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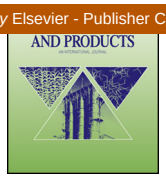

Use of dried sweet sorghum for the efficient production of lipids by the yeast *Lipomyces starkeyi* CBS 1807

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ABSTRACT

The ability of the oleaginous yeast *Lipomyces starkeyi* to efficiently produce lipids when cultivated on saccharified sweet sorghum stalks juice was evaluated. Initially the production of lipids using synthetic media mimicking sweet sorghum stalks has been studied and optimized concerning the nitrogen source and the C:N ratio. Under optimum conditions (yeast extract as nitrogen source and C:N ratio of 190) the lipid production reached 5.81 g/L with a lipid content of 47.3% (w/w) from a mixture of sucrose, glucose and fructose, mimicking the sugar composition of sorghum. When cultivated on sweet sorghum stalks juice, it was observed that no external nitrogen addition was necessary which could result in substantial decrease of the initial C:N ratio. Moreover a distinct saccharification process prior to yeast cultivation improved the lipid production yield as it resulted in an increase of the C:N ratio. The highest lipid production, which was 6.40 g/L with a lipid content of 29.5% (w/w), was obtained when juice from saccharified sweet sorghum stalks at an initial sorghum content of 12% (w/w) was used as feedstock.

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1. Introduction

The imminent shortage of fossil fuels and the rising environmental problems caused by the extended utilization of fossil raw materials had made the discovery of renewable alternatives first priority (Matsakas et al., 2014; Sarris et al., 2014). For this reason, a large part of research conducted nowadays is focusing on this target. Concerning the transport sector, which according to the European Commission constitute of 32.6% of the total consumption of energy in EU (European Commission, 2010), energy is derived from liquid fossil fuels, either gasoline or petroleum. In order to decrease the consumption of liquid fossil fuels, alternatively renewable fuels needs to be more extensively utilized, where the most common used are ethanol and biodiesel.

Biodiesel is the product of the transesterification of triacylglycerols (TGAs) with an alcohol in the presence of a catalyst and consist of a mixture of fatty acid esters (Economou et al., 2010; Agarwal, 2007). The similar properties that present comparing to the fossil petroleum, the low toxicity, its renewable characteristics as well as the low formation of pollutants during combustion make biodiesel an excellent substitute to fossil petroleum (Zhu et al., 2008; Zhao et al., 2012). Different kind of lipid sources can be utilized for the production of biodiesel, for example plant oils or animal fats (Agarwal, 2007). As most of these sources are edible, this can result in a direct competition between fuel or food production. The production of the first generation biofuel has been severely criticized to have contributed to the increase of food prices worldwide, which in turn has raised public awareness about biofuels production (Sims et al., 2010; Papanikolaou and Aggelis, 2011a; Pinzi et al., 2013; Leiva-Candia et al., 2014). This reason has made the researchers to turn their focus on the utilization on non-edible sources of TGAs for the production of biodiesel. These non-edible sources of lipids could for example be waste such as used cooking oils or be derived from microorganisms capable of accumulating lipids (Zhao et al., 2012; Papanikolaou and Aggelis, 2011a; Pinzi et al., 2013).

A microorganism is generally considered as oleaginous when is capable of accumulating more than about 20% (w/w) of lipids of its total dry biomass weight (Hu et al., 2009). Oleaginous microorganisms can be found in bacteria, yeasts, fungi and the algae genera

