Enzyme and Microbial Technology 46 (2010) 38–42

Contents lists available at ScienceDirect

Enzyme and Microbial Technology

journal homepage: www.elsevier.com/locate/emt

Industrial scale of optimization for the production of carboxymethylcellulase from rice bran by a marine bacterium, *Bacillus subtilis* subsp. *subtilis* A-53

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ARTICLE INFO

Article history:
Received 26 June 2009
Received in revised form 14 July 2009
Accepted 15 July 2009

Keywords:
Carboxymethylcellulase
Bacillus subtilis subsp. *subtilis*
Rice bran
Industrial-scale production

ABSTRACT

Rice bran and yeast extract were found to be the best combination of carbon and nitrogen sources for the production of carboxymethycellulase (CMCase) by *Bacillus subtilis* subsp. *subtlis* A-53. Optimal concentrations of rice bran and yeast extract for the production of CMCase were 5.0% (w/v) and 0.10% (w/v), respectively. Optimal temperature and initial pH of medium for cell growth of *B. subtilus* subsp. *subtilis* A-53 were 35 °C and 7.3, whereas those for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 were 30 °C and 6.8. Optimal agitation speed and aeration rate in a 7 L bioreactor were 300 rpm and 1.0 vvm, respectively. The optimal agitation speed and aeration rate for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 were lower than those for cell growth. The highest productions of CMCase by *B. subtilus* subsp. *subtilis* A-53 in 7 and 100 L bioreactors were 150.3 and 196.8 U mL^{-1}, respectively.

1. Introduction

Ethanol production from lignocellulosic biomass is emerging as one of the most important technologies for sustainable transportation fuels [1]. The production of ethanol from lignocellulosic biomass involves four processes – feedstock pretreatment, enzymatic saccharification, fermentation, and ethanol recovery [2]. A major constrain in enzymatic saccharification of cellulosic materials for the production of fermentable sugars is low productivity and the cost of cellulases [1].

The complete enzymatic hydrolysis of cellulosic materials needs at least three different types of cellulases; endoglucanase (1,4-β-D-glucan-4-glucanohydrolase; carboxymethylcellulase), exocellobiohydrolase (1,4-β-D-glucan glucohydrolase; avicelase), and β-glucosidase (β-D-glucoside glucohydrolase) [3]. The enzymatic saccharification of lignocellulosic materials for the production of ethanol was performed by commercial cellulases, in which the major cellulase was carboxymethylcellulase [4,5]. Most commercial cellulases have been produced by *Aspergillus* and *Trichderma* species with solid-state cultures [6–8]. Bacterial cellulase systems of *Clostridium*, *Cellomonas*, *Bacillus*, *Thermonospora*, *Ruminococcus*, *Bacteriodes*, *Erwinia*, and *Acetivibrio* species have been reported [9,10]. Production of carboxymethylcellulase (CMCase) from rice hulls by *B. amyloliquefaciens* DL-3 under a liquid culture was reported [11]. Production of CMCase by thermophilic *Bacillus* sp. was reported and cellulases produced by *Bacillus* sp. isolated from hot springs were purified [12,13].

Enzymes produced by marine microorganisms can provide numerous advantages over traditional enzymes due to the wide range of environments [14,15]. Cold-adapted peptidases were isolated from marine bacteria and a halo-tolerant marine bacterium, which produced κ-carrageenase, was studied [16,17]. We had reported identification of a marine bacterium, *Bacillus subtilis* subsp. *subtilis* A-53 and characterization of the CMCase produced by this strain [18]. It showed thermal stability at low temperatures and a high tolerance for metal ions. In this study, optimal conditions for the production of CMCase by *B. subtilis* subsp. *subtilis* A-53 were examined.

2. Materials and methods

2.1. Bacterial strain and medium

B. subtilis subsp. *subtilis* A-53 was isolated from seawater and identified in a previous study [18]. It utilized rice bran as a carbon source and produced carboxymethylcellulase (CMCase). The strain was maintained on an agar medium containing 2.0% (w/v) glucose, 0.25% yeast extract, 0.5% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4 \cdot 7H_2O$, 0.06% $(NH_4)_2SO_4$ and 1.5% (w/v) agar.

