3. Moser, H.A., H.J. Dutton, C.D. Evans and J.C. Cowan, Food Technol. 4:105 (1950).
4. Evans, C.D., JAOCS 32:596 (1955).
5. Fioriti, J.A., M.J. Kanuk and R.J. Sims, JAOCS 51:219 (1974).
6. Gray, J.I., JAOCS 55:539 (1978).
7. Smouse, T.H., JAOCS 56:747A (1979).
8. Jackson, H.W., JAOCS 58:227 (1981).
9. Scholz, R.G., and L.R. Ptak, JAOCS 43:596 (1966).
10. Evans, C.D., G.R. List, R.L. Hoffman and H.A. Moser, JAOCS 46:501 (1969).
11. Selk, E., H.A. Moser and W.K. Rohwedder, JAOCS 47:393 (1970).
12. Jarvi, R.K., G.D. Lee, D.R. Erickson and E.A. Butkus, JAOCS 48:121(1971).
13. Herman, K.T., L.C. Rose and R.L. Vandaveer, JAOCS 48:178 (1971).
14. Dupuy, H.P., S.P. Fore, and L.A. Goldblatt, JAOCS 48:876 (1971).
15. Dupuy, H.P., S.P. Fore and L.A. Goldblatt, JAOCS 50:340 (1973).
16. Dupuy, H.P., E.T. Rayner and J.I. Wadsworth, JAOCS 53:628 (1976).
17. Dupuy, H.P., E.T. Rayner, J.I Wadsworth and M.G. Legendre, JAOCS 54:445 (1977).
18. Williams, J.L., and J.H. Willie, JAOCS 53:634 (1976).
19. Williams, J.L., and T.H. Applewhite, JAOCS 54:461 (1977).
20. Warner, K., C.D. Evans, G.R. List, H.P. Dupuy, J.L. Wadsworth and G.E. Goheen, JAOCS 55:252 (1978).
21. Waltking, A.E., and H. Zmachinski, JAOCS 54:454 (1977).
22. Jackson, H.W., and D.J. Giacherio, JAOCS 54:458 (1977).
23. Seals, R.G., and E.G. Hammond, JAOCS 43:401 (1966).
24. Seals, R.G., and E. G. Hammond, JAOCS 47:278 (1970).
25. O'Mahony, M., J. Dairy Sci. 62:1954 (1979).
26. Dutton, H.J., C.R. Lancaster, C.D. Evans and J.C. Cowan, JAOCS 28:115 (1951).
27. Moser, H.A., P.C. Cooney, C.D. Evans and J.C. Cowan, JAOCS 43:632 (1966).

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Lipid from Yeast Fermentation: Effects of Cultural Conditions on Lipid Production and Its Characteristics of *Rhodotorula glutinis*

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ABSTRACT

To produce lipids from microbial origins, *Rhodotorula glutinis* (syn. *Rhodotorula gracilis*) NRRL Y-1091 was cultured in batch and continuous systems under nitrogen- and carbon-limited conditions. The lipid production patterns are shown to be different from each other depending on growing conditions. In continuous cultures under nitrogen-limited conditions, the maximum lipid accumulation was observed at the lowest dilution rate examined, giving the efficiency of substrate conversion of 16.4 g lipid per 100 g glucose consumed. As the dilution rate increased, cell biomass, lipid content, lipid productivity and lipid yield decreased. In carbon-limited continuous cultures, cell biomass decreased with increasing dilution rate, but lipid content remained almost constant. Neutral lipid portions in nitrogen-limited cultured yeast cells decreased as the dilution rate increased, and glyco- and phospholipid portions showed the reverse trend. Major components in the neutral lipid portions in yeast cells are triglyceride, free fatty acid, steryl ester and sterol. Phosphatidylserine was the predominant phospholipid in yeast cells. The dilution rate also affected the fatty acid composition of all lipid portions; polyunsaturated fatty acids increased and saturated and monounsaturated fatty acids decreased with increasing dilution rates. The degrees of unsaturation of each lipid class and total lipids were also increased by increasing the dilution rate.

INTRODUCTION

With the increasing demands for fats and oils for edible and industrial purposes, many assessments have been made to find new possible lipid sources other than the conventional plant and animal sources. As a result of such assessments, the lipid from microbial origins was found to be a possible source. As a matter of fact, the microbial lipid production has long been an attractive subject of research interests in both laboratories and industries (1–4).

