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Feasibility of microbial biodiesel and carotenoid production considering the potential of food processing wastewaters as low cost carbon sources using the example of red yeast *Rhodotorula glutinis*

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List of Acronyms

BOD	biological oxygen demand
C 16:0	palmitic acid methyl ester
C 18:0	stearic acid methyl ester
C 18:1	oleic acid methyl ester
C 18:2	linoleic acid methyl ester
C 18:3	α-linolenic acid methyl ester
C/N ratio	carbon to nitrogen ratio
CO_2	carbon dioxide
COD	chemical oxygen demand
DHA	docosahexaeneoic acid
e.g.	*exempli gratia*, for example
ER	energy ratio
et al.	*et alia*, and others
EU	European Union
FAME	fatty acid methyl ester
FER	fossil energy ratio
GHG	greenhouse gas
GLA	γ-linolenic acid
GWP	global warming potential
ha	hectare
i.e.	*id est*, that is
LCA	life cycle assessment
LUC	land use change
Mio.	millions
MJ	Megajoule
Mtoe	million tonnes oil equivalent
N	nitrogen
N_2O	nitrous oxide
NH_4^+	ammonium
PUFA	polyunsaturated fatty acid
SCO	single cell oil
TAG	triacylglycerol
US$	U.S. Dollar
YOB	“yeast oil for biodiesel”

1 Introduction

Against the background of rising prices for fossil energy sources, their overall finiteness and their role as leading source of carbon dioxide emissions, renewable resources for both energetic and material utilization have gained importance over the past decades. In the field of bioenergy, liquid biofuels have been developed as renewable and sustainable substitutes to petroleum derived transportation fuels like gasoline and diesel. Within a broad range of possible conversion technologies from biomass to biofuel, bioethanol from cornstarch (U.S.) and from sugarcane (Brazil) as well as biodiesel manufactured from plant oils like rapeseed (Europe) and soybean (U.S.) are currently the predominantly produced biofuels (Solomon, 2010). Accordingly, in these countries the production and consumption of biofuels have strongly been promoted by means of market instruments like subsidies or tax exemptions, as for example laid down in the *Directive 2003/30/EC*, which has been adopted by the European Union in 2003. This promotion can basically be attributed to the aspects of **CO_2 emission reduction** and **energy security**. Globally, about a quarter of all energy-related CO_2 emissions arise from the transport sector (IEA, 2010), in Europe 19 % of total greenhouse gas (GHG) emissions are produced in this sector (EU, 2012). Thus, a substitution of conventional fuels with biofuels is seen as a promising way to substantially reduce the overall GHG emissions and particularly the CO_2 emissions. Both Europe and the United States highly depend on energy imports – a fact that holds a certain potential for conflict. Since biomass as feedstock for biofuel production is geographically more evenly distributed as compared to fossil resources, the application of biofuels is seen as chance to gain **energy independency**.

As a result, the production of 1st generation biofuels increased significantly over the past decade (**Fig. 1**). However, with 2 % in 2010, the overall worldwide share of biofuels in transport remained comparatively low (IEA, 2012). In some countries, including first of all Brazil but also Cuba, Sweden and Germany, this share is considerably higher (IEA, 2006). Nevertheless further government incentives are necessary in order to increase the contribution of biofuels to the total energy required in the transport sector.

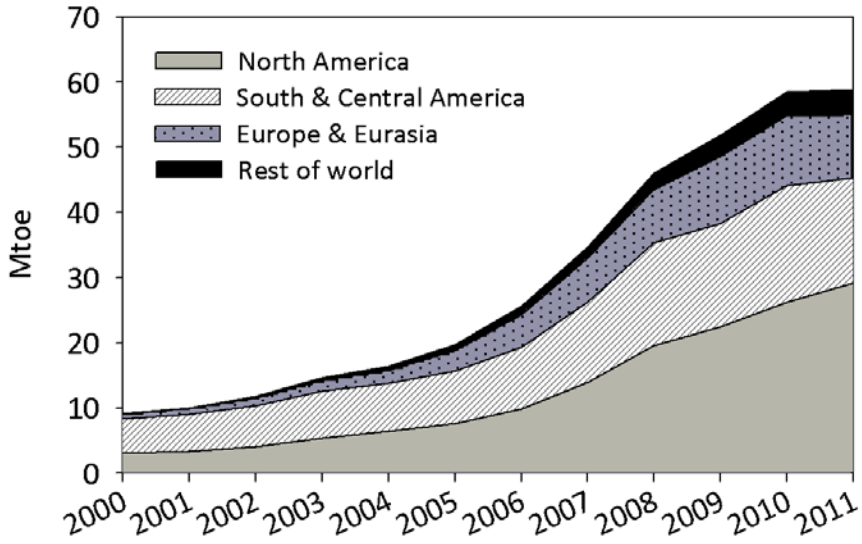

Figure 1: Development of worldwide biofuel production as million tonnes oil equivalent (Mtoe). Adapted from BP Statistical Review (2012).

Even if biofuels seem to offer some potential benefits, recently the impact of an ever increasing 1st generation biofuel production has been discussed controversially. Within this debate, the question was raised whether 1st generation biofuels can actually be characterized as sustainable. These concerns mainly address the influence of biofuel production on food prices and their actual GHG mitigation potential.

Since 1st generation biofuels are produced from agricultural food crops, there is a certain potential for a competition between the utilization for energetic and food purposes. Thus, the **impact of biofuels on food prices** has been widely assessed. Timilsina and Shrestha (2011) reviewed and summarized the literature in this context and concluded that biofuel production does have a significant effect on global agricultural markets, commodity and food prices. Especially the extensive production of biofuels in the United States considerably contributed to the rise in prices for corn and soybean. However, Baier *et al.* (2009) exemplary stated that the rise of food prices before and during the food crisis in 2008 can only be attributed to the concurrent expansion in biofuel production to a rather small extend of 12 %. Still, when considering the world's nutritional situation, it can be agreed that the production of biofuels from food crops bears a certain moral dilemma. Also, with continuously growing world population and global energy demand together with the on-going degradation of agricultural land, this conflict will most likely be reinforced in the next years. Regarding the case of biodiesel, Schenk *et al.* (2008) calculated that even a cultivation of oil crops for biodiesel production on the entire worldwide available arable land could only cover less than half of the current energy demand. This shows again that 1st generation biofuels are not the ultimate solution as a sustainable alternative to petroleum fuels.

Also the **environmental impact of biofuels**, especially with regard to **GHG emission savings** has been scrutinized. One major reason for the promotion of biofuels lies in their potential to reduce GHG emissions. The energetic utilization of biomass is considered "carbon neutral" because the CO_2 release during fuel combustion equals the amount of CO_2 captured during biomass growth, whereas fossil fuels release CO_2 that has been stored over millions of years. However, in some areas like feedstock production, handling, and processing always a certain amount of fossil fuel inputs are required. Taking into account these aspects, most Life Cycle Assessments (LCA) found that biodiesel offers emission savings - depending on the underlying assumptions and the type of feedstock - in a range between 50 and 80 % compared to its fossil counterpart (Timilsina and Shrestha, 2011; Cherubini *et al.*, 2009). Most LCAs however do not include the effect on GHG and especially on CO_2 emissions from direct and indirect land use changes connected with the expansion of crops for biofuel production. Direct LUC occurs when previously non-agricultural land (*e.g.* natural forest) is taken into agricultural production for biofuel feedstocks, whereas indirect LUC describes the diversion of land previously used for feed or food production into cultivation with crops for energy purposes. Both changes in use will influence the soil carbon pool and mostly – depending on the previous use of land - lead to a loss in carbon stock. So for example Hamelinck *et al.* (2008) calculated a reduction in soil carbon stock of 9 t C ha^{-1} for a conversion of temperate grassland into cultivation with rapeseed. An accurate assessment of LUC effects is rather difficult. Still it can be assumed, that if these changes are taken into account, conventional biofuels cannot longer hold the promise of GHG savings. Indeed rather contrary projections can be found in the literature. Fargione *et al.* (2008) calculated a

carbon payback period[1]of 37 years for the conversion of *cerrado* grassland in Brazil into cropping land for soybean biodiesel. The conversion of Indonesian peatland rainforest into palm oil plantations involves a carbon payback period of 423 years. Also in temperate regions the carbon storage potential is decreased by ca. 50 % when grassland is converted into arable land (Guo and Gifford, 2002). These LUC connected issues can only be overcome when abandoned or marginal cropland is taken into production for biofuel crops.

Besides the CO_2 emissions related to a change in land cultivation, also other GHGs need to be considered. Butterbach-Bahl et al. (2010) emphasize in this context the significant role of N_2O emissions, which mainly derive form the applied nitrogen fertilization. Since N_2O has a climate warming potential about 300 times as high as CO_2 and in most studies these emissions are either underestimated or not considered at all, it is obvious that even if LUC effects are included, the potential GHG savings of conventional biofuels are still overestimated. This especially holds true for crops that require high nitrogen fertilization rates like maize and rapeseed. All this shows that conventional 1st generation biofuels can hardly contribute to GHG savings at all. At the same time it needs to be considered, that next to climatic effects also other environmental parameters like local air pollution or eutrophication can be negatively affected and need to be taken into consideration as well when assessing the environmental impact of biofuel production. In this context, again the negative impact of intensive nitrogen fertilization on nitrate leaching and eutrophication can be cited as an example.

Next to other issues, the ambiguous role of biofuels for food prices or GHG savings show, that the potential of 1st generation biofuels in general and biodiesel in particular to contribute to future transport fuel requirements is rather limited.

This can also be concluded when looking at the recently developed biofuel support policies in the U.S. and in Europe, which promote the use of 2nd generation biofuels that are derived from lignocellulosic biomass. The *Renewable Fuels Standard* in the United States dictates a blending mandate as well as minimum GHG savings of 50-60 % for these advanced biofuels. In Europe *Directive 2009/28/EC* has been adopted to further promote biofuels by setting mandatory targets (*e.g.* 10 % of renewable energy in the transport sector by 2020) and certain sustainability standards. These include minimum GHG savings of 35 % from 2013 onwards (50 % in 2017; 60 % in 2018) and other criteria related to social sustainability and indirect land use change, thus indirectly also promoting the development and implementation of advanced 2nd generation lignocellulosic biofuels. All currently used conventional biofuels would already fail to achieve the goal of 35 % GHG savings (Butterbach-Bahl, 2010). In the recent years the amount of reports, which held the view that 1st generation biofuels are unsustainable and not able to reduce GHG emissions, increased. Due to this development, in fall 2012 the European Commission reacted by introducing several new proposals regarding future arrangements of biofuel policy, which included the cessation of subsidies for 1st generation biofuels after 2020 and the inclusion of indirect land use changes. Furthermore, the maximal supported production of biofuels, which is credited against the required share of 10 % of renewables

[1] The carbon payback period describes the number of years required until the amount of GHG released in course of land conversion for biofuel production equals the amount of GHG savings through the substitution of fossil fuels with biofuels.

in the transportation sector, is capped to 5 %. Thus, the remaining 5 % will need to be provided by novel 2nd and 3rd generation biofuels.

Since **2nd generation biofuels** utilize the whole plant biomass of mostly designated energy crops, they offer increased land use efficiencies and lessen the pressure on food production and land use. However, also for the cultivation of lignocellulosic biofuel feedstocks certain inputs, especially agricultural land, are required. Thus, also here is potential for direct and indirect land use changes affecting both the availability of land for food production and the carbon balance. Lignocellulosic biofuels have been reported to have a higher GHG mitigation potential than 1st generation biofuels (IEA, 2010) but also in this case land use changes are difficult to evaluate and moreover all previous reports on lignocellulosic biofuels are mainly of theoretical nature since none of these concepts have reached commercial implementation yet.

3rd generation biofuels, derived from microbes and microalgae have more recently been investigated as next step toward the development of sustainable transportation fuels. Due to their modest cultivation requirements regarding land space and their independence of location, season and climate (Subramaniam, 2010), they are less susceptible to the previously described major drawbacks linked to 1st and 2nd generation biofuels (Nigam and Singh, 2011). Next to other potential approaches to utilize microorganisms for biofuel production, this work concentrates on the production of **biodiesel from microbial fatty acids**, because in the European framework biodiesel is the predominant biofuel, with 63 % of total biofuel production in 2010 (EU, 2012). Conventional biodiesel is manufactured by the transesterification of triacylglycerols[2] (TAG) from plant oils with an alcohol (mostly methanol) into fatty acid alkyl (methyl) esters, whereas the terms fatty acid methyl esters (FAMEs) and biodiesel are often used synonymously (Knothe, 2005). The composition of these FAMEs complies with the fatty acid composition of the original plant oil and depicts an important parameter for the quality of the resulting biodiesel. Certain microbial species such as yeast, fungi and microalgae belonging to the group of oleaginous microorganisms are able to synthesize and accumulate large amounts of TAG in their biomass that exhibit similar characteristics to plant oil and thus can likewise be used as feedstock for biodiesel production. Depending on the organism and the cultivation conditions the lipid content ranges between 20 and 70 % of the biomass (Ratledge, 2010). In order to trigger the process of lipid synthesis and achieve high lipid contents, conditions of nutrient (mostly nitrogen) limitation and carbon excess should be present in the microbial growth medium. Under these conditions cell proliferation processes cease and the excess carbon is channeled into the synthesis of storage lipids (Ratledge, 2004). These lipids can then be extracted and transesterified into biodiesel. However, the main challenge attached to this approach lies in the economical dimension. Usually easy degradable sugars like glucose and fructose are used as carbon sources for microbial lipid production with the highest practical conversion considered to be at 20-22 %, *i.e.* 5 tonnes of sugar yield 1 tonne of oil (Ratledge, 2010). In 2011 glucose syrup was priced at approximately US$ 660 t^{-1}, while canola and soybean oil cost around US$ 1300 and 1200 t^{-1}, respectively (USDA, 2012). This makes clear, that any conversion from one into the other commodity can only be economically feasible when **low or zero-cost carbon sources** can be provided, because even if the boom in 1st generation biofuels led to an increase in plant oil prices over the last years, a further rapid rise in prices is rather unlikely. In this context a broad range of

[2] Triacylglycerols (TAG) are esters of three fatty acids and a glycerol backbone.

potential carbon sources including by-products like molasses (Alvarez *et al.*, 1992) and glycerol (Easterling *et al.*, 2009), residues like lignocellulosic hydrolyzates (Yu *et al.*, 2011) and waste like sewage sludge (Mondala *et al.*, 2011) have already been tested.

Also **wastes or residues from the agricultural sector** and the linked processing industry could be taken into consideration here. According to the third objective of the EU biofuels policy, which was to support farmers by diversifying the market outlets for agricultural products, the increased demand for 1st generation biofuels derived from agricultural crops had some beneficial effects on the agricultural sector. Next to the stabilization or even the increase of real commodity prices, it offered the **generation of new sources of income** as well as a **diversification** of the latter. In Europe the future framework for biofuels as described previously, will most likely lead to a reduction in 1st generation biodiesel production, since they will no longer be subject to any kind of monetary subsidy, which is however still necessary to make the production economically attractive. The supply of carbon sources for microbial 3rd generation biodiesel by the agricultural sector and connected downstream industries could help to further benefit from the promotion of biofuels beyond the era of 1st generation biodiesel. In this context this study screened different wastewaters from the food and beverage processing industry in order to evaluate their suitability to support growth and production of secondary metabolites by the oleaginous red yeast *Rhodotorula glutinis*.

Wastewaters from the food and beverage industry were chosen as potential carbon sources because they generally contain high contents of organic substances, which is reflected by a high chemical (COD) and biological (BOD) oxygen demand (**Tab. 1**). Moreover these sources usually do not require an additional pretreatment, do not compete with other potential utilization pathways and accrue in large amounts (**Tab. 2**).

Table 1: Chemical oxygen demand of wastewaters from food and beverage production.

Origin of wastewater	Chemical oxygen demand [mg L^{-1}]	Source
Sugar refinery	6000 - 30,000	DWA, 2009
Potato processing	20,000 - 30,000	Huang *et al*., 2003
Potato processing	3500 - 9000	DWA, 2005
Fruit juice production	2500 - 4500	DWA, 1999
Beer production	1800 - 3000	Rüffer and Rosenwinkel, 1991
Beer production	2000 - 6000	Driessen and Vereijken, 2003

Table 2: Wastewater production [1000 m^3] from different food and beverage industries in Germany in 2007 (Statistisches Bundesamt, 2011).

Origin of wastewater	Number of companies	Total wastewater	Disposal to public WWT*	Disposal to in-company WWT*
Potato processing	29	5413	969	3696
Fruit- and vegetable juice production	67	5974	1902	3297
Fruit and vegetable processing	175	21,225	5662	10,361
Sugar production	25	27,165	442	15,516
Beer production	292	31,748	10,915	18,091

*WWT: Wastewater treatment

Since these wastewaters can lead to severe water pollution if directly released into the environment, in-company wastewater treatment facilities are necessary to reduce the COD below 110 mg L^{-1}, which reflects the legally set threshold for wastewater disposal in Germany. A reduction in both COD and BOD through microbial fermentation has been observed before (Hall *et al.*, 2011; Xue *et al.*, 2010). Thus, microbial cultivation could also to some extend contribute to in-company wastewater treatment systems and hence offer a certain cost-saving potential

The **oleaginous red yeast *Rhodotorula glutinis*** was selected as test organism for this study due to its high potential lipid content of up to 70 % (Meng *et al.*, 2009; Ratledge and Cohen, 2008) as well as its non-pathogenic character and the comparatively easy handling (Kaiser *et al.*, 2007). Additionally, *R. glutinis* is able to synthesize certain carotenoids, namely β-carotene, torulene and torularhodin (Buzzini *et al.*, 2007). These carotenoids, especially β-carotene, face increasing economic significance since they can commercially be used as natural colorants for food, feed and cosmetic products as well as for nutritional and pharmaceutical purposes due to their antioxidant properties and their metabolic function as precursor for Vitamin A (Malisorn and Suntornsuk, 2008). Thus, the value of these products is considerably higher than that of microbial lipids for biodiesel production. Since **microbial carotenoid synthesis** also requires a sufficient supply of carbon, several wastes and by-products have been tested as potential nutrient and carbon sources (Malisorn and Suntornsuk, 2008; Buzzini and Martini, 2000; Frengova *et al.*, 1994). In this context, next to the aspect of lipid production, this study also investigated the suitability of wastewaters to support microbial carotenoid production as well as the possibility of a concomitant production of lipids and carotenoids by *R. glutinis*.

Against the previously described background the thesis aimed to revise the following **experimental hypotheses**:

1. Wastewaters collected from the food and beverage industry contain high amounts of organic compounds, which can potentially be utilized by the yeast *Rhodotorula glutinis* as carbon and/or nutrient sources.
2. The yeast *R. glutinis* is able to grow on these wastewaters and to convert the available carbon and nutrient sources into biomass and the production of lipids.
3. Microbial lipids from *R. glutinis* exhibit a similar fatty acid profile to common plant oil and can be extracted and subsequently transesterified into biodiesel that complies with all relevant quality criteria.
4. Next to the production of lipids, *R. glutinis* synthesizes the carotenoids β-carotene, torulene and torularhodin when cultivated on selected industrial wastewaters.

For the investigation of these hypotheses different wastewaters have been selected and screened for their ability to serve as adequate nutrient and carbon sources for growth and product formation by *R. glutinis*. Laboratory test procedures for the extraction of *e.g.* carotenoids have been developed accordingly.

Chapter I deals with the assessment of wastewaters from potato and lettuce processing as well as fruit juice production. This screening was extended by brewery and sugar refinery effluents as described in **chapters II and III**. In this context the ability of *R. glutinis* to degrade and utilize the disaccharide maltose has been examined as well. Based on the previous results, in **chapter V** the ability of *R. glutinis* to degrade starch as a rather complex polysaccharide was investigated in order to achieve a better understanding of the hitherto obtained results and to give possible directions for a further evaluation of potential wastewaters as carbon source. Regarding the amendment of wastewaters to optimize lipid and carotenoid yields and to further investigate to possibility for a simultaneous production of lipids and carotenoids, **chapter IV** examines the influence of different C/N ratios as well as different initial glucose and ammonium contents on the synthesis of both lipids and carotenoids. In the general discussion the previous results will be briefly summarized and discussed in the context of a general evaluation of the approach. Moreover the potential performance of microbial biodiesel in general in terms of economic, environmental and energetic aspects will be assessed.

The primary results as presented in chapters I-V have been submitted to peer-reviewed journals or have already been published. The status of the submission can be gathered from chapter 2. These scientific articles form the body of the dissertation. Further publications in terms of non-peer-reviewed publications or presentations are given in Table A8 in the appendix.

2 Publications

The present thesis consists of five scientific papers as reflected by chapters I-V, which represent the key element of the dissertation. Four of these papers have been published or accepted by peer-reviewed, international referenced journals, whereas chapter III has been published within the conference proceedings of an international conference. For citation of chapters I-V please use the references given below.

Chapter I

Schneider, T., Graeff-Hönninger, S., French, W.T., Hernandez, R., Claupein, W., Holmes W.E., Merkt, N. (2012): Screening of Industrial Wastewaters as Feedstock for the Microbial Production of Oils for Biodiesel Production and High-Quality Pigments. Journal of Combustion, Article ID 153410, 9 pages. doi:10.1155/2012/153410

Chapter II

Schneider, T., Graeff-Hönninger, S., French, W.T., Hernandez, R., Merkt, N., Claupein, W., Hetrick, M., Pham, P. (2013): Lipid and carotenoid production by oleaginous red yeast *Rhodotorula glutinis* cultivated on brewery effluents. Energy (accepted). doi: 10.1016/j.energy.2012.12.026

Chapter III

Schneider, T., Graeff-Hönninger, S., French, W.T., Hernandez, R., Claupein, W., Merkt, N. (2012): Microbial lipids for biodiesel production and carotenoids as value added by-products – Screening of industrial wastewaters as suitable feedstock for oleaginous red yeast *Rhodotorula glutinis*. Proceedings of the 20th European Biomass Conference and Exhibiton, Milan, 18-22 June 2012, pp. 329-335.

Chapter IV

Braunwald, T., Schwemmlein, L., Graeff-Hönninger, S., French, W.T., Hernandez, R., Holmes W.E., Claupein, W. (2013): Effect of different C/N-ratios on carotenoid and lipid production by *Rhodotorula glutinis*. Applied Microbiology and Biotechnology (accepted). doi 10.1007/s00253-013-5005-8

Chapter V

Schneider, T., Rempp, T., Graeff-Hönninger, S., French, W.T., Hernandez, R., Claupein, W. (2013): Utilization of soluble starch by oleaginous red yeast *Rhodotorula glutinis*. Journal of Sustainable Bioenergy Systems 3, 57-63.

3 Chapter I

Screening of industrial wastewaters as feedstock for the microbial production of oils for biodiesel production and high-quality pigments

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Publication I:

Schneider, T., Graeff-Hönninger, S., French, W. T., Hernandez, R., Claupein, W., Holmes W. E., Merkt, N. (2012): Screening of Industrial Wastewaters as Feedstock for the Microbial Production of Oils for Biodiesel Production and High-Quality Pigments. Journal of Combustion, Article ID 153410, 9 pages. doi:10.1155/2012/153410

Based on the assumption that an economically feasible production of microbial lipids and carotenoids requires a cheap and abundant source of carbon, wastewaters from potato, fruit juice and lettuce processing have been examined for their ability to support growth and formation of secondary metabolic products by the red yeast R. glutinis. Next to the determination of organic contents in these wastewaters as reflected by the Chemical Oxygen Demand (COD), the parameters of cell growth, lipid and carotenoid production as well as fatty acid composition have been assessed experimentally through the cultivation of R. glutinis on the respective wastewaters.

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Research Article

Screening of Industrial Wastewaters as Feedstock for the Microbial Production of Oils for Biodiesel Production and High-Quality Pigments

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The production of biodiesel has notably increased over the past decade. Currently, plant oil is the main feedstock for biodiesel production, but, due to concerns related to the competition with food production, alternative oil feedstocks have to be found. Oleaginous yeasts are known to produce high amounts of lipids, but no integrated process from microbial fermentation to final biodiesel production has reached commercial realization yet due to economic constraints. Therefore, growth and lipid production of red yeast *Rhodotorula glutinis* was tested on low-cost substrates, namely, wastewaters from potato, fruit juice, and lettuce processing. Additionally, the production of carotenoids as high-value by-products was examined. All evaluated wastewaters met the general criteria for microbial lipid production. However, no significant increase in lipid content was observed, probably due to lack of available carbon in wastewaters from fruit juice and lettuce processing, and excess of available nitrogen in potato processing wastewater, respectively. During growth on wastewaters from fruit juice and lettuce processing the carotenoid content increased significantly in the first 48 hours. The relations between carbon content, nitrogen content, and carotenoid production need to be further assessed. For economic viability, lipid and carotenoid production needs to be increased significantly. The screening of feedstocks should be extended to other wastewaters.

1. Introduction

In the course of the ongoing endeavor to find alternatives for fossil energy, significant effort has been put into expanding the utilization of renewable resources. Accordingly, there is already a considerable variety of products and commodities based on renewable resources available, which are fed both into energetic and material utilization pathways. Especially in the field of energy supply, the progressive depletion of conventional fossil fuels along with a worldwide growing demand for petroleum-based fuels has put high pressure on science and industry to find alternative energy sources [1]. Over the last decade, research has successfully managed to develop a broad range of sustainable and cost-effective techniques to produce renewable energy, one of them being the conversion of biomass into biofuels. While biofuels in general also include, for example, firewood or woodchips for direct combustion and use for heating or electricity production, liquid biofuels are mainly researched in order to replace conventional liquid fuels like diesel and petroleum [1].

Within this class, biodiesel is a renewable fuel, which is produced through the transesterification of oils to generate fatty acid methyl/ethyl esters (FAME/FAEE) [2]. Depending on the origin of the oily feedstock, it is referred to as 1st generation, 2nd generation, or meantime 3rd generation biodiesel.

While 1st generation biodiesel uses plant oils from for example, rapeseed and soybean for transesterification, 2nd generation biodiesel is derived from, lignocellulosic biomass that underlies biological or thermochemical processing thus increasing the land use efficiency [1]. Nevertheless, both approaches amplify the highly discussed competition with

food production either due to the direct use of edible crops for energetic purposes thus causing worldwide rising commodity prices, or because of shifting high-quality agricultural land away from food production to the cultivation of crops for energy production [3]. Biodiesel production of the 3rd generation tries to overcome this conflict by using fatty acids produced by oleaginous microorganisms for the transesterification into the FAMEs/FAEEs. In general, microorganisms that accumulate more than 20–25% of their cell dry weight in the form of lipids are referred to as oleaginous species [4], which include different types of yeast, molds, and algae. Especially the use of oleaginous yeast is a promising approach since they stand out from other heterotrophic microorganisms in terms of fast growth rate and high lipid content [3, 5, 6]. In addition, their lipids are mainly composed of triacylglycerols, which make their chemical characteristics and thus the potential applications comparable to plant oil [3]. Further advantages of yeasts compared to other microorganisms like fungi or microalgae include the low duplication times and the more easily scale up [5]. Commercial oil production using autotrophic algae, for example, might be restricted due to the need for sunlight during the cultivation process [1, 6].

The idea to use oleaginous yeast as source for microbial oil is not new [4]. Also, the various advantages over plants for the production of lipids such as short life cycles, low demand on space and independence of location, season, and climate [3, 5] have been widely recognized. Nevertheless, so far hardly any approach reached commercial realization, because the economics of production cannot compete yet with the comparable low costs of agricultural plant oil production [4]. Moreover, the comparatively costly process of microbial oil production and extraction results in biodiesel, which is considered as a rather low-value product. However, in the last years, interest in microbial oil production increased again due to the serious need for feedstock alternatives and biotechnological progress regarding the efficiency and scale up of the process of lipid production, extraction, and conversion to the desired FAMEs/FAEEs. Still, in order to reach economic feasibility, it is unavoidable to find low-cost and abundant feedstocks as well as low-cost and efficient extraction methods for microbial oil production.

The biochemical pathway of lipid accumulation in oleaginous yeasts is a secondary anabolic activity, which takes place under the preconditions of nitrogen depletion and carbon excess conditions in the culture medium, leading to a "metabolic overflow," where products from carbon degradation are not channeled into cell growth due to the lacking nitrogen, but into the synthesis of cellular storage lipids [7]. Regarding the characteristics of the carbon source, yeasts have only few requirements [6]. Consequently, they can utilize a broad range of substrates ranging from monosaccharides like glucose and xylose and polysaccharides like starch over cellulose hydrolysates to industrial and municipal organic waste with the latter fulfilling the criteria of cheap and vast availability [3]. Against this background, a variety of fermentation substrates have been tested for their ability to serve oleaginous yeast as adequate growth and production media, including, for example, effluent from steam fish processing [8], whey [9], municipal wastewater and sewage sludge [10–12], lignocellulosic materials [13], molasses [14–16], glycerol [17], and starch wastewater [18].

Still these feedstocks, even if renewable and abundant, have some negative aspects. Agricultural by-products like molasses from sugar beet and sugarcane can be used for other purposes like animal feed, thus resulting in a certain conflict of utilization. Other substrates like hydrolysates from lignocellulosic materials require a treatment prior to fermentation thus causing additional cost. That is why this paper looks at substrates—namely, wastewaters from food and beverage industry with a supposable high organic load—that have no alternative utilization and generally do not need an additional pretreatment.

In addition, the fermentation process with the microorganisms could possibly be included in an already existing wastewater treatment process, thus leading to a decrease in both chemical (COD) and biological oxygen demand (BOD) of the wastewater, bearing an additional cost-saving potential [8, 11, 18].

In order to reach the economic feasibility of microbial biodiesel production, the marketing of higher added value products that are formed in the course of microbial fermentation of the feedstock is another option. Depending on the substrate and microorganism used, this can be, for example, specialty chemicals, biosurfactants, or certain pigments. The red yeast *Rhodotorula glutinis* is a very efficient oleaginous yeast, that is able to accumulate up to 70% of its cell dry weight in the form of lipids [19] with a biomass production up to 180 g L^{-1} in rich fermentation media [20]. Additionally the fatty acid profile is rich in palmitic, oleic, and linolenic acid [21] and therefore comparable to vegetable oils allowing for biodiesel production. In addition, it is comparatively unproblematic to handle, being nontoxic and easy to grow and harvest [16]. Furthermore, *Rhodotorula glutinis* is able to produce certain carotenoids, namely, β-carotene, torularhodin, and torulene [22]. Especially, β-carotene, displaying an orange-yellow color, is commercially used as a natural colorant in food, feed, and cosmetic industry and, being the main source of provitamin A, as nutritional supplement [23]. It also finds application in the pharmaceuticals industry due to the ability to act as antioxidant and anticancer agent [24]. The production of carotenoids by *Rhodotorula glutinis* has also already been tested on a broad range of different waste and residue substrates including radish brine [24], whey [25], molasses, grape must, and different flour extracts [26]. Based on numbers of the Brazilian market, 1 kg of pure β-carotene corresponds to US \$600, making it a high-value product [23]. The natural production of carotenoids via microorganisms also takes into account the shift in consumer preferences from synthetically produced towards naturally produced food additives [27].

This shows that the economic restrictions regarding the application of microbial oils as renewable resource for biodiesel production can be overcome by the use of a cheap feedstock and the production of two products including a high value pigment. Since pigments will mostly be used for applications in the food and cosmetic sector, feedstocks like municipal wastewater or sewage sludge, which hold the same

economic advantages as wastewaters from food and beverage processing, have to be ruled out since the mentioned products could hardly be marketed if they are derived from human or animal waste.

In order to combine the production of biodiesel with In order to combine the production of biodiesel with the production of carotenoids as high value by-product and with the utilization of a cheap and abundant carbon source, the goal of this study was to examine cell growth, lipid and carotenoid production of *Rhodotorula glutinis* grown on wastewaters from fruit juice production, potato processing, and lettuce processing.

2. Materials and Methods

2.1. Wastewater. Three different process wastewaters were tested as growth substrate for *Rhodotorula glutinis*. Samples of potato process water were taken from a potato processing company directly after mechanical peeling. Wastewater from lettuce processing was collected after the processing steps of washing and cutting. Samples of fruit juice processing wastewater, mostly from apple processing, were obtained from a small fruit juice producer. This wastewater included all process waters from washing and pressing as well as any runoff water within the production. Chemical oxygen demand (COD) and total nitrogen content (N_t) were determined photometrically using reagent vials from Macherey-Nagel (Düren, Germany). For COD measurement, 2 mL of wastewater were added to the vials and digested for 2 h. COD values were then determined using a spectrophotometer (NANOCOLOR 400D; Macherey-Nagel, Düren, Germany) operated at 620 nm. Total nitrogen was determined according to DIN EN ISO 11905-1 using a wavelength of 385 nm. pH was measured potentiometrically using a pH-meter with glass electrode (SenTix 61, WTW GmbH, Weilheim, Germany) (Table 1). For the determination of lipid production, all wastewaters were sterile filtered prior to inoculation in order to maintain *Rhodotorula glutinis* as only microorganism. The potato wastewater had an initial high starch content, which decreased after sterile filtration. Therefore, 100 mg L^{-1} soluble starch was added to the wastewater according to the difference in starch content prior and after filtration.

