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Effect of synthetic and natural media on lipid production from *Fusarium oxysporum*

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ABSTRACT

Background: Dependence on fossil resources, for the production of fuels and energy, has resulted in environmental and financial problems, which require our immediate action in order to reverse the situation. Use of renewable sources for the production of fuels and energy is an important alternative with biodiesel remains as one of the promising options. Aim of this work is to evaluate the fungus *Fusarium oxysporum* for its potentials to accumulate microbial lipids when grown on synthetic media and saccharified sweet sorghum stalks.
Results: The effect of different carbon sources, nitrogen sources and C/N ratio on the lipid production was initially examined, which resulted in a lipid concentration of 4.4 g/L, with lipid content of 42.6% *w*/*w*. Sweet sorghum stalks were able to support growth and lipid production of the fungus, both as carbon source and as nitrogen source. It was also shown that saccharification of the dried stalks is an important step to increase lipid production. Removal of the remaining stalk solids enabled the lipid production during cultivation in increased initial solids of up to 16 *w*/*w*. This resulted in a lipid production of 3.81 g/L.
Conclusions: It was demonstrated that *F. oxysporum* can be used as an efficient oleaginous microorganism, with sweet sorghum serving as an excellent raw material for the cultivation of the fungus. The lipids obtained during this work were also found to have a fatty acid profile with good potentials to be used for biodiesel production.

1. Introduction

Our society strongly depends on fossil fuels, with 81% of world's energy derived from fossil fuels (cruel oil, coal and natural gas); with oil being the most consumed fuel in the world and used primarily for transportation [1]. The drawbacks of using fossil sources are that they are being depleted and their usage contributes to greenhouse gas (GHG) emission, which leaves a great negative impact on environment. Moreover, the need for importation of fossil fuels results in energy insecurity. Progressive decrease of fossil fuels, environmental problems, and increased energy consumption lead to a demand for more alternative and renewable energy sources [2]. Among many alternatives, biofuels have been showed to be a promising solution to replace nonrenewable fuels. They have attracted much attention due to their renewability, biodegradability and improved quality of exhaust gases [3]. Biofuels can be categorized into liquid, gas and solid fuels that are mainly produced from biomass [4]. In the last decade, biodiesel has attracted attention as a renewable and environmentally friendly fuel that can replace petroleum derived fuels [5].

Biodiesel is a mixture of fatty acid methyl esters (FAMEs) produced by transesterification of triacylglycerols (TAGs) in the presence of an alcohol (the most commonly used is methanol) and an acidic or basic catalyst, with vegetable oil often serving as the source of TAGs [6,7]. Biodiesel is a renewable, non-toxic, biodegradable, nonflammable, environmentally friendly fuel and does not contain sulfur or aromatic compounds [8]. Moreover, as biodiesel contains higher oxygen content compared to conventional diesel, when used in diesel engine the exhaust emissions have lower concentration of particles, carbon monoxide, sulfur, polyaromatics, hydrocarbons and smoke. Finally, the use of fuels derived from vegetable oils can be considered as “carbon neutral”, as their burning does not contribute to the net production ofatmospheric CO_2 since the plants capture atmospheric CO_2 via photosynthesis [3,9]. Different sources of TAGs can be utilized forbiodiesel production. Most common sources are vegetable oils (e.g. sunflower oil, soybean oil) and animal oils (e.g. beef and sheep tallow and poultry oil) [4]. However, using edible raw materials for biodiesel production is controversial and it raised a lot of criticism as it is the main cause of increased global food market prices. The main factor affecting the economic viability of biodiesel market is the price of the

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feedstock [10]. In order to make biodiesel more competitive and at the same time avoid the fuel vs food dilemma, utilization of non-food crops, agro-industrial wastes and other non-edible renewable resources is needed [10,11].

One of the alternative sources of TAGs is microbial oils or single cell oils (SCOs). SCOs are produced by microorganisms with the ability to accumulate lipids more than about 20% (*w*/*w*) of its total dry biomass weight and are considered as oleaginous [12]. Microbial sources of TAGs present a promising feedstock for biodiesel, because of the short production time, little labor required and the potentials so scale up the process [13]. Storage of lipids in cells of oleaginous microorganisms occurs during secondary metabolic growth, under conditions where carbon is in excess and another essential nutrient (most often nitrogen) is limiting [14]. Oleaginous microorganisms can be found in bacteria, yeasts, algae and fungi genera [15], with some filamentous fungi able to accumulate lipids as high as 80% of their cell biomass [16]. Fungi present some positive characteristics such as short life cycles, no need of light energy, they are easily scalable and can use a wide range of carbon sources, such as lignocellulosic material, agro-industrial residues and wastewater [14]. There are several filamentous fungi that can accumulate lipids such as *Aspergillus oryzae*, *Claviceps purpurea*, *Humicola lanuginose*, *Mortierella isabellina*, *Mortierella vinacea* and *Mucor circinelloides* [10,15]. Some fungi strains are also capable of accumulating polyunsaturated fatty acids such as: docosahexaenoic acid (DHA), γ-linolenic acid (GLA), eicosapentaenoic acid (EPA) and arachidonic acid (ARA) [13].

