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Properties of Lipases and Palm Oil Assimilating Patterns in Palm Oil Fermentation

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팜유발효에 있어서 리파제의 특성과 팜유자화와의 관계

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In order to elucidate the patterns of natural oils and fats assimilation by microorganisms, lipases properties of yeast and bacterium strain, *Torulopsis candia* Y-128 and *Acinetobacter calcoaceticus* KB-2, which could assimilate palm oil efficiently, were investigated. *T. candida* Y-128 attached palm oil droplets directly, and assimilated unsaturated fatty acid more easily than saturated acids liberated by the action of its lipase. Lipase of *A. calcoaceticus* KB-2 was extracellular and appeared quickly from the beginning of log phase of growth, whereas lipase of *T. candida* Y-128 appeared intracellular. The lipases of two strains seem to be only enough to utilize the lipid materials for their own growth, without accumulation of lipases in the culture broth. Lipases of the strains have 1(3-)-positional specificities on triglycerides. The patterns of palm oil assimilation showed that two strains attached droplets of lipid materials directly and split off fatty acids at 1(3-)-position of triglycerides first, and assimilated the reaction products via fatty acids metabolic pathway.

The great increase of vegetable oil production recently has opened up subjects of research for microbial utilization of the oils. Few reports, however, have been published on the utilization of natural oils and fats as fermentation sources[1]. Most microorganisms capable of assimilating oils and fats are lipolytical or hydrocarbon-assimilating. It is generally supposed that the species assimilate oils and fats via fatty acids by the action of lipase. Therefore, the species would produce lipase in a degree to utilize the lipids as carbon or energy sources, but assimilating patterns of oils and fats by microorganisms have not been demonstrated clearly yet.

In order to elucidate the patterns of natural oils and fats assimilation by microorganisms, physiological properties of yeast and bacterium strain[2-4] which could assimilate palm oil efficiently, production and localization of lipases, and positional specificities of lipases of the species during fermentation were investigated.

Materials and Methods

Organisms

The organisms used in this study were *Torulopsis candida* Y-128[2] and *Acinetobacter calcoaceticus* KB-2[3], and cultures were maintained on slant medium described previously[2-4].

Media and Culture

Media of *T. candida* Y-128 and *A. calcoaceticus* KB-2 were described previously[2-4]. Cultures of the strains were

Key words: Lipase, Palm oil, Fermentation *Torulopsis candida, Acinetobacter calcoaceticus*

carried out in suitable conditions of a shake or jar fermentor cultures, respectively.

Enzyme localization studies

The procedure used in this study are outlined in Fig. 1. Cell suspensions were cooled in ice, and cell wall was disrupted by a French press with two passes at 18,000 pounds per square inch (psi).

Determination of lipase activity

Lipase activity was determined by the modified agar diffusion method[5] and the method of Ota and Yamada[6]. The procedure of agar diffusion method was as follows. Polyvinyl alcohol (10 w/v%) suspended in a deionized water was mixed with 10 v/v% tributyrin, and then homogenized with a homogenizer (Nihon Seiki Kogyo Co., Max. speed for 5 min). To 30 ml of emulsion, 270 ml of tris-maleate buffer (pH 7.0) and 1.2% agar were added. After preparing the plate culture, stainless steel cup (dia 6 mm, H 10 mm) was set on the plate culture lightly. One ml of cell suspension, incubated for 24 hr at 30°C in a slant culture and diluted in a suitable concentration, was inoculated in a stainless steel cup and incubated. Lipase activity was determined by the zones of clearings on a plate culture. *Saccharomycopsis lipolytica,* lipase-producing strain[6], was used as a reference strain.

Lipase activity in non-emulsified (shaken) system was determined by the method described as Ota and Yamada[6]. Into a L-shaped test tube were added 1 ml of olive oil, 2 ml of 0.2M tris-maleate-0.2 M sodium hydroxide buffer (pH 7.0), 1 ml of 0.1 M $CaCl_2$ and 5 ml of deionized water. The mixture was preheated at 40°C with constant shaking (60 oscillation/min) for ten min and 1 ml of enzyme solution was added. The reaction was carried out for 50 min, and was stopped with 20 ml of a mixture of acetone and ethanol (1:1). The mixture was titrated with 0.05 N NaOH solution with phenolphthalein as an indicator. A blank was shaken without enzyme, and 1 ml of enzyme solution was added immediately before titration. One unit of lipase activity is defined as that quantity of the enzyme liberating 1 μeq of fatty acid from olive oil per minute.

