The Journal of Applied Science
วารสารวิทยาศาสตร์ประยุกต์
ISSN 1513-7805 (Printed in Thailand)

Vol. 17 Special issue: 18-26 [2018]
doi: 10.14416/j.appsci.2018.02.S03
ISSN 2586-9663 (Online)

Research Article

Enhanced succinic acid production from L-arabinose by *Corynebacterium glutamicum* CS176-*ΔldhA* disruptant

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Abstract

Corynebacterium glutamicum CS176 (CS176) was previously isolated from chicken feces contaminated soil from Suwanvajokkasikit Farm at Kasetsart University. The CS176 strain is among few native strains of industrial bacteria which can utilize L-arabinose, a pentose sugar as fermentative substrate. Recently, many attempts have been made to engineer *C. glutamicum* for efficient succinic acid production. In this research, we aimed to improve succinic acid production of CS176 strain. To do this, the gene encoded lactate dehydrogenase enzyme (*ldhA*) of CS176 was disrupted by construction of plasmid pK19*mobsacB-ΔldhA* harboring deleted *ldhA* and cloning to CS176. Subsequently, the disruptant, CS176-*ΔldhA*-C12 (C12) was obtained and its succinic acid production was investigated. The production was carried out by two-stage fermentation in 16x125 mm screw-cap tube containing 15 ml basal salt (BT) medium without nitrogen source. The results indicated the succinic acid concentration in BT-arabinose of C12 disruptant reached 1.74 g/l with a productivity yield of 0.89 g g^{-1}dried cell within 2 h after 10.7 mM sodium bicarbonate was added. C12 disruptant enhanced succinic acid in arabinose with 1.62-fold as compared with the wild type. In addition, the succinic acid concentration in BT-mixed glucose and arabinose reached 1.31 g/l with a productivity yield of 0.63 g/g dried cell which was 2.52-fold higher than that of wild type. Thus, this study suggested that disruption of *ldhA* gene of CS176 strain can improve succinic acid production especially in arabinose. The C12 disruptant would be suitable for starting as succinic acid producer and further works required for successful fermentative production especially from pentose sugar derived from lignocellulosic biomass.

Keywords: arabinose-utilizing *C. glutamicum*, bio-based succinic acid production, gene disruption

Introduction

Succinic acid has been used as an important building block for deriving high-value chemicals including 1,4-butanediol, γ-butyrolactone, adipic acid and tetrahydrofuran as well as biodegradable polymer such as polybutylene succinate (PBS) and polybutylene succinate adipate (McKinlay et al., 2007; Zeikus et al., 1999). Because succinic acid synthesized by petroleum chemical process may release CO_2 into environment and make a high environmental cost, bio-based production a green technology, has been considered for succinic acid production.

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Corynebacterium glutamicum, the facultative anaerobic non-pathogenic bacteria has been used for amino acids production industry (Kinoshita et al., 1985). Under O_2 deprivation condition, the cell growth was arrested but it could produce organic acids such as lactic acid, succinic acid and acetic acid from glucose-minimal medium passed by reductive TCA cycle pathway (Inui et al., 2004; Okino et al., 2005; 2008, Cheng et al., 2013; Wang et al., 2014).

C. glutamicum CS176 was previously isolated from chicken feces contaminated soil (Kitjakarn and Trakulnaleamsai, 2007). This is a few native strains which can utilize L-arabinose, pentose sugar as fermentative substrate. The aim of this study was to disrupt *ldhA* gene encoded lactate dehydrogenase enzyme of *C. glutamicum* CS176 to block lactic acid formation and characterize the capability of the Δ*ldhA* disruptant on succinic acid production using two-stage fermentation. The first stage was aerobic condition to increase the cell mass and the second stage was O_2 deprivation condition to produce the succinic acid. At the second stage, sodium bicarbonate was added to generate CO_2, the co-substrate of pyruvate carboxylase and phosphoenol-pyruvate carboxylase activities for improvement of the bio-based succinic acid production by *C. glutamicum.*

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work were shown in Table 1.

