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Construction of a whole-cell catalyst displaying a fungal lipase for effective treatment of oily wastewaters

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ABSTRACT

To combine the advantage of the oleaginous yeast *Yarrowia lipolytica* with the high activity of some fungal lipases for oily wastewater treatment, an effective lipase-displaying arming yeast was constructed using the flocculation functional domain of *Saccharomyces cerevisiae* as the protein anchor. To estimate the effect of the whole-cell oily wastewater treatment, the lipase-displaying arming yeast was added into an open activated sludge bioreactor. Within 72 h of whole-cell treatment, 96.9% of oil and 97.6% of chemical oxygen demand (COD) were removed, while only 87.1% of oil and 91.8% of COD were removed in control A (*Y. lipolytica* Polg was added), 45.1% of oil and 67.5% of COD were removed in control B (no cell was added) in 72 h. The lipase-displaying arming yeast exhibited remarkable oil removal and COD degradation effect compared with the control samples, exemplifying its application potential.

1. Introduction

Oily wastewater causes serious problems due to its high oil content and chemical oxygen demand (COD). Several methods have been proposed for oily wastewater treatment, including physico-chemical processes such as decantation, chemical oxidation, concentration, ultra filtration and reverse osmosis [1]. Compared with those methods, biological methods are also useful ones. To improve the biodegradability and to reduce oily wastewater toxicity, a variety of microorganisms and lipases have been used in oily wastewater treatment processes [2–7]. These microorganisms and lipases are usually used in the pre-treatment, so that some especial equipment and process must be added, implying excess wastewater pre-treatment process, long wastewater treatment cycle and high cost.

Lipases have been widely used in oily wastewater pre-treatment, but they are generally soluble and unstable. To overcome these problems, the lipases used for oily wastewater treatment are immobilized [4,5]. *Yarrowia lipolytica* is an useful microorganism for oily wastewater treatment [8], and it can stay alive in oily wastewater for a long time [9,10]. So *Y. lipolytica* can be directly added into activated sludge used for oily wastewater treatment, and become the microbial population of the activated sludge. This constitutes a convenient technique for oily wastewater treatment, but the lipase activity secreted by *Y. lipolytica* maybe too low for effective oil degradation. In the present work, we combine the advantage of *Y. lipolytica* as an oleaginous yeast with the high activity of a fungal lipase for oily wastewater treatment.

To increase the lipase activity of *Y. lipolytica*, we had the heterologous lipase lipRS displayed on *Y. lipolytica* Polg cell surface using the flocculation functional domain of *Saccharomyces cerevisiae* (Flo1p, encoded by *FLO*) as the protein anchor. The lipase lipRS was from the fungus *Rhizopus stolonifer*, and its characteristic was fit for oily wastewater treatment. The lipase-displaying arming yeast exhibited higher activity than the *Y. lipolytica* Polg strain, and proved effective for whole-cell oily wastewater treatment.

2. Materials and methods

2.1. Strains, vectors, enzymes, culture media and chemicals

Escherichia coli DH5α, used as the recipient strain for recombinant plasmids, was grown in LB medium (1% tryptone, 0.5% yeast

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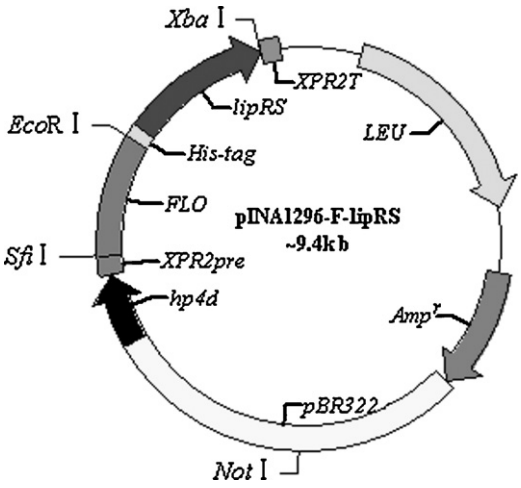

Fig. 1. Construction of the recombinant plasmid pINA1296-F-lipRS. hp4d, hybrid promoter derived from p*XPR2* (promoter from the alkaline extracellular protease – AEP – from *Y. lipolytica*); *XPR2* pre and *XPR2T*, secretion signal and transcriptional terminator from the AEP; *FLO*, flocculation functional domain Flo1p from *S. cerevisiae*; His-tag, the sequence encoding 6 × His-tag for immunostaining; *lipRS*, lipase gene isolated from *R. stolonifer*; *LEU2*, beta-isopropylmalate dehydrogenase from *Y. lipolytica*; *Amp*^{r}, Ampicillin resistance gene for *E. coli*; pBR322, provides a pBR docking platform for ulterior integration of pBR-based vectors.

