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Fatty Acid and Carotenoid Production by *Sporobolomyces ruberrimus* when Using Technical Glycerol and Ammonium Sulfate

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Abstract The production of carotenoids, lipid content, and fatty acid composition were all studied in a strain of *Sporobolomyces ruberrimus* when using different concentrations of technical glycerol as the carbon source and ammonium sulfate as the nitrogen source. The total lipids represented an average of 13% of the dry weight, and the maximum lipids were obtained when using 65.5 g/l technical glycerol (133.63 mg/g). The optimal conditions for fatty acid production were at 27°C using 20 g of ammonium sulfate and a pH range from 6 to 7, which produced a fatty acid yield of 32.5±1 mg/g, including 1.27±0.15 mg of linolenic acid (LNA), 7.50±0.45 mg of linoleic acid (LLA), 5.50±0.35 mg of palmitic acid (PA), 0.60±0.03 mg of palmitoleic acid (PAL), 1.28±0.11 mg of stearic acid (SA), 9.09±0.22 mg of oleic acid, 2.50±0.10 mg of erucic acid (EA), and 4.25±0.20 mg of lignoceric acid (LCA), where the palmitic, oleic, and linoleic acids combined formed about 37% of the total fatty acids. The concentration of total carotenoids was 2.80 mg/g when using 20 g of ammonium sulfate, and consisted of torularhodin (2.70 mg/g) and β-carotene (0.10 mg/g), at 23°C and pH 6. However, the highest amount with the maximum specific growth rate was obtained (μ_{max}=0.096 h^{-1}) with an ammonium sulfate concentration of 30 g/l.

Keywords: Carotenoids, technical glycerol, *Sporobolomyces ruberrimus*, ammonium sulfate, fatty acid

Lipid production by oleaginous microorganisms has already been studied by many researchers, along with their potential as alternatives to animal fats and plant oils [2, 6, 16]. With a greater understanding of the effects of animal fats and plant oils and their composition on human metabolism and health, researchers have started focusing on the use of certain lipids for disease prevention and treatment, and the promotion of good health [5, 23]. The medical effects of polyunsaturated fatty acids (PUFA), especially those with 20 carbon atoms, have been shown to include preventing blood platelet aggregation [4] and lowering plasma cholesterol [5, 14, 23]. PUFAs are also required for normal cellular functions and play a role in regulating membrane fluidity and as signal molecules [15, 21, 24]. Yeasts contain a variety of lipids, where the lipid content and composition are affected by the growth conditions and/or genetic make-up of the yeast, making yeasts suitable for the production of highly specific lipids [25, 26]. Since industrial yeasts are already significant suppliers of enzymes, flavors, essences, and proteins, there is no reason why they could not also serve as effective lipid sources [21, 23]. For example, certain yeasts belonging to the genus *Rhodotorula* have already been shown to contain notable amounts of linoleic and linolenic acids [10]. Lipid accumulation in cells requires the exhaustion of nitrogen (N), phosphorus (P), zinc (Zn), iron (Fe), or magnesium (Mg) to allow the excess carbon (C) to be changed into lipids. Thus, the best lipid synthesis yields have been obtained under N-limiting conditions [6, 24]. Certain species from various taxonomic group bacteria and yeasts have also been shown to be efficient natural producers of carotenoids [7, 10–12]. Moreover, certain yeasts, such as *Rhodotorula glutinis* strains, have been shown to be able to synthesize carotenoids in addition to lipids during fermentation [7]. Raw materials and by-products of agroindustrial origin have recently been proposed as low-cost alternative carbohydrate sources for microbial metabolite production. [3]. To improve the carotenoid production from *Phaffia rhodozyma*, the use of inexpensive substrates, such as corn wet-milling co-products [8] and peat hydrolysates [16], have also been studied, whereas pure glycerol has been used for astaxanthin production by *Phaffia rhodozyma* [9]. Yet, only a few

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microorganisms are known to be able to grow on technical glycerol, as the salts released from the oil transesterification in diluted technical glycerol exert significant inhibitory effects on most microorganisms. However, technical glycerol is a by-product and can become an important feed stock when biodiesel is applied on a large commercial scale. Accordingly, since fermentation conditions, such as the cultivation temperature and carbon and nitrogen sources, have a significant effect on microorganism metabolites, this study investigated the effect of ammonium sulfate, the initial pH, different concentrations of technical glycerol, and different temperatures on the fatty acid and carotenoid pigment compositions produced by *S. ruberrimus*, a red yeast producing carotenoids, in a batch bioreactor.

