

# **Production of single cell oils by the cold-adapted oleaginous yeast *Rhodotorula glacialis* AS 4.7: effects of the growth temperature and the C:N ratio**

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*Rhodotorula glacialis* AS 4.7 is an oleaginous psychrophilic yeast which was isolated from glacial environments. Despite its origin, the strain abundantly grew and accumulated lipids up to 20°C. The growth temperature did not influence the yield coefficients of both biomass and lipids production, but had significant effects on the growth rate and thus on volumetric productivity of lipid. 15°C were identified as the optimum temperature for lipid production. As the growth temperature decreased, the abundance of C18 fatty acids (FA) increased at the expenses of C16 FA and the unsaturation degree increased as well. In particular, remarkable amounts of linolenic acid (C18:3  $\omega$ -3) were produced at -3°C, accounting for 29% of FA. Lipid production by *R. glacialis* AS 4.7 especially occurred in carbon rich media, through a two-stages process. The first stage resulted in multiplication of cells and finished with the exhaustion of a nutrient other than the carbon source. During the second stage, the excess glucose was converted into intracellular storage lipids. The extent of the carbon excess had major positive effects on lipid production. The lipid content of biomass, glucose conversion into lipids, lipid concentration, and lipids productivity were all maximum with 120 g L<sup>-1</sup> glucose (68%, 16%, 19 g L<sup>-1</sup>, and 0.054 g L<sup>-1</sup> h<sup>-1</sup>, respectively). The results herein reported suggest that *R. glacialis* AS 4.7 could be considered as an interesting microorganism for the production of single cell oils and represent the first proposed biotechnological application for this yeast species.

## **1. Introduction**

Oleaginous microorganisms, such as yeasts, fungi, and microalgae, can accumulate high amounts of neutral storage lipids under appropriate cultivation conditions and their potential as sources of triacylglycerols (TAGs) has been investigated (Daum et al. 2007; Czapany et al. 2007). Interest in microbial TAGs focuses on those containing high amounts of the essential poly-unsaturated fatty acids of  $\omega$ -3 and  $\omega$ -6 series with nutritional importance (e.g. linoleic,  $\alpha$ -linolenic, and  $\gamma$ -linolenic, arachidonic, eicosapentaenoic, docosapentaenoic and docosohexaenoic acids) (Ratledge and Wynn 2002). Moreover, the utilization of microbial TAGs has been increasingly explored during the past decade in the biofuels industry (Antoni et al. 2007). Microbial lipids can

be successfully used to produce biodiesel by catalyzed transesterification with short chain alcohols, although they are not exploited industrially until now. Plant oils are the major feedstock for biodiesel production but encounter limitations regarding their availability at competitive price. These limitations decrease the attractiveness of biodiesel as a competitive alternative to petroleum-based fuel, but the microbial production of lipids promises to overcome them. In fact, the exploitation of microbial systems to produce lipids from cheap carbon sources has many advantages (short life cycle, low affection by venue, season and climate, easy to scale-up) and might be used for biodiesel production in the future (Li et al 2008).

Many yeast species were found to be oleaginous and accumulated triacyl-glycerol from 20 to 70% of biomass under appropriate cultivation conditions. They include *Cryptococcus albidus*, *Lipomyces lipofera*, *Lipomyces starkeyi*, *Rhodospiridium toruloides*, *Rhodotorula glutinis*, *Trichosporon pullulan*, and *Yarrowia lipolytica* (Ratledge and Wynn 2002, Daum et al. 2007). In this study we analyzed lipid accumulation of the cold adapted yeast *Rhodotorula glacialis* AS 4.7 grown on glucose as carbon source, at different temperatures ranging between -3 and 20°C, and with diverse ratios of carbon vs. nitrogen sources.

## **2. Materials and methods**

### **2.1 Strain and culture conditions**

*Rhodotorula glacialis* AS 4.7 was kindly provided by Prof P. Buzzini (University of Perugia, Italy). The strain was aerobically cultured in GMY medium, which contained 8 g L<sup>-1</sup> KH<sub>2</sub>PO<sub>4</sub>, 0.5 g L<sup>-1</sup> MgSO<sub>4</sub> · 7H<sub>2</sub>O, and 3 g L<sup>-1</sup> yeast extract (Difco Laboratories, Sparks, MD, USA), final pH 5.5 (Buzzini, 2001). Glucose was autoclaved separately from the other components (121°C for 15 min) and supplied at 40 g L<sup>-1</sup> unless otherwise stated.

