

Article

Efficient Co-Utilization of Biomass-Derived Mixed Sugars for Lactic Acid Production by *Bacillus coagulans* Azu-10

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Abstract: Lignocellulosic and algal biomass are promising substrates for lactic acid (LA) production. However, lack of xylose utilization and/or sequential utilization of mixed-sugars (carbon catabolite repression, CCR) from biomass hydrolysates by most microorganisms limits achievable titers, yields, and productivities for economical industry-scale production. This study aimed to design lignocellulose-derived substrates for efficient LA production by a thermophilic, xylose-utilizing, and inhibitor-resistant *Bacillus coagulans* Azu-10. This strain produced 102.2 g/L of LA from 104 g/L xylose at a yield of 1.0 g/g and productivity of 3.18 g/L/h. The CCR effect and LA production were investigated using different mixtures of glucose (G), cellobiose (C), and/or xylose (X). Strain Azu-10 has efficiently co-utilized GX and CX mixture without CCR; however, total substrate concentration (>75 g/L) was the only limiting factor. The strain completely consumed GX and CX mixture and homofermentatively produced LA up to 76.9 g/L. On the other hand, fermentation with GC mixture exhibited obvious CCR where both glucose concentration (>25 g/L) and total sugar concentration (>50 g/L) were the limiting factors. A maximum LA production of 50.3 g/L was produced from GC mixture with a yield of 0.93 g/g and productivity of 2.09 g/L/h. Batch fermentation of GCX mixture achieved a maximum LA concentration of 62.7 g/L at LA yield of 0.962 g/g and productivity of 1.3 g/L/h. Fermentation of GX and CX mixture was the best biomass for LA production. Fed-batch fermentation with GX mixture achieved LA production of 83.6 g/L at a yield of 0.895 g/g and productivity of 1.39 g/L/h.

Keywords: lactic acid; designed biomass; lignocellulose-derived sugars; mixed sugars; *B. coagulans*; CCR; homofermentation



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1. Introduction

Biomass materials are promising carbon sources for the production of various value-added products. Among these products, lactic acid (LA) is an essential organic acid that provides a broad range of applications in food, cosmetic, pharmaceutical, and chemical conversion industries [1]. It is also an important monomer for the production of polylactic acid (PLA) that has the potential to serve as promising biodegradable commodity plastics used for food packaging, textiles, trays, plates, and trash bags [2]. Besides, this polymer has recently achieved a great interest as an additive manufacturing technology, for 3D printing, manufacturing of implants as biomedical components [2,3]. However, the high production cost of LA is hampering the commercial availability of PLA materials. The main expenses lie within the efficient fermentation processes (either microbial strain or utilized substrate) or the downstream processes of LA production [4].

Industrially, LA can be manufactured by chemical or microbial fermentation routes. The chemical route is not preferred as it produces a racemic mixture of LA and involves the utilization of petrochemical resources [5]. Therefore, the microbial route is preferred not only because of the high optical purity enantiomers of LA that can be achieved via fermentation, but also due to the utilization of renewable substrates that significantly improved environmental sustainability and serve as relatively cheap raw materials [6].

The utilization of expensive raw materials has a significant effect on the overall LA production cost. Pure and crop sugars are utilized for production of LA; however, these sugars are considered as non-ideal substrates as compared to cellulosic materials, i.e., lignocellulosic and algal biomass act as the second and third-generation feedstock [7]. Cellulosic materials are preferred due to their low price, abundance, sustainability, and does not compete with food crops [8]. However, several technical limitations must be overcome to efficiently convert lignocellulosic biomass to LA. Of those, the direct utilization of these biomasses is difficult owing to their complex structure [4]. The main carbohydrates present in microalgae and macroalgae are significantly different from those of plant biomass. The main structural polysaccharides are composed of cellulose and hemicellulose with varied ratios based on the source and type of biomass [9,10]. These biomasses are mostly pretreated and saccharified for the release of fermentable sugars that yield mixtures of hexoses and pentoses including glucose, cellobiose, and xylose with little amounts of arabinose, mannose, and oligosaccharides [11,12].

Lactic acid-producing bacteria are efficient consumers for glucose while xylose could be utilized by few strains [4]. However, within fermentation of mixed sugars, most of these strains consume glucose preferentially to other sugars via a phenomenon known as carbon catabolite repression (CCR) resulting in extended fermentation time, wasting available substrates, lower LA titer, yield, and productivities [11,13]. Therefore, efficient utilization of all types of biomass-derived sugars in biomass hydrolysates is essential for economic conversion. In literature, very few wild type LA-producing strains could achieve efficient utilization of mixed-sugars without CCR achievable high LA titers, yields, and productivities [11,12,14]. Various approaches have been done to rescue the CCR effect for the ultimate goal of relaxed control of sugar utilization and enhanced LA fermentations including genetic engineering and utilization of co-cultures [15,16].

Most reported studies on fermentative LA production from biomasses are focused on strain isolation and selection, mutagenesis or molecular breeding, technologies for pre-treatment of cellulosic biomass, or optimization of LA fermentation from the hydrolysate regardless of their sugar composition [1,4,6–8,11,12,14–17]. However, the present investigation focused on the effect of various sugar concentrations on LA production in order to select the best sugar composition alleviating CCR. This would greatly help to design the best biomass hydrolysate composition that achieve the highest sugar utilization and LA production in term of titer, yield, and productivity. This step is highly essential for further modification of the saccharification processes feasible for the microbial strain to establish an adaptive process maximizing LA fermentation from biomass substrates.

We have recently reported a new thermophilic and homofermentative xylose utilizing LA producer, *Bacillus coagulans* Azu-10 that could ferment xylose efficiently at pH 7.0 and 50 °C [17]. This strain showed the capability of utilizing various sugars existing in the biomass hydrolysates such as glucose, cellobiose, xylose and arabinose as a sole carbon source. Besides, it showed high resistance to most microbial inhibitors derived from biomass pretreatments including furans, carboxylic acids, and phenolic compounds [17]. Therefore, the current investigation aimed to study the effect of various mixed-sugars derived from cellulosic biomass on the sugar utilization, CCR, and LA fermentation by the strain Azu-10 for the ultimate goal of establishing the optimally designed biomass hydrolysate suitable for efficient fermentation for economical LA industry-scale production.

2. Materials and Methods

2.1. Bacterial Strain and Fermentation Media

Modified de Man, Rogosa, and Sharpe (mMRS) medium supplemented with xylose instead of glucose was used for bacterial growth and inoculum preparation. mMRS is composed of g/L: xylose, 22.0; yeast extract, 5.0; peptone, 10.0; beef extract, 8.0; K_2HPO_4 , 2.0; $MgSO_4$, 0.1; $MnSO_4$, 0.05; sodium acetate, 5.0; ammonium citrate, 2.0, and tween 80, 1.0 mL. The medium pH was adjusted to 7.0 using 5 N HCl and 5 N NaOH as indicated in each experiment. All chemicals were of analytical grade and purchased from Sigma Aldrich except yeast extract and peptone that were purchased from Oxoid, Basingstoke, Hampshire, UK. Strain *Bacillus coagulans* Azu-10 isolated and characterized by our group [17] was used in this study. It was maintained in mMRS medium (containing 22.0 g/L xylose) for immediate use or preserved in 50% glycerol at -80°C .

2.2. Inoculum Preparation and Fermentations

For inoculum preparation, 1.0 mL of glycerol stock was inoculated into a test tube containing 9 mL mMRS medium and refreshed for 24 h at 50°C . A pre-culture was prepared by transferring refreshment culture into a flask containing sterilized mMRS medium at 10% (v/v), then incubated at 50°C for 18 h. The main fermentation media are mMRS supplemented with sugar at specific concentrations as described in each experiment. All fermentation experiments were performed in a 1-L fermenter (Biott, Tokyo, Japan) with a 0.4-L working volume as described previously [17]. The fermenters were inoculated with 10 % from the pre-culture. The fermentation temperature was 50°C , agitation at 200 rpm, and pH was controlled at 7.0 using 10 N NaOH.

To investigate the effect of different xylose concentrations, mMRS media were prepared and supplemented with 50, 75, 100, 125, and 150 g/L of xylose. For fermentation from the mixed sugars, different concentrations of glucose/xylose, cellobiose/xylose, glucose/cellobiose, and glucose/cellobiose/xylose mixture in mMRS medium were used as shown in Table 1 as previously described [11,12,14,15]. Batch fermentations were conducted using 1-L jar fermenter with a 0.4-L working volume under the same conditions as described above. Samples were collected at different time intervals to analyze biomass, sugar, lactic acid, acetic acid, formic acid, and ethanol concentrations.

