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Expression of native and mutant extracellular lipases from *Yarrowia lipolytica* in *Saccharomyces cerevisiae*

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Summary

***Saccharomyces cerevisiae* cannot produce extracellular lipase and utilize low-cost lipid substrates. This study aimed to express extracellular lipase from *Yarrowia lipolytica* in *S. cerevisiae*, construct recombinant oily substrate consumer strains, and compare the roles of native and mutant *Y. lipolytica* extracellular lipases in *S. cerevisiae*. The *LIP2* gene of *Y. lipolytica* DSM3286 and its mutant *Y. lipolytica* U6 were isolated and cloned by expression vector in *S. cerevisiae*. New recombinant *S. cerevisiae* strains FDS100 containing the native *LIP2* gene, and FDS101 containing the mutant *LIP2* gene were produced 10 and 15 U ml^{-1} extracellular lipase respectively, on a production medium containing olive oil. New recombinant *S. cerevisiae* strains produce acceptable amount of extracellular lipase in comparison with *Y. lipolytica* wild-type strains. These strains can utilize olive oil and lipids as low-cost substrates to produce bioethanol, single cell protein and other biotechnologically valuable products. The recombinant *S. cerevisiae* strain with mutant *LIP2* produced lipase with 1.5-fold higher activity. The *LIP2* gene of *Y. lipolytica* was expressed in *S. cerevisiae* as a heterologous protein without any modifications. Strong components of the *Y. lipolytica* expression/secretion system could be used for high-level production of recombinant proteins in *S. cerevisiae*.**

Introduction

For several centuries, *Saccharomyces cerevisiae* has been used in the production of food and ethanol, and today, this organism is also used as a platform for recombinant protein production (Nevoigt, 2008). *Saccharomyces cerevisiae* cannot acquire low-cost lipid substrates such as triacylglycerols from the growth media; therefore, this microorganism does not utilize these substrates for bioconversion to biotechnologically valuable products (Roermund *et al*., 2003; Ciafardini *et al*., 2006).

The yeast *Yarrowia lipolytica* utilizes hydrophobic substrates, including alkanes, fatty acids, fats and oils by activating several enzymes such as lipases/esterases (*LIP* genes), cytochromes P450 (*ALK* genes), and peroxisomal acyl-CoA oxidases (*POX* genes). Triglycerides are first hydrolysed by lipase into free fatty acids, which are then taken up by the cell (Hadeball, 1991; Iida *et al*., 1998; Sumita *et al*., 2002; Fickers *et al*., 2005a; Thevenieau *et al*., 2007).

Depending on media composition and environmental conditions, *Y. lipolytica* is able to produce several extracellular, membrane-bound and intracellular lipases (Barth and Gaillardin, 1997; Pereira-Meirelles *et al*., 2000; Guieysse *et al*., 2004; Cancino *et al*., 2008). Extracellular lipase Lip2 (38.5 kDa) is encoded by the *LIP2* gene (GenBank Accession No. AJ012632). This gene is responsible for all the extracellular lipase activity of *Y. lipolytica.* It is synthesized as a 334-amino acid (aa) preproprotein containing a signal sequence, a stretch of four dipeptides X-Ala, X-Pro as cleavage sites of diaminopeptidase, followed by a 12-aa pro-region ending with a Lys-Arg dipeptide, which is the cleavage site for the endoprotease encoded by *XPR6* (Pignede *et al*., 2000). This extracellular lipase Lip2p was reported to hydrolyse long-chain triglycerides, with preference for oleyl residues. The enzyme is efficient for the hydrolysis of natural oils and fats, and stays active at low temperatures (4°C) and pH 3–4 (Barth and Gaillardin, 1997; Destain *et al*., 1997).

Extracellular lipase Lip2 of *Y. lipolytica* was expressed and characterized in *Pichia pastoris* (Yu *et al*., 2007). More recently, the *LIP2* gene of *Y. lipolytica* was expressed in *S. cerevisiae* with some modification containing the replacement of native Lip2p prepro sequence with the *S. cerevisiae* carboxypeptidase Y (CPY) signal sequence, as well as an additional serine residue for optimal recognition and cleavage by the *S. cerevisiae* Kex2 protease (*LIP2*+*Ser*) with an inserted serine codon at aa position 34 by site-directed mutagenesis in the N-terminal *LIP2* sequence. Furthermore, the synthetic gene *LIP2* was codon-optimized for expression in *S. cerevisiae* (Shockey *et al*., 2011). Fermentation of fatty low-value renewable carbon sources aiming at the production of various added-value metabolites presents a noticeable interest in the sectors of industrial microbiology and

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biotechnology (Darvishi *et al*., 2009). The aims of the study were to express native and mutant extracellular lipases from *Y. lipolytica* in *S. cerevisiae* and to construct recombinant oily substrate consumer strains by different and easier strategies, and then compare these enzyme operations.

