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Master Thesis

The influence of different carbon sources on the lipid composition and production rate in *Apiotrichum brassicae*

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Statutory declaration

I hereby declare that I am the sole author of this work. No assistance other than that which is permitted has been used. Ideas and quotes taken directly or indirectly from other sources are identified as such. This written work has not yet been submitted in any part.

Abstract

The increasing global energy demand and the current climate crisis led to an increased production of biofuel from edible plant oils. These oils can be used to replace parts of the conventional petroleum fuel. However, due to the competition with the use as food and other disadvantages, alternative sources of non-edible oil for biofuel production are under research. Since many years, oleaginous microorganisms, such as yeasts, have been investigated as renewable source of oils as starting material for the synthesis of biodiesel. Substrate costs are a major factor in the cultivation of oleaginous yeasts. The yeasts are thus grown on cheap, waste derived substrates, to be able to economically compete with plant-derived oils. The aim of this master thesis was to investigate the influence of different carbon sources on the growth and single cell oil production of *Apiotrichum brassicae*. For that, *A. brassicae* was grown on different kinds of synthetic substrates and finally on a mixed volatile fatty acid substrate, derived from a pre-digestion of organic waste material, in fed batch and batch processes. The dry cell weight concentration, the single cell oil content and the lipid composition were determined for the biomass derived from the respective substrates. With acetic acid as sole carbon source a biomass concentration of 43.46 g L^{-1} with a single cell oil content of 67.99% and with the mixed substrate a biomass concentration of 15.07 g L^{-1} with a single cell oil content of 23.47 % could be reached. The results obtained during this work are well comparable with the data found in the literature and *A. brassicae* reached remarkably good results when grown on volatile fatty acids.

Abstract

Der weltweit steigende Energieverbrauch sowie die aktuelle Klimakrise führen zu einer steigenden Produktion an Biotreibstoffen aus pflanzlichen Speiseölen. Diese Öle können dazu verwendet werden, um Teile des konventionellen Petroleumtreibstoffs zu ersetzen. Aufgrund des Wettbewerbs mit der Verwendung als Nahrungsmittel und anderen Nachteilen wird jedoch nach alternativen Quellen für nicht essbare Öle für die Biotreibstoffproduktion geforscht. Seit mehreren Jahren werden ölhaltige Mikroorganismen, wie zum Beispiel Hefen, untersucht, um als erneuerbare Ölquelle für die Synthese von Biodiesel zu dienen. Da die Substratkosten ein großer Faktor bei der Kultivierung von ölhaltigen Hefen sind, wird an günstigen Substraten aus Abfallstoffen geforscht, um konkurrenzfähig mit der Herstellung von Pflanzenölen zu sein. Das Ziel dieser Masterarbeit war die Untersuchung des Einflusses verschiedener Kohlenstoffquellen auf das Wachstum und die Single cell oil Produktion von *Apiotrichum brassicae*. *A. brassicae* wurde dafür auf verschiedenen synthetischen Substraten und schließlich auf einem flüchtigen Fettsäuren-Substratgemisch, stammend aus einem Vorverdau von organischen Abfallstoffen, in Fed-Batch- und Batch-Prozessen kultiviert. Die Zelltrockenmassenkonzentration, der Single cell oil Gehalt sowie die Fettzusammensetzung wurden für die jeweiligen Biomassen, erzeugt mit Hilfe der unterschiedlichen Substrate, bestimmt. Mit Essigsäure als alleinige Kohlenstoffquelle konnte eine Biomassenkonzentration von 43,46 g L^{-1} mit einem Single cell oil Gehalt von 76,99 % erreicht werden. Mit dem gemischten Substrat wurde eine Biomassenkonzentration von 15,07 g L^{-1} mit einem Single cell oil Gehalt von 23,47 % erreicht. Die in dieser Arbeit gewonnenen Resultate waren gut mit Daten aus der Literatur vergleichbar und *A. brassicae* erreichte, wachsend auf flüchtigen Fettsäuren, bemerkenswert gute Resultate.

Contents

List of abbreviations

ACP	acyl carrier protein
ADP	adenosine diphosphate
AMP	adenosine monophosphate
Aox	Acetyl-CoA oxidase
ATP	adenosine triphosphate
CoA	coenzyme A
COD	chemical oxygen demand
DO	dissolved oxygen
DCW	dry cell weight
FAME	fatty acid methyl ester
HPLC	high-performance liquid chromatography
MUFA	monounsaturated fatty acid
P_i	inorganic phosphate
PUFA	polyunsaturated fatty acid
res. DCW	residual dry cell weight; dry cell weight without single cell oil
RO	reverse osmosis
SCO	single cell oil
SFA	saturated fatty acids
TAG	triacylglycerol
TCA	tricarboxylic acid
VFA	volatile fatty acid
YE	yeast extract

1 Introduction

1.1 General information on Single Cell Oils

Microorganisms, like yeasts, bacteria, microalgae and fungi, are able to produce lipids in their cellular compartment for different physiological purposes. The lipids include glycolipids, lipids that are anchored in the microorganism membrane, phospholipids, lipids that build up the cell membrane and triacylglycerols (TAGs). The latter are often synthesised to store energy to maintain cellular functions during stress situations and are denoted as single cell oils (SCO) (Carsanba et al. 2018). Depending on the used metabolic pathway, lipid production is often triggered by a lack of one nutrient source (for example nitrogen) and the excess of carbon. Lipid composition and properties depend on species, culture conditions and the substrate the microorganisms are grown on. Other influencing factors are the concentration of carbon source, the ratio of available carbon to nitrogen, the pH, inorganic salts, aeration and the temperature (Viñarta et al. 2020).

Microorganisms that are able to accumulate lipids more than 20 % *w/w* of the dry cell weight are called oleaginous microorganisms. The produced neutral lipids are mainly triacylglycerides, composed of various fatty acids from short chain fatty acids with six carbon atoms to long chain fatty acids with up to 36 carbon atoms. The synthesised fatty acids can be saturated, containing no double bond between two carbon atoms, monounsaturated, containing one double bond, or polyunsaturated, containing multiple double bonds. They are therefore called saturated fatty acids (SFA), monounsaturated fatty acids (MUFA) or polyunsaturated fatty acids (PUFA), respectively (Patel et al. 2020).

While SFA and MUFA can be used for biodiesel production, PUFA can be used as food supplements (Patel et al. 2020). Due to the similarity in fat composition and other advantages, like the independence on geographical or seasonal factors, the interest to use these single cell oils as an alternative to lipids derived from plants has increased over the last years (Ward and Singh 2005). At the moment biofuels are mainly produced from edible and non-edible plants that are rich in TAGs. This, however, leads to rising prices for plant oils and a competition between food and fuel. The use of SCO as starting material for biodiesel production can prevent these issues.

However, compared to the production of plant oils, draw backs in using microbial oils are often a lower yield and higher process complexity. To compete with oils derived from plants, cheap carbon sources like glycerol and volatile fatty acids, derived from waste streams can be used as substrates for microorganisms to reduce the production costs (Diwan et al. 2018; Fei et al. 2011).

Examples for the use of SCOs as suitable nutrition supplements are docosahexaenoic acid (22:6) and arachidonic acid (20:4). These fatty acids have beneficial effects on the neural and retinal development of infants and are present in human breast milk, but not in cow milk, which is often used as substitute. Today fermentation procedures with different fungus and microalgae strains are available to produce

both docosahexaenoic acid and arachidonic acid and provide an alternative source to fish oils (Ratledge 2004). Besides docosahexaenoic acid, a second omega-3-fatty acid, eicosapentaenoic acid, which has several health benefits, can be produced as single cell oil in oleaginous microorganisms. Therefore, omega-3 and omega-6 fatty acids microbial origins can be part of the solution to prevent overfishing and could become economical with increasing market prices of fish oil (Armenta and Valentine 2013). A further application field of oleaginous yeasts is the chemical industry. Fatty acids and fatty acid-based chemicals, like fatty alcohols or fatty acid ethyl esters, are starting materials for the production of detergents, surfactants, lubricants and cosmetic products. Due to the increased demand of such products, oleaginous microorganism are promising candidates as a renewable source of such substances (Adrio 2017).

1.2 Oleaginous microorganisms

Oleaginous microorganisms are able to accumulate more than 20 % *w/w* of their dry cell mass. They can be found throughout the kingdoms of yeasts, bacteria, microalgae and filamentous fungi.

Filamentous fungi are heterotrophic organisms, which means they consume organic compounds to provide them with nutrients and energy. When it comes to the production of single cell oil for biodiesel synthesis, they are promising microorganisms. They have a unique fatty acid profile and some of them contain high amounts of linoleic acid. An advantage over other oleaginous microorganisms is the growth on cheap carbon sources, like sewage sludge, agricultural residues and waste molasses. This is important, since substrate costs are one of the main cost driving factors in single cell oil production. Examples for oleaginous fungi are: *Cunninghamella echinulate*, *Mortierella alpina*, and *Aspergillus niger* (Patel et al. 2020).

In contrast to filamentous fungi, algae are phototrophic organisms, which means they perform photosynthesis for the supply of energy and for the fixation of carbon dioxide. The synthesised carbohydrates are used to build up cell material and produce storage lipids under nitrogen limitation. However, this process requires much more energy compared to converting carbohydrates into single cell oils like fungi or yeasts do and makes algae not suitable to produce high amounts of lipids for biodiesel production. Nevertheless, instead of serving as alternative to plant derived oils, algae can compete with fish oils, since their high amount of PUFAs may originate from eating the algae. Besides that, algae are already used to produce commercial products that can be sold for a higher price. For example, *Dunaliella salina* is used in fermentation processes to synthesise beta-carotenes or *Haematococcus plivialis* for the production of astaxanthin. A further difficulty is the cultivation of algae. Algae are often grown in aquatic cultures and although they grow faster compared to conventional plants, monocultures are advantageous to protect them from predators. Tubular reactors, which are used to produce beta-carotenes and astaxanthin, cannot be used for the large production of lipids used for biodiesel synthesis, due to the

high costs. Instead, open ponds are a better choice to cultivate algae on a larger scale. This would reduce the costs, but sufficient CO_2 input and stirring is needed to grow them efficiently, since the carbon dioxide content in the atmosphere is too low. Additionally, the cultivation is limited by environmental factors and temperature and light level changes (Ratledge and Cohen 2008).

Bacteria, like *Rhodococcus*, *Gordonia*, *Acinetobacter* and *Arthrobacter* species are known to be oil producers and count as oleaginous bacteria, but are not the first choice for single cell oil producers used for biodiesel synthesis, because most bacteria have a lower capability to accumulate lipids compared to other microorganisms (Patel et al. 2020).

The most promising microorganisms for SCO production are yeasts. Approximately 600 yeast species are known, of which 30 are considered oleaginous. The most important ones belong to the genera *Yarrowia*, *Candida*, *Cryptococcus*, *Rhodotorula*, *Rhodosporidium*, *Trichosporon* and *Lipomyces*. Yeasts are heterotrophic organisms and come with several advantages. Compared to filamentous fungi, they grow faster and are easier to cultivate on large scale. They have a higher tolerance to metal ions and are less affected by low oxygen concentrations. In contrast to microalgae, yeasts grow faster, are able to reach higher cell densities in the bioreactor and can be grown on different types of carbon sources. They are also less affected by climate and do not need sunlight to grow. Compared to bacteria, yeasts are bigger and are therefore easier for harvesting procedures. Beside these advantages to other organisms, there are some points that have to be considered for the cultivation of yeasts. Stirred tank reactors with aeration and pH control are required, which can make the fermentations procedure more complex and labour intensive. Also sterilisation, to prevent contamination and outgrowth by bacteria, and time consuming inoculation procedures, going from smaller fermenters to bigger ones, are often required (Ratledge and Cohen 2008; Quin et al. 2017).

1.3 Metabolic pathways

Oleaginous yeasts produce neutral lipids to store energy and carbon if one essential nutrient compound is absent. This can for example be nitrogen or phosphorus, elements that are essential for the growth of microorganisms. The reason for the synthesis of those lipids is to provide an easily accessible energy source, when nitrogen or phosphorus becomes available again and to prevent other organisms from consuming the nutrients. These storage lipids are usually present in the form of triacyl glycerides or free fatty acids, but also monoacylglycerols, diacylglycerols and steryl-esters. In contrast to polar lipids like phospholipids, these cannot be inserted into membranes (phospholipid bilayer). Therefore, lipid bodies or oil bodies with a hydrophobic core are formed. The pathway to synthesise these storage lipids by oleaginous yeasts is very similar to that of non-oleaginous yeasts, which have to synthesise lipids for the phospholipid membrane and other functional and structural purposes. However, oleaginous yeast

have specific key enzymes that allow them to accumulate a higher level of lipids than non-oleaginous yeasts.

In general, there are two different ways for oleaginous yeasts to synthesise storage lips. The *de novo* pathway, the pathway used when the microorganisms are grown on hydrophilic carbon sources like glucose, lactose or fructose and the *ex novo* pathway. This pathway is used when the yeasts are grown on hydrophobic substrates like, free fatty acids, triacyl glycerides and other fats and oils (Athenaki et al. 2018; Papanikolaou and Aggelis 2011).

1.3.1 *De novo* lipid accumulation

As already mentioned, storage lipid accumulation occurs under the absence of one essential nutrient compound, for example nitrogen, while carbon is present in excess amounts. Under these conditions, the carbon source is used to synthesise storage lipids instead of building up cell material for growth and cell division. One essential part of the *de novo* lipid accumulation, induced by nitrogen depletion, is the accumulation of tricarboxylic acid cycle intermediates, caused by an enzyme called AMP deaminase. This enzyme tries to compensate the deficiency of nitrogen by cleaving AMP, which has a regulatory function in the TCA cycle, to generate NH_3.

$$AMP \rightarrow \text{inosine-5'- monophosphate} + NH_3$$

Because of the decrease of intracellular AMP, the activity of isocitrate dehydrogenase is reduced. Therefore, less D-isocitrate is converted to α-ketoglutarate and because isocitrate and citrate are in equilibrium, citrate starts to accumulate. The increased level of citrate in the mitochondria is counteracted by the citrate-malate shuttle system, which transports citrate across the mitochondrial membrane in exchange with malate. In the cytoplasm, citrate is cleaved by an enzyme called ATP-citrate lyase (ACL) to generate acetyl-CoA.

$$\text{citrate} + CoA + ATP \rightarrow \text{acetyl-CoA} + \text{oxaloacetate} + ADP + P_i$$

Because of this breakdown of citrate, oleaginous yeasts are able to accumulate high levels of acetyl-CoA, which is the basis for the synthesis of fatty acids. This mechanism is one of the main differences between oleaginous and non-oleaginous yeasts, because the enzyme complex ATP-CL is not present in non-oleaginous yeasts.

The acetyl-CoA production is the starting point to build up various fatty acids. The first step, and also the first restricting step in fatty acids biosynthesis, is the formation of malonyl-CoA catalysed by acetyl-CoA carboxylase, a biotin and ATP requiring enzyme.

$$\text{acetyl-CoA} + HCO_3^- + ATP \rightarrow \text{malonyl-CoA} + ADP + P_i$$

The formed malonyl-CoA, as well as acetyl-CoA, are then attached to acyl-carrier protein (ACP) to generate malonyl- and acetyl-ACP. The following sequential additions of acetyl- ACPs to malonyl-ACP are performed by the fatty acid synthase, a multienzyme complex. This process yields palmitoyl-CoA.

$$\text{acetyl-CoA} + 7\ \text{malonyl-CoA} + 14\ \text{NADPH} \rightarrow \text{palmitoyl-CoA} + 7\ CO_2 + 14\ \text{NADP} + 7\ \text{CoASH} + 6\ H_2O$$

Because the formation of fatty acids is a reductive process, NADPH has to be constantly supplied, which is a further key process in storage lipid synthesis. Per cycle of fatty acid biosynthesis (condensation of one acetyl-CoA to the growing acyl-CoA chain), two NADPH are necessary. One of the possible mechanisms for delivering NADPH is the malic enzyme, which catalyses the conversion of malate into pyruvate under formation of CO_2 and NADPH.

$$\text{malate} + \text{NADP} \rightarrow \text{pyruvate} + CO_2 + \text{NADPH}$$

After the described biosynthesis of fatty acid esters, triacylglycerols are formed by esterification with glycerol. The alpha-glycerol phosphate acylation pathway starts with the activation of free fatty acids with CoA followed by acylation of glycerol.

The first acylation step at the sn-1 position is performed by glycerol-3-phosphate acyltransferase. The resulting metabolite, 1-acyl-glycerol-3-phosphate, is further acylated at the sn-2 position to form phosphatidic acid. This step is catalysed by the enzyme lysophosphatidic acid acyltransferase. After the dephosphorylation of phosphatidic acid, catalysed by phosphatidic acid phosphorylase, the resulting diacylglycerol is again acetylated, and triacylglycerol is formed. The final acetylation step is performed by phospholipid diacylglycerol acetyltransferase. In addition to the just described α-glycerol phosphate acylation pathway, which is used by oleaginous microorganisms, the dihydroxyacetone-phosphate pathway, is an alternative pathway to produce phosphatidic acid.

The synthesised TAGs can be composed of theoretically random acyl groups, but in most cases of oleaginous microorganisms unsaturated fatty acids are attached on the sn-2 position. This is responsible for the similarity to vegetable oils (Athenaki et al. 2018; Papanikolaou and Aggelis 2011).

1.3.2 *Ex novo* lipid accumulation

Some oleaginous yeasts are able to grow on hydrophobic substrates and accumulate high quantities of lipids via the *ex novo* pathway. They are able to metabolise fatty esters, free fatty acids, vegetable oils, industrial fats or TAGs as sole carbon and energy source.

If the substrate are free fatty acids, they are directly transported into the cell via active transport. All other hydrophobic substrates, like TAGs, are hydrolysed prior by secreted lipases to produce free fatty acids. The fatty acids are degraded to generate energy and metabolites for cell growth or are the starting

point for to the synthesis of new lipids. The intracellular free fatty acids are dissimilated by β-oxidation to single acetyl-CoA and small chain acyl-CoAs, catalysed by various acyl-CoA oxidases (Aox).

The β-oxidation process consists of four steps. At first acyl-CoA is dehydrogenated and converted to trans-Δ2-enoyl-CoA under the production of $FADH_2$ catalysed by acyl-CoA oxidase/acyl-CoA dehydrogenase. Then the bond between the C-2 and C-3 carbon atom is hydrogenated and L-3-hydroxyacyl-CoA is formed. This step is catalysed by the enzyme enoyl-CoA-hydratase. Through oxidation, catalysed by hydroxyacyl-CoA-dehydratase, 3-ketoacyl-CoA is formed and in the final step cleaved into acyl-CoA and acetyl-CoA. This final cleavage of the βoxidation is catalysed by the thiol group of coenzyme A and the resulting acyl-CoA chain is two carbon atoms shorter than the initial one. During this process, one mole NADPH and on mole $FADH_2$ are generated per mole acetyl-CoA, which can now enter the TCA-cycle or the glyoxylate bypass pathway for gluconeogenesis.

The lipid synthesis via *ex novo* synthesis has differences compared to the *de novo* synthesis. When the microorganisms are grown on hydrophobic substrates, lipid accumulation and growth take place at the same time. The production of storage lipids is growth-associated and independent of nitrogen, phosphor or sulphur depletion, which is necessary in the *de novo* pathway. In the *de novo* pathway, lipid formation is not growth-associated. Despite the simultaneous growth and lipid synthesis, the oxygen demand is higher compared to the growth on hydrophilic substrates (Athenaki et al. 2018; Papanikolaou and Aggelis 2011).

1.4 Substrates used in single cell oil production

One of the major problems with using single cell oils for the production of biofuels are the high costs of the substrates oleaginous microorganisms grow on. Since up to 75% of the total costs are assigned to raw material costs, using cheap carbon sources provides an easy way to reduce the costs for single cell oil production. Several biomass derived substrates and especially waste derived substrates, have already been investigated as carbon sources for the cultivation of oleaginous yeasts.

The largest biomass resource in the world is lignocellulosic biomass, like rice straw, pine wood, aspen wood and different types of agricultural wastes. These can be used as cheap substrates for oleaginous yeast. Lignocellulosic substrates often have to be pre-treated, for example with sulphuric acid. The obtained hydrolysates contain free sugars like glucose, xylose or arabinose, which can now be utilized by yeasts. Further substances like furfural and hydroxymethylfurfural can also be present in the hydrolysate and potentially inhibit the growth and SCO production of the yeast. With appropriate pre-treatments, the obtained hydrolysate is detoxified and therefore not only the inhibitors are removed, but the sugar concentration is increased as well. It has been reported that *Trichosporon fermentans*, an oleaginous yeast, reached a total biomass concentration of 28.6 g L^{-1} with a lipid content of 40.1% after 8 day of fermentation when grown on a rice straw hydrolysate (Huang et al. 2009).

A further lignocellulosic substrate that can be used for the cultivation of oleaginous yeast and single cell oil production is wheat straw. Similar to rice straw, the hydrolysates contain acetic acid, furfural and hydroxymethylfurfural. The main component, however, is xylose, but also arabinose, glucose and galactose are present. Experiments with five different oleaginous yeasts (*Cryptococcus curvatus, Rhodutorula glutinis, Rhodosporium toruloides, Lipomyces starkeyi,* and *Yarrowia lipolytica)* showed the applicability of wheat straw hydrolysates for lipid production. With 17.2 g L^{-1} dry cell weight and a lipid concentration of 33.5%, *C. curvatus* achieved the highest results growing on a non-detoxified wheat hydrolysate (Yu et al. 2011).

Wastewater is also a promising candidate as cheap and abundantly available substrate for the single cell oil production. An example for this is wastewater from the monosodium glutamate industry. With a high chemical oxygen demand (COD), ammonium and sulphate content and a low pH, wastewater from glutamate industry requires extensive and high energy demanding processing procedures. With the use of such wastewater as substrate, one can not only produce renewable oils for biodiesel production, but also reduce the cost of waste management. Xue et al. (2008) performed experiments with monosodium glutamate wastewater and added glucose to optimize the lipid production. Three different feeding strategies, batch, fed batch and feedback addition, were chosen to investigate the change of lipid formation in *Rhodutorula glutinis*. Of these three feeding strategies, feedback addition, by holding the glucose concentration at 15 g L^{-1}, was the most effective one. After 72 hours, a dry cell weight (DCW) concentration of 25 g L^{-1} was reached with a lipid content of 20%. Additionally, the COD was reduced by 40%.

Similar to the wastewater of the glutamate industry, sewage sludge can contain growth inhibiting substances and often has to be pre-treated to be used any further or has to be disposed, which is very costly. Raw sewage sludge consists of proteins, carbohydrate, lipids and bacterial extracellular polysaccharides, but also contains heavy metals, chlorine-paraffins or hazardous organic substances. The use for agricultural purposes is therefore often limited. Commonly, sewage sludge is digested in anaerobic fermenters to methane and carbon dioxide, but this requires large reactors and the conversion takes a long time. Using sewage sludge as substrate for single cell oil production can therefore be an alternative to anaerobe fermentation processes. Angerbauer et al. (2008) performed fermentation experiments with *Lipomyces starkeyi* on pre-treated sewage sludge. By adjusting the C:N ratio with glucose, a lipid concentration of 6.7 g L^{-1} could be reached.

Due to the high C:N ratio, molasses from different origin are also an interesting substrate. Molasses are a side product of the sugar industry (sugar beet and sugar cane) and are considered as low-cost substrate. The composition of sugar beet molasses is around 25% water and 50% sugar. Also, minerals and nitrogen are constituents of molasses. For both, biomass and lipid formation, the substrate concentration play a significant role and therefore the molasses concentration in the fermenters has to be adjusted. Another factor that influences the lipid yield is the pH. It was reported that with an initial pH of 5 and

high molasses concentrations, unsterile SCO production processes become possible, which reduces operation costs (Taskin et al. 2016). Taskin et al. (2016) investigated the growth and lipid formation of the yeast *Rhodutorula glutinis* on different molasses concentrations. The highest biomass concentration of 14.9 g L^{-1} was reached at a pH of 5.5 and a molasses concentration of 20%. The highest lipid content was 52.6% at a pH of 5.

Beside sugars, volatile fatty acids (VFA) are also a promising low-cost substrate for lipid production. Using organic waste, an abundant source of substrate, in anaerobic fermentation processes provides a good option to obtain big amounts of VFAs. The advantages over sugars are a shorter transformation pathway and a higher lipid conversion efficiency. Growth and lipid production depend on the type and mixture of VFAs, like acetic acid, propionic acid, iso-butyric acid, butyric acid, iso-valeric acid, valeric acid and caproic acid, but also on the respective amounts and concentrations. One disadvantage of VFAs can be their inhibitory effect on growth at high concentrations. Undissociated fatty acids are able to diffuse through the cell membrane and lead to pH changes within the cytoplasm. This can reduce biomass and single cell oil production. The extent of this effect depends on the type of acid used (pK_a value) and specifically on the concentration of undissociated acid depending on the pH (Llamas et al. 2020). Llamas et al. (2020) investigated the lipid formation of *Yarrowia lipolytica* grown on a digestate of microalgae biomass. The growth of the oleaginous yeast was investigated first for all VFAs present in the digestate individually. Subsequently, experiments were performed with mixed substrates. For the utilization of the mixed substrates the growth of *Y. lipolytica* depended on the acid profile, preferring short chain acids above a VFA concentration of 4 g L^{-1} and preferring long chain acids below a substrate concentration of 4 g L^{-1}. Using a real digestate, the amount of formed biomass was 2.5 times higher than the biomass that was produced using synthetic media. An explanation for this behaviour might be the presence of substances in the digestate that have a positive effect on the growth of the yeast and therefore higher VFA concentrations could be used in the fermentation. This indicates the suitability of digestates obtained by anaerobic fermentations for the growth of oleaginous yeasts and single cell oil production.

A possible source for VFAs are raw food wastes, because of their high amount of degradable organic compounds. Gao et al. 2017 conducted batch fermentations using the supernatant of a food waste fermentation effluent containing mostly acetic acid, propionic acid and butyric acid. The medium was diluted to a suitable VFAs concentration to circumvent the inhibitory effect of high volatile fatty acid concentrations. Again, *Y. lipolytica* was used as microorganism. The highest dry cell weight was achieved at 28°C with 2.0 g L^{-1} and a lipid content of 18.2%. Another substrate that can be used for the production of SCO, is waste activated sludge, which is produced in abundance in municipal sewage plants. The sludge contains a lot of organic materials which can be converted to volatile fatty acids, methane, hydrogen and lipids. By using sludge derived VFAs in fermentations with oleaginous microorganisms, the lipid content can be increased to optimize the outcome in biodiesel production. Liu et al. 2016 performed five cycles of a sequencing batch cultivation (exchanging culture medium until stationary growth phase is reached) using *Cryptococcus curvatus*. As carbon source, a VFA solution

derived from an anaerobic fermentation of waste activated sludge was used. After the removal of nitrogen from the substrate medium by pre-processes and therefore increasing the C/N ratio, a lipid content of 39.6% could be reached. The biomass concentration was 4.5 g L^{-1}.

An overview of the reached biomass concentrations and lipid contents of the above-mentioned experiments can be found in Table 1.

Table 1: Summary of biomass and lipid production of different oleaginous yeasts on different substrates

Strain	Carbon source	Biomass [g L^{-1}]	Lipid content [%]
[a] *T. fermentans*	Rice straw hydrolysate	28.6	40.1
[b] *C. curvatus*	Wheat straw hydrolysate	17.2	33.5
[c] *R. glutinis*	Monosodium glutamate wastewater	25	20
[d] *L. starkeyi*	Sewage sludge + glucose	8.9	75.2
[e] *R. glutinis*	Sugar beet molasses	14.9	52.6
[f] *Y. lipolytica*	Food waste fermentation effluent	2.0	18.2
[g] *C. curvatus*	Waste activated sludge	4.5	39.6

[a] Huang et al. (2009); [b] Yu et al. (2011); [c] Xue et al. (2008) [d] Angerbauer et al. (2008) [e] Taskin et al. (2016); [f] Gao et al. (2017) [g] Liu et al. (2016)

1.5 Economic considerations

With over 14 million metric tons of biodiesel per year, corresponding to 34% of the worldwide production, the European Union is the biggest producer of biodiesel worldwide (Deter, A. 2019).

The interest in using SCOs in the lipid industry and especially for biodiesel production has increased over the last years. The higher lipid and biomass productivity of oleaginous microorganisms, plus their shorter cultivation time and space requirements compared to agricultural products, which are currently the main raw material for lipid production, indicate good alternative lipid sources to plant oils (Llamas et al. 2020).

Several estimations of the SCO production costs and the costs for SCO derived biofuels have been performed. Bonatsos et al. (2020) published a cost estimation for a production plant with an output of 10 000 metric tons SCO per year. Including the fixed capital investment, the operating labour costs, the material costs and the utility costs, a final selling price of 4.613 \$ kg^{-1} SCO was estimated.

Compared to the costs of plant oils, like palm oil, the biggest obstacle in using SCO in the lipid industry is the high production cost. This is the reason why commercialisation and scale-up are still challenging. Beside the use of low-cost substrates, the utilization of co-products along the production line of SCOs

are crucial and necessary to be economically and environmentally competitive. The biomass of the defatted microorganisms can be used in energy conversion processes, like anaerobic digestions or further fermentations to use the residual stored energy (energy co-products). A second co-product are proteins. The defatted biomass provides the possibilities to use them as feed or feed additives. In the fisheries, single cell proteins (proteins originated from microorganisms) are already used as high value feed. However, also in this area the competition between plant derived nutrients and yeast derived nutrients plays a big role. The use of soybean meal is for instance quite common and well established. Other options are the co-production of for example carotenoids, fragrance chemicals and pigments. Also, recombinant proteins and polysaccharides can be high value side products. Nevertheless, the extraction and purification are laborious and can be expensive and the benefit strongly depends on the market price. For example, the market demand for carotenoids is rather low and production of these compounds as side product can lead to an excess amount and low market prices. Another challenging factor is the influence of these substances on lipid production (Parsons et al. 2020).

2 Objectives

The objective of this master thesis is to evaluate the influence of different carbon sources on the lipid production in the oleaginous yeast *Apiotrichum brassicae*. For this purpose, four different VFA were used as substrate in fed batch fermentations to examine their effects on biomass production, lipid content and fatty acid composition. The fatty acid profile and their percental amount of the total fatty acids were assessed for two odd-numbered (propionic and valeric acid) and two even-numbered (acetic and butyric acid) VFAs.

Additionally, the growth behaviour and lipid production of *A. brassicae* on other substrates, like lactic acids and sugars, were investigated in fed batch and batch fermentations. To evaluate the suitability of each individual carbon source, the substrates were compared by the reached volumetric SCO productivity and lipid yield, based on the total amount of consumed substrate and carbon.

To assess the ability of *A. brassicae* to produce lipids on a non-synthetic medium, an anaerobic digestion derived mixed VFA served as carbon source.

Finally, by comparing the obtained results with literature data of other oleaginous yeasts, the suitability of *A. brassicae* as lipid producer was assessed.

3 Materials and Methods

3.1 Microbial strain

For all experiments, the oleaginous yeast *Apiotrichum brassicae V134* was used. *A. brassicae* was first described in 1971 by Takashi Nakase and was originally named *Trichosporon brassicae*. Nakase (1971) describes the yeast as round to short oval. On plates they form pale yellow to grayish white colonies, with a smooth, mucous and shiny appearance. The strain lacks fermentative abilities and can grow on glucose, galactose, sucrose, xylose and glycerol, but is not able to assimilate lactose. Nitrate cannot be used as sole nitrogen source. The yeast was later renamed to *Apiotrichum brassicae* (Liu et al. 2015).

