A critical review of challenges and advances to produce 2G biodiesel with oleaginous microorganisms and lignocellulose.

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Abstract

Lignocellulose as carbon source and oleaginous microorganisms including yeast, bacterium, and fungi, have been studied extensively for oil and biodiesel production, however, several bottlenecks and challenges remain unresolved. Today the commercial biodiesel production is stuck at the first generation using oleaginous seeds as palm, soybean and others, but commercial oil production from oleaginous microorganisms is not cost-effective and sustainable. Before fermentation is necessary get rich-sugars liquor from lignocellulose, but this is very difficult because lignin affects both enzyme activity and oleaginous microorganism growth, then hydrolysis is a process that easily could be stopped by several changes in adequate conditions that sometimes is almost impossible has the control as the strain ability to produce enzymes or cellulose polymerization degree by origin of lignocellulose. The oil production in the bioprocess is a challenge too because fermentations of oleaginous microorganisms are aerobic which means need to supply oxygen constant during fermentation, this consume energy, besides lipid production is low, and the lipid yield decreases in the extraction process. In this small review discuss about those troubles related to sustainable lipid production with oleaginous microorganisms and lignocellulose as carbon source.

Keywords

Biodiesel 2G, oil, lignin, enzymatic hydrolysis.

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1. Introduction

Lignocellulose biofuel production mainly bioethanol and biodiesel has been a challenge since the scientific community and global policy government glimpsed lignocellulose as a carbon source to reach 2G biofuels due to its cheap and abundant material. However, the successful models at a commercial scale in biofuels implemented many years ago, do not use lignocellulose as a carbon source, the biofuels commercial production remains stuck in 1G biofuels, those successful models are bioethanol production in the USA and Brazil, where most of the commercial gasoline is a blend of 10 and 15% of bioethanol and fossil-derived gasoline [1], the question is ¿why bioethanol production is possible, but biodiesel production not? Firstly raw material for bioethanol is grain corn an abundant resource in the USA, the first place in worldwide corn production (USDA-ERS, 2023), and juice sugarcane in Brazil, sugarcane`s largest producer, however, corn grain and juice sugarcane is not enough for biofuels production because they are used for several purposes as animal feed, human consumption and others industrial applications. Lignocellulose is an abundant and economical raw material too, although farmers argue that they need corn stover to maintain and increased soil fertility, actually, lignocellulose is the most abundant raw material from different sources such as corn stover, wheat straw, barley straw, forest and industrial residues, energy plant cultures, etc., [2–5] However, today is not possible the sustainable and commercial biodiesel production.

Grain corn requires pretreatment to get corn liquor, but an acid or enzymatic pretreatment is enough to relatively easily reach a sugars high concentration liquor, the juice sugarcane even does not need acid or enzymatic pretreatment, this is 1G biofuels production, however, to hydrolysis lignocellulose the energy consumed is greater than corn grain, the

energy consumed is for mill, chemical pretreatment for lignin separation, enzymes production, agitation along 2-3 days, heat to 50 ºC, centrifuge 5000rpm [6] and for increase sugar concentration evaporate in a distiller or rotavapor, also spend energy and resource for it, making very expensive the process. Then for bioethanol, the fermentation is an anaerobic process, which means no need for agitation or oxygen supply, this is one of the main differences between bioethanol and biodiesel, oleaginous microorganisms are aerobic, to get biomass as pellets, mycelium or chlamydospores, is required supply of oxygen and agitation this increases energy spend for the biodiesel production process.

After fermentation of fungus or yeasts, the biomass recovery process from culture broth is an easy way by filtration or sedimentation, due to formation of pellets or any form of cells such as mycelium or chlamydospores, however, fatty acids are intracellular therefore is needed a cell disruption process for oil extraction [7], this stage increase the energy cost process too, because to separate protein fraction and lipid phase need centrifugation, then the solvent separation from oil usually is through a distillation process and more energy consumption for this stage [8].

Introduction in the global market electric vehicles since some years ago, has being demonstrated as a viable alternative to petroleum fuels for powering small road vehicles and this help for reach neutral carbon emissions, however biofuels likely to continue for some time more, because of are necessary for powering large vehicles as those required for aviation, shipping and heavy good vehicles [9]. Even today if industrial production of biodiesel could be cost-effective, biodiesel from oleaginous microorganism should be running on actuality.

