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Characterization of two novel lipase genes isolated directly from environmental sample

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Abstract Two novel lipase genes (*lipJ02*, *lipJ03*) were isolated directly from environmental DNA via genome-walking method. Lipase gene *lipJ02* contained an open reading frame (ORF) of 1,425 bp and encoded a 474-amino acids lipase protein, while lipase gene *lipJ03* contained an ORF of 1,413 bp and encoded a 470-amino acids lipase protein. The lipase genes were cloned into expression vector pPIC9K and successfully integrated into a heterologous fungal host, *Pichia pastoris* KM71, and the recombinant *P. pastoris* were screened via a high-throughput method. The recombinants were induced by methanol to secrete active lipases into cultural medium. The recombinant lipases were also purified and characterized. The optimum temperature for the purified lipase LipJ02 and LipJ03 was 30 and 35°C, respectively, at pH 8.0. They exhibited similar thermostability, but LipJ02 exhibited better pH stability than LipJ03.

Introduction

Lipolytic enzymes are ubiquitous in nature, being widely distributed in many kinds of organisms (Fojan et al. 2000). Lipases (E.C.3.1.1.3), which are esterases capable of hydrolyzing water-insoluble esters, have a wide range of potential uses in industry (Dharmsthiti and Kuhasuntisuk 1999; Jaeger et al. 1999). Lipase can also be used in enantioselective transesterification (Matsumoto et al. 2004). The classical approach to isolate new lipases is to screen a wide variety of microorganisms for lipolytic activity. The enzymes and the corresponding genes are then recovered from the identified organisms. Typically, however, only a small fraction (<1%) of microorganisms observed in nature can be cultivated using standard techniques (Amann et al. 1995). Thus, a large fraction of microbial diversity in the environment is lost due to the difficulties in enriching and isolating microorganisms in pure culture. Several approaches were recently developed to overcome this limitation. One is based on the use of environmental DNA for the construction of a DNA library and direct screening for functional gene products (Cottrell et al. 1999; Henne et al. 2000). Another method is to use PCR primers based on conserved regions of known genes to amplify novel genes from DNA obtained from uncultured biomass (Seow et al. 1997; Cottrell et al. 2000). We successfully isolated two lipase genes, *lipJ02* and *lipJ03*, directly via genome-walking method from environmental DNA. In this work, we report the cloning, sequencing and expression of the lipases, along with some biochemical properties of the purified enzymes.

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Materials and methods

Strains, vectors and culture medium

Escherichia coli DH5α, used as the host strain for recombinant plasmids, was grown in LB medium (1% tryptone, 0.5% yeast extract, 1%NaCl, pH 7.0) at 37°C. The pMD18-T vector, used as TA cloning vector for PCR product, was purchased from TaKaRa Biotechnology (Dalian, China). *Pichia pastoris* KM71 and pPIC9k, purchased from Invitrogen Corporation, were used as fungal host and expression vector, respectively. *P. pastoris* KM71 was grown in YEPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C. MD medium (1.34% YNB, 4×10^{-5}% biotin, 2% dextrose) plates were used to screen His^+ transformants. The recombinant *P. pastoris* was grown on BMGY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% glycerol) plates and BMMY medium (1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 0.5% methanol) plates at 30°C. BMGY

and BMMY medium were also used for scale-up of expression.

Nucleic acid manipulation

DNA was purified and manipulated essentially as described in a previous work (Sambrook et al. 1989). DNA was sequenced by the dideoxy chain-termination method on an ABI 377 automated sequencer. Sequence alignment was performed through BLAST (Altschul et al. 1997).

PCR amplification, cloning and sequencing of partial sequence of lipase genes

By comparing the amino acid sequences of over 20 lipases from *Pseudomonas*, two highly conserved regions (P-W-N-P-D-S-E and T-W-V-Q-D-L-N-R) flanking a fragment of about 280 amino acids were identified. Degenerate primers JBPF1 and JBPR1 were designed according to two highly conserved regions and CODEHOP (COnsensus-DEgenerate Hybrid Oligonucleotide Primer) primer design principles (Rose et al. 1998) (Table 1). The environmental sample DNA was prepared from soil samples (from Shanghai, China) as previously described (Zhou et al. 1996; Henne et al. 1999). Environmental DNA was used as template for amplifying partial sequence of lipase genes. PCR was performed as follows: 95°C 10 min, followed by 30 cycles of amplification (95°C 1 min, 60°C 45 s and 72°C 1 min) and 72°C 4 min after that. The purified PCR product was cloned into pMD18-T and sequenced with M13 primers from both strands by BioAsia Biotechnology (Shanghai, China).

