Enhanced Production of Cellulase from the Agricultural By-product Rice bran by *Escherichia coli* JM109/LBH-10 with a Shift in Vessel Pressure of a Pilot-scale Bioreactor

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The optimal vessel pressure of the bioreactor for cell growth and the production of cellulase, as well as the effect of a shift in pressure within the reactor on cellulase production were investigated. The optimal vessel pressure for the cell growth of *E. coli* JM109/LBH-10 was 0.08 MPa, whereas that for the production of cellulase was 0.04 MPa. The maximal production of cellulase by *E. coli* JM109/LBH-10 with a shift in the vessel pressure from 0.08 to 0.04 MPa after 24 h was 636.8 U/mL, which was 1.2 times higher than that without a shift. The shift in vessel pressure optimized for cell growth to that for the production of cellulase after the mid-term log-phase resulted in higher cell growth and cellulase production. A simple process with a shift in the vessel pressure of bioreactors to enhance the production of cellulase from agricultural by-products has been developed and can be directly applied to the industrial-scale production of cellulases.

Keywords: Cellulase; Escherichia coli JM109; Vessel pressure; Rice bran

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INTRODUCTION

Agricultural by-products such as rice hulls, rice bran, wheat straws, corn stover, and cotton stalks are renewable resources (Han *et al*. 2009; Wei *et al*. 2010). Rice bran, a by-product of rice milling, constitutes about 10% of the total weight of rough rice (Lee *et al*. 2010; Gul *et al*. 2015). Each year, 90% of the rice bran produced in the world is utilized cheaply as a feedstock, and the remainder is used for the extraction of rice bran oil (Kim *et al*. 2012a; Todhanakasem *et al*. 2014). The complete enzymatic hydrolysis of agricultural by-products into fermentable sugars requires the synergistic action of three types of cellulases: endoglucanses, exoglucanases, and cellobiases (Lee *et al*. 2008; Kim *et al*. 2011; Cao *et al*. 2013a). The enzymatic saccharification of agricultural by-products for the production of ethanol uses commercial cellulases. Because such enzymes also have the ability to degrade carboxymethylcellulose (CMC), they are sometimes evaluated by their ability to decompose CMC, and they have been called carboxymethylcellulase (CMCase) by some authors (Wei *et al*. 2009).

Complete enzymatic hydrolysis of cellulose requires the synergistic action of three types of enzymes: endoglucanses (cellulase, EC 3.2.1.4), exoglucanases (Avicelase, EC 3.2.1.91), and cellobiases (β-glucosidase, EC 3.2.1.21) (Lee *et al*. 2010). *Psychrobacter* *aquimaris* utilizes carboxymethyl cellulose (CMC); it was isolated from seawater and identified by its 16S rDNA sequence (Kim *et al*. 2010). The gene encoding the cellulase of *P. aquimaris* LBH-10 was cloned into *E. coli* JM109 to construct the recombinant *E. coli* JM109/LBH-10 (Lee *et al*. 2014). The optimal conditions for the production of CMCase by *E. coli* JM109/LBH-10 have been established. Unlike the traditional production of cellulases using *Aspergillus* and *Trichoderma* species with solid-state cultures, cellulase from *E. coli* JM109/LBH-10 is produced *via* batch fermentation in a stirred tank bioreactor (Lee *et al*. 2014).

In addition to optimized medium (Gao *et al*. 2012), the parameters involved in dissolved oxygen for mass production must be optimized to improve productivity and cost-efficiency (Cao *et al*. 2013b). The concentration of dissolved oxygen in the medium is influenced by agitation speed, aeration rate, and the vessel pressure of bioreactors (Jo *et al*. 2008; Han *et al*. 2014; Gao *et al*. 2015). In this study, the optimal vessel pressure of pilot-scale bioreactors for the cell growth and the production of cellulase by *E. coli* JM109/LBH-10 was investigated. The simple process for the enhanced production of cellulase from rice bran was developed based on the fact that the optimal vessel pressure for cell growth was different from that for cellulase production.

EXPERIMENTAL

Microorganisms and Medium

E. coli JM109/LBH-10 contains a gene encoding the cellulase of *Psychrobacter aquimaris* LBH-10 (Lee *et al*. 2014). The medium used for the production of cellulase contained the following components: 57.1 g/L rice bran, 6.40 g/L ammonium chloride, 5.0 g/L K_2HPO_4, 1.0 g/L NaCl, 0.6 g/L $MgSO_4 \cdot 7H_2O$, and 0.6 g/L $(NH_4)_2SO_4$. All chemicals and media used in this study were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA) and Difco Lab. (Sparks, MD, USA), respectively.