DNA Techniques

Genomic DNA was extracted from *C. glutamicum* CS176 followed by Sambrook et al. (2001) method. Plasmid DNA was isolated by using a GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's instructions. Restriction endonucleases were purchased from Takara. PCR (Perkin Elmer GeneAmp PCR System 9700) was performed using total volume of 25 µl with 100 ng of genomic DNA, 0.16 mM dNTPs and 0.75 units of USB® *Taq* DNA Polymerase in 10X PCR buffer (10X PCR buffer with provided 1.5 mM $MgCl_2$ in the final reaction volume, Affymetrix, Inc.) for 25 cycles at 95 °C for denaturation (30 sec), 56 °C for annealing (30 sec) and 72 °C for polymerization (1 min). T4 DNA ligase and 100 bp Plus DNA ladder were purchased from Thermo Scientific.

Construction of *ldhA* deletion mutant and plasmids

A portion of *ldhA* gene (1.1-kb) from genomic DNA of *C. glutamicum* CS176 was amplified with ldhF (5'-CTCTGTCGACATCAGGAAGTGGGATCGAAA-3') and ldhR (5'-CTCTGTCGAC TTCCATCCAACAGTTTCATT-3') primers (Inui et al, 2004). The 1.1-kb PCR product was ligated into pGEM-T easy vector, yielding pGEM-*ldhA* plasmid. The *ldhA* fragment was separated from pGEM-T easy vector by cutting with *Sal*I enzyme and inserted into the unique *Sal*I site of pUC18 vector, yielding pUC18-*ldhA*. The 3.8-kb pUC18-*ldhA* was digested with *EcoR*V and *Nae*I enzymes to remove 322 bp fragment and self-ligated yielding pUC18-*ΔldhA.* The 730 bp *ΔldhA* fragment was digested with *EcoR*I/*Hind*III and ligated into *EcoR*I/*Hind*III-digested pK19*mobsacB* plasmid yielding pK19*mobsacB*-*ΔldhA* and all of positive clones were confirmed by DNA sequencing method.

C. glutamicum CS176 was transformed by electroporation with plasmid pK19*mobsacB*-*ΔldhA* and selection for the first and second recombination events on brain heart infusion/ sorbitol (BHIS) plate containing kanamycin and S10 medium containing 10% sucrose, respectively (S10 medium per litre containing 10 g peptone, 10 g yeast extract, 5 g NaCl and

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100 g sucrose). Transformation of *E. coli* was performed by $CaCl_2$ procedure and selection on LB plate containing appropriate of antibiotic (Sambrook et al., 1989).

Table 1. Bacterial strains and plasmids used in the work

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
HIT-DH5α	*F-(80d lacZ M15)(lacZYA-argF)U169 hsdR17 (r- m+) recA1 endA1 relA1 deoR*	RBC Bioscience
Stellar (dam-/dcm-)	*F- ara Δ(lac-proAB)[Φ80d lacZΔM15] rpsL(str) thi Δ(mrr-hsdRMS-mcrBC) ΔmcrA dam dcm*	Clonetech
C. glutamicum		
CS176	Wild type, the arabinose utilizing strain	Kitjakarn & Trakulnaleamsai, (2007)
C12	*ldhA* disrupted mutant (*ΔldhA*)	This work
Plasmids		
pGEM-T easy	Ap^r; α-lac MCS, M13 ori	Promega
pGEM-T easy-*ldhA*	Ap^r; pGEM-T easy containing the *C. glutamicum* CS176 *ldhA* gene	This work
pUC18	Ap^r; α-lac MCS, M13 ori	Takara
pUC18-*ldhA*	Ap^r; pUC18 with 1.1 kb *Sal*I-*Sal*I fragment containing the *C. glutamicum* CS176 *ldhA* gene	This work
pUC18-**Δ***ldhA*	Ap^r; pUC18 with 730 bp *Sal*I-*Sal*I fragment containing *ΔldhA* gene	This work
pK19*mobsacB*	Km^r; plasmid for allelic exchange in *C. glutamicum*	Schäfer et al. (1994)
pK19*mobsacB*-**Δ***ldhA*	Km^r; with 730 bp *EcoR*I-*Hind*III fragment containing *ΔldhA* gene	This work

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Two-stage fermentation for succinic acid production

The CS176 and its disruptant were activated in 5 ml of A medium and incubated at room temperature with shaking at 350 rpm for 16-18 h (A medium per litre in dH_2O containing 2 g urea, 2 g yeast extract, 7 g casamino acid, 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O_2$ 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin and 0.2 mg thiamine).

