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Continuous Cellulosic Bioethanol Fermentation by Cyclic Fed-Batch Cocultivation

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Cocultivation of cellulolytic and saccharolytic microbial populations is a promising strategy to improve bioethanol production from the fermentation of recalcitrant cellulosic materials. Earlier studies have demonstrated the effectiveness of cocultivation in enhancing ethanolic fermentation of cellulose in batch fermentation. To further enhance process efficiency, a semicontinuous cyclic fed-batch fermentor configuration was evaluated for its potential in enhancing the efficiency of cellulose fermentation using cocultivation. Cocultures of cellulolytic *Clostridium thermocellum* LQRI and saccharolytic *Thermoanaerobacter pseudethanolicus* strain X514 were tested in the semicontinuous fermentor as a model system. Initial cellulose concentration and pH were identified as the key process parameters controlling cellulose fermentation performance in the fixed-volume cyclic fed-batch coculture system. At an initial cellulose concentration of 40 g liter^{-1}, the concentration of ethanol produced with pH control was 4.5-fold higher than that without pH control. It was also found that efficient cellulosic bioethanol production by cocultivation was sustained in the semicontinuous configuration, with bioethanol production reaching 474 mM in 96 h with an initial cellulose concentration of 80 g liter^{-1} and pH controlled at 6.5 to 6.8. These results suggested the advantages of the cyclic fed-batch process for cellulosic bioethanol fermentation by the cocultures.

Bioethanol remains an important renewable energy alternative to petroleum-based liquid transportation fuels (1). While bioethanol derived from food crops such as corn and sugarcane has dominated the current biofuel market, recent efforts have focused on the conversion of lignocellulosic biomass to bioethanol, i.e., cellulosic bioethanol, which is considered to be socioeconomically and environmentally more sustainable (2, 3).

However, the recalcitrance of cellulosic feedstock to bioconversion has posed a major challenge to the development of effective processes for cellulosic bioethanol, which typically include separate steps of enzymatic cellulose hydrolysis and microbial ethanologenic fermentation. One strategy to improve the cellulose utilization efficiency and then the ethanol production rate is the development of microbial consortia capable of simultaneously carrying out both cellulose hydrolysis and ethanologenic fermentation, representing an implementation of the consolidated bioprocessing (CBP) concept (4). Indeed, it has been demonstrated in earlier studies that cocultivation of cellulolytic and saccharolytic microbial populations could be successfully developed in batch cultures to improve cellulose utilization and ethanol production (5–7). Subsequent studies further identified metabolic mutualism, such as the supply of growth factors and utilization of excess metabolites, as the mechanism contributing to these improvements in ethanolic cellulose fermentation by cocultivation (5, 8, 9), further supporting the potential of cocultivation for enhancing cellulosic bioethanol fermentation.

Compared to mesophilic fermentation, direct fermentation of cellulosic biomass to ethanol by thermophilic bacteria has attracted increasing attention as thermophilic bioprocessing offers several advantages, such as a high cellulose utilization rate, facilitation of ethanol removal and recovery, reduction of cooling cost, and less chance of contamination (2). The thermophilic cellulolytic *Clostridium. thermocellum* strain LQRI and the saccharolytic *Thermoanaerobacter pseudethanolicus* strain X514 have been shown to exhibit enhanced ethanolic fermentation from cellulose in batch operation through cocultivation (5). However, the fermentation performance for the coculture in fed-batch operation was unknown. Fed-batch fermentation is a production technique between batch and continuous fermentation and is so called as a semicontinuous system with relative operational simplicity and superior industrial feasibility (10–12). As a new, attractive alternative strategy of semicontinuous cultivation, cyclic fed-batch fermentation has an additional advantage in that the productive phase of a process can be extended (13).

Thus, the objectives of this study were to (i) assess the efficiency of cellulosic bioethanol production in semicontinuous cyclic fed-batch cellulose fermentation with cellulolytic and saccharolytic cocultures and (ii) identify key process parameters controlling cellulose fermentation performance in the cyclic fed-batch coculture system. Toward those objectives, the cocultures of cellulolytic *Clostridium thermocellum* LQRI and saccharolytic *Thermoanaerobacter* sp. strain X514 were developed in a fixed-volume cyclic fed-batch mode system and the efficiency of cellulosic bioethanol

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TABLE 1 TaqMan probes and primers for the quantification of the 16S rRNA genes of *C. thermocellum* strain LQRI and *T. pseudoethanolicus* strain X514

Primer/probe	Sequence (5′–3′)	Target gene	Amplicon length (bp)	Optimum concn (nM)
PcqF	AAAGGAGAAATCCGGTATGA	*C. thermocellum* strain LQRI 16S rRNA gene	61	600
PcqR	AGCCGTTACCTCACCAACT			900
Probe	(6-FAM)-ATGGGCCCGCGTCCGATTAGC-TAMRA			400
PtxF	AACCCCTGCCTCTAGT	*T. pseudethanolicus* strain X514 16S rRNA gene	107	100
PtxR	GCCCAGGGCATATAGG			300
Probe	(6-FAM)-CTAGAGGGACTGCCGTGGACAACACG			400

fermentation was evaluated. As a type of extended fed-batch cultivation process, the fixed-volume cyclic fed-batch system used in this study refers to the periodic withdrawal and replacement of a portion of the reactor volume with an equal volume of fresh medium, with the residual culture functioning as the inoculum for subsequent fed-batch cycles (13). Results from this study show that efficient cellulosic bioethanol production by the cocultures was sustained in cyclic fed-batch fermentation.

MATERIALS AND METHODS

Chemicals and strains. Solka Floc (International Fiber Co., Urbana, OH) was used as the cellulosic substrate. All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO). *C. thermocellum* strain LQRI (ATCC 35609) was obtained from the American Type Culture Collection (Manassas, VA). *Thermoanaerobacter* sp. strain X514 was originally isolated from the deep subsurface in the Piceance Basin, CO (14), and has been maintained in our laboratory culture collection. Strain X514 has been deposited at the American Type Culture Collection (ATCC BAA-938). Strain LQRI is cellulolytic (15), while strain X514 is noncellulolytic but saccharolytic (14). Both strains are thermophilic and have been shown to exhibit enhanced ethanolic fermentation from cellulose in cocultivation (5).

