Efficient production of cellulase in the culture of Acremonium cellulolyticus using untreated waste paper sludge

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Abstract

Cellulase was produced by *Acremonium cellulolyticus* using untreated waste paper sludge (PS) as the carbon source. The clay present in PS did not show any inhibitory effect on cellulase production but did alter the pH during fermentation. On the flask scale, the maleate buffer concentration and pH were key factors that affected the efficiency of cellulase production from PS cellulose. Optimum cellulase production in a 3-L fermentor of working volume 1.5 L was achieved by controlling the pH value at 6.0 using 2 M NaOH and 2 M maleic acid, and the productivity reached 8.18 FPU/ml. When 40.89 g/l PS cellulose, 2.2 g/l $(NH_4)_2SO_4$, and 4.4 g/l urea were added to a 48-h culture, the cellulase activity was 9.31 FPU/ml at the flask scale and 10.96 FPU/ml in the 3-L fermentor. These values are approximately 80% of those obtained when pure cellulose is used as the carbon source. The method developed here presents a new route for the utilization of PS.

Keywords: *Acremonium cellulolyticus*, cellulase, paper sludge, biomass

Introduction

Paper sludge (PS) is the largest solid waste stream produced by the "pulp and paper industry." It is an attractive feedstock for emergent technologies that are based on the processing of cellulosic biomass. PS consists of 24.5% lignocelluloses (90% cellulose and 10% hemicellulose), 10.5% clay, and 65% water. More than 40% of clay is composed of kaolin and silica. Other minerals such as Si, Ti, Al, Fe, Mn, Na, and K are also present. Some PS materials also contain nonglucan carbohydrate (xylan and mannan) (*1*). Incineration and on-site landfills used for PS disposal are running out of storage space and are becoming an environmental concern (*2*). Another possible way to utilize PS based on its high content of polysaccharides (mainly cellulose), it can be further processed to produce cellulase which can be utilized again to hydrolysis PS enzymaticly.

Lignocellulose can be converted to value-added bioproducts. However, the presence of impurities such as clay hinders the accessibility of cellulase to lignocellulose. In many previous studies, a pretreatment step was incorporated to improve the accessibility of hydrolytic enzymes to cellulose. Pretreatment of the main parts of the lignocellulosic material was carried out by various methods such as steam pretreatment, lime pretreatment, liquid hot water pretreatment, and ammonia-based pretreatment (*3*). However, these processes are not environmentally friendly because they require high temperatures and the disposal of acid wastes. Moreover, such pretreatment processes are costly.

Landfills with PS are an environmental and economic problem. The current legislative trend in many countries is to restrict the amount and types of materials permitted in landfills. Therefore, finding alternative uses for PS would be of economic benefit to paper mills and would have a positive environmental effect (*4*). Many countries utilize PS in other ways. For example, in the UK, the annual PS output is nearly 1 million tons wet PS. This PS was treated by combustion to produce paper sludge ash (PSA). 70% of PSA was sold to end users and 30% of it being recycled in landfills since PSA acts as a liming agent and adds to the organic matter levels of soil(*5*). However, combustion of PSA is energy intensive process and one of reasons carbon dioxide evolution. In USA, the use of various pulping processes to convert PS to ethanol fuels is recommended in the Pacific-Northwest (*6*), and the ethanol yields and reactivity were determined by the simultaneous saccharification and fermentation culture (SSF) method. Optimization of the conditions for saccharification by Meicelase (a cellulase from *Trichoderma viride*) was studied, and the optimal conditions were determined to be pH 4.0 and 40°C at an enzyme concentration of 0.1%. This SSF process was used for organic acid production by *Corynebacterium glutamicum* (*7*) or for bioethanol production Untreated PS in the SSF (*8*) was desirable for the bioethanol production. However, to improve yields of glucose and ethanol, pretreatment of PS was applied: Mechanical grinding in a ball mill followed by chemical swelling in phosphoric acid. This method significantly enhances the enzyme saccharification rate and ethanol productivity (*9*).

Filamentous fungi, typically *Trichoderma reesei*, have been used for industrial cellulase production due to their high extracellular protein production capabilities.

Unfortunately, the amount of β-glucosidase secreted by *T. reesei* is insufficient (*10*). *Acremonium cellulolyticus*, which was isolated in 1987 and subsequently engineered to enhance its performance, produces both cellulase and β-glucosidase in addition to CMCase and small amounts of xylanase, β-1,3-glucanase, and amylase, and this organism is an alternative cellulase producer (*11*)(*12*)(*13*). Indeed, the commercial enzymes produced from *A. cellulolyticus* have higher activity than the *Trichoderma* enzymes in terms of hydrolyzing cellulolytic wastes (*13*). However, the *A. cellulolyticus* strain uses Solka Floc (SF) containing 100% cellulose which comprised of 70-80% crystalline cellulose and amorphous cellulose for the rest was used as the carbon source for cellulase production. This is an obstacle in the industrial production of cellulase from *A. cellulolyticus* because the SF as carbon source is expensive. Some researchers produced cellulase from bleached wood pulp and rice straw but they did mechanical, physical or chemical pretreatment (*14*)(*15*). Other researchers who work with *A. cellulolyticus* improved cellulase activity with classical mutagenesis using UV and SF as carbon source (*16*).

In this study, we aimed to produce cellulase from *A. cellulolyticus* using PS cellulose as the carbon source. Due to its composition, PS has not been used for cellulase production by *A. cellulolyticus* cultures. Use of SF as the carbon source in a conventional culture produced 13–15 FPU/ml cellulase at the flask scale, while use of PS yielded only 0.5 FPU/ml (data not shown). PS affects cellulase production because it contains clay, salts, and unknown impurities. This study was undertaken with the objective of developing a method for cellulase production using PS (especially wet PS) cellulose as the carbon source for *A. cellulolyticus* in culture.

Materials and Methods

Paper sludge (PS)

PS was provided by Tomoegawa Paper Co., Ltd. (Shizuoka, Japan). It contained 65% water, 10.5% clay, and 24.5% cellulose on a weight basis. Solka Floc (SF; CAS #9004-34-6; International Fiber Corp., New York, USA) was used as the carbon source for the *A. cellulolyticus* culture. SF is a fine white powder that is used as an industrial filtration material, and contains 100% cellulose which is comprised of approximately 70% - 80% crystalline cellulose and amorphous cellulose for the rest..

