Estudo de diferentes combinações de melaço/ vinhaça como substrato para produção de proteínas e lipídios por microrganismos

Study of molasses / vinasse waste ratio for single cell protein and total lipids production by

Marcia Luciana Cazetta[1]; Maria Antonia P. C. Celligoi[2]

Resumo

Diferentes combinações de melaço e vinhaça de cana-de-açúcar foram utilizadas como substrato para produção de biomassa protéica e lipídica por cinco microrganismos: quatro linhagens de leveduras: *Candida lipolytica, Rhodotorula mucilaginosa, Saccharomyces cerevisiae*, uma levedura isolada das lagoas de vinhaça (denominada LLV98) e uma bactéria, *Corynebacterium glutamicum.* Os meios utilizados foram: a) 50% de melaço e 50% de vinhaça; b) 25% de melaço e 75% de vinhaça e c) 75% de melaço e 25% de vinhaça. O objetivo deste trabalho foi estudar o crescimento celular e a produção de proteínas e lipídios na biomassa obtida a partir desses subprodutos. A maior produção de proteína na biomassa foi obtida por *S. cerevisiae*, 50,35%, seguida por *R. mucilaginosa*, 41,96%, em 25% de melaço e 75% de vinhaça. As menores produções de proteína foram obtidas por *C. glutamicum.* A melhor produção de lipídios totais, acima de 26%, foi obtida no meio de 50% de melaço e 50% de vinhaça por *S. cerevisiae* e *C. glutamicum.*

Palavras-chave*:* Vinhaça.Melaço. Biomassa. Proteína. Lipídios. Microrganismos.

Abstract

Different molasses/ vinasse ratio were used as substrate to investigate single cell protein and total lipids production by five microorganisms: four yeasts strains: *Candida lipolytica*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, a yeast isolated from vinasse lake (denominated LLV98) and a bacterium strain, *Corynebacterium glutamicum*. The media utilized were: a) 50% molasses and 50% vinasse; b) 25% molasses and 75% vinasse and c) 75% molasses and 25% vinasse. The objective of this work was to study the growth of microorganisms and also evaluate protein and lipids content in the biomass obtained from these by-products. The highest single cell protein production was obtained by *S. cerevisiae*, 50.35%, followed by *R. mucilaginosa*, 41.96%. The lowest productions were obtained by *C. glutamicum.* The higher total lipids productions, more than 26%, were founded in molasses plus vinasse at 50%/50% by *S. cerevisiae* and *C. glutamicum*.

Key word*s:* Vinasse. Molasses. Single cell protein. Total lipids. Microorganisms

[1] Doutora em Ciências Biológicas - Área de concentração em Microbiologia Aplicada. Itápolis-SP.- Email: malulz@yahoo.com.br
[2] Docente do Departamento de Bioquímica e Biotecnologia – Universidade Estadual de Londrina.

Introduction

Interest in industrial wastes as substrate for single cell protein production has increased recently (EMTIAZI; ETEMADIFAR; TAVASSOLI, 2003) for application as protein sources of high nutrition value and for environmental pollution control (NAVARRO; SEPÚLVEDA; RUBIO, 2000; PARASKEVOPOULOU et al., 2003). Many by-products or wastes, such as cheese whey (PARASKEVOUPOLOU et al., 2003), wastewater from glutamate fermentation (ZHENG et al., 2005) and cellulosic material are abundant in several countries and can be used as raw material for developing biotechnological process of industrial interest (ALTAF et al., 2005) by many microorganisms as yeasts, bacteria, algae and fungi (ARNOLD; KNAPP; JONHSON, 2000; ANUPAMA; RAVINDRA, 2000).

Furthermore, the interest for new sources of essential fatty acids has increased. Recently, the attention for yeasts and other microorganisms which are called oleaginous or "oil bearing", has been renewed, as source of oils and fats (RUPCIC; BLAGOVIC; MARIC, 1996; DYAL; NARINE, 2005).

Molasses is an agro-industrial by-product often used in alcohol distilleries due to the presence of fermentative sugars, being a good carbon source for the microorganism metabolism. Sugar cane molasses and vinasse are an abundant agro-industrial material produced in Brazil and other tropical countries and their low cost is an important factor for the economical viability of substances produced by fermentation. Its composition is rich in organic salts like nitrogen, phosphates, calcium and magnesium as well as micronutrients as zinc, manganese, copper and iron, besides many amino acids (WALISZEWSKI; ROMERO; PARDIO, 1997; CORTÉZ; BROSSARD-PÉREZ, 1997).

The vinasse is the major effluent from the ethanol industry and represents a major environmental problem. This black liquid is produced at a rate of 10 to 15 liters for each ethanol liter and it is a mixture of water and organic and inorganic compounds (MENEZES, 1980; ANCIÃES, 1981; POLACK; DAY; CHO, 1981).

