Identification and application of keto acids transporters in *Yarrowia lipolytica*

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Production of organic acids by microorganisms is of great importance for obtaining building-block chemicals from sustainable biomass. Extracellular accumulation of organic acids involved a series of transporters, which play important roles in the accumulation of specific organic acid while lack of systematic demonstration in eukaryotic microorganisms. To circumvent accumulation of by-product, efforts have been orchestrated to carboxylate transport mechanism for potential clue in *Yarrowia lipolytica* WSH-Z06. Six endogenous putative transporter genes, YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g and YALI0E32901g, were identified. Transport characteristics and substrate specificities were further investigated using a carboxylate-transport-deficient *Saccharomyces cerevisiae* strain. These transporters were expressed in *Y. lipolytica* WSH-Z06 to assess their roles in regulating extracellular keto acids accumulation. In a *Y. lipolytica* T1 line over expressing YALI0B19470g, \(\alpha\)-ketoglutarate accumulated to 46.7 g L\(^{-1}\), whereas the concentration of pyruvate decreased to 12.3 g L\(^{-1}\). Systematic identification of these keto acids transporters would provide clues to further improve the accumulation of specific organic acids with higher efficiency in eukaryotic microorganisms.
Results

Bioinformatics analysis of potential carboxylate transporters in Y. lipolytica. To screen putative carboxylate transporters, 6611 proteins encoded by Y. lipolytica CLIB122 genome were obtained from the UniProt database. Of these, 1104 proteins (Supplementary S1) were predicted as transmembrane proteins by TMHMM v.2.0 (Fig. 1A). Subsequently, 117 proteins were excluded from this set, due to the presence of a possible signal peptide at the N terminus, as predicted by SignalP 4.1. A sequence search using a conserved carboxylate transporter signature of JEN family, NXXX/THXS/TQDXXXT, identified six putative proteins encoded by the YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g, and YALI0E32901g genes, which exhibited high similarity to the signature sequence. A multiple sequence alignment of these sequences using homologous carboxylate transporter sequences from other fungi confirmed the presence of the conserved sequence (Fig. 1B).

In addition, a high level of sequence similarity among these putative transporters and the characterized carboxylate transporters was illustrated by two BLAST (http://blast.ncbi.nlm.nih.gov/) protocols, in which ScJen1p and KlJen2p were used as query sequences22–24. In these cases, the identity to ScJen1p and to KlJen2p were prospected, respectively (Fig. 2). In silico analysis of these protein sequences using the TMRPres2D tool revealed that the conserved sequence was located in the predicted transmembrane helices (Fig. 3). Hence, these transmembrane proteins were suspected to be potential carboxylate transporters.

Exogenous keto acid treatment. To uncover the response of carboxylate transporter candidates for uptake of exogenous keto acids, glucose-grown (repression condition) Y. lipolytica WSH-Z06 cells were transferred at the exponential phase to YPK or YPP medium (A large amount exogenous organic acids were accumulated in yeast cells, Supplementary S2). Initially, neither α-KG nor PA was detected in these cultures. During the first hour, the intracellular concentration of both carboxylates increased quickly to maximums of 3.90 μmol·(mg DCW)−1 for α-KG and 1.67 μmol·(mg DCW)−1 for PA in α-KG- and PA-treated cells, respectively. After incubation in YPK or YPP medium for 1 h, the concentration of α-KG gradually decreased 1 h to 3 h, until the minimum of 0.85 μmol·(mg DCW)−1 was reached. During this period, the content of PA also decreased to 0.23 μmol·(mg DCW)−1 (Fig. 4A).

To assay the effects of exogenous carboxylates on the expression profiles of these potential transporters, the expression of these genes were examined by qRT-PCR, using RNA extracted at 0 h as the control. When intracellular α-KG levels increased, the expression of YALI0D20108g, YALI0C21406g, YALI0D24607g, and YALI0E32901g decreased by 3.72 ± 0.91-fold, 3.88 ± 0.34-fold, 2.47 ± 0.52-fold, and 1.60 ± 0.18-fold, respectively, whereas the expression of YALI0B19470g and YALI0C15488g increased by 9.39 ± 0.82-fold and 4.32 ± 0.83-fold, respectively. Exogenous PA had a similar effect on expression, albeit to different levels. When incubated with PA, expression of YALI0D20108g, YALI0C21406g, YALI0D24607g, and YALI0E32901g decreased by 5.83 ± 0.81-fold, 4.04 ± 1.04-fold, 6.44 ± 0.98-fold, and 15.88 ± 1.94-fold, respectively, whereas expression of YALI0B19470g and YALI0C15488g increased by 2.83 ± 0.21-fold and 9.72 ± 0.22-fold, respectively (Fig. 4B). Hence, we concluded that transport of both keto acids should be predominantly mediated by YALI0B19470g and YALI0C15488g.