Production of Cellulase

Starter cultures for the production of cellulase by *E. coli* JM109/LBH-10 were prepared as described previously (Lee *et al*. 2014). The cultures were incubated at 35 °C for 2 days under aerobic conditions. Each starter culture was used to inoculate 150 mL of medium in 500-mL Erlenmeyer flasks. The main culture was carried out in the above-mentioned medium for 3 days under aerobic conditions. Samples were periodically withdrawn from the cultures to examine cell growth and the production of cellulase.

Batch fermentations for the production of cellulase by *E. coli* JM109/LBH-10 were performed in a 100-L bioreactor (Ko-Biotech Co., Inchen City, Korea) with a working volume of 70-L. The 100-L bioreactor was made of the stainless steel. The medium in the bioreactor is automatically sterilized and its vessel pressure can be controlled with ranges from 0.00 to 0.10 MPa. The inoculum sizes of batch fermentations for the production of cellulase were 5% (v/v). The temperature for batch fermentations was maintained at 35 °C (Lee *et al*. 2014).

Analytical Methods

Dry cell weight was measured as described previously (Jo *et al*. 2008; Kim *et al*. 2013). The activity of cellulase was measured by the 3,5-dinitrosalicylic acid (DNS) method (Kim *et al*. 2012b). Glucose was used to prepare a calibration curve. One unit of each cellulase was defined as the amount of enzyme that released 1 μmol of reducing sugar equivalent to glucose per minute under the assay conditions.

RESULTS AND DISCUSSION

Effect of Vessel Pressure on the Production of Cellulase

The effect of vessel pressure on cell growth and the production of cellulase by *E. coli* JM109/LBH-10 was investigated in a 100-L bioreactor. The vessel pressure ranged from 0.00 to 0.08 MPa. The agitation speed and aeration rate of the bioreactor were 270 rpm and 1.00 vvm, respectively. The radius of the impeller in the 100-L bioreactor was bigger than that in a 7-L bioreactor. The angular velocity of a 100-L bioreactor at 270 rpm was almost the same as that of a 7-L bioreactor at 480 rpm (Lee *et al*. 2015). During batch fermentation, the concentration of dissolved oxygen in the culture media decreased (Fig. 1). The concentration of dissolved oxygen in the medium decreased until 36 h after cultivation with vessel pressures of 0.00, 0.02, and 0.04 MPa, whereas it decreased until 24 h after cultivation with vessel pressures of 0.06 and 0.08 MPa. The cell growth of *E. coli* JM109/LBH-10 rapidly increased until 36 h after cultivation. The production of cellulase by *E. coli* JM109/LBH-10 was not correlated with cell growth but occurred during the mid-log and stationary phases.