Positional specificity of lipase

Triolein was purified from commercial triolein (Tokyo Kasei Kogy Co.) by precoated silica gel plate (type 60, Merck), developed with hexane/diethylether/methanol (80: 20: 5). Into a L-shaped test tube were introduced 0.1 ml of triolein, ml of 0.2 M-tris-maleate-0.2 M NaOH buffer (pH 8.0), 0.5 ml of 0.1 M $CaCl_2$ and 2.4 ml of deionized water. The mixture was preheated at 40°C for ten min, and 1 ml of enzyme solution was added. The enzyme solution was prepared as follows; salted out the supernatant I of *A. calcoaceticus* KB-2 and the supernatant IV of *T. candida* Y-128 with saturation of ammonium sulfate between 20% and 70%, and dialyzed against deionized water at 4°C. The reaction mixture was incubated at 40°C for 150 min with constant shaking. The hydrolyzed products were extracted by the method of Bligh and Dyer[7], and developed with chlorofrom/acetone (96: 4) on a precoated silica gel plate. A detector was iodine vapor.

Analytical method

Triolein assimilation by the strain was determined with HPLC by the modified mothod of Riisom and Hoffmeyer[8]. HPLC analyses were performed on a Hitachi LC-635A Chromatograph equipped with a differential refractometer (Shodex model RI SE-11). The separations were achieved on 300 × 8 mm ID steel column prepacked with 10 μm LiChromosorb DIOL. The samples were eluted with 95% isooctane-5% isopropanol (v/v) at a flow rate of 3 ml/min and a pressure of about 100 kg/cm^2. The samples were prepared as follows. After extraction of culture broth with isooctane,

Table 1. Assimilation of fatty acids as carbon sources by the strains*

Strain	*T. candida* Y-128				*A. calcoaceticus* KB-2			
Carbon source	6 hr		12 hr		6 hr		12 hr	
(2%)	pH	Dry cell	pH	Dry cell	pH	Dry cell	pH	Dry cell
Palm oil	2.5	6.0	2.3	10.6	6.2	11.2	4.9	12.2
Oleic acid	2.5	6.5	2.3	11.7	6.1	11.1	4.8	11.6
Linoleic acid	2.8	5.0	2.5	6.6	6.3	8.8	5.5	11.0
Palmitic acid	4.3	4.5	3.4	5.2	6.1	10.8	4.8	11.4
Stearic acid	4.9	4.2	4.0	5.0	6.2	11.0	5.0	11.2

*Fermentation of *T. candida* Y-128 and *A. calcoaceticus* KB-2 was at 30℃, pH 5.5 and 37℃, pH 7.0, respectively on a reciprocal shaker (120 strokes/min). Dry cell was as g dry cell/*l* of culture broth.

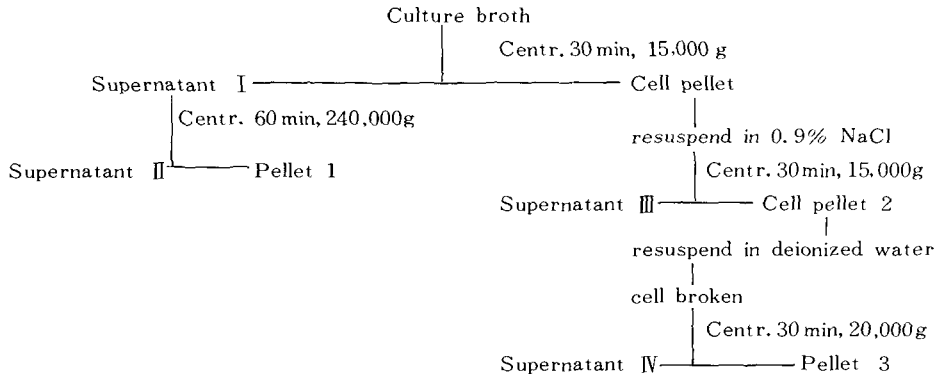

Fig. 1. Procedure of cell fractionation for determining the location of lipase.
Centr., Centrifugation. cell broken; cell suspension was passed at 18,000 psi with a French press.

filtered through silica gel G60 and evaporated. The residue was dissolved in the eluent and 100 μl of the solution was injected via loop injector. Residual triolein was calculated by comparision of the height of each peak.

