Lipid and Fatty Acid Biosynthesis by *Rhodotorula minuta*

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ABSTRACT: Demand for fatty acids is increasing at an annual rate of 17%, due to their increased use in the oleochemical and transport industries. Presently, vegetable oils are the major source of fatty acids, whereas lipids with fatty acids similar to those of some vegetable oils have been reported to be synthesized by oleaginous microorganisms. In the present study, the culturing conditions for the oleaginous yeast *Rhodotorula minuta* IIP-33 have been optimized. In contrast to the lipid accumulation characteristics of most oleaginous yeasts, a carbon-to-nitrogen ratio of 30 was favorable for maximal accumulation of lipids (48%) with 22.5% conversion of glucose as carbon substrate. The lipids contained fatty acids in the C_7–C_{18} range, the relative composition of which varied with culture temperature.

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KEY WORDS: Fatty acids, fed-batch, lipid, oleaginous, *Rhodotorula minuta.*

Natural fatty oils are the major source of fatty acids used as feedstock in the oleochemical industries. With the growing demand, at 17% per annum, for fatty acids in the oleochemical industries and the recent application of fatty acid esters as biodiesel and biodegradable lube oils (1,2), the feasibility of using microorganisms as the alternate source of fats and oils would be an attractive proposition. Biosynthesis of lipids, such as triglycerides, phospholipids and glycolipids, by some oleaginous yeasts has been well documented (3,4). Synthesis of fatty acids in oleaginous yeasts is facilitated by a decrease in the activity of the isocitrate dehydrogenase enzyme under diminished nitrogen levels in the culture (5).

Temperature-induced changes are reported in the fatty acid composition of the accumulated lipids of various oleaginous yeasts (6). Thus, synthesis of lipids with varying fatty acid composition should be feasible by culturing oleaginous microorganism under different conditions.

We report here the growth and lipid accumulation characteristics of a rarely explored yeast strain of *Rhodotorula minuta*. The effect of growth temperature on the regulation of fatty acid synthesis by the strain has also been investigated and reported.

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EXPERIMENTAL PROCEDURES

Yeast strain and culture media. The yeast strain was isolated from mineral oil-contaminated local soil and was identified as *R. minuta*. The strain grows in red-pigmented colonies on YPD agar plates or in YPD aqueous medium within a temperature range of 28–36°C. The strain was preserved on YPD agar slants and stored at 5°C. Preparation of inocula and growth studies of the strain were conducted in a synthetic medium (OP24) that contained the following major constituents (w/w %): D-Glucose, 2; $MgSO_4 \cdot 7H_2O$, 0.07; $(NH_4)_2SO_4$, 0.2; $Na_2HPO_4 \cdot 2H_2O$, 0.07; $KH_2PO_4 \cdot 2H_2O$, 0.12; and $ZnSO_4$, $CuSO_4$, and $FeSO_4$ in traces. Carbon-to-nitrogen (C/N) ratio was increased by adding excess glucose in the medium at the end of the growth phase.

Preparation of inocula. The yeast strain *R. minuta* IIP33 was grown to the exponential growth phase (o.d. 0.8) in 250-mL Erlenmeyer flasks that each contained 50 mL of growth medium (OP24), incubated at a temperature of 32°C and 240 rpm in an environmental shaker from INFORS AG (CH-4130; Bottmingen, Switzerland).

Fermentation conditions. Fermentation studies on growth and lipid accumulation characteristics of the strain were conducted in a 2.5-L bioreactor (INCELTECH LH Series 210 Fermentor, Berkshire, England). The pH was maintained at 4.5 by adding 1 N NaOH, agitation was at 600 rpm, aeration at 1 vvm, and the temperature was varied from 30 to 38°C for different batch experiments. Specific growth rate (μ h^{-1}) of the strain was determined by measuring the change in the natural-log values of the cellular dry weight per unit time while culturing at a preset temperature between 30 and 38°C. The fermentation was carried out in two phases, which included a growth phase of 25 h in batch mode, under excess of nitrogen, and a lipid accumulation phase of 30 h in fed-batch mode. The required quantity of glucose was added in fed-batch mode at 24-h intervals to maintain the initial C/N ratio at 30. The accumulation phase was extended for a further 20 h while maintaining the initial C/N ratio at 40. Periodically, samples of lipid-accumulated cells were drawn, and cellular yield on sugar (Yx/s), lipid yield on biomass (Yp/x) and lipid yield on consumed sugar, known as fat coefficient (Yp/s × 100), were determined. The degree of fatty acid unsaturation (DUS $mole^{-1}$) in the lipid was calculated as [1.0 (% monoene) + 2.0 (% diene) + 3.0 (% triene)]/100 (6).