MATERIALS AND METHODS

Microorganism and Growth Media

The *Sporobolomyces ruberrimus* (ATCC 66500) used in this study was isolated by Hadi Razavi (L.S.GC. - GP.B.A, France). The first preculture was conducted in a 300-ml baffled Erlenmeyer flask containing 50 ml of a medium composed of (per liter) 10 g of glucose, 5 g peptone, 3 g of yeast extract, and 3 g of malt extract, on a rotary shaker at 210 rpm for 24 h at 23°C. The pH was adjusted to 6 before sterilization. This preculture (10 ml) was then used to inoculate a second preculture phase in a 1-l baffled Erlenmeyer flask containing 200 ml of a medium comprising (per liter) 1.0 g of yeast extract, 0.5 g of peptone, 3 g of $(NH_4)_2SO_4$, 32.5 g of technical glycerol, 2 g of Na_2HPO_4, 1.5 g of $MgSO_4 \cdot 7H_2O$, and 4 g of KH_2PO_4, on a rotary shaker at 210 rpm for 24 h at 23°C with the pH adjusted to 6. The second preculture (150 ml) was then used as the inoculum in the bioreactor. The composition of the technical glycerol was 67% pure glycerol, 2% fatty acids, 3% salts, and 28% water.

To investigate the effect of ammonium sulfate, batch cultures were performed in a 3-l fermentor (Applikon, ADI 1030, Holland) that contained 1.5 l of the following medium (per liter): 3, 10, 20, or 30 g of $(NH_4)_2SO_4$, 67 g of glycerol (from technical glycerol), 0.5 g of peptone, 1 g of yeast extract, 2 g of Na_2HPO_4, 1.5 g of $(MgSO_4 \cdot 7H_20)$, and 4 g of KH_2PO_4. The initial pH was adjusted with a 2 M KOH or H_3PO_4 (42.5%) solution before sterilization (121°C for 20 min). After inoculation, the temperature, pH, and agitation were maintained at 23°C, 6, and 300 rpm, respectively, while the dissolved oxygen partial pressure was maintained at 50% air saturation by controlling the air flow-rate and agitation speed (300–900 rpm). The same culture as above was also used to study the effect of different concentrations of technical glycerol (per liter: 5.21, 10.33, 15.42, 20.20, 25.11, 30.15, 34.30, 40.40, 45.50, 50.80, 55.00, 60.53, 65.10, 70.68, and 75.80 g) on the cellular lipids. Biospumex 153 (Biosoph, Peronne, France) was used as the antifoam agent.

Analytical Methods

The yeast concentration was determined by filtering the samples through a 0.45-μm-pore-size polyamide membrane filter, and then washing the filter twice with physiological water (0.9% NaCl) and drying at 105°C for 12 h. The biomass was also measured based on the optical density using a spectrophotometer (LKB-Biochrom, Cambridge, England) at 600 nm. The glycerol concentration was determined using HPLC on a polypore column H (250 mm ×7 mm) using 0.04 N H_2SO_4 as the eluent at 65°C.