Microorganisms and media

A. cellulolyticus Y94 was seeded on SF agar slants, incubated at 28°C for 3.5 days until the colonies grew, and then stored at 4°C. The colonies were brown to reddish-brown. SF cellulose powder was used as the carbon source for *A. cellulolyticus*. The preculture medium contained 40 g/l cellulose powder, 24 g/l KH_2PO_4, 1 ml/l Tween 80, 5 g/l $(NH_4)_2SO_4$, 4.7 g/l potassium tartrate, 1.2 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $ZnSO_4 \cdot 7H_2O$, 0.01 g/l $MnSO_4 \cdot 6H_2O$, 0.01 g/l $CuSO_4 \cdot 7H_2O$, and 2 g/l urea (pH 4.0). The cellulase production medium contained 50 g/l PS cellulose, 1 g/l KH_2PO_4, 1 ml/l Tween 80, 5 g/l $(NH_4)_2SO_4$, 4.7 g/l potassium tartrate, 1.2 g/l $MgSO_4 \cdot 7H_2O$, 0.01 g/l $ZnSO_4 \cdot 7H_2O$, 0.01 g/l $MnSO_4 \cdot 6H_2O$, 0.01 g/l $CuSO_4 \cdot 7H_2O$, and 4 g/l urea.

For flask cultures, 4 colonies were inoculated in a 500-ml Erlenmeyer flask containing 50 ml of preculture medium. The preculture was incubated for 84 h at 28°C

and 220 rpm in a rotary shaker (Takasaki Sci. Instru. Co., Japan). Cellulase production in flasks was carried out by adding 5 ml (10%) of preculture to a 500-ml Erlenmeyer flask containing 50 ml of production medium. The cultures were incubated for 5 days at 28°C and 220 rpm on a rotary shaker (Takasaki Sci. Instru. Co., Japan).

Buffer in media

Acetate, Na-phosphate, K-phosphate, and maleate buffers were used in the medium (*17*). Acetate buffer was used for studies at pH 4.4 and 4.6; maleate buffer at pH 5.2, 5.6, 6.0, and 6.6; sodium phosphate buffers at pH 6.0; and sodium and potassium phosphate buffer at pH 7.0.

pH-controlled cellulase production in a fermentor

Cellulase production was carried out in 3-L jar fermentor equipped with Labo-controller (MDL-80, Marubishi, Tokyo Japan). The working volume was 1.5 L, and the pH was controlled. Distilled water was used to dissolve all components of the production medium, and the system was sterilized at 121°C for 20 min. The appropriate amount of urea was added to 50 ml of distilled water, and the solution was filtered through a 0.45-μm filter. Urea was added to the bioreactor prior to inoculation. Inoculums (15 ml) was added to the starter (150 ml) that contained 50 g/l PS cellulose as the carbon source. Next, 150 ml of starter was added to the bioreactor that contained 1350 ml of production medium. The broth was cultured at 28°C. The pH value was controlled at 4.5, 5.2, 5.7, 6.0, or 6.5 using 2 N NaOH and 2 N H_2SO_4 or 2 M maleic acid. Air was supplied at 1.5 volume of air per volume of medium per minute (vvm) at an agitation rate of 600 rpm. An antifoam agent (Silicone 72, KM-70, Shin-Etsu

142 Chemical Co. Ltd., Japan) was added in small amounts as required. Each experiment
143 was carried out in triplicate; the data are presented as averages.

144 Fed-batch culture

145 To optimize the fed-batch culture conditions, three variables of the feed
146 composition were set (PS cellulose, $(NH_4)_2SO_4$, and urea) at various levels in an
147 Erlenmeyer flask as stated at Table 1.

148 The pH was adjusted every 8–12 h to pH 5.9–6.0. A sample was removed each
149 day, and the cellulase activity was analyzed. The optimized feed conditions were used
150 for culture in the 3-L fermentor, and cellulase production was confirmed.

151 Analytical methods

152 The total cellulase activity was determined by the standard IUPAC procedure
153 using Whatman No. 1 filter paper, and the activity was expressed in filter paper units
154 (FPUs) (*18*). The FPU unit is based on the International Unit (IU), in which the absolute
155 amount of glucose in the FPU assay at a critical dilution is 2 mg for 0.5 ml critical
156 enzyme concentration in 60 min.

157 The cellulase components were analyzed as follows. Avicel (1% w/v, Sigma-
158 Aldrich Chem. GmbH) in acetate buffer (50 mM, pH 5.5) and the diluted enzyme
159 solution were mixed in equal volumes and reacted at 30°C for 120 min with 160 strokes
160 per minute (spm). Sampling was carried out every 15 min. A 180-µl aliquot of CMS
161 (1% w/v, Sigma-Aldrich Chem.) was mixed with 20 µl of diluted enzyme solution in
162 acetate buffer (50 mM, pH 5.5) and reacted at 40°C for 15 min. Sampling was carried

out every 3 min. The reducing sugars that were released were analyzed by the Somogyi-Nelson method (*19*). One unit of enzyme activity was defined as the amount of enzyme required to produce 1 μmol of reducing sugars in 1 min. The β-glucosidase activity was measured using glucose β-para-nitrophenyl (Glcβ-pNP) as the substrate. Aliquots (25 μl) of Glcβ-pNP (20 mM) and the diluted enzyme solution were mixed in acetate buffer (50 mM, pH 5.5) and reacted at 40°C for 10 min. Samples were removed every 3 min. The reaction was stopped with 380 μl of Na_2CO_3 (1 M), and the absorbance at 405 nm was measured. One unit of enzyme activity was defined as the amount of enzyme that released 1 μmol of pNP per min.

Due to the difficulty in separating the mycelium from the medium, the cell weight was measured by the inner nucleic acid (INA) method, and the dry cell weight (DCW) was calculated by the following two equations: INA concentration (g/l) = 1.72 × absorbance and DCW (g/l) = 16:565 × INA (*11*). The absorbance was measured spectrophotometrically at a wavelength of 260 nm.

The protein concentration in the supernatant was determined by the Lowry method using a spectrophotometer (UV-1800, Shimadzu, Kyoto). The concentration of reducing sugars in the medium was determined by the dinitrosalicylic acid method or by high-performance liquid chromatography (HPLC) (PU-980, JASCO, Co. Ltd., Japan) with Shim-pack CLC-NH2 columns (Shimadzu Co., Kyoto, Japan). The mobile phase was 75% acetonitrile solution that was pumped at a flow rate of 2 ml/min. Detection was carried out with a refractive index detector (RI-930, JASCO, Co. Ltd., Tokyo, Japan).