Analytical methods. Lipids were extracted from cells with the solvent mixture choloroform/methanol (1:3) in a Soxhlet extractor as described by Folch *et al*. (7). Sugar concentration of the broth was estimated by the Anthron method (8), and nitrogen by the Kjeldahl method (9). The methyl esters of the fatty acid samples were prepared by treating the lipid samples with BF_3 in methanol as described by Schmitz and Metcalfe (10), but we replaced petroleum ether with *n*-hexane as solvent for the recovery of methyl esters. Methyl esters of fatty acids were analyzed with a gas chromatograph (Perkin-Elmer Sigma-2000; Norwalk, CT), fitted with a Hewlett-Packard 3396 Series II integrator (Palo Alto, CA) and a packed column (20% OV-275 on Chromosorb 'P' 6′ × 1/8″) with oven programming from 155 (15 min) to 180°C (20 min) at 0.5°C per minute, while keeping injector and flame-ionization detector (FID) at 230 and 260°C, respectively. The relative fatty acid composition was estimated as percentages of the total peak area. Standard samples of fatty acid methyl esters (Aldrich Chemical Company, Inc., Milwaukee, WI) were used for identification of the fatty acids.

RESULTS AND DISCUSSION

Effect of carbon source on growth rate and lipid yield. The specific growth rate of an oleaginous strain on different substrates indicates the affinity of the strain toward the substrate used as carbon source. The specific growth rate and lipid yield of the strain were determined by using carbohydrates, e.g., glucose, fructose, galactose, and lactose. The strain grew at a maximal specific growth rate of 0.34 h^{-1} on glucose. Growth on other substrates was comparatively slow, and no growth was observed on lactose (Table 1). The specific growth rate (0.34 h^{-1}) of *R. minuta* IIP33 on glucose was comparatively higher than that of other oleaginous yeasts, e.g., *R. glutinis* CFR-1 (0.16 h^{-1}) and *Lipomyces starkeyi* (0.12 h^{-1}) as reported by Jacob and Krishnamurthy (11) and Suutari *et al.* (12). However, the specific growth rate of *R. minuta* IIP-33 on glucose was the same as that of *R. glutinis* IIP-30 on glucose (0.34 h^{-1}) but comparatively slower (0.22 h^{-1}) on sucrose compared to that of *R. glutinis* IIP-30 (13). Lipid accumulation of *R. minuta* IIP-33 was maximal on glucose (0.48) at the end of fed-batch fermentation, compared to other carbohydrates, e.g., sucrose (0.36), fructose (0.30) and galactose (0.11), as carbon substrate (Table 1). However, the strain was unable to grow on lactose. Lipid-synthesizing characteristics of the strain on different carbohydrates have indicated that whey would be an unsuitable feedstock for lipid synthesis because lactose was not assimilated by the strain. However, another strain of the same species, namely *R. minuta* NCIM- 3359, has been reported to grow on lactose (14).

TABLE 1
Growth and Lipid Accumulation by *Rhodotorula minuta* IIP-33

Carbon substrate	Sp. growth rate (μ h^{-1})	Lipid yield Y p/x (w/w%)
Glucose	0.34	0.48
Fructose	0.30	0.30
Sucrose	0.22	0.36
Galactose	0.15	0.11