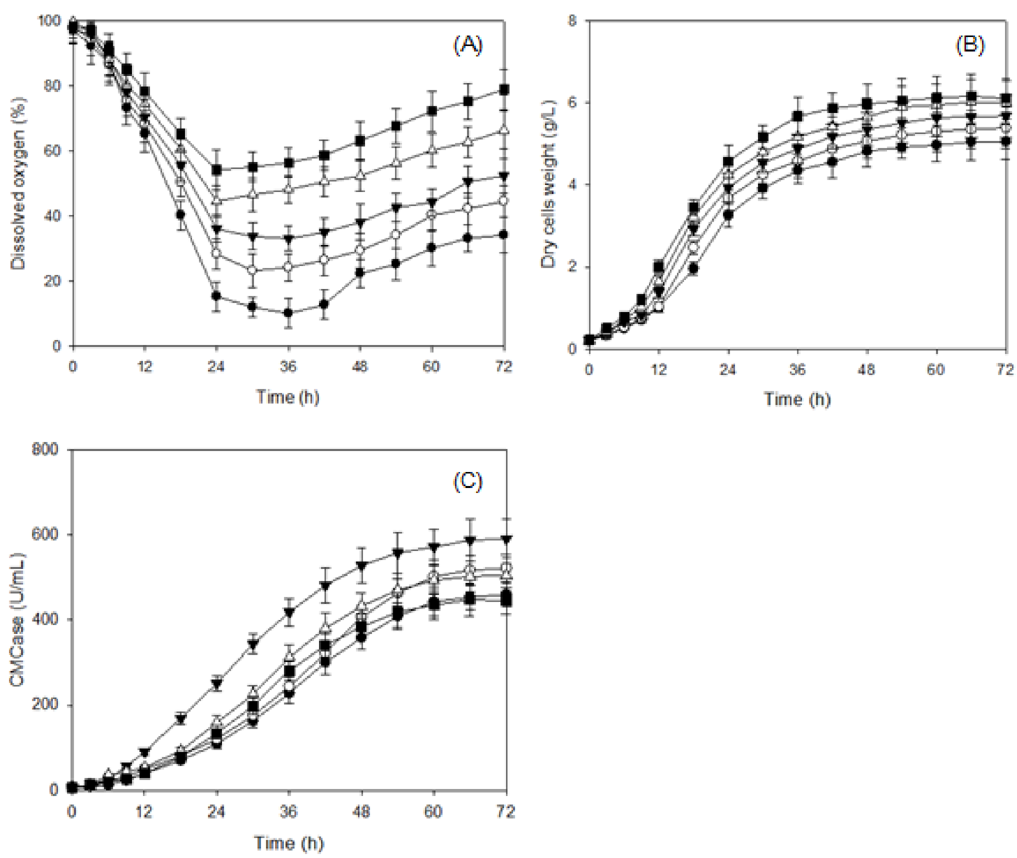

Fig. 1. Effect of the pressure within the reactor on dissolved oxygen (A), cell growth (B), and production of cellulase (C) by *E. coli* JM109/LBH-10 in a 100-L bioreactor (●, 0.00MPa; ○, 0.02 MPa; ▼, 0.04 MPa; △, 0.06 MPa; and ■, 0.08 MPa)

Based on the relationship between cell growth and their production, the production kinetics can be classified by 1) the growth associated production, 2) the non-growth associated production, and 3) the mixed-growth associated production (Shuler and Kargi 2001). The production pattern of cellulase by *E. coli* JM109/LBH-10 was the mixed-growth associated production, in which production takes place during the stationary phase. However, that of its wild type, *P. aquimaris* LBH-10 was the growth associated production, which means that CMCase was produced at the same time as cell growth.

The optimal vessel pressures for *E. coli* JM109/LBH-10 cell growth was 0.08 MPa, whereas cellulase production was best at 0.04 MPa (Table 1). The yield of cellulase from *E. coli* JM109/LBH-10 cultures with vessel pressures of 0.00, 0.02, 0.04, 0.06, and 0.08 MPa were 459.6, 521.3. 590.0, 504.1, and 444.5 U/mL, respectively. The production of cellulase in the culture with vessel pressure of 0.04 MPa was 1.3 times higher than the culture without increasing the vessel pressure. Increased vessel pressure of bioreactors can yield higher concentrations of dissolved oxygen in the medium and protect the culture from contamination (Seo *et al*. 2004; Jung *et al*. 2013). As shown in Table 2, the optimal bioreactor vessel pressure for the growth of some microorganisms was different from those for production of cellulase (Kim *et al*. 2012; Lee *et al*. 2012). The production of cellulase by *B. velezensis* A-68 with vessel pressure of 0.04 MPa was 1.2 times higher than that without vessel pressure (Gao *et al.* 2014). The production of cellulase by the recombinant *E. coli* JM109/A-53 with an optimized vessel pressure of 0.06 MPa was 1.4 times higher than that without vessel pressure (Lee *et al*. 2013). The higher concentration of dissolved oxygen in the medium caused by increased vessel pressure seems to result in higher cell growth, which enhances cellulase production.

