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Efficient production of 2,3-butanediol by recombinant *Saccharomyces cerevisiae* through modulation of gene expression by cocktail δ-integration

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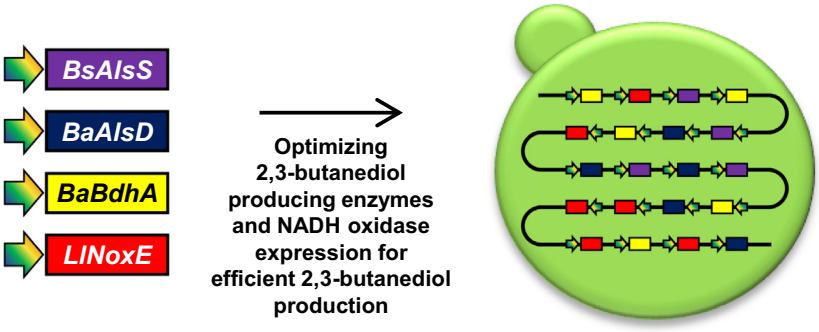
HIGHLIGHTS

- 2,3-Butanediol (BDO) is useful metabolites produced by microorganisms.
- BDO production by *Saccharomyces cerevisiae* was examined.
- Expression of 4 genes was optimized by cocktail δ-integration strategy.
- BDO productivity was improved by cocktail δ-integration strategy.
- The highest BDO production rate and yield were achieved in fed-batch cultivation.

GRAPHICAL ABSTRACT



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ABSTRACT

In this study, the expression of 4 genes encoding α-acetolactate synthase, α-acetolactate decarboxylase, 2,3-butanediol dehydrogenase, and NADH oxidase was modulated using a previously developed cocktail δ-integration strategy. The resultant strain, YPH499/dPdAdG/BD6-10, was used in a fed-batch cultivation for the production of 2,3-butanediol. The concentration, production rate, and yield obtained were 80.0 g/L, 4.00 g/L/h, and 41.7%, respectively. The production rate and yield of the compound obtained are higher for this strain compared to reports published for *Saccharomyces cerevisiae* so far. The cocktail δ-integration strategy allows for modulation of multiple gene expression, without the exact knowledge of rate-limiting steps, and therefore, could be used as a promising strategy for the production of bio-based chemicals in recombinant *S. cerevisiae*.

1. Introduction

The use of renewable feedstock such as cellulose and hemicellulose, and their constituent sugars glucose and xylose, for the production of bio-based chemicals by engineering metabolic pathways in various kinds of microorganisms has recently gained prominence because of the environmental problems associated with the combustion of fossil fuels (Jullesson et al., 2015). Among the host microorganisms used for metabolic engineering, the yeast *Saccharomyces cerevisiae* is particularly attractive due to its safety and convenience of use. *S. cerevisiae* is nonpathogenic, classified as a "generally regarded as safe" organism, and has long been used to produce organic compounds like ethanol (Ostergaard et al.,

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2000). Thus, the fermentation and processing technology for large-scale production is well established for *S. cerevisiae*.

2,3-Butanediol is one of the most useful metabolites produced by microorganisms, as both the compound and its derivatives can be used as fuels, resins, solvents, food additives, cosmetics, drugs, and fumigants (Bialkowska, 2016; Molitor et al., 2016). Thus, it has several applications in the chemical, food, cosmetic, and pharmaceutical industry, as well as in agriculture (Kim et al., 2016). The most efficient 2,3-butanediol-producing bacteria are *Klebsiella pneumoniae* and *Klebsiella oxytoca* (Bialkowska, 2016; Kumar et al., 2016). However, these bacteria are pathogenic, thereby restricting large-scale production because of strict safety regulations (Bialkowska, 2016; Celinska and Grajek, 2009). Although, alternative non-pathogenic 2,3-butanediol producers such as *Bacillus subtilis* (Biswas et al., 2012; Fu et al., 2016), *Bacillus amyloliquefaciens* (Sikora et al., 2016; Yang et al., 2011), and *Lactococcus lactis* (Gaspar et al., 2011; Kandasamy et al., 2016) have also been reported, the productivity does not fulfil industrial requirements. Thus, there is a need to search for an efficient non-pathogenic microorganism with the ability to produce 2,3-butanediol.

Non-pathogenic *S. cerevisiae* is a promising host microorganism that could be used for the production of 2,3-butanediol. There have been several reports about the production of the organic compound with metabolically engineered *S. cerevisiae* harboring the bacterial 2,3-butanediol biosynthetic pathway (Fig. 1) (Kim et al., 2013, 2016, 2017; Kim and Hahn, 2015; Ng et al., 2012). Expression of a *B. subtilis* α-acetolactate synthase and α-acetolactate decarboxylase and over-expression of the endogenous 2,3-butanediol dehydrogenase could lead to the conversion of pyruvate into optically pure (2R,3R)-butanediol by the Pdc-deficient *S. cerevisiae* (Kim et al., 2013). High yielding production, however, causes a redox imbalance (Kim and Hahn, 2015). This is due to the production of an extra molecule of NADH in a chemical reaction, where one molecule of glucose is converted into one molecule of 2,3-butanediol (Fig. 1). To address this issue, NADH oxidase from *L. lactis* was co-expressed along with the 2,3-butanediol producing enzymes in *S. cerevisiae* (Kim and Hahn, 2015; Kim et al., 2016). However, the productivity in this metabolically engineered *S. cerevisiae* is still low when compared to that obtained using the aforementioned pathogenic bacteria (Kim et al., 2012; Ma et al., 2009).

Recently, the cocktail δ-integration strategy has been developed, which involves the simultaneous integration of various multi-copy genes via δ-integration, followed by the selection of desirable transformants in *S. cerevisiae*. Using this strategy, expression of genes encoding cellulase (Yamada et al., 2010a,2011; Liu et al., 2017), those related to glycolysis (Yamada et al., 2017a), and genes involved in lactate production (Yamada et al., 2017b), and xylose assimilation (Kato et al., 2013) in *S. cerevisiae* were optimized. The strategy allows for modulation of more than ten different kinds of gene expression simultaneously, without the exact knowledge of the rate-limiting steps. Hence, this strategy could be useful for optimizing complicated metabolic pathway including the redox imbalance issue.

