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Two consecutive step process for ethanol and microbial oil production from sweet sorghum juice

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ABSTRACT

The juice extracted from sweet sorghum stalks has been previously explored to produce ethanol and also to grow oleaginous yeasts and algae with the objective of producing microbial oil. In this paper we propose a different process route in order to produce ethanol and microbial oil in two consecutive fermentation steps. Ethanol is produced first followed by the growth of oleaginous yeast employing the residual carbon and nitrogen left from the first step. Two yeasts were compared for ethanol production. *Trichosporon oleaginosus* was cultivated for lipid production. The yeast selection for the first step was the most important factor for achieving a high ethanol yield and the effect of inorganic nitrogen addition was not significant. The remaining sugars consisted of a mixture of sucrose and fructose and no residual glucose was detected in any of the runs. In the second step *T. oleaginosus DSM 11815* grew in the pooled juices remaining from the first step for 168 h and produced biomass with 28% lipid content. Glucose showed the highest uptake rate, sucrose was utilized until low glucose values prevailed, and fructose was slowly metabolized and a substantial amount remained. Although the two step process has flexibility in choosing the proper microorganism for each step, it is necessary to look for a rapid fructose uptake strain for both fermentations.

1. Introduction

Sweet sorghum is a sugar rich crop that has been considered an alternative feedstock to sugarcane for first generation ethanol production in tropical countries, due to its efficient C4-photosynthesis, short production cycle, and nitrogen and water use efficiency, high tolerance to environmental stress and adaptability to marginal lands [1]. Sweet sorghum presents additional advantages: (a) it can be considered a multiproduct crop due to its high sugar productivity and its grain with adequate nutritional characteristics [2], (b) it can sustain a full year production cycle as sweet sorghum is capable in the tropics of ratoon crops [3], and c) it can be grown without the addition of chemical fertilizers [4].

A sweet sorghum disadvantage is a short harvest window, which can be overcome by employing cultivars with different plant cycles or using the same variety sowed at different dates, or both [1]. Moreover, the harvested stalk sugar content and proportions of individual sugars deteriorates rapidly at ambient temperatures with negative consequences for further processing. A practical suggestion is to extract and clarify the juice, then concentrate and store the syrup [5].

Fuel ethanol production from sugar or starch rich crops, byproducts or wastes has been investigated thoroughly; the research findings and the present industrial practice have been summarized in several recent reviews [6,7]. There are two mature technologies producing in an unprecedented scale ethanol as a biofuel, using corn starch as a raw material in the USA and cane juice and molasses in Brazil. In the former, glucose is the main carbon source, in the latter, a mixture of sucrose and reducing sugars. In Europe beet molasses, alone or in mixtures with cane molasses, are used as raw material for ethanol production; sucrose being the predominant sugar in beet molasses.

Other crops have also been considered; among those that excel are: (a) Jerusalem artichoke [8] which accumulates inulin as the major carbohydrate that can be hydrolyzed by inulinase to a mixture of fructose, glucose and fructo-oligosaccharides; (b) carob pods [9] sugars which are a mixture of sucrose, glucose and fructose; and (c) sweet sorghum which accumulates in the mature stem a mixture of sucrose, glucose and fructose [1–4].

Sweet sorghum has been studied extensively as a raw material for ethanol production [10,11]. However, there is presently no industrial ethanol production from this raw material. The extensive research published employing sorghum juice has been done

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in the laboratory or small pilot units in which juice extraction is done in small roller mills with very poor performance. In order to increase sugar extraction efficiency, the simultaneous extraction-fermentation technique employing sweet sorghum stem particles suspended in water has been employed in small scale [2–4]. Developments have taken place in solid substrate fermentation of milled sorghum stalks and continuous pilot units have been designed, built and operated [12,13]. New strategies have been proposed to transform simultaneously not only the soluble sugars but the carbohydrates produced by the hydrolysis of the lignocellulosic matrix [14,15]. Several researchers have investigated the very high gravity (VGH) ethanol fermentation from sweet sorghum syrups [16,17] in order to find the maximum sugar conversion into ethanol and the minimization of fermentation byproducts, by testing different ethanol tolerant yeasts, several nitrogen sources and adding other nutrients.