Table 1. Effect of Vessel Pressure on the Growth and Production of Cellulase by *E. coli* LBH-10 in a 100-L Bioreactor

Inner Pressure (MPa)	DCW (g/L)	Cellulase (U/mL)		$Y_{x/s}$ (g/g)	$Y_{p/s}$ (U/g)	$Y_{p/x}$ (U/g)	μ (/h)	$μ_{max}$ (/h)
0.00	5.06 ± 0.44	459.6 ± 25.7		0.09	8.05	90.8	0.12	0.52
0.02	5.39 ± 0.50	521.3 ± 32.5		0.09	9.13	96.7	0.14	0.55
0.04	5.68 ± 0.52	590.9 ± 46.2		0.10	10.35	104.0	0.15	0.62
0.06	6.01 ± 0.54	504.1 ± 29.5		0.11	8.83	83.9	0.15	0.67
0.08	6.11 ± 0.49	444.5 ± 31.4		0.11	7.78	72.7	0.15	0.73

Table 2. Comparison of Optimal Vessel Pressure for Cell Growth and Production of Cellulases in Pilot-scale Bioreactors

Microorganism	Cellulase	Cell Growth		Production		Reference
		Inner pressure (MPa)	DCW (g/L)	Inner pressure (MPa)	Cellulase (U/mL)	
B. atrophaeus LBH-18	CMCase	0.06	2.96	0.06	127.5	Kim *et al.* 2012
B. velezensis A-68	CMCase	0.00	1.46	0.04	108.1	Gao *et al.* 2014
Cellulophaga lytica LBH-14	CMCase	0.00	3.51	0.06	153.1	Cao *et al.* 2013
Cellulophaga lytica LBH-14	cellobiase	0.00	3.20	0.06	140.1	Gao *et al.* 2015
E. coli JM109/DL-3	CMCase	0.08	3.05	0.06	871.0	Lee *et al.* 2012
E. coli JM109/A-53	CMCase	0.06	5.42	0.06	880.2	Lee *et al.* 2013
E. coli JM109/LBH-10	CMCase	0.08	6.11	0.04	590.9	This study

Effect of Shifts in Vessel Pressure on the Production of Cellulase

The culture conditions used to investigate the effect of shifts in the bioreactor pressure were 1) constant at 0.08 MPa, 2) shift from 0.08 to 0.04 MPa after 12 h, 3) shift from 0.08 to 0.04 MPa after 24 h, 4) shift from 0.08 to 0.04 MPa after 36 h, and 5) constant at 0.04 MPa. The time periods of 12, 24, and 36 h represented the early log-phase, mid-term log-phase, and late log-phase, respectively. As shown in Table 3, maximal cell growth was obtained at a pressure 0.08 MPa, which is the optimal vessel pressure for the cell growth of *E. coli* JM109/LBH-10. However, the maximal production of cellulase was with a shift in the vessel pressure from 0.08 to 0.04 MPa after 24 h. The yield of cellulase was 636.8 U/mL, which was 1.5 times higher than the yield under the optimized vessel pressure for cell growth and 1.2 times higher than that under the optimized vessel pressure for the production of cellulase. The enhanced production of cellulase with a shift in vessel pressure was due to the production pattern of *E. coli* JM109/LBH-10 (Fig. 2). Unlike the parental strain *P. aquimaris* LBH-10 with the growth-associated production of cellulase, *E. coli* JM109/LBH-10 showed mixed-growth associated production (Kim *et al*. 2010). The shift in vessel pressure from the optimal one for cell growth to that for production of cellulase at the mid-log phase resulted in more cells participating in making cellulase during the stationary phase. Shifts in culture pH, temperature, agitation speed, and aeration rate have been reported to enhance the production of cellulase (Kim *et al*. 2013; Lee *et al*. 2015).

