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# Inhibition of Lipid Accumulation and Lipid Body Formation in Oleaginous Yeast by Effective Components in Spices, Carvacrol, Eugenol, Thymol, and Piperine

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We screened natural organic compounds, which affected the lipid accumulation and the lipid body formation in oleaginous yeast, *Lipomyces starkeyi*, generating large lipid bodies. We found that four natural components in spices, carvacrol, thymol, eugenol, and piperine, inhibited the lipid accumulation at concentrations of 20−50 mg/L with a slight growth inhibition. The inhibitory effects were quantitatively represented by the total lipid accumulation amount, the triacylglycerol accumulation amount, and the average lipid body size. At 50 mg/L, the effects of these compounds were not identical and exhibited 11−37% decrease in lipid amount and 15−21% decrease in lipid body size with 13−39% decrease in cell growth. The inhibitory effect of these compounds lead to 30−69% decrease in triacylglycerol accumulation without any additional accumulation of its intermediates, suggesting that they will suppress the total carbon inflow into the triacylglycerol biosynthesis.

**KEYWORDS: Lipid accumulation; lipid body; oleaginous yeast; *Lipomyces starkeyi*; spice; carvacrol; eugenol; thymol; piperine**

## INTRODUCTION

Oleaginous microorganisms, which accumulate lipids in cells at greater than 50% of the dry weight, have the possibility to be commercial oil producers for foods and energy resources (*1*−*4*). All the oleaginous microorganisms contain lipid bodies in the cells where most of the lipids are concentrated as neutral lipids (*5*). The enlargement of the lipid body is always accompanied with lipid accumulation in oleaginous microorganisms. To understand and to control the formation of the lipid body is crucial for the design of microbial oils. We have investigated lipid body formation in the oleaginous fungi (*6*, *7*) and yeasts (*8*) to know the mechanisms underlying lipid body formation. Known factors controlling lipid accumulation in oleaginous microorganisms are the composition of the medium, such as the C/N ratio and nitrogen source (*1*, *9*), and the culture conditions, such as temperature and dissolved oxygen (*1*, *6*).

*Lipomyces starkeyi* is a typical lipid-accumulating yeast that produces lipids at more than 40%/dry cell weight (*8*, *12*). Since the produced lipids were concentrated into one or two large lipid bodies in a cell, the yeast is suitable to analyze lipid body formation visually. Some natural organic compounds, which were contained in plant seeds, were shown to have inhibitory effects on the lipid accumulation of this yeast (*10*, *11*). Furthermore, for contemporary human health it is important to control the lipid content in the body to prevent the adiposis which will lead to various diseases (*10*, *11*). Thus, the discovery of natural compounds which can control lipid accumulation will contribute to improve lipid production and to regulate excess lipid accumulation. Therefore, we screened a number of compounds among effective components in edible herbs and spices to find materials which affect the lipid accumulation in this yeast. Most of these compounds seriously inhibited the yeast growth at the higher concentrations, as supposed from their antibacterial activities (*13*). Four compounds, carvacrol, thymol, eugenol, and piperine, exhibited a considerable decrease in lipid accumulation with some growth inhibition. The inhibitory effects of these compounds on the lipid accumulation are described in this paper.

## MATERIALS AND METHODS

**Chemicals.** All natural compounds were purchased from Naclai Tesque, Inc., Kyoto, Japan, except piperine, which was from Aldlich Chemical Co., Milwaukee, WI.

**Organism and Culture.** Oleaginous yeast, *Lipomyces starkeyi* IFO10381 (IFO, Osaka, Japan) was maintained on YM agar (glucose, 10 g/L; peptone, 5 g/L; yeast extract, 3 g/L; malt extract, 3 g/L; agar, 20 g/L) at 25 °C. One loopful of yeast well grown on a YM agar slant was inoculated into 5 mL of YM broth and cultivated with 120 rpm rotary shaking at 27 °C for 2 days. The precultured broth was

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inoculated with 1% (v/v) inoculum into the MLA broth (glucose, 30 g/L; $NH_4Cl$, 0.5 g/L; $KH_2PO_4$,7.0 g/L; $Na_2HPO_4 \cdot 12H_2O$, 5.0 g/L; $MgSO_4 \cdot 7H_2O$, 1.5 g/L; $FeCl_3 \cdot 6H_2O$, 0.08 g/L; $ZnSO_4 \cdot 7H_2O$, 0.01 g/L; $CaCl_2 \cdot 2H_2O$, 0.1 g/L; $MnSO_4 \cdot 5H_2O$, 0.1 mg/L; $CuSO_4 \cdot 5H_2O$, 0.1 mg/L; $Co(NO_3)_2 \cdot 6H_2O$, 0.1 mg/L (pH 5.5) (*14*)). For the screening of natural organic compounds, each compound as a methanol solution was added to 5 mL of broth in a test tube to produce final concentrations of 20 or 200 mg/L compound and 1% (v/v) methanol at the start of the culture. As a control, 1% methanol was added to the broth. Yeasts were cultivated using a rotary shaker (120 rpm) at 27 °C for 7 days. For the detailed observations, 1 mL of the preculture of YM broth was inoculated into 100 mL of broth in a 500 mL Erlenmeyer flask with three baffles and cultivated using a rotary shaker (120 rpm) at 27 °C. The compound was added as a methanol solution to give a final concentration of 4−50 mg/L compound and 0.2% (v/v) methanol at the start of the culture. As a control, 0.2% methanol was added to the broth. Data concerning inhibitory effects of the compounds were obtained from a typical experiment among four independent experiments except for thymol (two experiments). Data were presented as average values of duplicates. Among four (or two) independent experiments, the general tendencies of inhibitory effects were constant, although variations concerning the cell yield and the total lipid yield were observed due to unknown reasons of seed culture.

