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Review

Cutaneotrichosporon oleaginosus: a versatile whole-cell biocatalyst for the production of single cell oil from agro-industrial wastes

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Abstract: *Cutaneotrichosporon oleaginosus* is an oleaginous yeast with several favourable qualities: it is fast growing, accumulating high amounts of lipids and having a very broad substrate spectrum. Its resistance to hydrolysis by-products makes it a promising biocatalyst for custom tailored microbial oils. *C. oleaginosus* can accumulate up to 60 wt% of its biomass as lipids. This species is able to grow by using as a substrate several compounds, such as acetic acid, biodiesel-derived glycerol, N-acetylglucosamine, lignocellulosic hydrolysates, wastepaper and other agro-industrial wastes. This review is focused on the state of the art about innovative and sustainable biorefinery schemes involving this promising yeast and second- and third-generation biomasses. Moreover, this review offers a comprehensive and updated summary of process strategies, biomass pretreatments and fermentation conditions for enhancing lipid production by *C. oleaginosus* as a whole-cell biocatalyst. Finally, an overview of the main industrial applications of single cell oil is reported together with future perspectives.

Keywords: *Cutaneotrichosporon oleaginosus*; oleaginous yeasts; whole-cell biocatalysis; biorefinery; single cell oil; biodiesel

Citation: Lastname, F.; Lastname, F.; Lastname, F. Title. *Catalysts* **2021**, *11*, x. https://doi.org/10.3390/xxxxx

Academic Editor: Firstname Lastname

Received: date
Accepted: date
Published: date

1. Introduction

The transition from a linear fossil-based economy to a new circular biobased one is a current global goal that requires the replacement of traditional refineries with innovative and sustainable biorefineries. Biorefinery processes aim to convert a wild range of low or negative values biomasses into marketable bio-based energy, materials and products [1]. Thus, the industrial production of biofuels and bioproducts from renewable resources is significantly rising in the last decades [2]. These renewable resources are often represented by agro-industrial side-streams which have to be converted to added-value compounds in the perspective of integrated industrial models and circular economy [3]. Moreover, in order to ensure the economic and environmental sustainability of biorefineries, the complete valorisation of the starting raw material by green process technology should be implemented [4,5].

Part of the most innovative biorefinery schemes is focused on the production of biofuels [2,6]. In the literature, several studies reported various chemical and/or biological catalytic approaches for the production of liquid bio-based fuels [7-9]. Among biofuels, biodiesel is one of the most promising for transporting since it does not require a dedicated technology and engines, differently from bio-based ethanol [10]. It is a crucial renewable energy source for heavy transport systems which cannot be easily electrified and decarbonised. Conventional biodiesel is produced from vegetable oils, such as palm, rapeseed and sunflower oil. Various homogeneous and heterogeneous catalysts, as well as

various enzymes, have been studied for the upgrading of vegetable oils, in particular for more sustainable production of first-generation biodiesel [11-13].

However, vegetable oils used for industrial purposes are often edible oils, thus determining an ethical debate on their use between industrial/energetic and food applications. In this context, a sustainable solution is represented by a new source of oil that corresponds to the so-called single cell oil (SCO). It is produced by oleaginous microorganisms, such as bacteria, microalgae and yeasts [14-16]. Oleaginous microorganisms are characterised by the ability to accumulate high concentrations of lipids into their cells, as energy storage. Several studies demonstrated the potential use of SCO as a good alternative to vegetable oil to produce new generation biodiesel and other bioproducts [3,7,17,18], as biosurfactants, biopolymers, cosmetics and animal feed (e.g. fish cultures) [14,19,20].

Among the most promising biocatalysts able to perform the conversion of various organic substrates, such as sugars, organic acid, glycerol, aromatics and amino acids into lipids, an important place is ascribed to oleaginous yeasts. These last are safe microorganisms able to accumulate triacylglycerols (TAGs) up to 70-80% of their dry cell weight [21]. The metabolic pathway for the synthesis of lipids is defined as lipogenesis, which occurs when the yeast is in an environment poor in nitrogen source and rich in carbon source, i.e. in the presence of a high C/N ratio [21].

A crucial aspect to guarantee the economic sustainability of yeasts oil production is represented by the use of low- or negative-value carbon sources deriving from second-generation biomasses and/or agro-industrial side-streams [22]. For example, the use of sugars-containing hydrolysates from lignocellulosic crops is one the most common approaches [3,4,7,23,24]. However, the quality of biomass hydrolysates is essential to have a good production of TAGs, as the presence of furanic compounds, such as furfural and 5-hydroxymethylfurfural, deriving from the degradation of sugars, can significantly alter the yeast growth and the oil yield [25,26]. In fact, some organic compounds, such as furaldehyde, formaldehyde, phenols, aliphatic acids, vanillic acid, uronic acid, 4-hydroxybenzoic acid, acetic acid and cinnamaldehyde, can act as inhibitors for different metabolic activities of oleaginous yeasts [27,28]. The susceptibility of microorganisms to inhibitors strictly depends on the strain they belong to, so it is necessary to adapt the fermentation process to the type of microorganism used, in order to increase the cell biomass and lipids productions [4,7,17].

Among oleaginous yeasts, the species *Cutaneotrichosporon oleaginosus*, also known as *Cryptococcus curvatus*, is one of the most studied. A comparative screening study of 1189 strains of oleaginous yeasts demonstrated that only 12 strains can co-ferment a mixture of sugars including glucose, xylose and arabinose [29]. *C. oleaginosus* is one of them [30], confirming itself as a promising and versatile whole-cell biocatalyst for the production of SCO. It is a GRAS (Generally Recognized As Safe) microorganism, widely diffused in nature, and was isolated from foodstuffs, like raw milk and lettuce, or from marine sediments. It presents several important metabolic features that make this species very promising for potential SCOs production at industrial scale. In particular, *C. oleaginosus* is fast growing, is able to use a wide range of carbon sources, can accumulate high content of TAGs (up to 60% of its dry cell weight) [27] and presents a good tolerance to the main growth inhibitors. In particular, this yeast can grow on carbon-rich media obtained from lignocellulosic biomasses [17], organic acids [31], organic wastes from the agriculture and food industry [32] as well as active sludge [33]. Moreover, the lipid profile is characterised by over 50% of unsaturated long-chain fatty acids, with a high quantity of oleic acid and linolenic acid. The first draft genome sequence of *C. oleaginosus* was published in 2016 [34], allowing significant progress in the understanding of both the metabolism of this biocatalyst and the development of genetic and molecular tools for increasing the lipid production [35]. Although it is well studied, there is no updated review regarding its use for the production of SCOs in the literature up to now. For this reason, similarly to other review works related to other species of oleaginous yeasts [36], the present review summarises

for the first time recent biorefinery approaches involving the whole-cell biocatalyst *C. oleaginosus*. In particular, the present work is focused on process reaction conditions, catalyst performances in terms of cell production and yield as well as oil production, content and yield, and lipid profile for each kind of substrate. The main aim of this review is to be a useful support for the study and the implementation of new and/or more efficient biorefinery models based on the principles of Green Chemistry and Circular Economy.

2. Taxonomy of biocatalyst

The *Trichosporonaceae*'s family of basidiomycete fungi belongs to the order *Trichosporonales*, the class *Tremellomycetes* and the subphylum *Agaricomycotina* which includes very different yeasts [37]. Recently, the taxonomy of this family has been revised to include six genera, called *Apiotrichum*, *Cutaneotrichosporon*, *Effuseotrichosporon*, *Haglerozyma*, *Trichosporon*, and *Vanrija*. The members of the *Trichosporonaceae* show a global distribution and have been recovered from various types of environments. *Cutaneotrichosporon spp.* are often associated with humans as hosts and can represent opportunistic pathogens for humans, but among these, some species are studied for important biotechnological applications. In particular, members of the *Trichosporonaceae* are known to be capable of producing and accumulating large quantities of single-cell oil with respect to their dry biomass [38-45].

Although this species was known under the name of *Candida curvata D* at the American Type Culture Collection (ATCC 20509), it has been identified in the scientific literature under various names, including *Apiotrichum curvatum* [46], *Cryptococcus curvatus* [47], *Trichosporon cutaneum* [48] and *Trichosporon oleaginosus* [49]. First, various names have been used over the years to indicate the ATCC 20509 strain and this has obviously made the collection of data more complex to delineate the taxonomic profile of this strain. Based on a multi-gene sequencing analysis, the phylogeny of the genus *Trichosporon* has recently been reviewed [50]. This review was based on a phylogenetic analysis of seven markers, more precisely LSU rRNA (domains D1/D2), SSU rRNA, Internal Transcribed Spacer (ITS), genes encoding the proteins RPB1, RPB2, TEF1, CYTB as well as a combination of morphological, biochemical and physiological characteristics [50]. Together with the previous data [51,52], this comprehensive multi-gene dataset led to a taxonomic revision of the genus. More recently, a phylogenomic study including genomic information from 17 species also revealed the phylogenetic heterogeneity of the genus [53]. Therefore, the previous genus *Trichosporon* has been inserted to the order of the *Trichosporonales* which now includes *Trichosporon* in feeling narrow, *Apiotrichum*, *Cutaneotrichosporon*, *Effuseotrichosporon*, *Haglerozyma* and *Vanrija* respectively [50]. During the phylogenetic restructuring, *T. oleaginosus* was put into the genus *Cutaneotrichosporon* and renamed *C. oleaginosus* [54]. The new genus *Cutaneotrichosporon* currently contains 13 species and half of them have been found cultivated as pathogenic or opportunistic on humans. The most recent literature of *C. oleaginosus* is focused on the biotechnological perspectives that characterise this microorganism [41]. Species found in the genus *Cutaneotrichosporon* do not form basidiocarps and do not exhibit sexual reproduction. In addition to its commonly described yeast state, *C. oleaginosus* also grows in a filamentous form and produces arthroconidia. In nature, it presumably grows as a filamentous fungus in soil and on leaf waste. The oleaginousity appears to be an adaptation to the highly variable supply of nutrients, supported by the very low maintenance energy of the yeast.

3. Applications of *C. oleaginosus* in the bioconversion of agro-industrial wastes to single cell oils

The combination of environmental and social issues related to the disposal of waste materials, climate change and global warming, and the exponential growth of the world population, make it necessary to accelerate the development and the scaling-up of systems for the sustainable conversion of municipal and agro-industrial wastes, namely renewable

resources, to biofuels, bioproducts and biomaterials. Biorefinery processes and technologies aim to implement this global goal [1]. In this context, *C. oleaginosus* is a promising and versatile biocatalyst for the fermentation of various carbon sources to bio-based oil [18,27]. In many cases, the substrate needs pretreatment in order to release reducing sugars, short-chain organic acids or alcohols which can be directly used by the microorganism [2,15]. The conversion of the raw material into the final carbon source often implies the production of biomass degradation side-products that could inhibit microbial growth during the fermentation [25]. The specific process to achieve the final carbon source from each agro-industrial waste is very important since it not only affects the medium composition, the yeast growth, the lipid yield and productivity and the lipid profile of SCOs but, above all, the overall process costs. Based on these considerations, in the following paragraphs, for each kind of agro-industrial waste a detailed description of the literature studies on *C. oleaginosus* will be presented, by focusing on the main process parameters such as the nature of the carbon source, its concentration, the carbon to nitrogen weight ratio, the fermentation technology, pH and temperature values, and the output information related to biomass concentration, lipid concentration, yield and productivity and the oil profile.