Results

Assimilation of fatty acids and palm oil by the strains

Table 1 shows the assimilation of fatty acids, main components of palm oil, as carbon and energy sources by the strains in a shake culture. While *A. calcoaceticus* KB-2 assimilated saturated and unsaturated fatty acids easily, *T. candida* Y-128 assimilated unsaturated fatty acids more easily than saturated fatty acids. Furthermore, with *T. candida* Y-128, most of saturated fatty acids adhered to the neck of flask or formed small globules in the culture broth. The physiological property, which assimilate fatty acids as carbon sources, was different between two strains.

Production and localization of lipases

Fig. 2 shows the growth curve and lipase production of *A. calcoaceticus* KB-2 in a jar fermentor culture. Lipase measured through the hydrolysis of olive oil appeared quickly from the beginning of log phase of growth. As growth slowed and became stationary, activity decreased.

The zones of clearings of *T. candida* Y-128 and *A. calcoaceticus* KB-2 on plate culture for 3 days incubation were very small, compared with lipase-producing strain, *S. lipolytica*[6]. Furthermore, at temperature lower than 30°C, the zones were very small and not so clear. According to the incubation time, the zones were enlarged around the colonies of the strains. Although the zones of clearings of *A. calcoaceticus* KB-2 were larger than those of *T. candida* Y-128, quantitative estimation of lipase activity was not possible. Lipase activities of the strains employed in this study seemed to be low.

Further studies on intracellular enzyme location were carried out by disrupting cells using a French press and fractionating broken material with differential centrifugation, as outlined in Fig. 1. The results show that most of extracellular lipase of *A. calcoaceticus* KB-2 obtained from supernatant by high-speed centrifugation (15,000 g, 30 min) was still soluble part after ultrahigh-speed centrifugation (240,000 g, 60 min). By washing the cells with 0.9% NaCl, about half of the enzyme was solubilized. Lipase of *A. calcoaceticus* KB-2 was extracellular, whereas most of lipase of T. candida Y-128 appeared intracellular. Cells of *T. candida* Y-128 were not easily broken with a French press, so lipase activity remained in the precipitate after centrifugation of broken material as shown in Table 2.

Table 2. Localization of lipase activity(munit/ml)

Fraction*	Lipase activity	
	T. candida Y-128	*A. calcoaceticus* KB-2
Supernatant I	24	160
Supernatant II	16	120
Pellet 1	0	48
Supernatant III	0	32
Pellet 2	143	24
Supernatant IV	80	20
Pellet 3	40	0

*For explanations of these fractions, see Fig. 1.

Positional specificities of lipases

TLC of hydrolyzed products of triolein by the lipases of *A. calcoaceticus* KB-2 and *T. candida* Y-128 is shown in Fig. 3. 1,3-diolein was not accumulated in the reaction mixture of the lipase. Lipases of the strains have 1(3-)-positional specificities on triglycerides.

Assimilation of triolein

In order to elucidate the patterns of lipase and palm oil assimilation by the strains employed in this study, residual oil during cultivation was extracted and analyzed. Fermentation was carried out in a jar fermentor to control the pH of culture broth, because the growth of *A. calcoaceticus* KB-2 inhibited in acidic conditions. Triolein was assimilated almost completely, and cell yields were increased, compared with palm oil fermentation. This also showed that unsaturated fatty acids assimilated more easily than saturated acids, especially in *T. candida* Y-128. The growth of *T. candida* Y-128 continued in a very acidic conditions of the broth.

