MINIREVIEW – Biotechnology & Synthetic Biology

Waste-derived volatile fatty acids as carbon source for added-value fermentation approaches

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One sentence summary: Waste valorization: volatile fatty acids for the microbial production of bioplastics, single-cell oil and omega-3 fatty acids.

Editor: Rich Boden.
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ABSTRACT

The establishment of a sustainable circular bioeconomy requires the effective material recycling from biomass and biowaste beyond composting/fertilizer or anaerobic digestion/bioenergy. Recently, volatile fatty acids attracted much attention due to their potential application as carbon source for the microbial production of high added-value products. Their low-cost production from different types of wastes through dark fermentation is a key aspect, which will potentially lead to the sustainable production of fuels, materials or chemicals, while diminishing the waste volume. This article reviews the utilization of a volatile fatty acid platform for the microbial production of polyhydroxyalkanoates, single cell oil and omega-3 fatty acids, giving emphasis on the fermentation challenges for the efficient implementation of the bioprocess and how they were addressed. These challenges were addressed through a research project funded by the European Commission under the Horizon 2020 programme entitled 'VOLATILE—Biowaste derived volatile fatty acid platform for biopolymers, bioactive compounds and chemical building blocks'.

INTRODUCTION

The constant depletion of finite earth's resources creates the need for a circular economy, which will ensure the efficient utilization of the waste streams of current conventional industrial processes. Such 'green' processes should not only accept a feedstock of waste streams, but also ensure the minimum waste production for themselves and therefore be carried out as environmentally friendly as possible (Korhonen, Honkasalo and Seppälä 2018). Under these circumstances, the bioconversion of waste utilizing microorganisms appears to be a very promising solution. Bioprocesses normally result in less byproducts and higher production yields, thus being easily incorporated in a 'green' strategy.

The use of microorganisms as bioconversion tools represents a promising and environmentally friendly alternative to conventional chemical processes. Established bioprocesses already exist, for producing added-value metabolites for diverse industrial and nutritional applications. Lipids, a specific category of metabolites, produced by single cell microorganisms (SCM) offer several advantages over plant oils or animal fats. Furthermore, they are constantly gaining popularity because SCM can produce a great diversity of lipids, while storing higher percentages than animal tissues (Park *et al.* 2018a; Garay, Boundy-Mills and German 2014). Oleaginous microorganisms can naturally accumulate more than 20% of lipid per cell dry weight (CDW)—some of them even exceeding 70%—and include microalgae, yeasts and some bacteria (Papanikolaou and Aggelis 2011a; Fontanille *et al.* 2012). One characteristic of eukaryotes like oleaginous yeasts or heterotrophic microalgae is the production of neutral lipids in the form of triacylglycerols (TAG), whereas in specific bacteria polymeric lipid accumulation occurs preferentially in the form of polyhydroxyalkanoates (PHA; Garay, Boundy-Mills and German 2014). Both microbial lipids and PHA are functional commodities of high value and production cost. It is therefore a matter of importance to investigate alternative, cost-effective production methods.

THE VOLATILE FATTY ACID PLATFORM

In the context of the establishment of a circular bioeconomy, the selection of sustainable, non-expensive carbon sources to produce lipids from microorganisms is the key. Therefore, volatile fatty acids (VFA), intermediates of anaerobic digestion, attract increasing attention as feed stock. VFA are short-chain organic acids (C2–C6) that can be found in dark fermentation (DF) effluents. DF follows the same pattern as anaerobic digestion, except that it eliminates the last stage of methanogenesis (Fig. 1), thus enabling the production of VFA and emission of hydrogen as biofuel (Lukajtis *et al.* 2018). What is more, VFA can be assimilated by various microorganisms (Chalima *et al.* 2019; Reddy *et al.* 2020; Llamas *et al.* 2020a).

The implementation of viable fermentative production processes using VFA as carbon source requires a thorough understanding of VFA uptake and cellular metabolism. Compared to sugar-based substrates, VFA trigger shorter metabolic pathways, which could theoretically lead to higher lipid conversion efficiencies. In contrast to metabolic pathways and key factors involved in lipid accumulation when using sugar-based carbon sources, information about VFA metabolism is scarce (Llamas *et al.* 2020a). Recent research in microbial lipid production has investigated the conversion of VFA into storage lipids by oleaginous yeasts (Llamas *et al.* 2020b; Miranda *et al.* 2020), microalgae (Chalima *et al.* 2019) and bacteria (Chakraborty, Gibbons and Muthukumarappan 2009; Mengmeng *et al.* 2009).

MICROBIAL LIPID METABOLISM

Storage lipids are normally produced in the presence of excess carbon source and when cell growth is hampered due to another nutrient limited availability. In the case of TAG the accumulation occurs through four main stages. In a first step, a pool of acetyl-coenzyme A (acetyl-CoA) and NADPH is generated through catabolic activities. These are the main precursors for intracellular fatty acid (FA) synthesis, since acetyl-CoA usually acts as initial building block for FA. The elongation of the FA chain by two carbon atoms requires malonyl-coenzyme A (malonyl-CoA), which is formed in the second step by a condensation reaction of acetyl-CoA and a bicarbonate ion. This way acetyl-CoA is carboxylated to malonyl-CoA, transferred to the acyl carrier protein (ACP) and further transformed by sequential turns within the FA synthase into acyl-ACP. Depending on the type of SCM, the acyl chain might end up, in a third step, being used for the formation of specific lipid pool or it may be incorporated in a neutral lipid droplet. The fourth step consists of the formation of intracellular lipid droplets (Fig. 2).

The *ex-novo* lipid accumulation pathway of yeast *Yarrowia lipolytica* is described, able to efficiently up-take hydrophobic substrates including FA, by modifying its cell surface to facilitate adhesion and transport of substrate into the cell (Beopoulos *et al.* 2009). These free FA will be activated by acyl-CoA synthetase into acyl-CoA and used in lipid metabolism directly or after undergoing a series of transformations. More specifically, acetic acid can be directly transformed to acetyl-CoA, a central intermediate of cell metabolism and lipid synthesis, whereas butyrate must undergo several biochemical transformations, including β-oxidation to acetoacetyl-CoA, and further cleavage to acetyl-CoA (Gao *et al.* 2020). Therefore, cell growth rates are directly associated with the different metabolic pathways of the specific single VFA.

In contrary to the formation of TAG, PHA formation can be considered as more common in prokaryotes and can also be divided in four steps. As in the case of eukaryotes, the first step generates a pool of acetyl-CoA that is used to synthesize hydroxyalkanoate monomers. These monomers depend on the species, as well as on the substrate consumed and are polymerized through various enzymatic processes into PHA. Finally, the PHA polymers are used for the formation of intracellular PHA lipid granules (Fig. 2).