Assessment of putative genes using heterologous expression in S. cerevisiae. To assess their possible roles in carboxylate transport, null mutants of endogenous transporters were constructed by gene disruption in S. cerevisiae CEN.PK2-1D. The ScJEN1 deletion mutant displayed reduced growth on lactate, acetate, PA, malate,
Figure 2 | Phylogenetic analysis of putative transporters. The phylogenetic tree of carboxylate proteins was constructed using the neighbor-joining method. Bootstrap values >50% are shown at the branch points. One thousand bootstrap replications were performed using the MEGA 5.0 software.

Figure 3 | Sequence-structure mapping of putative transporters. (A) YALI0B19470p, (B) YALI0C15488p, (C) YALI0C21406p, (D) YALI0D24607p, (E) YALI0D20108p, (F) YALI0E32901p. The conserved signature sequence was illustrated in red in every putative carboxylate transporter.
and α-KG compared to the parental strain. To completely abolish the uptake by endogenous transporters, the Ady2p was also disrupted in W1 strain. In contrast, the W2 strain, in which both ScJEN1 and ScADY2 were disrupted, cannot grow on lactate, acetate, PA, malate, and α-KG containing media. Moreover, the parental strain as well as the W1 and W2 strains did not grow on citrate (Fig. 5). All putative transporter genes were introduced into the W2 strain by genetic transformation based on Δhis3 complementation. The resulting strains grew well on all sources including citrate, supporting the conclusion that these genes encode carboxylate transporters (Fig. 5).

**Substrate specificity assay.** To investigate the substrate specificity of these proteins, glucose-grown S. cerevisiae CEN.PK2-1D and mutants were incubated in YPA, YPL, YPP, YPM, YPK, and YPC medium for 2 h after exhaustion of the endogenous carbon source in saline water. The intracellular carboxylate content in S. cerevisiae CEN.PK2-1D cells was measured (Table 1), revealing that 2.22 ± 0.21 μmol·(mg·DCW)^{-1} lactate, 0.94 ± 0.14 μmol·(mg·DCW)^{-1} PA, 3.14 ± 0.24 μmol·(mg·DCW)^{-1} malate, and 1.29 ± 0.12 μmol·(mg·DCW)^{-1} α-KG were present. In addition, acetate and citrate were not detected in cells incubated in the corresponding carboxylate-containing medium. As no carboxylates were detected in the double deletion cells, we concluded that ScJen1p and ScAdy2p deletions influenced the transport of PA, malate, and α-KG.

Figure 4 | Time courses of intracellular carboxylate content in Y. lipolytica WSH-Z06. (A) Open square: α-KG, open circle: PA. The change of concentration of α-KG and PA exhibited the same trend during throughout the treatment. (B) Expression profile of putative transporter genes at 1 h, at which time the intracellular contents of α-KG and PA were greatest. White: PA treatment; black: α-KG treatment.

Figure 5 | Growth test of S. cerevisiae strains under different carboxylates. WT: S. cerevisiae CEN.PK2-1D; W1: S. cerevisiae CEN.PK2-1D Δjen1; W2: S. cerevisiae CEN.PK2-1D Δjen1Δady2; W3: S. cerevisiae CEN.PK2-1D Δjen1Δady2 (pY13-TEF1-470); W4: S. cerevisiae CEN.PK2-1D Δjen1Δady2 (pY13-TEF1-488); W5: S. cerevisiae CEN.PK2-1D Δjen1Δady2 (pY13-TEF1-406); W6: S. cerevisiae CEN.PK2-1D Δjen1Δady2 (pY13-TEF1-607); W7: S. cerevisiae CEN.PK2-1D Δjen1Δady2 (pY13-TEF1-108); W8: S. cerevisiae CEN.PK2-1D Δjen1Δady2 (pY13-TEF1-901). (A) YPD, (B) YPA, (C) YPL, (D) YPP, (E) YPM, (F) YPK, (G) YPC.
The transport of α-KG was restored in all heterogeneous transporter-containing strains, with the highest accumulation (1.41 μmol·(mg·DCW)⁻¹) occurring in the W4 strain. Among the set, only the W4 could accumulate PA intracellularly (0.17 μmol·(mg·DCW)⁻¹). Based on these observations, we conclude that YALI0B19470g and YALI0D24607g encode proteins that transport dicarboxylates and tricarboxylates. In addition to PA, W4 cells (containing YALI0C15488p) also accumulated acetate, malate, α-KG, and citrate, indicating the corresponding protein also transported these carboxylates. In a similar manner, we conclude the proteins encoded by the corresponding genes transport the following carboxylates: YALI0C21406p for lactate, malate, α-KG, and citrate; YALI0D20108p for lactate, α-KG and citrate, and YALI0E32901p for acetate, malate, and citrate.

