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Lipids from *Lipomyces starkeyi*[#]

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Summary

The oleaginous yeast *Lipomyces starkeyi* is interesting for industrial-scale production of biodiesel. Here it is reported that this yeast is able to digest unsaccharified, soluble potato starch. In these experiments, the cultivation time of *Lipomyces starkeyi* was reduced to half of the previously reported values. The effect of C:N molar ratio was studied on growth and lipid content of *L. starkeyi* in media containing glucose or potato starch as C-source. At a C:N molar ratio of 61.2 in glucose medium, the lipid content was measured at 30 % of the dry matter. It was established that the cellular lipid content increased with increasing C:N molar ratio, but the cell yield decreased. In a pH-controlled 1-litre agitated and aerated bioreactor, batch cultivation on glucose resulted in 23 % lipids in cells on dry mass basis (dm), cell yield of 0.25 g/g glucose, and lipid yield of 0.06 g/g glucose. Under the same operating conditions, fed-batch cultivation with discrete glucose additions resulted in 27 % lipids, cell yield of 0.28 g/g glucose, and lipid yield of 0.08 g/g glucose. On soluble but unsaccharified sweet potato starch, cell yield was 0.41 g/g glucose equivalent, lipid yield 0.16 g/g glucose equivalent, and lipid content in cells 40 %. The major fatty acids in the cells were C16:0 and C18:1 (accounting for 85–90 % by mass of the total lipids) with the rest being C16:1 and C18:0. All of these are valuable lipids for biodiesel production. Based on these results, wastes and wastewater from food industry and sewage sludge could serve as sources for the production of biodiesel.

Key words: Lipomyces starkeyi, biodiesel, lipids, yeast, starch, fatty acid methyl esters

Introduction

Limited nature of fossilized resources and increasing concentration of greenhouse gases in our environment due to the indiscriminate use of fossil fuels have sparked a frantic race to harvest renewable resources to replace at least significant proportion of energy derived from fossil fuels. These efforts include utilization of solar, wind, hydro-, and geothermal energies. For the transportation sector, however, finding substitutes for petroleum fuels (gasoline, diesel, aviation fuel) using natural and renewable resources is turning out to be quite difficult. This difficulty stems from the high energy density of petroleum fuels and the vast amounts of their consumption. In the USA alone, 60 billion gallons of diesel oil, 138 billion gallons of gasoline, and 24 billion gallons of jet fuel were consumed in 2008 (*1*). On the other hand, 9.3 billion gallons of ethanol (2) (mostly by fermentation of corn starch) and 678 million gallons of biodiesel (mostly from soybean and corn oils) were produced during

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[#]Dedicated to the memory and respect of Prof Dr Sc Vladimir Marić

the same period (*3*), and this consumption is already straining the market prices of corn starch and soybean oil (*4*). Lignocellulosic biomass is presently being targeted as a major non-food source of carbohydrates for the production of bioethanol and liquid fuels from gasification/liquefaction intermediates (*5*). Similarly, algae are being targeted for the production of lipids that can be transformed into fatty acid esters, followed by blending into diesel and other fuels (*6*). Both of these technologies are still not commercial. On the other hand, there are several smaller but still significant sources of carbohydrates and lipids that can be readily used for production and consumption of fuels in local markets. These include waste grease and wastes from municipal and industrial enterprises. Specifically, the carbohydrate-rich wastes from food canning industries have a high potential as carbon source for production of lipids. Substantial quantities are available also as culled potato and sweet potato that are deemed too small, too large, or too unsightly for further processing (*7*).

Carbohydrates in the raw materials mentioned above are mostly starchy in nature and these can be microbially transformed into lipids. Several heterotrophic microorganisms, classified as oleaginous, are able to accumulate over 20 % of their dry mass as lipids (*8*). Some, such as *Rhodosporidium* sp., *Rhodotorula* sp., and *Lipomyces* sp., accumulate as much as 70 % of their dry biomass as intracellular lipids (*8*,*9*). The majority of those lipids are triacylglycerides (TAG) containing long chain fatty acids that are comparable to vegetable oils (*10*). The pattern of lipid accumulation in microorganisms has been well studied. It is known that lipid production requires a medium with an excess of sugars or similar components and limited amount of other nutrients, usually nitrogen and phosphate (*11*). Thus, oleaginous potential is critically affected by the carbon/nitrogen (C:N) molar ratio of the culture and other factors like aeration, inorganic salts, *etc*. Recently, a lipid-producing yeast, *Lipomyces starkeyi*, has attracted considerable attention (*11–16*). It has been grown to high cell densities using fed-batch techniques and ethanol as a carbon source (*16*). *Lipomyces starkeyi* has been shown to possess an ability to digest carbohydrates present in wastes (*12*). Although the costs of microbial oil production are currently higher than those of vegetable oil, utilization of waste containing carbohydrates has the potential to improve the techno-economics of microbial oil production.