The strain that was used in this master thesis was provided by Prof. Celia Pais of the University of Minho in Portugal and was stored in yeast extract peptone dextrose (YPD) containing 20% (*v/v*) glycerol at -80 °C.

3.2 Chemicals

In Table 2 the used chemical compounds and the companies where they were purchased or delivered from are listed.

Table 2: Used chemical compounds

Chemical	Company	Degree of purity
Yeast extract KAT	Ohly (DE)	-
Casein peptone E1 19546	Organotechnie (FR)	-
Acetic acid	Carl Roth (DE)	100 %
Lactic acid		90 %
Lactose		for microbiology
Glycerol		≥ 99.5 %
Propionic acid	Merck (DE)	≥ 98 %
Butyric acid		≥ 99 %
Valeric acid		≥ 98 %
Crude glycerol	Münzer (AUT)	-
Xylose	Ducefa Biochemie (NL)	99 %
Galactose	Honeywell Fluka (USA)	> 98 %
Sucrose	Agrana (AUT)	-

3.3 Shake flask experiment

To investigate the utilization of different carbon sources by *A. brassicae*, a shake-flask experiment with different substrates was initially performed. To match a concentration of 20 g L^{-1} in the shake flask, 200 g L^{-1} stock solutions of glucose, galactose, lactose, sucrose, xylose, glycerol and crude glycerol were prepared and autoclaved. 5.43 g yeast extract (YE) and 3.54 g peptone were dissolved in 1.26 L of water, autoclaved and then portioned into 300 mL shake flasks with 3 baffles with 90 mL each. Then 10 mL of the substrate stock solutions were added to the shake flasks. For each substrate two shake flasks were prepared. Except for glycerol and crude glycerol, the substrate was added to the YE and peptone solution after autoclaving the stock solutions separately. All shake flasks were inoculated with 100 µL *A. brassicae* cryo-culture under a laminar flow workbench. The experimental setup for the shake flask experiment is shown in Table 3.

Table 3: Shake flask experiment setup

working volume	100 mL
substrate conc.	20 g L^{-1}
yeast extract conc.	3.88 g L^{-1}
peptone from casein conc.	2.53 g L^{-1}

To determine the exact starting substrate concentration, 200 µL samples of each shake flask were taken and stored at -22°C in the freezer for later HPLC analysis. Then all 14 shake flasks were incubated at 30°C and 130 rpm in a Multitron standard incubator shaker (Infors).

After 18, 20, 22, 24, 42, 44 and 46 hours, 1 mL of sample was taken from each shake flask and the optical density was measured at a wavelength of 600 nm to follow the growth trend on each substrate. The residual amounts of samples were centrifuged at 12500 rpm (14324 g) for 10 minutes using a Beckman GS-15 centrifuge. The supernatant was stored in the freezer at -22°C. After 46 hours of fermentation, 4 mL samples were taken and centrifuged at 3500 rpm (2424 g) for 10 minutes using an Eppendorf 5810 centrifuge. The supernatant was collected and frozen. After two washing steps with RO water, the pellets were also stored at -22°C.

3.4 Experiments in parallel bioreactors

The DASGIP system (Eppendorf) is a parallel bioreactor system that consists of up to four 1.5 L reactors, a control unit and the DASware control software. Each reactor is equipped with a stirrer, a sparger, a pH electrode, a DO electrode, a temperature sensor, two tube connections for the two pumps, a sampling valve and an off-gas condenser.

The eight peristaltic pumps (two per reactor) were used to pump the feed medium, if needed, and to maintain a pH of 6.0 with a 2 M sterile NaOH (dilution of a 50 % NaOH solution) or H_2SO_4 solution (dilution of a concentrated H_2SO_4 solution). In case of glucose and xylose, H_2SO_4 was used to counteract pH changes. For all other substrates, NaOH was used.

The temperature was maintained at 30°C using a platinum temperature sensor to measure the actual temperature in the reactors and a heating block, in which the reactors were placed, to control the temperature.

To reduce evaporation of water, the off-gas was cooled in a condenser by a cooled water stream. Instead of using a filter, which can be easily blocked by excess foam formation, the tube of the off-gas condenser was dipped into a 2 M NaOH solution, to prevent contamination of the fermentation broth and also of the laboratory.

The DO of 20 % was maintained by varying the stirrer speed between 400 and 1200 rpm. The airflow was kept constant at 35 L h^{-1} through a sterile filter attached to the sparger.

3.4.1 Inoculum preparation

For each experiment, three 300 mL shake flasks with 3 baffles were prepared. The media used was composed of 10 g L^{-1} peptone of casein, 5 g L^{-1} yeast extract and 20 g L^{-1} glucose. To reach these concentrations, 3.3 g peptone and 1.65 g yeast extract were dissolved in 297 mL of distilled water and split into the three shake flasks with 99 mL each. 6.6 g of glucose were dissolved in 33 mL of RO water and autoclaved separately. 11 mL of sterile glucose stock solution were pipetted into each flask under the laminar flow workbench. All flasks were inoculated with 330 µL of *A. brassicae V134* cryo-culture and incubated for 24 h at 30 °C and 130 rpm in a Multitron standard incubator shaker.

After one day of incubation, the three inoculation flasks were checked under the microscope for possible contamination and the two flasks with the highest optical density were poured together under the laminar flow workbench.

3.4.2 Reactor preparation and setup

Before each fermentation, the clean reactor parts were assembled, and several preparation and calibration steps were performed.

The first step was a two point pH calibration. The four pH electrodes were put into a pH 7.0 buffer solution and after the DASGIP software showed stable values, the offset was calibrated. To calibrate the slope, the same procedure was performed with a pH 4.0 buffer solution.

The next step was to assemble and autoclave the bioreactor. The prepared yeast extract and peptone medium (described in section 3.3.3) was filled into each reactor and, the pH and DO sensors, the cooling device as well as the filter for aeration were placed into the lid of the reactor. To make sure no medium is pressed through the filter and the off-gas tubes, clamps were used to seal the tubes. After autoclaving at 121°C for 20 minutes using a Systec VE-120 autoclave, the lids of the reactors were closed tightly and to check if the reactors are tight, the clamp of the aeration tube was removed.

The pump calibration of the DASGIP system was performed using the integrated calibration method. 10 mL tubes were weighed before and after pumping the respective medium through the pipes into the tubes. With the set flow rate, the time, and the difference of the weight of the pumped medium, the software calibrated the pumps.

In order to clean the pump tubes before they are connected to the reactors, a cleaning in place procedure was done. The tubes were first flushed with 70% ethanol, then with a sterile 2 M NaOH solution and rinsed with RO water. As a last step the tubes of pump A were filled with the respective sterile feed medium and the tubes of pump B with a sterile 2 M $NaOH/H_2SO_4$ solution.

After the reactors were autoclaved, everything needed to control the fermentation was connected to the DASGIP control system. This includes the stirrer motors, the DO electrodes, pH sensors, the heating blocks, the off-gas cooling system and the aeration tubes. The off-gas tubes were put into a NaOH solution and the aeration was switched on.

After the addition of antifoam (see section 3.3.3), the DO sensors were ready to be calibrated. For that, the stirrers were set to 1200 rpm and when the medium was saturated with air (stable values in the DASGIP control software) the 100% DO point was calibrated. For the 0% DO point calibration, nitrogen was used.

The last step was to turn on the pH, the DO, the temperature and agitation control unit in the DASGIP software. The aeration was set to 35 L h^{-1} and if acidic feed medium was added to the reactor before the start of the fermentation, pump B was turned on for pH regulation.

3.4.3 Feed medium and feed strategy

For the DASGIP experiments, eight different synthetic carbon sources were used to investigate the single cell oil production: acetic acid, propionic acid, butyric acid, valeric acid, lactic acid, glucose, xylose and glycerol (two different feed rates). Finally, fermentations with a sterile and non-sterile mixed VFA substrate were performed. For all setups two parallel fermentations were done.

In case of acetic acid, propionic acid, butyric acid and lactic acid, a 500 g L^{-1} feed solution was prepared. The flasks were autoclaved and weighed to trace how much feed is consumed during the fermentation. Because valeric acid does not mix properly with water, it was used in pure form. For all five carbon

sources a feedback fed batch process was used, controlling the addition of substrate based on the pH in the reactors. Before inoculation, 2 mL feed solution were added to the respective reactors using the sampling valves (in case of valeric acid the 1 mL were added via pump) and the pH was adjusted. After inoculation, carboxylic acid used as feed, is consumed leading to a decrease of the pH. The control system counteracts this by adding more feed, containing an acidic carbon source.

The medium preparation for acetic acid, propionic acid, butyric acid, valeric acid and lactic acid was as followed: Each reactor should have a starting volume of 500 mL with a starting concentration of peptone of casein and yeast extract of 2.53 g L^{-1} and 3.88 g L^{-1} respectively. Because antifoam and distilled water, to rinse the sampling valve and tube, were needed, the yeast extract and peptone medium stock solution was prepared in a higher concentration. In Table 4, the volumes of the single components at the start of the fermentations are listed.

Table 4: Acetic, propionic, butyric, valeric and lactic acid - reactor starting volume setup

Added substance	[mL]	Comment
Peptone and YE medium	440	added before DO calibration
Antifoam	1	
Sterile RO water	2	
Feed medium	2	added before inoculation
Sterile RO water	3	
Inoculum	50	Inoculation start
Sterile RO water	2	
Total	500	

For the two sugars, glucose and xylose, 450 g L^{-1} solutions were prepared and autoclaved, but instead of using a fed batch process, a batch process was performed by adding 100 mL of substrate at the start of the fermentation via syringes. The starting volume was 500 mL with a sugar concentration of 90 g L^{-1} and a peptone of casein and yeast extract concentration of 2.53 g L^{-1} and 3.88 g L^{-1}, respectively. The reactor setup with the respective amounts of the single components is shown in Table 5.

Table 5: Glucose and xylose - reactor starting volume setup

Added substance	[mL]	Comment
Peptone and YE medium	340	added before DO calibration
Antifoam	1	
Sterile RO water	2	
Substrate	100	added before inoculation
Sterile RO water	4	
Inoculum	50	Inoculation start
Sterile RO water	3	
Total	500	

In case of glycerol a fed batch process with a feed medium concentration of again 500 g L^{-1} was performed. This time, four reactors were used with a constant feed rate. Two reactors were operated with a feed rate of 0.625 mL h^{-1}, which is equal to 0.3125 g h^{-1} and two with a feed rate of 1.25 mL h^{-1}, which is equal to 0.625 g h^{-1}.

The preparation of the peptone and yeast extract medium for the experiment using glycerol as substrate was the same as for the acetic acid. Again 440 mL were filled into each reactor before autoclavation. Because a constant feed was used, no substrate was added before inoculation. The reactor setup, using glycerol as feed, is shown in Table 6.

Table 6: Glycerol - reactor starting volume setup

Added substance	[mL]	Comment
Peptone and YE medium	440	added before DO calibration
Antifoam	1	
Sterile RO water	4	
Inoculum	50	Inoculation start
Sterile RO water	5	
Total	500	

For the fermentations using the mixed VFA substrate, sterile and non-sterile media were prepared. Parts of the VFA solution were pumped through a sterile filtration apparatus and after that, the pH of both

solutions was adjusted to 4. The feeding strategy was a fed batch process controlled by pH changes, similar to the synthetic volatile fatty acid media. The VFA compositions of both media are shown in Table 7, the YE and peptone starting concentration was 3.05 g L^{-1} and 1.99 g L^{-1}. The reactor setup, using the mixed VFA substrate, is shown in Table 8.

Table 7: VFA composition of the sterile and non-sterile mixed VFA substrate

	sterile	non-sterile
Acetic acid [g L^{-1}]	21.71	20.80
Propionic acid [g L^{-1}]	13.10	12.53
Butyric acid [g L^{-1}]	5.79	5.55
Isobutyric acid [g L^{-1}]	0.46	0.44
Valeric acid [g L^{-1}]	3.45	3.35
Isovaleric acid [g L^{-1}]	0.50	0.56
Caproic acid [g L^{-1}]	0.94	0.90
Total VFA [g L^{-1}]	45.95	44.13

Table 8: Mixed VFA substrate - reactor starting volume setup

Added substance	[mL]	Comment
Peptone and YE medium	240	added before DO calibration
Antifoam	1	
Sterile RO water	2	
Substrate	20	
Sterile RO water	3	
Inoculum	50	Inoculation start
Sterile RO water	2	
Total	318	

3.4.4 Fermentation and sampling

With a syringe, 50 mL of the inoculum (see section 3.4.1) were injected into each reactor via the Luer lock injection valve. In case of the fed batch processes, pump A for the addition of medium was turned on.

After the inoculation as well after 20, 22, 24, 26, 42, 44, 46, 48, 50, 68, 70 and 72 hours, samples were taken through the sampling valve. The first 2 mL, which were collected with a syringe, were discharged to remove the suspension within the sampling tube that is not representative for the suspension broth in the reactor at the specific time point. Then approximately 5 mL of sample were taken. 4 mL of this sample were volumetrically transferred into a Pyrex tube. With the residual fermentation broth, the OD_{600} was measured. The Pyrex tube was centrifugated at 3700 rpm (2700 g) for 7 minutes using an Eppendorf 5810 centrifuge. The supernatant was transferred into a 10 mL tube and stored in the freezer at -22°C for subsequent determination of carbon source concentration, phosphate concentration and TKN. The pellet was then washed twice with RO water and also stored for subsequent determination of dry cell weight and SCO content.

During the fermentation, the pH was checked externally to guarantee that the pH electrodes in the reactors work properly. Once a day, the samples were checked under the microscope for possible contamination.

After 72 hours the fermentation was stopped. All sensors and feed tubes were disconnected, the reactors were disassembled and before the fermentation broth was autoclaved the end volume of each reactors was measured. After autoclavation all parts were cleaned and dried for the next fermentation. In case of the four fermentations using the sterile und non-sterile mixed VFA substrate, the fermentations were stopped as soon as the feed flask were empty. Because of the higher dilution, the feed was consumed faster than the previous fermentations and the reactors were stopped after 48 hours.

3.5 Analytical methods

The following analytical steps were performed for all DASGIP Experiments using different substrates. The HPLC and GC analysis protocols were also used for the shake flask experiment.

3.5.1 Microscopy

To check possible contaminations, samples were examined using an Olympus Vanox AHBT3 microscope with 100x objective. The optical microscopy technic was phase-contrast microscopy.

3.5.2 Optical density

All optical densities were determined at a wavelength of 600 nm using a DR3900 Hach Lange photometer. Samples were diluted with RO water in a respective ratio to measure in an OD_{600} range between 0.2 and 1.

3.5.3 Cell dry weight

For each sampling step, the cell dry weight was determined. For this purpose, cleaned Pyrex tubes were dried at 105°C in a compartment drier and the weight of each tube was noted. After the sampling procedure (described in section 3.4.4) the Pyrex tubes containing the cell pellet were covered with parafilm (holes were punctured for water evaporation) and frozen at -80°. Subsequently the samples were freeze dried using a Christ, Alpha 2-4 LSC plus freeze drier. After the samples were dried, the Pyrex tubes was weighed again to get the dry cell weight.

3.5.4 Total Kjeldahl nitrogen determination

For the total Kjeldahl nitrogen determination, the BÜCHI Digest Automat K-438 digestor and the BÜCHI AutoKjeldahl Unit K-370 distillation unit were used.

2 mL of supernatant (see section 3.4.4) were pipetted into the BÜCHI glass tubes. The exact weight was noted and after rinsing the tubes with RO water, a Kjeldahl tablet was added to each tube. For the digestion, 20 mL of a 96% H_2SO_4 acid were added and the tubes were heated up by the BÜCHI digestor to 370°C. During this step, the organically bound nitrogen is converted into free ammonium ions (NH_4^+). After 4 hours the digestion was completed and the digestor was turned off.

The determination of free ammonium using the Kjeldahl distillation unit was always performed the following day. The apparatus pumps a 30 % sodium hydroxide solution into the BÜCHI tubes which are heated up and ammonium ions are converted into ammonia. The ammonia evaporates and condensed into a solution of a 2 % boric acid. Boric acid converts ammonia back into ammonium ions and can then be titrated with a 0.05 M hydrochloric acid. Based on the amount of HCl needed, the ammonium content can indirectly be determined.

3.5.5 Phosphate Determination

For the phosphorous determination two kits were used: LCK 350 kit and LCK 349 kit by HACH. Proper dilutions of the thawed supernatant of the taken samples (see section 3.4.4) with MQ water were prepared to stay within the measuring range (0.05-1.5 mg L^{-1} PO_4-P for LCK 349; 2.0-20.0 mg L^{-1} for LCK 350 kit). The residual steps were performed according to the work instructions of the respective determination kits (Hach Lange GMBH, 2020). Phosphate reacts with molybdate anions and form a yellow complex. By reduction with ascorbic acid the colour changes to molybdenum blue, which is then measured by the photometer (DR3900 Hach Lange) at 850 nm. Because a hydrolysis step was performed prior measurement, the total phosphate was determined.

3.5.6 Residual substrate concentration measurement via HPLC-Analysis

In order to measure the substrate concentration over the fermentation time, a Carrez-precipitation followed by HPLC-Analysis was performed. For the Carrez-precipitation a 2% (*v/v*) $K_4[Fe(CN)_6]\cdot 3H_2O$ solution and a 2% (*v/v*) $ZnSO_4\cdot 7H_2O$ solution were mixed. The formed $K_2Zn_3[Fe(CN)]_6]_2$ precipitates and removes proteins from the solution. Prior to the precipitation, proper dilutions of the samples were prepared to prevent overloading of the column and to stay within the measuring range of 10 mg L^{-1} and 1000 mg L^{-1}. Therefore, to all samples diluted sulfuric acid with a pH of 4 was added. For example, for a 1 to 5 dilution, 760 µL of diluted sulfuric acid were mixed with 200 µL sample. Then 20 µL of the first Carrez-precipitation solution were added, mixed and after one minute 20 µL of the second solution were added to a total volume of 1 mL. Then, after mixing and five minutes, all samples were centrifugated at 12500 rpm (14324 g) for 30 minutes using a Beckman GS-15 centrifuge.

After centrifugation, the supernatant was filtered through a 0.2 µm filter membrane into glass vials for HPLC. For concentration determination, two HPLC setups were used which are described in Table 9.

Table 9: HPLC setup

	Setup 1	**Setup 2**
Column type	ION 300 (Transgenomic, Omaha USA)	CARBOSep COREGEL 87H3 (Transgenomic, Omaha USA)
Detector	Refractive index detector (Agilent 1100)	Refractive index detector (Agilent 1100)
Temperature	45°C	65°C
Flow rate	0.325 mL min^{-1}	0.900 mL min^{-1}
Solvent	0.005 M H_2SO_4	0.005 M H_2SO_4

To analyse the residual sucrose concentrations, the sucrose had to be split prior into glucose and fructose. 33 µL sample were mixed with 67 µL of a 10 g L^{-1} invertase (SIGMA, I9253, ≥30 units/mg solid) solution and incubated at 50°C for 30 minutes. The samples were then diluted with RO water in a proper ratio, to prevent overloading of the HPLC column, and filtered into HPLC vials.

3.5.7 Quantification of FAME via GC-analysis

To determine the composition of the fatty acids contained in the lipid fraction of the cells and their respective amounts, a fatty acid methyl ester analysis via gas chromatography was performed. This was done according to the method described by Meesters et al (1996).

For the determination, 10 to 20 mg of dried biomass were weighed in. For each selected sample, a double determination was performed.

The next step was adding 2 mL of a 15/85 % (*v/v*) mixture of concentrated H_2SO_4 and methanol (99.9%) under the fume hood. After that, 2 mL of 1 mg mL^{-1} methyl benzoate as internal standard in chloroform were added. The tubes were then sealed tightly, mixed with a vortex mixer and placed in a preheated 100°C water bath for two hours. After that, the tubes were taken out and cooled to room temperature under the fume hood. To be able to take into account potential evaporation of chloroform, four samples of the internal standard solution were taken for subsequent analysis.

After the temperature of the Pyrex tubes reached room temperature, 1 mL of MQ-water was added to induce phase separation. After mixing, the tubes were centrifuged at 2800 rcf for 10 minutes using the Eppendorf 5810 centrifuge to reach full phase separation. The upper aqueous phases containing H_2SO_4 and methanol were removed carefully with Pasteur pipettes and discarded. To remove residual water from the organic phase and to neutralize residual acid, a spoonful Na_2SO_4 and Na_2CO_3 were added, mixed and centrifuged again at 2800 rcf for 10 minutes. After the centrifugation step, the supernatant was transferred to glass vials for GC analysis.

To determine the recovery rate, four Pyrex tubes were weighed in with a specific amount of palm oil (between 5 and 10 mg) and treated the same as described above and also prepared for the GC measurement.

The samples were then analysed via gas chromatography. The specifications are listed in Table 10.

Table 10: Gas chromatography specification

Column	type	HP-88
	length	100 m
	diameter	250 µm
	film thickness	0.2 µm
Temperature program	initial setpoint	120°C
	hold time	1 min
	#1 rate	10 °C min^{-1}
	#1 value	175 °C
	#1 hold time	12 min
	#2 rate	3 °C min^{-1}
	#2 value	190 °C
	#2 hold time	0 min
	#3 rate	5 °C min^{-1}
	#3 value	210 °C
	#3 hold time	5 min
	#4 rate	5 °C min^{-1}
	#4 value	230 °C
	#4 hold time	10 min
Injection	volume	1 µL
	temperature	230 °C
Flow rate	2 mL min^{-1}	
Split mode ratio	1:50	
Carrier gas	H_2	
Detector	flame ionization detector (Agilent 7890B)	

3.6 Calculations

With the weight of the biomass of the taken sample (see section 3.4.4) and the sampling volume of 4 mL, the dry cell weight concentration was calculated.

For the fat content, the gas chromatography data were evaluated with the help of the software: ChemStation for LC 3D system by Agilent Technologies. The FAME concentrations (derived by peak integration) were exported into an excel data sheet and corrected as follows. First the area of the internal standard solution was divided by the area of the internal standard of each sample. With this factor, the concentration of each individual fatty acid was multiplied to correct concentration changes through

chloroform evaporation. To calculate the concentration of the triglycerides from the corresponding fatty acid methyl esters, conversion factors of the "AOAC Official Method 996.06 Fat (Total, Saturated, Unsaturated) in Food" were used (House et al., 1997). The total amount of fat in each sample was calculated by multiplying the concentration of triacyl glycerides by the volume of chloroform used in the digestion step and then summed up. At last, the total amount lipids were multiplied with the recovery rate. For the transesterification efficiency (recovery rate), the palm oil samples were evaluated the same way and by comparing it with the initial weight the recovery rate could be calculated. The lipid content was calculated by dividing the calculated amount of lipids by the DCW used for the analysis.

$$\text{Lipid content} = \frac{\sum(C_i * \text{internal standard factor} * \text{conversion factor} * V_{solvent}) * \text{recovery rate}}{X}$$

C_i [g L^{-1}]: concentration of the respective fatty acids derived from the ChemStation software

$V_{solvent}$ [L]: volume of the used chloroform

Internal standard factor: peak area of the internal standard of the respective chromatogram divided by the average peak area of the internal standards

Conversion factor: respective Tri/FAME factors for the respective fatty acids

Recovery rate [g g^{-1}]: amount of palm oil, determined with this method, divided by the initial weight

X [g]: amount of dry cell weight used for the analysis

The SCO yield, the amount of single cell oil produced per the amount of consumed substrate, was calculated as follows. First the total amount of produced dry cell weight, and with that the total amount of produced single cell oil, was calculated by determining the liquid level of the reactor at the respective time point. For this, the amount of water added with the feed and the medium removed during sampling was included. It has to be noted that this calculation of the liquid level is only an attempt to get as close as possible to the actual fermentation broth volume, since other factors like evaporation could not be included. With the total volume of fermentation broth, the total amount of produced lipids could be calculated. With that and the total amount of consumed substrate, obtained by comparing the start and end concentrations by HPLC analysis, the SCO yield and the volumetric SCO productivity was then calculated.

$$\text{SCO yield} = \frac{C_{DCW} * V * \text{Lipid content}}{(S_0 - S) * V}$$

C_{DCW} [g L^{-1}]: dry cell weight concentration at the respective time point

V [L]: volume of the fermentation broth at the respective timepoint

S_0 [g L^{-1}]: substrate concentration/carbon content at the beginning of the fermentation

S [g L^{-1}]: substrate concentration/carbon content at the respective timepoint of the fermentation

$$\text{Volumetric SCO productivity} = \frac{C_{DCW} * \text{Lipid content}}{t}$$

t [h]: time of fermentation

4 Results

4.1 Utilization of different carbon sources by *Apiotrichum brassicae*

To assess the utilization of different carbon sources, *A. brassicae* was cultivated in 300 mL shake flasks, with a total media volume of 100 mL. The growth and single cell oil production were examined using following substrates: glucose, galactose, lactose, xylose, sucrose, glycerol and crude glycerol.

Experiment conditions: 20 g L^{-1} of the respective carbon source

3.88 g L^{-1} yeast extract; 2.53 g L^{-1} peptone from casein

30°C; 130 rpm

To follow the growth trend on each substrate, the optical density and the residual substrate concentration were determined. In Figure 1 - Figure 7, these two parameters are compared over the time of incubation. Except for lactose, the increasing OD_{600} along with decreasing substrate concentration shows that all carbon sources could be utilized by *A. brassicae*, although the extent and substrate consumption rate differs.

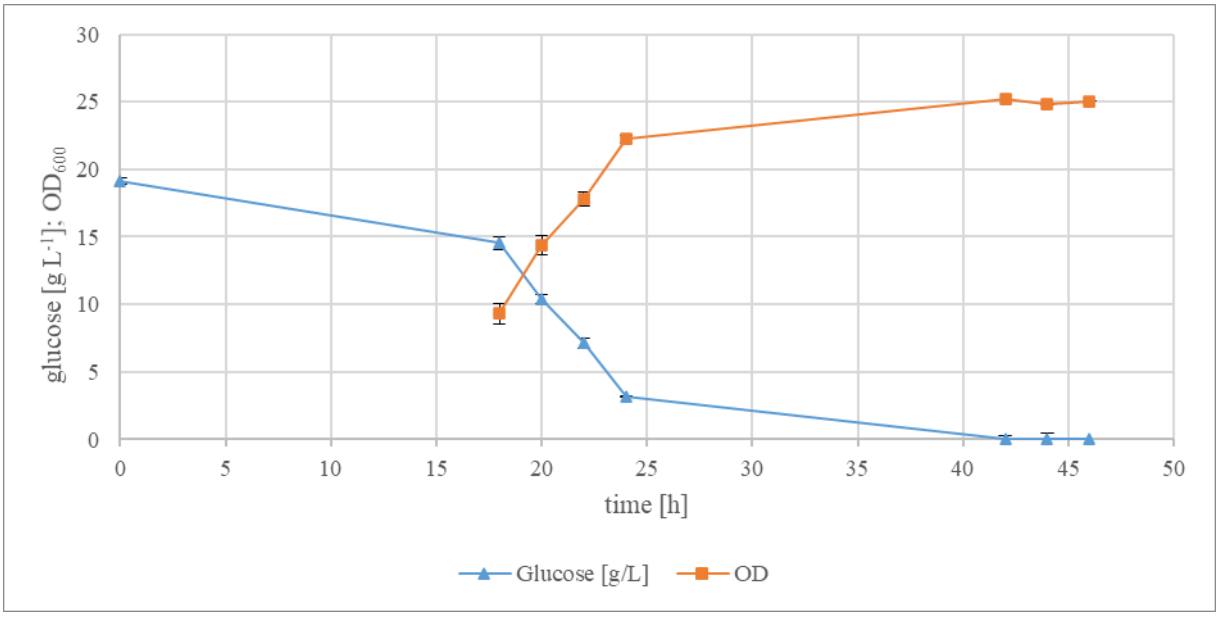

Figure 1: Change of OD_{600} (indicating growth behaviour) and glucose concentration (substrate consumption) over time (the strain was cultivated in duplicate)

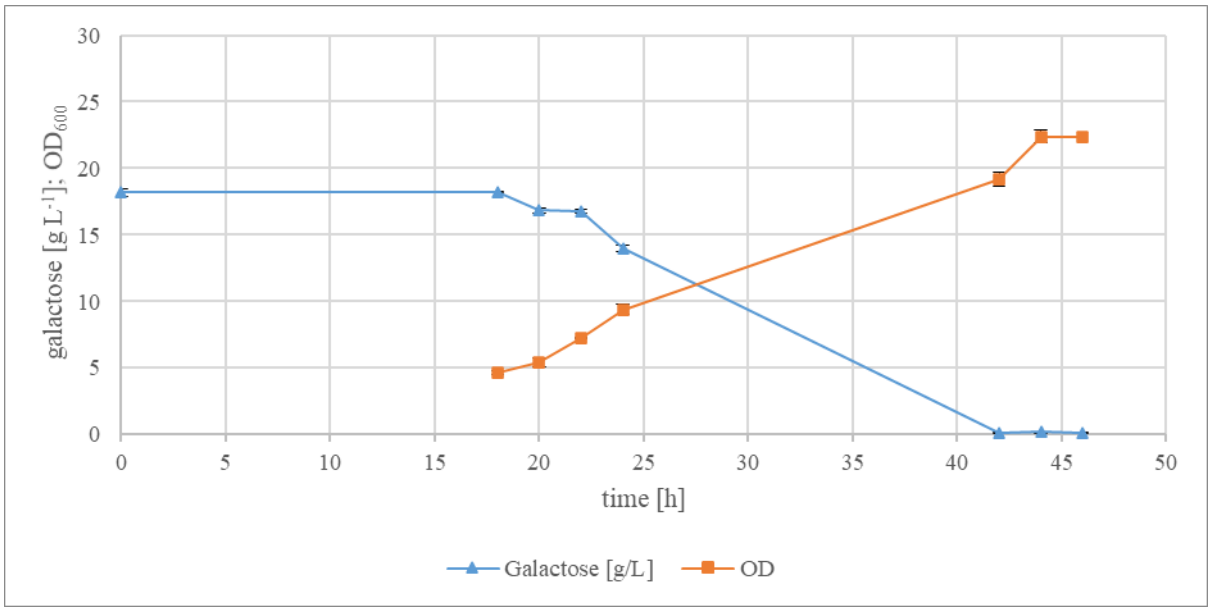

Figure 2: Change of OD_{600} (indicating growth behaviour) and galactose concentration (substrate consumption) over time (the strain was cultivated in duplicate)

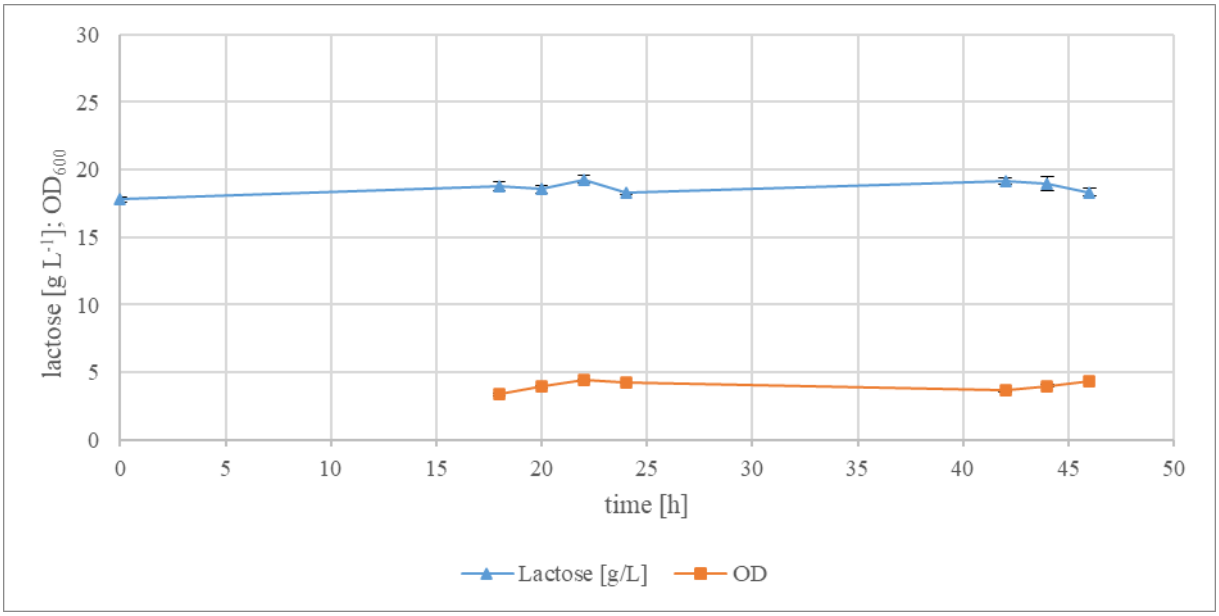

Figure 3: Change of OD_{600} (indicating growth behaviour) and lactose concentration (substrate consumption) over time (the strain was cultivated in duplicate)