Results

Recombinant S. cerevisiae *strains with* Y. lipolytica *extracellular lipase* LIP2 *gene*

The yeast *Y. lipolytica* efficiently degrades hydrophobic substrates such as fatty acids, fats and oils. The *LIP2* gene encodes the major extracellular lipase Lip2p, which is responsible for all the extracellular lipase activity of *Y. lipolytica* (Fickers *et al*., 2011).

More recently, Shockey *et al*. expressed codon-optimized *LIP2* in *S. cerevisiae* under the control of the galactose-inducible GAL1 and fatty acid inducible *PEX11* promoters with some modifications, including replacement of native Lip2p prepro sequence with the *S. cerevisiae* carboxypeptidase Y (CPY) signal sequence, and adding a serine residue for optimal recognition and cleavage by the *S. cerevisiae* Kex2 protease (*LIP2*+*Ser*) with an inserted serine codon at the aa position 34 by site-directed mutagenesis in the N-terminal *LIP2* sequence (Shockey *et al*., 2011).

In this study, the *S. cerevisiae* expression vector p426GPD with strong constitutive glycerol phosphate dehydrogenase (GPD) promoter was used for *LIP2* gene expression (Fig. 1B). The native *LIP2* gene from *Y. lipolytica* DSM3286 and the mutant *LIP2* gene from the mutant *Y. lipolytica* U6 were cloned into the vector without any modifications. The resulting constructs designated pFDP100 and pFDP101 contained the native and mutant *LIP2* gene respectively (Fig. 1C and D). The vectors were transformed to the *S. cerevisiae* strain CEN.PK 113-5D, and then, the new recombinant *S. cerevisiae* containing native and mutant extracellular lipase named *S. cerevisiae* FDS100 and FDS101 respectively.

Expression and secretion of Y. lipolytica *extracellular lipase in* S. cerevisiae

Extensive studies of the physiological behaviour of *S. cerevisiae* as well as the important ability of this yeast to express foreign genes in conjunction with its secretory apparatus make *S. cerevisiae* an attractive host for the production of certain heterologous proteins. Nevertheless, *S. cerevisiae* exhibits some disadvantages when used for the production of certain recombinant heterologous proteins (Ostergaard *et al*., 2000). To overcome these undesired modulations of the recombinant protein of interest, some modifications were proposed, including fusion of the prepro-leader sequence of *S. cerevisiae* to secrete the heterologous protein, codon optimization, etc. (Böer *et al*., 2007).

A different and easier strategy was used to investigate *Y. lipolytica LIP2* gene expression elements in the *S. cerevisiae* expression system. For example, using a secretion signal peptide and pro-region cleavage site of the *Y. lipolytica* KEX2-like XPR6 endoprotease, and not using codon optimization to ensure similar *S. cerevisiae* codon usage.

Surprisingly, hydrolysis halo was detected during phenotypic analysis of the recombinant *S. cerevisiae* strains FDS100 and FDS101 on YNBT medium, indicating the production of extracellular lipase in these strains. The *S. cerevisiae* strain 5D had no lipase activity on the YNBT medium, which contained p426GPD vector without a *Y. lipolytica* extracellular lipase gene (Fig. 2). These results show that *Y. lipolytica* heterologous proteins, especially extracellular lipase *LIP2*, can be expressed in *S. cerevisiae* without any modifications. This is significant because strong components of the *Y. lipolytica* expression/secretion system could be used for high-level production of recombinant proteins in *S. cerevisiae*. These components include fatty acids, derivative-inducible *POX* promoters, peptone-inducible *XPR2* promoter, fatty acids and derivatives, alkanes, ethanol- and acetate-inducible *ICL1* promoter, *LIP2* prepro and *XPR2* prepro secretion signals, and *LIP*2t, *XPR*2t and *PHO5*t terminators (Madzak *et al*., 2004).