This small review paper discusses the oil production from lignocellulose and fermentation with oleaginous microorganisms, on main factors that hinder this bioprocess, to summarize are high energy consumption and resources, difficult to scale to large volumes, and low lipid yield, the deep analysis could give guidelines for continuing research to overcome bottlenecks that today hinder biodiesel production with lignocellulose and oleaginous microorganisms.

1.1.1. Lignocellulose from agricultural and forest residues (2G Biofuels).

The world's annual lignocellulose production is 10^9-10^{12} Ton, composed of 20-40% hemicellulose, 10-25% lignin and 40-60% cellulose [61,62], due to this abundance has been suggesting the production of biodiesel from single cell oil, produced from oleaginous microorganisms and rich sugars hydrolysate from lignocellulose. The hydrolysis of cellulose to glucose is performed by the system called cellulase which includes 3 enzymes [63], consequently, a three stages process is required: 1. Hydrolysis of amorphous areas by endo-glucanases, which causes short chains of cellulose (Cellodextrins), 2. Hydrolysis of cellodextrins by exo-glucanases enzymes (Cellobiohydrolases) to cellobiose and 3. Hydrolysis of cellobiose by the enzyme beta-1,4-glucosidase originating glucose units [64]. For hemicellulose the hydrolysis of xylan is performed with the enzymes endo 1,4-Beta-Xylanases and Beta 1,4-Xylidases [65]. The main cellulolytic microorganisms are *Aspergillus niger, Aspergillus oryzae* [66] *Aspergillus fumigatus* [67], *Trichoderma reesei* [68], *Myceliopthora thermophila* [69], *Cellulomonas flavigena* [70] among others.

Although lignocellulose production is abundant, the real situation for oil production is that difficulties and bottlenecks do not overcome yet due to a physicochemical phenomenon called recalcitrance, the meaning of the concept is possible understand when despite of submit lignocellulose in aggressive depolymerization process, could be hydrothermal treatments at very high temperatures as steam explosion, or chemical treatment with highly oxidant reagents or acids and biological treatment with cellulolytic, xilanolytic and ligninolytic enzymes, always remain a lignocellulosic residue without degrading [71], just combustion is the process able to transform completely the lignocellulose in small molecules in one stage, acid or enzymatic process are suitable for liquors with high sugars concentration from starch or cellulose and xylan purified polymers but not for lignocellulose.

Lignin is the first trouble, because lignin hydrolysis release phenolic compounds, whose structural basis is benzoic acid or similar (See fig. 1), the salt of this acid is sodium benzoate used as food conservator in very small concentrations due to inhibit microbial growth, in fermentative process exactly that effect has the phenolic compounds, does not allow microbial growth of oleaginous microorganisms, and those is not easy problem to solve because of pretreatment by itself release phenolic compounds, sodium hydroxide is good pretreatment to remove lignin however has two inconvenient produce a contaminant and toxic liquor and does not remove completely the lignin, therefore one step for filtration should be add at the process or reagents as the polyethylene glycol could reduce inhibitor effect of phenolic compounds [72]. All those high energy and chemical requirements, also environment challenges, joined at remain lignin due to partial lignin fraction still hindered its further utilization.

Since lignin hydrolysis releases the most toxic compounds that represent a real risk to fermentation by yeasts and oleaginous fungi, the feasible option is use lignin as a compound from which obtains derivatives of high value with applications in textiles, lubricants, adhesives and personal care products and even some bacteria can use it as a carbon source for the production of PHA (Polyhydroxyalkanoate) [73].

Some pretreatments to remove lignin are sodium hydroxide extraction, microwave assisted depolymerization, supercritical fluids depolymerization, electrostatic separation [74], attrition mill to reduce corn stover particles less than 100µm [75], ionic liquids and deep eutectic solvents extraction [76] and potent ligninolytic enzymes to remove lignin as a biological pretreatment with *Pleurotus stratus, Phanerochaete crysosporium* and *Aspergillus oryzae* [77,78]. If three lignocellulose components are usable for high-value chemicals or biofuels, it could be a zero-residue and environmentally friend process [79]. However the bottleneck is that separation of three components of lignocellulose is not completely selective and always remain a fraction of every one of them, and the phenolic released are extremely toxic for microorganisms growth and for enzyme activity still in very low concentrations as 50µg-phenolics/mL [72].

