MICROBIAL LIPIDS PRODUCTION BY *LIPOMYCES STARKEYI* USING BYPRODUCTS FROM THE SUGAR AND ALCOHOL INDUSTRY

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ABSTRACT

Microbial oils are promising raw materials for the production of biodiesel, chemicals, pharmaceuticals and foods. However, its production is limited by the high cost of its cultivation medium composed by high-value substrates such as glucose. Nevertheless, the industrial production of sugar and alcohol generates great volumes of vinasse and molasses, low-cost byproducts with nutrients and carbon sources that can be suitable as a culture medium for the yeast *Lipomyces starkeyi* DSM 70296, aiming at the production of lipids. In this study, culture media composed by vinasse (V) and molasses (M) in proportions 90:10 and 70:30 (V:M) were tested for lipids production by *L. starkeyi.* These culture media produced 7 g/L of biomass and 41% of lipids and 5 g/L biomass and 38% lipids, respectively. Furthermore, 11 g/L of biomass and 43% lipid were obtained from a synthetic medium containing only sucrose (the main substrate present in molasses, C/N=50). Therefore, *L. starkeyi* was able to use molasses and vinasse, without supplementation, for cell growth and to achieve efficient lipid production, demonstrating that these byproducts are favorable low-cost substrates for microbial oils production.

Keywords: Vinasse, Molasses, *Lipomyces starkeyi*, Biodiesel.

1 INTRODUCTION

The demand for vegetable oils for biodiesel production has increased due to the depletion of oil reserves and the increase in greenhouse gas emissions. However, these oils are mainly intended for food production. Because of this, microbial oils with a fatty acid profile similar to vegetable oils have been considered promising raw materials for the production of biodiesel and other chemicals[1]. These oils can be produced by oleaginous microorganisms, which are capable of accumulating more than 20% (w/w) of lipids by dry cell weight, although certain species of microorganisms can synthesize up to 70% (w/w) of lipids by dry cell weight. Principally, microbial lipids called Single Cell Oils (SCO's) are produced by algae, fungi and bacteria[3], and have diverse applications, such as the production of biodiesel, pharmaceuticals and foods[4,5]. However, the yeast *Lipomyces starkeyi* DSM 70296 has stood out for consuming a wide variety of substrates, accumulating more than 40% (w/w) of lipids, and for being tolerant to inhibitors present in sugarcane bagasse hydrolyzate[6,7]. This microorganism has attained efficient lipid production using low-cost carbon sources, which is an important alternative to reduce the high cost of lipid production caused by the use of glucose as substrate.[7]

Sugarcane molasses and vinasse, obtained as by-products from sugar and ethanol production, respectively, are interesting substrates for the production of microbial lipids. Molasses is rich in sugars and mainly used in the production of ethanol, while vinasse is an abundant effluent mainly used in soil fertilization. Furthermore, inadequate disposal of vinasse can cause severe environmental problems, such as eutrophication[8]. This work evaluated the production of lipids by *L. starkeyi* using different mixtures of molasses and vinasse as substrate, focusing on greater use of vinasse aiming to promote lipid production at lower cost and with environmental benefits.