**Lipids Analysis.** The total lipid concentration was determined by gas chromatographic analysis of the total fatty acids (TFA) directly transmethylesterified from the dried cells (*15*). A 1 mL volume of 10% methanolic HCl and 0.5 mL of dichloromethane were added to the dried cells and kept at 60 °C for 3 h for the direct methylesterification. The reaction was stopped by the addition of 2 mL of saturated NaCl solution and 1 mL of *n*-heptane. The resultant methyl esters recovered in the heptane layer were then applied to a gas chromatograph (GC-17A; Shimadzu, Kyoto, Japan) equipped with a TC-70 capillary column (30 m × 0.25 mm i.d., GL Science, Tokyo, Japan) with temperature programming (180−220 °C at 4 °C/min increments). Heptadecanoic acid (C17:0) of 1 g/L in dichloromethane was transmethylesterified and used as the reference material for the determination of the fatty acids. The lipid classes were separated into triacylglycerol (TG), diacylglycerol (DG), monoacylglycerol (MG), phospholipids (PL), and free fatty acids (FFA) by TLC (*7*). Cells from the 1−7 mL culture broth after washing with the same volume of water were resuspended in 1 mL of distilled water. The cell suspension was mixed with 3 mL of chloroform/methanol (1:2, v/v) by shaking vigorously for 10 min. A 1 mL volume of chloroform and 1 mL of 10 mM potassium phosphate buffer (pH7) were added and mixed by shaking vigorously for 5 min. After the phase separation by centrifugation, the lower chloroform layer was collected. A 1 mL volume of chloroform was added to the remaining upper layer and mixed by shaking vigorously for 5 min. After the phase separation by centrifugation, the lower chloroform layer was collected. The chloroform layers were mixed and dried under nitrogen gas. The resultant lipid extract was dissolved in chloroform for loading on a Silica gel 60 plate (Merck, Darmstadt, Germany). The TLC was first developed using a plate with chloroform/acetone/methanol/acetic acid/$H_2O$ (10:4:2:2:1, by volume) to a height of about 8 cm above the origin and, after drying, subsequently with hexane/diethyl ether/acetic acid (80:40:1, by volume) to about 18 cm above the origin. The dried plate was sprayed with 2 g/L $CuSO_4$ in 13% (v/v) sulfuric acid and heated in an oven at 150 °C for 15 min. The carbonized spots were digitalized and converted to the chromatogram for the quantitative analysis by an image analyzer (LAS-1000plus, Fuji Photo Film Co., Tokyo, Japan). The standard solution of each class was used to correct the difference in carbonization efficiency for each class. As a standard, the mixed solution (1:1, w/v) of saturated and unsaturated fatty acid esters, such as the mixture of tristearin and triolein, was used to prevent errors from the difference in carbonization efficiency by unsaturation degree of fatty acid residues of esters. Soy bean lecithin oil (Lecithin DX, Nisshin Oillio, Ltd., Tokyo, Japan) was simultaneously applied to each plate as the control oil to normalize the difference in carbonization efficiency among the plates. All the intensity values isolated and identified by the chromatographic method were normalized by the values of the control oil for each plate, and the amounts were determined quantitatively by using the standard solution. Total lipids amount given by TLC analysis should theoretically agree with the GC analysis; however, the former was actually lower than the latter, presumably by the loss of lipid extraction. Then we obtained the relative composition of each lipid class by TLC analysis and the concentration of each class was calculated from the total lipids measured by GC analysis.

For the screening of compounds, rapid estimation method by the direct staining of the broth using a fluorescent dye, Nile Red, was used (*8*). A 0.1 mL amount of broth was mixed with 2 mL of 10 mM potassium phosphate buffer with 0.15 M KCl (pH 7.0; PBS) in a 10 mm acryl cuvette. The spectrum in a wavelength region of 400 to 700 nm for the cell suspension was recorded before and after the addition of 10 $\mu$L of Nile red solution. Spectra were corrected by subtracting the spectra before and after the Nile red addition using the computer software to determine the intensity of the peak of the corrected spectrum. The fluorescence intensity was proportional to the lipid content.