Transferred 0.1 ml culture broth from A medium into 5 ml BTN medium (BTN medium per litre in dH_2O containing 10 g glucose, 3 g NH_4Cl, 2 g urea, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin and 0.2 mg thiamine). Cultivation was performed under aerobic stage at room temperature, 350 rpm for 16-18 h. Under oxygen deprivation stage, 11 ml of culture broth from aerobic phase was transferred to screw cap tube (size 16×125 mm) containing 4 ml BT medium (BTN without N source). Cultivation was performed at room temperature, 120 rpm and 10.7 mM sodium bicarbonate was added at 4 h after cultivation and further incubation in CO_2 condition for 0-10 h. The carbon sources were varied (per litre): 10 g arabinose, 10 g glucose, 10 g sucrose, 5 g glucose and 5 g arabinose, 5 g glucose and 5 g sucrose, 5 g arabinose and 5 g sucrose, 0.33 g each of glucose, arabinose and sucrose.

Analytical methods

Cell growth was measured by spectrophotometry at optical density 600 nm (OD_{600}) (UV/VIS Spectrophotometer U-3310, Hitachi, Japan) or Klett Summerson colorimeter. Samples were collected at 0-10 h after bicarbonate was added and centrifuged at 10,000 rpm, 4 °C for 5 min. Supernatants were analyzed for sugars and organic acids. The qualitative organic acids were detected by Thin-layer chromatography (TLC) on TLC Silica gel 60 F_{254} (Merck, Germany) (Kraiker and Burch, 1973). The quantitative organic acids were quantified by HPLC (Varian ProStar, USA) equipped with UV detector and C18 ChromSep HPLC Column SS (Varian, Inc., USA) operating at 210 nm at 25 °C using 1 mM H_2SO_4 and 8 mM Na_2SO_4 pH 2.8 as mobile phase at flow rate 1 ml/min. Sugars concentrations were determined by HPLC (brand and model) equipped with ELSD detector and Rezex RPM-Monosaccharide Pb^{2+} Column (Phenomenex, USA) operating at 60 °C using deionized water as mobile phase at flow rate 0.6 ml/min.

Results and discussion

Screening of CS176-*ΔldhA* disruptant

In general, *C. glutamicum* could produce L-lactic acid as a major product and also produced succinic acid under oxygen deprivation condition via reductive branch of TCA cycle pathway. Many attempts have been drawn to improve succinic acid production in *C. glutamicum* by using metabolic engineering. The native *ldhA* gene was disrupted to block L-lactic acid formation pathway and shifted to succinic acid formation pathway.

Construction of recombinant *ΔldhA* plasmid was confirmed by DNA sequencing analysis. The result showed that *ΔldhA* fragment consists of 727 bp and the nucleotide positions from 271-603 was removed as compared with the *ldhA* gene from CS176 wildtype (Figure 1).

