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RESEARCH

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Enhancing D-lactic acid production by optimizing the expression of *D-LDH* gene in methylotrophic yeast *Komagataella phaffii*

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Abstract

Background Currently, efficient technologies producing useful chemicals from alternative carbon resources, such as methanol, to replace petroleum are in demand. The methanol-utilizing yeast, *Komagataella phaffii*, is a promising microorganism to produce chemicals from methanol using environment-friendly microbial processes. In this study, to achieve efficient D-lactic acid production from methanol, we investigated a combination of D-lactate dehydrogenase (*D-LDH*) genes and promoters in *K. phaffii*. The yeast strain was constructed by integrating a gene cassette containing the identified gene and promoter into the rDNA locus of *K. phaffii*, followed by post-transformational gene amplification. Subsequently, D-lactic acid production from methanol was evaluated.

Results Among the five *D-LDH* genes and eight promoters tested, the combination of LlDLDH derived from *Leuconostoc lactis* and *CAT1* and *FLD1* promoters was suitable for expression in *K. phaffii*. GS115_CFL/Z3/04, the best-engineered strain constructed via integration of LlDLDH linked to *CAT1* and *FLD1* promoters into the rDNA locus and post-transformational gene amplification, produced 5.18 g/L D-lactic acid from methanol. To the best of our knowledge, the amount of D-lactic acid from methanol produced by this engineered yeast is the highest reported value to date when utilizing methanol as the sole carbon source.

Conclusions This study demonstrated the effectiveness of combining different enzyme genes and promoters using multiple promoters with different induction and repression conditions, integrating the genes into the rDNA locus, and further amplifying the genes after transformation in *K. phaffii*. Using our established method, other *K. phaffii* strains can be engineered to produce various useful chemicals in the future.

Keywords D-Lactic acid, *Komagataella phaffii*, Methanol, Metabolic engineering, Yeast

Background

Currently, technologies producing various useful chemicals from alternative carbon resources to replace petroleum are in demand [1]. Methanol has attracted considerable attention as an alternative carbon source as it is a liquid at room temperature, easy to transport, and stable [2]. In recent years, development of new production technologies with low environmental impact, such as the production of methanol from CO_2 and methane, has increased [3, 4]. Currently, production of chemicals from methanol is performed using chemical processes with high environmental impact, and methanol is converted into various chemical products via ethylene and propylene.

Chemical processes require high-temperature and high-pressure conditions, exhibit low reaction specificities, and are not suitable for producing compounds with complex structures or high optical purity. In contrast,

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chemical production using microbial metabolism allows reactions to proceed under mild conditions and facilitates the selective production of compounds with high reaction specificity and optical purity. Because of these advantages, research on the production of useful chemicals from methanol using methanol-utilizing microorganisms has increased in recent years [5]. Many microorganisms can utilize methanol, including bacteria, such as *Methylobacterium extorquens* and *Bacillus ethanolicus*, and yeasts, such as *Komagataella phaffii* (formerly known as *Pichia pastoris*), *Candida boidinii*, and *Ogataea polymorpha*. These microorganisms metabolize methanol via the serine, ribulose phosphate, and xylose phosphate pathways [6–8]. These methylotrophic microorganisms can be used to produce useful compounds, such as mevalonic acid [9] and fatty alcohols [10], from methanol.

Lactic acid is used as a chemical in the food and pharmaceutical industries and as a raw material for polylactic acid, a biodegradable plastic [11–14]. Thermal stability of the polymer is improved when the enantiomers, poly(L-lactic acid) and poly(D-lactic acid), are mixed to form a stereocomplex structure [15]. Consequently, the demand for both L- and D-lactic acids is increasing [16], but the availability of D-lactic acid is lower than that of L-lactic acid in the market as D-lactic acid is more expensive. Therefore, an efficient method for D-lactic acid production is required.

K. phaffii, a type of methanol-utilizing yeast, is safe and used in well-established genetic engineering techniques as a host for protein production [17]. Recently, research on the development of recombinant *K. phaffii* capable of producing useful chemicals, such as malic acid [18] and lycopene [19], from methanol is attracting attention [20]. In *K. phaffii*, integration of a multicopy plasmid into the non-transcribed spacer of the ribosomal DNA (rDNA) locus leads to high mitotic stability and gene expression [21]. Additionally, a method for increasing gene expression based on the integration of genes into the rDNA locus and amplification of the gene copy number after transformation via repeated selection in media containing increasing antibiotic concentrations has been reported [22]. Lopes et al. reported that intracellular protein production is highly correlated with the copy number of the gene encoding the protein [23]. Yamada et al. first reported the production of D-lactic acid from methanol by integrating multiple copies of a gene cassette linking the *AOX1* promoter to the D-lactate dehydrogenase (*D-LDH*) gene derived from *Leuconostoc mesenteroides* at the rDNA locus [24]. However, the maximum reported titer of D-lactic acid from methanol is 3.48 g/L, which is lower than that obtained with sugar as the carbon source [25, 26].

Gene expression can be improved by optimizing the origin of the expressed gene and combination of the promoter, terminator, and secretion signal sequences. Zhong et al. [27] and Watcharawipas et al. [28] investigated the expression of *D-LDH* gene in yeast *Saccharomyces cerevisiae* and reported that the origin of *D-LDH* gene affects D-lactic acid production. Furthermore, previous studies reported that the combination of promoters, terminators, and secretory signal sequences in *S. cerevisiae* and *K. phaffii* significantly affects lipase expression [29, 30].

In this study, we investigated the combination of five *D-LDH* genes of different origins and eight promoters in *K. phaffii* to achieve efficient D-lactic acid production from methanol. The five *D-LDH* genes were *LdDLDH* (*Lactobacillus delbrueckii*) [31], *LmDLDH* (*L. mesenteroides*) [24], *LlDLDH* (*L. lactis*) [28], *LpDLDH* (*L. pseudomesenteroides*) [28], and *SlDLDH* (*Sporolactobacillus laevolacticus*) [32]. These *D-LDH* genes are also expressed in *S. cerevisiae* or *Escherichia coli* [28, 33, 34]. The eight promoters consist of four methanol-inducible promoters (*AOX1* promoter [pAOX1], *CAT1* promoter [pCAT1], mutant *CAT1* promoter [pCATm] [35], and *FLD1* promoter [pFLD1] [36]) and four constitutive promoters (*GAP1* promoter [pGAP1], A*DH3* promoter [pADH3] [37], mutant *GAP1* promoter [pGAP1m] [38], and *TEF1-α* promoter [pTEF1] [39]). Using these combinations, we identified the *D-LDH* gene and promoter combination suitable for expression in *K. phaffii*. Finally, the engineered yeast was constructed by integrating a gene cassette containing the identified gene and promoter into the rDNA locus of *K. phaffii*, followed by post-transformational gene amplification, and D-lactic acid production from methanol was evaluated.

