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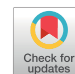
Adaptive evolution of *Gluconobacter oxydans* accelerates the conversion rate of non-glucose sugars derived from lignocellulose biomass



Ci Jin[a,1], Weiliang Hou[a,1], Ruimiao Yao[a,1], Pingping Zhou[a], Hongsen Zhang[a,b], Jie Bao[a,*]

[a] State Key Laboratory of Bioreactor Engineering, East China University of Science and Technology, 130 Meilong Road, Shanghai 200237, China
[b] Key Laboratory of Enzyme Engineering of Agricultural Microbiology, Ministry of Agriculture, College of Life Science, Henan Agricultural University, 63 Nongye Road, Zhengzhou, Henan 450002, China

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ABSTRACT

Gluconobacter oxydans is capable of oxidizing various lignocellulose derived sugars into the corresponding sugar acids including glucose, xylose, arabinose, galactose and mannose, but simultaneous utilization of these sugars is difficult. This study attempted an adaptive evolution of *G. oxydans* by alternate transfer in inhibitors containing hydrolysate and inhibitors free hydrolysate for intensifying sugars simultaneous utilization. After 420 days' continuous culture, the conversion rate of all non-glucose sugars significantly improved by several folds and achieved complete conversion of lignocellulose-derived sugars to the corresponding sugar acids. The significant up-regulation of mGDH gene in the adapted *G. oxydans* strain (more than 40-fold greater than the parental) was considered as the decisive factor for the improvement of strain performance. This evolution adaptation strategy also could be used to accelerate robust sugars utilization for other fermented strains in lignocellulose biorefinery.

1. Introduction

Gluconobacter oxydans is capable of oxidizing various sugars into the corresponding sugar acids including the lignocellulose derived non-glucose sugars such as xylose, arabinose, mannose and galactose (Zhang et al., 2016a; Zhou et al., 2015). These sugar acids are important chemicals used in food, pharmaceuticals and construction industry (Zhang et al., 2016b), showing efficient cement retarding properties as cement addition (Ma et al., 2015; Hou and Bao, 2019). However, the conversion rate of non-glucose sugars by *G. oxydans* is relatively slow comparing to glucose conversion rate (Yao et al., 2017). The outcome of the difference in sugar conversion leads to the prolonged fermentation time for completely oxidizing robust sugar into its sugar acid form during cellulosic sugar acids fermentation (Hou et al., 2018; Hou and Bao, 2019).

For directly improving the efficiency of non-glucose sugar acids conversion, two simple methods could be used. One is whole cell catalysis, but the drawback is the requirement of high cell mass, which inevitably increases the operation cost for cell culture and harvest (Yao et al., 2017; Zhou et al., 2019a,b). Another method is adaptive evolution of the fermenting strain, *G. oxydans*, by mimicking natural selection under specific stress. Adaptive evolution has been extensively used to modify tolerance and metabolism of microorganisms in complicated lignocellulose system (Almario et al., 2013; Wang et al., 2018; Wisselink et al., 2009). A specific case is that adaptive evolution of an engineered xylose-utilizing strain *Pediococcus acidilactici* (27 days) achieved 10-fold improvement in xylose conversion rate (Qiu et al., 2017). Another typical case is that a short term evolution of an engineered *Saccharomyces cerevisiae* led to the improvement of arabinose utilization rate by 4.2 folds (Wang et al., 2013). These successful examples showed that assimilation of non-glucose sugars could be efficiently accelerated by adaptive evolution.

In this study, the *G. oxydans* strain used for fermentative production of sugar acids from lignocellulose biomass was adaptively evolved by alternate culture between the inhibitors containing hydrolysate and the inhibitors free hydrolysate for 420 days. The long term adaptive evolution resulted in the significantly accelerated conversion rate of non-glucose sugars to the corresponding sugar acids. This study provided an effective method of adaptive evolution for improving the non-glucose sugars conversion rate in biorefinery fermentations.

2. Materials and methods

2.1. Raw materials

Corn stover (CS) was harvested from Bayan Nur League, Inner

* Corresponding author.
E-mail address: jbao@ecust.edu.cn (J. Bao).
[1] These authors are equally contributed to this work.