Carotenoid Extraction and Analysis

Five ml of the medium was harvested by centrifugation (4,500 ×*g* for 10 min), and then washed in physiological water (three times) and resuspended in 10 ml of distilled water. Thereafter, the suspension was broken with glass beads (0.4 mm, 10% w/v) for 10 min with cooling (−18°C) in a bead beater (Vibrogen-Zellmuhle, Bioblock, France), the bead-cell mixture harvested by centrifugation (4,500 ×*g* for 10 min), and then after washing and decanting, the yeast cells were resuspended in 5 ml of ethanol and vortexed for 1 min. Finally, the pigment contained in the ethanol was recovered. This procedure was repeated until the cells became colorless. The resulting samples were then filtered through P.T.F.E. membranes and stored at −80°C under a vacuum in the dark. The carotenoids were analyzed according to Razavi *et al.* [22] by high performance liquid chromatography (HPLC) using a Symmetry analytical C18 column (150 mm×4.6 mm, 300 Å) with a 3.5 μm sphere diameter (Waters, U.S.A.). The UV detector was operated at 420–500 nm and the column temperature was maintained at 35°C. The mobile phase was 1 ml/min of an isocratic acetonitrile-methanol-dichloromethane mixture solvent (71:22:7, v/v/v).

Lipid Extraction and Fatty Acid Analysis

The lipid extraction was performed according to the modified method of Aggelis *et al.* [1], and the fatty acid methyl esters were prepared using the method of Nor [18]. The fatty acid methyl esters were then analyzed by gas chromatography on a fused silica capillary column (Carbowax; 30 m×32 mm, 0.25 μm), where the temperature program ranged from 210°C to 240°C at a rate of 10°C/min, the carrier gas was He, and the peaks were identified using fatty acid methyl ester standards.

RESULTS

Fatty Acids Composition of Cellular Lipids of *Sporobolomyces ruberrimus*

Table 1 presents the fatty acid (FA) composition of the total lipids (fatty acid profiles). As shown, oleic acid (C18:1) was the main fatty acid with a very high concentration

Table 1. Fatty acid composition of cellular lipids produced by *Sporobolomyces ruberrimus* cultivated at 23°C and pH 6 using 65 g/l technical glycerol and 10 g/l ammonium sulfate.

Fatty acid chain	Retention time (min)	Fatty acid dry weight (mg/g)	Fatty acid (%)
C16:0	2.84	5.42	9.1
C16:1	3.89	0.66	1.1
C18:0	4.18	1.26	2.1
C18:1	4.41	9.27	15.4
C18:2	4.91	7.70	12.8
C18:3	5.73	1.38	2.3
N.D.[a]	11.21	8.18	1.6
C22:1	13.23	2.48	4.0
C24:0	13.55	4.21	7.0
N.D.[a]	14.92	2.82	4.7
N.D.[a]	15.57	10.53	17.5
N.D.[a]	16.87	6.32	10.5

[a]N.D.: not detected.

(15.4% of the total fatty acid dry weight), along with an appreciable presence (15.4%) of linoleic acid (C18:2), meaning 18-carbon atom fatty acids represented about 30% of the total FAs. In addition, two very long long-chain fatty acids were also present, erucic acid (C22-1) and lignoceric acid (C24:0). The 16-carbon atom fatty acids accounted for about 10% of the total FAs, and the last three long long-chain fatty acids with three different retention times in Table 1 were not detected. The time course of the fatty acid production and production kinetics of four fatty acids (palmitic acid, oleic acid, linoleic acid, and linolenic acid) in the batch fermentor are presented in Fig. 1. Although the concentration levels were different for the production of different fatty acids, similar evolution patterns were observed among their production curves. Oleic acid reached a maximum content of 9.4 mg/g after approximately

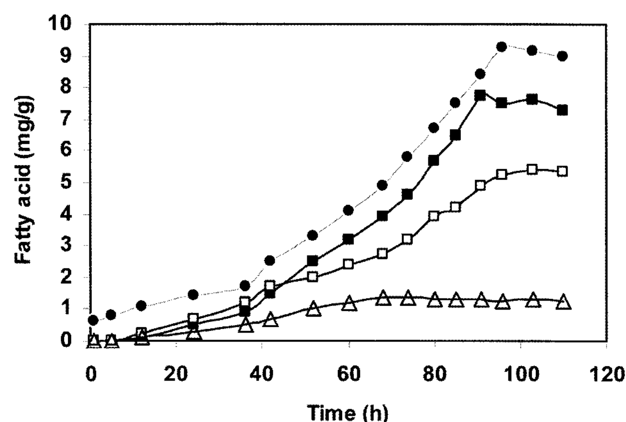

Fig. 1. Time course of fatty acid production by *S. ruberrimus* when using 65.10 g/l technical glycerol and 10 g/l ammonium sulfate at pH 6.0 and 23°C.