2.2. Microorganism, Cultivation, and Analysis. *R. glutinis* (CBS 20) from the collection of the Centraalbureau voor Schimmelcultures (CBS-KNAW, Utrecht, The Netherlands) was used for all experiments. The yeast was grown in a 1000 mL Erlenmeyer flask containing a yeast malt broth (YMA) (3 g L^{-1} yeast extract, 3 g L^{-1} malt extract, 10 g L^{-1} glucose and 5 g L^{-1} vegetable peptone) for 5 days at room temperature on a rotary shaker at 115 rpm. After 5 days, 15 mL of this media was used for inoculation of the wastewaters. The fermentation was carried out in 1000 mL Erlenmeyer flasks containing 500 mL of the respective wastewaters, which were cultivated under the same conditions as the initial YMA for 168 h. For analysis of cell dry mass, lipid content and fatty acid composition 40 mL samples were taken regularly every 24 h. For the analysis of carotenoid content and composition, 40 mL samples were taken at $t_{0\,h}$, $t_{48\,h}$, $t_{96\,h}$, and $t_{120\,h}$. From wastewater media from fruit juice and lettuce processing, additional samples were taken at $t_{168\,h}$. The experiment was carried out in triplicate.

Table 1: Initial chemical oxygen demand (COD), total nitrogen content (N_t), and pH value measured in the collected wastewaters.

Wastewater origin	COD (mg L^{-1})	N_t (mg L^{-1})	pH
Potato processing	13,200	210	7.10
Fruit juice production	1,068	10	8.18
Lettuce processing	885	30.5	7.98

For determination of cell dry weight, the samples were centrifuged at 2750 rpm. The pH of the supernatant was determined, and the supernatant discarded afterwards. Cells were freeze-dried to constant weight. The cell mass was then measured using a calibrated balance.

The lipid content of the cellular material was determined using a modification of the method described by Bligh and Dyer [28]. Cells were disrupted using 12.5 mL methanol added to 5 mL aqueous mixture of water and cell material. Lipids were extracted with chloroform following the procedure described by Zhang et al. [29]. After N_2 evaporation at 50°C for 60 min, the weight of the extracted lipids was determined by a calibrated balance.

Afterwards the microbial lipids from sampling times $t_{0\,h}$, $t_{48\,h}$, and $t_{120\,h}$ were transesterified to FAMEs to allow for chromatographic analysis of the fatty acid composition. Transesterification was carried out using methanol with 2% H_2SO_4 as catalyst at 60°C for 2 h. After quenching the reaction with $NaHCO_3$ solution, the FAMEs were extracted by toluene and analyzed using an Agilent (Santa Clara, CA, USA) 6890 gas chromatograph with flame ionization detector (GC-FID) equipped with a Stabilwax-DA capillary column (30 m × 0.25 mm ID and 0.25 μm film thickness). The different peaks were identified and quantified by comparison to responses from known FAME standards.

The samples for carotenoid analysis were centrifuged at 2750 rpm, freeze-dried, and hydrolysed with 1 N HCl in a water bath at 70°C for 0.5 h [30]. The HCl solution containing the hydrolysed cells was filtered through a 0.7 μm glass fiber filter. The filter paper with the residual cells was thoroughly rinsed with distilled water to remove the acid. The cells were extracted over the night in 35 mL acetone : methanol (1 : 1) solution. The acetone : methanol solvent was evaporated under N_2 blow at 50°C for 60 min, the extract then redissolved in 2 mL acetone, filtered, and prepared for HPLC analysis. For analysis, a Merck-Hitachi (Darmstadt, Germany) system was used, including interface module D-7000, pump L-7100, autosampler L-7200 and DAD detector L-7450A, and an Agilent (Böblingen, Germany) Eclipse Plus C18 column (5 μm; 250 mm × 4,6 mm). A gradient from 70 to 100% acetone was used as the mobile phase [31]. Chromatographic results were quantified by using response factor of β-carotene standards. Three distinct peaks were identified as compared to known retention time profiles

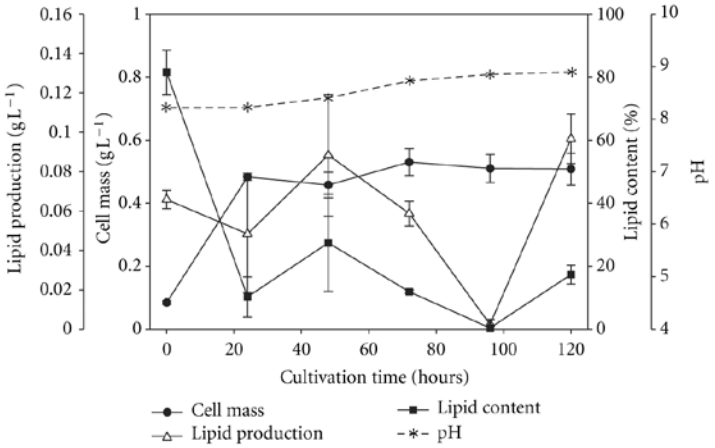

FIGURE 1: Cell mass production, lipid production, and lipid content of *R. glutinis* during cultivation on wastewaters from fruit juice processing.

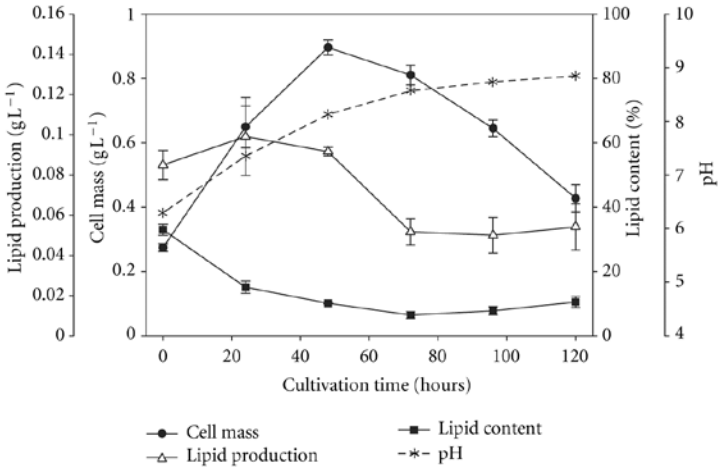

FIGURE 2: Cell mass production, lipid production, and lipid content of *R. glutinis* during cultivation on wastewaters from potato processing.

[22, 31]. Amounts of the three peaks were summed to provide the total carotenoid content.

2.3. Statistical Analysis. All data was subject to statistical analysis using SigmaStat version 10.0 (Jandel Scientific, USA). The statistical significance of differences in mean values of cell mass, lipid production, and lipid content over the course of cultivation time was calculated using single factor variance analysis with the Tukey-Test at the 5% level of probability.

3. Results and Discussion

3.1. Cell Growth of R. glutinis Grown in Wastewater Media. As shown on Figures 1–3, *R. glutinis* realized a significant increase in cell mass production on all wastewaters in the first hours of cultivation. Grown on the wastewater from fruit juice processing (Figure 1), cell mass rapidly increased sixfold from 0.08 g L^{-1} to 0.48 g L^{-1} during the first 24 h of cultivation and more or less stagnated for the remaining growth time with only slight but not significant increases to a maximum of 0.59 g L^{-1} at 96 h. Wastewater from potato processing (Figure 2) also allowed for fast and significant cell growth until 48 h of cultivation, reaching a 3.5 higher weight (0.91 g L^{-1}) compared to the initial cell mass (0.26 g L^{-1}). After this peak, cell mass constantly decreased to a final value of 0.51 g L^{-1} at t_{120}. When grown on wastewater from lettuce processing (Figure 3), cell mass increased rather slowly from 0.11 g L^{-1} (t_0) to 0.31 g L^{-1} (t_{72}) until t_{72} and stagnated after this. Due to different compounds in the wastewaters and possible differences in the cell mass of the inoculum, the initial cell mass concentration of the wastewaters was not the same for all three wastewaters. When compared on a relative

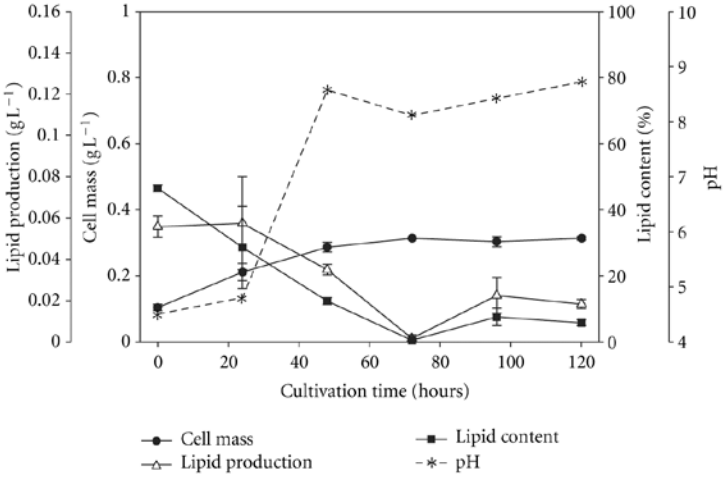

FIGURE 3: Cell mass production, lipid production, and lipid content of *R. glutinis* during cultivation on wastewaters from lettuce processing.

basis, *R. glutinis* realized the highest increase of cell mass on wastewaters from fruit juice processing. In this case, cell mass increased sixfold, whereas it only increased ca. threefold regarding wastewaters from potato and lettuce processing. These different patterns of cell growth are probably caused by the different levels and characteristics of the carbon sources available from the wastewaters. HPLC sugar analysis showed that fruit juice wastewaters contained small amounts of easily degradable glucose (0.31 g L^{-1}) and xylose (0.47 g L^{-1}), allowing for a quick and strong increase in cell mass during the first 24 h of cultivation. After this period, the carbon in the wastewater was exhausted leading to stagnation in cell growth. For wastewaters of potato and lettuce processing, HPLC analysis could not detect any glucose or xylose. Nevertheless, COD measurement showed that some organic but probably less degradable substances were present in the wastewaters, leading to lower and slower growth rates. This could also explain the similarity of relative growth rates realized on potato and lettuce processing wastewaters, despite the high difference in measured COD values.

3.2. Lipid Production of R. glutinis Grown in Wastewater Media. None of the wastewaters allowed for significant lipid production by *R. glutinis*. When grown on wastewaters from potato (Figure 2), lettuce (Figure 3), and fruit juice processing (Figure 1), the initial lipid content, the initial lipid content, which is explained by the lipid saturated cells of the inoculum, constantly decreased. The lipid content was calculated as ratio of cell mass and lipid production. Thus, in the first cultivation period, the decline in lipid content was probably mostly caused by the increase of cell production along with a stagnation in lipid production. On the contrary, during the second phase of cultivation, cell production stagnated while the lipid production decreased.

Oleaginous microorganisms start to produce lipids when a growth required nutrient, mostly nitrogen, is exhausted in the medium [1, 7]. The excess carbon is then channeled into lipid production. Accordingly, it has been shown that the lipid content is constantly low when nitrogen is present in the culture medium [32]. Thus, the carbon to nitrogen ratio (C/N ratio) of the culture medium is a very important parameter for the potential lipid production. As shown in Table 1, wastewaters from fruit juice and lettuce processing contain only a small amount of carbon as reflected by the low COD value. With comparatively low total nitrogen content of 10 mg L^{-1}, it can be assumed that the fruit juice wastewater has a beneficially high C/N ratio. Wastewater from lettuce processing showed a lower COD combined with a higher nitrogen content therefore indicating a lower C/N ratio. However, the increases in cell mass in Figures 1 and 3 suggest that nitrogen was still available and the carbon sources were used in favor to cell production. The stagnation of cell growth along with the low lipid production indicated that most of the available carbon was exhausted before nitrogen became the growth limiting factor. The wastewater from potato processing is characterized by a relatively high COD value (Table 1). However, with simultaneously high total nitrogen content, this leads to an unfavorable C/N ratio, which probably hindered lipid production. Also, here, it can be assumed that the carbon in the media was consumed before nitrogen was exhausted. Under favorable conditions regarding carbon and nitrogen content, *R. glutinis* was observed to display an *S*-shaped growth curve including a lag phase followed by an exponential phase and a stationary phase [18, 29]. In this context, Xue at al. [18] suggest an initial COD concentration of over 30,000 mg L^{-1} to allow for lipid production which indicates that the COD of all chosen wastewaters in this experiment was to low to facilitate the classical course of lipid production.

As described by Papanikolaou and Aggelis [7], previously stored lipids are to some extent degraded to generate lipid-free material (lipid turnover) when the carbon sources in the fermentation medium are completely exhausted. The same observation could be made in this study, since lipid content

further decreased after cell growth stagnated. The variability of lipid production in Figure 1 can be explained by the various anabolic and especially catabolic processes during cultivation. In the course of cell death and cell decay, new carbon sources are released into the media to some extent. In addition, the fluctuations of data for lipid production are ranging on a very low scale between 0 and 0.1 g L^{-1} and are also characterized by a high variance as reflected by the standard error.

The pH value is also an important factor influencing lipid production. Ranging between pH 7 and pH 8 (Table 1), all wastewaters were slightly alkaline. Since the medium was not buffered, the pH further increased in the course of cultivation. This could be due to organic acids being consumed from the medium. Since the optimum pH values for lipid accumulation of yeast cells were reported to be between pH 5.0 and 6.0 [14], the high pH value of the wastewaters could be another limiting factor for lipid production. In subsequent experiments, the impact of pH adjustment and pH buffering during cultivation through the addition of alkaline (NaOH) and acidic (HCl) solutions should be investigated.

However, the most important limiting factors for lipid production in the screened wastewaters seem to be the low content of available carbon sources in fruit juice and lettuce processing waters and the high content of nitrogen in potato processing waters, respectively. Potential options to overcome these inhibitory factors could be the supplementation with additional carbon sources (e.g., monosaccharide) in order to increase the carbon content on the one hand or the reduction of nitrogen content on the other hand. However, it has to be carefully assessed if these actions would still be consistent with the premise of economic feasibility. Likewise, the screening of feedstocks has to be extended to other wastewaters like wastewaters from vegetable processing (especially blanching water), sugar refining, and beer production. Even though lipid production and lipid content were low, all wastewaters met the general criteria for microbial oil production. There was no evidence for the existence of any inhibitory wastewater components found.

Regarding the aspect of economic viability, it is difficult to determine a certain minimum necessary lipid production target, since this is strongly influenced by the substrate and the cost of the selected process to convert the oleaginous microorganisms into biodiesel. Mondala et al. [33] calculated for their work with activated sewage sludge that a lipid content of approximately 10% (wt/wt) is necessary to produce biodiesel at a break-even price of approximately \$3.20 per gallon. This price would be economically competitive with that of petroleum-based diesel which is around \$3.82 per gallon (US Energy Information Administration, January, 2012) and with that of conventionally produced biodiesel (B 100) which is around \$4,18 per gallon (US Department of Energy, October 2011).

According to Revellame et al. [34], a biodiesel yield of more than 10% (wt/wt) will make the utilized feedstock cheaper than petroleum-based diesel (Figure 4). While in latter papers, the process of *in situ* transesterification was taken as the basis for calculation, this study uses the conventional process of drying, oil extraction, and conversion. Accordingly, it is difficult to apply these results to the approach described in this paper. Nevertheless, looking at the obtained results in this study, the lipid production needs to be significantly increased in order to reach a financially viable stage, even if the carotenoids as high value by-products are taken into consideration.

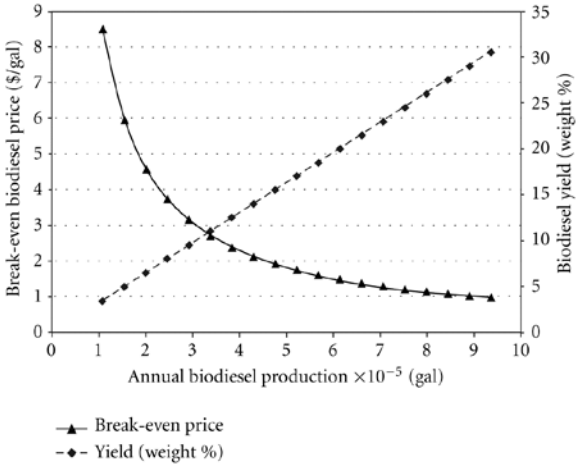

FIGURE 4: Influence of yield on the break-even price of biodiesel from wet activated sludge [34].

3.3. Fatty Acid Composition of Microbial Oils Produced in Wastewater Media. The gas chromatographic analysis of the FAMEs from microbial lipid showed a significant increase in the FAME content in the first 48 hours of cultivation for all three wastewaters (data not shown). The FAME composition, shown exemplary for sampling time t_{48} in Figure 5, also varied in the course of cultivation, whereas it was always predominated by long chain C 16 and C 18 fatty acid methyl esters, with oleic (C 18 : 1) and palmitic (C 16 : 0) acid methyl ester being the most important. This makes the properties of the microbial lipid comparable to plant oil, which mainly consists of palmitic and oleic acid [35]. Especially when wastewater form fruit juice and lettuce processing was used as growth substrate, linoleic (C 18 : 2) acid methyl ester was a main component of the overall FAME content as well. This is in accordance with Papanikolaou and Aggelis [7], who state that yeast lipid, which was produced utilizing sugar-based substrates, is generally composed of C 16 and C 18 fatty acids, while oleic acid (C 18 : 1) is the principal fatty acid followed by linoleic (C 18 : 2) and palmitic (C 16 : 0) acid. For the specific strain of *R. glutinis* (CBS 20) used in the present study, this fatty acid profile has been confirmed as well [36]. When grown on wastewaters from potato processing, the FAME composition also exhibited a high percentage of palmitoleic (C 16 : 1) and stearic (C 18 : 0) acid methyl esters (Figure 5).

3.4. Carotenoid Production of R. glutinis Grown in Wastewater Media. The total carotenoid production per Liter of

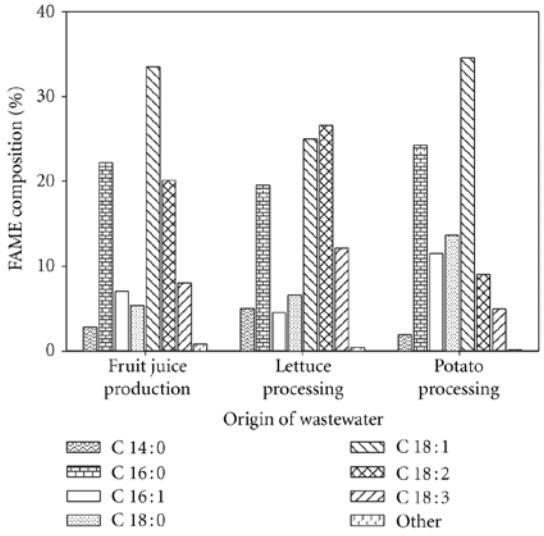

FIGURE 5: FAME composition of transesterified microbial lipid after 48 hours of cultivation with *R. glutinis* on different wastewaters.

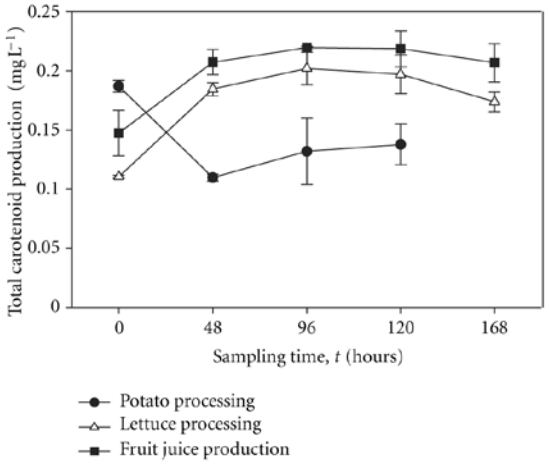

FIGURE 6: Total carotenoid production of *R. glutinis* during cultivation on wastewaters from potato, lettuce, and fruit juice processing.

wastewater in course of cultivation as displayed in Figure 6 showed great similarities for growth on wastewater media from fruit juice and lettuce processing. Grown on fruit juice wastewater media, *R. glutinis* realized a significant increase of almost 50% from 0.15 mg L^{-1} to 0.22 mg L^{-1} within the first 96 h of cultivation. Grown on lettuce wastewater the relative increase was 66% from 0.11 mg L^{-1} to 0.18 mg L^{-1} within the first 48 h. The increases were followed by a stagnation of carotenoid production for both wastewaters. On the contrary, the results from cultures grown on potato processing wastewater displayed a decrease of carotenoid production in the first 48 hours, followed by stagnation at around 0.13 mg L^{-1}. Yet, the differences in total carotenoid production were not at a significant level. Regarding the carotenoid composition, the wastewaters varied slightly from each other at the inoculation time t_0. After 48 h of cultivation, the shares of the different carotenoids remained at constant proportions.

Again the similarities between wastewaters from fruit juice production (32% β-carotene, 39% torulene, 29% torularhodin) and lettuce processing (33% β-carotene, 42% torulene, 25% torularhodin) could be observed, while results from potato processing wastewaters (43% β-carotene, 13% torulene, 44% torularhodin) differed.

In consideration of the initially measured wastewater properties COD, total *N*, and pH (Table 1), it is difficult to evaluate these results, since the particular influence of these parameters on carotenoid production on *R. glutinis* has not been fully investigated yet. Of course the availability of a carbon source is a prerequisite for all metabolic processes. Thus, the limited amount of carbon in fruit juice and lettuce processing wastewaters probably caused the early stagnation of carotenoid production and the associated comparatively low level of carotenoid content. Accordingly as already stated for lipid production, the tested wastewaters have to be improved if they should serve as medium for lipid and carotenoid production. However, although both lipid and carotenoid biosynthesis have the same precursor [37], results cited in literature are somewhat ambiguous when it comes to the influence of carbon and nitrogen source and the related C/N ratio on carotenoid production. While some authors [37, 38] report an enhancement in carotenoid production with increasing C/N ratio, others [39] found the opposite. Tinoi et al. [40] found that both increases in nitrogen and carbon source led to an increase in carotenoid content. The interactions between carbon and nitrogen source could also help to explain the drop of carotenoid production when grown on potato processing wastewaters. Based on the COD value, it can be suggested that carbon was available in sufficient quantities. Thus, it was probably the amount of nitrogen which influenced the carotenoid production or other substances in the wastewater that hindered carotenoid formation. Also, the relatively high pH value of the wastewaters could be a reason for the poor carotenoid production, since the optimum is reported to be around pH 6 [40]. Accordingly, it needs to be further investigated how carbon and nitrogen contents are related to carotenoid production before wastewaters can be effectively amended or specifically screened regarding their carbon and nitrogen content. If lipid and carotenoid production should be combined, it also needs to be considered that the optimization of process parameters and wastewater properties for the improvement of lipid yield will most likely be at the expense of carotenoid yield and vice versa.

4. Conclusion

All wastewaters showed the essential properties, which are mainly the presence of a degradable carbon source and the absence of any inhibitory substances, to allow for growth and second metabolism production of *R. glutinis* for the production of biodiesel and high-value pigments. Nevertheless, lipid

production was very low and lipid turnover processes already started in early stages of cultivation time. This indicated that the amount of degradable carbon in the wastewaters was too low, especially in wastewaters from fruit juice and lettuce processing, using all carbon for cell proliferation processes at the onset of cultivation when nitrogen was still available. Also, in the case of potato processing wastewater, where the COD and accordingly the carbon content was expected to be comparably higher, no significant lipid production was observed, probably owing to the high nitrogen content. Thus, if lipid production should be enhanced, the wastewater properties have to be modified regarding their carbon and nitrogen content, respectively. This could be achieved by the supplementation with additional low-cost carbon sources (e.g., biomass hydrolysates) or the removal of nitrogen through chemical or biological measures but yet needs to be in accordance with the aim of financial viability. Also, the reduction of initial pH and buffering of the pH during cultivation could be a way to enhance lipid and carotenoid production. The production of carotenoids was quantitatively confirmed, showing the general compatibility of lipid and carotenoid production. However, the influence of carbon and nitrogen content on carotenoid production needs to be further investigated in order to optimize both lipid and carotenoid production. For economic feasibility, lipid and carotenoid production needs to be increased significantly. Likewise, the screening of wastewaters should be extended with additional promising wastewaters like wastewaters from sugar refining, vegetable processing (i.e., blanching water), or beer production.

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4 Chapter II

Lipid and carotenoid production by oleaginous red yeast *Rhodotorula glutinis* cultivated on brewery effluents

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The results from the first chapter showed, that all three examined wastewaters were not able to substantially support the production of lipids and/or carotenoids by R. glutinis. However, it could be assumed, that the wastewaters did not contain any inhibitory substances and that they provided all necessary micro- and macronutrients for yeast growth. The major drawback was the limitation of yeast available carbon. For the wastewaters from fruit juice and lettuce processing this was in accordance with the low COD values. However, for wastewater from potato processing higher COD values were determined. Since the content of easy available monosaccharides remained on a low level, it was suggested that organic carbon was present in the form of starch, which was not utilized by R. glutinis. As a result, the range of wastewaters was extended by brewery effluents. Based on the literature and the characteristics of the brewing process it was assumed that these effluents have a high organic load and that the carbon is available in form of easy degradable mono- and disaccharides. However, after a first measurement of the COD of the end-of-pipe wastewater which resulted in a value as low as 6000 mg L^{-1}, it was decided to sample wastewaters from specific production processes. The process wastewater after lautering, where wort and spent grains are separated, showed a significantly higher COD value and thus was selected as cultivation medium for the subsequent experiment. In order to ensure an adequate supply with carbon, next to the raw, unaltered wastewater a second experimental treatment was introduced where the wastewater was additionally supplemented with glucose. By this supplementation it could also be examined if the wastewater served as a suitable source of nutrients for microbial growth.

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Lipid and carotenoid production by oleaginous red yeast *Rhodotorula glutinis* cultivated on brewery effluents

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ABSTRACT

This study investigated the production of microbial lipids for biodiesel production and high-value carotenoids by *Rhodotorula glutinis* combined with the use of brewery wastewater as carbon source for three treatments: (raw wastewater) WW_{raw}, (glucose supplemented raw wastewater) WW_{glu} and a (synthetic sugar medium) WW_{synth}. The collected brewery effluents showed high contents of sugars (maltose 24.34 g L^{-1}; glucose 5.77 g L^{-1}), but the low utilization of maltose led to a limitation of carbon in WW_{raw} and WW_{glu}. Since nitrogen was still available, carbon was channeled into cell growth instead of lipid formation, reaching an overall biomass production of 5.22 g L^{-1}, 7.38 g L^{-1}, and 9.55 g L^{-1}, respectively. Carotenoids were synthesized in all treatments with total average carotenoid contents between 0.6 and 1.2 mg L^{-1} and with high proportions of β-carotene (~50%) in the wastewater treatments. Suboptimal culture conditions (pH; aeration) have been identified as obstacles for higher lipid and carotenoid yields. Nevertheless, brewery wastewaters can be considered as carbon source for microbial fermentation, since they can be assumed to be an adequate source of nitrogen and other nutrients, whereas the utilization of maltose needs to be increased to achieve considerable amounts of lipid and carotenoid production.

1. Introduction

The worldwide energy consumption has been amplified in the past decades, mainly due to factors of population growth and economic development attended with an increasing level of mechanization [1]. To cover this demand mainly non-renewable fossil fuels such as petroleum, coal and natural gas are exploited. Especially in the transport sector almost the entire energy consumption (~95%) is covered by petroleum based fuels [2]. However, the finiteness along with the uneven distribution of this resource, mostly in favor to politically instable regions, has led to issues related with the security of energy supply as well as a strong volatility of the oil price [3]. So the scientific and political interest into biofuels as renewable alternatives to fossil fuels increased and was accelerated by events as for example the first oil crisis of the 1970s. More recently the aspect of the climate change mitigation potential of biofuels has led to a vital investment in the development of biofuel applications [4].

Among the renewable biofuels, biodiesel is produced by the transesterification of natural triglycerides typically from plant oils or animal fats with an alcohol – mostly methanol – into fatty acid alkyl (methyl) esters [5]. The biodiesel from this process has similar characteristics to that of mineral diesel. Taking into account certain advantages, e.g. a better lubricity and a higher cetane value and disadvantages e.g. lower energy content, higher cloud point and degradation problems of biodiesel, its overall fuel economy can be compared to conventional diesel [4]. Based on the oily feedstock, which is used for the transesterification process, biodiesel can be classified into certain categories. So-called 1st generation biodiesel is mainly derived from vegetable oils from crops like rapeseed or soybean and is globally produced at a significant commercial scale. 2nd generation biodiesel is produced using oils from non-edible plants (e.g. *Jatropha* or *Ricinus*), whereas 3rd generation biodiesel is considered as processing oils from certain microorganisms (e.g. microalgae or yeast) [6]. Due to the previously described driving forces, the global production of 1st generation biodiesel has

Abbreviations: FAME, fatty acid methyl ester; COD, chemical oxygen demand; SCO, single cell oil; GHG, greenhouse gas.

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increased significantly in the last years, with Germany and the US being the biggest producers. At the same time, despite this substantial growth, the share of biofuels in general and of biodiesel in particular in total transportation fuel demand is quite low, with only Sweden (ca. 2.5%), Cuba (ca. 6.5%) and Brazil (ca. 13.5%) yielding more than 2% in 2006 [7]. However, owing to specific governmental incentives this share could be increased and is targeted to further increase. For example, in Germany the biofuel share accounted for 5.8% (biodiesel share 4.3%) of total primary fuel consumption in 2011 [8]. This is in accordance with the 2003 EU Biofuels Directive, which defined a biofuel share of 5.75% in 2010 and 10% in 2020 as future goals. Despite soaring oil prices and continuously declining cost of biofuel production this can only be reached by subsidies e.g. in the form of tax exceptions.

However, the production of first generation biodiesel does not come without problems. Since currently food crops like rapeseed and soybean are the major feedstock for biodiesel in Europe and the United States, respectively [9], biodiesel production was partially made responsible for globally rising commodity prices, enforcing the controversy about the conflict between food and fuel production [4,6,10]. To decrease the direct competition with food production 2nd generation biodiesel was introduced, which, in terms of the feedstock, substitutes food crops with non-edible crops. Additionally, through the utilization of the whole plant biomass, a higher land use efficiency can be achieved. Still there are concerns about competition over arable land and land use changes required for these feedstocks [6]. Around 1% of the arable land is used for the production of biofuels [7]. Looking at the biofuel production targets it can be assumed that this share will increase substantially in the future, which will lead to an even more keen competition between biodiesel and food industry over limited land availability. Considering 2nd generation feedstocks, which are considered to be able to grow on marginal sites, it will be necessary to convert uncultivated land areas into arable sites for crop production. If this aspect of land use change is taken into account, also the potential of biofuels to save (greenhouse gas) GHG emissions has to be questioned. If land use changes are not considered within the life-cycle analysis of biodiesel, the GHG reductions compared to conventional diesel range between 40 and 80%, depending on the feedstock and the different assumptions used for the study. If the effects from land use change are considered, the total GHG emissions actually increase [4]. Additionally Schenk et al. [11] calculated that even if current oilseed crops would be cultivated on all available arable land, they would only provide enough feedstock to cover less than half of the present energy demand.

This makes clear that further approaches for a sustainable and environmental friendly biodiesel production need to be explored. Accordingly microbial oils have gained substantial interest as new feedstock for a 3rd generation of biodiesel. These so-called (single cell oils) SCO, mostly consisting of (triacylglycerols) TAG, are extracted from oleaginous microorganisms, which are defined as accumulating more than 20% of their cell dry weight in the form of lipids [12]. The basic precondition for oil accumulation is a nutrient limitation (mostly nitrogen) in the growth medium accompanied by excess carbon conditions, so that carbon is channeled into lipid synthesis rather than in the formation of biomass [13]. These microorganisms (yeast, fungi and algae) and their ability to synthesize lipids are already known for several decades but only recently, due to better and less expensive technological and biotechnological opportunities, gained new interest regarding the exploitation of SCO for biodiesel production [14]. The utilization of microorganisms, especially yeasts, for lipid production offers various advantages, including short life cycles, low demand on space and independence of location, season and climate [10,15]. However, no approach has reached commercial realization yet due to the high cost of the process. This is particularly challenging since microbial oils have to economically compete against comparatively cheap (vegetable oils) and highly price-volatile (crude oil) diesel feedstocks. So, for example Ratledge and Cohen [13] calculated a price of US$ 3000 per tonne yeast SCO, which is around double the price of common rapeseed oil. For 2006 Chisti [16] assumed a production cost of US$ 1.40–2.80 per liter algae derived oil, which summed up to US$ 1.54–2.94 L^{-1} algal biodiesel, on the contrary of US$ 0.66 per liter palm oil derived biodiesel and US$ 0.49 (taxes and distribution cost excluded) per liter conventional diesel. The main cost of microbial cultivation (up to 80%) is caused by the required carbon source. Thus, the utilization of a cheap and abundant organic carbon source can substantially contribute to the cost reduction of the overall approach [17]. Accordingly, several low cost fermentation substrates like glycerol [18,19,64], palm oil mill effluent [20], municipal wastewater [21], molasses [22], starch wastewater [23], and cellulose hydrolyzates [24] have already been tested successfully for their ability to support growth and lipid production by oleaginous yeasts. However, even if low-cost carbon sources are used as substrate, up to now microbial biodiesel production cannot compete with conventional petroleum diesel on a commercial scale, due to the high cost of lipid production, extraction and conversion.