### Copy number analysis of putative transporter genes in recombinant strains

In order to estimate copy number of each expression cassette that integrated in genome of recombinant strains, quantitative-PCR (qPCR) analysis was carried out. ACT1 was utilized as an endogenous control. With comparison to ACT1, no obvious relative changes of these transportsers were observed (Fig. 6). This indicated that these transporter genes were presented in a single copy in parental strain, respectively. After individual transportation of each endogenous transporters gene into cells of parental strain, relative changes of 2.54 ± 0.22-fold for YALI0B19470g, 2.33 ± 0.27-fold for YALI0C15488g, 2.62 ± 0.33-fold for YALI0C21406g, 2.28 ± 0.19-fold for YALI0D24607g, 2.39 ± 0.26-fold for YALI0D20108g and 2.55 ± 0.32-fold for YALI0E32901g were observed, which indicated one more copy of each transporter gene existed per genome in corresponding recombinant strain (Fig. 6).

### The effects of transporter genes on carboxylate accumulation in Y. lipolytica

As previously reported carboxylate transporter possess bidirectional functions for carboxylate influx and efflux. To clarify the roles of these transporters on carboxylate extracellular accumulation, they were overexpressed in Y. lipolytica WSH-Z06. The hp4d promoter was used to increase the transcription of these genes, and YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g and YALI0E32901g expression was improved by 23.34 ± 2.67-fold, 8.53 ± 0.90-fold, 9.32 ± 0.82-fold, 11.79 ± 1.32-fold, 3.37 ± 0.49-fold and 10.50 ± 0.97-fold, respectively (Fig. 7). Due to the flexible substrate specificity of these transporters, the accumulation of PA and α-KG increased in T5 cells, and the ratio of extracellular α-KG/PA decreased from 2.06 to 1.87 compared to the wild-type strain. This ratio also decreased in YALI0C15488g, YALI0C21406g, YALI0D24607g, and YALI0E32901g overexpressing strains, as only extracellular PA increased for T2, T3, T4, and T6. A competitive dual effect was observed for strain T1: the transport of α-KG increased dramatically, whereas the concentration of PA dropped by 30.6%, resulting in an increase in the ratio of extracellular α-KG/PA from 2.06 to 3.79 (Fig. 8).

The intracellular carboxylate content, Cin (μmol·(mg·DCW)⁻¹), was also determined (Table 2). The intracellular accumulation of α-KG decreased from 0.026 ± 0.005 μmol·(mg·DCW)⁻¹ to 0.014 ± 0.003 μmol·(mg·DCW)⁻¹ in T1.

### Table 1 | Intracellular carboxylate accumulation in S. cerevisiae CEN.PK2-1D and derivatives

| Strain | Genotype | Acetate | Lactate | PA | Malate | α-KG | Citrate |
|--------|----------|---------|---------|----|--------|------|--------|
| WT     | S. cerevisiae, CEN.PK2-1D | * | 2.22 ± 0.21 | 0.94 ± 0.12 | 3.14 ± 0.24 | 1.29 ± 0.12 | / |
| W1     | S. cerevisiae, CEN.PK2-1D Δjen1 | / | / | / | / | / | / |
| W2     | S. cerevisiae, CEN.PK2-1D Δjen1Δady2 | / | / | / | / | / | / |
| W3     | S. cerevisiae, CEN.PK2-1D, Δjen1Δady22pY13-TEF1-470 | / | / | / | / | / | / |
| W4     | S. cerevisiae, CEN.PK2-1D, Δjen1Δady22pY13-TEF1-488 | 0.81 ± 0.07 | / | 0.17 ± 0.03 | 0.11 ± 0.01 | 1.41 ± 0.19 | 0.08 ± 0.01 |
| W5     | S. cerevisiae, CEN.PK2-1D, Δjen1Δady22pY13-TEF1-406 | / | 0.53 ± 0.04 | / | 0.09 ± 0.01 | 0.77 ± 0.06 | 0.04 ± 0.01 |
| W6     | S. cerevisiae, CEN.PK2-1D, Δjen1Δady22pY13-TEF1-607 | / | / | 0.15 ± 0.01 | 0.09 ± 0.01 | 0.04 ± 0.01 | / |
| W7     | S. cerevisiae, CEN.PK2-1D, Δjen1Δady22pY13-TEF1-108 | 0.66 ± 0.04 | / | / | / | / | / |
| W8     | S. cerevisiae, CEN.PK2-1D, Δjen1Δady22pY13-TEF1-901 | / | / | / | / | / | / |

* Carboxylate content less than 0.005 μmol·(mg·DCW)⁻¹ or undetected. The intracellular carboxylate content was measured during substrate specificity tests and presented as μmol·(mg·DCW)⁻¹.