1.1.1.1. Inhibitors: Furfural and hidroximetilfurfural

The furan aldehydes compounds mainly furfural and hydroxymethylfurfural are produced due to pentose and hexose dehydration, they are very strong growth cell inhibitors, are released while lignocellulose hydrolysis release reducing sugars. Maybe lipid yields produced from lignocellulose biomass decreases, because of inhibitors released from pretreatment and enzymatic hydrolysis, however *M. isabellina* has some resistance to

furfural and hydroxymethylfurfural, the yield decreases, but the fungus can growth up to 88.8% with furfural released and 76.9% with hydroxymethylfurfural compared to a test control without inhibitor, resistance to these inhibitors maybe due to the ability of the fungus to convert them to less toxic compounds [80].

1.1.1.2. Inhibitors: phenolic compounds and aldehydes.

p-coumaric acid, syringic acid and ferulic acid,

The acids p-coumaric acid, syringic acid and ferulic acid, are main inhibitors of fermentation process, however could be used as carbon sources to produce lipids in some bacteria, the key intermediate compound is protochatechuic acid, which is obtained by specific enzymes activity, for example, enzyme 4-acid hidroxibenzoic, 3-hydrolase, react on p-coumaric acid to produce afore mentioned protochatechuic acid, that is converted to acetyl CoA, the syringic acid also is converted to acetyl CoA, through a demetylation to covert first to 3-Omethyl gallic acid and then transformed into 2-pyranone-4,6 dicarboxylic acid to finally enter the cycle TCA. The ferulic acid has commonly an intermediate, the vanillic acid, demethylated by a demethylase and transformated in protochatechuic acid, then acetyl CoA and enter the cycle TCA [81].

Bacterium *Rhodococcus opacus,* and others like *Acinetobacter calcoaceticus* and *Pseudomonas putida* can metabolize phenolic compounds, however Wang et al. [51] used as carbon source liquor of corn stover pretreated with ammonia, the lignin concentration was 4g-lignin/L and inoculated with *Rhodococcus opacus*, after 114h of fermentation the intake of lignin by the microorganism just was 20%. The pointed out key factor to phenolic compounds, overall is that inhibition effect strongly depend of phenolic

compounds concentration in hydrolysate, when concentration is around of 0.2g-phenolic-compoundss/L, several microorganisms could resist and continue growing, but when concentration around 2g-phenolic-compounds/L the most of microorganisms stop growing completely [82].

Table 1
Lignin extraction methods.

Lignin extraction method.	Lignin extracted %	Sugar concentration g/L	Disadvantage	Refs
Sodium hydroxide NaOH	55	87.9	Toxic and expensive	[6]
Sodium chlorite $NaClO_2$	92.1	22	Highly toxic	[83]
Steam explosion	3	47.6	Small lignin extraction percentage	[6]
Attrition mill	84	20	High energy cost	[84]
Deep eutectic solvent	44.65	2.29	High solvent cost	[85]
Electrostatic separation	54	43.5	Patented process	[74]
Ligninolytic enzymes	30	12.7	Very slow process Small lignin extraction	[86,87]
Ammonia fiber expansion	47.6	44.8	Expensive	[51]

Besides phenolic compounds also inhibit enzyme activity of CMCase and xylanase, little concentrations as 0.05g-phenolic-compounds/L has direct effect on hydrolysis, therefore pretreatment of lignocellulose should remove at least 95% of lignin to avoid phenolic compounds release and other compounds like furfural, which also has toxic effect, but in real practice every stage release phenolic compounds, and as mentioned above does not exist any pretreatment to remove lignin completely just in one stage and the highest

removal lignin percentage do not reach 95% (See table 1), even for every one of the lignocellulose components is not possible complete separation and/or hydrolysis in pretreatment, according with Zhao et al. [6] the pretreatment to remove lignin with sodium hydroxide 2% pretreatment, however need 120 ºC for 30min, and for reach 120ºC is necessary increases pressure for it, due to the low density, the lignocellulose takes up a lot of space, therefore large volume containers will require huge energy quantity for reach high pressure and actually is not economically viable, to reduce space needs, the mill through different size grids to get less than 1mm particles of lignocellulose could reduce volume of lignocellulose but bring other difficulties as high energy consume and the suspension as a fluid easily remain stuck in tubing causing troubles for handle.