Most of the organisms used for lipid production were yeast, fungi and algae (5, 6). Lipid production with *Rhodotorula glutinis* (syn. *Rhodotorula gracilis*), one of the oleaginous yeasts used widely, have been carried out for a long time and continued to recent years (7–9). The characteristics of lipids of *R. glutinis* obtained from batch cultures were well established with respect to the effects of nutrient sources, pH and temperature (10–12).

The continuous cultivation of *R. glutinis*, however, was first performed by Ratledge and Hall to study the oxygen demands under nitrogen- and carbon-limited conditions (13). In another work of Ratledge and Hall (14), they reported that the fatty acid compositions of total lipids were unchanged depending on the growth rates under nitrogen-limited conditions, but slight changes were observed under carbon-limited conditions. In contrast to the results of Ratledge and Hall (14), we found that the fatty acid compositions of total lipids were changed as the growth rate altered under nitrogen-limited conditions and, therefore, the degrees of fatty acid unsaturation were also changed (15).

The analyses of lipids from *R. glutinis* in the abovementioned studies (14, 15) were all aimed at the analyses of total lipids. The characterization of fractionated lipid classes in *R. glutinis* and fatty acid compositions of each lipid class have not been reported elsewhere to our knowledge. In this paper, we report the lipid production data obtained from batch and continuous cultures and the results of analyses of lipid classes in *R. glutinis*; we believe this is the first report to elucidate the quantitative and qualitative changes in lipid classes depending on the growth conditions.

EXPERIMENTAL PROCEDURES

Materials

Standard materials used in thin layer chromatography

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(TLC) and high performance liquid chromatography (HPLC) such as cholesteryl oleate, cholesterol, triolein, diolein, monoolein, cerebroside, all fatty acids and phospholipids were purchased from Sigma Co. (St. Louis, MO). Stearyl glucoside, monogalactosyl diglyceride and digalactosyl diglyceride were obtained from Applied Science Lab. (State College, PA). Solvents of chromatographic grade were purchased from Burdick & Jackson (Muskegon, MI). All other reagents and chemicals used were of analytical grade.

Microorganism and Medium

A strain of the yeast *Rhodotorula glutinis* NRRL Y-1091 was used in this study. For nitrogen-limited cultivation of the organism, the synthetic medium was used and it contained (per liter): 0.134 g $CaCl_2 \cdot 2H_2O$; 0.73 g $MgSO_4$; 2 g Na_2HPO_4; 7 g KH_2PO_4; 0.5 g yeast extract; 1 g $(NH_4)_2SO_4$; and 35 g glucose. For carbon-limited cultivation, compositions of all components were the same as those for nitrogen-limited medium except $(NH_4)_2SO_4$ and glucose: 3 g $(NH_4)_2SO_4$ and 15 g glucose per liter. The nitrogen content in yeast extract used (Difco, Detroit, MI) was 7.2% (wt) determined by micro-Kjeldahl method (16). Carbon to nitrogen ratios in the nitrogen- and carbon-limited media were 56.5 and 8.9, respectively. Glucose solution (1.5 or 3.5%, w/v) was made up in distilled water and mixed with the salt medium after sterilization for the batch- and continuous-culture run. The silicone antifoam agent used was Antifoam A Compound (Dow Corning, Midland, MI).

Batch and Continuous Cultures

A 5-L jar fermenter (New Brunswick Scientific Co., Edison, NJ) system was employed in this study. The operation conditions and control set points were: 2 L working volume, 30 ± 0.5°C, pH 5.5 ± 0.1, 2 L air/min, and 700–800 rpm agitation. Medium flow rate was adjusted and controlled as needed using auxiliary Masterflex pumps (Cole-Parmer, Chicago, IL). pH was maintained at 5.5 with 2N HCl and 2N NaOH by use of an automatic pH controller (New Brunswick Scientific Co.). In continuous cultivations, the steady-state conditions could be obtained after continuous flow of 4–5 working volume of medium.