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Figure 6 | Copy number analysis of putative transporter genes in recombinant strains. Copy number of YALI0B19470g in T1, YALI0C15488g in T2, YALI0C21406g in T3, YALI0D24607g in T4, YALI0D20108g in T5 and YALI0E32901g in T6 were determined through qPCR analysis. As ACT1 existed in one copy per Y. lipolytica genome, copy number of putative transporter genes in wild-type strain (white) and recombinants (grey) was expressed as relative change. With comparison to ACT1, one more copy of each transporter gene was determined in corresponding recombinants.
The object of the current work was to screen and identify carboxylate transporters in Y. lipolytica WSH-Z06. RNA extracted from glucose-grown cells of the T1, T2, T3, T4, T5, and T6 strains was subjected to qRT-PCR. The strains T1-T6 were mutant variants in which plasmid p0(hph)-470, p0(hph)-488, p0(hph)-406 p0(hph)-607, p0(hph)-108 and p0(hph)-901 were individually expressed in the wild-type strain. The transcription of YALI0B19470g, YALI0C15488g, YALI0C21406g, YALI0D24607g, YALI0D20108g, and YALI0E32901g increased by 23.34 ± 0.002, 3.92 ± 0.029, 0.017 ± 0.003, and 0.002 ± 0.025 μmol·(mg·DCW)^{-1} in strains T1, T4, T5 and T6 respectively. Compared to the wild-type strain, the growth yield decreased from 11.17 ± 1.08 g·L^{-1} to 8.45 ± 0.72, 8.27 ± 0.89, 8.34 ± 0.76, 8.47 ± 0.61, 8.36 ± 0.88 and 7.40 ± 0.71 g·L^{-1} for strains T1, T2, T3, T4, T5 and T6, respectively, suggesting that enhanced carboxylate transportation caused the shift in carbon flux from cellular growth to extracellular carboxylate accumulation. The combination of increased carboxylate accumulation with decreased cell growth lead to a sharp increase in the yield of α-KG and PA, Y_{α-KG/DCW} and Y_{PA/DCW}. Finally, the ratio of extracellular carboxylate to intracellular carboxylate increased, and maximum values of 2399.71 ± 241.63 for α-KG and 1685.20 ± 208.56 for PA were observed for the T1 strain. Based on these observations, overexpression of YALI0A9470g was considered the best strategy to enhance α-KG transport and reduce PA accumulation.

**Discussion**

The object of the current work was to screen and identify carboxylate transporters, then determine whether the identified proteins regulate accumulation of non-target carboxylates. The duplication of transporters and the flexible substrate specificity demonstrated by the identified carboxylate transporters facilitated extracellular accumulation of carboxylates. Moreover, knowledge of the examined carboxylate transport mechanism is a prerequisite for improving carboxylate synthesis through metabolic engineering. The results provide new insights for regulating extracellular carboxylate accumulation in similar eukaryotic microorganisms.

To circumvent the issue of PA accumulation, previous studies have focused mainly on the regulation of intrinsic forces that redistribute carbon flux from other intermediates to α-KG production. Expression of PA carboxylase10, malate dehydrogenase, and fumarase2 dramatically decreased the accumulation of PA. A strategy to regulate co-factor regeneration resulted in remarkable reduction of extracellular PA13. However, as PA has a pivotal role in the regulation of carbon metabolism10, these modifications could not entirely overcome PA accumulation. As accumulation of carboxylate is believed to be a yeast defense response to severe environmental conditions22,

**Table 2 | The effects of transporter overexpression on carboxylate production in Y. lipolytica and derivatives**

| Strain | DCW(g·L^{-1}) | α-KG | PA | Y_{α-KG/DCW}(g·g^{-1}) | Y_{PA/DCW}(g·g^{-1}) | R_{α-KG/PA} | PA |
|--------|----------------|------|----|------------------------|------------------------|--------------|-----|
| WSH-Z06 | 11.17 ± 1.08   | 0.026 ± 0.005 | 0.034 ± 0.006 | 3.28 ± 0.32             | 0.23 ± 0.03            | 860.35 ± 78.45 | 531.45 ± 48.97 |
| T1     | 8.45 ± 0.72    | 0.014 ± 0.002 | 0.009 ± 0.002 | 4.94 ± 0.52             | 0.30 ± 0.04            | 2399.71 ± 241.63 | 1685.20 ± 208.56 |
| T2     | 8.27 ± 0.89    | 0.027 ± 0.003 | 0.032 ± 0.004 | 3.90 ± 0.42             | 0.25 ± 0.03            | 975.27 ± 109.21 | 769.61 ± 84.21 |
| T3     | 8.34 ± 0.76    | 0.020 ± 0.005 | 0.033 ± 0.005 | 4.13 ± 0.44             | 0.24 ± 0.02            | 938.76 ± 96.32  | 779.42 ± 94.38 |
| T4     | 8.47 ± 0.61    | 0.023 ± 0.003 | 0.029 ± 0.003 | 3.91 ± 0.38             | 0.31 ± 0.04            | 1161.29 ± 125.76 | 865.52 ± 90.76 |
| T5     | 8.36 ± 0.88    | 0.013 ± 0.002 | 0.017 ± 0.002 | 4.71 ± 0.46             | 0.59 ± 0.06            | 2358.20 ± 359.79 | 1641.53 ± 178.94 |
| T6     | 7.40 ± 0.71    | 0.020 ± 0.002 | 0.025 ± 0.002 | 5.27 ± 0.61             | 0.36 ± 0.04            | 1756.27 ± 193.1 | 1343.41 ± 147.89 |