Although the growth patterns of the strains on triolein as a substrate were similiar to each other, intermediate products such as diolein and monoolein were different. Whereas very small amount of di-and monoolein were detected in the culture broth of *T. candida* Y-128, some amount of dioleins formations were recognized in that of *A. calcoaceticus* KB-2 as shown in Fig. 4. In early phases of growth, di- and monoolein contained in the substrate (reagent grade of triolein, Tokyo Kasei Kogyo Co.) were assimilated, and then dioleins, especially 1,2-diolein, were accumulated before utilization for cell mass as a carbon source. Small amount of 1,3-diolein appeared in Fig. 4 (second peak in Ohr) was assimilated very slowly during fermentation, whereas 1,2-diolein was formed and assimilated quickly (third peaks in Fig. 4.). Triolein was assimilated for a short cultivation time by the strain (first peak in Fig. 4.).

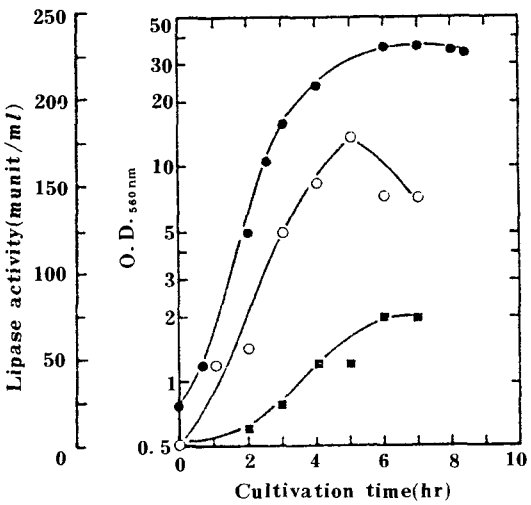

Fig. 2. Cell growth and lipase production of *A. calcoaceticus* KB-2 on Palm Oil(2%).

Fermentation was at 39℃, pH 6.8. Aeration and agitation speed were 1.0 vvm and 1,000 rpm. Lipase was measured by hydrolysis of olive oil. ● growth ; ○ activity in supernatant; ■ activity in washed cells.

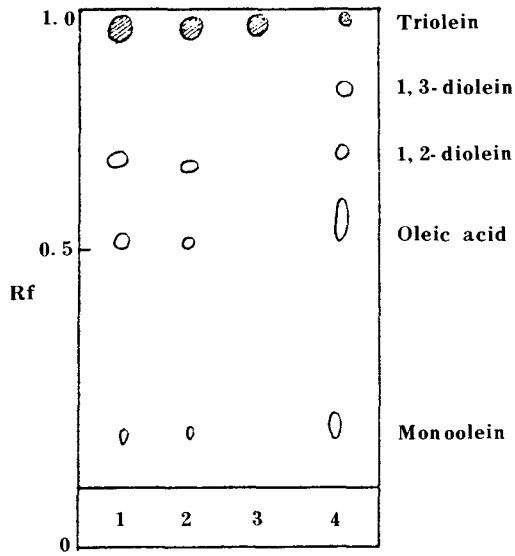

Fig. 3. Hydrolysis products of triolein by lipases of *A. calcoaceticus* KB-2 and *T. candida* Y-128.

Hydrolysis products of *A. calcoaceticus*(1) and *T. candida*(2); substrate(3) and standard(4). Solvent system: chloroform/acetone (96:4).

Discussion

Lipase activities of the strains were very low and unstable, compared with that of *S. lipolytica*[6]. Although the activity of *A. calcoaceticus* KB-2 was somewhat higher than that of other *Acinetobacter* strain reported by Breuil and Kushner[9], the lipase of this strain seems to be only enough to utilize the lipid materials for its own growth, without accumulation of lipase in the culture broth. Therefore, the strains attached palm oil droplets directly with microscopic observation during cultivation[4] and split off fatty acids at 1(3-)-position of triglycerides first and assimilated. When the strain could not produce an emulsifying agent, the emulsion state of the substrate would affect the cell growth[2].