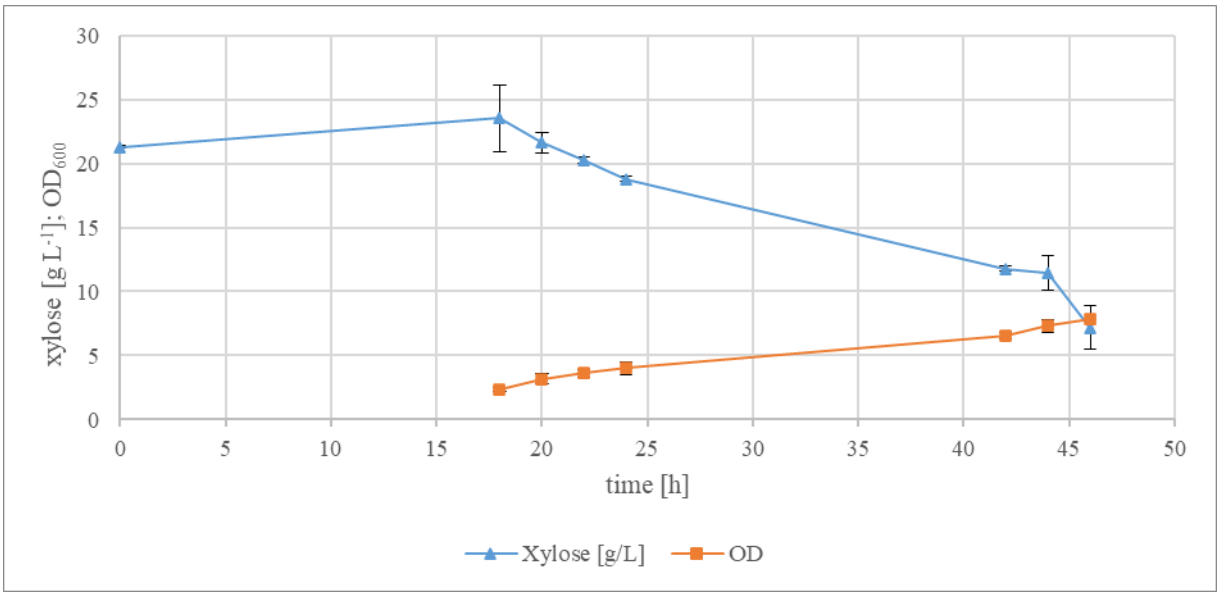

Figure 4: Change of OD_{600} (indicating growth behaviour) and xylose concentration (substrate consumption) over time (the strain was cultivated in duplicate)

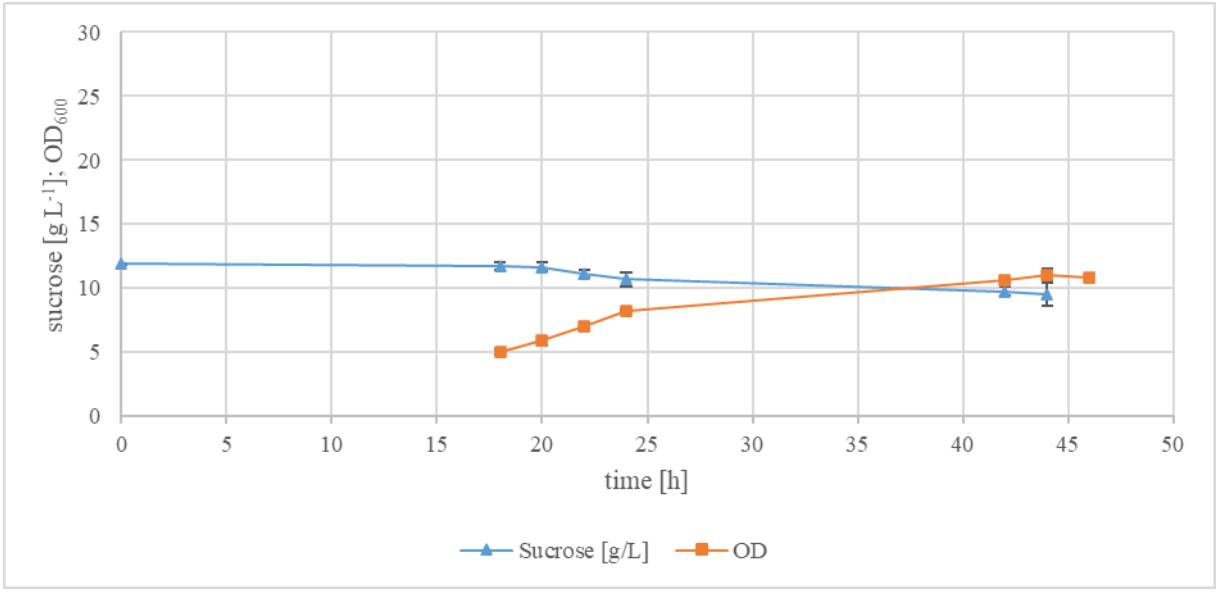

Figure 5: Change of OD_{600} (indicating growth behaviour) and sucrose concentration (substrate consumption) over time (the strain was cultivated in duplicate)

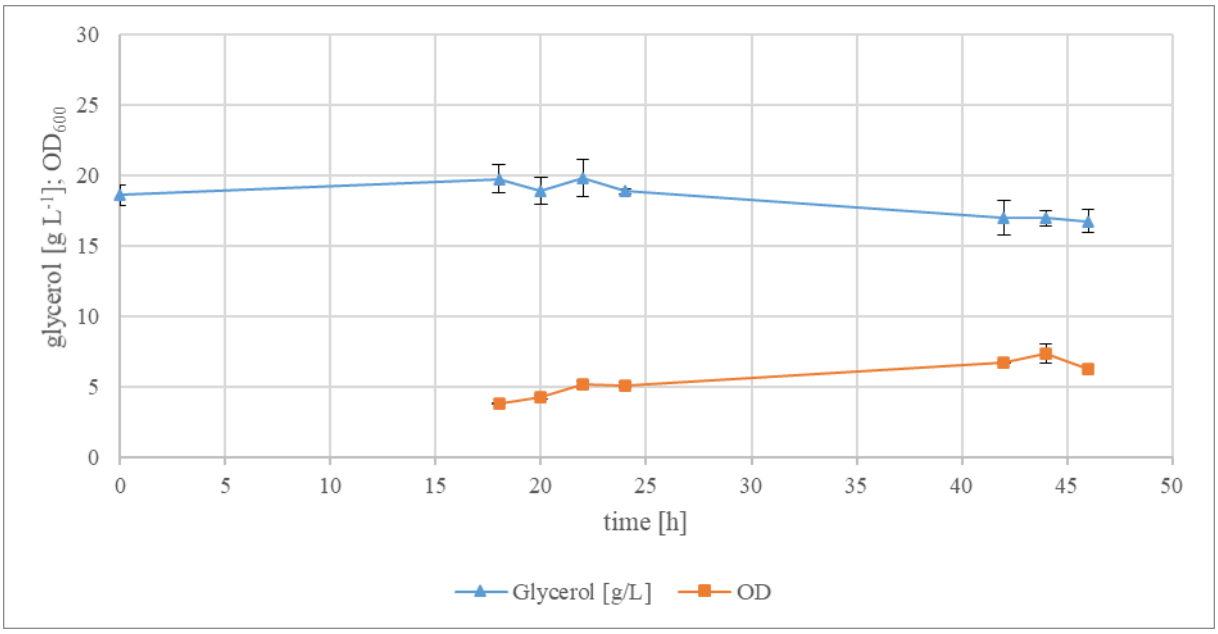

Figure 6: Change of OD_{600} (indicating growth behaviour) and glycerol concentration (indicating substrate consumption) over time (the strain was cultivated in duplicate)

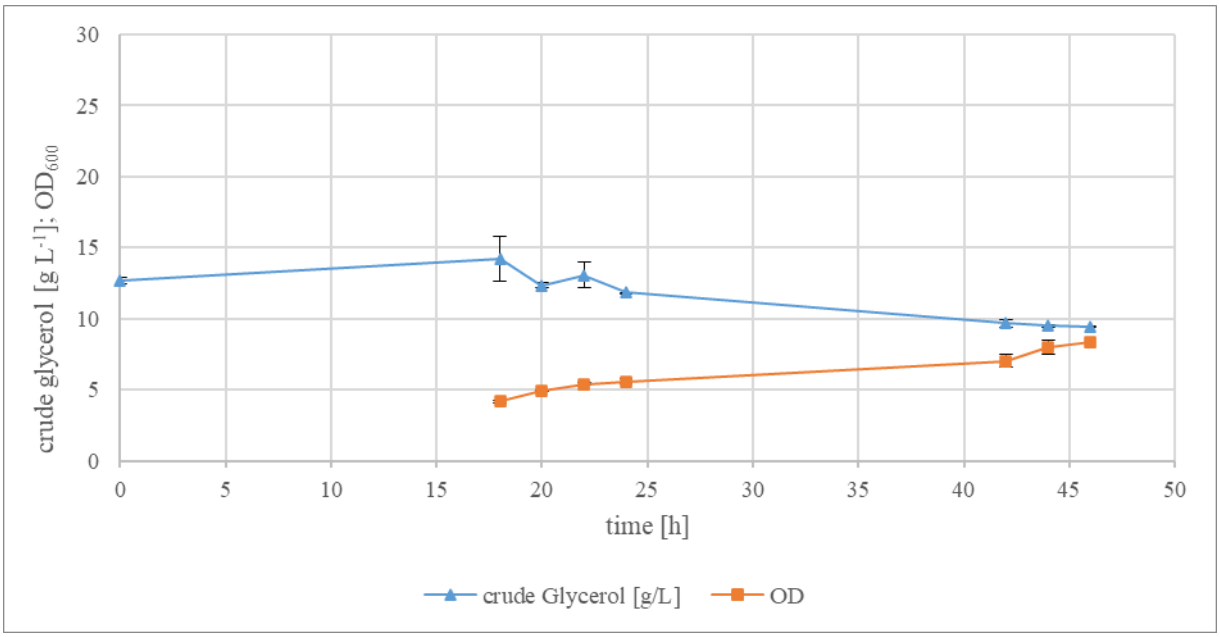

Figure 7: Change of OD_{600} (indicating growth behaviour) and crude glycerol concentration (substrate consumption) over time (the strain was cultivated in duplicate)

A further parameter, to compare the utilization of different substrates, are the substrate consumption rates, which are illustrated in Table 11. Glucose showed the best utilization in *Apiotrichum brassicae*, with and OD_{600} of 25.06 and a dry cell weight (DCW) concentration of 11.75 g L^{-1} after 46 hours and a substrate depletion after 42 hours. The utilization of galactose showed similar results with a DCW of 10.21 g L^{-1} after 46 hours and a slightly lower substrate consumption rate. The cell dry weight end concentrations of xylose, sucrose, glycerol and crude glycerol as substrate, were much lower and were

in a range of 3.46 g L^{-1} (glycerol) and 4.93 g L^{-1} (sucrose). Xylose, however, showed a much higher substrate consumption rate with 0.307 g L^{-1} h^{-1} compared to the other three substrates with similar DCW end-concentrations. Lactose as single carbon source showed no growth of *A. brassicae*.

Table 11: DCW concentration, SCO concentration, lipid content, substrate consumption rate and substrate end concentration after 46h of all carbon sources used in the substrate utilization experiment (average of duplicates)

C-source	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	Substrate consumption [g L^{-1} h^{-1}]	Substrate end concentration [g L^{-1}]
Glucose	11.75	2.22	18.93	0.416	0.00
Galactose	10.21	0.92	9.01	0.391	0.17
Lactose	2.10	0.08	4.00	-	18.34
Xylose	4.28	0.20	4.73	0.307	7.19
Sucrose	4.93	0.20	4.00	0.054	9.52
Glycerol	3.46	0.14	4.03	0.040	16.78
Crude glycerol	4.19	0.18	4.34	0.070	9.48

Table 12: Fatty acid composition in % of A. brassicae growing on different carbon sources; fatty acids with an amount of less than 2% of the total fat content are summarised in "others" and are left blank or are not listed (average of duplicates)

C-source	C12:0 [%]	C15:0 [%]	C16:0 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	C18:3 n3 [%]	C24:0 [%]	Others [%]
Glucose	-	-	22.4	22.0	36.8	10.7	-	-	8.1
Galactose	-	-	15.9	7.8	36.9	28.9	3.1	-	10.5
Lactose	4.8	2.5	11.7	-	20.0	53.7	7.4	-	-
Xylose	2.7	-	13.5	5.3	24.9	41.7	3.8	2.1	6.1
Sucrose	3.2	-	10.6	2.0	21.3	48.5	6.3	-	8.1
Glycerol	3.5	-	11.2	3.1	21.9	46.3	5.6	2.2	6.2
Crude glycerol	2.9	-	12.4	3.5	24.8	45.1	4.0	-	7.4

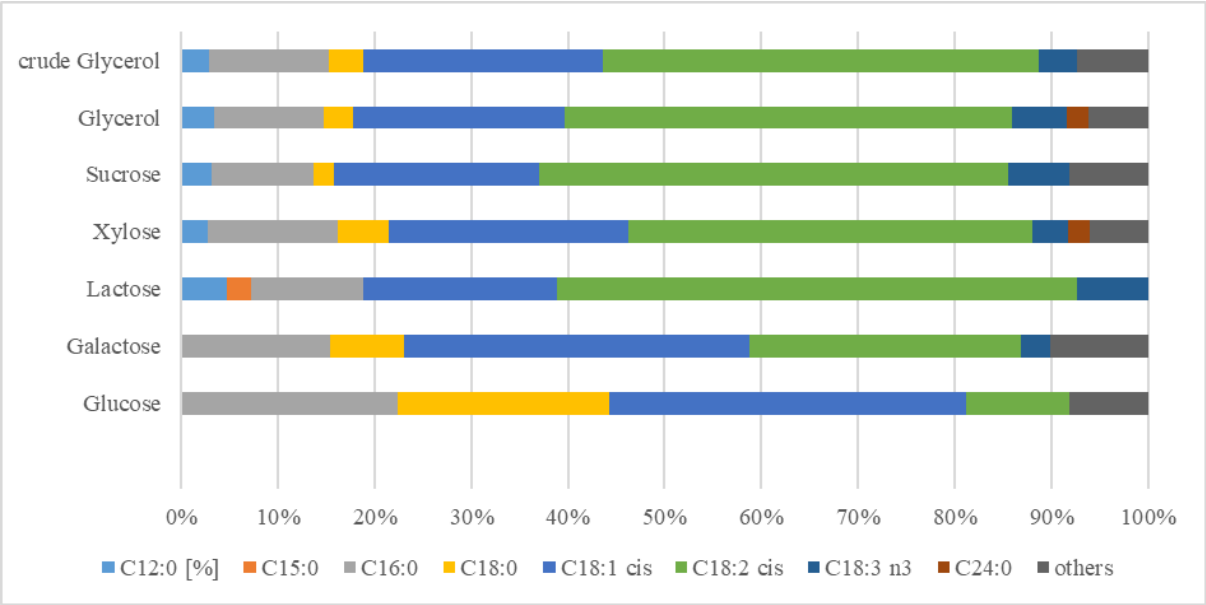

Figure 8: Fatty acid composition in % for A. brassicae growing on different carbon sources (average of duplicates)

Table 12 and Figure 8 show the percental amount of the single fatty acids as percentage of the total fatty acid amount for the yeast *A. brassicae* grown on different carbon sources. Fatty acids with a share of 2% or less are summarised as "others" and are therefore not listed or left blank in the table and figure.

With a lipid content of 18.9 % and a total single cell oil concentration of 2.22 g L^{-1}, using glucose as carbon source resulted in the highest fat content after 46 hours. The most common fatty acids for all substrates are oleic (C18:1) and linoleic acid (C18:2), with more C18:1 than C18:2 using glucose and galactose and vice versa for all other substrates. The results for crude glycerol and glycerol are very similar to each other with a slightly higher fat content for the non-purified one (crude glycerol 4.3 %; glycerol 4.0%).

4.2 SCO production using volatile fatty acids as carbon source

4.2.1 Acetic acid

To investigate the single cell oil production using acetic acid as carbon source, two fed batch fermentations with a feed concentration of 581.37 g L^{-1} were performed. Feed addition was regulated by the pH, which was set to 6.0 (described in section 3.4.3). DO level was set to 20 % and was controlled by the stirrer speed. The temperature of 30 °C was kept constant during the whole fermentation.

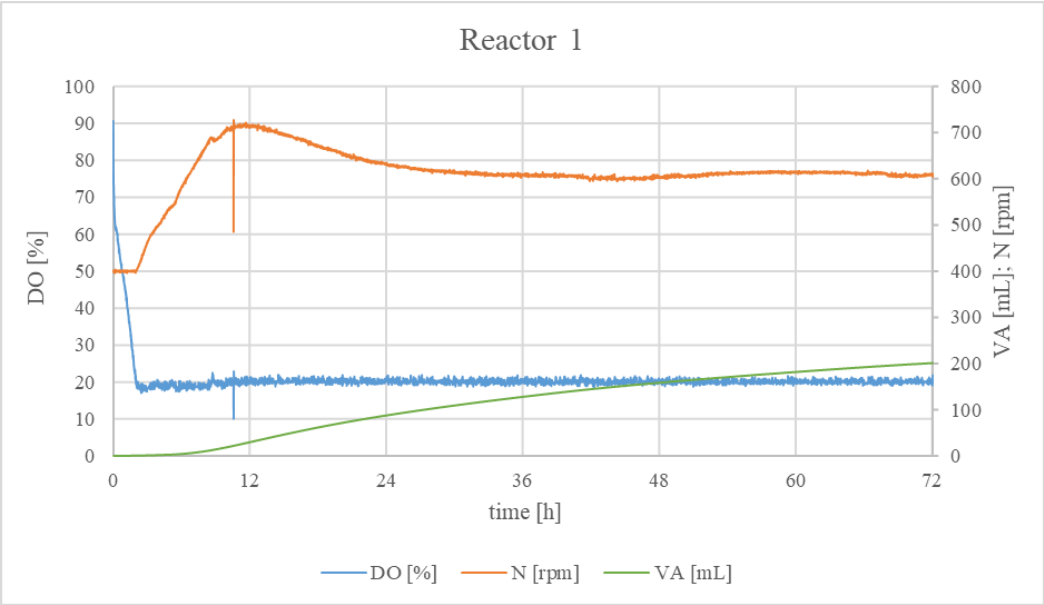

Figure 9: The course of the fed batch fermentation using acetic acid as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = acetic acid in mL

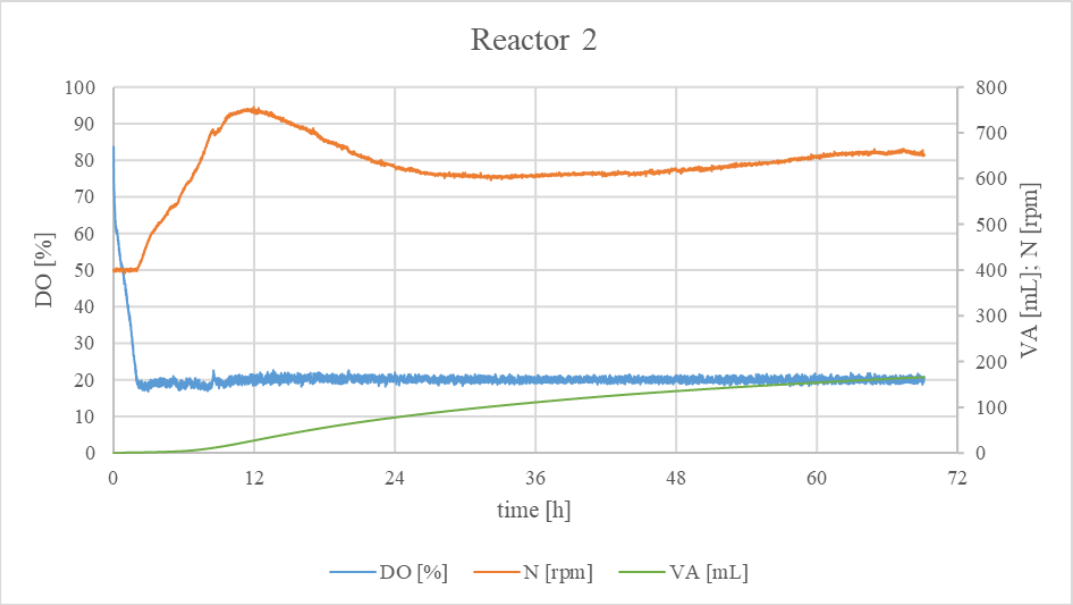

Figure 10: The course of the fed batch fermentation using acetic acid as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = acetic acid in mL

The temporal courses of both fermentations are illustrated in Figure 9 and Figure 10. At the beginning of the fermentation the stirrer speed is increased due to the growth of the yeast. After 12 hours, the stirrer speed needed to maintain a DO level of 20% (20% of the maximal DO reached with this setup) decreases, indicating a decreasing oxygen consumption. After 24 hours, the stirrer speed remains constant with a minor increase to the end of fermentation. A DO level of 20% after 72 hours (end of fermentation), indicates that the yeast is still consuming oxygen and therefore metabolising substrate.

The temperature and the pH remained constant over the fermentation time and are therefore not shown in both figures.

The similarity of both diagrams is a result of the robust DASGIP system, which can deliver very reproducible results. Therefore, the average of the evaluated results of both reactors was calculated and used for the presentation of the results.

In Table 13 the dry cell weight, the single cell oil concentration and the lipid content are listed. Also, the amount of SCO produced per the amount of consumed substrate (SCO/substrate) and carbon (SCO/carbon) as well as the volumetric productivity are listed. Due to time restrictions, only every second sample was used to determine the SCO content.

Table 13: Dry cell weight, single cell oil, fat content, SCO/substrate, SCO/carbon and volumetric SCO productivity using acetic acid as substrate (average of duplicates)

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	2.19	-	-	-	-	-
20	21.20	9.33	35.79	-	-	-
22	23.51	-	-	-	-	-
24	25.08	9.29	37.14	0.12	0.29	0.39
26	26.21	-	-	-	-	-
44	34.44	16.19	46.98	0.12	0.30	0.37
46	34.53	-	-	-	-	-
48	35.25	21.48	60.95	0.15	0.37	0.45
50	36.63	-	-	-	-	-
68	40.35	26.70	66.17	0.15	0.37	0.39
70	41.43	-	-	-	-	-
72	43.46	29.52	67.99	0.15	0.39	0.41

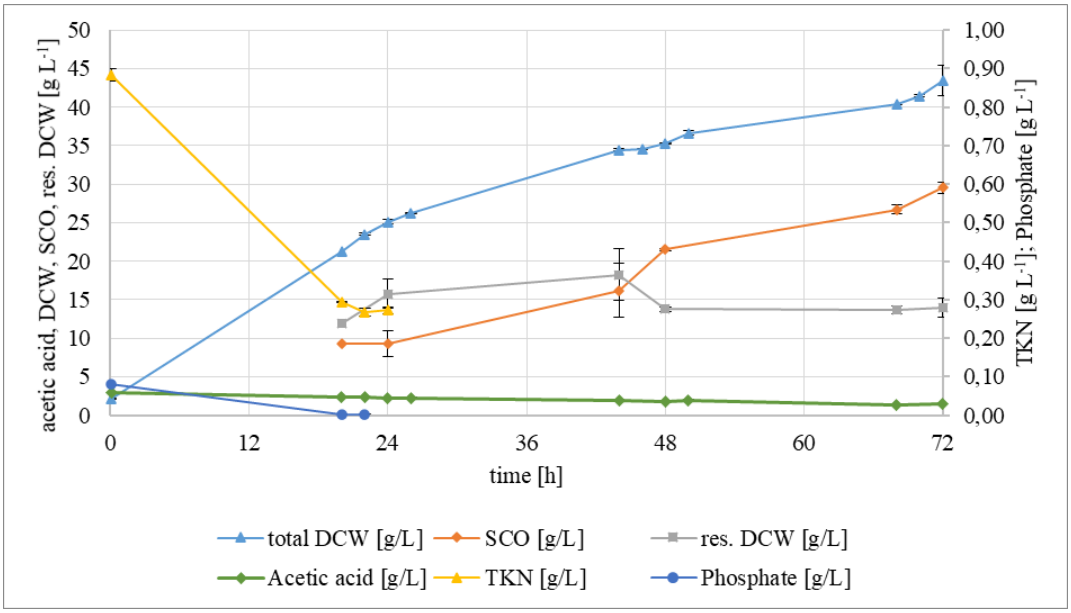

Figure 11: DCW, SCO, res. DCW, acetic acid, nitrogen and phosphorous changes over the time of fermentation using acetic acid as substrate (average of duplicates)

Figure 11 shows the dry cell weight concentration, the single cell oil concentration, the residual dry cell weight concentration, the acetic acid concentration, the total Kjeldahl nitrogen and phosphate content changes over the fermentation time. Because no samples were taken during the night, the first samples were taken on the day following the inoculation. Due to this, the decrease or depletion of nitrogen and phosphate in the fermentation medium can only be illustrated roughly. The graph shows a simultaneous increase of cell material and production of single cell oil, with a dry cell weight of 43.46 g L^{-1} and a single cell oil concentration of 29.52 g L^{-1}, which is equal to a fat content of 67.99% after 72 hours. Between 44 and 48 hours of fermentation, a point was reached where the cells contain more single cell oil than other cell material. The acetic acid concentration in both reactors varied over the fermentation time between approximately 1 and 3 g L^{-1}, showing that no substrate accumulation occurred. At the last measurement point the acetic acid concentration was 1.46 g L^{-1} in reactor 1 and 1.45 g L^{-1} in reactor 2.

Table 13 shows that the volumetric single cell oil productivity remains relatively constant over the time of fermentation, with an increase until hour 48, reaching a productivity of 0.45 g L^{-1} h^{-1}, and a subsequent decrease. The single cell oil production per used substrate reached also a maximum after 48 hours and remained at this level.

Table 14: Fatty acid composition in % using acetic acid as carbon source (average of duplicates)

	C16:0 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	Others (<2%) [%]
20 h	15.6	21.2	50.4	7.2	5.5
24 h	15.9	22.3	50.4	6.0	5.4
44 h	15.8	28.2	45.4	5.5	5.1
48 h	15.8	29.6	43.7	6.1	4.8
68 h	14.9	32.5	41.2	6.4	5.1
72 h	14.8	32.9	41.1	6.4	4.9

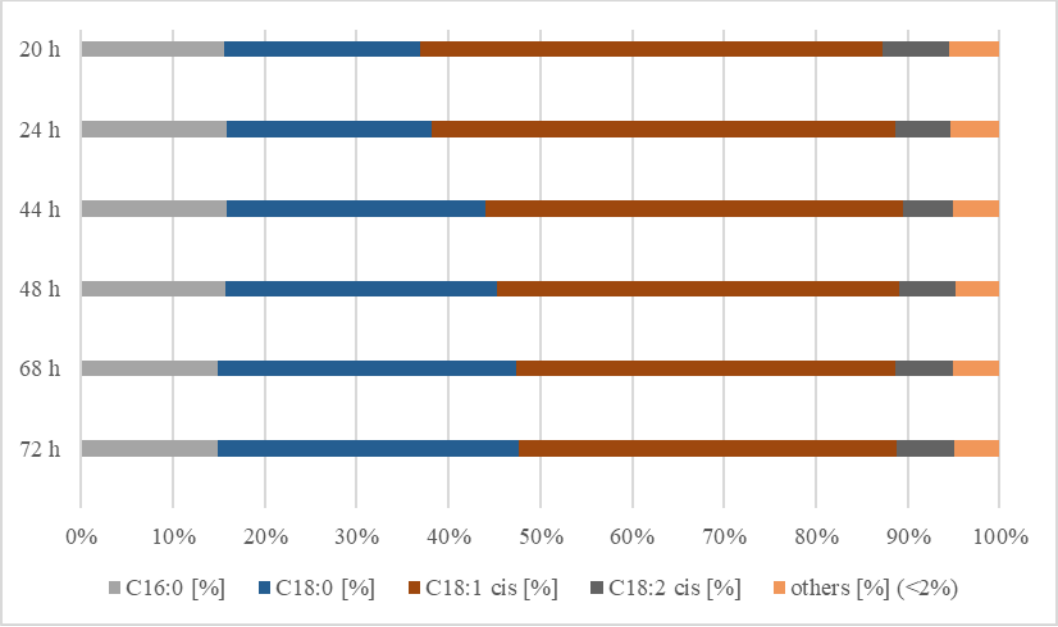

Figure 12: Fatty acid composition in % using acetic acid as carbon source (average of duplicates)

Table 14 and Figure 12 show the changes of the fat composition over the time of fermentation. With 41.1 % oleic acid (C18:1) is the most common fatty acid after 72 hours, followed by stearic acid (C18:0) with 32.9%, palmitic acid (C16:0) with 14.8 % and linoleic acid (C18:2) with 6.4%. Fatty acids with a content of less than 2% are summed up in “others” and make up 4.9%. Over the time of fermentation, the amount of palmitic and linoleic acid does not change much. In contrast, the percental amount of stearic acid increases and oleic acid decreases the longer the fermentation lasts.

4.2.2 Propionic acid

With propionic acid also two fermentations were performed with a fed concentration of 606.57 g L^{-1}. These two fermentations were executed in the same way as the two acetic acid fermentations.

