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Engineering *Saccharomyces cerevisiae* and Controlling Conditions for 2,3-Butanediol Production from Glycerol

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2,3-Butanediol (2,3-BDO) has gained much attention due to its bulk chemical uses in numerous applications such as the production of pharmaceuticals, cosmetics, synthetic rubber, inks, resins, perfumes, foodstuffs, fuel additives, and aviation fuel. Additionally, 2,3-BDO can be used as an antifreeze, enhancer of plant drought resistance, plant growth stimulator, and plant pest-virus control agent. Engineered *Saccharomyces cerevisiae* has exhibited superb potential for converting glucose to 2,3-BDO, but it lacks the native capability to do so from glycerol. Glycerol, an affordable by-product of biodiesel, is a green solvent with high permittivity and low inhibitory effects, making it a cost-effective option for lignocellulose biorefinery. Therefore, we aimed to further engineer and expand substrate utilization to glycerol or co-utilization of glycerol with glucose for 2,3-BDO production using the engineered *S. cerevisiae* SK-FGG4 strain, which was previously engineered to convert glycerol to ethanol. The 2,3-BDO biosynthesis pathway, consisting of codon-optimized *Bacillus subtilis* acetolactate synthase (*BsAlsS*), acetolactate decarboxylase (*BsAlsD*), and *S. cerevisiae* butanediol dehydrogenase (*ScBDH*), was assembled into one module and integrated into the genomic DNA of SK-FGG4, generating the SK-MA1 strain. Interestingly, SK-MA1 completely shifted glycerol conversion under microaerobic conditions from ethanol to a mixture of acetoin and 2,3-BDO at 0.43 g acetoin+2,3-BDO/g glycerol, with 2,3-BDO accounting for 0.28 g 2,3-BDO/g glycerol. The SK-MA2, SK-MA3, and SK-MA4 strains, which were generated using CRISPR by replacing alcohol dehydrogenase *ScADH1*, *ScADH2*, and *ScADH5* enzymes individually with the 2,3-BDO biosynthesis enzymes, exhibited enhanced conversion parameters on a complex medium under aerobic conditions compared to the SK-MA1 strain. Double replacement of *ScADH1* and *ScADH5* with 2,3-BDO biosynthesis enzymes in the SK-MA7 strain promoted higher accumulation of 2,3-BDO over other strains in aerobic conditions, with total conversion reaching 0.41 g acetoin+2,3-BDO/g glucose+glycerol, which is over 3.2-fold higher than the reference SK-MA6 strain, a native strain (D452-2) with *ScADH5* replaced with 2,3-BDO biosynthesis enzymes. Under these aerobic conditions, acetoin was over 2-fold of the 2,3-BDO. Lowering the aeration to microaerobic levels decreased acetoin accumulation and increased the production of 2,3-BDO. Strain SK-MA3 produced 0.34 g 2,3-BDO/g glucose+glycerol with a total conversion of 0.455 g acetoin+2,3-BDO/g glucose+glycerol, representing 92% of the theoretical yield at 96 hours. This study is the first to explore the conversion of glycerol to 2,3-BDO using engineered *S. cerevisiae* and supports broadening glycerol-based biorefinery approaches.

1. Introduction

There is a growing emphasis on advancing biorefinery technologies that utilize renewable resources for biofuel and bio-based chemical production to reduce petroleum dependence and slow global warming. Plant oil industries such as that of biodiesel generate considerable amounts of glycerol.¹ As a green organic solvent, glycerol was used to develop efficient and eco-friendly lignocellulosic biorefineries for enhanced dissociation of antiviral glycerolysis-lignin and biomass polysaccharide components during glycerolysis pretreatment.² Besides its renewability from plant oil industries, the high permittivity, nontoxicity, stability, and affordability of glycerol make it an advantageous candidate for promoting efficient processing and cost-effective biorefineries.^{3, 4}

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diverting the glycerol metabolic flux to 2,3-BDO in a novel set of engineered *S. cerevisiae* derived from SK-FGG4. These strains express *BsAlsS* and *BsAlsD*, while also overexpressing *ScBDH* without any deficiencies in *ScPDCs*. Consequently, the engineered strains were able to convert glycerol or co-convert it with glucose to produce 2,3-BDO. (Fig. 1). This is the first study converting glycerol to 2,3-BDO using a microbial cell factory, *S. cerevisiae*, and presents the foundation for further metabolic engineering to enhance 2,3-BDO production from glycerol.

2. Materials and methods

2.1 Media used

In this study, several media were used; YPD medium (10 g containing yeast extract; 20 g peptone; 20 g glucose, per liter) was utilized for either preserving or cultivating the yeast strains. Agar was used to solidify the media at 18 g/liter when preparing agar plate media. A selective medium for positive integration of *ScHIS3* (imidazoleglycerol-phosphate dehydratase) was prepared by supplementing with 6.78 g/liter Yeast Nitrogen Base without amino acids (YNB) in 20 g/liter glucose, and 20 mg/liter of leucine (YNB_{D20}) media, as reported in the literature.²⁹ YPD medium enriched with geneticin (G418), referred to as YPD_{G418}, was employed as a selective medium using the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) system. In the case of fermenting glycerol as the sole carbon source, YNB was supplemented with 40 g/L glycerol alongside 0.5 g/L leucine (referred to as YNB_{G40}). For fermentation on a complex medium, 40 g/L glycerol was replaced with the glucose in the YPD to prepare YP_{G40}. Also, the co-fermentation medium was prepared by supplementing with 40 g/L glycerol in YPD medium, preparing YP_{D20G40}. To avoid interference of any degradation products during autoclaving, all liquid media utilized in this study was filter sterilized (TPP vacuum filtration, "rapid-Filtermax", 0.2µm PES, Switzerland). Glycerol, glucose, and amino acids were also filtered before adding to the agar media. Luria-Bertani (LB) medium, which is composed of 1% tryptone, 1% sodium chloride (NaCl), and 0.5% yeast extract, was used for cultivating *Escherichia coli*. Ampicillin was supplemented to the LB medium at 150 µg/mL to select for *E. coli* carrying ampicillin resistance plasmids, whereas Kanamycin was used at 75 µg/mL for selecting and cultivating *E. coli* harboring the pCas9 multiplex plasmid.³⁰

2.2 Constructing 2,3-BDO modules and their harboring plasmids

The primers utilized in this investigation were designed using the sequences accessible in the S288C *Saccharomyces* Genome Database, which are itemized in Table S1. The 2,3-BDO module, the complete sequence of which is noted in Table S2, was assembled from 3 cassettes (9 fragments). First, every single fragment was generated by PCR using high-fidelity polymerase KOD-plus neo (Toyobo, Osaka, Japan). PCR products were highly purified through a 2% agarose gel and the FastGene Gel/PCR extraction kit (Nippon Genetics, Tokyo, Japan). *Bacillus subtilis* acetolactate synthase (*BsAlsS*) and acetolactate decarboxylase (*BsAlsD*) were codon-optimized and purchased from Twist Bioscience, Japan. The mutated *DIT1_{d22}* terminator was obtained by PCR from the M1 plasmid used

previously by authors.²⁹ The rest of the module fragments were obtained from the genomic DNA of the native strain, D452-2³¹ using related primers listed in Table S1.

To construct the *BsAlsS* cassette, the homologous region (HR) upstream of *ScADH1* was added to the *TDH3* promoter by PCR, and then cleaved by *NotI*. The terminator *DIT1_{d22}*, which is immediately followed by *PGKp*, was also generated by PCR and cleaved by *BamHI*. The *BsAlsS* gene was amplified by PCR and cleaved by *NotI/BamHI* and ligated between cleaved HR-*TDH3p-NotI* and *BamHI-DIT1_{d22}* upstream of *PGKp*. After a 15 min. ligation, the *BsAlsS* cassette, flanked by *ScADH1*-HR and the upstream region of *PGKp* (Fig. S2), was amplified by PCR.

BsAlsD was similarly constructed; a sequence of conjoined *DIT1_{d22}* and *PGKp* was generated by PCR using primers listed in Table S1, and the fragment was cleaved by *NotI*. Similarly, a fragment of terminator *DIT1_{d22}* followed by *ScFABp* was prepared by PCR and cleaved by *BamHI*. After that, *BsAlsD* which had been cleaved by *NotI/BamHI* was ligated between the promoter and the terminator, and the ligation mixture was used as a template for generating the *BsAlsD* cassette (Fig. S2). In parallel, the *ScBDH* cassette was generated as follows: a downstream sequence of *DIT1_{d22}* connected to the *ScFBA* promoter was generated by PCR and the *ScRPL41BT* terminator was amplified with the HR region downstream of *ScADH1*. Then the generated fragments were cleaved by *NotI* and *BamHI*. The *ScBDH* fragment that was amplified by PCR was cleaved by *NotI* and *BamHI* and ligated, generating the third *ScBDH* cassette.