**Measurement of Cell Size and Lipid Body Size.** An aliquot of the cell suspension mixed with the same volume of 0.2 g/L Nile Blue A solution in water and the same volume of 0.1 M glycine buffer (pH 10) was immediately observed with a microscope (*16*). Some intracellular organelles were stained in colors such as nuclear components in purple, cytoplasm in blue-green, and lipid body in orange. The lipid body was identified by the fluorescence observation as the lipid body was the sole organelle showing fluorescence by this staining. Microscopic DIC photographs were taken using a Nikon E600 microscope (Nikon, Tokyo, Japan) equipped with a color CCD digital camera (DP12, Olympus, Tokyo, Japan). A fluorescence photograph of the same field was also taken with the same microscope using a 450−490 nm excitation filter, a 505 nm diachronic mirror, and a 520 nm barrier filter with ×60 objective lens. The digital color pictures from the digital camera were converted to the binary image by manually tracing on the digital image. The area and perimeter of a cell and a lipid body were measured from the binary image using Scion Image software (Scion Corp., Frederick, MD). The shapes of this yeast cell and the lipid body are almost spherical or slightly oblate. Under the assumption that the shapes of the cell and the lipid body are spherical, the diameters of the cell and the lipid body were estimated individually from the area of the binary image and from the circumference of the binary image and averaged. The occupied volume percent of lipid body in a cell was estimated from the above diameters of the cell and the lipid body. The diameters of the cell and the lipid body and the occupied volume percent of lipid body were estimated for every cell in one picture field. Since all the values of the cells in the same photograph were measured and averaged without exception, the values indicate the average sizes of yeasts in a part of the broth, including the well-grown cells with large lipid bodies and also the small cells just separated from their mother cells. If one picture field contained less than 50 cells, all the cells in two or more pictures were used for the estimation so that the total cell number exceeded 50 cells to be averaged. When the plural number of lipid bodies existed in a cell, the diameter value was obtained from the largest one and the lipid body volume was estimated from the total volume of all the lipid bodies in a cell.

**Other Analysis.** The glucose concentration was determined using the Glucose CII Test Wako (Wako Pure Chemical Industries, Osaka, Japan). The cell growth was based on the dry cell weight. Cells in the 1−5 mL culture broth were collected after washing with the same volume of water by centrifugation and weighed after being dried at 105 °C overnight.

## RESULTS AND DISCUSSION

**Screening of Natural Organic Compounds.** *Lipomyces starkeyi* IFO10381 aerobically cultivated in the MLA broth accumulates lipids in cells with a lipid content of greater than 40%/dry cell weight in 4−5 days. Under this condition, this strain shows a unicellular growth in the shape of a spherical to oval cell having a spherical lipid body. We tested many natural organic compounds for their effects on the lipid accumulation in *L. starkeyi*: acetophenone, allyl isothiocyanate, *m*-anisalde-