Fusarium oxysporum is a filamentous fungus that is capable of excreting cellulases and hemicellulases, making it an ideal candidate to be cultivated on different lignocellulosic materials [17]. *F. oxysporum* has been extensively evaluated for the production of cellulolytic and hemicellulolytic enzymes [18,19,20,21], and as a fermenting microorganism for the production of ethanol from various lignocellulosic raw materials [17,22,23,24]. Apart from ethanol, *F. oxysporum* was also evaluated as an oleaginous microorganism [25,26, 27,28] although not as extensively as in ethanol. A potential advantage of using *F. oxysporum* as oleaginous microorganism is the ability of the fungus to secrete cellulolytic and hemicellulolytic enzymes. These enzymes can facilitate the hydrolysis of cellulose and hemicellulose of lignocellulosic raw materials and in turn reduce the amount of enzymes required for the preparation of lignocellulosic hydrolysates. Lower enzyme loads are beneficial for reducing the production cost of the process.

One of the main challenges in the commercialization of SCOs production is the high cost of the feedstock for the cultivation of the oleaginous microorganisms [29]. It is estimated that using commercial glucose for the cultivation of oleaginous microorganisms can account for the 80% of the total material cost, which is equal to 35% of the overall biodiesel production cost [11]. To avoid high production costs, utilization of low-cost material such as lignocellulosic material, which do not compete with food production, has been employed [29]. Among the different options the use of energy crops, such as sweet sorghum, offers a sustainable solution. Sweet sorghum (*Sorghum bicolor* L. Moench) is a C4 crop in the grass family that can grow to heights from 120 cm to above 400 cm [30]. Sweet sorghum possesses an efficient photosynthetic system, is rich in soluble sugars, can grow rapidly, has great water- and nitrogen- use efficiency, requires little chemical fertilizers, can tolerate harsh environments and can adapt to marginal lands [31,32,33]. One of the challenges of using sweet sorghum is that, due to the high concentration of sugars present in sweet sorghum, the stalks can easily get contaminated if stored at room temperature. To prevent microbial contamination and also to reduce the total volume of the stalks, a drying step, under mild conditions, was previously proposed [34]. Another challenge of using sweet sorghum for the cultivation of oleaginous microorganisms is the relatively low carbon to nitrogen (C/N) ratio which was estimated to be approximately 60–65 [35]. Increase of the concentration of carbon and subsequent of the C/N can be achieved by partially hydrolyzing the insoluble carbohydrates (such as cellulose) that are present in the sweet sorghum stalks.

The aim of this work is to examine the use of the fungus *F. oxysporum* F3 as an oleaginous microorganism. Initially, a study of the potential of the fungus for accumulating lipids took place on synthetic media mimicking the composition of sweet sorghum to examine and optimize different cultivation parameters. Finally, the ability of the fungus to grow on saccharified sweet sorghum stalks was evaluated in terms of optimizing the accumulation of lipids and the results were compared with other oleaginous fungi growing on lignocellulosic raw materials.

2. Materials and methods

2.1. Raw material, microorganism and its maintenance

Sweet sorghum (*S. bicolor* (L.) Moench) belonging to the Keller cultivar was used in the current work and was kindly provided by Prof. George Skarakis (Department of Crop Science, Agricultural University of Athens). Sweet sorghum stalks were stored at -20°C, after the leaves and seeds were removed by hand. Dried stalks were prepared as previously described [34] and were milled to 0.75 mm particles. The carbohydrate content of dried sweet sorghum stalks was as follows (% *w*/*w*): cellulose, 19.6; fructose, 8.1; glucose, 8.2; hemicellulose, 15.2 and sucrose, 34.4 [34]. The fungus strain used in this work was *F. oxysporum* F3, which was previously isolated from cumin [36]. The fungus was maintained on agar plate with following composition: 39 g/L potato dextrose agar and 2 g/L yeast extract.

2.2. Pre-culture media

Prior to each experiment, the fungus was inoculated into 250 mL Erlenmeyer flasks containing 50 mL of pre-culture broth with following composition: $CaCl_2 \cdot 2H_2O$, 0.3 g/L; KH_2PO_4, 1 g/L; $MgSO_4 \cdot 7H_2O$,0.3 g/L; $NaH_2PO_4 \cdot 2H_2O$, 6.94 g/L; $Na_2HPO_4 \cdot 2H_2O$, 9.52 g/L; glucose, 20 g/L; $(NH_4)_2HPO_4$, 10 g/L [17]. The pH of the pre-culture broth was adjusted to 6, followed by sterilization at 121°C for 20 min. Inoculation was done using a loop containing cells from agar plates and incubation of the pre-culture media was carried out at 30°C and 160 rpm for 48 h.