A general interest in producing microbial oils for further transformation into biodiesel has identified promising oleaginous algae, bacteria, fungi and yeast and has encouraged studies concerning their growth and lipid accumulation kinetics summarized in recent reviews [18,19]. Sweet sorghum juice and syrup have been used as carbon substrates for microbial oil production. Cui and Liang [20] employed the oleaginous yeast *Cryptococcus curvatus.* Gao et al. [21] grew heterotrophically the alga *Chlorella protothecoides*. In both cases the resulting microbial biomass had a high proportion of lipids. Oleaginous yeasts have been grown on sucrose, glucose and fructose containing raw materials. *Rhodotorula glutinis* and *Trichosporon fermentans* have been grown on cane molasses with acceptable growth and lipid accumulation [22,23]. *Rhosporidium toruloides*, *Rhodotorula mucilaginosa* and *Cryptococcus* sp. were grown successfully in hydrolyzed extracts of Jerusalem artichoke tubers, either by inulinase or by acids, producing cells with high lipid content [24–26].

Instead of employing sweet sorghum juice as raw material in different processes to produce either ethanol or biodiesel, we are proposing a different alternative route in order to obtain ethanol and microbial oil as main products in two consecutive fermentation steps. The first step consists of ethanol production and the second step the growth of oleaginous yeast employing the residual carbon and nitrogen left from the first step. In between the steps, *Saccharomyces cerevisae* is separated by centrifugation and ethanol by distillation. At the end of the production line the oleaginous yeast biomass oil can be converted to fatty acids ethyl esters or FAEE employing part of the ethanol produced in the first step. In this paper we present experimental results of the two fermentation steps with the main objective of providing a proof of concept.

2. Material and methods

2.1. Sweet sorghum juice

Five stalks from four sweet sorghum varieties, Della, M81-E, Sugar Drip, Top 76-6 and Umbrella were brought from our experimental agricultural station stripped of adhering leaves, leaf sheaths, and top and were kept at −10 °C until processed. The thawed stalks were pressed employing a stainless steel pilot three roll crushing mill (*Vencedora Maqtron Model 721*) with a 2-HP motor. Juice Brix was measured with a digital refractometer (*Model 300034 Sper Scientific Ltd*) and a juice sample was used for sugar analysis as described below. The juice mixture soluble solids were 17.4 Brix. Three different concentrations were prepared diluting with distilled water, 17, 15 and 13 Brix. Their total sugar content was 15.3, 13.5 and 11.7 wt%, respectively of which 69% was sucrose, 19% was glucose and 12% fructose. Kjeldahl nitrogen was 0.42 g/L and pH 5.2. Starch, mannitol, phosphorus and organic acids contents were not quantified.

2.2. Microorganisms and inoculum preparation

The ethanol production first step was done employing two *Saccharomyces cerevisae* yeasts separately as explained below, CBS 400 and CBS 459 (Centraalbureau voor Schimmelcultures, Utrecht, The Netherlands). In the second step, the oleaginous yeast *Trichosporon oleaginosus DSM 11815* (Leibnitz Institut DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH, Braunschweig, Germany) was cultivated for lipid production.

The inoculum for all yeasts was prepared as follows: a pure culture sample was grown in a 30 g/L Sabouraud broth (Merck, 2% glucose, 0.5% animal peptone, and 0.5% casein peptone) plus 1% additional sucrose. One hundred and twenty-five milliliters of broth was added into a 250 mL flask, sterilized at 121 °C for 20 min, cooled, inoculated, and agitated at 250 rpm at 30 °C for 48 h (Incubator Shaker Lab Companion Model SI-600). The suspension was centrifuged at 1600*g* for 5 min at 10 °C (Eppendorf Table-top Refrigerated Centrifuge Model 5804R). The solid pellet was suspended in deionized water and the optical density adjusted approximately to a value of 1.8.