In this study, genes encoding α-acetolactate synthase from *B. subtilis* (*BsAlsS*), α-acetolactate decarboxylase (*BaAlsD*) and butanediol dehydrogenase (*BaBdhA*) from *B. amyloliquefaciens*, and NADH oxidase from *L. lactis* (*LlNoxE*) were expressed in *S. cerevisiae* deficient in genes that code for alcohol dehydrogenase (*ADH1*), pyruvate decarboxylase (*PDC1*), and glycerol-3-phosphate dehydrogenase (*GPD1*) using the cocktail δ-integration strategy. Fed-batch cultivation was then carried out to produce 2,3-butanediol using the modified *S. cerevisiae* strain.

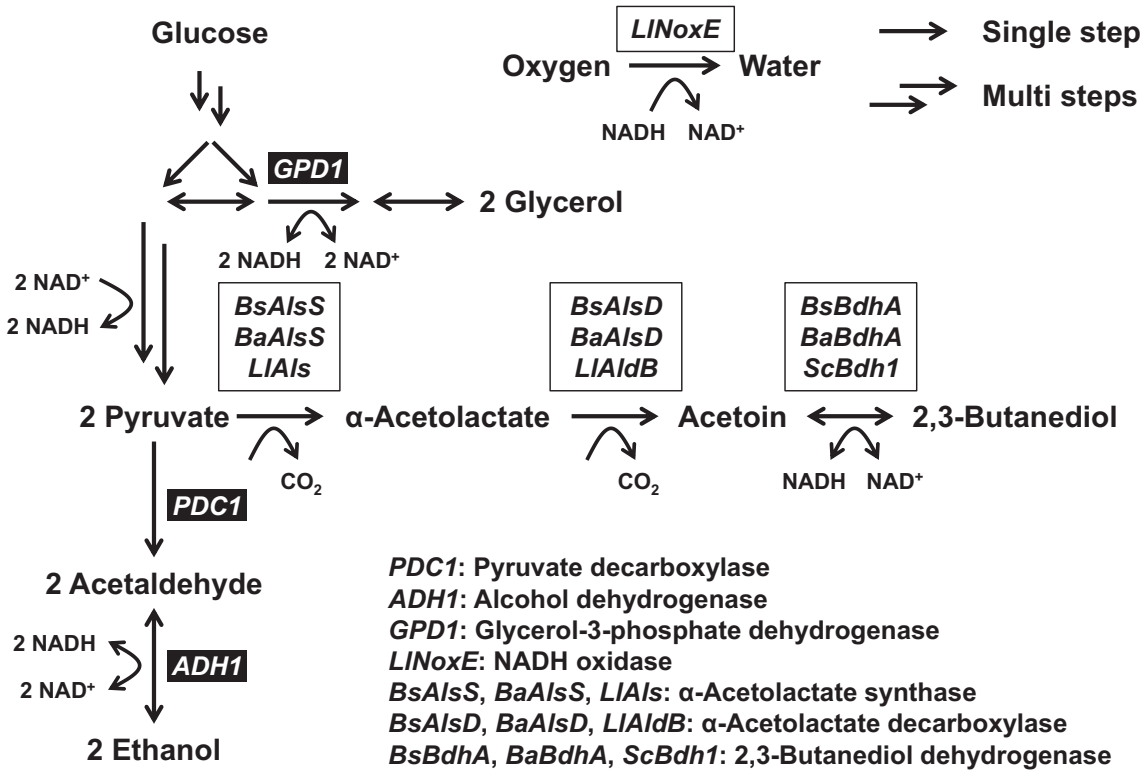

Fig. 1. Metabolic pathway for 2,3-butanediol production. Directional arrows and bidirectional arrows represent irreversible and reversible reactions, respectively. Genes indicated in the white boxes were overexpressed, and genes in the black boxes were deleted in this study.

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2. Materials and methods

2.1. Strains and media

Table 1 summarizes the relevant features of *S. cerevisiae* strains used in this study. The strain *S. cerevisiae* YPH499 (NBRC 10505) was used as a host for metabolic engineering. Yeast cells were cultivated in the following media supplemented with appropriate amino acids and nucleic acids: yeast/peptone/dextrose (YPD) medium (10 g/L yeast extract [Formedium, Norfolk, UK], 20 g/L peptone [Formedium], and 20 g/L glucose), YPD100 medium (10 g/L yeast extract, 20 g/L peptone, and 100 g/L glucose), synthetic dextrose (SD) medium (6.7 g/L yeast nitrogen base without amino acids [Formedium] and 20 g/L glucose), or synthetic complete drop-out (SC) medium (6.7 g/L yeast nitrogen base without amino acids, 20 g/L glucose, and 2 g/L SC multiple drop-outs [DSCK1028, Formedium]).

2.2. Yeast cultivation

Microplate cultivation was performed in 1.0 mL of SD or YPD medium, using a 2-mL 96-well deep-well plate, equipped with a gas permeable seal and a rotary plate shaker set to 30 °C and 1500 rpm. Fresh culture was mixed with (5% [vol/vol]) preculture grown in a microplate (1.0 mL of SD or YPD medium) for 48 h at 30 °C and 1500 rpm.

Batch flask cultivation was performed in 150 mL of SC or YPD medium in a 500-mL flask with baffles and maintained in a rotary shaker set to 30 °C and 150 rpm. Cultivation was begun by inoculating (initial OD_{600}, 0.05) it with a preculture grown in a test tube for 48 h at 30 °C and 150 rpm in SC or YPD medium

Fed-batch flask cultivation was performed in 70 mL of YPD100 medium in a 500-mL flask with baffles and maintained in a rotary shaker set to 30 °C and 150 rpm. The precultured cells, grown in a flask containing 70 mL of YPD medium for 24 h at 30 °C and 150 rpm, were collected by centrifugation (10,000*g* for 2 min at 4 °C), and the fed-batch flask cultivation was initiated by suspending the collected cells in fresh YPD100 medium (initial OD_{600}, 5.0). After glucose was depleted in the medium, 700 g/L glucose was fed to the medium up to a final glucose concentration of 100 g/L. The glucose concentration in the culture supernatant was measured every 1 or 2 h throughout the 5 to 15 h timeframe of each cultivation phase.