Analysis of Y. lipolytica *native and mutant extracellular lipases expression in* S. cerevisiae

Overexpression of the extracellular lipase in *Y. lipolytica* was initially achieved by two different approaches. In the Nicaud group, the *LIP2* gene was cloned under the control of the strong, oleic acid-inducible *POX2* promoter, by using multiple copies of the gene. The resulting strains are actually constructed by metabolic engineering, producing unstable amounts of lipase on expensive laboratory medium. In the Thonart's group, overproducing mutants were isolated from the wild-type strain CBS6303 by successive rounds of chemical mutagenesis using N-methyl-N′-nitro-N-nitrosoguanidine. This led to the selection of the second-generation mutant LgX64.81, which produced stable amounts of lipase in cheap medium and is now used as an industrial strain (Fickers *et al*., 2011).

In the first step of our project on inverse metabolic engineering of *Y. lipolytica* extracellular lipase, an UV mutant strain was obtained that produced 10.5-fold lipase compared with the wild-type strain DSM3286, and could be used at the industrial scale (Darvishi *et al*., 2011). Sequence analysis showed strict identity between the

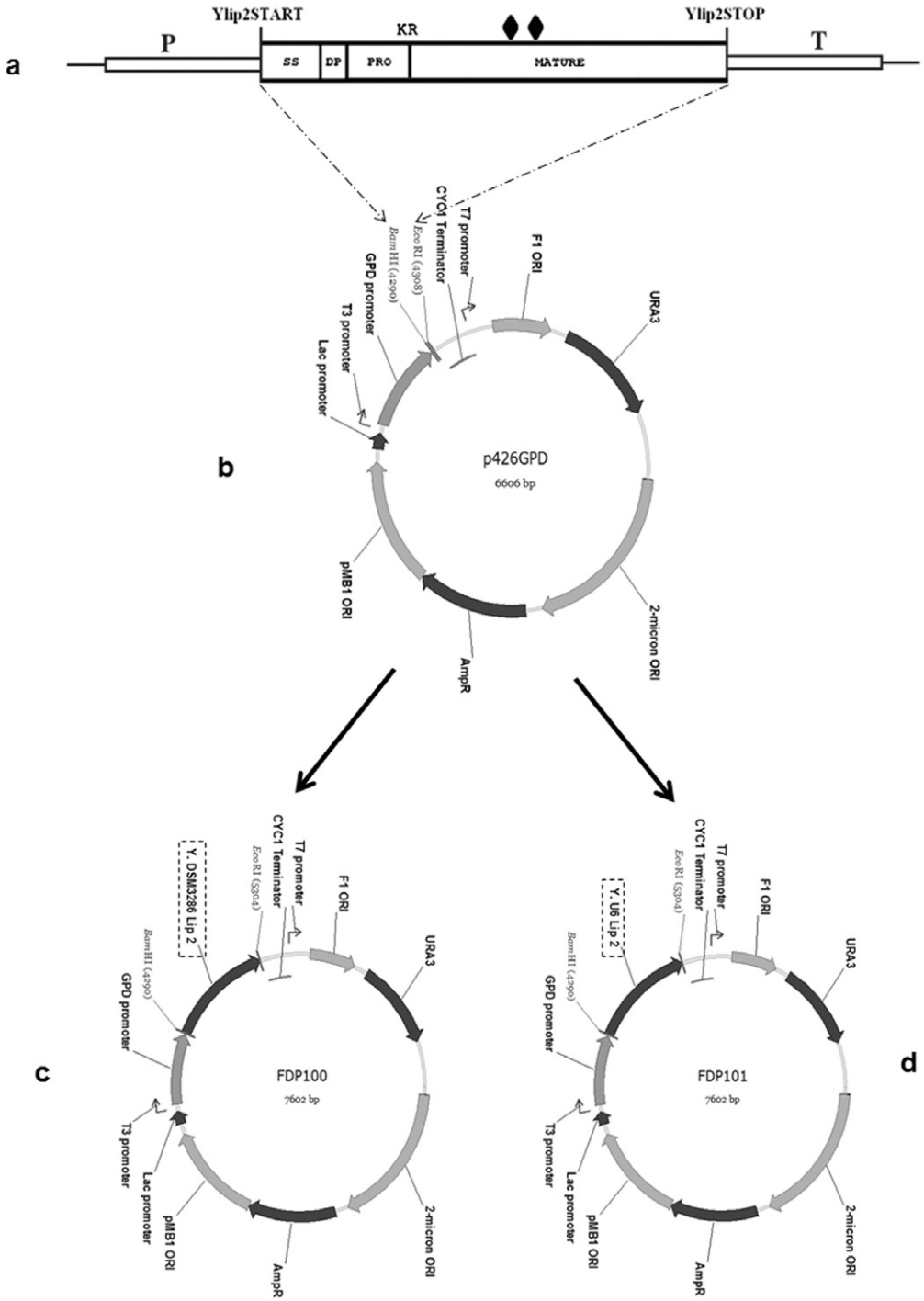

Fig. 1. Schematic representation of extracellular lipase gene (*LIP2*), p426GPD, pFDP100 and pFDP101 vectors. A. Schematic representation of extracellular lipase gene (*LIP2*) and situation of forward primer (Ylip2START) and reverse primer (Ylip2STOP). Shown are the putative 13-aa signal sequence (SS), followed by a stretch of four dipeptides (DP); a short 12-aa pro region (PRO), including the Lys-Arg (KR) cleavage site of the KEX2-like XPR6 endoprotease; and the mature 301-aa lipase (MATURE). The