Results

Influence of clay

Cellulase production was investigated by varying the ratios of SF and PS cellulose, which served as the carbon sources. Cellulase activity decreased linearly with an increase in the amount of PS cellulose in the carbon source (Fig. 1). When PS was suspended in the medium, it altered the pH of the culture to neutral or higher pH values. At 20% and 40% PS cellulose, the pH value of the culture was close to that observed at 0% PS cellulose, and the cellulase production was almost the same. However, at PS cellulose values higher than 60%, the pH value reached 6.7, where the cellulase activity decreased. The pH values of 80% and 100% PS cellulose were higher than 7.9 in the cultures, and the cellulase activity was one-third that of the control.

Effects of the buffered medium and pH on cellulase production in flask cultures

To avoid pH changes in the culture, we examined the effects of adding acetate buffer, phosphate buffer, and maleate buffer. When phosphate buffer was added, the initial pH was 6.5, but the value increased to 8 during the course of fermentation (data not shown). Although the cells had grown, cellulase activity was not detected because the produced enzyme was deactivated by the increase in pH. In the case of acetate buffer, the pH value was 4.5, but cell growth and cellulase production were not detected. This is probably because the acetyl groups change the hydrophobicity of cellulose and thereby affect the binding of the cellulose-binding domain (CBD) of cellulase to cellulose. Later, the introduction of acetyl groups increases the dimension (diameter) of the cellulose chain, due to which the chain becomes too big to fit through the tunnel of

207 the enzyme (*20*). Consequently, *A. cellulolyticus* cells could not utilize the carbon
208 source. The pH was maintained in the range of 5.5–6.5 using maleate buffer, and the
209 cellulase activity increased to 6.87 FPU/ml after 4 days of culture (data not shown).
210 Maleate buffers (0.2 M) at pH 5.2, 5.6, 6.0, and 6.6 were used for cellulase production
211 on the flask scale. On the 2nd day of culture, the pH increase in all cultures was
212 approximately 2 because the existence of clay and the buffer capacity was not strong
213 enough to stabilize the pH of culture. The maximum cellulase activities in pH 5.2-, 5.6-,
214 6.0-, and 6.6-maleate buffered media were 6.0, 3.0, 0.3, and 0.1 FPU/ml, respectively
215 (data not shown).

216 Maleate buffer had no inhibitory effect on cell growth and cellulase production
217 in *A. cellulolyticus* cultures, but the culture pH was not maintained at a constant level.
218 To stabilize the pH in the flask culture, the buffer capacity was increased from 0.2 M to
219 0.4M, 0.6M, 0.8M and 1.0 M with initial pH 5.2. When the buffer capacity of 0.8 and
220 1.0 M was in the cultures, the pH at the end of culture was 6.2-6.3. However, at a buffer
221 capacity of 0.4 and 0.2 M, the pH increased to more than 8 in a culture time of 6 d (Fig.
222 2A). The cellulase activity increased until the 3rd day of culture in all cases, but after the
223 3rd day, the activity in cultures with a buffer capacity of 0.2, 0.4, and 0.6 M decreased
224 significantly (Fig. 2B). At 0.8 M to 1 M, the pH did not increase significantly, and the
225 cellulase activity reached 6.83 FPU/ml and 5.68 FPU/ml, respectively. This indicates
226 that the buffer capacity and pH of the culture are important factors in the production of
227 cellulase from PS cellulose.

228 **Cellulase production in a pH-controlled culture in a jar fermentor**

The pH was controlled at five values, i.e., 4.5, 5.2, 5.7, 6.0, and 6.5, using maleic acid as the pH controlling agent. The DCW of the pH 5.2-controlled culture reached 20 g/l (Fig. 3B), but the highest cellulase activity was obtained in the pH 6.0-controlled culture (8.16 FPU/ml) (Fig. 3A). The highest specific enzyme activity was 0.85 FPU/mg protein in the pH 5.2-controlled culture (Fig. 3C). The optimum pH value was the same when Solka Floc was used as the carbon source (*12*).

The concentrations of sub cellulase, CMCase, avicelase, and β-glucosidase were measured and are shown in Fig. 4. The highest CMCase activity was found in the pH 5.2-controlled culture, and the highest avicelase and β-glucosidase activities were observed in the pH 5.7-controlled culture. Sub cellulase activity could not be detected in the pH 6.5-controlled culture.

Optimization of cellulase production using untreated PS

To increase cellulase production, the PS cellulose concentration was increased to 75 g/L. Unfortunately, this had a negative impact on the culture due to mass transfer limitations (*21*). The mass transfer coefficient is a function of the viscosity (μ), particle size (d_p) and velocity flow (U) around the particle, fluid density (ρ), and diffusion coefficient (D_{AB}).

$$k_c \approx 0.6 \frac{U^{0.5} \rho^{1/6} D_{AB}^{2/3}}{\mu^{1/6} d_p^{0.5}}$$

In the initial culture, the high viscosity and large particle size resulted in a very low mass transfer coefficient. This would obstruct the aeration of the culture, due to which *A. cellulolyticus* would not grow well. Therefore, fed-batch cultivation was carried out.

The feed time was set at 48 h of culture (data not shown), and the feed composition was managed at 4 levels each for PS cellulose (from 5 g/l to 60 g/l), $(NH_4)_2SO_4$ (from 0.1 g to 0.4 g), and urea (from 0.4 ml to 1.6 ml). The experimental model is described in the quadratic model developed by Design Expert (ver. 7.1.6). Table 2 shows the ANOVA results. The model that has an F value of 5.51 appears to be significant because there is only 0.01% possibility that such a large model F value could be due to noise. The data shows also the dependent of cellulase production was influenced firstly by PS cellulose which has F value 11.26809 for 1st order and F value 18.60903 for 2nd order or quadratic form. The next variable that influence cellulase production is $(NH_4)_2SO_4$ with F value 4.718047 for 1st order. Urea did not influence cellulase production significantly at 1st order but influence cellulase production in quadratic form or 2nd order. However, the interaction variables among PS cellulose, $(NH_4)_2SO_4$, and urea were not significant. Statistical analysis suggested the use of a quadratic model for the experiments, as shown in Fig. 5. Furthermore, cellulase production of a fed-batch culture in terms of PS cellulose (*A*), $(NH_4)_2SO_4$ (*B*), and urea (*C*) can be represented in mathematical equation as follows:

$$\text{Cellulase activity (FPU/ml)} = 5.42876+0.15672A-2.64217B+4.03016C-0.00204471A^2 -2.22195C^2$$

Based on the results, the optimal feed composition was set as 40.89 g/l PS cellulose, 0.11 g $(NH_4)_2SO_4$, and 1.01 ml urea. Using this feed composition, the predicted cellulase production was 9.93 FPU/ml using the 99% Prediction Interval (PI) (high = 12.69 FPU/ml and low = 7.16 FPU/ml). This was confirmed by the value from the flask culture (9.31 FPU/ml), which was within the predicted range.