**Table 1.** Effect of Compound Addition on Growth and Lipid Accumulation in *L. starkeyi*[a]

| compd | concn (mg/L) | growth[b] (%) | glucose consumptn[c] (%) | lipid accumulatn[d] (%) | compd | concn (mg/L) | growth[b] (%) | glucose consumptn[c] (%) | lipid accumulatn[d] (%) |
|---|---|---|---|---|---|---|---|---|---|
| isovaleric acid | 20 | 98 | 100 | 90 | anise alcohol | 20 | 86 | 100 | 98 |
| | 200 | 77 | 100 | 90 | | 200 | 86 | 100 | 52 |
| methyl isobutyl ketone | 20 | 102 | 100 | 107 | *o*-anisaldehyde | 20 | 81 | 100 | 99 |
| | 200 | 100 | 100 | 111 | | 200 | 81 | 100 | 57 |
| linalool | 20 | 94 | 100 | 94 | *m*-anisaldehyde | 20 | 84 | 100 | 93 |
| | 200 | 66 | 100 | 37 | | 200 | 80 | 100 | 65 |
| geraniol | 20 | 92 | 100 | 97 | *p*-anisaldehyde | 20 | 89 | 100 | 104 |
| | 200 | 1 | 0 | 0 | | 200 | 83 | 100 | 58 |
| citral | 20 | 92 | 100 | 95 | benzaldehyde | 20 | 88 | 100 | 97 |
| | 200 | 3 | 0 | 0 | | 200 | 95 | 100 | 93 |
| citronellal | 20 | 97 | 100 | 92 | acetophenone | 20 | 97 | 100 | 116 |
| | 200 | 1 | 0 | 0 | | 200 | 94 | 100 | 89 |
| $\beta$-myrcene | 20 | 107 | 100 | 64 | *p*-methylacetophenone | 20 | 96 | 100 | 112 |
| | 200 | 69 | 46 | 40 | | 200 | 91 | 100 | 95 |
| carvacrol | 20 | 109 | 100 | 26 | *p*-methoxyacetophenone | 20 | 105 | 100 | 98 |
| | 200 | 2 | 3 | 1 | | 200 | 93 | 100 | 50 |
| thymol | 20 | 95 | 100 | 66 | *m*-methoxyacetophenone | 20 | 103 | 100 | 98 |
| | 200 | 3 | 0 | 0 | | 200 | 104 | 100 | 52 |
| D-limonene | 20 | 102 | 100 | 95 | *o*-methoxyacetophenone | 20 | 103 | 100 | 97 |
| | 200 | 70 | 27 | 58 | | 200 | 107 | 100 | 70 |
| L-perillaldehyde | 20 | 94 | 100 | 86 | 1,8-cineole | 20 | 99 | 100 | 72 |
| | 200 | 6 | 4 | 1 | | 200 | 103 | 100 | 120 |
| *p*-cymene | 20 | 105 | 100 | 96 | borneol | 20 | 92 | 100 | 96 |
| | 200 | 54 | 46 | 33 | | 200 | 86 | 100 | 26 |
| $\alpha$-terpineol | 20 | 100 | 100 | 120 | DL-camphor | 20 | 95 | 100 | 93 |
| | 200 | 59 | 100 | 12 | | 200 | 90 | 100 | 86 |
| menthone | 20 | 99 | 100 | 110 | $\alpha$-pinene | 20 | 98 | 100 | 118 |
| | 200 | 97 | 100 | 78 | | 200 | 101 | 92 | 87 |
| (+)-pulegone | 20 | 101 | 100 | 91 | $\beta$-pinene | 20 | 96 | 100 | 119 |
| | 200 | 83 | 100 | 13 | | 200 | 58 | 45 | 35 |
| *t*-cinnamaldehyde | 20 | 56 | 100 | 96 | diacetyl | 20 | 101 | 100 | 111 |
| | 200 | 4 | 3 | 1 | | 200 | 92 | 84 | 66 |
| *t*-cinnamic acid | 20 | 49 | 100 | 65 | benzyl benzoate | 20 | 85 | 100 | 81 |
| | 200 | 2 | 5 | 1 | | 200 | 0 | 0 | 0 |
| anethole | 20 | 111 | 100 | 146 | piperine | 20 | 96 | 100 | 22 |
| | 200 | 3 | 1 | 1 | | 200 | 81 | 100 | 25 |
| eugenol | 20 | 94 | 100 | 71 | capsaicin | 20 | 99 | 100 | 110 |
| | 200 | 6 | 16 | 2 | | 200 | 4 | 2 | 1 |
| isoeugenol | 20 | 104 | 100 | 97 | allyl isothiocyanate | 20 | 96 | 75 | 83 |
| | 200 | 4 | 0 | 0 | | 200 | 2 | 1 | 1 |
| vanillin | 20 | 100 | 100 | 106 | | | | | |
| | 200 | 98 | 100 | 103 | | | | | |

[a] Data shown are values at 7 day culture relative to the control, into which only 1% methanol was added. [b] Relative value of dry cell weight to the control. [c] Relative value of glucose consumed to the control. [d] Relative value of fluorescent intensity of the broth to the control.

hyde, *o*-anisaldehyde, *p*-anisaldehyde, anethole, anisealcohol, benzaldehyde, benzyl benzoate, borneol, capsaicin, carvacrol, 1,8-cineole, *t*-cinnamaldehyde, *t*-cinnamic acid, citral, citronellal, DL-camphor, *p*-cymene, diacetyl, eugenol, geraniol, isoeugenol, isovaleric acid, D-limonene, linalool, menthone, methylisobutyl ketone, *m*-methoxyacetophenone, *p*-methoxyacetophenone, *o*-methoxyacetophenone, *p*-methylacetophenone, $\beta$-myrcene, *l*-perillaldehyde, $\alpha$-pinene, $\beta$-pinene, piperine, (+)-pulegone, $\alpha$-terpineol, thymol, and vanillin (**Table 1**). All of them are natural organic compounds which are known as effective components in edible herbs and spices. Half of them showed no significant effect on the growth and lipid accumulation even under the addition at 200 mg/L. Among the remains, we selected the compounds which kept good growth at 20 mg/L and inhibited the lipid accumulation considerably at 200 mg/L and moderately at 20 mg/L. We also considered the microscopic observation of the broth. After all, four compounds, carvacrol, thymol, eugenol, and piperine, were selected as they showed inhibition of the lipid accumulation without a significant growth inhibition at 20 mg/L. When the compounds were added at 200 mg/L, carvacrol, thymol, and eugenol significantly inhibited cell growth and, consequently, caused no lipid accumulation. On the other hand, the addition of piperine at 200 mg/L decreased cell growth and lipid accumulation by about 20%. We then investigated the lipid accumulation of these four compounds.