Medium formulation and preparation. All cellulose fermentation experiments were conducted in an anaerobic medium modified from a previously described mineral salts formula (16). The medium for an initial Solka Floc concentration of 10 g liter^{-1} contained the following (per liter): 10.0 g NaCl, 0.5 g $MgCl_2 \cdot 6H_2O$, 0.2 g KH_2PO_4, 0.3 g NH_4Cl, 0.3 g KCl, 0.015 g $CaCl_2 \cdot 2H_2O$, 0.2 g yeast extract, 1 ml trace element solution, 1 ml selenium-tungsten solution, 2.52 g $NaHCO_3$, 0.05 mg resazurin. The amounts of KH_2PO_4, NH_4Cl, and yeast extract in the medium increased proportionally with the increase in the initial Solka Floc concentrations used for fermentation. The trace element solution contained the following (per liter): 1.5 g $FeCl_2 \cdot 4H_2O$, 0.19 g $CoCl_2 \cdot 6H_2O$, 0.1 g $MnCl_2 \cdot 4H_2O$, 70 mg $ZnCl_2$, 6 mg H_3BO_3, 36 mg $Na_2MoO_4 \cdot 2H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, and 2 mg $CuCl_2 \cdot 2H_2O$. The selenium-tungsten solution contained 6 mg $Na_2SeO_3 \cdot 5H_2O$ per liter, 8 mg $Na_2WO_4 \cdot 2H_2O$ per liter, and 0.5 g NaOH per liter.

The pH of the medium was adjusted to 7.2 to 7.3 by purging with an oxygen-free nitrogen-CO_2 gas mix. The anoxic condition was maintained by the addition of sulfide (0.048 g $Na_2S \cdot 9H_2O$) and cysteine (0.031 g L-cysteine) as reductants as previously described (8). Following autoclaving, the medium was supplemented with 1% (vol/vol) filter-sterilized vitamin solution containing the following (per liter): 20 mg biotin, 20 mg folic acid, 100 mg pyridoxine hydrochloride, 50 mg riboflavin, 50 mg thiamine, 50 mg nicotinic acid, 50 mg pantothenic acid, 1 mg vitamin B_{12}, 50 mg *p*-aminobenzoic acid, and 50 mg thioctic acid.

Configuration of batch and cyclic fed-batch fermentation. Triplicate batch fermentations were set up in 70-ml anaerobic culture bottles with a 30-ml working volume as previously described (17). Cellulose fermentation was initiated by a 1% (vol/vol) inoculum of log-phase cultures (optical density at 600 nm [OD_{600}], ~0.5). Monoculture was inoculated with *C. thermocellum* strain LQRI only, also referred to as the CT monoculture. Cocultures were inoculated with *Thermoanaerobacter* sp. strain X514 in addition to *C. thermocellum*, also referred to as the CT-X514 cocultures. All batch fermentation experiments were performed at 60°C without constant agitation.

Cyclic fed-batch fermentations were carried out in two 7-liter BioFlo 110 fermentors (New Brunswick Scientific Co., Edison, NJ) with agitation at 50 rpm and temperature set at 60 ± 0.5°C. The working volume for each fermentor was 3 liters. One fermentor was inoculated with the CT monoculture, while the other fermentor was inoculated with the CT-X514 cocultures. Both fermentors were operated in cyclic fed-batch mode with fixed volume, as described previously (13). The duration of one fed-batch cycle was 96 h when cellulose was fed at concentrations not higher than 40 g liter^{-1}; the duration of fed-batch cycles was extended to 240 h for 60 g liter^{-1} cellulose and 264 h for initial cellulose concentrations of 80 to 100 g liter^{-1} to maximize cellulose utilization. Following the completion of bioethanol fermentation in each feeding cycle, 90% of the culture suspension was discharged within 15 min and subsequently replaced with an equal volume of fresh medium including cellulose within 15 min. Prior to replacement, the fresh medium was autoclaved and cooled to around 60°C. Nitrogen was sparged into the fermentors for 10 min every 8 h. Gas effluent was linked to a water trap to avoid oxygen going into the fermentors, and the fermentors were inoculated only at the beginning of experiments.

At each initial cellulose concentration, the fermentor was considered to have reached stable performance when the variation in cellulose utilization and ethanol concentration in three continuous operational cycles was less than 5%. The bioreactors could be operated with or without automatic pH control. The control of pH was achieved by automatic addition of 5 M NaOH. All cyclic fed-batch fermentation experiments were repeated as duplicates. The operation of fermentors was started with an initial cellulose concentration of 10 g liter^{-1} without pH control. Under this condition only, the fermentors were operated for 15 cycles. Under all other conditions, the fermentors were operated for 6 to 8 cycles, as fermentors reached stable operation after 4 to 5 operational cycles according to cellulose and ethanol concentrations at the end of each cycle. Detailed chemical analysis was done only after fermentors became stable.

Quantitative real-time PCR. Genomic DNA of cells for each strain and cocultures was extracted using a method modified from the work of Zhou et al. (18). For preparation of standards, strains LQRI and X514 were first grown on cellobiose and glucose in 70-ml anaerobic batch culture bottles, respectively. Subsequently, cells were harvested, washed, and resuspended to an OD_{600} of less than 1.0 with TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0). After the addition of lysozyme, samples were incubated at 30°C for 30 min with RNase A (0.1 mg ml^{-1}) to remove RNA. To extract DNA from cocultures in the fed-batch fermentor, the agitation speed in the fermentor was increased to make the suspension and cellulose well mixed, and then 2-ml samples were taken out.

The 16S rRNA gene sequences of *C. thermocellum* strain LQRI and *Thermoanaerobacter pseudethanolicus* strain X514 were aligned for TaqMan probe and primer design with Primer Express software (Applied Biosystems, Foster City, CA). All primers and probes were synthesized and labeled by Applied Biosystems (Table 1). The fluorogenic probe was 5′ labeled with FAM (6-

carboxyfluorescein) and 3′ labeled with TAMRA (6-carboxytetramethylrhodamine), which served as a quenching dye.