Symbols: □, Palmitic acid; ●, Oleic acid; ■, Linoleic acid; △, Linolenic acid.

90 h of fermentation, and the maximum production of fatty acids seemed to occur during the exponential growth phase. The fatty acid production was also somewhat correlated with the amount of yeast growth in each experiment, where the maximum fatty acid production was observed to occur around the end of the exponential growth phase. The minimum fatty acid content was observed for linolenic acid.

Effect of Different Concentrations of Technical Glycerol on Production of Carotenoids and Lipids by *S. ruberrimus* in a 3-l Fermentor

The influence of different concentrations of technical glycerol (5.21, 15.42, 20.20, 30.15, 40.40, 45.50, 50.80, 55.00, 60.53, 65.10, 70.68, and 75.80 g/l) on the production of carotenoids and lipids was studied using *S. ruberrimus*

Table 2. Effect of different concentrations of technical glycerol on production of carotenoids and lipids by *S. ruberrimus* in a 3-l fermentor.

Technical glycerol (g/l)	Biomass (g/l)	Dried material after extraction (Lipid+Pigment)		Total lipids			Total carotenoids			Individual carotenoids	
		(mg/g)	(%)	(mg/g)	(g/l)	(%)	(mg/g)	(mg/l)	(%)	Torularhodin (mg/g)	β-Carotene (mg/g)
5.21	2.80	100.20[a]	10.02	97.12[a]	0.27	9.71	2.88[a,b]	8.06	0.28	2.70[b]	0.18
15.42	8.10	103.70[a]	10.37	100.73[a]	0.81	10.07	2.93[a,b]	23.73	0.29	2.79[b]	0.14
20.20	10.80	104.90[a]	10.49	102.22[a]	1.10	10.22	2.68[b]	28.94	0.26	2.55[a]	0.13
30.15	16.20	117.44[a,b]	11.74	114.76[a,b]	1.85	11.47	2.68[b]	43.44	0.26	2.50[a]	0.18
40.40	21.50	122.97[a,b]	12.29	120.42[a,b]	2.58	12.04	2.55[b]	54.82	0.25	2.41[a]	0.14
45.50	24.00	125.00[a,b]	12.50	122.27[a,b]	2.93	12.22	2.73[a]	65.52	0.27	2.58[a]	0.15
50.80	26.30	128.88[b,c]	12.88	125.90[a,b,c]	3.31	12.59	2.98[a,b]	78.37	0.29	2.84[b]	0.18
55.00	28.60	130.40[b,c]	13.04	127.97[a,b,c]	3.65	12.79	2.43[b]	69.49	0.24	2.30[c]	0.13
60.53	31.00	131.87[b,c]	13.18	129.15[a,b,c]	4.00	12.91	2.72[b]	84.32	0.27	2.55[a]	0.17
65.10	33.50	133.63[b,c]	13.36	130.66[a,b,c]	4.37	13.06	2.97[a,b]	99.49	0.29	2.80[b]	0.17
70.68	33.61	132.78[b,c]	13.27	130.68[a,b,c]	4.39	13.06	2.10[b]	70.56	0.21	1.93[d]	0.17
75.80	33.30	132.00[b,c]	13.20	129.99[a,b,c]	4.32	12.99	2.01[b]	66.93	0.20	1.89[d]	0.12

The pH was controlled at 6.0 using 20 g/l $(NH_4)_2SO_4$ and the temperature maintained at 23°C. The means are in the same column with different letters and significantly different at a 5% level according to Duncan's multiple range test. n=3.

Table 3. Effect of $(NH_4)_2SO_4$ supplement on lipid and fatty acid production by *S. ruberrimus* in a 3-l batch fermentor.