extract, 1% NaCl, pH 7.0) at 37 °C. The *Y. lipolytica* Polg strain (Leu$^-$, ΔAEP, ΔAXP, Suc$^+$) and the expression plasmid pINA1296 were kindly supplied by Prof. Catherine Madzak [11,12]. The activated sludge was obtained from Shahu Lake sewage farm (Wuhan, China), and cultured in an open bioreactor system with synthetic oily wastewater consisting of measurable glucose, peptone and phosphate. The lipase gene *lipRS* (GenBank: DQ139862) isolated from *R. stolonifer* was kindly supplied by Dr. Yinbo Zhang [13], and the *FLO* gene was cloned from *S. cerevisiae* ATCC 60715. Yeast were grown in complete medium (YPD: 1% yeast extract, 2% peptone, 2% glucose) or selective medium (MD: 1.34% yeast nitrogen base, 4×10^{-5}% biotin, 2% dextrose, 2% agar). The *exTaq* DNA polymerase, restriction enzymes, T4 DNA ligase and modification enzymes were purchased from TaKaRa Biotechnology (Dalian, China) Co., Ltd. All the other chemicals used in this experiment were purchased from China National Medicines Corporation Ltd.

2.2. Nucleic acid manipulation

DNA was purified and manipulated essentially as described by Sambrook et al. [14]. DNA was sequenced with the ABI 3730 automated sequencer from both strands by Sunny-bio Biotechnology Ltd. (Shanghai, China).

2.3. Construction of the recombinant plasmid

The *FLO* gene was amplified using the genomic DNA of *S. cerevisiae* ATCC 60715 as a template, the primers were designed according to the *FLO* gene sequence (GenBank accession no. NC_001133): FLO-f-*Sfi*I, 5′-tttggccgttctggccatgacaatgcctcatcgctatatgt-3′ (the underlined bases encode the *Sfi*I site); FLO-r-*Eco*RI-His, 5′-gatgaattc*atggtgatggtgatgatg*ggtgatttgt-3′ (the underlined and italic bases encode *Eco*RI and 6 × His-tag sites respectively). The *lipRS* gene was amplified with the primers: lipRS-f-*Eco*RI, 5′-gcggaattcatggtttcattcatttccattactcaag-3′ (the underlined bases encode *Eco*RI); lipRS-r-*Xba*I, 5′ -gcgtctagattacaaacagcttccttcgttgatacc-3′ (the underlined bases