2.3. Cultivation of F. oxysporum on synthetic media

Cultivation of the fungus on synthetic media to evaluate its ability to accumulate lipids took place in 1 L Erlenmeyer flasks containing 200 mL of cultivation broth with a medium of the following composition: $CaCl_2 \cdot 2H_2O$, 0.3 g/L; $MgSO_4 \cdot 7H_2O$, 0.3 g/L; $NaH_2PO_4 \cdot 2H_2O$, 6.94 g/L; $Na_2HPO_4 \cdot 2H_2O$, 9.52 g/L. The pH of the cultivation broth was adjusted to 6 and sterilized at 121°C for 20 min. To determine the effect of the sugars present in sweet sorghum stalks on the growth and lipid accumulation of the fungus, preliminary tests were done using glucose, fructose and sucrose alone or in a combination similar to the one found in sweet sorghum stalks. The initial sugar concentration was 40 g/L and a mixture of yeast extract and ammonium sulfate was used as nitrogen source, at a concentration resulting in a C/N ratio of 100. For the trials determining the effect of nitrogen source and of the C/N ratio on the fungus growth and lipid accumulation ability, the mixture of the sugars was used as a carbon source at a concentration of 40 g/L, whereas the concentration of the nitrogen source varied in order to achieve the desired C/N ratio, which, during the study of the nitrogen source effect, was set as 100. Inoculation took place with 5% v/v of pre-culture media. All the cultivations were performed in duplicates.

2.4. Preparation of saccharified sweet sorghum stalks and lipid production

Prior to cultivation on sweet sorghum stalks, the stalks were diluted in a broth with minerals of the same composition as the synthetic media (excluding any carbon and nitrogen source). Saccharification of the stalks took place at 50°C and pH of 5, with the use of a mixture of the commercial enzymatic solutions Celluclast® 1.5 L and Novozym® 188 (Novozymes A/S Denmark) at a ratio of 5:1 (*v*/*v*) by applying an enzyme load of 8.32 FPU/g solids for 8.6 h; conditions that were previously found to be optimal for sweet sorghum saccharification [34]. Saccharification was performed only by using the Celluclast® 1.5 L, whereas Novozym® 188 was added during inoculation as it was previously found that it also shows invertase activity [34]. The presence of invertase would result in the hydrolysis of sucrose and in turn higher glucose concentrations, which will inhibit the action of cellulases. At the end of the saccharification, the pH was increased to 6, followed by sterilization at 121°C for 20 min and finally the saccharified stalks were inoculated with 5% *v*/*v* of pre-culture media.

In another series of experiments, the effect of solid removal was also examined. For this reason, at the end of the saccharification the solids were removed by squeezing the solution through a nylon filtration cloth followed by centrifugation. Finally, to evaluate the effect of the enzymatic saccharification, control cultivations without prior saccharification were also included. When cultivation took place with the solids present, inoculation took place directly in the solution, whereas for the control cultivation on sweet sorghum juice, the solution was left to soak for 2 h at 50°C to facilitate better sugar extraction prior to solids removal. All the cultivations were performed in duplicates.

2.5. Analytical methods

Samples were retrieved from the cultivation broths once per day. Prior to analysis, the retrieved samples were filtered through a filter paper (70 mm; Munktell, Falun, Sweden) to separate the fungi biomass and solid particles (if present) from the cultivation broth. Biomass was washed with distilled water to remove any remaining particles from the broth. The obtained liquid was used for total sugars quantification by the 3,5-dinitrosalicylic acid (DNS) method [37]. Prior to DNS analysis, sucrose was hydrolysed to glucose and fructose under acidic conditions (12 M HCl) at 70°C for 15 min, followed by neutralization with 12 M NaOH. When synthetic media or sorghum juice was used, the fungus biomass was transferred to pre-weighted glass vials and dried at 80–90°C until constant weight, and finally biomass concentration was determined gravimetrically. When solids were also present, *F. oxysporum* biomass was determined based on the glucosamine concentration of the cell wall of the fungus, as previously described [38]. Lipid extraction was conducted with the use of a mixture of chloroform:methanol at a 2:1 (*v*/*v*) ratio [39]. Lipid concentration was determined gravimetrically in pre-weighted glass spherical flasks after solvents evaporation under vacuum in rotary evaporator. It is worth mentioning that when sweet sorghum solids were present, lipid content of the solids was also determined and removed from the total lipid in order to obtain the net lipids produced by the fungus.

The fatty acid profile was determined by initially converting the lipids to their corresponding FAMEs [40]. Finally, the FAMEs were analyzed by gas-chromatography (Varian CP-3800, Agilent Technologies, Santa Clara, CA, USA) equipped with a capillary column WCOT fused silica 100 m × 0.25 mm coating CPSIL 88 for FAME (Agilent Technologies, Santa Clara, CA, USA) at operating conditions that were previously described [41].