### **2.2 Growth experiments and bioreactor operation**

Different glucose concentrations and different temperatures were tried out in bioreactor batch cultures using a Labfors apparatus (Infors AG, Bottmingen, Switzerland) with 2 L working volume. *R. glacialis* AS 4.7 was cultured at 10°C in GMY medium containing 1.6, 4, 8, 16, 40, 80, 120, and 160 g L<sup>-1</sup> glucose, or at -3, 0, 5, 10, 15, and 20°C with 40 g L<sup>-1</sup> glucose. At -3°C, 30 g L<sup>-1</sup> NaCl were used to prevent freezing. In any case, the bioreactor was inoculated (10% v/v) with a 48-h seed culture grown at the target temperature on 4 g L<sup>-1</sup> glucose. The culture was stirred at 400 rpm and sparged with 0.5 v/v/min filter-sterilized air. Samples were collected periodically to monitor the growth and to analyze glucose and lipids.

The cells were counted in a Bürker chamber and biomass dry weight was determined gravimetrically using pre-weighed cellulose nitrate membrane filters. Glucose was analyzed by HPLC with a refractive index detector (HPLC System, 1200 Series, Agilent Technologies, Santa Clara, CA). The analysis was performed with an Aminex HPX-87H ion exclusion column and 0.005 M H<sub>2</sub>SO<sub>4</sub> (0.6 ml min<sup>-1</sup>) as the mobile phase.

### **2.3 Lipid analysis**

Biomass from 50 ml culture samples was harvested, washed with distilled water, frozen at -80°C and lyophilized. Lipids were extracted from 1 g of lyophilized biomass

extracted with 50 ml chloroform:methanol mixture (Rossi et al. 2009). Solvents were removed and lipids were determined gravimetrically. The lipids were subjected to methanolysis and the fatty acyl methyl esters were analyzed by GC-MS (Morrison et al. 1964; Rossi et al. 2009). Quadrupole GC-MS system (HP5890 Series II gas chromatograph - HP5972 mass selective detector) equipped with HP-5 capillary column (Agilent Technologies) and EI ionisation detector (70 eV) was used. The injection temperature was 280°C and oven temperature was programmed from 80°C (1 min isotherm) to 130°C at a rate of 50°C min<sup>-1</sup>, then to 280°C at a rate of 5°C min<sup>-1</sup> (20 min isotherm at 280°C). The unsaturation index (UI) was calculated as the number of the double bonds of fatty acids multiplied by their relative amount.

#### 2.4 Statistical analysis

All values are means of three separate experiments. Differences in means among the growth temperatures were analyzed using two-way ANOVA with repeated measures with the group as the first factor and temperature as the second factor, followed by Bonferroni *post hoc* comparisons. Differences were considered statistically significant for  $P \leq 0.05$ .

### 3. Results

#### 3.1 Growth kinetics and lipid composition at different temperatures

*R. glacialis* AS 4.7 was cultured batchwise at different temperatures with 40 g L<sup>-1</sup> glucose and biomass was analyzed when the carbon source was exhausted. The strain abundantly grew at all the temperatures in the range between -3 and 20°C (table 1), whereas the growth was hampered at 25°C. The temperature did not influence both the final biomass and lipids concentration. Therefore, biomass/substrate ( $Y_{X/S}$ ), lipid/biomass ( $Y_{L/X}$ ), and lipid/glucose ( $Y_{L/S}$ ) yield coefficients were not affected by temperature. On the average, 14 g L<sup>-1</sup> dry biomass and 5.0 g L<sup>-1</sup> lipids were obtained and the mean values of  $Y_{X/S}$ ,  $Y_{L/X}$ , and  $Y_{L/S}$  were 0.35, 0.34, and 0.12 g g<sup>-1</sup>, respectively. The specific growth rate was markedly affected by the temperature and was highest (0.075 h<sup>-1</sup>) at 15°C (tab. 1). Therefore, volumetric productivities of both biomass ( $Q_X$ ) and lipids ( $Q_L$ ) depended by the temperature and were the maximum at 15°C (0.101 and 0.034 g L<sup>-1</sup> h<sup>-1</sup>, respectively).

Fatty acid (FA) composition revealed that saturated and unsaturated FA with chain length ranging from 14 to 18 carbons always accounted for more than 99%, independently from the growth temperatures. The relative amount of unsaturated FA other than palmitoleic (C16:1 Δ9), oleic (C18:1 Δ9), linoleic (C18:2 Δ9,12), and α-linolenic (C18:3 Δ9,12,15) was negligible. The C18 FA (C18 + C18:1 + C18:2 + C18:3) were the most abundant, but they decreased as the growth temperature increased, from 80.6% at -3°C to 67.5% at 20°C (tab. 1). The C16 FA (C16 + C16:1) were less abundant than C18 FA, but increased as the growth temperature increased (from 15.9% at -3°C to 27.4% at 20°C). The highest concentration of myristic acid (C14) was observed at 15°C (6.9%). The UI progressively increased from 0.81 to 1.49 as the growth temperature decreased from 20 to -3°C, mostly due to α-linolenic acid, which was absent at 20°C and accounted for 29% at -3°C. Unlike α-linolenic acid, the other

unsaturated FA (C16:1, C18:1, and C18:2, accounting on average for 1.5, 41.2, and 17.3%, respectively) did not exhibit any significant correlation with temperature.