2.2. Production of carboxymethylcellulase (CMCase)

Starter cultures were prepared by transferring cells from agar slants to 50 mL of the same medium, for maintenance, except for agar in 250 mL Erlenmeyer flasks. The resulting cultures were incubated for 2 days at 30 °C under aerobic conditions. Each

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doi:10.1016/j.enzmictec.2009.07.009

Table 1
Effect of carbon and nitrogen sources on cell growth of *B. subtilis* subsp. *subtlis* A-53.

DCW[a] ($g\,L^{-1}$)						
Nitrogen sources	Carbon sources					
	Glucose	Fructose	Maltose	Sucrose	Rice bran	Rice hulls
Malt extract	1.86 ± 0.21	1.54 ± 0.18	1.54 ± 0.24	2.14 ± 0.27	0.80 ± 0.11	0.84 ± 0.21
Peptone	1.78 ± 0.19	1.66 ± 0.14	2.08 ± 0.33	2.68 ± 0.35	0.80 ± 0.18	0.94 ± 0.15
Tryptone	2.40 ± 0.22	1.90 ± 0.17	2.04 ± 0.21	3.02 ± 0.29	1.30 ± 0.15	0.72 ± 0.24
Yeast extract	2.28 ± 0.31	2.34 ± 0.22	1.66 ± 0.13	3.40 ± 0.43	1.24 ± 0.24	0.92 ± 0.18
Ammonium chloride	1.72 ± 0.16	1.70 ± 0.15	1.40 ± 0.16	2.58 ± 0.24	1.54 ± 0.22	1.00 ± 0.17
Ammonium nitrate	1.84 ± 0.14	1.86 ± 0.19	1.30 ± 0.14	1.60 ± 0.19	1.34 ± 0.18	1.36 ± 0.19

[a] Dry cells weight.

starter culture was used as an inoculum for 100 mL of medium in 500 mL Erlenmeyer flasks. The main culture was carried out in a medium containing 2.0% (w/v) rice bran, 0.25% yeast extract, 0.5% K_2HPO_4, 0.1% NaCl, 0.02% $MgSO_4{\cdot}7H_2O$, and 0.06% $(NH_4)_2SO_4$ at 30 °C for 72 h under aerobic conditions. Samples were periodically withdrawn from the cultures to examine cell growth and production of CMCase by *B. subtilis* subsp. *subtilis* A-53.

Batch fermentations for the production of CMCase by *B. subtilis* subsp. *subtilis* A-53 were performed in 7 and 100 L bioreactors (Ko-Biotech Co., Korea). Working volumes of the 7 and 100 L bioreactors were 5 and 70 L, respectively, and inoculum size of batch fermentations for the production of CMCase by *B. amyloliquefaciens* DL-3 was 5% (v/v). Carbon and nitrogen sources for batch fermentations were 5% (w/v) rice bran and 0.1% (w/v) yeast extract. Temperatures for batch fermentations with the 7 and 100 L bioreactors were maintained at 30 °C. The agitation speed of a 7 L bioreactor ranged from 200 to 500 rpm and its aeration rate ranged from 0.5 to 2.0 vvm. Agitation was provided by three six-flat-blade impellers in a 7 L fermentor. Agitation speed and aeration rate for a 100 L bioreactor were 200 rpm and 1.0 vvm. The inner pressure in a 100 L bioreactor was 0.2 $kgf\,cm^{-2}$.

2.3. Analytical methods

Dry cells weight was measured by directly weighing the biomass after drying to a constant weight at 100–105 °C, after collection of cells by centrifugation at 12,000 × *g* for 10 min. The activity of CMCase produced by *B. subtilis* subsp. *subtlis* A-53 was determined based on the release of reducing sugars from carboxymethylcellulose (CMC) using the 3,5-dinitrosalicylic acid (DNS) method [19]. A mixture of dialyzed culture broth after removal of cells and 1.0% (w/v) CMC dissolved in a 50 mM Tris–HCl buffer, pH 7.0, was incubated at 50 °C for 20 min, and the reaction was stopped by adding DNS reagent. The treated samples were boiled for 10 min, cooled in water for color stabilization, and optical density was measured at 550 nm. The activity of CMCase was determined by using a calibration curve for glucose (Sigma–Aldrich, UK). One unit of CMCase activity was defined as the amount of enzyme that releases 1 μmol of reducing sugar equivalent to glucose, per minute, under the assay condition.

