CHEMICAL ENGINEERING TRANSACTIONS
VOL. 65, 2018
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Eliseo Ranzi, Mario Costa

ISBN 978-88-95608-62-4; **ISSN** 2283-9216
DOI: 10.3303/CET1865048

The Effect of Feeding Strategy on Butanol Production by *Clostridium beijerinckii* NCIMB 8052 using Glucose and Xylose

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We performed fed-batch fermentations of glucose and xylose mixtures producing butanol. Our aim was to develop a feeding strategy for coping with carbon catabolite repression (CCR) and sequential utilization problems as well as understanding the effect of feeding strategy on fermentation kinetics. Experimental results showed that fermenter 1 with only xylose as the initial carbon source could co-utilize sugars for all mixed sugar feeds. On the other hand, fermenter 2 with only glucose as the initial sugar showed sequential utilization. Xylose in fermenter 2 accumulated while glucose was present; it was only utilized after the glucose was completely exhausted. Besides the sugar utilization profile, the feeding strategy had an impact on the fermentation kinetics. Maximum specific growth rates were 0.68 h^{-1} and 0.94 h^{-1}, for fermenter 1 and 2, respectively. Fermenter 1 produced 4.98 g/l butanol and yield was 0.28 g/g, while fermenter 2 produced 0.5 g/l butanol with a yield value of 0.05 g/g. Total sugar utilization was also higher for fermenter 1, 81 % and 46 % for fermenter 2. The feeding strategy we proposed showed that wild type *Clostridium beijerinckii* NCIMB 8052 can co-utilize glucose and xylose, and produce butanol. Our observation suggests that we can tackle sequential utilization problem and enhance fermentation process with the proposed feeding strategy without having to manipulate the strain.

1. Introduction

Butanol has become a popular renewable energy carrier due to the depletion of fossil fuel resources and raising environmental concerns (Ezeji et al., 2014). However, its production faces feedstock availability and low yield problems. In this study, we focus on lignocellulosic biomass as the feedstock, since it is the most abundant renewable biomass resource on the planet, and it avoids the direct fuel-versus-food competition caused by the use of e.g. corn and sugar cane in biofuel production. The composition of lignocellulose depends on plant species, age and growth conditions with typical dry weight of 34.2-46.4 % glucose, 4.9-24.9 % xylose, 1.1-2.9 % arabinose, 0.3-12 % mannose, and 11.9-29.4 % lignin as reported in literature (Jørgensen et al., 2007). Even though lignocellulosic sugars is a mixture of pentose and hexose sugars, current methodologies still mainly focus on the fermentation of hexose sugars, mainly glucose while discarding the rest of the feedstock or using it as a source for process energy. Therefore, full exploitation of all the sugars bound in lignocellulosic biomass can contribute to solving the low yield problem. However, the cells' efficiency at utilizing different sugars in mixed form tends to decrease due to a phenomenon called carbon catabolite repression (CCR). CCR reduces or prevents the utilization of pentose sugars in the presence of a preferred carbon source such as glucose (Ren et al., 2010). In addition, sequential utilization of sugars extends residence time, which increases the process operating costs. There is an ongoing research on metabolic engineering (Grimmler et al., 2010; Gu et al., 2010; Ren et al., 2012; Servinsky et al., 2010; Xiao et al., 2012; Xin et al., 2014; Zhang et al., 2012a) to develop Clostridial strains capable of simultaneously fermenting both hexose and pentose as substrates for solvent production. Lee et al., (2016) stated that metabolic engineering is necessary to achieve co-utilization of sugars. Therefore, our objective is twofold: i) understanding the effect

Please cite this article as: Birgen C., Markussen S., Wentzel A., Preisig H.A., 2018, The effect of feeding strategy on butanol production by clostridium beijerinckii ncimb 8052 using glucose and xylose, Chemical Engineering Transactions, 65, 283-288 DOI: 10.3303/CET1865048

of the feeding strategy on butanol fermentation using mixtures of glucose and xylose, and ii) developing a feeding strategy to tackle CCR and sequential utilization problems without having to manipulate the strain.