Real-time PCR was performed in a 25-μl reaction mixture that consisted of 2 μl of template DNA, 12.5 μl of TaqMan Universal Master Mix (Applied Biosystems), and primers and probe. The PCR protocol for bacterial 16S rRNA gene quantification was as follows: 2 min at 50°C, 10 min at 95°C, and then 45 cycles consisting of 15 s at 95°C and 1 min at 60°C. Reactions were carried out in an iQ5 real-time PCR detection system (Bio-Rad). The fluorescence signal was normalized by dividing the reporter dye (6-carboxyfluorescein) emission by the passive reference dye emission. The parameter C_T (threshold cycle) is the cycle number at which the fluorescence emission crossed a threshold within the logarithmic increase phase. The threshold was defined as 10 times the standard deviation around the average intensity of background fluorescence from nontemplate controls.

Primer and probe concentrations were optimized, and the optimum primer and probe concentrations used in this study are summarized in Table 1. Standard curves, based on the log transformation of known cell concentration (cells ml^{-1}) versus threshold cycle, were obtained through extracting DNA of pure culture samples after counting cell numbers by light microscopy, as shown in Fig. S1 in the supplemental material.

Analytical methods. To monitor the production of fermentation products, samples (2 ml) from the culture broth were taken periodically using degassed sterile syringes followed by filtration through an 0.2-μm membrane filter. Ethanol in the samples was quantified with gas chromatography using a previously described protocol (17). Quantification of acetate, lactate, formate, glucose, cellobiose, and xylose was conducted with a high-performance liquid chromatography (HPLC) apparatus (Agilent Technologies, Santa Clara, CA) equipped with a Bio-Rad HPX-87H column at 55°C. The mobile phase was 0.025% (vol/vol) H_2SO_4 at a flow rate of 0.6 ml min^{-1}. Total soluble reducing sugar in the fermentation broth was determined as glucose equivalent value by the phenol-sulfuric acid method (19).

The residual cellulose concentration was determined through removal of noncellulosic materials using acetic acid-nitric acid reagent, and then quantification was done through measuring the total sugar content (20). Carbon recovery was calculated by assuming stoichiometric production of CO_2 with ethanol, acetate, and lactate (21). Briefly, carbon recovery was calculated as a function of the concentrations (moles per liter) of ethanol (E), acetic acid (A), lactic acid (L), initial cellulose (C_i) and final cellulose (C_f), initial total soluble sugar (S_i), and final total soluble sugar (S_f) by using the following equation: carbon recovery = $[3 \times (E + A + L)]/[6 \times (C_i - C_f) + 6 \times (S_f - S_i)]$. The calculated carbon recovery acted as an index reflecting the extent to which consumed substrate was accounted for in fermentation products (21).

RESULTS

Cyclic fed-batch fermentation of cellulose without pH control: bioethanol production. To evaluate the effectiveness of semicontinuous cyclic fed-batch fermentation for cellulosic bioethanol fermentation, the decomposition of Solka Floc at an initial cellulose concentration of 10 g $liter^{-1}$ and subsequent conversion to bioethanol by the CT-X514 cocultures as well as the CT monoculture were monitored in cyclic fed-batch bioreactors without pH control. Stable fermentation was achieved in the fermentors after five 96-h fed-batch cycles, as evidenced by the identical cellulose utilization and ethanol yields at the end of fermentation cycles (see Fig. S2 in the supplemental material). Stable operation was continued for another 10 fed-batch cycles, and temporal samples were taken throughout the 15th fed-batch cycle to monitor the dynamics of pH, soluble sugars, and fermentation products in a single fed-batch cycle, as shown in Fig. 1.

Bioethanol production in the fermentor inoculated with the CT-X514 cocultures was much higher than that with the CT monoculture. While the bioethanol concentration in the monoculture fermentor peaked at approximately 10 mM, the bioethanol level in the CT-X514 coculture fermentor reached 42.8 mM at 28 h of the 15th operational cycle (Fig. 1A). However, the production of bioethanol diminished beyond 28 h of the fed-batch cycle (Fig. 1A). In comparison, the concentration of acetate as the fermentation product continued to rise past 28 h of the fed-batch cycle. No fermentation end product other than ethanol and acetate was detected in the fermentors.

Cyclic fed-batch fermentation of cellulose without pH control: cellulose utilization and accumulation of soluble sugars. As shown in Fig. 1B, the monoculture had a 7-hour lag phase for cellulose utilization, but there was no lag phase for cocultures. However, cellulose utilization rates for monoculture and cocultures did not show much difference between 10 and 30 h, as suggested by similar slopes of cellulose concentration during this period. At the 34th hour, cellulose utilization reached ~62% in the coculture fermentor while only 46% cellulose utilization was achieved in the monoculture fermentor (Fig. 1B). Interestingly, utilization of cellulose ceased in the coculture fermentor beyond 34 h into the fed-batch cycle. In contrast, cellulose utilization in the monoculture fermentor continued throughout the 96-h fed-batch cycle. At the end of the cycle, residual cellulose levels in the monoculture and coculture fermentors were 2.3 and 3.5 g $liter^{-1}$, respectively. Thus, at the end of the fed-batch cycle, cellulose utilization in the monoculture fermentor became greater than that in the coculture fermentor, despite the much higher ethanol production in the coculture fermentor than in the monoculture fermentor (Fig. 1A and B). This discrepancy between cellulose utilization and ethanol production in the monoculture fermentor suggested the potential accumulation of other intermediates from cellulolysis.

Indeed, soluble reducing sugars, presumably the product of cellulolysis, accumulated steadily in the monoculture fermentor throughout the fed-batch cycle, reaching 5.8 g $liter^{-1}$ at the end of the cycle. Further analysis of the composition of the soluble sugar showed that the concentrations of glucose, cellobiose, and xylose in the monoculture were 4.3, 11.5, and 2.2 mM, respectively, representing 67.6% of the total soluble reducing sugar. It could be presumed that the remaining 32.4% of the soluble sugars consisted of other short-chain cellodextrins. In contrast, the concentration of reduced sugars was minimal in the coculture fermentor, detected at only 0.17 g $liter^{-1}$ (Fig. 1B), which might be due to consumption of reduced sugars by X514. In addition, it should be mentioned that higher soluble sugar concentrations in monoculture than in coculture fermentation at the beginning of one operational cycle mainly resulted from higher residential soluble sugar concentrations at the previous operational cycle. The low initial soluble sugar concentration in coculture fermentation also favored the long-term operation of the cyclic fed-batch fermentors.