Analytical Methods

Cell concentration was measured by dry cell weight. Residual glucose concentration in the fermenter vessel was determined by dinitrosalicylic acid method (17). Residual ammonium nitrogen concentration and protein content in yeast cells were measured by the micro-Kjeldahl determination (16). Lipids were extracted from the dried and ground yeast cells by modified percolation method (9) and were purified by Folch's washing method (18). Then the purified lipids were fractionated into neutral-, glyco- and phospholipid with silicic acid column chromatography (19). The fractionated lipids were characterized by TLC and HPLC. In carrying out TLC, the precoated silica gel plates (20 cm × 20 cm, 250 μm thickness, E. Merck) were used and solvent systems used were as follows: neutral lipid, petroleum ether/diethyl ether/acetate (100:15:1, v/v/v); glycolipid, chloroform/methanol (110:40, v/v); phospholipid, chloroform/methanol/water/18.4% ammonium hydroxide (130:70:8:0.5, v/v/v/v). TLC plates developed in appropriate developing solvents were air-dried and visualized by one of the following methods: iodine vapor adsorption, charring with 50% sulfuric acid in methanol and with 20% perchloric acid. The free fatty acids of neutral lipid and glycolipid were obtained by alkali hydrolysis (20) and those of phospholipid by acid hydrolysis (21). The fatty acid compositions of each lipid class were analyzed by HPLC (Waters Associates ALC/GPC-244, Milford, MA) equipped with R401 RI detector. The column used was μ-Bondapak C18 and the specification and operating conditions were as follows: elution solvent, methanol/chloroform/water (70:19:10, v/v/v); sample solvent, chloroform; flow rate, 2 mL/min. The quantification of peaks was done by comparison of peak height. The conversion factors, height to weight, for each fatty acid were previously calculated.

RESULTS AND DISCUSSION

Batch and Continuous Cultivation

In the fermentation system to obtain more microbial lipids, the cultural medium should involve the minimum nitrogen sources only for cell proliferation and excess carbon sources being channeled to lipid biosynthesis. In this study, however, the cultivations in nitrogen-limited and carbon-limited conditions were conducted to compare such cellular responses as biomass and lipid production and alteration of lipid characteristics depending on the growth environments.

The profiles of cell growth and lipid production in nitrogen-limited batch culture are shown in Figure 1. The maximum specific growth rate measured was 0.13 hr^{-1} under the given conditions. In this culture, the lipid accumulation was initiated at 20 hr after the beginning of fermentation and continued to 70 hr. At the end of 70 hr fermentation, the maximum biomass concentration (14 g/L) was reached with 58% lipid content. Lipid production (g lipid/L fermentation broth) also reached its maximum value at 70 hr fermentation time.

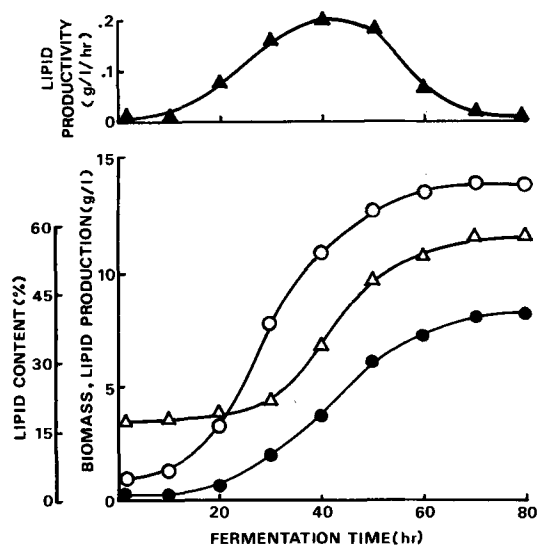

FIG. 1. Relationship between cell biomass, lipid content, lipid production, and lipid productivity and fermentation time of *R. glutinis* grown in nitrogen-limited batch culture: ○—○, cell biomass; △—△, lipid content; ●—●, lipid production; and ▲—▲, lipid productivity.

Lipid productivity (g lipid/L fermentation broth/hr) was computed by graphical differentiation of lipid production curve. The proliferation of cells was curtailed at ca. 40 hr fermentation time due to the depletion of nitrogen sources (not shown in Fig. 1). In spite of the cessation of cell proliferation, the biomass concentration continued to increase. This increase was due to the accumulation of extra lipid synthesized from surplus carbon sources. This accumulation pattern of extra lipid was strongly confirmed by

the profile of lipid productivity which showed the occurrence of the continuous lipid production even after the cessation of cell propagation.

As shown in Figure 2, the biomass concentration, lipid content, lipid production and lipid productivity were significantly lower in carbon-limited culture than in nitrogen-limited culture. The maximum specific growth rate measured was 0.24 hr^{-1} under carbon-limited condition. The maximum lipid productivity in carbon-limited culture was about one third of that in nitrogen-limited culture. Lipid production in carbon-limited culture, in contrast to nitrogen-limited culture, ceased at the late logarithmic phase, that is, there were no surplus carbon sources in the medium which could be utilized for storage lipid biosynthesis.