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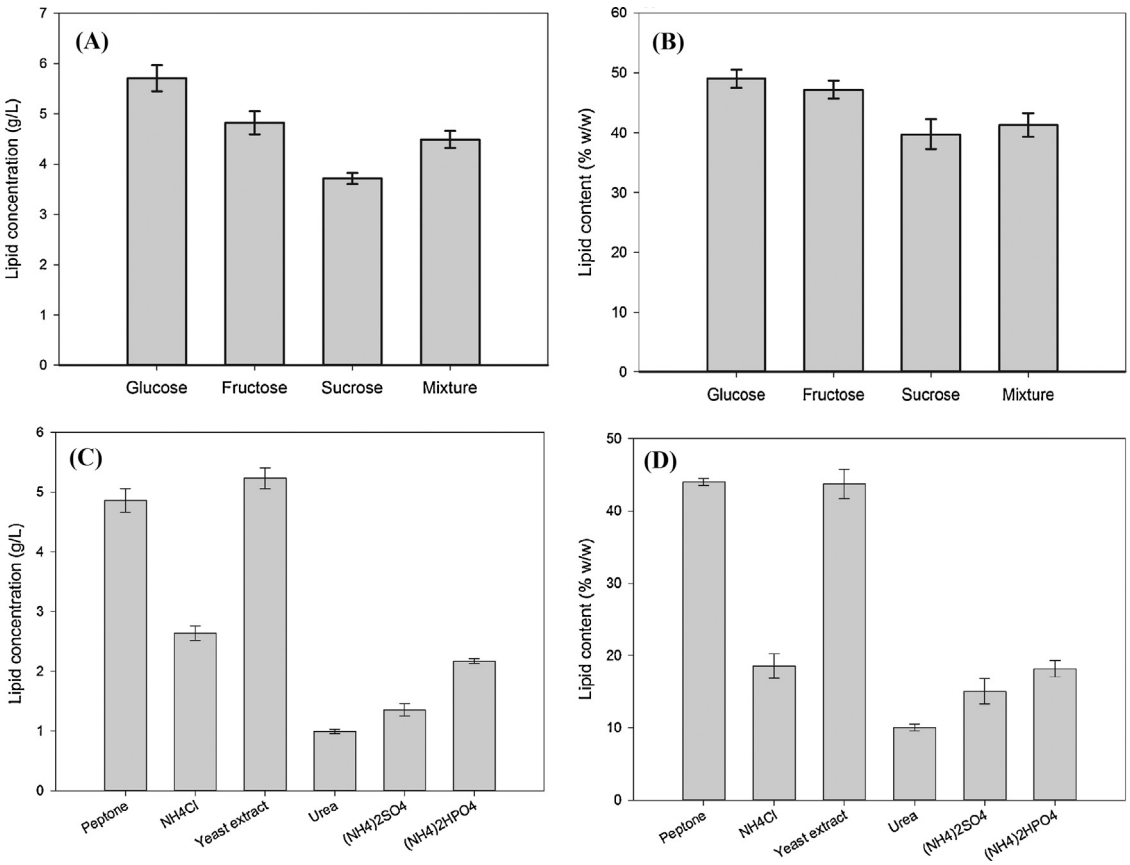

Fig. 1. Effect of different sugars on the lipid concentration (A) and lipid content (B), under constant C:N ratio of 100 by the addition of a mixture of yeast extract and ammonium sulfate. Effect of nitrogen source the on lipid concentration (C) and lipid content (D) using sugar mixture for the yeast cultivation under constant C:N ratio of 100.

(Leiva-Candia et al., 2014; Li et al., 2008). Oleaginous yeasts have some attractive characteristics such as their high growth rates, their cultivation is not affected by climate conditions and do not require big areas and the most important matter is that they can use low cost fermentation medium such as waste agricultural materials and also some industrial byproducts (Ageitos et al., 2011; Zhao et al., 2012). There are several yeast species that are capable of efficiently accumulate lipids, such as *Cryptococcus curvatus*, *Lipomyces starkeyi*, *Rhodotorula glutinis*, *Rhodosporidium toruloides*, *Yarrowia lipolytica*, etc. (Li et al., 2008; Ageitos et al., 2011). Among the oleaginous organisms, *L. starkeyi* represents an excellent candidate for efficient lipid production from a wide variety of raw materials such as glucose and xylose (Zhao et al., 2008), sewage sludge (Angerbauer et al., 2008) wheat straw hydrolysates (Yu et al., 2011), glucose enriched fishmeal wastewater (Huang et al., 2011) and olive oil mill wastewaters (Yousuf et al., 2010).

One major obstacle that hinders commercialization of microbial lipid production is the high cost of the raw materials (Zhao et al., 2012; Chen et al., 2013). In order to decrease this cost, low-cost raw materials such as lignocelluloses have to be exploited. Utilization of these raw materials not only decreases the production cost but also do not compete with food production, as for example when sucrose or starch are used.