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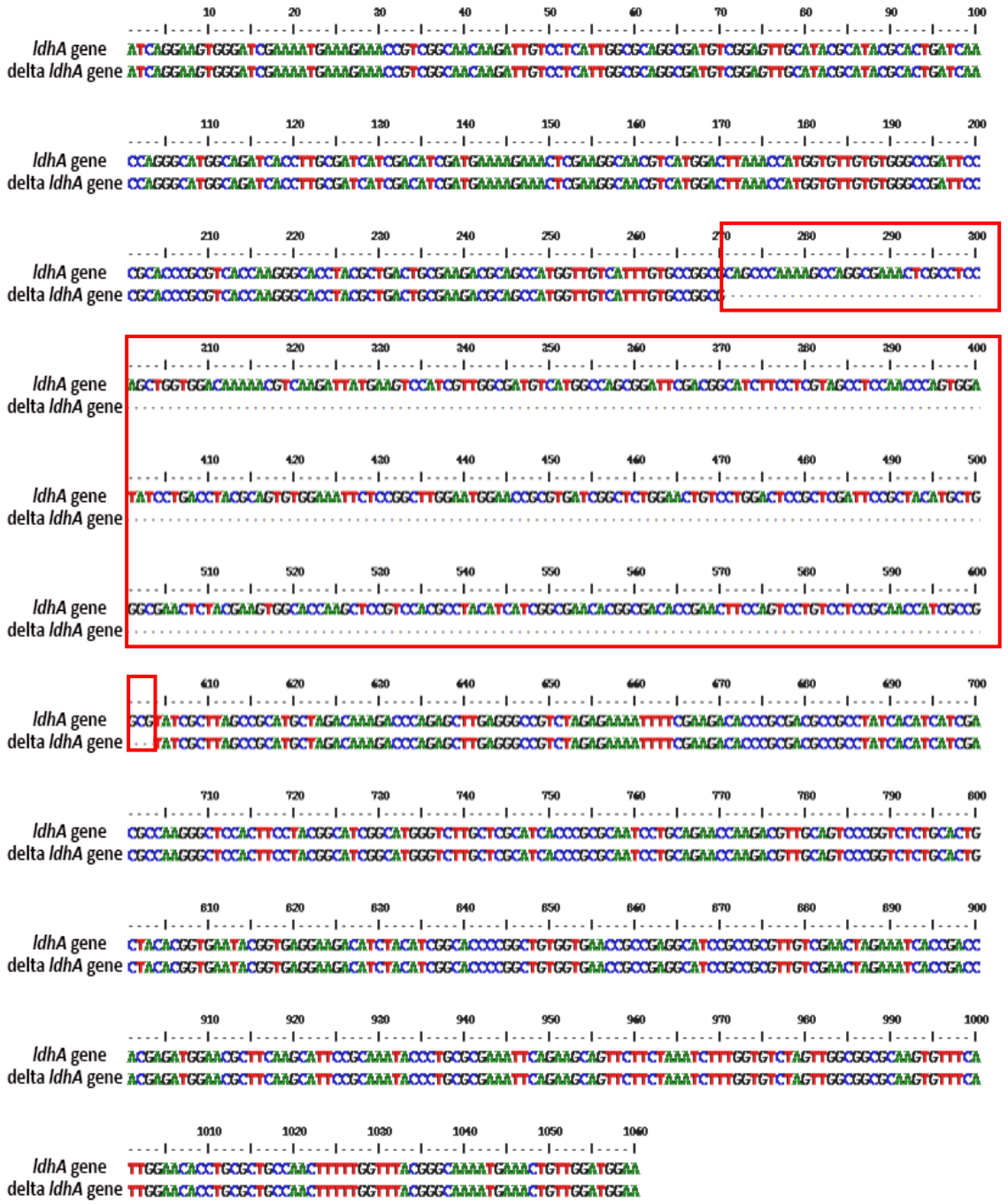

Figure 1. Comparison of nucleotide sequences between *ldhA* gene (top line) from *C. glutamicum* CS176 and *ΔldhA* fragment (bottom line) from recombinant pK19*mobsacB-ΔldhA* plasmid. Region in the red box was deleted.

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The *ldhA* disruption in *C. glutamicum* CS176 was constructed in two-step homologous recombination with pK19*mobsacB-ΔldhA* plasmid. The first step of recombination event, the clones harboring two bands of 1.1 kb (full length of *ldhA* gene from CS176, wild type) and 730 bp of *ΔldhA* gene were selected by kanamycin resistant trait on BHIS plate containing kanamycin (50 µg/ml). Then, the second recombination event was selected by sucrose resistant growth on S10 plate containing 10% sucrose. 10 out of 23 sucrose resistant colonies (clone C11-C20) which appeared on S10 plate were randomly picked up to screen positive disruptant by colony PCR. As a result, 9 clones possessed 750 bp PCR fragment indicating positive clones harboring deleted *ldhA* gene while only C17 was not detected due to no amplification (Figure 2). Then, 9 disruptants (except C17) were screened for succinic acid production ability on BTN-glucose plate containing 1% $MgCO_3$ under O_2 deprivation condition to observe clear zone after $MgCO_3$ hydrolized by organic acid. The clone named C12 showed the largest of clear zone on screening plate (data not shown) thus, C12 disruptant was selected for further characterization on succinic acid production.

