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The impact of *MIG1* and/or *MIG2* disruption on aerobic metabolism of succinate dehydrogenase negative *Saccharomyces cerevisiae*

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Abstract The zinc finger proteins Mig1 and Mig2 play important roles in glucose repression of *Saccharomyces cerevisiae*. To investigate whether the alleviation of glucose effect would result in an increase in aerobic succinate production, *MIG1* and/or *MIG2* were disrupted in a succinate dehydrogenase (SDH)-negative *S. cerevisiae* strain. Moreover, their impacts on physiology of the SDH-negative *S. cerevisiae* strain were studied under fully aerobic conditions when glucose was the sole carbon source. Our results showed that the succinate production for the SDH-negative *S. cerevisiae* was very low even under fully aerobic conditions. Furthermore, deletion of *MIG1* and/or *MIG2* did not result in an increase in succinate production in the SDH-negative *S. cerevisiae* strain. However, the synthesis of acetate was significantly affected by *MIG1* deletion or in combination with *MIG2* deletion. The acetate production for the *mig1*/*mig2* double mutant BS2M was reduced by 69.72% compared to the parent strain B2S. In addition, the amount of ethanol produced by BS2M was slightly decreased. With the *mig2* mutant BSM2, the concentrations of pyruvate and glycerol were increased by 26.23% and 15.28%, respectively, compared to the parent strain B2S.

Keywords *Saccharomyces cerevisiae* · Succinate dehydrogenase · *MIG1* · *MIG2* · Aerobic metabolism · Glucose repression

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Introduction

The yeast *Saccharomyces cerevisiae* is well established as a eukaryotic model organism for the study of metabolic engineering (Ostergaard et al. 2000; Nevoigt 2008). In recent years, redirection of metabolic fluxes in *S. cerevisiae* away from alcoholic fermentation towards other desired chemicals has attracted more and more attention (Saitoh et al. 2005; Zelle et al. 2008). Succinic acid is an essential flavor component produced by *S. cerevisiae* in sake fermentation (Arikawa et al. 1999), and it is also a potential building block for a wide variety of value-added chemicals made from biomass (Anonymous 2004). As a C4 metabolite mainly produced from the TCA cycle and the glyoxylate cycle, it is a good case study for implementing a metabolic engineering strategy for redistribution carbon fluxes in *S. cerevisiae*.

Succinate dehydrogenase (SDH) is a key enzyme of the Krebs cycle and the electron transport chain, oxidizes succinate, and reduces ubiquinone using a flavin adenine dinucleotide cofactor (Oyedotun and Lemire 2004). The SDH of *S. cerevisiae* consists of five subunits encoded by the *SDH1*, *SDH2*, *SDH3*, *SDH4*, and *SDH5* genes, respectively (Oyedotun and Lemire 2004; Hao et al. 2009). An increase of succinate production was observed when succinate dehydrogenase activity was lost by the double deletion of *SDH1* and *SDH2* when *S. cerevisiae* was cultivated under shake flask conditions; however, the succinate production was still very low (Kubo et al. 2000). In fact, *S. cerevisiae* has a strong tendency to produce ethanol when external glucose is in excess even under fully aerobic conditions (van Dijken et al. 1993). Moreover, a large number of genes involved in the Krebs cycle, the glyoxylate cycle, and in oxidative phosphorylation are repressed in *S. cerevisiae* by a regulatory

mechanism under high glucose level, a phenomenon known as glucose repression (Santangelo 2006; Rolland et al. 2002; Westholm et al. 2008). Therefore, manipulating the activities of regulatory proteins involved in glucose repression seems to be a rational approach for redirection of carbon fluxes to succinic acid production (Blom et al. 2000).

Mig1 and Mig2 have been recognized as two main effectors in glucose repression. Mig1 is a zinc finger protein that binds to the promoters of many genes and represses their transcription when glucose is added to the medium (Klein et al. 1998; Westholm et al. 2008). Mig2, showing 71% homology to Mig1, also performs a role in glucose repression (Lutfiyya et al. 1998). Deletion of *MIG1* gene alone or in combination with *MIG2* gene has been shown to alleviate the glucose effect and increase the respiratory activity of *S. cerevisiae* (Klein et al. 1996; Klein et al. 1999). We wondered whether the deletion of *MIG1* and/or *MIG2* would improve succinate production in a SDH negative *S. cerevisiae* strain under aerobic conditions when high concentration of glucose was present.