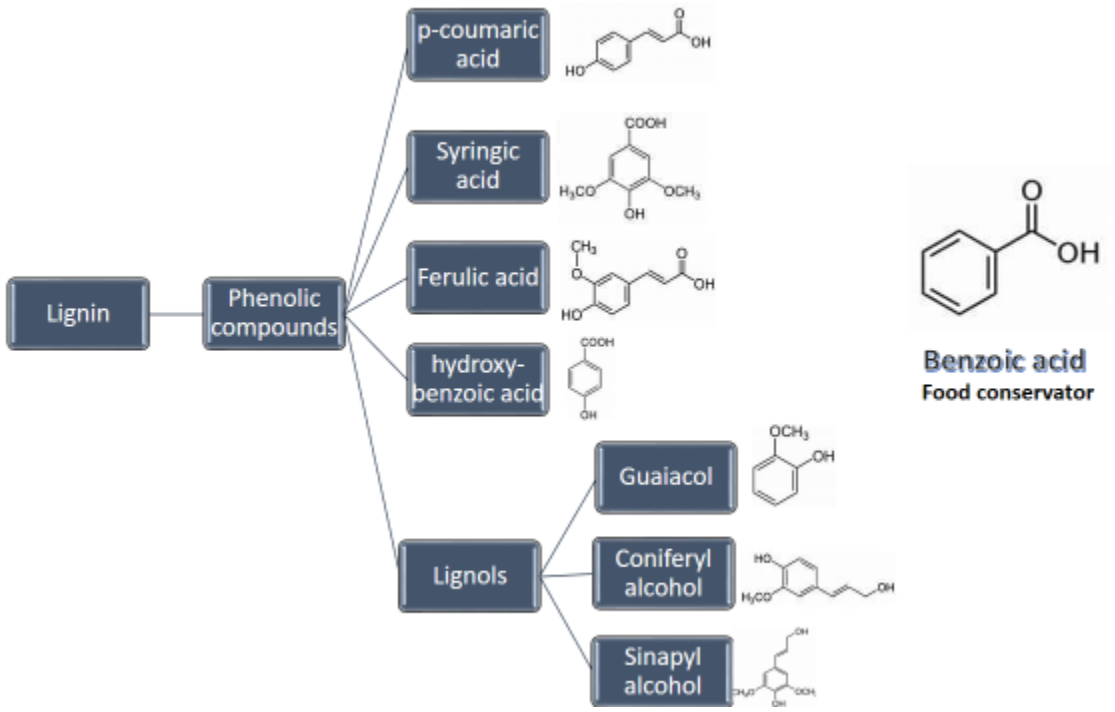

Fig. 1. Phenolic compounds derived from the lignin, the base of chemical structure is similar to that of the benzoic acid.

The bio-refining process means fraction, separation and essentially purification, therefore the component of lignocellulose that should be firstly purified is cellulose, to overcome technical problems and bottlenecks this is the critical step, due to its importance a physicochemical purification is more appropriate and more efficient than biological, However, also the clear disadvantages are visible, the most hemicellulose fraction is loosed together lignin, a liquor toxic is produced, but this liquor also contains sugars in low concentrations. After alkali pretreatment lignin residues are remain and at least two unit operations are needed for whole cellulose purification, this process waste almost 60% of origin lignocellulose raw material and this demand a lot of energy but, as aforementioned the lignocellulose pretreatment unavoidably needs energy, it can be proposed that due to the abundance of lignocellulose the most of energy consumed could be get from the lignocellulose combustion, for example, to lignin extraction should be heat 120ºC, lignocellulose combustion could be used for that purpose, and any process like dried or heat. Under this assumption in reference to abundance of lignocellulose ever stage that needs heat could be solve in this way.