3. Results and discussion

3.1. Cultivation of F. oxysporum on synthetic media for lipid production

Prior to the cultivation on sweet sorghum, the ability of the fungus *F. oxysporum* to accumulate lipids was evaluated on synthetic media, mimicking the composition of sweet sorghum. Initially the ability of the fungus to convert the sugars present in sweet sorghum to lipids was evaluated. For this reason, the fungus was cultivated on glucose, fructose and sucrose alone and on a mixture of them at a ratio found in sweet sorghum (glucose, 16%; fructose, 16% and sucrose 68%) to evaluate sugar consumption, cell growth and lipid production. All the tested sugars were found to support the growth of the fungus and accumulation of lipids (Table 1). The highest biomass concentration (7.35 g/L) was obtained when glucose was used as a single carbon source, whereas when cultivating the fungus on media with sucrose resulted in the lowest biomass concentration. Biomass concentration reached 6.23 g/L when the fungus was cultivated on the sugar mixture, which is between the concentration achieved with the glucose and sucrose. Lipid production was also affected by the carbon source, with the highest concentration obtained on the sugar mixture (3.30 g/L), which was slightly higher compared to glucose (3.10 g/L) and the lowest on fructose (1.83 g/L). The lipid content varied between 25.5% of CDW (cell dry weight) and 52.9% of CDW, with the highest content obtained in the mixture of sugars and the lowest on fructose. No significant difference was observed in the duration of cultivation, with all the cultivation to last 8 d except from the sugar mixture which lasted 7 d (Table 1). The biomass formation yield was higher when simple sugars were used (glucose and fructose), whereas the addition of the disaccharide sucrose had a negative impact in the yield (Table 1). Finally, the highest lipid formation yield was observed on the sugar mixture. These results show that sugars found in sweet sorghum are suitable for cultivation of fungi *F. oxysporum* and the production of single cell oils.

The main factors affecting the *de novo* lipid accumulation are the depletion of nitrogen source and to a lesser extent other essential nutrients such as phosphorus and sulfur. It has also been shown that the source of nitrogen (organic or inorganic) plays a role in the process of lipid accumulation [42]. It is generally considered that a C/N ratio higher than 20 is required for lipid accumulation, with the optimal ratio to vary a lot and being dependent on the cultivating microorganism [42]. High C/N ratio has been reported to be essential for high lipid production [43], although in some cases very high C/N ratios can result in decreased lipid accumulation [42]. Thus, in this work, we evaluated the effect of different organic (peptone, urea and yeast extract) and inorganic (ammonium sulfate, ammonium phosphate and ammonium chloride) nitrogen sources with a constant C/N ratio equal to 100 (Table 2). It can be observed that all the tested organic sources were suitable for the growth of the fungus and resulted in high biomass, whereas the inorganic nitrogen sources (except from the ammonium phosphate) were not as favorable for the formation of biomass. The highest biomass was observed on ammonium phosphate (8.48 g/L), followed by urea (8.16 g/L). Complex organic nitrogen sources (peptone and yeast extract) resulted in lower biomass concentration compared to urea. In general, organic nitrogen sources were more favorable for the growth of the fungus under nitrogen limitation conditions (with exception of ammonium phosphate) and this can be evident also by the biomass formation yields (Table 2). However, with exception of yeast extract, inorganic source of nitrogen promoted better lipid production; with the lipid concentrations being 2.75 g/L for ammonium chloride; 2.93 g/L for ammonium sulfate and 3.03 g/L for ammonium phosphate. Inorganic nitrogen sources were found to be favorable for lipid accumulation during the cultivation of other fungi such as *Aspergillus versicolor* [44] and *Cunninghamella japonica* [45] with potassium nitrate and ammonium nitrate, respectively, resulting in the highest lipids accumulation. Although inorganic sources of nitrogen seem to be more favorable for lipids accumulation from *F. oxysporum*, yeast extract as a nitrogen source resulted in the highest lipid production reaching 3.68 g/L with a lipid content 49.5% of CDW. The source of nitrogen has also an impact on the duration of the cultivation, as can been also evident by the lipids productivity (Table

Table 1
Effect of carbon source on biomass and lipid production under a C/N ratio equal to 100 (mixture of yeast extract and ammonium sulfate) and sugar concentration of 40 g/L.

Sugar source	Biomass concentration (g/L)	Lipids concentration (g/L)	Lipids content (% of DCW)	Duration (d)	Biomass yield (g/g)[a]	Lipids yield (g/g)[a]	Lipids productivity (g/L·d)
Glucose	7.35 (0.51)	3.10 (0.42)	42.2 (6.4)	8	0.206 (0.025)	0.087 (0.015)	0.39 (0.05)
Fructose	7.18 (0.37)	1.83 (0.14)	25.5 (2.3)	8	0.283 (0.016)	0.072 (0.006)	0.23 (0.02)
Sucrose	5.07 (0.13)	2.47 (0.08)	48.7 (1.9)	8	0.151 (0.009)	0.073 (0.005)	0.31 (0.01)
Mixture	6.23 (0.22)	3.30 (0.30)	52.9 (5.1)	7	0.175 (0.012)	0.092 (0.010)	0.47 (0.04)

Parenthesis indicate the standard deviation of the measurement.
[a] Biomass and lipids yield calculated as grams produced per grams of consumed sugars.

2), with the lowest duration to be achieved on peptone (5 d) and the highest duration on urea (14 d).