**Inhibition of Lipid Accumulation. Figure 1** shows the time course of the growth and lipid accumulation of *L. starkeyi* with and without compounds. Without any compounds, *L. starkeyi* consumed the initial 30 g/L glucose after 70−90 h and reached the maximum growth after 100 h (**Figure 1A**). After the rapid glucose consumption (40−70 h), the lipid accumulation was significantly accelerated during 50−100 h. The accumulated lipid amount finally increased to 40−43% of the dry cell weight at the maximum. At the beginning of the culture, cells had several small (approximately 0.5 $\mu$m) lipid bodies as shown in **Figure 2A**. After the rapid glucose consumption, one of the lipid bodies, sometimes two of them, began to enlarge. Then almost all the cells came to contain large lipid bodies, many of which reached more than 4 $\mu$m in diameter (**Figure 2A**). Some cells enlarged to about 10 $\mu$m in diameter with a large lipid body of more than 8 $\mu$m in diameter after 150 h. In **Figure 3**, the average sizes of the cell and lipid body are shown by the measurement from the microscopic observations. These values well demonstrated that the lipid body rapidly enlarged from 1.5 to 4 $\mu$m while the cell size slightly increased from 50 through 100 h. The occupied volume percent of the lipid body also

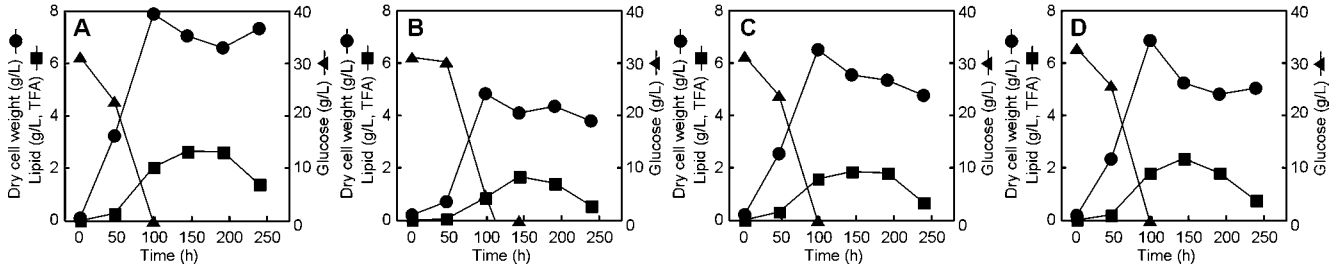


**Figure 1.** Effect of the addition of natural organic compounds on the growth of *Lipomyces starkeyi* and its lipid accumulation. Only methanol at 0.2% (v/v) was added as the control experiment (**A**). Carvacrol (**B**), eugenol (**C**), and piperine (**D**) were added at 50 mg/L with 0.2% methanol. Key: dry cell weight (●); lipid content as a concentration of total fatty acids (■); glucose concentration (▲). Data are presented as average values of duplicates in the typical experiment.

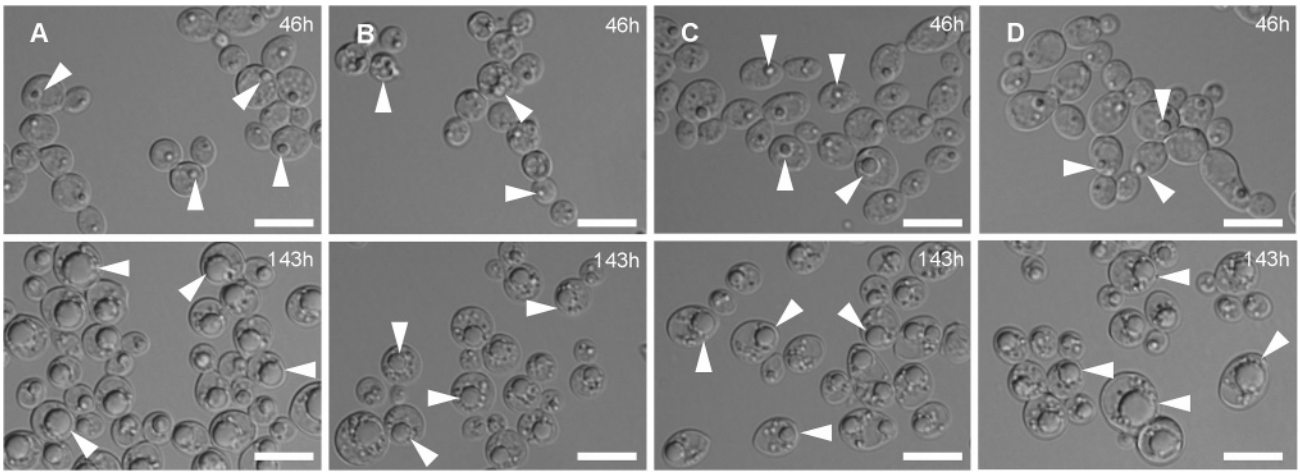


**Figure 2.** Effect of the addition of natural organic compounds on the enlargement of lipid bodies in *Lipomyces starkeyi*. Only methanol at 0.2% (v/v) was added as the control experiment (**A**). Carvacrol (**B**), eugenol (**C**), and piperine (**D**) were added at 50 mg/L with 0.2% methanol. A culture broth of the experiment of **Figure 1** was taken at the log phase (46 h) and the stationary phase (146 h). Arrows indicate lipid bodies. Bar = 10 $\mu$m.