The three cassettes were further purified on an agarose gel then the *BsAlsD* cassette was inserted before the *ScBDH* cassette through overlapping regions with the PCR and primers shown in Table S1. The connected *BsAlsD-ScBDH* sequence was purified before connection with the *BsAlsS* cassette by PCR to generate the whole module as depicted in Fig. S2. The completed 2,3-BDO synthesis pathway fragment digested with *XhoI/SalI* and cloned into a truncated and dephosphorylated *XhoI/SalI* *HIS3* plasmid to generate the pBDO/ADH1-*HIS3* plasmid (Table 2 and Fig. S2). Changing the HR from *ScADH1* to *ScADH2* or *ScADH5* was carried out by PCR and the primers are listed in Table S1. These variants were re-cloned into a truncated and dephosphorylated *XhoI/SalI* *HIS3* plasmid to generate the pBDO/ADH2-*HIS3* and pBDO/ADH5-*HIS3* plasmids (Table 2), respectively. The plasmids were then transformed into NEB 10-beta *Escherichia coli* (New England BioLabs, Tokyo, Japan) via the heat shock method for storage and further experimentation, following the manufacturer's instructions. After culturing overnight in LB broth, the plasmids were extracted from the *E. coli* using the QIAprep Spin miniprep kit (QIAGEN, Hilden, Germany). The whole 2,3-BDO synthesis pathway sequence was confirmed by sequencing, and these verified plasmids were digested with *SalI* and *XhoI* to afford the donor DNA used for repairing the double-strand break made by the pCas9 multiplex.

2.3 Constructing pCas9 multiplex plasmids

The modified construction procedure outlined by Ryan et al.³⁰ was followed with previously described modifications²⁹, to generate the pCAS-gRNA-CRISPR multiplex targeting *ScADH1*, *ScADH2*, and *ScADH5*. In summary, the online tool <https://chopchop.cbu.uib.no/> was utilized to select specific guides positioned 20 base pairs (bp) upstream of the protospacer-adjacent motif (PAM) for *ScADH1*,

ScADH2, and *ScADH5*, and primers were designed accordingly (refer to Table S1). Subsequently, a universal scaffold was constructed through two rounds of PCR and inserted into a multiplex plasmid at the *SmaI/PstI* sites. Following plasmid extraction from *E. coli*, the sequence of the universal scaffold was confirmed via sequencing.

2.4 Engineering *S. cerevisiae* strains

To integrate the 2,3-BDO module into the *HIS3* locus, the pBDO/ADH1-*HIS3* plasmid was linearized at the *BsiWI* site within the *HIS3* gene before transformation, enabling recombination at the *HIS3* locus. The transformation process followed the lithium acetate method described previously³⁰ with some modifications. Specifically, approximately 2 µg of completely linearized pBDO/ADH1-*HIS3* plasmid in less than a 5 µl volume was combined with a final concentration of the transformation solution consisting of 34% PEG₃₀₀₀ containing 100 mM lithium acetate, 100 mM Tris-Cl, 1 mM EDTA, and 1% ssDNA from Salmon testes. The mixture and competent SK-FGG4 cells were then incubated for 30 minutes at 30 °C, followed by a static heat shock at 42 °C for 50 minutes. Subsequently, 200 µl of the mixture was directly plated onto 12 cm Petri dishes with YNB_{D20} medium and incubated at 30 °C for 4–7 days. Up to 12 of the largest colonies were selected and transferred to new YNB_{D20} plates to prevent the growth of the parental strain. From these colonies, four were randomly chosen for further fermentation studies.

The CRISPR-Cas9 multiplex genome editing method described previously^{29,30} was employed to create double-strand breaks in either the *ScADH1*, *ScADH2*, or *ScADH5* genes. These breaks facilitated homologous recombination with the 2,3-BDO modules

while simultaneously excising the genomic DNA sequence of the target genes. Transformants that successfully underwent this process were chosen randomly from colonies grown on YPD_{G418}, a selective medium. PCR verification was conducted using a primer located on the 2,3-BDO module and another on the genomic sequence upstream or downstream of the homologous loci. Following verification, positive colonies were further cultured on YPD_{G418} plates to inhibit the growth of negative cells. The engineered strains were then propagated at 220 rpm in liquid YPD-free G418 medium for several generations to ensure the elimination of the pCas9 multiplex. Once confirmed to harbor the desired genes, the generated strains and their respective genotypes were documented, as shown in Table 1.

2.5 Yeast growth conditions and precultures

The yeast cells were cultured following a method similar to those outlined in previous studies²⁹ with small modifications. Specifically, all strains were cultivated on YPD plates before propagation in 10 mL of YPD liquid medium in 50-mL Falcon tubes (VIOLAMO, Japan) to simplify cell recovery procedures, avoid the autoclaving process, and minimize the chances of contamination. The preculture was incubated at 30 °C with agitation at 200 rpm overnight using the TAITEC BioShaker BR-42FL (Japan), positioned at an angle of approximately 45°. During cultivation, the caps of the Falcon tubes were closed loosely to allow CO₂ ventilation. Cell density was assessed by measuring the optical density at 600 nm (OD₆₀₀) using a spectrophotometer (AS ONE, Japan) following a forty-fold dilution. The grown cells were used as preculture for the fermentation process.

Table 1 Plasmids and *S. cerevisiae* strains used in this study and their relevant genotypes.

Plasmids/strains	Relevant genotype	Reference
Plasmids		
pTDH3 _p -DIT1 _{d22t} -HIS3	<i>HIS3</i> ; <i>TDH3</i> promoter (p) and mutated <i>DIT1</i> _{d22} terminator (t)	This study
pBDO/ADH1- <i>HIS3</i>	<i>HIS3</i> ; expresses <i>BsAlsS</i> , <i>BsAlsD</i> , and <i>ScBDH</i> /homologous regions to <i>ScADH1</i> p and t.	This study
pBDO/ADH2- <i>HIS3</i>	<i>HIS3</i> ; expresses <i>BsAlsS</i> , <i>BsAlsD</i> , and <i>ScBDH</i> /homologous regions to <i>ScADH2</i> p and t.	This study
pBDO/ADH5- <i>HIS3</i>	<i>HIS3</i> ; expresses <i>BsAlsS</i> , <i>BsAlsD</i> , and <i>ScBDH</i> /homologous regions to <i>ScADH5</i> p and t.	This study
Multiplex pCas9/ <i>ScADH1</i>	Multiplex expresses Cas9 and gRNA to base No. 146 of <i>ScADH1</i>	This study
Multiplex pCas9/ <i>ScADH2</i>	Multiplex expresses Cas9 and gRNA to base No. 640 of <i>ScADH2</i>	This study
Multiplex pCas9/ <i>ScADH5</i>	Multiplex expresses Cas9 and gRNA to base No. 160 of <i>ScADH5</i>	This study
Strains		
D452-2	<i>MATα leu2 his3 ura3 can1</i>	31
SK-FGG4	<i>ΔGPD1::TDH3_p-LlnoxE-DIT1_{d22b} AUR1::PGK_p-CuFPS1-RPL41B_t PGK_p-ScTPI1-PGK_t PGK_p-ScDAK2-PGK_t PGK_p-ScDAK1-PGK_t ΔGUT1::TEF_p-CuFPS1-CYC1_t TYS1_p-OpGDH-ATP15_t TDH3_p-ScDAK1-DIT1_{d22b} FBA1_p-ScTPI1-TDH3_t</i>	29
SK-MA1	SK-FGG4, <i>HIS3::TDH3_p-BsAlsS-DIT1_{d22b} PGK_p-BsAlsD-DIT1_{d22b} FBA1_p-ScBDH-RPL41B_t</i>	This study
SK-MA2	SK-FGG4, <i>ΔADH1::TDH3_p-BsAlsS-DIT1_{d22b} PGK_p-BsAlsD-DIT1_{d22b} FBA1_p-ScBDH-RPL41B_t</i>	This study
SK-MA3	SK-FGG4, <i>ΔADH2::TDH3_p-BsAlsS-DIT1_{d22b} PGK_p-BsAlsD-DIT1_{d22b} FBA1_p-ScBDH-RPL41B_t</i>	This study
SK-MA4	SK-FGG4, <i>ΔADH5::TDH3_p-BsAlsS-DIT1_{d22b} PGK_p-BsAlsD-DIT1_{d22b} FBA1_p-ScBDH-RPL41B_t</i>	This study
SK-MA5	D452-2, <i>ΔADH1::TDH3_p-BsAlsS-DIT1_{d22b} PGK_p-BsAlsD-DIT1_{d22b} FBA1_p-ScBDH-RPL41B_t</i>	This study
SK-MA6	D452-2, <i>ΔADH5::TDH3_p-BsAlsS-DIT1_{d22b} PGK_p-BsAlsD-DIT1_{d22b} FBA1_p-ScBDH-RPL41B_t</i>	This study
SK-MA7	SK-MA4, <i>ΔADH1::TDH3_p-BsAlsS-DIT1_{d22b} PGK_p-BsAlsD-DIT1_{d22b} FBA1_p-ScBDH-RPL41B_t</i>	This study

2.6 Fermentation conditions and procedures

Precultures were centrifuged at 2500 g to obtain the cell pellets which were washed with ultrapure sterilized Milli Q water and used as a starter for fermentation experiments. For fermenting glycerol as the sole carbon source, YNB_{G40} was used for diluting the preculture of strain SK-MA1. The OD₆₀₀ was adjusted to 0.02 by addition of media. Three sets of experiments were prepared to investigate different aeration levels as previously reported²⁹, with some modification. The modification included using 10 mL, 20 mL, and 30 mL culture-medium /100-mL Erlenmeyer flasks, which are referred to here as aerobic, semi-aerobic, and microaerobic conditions, respectively.

To avoid any limitations in the nutrient content, YNB_{G40} was replaced by YP_{G40} before conducting the comparative aerobic fermentations of SK-MA1, SK-MA2, SK-MA-3, and SK-MA4 strains. Similarly, the co-fermentation medium YP_{D20G40} was also used aerobically for comparing SK-MA3, SK-MA4, SK-MA5, SK-MA6, and SK-MA7 strains. Also, YP_{D20G40} was used to compare SK-MA3 and SK-MA7 strains under semi- and microaerobic conditions.