Cloning of upstream and downstream sequences by genome-walking

Homology analysis through BLAST (Altschul et al. 1997) confirmed that the partial sequences obtained were fragments of two novel lipase genes. To obtain the upstream and downstream sequences flanking the fragments, the Gene Special Primers (GSP) GSPJ02up1, GSPJ02up2, GSPJ03up1, GSPJ03up2 and GSPJ02down1, GSPJ02down2, GSPJ03down1, GSPJ03down2 (Table 1) were designed for upstream and downstream sequences genome-walking of the two lipase genes, respectively. The primers AP1 and AP2 (Table 1) could be annealed with Genome-Walking Adaptor (GWA) (Table 1). Genomic DNA was ligated with GWA after digestion with blunting restriction enzymes *Sna*BI, *Eco*RV, *Sca*I and *Sma*I, and the genome-walking libraries were constructed. Genome-walking PCR was performed as previously described (Morris et al. 1998). Two-step cycle PCR and nested PCR were performed to elevate the specificity. The first PCR with primers AP1 and GSPJ02up1 or GSPJ03up1 (or

Table 1 Oligonucleotides used in this study[a]

Function	Names	Sequences(5′→3′)
Primers for partial sequence of lipase gene	JBPF1	ccStggaaYccSgaYtcggaa
	JBPR1	gcggttSaggtcYtgSaccca
Primers for genome-walking	GSPJ02up1	cccctggcgtcgaccttgccggtata
	GSPJ02up2	tgataggtgtccagccggcgtgttgca
	GSPJ02down1	atggcgacggcatgacgcgtgttctg
	GSPJ02down2	gcgggccaacacgtgggtccaagac
	GSPJ03up1	cgcgcatcgaccttgccaccgtagc
	GSPJ03up2	cgcgctggcactgatgggggtccaa
	GSPJ03down1	gcgggtgctcgaatcgggtttttacg
	GSPJ03down2	cagatgacccgcgattccaccgtgat
	Ap1	gtaatacgactcactatagggc
	Ap2	actatagggcacgcgtggt
Genome-walking adaptor	GWA	gtaatacgactcactatagggcacgcgtggtcgacggcccgggctggt H_2N-cccgacca-PO_4
Primers for lipase gene *lipJ02*, *lipJ03*	LipJ02Pf	aaa*gaattc*caacaaaagagaggcagtaccatg
	LipJ02Pr	aaa*gcggccgc*cccctcccaccgttttaactgat
	lipJ03Pf	aaa*gaattc*gacaagtccaacaacagagagg
	lipJ03Pr	aaa*gcggccgc*cttcctcacgcgataacc

[a]Base abbreviations: Y (c or t), S (g or c); primers JBPF1 and JBPR1 were used to amplify the partial sequence of lipase gene; primers GSPJ02up1, GSPJ02up2 and GSPJ02down1, GSPJ02down2 were used to amplify the upstream and downstream sequences of lipase gene *lipJ02*, respectively; primers GSPJ03up1, GSPJ03up2 and GSPJ03down1, GSPJ03down2 were used to amplify the upstream and downstream sequence of lipase gene *lipJ03*, respectively; primers Ap1 and Ap2 were the universal primers, which could be annealed with the Genome-Walking Adaptor; GWA is a Genome-Walking Adaptor consisting of two complementary oligonucleotide chains, with the 3′ end of the shorter chain modified by amido and 5′ end phosphorylated, and it could be ligated with the genomic DNA digested with blunting restriction enzyme to construct genome-walking library; primers LipJ02Pf and LipJ02Pr were used to amplify the full-length *lipJ02* gene with *Eco*RI and *Not* I restriction sites; primers LipJ03Pf and LipJ03Pr were used to amplify the full-length *lipJ03* gene with *Eco*RI and *Not*I restriction sites

GSPJ02down1 or GSPJ03down1) was performed as follows: 7 cycles of 35 s at 95°C, 4 min at 72°C, followed by 32 cycles of 35 s at 95°C, 4 min at 67°C, another period of 67°C for 10 min was added after the final cycle. The 1-μl genome-walking library was used as a template in the first PCR. The nested PCR was performed as previously described with primers AP2 and GSPJ02up2 or GSPJ03up2 (or GSPJ02down2 or GSPJ03down2), but the 1-μl product of the first PCR was used as template. Purified products of the nested PCR were cloned into pMD18-T, and sequenced with M13 primers from both strands obtained from BioAsia Biotechnology (Shanghai, China).