Sweet sorghum is an excellent renewable feedstock as it contains high amounts of soluble and insoluble carbohydrates, presents fast growth, has high resistance to harsh climate conditions (like drought) and requires low fertilization and irrigation (Gnansounou et al., 2005; Wu et al., 2010; Matsakas and Christakopoulos, 2013a; Whitfield et al., 2012). However, the low carbon to nitrogen (C:N) ratio (60–65) (Economou et al., 2010) can partially inhibit lipid accumulation. In order to increase the lipid production yield of *L. starkeyi*, one aim of the current investigation was to increase the C:N ratio of sweet sorghum by incorporating a distinct enzymatic treatment prior to cultivation by employing commercial cellulase solutions. Another drawback for the utilization of sweet sorghum stalks is the low storage stability due to the presence of soluble sugars which can been easily degraded by contaminating microorganisms. The low storage stability together with the seasonal character of the sweet sorghum harvest can result in a non-annual availability of stalks. Drying of stalks as previously demonstrated (Matsakas and Christakopoulos, 2013b; Shen and Liu, 2009), can efficiently prevent sugar degradation and hence improve the storage stability of the stalks enabling an annual availability.

2. Materials and methods

2.1. Raw material and microorganism

The cultivar of sweet sorghum utilized was Keller, offered by Prof. George Skarakis, Agricultural University of Athens, Greece. Fresh stalks were preserved at −20 °C until used after removing the leaves by hand. Preparation of dried sweet sorghum stalks was done as previously described (Matsakas and Christakopoulos, 2013b). The dried sweet sorghum stalk particles were milled at 0.75 mm prior to usage. The composition of the dried sweet sorghum stalks was as follow (%, w/w): sucrose, 34.4; glucose, 8.2; fructose, 8.1; cellulose, 19.6; hemicellulose, 15.2 (Matsakas and Christakopoulos, 2013b).

The yeast strain utilized during this work was *L. starkeyi* CBS 1807 and pursued from CBS-KNAW Fungal Biodiversity Centre (Utrecht, The Netherlands).

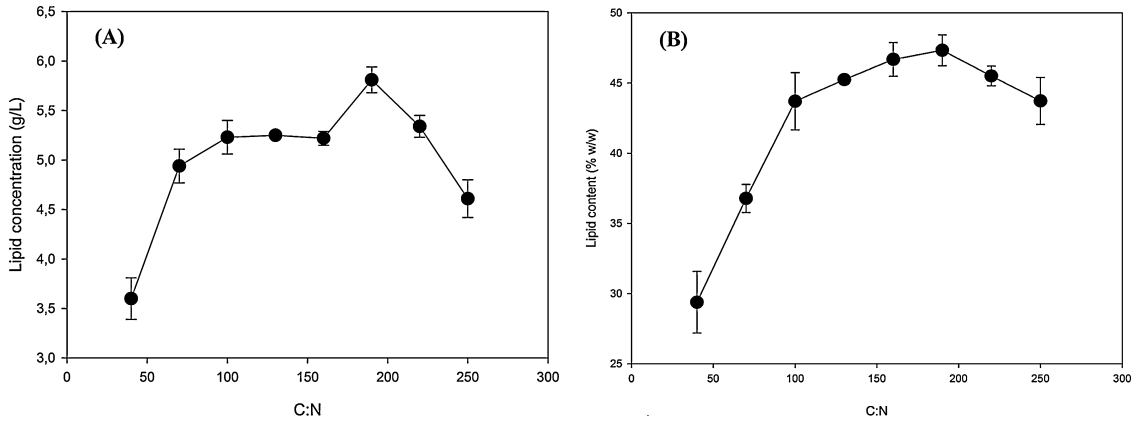

Fig. 2. Effect of different C:N ratios on lipid concentration (A) and lipid content (B), when cultivation was performed using sugar mixture with the addition of yeast extract as nitrogen source.

2.2. Pre-culture media

Prior to each experiment, yeast was grown in 250 mL Erlenmeyer flasks containing 50 mL of pre-culture broth with the following composition: Glucose, 20 g/L; Meat peptone, 10 g/L; Yeast extract, 10 g/L; KH_2PO_4, 6 g/L; Na_2HPO_4, 2 g/L. The pH of the media was adjusted to 6 and prior to inoculation, the pre-culture broth was sterilized at 121 °C for 15 min. Cultures for lipid production were inoculated with 5% (v/v) of the pre-culture media. The incubation of the pre-culture took place at 30 °C and 200 rpm for 48 h.

