Effect of Non-ionic Surfactants on the Growth of 
*Candida rugosa* JF–101 with Several *n*-Alkanes

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The growth of *Candida rugosa* JF–101 was either stimulated or not affected by Tween 80 in *n*-decane (*n*-C10)–*n*-dodecane (*n*-C12) media and inhibited in *n*-C13–*n*-C16 media. The growth of *Saccharomyces lipolytica* IAM 4947 was stimulated by the surfactant in *n*-C10–*n*-C13 and inhibited in *n*-C14–*n*-C16. Dodecyl poly(oxyethylene) ether (*C*12P*O*E*10*) strongly inhibited the growth of *C. rugosa* JF–101 in glucose medium and slightly so in *n*-alkane medium. *C*14P*O*E*20*, *C*16P*O*E*30* and *C*18P*O*E*20* strongly inhibited its growth in *n*-C16 medium but failed to do so in glucose or *n*-C16 media. Several alcohols corresponding to *C*16P*O*E*0* were also assessed for their ability to promote or inhibit growth. 1-Decanol and 1-dodecanol inhibited growth in glucose but not so in *n*-alkane. *C. rugosa* JF–101 and *S. lipolytica* IAM 4947, all having strong *n*-alkane-assimilating activity, showed greater affinity for *n*-C16 than *C. tropicalis* IAM 4826 having weak activity. The affinity of *C. rugosa* JF–101 was reduced 76% by Pronase but its respiratory activity (RA) did not decrease significantly. Speroplast of the yeast lost almost all RA on *n*-alkane and it subsequently could hardly be recovered by Tween 80.

1 Introduction

The emulsification of *n*-alkane is one of the important factors for the fermentation of its assimilating microorganisms by using *n*-alkane and there are many investigators on it and various findings have been reported. Although the effect of surfactants on the growth of yeasts has been fairly studied, the results were varied (stimulatory, inhibitory or unaffected) depending on the investigators. It may be due to the differences of strains, surfactants and the *n*-alkane used. Many studies on the sort of surfactants were performed but there were few on alkyl POE ethers in *n*-alkane fermentation. For series of *n*-alkanes having various carbon chain lengths, however, few studies were performed on the relation with surfactants under the same condition despite the crucial problem. Merely, Withworth *et al.* partially investigated on it with *C. (S.) lipolytica*. On the other hand, some investigators pointed out that the binding affinity of the cell surface for *n*-alkane is also one of the crucial factors for *n*-alkane assimilation by yeasts.

In this paper, we describe the relation between the carbon chain lengths of *n*-alkanes and the effects of several surfactants on the growth of yeasts, especially in *C. rugosa* JF–101, the relation between the binding affinities of several yeasts for *n*-C14 and their properties, and the RA of the cells after degradation of cell surface and cell wall in *C. rugosa* JF–101.

2 Experimental

2.1 Materials

*n*-Alkanes (*n*-C10–*n*-C16, 99% pure), Triton X-100, nonylphenyl POE ether (POE : *n*=10) and *n*-alcohols (1-decanol–1-octadecanol) were purchased from Tokyo Chemical Industry Co., Ltd., Tokyo. Tween 20 and Tween 80 were purchased from Kao-Atlas Co., Ltd., Tokyo. Emulgen 123 P (dodecyl POE ether) was a gift from Kao Co., Ltd., Tokyo. Alkyl POE ethers *C*12P*O*E*10*: *C*12H24O*10* (CH2=CH2O)*n*H; *m*=10, 12, 14, 16, 18, and 22, *n*=20; *m*=12, 14, and 16, *n*=10 and 30 were supplied by Nihon Surfactant Ind. Co., Ltd., Tokyo, and have a narrow distribution of molecular weight. Pronase and Zymolase were purchased from Kaken Seiyaku Co. Ltd., Tokyo and Kirin Brewery Co. Ltd., Tokyo, respectively.