Fed-batch culture in a jar fermentor

The optimal feed condition was confirmed using a 3-L jar fermentor of working volume 1.5 L. As shown in Fig. 6, when optimal amounts of PS cellulose, $(NH_4)_2SO_4$ and urea were fed at 48 h, the DCW and cellulase activity were 20 g/l and 10.96 FPU/ml, respectively. The amount of residual PS cellulose in the reactor was still 2% or about 20 g/l. When the feed that contain PS cellulose was 40.89 g/l was included into fermentor, the total of PS cellulose was 6.1% and consider not so high. Therefore, the mass transfer limitations were still in the tolerable range. The specific enzyme activity of 0.63 FPU/mg was also high for a fed-batch culture and cellulase production was within the predicted range, indicating successful scale-up of the flask culture.

Discussion

There are no specific reports in related literature on cellulase production from PS. In this study, we present a method for utilizing PS as the carbon source to produce cellulase. This product, cellulase, can be used for the saccharification of any carbon source, including PS itself, without any pretreatment. Further studies will be undertaken

289 to examine the application of this cellulase in the SSF method to generate ethanol fuel.
290 In this study, it was possible to produce cellulase using untreated PS. Dissolved oxygen
291 concentration (DO), PS amount, feeding time, pH, buffer, and nutrients affected
292 cellulase production. Referring to DO, minimum DO level in different pH-controlled
293 culture was higher than 30% (data not shown), suggesting that DO is not a limiting
294 factor in cellulase production. Since pH and buffer was important factors we
295 investigated intensively already. Feeding time, nutrient and PS amount are the most
296 signitificant factors. The feeding time and nutrient can be controllable, but the PS
297 amount causes problem, which were encountered due to the viscosity of the culture,
298 which resulted in mass transfer limitations. However, these could be overcome by fed-
299 batch culture. More than 2 additions of feed resulted in a very high increase in the
300 amount of clay (more than 30%), which affected cell growth. We are still studying the
301 effects of the clay concentration in PS to determine the quality of PS required for
302 cellulase production.

303 Clay may immobilize or adsorb cellulase on its surface or pores. To confirm this,
304 the clay was mixed with cellulase solution and precipitated by centrifugation at 4000
305 rpm for 10 min. It was then washed with buffer and used for the saccharification of PS
306 for 60 min. However, the formation of reducing sugars was not detected. Cellulase
307 activity in the supernatant was not significant different from before mixing. This
308 indicates that the clay constituent of PS had not adsorbed or immobilized the cellulase
309 present in the culture.

310 We compared the cellulase produced from PS cellulose with the commercial
311 enzyme from *A. cellulolyticus*. After saccharification for 48 h, the saccharification

yields of the culture broth were 48.5% and 51.1% using the enzyme from PS cellulose and commercial cellulase, respectively (data not shown). Thus, the results of this study showed that cellulase can be produced from PS in the culture of *A. cellulolyticus*, and the enzyme levels reached 10.96 FPU/ml in a fed-batch operation. The produced cellulase can be used for PS saccharification.

Cellulase production is a key step in biorefining, especially in the production of second-generation bioethanol as an alternative fuel (*22*). PS is a waste material that should be recovered and reused.. It is cheap and abundant, but its disposal is a problem in environmental terms. Therefore, it would be useful to bio-convert PS to the high-value product cellulase.

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330 **Legends for figures**

331 **Fig. 1.** Effect of PS on cellulase production (black bars) and culture pH (gray bars) in *A.*
332 *cellulolyticus* cultures. A mixture of PS cellulose and SF was used as the carbon
333 source.

334 **Fig. 2.** Effect of buffer capacity of the medium on culture pH (A) and cellulase activity
335 (B) in flask cultures of *A. cellulolyticus.* The maleate buffer concentration was 0.2
336 M (closed circles), 0.4 M (closed squares), 0.6 M (closed triangles), 0.8 M (open
337 triangles), or 1 M (open circles).

338 **Fig. 3.** The cellulase activity (A), DCW (B), and specific cellulase activity (C) in pH-
339 controlled cultures in a 3-L fermentor. The pH was controlled at 4.5 (open circle),
340 5.2 (closed circle), 5.7 (closed triangles), 6.0 (closed square), and 6.5 (open
341 square) using NaOH and maleic acid.

342 **Fig. 4.** Effect of culture pH on cellulase activity in the pH-controlled culture of *A.*
343 *cellulolyticus* using untreated PS as carbon source. A, B, and C denote CMCase,
344 avicelase and β-glucosidase, respectively. The culture broth obtained as shown
345 in Fig. 3 was used to measure the sub cellulase activity.

346 **Fig. 5.** Surface response of the experimental model for cellulase production. (A)
347 $(NH_4)_2SO_4$ and urea, (B) PS cellulose and urea, and (C) PS cellulose and
348 $(NH_4)_2SO_4$.

Fig. 6. Fed-batch culture of *A. cellulolyticus*. The following components were added after 48 h of culture: 40.89 g/l of PS cellulose, 0.11 g of $(NH_4)_2SO_4$, and 1.01 ml of urea. Symbols: closed squares, residual PS cellulose concentration; closed circles, cellulase activity; open circles, DCW; open squares, specific enzyme activity.

355 Table 1-Feeding composition for fed batch fermentation in 50 ml culture

C source	N source	
PS cellulose (g/l)	$(NH_4)_2SO_4$ (g in 2 ml distilled water)	200 g/l Urea (ml)
5	0.1	0.4
20	0.2	0.8
40	0.3	1.2
60	0.4	1.6

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357

358

359 Table 2-Analysis of the variance in optimized cellulase production

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F	
Model	44.20135	9	4.911261	5.509815	0.0001	significant
A-PS	10.04399	1	10.04399	11.26809	0.0020	
B-$(NH_4)_2SO_4$	4.205507	1	4.205507	4.718047	0.0374	
C-urea	0.911256	1	0.911256	1.022314	0.3196	
AB	0.788162	1	0.788162	0.884219	0.3541	
AC	0.921226	1	0.921226	1.033499	0.3170	
BC	0.202082	1	0.202082	0.22671	0.6372	
A^2	16.58746	1	16.58746	18.60903	0.0001	
B^2	0.578272	1	0.578272	0.648748	0.4265	
C^2	4.737049	1	4.737049	5.314371	0.0278	