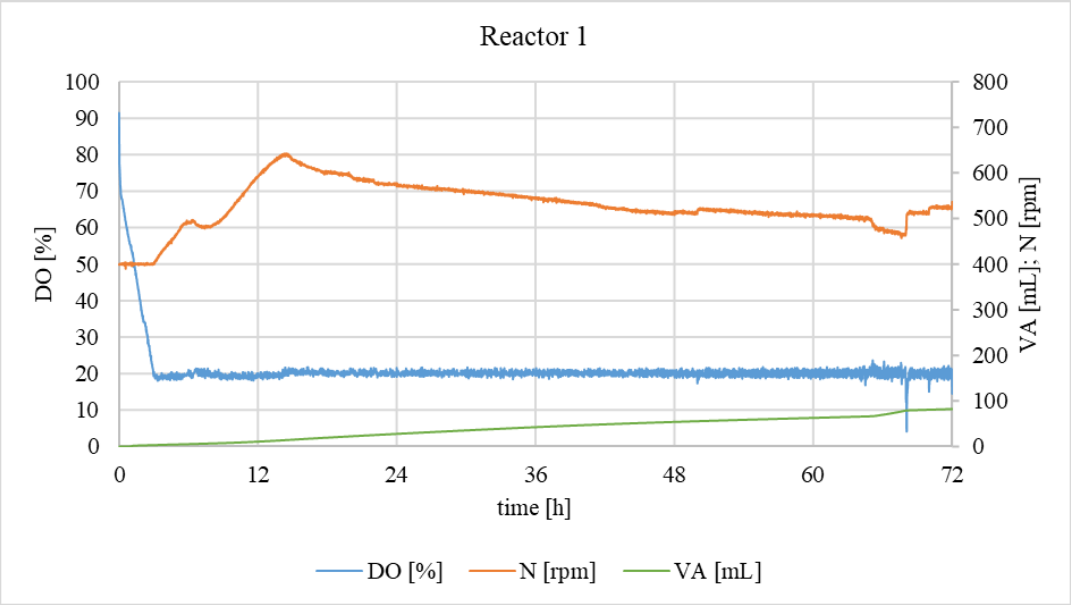

Figure 13: The course of the fed batch fermentation using propionic acid as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = propionic acid in mL

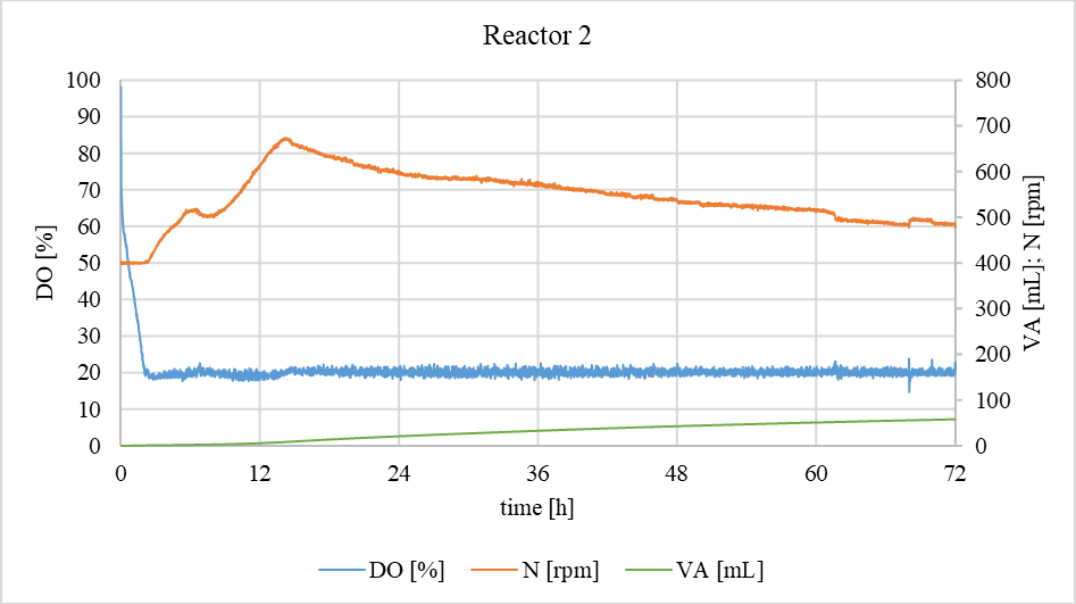

Figure 14: The course of the fed batch fermentation using propionic acid as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = propionic acid in mL

As shown in Figure 13 and Figure 14, the stirrer speed increased at the beginning of both fermentations. After about 14 hours, the stirrer speed required to hold the DO level at 20 % continuously decreases, which indicates a reduced oxygen consumption and reduced metabolic activity. The fermentation was

stopped after 72 hours, although the ongoing consumption of oxygen indicates that there may still have been microbial activity. The temperature and pH were constant over the fermentation time and are not shown in both figures.

Table 15: Dry cell weight, single cell oil and fat content, SCO/substrate, SCO/carbon and volumetric SCO productivity using propionic acid as substrate (average of duplicates)

Duration [h]	CDW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	2.07	-	-	-	-	-
20	9.39	0.74	7.91	0.03	0.07	0.04
22	8.45	-	-	-	-	-
24	11.47	1.11	9.66	0.04	0.08	0.05
26	11.63	-	-	-	-	-
44	16.75	4.42	25.99	0.09	0.18	0.10
46	17.40	-	-	-	-	-
48	17.32	5.21	30.13	0.09	0.18	0.11
50	17.89	-	-	-	-	-
68	22.23	8.31	37.73	0.10	0.20	0.12
70	21.53	-	-	-	-	-
72	21.57	7.49	34.68	0.08	0.17	0.10

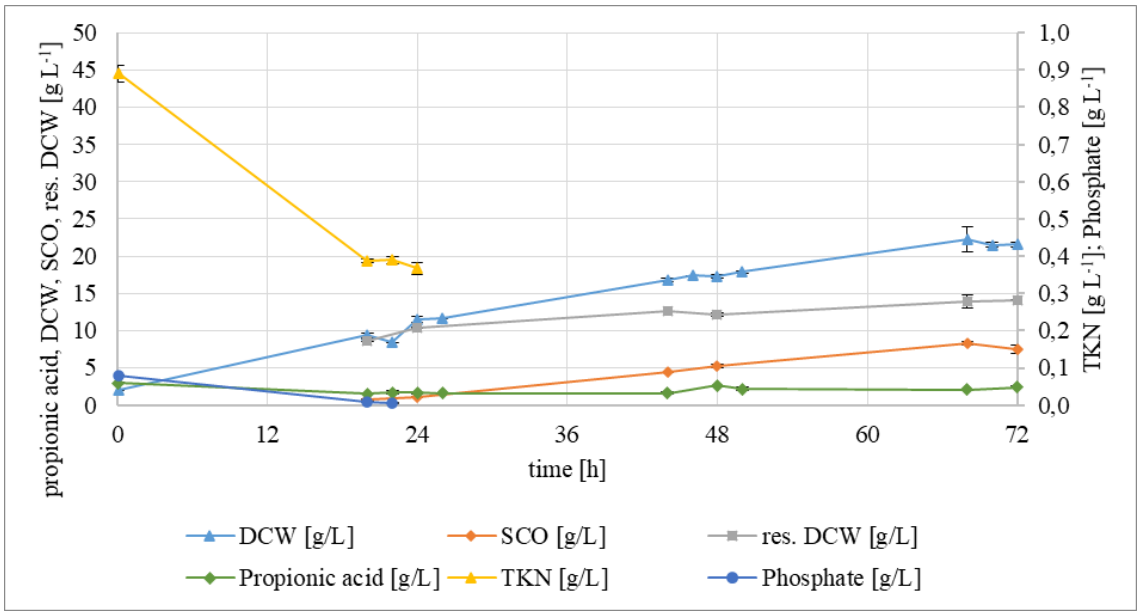

Figure 15: DCW, SCO, res. DCW, propionic acid, nitrogen and phosphorous changes over the time of fermentation using propionic acid as substrate (average of duplicates)

In Figure 15, the increase of cell material and single cell oil are plotted against the fermentation time. The highest concentration of DCW as well as the highest concentration of SCO, which are listed in

Table 15, were reached after 68 hours with 22.23 g L^{-1} and 8.31 g L^{-1}. The fat content was 37.73%. After 68 hours, the cell concentration seems to be decreasing, but because of the high standard deviation at hour 68, this value is subject to uncertainty and a still increasing biomass is possible. This is also indicated by a DO level of 20% after 68 hours, which can be seen in Figure 13 and Figure 14. However, the SCO concentration after 68 hours is decreasing. During the whole fermentation, the SCO content did not exceed the residual cell material but if both graphs are compared, it can be seen that the production of SCO is faster than other cell components. Phosphate depletion was observed, which possibly induced the synthesis of SCO. Similar to the fermentation with acetic acid, no substrate accumulated over the time of fermentation. The propionic acid concentration after 72 hours was 2.27 and 2.52 g L^{-1} in reactor 1 and 2.

Table 16: Fatty acid composition in % using propionic acid as carbon source (average of duplicates)

	C15:0 [%]	C16:0 [%]	C17:0 [%]	C17:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]
20 h	7.0	5.8	38.3	24.9	1.3	11.2	11.3
24 h	7.0	5.3	41.4	26.5	1.3	10.5	8.0
44 h	5.9	4.2	46.1	29.0	0.8	9.8	4.2
48 h	5.9	4.2	46.0	30.0	0.8	9.4	3.7
68 h	5.5	3.8	47.4	30.8	0.6	9.0	2.9
72 h	5.6	3.7	47.5	30.8	0.6	9.0	2.8

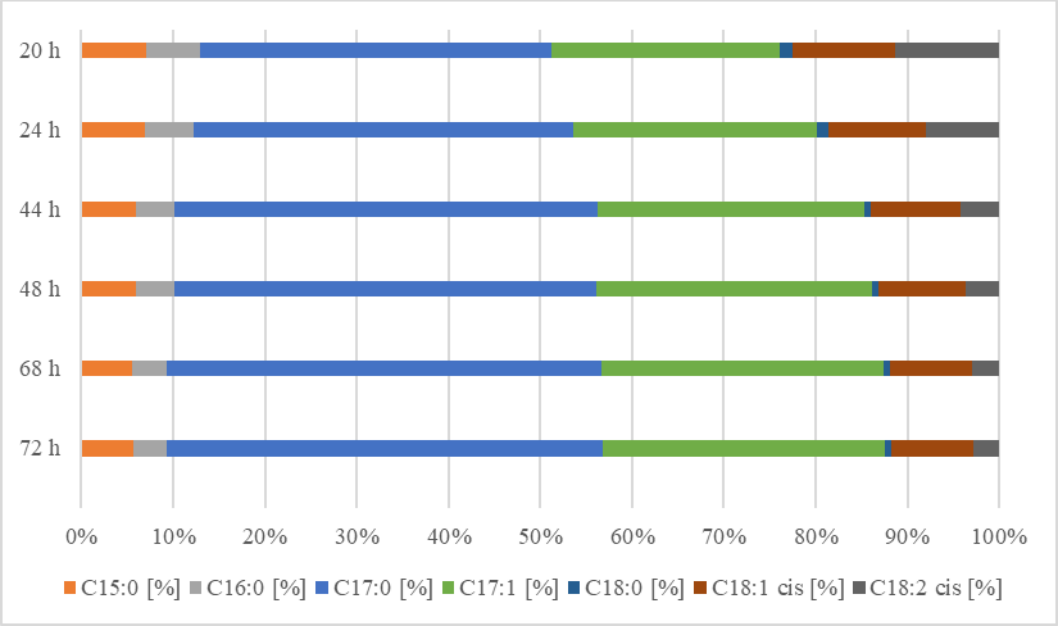

Figure 16: Fatty acid composition in % using propionic acid s carbon source (average of duplicates)

The changes of the fatty acid composition in % using propionic acid as carbon source over the fermentation time is visualised in Figure 16. Using propionic acid as carbon source, margaric (C17:0) and heptadecenoic acid (C17:1) are produced in the highest amounts after 72 hours with 47.5% and 30.8% respectively. For both, the percental amount is increasing during the time of fermentation. The percentage of pentadecanoic (C15:0), palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic (C18:2) acid is decreased during fermentation.

4.2.3 Butyric acid

With butyric acid, two fed batch fermentations were performed. During the two fermentations, no problems occurred and at the end of fermentation the DO was still at 20 %, which indicates that the yeast was still metabolising and consuming oxygen (Figure 17 and Figure 18).

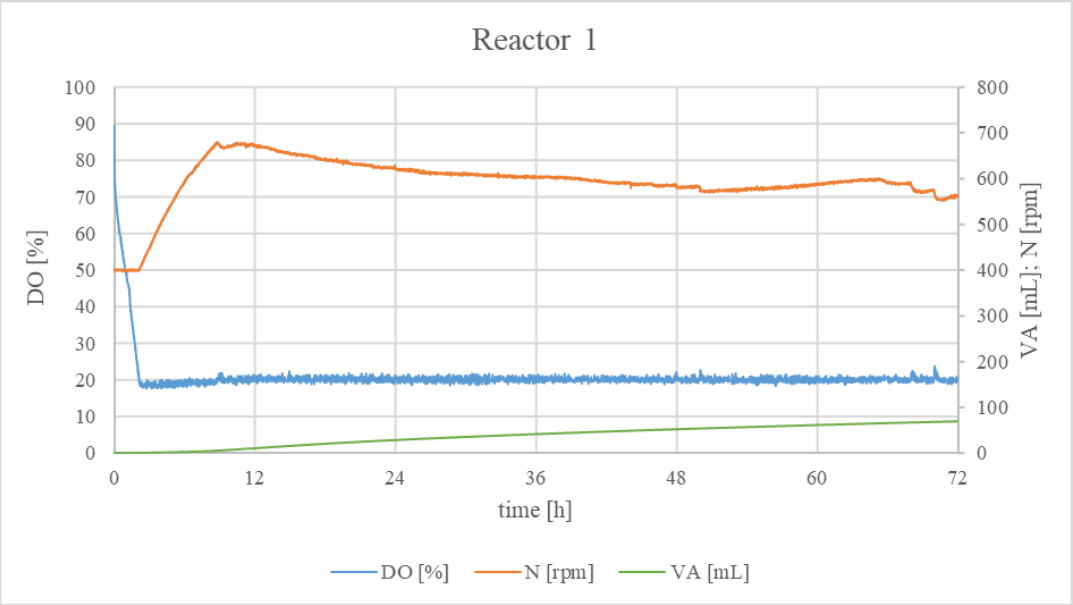

Figure 17: The course of the fed batch fermentation using butyric acid as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = butyric acid in mL

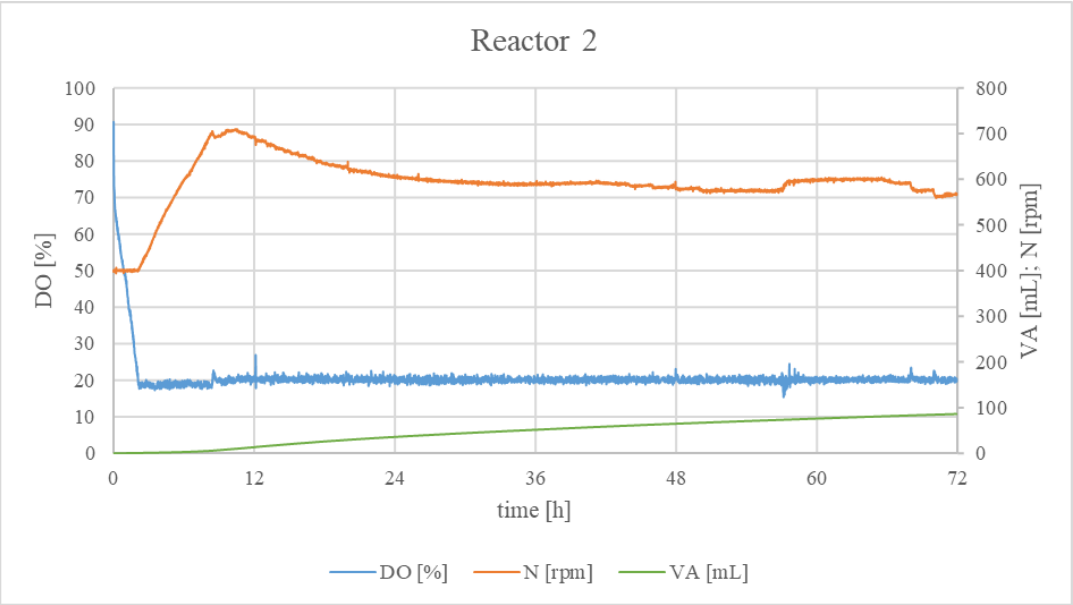

Figure 18: The course of the fed batch fermentation using butyric acid as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = butyric acid in mL

After 20 hours, a dry cell weight concentration of 43.06 g L^{-1} was reached and remained more or less constant until the end of fermentation (Table 17). The highest DCW concentration, however, was reached after 50 hours with 46.49 g L^{-1}. The highest lipid content was reached after 68 hours with a percental amount of 52.01%. At this point, the lipid content exceeded the residual cell material, which can be seen in Figure 19. The single cell oil concentration increased until hour 68, then slightly decreased.

After 20 hours, the phosphate content was limited, which probably induced SCO production.

With a butyric acid concentration of 1.09 g L^{-1} in reactor 1 and 0.84 g L^{-1} in reactor 2 at the end of the fermentation and a gradually decrease over the fermentation time, no substrate accumulation occurred.

The volumetric productivity was the highest between hour 20 and 24 with 0.54 g L^{-1} h^{-1} and decreased to 0.31 g L^{-1} h^{-1} after 72 hours. The SCO yield was also the highest after 20 hours, with 0.71 g SCO produced per g substrate and decreased to 0.23 g g^{-1} after 72 hours.

Table 17: Dry cell weight, single cell oil, fat content, SCO/substrate, SCO/carbon and volumetric SCO productivity using butyric acid as substrate (average of duplicates)

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	5.14	-	-	-	-	-
20	43.06	10.86	25.26	0.38	0.71	0.54
22	43.23	-	-	-	-	-
24	44.33	12.88	29.08	0.36	0.66	0.54
26	44.23	-	-	-	-	-
44	42.48	18.41	43.35	0.29	0.54	0.42
46	44.61	-	-	-	-	-
48	42.52	18.57	43.69	0.27	0.49	0.39
50	46.49	-	-	-	-	-
68	45.15	23.52	52.01	0.26	0.47	0.35
70	45.87	-	-	-	-	-
72	43.40	22.41	51.63	0.23	0.42	0.31

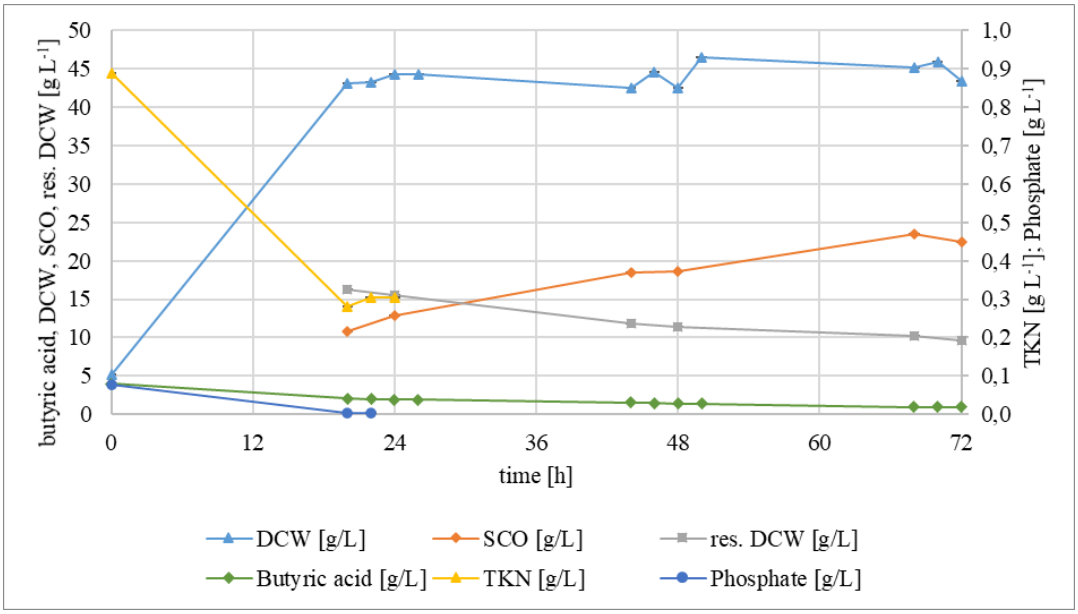

Figure 19: DCW, SCO, res. DCW, butyric acid, nitrogen and phosphor change over the time of fermentation using butyric acid as substrate (average of duplicates)

The results of FAME analysis are listed in Table 18 and visualised in Figure 20. Using butyric acid as substrate, mainly palmitic (C16:0), stearic (C18:0), oleic (C18:1) and linoleic acid (C18:20) are produced. Oleic acid (C18:1) was the most frequently produced fatty acid, with 38.5% and 45.6% after

20 and 72 hours. With a fatty acid content of 32.6% after 20 hours and a decrease to 25.1% of the total amount of fatty acids, palmitic acid was the second most common fatty acid produced. Stearic acid (18:0) had a percental amount of 15.2% after 72 hours (increasing with fermentation time) and linoleic acid had a percental amount of 8.4% after 72 hours (decreasing with fermentation time).

Table 18: Fatty acid composition in % using butyric acid as carbon source (average of duplicates)

	C16:0 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	others (<2%) [%]
20 h	32.7	8.2	38.5	13.2	7.3
24 h	32.2	8.3	41.2	11.9	6.6
44 h	28.7	11.1	45.1	9.1	6.0
48 h	27.7	11.9	45.9	8.8	5.7
68 h	25.9	14.4	45.6	8.4	5.7
72 h	25.1	15.2	45.6	8.4	5.6

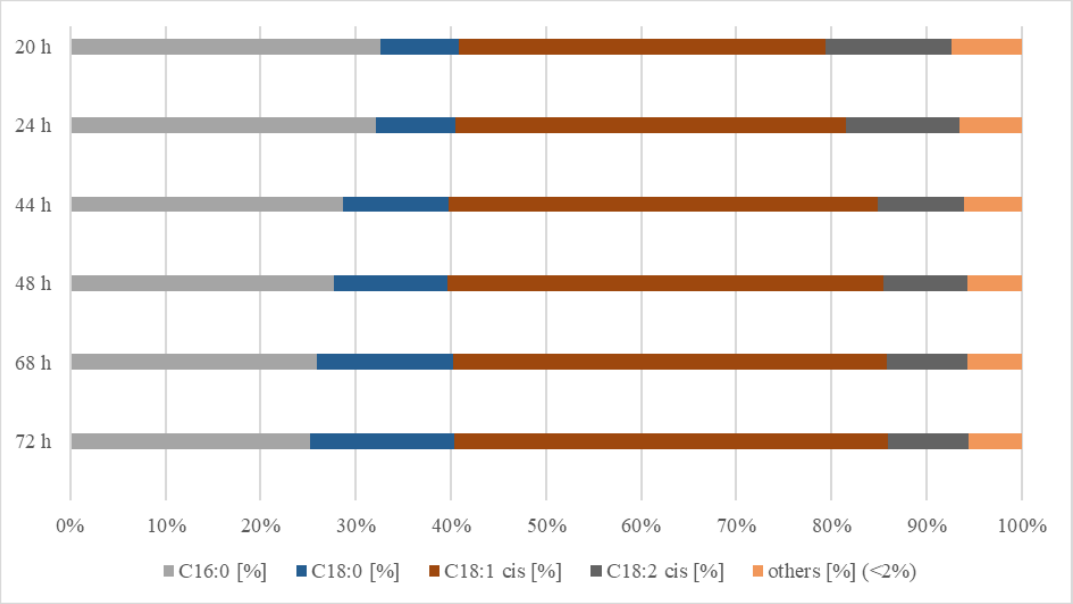

Figure 20: Fatty acid composition in % using butyric acid as carbon source (average of duplicates)

4.2.4 Valeric acid

For the two fermentations using valeric acid as feed, the substrate was used in pure form, because of the poor miscibility with water. Figure 22 shows there were problems with feed addition to the reactor between hour 10 and 20. One of the pump tubes became leaky, maybe because valeric acid was used in pure form, and caused the tube to leak. Because of this no feed was pumped out of the flask into the

reactor. This can be seen by the steep slope of the volume of pump A in the graph. For the evaluation, the amount of used feed that was recorded by the DASGIP control system but not added to the reactor was subtracted. For the results, the average of both fermentations was considered despite the problems with reactor two.

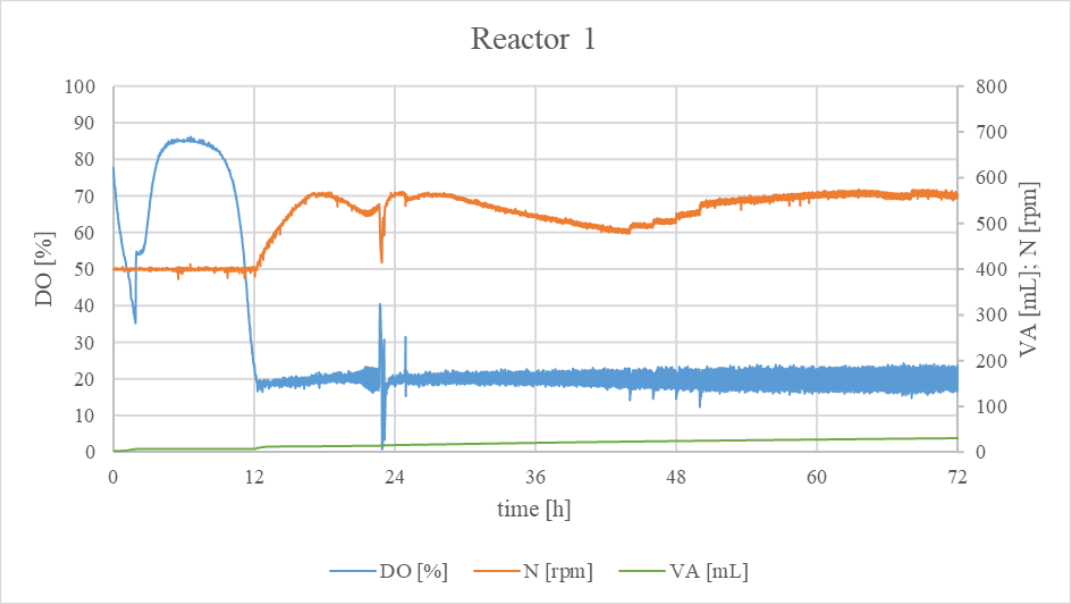

Figure 21: The course of the fed batch fermentation using valeric acid as substrate in reactor 1; DO = dissolved oxygen in, N = stirrer speed in rpm, VA (volume of pump A) = valeric acid in mL

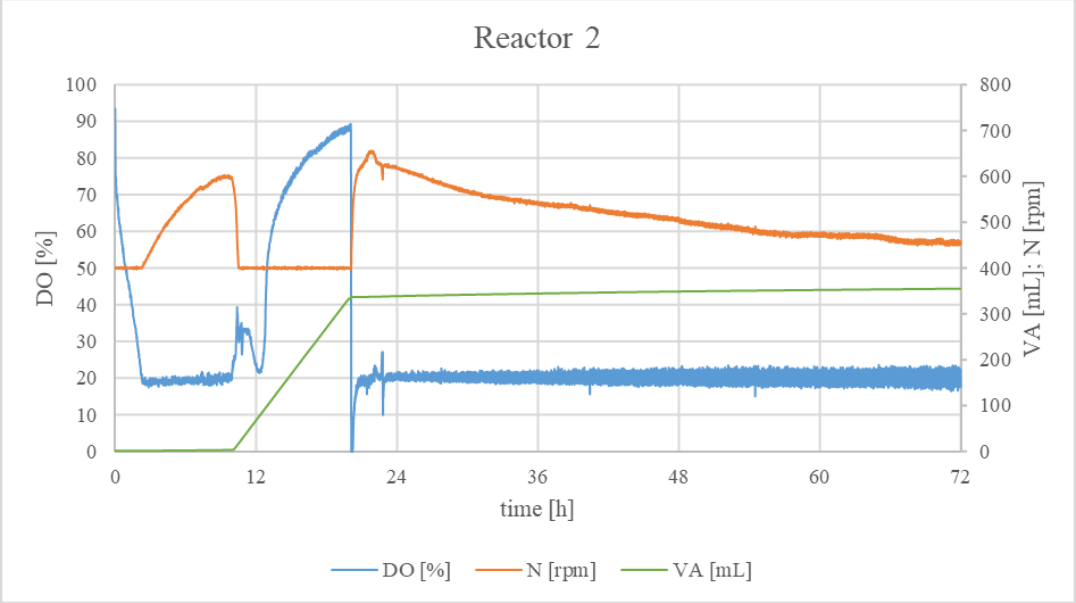

Figure 22: The course of the fed batch fermentation using valeric acid as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = valeric acid in mL

Compared to other volatile fatty acids, valeric acid was utilized in lower amounts by *A. brassicae*. Therefore, the DCW end concentration and the SCO concentration are low, reaching 23.61 g L^{-1} and

7.95 g L^{-1} respectively. This is equal to a SCO content of 33.51%. The course of the increase of DCW and SCO concentration over the whole fermentation time can be seen in Figure 23. The values are listed in Table 19. Depletion of the phosphate content was reached after 20 hours. The nitrogen concentration was more than halved after 24 hours. During the whole fermentation, the valeric acid concentration did not exceed 1 g L^{-1} in both reactors.

The volumetric SCO productivity and the ration of SCO produced per consumed substrate were increased over the fermentation time with 0.11 g L^{-1} h^{-1} and 0.14 g g^{-1} after 72 hours.

Table 19: Dry cell weight, single cell oil, fat content, SCO/substrate, SCO/carbon and volumetric SCO productivity using valeric acid as substrate (average of duplicates)

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	2.52	-	-	-	-	-
20	6.28	0.32	5.12	0.01	0.02	0.02
22	7.55	-	-	-	-	-
24	9.25	0.84	9.00	0.06	0.10	0.04
26	10.39	-	-	-	-	-
44	18.24	3.67	19.83	0.11	0.19	0.08
46	19.11	-	-	-	-	-
48	19.38	4.70	23.94	0.12	0.21	0.10
50	20.16	-	-	-	-	-
68	23.38	7.01	29.83	0.14	0.23	0.10
70	24.50	-	-	-	-	-
72	23.61	7.95	33.51	0.14	0.24	0.11

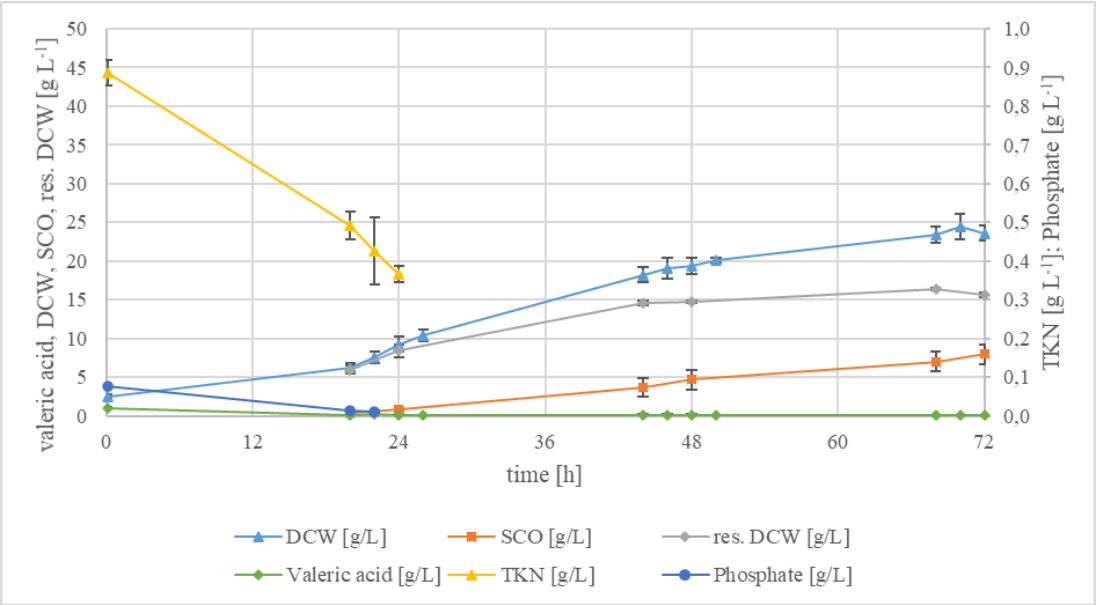

Figure 23: DCW, SCO, res. DCW, valeric acid, nitrogen and phosphor change over the time of fermentation using valeric acid as substrate (average of duplicates)

The composition of the single cell oil consisted of pentadecanoic (C15:0), palmitic (C16:0), margaric (C17:0), heptadecenoic acid (C17:1), stearic (C18:0), oleic (C18:1) and linoleic acid (C18:2) and varied over the fermentation time (see Table 20 and Figure 24). The most frequent fatty acid was margaric acid (C17:0), with 10.6% at the beginning, increasing to 38.1 % after 72 hours. The highest percental amount was after 44 hours with 42.7%. Oleic acid (C18:1) made 19.0% of the total amount of single cell oil after 20 hours, decreased to 15.6% after 44 hours and increased again to 24.3% after 72 hours and is therefore the second most common fatty acid produced. Similar to C17:0, C17:1 increased in the first 44 hours and then decreased to 18.3% after 72 hours. The linoleic acid content decreased dramatically in the first 44 hours, with 40.3% after 20 hours and 7.3% after 44 hours. The end content was 5.6% after 72 hours. The residual percental amounts were pentadecanoic acid, palmitic acid and stearic acid with 2.3%, 7.6% and 3.7% after 72 hours.