2.3. Gene deletion and detection in yeast

GPD1, the key enzyme of glycerol synthesis (Remize et al., 2003) in YPH499/dPdA, was deleted as per the method described by Yamada et al. (2017a). All the primers used in this study are summarized in Supplementary Table 1. *LlNoxE* detection was performed via yeast colony-directed PCR (Akada et al., 2000), using the primers noxE_(F) and noxE_(R).

2.4. Plasmid construction and yeast transformation

The plasmids used in this study were constructed as described in the Supplementary Material. The episomal plasmids were transformed into *S. cerevisiae*, following the lithium acetate method (Chen et al., 1992). For the cocktail δ-integration of plasmid libraries, identical moles of δ-integrative plasmid libraries were linearized by using restriction enzyme AscI before transformation. Transformants were separated on SD medium containing 20 g/L agar and appropriate amino acids and nucleic acids.

2.5. Analysis of growth and metabolites

The OD_{600}, yeast dry cell weight, and glucose, ethanol, and glycerol concentrations of each culture broth were measured as described previously (Yamada et al., 2017a). The carbon yield was calculated using the conversion coefficient equal to 0.47 g-carbon/g-dry cell weight (Xie et al., 2006). The acetoin concentration was determined following the previously described colorimetric method (Westerfield, 1945), or the high performance liquid chromatography (HPLC) method (Yamada et al., 2017a). The 2,3-butanediol concentration was also determined by HPLC

Table 1
S. cerevisiae strains and plasmids used in this study.

Strains and plasmids	Relevant features	Reference
Saccharomyces cerevisiae strain		
YPH499	*MAT*a *ura3-52 lys2-801_amber ade2-101_ochre trp1-Δ63 his3-Δ200 leu2-Δ1*	NITE Biological Resource Center
YPH499/dPdA	YPH499 Δ*PDC1*/Δ*ADH1*	Yamada et al., 2017a
YPH499/dPdAdG	YPH499 Δ*PDC1*/Δ*ADH1*/Δ*GPD1*	This study
YPH499/dPdA/BD1-2	YPH499/dPdA transformed with pEWPG-BsAlsS and pEUPG-BaAlsD	This study
YPH499/dPdA/BD1-2-2	YPH499/dWBD1-2 transformed with pEHPG-BaBdhA	This study
YPH499/dPdA/BD53	YPH499/dPdA cocktail δ-integration of pδU_LibBsAlsS, pδU_LibBaAlsD, and pδU_LibBaBdhA	This study
YPH499/dPdAdG/BD6-6	YPH499/dPdA cocktail δ-integration of pδU_LibBsAlsS, pδU_LibBaAlsD, and pδU_LibBaBdhA	This study
YPH499/dPdAdG/BDN6-10	YPH499/dPdAdG cocktail δ-integration of pδU_LibBsAlsS, pδU_LibBaAlsD, pδU_LibBaBdhA, and pδU_LibLlNoxE	This study
Plasmid and plasmid libraly		
pδU	δ-Integrative vacant plasmid with *URA3* as selection marker	Yamada et al., 2010b
pRS403	Integrative vacant plasmid with *HIS3* as selection marker	Stratagene, CA, USA
pRS404	Integrative vacant plasmid with *TRP1* as selection marker	Stratagene, CA, USA
pRS406	Integrative vacant plasmid with *URA3* as selection marker	Stratagene, CA, USA
pEHPG	pRS403 harboring PGK1 promoter and PGK1 terminator and 2 μ ori	This study
pEWPG	pRS404 harboring PGK1 promoter and PGK1 terminator and 2 μ ori	This study
pEUPG	pRS406 harboring PGK1 promoter and PGK1 terminator and 2 μ ori	This study
pEWPG-BsAlsS	pEWPG harboring *BsAlsS*	This study
pEUPG-BaAlsD	pEUPG harboring *BaAlsD*	This study
pEHPG-BaBdhA	pEHPG harboring *BaBdhA*	This study
pδU_LibBsAlsS	Plasmid libraly for expression of BsAlsS by 15 kinds of promoters	This study
pδU_LibBaAlsD	Plasmid libraly for expression of BaAlsD by 15 kinds of promoters	This study
pδU_LibBaBdhA	Plasmid libraly for expression of BaBdhA by 15 kinds of promoters	This study
pδU_LibLlNoxE	Plasmid libraly for expression of LlNoxE by 15 kinds of promoters	This study

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(Yamada et al., 2017a). The yield of metabolites was calculated following the previously described equation (Yamada et al., 2017b).

2.6. Real-time PCR quantification

Total RNA was isolated from yeast cells cultivated in SC medium for 38 h at 30 °C, and cDNA was synthesized as previously described (Yamada et al., 2017a). This cDNA was used as a template to quantify the transcription levels of the 2,3-butanediol producing enzymes using real-time PCR as described previously (Yamada et al., 2017a). The primers used are also listed in Supplementary Table 1.

3. Results and discussion

3.1. Screening for high-yielding, 2,3-butanediol-producing strains after δ-integration of BsAlsS, BaAlsD, and BaBdhA genes

There were nine enzymes contributing to the production of 2,3-butanediol tested in this study (Fig. 1 and Supplementary Table 2). Co-expression of three of those genes, *BsAlsS*, *BaAlsD*, and *BaBdhA,* resulted in a superior production of 2,3-butanediol (Supplementary Fig. 1). Thus, to modulate the expression of *BsAlsS*, *BaAlsD*, and *BaBdhA*, three δ-integrative plasmids pδU_LibBsAlsS, pδU_LibBaAlsD, and pδU_LibBaBdhA were co-transformed into strain YPH499/dPdA and the amount of 2,3-butanediol produced in SD medium after 72 h was evaluated.

In 90 transformants, 68% of transformants produced detectable amounts of 2,3-butanediol, and 42% of transformants produced higher amounts of the compound when compared to the control YPH499/dPdA/BD1-2-2 (which expressed *BsAlsS*, *BaAlsD*, and *BaBdhA,* episomally) (data not shown). The strain producing the highest amount of the compound was named YPH499/dPdA/BD53.

3.2. Evaluation of cell growth and metabolite production in the engineered yeast strain YPH499/dPdA/BD53

To determine the culture characteristics, both the modified and control strains were cultivated in flasks and the cell growth and production of metabolites were measured.