2. Materials and Methods

2.1 Microorganism and medium

Wild type *Clostridium beijerinckii* NCIMB 8052 was used in this study due to its ability to utilize both sugars (Zhang et al., 2012). We chose xylose as a representative pentose sugar and glucose as hexose sugar. We used a medium containing 5 g/l sugar, 2.5 g/l Na-acetate, 5 g/l yeast extract, 2 g/l $(NH_4)_2SO_4$, 0.01 g/L NaCl, 0.75 g/l KH_2PO_4, 1.5 g/l K_2HPO_4, 0.2 g/l $MgSO_4.7H_2O$, 0.01 g/l $MnSO_4.H_2O$, 0.01 g/l $FeSO_4.7H_2O$, 0.01 g/l p-aminobenzoic acid, 0.01 g/l biotin and 0.1 g/l thiamine. The fermenters were inoculated with the same culture grown over night on reinforced Clostridial medium (CM0149, Oxoid).

2.2 Fed-batch fermentation experiments

We conducted fed-batch butanol production experiments in 2 l Applicon fermenters with 1 l working volume, an inoculum size of 2% v/v in anaerobic conditions, stirred at 150 rpm and temperature controlled at 37°C. There was no pH-control applied. We performed two fed-batch fermentation experiments: one had only xylose as the initial sugar (fermenter 1) and the other one had only glucose (fermenter 2). We then fed different mixed sugar solutions of glucose and xylose to fermenter 1 by gradually increasing the glucose to xylose ratio in the feed 1:4, 2:3, 3:2, 4:1 and 1:0 (Glucose:Xylose). In a similar manner, we fed different mixed sugar solutions of glucose and xylose to fermenter 2 by gradually decreasing the glucose to xylose ratio in the 4:1, 3:2, 2:3, 1:4. We changed the sugar ratio in the feed to observe the response of the culture to changing composition. We kept the total sugar concentration in the fermenters at 5 g/l so that we could clearly observe the sugar utilization patterns. We kept the liquid level in the fermenters constant by using concentrated sugar solutions and removing equal amounts as feed during sampling to reduce dilution effects.

2.3 Analytical methods

Optical density (OD) was used as a measure for cell concentration, measured at 660 nm with a UV-vis spectrophotometer UV-1700 (Shimadzu) with water as the reference. Samples exceeding 0.4 OD were diluted with water so that the Beer-Lambert Law is applied. The OD readings were correlated with dry cell mass measurements from 10 ml culture samples taken at the time of the OD reading. The dry cell mass samples were centrifuged (10 000 rpm) at 4°C for 5 minutes and pelleted cell mass washed with deionized water for three times. The cells were then dried at 120°C until constant weight was obtained. The samples for determining concentrations of glucose, xylose, butanol, acetic acid and butyric acid were filtrated (Millipore filter, 0.2 µm) before analysis by using a HPLC system (Shimadzu Model 9) equipped with UV (210 nm) and RI detector and an Aminex HPX-87H column (Biorad). Samples were eluted with 5 mM H_2SO_4 buffer, flow rate 0.6 ml/min at 45°C. Quantification was performed using standards for each component.

2.4 Estimation of kinetic coefficients

We estimated the maximum specific growth rate, μ_{max} (h^{-1}) during the exponential growth phase where the nutrient concentration is high enough so that the growth rate is independent of nutrient concentration. Therefore, μ_{max} is equal to specific growth rate, μ (h^{-1}), which is shown in Eq(1).

$$\frac{dX}{dt} = \mu X \tag{1}$$

where X is the cell mass concentration (g/l) and t is time (h). The specific growth rate is determined during the exponential growth phase by estimating the slope of the cell concentration versus time plot.
Yield based on sugar consumption is one of the most common coefficients that indicates how efficient the process is. We estimated cell mass yield, $Y_{X/S}$ (g cell/g sugar) and butanol yield, $Y_{B/S}$ (g butanol/g sugar) on sugar shown in Eq(2) and Eq(3), respectively, and butanol yield on cell mass, $Y_{B/X}$ (g butanol/g cell) shown in Eq(4).