Finally, the effect of different C/N ratios (50, 100 and 150) on lipid accumulation was evaluated with yeast extract as nitrogen source. As shown in Table 3 the biomass of *F. oxysporum* decreased when increasing the C/N ratio with the highest biomass to be 10.44 g/L when C/N ratio was 50. The negative impact of the increasing C/N ratio on biomass formation could be attributed to the reduction of the available nitrogen which is necessary for the formation of the biomass. Increasing of C/N ratio also had a negative impact in the concentration of lipids, although the decrease in lipid concentration was lower compared to the decrease in the biomass concentration. The high lipid concentration obtained at relatively low C/N ratio is a positive characteristic, since, due to the protein content of sweet sorghum, the C/N of the stalks can be relatively low. Similar optimal C/N ratio (57) was found to be optimal for lipid accumulation of *M. isabellina* [46], whereas in other works the optimal C/N ratio was considerably higher, such as for *Cunninghamella echinulata* and *Mortierella isabellina* [47]. Finally, with increasing of the C/N ratio, the duration of the cultivation increased from to 6 d (C/N 50) to 8 d (C/N 100) and 9 d (C/N 150). The highest lipid concentration at a C/N of 50 reached 4.45 g/L after 6 d of cultivation (Fig. 1), which corresponds to a lipid content of 42.6% of CDW. Under these conditions the yield of biomass production was $Y_{B/S} = 0.266$ g/g and the yield of lipid production per gram of consumed sugars was $Y_{L/S} = 0.113$ g/g. The lipid production was 0.74 g/L·d.

3.2. Cultivation of F. oxysporum on sweet sorghum for lipid production

3.2.1. Use of saccharified sorghum stalks

After evaluating the potentials of *F. oxysporum* F3 to serve as oleaginous microorganism on synthetic media, the fungus was also studied for its ability to grow and accumulate lipids on saccharified sweet sorghum stalks. As discussed in the introduction, sweet sorghum stalks also contain protein and not just sugars. It was also previously mentioned that sweet sorghum stalks contain a significant amount of protein which was found to be approximately 11.4 g of protein per 100 g of total sugars in sweet hydrolysate juice [48]. On the other hand, proteins are not always readily assimilable for all the cultivated microorganism. At the same time, the free amino nitrogen content of sorghum was found to be approximately 194 mg per 100 g sugars [48]. To examine whether the proteins present in saccharified sweet sorghum stalks can support growth and lipid accumulation of *F. oxysporum*, the fungus was cultivated at a solids content of 8.7% *w*/w, without any external nitrogen source addition and with the addition of yeast extract at a concentration of 0.2 g/100 g of solids. The addition of yeast extract had a positive impact on the cell growth of *F. oxysporum*, increasing the biomass concentration from 10.99 g/L to 13.46 g/L. On the other hand, addition of external nitrogen source did not affect the lipid production which was 1.91 g/L in both cultivations. This indicates that the nitrogen present in the stalks as proteins is adequate for the efficient lipid production from *F. oxysporum* and no external nitrogen addition is necessary. The ability of the stalks to support the growth of fungi without external nitrogen addition is a positive characteristic with an important impact on process economics. More specifically, it was previously found that use of yeast extract as nitrogen source accounts for 16% of the total raw material cost of biodiesel production (which is equal to approximately 7% of the overall biodiesel production cost), and is the second most important raw material cost after the carbon source [11].

In the next stage of this investigation, the effect of enzymatic saccharification on the lipid production yields was examined, by cultivating the fungus on sweet sorghum stalks without prior saccharification, at solids content of 8.7% *w*/w. The presence of the saccharification step improved the lipids production from 0.71 g/L (non-saccharified) to 1.91 g/L (saccharified stalks). Biomass production also improved from 7.11 g/L to 13.46 g/L. It can be concluded that the saccharification step is necessary to improve the lipid production yield from sweet sorghum stalks. During the saccharification, the enzymes act on the insoluble carbohydrates releasing sugars that can be utilized by the fungus. Apart from increasing the available sugars, saccharification also improves the C/N ratio making it more favorable for accumulation of lipids.

Finally, in an attempt to increase the concentration of the produced lipids, the effect of increasing the initial concentration of sweet sorghum stalks (8.7%, 12% and 16% *w*/w) was also tested. Increasing the solids content had a negative impact on the lipid production which reduced to 0.62 g/L for the 12% w/w and to 0.82 g/L for the 16% w/w. The most apparent reason can be that the increasing concentration of solids resulted in inefficient broth mixing which lead to a poor aeration.

3.2.2. Use of sorghum juice

From the previous part of this investigation it was concluded that increased concentration of solids in the cultivation broth had a negative

Table 2
Effect of nitrogen source on biomass and lipid production under a C/N ratio equal to 100 and sugar concentration of 40 g/L (containing a mixture of glucose, fructose and sucrose).

Sugar source	Biomass concentration (g/L)	Lipids concentration (g/L)	Lipids content (% of DCW)	Duration (d)	Biomass yield (g/g)[a]	Lipids yield (g/g)[a]	Lipids productivity (g/L·d)
Peptone	6.26 (0.08)	1.69 (0.26)	27.1 (4.1)	5	0.251 (0.003)	0.068 (0.010)	0.34 (0.05)
Urea	8.16 (0.74)	2.21 (0.06)	27.0 (2.6)	14	0.211 (0.021)	0.057 (0.003)	0.16 (0.00)
Yeast extract	7.43 (0.20)	3.68 (0.33)	49.5 (4.6)	8	0.201 (0.007)	0.099 (0.009)	0.46 (0.04)
Ammonium sulfate	5.37 (0.10)	2.93 (0.16)	54.5 (3.1)	10	0.166 (0.008)	0.091 (0.007)	0.29 (0.02)
Ammonium phosphate	8.48 (1.44)	3.03 (0.73)	35.7 (10.5)	8	0.230 (0.040)	0.082 (0.020)	0.38 (0.09)
Ammonium chloride	4.14 (0.61)	2.75 (0.36)	66.4 (13.0)	9	0.176 (0.027)	0.117 (0.016)	0.31 (0.04)

Parenthesis indicate the standard deviation of the measurement.
[a] Biomass and lipids yield calculated as grams produced per grams of consumed sugars.