2.3. Lipid production on synthetic media

Cultivation of the yeast using synthetic media for lipid production took place in 1 L Erlenmeyer flasks containing 200 mL of cultivation broth, at 30 °C and 200 rpm. The composition of the broth (except for the nitrogen and carbon source) was the same as elsewhere described (Papanikolaou and Aggelis, 2002). The pH of the broth was adjusted to 6 and the initial sugar concentration in all the experiments was set to 40 g/L. The carbon sources were commercial sugars such as glucose, fructose and sucrose, alone or in mixtures. During the experiments of the effect of different sugars, the nitrogen source was a mixture of yeast extract and ammonium sulfate at a concentration corresponding to a C:N ratio equal to 100. During the experiments where the nitrogen source or the C:N ratio was evaluated, the concentration of the sugars remained constant at 40 g/L, whereas the concentration of the nitrogen source varied in order to achieved the desired C:N ratio.

2.4. Sweet sorghum saccharification and lipid production

Sweet sorghum stalks were saccharified for 8.6 h by applying enzyme loading equal to 8.32 FPU/g solid at 50 °C, as previously identified as optimal saccharification conditions (Matsakas and Christakopoulos, 2013b). Saccharification was performed by the addition of a mixture of Celluclast® 1.5 L and Novozym® 188 (Novozymes A/S, Denmark) at a 5:1 (v/v) ratio. Novozym® 188 was added at the start-up of the yeast cultivation in order to minimize sucrose hydrolysis and in turn inhibition of cellulases from the glucose. After saccharification the solids were removed by squeezing through a coating sheet and centrifugation. During the experiments where no enzymatic treatment was applied, sweet sorghum was soaked at 50 °C for 2 h prior to solids separation in order to facilitate the extraction of sugars from the stalks. After adjusting the pH to 6 the liquid fraction was sterilized and used as a broth for yeast cultivation.

In order to prepare sweet sorghum juice, the stalks were dissolved in the same mineral solution that was used in synthetic medium without the addition of either carbon or nitrogen source (unless otherwise stated).

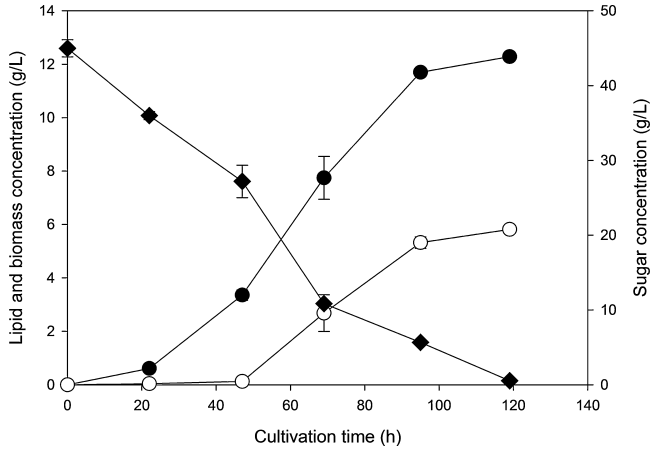

Fig. 3. Time course of biomass (•) and lipid (○) concentration and sugars consumption (♦) when *L. starkeyi* was cultivated on sugar mixture, at a C:N ratio of 190 and yeast extract as nitrogen source.

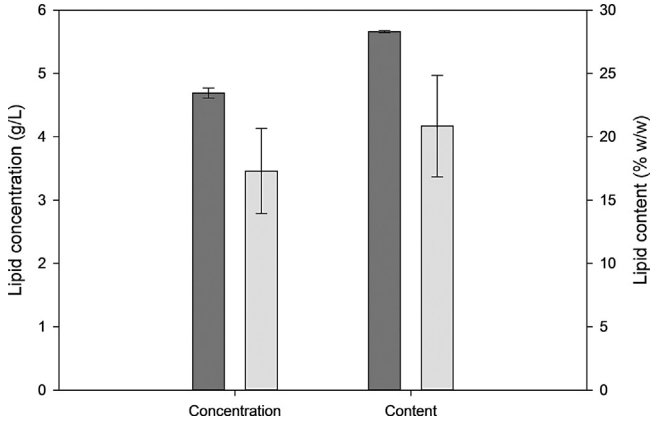

Fig. 4. Effect of addition of external nitrogen source (0.2 g yeast extract per 100 g of sorghum) on lipid concentration and content, when *L. starkeyi* was cultivated on sweet sorghum juice from a sweet sorghum initial concentration of 8.7% (w/w). Experiment with the addition of external nitrogen source is represented by the light gray bar, whereas the control by the dark gray bar.