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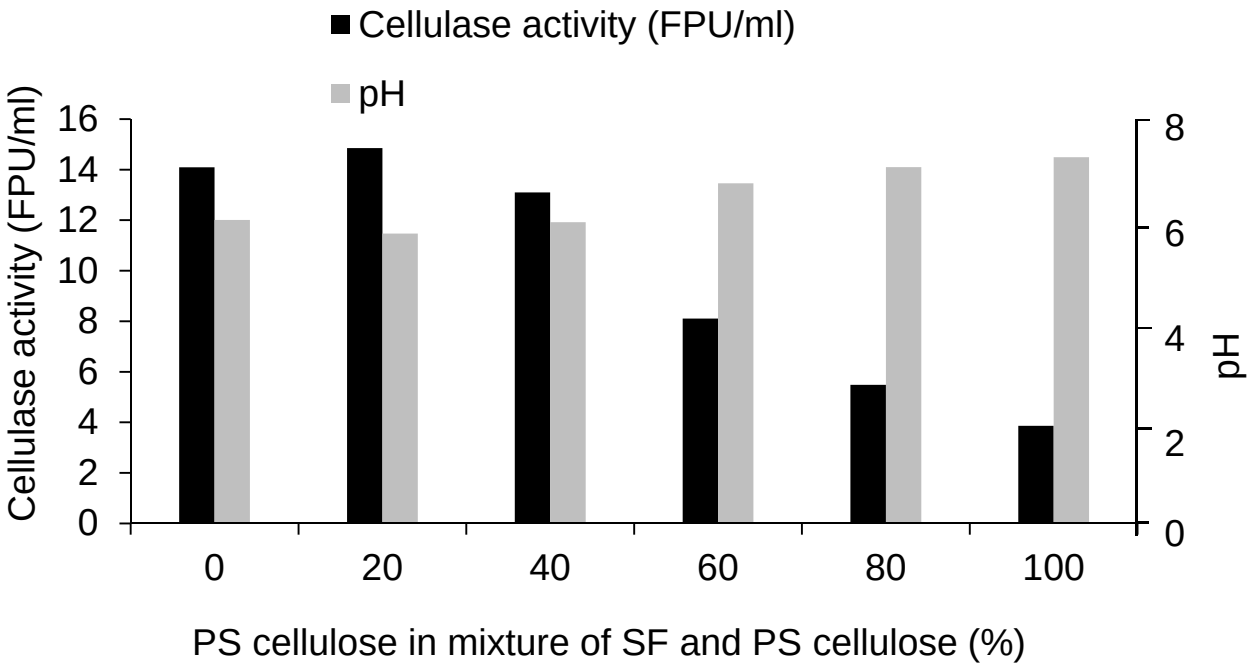
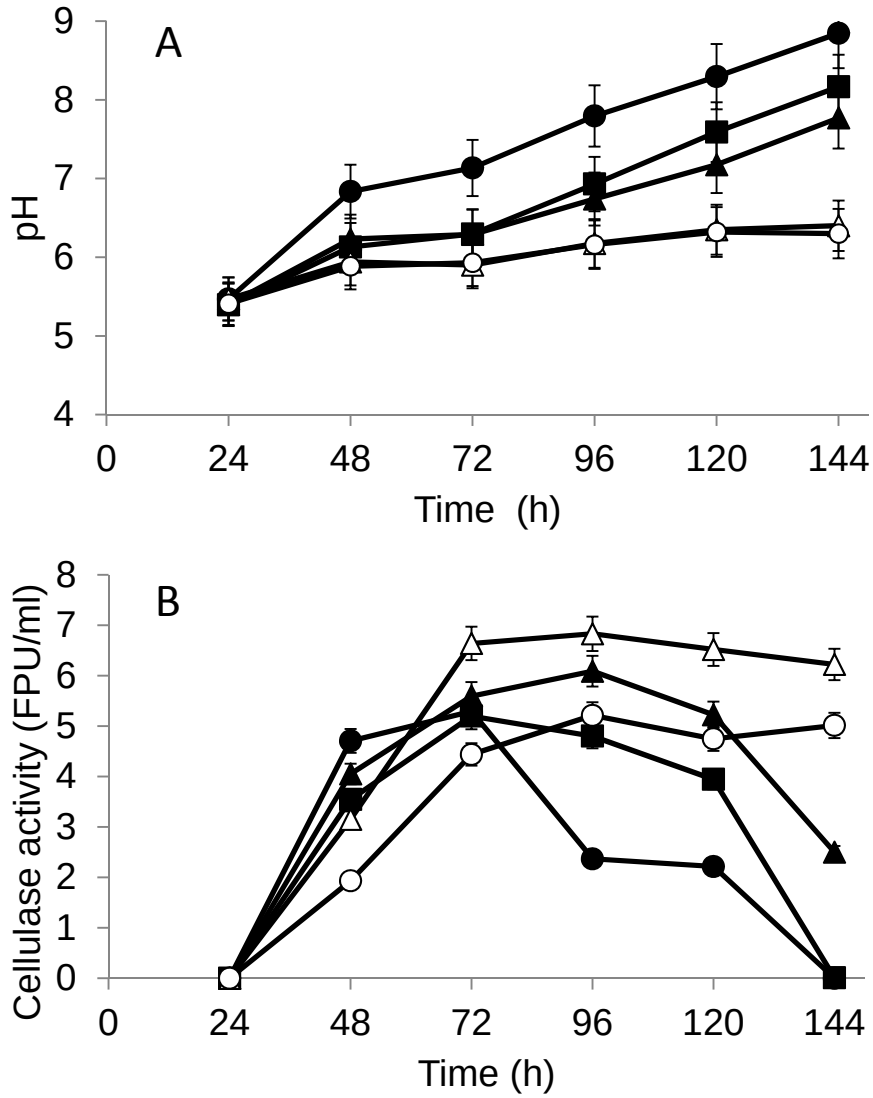