Role of C/N ratio in lipid accumulation. Growth and lipid accumulation profiles of *R. minuta* IIP-33 on glucose in batch and fed-batch fermentation are presented in Figure 1. The yeast strain displayed a growth-associated lipid yield of 0.25 under growth-phase batch fermentation, where the initial C/N ratio of the batch was maintained at 17, and the temperature at 32°C. The lipid yield of the cells increased to 0.48 at the end of fed-batch mode, where the initial C/N was maintained at 30. On further increasing the C/N to 40 in the fed-batch mode, lipid yield was decreased to 0.33. Thus, severe nitrogen limitation is not favorable for lipid accumulation by the strain, which is in agreement with the results observed by Patel *et al*. (14) with *R. minuta* NCIM-3359 on glucose. A similar decrease of lipid accumulation by *Cunninghamella echinulata* CCRC 31840 at higher C/N (40) has been reported by Chen and Chang (15). However, *R. minuta* IIP-33 produced an extracellular compound in the culture broth at C/N 40 with the consumption of sugar.

Function of temperature on lipid yield and fat coefficient. Temperature-induced changes in lipid biosynthesis are significant to know whether the organism is capable of adapting itself to wide ranges of temperature and how it affects its lipid yield and fat coefficient besides its fatty acid composition. Lipid yield and the fat coefficient profile of *R. minuta* IIP-33, cultured at different growth phase temperatures, i.e., 30 to 38°C, are illustrated in Figure 2. A maximal lipid yield of 0.25 and a fat coefficient of 14% in the growth phase were observed at a temperature of 32°C. The overall lipid yield and fat coefficient at the end of the accumulation phase were also maximal, i.e., 0.48 and 20.2%, respectively, at the same temperature. However, a temperature above 34°C was unfavorable for overall lipid biosynthesis, and the lipid yield and fat coefficient decreased to 0.12 and 3%, respectively, at 38°C. At the optimal growth temperature (32°C) of *R. minuta* IIP-33, the fat coefficient (20.2%) was higher than that of 14% for *L. starkeyi* (12) and 11% for *R. minuta* NCIM-3359 (14), observed at their optimal growth temperature.

Effect of temperature on fatty acid composition. Temperature plays an important role in regulation of fatty acid composition of membrane lipids of a microoganism (16). The variation of the fatty acid composition of lipids, accumulated in the growth phase of *R. minuta* cultured at different temperatures (30 to 38°C), is shown in Figure 3. The general fatty acid profile of *R. minuta* grown at different temperatures shows a wide range of fatty acids (C_7 to C_{18}), presumably owing to temperature-sensitive acyl-carrier protein, part of a key enzyme associated with chain elongation of fatty acids. Synthesis of long-chain fatty acids, e.g., C_{16}, C_{18}, $C_{18:1}$, and $C_{18:2}$, was predominant at 30–32°C, i.e., near the optimal growth temperature (Fig. 3A), whereas short-chain acids, e.g., C_7, C_8, C_9, were predominantly synthesized at 38°C (Fig.