Concentration (g/l)	C/N ratio	Total lipids (mg/g)	Fatty acids (mg/g)								Total (mg/g)
			LNA	LLA	PA	PAL	SA	OA	EA	LCA	
1	65.00	102.45	0.75^{a}	4.45^{a}	2.21^{c}	0.11^{b}	0.57^{d}	4.10^{a}	0.86^{e}	1.07^{b}	14.12^{a}
3	21.66	104.56	1.07^{b}	5.18^{d}	3.08^{b}	0.32^{c}	0.75^{a}	5.03^{c}	1.16^{a}	2.04^{c}	18.63^{e}
5	13.00	105.44	1.45^{c}	6.23^{c}	4.34^{d}	0.39^{c}	1.02^{c}	6.87^{d}	1.98^{b}	2.78^{a}	25.06^{d}
10	6.50	123.55	1.23^{c}	7.10^{f}	5.34^{e}	0.45^{d}	1.06^{c}	9.03^{e}	2.13^{c}	3.56^{e}	29.90^{b}
20	3.25	131.87	1.37^{c}	7.82^{e}	5.87^{e}	0.67^{e}	1.28^{b}	9.02^{e}	2.51^{d}	4.25^{d}	32.79^{c}
30	2.16	112.39	1.02^{b}	6.98^{f}	4.08^{d}	0.49^{d}	1.12^{c}	8.56^{e}	2.03^{b}	3.98^{e}	28.26^{f}

The pH was controlled at 6.0 using 65 g/l technical glycerol and the temperature maintained at 23°C. The means are in the same column with different letters and significantly different at a 5% level according to Duncan's multiple range test. *n*=3. LNA, linolenic acid; LLA, Linoleic acid; PA, Palmitic acid; PAL, Palmitoleic acid; SA, Stearic acid; OA, Oleic acid; EA, Erucic acid; LCA, Lignoceric acid.

in a 3-l fermentor (Table 2). Although glycerol was found to have an important effect on the biomass and lipid content, it did not affect the production of carotenoids. When adding the initial technical glycerol, the biomass and total lipid concentration increased significantly, and the maximum biomass (33.5 g/l) and total lipid (130.66 g/l) were obtained in a concentration of about 65 g/l glycerol. Thereafter, the biomass and total lipid concentration did not increase any further. The fatty acid composition of the cultures with different concentrations of technical glycerol remained the same. The carotenoid biomass increased to a maximum of 99.49 mg/l when increasing the concentration of glycerol, and thereafter decreased significantly. The carbon source was also exhausted after the biomass reached 33 g/l. The glycerol concentration did not affect the proportions of individual carotenoids.

Effect of $(NH_4)_2SO_4$ on Lipid and Fatty Acid Production by *S. ruberrimus* in a 3-l Batch Fermentor

The effect of different concentrations of $(NH_4)_2SO_4$ on the total lipids and fatty acids is presented in Table 3. The production of individual fatty acids was found to depend on the nitrogen concentration. Thus, the concentration of the nitrogen source $(NH_4)_2SO_4$ in the substrate was very important as regards the level of lipid production, especially for individual fatty acids.

Effect of Different Initial pHs on Fatty Acid Production by *S. ruberrimus* in a 3-l Batch Fermentor

Media cultures were run at pH 3.5, 4, 4.4, 5, 5.5, 6, 6.6, 7, 7.6, and 8.2, at 23°C. The optimum initial pH was identified as between 6 and 7 for the production and yield of fatty acids, as the total fatty acids decreased when the initial pH was higher than 7.6 and lower than 5.5 (Table 4). The maximum content of fatty acids was 32.54 mg/g, including 1.38 mg of LNA, 7.70 mg of LLA, 5.42 mg of PA, 0.66 mg of PAL, 1.31 mg of SA, 9.25 mg of OA, 2.51 mg of EA, and 4.31 mg of LCA. The minimum amount and yield of fatty acids were obtained at pH 3.5 (8.41 mg/g and 6.81 g/l). The pH level also had an affect on the proportion of fatty acids (LNA, LLA, PA, PAL, SA, OA, EA, and LCA), which decreased and reached a minimum when the pH was 3.5, and increased (maximum) when the pH was 6. The final pH for all the fermented cultures was between 3.2–4.

Table 4. Effect of different initial pHs on fatty acid production by *S. ruberrimus* in a 3-l batch fermentor.