3. Results and discussion

3.1. Effect of carbon and nitrogen sources on production of CMCase

The effect of carbon and nitrogen sources on cell growth and the production of CMCase by *B. subtilis* subsp. *subtlis* A-53 was investigated. Carbon sources tested for production of CMCase by *B. subtilis* subsp. *subtlis* A-53 were 2.0% (w/v) glucose, fructose, maltose, sucrose, rice bran, and rice hulls. Nitrogen sources tested were 0.25% (w/v) malt extract, peptone, tryptone, yeast extract, ammonium sulfate, and ammonium nitrate.

Sucrose and yeast extract were found to be the best combination of carbon and nitrogen sources for cell growth of *B. subtilis* subsp. *subtlis* A-53, as shown in Table 1. Combinations of sucrose and tryptone and sucrose and peptone were also better for cell growth of *B. subtilis* subsp. *subtlis* A-53, whereas those of rice hull and tryptone, rice bran and malt extract, and rice bran and peptone were not good for cell growth. Rice bran and yeast extract were the best combination of carbon and nitrogen sources for production of CMCase by *B. subtilis* subsp. *subtlis* A-53, as shown in Table 2. Combinations of rice hull and tryptone and rice bran and tryptone were found to be better for production of CMCase by *B. subtilis* subsp. *subtlis* A-53, whereas those of fructose and tryptone, sucrose and peptone, and glucose and yeast extract were not good for production of CMCase. The best combination of carbon and nitrogen sources for cell growth of *B. subtilis* subsp. *subtlis* A-53 was different from that for production of CMCase. The production of CMCase by *B. subtilis* subsp. *subtlis* A-53 was 89.6 $U\,mL^{-1}$ from 2.0% (w/v) rice bran and 0.25% yeast extract. The best carbon source for production of CMCase produced by *B. amyloliquefaciens* DL-3 was rice hulls and those by *Bacillus* sp. CH43 and HR68 were rice bran [11,12]. A major carbon source for the production of CMCases by *Aspergillus* and *Trichoderma* species was reported to be wheat bran [8,20,21]. Cellulases are inducible enzymes, which is the reason why most carbon sources for the production of CMCase are cellulosic materials such as rice hulls, rice bran or wheat bran.

3.2. Effect of rice bran and yeast extract on production of CMCase

The effect of rice bran and yeast extract as carbon and nitrogen sources on cell growth and production of the CMCase by *B. subtilus* subsp. *subtilis* A-53 was examined. The composition of the rice bran used in this study was as follows: 48.0% carbohydrate, 6.9% fiber, 14.9% crude lipid, 13.1% crude protein, 7.6% ash, and 9.5% water. The concentration of rice bran ranged from 0.0 to 10.0% (w/v), whereas that of yeast extract ranged from 0.0 to 1.0% (w/v). Cell growth of *B. subtilus* subsp. *subtilis* A-53 was enhanced with higher concentrations of rice bran and yeast extract, as shown in Table 3. Production

Table 2
Effect of carbon and nitrogen sources on production of CMCase by *B. subtilis* subsp. *subtlis* A-53.

CMCase ($U\,mL^{-1}$)						
Nitrogen sources	Carbon sources					
	Glucose	Fructose	Maltose	Sucrose	Rice bran	Rice hulls
Malt extract	27.9 ± 3.4	22.6 ± 4.4	38.6 ± 5.6	30.6 ± 2.8	41.5 ± 6.5	45.2 ± 7.2
Peptone	17.1 ± 2.6	33.1 ± 5.8	24.1 ± 3.5	10.7 ± 3.1	31.9 ± 7.2	65.1 ± 8.4
Tryptone	22.2 ± 4.8	10.2 ± 3.1	31.6 ± 4.8	4.6 ± 1.9	74.6 ± 6.1	69.0 ± 7.6
Yeast extract	11.9 ± 2.3	13.0 ± 2.7	23.7 ± 5.4	19.1 ± 3.1	83.6 ± 6.8	74.7 ± 6.8
Ammonium chloride	30.1 ± 3.8	31.5 ± 4.6	43.5 ± 6.1	19.4 ± 4.6	29.6 ± 5.1	38.3 ± 5.3
Ammonium nitrate	29.7 ± 2.2	59.1 ± 7.3	36.2 ± 4.7	28.2 ± 3.7	52.3 ± 5.4	57.1 ± 7.6

Table 3
Effect of rice bran and yeast extract as carbon and nitrogen sources on cell growth of *B. subtilis* subsp. *subtlis* A-53.

