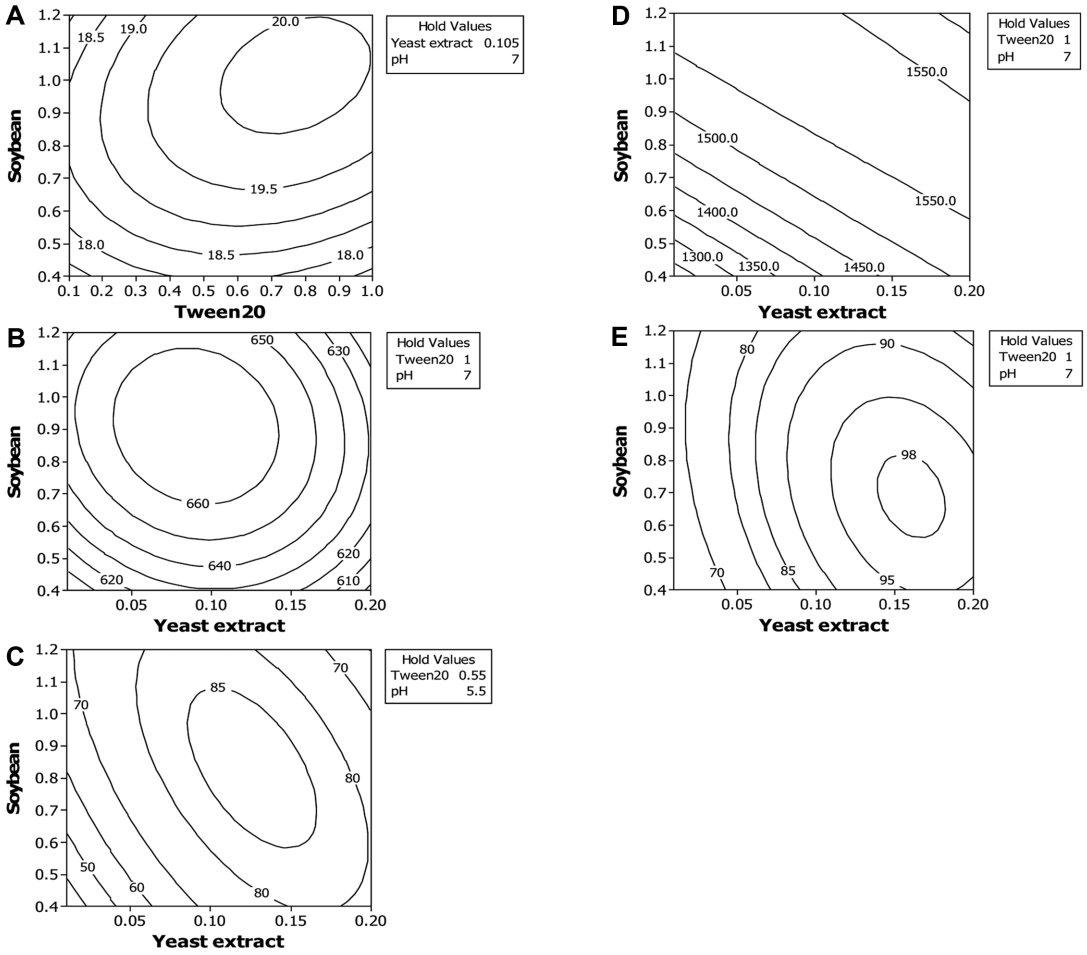


**Fig. 1.** Contour plots showing the effects of independent variables on FPase (**A**), endoglucanase (**B**), β-glucosidase (**C**), xylanase (**D**), and β-xylosidase (**E**) production (U/gds) by *A. aculeatus* BCC199.

efficient co-production of both cellulolytic and hemicellulolytic enzymes by BCC199 in this study was different to that previously reported from solid-state fermentation of *A. foetidus*, where low levels of cellulase and β-xylosidase were found under the optimized condition for xylanase production [5]. The optimized condition was then used for enzyme production for subsequent experiments.

### Multi-Enzyme Characterization

The multi-enzymes produced by solid-state fermentation with *A. aculeatus* BCC199 were partially characterized in terms of influence of temperature and pH. As shown in Fig. 2A, the maximum enzyme activities of FPase, endoglucanase, and xylanase were found at 50–55°C, whereas high activities of β-glucosidase and β-xylosidase were found at higher temperature up to 65–70°C. Similar results have been reported for *A. nidulans*, in which optimum endoglucanase, exoglucanase, and xylanase activities were observed at 50–56°C, and maximum β-glucosidase II activity was at 65°C [2, 9].