The composition of VFA in the substrate correspondingly affects the yield, productivity and monomer composition of the produced PHA. Generally, even number of carbon chain VFA tend to generate the 3-hydroxybutyrate (3HB) monomer, whereas odd carbon chain VFA yield 3-hydroxyvalerate (3HV) and other longer-chain monomers (Hao, Wang and Wang 2017). VFA with odd and even number of carbon atoms follow different pathways to be converted to acetyl-CoA and propionyl-CoA. Still some researchers indicate that the conversion of butyrate and valerate could also proceed directly to the corresponding hydroxyacyl-CoA (Huang *et al.* 2018a).
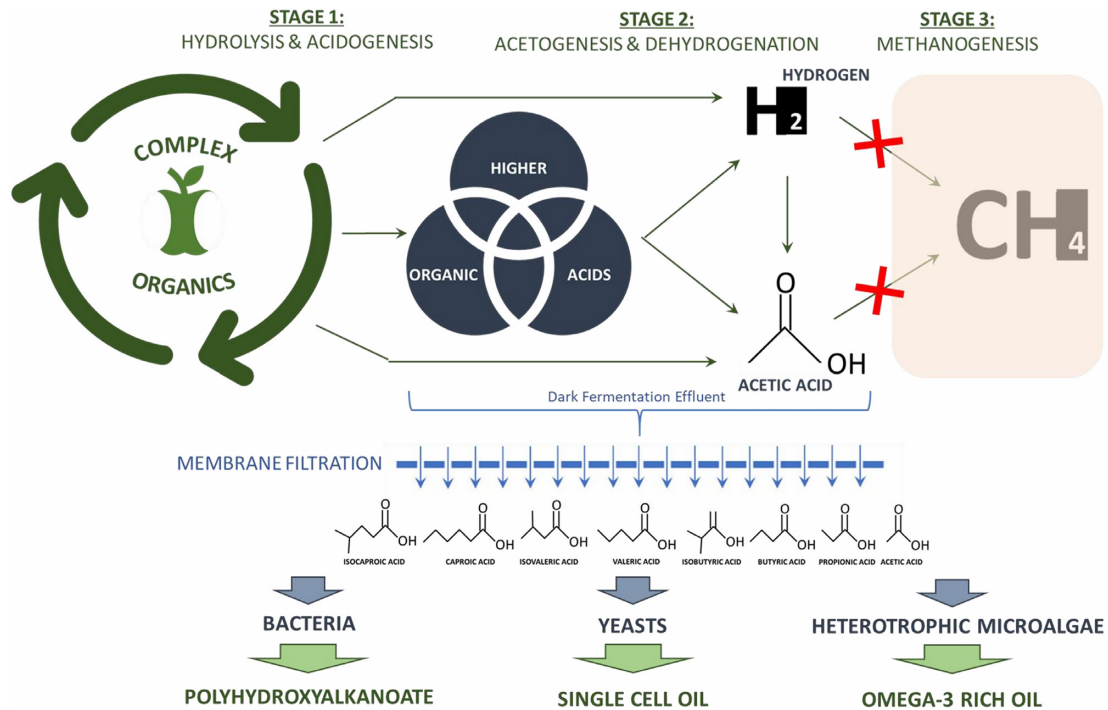
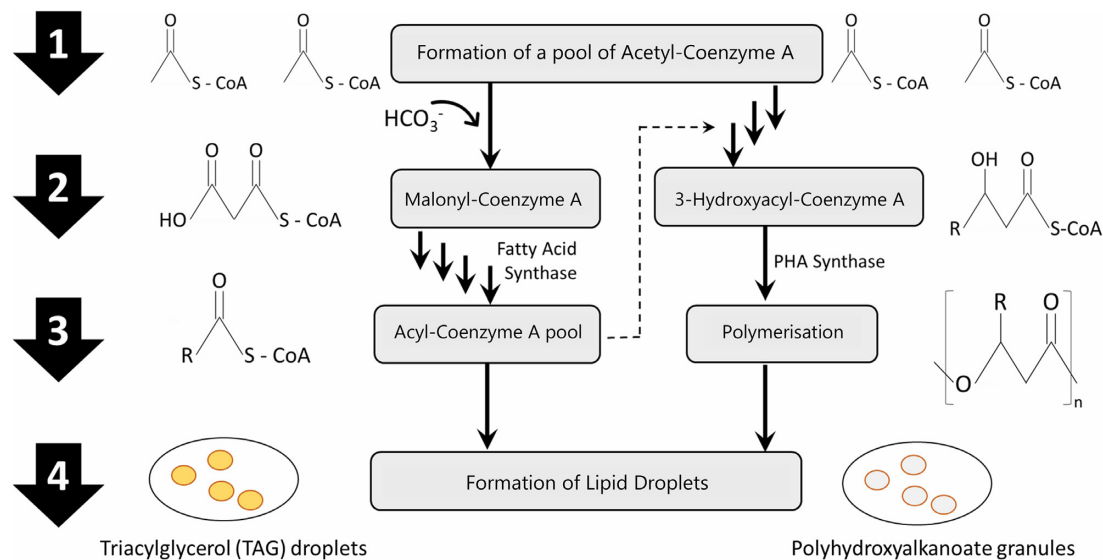**Figure 1.** Schematic representation of volatile fatty acid platform.

Figure 2. Schematic metabolic steps involved in lipid accumulation (adapted from Garay, Boundy-Mills and German 2014).

BIOCONVERSION OF VFA TO PHA

PHA use as plastics

In the last decades, plastics have been playing an increasingly important role in almost all sectors of our life, with innovative applications being developed as stand-alone products or as parts of other devices. In 2018, almost 360 million tons of plastics were produced worldwide, generating over 1.6 million jobs in Europe alone (Europe P, GMR 2019). However, the vast majority of the currently used plastics is derived from non-renewable fossil resources and is not biodegradable. Although a significant effort has been made in the recent years to find solutions for

their safe disposal, fossil fuel-based plastics' toll to the environment is still dramatic, with around 60 million tons per year, ending up in soils, lakes, rivers or the sea.

One promising alternative to the traditional plastics are biopolyesters such as PHA, produced by naturally occurring microorganisms. PHA have physicochemical properties similar to conventional plastics but, contrary to the latter, they are biodegradable and/or bio compostable, given the appropriate conditions.

PHA can be homopolymers containing only one monomeric unit, co-polymers that are composed by two different monomeric units, or heteropolymers composed of more

than two different monomeric units. In addition, they can be classified based on the length of the alkyl side chain of the polymer. Depending on the carbon source, the metabolic pathway and the specificity of the PHA-synthase employed in their production, biopolymers with different structure and mechanical properties are obtained, which will have the according impact on the final application of the plastic produced (Nielsen *et al.* 2017). For example, the homopolymer polyhydroxybutyrate (PHB) is brittle and stiff, while the co-polymer poly-(3-hydroxybutyrate-co-3-hydroxyvalerate; PHB-co-PHV) has improved thermomechanical properties, leading to a more flexible and impact-resistant material, as its crystallinity, crystallization rate, glass transition temperature (T_g) and melting temperature (T_m) are reduced (Al Battashi *et al.* 2020).

Despite of the advantages of the bio-based plastics, their current high production cost, when compared to the fossil alternative, has been hampering the efforts for a large-scale PHA production and widespread application of the more environmentally friendly polymers. The main contributions to production cost are the raw material used as carbon source for bacterial growth, as well as the extraction processes (Yun, Sawant and Kim 2013; Koller 2020). Furthermore, most of the current production processes implemented commercially use of sugars or oils as main carbon source, competing with food and feed industry. One way to reduce the cost and improve the sustainability of production is to replace refined sugars with carbon sources derived from existing wastes or side-streams, such as VFA (Fig. 3).

Bacteria for PHA production

The production of PHA from VFA could be achieved by fermentative processes using either mixed or pure bacterial cultures, which accumulate PHA intracellularly as carbon and energy reserve. More than 150 different structures of PHA are generated by various genera of bacteria under different growth conditions (Kumar *et al.* 2019). The use of axenic cultures allows the implementation of a process with increased robustness and leads to a more homogeneous and uniform polymer. It is of utmost importance to produce a biopolymer with a predictable monomeric composition and thus with known properties, suitable for the intended end-application. A variety of bacteria are capable of naturally accumulating PHA, with *Pseudomonas putida* and *Cupriavidus necator*-utilizing oils or sugars, respectively, as carbon source—being the most widely used for industrial production. When it comes to the different processes, the use of sugars as carbon source will inevitably result in the PHB homopolymer. If the production of co-polymers is desired, precursors of other monomers need to be additionally provided to the fermentation broth, such as the addition of propionic acid to generate 3HV monomers.

Utilization of VFA as substrate for PHA production

When using VFA as carbon source, the feed-rate needs to be carefully adjusted to minimize the accumulation of acid in the broth, since high concentrations of these compounds are toxic to the cells. In order to do so, the feed-rate profile can be pre-programmed, or a pH-stat strategy can be used, in which the acidic VFA stream is used to control the pH at the specific set-point of the fermentation. Within the VOLATILE project, a process was implemented at Biotrend SA (Portugal) by feeding VFA produced by DF of municipal solid waste that contained acetic, propionic, butyric, valeric and caproic acid, resulting in

a promising PHA productivity of 0.2 g/L/h. A PHB-co-PHV was produced, with 20% 3HV, confirming the incorporation of valeric acid and propionic acid in the polymer. Furthermore, the content of 3HV in the polymer is in line with others reported using the co-addition of concentrated streams of pure, commercial VFA to the otherwise sugar-based fermentation (Yun, Sawant and Kim 2013; Mendonça *et al.* 2014).

BIOCONVERSION OF VFA TO SINGLE CELL OIL

Oleaginous yeasts for VFA valorization

Oleaginous yeasts are per definition yeast strains that can accumulate lipids to more than 20% of their total CDW. These lipids—also called single cell oils (SCO)—act as storage compound for carbon and energy (Papanikolaou and Aggelis 2011a). Oleaginous yeast species extensively studied include e.g. *Lipomyces starkeyi*, *Y. lipolytica*, *Cutaneotrichosporon curvatum* (formerly *Cryptococcus curvatus*), *Naganishia albida* (formerly *Cryptococcus albidus*; Liu *et al.* 2015), *Rhodotorula toruloides* (formerly *Rhodosporidium toruloides*; Wang *et al.* 2015) and *Rhodotorula mucilaginosa* (Papanikolaou and Aggelis 2011b). Following a recently high-throughput screening for yeasts, several new species were described as oil producers namely *Millerozyma farinosa, Trigonopsis cantarelli* and *Geotrichum candidum* (Miranda *et al.* 2020; TRANSBIO project funded under FP7-KBBE, grant agreement ID 289603).