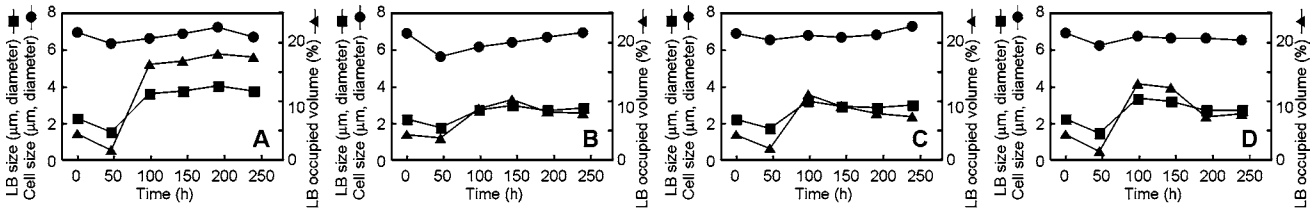


**Figure 3.** Effect of the addition of natural organic compounds on the increase in size of cell and lipid body (LB) in *Lipomyces starkeyi*. Only methanol at 0.2% (v/v) was added as the control experiment (**A**). Carvacrol (**B**), eugenol (**C**), and piperine (**D**) were added at 50 mg/L with 0.2% methanol. Each value for more than 50 cells was individually estimated for each cell taken from the experiment of **Figure 1** and averaged. Key: cell size in average diameter (●); lipid body size in average diameter (■); occupied volume percent of lipid body in a cell (▲).

drastically increased. After a long time culture (over 200 h), the lipid body size, once enlarged, did not decrease so much although the lipid content in the cell decreased.

The effects of four compounds, carvacrol, thymol, eugenol, and piperine, were examined at 4−50 mg/L. The effects of these compounds depended on their concentrations. The lipid amount accumulated was significantly decreased with the addition at 20−50 mg/L, while the effects of these compounds at 4−10 mg/L on the lipid accumulation were not apparent. In **Figure 1B−D**, the effects of the addition of carvacrol, eugenol, and piperine at 50 mg/L are shown.

When carvacrol was added to the culture, glucose consumption and cell growth were not observed in the first 50 h (**Figure 1B**). When the glucose began to be consumed after 50 h incubation, the cells drastically grew during the next 50 h (50−100 h) and the lipid accumulation increased during the next 100 h (50−150 h). However, both of them could not reach the level of the control. The size of lipid body was smaller than the control, while the size of the cell was slightly smaller than the control. The addition of carvacrol at 50 mg/L suppressed the enlargement of the lipid body, and it represented the reduction of the lipid accumulation. Finally, the carvacrol addition at 50 mg/L showed a 39% decrease in the cell growth, a 37% decrease in the lipid amount, and a 20% decrease in the lipid body size at the maximum lipid accumulation, compared to the control. On the other hand, the lipid content/the dry cell weight reached the same level (41% at 143 h) as the cell growth and lipid accumulation were simultaneously decreased. For the thymol addition, the inhibition was almost the same as for the carvacrol addition (data not shown). This may reflect the fact that thymol and carvacrol are structural isomers which were different in the position of the hydroxyl group. In the case of the addition of carvacrol or thymol at 5−20 mg/L, the lag time of the growth was about 20 h and the lipid accumulation showed no or a less than 9% decrease, while the addition at 200 mg/L showed a 97−98% decrease in the cell growth and 96−99%

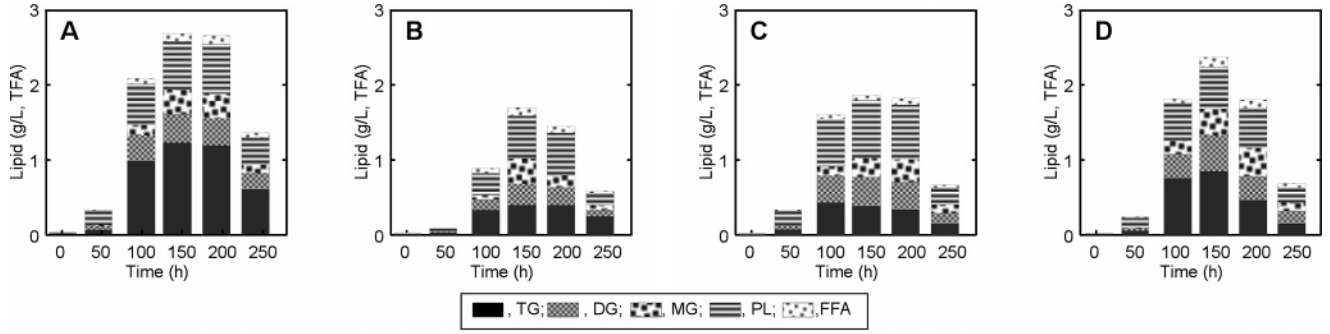


**Figure 4.** Effect of natural organic compounds addition on the accumulating distribution for each lipid class: added only methanol at 0.2% (v/v) (control, **A**); carvacrol added at 50 mg/L with 0.2% methanol (**B**); eugenol added at 50 mg/L with 0.2% methanol (**C**); piperine added at 50 mg/L with 0.2% methanol (**D**). Data are presented as average values of duplicates in the typical experiment which correspond to the same experiment of **Figure 1**.

decrease in the lipid accumulation (data not shown). The length of the lag time increased and the lipid accumulation amount decreased with an increase in the added concentration of carvacrol or thymol in the range of 5−50 mg/L, whereas the cell growth was almost depressed at 200 mg/L.