Table 3
Effect of the C/N ratio on biomass and lipid production when *F. oxysporum* was cultivated on a 40 g/L sugar mixture with yeast extract as nitrogen source.

C/N ratio	Biomass concentration (g/L)	Lipids concentration (g/L)	Lipids content (% of DCW)	Duration (d)	Biomass yield (g/g)[a]	Lipids yield (g/g)[a]	Lipids productivity (g/L·d)
50	10.44 (0.13)	4.45 (0.06)	42.6 (0.7)	6	0.266 (0.003)	0.113 (0.001)	0.74 (0.01)
100	7.43 (0.20)	3.68 (0.33)	49.5 (4.6)	8	0.201 (0.007)	0.099 (0.009)	0.46 (0.04)
150	7.55 (0.27)	3.72 (0.68)	49.2 (9.1)	9	0.249 (0.010)	0.123 (0.022)	0.41 (0.08)

Parenthesis indicate the standard deviation of the measurement.
[a] Biomass and lipids yield calculated as grams produced per grams of consumed sugars.

impact on the lipid production yields. For this reason, in the next stage of the study the effect of solids removal was investigated in the absence or presence of the enzymatic saccharification step. Removal of solids had a positive impact on the lipid production which, at an initial solids concentration of 8.7% *w*/w, increased from 1.91 g/L to 2.39 g/L. Moreover, the use of the sorghum juice enabled successful cultivation of the fungus and efficient lipid production under high solids content of 12% *w*/*w* and 16% *w*/*w*, with the lipid production being 3.29 g/L and 3.81 g/L, respectively (Table 4). The presence of the saccharification step had a positive impact on the lipid production, with the lipid concentration being higher in all the initial solids concentrations that were tested (Table 4). The higher lipid concentration obtained in the presence of the enzymatic saccharification can be mainly attributed to the increase of the soluble sugars at the start-up of the cultivation. More specifically, sugars concentration increased from 41.3 g/L to 47.0 g/L for the 8.7% *w*/w, from 63.6 g/L to 74.5 g/L for the 12% w/w and from 76.6 g/L to 86.3 g/L for the 16% *w*/w. Presence of saccharification was found to improve the lipid yields in other works employing *Rhodosporidium toruloides* [41] and *Lipomyces starkeyi* [29]. Other researchers have evaluated the effect of the stage of hydrolysis on the lipids yield, namely prior to cultivation or simultaneously, with the simultaneous application of enzymes to result in higher lipid production during growth of *Cryptococcus curvatus* on corn stover hydrolysates [49].

Growth and lipid accumulation kinetics during the growth of *F.oxysporum* on the juice from the saccharified sorghum at 16% *w*/*w*solid content is shown in Fig. 2. The highest lipid and biomass concentration obtained after 7 d of cultivation were 3.81 g/L and 21.52 g/L, respectively. Under these conditions the biomass yield was 0.272 g/g consumed sugar and the lipid yield was 0.048 g/g consumed sugar with lipid productivity 0.54 g/L per day.

Oleaginous fungi have been used for lipids production when cultivated on lignocellulosic raw materials in other works. For example, Zheng et al. cultivated a variety of fungi strains (*Aspergillus terreus*, *Cunninghamella elegans*, *Mortierella isabellina*, *Mortierella vinacea*, *Rhizopus oryzae* and *Thermomyces lanuginosus*) on detoxified and undetoxified wheat straw hydrolysates, where lipids production varied between 0.77 g/L and 2.63 g/L [50]. Other researchers have used rice hull hydrolysates for the cultivation of *M. isabellina*, which produced 3.6 g/L lipids [46]. Ruan et al. [51] cultivated *M. isabellina* in different hydrolysates (corn stover, switchgrass, miscanthus and giant reed) with the lipid production being lower (3.02–3.71 g/L) in comparison to the lipid production of the current work, except using switchgrass hydrolysate, which reached 4.4 g/L. On the other hand, in another work a higher lipid concentration wasachieved (5.1 g/L, which was further improved to 6.9 g/L when the cultivation performed at bioreactor) from combined acid- and alkali- pretreated corn stover hydrolysate [52]. Acid and alkaline pretreated corn stover was also used for the cultivation of *M. isabellina* with the lipids concentration to be 2.48 g/L (alkaline pretreated) and 4.78 g/L (acid pretreated) [53]. Apart from the high lipid concentration achieved during this work, when compared to other results when cultivating oleaginous fungi on lignocellulosic raw materials, the use of sweet sorghum does not involve chemical treatment steps (e.g. acid hydrolysis) and therefore there is no need for detoxification of the hydrolysates prior to fermentation. Moreover, sweet sorghum stalks were found to be capable of supporting the growth of the fungi in terms of the required nitrogen and no external nitrogen addition is needed.