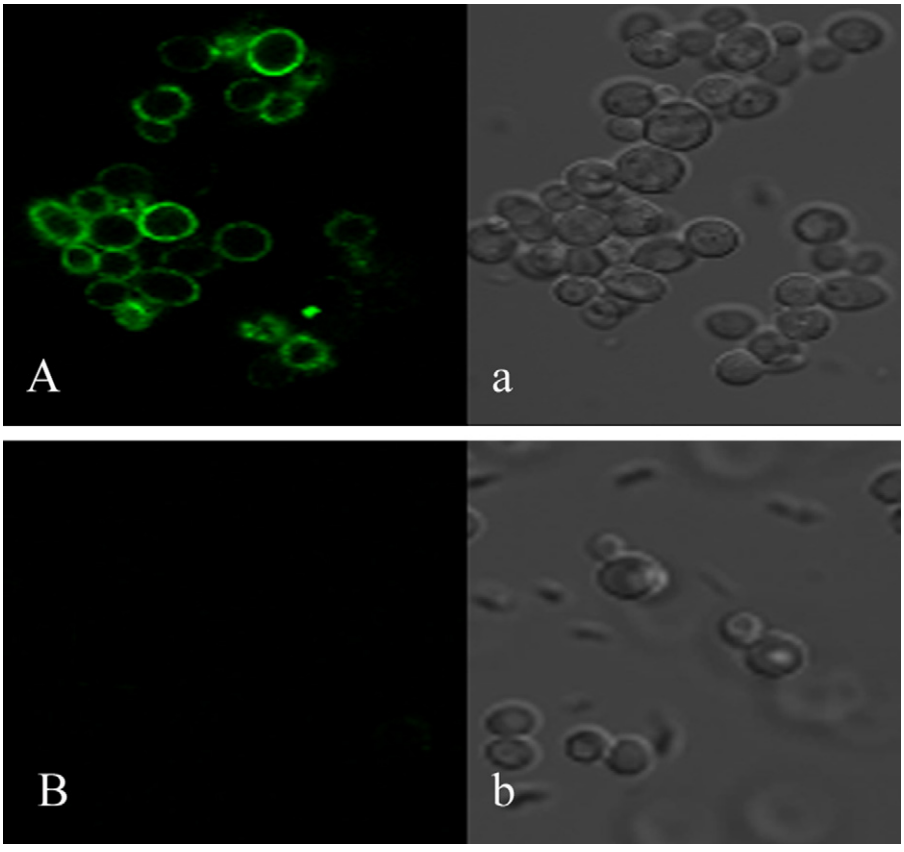

Fig. 2. Detection of the recombinant *Yarrowia lipolytica* with immunofluorescence. Fluorescence micrographs (panels A and B) and differential interference contrast micrographs (panels a and b) are shown. Panels A and a, *Yarrowia lipolytica* (pINA1296-F-lipRS); panels B and b, *Yarrowia lipolytica* Polg transformed with pINA1296 (control).

Table 1
Summary of oil removal in the open bioreactor system.

Parallel experiments	Oil concentration (g/L)/oil-degradation rate (%)						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
1[a]	5.00×10^3/0	1.14×10^3/77.2	873/82.5	682/86.4	466/90.7	311/93.8	153/96.9
2[b]	5.00×10^3/0	2.10×10^3/57.9	1.54×10^3/69.3	1.20×10^3/75.9	983/80.3	761/84.8	643/87.1
3[c]	5.00×10^3/0	4.47×10^3/10.7	4.08×10^3/18.4	3.67×10^3/26.7	3.05×10^3/38.9	2.89×10^3/42.2	2.75×10^3/45.1

[a] Whole-cell lipase *Yarrowia lipolytica* (pINA1296-F-lipRS).
[b] Control A, *Yarrowia lipolytica* polg.
[c] Control B, no cell added.

encode *Xba*I). The fragments *FLO* and *lipRS* were digested with *Eco*RI and ligated by T4 DNA ligase. Subsequently, the ligated product served as a template to amplify the fusion fragment *FLO–lipRS* using FLO-f-*Sfi*I as the forward primer and lipRS-r-*Xba*I as the reverse primer. The resultant 2.2 kb PCR product was recovered and digested with *Sfi*I and *Xba*I, and then ligated with the expression vector pINA1296 digested with the same restriction enzymes. The fusion fragment *FLO–lipRS* was cloned into the expression vector pINA1296, and the recombinant plasmid was named pINA1296-F-lipRS.

2.4. Yeast transformation and screening of the recombinant strains

The recombinant plasmid pINA1296-F-lipRS prepared as described above was linearized with *Not*I and transformed into *Y. lipolytica* polg as described by Madzak et al. [15]. Transformants were obtained by plating on MD selective medium plates and incubated at 28 °C for 36–48 h. After the transformants were identified, they were grown in YPD culture media for 6 days to have the heterologous lipase expressed and surface displayed.

2.5. Immunofluorescence assay

Immunostaining was performed as follows: a total of 2×10^7 yeast cells that displayed fusion protein FLO-lipRS were collected by centrifugation at 4×10^3 *g* for 1 min at 4 °C. The collected cells were washed three times with PBS containing 0.1% (w/v) BSA and then the cells were incubated with the 6 × His-tag mouse monoclonal antibody (Novagen; diluted to 1:50 with 1% BSA) on ice for 2 h with occasional mixing. After the cells had been washed three times with PBS, the secondary antibody, fluorescein isothiocyanate (FITC)-conjugated goat anti-mouse immunoglobulin G (diluted 1:200 in PBS with 1% BSA), was added and allowed to react with the cells on ice for 30 min in the dark. Finally, the cells were washed three times with PBS and detected by laser-scanning confocal microscopy (Leica TCS SP2).

2.6. Assay of the recombinant lipase activity

Yeast cells were collected by centrifugation at 4×10^3 *g* at room temperature for 1 min. After the cells had been washed twice with 1 ml of 20 mM Tris–Hcl buffer (pH 8.0), the cell suspension was adjusted to an OD_{600} of 6.0. 100 μl of the cell suspension and 50 μl of 25 mM *p*-nitrophenyl palmitate were mixed and allowed to react at 37 °C for 10 min. The reaction was stopped by the addition of 500 μl of ethanol. Then the reaction mixture was centrifuged at 5×10^3 *g* at room temperature for 1 min. 200 μl of the resulting supernatant was placed in a 96-well plate, and the activity of lipase was assayed by measuring the absorbance of liberated ρ-nitrophenol (ρNP) at 405 nm using a kinetic microplate reader [16,17]. One unit (U) of hydrolysis activity was defined as the amount of enzyme that released 1 μmol of ρ-NP per minute under the assay conditions.