In the present study, we constructed a SDH-negative *S. cerevisiae* strain B2S by double deletion of *SDH1* and *SDH2* from a haploid laboratory strain BY4742, and we further deleted *MIG1* and/or *MIG2* in strain B2S. Then, the physiological characteristics of these genetically engineered strains were compared, and the impact of *MIG1* and/or *MIG2* disruption on aerobic carbohydrate metabolism of the SDH-negative *S. cerevisiae* strain was elucidated in this paper.

Materials and methods

Yeast strains and medium

The yeast strains used are listed in Table 1. Strains were routinely maintained and grown on YPD medium (1% yeast extract, 2% Bacto peptone, and 2% glucose; solid media contained 2% agar). For selection of yeast transformants, G418 or phleomycin was added with the final concentration at 200 and 10 μg/mL, respectively. The mineral salts medium for preculture and batch cultivations was prepared as described by Verduyn et al. (1992). The concentration of glucose was 100 mM. In addition, uracil, histidine, lysine, and leucine were added at a final concentration at 200 mg/L each.

Mutant construction

The SDH-negative strain B2S was constructed by subsequent deleting *SDH1* and *SDH2* genes in BY4742 with the Cre/*loxP* recombination system according to the method described by Güldener et al. (1996, 2002). Initially, the *SDH1* gene was deleted by replacement of the *loxP-ble-loxP* cassette amplified from pUG66 (from EUROSCARF) using the oligonucleotide primers F-KOsdh1 and R-KOsdh1 (Table 2). The *sdh1* mutant was selected from geneticin-resistant transformants and verified by PCR analysis. Then, the *loxP-kanMX-loxP* cassette amplified from pUG6 (from EUROSCARF) using the oligonucleotide primers F-KOsdh2 and R-KOsdh2 for disrupting the *SDH2* gene was transformed into the *sdh1* mutant strain. The *sdh1* and *sdh2* double mutant strain was isolated from phleomycin-resistant transformants and confirmed by PCR analysis. The markers were popped out by cre recombinase expressed by pSH47 (from EUROSCARF). Strain B2S was identified as a SDH-negative strain and was used to construct all further strains. To delete the *MIG1* gene, a deletion cassette that confers geneticin resistance was made by performing a PCR on plasmid pUG6 using the oligonucleotide primers F-KOmig1 and R-KOmig1 (Table 2). The *MIG2* gene was deleted by replacement of the *loxP-ble-loxP* cassette amplified from pUG66 using the oligonucleotide primers F-KOmig2 and R-KOmig2 (Table 2). The replacement of the *MIG1* and the *MIG2* ORFs by the amplified modules were verified by PCR analysis of total DNA isolated from the G418 and the phleomycin-resistant transformants, respectively.

Batch cultivations

A single colony from a fresh YPD plate was inoculated into 3 mL YPD and incubated overnight at 30°C shaking at 220 rpm. Cells from 1 mL overnight culture were washed twice with sterilized deionized water, then transferred to a 250-mL flask contain 50 mL mineral salts medium and kept in an orbital shaker set at 220 rpm for 24 h. Batch

Table 1 Yeast strains used in this study

Strain	Genotype	Source
BY4742	*Matα, ura3Δ0, his3Δ1, lys2Δ0, leu2Δ0*	EUROSCARF
B2S	*sdh1Δ::loxP, sdh2Δ::loxP*	This work
BSM1	*sdh1Δ::loxP, sdh2Δ::loxP, mig1Δ::KanMX*	This work
BSM2	*sdh1Δ::loxP, sdh2Δ::loxP, mig2Δ::ble*	This work
BS2M	*sdh1Δ::loxP, sdh2Δ::loxP, mig1Δ::KanMX, mig2Δ::ble*	This work