DCW ($g L^{-1}$)						
Yeast extract (%)	Rice bran (%)					
	0.0	1.0	2.0	3.0	5.0	10.0
0.00	0.37 ± 0.06	0.86 ± 0.18	1.11 ± 0.16	1.34 ± 0.11	1.86 ± 0.15	2.26 ± 0.26
0.05	0.50 ± 0.08	1.15 ± 0.23	1.62 ± 0.18	2.18 ± 0.24	2.63 ± 0.23	3.31 ± 0.32
0.10	0.63 ± 0.11	1.23 ± 0.18	2.03 ± 0.16	2.64 ± 0.28	3.13 ± 0.30	3.42 ± 0.31
0.25	0.65 ± 0.09	1.66 ± 0.21	2.46 ± 0.22	2.96 ± 0.35	3.43 ± 0.23	4.30 ± 0.38
0.50	0.75 ± 0.14	1.87 ± 0.24	2.63 ± 0.18	3.26 ± 0.22	3.66 ± 0.35	5.24 ± 0.42
1.00	0.63 ± 0.16	1.20 ± 0.17	1.54 ± 0.17	2.20 ± 0.27	3.35 ± 0.27	5.46 ± 0.47

Table 4
Effect of rice bran and yeast extract as carbon and nitrogen sources on production of CMCase by *B. subtilis* subsp. *subtlis* A-53.

CMCase ($U mL^{-1}$)						
Yeast extract (%)	Rice bran (%)					
	0.0	1.0	2.0	3.0	5.0	10.0
0.00	12.9 ± 3.5	24.6 ± 3.4	35.7 ± 4.6	44.2 ± 3.8	52.6 ± 5.7	39.2 ± 4.3
0.05	18.9 ± 4.2	43.7 ± 5.6	52.4 ± 4.4	71.4 ± 6.5	89.3 ± 7.7	54.3 ± 6.1
0.10	26.2 ± 3.4	56.4 ± 7.4	68.9 ± 7.2	115.0 ± 13.5	131.7 ± 10.5	70.4 ± 6.8
0.25	22.1 ± 3.0	38.8 ± 4.8	65.2 ± 6.8	98.6 ± 10.2	121.2 ± 14.2	70.1 ± 8.2
0.50	17.8 ± 2.7	32.7 ± 5.2	53.2 ± 7.1	74.2 ± 6.4	104.7 ± 11.3	44.2 ± 5.2
1.00	13.1 ± 2.4	24.5 ± 4.3	49.2 ± 3.9	66.3 ± 7.3	84.6 ± 6.5	39.3 ± 4.7

Table 5
Effect of initial pH of medium and temperature on cell growth of *B. subtilis* subsp. *subtlis* A-53.

DCW ($g L^{-1}$)						
Temperature (°C)	Initial pH					
	5.8	6.3	6.8	7.3	7.8	8.3
25	1.62 ± 0.18	2.04 ± 0.23	2.45 ± 0.18	2.86 ± 0.32	2.53 ± 0.17	1.42 ± 0.20
30	2.42 ± 0.21	3.05 ± 0.25	3.30 ± 0.41	3.50 ± 0.37	3.35 ± 0.31	2.25 ± 0.18
35	3.20 ± 0.20	3.64 ± 0.24	4.18 ± 0.35	4.60 ± 0.28	4.18 ± 0.37	3.21 ± 0.26
40	1.34 ± 0.16	2.02 ± 0.17	2.65 ± 0.24	2.85 ± 0.34	2.15 ± 0.23	1.43 ± 0.21

of CMCase by *B. subtilus* subsp. *subtilis* A-53 also increased with increased concentrations of rice bran and yeast extract, but production of CMCase did not increase with concentrations higher than 5.0% in rice bran and 0.10% in yeast extract. The highest production of CMCase was 131.7 U mL^{-1} at 30 °C under aerobic conditions when concentrations of rice bran and yeast extract were 5.0% and 0.10%, respectively, as shown in Table 4.