2.7. Culture of activated sludge and analytical methods

The activated sludge was cultured in a 15 L open bioreactor system with synthetic oily wastewater consisting of glucose, peptone and phosphate. For investigating the effect of the whole-cell lipase accompanying oil degradation, synthetic oily wastewater containing 5×10^3 mg/L oil was placed in 15 L open bioreactor system, and 500 ml whole-cell lipase culture with YPD (about 10^8 cell/ml, removing the culture medium before use) was put into the open bioreactor system. Two control experiments were performed: control A, 500 ml *Y. lipolytica* Polg culture with YPD (about 10^8 cell/ml, removing the culture medium before used) were added; control B, no cell were added. Each open bioreactor system was carried out at room temperature, and aeration was supplied at 0.1 L air/min by an air pump. Oil and COD degradation rates were measured every 12 h.

Oil concentration was determined according to the gravimetric method [18]. Oil-degradation rate (%) was defined as the amount of oil degraded versus the amount of initial oil. COD was determined according to the dichromate titration [19]. The COD degradation efficiency was defined as the amount of COD decreased versus the amount of initial COD. All the experiments were performed in triplicate, and the average was calculated.

3. Results

3.1. Construction of the lipase-displaying arming yeast

The recombinant plasmid pINA1296-F-lipRS containing *FLO–lipRS* fusion gene was constructed. The fusion gene *FLO–lipRS* was cloned into the plasmid pINA1296, downstream from the 3′-end of the secretion signal sequence (*XPR2* pre). The sequence

Table 2
Summary of COD degradation in the open bioreactor system.

Parallel experiments	COD (mg/L)/COD degradation rate (%)						
	0 h	12 h	24 h	36 h	48 h	60 h	72 h
1[a]	1.50×10^4/0	8.56×10^3/42.9	6.53×10^3/56.4	5.33×10^3/64.5	2.31×10^3/84.6	956/93.6	356/97.6
2[b]	1.50×10^4/0	1.13×10^4/24.5	9.97×10^3/33.5	7.63×10^3/49.1	5.70×10^3/62.0	3.53×10^3/76.5	1.24×10^3/91.8
3[c]	1.50×10^4/0	1.43×10^4/4.6	1.13×10^3/24.5	9.99×10^3/33.4	7.96×10^3/46.9	6.54×10^3/56.4	4.87×10^3/67.5

[a] Whole-cell lipase *Yarrowia lipolytica* (pINA1296-F-lipRS).
[b] Control A, *Yarrowia lipolytica* polg.
[c] Control B, no cell added.

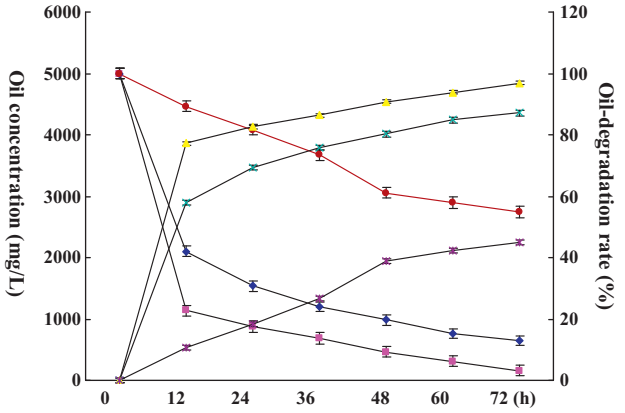

Fig. 3. Oil degradation in the open bioreactor system. Whole-cell lipase, *Yarrowia lipolytica* (pINA1296-F-lipRS); control A, *Yarrowia lipolytica* Polg; control B, no cell added. , whole-cell lipase oil concentration; , control A oil concentration; , control B oil concentration; , whole-cell lipase oil degradation rate; , control A oil degradation rate; , control B oil degradation rate.

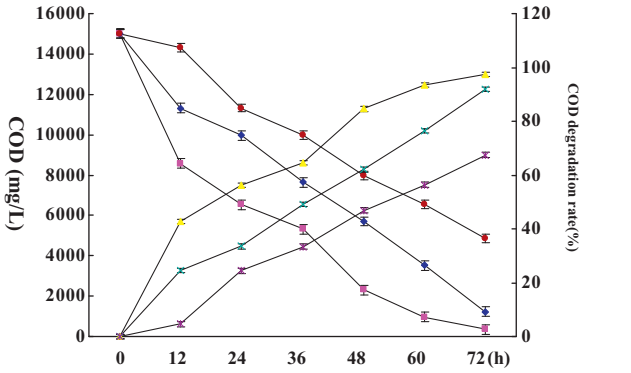

Fig. 4. The COD degradation in the open bioreactor system. Whole-cell lipase, *Yarrowia lipolytica* (pINA1296-F-lipRS); control A, *Yarrowia lipolytica* Polg; control B, no cell added. , whole-cell lipase COD; , control A COD; , control B COD; , whole-cell lipase COD degradation rate; , control A COD degradation rate; , control B COD degradation rate.