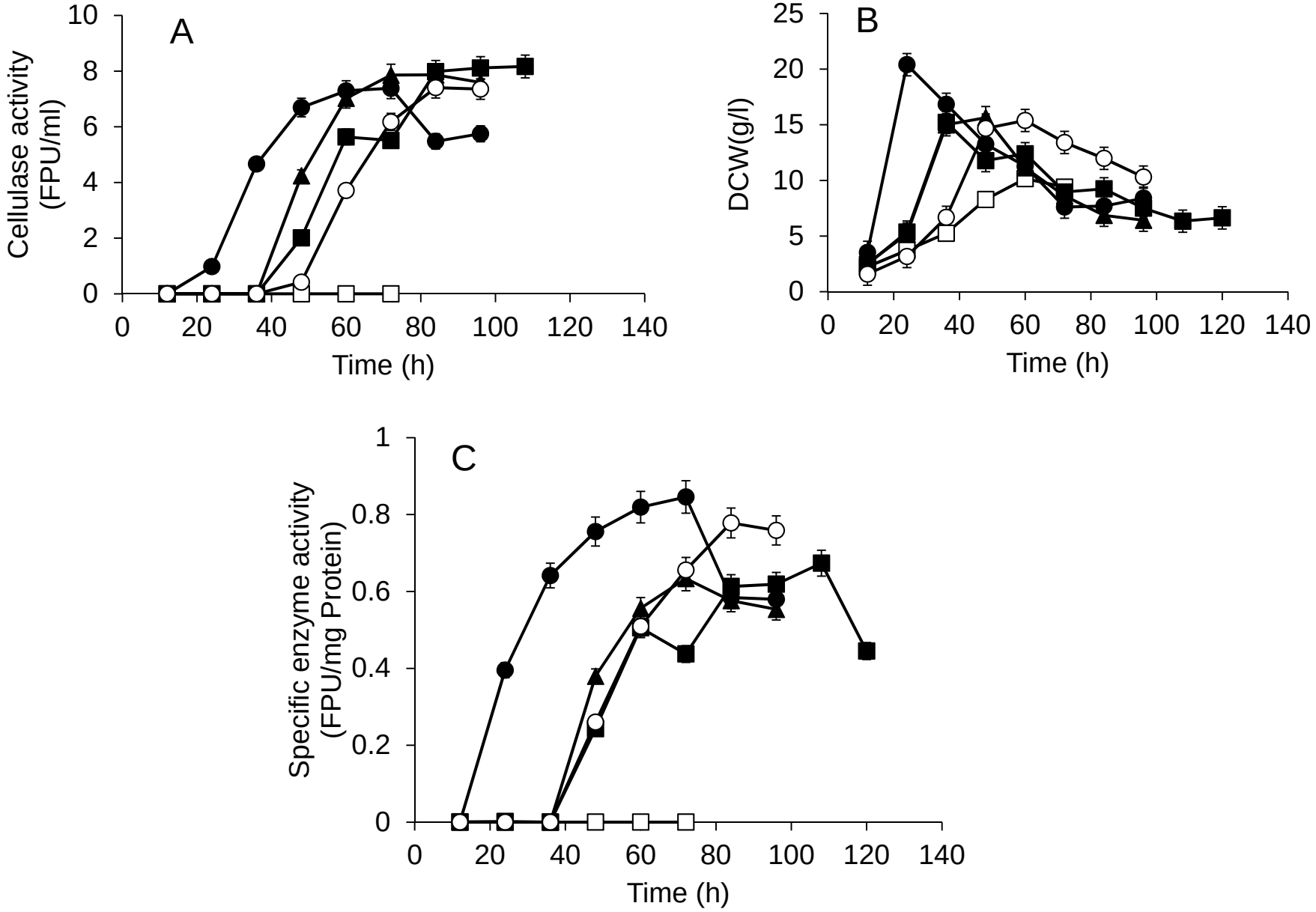
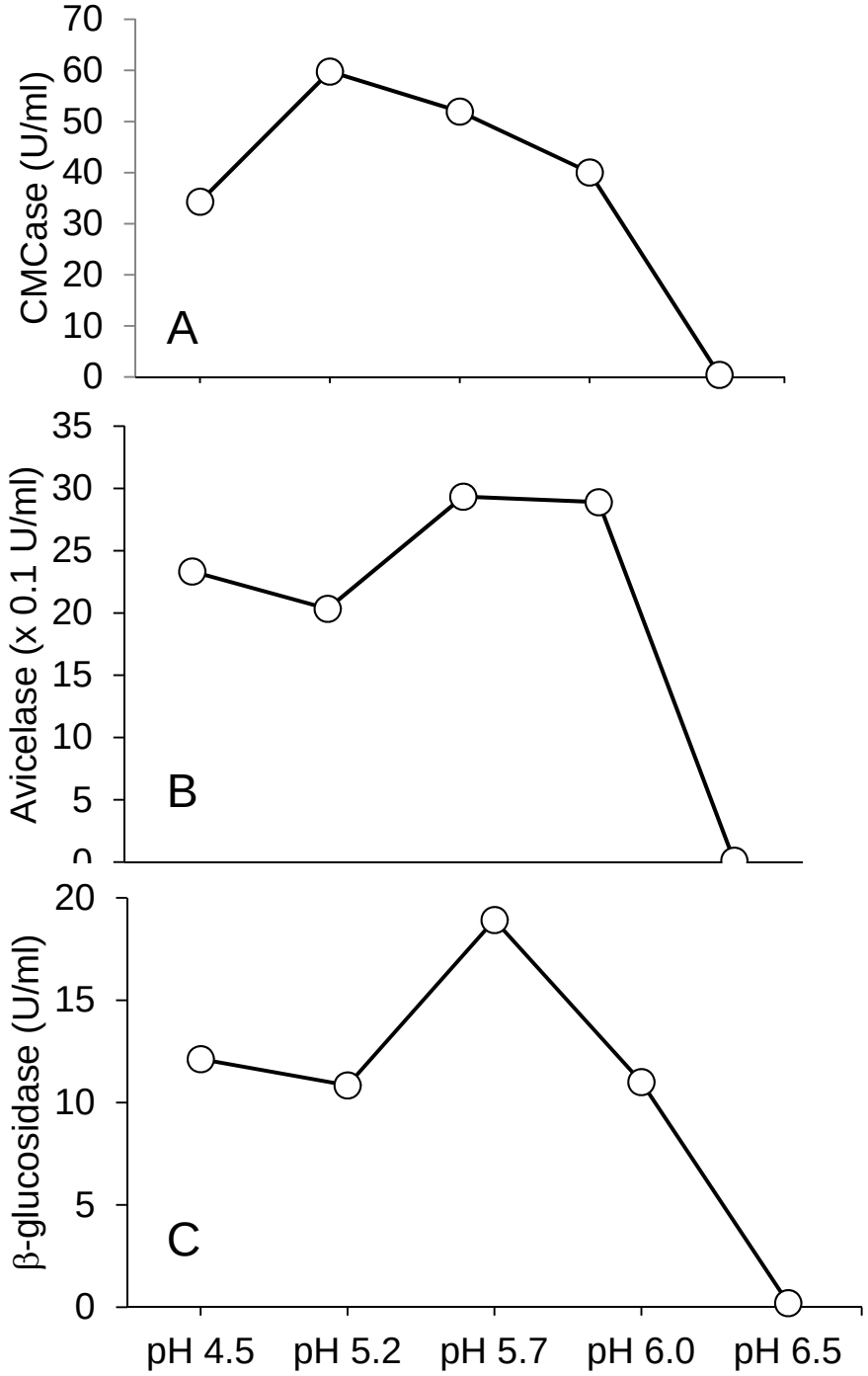
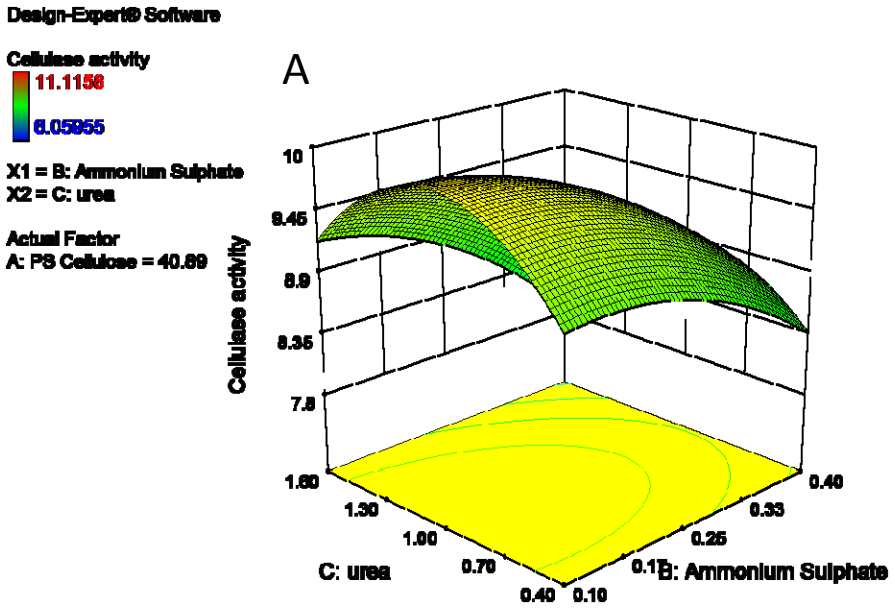