Methods

Strains and media

E. coli strain NEB5α (New England Biolabs Japan, Tokyo, Japan) was used as a host for recombinant DNA manipulation. Genetically modified *E. coli* cells were cultured in the Luria–Bertani medium (20 g/L Luria–Bertani broth powder [Nacalai Tesque, Kyoto, Japan]) supplemented with 100 μg/mL ampicillin sodium salt (Nacalai Tesque).

K. phaffii GS115 (Thermo Fisher Scientific, Yokohama, Japan) was used as the host for D-lactic acid production. *K. phaffii* cells were cultured in the yeast/peptone/glucose (YPD) medium (10 g/L yeast extract [Formedium, Norfolk, UK], 20 g/L peptone [Formedium], and 20 g/L glucose [Nacalai Tesque]), yeast/peptone/methanol (YPM) medium (10 g/L yeast extract, 20 g/L peptone, and 30 g/L methanol [Nacalai Tesque]), yeast/peptone/dextrose/sorbitol (YPDS) medium (10 g/L yeast extract, 20 g/L peptone, 20 g/L glucose, and 1 mol/L sorbitol [Nacalai Tesque]), RDB

medium (182 g/L sorbitol, 10 g/L glucose, 13.4 g/L yeast nitrogen base without amino acid [Formedium], and 4×10^{-4} g/L biotin [Nacalai Tesque]), MD medium (13.4 g/L yeast nitrogen base without amino acids, 20 g/L glucose, and 4×10^{-4} g/L biotin), or MM medium (13.4 g/L yeast nitrogen base without amino acids, 30 g/L methanol, and 4×10^{-4} g/L biotin). As required, 20 g/L agar (Nacalai Tesque) and a predetermined amount of zeocin (InvivoGen, San Diego, CA, USA) were added.

Yeast cultivation

Ninety-six deep-well culture was performed using 1 mL of YPM medium and Deep Well Maximizer (Taitec, Nagoya, Japan) at 30 °C and 1200 rpm. Cultivation was initiated by culturing the cells in a well containing 1 mL of YPD medium at 30 °C and 1200 rpm for 24 h, harvesting and washing the cells, and suspending them in 1 mL of fresh medium.

Test tube cultures were performed using 5 mL of the medium in test tubes (165 mm height and 16 mm diameter) and reciprocating shaker (Taitec) at 30 °C and 150 rpm. Cultures were initiated by inoculating the precultures grown in test tubes containing the MD medium at 30 °C and 150 rpm for 72 h (initial OD_{600}: 3.0).

Flask cultures were performed using a rotary shaker (Taitec) at 30 °C and 200 rpm, with 250-mL flasks equipped with a gas-permeable seal (EXCEL scientific, Victorville, CA, USA) containing 50 mL of medium. Cultures were initiated by inoculating (initial OD_{600}: 11.0) the precultures grown in 250 mL flasks containing the YPD medium for 24 h at 200 rpm and 30 °C.

Plasmid construction and yeast transformation

All plasmids and primers used in this study are listed in Table 1 and Table S1, respectively. pPPE_AOX_XDLDH (X, Ld, Ll, Lp, or Sl), the plasmid used for *D-LDH* expression, was constructed as described below. Plasmid pPPE [30] was linearized using the restriction enzymes, *EcoRI* and *NotI*. Fragments of *D-LDH* gene from *L. mesenteroides* linked to pAOX1 were obtained via polymerase chain reaction (PCR) amplification using plasmid pRDZ_AO_DLDH [24] as the template and pAOX_pPPE(F)_ASS and LmeDLDH_pPPE(R)_ASS as the primers. The resulting gene fragments were ligated using the NEBuilder HiFi DNA Assembly Master Mix (New England Biolabs Japan) to construct the pPPE_AOX_LmDLDH plasmid.

Genes encoding LdDLDH, LlDLDH, LpDLDH, and SlDLDH were PCR-amplified using synthetic genes (Twist Bioscience, South San Francisco, CA, USA) as templates and LdDLDH(F)_ASS and LdDLDH(R)_ASS, LlDLDH(F)_ASS and LlDLDH(R)_ASS, LpDLDH(F)_ASS and LpDLDH(R)_ASS, and SlDLDH2Mu(F)_ASS and SlDLDH2Mu(R)_ASS, respectively, as primers. pPPE_AOX_LmDLDH was linearized using the restriction enzymes, *BamHI* and *NotI*. The resulting gene fragments were used to construct the pPPE_AOX_LdDLDH, pPPE_AOX_LlDLDH, pPPE_AOX_LpDLDH, and pPPE_AOX_ SlDLDH plasmids, respectively, by ligating the gene fragments using the NEBuilder HiFi DNA Assembly Master Mix.

pPPE_X_LlDLDH (X; ADH, CAT, CATm, FLD, GAP, GAPm, TEF), the plasmid used for promoter

Table 1 List of plasmids used in this study

Plasmid name	Relevant features
pPPE_AOX_Ld	Episomal plasmid for expression of LdDLDH (derived from *Lactobacillus delbrueckii*) by *AOX1* promoter
pPPE_AOX_Ll	Episomal plasmid for expression of LlDLDH (derived from *Leuconostoc lactis*) by *AOX1* promoter
pPPE_AOX_Lm	Episomal plasmid for expression of LmDLDH (derived from *Leuconostoc mesenteroides*) by *AOX1* promoter
pPPE_AOX_Lp	Episomal plasmid for expression of LpDLDH (derived from *Leuconostoc pseudomesenteroides*) by *AOX1* promoter
pPPE_AOX_Sl	Episomal plasmid for expression of SlDLDH (derived from *Sporolactobacillus laevolacticus*) by *AOX1* promoter
pPPE_ADH_Ll	Episomal plasmid for expression of LlDLDH by *ADH3* promoter
pPPE_CAT_Ll	Episomal plasmid for expression of LlDLDH by *CAT1* promoter
pPPE_CATm_Ll	Episomal plasmid for expression of LlDLDH by mutant *CAT1* promoter
pPPE_FLD_Ll	Episomal plasmid for expression of LlDLDH by *FLD1* promoter
pPPE_GAP_Ll	Episomal plasmid for expression of LlDLDH by *GAP1* promoter
pPPE_GAPm_Ll	Episomal plasmid for expression of LlDLDH by mutant *GAP1* promoter
pPPE_TEF_Ll	Episomal plasmid for expression of LlDLDH by *TEF1* promoter
pRDZ_CAT_Ll	Multicopy integrative plasmid for expression of LlDLDH by *CAT1* promoter
pRDZ_FLD_Ll	Multicopy integrative plasmid for expression of LlDLDH by *FLD1* promoter