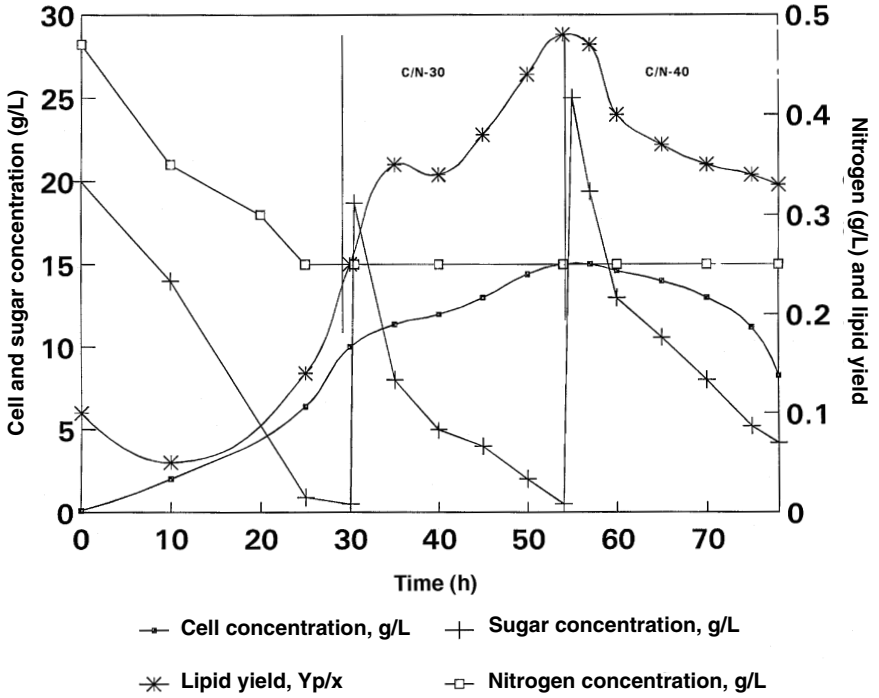

FIG. 1. Growth and lipid accumulation profile of *Rhodotorula minuta* IIP-33 on glucose at different carbon-to-nitrogen (C/N) ratio.

3B). However, a negligible variation in composition of middle-ranged (C_{10}–C_{14}) fatty acids was observed as a function of temperature. The degree of unsaturation (DUS mole^{-1}) was maximal (0.57) at 32°C and decreased to 0.02 at 38°C (Fig. 3B). The decrease in degree of unsaturation is probably due to a decrease in activity of desaturase enzymes at 38°C, which results in an increase of saturated short-chain (C_7–C_9) fatty acids in the lipids. The strain *R. minuta* IIP-33 exhibits bi-

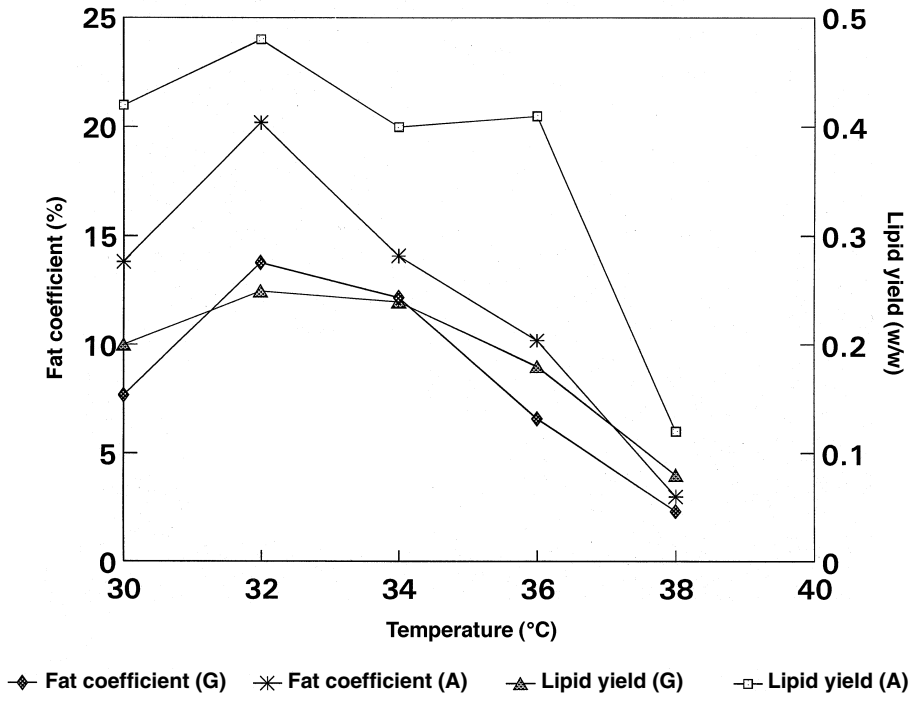

FIG. 2. Effect of temperature on fat coefficient and lipid yield of *R. minuta* in growth (G) and accumulation (A) phases. See Figure 1 for other abbreviation.

