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# The Possible Involvement of the Cell Surface in Aliphatic Hydrocarbon Utilization by an Oil-Degrading Yeast, *Yarrowia lipolytica* 180

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**Abstract** An oil-degrading yeast, *Yarrowia lipolytica* 180, exhibits interesting cell surface characteristics under the growth on hydrocarbons. An electron microscopic study revealed that the cells grown on crude oil showed protrusions on the cell surface, and thicker periplasmic space and cell wall than the cells grown on glucose. *Y. lipolytica* cells lost its cell hydrophobicity after pronase (0.1 mg/ml) treatment. The strain produced two types of emulsifying materials during the growth on hydrocarbons; one was water-soluble extracellular materials and the other was cell wall-associated materials. Both emulsifying materials at lower concentration (0.12%) enhanced the oil-degrading activity of *Moraxella* sp. K12-7, which had medium emulsifying activity and negative cell hydrophobicity; however, it inhibited the oil-degrading activity of *Pseudomonas* sp. K12-5, which had medium emulsifying activity and cell hydrophobicity. These results suggest that the oil-degrading activity of *Y. lipolytica* 180 is closely associated with cell surface structure, and that a finely controlled application of *Y. lipolytica* 180 in combination with other oil-degrading microorganisms showed a possible enhancing efficiency of oil degradation.

**Key words:** *Yarrowia lipolytica* 180, bioemulsification, cell hydrophobicity, aliphatic hydrocarbon, biodegradation

The growth of yeast and other microorganisms on hydrocarbon substrate requires the transport of hydrocarbon from the oil phase into the cells. Microorganisms can utilize liquid hydrocarbons in the solution form, in the accommodation form, or in the large drop form [2, 22]. Because most hydrocarbons are poorly soluble in water, the concentration of hydrocarbons in water solution is extremely low. Therefore, it was suggested that hydrocarbons dissolved in water phase were not the source of hydrocarbons for the cells of *Candida lipolytica*, but rather those attached to cells were the source [16] for the growth on hexadecane. Hydrocarbons in accommodation form, which is formed by emulsifying agents, are much smaller than microorganisms and hence the hydrocarbon droplets attach to the surface of the cells, instead of the cells attaching to the surface of the hydrocarbon drops. In the case of the larger drop form, microorganisms directly attach to the surface of hydrocarbon drops which are much larger than the cells, and hydrocarbon uptake presumably takes place through diffusion or active transport at the point of contact [14]. Therefore, the availability of hydrocarbon surface area for cell attachment would be the limiting factor for hydrocarbon uptake. The interfacial area between hydrocarbon and aqueous phase can be greatly increased with the decrease in hydrocarbon droplet size, leading to increased hydrocarbon uptake by microorganisms [2, 9]. The effects of emulsifying materials on the growth of hydrocarbon-degrading microorganisms have been reported to be both positive and negative [1, 23, 24], but the reason is not yet clear.

In addition to the production of emulsifying materials, cell hydrophobicity can be considered as one of the important factors controlling hydrocarbon assimilation. Cells with higher hydrophobicity have better chances to adhere to oil droplets [20]. The cell surface properties of hydrocarbon-degrading microorganisms to adhere to hydrocarbon surface have been well documented [17, 20, 25], however, the processes by which the hydrocarbons are transported to the cells and subsequently assimilated by the cells are still not fully understood. There have been several studies on the surface structure of yeast cells by the use of scanning electron microscopy and transmission electron microscopy to understand the mode of transportation [10, 18, 21]. These studies revealed many interesting features on the physiological activities and the ultrastructure of the cells.

In order to understand the role of cell surface of *Yarrowia lipolytica* 180 in hydrocarbon degradation, the surface structure of *Y. lipolytica* 180 in relationship with its degradation of hydrocarbons was studied. For possible

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application of *Y. lipolytica* 180 in bioremediation of oil spilled environments, the enhancement of oil degradation by *Y. lipolytica* 180 in combination with other oil-degrading bacterial strains was determined.