Cyclic fed-batch fermentation of cellulose without pH control: pH shift with cellulose fermentation. It was expected that pH would drop during cellulose fermentation due to the production of organic acids as fermentation end products. Indeed, pH decreased rapidly from neutral pH in the first 28 h of the fed-batch cycle, the same time period when rapid bioethanol production was observed in both the monoculture and coculture fermentors (Fig. 1C). The pH shift was more pronounced in the coculture fermentor than in the monoculture fermentor, with the pH declining to 4.9 and 5.6 in the coculture and monoculture fermentors at 28 h into the fed-batch cycle, respectively. While the pH in

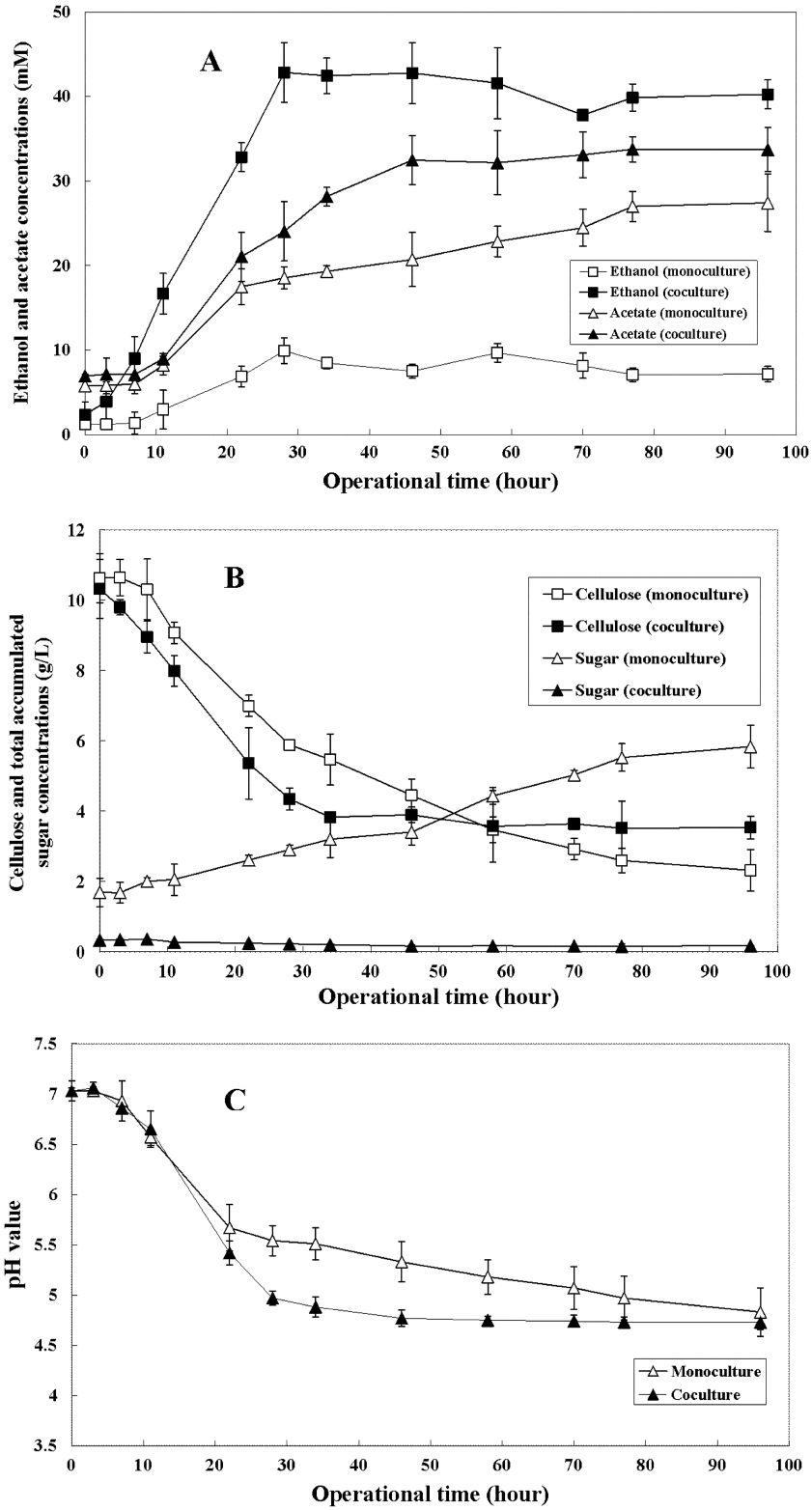

FIG 1 Ethanol and acetate concentrations (A), cellulose and total accumulated sugar concentrations (B), and pH values (C) in monoculture LQRI and coculture fermentations at an initial cellulose concentration of 10 g liter^{-1} without pH control in fed-batch fermentors at the 15th cycle. Data are means of duplicate fermentation runs with the error bars showing the standard deviations.