The effects of pH on *A. aculeatus* BCC199 multi-enzyme activities were also determined (Fig. 2B). The activities of FPase, endoglucanase, and xylanase were optimum at pH 5–5.5, whereas the maximum activity of β-glucosidase and β-xylosidase was found at pH 3–3.5. It was also found that cellulolytic and hemicellulolytic activities were decreased by 50% of the highest activity when the pH was higher than 7.0–7.5. At pH 10.0, all enzyme activities remained less than 1.9% residual activity, except for FPase where 29.3% residual activity was detected under this condition. These results suggested that the multi-enzyme complex produced by *A. aculeatus* BCC199 was active at mild acidic pH and had a negative effect at neutral or alkaline pH, which is similar to reports for cellulases and hemicellulases from

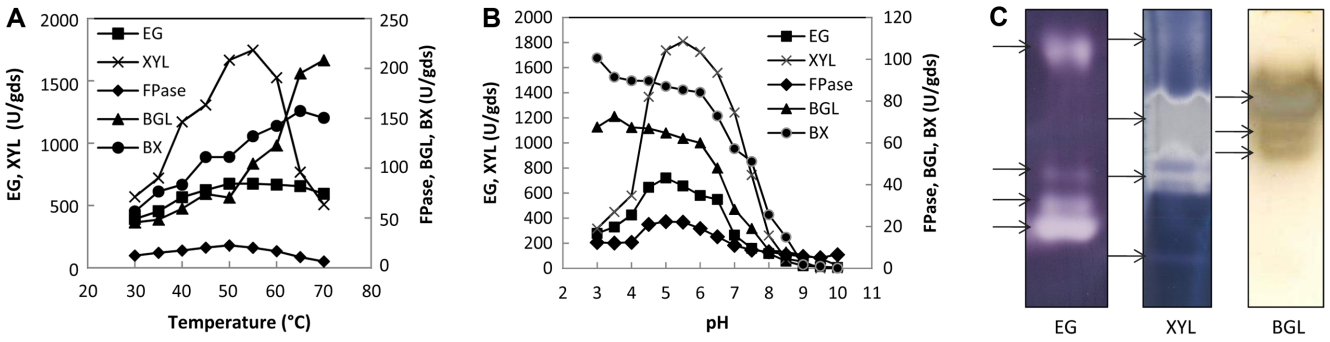


**Fig. 2.** Influence of (**A**) temperature and (**B**) pH on the activities of FPase, endoglucanase (EG), β-glucosidase (BGL), xylanase (XYL), and β-xylosidase and (BX) (**C**) The multiplicity of endoglucanase, β-glucosidase, and xylanase of the *A. aculeatus* BCC199 crude enzyme preparation.

Arrows indicate the multiple isoforms of those enzymes on the polyacrylamide gel.

*A. fumigatus*, *A. japonicus*, and *Chrysoporthe cubensis* [7, 8, 13]. With this evidence, it is important to consider the pH and temperature ranges of the reaction process before utilizing these enzymes for industrial application. Moreover, the temperature and pH ranges for active activity of the BCC199 multi-enzyme resembled that of several commercial cellulase preparations. This would offer the potential use of the BCC199 multi-enzyme as supplementation of auxiliary enzymes to commercial cellulases during the saccharification process.

Endoglucanase, xylanase, and β-glucosidase isoforms of *A. aculeatus* BCC199 crude enzyme were examined through zymogram analysis. On the activity-stained PAGE gel, at least four endoglucanase isoforms, four xylanase isoforms, and three β-glucosidase isoforms were detected (Fig. 2C). The multiplicity of cellulases and hemicellulases in filamentous fungi has been widely accepted as a common occurrence that may reflect the need of suitable enzymes with different specificities to efficiently hydrolyze heterogenic lignocellulosic substrates [4, 7]. Two isoforms of endoglucanase, xylanase, and β-glucosidase were found in the *A. aculeatus* BCC199 crude enzyme produced from submerged cultivation using rice straw as the carbon source (data not shown). Based on this result, the different compositions of lignocellulosic substrates might be one factor regulating the expression of these isozymes. In *A. fumigatus fresenius*, five forms of endoglucanase were expressed when the fungus was grown on rice straw, wheat bran, and wheat straw, whereas four forms of endoglucanase were produced when the carbon source was changed to corn cob [30]. Moreover, differential expression of the endoglucanase and β-glucosidase isoforms during growth on different carbon sources was reported in *A. terreus* and *Myceliophthora* sp. IMI 387099 [1, 23].