2.3. Experimental strategy

A two level factorial design for three factors was used for the ethanol production step (Design Expert 9.0.5). The factors were: (a) the yeast culture, (b) sweet sorghum juice initial Brix and (c) the quantity of ammonium phosphate added. Note that the first factor is categorical and the other two are numerical. The design consisted in 14 runs including six central point repetitions. Center points were duplicated at both the low and high value of the categorical factor. We decided to include the yeast strain as an experimental factor in order to compare its effect to those effects from the other two design variables. Previous research on industrial ethanol fermentations from sugarcane byproducts under normal and high gravity conditions have shown the importance of the yeast strain on the fermentation outcome [27].

For each run, 100 mL of the prepared sweet sorghum juice were placed in 250 mL Erlenmeyer flasks and 10 mL of the yeast inoculum were added. The flasks were kept for 48 h at 30 °C under static conditions. The yeast was separated by centrifugation at 2000*g* for 5 min at 10 °C (Sorvall RT7 refrigerated centrifuge) and a liquid sample was sent for sugar and ethanol analysis as explained below. All remaining liquids were then pooled and ethanol was separated by distillation. The hot residual liquid was placed in a 1 Liter bioreactor (BioStat® A Plus-Sartorius Stedim), cooled, had a pH of 4.8 and inoculated with the oleaginous yeast. Temperature was set at 30 °C, an air flow of 1.2 vvm, a controlled pH of 4.80, 350 rpm and a working liquid volume of approximately 750 mL. It was operated for 168 h and samples were taken periodically. Yeast dry weight and total sugars were determined as explained below. Yeast biomass was recuperated by centrifugation at 1600*g* for 5 min at 10 °C (Eppendorf Table-top Refrigerated Centrifuge Model 5804R). It was kept frozen for two days at −10 °C. It was thawed, placed on a tray and dried with air at 65 °C. Moisture and oil content were determined as explained below.

2.4. Analysis

Sugars in the filtrate were determined with an Agilent 1100 high pressure liquid chromatograph, an Agilent 1200 refractive index detector, a Zorbax NH2, 25 cm long, 4.6 mm internal diameter column, employing acetonitrile in water (70–30), as the solvent phase. Ethanol was quantified employing an Agilent 6890 N gas chro-

matograph, with an HP-Plot/Q, 30 m long, 32 mm internal diameter column. Moisture of the dry yeast biomass was determined gravimetrically by placing a sample in an oven at 65 °C until constant weight. The oil content in the dry biomass was determined by a modification of the Blight-Dyer method. Briefly, the procedure was as follows: a known weight of yeast, close to 80 mg, was placed in 15 mL tube. Two mL of ethanol and 1 mL of chloroform were added and placed on a Vortex Mixer for 1 min. The tube was left for 24 h at ambient temperature. One mL of chloroform was added and the contents were mixed for 1 min, then 1.8 mL of distilled water was added and the tube was mixed for another 2 min. The contents were centrifuged at 1600*g* for 5 min at 10 °C (Eppendorf Table-top Refrigerated Centrifuge Model 5804R). The upper layer was discarded and the lower layer was filtrated employing Whatman paper No.1 into a small beaker of known weight. The beaker was placed in an oven at 65 °C for 24 h. It was cooled and weighed.

3. Results and discussion

3.1. First step

The factorial design matrix is shown in Table 1. The three factors are shown as coded variables in columns 2–4 and also as actual values in columns 5–7. The next three columns list the response variables for each of the 14 runs, the ethanol concentration in g/100 mL, the sugar consumption as% of the initial value and the ethanol yield in terms of sugar consumed.