3.1. Acetic acid

Acetic acid is produced by the hydrothermal processing of lignocellulosic biomass, due to the hydrolysis of the acetyl group of the hemicellulose fraction, and by anaerobic digestion performed by bacteria. It is usually considered a by-product and can act as a growth inhibitor for several microorganisms. The conversion of acetic acid into lipids by oleaginous yeasts represents a prominent strategy for upgrading this waste to a marketable and valuable chemical. Acetic acid can be assimilated into microbial cells and converted to Acetyl-CoA, which represents the precursor of lipid biosynthesis [55,56]. When weak organic acids, including acetic acid, are added to the culture medium at pH 5-6 appear in their undissociated form, which tends to enter the cell by passive diffusion. On the other hand, the dissociated form of organic acids can enter the cell by the active transport mechanism of carboxylic acids which occurs through the use of an acetate/proton symport [57]. A similar transport system is also present in the cell membrane of *C. oleaginosus*. Once inside, the undissociated form of the acid dissociates, releasing protons. This causes a reduction in the intracellular pH and if this reduction is significant it could affect the metabolic functions by altering the normal cell growth [58]. The acid dissociation constant (pKa) for acetic acid is 4.75 favouring the undissociated form at pH 5-6 and the dissociated form at pH values higher than 7. Its dissociated form is much less toxic than the undissociated one [59,60]. Acetic acid has been studied as a potential substrate for the cultivation of *C. oleaginosus*. When acetic acid is used as the sole carbon source, a significant influence of pH on the ability of *C. oleaginosus* to grow on this waste is observed. Furthermore, the composition of SCOs obtained from the fermentation of acetic acid is very similar to that of vegetable oils, confirming the potential of this by-product to be used as a cheap raw material for the synthesis of biodiesel, biosurfactants, biopolymers and other bio-based molecules of industrial interest. Table 1 shows the main process information regarding the SCOs production starting from acetic acid by the biocatalyst *C. oleaginosus*.

Table 1. Bioconversion of acetic acid to single cell oil by the biocatalyst *C. oleaginosus*.

AA (g/L)	FT	C/N (g/g)	pH	T (°C)	C_X (g/L)	C_L (g/L)	Y_{LX} (w/w%)	Y_{LS} (w/w%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	Ref.
	F	10	6	30	1.7	0.3	15.0	6.0	17.5	20.5	49.1	
n.a.	F	10	7	30	2.2	0.4	18.0	9.0	19.3	19.0	47.5	[61]
	F	10	7	30	80.0	12.0	15.0	15.0	20.2	17.6	44.6	
n.a.	B	50	7	30	8.1	4.2	49.9	15.0	32.0	23.6	39.5	[62]
30	B	100	7	30	7.2	4.2	58.0	40.4	17.5	15.8	29.5	[31]
30	B	62.5	9	30	8.4	4.9	58.3	17.2	n.a.	n.a.	n.a.	[63]
40	B	62.5	9	30	9.7	6.1	62.5	15.9	n.a.	n.a.	n.a.	
6	F	40	5.5	25	3.2	1.0	32.2	17.0	12.3	17.4	50.3	[64]
5	B	10	7	30	2.1	0.5	24.0	30.0	9.4	19.4	51.6	
10	B	10	7	30	3.4	1.2	35.6	30.0	9.0	20.4	55.2	
20	B	10	7	30	4.8	2.9	60.2	35.0	8.0	22.5	55.3	[65]
30	B	10	7	30	6.3	3.7	58.9	30.0	7.8	22.2	54.0	
40	B	10	7	30	7.0	5.0	71.7	32.0	8.4	29.8	50.4	
30	B	59.0	8	34.7	6.7	5.5	82.1	25.0	7.0	31.2	44.8	
30	B[a]	60.5	8	30.8	7.9	6.2	78.3	23.0	6.5	28.8	49.7	
30	B[b]	59.4	8	29.4	8.8	5.2	59.1	19.0	9.5	27.3	49.4	[66]
40	B	58.7	8	36.6	6.2	3.8	61.1	18.0	5.6	32.6	41.4	
40	B[a]	59.2	8	39.0	6.3	4.9	77.8	17.0	6.0	34.1	42.3	
40	B[b]	58.7	8	27.5	12.8	7.2	56.3	20.0	8.9	26.2	50.3	

AA = acetic acid concentration; FT = fermentation technology, F = fed-batch mode fermentation, B = batch-mode fermentation; C/N = carbon to nitrogen weight ratio; C_x = cell biomass concentration; C_L = lipids concentration; Y_{LX} = intracellular lipid content; Y_{LS} = lipids yield; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = oleic acid; n.a. = not available.
[a] addition of delignified cellulose without catalyst immobilisation [b] immobilisation of biocatalyst on solid support.

In the studies reported in the literature so far, the temperature was mostly set at 30 °C, pH ranged from 6 to 9 and C/N weight ratios from 10 to 100 g/g, while the employed acetic acid concentration was in the range 5-40 g/L. Most of the fermentations were conducted in batch-mode with the sole exception of the studies of Béligon et al. [61] and Park et al. [64]. The maximum lipid yield reached on acetic acid was 40.4 w/w%. It was obtained by Liu et al. [31] in batch-mode in the presence of 30 g/L substrate as a single carbon source with a C/N ratio of 100 g/g, pH 7, 30 °C. The yeast strain mostly adopted as biocatalyst was *C. oleaginosus* ATCC 20509. The equilibrium of the acetic acid/acetate species appeared to be a determining parameter for facilitating active transport across the membrane and significantly improving cell growth. The consumption of acetate is directly related to the increase in pH. At basic pH, the dissociated form prevails, reducing the inhibitory effect that the substrate could have in favour of cell growth [31]. This behaviour was confirmed by the studies of Huang et al. [63] and Xu et al. [66] who performed the fermentation at pH 9 and 8, respectively, facilitating the consumption of acetic acid and reaching an average lipid content of 60.4 and 60.0 w/w%, respectively. Furthermore, the high lipid accumulation was favoured by using a high C/N ratio of around 60 g/g. Huang et al. [65] employed as biocatalyst the yeast strain JCM 1532 which is an acid-tolerant microorganism. Its ability to grow on different concentrations of acetic acid was investigated, as reported in Table 1. The cell biomass and lipids production as well as the lipid content increased as a function of the increase of acetic acid concentration, from 5 to 40 g/L. The lipid content rapidly increased from 24.0 to 60.2 w/w% by ranging from 5 to 20 g/L carbon

source. When the yeast was cultured in the presence of 40 g/L acetic acid, the highest lipid content of 71.7 w/w% was obtained. At the same time, the lipid production increased from 0.5 to 5.0 g/L, moving from 5 to 40 g/L acetic acid. The different concentrations of acetic acid did not significantly affect the SCOs composition. Palmitic acid methyl ester (C16:0) ranged from 7.8 to 9.4%, stearic acid methyl ester (C18:0) ranged from 19.4 to 29.8% and oleic acid methyl ester ranged from 50.4 to 55.3%. Xu et al. [66] studied the effect of biocatalyst immobilisation on the metabolic performances of the yeast strain *C. oleaginosus* ATCC 20509. Authors immobilised cells on delignified cellulose and investigated the lipid production under different process conditions as reported in Table 1. In the presence of 30 and 40 g/L acetic acid and in the absence of cells immobilisation, the dry cell weight of 6.7 and 6.2 g/L and the lipid production of 5.5 and 3.8 g/L were achieved, respectively. The lipid yield was 25 w/w% in the presence of 30 g/L acetic acid, while in the presence of 40 g/L it was 18.0 w/w%. Under the same process conditions, by adding the delignified cellulose in the culture medium without immobilising, the dry cell weight of 7.9 and 6.3 g/L and the lipid production of 6.2 and 4.9 g/L were achieved in the presence of 30 and 40 g/L acetic acid, respectively. Lipid yields were 23.0 and 17.0 w/w%, respectively. Finally, by immobilising the biocatalyst on the cellulose the cell biomass production increased up to 8.8 and 12.8 g/L at 30 and 40 g/L acetic acid, respectively, the production of the lipid resulted 5.2 and 7.2 g/L, respectively, while lipids yields were 19.0 and 20 w/w%. Similarly to the study of Huang et al. [65], the increase in the acetic acid concentration and the immobilisation of *C. oleaginosus* cells on the solid support did not affect the SCOs profile.

Acetic acid belongs to volatile organic acids (VOAs), together with propionic and butyric acids. VOAs are a mixture of different types of short-chain acids (C1-C4) that are usually produced during the anaerobic digestion of organic materials and wastes. Yields and chemical compositions of VOAs are significantly influenced by the substrate and the conditions adopted in anaerobic fermentation process. Interest in this class of by-products has increased in the last years since they represent a potential low- or negative-value substrate for the SCOs production by *C. oleaginosus*. VOAs from food, human and animal waste can reach a high concentration in the range of 10-40 g/L [67], while VOAs from activated sludge can reach a low concentration in the range of 2-8 g/L [68]. Similarly to acetic acid, other VOAs can be transported through the membrane by passive diffusion or active acetate/proton symport. Several studies used low concentrations of VOAs (2-10 g/L) as carbon source because high concentrations cause the inhibition of the yeast growth [31,64,69]. Table 2 summarised the main biorefinery processes for the conversion of VOAs into SCOs by the strain *C. oleaginosus* ATCC 20509.

Table 2. Bioconversion of volatile organic acids (VOAs) to single cell oil by the biocatalyst *C. oleaginosus* working at 30 °C and pH 7.

VOAs (g/L)	FT	C/N (g/g)	C_X (g/L)	C_L (g/L)	Y_{LX} (w/w%)	Y_{LS} (w/w%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	Ref.
5[a]	F	10	7.7	1.1	14.0	5.0	19.0	25.3	47.7	[69]
5[a]	C	50	26.7	13.6	51.0	13.4	13.5	26.9	51.4	
18:9:3[b]	B	62.5	9.0	4.8	53.3	18.7	n.a.	n.a.	n.a.	[63]
24:12:4[b]	B	62.5	11.8	7.5	63.2	18.7	n.a.	n.a.	n.a.	
15:0:15[b]	B	100	8.3	4.8	57.1	41.4	16.3	22.4	35.3	[31]
15:5:10[b]	B	100	8.7	4.9	56.9	37.6	12.7	14.1	31.6	
15:10:5[b]	B	100	8.0	4.6	57.2	33.4	11.1	9.9	33.2	
15:15:0[b]	B	100	7.6	4.0	52.1	27.1	9.8	9.4	30.0	
10:5:15[b]	B	100	8.4	4.7	56.5	33.2	11.0	8.0	31.5	
10:10:10[b]	B	100	8.1	4.3	52.5	31.2	9.7	7.9	30.7	
10:15:5[b]	B	100	6.9	3.3	48.2	25.6	9.2	8.1	29.6	
5:10:15[b]	B	100	8.2	4.0	48.4	28.1	9.1	5.2	27.3	
5:15:10[b]	B	100	8.2	3.8	46.5	27.3	8.8	3.5	26.1	
0:15:15[b]	B	100	7.4	2.4	32.4	16.7	9.3	3.4	26.4	

VOAs = volatile organic acids concentration; FT = fermentation technology, F = fed-batch mode fermentation, C = continuous mode fermentation, B = batch-mode fermentation; C/N = carbon to nitrogen weight ratio; C_x = cell biomass concentration; C_L = lipids concentration; Y_{LX} = intracellular lipid content; Y_{LS} = lipids yield; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = oleic acid. n.a. = not available.

[a] acetic acid, [b] acetic acid : propionic acid : butyric acid concentrations.