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Mongolia Autonomous Region, China in fall 2015. After collection, corn stover was milled to pass a mesh with the circle diameter of 10 mm, then washed to remove field dirt, stones and metals and dried to constant weight. The raw corn stover contained 35.4% of cellulose and 24.6% of hemicellulose measured according to NREL protocols (Sluiter et al., 2008, 2012).

2.2. Enzymes and chemicals

Cellulase Cellic CTec 2.0 was purchased from Novozymes (China), Beijing, China. The filter paper activity was 203.2 FPU/mL determined according to the NREL protocol LAP-006 (Adney and Baker, 1996) and the protein concentration was 87.3 mg/mL determined by Bradford method (1976). Yeast extract was from Angel Yeast Co., Yichang, Hubei, China. Agar was from Biosharp Co., Shanghai, China. All other chemicals NaOH, $Ca(OH)_2$, $(NH_4)_2SO_4$, $MgSO_4 \cdot 7H_2O$ and KH_2PO_4 were purchased from the local supplier Linfeng Chemical Reagent Co., Shanghai, China.

2.3. Strains and media

Gluconobacter oxydans DSM 2003 was purchased from German Collection of Microorganisms and Cell Cultures (DSMZ), Braunschweig, Germany. Seed medium used for *G. oxydans* DSM 2003 including 80.0 g/L sorbitol, 10.0 g/L yeast extract, 1.5 g/L KH_2PO_4, 1.5 g/L $(NH_4)_2SO_4$ and 0.5 g/L $MgSO_4 \cdot 7H_2O$.

Biodetoxification fungus *Amorphotheca resinae* ZN1 was stored in Chinese General Microorganisms Collection Center (CGMCC) with the registration number 7452 (Zhang et al., 2010).

2.4. Pretreatment, biodetoxification and hydrolysis operations

Corn stover was pretreated using the dry sulfuric acid pretreatment according to Zhang et al. (2011) and He et al. (2014). The pretreated corn stover slurry contained approximately 50% of solids and no wastewater was generated, then was neutralized, milled and then detoxified using *A. resinae* ZN1 according to Zhang et al. (2010) and He et al. (2016).

The inhibitors containing hydrolysate was prepared by enzymatic hydrolysis of pretreated but not detoxified corn stover using cellulase at dosage of 4 mg protein/g dry solids matter (DM) and 15% (w/w) solids content for 48 h at 50 °C. The hydrolysate was centrifuged at 16,125 × *g* for 10 min to remove solid residues and the supernatant was used for adaptive evolution and flask fermentation. The inhibitors free corn stover hydrolysate was prepared using pretreated and detoxified corn stover at same operation conditions.

2.5. Evolutionary adaptation

The adaptive evolution of *G. oxydans* DSM 2003 was conducted by alternate switch between the inhibitors containing hydrolysate and the inhibitors free hydrolysate. Briefly, *G. oxydans* was cultured into the inhibitors containing hydrolysate in 100 mL flask containing 20 mL of hydrolysate at 30 °C and 220 rpm for 24 h. Then the culture was transferred into the inhibitors free hydrolysate at inoculum ratio of 10% (v/v) for another 24 h. The same procedure was conducted by back to the inhibitors containing hydrolysate and then the inhibitors free hydrolysate. The alternate culture was successively conducted for 420 days. The pH was maintained at 5–6 by adding 0.16 g $CaCO_3$ at every transfer. The inhibitors containing hydrolysate used for the first 34 transfers contained 54.5 g/L of glucose, 23.5 g/L of xylose, 3.2 g/L of acetic acid, 0.3 g/L of furfural, 0.2 g/L of 5-hydroxymethylfurfural (HMF). Another inhibitor containing hydrolysate contained 62.9 g/L of glucose, 29.7 g/L of xylose, 6.1 g/L of acetic acid, 0.2 g/L of furfural and 0.2 g/L of HMF. The inhibitors free hydrolysate contained 67.2 g/L of glucose, 21.8 g/L of xylose and 0.6 g/L of acetic acid.