The continuous cultivations of *R. glutinis* were carried out in the dilution rate range of 0.02–0.1 hr^{-1} in nitrogen-limited conditions and 0.03–0.16 hr^{-1} in carbon-limited conditions. Table I shows the effects of dilution rate (D) on biomass concentration, lipid content, lipid productivity and lipid yield of *R. glutinis* grown in nitrogen- and carbon-limited conditions. In the dilution rates examined, maximum lipid productivity was observed to be 0.077 g lipid/L fermentation broth/hr at D = 0.02 hr^{-1} in nitrogen-limited cultures. The efficiency of conversion from carbon source to lipid, designated by lipid yield, was also shown to be highest at D = 0.02 hr^{-1}. With the increase of dilution rate, biomass concentration and lipid content decreased gradually in nitrogen-limited cultures. However, in carbon-limited cultures, lipid contents did not change significantly depending on the changes of dilution rate, indicating that there were no cellular storage lipids and that the yeast cells contained only structural and functional lipids at almost constant level. Similar results were also reported with *R. glutinis* (14) and with *Candida* 107 (22).

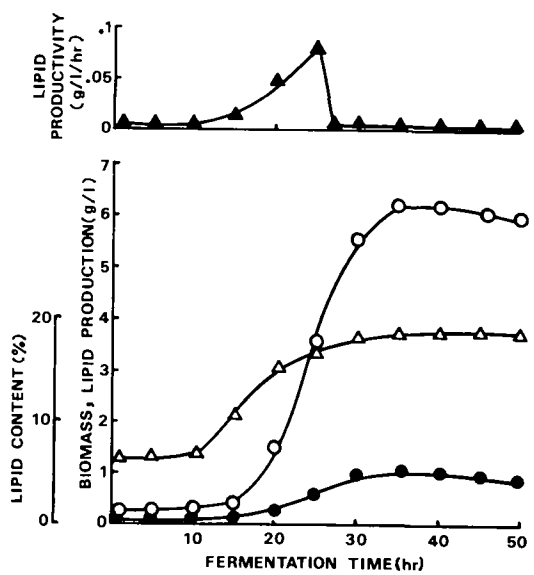

FIG. 2. Relationship between cell biomass, lipid content, lipid production and lipid productivity and fermentation time of *R. glutinis* grown in carbon-limited batch culture: ○—○, cell biomass; △—△, lipid content; ●—●, lipid production; and ▲—▲, lipid productivity.

Relationship between Dilution Rate and Lipid Classes

The lipids obtained from batch and continuous cultivations were fractionated into neutral-, glyco- and phospholipid using silicic acid column chromatography and the results are shown in Table II. The average recovery yield of column chromatography was in the range of 96–103%. Purity of each fractionated lipid was checked by TLC.

In the lipids obtained from nitrogen-limited cultures, the neutral lipid was found to be the predominant one and the proportions of neutral lipid decreased as dilution rate increased. However, the proportion of glycolipid and phospholipid increased with the increase of dilution rate. The proportions of neutral-, glyco- and phospholipid in total lipid of *R. glutinis* grown in nitrogen-limited batch culture after 80 hr fermentation time were 84.1%, 8.3% and 7.6%, respectively.

The proportion of neutral lipid in carbon-limited cultured yeasts also depended on the dilution rate: the amount of neutral lipid increased as the dilution rate increased. On the other hand, the proportions of glyco- and phospholipid showed the reverse trend. The contents of neutral-, glyco- and phospholipid in *R. glutinis* grown in carbon-limited batch culture after 50 hr fermentation time were 65.4%, 28.2% and 6.4%, respectively.

The major lipid class included in microbial storage lipid was known to be neutral lipid, especially triglyceride (23). The maximum lipid accumulation occurred at the lowest dilution rate in nitrogen-limited cultures and the proportion of neutral lipid was the highest at 83.6%. At higher dilution rates in nitrogen-limited cultures, more glyco- and phospholipid were present than at lower dilution rates.