The SCOs composition and concentration are affected by the chemical composition and the concentration of VOAs. Usually, acetic acid content is the highest compared to other organic acids. It counts for around 43-69% [70], followed by propionic and butyric acids, which account for 10-54% [71] and 9-47% [72], respectively. Béligon et al. [69] and Liu et al. [31] reported that acetic acid is generally more reactive, representing a better carbon source than propionic and butyric acids, ensuring a higher growth of *C. oleaginosus*. As reported in Table 2, different fermentation technologies (batch, fed-batch and continuous mode) as well as various C/N ratios in the range 10-100 g/g were studied. The employment of continuous process setups favoured the cell biomass and lipids productions, reaching the maximum values of 26.7 and 13.6 g/L, respectively, in the study of Béligon et al. [69]. Moreover, as widely reported in the literature for all the oleaginous yeasts, the increase in the C/N ratio, namely the presence of a low concentration of nitrogen content in the fermentation medium, resulted in an increase of the lipids yield. This last was 5 w/w% at C/N of 10, 13.4 w/w% at C/N of 50, 18.7 w/w% at 62.5 and in the range 25-42 w/w% at C/N of 100, starting from several composition of VOAs. Regarding this aspect, Liu et al. [31] studied different mixtures of VOAs as the substrate for the production of lipids, by changing the relative ratios of acetic, propionic and butyric acids. The best results were obtained in the presence of 15:0:15 and 15:5:10 (g/L) ratios, with a biomass production of around 8.6 g/L, the lipid concentration of around 5 g/L, the lipid content of around 57 w/w% and the lipids yield respect to the consumed substrate of around 40 w/w%. These values were similar to those obtained by Liu et al. [31] (Table 1) under the same process conditions (batch mode, pH 7, C/N = 100) in the presence of the same concentration (30 g/L) of the sole acetic acid. The obtained results were higher than those

obtained by Huang et al. [65] in batch mode, at pH 7 in the presence of the C/N of 10 and the sole acetic acid (30 g/L) as carbon source. As reported in Table 2, by replacing the acetic acid with propionic and butyric acid (e.g., ratio 0:15:15), the performance of *C. oleaginous* significantly got worse. In particular, in the presence of the ratios 10:5:15, 10:10:10, 10:15:5, 5:10:15 and 5:15:10 the lipids yield decreased to 30 w/w%, while at the ratios of 0:15:15, namely in the absence of acetic acid, the value was only 16.7 w/w%, demonstrating that among VOAs, acetic acid represents the preferred carbon source by the adopted biocatalyst. Metabolic studies on the use of VOAs by *C. oleaginous* demonstrated that this yeast species used firstly acetic acid and subsequently butyric acid and propionic acid [31,73]. The preference for butyric acid was confirmed by the results obtained in the fermentation of 15:0:15 and 15:15:0 g/L of VOAs. In the presence of 15 g/L of both acetic and butyric acid the lipids yield was 41.4 w/w%, while in the presence of 15 g/L of both acetic and propionic acid the value was 27.1 w/w% as reported in Table 2. Moreover, the internal composition of the fermented VOAs significantly affected the SCOs composition. In the presence of the ratio 15:0:15 g/L, namely in the presence of acetic acid and butyric acid, C16:0 was 16.3%, C18:0 was 22.4% and C18:1 was 35.3%. By fermenting acetic acid and propionic acid (15:15:0 g/L), C16:0, C18:0 and C18:1 were 9.8, 9.4 and 30.0%, respectively. Finally, in the presence of propionic and butyric acid (0:15:15 g/L), all the three fatty acid methyl esters decreased to 9.3, 3.4 and 26.4%, respectively. These results confirmed the modularity of SCO profile as a function of the nature of the fermentation substrate. This represents an outstanding advantage of oil production by oleaginous yeasts since it allows to produce tailored oil composition in the perspective of final industrial application by changing the process parameters. Based on these studies, acetic acid represents the best substrate among the short-chain organic acids studied up to now as a carbon source for the production of microbial oil by *C. oleaginosus*, while butyric acid improves lipid production by facilitating the cell mass production. The mixing of acetic acid and butyric acid presented benefits for SCO production.

3.2. Crude glycerol

Glycerol is the main co-product of fatty acid methyl esters synthesis in the transesterification reaction of triglycerides. Currently, this reaction is widely used at the industrial scale for the production of traditional biodiesel starting from vegetable oils. For this reason, crude glycerol is one of the most abundant and low-cost wastes of the energy industries which can be potentially recycled in innovative biorefinery schemes for the production of new generation biodiesel, according to the new concept of the Circular Economy. Regarding the stoichiometry of the glycerol production process, 3 moles of methanol are necessary to convert 1 mole of triglyceride into 3 moles of fatty acid methyl esters (FAMEs) and 1 mole of glycerol. However, at the industrial scale, the addition of methanol in excess is adopted to maximise the oil conversion to biodiesel [74]. Glycerol is generated as a by-product of the biodiesel production process in a ratio of approximately 1:10 by weight to biodiesel. With the increase in biodiesel production in the recent years, the annual amount of crude glycerol obtained as a by-product from this reaction is currently approximately 1.9 Mton and this value is expected to exponentially increase in the coming years. In addition to methanol, other impurities are also present in the crude glycerol, including saponification products, water, catalysts (acids or bases) and salts (NaCl) [75]. Due to the presence of these impurities, its purification is not economically sustainable at the industrial level. In the last few years, raw glycerol resulting from biodiesel production has been used as a carbon source for the production of lipids by adopting several oleaginous species as biocatalysts, including *C. oleaginosus* [76-79]. From a biochemical point of view, once glycerol has entered the cell through an antiport carrier on the cell membrane, it is phosphorylated by the enzyme glycerol kinase to form glycerol-3-phosphate which is subsequently used in the synthesis of triglycerides. The maximum theoretical lipid yield from glycerol is 30 w/w%, slightly lower than that obtainable from glucose equal to 32 w/w%,

due to the different metabolic pathways [56]. The co-fermentation of sugars (glucose and xylose) and glycerol was also investigated to accelerate the cell growth and favour the production of the lipid by *C. oleaginosus* [77]. Glycerol is simultaneously assimilated with sugars by the yeast [77]. Table 3 reports the literature review on recent biorefinery processes for the conversion of pure glycerol, crude glycerol or mixtures of glycerol and reducing sugars (pure or in lignocellulosic hydrolysates) to SCOs by *C. oleaginosus*.

Table 3. Bioconversion of glycerol to single cell oil by the biocatalyst *C. oleaginosus* at 30 °C.

Carbon source (g/L)	FT	C/N (g/g)	pH	C_X (g/L)	C_L (g/L)	Y_{LX} (w/w%)	Y_{LT} (g/L/h)	Y_{LS} (w/w%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	Ref.
CG 10.3	B	20	5.5	13.4	3.1	23.0	0.05	n.a.	n.a.	n.a.	n.a.	[79]
CG 15.3	B	30	5.5	23.7	11.3	47.5	0.21	n.a.	n.a.	n.a.	n.a.	
CG 22.8	B	45	5.5	24.8	12.1	49.0	0.22	n.a.	n.a.	n.a.	n.a.	
CG 29.7	B	60	5.5	19.3	10.0	52.0	0.18	n.a.	n.a.	n.a.	n.a.	
CG 46.3	F	45	5.5	43.8	21.9	49.9	0.42	n.a.	n.a.	n.a.	n.a.	
CG 46.3	F[a]	45	5	43.2	20.8	48.1	0.35	n.a.	30.0	n.a.	46.0	[76]
CG 20.0	B	30	7	5.6	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	[80]
CG 40.0	B	30	7	2.2	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
CG 60.0	B	30	7	0.4	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
CG 80.0	B	30	7	0.5	n.a.	n.a	n.a.	n.a.	n.a.	n.a.	n.a.	
CG 25.8	F	30	5.5	31.2	n.a.	44.6	1.2	n.a	23.0	16.7	39.6	
CG 32.0	F	30	5.5	32.9	n.a.	52.9	1.5	n.a.	n.a.	n.a.	n.a.	
Glu 40 + Xyl 20	B	27	5.5	25.5	10.4	40.7	0.14	16.5	29.0	12.5	46.8	[77]
Glu 40 + Xyl 20 + CG 30	B	27	5.5	34.4	16.8	48.7	0.17	18.3	27.2	12.7	49.8	
Xyl 30 + CG 30	B	27	5.5	25.8	10.0	38.8	0.12	16.3	28.7	13.7	45.2	
CG 30	B	27	5.5	15.4	4.2	27.3	0.09	13.6	23.3	18.0	48.4	
CSEH	B	27	5.5	11.8	4.6	39.4	0.08	15.9	29.6	13.4	47.2	
CSEH + CG 30	B	27	5.5	21.7	10.8	49.7	0.13	18.0	34.2	13.4	46.3	

CG = crude glycerol; Glu = glucose; Xyl = xylose; CSEH = Corn stover enzymatic hydrolysate (glucose 18.8 g/L + xylose 14.5 g/L); FT = fermentation technology, B = batch-mode fermentation, F = fed-batch mode fermentation; C/N = carbon to nitrogen weight ratio; C_x = cell biomass concentration; C_L = lipids concentration; Y_{LX} = intracellular lipid content; Y_{LT} = lipids productivity; Y_{LS} = lipids yield; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = oleic acid. n.a. = not available.

[a] Non-sterilised fermentation medium.

As previously mentioned, methanol represents an impurity of crude glycerol resulting from traditional biodiesel production. Generally, energy companies are reluctant to recover excess methanol, due to its low price and the high energy demand for its recycling [81]. It has been demonstrated that methanol can be used, within certain threshold concentrations, to selectively inhibit the growth of contaminating microorganisms, such as bacteria, which can contaminate fermentation and limit the yeast biomass production [76]. This strategy can be used when the fermentation medium and fermenter are not sterilised [82]. The presence of methanol in the medium influences the growth of *C. oleaginosus* and contaminating species but its extent is a function of the concentration of the different impurities present in the crude glycerol [76]. It has been reported that methanol can inhibit the cell growth of *C. oleaginosus* at concentrations higher than 14 g/L, while values just higher than 15 mg/L can completely inactivate the microbial activity of bacteria [83]. Therefore, the optimisation of the methanol concentration in the formulation of the fermentation medium can lead to the development of a process strategy that allows fermentation to be carried out in non-sterile conditions, thus reducing process costs without significantly affecting the growth and the performance of the biocatalyst. In this regard, in the studies of Chen et al. [76,79], the effect of both glycerol and methanol concentration on SCOs production was investigated. Different glycerol concentrations in the range 10.3-46.3 g/L and methanol concentrations of 0, 14, 22, 33 and 44 g/L were tested as substrate for lipid production by *C. oleaginosus* ATCC 20509. In the presence of a high concentration of methanol in the crude glycerol, it can be evaporated to reach the desired concentration before using the obtained raw glycerol as a carbon source. The C/N ratios 20, 30, 45 and 60, in the batch-mode fermentation, were investigated to evaluate the impact of this important process parameter on lipid production. The best results were achieved in the presence of the C/N of 45 which led to obtaining the biomass production of 24.8 g/L, the lipid production of 12.1 g/L, the lipids content of 49 w/w% and the lipids productivity of 0.22 g/L/h. By replicating the same process conditions in combination with the fed-batch mode fermentation, the authors obtained better results than the batch-mode. In particular, the cell biomass concentration was 43.8 g/L, the lipid production was 21.9 g/L, the lipids content was 49.9 w/w% and the lipids productivity was 0.42 g/L/h. The improvement of biocatalyst performance in the fed-batch mode is related to the possibility to add fresh substrate, properly tune the C/N ratio favouring different metabolic pathways, such as the cell growth and the lipogenesis, and to dilute toxic compounds during the fermentation.

As reported in the study of Chen et al. [79], from 1 L of crude glycerol it was possible to produce 109.4 g of lipids and 103.9 g of biodiesel assuming that the yield of the transesterification reaction was 95 wt%. The same authors performed the optimised fermentation process in a non-sterilised fermentation medium by taking advantage of the presence of methanol in the crude glycerol used as carbon source [76]. Surprisingly, the fermentation effectively occurred and similar results with respect to the process carried out in a sterilised medium were achieved. The cell biomass concentration resulted 43.2 g/L, the lipid production 20.8 g/L, the lipids content 48.1 w/w% and the lipids productivity 0.35 g/L/h (Table 3).