Several physiological studies relating to growth and lipid production by *Lipomyces starkeyi* have been reported in literature (*13*). These studies are concerned with growth on glucose (*14*), glucose and xylose (*15*), ethanol (*16*), sewage sludge (*12*), and cheese whey permeate (*17*). Lipid content in cells grown on glucose has been reported. However, fatty acid profiles of lipids produced by *Lipomyces* grown on glucose have not been reported. Lately, most of the published work involving *Lipomyces starkeyi* has included substrates such as ethanol and sewage sludge, and fatty acid profile of lipids in cells grown on these substrates has been reported (*12*,*16*). Still, information concerning the effect of medium composition and conditions on the cell growth, lipid production and fatty acid profile in *Lipomyces starkeyi* NRRL Y-11557 grown on carbohydrates such as glucose and starch is conspicuously missing from published literature.

We have explored the potential of *Lipomyces starkeyi* to utilize starch-bearing wastes for the production of microbial oils. Cell growth and lipid production on glucose and soluble starch have been investigated and lipids produced have been characterized. The results of this work are presented in this paper. Specifically, the results dealing with selection of a suitable medium, environmental conditions for the growth of cells, the effect of C:N molar ratio on the growth and lipid accumulation of *Lipomyces starkeyi* in shake flasks, and experimental results in agitated and aerated bioreactor have been described.

Materials and Methods

Lipomyces starkeyi NRRL Y-11557 was obtained from National Center for Agricultural Utilization Research, United States Department of Agriculture Laboratory, Agricultural Research Service (USDA, ARS), Peoria, IL, USA. Colonies of *Lipomyces starkeyi* were grown on agar slants at 30 °C. The slants were prepared with 3 g/L of malt extract, 2.5 g/L of yeast extract, and 2 % agar in deionized water (*14*). For growth on starch, the composition of agar slant was modified to contain 2 % (*m/V*) soluble potato starch, 0.5 % (*m/V*) yeast extract, 1 % peptone, and 2 % agar (*18*). The agar slants were stored in the refrigerator and propagated monthly. Inocula for experiments were prepared by incubating the refrigerated cells on fresh agar slants for 48 h at 30 °C, followed by two successive cell propagations in liquid media at 30 °C, once for 48 h and the second time for 30 h. This inoculum preparation protocol resulted in reproducible growth in experimental shake flasks. The composition of the fermentation (F) medium (n(C):n(N)=61.2) containing glucose was: 30 g/L of glucose, 1 g/L of $(NH_4)_2SO_4$, 1.5 g/L of yeast extract, 2.5 g/L of $Na_2HPO_4{\cdot}7H_2O$, 7 g/L of KH_2PO_4, 0.15 g/L of $MgSO_4{\cdot}7H_2O$, 0.1 g/L of $CaCl_2{\cdot}2H_2O$, 8.2 mg/L of $FeSO_4$, 10 mg/L of $ZnSO_4{\cdot}H_2O$, 7 μg/L of $MnSO_4{\cdot}H_2O$, 9.41 μg/L of $CoCl_2{\cdot}6H_2O$ and 10 mg/L of $CuSO_4$. In starch medium, 30 g/L of glucose were replaced with 27.3 g/L of starch (equivalent to 30 g/L of glucose). Different C:N molar ratios were achieved by varying the amount of ammonium sulphate. The pH of the media was adjusted using 1 M NaOH and/or 1 M HCl.

Shake flask experiments were carried out in 500-mL baffled flasks containing 125 mL of media. Inoculum level was 4 % (by volume). Incubations were done at 30 °C and 140 rpm in an orbital shaker. Cultivations were carried out in a controlled-pH 1-litre working volume Multigen Convertible Bench-Top Culture Apparatus Model F-1000 (New Brunswick Scientific Co, Edison, NJ, USA). Aeration rate was 1 vvm. Agitation rate in the bioreactor was 1000 rpm. pH was controlled at 5.5 by the addition of 1 M NaOH solution.