Analysis of the lipid profile obtained from *F. oxysporum* when grown on 16% (*w*/w) sweet sorghum juice is shown in Table 5. The dominant fatty acid is oleic acid, followed by palmitic acid and linoleic acid. Lipids that contain high amounts of oleic acid are considered to be

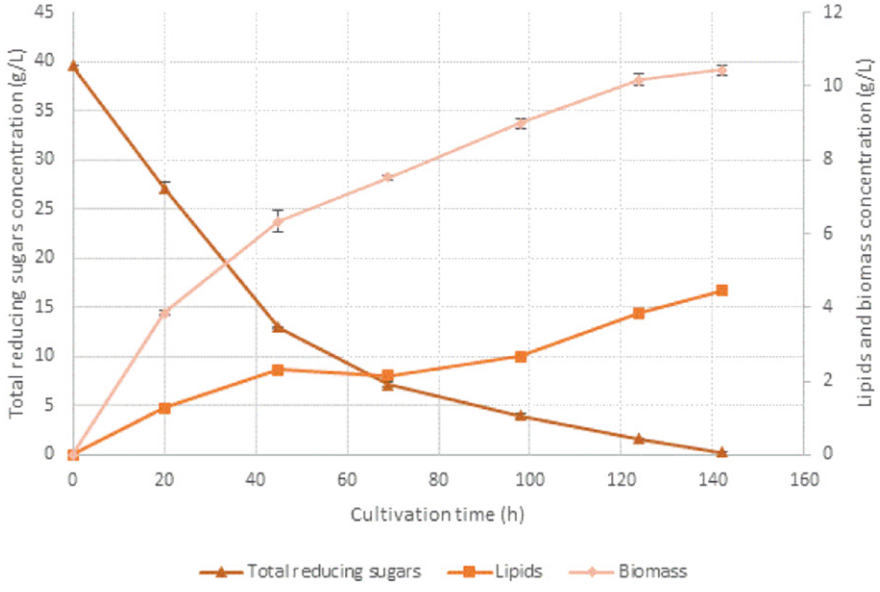

Fig. 1. Time course of the growth and lipid production of *F. oxysporum* when cultivated on a 40 g/L sugar mixture with yeast extract at a C/N ratio of 50.

Table 4
Effect of the solids content and enzymatic saccharification on the lipid production when *F. oxysporum* was cultivated on sweet sorghum stalks without the addition of any nitrogen source. Solids were removed prior to inoculation.

Solids load (% w/w)	Enzymatic saccharification	Biomass concentration (g/L)	Lipids concentration (g/L)	Lipids content (% of DCW)	Duration (d)	Biomass yield (g/g)[a]	Lipids yield (g/g)[a]	Lipids productivity (g/L·d)
8.7	+	11.23 (0.50)	2.39 (0.19)	21.3 (1.9)	6	0.244 (0.016)	0.052 (0.005)	0.40 (0.03)
	-	11.55 (0.46)	2.06 (0.10)	17.8 (1.1)	7	0.306 (0.030)	0.055 (0.006)	0.29 (0.01)
12	+	16.06 (0.09)	3.29 (0.02)	20.5 (0.2)	7	0.220 (0.036)	0.045 (0.007)	0.47 (0.00)
	-	12.84 (0.80)	2.83 (0.05)	22.0 (1.4)	5	0.237 (0.037)	0.052 (0.007)	0.57 (0.01)
16	+	21.52 (0.37)	3.81 (0.23)	17.7 (1.1)	7	0.272 (0.012)	0.048 (0.004)	0.54 (0.03)
	-	17.84 (0.51)	3.48 (0.32)	19.5 (1.9)	8	0.253 (0.025)	0.049 (0.006)	0.44 (0.04)

Parenthesis indicate the standard deviation of the measurement.
[a] Biomass and lipids yield calculated as grams produced per grams of consumed sugars.

good candidates for the production of biodiesel due to their beneficial properties [54].