2.2 Microorganisms and cultivation

sp. EP 4-8. These yeasts were maintained on yeast malt extract-agar slants, and were cultivated on 1 % (wt/vol) glucose or 1 % (vol/vol) n-alkane as sole carbon and energy sources as described by Iida and Finnerty with or without non-ionic surfactant (0.02 or 0.1 %) at 27°C on a rotary shaker (220 rpm).

2.3 Determination of cell yield
Cell concentration was determined by optical density at 660 nm with a Shimazu-Bausch & Lomb Spectronic 20 colorimeter. Thereading of cell suspension could be covered to dry cell mass per mL referring to a calibration chart. When n-alkane was used as carbon source, the dry cell yield was determined as follows. Samples were treated with the mixed solvent of ethanol-butanol-chloroform (10:10:1, vol/vol) and dried to constancy at 105°C.

2.4 Measurement of affinity of cells for n-alkane
The affinity of cells for n-alkane was measured by the method of Kaeppeli and Fiechter with modifications. Cells harvested at early stationary phase were washed with distilled water and were suspended in M/15 phosphate buffer (pH 7.2) containing 5 mM KCN to prevent the oxidation of the n-alkane. Then n-C16 emulsion was added to the cell suspension. After an incubation for 2 min, cells were sedimented at 18,000 x g for 5 min. n-C16 floated to the top of tube and the supernatant were removed with Pasteur pipette and Kimwipe. The sediment was washed with distilled water, then n-C16 attached to the cell surface was removed with n-hexane after 1 mL of n-C14 was added as internal standard, n-C16 removed was quantified with gas-liquid chromatograph Hitachi 163, equipped with a flame ionization detector. A glass column (Apiezon grease L, 1.5 m x 3 mm) was used at 200°C (carrier: N2, 25 mL/min).

2.5 Pronase treatment of the cells
The cells were treated with Pronase to modify the cell surface. To the 4.5 mL of cell suspension (corresponding 50 mg of dry cells in M/15 phosphate buffer, pH 7.5), 0.5 mL of Pronase solution (5 mg in the same buffer) was added and incubated at 37°C with shaking (100 rpm).

2.6 Formation of spheroplasts and measurement of oxygen uptake
Spheroplasts of yeast cells were formed by Zymolyase and respiratory activity (RA: oxygen uptake rate, μL/h/mg-cells) was measured by Warburg manometric technique as previously.

3 Results and Discussion
3.1 Effect of Tween 80 on the growth of yeasts on various n-alkanes
The effect of 0.02 % of Tween 80 on the cell growth was tested in C. rugosa JF-101, C. rugosa JF-114, C. tropicalis IAM 4826, S. lipolytica IAM 4947, S. lipolytica No. 6-20, T. sericeum EP 4-3, and T. sp. EP 4-8. Although Tween 80 did not affect the growth of these yeasts on glucose except C. rugosa JF-101 (stimulated), it stimulated the growth of all these yeasts on n-C16. At the same time, the growth of these yeasts on n-C16 was drastically inhibited by Tween 80 except in two strains of C. rugosa. In C. rugosa JF-101, the growth on n-C16 was inhibited by Tween 80 when the concentration of the surfactant was increased to 0.1 %, but at this concentration, the growths on glucose and n-C16 were still stimulated (Fig.-1). It is not clear why C. rugosa was not so sensitive for Tween 80 below 0.08 % when it grew on n-C16.