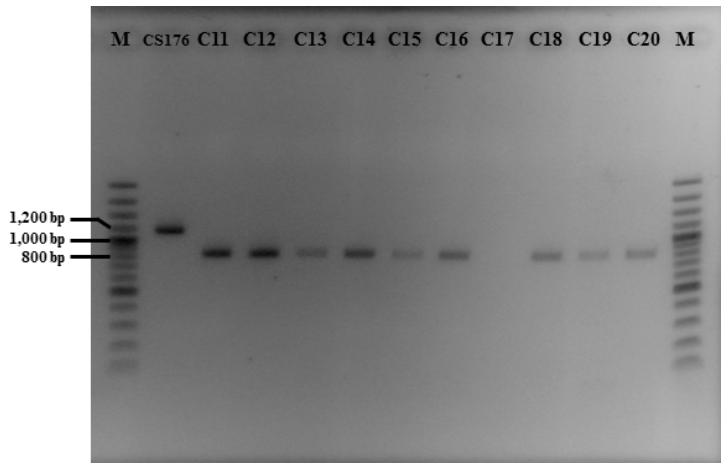

Figure 2. PCR product of *ldhA* gene from CS176, wild type and sucrose resistant clones on 1.2% agarose gel; M: DNA marker, 100 bp Plus DNA ladder.

Succinic acid production of CS176 wild type and C12 disruptant

Both of CS176 and selected C12 disruptant could grow and increase the number of cell mass in BTN medium containing glucose, arabinose or sucrose as sole and mixed carbon source(s) at high aeration condition and produced succinic acid under oxygen deprivation condition (as described in material and methods). However, high carbon dioxide has been shown to elevate succinic acid in *Anaerobiospirillum succiniciproducens* and *C. glutamicum* (Samuelov et. al. 1991, Okino et. al., 2005). Thus, the succinic acid production in various sugars of *C. glutamicum* CS176 and C12 disruptant were analyzed in the conventional fermentation with adding 10.7 mM bicarbonate as a maximum concentration in tested condition. The results are shown in Figure 3 and Table 2. The results revealed that C12

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disruptant could produce succinic acid in BT medium containing either arabinose or mixed sugars better than that of CS176 wild type. The succinic acid production in BT-arabinose of C12 disruptant reached 1.74 g/l within 2 h after adding 10.7 mM sodium bicarbonate with a yield of 0.89 g/gCDW while CS176 reached the highest succinic acid concentration of 1.07 g/l at 8 h. The succinic acid yield of C12 disruptant was increased 1.62 folds with highest production rate of 0.45 g/gCDW/h. The result revealed that the consumption of arabinose to succinic acid in C12 was increased as compare to that of CS176. In term of mix carbon sources, succinic acid yield from C12 disruptant was higher 2-4.4 folds. The highest succinic acid concentration in BT medium containing glucose and sucrose from C12 disruptant was 3.31 g/l. Moreover, other organic acids as observed by shorter retention time peaks appeared on HPLC profile could be detected along with succinic acid analysis (profile not shown).