diamonds indicate the positions of the potential signals for asparagine-linked glycosylation (Asn-X-Thr/Ser). The promoter (P) and terminator (T) regions, consisting of 1.06 and 0.97 kb fragments situated upstream and downstream from the y*LIP2* ORF respectively. B. Schematic representation structure of the p426GPD *Saccharomyces cerevisiae* expression vector. GPD promoter, glycerol phosphate dehydrogenase promoter; Lac promoter, lac promoter; T3, T3 phage promoter; T7 promoter, T7 phage promoter; AmpR, ampicillin resistance gene; URA3, *URA3* marker; 2-micron ORI, yeast 2 μ expression replication origin; pMB1 ORI, origin replication of *E. coli*; F1 ORI, origin of replication. C. The structure of pFDP100 vector containing extracellular lipase ORF of native *Y. lipolytica* strain DSM3286 (Y.DSM3286 Lip2). D. The structure of pFDP101 vector containing extracellular lipase ORF of the mutant *Y. lipolytica* strain U6 (Y.U6 Lip2).

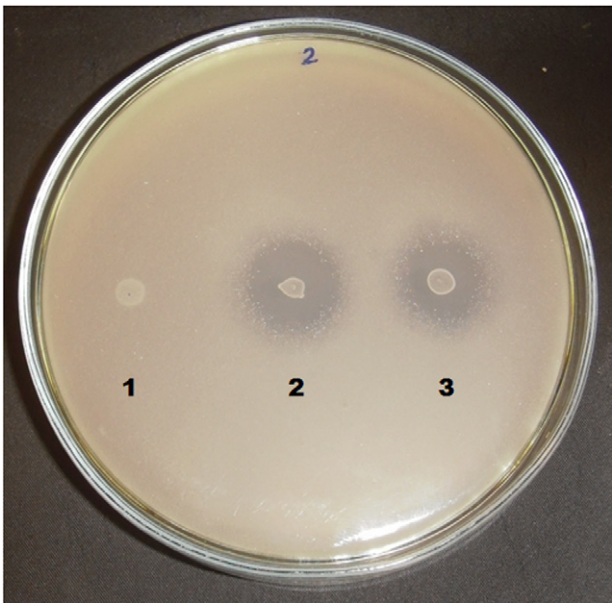

Fig. 2. Qualitative evaluation of lipolytic activity of the Ura$^+$ transformants on YNBT agar containing tributyrin; Lipase detection was performed by spotting 2 µl of each cell suspension (10^7 cell ml^{-1}) on YNBT agar. The plates were incubated 48 h at 28°C. The size of the clear zone around the colonies, which reflects tributyrin hydrolysis due to extracellular lipase production, was measured. (1) *S. cerevisiae* 5D transformed by p426GPD vector (without *Y. lipolytica* extracellular lipase gene) as control; (2) *S. cerevisiae* FDS100 transformed by pFDP100 vector (containing *Y. lipolytica* DSM3286 native extracellular lipase gene); (3) *S. cerevisiae* FDS101 transformed by pFDP101 vector (containing *Y. lipolytica* U6 mutant extracellular lipase gene).