Table 2 PCR Primers used for gene knock out in this study

Primer	Sequence (5′→3′)[a]
F-KOsdh1	TTTCATAGTACGAAGAAGAACGAGAATAAAGATGCTATCGagctgaagcttcgtacgc
R-KOsdh1	CGTAAAATACAATGAGGTTCAAATTAGTAGGCTCTTACAGgcataggccactagtggatc
F-KOsdh2	TATACTTTCTTGAAATACTGGAGTATACATATTTATAGGGCagctgaagcttcgtacgc
R-KOsdh2	AGGCTTCTGAGTTCTTCGGGGCTAGCTGTTTTTCTGATAgcataggccactagtggatc
F-KOmig1	ACGAGAGTTGAGTATAGTGGAGACGACATACTACCATAGCCagctgaagcttcgtacgc
R-KOmig1	ATTGTCTTTTGATTTATCTGCACCGCCAAAAACTTGTCAgcataggccactagtggatc
F-KOmig2	ACCTCGAGAACAAACAAAATAAAAATAAAAAAAGAGAATGCagctgaagcttcgtacgc
R-KOmig2	GTTAGAGGAAAAATGGTGAGATAAAAAGGGGCCGTAAAGgcataggccactagtggatc

[a] The capital letters indicate nucleotides homologous to upstream and downstream of the corresponding gene ORFs; the lowercase letters indicate nucleotides complementary to sequences in the pUG plasmids flanking the disruption cassettes

experiments were carried out at 30°C in 5-L bioreactors with a working volume of 3 L and performed by inoculation of the precultured cells to give a final optical density at 600 nm of 0.1. The aeration rate employed for the batch cultivation was 3.5 L/min and maintained the dissolved oxygen tension above 50% of air saturation. The pH was controlled at 4.5 by the automatic addition of 2 M NaOH or 2 M HCl. The agitation rate was maintained at 400 rpm.

Analytical techniques

Cell growth was monitored by measuring the optical density (OD) at 600 nm in a spectrophotometer. Acetate, pyruvate, and succinate were determined by an ion chromatography system ICS3000 (Dionex,USA) as described by Geng et al. (2008). Glycerol was analyzed by a high-performance liquid chromatography system equipped with a charged aerosol detector (ESA, USA), using a Shodex Asahipak NH_2P-50 4E column eluted with 75% (*v*/*v*) acetonitrile at a flow rate of 1 mL min^{-1} at 25°C. Glucose was analyzed by a high-performance liquid chromatography system equipped with a pulsed amperometric detector (Dionex, USA), using a PA-1 sugar analytic column (Dionex, USA) eluted with 100 mM NaOH at a flow rate of 1 mL min^{-1} at 30°C. Ethanol was determined by an automatic ethanol analyzer SBA-40E (Biology Institute of Shandong Academy of Sciences, China) equipped with an immobilized enzyme electrode.

Analysis of oxygen consumption rates

For oxygen consumption capacity measurements, the cells were grown to mid-log phase in bioreactors, harvested by centrifuging at 1,000×*g* for 5 min, washed three times with ice-cold deionized H_2O, and resuspended in oxygraph buffer [1% yeast extract (*w*/*v*), 0.1% KH_2PO_4 (*w*/*v*), 0.12% $(NH_4)_2SO_4$ (*w*/*v*)] at a cell density of 20 OD_{600} (the value of optical density at 600 nm) units per milliliter. Oxygen consumption rates of the cells were measured with a Clark-type oxygen electrode (Dalian Sane Analytical Instruments Ltd. China), with 100 mM glucose as substrate.

Results

The physiological characteristics of a SDH-negative strain under aerobic cultivation

For this study, the physiological characteristic of a SDH-negative strain B2S was first investigated under fully aerobic conditions in well-controlled bioreactors. Compared to the parent strain BY4742, the specific growth rate of B2S (0.27±0.01 h^{-1}) was decreased by 6.9% in the exponential phase, while the ultimate biomass yield was elevated following the full utilization of glucose (Fig. 1). In addition, a 56.25% increase in succinate production was observed for B2S (Table 3). Moreover, the amounts of acetate and glycerol produced by B2S were reduced by 28.15% and 16.43%, respectively, compared to BY4742 (Table 3). However, no significant difference in amounts of other metabolites between BY4742 and B2S was observed (Table 3).

