BIOENERGY AND BIOFUELS

Effect of different C/N ratios on carotenoid and lipid production by *Rhodotorula glutinis*

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Abstract Due to the increasing demand for sustainable biofuels, microbial oils as feedstock for the transesterification into biodiesel have gained scientific and commercial interest. Also, microbial carotenoids have a considerable market potential as natural colorants. The carbon to nitrogen (C/N) ratio of the respective cultivation media is one of the most important parameters that influence the production of microbial lipids and carotenoids. Thus, in the present experiment, the influence of different C/N ratios, initial glucose loadings, and ammonium concentrations of the cultivation medium on microbial cell growth and lipid and carotenoid production by the oleaginous red yeast *Rhodotorula glutinis* has been assessed. As a general trend, both lipid and carotenoid production increased at high C/N ratios. It was shown that not only the final C/N ratio but also the respectively applied initial carbon and nitrogen contents influenced the observed parameters. The lipid yield was not affected by different ammonium contents, while the carotenoid production significantly decreased both at low and high levels of ammonium supply. A glucose-based increase from C/N 70 to 120 did not lead to an increased lipid production, while carotenoid synthesis was positively affected. Generally, it can be asserted that lipid and carotenoid synthesis are stimulated at higher C/N ratios.

Keywords Microbial lipid · Microbial carotenoid · *Rhodotorula glutinis* · C/N ratio

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Introduction

The finiteness of natural oil reserves, soaring oil prices, and environmental concerns have led to an increasing propagation of renewable resources in the past decades, both in terms of energetic and material utilization. Accordingly, political stakeholders set ambitious goals regarding the integration of alternative energy sources into established energy systems. For example, in the European Union by 2020, 20 % of total energy consumption should be covered by renewable resources, whereas in the transportation sector, 10 % of petrol and diesel consumption should be substituted by biofuels (EU 2009). Regarding the production of biofuels, certain sustainability criteria apply, which include a mandatory minimum saving in greenhouse gas emissions of 35 % (by 2017, 50 %; by 2018, 60 %) compared to the utilization of conventional fossil energy sources. However, if all desired parameters, e.g., direct and/or indirect land use change effects, are taken into account for this calculation, it is unlikely that any of the currently commercially produced first-generation biofuels will be able to meet these standards. This, along with the often debated food vs. fuel issue and other environmental and efficiency objections, accelerated the research into new pathways for biofuel production.

One of these approaches is the production of biodiesel through the transesterification of microbial lipids obtained from oleaginous microorganisms (e.g., microalgae or yeast) with methanol into fatty acid methyl esters (FAMEs). These microorganisms, which are considered as accumulating 20 % or more of their cell weight in the form of lipids, need to be cultivated under nutrient-limited and excess carbon conditions in order to trigger the synthesis of lipids, when carbon is used for lipid accumulation instead of cell proliferation processes (Ratledge and Cohen 2008). With lipid contents ranging between 20 and 70 %, short life cycles, low space requirements,

and their independence from location, season, and climate, these microorganisms offer some potential advantages over conventional oil crops (Subramaniam et al. 2010). However, the high cost of this process is a major constraint regarding its implementation on a commercial scale. Thus, it is crucial to use cheap organic carbon sources for fermentation. In this context, different waste substrates have already been successfully tested for their performance as a carbon and nutrient source in microbial growth media (Cheirslip et al. 2011; Chi et al. 2011; Alvarez et al. 1992; Xue et al. 2010). Some oleaginous microorganisms are also capable to produce other value-added products. The oleaginous red yeast *Rhodotorula glutinis* can accumulate lipids up to 70 % of its cell dry weight (Meng et al. 2009) as well as certain carotenoids during the cultivation (Buzzini et al. 2007), whereas the value of the latter is considerably higher than that of microbial lipids for biodiesel production. Also, for microbial carotenoid production, a variety of cheap carbon sources has been investigated (Buzzini and Martini 2000; Malisorn and Suntornsuk 2008; Frengova et al. 1994). In order to screen further potential, especially waste-derived carbon sources, it is necessary to develop certain criteria, which can be used for an evaluation in terms of their suitability to facilitate the production of lipids and/or carotenoids.