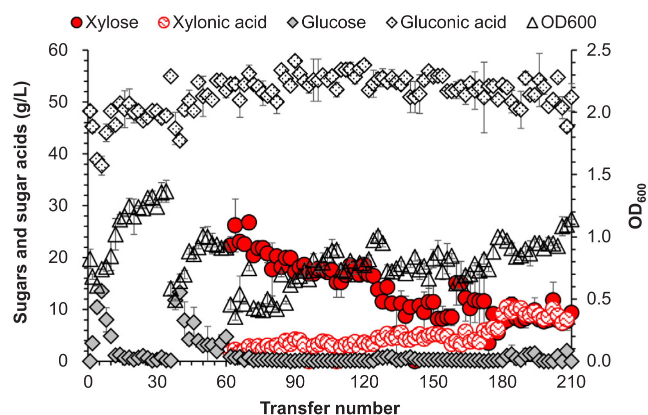

Fig. 1. Adaptive evolution profile of *G. oxydans* DSM 2003. The cells were by alternatively transferred between the inhibitors containing hydrolysate and the inhibitors free hydrolysate every 24 h. The pH was maintained by adding 0.16 g $CaCO_3$ into the fresh hydrolysate. The inhibitors free hydrolysate contained 67.2 g/L of glucose, 21.8 g/L of xylose, and 0.57 g/L of acetic acid. The inhibitor containing hydrolysate used in the first 34 transfers contained 54.5 g/L of glucose, 23.5 g/L of xylose, 3.24 g/L of acetic acid, 0.31 g/L of furfural, 0.20 g/L of 5-hydroxymethylfurfural (HMF). The inhibitor containing hydrolysate used after the 34 transfers contained 6 2.9 g/L of glucose, 29.7 g/L of xylose, 6.07 g/L of acetic acid, 0.15 g/L of furfural and 0.24 g/L of HMF. The hydrolysates were prepared under 15% (w/w) of the dry acid pretreated corn stover or dry acid pretreated/biodetoxified corn stover, 4 mg cellulase protein/g dry solid matter (DM), 50 °C for 48 h.

2.6. Aerobic fermentation

One vial (2 mL) of *G. oxydans* stock was inoculated into 20 mL of seed medium in 100 mL flask at 30 °C and 220 rpm for 15 h. For flask fermentation, seed broth was inoculated at 10% volume ratio into 45 mL of inhibitor containing hydrolysate and fermented at 30 °C and 220 rpm for 72 h. The pH was controlled to a range of 5–6 by adding 5 M NaOH every 6 h.

For fermentor fermentation, corn stover hydrolysate slurry at 30% of solids loading and seed broth at 5% (v/v) of inoculation ratio were together fed into a 5 L fermentor (Hou et al., 2017). Fermentation conditions were 35 °C, 1 vvm and 500 rpm for 72 h. The pH was maintained at 4.8 by 5 M NaOH.

2.7. Analysis of sugars and acids

D-glucose, D-xylose, D-galactose, L-arabinose, D-mannose were analyzed by HPLC (LC-20AD, refractive index detector RID-10A, Shimadzu, Kyoto, Japan) equipped with HPX-87P column (Bio-rad, Hercules, CA, USA) at 80 °C with the sterilized deionized water as mobile phase at a flow rate of 0.6 mL/min. D-gluconate and D-xylonate were determined by HPLC (LC-20AT, UV/VIS detector SPD-20A, Shimadzu, Kyoto, Japan) fitted with an Aminex HPX-87H column (Bio-rad, Hercules, CA, USA) at 55 °C using mobile phase of 5 mM H_2SO_4 at the rate of 0.4 mL/min and the detection wavelength of 210 nm. Other sugar acids (D-galactonic acid, L-arabonic acid, D-mannonic acid) were identified by Agilent 1200 HPLC with Quadruple LC-MS 6120 detector (Santa Clara, California, USA) (Yao et al., 2017).

2.8. qRT-PCR analysis and DNA sequencing

The total RNA was extracted using Trizol reagent (RNAiso Plus, TAKARA, Otsu, Japan) according to the protocol. Reverse transcription reactions were carried out using ReverTra Ace qPCR RT Master Mix with gDNA Remover kit (Toyobo, Osaka, Japan) according to the protocol. For each real-time qPCR (qRT-PCR) reaction, a SYBR Green Realtime PCR Master Mix kit (Toyobo, Osaka, Japan) was used on a