comparison, was constructed as described below. PCR amplification was performed using pPPE_AOX_LlDLDH as the template and pPPE_Ll(F)_ASS and pPPE_Ll(R)_ASS as the primers. The genes encoding pADH3, pCAT1, pFLD1, pGAP1, and pTEF1 were PCR-amplified using *K. phaffii* genomic DNA as the template and ADH3(F)_ASS and ADH3(R)_ASS, CAT1(F)_ASS and CAT1(R)_ASS, FLD1(F)_ASS and FLD1(R)_ASS, GAP1(F)_ASS and GAP1(R)_ASS, and TEF1(F)_ASS and TEF1(R)_ASS, respectively, as the primers. The genes encoding pCAT1m and pGAP1m were PCR-amplified using CAT1(F)_ASS and CAT1(R)_ASS and GAP1(F)_ASS and GAP1(R)_ASS with synthetic genes (Twist Bioscience) as the templates. The gene fragments were ligated using the NEBuilder HiFi DNA Assembly Master Mix to construct the pPPE_X_LlDLDH plasmid.

Multicopy integration plasmids pRDZ_CAT_LlDLDH and pRDZ_FLD_LlDLDH for *D-LDH* expression were constructed as described below. PCR amplification was performed using prDZ_AO_DLDH as the template and pRDZ_CAT(F)_ASS and pRDZ_CAT(R)_ASS and pRDZ_FLD(F)_ASS and pRDZ_FLD(R)_ASS, respectively, as the primers. Gene fragments containing pCAT1 and pFLD1 were PCR-amplified using pPPE_CAT_LlDLDH and pPPE_FLD_LlDLDH, respectively, as the templates and pRDZ_CAT_Ll(F)_ASS and pRDZ_CAT_Ll(R)_ASS and pRDZ_FLD_Ll(F)_ASS and pRDZ_FLD_Ll(R)_ASS, respectively, as the primers. The gene fragments were ligated using the NEBuilder HiFi DNA Assembly Master Mix to construct the pRDZ_CAT_LlDLDH and pRDZ_FLD_LlDLDH plasmids. The resulting plasmids were linearized using restriction enzyme *AscI* prior to transformation.

The resulting plasmids were transformed into *K. phaffii* GS115 using a previously described electroporation method [40]. Transformants were selected on the RDB medium containing 20 g/L agar or YPDS medium containing 20 g/L agar and 0.1 g/L zeocin.

Analyses of growth and metabolite levels

OD_{600} of each culture was determined by measuring the absorbance at 600 nm using a spectrophotometer (Shimadzu, Kyoto, Japan).

D-Lactic acid concentration was calculated based on NADH concentration determined from the absorbance at 340 nm using a partially modified enzymatic reaction method with *D-LDH* described by Rosenberg et al. [41]. Briefly, the culture broth was centrifuged at 10,000×*g* for 2 min at 4 °C and diluted appropriately with water. Then, 140 µL of glycine-semicarbazide buffer (15 g/L glycine (Nacalai Tesque) and 22 g/L semicarbazide hydrochloride (Nacalai Tesque), adjusted to pH 8.7 using NaOH), 30 µL of 30 mM NAD^+ (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan), 5 µL of 2 mg/mL D-LDH (Toyobo, Osaka, Japan), and 10 µL of diluted supernatant were mixed and incubated at 30 °C for 3 h. After the reaction, absorbance of the sample was measured at 340 nm using a microplate reader (Thermo Fisher Scientific, Waltham, MA, US). D-Lactic acid concentration was calculated using a calibration curve plotted using D-lactic acid as the standard.

Methanol concentration was determined using a colorimetric method [42] using 4-amino-3-hydrazino-5-mercapto-1,2,4-triazole (AHMT) (Nacalai Tesque). The culture broth was centrifuged at 10,000×*g* for 2 min at 4 °C, and the resulting culture supernatant was diluted appropriately. Then, 48 µL of the diluted culture supernatant was mixed with 2 µL of 200 mM potassium phosphate buffer (pH 8.0) containing 0.25 U alcohol oxidase (A2404; Sigma-Aldrich Japan, Tokyo, Japan) and incubated at 25 °C for 80 min. Subsequently, 50 µL of 5 mol/L potassium hydroxide and 50 µL of AHMT solution (5 g/L AHMT [Tokyo Chemical Industry, Tokyo, Japan] and 50 g/L hydrochloric acid [Nacalai Tesque]) were added to the reaction solution and incubated at 25 °C for 20 min. The reaction solution was then mixed with 250 µL of potassium periodate solution (7.5 g/L potassium periodate [Nacalai Tesque] and 0.2 mol/L potassium hydroxide [Nacalai Tesque]), and absorbance at 550 nm was measured. Methanol concentration was calculated based on the calibration curve plotted using methanol as the standard.

D-LDH activity in yeast cells was measured following the method described by Tokuhiro et al. [43]. Yeast cells were cultured in test tubes containing 5 mL of MM medium for 48 h, after which the culture medium was centrifuged at 10,000×*g* and 4 °C for 1 min to collect the cells. The collected cells were washed twice with water and resuspended in 300 µL of 100 mM potassium phosphate buffer (pH 7.0). The cell suspension was mixed with 500 µL of zirconia beads (particle size: 0.45 mm) and passed through a bead crusher (Bio Medical Science, Tokyo, Japan) for 900 s. The cell lysate was centrifuged at 10,000×*g* and 4 °C for 5 min, and the supernatant was collected. Then, 5 µL of the supernatant was mixed with 180 µL of 100 mM glycine NaOH buffer (pH 10.0), 10 µL of 0.5 M D-lactic acid, and 10 µL of 20 mM NAD^+ solution, and the change in absorbance at 340 nm and 30 °C was measured. One unit of *D-LDH* activity was defined as the amount of enzyme releasing 1 µmol of NADH per minute at 30 °C.