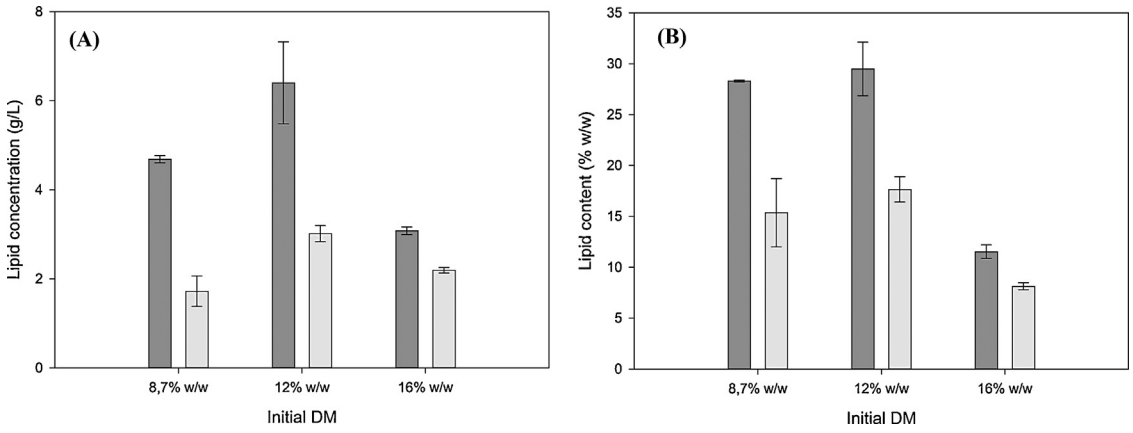

Fig. 5. Effect of different initial sweet sorghum contents on lipid concentration (A) and content (B), with the presence (dark gray bars) or absence (light gray bars) of a distinct enzymatic saccharification step.

2.5. Analytical methods

Samples were centrifuged in order to separate the yeast biomass from the cultivation broth and the supernatant was used for the quantification of total sugars concentration by the DNS method (Miller, 1959). In order to enable sucrose quantification by the DNS method, samples were incubated at 70 °C for 15 min in the presence of HCl for sucrose hydrolysis. Biomass was washed with distilled water to remove residual sugars and salts and centrifuged again. Finally the biomass was transferred to pre-weighted glass vials and the biomass concentration was estimated by the weight difference after being dried at 80–90 °C until constant weight. Lipid extraction from the dried biomass was performed by using a mixture of chloroform and methanol at 2:1 volumes (Folch et al., 1957) and quantification was done gravimetrically after solvent evaporation under vacuum using a rotary evaporator.

The fatty acid profile was determined by conversion of lipids to methyl-esters (Appelqvist, 1968) and analyzed by gas chromatography (Varian CP-3800, Agilent Technologies, USA) with a capillary column (WCOT fused silica 100 m × 0.25 mm coating CPSIL 88 for FAME) using helium as carrier gas at a flow rate of 30 mL/min. The initial oven temperature was set to 175 °C for 26 min, following by an increase to 205 °C with a rate of 2 °C/min, where it remained for 24 min. The temperatures of the injector and detector were set to 270 and 300 °C, respectively.

3. Results and discussion

3.1. Evaluation of lipids production on synthetic media

To characterize cell growth, sugar utilization and lipid production patterns of *L. starkeyi* CBS 1807, the yeast was cultivated in a medium with glucose, fructose and sucrose, either as single carbon and energy source or in mixtures at the ratio that are present in sweet sorghum stalks (glucose, 16%; fructose, 16% and sucrose 68%). As can been seen in Fig. 1, all three sugars were suitable for the cultivation of the yeast and efficient lipid production. The highest lipid production was observed during cultivation on glucose and reached 5.71 g/L with a lipid content of 49% (w/w), whereas cultivation on sucrose resulted in the lowest lipid production and content. Cultivation on the sugar mixture resulted in a lipid concentration of 4.49 g/L and a lipid content of 41.3% (w/w), indicating that the sugar composition of sweet sorghum stalks is suitable for the cultivation and lipid production of *L. starkeyi*.