Impact of *MIG1* or *MIG2* deletion on SDH-negative *S. cerevisiae*

To investigate the impact of a single deletion of *MIG1* or *MIG2* on SDH-negative *S. cerevisiae*, we constructed strains BSM1 and BSM2 from B2S, with a gene complete deletion of *MIG1* and *MIG2*, respectively. Then, aerobic batch culture growth of the strains B2S, BSM1 and BSM2 was investigated (Fig. 1a). During the exponential growth phase, the specific growth rate for BSM1 (0.29±0.01 h^{-1}) was higher than for B2S (0.27±0.01 h^{-1}), while the specific growth rate for BSM2 (0.24±0.01 h^{-1}) was significantly decreased. The glucose consumption rates corresponded well to the growth characteristics in each strain (Fig. 1b). In addition, the concentrations of acetate, ethanol, pyruvate, glycerol, and succinate in the media were measured when glucose was exhausted (Table 3). The production of acetate for BSM1 was 42.69% lower than for B2S, but no difference was noticed between B2S and BSM2. In contrast, the productions of pyruvate and glycerol were similar for B2S and BSM1 but were increased by 26.23%

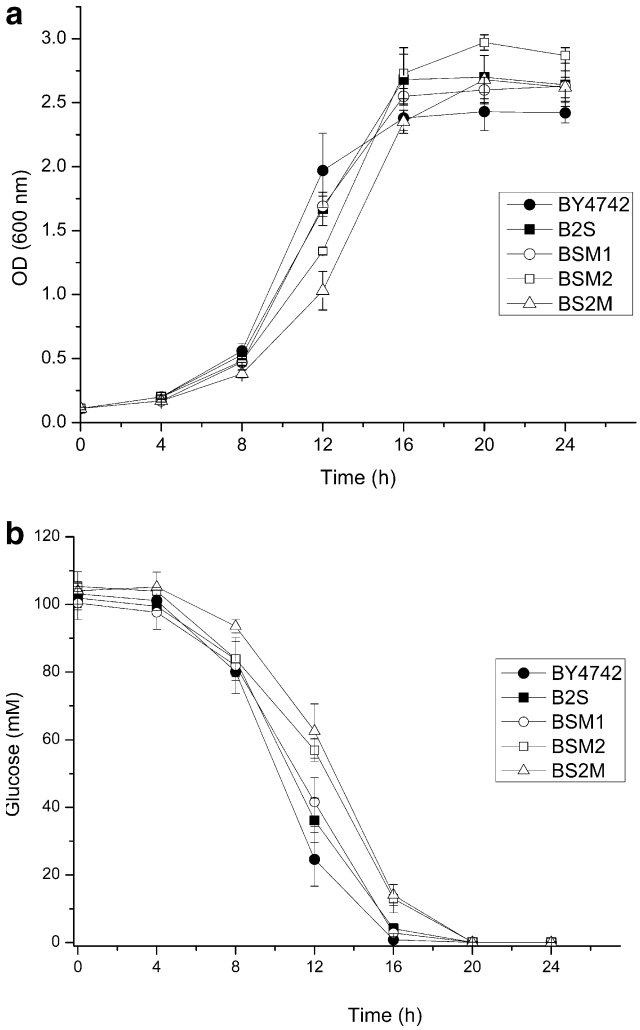

Fig. 1 **a** The growth curves of the strains BY4742, B2S, BSM1, BSM2, and BS2M; **b** the glucose consumption curves of the strains BY4742, B2S, BSM1, BSM2, and BS2M. Strains were grown in mineral salts medium in 5 L bioreactors with a working volume of 3 L. The agitation rate was maintained at 400 rpm. The aeration rate was set at 3.5 L/min and maintained the dissolved oxygen tension above 50% of air saturation. The pH was controlled at 4.5. *Closed circles* represent strain BY4742; *closed squares* represent strain B2S; *open circles* represent strain BSM1; *open squares* represent strain BSM2; *open triangles* represent strain BS2M. Mean values and standard deviations of at least three independent experiments are shown

and 15.28%, respectively, for BSM2 compared to B2S. However, no significant difference was observed in amount of ethanol formed by the strains B2S, BSM1, and BSM2. In addition, a significant increase in succinate production was not observed either in BSM1 or in BSM2. To test whether the deletion of *MIG1* and/or *MIG2* would result in an increase of respiratory capacity, we measured oxygen consumption rates of the cells grown to mid-logarithmic phase (Fig. 2). The oxygen consumption rate of B2S was not significantly affected by deletion of *MIG1*, while it was drastically decreased by deletion of *MIG2*.