In the study of Liang et al. [80], different concentrations of crude glycerol derived from yellow grease in the range 20-80 g/L were tested in batch-mode fermentation, adopting pH 7 and the C/N ratio equal to 30. These preliminary tests were carried out for 72 hours. In the presence of the highest glycerol concentrations, namely 60 and 80 g/L, significant cell growth was not observed, with a cell biomass production of 0.4 and 0.5 g/L, respectively. In the presence of the carbon source of 40 g/L, a moderate yeast growth was observed with a cell biomass production of 2.2 g/L. Finally, the best results were achieved in the presence of the lowest glycerol concentration (20 g/L), resulting in a cell biomass concentration of 5.6 g/L. This value resulted double respect to that obtained at 40 g/L and 10-folds higher with respect to the test carried out at 60 and 80 g/L of crude glycerol. Moreover, the authors compared the performance of *C. oleaginosus* on crude glycerol with respect to that observed on pure glycerol under the same process conditions. A higher cell

biomass production was achieved on pure glycerol. In particular, the value obtained on crude glycerol resulted in 67% of that researched on pure glycerol, due to the presence of toxic impurities for the biocatalyst. In the same study, in order to overcome substrate inhibition, a fed-batch fermentation strategy was implemented. Two different processes were carried out as reported in Table 3. For both fermentations pH was keep constant at 5.5, the starting C/N was 30 and the processing time was 12 days. However, for the first fed-batch fermentation, the starting glycerol concentration was 25.8 g/L and the C/N was keep constant during the whole process by the addition of a precise volume of crude glycerol and NH_4Cl, as inorganic nitrogen source, at different times, such as 3, 6.25, 7, 9.1 and 10 days. On the contrary, for the second fed-batch fermentation, the starting glycerol concentration was 32 g/L and the C/N of 30 was maintained constant for the first 6 days by the addition of the sole nitrogen source. In the second part of the process, the nitrogen addition was interrupted while the glycerol and medium supplements were added, thus determining the increase of the C/N ratio. The fed-batch mode ensured good cell biomass production of around 32 g/L in both cases. Moreover, in the second fed-batch fermentation, the decrease in the nitrogen content in the culture medium favoured the intracellular lipids accumulation. In fact, in the first bioconversion approach, the cell content was 44.6 w/w% with a productivity of 1.2 g/L/h, while in the second one it resulted 52.9 w/w% with a productivity of 1.5 g/L/h.

In the study of Gong et al. [77] a mixture of corn stover enzymatic hydrolysate (CSEH) and crude glycerol was used as a carbon source. The strategy of co-using two waste substrates was implemented in order to improve the lipid production by *C. oleaginosus* because sugars are a better carbon source with respect to glycerol, favouring cell growth. The authors tested different medium compositions in terms of the nature of the carbon source and its total content. In all the runs, the C/N was equal to 27, pH was 5.5 and batch-mode fermentation was adopted. Six culture media were tested: (1) glucose 40 g/L + xylose 20 g/L; (2) glucose 40 g/L + xylose 20 g/L + crude glycerol 30 g/L; (3) xylose 30 g/L + crude glycerol 30 g/L; (4) crude glycerol 30 g/L; (5) CSEH (glucose 18.8 g/L + xylose 14.5 g/L); (6) CSEH + crude glycerol 30 g/L. By comparing the tests carried out on culture media (1), (2) and (3), namely on pure sugars and sugars + crude glycerol, it is possible to observe the limited influence of the presence of glycerol in the different synthetic media on the performances of the biocatalyst. This result was not foregone as glycerol is not the optimal carbon source for oleaginous yeasts. Under conditions (1), the lipid production was 10.4 g/L and the lipid yield was 16.5 w/w%. The sugars were completely consumed within 72 hours. Under conditions (2), namely in the presence of higher carbon content, the lipid production increased to 16.8 g/L and the lipid yield was 18.3 w/w%. Also in this case, sugars were consumed within 72 hours while the glycerol consumption started later, from 72 to 84 hours. Finally, under conditions (3), the lipid concentration and yield were 10.0 g/L and 16.3 w/w%, respectively. All these findings confirmed that *C. oleaginosus* prefers the use of hexose and pentose before glycerol. Moreover, the results were slightly better than those obtained in the presence of sole reducing sugars (conditions (1)). Therefore, beneficial synergistic effects occur when glycerol is mixed with glucose and xylose. Probably, the combined use of glycerol, glucose and xylose triggers multiple biochemical pathways for the carbon source consumption that promote the accumulation of lipids. Differently, when the sole crude glycerol was used in the fermentation medium (30 g/L), the performance of the biocatalyst was worse than that performed in mixed culture media: it was consumed within 48 hours and ensured a cell biomass and lipids production of 15.4 and 4.2 g/L, respectively, the lipids productivity of 0.09 g/L/h and the lipid content and yield of 27.3 w/w% and 13.6 w/w%, respectively.

Finally, as reported in Table 3, Gong et al. also investigated the co-utilisation of corn hydrolysate and crude glycerol as carbon sources. In this process layout, the dry cell biomass production resulted 21.7 g/L, the concentration of lipids was 10.8 g/L, the lipid content was 49.7 w/w%, the productivity was 0.13 g/L/h and the lipid yield was 18.0 w/w%. The obtained results confirmed the efficacy of fermenting second-generation sugars from

lignocellulosic biomasses and crude glycerol. In fact, lipids production was higher than that obtained on sole crude glycerol and sole CSEH and, at the same time, the results were similar to those obtained on mixed pure sugars and crude glycerol. Moreover, microbial oils obtained by fermenting all the media tested by Gong et al. showed a similar FAMEs profile: C16:0 ranged from 23.3 to 34.2%, C18:0 ranged from 12.5 to 18.0% and C18:1 ranged from 45.2 to 49.8%. Therefore, the process strategy based on the simultaneous biological conversion of lignocellulosic hydrolysates and waste glycerol provides an extremely useful solution to ensure high lipid production upgrading cheap raw feeds.

3.3. N-acetylglucosamine

N-acetylglucosamine (NAG) is an amino-monosaccharide and represents the structural unit of chitin ($(C_8H_{13}O_5N)_n$). Chitin is formed by β-1-4 bonds between N-acetylglucosamine units. NAG is widely spread in nature that polymerises forming a polysaccharide (chitin) that makes up the exoskeleton of insects and arthropods. Since the chitin source is abundant and shellfish processing waste presents environmental problems, it is crucial to explore NAG as an alternative feedstock for innovative and sustainable biorefinery models. NAG is rarely used as a carbon source for the fermentation of oleaginous yeasts and a few studies have been conducted so far. *C. oleaginosus* is able to assimilate this molecule and use it for SCOs production [84,85]. In the literature, a biochemical pathway for the synthesis of triglycerides starting from NAG was proposed [84,85]. NAG is transported into the cell across the cell membrane; once it is in the cytoplasm, NAG is phosphorylated by the NAG kinase to form NAG-6-phosphate. The NAG-6-phosphate is then deacetylated to release acetate followed by the deamination to generate fructose-6-phosphate by the NAG deaminase. Finally, fructose-6-phosphate and acetate are channelled into the traditional pathway of lipogenesis. A small part of the released ammonium (NH_4^+) is used intracellularly for nitrogen metabolism, while most of it is secreted outside the cell. Table 4 shows the literature data on biocatalytic processes for the conversion of NAG to lipids by *C. oleaginosus*.

Table 4. Bioconversion of N-acetylglucosamine to single cell oil by the biocatalyst *C. oleaginosus* at pH 5.5.

N-acetylglucosamine (g/L)	FT	C/N (g/g)	T (°C)	C_X (g/L)	C_L (g/L)	Y_{LX} (w/w%)	Y_{LT} (g/L/h)	Y_{LS} (w/w%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	Ref.
70	B	7.2	22	17.6	8.8	50.0	0.07	17.1	31.3	21.9	41.6	[84]
70	B	7.2	26	16.9	9.1	54.0	0.08	16.0	24.0	18.2	47.4	
70	B	7.2	30	19.1	8.6	45.0	0.07	15.5	23.9	19.0	51.1	
70	B	7.2	35	13.8	7.2	52.0	0.06	14.2	24.2	19.5	51.6	
90	B	7.2	26	22.9	10.1	44.0	0.08	15.3	n.a.	n.a.	n.a.	
110	B	7.2	26	20.3	9.7	48.0	0.08	16.2	n.a.	n.a.	n.a.	
40	B	8.0	30	16.8	5.1	30.5	0.11	15.0	40.2	9.5	42.3	[85]
40	B[a]	8.0	30	16.2	8.4	51.6	0.12	22.0	n.a.	n.a.	n.a.	
40	B[a,b]	8.0	30	17.4	9.9	56.9	0.17	23.0	43.6	9.8	40.1	

FT = fermentation technology, B = batch-mode fermentation; C/N = carbon to nitrogen weight ratio; C_x = cell biomass concentration; C_L = lipids concentration; Y_{LX} = intracellular lipid content; Y_{LT} = lipids productivity; Y_{LS} = lipids yield; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = oleic acid. n.a. = not available.

[a] Two-stage fermentation process. [b] Non-sterilised fermentation medium.

In the study of Wu et al. [84], authors preliminarily investigated the effect of temperature on the bioconversion process. Different batch-mode fermentations were carried out at 22, 26, 30 and 35 °C. The NAG concentration, the C/N weight ratio and the pH were set at 70 g/L, 7.2 g/g and 5.5, respectively. The highest lipid production (9.1 g/L) was reached at 26 °C, which ensured a cell biomass production of 16.9 g/L, the lipid content of 54.0 w/w%, the lipid productivity of 0.08 g/L/h and the lipid yield of 16.0 w/w%. Thus, this temperature was selected by the authors for subsequent tests aiming at investigating the effect of substrate concentration on lipogenesis. Moreover, the variation in the temperature affected the FAMEs profile of SCOs. The microbial oil obtained at 22 °C contained 31.3% C16:0, 21.9% C18:0 and 41.6% C18:1 while that obtained at 35 °C was characterised by 24.2% C16:0, 19.5% C18:0 and 51.6% C18:1. In the same study, two different concentrations of 90 and 110 g/L of NAG were tested. The presence of 90 g/L NAG ensured the highest biomass production (22.9 g/L), even if working in the range 70-110 g/L, no significant differences were observed in *C. oleaginosus* performance. Based on the results reported in Table 4, it is possible to emphasise the possibility to use this biocatalyst in a wide range of fermentation conditions, in terms of temperature and substrate concentrations, for the conversion of this waste into oil. However, in all the cases, relatively low lipid yields were achieved in the range of 14-17 w/w%. These results agreed with the low C/N adopted. In fact, during the assimilation of N-acetylglucosamine by the yeast, the release of NH_4^+ ions is carried out by determining an excess in the nitrogen content which does not favour lipogenesis as widely reported in the literature.