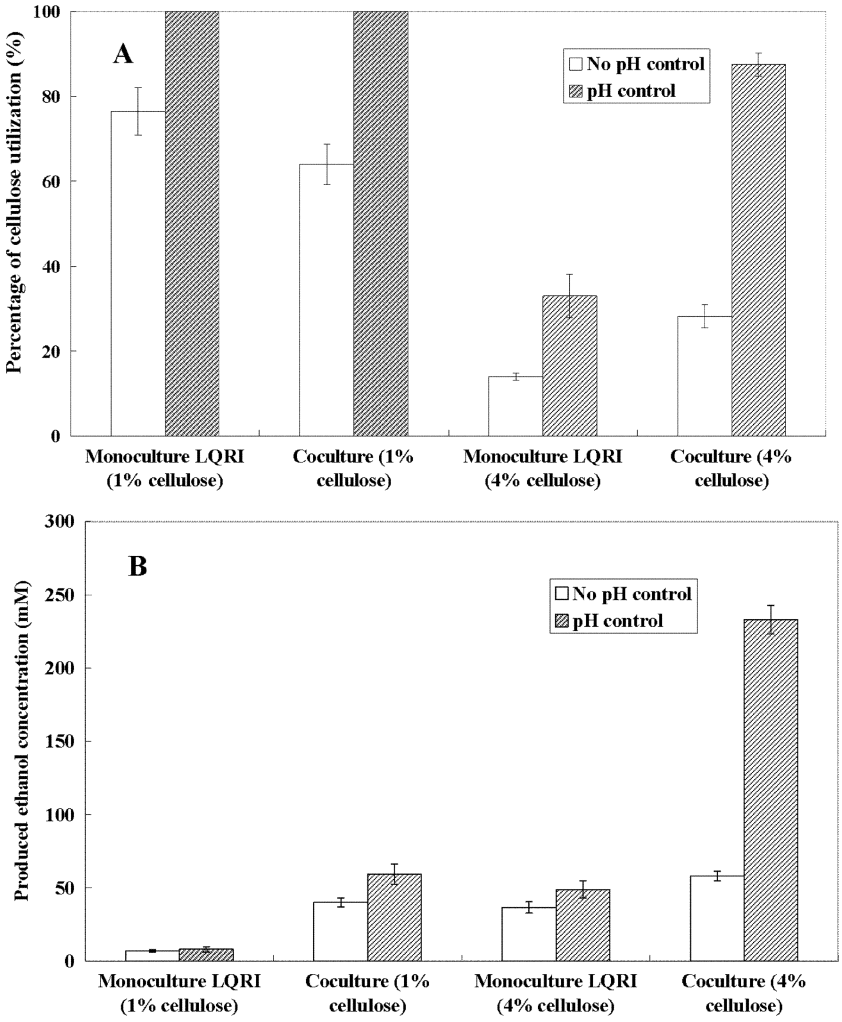

FIG 2 Effect of pH on cellulose utilization (A) and ethanol production (B) by the monoculture LQRI and coculture in cyclic fed-batch fermentors. Data were obtained after 96 h of fermentation. For both monoculture and cocultures, cellulose was completely utilized at an initial cellulose concentration of 10 g liter^{-1} under pH control.

the coculture fermentor remained steady, the pH in the monoculture fermentor continued to decline past 28 h into the fed-batch cycle. At the end of the fed-batch cycle, pHs in the two fermentors converged at 4.7 to 4.8. Notably, cellulose utilization in both fermentors diminished when pH dropped below 5.0 (Fig. 1B). Obviously, the decline of pH during cellulose fermentation appeared to be linked to cellulose utilization.

Effect of pH control in fed-batch fermentors. Cellulose fermentations by the monoculture or the coculture with or without pH control were compared between two different initial cellulose concentrations of 10 and 40 g liter^{-1}. pH was controlled around 6.5 to 6.8 by the addition of 5 M NaOH, as batch experiments showed that cultures of both strain LQRI and X514 were free of negative impact on substrate utilization at pH 6.5 or greater (see Fig. S3 in the supplemental material). As shown in Fig. 2, pH control enhanced cellulose utilization and ethanol production by both the monocultures and cocultures. The enhancement became more apparent at an initial cellulose concentration of 40 g liter^{-1} than at an initial cellulose concentration of 10 g liter^{-1}. Under pH control, the advantage of coculture fermentation over monoculture fermentation also became more obvious. While the ethanol concentration in coculture fermentation was only 1.6 times higher than that of the monoculture at the initial cellulose concentration of 40 g liter^{-1} without pH control, the ethanol concentration in coculture fermentation was 4.8 times that in monoculture fermentation at the same initial cellulose concentration with pH control. Meanwhile, pH control also significantly increased performance of coculture fermentation in terms of both cellulose utilization and ethanol production. At an initial cellulose concentration of 40 g liter^{-1}, the ethanol concentration produced with pH control was 4.5-fold higher than that produced without pH control. The results confirmed that pH was a key parameter affecting cellulose utilization and ethanol production, especially at higher initial cellulose concentrations.

Coculture fermentation in cyclic fed-batch fermentors with pH control: effect of initial cellulose concentrations. Fermentation performance obtained under low cellulose concentrations might not be achievable under high cellulose concentrations. Therefore, the response of coculture fermentation to high initial cellulose concentrations was studied at various initial cellulose

TABLE 2 Carbon recovery, ratio of ethanol to acetate, and ethanol production yield for coculture fermentation at the 96th hour of one operational cycle under various initial cellulose concentrations with pH control

Parameter	Value at initial cellulose concn (g liter^{-1}):					
	10	20	40	60	80	100
Carbon recovery	0.90 ± 0.02	0.79 ± 0.03	0.88 ± 0.03	0.91 ± 0.01	0.86 ± 0.04	0.79 ± 0.04
Ethanol/acetate (mM/mM)	1.31 ± 0.18	1.64 ± 0.14	3.45 ± 0.40	4.43 ± 0.42	4.87 ± 0.32	0.99 ± 0.08
Ethanol production/cellulose utilization (g/g)	0.29 ± 0.02	0.27 ± 0.02	0.31 ± 0.02	0.33 ± 0.03	0.32 ± 0.02	0.16 ± 0.01

concentrations ranging from 10 to 100 g liter^{-1} in the coculture fermentor with pH control at 6.5 to 6.8.

As shown in Table 2, carbon recovery rates after 96 h of fermentation under various initial cellulose concentrations ranged from 0.79 to 0.91. The performance under different initial cellulose concentrations at the 96th hour of fermentation cycles is shown in Fig. 3. Cellulose could be completely utilized within 96 h at initial cellulose concentrations of 10 and 20 g liter^{-1}. With further increases in cellulose concentration, cellulose utilization decreased. While cellulose utilizations at an initial cellulose concentration of 40 to 80 g liter^{-1} were between 79.4% and 87.3%, only 50.5% of the cellulose added was utilized at the initial cellulose concentration of 100 g liter^{-1}. With the increase in initial cellulose concentration to 80 g liter^{-1}, the accumulation of soluble sugar also increased. However, a further increase in initial cellulose concentration to 100 g liter^{-1} did not lead to an increase in the accumulation of soluble sugar.