Initial pH	Biomass (g/l)	Final pH	Fatty acids (mg/g)								Total (mg/g)	LCC
			LNA	LLA	PA	PAL	SA	OA	EA	LCA		
3.5	6.81	3.21	0.42^{a}	1.65^{c}	1.13^{d}	0.03^{c}	0.11^{a}	3.20^{b}	0.22^{d}	1.65^{a}	8.41^{c}	2.59
4	8.12	3.34	0.85^{b}	3.65^{b}	2.23^{a}	0.23^{b}	0.17^{a}	3.75^{a}	0.54^{a}	1.98^{b}	13.40^{b}	0.49
4.4	9.20	3.32	1.10^{c}	3.90^{b}	2.85^{d}	0.31^{b}	0.23^{b}	4.08^{a}	0.87^{c}	2.08^{b}	15.42^{e}	0.64
5	9.19	3.45	$1.26^{d,e,c}$	4.20^{c}	3.20^{d}	0.43^{a}	0.53^{c}	5.98^{c}	1.84^{d}	2.97^{c}	20.41^{f}	0.85
5.5	10.5	3.63	$1.32^{d,e}$	5.23^{d}	3.87^{c}	0.52^{d}	0.98^{d}	6.83^{d}	2.09^{d}	3.67^{d}	24.51^{a}	1.16
6	11.3	3.81	$1.38^{d,e}$	7.70^{e}	5.42^{f}	0.66^{e}	1.31^{e}	9.25^{e}	2.51^{e}	4.31^{e}	32.54^{d}	1.66
6.6	11.0	3.87	$1.41^{d,e}$	7.10^{e}	5.12^{f}	0.5^{d}	1.38^{e}	9.11^{e}	2.48^{e}	4.28^{e}	31.39^{d}	1.57
7	10.8	3.85	$1.31^{d,e,c}$	7.12^{e}	4.89^{f}	0.34^{b}	1.19^{f}	8.09^{f}	2.09^{d}	3.08^{c}	28.11^{g}	1.38
7.6	10.2	3.93	1.04^{c}	6.01^{f}	4.09^{e}	0.41^{a}	$1.08^{f,d}$	7.86^{f}	1.08^{c}	2.07^{b}	23.64^{a}	1.10
8.2	9.70	3.98	0.32^{a}	4.08^{c}	2.08^{a}	0.12^{f}	0.43^{c}	5.98^{c}	0.92^{c}	1.08^{a}	15.01^{e}	0.66

The temperature was maintained at 23°C. The means are in the same column with different letters and significantly different at a 5% level according to Duncan's multiple range test. *n*=3.

Same abbreviations as described in Table3.

LCC: lipid conversion coefficient.

Table 5. Effect of different temperatures on fatty acid production by *S. ruberrimus* in a 3-l batch fermentor.

Temperature (°C)	Biomass (g/l)	Fermentation time (h)	Fatty acids (mg/g)								Total (mg/g)
			LNA	LLA	PA	PAL	SA	OA	EA	LCA	
19	34.42	97	0.35[a]	1.45[a]	2.16[c]	0.24[b]	1.01[a]	3.27[c]	1.43[d]	2.65[a]	12.65[d]
23	34.02	58	1.36[b]	7.04[c]	5.86[a]	0.71[c]	1.38[d]	9.21[a]	2.49[c]	4.42[b]	32.47[a]
27	33.89	44	1.27[b]	7.98[c]	5.25[a]	0.68[c]	1.30[d]	9.19[a]	2.65[c]	4.74[b]	33.06[a]
31	25.93	42	0.26[a]	1.27[a]	2.20[c]	0.11[a]	0.87[b]	2.94[d]	1.01[a]	1.98[d]	10.64[b]

The pH was controlled at 6.0 using 65 g/l technical glycerol. The means are in the same column with different letters and significantly different at a 5% level according to Duncan's multiple range test. n=3. Same abbreviations are used as described in Table 3.