2 MATERIAL & METHODS

Sugarcane molasses and vinasse were supplied by Raízen-Usina Costa Pinto (Piracicaba, São Paulo, Brazil). Soluble solids (SS) were determined by refractometry (°Brix) with an Abbe refractometer (PZO WARSZAWA RL1®) calibrated with deionized water (refractive index = 1.3330; 0º Brix at 20ºC). The pH was determined with a pH meter (Sensoglass®). The yeast *Lypomyces starkeyi* DSM 70296 was preserved on YPD Agar (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose, 20 g/L Agar, pH 5.5) at 4°C. The pre-inoculum was cultivated in YPD medium (10 g/L yeast extract, 10 g/L peptone, 20 g/L glucose) at 28°C, 180 rpm for 48 h in an orbital shaker (Lucadema®). For cell cultivation, the inoculum was prepared by transferring 10% (v/v) of the pre-inoculum to the inoculum medium, incubated for 24h, and then transferred to the cultivation medium (10% v/v) during the exponential phase. The composition of the inoculum and cultivation culture media, in g/L, was: 20 g of sucrose, 0.67 g of yeast extract, 0.48 g of $(NH_4)2SO_4$, 0.4 of Mg_2SO_4. $7H_2O$, 3.5 g KH_2PO_4, 1 g Na_2HPO_4, 0.04 g $CaCl_2.2H_2O$, 0.08 g $ZnSO_4 \cdot 7H_2O$, 0.001 g $CuSO_4 \cdot 5H_2O$, 0.001 g $CoCL_2 \cdot 6H_2O$ and 0.005 g $(NH_4)2Mo_2O_7$ (pH 5.5). The carbon/nitrogen ratio (C/N) was 50. Cultivations were carried out in triplicate in 125 mL Erlenmeyer flasks with 50 mL of culture medium, at 28°C, 180 rpm and pH 5.5. The pH was adjusted with HCl (1 M). The sugar and salt solutions were sterilized separately. The samples collected during cultivation were centrifuged at 10000 rpm for 15 minutes at 4°C (SL-706 centrifuge, Solab®), and the supernatants were stored at -10 °C.

Cultures with 10% molasses in distilled water and a mixture of molasses and vinasse (10:10, w/v) were supplemented with 0.229 g/L yeast extract and 0.164 g/L ammonium sulfate to maintain C/N ratio=50. For proportions of molasses (M) and vinasse (V) of 10:90 (V:M=90:10) and 30:70 (V:M=70:30), a working volume of 100 mL, pH 5 was used. Cell biomass was quantified by reading a spectrophotometer (Kasuaki®) at 600 nm and dry weight was determined after centrifuging the fermented medium at 10000 rpm for 15 minutes at 4 °C (SL-706 centrifuge, Solab®), washing the cells with distilled water, new centrifugation and drying in an oven (Solab®) at 60 °C until constant weight. The ammoniacal nitrogen was quantified by the Berthelot reaction at 630 nm in a spectrophotometer (Kasuaki®) and the standard curve was constructed with different concentrations of ammonium sulfate (4 - 20 mg/L NNH4). The microbial biomass was lyophilized at 400 mmHg and -40 °C using a LJJ04 lyophilizer (JJcientific®). The microbial lipid content was determined by the Bligh-Dyer method using methanol and chloroform as solvents for extraction of the lipids present in cells previously treated with 2 M HCl for 1h at 80 °C[7,9].

3 RESULTS & DISCUSSION

Molasses and vinasse showed pH values of 5.14 (±0.02) and 4.78 (±0.03), respectively. The °Brix scale showed that molasses has higher soluble solids content (71.6°Brix) than vinasse (1.7°Brix). This can be explained by the high sugar content in molasses.

The use of sucrose as the only carbon source by *Lipomyces starkeyi* was evaluated, since sucrose is the main sugar in sugarcane molasses[10]. Although *L. starkeyi* can metabolize sucrose more slowly than glucose and xylose[11], there was no difficulty in consuming sucrose, which can be observed by the absence of lag phase (Figure 1). *L. starkeyi* reached 11.22 (±0.67) g/L cell biomass (CDW) (Figure 1). It is observed that ammoniacal nitrogen was consumed up to 24 hours. For oleaginous microorganisms, when nitrogen is limited and there is excess carbon, lipid accumulation occurs. The lipid content obtained from this cultivation was 43% (w/w), which demonstrates efficient lipid production from sucrose.

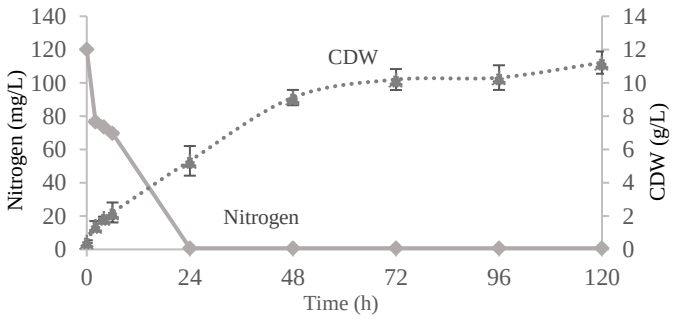

Figure 1 Kinetics profile of *L. starkeyi* growth (CDW: Cell Dry Weight) and nitrogen consumption in synthetic sucrose medium (C/N=50).