## MATERIALS AND METHODS

### Microorganism and Growth Condition

The yeast strain used in this study, *Y. lipolytica* 180 (KCTC 0533BP), was isolated from oil-contaminated soil sampled in Panweol industrial area, Republic of Korea [6]. The strain was cultured on Yeast Malt Extract medium (YM medium, Difco Laboratories, Detroit, U.S.A.) or on the mineral salts medium (MSM) containing Arabian light crude oil as described by Kim *et al.* [6].

### Electron Microscopy

To prepare samples for scanning electron microscopy (SEM), centrifuged cells were overlayed on 2.0% (w/v) agar, and then fixed with 2% glutaraldehyde in 0.1 M potassium phosphate buffer (pH 7.2). Cells were postfixed with 1% osmium tetroxide in the same buffer for 3 h. Cells were dehydrated through a graded alcohol series of alcohol-water mixture, and then reacted with isoamyl acetate and dried at room temperature. The blocks were coated with gold (Edwards S150B) after drying at room temperature, and then the blocks were observed using SEM (Hitachi, S-507, Japan).

For transmission electron microscopy (TEM), cells were postfixed with 1% osmium tetroxide in the same buffer for 40 min, washed, and then dehydrated. Reacted blocks with propylene oxide were finally embedded in Epon 812 [11, 12]. Thin sections were cut with a diamond knife on ultratome (LKB2088) and mounted on grids. The sections were stained with uranyl acetate and lead citrate before an electron microscopic examination (TEM 109 CARL ZEISS, Germany). A total of 30 transmission electron microphotographs of the cells were taken, and the whole cell diameter, the thickness of cell wall and periplasmic space were measured.

### Change in Cell Wall Hydrophobicity after Pronase Treatment

Yeast cells grown on glucose (2%, w/v) or hexadecane (2%, v/v) at 30°C for 2 days were harvested (15 min at 4,000 rpm). After washing twice with distilled water, the cells were suspended in 0.01 M Tris-HCl buffer to give an $A_{400}$ of 10. Pronase was added to the cell suspension at a concentration of 0.1 mg/ml, and the cells were harvested after incubation for 60, 90, and 120 min in a shaking water bath at 37°C. The cell pellet was resuspended in buffer salt solution (BSS; $K_2HPO_4$ 16.9 g, $KH_2PO_4$ 7.3 g, urea 18 g, $MgSO_4 \cdot 7H_20$, DW 1 l, pH 7.0) to give an $A_{400}$ of 1.0 for the measurement of hydrophobicity as described by Rosenberg *et al.* [19]. Hydrophobicity was expressed as the percentage of adherence to hexadecane, which was calculated using the following equation:

$$\% \text{ Hydrophobicity} = \frac{1 - A_{400} \text{ of aqueous phase}}{A_{400} \text{ of the cell suspension}} \times 100$$

### Preparation of Cell-Free Culture Broth (CFCB) and Cell Debris (CD) of *Y. lipolytica* 180

For the preparation of CFCB, *Y. lipolytica* 180 was cultured in MSM containing 1% (v/v) of heptadecane for 4 days at 25°C with shaking at 120 rpm. The cells were removed by centrifugation at 9,000 rpm for 20 min and the supernatant was filtered through a membrane filter (pore size 0.2 μm, HiFiL MF Memb., Seoul Sciences Co., Korea).

For the preparation of CD, cell pellets obtained during the preparation of CFCB was washed twice with Ringer solution (pH 7.0) and resuspended in 250 ml of 0.25 M sucrose-BSS. The suspension was treated with a Mini-Beadbeater (Biospec Products, Bartlesville, U.S.A.) in the presence of 0.5 mm glass beads for cell breakage. After washing the treated cells twice with Ringer solution at 5,000 rpm for 15 min, the pellets were weighed and resuspended in 50 ml sterile aged sea water.

### Effects of CFCB and CD on Oil Degradation Activity of Oil-Degrading Bacteria

Fifty milliliter aliquots of CFCB were transferred to 250-ml Erlenmeyer flasks and crude oil was added to a final concentration of 2% (v/v). The suspension of CD was transferred to 50 ml of MSM with 2% (v/v) crude oil to a final concentration of 0.12% and 0.24% (w/v). One milliliter of culture solutions ($A_{400}$ of 10) of other oil-degrading bacteria, *Pseudomonas* sp. K12-5 or *Moraxella* sp. K12-7 [5], were added to the prepared solutions. The prepared CFCB and CD solutions were incubated at 25°C for 3 days with shaking at 120 rpm.