Effect of Different Temperatures on Fatty Acid Production by *S. ruberrimus* in a 3-l Batch Fermentor

The influence of temperature on the biomass, lipid content, and proportion of fatty acids was studied at 19°C, 23°C, 27°C, and 31°C (Table 5). The fatty acids decreased with a decrease of temperature, whereas the proportion of fatty acids decreased when the temperature was lowered from 23°C to 19°C, and then increased when the temperature was increased from 27°C to 31°C. The growth rate decreased with a decrease of temperature, where the time taken to achieve a biomass of 33.89 g/l increased from 44 h at 27°C to 97 h at 19°C. The maximum content of total fatty acids (about 33 mg/g) was obtained between 23–27°C, and when the temperature was increased, a corresponding increase was noted for all fatty acids (LNA, LLA, PA, PAL, SA, OA, EA, and LCA). The content of OA was at a maximum between 23–27°C (about 9.20 mg/g), whereas the LNA and SA contents were at a minimum (about 1.30 mg/g).

Effect of Initial Ammonium Sulfate Concentration on Carotenoid Production

Under controlled pH conditions (set at 6.0), the growth and pigment production by *S. ruberrimus* were investigated with different initial concentrations of ammonium sulfate. When increasing the initial ammonium sulfate concentration, the period for complete glycerol consumption was significantly shortened. Fig. 2 shows the varying growth, glycerol consumption, and carotenoid production by the strain. For all runs, the glycerol consumption curve showed a uniform reduction in the substrate concentration. The maximum carotenoid production was also reached when the maximum growth was obtained. Nonetheless, although the production kinetics for each carotenoid (torularhodin and β-carotene) showed a similar evolution, different concentrations were produced when the initial ammonium sulfate concentration was varied. The torularhodin accumulation reached a maximum concentration of 41.4, 90.5, and 69 mg/l when 3, 20, and 30 g/l of initial ammonium sulfate was employed, respectively. When 30 g/l of ammonium sulfate was used in the culture medium, this significantly increased the specific growth rate and shortened the run time (about 46 h) taken for complete glycerol uptake. The μ_{max} was also high, reaching 0.096 h^{-1} after approximately 12 h when the pigments began to accumulate. The maximum specific growth rate was obtained (0.072 h^{-1}) when using 20 g/l ammonium sulfate, and the highest carotenoid concentration was obtained on the third day of cultivation (Table 2). When using 3 g/l of ammonium sulfate, the biomass production slowly increased, yet the time taken to reach a full glycerol uptake was unexpectedly extended (172 h). In this case, the μ_{max} decreased to 0.058 h^{-1} when the nitrogen concentration was approximately exhausted,

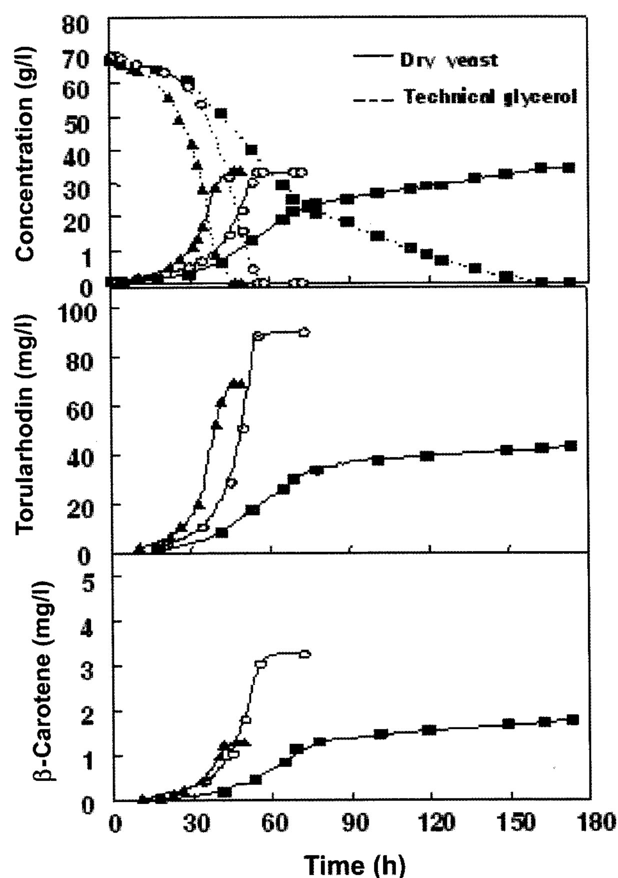

Fig. 2. Growth and production of torularhodin and β-carotene (mg/l) by *S. ruberrimus* with different initial ammonium sulfate concentrations, pH controlled at 6.0, and temperature maintained at 23°C.