Impact of *MIG1* and *MIG2* double deletion on SDH-negative *S. cerevisiae*

To investigate the combination effects of Mig1 and Mig2 on aerobic carbohydrate metabolism in a SDH-negative strain, we constructed BS2M from B2S by double deletion of *MIG1* and *MIG2*. Under fully aerobic batch cultivations, the specific growth rate of BS2M (0.22±0.02 h^{-1}) was similar to that of BSM2 (0.24±0.01 h^{-1}) but was 16.25% and 22.09% lower than that of B2S (0.27±0.01 h^{-1}) and BSM1 (029±0.01 h^{-1}), respectively (Fig. 1a). The concentrations of ethanol, acetate, pyruvate, glycerol, and succinate produced by BS2M were also determined (Table 3). Interestingly, the acetate production in BS2M was drastically reduced further. Compared to strain BSM1 and B2S, the acetate production in BS2M was reduced by 47.31% and 69.72%, respectively. Furthermore, the ethanol production in BS2M was slightly decreased, whereas the concentration of pyruvate produced by BS2M was increased by 14.75%, compared to B2S. However, no significant difference between B2S and BS2M in succinate production was observed (Table 3). In addition, the respiratory capacity of BS2M was also not increased, compared to B2S (Fig. 2).

Discussion

As it is well known, succinate is an intermediate product of the TCA cycle. The ablation of succinate dehydrogenase activity would be expected to result in accumulation of succinate. Previously, several groups have studied the metabolism of SDH-negative *S. cerevisiae* cells under anaerobic conditions or oxygen-limited shake flask conditions (Arikawa et al. 1999; Kubo et al. 2000; Camarasa et al. 2003). However, studies under fully aerobic metabolism of a SDH-negative *S. cerevisiae* strain have not been reported so far. Because oxygen limitation might hamper the carbon fluxes into the TCA cycle in *S. cerevisiae*, we first examined the metabolism of the SDH-negative *S. cerevisiae* B2S under fully aerobic conditions on 100 mM glucose in well-controlled bioreactors. Compared to an earlier study in which a SDH-negative *S. cerevisiae* strain showed a 51.9% increase in succinate production when cultivated under oxygen-limited shake flask conditions (Kubo et al. 2000), the succinate production for the SDH-negative strain B2S was only increased by 56.25% under fully aerobic conditions in our study. This finding indicated that the oxygen limitation might not be the key restricting factor for succinate production when high glucose was present. Moreover, a drastic reduction of acetate was also observed in B2S. Because succinate dehydrogenase is an important component of electron transport system in mitochondria, the inactivation of SDH will result in

Table 3 The concentration of metabolites produced by BY4742 and engineered strains under aerobic conditions

Metabolite	Metabolite concentration (mM)[a]				
	BY4742	B2S	BSM1	BSM2	BS2M
Ethanol	156.16±6.64	151.33±11.10	135.12±0.17	147.10±12.62	130.77±7.76
Acetate	2.38±0.21	1.71±0.02	0.98±0.04	1.69±0.06	0.51±0.03
Pyruvate	1.26±0.09	1.22±0.03	1.27±0.08	1.54±0.08	1.40±0.03
Glycerol	21.85±0.06	18.26±0.39	19.06±0.91	21.05±1.35	20.11±0.31
Succinate	0.16±0.00	0.25±0.02	0.20±0.04	0.16±0.02	0.22±0.05

[a] The concentrations of metabolites were determined after glucose was completely exhausted. For BY4742, samples for metabolites measurement were taken at 16 h after starting batch cultivations; for other genetically engineered strains, samples for metabolites measurement were taken at 20 h after starting batch cultivations. Values are given as means ± standard deviations in at least three independent experiments

respiratory deficiency and affect the efficiency of the electron transport chain under aerobic conditions (Cooley and Vermaas 2001). Consequently, the capacity of mitochondrial NADH oxidation might be indirectly affected and should result in NAD^+/NADH imbalance. Therefore, the decreased acetate production observed in B2S might partly be due to surplus NADH. However, in conflict with our result, an earlier study had shown that acetate production of a SDH-deficient strain was significantly increased compared to the wild strain (Romano and Kolter 2005). One possible reason might be the different cultivation conditions between the two studies.

