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Induction and Characterization of Mutants Enhanced in Assimilability of *n*-Alkanes in Shake Cultures from a Strain of *Candida* sp.

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A newly devised enrichment-culture technique made it possible to derive mutants, which were enhanced in assimilability of liquid *n*-alkanes in shake cultures, from an itaconate-producing yeast, *Candida* sp. strain S-10, being poor in the ability. Although the mutants were still able to produce itaconic acid from glucose, they produced succinic and malic acids instead of itaconic acid from *n*-alkanes. The mutant, which showed the best growth on *n*-alkanes in shake cultures, was found to produce a surface-active metabolite. The metabolite even stimulated the growth of the parent on *n*-alkanes in shake cultures. From these findings it is concluded that the mutant was enhanced in the productivity of the surface-active metabolite and, consequently, was able to grow well on liquid *n*-alkanes in shake cultures.

In a previous paper, we described the production of itaconic acid from glucose by a screened strain of genus *Candida*.[1] We attempted to produce the acid from *n*-alkanes with the strain. The strain, however, showed poor growth on liquid *n*-alkanes in shake cultures, although it was able to grow well on the *n*-alkanes supplied in vapor form in plate cultures. Then we tried to induce mutants able to grow well on *n*-alkanes in shake cultures, and obtained several mutants by a newly devised enrichment-culture technique. The mutants could not produce itaconic acid from *n*-alkanes. The mutant which showed the best growth on *n*-alkanes in shake cultures was found to produce a surface-active metabolite which was proved to be responsible for the growth of the mutant on the *n*-alkanes. This paper describes the induction of and selection method for the mutants and the characterization of the best mutant among them.

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Materials and Methods

Microorganisms *Candida* sp. strain S-10,[1] and its mutants were used. Strain B-7 showed the best growth on *n*-alkanes in shake cultures. *Yarrowia* (*Saccharomycopsis*) *lipolytica* IFO 1659 was also used.

Induction and enrichment-culture of mutants Mutants, which were able to grow well on liquid *n*-alkanes in shake cultures, were derived from the parent, strain S-10, by the following enrichment-culture. Cells of the parent grown on potato-dextrose agar were suspended in 0.05 M phosphate buffer (pH 6.5). To 30 ml of the suspension (about 1×10^8 cells per ml) was added 12 mg of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine(NTG) and the mixture was incubated at 26°C for 30 min. After washing by centrifugation, the cells were inoculated into Monod test tubes containing 8 ml of the medium composed of 10% (v/v) *n*-alkane-mixture, 0.3% $NaNO_3$, 0.02% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$ and 0.02% Span 80, and incubated with shaking for several days at 26°C. After the test tubes had been allowed to stand for 5 min, the cells which had entered the *n*-alkane layer or come to the interface between the *n*-alkane and aqueous layers were taken out with capillaries and transferred to Monod test tubes containing 8 ml of the same medium. The cells were again

incubated under the same conditions as described above. After these steps had been repeated 3 to 5 times, the cells were spread on potato-dextrose agar. Colonies which appeared after incubation at 26°C for 2 to 3 days were isolated.

Cultural conditions To prepare the seed culture for the growth test, a loopful of cells of the parent or the mutants grown on potato-dextrose agar were inoculated into a 500-ml Sakaguchi flask containing 50 ml of YM medium, which was composed of 0.5% peptone, 0.3% yeast extract, 0.3% malt extract and 1% glucose, and incubated for 24 h at 26°C with shaking. The seed culture (1 ml) was transferred to each of 500-ml Sakaguchi flasks containing 50 ml of a basal medium supplemented with or without 0.02% Span 80, and incubated for 48 h at 26°C with shaking. The basal medium contained 1% (v/v) *n*-alkane or 2% glucose, 0.2% NH_4Cl, 0.05% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract and 0.2% $CaCO_3$ (sterilized separately).

For studies of organic acid production, cells grown on potato-dextrose agar slants were inoculated into 25 ml of medium composed of 5% *n*-alkanes or 10% glucose, 0.3% NH_4Cl, 0.02% KH_2PO_4, 0.05% $MgSO_4 \cdot 7H_2O$, 0.1% yeast extract and 5% $CaCO_3$ (sterilized separately) 300-ml Erlenmeyer flasks, and incubated for 5 days at 26°C on a rotary shaker (220 rpm). When *n*-alkane was used as carbon source, 0.02% Span 80 was added to the medium.