Construction of the recombinant plasmids containing lipase gene and expression in *P. pastoris*

Primers LipJ02Pf, LipJ02Pr and LipJ03Pf, LipJ03Pr (Table 1), with *Eco*RI and *Not*I restriction sites, respectively, were synthesized (based on the analysis of upstream and downstream sequences obtained from genome-walking) to amplify the complete ORF of *lipJ02* and *lipJ03* gene directly from the environmental DNA. The PCR products coding for the mature *lipJ02* and *lipJ03* were digested with *Eco*RI and *Not*I and subsequently cloned into the expression vector pPIC9k.

Electrocompetent cells of *P. pastoris* KM71 were prepared according to the supplier's instruction (Invitrogen). Ten micrograms of recombinant plasmid linearized with *Sac*I was mixed with 80 μl of electrocompetent cells, and electroporated via pulse discharge (1,500 V, 25 μF; Bio-Rad Gene Pulser) for 5 ms. After pulsing, 1 ml ice-cold 1 M sorbitol was immediately added to the cuvette. Then, 200-μl aliquots were spread on MD plates, and the plates were incubated at 30°C to screen for His^+ transformants according to their capacity to grow in the absence of histidine. His^+ clones were grown on BMGY plates at 30°C overnight, and then transferred onto BMMY plates supplemented with 1% olive oil and 0.0002% rhodamine B at 30°C. Fresh methanol (150 μl) was added in the lid of plates every 24 h to induce the lipase protein expression. The recombinant strains, which secreted functional lipases, were screened by BMMY plates supplemented with olive oil and rhodamine B.

Purification and characterization of the recombinant lipases

Scale-up of expression was performed according to the supplier's instruction (Invitrogen). The recombinant strains were grown in 100 ml BMGY medium at 30°C and 250 rpm until the OD_{600} of the culture reached 2.0–6.0. The cells were harvested by centrifugation and resuspended at a fivefold concentration in 20 ml BMMY medium to induce protein expression. The cells were incubated for 6 days at 30°C and 250 rpm, and fresh methanol was added to a final concentration of 0.5% to maintain induction every 24 h. Aliquots of culture supernatant were taken daily and examined for protein production by SDS-PAGE, and the lipase activity was assayed at the same time.

The lipases were purified on a DEAE-agarose column essentially as described by Abelson et al. (1990). Culture supernatants were desalted with a desalting column (16× 100 mm, Amersham-Pharmacia Biotech, Sweden) in buffer A (20 mM Tris–HCl, pH 7.0) at 10 ml/min as recommended. Next, the culture supernatants were loaded to a 5-ml DEAE-agarose column (Amersham-Pharmacia Biotech) run by AKTA Prime (Amersham-Pharmacia Biotech). Then, the column was washed with buffer A to remove the unbound proteins. A linear gradient of NaCl (50–600 mM) in buffer A was performed at 4 ml/min for 30 min. Eluates of every 5 ml were collected and analyzed by 12% SDS-PAGE. After protein separation, the characteristics of lipase LipJ02 and LipJ03 were determined. The release of *p*-nitrophenol (*p*-NP) from *p*-NP-derivative substrates were measured as previously described (Maurich and Zacchigna 1991; Prim et al. 2000). One unit of activity was defined as the amount of enzyme that released 1 μmol of *p*-NP per minute under the assay conditions.

GenBank accession number

Nucleotide sequence data of *lipJ02* and *lipJ03* are available in the GenBank database under accession numbers AY673674 and AY700013, respectively.