The strains T1–T6 were mutant variants in which plasmid p0(hph)-470, p0(hph)-488, p0(hph)-406 p0(hph)-607, p0(hph)-108 and p0(hph)-901 were individually expressed in the wild-type strain. R_{α-KG/PA} is the mass ratio of extracellular carboxylate to intracellular carboxylate. The yield of Y_{α-KG/DCW} and Y_{PA/DCW} is the mass ratio of α-KG to DCW and PA to DCW, respectively.
regulation of carboxylate transportation process might be another potent route for enhancement of α-KG production.

The carboxylate transport process of yeast is an intensively investigated field[28]. The *S. cerevisiae* ScJEN1 and ScADY2 genes were identified as key carboxylate transporters[29]. The duplication of transporters has been strongly implicated in the utilization of organic acids as a carbon source[24]. In *Kluyveromyces lactis*, the presence of two carboxylate transporters, Jen1p and Jen2p, guaranteed efficient uptake of lactic acid as a substrate from a lactic-acid-producing habitat[31]. As the cells of *Y. lipolytica* harbored a powerful potential to use a wide range of substrates as a sources of carbon and energy[31], these results indicated that powerful carboxylate transporters that maintain intracellular environment homeostasis. A previous study reported that reduction of by-product resulted in enhanced synthesis of target carboxylate by de-repression of the feedback inhibition[3]. It was speculated that the enhanced synthesis of α-KG could be achieved through de-repression of the feedback inhibition by efflux of intermediates.

In *Y. lipolytica*, efficient carboxylate transport was achieved by the duplication of *iso*-functional transporters. Evolution analysis and motif identification confirmed that a precursor form of Jen1p, preJen1p, arose from the duplication of an ancestral Jen2p[29,32]. In *S. cerevisiae*, the transport capacity and substrate affinity of Jen1p were determined by the conserved NXXS/THXS/TQDXXT sequence[33]. Presence of this signature sequence also determined the flexibility of substrate specificity for these transporters. Previously, Jen1p was induced by lactate, PA, and propionate, whereas Ady2p and Jen2p were induced by acetate[29,30]. Expression of transporters from *Y. lipolytica* displayed different responses to exogenous carbon source, and single carboxylate induced multiple transporters.

The roles of these carboxylate transporters were assayed via double their copy numbers in the genomic DNA of α-KG producer. One more copy of each endogenous carboxylate transporter was observed (Fig. 6). Our observation that overexpression of carboxylate transporters resulted in enhanced accumulation of extracellular carboxylates (Fig. 7). Previously, uncovered the mechanism for malate efflux was mediated by monoanionic malate concentration gradient, in which the proton symport was major force[25]. Similar to the observations that production of carboxylate was benefited from over-expression of carboxylate transporter[12,24], the efflux of carboxylate accompanied by symport of proton was speculated[25]. Based on measurement of total content of mixture of monoanionic, dianionic and undissociated form of the carboxylates, the intracellular content of carboxylates was not correlated with extracellular carboxylate content. While, the reported carboxylate transporter was specific for monoanionic form of carboxylate for efflux, the efflux specificity of this series of transporter would be the key to the contradictory observation in future studies.

Methods

Strains and plasmids. All strains used in this study were summarized in Table 3. *Y. lipolytica* WSH-20b, an α-KG producing wild-type strain, was previously screened by our lab[12]. The plasmid p0(hph) was previously constructed based on plasmid p0. The p0 could integrate a single copy of exogenous sequence via recombinant at the locus of *XRP2* in the genome of *Y. lipolytica*[24]. The hygromycin phosphotransferase encoded by *hph* was amplified from plasmid pUB4-CRE and was used to replace the *URA3* sequence in *Y. lipolytica*[24]. The *pY13-TEF1* plasmid was used for heterologous expression of potential transporters.