3.3. Effect of initial pH of medium and temperature on production of CMCase

The effect of temperature and initial pH of medium on cell growth and production of CMCase was investigated. Carbon and nitrogen sources were 5.0% rice bran and 0.1% yeast extract. Temperatures ranged from 25 to 40 °C and the initial pH of the medium ranged from 5.8 to 8.3. The optimal temperature and initial pH of medium for cell growth of *B. subtilus* subsp. *subtilis* A-53 were 35 °C and 7.3, as shown in Table 5. Those for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 were 30 °C and 6.8, as shown in Table 6. The highest production of CMCase from 5.0% rice bran and 0.10% yeast extract with optimal temperature and initial pH of the medium was 136.8 U mL^{-1}. Optimal temperature and initial pH of medium for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 were also different from those for cell growth such as other bacterial productions of CMCases. The optimal temperature and initial pH of the medium for the production of CMCase by *B. amyloliquefaciens* DL-3 were 37 °C and 6.8, whereas those for cell growth were 32 °C and 7.2 [11].

3.4. Effect of agitation speed on production of CMCase

The effect of agitation speed on cell growth and production of CMCase was investigated in a 7 L bioreactor (Ko-Biotech Co., Korea). Agitation speed ranged from 200 to 500 rpm and the aeration rate was 1.0 vvm. The temperature and initial pH of medium for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 were 30 °C and 6.8, respectively. Higher agitation speed, which resulted in an increase in concentration of dissolved oxygen in the medium in

Table 6
Effect of initial pH of medium and temperature on production of CMCase by *B. subtilis* subsp. *subtlis* A-53.

CMCase ($U mL^{-1}$)						
Temperature (°C)	Initial pH					
	5.8	6.3	6.8	7.3	7.8	8.2
25	55.6 ± 5.5	62.1 ± 5.8	95.5 ± 10.4	72.4 ± 6.8	55.3 ± 4.2	44.5 ± 7.2
30	65.6 ± 4.9	108.9 ± 12.5	136.8 ± 11.7	112.9 ± 9.4	82.6 ± 6.7	61.5 ± 6.6
35	53.4 ± 6.4	81.2 ± 6.2	111.7 ± 8.9	94.3 ± 8.6	68.9 ± 6.1	35.3 ± 4.6
40	37.2 ± 4.2	53.3 ± 5.4	75.7 ± 8.2	62.8 ± 7.3	42.6 ± 5.6	22.7 ± 3.8

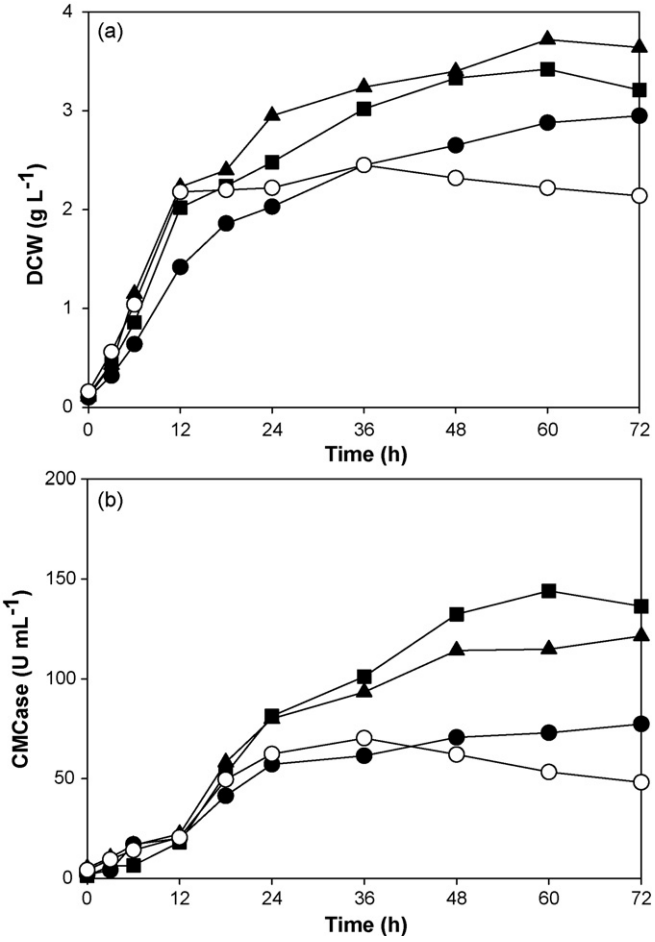

Fig. 1. Effect of agitation speed on cell growth (a) and production (b) of CMCase by *B. subtilis* subsp. *subtilis* A-53 in a 7 L bioreactor (●, 200 rpm; ■, 300 rpm; ▲, 400 rpm; and ○, 500 rpm).