When eugenol was added to the culture, the glucose consumption and the cell growth started from the beginning of the incubation without a lag time, but their rates were lower than the control (**Figure 1C**). At 50−100 h, the lipids were drastically accumulated. Around 100 h, the cell growth and lipid accumulation reached maximum values. The average cell size changed with time course almost in the same level as the control. The lipid body size which reflected the lipid accumulation drastically increased at 50−100 h, but its size and the accumulated amount were lower than the control (**Figure 3C**). The maximum lipid content was 34% of the dry cell weight at 191 h. Finally, the eugenol addition at 50 mg/L showed an 18% decrease in the cell growth, a 31% decrease in the lipid amount, and a 21% decrease in the lipid body size. The eugenol addition at 4−20 mg/L showed a 10% decrease in the lipid amount while at 200 mg/L a 90% decrease in the lipid amount with a 94% decrease in the cell growth (data not shown).

When piperine was added, the glucose consumption and cell growth started without a lag time (**Figure 1D**). The cell growth reached a maximum value after 100 h of incubation, while the lipid accumulation continued until 150 h. The enlargement of the lipid body and the lipid accumulation stopped at less than the level of the control (**Figures 1D** and **3D**). The maximum lipid content was 45% of the dry cell weight at 143 h, where the decrease in the dry cell weight was rather rapid but the lipid accumulation was still increased. Finally, the piperine addition at 50 mg/L showed a 13% decrease in the cell growth, an 11% decrease in the lipid amount, and a 15% decrease in the lipid body size. The piperine addition at 10−20 mg/L showed a 15−25% decrease in the lipid accumulation while at 200 mg/L a 26% decrease in the lipid accumulation with a 19% decrease in the cell growth (data not shown).

Despite the addition of compounds, the average cell sizes were within the range of 6.6 and 7.0 $\mu$m except for the log phase of the growth. This would indicate that the cell size increased independently of the lipid body enlargement. The occupied volume percent of the lipid body is also estimated in **Figure 3**. Without these compounds the lipid body occupied 18% of the cell volume at the maximum, whereas with these compounds it occupied 10−13%. The fact that these compounds made the occupied volume percent of the lipid body less than two-thirds of the control means that these inhibitors suppressed the lipid body enlargement. With inhibitor addition, many small lipid bodies less than 0.5 $\mu$m other than the enlarged lipid body were often observed in the cells after the start of the lipid accumulation, as shown in **Figure 3B−D** (143 h). These observations suggest that these compounds did not block the initiation of the lipid body formation but suppressed the lipid body enlargement and/or the fusion of small lipid bodies into the enlarged one. The effects of carvacrol and thymol were similar and seemed remarkable compared to the effects of eugenol and piperine.

Carvacrol, thymol, and eugenol, which are phenolic compounds, are known to have antibacterial activities. The antibacterial activities of carvacrol and thymol seem to depend on their properties that make the cell membrane permeable (*13*, *17*). Thymol and eugenol showed antifungal activities; the surfaces of the *Saccharomyces cerevisiae* cells were significantly damaged by these compounds at a concentration near the minimum inhibitory concentration (MIC) (*18*). It is also possible that these phenolic compounds affected the cell surface of this yeast and consequently inhibit the growth and the lipid accumulation. In this study, the glucose consumption and the cell size were not so much affected but the lipid amount and the lipid body size were considerably decreased by these compounds at concentrations lower than the MIC. From these facts, we conclude that these compounds affect not only cell growth but also lipid accumulation: the inhibitory effects are apparent on the decrease in the lipid amount accompanying with the reduced size of lipid bodies and the existence of unenlarged lipid bodies at concentrations lower than the lethal concentration.

**Effect on Lipid Composition. Figure 4** shows the lipid composition of the accumulated lipids in *L. starkeyi*. Without inhibitors, 45% of the accumulated lipids were TG and about 25% was PL after a 100 h incubation when the lipids rapidly accumulated (**Figure 4A**). TG was the main component in any event. The TG amount significantly increased during 50−100 h incubation, whereas the amounts of the other classes, such as PL, DG, MG, and FFA, did not show any drastic increase as compared with the TG amount. In any case, phosphatidylethanolamine and phosphatidylcholine were dominant in PL (data not shown). Sterols and sterol esters were not detected throughout the culture under this condition.