In order to overcome the limitations in the use of NAG as the carbon source for oleaginous yeasts, due to the high nitrogen content, Tang et al. [85] developed an innovative two-stage process. It aimed to enhance the ability of *C. oleaginosus* by implementing two different culture conditions in terms of inoculum age, inoculum size and C/N ratio. In fact, when both cell biomass growth and lipid production processes occur at the same time, neither of them reaches the maximal capacity, reflecting the dilemma of different nutritional demands between them. The implementation of two spatially separated processes, the first one for the cell propagation and the second one for the lipid accumulation, allows the intensification of lipids production by oleaginous species. At first, a control batch-mode fermentation was carried out at pH 5.5, 30 °C and C/N of 8. Under these conditions, the cell biomass production, lipid production, lipid content, productivity and yield were 16.8 g/L, 5.1 g/L, 30.5 w/w%, 0.11 g/L/h and 15.0 w/w%, respectively. Then a two-stage fermentation process was adopted. In the first step, a nutrient-rich synthetic medium (yeast extract 10 g/L, peptone 20 g/L, NAG 16 g/L) was used to favour the cell biomass production, by using a low C/N ration, while in the second step a nitrogen-limited culture medium (NAG 40 g/L) was used in order to increase the C/N and favour the lipid accumulation. Moreover, the authors demonstrated that an inoculum size of 4.5 g/L and an inoculum age of 24 hours maximised the production of lipids by *C. oleaginosus* on NAG. Under the optimal process conditions, the cell biomass production, the lipid production, the lipid content, productivity and yield were 16.2 g/L, 8.4 g/L, 51.6 w/w%, 0.12 g/L/h and 22.0 w/w%, respectively. Finally, the same two-stage process was performed in non-sterile conditions. NAG solutions and water were not sterilised, and all the operating procedures were performed in a non-aseptic environment. Under these cost-saving conditions, similar results were obtained (Table 4), demonstrating the possibility to work in non-sterile conditions without penalise the lipids production. Moreover, these results agreed with those reported in previous studies performed on cellulose-deriving glucose under non-sterile conditions by *C. oleaginosus* [86]. The two-stage fermentation in non-sterile conditions did not affect the chemical composition of SCO with respect to the traditional batch-mode fermentation (in sterile conditions) carried out under the same process parameters, as reported in Table 4. However, the lipid profile obtained by Tang et al. [85] was different than that obtained by Wu et al. [84] probably due to the different concentrations of NAG

in the media since the adopted yeast strain was the same, namely *C. oleaginosus* ATCC 20509.

3.4. Lignocellulosic biomasses

Plant-based biomasses are one of the most abundant renewable sources in the world. Vegetable biomasses are classified in first-, second- and third-generation biomass as a function of their nature [1,87]. Starch-rich and edible crops, namely first-generation biomass, are the most adopted feedstock in industrial-scale biorefinery processes due to their high productivity and easiness of hydrolysis to reducing sugars. However, their use is characterised by an ethical debate about the so-called "food-feed-fuel competition" [88,89]. For this reason, in the last decades, a huge effort was performed in order to replace the use of first-generation biomass with the exploitation of lignocellulosic non-food crops. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose and lignin, together with a low amount of extractives, proteins and ash. Based on this chemical composition, lignocellulosic biomasses represent a promising alternative to food crops as a source of sugars and other biobased molecules. Lignocellulosic biomass can be further segmented into different types, such as forestry (birch, eucalyptus, spruce, oak, pine, poplar), forestry waste (the residues of trees and shrubs, sawdust), energy crops (sorghum, miscanthus, giant reed, kenaf, switchgrass, corn, sugarcane), agricultural residues (corn stover, wheat straw), algae, industrial and domestic waste (fruit or vegetable waste), and any other animal manure (cattle, swine, poultry) [5]. These lignocellulosic biomass resources significantly differ in the contents, compositions and structures of cellulose, hemicellulose and lignin with completely different properties. The relative abundance of each biopolymer is strongly related to the source of the biomass and, in the case of hardwood, softwood and grasses, also to the soil and environmental conditions. Cellulose, hemicellulose, and lignin are closely associated with each other through covalent linkages and each component has a specific functionality. From a structural point of view, cellulose represents the internal structure, composing the fibres of the cell walls; hemicellulose links the cellulose chains to the lignin fraction through covalent linkages, and finally lignin represents the external protective layer providing mechanical strength to the biomass. Cellulose and hemicellulose are intimately associated together through hydrogen bonds, meanwhile lignin is covalently linked to hemicelluloses to form lignin-carbohydrate complex [5]. There are five different types of lignin-carbohydrate bonds, namely, phenyl glycosides, benzyl ethers, γ-esters, ferulate/coumarate esters and hemiacetal/acetal linkages that are linked to lignin at 4-OH and 4-O positions [90]. It has been suggested that the interactions between the microfibers from cellulose and hemicelluloses, as well as the lignin-carbohydrate complexes linkages, play a significant role in the wood structure and significantly affect its susceptibility to the hydrolysis by reducing the area of cellulose accessible for chemical or biological catalysts, such as inorganic acids and enzymes. The complex structure of lignocellulosic biomass and its innate recalcitrance to chemical and/or biological depolymerisation require multi-step processes in order to convert the starting biomass into added-value products. The most common biorefinery schemes are based on the initial pretreatment of lignocellulosic biomass in order to deconstruct the lignocellulose matrix and make it more prone to subsequent chemical or biological hydrolysis [4,7,15,91,92]. Table 5 shows a short list of the most common pretreatment approaches of lignocellulosic biomass together with a brief description of their main advantages and disadvantages.

Table 5. Summary of the most common pretreatment approaches for lignocellulosic biomasses.

Pretreatment	Optimal conditions	Advantages	Disadvantages	Ref.
Dilute acid pretreatment	H_2SO_4, HCl, CH_3COOH 1-4 wt%, 50-60 °C, 30-120 min	High availability of catalyst, low consumption and cost of the catalyst, high efficiency of the catalyst	Corrosion of the reactor, downstream process for acidic streams, high environmental impact, difficult catalyst recycling, production of sugars-degradation by-products with an inhibitory effect on yeast growth, effect on the SCOs profile	[4,93,94]
Microwave irradiation	H_2SO_4 0.5 wt%, 135-190 °C, 2-20 min; 635-1400 W, 2450 MHz	Energy- and time-saving approach, versatile application in combination with others chemical, physical and biological processes	Low technology readiness level	[95-98]
Alkaline pretreatment	NaOH, KOH, $Ca(OH)_2$, 1-3 wt%, 30-60 °C, 6-24 h	High availability of catalyst, low consumption and cost of the catalyst, good efficacy, good lignin destructuration	Downstream process for basic streams, high environmental impact, difficult catalyst recycling	[99,100]
Steam explosion	160-260 °C, 5–50 atm, 2-10 min	Low environmental impact, very effective in biomass destructuration, high improvement of enzymatic digestability of treated solid residue, no catalyst need, very short process time	Energy-intensive process, production of sugars-degradation by-products with an inhibitory effect on yeast growth	[17,101]
Ionic liquid	[Bmim][Cl] + [Bmim][OAc], [Emim][OAc], [triethylammonium]), 5-10 wt%, 80–160 °C, 1-22 h	Versatile application in combination with other chemical and biological processes, low environmental impact, decrease in crystallinity of fibres, increase in biomass porosity, good improvement of enzymatic digestability of treated solid residue	High cost, difficult manipulation, negative effect on hydrolytic enzymes, difficult recycling	[102,103]

Organosolv	Acetone, ethanol; γ-valerolactone, 60-80 v/v%, 120-180 °C, 60-120 min	Low environmental impact if based on green solvent, no catalyst need, high delignification degree, high improvement of enzymatic digestibility of treated solid residue	Use of high volume of solvents, loss of a part of the hemicellulose fraction, downstream process for solvent recycling	[104-106]

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Chemical pretreatments are usually based on the use of acids, bases, solvents and ionic liquid. Thermal or thermochemical approaches are usually based on microwave irradiation and steam explosion. Each of these pretreatments presents important advantages and disadvantages from a technical and/or environmental point of view. For this reason, there is not an ideal pretreatment strategy for lignocellulosic biomass, but it should be selected as a function of the adopted biomass, the implemented conversion technology and final added-value bioproducts. One of the most important aspects for the selection of the useful pretreatment and its operating conditions is the production of sugars-degradation by-products since their production during pretreatment or cellulose and hemicellulose depolymerisation can strongly affect the biological conversion of fermentable sugars into valuable compounds such as SCOs. In fact, harsh process conditions favour the subsequent enzymatic digestibility of cellulose and hemicellulose to glucose and xylose, respectively, but they can also favour the production of 5-hydroxymethylfurfural, furfural, levulinic acid, formic acid, and aromatic compounds. These last molecules can partially or totally inhibit yeast growth during the fermentation of lignocellulosic hydrolysates. For these reasons, it is very important to optimise the reaction conditions of the pretreatment step in order to increase the carbohydrates availability to depolymerisation but limiting at the same time the synthesis of toxic by-products for the whole-cell biocatalyst. As an alternative, a pretreatment approach can be performed under harsh process conditions to maximise the biomass destructuration but it should be associated with one or more detoxification approaches in order to selectively remove undesired products in the biomass hydrolysates. Table 6 shows the main strategies for the detoxification of lignocellulosic hydrolysates before their use as a carbon source in bioprocesses. Also in this case, each approach presents advantages and disadvantages, as briefly described in Table 6. Detoxification approaches are based on chemical, physical or biological methods. Similarly to the selection of the pretreatment step, also the selection of the detoxification step is strictly related to the nature of the lignocellulosic hydrolysate, the concentration of by-products and sugars and the biocatalyst used in the following fermentation step. For the production of SCOs by *C. oleaginous* several biorefinery layouts are reported in the literature. Table 7 shows the most recent studies on biocatalytic processes for the conversion of various lignocellulosic biomasses to lipids by *C. oleaginosus*.

In the paper of Caporusso et al. [7], cardoon stalks hydrolysate was used as the carbon source in batch-mode fermentation. The lignocellulosic hydrolysate was produced by steam explosion pretreatment coupled with enzymatic hydrolysis. A sugars-rich liquid phase was obtained containing 90 g/L glucose and around 10 g/L xylose. Both undetoxified hydrolysate and simulated cardoon hydrolysate (prepared with pure glucose and xylose in the same concentration of the hydrolysate) were tested as fermentation medium with a C/N of 85. Moreover, the effect of the inoculum age was also investigated on yeast growth and lipids production. In particular, the inoculum in the exponential growth phase (50 h) was compared to the inoculum in the stationary growth phase (100 h) of *C. oleaginosus*. As reported in Table 7, no growth was observed on the undetoxified cardoon hydrolysate due to the synergistic toxic effect of various by-products produced during the steam explosion pretreatment, such as acetic acid (2.1 g/L), 5-HMF (0.32 g/L), furfural (0.42 g/L) and total phenolic compounds (5 g/L). As expected, the biocatalyst showed better performance on the simulated hydrolysate with the inoculum at the stationary growth phase. Under these conditions, the cell biomass production, lipid production, lipid content, productivity and yield were 22.8 g/L, 7.5 g/L, 32.9 w/w%, 0.02 g/L/h, 13.6 w/w%, respectively. By inoculating the biocatalyst at the exponential growth phase, the cell biomass production, the lipid production, the lipid content, productivity and yield were 26.0 g/L, 6.8 g/L, 26.2 w/w%, 0.02 g/L/h, 9.9 w/w%, respectively. Based on these results, the inoculum in the stationary phase favoured the lipids production, representing a useful approach in order to improve the biocatalyst performance by maintaining the same FAMEs profile.

Table 6. Summary of the main detoxification approaches used for the bioconversion of lignocellulosic hydrolysates.