Biochemistry of SCO synthesis in yeasts

There are two different ways of lipid accumulation in yeasts; *ex-novo* and *de-novo*. Biotransformation of specific hydrophobic substrates to FA and subsequently lipids is called *ex-novo* lipid accumulation. The process is growth associated and can be exploited for the biotechnological production of higher value lipids from low-value or waste lipids. *De-novo* lipid accumulation, on the other hand, is not growth associated and is induced by limitation of crucial nutrients like nitrogen and phosphorous, while carbon is available in excess. Although *ex-novo* synthesis is restricted to selected hydrophobic carbon sources, *de-novo* synthesis allows the utilization of a wide range of broadly available substrates, such as glucose-rich wastes, lactose containing whey, xylose-rich lignocellulose hydrolysates, crude glycerol from biodiesel production, as well as short carboxylic acids (Papanikolaou and Aggelis 2011a). While oleaginous yeasts have widely been cultivated on sugars, polysaccharides and glycerol, the use of VFA for SCO production received only modest attention until recently (Fontanille *et al.* 2012). In the last years, however, several reports on the use of acetic, propionic and butyric acid, as well as mixtures of these three VFA, for lipid production have been published (Fei *et al.* 2011a; Béligon *et al.* 2015; Kolouchová *et al.* 2015; Řezanka, Kolouchová and Sigler 2015; Liu *et al.* 2016a; Park *et al.* 2017). In addition, the utilization of VFA mixtures containing iso-butyric, valeric, iso-valeric and caproic acid for SCO production has been also reported (Llamas *et al.* 2020b).

In contrast to other carbon sources, acetate can be metabolized along a very direct pathway in the cell, as already mentioned. The crucial enzyme in this pathway is acetyl-CoA synthetase (ACS; Vorapreeda *et al.* 2012). ACS activates acetate to acetyl-AMP, which is subsequently converted to acetyl-CoA (Starai and Escalante-Semerena 2004). In a similar fashion, ACS can also activate propionate to propionyl-CoA. In addition, the presence of a specific propionyl-CoA synthetase has been reported for *Saccharomyces cerevisiae* (Pronk *et al.* 1994). This
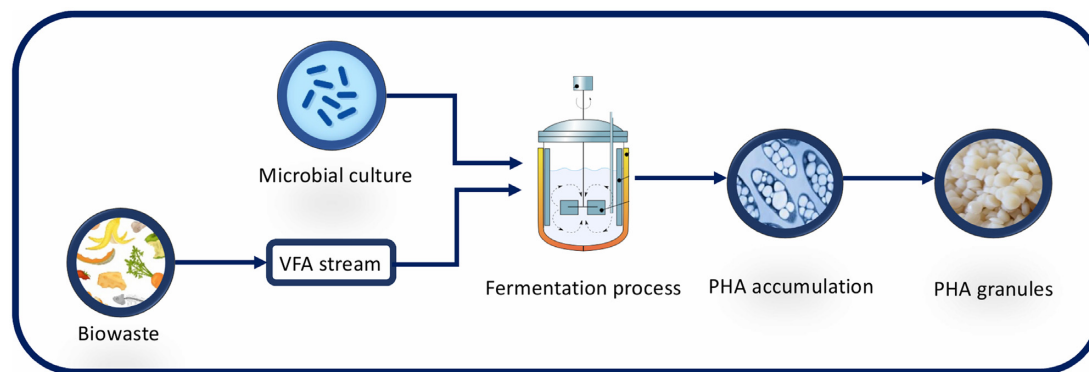
Figure 3. Flow diagram of PHA production from biowaste and microbial cultures.

enzyme also facilitates the activation of propionate to propionyl-CoA (Wu and San 2014), but, to the best of our knowledge, its presence in oleaginous yeasts has not yet been verified.

The presence of ACS in non-oleaginous and oleaginous yeasts has been continuously reported (De Jong-Gubbels *et al.* 1997; Jogl and Tong 2004; Vorapreeda *et al.* 2012; Liu *et al.* 2016b). Since there are several reports on the production of odd numbered FA from propionate, it can, however, be assumed that a metabolic pathway for activation of propionate is present in various oleaginous yeasts species (Park *et al.* 2018a; Kolouchová *et al.* 2015; Řezanka, Kolouchová and Sigler 2015). Propionate is activated to propionyl-CoA, which is condensed with acetyl-CoA to subsequently form odd numbered FA (Park *et al.* 2018b). It follows that the substrate has a direct effect on the composition of the storage lipids. Usually, SCOs contain high levels of C16:0 (up to 25% of the total FA), C18:1 (up to 70% of FA) and C18:2 (up to 25% of FA; Papanikolaou and Aggelis 2011a). However, shares of above 30% of the total FA have been reported for C17:1 with propionate as carbon source (Řezanka, Kolouchová and Sigler 2015; Park *et al.* 2018b). The FA composition is relevant, as it has a considerable impact on the material properties of SCO (Alvarez 2002). Furthermore, individual FA, especially those otherwise uncommon in nature like C17:1, may have value adding properties like antimicrobial effects or medical uses (Ratledge *et al.* 2010; Avis, Boulanger and Elanger 2000). In the case of the SCO produced by yeast or microalgae in the VOLATILE project, a significant part of odd chain fatty acids of C15 and C17 can be found, most likely due to propionic acid inside DF effluent.

Utilization of VFA by oleaginous yeasts

Within the past years, research on SCO aimed mainly towards biodiesel applications. While this resulted in a focus on (crude) glycerol or sugar-rich substrates as carbon source (Papanikolaou and Aggelis 2011b), VFA-rich substrates were and still are, a topic of interest. While glucose is a convenient carbon source, it is simply too expensive for the production of a bulk product like biodiesel. Waste-derived VFA or VFA-rich hydrolysates, on the contrary, offer a low-cost alternative (Fei *et al.* 2011a). Still, several factors have to be considered when they are used as carbon source. For instance, VFA concentrations in these substrates are often below 20 g/L (Chi *et al.* 2011; Lian *et al.* 2012; Peng *et al.* 2013; Xu et al. 2014, 2015; Gong *et al.* 2015, 2016), which renders them unsuitable for fed-batch processes due to volumetric constraints. Furthermore, such waste-derived effluents often contain growth limiting or even inhibiting compounds (Chi *et al.* 2011; Lian *et al.* 2012; Yu *et al.* 2020). In

addition, high VFA concentrations themselves can have adverse effects on the yeast cells, especially at VFA concentrations above 5 g/L (Fontanille *et al.* 2012) and low pH-values. A summary of several studies addressing the use of VFAs for lipid production in various yeast strains is given in Table 1.

Microalgae for VFA valorization

Microalgae is another category of oleaginous microorganisms, capable of accumulating high amounts of SCO, which, in specific species include valuable FA, such as omega-3. They comprise a vast category of microorganisms that has attracted industrial attention during the last decades owning to their ability to accumulate valuable bioactive metabolites, coupled with their easy fermentation. Most of the strains are found in saline or freshwater ecosystems and grow autotrophically, absorbing the dissolved carbon dioxide from water. However, some microalgae lack the ability of photosynthesis and therefore grow heterotrophically by consuming conventional carbon sources (Singh and Saxena 2015). Also, specific autotrophic strains will grow heterotrophically if they are provided a carbon source, while combined fermentation is referred to as mixotrophy.

Both autotrophic and heterotrophic fermentation techniques have been developed for microalgae in pilot or industrial units. Although the first category allows for low cost production, being able to utilize resources abundantly available, such as the solar energy and the atmospheric CO_2, heterotrophic fermentation gains momentum, as means of controlling more efficiently the culture conditions and absolving the process from the—otherwise necessary—weather and climate dependence (Perez-Garcia *et al.* 2011). What is more, this type of fermentation leads to much higher biomass productivities, consequently enabling higher final product yields (Liang, Sarkany and Cui 2009; Bumbak *et al.* 2011). In truth, the only drawback of heterotrophic cultures-assuming that proper techniques to avoid any culture contamination are a prerequisite- is the cost of the raw material, that is, the feed of the culture.

In order to overcome this constrain, new low-, or even zero-cost, resources are searched. DF-derived VFA have repeatedly been examined as a promising carbon source for microalgae fermentation (Liu *et al.* 2013; Chalima *et al.* 2019), with researchers focusing mostly on the strains and feeding method that can effectively support VFA bioconversion.