Results

Cloning and sequencing of lipase genes *lipJ02* and *lipJ03*

Two partial sequences of 870 nucleotides were obtained from environmental DNA as described in Materials and methods. Sequence alignment through BLAST (Altschul et al. 1997) showed that one nucleotide sequence was 79% identical to the partial sequence of lipase gene (Genbank AB025596) from *Pseudomonas* sp. MIS38 (Amada et al. 2000), 80% identical to that of lipase gene (Genbank AF307943) reported by Beven et al. (2001) and 85% identical to that of lipase gene (Genbank AB109039) from *Pseudomonas fluorescens* HU380 (Kojima et al. 2003); the other was 80, 83 and 85% identical to the same lipases mentioned, respectively. This confirms that the two nucleotide fragments were partial sequences of two novel lipase genes. The two highly conserved amino acids could be found in the translated partial sequences. Thus the gene special primers (GSP) were designed for genome-walking as described in Materials and methods. The sequences obtained by genome-walking were analyzed using GeneTool Lite software and two DNA sequence of 1,760 and 1,936 bp were reconstructed. One contained an open reading frame (ORF) of 1,425 bp, which was 83% identical to the lipase gene from *Pseudomonas* sp. MIS38 (Amada et al. 2000), 81% identical to that reported by Beven et al. (2001), 85% identical to that from *P. fluorescens* HU380

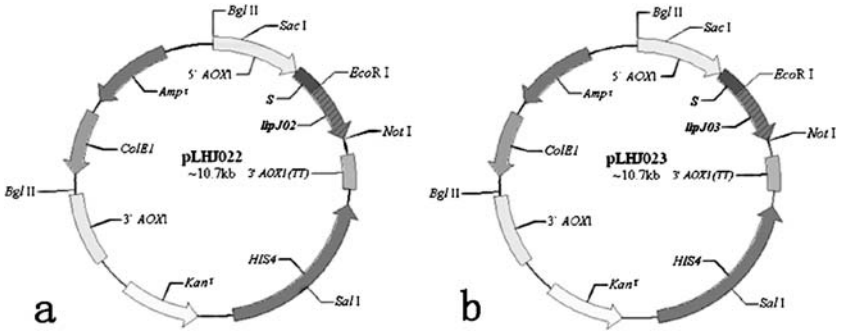

Fig. 1 The structure of the expression plasmids pLHJ022 and pLHJ023. *5'AOX1* Alcohol oxidase 1 promoter for methanol-inducible high level expression in *Pichia*, *S* α-factor signal sequence for secretion in *Pichia*, *lipJ02* lipase gene *lipJ02* cloned in frame and downstream of the α-factor signal sequence (**a**), *lipJ03* lipase gene *lipJ03* cloned in frame and downstream of the α-factor signal sequence (**b**), *3'AOX1(TT)*, transcriptional terminator from *Pichia pastorisAOX1* gene, *HIS4Pichia* wild-type gene coding for histidinol dehydrogenase and used to complement *Pichia* his4$^-$ strains, *kan^r* Kanamycin resistance gene from Tn903 which confers resistance to G418 in *Pichia*, *3'AOX1* sequences from the *AOX1* gene that are further 3′ to the TT sequences, *CoE1E. coli* origin of replication, *Amp^r* Ampicillin resistance gene

(Kojima et al. 2003) and encoded a 474-amino acids protein. The other contained an ORF of 1,413 bp, which was 84% identical to the lipase gene from *Pseudomonas* sp. MIS38 (Amada et al. 2000), 83% identical to that reported by Beven et al. (2001), 85% identical to that from *P. fluorescens* HU380 (Kojima et al. 2003), and encoded a 470-amino acids protein. The two sequences shared up to 86% identity between them, and were named *lipJ02* and *lipJ03*, respectively. The DNA sequences of them have been submitted to Genbank (accession number AY673674<*lipJ02*>, AY700013<*lipJ03*>).

Expression and purification of the lipases

Primers LipJ02Pf, LipJ03Pf and Lip02Pr, Lip03Pr (Table 1) with restriction sites *Eco*RI and *Not*I, respectively, were designed to amplify the complete sequence of *lipJ02* and *lipJ03* gene by using the environmental DNA as template. The PCR products were cloned into the expression vector pPIC9k with the ORF of mature genes being cloned in frame and downstream of the α-factor signal sequence to allow secretion of recombinant proteins into medium. The recombinant plasmids with *lipJ02* and *lipJ03* were named pLHJ022 (Fig. 1a) and pLHJ023 (Fig. 1b), respectively.