Table 1. Design of mixed sugars used for lactic acid production in batch fermentations in the present study.

Mixture		Glucose (g/L)	Cellobiose (g/L)	Xylose (g/L)
Glucose/Xylose (G/X)	G25X25	25	–	25
	G25X50	25	–	50
	G50X25	50	–	25
	G50X50	50	–	50
Cellobiose/Xylose (C/X)	C25X25	–	25	25
	C25X50	–	25	50
	C50X25	–	50	25
	C50X50	–	50	50
Glucose/Cellobiose (G/C)	G25C25	25	25	–
	G25C50	25	50	–
	G50C25	50	25	–
	G50C50	50	50	–
	G10C10	10	10	–
	G10C20	10	20	–
	G15C15	15	15	–
	G20C10	20	10	–
Glucose/Cellobiose/Xylose (G/C/X)	G25C25X25	25	25	25
	G50C50X50	50	50	50

For fed-batch fermentation, mMRS medium was used and supplemented with 25 g/L glucose/25 g/L xylose (G25X25) as initial sugar, and then feeding was conducted with G25X25 after 20 h of fermentation. Fermentation conditions were conducted as described above. Samples were withdrawn at various time intervals and analyzed for cell growth, sugar concentrations, lactic acid, acetic acid, formic acid, and ethanol concentration.

2.3. Analytical Methods

Growth of Azu-10 cells was monitored by measurement of the optical density using a spectrophotometer at a wavelength of 562 nm ($OD_{562\text{ nm}}$). Sugars (glucose, xylose, and cellobiose), and fermentation products (lactic acid, acetic acid, formic acid, and ethanol) were analyzed using high-performance liquid chromatography system (HPLC, Agilent 1200 series chromatograph, USA) using a refraction index detector (RID-6A) and Biorad Aminex HPX-87H column (300×7.8 mm) at 50 °C. The mobile phase was sulfuric acid (0.01 N) at a flow rate of 1.0 mL/min. The samples were filtered through a Sep Pak C18 filter then injected into a column at 20 μ L injection volume. The average calibration curves were constructed with the series of glucose, xylose, cellobiose, and lactic acid standard solutions in the range 5.0–20 g/L. While standard solutions for acetic acid, formic acid, and ethanol were prepared in the range of 2.5–10 g/L. Total consumed sugars (g/L) were calculated based on the differences between initial and final sugar concentration. The yield of LA (g/g) was calculated as the ratio of the produced LA (g/L) to consumed sugar concentration (g/L); LA productivity (g/L/h) was calculated as the ratio of produced LA (g/L) to the fermentation time (h); maximum LA productivity (g/L/h) was determined by the difference between LA concentrations of two respective samples divided by the time difference.

2.4. Statistical Analysis

One-way analysis of variance (ANOVA) was used to show the significant differences between treatments. The mean difference comparison between the treatments was subsequently analyzed by the Tukey HSD (honestly significant difference) test at $p < 0.05$. Data analysis was subjected using statistical package SPSS v17 (SPSS Inc., Chicago, IL, USA). All results presented are the means of three independent replicates.

3. Results

3.1. Effect of Xylose Concentration on Lactic Acid Fermentation

Xylose is metabolised homofermentatively or heterofermentatively depending on microbial LA-producer strain and/or xylose concentration [18]. In our previous study, we reported that strain Azu-10 metabolized xylose homofermentatively using 22 g/L of xylose. To study the effect of higher xylose concentration on LA production and by-product formation and to investigate substrate inhibition effect, various xylose concentrations (50–150 g/L) were utilized in batch fermentations (Table 2). High cell biomass ranged $OD_{562\text{ nm}}$ of 14.7–17.8 was obtained at xylose concentration of 50–125 g/L while a sharp decrease in $OD_{562\text{ nm}}$ of 10.2 was obtained at 150 g/L xylose concentration. Up to 100 g/L, xylose was completely consumed while 31.1 g/L and 72.5 g/L was left in the fermentation media at initial xylose concentration of 125 g/L and 150 g/L, respectively. At 50 g/L xylose, 50.7 g/L of LA was produced with an LA yield of 0.99 g/g and productivity of 4.22 g/L/h and little formic acid at 2.6 g/L while no acetic acid or ethanol was produced. At 75 g/L xylose, high LA production of 76.5 g/L with a yield of 0.989 g/g, LA productivity of 4.25 g/L/h, and without ethanol production was obtained. Little formic acid (2.91 g/L) and acetic acid (2.61 g/L) were produced. Additionally, complete conversion of xylose to LA was obtained at 100 g/L xylose with 102 g/L of LA production at LA yield of 1.0 g/g with little by-product formation. However, lower LA productivity than obtained at 50–75 g/L xylose at 3.19 g/L/h was obtained due to longer fermentation time. On the other hand, the highest maximum LA productivity was obtained at 100 g/L xylose with 8.55 g/L/h as compared to 5.63–8.03 g/L/h obtained at 50–75 g/L xylose. Lower

LA production of 101.2 g/L and 75.7 g/L were obtained at 125 g/L and 150 g/L xylose, respectively. These results indicated that a higher initial xylose concentration resulted in a higher concentration and yield of lactic acid in the fermentation up to 100 g/L with little by-product and high fermentation efficiency.

Table 2. Effect of xylose concentrations on lactic acid production by *B. coagulans* Azu-10.

Xylose Conc. (g/L)	Max. Biomass (OD _{562 nm})	Residual Xylose (g/L)	LA (g/L)	Formic Acid (g/L)	Acetic Acid (g/L)	Ethanol (g/L)	Y _{LA} (g/g)	P _{LA} [g/(L·h)]	Max. P _{LA} [g/(L·h)]
50	14.7	0.0	50.7 ± 1.38	2.60	0.0	0.0	0.999	4.22	5.63
75	17.8	0.0	76.5 ± 1.56	2.91	2.61	0.0	0.988	4.25	8.03
100	15.5	0.0	102.2 ± 0.661	2.21	2.50	0.0	1.0	3.18	8.55
125	15.7	31.1	101.2 ± 1.2	0.0	1.98	0.0	1.0	2.11	7.33
150	10.2	72.5	75.7 ± 2.78	0.0	1.56	0.0	0.920	1.57	3.42

LA, Lactic acid; Y_{LA}, Lactic acid yield; P_{LA}, Lactic acid productivity; Max. P_{LA}, maximum lactic acid productivity.

3.2. Effect of Different Ratios of Glucose and Xylose on CCR and LA Production

To investigate the effects of sugar mixture on LA fermentation, *Bacillus coagulans* Azu-10 was firstly grown in batch fermentations with four ratios of glucose/xylose mixtures: G25X25, G25X50, G25X50, and G50X50. In all fermentations, glucose was completely utilized within 10 h with almost similar consumption rates, while xylose consumption was quite different (Table 3, Figure 1). With G25X25 and G25X50 (Figure 1A,B), simultaneous utilization of glucose and xylose occurred with complete consumption of both sugars within 30 h. A lower xylose consumption rate was achieved in the presence of glucose compared with that obtained after glucose depletion. When xylose concentration was increased, a significant decrease in LA yield was obtained with G25X50 at 0.77 g/g than 0.956 g/g obtained with G25X25 (Table 3). Besides, fermentation of G25X25 exhibited LA concentration (54.2 g/L) with higher LA productivity (4.93 g/L/h) and maximum LA productivity (8.29 g/L/h) than G25X50 at 1.97 g/L/h and 7.13 g/L/h, respectively. With G50X25 (Figure 1C), simultaneous utilization of both sugars was achieved with complete sugar consumption within 24h. A higher glucose consumption rate was obtained that completely utilized within 8 h of fermentation. Higher LA titre (76.9), yield (0.97 g/g), productivity (3.2 g/L/h), and maximal productivity (11.49 g/L/h) were obtained than with the same total sugar concentration of 75 g/L (G25X50). With G50X50 (Figure 1D), glucose was completely consumed within 10 h while high residual xylose concentration (20.8 g/L) was left in the fermentation media. Only 80.5 g/L of LA was produced with a yield of 0.97 g/g, productivity of 1.67 g/L/h, and maximal LA productivity of 12.2 g/L/h. As shown (Figure 1D), the CCR was only appeared at high mixed sugar concentration (G50X50).

Table 3. Effect of different ratios of glucose/xylose mixtures on sugar utilization and lactic acid fermentation efficiency by *B. coagulans* Azu-10.