TABLE I

Effect of Dilution Rate on Biomass and Lipid Production of *R. glutinis* Grown in Continuous Cultures

	Dilution rate (hr^{-1})	Biomass (g/L)	Lipid content (%)	Lipid productivity (g/L/hr)	Specific lipid production rate (g lipid/g lipid-free biomass/hr)	Lipid yield (g lipid/ 100 g glucose consumed)
Nitrogen-limited	0.02	6.7	57.2	0.077	0.027	16.4
	0.04	3.9	37.0	0.058	0.023	7.1
	0.06	2.6	16.9	0.026	0.012	2.6
	0.08	1.6	16.2	0.021	0.015	2.5
	0.10	1.4	13.6	0.019	0.016	1.8
Carbon-limited	0.03	4.1	13.3	0.016	0.005	3.7
	0.06	3.4	15.5	0.032	0.011	3.8
	0.10	2.1	11.2	0.024	0.013	2.6
	0.13	1.6	15.0	0.031	0.023	3.6
	0.16	1.1	12.0	0.021	0.022	2.1

TABLE II

Relationship between Dilution Rate and Proportion of Lipid Classes in *R. glutinis*

	Dilution rate (hr^{-1})	Neutral lipid (%, wt)	Glycolipid (%, wt)	Phospholipid (%, wt)
Nitrogen-limited	0.02	83.6	5.7	10.7
	0.04	83.3	5.8	10.9
	0.06	75.9	12.3	11.8
	0.08	70.5	12.2	17.3
	0.10	54.7	21.1	24.2
Carbon-limited	0.03	38.9	34.7	26.4
	0.06	51.1	31.0	17.9
	0.10	51.8	25.0	23.2
	0.13	62.0	23.7	14.3
	0.16	67.8	19.6	12.6

This might indicate that yeast cells in higher dilution rates, i.e., in higher growth rate, needed more structural and functional lipids. However, the increase of neutral lipid proportion in *R. glutinis* grown in carbon-limited conditions with increasing dilution rate seemed to be due to the synthesis of more functional and structural lipids such as sterol and steryl ester which were involved in the pool of neutral lipids (24) (see also TLC section). Similar results were reported by Gill et al. with another oleaginous yeast, *Candida* 107 (22).

TLC of Fractionated Lipid Classes

With the lipid classes obtained from yeast cells grown in nitrogen- and carbon-limited batch cultures (see above), the characterization of each lipid class was performed by TLC. With comparison of R_f values of standard materials, it was found that steryl ester, sterol, free fatty acid, mono-, di- and triglycerides were present in neutral lipid portion of both batch-cultured yeasts. Tocopherol was not detected in neutral lipids of any cultures.

For quantification of the components, the charred TLC plates were applied to the Fiber Optic Scanner (Kontes, Model 800, Vineland, NJ). Emission peak of phosphorus-coated disc of the scanner was 440 nm and the results are given in Table III. The presence of triglyceride was more pronounced in nitrogen-limited culture than in carbon-limited culture. But in the cases of free fatty acid, steryl ester and sterol, the reverse trends were observed. Mono- and diglycerides were also detected only in trace amounts. The compositions of phospholipids obtained from both cultures were similar to each other except phosphatidylcholine; phosphatidylcholine was more pronounced in carbon-limited cultures than in nitrogen-limited culture.

In glycolipid portions obtained from both cultures, 9 or 10 spots were detected. Because of the unavailability of glycolipid standard samples except sterylglycoside, mono- and digalactosyl diglyceride and cerebroside, we could not identify all spots detected. These four glycolipids were detected in both cultures and the sum of them was in the range of 11–15%.

Fatty Acid Compositions of Lipid Classes

The fatty acid compositions of each lipid class are shown in Table IV. In neutral lipids of nitrogen- and carbon-limited cultures, the contents of polyunsaturated fatty acids (linoleic and linolenic acid) increased but mono-unsaturated (oleic acid) and saturated fatty acids (palmitic and stearic acid) decreased as dilution rate increased. The trend of variation in fatty acid composition of glycolipid was almost the same as that of neutral lipid. Under carbon-limited conditions, the content of linoleic acid in phospholipid decreased and oleic acid increased as dilution rate increased. Linolenic acid was detected only in trace amount.

TABLE III

Compositions of Neutral Lipid and Phospholipid of *R. glutinis* Grown in Nitrogen- and Carbon-Limited Batch Cultures

	Nitrogen-limited (%)[a]	Carbon-limited (%)
Neutral lipid		
Triglyceride	79.8	33.9
Diglyceride	tr[b]	tr
Monoglyceride	tr	tr
Free fatty acid	5.4	38.1
Steryl ester	8.6	11.3
Sterol	3.1	12.9
Unknown	2.9	3.5
Phospholipid		
Phosphatidylserine	47.9	39.9
Phosphatidylethanolamine	14.6	12.3
Phosphatidylinositol	12.1	10.7
Lysophosphatidylcholine	12.0	10.4
Phosphatidylcholine	6.9	18.7
Cardiolipin	2.2	4.0
Phosphatidic acid	tr	tr
Unknown	4.2	3.7

[a]Percentage distribution by area, not weight.
[b]Trace amount.