The recombinant plasmids pLHJ022 and pLHJ023 linearized with *Sac*I were transformed into fungal host, *P. pastoris* KM71. A total of 50 His$^+$ transformants of each sample were transferred onto BMGY plates and incubated at 30°C for 24 h. Then the transformants were transferred onto BMMY plates supplemented with 1% olive oil and 0.0002% rhodamine B and induced by fresh methanol as described in Materials and methods. Because the functional lipase secreted from the transformants could react with olive oil and rhodamine B in particular (Kouker and Jaeger 1987), the expression patterns could be determined via the fluorescent halo around some His$^+$ transformants under UV light (Fig. 2). The transformant with the highest lipase activity (bigger fluorescent halo implied higher lipase activity) was inoculated from each sample and named KM71-pLHJ022 and KM71-pLHJ023, respectively. By using the total DNA of KM71-pLHJ022 as a template, a DNA fragment with the same size as lipase gene *lipJ02* was obtained with primers LipJ02Pf and LipJ02Pr by PCR amplification (total DNA of *P. pastoris* KM71 transfected with the plasmid pPIC9k was used as negative control template), which convinced us that the KM71-pLHJ022 was a recombinant *P. pastoris* with lipase gene *lipJ02*. KM71-pLHJ023 was a recombinant *P. pastoris* with lipase gene *lipJ03*, which was identified via the same method with primers LipJ03Pf and LipJ03Pr.

Scale-up of expression was performed as described in Materials and methods. Aliquots of culture supernatants were taken daily and analyzed by SDS-PAGE, and the lipase activity was assayed at the same time. The protein expressed by recombinant strain KM71-pLHJ022 and KM71-pLHJ023 (along with *P. pastoris* KM71 cells transfected with the control plasmid pPIC9k) were analyzed by SDS-PAGE, which revealed a new band in the medium

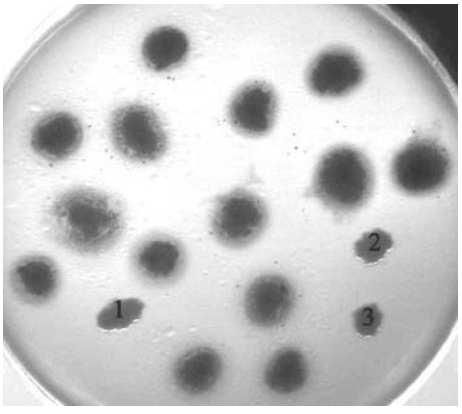

Fig. 2 Recombinants screened by BMMY plates supplemented with olive oil and rhodamine B (observation under UV light) 1–3: negative control (*Pichia pastoris* KM71 transfected with the control plasmid pPIC9k); the others: recombinants, which secreted functional lipase (fluorescent halo around them under UV light)

with an apparent molecular mass corresponding to the predicted size (about 52 kDa) of LipJ02 and LipJ03 (Fig. 3a), respectively. Lipase activity reached its maximum level (13.6 and 15.3 U/ml, respectively, assayed at 37°C, pH 8.0; with *p*-nitrophenol-laurate used as a substrate) in an induction period of 4 days, and the culture supernatants were harvested for further analysis.

As the predicted isoelectric point of the lipase LipJ02 and LipJ03 were about pH 4.71 and 4.72, respectively, using Gene Runner software, anion-exchange chromatography was used to purify the recombinant proteins from culture supernatants essentially according to Abelson et al. (1990). The recombinant proteins were successfully purified by anion-exchange chromatography with a linear gradient of NaCl (50–600 mM). The recombinant proteins were released by the elution buffer of about 200 mM NaCl. Every 5 ml of eluate was collected and analyzed by SDS-PAGE, and only one band of about 52 kDa could be detected, which indicates the high purity and homogeneity of the sample (Fig. 3b). The yield of purification was about 363.4 and 386.3 mg per liter of culture supernatants, respectively, determined spectrophotometrically at 280 nm using calculated extinction coefficients (Stoscheck 1990).

Characterization of the lipase LipJ02 and LipJ03

p-Nitrophenol-laurate was used as a substrate to assay the lipase activity. Lipase LipJ02 and LipJ03 had a temperature optimum of 30 and 35°C, respectively, at pH 8.0. A total lipase activity of 42.6 and 45.1 U could be detected per 1 mg purified protein under optimum reaction conditions. LipJ02 retained over 80% activity after being incubated at a pH range (5.0–10.0) for 3 h at room temperature, whereas lipase LipJ03 only retained about 65%. They retained about 65% activity after being incubated at 60°C for 3 h at pH 7.0.