The analysis of the factorial design indicated that the major effects on the ethanol production data were the initial sugar content (p: 0.0346) and the yeast strain (p: 0.0645). The ethanol averages for the 17, 15 and 13 initial Brix flasks were 8.06, 6.65 and 6.58 g/100 mL respectively. The yeast strain ($p < 0.0001$) and the initial sugar content (p: 0.0687) influenced the sugar consumption and the yeast strain (p: 0.0247) had the largest effect on the ethanol yield values. The CBS 459 strain had higher ethanol yield values than the CBS 400 strain as shown in Table 1. Hence it showed a superior performance as it produced more ethanol with less sugar consumed. However in some cases the yield coefficient was 0.50, close to the theoretical figure. The only suggestion that we have is that during the initial flask sterilization, a fraction of the starch present in the sweet sorghum juice could have been hydrolyzed providing extra glucose that was fermented but was not taken into account in the initial analysis done prior the flask sterilization. It has been reported that the efficiency in converting sweet sorghum juice sugars into ethanol was dependent on the yeast strain employed [28]. The importance of the yeast strain is more than evident in the production of ethanol at industrial scale from sugarcane molasses where selected yeast strains have increased the ethanol yield, reducing glycerol and foam formation and showing high viability during recycling [29,30]. Most of the experimental data in the literature employing sweet sorghum juice, as such or concentrated, report yield values below or close to the theoretical yield coefficient, although many different alternatives of handling the juice have been reported and in some cases the estimated yield is based on the initial sugar content and not the amount of sugar consumed [16,28,31–36]. However there were a few reports where ethanol yield coefficients were above 0.51 [37–40], and in one of those [40] the authors suggested that other sugars present, not detected by HPLC, could have been transformed into ethanol. In any event, we believe that the starch fate during juice handling, clarification, centrifugation and sterilization, should be studied in the future with more detail in order to confirm if glucose is produced prior to juice fermentation.

Sugar consumption in all flasks was above 94%. The remaining sugars consisted mainly of a mixture of similar proportions of sucrose and fructose. No residual glucose was detected in any of the 14 runs. Most of the data published on sweet sorghum fermentations shows incomplete sugar utilization. Some authors indicated that residual sugars were mainly reducing sugars [35,41]. Other authors have pointed out that fructose is the reducing sugar not utilized [33,42]. Similar results have been obtained with carob pod extracts [43] which, as mentioned before, resembles sweet sorghum juice in terms of sugar composition. Lima-Costa et al. [9] followed the uptake of the different sugars in carob pod and found that in the exponential growth phase glucose and fructose were consumed in that order. Then sucrose hydrolysis took place and the monosaccharides produced were also consumed, producing a dynamic balance in solution. Recent results with sugarcane molasses also report residual sugars after fermentation under different conditions but no data was given on the individual sugars uptake [44]. Total reducing sugar consumption was reported for fermentations of olive mill waste water initially containing glucose and fructose in a medium enriched with glucose [45].

It is well known that glucose is a preferred uptake substrate for common ethanol producing yeasts over sucrose and fructose and that sucrose is hydrolyzed by invertase in the yeast cell membrane into glucose and fructose which are then transported inside the cell [46]. *S. cerevisae* strains possess different glucose transporters of the Hxt family [46] in contrast, in *S. bayanum* [47] a specific fructose transporter exists and in the osmotolerant *Z. rouxii* fructose is consumed faster than glucose [48]. In other words, how and to what extent sugar utilization takes place from an initial mixture of sucrose, glucose and fructose will depend on the yeast strain employed.

The ammonium phosphate concentration was not a significant factor on the three response variables. This salt has not been commonly employed before; instead ammonium sulphate has been preferred over other inorganic nitrogen sources [49]. In normal and VHG fermentations with sweet sorghum juice the addition of organic nitrogen sources like yeast extract and peptone has improved sugar consumption and ethanol yield [16,37,38] however these compounds are relatively expensive. An interesting approach has been the fermentation of sweet sorghum juice mixed with malted [50] or mashed [51] sweet sorghum grains which provide protein nitrogen.