In order to further evaluate and improve the lipid production from the sugar mixture, other factors that affect lipid accumulation were studied and optimized. Generally, lipid accumulation is affected by several factors such as nitrogen, temperature etc. (Zhu et al., 2008). Of all these factors, nitrogen plays a very important role as the accumulation of lipids starts after depletion of nitrogen from the cultivation broth (Papanikolaou et al., 2007). In addition, the form of nitrogen (organic or inorganic) highly affects the production of lipids (Papanikolaou and Aggelis, 2011b). For these reasons it is of great importance to evaluate the nitrogen source as well as the ratio of the carbon to nitrogen concentrations (C:N). During this work different organic (yeast extract, meat peptone and urea) and inorganic (NH_4Cl, $(NH_4)_2SO_4$ and $(NH_4)_2HPO_4$) nitrogen sources were evaluated at a constant C:N ratio equal to 100. As is shown in Fig. 1, complex organic nitrogen sources (yeast extract and peptone) were more favorable for the production of lipids. More specifically, the presence of yeast extract resulted in the production of 5.23 g/L lipids with a lipid content of 43.7% (w/w). The same positive effect of the presence of organic nitrogen source was reported for different microorganisms like *Cunninghamella echinulata* (Certik et al., 1999), *Trichosporon fermentans* (Zhu et al., 2008) and *R. toruloides* (Evans and Ratledge, 1984).

Finally, the effect of different C:N ratio in a wide range (40–250) was evaluated by using yeast extract as nitrogen source. As can be seen in Fig. 2, lipid production increased gradually with the increase of the C:N ratio and reached a maximum of 5.81 g/L and 47.3% (w/w) of lipid production and lipid content, respectively, at ratio of 190. A further rise of the C:N ratio resulted in a slight drop of both lipid production and lipid content. Similar results concerning the %

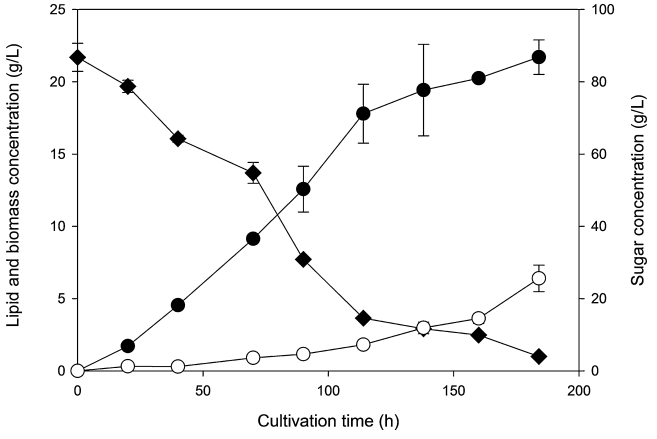

Fig. 6. Time course of biomass (●) and lipid (○) concentration and sugars consumption (♦) when *L. starkeyi* was cultivated on sweet sorghum juice that came from 12% (w/w) sweet sorghum concentration without the addition of an external nitrogen source.

Table 1
Comparison of lipid concentrations obtained during this work with other reported in literature.

Microorganism	Raw material	Lipid concentration (g/L)	Productivity (g/L·day)	Reference
L. starkeyi	Non-detoxified dilute sulfuric acid pretreated wheat straw	4.6	n.a.	Yu et al. (2011)
L. starkeyi	Detoxified dilute sulfuric acid pretreated wheat straw	3.7	n.a.	Yu et al. (2011)
L. starkeyi	Ultrasonic treated sewage sludge	1.0	n.a.	Angerbauer et al. (2008)
L. starkeyi	Sweet potato starch	4.8	2.40	Wild et al. (2010)
L. starkeyi	Glucose-enriched fishmeal wastewater	2.7	0.45	Huang et al. (2011)
L. starkeyi	Detoxified corncob hydrolyzates treated with dilute sulfuric acid	8.1	1.01	Huang et al. (2014)
C. curvatus	Sweet sorghum bagasse	2.6	0.87	Liang et al. (2012)
Chlorella protothecoides	Sweet sorghum juice	2.9	0.59	Gao et al. (2010)
Schizochytrium limacinum	Sweet sorghum juice	6.9	1.38	Liang et al. (2010)
L. starkeyi	Juice from saccharified sweet sorghum	6.4	0.80	Present work

(w/w) lipid accumulation at low C:N ratio were reported by Wild et al. (2010) for the same microorganism. In order to achieve high lipid production our data reveals that the C:N ratio should preferably be kept above 100. Under optimum conditions the yield of lipid productions per gram of consumed sugars was $Y_{L/S}$ = 0.131 g/g, whereas the yield of biomass formation was $Y_{X/S}$ = 0.276 g/g with a productivity of 1.162 g/L·day (Fig. 3).