Results

Effect of *D-LDH* gene type on D-lactic acid production

To express five *D-LDH* genes *(LdDLDH*, *LlDLDH*, *LmDLDH*, *LpDLDH*, and *SlDLDH*) under the control of

pAOX1, pPPE_AOX_XDLDH plasmids were constructed by connecting pAOX1 to each *D-LDH* gene. These plasmids were transformed into *K. phaffii* GS115, resulting in strains designated as GS115_AOX_X (X; Ld, Ll, Lm, Lp, or Sl; Table 2). Figure 1 shows the D-lactic acid concentration in the culture after cultivating the GS115_AOX_X transformants in MM medium for 288 h. Compared with GS115_AOX_Lm (83 mg/L), which expressed the conventional *LmDLDH* gene, GS115_AOX_Ld (59 mg/L) and GS115_AOX_Sl (35 mg/L) exhibited lower D-lactic acid concentrations. In contrast, GS115_AOX_Ll (186 mg/L) and GS115_AOX_Lp (155 mg/L) exhibited high D-lactic acid concentrations (2.25- and 1.88-times higher than those of GS115_AOX_Lm, respectively). These results suggest that, among the five types of *D-LDH* examined, *LlDLDH* is the most suitable *D-LDH* for expression in *K. phaffii*.

Effect of *D-LDH*-expressing promoter on D-lactic acid production

To select a suitable promoter to express LlDLDH, which is optimal for *D-LDH* gene expression in *K. phaffii*, pPPE_X_LlDLDH plasmids were constructed by linking LlDLDH to eight different promoters: four methanol-inducible type (pAOX1, pCAT1, pCATm, and pFLD1) and four constitutive type (pADH3, pGAP1, pGAP1m, and pTEF1) promoters. These plasmids were transformed into *K. phaffii* GS115, resulting in strains designated as GS115_X_Ll (X: AOX, CAT, CATm, FLD, ADH, GAP, GAPm, and TEF; Table 2). Figure 2A shows the time course of D-lactic acid concentration in the GS115_X_Ll transformants cultured in MM medium. D-Lactic acid production was confirmed in five strains: GS115_AOX_Ll, GS115_CAT_Ll, GS115_GAP_Ll, GS115_FLD_Ll, and GS115_TEF_Ll. In contrast, GS115_ADH_Ll, GS115_CATm_Ll, and GS115_GAPm_Ll produced small amounts of D-lactic acid. Among the five strains producing D-lactic acid, GS115_GAP_Ll produced 69 mg/L D-lactic acid for up to 48 h of cultivation, after which the concentration remained constant. GS115_TEF_Ll produced increased D-lactic acid concentration over time, but the concentration remained at 48 mg/L after 288 h, which was lower than that produced by GS115_AOX_Ll (186 mg/L), which expressed LlDLDH using conventional

Table 2 List of yeast strains used in this study

Strain name	Relevant features
GS115	*Komagataella phaffii* host strain
GS115_AOX_Ld	GS115 harboring pPPE_AOX_Ld
GS115_AOX_Lm	GS115 harboring pPPE_AOX_Lm
GS115_AOX_Ll	GS115 harboring pPPE_AOX_Ll
GS115_AOX_Lp	GS115 harboring pPPE_AOX_Lp
GS115_AOX_Sl	GS115 harboring pPPE_AOX_Sl
GS115_ADH_Ll	GS115 harboring pPPE_ADH_Ll
GS115_CAT_Ll	GS115 harboring pPPE_CAT_Ll
GS115_CATm_Ll	GS115 harboring pPPE_CATm_Ll
GS115_FLD_Ll	GS115 harboring pPPE_FLD_Ll
GS115_GAP_Ll	GS115 harboring pPPE_GAP_Ll
GS115_GAPm_Ll	GS115 harboring pPPE_GAPm_Ll
GS115_TEF_Ll	GS115 harboring pPPE_TEF_Ll
GS115_CL/Z1/*[a]	GS115 transformed with pRDZ_CAT_Ll and separated using 0.1 g/L zeocin
GS115_CL/Z2/*[a]	GS115 transformed with pRDZ_CAT_Ll and gene amplified using 0.5 g/L zeocin
GS115_CL/Z3/*[a)]	GS115 transformed with pRDZ_CAT_Ll and gene amplified using 1.0 g/L zeocin
GS115_CL/Z4/*[a)]	GS115 transformed with pRDZ_CAT_Ll and gene amplified using 2.0 g/L zeocin
GS115_FL/Z1/*[a]	GS115 transformed with pRDZ_FLD_Ll and separated using 0.1 g/L zeocin
GS115_FL/Z2/*[a)]	GS115 transformed with pRDZ_FLD_Ll and gene amplified using 0.5 g/L zeocin
GS115_FL/Z3/*[a)]	GS115 transformed with pRDZ_FLD_Ll and gene amplified using 1.0 g/L zeocin
GS115_FL/Z4/*[a)]	GS115 transformed with pRDZ_FLD_Ll and gene amplified using 2.0 g/L zeocin
GS115_CFL/Z1/*[a)]	GS115 simultaneously transformed with pRDZ_CAT_Ll and pRDZ_FLD_Ll and separated using 0.1 g/L zeocin
GS115_CFL/Z2/*[a)]	GS115 simultaneously transformed with pRDZ_CAT_Ll and pRDZ_FLD_Ll and gene amplified using 0.5 g/L zeocin
GS115_CFL/Z3/*[a)]	GS115 simultaneously transformed with pRDZ_CAT_Ll and pRDZ_FLD_Ll and gene amplified using 1.0 g/L zeocin
GS115_CFL/Z4/*[a)]	GS115 simultaneously transformed with pRDZ_CAT_Ll and pRDZ_FLD_Ll and gene amplified using 2.0 g/L zeocin

[a)] *;1–90

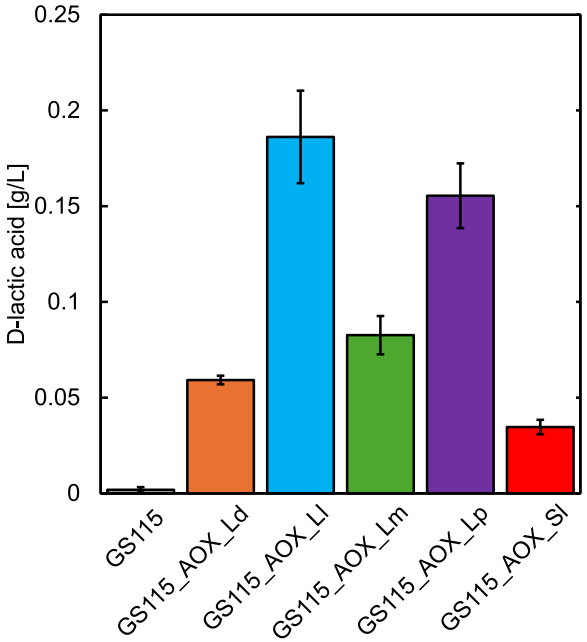

Fig. 1 Comparison of D-lactic acid production by engineered yeasts expressing D-lactate dehydrogenase (D-LDH) from five different microorganisms under the control of pAOX1

pAOX1. GS115_CAT1_Ll and GS115_FLD_Ll produced increased D-lactic acid concentrations for up to 48 h, reaching 185 and 182 mg/L, respectively. These values were 6.35- and 6.23-times higher than those produced by GS115_AOX_Ll (29 mg/L) at the same time point. After 288 h of cultivation, D-lactic acid levels by GS115_CAT1_Ll and GS115_FLD_Ll reached 212 and 220 mg/L, respectively, which were 1.24- and 1.29-times higher than those produced by GS115_AOX_Ll.