### Table 3 | Strains used in this study

| Strain | Genotype and remarks |
|--------|---------------------|
| *S. cerevisiae* CEN.PK2-1D | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2 |
| W1     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δjen1*:URA3 |
| W2     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δadj2*:LEU2 |
| W3     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δjen1*:URA3; Δadj2*:LEU2[pY13-TEF1:470] |
| W4     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δjen1*:URA3; Δadj2*:LEU2[pY13-TEF1:488] |
| W5     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δjen1*:URA3; Δadj2*:LEU2[pY13-TEF1:406] |
| W6     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δjen1*:URA3; Δadj2*:LEU2[pY13-TEF1:607] |
| W7     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δjen1*:URA3; Δadj2*:LEU2[pY13-TEF1:108] |
| W8     | MATα *ura3-52, Δtrp1-289; Δleu2-3,112; Δhis3-1; MAL28*; SUC2; Δjen1*:URA3; Δadj2*:LEU2[pY13-TEF1:901] |
| *Y. lipolytica* WSH-Z06 | Wild-type |
| T1     | Wild-type[p(hph)-470] |
| T2     | Wild-type[p(hph)-488] |
| T3     | Wild-type[p(hph)-406] |
| T4     | Wild-type[p(hph)-607] |
| T5     | wild-type[p(hph)-108] |
| T6     | Wild-type[p(hph)-901] |

### Table 4 | Oligonucleotide primers used for qRT-PCR

| Gene     | Primers          | Sequence (5’-3’) | Product size (bp) |
|----------|------------------|-----------------|------------------|
| YALI0B19470g | YALI0B19470F | CAAAAGGAGAACAAAGAGC | 153 |
| YALI0C15488g | YALI0C15488F | CAAAACCATCAGCCATC | 199 |
| YALI0C21406g | YALI0C21406F | GCAGACCATCAGCAGC | 171 |
| YALI0D20108g | YALI0D20108F | TGCTACAGGAGGCTATGC | 135 |
| YALI0D24607g | YALI0D24607F | GCAGTCCAGTGAGGAGC | 104 |
| YALI0E32901g | YALI0E32901F | GACCTCCCTAAGGTCCT | 188 |
| ACT1     | ACT1-F          | AAGGCAAGGAGGAGATG | 132 |
| ACT1     | ACT1-R          | ACCAGAGTCAAGAAGC |
**Table 5 | Oligonucleotides used for gene disruption in S. cerevisiae**

| Primers | Sequence(5'→3') | Description |
|---------|-----------------|-------------|
| JEN-LF  | GGGATCCATGCGTCTGCAATTACAGATG | BamHI |
| JEN-LN  | ATGGCGCTAAAGGACGTAACATC | EcoRI |
| JEN-RF  | AAAGGCTATATATTGGGCGG |  |
| JEN-RN  | GAAATTTGTCACAAACTCTCAATTC |  |
| JEN-MF  | GATCCACCTCTACTTGGACTCGTAGGGGA | AvrII |
| JEN-MR  | CGGCAACATTAAATGCGCTTTGAGTGATGAATACC |  |
| ADY-LF  | GGTATCCATGTCGTAATTACAGATG | BamHI |
| ADY-LR  | CCACCTAAAAACATGACACACC |  |
| ADY-RF  | GATTGCTGGTATTTGGGAGATAG |  |
| ADY-RN  | CCTAGGGCTCTTGGATAGTGAATGG |  |
| ADY-MF  | CGGTGCTATGTTTATGGTGGGCAAGAAC |  |
| ADY-MR  | CATACTCCAAAATACGAACTCGTGAAGCTTCGTACGC |  |
| ADY-RI  | GAAATTCTTATCGAGTATCGCTCGAAGAAC |  |

**Media and culture conditions.** E. coli was cultured in Luria broth (LB) medium (5 g L⁻¹ yeast extract, 10 g L⁻¹ peptone, 10 g L⁻¹ NaCl) supplemented with 100 mg L⁻¹ ampicillin when necessary. Yeast strains were cultured in YPD medium (10 g L⁻¹ yeast extract, 20 g L⁻¹ peptone, and 20 g L⁻¹ dextrose) or YNB medium (20 g L⁻¹ glucose, 1.7 g L⁻¹ yeast nitrogen base without amino acids, 5 g L⁻¹ (NH₄)₂SO₄ supplemented with 50 mg mL⁻¹ uracil, leucine, tryptophan and histidine when necessary. The following media were used for carbohydrate treatment: YPA (1.7 g L⁻¹ yeast nitrogen base without amino acids, 5 g L⁻¹ (NH₄)₂SO₄, 50 g L⁻¹ malt, pH 7.0), YPM (1.7 g L⁻¹ yeast nitrogen base without amino acids, 5 g L⁻¹ (NH₄)₂SO₄, and 100 g L⁻¹ α-KG, pH 7.0), YPC (1.7 g L⁻¹ yeast nitrogen base without amino acids, 5 g L⁻¹ (NH₄)₂SO₄, 30 g L⁻¹ malt, pH 7.0), YPK (10 g L⁻¹ yeast extract without amino acids, 5 g L⁻¹ (NH₄)₂SO₄, and 100 g L⁻¹ α-KG, pH 7.0), YPA, YPL, YPP, YPM, YPK, and YPC media, supplemented as necessary with 50 mg L⁻¹ uracil, leucine, tryptophan, and histidine, were used to assay carbohydrate transportation of S. cerevisiae CEN.PK2-1D cells and derivatives.