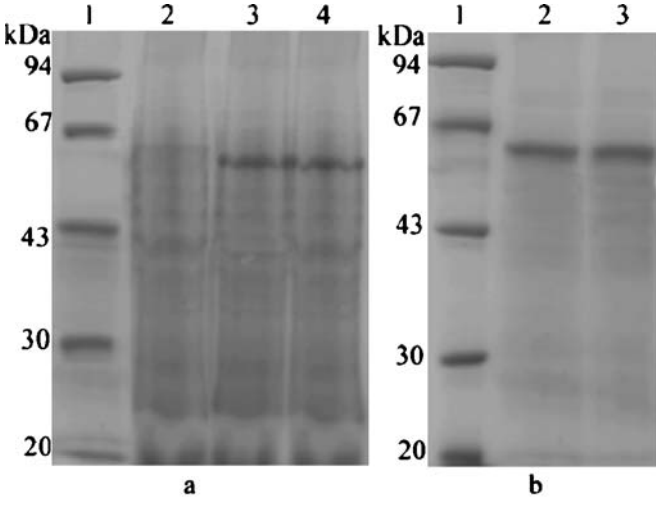

Fig. 3 SDS-PAGE analysis on samples of expression and pure proteins. (Samples were resolved on 12% polyacrylamide gel and then stained with Coomassie Blue R-250.) **a** *Lane 1*, protein molecular weight markers; *lane 2* culture supernatants of *Pichia pastoris* KM71 transfected with the control plasmid pPIC9k induced as negative control; *lane 3* culture supernatants of recombinant *Pichia pastoris* KM71-pLHJ022 after being induced for 4 days; *lane 4* culture supernatants of recombinant *Pichia pastoris* KM71-pLHJ023 after induced for 4 days. **b** *Lane 1*, protein molecular weight markers; *lane 2*, purified lipase LipJ02; *lane 3*, purified lipase LipJ03

Discussion

Many lipase genes have been isolated from genomic libraries of cultured microorganisms (Chung et al. 1991; Oh et al. 1999; Martinez and Soberon-Chavez 2001; Rahman et al. 2003; Ruiz et al. 2003). However, a large fraction of the microbial diversity in an environment is lost due to the difficulties in enriching and isolating microorganisms in pure culture. Several approaches were developed to overcome this problem, as mentioned in Introduction. To screen a DNA library of environmental sample, enough purified environmental DNA and gene-specific oligonucleotide probes should be prepared. Otherwise, a sensitive assay on functional gene products should be developed. All this means lots of hard work and higher costs. Fortunately, two highly conserved regions were found by comparing the amino acid sequences of over 20 lipases from *Pseudomonas* sp. Based on the two highly conserved regions, degenerate primers JBPF1 and JBPR1 (Table 1) were designed to amplify partial sequence of lipase genes. Then the full-length lipase genes were obtained by genome-walking method. It was an effective method for isolating lipase genes from environmental sample, and fewer environmental DNAs were needed than for constructing a DNA library of environmental sample. We successfully isolated two novel lipase genes (*lipJ02* and *lipJ03*) from environmental sample DNA via this method. Sequence alignment through BLAST (Altschul et al. 1997) showed that *lipJ02* and *lipJ03* were two novel lipase genes from uncultured *Pseudomonas* sp. If conserved regions can be found from different genera, different lipase gene can be obtained by this method.

The lipase genes *lipJ02* and *lipJ03* have been expressed in a heterologous fungal host, *P. pastoris* KM71. Generally, recombinants are screened by PCR from numerous transformants, and the expression pattern should be analyzed individually. We screened the recombinants by BMMY plates supplemented with olive oil and rhodamine B, and PCR was only used to identify the recombinant. Our main concern was the functional lipases expressed and secreted by the recombinants, which made screening recombinants easier and more direct, and less costly, too. It is a high throughput method for screening the recombinant *P. pastoris*. Since the major protein expressed by recombinant *P. pastoris* were secreted lipases, the recombinant protein could be conveniently purified from culture supernatants.

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