Figure 2B shows the intracellular *D-LDH* activity of the GS115_X_Ll strains after 48 h of cultivation. *D-LDH* activities of GS115_CAT_Ll and GS115_FLD_Ll were 993 and 1026 U/mL-culture, respectively, which were higher than those of GS115_AOX_Ll (639 U/mL-culture). In general, a positive correlation was observed between *D-LDH* activity and D-lactic acid production in the top three strains with high *D-LDH* activity. In the three strains showing low D-lactic acid production (GS115_ADH_Ll, GS115_CATm_Ll, and GS115_GAPm_Ll), *D-LDH* activity was lower than that in GS115_AOX_Ll. These results indicate that pCAT1 and pFLD1 are suitable for the expression of LlDLDH in *K. phaffii*.

Construction of a strain with *D-LDH* gene integrated into the rDNA locus and selection of a D-lactic acid-producing strain

Examination of the types of *D-LDH* genes and promoters revealed that the use of *LlDLDH* as the *D-LDH* gene and pCAT1 or pFLD1 as the promoter was suitable for D-lactic acid production in *K. phaffii*. Therefore, multicopy integrative plasmids pRDZ_CAT_LlDLDH (containing LlDLDH and pCAT1) and pRDZ_FLD_LlDLDH (containing LlDLDH and pFLD1) targeting the rDNA locus were constructed (Table 1) and transformed into *K. phaffii* GS115. Transformants were separated on YPDS

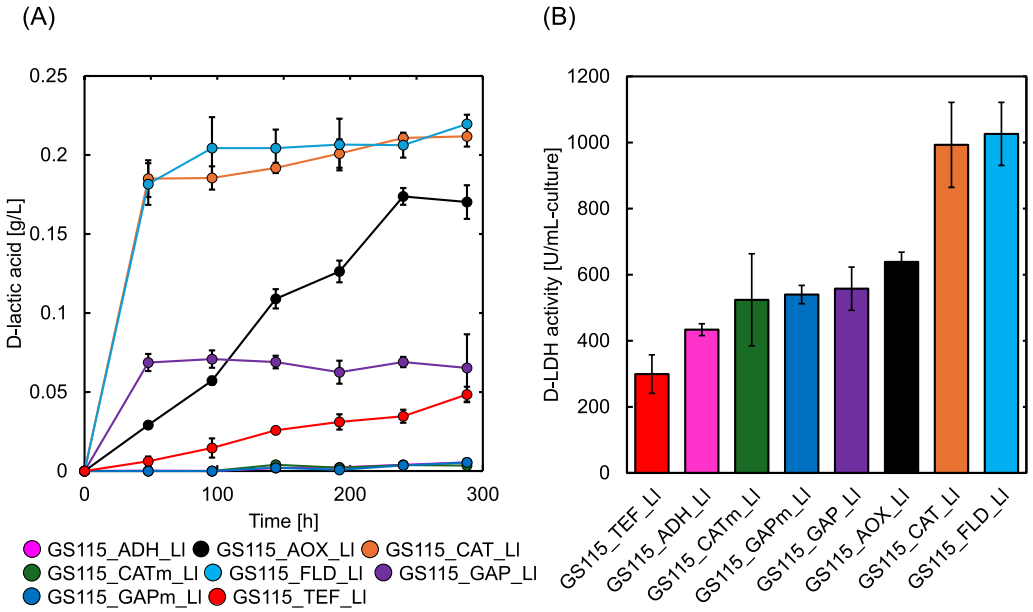

Fig. 2 **A** Time course of D-lactic acid production by engineered yeast expressing LlDLDH under the control of eight different promoters. **B** D-LDH activity in the culture supernatant after 48 h of culture

containing 0.1 g/L zeocin, and 90 colonies were randomly isolated. The transformants were named GS115_CL/Z1/* (transformed with pRDZ_CAT_LlDLDH), GS115_FL/Z1/* (transformed with pRDZ_FLD_LlDLDH), and GS115_CFL/Z1/* (transformed with identical amounts of pRDZ_CAT_LlDLDH and pRDZ_FLD_LlDLDH) (*1–90; Table 2). Figure 3 shows the concentration of D-lactic acid in the culture after 48 h of cultivation in a 96-well plate for each of the 90 strains. Among the transformants obtained using pRDZ_CAT_LlDLDH, the highest D-lactic acid-producing strains were GS115_CL/Z1/38 (2.83 g/L), GS115_CL/Z1/83 (2.89 g/L), GS115_CL/Z1/63 (2.96 g/L), and GS115_CL/Z1/70 (3.17 g/L). Similarly, among the transformants obtained using pRDZ_FLD_LlDLDH, the highest producing strains were GS115_FL/Z1/21 (4.00 g/L), GS115_FL/Z1/11 (4.02 g/L), GS115_FL/Z1/63 (4.30 g/L), and GS115_FL/Z1/33 (4.34 g/L). For the transformants constructed using a mixture of both plasmids, the highest producing strains were GS115_CFL/Z1/51 (3.03 g/L), GS115_CFL/Z1/44 (3.44 g/L), GS115_CFL/Z1/62 (3.71 g/L), and GS115_CFL/Z1/03 (4.07 g/L).