It was ascertained that the effect of Tween 80 on the growth of yeast varied with the species of yeast and the growth substrate as mentioned above. So, experiments were performed with various n-alkanes (n-C16~n-C18) as the growth substrate in C. rugosa JF-101 (0.1 % of Tween 80) and S. lipolytica IAM 4947 (0.02 % of Tween 80). As shown in Table-1, the growths of C. rugosa on n-C16~n-C17 were slightly stimulated or not affected, but the growths on n-C13~n-C15 were slightly inhibited and the growth on n-C16 was strongly inhibited by 0.1 % of Tween 80. On the other hand, the growths of S. lipolytica on n-C16~n-C13 were slightly stimulated but the growths on n-C14~n-C16 were strongly inhibited by 0.02 % of Tween 80. In our previous investigation, the RA of C. rugosa for various n-alkanes could be grouped to high for n-C16~n-C17 and low for n-C13~n-C18, and that of C. (S.) lipolytica could be grouped to high for n-C10~n-C12, middle for n-C13, and low for n-C14~n-C16. In our study for cellular fatty acids derived from n-alkanes in yeasts, it was suggested that n-C13~n-C14 were incorporated by direct incorporation system in C. rugosa and n-C14~
3.2 Effect of several surfactants on the growth of yeast

The surfactants were used at the concentration of 0.02% for C. rugosa JF-101 and S. lipolytica IAM 4947 on n-C_{10} and n-C_{16}, which were typical substrates for the effect of surfactants. In the former yeast, 0.1% of surfactants were also used because 0.02% of Tween 80 was not effective for the growth on n-C_{16} (Fig.-1).

In the growth on n-C_{16}, both yeasts were stimulated or unaffected by Tween 80, Tween 20, Emulgen 123 P and nonylphenyl POE ether as shown in Table-2. It was notable that Triton X-100 inhibited the growth of S. lipolytica in stationary phase, This result was similar to the inhibitory effect of the surfactant on RA of C. (S.) lipolytica ATCC 8661 on n-C_{12} by Whitworth et al., but it did not inhibit the growth of C. rugosa JF-101 even at 0.1%. In nonylphenyl POE ether, the growth rate was stimulated and the growth phase had already run into death phase when the control arrived at stationary phase. The stimulative effects for both yeasts were not remarkable in stationary phase but in log phase, Remarkable differences did not occur between 0.02% of surfactants and 0.1% in C. rugosa. On the other hand, the growth of C. rugosa on n-C_{16} was strongly inhibited by each surfactant at 0.1%. At 0.02%, only Emulgen 123 P slightly inhibited the growth but other surfactants used did not affect it. In S. lipolytica, all of these

Table-1 Effect of Tween 80 on the growth of hydrocarbon-assimilating yeast.

<table>
<thead>
<tr>
<th>Growth substrate</th>
<th>C. rugosa JF-101*</th>
<th>S. lipolytica IAM 4947**</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Control</td>
<td>Effect of Tween 80 on growth</td>
</tr>
<tr>
<td></td>
<td>Log</td>
<td>St.</td>
</tr>
<tr>
<td>n-C_{10}</td>
<td>150</td>
<td>270</td>
</tr>
<tr>
<td>n-C_{11}</td>
<td>160</td>
<td>330</td>
</tr>
<tr>
<td>n-C_{12}</td>
<td>150</td>
<td>300</td>
</tr>
<tr>
<td>n-C_{13}</td>
<td>170</td>
<td>330</td>
</tr>
<tr>
<td>n-C_{14}</td>
<td>160</td>
<td>320</td>
</tr>
<tr>
<td>n-C_{15}</td>
<td>170</td>
<td>350</td>
</tr>
<tr>
<td>n-C_{16}</td>
<td>170</td>
<td>350</td>
</tr>
</tbody>
</table>

mg−dry cells/flask (80 mL of medium). Cells cultivated under existence of Tween 80 were harvested at the same time with the harvest in control.

* Tween 80 : 0.1% ; growth phase : Log (24~34 h), St. (Stationary, 36~54 h).

** Tween 80 : 0.02% ; growth phase : Log (30~80 h), St. (50~120 h).
surfactants strongly inhibited the growth on \( n-C_{16} \) at 0.02%. Both yeasts could assimilate Tween 80 and Tween 20, but could not Emulgen 123 P, nonylphenyl POE ether and Triton X-100.