LIP2 sequences of *Y. lipolytica* DSM3286 and its mutant strain U6. However, only two silent substitutions at the positions 362 and 385 were observed in the open reading frame (ORF) region of *LIP2* gene. Two single substitutions and two duplications of the T nucleotide were also detected in its promoter region (Darvishi *et al*., 2011).

The effects of these genetic changes on *LIP2* expression must be analysed stepwise in a suitable host. To address this problem, the ORF regions in the mutant and native *LIP2* genes were selected for expression in *S. cerevisiae*. Multiple sequence alignment obtained with ClustalW program of the extracellular lipase *LIP2* from *Y. lipolytica* strains CLIB122, DSM3286 and U6 show that threonine at the positions 121 and 129 is replaced with isoleucine and serine in the mutant lipase of *Y. lipolytica* U6 mutant strain compared with the native lipase of wild-type strains (Fig. 3).

The phenotype of the recombinant lipase strains were further investigated by following the growth and lipase activity of strains in liquid medium containing olive oil as the sole carbon source. The *S. cerevisiae* FDS101 containing mutant *LIP2* produced 1.5-fold higher levels of lipase than *S. cerevisiae* FDS100 with the wild-type gene (Fig. 4). Lipase production increased only 1.5-fold by using substitutions in the ORF region. Figure 5 shows the time course of biomass production of *S. cerevisiae* strains 5D, FDS100 and FDS101 during culture in olive oil medium. The growth curves were similar for *S. cerevisiae* strains FDS100 and FDS101. Crude supernatant preparations of enzyme analysed by SDS-PAGE show one main band corresponding to 38 kDa, similar to *Y. lipolytica* extracellular lipase enzyme (data not shown).

Discussion

Current agricultural and industrial practices have led to the generation of large amounts of various low-value or negative-cost crude fatty materials. On the other hand, the fermentation of these renewable materials as carbon sources for the production of various added-value metabolites such as enzymes and recombinant protein is of great interest in the industrial microbiology and biotechnology sectors (Darvishi *et al*., 2009; 2011; Darvishi, 2012).

Free fatty acids are readily taken up by *S. cerevisiae*. Most oils exist as triacylglycerols, a form of lipid that cannot be directly taken up by *S. cerevisiae*. The first step of their catabolism involves hydrolysis into free fatty acids and glycerol by lipolytic enzymes. *S. cerevisiae* cannot produce extracellular lipases and utilize low-cost lipid substrates (Roermund *et al*., 2003; Fickers *et al*., 2005a).

The *Y. lipolytica* extracellular lipase Lip2p was reported to hydrolyse long-chain triglycerides, with a preference for oleyl residues, and it is the best candidate gene to equip *S. cerevisiae* for utilizing low-cost lipid substrates. In the previous studies, researchers have reported that codon usage in *Y. lipolytica* appears to be rather different compared with that in *S. cerevisiae*, but similar to that in *Aspergillus* genus (Gaillardin and Heslot, 1988; Spencer *et al*., 2002). However, the heterologous genes expressed in *Y. lipolytica* were not modified to fit this peculiar codon usage (Madzak *et al*., 2004). The native *LIP2* gene from *Y. lipolytica* DSM3286 and mutant *LIP2* gene from its mutant *Y. lipolytica* U6 were cloned into the vector without any modifications, unlike the work done by Shockey *et al*., where superabundant modifications for the cloning and expression of *LIP2* gene in *S. cerevisiae* were used (Shockey *et al*., 2011).

Surprisingly, recombinant *S. cerevisiae* strains expressed and secreted *Y. lipolytica* extracellular lipase, especially on olive oil medium. These results show that heterologous proteins of *Y. lipolytica* could be expressed in *S. cerevisiae* without any modifications. It is important because strong components of the *Y. lipolytica* expression/secretion system could be used for high-level production of recombinant proteins in *S. cerevisiae*. Engineered *S. cerevisiae* strains by Shockey *et al*. produced lipase on semisynthetic mineral media containing appro-