After the incubation, the residual oil in the culture medium was extracted and determined as described by Kim *et al.* [6].

## RESULTS AND DISCUSSION

### Cell Surface Structure of *Y. lipolytica* 180 Grown on Crude Oil

To observe changes in cell surface structure, cells grown on glucose or crude oil were morphologically compared by scanning electron microscopy. Figure 1 shows that the surface of cells grown on crude oil was tougher than that of cells grown on glucose. Small extrusions were radially distributed over the surface of cells grown on crude oil although these were not observed on that of glucose-grown cells. These results were in good agreement with that

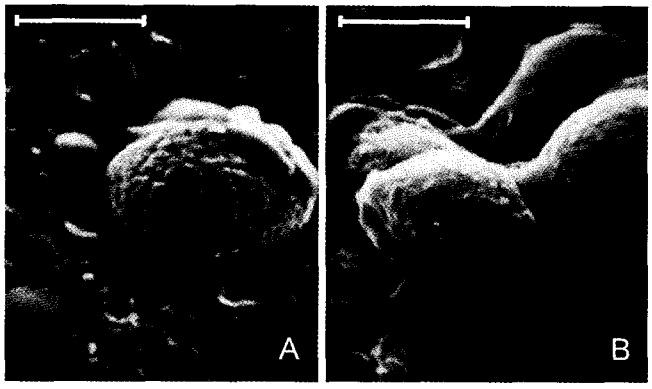


**Fig. 1.** Scanning electron microphotographs (SEM) of *Y. lipolytica* 180 cells.
Cells were cultured on crude oil (A) and glucose (B) as a sole carbon source for 5 days. The bars represent 2.5 μm (Magnification ×12,000).

obtained by Käppeli *et al.* [10] who observed unique radial protrusions with alkane-binding affinity on the cell surfaces of the cells grown on *n*-alkane. From the TEM observation, differences in the thickness of cell wall and periplasmic space between the cells grown on glucose and crude oil were also significant (Fig. 2). Table 1 shows the ratio of cell wall thickness and periplasmic space thickness to the diameter of the cells, grown on glucose or crude oil during 5 days of incubation. The ratios of cell wall thickness to the whole cell diameter of the cells grown on crude oil were higher than those of the cells grown on glucose. The ratios of periplasmic space thickness to the whole cell diameter also showed the same tendency as the ratios of cell wall thickness to the whole cell diameter. Others also reported that periplasmic membranes of oil-grown cells were thicker and that there was more endoplasmic recticulum and ribosome in the case of oil-grown cells [11, 15, 21]. Mineki *et al.* [13] showed that the cell wall portion of *Candida rugosh* and *Candida lipolytica* was essential in the metabolism of *n*-alkanes. Käppeli and Fiechter [8] also demonstrated that the peripheral portion of the cell wall involved components to which the insoluble, hydrophobic substrate was adsorbed. These observations suggest that the cell surface structures aid in the hydrophobic interaction between the cell surface and hydrocarbon, thereby facilitating transport of hydrocarbons into cells.

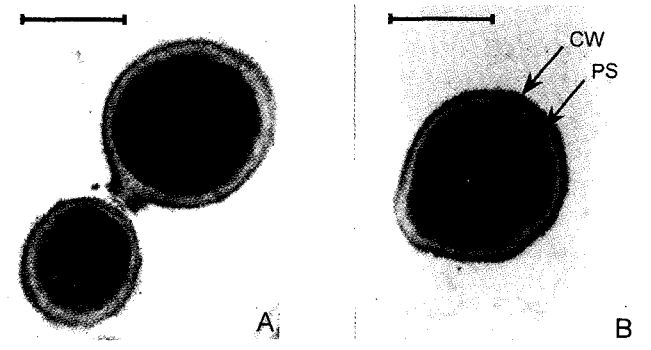


**Fig. 2.** Transmission electron microphotographs (TEM) of *Y. lipolytica* 180 cells.
Cells were cultured on crude oil (A) and glucose (B) as a sole carbon source for 5 days. The bars represent 1 μm. CW: cell wall; and PS: periplasmic space.