Detoxification method	Advantages	Disadvantages	Ref.
Vacuum evaporation	Efficient for volatile compounds such as acetic acid, furfural and vanillin	Not effective in the removal of non-volatile compounds (e.g. extractives and lignin derivatives)	[17,107]
Membrane extraction	Effective for the reduction in the concentration of sulfuric acid, acetic acid, formic acid, levulinic acid, furfural and 5-HMF	Increase in the viscosity of the organic phase, energy-intensive process	[108]
Overliming with $Ca(OH)_2$	Cost-effective method, effective for removal of organic acids	A slight loss of sugars (about 10 wt%) by adsorption on the precipitate	[109]
Activated carbon treatment	High adsorption capacity of inhibitory compounds, without affecting the sugars concentration	The effectiveness of activated carbon treatment is strongly related to various process variables such as pH, contact time, temperature and liquid to solid weight ratio	[110]
Ion-exchange resins	Greater specificity for organic and inorganic compounds	Expensive method	[111]
Ethyl acetate extraction	Effective removal of phenolic compounds	Not effective for hydrophilic compounds	[112]
in situ microbial detoxification	Cost-effective and more sustainable method than chemical-physical processes, low number of side reactions due to the great selectivity of microorganisms, energy-saving approach	Considerable consumption of fermentable sugars	[113]
Enzymatic purification	Effective removal of phenolic compounds, sustainable and energy-saving method, high selectivity	Low technology readiness level, high cost of enzymes	[114]

Table 7. Bioconversion of lignocellulosic biomass hydrolysates to single cell oil by the biocatalyst *C. oleaginosus*.

Carbon source (g/L)	FT	C/N (g/g)	pH	T (°C)	C_X (g/L)	C_L (g/L)	Y_{LX} (w/w%)	Y_{LT} (g/L/h)	Y_{LS} (w/w%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	Ref.
Cardoon stalks (Glu 90.0 + Xyl 9.4)	B[a,1]	85	5.5	30	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	[7]
	B[a,2]	85	5.5	30	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	
	B[b,1]	85	5.5	30	26.0	6.8	26.2	0.02	9.9	22.6	10.0	39.5	
	B[b,2]	85	5.5	30	22.8	7.5	32.9	0.02	13.6	21.6	11.5	37.8	
Corncob (Glu 40 + Xyl n.a.)	B	60	6.0	25	8.2	4.7	59.1	0.05	12.0	21.1	7.1	58.9	[115]
Corncob (Glu 60 + Xyl n.a.)	B	90	6.0	25	12.6	7.6	60.2	0.08	13.0	21.0	7.5	57.5	
Beech wood (Glu 41.7 + Xyl 6.2 + Cell 2.1)	F	10	5.0	28	44.9	21.9	47.9	0.23	10.0	35.1	12.6	44.6	[116]
Vegetable waste (TRS 472.4)[c]	B	45	6.5	28	9.5	2.7	28.3	0.01	n.a.	26.5	9.2	53.2	[117]
Vegetable waste (TRS 439.1)[d]	B	45	6.5	28	8.1	2.1	26.0	0.01	n.a.	25.3	10.6	57.7	
Poplar wood (Glu 7.1 + Xyl 143.9)	B	200	5.5	30	13.9	5.1	37.0	0.05	19.0	15.7	9.0	45.9	[118]
Giant reed (Glu 90.1 + Xyl 20.8)	F	24	5.5	30	44.7	28.4	63.6	0.09	19.8	26.9	8.2	33.3	[17]
Sugarcane bagasse (TRS 80.0)	B	n.a.	5.0	30	25.3	10.3	40.5	0.09	14.0	22.9	10.4	54.7	[119]
Sweet sorghum stalks (n.a.)	B	60	6.5	30	n.a.	2.0	n.a.	0.02	6.7	23.0	4.6	37.9	[120]
Water hyacinth (TRS 35.0)	B	0.5	7	30	12.7	1.4	10.7	0.02	5.6	n.a.	n.a.	n.a.	[121]
	B[e]	12.3	7	30	12.4	4.5	35.8	0.05	17.9	n.a.	n.a.	n.a.	
	B[f]	13.6	7	30	11.4	3.6	31.4	0.04	9.2	n.a.	n.a.	n.a.	
	B[e,f]	13.6	7	30	12.2	7.3	59.7	0.09	19.6	48.4	3.0	43.0	
Corn stover (TRS 70.0)	F	n.a.	6	30	50.7	31.3	61.7	0.12	18.0	27.5	12.0	49.0	[92]

Glu = glucose; Xyl = xylose; Cell = cellobiose; TRS = total reducing sugars; FT = fermentation technology, B = batch-mode fermentation, F = fed-batch mode fermentation; C/N = carbon to nitrogen weight ratio; C_x = cell biomass concentration; C_L = lipids concentration; Y_{LX} = intracellular lipid content; Y_{LT} = lipids productivity; Y_{LS} = lipids yield; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = oleic acid. n.a. = not available. [a] Undetoxified cardoon hydrolysate as fermentation medium; [b] Simulated cardoon hydrolysate as fermentation medium; [c] H_2SO_4-pretreatment; [d] HCl-pretreatment; [e] phosphate-free hydrolysate; [f] = acetate-supplemented hydrolysate; [1] Yeast inoculum in the exponential growth phase; [2] Yeast inoculum in the stationary growth phase.

In the study of Chang et al. [115], corncob hydrolysate was adopted as the carbon source for SCO production. Acid pretreatment of biomass was performed with 1 wt% sulfuric acid for 30 min at a solid-to-liquid weight ratio of 1:10. The acidic liquid filtrate containing cellulose and hemicellulose fractions was further hydrolysed by autoclaving at 121 °C for 60 min. Finally, the enzymatic hydrolysis of cellulose was performed. The authors firstly investigated the effect of glucose concentration and C/N weight ratio on the lipogenic performance of *C. oleaginous*. Then, they adopted the optimal reaction conditions in the lignocellulosic hydrolysate fermentation carried out at pH 6.0 and 25 °C. Two different corncob hydrolysates were tested as culture medium: the first one containing the glucose concentration of 40 g/L with a C/N ratio of 60; the second one containing the glucose concentration of 60 g/L with a C/N of 90. The xylose content in the corncob hydrolysates and its contribution to the C/N ratio and lipogenesis were not considered by the authors who did not report any information on hemicellulose fate in the paper. As reported in Table 7, in the first case the cell biomass production was 8.2 g/L, the production of the lipids was 4.7 g/L, the lipid content was 59.1 w/w%, the lipids productivity and yield were 0.05 g/L/h and 12.0 w/w%, respectively. In the second case, the cell biomass production, the lipid production, the lipid content, productivity and yield increased to 12.6 g/L, 7.6 g/L, 60.2 w/w%, 0.08 g/L/h and 13.0 w/w%, respectively. According to the literature, the increase of the C/N ratio (from 60 to 90) in combination with the increase of the carbon source concentration (from 40 to 60 g/L) favoured the lipogenesis. Moreover, in this case, the variation of C/N and sugars concentration did not affect the SCO profile even if this last was different with respect to the profile obtained by Caporusso et al. [7] in terms of C18:1 relative content.

In the study of Siebenhaller et al. [116], beech wood cellulose fibre hydrolysate was used as the substrate for the bioconversion of second-generation sugars to oil. Acid-catalysed organosolv pretreatment was adopted to selectively remove the lignin fraction and part of the hemicellulose fraction. After washing the solid residue, it was used as the substrate for the enzymatic hydrolysis of the cellulose fraction. The obtained hydrolysate contained 30.8 g/L cellobiose, 89.7 g/L xylose and 608.3 g/L glucose. However, it was diluted in order to obtain the concentration of total sugars of 50 g/L, corresponding to 41.7 g/L glucose, 6.2 g/L xylose and 2.1 g/L cellobiose. Fed-batch mode fermentation was performed at 28 °C, pH 5.0, with a very low C/N ratio, equal to 10. Every 24 h fresh sugars-rich hydrolysate was added to the bioreactor in order to maintain the total sugars concentration at around 50 g/L. After 96 h fermentation, the total consumption of sugars in the medium was achieved. The cell biomass production, lipid production, lipid content, productivity and yield were 44.9 g/L, 21.9 g/L, 47.9 w/w%, 0.23 g/L/h and 10.0 w/w%, respectively. As expected, the very low value of the C/N ratio favoured cell biomass production instead of lipogenesis and a low lipid yield was reached despite the fed-batch mode fermentation, which usually favours the lipids production due to the C/N increase during the process. Despite the C/N ratio was lower than that adopted in the previous works [7,115] and the similar lipid yield values, the productivity of 0.23 g/L/h obtained in the fed-batch configuration was significantly higher than the value of 0.08 g/L/h reached by Chang et al. [115] and the value of 0.02 g/L/h achieved by Caporusso et al. [7] in batch-mode. Thus, the study of Siebenhaller et al. [116] demonstrated that *C. oleaginous* improves its catalytic performance in the fed-batch fermentation.

In the study of Chatterjee et al. [117], vegetable waste hydrolysate was converted into SCOs. Vegetable waste was mainly composed of left over potatoes (~35 wt%), carrot (~18 wt%), cucumber (~13 wt%), tomato (~12 wt%), brinjal (~7 wt%), lady finger (~6 wt%), cabbage leafs (~5 wt%) and bottle gourd peels (~4 wt%). This biomass, appearing as a slurry, was firstly pretreated with different concentrations of acids (H_2SO_4, HCl, HNO_3, H_3PO_4) or bases (NaOH, KOH) in the autoclave at 120 °C, 15 psi for 15 min. The highest total reducing sugars concentration was obtained by acid pretreatment. In particular, the pretreatment with 1.5 wt% H_2SO_4 yielded 472.4 g/L of reducing sugars while the pretreatment with 2 wt% HCl yielded 439.1 g/L of sugars. These last hydrolysates were used as

fermentation medium for *C. oleaginosus* growth. The process was carried out at 28 °C and pH 6.5 with a C/N ratio of 45. By fermenting the H_2SO_4-catalysed hydrolysate the cell biomass production, the lipid production, the lipid content and productivity were 9.5 g/L, 2.7 g/L, 28.3 w/w%, 0.01 g/L/h, respectively. By fermenting the HCl-catalysed hydrolysate the cell biomass production, lipid production, lipid content and productivity were very similar. Although the abundant availability of the carbon source (more than 400 g/L as total reducing sugars), the catalytic performance of *C. oleaginosus* was not valuable. The microbial oils obtained by fermenting the two hydrolysates were very similar. Moreover, the FAMEs profile was similar to that obtained by fermenting corncob hydrolysates [115], namely rich in oleic acid methyl esters.

Samavi et al. [118] adopted poplar wood hemicellulose hydrolysate as the substrate for SCOs production. Poplar wood chips were firstly pretreated by steam explosion. From this process, a solid residue rich in cellulose and lignin fractions and a liquid phase rich in xylose deriving from hemicellulose fraction were obtained. This hydrolysate contained 143.9 g/L xylose, 7.1 g/L glucose, 3.3 g/L arabinose, 9.6 g/L acetic acid and 0.5 g/L 5-HMF. It was fermented without any detoxification step with a C/N ratio very high, equal to 200. The bioconversion process was kept for 8 days even if the complete consumption of xylose and glucose was observed after 5 days. The lipid concentration increased in the second stage of the fermentation and reached its maximum by the end of the seventh day. The achieved lipids yield of 19.0 w/w% was higher than the values of 13.0, 10.0, 14.0 and 6.7 w/w% obtained from corncob, beech wood, sugarcane bagasse and sweet sorghum stalks, respectively, probably due to the adoption of the very high C/N.