For both metabolic pathways, the carbon to nitrogen (C/N) ratio of the growth medium plays a crucial role. However, whereas it is agreed that with high C/N ratios, high lipid yields can be achieved, there is some ambiguity regarding the influence on carotenoid production, with some results indicating a positive correlation between C/N ratio and carotenoid production (Saenge et al. 2011) and other authors stating an inverse relationship (Somashekar and Joseph 2000). Additionally, since carotenoid and lipid production share acetyl CoA as common precursor, it cannot be excluded that there are certain interactions between both production pathways at certain C/N ratios. A more detailed knowledge about the effect of the C/N ratio on carotenoid production would be helpful in order to design or evaluate fermentation media for the formation of the desired product. Therefore, this work investigated the effect of three different C/N ratios with three different carbon and nitrogen levels on biomass, lipid, and carotenoid production by the yeast *R. glutinis*.

Material and methods

Microorganism, media preparation, and cultivation

The oleaginous red yeast *R. glutinis* (ATCC 15125) was cultivated for 10 days in 1 L of yeast mold broth (BD Difco™ 271120) on a rotary incubator at 130 rpm and 30 °C. These media were then used as an inoculum for the experimental media treatments. The first treatment was a basic sugar medium with a C/N of 70, which contained (per liter) 57.0 g glucose, 1.0 g yeast extract, 1.0 g Na_2HPO_4 12 H_2O, 1.0 g KH_2PO_4, 0.4 g $MgSO_4$ 7 H_2O, 1.0 g $(NH_4)_2SO_4$, 10.0 mL trace mineral solution, and 6.0 mL $FeSO_4$ solution (4 g L^{-1} $FeSO_4$ 7 H_2O). The trace mineral solution consisted of (per liter) 3.6 g Ca_2Cl_2 2 H_2O, 0.75 g $ZnSO_4$ 7 H_2O, 0.13 g $CuSO_4$ 5 H_2O, 0.5 g $MnSO_4$ H_2O, 0.13 g $CoCl_2$ 6 H_2O, and 0.17 g Na_2MoO_4 2 H_2O. The four other treatments were designed by varying this basic medium either in terms of the glucose or the ammonium sulfate content according to the specifications listed below (Table 1). All other properties of the media remained the same. For the calculations of the C/N ratio, a carbon content in glucose of 40 % and a nitrogen content of 21.205 % in ammonium sulfate and 11.4 % in yeast extract were assumed. The respective media (500 mL) was autoclaved (glucose was autoclaved separately), inoculated with 30 mL of the stock culture, and cultivated in 1-L baffled Erlenmeyer flasks for 216 h in a rotary incubator at 130 rpm and 30 °C. The respective change of total media quantity from 500 to 530 mL was not incorporated into the calculations for the initial glucose and ammonium contents; thus, the final measured glucose and ammonium contents and associated C/N ratios could be subject to minor changes. The experiment was carried out in triplicate. Samples for lipid extraction (40 mL) were taken at 0, 48, 120, and 216 h. For carotenoid extraction, also 40 mL samples were taken at 0, 48, 96, 120, 168, and 216 h. Thus, at the end of cultivation, about 75 % of the total volume was used for the assessment of biomass, lipids, and carotenoids. For the determination of cell dry weight, the samples intended for both lipid and carotenoid extraction were centrifuged at RCF 1,522×*g* (Eppendorf 5810R, Rotor A-4-62, Eppendorf AG, Hamburg, Germany) for 10 min and then freeze-dried and weighted. The supernatant of these samples was used for the HPLC analysis of the glucose content of the media.

Lipid and carotenoid extraction

The lipid content of the cells was determined according to the method by Bligh and Dyer (1956). The cells were disrupted

Table 1 Contents of glucose and ammonium sulfate and the resulting C/N ratio in the experimental treatments

Treatment	Glucose (g L^{-1})	$(NH_4)_2SO_4$ (g L^{-1})	C/N ratio
C/N 20c	16.2	1.0	20:1
C/N 20n	57.0	4.885	20:1
C/N 70	57.0	1.0	70:1
C/N 120c	97.4	1.0	120:1
C/N 120n	57.0	0.362	120:1

c, changes in C/N ratio by altering glucose contents at a constant amount of ammonium; n, changes in C/N ratio by altering ammonium contents at a constant amount of glucose

with methanol and lipids extracted with chloroform following a modification of the method as described by Zhang et al. (2011). Afterwards, the solvents were evaporated in the TurboVap LV (Caliper Life Sciences, Hopkinton, MA, USA) under nitrogen blow (10–15 psi) at 50 °C for 1 h. The extract was weighted and is later referred to as total lipids. For transesterification into FAMEs, the extract was mixed with methanol that contained 2 % H_2SO_4 as catalyst and incubated in a water bath at 60 °C for 2 h, and then, the reaction was quenched by adding $NaHCO_3$ solution. FAMEs were extracted with toluene and analyzed using GC-FID (Agilent 6890, Santa Clara, CA, USA) equipped with the Restek Stabilwax-DA capillary column (30 m×0.25 mm ID and 0.25 μm film thickness). The different peaks were identified by comparison to responses from a known FAME standard.