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CLIB122   1    MKLSTILFTACATLAAALPSPITPSEAAVLQKRVYTSTETSHIDQESYNFFEKYARLANI  60
DSM3286   1    MKLSTILFTACATLAAALPSPITPSEAAVLQKRVYTSTETSHIDQESYNFFEKYARLANI  60
U6        1    MKLSTILFTACATLAAALPSPITPSEAAVLQKRVYTSTETSHIDQESYNFFEKYARLANI  60

CLIB122   61   GYCVGPGTKIFKPFNCGLQCAHFPNVELIEEFHDPRLIFDVSGYLAVDHASKQIYLVIRG  120
DSM3286   61   GYCVGPGTKIFKPFNCGLQCAHFPNVELIEEFHDPRLIFDVSGYLAVDHASKQIYLVIRG  120
U6        61   GYCVGPGTKIFKPFNCGLQCAHFPNVELIEEFHDPRLIFDVSGYLAVDHASKQIYLVIRG  120

                    *       *
CLIB122   121  THSLEDVITDIRIMQAPLTNFDLAANISSTATCDDCLVHNGFIQSYNNTYNQIGPKLDSV  180
DSM3286   121  THSLEDVITDIRIMQAPLTNFDLAANISSTATCDDCLVHNGFIQSYNNTYNQIGPKLDSV  180
U6        121  IHSLEDVISDIRIMQAPLTNFDLAANISSTATCDDCLVHNGFIQSYNNTYNQIGPKLDSV  180

CLIB122   181  IEQYPDYQIAVTGHSLGGAAALLFGINLKVNGHDPLVVTLGQPIVGNAGFANWVDKLFFG  240
DSM3286   181  IEQYPDYQIAVTGHSLGGAAALLFGINLKVNGHDPLVVTLGQPIVGNAGFANWVDKLFFG  240
U6        181  IEQYPDYQIAVTGHSLGGAAALLFGINLKVNGHDPLVVTLGQPIVGNAGFANWVDKLFFG  240

CLIB122   241  QENPDVSKVSKDRKLYRITHRGDIVPQVPFWDGYQHCSGEVFIDWPLIHPPLSNVVMCQG  30
DSM3286   241  QENPDVSKVSKDRKLYRITHRGDIVPQVPFWDGYQHCSGEVFIDWPLIHPPLSNVVMCQG  300
U6        241  QENPDVSKVSKDRKLYRITHRGDIVPQVPFWDGYQHCSGEVFIDWPLIHPPLSNVVMCQG  300

CLIB122   301  QSNKQCSAGNTLLQQVNVIGNHLQYFVTEGVCGI  334
DSM3286   301  QSNKQCSAGNTLLQQVNVIGNHLQYFVTEGVCGI  334
U6        301  QSNKQCSAGNTLLQQVNVIGNHLQYFVTEGVCGI  334
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Fig. 3. Multiple sequence alignment obtained with the ClustalW program of the extracellular lipase *LIP2* from *Y. lipolytica* strains CLIB122 (GenBank Accession No. XP500282), DSM3286 (GenBank Accession No. ADL57414), and U6 (GenBank Accession No. ADL57415). Asterisks with grey boxes indicate that threonine at the positions 121 and 129 is replaced with isoleucine and serine for the mutant lipase of *Y. lipolytica* U6 mutant strain in comparison with the native lipase of wild-type strains.

priate amino acid supplements, and either free linoleic acid or trilinolein as well as on laboratory and expensive media (Shockey *et al*., 2011).

Yarrowia lipolytica wild-type strains generally have lipase activities ranging from 1.8 U ml^{-1} to 45.5 U ml^{-1} (Papanikolaou *et al*., 2007). Hence, new recombinant *S. cerevisiae* strains produce acceptable amount of extracellular lipase in comparison with *Y. lipolytica* wild-type strains. Furthermore, the recombinant *S. cerevisiae* strains constructed in this study can utilize olive oil and

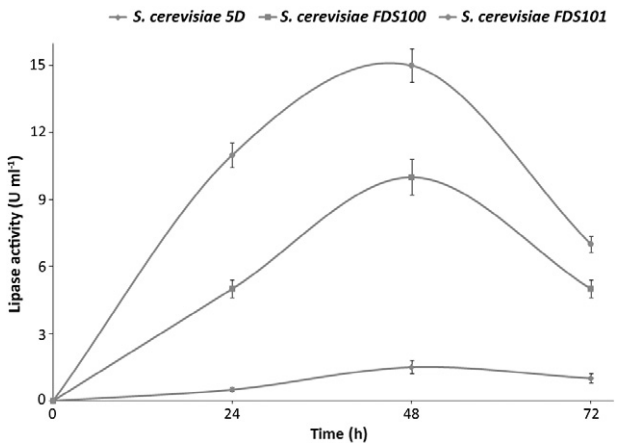

Fig. 4. Time course of lipase activity of *Saccharomyces cerevisiae* strain 5D, *S. cerevisiae* strain FDS100, and *S. cerevisiae* strain FDS101 during culture in olive oil medium (as a basal medium for lipase production). Error bars in graph represent the mean standard error from three independent experiments.