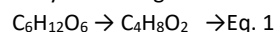
2.7 Analytical procedures of the fermentation elements

The concentrations of the substrates, glycerol and glucose, as well as various metabolites such as acetoin, 2,3-BDO, ethanol, acetic acid, succinic acid, pyruvate, and acetaldehyde were measured following previously reported methods.²⁹ In short, the samples were assessed using an Aminex HPX-87H column for HPLC analysis, incorporating a refractive index detector and a prominence diode array. The sample was automatically injected into a mobile phase containing 5 mM H₂SO₄ at a flow rate of 0.6 mL/min. To prepare the samples, 100 µL was withdrawn from the fermentation flasks under sterile conditions, diluted with 900 µL of Milli-Q water in Eppendorf tubes, mixed, and then centrifuged at 21500 g for 5 minutes using a Hitachi Himac CT15RE centrifuge. The resulting supernatants were filtered using a hydrophilic FILTSTAR PTFE 0.45 µm syringe filter (Hawach Scientific, China) and transferred into fresh 2 mL HPLC glass vials using a 1 mL syringe.

2.8 Conversion efficiency calculations

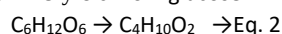
Theoretical (Theo.) yield= Molar mass of product/ Molar mass of substrate (glucose and/or glycerol)

Theo. acetoin yield from glucose:



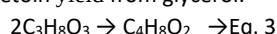
$$1 \text{ g} \rightarrow 0.488 \text{ g} \rightarrow \text{Eq. 1}$$

Theo. 2,3-BDO yield from glucose:



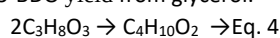
$$1 \text{ g} \rightarrow 0.50 \text{ g} \rightarrow \text{Eq. 2}$$

Theo. acetoin yield from glycerol:



$$1 \text{ g} \rightarrow 0.478 \text{ g} \rightarrow \text{Eq. 3}$$

Theo. 2,3-BDO yield from glycerol:



$$1 \text{ g} \rightarrow 0.489 \text{ g} \rightarrow \text{Eq. 4}$$

Theo. acetoin yield (g/L) = [Initial concentration (IC) of glucose (g/L) – residual concentration (RC) of glucose (g/L)] * 0.488

Theo. acetoin (g/L) = [IC of glycerol (g/L) – RC of glycerol (g/L)] * 0.478

Theo. 2,3-BDO (g/L) = [IC of glucose (g/L) – RC glucose (g/L)] * 0.50

Theo. 2,3-BDO (g/L) = [IC of glycerol (g/L) – RC of glycerol (g/L)]

*489

3. Results

3.1 2,3-BDO module construction and recombination of the SK-MA1 strain

The 2,3-BDO module was assembled as depicted in Fig. S2 with details described in the methods section. The A195T *BsA/sS* single mutant was generated during the construction of 2,3-BDO module. We found that the mutated residue is approximately 40 Å away from the active site, suggesting that it is not directly related to enzyme activity though the region around the residue seems to be important for tetramer formation. Nonetheless, the exact effect of this mutation may require further study in the future.

The previously engineered SK-FGG4 strain, which converts glycerol to ethanol and has the *URA3* maker restored²⁹, was used as the recipient strain to generate SK-MA1 (Table 1). In this strain, successful integration of 2,3-BDO into *HIS3* serves as the auxotrophic marker by recovering the sixth step in histidine biosynthesis, allowing growth on YNB_{D20} media without histidine.

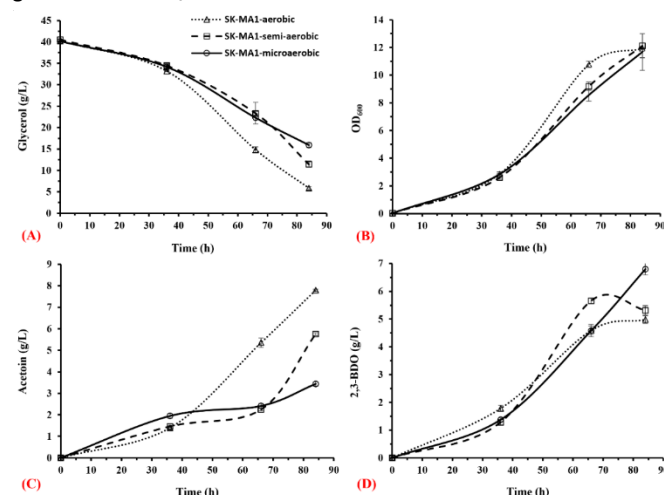


Fig. 2 Time course fermentation of glycerol as the sole carbon source by the SK-MA1 strain in which one copy of the 2,3-BDO pathway is heterologously expressed in the *HIS3* locus, under three different aeration conditions: aerobic, semi-aerobic, and microaerobic. The fermentation was conducted in YNB_{G40} with 200 rpm orbital shaking at 30 °C. Glycerol consumption (A), optical density, OD₆₀₀ (B), acetoin production (C), and 2,3-BDO production (D). The data represents the SD of the average of two independent experiments.

3.2 Conversion of glycerol as sole carbon source to 2,3-BDO by the recombinant SK-MA1 strain

The recombinant *S. cerevisiae* SK-MA1 strain was able to convert glycerol as the sole carbon source to 2,3-BDO under three different aeration conditions. Under aerobic conditions, glycerol consumption was faster than under semi-aerobic and microaerobic conditions (Fig. 2A) but accumulated a higher concentration of acetoin (Fig. 2C). The

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Table 2 Fermentation characteristics of glycerol as sole carbon source by the recombinant SK-MA1 strain under three different aeration conditions: aerobic, semi-aerobic, and microaerobic, at 66 and 84 hours of fermentation*

Parameters	Strains/time	SK-MA1-aerobic		SK-MA1-semi-aerobic		SK-MA1-microaerobic	
		66 h	84 h	66 h	84 h	66 h	84 h
Consumed glycerol (g/L)		25.7 ± 0.3	34.5 ± 0.1	15.9 ± 1.2	28.7 ± 0.3	18.6 ± 0.8	23.9 ± 0.3
Consumption ratio %		64.0 ± 0.8	85.9 ± 0.4	39.3 ± 3.1	70.9 ± 0.9	46.3 ± 1.9	59.6 ± 0.7
Acetoin production (g/L)		5.6 ± 0.2	7.8 ± 0.1	2.3 ± 0.1	5.8 ± 0.1	2.4 ± 0.1	3.5 ± 0.1
2,3-Butanediol production (g/L)		4.6 ± 0.2	5.0 ± 0.1	5.6 ± 0.1	5.3 ± 0.2	4.6 ± 0.2	6.8 ± 0.2
Total production (g/L)		10.2 ± 0.4	12.8 ± 0.2	7.9 ± 0.2	11.1 ± 0.3	7.0 ± 0.3	10.3 ± 0.3
Total conversion $\text{g}_{\text{acetoin}+2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$		0.40 ± 0.01	0.37 ± 0.01	0.5 ± 0.1	0.39 ± 0.01	0.38 ± 0.1	0.43 ± 0.01
Total conversion efficiency %		81.6 ± 0.5	75.5 ± 0.3	100 ± 2	79.6 ± 0.3	77.5 ± 0.3	87.7 ± 0.7
Specific growth rate per h		0.16 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.14 ± 0.00	0.13 ± 0.00	0.14 ± 0.00

*The fermentation was conducted in YNB_{G40} with 200 rpm orbital shaking at 30°C. The data represents the SD of the average of two independent experiments.

acetoin product reached 7.8 g/L which corresponds to 0.23 $\text{g}_{\text{acetoin}}/\text{g}_{\text{glycerol}}$, while the 2,3-BDO product was 5 g/L, which is equal to 0.15 $\text{g}_{2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$ (Table 2). The specific growth rate was almost 14% and 23% higher in aerobic conditions compared to semi-aerobic and microaerobic conditions at 66h, respectively (Fig. 2B, Table 2). On the other hand, microaerobic conditions reduced acetoin accumulation and promoted 2,3-BDO production which reached 0.28 $\text{g}_{2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$ (Fig. 2D, Table 2). Interestingly, under semi-aerobic conditions at 66 h, the SK-MA1 strain produced 0.35 $\text{g}_{2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$ and 0.14 $\text{g}_{\text{acetoin}}/\text{g}_{\text{glycerol}}$ which approaches the theoretical yield. However, the strain's performance dropped to 79.6% at 84 h (Table 2).

3.3 Replacing the *ScADH* genes with the 2,3-BDO synthesis pathway in the CRISPR-engineered strains

The selected gRNAs listed in Table S1 within the CRISPR-Cas9 multiplex were able to induce a double-strand break in the target *ScADH* gene. With the genome editing protocol³⁰, the 2,3-BDO module successfully replaced the *ScADH* genes. The list of generated strains and their genomic modifications are listed in Table 1. Replacement of *ScADH1* of the SK-FGG4 strain with the 2,3-BDO synthesis genes generated SK-MA2. Substitution of *ScADH2* of the SK-FGG4 strain with the 2,3-BDO synthesis genes generated SK-MA3. Substitution of *ScADH5* propagated SK-MA4. Similarly, we replaced *ScADH1* and *ScADH5* individually in the native D452-2 strain, producing SK-MA5 and SK-MA6, respectively. Finally, the SK-MA7 strain was bred by double replacement of *ScADH1* and *ScADH5* with 2 copies of the 2,3-BDO synthesis genes in the SK-FGG4 strain (Table 1).