**Enzymatic Hydrolysis by Multi-Enzyme from *A. aculeatus* BCC199**

In order to examine the efficiency of *A. aculeatus* BCC199 multi-enzyme on hydrolysis of agricultural residues, comparative analysis with various commercial cellulase preparations was carried out. In this hydrolysis experiment, the *A. aculeatus* BCC199 enzyme preparation was produced in scale-up amount in a 1,000 ml flask under the optimized conditions and then concentrated to 10-folds of the original enzyme volume. The composite enzyme activity profile of the concentrated BCC199 enzyme was compared with other commercial cellulase preparations in regard to enzyme activity units relative to the IUPAC unit of total cellulase activity (U/FPU). This U/FPU index would reasonably facilitate a comparison between enzymes produced in laboratory scale and industrial scale, since they have remarkable differences in size of bioreactors, concentration folds, and additives [8].

From Table 3, it was shown that the concentrated BCC199 multi-enzyme produced in this study contained higher relative activities of endoglucanase, β-glucosidase, xylanase, and β-xylosidase than the other commercial enzymes tested. The main focus is that the concentrated BCC199 enzyme contained up to 11.5 U/FPU of β-glucosidase activity, which was 2.0–11.5 times more compared with those produced from *Trichoderma reesei*-based commercial enzymes (Celluclast1.5L, Accellerase1500, and SpezymeCP). For commercial enzymes, the limited amount of β-glucosidase activity causes the accumulation of cellobiose, leading to the repression of enzyme biosynthesis and end-product inhibition of upstream enzymes, which result in a limited hydrolysis yield [35]. Furthermore, 255.9 U/FPU of xylanase and 11.1 U/FPU of β-xylosidase were expressed in the

**Table 3.** Activity profile of concentrated crude enzymes from *A. aculeatus* BCC199 and different commercial enzyme preparations used in the hydrolysis experiment.

| Enzyme | Unit enzyme activity/ unit FPase | | | | |
|---|---|---|---|---|---|
| | FPase | EG | BGL | XYL | BX |
| BCC199 | 1.0 | 153.1 | 11.5 | 255.9 | 11.1 |
| Celluclast | 1.0 | 37.5 | 1.0 | 13.6 | 1.0 |
| Accellerase1500 | 1.0 | 54.2 | 5.7 | 17.4 | 0.2 |
| SpezymeCP | 1.0 | 57.9 | 1.6 | 34.9 | 0.6 |

The data represent the ratio of units of enzyme activity to one unit of FPase activity (FPU).

concentrated BCC199 enzyme preparation, which are very high compared with the commercial enzymes. The xylanase and β-xylosidase activities are essential for hydrolysis of xylan polymer into monomeric xylose sugar, and addition of xylanases in the hydrolysis reaction was found to improve the glucose yield that was released by the action of cellulases [17].

Comparative study of the concentrated BCC199 enzyme and the selected commercial enzymes on hydrolysis of various biomass feedstocks, including rice straw, corn cob, corn stover, sugarcane bagasse, and Napier grass, was then carried out. To disrupt the recalcitrant lignin structure, all biomass feedstocks were subjected to alkaline pretreatment. The specific activity of the concentrated BCC199 multi-enzyme was 0.5 FPU/mg protein, which was relatively lower than the commercial enzymes (1.6–2.7 FPU/mg protein). Interestingly, the hydrolysis profile showed that the highest total reducing sugar products were obtained with the concentrated BCC199 enzyme in the hydrolysis of pretreated rice straw, corn cob, and corn stover (Fig. 3). As a result of the presence of the relatively high β-glucosidase activity compared with other commercial enzymes tested, the glucose yields obtained by the action of the concentrated BCC199 enzyme on these biomass feedstocks were higher than that of the commercial enzymes. Moreover, the highest xylose yield was produced by the concentrated BCC199 enzyme hydrolysis, which corresponds to its high ratio of xylanase and β-xylosidase activities. The high levels of total reducing sugars released by the concentrated BCC199 enzyme hydrolysis is in agreement with a report that supplementation of xylanase and β-xylosidase into a cellulase preparation (SpezymeCP) was able to boost up the conversion of cellulose and hemicellulose in pretreated biomass [27]. In addition, the presence of xylan and xylooligomers was normally found to dramatically decrease cellulose conversion rates and yields during hydrolysis reaction, by competitively inhibiting cellulase activity [28]. However, for the hydrolysis of pretreated bagasse and Napier grass, Celluclast and SpezymeCP were slightly