encoding the 6 × His-tag was inserted between *FLO* and *lipRS* for immunostaining, as shown in Fig. 1, the C-terminal of the Lip RS was free to allow the protein fold correctly. The recombinant plasmid structure was confirmed by sequence analysis.

The recombinant plasmid pINA1296-F-lipRS was linearized with *Not*I, and transformed into *Y. lipolytica* Polg strain. The *lipRS* gene was expressed under the control of the quasi-constitutive *hp4d* hybrid promoter. The recombinant strain was named *Y. lipolytica* (pINA1296-F-lipRS). Expression of the whole-cell lipase was performed by flask cultivation.

3.2. Detection and characterization of the whole-cell lipase

To detect the displayed lipase on the recombinant *Y. lipolytica* (pINA1296-F-lipRS) cell surface, the immunofluorescence assay was performed. Simultaneously, the *Y. lipolytica* cells transformed with pINA1296 plasmid were used as a negative control. Observed with the laser scanning confocal microscope, the recombinant *Y. lipolytica* cells emitted green fluorescence, as shown in Fig. 2. In contrast, the negative control was not luminescent. This confirmed that the recombinant lipases were displayed on the cell surface successfully.

To investigate the activity of the whole-cell lipase, the recombinant *Y. lipolytica* (pINA1296-F-lipRS) and *Y. lipolytica* Polg were cultivated for 6 days before use as whole-cell enzyme, and 25 mM *p*-nitrophenyl palmitate was used as substrate. The activity of *Y. lipolytica* (pINA1296-F-lipRS) and *Y. lipolytica* Polg were respectively 293 and 191 U/g dry cell at 30 °C, pH 7.0. The *Y. lipolytica* cells displaying the heterologous lipase lipRS thus exhibited higher hydrolysis activity than the host (*Y. lipolytica* Polg).

3.3. Application of the whole-cell lipase in oily wastewater treatment

To estimate the effect of the whole-cell lipase for oily wastewater treatment, three parallel experiments were performed. Synthetic oily wastewater containing 5×10^3 mg/L oil was used in each experiment; its initial COD was 1.5×10^4 mg/L. Table 1 summarizes the oil removal results in parallel experiments. In the first 12 h, 77.2% of the oil was degraded and after 72 h treatment, 96.9% of the oil was removed by the whole-cell lipase. While only 87.1% and 45.1% oil were degraded in controls in 72 h. As shown in Fig. 3, oil removal in the open activated sludge bioreactor system with the lipase-displaying arming yeast appears more effective than that with *Y. lipolytica* Polg. The whole-cell lipase exhibited remarkable oil removal effect.

At the same time, the change of the COD was inspected. The trend of COD change was similar to the trend of oil concentration, as shown in Fig. 4. The COD degradation efficiency was 42.9% during the first 12 h, and it reached 97.6% after 72 h in the open activated sludge bioreactor system with the whole-cell lipases, while the COD degradation efficiency of the controls were only 91.8% and 67.5% in 72 h. As shown in Table 2, the whole-cell lipase was effective for oil removal and COD degradation.

4. Discussion and conclusion

Y. lipolytica [8] and lipases [4,5] have been previously used for oily wastewater treatment. To avoid the excess wastewater pre-treatment process and high cost, we constructed a whole-cell lipase. It combined the advantages of *Y. lipolytica* (which can be used directly in activated sludge) with the high activity of a fungal lipase for oily wastewater treatment. The oil was hydrolyzed by whole-cell lipase fleetly, and became small organic compounds which could be used easily by the microorganisms in activated sludge. So the whole-cell lipase exhibited remarkable oil removal and COD degradation effects, showing its important application potential. The *Y. lipolytica* displayed with the lipase was a GMO (Genetically Modified Organism), so its bio-safety must be evaluated before its practical application in wastewater treatment.

Y. lipolytica constitutes an effective expression system [11,12], the hybrid promoter (*hp4d*) having already been applied to the production or secretion of a variety of recombinant proteins. But it has never before been used for reconstructing the microbial population in an activated sludge. In this study, we successfully constructed a whole-cell lipase for oily wastewater treatment and demonstrated its potential.

Acknowledgements

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