The degree of fatty acid unsaturation for each lipid class was calculated by the method of Kates and Baxter (25) and is shown in Table IV. In nitrogen-limited cultures, the degree of unsaturation of neutral- and glycolipid varied significantly, but phospholipid varied slightly depending on the dilution rates. The similar trends were also observed in carbon-limited cultures.

Choi et al. (15) reported that the fatty acid composition varied depending on the change of dilution rate, whereas Ratledge and Hall (14) reported that the composition did not change at all. Therefore, the effect of cultural conditions on fatty acid composition appears to be strain-specific.

The degree of unsaturation of total lipid was estimated from the results of proportion and degree of unsaturation for each lipid class as shown in Tables II and IV, and illustrated in Figure 3. In general, the degree of unsaturation of carbon-limited cultures was higher than that of nitrogen-limited cultures. The changes in the values depending on

TABLE IV

Effect of Dilution Rate on Fatty Acid Composition and Degree of Unsaturation of Lipid Classes of *R. glutinis* Grown in Continuous Cultures[a]

	D (hr^{-1})	C_{14}			C_{16}			C_{18}			$C_{18:1}$			$C_{18:2}$			$C_{18:3}$			Δ/mole[b]		
		N	G	P	N	G	P	N	G	P	N	G	P	N	G	P	N	G	P	N	G	P
Nitrogen-limited	0.02	9.1	29.0	13.4	24.0	tr[c]	39.7	14.4	16.2	6.0	26.6	30.0	22.9	22.1	10.4	12.2	3.8	14.6	5.8	0.822	0.946	0.647
	0.04	9.8	28.5	20.4	20.6	tr	31.6	13.4	14.8	7.3	22.2	17.4	23.5	29.5	20.9	9.9	4.5	18.4	7.3	0.947	1.144	0.652
	0.06	6.5	30.3	24.9	18.5	tr	29.0	11.7	13.1	4.7	24.7	16.0	20.3	34.2	16.5	14.2	4.4	24.1	6.9	1.063	1.213	0.694
	0.08	12.6	39.2	15.4	14.9	tr	36.2	8.9	6.5	5.0	20.4	12.3	27.8	37.7	20.8	10.3	5.5	21.4	5.3	1.123	1.181	0.643
	0.10	10.2	31.8	20.0	14.3	tr	34.1	8.6	4.3	3.9	20.2	10.7	22.6	39.6	28.2	11.5	7.1	25.0	7.9	1.207	1.421	0.693
Carbon-limited	0.03	tr	22.5	22.7	25.8	tr	33.3	10.6	tr	4.0	28.9	25.0	18.8	30.1	28.3	21.2	4.6	24.2	tr	1.029	1.542	0.612
	0.06	tr	42.1	22.4	21.2	tr	35.8	12.2	tr	8.3	28.7	9.1	21.6	27.0	18.1	11.9	10.8	30.7	tr	1.151	1.374	0.454
	0.10	tr	32.7	19.7	22.7	tr	36.2	12.5	tr	5.9	25.0	9.7	24.8	28.6	21.3	13.4	12.4	36.3	tr	1.194	1.612	0.516
	0.13	tr	15.2	22.3	18.9	tr	40.8	9.2	tr	6.4	20.6	18.2	22.8	37.4	38.0	7.6	13.9	28.6	tr	1.371	1.800	0.380
	0.16	tr	tr	29.8	22.2	tr	27.6	6.5	tr	5.9	19.7	30.0	20.2	37.7	42.8	16.5	13.9	27.2	tr	1.368	1.972	0.532

[a]Some other fatty acids (lauric, palmitoleic and arachidic acid) were also detected in trace amounts and were not included in this table.
[b]Δ/mole designates the degree of unsaturation (25).
[c]Trace amount detected less than 1%.