Mixed Sugars	Max. Biomass (OD _{562 nm})	Residual Glucose (g/L)	Residual Xylose (g/L)	LA (g/L)	Formic Acid (g/L)	Acetic Acid (g/L)	Ethanol (g/L)	Y _{LA} (g/g)	P _{LA} [g/(L·h)]	Max. P _{LA} [g/(L·h)]
G25X25	15.3	0.0	0.0	54.2 ± 0.898	0.80	0.420	0.0	0.956	4.93	8.29
G25X50	13.8	0.0	0.0	59.3 ± 3.16	1.0	1.14	0.0	0.770	1.97	7.13
G50X25	15.1	0.0	0.0	76.9 ± 2.91	0.0	0.280	0.0	0.970	3.20	11.4
G50X50	14.5	0.0	20.8	80.5 ± 1.45	0.0	0.0	0.0	0.890	1.67	12.2

[G25X25], fermentation of 25 g/L glucose and 25 g/L xylose; [G25X50], fermentation of 25 g/L glucose and 50 g/L xylose; [G50X25], fermentation of 50 g/L glucose and 25 g/L xylose; [G50X50], fermentation of 50 g/L glucose and 50 g/L xylose; LA, Lactic acid; Y_{LA}, Lactic acid yield; P_{LA}, Lactic acid productivity; Max. P_{LA}, maximum lactic acid productivity.

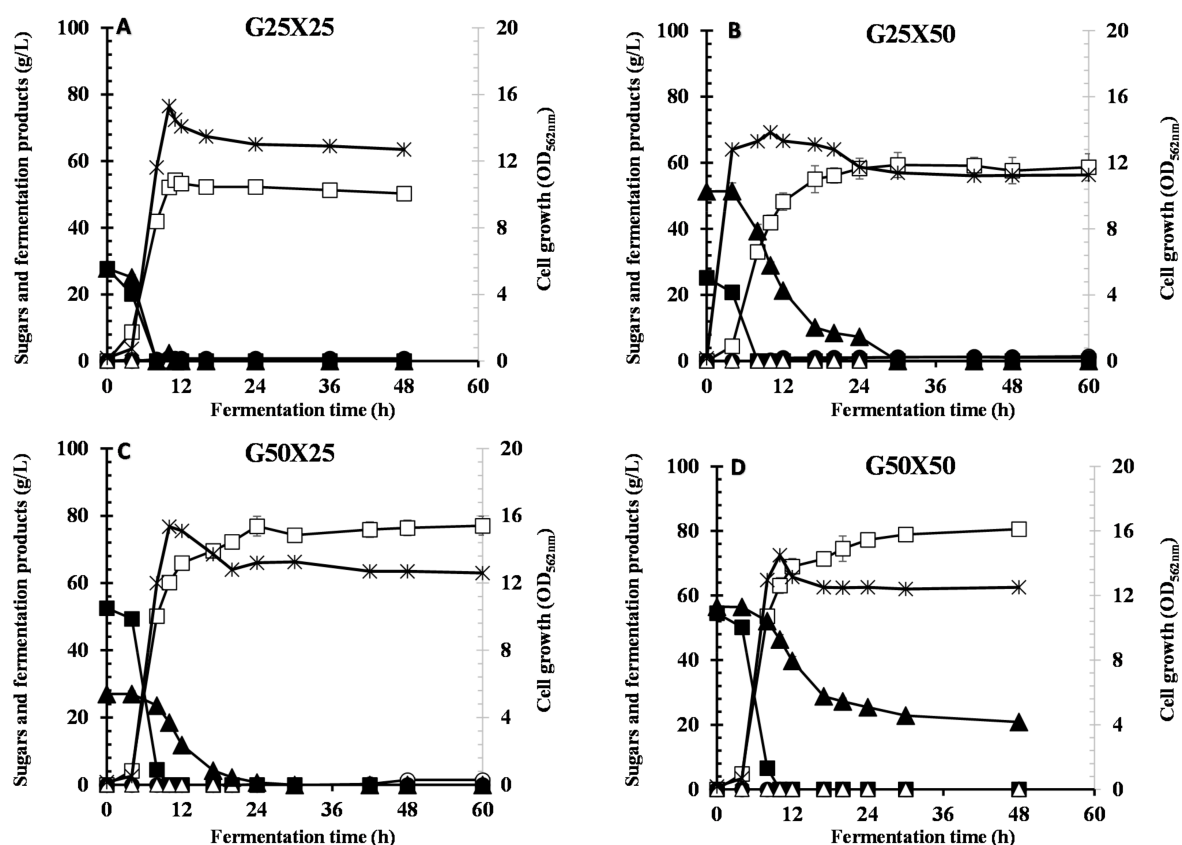


Figure 1. Effect of different glucose/xylose concentrations on the growth and lactic acid production by *B. coagulans* Azu-10. (A) fermentation of 25 g/L glucose and 25 g/L xylose [G25X25]; (B) fermentation of 25 g/L glucose and 50 g/L xylose [G25X50]; (C) fermentation of 50 g/L glucose and 25 g/L xylose [G50X25]; (D) fermentation of 50 g/L glucose and 50 g/L xylose [G50X50]. Symbols: ■, glucose (g/L); ▲, xylose (g/L); □, lactic acid (g/L); ○, acetic acid (g/L); ●, formic acid (g/L); Δ, ethanol (g/L); *, growth ($OD_{562\text{ nm}}$).

3.3. Effect of Different Ratios of Cellobiose and Xylose on CCR and LA Production

To investigate the effects of the replacement of glucose by cellobiose in sugar mixture on CCR and LA fermentation, *Bacillus coagulans* Azu-10 was grown with different ratios of cellobiose/xylose mixtures: C25X25, C25X50, C50X25, and C50X50 (Table 4). High cell biomass ranged $OD_{562\text{ nm}}$ of 12.6–15.8 was obtained at a total mixed-sugar concentration of 50–75 g/L while a sharp decrease in $OD_{562\text{ nm}}$ of 10.7 was obtained at 100 g/L mixed-sugar concentration. In all fermentations, cellobiose was consumed completely within 48 h. The sugar consumption rates were lower than obtained with glucose/xylose mixture (Figure 2). With G25X25 (Figure 2A), both sugars were simultaneously utilized within 14 h with the production of 51.5 g/L of LA at a yield of 1.0 g/g, productivity of 3.22 g/L/h, and maximal productivity of 6.60 g/L/h (Table 4). Little by-products were produced (1.31 g/L of formic acid and 1.11 g/L acetic acid). With C25X50 (Figure 2B), both sugars were simultaneously consumed with almost the same consumption rates. Cellobiose was consumed within 12 h while xylose was consumed within 24 h. Complete conversion of sugars to LA was achieved with production of 74.2 g/L LA at a yield of 1.02 g/g; productivity of 3.12 g/L/h, and maximal LA productivity of 8.68 g/L/h. On the other hand, xylose consumption rate was decreased when cellobiose concentration was increased to 50 g/L (Figure 2C,D). With C50X25, all sugars were completely consumed within 24 h, with the production of 74.5 g/L LA at a yield of 0.964 g/g; productivity of 2.69 g/L/h, and maximal LA productivity of 6.91 g/L/h. With C50X50, cell biomass was greatly decreased to $OD_{562\text{ nm}}$ of 10.7 and simultaneous sugar consumption was achieved but with lower rates than previous fermentations. Cellobiose was consumed within 30 h while xylose was not completely

consumed and 8.58 g/L was left in the medium after 48 h (Figure 2D). Only 87.1 g/L of LA at yield of 1.02 g/g; productivity of 1.81 g/L/h, and maximal LA productivity of 7.34 g/L/h was obtained. Little acetic acid at 1.86 g/L was produced without formic acid or ethanol production. As shown by Figure 2, no CCR occurred at all fermentations. Strain Azu-10 achieved better fermentation efficiency with cellobiose/xylose mixture than glucose/xylose mixture in terms of sugar co-utilization, high LA titre, and yields.

Table 4. Effect of different ratios of cellobiose/xylose mixtures on sugar utilization and lactic acid fermentation efficiency by *B. coagulans* Azu-10.