Cellulase activity (FPU/ml)
pH
Cellulase activity (FPU/ml)
16
14
12
10
8
6
4
2
0
8
6
4
2
0
pH
0
20
40
60
80
100
PS cellulose in mixture of SF and PS cellulose (%)


A
pH
9
8
7
6
5
4
0
24
48
72
96
120
144
Time (h)
B
Cellulase activity (FPU/ml)
8
7
6
5
4
3
2
1
0
0
24
48
72
96
120
144
Time (h)

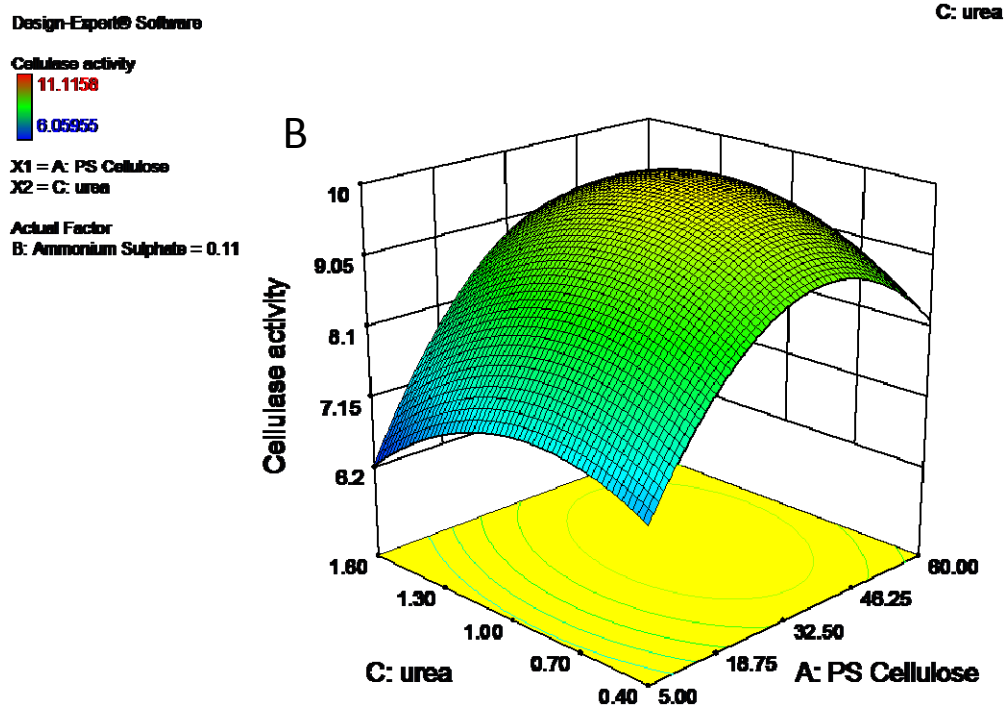

A
Cellulase activity (FPU/ml)
Time (h)
B
DCW(g/l)
Time (h)
C
Specific enzyme activity (FPU/mg Protein)
Time (h)


CMCase (U/ml)
70
60
50
40
30
20
10
0
A
Avicelase (x 0.1 U/ml)
35
30
25
20
15
10
5
0
B
β-glucosidase (U/ml)
20
15
10
5
0
C
pH 4.5
pH 5.2
pH 5.7
pH 6.0
pH 6.5