3.4 Explore glycerol conversion to 2,3-BDO in a complex medium by strains SK-MA1–SK-MA4

In this part, the aerobic conversion of glycerol to 2,3-BDO was explored in the YP_{G40} medium by strains SK-MA1, SK-MA2, SK-MA3, and SK-MA4 in comparison to the reference strain, SK-FGG4 (Fig. 3). SK-FGG4 converted glycerol efficiently to ethanol. The glycerol consumption by SK-FGG4 is significantly faster than 2,3-BDO-producing strains (Fig. 3A). SK-FGG4 is the only strain that produces ethanol (Fig. 3B). SK-MA1 at 36 h produced the highest 2,3-BDO concentration at 3.6 g/L, after which the glycerol flux accumulated at acetoin with further decreasing the 2,3-BDO concentration. The acetoin accumulation rose from 0.9 g/L to 8 g/L at 66 h (Fig. 3C and D). The combined conversion of acetoin and 2,3-BDO reached 0.31 $\text{g}_{\text{acetoin}+2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$ (Table 3). The replacement of *ScADH1* with the 2,3-BDO module in the SK-MA2 strain increases the production of 2,3-BDO by 60% and acetoin by 11.4% compared to SK-MA1 (Fig. 3C and D), and the total conversion reached 0.39 $\text{g}_{\text{acetoin}+2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$, which corresponds to 80.4 % conversion efficiency (Table 3). SK-MA3 which replaced its *ScADH2* with the 2,3-BDO module accumulated the highest acetoin content reaching 10.3 g/L representing 0.26 $\text{g}_{\text{acetoin}+2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$, while the end-product 2,3 BDO at 3 g/L was the lowest among all strains. On the other hand, the highest production of 6.6 g/L 2,3-BDO was obtained by the substitution of *ScADH5* (SK-MA4), and the accumulation of acetoin reached 8 g/L, which increased the total conversion to 0.45 $\text{g}_{\text{acetoin}+2,3\text{-BDO}}/\text{g}_{\text{glycerol}}$ (94.2% conversion efficiency) as shown in Table 3. There were variations in the growth rate among all strains; the optical density (OD₆₀₀) of SK-MA3 showed the highest growth at 16.2 (Fig. 3E).

Table 3 Comparison of aerobic glycerol fermentation characteristics by SK-MA1 to SK-MA4 strains*

Parameters	Strains	SK-MA1	SK-MA2	SK-MA3	SK-MA4
Consumed glycerol (g/L)		37.3 ± 0.2	37.1 ± 0.1	39.0 ± 0.1	32.2 ± 1.1
Consumption ratio %		92.6 ± 0.4	92.1 ± 0.2	97.0 ± 0.2	79.5 ± 2.4
Acetoin production (g/L)		7.9 ± 0.1	8.8 ± 0.1	10.3 ± 0.1	8.0 ± 0.1
2,3-Butanediol production (g/L)		3.5 ± 0.1	5.6 ± 0.1	3.0 ± 0.1	6.6 ± 0.4
Total production (g/L)		11.4 ± 0.2	14.4 ± 0.2	13.3 ± 0.2	14.6 ± 0.5
Total conversion g _{acetoin+2,3-BDO} /g _{glycerol}		0.31 ± 0.01	0.39 ± 0.01	0.34 ± 0.1	0.45 ± 0.01
Total conversion efficiency %		64.6 ± 1.0	80.4 ± 0.4	69.5 ± 0.4	94.2 ± 1.1

*The fermentation was conducted in YP_{G40} with 200 rpm orbital shaking at 30°C. The data represents the SD of the average of two independent experiments. The data was obtained after 66 hours.

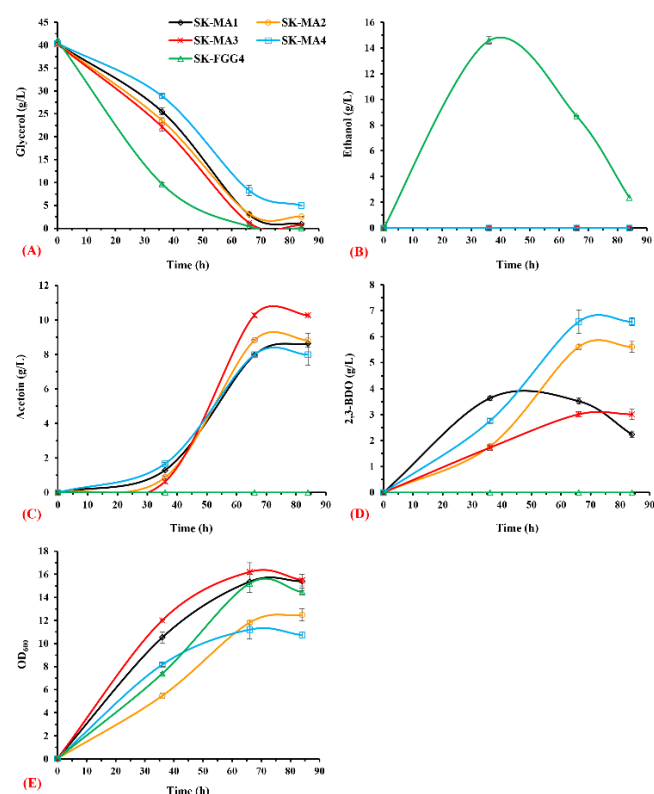


Fig. 3 The progression of aerobic glycerol conversion by yeast heterologously expressing one copy of the 2,3-BDO synthesis enzymes which replaced the ScADH1, 2, or 5 enzymes to generate the SK-MA2, SK-MA3, or SK-MA4 strains, respectively, in comparison with SK-MA1 (harboring 2,3-BDO synthesis enzymes with all ScADH isoforms). Glycerol consumption (A), ethanol production (B), acetoin production (C), 2,3-BDO production (D), and OD₆₀₀ (e). The fermentation was carried out in 100 Erlenmeyer flasks containing a 10 mL culture of YP_{G40} media with 200 rpm orbital shaking at 30 °C. The data represents the SD of the average of two independent experiments.

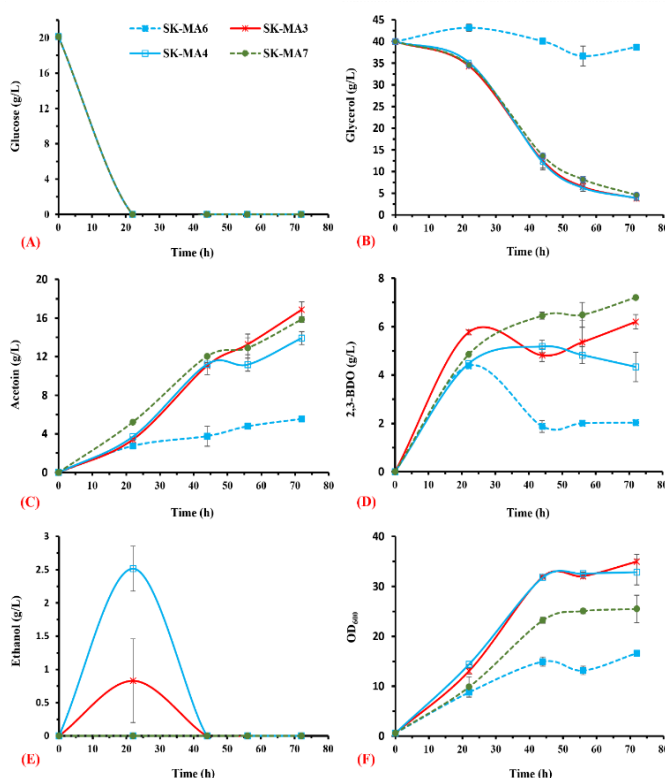


Fig. 4 Comparison of the aerobic fermentation of a glycerol-glucose mixture by SK-MA6 (*ScADH5* of a native strain replaced by one copy of the 2,3-BDO synthesis enzymes), SK-MA3 (*ScADH2* replaced with 2,3-BDO synthesis enzymes), SK-MA4 (*ScADH5* replaced with 2,3-BDO synthesis enzymes), and SK-MA7 (*ScADH1* and *ScADH5* each replaced by 2,3-BDO synthesis enzymes). Glucose consumption (A), glycerol consumption (B), acetoin production (C), 2,3-BDO production (D), ethanol production (E), and OD₆₀₀ (F). The fermentation was carried out in 100-ml Erlenmeyer flasks containing a 10 mL culture of YPD_{D20G40} with 200 rpm orbital shaking at 30 °C. The data represents the SD of the average of two independent experiments.

Table 4 Comparison of aerobic glycerol-glucose mixture fermentation characteristics by SK-MA3 to SK-MA7 strains*

Parameters	Strains	SK-MA3	SK-MA4	SK-MA5	SK-MA6	SK-MA7
Consumed glycerol (g/L)		36.2 ± 0.5	36.1 ± 0.1	1.9 ± 0.3	1.3 ± 0.1	35.4 ± 0.1
Consumed glucose (g/L)		20.1 ± 0.1	20.1 ± 0.1	20.1 ± 0.1	20.1 ± 0.1	20.1 ± 0.1
Total consumption (g/L)		56.3 ± 0.6	56.2 ± 0.2	22.0 ± 0.4	21.4 ± 0.2	55.5 ± 0.2
Acetoin production (g/L)		16.9 ± 0.8	13.9 ± 0.7	5.1 ± 0.1	5.5 ± 0.1	15.8 ± 0.3
2,3-Butanediol production (g/L)		6.2 ± 0.3	4.3 ± 0.6	1.2 ± 0.2	2.0 ± 0.1	7.2 ± 0.1
Total production (g/L)		23.1 ± 1.1	18.2 ± 1.3	6.3 ± 0.3	7.5 ± 0.2	23.0 ± 0.4
Total conversion g _{acetoin+2,3-BDO} /g _{glycerol+glucose}		0.41 ± 0.01	0.32 ± 0.01	0.29 ± 0.1	0.35 ± 0.01	0.41 ± 0.01
Total conversion efficiency %		84.0 ± 1.0	65.0 ± 0.5	58.0 ± 0.3	70.0 ± 0.7	84.9 ± 1.0

*The fermentation was conducted in YP_{D20G40} with 200 rpm orbital shaking at 30°C. The data represents the SD of the average of two independent experiments. The data was obtained after 72 hours.