In Brazil, most of the vinasse that results from ethanol production is being used as fertilizer due to its organic matter content and nutrients like high potassium and nitrogen contents (30 g Kg^{-1} dry weight) Diaz et al. (2003). The problem occurs when some soils do not respond positively to the application of this acid material (WARD, 1991; CORTEZ; BROSSARD PEREZ, 1997) reducing the alkalinity of the soil, destroying the crops (KUMAR; VISWANATHAN, 1991), causing manganese deficiency in the soil (AGRAWAL; PANDEY, 1994) and inhibiting seed germination (KANNABIAN; PRAGASAN, 1993).

In this work, our objective was to study the growth of microorganisms in a mixture of molasses and vinasse and also evaluate protein and lipid content in the biomass obtained from these by-products.

Material and Methods

Microorganisms: Microorganisms utilized were *Candida lipolytica*, *Rhodotorula mucilaginosa*, *Saccharomyces cerevisiae*, a yeast isolated from vinasse lake (denominated LLV98) and bacterium *Corynebacterium glutamicum*.

Fermentation medium: For fermentation medium sugar cane molasses and vinasse were utilized with different rates: a) 50% molasses and 50% vinasse, b) 25% molasses and 75% vinasse and c) 75% molasses and 25% vinasse. Molasses and vinasse were obtained from Cooperativa Agrícola de Rolândia - COROL - Paraná, Brazil.

Fermentations: Fermentations were carried out in 250 mL Erlenmeyer flasks containing 50 mL fermentation medium, in duplicate, at $28^0C \pm 2^0C$ and agitation rate of 180 rpm.

Analytical methods: The single cell protein was assayed for its moisture content by drying of the sample at 105^0C until constant weight and against standard curve of dry mass x optic density. Its protein content was determined by applying the Kjeldahl method (N x 6.25) (ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS, 1984).

Determination of reducing sugars: The reducing sugars from cultures were firstly hydrolyzed by HCl 2M and boiled during 5 minutes. After that, the solution was neutralized with NaOH 2M. Reducing sugars were determined by Somogy (1945) and Nelson (1944) method.

Determination of total lipids: Lipids were extracted with a mixture of chloroform-methanol (1:1, v/v) according to ASSOCIATION OF OFFICIAL ANALYTICAL CHEMISTS method (1975). The total lipids content in the extract was quantified by Frings e Dunn's method (1971).

Results and Discussion

a) Fermentation in 50% molasses and 50% vinasse

This culture condition was unfavorable for biomass production by both yeast LLV98 and *C. glutamicum*, achieving concentrations lower than 1.00 g/L. *Rhodotorula mucilaginosa* also showed low biomass production, 2.63 g/L. Highest biomass were obtained by both *S. cerevisiae* and *C. lipolytica*, 7.49 g/L and 6.90 g/L, respectively. *Saccharomyces cerevisiae* obtained highest biomass protein yield, 2.55 g/L and highest productivity, $0.15 g/L.h^{-1}$ (figure 1). Sugar consumption was low for all microorganisms, except for *S. cerevisiae*, which consumed 98.25% (Table 1 and figure 2).

The total lipid content analysis showed that molasses and vinasses at 50%/ 50% stimulated lipid production by both *S. cerevisiae* and *C. glutamicum*, which obtained more than 26%. Lipid values obtained by both *S. cerevisiae* and *C. glutamicum* in this culture condition are close to described for oleaginous microorganisms. As described by Murphy (1991) and Ratledge (1996), yeasts and fungi that contain more than

Table 1 - Biomass yield (g/L), total lipid yield (%) and substrate consumption (%) in molasses 50% and vinasse 50% after 48 hours.

Microorganism	Biomass (g/L)	Substrate consumption (%)	Total lipids (%)
Candida lipolytica	6.90	30.05	17.68
Yeast LLV98	0.41	14.50	8.98
Rhodotorula mucilaginosa	2.63	37.48	19.50
Saccharomyces cerevisiae	7.49	98.25	26.90
Corynebacterium glutamicum	0.51	14.02	26.50

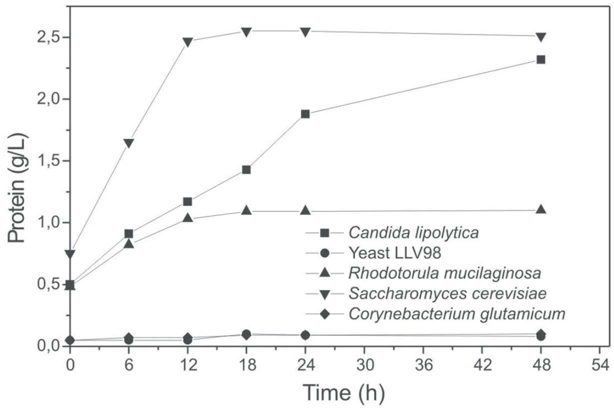

Figure 1. Protein production (g/L) in 50% molasses and 50% vinasse after 48 hours.