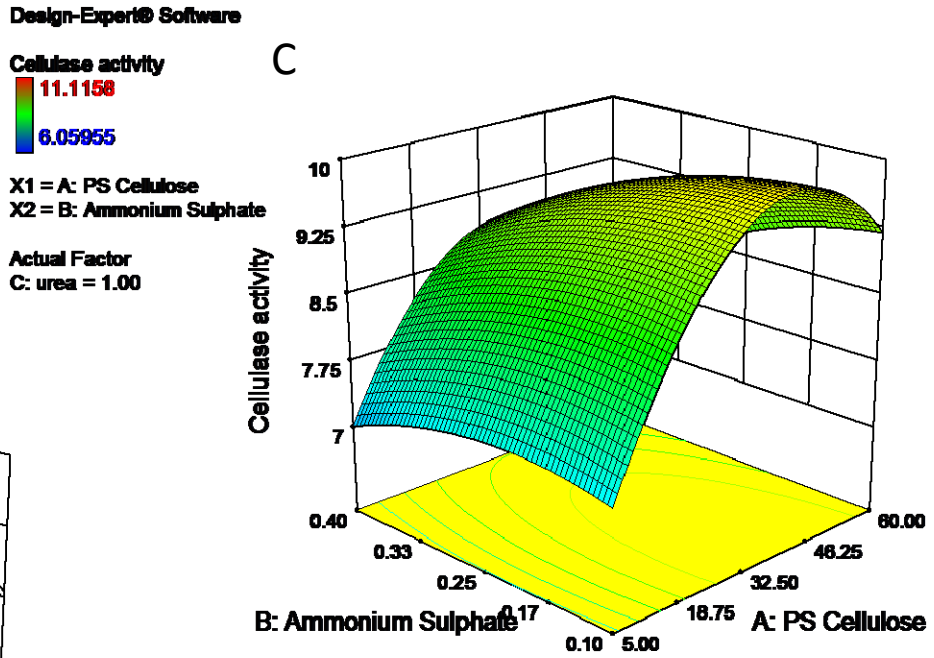
A

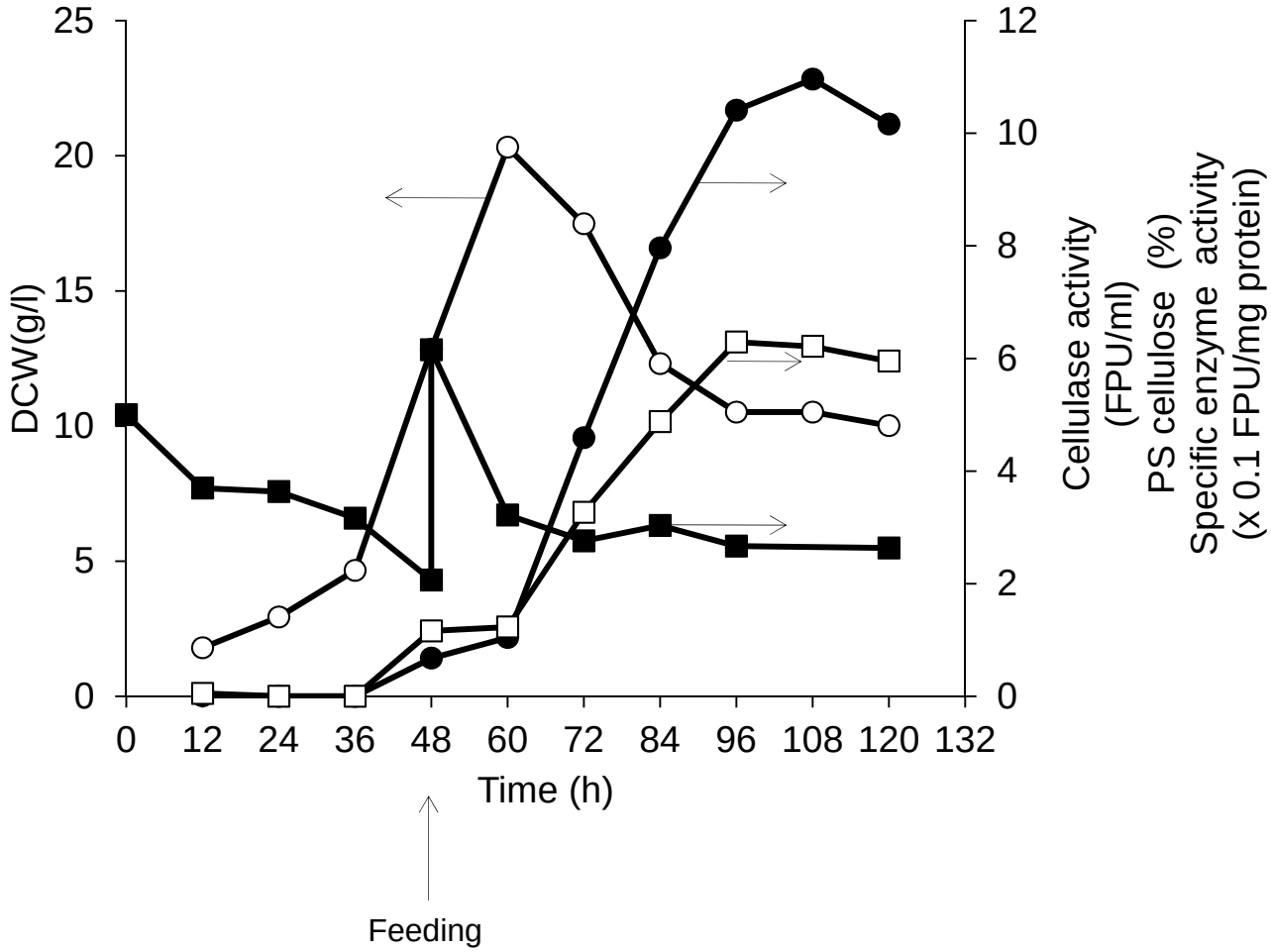

Design-Expert® Software
Cellulase activity
11.1158
6.05955
X1 = B: Ammonium Sulphate
X2 = C: urea
Actual Factor
A: PS Cellulose = 40.89
Cellulase activity
10
9.45
8.9
8.35
7.8
1.60
1.30
1.00
0.70
0.40
C: urea
0.10
0.17
0.25
0.33
0.40
B: Ammonium Sulphate

B


Design-Expert® Software
Cellulase activity
11.1158
6.05955
X1 = A: PS Cellulose
X2 = C: urea
Actual Factor
B: Ammonium Sulphate = 0.11
Cellulase activity
10
9.05
8.1
7.15
6.2
1.60
1.30
1.00
0.70
0.40
C: urea
5.00
18.75
32.50
46.25
60.00
A: PS Cellulose

C


Design-Expert® Software
Cellulase activity
11.1158
6.05955
X1 = A: PS Cellulose
X2 = B: Ammonium Sulphate
Actual Factor
C: urea = 1.00
Cellulase activity
10
9.25
8.5
7.75
7
0.40
0.33
0.25
0.17
0.10
B: Ammonium Sulphate
5.00
18.75
32.50
46.25
60.00
A: PS Cellulose


DCW(g/l)
Cellulase activity (FPU/ml)
PS cellulose (%)
Specific enzyme activity (x 0.1 FPU/mg protein)
Time (h)
Feeding
0
5
10
15
20
25
0
2
4
6
8
10
12
0 12 24 36 48 60 72 84 96 108 120 132