The cell density was measured as dry mass. Glucose, sucrose and maltose concentrations were measured using HPLC. Starch was analyzed using iodine staining (*19*). The lipids were extracted from the centrifuged cells using a modified Bligh and Dyer method (*20*), and from the freeze-dried cells using Accelerated Solvent Extractor (ASE300 from Dionex, Sunnyvale CA, USA). In both

cases, C13:0 fatty acid was added before extraction as an internal standard. Bligh and Dyer extraction involved chloroform-methanol solvent system. The conditions used for ASE extraction were as follows: volume of extraction cell – 40 mL; solvent system – either hexane or chloroform-methanol mixture; sample size – 2 g of freeze-dried microbial cells with hexane and 1 g of freeze-dried microbial cells with chloroform-methanol; operating pressure – 103.4 bar; oven temperature – 125 °C for hexane and 40 °C for chloroform-methanol system; cycles – two, each consisting of filling the extraction cell with a solvent, oven heat up time of 6 min, static time of 5 min, purging with nitrogen at 10.34 bar for 60 s, flushing with solvent (60 % of cell volumes) followed by nitrogen purge for 60 s. Lipid content in the cells was analyzed by gravimetric analysis following the extraction. Fatty acid content in the lipids was determined by formation and analysis of methyl esters of extracted lipids in a GC-MS. The details of experimental and analytical procedures were reported by Wild (*21*).

Results and Discussion

Selection of growth medium through shake flask experiments

Shake flasks were used to identify a desirable growth medium and pH value for cultivation. Three nutrient media, yeast malt extract (YM) medium of Uzuka *et al.* (*22*), S medium of Yamauchi *et al.* (*16*), and fermentation (F) medium of Suutari *et al.* (*23*), were evaluated for growth and lipid production. F medium, composition reported in Materials and Methods section, with initial pH value adjusted to 5.5, resulted in the highest maximum specific growth rate of cells and shortest cultivation times. It is a semi-synthetic medium in which a major portion of nitrogen (N) is supplied in the form of ammonium ions; some N is supplied by the yeast extract as well. As reported by several authors, nitrogen limitation triggers the accumulation of lipids in lipid-producing cells (*16*). F medium was used in all the subsequent experiments as the use of this medium permits easy manipulation of C:N molar ratio in the medium.

Results of two shake flask experiments with medium F (n(C):n(N)=61.2) are presented in Fig. 1. The shake flask results were highly reproducible. Similar observations were made in all the other shake flask experiments as well. The solid lines in Fig. 1 represent trend lines and these show that the cells grew vigorously after a short lag phase and essentially reached a stationary phase in 48 h, when all the sugar in the medium was exhausted. pH values in the flasks without pH control dropped to around 3.5 over this time, while the cell dry matter concentration increased to 8.4 g/L.

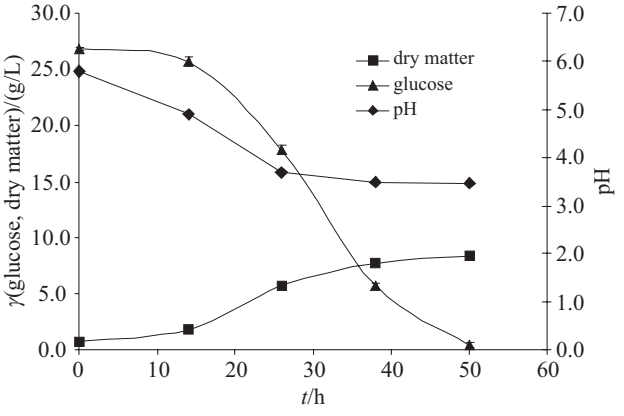

Fig. 1. Growth of *Lipomyces starkeyi* on glucose in F medium at C:N molar ratio of 61.2