Post-transformational gene amplification and selection of strains with high D-lactic acid production

Among the 90 strains obtained via transformation with the pCAT-containing plasmid, pRDZ_CAT_LlDLDH, in the presence of 0.1 g/L zeocin, the top four strains with the highest D-lactic acid production (GS115_CL/Z1/38, GS115_CL/Z1/63, GS115_CL/Z1/83, and GS115_CL/Z1/70; Fig. 3) were separately cultured in test tubes containing YPD medium and 0.1 g/L zeocin for 24 h. Approximately 150 cells were collected from each culture and mixed, and colonies were isolated on YPD agar medium with a zeocin concentration of 0.5 g/L. Ninety randomly isolated colonies were cultured in 96-deep-well plate containing YPM, and the top four strains with the highest D-lactic acid production were selected. The same procedure was performed in a stepwise manner on YPD medium containing 1.0 and 2.0 g/L zeocin. The strains that were subjected to gene amplification in YPD medium containing 0.5, 1.0, and 2.0 g/L zeocin were designated as GS115_CL/Z2/*, GS115_CL/Z3/*, and GS115_CL/Z4/* (*;1–90), respectively (Table 2). The same procedure was performed using the pFLD-containing plasmid pRDZ_FLD_LlDLDH or the pCAT-containing plasmid pRDZ_CAT_LlDLDH and pFLD-containing plasmid pRDZ_FLD_LlDLDH simultaneously and the strains were named as GS115_CL/Z2/*, GS115_CL/Z3/*, GS115_CL/Z4/* (*;1–90) or GS115_CFL/Z2/*, GS115_CFL/Z3/*, and GS115_CFL/Z4/* (*;1–90), respectively (Table 2).

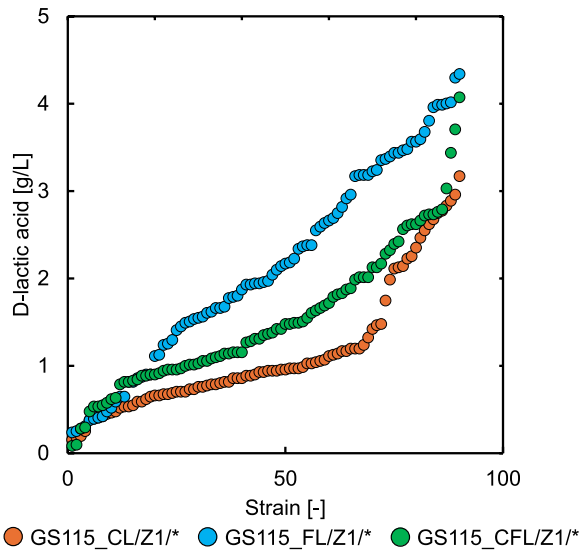

Fig. 3 D-Lactic acid production by engineered yeast constructed via multicopy integration after 48 h of deep-well culture. GS115_CL/Z1/* possessed LlDLDH linked to pCAT1, GS115_FL/Z1/* possessed LlDLDH linked to pFLD1, and GS115_FL/Z1/* possessed LlDLDH linked to pCAT1 and pFLD1

D-Lactic acid concentrations of the strains obtained via the post-transformational gene amplification procedure described above and those of the strains before gene amplification (GS115_CL/Z1/*, GS115_FL/Z1/*, and GS115_CFL/Z1/*) cultured for 24 h in 96-deep-well plates are shown in Fig. 4A, B, and C. The values of the strains with the highest D-lactic acid production among the 90 selected strains at each zeocin concentration are shown in Fig. 4D.

As shown in Fig. 4A, B, and D, when transformed with plasmids carrying pCAT1 or pFLD1, gene amplification at a zeocin concentration of 0.5 g/L improved D-lactic acid production. The strains showing the highest D-lactic acid production were CL/Z2/71 (11.12 g/L) and FL/Z2/38 (9.62 g/L). In contrast, when gene amplification was performed at increased zeocin concentrations of 1.0 and 2.0 g/L, D-lactic acid production was decreased in both transformants.

As shown in Fig. 4C and D, when two types of plasmids carrying pCAT1 or pFLD1 were transformed simultaneously, D-lactic acid production was improved via gene amplification with increasing zeocin concentrations from 0.1 to 0.5 and 1.0 g/L, respectively. The strains showing the highest D-lactic acid production in the presence of 0.5 and 1.0 g/L zeocin were CFL/Z2/57 (6.80 g/L) and CFL/Z3/04 (10.34 g/L), respectively. However, when gene amplification was performed in the presence of 2.0 g/L zeocin, D-lactic acid production was decreased.