3.3 Effect of alkyl POE ethers on the growth of \( C. \) rugosa JF-101

Emulgen 123 P \((C_{12}POE_n)\) was the most inhibitive for the growth of the yeast in the surfactants used as mentioned above (Table-2). So, the effect of many kinds of \( C_mPOE_n \), which have a narrow distribution of molecular weight, on the growth of \( C. \) rugosa JF-101 on glucose, \( n-C_{10} \) and \( n-C_{16} \) was examined. As shown in Table-3, the growth of the yeast on glucose was not affected by these surfactants except \( C_{16}POE_{10} \) (strongly inhibited), \( C_{14}POE_{18} \) (moderately inhibited), \( C_{16}POE_{20} \) and \( C_{16}POE_{10} \) (slightly inhibited). On the other hand, the growth on \( n-C_{16} \) were stimulated by many surfactants in log phase and not affected in stationary phase as \( C_{10-18}POE_{20} \) and \( C_{16}POE_{10} \), but \( C_{14}POE_{16} \) and \( C_{22}POE_{20} \) slightly inhibited the growth in both growth phases. In the case of the growth on \( n-C_{16} \), a number of surfactants affected inhibitory. \( C_{16}POE_{20,30} \) and \( C_{18}POE_{20} \) specifically and strongly inhibited the growth. Namely, these three ethers did not inhibit the growths on glucose and on \( n-C_{16} \). \( C_{22}POE_{20} \) moderately inhibited the growth, \( C_{12}POE_{16} \) and \( C_{16}POE_{10} \) slightly inhibited it. It is a new finding that the effects of several surfactants on the growths of yeasts depended on the carbon chain lengths of \( n \)-alkanes and it was also shown in two paragraphs (3.1 and 3.2). It is unclear whether these inhibitions are caused by interruption of \( n-C_{16} \) uptake or by inhibition of the enzyme18-20 which participates in the assimilation of \( n-C_{16} \). The relation between hydrophile-lipophile balance (HLB) of alkyl POE ethers and effects on the growth of the yeast was examined. As shown in Table-3, HLB values and the effects of surfactants showed no apparent correlation.

Furthermore, the effect of alcohols \((C_mPOE_o, m=10, 12, 14, 16, \text{ and } 18)\) was examined to obtain the information on the role of alkyl moiety. These alcohols did not affect the growth on \( n-C_{10} \) and \( n-C_{16} \) at the concentration of 0.07%. On the other hand, the growth on glucose was strongly inhibited by \( C_{10}POE_o \) \((C_{10}H_{21}OH)\) and \( C_{16}POE_o \) \((C_{16}H_{33}OH)\). Antibacterial activities of 1-decanol

<table>
<thead>
<tr>
<th>Surfactant (0.02%)</th>
<th>( C. ) rugosa JF-101</th>
<th>( S. ) lipolytica IAM 4947</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Relative cell yield (%)</td>
<td>Relative cell yield (%)</td>
</tr>
<tr>
<td>Growth substrate</td>
<td>( n-C_{10} )</td>
<td>( n-C_{16} )</td>
</tr>
<tr>
<td>Log St.</td>
<td>Log St.</td>
<td>Log St.</td>
</tr>
<tr>
<td>Tween 80</td>
<td>124 (123)**</td>
<td>116 (105)</td>
</tr>
<tr>
<td>Tween 20</td>
<td>114 (105)</td>
<td>90 (98)</td>
</tr>
<tr>
<td>Emulgen 123 P</td>
<td>116 (113)</td>
<td>60 (99)</td>
</tr>
<tr>
<td>NP POE ether***</td>
<td>119 (125)</td>
<td>105 (102)</td>
</tr>
<tr>
<td>Triton X-100</td>
<td>114 (119)</td>
<td>103 (110)</td>
</tr>
</tbody>
</table>

Table-2 Effect of several surfactants on the growth of yeasts.

Dry cell weight obtained in the culture without surfactant in each case was basis (100%).