The cell growth curve and the produced metabolite concentrations are shown in Fig. 2, and the values are presented in Table 2. The modified strain led to a higher cell growth, had a higher glucose consumption rate, and resulted in higher 2,3-butanediol production rate than in the control yeast. YPH499/dPdA/BD53 produced 5.96 g/L of 2,3-butanediol after 64 h cultivation, and the production rate and yield were 0.09 g/L/h and 30.5%, respectively. The main byproduct obtained was glycerol.

The sum of the carbon yields in YPH499/dPdA/BD1-2-2 was a little lower than that in YPH499/dPdA/BD53 (Table 2). This would be due to the difference of by-products formation performance of the two strains. The host strain YPH499 used in this study can produce and accumulate small amount of organic acids such as pyruvic acid, acetic acid, and succinic acid naturally (Mimitsuka et al., 2015). Those products formation would lead to the lower carbon yields in YPH499/dPdA/BD1-2-2.

Although, *ADH1* and *PDC1* were deleted in both the control YPH499/dPdA/BD1-2-2 and YPH499/dPdA/BD53, only the control strain produced large amounts of ethanol as by-product (Fig. 2 and Table 2). It should be noted that the amount of 2,3-butanediol produced in the control strain was lower than the amount obtained in the modified strain. This would explain the production of ethanol to compensate for the depletion of NAD^+. Although, both *ADH1* and *PDC1* deletion is effective to reduce ethanol production in *S. cerevisiae* (Tokuhiro et al., 2009; Yamada et al., 2017a), the isozymes of alcohol dehydrogenase (e.g. *ADH2*, *ADH3*, *ADH4*, *ADH5*, and *SFA1*) and pyruvate decarboxylase (e.g. *PDC5*) expression could be up-regulated spontaneously to maintain redox balance (Hohmann and Cederberg, 1990; Ida et al., 2012). Thus, the isozymes for ethanol production could be up-regulated leading to ethanol production in the control strain. The major by-product obtained in the modified yeast was glycerol and acetoin. The formation of glycerol is one of the main redox balancing strategies employed by *S. cerevisiae* (Nordström, 1968; Van Dijken and Scheffers, 1986). Hence, deletion of *ADH1* and *PDC1* led to the production of glycerol as the main by-product. To improve the obtained yield of 2,3-butanediol in the modified yeast, glycerol and acetoin production should be reduced.

3.3. Transcription levels of transformants producing 2,3-butanediol

To investigate the reason for a higher yield of the compound, transcription levels of the genes *BsAlsS*, *BaAlsD*, and *BaBdhA* were quantified in both the control and in the modified strain.

As shown in Table 3, the transcription levels of *BsAlsS* and *BaAlsD* was 1.27 and 1.56-fold higher, respectively, in the modified strain when compared to the control strain. In contrast, the *BaBdhA* transcription level was similar between the two strains.

The cocktail δ-integration strategy led to the upregulation of *BsAlsS* and *BaAlsD* genes in the modified strain (Table 3), which resulted in higher production rate of 2,3-butanediol. Hence, the conversion rate of pyruvate to α-acetolactate, catalyzed by *BsAlsS,* and of α-acetolactate into acetoin (catalyzed by *BaAlsD)* must be slower compared to that of acetoin being converted to 2,3-butanediol catalyzed by *BaBdhA* in the control strain. Choi et al. (2016) had also reported that the conversion of α-acetolactate into acetoin catalyzed by α-acetolactate decarboxylase might be the rate limiting step in 2,3-butanediol production in recombinant *S. cerevisiae*. Although, acetoin accumulation was observed in the cocktail δ-integrant strain (Fig. 2), the cocktail δ-integration strategy could be useful. This is because, it allows for modulation of enzyme expression and improvement of target bio-based chemicals productivity without the need for complete knowledge of the rate-limiting steps.

3.4. Screening for strains producing a high yield of 2,3-butanediol after δ-integration of BsAlsS, BaAlsD, and BaBdhA genes and LlNoxE

To reduce the formation of glycerol as byproduct, *LlNoxE* was co-expressed along with *BsAlsS*, *BaAlsD*, and *BaBdhA* genes in the *GPD1* deleted strain YPH499/dPdAdG. Four δ-integrative plasmid libraries, pδU_LibBsAlsS, pδU_LibBaAlsD, pδU_LibBaBdhA, and pδU_LibLlNoxE were co-transformed into YPH499/dPdAdG and the amount of 2,3-butanediol produced was evaluated.

First, 548 transformants were cultivated in a microplate for a long-term of 48 h to induce re-conversion of 2,3-butanediol into acetoin. This was followed by measuring the concentration of acetoin. In 548 transformants, 20.1% of the transformants produced detectable amount of acetoin (data not shown). Of the pool, 93 transformants, which produced more than 0.2 g/L of acetoin in 48 h of cultivation, were further cultivated in a microplate for 18 h and the 2,3-butanediol concentration was measured by HPLC. All 93 transformants produced detectable amount of the compound (data not shown). The strains yielding the highest amount of 2,3-butanediol are YPH499/dPdAdG/BD6-6, YPH499/dPdAdG/BD3-98, YPH499/dPdAdG/BD6-10, YPH499/dPdAdG/BD5-58, YPH499/dPdAdG/BD2-23, YPH499/dPdAdG/BD5-59, YPH499/dPdAdG/BD5-1, and YPH499/dPdAdG/BD2-58; listed in the decreasing order of production of the compound.