1.2. Enzymatic hydrolysis

As aforementioned several microorganisms has the cellulolytic enzyme production ability, and has been studied extensively, the specific and specialized process make the enzyme cost very expensive and frequently is recommend that enzyme production be with same substrate due to enzyme specificity, this mean on site enzyme production. However the total reducing sugars yield in hydrolysate can be from 1.19 to 180 g-glucose/L [88,89] this huge difference could be caused by culture conditions, microbial strain, lignocellulose origin, cellulose polymerization degree and even metabolic state of the microorganism

[90] due to it a bottleneck in real lab or industrial conditions could be that enzymes has not good performance. According to Gutierrez et al. , cellulose is not soluble when there are 6-8 glucose molecules joined, this feature and considering that gen regulating mechanisms are not similar for all those cellulolytic microorganisms, and the structure complex of lignocellulose pretreated, where lignin and xylan residual polymers are hindering enzyme activities, is highly possible that enzymes will be inhibit, even two process has been studied as enhancers to hydrolysis of cellulose, first cellulose processing and its derivatization into carboxymethylcellulose [91] and second acid maleic treatment as mimetic enzyme to xylanase and cellulase [92], the disadvantages as aforementioned could be that for this pretreatments first cellulose should be purified which is a laborious and highly energy and resources consume process, plus the chemical reagents needed for carboxymethylcellulose preparation increase cost, and unavoidably always remain a lignocellulose residue.

2. Oleaginous microorganisms fermentation process

2.1. Oleaginous microorganisms

An oleaginous microorganism is able to store at least 20% of lipids by dry cell weight [10–12] although some fungi and yeast species store up to 85% of lipids [13], due to theoretical yield, they are not able to efficiently convert sugars into lipids, as ethanologenic yeasts convert sugars into ethanol [14]. For example *Sacharomyces cerevisiae* is able to reach 0.47g-ethanol/g-glucose consumed [15], thus, the research carried out with oleaginous yeasts focuses on lipid yield increase; mainly by metabolic engineering [16,17] although have also been used other strategies such as, alternative carbon sources and

fermentation systems in semi-continuous mode, [18] high cell density cultures in fed-batch mode [19–21] and extracellular oil capture [22].

2.1.1. Fungi and yeast

The most studied oil fungi are: *Mortierella alpine* [23,24], *Mortierella isabellina* [25–27], *Cunnhinghamella echinulata* [28,29], *Aspergillus oryzae* [30,31] and *Mucor circinelloides* [32,33]. The oleaginous yeasts workhorses are *Yarrowia lipolytica* [22,34], *Lipomyces starkeyi* [19,35–38], *Rhodosporidium toruloides* [39,40], *Trichosporon fermentans* [41] *Rhodotorula* [42–44] *Candida* sp. [45] *Cryptococcus curvatus* [46], *Trichosporon cutaneum* [47,48] and *Cutaneotrichosporon oleaginosus* [49], recently *Cryptococcus curvatus, Trichosporon cutaneum* and *Cutaneotrichosporon* oleaginosus are considered same microorganism [49,50] .

2.1.2. Bacteria

The bacteria able to produce lipids are *Rhodococcus, Streptomyces, Bacillus, Acinetobacter, Nocardia*, among others, some authors consider that bacteria are poor lipid producers. However, this potential is argued in other studies, because they are fast-growing microorganisms, in addition to, their flexibility to grow in various carbon sources, for example, *Rhodococcus opacus*, can use lignin as carbon source [51].

Rhodococcus opacus is the most tested for lipid synthesis, high cell density cultivation process at pilot-scale in fed-batch mode was developed, the biomass yield can reach 37.5g-biomass/L, and triacylglycerol content 52% in fed-batch fermentations from 3% w/v of sugar molasses as carbon source [52], this lipid yield is similar to oleaginous fungi genus *Mortierella isabellina* [25,27].

The oleaginous microorganisms produce a lipid yield actually very low (less than 20g-lipids/L), except for metabolic engineering yeast, but in industrial terms scaling up for great volumes as 10 m^3 or even more, results in a real oil quantity get from dry cell weight that is not enough for cover the energy and resources spend to whole production process.