To investigate whether the alleviation of glucose repression in the SDH-negative *S. cerevisiae* strain would prompt the succinate production, we further deleted *MIG1* and/or *MIG2* in B2S. Previously, several studies have demonstrated that the expressions of a number of genes involved in the TCA cycle, the glyoxylate shunt and the respiration were upregulated in a *mig1* mutant or in a *mig1*/*mig2* double mutant (Westholm et al. 2008; Westergaard et al. 2007). However, the carbon fluxes toward the TCA cycle and the glyoxylate cycle did not seem to be remarkably increased in our study, because the accumulation of succinate was not significantly enhanced by further deletion of *MIG1* and/or *MIG2* in B2S (Table 3). Moreover, the ethanol production was also predominant in all the mutants constructed in our study. Because a limited respiratory capacity of *S. cerevisiae* has been considered as the one of main causations which lead to aerobic alcoholic fermentation (Vemuri et al. 2007), we measured oxygen consumption rates of the cells to test whether the deletion of *MIG1* and/or *MIG2* would result in an increase of respiratory capacity of B2S. Indeed, the respiratory capacity was not improved by deletion of *MIG1* and/or *MIG2* in our study (Fig. 2). This might be one of the reasons why the succinate production was not further improved by deletion of *MIG1* and/or *MIG2* in B2S.

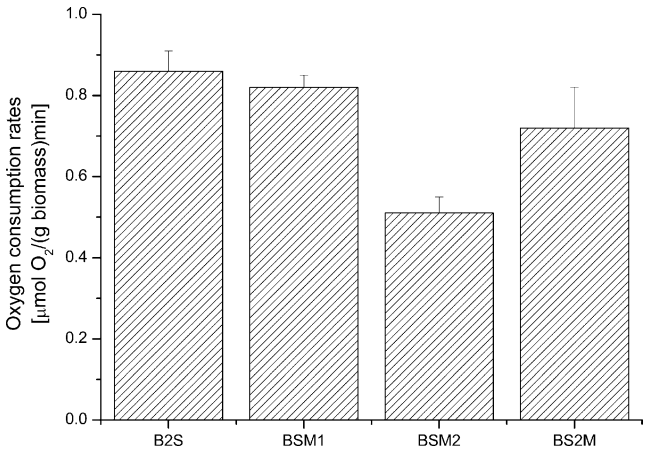

Fig. 2 The oxygen consumption rates of the strains B2S, BSM1, BSM2, and BS2M. Batch-grown cells in 5 L bioreactors were taken at mid-log phase and washed three times with ice-cold deionized H_2O. Oxygen consumption rates of the cells were measured with a Clark-type oxygen electrode in oxygraph buffer at a cell density of 20 OD_{600} units mL^{-1}. The substrate was 100 mM glucose

Compared to Mig1, Mig2 has been elucidated to have minor impact on carbohydrate metabolism of *S. cerevisiae* on 2% glucose, because only a few genes were significantly affected in a *mig2* single mutant at transcription level (Westholm et al. 2008). However, the change in gene expression cannot always reflect the real change of metabolism. In the present study, we found the respiratory capacity was significantly decreased by deletion of *MIG2* in B2S (Fig. 2). Furthermore, the production of glycerol and pyruvate were also significantly affected by *MIG2* disruption. These results indicated that Mig2 also played an important role in the regulation of carbohydrate metabolism in *S. cerevisiae*, and the mechanism of Mig2 in the regulation of metabolism should be further investigated at multilevel (RNA, proteins, metabolites).

In the present study, we have demonstrated that aerobic succinate production in the SDH-negative *S. cerevisiae* strain was not restricted by limited oxygen. Furthermore, deletion of *MIG1* and/or *MIG2* did not result in an increase in aerobic succinate production for the SDH-negative *S. cerevisiae* strain. This might be partly due to the limited

respiratory capacity, which was also not improved by deletion of *MIG1* and/or *MIG2*. However, a significant decrease in acetate production was observed in B2S, BSM1, and BS2M, compared to BY4742. Especially, the acetate production in BS2M was reduced by 69.72% and 78.29%, respectively, compared to B2S and BY4742. In addition, a slight decrease in ethanol production was also observed in BS2M, compared to B2S. These findings indicated that the double deletion of *MIG1* and *MIG2* could potentially have a positive effect on redistribution of carbon fluxes to maximum succinate production in further studies.

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