To overcome these economic constraints this study looks at the possibility of the simultaneous production and the marketing of additional high value added products. For this purpose the red yeast *Rhodotorula glutinis* was selected for the present study. Under optimal conditions regarding nutrient and carbon supply, type of carbon source (preferably glucose) as well as other cultivation parameters, this yeast can achieve a maximum lipid content of 72% [13,25] with a fatty acid profile similar to plant oil [20]. In experiments with starch wastewater [23] lipid contents up to 40% of cell dry weight have been reported. Additionally, this yeast is able to produce certain carotenoids, namely β-carotene, torularhodin, and torulene, in the course of microbial fermentation [26]. Carotenoids are important natural pigments of increasing economic significance, which are commercially used as colorant for food, feed, and cosmetic products, as nutritional supplement due to their pro-Vitamin A character and in pharmaceutical products for their anticancer and antioxidant properties [27,28]. The production of carotenoids by *R. glutinis* has also already been confirmed on several waste and residue based fermentation substrates such as flour extracts, grape must, molasses [29], radish brine [27], and whey [30]. Through the combined production of biodiesel and high-value carotenoids the overall approach of microbial product formation could be brought one step closer to economic feasibility and commercial realization.

All previously mentioned fermentation substrates exhibit the feature of cheap and vast availability. However, some substrates like molasses from sugar beet and sugar cane or hydrolyzates from lignocellulosic materials can be used for alternative purposes like animal feed which increases the price of the carbon source or require a thermochemical or biological treatment prior to fermentation increasing the production costs, respectively. Other substrates, which are derived from human or animal waste, like sewage sludge or municipal wastewaters, can hardly be used in applications related to human nutrition or in the cosmetic industry, due to consumer preferences and requirements. That is why this study looks at wastewater from beer production, which has no alternative utilization, generally does not need any additional pretreatment and should not cause any concerns when used in processes related to human consumption.

Wastewaters from food and beverage industry in general have high organic contents, reflected by a high chemical (COD) and biological (BOD) oxygen demand. If released into the environment

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without sufficient treatment, these wastewaters can cause severe pollution. Consequently there are certain statuary thresholds for wastewaters that are disposed into public drainage systems or directly into surface waters. On the other hand the high nutrient and carbon load leads to the assumption that these industrial wastewaters could serve as an adequate substrate for microbial growth. Accordingly, the microbial cultivation could be integrated in and contribute to in-company wastewater treatment systems and thus offer a certain cost saving potential with energy recovery. A reduction of both COD and BOD through microbial fermentation has been reported before [23,31]. The COD of brewery wastewaters ranges between 2000 and 6000 mg L^{-1} [32,33], unlike a COD of 110 mg L^{-1} as legally set limit for wastewater disposal in Germany. Effluents from specific processes (e.g. wort separation) can exhibit considerably higher COD values from 15,600 [34] up to over 50,000 mg L^{-1} [35] with high contents of dissolved sugars, mainly maltose. The possibility of fungal growth on these effluents has already been confirmed [35].

The objective of this study was to examine cell growth and second metabolism production of lipids and carotenoids by *R. glutinis* cultivated on actual brewery effluents and synthetic media. Also the rate of COD and nitrogen removal was observed, with special focus put on the degradation of maltose as main carbon source in brewery wastewaters.

2. Materials and methods

2.1. Wastewater and media

Process wastewater from beer production was collected at a local brewery. Samples were taken after the process of lautering, in which wort and spent grain are separated. After filtering, the spent grains are rinsed with water, to ensure that the maximum amount of sugar is transferred to the wort. This process always leads to certain amount of excess water. Samples were taken from this excess water before it was channeled into the wastewater system. To identify basis characteristics of the wastewater, (chemical oxygen demand) COD, (total nitrogen) content N_t and (ammonium content) NH_4^+-N were determined photometrically using reagent vials from Macherey–Nagel (Düren, Germany). For COD measurement, 0.2 mL of wastewater were added to the vials and digested for 2 h at 148 °C. COD values where then determined using a spectrophotometer (NANOCOLOR 400D; Macherey–Nagel, Düren, Germany) operated at 620 nm. Total nitrogen was measured at a wavelength of 385 nm, according to DIN EN ISO 11905-1. The pH was measured potentiometrically using a pH-meter with glass electrode (SenTix 61, WTW GmbH, Weilheim, Germany).

Sugars, such as sucrose, maltose, glucose, and fructose were determined via HPLC. The sum of these sugars is later referred to as total sugar content. Measured characteristics of the wastewater were: COD 40 g L^{-1}, (total nitrogen) content N_t 210 mg L^{-1}, pH 7, maltose 24.34 g L^{-1}, glucose 5.77 g L^{-1}, sucrose 4.01 g L^{-1}, and fructose 0.30 g L^{-1}. This raw wastewater was autoclaved and taken as media for the first treatment (**WW_{raw}**). For treatment 2 (**WW_{glu}**) the raw wastewater was supplemented with 9.38 g L^{-1} glucose in order to adjust the COD to 50 g L^{-1}, assuming that 1 g glucose equals a COD of 1066 g L^{-1}.

In order to have a direct comparison to an artificial medium, in treatment 3 (**WW_{synth}**) a synthetic sugar medium was composed based on the total nitrogen value (210 mg L^{-1}) of the raw wastewater and, same as in treatment 2, a COD of 50 g L^{-1}. It contained (per liter) 1.0 g yeast extract; 1.0 g $Na_2HPO_4 \cdot 12H_2O$; 1.0 g KH_2PO_4; 0.4 g $MgSO_4 \cdot 7H_2O$; 0.455 g NH_4SO_4; 46.9 g glucose; 6 mL $FeSO_4$ solution (4 g L^{-1} $FeSO_4 \cdot 7H_2O$), and 10 mL trace mineral solution. The trace mineral solution consisted of (per liter) 3.6 g $Ca_2Cl_2 \cdot 2H_2O$; 0.75 g $ZnSO_4 \cdot 7H_2O$; 0.13 g $CuSO_4 \cdot 5H_2O$; 0.5 g $MnSO_4 \cdot H_2O$; 0.13 g $CoCl_2 \cdot 6H_2O$, and 0.17 g $Na_2MoO_4 \cdot 2H_2O$. Based on a nitrogen content of 21.205% in ammonium sulfate and 11.45% in yeast extract this would sum up to a nitrogen content of 210.98 mg L^{-1}.

All solutions were autoclaved prior to inoculation to maintain *R. glutinis* as only microorganism. To eliminate inhibitory effects due to non optimal pH values, the pH in all solutions was adjusted to 5.5 prior to autoclaving using HCl (1 mol L^{-1}). Additionally, when WW_{synth} showed a strong drop in pH in the first stage of cultivation, NaOH was added after 72 h to buffer the medium. The experiment was carried out in triplicate.

2.2. Microorganism, cultivation and analysis

R. glutinis (ATCC 15125) was grown in a 1000 mL Erlenmeyer flask containing a (yeast malt broth) YMA (3 g L^{-1} yeast extract; 3 g L^{-1} malt extract; 10 g L^{-1} glucose; 5 g L^{-1} vegetable peptone) for 5 days at 25 °C on a rotary shaker at 115 rpm. This media was then used as inoculum for the wastewater treatments, which were fermented in 1000 mL Erlenmeyer flasks, containing 500 mL of the respective wastewater media. All treatments were cultivated on a rotary shaker (115 rpm) at 25 °C for 168 h. Samples for lipid extraction and determination of lipid content were taken regularly every 24 h between 0 and 120 h. The carotenoid content was measured at the four cultivation times 0 h, 48 h, 96 h, and 168 h.

Cell dry mass was determined both prior to lipid and carotenoid extraction. For this, the samples of the cultivation media (40 mL for lipid extraction; 50 mL for carotenoid extraction) were centrifuged at 3000 rpm for 10 min, freeze-dried and weighted. The supernatant after centrifugation was used for the determination of pH, COD, total N, NH_4^+-N, and sugar composition and afterwards discarded. Since the wastewater contained sediments and residuals that contributed to the overall cell dry mass, the actual biomass production was calculated by the difference between the cell pellets at the respective sampling times and the weight after 0 h. [Biomass production at t_n = Cell pellet weight at t_n − (Cell pellet weight at t_0 − 0.39 g L^{-1})]

With 0.39 g L^{-1} being the cell pellet weight after 0 h of treatment WW_{synth} which is taken as value for the inoculum derived cell mass. These data are later referred to as biomass production.

The lipid content of the dried cell pellet was determined gravimetrically using a modification of the method by Bligh and Dyer [36]. Cells were therefore disrupted with methanol and lipids extracted with chloroform following the procedure described by Zhang et al. [37]. After solvent evaporation under nitrogen blow at 50 °C the weight of the remaining extract was determined by a calibrated balance. This extract from the Bligh and Dyer procedure is later referred to as total lipids. Afterwards the microbial lipids from sampling times 0 h, 48 h, and 120 h were transesterified to FAMEs to allow for chromatographic analysis of the fatty acid composition. Methanol with 2% H_2SO_4 as catalyst was used for the transesterification process which was carried out in a waterbath at 60 °C for 2 h. The reaction was quenched with $NaHCO_3$ solution, the FAMEs were extracted with toluene, stabilized with butylated hydroxytoluene and analyzed using an Agilent 6890 (gas chromatograph with flame ionization detector) GC-FID equipped with an Agilent J&W GC capillary column (30 m × 0.252 mm ID and 0.25 μm film thickness). The different peaks were identified by comparison to responses from a known FAME standard (Supelco® 37 Component FAME Mix, SigmaAldrich, München, Germany). For percentage FAME composition all identified fatty acid peaks were summed up and set as 100%. For quantitative determination of FAME production, the peak areas of fatty acids in the samples were compared with the peak areas of fatty acids in a known amount of FAME standard.

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For carotenoid analysis the freeze-dried cell pellets were hydrolyzed with HCl (1 mol L^{-1}) in a waterbath at 70 °C for 0.5 h [38]. Afterwards the HCl solution was filtered through a 0.7 μm glass fiber filter. The pigmented cells remained on the filter and were thoroughly rinsed with distilled water to remove the acid. The cells were then extracted overnight in 30 mL acetone:methanol (1:1) solution. The solvent was afterwards evaporated under N_2 blow at 50 °C, the extract redissolved in acetone and analyzed using a Merck-Hitachi HPLC system with DAD detector equipped with an Agilent Eclipse Plus C18 column (5 μm, 250 mm × 4.6 mm). A gradient from 70 to 100% acetone was used as the mobile phase [39]. Chromatographic results were quantified by using response factor of β-carotene standards. Three distinct peaks were identified as compared to known retention time profiles [27,39]. Amounts of the three peaks were summed to provide the total carotenoid production.

All data was statistically analyzed using Sigma Stat 3.5 (Jandel Scientific, USA). The statistical significance of differences in mean values of cell mass, lipid production, lipid content, carotenoid production and FAME production in course of cultivation time was calculated using single factor variance analysis with the Tukey-Test at the 5% level of probability.

3. Results and discussion

3.1. Sugar consumption, cell growth and COD removal

As shown in Table 1, the COD values measured at the inoculation time in WW_{glu} and WW_{synth} were 39.4 g L^{-1} and 36.4 g L^{-1} respectively, indicating that the initial COD contents of the different media could not be adjusted as calculated. The same problem appeared with the total nitrogen content which was meant to be 210 mg L^{-1} in all three treatments. These deviations can be explained by microbial processes in the wastewater after measurement and prior to autoclaving or the process of autoclaving itself, especially for nitrogen since it is known to degrade at high temperatures.

When the carbon content was directly calculated from the total sugar content, WW_{glu} (16.66 g C L^{-1}) and WW_{synth} (17.32 g C L^{-1}) showed similar values, leading to an overall C/N ratio of around 100:1 for both treatments. The C/N ratio for WW_{raw} was 55:1. However, as the results regarding the sugar consumption (Fig. 1) indicate, not all detected sugars were utilized by *R. glutinis*. In all treatments the monosaccharides glucose and fructose were readily consumed with an average rate of 5.5 g, 3.27 g, and 5.89 g of glucose per 24 h of cultivation time in treatments WW_{raw}, WW_{glu}, and WW_{synth}, respectively. The sucrose content was not significantly reduced in any of the treatments. Also the utilization of the disaccharide maltose as main carbon source in brewery wastewaters was only unsatisfactory. Only in WW_{raw} a slight but significant decrease of maltose content of 1.42 g L^{-1} between 72 h and 120 h could be observed. This means that in the present experiment maltose was not available as carbon source for microbial growth and production processes. If in this context the carbon content of the wastewater treatments is calculated again, based on the amount of the actual utilized sugars glucose and fructose, WW_{raw} yields a C/N ratio of 13:1 and WW_{glu} a C/N ratio of 33:1, while the C/N of WW_{synth} remains at 103:1. Since fructose was only detected in very small quantities, these results suggest that *R. glutinis* was de facto growing under carbon limited conditions once glucose was exhausted, which was the case after 24 h in WW_{raw} and after 96 h in WW_{glu}. In the synthetic sugar medium glucose was available throughout the cultivation with 8 g L^{-1} remaining after 168 h.

Table 1
(Chemical oxygen demand) COD, (total N) N_t, (ammonium N) NH_4^+-N and sugar content of wastewater media at (inoculation time) t_0.

	COD (g L^{-1})	N_t (mg L^{-1})	NH_4^+-N (mg L^{-1})	Sucrose (g L^{-1})	Maltose (g L^{-1})	Glucose (g L^{-1})	Fructose (g L^{-1})
WW_{raw}	36.0	244	21	3.36	22.62	5.50	0.56
WW_{glu}	39.4	165	25	3.54	22.96	13.06	0.71
WW_{synth}	36.4	168	51	0	0	43.29	0

The carbon limitation, which was caused by the incapability of *R. glutinis* to substantially utilize maltose under the present conditions, was also confirmed by the patterns of cell growth (Fig. 2). In all treatments cell mass increased constantly and equally until 48 h. Due to the de facto carbon limitation in the raw wastewater after this point, the growth rate decreased and finally stagnated with an absolute biomass yield of 5.22 g L^{-1} after 168 h. In the other treatments a further increase in cell mass was observed, with WW_{glu} (7.38 g L^{-1}) and WW_{synth} (6.77 g L^{-1}) yielding a similar biomass after 96 h of cultivation. At this point glucose is exhausted in WW_{glu}, leading to stagnation in cell growth. In the synthetic sugar medium the cells continue to grow, reaching a cell mass of 9.55 g L^{-1} after 168 h of cultivation. These results show that glucose from brewery wastewater and glucose from the synthetic media were both converted into cell growth at a similar rate. Additionally the brewery wastewater seemed to provide sufficient macro- and micronutrients to allow for cell growth processes. Thus, it can be assumed that brewery wastewaters can serve as nitrogen source for microbial growth and need no supplementation of other nutrients, when compared to the growth on an optimized artificial sugar medium. The measured final biomass yields (WW_{raw} 5.22 g L^{-1}; WW_{glu} 7.82 g L^{-1}; WW_{synth} 9.55 g L^{-1}) are in accordance with other results in the literature, in which *R. glutinis* in batch culture yields – depending on the substrate – between 2 g L^{-1} and 13 g L^{-1} [18,20,23,37]. Reported values of 30–60 g L^{-1} [23,40,41] are only reached when fermenters with e.g. aeration, pH control, and continuous carbon supplementation were used for the cultivation. This shows that the biomass yields of *R. glutinis* grown on brewery effluents could potentially also be increased, when cultivation is carried out in a fermenter.

When working with wastewaters, the COD removal is an interesting aspect, since a decrease in the COD of industrial wastewaters contributes to the necessary treatment of wastewaters and can help to reduce costs. In WW_{synth} the COD value was clearly reduced through the consumption of glucose, as indicated by the compliance of glucose (Fig. 1) and COD (Fig. 3) removal. In the actual wastewater media WW_{raw} and WW_{glu} the COD was hardly reduced (Fig. 3), as only an insignificant amount of maltose, which contributed the largest part to the COD value, was consumed. Accordingly a significant COD removal can only be achieved if the utilization of maltose is increased.

The observed pattern of sugar consumption, cell growth and COD removal showed that the insufficient utilization of maltose and the resulting carbon limitation were the main limiting factors regarding the suitability of brewery wastewaters as carbon source for *R. glutinis*. While some yeasts, especially brewer's yeast, are well known for their capability to degrade maltose, no explicit statement is made in the literature regarding *R. glutinis*. Janda and Hedenström [42] reported the consumption of maltose from the media by *R. glutinis*. However, in their trials the maximum maltose content was only 200 mg L^{-1}, which was almost completely consumed after ca. 90 min. In the present trial almost 2 g L^{-1} of maltose were consumed. Buzzini [43] results suggest that *R. glutinis*, strain DBVPG 3853 is able to utilize maltose for biomass and carotenoid production, while Latha et al. [38] observed a strong decrease in cell dry weight and carotenoid yield when maltose – in comparison to glucose or sucrose – was used as carbon source for *R. glutinis*, strain

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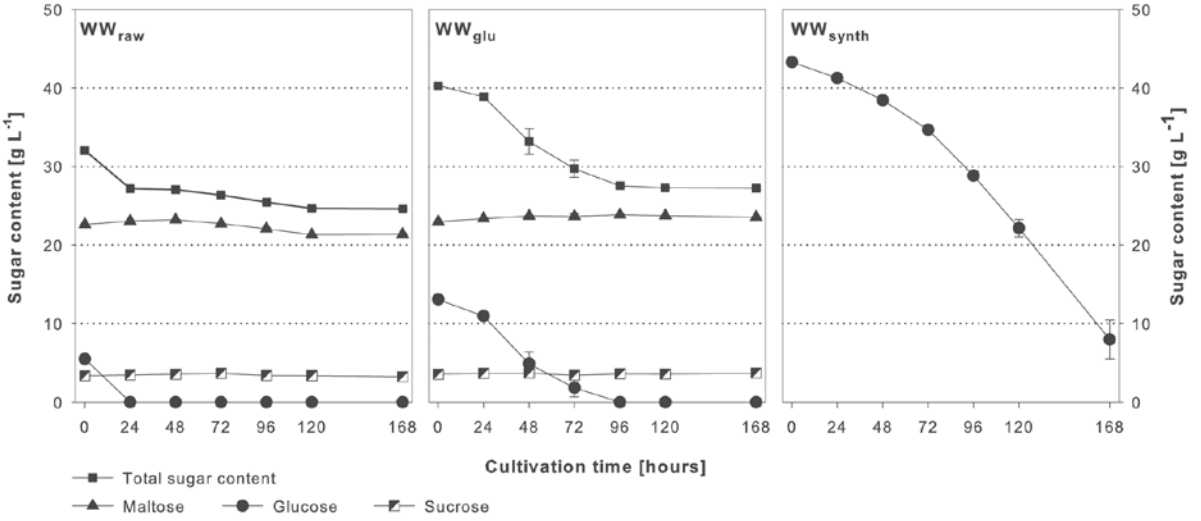

Fig. 1. Sugar consumption by *R. glutinis* cultivated on brewery wastewater media (WW_{raw}, WW_{glu}) and (synthetic sugar media) WW_{synth}.

DFR-PDY. Therefore, it can be assumed that *R. glutinis* does not generally lack the ability to utilize maltose. Thus, in a subsequent experiment the cultivation time was increased to 264 h to check whether the yeast simply needs more time for the built up of the enzymatic system for maltose degradation [44]. The results showed that maltose consumption was slightly increased to 2.62 g L^{-1}, which was still only a small part of the overall maltose content. Since maltose consumption appeared at the end of cultivation, when all other sugars were exhausted, a further prolongation of cultivation time could be useful. Also the option of acid hydrolysis was tested, showing that maltose was to some extend converted into easy available glucose. However, this did not lead to an increase in biomass, lipid or carotenoid yield. Also a lot of hydroxide was needed to neutralize the solution after hydrolysis to a suitable pH. A possible solution to this could be an approach with enzymatic hydrolysis of maltose. However, these options would require a certain additional input in terms of cost for equipment, certain chemicals and/or enzymes. Accordingly an additional hydrolysis step to break down maltose into glucose molecules would in turn increase the processing cost. Another option, if available, would be the use of specific strains of *R. glutinis* with the ability to degrade maltose or the application of maltose acclimated *R. glutinis* via grown solely on this substrate for numerous generations. Also the co-fermentation with other microorganisms that excrete the corresponding hydrolases could be a possible option.

3.2. Lipid production and FAME composition

With regard to the actual utilized sugars, the C/N ratios for the different treatments were calculated as 13:1 (WW_{raw}), 33:1 (WW_{glu}) and 103:1 (WW_{synth}) (see 3.1). Generally, a high C/N ratio is considered as optimal for lipid accumulation [14], whereas the optimum is often reported to be close to C/N 100 [15,45,46], which would match the C/N of the synthetic sugar medium. Then again some authors determined the optimum C/N at a lower range between 30 and 80 [21,47]. A C/N of >20 is generally seen as necessary condition for lipid induction in oleaginous microorganisms [21,48]. This could explain why *R. glutinis* did not pass into the lipid accumulation phase in WW_{raw} and WW_{glu}, as indicated by the decreasing values for lipid production (Fig. 2). According to the results of Angerbauer et al. [46], who reported an oil production of 4.1 g L^{-1} in *Lipomyces starkeyi* cultivated at C/N 15 and Easterling et al. [19], who achieved an oil content of over 30% in *Rhodotorula glutinis* cultivated at C/N 10, a lipid production at low C/N ratios seems to be possible. Still, it is agreed that only under excess carbon conditions, lipids are synthesized. Also, it has been shown that the lipid content of oleaginous cells is constantly low when nitrogen is present in the culture medium, because excess carbon is only channeled into lipid production when a growth required nutrient is exhausted in the medium [48]. The observed limitation of available carbon in WW_{raw} and WW_{glu}, along with the pattern of cell growth and the data for total nitrogen (Fig. 3), which indicate the availability of nitrogen throughout the cultivation time, can be assumed as reasons for the lack of lipid production. The lipids detected at the

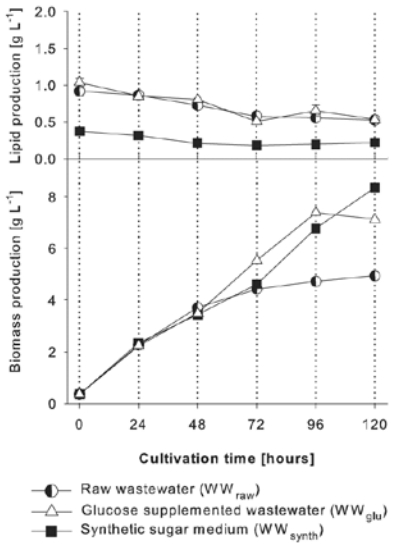

Fig. 2. Biomass and lipid production by *R. glutinis* in course of cultivation time.

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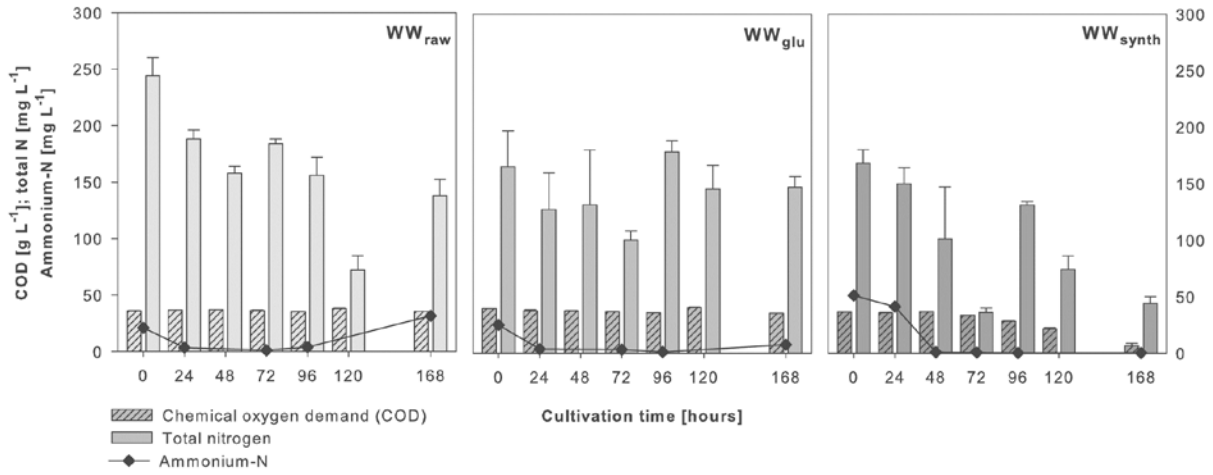

Fig. 3. (Chemical oxygen demand) COD, total nitrogen and NH_4^+-N in course of cultivation of *R. glutinis* on brewery wastewater media (WW_{raw}, WW_{glu}) and (synthetic sugar media) WW_{synth}.

beginning of cultivation derive from the inoculum or, as later explained, directly from the wastewater, whereas the decline in lipid production, especially at the onset of cultivation, is probably caused by lipid turnover processes [48]. According to the C/N ratio and the carbon availability, treatment WW_{synth} provided ideal conditions for lipid accumulation. However, only in the FAME analysis a slight increase in FAME production was observed (Fig. 4), whereas the gravimetrical determination of lipids via Bligh and Dyer extraction gave declining values for lipid production and lipid content. This could also be explained by the lack of nitrogen limitation as reflected by the constantly increasing cell mass. As another aspect Papanikolaou et al. [49] assumed that ATP-citrate lyase as key enzyme for lipid synthesis is inactive at high glucose and C/N levels, thus resulting in a low cellular lipid accumulation. Additionally, the pH value of WW_{synth} declined sharply to less than pH 3 after 48 h of cultivation (data not shown), which is considered as unfavorable for lipid production [50,51], with the optimum pH ranging between 5 and 6.

Regarding the potential of lipid production utilizing brewery wastewaters, again it can be concluded that either maltose needs to be made available to the yeast or the media has to be supplemented with other carbon sources in order to achieve a considerable amount of lipid production. Also, in order to evaluate the quantity and quality of lipid production on brewery wastewaters in further experiments, the cultivation time should be extended to ensure the consumption and exhaustion of nitrogen in the media.

The FAME composition and the total amount of FAMEs produced differed strongly between the actual wastewater treatments WW_{raw} and WW_{glu} and the synthetic sugar media WW_{synth} (Fig. 4). Already at inoculation time samples of WW_{raw} and WW_{glu} showed a significant higher FAME content than those of WW_{synth} treatment. Since the inoculum was identical for all treatments, these differences are probably derived from sediments and other residues present in the brewery wastewater, which have not been removed before cultivation. It can be suggested that these constituents contained a directly extractable lipid fraction, which may originate from cell wall membrane lipids of microorganisms in the wastewater. In accordance with the gravimetrically determined values for lipid production the FAME content then decreased in course of cultivation. In treatment WW_{synth} the FAME content increased from 0 to 120 h of cultivation, but with about 50 mg L^{-1} after 120 h only reached a low level. This is somewhat contradictory to the observed decrease in total lipid production. However, since there is a large gap between the gravimetrically determined total lipids and the chromatographically determined FAMEs it could be possible that while the total lipid biomass decreased the content of saponifiable lipids was increased.

Lipids from yeast in general and from *Rhodotorula* strains in particular are generally characterized by a domination of C16 and C18 long-chain fatty acids [10,20,52]. Also in this investigation the primary fatty acids palmitic acid (C16:0), stearic acid (C18:0), oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) account for over 90% (except WW_{synth} at t_0 with 86%) of the total fatty acids. However, fatty acid profiles from lipids produced by *R. glutinis* have mostly been found to be dominated by palmitic and oleic acid (Table 2), which is also the case in WW_{synth} with a 46.54% share of oleic acid and a 13.9% share of palmitic acid after 120 h of cultivation. Additionally in this treatment the amount of polyunsaturated fatty acids was constantly increasing, opposite to a decrease in saturated fatty acids (Fig. 5). These elongation and desaturation processes are typical for the lipid metabolism of yeast, thus indicating that the process of lipid synthesis and accumulation has been triggered in this treatment [53,64]. As Table 2 shows, the fatty acid profiles of oils obtained from *R. glutinis* in this and other investigations are similar to conventional plant oils, whereas the fatty acid composition is particularly important regarding the quality and properties of biodiesel. For example, with increasing chain length positive attributes like cetane number, heat of combustion and melting point will increase. Saturated fatty acids also lend higher cetane numbers, shorter ignition delay times and improved oxidative stability, while unsaturated fatty acids give favorable characteristics in terms of cold flow and pour point properties [54]. In the wastewater treatments WW_{raw} and WW_{glu} linoleic acid is the prevailing fatty acid with a share of over 50% throughout the cultivation. Since linoleic acid is often found in the lipids of cell membranes, this high proportion supports the assumption that in these treatments lipids were directly derived from cell membranes. This suggests that no *De novo* lipid synthesis appeared, which is defined as the production of lipids from sugar as a carbon source. In addition no increase in polyunsaturated fatty acids was observed, whereas similar to WW_{synth} the fraction of saturated fatty acids, especially C16:0, decreased.

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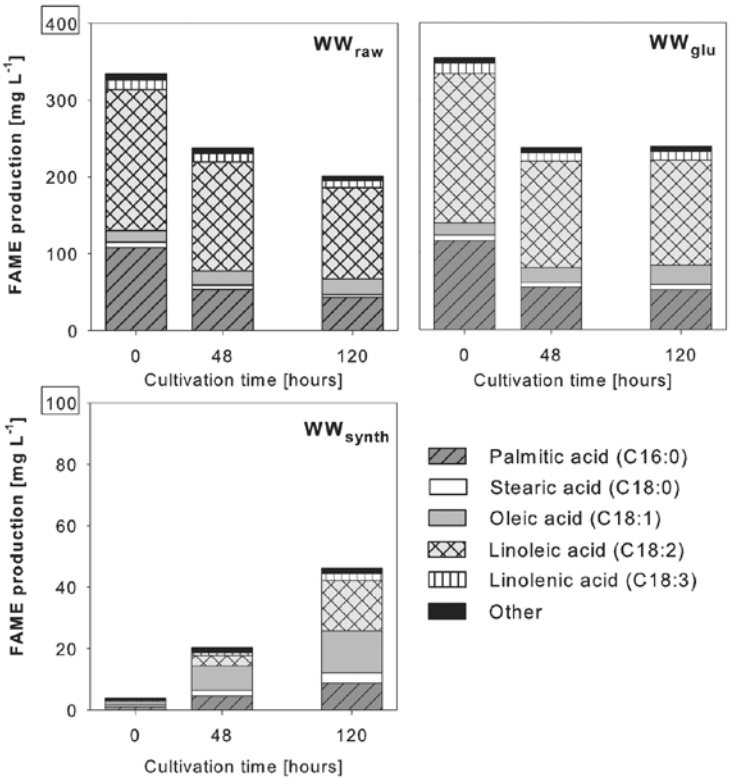

Fig. 4. FAME production and fatty acid composition by *R. glutinis* cultivated on brewery wastewater media (WW_{raw}, WW_{glu}) and (synthetic sugar media) WW_{synth}.

With these results is can be summarized that lipid synthesis and accumulation of *R. glutinis* was not triggered in the wastewater treatments WW_{raw} and WW_{glu}, due to an early limitation of carbon. The – in comparison to WW_{synth} – higher FAME contents at the beginning of cultivation are probably derived from a directly extractable fraction of lipids in the brewery wastewaters. Inconsistent with the gravimetrically determined total lipid production, the FAME contents and the FAME composition regarding the sugar medium WW_{synth} indicate that *R. glutinis* began to synthesize lipids. However, the amount of FAMEs produced was on a negligible level, which could be explained by the continuous availability of nitrogen, the high glucose loading, and an unfavorable pH value.