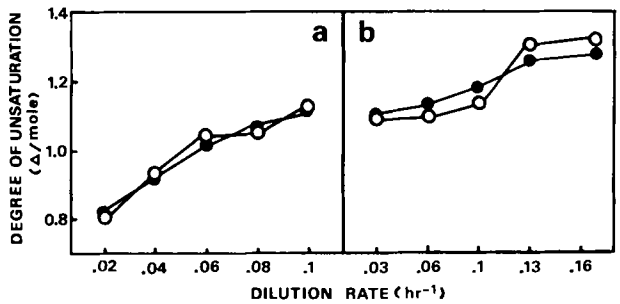

FIG. 3. Effect of dilution rate on the degree of unsaturation of total lipid of *R. glutinis* grown in nitrogen- (a) and carbon-limited continuous culture (b): ○—○, calculated; ●— ●, experimental.

dilution rates were observed more significantly in nitrogen-limited cultures than in carbon-limited cultures. The degrees of unsaturation of total lipids were also obtained by total lipids analyses with HPLC and are also shown in Figure 3. Two values, calculated and experimental, were very close to each other at different dilution rates.

All the works described above show that the productivity and characteristics of lipid in *R. glutinis* can be altered by cultural conditions. The possibility of commercial lipid productions by yeast fermentation is still remote due to the cost of carbon source and lipid extraction. However, if these problems are solved by broadening the substrate spectrum of the organism and weakening the cell wall by the cell fusion method or other possible means, the oleaginous yeasts possess great potential as valuable lipid sources. Furthermore, the defatted yeast residues contain the good quality proteins (26), suggesting that the defatted yeast cells can be used as the single cell protein.

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REFERENCES

1. Woodbine, M., Progress in Industrial Microbiology, Vol. 1, 1959, p. 179.
2. Moon, N.J., and E.G. Hammond, JAOCS 55:683 (1978).
3. Ratledge, C., DECHEMA 83:165 (1979).
4. Ratledge, C., Progress in Industrial Microbiology, Vol. 16, 1982, p. 120.
5. Ratledge, C., in New Sources in Fats and Oils, edited by E.H. Pryde, L. H. Princen and K.D. Mukerjee, AOCS, Champaign, IL, 1981, p. 159.
6. Ackman, R.G., in New Sources in Fats and Oils, edited by E.H. Pryde, L. H. Princen and K.D. Mukerjee, AOCS, Champaign, IL, 1981, p. 189.
7. Enebo, L., L.G. Anderson and H. Lundin, Arch. Biochem. 11: 383 (1946).
8. Pan, S.C., A.A. Andreasen and P. Kolachov, Arch. Biochem. Biophys. 23:419 (1949).
9. Yoon, S.H., J.W. Rhim, S.Y. Choi, D.D.Y. Ryu and J.S. Rhee, J. Ferment. Technol. 60:243 (1982).
10. Enebo, L., and H. Iwamoto, Acta Chem. Scand. 20:439 (1966).
11. Kessel, R.H.J., J. Appl. Bacteriol. 31:220 (1968).
12. Krumphanzl, V., V. Gregr, J. Pelechova and J. Uher, Biotechnol. Bioeng. Symp. No. 4, 245 (1973).
13. Ratledge, C., and M.J. Hall, Appl. Env. Microbiol. 34:230 (1977).
14. Ratledge, C., and M.J. Hall, Biotechnol. Lett. 1:115 (1979).
15. Choi, S.Y., D.D.Y. Ryu and J.S. Rhee, Biotechnol. Bioeng. 24: 1165 (1982).
16. Official Methods of Analysis of the Association of Official Analytical Chemists, 13th edn., AOAC, Washington, DC, 1980, p. 858.
17. Miller, G.L., Anal. Chem. 31:426 (1959).
18. Folch, J., M. Lees and G.H. Sloane-Stanley, J. Biol. Chem. 226:497 (1957).

19. Rouser, G., G. Kritchevsky and A. Yamamoto, in Lipid Chromatography Analysis, Edward Arnold, London, 1967, p. 99.
20. Christie, W.W., in Lipid Analysis, Pergamon, Oxford, 1973, p. 85.
21. Lee, F.A., and L.R. Mattick, J. Food Sci. 26:273 (1961).
22. Gill, C.O., M.J. Hall and C. Ratledge, Appl. Env. Microbiol. 33:231 (1977).
23. Uzuka, Y., T. Kanamori, T. Koga, K. Tanaka and T. Naganuma, J. Gen. Appl. Microbiol. 21:157 (1975).
24. Rattray, J.B.M., A. Schibeci and D.K. Kidby, Bacteriol. Rev. 39:197 (1975).
25. Kates, M., and R.M. Baxter, Can. J. Biochem. Physiol. 40: 1213 (1962).
26. Rhim, J.W., M.S. Thesis, Korea Adv. Inst. Sci., 1980.