Mixed Sugars	Max. Biomass (OD _{562 nm})	Residual Cellobiose (g/L)	Residual Xylose (g/L)	LA (g/L)	Formic Acid (g/L)	Acetic Acid (g/L)	Ethanol (g/L)	Y_{LA} (g/g)	P_{LA} [g/(L·h)]	Max. P_{LA} [g/(L·h)]
C25X25	12.6	0.0	0.0	51.5 ± 1.46	1.31	1.11	0.0	1.00	3.22	6.60
C25X50	15.8	0.0	0.0	74.2 ± 0.438	2.40	2.36	0.0	1.20	3.12	8.68
C50X25	14.6	0.0	0.0	74.5 ± 0.799	0.0	1.67	0.0	0.964	2.69	6.91
C50X50	10.7	0.0	8.58	87.1 ± 1.59	0.0	1.86	0.0	1.02	1.81	7.34

[C25X25], fermentation of 25 g/L cellobiose and 25 g/L xylose; [C25X50], fermentation of 25 g/L cellobiose and 50 g/L xylose; [C50X25], fermentation of 50 g/L cellobiose and 25 g/L xylose; [C50X50], fermentation of 50 g/L cellobiose and 50 g/L xylose; LA, Lactic acid; Y_{LA} , Lactic acid yield; P_{LA} , Lactic acid productivity; Max. P_{LA} , Maximum lactic acid productivity.

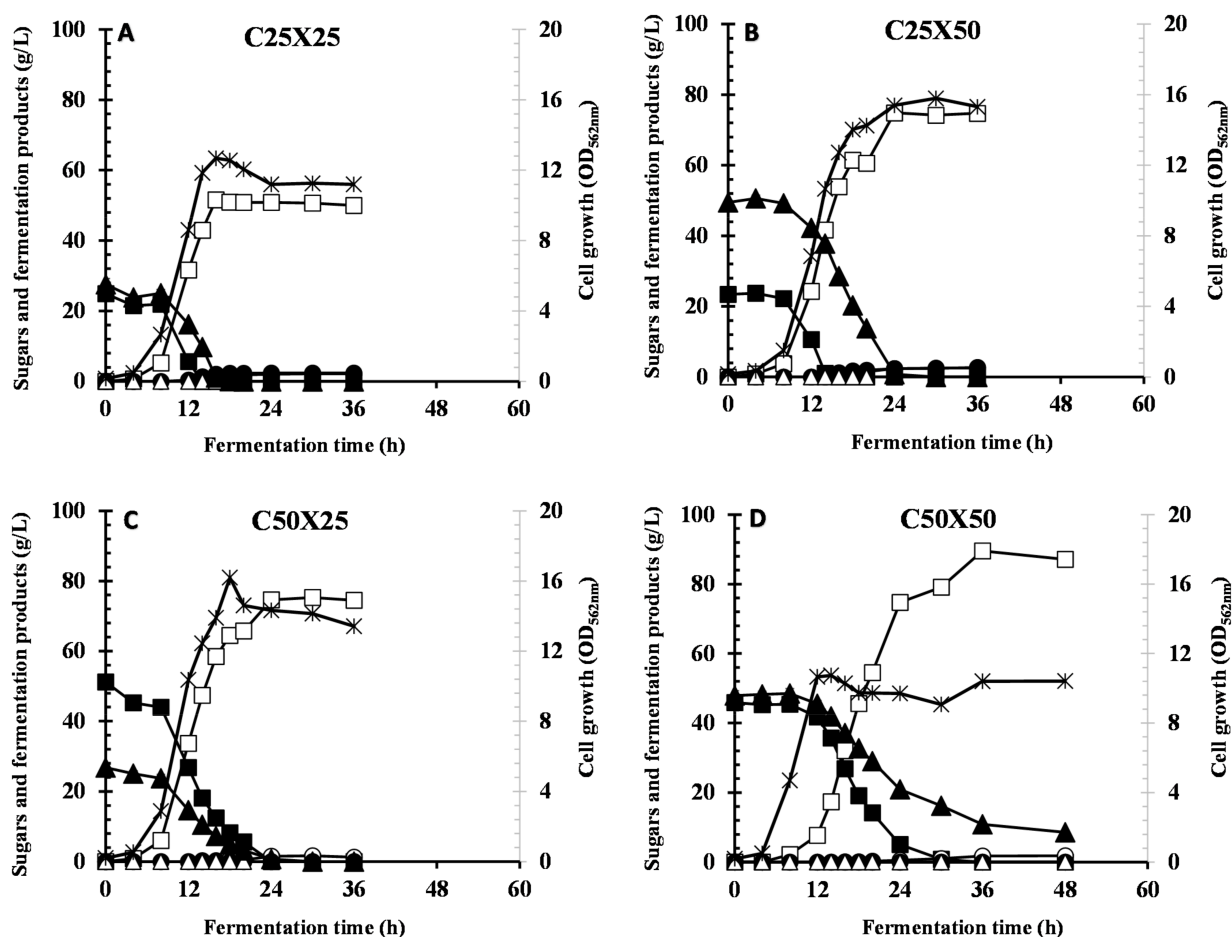


Figure 2. Effect of different cellobiose/xylose concentrations on growth and lactic acid production by *B. coagulans* Azu-10. (A) fermentation of 25 g/L cellobiose and 25 g/L xylose [C25X25]; (B) fermentation of 25 g/L cellobiose and 50 g/L xylose [C25X50]; (C) fermentation of 50 g/L cellobiose and 25 g/L xylose [C50X25]; (D) fermentation of 50 g/L cellobiose and 50 g/L xylose [C50X50]. Symbols: ■, cellobiose (g/L); ▲, xylose (g/L); □, lactic acid (g/L); ○, acetic acid (g/L); ●, formic acid (g/L); △, ethanol (g/L); *, growth (OD_{562 nm}).

3.4. Effect of Different Ratios of Glucose and Cellobiose on CCR and LA Production

To investigate the effects of glucose/cellobiose mixture on CCR and LA fermentation, *Bacillus coagulans* Azu-10 was firstly grown with different ratios of glucose/cellobiose mixtures: G25C25, G25C50, G50C50, and G50C25 (Figure 3). Comparable cell biomass ranged OD_{562 nm} of 8.74–9.38 was obtained when 25 g/L glucose was used while higher biomass at OD_{562 nm} of 13.7–15.3 were obtained when 50 g/L glucose was used (Table 5). In contrast to the previous fermentations with G/X or C/X mixture, obvious CCR occurred in G/C sugar mixture where glucose is completely consumed within 4–8 h and cellobiose is hardly utilized (Figure 3A–D). On the other hand, a significant increase in glucose consumption rate was obtained in the presence of cellobiose achieving a maximal LA productivity ranged 12.4–14.5 g/L/h as compared to that obtained with G/X and C/X mixtures.