Table 2
Some reported fatty acid profiles of *R. glutinis* cultivated on different carbon sources in comparison to plant seed oils commonly used for biodiesel production.

C16:0	C18:0	C18:1	C18:2	C18:3	Carbon source/ Oil source	Reference
20.37	10.33	47.88	7.31	0.85	Palm oil mill effluent	Saenge et al. [20]
13.64	2.83	32.35	20.41	8.63	Glucose	Perrier et al. [52]
16.01	21.86	18.05	15.91	1.76	Glycerol	Easterling et al. [19]
18.7	13.2	43.8	12.7	5.5	Acid hydrate (Switchgrass)	Zhang et al. [37]
18.74	1.16	66.96	4.57	n.m.	Lignocellulosic hydrolyzates	Dai et al. [55]
37	3	47	8	0	n.m.	Ratledge [56]
13.9	5.75	46.54	26.57	3.37	Glucose	This work
12	3	23	55	6	Soybean oil	Fassinou et al. [65]
3	1	64	22	8	Rapeseed oil	Fassinou et al. [65]

n.m.: Not mentioned.

3.3. Carotenoid production

There was no carotenoid production observed in the early phase of cultivation. In WW_{raw} the synthesis of carotenoids started after 48 h and further increased to a final amount of ca. 0.6 mg L^{-1} total carotenoids after 168 h (Fig. 6). In the other two treatments carotenoids were not detected until 96 h of cultivation. After 96 h the total carotenoid content increased strongly in WW_{glu} and WW_{synth}, yielding around 1.2 and 1.0 mg L^{-1} at the end of cultivation, respectively. However, all gains in carotenoid production were accompanied by an increasing variance of the measured data, leading to high standard errors. Thus none of the detected increases in total carotenoid production were on a statistically significant level. Regarding the carotenoid composition it can be differentiated between the actual wastewater treatments WW_{raw} and WW_{glu} and the sugar medium WW_{synth}. In the first two treatments torularhodin (46.9% and 47.7%, respectively) and β-carotene (51.6% and 48.8%, respectively) were the major carotenoids, whereas in WW_{synth} the shares of all 3 carotenoids were almost evenly distributed (32.8% β-carotene, 28.5% torulene, and 38.7% torularhodin).

Considering the high standard errors, the measured total carotenoid contents in all treatments were comparable low. However, as Table 3 shows, in literature depending on substrate type, cultivation condition and strain, a broad range of carotenoid yields for *R. glutinis* is reported, ranging between 0.5 and 185 mg L^{-1} of culture fluid (see also Ref. [57]). In WW_{raw} and WW_{glu} the carotenoid production started once cell growth reached the stationary phase, which indicates that carotenoid synthesis was not related to cell growth. That would explain the earlier onset of carotenoid

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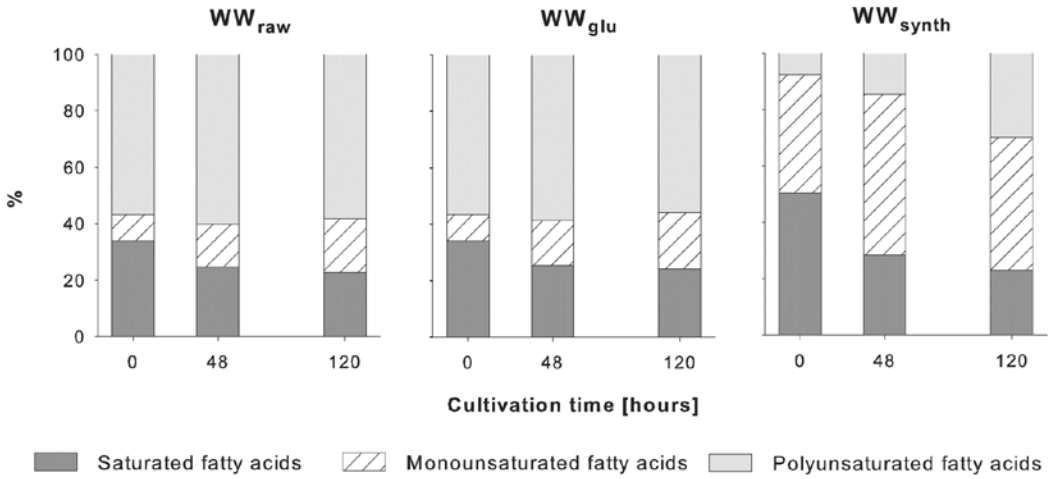

Fig. 5. Degree of saturation of fatty acids by *R. glutinis* cultivated on brewery wastewater media (WW_{raw}, WW_{glu}) and (synthetic sugar media) WW_{synth}.

production in WW_{raw}. But this pattern has only rarely been reported [38,62] and is contradicted by most authors that observed a growth-associated carotenoid production [60,61].

However, the presence of a suitable carbon source for carotenoid synthesis is required, so that the lack of available carbon probably inhibited a higher carotenoid content in WW_{raw} and WW_{glu}. Regarding the sugar medium in WW_{synth} the comparable high glucose content could have had a negative impact on carotenoid production, since some authors observed the Crabtree effect in batch cultures of *R. glutinis* [20,57], which describes the production of fermentation products such as organic acids at high carbon concentrations which repress the production of carotenoids. In *Phaffia rhodozyma* for example a reducing pigment yield at carbon concentrations higher than 12 g L^{-1} has been observed [63]. This rationale is supported by the strongly decreasing pH in WW_{synth}, whereas low pH values are generally suboptimal for carotenoid synthesis with the optimum being around pH 6 [59,60]. In addition, Latha et al. [38] found maltose to be an unfavorable carbon source for pigment production, with carotenoid yields almost halved compared to glucose-based media. Glucose was found to be one of the highest yielding carbon sources [58] which could explain the higher carotenoid contents in WW_{glu} and WW_{synth}.

Regarding the compatibility of carotenoid and lipid production, Saenge et al. [20] found that at high C/N ratios both lipids and carotenoids are synthesized by *R. glutinis*. However, it seems that at low C/N ratios the production pathways are competing over the scarce carbon. Also, both production pathways require Acetyl Co-A as common precursor, which might add as competitive factor. However, since the total carotenoid yield was low and afflicted with high standard errors it is difficult to assess the kinetics of pigment production as well as its correlation to lipid production. Still it can be concluded that brewery wastewaters as substrate allow for microbial carotenoid production by *R. glutinis*, even as raw, not supplemented fermentation medium.

Also the high proportion of β-carotene in the wastewater treatments is favorable since currently it quickens the highest commercial interest, whereas the carotenoids composition also highly depends on the used yeast strain and carbon source [57]. Additionally it can be expected that the total carotenoid yield and the β-carotene proportion of *R. glutinis* growing on such

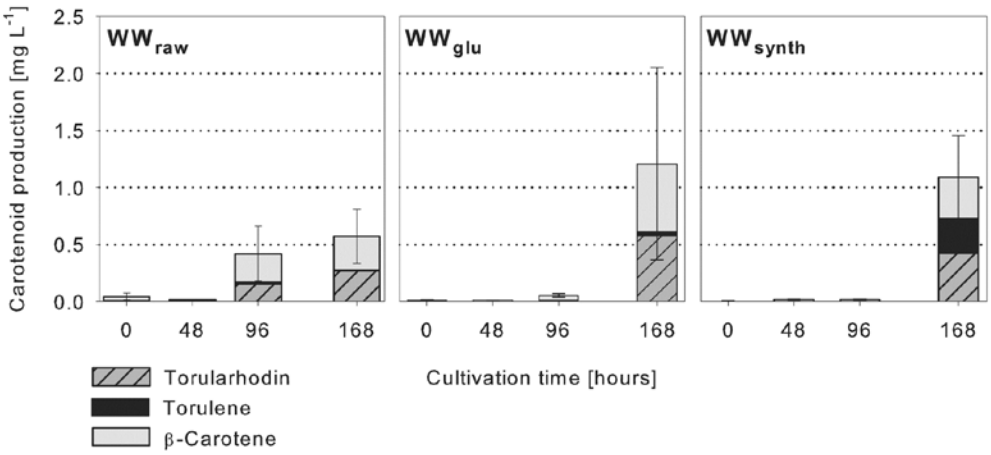

Fig. 6. Carotenoid production and composition by *R. glutinis* cultivated on brewery wastewaters (WW_{raw}, WW_{glu}) and sugar medium (WW_{synth}).

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Table 3
Some reported carotenoid yields of different *R. glutinis* strains cultivated on different carbon sources.

Total carotenoids	Carbon source	Strain	Reference
0.63 mg L^{-1}	Glucose	DBVPG 6081	Buzzini et al. [26]
1.80 mg L^{-1}	Glucose syrup	DBVPG 3853	Buzzini & Martini [29]
2.20 mg L^{-1}	Glucose	Wild strain[a]	Bhosale & Gadre [58]
3.48 mg L^{-1}	Hydrolyzed mung bean waste flour/sweet potato extract	n.m.[b]	Tinoi et al. [59]
5.95 mg L^{-1}	Rectified grape must	DBVPG 3853	Buzzini & Martini [29]
33 mg L^{-1}	Glucose	Mutant 32[b]	Bhosale & Gadre [58]
125 mg L^{-1}	Molasses sucrose	Own isolate	Aksu & Eren [60]
185 mg L^{-1}	Sugar cane molasses	Mutant 32[b]	Bhosale & Gadre [61]

[a] NCIM 3353.
[b] n.m.: Not mentioned.

wastewaters can be significantly improved by using specific hyper producing mutants or by cultivation in fed-batch or continuous fermentation systems, in which crucial cultivation parameters like pH and especially aeration can be controlled.

4. Conclusion

Regarding the assessment of brewery wastewaters as suitable fermentation substrate for lipid and carotenoid production by red yeast *Rhodotorula glutinis*, the analysis of the collected wastewaters showed, that they contained a high amount of sugars as potential carbon source for microbial fermentation. Maltose as largest fraction of these sugars however was hardly utilized by *R. glutinis* under the culture conditions. The resulting carbon limitation potentially inhibited lipid accumulation and hampered carotenoid formation. Still it can be concluded that brewery wastewaters can serve as sole nitrogen source and also provide other required nutrients for microbial growth. There was no evidence for any inhibitory substances present in the brewery effluents, as both biomass and carotenoid production was observed in the wastewater treatments. However to increase lipid and pigment yields it will be necessary to increase the availability of the maltose fraction in the wastewaters, which could be achieved by hydrolyzation measures or by the use of a *R. glutinis* strain with the capacity to degrade maltose. Also the optimization and the control of culture conditions, especially with regard to pH and aeration as provided by a fermenter, will help to increase cell growth as well as lipid and carotenoid formation. Despite these constrains brewery effluents are an encouraging substrate for the simultaneous microbial production of lipids and carotenoids and should be subject to further investigation.

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5 Chapter III

Microbial lipids for biodiesel production and carotenoids as value added by-products – Screening of industrial wastewaters as suitable feedstock for oleaginous red yeast *Rhodotorula glutinis*

Publication III:

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The cultivation of R. glutinis on brewery process wastewaters as described in the previous chapter showed that these effluents indeed contain high amounts of sugars, mainly maltose, but also that the yeast was not capable to substantially utilize this carbon source. However, the results for biomass and carotenoid production suggest that nitrogen and other nutrients were sufficiently provided. Thus, in this chapter a second experiment with brewery wastewaters was designed, where (i) the cultivation time was increased in order to survey if R. glutinis just requires more time for the adaptation of its enzyme systems for maltose utilization, (ii) raw wastewater was hydrolyzed with hydrochloric acid in order to convert maltose into easy available glucose and (iii) maltose degradation and utilization was tested on the basis of an artificial wastewater containing maltose.

As a second aspect yet another industrial wastewater from a sugar refinery was evaluated in terms of carbon content, biomass and lipid production during cultivation with R. glutinis.

MICROBIAL LIPIDS FOR BIODIESEL PRODUCTION AND CAROTENOIDS AS VALUE ADDED BY-PRODUCTS – SCREENING OF INDSUTRIAL WASTEWATERS AS SUITBALE FEEDSTOCK FOR OLEAGINOUS RED YEAST *RHODOTORULA GLUTINIS*

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ABSTRACT: The synthesis of single cell oil (SCO) as alternative feedstock for the production of biodiesel as well as the formation of carotenoids as value added by-product of red yeast *Rhodotorula glutinis* cultivated on wastewaters from sugar refinery and beer production was investigated. Additionally, the ability of *R. glutinis* to utilize maltose as main carbon source in brewery wastewaters was examined. *R. glutinis* was able to produce biomass both on sugar refinery (4.86 g L^{-1}) and brewery (4.25 g L^{-1}) wastewaters. No lipid production was observed during growth on sugar refinery wastewaters probably due to low amounts of yeast available carbon sources. Cultivated on different brewery wastewater treatments, lipid production reached a maximum of 2 g L^{-1}. Maltose was consumed in small quantities. No carotenoids were produced, probably because carbon was utilized in favor of lipid production.

Keywords: *Rhodotorula glutinis*, second generation biodiesel, wastewater, single cell oil, carotenoids

1 INTRODUCTION

Single cell oil (SCO) produced by oleaginous microorganisms has been found to be a sustainable alternative feedstock for the production of biodiesel. The use of conventional feedstocks like plant oils from rapeseed and soybean has been criticized in recent years as is the production of these oils may increase the competition with food production and seen to be responsible for rising commodity prices [1]. Also the price increase of petroleum fuels owing to the finiteness crude oil has led to a renewed interest into this field of research. Oleaginous microorganisms are defined as producing more than 20 % of their cell dry weight in the form of lipids [2] with a fatty acid composition comparable to plant oil [1]. The oils are rich in polyunsaturated fatty acids which can be used for oleochemical or nutritional applications [3]. Oleaginous yeasts in particular hold the advantage of low duplication times, fast growth rate, high oil content, and an easy scale up [1, 4]. The basic requirement for growth and lipid production of these organisms is a medium with an excess of carbon source and a limited amount of nitrogen, whereas a broad range of possible carbon sources have already been tested, including glucose, xylose, arabinose, mannose or glycerol. Also the importance of the nitrogen source, the C/N ratio, pH, temperature, and aeration has been demonstrated [3]. Recently research has focused on the utilization of low cost substrates like agricultural and industrial waste and residues for microbial fermentation, since this could help to reduce the cost of microbial oil production and make this approach more competitive to conventional plant oil derived biodiesel.

Among the family of oleaginous yeasts, *Rhodotorula glutinis* has one of the highest lipid contents of up to 72 % [5]. It additionally synthesizes certain carotenoids, namely β-carotene, torulene, and torularhodin [6]. These natural pigments with antioxidant properties and pro Vitamin A character are used as natural colorant or dietary supplement in food, cosmetic and pharmaceutical industry. Compared to oil for biodiesel production, carotenoids are a comparatively high value product. With the simultaneous production of lipids and carotenoids combined with the use of a low cost fermentation substrate the economic features of microbial product formation could be enhanced.

Wastewaters from food and beverage industry have a high organic load and can lead to severe pollution if released into the environment without an adequate treatment. Accordingly there are certain national laws and regulations that set certain standards for wastewaters that are disposed into public drainage systems or directly into surface waters. Due to their nutrient load it can be supposed that these wastewaters have good qualities as feedstock for microbial growth. It has been shown that the organic load, reflected by the chemical (COD) and biological (BOD) oxygen demand can be reduced through microbial fermentation [7, 8]. Hence, the fermentation process could contribute to in-company wastewater treatment systems and help to reduce costs that occur with regard to wastewater treatment, management and disposal. Additionally, wastewaters in food and beverage are a low cost substrate without alternative utilization and accrue in large amounts.

In a first study the growth, lipid and carotenoid production of *Rhodotorula glutinis* on wastewaters from lettuce processing, potato processing and fruit juice production have been examined [9] and the obstacles for product formation were identified in the lack of (yeast available) carbon sources and in the high nitrogen content, respectively. Therefore, the screening of wastewaters was extended to sugar refinery and brewery wastewaters.

The COD of sugar refinery wastewaters is reported to range between 6,000 and 30,000 mg L^{-1} [10]. The COD of brewery wastewaters generally ranges between 2,000 and 6,000 mg L^{-1} [11, 12], whereas wastewaters from certain processes (e.g. wort separation) can have considerable higher amounts of COD ranging from 15,600 [13] to over 50,000 mg L^{-1} [14] with a high content of dissolved sugars, mostly maltose. Fungal growth on these process waters has already been described [14]. In a first experiment with brewery wastewaters despite high concentrations of sugars in the medium, no lipid production was observed [15]. It was suggested that this was caused by an insufficient degradation and utilization of maltose. Thus, the experiment was repeated with a longer cultivation time to examine the ability of *R. glutinis* to degrade maltose, with the results being presented in this paper.

2 MATERIALS AND METHODS

2.1 Wastewater and Media

Wastewater samples from sugar refinery were taken from wastewater ponds before being fed into the in-company wastewater treatment plant. The process wastewater from beer production was collected at a local brewery after the process of wort separation. To identify basic characteristics of the wastewaters, chemical oxygen demand (COD) and total nitrogen content (Nt) were determined photometrically using reagent vials from Macherey-Nagel (Düren, Germany). pH was measured potentiometrically using a pH-meter with glass electrode. The content of the sugars sucrose, glucose, fructose and for brewery wastewater also maltose was measured via HPLC. The sum of these sugars is later referred to as total sugar content. The basic characteristics of the wastewaters are summarized in Table I.

Table I: Basic characteristics of tested raw wastewaters

Wastewater source	COD (g L^{-1})	total N (mg L^{-1})	pH
Sugar refinery	14.1	33.0	6.7
Brewery	44.8	52.0	5.9

In order to observe microbial growth and lipid production on sugar refinery wastewaters 500 ml of the wastewater was autoclaved to eliminate other microorganisms, cooled down and inoculated with R. glutinis. This experiment was carried out in quadruplicate.

For the experiment with brewery wastewaters three different treatments were designed with particular focus on maltose degradation by R. glutinis. For treatment 1 (T 1) 500 ml of raw wastewater was centrifuged to remove sediments and other residues. Afterwards 150 ml of distilled water was added to give a total media volume of 650 ml which was then autoclaved. For Treatment 2 (T 2) 500 ml of centrifuged raw wastewater was hydrolyzed with 150 mL 3 N HCl at 70 °C for one hour as an attempt to convert maltose into glucose. For treatment 3 (T 3), an artificial medium was designed according to the wastewater characteristics from the first run of the experiment [15]. It contained (per liter) 1.0 g yeast extract; 1.0 g $Na_2HPO_4 \cdot 12\ H_2O$; 1.0 g KH_2PO_4; 0.4 g $MgSO_4 \cdot 7\ H_2O$; 0.4 g NH_4SO_4; 24 g maltose; 5.5 g glucose; 4 g sucrose; 6 ml $FeSO_4$ solution (4 g L-1 $FeSO_4 \cdot 7\ H_2O$), and 10 ml trace mineral solution. The trace mineral solution consisted of (per liter) 3.6 g $Ca_2Cl_2 \cdot 2\ H_2O$; 0.75 g $ZnSO_4 \cdot 7\ H_2O$; 0.13 g $CuSO_4 \cdot 5\ H_2O$; 0.5 g $MnSO_4 \cdot H_2O$; 0.13 g $CoCl_2 \cdot 6\ H_2O$, and 0.17 g $Na_2MoO_4 \cdot 2\ H_2O$. 500 ml of this synthetic medium was diluted with 150 ml of distilled water and autoclaved. To eliminate inhibitory effects due to non-optimal pH values, the pH was adjusted to ~pH 6 prior to autoclaving. The experiment was carried out in triplicate.

2.2 Microorganism, Cultivation and Analysis

R. glutinis (ATCC 15125) was grown in a 1000 mL Erlenmeyer flask containing a yeast malt broth (YMA) (3 g L-1 yeast extract; 3 g L-1 malt extract; 10 g L-1 glucose; 5 g L-1 vegetable peptone) for 5 days at 25 °C on a rotary shaker at 115 rpm. This media was then used as inoculum for the wastewater treatments, which were fermented in 1000 mL Erlenmeyer flasks, containing 500 mL (sugar refinery medium) and 650 mL (brewery media) of the respective wastewater media, respectively. All treatments were cultivated on a rotary shaker (115 rpm) at 30 °C for 120 h (sugar refinery) and 264 h (brewery), respectively. For determination of cell dry mass 50 mL samples were taken regularly every 24 h, centrifuged at 3000 rpm, freeze-dried and weighted. The supernatant after centrifugation was used for the determination of pH, COD, Nt, and sugar composition. The lipid content of the dried cell pellet was determined gravimetrically using a modification of the method by Bligh and Dyer [16]. This extract is later referred to as lipids. For

carotenoid analysis, 50 mL samples were taken, centrifuged, freeze dried and hydrolyzed with 1 N HCl in a water bath at 70 °C for 0.5 h. After removal of the acid, the cells were extracted in 30 mL acetone:methanol (1:1) solution. The solvent was afterwards evaporated under N2 blow at 50 °C, the extract redissolved in acetone and analyzed using a Merck-Hitachi HPLC system with DAD detector equipped with an Agilent Eclipse Plus C18 column (5 µm, 250 mm x 4.6 mm). Chromatographic results were quantified by using response factor of β-carotene standards. Three distinct peaks were identified as compared to known retention time profiles [6]. Amounts of the three peaks were summed to provide the total carotenoid production.

All data was statistically analyzed using Sigma Stat 3.5. The statistical significance of differences in mean values was calculated using single factor variance analysis with the Tukey-Test at the 5% level of probability.

3 RESULTS AND DISCUSSION

3.1 Microbial growth on sugar refinery wastewater

Even though the wastewater had a comparatively high chemical oxygen demand (COD), only a small amount of sugars was detected with HPLC. At inoculation time the wastewater only contained 0.03 g L^{-1} sucrose, 0.01 g L^{-1} glucose and 0.22 g L^{-1} fructose. Since the COD also includes organic compounds, which are difficult to degrade it was questionable if the medium contained a sufficient amount of yeast available carbon. However, the results showed that the wastewater allowed for continuous cell growth with cell mass increasing significantly from 0.82 g L^{-1} at inoculation time to 4.86 g L^{-1} after 120 h of cultivation (Fig. 1). At the same time sugars and nitrogen were consumed from the medium, with a residual content of 0.05 g L^{-1} and 18.8 mg L^{-1}, respectively (Fig. 1). The COD was halved from 12.8 to 6.3 g L^{-1}. This suggests that *R. glutinis* also consumed other carbon sources from the medium.

The **lipid production** and the lipid content of the cells did not differ significantly between the different sampling times. Even though the lipid content seemed to increase during the first 48 h of cultivation, these values were afflicted with a high standard error. After 72 h the lipid content started to decrease, however also this development was not significant. This was probably caused by the low amount of yeast available carbon and the constant availability of nitrogen in the medium, since it is well established, that lipid accumulation only starts when nutrient limitation coincides with excess carbon in the medium [3].

The continuous increase of cell mass indicated that cell proliferation and thus nutrient availability was not limited. The very low amount of sugars in the medium suggested that all carbon sources were consumed for the production of biomass without any capacity to channel some excess carbon into the lipid accumulation pathway. The tendency of decreasing lipid content after 48 h furthermore indicated that previously produced storage lipids have been mobilized by the cells to compensate for the lack of carbon from the medium [3]. Moreover, the pH value of the medium, ranging between pH 7 and pH 8 (Fig. 1) was not optimal for lipid accumulation. Since the optimal pH for lipid accumulation was reported to be between pH 5.0 and 6.0 [17] this could be another factor contributing to the low lipid content.

However, it was shown that sugar refinery wastewaters fulfilled the basic criteria to support growth of *R. glutinis*. To allow for lipid accumulation it will be necessary to increase the carbon content – and accordingly the C/N ratio – of the medium. This could be achieved by choosing other, more polluted wastewater streams within the sugar refinery or by adjusting the sampling to times with particularly high organic loads in the wastewater. Furthermore it needs to be assessed which other carbon sources in the wastewater can be consumed by *R. glutinis* and to which extend they contribute to the wastewater composition.

3.2 Microbial growth on brewery wastewater

Based on the results from a first experiment with brewery wastewaters [15], the three different treatments in this experiment were designed in order to examine different aspects: (i) can *R. glutinis* utilize maltose from the medium when other carbon sources are exhausted?, (ii) can the growth and/or lipid accumulation rate be improved by increasing the amount of easily available glucose in the medium through acid hydrolysis of maltose?

Looking at the sugar content of the raw wastewater it was found that the sugar content in the first experiment was higher than in the second experiment. Therefore T 3 (s. 2.1), which was designed according to the parameters of the first experiment, contained higher contents of maltose, glucose and fructose (Tab. II). Moreover, when comparing T 1 to the raw wastewater treatment from the first experiment, it has to be taken into account, that for T 1 wastewater was diluted with 150 ml distilled water, which gives a lower sugar concentration. Values for T 2 showed that acid hydrolysis led to a reduction in maltose content and an increase in glucose content.

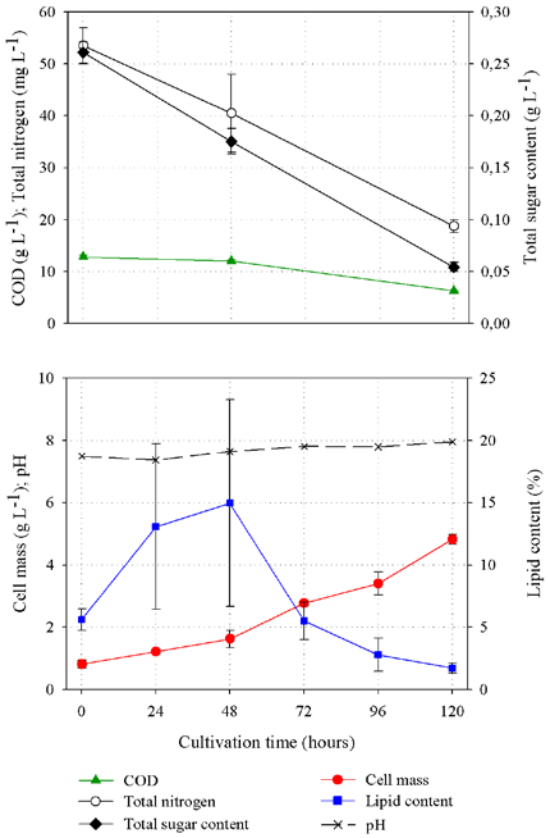

Figure 1: Chemical oxygen demand (COD) [g L^{-1}], total nitrogen [mg L^{-1}], total sugar content [g L^{-1}], cell growth [g L^{-1}], lipid content [%] and pH of *R. glutinis* cultivated on sugar refinery wastewaters

Table II: Total N (N_t) and sucrose (Suc), maltose (Mal), glucose (Glu), and fructose (Fru) content of wastewater media at inoculation time

	N_t (mg L^{-1})	Suc (g L^{-1})	Mal (g L^{-1})	Glu (g L^{-1})	Fru (g L^{-1})
T 1	140.5	4.37	13.78	2.22	0.32
T 2	168.5	2.92	8.75	5.06	0.96
T 3	111.5	0	17.33	4.44	0.66

The absolute **biomass yield** (T 1: 4.26 g L^{-1}; T 2: 4.04 g L^{-1}; T 3: 3.96 g L^{-1}) at the end of cultivation was quite similar for all treatments. On a relative basis, calculated by setting the cell dry mass at inoculation time equal to one, T 3 showed by far the strongest increase by a factor of almost 22 in the first 120 h of cultivation (Fig. 2). The course of biomass production fitted well with the pattern of sugar consumption in all treatments. The exponential cell growth ceased at 48 h in T 1 and at 120 h in T 2 and T 3, which in every case coincided with the exhaustion of the monosaccharides glucose and fructose from the medium (data not shown).

As shown in Fig. 2, the **lipid production** in all treatments increased significantly. In T 2 and T 3 the lipid production increased rapidly from inoculation time on. These two treatments also had the highest share of easily degradable glucose and fructose (Tab. 2). As already stated previously, all carbon that is not needed for biomass production is channeled into lipid production [3]. Since the nitrogen content with 168.5 and 111.5 mg L^{-1} respectively was quite low it can be suggested that in this first phase of cultivation excess carbon was channeled into lipid synthesis. As cell growth and sugar consumption continued, a certain threshold of carbon flux was reached, where all carbon is needed for biomass production. In T 2 this point was reached at 48 h. Cell growth at this time was still increasing strongly and all carbon sources as well as some of the storage lipids were consumed by the cells to support the biomass production. After 120 h all monosaccharides were consumed from the media and no maltose degradation could be observed until the end of cultivation. Due to this lack of carbon, more storage lipids were degraded, which is reflected by the continuously decreasing lipid production.

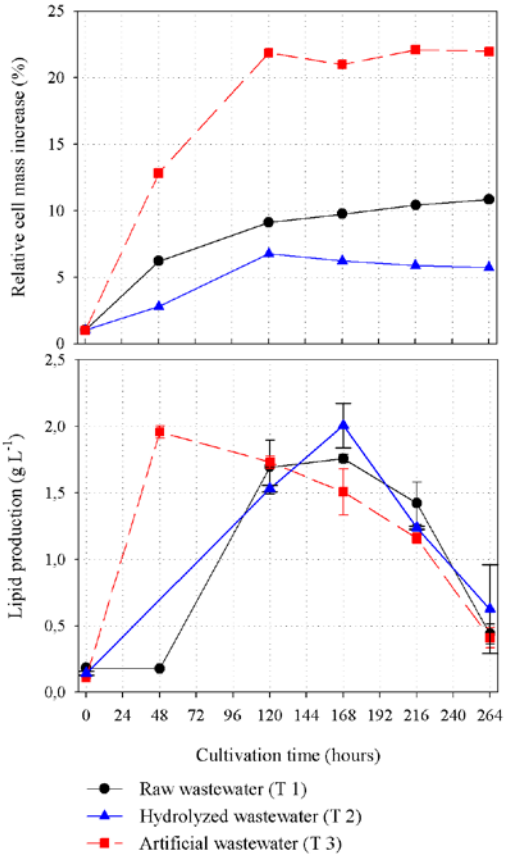

Figure 2: Relative cell mass increase [%] und lipid production [g L^{-1}] of *R. glutinis* cultivated on different brewery wastewater treatments

In T 3 lipids were produced until 168 h, supported by the consumption of glucose, fructose and sucrose. In comparison to T 2 the amount of cell mass production was lower, so that more carbon was available for the production of storage lipids. However, after this point lipid production started to decrease, even though no more cell growth processes occurred at this point.

In T 1 the amount of glucose and fructose was lower and probably just enough to support the cell growth processes. However, after the exponential growth phase at 48 h the lipid production started to increase until 168 h where glucose, fructose and sucrose were exhausted from the medium. Maltose decreased significantly from 13.38 g L^{-1} at 168 h to 11.17 g L^{-1} at 264 h, but this did not provide enough energy to stop lipid turnover in the cells. Still, the results indicate that *R. glutinis* was able to degrade and assimilate maltose from brewery wastewaters as carbon source to some extent. For a definite judgment on the maltose assimilating capacity of *R. glutinis* it could be advisable to further increase the cultivation time.

Regarding the values for cell mass production and lipid accumulation these findings comply with reports of other authors that cultivated *R. glutinis* on waste derived substrates. Cheirslip et at. [18] observed a biomass yield around 2 g L^{-1} and a lipid production around 1.5 g L^{-1} with *R. glutinis* cultivated on effluents from steam fish processing. Grown on palm oil mill effluent and different nitrogen sources *R. glutinis* achieved a biomass production between 4.15 and 6.33 g L^{-1} and a lipid content between 20.97 and 32.63 % [19]. Compared to the maximum values of lipid production by *R. glutinis* obtained under optimum culture condition and carbon sources in a bioreactor, where lipid yields of up to 15 g L^{-1} could be achieved [20], the present results seem to be very low (s. Tab.3). Yet, in Germany every year over 18 million m³ of brewery wastewaters are fed into in-company wastewater treatment facilities [21]. If this amount of wastewater would completely be utilized as medium for lipid production with an average lipid yield of 2 g L^{-1}, over 36,000 t of lipids could be produced. In 2010 in Germany 1.46 mio t of rapeseed oil were used for biodiesel production.

Table III: Maximum lipid production and corresponding lipid content of *R. glutinis* on different brewery wastewater treatments

	Time (hours)	Lipid production (g L^{-1})	Lipid content (%)
T 1	168	1.76	45.88
T 2	168	2.01	45.59
T 3	48	1.96	91.00

The microbial biodiesel from brewery effluents could substitute around 2.5 % of these plant derived oils, sparing over 50,000 ha of agricultural land, which would not be needed for the production of rapeseed for biodiesel production anymore.