2.2. Carbon source

Carbon source have a roleplay key in fermentation of oleaginous microorganisms, this is due to oil quantity obtained has a direct relationship with sugar quantity consumed, the sugars like disaccharides for example sucrose, lactose, cellobiose, and monosaccharides as glucose, xylose, fructose and arabinose, are refined sugars that allow easy and fast consumption by oleaginous microorganisms, the refining process increases the cost besides is argued that should be used for human consumption and not use for biofuels production, however human health troubles as obesity and hypertension related with excessive sugar intake should redirect sugars refined production for biofuels, as also sugars used for alcohol beverages like beer, tequila and others could be convert in oil for biodiesel, and reduce human health troubles related with alcohol intake too, this option proposed is not possible as market prices of beverages are higher than biofuels and government policies not allow it.

2.2.1. Corn and sugarcane (1G Biofuels)

Corn starch and sugarcane could be useful to oil production with oleaginous microorganisms, but the availability of them is specific for every country, USA is the greatest country corn producer worldwide and Brazil get sugarcane surplus at a certain time of the year. Therefore ¿Why not produce oil as raw material to biodiesel from

sugarcane juice surplus? Socol et al. [40] made a research on biodiesel production in pilot scale with sugarcane juice as carbon source and the findings were economically viable oil production and that even compete with fossil biodiesel prices, this is a cheaper bioprocess than bioplastics production as polyhydroxy-alcanoate.

However besides of USA, Brazil, China, Russia and Argentina in the most of countries demand of corn and sugarcane is greater than offer, and the governments in Latin America for example, Mexico and Colombia every year should import huge amounts of corn grain from countries big producers to satisfy the demand, besides even in countries as Brazil needs corn and sugarcane for ensure the security for bioethanol demand due to that, the corn and sugarcane production is not enough for biodiesel production. Also the corn grain is used as animal feed for meat production, the suddenly change to biofuel production could be a risk for food security [60].

2.3. Submerged fermentation

2.3.1. Agitation and aeration

Agitation and aeration are both essential due to microorganisms are aerobics, in research´s lab for small bioreactors oxygen supply and agitation is possible, for example in airlift bioreactors of 2L even oxygen supply also has agitation function, however for oil production with oleaginous microorganisms the scaling up 10-100m^3 is essential, and airlift no looks like an scalable option due to, although the best oxygen supplier or air compressor could satisfy oxygen necessary for agitation in a 100m^3 bioreactor or even 600m^3 Bisgaard et al. [95], today there is not an industrial process for oil production with oleaginous microorganisms, this could be due to high energy cost and also because in the stationary phase of fermentation the high biomass cell concentration do not allow

broth recirculation. For continuous stirrer tank bioreactors scaling up could be possible however for them, also high energy consume is required, because of agitation and aeration should be continuous. The high expense for fermentation process such as aeration and agitation, coupled with relative low lipid productivity, have limited the economic competitiveness of this technology.

2.3.2. Sterilization

An relevant point is the energy cost due to sterilizing the bioreactor with culture medium therefore the fermentation should be carried out in an unsterilized bioreactor to reduce production costs [96]. Some few studies address this topic, but in real life conditions, for produce biomass from a microorganism in bioreactors, unavoidably the contamination risk is present, scalable options increased that risk, great volume bioreactor more complicated to sterilize. Very easy contamination by any microorganism if bioreactor is not sterilized.

It could be possible increase the percentage of inoculum to avoid contamination effect, however although the target microorganism could win for highest biomass production, the contaminant microorganism is present in less quantity but enough for decrease biomass and lipid production.

2.4. Solid state fermentation

Since solid state fermentation do not need continuous agitation and aeration as submerged fermentation, this great advantage looks like the best way for oil production with oleaginous microorganisms however it is very far to be true, actually this way is too

complex to get single cell oil due to more time for fungal growth, small percentage of bioconversion, need for high contact surface and lipid recovery.