In plate and slant cultures, *n*-alkane was supplied in vapor form to the cells: a filter paper soaked with *n*-alkane was put on the lid of a petri-dish or in a test tube.

Determination of cell concentration Cell concentration was determined by optical density at 580 nm with a Shimadzu-Bausch & Lomb Spectronic 20 colorimeter. When *n*-alkane was used as carbon source, the optical densities of cultures were measured after the following treatment. One part of the culture was combined with two parts of a solvent-mixture composed of ethanol, butanol and chloroform (10 : 10 : 1, v/v). The mixture was vigorously shaken and centrifuged at $1450 \times g$ for 15 min. The collected cells were washed with distilled water, centrifuged, and resuspended in distilled water.

Determination of organic acids Organic acids were assayed by paper and gas-liquid chromatography as described previously.[1]

Determination of affinity of cells for *n*-hexadecane The affinity of cells for *n*-hexadecane was measured by the same method as described previously.[2] Cells grown on *n*-hexadecane- or glucose-agar slants for 48 h at 30°C were suspended in 8 ml of 0.05 M phosphate buffer (pH 6.0), and the optical density of the cell suspension was measured at 580 nm (defined as OD_1). Two ml of *n*-hexadecane was added to the cell suspension and the mixture was mixed well with a vibrator. After the mixture had been allowed to stand and the aqueous and *n*-hexadecane layers had separated, the optical density of the aqueous layer was measured at 580 nm (defined as OD_2). The affinity of cells for *n*-hexadecane was expressed as $(OD_1-OD_2)/OD_1$; the value of 1.0 represents the highest affinity of cells for *n*-hexadecane.

Measurement of surface tension Surface tension was measured by the method of Wilhelmy with a Kyowa Surface Tensiometer (Kyowa Kagaku Co.).

Extraction and purification of a surface-active metabolite from culture broth The culture broth of strain B-7 was centrifuged and cells were removed. The supernatant was acidified to pH 5 with 2N HCl and extracted twice with methyl ethyl ketone. The extract was purified by silica gel column chromatography, which will be described in the next paper.

Taxonomical examination Taxonomical examination of the mutants was carried out according to the methods described by Lodder.[3]

Chemicals *n*-Alkanes (C_{10}–C_{17}), purchased from Kanto Chemical Co., were certified as being 98% pure. Emulgens, nonionic surfactants, were purchased from Kao Soap Co.; their chemical structures are shown in Table 1.

Results

Induction and selection of *n*-alkane-assimilable mutants in shake cultures The cells treated with NTG were observed to grow slowly in half of the Monod test tubes containing the *n*-alkane-mixture medium. After the tubes had been allowed to stand, it was observed that the cells were distributed among *n*-alkane layers, aqueous layers and their interfaces. The cells in the *n*-alkane layers or on the interfaces were transferred to Monod test tubes containing fresh medium and incubated under the same conditions. Sixteen mutants were isolated after 2 to 4 successive enrichment-culture steps and plating.

Taxonomical examination of the mutants Morphological and physiological properties of the mutants were examined to confirm whether the mutants were derived from *Candida* sp. strain S-10, the parent, or had originated from contaminants. Except for the assimilability of liquid *n*-alkanes in shake cultures, the tested morphological and

physiological properties coincided with those observed in the parent. These observations proved that the mutants were clearly derived from *Candida* sp. strain S-10.

Acid production from *n*-alkanes by the mutants All the mutants were still able to produce itaconic acid from glucose, but they were unable to produce the acid from *n*-alkanes. They were found to produce malic and succinic acids instead of itaconic acid. Mutant strain B-7 produced 4.8 g/*l* of malic acid and 3.2 g/*l* of succinic acid from *n*-hexadecane on 5 days cultivation.

Difference in *n*-alkane-assimilability between plate and shake cultures The growth of the best mutant, strain B-7, was compared with that of the parent. Both strains grew similarly on glucose. The mutant was able to grow well in *n*-hexadecane medium containing a surfactant, Span 80, while the parent grew poorly in the same *n*-hexadecane medium in shake cultures.

Although the parent grew poorly on liquid *n*-alkanes ranging from *n*-undecane to *n*-heptadecane in shake cultures, it was able to grow well on the *n*-alkanes in plate cultures, where the *n*-alkanes were supplied in vapor form. These results suggest that the parent had the enzymatic activities responsible for *n*-alkane oxidation and that the activity of uptake of *n*-alkanes in the liquid state was improved in the mutant.