Effect of C:N molar ratio on growth and lipid production

C:N molar ratio was changed in F medium from 19.8 to 61.2 by changing either the initial concentration of glucose or that of ammonium sulphate in the medium, and the results from shake flask experiments (pH uncontrolled) are presented in Table 1. All the numbers reported in Table 1 refer to the end of the experiments. In all the cases, glucose in the medium was exhausted within 50 h, when the experiments were terminated. As expected, increasing C:N molar ratio resulted in higher lipid content in the cells, but with reduced yield and reduced specific growth rate of the cells. Yield of lipid on carbon source did not show much influence of C:N molar ratio. Similar data were reported by Yamauchi *et al.* (*16*) also. In a study involving growth of *Lipomyces starkeyi* DSM 70295 on sewage sludge, Angerbauer *et al.* (*12*) found that the cells had a lipid content of 40 % at a C:N molar ratio of 60. In a recent paper, Zhao *et al.* (*15*) optimized medium composition for growth of *Lipomyces starkeyi* AS 2.1560 on a mixture of glucose and xylose (2:1 by mass). In the base case, lipid content in their cells (35 %) was similar to that observed in this paper, but optimization of the medium for concentration of sugars, yeast extract and $FeSO_4$ resulted in 60 % increase in lipid content within the cells. Cultivation time to achieve this lipid content was 120 h.

Table 1. Growth parameters and lipid content of *Lipomyces starkeyi* on glucose in F medium with different C:N molar ratios (shake flasks, pH uncontrolled)

C:N ratio	Maximum specific growth rate	Cell yield m(cell dm)/m(glucose)	Lipid content	Lipid yield m(lipid)/m(glucose)
	h^{-1}	g/g	%	g/g
19.8	0.10	0.44	19.4	0.09
39.7	0.08	0.38	22.1	0.09
61.2	0.06	0.29	30.0	0.10

Fatty acid profile of lipids

Fatty acid composition of lipids extracted from the cells using Bligh and Dyer method is presented in Fig. 2. The main fatty acids in the lipids from *L. starkeyi* were C16:0 (palmitic acid) and C18:1 (oleic acid) (accounting for 85–90 % by mass of the total lipids; 39–45 and 40–50 %, respectively). Other fatty acids present were C16:1 (palmitoleic acid), amounting to 10–15 %, and C18:0 (stearic acid), approx. 2–4 %. A slight shift from palmitic to oleic acid would be observed as the C:N molar ratio was increased.

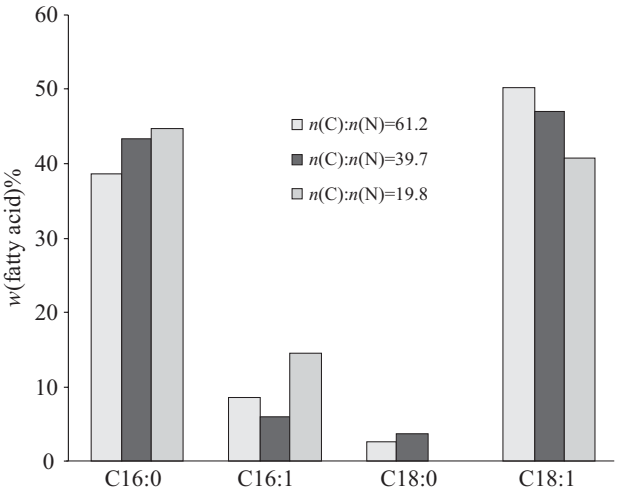

Fig. 2. Fatty acid composition of *Lipomyces starkeyi* cells grown in media containing different C:N molar ratios

Lipid profiles in *Lipomyces starkeyi* and other cells have been reported by a number of researchers (*12*,*16*). Angerbauer *et al.* (*12*) reported lipid composition in *L. starkeyi* cells grown on sewage sludge; the main fatty acids were C16:0 (56 %), C16:1 (2 %), C18:0 (14 %), and C18:1 (26 %). Small amounts of C14:0 (myristic acid), C18:3 (linolenic acid), C20:0 (arachidic acid), C20:1 (gadoleic acid), C22:0 (behenic acid) were also present; these were all under 1 % by mass and mostly under 0.5 % by mass. The overall profile thus was similar to the findings observed in this work. Differences in the relative proportions of palmitic and oleic acids are perhaps due to the differences in carbon sources, medium composition, and duration of cultivation. Yamauchi *et al.* (*16*) reported fatty acid profile of lipids produced by *L. starkeyi* cells grown on ethanol in a fed-batch culture. These authors found that fatty acid composition in the cells undergoes change with time during fed-batch cultivation; fractions of C16:0 and C18:1 fatty acids in intracellular lipids increased with time up to 90 h. The fractions of C18:0 and C18:2 went down while that of C16:1 remained unchanged. C16:0 accounted for 28–32 % of all the fatty acids and C18:1 for 50–54 %. These percentages are similar to those found in this work.