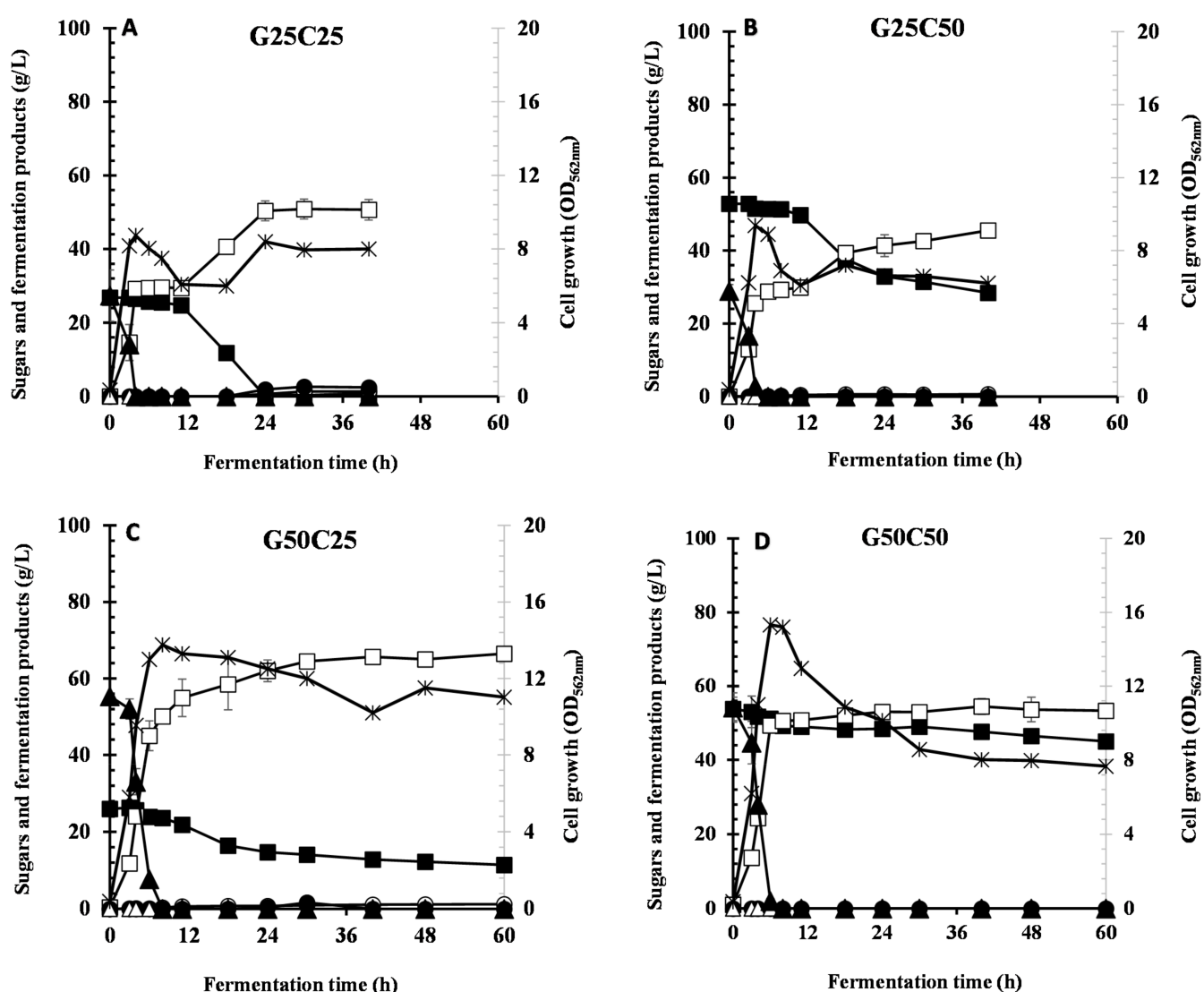


Figure 3. Effect of different glucose/cellobiose concentrations on growth and lactic acid production by *B. coagulans* Azu-10. (A) fermentation of 25 g/L glucose and 25 g/L cellobiose [G25C25]; (B) fermentation of 25 g/L glucose and 50 g/L cellobiose [G25C50]; (C) fermentation of 50 g/L glucose and 25 g/L cellobiose [G50C25]; (D) fermentation of 50 g/L glucose and 50 g/L cellobiose [G50C50]. Symbols: ■, cellobiose (g/L); ▲, glucose (g/L); □, lactic acid (g/L); ○, acetic acid (g/L); ●, formic acid (g/L); Δ, ethanol (g/L); *, growth (OD_{562 nm}).

With G25C25 (Figure 3A), glucose was consumed within 4 h while cellobiose was consumed within 24 h. A maximum LA production of 50.3 g/L with LA yield of 0.93 g/g,

productivity of 2.09 g/L/h was obtained. The maximal productivity achieved the highest value at 14.5 g/L/h among all tested sugar mixtures. Few by-products of acetic acid (0.61 g/L), formic acid (1.85 g/L), and ethanol (0.590 g/L) were produced. In the other fermentations with G25C50, G50C25, and G50C50, only glucose was completely utilized with high residual cellobiose at 28.4, 11.4, and 45 g/L, respectively (Figure 3B–D). Additionally, lower LA concentrations (45.5–65.6 g/L) with yields ranged 0.846–0.959 g/L were obtained as compared to the same total sugar with other sugar mixtures. No formic acid or ethanol was produced while little acetic acid of 0.662 g/L and 1.08 g/L was produced from G25C50 and G50C25, respectively.

In a trial to rescue the effect of CCR, fermentations with lower sugar concentrations of G10C10, G10C20, G15C15, G20C10, and G20C10 were investigated (Figure S1, see supplementary data). CCR was apparently relieved upon the depletion of glucose that was utilized within 4 h while cellobiose was consumed within 18 h. Almost all sugar mixtures are converted to LA (ranged 23.7–40.5 g/L) with ~1.0 g/g yield, productivities ranged 1.31–2.25 g/L/h, and maximum LA productivities ranged 9.85–11.6 g/L/h.

3.5. Utilization of Glucose/Xylose/Cellobiose Mixture

To investigate LA fermentation from glucose/xylose/cellobiose mixture, batch fermentation of G25X25C25 and G50X50C50 were performed as shown in Figure 4. With G25X25C25 (Figure 4A), strain Azu-10 achieved high cell biomass ($OD_{562\text{nm}}$ of 14.1) with complete consumption of glucose within 12 h. As indicated, CCR existed and is relieved after glucose depletion where xylose and cellobiose were then simultaneously consumed. Xylose was consumed at a higher rate compared with cellobiose. At the end of fermentation, 2.22 g/L of xylose and 4.39 g/L of cellobiose was remained unutilized (Table 6). A maximum LA concentration of 62.7 g/L was produced at a LA yield of 0.962 g/g, a productivity of 1.3 g/L/h, and maximal productivity of 5.65 g/L/h. No ethanol was produced while very little formic acid (0.414 g/L) and acetic acid (0.431 g/L) were produced. On the other hand, high glucose concentration drastically affected G50X50C50 fermentation (Figure 4B) as only 80.4 g/L of LA was produced with very low yield (0.71 g/g) and high residual sugar (6.22 g/L xylose, and 36.3 g/L cellobiose).

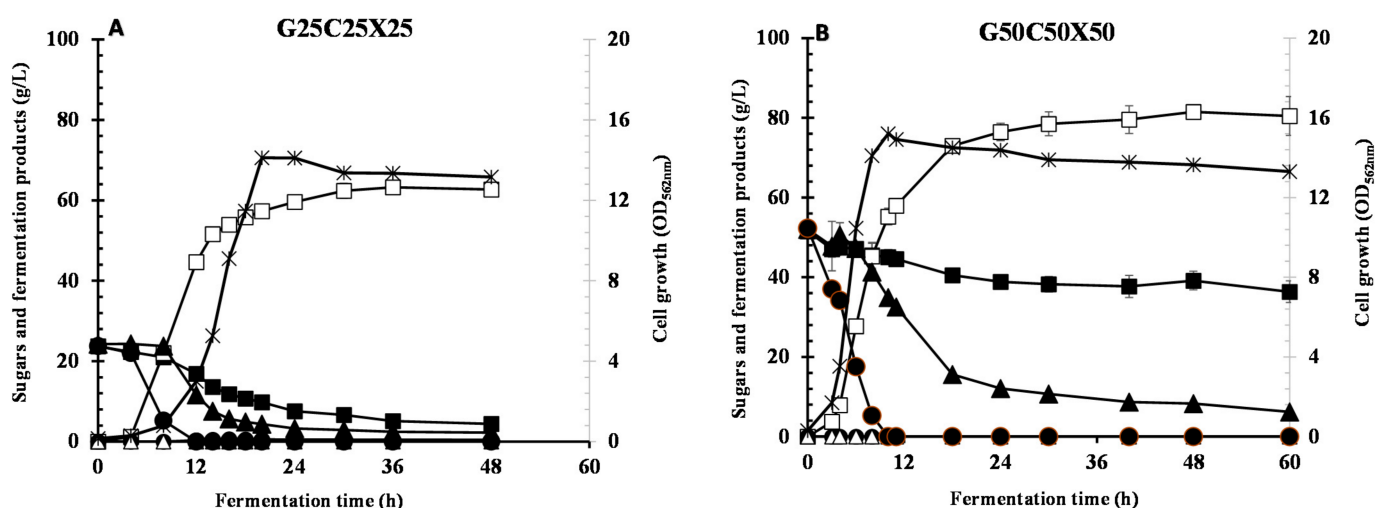


Figure 4. Effect of different glucose/cellobiose/xylose concentrations on growth and lactic acid production by *B. coagulans* Azu-10. (A) fermentation of 25 g/L glucose, 25 g/L cellobiose, and 25 g/L xylose [G25C25X25]; (B) fermentation of 50 g/L glucose, 50 g/L cellobiose, and 50 g/L xylose [G50C50X50]. Symbols: ●, glucose (g/L); ■, cellobiose (g/L); ▲, xylose (g/L); □, lactic acid (g/L); ○, acetic acid (g/L); ◆, formic acid (g/L); Δ, ethanol (g/L); *, growth ($OD_{562\text{nm}}$).

Table 5. Effect of different ratios of glucose/cellobiose mixture on sugar utilization and lactic acid fermentation efficiency by *B. coagulans* Azu-10.