Table 20: Fatty acid composition in % using valeric acid as carbon source (average of duplicates)

	C15:0 [%]	C16:0 [%]	C17:0 [%]	C17:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]
20 h	4.8	11.6	10.6	10.0	3.6	19.0	40.3
24 h	4.1	7.6	29.7	17.3	2.62	17.5	21.4
44 h	3.4	5.5	42.7	23.4	2.0	15.6	7.4
48 h	3.1	6.0	42.0	22.3	2.4	17.7	6.5
68 h	2.4	7.5	38.4	19.0	3.7	23.2	5.7
72 h	2.3	7.6	38.1	18.3	3.7	24.3	5.6

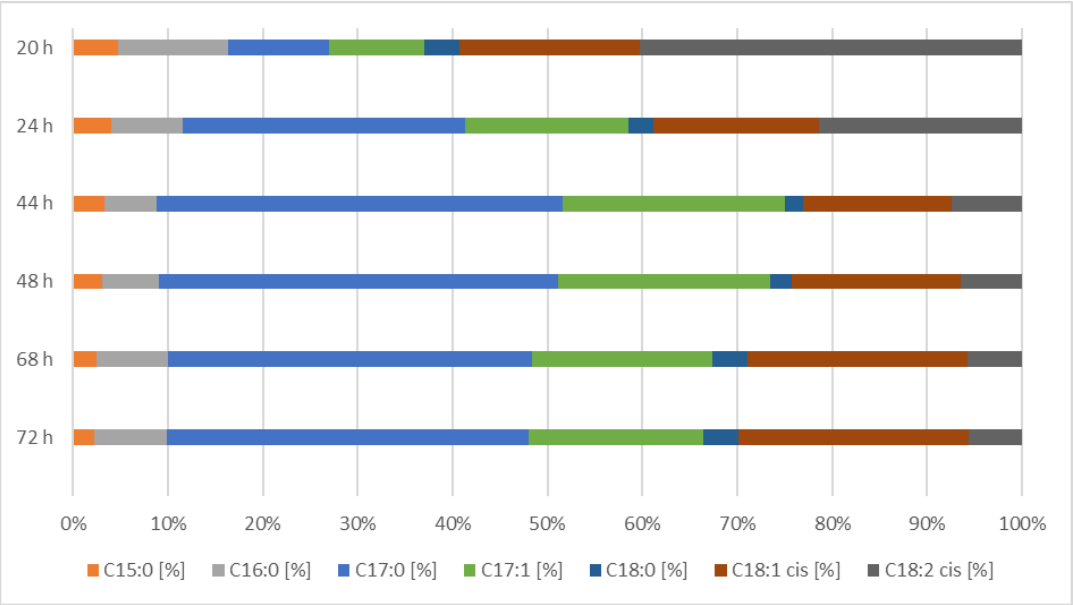

Figure 24: Fatty acid composition in % using valeric acid as carbon source (average of duplicates)

4.3 SCO production using lactic acid as feed

The lactic acid feed concentration was 368.13 g L^{-1} and all fermentation parameters, like DO, temperature and pH, were the same as the fermentations using VFA as carbon source. In Figure 25 and Figure 26 the courses of the two fermentations are illustrated. Lactic acid was utilized badly and the oxygen consumption was very low. Because of this the stirrer speed did not increase from the minimum stirrer speed at all. At the beginning, the DO concentration decreased to 20 % and the yeast started to adapt to the new cultivation conditions. Till hour 12 and 15, the DO remained at 20%, although no increased stirrer speed was required, and then started to rise. This behaviour is maybe caused by the present YE, peptone and maybe residual glucose residues in the starting phase. These substances are utilized by the yeast and as soon as only lactic acid is available, which is only poorly metabolised, the DO level increases.

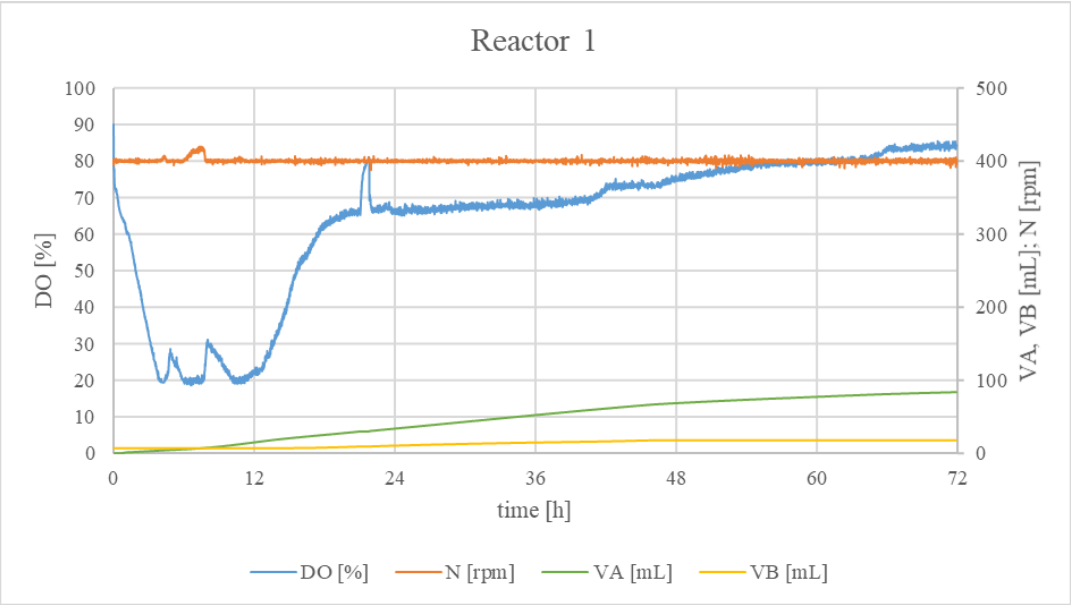

Figure 25: The course of the fed batch fermentation using lactic acid as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = lactic acid in mL, VB (volume of pump B) = NaOH consumption in mL

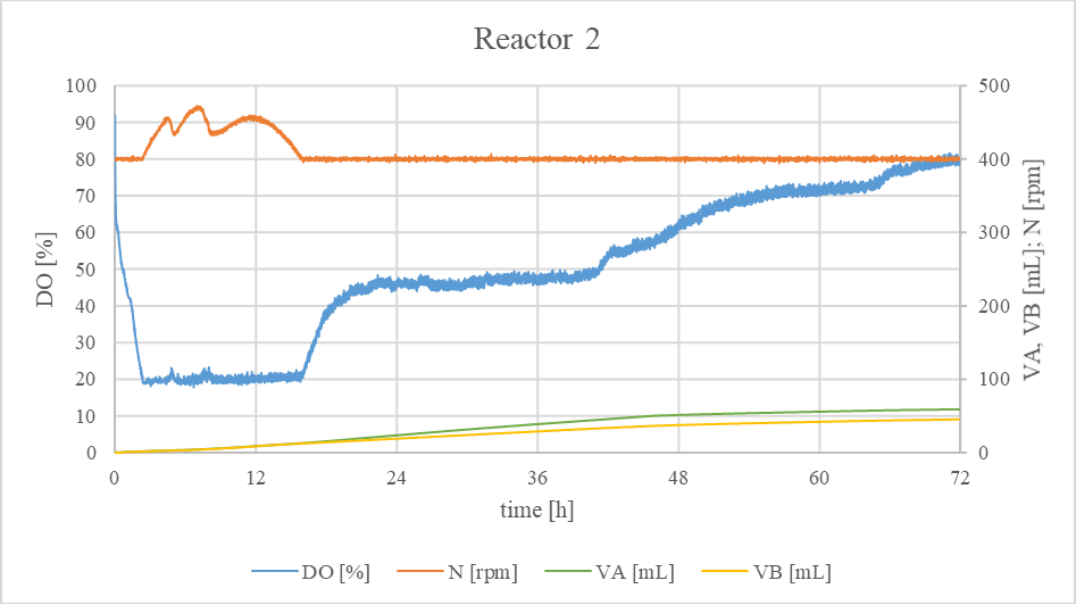

Figure 26: The course of the fed batch fermentation using lactic acid as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = lactic acid in mL, VB (volume of pump B) = NaOH consumption in mL

In

Table 21 the DCW and SCO concentrations over the fermentation time are listed. The end concentrations after 72 hours of both are 13.76 g L^{-1} and 2.80 g L^{-1} respectively, which is equal to a lipid content of 20.40%. Although *A. brassicae* metabolised lactic acid very slowly, there is a nearly linear increase of cell material as well as single cell oil, which can be seen in Figure 27. The volumetric single cell oil productivity is increased from 0.02 g L^{-1} h^{-1} after 20 hours to 0.04 g L^{-1} h^{-1} and the maximum amount of

SCO produced per g substrate was 0.09g. In both reactors, the lactic acid concentration varied between approximately 1 and 2 g L^{-1}. At the end of fermentation, the substrate concentration in reactor 1 and 2 was 1.82 and 1.92 g L^{-1}, showing that no substrate accumulation occurred.

Table 21: DCW concentration, SCO concentration, Lipid content, SCO/substrate, SCO/carbon and volumetric SCO productivity using lactic acid as substrate (average of duplicates)

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g $^{-1}$L h^{-1}]
0	5.00	-	-	-	-	-
20	6.01	0.43	7.11	0.05	0.12	0.02
22	5.69	-	-	-	-	-
24	7.25	0.57	7.83	0.05	0.13	0.02
26	6.91	-	-	-	-	-
44	9.53	1.48	15.58	0.07	0.17	0.03
46	9.40	-	-	-	-	-
48	9.81	1.63	16.59	0.07	0.17	0.03
50	9.69	-	-	-	-	-
68	12.94	2.67	20.67	0.09	0.23	0.04
70	13.00	-	-	-	-	-
72	13.76	2.80	20.40	0.09	0.22	0.04

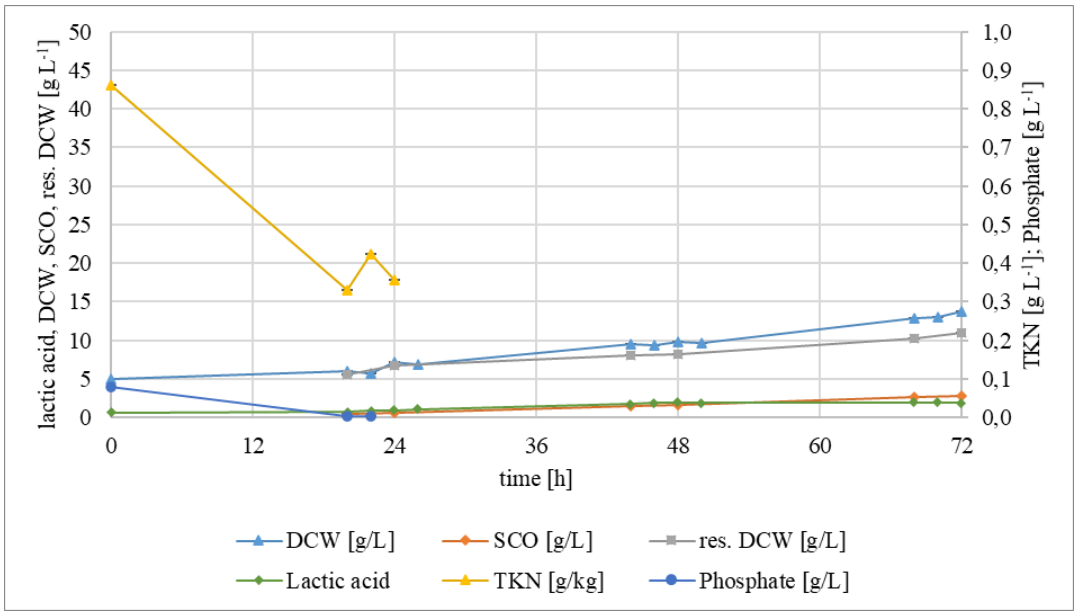

Figure 27: DCW, SCO, res. DCW, lactic acid, nitrogen and phosphor change over the time of fermentation using lactic acid as substrate (average of duplicates)

Table 22 shows the percental amounts of fatty acids with a content above two percent, other fatty acids are summed up in “others”. During the fermentation, oleic acid (18:1 cis) is produced in highest amount with up to 54.4% after 44 hours and 54.0% after 72 hours. The second most frequent fatty acid is palmitic acid (C16:0) with 20.2% after 72 hours followed by linoleic acid (C18:2) with 14.8%. After 20 hours the linoleic acid content was 37.2%. The percental amount of stearic acid (C18:0) only increased slightly with 7.8% after 72 hours. Generally, after 44 hours the composition only had minor changes. Between hour 24 and 44 there is a strong increase in C18:1 and a strong decrease in C18:2. In Figure 28, these results are visualized.

Table 22: Fatty acid composition in % using lactic acid as carbon source (average of duplicates)

	C16:0 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	others (<2%) [%]
20 h	15.4	6.0	36.5	37.2	4.9
24 h	15.4	6.1	39.6	34.5	4.4
44 h	17.8	6.9	53.9	17.9	3.5
48 h	18.2	7.0	54.4	16.9	3.4
68 h	19.9	7.7	54.1	15.0	3.3
72 h	20.2	7.8	54.0	14.8	3.1

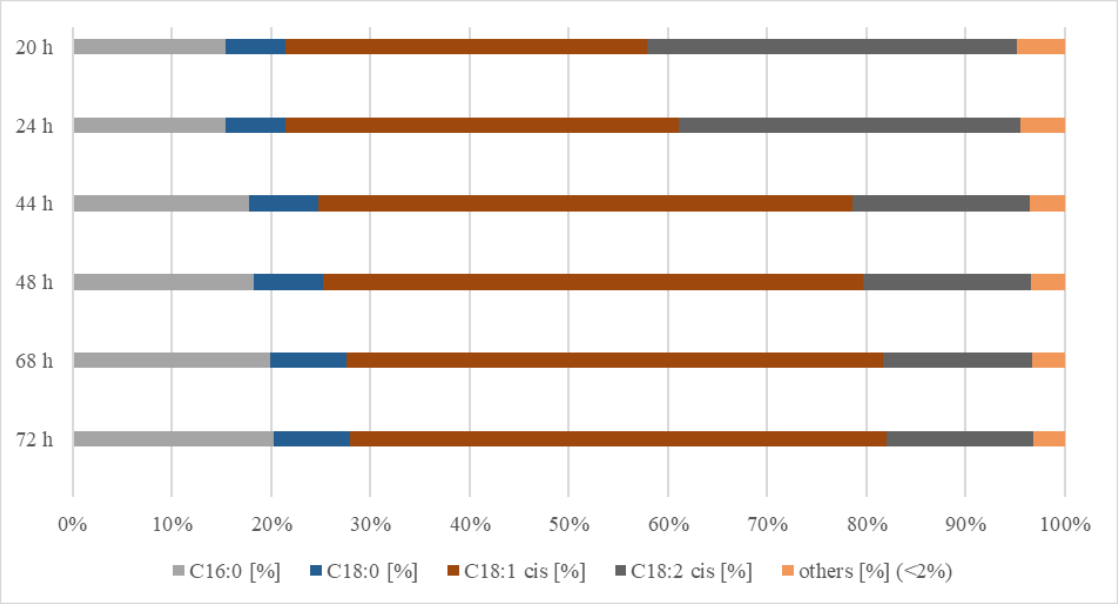

Figure 28: Fatty acid composition in % using lactic acid as carbon source (average of duplicates)

4.4 SCO production using sugar as carbon source

4.4.1 Glucose

Two batch fermentation with glucose as substrate were performed with a substrate concentration of 76.81 g L^{-1} in each reactor. Because of the fast glucose consumption of *A. brassicae*, a high stirrer speed was necessary to provide enough oxygen. Due to the high stirrer speed and because the addition of 1 mL antifoam was not sufficient, a lot of foam was formed between the first sampling point at the start of the fermentation and the second after 20 hours. Because of this, a lot of fermentation broth was pressed though the off-gas tube out of the reactor. To reduce foaming in the reactors 2 mL of antifoam and 1 mL of water for rinsing were added to each reactor via the sampling valve. This led to a drop of the measured DO level and an increased stirrer speed in both reactors, which can be seen in Figure 29 and Figure 30. Although the volume of the fermentation broth was significantly reduced, the fermentation was continued because of the batch modus the concentrations of all substances should remain the same. The reduced volume is not expected to have a great impact on the outcome of the fermentations, but nevertheless the results have to be interpreted with caution. After 28 hours the stirrer speed reached the minimum of 400 rpm and the DO level increased. This indicates that all substrate was consumed. The excessive foam formation probably led to measuring errors of the DO values and is therefore the reason for the stirrer speed variations within the first day.

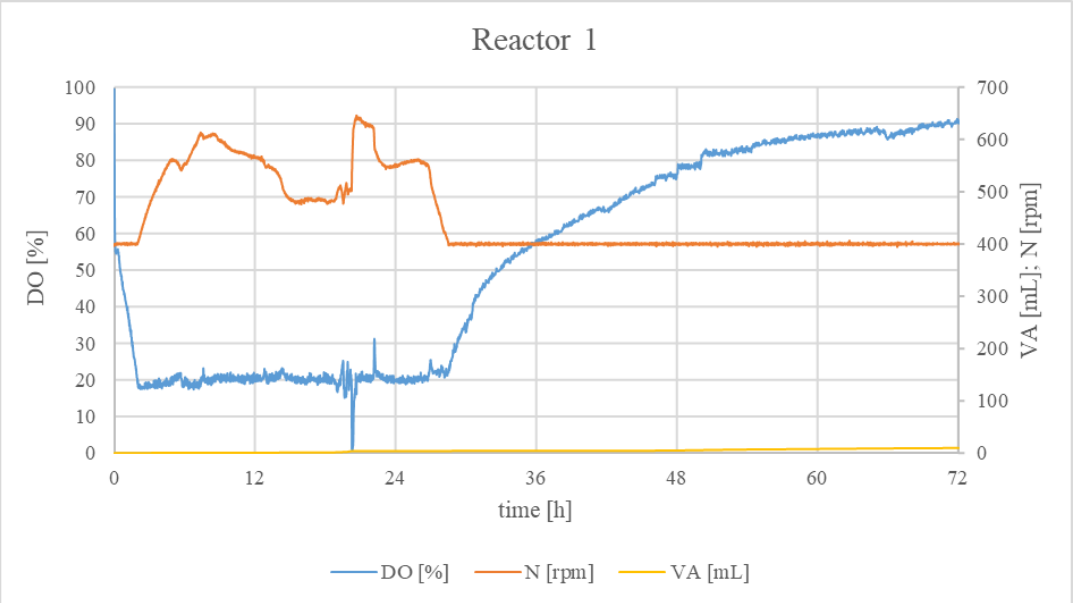

Figure 29: The course of the batch fermentation using glucose as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = H_2SO_4 consumption in mL

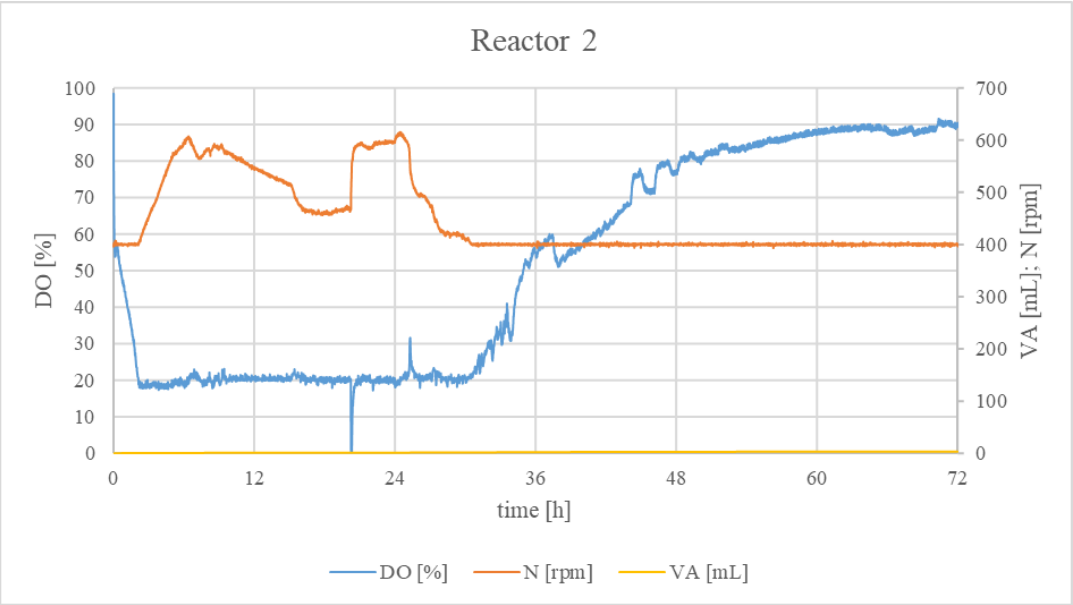

Figure 30: The course of the batch fermentation using glucose as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = H_2SO_4 consumption in mL

The average of both final DCW concentration of *A. brassicae* growing on glucose is 43.40 g L^{-1}. This is nearly the same concentration as already after 20 hours with 43.06 g L^{-1}. Between these two sampling points, the concentration changed only slightly with the highest value after 50 hours with 46.49 g L^{-1} (see Figure 31). In contrast to that, the single cell oil concentration increased over the fermentation time, reaching a maximum of 14.63 g L^{-1} after 68h. The highest fat content was 32.30% after 72 hours. The amount of produced SCO per g utilized substrate was 0.18 g after 72 hours and increased with time. The volumetric SCO productivity decreased with time, with 0.43 g L^{-1} h^{-1} after 20 hours and 0.27 g L^{-1} h^{-1} after 72 hours (shown in Table 23).

The nitrogen and phosphate concentration reduction were similar to the previous performed fermentations.

Table 23: DCW concentration, SCO concentration, lipid content, SCO/substrate, SCO/carbon and volumetric SCO productivity using glucose as substrate (average of duplicates)

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	5.14	-	-	-	-	-
20	43.06	8.58	19.92	0.13	0.31	0.43
22	43.23	-	-	-	-	-
24	44.33	10.03	22.63	0.13	0.31	0.42
26	44.23	-	-	-	-	-
44	42.48	12.80	30.15	0.16	0.40	0.29
46	44.61	-	-	-	-	-
48	42.52	12.83	30.20	0.16	0.40	0.27
50	46.49	-	-	-	-	-
68	45.15	14.63	32.30	0.18	0.45	0.22
70	45.87	-	-	-	-	-
72	43.40	14.07	32.44	0.18	0.44	0.20

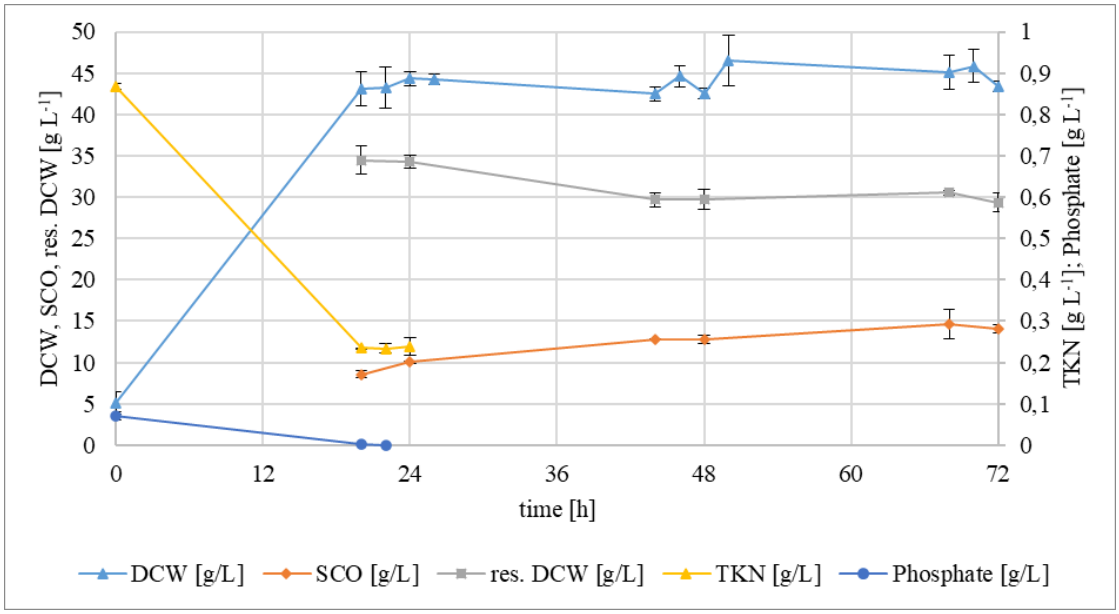

Figure 31: DCW, SCO, res. DCW, nitrogen and phosphor change over the time of fermentation using glucose as substrate (average of duplicates)

The glucose concentration rapidly decreased in both reactors, with almost no substrate left after 26 hours of fermentation (0.05 g L^{-1} in reactor 1 and 0.15 g L^{-1} in reactor 2). The course of the substrate concentration is shown in Figure 32.

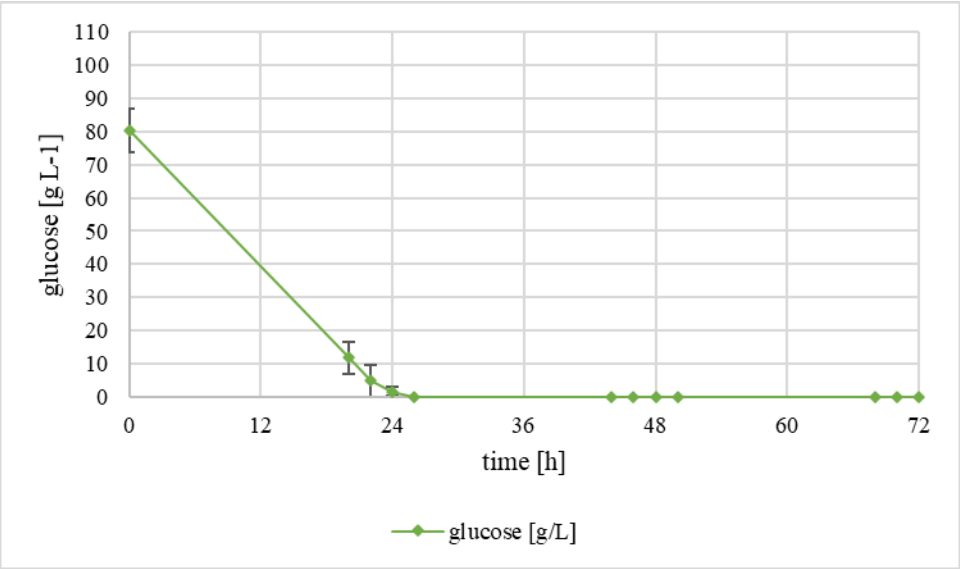

Figure 32: Glucose concentration change over the time of fermentation using glucose as carbon source (average of duplicates)

Fatty acid composition did not change much over time when glucose was used as carbon source for SCO production. Oleic acid (C18:1) was the most common fatty acid with 48.4%, followed by palmitic acid (C16:0), stearic acid (C18:0) and linoleic acid (C18.2) with 20.8%, 15.2% and 12.1% after 72 hours (shown in Table 24 and Figure 33).

Table 24: Fatty acid composition in % using glucose as carbon source (average of duplicates)

	C16:0 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	others (<2%) [%]
20 h	21.3	14.5	49.2	11.6	340
24 h	21.1	15.1	50.0	10.6	3.3
44 h	20.9	15.2	49.2	11.2	3.4
48 h	20.9	15.1	49.4	11.3	3.3
68 h	20.8	15.2	48.7	11.9	3.4
72 h	20.8	15.2	48.4	12.1	3.5

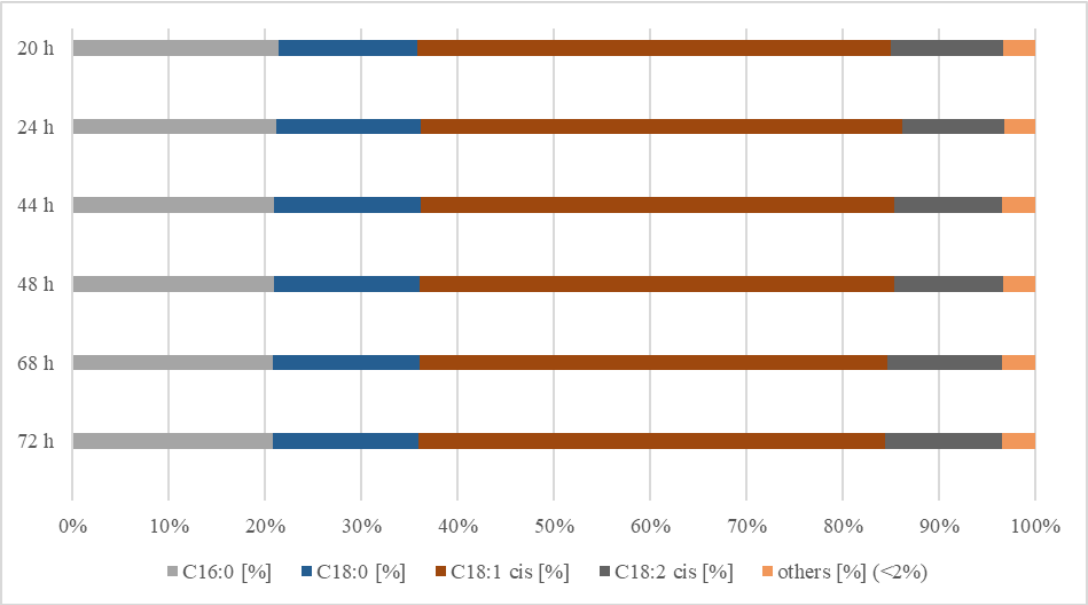

Figure 33: Fatty acid composition in % using glucose as carbon source (average of duplicates)

4.4.2 Xylose

The two batch fermentations with xylose had a substrate concentration of 91.81 g L^{-1}. Similar to glucose, the utilization of xylose lead to the formation of foam that could not be prevented by 1 mL antifoam at the start of the fermentation. In contrast to glucose, excessive foam formation occurred later, and fermentation broth loss was prevented by addition of antifoam to each reactor. This led to a drop in the measured DO values, which can be seen in Figure 34 after 26 and 44 hours (1mL antifoam added respectively) and in Figure 35 after 26 hours (2 mL antifoam added). It has to be mentioned that the formation of foam distorted the measurement of the DO values, which led to an unusual DO curve. Nevertheless, at the beginning of the fermentation, the DO values decreased to nearly 20%. In this phase the yeast probably started to adapt to the use of xylose as carbon source. The low stirrer speed over the whole time of fermentation was possibly caused by the incorrect measurement of the DO values caused by foam formation, but even after the addition of antifoam and a DO levels of 20%, the minimum stirrer speed was sufficient which indicates the poor xylose utilization ability of *A. brassicae*.