Although many microalgae are able to assimilate acetate, which is the most abundant organic acid in a DF effluent, there are just a few candidates that are able to consume or even survive, small quantities of other VFA, such as butyric or

Table 1. Use of VFA (mixture, or isolated acetic, propionic and butyric acid) as alternative carbon sources for oleaginous (*Cryptococcus albidus*, *Candida sp*, *Trichosporon cutaneum*, *Rhodosporidium toruloides*, *Rhodotorula glutinis*, *Yarrowia lipolytica* and *Cryptococcus curvatus*) and non-oleaginous (*Saccharomyces cerevisiae* and *Torulaspora delbrueckii*) yeast species, and their performance regarding the capacity of each of them to accumulate lipids.

Yeast species	Carbon source (g/L)	Cultivation mode	Lipid conc. (g/L)	Lipid content (% w/w)	$Y_{L/S}$ (g/g)	Ref.
Cryptococcus albidus	VFAs[a]	Two-stage batch	14.5	55.0	0.17	Fei *et al.* (2011b)
Cryptococcus curvatus	Acetic acid (40)	Batch	5.01	71.7	NA	Huang *et al.* (2018b)
Candida sp.	Acetic acid (4)	Batch	0.76	24.2	NA	Kolouchová *et al.* (2015)
Trichosporon cutaneum	Propionic acid (4)	Batch	0.38	23.9	NA	Kolouchová *et al.* (2015)
Saccharomyces cerevisiae	Acetic acid (4)	Batch	0.05	2.80	NA	Kolouchová *et al.* (2015)
Torulaspora delbrueckii	Propionic acid (4)	Batch	0.08	2.70	NA	Kolouchová *et al.* (2015)
Rhodotorula glutinis	Acetic acid (4)	Batch	0.55	19.50	NA	Kolouchová *et al.* (2015)
Rhodosporidium toruloides	Acetic acid (10)	batch	NA	19.1	0.16	Huang *et al.* (2016)
Yarrowia lipolytica	Acetic acid (12)	Fed-batch	1.84	30.8	0.15	Fontanille *et al.* (2012)
Yarrowia lipolytica	Propionic acid (8)	Fed-batch	0.87	25.7	0.11	Fontanille *et al.* (2012)
Yarrowia lipolytica	Butyric acid (12)	Fed-batch	0.85	25.1	0.07	Fontanille *et al.* (2012)
Cryptococcus curvatus	Acetate (5)/acetic acid (700)[b]	Fed-batch	28.4	60.0	0.18	Béligon *et al.* (2015)

$Y_{L/S}$: lipid yield (g/g C); NA: not available.
[a] Combination of acetic, propionic and butyric acids at a ratio of 6:1:3.
[b] Acetate used as initial carbon source, acetic acid used as feed.

propionic acid (Table 2). Some *Chlorella* species, such as *Chlorella vulgaris* and *Chlorella sorokiniana* have been known to assimilate butyrate, but only under specific conditions (Moon *et al.* 2013; Ren *et al.* 2013). Also *Auxenochlorella protothecoides* (Turon *et al.* 2015), *Scenedesmus* sp. (Ren *et al.* 2013) and *Chlamydomonas reinhardtii* were able to proliferate in mixtures of VFA, with the latter being cultivated also mixotrophically (Moon *et al.* 2013).

VFA growth inhibition

According to previous research results, each microalga exhibits a different tolerance to VFA, when one or more of them are used as main carbon source. This strain dependent VFA assimilation seems to be also affected by culture conditions, such as the pH of the medium, since all microalgae reported so far achieve a better acid assimilation when cultivated in rather neutral pH values (Table 2). Therefore, the maintenance of a proper pH appears to be of great importance for the effective microalgae fermentation.

So far it is obvious that the main obstacle for basic VFA consumption is butyrate, rather than acetate assimilation. When VFA mixtures are examined, researches have repeatedly observed that acetate and butyrate are consumed in a diauxic manner, that is, the preferred substrate—acetate—is consumed first, before butyrate assimilation begins. To the best of our knowledge there is, but one microalga, reported in literature capable of withstanding high concentrations of butyrate. On behalf of VOLATILE project, *Crypthecodinium cohnii* has been successfully cultivated in butyrate and even propionate at initial concentrations as high as 30 g/L or 20 g/L, respectively (Chalima *et al.* 2019).

Utilization of VFA for omega-3 production

The majority of microalgae produced industrially are exploited as dietary supplements in the form of dried biomass (Brasil *et al.* 2017). However, another popular application involves the fermentation of specific oleaginous strains for the extraction of lipids. Microalgal lipids can be used either as alternative source

of esters for biodiesel production (Wen *et al.* 2013), or as functional commodities of nutritional value, especially when they are comprised of a significant percentage of omega-3 FA (Lopes and Silva 2019). Industrial production of omega-3 FA by the utilization of biowaste-derived VFA is indeed a very attractive option, which allows the establishment of a circular process with fewer waste streams. Furthermore, developing a process for a final high added-value product, allows some liberties on the fermentation and downstream process strategy, which is not possible in the case of a bulk product such as biodiesel.

Omega-3 FA are comprised of four FA; α-linolenic acid (ALA), eicosapentaenoic acid (EPA), docosapentaenoic acid (DPA) and docosahexaenoic acid (DHA; Ghasemifard, Turchini and Sinclair 2014; Byelashov, Sinclair and Kaur 2015). The human organism is incapable of synthesizing substantial amounts of EPA and DHA and therefore, these FA must be taken up through our nutrition. Although fish oil is, for the present, the basic source of EPA and DHA, it cannot be deemed as a sustainable choice, due to constant fish stock depletion. What is more, fish tissue contains always a mixture of EPA/DHA, when in specific food applications, the addition of only one FA is desirable (Chalima *et al.* 2017). For the above reasons, the substitution of fish with algaloil is constantly gaining momentum. Currently some microalgae strains cultivated for omega-3 production include *C. cohnii* and *Schizochytrium limanicum* (Chalima *et al.* 2017). *C. cohnii* is being cultivated since the 1990s as an industrial producer of DHA and its oil has long received a GRAS (generally recognized as safe) certificate (Ryan and Nelson 2010). Chalima *et al.* (2020) succeeded, within the VOLATILE initiative, to cultivate the strain *C. cohnii* ATCC 30772 in a fed-batch process with a feed of a DF effluent from a vegetable, garden and food biowaste stream. The cells grew effectively, assimilating the available carbon of the VFA content, while accumulating 35.6% DHA of total FA.

FERMENTATION TECHNIQUES

PHA producing bacteria

Fermentation can greatly influence the total product of a bioconversion process, while, depending on the PHA producer, the

Table 2. Microalgae strains that have been known to assimilate a mixture of VFA (pure or waste-derived).

Microalgae species	Carbon source	Process	Production of dry biomass	Ref.
Chlorella vulgaris ESP6	Mixture of acetate, lactate, butyrate and HCO-	Photoheterotrophic, constant pH = 7.5	0.87 g/L	Liu *et al.* (2013)
Chlorella protothecoides FACHB-3	WAS hydrolysate containing a mixture of VFA	Heterotrophic	0.5 g/L	Wen *et al.* (2013)
Arctic Chlorella sp. ArM0029B	Acetic, propionic and butyric acids in ratio 6:1:3 (by mass)	CO_2 supplied- mixotrophic followed by the VFA supply	0.38 g/L/d	Ryu *et al.* (2015)
Micractinium inermum F014	Acetic, propionic and butyric acids in ratio 6:1:3 (by mass)	CO_2 supplied- mixotrophic followed by the VFA supply	0.37 g/L/d	Ryu *et al.* (2015)
Ettlia sp. YC001	Acetic, propionic and butyric acids in ratio 6:1:3 (by mass)	CO_2 supplied- mixotrophic followed by the VFA supply	0.17 g/L/d	Ryu *et al.* (2015)
Chlorella protothecoides UTEX 25	Acetic acid: propionic acid: butyric acid in a ratio 8:1:1	Heterotrophic	0.61 g/L	Fei *et al.* (2015)
Chlamydomonas reinhardtii wild-type strain CC-124	Acetic acid: propionic acid: butyric acid in a ratio 8:1:1	Mixotrophic	2.05 g/L	Moon *et al.* (2013)
Chlorella sorokiniana (CCAP 211/8K)	Acetate and butyrate	Mixotrophic	1.14 g/L	Turon *et al.* (2015)
Scenedesmus sp. strain R-16	Butyrate	Heterotrophic	0.79 g/L	Ren *et al.* (2013)
Crypthecodinium cohnii strain ATCC 30 772	DF effluent from biowaste	Heterotrophic (pH-auxostat culture)	6.5 ± 0.3 g/L	Chalima *et al.* (2019)
Acutodesmus obliquus	Butyrate	mixotrophic	4.3 g/g*	Lacroux *et al.* (2020)

*The yield due to heterotrophy alone under mixotrophic conditions is estimated at 1.06 g DCW/g.

fermentation method differs. A possible approach, which has been applied for enhanced production of antibiotics, recombinant proteins and PHA, is a cyclic fed-batch fermentation strategy. Singh *et al.* (2021), reported the employment of such strategy for PHA production by *Bacillus thuringiensis* using a glucose-rich hydrolysate from dry de-ashed pulp and paper mill sludge as carbon source, entailing the partial withdrawal of fermentation medium and subsequent re-filling with an equal volume of fresh fermentation medium (Singh *et al.* 2021). As a result, the concentrations of toxic by-products remained low, allowing enhanced cell growth to achieve high concentrations of final biomass and increased PHA yields.