As shown in Fig. 3B, the highest ethanol concentration was achieved at the initial cellulose concentration of 80 g liter^{-1}. A nearly linear correlation was observed between ethanol concentration and initial cellulose concentration from 10 and 80 g liter^{-1}. However, a further increase in the initial cellulose concentration

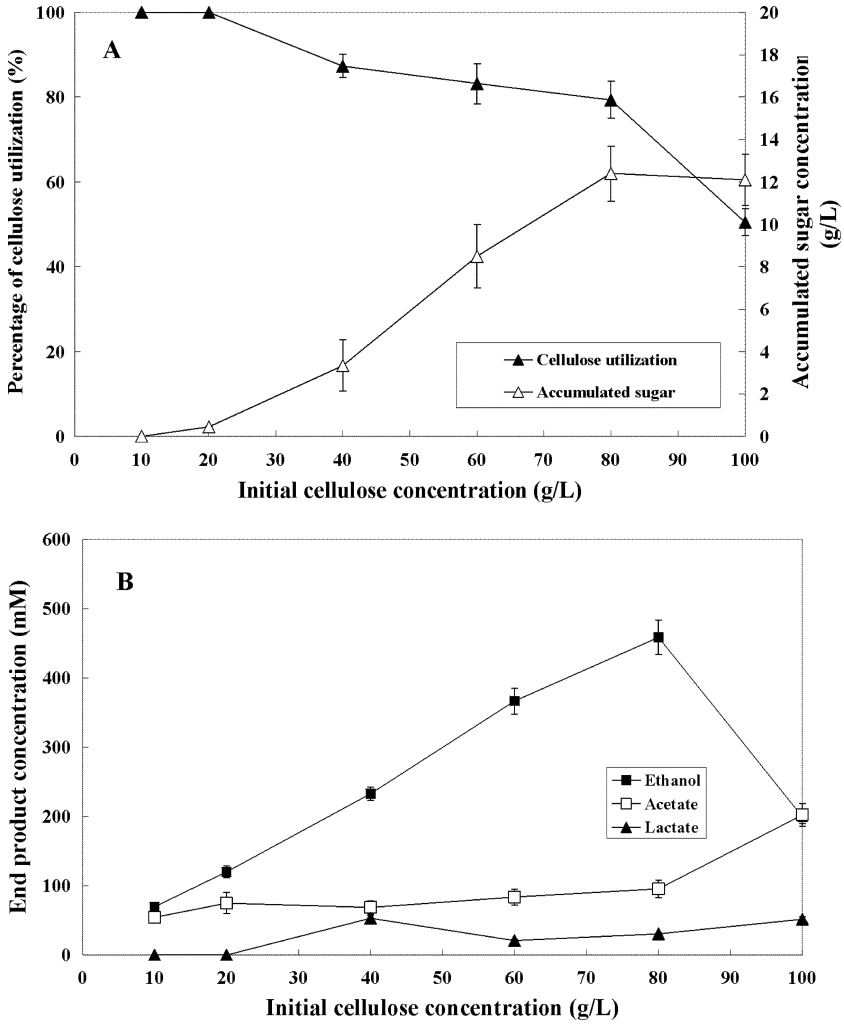

FIG 3 Effect of initial cellulose concentration on cellulose utilization and sugar accumulation (A) and end product concentration (B) in the cyclic fed-batch fermentor with pH control. Data were obtained after 96 h of fermentation.

to 100 g liter^{-1} resulted in a sharp decrease in ethanol concentration and an increase in acetate concentrations. The ethanol/acetate ratio, an important measure of the efficiency of ethanolic fermentation, reached the maximum value at the end of the fermentation cycle when the initial cellulose concentration was 80 g liter^{-1} (Table 2). The above results indicate that the optimal initial cellulose concentration for ethanol production in the coculture was 80 g liter^{-1} with pH control at 6.5 to 6.8.

Lactate production was found to depend on initial cellulose concentrations. While not detected at initial cellulose concentrations of 10 and 20 g liter^{-1}, lactate was detected at concentrations of 21 to 53 mM when cellulose was fed at higher concentrations (Fig. 3B).

Coculture fermentation performance in cyclic fed-batch fermentation with pH control under an initial cellulose concentration of 80 g liter^{-1}. Since the initial cellulose concentration of 80 g liter^{-1} resulted in the most efficient ethanolic fermentation, the fed-batch fermentation of cellulose at 80 g liter^{-1} was characterized in more detail to identify the processes underlying efficient cellulose fermentation (Fig. 4). Cellulose was utilized mainly within the initial 120 h without a lag phase. At the 120th hour, the residual cellulose concentration in the fermentor was only 4.9 g liter^{-1}. After 48 hours of fermentation, the concentrations of total soluble sugars rose quickly and reached 14.5 g liter^{-1} at the 120th hour. As shown in Fig. 4B, cellobiose, glucose, and xylose could all be detected after 48 hours of fermentation, and glucose concentrations were higher than cellobiose and xylose concentrations.

Ethanol was mainly produced within the initial 72 hours of fermentation. Subsequently, the ethanol concentration increased only slowly, reaching a maximum concentration of 474 mM at the 168th hour. Similarly, the acetate concentration had a relatively quick increase to 81.2 mM during the initial 48 h of fermentation, followed by a slower increase, eventually reaching 124.8 mM at the end of the operational cycle. While lactate concentrations also had a relatively quick increase during the initial 48-hour fermentation, further operation did not cause much change in lactate concentrations. In contrast, formate was detected only during the initial 48 h at concentrations less than 5 mM.

The population dynamics of the coculture partners were monitored by real-time PCR (Fig. 5). The cell density of strain LQRI increased quickly during the initial 72 h, followed by a lower rate of increase until the 120th hour and then a decrease with further operation. The cell density of strain X514 was found to increase quickly during an initial period, with the maximum number of $5.65 \times 10^9 \pm 0.61 \times 10^9$ liter^{-1} occurring at the 72nd hour, and then decreased with further operation. Interestingly, the decrease in cell density of strain X514 coincided with the cessation of ethanol production (Fig. 4C). Although the growth patterns of the two strains within the cycle showed differences, it was apparent that the two strains were able to coexist throughout the experimental period in the fed-batch fermentation with cellulose as the substrate.

Both the ratio of ethanol production to cellulose utilization and the ethanol production rate changed during the fermentation cycles (Fig. 6). From the 24th to the 72nd hour of the operational cycle, the ratio of ethanol production to cellulose utilization reached 0.43 with a 75.4% theoretical conversion. Subsequently, the ratio decreased significantly with further fermentation operation and became stable after 120 h with an average value of only 0.26. The ethanol production rate increased first with fermentation operation and reached the highest value of 0.8 g h^{-1} at the 72nd hour, followed by a quick decrease with further operation. At the end of the operational cycle, the ethanol production rate was only 0.22 g h^{-1}. The mechanisms related to such a phenomenon need to be further investigated in the future.