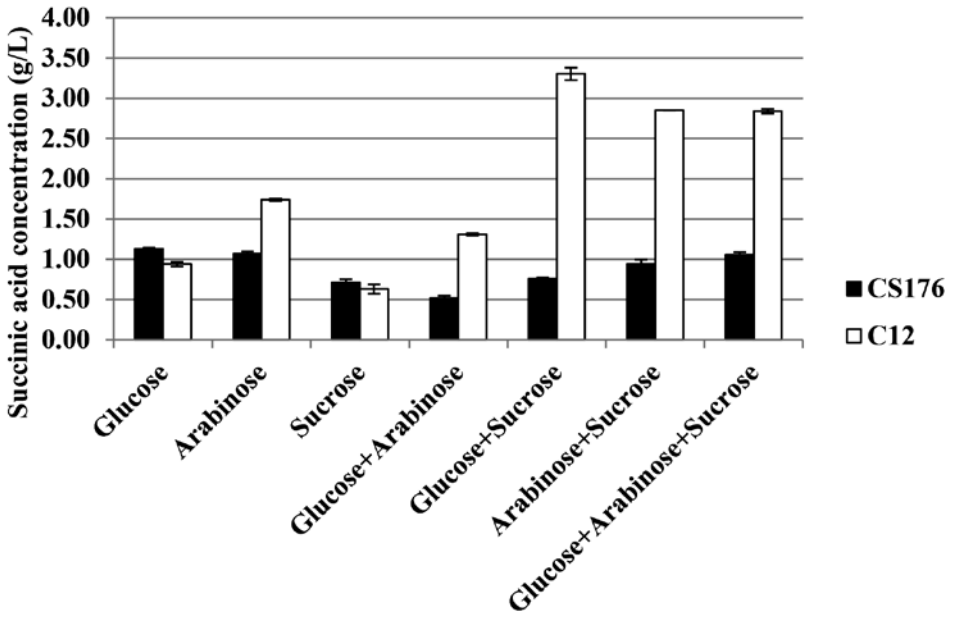

Figure 3. Succinic acid production under oxygen deprivation condition with addition of 10.7 mM bicarbonate by *C. glutamicum* CS176 wild type (black bar) and C12 disruptant (white bar) in medium with various sugars. Data were average of duplicate experiments.

In this study, the disruption of lactate dehydrogenase gene (Δ*ldhA*) of *C. glutamicum* CS176, an arabinose utilizing strain, by homologous recombination was successful. The disruptant, namely, *C. glutamicum-ΔldhA*-C12 was obtained. The result reveals C12 disruptant improved succinic acid production as compare to CS176 wild type under conventional two-stage fermentation supplemented with 10.7 mM bicarbonate. The maximum succinic acid production rate of 0.45 g/gCDW/h was achieved in BT-arabinose within two hours after adding bicarbonate. The succinic acid yield was enhanced 1.62 folds as compared to that of wild type. Thus, this study indicated that disruption of *ldhA* gene of CS176 strain can improve succinic acid production in arabinose. The C12 disruptant would be suitable strain for further

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improvement required for successful fermentative production of succinic acid especially from pentose sugars derived from lignocellulosic biomass.

Table 2. Summary of succinic acid production by *C. glutamicum* CS176 and C12 disruptant in various carbon sources in the presence of 10.7 mM bicarbonate

Strain	Carbon source(s)	Titer (g/l)	Time giving highest succinic acid after adding bicarbonate (h)	Productivity (g/l/h)	Succinic acid yield (g/gCDW)	Production rate (g/gCDW/h)
C. glutamicum CS176	Glucose	1.13±0.01	8	0.14	0.47	0.06
	Arabinose	1.07±0.03	8	0.13	0.54	0.07
	Sucrose	0.71±0.04	6	0.12	0.34	0.06
	Glucose and arabinose	0.52±0.03	4	0.13	0.24	0.06
	Glucose and sucrose	0.76±0.01	6	0.13	0.33	0.06
	Arabinose and sucrose	0.94±0.06	8	0.12	0.42	0.05
	Glucose, sucrose and arabinose	1.10±0.03	8	0.14	0.53	0.06
C. glutamicum-*ΔldhA*-C12 disruptant	Glucose	0.94±0.03	6	0.16	0.47	0.08
	Arabinose	1.74±0.01	2	0.87	0.89	0.45
	Sucrose	0.63±0.06	8	0.08	0.33	0.04
	Glucose and arabinose	1.31±0.01	6	0.22	0.63	0.11
	Glucose and sucrose	3.31±0.08	8	0.41	1.48	0.19
	Arabinose and sucrose	2.85±0.00	8	0.36	1.28	0.16
	Glucose, sucrose and arabinose	2.84±0.03	8	0.36	1.17	0.15

Acknowledgements

Plasmid pK19*mobsacB* was kindly provided by Associate Professor Dr. Toshiharu Yakushi, Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan. This work was financial supported by The Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University and Graduate Thesis Grant from The National Research Council of Thailand (NRCT).

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