3.5 Aerobic co-conversions of glycerol and glucose to 2,3-BDO by strains SK-MA3–SK-MA7

The modified strains SK-MA3–SK-MA7 were assessed for their capacity to convert a mixed carbon source - glycerol and glucose - under aerobic conditions using the YP_{D20G40} medium. Strain SK-MA6, a native strain with *ScADH5* replaced with 2,3-BDO synthesis enzymes, at 20 h, produced 4.4 g/L of 2,3-BDO, and 2.8 g/L acetoin with increased glycerol concentration at 3.2 g/L (Fig. 4). Strain SK-MA5, a native strain with *ScADH1* substituted with 2,3-BDO synthesis enzymes, produced similar results (Fig. S1). SK-MA3 exhibited the ability to convert a mixture of glycerol and glucose into acetoin and 2,3-BDO under aerobic conditions, yielding 16.9 g/L acetoin, equivalent to 0.30 g_{acetoin}/g_{glycerol+glucose}. With 2,3-BDO production at 0.12 g_{2,3-BDO}/g_{glycerol+glucose}, the total conversion reached 42 g_{acetoin+2,3-BDO}/g_{glycerol+glucose}. SK-MA4 demonstrated a reduction in conversion products, with acetoin decreasing to 13.9 g/L and 2,3-BDO decreasing to 4.3 g/L, corresponding to 0.32 g_{acetoin+2,3-BDO}/g_{glycerol+glucose} (Table 4).

By double replacement of *ScADH1* and *ScADH5* with the 2,3-BDO synthesis pathway in the SK-MA7 strain, 2,3-BDO production was significantly enhanced by 67% compared to SK-MA4, with the 2,3-BDO and acetoin concentrations reaching 7.2 g/L and 15.8 g/L respectively, representing 0.41 g_{acetoin+2,3-BDO}/g_{glycerol+glucose} (Table 4). Interestingly, SK-MA7 did not exhibit the ethanol production observed in SK-MA3 and SK-MA4 (Fig. 4E). Remarkably, the OD₆₀₀ values of SK-MA3 and SK-MA4 increased substantially to 35 and 32.9, respectively, while the growth of SK-MA7 reached 25.5 (Fig. 4F).

3.6 Fermentation of glycerol-glucose mixture to 2,3-BDO under semi and microaerobic conditions

Strains SK-MA3 and SK-MA7, which produced the highest 2,3-BDO levels from the glycerol-glucose mixture in aerobic conditions on YP_{D20G40} medium, were examined further under semi- and microaerobic conditions. There was a positive correlation between aeration level, growth rate, and glycerol consumption (Fig. 5). Strain SK-MA3 consumed 38.5 g/L versus 32.8 g/L of glycerol with semi and

microaerobic conditions, respectively. In contrast, glycerol consumption decreased from 37.7 g/L to 34.5 g/L in the SK-MA7 strain under semi- and microaerobic conditions, respectively (Table 5). Glucose was consumed rapidly in both strains (Fig. 5A). Interestingly, decreasing the aeration level increases the 2,3-BDO concentration in both strains. For SK-MA7 under aerobic conditions, the maximum production of 2,3-BDO was 7.2 g/L (Fig. 4D), which increased to 8.9 g/L in a semi-aerobic environment and raised to 12.9 g/L in microaerobic conditions (Fig. 5D). In contrast, the acetoin concentration decreased from 15.8 g/L in aerobic conditions (Fig. 4C) to 13 g/L and 5 g/L in semi-, and microaerobic conditions, respectively (Fig. 5C). SK-MA3 preferentially produced 2,3-BDO. The maximum production reached 15.5 g/L under microaerobic conditions compared to 11.2 g/L under semi-aerobic conditions (Fig. 5D) and 6.2 g/L under aerobic conditions (Fig. 4D). Acetoin accumulated by SK-MA3 in microaerobic conditions was 5.5 g/L at 96 h, a maximum point for 2,3-BDO production, which increased to 11.5 g/L at 120 h (Fig. 5C, Table 5). SK-MA3 and SK-MA7 produced higher concentrations of acetoin (18.4 g/L and 18.7 g/L respectively) under semi-aerobic conditions.

The total conversion of SK-MA7 reached 0.46 g_{acetoin+2,3-BDO}/g_{glycerol+glucose}, which represents 93% of the total conversion efficiency under microaerobic conditions. A slightly lower conversion (0.45 g_{acetoin+2,3-BDO}/g_{glycerol+glucose}, equal to 91% of the total conversion efficiency) was obtained under semi-aerobic conditions. A comparable strain SK-MA3 produced 0.41 g_{acetoin+2,3-BDO}/g_{glycerol+glucose} and 0.43 g_{acetoin+2,3-BDO}/g_{glycerol+glucose} with semi-, and microaerobic conditions, respectively, which is equivalent to 83% and 87% total conversion efficiency, respectively (Table 5).

Fig. 6 shows the comparative data of semi- and microaerobic glycerol fermentation on YP_{D20G40} media of the SK-MA3 and SK-MA7 strains at 96 h before the metabolic flux is reverted from 2,3-BDO back to acetoin. At this time point, strain SK-MA3 under microaerobic conditions revealed higher conversion efficiency. The production of 2,3-BDO represents 0.34 g_{2,3-BDO}/g_{glycerol+glucose} while the total conversion with acetoin reached 0.46 g_{acetoin+2,3-BDO}/g_{glycerol+glucose} corresponding to 92% total conversion efficiency. SK-MA7 at the same point produced

Table 5 Comparison of semi-aerobic and microaerobic glycerol-glucose mixture fermentation characteristics by SK-MA3 (one copy of 2,3-BDO module), and SK-MA7 (two copies of 2,3-BDO module) strains*

Parameters	Strain	Semi-aerobic		Microaerobic	
		SK-MA3	SK-MA7	SK-MA3	SK-MA7
Consumed glycerol (g/L)		38.5 ± 0.3	37.7 ± 0.2	32.8 ± 0.6	34.5 ± 0.1
Consumed glucose (g/L)		19.9 ± 0.1	19.9 ± 0.1	19.9 ± 0.1	19.9 ± 0.1
Total consumption (g/L)		58.4 ± 0.4	57.6 ± 0.3	52.7 ± 0.7	54.4 ± 0.2
Acetoin production (g/L)		18.4 ± 0.1	18.7 ± 0.2	11.5 ± 1.7	16.7 ± 1.4
2,3-Butanediol production (g/L)		5.6 ± 0.2	7.3 ± 0.5	10.9 ± 1.4	8.1 ± 0.5
Total production (g/L)		24.0 ± 0.3	25.9 ± 0.7	22.4 ± 2.1	24.8 ± 1.9
Total conversion $\frac{\text{g acetoin}+2,3\text{-BDO}}{\text{g glycerol}+\text{glucose}}$		0.41 ± 0.01	0.45 ± 0.01	0.43 ± 0.01	0.46 ± 0.01
Total conversion efficiency %		83 ± 1.0	91.0 ± 1.0	87.0 ± 2.0	93.0 ± 1.0

*The fermentation was conducted in YP_{D20G40} with 200 rpm orbital shaking at 30 °C. The data represents the SD of the average of two independent experiments. The data was obtained after 120 hours.

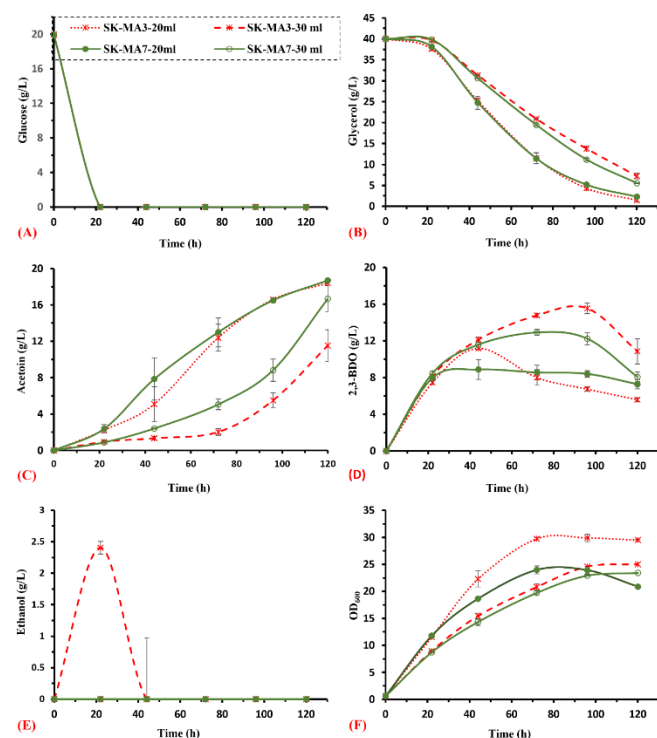


Fig. 5 Flux profiles and products of the fermentation of a glycerol-glucose mixture by SK-MA3 (*ScADH2* replaced by one copy of the 2,3-BDO synthesis enzymes) and SK-MA7 (*ScADH1* and *ScADH5* each replaced by one copy of the 2,3-BDO synthesis enzymes) under semi- and microaerobic conditions. The fermentation of YP_{D20G40} was conducted with 200 rpm orbital shaking at 30 °C. Glucose consumption (A), glycerol consumption (B), acetoin production (C), 2,3-BDO production (D), ethanol production (E), and OD₆₀₀ (F). The data represents the SD of the average of two independent experiments.