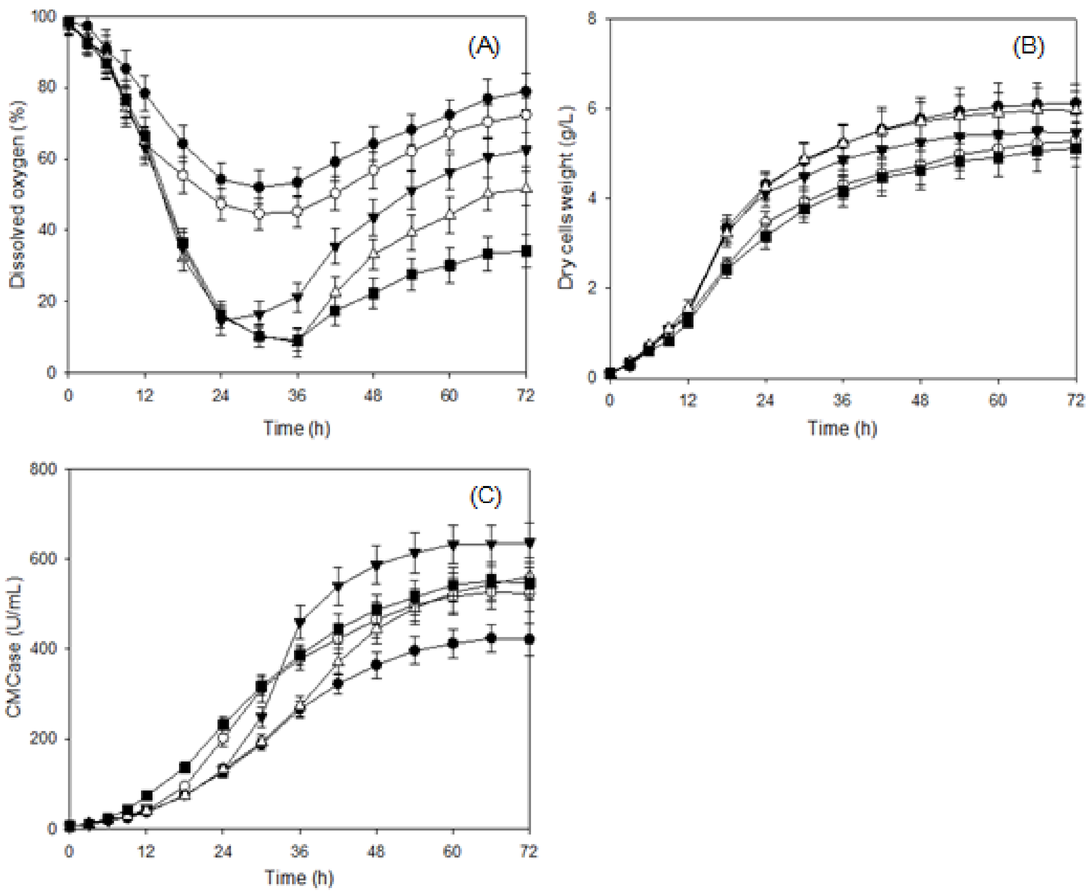

Fig. 2. Effect of shift in vessel pressure on dissolved oxygen (A), cell growth (B), and production of cellulase (C) by *E. coli* JM109/LBH-10 in a 100-L bioreactor (●, constant at 0.08 MPa; ○, shift from 0.08 to 0.04 MPa after 12 h; ▼, shift from 0.08 to 0.04 MPa after 24 h; △, shift from 0.08 to 0.04 MPa after 36 h; and ■, constant at 0.04 MPa)

Table 3. Effect of Shift in Vessel Pressure of Bioreactor on the Cell Growth and Production of Cellulase by *E. coli* JM109/LBH-10

Shift in Vessel Pressure		DCW (g/L)	Cellulase (U/mL)	$Y_{x/s}$ (g/g)	$Y_{p/s}$ (U/g)	$Y_{p/x}$ (U/g)	μ (/h)	$μ_{max}$ (/h)
Inner Pressure (MPa)	Time (h)							
0.08	0	6.13 ± 0.42	421.0 ± 35.9	0.11	7.37	68.7	0.19	0.25
0.08 to 0.04	12	5.29 ± 0.39	523.3 ± 40.1	0.09	9.16	98.9	0.17	0.24
0.08 to 0.04	24	5.47 ± 0.41	636.8 ± 44.1	0.10	11.15	116.4	0.19	0.25
0.08 to 0.04	36	5.97 ± 0.42	562.2 ± 40.5	0.10	9.85	94.2	0.19	0.27
0.04	0	5.12 ± 0.40	545.6 ± 36.2	0.09	9.60	106.6	0.17	0.21

CONCLUSIONS

1. Rice bran and low-cost ammonium chloride were used as carbon and nitrogen sources for the production of cellulose. This approach to the production of cellulase can overcome a major constraint in the enzymatic saccharification of agricultural by-products for fermentable sugars.
2. In this study, the simple process of shift in the vessel pressure of bioreactors resulted in the enhanced production of cellulase. This process can be directly applied to the industrial-scale production of cellulases.
3. The cellulase produced by *E. coli* JM109/LBH-10 was a mixed-growth-associated product, unlike that from the parental strain, *P. aquimaris* LBH-10, which was a growth-associated product. The shift from the optimal inner pressure for cell growth to that for the production of cellulase after the mid-term log-phase resulted in relatively higher cell growth and production of cellulase.

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