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Effect of Selected Antioxidants on the Stability of Virgin Olive Oil

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ABSTRACT

Virgin unrefined olive oil was protected from oxidation with the antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), and tertiary butylhydroquinone (TBHQ) and in one case propyl gallate (PG). All the antioxidants improved the stability of olive oil under accelerated conditions (oven test) and storage conditions at 50 C. In the oven test, where the type of oil used was the same as that used in long-term storage studies (room temperature and 50 C), the relative inhibition effect of the antioxidants was in the following order: TBHQ = BHA > BHT. The combinations of BHA and BHT with TBHQ displayed better stabilizing qualities. Antioxidants did not prevent peroxide formation in olive oil stored at room temperature in daylight; these samples oxidized to a high degree, probably due to the catalytic action of chlorophyll. Citric acid (CA) used alone did not affect the oxidative stability of the oil in the oven test and at room temperature in the dark, but exhibited a negative effect at 50 C. The reduction in peroxide content with teritary butylhydroquinone (TBHQ) in the dark at 50 C was greater than anticipated from the oven studies. Potency of the antioxidants under these conditions (50 C) was in the following order: TBHQ > BHT > BHA. The combinations of BHA 0.01% or BHT 0.01% with TBHQ 0.005% used in the dark at 50 C were less effective than TBHQ 0.01%.

INTRODUCTION

Olive oil extracted from the fruits of the tree *Olea europaea* is one of the very few if not the only plant oil in the world which can be consumed in its natural state without being further treated or refined. The oil obtained from healthy mature olive fruits by mechanical means, without any chemical treatment, is called "virgin". This oil is of the highest quality, and is the type used in this study.

Like other vegetable oils, olive oil undergoes oxidative deterioration as a result of many factors. The autoxidation of olive oil results in development of off-flavors and odors and some of its physical properties may also be altered (1). The prevention of autoxidation in olive oil is of great importance from the standpoint of palatability and economy.

Polyphenols, which are natural inhibitors of oxidation in olive leaves, were found to favor the stability of olive oil (2, 3). Hirahara (4) observed an antioxidant effect when alcohol or ether extract from cloves were added to olive oil. The effect of some synthetic antioxidants on the stability of olive oil has been studied (5, 6).

The comparative effect of the antioxidants butylated hydroxyanisole (BHA), butylated hydroxytoluene (BHT), tertiary butylhydroquinone (TBHQ), and propyl gallate (PG) (Eastman Chemical Company, Kingsport, TN) with respect to retardation of autoxidation of virgin olive oil stored at different conditions, was studied. The effect of citric acid used alone was also investigated. Photooxidation of the oil exposed to daylight at room temperature storage was observed.

EXPERIMENTAL PROCEDURE

The olive oil used in this study was derived from the fruits of the olive tree cultivar *Tsounati*, grown on the island of Crete, and was extracted by hydraulic pressing. Three samples of virgin unrefined olive oil from different harvesting periods and having different initial stability and free fatty acid (FFA) content were obtained. They were numbered as olive oil no. 1, no. 2, and no. 3 and had a peroxide value (PV) of 16, 35 and 12 and FFA content expressed as oleic acid of 0.5%, 2.1% and 1.0%, respectively.

The fatty acid composition of the oil was determined by gas liquid chromatography (GLC) in a Beckman GC-4 gas chromatograph. Boron trifluoride-methanol (Sigma Chemical Company, St. Louis, MO) was used for preparing methyl esters (7). The percentage fatty acid composition of the examined samples varied from 7.6 to 13.6 palmitic, 3.0 to 3.3 stearic, 75.6 to 83.9 oleic, and 5.3 to 7.6 linoleic. Traces of palmitoleic and linolenic acids were detected.

The antioxidants were evaluated under accelerated oxidative conditions (oven test at 65 C and 100 C) and under less stressful conditions (room temperature and 50 C). The oil was heated to 60 C and after the addition of antioxidant was held at room temperature for 24 hr with occasional stirring to ensure complete solution of the antioxidants. Citric acid was added to the oil as a solution in a mixture of ethanol and distilled water 1:1 (v/v).

For the oven test (usually referred to as the Schaal oven test), the oil was placed in petri dishes (5.5" in diameter and 3/4" in height) and kept in a constant temperature oven. Samples were removed periodically for peroxide determination. The oven stability of the oil was taken as the number of days needed for the peroxide content of the

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