Effect of the addition of surfactants on growth of the mutant in shake cultures Since the addition of Span 80 to *n*-alkane-medium markedly stimulated the growth of the mutant in shake cultures, the effects of addition of various surfactants on the growth of the mutant were examined. Table 1 shows that Span 20 and Emalgen 903 also stimulated the growth of the mutant among the surfactants tested.

Table 2 shows the growth of the mutant on *n*-alkanes ranging from *n*-decane to *n*-heptadecane in shake cultures. The mutant was able to grow well on every *n*-alkane except *n*-decane.

Affinities of the parent- and mutant-cells for *n*-hexadecane Affinities of the parent- and mutant-cells for *n*-hexadecane were measured, because the improved ability to assimilate liquid *n*-alkanes of the mutant was thought to be ascribable to an increase in the affinity of the mutant-cells for liquid *n*-alkanes. Table 3 shows that when *n*-hexadecane was used as carbon source in the growth medium the affinity of the mutant-cells was about 1.5 times as high as that of the parent-cells, but was only half that of *Yarrowia lipolytica* IFO 1659, a good *n*-alkane-assimilating yeast. When glucose was used as carbon source in the growth medium, the affinities of both the parent- and mutant-

Table 1. Effect of various surfactants on growth of the mutant, strain B-7, in shake cultures with *n*-hexadecane-medium.

Surfactant*		Growth (OD)
None		2.1
Span 20	(S-monolaurate)	8.7
Span 80	(S-monooleate)	8.2
Tween 20	(POES-monolaurate)	2.5
Tween 80	(POES-monooleate)	3.2
Emulgen 105	(POE-lauryl ether)	2.3
Emulgen 404	(POE-oleyl ether)	1.1
Emulgen 903	(POE-nonyl phenol ether)	9.8

The mutant was cultured at 26°C for 48 h in 50 ml of the basal medium containing 1% of *n*-hexadecane as carbon source and 0.02% of the indicated surfactant.

* S, sorbitan; POES, polyoxyethylene sorbitan; POE, polyoxyethylene.

Table 2. Growth of the mutant, strain B-7, on different *n*-alkanes in shake cultures.

Carbon source	Growth (OD)
n-Decane	0.4
n-Undecane	5.1
n-Dodecane	7.3
n-Tridecane	7.2
n-Tetradecane	7.5
n-Pentadecane	7.7
n-Hexadecane	8.4
n-Heptadecane	6.8

The mutant was cultured at 26°C for 48 h in the basal medium containing 1% of different *n*-alkanes as carbon source and 0.02% of Span 80.

Table 3. Affinities of the parent, strain S-10, and the mutant, strain B-7, cells for *n*-hexadecane.

Strain	Carbon source in growth medium	Affinity of cells for *n*-hexadecane*
The parent	Glucose	0.04
	n-Hexadecane	0.25
The mutant	Glucose	0.05
	n-Hexadecane	0.39
Yarrowia lipolytica IFO 1659	Glucose	0.87
	n-Hexadecane	0.88

* The value of 1.0 represents the highest affinity for *n*-hexadecane.

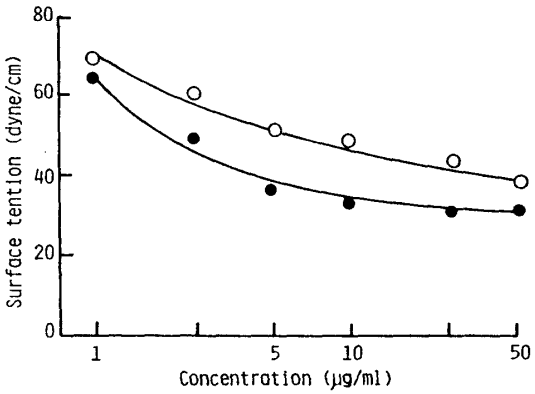

Fig. 1. Surface tension of solutions of Span 80 and the metabolite extracted from the culture broth of the mutant, strain B-7.
○, Span 80; ●, the metabolite.

cells were very weak. For the *Y. lipolytica* strain, the affinity of the cells grown on glucose was almost equal to that of the cells grown on *n*-hexadecane.