For carotenoid analysis, the freeze-dried cells were hydrolyzed by incubation in HCl (1 mol L^{-1}) at 70 °C in a water bath for 0.5 h (Latha et al. 2005). The solution with the disrupted cells was then filtered (0.7-μm glass fiber filter), and the cells were thoroughly rinsed with distilled water to remove the acid. Afterwards, carotenoids were extracted over night in 30 mL acetone/methanol (1:1) solution. The solvents were evaporated under N_2 blow at 50 °C, and the remaining extracts were redissolved in 2 mL acetonitrile/isopropyl–alcohol/ethyl acetate (40:40:20) solution. The analysis was conducted using the HPLC device (Agilent 1100, Santa Clara, CA, USA) equipped with the Evaporative Light Scattering Detector (Varian 385-LC, Palo Alto, CA, USA) and the Restek Ultra C18 column (5 μm, 250×4.6 mm). Acetonitrile/isopropyl–alcohol/ethyl acetate (40:40:20) was used as a mobile phase at a flow rate of 1 mL min^{-1}. All indentified peaks were quantified by using response factor of β-carotene standards. The different carotenoids were identified according to known retention time profiles (Malisorn and Suntornsuk 2008; Weber et al. 2007). The total carotenoid content was calculated as the sum of the four identified carotenoids.

The statistical analysis of the data was conducted using Sigma Stat 3.5 (Jandel Scientific, USA). In One-way ANOVAs, the statistical significance of differences in mean values of the different measured parameters was calculated and compared with the Tukey's test at the 5 % level of probability.

Results

Glucose utilization and biomass production

The pattern of glucose utilization (Fig. 1) showed that glucose was consumed strongly in all treatments in the first half of cultivation. At low glucose concentrations, (C/N 20c) the rate of consumption was lower, but still, glucose was already exhausted after 96 h. The treatments with initial carbon contents
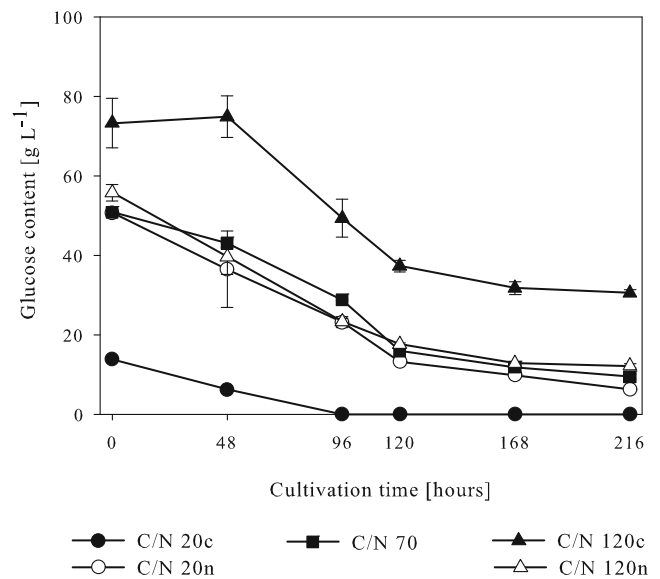

Fig. 1 Glucose utilization by *R. glutinis* during cultivation at different C/N ratios, initial glucose, and nitrogen contents

of 57 g L^{-1} exhibited a synchronous course of glucose utilization with a strong, almost-linear decrease within the first 120 h and a more leveled curve progression between 120 and 216 h. Except for the stagnation in the first 48 h, C/N 120c showed a similar pattern of glucose utilization. However, the initial glucose content of 73.33 g L^{-1} indicated that glucose may has been degraded to some extend during autoclaving, since initially, 97.4 g L^{-1} of glucose was added to the medium in this treatment. Since no observations indicated the caramelization of glucose, it was suggested that glucose at high concentrations was partly converted to fructose during autoclaving. However, this thesis could not be further examined, since only glucose and xylose were determined during HPLC.