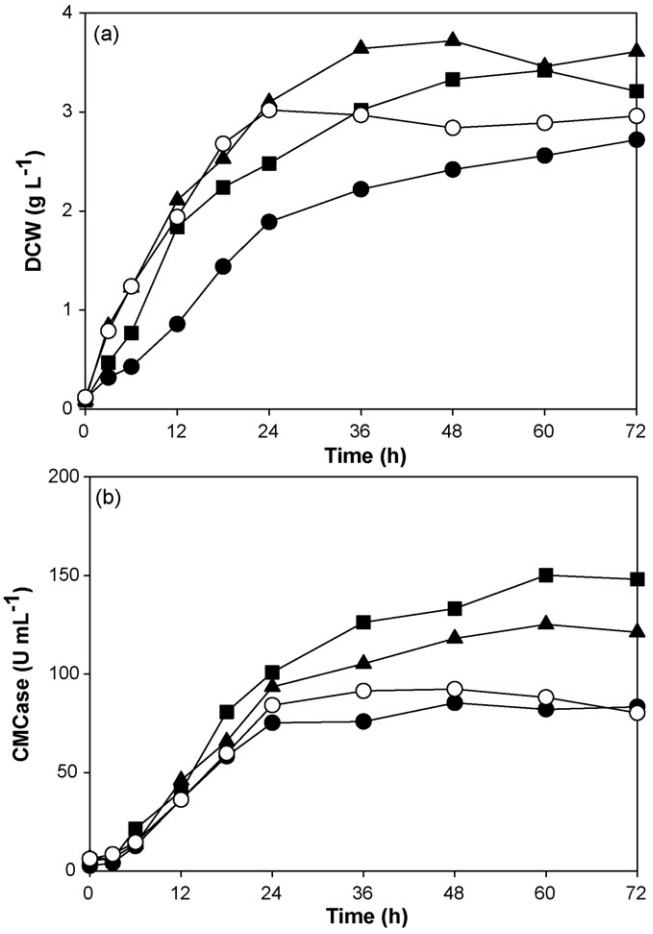

Fig. 2. Effect of aeration rate on cell growth (a) and production (b) of CMCase by *B. subtilis* subsp. *subtilis* A-53 in a 7 L bioreactor (●, 0.5 vvm; ■, 1.0 vvm; ▲, 1.5 vvm; and ○, 2.0 vvm).

a 7 L bioreactor, enhanced cell growth of *B. subtilus* subsp. *subtilis* A-53, as shown in Fig. 1. The optimal agitation speed for the production of CMCase by *B. amyloliquefaciens* DL-3 was lower than that for cell growth. The highest production of CMCase at 144.1 U mL^{-1} was observed at an agitation speed of 300 rpm [11]. Production of cellulases by *T. reesei* drops at higher agitation rates [22]. It seems that higher concentrations of dissolved oxygen in the medium promote cell growth of *B. subtilus* subsp. *subtilis* A-53 while inhibiting the production of CMCase.

3.5. Effect of aeration rate on production of CMCase

The effect of aeration rate on cell growth and production of CMCase was also investigated. Aeration rate ranged from 0.5 to 2.0 vvm and the agitation speed was 300 rpm. A higher aeration rate, as well as higher agitation speed, also enhanced cell growth of *B. subtilus* subsp. *subtilis* A-53, as shown in Fig. 2. The optimal aeration rate for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 was lower than that for cell growth. The optimal aeration rate for cell growth was 1.5 vvm, whereas that for production of CMCase was 1.0 vvm. The highest production of CMCase by *B. subtilus* subsp. *subtilis* A-53 in a 7 L bioreactor was 150.3 U mL^{-1} when agitation speed and aeration rate were 300 rpm and 1.0 vvm, respectively. Cell growth and the production of cellulases by *B. amyloliquefaciens* DL-3 and *T. reesei* were affected by the dissolved oxygen concentration [11,22]. The concentration of dissolved oxygen in the medium can be influenced by agitation speed, aeration rate, and the inner pressure of bioreactors [23,24]. It seems that a higher than optimal concentration of dissolved oxygen for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 due to higher aeration rates and agitation speeds, led the biosynthetic pathway to cell growth and not to the production of CMCase.