Finally, these 8 transformants were cultivated and *LlNoxE* was detected by colony directed PCR. As shown in Fig. 3, YPH499/

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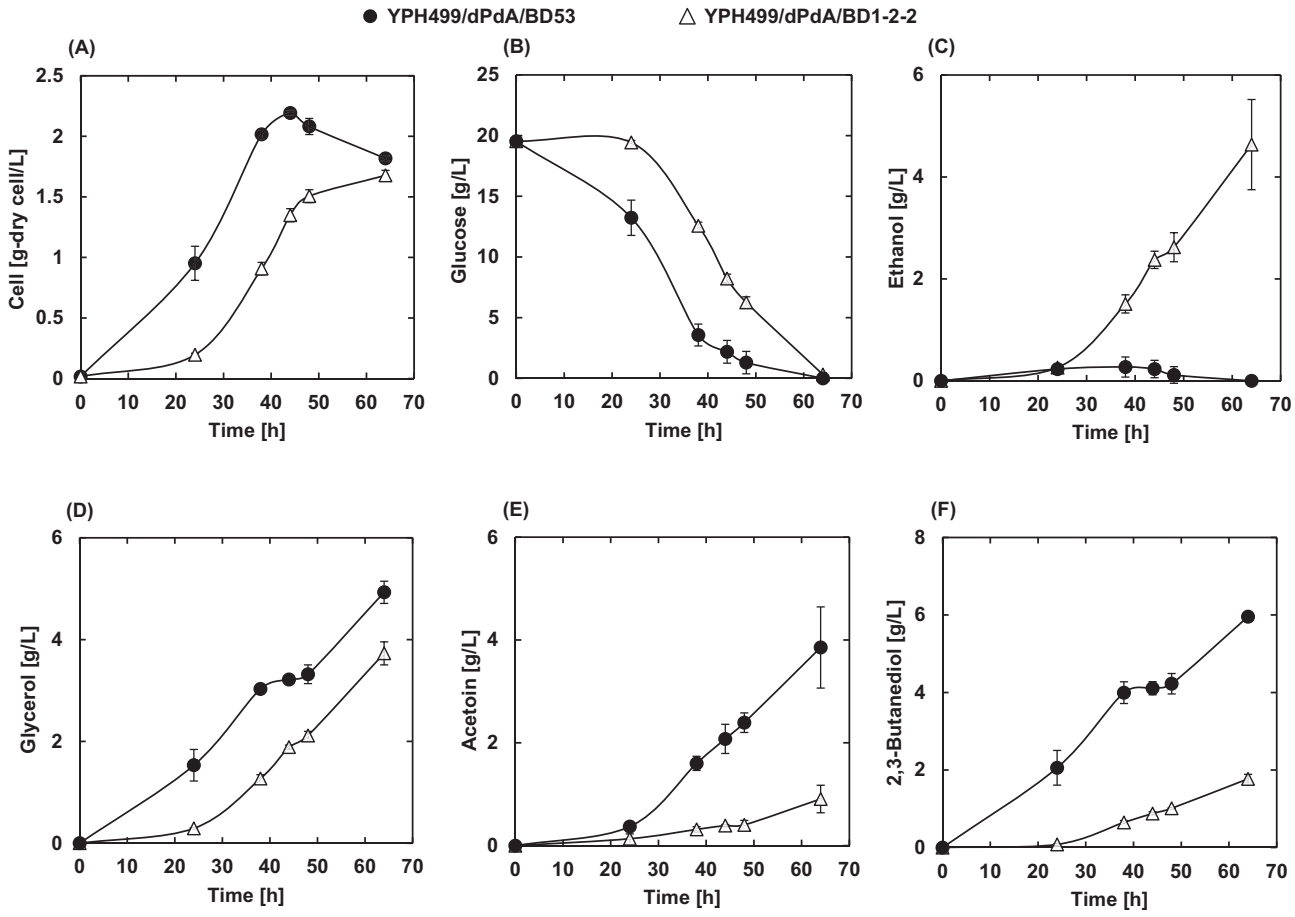

Fig. 2. Time course of metabolite concentrations and dry cell weight of the cocktail δ-integrant strain YPH499/dPdA/BD53 and episomal strain YPH499/dPdA/BD1-2-2. (A) Dry cell weight, (B) glucose concentration, (C) ethanol concentration, (D) glycerol concentration, (E) acetoin concentration, (F) 2,3-butanediol concentration are shown. Data is presented as the average of 3 independent experiments. Error bars represent means ± standard deviation.

Table 2
Properties of 2,3-butanediol producing recombinant *S. cerevisiae* strains.

Strain	Medium	Cultivation duration [h]	2,3-Butanediol production [g/L]	2,3-Butanediol production rate [g/L/h]	Carbon yield to 2,3-butanediol [%]	Carbon yield to glycerol [%]	Carbon yield to acetoin [%]	Carbon yield to ethanol [%]	Carbon yield to cell [%]
YPH499/dPdA/BD1-2-2	SC	64	1.77	0.03	12.3	19.0	6.4	31.4	10.1
YPH499/dPdA/BD53	SC	64	5.96	0.09	40.6	24.7	26.9	0.0	10.8
YPH499/dPdAdG/BD6-6	YPD	40	7.27	0.18	49.5	20.7	2.1	6.1	13.0
YPH499/dPdAdG/BDN6-10	YPD	25	7.08	0.28	47.4	5.3	14.0	2.5	15.3

dPdAdG/BD6-10 led to the maximum amount of 2,3-butanediol when produced in a flask-cultivation for 18 h. All transformants were found to harbor the *LlNoxE* gene, except for YPH499/dPdAdG/BD6-6.

YPH499/dPdA/BD53 was selected from only 90 transformants because of the poor throughput of HPLC analysis based screening. In contrast, 8 transformants expressing 2,3-butanediol producing enzymes and *LlNoxE* were selected from 548 transformants. One of the main limitations of the cocktail δ-integration strategy is the difficulty in generating high-throughput screening method. In this study, by using high-throughput colorimetric analysis based screening, acetoin productivity of many transformants could be analyzed. When glucose is depleted from the media, the recombinant *S. cerevisiae* consumes the produced 2,3-butanediol by a reverse reaction (Kim and Hahn, 2015). And thus, acetoin concentration in the supernatants of long-term cultures is well correlated to the 2,3-butanediol productivity in short-term cultivation (data not shown). The utility of cocktail δ-integration could be enhanced by constructing a high-throughput screening strategy.

Table 3
Transcription levels of *BsAlsS*, *BaAlsD*, and *BaBdhA* in the transformants YPH499/dPdA/BD53 relative to the episomal strain YPH499/dPdA/BD1-2-2.

Gene	Relative transcription level [–]
BsAlsS	1.27 ± 0.07*
BaAlsD	1.56 ± 0.09**
BaBdhA	1.06 ± 0.20

Data is presented as the average of 3 independent experiments. Error bars represent means ± standard deviation. Significant differences were calculated using Student's *t*-test (**; $p < 0.01$, *; $p < 0.05$).