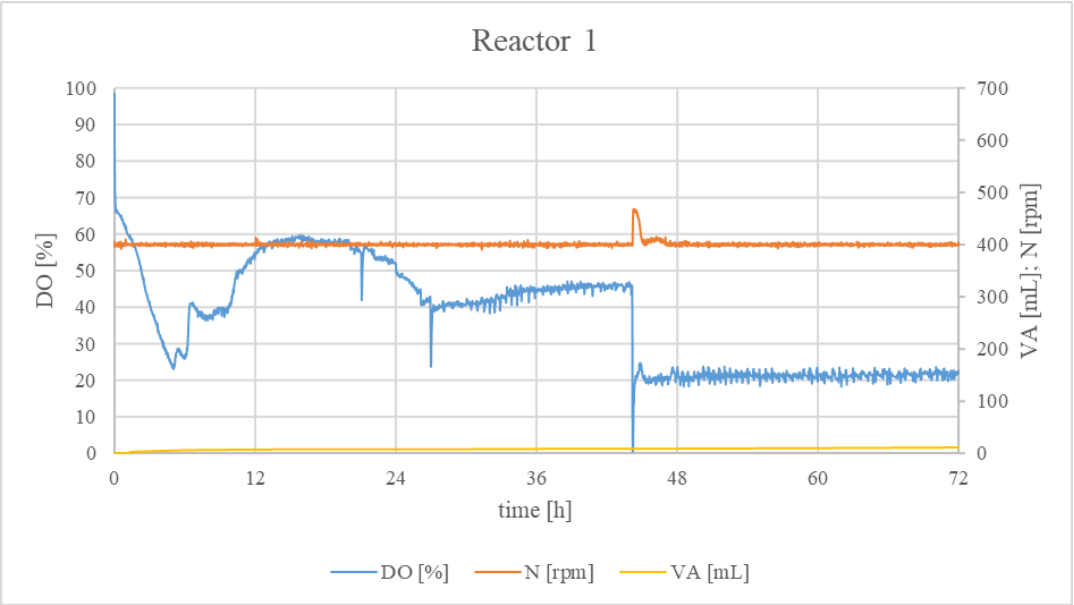

Figure 34: The course of the batch fermentation using xylose as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = H_2SO_4 in mL,

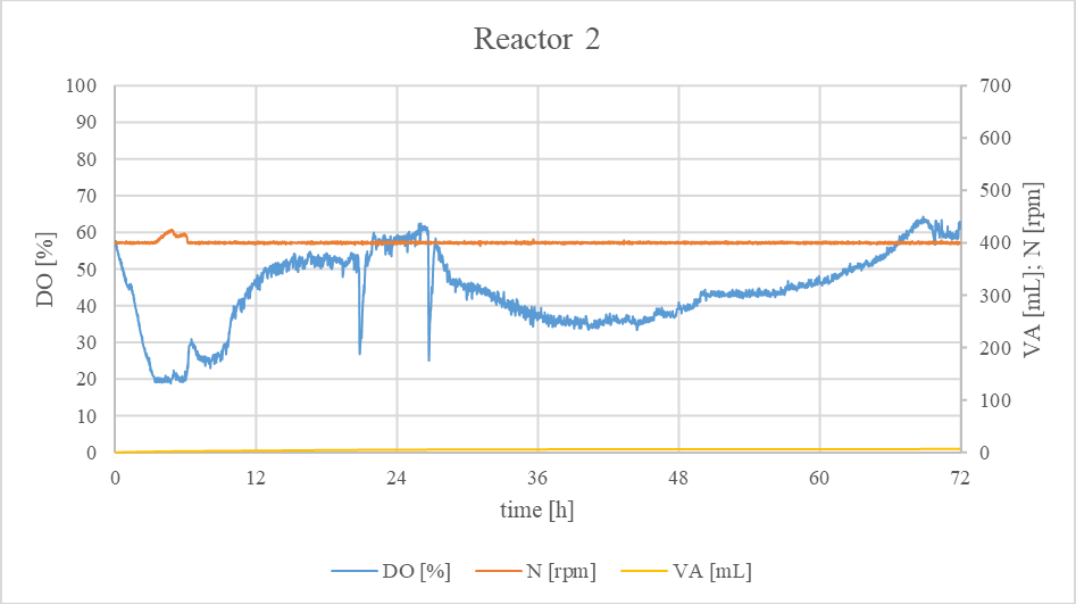

Figure 35: The course of the fed batch fermentation using xylose as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) H_2SO_4 consumption in mL

Table 25 and Figure 36 show the course of fermentation using xylose as substrate. The biomass increased over the whole fermentation time, with some fluctuations between hour 0 and hour 26. After 72 hours a DCW concentration of 13.76 g L^{-1} was reached with a lipid content of 8.75%. Phosphate was depleted after 72 h, while the nitrogen concentration was 0.25 g L^{-1}. This indicates that SCO production was induced by phosphate limitation. Volumetric SCO productivity was low with fluctuations between 0.01 and 0.02 g L^{-1} h^{-1}. The yield was reduced from 0.04 after 44 hours to 0.01 after 72 hours. The negative yields after 20 and 24 hours were probably caused by errors during determination of substrate

concentration at the respective time point. Since the determined substrate concentrations increases, the SCO yield is negative for the first two sampling points.

Table 25: Dry cell weight concentration, single cell oil concentration, lipid content, SCO/substrate, SCO/carbon and volumetric SCO productivity using xylose as substrate (average of duplicates)

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	volumetric SCO productivity [g L^{-1} h^{-1}]
0	5.00	-	-	-	-	-
20	6.01	0.31	5.11	-0.03	-0.09	0.02
22	5.69	-	-	-	-	-
24	7.25	0.31	4.30	-0.20	-0.50	0.01
26	6.91	-	-	-	-	-
44	9.53	0.57	6.01	0.04	0.10	0.01
46	9.40	-	-	-	-	-
48	9.81	0.64	6.47	0.03	0.07	0.01
50	9.69	-	-	-	-	-
68	12.94	1.09	8.40	0.02	0.05	0.02
70	13.00	-	-	-	-	-
72	13.76	1.21	8.75	0.01	0.03	0.02

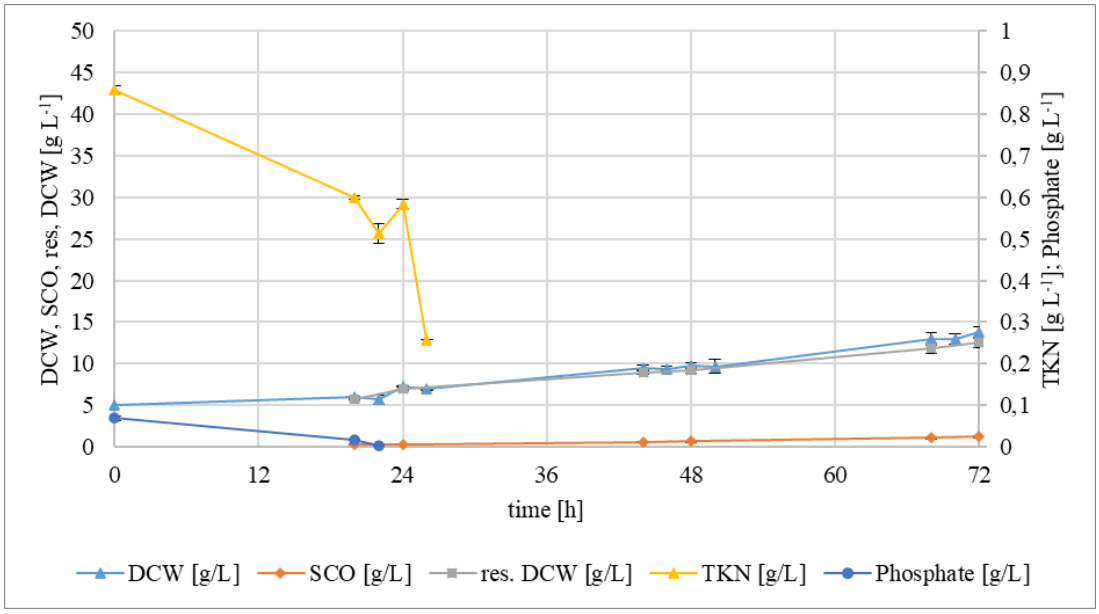

Figure 36: DCW, SCO, res. DCW, nitrogen and phosphor change over the time of fermentation using xylose as substrate (average of duplicates)

The xylose concentration in both reactors, shown in Figure 37, gradually decreased over the time of fermentation, with 17.80 g L^{-1} left in reactor 1 and 20.00 g L^{-1} in reactor 2.

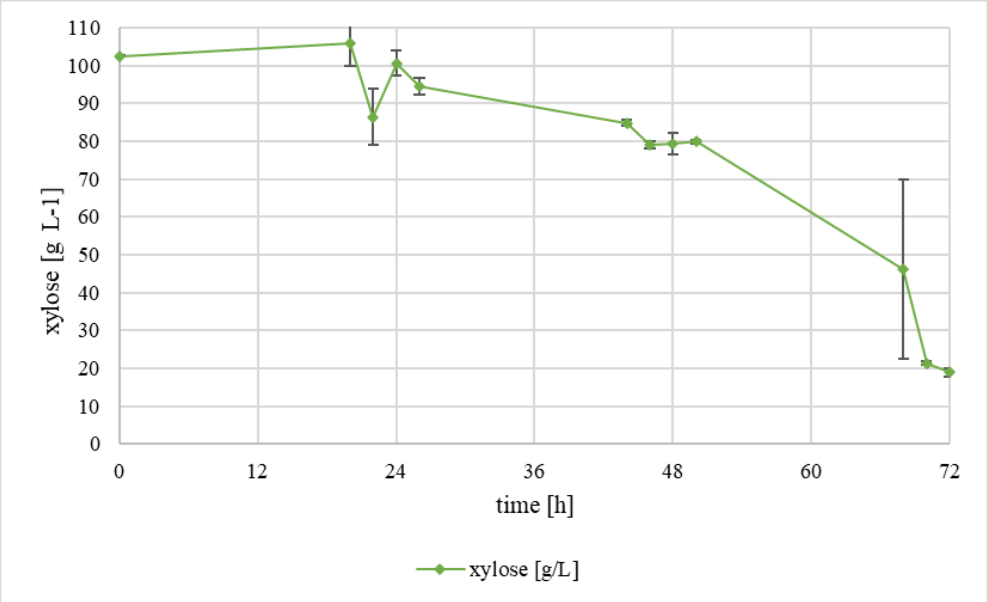

Figure 37: Xylose concentration change over the time of fermentation using xylose as carbon source (average of duplicates)

Using xylose as substrate, *A. brassicae* was able to produce only low amounts of SCO, which consists of C16:0, C18:0, C18:1 cis, C18:2 cis and C24:0 fatty acids. Oleic acid (C18:1) and linoleic acid (C18:2) were the most common fatty acid, with 36.1% (increasing percental amount with time) and 35.9% (decreasing percental amount with time) of the total lipids. With 14.8%, 9.9% and 3.2% of the total amount of fatty acids, palmitic acid, stearic acid and lignoceric acid were also found. The composition changes over the fermentation time can be seen in Table 26 and are visualized in Figure 38.

Table 26: Fatty acid composition in % using xylose as carbon source (average of duplicates)

	C16:0 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	C24:0 [%]
20 h	17.6	5.4	33.8	41.0	2.2
24 h	16.8	5.1	32.5	43.3	2.4
44 h	15.6	8.1	30.5	43.1	2.7
48 h	15.3	9.0	31.5	41.1	3.1
68 h	14.7	9.5	35.7	36.7	3.4
72 h	14.8	9.9	36.1	35.9	3.2

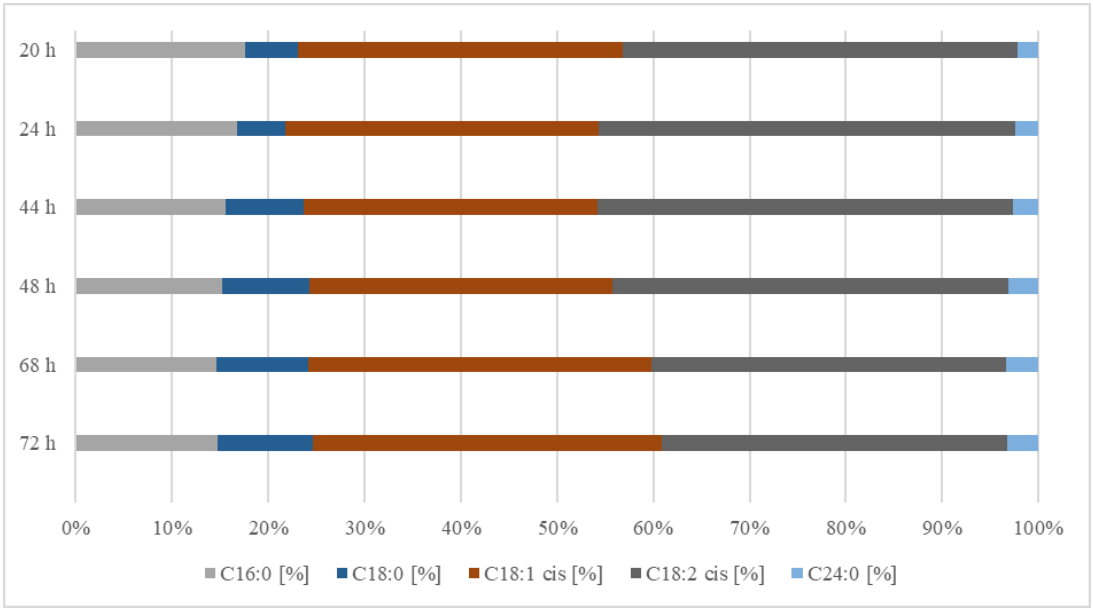

Figure 38: Fatty acid composition in % using xylose as carbon source (average of duplicates)

4.5 SCO production using glycerol as feed

4.5.1 Feed rate 0.625 mL h^{-1}

For the first two fed batch fermentations with glycerol, a linear feed with 0.625 mL h^{-1} was chosen. A glycerol feed with a concentration of 500 g L^{-1} was prepared, but because there are inaccuracies when weighing in the carbon source and because the exact concentration of the prepared feed was not determined, the calculations were performed using the 500 g L^{-1}. This has to be considered when interpreting the evaluated results. In Figure 39 and Figure 40 (the course of the fermentation), it can be seen, that after 44 hours the base consumption (VB) is increased in both diagrams. This was caused, because the tubes of the base flask were disconnected overnight and the pump tried to compensate the drop of the pH, but no base was pumped into the reactors. After 44 and 68 hours, 1 mL of antifoam was added to both reactors, which lead to an abrupt drop of the DO level. At the beginning, the DO level dropped to 20%, caused by metabolising residual YE, peptone and maybe glucose from the inoculum. After that, the DO level increased rapidly and decreased towards the end of fermentation. The stirrer speed was always on the minimum rpm, which can be traced back to a low glycerol utilization ability of *A. brassicae*.

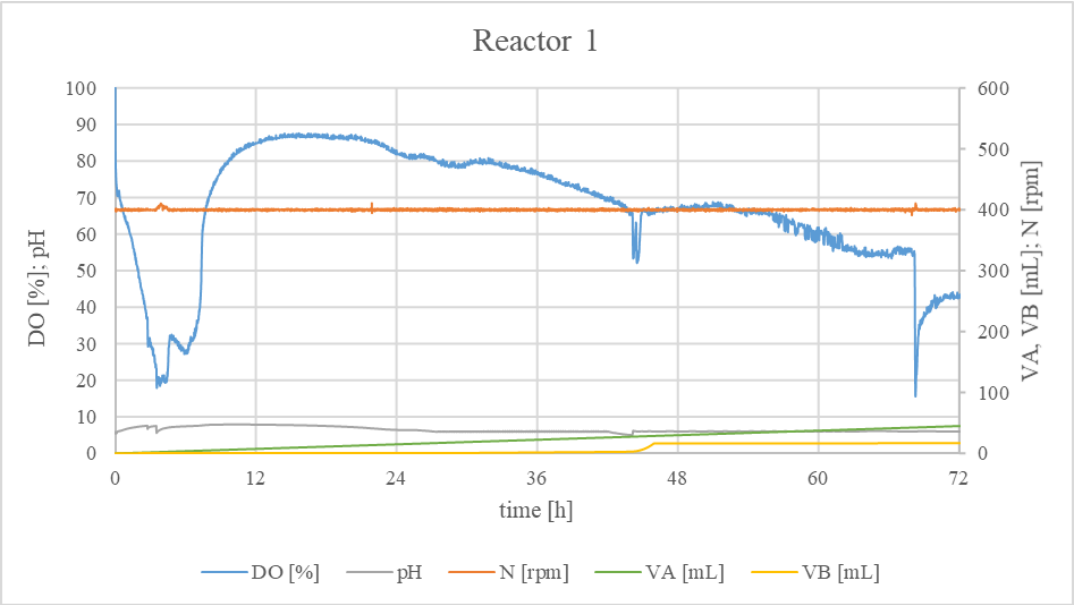

Figure 39: The course of the fed batch fermentation with a feed rate of 0.625 mL h^{-1} using glycerol as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = glycerol in mL, VB (volume of pump B) = NaOH consumption in mL

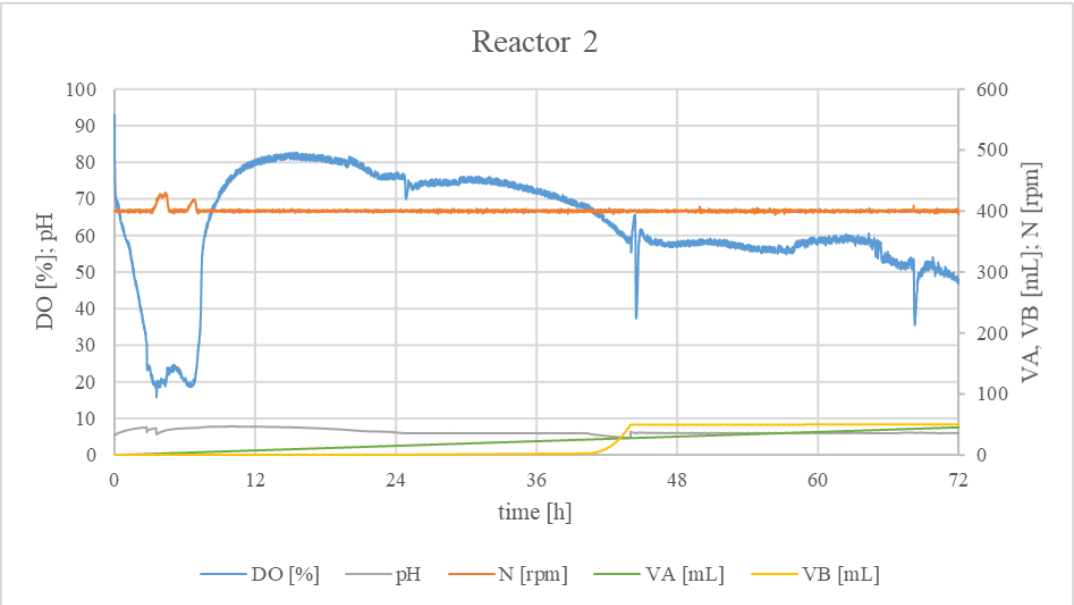

Figure 40: The course of the fed batch fermentation with a feed rate of 0.625mL h^{-1} using glycerol as substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = xylose in mL, VB (volume of pump B) = NaOH consumption in mL

Table 27 gives an overview of the DCW and SCO concentration over the fermentation time. After 72 hours the DCW concentration was 19.91 g L^{-1} with a fat content of 13.08%. The low increase of lipid content over time is also visualized in Figure 41. The SCO volumetric productivity and the ratio of SCO produced per substrate are increasing with the time of fermentation and are 0.04 g L^{-1} and 0.05 g g^{-1} after 72 hours. In both reactors, glycerol accumulated over the time of fermentation, with an end concentration of 22.45 and 18.87 g L^{-1} in reactor 1 and 2.

Table 27: Dry cell weight, single cell oil and lipid content SCO/substrate, SCO/Carbon and volumetric SCO productivity using glycerol as carbon source with a feed rate of 0.625 mL h^{-1}

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	2.04	-	-	-	-	-
20	5.64	0.26	4.64	0.02	0.06	0.01
22	5.90	-	-	-	-	-
24	5.97	0.27	4.50	0.02	0.05	0.01
26	3.10	-	-	-	-	-
44	10.20	0.63	6.13	0.02	0.06	0.01
46	10.49	-	-	-	-	-
48	12.80	0.93	7.27	0.03	0.08	0.02
50	12.41	-	-	-	-	-
68	18.89	2.23	11.82	0.05	0.13	0.03
70	19.15	-	-	-	-	-
72	19.91	2.61	13.08	0.05	0.14	0.04

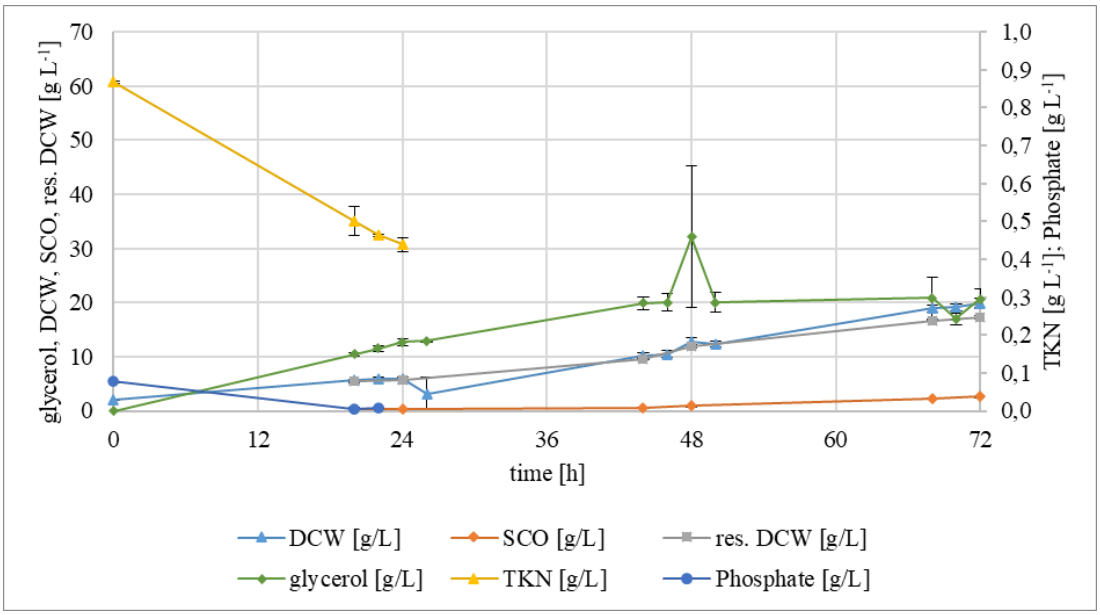

Figure 41: DCW, SCO, res. DCW, glycerol, nitrogen and phosphorous change over the time of fermentation using glycerol with a feed rate of 0.625 mLh^{-1} as substrate (average of duplicates)*

With 26.8% after 20 hours and with 44.7% after 72 hours, oleic acid (C18:1) was the most common fatty acid in the SCO produced from glycerol. The linoleic acid content decreased from 49.9% after 20 hours to 22.5% after 72 hours. The next most common fatty acids were palmitic acid (C16:0) (increase

of percental amount), stearic acid (C18:0), eicosadienoic acid (C20:2) and palmitoleic acid (C16:0) with 22.1%, 7.2%, 1.9% and 1.5% at the end of the fermentation.

Table 28: Fatty acid composition in % using glycerol as carbon source with a federate 0f 0.625 mL h^{-1} (average of duplicates)

	C16:0 [%]	C16:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	C20:2 [%]
20 h	15.0	1.4	4.5	26.8	49.9	2.4
24 h	14.5	1.5	4.2	25.6	52.1	2.0
44 h	17.5	1.2	6.1	33.0	40.0	2.3
48 h	19.1	0.9	7.5	36.6	33.8	2.0
68 h	21.7	1.5	6.9	44.1	23.9	1.8
72 h	22.1	1.5	7.2	44.7	22.5	1.9

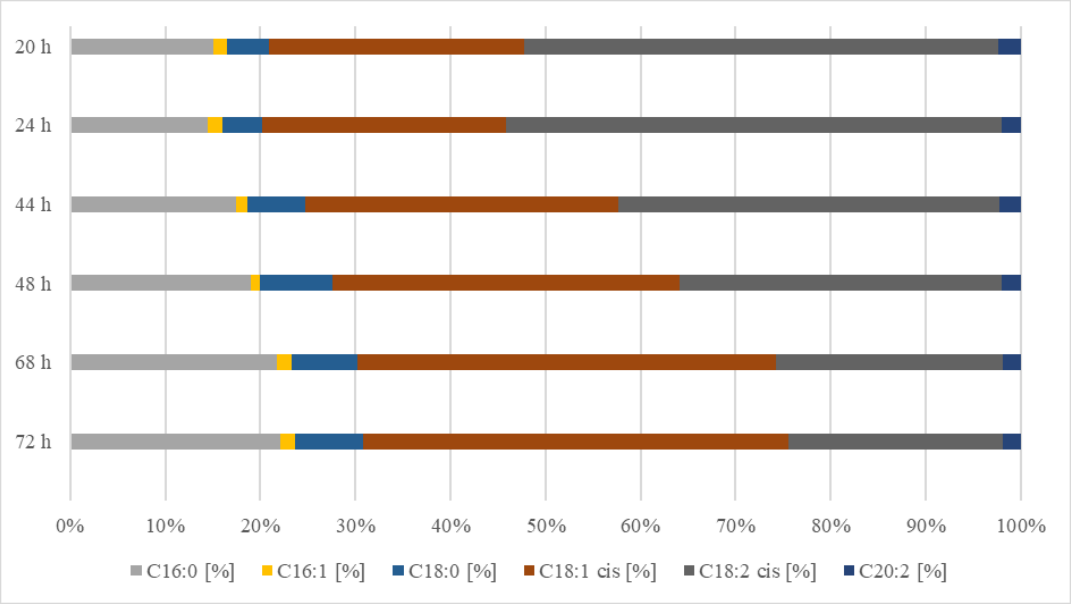

Figure 42: Fatty acid composition in % using glycerol as carbon source with a feed rate of 0.625 mL h^{-1} (average of duplicates)

4.5.2 Feed rate 1.25 mL h^{-1}

The two glycerol fed batch fermentations with a feed rate of 1.25 mL h^{-1} were performed in parallel to the two fermentations with a lower feed rate and were very similar regarding the course of fermentation. At hour 36, the connection to the base was detached and at hour 44 and 68, 1 mL antifoam was added to the reactors. Both events can be observed on the basis of the pH and VB curve and the DO curve in Figure 43 and Figure 44.

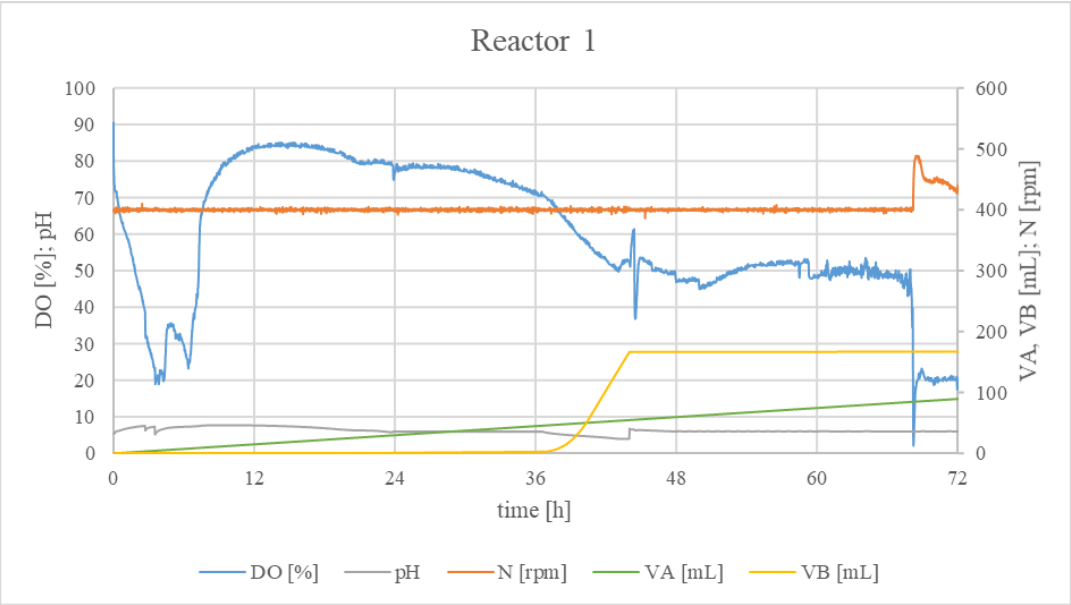

Figure 43: The course of the fed batch fermentation with a feed rate of 1.25 mL h^{-1} using glycerol as substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = glycerol in mL, VB (volume of pump B) = NaOH consumption in mL

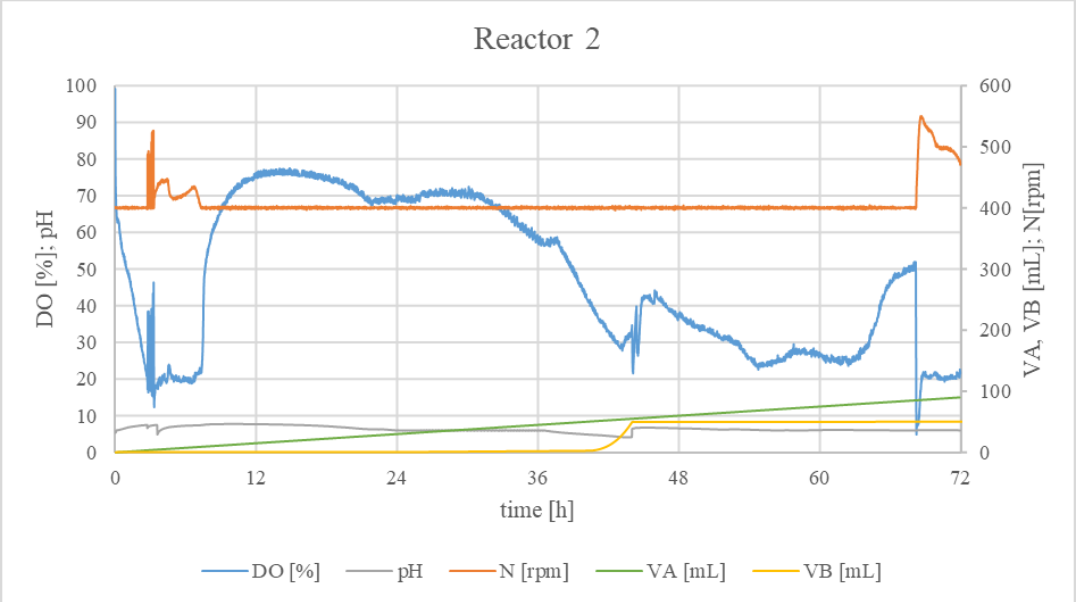

Figure 44: The course of the fed batch fermentation with a feed rate of 1.25 mL h^{-1} using glycerol as substrate in reactor 2; DO = dissolved oxygen in %, T = temperature in °C, N = stirrer speed in rpm, VA (volume of pump A) = glycerol in mL, VB (volume of pump B) = NaOH consumption in mL

At the end of the fermentation, the DCW concentration was 23.87 g L^{-1} with a lipid content of 13.40%. As it can be seen in Table 29 and in Figure 45, the cells were growing over the whole fermentation time and produced simultaneously single cell oils, but in low quantity. The volumetric SCO productivity was 0.04 g L^{-1} h^{-1} and the 0.04 g lipids were produced utilizing 1 g glycerol after 72 hours. The increase of the parameters shown in Table 29 are very similar to the fermentations with the lower feed rate.