3.2. Second step

The residual sugars from the first step were estimated for each flask and the overall quantity in the pooled liquid was approximately 91 g of a mixture of similar proportions of sucrose and fructose. During centrifugation liquid was lost and remained within the separated yeast biomass. The sugar loss estimated by the difference in volumes was approximately 40 g. After ethanol distillation and the addition of yeast the final working volume of the residual liquid was 750 mL. Hence the sugar concentration at the start of the oleaginous yeast growth was approximately 52 g, which in 750 mL give a concentration of 69 g/L. The analyzed sample of this liquid gave a total sugar of 71.2 g/L consisting of approximately 30% sucrose, 21% glucose and 49% fructose. During ethanol distillation some sucrose inversion to glucose and fructose took place due to the high temperature and acid conditions. The same effect has been reported before during sugar beet juice concentration [52]. However no major sugar thermal degradation or oligosaccharides formation took place as was observed in the chromatograms. This fact has also been reported during sweet sorghum juice concentration [53]. The total nitrogen concentration at the start of the oleaginous yeast growth was not measured. However it can be estimated as follows. The total amounts of organic nitrogen in the sweet sorghum juice and the nitrogen added in the ammonium salt at the start of the ethanol fermentation were 0.19 g and 0.41 g respec-

Table 1
Design matrix for the two level three factors 14 runs factorial design.

Run	Coded factors			Actual values			Responses		
	Yeast	Brix	$(NH_4)PO_4$	Yeast	Brix	$(NH_4)PO_4$ g/L	Ethanol g/100 mL	Sugar consumption%	Yield coefficient
1	−1	−1	+1	400	13	2	3.65	97.06	0.32
2	−1	−1	−1	400	13	0	4.58	97.49	0.40
3	+1	0	0	459	15	1	6.12	94.79	0.50
4	+1	+1	−1	459	17	0	6.76	94.28	0.46
5	+1	0	0	459	15	1	6.05	92.71	0.49
6	−1	0	0	400	15	1	5.07	97.45	0.40
7	−1	+1	+1	400	17	2	5.75	96.62	0.39
8	−1	+1	−1	400	17	0	4.63	96.23	0.31
9	−1	0	0	400	15	1	5.40	95.91	0.43
10	+1	−1	+1	459	13	2	4.73	94.90	0.43
11	+1	+1	+1	459	17	2	6.20	94.68	0.43
12	+1	0	0	459	15	1	3.89	95.03	0.32
13	−1	0	0	400	15	1	4.63	97.48	0.37
14	+1	−1	−1	459	13	0	5.48	94.77	0.50

Table 2
Lipid productivity experimental data for oleaginous yeast grown on substrates containing sucrose, glucose and fructose mixtures.

Yeast	Carbon source	Biomass produced g/L	Biomass oil content% dry weight	Fermentation time h	Lipid productivity g/L per day	Reference
Trichosporon oleaginosus DSM 11815	Remaining liquid from ethanol production from sweet sorghum juice	21	28	168	0.86	This work
Cryptococcus curvatus ATCC 20509	Sweet sorghum juice	22	51	72	3.74	20
Rhodotorula glutinis IIP-30	Cane molasses	18	22	120	0.79	22
Trichosporon fermentans CICC 1368	Cane molasses	36	35	264	1.15	23
Rhodotorula glutinis CCT 2182	Cane molasses	32	36	48	5.79	64
Rhodosporidium toruloides CCT 0783	Cane molasses	35	44	37	9.94	64
Rhodotorula minuta CCT 1751	Cane molasses	21	27	55	2.50	64
Lipomyces starkeyi DSM 70296	Cane molasses	21	32	57	2.87	64

tively. Nitrogen consumption in sweet sorghum juice fermentation has been reported to be in the order of 86% for ammonium salts [49] and 71% for organic nitrogen [16]. Assuming these conversions, the total residual nitrogen was 0.6 g. The initial C/N ratio was then around 86. This value is close to the optimum value of 100 for oleaginous yeast growth and lipid accumulation, which normally is a long process of about 90 h [18].