Batch vs. fed-batch operation of bioreactors

Cells were cultivated in a 1-litre working volume stirred bioreactor in which agitation was set at 1000 rpm and aeration was 1 vvm. pH in the medium was controlled at 5.5 by the addition of 1 M NaOH solution. The experiments were carried out with F medium (n(C):n(N)= 61.2) and 30 g/L of glucose in two ways. In the first, all the 30 g/L of glucose were added at the start of the experiment. In the second, it was added in five installments with each addition made when glucose concentration in broth was measured below 1 g/L. The results of these experiments are plotted in Fig. 3 and presented in Table 2. The fed-batch experiment was carried out to simulate the behaviour of cells when grown in the presence of low concentrations of carbon source. Glucose concentration remained below 9 g/L throughout the fed-batch operation.

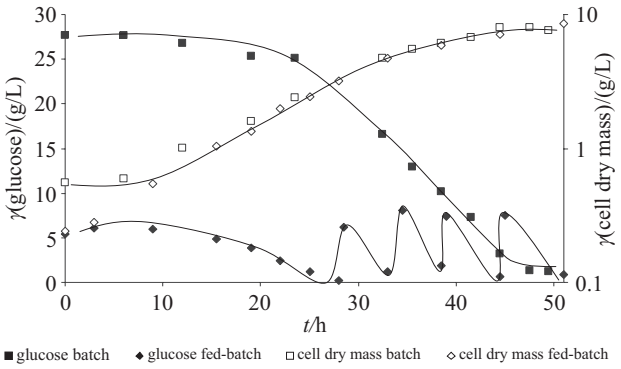

Fig. 3. Effect of carbon feeding on cell growth and glucose consumption in bioreactor cultivations

Table 2. Effect of carbon source and feeding strategy on growth and lipid production in a pH-controlled bioreactor

Experiment	C:N ratio	Maximum specific growth rate	Lipid content	Lipid yield m(lipid)/ m(glucose)
		h^{-1}	%	g/g
glucose batch	61.2	0.08	23.7	0.06
glucose fed-batch	61.2	0.10	27.0	0.08
starch batch	61.2	0.08	40.3±3.6	0.16±0.02

Although the growth behaviour of cells did not show any difference from that observed when all the glucose was added at the start (Fig. 3), the lipid content in cells and the lipid yield registered improvement (Table 2). These results point to a potentially desirable fed-batch operation for the production of lipids even when C:N molar ratio is predecided. This would also permit the manipulation of the C:N molar ratio in the broth as the cells are directed from growth to lipid accumulation. This would be in agreement with the observations of Yamauchi *et al.* (*16*), Pan *et al.* (*24*), and Li *et al.* (*10*), where fed-batch operation was extolled for production of microbial lipids. Yamauchi *et al.* (*16*) obtained cell density of 153 g/L with lipid content of 54 % (*m/V*) in fed-batch culture of *Lipomyces starkeyi* grown on ethanol for over 140 h. Li *et al.* (*10*) increased the lipid productivity in their efforts to produce lipids from yeast *Rhodosporidium toruloides* on glucose, by utilizing a flask fed-batch culture in which the final cell density was 151 g/L and lipid content was 48 %. A pilot-scale fed-batch culture resulted in cell density of 106.5 g/L, lipid content 67.5 %, and lipid productivity of 0.54 g/(L·h) in a 134-hour experiment. Pan *et al.* (*24*) obtained cell density of 185 g/L in 84-hour fed-batch culture of *Rhodotorula glutinis*.

Lipid production with soluble sweet potato starch as carbon source

For starch as substrate, glucose (30 g/L) in F medium was replaced by an equivalent amount of starch (27.3 g/L). Soluble sweet potato starch obtained from Sigma Aldrich, St. Louis, MO, USA was used for this purpose. C:N molar ratio was maintained at 61.2. The results of the measurements of starch and cell dry mass

concentrations during batch cultivation in a pH-controlled bioreactor are presented in Fig. 4.