However, a final assessment of the lipid production and its value for microbial biodiesel production can only be made after transesterification of the lipids into fatty acid methyl esters (FAME). Next to the total FAME yield, the analysis of the fatty acid composition will help to evaluate the quality of the synthesized lipids with regard to their utilization as biodiesel feedstock or for oleochemical applications.

The **carotenoid production** was very low in all treatments and at all sampling times. The low and insignificant changes in carotenoid production in the course of cultivation (Fig. 3) suggest that no carotenoid production occurred. Other authors [22] implied that at high C/N ratios lipids are produced in preference to carotenoids. The C/N ratios of the different treatments were comparatively high, ranging approximately between 40:1 (T 2) and 80:1 (T 3), which might have triggered the production of lipids rather than that of carotenoids. Saenge [19] reported the optimum condition for both lipid and carotenoid production at high C/N ratios of 180:1 and 170:1, respectively. This shows that the influence of the C/N ratio on the relation between lipid and carotenoid production needs to be further assessed, in order to *a priori* evaluate certain feedstocks and cultivation media based on their C/N ration for their suitability for either lipid or carotenoid production.

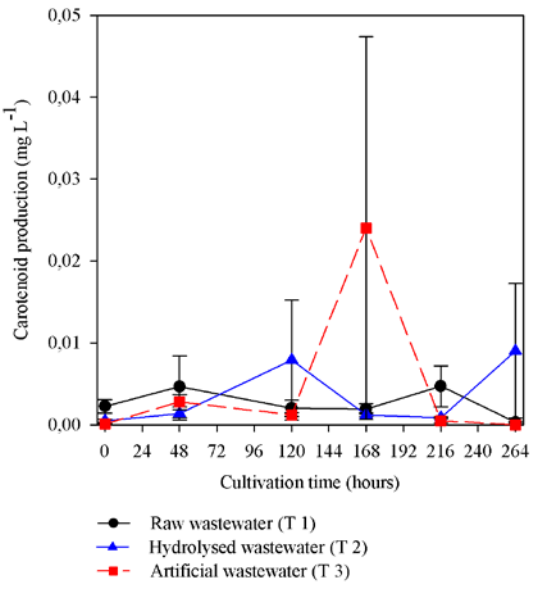

Figure 3: Carotenoid production [mg L^{-1}] of *R. glutinis* cultivated on different brewery wastewater treatments

4 CONCLUSION

The significant increase of cell mass production of *R. glutinis* grown on effluents from a sugar refinery plant and a brewery indicated that the wastewaters were a potential source of yeast available carbon. However, no lipid production was observed when *R. glutinis* was cultivated on sugar refinery wastewaters, probably due to the continuous availability of nitrogen in the medium on the one hand and a only limited availability of carbon on the other hand. Brewery wastewater contained a comparatively high amount of sugars, which contributed to a significant lipid production. Yet, lipid production decreased rather quickly in the second half of cultivation, since maltose as main sugar source in the wastewaters could not sufficiently be utilized. There was no simultaneous production of lipids and carotenoids, probably because the conditions favored the synthesis of lipids. Nevertheless wastewater can play a role as low cost carbon sources for the production of microbial metabolites and contribute to a more sustainable production of biodiesel. Still, more research is needed in order to examine the effect of certain wastewater characteristic – particularly the C/N ratio – on lipids and carotenoid production, to improve cultivation and extraction methods, and to reduce the cost of the overall process. Moreover, further industrial wastewaters with a supposedly high organic content should be tested as potential sources of carbon for microbial growth.

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6 Chapter IV

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

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In chapter II and chapter III R. glutinis was cultivated on brewery wastewaters. The results of the two experiments differed regarding the results of lipid and carotenoid production: In chapter II no lipids were produced, but carotenoid production was observed, while in chapter III it was vice versa. This indicates that there might be a certain competition over the available carbon between the two metabolic pathways, which would hamper the simultaneous production of microbial lipids and carotenoids. Reports from other researchers clearly state that a high C/N ratio is required for lipid production. For the synthesis of carotenoids however there is some ambiguity regarding the effect of the C/N ratio. Thus, in this chapter the influence of different C/N ratios on both lipid and carotenoid yield was assessed. Furthermore the composition of the C/N ratio in terms of initial carbon and nitrogen contents was evaluated. Since the results from the previous cultivations on raw wastewaters were quite disappointing, for this experiment synthetic sugar media was selected for cultivation with R. glutinis. The investigation of the influence of the C/N ratio on lipid and carotenoid production could also help to develop decision parameters to a priori evaluate the suitability of wastewaters for the production of either lipids or carotenoids.

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 C/N (carbon to nitrogen) 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, 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 C/N 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*

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 1st 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 – 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 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 Co-A 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 *Rhodotorula 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. This media was then used as 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 $Na_2HPO_4 \cdot 12\ H_2O$, 1.0 g KH_2PO_4, 0.4 g $MgSO_4 \cdot 7\ H_2O$, 1.0 g $(NH_4)_2SO_4$, 10.0 mL trace mineral solution and 6.0 mL $FeSO_4$ solution (4 g L^{-1} $FeSO_4 \cdot 7\ H_2O$). The trace mineral solution consisted of (per liter) 3.6 g $Ca_2Cl_2 \cdot 2\ H_2O$; 0.75 g $ZnSO_4 \cdot 7\ H_2O$; 0.13 g $CuSO_4 \cdot 5\ H_2O$; 0.5 g $MnSO_4 \cdot H_2O$; 0.13 g $CoCl_2 \cdot 6\ H_2O$, and 0.17 g $Na_2MoO_4 \cdot 2\ H_2O$. 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 (Tab. 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. 500 mL of the respective media were 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 mL 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.

Tab. 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

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 x g (Eppendorf 5810R, Rotor A-4-62, Eppendorf AG, Hamburg, Germany) for 10 minutes, 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 with methanol and lipids extracted with chloroform following a modification of the method as described by Zhang et al. (2011). The solvents were afterwards evaporated in a TurboVap LV (Caliper Life Sciences, Hobkinton, 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 waterbath 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 a Restek Stabilwax-DA capillary column (30 m x 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 waterbath 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 thoroughly rinsed with distilled water to remove the acid. Afterward carotenoids were extracted overnight in 30 mL acetone:methanol (1:1) solution. The solvents were evaporated under N_2 blow at 50 °C and the remaining extracts redissolved in 2 mL acetonitril: isopropyl-alcohol:ethyl acetate (40:40:20) solution. The analysis was conducted using a HPLC device (Agilent 1100, Santa Clara, CA, USA) equipped with an Evaporative Light Scattering Detector (Varian 385-LC, Palo Alto, CA, USA) and a Restek Ultra C18 column (5 µm, 250 mm x 4.6 mm). Acetonitril: isopropyl-alcohol:ethyl acetate (40:40:20) was used as mobile phase at a flow rate of 1 mL min^{-1}. All identified 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-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 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 levelled curve progression between 120 h 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 were added to the medium in this treatment.

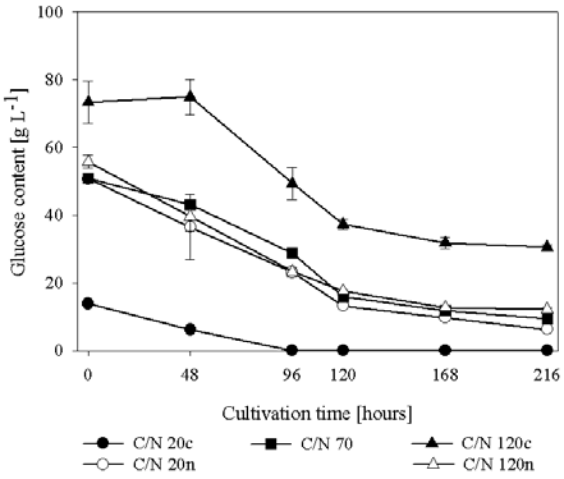

Fig. 1 Glucose utilization by *R. glutinis* during cultivation at different C/N ratios, initial glucose and nitrogen contents

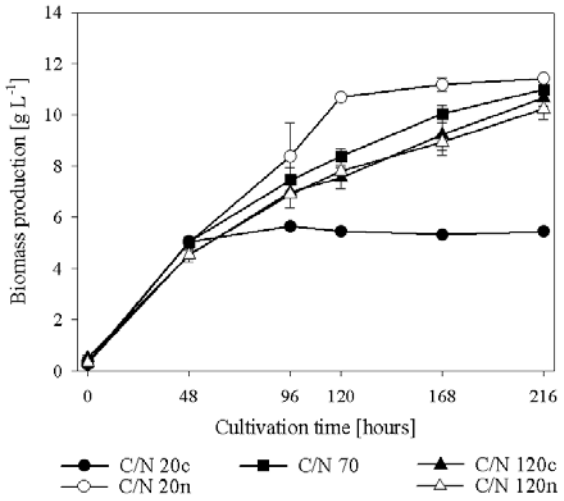

Fig. 2 Biomass production by *R. glutinis* during cultivation at different C/N ratios, initial glucose and nitrogen contents

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 g L-1 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 (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 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 treatments only varied regarding the temporal distribution of lipid production, with C/N 20n (4.855 g L^{-1} $(NH_4)_2SO_4$) mainly producing lipids between 48 and 120 h of cultivation and C/N 70 (1.0 g L^{-1} $(NH_4)_2SO_4$) and C/N 120n (0.362 g L^{-1} $(NH_4)_2SO_4$) focusing the lipid production on the end of cultivation from 120 h on.

The fatty acid composition was generally similar in all treatments (Tab. 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 C/N treatments 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.

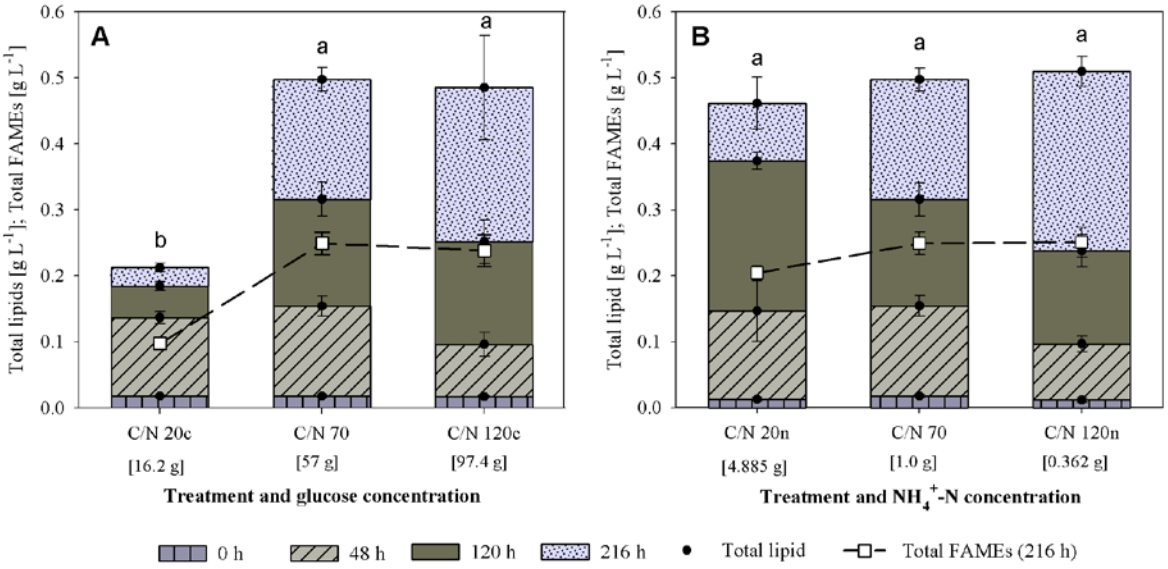

Fig. 3 Temporal break down of total lipid yield and FAME yield at the end of cultivation of *R. glutinis* grown at different C/N ratios and effects of different initial glucose concentrations at constant ammonium contents (A) and different initial ammonium concentrations at constant glucose contents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

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

	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	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}

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

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 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.

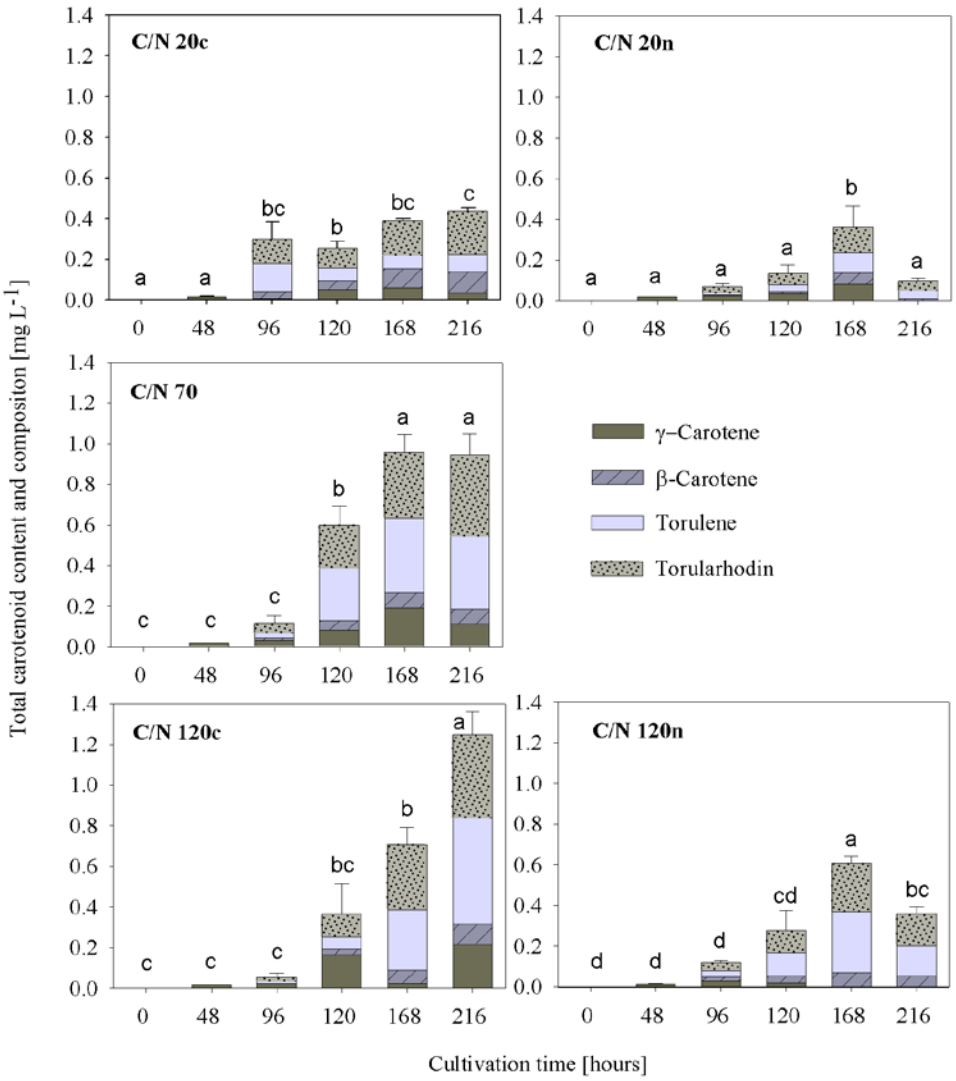

Fig. 4 Total carotenoid content and carotenoid composition of *R. glutinis* cultivated at different C/N ratios, initial carbon and nitrogen contents. Different lower case letters indicate significant differences between sampling times at 5 % levels of probability.

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 type 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 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 channelled 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 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 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 & 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 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 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 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 *Phaffia 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} $(NH_4)_2SO_4$. Someshekar and Joseph (2000), who also observed lower carotenoid yields with decreasing nitrogen contents, suggested that under these conditions available carbon will be utilized in favour 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 favoured over β-carotene production. In the group of carotenoids synthesized by *R. glutinis*, γ-carotene can be considered as primary carotenoid, which acts as 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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7 Chapter V

Utilization of soluble starch by oleaginous red yeast *Rhodotorula glutinis*

Publication V:

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The analysis of the raw wastewaters which have been assessed in the first three chapters showed, that especially wastewater from potato processing, but also from the sugar refinery and brewing process contained a fraction of organic carbon that was represented by the high COD values, but was not available in the form of sugars as detected by HPLC. This fraction was determined to be native starch, since in all of these wastewaters different amounts of starch have been detected. Especially in wastewaters from potato processing it can be assumed that starch is abundant. In order to utilize starch as carbon source, microorganisms need to be capable to develop certain enzyme systems that excrete enzymes such as amylases. In order to examine whether R. glutinis is able to utilize starch as carbon source, in this chapter the yeast was cultivated on synthetic media with different starch contents. Starch is an abundant carbon source in a lot of food industry wastewaters. Thus, the ability to utilize starch is an important aspect for the selection of suitable microorganisms, if the starch in these wastewaters should be efficiently utilized. If R. glutinis was not able to degrade starch, an additional pretreatment of the respective wastewaters or a respective amendment of the yeast itself would be necessary to provide an adequate carbon supply for cell growth, lipid and carotenoid production.

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Utilization of Soluble Starch by Oleaginous Red Yeast *Rhodotorula glutinis*

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ABSTRACT

Starch containing wastewaters from the food and feed industry have been identified as potential cheap carbon sources for the production of microbial lipids. Due to its high potential lipid content the oleaginous yeast *Rhodotorula glutinis* is often used for fermentations in this field. Moreover it is investigated in the context of microbial carotenoid production, which also requires a cheap source of carbon. Thus, the ability of *R. glutinis* (ATCC 15125^{TM}) to degrade and utilize soluble starch for the production of lipids has been assessed in this study. While glucose and fructose were readily consumed from the medium, starch was only slightly reduced in one treatment. The yield of fatty acid methyl esters (FAME) was graduated corresponding to the initial sugar contents, with the highest FAME yield (1.5 g·L^{-1}) at the highest initial sugar content. In the treatment that contained starch as single carbon source, no FAME production was realized. Accordingly, if starchy wastewaters should be used for microbial cultivation with *R. glutinis*, an enzymatic or chemical pretreatment for starch hydrolysis should be applied, to increase the availability of this carbon source.

Keywords: *Rhodotorula glutinis*; Microbial Lipid Production; Starch

1. Introduction

Worldwide, renewable biofuels such as bioethanol and biodiesel contribute a significant share to the overall fuel consumption [1]. Against the background of rising crude oil prices and environmental concerns related with the exploitation of fossil oil resources, politics set strong incentives to further increase the production of biofuels—in Europe mainly biodiesel. So-called 1st generation biodiesel is produced by the transesterification of plant oils like rapeseed and soybean oil with an alcohol—mostly methanol—into fatty acid alkyl (methyl) esters (FAMEs) [2], whereas the terms "FAME" and "biodiesel" are often used synonymously. However, the 1st generation biodiesel derived from agricultural oil crops has been facing increasing criticism in terms of its impact on food prices, climate and ecosystems, so that the question was raised if 1st generation biofuels should actually be further promoted as sustainable energy alternative in the future. Accordingly, research and industry strived to find alternative oil sources for the transesterification into biodiesel. In this context the utilization of oils produced by oleaginous microorganisms has been identified and investigated as one possible approach. These organisms are able to accumulate significant amounts of lipids inside their cells, some as high as 80% of their respective cell dry weight, with similar characteristics as plant oil, when cultivated under nutrient limited and carbon excess conditions [3,4]. In terms of microbial lipid production, the red yeast *Rhodotorula glutinis* has been studied due to its high potential oil content of up to 72% [4]. It has also been investigated for its ability to produce high value carotenoids, namely β-carotene, torulene and torularhodin, which can be utilized as natural colorants or as ingredients of pharmaceutical products due to their antioxidant and pro-vitamin A properties. For both production pathways the cost of the carbon source has been identified as one of the main cost factors. Thus, the utilization of a cheap carbon source could significantly enhance the economic features of both approaches. Hence, a broad range of potential substrates have been tested for 1) lipid production, ranging from industrial by-products like glycerol [5] and molasses [6] over hydrolyzates of agricultural residues [7] to original waste products like municipal wastewater [8] and 2) carotenoid production, including flour extracts, grape must [9], radish brine [10] and whey [11]. Also wastewaters from the food industry with supposedly high organic contents have gained attention [12,13]. A lot of these substrates contain starch as a

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principal carbon source. These starch containing wastewaters bear a large potential as cheap carbon sources for the microbial lipid production, if the respective microorganisms are able to utilize starch as rather complex polysaccharide in comparison to easily available monosaccharides. Some molds have shown good results when cultivated on starch as main carbon source [14,15], whereas it has to our knowledge not yet clearly been stated if *R. glutinis* is actually capable of degrading and utilizing starch as carbon source. Rubio *et al.* [16] found that strains of *R. glutinis* did not grow on starch and also its poor performance on starchy wastewaters from potato processing [17] suggest, that starch should be hydrolyzed prior to cultivation [18]. In contrast to these findings, Bhosale and Gadre [19] observed cell growth and carotenoid production by *R. glutinis* on starch as carbon source. Elsewhere [20,21] it is stated that starch assimilation of *R. glutinis* is strain specific. Thus, for the present study *R. glutinis* (ATCC 15125™) was cultivated on starch and glucose as carbon sources and the production of biomass and microbial lipids was determined in course of the cultivation to evaluate the suitability of starch as carbon source for this specific yeast strain.

2. Materials and Methods

Three treatments were designed which differed in the type and composition of the carbon source used. Apart from this, all treatments shared the same basal media, which contained (per liter) 0.5 g yeast extract; 1.0 g $Na_2HPO_4 \times 12\ H_2O$; 1.0 g KH_2PO_4; 0.4 g $MgSO_4 \times 7\ H_2O$; 1.0 g $(NH_4)_2SO_4$; 6 ml $FeSO_4$ solution (4 $g \cdot L^{-1}$ $FeSO_4 \times 7\ H_2O$), and 10 ml trace mineral solution. The trace mineral solution consisted of (per liter) 3.6 g $Ca_2Cl_2 \times 2\ H_2O$; 0.75 g $ZnSO_4 \times 7\ H_2O$; 0.13 g $CuSO_4 \times 5\ H_2O$; 0.5 g $MnSO_4 \times H_2O$; 0.13 g $CoCl_2 \times 6\ H_2O$, and 0.17 g $Na_2MoO_4 \times 2\ H_2O$. **Table 1** shows the type and amount of carbon source used for the respective treatment. "G100" was set as a control treatment and contained only glucose. In the other treatments fructose was used as easily available monosaccharide in order to be able to distinguish between the initially added carbon source and the glucose resulting out of starch degradation. Generally, the suitability of glucose and fructose as carbon source for *R. glutinis* was assumed to be equal.

Table 1. Type and composition of carbon sources for the experimental treatments.

Treatment	Glucose [$g \cdot L^{-1}$]	Fructose [$g \cdot L^{-1}$]	Starch [$g \cdot L^{-1}$]
G 100	40.0		
F + S 50:50		20.0	19.0
S 100		5.0	33.25

In "S 100" 5 $g \cdot L^{-1}$ of fructose were added in order to facilitate the initial adaption of the yeast to the utilization of starch as carbon source. All media were autoclaved and afterwards inoculated with 20 mL of *R. glutinis* seed culture, which has been cultivated on yeast malt broth (3 $g \cdot L^{-1}$ yeast extract, 3 $g \cdot L^{-1}$ malt extract, 10 $g \cdot L^{-1}$ glucose, 5 $g \cdot L^{-1}$ vegetable peptone) for 48 h at 25°C on a rotary shaker at 115 rpm. *Rhodotorula glutinis* ((Fresenius) Harrison, anamorph) was obtained from the American Type Culture Collection (ATCC 15125™). Fermentation was carried out in 1000 mL Erlenmeyer flasks, containing 700 mL of the respective medium, on a rotary shaker at 115 rpm and 25°C for 240 h. The experiment was carried out in triplicate. 50 mL samples were taken after 0, 48, 120, 168, 216, and 240 h of cultivation and centrifuged at 3000 rpm for 10 min. The supernatant was used for the determination of pH, NH_4^+-N and sugar content. NH_4^+-N was measured photometrically using reagent vials from Macherey-Nagel (Düren, Germany). 200 µL of the sample is added to the test tube. Ammonium reacts with hypochlorite and salicylate in the presence of sodium nitroprussiate as catalyst to form a blue indophenol. The color strength is then measured with a photometer (NANOCOLOR 400 D, Macherey-Nagel, Düren, Germany) at a wavelength of 690 nm. The sugar content was analyzed via HPLC (Merck-Hitachi HPLC system with DAD detector) using a Phenomenex Rezex™ RPM-Monosaccharide Pb + 2 (8%) column (300 × 7.8 mm) with water as mobile phase at a flow rate of 0.6 $ml \cdot min^{-1}$. The sugar content was determined by comparison to known standards (**Figure 1**). The remaining cell pellet was freeze-dried and weighted to determine the cell dry mass. It was observed that the pellet also contained a fraction of undissolved starch.

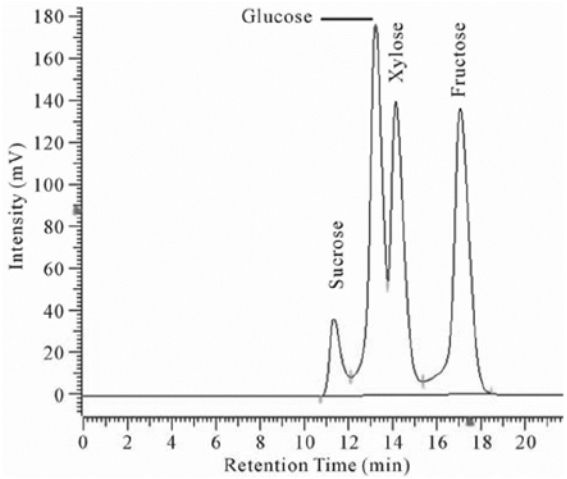

Figure 1. HPLC Chromatogram of standard for sugar analysis containing 10 $g \cdot L^{-1}$ sucrose, glucose, xylose, and fructose each.

Thus, the actual biomass production at the respective sampling times was calculated by subtracting the initially measured weight after 0 h, assuming that the fraction of undissolved starch will remain constant over the cultivation time. The lipid content of the dried cell pellet was determined using a modification [22] of the classical Bligh and Dyer [23] extraction with methanol and chloroform as solvents. The final lipid extract was weighted after the solvents were evaporated under nitrogen blow at 50˚C. This extract from the Bligh and Dyer procedure is later referred to as total extractable lipids. Afterward the extracts from sampling times 48 h and 240 h were transesterified with methanol with 2% H_2SO_4 as catalyst in a waterbath at 60˚C for 2 h. The reaction was quenched with $NaHCO_3$ solution and the resulting FAMEs were extracted with toluene and analyzed using an Agilent 6890 gas chromatograph with flame ionization detector (GC-FID) equipped with an Agilent J&W GC capillary column (30 m × 0.252 mm ID and 0.25 µm film thickness). The different fatty acids were identified by comparison to responses from a known FAME standard (Supelco® 37 component FAME Mix, Sigma Aldrich, München, Germany) (**Figure 2**). For quantitative determination of FAME production, the peak areas of fatty acids in the samples were compared with the peak areas of fatty acids in a known amount of FAME standard.

For starch analysis samples of the cultivation media were heated in a waterbath to completely dissolve all starch, then diluted with distilled water, mixed with amyloglucosidase (AMG) solution and incubated at 60˚C for 1.5 h in order to degrade starch into glucose. The amyloglucosidase solution was prepared daily by (per 12 samples) dissolving 10.6 mg of AMG (Amyloglucosidase from *Aspergillus niger*, ~70 U·mg^{-1}, Sigma Aldrich, München, Germany) in 12 ml buffer solution (pH 4.6). The samples were then filtered and analyzed for their glucose content using HPLC according to the method previously described for the sugar analysis. From the measured glucose contents, the starch content was calculated based on a 90% conversion rate from starch to glucose.

All data was statistically analyzed using Sigma Stat

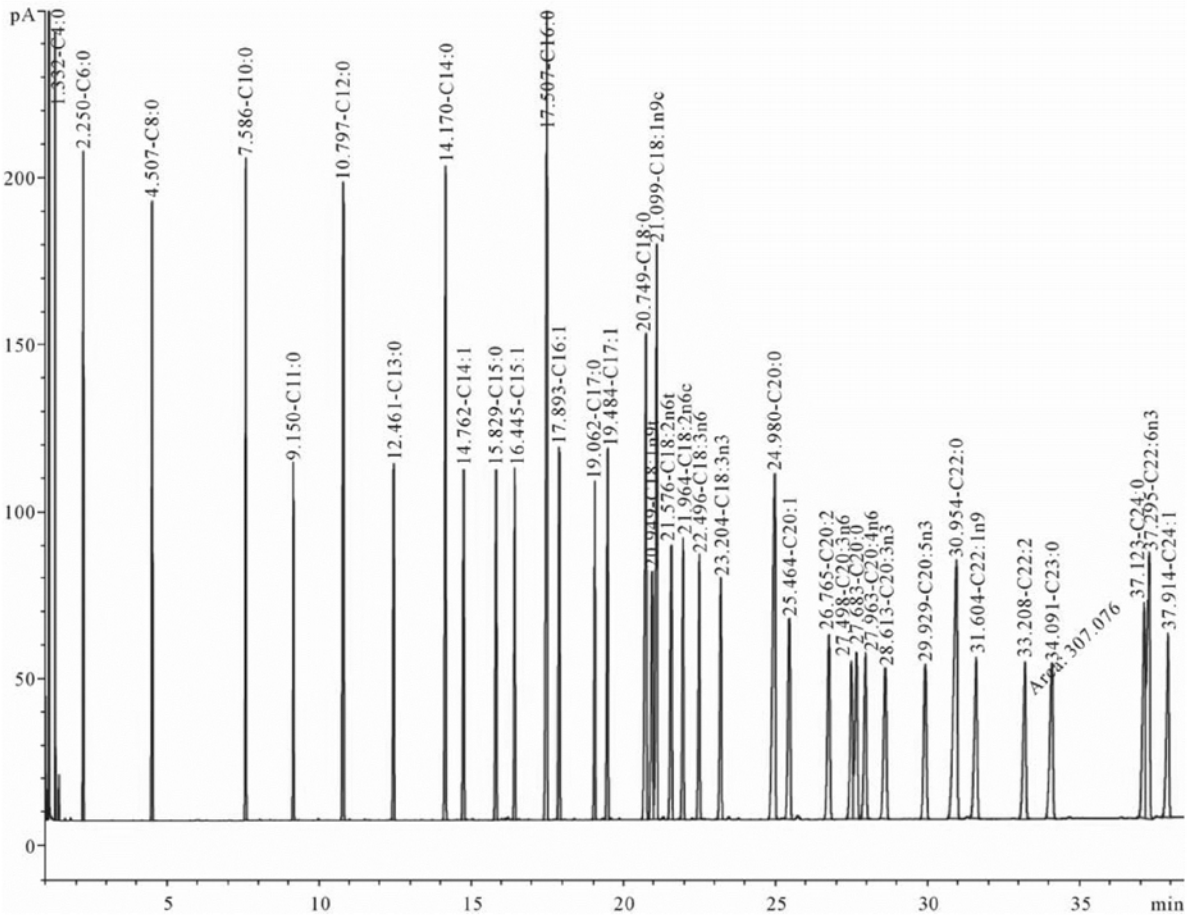

Figure 2. GC chromatogram of FAME standard solution.

3.5 (Jandel Scientific, USA). The statistical significance of differences in mean values was calculated using single factor variance analysis with the Tukey-Test at the 5% level of probability.

3. Results and Discussion

3.1. Sugar and Starch Consumption

As **Figure 3** shows, the monosaccharides glucose and fructose were steadily consumed in course of cultivation. Thus, based on the different initial sugar contents, the easily available carbon sources were exhausted after 48 h in S 100 and after 168 h in F + S 50:50. In treatment G 100 glucose was available throughout the cultivation with 75 % of total glucose consumed after 240 h.

The initially measured starch content of 18.1 $g \cdot L^{-1}$ in F + S 50:50 did not vary significantly over the duration of the fermentation. In treatment S 100 the starch content decreased significantly from 33.2 $g \cdot L^{-1}$ at 120 h to 27.5 $g \cdot L^{-1}$ at 168 h. This indicates that some type of mechanism for starch degradation must be available in *R. glutinis*. Verstraete *et al.* [24] reported the presence of an amylolytic *Rhodotorula* strain in activated sludge. However, at the same time it is rather incomprehensible that after this point no further decrease in starch was detected. If different carbon sources are offered for microbial growth, the easily available sugars are preferentially utilized. If this source is exhausted, cells switch to the second source, whereas at that time often a certain lag period can be observed, since it needs some time until the required enzymes for the degradation of the respective carbon source are produced. In the present study this lag phase was quite long and could be observed in S 100 between 48 h (fructose exhaustion) and 168 h (starch utilization). In F + S 50:50 fructose was available until 168 h. Thus, it can be assumed that in this treatment after a lag phase a certain degree of starch utilization might occur. To verify this assumption, the cultivation time should be increased in further studies.