In the study of Di Fidio et al. [17], SCO was produced starting from giant reed (*Arundo donax* L.). Biomass was pretreated by the acid-catalysed steam explosion followed by enzymatic hydrolysis. The sugars-rich hydrolysates contained 90.1 g/L glucose, 20.8 g/L xylose, 3.2 g/L acetic acid, 0.3 g/L 5-HMF and 0.7 g/L furfural. Fed-batch fermentation of undetoxified hydrolysate was carried out in a 2 L bioreactor for 13 days at pH 5.5 and 30 °C. The starting C/N ratio was set at 24 but it was increased up to 300 at 192 h (8^{th} day) by feeding the bioreactor with a concentrated hydrolysate in order to favour the lipogenesis after the initial cell biomass production. This approach was similar to the spatially separated two-stage fermentation process developed by Tang et al. [85] for N-acetylglucosamine which aimed to firstly maximise the yeast growth and then the lipids production by using a low C/N ratio in the first stage and a very high C/N in the second stage. At the end of the fed-batch fermentation, the cell biomass production, the lipid production, the lipid content, productivity and yield were 44.7 g/L, 28.4 g/L, 63.6 w/w%, 0.09 g/L/h, 19.8 w/w%, respectively. These results confirmed the higher efficacy of the fed-batch mode fermentation respect to the batch configuration, according to the studies of Siebenhaller et al. [116] and Gong et al. [92], as well as the increase of lipids yield through the adoption of a very high C/N ratio (up to 300 g/g), similarly to the study of Samavi et al. [118]. The yield of 19.8 w/w% reached from giant reed hydrolysate was the highest value reported in Table 7 for the biorefinery processes concerning the bioconversion of lignocellulosic biomasses to SCOs by *C. oleaginosus*. However, SCO produced by fermenting giant reed hydrolysate was characterised by a significantly lower content of C18:1 than microbial oils obtained from other lignocellulosic biomasses. In particular, it contained only 33.3% oleic acid while the average relative amount was around 50%.

Brar et al. [119] adopted the xylose-rich acid hydrolysate of sugarcane bagasse as the culture medium for SCOs production by *C. oleaginosus*. Biomass was pretreated with dilute acid (1 wt% H_2SO_4) at a solid to liquid ratio of 1:15 at 121 °C, 15 psi for 30 min. After pretreatment, the cellulose-rich solid residue was recovered by filtration while the xylose-rich hydrolysate was detoxified by 2 wt% activated charcoal in order to reduce the concentration of inhibitors. The detoxified lignocellulosic hydrolysate contained around 80 g/L total reducing sugars. The batch-mode fermentation continued for 144 h. The efficient and selective removal of inhibitors favoured the cell biomass production (25.3 g/L) and the lipogenesis with a lipids productivity of 0.09 g/L/h that represented the maximum

value reported in the literature for the bioconversion of lignocellulosic hydrolysates to SCO by *C. oleaginosus* in batch-mode configuration (Table 7).

In the study of Antonopoulou et al. [120], sweet sorghum stalks were used as raw material for microbial lipids production. Direct enzymatic hydrolysis was performed on dry biomass without any pretreatment step. The composition of the obtained hydrolysate was not reported in the study. It was adopted as a fermentation medium without any detoxification step and addition of a nitrogen source. The estimated C/N of the hydrolysate was 60. The bioprocess was carried out at 30 °C and pH 6.5 in batch-mode configuration. The cell biomass production and the intracellular lipids content were not reported. The lipids production and yield were very low, equal to 2.0 g/L and 6.7 w/w%, respectively, probably due to the presence of inhibitors in the hydrolysate or the extremely low nitrogen content in the culture medium since the absence of supplementation of external N sources such as peptone, yeast extract or ammonium sulphate. Moreover, these process conditions also affected the FAMEs profile since it contained a low relative percentage of C18:0 and C18:1, equal to 4.6 and 37.9%, with respect to the other SCOs profiles reported in Table 7.

In the study of Zhou et al. [121], water hyacinth hydrolysate was converted into SCO by *C. oleaginosus*. Water hyacinth was pretreated by using 0.5-2.0 wt% sulfuric acid at 120 °C for 60 min. The solid-to-liquid weight ratio was 1:10. The pretreated biomass was then subjected to enzymatic hydrolysis. In order to favour the bioconversion of the sugars into SCO by *C. oleaginosus*, phosphate removal was performed. Calcium hydroxide powder was added into the hydrolysate until the pH reached 10.0. Then, the suspension was magnetically stirred for 30 min at room temperature and set for 2 h. The resulting solids were removed by centrifugation. In this way, the KH_2PO_4 concentration was reduced from 2 to 0 g/L in the medium. Batch-mode fermentation tests were carried out on both phosphate-rich and phosphate-free water hyacinth hydrolysates, under the same process conditions (pH 7, 30 °C). C/N ratio differed in the two hydrolysates. In the phosphate-rich one, it was very low (0.5) while in the second one it was 12.3. These low values were due to the high protein content of biomass (around 20 wt% on the dry matter). By fermenting the phosphate-rich hydrolysate the cell biomass production, the lipid production, the lipid content, productivity and yield were 12.7 g/L, 1.4 g/L, 10.7 w/w%, 0.02 g/L/h, 5.6 w/w%, respectively. Differently, by fermenting the phosphate-free hydrolysate the performance of *C. oleaginosus* significantly improved. These results clearly demonstrated the effect of nutrients, such as nitrogen and phosphorous, on lipogenesis. In the presence of the same carbon content and cell biomass production (around 12.5 g/L), the complete removal of phosphorus and the increase of the C/N ratio from 0.5 to around 12 significantly increased lipids production. The lipids content increased from 10.7 to 35.8 w/w% and the lipids yield increased from 5.6 to 17.9 w/w%. The biochemical mechanism of lipid overproduction under phosphate limitation conditions was widely reported in the literature [122]. The phosphate relevant metabolism, ribonucleic acid (RNA) degradation and triacylglycerols (TAG) biosynthesis are activated, whereas the tricarboxylic acid (TCA) cycle and ribosome biosynthesis are inhibited under phosphate limitation, which channels carbon flux to lipid biosynthesis. These authors also investigated the effect on water hyacinth enzymatic hydrolysates of both acetate supplementation and P removal from the same medium, as reported in Table 7. By just adding acetate into the hydrolysate an increase of lipids content and yield was observed up to 31.4 w/w% and 9.2 w/w%, respectively. The best results were reached by combining the acetate supplementation with the P removal. The lipids production, content and yield significantly increased up to 7.3 g/L, 59.7 w/w% and 19.6 w/w%, respectively. By comparing the results from the fermentation of water hyacinth hydrolysates with and without the acetate supplementation, it was concluded that co-utilization of lignocellulosic hydrolysate and acetate was an effective method to promote lipid production. Moreover, the combination of acetate supplementation and P removal significantly improved the lipogenic performance of the biocatalyst *C. oleaginosus* but modified the SCO composition. In fact, the obtained oil contained a very high relative

content of palmitic acid (48.4%) and a very low amount of stearic acid (3.0%), while the relative percentage of oleic acid (43.0%) was similar to the other SCOs profile. Similarly to the fermentation of VOAs, the fermentation of different lignocellulosic biomasses under various process conditions allows the production of oils with a tailored and desired chemical composition in the perspective of their applications.

In the study of Gong et al. [92], corn stover was used as starting lignocellulosic biomass. Alkaline organosolv pretreatment was performed by using sodium hydroxide-methanol solution. The pretreated biomass was then used as the substrate for second-generation sugars production by enzymatic hydrolysis. Corn stover hydrolysate contained 70.0 g/L total reducing sugars, composed of 40 g/L glucose, 20 g/L xylose and 10 g/L as the sum of arabinose, cellobiose and other minor monosaccharides. Fed-batch fermentation was carried at pH 6 and 30 °C in order to enhance the production of the lipids in the perspective of industrial-scale applications. Two feedings were performed at 72 and 156 h, namely when reducing sugars concentration was lover than 10 g/L. The fermentation process continued for 11 days. As reported in Table 7, the results obtained in fed-batch mode fermentation of different lignocellulosic hydrolysates were quite similar [17,92,116] and demonstrated that this process configuration improved the catalytic performance of the yeast *C. oleaginosus*. Moreover, the obtained FAMEs profile was similar to that reported in most of the studies on the fermentation of lignocellulosic biomasses.

3.5. Wastepaper

Cellulosic wastepaper is approximately composed of 70-80 wt% cellulose, 5-15 wt% hemicellulose and a negligible amount of lignin, proteins, additives and ash [3,123]. Wastepaper can derive from the paper-making process of pulp and paper industries or by the recycling of paper deriving from offices, newspapers, notebooks, cardboard, etc. Over 400 million tonnes of cellulosic waste are generated in Europe each year and only around 50-65% is recycled due to the shortening of fibres during recycling which reduces the quality of the paper [124]. In order to favour the complete exploitation of this kind of renewable waste deriving from plants by the production of add-value molecules, various catalytic approaches and biorefinery processes have been studied and optimised. In the literature, several works investigated the conversion of wastepaper into ethanol, methane and biodiesel [125]. To date, wastepaper has scarcely been studied as a negative-value raw material for the production of second-generation sugars which are subsequently converted into new generation lipids. There are two main advantages of using cellulosic waste over lignocellulosic waste for lipid production. On one hand, energy-intensive pretreatments (e.g. steam explosion) or corrosive and dangerous reagents (e.g. strong inorganic acids) are not necessary to deconstruct the lignocellulosic structure which is scarcely recalcitrant to hydrolysis due to the low lignin content and the very high abundance of pure cellulose and hemicellulose [126]. On the other hand, most of the inhibitors deriving from lignocellulosic biomass and nitrogen components are removed through the washing step during the paper-making process. Therefore, wastepaper is a suitable raw material for the production of high-quality hydrolysates, rich in monosaccharides and deficient in nitrogen and inhibitors, to be exploited as substrates in more economically sustainable production of SCOs [127]. Up to now, in the literature, only two studies have investigated the use of wastepaper hydrolysates for the production of new generation oil by *C. oleaginosus* [127,128]. Table 8 reports the main information related to the proposed biorefinery schemes.

Table 8. Bioconversion of wastepaper to single cell oil by the biocatalyst *C. oleaginosus*.

Carbon source	FT	C/N (g/g)	pH	T (°C)	C_X (g/L)	C_L (g/L)	Y_{LX} (w/w%)	Y_{LT} (g/L/h)	Y_{LS} (w/w%)	C16:0 (%)	C18:0 (%)	C18:1 (%)	Ref.
OPEH	B	45.9	5.5	30	17.3	9.1	52.5	0.19	20.1	32.9	7.6	52.3	
NPEH	B	47.2	5.5	30	14.7	7.5	51.4	0.16	20.9	32.6	6.8	51.5	[127]
CBEH	B	53.7	5.5	30	12.5	7.1	56.4	0.15	22.4	30.2	6.0	55.2	
WOP-H_2O_2	B	80.0	6.0	30	15.2	5.8	37.8	0.08	23.5	21.6	12.4	52.3	[128]

OPEH = Office Paper Enzymatic Hydrolysates (glucose 37.3 g/L + xylose 7.3 g/L); NPEH = Newspaper Enzymatic Hydrolysates (glucose 29.0 g/L + xylose 6.5 g/L); CBEH = Cardboard Enzymatic Hydrolysates (glucose 27.7 g/L + xylose 4.9 g/L); WOP-H_2O_2 = Waste Office Paper pre-treated with H_2O_2 (glucose 22.7 g/L + xylose 1.8 g/L); FT = fermentation technology, B = batch-mode fermentation; C/N = carbon to nitrogen weight ratio; C_x = cell biomass concentration; C_L = lipids concentration; Y_{LX} = intracellular lipid content; Y_{LT} = lipids productivity; Y_{LS} = lipids yield; C16:0 = palmitic acid; C18:0 = stearic acid; C18:1 = oleic acid.