Despite the differences in glucose consumption during the first 48 h, biomass production within this period was almost identical in all treatments (Fig. 2). After that point, biomass production stagnated at around 5.5 g L^{-1} in C/N 20c due to the early exhaustion of the carbon source in the medium. In all other treatments, the biomass yield was almost similar and ranged between 10.2 and 11.4 g L^{-1} after 216 h. Treatment C/N 20n stood out with the highest overall biomass yield and with the highest production rate of cell mass. This advantage is probably caused by the high nitrogen content in this treatment. Regarding the different glucose levels, an increase from 16.2 g L^{-1} (C/N 20c) to 57.0 g L^{-1} (C/N 70) led to a significant increase in cell mass production. A further increase to 97 g L^{-1} showed no positive effect on the total biomass yield.

Lipid production and fatty acid composition

Figure 3 shows the total lipid and FAME yield of *R. glutinis* at different C/N ratios as well as at different initial glucose
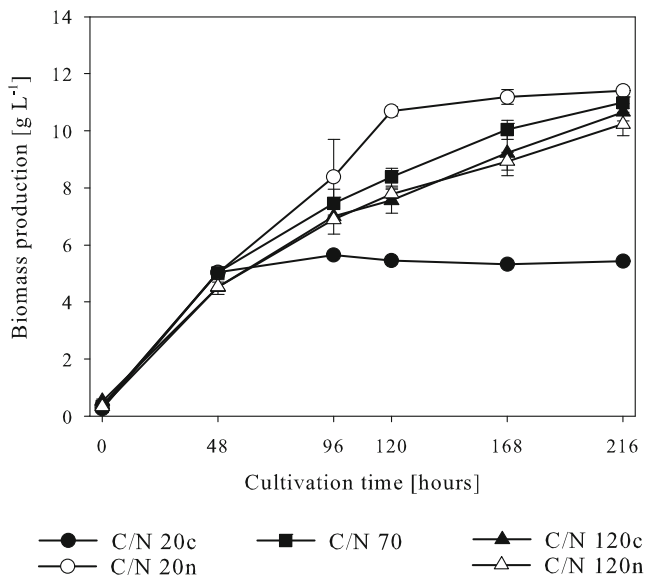
Fig. 2 Biomass production by *R. glutinis* during cultivation at different C/N ratios, initial glucose, and nitrogen contents

treatments only varied regarding the temporal distribution of lipid production, with C/N 20n (4.855 g L^{-1} (NH_4)$_2$$SO_4$) mainly producing lipids between 48 and 120 h of cultivation and C/N 70 (1.0 g L^{-1} (NH_4)$_2$$SO_4$) and C/N 120n (0.362 g L^{-1} (NH_4)$_2$$SO_4$) focusing the lipid production at the end of cultivation from 120 h on.

The fatty acid composition was generally similar in all treatments (Table 2). With average shares of over 30 %, oleic acid (C 18:1) and linoleic acid (C 18:2) stand out as major contributors to the overall FAME content. The fatty acid composition is similar to that of plant oils which are usually used for biodiesel production.

Looking at the fatty acid profiles as a function of the different C/N-based treatments, a certain trend between low and high C/N treatments could be observed. C/N 20c and C/N 20n showed significantly lower contents in saturated fatty acids C 16:0 and C 18:0 when compared to the other treatments. Regarding the unsaturated fatty acids, a tendency of higher proportions of C 18:1 and C 18:2 at low C/N ratios could be observed, whereas for C 18:3, the opposite holds true.

Carotenoid production

The production of carotenoids was observed in all experimental treatments (Fig. 4). The results showed that as a general trend, the carotenoid content increased at high C/N ratios. The highest yielding treatment after 216 h of cultivation was C/N 120c (1.247 mg L^{-1}) opposite to C/N 20n with the lowest carotenoid content (0.097 mg L^{-1}) at the end of cultivation. Treatment C/N 20c exhibited the strongest initial