When using low concentration feedstocks, the productivity of the process is greatly affected by the dilution of the broth due to the addition of large volumes of substrate. A promising way to obtain high cell densities and productivities would be the use of cell recycling systems coupled to fed-batch processes (Kourmentza *et al.* 2017). This strategy, employing cross-flow membranes to recirculate cells into the fed-batch reactor using a recombinant *E. coli* resulted in cell densities up to 200 g/L with a productivity of 4.6 g PHA/L/h, using as carbon source a whey solution rich in lactose (Ahn, Park and Lee 2001).

Although several bacteria are able to produce PHA during growth and do not require growth-limiting conditions (Kourmentza *et al.* 2017), usually a two-stage process could be necessary, the first for biomass build-up, followed by a PHA accumulation stage triggered by the limitation of a growth-essential nutrient and supply of excess carbon. This is the case of another fermentation strategy in which a fed-batch process using wheat straw hydrolysate as carbon source was developed to produce PHA using a Gram-negative bacterium from the genus *Paraburkholderia* (Cesário *et al.* 2014).

Oleaginous yeasts

Due to naturally low VFA concentrations found in effluents, simple batch processes are widely reported at lab scale. Low VFA concentrations prevent substrate inhibition to a certain extent; biomass concentrations reached, however, are usually below 10 g/L. Even though lipid contents of above 50% of the cell dry weight have been reported for such processes, volumetric lipid productivities are deemed too low for industrial scale applications (Fei *et al.* 2011a; Zheng *et al.* 2012; Kolouchová *et al.* 2015; Huang *et al.* 2016, 2018b; Park *et al.* 2017). Increases in lipid content (Xu *et al.* 2015) and volumetric lipid productivities (Huang *et al.* 2016) have also been reported for sequencing batch processes, in which biomass is harvested after a certain amount of time and re-suspended in fresh VFA-rich medium. Overall volumetric lipid productivities, however, remain low. Therefore, several studies propose the use of a two-stage batch process; glucose is used to facilitate initial cell growth and after its depletion, VFA are added to the medium for lipid production in nutrient limited conditions. While specific parameters could be improved, overall performance in such cases was nevertheless comparable to single stage batch processes (Fei *et al.* 2011b; Huang *et al.* 2016).

Two-stage approaches have also been used for fed-batch fermentation of oleaginous yeasts, which allow high volumetric productivities by utilizing VFA, without the drawback of reaching inhibitory acid concentrations. In conventional processes, glucose or glycerol are commonly used as carbon source in the first stage of the process, in order to promote initial cell growth—a major factor for determining the maximum final lipid production. In the second stage, VFA are subsequently added as feed for lipid production. This way, biomass concentrations of up to 41 g/L, with lipid contents of 55% CDW and volumetric lipid productivities up to 0.33 g/L·h have been reached (Fei *et al.* 2011b; Christophe *et al.* 2012; Fontanille *et al.* 2012). The drawback of the initial use of a different substrate can be eliminated only by utilizing a VFA containing carbon source or pure VFA both for initial growth and subsequent SCO production. Béligon *et al.* (2015) observed that *C. curvatus* was able to reach biomass concentrations of 80 g/L in a pH-stat fed-batch process when solely grown on acetate. SCO content of the biomass was, however,

significantly low. By varying fermentation conditions, the lipid concentration could be increased from 12 to 28.4 g/L. In a similar setup, Chi *et al*. (2011) demonstrated that an even higher biomass concentration of 186 g/L was possible with the same strain (Chi *et al*. 2011; Béligon *et al*. 2015). Furthermore, a SCO content of 75% could be obtained, resulting in volumetric lipid productivity of 0.66 g/L·h. This was achieved by using VFA containing DF effluent as initial medium. Data on the economic feasibility of VFA based SCO production processes is limited. Based on calculations for other carbon sources, a minimum SCO price of 2.4\$/kg is estimated (Bonatsos *et al*. 2020). For many applications, this is not competitive to the price of vegetable oils (Koutinas *et al*. 2014). However, there is potential for improvement of the production parameters (product yield, titer, volumetric productivity), especially for VFA based processes, through the use and further development of continuous or semi-continuous systems with cell retention (Llamas *et al*. 2020b).

Microalgae

To overcome the apparent inhibitory effect of butyrate, mentioned previously, and even acetate at higher concentrations, researchers have examined various fermentation techniques. It is common that the enrichment of cell biomass increases the tolerance of the culture to less preferable substrates. Such techniques, that in the past involved enrichment of the medium with the preferred acetate, or growth under mixotrophic conditions, promote the early proliferation of the cells and allow a more efficient utilization of butyrate later (Ren *et al*. 2013; Ryu *et al*. 2015). The mixotrophic fermentation strategy increased the biomass and acid consumption in the case of *C. reinhardtii* and *C. sorokiniana*. Furthermore, the initial addition of a higher acetate percentage has repeatedly been proven effective towards combined higher assimilation of butyrate (Turon *et al*. 2016). This fact can be the result of more cells being initially produced by acetate consumption.

In the case of a fed-batch culture, the ability to maintain a non-inhibitory acid concentration is enhanced. Many proposed fermentation techniques therefore support the use of a fed-batch pH-stat system. That way microalgae have been effectively cultivated, while a feed of organic acids is added in a controlled manner, in order to ensure both the continuously low concentration of the acids inside the bioreactor vessel and also the maintenance of a stable, favorable pH (Ratledge *et al*. 2001). In the case of a feed of a DF effluent, the fed-batch fermentation was proven to be even more productive, in terms of biomass and lipids, compared to the corresponding feed of pure acids (Chalima *et al*. 2019). This is a result of the residual organic content present in the liquid fraction of the pretreated waste, apart from VFA,-such as lactic and formic acid or ethanol (Bastidas-Oyanedel *et al*. 2015)-as well as the high concentration of ammonium that enhances cell growth. It is therefore, safe to assume, that when the proper cultivation mode eliminates the inhibitory effects of a DF liquid fraction, its organic residue acts favorably for the fermentation outcome, offering one more advantage to the VFA platform concept.

OTHER POSSIBLE END-PRODUCTS

VFA are characterized as platform chemicals, which means that a wider variety of applications can be considered, even for waste-derived, mixed VFA. Direct use after separation and concentration is possible, e.g. as carbon source in denitrification processes or alternatively, as preservative in food and feed,

since VFA are known to have antimicrobial properties (Zacharof and Lovitt 2013; Lee *et al*. 2014). When coupling a secondary microbial process to the initial VFA production platform, other high added-value applications are possible. Two possible routes can be microbial protein production and chain elongation to medium chain fatty acids (MCFA).

Microbial protein is considered as a sustainable and future-proof route to meet the increasing demand of protein-rich feed and food. It consists of microbial biomass from aerobic heterotrophic bacteria or photoheterotrophic bacteria, with a high amino-acid content (Matassa *et al*. 2016; Alloul *et al*. 2019). VFA obtained from the fermentation of diverse waste streams can be used directly as carbon source for microbial protein production (Alloul *et al*. 2018). This route furthermore valorizes the nutrients solubilized in the organic waste fermentation.

Chain elongation to MCFA is a microbial process leading to VFA elongation, via reverse β-oxidation. The process requires an electron donor, typically ethanol. It is often observed as side-process in anaerobic mixed microbial fermentation, and is now developed as a separate biotechnological process (Angenent *et al*. 2016). The usual end-product, caproic acid, has a low solubility in water in its acid form, thus facilitating product recovery, compared to shorter chain VFA. Caproic acid has applications in the feed industry, chemical industry and as fuel precursor (De Groof *et al*. 2019). However, it must be noted that sustainable caproic acid production from VFA requires a sustainable ethanol source (Chen *et al*. 2017). A second chain elongation pathway is now gaining attention, which does not require an external electron donor source; chain elongation with lactic acid as electron donor (De Groof *et al*. 2019). Lactic acid is another by-product of DF, which appears naturally in small amounts in effluents. Therefore, for such a plan, no external addition is needed. In terms of organic waste valorization, this implies broadening of the VFA platform term to a carboxylate platform, in which also lactic acid metabolism is included. In order to establish such a platform, research efforts should be directed towards understanding the conditions in which lactic acid is efficiently produced, and which conditions favor VFA production during waste fermentation. This approach will greatly increase the applicability of the VFA/carboxylate platform.