The changes in time of the oleaginous yeast *T. oleaginosus DSM 11815* dry weight and the sucrose, glucose and fructose concentrations from the pooled, ethanol stripped, remaining juice are shown in Fig. 1. The yeast oil accumulation with time was not followed. Lipid accumulation is usually not associated with cell growth, although there are exceptions [19], and occurs either under limiting nitrogen conditions or after nitrogen depletion which happen during the last phase of the process [18,19]. After 168 h the yeast dry weight was 21.23 g/L with an oil content of 28.33%. The corresponding yield coefficient in terms of consumed sugars was 0.47. The oil productivity was 0.86 g per liter per day and the overall lipid yield was 0.13 g of oil per g of sugar consumed.

The growing medium had an initial pH of 4.8 and was controlled at that value during the fermentation. An acid pH effect on the growth and lipid accumulation phases of oleaginous yeasts is still under debate as different strains have showed specific behavior. For example, the early work of Kessell [54] showed that low pH values retarded growth of *Rhodotorula gracilis* but the lipid production rate increased, although the final lipid concentration did not change. Naganuma et al. [55] grew *Lipomyces starkeyi* on glucose and showed that the cultural temperature and the initial pH

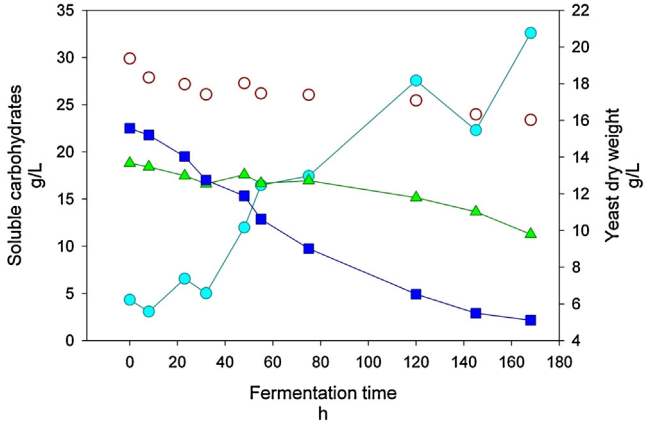

Fig. 1. Changes in time of the yeast biomass dry weight, and the concentrations of sucrose, glucose and fructose in the bioreactor. Filled circles stand for dry weight. Triangles up identify sucrose. Squares are for glucose and hollow circles are for fructose.

value of the medium affected the total cell number and lipid content; the optimum temperature ranged from 25.5 to 29.5 °C and the optimum pH value was 4.9. These results were similar to the ones reported by Angerbauer et al. [56] which reported for the same yeast grown on sewage sludge that the highest lipid accumulation was found at pH 5.0. No deleterious effects of low pH on oil accumulation were reported by Turcotte and Kosaric [57] employ-

ing *Rhodosporidium toruloides* in a glucose medium. Johnson et al. [58] showed that for *Rhodotorula glutinis* lipid accumulation was strongly affected by the medium pH when fed-batch on glucose and the maximum lipid yield was obtained at pH 4.0. Looking specifically to Trichosporon strains Gao et al. [59] employing *T. cutaneum* and corncob hydrolysates used a pH of 5.0. Shen et al. [60] found for *T. fermentans* in a molasses medium acceptable growth and lipid accumulation for pH 3.0, 5.0 and 7.0. Liu et al. [61] devised a SSF scheme employing corn stover hydrolyzsates and *T. cutaneum*. The enzymatic step was performed at pH 4.8 and the microbial oil production at pH 5.0. Kitcha and Cheirsilp [62] reported an interesting fact for the yeast *Trichosporonoides spathulata*, in which a culture without pH control, starting at pH of 6.0 dropped this value to approximately 4.5 without deleterious effects on biomass growth or lipid accumulation. Zhu et al. [23] employing *T. fermentans* on glucose or molasses found that growth and lipid accumulation could be achieved at pH 4.5, 5.0 and 5.5, the results were slightly lower than those obtained at pH 6.0–6.5. In summary then, experimental evidence demonstrates that an acid pH for growth and lipid accumulation is possible.