Mixed Sugars	Max. Biomass (OD _{562 nm})	Residual Glucose (g/L)	Residual Cellobiose (g/L)	LA (g/L)	Formic Acid (g/L)	Acetic Acid (g/L)	Ethanol (g/L)	Y _{LA} (g/g)	P _{LA} [g/(L·h)]	Max. P _{LA} [g/(L·h)]
C25G25	8.74	0.0	0	50.3 ± 2.68	1.85	0.610	0.590	0.930	2.09	14.5
G25C50	9.38	0.0	28.4	45.5 ± 1.31	0.0	0.662	0.0	0.853	1.13	12.6
G50C25	13.7	0.0	11.4	65.6 ± 0.459	0.0	1.08	0.0	0.959	1.64	12.7
G50C50	15.3	0.0	45.0	53.3 ± 1.96	0.0	0.0	0.0	0.846	0.889	12.4
G10C10	4.58	0.0	0.0	23.7 ± 1.34	0.0	0.62	0.0	1.04	1.31	9.98
G10C20	5.80	0.0	0.0	32.8 ± 0.212	0.0	1.34	0.0	1.05	1.82	10.3
G15C15	8.02	0.0	0.0	31.1 ± 1.69	1.65	0.960	0.671	1.05	1.72	10.1
G20C10	8.50	0.0	0.0	32.1 ± 0.141	2.20	1.06	0.820	1.03	1.78	11.6
G20C20	7.76	0.0	0.0	40.5 ± 0.212	0.658	0.560	0.611	1.02	2.25	9.85

[G25C25], fermentation of 25 g/L glucose and 25 g/L cellobiose; [G25C50], fermentation of 25 g/L glucose and 50 g/L cellobiose; [G50C25], fermentation of 50 g/L glucose and 25 g/L cellobiose; [G50C50], fermentation of 50 g/L glucose and 50 g/L cellobiose; [G10C10], fermentation of 10 g/L glucose and 10 g/L cellobiose; [G10C20], fermentation of 10 g/L glucose and 20 g/L cellobiose; [G15C15], fermentation of 15 g/L glucose and 15 g/L cellobiose; [G20C10], fermentation of 20 g/L glucose and 10 g/L cellobiose; [G20C20], fermentation of 20 g/L glucose and 20 g/L cellobiose; LA, Lactic acid; Y_{LA}, Lactic acid yield; P_{LA}, Lactic acid productivity; Max. P_{LA}, Maximum lactic acid productivity.

Table 6. Effect of different ratios of glucose/cellobiose/xylose mixtures on sugar utilization and lactic acid fermentation efficiency by *B. coagulans* Azu-10.

Mixed Sugars	Max. Biomass (OD _{562 nm})	Residual Glucose(g/L)	Residual Cellobiose (g/L)	Residual Xylose (g/L)	LA (g/L)	Formic Acid (g/L)	Acetic Acid (g/L)	Ethanol (g/L)	Y _{LA} (g/g)	P _{LA} [g/(L·h)]	Max. P _{LA} [g/(L·h)]
G25X25C25	14.1	0.0	4.39	2.22	62.7 ± 0.424	0.414	0.431	0.0	0.962	1.30	5.65
G50X50C50	15.2	0.0	36.3	6.22	80.4 ± 4.87	0.0	0.058	0.0	0.710	1.34	9.90

[G25C25X25], fermentation of 25 g/L glucose, 25 g/L cellobiose, and 25 g/L xylose; [G50C50X50], fermentation of 50 g/L glucose, 50 g/L cellobiose, and 50 g/L xylose; LA, Lactic acid; Y_{LA}, Lactic acid yield; P_{LA}, Lactic acid productivity; Max. P_{LA}, Maximum lactic acid productivity.

3.6. Fed-Batch Fermentation with Glucose/Xylose Mixture

Based on the above results, the GX mixture was selected as the best suitable substrate in terms of easier preparation based on the complete hydrolysis process. To enhance LA fermentation, fed batch was conducted starting with G25X25 as shown in Figure 5. As indicated, high cell biomass was achieved at $OD_{562\text{ nm}}$ of 14.1 with simultaneous sugar utilization. While glucose was completely consumed, 12 g/L of xylose remained unutilized in the fermentation broth. Homofermentative LA production of 83.6 was produced after 60 h with a yield of 0.895 g/g, a productivity of 1.39 g/L/h, and maximum productivity of 8.11 g/L/h.

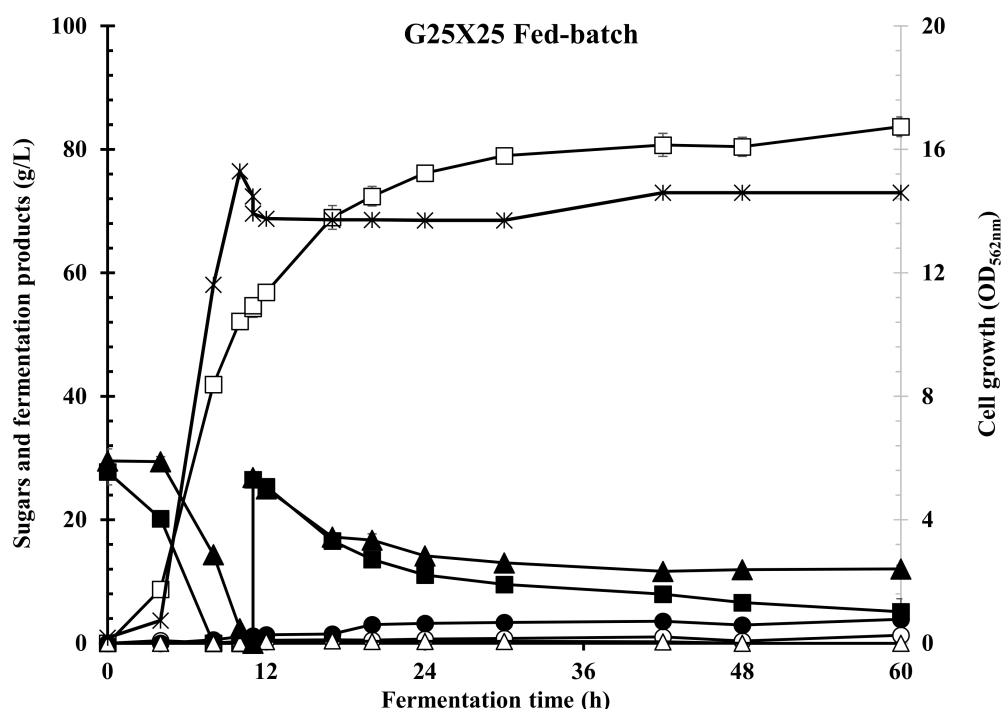


Figure 5. Fed-batch fermentation of glucose/xylose mixture for lactic acid production by *B. coagulans* Azu-10. Initial sugars were 25 g/L glucose and 25 g/L xylose [G25X25] and fed with same sugar concentrations after 12 h of fermentation. Symbols: ■, glucose (g/L); ▲, xylose (g/L); □, lactic acid (g/L); ○, acetic acid (g/L); ●, formic acid (g/L); △, ethanol (g/L); *, growth ($OD_{562\text{ nm}}$).

4. Discussion

Cellulose biomasses are varied greatly in their sugar content and therefore, yield diverse ratios of sugar mixtures upon hydrolysis that should affect sugar utilization and LA fermentation efficiency owing to the carbon catabolite repression effect [14,15]. Biomass hydrolysates are composed of a mixture of sugars; therefore, co-utilization of various sugars is essential for economically feasible LA fermentation processes. However, LA fermentation by microbial producers is associated with several challenges including the un-consumption of existed sugars especially xylose. Otherwise, when sugars have been consumed, the utilization of preferred carbon and energy source such as glucose represses the consumption of alternative sugars that is known as carbon catabolite repression (CCR). Simultaneous utilization of biomass-derived mixed sugars (glucose, cellobiose, and xylose) results from partial or complete saccharification process has been demonstrated by means of few strains [11,12,15]. Therefore, there is an urgent need to select the optimal substrates (sugars mixture) suitable for specific microbial strain for an effective LA fermentation process. This is known as “designed biomass” that can be used to establish the consolidated bioprocessing systems [18].

We have recently isolated a robust LA-producer strain, *Bacillus coagulans* Azu-10, capable of utilizing xylose homofermentatively and could tolerate several biomass-derived

microbial inhibitors [17]. This study firstly investigated the effect of different xylose concentrations on the LA fermentation in order to determine the optimal substrate concentration and the substrate inhibition. Strain Azu-10 has efficiently utilized up to 100 g/L xylose to LA at a yield of 1.0 g/g in a homofermentative pattern.