$$Y_{X/S} = \frac{\Delta X}{\Delta S} \tag{2}$$

$$Y_{B/S} = \frac{\Delta B}{\Delta S} \tag{3}$$

$$Y_{B/X} = \frac{\Delta B}{\Delta X} \tag{4}$$

where ΔX is the total change in cell mass concentration (g/l), ΔS is the total sugar consumption (g/l), ΔB is the total butanol production (g/l).
Sugar utilization can be a good indicator of the fermentation performance as well. Complete utilization of the sugars is desirable to improve overall process economy. Sugar utilization, S (%) is calculated for both glucose, S_G and xylose, S_X as well for the total sugar by using Eq(5).

$$S\,(\%) = \frac{S_{consumed}}{S_{fed}} * 100 \qquad (5)$$

3. Results

3.1 Fermentation kinetics

Figure 1 shows the fermentation kinetics of fermenter 1 in terms of total sugar fed and consumed a), and metabolites and cell mass concentrations b) over time. Initial sugar was xylose only in fermenter 1, and it was utilized completely within 6 hours. In the first feed interval, the cell mass concentration increased exponentially, and produced butyric acid due to depletion of xylose. Then, we fed a mixture of glucose and xylose with 1:4 (Glucose:Xylose) 6 hours after inoculation and start of fermentation. During the second feed interval, sugars were co-utilized, but xylose utilization rate was higher. The culture exhibited steady growth in cell mass, so did the production of butyric and acetic acid. At hour 7, we detected butanol for the first time. We then fed the culture with 2:3 (Glucose:Xylose) at hour 8. In the third feed interval, glucose and xylose were co-utilized almost at the same rate. Butyric and acetic acid concentrations decreased due to re-assimilation to produce butanol. The next feed containing 3:2 (Glucose:Xylose) was fed at hour 13 after start. During the fourth feed interval, sugars were again co-utilized. Butanol production rate was the highest obtained, and cell mass continued to increase steadily. We observed a sharper decrease in butyric and acetic acid concentrations due to rapid butanol production. We fed the feed with 4:1 (Glucose:Xylose) at hour 22. In this fifth feed interval, even though sugars were again co-utilized, we observed a metabolic switch. Butanol was produced at a slower rate together with butyric acid, and cell mass concentration remained constant. We fed the final feed at hour 29, which consisted of glucose (1:0) only. During the sixth feed interval, glucose utilization rate was slower compared to previous feed intervals. Butanol and butyric acid were produced simultaneously again, and cell mass concentration decreased.

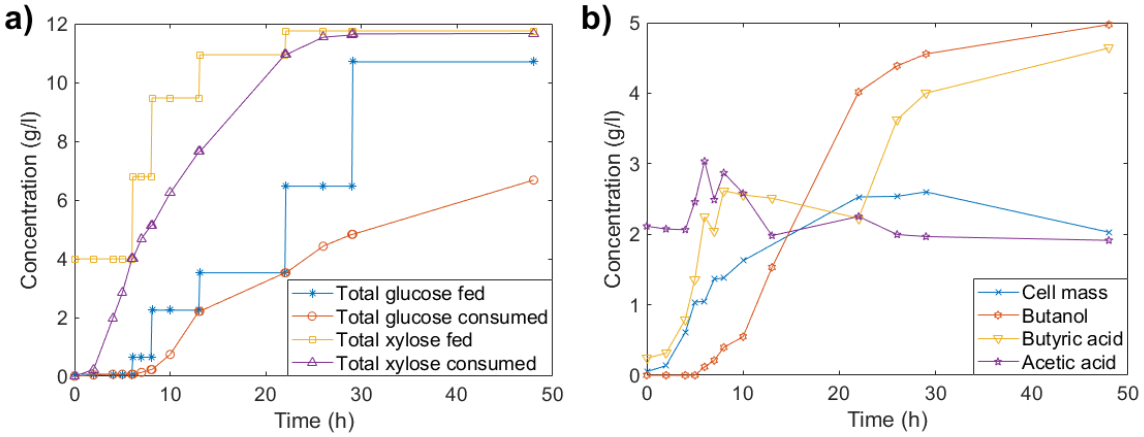

Figure 1: Sugar a), and metabolite and cell mass b) profiles for fermenter 1 with xylose as the initial sugar.