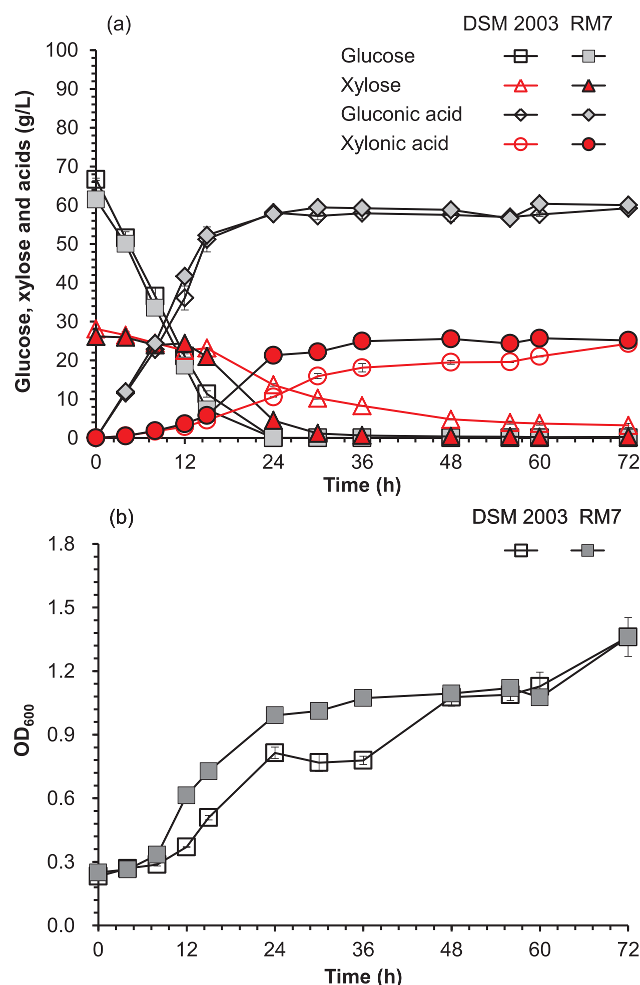

Fig. 2. Fermentation performance evaluation of the adapted *G. oxydans* RM7 using the low sugars and inhibitors containing corn stover hydrolysate in flask fermentation. (a) Sugars consumption and conversion; (b) Cell viability. The fermentation was carried out at 30 °C, pH 5.5, 220 rpm and the inoculum size 10% in a 250 mL flask containing 45 mL hydrolysate. The NaOH (5 M) was added every 6 h to neutralize the produced sugar acids for controlling the pH to a range of 5–6. The hydrolysate was prepared at 15% solids loading, 4 mg protein/g DM enzymatic loading, 50 °C, 150 rpm for 48 h and contained 6.07 g/L of acetic acid, 0.15 g/L of furfural and 0.24 g/L of 5-hydroxymethylfurfural (HMF).