and nitrogen contents. When the C/N ratio was increased through higher glucose contents, it clearly influenced the total lipid and FAME yield. The yield was more than doubled, when the glucose content was raised from 16.2 g L^{-1} (C/N 20c) to 57.0 g L^{-1} (C/N 70). This was mostly due to the only small gains in lipid production after 48 h of cultivation in treatment C/N 20c. However, a further increase to 97.4 g L^{-1} (C/N 120c) did not lead to higher lipid yields. The amount of added ammonium sulfate had no significant effect on total lipid or total FAME production (Fig. 3). The
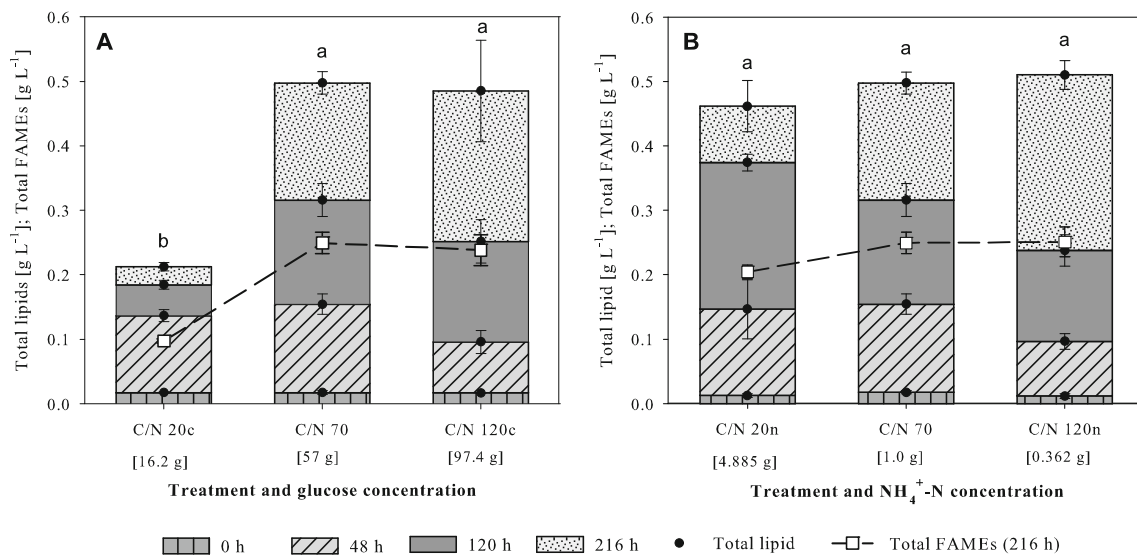

Fig. 3 Temporal breakdown of total lipid yield and FAME yield at the end of cultivation of *R. glutinis* grown at different C/N ratios and effects of different initial glucose concentrations at constant ammonium contents (**a**) and different initial ammonium concentrations at constant glucose contents (**b**). Different *lower case letters* indicate significant differences of total lipids after 216 h of cultivation

Table 2 Fatty acid composition of lipids from *R. glutinis* cultivated at different C/N ratios, initial glucose, and nitrogen contents

Values in the same column that do not share the same alphabetic superscript are significantly different at 5 % levels of probability

	Palmitic acid C 16:0 (%)	Stearic acid C 18:0 (%)	Oleic acid C 18:1 (%)	Linoleic acid C 18:2 (%)	Linolenic acid C 18:3 (%)
C/N 20c	15.07±0.08[c]	4.07±0.13[b]	44.38±0.32[a]	31.17±0.36[ab]	3.97±0.02[b]
C/N 20n	05.85±2.60[b]	6.75±0.53[c]	39.95±1.64[ac]	42.29±0.71[a]	3.67±0.09[b]
C/N 70	21.09±1.58[ac]	9.25±0.68[a]	33.65±2.47[bc]	26.68±5.34[b]	7.35±0.44[a]
C/N 120c	19.52±0.29[ac]	8.75±0.30[a]	30.45±0.47[b]	32.36±0.87[ab]	6.97±0.27[a]
C/N 120n	24.30±0.59[a]	9.15±0.14[a]	34.50±0.67[bc]	22.94±1.06[b]	6.54±0.07[a]

increase in carotenoid content after 96 h but stagnated afterwards. The medium and high C/N treatments C/N 70 and C/N 120c showed a similar temporal course of carotenoid formation with no significant differences between 0 and 168 h. After that point, C/N 70 stagnated, while C/N 120c further increased to the significantly highest yield of all treatments. In all treatments that initially contained 57 g L^{-1} glucose, there was no further increase in carotenoid production after 168 h when around 10 g L^{-1} glucose remained in the media. Regarding the amount of ammonium, it could be observed that both low and high ammonium concentrations had a detrimental effect on overall carotenoid production, as C/N 70 yields significantly more carotenoids than C/N 20c and C/N 120n. Regarding the carotenoid composition, the distribution of the single carotenoids showed a predominance of torulene and torularhodin over β-carotene in all treatments.