Most oleaginous yeasts show relatively low growth rates, so that in order to optimize lipid production a high amount of lipid containing biomass must be produced in the shortest possible time period [18]. Some lipid productivity experimental data from raw materials containing a mixture of sucrose, glucose and fructose has been summarized in Table 2. It can be seen that our results are comparable in terms of lipid productivity to those of Johnson et al. [22] and Zhu et al. [23]. However they are much lower than the productivities reported by Vieria et al. [63]. There is still more room for improvement as an example Karatay and Donmez [64] report high lipid content dry weight values of 46.8, 59.9 and 69.5% for *Candida tropicalis*, *Candida lipolytica* and *Rhodotorula mucilaginosa* biomass respectively grown on diluted molasses with 1 g/L of ammonium sulphate added.

The fatty acid composition of the microbial oil produced was: 43% linoleic acid (18:2), 36% palmitic acid (16:0), 9% oleic acid (18:1), 7% stearic acid (18:0), 4% linolenic acid (18:3) and 1% other minor components, results which are typical of oleaginous yeast oil [18,19].

As shown in Fig. 1 glucose and fructose were not only assimilated by yeast but were also produced by enzymatic sucrose hydrolysis, thus establishing a dynamic situation in the pooled juice. Glucose showed the highest uptake rate by the oleaginous yeast. Sucrose was utilized until low glucose values prevailed. Fructose showed the slowest uptake rate. To our knowledge there are no reported values in the literature for invertase activity in oleaginous yeasts. However, there is ample experimental evidence that they can use sucrose as a carbon source [20,22,23,63,64]. In fact, only five of 48 oleaginous yeast strains failed to grow in sucrose as their only carbon source and Trichosporon strains were not among them [65]. Cui and Liang [20] grew in batch and fed-batch modes the oleaginous yeast *Cryptococcus curvatus* in diluted sweet sorghum syrup with four different initial sugar concentrations for 72 h. Sugars were totally consumed in batch mode only when the initial concentrations were 11.7 and 17.4 g/L. In fed batch mode a significant amount of sugars was not utilized. Glucose was the preferred substrate, followed by fructose and finally by sucrose, which at higher initial concentrations was not utilized at all in batch mode. Zhu et al. [23] employed cane molasses to support growth of *T. fermentans*, and then added sucrose, glucose or fructose for lipid synthesis with excellent results. On the other hand several articles have been published in which oleaginous yeasts have been grown in distillery waste waters, however due to their low carbon content the amount of lipid rich biomass obtained has been below 10 g/L [66]. A wider choice of oleaginous yeast is available [65] so that further research should look for an oleaginous microorganism capable of totally utilizing a mixture of sucrose, glucose and fructose in a reasonable time span with high lipid productivity.

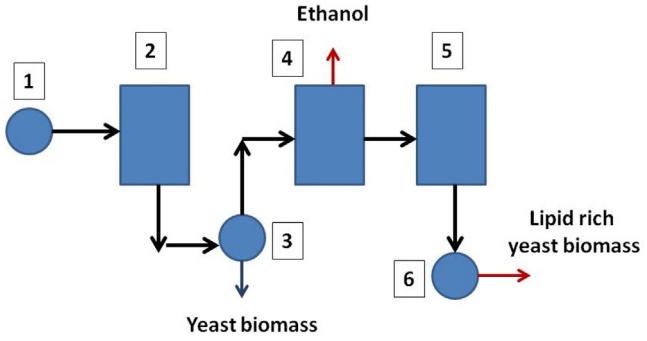

Fig. 2. Two consecutive step process for ethanol and microbial oil production from sweet sorghum juice. 1. Juice extraction, concentration and storage. 2. Ethanol fermentation. 3. Yeast separation. 4. Ethanol distillation. 5. Oleaginous yeast production. 6. Separation of lipid rich biomass.