Yeast cells were harvested by centrifugation at 10,000 × g for 10 min, and washed twice with cold distilled water. The dry cell weight was determined according to protocol described previously. The intracellular carbohydrate content was determined according to the method previously, with little modification. Harvested cells were stored in liquid nitrogen until extraction of intracellular organic acid which was followed by cell disruption of according to a protocol described previously. Supernatants were used for determination of intracellular carbohydrate concentrations by high-performance liquid chromatography (HPLC). The intracellular concentration of carbohydrate was expressed in μmol·(mg DCW)⁻¹·h⁻¹.

**Quantitative real-time PCR analysis.** The cells of Y. lipolytica WSH-2 were harvested, centrifuged at 10,000 × g for 10 min, and immediately frozen in liquid nitrogen until RNA extraction. Total RNA was extracted using Trizol reagent (Life Technologies, Carlsbad, CA), according to the manufacturer’s instructions. cDNA was synthesized from 5 μg total RNA using the PrimeScript RT Reagent Kit Perfect Real Time (Takara, Dalian, China). qRT-PCR was performed with the synthesized cDNA and primers listed in Table 4 using the SYBR Premix Ex Taq™ kit (Takara, Dalian, China) and a LightCycler 480 II Real-time PCR instrument (Roche Applied Science, Mannheim, Germany). All experiments were performed in triplicate and triplicate values were used for further calculations. Fold changes were determined by the 2⁻ΔΔCt method and normalized to the ACT1 gene.

**Disruption of the ScJEN1 and ScADY2 genes in S. cerevisiae.** To disrupt the S. cerevisiae CEN.PK2-1D JEN1 and ADY2 genes, two disruption cassettes were constructed using a protocol described previously. TheloxP-URA3-loxP and loxP-LEU2-loxP modules were amplified from plasmids pUG72 and pUG73, respectively, with oligonucleotides JEN-MF/JEN-MR, ADY-MF/ADY-MR, respectively (Table 5). Two fragments ofScJEN1 were amplified from S. cerevisiae genomic DNA and were subsequently flanked by theloxP-URA3-loxP module to generate the JEN1 disruption cassette. ScADY2 fragments, PCR-amplified from S. cerevisiae genomic DNA, were flanked with theloxP-LEU2-loxP module to generate theADY2 disruption cassette. The disruption cassettes were introduced into S. cerevisiae CEN.PK2-1D with a previously described protocol, and the resulting line, S. cerevisiae CEN.PK2-1D Δjen1, is referred to as W1. The ADY2 disruption cassette was introduced into this W1 strain, and the resulting double deletion strain is referred to as W2.

**Table 6 | Oligonucleotides used for gene expression in S. cerevisiae**

| Primers | Sequence(5'→3') | Description |
|---------|-----------------|-------------|
| YAU0D20108gF2 | CGGGATCCATGCGTCTGCAATTACAGATG | BamHI |
| YAU0D20108gR2 | GGAATTCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0C21406gF2 | CGGGATCCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0C21406gR2 | GGAATTCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0B19470gF2 | CGGGATCCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0B19470gR2 | GGAATTCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0C15488gF2 | CGGGATCCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0C15488gR2 | GGAATTCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0D224607gF2 | CGGGATCCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0D224607gR2 | GGAATTCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0E32901gF2 | GGAATTCATGCGTCTGCAATTACAGATG | EcoRI |
| YAU0E32901gR2 | GGAATTCATGCGTCTGCAATTACAGATG | EcoRI |