Numerous cellulosic biomasses have been widely used for LA fermentation, including woody biomasses, algal biomass, and grass biomass. In general, woody biomass contains higher cellulose content (40–55% cellulose, and 8–25% hemicellulose) than grass biomass (25–50% cellulose, 20–50% hemicellulose) [19] while the structural polysaccharides of algae are different. These varying contents result in differential performances in enzymatic hydrolysis and LA fermentation. For example, 82% glucose was obtained from enzymatically hydrolyzed NaOH-pretreated Napier grass [20], whereas 46.2% glucose was obtained from NaOH-pretreated rice straw [21], 28% glucose from NaOH pretreated phragmites, and 33% glucose from switchgrass [22].

Besides, one of the major challenges in fermentative LA production using cellulosic biomass is the release of microbial growth and LA production inhibitors such as furfural and 5-hydroxy methyl furfural, vanillin, acetic acid, and formic acid at concentrations ranging from 0.08 g/L to 5.3 g/L [23]. Interestingly, strain Azu-10 achieved efficient LA production in the presence of different inhibitory compounds [17]. This would overcome the extra required steps for substrate preparation including the removal of the inhibitors using chemicals, such as over-liming [15,24] or conversion of these inhibitory materials into less-toxic substances with a view to simplify the overall fermentation processes in an eco-friendly method. Some *B. coagulans* strains have been reported to be resistant to certain levels of these inhibitors [24–27]; for example, *Bacillus* sp. P38 could tolerate up to 6 g/L 2-furfural in corn stover hydrolysate [27].

A recent study showed that the pretreatment methods affected the sugar ratio. Papayannakos et al. [28] compared various chemical and physicochemical pretreatment methods (alkali, microwave-assisted acid, organosolv, hydrothermal treatment, and combination of the organosolv and hydrothermal treatment) using 15% solid loading of cotton stalks. Cellulose, hemicellulose, and lignin content of the pretreated materials were varied depending on the type of pretreatment applied, which might affect the fermentable sugar release. The order of pretreatments of cotton stalks was organosolv-hydrothermal, organosolv, alkali, microwave-assisted acid, and hydrothermal treatments with cellulose contents of 79.6, 77.0, 55.3, 48.6, and 47.3 g/100 g of pretreated cotton stalks, whereas the hemicellulose contents were 0.4, 1.4, 21.2, 9.6, and 3.9 g/100 g of pretreated cotton stalks, respectively [28]. The cellulose hydrolysis yield of wheat straw was enhanced using a combination of steam explosion pretreatment and physical mill or chlorite pretreatment [29].

The concentration of glucose/cellobiose/xylose mixture in cellulosic hydrolysate is greatly varied based on the hydrolysis process. Zhao et al., [30] obtained cellobiose (7.29 g/L)/glucose (5.40 g/L)/xylose (10.2 g/L)/arabinose (1.89 g/L) mixture in separate hydrolysis process and cellobiose (5.31 g/L)/glucose (3.22 g/L)/xylose (10.3 g/L)/arabinose (1.91 g/L) mixture in repeated hydrolysis process from H₂SO₄ pretreated rice straw that hydrolysed by 2.6 U/g-substrate of cellulase while they obtained glucose (14.8 g/L)/xylose (9.7 g/L)/arabinose (1.31 g/L) mixture using 2.6 U/g-substrate of cellulase and 42.1 U/g-substrate of β -glucosidase. Moradi et al. [31] obtained glucose (5.40 g/L)/xylose (1.2 g/L)/arabinose (0.3 g/L) mixture from 85% (v/v) H₃PO₄-pretreated rice straw that treated with 25 U/g-substrate of cellulase and 50.0 U/g-substrate of β -glucosidase while they obtained glucose (7.30 g/L)/xylose (2.20 g/L)/arabinose (0.400 g/L) mixture from 12% (v/v) NaOH-pretreated rice straw. On the other hand, Amiri et al. [21] obtained glucose (15.0 g/L)/xylose (5.5 g/L)/arabinose (1.0 g/L) mixture from ethanol organosolve-pretreated rice straw using 25 U/g-substrate of cellulase and 40.0 U/g-substrate of β -glucosidase. He et al., [20] obtained only glucose (34.8 g/L)/xylose (16.4 g/L) mixture from enzymatically hydrolyzed NaOH-pretreated Napier grass using 40 U/g-substrate of cellulase. Additionally, Gao and Rehmann [32] obtained glucose (35.1 g/L)/xylose (20.1 g/L) mixture from enzymatically hydrolyzed NaOH-pretreated corncob using 15 U/g-substrate of cel-

lulase. Amiri and Karimi [33] obtained glucose (15.8 g/L)/xylose (6.47 g/L)/arabinose (0.89 g/L) mixture from ethanol organosolve-pretreated elm wood using 25 U/g-substrate of cellulase and 40.0 U/g-substrate of β -glucosidase while they obtained glucose (5.8 g/L)/xylose (1.07 g/L)/galactose (0.06 g/L)/mannose (2.30 g/L) mixture from pine using same hydrolysis process. Zhang et al., [34] obtained cellobiose (1.2 g/L)/glucose (35.1 g/L)/xylose (3.0 g/L)/arabinose (10.1 g/L) mixture from $\text{Ca}(\text{OH})_2$ -pretreated corncob and enzymatically hydrolyzed using 48.0 U/g-substrate of cellulase and 20.0 U/g-substrate of β -glucosidase. Gao et al., [22] obtained glucose (40.4 g/L)/xylose (16.5 g/L) mixture from NaOH-pretreated switchgrass (*Panicum virgatum*) using 15 U/g-substrate of cellulase while they obtained glucose (35.7 g/L)/xylose (15.2 g/L) mixture from NaOH-pretreated phragmites (*Phragmites australis*) using 15 U/g-substrate of cellulase. Zhang et al., [35] obtained cellobiose (1.68 g/L)/glucose (39.5 g/L) from steam explosion-pretreated eucalyptus using 175 U/g-substrate of cellulase. Besides, various strategies can be used to control specific sugar concentrations to be used for specific fermentation processes including using a specific hydrolysis process, mixing different biomasses, and adding other resources. A semi-hydrolysis process using cellulase with low β -glucosidase loading can control the disaccharide cellobiose concentration in a sugar mixture [30]. The inhibition of cellulases by cellobiose (as a potent inhibitor) was controlled using multifunctional cellulases such as Umcel9y-1, Td2F2, and CoGH1A [36]. High solid loading can be also used for generating a satisfactory amount of sugar. A starchy slurry was mixed with salix hydrolysate that improved the xylose consumption from 29 to 81% and enhanced the yield of fermentation end-product yield [37].

Another problem with the use of mixed sugars in cellulosic biomass is carbon catabolite repression (CCR). Interestingly, when strain Azu-10 was cultivated in different ratios of glucose–xylose mixtures, CCR was alleviated where both sugars were consumed simultaneously. The only limitation of G/X fermentation is the total substrate concentration greater than 75 g/L of mixed sugars. The intensities of CCR were previously reported as dependent on the concentration of the preferred sugar [12]. In contrast, high LA yield was achieved when xylose concentration was 25 g/L while decreased yield was obtained at 50 g/L xylose. Our results suggest that the by-products (acetate, format, and ethanol) are not increased concerning the substrate conversion to cell mass rather than metabolic shift. *Lb. brevis* ATCC 367 was reported to possess a relaxed CCR mechanism with glucose/xylose mixture; however, it produced LA at low yield (0.52 g/g) and high by-product formation [15]. At concentrations higher than G25X25, glucose is preferentially consumed while xylose is consumed at a lower rate until completely utilized. At concentrations higher than 75 g/L (G50X50), only glucose was consumed while high residual xylose was left. This may be attributed to the limitation of some nutritional elements and/or accumulation of fermentation end-products post-glucose depletion, that slow and incomplete fermentation of non-glucose sugars [23,38]. Our results demonstrate that the initial concentration of glucose is a key factor determining xylose utilization for LA fermentation from mixed sugars using strain Azu-10, and G25X25 exhibited the best LA fermentation efficiency. *Enterococcus mundtii* QU 25 achieved homo-LA production with low by-product formation and simultaneous glucose/xylose utilization by maintaining the concentrations of xylose over 10 g/L and glucose below 25 g/L [14]. Besides high glucose concentration (50 g/L) did not repress enzymes responsible for xylose metabolism by strain Azu-10 where 76.9 g/L of LA could be produced from G50X25 after 24 h. In contrast, only 55.9 g/L of LA with a yield of 0.776 g/g was produced after 72 h using G50X25 by *E. mundtii* QU 25 [14]. On the other hand, *E. faecium* QU 50 showed relaxed CCR in the utilization of G20X20 with the production of 41.0 g/L of LA at a yield of 1.01 g/g [11]. *B. coagulans* JI12 utilized empty fruit bunches hydrolysate (containing glucose, 4.7 g/L; xylose, 48.8 g/L; and arabinose, 9.6 g/L) producing 59.2 g/L of LA at a yield of 0.97 g/g [39]. Zhang et al., [26] used membrane integrated repeated batch fermentation to mitigated the CCR feature of *B. coagulans* IPE22 and the feedback inhibition during fermentation of wheat straw hydrolysates (29.72 g/L glucose, 24.69 g/L xylose and 5.14 g/L arabinose). They could achieve 54.5–56.4 g/L of LA