Fig. 4 Total carotenoid content and carotenoid composition of *R. glutinis* cultivated at different C/N ratios, initial carbon, and nitrogen contents
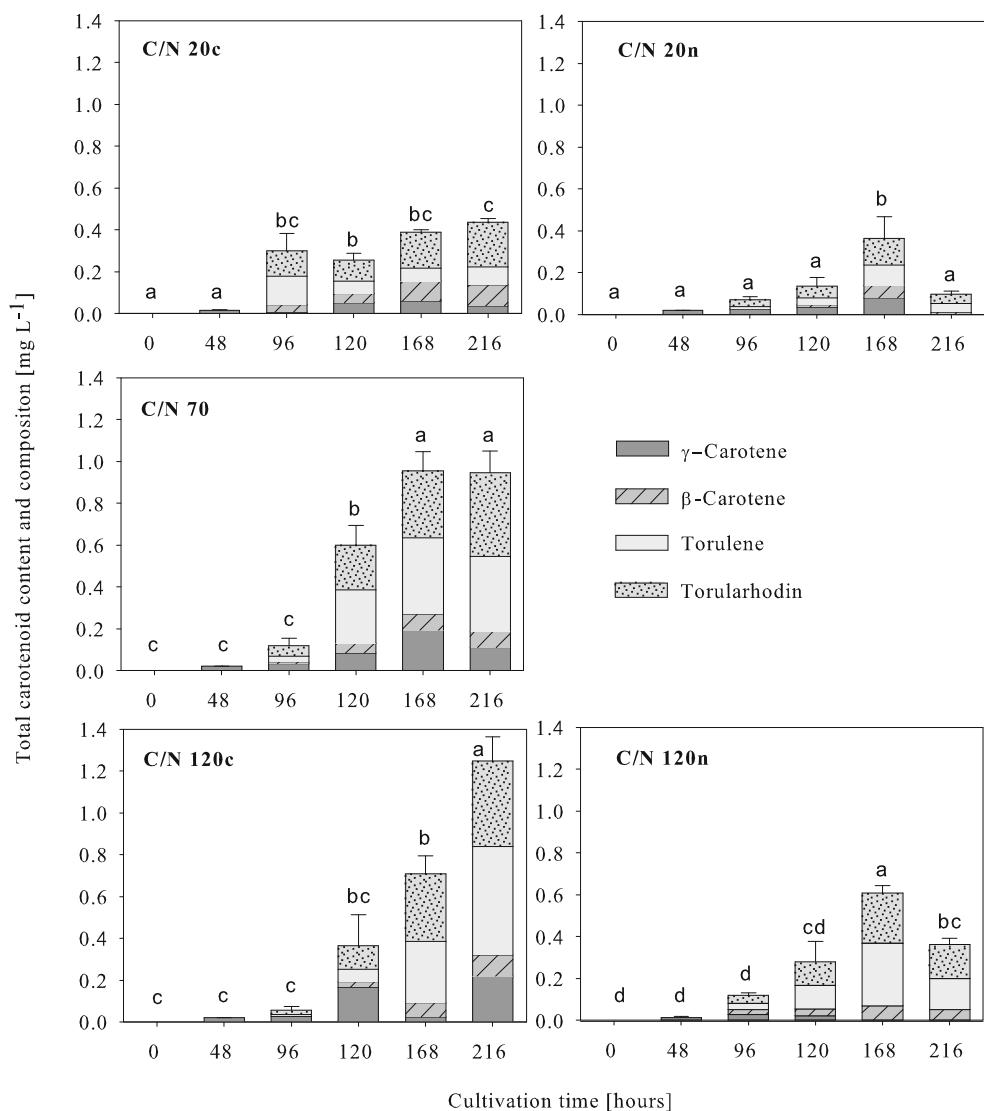
Discussion