**Table 1.** Changes in ultrastructure of *Y. lipolytica* 180 grown on different carbon sources.

| Incubation time (days) | Ratio of CW[a] to whole cell diameter (%) | | Ratio of PS[b] to whole cell diameter (%) | |
|---|---|---|---|---|
| | Glucose | Crude oil | Glucose | Crude oil |
| 1 | 2.3±0.4 | - | 2.7±0.1 | - |
| 2 | - | 6.3±0.3 | 3.4±0.6 | 5.7±0.2 |
| 3 | 3±0.1 | 6.8±0.5 | 5.5±0.5 | 6.8±0.2 |
| 5 | 4.6±0.1 | 8.5±0.1 | 4.4±0.4 | 11.5±0.9 |

[a]CW: Cell wall thickness.
[b]PS: Periplasmic space thickness.
-: not determined.
Results represent the mean value ± standard deviation.

## Change in Cell Wall Hydrophobicity After Pronase Treatment

As reported by Kim *et al.* [6], *Y. lipolytica* 180 showed strong cell hydrophobicity, which did not require hydrocarbon substrates for its induction. However, cell hydrophobicity was greatly reduced after pronase treatment, since polysaccharide-fatty acid complexes were removed, and hexadecane was no longer easily adsorbed to the cell surface after 2 h (Fig. 3). Glucose-grown cells lost their cell hydrophobicity immediately after pronase treatment, whereas it took an hour for hexadecane-grown cells to begin losing their hydrophobicity. These results indicate that the polysaccharide-fatty acid complex on the cell surface of *Y. lipolytica* represents a part of the alkane-binding system. Käppeli and Fiechter reported a similar phenomenon [8] on the components from the cell surface of *Candida tropicalis*. Mineki *et al.* [13] showed

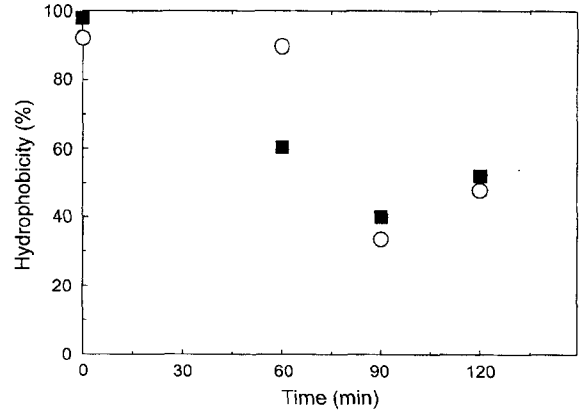


**Fig. 3.** Change in cell hydrophobicity after treatment with pronase.
■, cells grown on glucose; ○, cells grown on *n*-hexadecane.

that spheroplasts of *C. lipolytica* maintained the respiration rate of glucose in the range of 32% to 34%, however, they did not show any respiration activity with *n*-decane.

**Effect of CFCB and CD of *Y. lipolytica* 180 on the Oil Degrading Activity of Petroleum-Degrading Bacteria**

CFCB and CD were obtained to determine their roles in hydrocarbon uptake and metabolism. Both the CFCB and CD of *Y. lipolytica* 180 strain showed strong emulsification activities and high oil degradation activities (Table 2). These results indicated that some components in the cell wall or membrane as well as certain extracellular materials released from the cells of *Y. lipolytica* 180 had activities of emulsification and transformation/degradation of crude oil.