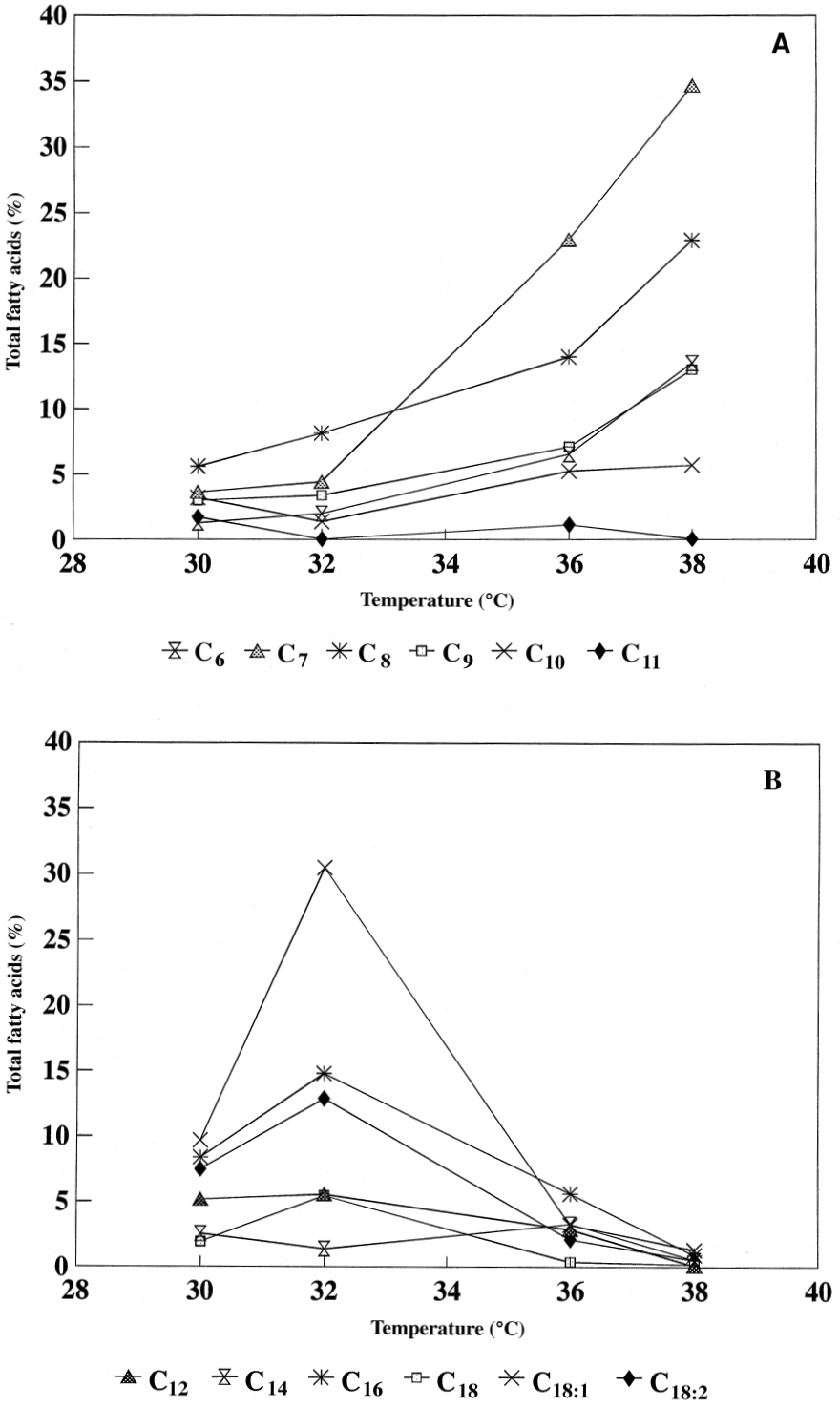

FIG. 3. (A) C_6–C_{11} fatty acid composition of *R. minuta* at different growth temperatures. (B) C_{12}–C_{18} fatty acid composition of *R. minuta* at different growth temperatures. See Figure 1 for abbreviation.

phasic behavior of the temperature-dependent degree of unsaturation, similar to *L. starkeyi,* which showed a maximal degree of unsaturation, 1.2, at 20°C, which decreased to 0.8 at 32°C (6).

Lipid biosynthesis under a comparatively low C/N ratio (30) by *R. minuta* IIP-33 seems to be favorable for using molasses as a cheap carbohydrate source. Biosynthesis of saturated and unsaturated fatty acids, either long-chain (C_{16}–C_{18}) or short-chain (C_8–C_{12}), can be manipulated by regulation of culture temperature.

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