Growth phase: Log, 29 h \((n-C_{10})\) and 24 h \((n-C_{16})\), St., 42 h \((n-C_{10})\) and 36 h \((n-C_{16})\) in \( C. \) rugosa; Log, 30 h \((n-C_{10} \text{ and } n-C_{16})\), St., 50 h \((n-C_{10} \text{ and } n-C_{16})\) in \( S. \) lipolytica.

*Assimilation activity: +++, assimilated well; +, assimilated; -, not assimilated; these yeasts were cultivated on 2.5% of each surfactant as sole carbon source for 2 days (+ and +) or 4 weeks (-).

**The figure in parentheses shows the value for 0.1% of the surfactant.

***Nonylphenyl poly (oxyethylene) ether, DP of POE is 10.
and 1-dodecanol have been reported by Kato et al. These alcohols also inhibited the growth of the yeast of C. rugosa on glucose in this investigation. The inhibitory effect was retained by C_{12}POE_{14}, which indicated this surfactant was harmful for the yeast itself, but it was lost by increasing the degree of polymerization (DP) of POE group to 20 and 30. The effect of alkyl POE ethers depended not only on alkyl group but also on DP of their POE group, and the most crucial factor for inhibitory effect was suggested in alkyl group-in-itself.

3.4 Relation between binding affinity for n-C_{16} and effect of Tween 80 on the growth in several yeasts

Nakahara et al. reported that the growths of yeasts having high binding affinities were inhibited or not affected by the surfactant but the growth of a mutant of C. sp. having low binding affinity was stimulated by it in n-alkane medium. So, we measured the binding affinities of several yeast cells. As shown in Table-4, good n-alkane-assimilating yeasts as both strains of C. rugosa, both strains of S. lipolytica, T. sericeum EP 4-3, and T. sp. EP 4-8 had higher affinities compared with that of C. tropicalis IAM 4826 which took comparatively a long time to assimilate n-alkanes. In C. rugosa JF-101 and S. lipolytica IAM 4947 having high affinities, the effects of 0.02 % of Tween 80 on the growth on n-C_{16} differed in each other (Table-2), but C. tropicalis IAM 4826 having a low affinity was identically inhibited by the surfactant as in both strains of S. lipolytica. Therefore, no relation was found between the affinities of these yeasts and the effects

<table>
<thead>
<tr>
<th>Yeasts</th>
<th>Binding affinity (g/mg-dry cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Candida rugosa JF-101</td>
<td>1.9×10^{-4}</td>
</tr>
<tr>
<td>C. rugosa JF-114</td>
<td>1.9×10^{-4}</td>
</tr>
<tr>
<td>C. tropicalis IAM 4826</td>
<td>1.0×10^{-4}</td>
</tr>
<tr>
<td>Saccharomyces lipolytica IAM 4947</td>
<td>2.1×10^{-4}</td>
</tr>
<tr>
<td>S. lipolytica No. 6-20</td>
<td>1.9×10^{-4}</td>
</tr>
<tr>
<td>Trichosporon sericeum EP 4-3</td>
<td>2.2×10^{-4}</td>
</tr>
<tr>
<td>T. sp. EP 4-8</td>
<td>1.9×10^{-4}</td>
</tr>
</tbody>
</table>

Cells were harvested at early stationary growth phase on n-C_{16}.
of surfactants on the growth of the strains.

3.5 Relation between binding affinity and 
\( n-C_{16} \) uptake in \( C. \) rugosa JF-101

Kaeppeli and Fiechter\(^2\) pointed out that the cell surface (the peripheral part of the cell wall) participated binding affinity of the yeast for \( n-C_{16} \). So, the relation between the affinity and \( n \)-alkane uptake, which is the first step in \( n \)-alkane assimilation and is presumably affected by the surfactant, was investigated after degradation of the cell surface with Pronase. RA was measured as the index for \( n \)-alkane uptake by the yeast. As shown in Fig.-2, the great reduction (76 \%) in binding affinity occurred after 60 min of incubation with Pronase. Under this condition, lysis hardly occurred and the RA of the cells treated by Pronase on \( n-C_{16} \) merely reduced 26 \% compared with that of intact cells (100 \( \mu \text{L}/\text{h/mg-cells} \)). At the same time, the RA on glucose did not change between these cells. These results indicated that the binding affinity did not considerably relate with \( n \)-alkane uptake into the cell in \( C. \) rugosa JF-101.