Effect of the addition of the metabolite produced by the mutant on growth of the parent in shake cultures Preliminary experiments suggested that the culture broth of the mutant contained a metabolite having a stimulatory effect on the growth of the mutant on *n*-hexadecane in shake cultures. The metabolite was found to be extractable with methyl ethyl ketone from the culture broth. Removal of the solvent left an oily residue, a surface-active substance (Fig. 1). In order to examine the effect of the addition of the metabolite on growth, the purified metabolite was added to *n*-hexadecane medium at a concentration of 0.02%. As Fig. 2 shows, the metabolite stimulated markedly not only the growth of the mutant but also that of the parent in shake cultures.

Discussion

Mutants, which were able to grow well on liquid *n*-alkanes in shake cultures, were derived from *Candida* sp. strain S-10, being poor in the ability, by a newly devised method of enrichment-culture.

The parent grew well on liquid *n*-alkanes in plate cultures, where the *n*-alkanes were supplied in vapor form, but grew poorly on the *n*-alkanes in shake cultures. Consequently, the parent was assumed to have the enzymatic activities of *n*-alkane oxidation but little activity of uptake of liquid *n*-alkanes in shake cultures. This assumption was confirmed by the results that the addition of the metabolite of the mutant stimulated the growth of the parent as well as that of the mutant.

The metabolite was found to be surface-active. Therefore, the growth-stimulating effect of the metabolite seemed to be ascribable to its potent activity of dispersing liquid *n*-alkanes in culture media.

Extracellular metabolites have been reported to be an important factor for the assimilation of *n*-alkanes in some microorganisms. Hisatsuka *et al.*[4] described that the growth of *Pseudomonas aeruginosa* S7B1 was stimulated by its own extracellular product, a surface-active rhamnolipid. Itoh and Suzuki[5] derived a mutant being deficient in the activity of *n*-alkane assimilation from *P. aeruginosa* KY 4205, a good *n*-alkane-assimilating bacterium, and suggested that the mutant was

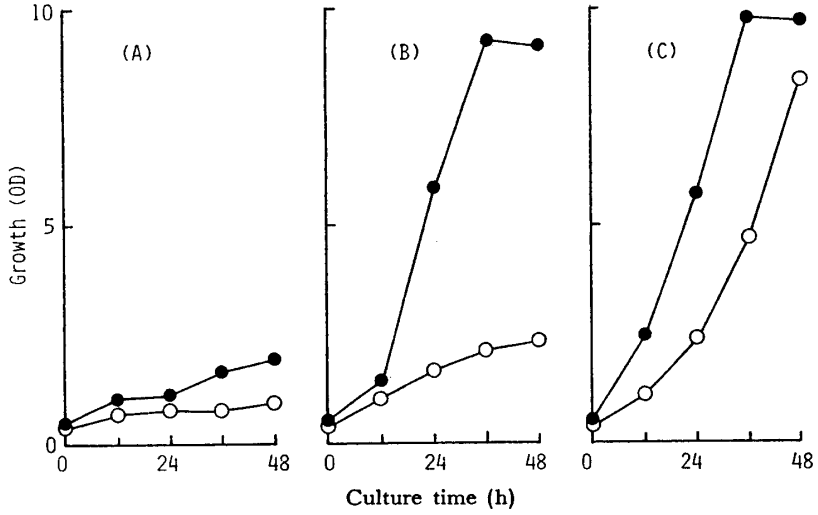

Fig. 2. Stimulatory effect of Span 80 and the metabolite extracted from the culture broth of the mutant, strain B-7, on growth of the parent and the mutant.
The parent and the mutant were cultured at 26°C in 50 ml of the *n*-hexadecane-medium (A), in the medium supplemented with Span 80 (B), and in the medium supplemented with the metabolite (C).
○, the parent; ●, the mutant.

deficient in the activity of the extracellular production of rhamnolipid.

From these descriptions and the present observations, it is concluded that the present mutant was improved in the productivity of the surface-active metabolite and, consequently, was able to grow well on liquid *n*-alkanes in shake cultures. Further characterization of the metabolite will be described in separate papers.

It is well known that *n*-alkanes are degraded by *β*-oxidation into acetyl-units, and C_4-acids being required for cell synthesis are supplied through the glyoxylate cycle from the acetyl-units. Itaconic acid has been reported to inhibit the activity of isocitrate lyase, one of the key enzymes of the glyoxylate cycle, in *Pseudomonas indigofera*[6] and *Yarrowia lipolytica*.[7] These facts may explain in part the results that all the present mutants produced malic and succinic acids, instead of itaconic acid, from *n*-alkanes, although they still produced itaconic acid from glucose.

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