0.25 g $\frac{2,3\text{-BDO}}{\text{g glycerol}+\text{glucose}}$ and total conversion including acetoin reached 0.43 $\frac{\text{g acetoin}+2,3\text{-BDO}}{\text{g glycerol}+\text{glucose}}$ equal to 87.2% conversion efficiency. Under semi-aerobic conditions at 96h, SK-MA7 showed a higher conversion efficiency which reached 91.7%, with acetoin as the main product approaching 0.30 $\frac{\text{g acetoin}}{\text{g glycerol}+\text{glucose}}$. In comparison to SK-MA3 at 96 h under semi-aerobic conditions, the acetoin

production rate was the same as SK-MA7 although the conversion efficiency was lower at 84.8%.

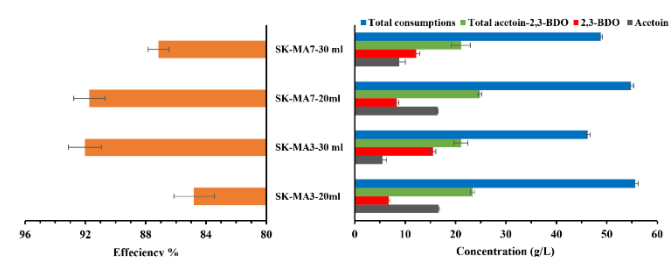


Fig. 6 Comparison of parameters at 96 h of a glycerol-glucose fermentation by SK-MA3 (*ScADH2* replaced by one copy of the 2,3-BDO synthesis enzymes) and SK-MA7 (*ScADH1* and *ScADH5* each replaced by one copy of the 2,3-BDO synthesis enzymes) under semi- and microaerobic conditions. The fermentation of YP_{D20G40} with 200 rpm orbital shaking at 30 °C. The data represents the SD of the average of two independent experiments.

4. Discussion

Increasing apprehensions regarding sustainability and global climate change have propelled the advancement of sustainable production routes for biofuels and chemicals derived from lignocellulosic biomass through integrated biological and chemical processes.³² Utilizing glycerol, a renewable surplus resource from the production of biodiesel, as a green organosolvent with high permittivity and low inhibitory effects makes it a cost-effective option for lignocellulose biorefinery, and it has attracted considerable attention. *S. cerevisiae* was engineered comprehensively to create the SK-FGG4 strain which converts glycerol efficiently to ethanol.²⁹ This novel strain successfully converted a mixture of glycerol and the hydrolyzed sugars of sugarcane trash (SCT) to bioethanol alongside the conversion of 29% of the SCT lignin to non-cytotoxic antiviral substances.² Such a sustainable approach can be broadened to produce 2,3-BDO by redirecting the end-product of the engineered SK-FGG4 yeast strain from ethanol to 2,3-BDO. Interestingly, the SK-

MA1 strain harboring the 2,3-BDO module (Fig. S2) in the *ScHIS3* locus completely shifted the metabolic flux of glycerol to the 2,3-BDO biosynthesis pathway (Figs. 1, 2). Intensive metabolic engineering is required to shift the flux from ethanol to 2,3-BDO with glucose feeding. Deleting *ScPDC1*, *ScPDC5*, and *ScPDC6* was essential for abolishing ethanol production.^{24–28} However, removing the *ScPDC* genes results in growth defects due to a lack of cytosolic acetyl CoA and C2-auxotrophy which created a need for acetate or ethanol, or evolutionary adaptation.^{24, 27, 33} Glycerol as a substrate has a unique feature and might downregulate the *ScPDCs*, and in the strains generated here in this work, though this needs further investigation. This idea is supported by a previous study where transcription levels of *ScPDC1*, *ScPDC5*, *ScPDC6*, and *ScTHI3*, a regulatory protein that interacts with *ScPDC2p* and *Thi2p* transcription factors, in one acclimatized glycerol grower *S. cerevisiae* strain were lower than those in the wild type when glycerol was used as the sole carbon source.³⁴ However, it was found in the same work that dihydrolipoamide dehydrogenase *ScLPD1* (a component (E3) within the multi-enzyme complexes of pyruvate dehydrogenase and 2-oxoglutarate dehydrogenase which plays a role in the conversion of pyruvate to oxaloacetate) was upregulated.³⁴ This upregulation of the tricarboxylic acid cycle may partly explain the glycerol growth observed in the acclimatized strains. Conversely, the downregulation of *ScPDCs* and the associated NADH/NAD⁺ redox imbalance helps to explain why those acclimatized strains are unable to produce ethanol from glycerol. A previously engineered strain SK-FGG4, which was used as a starting point for this study, has overcome such limitations when converting glycerol to ethanol efficiently.²⁹ Therefore, the heritage of genome-edited SK-FGG4 made it possible for it to convert glycerol to 2,3-BDO with a simple introduction of the 2,3-BDO biosynthesis pathway without a need for any deletion of *ScPDCs* (Fig. 2).

The data obtained from the aerobic fermentation of glycerol using strains engineered to replace their cytosolic alcohol dehydrogenases (*ScADH1* (SK-MA2), *ScADH2* (SK-MA3), and *ScADH5* (SK-MA4)) with the 2,3-BDO module compared to SK-MA1(2,3-BDO/*ScHIS3*) implied that increased levels of NADH for proper *ScBDH* function support the conversion of glycerol to 2,3-BDO. Ethanol and acetaldehyde were produced in undetectable quantities by all strains when glycerol was utilized as the carbon source. Strains SK-MA2 and SK-MA4 produced higher levels of 2,3-BDO compared to SK-MA1. SK-MA3 accumulated higher levels of acetoin compared to other strains (Fig. 3). The absence of *ScADH2* may contribute to a NADH deficit and thereby attenuate acetoin conversion to 2,3-BDO, but its role related to increased acetoin accumulation is still unclear. Deleting *ScADH1* in JHY608 yeast harboring the 2,3-BDO metabolic pathway decreased ethanol production from 5.97 g/L to 3.44 g/L when 50 g/L of glucose was utilized.³⁵ We have found that conversion to 2,3-BDO from glycerol has an advantage over that of glucose, where ethanol production is diminished. To further confirm this finding, a fermentation of YP_{D20G40} medium by SK-MA5 and SK-MA6, in which the 2,3-BDO module replaced *ScADH1* and *ScADH5*, respectively, of the native strain D452-2, shifted the metabolic fluxes of glucose to the 2,3-BDO biosynthesis pathway without ethanol production (Figs.

4, and S1). This finding prompted further transcriptional studies to determine the limitation of the fluxes using glycerol compared to glucose, the results of which also support the hypothesis mentioned above. Phosphoglycerate kinase (*ScPGK1*) was found to be downregulated in an acclimatized glycerol-growing strain grown on a glycerol-based medium.³⁴ Such limitations must be addressed and overcome in the future as the process of 2,3-BDO production from glycerol is optimized.

At the onset of YP_{D20G40} medium fermentation by SK-MA3 and SK-MA4 strains, 0.83 g/L and 2.5 g/L of ethanol were produced, respectively, which was rapidly consumed. This data supports the feature of utilizing glycerol as a carbon source. By double replacement of *ScADH1* and *ScADH5* with 2,3-BDO synthesis enzymes in the SK-MA7 strain, this initial ethanol production was not observed (Fig. 4E). It is not clear whether the introduction of two copies of the 2,3-BDO biosynthesis pathway enzymes or the deletion of both *ScADH* isoforms or the availability of NADH for *ScBDH1* or their synergetic effects prevent this early ethanol production. Biosynthesis of 2,3-BDO from glucose causes a NADH/NAD⁺ redox imbalance²³ as two NADH molecules are generated by glycolysis and only one is oxidized when *BsAlsD* is utilized (Fig. 1). Most importantly, glycerol has two additional hydrogens that require reduction and therefore additional equivalents of NADH compared to glucose during its conversion to 2,3-BDO (Fig. 1). This redox imbalance has been reported to be one of the major bottlenecks for the fermentation of glycerol to ethanol by native *S. cerevisiae*²⁹ or glycerol-acclimatized *S. cerevisiae*.³⁴ The *LnoxE* shuttle was heterologously expressed in SK-FGG4 to recycle one NADH/NAD⁺ with *OpGDH* for every molecule of ethanol. Theoretically, two NADH molecules should be recycled per 2,3-BDO molecule through the native *ScBDH* route while three NADH molecules are recycled per 2,3-BDO molecule through integrated *BsAlsD* (Fig. 1). Furthermore, excess NADH is generated through the fluxes to the tricarboxylic acid cycle and cell biomass formation. *LnoxE* has been reported to maintain redox balance during 2,3-BDO production from glucose.^{24–26} Therefore, it was expected that aerobic conditions would be appropriate to oxidize excess NADH through native oxidizing shuttles, *ScNDE1*, and *ScNDE2* alongside *LnoxE*. Scrutinizing the higher acetoin accumulation during aerobic conditions (Figs. 2–5) alongside the dual substrate specificity of *OpGDH*, which prefers 2,3-BDO over glycerol³⁶, it was proposed that lower aeration may lower by-product accumulation. Interestingly, lowering aeration significantly decreased acetoin levels in SK-MA1, SK-MA3, and SK-MA7 strains, indicating that NADH was the limiting factor for the forward reaction of *ScBDH* (Figs. 2, 5). Indeed, acetoin also accumulated during glucose conversion to 2,3-BDO, and heterologous expression of five copies of *BsBdhA* were introduced to overcome this obstacle.²⁸