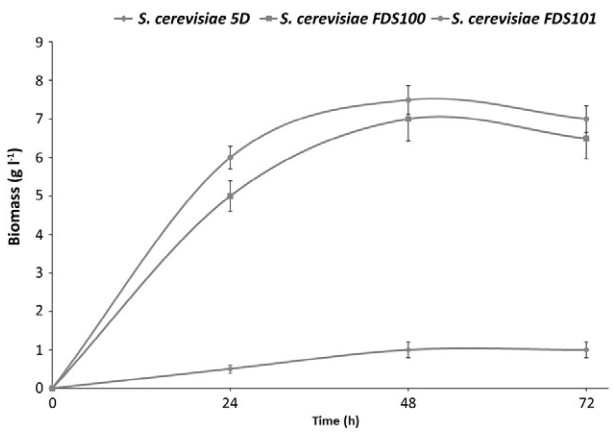

Fig. 5. Time course of biomass production of *Saccharomyces cerevisiae* strain 5D, *S. cerevisiae* strain FDS100, and *S. cerevisiae* strain FDS101 during culture in olive oil medium (as a basal medium for lipase production). Error bars in graph represent the mean standard error from three independent experiments.

lipids as low-cost substrates to produce bioethanol and other biotechnologically valuable products.

In the literature, it is suggested that lower biomass yield on substrate ($Y_{X/S}$) values around 0.5–0.7 g g^{-1} obtained from high-lipase producing wild *Y. lipolytica* strains are very satisfactory for single-cell protein fermentation from fatty substrates (Darvishi *et al*., 2009). The recombinant *S. cerevisiae* strains have lower $Y_{X/S}$ values around 0.7 g g^{-1} and can be used for single-cell protein production.

Inverse metabolic engineering by elucidation of a metabolic engineering strategy involves the following steps: first, identifying, constructing or calculating a desired phenotype; second, determining the genetic or the particular environmental factors conferring that phenotype; and third, endowing that phenotype on another strain or organism by directed genetic or environmental manipulation (Bailey *et al*., 2002). Now, inverse metabolic engineering is a good strategy in microbial enzyme biotechnology. To successfully attain extracellular lipase inverse metabolic engineering in *Y. lipolytica*, we constructed the high-level lipase producer strain U6 by UV mutagenesis (Darvishi *et al*., 2011). The effects of genetic changes in *LIP2* expression must be analysed stepwise in a suitable host. To address this problem, the ORF regions of mutant and native *LIP2* were selected for expression in *S. cerevisiae.*

The recombinant *S. cerevisiae* with mutations in the *LIP2* ORF region produced lipase with 1.5-fold higher activity compared with the wild-type ORF region. Therefore, the ORF region is not a good target for inverse metabolic engineering and site-directed mutagenesis of extracellular lipase in *Y. lipolytica*. Fickers *et al*. detected a single silent substitution of T for C at the *LIP2* coding region as well as six single substitutions and duplication of the ACAGATCAT sequence in the promoter region of the industrial mutant strain LgX64.81 (Fickers *et al*., 2003). In conclusion, instead of the ORF region, it will be more useful if the studies on mutant *LIP2* promoters from the industrial *Y. lipolytica* strains propose to construct stable and high-level extracellular lipase producing yeast strains.