*Note: S. cerevisiae refers to the yeast species used in the experiments described in the table.*
Table 7 | Oligonucleotides used for gene expression in Y. lipolytica

| Primers | Sequence (5'-3') | Description |
|---------|-----------------|-------------|
| YAL10D20108gF1 | CCGAGATCCTAGATTGACACTACCCCG | BamHI |
| YAL10D20108gR1 | GGAATCTCTAGAGATGCTGTCGCAAGAC | EcoRI |
| YAL10D21406gF1 | GGAATCTCTAGAGATGCTGTCGCAAGAC | EcoRI |
| YAL10D21406gR1 | TIGCGCGCGCTGCACTTTTTGGATTGCGG | NotI |
| YAL10B19470gF1 | CGGAGATCCTAGATTGACACTACCCCG | BamHI |
| YAL10B19470gR1 | GGAATCTCTAGAGATGCTGTCGCAAGAC | EcoRI |
| YAL10C15488gF1 | GGATCCATGACCCAGTCGTCAAGTC | EcoRI |
| YAL10C15488gR1 | GGATCCATGACCCAGTCGTCAAGTC | EcoRI |
| YAL10E23901gF1 | GGAATCTCTAGAGATGCTGTCGCAAGAC | BamHI |
| YAL10E23901gR1 | GGAATCTCTAGAGATGCTGTCGCAAGAC | EcoRI |
| VB-F | CGTTTTCGACCCGACCAGAT | |
| VB-R | GCGTGTGCAAGCCGGACAGAT | |
| YAL10C15488gF1 | TTGATAGCTGCAAGCCGGACAGAT | |
| YAL10C15488gR1 | TTGATAGCTGCAAGCCGGACAGAT | |
| YAL10E23901gF1 | GAAAGCAAGGCGGTGATACCG | |
| YAL10E23901gR1 | GAAAGCAAGGCGGTGATACCG | |
| YAL10E23901gF1 | GAAAGCAAGGCGGTGATACCG | |
| YAL10E23901gR1 | GAAAGCAAGGCGGTGATACCG | |

**Heterologous expression of putative transporter genes in S. cerevisiae W2.** Six putative transporter genes, YAL10B19470g, YAL10C15488g, YAL10C21406g, YAL10D24607g, YAL10D2108g, and YAL10E23901g, were amplified from Y. lipolytica WSH-Z06 genomic DNA with the primers listed in Table 7. The amplified fragments were digested into BamHI–EcoRI sites of the pY13-TEF1 expression vector, resulting in the pY13-TEF1-470, pY13-TEF1-488, pY13-TEF1-406, pY13-TEF1-607, pY13-TEF1-108, and pY13-TEF1-901 plasmids, respectively. These vectors were introduced into S. cerevisiae W2 using a previously described protocol. The resulting transformants, Y. lipolytica T1, T2, T3, T4, T5, and T6, respectively, were screened on YPD plates containing 400 µg mL⁻¹ hygromycin B and verified with the oligonucleotides listed in Table 7.

**Copy number analysis.** In order to determine the copy number of the integrative expression cassettes in the recombinant strains, a qPCR analysis was performed on the genomic DNA template using ACT1 as the internal control. Genomic DNA from parental strain (WSH-Z06) and six recombinant strains (T1, T2, T3, T4, T5, and T6) were isolated after disruption of yeast cells with glass beads (Sigma-Aldrich, St. Louis, MO) by FastPrep 24 MP Biomedicals, Santa Ana, CA). qPCR was performed with the 5 ng genomic DNA and primers listed in Table 4 using the SYBR Premix Ex Taq™ Kit (Takara, Dalian, China) and a LightCycler 480 II Real-time PCR instrument (Roche Applied Science, Mannheim, Germany). All experiments were performed in triplicate and mean values were used for further calculations. Fold changes were determined by the 2⁻ΔΔCT method and normalized to the ACT1 gene.

**Shake flask culture.** Shake flask culture was performed in 500 mL flasks containing 50 mL fermentation medium following the protocol stated previously. A yeast seed culture was performed in 500 mL flasks containing 50 mL fermentation medium. An inoculum volume of 10% (v/v) was used for 18 h on a rotary shaker at 28°C. The culture was used to inoculate 500 mL flasks containing 50 mL fermentation medium. An inoculum volume of 10% (v/v) was used for 0.02 µm filtration. Cell suspensions were centrifuged at 100 × g for 10 min. The supernatant was diluted 50 times and filtered through a membrane (pore size = 0.22 µm). α-KG, pyruvic acid, acetate, lactate, and citrate present in the supernatant were simultaneously determined by HPLC (Aglient 1200 series, Santa Clara, CA, USA) with an Aminex HPX-87H column (300 mm × 7.8 mm; Bio-Rad Laboratories Inc., Hercules, CA, USA). The mobile phase was 5 mmol L⁻¹ sulfuric acid in distilled, de-ionized water filtered to 0.22 µm. The mobile phase flow rate was 0.6 mL min⁻¹. The column temperature was maintained at 35°C, and the injection volume was 10 µL. α-KG, pyruvic acid, acetate, lactate, and citrate were detected with a UV detector (wavelength at 210 nm). To determine the intracellular carbohydrates, cells taken from shake flask culture were disrupted and lysates were centrifuged at 10,000 × g for 10 min. The carbohydrate content in supernatant was determined by HPLC followed the protocol above.

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