Table 29: Dry cell weight, single cell oil and lipid content, SCO/substrate, SCO/carbon and volumetric SCO productivity using glycerol as carbon source with a feed rate of 1.25 mL h^{-1}(average of duplicates)

Duration [h]	CDW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	2.05	-	-	-	-	-
20	5.75	0.27	4.70	0.01	0.03	0.01
22	5.99	-	-	-	-	-
24	3.10	0.28	4.53	0.01	0.03	0.01
26	3.18	-	-	-	-	-
44	10.88	0.72	6.61	0.01	0.04	0.02
46	11.99	-	-	-	-	-
48	12.84	0.91	7.05	0.02	0.04	0.02
50	13.91	-	-	-	-	-
68	22.72	2.78	12.25	0.04	0.09	0.04
70	21.55	-	-	-	-	-
72	23.87	3.19	13.40	0.04	0.09	0.04

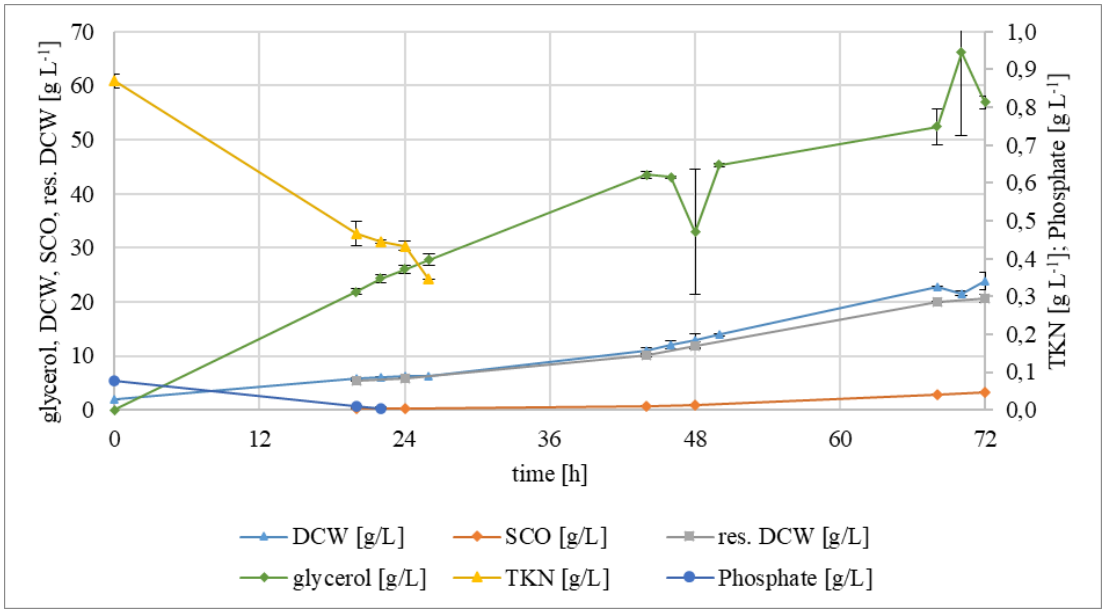

Figure 45: DCW, SCO, res. DCW, glycerol, nitrogen and phosphorous change over the time of fermentation using glycerol with a feed rate of 1.25 mL h^{-1} as substrate (average of duplicates)

The fatty acid composition and the increase and decrease of the individual fatty acids are again very similar to the fermentations with a lower feed rate. The results are listed in Table 30 and are visualised in Figure 46.

In both reactors, glycerol accumulated over the time of fermentation, with an end concentration of 55.77 and 58.09 g L^{-1} in reactor 1 and 2.

Table 30: Fatty acid composition in % using glycerol as carbon source with a feed rate of 1.25 mL h^{-1} (average of duplicates)

	C16:0 [%]	C16:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	C20:2 [%]
20 h	15.2	1.4	4.6	26.2	50.5	2.1
24 h	14.6	1.4	5.2	26.0	50.9	2.0
44 h	17.6	0.9	8.9	35.7	34.9	2.0
48 h	18.4	1.1	8.4	36.4	33.7	2.0
68 h	22.3	1.3	9.1	44.5	21.1	1.8
72 h	22.2	1.2	9.4	45.2	20.2	1.7

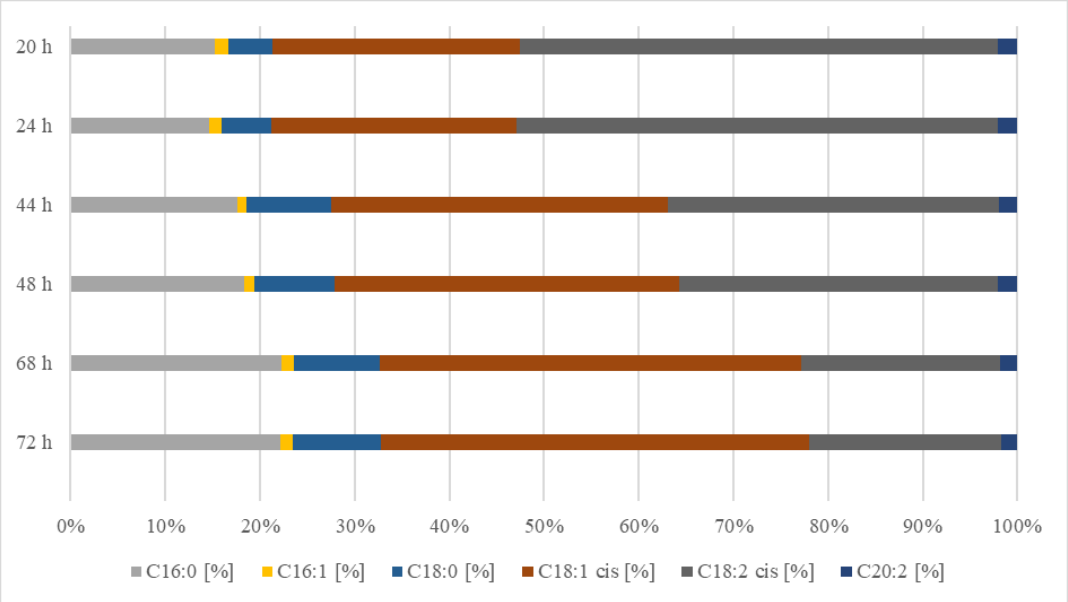

Figure 46: Fatty acid composition in % using glycerol as carbon source with a feed rate of 1.25 mL h^{-1} (average of duplicates)

4.6 SCO production using a mixed VFA substrate as feed

4.6.1 Sterile feed

In Figure 47 and Figure 48 the course of fermentation using the sterile mixed substrate as feed are shown. In Reactor 1 no problems occurred, and the feed flask was empty after about 39/40 hours. This is indicated by the decreased stirrer speed. After that timepoint residual volatile fatty acids are metabolized as the DO values are still at 20%, although the stirrer speed is decreasing gradually. In contrast to that, after about 7 hours of fermentation, the connection to the feed flask of reactor 2 was disconnected and

no feed was added. Because there was no substrate added, the DO values increased, and the stirrer speed was reduced to the minimum 400 rpm. On the next day, at hour 20, the problem was recognised, and the feed flask was reconnected. After about 50 hours, the feed flask was empty and after about 65 hours substrate depletion occurred and the DO values started to rise. For the calculations, only the results obtained by reactor 1 were considered.

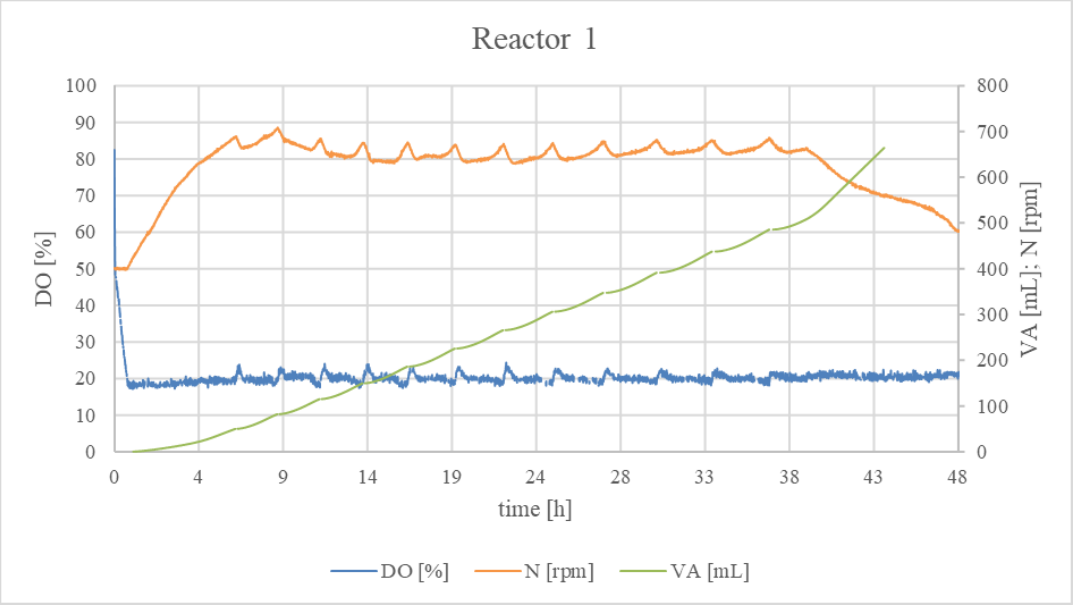

Figure 47: The course of the fed batch fermentation with the sterile mixed substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = mixed substrate in mL

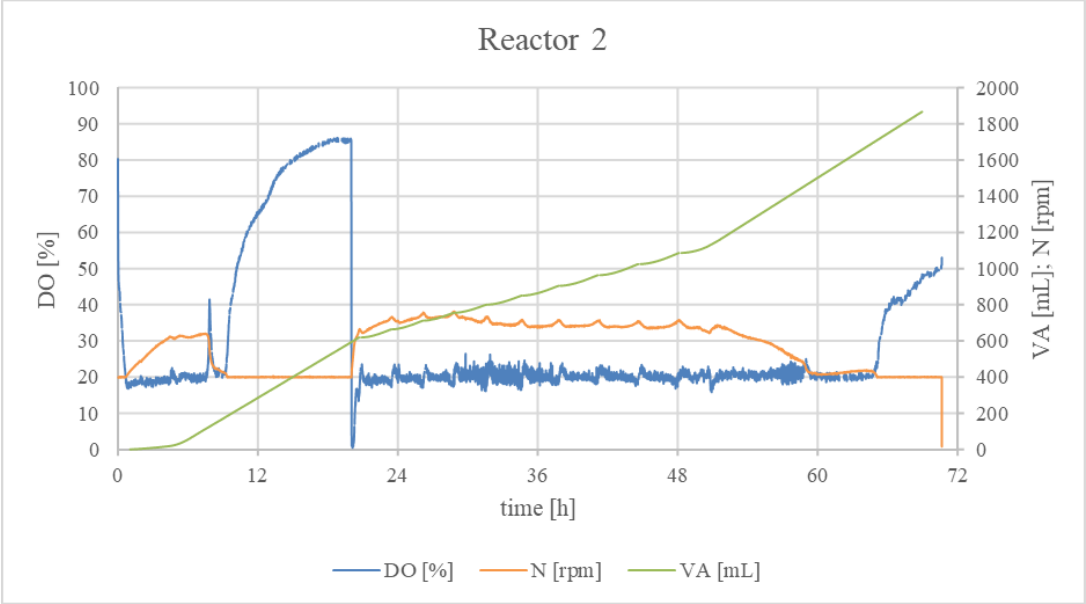

Figure 48: The course of the fed batch fermentation with the sterile mixed substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = mixed substrate in mL,

Table 31 shows the DCW and the SCO concentrations along with the SCO yield and volumetric productivity. The highest DCW was reached after 44 hours, approximately 4 hours after the feed flask was empty, indicating that there was still enough substrate to build up biomass. While the biomass concentration started to decrease at this timepoint, the highest lipid content, with 24%, was reached at the end of fermentation. The SCO yield increased till hour 44 to 0.11 g SCO per g substrate or 0.21 g SCO per g carbon. The volumetric SCO productivity started to decrease after already 24 hours. Figure 49 shows, that the nitrogen content stayed more or less constant of the fermentation time. This is maybe because of ammonia present in the feed. Phosphate depletion was reached at least after 20 hours. No substrate accumulation occurred.

Table 31: DCW concentration, SCO concentration, lipid content, SCO/substrate, SCO/carbon and volumetric SCO productivity using the sterile mixed substrate as feed

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/Substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
0	4.00	-	-	-	-	-
20	11.97	1.97	16.49	0.09	0.17	0.10
22	12.45	-	-	-	-	-
24	12.95	2.49	19.25	0.10	0.19	0.10
26	13.02	-	-	-	-	-
44	15.60	3.62	23.22	0.11	0.21	0.08
46	15.45	-	-	-	-	-
48	15.35	3.68	24.00	0.11	0.20	0.08

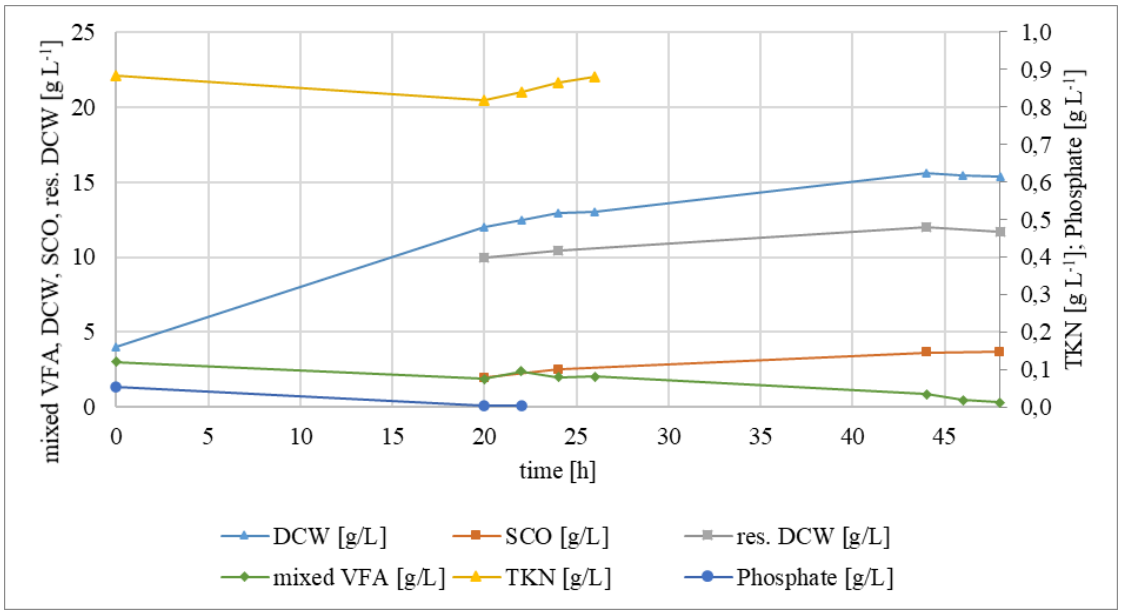

Figure 49: DCW, SCO, res. DCW, mixed VFA, nitrogen and phosphor change over the time of fermentation using the sterile mixed substrate as feed

The most common fatty acids produced, were palmitic (C16:0) and oleic acid (C18:1) with 35.5% and 34.4% respectively after 48 hours. Further fatty acids were C17:0, C17:1, C18:0 and C18:2. The overall composition did not change very much over the fermentation time, despite a slightly increase of palmitic and a slightly decrease of linoleic acid. The results of the lipid composition and their changes over the fermentation time are shown in Table 32 and Figure 50.

Table 32: Fatty acid composition in % using the sterile mixed substrate as feed

	C16:0 [%]	C17:0 [%]	C17:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	others (<2%) [%]
20 h	31.0	8.8	1.8	8.9	35.1	7.8	6.6
24 h	34.2	7.5	1.8	8.3	34.8	6.7	6.8
44 h	36.4	7.9	2.5	7.7	35.0	3.8	6.8
48 h	35.5	8.4	2.7	7.7	34.4	4.3	7.1

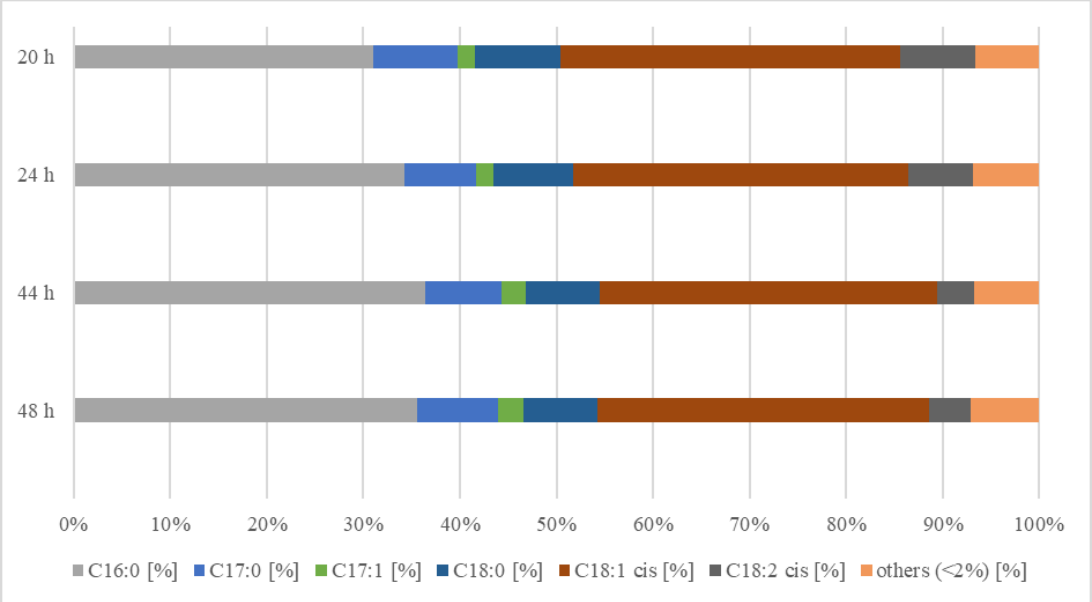

Figure 50: Fatty acid composition in % using the sterile mixed substrate as feed

4.6.2 Non-sterile feed

The two fermentations with the non-sterile feed were performed in parallel to the sterile mixed feed fermentations. The course of the fermentation of both reactors are illustrated in Figure 51 and Figure 52 and are very similar to the first reactor of the sterile feed. For both reactors, no problems occurred, the feed flasks were empty after about 36 hours and the reactors were shut down after 48 hours of fermentations. In both reactors no contamination occurred.

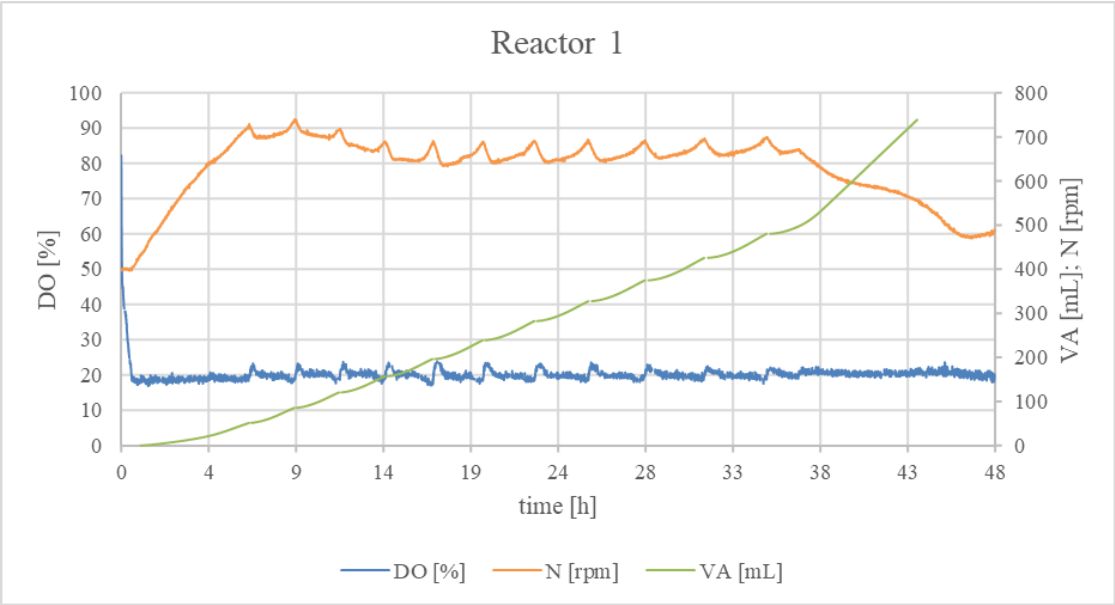

Figure 51: The course of the fed batch fermentation with the non-sterile substrate in reactor 1; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) =non-sterile mixed substrate in mL

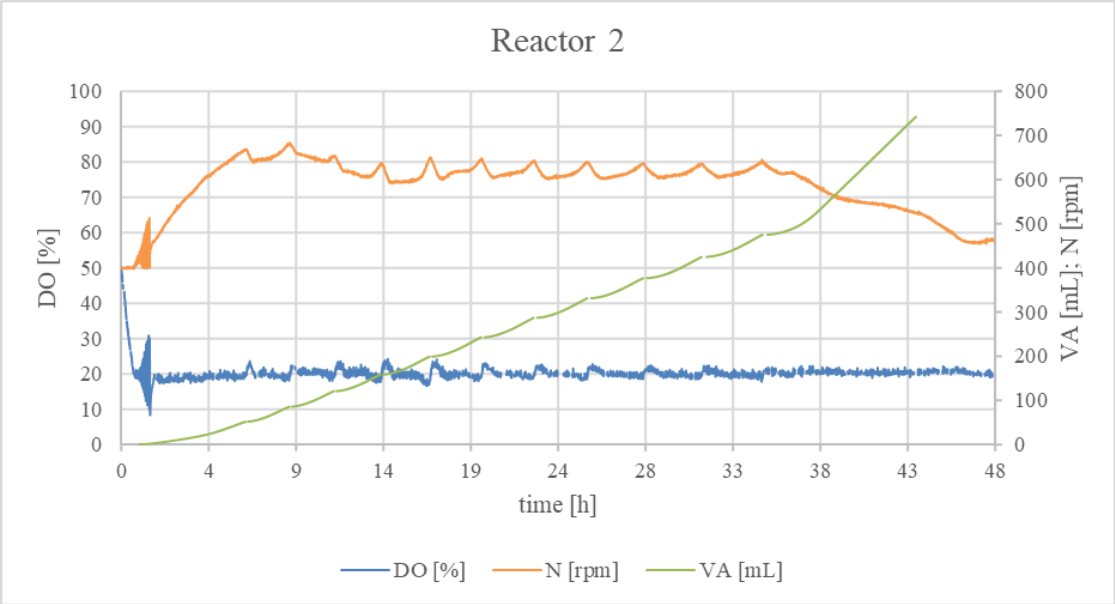

Figure 52: The course of the fed batch fermentation with the non-sterile mixed substrate in reactor 2; DO = dissolved oxygen in %, N = stirrer speed in rpm, VA (volume of pump A) = non-sterile mixed substrate in mL

The highest DCW concentration, as well as the highest lipid content were reached after 48 hours with 15.07 g L^{-1} and 23.47 %. The lipid yield increased to 0.21 g SCO per g carbon, the volumetric productivity decreased from 0.10 g^{-1} L h^{-1} after 20 hours to 0.07 g^{-1} h^{-1} at the end of the fermentation. No substrate accumulation occurred. Phosphor depletion was reached at least after 20 hours and the nitrogen content stayed constant throughout the measuring period. The results are shown in Table 33 and Figure 53.

Table 33: DCW concentration, SCO concentration, lipid content, SCO/substrate, SCO/carbon and volumetric SCO productivity using the non-sterile mixed substrate feed (average of duplicates)

Duration [h]	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/Substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g ^{-1}L h^{-1}]
0	3.76	-	-	-	-	-
20	11.22	1.81	15.96	0.08	0.17	0.09
22	12.00	-	-	-	-	-
24	12.60	2.30	18.28	0.09	0.18	0.10
26	12.50	-	-	-	-	-
44	15.00	3.40	22.64	0.11	0.20	0.08
46	14.97	-	-	-	-	-
48	15.07	3.54	23.47	0.11	0.21	0.07

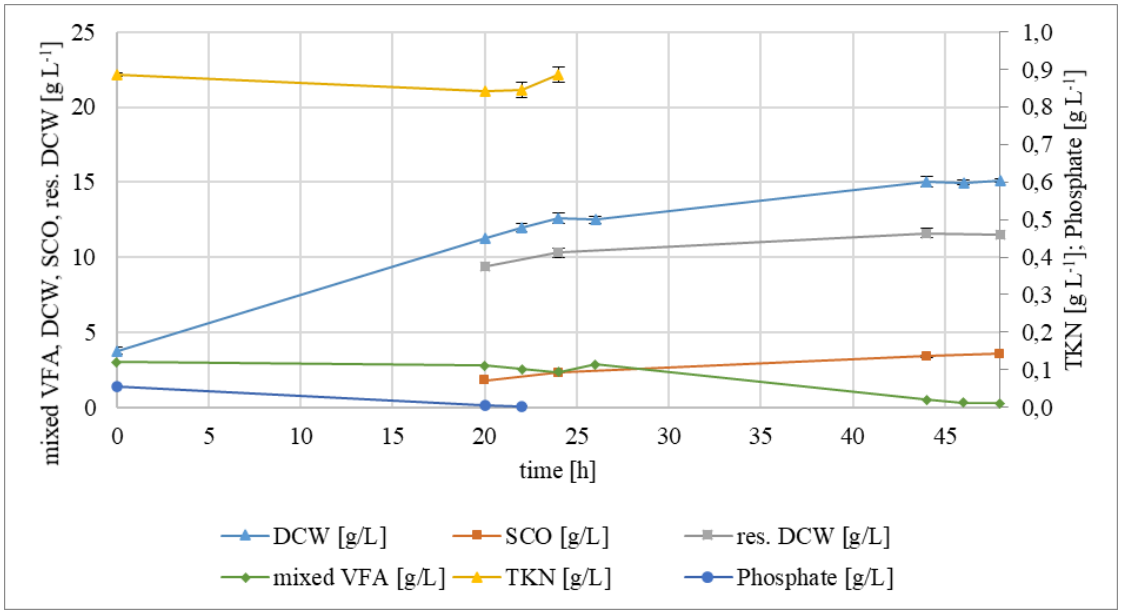

Figure 53: DCW, SCO, res. DCW, mixed VFA, nitrogen and phosphor change over the time of fermentation using the non-sterile mixed substrate as feed (average of duplicates)

The fatty acid composition, which is shown in Table 34 and in Figure 54 are very similar to the results obtained using the sterile feed.

Table 34: Fatty acid composition in % using the non-sterile mixed medium as feed (average of duplicates)

	C16:0 [%]	C17:0 [%]	C17:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	others (<2%) [%]
20 h	30.6	8.1	2.0	9.0	35.8	7.8	6.7
24 h	32.9	7.2	1.6	9.5	36.3	6.0	6.5
44 h	35.0	9.3	3.1	7.4	34.0	4.6	6.6
48 h	34.7	9.3	3.2	7.0	33.7	5.1	7.0

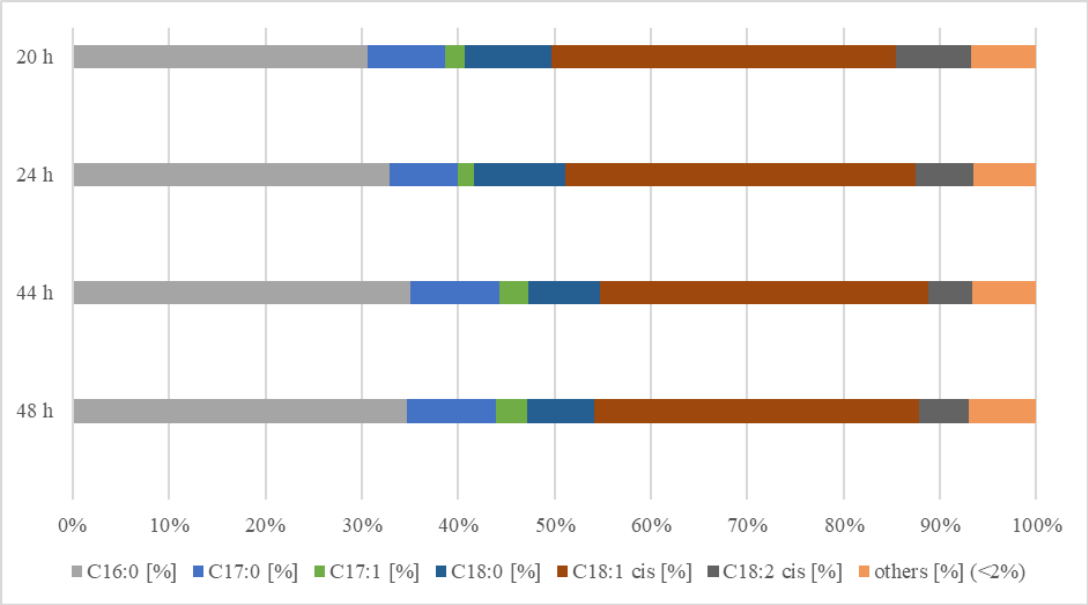

Figure 54: Fatty acid composition in % using the non-sterile mixed medium as feed (average of duplicates)

4.7 Summary of all bioreactor results

Table 35 gives an overview of the dry cell weight and single cell oil concentration, the lipid content, the single cell oil yield as well as the volumetric single cell oil productivity that were achieved after 72 or 48 hours of fermentation using the respective carbon sources.

Table 35: DCW concentration, SCO concentration, lipid content, SCO/substrate, SCO/carbon, volumetric SCO productivity of all synthetic carbon sources after 72 hours of fermentation and the non-sterile mixed substrate after 48 hours of fermentation

	DCW [g L^{-1}]	SCO [g L^{-1}]	Lipid content [%]	SCO/substrate [g g^{-1}]	SCO/C [g g^{-1}]	Volumetric SCO productivity [g L^{-1} h^{-1}]
Acetic acid	43.46	29.52	67.99	0.15	0.39	0.41
Propionic acid	21.57	7.49	34.68	0.08	0.17	0.10
Butyric acid	43.40	22.41	51.63	0.23	0.42	0.31
Valeric acid	23.61	7.95	33.51	0.14	0.24	0.11
Lactic acid	13.76	2.80	20.40	0.09	0.22	0.04
Glucose	43.40	14.07	32.44	0.18	0.44	0.20
Xylose	13.76	1.21	8.75	0.01	0.03	0.02
Glycerol [a)]	19.91	2.61	13.08	0.05	0.14	0.04
Glycerol [b)]	23.87	3.19	13.40	0.04	0.09	0.04
Mixed VFA substrate	15.07	3.54	23.47	0.11	0.21	0.07

[a)] 0.625 mL h^{-1} [b)] 1.25 mL h^{-1}

Table 36 gives an overview of the fatty acid composition of the SCO, that is produced using the respective carbon sources.