Palm oil assimilation patterns by the strains seem to be

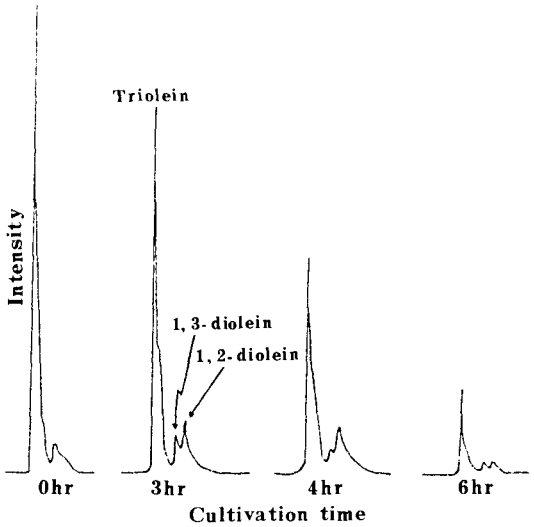

Fig. 4. HPLC Chromatograms of triolein assimilation by *A. calcoaceticus* KB-2.
Fermentation was at 39℃ and pH 6.8.

mainly derived from the physiological properties of organisms which assimilate lipid mateials. While *A. calcoaceticus* KB-2 could assimilate saturated and unsaturated fatty acids easily, *T. candida* Y-128 assimilated unsaturated fatty acids selectively such as other strains[1]. When the strain assimilated unsaturated fatty acids selectively, residual free saturated fatty acids and glycerides containing saturated acids mainly would cause the adhesion to the wall of fermentor gradually during cultivation in palm oil fermentation. These fractions could not be emulsified and assimilated easily at late stages of cultivation, and the residual palm oil fractions would affect the cell yield.

The main component of triglycerides composition in Malaysian palm olein (fractionating product of palm oil which contains triglycerides of high unsaturated fatty acids content in purification process), used in this study, were POO, POP, POL and PLP-MOP in order[10], and oleic and linoleic acids at the 2-position are 66.1% and 20.5% respectively[11]. Assuming the positional specificity of fatty acids in the substrate, the patterns of palm oil assimilation by the strains may be explained as follows. When the lipase of the species attacks the 1(3-)-position of the triglycerides of palm oil, palmitic acid and some amount of C18 unsaturated fatty acids are split off first and assimilated via ß-oxidation as in hydrocarbon-assimilating strains. When the strain can assimilate free fatty acids randomly like *A. calcoaceticus* KB-2, diglycerides were accumulated in a degree temporally as shown in Fig. 4. Fatty acid at 2-position of glycerides would be isomeriazed spontaneously to 1(3-)-position as reported by Okumura et al.[12] and Tsujisaka et al.[13].

Although the metabolic pathway of fatty acids by these strains is not known clearly, the selection of strain which could assimilate saturated and unsaturated fatty acids easily, seems to be an important point for cell production from palm oil and other natural fatty resources.

요 약

미생물에 의한 천연유지의 분해자화과정을 규명함으로써 값싼 유지를 발효원으로서 활용하기 위하여 팜유자화성 유용균주인 *Torulopsis candida* Y-128과 *Acinetobacter calcoaceticus* KB-2가 생산하는 리파제의 특성과 이들 균주의 생리적인 특성을 검토하였다. *T. candida* Y-128은 팜유입자에 부착·자화하며, 리파제의 작용에 의해 유리되는 불포화지방산을 포화지방산에 비해 쉽게 자화이용함으로써 균체증식이 이루어지고 있었다. *T. candida* Y-128의 리파제는 대부분 균체내에 존재하는데 비해, *A. calcoaceticus* KB-2는 배양시에 균체증식 대수기부터 균체외로 리파제가 생성됨을 알 수 있었으며, 리파제에 의해 유리된 포화 지방산도 다른 균주에 비해 자화이용이 용이함을 알 수 있었다. 두 균주는 배양액중에 리파제를 축적하지 않고 균체생육에 필요한 정도를 생산하며 천연유지중 1(3-)-위치의 지방산에 작용하는 위치특이성을 나타내었다. 따라서 두 균주는 천연유지입자에 부착하여 1(3-)-위치의 지방산을 분해하고, 분해생성물은 지방산대사경로를 거쳐 자화이용되는 것으로 보여진다.

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