TABLE 1. Specific growth rate ( $\mu$ ), volumetric productivities ( $Q_X$  and  $Q_L$ ), and relative composition and Unsaturation Index (UI) of fatty acids of *R. glacialis* AS 4.7 grown at different temperatures with 40 g L<sup>-1</sup> glucose.

T (°C)	$\mu$ (h <sup>-1</sup> )	$Q_X$ (g L <sup>-1</sup> h <sup>-1</sup> )	$Q_L$ (g L <sup>-1</sup> h <sup>-1</sup> )	Total C16 (%)	Total C18 (%)	UI
-3	0.010	0.015	0.005 <sup>b</sup>	15.9 <sup>a</sup>	80.6 <sup>a</sup>	1.49
0	0.019	0.023	0.007 <sup>b</sup>	16.7 <sup>a</sup>	80.3 <sup>a</sup>	1.15
5	0.053 <sup>b</sup>	0.050	0.016	22.8 <sup>b</sup>	72.5 <sup>b</sup>	0.99
10	0.071 <sup>a</sup>	0.077 <sup>a</sup>	0.026 <sup>a</sup>	22.7 <sup>b</sup>	72.6 <sup>b</sup>	0.93 <sup>a</sup>
15	0.075 <sup>a</sup>	0.101	0.034	27.0 <sup>c</sup>	66.1 <sup>c</sup>	0.87 <sup>ab</sup>
20	0.053 <sup>b</sup>	0.075 <sup>a</sup>	0.023 <sup>a</sup>	27.4 <sup>c</sup>	67.5 <sup>c</sup>	0.81 <sup>b</sup>

Within a column, superscripts indicate statistically similar means,  $P > 0.05$ ,  $n = 3$ .

### 3.2 Growth and lipid production at different C:N ratios

*R. glacialis* AS 4.7 was cultured at 10°C with the following initial glucose concentrations: 1.6, 4, 8, 16, 40, 80, 120, and 160 g L<sup>-1</sup>, corresponding to C:N ratios of 5.6, 8.5, 13, 23, 52, 101, 149, and 198, respectively. For initial glucose ranging from 1.6 to 40 g L<sup>-1</sup>, exponential growth occurred with the same specific rate (0.071 h<sup>-1</sup>), which decreased with higher initial glucose concentration (tab. 2).

Growth and lipid production by *Rhodotorula glacialis* AS 4.7 occurred through two stages. The first one resulted in multiplication of cells and finished with the exhaustion of a nutrient other than the carbon source. During the second one, the excess glucose was converted into intracellular lipids. If initial glucose ranged between 1.6 and 16 g L<sup>-1</sup>, the entrance into stationary phase corresponded to glucose exhaustion, and lipid accumulation was scarce. In this range, stationary-phase cell counts progressively increased from 1.2e+08 to 4.7e+08 ml<sup>-1</sup>, even though a direct relationship was not observed. Above 16 g L<sup>-1</sup> glucose, cell counts did not further increase beyond 8.3e+08 ml<sup>-1</sup>, and lipid accumulation was observed during the second stage.

At the entrance into the second stage, approximately 16 g L<sup>-1</sup> glucose were consumed by cultures that were not carbon limited. Then, residual glucose continued to be consumed even if cell counts did not increase. Glucose consumption was nearly linear and occurred at the rate of 0.34 g L<sup>-1</sup> h<sup>-1</sup>, without any relationship with initial glucose concentration. The carbon source was exhausted for initial concentration up to 120 g L<sup>-1</sup>; only with the initial concentration of 160 g L<sup>-1</sup>, 18 g L<sup>-1</sup> were not utilized. Along with glucose consumption, biomass dry weight continued to increase during the second stage, due to the accumulation of increasing amounts of intracellular lipids. Likewise glucose consumption, the rate of lipid production was linear and occurred at the rate of 0.080 g L<sup>-1</sup> h<sup>-1</sup>.

Diversely from cell counts, the final concentration of biomass and intracellular lipids increased due to increasing amounts of initial glucose (tab. 2). In particular, intracellular lipids accounted for a progressively greater portion of biomass and the overall conversion of glucose into lipids increased as well. With 120 and 160 g L<sup>-1</sup> glucose, the

highest lipid production was obtained in terms of lipid concentration (19 and 22 g L<sup>-1</sup>, respectively), lipid content of biomass (68%), lipid/glucose yield coefficient (16%), and productivity (0.054 g L<sup>-1</sup> h<sup>-1</sup>).

TABLE 2. Yield coefficients, volumetric productivities, and final concentration of cells, biomass, and lipids in batch cultures of *R. glacialis* AS 4.7 with different initial glucose concentration.