FUTURE PERSPECTIVES FOR VFA-BASED BIOPROCESSES

It is obvious that a VFA platform can offer a solution to the microbial valorization of carbon contained in waste biomass. Omega-3 FA for food and nutraceuticals, as well as PHA as a plastic alternative, are commodities of increasing value. As a result, their integration in a biowaste biorefinery, whose main goal is the release of added-value products coupled with a waste minimization (Chandra *et al*. 2019), is a realistic prospect. It must be noted that microalgal oil is a certified GRAS commodity that is already in the market of baby formulas and adult supplements (Wynn *et al*. 2010). The production of omega-3 from VFA has been shown to be feasible by microalgae fermentation, which results in a pure oil product that can be analyzed to its main components with simple techniques (Chalima *et al*. 2019). Therefore, it is possible that the use of a waste-derived feed does not reduce the quality or safety of the final extracted product and should be attempted in industrial scale.

Also, SCO offer many advantages against conventional oil sources. Although SCO production from many different carbon sources has been reported in literature, to our knowledge no

industrial process has been implemented to date. While considerable amount of literature focuses on the production of biodiesel, the production of low value products like biodiesel, fatty alcohols or fatty amines is not economically feasible (Probst *et al.* 2016). However, if it is possible to produce SCO with a specific fatty acid spectrum suitable for high value applications, the impact of comparably high production costs is lower due to the higher value of the product (Koutinas *et al.* 2014). Depending on substrate costs, as well as the intended use of the SCOs, VFA based SCO production poses an interesting opportunity for the production of speciality chemicals.

Finally, the potential markets for PHAs produced by VFAs derived from the treatment of waste are constrained by regulation, availability of VFAs as raw material and product specification. The most promising markets combine the willingness to pay a premium for the specific functionality of biodegradability, with the potential regulatory constraints caused by the raw material being a waste. The enhanced mechanical properties, due to the significant incorporation of 3HV, which imparts increased flexibility, allow the use of PHA in flexible materials, such as films, for example on-site biodegradable agricultural mulch films, biodegradable or compostable garbage bags, biodegradable pots for plant nurseries and use in self-healing concrete. Still, while a wide range of VFA-rich substrates is in principle available, most fed-batch processes described in literature- for bacteria, yeasts or microalgae- rely heavily on the utilization of commercially available chemical grade acetic acid. This discrepancy arises from the need for highly concentrated VFA feed solutions and the comparably low VFA concentrations in many substrates, which results in low productivities and a significant dilution of the broth. Potential ways to circumvent this issue include the concentration of the VFA stream using electrodialysis, or nanofiltration and reverse osmosis steps, ideally powered by renewable energy, or performing the fermentation using cell retention devices in order to keep high cell concentrations and hence productivities. In addition, other fermentation modes like continuous processes or a cell-recycling approach have to be explored. To our knowledge, the latter concept has not yet been realized for VFA based microbial oil production, however, continuous processes have occasionally been described for oleaginous yeasts (Gong *et al.* 2015; Vajpeyi and Chandran 2015).

ACKNOWLEDGMENTS

REFERENCES

Ahn WS, Park SJ, Lee SY. Production of poly(3-hydroxybutyrate) from whey by cell recycle fed-batch culture of recombinant *Escherichia coli. Biotechnol Lett* 2001;**23**:235–40.

Al Battashi H, Al-Kindi S, Gupta VK *et al.* Polyhydroxyalkanoate (PHA) production using volatile fatty acids derived from the anaerobic digestion of waste paper. *J Polym Environ* 2020. DOI: 10.1007/s10924-020-01870-0.

Alloul A, Ganigué R, Spiller M *et al.* Capture-ferment-upgrade: a three-step approach for the valorization of sewage organics as commodities. *Environ Sci Technol* 2018;**52**:6729–42.

Alloul A, Wuyts S, Lebeer S *et al.* Volatile fatty acids impacting phototrophic growth kinetics of purple bacteria: paving the way for protein production on fermented wastewater. *Water Res* 2019;**152**:138–47.

Alvarez MHM. Triacylglycerols in prokaryotic microorganisms. *Appl Microbiol Biotechnol* 2002;**60**:367–76.

Angenent LT, Richter H, Buckel W *et al.* Chain elongation with reactor microbiomes: open-culture biotechnology to produce biochemicals. *Environ Sci Technol* 2016;**50**:2796–810.

Avis TJ, Boulanger RR, Elanger RRB. Synthesis and biological characterization of (z) -9-heptadecenoic and (z) -6-methyl-9-heptadecenoic acids: fatty acids with antibiotic activity produced by *Pseudozyma flocculosa.* 2000;**26**:987–1000.

Bastidas-Oyanedel JR, Bonk F, Thomsen MH *et al.* Dark fermentation biorefinery in the present and future (bio)chemical industry. *Rev Environ Sci Biotechnol* 2015;**14**:473–98.

Béligon V, Poughon L, Christophe G *et al.* Improvement and modeling of culture parameters to enhance biomass and lipid production by the oleaginous yeast *Cryptococcus curvatus* grown on acetate. *Bioresour Technol* 2015;**192**:582–91.

Beopoulos A, Cescut J, Haddouche R *et al. Yarrowia lipolytica* as a model for bio-oil production. *Prog Lipid Res* 2009;**48**:375–87.

Bonatsos N, Marazioti C, Moutousidi E *et al.* Techno-economic analysis and life cycle assessment of heterotrophic yeast-derived single cell oil production process. *Fuel* 2020;**264**:116839.

Brasil B dos SAF, de Siqueira FG, Salum TFC *et al.* Microalgae and cyanobacteria as enzyme biofactories. *Algal Research* 2017;**25**:76–89.

Bumbak F, Cook S, Zachleder V *et al.* Best practices in heterotrophic high-cell-density microalgal processes: achievements, potential and possible limitations. *Appl Microbiol Biotechnol* 2011;**91**:31–46.

Byelashov OA, Sinclair AJ, Kaur G. Dietary sources, current intakes, and nutritional role of omega-3 docosapentaenoic acid. *Lipid Technol* 2015;**27**:79–82.

Cesário MT, Raposo RS, de Almeida MCMD *et al.* Enhanced bioproduction of poly-3-hydroxybutyrate from wheat straw lignocellulosic hydrolysates. *New Biotechnol* 2014;**31**:104–13.

Chakraborty P, Gibbons W, Muthukumarappan K. Conversion of volatile fatty acids into polyhydroxyalkanoate by Ralstonia eutropha. *J Appl Microbiol* 2009;**106**:1996–2005.

Chalima A, Hatzidaki A, Karnaouri A *et al.* Integration of a dark fermentation e ffl uent in a microalgal-based biore fi nery for the production of high-added value omega-3 fatty acids. *Appl Energy* 2019;**241**:130–8.

Chalima A, Oliver L, De Castro LF *et al.* Utilization of volatile fatty acids from microalgae for the production of high added value compounds. *Fermentation* 2017;**3**. DOI: 10.3390/fermentation3040054.

Chalima A, Taxeidis G, Topakas E. Optimization of the production of docosahexaenoic fatty acid by the heterotrophic microalga *Crypthecodinium cohnii* utilizing a dark fermentation ef fl uent. *Renew Energy* 2020;**152**:102–9.

Chandra R, Iqbal HMN, Vishal G *et al.* Algal biorefinery: a sustainable approach to valorize algal-based biomass towards multiple product recovery. *Bioresour Technol* 2019;**278**:346–59.

Chen WS, Strik DPBTB, Buisman CJN *et al.* Production of caproic acid from mixed organic waste: an environmental life cycle perspective. *Environ Sci Technol* 2017;**51**. DOI: 10.1021/acs.est.6b06220.

Chi Z, Zheng Y, Ma J *et al*. Oleaginous yeast *Cryptococcus curvatus* culture with dark fermentation hydrogen production effluent as feedstock for microbial lipid production. *Int J Hydrogen Energy* 2011;**36**. DOI: 10.1016/j.ijhydene.2011.04.124.

Christophe G, Deo JL, Kumar V *et al*. Production of oils from acetic acid by the oleaginous yeast *Cryptococcus curvatus*. *Appl Biochem Biotechnol* 2012;**167**:1270–9.

De Groof V, Coma M, Arnot T *et al*. Medium chain carboxylic acids from complex organic feedstocks by mixed culture fermentation. *Molecules* 2019;**24**. DOI: 10.3390/molecules24030398.