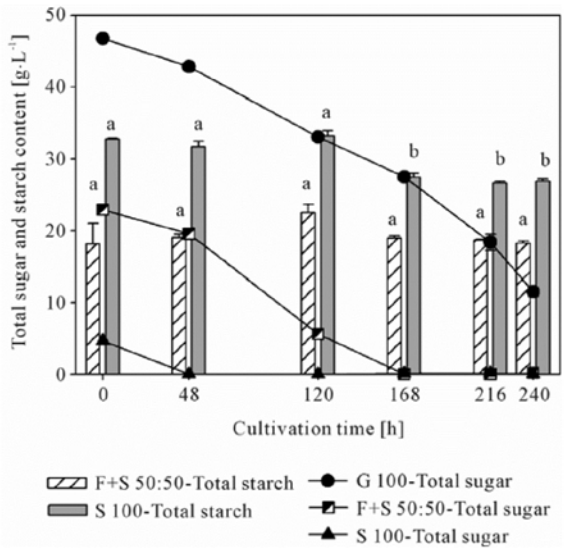

Figure 3. Utilization of monosaccarides and starch by *Rhodotorula glutinis* cultivated on glucose (G 100), a fructose/starch mixture (F + S 50:50), and starch (S 100). Different lower case letters within a treatment indicate significant differences at 5% level of probability.

3.2. Biomass Production

The results of sugar and starch consumption were in line with the data obtained from biomass and lipid production (**Figure 4**). Until 168 h the treatments G 100 and F + S 50:50 showed almost an identical increase in cell mass production along with a simultaneous decrease of the ammonium content, with ammonium as nitrogen source being completely exhausted after 120 h. The further increase in biomass was probably facilitated by the utilization of the yeast extract, which was initially added to the medium as secondary nitrogen source. In G 100 the cell mass continued to increase until 216 h, reaching a biomass yield of 12.3 $g \cdot L^{-1}$. Since glucose was still available at this point it can be assumed that nitrogen was the limiting factor for further biomass production. In F + S 50:50 biomass production ceased after 168 h at 9.1 $g \cdot L^{-1}$. This can be explained by the limitation of carbon, since the entire fructose was consumed from the medium and

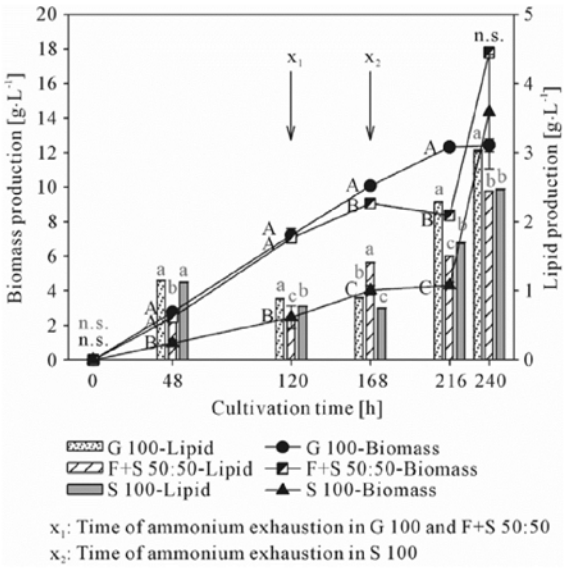

Figure 4. Biomass and lipid production of *Rhodotorula glutinis* cultivated with glucose (G 100), a fructose/starch mixture (F + S 50:50), and starch (S 100) as carbon source. Different lower case and capital letters indicate significant differences between the treatments for lipid and biomass production, respectively, at 5% level of probability.

starch as secondary carbon source was not utilized. In treatment S 100, fructose was exhausted after 48 h of cultivation, while no starch utilization could be observed until 120 h. Despite this limited availability of carbon the cell mass increased more or less steadily until 168 h.

However, compared to the other treatments biomass production was much lower, yielding less than half (4.0 $g \cdot L^{-1}$) of the cell mass compared to G 100 and F + S 50:50 after 168 h. Due to the fact, that nitrogen was utilized for the formation of biomass, ammonium was consumed from the media more slowly in S 100, being exhausted after 168 h of cultivation. The increase of biomass production after 216 h in F + S 50:50 and S 100 cannot be coherently explained. However, it is noticeable that the increase was almost identical for both treatments containing starch and it was also confirmed by photometrical cell density determination. Thus, it is unlikely that these increases are a matter of coincidence or flawed measurements. A production of actual cell mass is unlikely since nitrogen was exhausted at this point. Since the cell pellet after freeze drying was contaminated with residues of undissolved starch, the biomass production was calculated by subtracting the initially measured pellet weight at 0 h. For this, a stable proportion if residual starch was assumed. Thus, a decrease in the fraction of undissolved starch could appear as an increase in biomass production. However, this assumption is not supported by the fact, that no decrease in the starch content was detected.

3.3. Lipid and FAME Production

Regarding lipid production, it needs to be differentiated between the gravimetrically determined extractable lipid fraction as obtained by the Bligh and Dyer extraction (**Figure 4**) and the amount of saponifiable lipids represented by the amount of FAMEs as detected using GC analysis (**Figure 5**), because next to this saponifiable fraction of lipids the Bligh and Dyer extracts contain many other compounds that will dissolve in chloroform, e.g. certain carotenoids.

Still the gravimetrical results for lipid production are valuable indicators regarding the cycle of lipid production during microbial cultivation. Furthermore it needs to be considered, that not all extracted lipids are necessarily derived from the process of *De novo* lipid synthesis, which is defined as the production of storage lipids from sugar as carbon source. Since lipids are also found in cell membranes, an increasing lipid yield can be to some extend associated to a rise in biomass production. The gravimetrical lipid production (**Figure 4**) can roughly be divided into three phases, which are similar for all treatments: 1) an increase in lipid production during the first 48 h of growth, 2) a stagnation of lipid production in the middle of cultivation until 168 h, and 3) an increase in lipid production at the end of cultivation after 168 h (G 100 and S 100) and 216 h (F + S 50:50), respectively. The initial increase is probably related to the simultaneous onset of biomass production as described previously and thus cell growth induced. The second phase of lipid production 3) just starts after ammonium is exhausted from the medium (**Figure 4**). This is characteristically for *De novo* lipid synthesis, since carbon is only channelled into the production of storage lipids, when a limitation of a growth required nutrient (mostly nitrogen) restricts cell growth processes [4]. With 3.0 $g \cdot L^{-1}$ after 240 h, G 100 showed the highest lipid production. Other reports where shake flask cultivations of *R. glutinis* were applied observed similar yields [25-27]. However, treatments F + S 50:50 (2.4 $g \cdot L^{-1}$) and S 100 (2.5 $g \cdot L^{-1}$) had only slightly lower values for lipid production. This is rather surprising, since at that time in both treatments fructose was exhausted and starch was not or only slightly degraded, thus a carbon limitation could be assumed, which would usually hamper the production of biomass and lipids. The effect of the differences in the initial amounts of easily available sugars in the different treatments becomes obvious with the chromatographically determined FAME production (**Figure 5**). Since only glucose and fructose have been consumed as carbon sources, the FAME production follows the pattern of initial sugar content, whereas G 100 with the highest sugar content yields the highest amount of FAMEs, followed by F + S 50:50 with around half the initial sugar content and FAME production. S 100 had the lowest sugar content and only small amounts of starch were utilized. Thus, carbon was limited and no significant FAME production could be observed.

The fatty acid composition (**Table 2**) showed a pre-

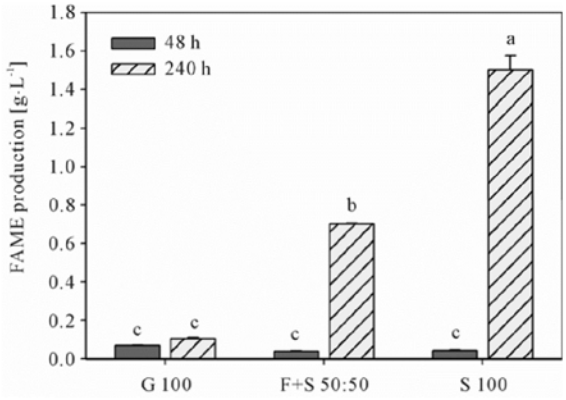

Figure 5. FAME production by *Rhodotorula glutinis* cultivated on glucose (G 100), starch (S 100) and a fructose/starch mixture (F + S 50:50) after 48 and 240 h of cultivation. Different lower case letters indicate significant differences at 5% level of probability.

Table 2. Fatty acid composition [%] of microbial lipids from *Rhodotorula glutinis* after 240 h of cultivation on different carbon sources.

Treatment	16:0	18:0	18:1	18:2	18:3	Other
G 100	27.9	11.6	43.7	10.7	3.5	2.6
F + S 50:50	30.2	8.8	44.0	11.9	1.6	3.5
S 100	12.6	5.8	42.7	33.9	2.8	2.2

dominance of palmitic (C 16:0) and oleic (C 18:1) acid, which is characteristic for *R. glutinis* [26,28]. Generally in all treatments the long-chain C 16 and C 18 fatty acid account for the vast majority with over 97% of total fatty acids. Thus, the microbial oils can be transesterified into biodiesel just like conventional plant oil. Moreover, the high share of oleic acid has beneficial effects on biodiesel fuel quality [29]. The comparably high content of linoleic acid (C 18:2) in S 100 and the fact that linoleic acid is often found in the lipids of cell membranes indicate that in this treatment biomass associated lipids derived from cell membranes prevailed.

The obtained results generally suggest that the mechanisms of starch utilization are poorly developed in *R. glutinis*. However, since in treatment S 100 a significant reduction of starch was measured, it cannot be ruled out, that this strain of *R. glutinis* over time develops the required enzymatic systems for starch utilization. For further experiments the cultivation time should be increased to observe if starch is further utilized. However, in the context of starch containing wastewaters as potential carbon source for microbial lipid and biodiesel production, it can be stated that a suitable enzymatic or thermochemical pretreatment for starch hydrolysis would increase the amount of available carbon and thus potentially increase the lipid yields. Other possible measures for increased starch utilization could be the co-cultivation with amylolytic yeast strains (e.g. [18]) or the use of specific strains of *R. glutinis*, which are adapted to the utilization of starch.

4. Conclusion

Wastewaters from food industry with high starch contents are a promising cheap carbon source as feedstock for microbial lipid and biodiesel production and also of potential interest for the microbial production of carotenoids. When red yeast *R. glutinis* was cultivated on soluble starch as carbon source, it yet showed a poor ability of starch degradation and utilization. Thus, before these wastewaters are used as fermentation medium it is advisable to conduct an additional pretreatment step for starch hydrolysis. However, since some literature sources report starch utilization by *R. glutinis*, the present results might be strain specific.

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8 General discussion

In the previous chapters different aspects of the utilization of industrial wastewaters as carbon source for microbial lipid and carotenoid production have been assessed. The primary results have been presented in the form of scientific articles. Thus, a detailed discussion of the respective experimental results can be found at the end of each chapter and is not included in the general discussion. In the first part of this general discussion, the results from chapters I-V are briefly summarized to finally examine the initially developed hypotheses and to draw implications for further research in this field.

The present chapter also aims to discuss the obtained results in a broader context and to evaluate the overall concept of microbial biodiesel production in terms of its potential to contribute an appreciable share in the future transportation fuel mix. In the introduction it has been shown that liquid biofuels in general are mainly promoted due to their potential to reduce GHG emissions from the transport sector. However, next to their **environmental benefits**, biofuels will also be judged regarding their **economic performance** and the **net energy gains** they provide over conventional fuels (Hill *et al.*, 2006). In addition, it is of importance if the proposed biofuels can actually be **produced in sufficient quantities** to displace conventional fossil fuels in significant amounts. Thus, in the second part of the general discussion the process of microbial biodiesel production will further be scrutinized according to these four aspects.

8.1 Experimental results and implications for further research

Wastewaters from lettuce processing, fruit juice production, potato processing, sugar refinery and beer production have been assessed in the previous chapters. They contained different amounts of organic substances as reflected by their Chemical Oxygen Demand (COD). According to this indicator the wastewaters could be divided into low (lettuce processing, fruit juice production), medium (sugar refinery, potato processing, total brewery wastewater) and high (process wastewater from lautering) COD wastewaters. However, in all wastewaters the COD was mainly derived from more complex carbon sources like disaccharides (maltose) and polysaccharides (starch), which could not be sufficiently utilized by *R. glutinis*. Due to the resulting carbon limitation microbial growth was hindered. Still yeast cell mass production was observed in all experiments, which indicated that the wastewaters did not contain any inhibitory substances and provided sufficient amounts of growth required micro and macro nutrients. Thus, regarding **hypothesis 1** it can be stated that wastewaters are a potential source of carbon and nutrients, whereas some aspects need to be taken into account. Firstly, the total end-of-pipe wastewaters are often diluted down to low contents of organic substances. Thus, is has been recommended to target specific wastewater streams from specific processes. Secondly, if wastewaters contain carbon sources other than monosaccharides, a hydrolytic pretreatment should be applied to degrade more complex carbon sources. Otherwise the selected microorganisms or the microbial consortium intended for fermentation needs to be adapted accordingly. With these findings in mind, a rerun of some experiments (sampling of specific fruit juice process wastewaters *e.g.* directly after pressing; enzymatic treatment of potato wastewaters prior to microbial fermentation) could deliver improved results.

Regarding **hypotheses 2-4** it needs to be stated that biomass production was realized by *R. glutinis* in all cases, but lipid and carotenoid production were only weakly developed or lacking completely in the wastewater treatments. The limitation of carbon due to (i) generally low carbon contents in the wastewaters and (ii) the poor performance of *R. glutinis* regarding the degradation of maltose and starch have been identified as major reasons for these results. All obtained microbial lipids by *R. glutinis* had a fatty acid profile comparable to plant oil with high contents of palmitic and oleic acid and are thus suitable for transesterification into biodiesel. The production of carotenoids was visually observed and also analytically confirmed for most wastewater experiments. A simultaneous production of carotenoids and lipids seems to be possible, while a certain competition over the common precursor Acetyl-CoA cannot be ruled out. Still it needs to be mentioned at this point, that even if a simultaneous production is metabolically possible, there is no protocol for the simultaneous extraction of lipids and carotenoids from biomass available. Moreover, since lipids are intended for biodiesel production and carotenoids are produced for nutraceutical applications, the production and processing of the two products need to comply with completely different production standards. Thus, it is probably not reasonable to aim at a combined production of lipids and carotenoids as co-products, but rather stick to the separate production pathways.

Relative to the initially set expectations, the tested wastewaters underachieved in their role as carbon source for *R. glutinis*. Still the results give valuable suggestions regarding further research in this field. Next to the already mentioned amendment of the wastewaters, the screening of wastewaters in general should be extended. For example, wastewater from vegetable processing, especially after the blanching process, exhibits high contents of organic substances. Additionally it needs to be considered that the present experiments were carried out as shake flask fermentations, with partly uncontrolled conditions regarding pH and aeration.

Table 3: Lipid yields of *R. glutinis* cultivated in different fermentation systems (a) and carotenoid yields of different *R. glutinis* strains (b) cultivated on different carbon sources.

a) Lipid yield	**Carbon source**	**Fermentation**	**Source**
2.5 g L^{-1}	Sugar cane molasses	Shake flask	Cheirslip *et al.*, 2011
3.0 g L^{-1}	Palm oil mill effluent	Shake flask	Saenge *et al.*, 2011
5.6 g L^{-1}	Glucose	Shake flask	Dai *et al.*, 2007
~ 20 g L^{-1}	Glucose	Fermenter	Dai *et al.*, 2007
~ 20 g L^{-1}	Corn waste syrup	Fermenter	Xue *et al.*, 2010

b) Carotinoid yield	**Carbon source**	**Strain**	**Source**
0.63 mg L^{-1}	Glucose	DBVPG 6081	Buzzini *et al.*, 2007
1.80 mg L^{-1}	Glucose syrup	DBVPG 3853	Buzzini & Martini, 1999
3.48 mg L^{-1}	Hydrolyzed mung bean waste flour / sweet potato extract	-	Tinoi *et al.*, 2005
5.95 mg L^{-1}	Rectified grape must	DBVPG 3853	Buzzini & Martini, 1999
33 mg L^{-1}	Glucose	Mutant 32*	Bhosale & Gadre, 2001
125 mg L^{-1}	Molasses sucrose	own isolate	Aksu & Eren, 2007
185 mg L^{-1}	Sugar cane molasses	Mutant 32*	Bhosale & Gadre, 2001

* NCIM 3353

It has been shown that yields can be significantly improved when the fermentation is conducted in a fermenter (**Tab. 3a**), where optimal conditions can be controlled and maintained during the cultivation. Regarding the carotenoid production, it has been shown that high carotenoid yielding strains can be established through specific breeding (**Tab. 3b**). Thus, whereas the present study assessed the general possibility of microbial cultivation on wastewaters, subsequent experiments are needed to optimize culture conditions, improve methodological features and maximize yields.

8.2 Does microbial biodiesel make sense?

The scientific knowledge, that certain microorganisms are capable of producing high amounts of lipids is not new. First reports about oil exploitation from yeasts are dated to the beginning of the 20th century. However, already at that time it was figured, that turning sugar into a plant oil substitute at poor conversion rates would make no economic sense and oleaginous organisms more or less remained a scientific curiosity (Ratledge, 2010). Accordingly, the future of microbial oils has been challenged over time. The renewed interest in this field of research within the late decades of the 20th century was mainly due to the finding, that some polyunsaturated fatty acids (PUFAs) were of high dietary value and that the exploitation of specific PUFAs like γ-linolenic acid (GLA) or docosahexaeneoic acid (DHA) yields considerably higher prices than conventional plant oil (Ratledge, 1993). In this context Ratledge (1993) asked "SCO[1] – have they a biotechnological future?" to find that the exploitation of high value fatty acids is the only economic feasible option for the application of oleaginous microorganisms and that the price erosion for alternate PUFA sources even handicaps this option. 15 years later, due to the current revival of microbial oils as source for biodiesel, Ratledge and Cohen (2008) raised the question again: "Do they have a future for biodiesel or as commodity oils?" Again, the economic dimension was described quite pessimistic, but still it is argued that further increases in the price of plant and crude oil could turn at least algal lipids into a viable alternative within the next 10-15 years (Ratledge and Cohen, 2008).

As shown in **Fig. 2**, the prices of plant oils as competitive product are indeed increasing. The price of crude oil as competitor for renewable fuels in general has been increasing as well, being still on a lower level compared to the plant oils. While microbial oils could profit from this trend, the prices for glucose as usually used carbon source are increasing at the same time, making the process of microbial fermentation also more expensive again. Thus, it is agreed that the economic restraints remain to be a major issue regarding the commercial up-scaling of the process. In this context, the next section assesses the cost structure of a potential microbial oil production plant, identifies certain bottlenecks and major cost sources within the process and evaluates different means to improve the economic performance.

[1] SCO = Single Cell Oils

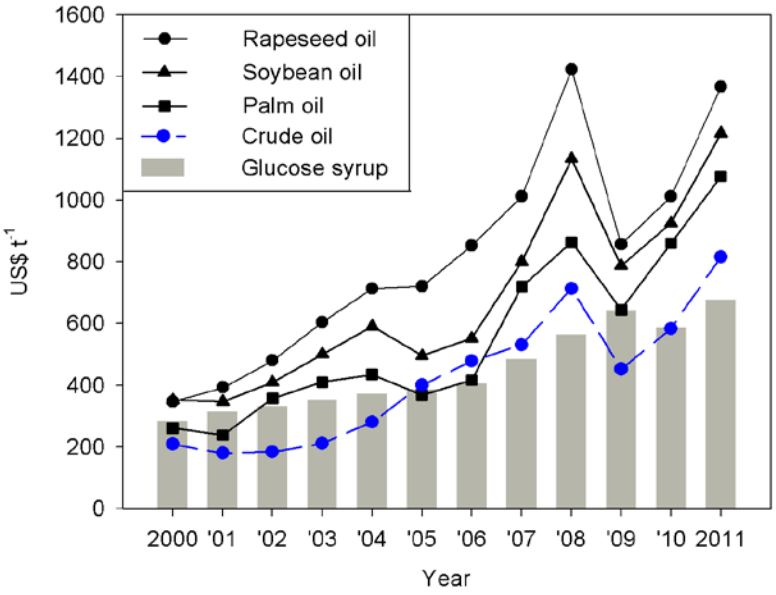


Figure 2: Price development of different plant oils (FOB Rotterdam), crude oil (brent) and glucose syrup.

8.2.1 Economic performance

It has already been stated multiple times, that the cost of microbial lipid production needs to be decreased to make it competitive to common plant oils. In this context it is inevitable to determine what kind of costs arise in the different process steps, to identify the major cost factors and to actually calculate different cost related economic parameters like the capital cost, the manufacturing cost and finally the break-even price of microbial biodiesel. Some cost calculations from the 1980s have been summarized by Ratledge (1987) and range between US$ 680 and 2500 per ton[2] of lipid. In their own calculation from 2008, Ratledge and Cohen assumed a price of US$ 3000 per tonne, excluding the cost of feedstock and the cost for extraction. However, these calculations are in some parts outdated and they also take different assumptions as basis.

For algal oil production different economic assessments have been published in the literature. Regarding some processes, these systems have similarities to those of microbial lipid production, *e.g.* in terms of biomass separation, drying and the subsequent lipid extraction. Depending on the underlying assumptions, the reported costs vary significantly (Sun *et al.*, 2011). Ratledge and Cohen (2008) concluded that algal oils cannot be produced for less than US$ 5600 – 7000 t^{-1}. Gallagher (2011) reported considerably lower prices (US$ 2 gal^{-1} (≈ US$ 587 t^{-1})), whereas other authors also assumed rather high prices around US$ 12 gal^{-1} (≈ US$ 3522 t^{-1}) (Sun *et al.*, 2011) and up to US$ 18 gal^{-1} (≈ US$ 5283 t^{-1}) (Davis *et al.*, 2011). However, since autotrophic algae utilize CO_2 as carbon source and the cultivations are mostly carried out in open pond systems, there are also cost relevant differences to microbial oil production. Thus, a simple cost analysis of the proposed microbial oil production process has been carried out. These results should help to draw a realistic picture of the current cost situation and to develop suggestions to reduce the cost related to the production process.

[2] Especially in Anglophone literature there is some discontinuity regarding the usage of the terms “ton” and “tonne” as reference values. Whereas “tonne” refers to the metric ton (≙ 1000 kg), the term “ton” refers to the American short ton (≙ 2000 lb ≈ 907.18 kg). The abbreviation “t” applies to a metric ton.

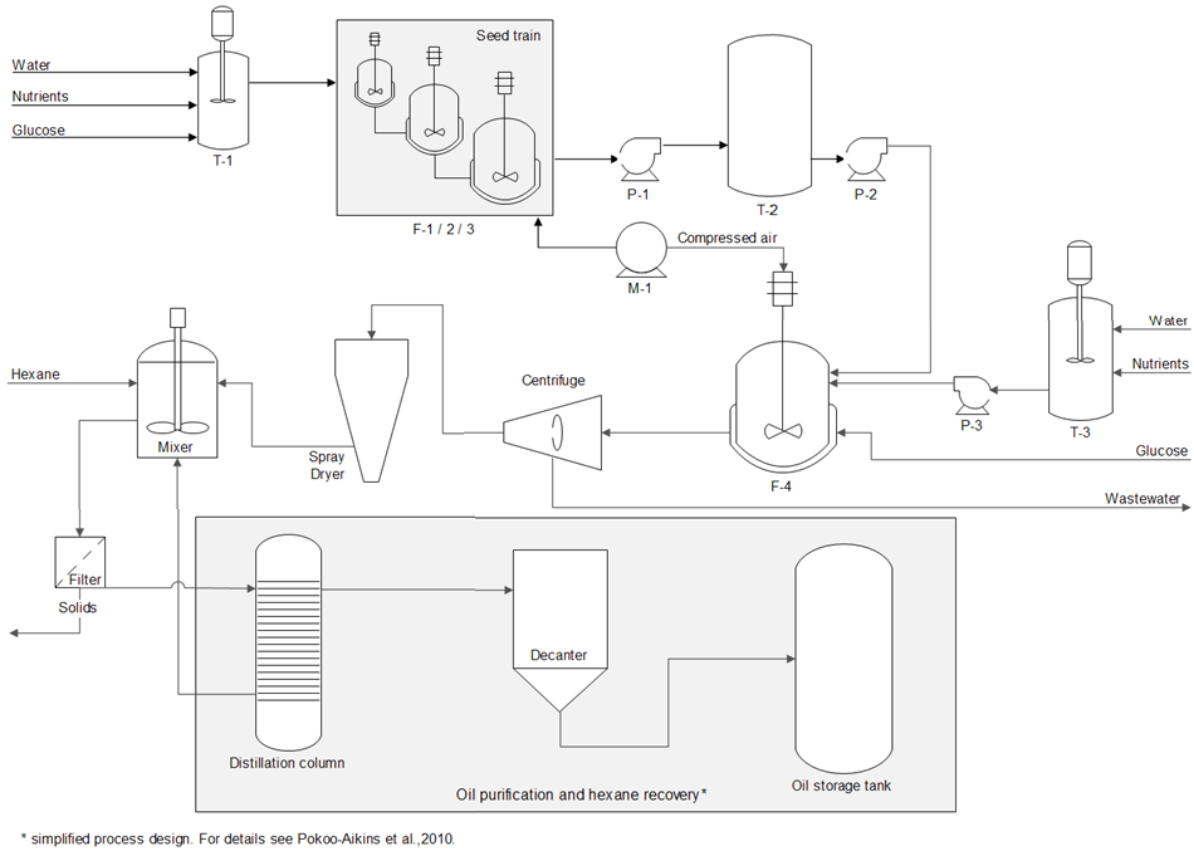


Figure 3: Simplified process design of the "yeast oil for biodiesel" process.

For this, a simple large scale process for microbial cultivation and lipid extraction has been designed (**Fig. 3**) and complemented with cost of equipment (**Tab. A 7**). The underlying methodology is listed in the "Infobox" below. When interpreting the obtained results from the cost calculation (**Tab. 4**), it needs to be considered that the design of the process and also the cost estimations for equipment or certain utilities are mere assumptions. Of course these are somewhat validated through the underlying literature sources, but still it needs to be considered that the calculated cost positions should rather be regarded as ballpark figures.

Under the given assumptions the annual manufacturing cost were calculated as US$ 24,234,717.78. Based on the estimated lipid production this leads to a break-even price of the microbial oil at US$ 2,362.02 t^{-1}. This means the oil must be sold at this price in order cover the cost of the production process, which also means that at this point no actual monetary profit has been gained. It has to be kept in mind, that the cost of the carbon feedstock for lipid production has not been included in this calculation. With prices of rapeseed and soybean oil around US$ 1300 and 1200 t^{-1}, respectively, it becomes clear, that the microbial oil process is not competitive to the conventional 1st generation biodiesel sources, which supports the results from economic assessments by other authors. In order to improve the economic situation, there are three possible approaches: (i) The reduction of production cost, (ii) an increase of lipid yields or increased prices for obtained by-products, or (iii) higher prices for the competitive product (*i.e.* plant oil).

Infobox: **Cost analysis – Assumptions and Methodology**

For the selection and the sizing of the different equipment components, a production plant with an annual production of around 10,000 t was targeted. The total operating time was assumed to be **360 days** (8640 h) per year. One fermentation cycle lasts 144 h (**120 h fermentation** + 24 h break for cleaning etc.), adding up to 60 cycles per year and fermenter. Under consideration of literature values, the **biomass yield** was set at **62.5 g L^{-1}** (Ratledge and Cohen, 2008; Yen and Yang, 2012), with an average **lipid content of 40 %** (Ratledge and Cohen, 2008) yielding **25 g L^{-1} lipid production**. Assuming a total of 12 fermenters and a working volume of 80 % (**600 m³**), the fermenters for lipid production were sized 750 m^3. The efficiency of the oil extraction process was set at 95 %, leading to an **annual oil production of 10,260 t**. The residual biomass after extraction contains considerable amounts of protein and can thus be used as animal feed. The selling of the **by-product** was presumed to yield **US$ 100 t^{-1}**.

The production process consist of four main parts: Seed production, fermentation, cell mass recovery, and lipid extraction. The final process of oil transesterification into biodiesel was omitted, since this process can be considered equal for both microbial and plant oils. Equipment cost for processes 1-3 were obtained from different literature sources*. All data were standardized on the basis of the cost year 2011 by scaling with cost factors according to the Chemical Engineering Plant Cost Index. For the calculation of the final installed cost, the purchase cost was multiplied by different Installation Factors as provided by Humbird *et al.*, 2011.

(1) Seed production: The process design for seed production was adapted from Humbird *et al.* (2011). To provide inoculum to the production fermenters, three trains with three seed fermenters each are batchwise operated. The cultivation media consist of glucose (10 g L^{-1}), yeast extract (3 g L^{-1}) and peptone (5 g L^{1}). The seed fermenters are each sized at 5 % of the next fermenter volume *i.e.* 0.1875 m³, 3.75 m³, and 75 m³.

(2) Fermentation: The fermentation was designed as fed-batch process with an initial working volume of 50 %. The basic medium contains $NaHPO_4$ (1.0 g L^{-1}), KH_2PO_4 (1.0 g L^{-1}), $MgSO_4$ (0.4 g L^{-1}), $(NH_4)_2SO_4$ (0.5 g L^{-1}), and glucose (50 g L^{-1}). Inoculum from seed production is fed to the broth at 10 vol. %. With proceeding glucose consumption, glucose syrup is over time added to a maximum working volume of 80 %. The fermenters are agitated, temperature controlled and sparged through with air.

(3) Cell mass recovery: Upon completion of the cultivation, the fermentation broth is centrifuged in a flow disc stack centrifuge to recover a cell paste with ca. 80 % moisture content (Molina-Grima *et al.*, 2003). In order to obtain a dry powder for lipid extraction, the biomass paste is further dewatered using a spray dryer.

(4) Lipid extraction: Hexane was selected as solvent for the current extraction process. Since the process design and the single equipment cost were difficult to acquire from the literature, the complete process step of lipid extraction with hexane was adapted from Pokoo-Aikins *et al.* (2010), who assessed the performance of different solvents for the extraction of lipids from sewage sludge. They calculated the total production cost (TPC) of the extraction. This was then directly incorporated in the cost calculation of the microbial oil production process. The extraction basically consists of three steps. (i) The dried cells are mixed with hexane and filtered to remove solids. (ii) In a distillation column the bulk of the oil and the solvent are recovered. (iii) Solvent and oil are separated in a decanter and oils stored in a storage tank.

The calculation of the different economic cost parameters as shown in Tab. 4 is adapted from You *et al.* (2008). The underlying assumptions for the **direct operating cost (DOC)** were as follows:

- The annual media requirement for seed preparation and fermentation is 27,000 m³ and 243,000 m³, respectively. For the cost calculation only glucose requirements for seed preparation were considered.
- Cost of electricity was calculated at US$ 0.0611 kWh^{-1} and an electricity demand of 5340 kW for seed production and fermentation (Humbird *et al.*, 2011), 30 kW for the centrifuge and 50 kW for the spray dryer were assumed. The electricity cost of lipid extraction is already incorporated in the TPC of the process as reported by Pokoo-Aikins *et al.* (2010).
- Cost for water only included water requirements for media preparation with US$ 0.22 m^{-3} (Peters and Timmerhaus, 1991) Wastewater was calculated as 90 % of fresh water input with US$ 0.59 m^{-3} (Molina-Grima *et al*., 2003).

* For details see appendix.

Table 4: Annual cost estimates (US$) for the process of microbial oil production.