Glucose (g L <sup>-1</sup> )	Cells (ml <sup>-1</sup> )	Biomass (g L <sup>-1</sup> )	Lipids (g L <sup>-1</sup> )	Y <sub>L/S</sub> (g g <sup>-1</sup> )	Y <sub>L/X</sub> (g g <sup>-1</sup> )	Y <sub>X/S</sub> (g g <sup>-1</sup> )	Q <sub>X</sub> (g L <sup>-1</sup> h <sup>-1</sup> )	Q <sub>L</sub> (g L <sup>-1</sup> h <sup>-1</sup> )
1.6	1.2e+08	2.1	0.14 <sup>a</sup>	0.09 <sup>a</sup>	0.07 <sup>a</sup>	1.31	0.045	0.0030 <sup>a</sup>
4	3.5e+08 <sup>a</sup>	3.8	0.19 <sup>a</sup>	0.05 <sup>a</sup>	0.05 <sup>a</sup>	0.94	0.052 <sup>a</sup>	0.0026 <sup>a</sup>
8	4.0e+08 <sup>ab</sup>	4.9	0.42	0.05 <sup>a</sup>	0.09 <sup>a</sup>	0.61	0.052 <sup>a</sup>	0.0044
16	4.7e+08 <sup>b</sup>	7.0	1.36	0.09	0.20	0.44	0.056	0.011
40	6.9e+08	14	4.74	0.12	0.34	0.35	0.075 <sup>b</sup>	0.025
80	8.1e+08 <sup>c</sup>	22	12	0.15 <sup>b</sup>	0.55	0.28	0.076 <sup>b</sup>	0.042
120	8.0e+08 <sup>c</sup>	28	19	0.16 <sup>b</sup>	0.68 <sup>b</sup>	0.23 <sup>a</sup>	0.079 <sup>c</sup>	0.054 <sup>b</sup>
160	8.2e+08 <sup>c</sup>	33	22	0.16 <sup>b</sup>	0.68 <sup>b</sup>	0.23 <sup>a</sup>	0.080 <sup>c</sup>	0.054 <sup>b</sup>

Within a column, superscripts indicate statistically similar means, P > 0.05, n = 3.

#### 4. Discussion

This study explored the production of intracellular lipids by the psychrophilic oleaginous yeast *Rhodotorula glacialis* AS 4.7 as a function of the growth temperature and the C:N ratio of the medium, with the perspective to determine whether this strain could be exploited for biotechnological applications. The strain is oleaginous and accumulates high amounts of lipids within lipid cultures when it is cultured in a medium with high C:N ratio. Even if *R. glacialis* AS 4.7 was isolated from alpine glacial environment, it grew abundantly and accumulated lipids up to 20°C. Similarly to other psychrophilic oleaginous yeasts (Rossi et al. 2009), the growth temperature did not influence the yield coefficients of both biomass and lipids production, but had significant effects on the growth rate and thus on volumetric productivity. Based on both the highest growth rate and volumetric productivity, the optimal temperature for lipid production was 15°C. Diversely from the amount of intracellular lipids, the composition of FA in the lipid fraction depended on the growth temperature. As the growth temperature decreased, the abundance of C18 FA increased at the expenses of the C16 FA and the unsaturation degree increased as well. In particular, remarkable amounts of linolenic acid (C18:3 ω-3) were produced at -3°C, accounting for 29% of FA. This observation is in agreement with previous studies on oleaginous species demonstrating that decreasing growth temperatures caused an acclimatory response and resulted in a higher length and degree of unsaturation of FA (Rossi et al. 2009).

Likewise other oleaginous yeasts (Gangar et al. 2002, Ratledge 2002, Czabany et al. 2007), most of lipid accumulation occurred when *R. glacialis* AS 4.7 exhausted a nutrient from the medium, but glucose still remained. Glucose continued to be assimilated by the cells and was converted into triacylglycerols at approximately the

same rate at which lipid was synthesized during the balance phase of growth. However, as the limitation in the supply of a nutrient prevented proliferation, the lipids were stored within the existing cells which progressively became obese. The extent of the carbon excess had major positive effects on lipid production. The lipid content of biomass, glucose conversion into lipids, lipid concentration, and lipids productivity were all maximum with 120 g L<sup>-1</sup> glucose (68%, 16%, 19 g L<sup>-1</sup>, and 0.054 g L<sup>-1</sup> h<sup>-1</sup>, respectively). The results herein reported suggest that *R. glacialis* AS 4.7 could be considered as an interesting microorganism for the production of single cell oils. This strain is intriguing since can grow and produce lipids over a wide range of temperatures, albeit it originate from a cold habitat. Furthermore, the present study is the first proposed biotechnological application for *Rhodotorula glacialis* species.

## 5. References

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