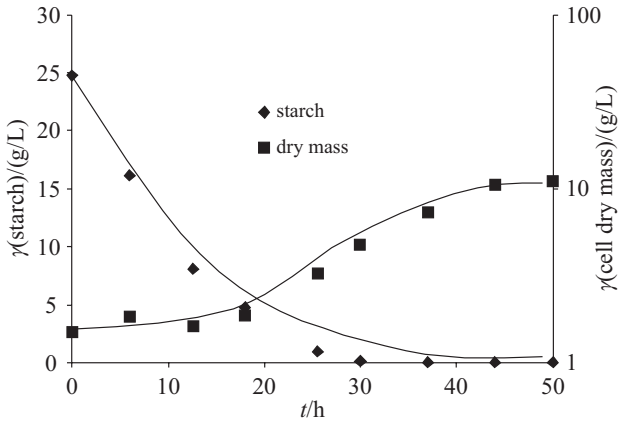

Fig. 4. Concentration of starch and cell dry mass during growth of *Lipomyces starkeyi* on sweet potato starch in F medium (*n*(C): *n*(N)=61.2, pH=5.5)

Even though soluble starch was used in this work, some particulate solids were present at the start of the runs and these interfered with cell dry mass measurements. As a result, the dry mass measurements at the start of the experiments were inflated. After 24 h of the experiment, the starch particulates were not present. Based on these measurements, 12 g/L were produced in 50 h from 27.3 g/L of starch, suggesting a cell yield of 0.44 g/g starch or 0.4 g/g glucose equivalent. All the starch, measured by iodine test, was gone within 30 h.

Concentrations of carbohydrates (glucose, maltose, and sucrose) found in the broth are presented in Fig. 5. The analysis of carbohydrates was conducted by HPLC and the peaks were identified using retention times of different carbohydrates. The profiles show typical patterns of intermediates of reaction (hydrolysis of starch and other carbohydrate polymers present in potato starch). None of these concentrations exceeded 6 g/L during the cultivation, and all were reduced to zero within 50 h. It is important to note that sweet potato contains not only starch, but also sugars that include glucose, fructose, maltose, and sucrose; sugar fraction in sweet potato ranges between 5 and 38 % on dry matter basis (*7*,*25*). The higher fractions are observed specially in Louisiana varieties of sweet potato (*7*) and most of it is present as sucrose.

The estimated cell growth and yield parameters with sweet potato starch are also listed in Table 2, along with those on glucose (batch and fed-batch). These data show that soluble sweet potato starch is an excellent substrate for production of microbial lipids by *Lipomyces starkeyi*. These observations with sweet potato starch are very interesting since the starch utilized in this work was not subjected to saccharification. Even though sugars are present in sweet potato, the fact that *Lipomyces starkeyi* utilized all the carbohydrates with high cell and lipid yields suggests that *Lipomyces starkeyi* may possess glycosidase activity. To the best of our knowledge, this is the first time that utilization of unsaccharified starch has been reported for cultivation of *Lipomyces starkeyi*. Moreover, the differences in cell and lipid yields with starch and glucose as substrates for the same C:N molar ratio in the medium are striking too. At the present stage, the reasons for this are not clear and further work involving analysis of additional intermediates, intracellular and extracellular enzymes, and cellular metabolism is underway. It has been reported that starch in sweet potato is easy to gelatinize, has a lower degree of polymerization, and does not retrograde easily (*7*). The role of these factors also needs follow-up.

Comparison of fatty acids in lipids produced from different processes

Fatty acid profiles of lipids produced by cells grown on glucose (batch and fed-batch) and on sweet potato starch are presented in Fig. 6. As suggested earlier, it is suspected that maintaining the glucose concentrations low during cultivation of cells may have beneficial effect on cellular production of lipids and perhaps shift the fatty acid profile towards production of unsaturated fatty acids. The use of unsaccharified soluble potato starch as substrate also resulted in sugar concentrations below 5 g/L and, as in fed-batch experiments, it resulted not only in

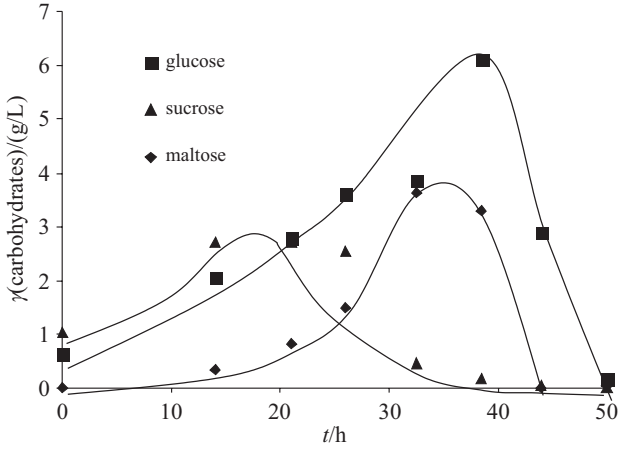

Fig. 5. Concentrations of carbohydrates in the broth during the growth of *Lipomyces starkeyi* on sweet potato starch