De Jong-Gubbels P, Van Den Berg MA, Steensma HY *et al*. The *Saccharomyces cerevisiae* acetyl-coenzyme a synthetase encoded by the a ACS1 gene, but not the a ACS2-encoded enzyme, is subject to glucose catabolite inactivation. *FEMS Microbiol Lett* 1997;**153**. DOI: 10.1016/S0378-1097(97)00236-X.

Fei Q, Chang HN, Shang L *et al*. Exploring low-cost carbon sources for microbial lipids production by fed-batch cultivation of *Cryptococcus albidus*. *Biotechnol Bioprocess Eng* 2011b;**16**. DOI: 10.1007/s12257-010-0370-y.

Fei Q, Chang HN, Shang L *et al*. The effect of volatile fatty acids as a sole carbon source on lipid accumulation by *Cryptococcus albidus* for biodiesel production. *Bioresour Technol* 2011a;**102**. DOI: 10.1016/j.biortech.2010.10.141.

Fei Q, Fu R, Shang L *et al*. Lipid production by microalgae Chlorella protothecoides with volatile fatty acids (VFAs) as carbon sources in heterotrophic cultivation and its economic assessment. *Bioprocess Biosyst Eng* 2015;**38**:691–700.

Fontanille P, Kumar V, Christophe G *et al*. Bioconversion of volatile fatty acids into lipids by the oleaginous yeast *Yarrowia lipolytica*. *Bioresour Technol* 2012;**114**. DOI: 10.1016/j.biortech.2012.02.091.

Gao R, Li Z, Zhou X *et al*. Enhanced lipid production by *Yarrowia lipolytica* cultured with synthetic and waste-derived high-content volatile fatty acids under alkaline conditions. *Biotechnol Biofuels* 2020;**13**. DOI: 10.1186/s13068-019-1645-y.

Garay LA, Boundy-Mills KL, German JB. Accumulation of high-value lipids in single-cell microorganisms: mechanistic approach and future perspectives. *J Agric Food Chem* 2014;**62**:2709–27.

Ghasemifard S, Turchini GM, Sinclair AJ. Omega-3 long chain fatty acid "bioavailability": a review of evidence and methodological considerations. *Prog Lipid Res* 2014;**56**:92–108.

Gong Z, Shen H, Zhou W *et al*. Efficient conversion of acetate into lipids by the oleaginous yeast *Cryptococcus curvatus*. *Biotechnol Biofuels* 2015;**8**. DOI: 10.1186/s13068-015-0371-3.

Gong Z, Zhou W, Shen H *et al*. Co-fermentation of acetate and sugars facilitating microbial lipid production on acetate-rich biomass hydrolysates. *Bioresour Technol* 2016;**207**. DOI: 10.1016/j.biortech.2016.01.122.

Hao J, Wang X, Wang H. Overall process of using a valerate-dominant sludge hydrolysate to produce high-quality polyhydroxyalkanoates (PHA) in a mixed culture. *Sci Rep* 2017;**7**. DOI: 10.1038/s41598-017-07154-3.

Huang L, Chen Z, Wen Q *et al*. Insights into Feast-Famine polyhydroxyalkanoate (PHA)-producer selection: microbial community succession, relationships with system function and underlying driving forces. *Water Res* 2018a;**131**. DOI: 10.1016/j.watres.2017.12.033.

Huang X-F, Shen Y, Luo H-J *et al*. Enhancement of extracellular lipid production by oleaginous yeast through preculture and sequencing batch culture strategy with acetic acid. *Bioresour Technol* 2018b;**247**:395–401

Huang XF, Liu JN, Lu LJ *et al*. Culture strategies for lipid production using acetic acid as sole carbon source by

Rhodosporidium toruloides. *Bioresour Technol* 2016;**206**. DOI: 10.1016/j.biortech.2016.01.073.

Jogl G, Tong L. Crystal structure of yeast acetyl-coenzyme A synthetase in complex with AMP. *Biochemistry* 2004;**43**. DOI: 10.1021/bi035911a.

Koller M. Established and advanced approaches for recovery of microbial polyhydroxyalkanoate (PHA) biopolyesters from surrounding microbial biomass. *EuroBiotech J* 2020;**4**:113–26.

Kolouchová I, Schreiberová O, Sigler K *et al*. Biotransformation of volatile fatty acids by oleaginous and non-oleaginous yeast species. *FEMS Yeast Res* 2015;**15**. DOI: 10.1093/femsyr/fov076.

Korhonen J, Honkasalo A, Seppälä J. Circular economy : the concept and its limitations. *Ecol Econ* 2018;**143**:37–46.

Kourmentza C, Plácido J, Venetsaneas N *et al*. Recent advances and challenges towards sustainable polyhydroxyalkanoate (PHA) production. *Bioengineering* 2017;**4**. DOI: 10.3390/bioengineering4020055.

Koutinas AA, Chatzifragkou A, Kopsahelis N *et al*. Design and techno-economic evaluation of microbial oil production as a renewable resource for biodiesel and oleochemical production. *Fuel* 2014;**116**. DOI: 10.1016/j.fuel.2013.08.045.

Kumar G, Ponnusamy VK, Bhosale RR *et al*. A review on the conversion of volatile fatty acids to polyhydroxyalkanoates using dark fermentative effluents from hydrogen production. *Bioresour Technol* 2019;**287**:121427.

Lacroux J, Trably E, Bernet N *et al*. Mixotrophic growth of microalgae on volatile fatty acids is determined by their undissociated form. *Algal Res* 2020;**47**:101870.

Lee WS, Chua ASM, Yeoh HK *et al*. A review of the production and applications of waste-derived volatile fatty acids. *Chem Eng J* 2014;**235**. DOI: 10.1016/j.cej.2013.09.002.

Liang Y, Sarkany N, Cui Y. Biomass and lipid productivities of *Chlorella vulgaris* under autotrophic, heterotrophic and mixotrophic growth conditions. *Biotechnol Lett* 2009;**31**: 1043–9.

Lian J, Garcia-Perez M, Coates R *et al*. Yeast fermentation of carboxylic acids obtained from pyrolytic aqueous phases for lipid production. *Bioresour Technol* 2012;**118**. DOI: 10.1016/j.biortech.2012.05.010.

Liu CH, Chang CY, Liao Q *et al*. Photoheterotrophic growth of *Chlorella vulgaris* ESP6 on organic acids from dark hydrogen fermentation effluents. *Bioresour Technol* 2013;**145**: 331–6.

Liu J, Yuan M, Liu JN *et al*. Microbial conversion of mixed volatile fatty acids into microbial lipids by sequencing batch culture strategy. *Bioresour Technol* 2016a;**222**. DOI: 10.1016/j.biortech.2016.09.100.

Liu XZ, Wang QM, Göker M *et al*. Towards an integrated phylogenetic classification of the *Tremellomycetes*. *Stud Mycol* 2015;**81**. DOI: 10.1016/j.simyco.2015.12.001.

Liu Y, Zhang M, Wang T *et al*. Two acetyl-CoA synthetase isoenzymes are encoded by distinct genes in marine yeast *Rhodosporidium diobovatum*. *Biotechnol Lett* 2016b;**38**. DOI: 10.1007/s10529-015-2006-y.

Llamas M, Dourou M, González-Fernández C *et al*. Screening of oleaginous yeasts for lipid production using volatile fatty acids as substrate. *Biomass Bioenergy* 2020a;**138**. DOI: 10.1016/j.biombioe.2020.105553.

Llamas M, Magdalena JA, González-Fernández C *et al*. Volatile fatty acids as novel building blocks for oil-based chemistry via oleaginous yeast fermentation. *Biotechnol Bioeng* 2020b;**117**. DOI: 10.1002/bit.27180.

Lopes T, Silva C. The Dark Side of Microalgae Biotechnology : A Heterotrophic Biorefinery Platform Directed to ω-3 Rich Lipid

Production, *Microorganisms* 2019;**7**:1–21. DOI: 0.3390/microorganisms7120670.

Lukajtis R, Hołowacz I, Kucharska K *et al*. Hydrogen production from biomass using dark fermentation. *Renew Sustain Energy Rev* 2018;**91**:665–94.

Matassa S, Boon N, Pikaar I *et al*. Microbial protein: future sustainable food supply route with low environmental footprint. *Microb Biotechnol* 2016;**9**. DOI: 10.1111/1751-7915.12369.

Mendonça TT, Gomez JGC, Buffoni E *et al*. Exploring the potential of *Burkholderia sacchari* to produce polyhydroxyalkanoates. *J Appl Microbiol* 2014;**116**:815–29.