The amounts of 2,3-butanediol produced in the microplate cultivation method and the flask cultivation method did not match

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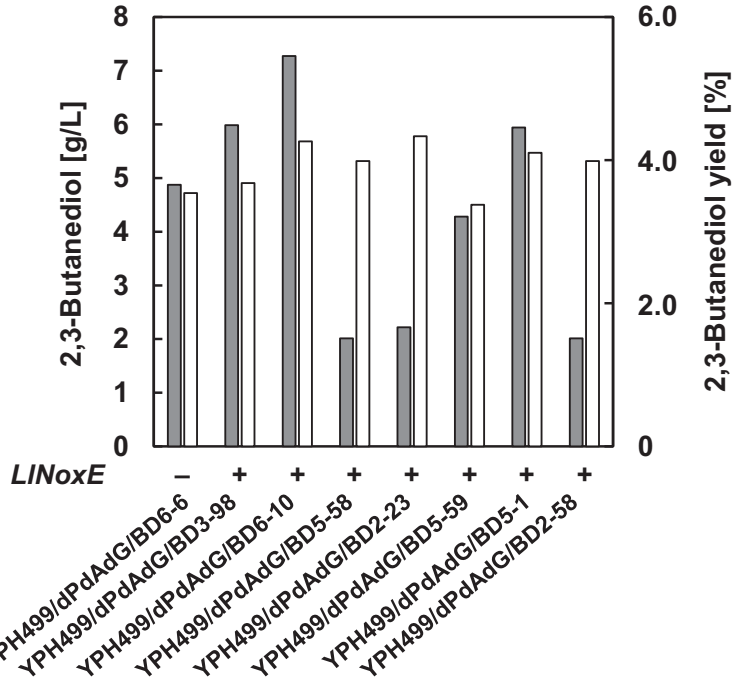

Fig. 3. 2,3-Butanediol and acetoin production in flask cultivation after 18 h by cocktail δ-integrant transformants with *BsAlsS*, *BaAlsD*, *BaBdhA*, and *LiNoxE*. Gray bars, 2,3-butanediol concentration; white bars, 2,3-butanediol yield.

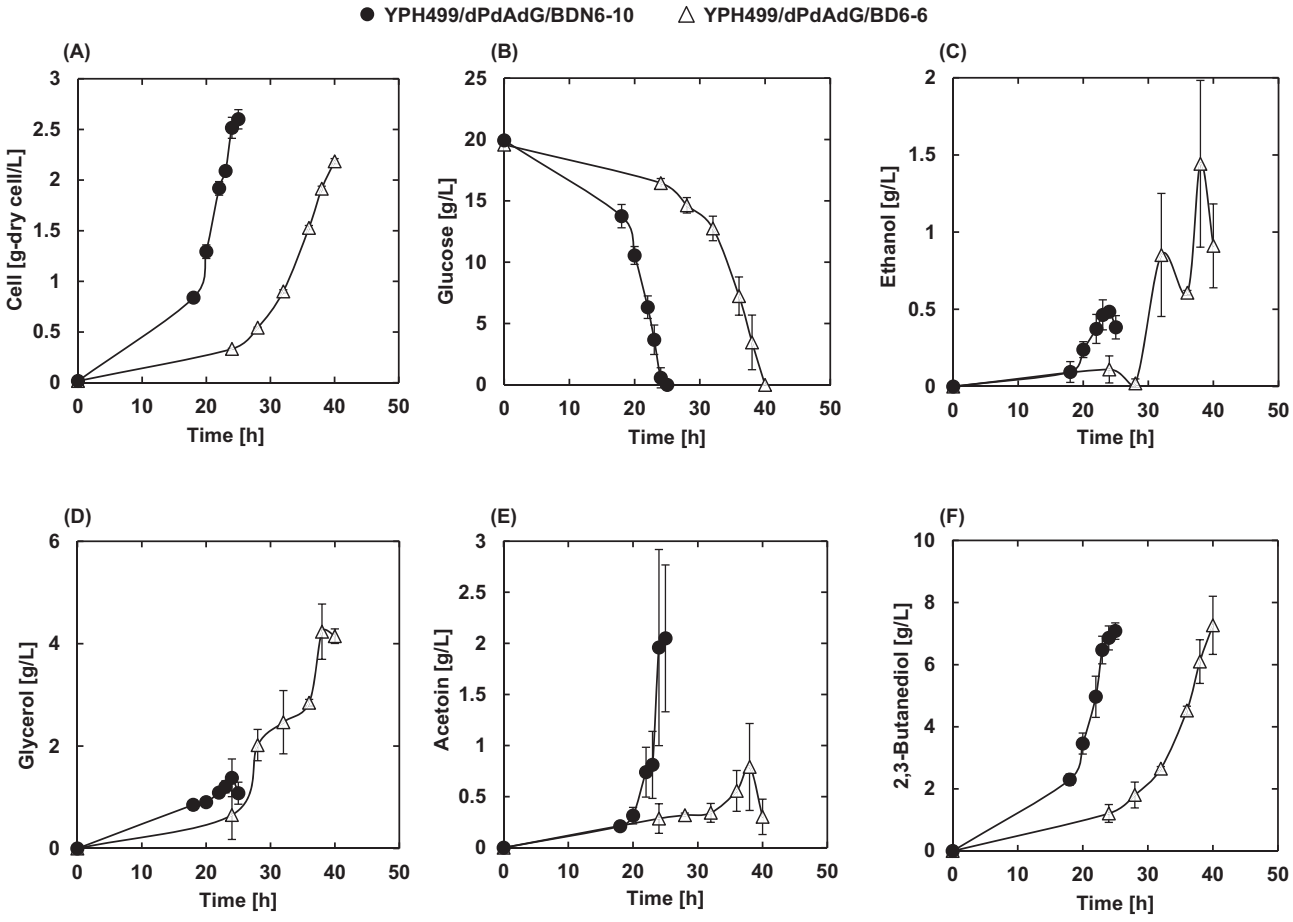

Fig. 4. Time course of metabolite concentrations and dry cell weight of the cocktail δ-integrant strain YPH499/dPdAdG/BD6-6 and YPH499/dPdAdG/BDN6-10. (A) Dry cell weight (B) glucose concentration, (C) ethanol concentration, (D) glycerol concentration, (E) acetoin concentration, (F) 2,3-butanediol concentration are shown. Data is presented as the average of 3 independent experiments. Error bars represent means ± standard deviation.