3.2. Cultivation of *L. starkeyi* on juice obtained after enzymatic treatment of sweet sorghum stalks

During initial experiments it was observed that *L. starkeyi* was not able to grow in the presence of the solids from sweet sorghum stalks (data not shown) and as a result the solids were removed after the enzymatic saccharification.

The ability of *L. starkeyi* to exploit sweet sorghum's proteins as nitrogen source was evaluated by studying the effect of the addition of an external nitrogen source (more specifically yeast extract) at a concentration equivalent to 0.2 g/100 g of sweet sorghum on lipid production (initial sweet sorghum content was 8.7%, w/w). This resulted in a significant decrease in lipid production from 4.69 g/L (lipid content of 28.3%, w/w) to 3.46 g/L (lipid content of 20.8%, w/w) (Fig. 4) probably caused by the substantial decrease of the initial C:N ratio of the medium after the addition of yeast extract. Moreover, even without addition of an external nitrogen source the lipid production was lower compared to those obtained in synthetic media, underpinning the importance of increasing the C:N ratio by increasing the initial sugars concentration.

In order to evaluate the effect of the distinct enzymatic saccharification step on lipid production, experiments were conducted at different initial sweet sorghum contents (8.7%, 12% and 16%, w/w) with or without enzymatic treatment prior to cultivation. The incorporation of a distinct saccharification step not only increases the initial concentration of sugars (approximately 3–12%) but also facilitates a better recovery of the liquid and in turn sugars by reducing the viscosity of the slurry, which is a result of the cellulolytic activity. The most probable reason for the viscosity reduction is the collapse of the structure of lignocellulose as well as the loss of the water-binding capacity due to the degradation of cellulose (Szijártó et al., 2011).

As can been seen in Fig. 5, the presence of a distinct saccharification step increased the production of lipids under all the initial sorghum consistencies. Optimum conditions for lipid production by *L. starkeyi* when cultivated on sweet sorghum juice where at 12% (w/w) initial sweet sorghum concentration with the incorporation of a distinct saccharification step and without any additional nitrogen source (Fig. 6). The lipid production reached 6.40 g/L with a lipid content of 29.5% (w/w). Under these conditions lipid yield per consumed sugars was $Y_{L/S}$ = 0.077 g/g, whereas the corresponding yield for biomass formation was $Y_{X/S}$ = 0.262 g/g which is comparable with that obtained when using the synthetic media (C:N ratio of 190). Moreover the lipid productivity was 0.8 g/L·day and the lipid yield per sweet sorghum solids reached 5.33 g/100 g of sweet sorghum. Lipid production obtained during this work was higher than most of the works reported in the literature when *L. starkeyi* was cultivated on renewable raw materials or sweet sorghum was used as raw material (Table 1).

Table 2
Fatty acid composition of the lipids produced during cultivation of *L. starkeyi* on juice from 12% (w/w) liquefied sweet sorghum stalks.

Fatty acid	% concentration (w/w)
C16:0	42.90
C16:1	2.15
C18:0	4.90
C18:1 (n-9)	49.85
C20:4 (n-6)	0.17

Analysis of the lipid profile obtained at the optimum conditions when yeast cultivated on sweet sorghum is shown in Table 2. Oleic acid is the dominant fatty acid in the lipids obtained by *L. starkeyi* followed by palmitic acid. High concentration of oleic acid in the obtained lipids is a desirable property for the production of biodiesel (Sitepu et al., 2013). Smaller amounts of palmitoleic and stearic acid are also present. Similar fatty acid composition of lipids obtained from *L. starkeyi* was also found when the yeast was cultivated on starch (Wild et al., 2010), mixtures of glucose and xylose (Zhao et al., 2008) and various mixtures of glucose, cellobiose and xylose (Gong et al., 2012).

4. Conclusions

During this work it was demonstrated that *L. starkeyi* could grow well in juice obtained by enzymatically pretreated dried sweet sorghum stalks and efficiently accumulate lipids. The incorporation of the distinct saccharification step could significantly enhance the accumulation of lipids, with the highest lipid concentration obtained during this work to be 6.40 g/L from juice coming from 12% (w/w) sweet sorghum concentration. Moreover, the addition of external nitrogen source was not necessary during sweet sorghum utilization, which is beneficial for the process economics.

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