Lipid production and fatty acid composition

The lipid metabolism in oleaginous yeasts is only triggered when a growth-required nutrient (mostly nitrogen) is limited, and carbon is still abundantly available (Papanikolaou and Aggelis 2001). From this, it can be deduced that a high C/N ratio of the growth media will positively affect the lipid accumulation in these organisms. Accordingly, the lipid and FAME yield was increased at high initial glucose loadings, whereas the limitation of lipid production in C/N 20c after 48 h can be explained by the carbon limitation after this point as indicated by the pattern of glucose consumption (Fig. 1) and biomass production (Fig. 2). However, the results also imply that at an ammonium level of 1.0 g L^{-1}, an increase from 57 g L^{-1} (C/N 70) to 97.4 g L^{-1} (C/N 120c) initial glucose has no positive effect on lipid production using batch fermentation and should thus be avoided, since the cost of carbon contributes a high share to the overall cost of the whole process, and any potential savings in carbon utilization will help to improve the economic features of the approach. Similar results have been presented by Karanth and Sattur (1991), who found that lipid production in batch fermentation was similar for initial sugar concentrations of 60 and 80 g L^{-1}, while these treatments yielded around twice as much lipids as treatments with an initial sugar concentration of 100 g L^{-1}, which again had about the same level of lipid production compared to 40 g L^{-1} initial sugar concentration at the end of cultivation time. Mondala et al. (2012) observed no difference in lipid yield between initial glucose loadings of 40 and 60 g L^{-1} and attributed this to some types of substrate inhibition caused by high initial glucose contents in batch fermentation. Elsewhere, it was pointed out that some key enzymes for lipid synthesis might be inactive at high glucose concentrations (Papanikolaou et al. 2002). However, in the present case, the manifest explanation could be that after 216 h of cultivation in both treatments C/N 70 (57 g L^{-1} glucose) and C/N 120c (97 g L^{-1} glucose), the fairly the same amount of sugar—42.4 vs. 42.8 g L^{-1}, respectively—was actually utilized. Accordingly, the difference in initial glucose loadings might have an effect on lipid production once the cultivation time is prolonged.

Regarding the influence of the initial nitrogen content, at high C/N ratios, the lipid production was shifted to the end of cultivation. Normally, an opposite pattern could be anticipated, since lower nitrogen levels would suggest an early shift to lipid synthesis.

The general low lipid production, combined with the continuous production of biomass in treatments with sufficient glucose supply suggest that nitrogen was not exhausted from the cultivation media. Accordingly, the lipid accumulation process was not completely triggered, and glucose not only channeled into the lipid synthesis pathway but also utilized for the formation of biomass.

Ranging between 0.2 and 0.5 g L^{-1} after 216 h of cultivation, the lipid production in this experiment was low. This was probably caused by other cultivation parameters, which could not be established at an optimal level. Due to the acidifying effect of ammonium uptake by the cell, the pH declined sharply to less than pH 3 at early stages of cultivation, which has previously been reported as detrimental to lipid production (Johnson et al. 1992). Also, the aeration of the growth medium, which was not provided in the present experiment, has been shown to positively affect the lipid yield of R. glutinis being an aerobic microorganism (Saenge et al. 2011). The chosen range of C/N ratios should have all been able to generally support lipid production, since a C/N ratio of 20 is seen as a minimum condition for lipid induction (Chi et al. 2011; Papanikolaou and Aggelis 2001), whereas most authors recommend a C/N close to 100 as ideal for lipid accumulation (Ageitos et al. 2001; Yousuf 2012; Angerbauer et al. 2008). The fatty acid composition was well in accordance with the typically reported predomination of palmitic (C 16:0) and oleic (C 18:1) acid (Saenge et al. 2011; Perrier et al. 1995) in strains of R. glutinis. As a general trend, it was observed that low C/N ratios led to low contents of saturated fatty acids and an increased content of unsaturated fatty acids. Similar findings were reported by Mondala et al. (2012) who found increasing levels of C 16:0 and C 18:0 with increasing initial C/N ratios. Generally, the lipids produced in each treatment are suitable for the production of biodiesel. Still, the accurate fatty acid profile influences crucial qualitative properties of biodiesel. Important aspects of diesel fuel quality like the cetane number, heat of combustion, and cold flow properties are differently influenced by chain length and the degree of saturation. Thus, it is difficult to give any recommendations whether low C/N conditions that tend to give more unsaturated fatty acids or high C/N conditions that have a larger share of saturated fatty acids should be preferred. To balance these parameters, generally oleic acid is seen as optimal fatty acid for improved fuel properties (Knothe 2005). In this case, the best yields for oleic acid were detected in the low C/N treatments. In all treatments, the total amount of produced FAMEs is around 50 % of the total lipid fraction extracted by the Bligh and Dyer method. This suggests that only a small but constant fraction of these extracts can be transesterified into biodiesel. Thus, if no transesterification is conducted, lipid yields should be assessed carefully to avoid an overestimation of potential biodiesel yields.