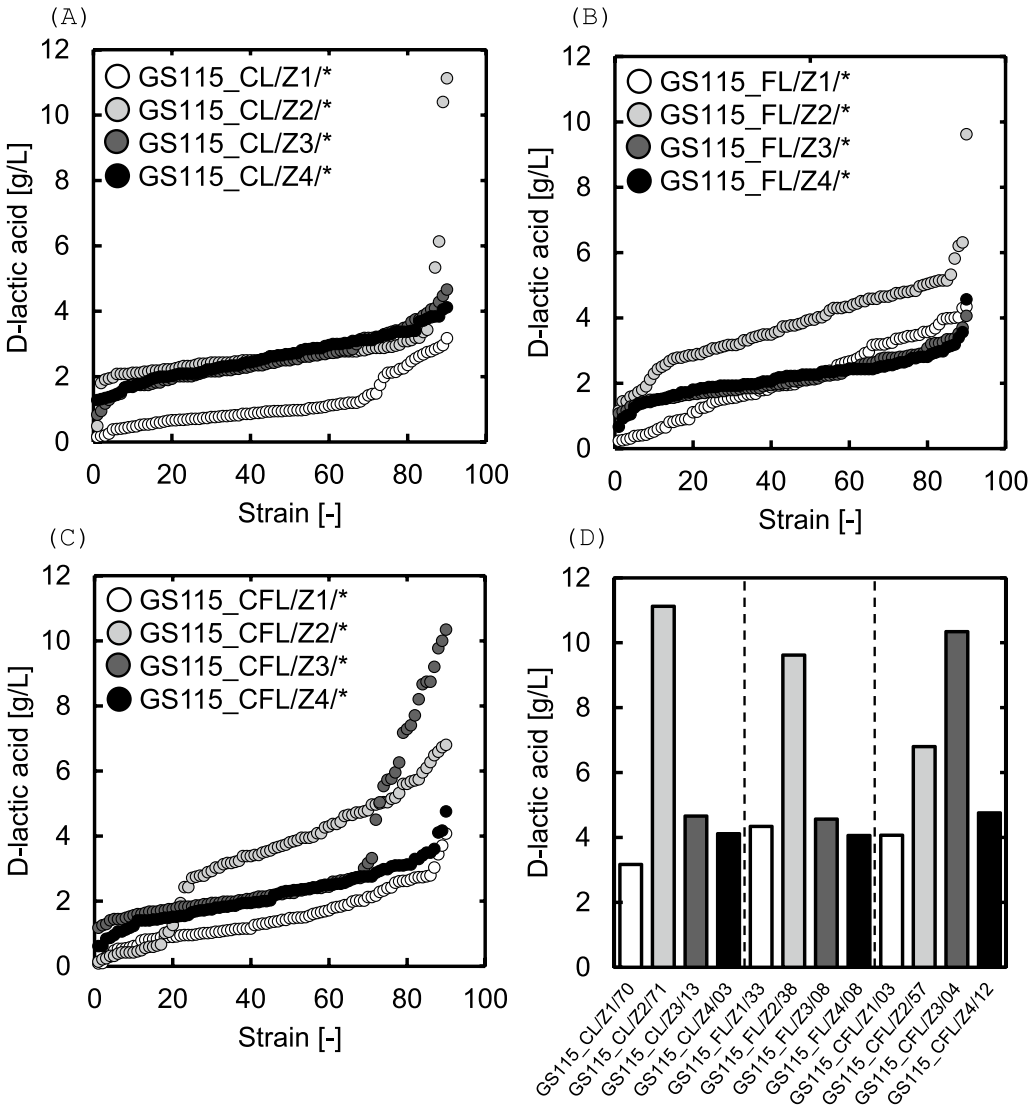

Fig. 4 D-lactic acid production by engineered yeast constructed via multicopy integration and post-transformational gene amplification after 48 h of deep-well culture. **A** Engineered strains possessing LIDLDH linked to pCAT1. **B** Engineered strains possessing LIDLDH linked to pFLD1. **C** Engineered strains possessing LIDLDH linked to both pCAT1 and pFLD1. **D** Comparison of strains showing the highest D-lactic acid production under each condition. Strains containing Z1 in their names were isolated in the presence of 0.1 g/L zeocin, and strains with Z2, Z3, and Z4 in their names were gene amplified in the presence of 0.5, 1.0, and 2.0 g/L zeocin, respectively

Time course of D-lactic acid production by post-transformational gene-amplified strains in a flask culture

To evaluate the culture behavior in flasks of strains GS115_CL/Z2/71 (transformed by plasmid carrying pCAT1 and gene amplified in the presence of 0.5 g/L zeocin), GS115_FL/Z2/38 (transformed by plasmid carrying pFLD1 and gene amplified in the presence of 0.5 g/L zeocin), and GS115_CFL/Z3/04 (transformed by plasmids carrying pCAT1 and pFLD1 and gene amplified in the presence of 1.0 g/L zeocin), which exhibited high D-lactic acid production in 96-deep-well plates (Fig. 4), they were cultured in flasks containing the YPM medium. The methanol concentrations, D-lactic acid concentrations, and OD_{600} values of the strains are shown in Fig. 5A, B, and C, respectively. As shown in Fig. 5A, no significant difference in methanol consumption was observed among the three strains, and all three strains completely consumed methanol after 192 h of cultivation. Figure 5B shows that GS115_CL/Z2/71 and GS115_FL/Z2/38 strains continued to produce D-lactic acid until 96 h of cultivation, with GS115_CL/Z2/71 and GS115_FL/

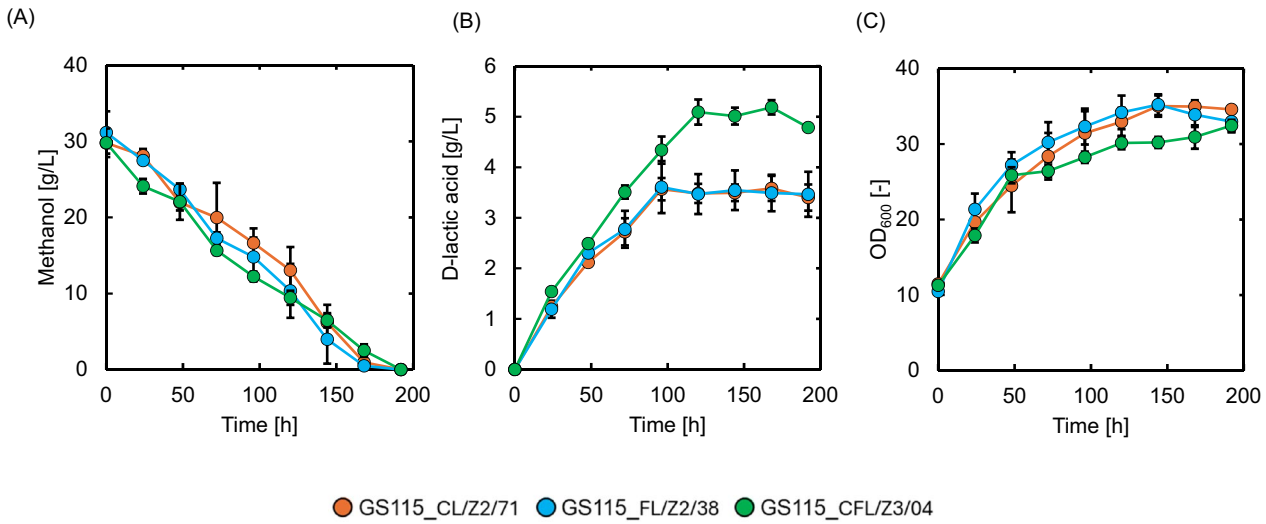

Fig. 5 Time course of **A** methanol concentrations, **B** D-lactic acid concentrations, and **C** OD_{600} values of strains constructed via post-transformational gene amplification in a flask culture

Z3/38 strains showing maximum D-lactic acid production of 3.48 and 3.61 g/L, respectively, after 96 h of cultivation. GS115_CFL/Z3/04 strain continued to produce D-lactic acid up to 120 h of cultivation, after which it produced 5.09 g/L of D-lactic acid. Figure 5C shows that cell proliferation was observed in strains GS115_CL/Z2/71 and GS115_FL/Z2/38 until 144 h of cultivation, after which cell density decreased slightly in both strains. In contrast, GS115_CFL/Z3/04 strain showed lower growth after 48 h of culture than the other two strains but continued to grow until 192 h of culture. After 192 h of cultivation, OD_{600} values of all three strains were similar.

Discussion

In this study, we investigated the type of *D-LDH* gene expressed and optimal combination of promoters to improve D-lactic acid production from methanol in *K. phaffii*. The highest D-lactic acid production was achieved by integrating the *LlDLDH* gene from *L. lactis* linked to pCAT1 and pFLD1 into the rDNA locus of the *K. phaffii* genome and performing post-transformational gene amplification. The results indicate that the combination of expressed genes and promoters, use of multiple promoters, and multicopy integration of genes are effective for the production of D-lactic acid from methanol by engineered *K. phaffii*.