Glycerol is commonly a by-product of previous studies that converted glucose to 2,3-BDO²⁷. Introducing the 2,3-BDO pathway in the D452-2 strain (SK-MA6) in this study increased glycerol concentration from 40 g/L to 43.2 g/L, while SK-MA3 and SK-MA7 decreased the glycerol concentration to 35.2 and 34.6 g/L, respectively (Fig. 4B). The co-conversion strategy demonstrates the potency of our engineered strains to prevent glycerol production from glucose and partially overcome the carbon catabolite

repression (CCR) of glucose to glycerol by consuming approximately 5 g/L of glycerol in the initial 24 hours (Fig. 4B). We have significantly improved CCR in our previous work²⁹, but further metabolic engineering is required to completely overcome CCR. Such a co-conversion strategy is important for biorefinery applications utilizing glycerolysis².

One of the major obstacles in glycerol conversion to 2,3-BDO is the broad substrate specificity of *OpGDH* which readily takes 2,3-BDO as a substrate to convert it back to acetoin, hindering high titer production. This has not been observed in the absence of *OpGDH* during glucose conversion to 2,3-BDO. In SK-FGG4, *OpGDH* has 61-fold substrate specificity toward (2R,3R)-2,3-BDO over glucose, which is over 95 % of the 2,3-BDO produced in this study. *OpGDH* also has a 1.6-fold specificity toward meso-(2R,3S)-2,3-BDO over glycerol at 500 mM of each substrate with 10 mM NAD⁺. The K_m for glycerol was 118 mM while the K_m for dihydroxyacetone was 4.87 μ M.³⁶ The *OpGDH* kinetics are consistent with the observed accumulation of acetoin, which increased remarkably at the end of the fermentation using the genetic modifications of strains generated in this study (Table 1). Lower aeration implies increasing NADH and promotes the unhindered reaction of *ScBDH* to increase 2,3-BDO production (Figs. 2, 5). When the 2,3-BDO level starts to exceed the concentration of acetoin and glycerol, the cells (*OpGDH*) tend to equilibrate acetoin and 2,3-BDO by reversing the reaction toward acetoin. This mixture of acetoin and 2,3-BDO may not be of significant concern as, recently, a one-pot synthesis of C₁₀ fuel precursor from acetoin condensation using similar acetoin, 2,3-BDO mixture ratios in the fermentation broth was developed using an ionic liquid-based salting-out extraction system.³⁷

Although we succeeded for the first time in converting glycerol to 2,3-BDO using *S. cerevisiae*, the flux of glycerol to ethanol using SK-FGG4 was faster than to 2,3-BDO by SK-MA1, SK-MA2, SK-MA3, and SK-MA4 (Fig. 3), which calls for further genetic modification to improve the productivity as well as the efficiency of 2,3-BDO synthesis, which is the aim of our future studies. *Schizosaccharomyces pombe* GDH has a much lower glycerol K_m value of 0.5 mM, and its substrate specificity toward 2,3-BDO is lower than that toward glycerol by 40%.³⁸ It may also be possible to adjust the substrate specificity of GDH as there is precedent where the coenzyme specificities of xylose reductase and xylitol dehydrogenase toward xylose and xylitol, respectively, were altered by site-directed mutagenesis^{39, 40} and the substrate preference of *Aspergillus sydowii* feruloyl esterase was similarly converted from methyl ferulate to methyl sinapate.⁴¹ Therefore, employing heterologous *SpGDH* or engineering the substrate specificity of *OpGDH* might overcome these limitations.

Conclusions

Glycerol, a renewable carbon source from the biodiesel industry, can be utilized to broaden carbon resources for the environmentally friendly production of various bio-based chemicals. This is the first study to explore the possibility of converting glycerol to 2,3-BDO using an engineered *S. cerevisiae* harboring 2,3-BDO biosynthesis enzymes without any *ScADH* or *ScPDC* deletions. Conversion of glycerol to 2,3-BDO without deleting *ScPDC* avoids *S. cerevisiae* growth deficiencies,

representing a unique example of glycerol utilization as the carbon source. Replacing *ScADH* with 2,3-BDO synthesis enzymes has higher conversion characteristics especially with the replacement of *ScADH2* in the SK-MA3 strain. Aerobic fermentation accumulates a higher concentration of acetoin, while microaerobic fermentation is a key to higher 2,3-BDO production and conversion efficiency. The engineered strain SK-MA3 showed the ability to co-convert glycerol and glucose, consuming 26.2 g/L and 19.9 g/L respectively, in YP_{D20G40} medium to a mixture of acetoin and 2,3-BDO. The maximum 2,3-BDO production titer was 15.5 g/L at 96 h while 5.5 g/L of acetoin was accumulated, representing 0.34g 2,3-BDO/g glycerol+glucose with total conversion reaching 0.456 g acetoin+2,3-BDO/g glycerol+glucose and 92% conversion efficiency. At the end of the fermentation, the 2,3-BDO concentration dropped to 10.8 g/L, while the acetoin concentration increased to 11.4 g/L with an extra 6.54 g/L of glycerol consumption. Dual substrate specificity of *OpGDH* toward glycerol and 2,3-BDO and their kinetics play a major role in the flux to 2,3-BDO from glycerol, acetoin accumulation, conversion rates, and efficiency.

Author contributions

S.M.R.K. conceived the research idea, planned the experiments, provided information regarding procurement of strains, chemicals, and toolboxes for genetic engineering, performed the experiments, and analyzed the results. S.M.R.K. also wrote, revised, and submitted the manuscript. M.A.A. conceived the research idea, planned the experiments, performed the experiments, analyzed the results, and revised the manuscript. M. K. in part provided the funds, discussed the results, and revised the manuscript. T.W. was responsible for financial support and provided the chemicals and equipment. T.W. was also involved in formulating the research idea, planning, and organization, discussion of the results, and manuscript revision and submission.

Conflicts of interest

There are no conflicts to declare.

Data availability

All necessary data required to assess our findings are available in this manuscript or its supplementary data. Further details related to this article may be requested from the authors

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Glycerol conversion to 2,3-butanediol by engineered *Saccharomyces cerevisiae*

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Supplementary Data

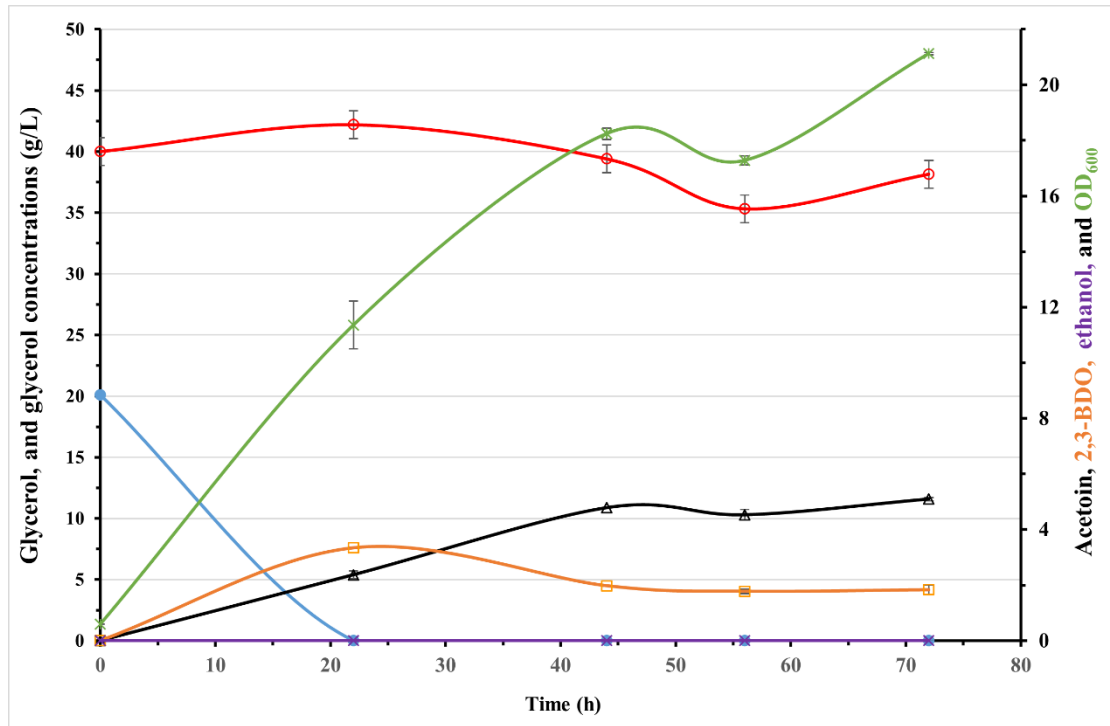


Fig. S1. Time course aerobic fermentation of glycerol-glucose mixture by SK-MA5, which replaced *ScADH1* with 2,3-BDO in the native strain (D452-2). The fermentation was carried out in 100-ml Erlenmeyer flasks containing a 10 mL culture of YPD_{D20G40} at 200 rpm orbital shaking and 30 °C. Glycerol (red open circular), glucose (sky blue closed circular), acetoin (black open triangular), 2,3-BDO (orange open squarer), and OD₆₀₀ (green star). The data represents the SD from the means of two independent experiments.