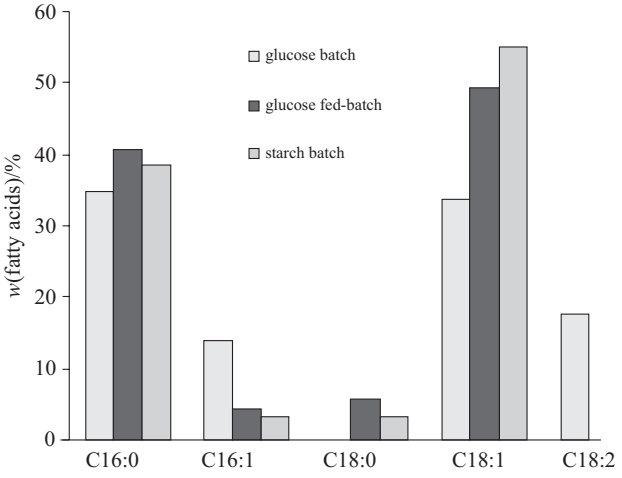

Fig. 6. Fatty acid profile of lipids produced by *Lipomyces starkeyi* in bioreactor (*n*(C):*n*(N)=61.2)

high concentrations of C18:1, but also in increased lipid content in the cells. This issue needs further follow-up, perhaps in the form of true fed-batch experiments with glucose and/or continuous operation, and analysis of lipid metabolism in the cells. These experiments are currently being planned.

The fatty acid profile of lipids in *Lipomyces starkeyi* grown on starch is compared in Table 3 with those reported in published literature (*12*,*16*,*26*,*27*) for this cell as well as with the lipid profile of some common vegetable oils.

Table 3. Comparison of fatty acid profiles of lipids observed in this work with those reported in literature

Lipid profiles of *L. starkeyi* on different substrates					
	C16:0	C18:0	C16:1	C18:1	C18:2
starch (this work)	39.0	3.0	3.0	55.0	
sludge (*12*)	55.9	13.8	1.8	25.8	0.1
ethanol (*16*)	31.8	6.7	2.3	53.2	5.0

Lipid profiles of some common vegetable oils						
	C16:0	C18:0	C20:0	C18:1	C18:2	C18:3
palm (*26*)	47.90	4.23		37.00	9.07	
corn (*27*)	11.67	1.85	0.24	25.16	60.60	0.48
soybean (*27*)	11.75	3.15	0.00	23.26	55.53	6.31
sunflower (*27*)	6.08	3.26	0.00	16.93	73.73	0.00

The lipid profile for starch-grown cells was similar to that of ethanol-grown cells as reported by Yamauchi *et al.* (*16*). In both cases, the unsaturated fatty acids comprised between 58 and 60 % and the rest were unsaturated. This, however, is quite different for sludge-grown cells reported by Angerbauer *et al.* (*12*), who found a preponderance (70 % by mass) of saturated fatty acids in the cellular lipids. This issue of the ratio of unsaturated to saturated fatty acids in the lipids is of great interest to biodiesel industry as it affects cloud point in the biodiesel strongly. In this respect, the fatty acid profile observed in this work points to characteristics of biodiesel between those obtained from plant oils (palm oil) and from vegetable oils (corn and soybean oils).

Conclusions

The results presented here show that *Lipomyces starkeyi* NRRL Y11557 has a potential for production of microbial lipids from starchy substrates. High values of C:N molar ratio resulted in increased fraction of lipids in cells, but reduced growth rates and cell yields. The use of soluble potato starch as a carbon source resulted in higher cell yield as well as higher fraction of lipids in the cells. The *L. starkeyi* cells were able to utilize unsaccharified soluble potato starch. Lipids in *L. starkeyi* cells contained mainly C16:0 and C18:1 fatty acids. Growth on starch increased the fraction of C18:1 fatty acid in the cells, a feature that will be desirable for improved cloud point in biodiesels produced from these lipids.

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