To evaluate the quality of biodiesel derived from the lipids of *F. oxysporum*, different biodiesel properties were estimated according to equations found in literature, using the fatty acid composition of the obtained lipids. One important biodiesel parameter is the cetane number, which characterizes the quality of a biodiesel and described the ignition quality of a diesel fuel [55]. This value was estimated to be 57, as previously described by Bonturi et al. [56], which fulfills the requirements of the cetane number needing to be higher than 51 (according to UNE-EN 14214). The iodine value is another significant parameter which is a description of the concentration of the lipids in unsaturated fatty acids [55] and is often correlated with the oxidative stability of the biodiesel [57]. According to the UNE-EN 14214, iodine value has to be below 120 g I_2/100 g. The iodine value of the lipids obtained during this work was calculated, based on the equation proposed by Kyriakidis and Katsiloulis [58], to be 73 g I_2/100 g, which complies with the standards required for biodiesel production. Moreover, the saponification value was calculated according to equations described by Islam et al. [59] and was found to be 199.3 mg KOH/g, which is similar to values that are mentioned in literature for other oleaginous microorganisms [57,59]. The energy value of biodiesel can be determined by calculating the high heating value (HHV). Estimation of this value can be performed based on the fatty acid composition [59,60]. The HHV of the lipids obtained during this work was estimated to be 40.17 MJ/Kg, a value that is generally higher than many SCOs derived from different oleaginous microorganisms [59]. Two other important properties of biodiesel are the kinematic viscosity and the density, which affect the fuel supply to the injector and to the combustion chamber [59]. These two values were estimated by the empirical equations proposed by Ramírez-Verduzco et al. [61] and were 3.94 mm^2/s and 0.86 g/cm^3, respectively; values that meet the standards of UNE-EN 14214 (kinematic viscosity, 3.5–5.0 mm^2/s; density, 0.86–0.90 g/cm^3). The oxidative stability was also calculated based on the composition of the lipids in C18:2 and C18:3 [60]. Oxidation of biodiesel can result in the formation of insoluble gums, organic acids and aldehydes which in turn can create problems in the engine and injection system [62]. The oxidation stability at 110°C of the lipids derived from *F. oxysporum* was estimated to be 8.5 h, which is above the minimum required value of 8 h according to the UNE-EN 14214. Finally, the CFPP (cold filter plugging point) was calculated as described by Islam et al. [59] to be 15°C. CFPP is affected by the composition of the microbial oil in saturated fatty acids and especially in higher chain saturated fatty acids (higher than C16). Saturated fatty acids of the oil obtained from *F. oxysporum* during thiswork were 35.4% of the total fatty acids. Such levels of saturated fatty acids are commonly found in oils obtained from fungus such as *Mucor circinelloides* (35.8% saturated fatty acids) [63], *Rhizopus oryzae* (30.5–36.8%) [50], *Aspergillus oryzae* (39.2%) [64] and *A. terreus* (46.4%) [65]. It can be concluded that the obtained lipids from the cultivation of *F. oxysporum* present good potentials to be used as raw material for the production of biodiesel.

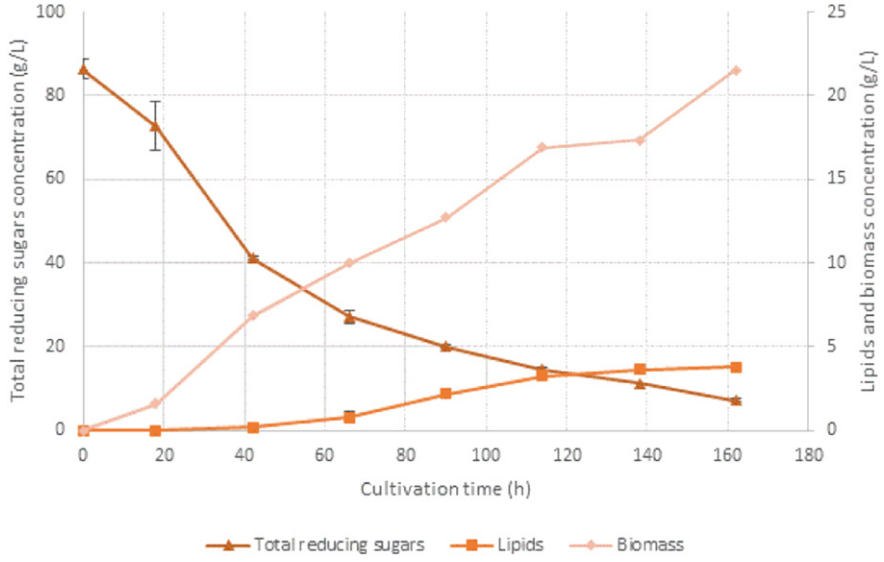

Fig. 2. Time course of the growth and lipid production of *F. oxysporum* when cultivated on saccharified sweet sorghum stalks of a solids concentration of 16% *w*/w without the addition of any external nitrogen source. Solids were removed prior to inoculation.

Table 5
Fatty acid composition of the lipids obtained during cultivation of *F.oxysporum* on juice from saccharified sorghum stalks at a solid concentration of 16% w/w.

Fatty acid	% concentration (w/w)
C14:0	0.6
C15:0	0.2
C16:0	22
C16:1	0.7
C18:0	11
C18:1	42
C18:2	20
C18:3	0.1
C20:0	0.7
C22:0	0.5
C24:0	0.4

4. Conclusions

The fungus *F. oxysporum* was evaluated as an oleaginous microorganism growing on synthetic media and saccharified sweet sorghum stalks. The effect of the carbon and nitrogen source as well as the C/N ratio was examined, with the highest lipids concentration obtained to be 4.4 g/L. Furthermore, the fungus was capable of growing on saccharified sweet sorghum stalks without the need of external nitrogen supplementation. Removal of the particles of sweet sorghum stalks enabled the cultivation of the fungus up to a solids content of 16% *w*/w, with the highest lipid production being 3.81 g/L. Analysis of the FAME composition of the obtained lipids showed that the dominant fatty acids were oleic acid, palmitic acid and linoleic acid. The composition of the FAMEs was used to estimate the fuel properties of the biodiesel, showing that the lipids obtained from *F. oxysporum* are suitable for biodiesel production.

Declaration of interest

Authors declare no competing of interest.

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