Figure 2 shows fermentation kinetics of fermenter 2 in terms of total sugar fed and consumed a), and metabolites and cell mass concentrations b) over time. Initial sugar was solely glucose in fermenter 2, and it was utilized almost completely within 5 hours with respect to the start of the fermenter. During the first feed interval, we observed that the cell mass concentration increased exponentially, and butyric acid concentration increased as glucose was consumed. We then fed a mixture of glucose and xylose with 4:1 (Glucose:Xylose) at hour 5 after inoculation and start of fermentation. In the second feed interval, only glucose was utilized and xylose concentration remained constant. Cell mass exhibited steady growth, so did butyric and acetic acids. At hour 7, we detected butanol for the first time. Then, we fed the culture with 3:2 (Glucose:Xylose) at hour 8. During the third feed interval, again only glucose was utilized and xylose accumulated. Butyric and acetic acid concentrations kept increasing. The next feed containing 2:3 (Glucose:Xylose) was fed at hour 13. In the fourth feed interval, sugars were finally co-utilized. Butanol concentration was constant, and cell mass decreased. Butyric and acetic acid concentrations increased as well. We added the final feed containing 1:4

(Glucose:Xylose) at hour 22. During the fifth feed interval, sugars were co-utilized almost at the same rate. All metabolite concentrations increased and cell mass concentration kept decreasing.

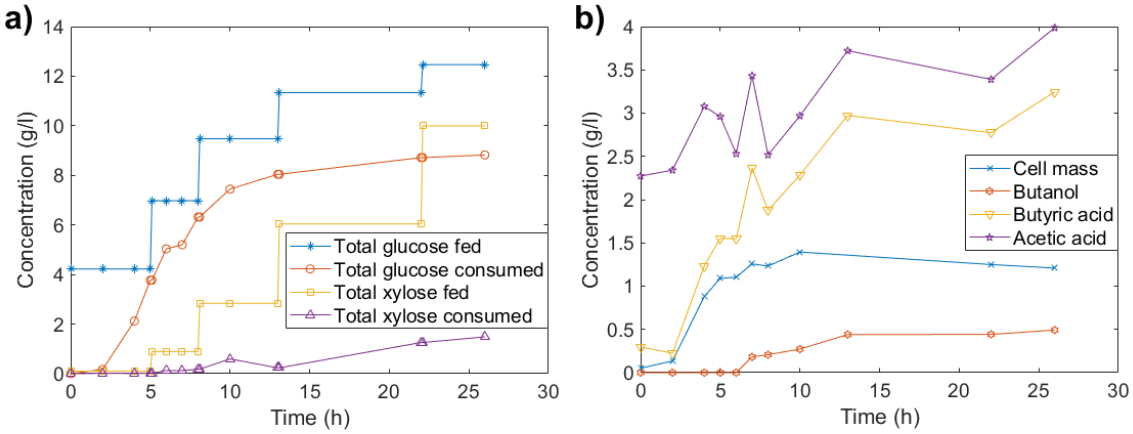

Figure 2: Sugar a), and metabolite and cell mass b) profiles for fermenter 2 with glucose as the initial sugar.

We observed co-utilization of sugars in fermenter 1, which had only xylose as the initial sugar, for all mixed sugar feeds added. However, only the last two feeds were utilized simultaneously in fermenter 2 with only glucose as the first sugar. Both fermentations terminated when the total concentration of acids reached 6 g/l. Sugar utilization was higher in fermenter 1, therefore more butanol was produced in fermenter 1. We obtained higher butanol concentration 4.98 g/l in fermenter 1 than in fermenter 2, 0.5 g/l.