In this study, two types of *D-LDH* genes, *LlDLDH* and *LpDLDH*, were expressed in *K. phaffii*, and they achieved higher D-lactic acid production than conventional *LmDLDH* [24]; the highest D-lactic acid production was achieved with *LlDLDH* (Fig. 1). Watcharawipas et al. also expressed these three *D-LDH* genes in *S. cerevisiae* and reported that the highest D-lactic acid production is achieved with *LpDLDH*, followed by *LlDLDH* and *LmDLDH* [28]. In contrast, in a study on *D-LDH* expression in *O. polymorpha*, almost no D-lactic acid was produced with *LmDLDH* [44]. This indicates that the effective *D-LDH* gene varies depending on the host microorganism. Therefore, type of *D-LDH* gene expressed in each host should be considered for high D-lactic acid production.

In this study, high D-lactic acid production was achieved when pCAT1 or pFLD1 was used to express *D-LDH* (Fig. 2A). Of the eight promoters used in this study, two promoters that improved D-lactic acid production over conventional pAOX1, pCAT1, and pFLD1 are known to be strong methanol-inducible promoters, along with pAOX1 [45]. Vogl et al. reported that pFLD1 is comparable to pAOX1 and that pCAT1 reaches expression levels up to 1.8-fold higher than those of pAOX1 in *K. phaffii* [46]. When pCAT1 or pFLD1 was used, the D-lactic acid concentration nearly reached a plateau at 48 h (Fig. 2A). This is likely due to a decrease in the pH of the medium or a lack of nitrogen sources in the medium. Similarly, Wu et al. reported that the pH decrease due to lactic acid accumulation reduced lactic acid productivity from methanol [47]. In addition, the nitrogen source in the minimal medium was limited [48], and the D-lactic acid production likely reached a plateau due to competition between the nitrogen source for cell growth and D-lactic acid production. In terms of cell proliferation, optimal methanol concentration for culturing *K. phaffii* is approximately 4.0 g/L [49]. In addition, methanol concentrations < 10 g/L have been used to produce heterologous

proteins and useful compounds using methanol-inducible promoters in *K. phaffii* [35, 50–54]. In contrast, methanol concentration used in this study (30 g/L) was higher than that used in other studies. Only a little is known about the expression intensity of methanol-inducible promoters at methanol concentrations > 10 g/L. However, similar to the galactose-inducible promoter, pGAL1, in *S. cerevisiae* [55], the level of gene expression driven by the methanol-inducible promoter in *K. phaffii* may be dependent on the methanol concentration in the medium. pCAT1 and pFLD1 may have higher expression levels than pAOX1 in the presence of high concentrations of methanol. Therefore, exploration of useful promoters at high methanol concentrations is important for the efficient production of various useful chemicals from methanol.

When *LlDLDH* was expressed in *K. phaffii* using two mutant promoters, pCAT1m and pGAP1m, almost no D-lactic acid production was observed with either promoter (Fig. 2A). pCAT1m and pGAP1m are mutants of pCAT1 and pGAP1, respectively, with a partial modification of the promoter sequence to improve GFP fluorescence [35, 38]. Gene expression levels vary depending on the gene and promoter combination [29, 30]. Therefore, mutant promoters effective for the expression of GFP were not suitable for the expression of *LlDLDH*, leading to low D-lactic acid production. In the future, identification of suitable promoters for target gene expression will be important for the production of chemicals from methanol.

GS115_CFL/Z3/04 strain, constructed by integrating the *LlDLDH* gene linked to two promoters (pCAT1 and pFLD1) and amplifying it after transformation, produced a maximum of 5.18 g/L of D-lactic acid after 168 h of cultivation (Fig. 5B). There have been several reports of lactic acid production by methylotrophic yeasts using methanol as a carbon source. Bachleitner et al. achieved L-lactic acid production of 17 g/L using methanol and glycerol as co-substrates [56]. On the other hand, lactic acid production using methanol as the sole carbon source has been reported in three studies, one for D-lactic acid production [24] and two for L-lactic acid production [44, 47]. The final lactic acid production levels in these studies were 3.48 g/L [24], 3.8 g/L [44], and 4.2 g/L [47], respectively. Therefore, to the best of our knowledge, this study achieved the highest lactic acid production when methanol was used as the sole carbon source.

Previous studies have reported that the combined use of the two promoters, pAOX1 and pGAP1, enhances the production of heterologous proteins in *K. phaffii* [57]. This is possibly because, even under conditions of repression of one promoter, gene expression is maintained at a high level by the action of the other promoter, thereby increasing the production of the target protein. Strengths of the promoters of some methanol metabolism-related genes in *K. phaffii* are reduced by amino acids in the culture medium [58], and the repression conditions of pCAT1 and pFLD1 used in this study were different [35, 59]. Combination of the two promoters with different repression conditions, pCAT1 and pFLD1, in this study enabled *D-LDH* to be constantly expressed at a high level and to continue to stably produce D-lactic acid over a long period, thereby achieving the high D-lactic acid production. Therefore, simultaneous expression of key metabolic enzymes using multiple promoters is a useful strategy for chemical production in *K. phaffii*.

Conclusions

In this study, *LlDLDH* gene from *L. lactis* was linked to pCAT1 and pFLD1, integrated into the rDNA locus of *K. phaffii*, and subjected to post-transformational gene amplification to construct an engineered yeast strain, GS115_CFL/Z3/04, capable of producing D-lactic acid from methanol. To the best of our knowledge, the amount of D-lactic acid produced by this engineered yeast is the highest reported to date when methanol is used as the sole carbon source. In lactic acid production from methanol, strategies such as overexpression of transcriptional activators [56] and inhibition of the reverse reaction from lactic acid to pyruvate [47] have been employed to increase lactic acid production. Integrating these genetic modification strategies with the strategy in this study likely further increases the production of D-lactic acid. On the other hand, it has been reported that the methanol assimilation ability of *K. phaffii* decreases when it is cultured for a long period of time using methanol as the sole carbon source [60]. Addressing this issue is also important for efficient production of D-lactic acid from methanol. This study demonstrated the effectiveness of combining different enzyme genes and promoters using multiple promoters with different induction and repression conditions, integrating the genes into the rDNA locus, and amplifying the genes after transformation in *K. phaffii*. This study outlines a method to engineer other *K. phaffii* strains capable of producing various useful chemicals in the future.

Abbreviations

AHMT	4-Amino-3-hydrazino-5-mercapto-1,2,4-triazole
D-LDH	D-Lactate dehydrogenase
PCR	Polymerase chain reaction

Supplementary Information

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Supplementary Material 1.

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Author contributions
YI performed the experiments and wrote the manuscript. RY conceptualized the study, wrote the manuscript, supervised the study, and acquired funding. TM and HO supervised the study.

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Availability of data and materials
The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethics approval and consent to participate
Not applicable.

Consent for publication
Not applicable.

Competing interests
The authors declare no competing interests.

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