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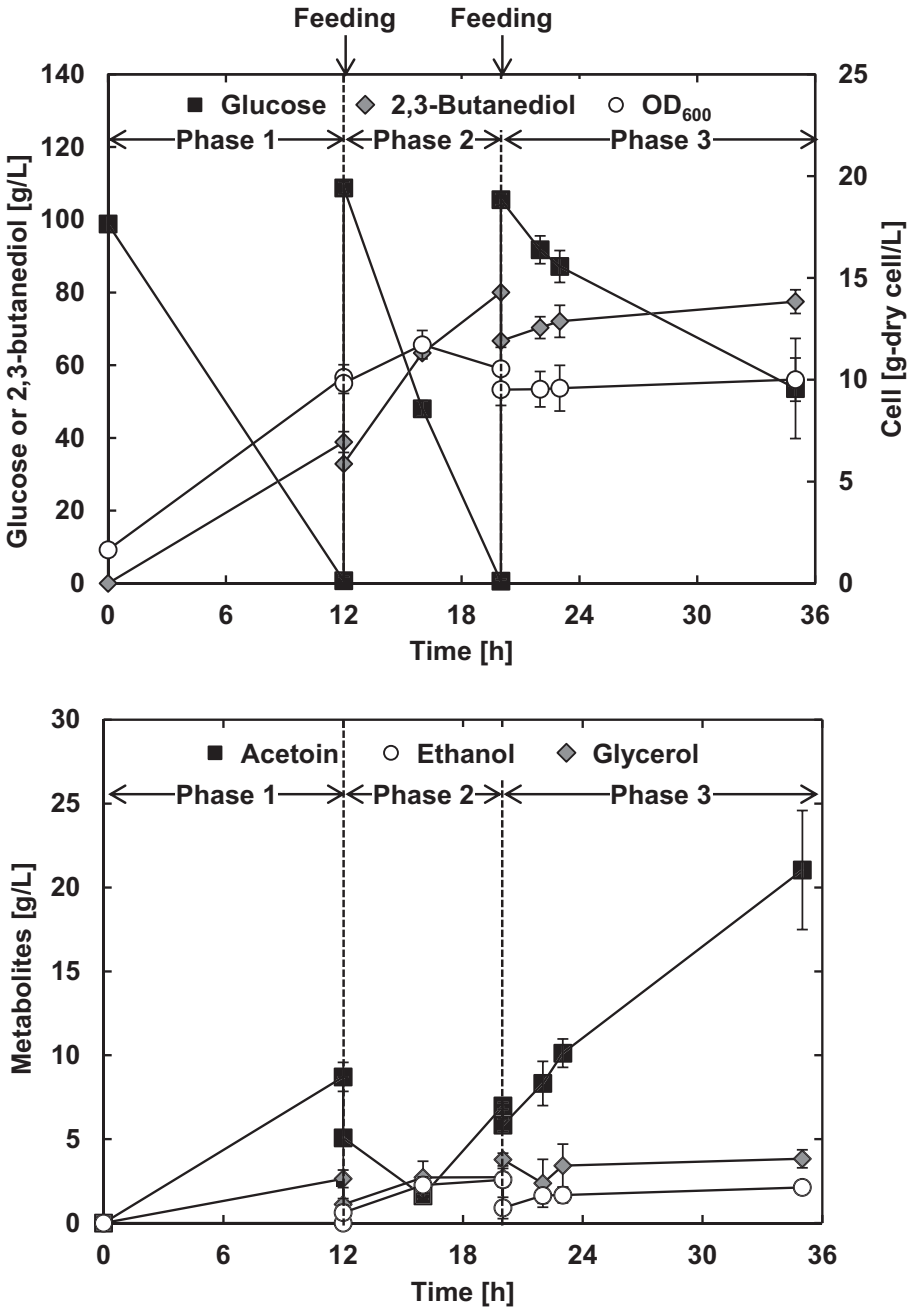

Fig. 5. Time course of metabolite concentrations and dry cell weight of the cocktail δ-integrant strain YPH499/dPdAdG/BDN6-10 in fed-batch cultivation. The dashed lines indicate glucose feeding points. Data is presented as the average of 3 independent experiments. Error bars represent means ± standard deviation.

Table 4
Fed-batch cultivation of the yeast strain YPH499/dPdAdG/BD6-10.

Phase	Cultivation duration [h]	2,3-Butanediol [g/L]	2,3-Butanediol production rate [g/L/h]	Carbon yield to 2,3-butanediol [%]	Carbon yield to glycerol [%]	Carbon yield to acetoin [%]	Carbon yield to ethanol [%]	Carbon yield to cell [%]
1	12	38.9	3.24	53.0	2.6	12.1	0.0	10.1
2	8	47.1	5.89	58.1	1.5	2.4	2.4	0.8
3	15	10.8	0.72	27.7	0.1	39.9	3.1	1.2
1–2	20	80.0	4.00	55.6	1.4	5.0	1.8	5.1
1–3	35	77.5	2.21	49.2	1.8	13.7	1.3	3.8

(Fig. 3). This is because the analysis for the compound was carried out only at the end, by which time, the produced 2,3-butanediol might have been re-converted into acetoin by some transformants because of glucose depletion. Besides, the expression of *LlNoxE* may affect the mismatch in the values obtained during the two cultivation methods. *LlNoxE* catalyzes the conversion of oxygen into water and is associated with the conversion of NADH to NAD^+ (Fig. 1). Thus, the concentration of dissolved oxygen affects redox balance.

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The difference of oxygen transfer coefficient between the microplate cultivation and the flask cultivation could also contribute to the difference in the amount of compound produced. A screening strategy for an industrial-scale cultivation needs to be developed and would be the focus of future works.

Interestingly, the top 7 of the 8 largest 2,3-butanediol- producing transformants, selected by microplate cultivation were harboring the *LlNoxE* gene. As mentioned above, although the activity of *LlNoxE* is affected by the mode of cultivation, expression of *LlNoxE* would have a positive effect on 2,3-butanediol production in most transformants.