3.3. Two-step process assessments

It is well known that in industrial ethanol production from sugar cane, raw material is the most important factor in the final production cost [67] so that yeast strains that are commonly used consume practically all sugars present with reduced byproducts [30]. In the present strategy an appropriate yeast strain should be able to produce ethanol at 8–10% by weight, which are normal values attained in an industrial process, from high sugar concentrated sweet sorghum juice in an optimized time span so that sugars are left for the second step. Previous research shows that this is possible as commented before. In the microbial oil process the capital investment and energy required for the fermentation units are the important production costs together with raw materials [68]. The latter factor does not exist in our present strategy as residuals sugars come from the ethanol fermentation step, but even with zero cost for raw materials, as shown by Koutinas et al. [68], the lipid productivity still must be increased in order to have a cost competitive biodiesel. The experimental data that has been reported so far in the literature refers mainly to the lipid yield in terms of sugar consumed and not necessarily to lipid productivity [18,19,69,70] factor which is still far from the desired value [68]. As discussed recently [71] it seems that lipid yield and lipid productivity, are close, or have reached their experimental upper limit. Hence, other alternatives must be considered, for example: (a) different process strategies, like solid substrate fermentation favored by Meeuwse et al. [68] or the one proposed here, (b) direct production of methyl or ethyl esters in redesigned cell factories [72], and (c) secretion of the lipid fraction from the cell [73] or combinations. At this moment is too early to make an economic evaluation of the two-step process, however producing two products from the same raw material is an alternative to look for in order to reduce production costs. As shown in Fig. 2 in the two-step process there are also several features in order to reduce costs; first, consolidation of ethanol separation and medium sterilization and second, no need for pH adjustment for the second step.

At the time of writing this article we became aware of the publication of Morikawa et al. [74] reporting ethanol and biodiesel production from wheat straw using dilute acid pretreatment; the residual cellulose rich solid was transformed into ethanol by simultaneous saccharification and fermentation and the hemicellulose rich hydrolyzate was used to grow *Rhodosporidium toruloides*. Although there are differences with our approach, the general concept is the same, hence the proposed process scheme is an alternative to consider either in first generation 1G, or in second

generation 2G, or in a combination of both process for ethanol production, in a two-step scheme with microbial oil production. From the biorefinery point of view, more valuable products than biodiesel obtained from the microbial oil could be considered as discussed elsewhere [75].

4. Conclusion

We have proposed a different process route in order to produce ethanol and microbial oil in two consecutive fermentation steps from sweet sorghum juice. The first step consisted of ethanol production and the second step in the growth of oleaginous yeast employing the residual carbon and nitrogen left from the first step. In this paper we presented experimental data with the main objective of providing a proof of concept. The yeast selection for the first step was the most important factor for achieving a high ethanol yield and the effect of inorganic nitrogen addition was not significant. The remaining sugars consisted mainly of sucrose and fructose as no residual glucose was detected. Sucrose inversion took place during ethanol separation so that sucrose, glucose and fructose were present in the residual liquid. In the second step, *T. oleaginosus DSM 11815* grew in the pooled juice remaining from the first step. The yeast took a relatively long time to grow and accumulate oil although the initial C/N ratio was acceptable. In order to consolidate process steps, no pH adjustment was made and growth took place at a pH of 4.8, no additional sterilization was performed in the residual liquid from the distillation step. Glucose showed the highest uptake rate, sucrose was utilized until low glucose values prevailed, and fructose was slowly metabolized and a substantial amount remained. Although the two step process has flexibility in choosing the proper microorganism for each step, it is necessary to look for a rapid fructose uptake strain for both fermentations.

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