Table 36: Fatty acid composition in % of all synthetic carbon sources after 72 hours of fermentation and the non-sterile mixed substrate after 48 hours

	C15:0 [%]	C16:0 [%]	C16:1 [%]	C17:0 [%]	C17:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	C20:2 [%]	C24:0 [%]	Others (<2%) [%]
Acetic acid	-	14.8	-	-	-	32.9	41.1	6.4	-	-	4.9
Propionic acid	5.6	3.7	-	47.5	30.8	0.6	9.0	2.8	-	-	-
Butyric acid	-	25.1	-	-	-	15.2	45.7	8.4	-	-	5.6
Valeric acid	2.3	7.6	-	38.1	18.3	3.7	24.3	5.6	-	-	-
Lactic acid	-	20.2	-	-	-	7.8	54.1	14.8	-	-	3.1
Glucose	-	20.8	-	-	-	15.2	48.4	12.1	-	-	3.5
Xylose	-	14.8	-	-	-	9.9	36.1	35.9	-	3.2	-
Glycerol [a)]	-	22.1	1.5	-	-	7.3	44.7	22.5	1.9	-	-
Glycerol [b)]	-	22.2	1.2	-	-	9.4	45.2	20.2	1.8	-	-
Mixed VFA substrate	-	34.7	-	9.3	3.2	7.0	33.7	5.1	-	-	7.0

[a)] 0.625 mL h^{-1} [b)] 1.25 mL h^{-1}

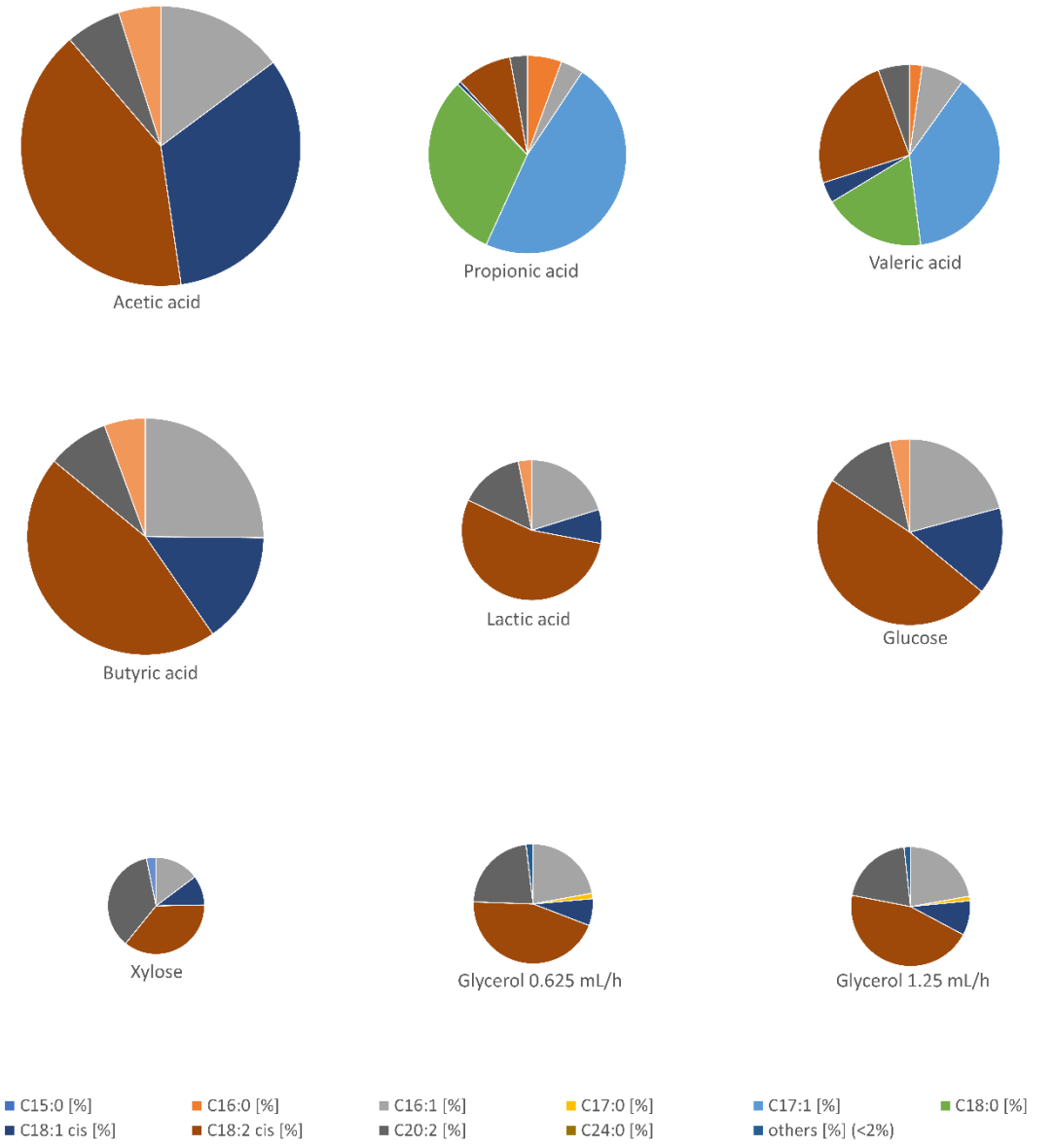

Figure 55: Fatty acid composition of all synthetic carbon sources after 72 hours of fermentation and the non-sterile mixed substrate after 48 hours; areas of the pie charts indicate the lipid content reached with the different carbon sources (in relation to each other)

5 Discussion

5.1 VFA fermentation process resume and improvements

The developed process shows high suitability for the VFA based SCO production. It was very robust and delivered stable results during double determinations, if no major problems, like disconnected feed tubes or leaky pump tubes, occurred. Comparing the two graphs of the course of fermentations, Figure 9 and Figure 10 for acetic acid and the residual graphics respectively for the other substrates, they look very similar or identical, which indicates the stable and robust fermentation procedures. However, an instrument to measure the liquid level/liquid volume or the weight of the reactors would improve the results. As already stated in 4.2.1, the volume of the reactors at the respective timepoints was only an approximation and the volume loss, mostly through evaporation, was in some cases very high. A better control of that would lead to more accurate results when it comes to the calculation of the volumetric productivity for example. In total, the used fermentation system worked very well for the lab-scale experiments and the obtained results provide a basis for further scale-up experiments that will allow a better estimation of the process parameters.

5.2 Substrate utilization in shake flasks

For an overview of utilizable substrates for *A. brassicae*, shake flask experiments were performed with different kind of sugars.

The results show that glucose was utilized very fast and in high amounts and the stationary phase was reached after about 24 hours. Compared to that, galactose was also consumed in high amounts but slower. An explanation for this is that *A. brassicae* was grown previously on glucose and therefore a lag phase (to adapt to the medium) occurred using galactose as substrate. The higher fat content of 18.93% growing on glucose compared to 9.01% using galactose as substrate indicates that glucose is converted more efficiently into lipids than galactose, because nearly the same amount of substrate was consumed during the shake flask experiment. Xylose was consumed also fast, with a substrate consumption rate only 0.25% lower than the substrate consumption rate of glucose or galactose. Remarkably, a much lower OD_{600} value and therefore a much lower cell density was reached, which indicates that xylose can be consumed in high amounts, but the utilization and conversion of substrate into biomass is less efficient. Nakase (1971) reported that lactose cannot be utilized by *A. brassicae*. This corresponds to the obtained results, since lactose was not consumed, and growth was not observed. The inability of utilizing lactose may be a result of the absence of lactase enzymes to cut the disaccharide, since the monosaccharides glucose and galactose could be utilized. The second disaccharide used in the shake flask experiment, sucrose, was metabolised by *A. brassicae*. The results for crude glycerol and glycerol were very similar, regarding substrate consumption, cell growth and lipid production, with a slightly

better yield using crude glycerol, which enables the application of glycerol without extensive purification procedures.

The shake flask experiments reached in general low DCW concentrations and lipid content, which is nevertheless normal for shake flask experiments. Growth under these conditions is slow, because the oxygen input is lower compared to stirred tank reactor fermentations. The results obtained by *A. brassicae* are comparable to previously performed studies with different oleaginous yeast strains. Shake flask experiments performed with the yeast *Rhodotorula graminis* under similar conditions lead to similar DCW concentrations (Galafassi et al. 2012). Growing on glucose and galactose, the yeast reached a biomass concentration of 16.09 g L^{-1} and 12.44 g L^{-1}. Slightly higher compared to our results with 11.75 g L^{-1} and 10.21 g L^{-1}. It has to be mentioned that with 50 g L^{-1}, the carbon source concentration was much higher, which is probably also responsible for the much higher lipid yield of 40% and 24% for glucose and galactose. With xylose as substrate the lipid content and biomass concentrations were higher with 10.78 g L^{-1} and 17% in *R. graminis*.

Tchakouteu et al. (2015) performed shake flask experiments with different oleaginous yeast growing on crude glycerol. With a biomass concentration between 3.1 and 11.8 g L^{-1} and a lipid content between 3.8% and 32.7% the results are again comparable to the results of this work.

The results of the shake flask experiment show that some sugars can be used for the growth and lipid production in *A. brassicae*. The efficiency of converting these sugars into biomass and SCO can probably be increased by adapting the yeast to the respective medium. Adaptation procedures have been described for many yeasts. For example *Saccharomyces cerevisiae* was successfully adapted to high galactose concentrations to increase ethanol production (Ra et al. 2015). However, *A. brassicae* was screened for the use of volatile fatty acids as substrate (Miranda et al. 2020), and therefore the focus of the next experiments was on VFA.

5.3 Single VFA as carbon source

The results of the eight fermentations (two with each volatile fatty acid) using acetic acid, propionic acid, butyric acid and valeric acid as substrate are discussed on the basis of the highest results obtained after the respective time.

Using acetic acid as substrate, a biomass concentration of 43.46 g L^{-1} was reached after 72 hours. With a lipid content of 67.99 % the highest amount of single cell oil was produced, compared to all other substrates. With butyric acid even more biomass per litre was produced and comparing it with acetic acid, *A. brassicae* was able to grow faster on butyric acid. However, less single cell oil was produced, although the SCO yield was significantly higher. While the yield as well as the volumetric productivity remained more or less constant over the time of fermentation using acetic acid, these values decreased

for butyric acid as substrate. This is because *A. brassicae* built up large amount of biomass in short amount of time and with that the lipid concentration increased rapidly, although the lipid content was low. In the later phase the yeast predominantly synthesised storage lipids and the lipid content increased.

Similar to the course of fermentation using acetic acid was the course of fermentation using propionic and valeric acid. The biomass and single cell oil content was built up more gradually and constant compared to butyric acid. With propionic acid, a fat content of 37.73% was reached, which was lower compared to acetic and butyric acid but higher compared to valeric acid as substrate. Although a higher biomass concentration could be reached using valeric acid, less single cell oil was produced, compared to propionic acid. Because valeric acid was used in pure form, no water was added with the feed and the fermentation broth was less diluted compared to fermentations with other VFA as feed.

Looking at these results, a higher amount of single cell oils was reached using even-numbered volatile fatty acids, making them probably more suitable to produce high amounts of the single cell oils.

Comparing the results obtained with *A. brassicae* with data from literature, indicates that *A. brassicae* is highly suitable for lipid production using VFA as carbon source. Christophe et al. (2012) used a two-stage bioreactor fermentation to produce single cell oils using *Cryptococcus curvatus*, an oleaginous yeast that is often used for the production of single cell oils. First, the yeast was grown on glucose to build up biomass. Then acetic acid was added under nitrogen limitation to produce single cell oil. In the second phase, a SCO yield per substrate of 0.15 g g^{-1} was reached, but the fat content of 50.89% was lower compared to the lipid content of 67.99% obtained in this work. Similar to that, Fontanille et al. (2012) performed two stage fermentations with *Yarrowia lipolytica*, known for high lipid production capacity. In the first phase glucose was utilized for biomass production and in the second phase different volatile fatty acids were added. With acetic acid, a lipid content, a lipid yield and a lipid productivity of 40.69%, 0.13 g g^{-1} and 0.16 g L^{-1} h^{-1} were reached respectively. The results for butyric acid were 24.00%, 0.11 g g^{-1} and 0.17 g L^{-1} h^{-1} and for propionic acid 38.06%, 0.20 g g^{-1} and 0.30 g L^{-1} h^{-1}. The results obtained with acetic acid as feed, show that the fat content, the yield of single cell oils and especially the volumetric productivity is lower compared to the results obtained in this thesis with *A. brassicae*. The values for butyric acid are more than twice as high for *A. brassicae* then for *Y. lipolytica*. The fat content reached with propionic acid, was nearly the same for both yeasts, only the lipid yield and the productivity were much higher in *Y. lipolytica*.

Another study performed with different types of oleaginous yeasts growing on acetic and propionic acid alone, stated comparable but mostly lower biomass concentrations and lipid contents (Kolouchová et al. 2015).

Using propionic and butyric acid as carbon source, the highest biomass concentrations as well as the highest fat content were reached after 68 hours. This suggests that the fermentation using propionic acid as feed should be stopped after 68 h, since the SCO yield as well as the volumetric SCO productivity

started decreasing at this time. For butyric acid, the highest DCW that was measured was already reached after 50 hours, but because the single cell oil content at this time point was not measured, the other values could not be calculated.

The different lipid composition achieved with the four different volatile fatty acids are discussed on the hand of the data obtained at the end of the fermentation after 72h (see Table 36).

Using acetic acid or butyric acid as substrate, which are even numbered volatile fatty acids, high amounts of stearic acid (C18:0) and oleic acid (C18:1) are produced but no margaric acid (C17:0) or heptadecanoic acid (C17:1) are formed, compared to the odd numbered volatile fatty acids, like propionic and valeric acid. Linoleic acid (C18:2) and palmitic acid (C16:0) are formed when using all substrates. However, the share of C16:0 is higher, when utilizing acetic and butyric acid. This fatty acid profile seems to be different compared to fatty acid composition profiles found in the literature using the same substrates. The fatty acid profiles described by Fontanille et al. (2012) did not show many variations between SCO produced from acetic, propionic and butyric acid as carbon source. Furthermore, the presence of fatty acids with seventeen carbons when using propionic acid as carbon source for SCO production is not reported. Compared to this Kolouchova et al. (2015) found that all studied oleaginous strains produce heptadecanoic acid when grown on propionic acid. However only low amounts of margaric acid are reported.

While only even numbered fatty acids were produced when using acetic and butyric acid, around 84% of the total fat is made up of odd numbered fatty acid when using propionic acid as carbon source. For valeric acid as carbon source around 59% the fatty acids produced were of odd numbered.

The big difference in percental lipid composition of the fatty acids that are formed for all substrates (palmitic acid: 3.7% - 25.1%, stearic acid: 0.6%- 32.9% and oleic acid: 9.0% - 45.6%) and the different types of produced fatty acids (C15:0, C17:0 and C17:1) indicate that there might be different pathways available for even and odd numbered carbon sources. Kolouchová et al. (2015) ascribed this to the presence of a specific metabolic pathway for the utilization of propionic acid. The propionic acid directly reacts with coenzyme A and forms propionyl-CoA, which is the precursor for the formation of odd numbered fatty acids.

While the type of synthesised fatty acids depends on the used substrate, there are also changes in the fatty acid profile over the fermentation time. This is a result of the changing ratio of phospholipids in the cell membrane that are produced along with biomass, to storage lipids, which are produced under nutrition limitation. The most common fatty acid present in the cell membrane are often C16:0, C16:1 and C18:1 (Henderson and Block 2014). These FA cannot be differentiated from the FAs from the storage lipids by fatty acid methyl ester GC analysis and influence the lipid profile. Table 14,

Table 16, Table 18 and Table 20 show, that during all four fermentations the percental amount of palmitic acid (16:0) decreases. This decrease might be explained by a decrease in the ratio of membrane

derived phospholipids to storage lipids throughout the fermentation. The higher the amount of storage lipid in the cells, the lower the effect of the phospholipids on the lipid profile, since their amount stays more or less constant.

Table 37: Fatty acid composition in % of plant oils (Orsavova et al. 2015) (sunflower, rapeseed and peanut) and of A. brassicae (acetic and propionic acid)

	C15:0 [%]	C16:0 [%]	C17:0 [%]	C17:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	C20:1 [%]	Others (>2%) [%]
Sunflower oil	-	6.2	-	-	2.8	28.0	62.2	-	0.8
Rapeseed oil	-	4.6	-	-	1.7	63.3	19.6	9.1	1.7
Peanut oil	-	7.5	-	-	2.1	71.1	18.2	-	1.1
SCO from acetic acid	-	14.8	-	-	32.9	41.1	6.4	-	4.9
SCO from propionic acid	5.6	3.7	47.5	30.8	0.6	9.0	2.8	-	-

Comparing the fatty acid composition in *A. brassicae* growing on acetic and butyric acid to the lipid composition in plant oils listed in Table 37 shows that they have some similarity. The most common fatty acids in these plant oils are fatty acids with 18 carbon atoms, but also palmitic acid is present. While the ration of unsaturated and saturated fatty acid is nearly the same, the percental amount of oleic and linoleic acid varies between the plant oils. The fatty acid profile obtained using acetic and butyric acid as carbon source show similar percental amount of C18 fatty acids, with a higher amount of saturated fatty acids. Also, the palmitic content is higher compared to the three plant oils. However, due to the similarities, lipids derived from *A. brassicae* can be used in some cases as alternative to plant derived oils, for example biodiesel production.

Growing on propionic and valeric acid, *A. brassicae* synthesises C17:0 and C17:1, which are not present in plant oils and provide a sustainable source for those fatty acids. Heptadecenoic acid (17:1) is known to exert antibiotics effect on fungi (Avis et al. 2000) and can possibly be used for the treatment of psoriasis or allergies (Degwert et al. 1998).

5.4 Lactic acid as carbon source

Lactic acid is a common fermentation product in biogenic waste materials, like whey or molasses. By converting not only the present sugars but also lactic acid into product, the yield is increased. Beside that, lactic acid also accumulates at the initial phase of the acidification process of biomass into VFA.

If lactic acid can be used as substrate to produce SCO, the acidification process can be stopped earlier, resulting in a more economical process.

With lactic acid as carbon source a much lower fat content was reached in *A. brassicae* compared to the volatile fatty acids. The SCO yield of 0.09 g g^{-1} is comparable to that of propionic acid. In comparison, lactic acid was however utilized much slower and in lower amounts. Due to this, the yeast builds up less biomass and therefore less lipids were produced. After 72 hours, a DCW concentration of 13.76 g L^{-1} was reached, which is around 30% of the biomass concentration that was achieved with glucose as feed, but approximately the same DCW concentration with xylose as feed. In terms of SCO concentration, only about 2.80 g L^{-1} were reached compared to 14.07 g L^{-1} using of glucose as carbon source. Both the yield of SCO and the volumetric SCO productivity increased till the end of fermentation. The fatty acid profile is comparable to the profile achieved with glucose or xylose. With 20.2% of palmitic acid, the percentage is similar to that of glucose. Also stearic, oleic and linoleic acids are found in the fatty acid profile, with more unsaturated (C18:1 and C18:2) fatty acids then saturated acids. More than 50% of the total fatty acids were oleic acid. While over the fermentation time the percentage of most fatty acids stayed more or less constant (the palmitic acid content increased only by 5%), the oleic and linoleic acid content changed. The two FA were present in equal amounts after 20 hours, later the ratio was shifted to the side of oleic acid.

The comparison of the data obtained from the fermentation with lactic acid with literature data was not possible, because no study was found growing oleaginous yeasts on lactic acid alone.

5.5 Glucose and Xylose as carbon source

The results for glucose and xylose used as substrates, are discussed and compared to the other results on the hand of the highest values reached during fermentation.

Glucose, as already discussed, was utilized very fast. After 20 hours, the biomass concentration reached 43.06 g L^{-1}. Similar to the growth on butyric acid, a lot of biomass and therefore a lot of lipids were produced at the start of fermentation. This can be seen on the high volumetric productivity at the beginning with 0.43 g L h^{-1}, which then decreased to 0.22 g L^{-1} h^{-1} after 68 hours. Despite the high volumetric productivity, the SCO yield was lower at the start compared to the yield after 68 hours which was 0.18 g g^{-1}. At the beginning, more energy, gained by utilizing glucose, was invested to build up biomass. After about 20 hours, phosphate was depleted, and glucose was probably metabolised to build up SCO. The fat content after 68 hours reached 32.3% and the lipid concentrations increased up to 14.63 g L^{-1}. The amount of produced lipids is therefore higher than the amount of lipids obtained with propionic or valeric acid as carbon source. Growing on xylose, a dry cell weight concentration of 13.76 g L^{-1} was reached. This value is lower than the DCW concentration reached using VFAs as carbon

source. The fat content was low reaching 8.75% and the SCO concentration was even lower than the concentration reached using lactic acid as substrate. A low volumetric SCO productivity and a decreasing yield of SCO with fermentation time, make *A. brassicae* not suitable for SCO production growing on xylose. However, adapting *A. brassicae* to xylose prior the fed batch fermentations, would give more precise indications to that.

In literature, many studies can be found where glucose and xylose are used in batch and fed batch fermentation processes for SCO production. Zhang et al. (2011) investigated the lipid production of *Cryptococcus curvatus* in a glucose fed batch process. With this oleaginous yeast, a DCW concentration of 104.1 g L^{-1} with a lipid content of 82.7% could be reached in 185 hours. DCW and SCO content reached in this fermentation done at in a 30 L scale were higher compared to the results of this work. However, fermentation time was also longer. The volumetric SCO productivity after 20 hours was 0.47 g L^{-1} h^{-1}, which is similar to the productivity reached in this work. Li et al. (2007) achieved a biomass concentration of 106.5 g L^{-1} and a lipid content of 67% in a fed-batch cultivation using glucose as carbon source. The volumetric productivity in this 15 L reactor reached 0.54 g L^{-1} h^{-1}. The used oleaginous yeast was *Rhodosporidium toruloides.* (Juanssilfero et al. 2018) performed batch fermentations using glucose and xylose as separate carbon sources for the lipid production in *Lipomyces starkeyi*. The concentration of both sugars was 50 g L^{-1}. Glucose led to a biomass concentration of 30.3 g L^{-1} with a lipid content of 79.6%. Xylose led to very similar results in this yeast with a DCW concentration of 28.7 g L^{-1} and 85.1% lipid content.

Comparing the fatty acid composition using glucose as substrate with the results Zhang et al. (2011) obtained by cultivating *C. curvatus* on glucose, shows that in our case less palmitic but more oleic acid was formed. In general, the results are however comparable. In *C. curvatus*, also C18:3 and C24:0 was found, but in very small amounts. Furthermore, the results of the fatty acid analysis correspond well to the fatty acid composition reported by Li et al. (2007) for *R. toruloides.* The fatty acid composition obtained by utilizing xylose showed more differences when compared to literature data. *L. starkeyi* grown on xylose in a batch culture, produced more than twice as much palmitic acid, but barely linoleic acid. The percental amount of stearic and oleic acid was very similar.

Unlike the fermentation using volatile fatty acids as carbon source, *A. brassicae* is less suitable for cultivation on sole glucose and xylose for massive SCO production. There are other oleaginous yeasts available, which are more efficient in utilizing these sugars. These results however do not come as a surprise, because *A. brassicae* was screened for acetic acid utilization and no optimization procedures were conducted for the growth on sugars.

5.6 Glycerol as carbon source

Especially for the production of biofuel, glycerol would be a very suitable carbon source for oleaginous yeasts. During the production process of biodiesel, crude glycerol accumulates as waste material and has to be purified for further used. By using glycerol as substrate for the production of new triacyl glycerides, recycling costs can be reduced and the biodiesel production process can be optimized.

For the fermentations using glycerol as carbon source, two different constant feed rates were chosen, based on previous experiments, to examine possible accumulations of glycerol during the fermentation. The results were obtained after 72 hours and are listed in **Fehler! Ungültiger Eigenverweis auf Textmarke.**.

Table 38: Highest results obtained after 72 h with glycerol as carbon source with a feed rate of 0.625 and 1.25 mL h^{-1}

	DCW [g L^{-1}]	Std. Dev.	SCO [g L^{-1}]	Std. Dev.	Lipid content [%]	Std. Dev.	SCO/substrate [g g^{-1}]	Std. Dev.	SCO/C [g g^{-1}]	Std. Dev.	Volumetric SCO productivity [g L^{-1} h^{-1}]	Std. Dev.
0.625 mL h^{-1}	19.91	0.91	2.61	0.16	13.08	0.22	0.05	0.01	0.14	0.01	0.04	0.00
1.25 mL h^{-1}	23.87	1.60	3.19	0.13	13.40	0.35	0.04	0.00	0.09	0.00	0.04	0.00

Comparing both feed rates shows that higher DCWs and SCO concentrations were reached with a feed rate of 1.25 mL h^{-1}. The lipid content, however, was comparable. Since the SCO yield per utilized carbon is lower for the higher feed rate, the lower feed rate is probable more suitable for the single cell oil production process. On the other hand, the volumetric productivity is nearly the same for both rates. If the substrate costs are not a big issue, the higher feed rate might be more suitable for SCO production, due to higher growth and lipid production. Nevertheless, at both feed rates glycerol started to accumulate. Comparing the biomass accumulation with other substrates, glycerol is comparable to propionic and valeric acid. Less biomass is formed compared to the use of acetic acid, butyric acid and glucose, but more than for the use of xylose. The fat content however was higher for all volatile fatty acid substrates.

Liang et al. 2010 used *Cryptococcus curvatus* in a fed batch fermentation to convert crude glycerol to lipids. For six days, specific amounts of glycerol and nitrogen were added to the reactors at defined time points. After that the nitrogen supply was stopped and only glycerol was added. After a total fermentation time of 12 days, a DCW concentration of 32.9 g L^{-1} with a lipid content of 52.9% was achieved. Despite the higher biomass and lipid concentration, a volumetric SCO productivity of 0.0625 g L^{-1} h^{-1} was reached, which is approximately one third higher than the results obtained with *A. brassicae* in this work.

Table 39: Fatty acid composition in % after 72 h using glycerol as carbon source with a feed rate of 0.625 and 1.25 mL h^{-1}

	C16:0 [%]	C16:1 [%]	C18:0 [%]	C18:1 cis [%]	C18:2 cis [%]	C20:2 [%]
0.625 mL h^{-1}	22.1	1.5	7.2	44.7	22.5	1.9
1.25 mL h^{-1}	22.2	1.2	9.4	45.2	20.2	1.7

The fatty acid composition after 72 hours, shown in Table 39, are very similar for both substrate feeding rates. Compared to the other substrates, the amount of unsaturated fatty acid is very high, similar to the fermentation with xylose. The palmitic acid content is comparable to the fermentations where glucose was used and the oleic acid content is comparable to the fermentations where acetic and butyric acid were used. Also C20:2 fatty acids were found, although their share was rather low.

5.7 VFA mixture derived from anaerobic digestion

The results of the fermentations using the mixed substrate were very similar for the sterile and non-sterile feed. No contaminations were observed under the microscope, which is also indicated by the similarity of the course of fermentation and results. Since only one fermentation with the sterile feed worked properly, only the results of the non-sterile feed are discussed. The mixed substrate contained acetic acid, propionic acid, butyric acid, iso-butyric acid, valeric acid, iso-valeric acid and caproic acid.

The obtained DCW and lipid content after 48 hours of 15.07 g L^{-1} and 23.47% are low, considering the lowest DCW, obtained by propionic acid as sole carbon source was 21.57 g L^{-1}. However, the total VFA concentration in the mixed feed was much lower compared to the concentration where only one type of VFA was used as carbon source. Instead, the lipid yield is more suitable to compare the results. At the end of the fermentation, the lipid yield was 0.11 g SCO per g VFA, compared to 0.08 g g^{-1} and 0.23 g g^{-1} utilizing propionic and butyric acid (lowest and highest lipid yield using VFA as carbon source). The volumetric SCO productivity reached its maximum after 24 h with 0.10 g L^{-1} h^{-1} and decreased to 0.7 g L^{-1} h^{-1}. Bettencourt et al. (2020) used a similar substrate in batch fermentations, containing acetic, propionic, butyric, iso-butyric, valeric, iso-valeric and caproic acid, obtained by anaerobic digestion. The starting VFA concentration in the Erlenmeyer flasks was 15 g L^{-1} and although a lower biomass concentration of only 8.3 g L^{-1} was reached, a lipid content of 43% lead to an almost equal SCO concentration. The lipid yield was 0.5 g SCO per g VFA after 120 hours.

Except for caproic acid and the respective iso-formes of butyric and valeric acid, statements for an expected lipid composition can be made. The utilization of acetic and butyric acid lead to an expected production of palmitic (C16:0), stearic (C18:0) and oleic acid (C18:1). Propionic and valeric acid led to

the formation of mostly C17:0 and C17:1 and additionally C18:1 for valeric acid. The results obtained after 48 hours using the non-sterile mixed substrate almost met these expectations. With 33.7% of the total lipids, oleic acid was the second most common fatty acid produced during the fermentation. Also, stearic and linoleic acid are produced in small amount with 7.0% and 5.1%. The most common fatty acid although is palmitic acid with 34.7%, which is higher than the percentual amount reached in the synthetic acetic and butyric acid media. With 9.3% and 3.2% respectively, C17:0 and C17:1 were also produced in small amounts but because of the lower lipid yields of propionic and valeric acid the results are as expected.

5.8 General discussion and further research

Comparing the evaluated carbon sources shows that volatile fatty acids are the most suitable substrate for the production of lipids in *A. brassicae*. Especially acetic and butyric acid appear to be a good choice when it comes to high SCO yields and volumetric productivities, which are necessary for an economical and efficient process. Odd numbered volatile fatty acids, like propionic and valeric acid, give rise to a unique fatty acid profile, which can be used to produce high-value lipid products. However, anaerobic digestion derived effluents always contain a mixture of VFA and the production of substrate solutions containing only one type of carbon source is labour intensive and expensive. For this reason, further research is necessary to investigate the influence of different VFA ratios and VFA concentrations on the lipid production. The use of a non-sterile carbon feed, which was successfully demonstrated in the experiments of this work, is only one optimization step that shows how further improvements can make the production process for single cell oil more cost-effective.

6 Conclusion

In this work the dependence of single cell oil production and fatty acid composition on the type of carbon source in *A. brassicae V134*, is shown. Generally, *A. brassicae* was able to metabolise several different kinds of carbon sources into SCO oils and is therefore a versatile production strain for SCO. With acetic and butyric acid, even numbered volatile fatty acids, the highest lipid contents and cell densities, were reached. The main fatty acids were C18 fatty acids. With odd numbered volatile fatty acids, like propionic and valeric acid, as substrate, the unusual fatty acids C17:0 and C17:1 are produced. In these cases, up to approximately 80% of total fatty acids were made up of C17:0 and C17:1. That said, growth and total lipid accumulation of *A. brassicae* V134 was worse compared to the before mentioned even numbered VFAs. Beside a high biomass concentration and a high total lipid accumulation, a high lipid yield and productivity are also important for optimizing SCO production. With acetic acid, a volumetric SCO productivity of 0.41 g L^{-1} h^{-1} after 72 hours was achieved. The highest lipid yield of 0.42 g produced SCO per g utilised carbon was achieved with butyric acid. Fermentations with an anaerobic digestion derived mixed VFA substrate showed that it is possible to cultivate *A. brassicae* on cheap carbon sources. The obtained results were lower than expected, but since only the influence of sterile and non-sterile substrate medium was examined and no optimization of the feeding strategy was performed, the lipid yield can probably still be increased, making *A. brassicae* V134 a very promising candidate for lipid production on VFAs. The results obtained by the residual substrates indicate less suitability for the cultivation and SCO production of *A. brassicae* V134.

Comparing these results to the results found in the literature, shows that *A. brassicae V134* is very suitable for cultivation on VFAs. This provides the opportunity for bigger scale fermentations on cheap substrates, since VFAs can be produced in anaerobic digestion of organic waste.

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