3.6. Industrial-scale production of CMCase in a 100 L bioreactor

A batch fermentation for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 was performed in a 100 L bioreactor with an inner pressure of 0.2 kgf cm^{-2}. Carbon and nitrogen sources for production of CMCase were 5.0% rice bran and 0.1% yeast extract. Temperature and initial pH of the medium were 30 °C and 6.8. Agitation speed and aeration rate of the 100 L bioreactor were 200 rpm and 1.0 vvm. The radius of the impeller in a 100 L bioreactor is bigger than that in a 7 L bioreactor. The angular velocity of a 100 L bioreactor with 200 rpm is almost the same as that of a 7 L bioreactor with 300 rpm.

During cultivation, the pH in the medium gradually decreased until 18 h then increased to 6.8, as shown in Fig. 3. Cell growth of *B. subtilus* subsp. *subtilis* A-53 rapidly increased until 24 h. Production of CMCase by *B. subtilus* subsp. *subtilis* A-53 started after a dramatic decrease in concentration of the dissolved oxygen until 9 h. The highest production of CMCase from 5.0% rice bran and 0.1% yeast extract as carbon and nitrogen sources in a 100 L bioreactor was 196.8 U/mL. Production of CMCase seemed to be parallel with cell growth of *B. subtilus* subsp. *subtilis* A-53.

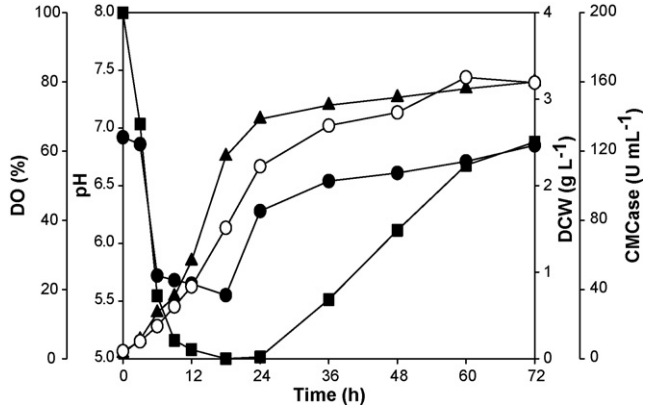

Fig. 3. Growth curve and production of CMCase by *B. subtilis* subsp. *subtilis* A-53 in a medium of 5.0% rice bran and 0.1% yeast extract in a 100 L bioreactor (●, pH; ■, DO; ▲, DCW; and ○, CMCase).

4. Conclusion

A major constrain in enzymatic saccharification of cellulosic materials for fermentable sugars is its low productivity and the cost of cellulases [1]. The enzymatic hydrolysis of cellulosic materials needs at least three different types of cellulases [3]. The enzymatic saccharification of rice hulls was performed by commercial cellulases, in which the major cellulase was CMCase [4]. Optimal conditions for the production of CMCase by *B. subtilus* subsp. *subtilis* A-53 were established in this study. The highest productions of CMCase by *B. subtilus* subsp. *subtilis* A-53 in 7 and 100 L bioreactors under optimized conditions were 150.3 and 196.8 U mL^{-1}, respectively. Productivities of CMCases produced by *Bacillus* species and *Cellomonas biazotea* under liquid cultures ranged from 9 to 211 U mL^{-1} [9,11,25]. Those of fungal CMCases produced by *Aspergillus*, *Sporotricum*, *Termiascus*, and *Trichoderma* species with solid-state cultures ranged from 45 to 1572 U g^{-1} carbon source [8,21,26]. The characterization of the CMCase produced by *B. subtilus* subsp. *subtilis* A-53 was previously reported that it had thermal stability at low temperatures and a high tolerance for metal ions [18]. It will be used for hydrolyzing cellulosic materials in severe conditions with low temperatures and a high concentration of metal ions. Future studies will be focused on the cloning of the CMCase gene from *B. subtilus* subsp. *subtilis* A-53 and its expression to enhance productivity of CMCase.

Acknowledgement

This paper was financially supported by Dong-A University Research Fund.

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