with a yield of 0.94–96 g/g. A mixed sugar of sucrose, glucose, and/or fructose was also consumed for LA production without CCR by *E. faecalis* RKY1 for LA production without CCR [40]. Yoshida et al., [41] integrated xylose-assimilating pathway into *Lb. plantarum* and the strain produced homo LA from G75X25 without CCR, achieving 74.2 g/L of LA at a yield of 0.78 g/g. Similarly, Zhang et al., [42] genetically modified *Lb. plantarum* *ldhL1-pCU-PxylAB* to simultaneously utilize xylose/glucose mixture. The obtained strain achieved 20% more LA than the parent strain from cellulosic biomass. Lu et al., [43] engineered *E. coli* strain devoid of the glucose effect by the elimination of the *ptsG* gene encoding PTS enzyme for glucose transport, followed by metabolic evolution. The obtained strain co-utilized mixed sugars (G50X50) with the production of 83.0 g/L of LA [43]. Other researchers used mixed culture to overcome CCR [16,35]. Co-cultivation of *Lb. plantarum* ATCC 21,028 and *Lb. brevis* enhanced LA production, yield (0.8 g/g-consumed sugar), and reduced by-products formation from mixed sugars. Additionally, co-cultivation of *Lb. rhamnosus* and *Lb. brevis* has been used for LA fermentation of xylose/glucose mixture and corn stover hydrolysate [16].

In this study, the replacement of glucose by cellobiose achieved higher LA production titer than G/X sugar mixture without CCR. This might indicate that cellobiose has a lower effect than glucose on the enzymes responsible for xylose metabolism by Azu-10 strain. This might be attributed to the higher enzymatic activities of xylose isomerase and xylulokinase in cells grown in the presence of C/X sugar mixture than those obtained with cells grown in G/X sugar mixture as previously reported [11]. This approach was also reported to rescue the apparent CCR by *E. mundtii* QU 25 by enhancing xylose isomerase and xylulokinase activities in cellobiose/xylose grown cells. The strain produced 122 g/L of LA at a yield of 0.766 g/g from C100X60 compared to 71.2 g/L LA with a yield of 0.603 g/g obtained from G100X60 [12]. Strain *E. faecium* QU 50 exhibited no CCR during LA fermentation of C20X20 with the production of 43.6 g/L of LA at a yield of 1.05 g/g after 18 h [11].

Cellulose fraction of cellulosic biomass can be hydrolyzed to glucose mainly in a complete hydrolysis process using cellulases and β -glucosidase or to a mixture of glucose and cellobiose in partial hydrolysis using cellulases only [30]. To reveal the glucose effect of mixed-sugars released from cellulose saccharification of partial hydrolysis utilizing only cellulases without β -glucosidase (forming cellobiose and glucose). It was found that glucose concentration greater than 25 and total sugar concentration greater than 50 g/L are limiting factors for CCR by strain Azu-10 in G/C mixture. This might be attributed to the fact that glucose is a potent inhibitor for beta-glucosidase and cellulase activities [44]. Supplementation of mannose and xylose during cellobiose hydrolysis did not show any inhibitory effects on beta-glucosidase activity as previously reported [44]. Therefore, controlling the ratio of glucose to cellobiose is of great importance for effective LA fermentation by Azu-10. Our results indicated that the existence of cellobiose enhance glucose consumption but glucose retard cellobiose consumption at the tested concentrations. The results presented in this study indicate that initial glucose concentration >25.0 g/L is a limiting factor for efficient utilization of mixed glucose/cellobiose and high LA fermentation by *Bacillus coagulans* Azu-10. Further studies should be conducted to improve the tolerance of β -glucosidase to glucose in order to maximize the sugar utilization and LA fermentation process.

In this study, fermentation of glucose/cellobiose/xylose mixture was also found to be dependent on the glucose and total substrate concentrations. It is clear that glucose has more effect on cellobiose metabolism than xylose metabolism; therefore, Azu-10 preferentially utilized xylose next to glucose and then cellobiose. Our results indicated that the existence of cellobiose in the sugar mixture of lignocellulosic biomass hydrolysate should be controlled as it reduces the overall LA fermentation process. Thereby, it is recommended to use complete biomass hydrolysis by using cellulase(s) and β -glucosidase to yield high glucose concentration prior to LA fermentation by Azu-10. Mixing hemicellulose hydrolysate and cellulose hydrolysate would simplify and maximize LA fermentation by

this strain. Taking together, fed-batch fermentation of glucose/xylose mixture resulted in homolactic fermentation with simultaneous sugar utilization. Further studies on the optimization of feeding solution and nutrient supplementation are required for achieving complete sugar conversion.

The homofermentative LA fermentation pattern and the obtained high yield by strain Azu-10 are mainly attributed to the metabolism of hexose by Embden–Meyerhof–Parnas pathway (EMP) and pentose sugars by the pentose phosphate pathway (PPP) [45]. In the EMP pathway, hexose sugars are converted to pyruvate that is further reduced by LDH to produce LA [8]. Each mole of glucose is converted to 2 mole LA; thus, the maximum theoretical yield of LA from glucose is 1 g/g. While in PPP, xylose is converted to xylulose 5-phosphate that is metabolized by transaldolase and transketolase to form GAP that is then converted to pyruvate and then to LA via EMP. In the PPP, every 3 moles of xylose are converted to 5 moles of LA, with a theoretical yield of 1 g/g [8,18,46]. This characteristic is much better suited for increasing LA yield. De Clerck et al., [47] investigated 15 *B. coagulans* strains and found that half of the strains can metabolize xylose. Besides, most of the reported *Bacillus* strains achieved homolactic fermentation from xylose [47,48]. On the other hand, most of the reported LAB metabolize xylose heterofermentatively via phosphoketolase (PK) pathway that produced LA and high byproducts (acetate or ethanol) with a theoretical LA yield of only 0.6 g/g (1 mol/mol) [46]. To the best of our knowledge, only two LAB stains, *Enterococcus mundtii* QU 25 and *E. faecium* QU 50 were reported to metabolize xylose via PPP/EMP [49,50].

5. Conclusions

The present study demonstrated that *Bacillus coagulans* Azu-10 has the potential for homofermentative LA production from cellulosic biomass-derived substrates, which contain a mixture of C6 and C5 sugars. This strain could simultaneously utilize glucose/xylose and cellobiose/xylose mixtures. However, glucose limits the utilization of cellobiose in the sugar mixture and consequently limits the fermentation process. A complete hydrolysis of biomass yielding glucose and xylose as major sugars with less cellobiose content should provide the best lactic acid fermentation by Azu-10. Therefore, the present study clarified the effect of specific sugars and substrate concentrations on the performance of homofermentative LA production by Azu-10. These findings contribute to the development of more efficient LA production within the context of second- and third-generation biomass.

Supplementary Materials: The following are available online at <https://www.mdpi.com/2311-5637/7/1/28/s1>, Figure S1: Effect of different glucose/cellobiose concentrations on the growth and lactic acid production by *B. coagulans* Azu-10. (A) fermentation of 10 g/L glucose and 10 g/L cellobiose [G10C10]; (B) fermentation of 10 g/L glucose and 20 g/L cellobiose [G10C20]; (C) fermentation of 15 g/L glucose and 15 g/L cellobiose [G15C15]; (D) fermentation of 20 g/L glucose and 10 g/L cellobiose [G20C10]; (E) fermentation of 20 g/L glucose and 20 g/L cellobiose [G20C20]. Symbols: ■, cellobiose (g/L); ▲, glucose (g/L); □, lactic acid (g/L); ○, acetic acid (g/L); ●, formic acid (g/L); Δ, ethanol (g/L); *, growth (OD_{562 nm}).

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