When carvacrol, eugenol, or piperine was added to the culture (**Figure 4B−D**), the accumulated amount of TG had apparently decreased compared to the control. **Table 2** summarized the inhibitory effects of these compounds when the total lipid reached to the maximum. The accumulated amount of TG showed a 68% decrease with carvacrol, a 69% decrease with eugenol, and a 30% decrease with piperine from the control. On the other hand, the other classes, PL, DG, MG, and FFA, showed amounts similar to that of the control throughout the time course. The PL amount increased with an increase in the

**Table 2.** Comparison of Inhibition of Compounds on Growth and Lipid Accumulation in *Lipomyces starkeyi*[a]

| compd | dry cell wt (g/L) | tot. lipids[b] (g/L) | TG[b] (g/L) | PL[b] (g/L) | DG[b] (g/L) | MG[b] (g/L) | FFA[b] (g/L) |
|---|---|---|---|---|---|---|---|
| control | 7.05 | 2.67 | 1.22 | 0.64 | 0.37 | 0.33 | 0.09 |
| carvacrol[c] | 4.10 | 1.69 | 0.39 | 0.56 | 0.28 | 0.34 | 0.11 |
| eugenol[c] | 5.55 | 1.85 | 0.38 | 0.77 | 0.39 | 0.26 | 0.05 |
| piperin[c] | 5.25 | 2.37 | 0.85 | 0.56 | 0.47 | 0.35 | 0.13 |

[a] Data were taken when the total lipids showed the maximum values at 143 h. [b] Total lipids, total fatty acid derived from transmethylation of dry cells; TG, triacylglycerol; PL, phospholipids; DG, diacylglycerol; MG, monoacylglycerol; FFA, free fatty acid. [c] Compound was added as methanol solution to give 50 mg/L compound and 0.2% methanol.

dry cell weight, but it had no relationship with the lipid accumulation. If the inhibitory effects of these compounds at the given concentration mainly resulted in the cell growth inhibition, the yields of lipid classes other than TG were also decreased. Especially, PL yield might be decreased much more, as PL amount would reflect the amount of the cell membrane and organelle. Considering the almost equivalent PL yield and almost the same cell size despite the inhibitory compounds, we think that these compounds inhibited the TG accumulation with somewhat growth inhibition.

It was noted that the accumulated amount of the total lipids mainly correlated to the TG amount; that is, the degree of the decrease in the lipid accumulation depended on the decrease in the TG accumulation. If these compounds inhibited some of the steps of the TG biosynthetic process, one or more of its intermediate compounds, such as DG, MG, or FFA, or one or more of the phospholipids, would be additionally accumulated instead of the decrease in the TG. However, the inhibition of these compounds resulted only in the decrease in the TG accumulation without an increase in any corresponding intermediates. These inhibitors did not affect the intermediate process of the TG biosynthesis but diminished the final amount of the TG accumulation. These facts suggest that these compounds depressed the total carbon inflow into the TG biosynthetic process and lead to the decrease in the TG accumulation amount and consequently to the decrease in the total lipid accumulation, although it is unclear where are functional sites of these compounds.

The decreased amounts of dry cell weight by inhibitors exceeded the decreased amounts of total lipids (**Table 2**). This is always observed, but the differences were not the same for each experiment. It seemed that the other cell components were also affected by the inhibitors. If any glucose exists in the broth where the nitrogen source is not enough for the growth, the excess glucose was metabolized into lipids and other storage materials. As this yeast is known to produce polysaccharides and polysaccharide-degrading enzymes (*19*, *20*), polysaccharide may be a candidate material as a carbon-rich storage. Thymol and eugenol were shown to damage the cell wall and membrane of *Saccharomyces cerevisiae* (*18*). Assuming that these compounds injured the cell surface of *Lipomyces*, the carbon sources for TG synthesis or other storage materials can be released from the cell. For the defense from these inhibitors energy consumption can be increased to consume glucose more than the control. Detail of this phenomenon is a future subject. For the present, it is apparent that the same amount of glucose was consumed but cell yield and TG yield were decreased by inhibitors by unknown mechanisms.

The fatty acid composition of the total lipids was almost constant after the rapid accumulation despite the addition of inhibitors: 47−49% oleic acid, 33−34% palmitic acid, 6−9% linoleic acid, 3−5% stearic acid, and 3% palmitoleic acid. These compounds did not affect the fatty acid composition. The composition of the lipid class shown here was not identical with those of the previous observations which showed a 77−79% TG, a 10−15% PL, and 1−7% sterols + sterol esters (*21*, *22*). The contradiction with the previous results may depend on the difference in the medium, strain, and growth conditions, as the culture condition in this study is conducted to the optimized lipid accumulation for this strain.

In this paper, the cell size and lipid body size were estimated as the average from the total yeast population which included cells from a small cell just separated from her mother cell with small lipid bodies to a well-grown cell with a large lipid body. **Figures 1** and **3** showed that the enlargement of the lipid body well reflected the increase in the lipid accumulation. By a comparison of the lipid accumulation with and without inhibitors, the decreases in the occupied volume percent of lipid body and in the TG accumulation were well correlated with the inhibitory effects of the compounds. The inhibitions by these four compounds were quantitatively represented by the decrease in the lipid body size and in the TG accumulation amount with a correlation.

In conclusion, carvacrol, thymol, eugenol, and piperine at 20−50 mg/L, concentrations lower than the lethal concentration, showed inhibitory effects on the lipid accumulation with some growth inhibition. These compounds depressed the carbon inflow into the TG biosynthesis and the enlargement of lipid body but did not stop the initiation of the lipid body formation or the intermediate process of TG biosynthesis.

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