Experimental procedures

Strains and culture conditions

Yarrowia lipolytica strain DSM3286 was obtained from the culture collection of the DSMZ, Germany. The *Y. lipolytica* U6 mutant strain was obtained by physical mutagenesis (with ultraviolet radiation) from the wild strain DSM3286 (Darvishi *et al*., 2011). *Saccharomyces cerevisiae* strain CEN.PK 113-5D (*MATA ura3-52 suc2 mal2-8*^{c}). Yeasts were grown on YPD (glucose, 1.5%; yeast extract, 1%; casein peptone, 1%) or YNB (yeast nitrogen base without amino acids and ammonium sulfate) supplemented according to auxotrophic requirements. For selection of Ura^+ clones, transformants were plated on YNBura (YNB with 0.01% uracil). YNBT (YNB with 1% of tributyrin) was used for the qualitative evaluation of lipolytic activity of the yeast strains.

Escherichia coli strain DH5α (Gibco-BRL, Rockville, MD, USA), used for transformation and amplification of recombinant plasmid DNA, were grown at 37°C in Luria–Bertani medium supplemented with ampicillin (100 μg ml^{-1}), when required (Fickers *et al*., 2005b).

The composition of the basal medium for lipase production was as follows: olive oil, 10 ml; yeast extract, 2 g; KH_2PO_4, 0.5 g; K_2HPO_4, 0.5 g; $MgSO_4$, 0.5 g; $CaCl_2$, 0.1 g; and NaCl, 0.1 g l^{-1}. Initial pH of the medium was adjusted to 6. At cultivation, the yeast strains were grown in 50 ml of YPD or production medium in 250 ml flasks at 30°C and 200 r.p.m. (Darvishi *et al*., 2009).

General genetic techniques

Standard molecular genetic techniques were used (Sambrook and Russell, 2001). Genomic DNA from *Y. lipolytica* strains were extracted and used as a template for polymerase chain reaction (PCR). Primers were designed and checked by OLIGO software (version 5.0, w. Rychlik) and blasted using BLAST facilities available on the NCBI web site against the *LIP2* nucleotide sequence from *Y. lipolytica* strain DSM3286 (GenBank Accession No. HM486899). Primers were as follows: Ylip2START (5′-ACG*GGATCC*ATGAAGC TTTCCACCATCC-3′) with *Bam*HI restriction site as the forward primer and Ylip2STOP (5′-TCC*GAATTC*CGCTTA GATACCACAGACACCCTC-3′) with the *Eco*RI restriction site as reverse primer (Fig. 1A).

The PCR amplification was performed in a thermal cycler (Eppendorf, Germany) with a final volume of 50 μl containing 5 μl of 10× buffer (100 mM Tris-HCl, pH 9, 500 mM KCl and 15 mM $MgCl_2$), 200 mM dNTP (200 mM each dATP, dCTP, dGTP and dTTP), 30–60 ng of template, 50 pmol appropriate primers and 0.5 U each of *Taq* or *Pfu* (Fermentas, Canada) DNA polymerases. Twenty-five PCR cycles were carried out with the following programme: denaturation at 95°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 1 min kbp^{-1}. DNA fragments were recovered from agarose gels by using a Bioneer gel extraction kit (Bioneer, Korea) (Darvishi *et al*., 2011).

Construction of LIP2 *expression vector and transformation of yeast cells*

The purified PCR product and *S. cerevisiae* expression vector p426GPD (Yeast 2 μ expression vector with *GPD* promoter and *URA3* marker; GenBank Accession No. DQ019861) were digested with *Bam*HI and *Eco*RI restriction enzymes, and then ligated with T4 ligase. The resulting vector was transformed into the *S. cerevisiae* strain CEN.PK 113-5D by using the lithium acetate method (Mumberg *et al*., 1995; Fickers *et al*., 2005b).

Analytical methods

Qualitative evaluation of yeasts was performed on H/C diameter of YNBT medium, where the H/C ratio represents the

ratio of the diameter of the hydrolysis halo (H) to the diameter of the cell colony (C). An H/C ratio of 1 means no tributyrin hydrolysis, and thus no lipase production, whereas a higher H/C ratio indicates lipase secretion. The H/C ratios of the mutant and wild-type strains were compared. Microbial growth was monitored by optical density measurements at 600 nm (Fickers *et al*., 2005c). The extracellular lipase activity was determined using a titrimetric assay performed in olive oil emulsion as the enzyme substrate. One unit (U) of lipase activity is defined as the amount of enzyme that produces 1 μmol of fatty acid per minute at 37°C and pH 7, as described previously (Kar *et al*., 2008). The molecular weight of the enzyme was determined by analysing the supernatant by using SDS-PAGE (Destain *et al*., 1997).

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