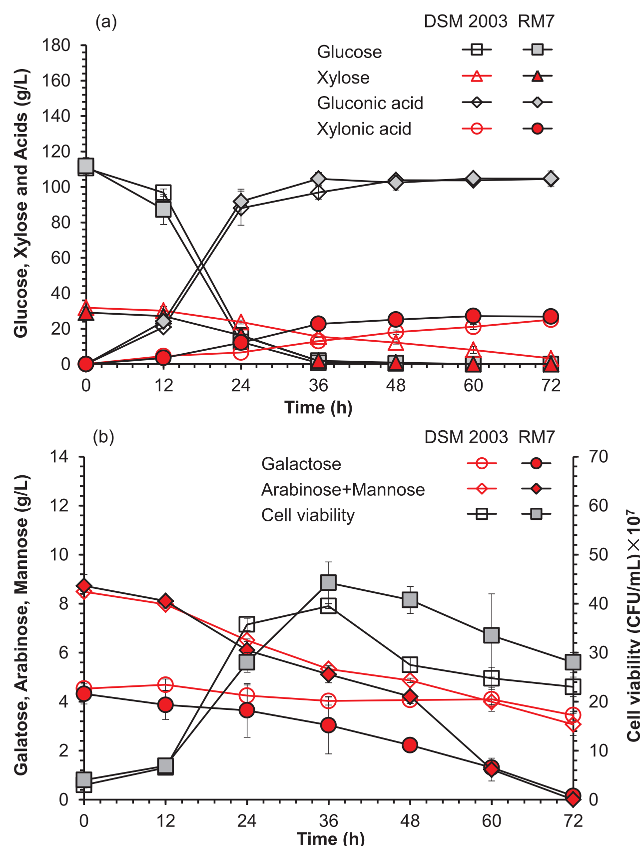

Fig. 3. Fermentation performance evaluation of the adapted *G. oxydans* RM7 using the high sugars and inhibitors free corn stover hydrolysate in fermentor. (a) Glucose and xylose conversion; (b) Other non-glucose sugars consumption and cell viability. Fermentation was carried out at pH 4.8, 35 °C, 500 rpm, 1 vvm of aeration rate, 5% (v/v) inoculum ratio, 2.5 L corn stover hydrolysate slurry in 5 L fermentor. The pH was maintained at 4.8 by automatic addition of 5 M NaOH. The hydrolysate slurry was prepared at 30% (w/w) of dry acid pretreated and biodetoxified corn stover solids, 4 mg cellulase protein/g dry solids matter (DM), 50 °C, 150 rpm for 48 h.

BioRad CFX 96 (Hercules, CA, USA). The 16S ribosomal RNA gene was served as an internal control to normalize for difference in total RNA quantity. Transcription level of the gene was quantified using the formula $2^{-\Delta\Delta Ct}$.

The genomic DNA was extracted using TIANamp Bacterial DNA Kit (Tiangen Biotech, Beijing, China) and sequenced by Majorbio (Shanghai, China). The promoter region (−35 and −10 region) was predicted by BPROM software (http://www.softberry.com/berry.phtml). The primers used for DNA amplification included a 156 bp fragment of 16s rRNA by the primer pair JC001F (CACTTTCAGGTGGGCACTC) and JC001R (GTCACCGCCATTGTAGCAC), a 135 bp fragment of mGDH by the primer pair JC002F (GGAATGTTCGAGTGGGGT) and JC002R (TACCCTTGGCGTCCTTTT), and a 500 bp upstream and CDS sequence of mGDH by the primer pair JC003F (ACTGCTTGATTGTCTTCGC) and JC003R (TCATTTCTGATCGGGCAG), from the genomic DNA of *G. oxydans* DSM 2003 and *G. oxydans* RM7.

3. Results and discussion

3.1. Long term adaptive evolution of G. oxydans in corn stover hydrolysate

G. oxydans DSM 2003 was used as parental strain of adaptive evolution for accelerating non-glucose sugars conversion rate. One transfer included a 24 h culture in the inhibitors containing hydrolysate and followed by another 24 h culture in the inhibitors free hydrolysate. Totally 210 transfers (equivalent to 420 days) was conducted as shown in Fig. 1. In the initial cultures (4 transfers), glucose consumption rate and cell growth rate of *G. oxydans* rapidly reduced, and then increased gradually. When the transfer came to 36 times, the inhibitor containing hydrolysate was replaced by a corn stover hydrolysate containing more acetic acid (from 3.24 g/L to 6.07 g/L) and sugars (from 54.49 g/L to 62.94 g/L). After 420 days' culture, the cell growth, sugar conversion rate, and sugar acid formation rate of *G. oxydans* DSM 2003 were stable and the obtained strain was defined as *G. oxydans* RM7.

The fermentation performance of *G. oxydans* RM7 was evaluated in the inhibitors containing hydrolysate in shake flask (Fig. 2). Glucose conversion to gluconic acid of *G. oxydans* RM7 was essentially the same (slightly improved) with parental *G. oxydans* DSM 2003, but the conversion rate of xylose to xylonic acid significantly improved. The total fermentation time was hence reduced to 36 h from 72 h (Fig. 2a). Cell growth rate was also accelerated due to the more rapid sugars utilization (Fig. 2b).

Table 1
Promotor mutation sequencing.

	Promoter	CDS (2427bp)
G. oxydans DSM2003	TCTGACATCTTGACCCTTGACGCCCATCCATGTGCTTTGTCCGGAAGTCACCGTGATGCATCAGAACAACAGATCCAGGAACATC	ATGAGCAC···
G. oxydans RM7	TCTGACATCTTGACCCTTGACGCCCATCCATGTACTTTGTCCGGAAGTCACCGTGATGCATCAGAACAACAGATCCAGGAACATC	ATGAGCAC···

The letters indicate putative core hexamers of −10 regions (red) and −35 regions (orange) predicted by BPROM software (http://www.softberry.com/berry.phtml). The blue letters indicate the mGDH coding region and the solid box indicates the mutation.