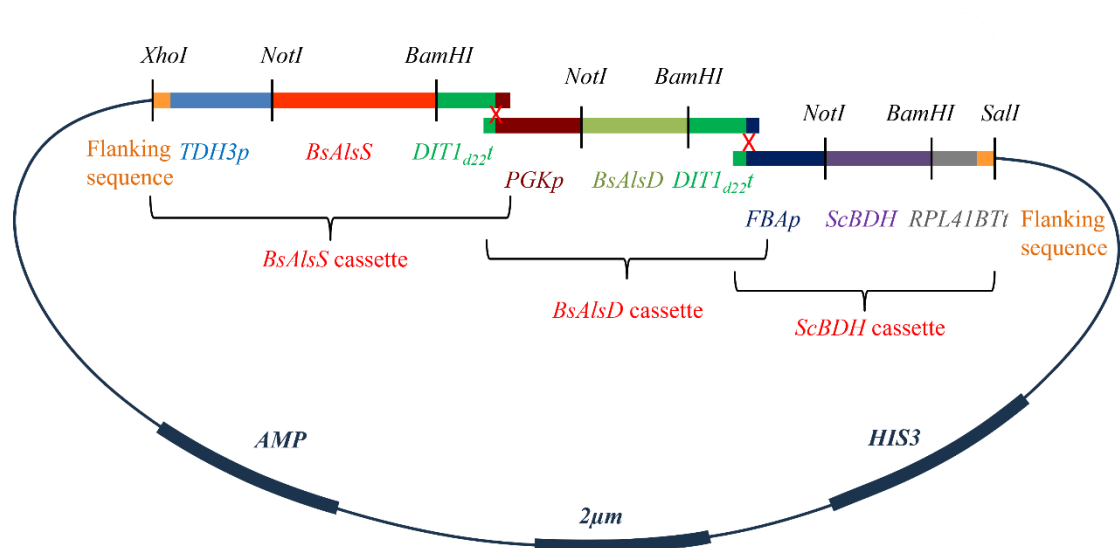


Fig. S2. Diagram illustrating the construction of the 2,3-BDO module in the HIS3 plasmid.

Table S1 Primers used in this study.

Primers used for assembling 2, 3 BDO modules/ plasmids.	
<i>XhoI</i> -upstream <i>ScADH1-TDH3p</i> FOR.	aaaactcgcagcacaatatctcaagctataccaagcatacaatcaactatctcatatacatcttcagttcgcagtttatca
<i>NotI</i> - <i>TDH3p</i> REV	tcatgcggccgcttggttgttatgtgtg
<i>NotI</i> - <i>ALsS</i> FOR	aaagcggccgcatgactaaagcaac
<i>BamHI</i> - <i>ALsS</i> REV	ttaggatccctacaaagccttgttttc
<i>NotI</i> - <i>BamHI-DIT1_{422t}</i> FOR.	ttttgcggccgcgatcctaaagtaagag
<i>PGKp</i> - <i>DIT1_{422t}</i> REV	cggcatcttggatgaaaagaaaggcaata
<i>DIT1_{422t}</i> - <i>PGKp</i> FOR	tcctttcatcaaatgacgatttgggcgc
<i>NotI</i> - <i>PGKp</i> REV	atgcggccgctgttttatattgtgttaa
<i>PGKp</i> - <i>AlsD</i> FOR	atataaacagcggccgcatgaaaagagaa
<i>BamHI</i> - <i>AlsD</i> REV	tttaggatccctactcagggagccctcag
<i>AlsD</i> - <i>BamHI</i> - <i>D22</i>	ccctgagtagggatcctaaagtaagagcgc
<i>FBA1p</i> - <i>DIT1_{422t}</i> REV	gtactgtcagttattgtatgatgaaaagaaaggc
<i>DIT1_{422t}</i> - <i>FBA1p</i>	ataacaatactgacagtactaaataattgcc
<i>BDH1</i> - <i>NotI</i> - <i>FBA1p</i> REV	atgcggccgcttgaatatgtattacttgg
<i>FBA1p</i> - <i>NotI</i> - <i>BDH1</i> FOR	catattcaaagcggccgcatgagagctttg
<i>BamHI</i> <i>BDH1</i> REV	cccggatccctacttcttccacgtgattg
<i>BamHI</i> - <i>RPL41BT</i> FOR	gtaaggatccgcggttagagcaaatcgt
Downstream <i>ScADH1-RPL41BT</i> REV.	ttgtcgcactattttttataacttatttaataaaaaatcataatcataagaaattcgcgaaaaatctttcaagc
Upstream <i>ScADH2-TDH3p</i> FOR.	atatcaagctcaaaaagcatacaatcaactatcaactattactatcgtaatacacatcttcagttcgcagtttatc
Downstream <i>ScADH2-RPL41BT</i> REV.	tactaatataggcatattgataatgaaaactataaatcgtaagacataagagatccgcgcgaaaaatctttcaagcac
Upstream <i>ScADH5-TDH3p</i> FOR.	ctctgattggaagatacctaaagaaattatttaactacatatctacaaatcaaagcatcttttcagttcgcagtttatc
Downstream <i>ScADH5-RPL41BT</i> REV.	tatctacaaaatagcttatataaaaaagtaaaaatatattcatcaaatcggtacaaaagaccgaaaaatctttcaagcac
<i>XhoI</i> -upstream <i>ScADH2</i>	aaaactcgcagatcaagctcaaaaagc
<i>Sall</i> -downstream <i>ScADH2</i>	ttgtcgcactactaatataggcatattg
<i>XhoI</i> -upstream <i>ScADH5</i>	aaaactcgcagctctgattggaagatacc
<i>Sall</i> -downstream <i>ScADH5</i>	ttgtcgcactatctacaaaatagcttat
Primers used for constructing multiplex pCas9 plasmids	
pCas9-gRNA target 146 <i>ScADH1</i> FOR.	tcacactgactgcacgctgttttagagctagaaatagc
pCas9-gRNA target 146 <i>ScADH1</i> REV.	agcgtgcaagtgcagtgtagacaaagtccttcgcccaccg
pCas9-gRNA target 640 <i>ScADH2</i> FOR.	gaattgtttacctgcctcgggttttagagctagaaatagc
pCas9-gRNA target 640 <i>ScADH2</i> REV.	ccgagcgcaggtaaacaattcaaaagtccttcgcccaccg
pCas9-gRNA target 160 <i>ScADH5</i> FOR.	agcgtgcaagtgcagtgtagacaaagtccttcgcccaccg
pCas9-gRNA target 160 <i>ScADH5</i> FOR.	tgccacgcgtgcaagtcactaaagtccttcgcccaccg
pCas9 FOR.	cggaaataggaaactcaagcg
pCas9 REV.	tttttctgcagcgaggagcc
Primers were used to confirm the homologous integration during the knocking out.	
271-downstream <i>ScADH1</i> REV.	gagattcatcaactcattgctggagtta
187- upstream <i>ScADH2</i> FOR.	ggtaaagctatagcatgcctatcacat
324-downstream <i>ScADH5</i> REV.	gcctatttcagttgtcttacgcacgc

Underlines indicate restriction sites and bold font points to the gRNA sequences.

Table S2. The sequence of 2,3-BDO module replacing *ScADH1*.

aaaa**ctc****gag****caca**atattt**caag**ctata**ccaag**cataca**aatca**ctat**ctc**tata**cat**tttt**tcag**t**cgag**tt**atc**attatca**actg**ccattt**caa**ga
 atac**g**taaa**ata**at**ta**atagtagtgatttt**cta**actt**tatt**ag**tca**aaaa**attag**cctt**ta**att**ctg**ctg**ta**acc**g**tacatg**cc**caaa**atag**gggg**g**cggt
 tacacag**aa**tata**ta**acat**cg**taggt**gt**ctgg**gtg**aacag**ttatt**cct**gg**catcc**act**aa**ata**at**gg**ag**ccc**gctt**ta**ag**ctg**gcat**cc**agaaaa
 aaa**ga**at**ccc**ag**cac**caaa**atatt**gttt**ctc**ac**ca**ac**cat**cag**tt**catag**gt**ccatt**ct**ctag**c**ga**act**acagag**aa**caggg**gc**acaa**cag**gc
 aaaa**a**cgg**gc**caca**ac**ct**aat**ggag**tgat**gca**acct**g**c**tg**ga**g**ta**at**gat**gacac**a**agg**ca**att**g**acc**ac**g**cat**gtat**ct**at**ct**at**tt**ct**tac**
 ac**tt**ct**tatt**ac**tt**ct**g**ct**ct**ct**g**att**gg**aaaa**ag**ctgaaaa**aa**ag**gtg**aa**acc**ag**tt**cc**ct**gaa**attatt**cc**ct**act**g**tact**aa**ag**at**atata**aa**
 gac**g**gtag**gt**att**g**att**g**ta**att**ct**g**ta**att**ct**att**ct**ta**aa**act**ct**ta**aa**tt**ct**act**tt**at**ag**ttag**ct**tttt**tt**ag**tt**ta**aa**cac**ca**ga**act**ag**tt**tc**ga
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