Mengmeng C, Hong C, Qingliang Z *et al*. Optimal production of polyhydroxyalkanoates (PHA) in activated sludge fed by volatile fatty acids (VFAs) generated from alkaline excess sludge fermentation. *Bioresour Technol* 2009;**100**. DOI: 10.1016/j.biortech.2008.09.014.

Miranda C, Bettencourt S, Pozdniakova T *et al*. Modified high-throughput Nile red fluorescence assay for the rapid screening of oleaginous yeasts using acetic acid as carbon source. *BMC Microbiol* 2020;**20**. DOI: 10.1186/s12866-020-01742-6.

Moon M, Kim CW, Park WK *et al*. Mixotrophic growth with acetate or volatile fatty acids maximizes growth and lipid production in *Chlamydomonas reinhardtii*. *Algal Res* 2013;**2**:352–7.

Nielsen C, Rahman A, Rehman AU *et al*. Food waste conversion to microbial polyhydroxyalkanoates. *Microb Biotechnol* 2017;**10**:1338–52.

Papanikolaou S, Aggelis G. Lipids of oleaginous yeasts. Part I: biochemistry of single cell oil production. *Eur J Lipid Sci Technol* 2011a;**113**. DOI: 10.1002/ejlt.201100014.

Papanikolaou S, Aggelis G. Lipids of oleaginous yeasts. Part II: technology and potential applications. *Eur J Lipid Sci Technol* 2011b;**113**. DOI: 10.1002/ejlt.201100015.

Park GW, Chang HN, Jung K *et al*. Production of microbial lipid by *Cryptococcus curvatus* on rice straw hydrolysates. *Process Biochem* 2017;**56**. DOI: 10.1016/j.procbio.2017.02.020.

Park YK, Dulermo T, Amaro RL *et al*. Biotechnology for biofuels optimization of odd chain fatty acid production by *Yarrowia lipolytica*. *Biotechnol Biofuels* 2018a:1–12.

Park YK, Dulermo T, Ledesma-Amaro R *et al*. Optimization of odd chain fatty acid production by *Yarrowia lipolytica*. *Biotechnol Biofuels* 2018b;**11**. DOI: 10.1186/s13068-018-1154-4.

Peng WF, Huang C, Chen XF *et al*. Microbial conversion of wastewater from butanol fermentation to microbial oil by oleaginous yeast *Trichosporon dermatis*. *Renewable Energy* 2013;**55**. DOI: 10.1016/j.renene.2012.12.017.

Perez-Garcia O, Escalante FME, de-Bashan LE *et al*. Heterotrophic cultures of microalgae: metabolism and potential products. *Water Res* 2011;**45**:11–36.

Plastics Europe GMR. Conversio Market & Strategy GmbH. *Plastics - the Facts 2019* 2019;**14**:35.

Probst K V., Schulte LR, Durrett TP *et al*. Oleaginous yeast: a value-added platform for renewable oils. *Crit Rev Biotechnol* 2016;**36**. DOI: 10.3109/07388551.2015.1064855.

Pronk JT, Van der Linden-Beuman A, Verduyn C *et al*. Propionate metabolism in *Saccharomyces cerevisiae*: implications for the metabolon hypothesis. *Microbiology* 1994;**140**. DOI: 10.1099/00221287-140-4-717.

Ratledge C, Kanagachandran K, Anderson AJ *et al*. Production of docosahexaenoic acid by *Crypthecodinium cohnii* grown in a pH-auxostat culture with acetic acid as principal carbon source. *Lipids* 2001;**36**:1241–6.

Ratledge IC, Gb B, Anderson J *et al. (12) United States Patent (10) Patent No.: (45) Date of Patent*. 2010, 2.

Reddy MV, Kumar G, Mohanakrishna G *et al*. Review on the production of medium and small chain fatty acids through waste valorization and CO2 fixation. *Bioresour Technol* **309**, 2020:123400.

Ren H-Y, Liu B-F, Ma C *et al*. A new lipid-rich microalga *Scenedesmus* sp. strain R-16 isolated using Nile red staining: effects of carbon and nitrogen sources and initial pH on the biomass and lipid production. *Biotechnol Biofuels* 2013;**6**:143.

Řezanka T, Kolouchová I, Sigler K. Precursor directed biosynthesis of odd-numbered fatty acids by different yeasts. *Folia Microbiol (Praha)* 2015;**60**. DOI: 10.1007/s12223-015-0388-9.

Ryan AS, Nelson EB.. Safety Evaluation of Single Cell Oils and the Regulatory Requirements for Use as Food Ingredients. In: Cohen Z, Ratledge C (eds.). *Single Cell Oils (Second Edition)*. AOCS Press, 2010, 317–50.

Ryu BG, Kim W, Heo SW *et al*. Advanced treatment of residual nitrogen from biologically treated coke effluent by a microalga-mediated process using volatile fatty acids (VFAs) under stepwise mixotrophic conditions. *Bioresour Technol* 2015;**191**:488–95.

Singh J, Saxena RC. An introduction to microalgae: diversity and significance. 2015:11–24. DOI: 10.1016/B978-0-12-800776-1.00002-9.

Singh S, Sithole B, Lekha P *et al*. Optimization of cultivation medium and cyclic fed-batch fermentation strategy for enhanced polyhydroxyalkanoate production by *Bacillus thuringiensis* using a glucose-rich hydrolyzate. *Bioresour Bioprocess* 2021;**8**. DOI: 10.1186/s40643-021-00361-x.

Starai VJ, Escalante-Semerena JC. Acetyl-coenzyme A synthetase (AMP forming). *Cell Mol Life Sci* 2004;**61**. DOI: 10.1007/s00018-004-3448-x.

Turon V, Baroukh C, Trably E *et al*. Use of fermentative metabolites for heterotrophic microalgae growth: yields and kinetics. *Bioresour Technol* 2015;**175**:342–9.

Turon V, Trably E, Fouilland E *et al*. Potentialities of dark fermentation effluents as substrates for microalgae growth: a review. *Process Biochem* 2016;**51**:1843–54.

Vajpeyi S, Chandran K. Microbial conversion of synthetic and food waste-derived volatile fatty acids to lipids. *Bioresour Technol* 2015;**188**:49–55.

Vorapreeda T, Thammarongtham C, Cheevadhanarak S *et al*. Alternative routes of acetyl-CoA synthesis identified by comparative genomic analysis: involvement in the lipid production of oleaginous yeast and fungi. *Microbiology* 2012;**158**. DOI: 10.1099/mic.0.051946-0.

Wang QM, Yurkov AM, Göker M *et al*. Phylogenetic classification of yeasts and related taxa within *Pucciniomycotina*. *Studies in Mycology* 2015;**81**. DOI: 10.1016/j.simyco.2015.12.002.

Wen Q, Chen Z, Li P *et al*. Lipid production for biofuels from hydrolyzate of waste activated sludge by heterotrophic *Chlorella protothecoides*. *Bioresour Technol* 2013;**143**:695–8.

Wu H, San KY. Efficient odd straight medium chain free fatty acid production by metabolically engineered *Escherichia coli*. *Biotechnol Bioeng* 2014;**111**. DOI: 10.1002/bit.25296.

Wynn J, Behrens P, Sundararajan A *et al*. Production of single cell oils by dinoflagellates. *Single Cell Oils Microb Algal Oils Second Ed* 2010:115–29.

Xu X, Kim JY, Cho HU *et al*. Bioconversion of volatile fatty acids from macroalgae fermentation into microbial lipids by oleaginous yeast. *Chem Eng J* 2015;**264**. DOI: 10.1016/j.cej.2014.12.011.

Xu X, Kim JY, Oh YR *et al*. Production of biodiesel from carbon sources of macroalgae, *Laminaria japonica*. *Bioresour Technol* 2014;**169**. DOI: 10.1016/j.biortech.2014.07.015.

Yun JH, Sawant SS, Kim BS. Production of polyhydroxyalkanoates by *Ralstonia eutropha* from volatile fatty acids. *Korean J Chem Eng* 2013;**30**:2223–7.

Yu Y, Xu Z, Chen S *et al*. Microbial lipid production from dilute acid and dilute alkali pretreated corn stover via *Trichosporon dermatis*. *Bioresour Technol* 2020;**295**. DOI: 10.1016/j.biortech.2019.122253.

Zacharof MP, Lovitt RW. Complex effluent streams as a potential source of volatile fatty acids. *Waste Biomass Valorization* 2013;**4**. DOI: 10.1007/s12649-013-9202-6.

Zheng Y, Chi Z, Ahring BK *et al*. Oleaginous yeast *Cryptococcus curvatus* for biofuel production: ammonia's effect. *Biomass Bioenergy* 2012;**37**. DOI: 10.1016/j.biombioe.2011.12.022.