Symbols: ■ 3 g/l, ○ 2 g/l, ▲ 30 g/l.

the growth rate decreased slightly, and growth became very slow.

DISCUSSION

When investigating the effects of technical glycerol and ammonium sulfate on fatty acid and carotenoid synthesis, the present study found that whereas technical glycerol and ammonium sulfate had a small effect on the proportion of fatty acids, the lipid content increased when increasing the technical glycerol concentration and ammonium sulfate. The lipid increase seemed to be due to the presence of different fatty acids (approximately 2%) in technical glycerol when compared with pure glycerol. The increased lipid yield also seemed to be coupled to an increased proportion of carbon converted into lipids. Ghanem *et al.* [6] reported the same results when using ammonium sulfate for the production of lipids by *Rhodotorula glutinis*, and also found that the utilization of nitrogen in the form of NH_4 radicals stimulated the accumulation the most. In the present study, the maximal cellular lipid content was achieved with $(NH_4)_2SO_4$, and the lipid contents produced by *S. ruberrimus* were markedly affected by the nitrogen level (ammonium sulfate) in the medium, where the productivity was decreased with an increased nitrogen level in the medium. Similar metabolic activities have also been previously reported in different molds and yeasts [6, 13], and certain yeasts can accumulate large amounts of lipids when grown with an excess of carbon and a deficiency of nitrogen [7]. In the present study, the growth rate decreased with a decrease in temperature, where the time taken to achieve a biomass of above 34 g/l increased from 42 h at 31°C to 97 h at 19°C, plus the lipid content also decreased when decreasing the temperature. The general trend at 27°C and 19°C was that the proportion of fatty acids was unaffected by the culture time. Environmental parameters, such as the pH, temperature, and good aeration, are known to support the growth and yield of fatty acids, as well as carotenoids, plus the biosynthesis of unsaturated fatty acids requires O_2, depending on the enzyme desaturase [20]. However, in the present study, when the aeration was changed from 20% to 50% air saturation by controlling the air flow-rate, this resulted in a high growth rate, yet no difference of unsaturation in the fatty acid composition.

After the nitrogen source was exhausted, it seemed that the yeast continued to grow slowly with the aid of cell metabolites either from dead cells or an intracellular pool containing nitrogen formed throughout the process. As shown above, the carotenoid content in the yeast reached a maximum when the cell growth had practically ended, except when the content of ammonium sulfate was 3 g/l. A high concentration of ammonium sulfate (30 g/l) in the culture medium was also observed to have a possible inhibitory effect on the red pigment content and thus on the carotenoid metabolism. As such, the inhibitory effect of the nitrogen substrate seemed to agree with previous results, where *P. rhodozyma* was cultivated in the presence of alfalfa residual juice (ARJ) as the nitrogen source [9], and an ARJ concentration above 1.25% (v/v) had an inhibitory effect on the pigmentation of the yeast. Similarly, Meyer and du Preez [17] also reported that an excess of ammonium decreased the intracellular astaxanthin content in *P. rhodozyma*. Therefore, the ammonium sulfate concentration appeared to control the growth rate and intracellular carotenoid concentration.

The present study also found a correlation between the biomass accumulation and the glycerol content, where yield remained equal to 0.52±0.01 g/g with a correlation value of 0.98, demonstrating that technical glycerol had an effect as a carbon source on the growth, and lipid and carotenoid production of *S. ruberrimus*. These observations also agreed with the results of Kusdiyantini *et al.* [13], where the use of glycerol increased microbial biosynthetic activity for the production of carotenoids by *P. rhodozyma*. Consequently, the present results demonstrated that *S. ruberrimus* is capable of high lipid and carotenoid production when using an inexpensive culture containing technical glycerol and ammonium sulfate.

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