The solid state fermentation as an alternative option to avoid submerged fermentation costs has the advantage that not required energy for the fermentation, but today neither is a sustainable and viable bioprocess, Liu et al. [97] studied a solid state fermentation process with *Phanerochaete crysosporium* to lipid production, this bioprocess does not need oxygen supply and agitation, however is a very slow process of around 12 days, besides the chlamydospores growth only occur in the surface layer where the microorganisms has oxygen, but in deeper layer fungus does not growth, the optimal thickness layer is 1.5cm, when layer increases thickness, deeper cells not growth due to lack of oxygen. To scaling up solid state fermentation, supply oxygen through remove daily as in compost process could be an option, but this break chlamydospores. To solid state fermentation with 1.5cm thickness layer is needed a huge superficial area, scaling up could be possible but first design a functional and good performance solid state bioreactor for great volumes as $100m^3$.

3. Lipid yield

An aspect important in oil production with oleaginous microorganisms is yield. To date, even the highest yield is insufficient for commercial lipid production [98], although advances in research tend to increase [16,99,100] the maximum stoichiometry yield limited by acetyl CoA, hinder enhance it, under ideal fermentation conditions the lipid yield for single cell oil (SCO) production is around 0.22g-lipids/g-glucose consumed [101], the theoretical yield is higher and has been calculated from sugars stoichiometry such as glucose and xylose, 100g of catabolized glucose generated 1.1 mol of acetyl CoA (MW =

809.57g/mol) so if all this acetyl CoA, is channeled into lipid synthesis, the theoretical maximum yield is 0.32 g-lipids/g-glucose consumed; in the case of xylose, the metabolic pathway of α-ketoglutarate is the most efficient 1.2 mol of acetyl CoA is produced per 100 g of catabolized xylose, with a maximum theoretical yield of 0.34g-lipids/g-xylose consumed [102] although the pentose phosphate pathway is the most common pathway used by microorganisms to catabolize xylose [103], this pathway produces 1 mol of Acetyl CoA for every 100g of xylose consumed, generating a maximum theoretical yield of 0.3 g-lipids/g-xylose consumed. Recently a synthetic pathway was found, which consists of recycling Nicotinamide Adenine Dinucleotide Phosphate (NADPH), with this, theoretical yield increases to 0.351g-lipids/g-glucose consumed [98].

Achieve theoretical yield is very difficult because acetyl CoA is used in several metabolic pathways [104], for example, the mevalonate pathway consumes acetyl CoA and ATP (Adenosine triphosphate) to produce carotenoids and sterols [44]; significant advances have been made with *Yarrowia lipolytica* by achieving 0.27g-lipid/g-glucose consumed [105] and later with an oil capture strategy, a yield of 0.33g-lipid/g-glucose consumed was achieved [22], but the oil production was 12g-lipid/L-culture medium, very low compared to 98.9g-lipid/L obtained in research carried out by Qiao et al. [105] and in a context of viable production of SCO, increase yield in g-lipid/L as well as yield in g-lipid/g-sugar consumed, are important to decrease economic and energy costs. The above-mentioned yields were obtained using pure glucose, however should be use lignocellulose sugars but yield is less, around 0.22 g-lipids/g-sugar (See table 2) and according to the NREL (National Renewable Energy Laboratory), to make biodiesel production viable it is

necessary to achieve a yield of 0.28 g-lipids/g-lignocellulose sugar, and 1.3 g/L/h productivity.

For fungal oleaginous microorganisms is very important reach at least 80-100g-glucose/L in hydrolysate to produce 18-22g-DCW/L and 12g-lipids/L, here a key point whereas several papers report 36g-biomass/L and 18-20g-lipids/L[27,93,94], is truth that around 10g-glucose/L, yield overall 3±0.5g-DCW/L, however increase to 100g-glucose/L and produce 30±5g-DCW/L, this is a surpassed and no reproducible or repeatable result, in several fermentations where we determine kinetics parameters (data no shown) resulted in 23±1.5g-DCW/L, this is similar results obtained by Zhao et. al. 2017, as the last advance published with oleaginous fungi *Mortierella isabellina.*

Table 2

Highest yield g-lipid/L of different oleaginous microorganisms in submerged fermentation.