3.5. Evaluation of cell growth and metabolite production in the modified yeast strain YPH499/dPdAdG/BD6-10

To characterize the culture properties, the modified strain and the *LlNoxE* non-expressing control strain YPH499/dPdAdG/BD6-6 were cultivated in flask and the cell growth and production of metabolites were evaluated.

Cell growth and metabolite concentrations are shown in Fig. 4, and the values are presented in Table 2. The modified strain demonstrated a higher cell growth and a higher glucose consumption rate when compared to that by the control. It produced 7.08 g/L of 2,3-butanediol after 25 h cultivation, and the production rate and yield were 0.28 g/L/h and 35.5%, respectively. The main byproducts obtained were acetoin and glycerol. In contrast, YPH499/dPdAdG/BD6-6 produced 7.27 g/L of 2,3-butanediol after 40 h cultivation, and the production rate and yield were 0.18 g/L/h and 37.1%, respectively. The main byproducts obtained in the control strain were glycerol and ethanol.

Although YPH499/dPdAdG/BD6-10 expressing *LlNoxE* produced lower amount of glycerol as by-product than the control strain, both *GPD1* deleted strains produced glycerol (Table 2). *GPD1* is major glycerol 3-phosphate dehydrogenase, and an isozyme *GPD2* exists in *S. cerevisiae* (Eriksson et al., 1995). Deletion of *GPD2* did not lead to any observable phenotype in wild type strain, whereas it was responsible for glycerol formation in the *GPD1* deleted strain (Eriksson et al., 1995; Michnick et al., 1997). Furthermore, double deletion of *GPD1* and *GPD2* hampered cell growth strongly (Hubmann et al., 2011). To further modulate redox balance in *GPD1* deleted strain, the by-product glycerol yield needs to be reduced, which would lead, in turn, to an increase in the yield of 2,3-butanediol.

Although, expression of several genes would be modulated in YPH499/dPdAdG/BD6-10, expression of *LlNoxE* in YPH499/dPdAdG/BD6-10 could cause crucial difference of the 2,3-butanediol production and also contribute to differences observed in other properties between the control and the modified YPH499/dPdAdG/BD6-10 strain (Fig. 4). Especially, YPH499/BDN6-10 showed rapid cell growth in latter cultivation period. In previous reports, redox balance has been shown to affect many culture characteristics, such as sugar consumption rate and biomass and fermentation products formation (Heux et al., 2006; Van Dijken and Scheffers, 1986). Thus, modulating the expression of 2,3-butanediol producing enzymes and *LlNoxE* is a challenging task. The results show that the cocktail δ-integration strategy could be used to modulate complicated enzyme expression including redox reaction.

3.6. Fed-batch cultivation of the modified strain YPH499/dPdAdG/BD6-10

To further investigate the application of modified YPH499/dPdAdG/BD6-10, fed-batch culture was carried out using 300 g/L of glucose with a 2-time feeding regime.

As shown in Fig. 5 and Table 4, glucose was consumed and 2,3-butanediol was produced steadily at phase 1 and 2. The concentration, production rate, and yield through phase 1–2 were 80.0 g/L, 4.00 g/L/h, and 41.7%, respectively. In contrast, at phase 3, the concentration, production rate, and yield were drastically decreased to 10.8 g/L, 0.72 g/L/h and 20.8% respectively.

The results show that efficient 2,3-butanediol production was achieved thorough fed-batch cultivation when using the strain YPH499/dPdAdG/BD6-10. There are dozens of reports about the 2,3-butanediol production by bacteria (Bialkowska, 2016; Ma et al., 2009) and recombinant *S. cerevisiae* (Kim et al., 2016, 2017). Although, there are some reports of comparable amounts of the compound being produced by recombinant *S. cerevisiae* and pathogenic bacteria, the 2,3-butanediol production rate of the former were lower than the value achieved by the latter. This study indicated the highest level of 2,3-butanediol production rate, so far in all microorganisms, including pathogenic bacteria and recombinant *S. cerevisiae*.

Although cocktail δ-integration strategy led to the highest yield of 2,3-butanediol among all recombinant *S. cerevisiae*, the yield was lower than that obtained using pathogenic bacteria. The major byproducts in the fed-batch culture (phase 1–2) in this study were acetoin and biomass. There are 2 innate butanediol dehydrogenase genes (*BDH1* and *BDH2*) in *S. cerevisiae*. Although BDH2 is inactive toward 2,3-butanediol, BDH1 reversibly transforms acetoin into 2,3-butanediol (Gonzalez et al., 2010). Thus, deletion of innate *BDH1* may contribute to improve the yield of 2,3-butanediol. Additionally, repeated cocktail δ-integration strategy could be used to further modulate the gene expression in an attempt to increase the yield of 2,3-butanediol.

Of note, although, Kim et al. (2016) reported large amount of 2,3-butanediol production by recombinant *S. cerevisiae*, the engineered strain constructed in this study could not produce over 80.0 g/L of 2,3-butanediol (Fig. 5). This would be because the low tolerance of the host *S. cerevisiae* strains for the compound. Indeed, engineered strain YPH499/dPdAdG/BD6-10 could not grow on YPD medium containing over 80 g/L of 2,3-BDO (data not shown). By improving the tolerance of *S. cerevisiae* or by using cocktail δ-integration strategy in more robust strains, more efficient 2,3-butanediol production could be achieved. Additionally, the evaluation of 2,3-butanediol production in well-controlled large scale cultivation and process optimization should also be required for an industrial application of an engineered strain (Dheskali et al., 2017).

4. Conclusion

To conclude, the cocktail δ-integration strategy, which allowed the modulation of enzyme expression without the exact knowledge of rate-limiting steps, was applied for the production of 2,3-butanediol. The resultant engineered strain YPH499/dPdAdG/BD6-10 expressing *BsAlsS*, *BaAlsD*, *BaBdhA*, and *LlNoxE* had the highest level of 2,3-butanediol production rate and yield in fed-batch cultivation reported so far. The cocktail δ-integration strategy could be promising for constructing metabolically engineered *S. cerevisiae* capable of producing bio-based chemicals.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2017.05.034.

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