In both the studies, a pulverisation process was proposed followed by enzymatic hydrolysis with cellulase, xylanase and β-glucosidase in order to produce reducing sugars to be fermented. In the work of Zhou et al. [127], three different wastes were adopted as raw materials: office paper, newspaper and cardboard. All of them presented a similar chemical composition. In particular, office paper was characterised by the following composition (wt% respect to the dry matter): glucan 60.3, xylan 11.7, lignin 1.4, crude proteins 0.4, ash 23.4. The newspaper showed the following composition (wt%): glucan 55.6, xylan 10.1, lignin 10.3, crude proteins 0.7, ash 15.2. Cardboard was composed of (wt%): glucan 56.8, xylan 6.2, lignin 13.1, crude proteins 1.3, ash 12.5. The direct enzymatic hydrolysis of the feedstocks produced similar hydrolysates. Office paper enzymatic hydrolysate (OPEH) was composed of glucose 37.3 g/L and xylose 7.3 g/L; newspaper enzymatic hydrolysate (NPEH) contained glucose 29.0 g/L and xylose 6.5 g/L, while cardboard enzymatic hydrolysate (CBEH) contained glucose 22.7 g/L and xylose 1.8 g/L. All of them were adopted as fermentation media in the bioconversion of sugars into SCO catalysed by *C. oleaginosus* in batch-mode fermentation. Similar C/N values characterised the wastepaper hydrolysates: OPEH 45.9, NPEH 47.2, CBEH 53.7. Based on similar fermentation conditions, especially in terms of nature and concentration of carbon source, all the processes reached similar results as reported in Table 8. The cell biomass production ranged from 12.5 to 17.3 g/L, the lipids production ranged from 7.1 to 9.1 w/w%, the lipids content ranged from 51.4 to 56.4 w/w%, the lipids productivity from 0.15 to 0.19 g/L/h and lipids yield from 20.1 to 22.4 w/w%. Also the SCOs profiles were similar and were characterised by a higher content of C16:0 (around 30%) than the FAMEs profiles obtained by fermenting lignocellulosic biomasses (Table 7). From the mass balance point of view, authors reported that from 1 kg of office paper, newspaper and cardboard waste 98.2, 80.8 and 75.7 g of SCO was produced, respectively.

Annamalai et al. [128] proposed a pretreatment of office paper waste based on the use of 0.5 wt% hydrogen peroxide in combination with a step in the autoclave at 121 °C for 30 min. This kind of pretreatment aimed to reduce the lignin content in the biomass before the enzymatic hydrolysis of cellulose and hemicellulose to glucose and xylose, respectively. In fact, hydrogen peroxide promotes rapid oxidative depolymerization of lignin from the lignocellulosic materials by increasing the cellulose and hemicellulose contents in the obtained solid residue [91]. The starting raw material contained 52.4 wt% cellulose, 9.5 wt% hemicellulose, 15.1 wt% lignin, 4.6 wt% moisture and 18.4 wt% ash. After the H_2O_2-catalysed pretreatment, the wastepaper composition became 73.4 wt% cellulose, 5.9 wt% hemicellulose, 2.5 wt% lignin, 6.2 wt% moisture and 10.4 wt% ash. By comparing the enzymatic digestibility of office paper waste before and after the pretreatment, the value was around 60% on unpretreated biomass and around 90% on pretreated one, confirming the efficacy of the H_2O_2-catalysed pretreatment on sugars production. The fermentation of the office paper hydrolysate was performed in batch-mode, at pH 6 with a high C/N of 80. Moreover, the effect of various organic and inorganic nitrogen sources on cell mass and lipid production was investigated. Yeast extract, peptone, ammonium sulphate and ammonium chloride were tested as nitrogen sources. The best component of the culture medium for enhancing the performance of *C. oleaginosus* resulted yeast extract, while the worst one was ammonium sulphate. These findings were in agreement with those reported in the literature for the same biocatalyst [17]. Under the optimal process conditions, the cell biomass production and the lipid production, content, productivity and yield were 15.2 g/L, 5.8 g/L, 37.8 w/w%, 0.08 g/L/h, 23.5 w/w%, respectively. Although, the relatively low lipids content, a very high yield was reached, especially if compared to the maximum theoretical yield of 32 w/w% regarding the conversion of glucose to triglycerides [56]. The SCO profile was slightly different with respect to those obtained in the study of Zhou et al. [127]: the C16:0 relative content was around 20% instead of 30% while C18:0 was around 12% instead of 6%.

Overall, the bioconversion of wastepaper hydrolysates to SCOs by *C. oleaginosus* (Table 8) ensured higher lipids yields than the fermentation of traditional lignocellulosic hydrolysates (Table 7) despite the same nature of the carbon source (glucose and xylose). The main difference between these two kinds of wastes, namely the absence of lignin and the possibility to avoid pretreatments for wastepaper, allows the obtaining of high-quality sugars-rich hydrolysates that are very similar to synthetic media based on pure sugars. This excellent chemical composition of hydrolysates maximises the activity of the whole-cell biocatalyst *C. oleaginosus* ensuring lipids yield closed to the maximum theoretical one, according to the literature [3].

7. Applications of single cell oils, strengths and weaknesses

In the last 30 years, the study of the biochemistry of lipids synthesis and accumulation by oleaginous yeasts and the investigation of innovative and sustainable biorefinery processes have become one of the most popular research topics in the field of industrial biocatalysis and biotechnology [129]. SCOs can play a crucial role in different fields, such as human health, nutraceuticals, cosmetics, animal feeding, oleochemical and biopolymers industries, and the bioenergy sector [19,20,78,130-132]. Actually, the biochemical pathways for oil accumulation in oleaginous yeasts are known and this favours the research work on finding low-cost and alternative feedstocks for SCOs production and on improving the bio-oil productivity by genetic tools or optimising the bioconversion process. SCOs will play a more critical role in the future, and low-cost substrates, such as municipal and agro-industrial wastes as well as dedicated lignocellulosic crops, will play a crucial role in the industrialization of SCOs production [2,6,15,27]. Some of the main advantages of SCOs are their similar composition to vegetable oils, the higher production rate with respect to vegetable oils, the independence from the seasons and the climate, and the lower land needing than traditional bio-oil. Microbial oil has potential for application in the production of new generation biofuels because of its similarity to oils obtained from oleaginous crops, such as palm, sunflower and rapeseed oils, used for the production of traditional biodiesel [3,4,7,18,133]. The use of microbial oils instead of food or vegetable oils for industrial and energetic purposes represents a key solution to the ethical debate "food versus fuel" [89]. Due to the versatile lipid profile of SCOs and the possibility to customise the oil composition by using a particular biocatalyst, namely a specific oleaginous yeast strain, yeast oils represent a promising raw material for the synthesis of tailored triacylglycerols, biosurfactants, food additives, bio-based lubricants and detergents [18,134-136]. The different fatty acid methyl esters obtained by the transesterification of single cell oils are characterised by various industrial applications. In particular, myristic acid methyl ester is a flavouring agent in soap and cosmetics manufacturing; palmitic acid methyl ester is an important constituent of biofuel, soap and cosmetics; palmitoleic acid methyl ester is an anti-thrombotic agent and it found application in pharmaceutics; stearic acid methyl ester is used for biofuels, manufacturing of soap, cosmetics and lubricating agents; oleic acid methyl ester is an effective emulsifying agent and it founds applications in pharmaceutical products; linoleic acid methyl ester is used in beauty products, antioxidants, and manufacturing of oil paints; linolenic acid methyl ester founds applications in the manufacturing of soap, emulsifiers and inflammatory agents [117]. Another important potential application of SCOs is represented by the sustainable production of polyunsaturated fatty acids (PUFAs) [14,130,132]. PUFAs of the ω-3 and ω-6 classes (e.g., α-linolenic acid, linoleic acid) are essential for maintaining biofunctions in mammalians like humans. Since humans cannot synthesise these essential fatty acids, they must be taken up from different food sources. Classical sources for these fatty acids are fish oil [137]. However, SCOs are a promising source as well, representing a solution to reduce the pressure on marine ecosystems. As a result, several approaches have focused on the supply of PUFAs from algae and yeast [138]. The last important application of SCOs regards animal and fish nutrition [19,139,140]. As reported in the pilot study of Blomqvist et al. [19] there were no significant differences regarding weight and length gain, feed

conversion ratio, specific growth rate, condition factor and hepatosomatic index between the control group of fishes fed with traditional vegetable oil and the group fed with microbial oil. The fatty and amino acid composition of diet from both groups was comparable. This study demonstrated that it is possible to replace vegetable oil with SCO produced from lignocellulosic biomasses.

Notwithstanding the above promising perspectives of the potential applications of SCOs some crucial technical aspects remain to be solved in order to make possible the industrial scale-up, such as the optimisation of the yeast cell lysis and of the following oil extraction. In general, there are no recovery methods that are equally efficient for different species of oleaginous yeasts. Each method is based on different mechanisms to disrupt cells and extract the lipids. There are mechanical (bead mill, ultrasonication, homogenization and microwave), chemical (acid or base digestions, osmotic shock, supercritical fluid extraction) and biological (enzymes) approaches for the disruption or permeabilization of oleaginous yeasts membrane [141]. To date each of them presents different advantages and disadvantages from the economic, environmental and/or technical point of view. Regarding the oil extraction after the cell lysis, lipids are typically recovered by liquid-liquid extraction based on the use of organic solvents. In particular, the combination of polar and nonpolar solvents for extraction is the most adopted method since it ensures the efficient and selective extraction of triglycerides. The most cited protocol in the literature is based on the combination of chloroform and methanol as developed by Folch and by Bligh and Dyer [142]. However, the use of these carcinogenic and hazardous chemicals severely limits the sustainability of the SCOs production to the commercial scale. Several studies investigated the substitution of chloroform and methanol with green solvents such as terpenes (*p*-cymene and *d*-limonene), esters (isoamyl acetate, butyl acetate, ethyl acetate), ethers (cyclopentyl methyl ether, 2-methyltetrahydrofuran), alcohols (isopropanol, ethanol) and amines (N-ethylbutylamine, N-dipropylamine and N, N-dimethylcyclohexylamine) [141,143-145]. Although some of them (*p*-cymene, *d*-limonene, α-pinene, ethyl lactate, isopropanol and ethanol) were not suitable for replacing petroleum-based solvents, mainly due to technical and economic reasons, the potential use of green solvents has been demonstrated for several oleaginous yeasts with comparable extraction performance and no significant impact on the composition of lipids. Further studies will be needed to estimate the energy consumption and environmental impact of each method in order to determine which approaches represent the promising alternatives for lipids recovery from different yeast species in the perspective of industrial scale-up.

8. Conclusions

To the best of our knowledge, the present work represents the first literature review on the main recent biorefinery processes catalysed by the whole-cell biocatalyst *Cutaneotrichosporon oleaginosus*. Among oleaginous yeasts, the species *C. oleaginosus* is one the most promising biocatalyst for the sustainable conversion of a wide range of agro-industrial waste to new generation oil, the so-called single cell oil. *C. oleaginosus* is fast growing, is able to use various carbon sources with high lipids yields, can accumulate high content of lipids and presents a good tolerance to the main growth inhibitors. In particular, this yeast can grow on carbon-rich media obtained from lignocellulosic biomasses, organic acids, organic wastes from the agriculture and food industry as well as wastepaper. Moreover, the lipid profile of this yeast is characterised by over 50% of unsaturated long-chain fatty acids, with a high quantity of oleic acid and linolenic acid. This chemical composition made SCO a strategic and versatile platform chemical for different industrial reactions and applications in the field of bioenergy, biopolymers, biomaterials, fine chemicals, cosmetics and animal feed. All the process information and oil profiles were summarised and deeply described in the present review. This work aims to provide a comprehensive and detailed description of the recent bioprocesses catalysed by *C. oleaginous* and, at the same

time, to provide a useful guide in the use of this biocatalyst for the advancement of new biorefinery models.

Author Contributions: Conceptualization, Di Fidio, N. and Raspolli Galletti, A. M.; writing—original draft preparation, Di Fidio, N. and Minonne, F.; writing—review and editing, Antonetti, C. and Raspolli Galletti, A. M.; supervision, Antonetti, C. and Raspolli Galletti, A. M. All authors have read and agreed to the published version of the manuscript.

Funding: This research received no external funding.

Conflicts of Interest: The authors declare no conflict of interest.

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