Type	**Oleaginous microorganism**	**Yield g-lipid/L**	**Yield g-lipid/g-sugar consumed**	**Lipid accumulation percentage %**	**Sugar intake g/L**	**Yield g-biomass/L**	**Refs.**
Fungi	*M. alpine*	18.6	0.19	53	98.2	35.12	[23]
	M. Isabellina	18.5	0.18	64.5	100	28.8	[27]
	M. Isabellina	18.1	0.18	55	100	35.9	[93]
	M. Isabellina	17.8	0.23	61	80	29.5	[94]
Yeasts	*Y. lipolytica*	115	0.16	59.3		194	[100]
	R toruloides	89.4	0.22	75.6	367	148	[107]
	Y. lipolytica	85	0.21	77	430	118.4	[108]
Algae	*C. vulgaris*	0.15		16.6		0.9	[56]
	S. incrassatulus	0.41		23.1		1.8	[109]
	D. tertiolecta	0.48		0.33		1.44	[110]
Bacterium	*R. opacus*	3.04	0.2	16	80	19	[111]

3.1. Lipid extraction

The fatty acids of oleaginous microorganisms are intracellularly, therefore cell disruption pretreatment is needed, sometimes acid treatment is enough for release lipids, but enzymatic pretreatment also be useful, both acid and enzymatic treatments requires agitation and temperature increased causing plus energy consumption (See table 3), next solvent extraction spend energy for agitation and centrifugation, and distillation in solvent recovery stage. To bacterium lipid extraction is highly difficult even more than algae, due to peptidoglycan of cell wall is very strong.

Table 3

Differences between several cell disruption and lipid extraction methods.

Cell disruption method	Recovery percentage	Oleaginous microorganism	Process with highly Energy spend		Disadvantages	Refs.
High pressure homogenization	55.9%	*Rhodosporidium toruloides*	High pressure	600bar	High energy spend	[112]
Mineral acids wetting	99%	*Rhodosporidium toruloides*	Agitation Centrifugation Distillation	60rpm 6000g	Environmentally toxic	[40]
Enzymes	86.9%	*Mortierella alpine*	Heat	80ºC	High enzymes cost	[7]
Extraction method						
Microwave-assisted extraction	77%		Microwave	95ºC	High cost equipment	[113]
Ultrasound-assisted extraction	19.49%	*Mortierella isabellina*	Distillation	70-80ºC	Low oil recovery	[114]
Blight and Dyer	88.6%	*Chlorella pyrenoidosa*	Distillation Agitation	70-80ºC	Laborious Time-consuming High toxic	[115,116]
Folch	77.15%	*Chlorella pyrenoidosa*	Distillation Agitation	70-80ºC	Laborious High toxic	[115,116]
Soxhlet	63.4%	*Mortierella isabellina*	Distillation	70-80ºC	High toxic No able for wet samples	[117]
Supercritical fluids	92%	*Scenedesmus obliquus*	High pressure	220 bar	High energy spend	[118]

Fig. 2. Diagram for biodiesel production process from lignocellulose and oleaginous microorganisms

4. Industrial Scaling up

The most problems in biodiesel production with oleaginous microorganisms aforementioned, are the bottleneck to scaling up the process. International companies or state enterprises, surely has the economic power to install the infrastructure need to biorefinery, but the oil production capacity is limited, and a continuous process development is highly difficult to coordinate every stage in a sequential process, for example if enzyme production is not enough, surely don`t get rich sugars liquor, this means time and resources lost. The plants of biofuels like bioethanol 2G was shut down due to technical and economic problems, the establishment of this industry has clearly been challenging [119].

5. Conclusions

Due to lignin is highly toxic for oleaginous microorganisms and enzyme activities, it is essential delete of the hydrolysate, this a critical step to release the bottlenecks that hinder cell growth and oil synthesis. The oil production with oleaginous microorganisms could not be sustainable by lipid yield increase, rather the way is a process development able easily scaling up and low energy cost and resources. It is necessary more research about a consolidated bioprocess for lignocellulose-lipids bioconversion. The lipid extraction method desirable is the one who does not need solvents and is low energy consume, the supercritical fluids extraction adheres to first one but consume high energy, therefore is needed to research out to reach those lipid recovery percentages from high toxic solvents.

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7. Bibliography

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