3.6 Effect of degradation of cell wall on 
\( n \)-alkane uptake in \( C. \) rugosa JF-101

To get information on the part participating \( n \)-alkane uptake, cell wall was removed by Zymolyase for forming the spheroplast. Although the RA of the spheroplasts on glucose was maintained 77 \% of that in intact cells (52 \( \mu \text{L}/\text{h/mg-cells} \)) after 1 h of incubation and oxygen uptake was saturated at 1.5 h, that for \( n-C_{16} \) reduced to 7.6 \% (intact cells: 170 \( \mu \text{L}/\text{h/mg-cells} \)) and was slightly recovered to 14.7 \% by Tween 80 (Fig.-3). The RA of the spheroplasts on \( n-C_{16} \) was completely lost and was not recovered by Tween 80. These results suggest that cell wall and/or surface of cell membrane (SCM) of \( C. \) rugosa JF-101 considerably participates the \( n \)-alkane uptake into the cell, and the surfactant cannot bring into full play of the stimulative effect on the growth of the yeast without cell wall and/or SCM. Further investigation will be carried to know the effect of surfactant on uptake of various \( n \)-alkanes by the yeast. On the other hand, it should be investigated whether the surfactant affect the assimilation of \( n \)-alkane after uptake of it as described above (paragraph 3.1).

Acknowledgement

We thank Dr. Y. Tabata, Nihon Surfactant Industries Company Ltd., Tokyo, for supplying alkyl POE ethers. We are also grateful to Dr. M. Abe in this university for his valuable advice, and to Mr. K. Ueno and Mr. A. Tachime for their technical assistance.

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References


**Candida rugosa** JF-101 の数種の n-アルカンでの生育に及ぼす非イオン界面活性剤の影響

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Tween 80 は**Candida rugosa** JF-101 の n-デカン (n-C_{10})~n-ドデカン (n-C_{12}) 培地での生育には促進的に働くが、あるいは影響を与えなかったが、n-C_{14}~n-C_{16} 培地では阻害的であった。また、*Saccharomyces lipolytica* IAM 4947 の生育を n-C_{16}~n-C_{18} 培地で促進し、n-C_{14}~n-C_{16} 培地では阻害した。アルキル＝ポリ（オキシエチレン）=エーテルの C. rugosa JF-101 の生育に及ぼす影響は、ドデシル＝ポリ（オキシエチレン）=エーテル (C_{12}POE_{16}) がグルコース培地で強い阻害を示したが、n-アルカン培地での阻害は弱かった。C_{15}POE_{16}, C_{16}POE_{16} 及び C_{14}POE_{16} は n-C_{14} 培地で強力な阻害を示したがグルコース培地や n-C_{16} 培地では阻害しなかった。C_{8}POE_{9} に相当する数種のアルコールについても実験を行ったが、1-デカノールと 1-ドデカノールがグルコース培地での生育を阻害したが、n-アルカン培地では影響を与えてなかった。n-アルカン資化力の強い C. rugosa JF-101 と S. lipolytica IAM 4947 は資化力の弱い C. tropicalis IAM 4826 よりも n-C_{16} に対する親和性が高かった。C. rugosa JF-101 をプロニューゼで処理すると、その n-C_{16} 親和性は 76 % 失われたが呼吸活性はそれほど下がらなかった。さらに、本酵母はステルールを最大量として n-アルカンに対する呼吸活性をほとんど失い、Tween 80 添加によっても回復はほとんどみられなかった。