Similar to the maximum concentration of ethanol produced from cellulose, the ethanol production rate is also one of the important parameters related to production costs for biofuel production. This study showed that operational condition was related to the end product production rate, and selection of adequate fermentation time could improve the ethanol production rate. According to Fig. 4 and Fig. 6, the duration of the fermentation cycle might be set as 72 h to achieve the maximum ethanol production rate without substantially sacrificing ethanol concentration. At this point of fermentation, the ethanol concentration and ratio of ethanol production to cellulose utilization reached 449 mM and 0.42, respectively.

DISCUSSION

This study demonstrated that the two thermophilic strains *C. thermocellum* LQRI and *Thermoanaerobacter* sp. strain X514 could coexist and work together in a cyclic fed-batch model with enhanced ethanol production from thermophilic cellulose fermentation compared to the monoculture LQRI. The two strains were isolated from different environments, with LQRI originating from sewage digester sludge and effluent (22) and X514 originating from the deep subsurface environment of the Piceance Basin in Colorado (14). The coexistence of the two strains might be due to a mutualistic relationship between them, as reported previously (5). Although the saccharolytic strain X514 could not utilize cellulose, the cellulolytic strain LQRI may supply soluble carbon sources as the substrate for X514. In turn, the removal of the hydrolytic end products by X514 in the coculture may facilitate the enzymatic degradation of cellulose by LQRI. Interestingly, the hemicellulose portion of Solka Floc was also metabolized by the coculture. This may be due to the combined metabolic activities of the two strains. *C. thermocellum* hydrolyzes xylan to xylobiose and xylose (2), and strain X514 further ferments these substrates to end products. The ability to ferment hemicellulose and pentose to ethanol reemphasizes the significance of the cocultures in the bioconversion of biomass that generally has high proportions of hemicellulose.

The metabolic pathways of anaerobic thermophilic bacteria for cellulose utilization are a complex process that involves adhesion of microbial cells to cellulose, cellulose hydrolysis, and fermentation of the resulting soluble sugars. Thermophilic cellulose fermentation was affected by different environmental conditions (24). This study found that application of pH control enhanced cellulose utilization and ethanol production by the cocultures especially under high initial cellulose concentrations. Of the process parameters influencing ethanologenic fermentation, pH has been considered to be among the most important (25). The growth of anaerobic cellulolytic bacteria was particularly sensitive to low pH, and most of those strains cannot grow at pHs of $<$6.0 (2, 20, 26, 27). Therefore, the pH control as applied in this study favored cell growth. Just through application of pH control, the cocultures were able to handle high cellulose concentrations and produced ethanol at a maximum concentration of 473 mM, which demonstrated the potential of the cocultures in cellulose utilization for ethanol production.

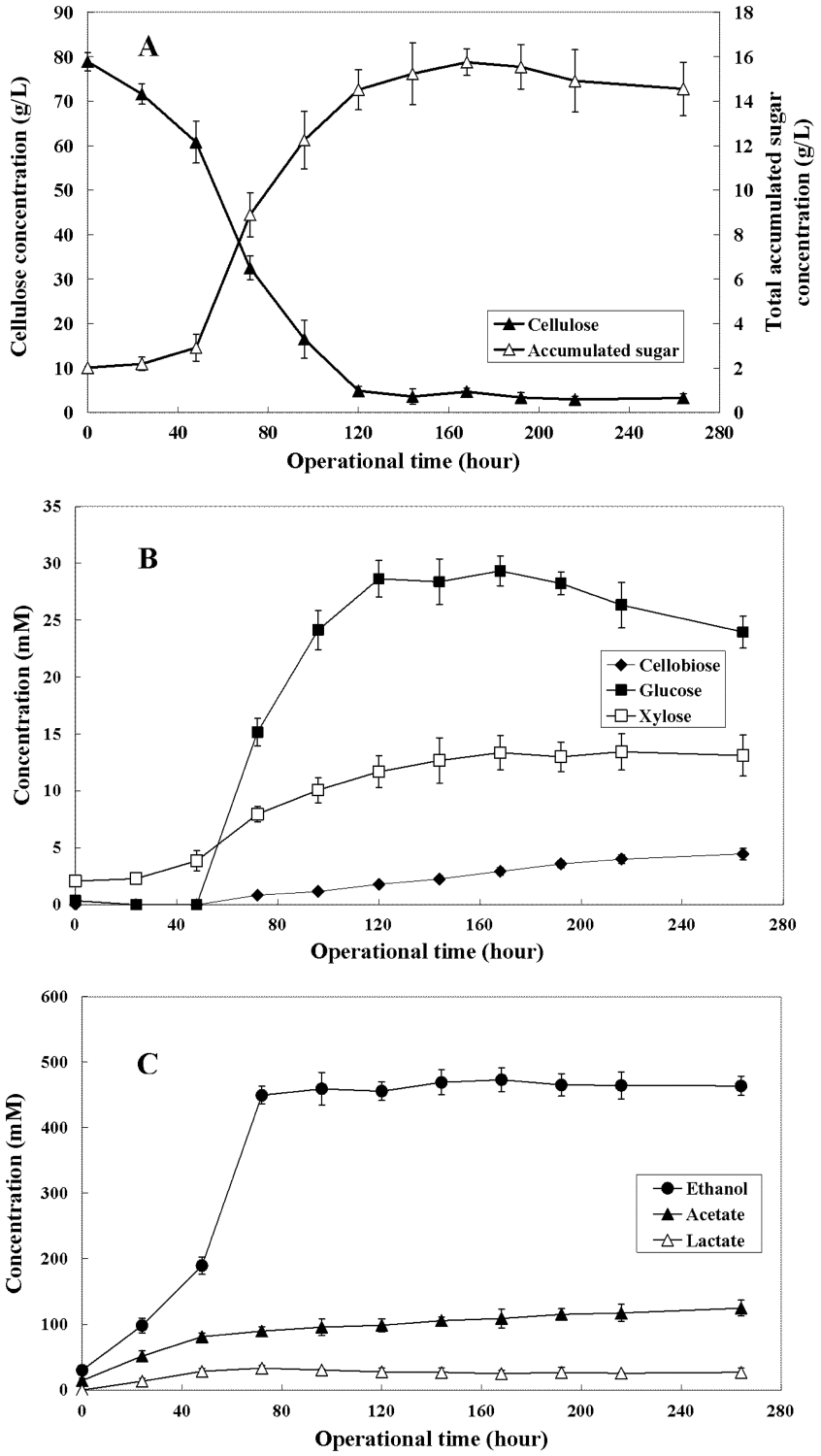

FIG 4 Cellulose and total accumulated sugar concentrations (A); cellobiose, glucose, and xylose concentrations (B); and fermentation end product concentrations (C) in coculture fermentation with pH control at an initial cellulose concentration of 80 g liter^{-1}.