Carotenoid production

Same as microbial lipids, microbial carotenoids are formed in the secondary metabolism of the yeast. Lipids and carotenoids even share acetyl CoA as a common precursor. While

it is fairly agreed that high C/N ratios are beneficial for the lipid yield, the effect of C/N ratio on carotenoid synthesis is somewhat contradictory. The results indicate that in the present experiment, carotenoid production was not growth associated, since C/N 20n showed the highest biomass production while yielding the lowest amount of carotenoids. A reason for the low carotenoid synthesis in C/N 20n might be the low pH value (<2), since optimum pH levels for carotenoid production have been reported to be around pH 6 (Tinoi et al. 2005; Aksu and Eren 2007).

Looking at the different levels of initial glucose, it could be stated that at low glucose loadings, the carotenoid synthesis was limited due to carbon exhaustion. Thus, the stagnation in carotenoid production after 96 h in C/N 20c accords with the point of glucose exhaustion from the medium.

With continuously increasing carotenoid yields, especially towards the end of cultivation in C/N 120c, it can be suggested that this treatment takes advantage of the comparatively high amounts of glucose still available at the end of cultivation time. The retained development of carotenoid synthesis at early cultivation stages could be attributed to inhibition caused by the initially high glucose content. It has been demonstrated that batch cultures of R. glutinis and Phaffia rhodozyma are affected by the Crabtree effect, which eventually leads to diminishing carotenoid yields at high initial glucose loadings (Saenge et al. 2011; Frengova and Beshkova 2009; Ramírez et al. 2001). Thus, also here, the optimum level of initial glucose concentration in the media in combination with the cultivation time needs to be assessed carefully.

Regarding the effect of initial ammonium content, it was observed that at high (C/N 20n) and low (C/N 120n) ammonium contents, no γ-carotene was synthesized in the late stages of cultivation. Being the precursor for all other carotenoids, this explains the drop in carotenoid content from 168 to 216 h in these treatments. C/N 20c contained the highest amount of nitrogen (4.885 g L^{-1} ($NH_4)_2SO_4$). With acetyl CoA as common major building block for both lipid and carotenoid synthesis, it can be suggested that the switch between the primary and secondary metabolism is initiated through the same conditions, namely, nitrogen limitation. This would explain the poor performance of C/N 20n. Similar results have been shown for P. rhodozyma, where accumulation of fatty acids and astaxanthin were decreased at high ammonium concentrations (Flores-Cotera et al. 2001). Since the ammonium concentration did not affect the lipid production, this could mean that carotenoid synthesis is more susceptible to the effect of nitrogen limitation than lipid synthesis.

At low initial ammonium contents (C/N 120n), the carotenoid production also decreased compared to medium ammonium contents (C/N 70) (Fig. 4). Also, Aksu and Eren (2007) reported an optimum curve for carotenoid production as a function of nitrogen concentration, with sharply decreasing total carotenoid production below 1.0 g L^{-1} ($NH_4)_2SO_4$.

Somashekar and Joseph (2000), who also observed lower carotenoid yields with decreasing nitrogen contents, suggested that under these conditions, available carbon will be utilized in favor to lipid production rather than carotenoid synthesis.

However, it could also be observed that generally, lipids and carotenoids can be produced simultaneously and that there is no "head or tails" relation between the two synthesis pathways.

Since β-carotene was only produced as minor carotenoid, it seems that under the present cultural conditions, the pathway of torulene production is favored over β-carotene production. In the group of carotenoids synthesized by R. glutinis, γ-carotene can be considered as primary carotenoid, which acts as a precursor and branch point for either β-carotene or torulene synthesis, whereas torulene again can further be metabolized into torularhodin (Frengova and Beshkova 2009). This can be regarded as an undesired development, since to date, mainly β-carotene gains commercial interest, while for torulene and torularhodin, suitable commercial applications are still rare. Interestingly, even though the absolute carotenoid yields at low C/N ratios are disappointing, C/N 20c showed the highest β-carotene proportion on a relative basis. Thus, low or moderate C/N ratios should not generally be ruled out when media for microbial carotenoid and especially β-carotene production are designed.

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