3.2. Kinetic coefficients

We estimated the kinetic coefficients as described in Section 2.4 for both fermenter 1 and fermenter 2, which are shown in Table 1. Maximum specific growth rate, μ_{max} (h^{-1}), 0.94 and cell mass yield on sugar, $Y_{X/S}$ (g/g), 0.12 were higher in fermenter 2 than in fermenter 1 with μ_{max} (h^{-1}) value of 0.68 and $Y_{X/S}$ (g/g) value of 0.1. In fermenter 1, butanol yield on sugar, $Y_{B/S}$ (g/g) and butanol yield on cell mass, $Y_{B/X}$ (g/g) were 0.28 and 2.8, respectively, while they were 0.05 g/g and 0.42 g/g in fermenter 2.

Table 1: Kinetic coefficients for fermenter 1 and fermenter 2.

Kinetic coefficient	Fermenter 1	Fermenter 2
μ_{max} (h^{-1})	0.68	0.94
$Y_{X/S}$ (g/g)	0.1	0.12
$Y_{B/S}$ (g/g)	0.28	0.05
$Y_{B/X}$ (g/g)	2.8	0.42
S_G (%)	62	71
S_X (%)	100	1
S (%)	81	46

All xylose fed to fermenter 1 was utilized while only a small amount of the total fed xylose was utilized in fermenter 2, therefore total xylose utilization, S_X (%) was 100% in fermenter 1, and 1% in fermenter 2. On the contrary, total glucose utilization, S_G (%) was higher in fermenter 2, 71%, than in fermenter 1, 62%. Overall sugar utilization with respect to the total sugar fed was greater in fermenter 2, 81%, than in fermenter 1, 46%.

4. Discussion

In this study, we presented a feeding strategy to cope with CCR and sequential utilization problems for *C. beijerinckii*, an important industrial *Clostridium* strain, for ABE (acetone-butanol-ethanol) production from lignocellulosic sugars, glucose and xylose mixtures. Our strategy resulted in simultaneous utilization of both sugars. To our knowledge, this is the first report focusing on tackling CCR and sequential utilization problems for *C. beijerinckii* without having to manipulate the strain. We used *C. beijerinckii* NCIMB 8052 as a model strain; therefore, this strategy can likely be extended to other *C. beijerinckii* strains to improve ABE fermentation of glucose and xylose as well as potentially also other hexose and pentose sugar mixtures.

A collection of literature values and our results is presented in Table 2. We obtained a high butanol yield (0.28 g/g, fermenter 1) for Clostridial ABE fermentation of glucose and xylose. Moreover, our sugar utilization, 81% (fermenter 1), was higher than previously reported values (63%, 70%, and 69%) (Zhang et al., 2016). The reason can be related to having xylose as the only carbon source initially. Even though glucose is the preferred carbon source, having only xylose in fermenter 1 as the initial sugar activated the xylose metabolism. Therefore, when we fed mixtures of glucose and xylose, sugars were co-utilized. We could confirm this statement with results of fermenter 2, since it had glucose as the initial sugar, and when we fed mixtures of glucose and xylose, only glucose was utilized initially. Simultaneous utilization of sugars enhanced the overall sugar utilization and contributed to the high butanol yield.

Besides the effect of initial carbon source, the operating mode can also have an influence on fermentation performance. Reported values in Table 2 show the results of batch fermentations, while we did fed-batch experiments by keeping the total sugar concentration in the broth (10 g/l) relatively low compared to common practice (60 g/l). Combined effects of the operating mode and limited sugar concentration might have also promoted better sugar utilization and higher butanol production.

Co-utilization of sugars does not guarantee that CCR was tackled. A recent transcriptional study showed that during fermentation of glucose and xylose to produce butanol by *C.beijerinckii* SE-2, glucose inhibition on xylose metabolism-related genes was still present even though the sugars were co-utilized (Zhang et al., 2016).

Table 2: Comparison of our results with previous studies of co-utilization of mixed sugars by Clostridia.