Table 2
The mGDH transcriptional level of the evolved strain relative to the parental strain in glucose medium and xylose medium.

Genes	Genes ID	Functional annotation	Fold change	
			Glucose	Xylose
mGDH	W826_RS0112255	Glucose dehydrogenase	39.88 ± 2.78	69.90 ± 7.59

G. oxydans RM7 was further evaluated in the inhibitor free (biodetoxified) hydrolysate with higher sugar concentration in fermentor with accurate control of temperature, aeration rate and pH value (Fig. 3). Similar to the flask fermentation, glucose conversion rate slightly improved and xylose conversion rate was accelerated by 4.1 folds (Fig. 3a). The measured results showed that arabinose, mannose and galactose were completely converted to the corresponding sugar acids by *G. oxydans* RM7 at 72 h, comparing to above 70% residual of the total sugars by *G. oxydans* DSM 2003 (Fig. 3b). These results clearly indicate that adaptive evolution efficiently enhanced the utilization efficiency of *G. oxydans* on the non-glucose sugars derived from lignocellulose feedstock.

This study showed that adaptive evolution is an easy, practical, and effective method to train the cells to a new environment such as lignocellulose hydrolysate. The fine and alternate tuning of culture strengthened the robustness of the strain during the adaptive evolution switched in the inhibitors containing/inhibitors free hydrolysates. The adapted strain *G. oxydans* RM7 showed high sugar acid productivity in the future industrial applications of lignocellulose biorefinery.

3.2. Genetic and transcriptional analysis of the evolved strain

The oxidation conversion of glucose, xylose, arabinose, galactose, and mannose in *G. oxydans* is catalyzed by the membrane bound PQQ-dependent glucose dehydrogenase enzyme (mGDH) (Buchert, 1991; Pronk et al., 1988; Zhang et al., 2013). The mGDH encoding gene W826_RS0112255 in parental strain *G. oxydans* DSM 2003 and adapted strain *G. oxydans* RM7 were both sequenced and a point mutation was detected at the putative promoter regional of the mGDH gene in *G. oxydans* RM7 (Table 1). The strength of the mutant promoter was checked by expressing GFP gene in *G. oxydans* DSM 2003 but no observable change in fluorescent brightness was found. Then the transcriptional level of mGDH was analyzed by real-time qPCR and a significant up-regulation of the mGDH gene was identified in *G. oxydans* RM7. The up-regulation level of the mGDH gene was respectively 39.88- and 69.90-fold greater in glucose containing medium and xylose containing medium comparing to that in *G. oxydans* DSM 2003 (Table 2). The increased mGDH biosynthesis might be the decisive factor on the increased non-glucose conversion in adapted strain *G. oxydans* RM7.

The expression of mGDH might be influenced by some intracellular cofactors, such as PQQ and $PQQH_2$ (Zhou et al., 2019a,b) and no change was observed in fluorescent strength by expressing GFP gene under the original and mutant promoter of mGDH. Therefore, the point mutation in the promoter region of mGDH gene in *G. oxydans* RM7 may not be the major reason of the increased regulation. The improved capability of mGDH could be caused by the global genome or regulation changes, instead of a single gene. To clarify the global changes, the whole genome analysis of the wild-type and adaptive strain will be further conducted.

In this study, a *G. oxydans* strain used for fermentative production of sugar acids from lignocellulose biomass was adaptively evolved by alternate culture between the inhibitors containing hydrolysate and the inhibitors free hydrolysate for 420 days and the long term adaptive evolution resulted in the significantly accelerated conversion rate of non-glucose sugars to the corresponding sugar acids.

4. Conclusion

The adaptive evolution of *G. oxydans* by the long term alternate culture in the inhibitors containing hydrolysate and inhibitors free hydrolysate significantly improved the non-glucose sugars conversion rate and hence achieved complete conversion of all sugars from lignocellulose biomass to the corresponding sugar acids in biorefining fermentation. The up-regulated transcription of mGDH was considered to the decisive factor of the improved non-glucose sugar conversion in adapted strain *G. oxydans* RM7.

Acknowledgement

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