With increases in initial cellulose concentrations from 10 to 80 g liter^{-1}, sugar concentrations increased in the coculture fermentation. The presence of accumulated extracellular sugars indicated that the rate of cellulose catabolism exceeded the rate of soluble sugar consumption, and the conversion of insoluble carbohydrate to soluble cello-oligosaccharides was not the rate-limiting step in cellulose fermentation by the cocultures. Meanwhile, the accumulation of sugar suggests that the activity of saccharolytic X514 did not keep pace with cellulolysis by strain LQRI. Corresponding to the accumulation of sol-

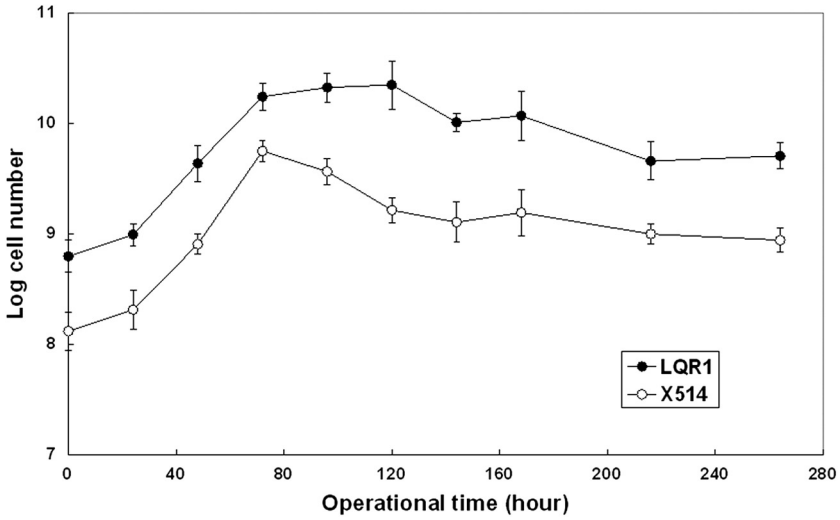

FIG 5 Cell number for the strains LQRI and X514 in coculture fermentation with pH control at an initial cellulose concentration of 80 g liter^{-1}.

uble sugars under high initial cellulose concentrations, lactate was produced by the coculture. In fact, it was found that lactate was produced by *Clostridium cellulolyticum* (28) and *C. thermocellum* ATCC 27405 (29) with an increase in initial cellulose concentrations. Therefore, initial cellulose concentrations might play a role in influencing metabolism pathways in fermentative thermophilic bacteria.

This study also found that fermentation performance by the cocultures became less efficient with the increase in initial cellulose concentration from 80 to 100 g liter^{-1}. The concentrations of both accumulated sugars and end products at an initial cellulose concentration of 100 g liter^{-1} were lower than those at an initial cellulose concentration of 80 g liter^{-1}, indicating that low bioconversion yields under 100 g liter^{-1} were not due to sugar accumulation and end product inhibition but mainly due to the inhibition of cellulose hydrolysis. In addition, under this high cellulose concentration, slurry rheological properties might undergo dynamic and dramatic changes as the conversion proceeded (30), and then mass transfer limitation became a main factor responsible for the low utilization rate (31), which might also contribute to the decrease in fermentation following the increase in initial cellulose concentrations from 80 g liter^{-1} to 100 g liter^{-1} in this study. The inhibitory effect of high cellulose concentrations suggested that cellulose concentrations for thermophilic fermentation by the cocultures LQRI and X514 should be controlled at levels not higher than 80 g liter^{-1}, which could be readily achieved by cyclic fed-batch operations (32).

In summary, thermophilic cocultivation of cellulolytic *Clostridium thermocellum* LQRI and saccharolytic *Thermoanaerobacter* sp. strain X514 was sustained in the semicontinuous configuration with efficient cellulosic bioethanol production. Initial cellulose concentration and pH were identified as the key process parameters controlling cellulose fermentation performance in the fixed-volume cyclic fed-batch coculture system. Further optimization of the fed-batch strategy could have potential to increase cellulose fermentation by the cocultures.

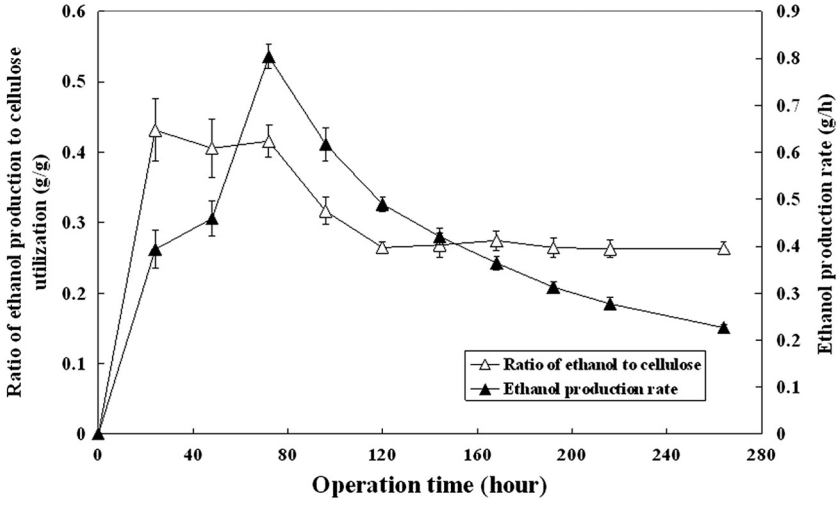

FIG 6 Profiles of the ratio of ethanol production to cellulose utilization and specific ethanol production rate under pH-controlled coculture fermentation at an initial cellulose concentration of 80 g liter^{-1}.

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