Cultivations in molasses 10% (w/v), molasses and vinasse 10:10 (w/v) and molasses 10% (w/v) with salts and molasses and vinasse 10:10 (w/v) with the addition of salts were carried out to verify the growth capacity of the microorganism under these conditions, as well as to compare the growth of yeast with and without supplementation. The cell biomass concentration was similar for culture media with and without salts for 54 h, as shown in Table 1. The addition of salts did not cause a major difference in the cell growth, however, it may have influenced the accumulation of lipids (not determined). This experiment also demonstrated that it is not necessary to hydrolyze sugarcane molasses to be used as substrate by the *L. starkeyi*.

Table 1 Cell biomass concentration of *L. starkeyi* in molasses and vinasse at 54 h of cultivation.

	Cultivation*			
	M (10%)	**M (10%)+S**	**M + V (10:10)**	**M + V (10:10) + S**
CDW (g/L)	6.19 ±0.06	6.23 ±0.73	6.18 ±0.04	6.16 ±1.01

*weight/volume (w/v)
M = molasses; V = vinasse; S = Salts

In the 30:70 (w/v) molasses:vinasse conditions, *L. starkeyi* had difficulty adapting, taking 72 h to reach the appropriate cell concentration, compared to 24 h in 10:90 (w/v) molasses:vinasse medium. This was probably due to the higher concentration of inhibitory compounds in the molasses. Figure 2 and Table 3 show that in M:V=70:30 and 90:10 media, the growth of *L. starkeyi* was lower than in the synthetic sucrose medium, possibly due to the presence of toxic compounds and nitrogen deficiency in vinasse. and molasses. Ammoniacal nitrogen analyses for these conditions resulted in values below zero. Therefore, other proportions of molasses and vinasse can be studied to improve the growth and production of lipids from these low-cost substrates, aiming to increase the economic competitiveness of microbial lipids and reduce environmental impacts caused by inadequate disposal of vinasse.

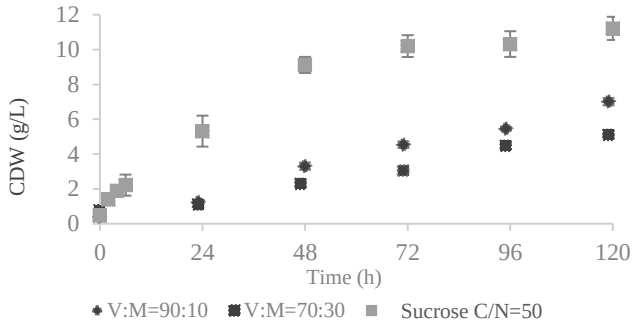

Figure 2 Kinetic profile of *L. starkey*i growth (CDW: Cell Dry Weight) in synthetic sucrose medium (C/N=50), V:M=90:10; V:M=70:30

Lipid content values were similar (Table 3), exceeding 20% of the dry weight of the microbial cell. In the 90:10 (V:M) condition, *L. starkeyi* had greater lipid accumulation and final biomass concentration compared to the 70:30 (V:M) condition, being the best condition studied. In a previous study, *L. starkeyi* DSM 70296 presented 32% lipid content with sugar cane molasses and $(NH_4)2SO_4$[12], thus representing an increase of 28% and 18% in lipid content, respectively.

Table 3 Effects of different culture media on cell biomass, concentration and lipid content.

Culture medium	Nitrogen source	Biomass (g/L)	Lipids (g/L)	Lipids (%)
Sucrose C/N=50	$(NH_4)_2SO_4$ and yeast extract	11.22±0.67	4.65±0.18	42.62±1.73
70:30 (V:M)	-	5.09±0.04	1.93±0.01	37.89±0.12
90:10 (V:M)	-	7.01±0.21	2.86±0.04	40.92±0.38

4 CONCLUSION

This work demonstrated that molasses and vinasse are promising substrates for microbial lipids production by *L. starkeyi*, which can contribute to a more economical production of biodiesel, pharmaceuticals and foods integrated to biorefinery processes, and decrease environmental impact caused by inadequate disposal of vinasse in soil.

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