A. Total capital investment cost, TCC (FCC + WCC)			**73,261,876.00**
1. Equipment cost, EC		**41,529,321.47**	
Seed production			
a. Seed fermenters	3,860,538.89		
b. Storage tanks	2,888,375.46		
c. Pumps	94,083.46		
d. Others	628,457.56		
Fermentation			
e. Fermenters	28,266,771.10		
f. Storage tanks	2,328,357.42		
g. Pumps	138,350.44		
h. Others	850,437.75		
Harvesting and Drying			
i. Centrifuge	1,505,829.18		
j. Spray Dryer	883,458.00		
2. Contingencies and fees, CFC (18 % of EC)		**7,475,277.86**	
3. Total basic module cost, TBMC (EC + CFC)		**49,004,599.33**	
4. Auxiliary facility cost, AFC (30 % of TBMC)		**14,701,379.80**	
5. Fixed capital cost, FCC (TBMC + AFC)		**63,705,979.13**	
6. Working capital, WCC (15 % of FCC)		**9,555,896.87**	
B. Total annual production cost, TPC (DOC + IOC + GE + DEPC)			**26,934,317.78**
TPC - Fermentation, harvesting and drying			*23,567,317.78*
1. Direct operating cost, DOC		**11,562,802.31**	
a. Glucose syrup (seed production only)	156,354.00		
b. Nutrients	1,827,835.33		
c. Electricity	2,892,913.92		
d. Water	53,460.00		
e. Operating labor	1,598,400.00		
f. Supervisory and clerical labor (15 % of operating labor)	239,760.00		
g. Maintenance and repairs (6 % of FCC)	3,822,358.75		
h. Operating supplies (15 % of maintenance and repairs)	573,353.81		
i. Laboratory charges (15 % of labor)	239,760.00		
j. Wastewater treatment	129,033.00		
2. Indirect operating cost, IOC		**2,233,159.58**	
a. Overhead, packing, storage (60 % of labor)	959,040.00		
b. Local taxes (1.5 % of FCC)	955,589.69		
c. Insurance (0.5 % of FCC)	318,529.90		
3. General expenses, GE		**3,198,605.34**	
a. Administrative expenses (25 % of overhead)	239,760.00		
b. Distribution and selling (≈ 10 % of TMC)	2,107,331.98		
c. Research and development (≈ 5 % of TMC)	1,053,665.99		
4. Depreciation, DEPC (10 % of FCC)		**6,370,597.91**	
TPC – Lipid extraction and processing[1]			*3,367,000.00*
C. Total manufacturing cost, TMC (TPC - CBP)			**24,234,317.78**
1. Credit of by-product, CBP		**2,700,000.00**	
D. Break-even price of oil [US$ t^{-1}]			**2,362.02**

[1]adopted from Pokoo-Aikins *et al.*, 2010

In order to reduce production costs, the main cost factors and bottlenecks within the production need to be identified. Looking at the cost compilation, the main costs arise during fermentation, harvesting and drying. Within these processes, DOC (49.06 %) and DEPC (27.03 %) are the main cost contributors. The TPC of lipid extraction and processing only accounts for 12.5 % of the total TPC. The DEPC and the cost of maintenance and repairs (accounting for 33.06 % of the DOC) are directly related to the equipment cost (EC). Accordingly, reducing the cost of equipment can strongly affect the overall production cost. Also Davies *et al.* (2011) conclude that cost improvements, in this case for algal cultivations, will rather be facilitated through capital cost than through operating cost reductions. However, also decreases in DOC offer a strong potential for overall cost reductions, whereas next to cost of maintenance and repairs as describes previously, the cost for electricity (25.05 % of DOC) plays an important role in this context. Thus, the economic and the energetic viability of the process are strongly connected with each other (de Boer *et al.*, 2012). This aspect will further be dealt with in the next chapter.

Looking at the equipment cost, the expenses for the fermenters can easily be indentified as the major cost factor. To find possible less expensive alternatives for this type of closed cultivation, again a look at autotrophic algae cultivation is helpful. In this field mostly two types of cultivation are considered, which are open fermentation in pond systems and closed cultivation in photobioreactors (PBR), which are principally similar to the fermenters modeled in the present lipid production process. Different economic assessments have shown that the use of PBRs cause to much cost to be feasible for the production of microalgal biodiesel and is thus restricted for applications in the production of high-value nutraceutical products (Greenwell *et al*., 2010). Open ponds on the contrary are simple and low-cost systems that are suitable for large scale production processes. Based on the data provided by Li *et al.* (2011), the production process was changed from closed to open cultivation by substituting the 12 fermenters for lipid production with 10 raceway pond units with 3000 m² each. The major drawback of these open cultivation systems is the risk of contamination with other microorganisms and the fact that the cultivation conditions cannot be fully controlled. To allow for these effects the biomass yield and the lipid content were adjusted downwards. In order to minimize the risk of contamination, the raceways should be operated at a high organic load and constantly fed with fresh contaminant-free inoculum (Greenwell *et al.*, 2010). To meet this increased demand for inoculum, the seed production process has been extended, now operating with six instead of three seed trains. The amount of operating labor has been reduced, since it can be suggested that the operation of raceway ponds will require less manpower. A brief summary of the underlying assumptions and cost positions is provided in **table 5**. In this scenario the equipment costs can approximately be cut in half and also direct and indirect operating costs decrease. Due to the lower yield assumptions the reduction of the break-even price is not as strong as maybe expected, but still at a considerable scale of around 20 %. If the initially assumed lipid yield of 25 g L^{-1} could be realized in this system, the break-even price can be beat down to less than US$ 1500. Again, these calculations do not include the cost of the carbon feedstock which once more emphasizes the importance of the utilization of low cost carbon sources as proposed at the beginning. The experimental results have shown that wastewaters can act as sufficient nutrient sources. If the purchase cost of nutrients is removed from the cost calculation this could further reduce the operating cost.

Table 5: Assumptions and cost estimates for microbial oil production in open raceway ponds.

Equipment assumptions[1]:			***Yield assumptions:***		
Raceway ponds (incl. paddlewheels)	30,000 m²	US$ 12 m^{-2}	Biomass production	55.00	g L^{-1}
Land area requirement	37,500 m²	US$ 12 m^{-2}	Lipid content	35.00	%
Air compressor system	10 kW	US$ 6000	Lipid production	19.25	g L^{-1}
Pond power input	400 W		Annual lipid production	7900.20	t
Pond medium depth	30 cm				

Annual cost estimates [US$]:		
Total capital investment cost, TCC		**36,833,026.85**
Equipment cost, EC	20,879,217.08	
Total manufacturing cost, TMC		**14,879,023.40**
Direct operating cost, DOC	7,204,734.94	
a. Nutrients	*2,700,655.33*	
b. Electricity	*361,297.50*	
c. Water	*59,400.00*	
d. *Operating labor*	*1,198,800.00*	
Indirect operating cost, IOC	1,359,654.38	
Break-even price of oil [US$ t^{-1}]		**1,883.37**

[1]adopted from Li *et al.*, 2011

However, when comparing closed fermenters with open pond systems it also needs to be considered that ponds require more land than fermenters. This is caused by the relatively shallow level of medium (or water in terms of algae cultivation) in then ponds. With a certain defined amount of medium or water necessary for cultivation, the depth of the ponds will be the key variable for the determination of the overall land requirement. For algae cultivation, the average water depth ranges between 10 and 30 cm. In deeper ponds algae could shade each other, leading to an unequal distribution of sunlight (Richardson *et al.*, 2010). However, since this approach works with heterotrophic yeasts that do not depend on sunlight, the pond depth could be increased. Whereas this leads to significant land savings, the reduction of the production cost is only marginal. When the medium depth is increased to 100 cm the land requirement decreases to 9 ha, whereas the break-even price is only diminished by around US$ 30 t^{-1}. Furthermore it needs to be considered, that open ponds are mostly operated outdoors without temperature control. For most oleaginous yeasts the optimum cultivation temperature is above 20 °C. This means that in most climatic regions it will not be possible to operate a facility throughout the year. Of course different technical measures to adjust the temperature can be taken, but this will again add to the production cost. All in all the application of open pond cultivation for yeast oil production offers a promising cost saving potential. But this type of cultivation has not been tested yet with oleaginous yeasts, so that it is difficult to assess their susceptibility to other contaminant organisms and unstable cultivation conditions and thus to estimate the realized biomass and lipid yields.

Also higher lipid yields and increased prices for both obtained by-products and the competitive products have been suggested as means to improve the economic characteristics. The cost analysis was conducted with a lipid content of 40 %. At the maximal possible biomass yield, this is assumed to be the optimum value. A further improvement in oil content can only be achieved by reducing the amount of produced

lipid-free biomass rather than increasing the amount of produced oil (Ratledge and Cohen, 2008). Thus, for the present calculation already quite optimistic yield assumptions have been made and it is unlikely that considerable yield improvements will be realized in the future.

In the first part of the discussion it has already been stated that the combined production of different co-products *e.g.* microbial lipids and carotenoids is not practicable, especially if the fields of application raise different requirements for the production process. Thus, independent of the product that is extracted from the microbial biomass, the residual biomass is the only feasible by-product. Due to its protein content, it was assumed to be sold as animal feed additive. However, it is not sure if the residual biomass can actually meet the criteria that apply for animal feed utilization. Furthermore it would need a certain treatment *e.g.* removal of all solvent residues in order to be marketed in this way. Otherwise the biomass could be utilized for direct combustion or anaerobic digestion, which would yet yield lower compensations than the use for animal feed. Thus, it seems that also on the topic of value of the by-product the full potential has been tapped.

Regarding the price of the competitive product *i.e.* plant oil, it has already been stated several times that a substantial rise in plant oil prices is rather unlikely. Also the crude oil prices as final benchmark for renewable oils are still on a lower level and also quite volatile, which makes them hard to compete with. Even though crude oil prices have been increasing, a strong escalation of prices as expected when “Peak-oil” becomes reality cannot be foreseen for the near-term future.

Thus it can be doubted that the process of microbial biodiesel production will become economic feasible within the next years. A commercial production will thus only be implemented through governmental financial support systems in terms of subsidies. Already over the past decade the European Union has allowed their Member States certain regulatory policy measures in order to promote the commercial launch of biofuels. These measures vary between the different Member States and include border protection, mandatory blending requirements and excise tax exemptions, with the latter contributing the largest share to the overall support. In 2006 a total transfer of € 2436 Mio has been supplied for biodiesel in the EU. In the same year subsidies and market price support accounted for a share of 65 % of total biodiesel market value (Kutas *et al.*, 2007). The implementation of subsidies generally involves some negative impacts. Firstly they cost financial resources and thus decrease the fiscal revenues and secondly they always lead to a suboptimal allocation of resources and a loss in economic efficiency. For example, regarding biodiesel from rapeseed the subsidy cost to mitigate one tonne of CO_2-equivalent is estimated to be over € 600 (Kutas *et al.*, 2007). From an economic point of view this makes hardly any sense, since reductions could be cheaper purchased over the market using the prospects of Clean Development Mechanism (CDM). Thus a subsidy of any type is only justified, if it is volitional to reach a certain economic or societal state, which is not realized through the market itself. This is the case for biofuels. The higher prices of biofuels would normally hinder an increased adoption of biofuels, which is necessary in order to achieve the goals of emission savings and biofuel shares set by the European Union. Lower prices for competing petroleum fuels can be achieved because negative externalities caused by petroleum fuel production and consumption are not reflected in their market price. So far, basically all types of biofuels are supported. However, it has been shown that microbial biodiesel and also 3rd generation biofuels in general need specific subsidies in order to be competitive

toward common 1st generation biodiesel/biofuels. The European Union already indicated their efforts to achieve exactly this. In fall 2012, the EU Commission proposed a revision of the current support framework, to react to an increasing number of reports that cast a rather bad light on 1st generation biofuels in terms of their sustainability and their GHG emission potential. While the general goals of 6 % GHG emission reduction and a share of 10 % renewables in the transport sector until 2020 remain, the amount of 1st generation biofuels that are actually credited against this share should be capped to 5 %. The remaining 5 % thus have to be achieved by 2nd and 3rd generation biofuels. Furthermore, it is planned to completely cease the support for 1st generation biofuels after 2020 and instead set specific market incentives for the production of 2nd and 3rd generation biofuels that provide significant emission cuts (EU press release, 2012). Still, in order to qualify for that kind of support, microbial biodiesel needs to provide **emission savings and environmental benefits** as well as contribute to energy independency in a sustainable way. The performance of microbial biodiesel regarding these aspects will thus be discussed in the following sections.

8.2.2 Environmental benefits

Microbial biodiesel and biodiesel in general have several environmental advantages over conventional petroleum diesel, which are considered to be for example reduced life cycle emissions of GHG and air pollutants like carbon monoxide, volatile organic compounds and oxides of sulfur (Hill *et al.*, 2006), whereas the potential GHG emission savings are the major reason for the promotion of biodiesel. However, as stated in the introduction, these emission savings are approaching zero or are even turning negative, when effects of direct and indirect land use change are taken into consideration. Furthermore, within the next years European standards will require GHG emission savings of up to 60 % for biodiesel compared to conventional diesel. Microbial biodiesel and other 2nd and 3rd generation biofuels will be judged by this threshold in the future, with the GHG emission potential being one of the most important benchmark for different biofuel processes.

In order to evaluate the **GHG emissions of yeast biodiesel**, it is necessary to examine the production chain within wide system boundaries, from “cradle to gate” or even from “well-to-wheel”. For the “yeast oil for biodiesel”-process (YOB) no such life cycle assessment (LCA) has been reported so far. To the authors knowledge in fact there are generally no descriptions of this process above bench scale available. However, within certain system boundaries the pathways of biodiesel production from both yeast and algae are similar and LCAs of algal biodiesel production can be used as valuable source of information. Same as for microalgae, the life cycle of yeast biodiesel can be divided into different categories. On a “cradle to gate” basis these would be microbial cultivation, lipid extraction and biodiesel production. When the yeast cultivation is carried out in raceway systems as suggested in the previous chapter, this even increases the affinity to algae cultivation systems. Yet, one difference, which is of particular importance for the emission of CO_2, is the fact that algae are autotrophic organisms that actually consume CO_2 as energy source, while yeasts are heterotrophic organisms that require an organic carbon source and produce CO_2 during cellular respiration. The carbon “fixation” by autotrophic algae is reported to range between 1.5 and 1.8 kg CO_2 per kg of dry algal biomass (Chisti, 2007; Patil *et al*., 2008). While this appears to be a grave advantage of algal cultivation, the process also suffers some limitations in comparison to yeast

cultivation. These are the need for sunlight and the supply of CO_2 in high concentrations. The former limits the achievement of maximum yield, limits the production to certain areas and also leads to the fact that up to 25 % of the biomass produced during daylight can be lost due to respiration at night time (Chisti, 2007). Regarding the latter it is often assumed that CO_2 is provided through flue gas from industrial facilities. This to some extend also limits the location of algae production to industry close sites. Other advantages of using yeasts are their higher lipid contents (Ratledge and Cohen, 2008), high growth rates due to their unicellular nature and their easier handling regarding the scale up (Bhosale and Gadre, 2001).

If the system boundaries should be further broadened, additional life cycle categories need to be considered. These would include the GHG emissions related to nutrient production, carbon feedstock provision/production, waste and wastewater treatment, as well as the processing of by-products. Also here certain parallels to algal cultivation systems can be drawn.

Several studies have assessed the performance of algae biodiesel with regard to life cycle GHG emissions. It lies in the nature of life cycle assessments that the results can vary strongly between different studies, depending on which system boundaries have been chosen and which underlying assumptions have been made. Nevertheless most authors agree, that with the currently available technologies the GHG emissions of the "microalgae to biodiesel" process are actually worse compared to petroleum diesel and also compared to conventional 1st generation biodiesel (Clarens *et al.*, 2010; Lardon *et al.*, 2009; Khoo *et al.*, 2011, Sanders and Murthy, 2010). Lardon *et al.* (2009) assessed a whole set of different potential environmental impacts and found that the global warming potential from algal biodiesel is considerably higher than that of biodiesel from rapeseed or palm, whereas it needs to be argued here that no land use change effects have been included in the modeling of the global warming potential of these 1st generation biodiesel sources. It was also shown that the climate relevant emissions were mainly driven by the **high energy requirements** connected to the extraction of the algal oil. This strong relationship between the energy demand within the oil extraction process and the overall GHG emissions was similarly reported by Stephenson *et al.* (2010) and Khoo *et al.* (2011). Since lipid extraction techniques are similar for algal and yeast cells, also the YOB process will suffer this limitation. The cited authors also agree that reduced energy consumption, especially within the extraction process (including drying), can substantially improve the GHG balance and the overall sustainability of algal - and accordingly also yeast – derived biodiesel. The aspect of energy savings will be further discussed within the next chapter, dealing with the overall energy balance. However, Stephenson *et al.* (2010) reported that the algal biodiesel would require 85 % less fossil-energy and emit 78 % less GHG compared to fossil-derived diesel. This good performance is based on the fact that high rates of energy production and CO_2 savings due to anaerobic digestion of residual biomass were assumed.

Clarens *et al*. (2010) identified the aspect of **fertilization** as another driving force of the global warming potential. This applies also for the YOB process, which requires sufficient amounts nutrients, mainly nitrogen and phosphorus. If these nutrients could be supplied from waste sources, *e.g.* certain wastewaters as shown in the results section, this could help to appreciably reduce GHG emissions. When the allocation of the nutrient source is included in the life cycle analysis, it is likewise necessary to consider the origin of the carbon feedstock as energy source for yeast cultivation. If, as intended, residual or waste

products are used as feedstock it will not greatly affect the GHG balance. However, if the carbon source is derived from *e.g.* agricultural products like sugar cane, corn starch or lignocellulosic biomass, also the emissions related to the production of these commodities would have to be incorporated. Needless to say this would put a further burden on the overall GHG balance.

Besides few exceptions it can be assumed that based on different life cycle evaluations of algal biodiesel the YOB process will currently not succeed to realize the GHG saving goals set by the European Union. Under these criteria microbial biodiesel will not be a viable alternative to displace 1st generation biofuel. Still it needs to be considered that this approach is still in its infancy. The central flaws of the process have been identified, showing that there is still room for further research and development. Thus it is reasonable to point out the possibilities to substantially improve the climatic impact of this process by (bio-) technological progress.

It has to be positively remarked, that among the other examined environmental factors microbial biodiesel could outcompete 1st generation biodiesel in terms of **eutrophication potential** and **land competition**. Compared to agricultural crops, the risk of nutrient leaching into ground- and surface waters is minimized, since nutrient supply and uptake as well as the treatment of spent nutrient broth can be surveyed and controlled. While the land use by agricultural crops and the connected land use change effects are highly discussed for 1st generation biodiesel, this issue does not apply for microbial biodiesel, because the production plants require only a fraction of land area. A facility with fermenters which are built vertically would require the least space. But even with raceway cultivation according to the previous model an oil production of 7900 t on only 30 ha pond area (ca. 260 t ha^{-1}) could be realized.

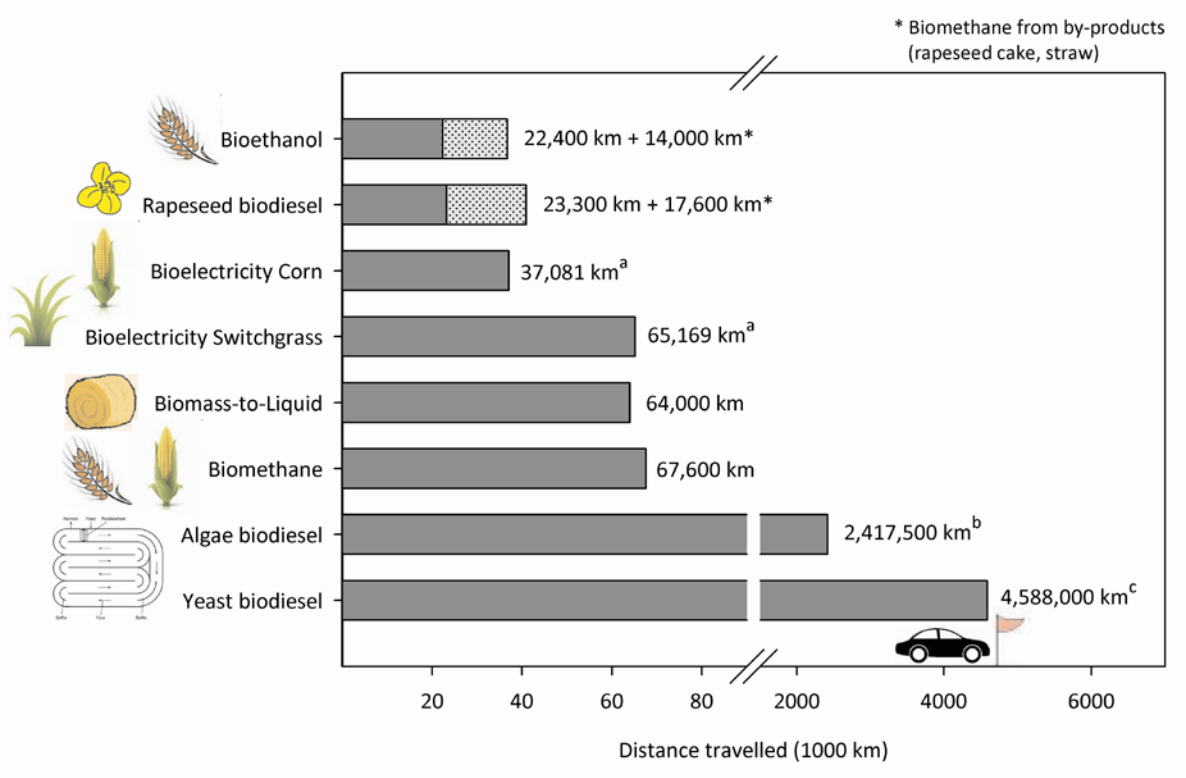


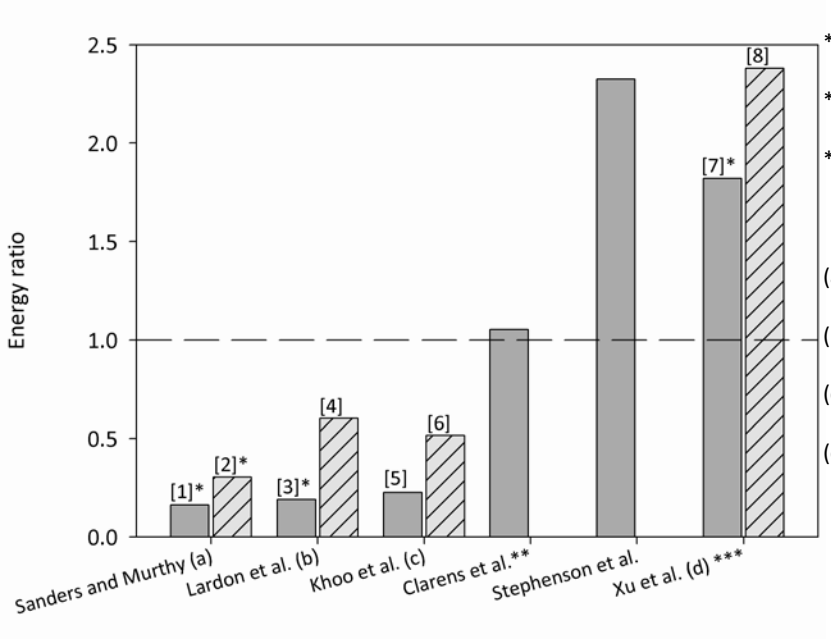
Figure 4: Possible accomplished distance by a passenger car on fuel cultivated on 1 ha land. Where not indicated differently data were adopted from FNR, 2012.
[a] Campbell *et al.*, 2009; [b] Calculated based on data by Chisti (2007); [c] Own calculations; annual production

For autotrophic microalgae Chisti (2007) calculated oil yields as high as 137 t ha^{-1}. The required area could even be further reduced when pond depth is increased. On a global average, allowing for different climatic conditions, crop varieties and crop management measures, the oil yield from temperate oil crops can be quoted at around 1 t ha^{-1}, whereas palm oil yields can reach up to 4 t ha^{-1} (Murphy, 2009). Thus, yeasts are several times more efficient oil producers than agricultural oil crops. Moreover, it is possible to even use non-agricultural and marginal land to build microbial production units. If these oil yields are converted into biodiesel and translated into the potential distance a passenger car can travel with the fuel produced on 1 ha land (**Fig. 4**), the land use efficiency of microbial biodiesel becomes even more obvious.

8.2.3 Energy balance

Any approach related to the allocation of energy is only efficient, when the energy produced exceeds the required energy input of the process. Thus, if a process is intended for implementation on a large scale, a positive energy balance is an inevitable prerequisite. As already indicated in the previous chapter, the process of lipid extraction acts as a major energy sink within the production process of algal - and thus presumably also yeast - derived biodiesel. This strongly influences the overall energy balance of the proposed process as shown in different energy based LCAs provided in the literature. Again, due to the thematic proximity it is possible to apply results from energy analysis of the "algae for biodiesel" process to the "yeast for biodiesel" process. **Figure 5** shows the results of different studies regarding the energy ratio of algal biodiesel production, which is calculated by dividing the energy provided by the obtained product (biodiesel) through the required input energy for this production. Thus, at an energy ratio above 1 the produced energy will offset the consumed energy and lead to a positive energy balance. Direct comparisons between these studies are difficult due to the different assumptions in terms of yield and process parameters, the selected system boundaries, the allocation of co-products, as well as the applied technologies. This also explains the partly considerable differences between the reported values.

Sander and Murthy (2010), Lardon *et al.* (2009) and Khoo *et al*. (2011) came to the conclusion that the energy demand within algae cultivation, lipid extraction and biodiesel production is greater than the energy provided by the resulting amount of biodiesel. However, they all identified major bottlenecks in energy consumption, suggested and assessed possible measures to substantially reduce the energy demand and showed that the energetic utilization of the obtained co-products can help taking the process to an energetically self-sufficient level. Both Sander and Murthy (2010) and Lardon *et al*. (2009) included several dewatering and drying steps in their model in order to achieve an algae solid content of at least 90 % prior to lipid extraction. Accordingly, both identified the drying step as largest energy sink within the process. Sander and Murthy (2010) also showed that the use of a filter press (Fig.5 [2]) for primary dewatering of the algal slurry requires only half the energy compared to a centrifuge (Fig. 5 [1]). They also suggested the application of solar drying to further reduce the energy demand and calculated that a solar drying to 19 % moisture content would be necessary to achieve a zero net energy balance. While Lardon *et al.* (2009) also mention solar drying as possible alternative they argue that the feasibility of this approach has not been demonstrated yet.



* Cells dried to ca. 90 % solid content prior to extraction.
** Lipid extraction and transesterification not included.
*** Fossil energy ratio including co-product allocation, and utilization of waste heat.

(a) Pre-drying with centrifuge [1] and filter press [2]
(b) Cultivation under normal [3] and low [4] nitrogen conditions
(c) Base case [5] and best case [6] scenario
(d) Cultivation under low nitrogen conditions with dry [7] and wet [8] extraction

Figure 5: Energy ratios of "algae to biodiesel" processes, calculated from different literature sources. Different numbers indicate different technologies applied within one study.
[Where not indicated differently, the process included algal cultivation and harvesting, wet lipid extraction, and biodiesel production, excluding any co-product allocation. Energy ratios were calculated by dividing the amount of energy (Joule) contained in biodiesel by the energy required to produce this defined amount of biodiesel.]

In order to reduce the energy for drying, they suggested shifting to a wet extraction system where lipids are only dewatered to a solid content of ca. 20 % prior to solvent extraction. Moreover they proposed the cultivation of algae under nitrogen deprived conditions to achieve higher lipid yields and to reduce fertilizer demands. If both measures were applied the cumulative energy demand could be reduced by around 70 % due to large savings in the fields of heat and fertilizer provision (Fig. 5 [4]). Next to energy savings related to technology changes, also the allocation of possible co-products needs to be considered. When algal meal was used to displace corn as feedstock for ethanol production (Sander and Murthy, 2010) or when the oil cake was channeled to anaerobic digestion (Lardon *et al.*, 2009) considerable amounts of energy can be provided, that – together with the energy contained in the biodiesel – will offset the total energy demand. At the same time it is somewhat controversial to include co-product credits in energy calculations (Xu *et al.*, 2011). Since there is no microbial biodiesel production on large scale yet, potential applications and the energetic evaluation of the co-products is based on assumptions only, leaving room for overestimations of the actual energy value. Also the energetic utilization of residual biomass competes with other possible uses, for example as animal feed as suggested previously.

Khoo *et al*. (2011) considered a dewatering to a solid content of only 15 % via centrifugation (Fig. 5 [5]). Because extensive drying was omitted, the main energy (85 %) was required during lipid extraction, whereas solvent evaporation and recovery can be considered as most energy intensive processes in this context. Also here a significant reduction (ca. 60 %) of energy requirements could be achieved by different measures. To constitute a certain best case scenario (Fig. 5 [6]), the hypothetical lipid content was increased to 45 %, the estimation of the heating value of algal biodiesel increased to

42 MJ kg^{-1} and the energy consumption in lipid extraction hypothetically reduced to 1.3 MJ per MJ biodiesel, assuming the prospective availability of more efficient extraction techniques. However, with an energy demand of 1.94 MJ per MJ of biodiesel the energy balance, leaving out any co-product allocation, remained negative.

Opposite to the previously assessed studies, Clarens *et al.* (2010), Stephenson *et al*. (2010) and Xu *et al.* (2011) reported a positive energy balance and accordingly an energy ratio above 1 for microalgal biodiesel. Clarens *et al.* (2010) only assessed algae as alternate source of biomass compared to agricultural crops. Thus he calculated the energy requirements related to the energy provided by algal biomass, omitting the steps of lipid extraction and biodiesel production. Since these have been identified as major energy sinks, the interpretations by this author should be approached with caution. Contrary to most other reports Stephenson *et al.* (2010) found the bulk of energy consumption (ca. 70 %) within cultivation and centrifugal dewatering of the algal slurry, which can be seen as the major explanation for the low overall energy requirements. To achieve this low energy demand they designed an extraction process where heat is only required for the separation of lipids and hexane. However, since increased temperatures and pressure can increase the extraction efficiency (de Boer *et al.*, 2012), it needs to be argued that the procedure proposed by Stephenson *et al*. (2010) could lead to lower lipid extraction yields.

Xu *et al.* (2011) modeled two biodiesel production pathways aimed at reducing the energy consumption. Since they also identified the dewatering of algal sludge as major energy sink, they proposed a dry (Fig. 5 [7]) and a wet (Fig. 5 [8]) route to reduce energy consumption related do drying. In the dry route energy savings should be achieved by improving the drying efficiency through the combination of different dewatering techniques, while the wet route omits the dewatering step completely, extracting lipids directly from the wet sludge. While in the dry route lipids will be transesterified into biodiesel, the wet route assumes the hydrotreating of lipids to obtain green diesel. They also modeled two scenarios with conditions of standard and low nitrogen supply. For all base case scenarios regardless of extraction route and nitrogen status a fossil energy ratio (FER) >1 was achieved. Here it needs to be considered that the energy yields from the co-products gas, pyrolysis oil and glycerol were fully included into the calculation of FER. Without the allocation of the co-products the FNR would decrease appreciably. Major energy sinks were identified as the dewatering step within the dry route and the extraction within the wet route. For the wet route the energy savings due the abdication of drying was offset by the increased energy requirements for extraction. Thus, a reasoned combination of different drying techniques can substantially decrease the energy requirements. Due to higher lipid yields and lower fertilizer requirements the low N conditions should be favored. If waste heat is used for thermal drying, solvent evaporation and extraction, the FER even further increased, reaching 2.38 and 1.82 for the dry and the wet route, respectively.

It can be summarized that based on different underlying assumptions the results from different LCAs vary considerably. However, even if some of them calculated a positive energy balance where others did not, they agree that through different improvements a positive energy balance for microbial biodiesel can be achieved. These have been identified as (i) the cultivation under low nitrogen conditions and recycling of spent medium and nutrients to increase lipid yields, reduce fertilizer demand and prevent further energy demand regarding wastewater handling, (ii) the wet extraction of biomass

in order to avoid energy intensive drying, (iii) the improvement of dewatering techniques to increase drying efficiency, and (iv) the utilization of waste heat for drying or solvent evaporation.

To reduce the energy related to lipid extraction, recently other technologies like application of supercritical fluids, *in-situ* transesterification and biological or thermochemical processing of the whole microbial biomass have emerged (de Boer *et al.*, 2012; Liu and Zhao, 2007). Still, at the current state of the art the energetic feasibility of these processes is also a close call and need continued research. However, all in all it can be stated that despite certain roadblocks and with consistent technological development is it possible to achieve an energetic feasible production of microbial biodiesel on a large scale.

8.2.4 Production potential

One of the central goals supposed to be achieved by the implementation of biofuels is an increase in energy self-sufficiency. In this context biofuels would need to contribute a considerably larger share in the transportation sector than the currently required 10 % to appreciably reduce the European dependency on foreign energy sources. It has already been shown several times, that oil crops for 1st generation biodiesel production would have to cover vast parts of agricultural land in order to contribute only marginally to the total biodiesel consumption (de Boer *et al.*, 2012, Schenk *et al*., 2008). Thus, the suitability of these biodiesel sources is also questioned in terms of the actual production potential. Assuming a potential annual biodiesel production of 7110 t of biodiesel using 30 ha land area for cultivation with the “yeast oil for biodiesel” process, a land area of around 90,000 ha would be required to cover 5 % of total energy consumption in the European energy sector. Thus, yeast derived biodiesel could help to achieve the margin of 10 % renewables contribution in the transport sector, assuming that the other 5 % until 2020 will be provided by 1st generation biofuels. If the contribution of yeast biodiesel is increased to 10 % and 50 % the land requirement increases to ca. 180,000 and 900,000 ha, respectively. Comparing this to the current land cover in the European Union, which displays *e.g.* around 25 Mio. ha defined as shrubland and 8.6 Mio. ha bare land (Eurostat, 2010), it becomes obvious that the land requirement, as one of the major limitations with regard to 1st generation biofuels, will not limit the potential of yeast biodiesel production.