Strain	Sugar consumed (g/l)			Butanol yield (g/g)	Butanol titer (g/l)	Comments & reference
	Glucose	Xylose	Arabinose			
C.beijerinckii NCIMB 8052	6.7	17.7	-	0.28	4.98	Wild type (This study, fermenter 1)
C.beijerinckii NCIMB 8052	8.8	1.5	-	0.05	0.5	Wild type (This study, fermenter 2)
C.beijerinckii NCIMB 8052	7.18	20.3	3.5	0.22	6.8	Wild type (Xiao et al., 2012)
C.beijerinckii NCIMB 8052	7.18	33.6	8.7	0.23	11.27	xylR down and *xylT* up regulation (Xiao et al., 2012)
C.beijerinckii SE-2	20.2	19.8	-	0.18	7.3	Wild type (Zhang et al., 2016)
	21.1	20.6	-	0.17	7	
	20	18.5	-	0.21	8	
C.acetobutylicum ATCC 824	19	13	-	0.18	5.8	*talA* up regulation (Gu et al., 2009)
	28	11	-	0.19	7.5	
	41	9	-	0.18	8.8	
C.acetobutylicum ATCC 824	33	30	-	0.19	12.05	*ccpA* disruption (Ren et al., 2010)
C.acetobutylicum DSM 792	40	6	-	NA	NA	*cre* sequence (Bruder et al., 2015)

We could detect only butanol as solvent in both fermenters. A more detailed analysis of the fermentation broth and off gas stream should be done to analyze if any other solvents are present. In that case, we would be able to calculate the actual butanol selectivity. Type of the sugar has an influence on the butanol selectivity. Raganati et al., (2014) reported that butanol selectivity was 55% and 75% for fermentations of glucose and xylose, respectively. Clostridial ABE production is typically inhibited by the toxic acids and solvents produced. The inhibitory concentration of butanol when the growth entirely stops and fermentation terminates is 16 g/l (Ezeji et al., 2007). Ballongue et al., (1987) have reported that the growth rate decreases by 50% when either acetic acid or butyric acid reaches 4 g/l, and the growth stops entirely when the total concentration of acids reaches approximately 5 g/l. Our results are in line with these earlier findings; both fermenter 1 and fermenter 2 terminated when the total concentration of acids was 6 g/l. Accumulation of acids in fermenter 1 was likely due to a metabolic switch. Typically, butyric acid concentration decreases as butanol is being produced. This was our observation until 22 hours after inoculation; however, after the metabolic switch, butyric acid and butanol concentrations increased at the same rates until the fermentation terminated. Sugar concentration decreased to zero around hour 22, and this might be the reason for metabolic switch as discussed in an earlier study (Okamoto et al., 1988).

5. Conclusions

We developed a feeding strategy to cope with carbon catabolite repression (CCR) and sequential utilization problems, as well as to understand the effect of the feeding strategy on fermentation kinetics. Fermenter 1 with only xylose as the initial carbon source could co-utilize sugars for all mixed sugar feeds. On the other hand, fermenter 2 with only glucose as the initial sugar suffered from sequential utilization. Xylose in fermenter 2 accumulated while glucose was present; it was only utilized after the glucose was completely exhausted. Maximum specific growth rates were 0.68 h^{-1} and 0.94 h^{-1}, for fermenter 1 and 2, respectively. Fermenter 1 produced 4.98 g/l butanol and yield was 0.28 g/g, while fermenter 2 produced 0.5 g/l butanol with a yield value of 0.05 g/g. Total sugar utilization was also higher for fermenter 1, 81%, and 46% for fermenter 2. Our observation suggests that we can tackle sequential utilization problem by the proposed feeding strategy. Transcriptional studies need to be performed to understand in depth if CCR is active or not, as well as to understand the co-utilization mechanism for improvement of the proposed feeding approach.

Acknowledgments

EcoLodge - Efficient production of Butyl Butyrate from Lignocellulose derived Sugars (NFR246821) has received funding from the Norwegian Research Council's ENERGIX programme and the Ministry of Science and Technology, Government of India (INT/NOR/RCN/P-06/2015).

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