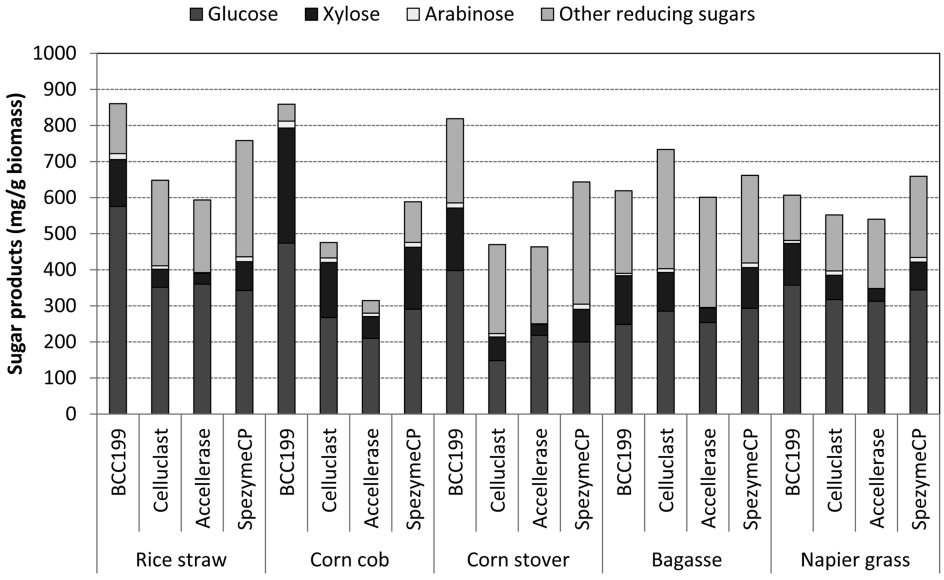


**Fig. 3.** Profile of enzymatic hydrolysis of alkaline-pretreated agricultural residues with secreted enzymes from *A. aculeatus* BCC199 or three commercial cellulases.

The reaction mixture contained 5% (w/v) pretreated biomass, 50 mM sodium acetate buffer (pH 5.0), 1 mM sodium azide, and 7.5 FPU/g enzyme (15 FPU/g for Napier grass). Total protein amounts of the concentrated BCC199 enzyme, Celluclast1.5L, Accellerase1500, and SpezymeCP were 6.8, 47.3, 18.3, and 35.2 g/l, respectively.

more efficient than the concentrated BCC199 enzyme. This result might be due to the different structure and composition of lignocellulosic substrates, which reflects the different requirements of composite enzymes and ratio on their efficient degradation. Our previous research suggested that the *A. aculeatus* BCC199 multi-enzyme complex has the ability to produce various hydrolytic enzymes, and thus offers the possibility of activity complementation with other enzyme systems [31]. Based on the present study, the BCC199 enzyme as a single crude enzyme is highly efficient in the hydrolysis of pretreated rice straw, corn cob, and corn stover. The enzyme extract possesses the balanced cellulolytic and xylanolytic activities required for efficient saccharification of these biomass feedstocks. Alternatively, application of this enzyme as a supplemented enzyme with commercial cellulase application is also valid.

This study demonstrates the optimization of *A. aculeatus* BCC199 multi-enzyme production in solid-state fermentation by using an experimental design approach, where various medium components as well as culture conditions were adjusted. With this approach, the production of core cellulases and hemicellulases was increased 1.2–2.1 times under the optimized condition. In contrast to commercial enzymes investigated in this study, the *A. aculeatus* BCC199 multi-enzyme preparation showed relatively high activity of β-glucosidase, xylanase, and β-xylosidase, which provide a balanced activity for saccharification of both cellulose and hemicellulose fractions of the lignocellulosic biomass. As shown in the hydrolysis of alkaline-pretreated rice straw, corn cob, and corn stover, the BCC199 multi-enzyme was able to produce high yields of both glucose and xylose compared with the commercial enzymes. Therefore, this work demonstrated the potential economical production of *A. aculeatus* BCC199 multi-enzyme for on-site enzyme production, and showed the great opportunity for application of this multi-enzyme in the biomass saccharification process for the biorefinery industry.

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