In the light of the ambitious biofuel and emission goals as well as the sustainability criteria set by the European Union, it is obvious that novel processes for biofuel production will be necessary in the future. While biodiesel from heterotrophic oleaginous microorganisms is one of the possible approaches in this context, also a broad range of other techniques regarding the provision of 2nd and 3rd generation biofuels, to displace both petroleum derived diesel and gasoline, are emerging and developing (Fig. 5). As described previously, the microbial biodiesel process itself must satisfy in economic, environmental and energetic terms to advance as feasible biofuel alternative. Moreover, in the same terms it also needs to prove itself competitive within the group of other next generation biofuels, to be promoted in the future. The discussion shows that the proposed process of microbial biodiesel production still has to tackle various problems, especially with regard to its feasibility in economic and

environmental terms. However, it has been shown that there are promising means of improvement. Thus, in the long term microbial biodiesel can become a commercial reality above laboratory and pilot scale. Due to the different restrictions this will probably not happen within the next 20-30 years.

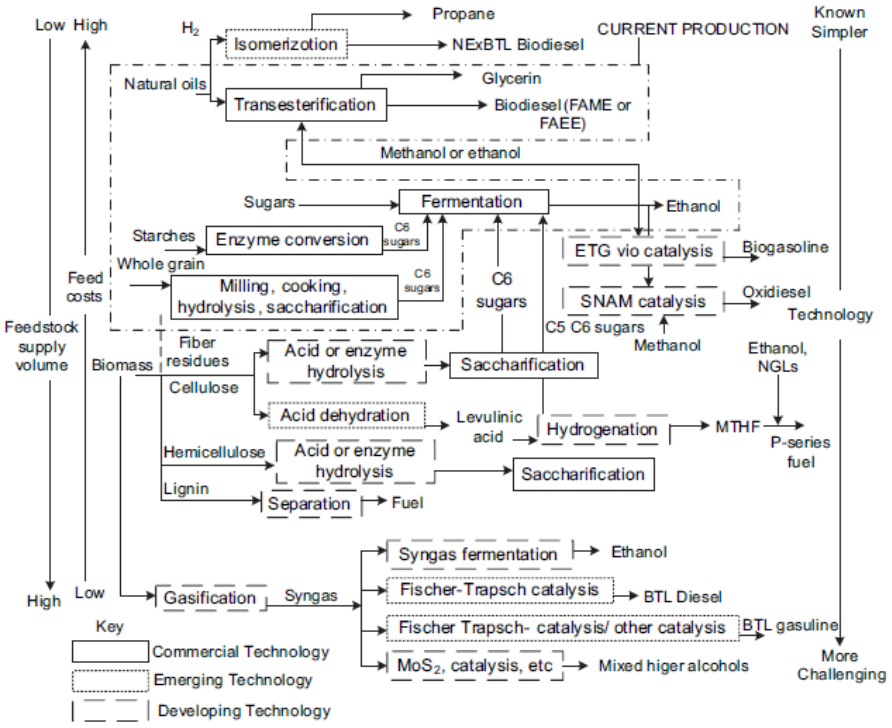


Figure 6: Various technological routes for biofuel production (Adopted from Nigam, 2011; Adapted from NEXANT, 2007).

8.3 Feasibility of carotenoid production by R. glutinis

The objective of the present thesis was not only to assess the microbial oil and biodiesel production, but also to look at the feasibility of carotenoid production by *Rhodotorula glutinis*. While initially a concept of a simultaneous production of lipids and carotenoids was pursued, it has been shown that a segregated production of lipids and carotenoids should be favored due to a certain potential metabolic competition and different requirements regarding the production standards as described in chapter 8.1.

The microbial production of natural carotenoids has gained increasing scientific interest in the past years. This was mainly based on the increased recognition of the carotenoids' nutraceutical properties and a shift in consumer preferences away from synthetically produced carotenoids. Also it was thought that production cost could be decreased by a microbial approach.

The global market for carotenoids is estimated to reach around US$ 1 billion in the next years (Chandi and Gill, 2011) and continues to increase, while β-carotene accounts for roughly one third of this market (Ribeiro, 2011). The other carotenoids synthesized by

R. glutinis, torulene and torularhodin, have no commercial application yet. Thus, when it comes to an evaluation regarding the large-scale production of *R. glutinis* carotenoids, these have to be left out so far. However, carotenoid related applications are a booming market, so new potential products and uses are steadily looked for.

Regarding the production of β-carotene, different commercial scale systems based on the pond cultivation of green algae *Dunaliella salina*, which is considered the richest natural source of β-carotene (Borowitzka, 2010), are currently available. The main commercial production sites are located in Australia, Israel and the United States. Large pond operations in Hutt Lagoon, Western Australia (520 ha) and Whyalla, South Australia (440 ha) operated by Cognis are said to provide around 80 % of the world's β-carotene market (Edye, 2012). Similar to microbial lipid production, the steps of harvesting, extraction and purification are the major cost contributors within the production of β-carotene products (Borowitzka, 2010). Additionally the application in the nutraceutical industry places even higher demands on the production process, *e.g.* in terms of hygienic aspects.

At the current market situations the high resulting production costs are offset by the high price of β-carotene, which is quoted at around \$US 600 kg^{-1}, while naturally produced β-carotene can yield even more (Ribeiro *et al.*, 2011; Johnson and Schroeder, 1996; Benemann and Oswald, 1996). However, according to the law of supply and demand it is questionable if prices will remain on this high level when the production of β-carotene is largely increased.

Currently there is no commercial scale process of β-carotene production by *R. glutinis*, since the process is not cost competitive yet. While the importance of a low cost carbon source for cultivation has been pointed out previously, also an increased β-carotene yield and improved extraction techniques provide considerable cost saving potential. Given further research and development within this topic and regardless of the issue of the actual market demand and possible price changes, it can be assumed that it will be possible to economically and sustainable produce β-carotene from *R. glutinis* in the near future. Putting it in the words of Borowitzka (2010) it can generally be stated that "[…] commercial carotenoid biosynthesis using microorganisms has a great and colorful future".

9 Summary

The bulk of today's world energy consumption is provided by fossil-derived energy sources. Due to increasing prices, their overall finiteness and environmental concerns regarding their role as leading source of greenhouse gas (GHG) emissions, many efforts aim to increase the share of renewable resources in the energy mix. Particular action has been taken to promote the implementation of sustainable biofuels as renewable alternatives to petroleum derived transportation fuels like gasoline and diesel. In this context, the production of so-called 1st generation biodiesel, which is obtained through the transesterification of plant oils, has increased strongly within the past decade. However, since this approach lately faced serious criticism in terms of its impact on the environment, GHG emissions and food prices, a transition to new biofuel production technologies has been proposed.

Against this background this thesis assessed the feasibility of biodiesel produced by heterotrophic microorganisms, particularly yeasts, using the example of oleaginous red yeast *Rhodotorula glutinis*. Within this approach certain oil-accumulating microorganisms are cultivated on an adequate carbon source. The oil is then extracted from the cells and can be subsequently transesterified into biodiesel. To improve the economic efficiency of this process, several studies have been conducted in order to test (i) whether wastewaters from the agricultural processing industry can be utilized as low-cost carbon and nutrient source for growth and lipid production by *R. glutinis* and (ii) if they also facilitate the simultaneous production of β-carotene and other carotenoids as high-value by-products.

Accordingly, different **hypotheses** have been examined, with the central results being listed in the following:

1. *Wastewaters from the food and beverage processing industry (fruit juice production, lettuce and potato processing, beer production, and sugar refinery) contain high amounts of organic compounds, which can be utilized by the yeast R. glutinis as carbon and/or nutrient sources.*

This hypothesis was partly falsified. Depending on their origin, the wastewaters provided different amounts of organic substances. The bulk of carbon was present in form of more complex disaccharides (maltose) and polysaccharides (starch), which could not sufficiently be utilized by *R. glutinis*. However, the wastewaters did not contain any inhibitory substances and provided sufficient amounts of growth required micro and macro nutrients.

2. *The yeast R. glutinis is able to grow on these wastewaters and to convert the available carbon and nutrient sources into biomass and the production of lipids.*

Again, this hypothesis was partly falsified. While biomass production was realized by *R. glutinis* in all cases, lipid production was only weakly developed or lacking completely in the wastewater treatments. This was attributed to (i) generally low carbon contents in some wastewaters and (ii) the poor performance of *R. glutinis* regarding the degradation of maltose and starch. To overcome these issues it was recommended to target highly loaded wastewater streams from specific processes, to apply a hydrolytic pretreatment

to degrade more complex carbon sources and to carefully select specifically adapted microorganisms or microbial consortia, respectively.

3. *Microbial lipids from R. glutinis exhibit a similar fatty acid profile to common plant oil and can be transesterified into biodiesel.*

This hypothesis was clearly approved. The obtained fatty acid profiles contained high amounts of palmitic and oleic acid, similar to plant oil.

4. *R. glutinis synthesizes the carotenoids β-carotene, torulene and torularhodin when cultivated on selected industrial wastewaters.*

Also this hypothesis was approved. The production of all carotenoids was analytically confirmed in most treatments. A simultaneous production with microbial lipids seems to be possible, whereas a certain competition over the common precursor Acetyl-CoA cannot be ruled out. However, due to different production standards for the two utilization pathways, it is probably not reasonable to aim at a combined production of lipids and carotenoids.

It has been shown at a small scale, that agricultural processing wastewaters can be used as feedstock for the microbial production of lipids and carotenoids. If this approach is further pursued, this could open new opportunities of income generation for the agricultural sector. Since microbial growth reduced the organic load of the wastewaters, the integration of oleaginous microorganisms into in-company wastewater treatment systems could also be a further valuable possibility.

The general discussion continued to assess the approach of **microbial biodiesel production** in a broader context in terms of its **economic, environmental and energetic performance**. A large scale "yeast oil for biodiesel" (YOB) process was modeled and relevant equipment and operating costs have been added. The break-even price of microbial oil, excluding the cost of the carbon source for fermentation, was calculated as US$ 2362.02 t^{-1}. This is around double the price of conventional plant oils as competing products. To reduce the costs, cultivation in open raceway ponds was proposed. This led to a cost reduction of around 20 % to a break-even price of US$ 1883.37 t^{-1}. These results once again emphasized the importance of a low cost carbon source and the issues regarding the economic feasibility. Thus, even if crude oil and plant oil prices continue to increase in the next years, it was supposed that a competitive price of microbial oils in the short term future can only be achieved by the implementation of market instruments like subsidies or tax exemptions.

In order to assess the potential **environmental benefits** different life cycle assessments from algae biodiesel production, which share common features with the proposed YOB process, have been analyzed. It was concluded, that microbial biodiesel cannot outperform common 1st generation biodiesel in terms of GHG emissions, whereas factors of eutrophication potential and land competition could be significantly improved, with the oil production per hectare of land being more than 100 times higher compared to conventional oil crops. The high climate relevant emissions were mainly driven by the high energy requirements connected to the extraction of microbial oil, which also puts a heavy burden on the **energetic efficiency** of the process. Depending on different underlying assumptions it could not be clearly evaluated whether the energy output of the process actually exceeds the required energy input. However, it can be stated that through different improvements, *e.g.* in terms of an increasing drying efficiency of yeast

biomass or less energy intensive extraction techniques, it can be possible to achieve an energetic feasible production of microbial biodiesel on large scale. Said improvements could then also considerably reduce the related GHG emissions. Considering the potential of continued research and technical development along with the political commitment to promote 2nd and 3rd generation biofuels, it was concluded, that in the long term microbial biodiesel could become a commercial reality above laboratory and pilot scale. Due to the different restrictions this will probably not happen within the next 20-30 years.

Finally the **feasibility of carotenoid production** by *R. glutinis* was evaluated. Contrary to biodiesel, carotenoids are high-value products, with β-carotene yielding around US$ 600 kg^{-1}, while torulene and torularhodin have no commercial application yet. Sustained by this high price and allowing for certain improvements regarding β-carotene yields and extraction techniques, it can be possible to economically and sustainable produce β-carotene from *R. glutinis* in the near future. However, given the fact that 80 % of today's β-carotene demand is already covered by large production sites in Australia, it is questionable how market prices will react on an increased supply of naturally produced β-carotene.

10 Zusammenfassung

Der Großteil des heutigen Energieverbrauchs wird durch fossile Energieträger abgedeckt. Aufgrund von steigenden Preisen, der Endlichkeit dieser Rohstoffe sowie umweltrelevanter Bedenken hinsichtlich ihrer Rolle als Hauptquelle von klimarelevanten Emissionen, sind die Bestreben groß, den Anteil von erneuerbaren Ressourcen im Energiemix zu erhöhen. Insbesondere der Ersatz der fossilen Kraftstoffe Benzin und Diesel im Transportsektor durch die Einführung nachhaltiger und erneuerbarer Alternativen wurde forciert. In diesem Zusammenhang ist die Produktion von sogenanntem Biodiesel der 1. Generation, der durch die Umesterung von Pflanzenölen gewonnen wird, innerhalb des letzten Jahrzehnts stark angestiegen. Allerdings wird in letzter Zeit verstärkt ein Wechsel zu neuen Produktionswegen für Biokraftstoffe gefordert, da sich Biokraftstoffe der 1. Generation starker Kritik bezüglich ihrer Wirkung auf Umwelt, Treibhausgasemissionen und Lebensmittelpreise ausgesetzt sehen.

Vor diesem Hintergrund beschäftigt sich die vorliegende Arbeit mit der Möglichkeit der Biodieselproduktion durch heterotrophe Mikroorganismen, insbesondere Hefen, am Beispiel der ölakkumulierenden Rothefe *Rhodotorula glutinis*. Dieser Ansatz beinhaltet die Kultivierung bestimmter ölakkumulierender Mikroorganismen auf geeigneten Kohlenstoffquellen, wobei das produzierte Öl anschließend extrahiert und in Biodiesel umgeestert werden kann. Um die Wirtschaftlichkeit dieses Ansatzes zu verbessern, wurden verschiedene Versuche durchgeführt, um zu überprüfen (i) ob Abwässer der landwirtschaftlichen Verarbeitungsindustrie und des Agribusiness als kostengünstige Kohlenstoff- und Nährstoffquellen für Wachstum und Lipidproduktion von *R. glutinis* genutzt werden können und (ii) ob sie darüber hinaus die simultane Produktion von β-Carotin und weiteren Carotinoiden als hochwertige Nebenprodukte ermöglichen.

Dementsprechend wurden verschiedene **Hypothesen** aufgestellt und untersucht. Die zentralen Ergebnisse sind im Folgenden aufgeführt.

1. *Abwässer der Lebensmittel- und Getränkeindustrie (Fruchtsaftproduktion, Salat- und Kartoffelverarbeitung, Bierproduktion und Zuckerraffination) enthalten hohe Gehalte organischer Bestandsteile, die von R. glutinis als Kohlenstoff- und Nährstoffquelle genutzt werden können.*

Ausgehend von der Herkunft enthielten die Abwässer unterschiedlich hohe Gehalte organischer Substanzen. Kohlenstoff lag hauptsächlich in Form von komplexeren Dissaccariden (Maltose) und Polysaccariden (Stärke) vor, welche nur unzureichend von *R. glutinis* abgebaut werden konnten. Die Abwässer enthielten jedoch keine wachstumshemmenden Stoffe und stellten gleichzeitig wachstumsrelevante Mikro- und Makronährstoffe in ausreichender Menge zu Verfügung.

2. *R. glutinis ist in der Lage auf diesen Abwässern zu wachsen und darüber hinaus die verfügbaren Kohlenstoff- und Nährstoffquellen in Biomasse- und Lipidproduktion umzusetzen.*

Während ein Biomasseaufbau in allen Versuchen beobachtet werden konnte, war die Lipidproduktion in den Abwasservarianten nur schwach oder gar nicht ausgeprägt. Dies wurde mit den generell geringen Kohlenstoffgehalten mancher Abwässer sowie mit der schlechten Abbauleistung für Maltose und Stärke durch *R. glutinis* begründet. Um diese

Limitierungen aufzuheben, wurde (i) die Auswahl spezifischer hoch belasteter Prozessabwasserströme, (ii) eine hydrolytische Vorbehandlung zum Abbau komplexer Kohlenstoffquellen und (iii) die Auswahl spezifisch adaptierter Mikroorganismen bzw. mikrobieller Konsortien empfohlen.

3. *Mikrobielle Öle von R. glutinis zeigen ein zu Pflanzenölen vergleichbares Fettsäureprofil und können daher in Biodiesel umgeestert werden.*

Die Möglichkeit der Biodieselproduktion aus mikrobiellen Ölen wurde bestätigt. Alle gemessenen Fettsäureprofile enthielten die für Pflanzenöl typischen hohen Mengen von Palmitin- und Ölsäure.

4. *R. glutinis synthetisiert die Carotinoide β-Carotin, Torulin und Torularhodin bei Kultivierung auf den ausgewählten Abwässern.*

Die Produktion aller genannten Carotinoide konnte in einem Großteil der Varianten analytisch bestätigt werden. Die simultane Produktion mit mikrobiellen Lipiden scheint möglich, wobei eine Konkurrenz um den gemeinsamen metabolischen Baustein Acetyl-CoA nicht ausgeschlossen werden kann. Allerdings ist es aufgrund der unterschiedlichen Produktionsstandards der beiden Nutzungswege wahrscheinlich nicht empfehlenswert eine kombinierte Produktion von Lipiden und Carotinoiden zu verfolgen.

In kleinem Maßstab konnte gezeigt werden, dass Abwässer aus der landwirtschaftlichen Verarbeitungsindustrie als Ausgangsstoff für die mikrobielle Produktion von Lipiden und Carotinoiden genutzt werden können. Falls dieser Ansatz weiter verfolgt wird, könnten sich dadurch neue Einkommensquellen für den landwirtschaftlichen Sektor ergeben. Da mikrobielles Wachstum die organische Belastung von Abwässern reduzieren kann, ist darüber hinaus eine Integration des Ansatzes in betriebseigene Abwasserbehandlungssysteme als weitere wertgebende Komponente denkbar.

Die Gesamtdiskussion führte die Bewertung der **mikrobiellen Biodieselproduktion** in einem größeren Gesamtzusammenhang fort, der **ökonomische, ökologische sowie energetische Parameter** mit einschloss. Dazu wurde ein Produktionsprozess für die Herstellung von Ölen aus Hefen im größeren Maßstab modelliert und mit den entsprechenden Geräte- und Produktionskosten versehen. Der so errechnete Break-even Preis, exklusive der Kosten für die Kohlenstoffquelle, lag bei US$ 2362,02 t^{-1} und war damit etwa doppelt so hoch wie der Preis für herkömmliches Pflanzenöl als relevantes Konkurrenzprodukt. Um Kosten einzusparen, wurde die Kultivierung der Hefen in offenen Teichsystemen (raceway ponds) vorgeschlagen, was zu einer Kostenminderung von ca. 20 % auf einen Break-even Preis von US$ 1883,37 t^{-1} führte. Diese Ergebnisse verdeutlichen wiederum die Notwendigkeit einer kostengünstigen Kohlenstoffquelle, sowie die generelle wirtschaftliche Problematik des Ansatzes. Daher kann davon ausgegangen werden, dass trotz steigender Pflanzenöl- und Rohölpreise ein konkurrenzfähiger Marktpreis für mikrobielle Öle nur durch ordnungspolitische Maßnahmen wie Subventionen oder Steuervergünstigungen in naher Zukunft erreicht werden kann.

Um eventuelle **umweltrelevante Vorteile** zu beurteilen, wurden verschiedene Ökobilanzen ausgewertet, die sich mit der thematisch nah verwandten Produktion von Biodiesel aus ölakkumulierenden Algen beschäftigten. Es wurde festgestellt, dass mikrobieller Biodiesel im Vergleich zu konventionellem Biodiesel der 1. Generation keine Emissionseinsparungen aufweisen kann, während er hingegen in den Bereichen

„Eutrophierungspotential“ und „Landnutzungseffizienz“ deutlich besser abschneidet. So können beispielsweise im Vergleich zu herkömmlichen Ölpflanzen je Hektar mehr als 100-mal so viel Öl produziert werden. Die hohen klimarelevanten Emissionen werden zum Großteil durch den hohen Energieverbrauch während des Ölextraktionsprozess verursacht, was gleichermaßen auch eine große Belastung hinsichtlich der **Energieeffizienz** des Prozesses darstellt. Aufgrund der variierenden Ausgangsannahmen der verschiedenen Bilanzierungen konnte nicht abschließend festgestellt werden, ob der Energiegewinn aus dem Produktionsprozess tatsächlich den benötigten Energieinput übersteigt. Es wurde jedoch gefolgert, dass verschiedene Prozessverbesserungen, wie z. B. eine gesteigerte Effizienz der Biomassetrocknung sowie weniger energieintensive Extraktionsmethoden, eine positive Energiebilanz ermöglichen können. Besagte Verbesserungen können ebenfalls dazu dienen, die klimarelevanten Emissionen des Prozesses entscheidend zu reduzieren. In Anbetracht des Potentials fortschreitender Forschung und technischer Entwicklung und einhergehend mit dem politischen Willen Biokraftstoffe der 2. und 3. Generation weiter zu fördern, kann die mikrobielle Biodieselproduktion langfristig zur wirtschaftlichen Realität werden. Aufgrund der genannten Restriktionen wird dies allerdings wahrscheinlich nicht innerhalb der nächsten 20-30 Jahre geschehen.

Abschließend wurde die **Realisierbarkeit der Produktion von Carotinoiden** durch *R. glutinis* bewertet. Im Gegensatz zu Biodiesel stellen Carotinoide hochwertige Produkte dar, wobei β-Carotin einen Marktpreis von ca. US$ 600 kg^{-1} erzielen kann, während es für Torulin und Torularhodin bislang noch keine kommerziellen Anwendungen gibt. Dieser hohe Preis sowie bestimmte Optimierungen bezüglich des β-Carotin Ertrags und der angewandten Extraktionsmethoden können in naher Zukunft eine wirtschaftliche und nachhaltige Produktion von β-Carotin durch *R. glutinis* ermöglichen. Angesichts der Tatsache dass ca. 80 % des aktuellen β-Carotin Marktes bereits durch große Produktionsstätten in Australien abgedeckt werden, bleibt es allerdings fraglich wie die Marktpreise auf eine weitere Ausweitung der natürlichen β-Carotin Produktion reagieren werden.

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Appendix

Table A 6: Media composition, nutrient cost and cost sources

Nutrient	Media composition [g L^{-1}]		Cost [US$ kg^{-1}]	Source
	Seed production	*Fermentation*		
Glucose[1]	10.0		0.68	USDA ERS, 2011
Yeast extract	3.0		7.78	Castilho *et al.*, 2000
Peptone	5.0		5.03	Kaylen *et al.*, 2000
$NaHPO_4$		1.0	1.60	CMR, 2006
KH_2PO_4		1.0	0.29	Wooley *et al.*, 1999
$MgSO_4$		0.4	0.42	CMR, 2006
$(NH_4)_2SO_4$		0.5	0.16	CMR, 2006

[1]Glucose syrup (98 % glucose content)

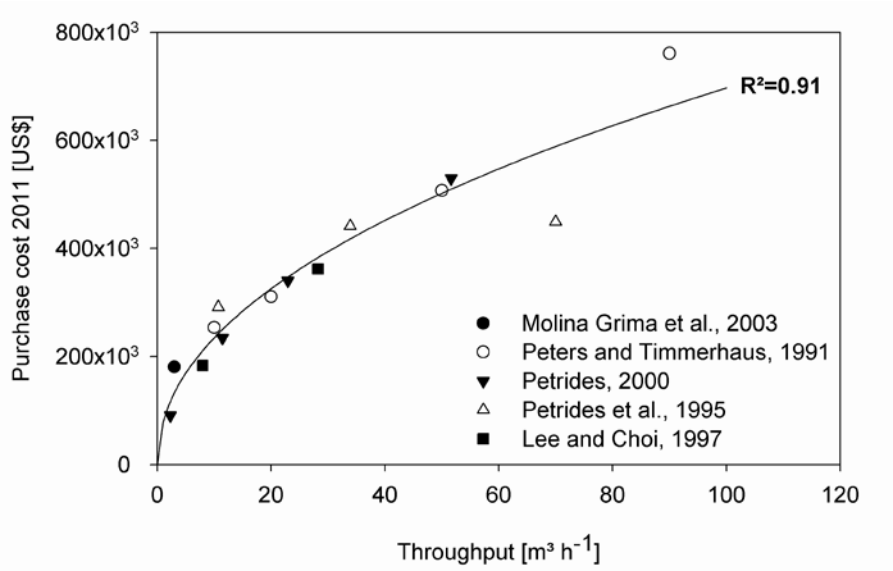

Figure A 7: Regression curve for cost calculation of disk stack centrifuges based on the required throughput (own calculation).

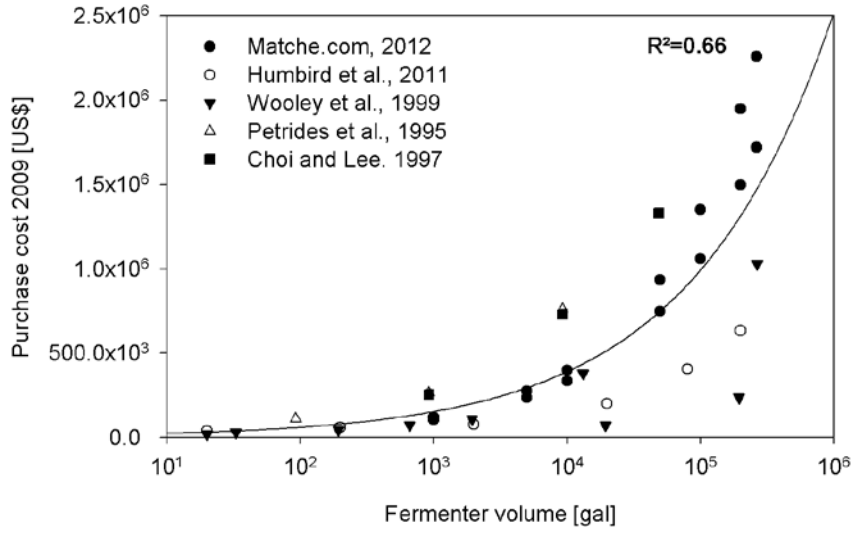

Figure A 8: Regression curve for cost calculation of fermentation vessels based on fermenter volume (own calculation).

Table A 7: **Overview over process equipment including equipment description, equipment cost and cost sources.**

	Equipment title	Description	Source	Purchase cost in 2011 [$]	No. reqd.	Install. factor	Installed cost in 2011 [$]
T-1	Medium tank	300 m^3, 1 atm, Internal coil	Humbird *et al.*, 2011	197,515.23	1	2.0	395,030.47
	Medium tank agitator	10 hp, 316 SS[1]	Humbird *et al.*, 2011	24,128.28	1	1.5	36,192.42
F-1	1st seed fermenter	0.1875 m^3, incl. temperature control and agitator	Regression	45,705.82	3	1.8	246,811.43
F-2	2nd seed fermenter	3.75 m^3, incl. temperature control and agitator	Regression	153,528.60	3	1.8	829,054.44
F-3	3rd seed fermenter	75 m^3, incl. temperature control and agitator	Regression	515,680.19	3	1.8	2,784,673.03
P-1	Seed transfer pump	45 $m^3\ h^{-1}$ flow rate, 40 hp	Humbird *et al.*, 2011	13,635.28	3	2.3	94,083.46
M-1	Fermenter air compressor package	13,500 $m^3\ h^{-1}$ at 16 psig	Humbird *et al.*, 2011	196,392.99	1	1.6	628,457.56
T-2	Seed hold tank	150 m^3, incl. temperature control and agitator	Regression	682,542.38	2	1.8	2,457,152.57
P-2	Seed hold transfer pump	45 $m^3\ h^{-1}$ flow rate, 10 hp	Humbird *et al.*, 2011	9,202.41	1	2.3	84,662.21
F-4	Lipid fermenter	750 m^3, incl. temperature control and agitator	Regression	1,308,646.81	12	1.8	28,266,771.10
T-3	Medium tank lipid production	1500 m^3, 316 SS	Humbird *et al.*, 2011	492,665.84	2	1.8	2,183,587.73
	Medium tank agitator	20 hp	Humbird *et al.*, 2011	48,256.56	2	1.5	144,769.69
P-3	Medium tank pump	485 $m^3\ h^{-1}$ flow rate, 125 hp	Humbird *et al.*, 2011	30,076.18	2	2.3	138,350.44
	CIP system	Clean in Place system for sterilization, 375 m^3	Humbird *et al.*, 2011	472,465.41	1	1.8	850,437.75
	Centrifuge	50 $m^3\ t^{-1}$, 30 kW	Regression	504,943.06	3	1.0	1,505,829.18
	Spray dryer	3.2 t h^{-1} evaporative capacity; installed	Peters and Timmerhaus, 1991	883,458.00	1		883,458.00

[1]Stainless Steel

Table A 8: **General overview over presentations and publications in context of the dissertation**

T. Schneider, S. Graeff-Hönninger, W. Claupein, W. T. French, R. Hernandez
Stoffliche Nutzung industrieller Abwässer zur Produktion hochwertiger mikrobieller Öle und Pigmente
Conference Proceedings. Mitteilungen der Gesellschaft für Pflanzenbauwissenschaften, Bd. 23; 54. Jahrestagung der GPW 27-29 September 2011 in Kiel, p. 282.

T. Schneider, S. Graeff-Hönninger, W. T. French, R. Hernandez, W. Claupein
Material utilization of industrial wastewaters for the production of high-quality microbial oils and pigments
Poster presentation. Mississippi Biofuels Conference, Starkville (MS), 5-7 October 2011

T. Schneider, S. Graeff-Hönninger, W. T. French, R. Hernandez, W. Claupein, N. Merkt
Microbial production of biodiesel and carotenoids using oleaginous red yeast *Rhodotorula glutinis* – Screening of industrial wastewaters as suitable feedstock
Conference Proceedings and Poster presentation. Proceedings of the 5th International Conference on Sustainable Energy & Environmental Protection, Dublin, 5-8 June 2012, pp. 119-124.

T. Schneider, S. Graeff-Hönninger, W. T. French, R. Hernandez, N. Merkt, W. Claupein
Microbial lipids for biodiesel production and carotenoids as value added by-products – Screening of industrial wastewaters as suitable feedstock for oleaginous red yeast Rhodotorula glutinis
Conference Proceedings and Oral presentation. Proceedings of the 20th European Biomass Conference and Exhibition, Milan, 18-22 June 2012, pp. 1541-1546.

L. Schwemmlein, T. Schneider, S. Graeff-Hönninger, W. T. French, R. Hernandez, W. Claupein, W. E. Holmes
Effects of different C/N-ratios of the growth media on carotenoid and lipid production by the red yeast *Rhodotorula glutinis*
Conference Proceedings and Poster presentation. Proceedings of the 20th European Biomass Conference and Exhibition, Milan, 18-22 June 2012, pp. 1778-1782.

T. Schneider, S. Graeff-Hönninger, W. T. French, R. Hernandez, N. Merkt, W. Claupein
Maltose degradation by Rhodotorula glutinis as precondition for the utilization of brewery wastewaters as carbon source for microbial growth
Poster presentation. Southeast Biofuels and Renewable Energy Conference, Jackson (MS), 8-9 August 2012.

T. Schneider, S. Graeff-Hönninger, W. T. French, R. Hernandez, W. Claupein, N. Merkt
Nutzung von Brauereiabwässern zur Produktion mikrobieller Öle und Pigmente
Conference Proceedings. Mitteilungen der Gesellschaft für Pflanzenbauwissenschaften, Bd. 24; 55. Jahrestagung der GPW, 24-27 September 2012 in Berlin, pp. 232-233.

T. Schneider, S. Graeff-Hönninger, W. T. French, R. Hernandez, W. Claupein
Nutzung industrieller Abwässer und lignocellulosereicher Biomasse zur Produktion von mikrobiellen Ölen und Pigmenten
Oral presentation. Kolloquium BioEconomy, Karlsruhe, 28-29 November 2012.

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Hiermit bestätige ich, dass ich die vorliegende Dissertation selbstständig angefertigt habe, nur die angegebenen Quellen und Hilfsmittel benutzt habe und wörtlich oder inhaltlich übernommene Stellen als solche gekennzeichnet habe. Die Hilfe einer kommerziellen Promotionsvermittlung oder –beratung wurde nicht in Anspruch genommen.

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