The effects of CFCB and CD on the oil-degrading activities of *Pseudomonas* sp. K12-5 and *Moraxella* sp. K12-7 were also determined (Table 2). *Pseudomonas* sp. K12-5 represented moderate hydrophobicity, although *Moraxella* sp. K12-7 showed absolutely negative hydrophobicity. Both strains showed similar surface tension values of 63 and 64 dynes/cm, respectively, after 3 days of incubation at 25°C. When the K12-7 strain was cultured in CFCB, the oil degradation rate was increased up to 57% compared with the combined degradation rate (34%) of the strain itself (7%) and the CFCB (27%). This may be due to the enhanced emulsification of hydrocarbons by the addition of CFCB. Because the strain K12-7 showed absolutely negative hydrophobicity, hydrocarbons in accommodation form seemed to be preferably utilized by the strain. Addition of CFCB, which decreased hydrocarbon droplet size through emulsification, might have increased the interfacial area between the hydrocarbon and the aqueous phase, thereby enhancing hydrocarbon uptake by the strain K12-7. Other studies also reported similar effects of emulsifying materials [2, 3, 9]. In the case of K12-5, however, addition of CFCB negatively affected oil degradation: CFCB having emulsifying activity might interfere with the emulsifying activity of the strain K12-5 due to incompatibility between two emulsifying materials, as reported in other studies [1, 4, 23, 24]. It is possible that the emulsifying activity of CFCB also interfered with cell hydrophobicity of the strain. According to Ko *et al.* [7], the oil degradation rate decreased when oil degraders having different modes of hydrocarbon uptake were mixed together. Zhang and Miller [25] also investigated the effect of a rhamnolipid biosurfactant on degradation rate. In this study, octadecane degraders with low cell hydrophobicities were positively affected, however, octadecane degraders with high cell hydrophobicities were negatively affected due to interference by the rhamnolipid in the interaction between the cells and octadecane.

**Table 2.** Effect of CFCB and CD of *Y. lipolytica* 180 on the oil-degrading activity of *Pseudomonas* sp. K12-5 and *Moraxella* sp. K12-7.

| Culture condition | % Degraded[a] | Emulsifying activity[b] |
|---|---|---|
| K12-7 in CFCB[c] | 57 | +++ |
| K12-5 in CFCB | 20 | +++ |
| CFCB alone | 27 | +++ |
| K12-7 in MSM with CD[d] (0.12%) | 39 | +++ |
| K12-5 in MSM with CD (0.12%) | 46 | +++ |
| CD (0.12%) | 34 | +++ |
| K12-7 in MSM with CD (0.24%) | 25 | +++ |
| K12-5 in MSM with CD (0.24%) | 37 | +++ |
| CD (0.24%) | 34 | +++ |
| K12-7 in MSM | 7 | ± |
| K12-5 in MSM | 31 | + |
| MSM alone | 0 | - |

[a]% Degraded=(amount of remained oil in test flask/amount of remained oil in control flask)×100, None of the microorganisms, CD, or CFCB was introduced into MSM in control flasks.
[b]Emulsifying activity; +++: Excellent; ++: Very good; +: Good; ±: Poor; -: No.
[c]CFCB, cell-free culture extract.
[d]CD, cell debris.

When CD was added into the MSM inoculated with the strain K12-7 at a concentration of 0.12%, the oil degradation rate was similar to the combined degradation rate of the strain itself and CD. However, the oil-degradation rate decreased at 0.24% of CD, probably due to a counteraction between the emulsifying activity of K12-7 and CD-mediated hydrophobicity. Addition of CD at a higher concentration into the MSM inoculated with the strain K12-5 further decreased the oil degradation rate. Similar to the effect of CFCB, the emulsifying activity of CD acted as an inhibitor on the oil degradation by K12-5, and the strong hydrophobicity of CD at higher concentration could make more hydrocarbon droplets which were not available to K12-5.

In summary, the results show that *Y. lipolytica* 180 showed interesting cell surface characteristics under the growth on hydrocarbons, which were closely associated with hydrocarbon utilization. *Y. lipolytica* 180 can easily degrade hydrocarbons through its high emulsifying activity and hydrophobic cell surfaces. Addition of cell materials obtained from *Y. lipolytica* 180 with emulsifying activity and hydrophobicity enhanced oil biodegradation in some cases but inhibited it in other cases. Therefore, the interactions among hydrocarbons, emulsifying materials, and microorganisms should be carefully investigated to estimate the potential of emulsifying materials prior to practical applications such as bioremediation. Understanding these interactions will also be helpful to find reasons for failure of introduction of emulsifying materials or oil-degrading microorganisms in bioremediation.

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