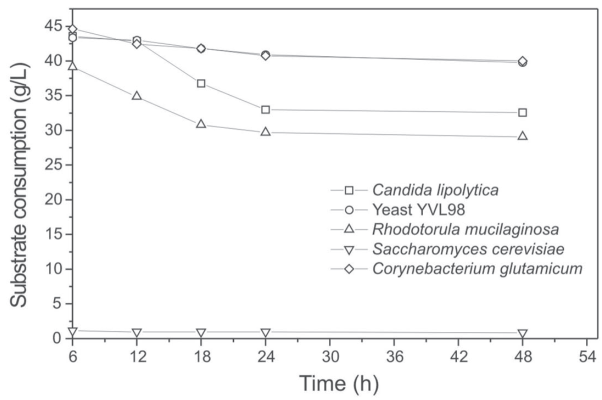

Figure 2. Substrate consumption (g/L) in 50% molasses and 50% vinasse after 48 hours.

25% of their biomass in the form of lipids are defined as oleaginous microorganisms. These results were higher than those obtained using molasses and vinasse separately (unpublished results).

The values reached by both *R. mucilaginosa* and *C. lipolytica* varied from 17 to 19.5%, which are higher than those obtained by Rupcic, Blagovic and Maric (1996), who produced 14% of lipids in glucose at 1%. Yeast LLV98 obtained 8.9%. *Saccharomyces cerevisiae* showed best total lipid yield and productivity, 2.0 g/L and 0.12 g/L.h^{-1}, respectively (Figure 3).

c) Fermentation in molasses 75% and vinasse 25%

.yeast LLV98 and *R. mucilaginosa* produced about 5.0 g/L while *C. lipolytica* achieved 4.14 g/L and *C. glutamicum* 2.78 g/L. The highest protein yield was obtained by *S. cerevisiae* (2.52 g/L) followed by *R. mucilaginosa* (1.80 g/L), while *C. lipolytica*

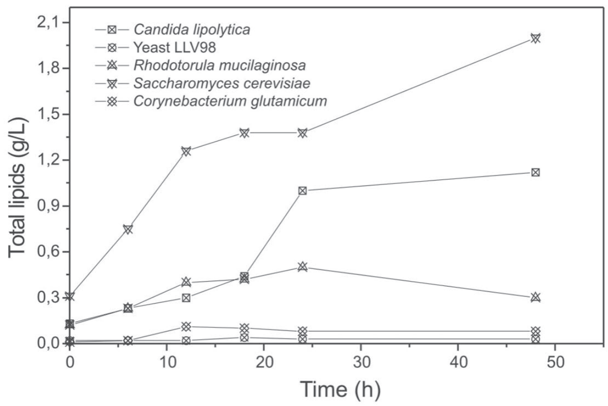

Figure 3. Lipid productions (g/L) in molasses 50% and vinasse 50% after 48 hours

b) Fermentation in 25% molasses and 75 % vinasse

Biomass production in 25% molasses and 75% vinasse ranged from 4.0 to 7.0 g/L. *Corynebacterium glutamicum, R. mucilaginosa* and yeast LLV98 reached higher biomass production than in the previous experiment. *Candida lipolytica* showed similar biomass production than in molasses and vinasse 50%/ 50%; for *S. cerevisiae* the biomass production decreased 22.3 %.

Highest protein production was obtained by *R. mucilaginosa*, 2.54 g/L (Figure 4) but the highest productivity was reached by *S. cerevisiae*, 0.22 g/ L.h^{-1}. This protein productivity was the same as the one found in the experiments of Rueger and Tauk-Tornizielo (1996), 0.202 g/L^{1}, in mixed culture of *Aspergillus niger* and *Penicillium fellutanum* in vinasse plus molasses at 50 g/L. Crude protein content was 50% of the *S. cerevisiae* dry weight, which was similar to values described by *Kefir* microflora (54%) in cheese whey (PARASKEVOPOULOU et al., 2003). *Saccharomyces cerevisiae* is classified among the most interesting microorganisms for their protein content, which can account 50% of the dry weight (ZIINO et al., 1999). *Rhodotorula mucilaginosa* achieved 41.96% of biomass protein percentage, while *C. lipolytica* and yeast LLV98 was about 33.5% and *C. glutamicum,* 25.18%.

Substrate consumption was higher than previous assays, except to *S. cerevisiae* that shown slight decrease (from 98% to 90%) (Table 2 and Figure 5).

Table 2.
Biomass yield (g/L), total lipid yield (%) and substrate consumption (%) in molasses 25% and vinasse 75% after 48 hours.

Microorganisms	Biomass (g/L)	Substrate consumption(%)	Total lipids (%)
Candida lipolytica	7.00	93.50	8.92
Yeast LLV98	6.88	94.93	10.64
Rhodotorula mucilaginosa	7.56	73.93	10.32
Saccharomyces cerevisiae	5.82	90.48	8.83
Corynebacterium glutamicum	4.20	18.95	14.40

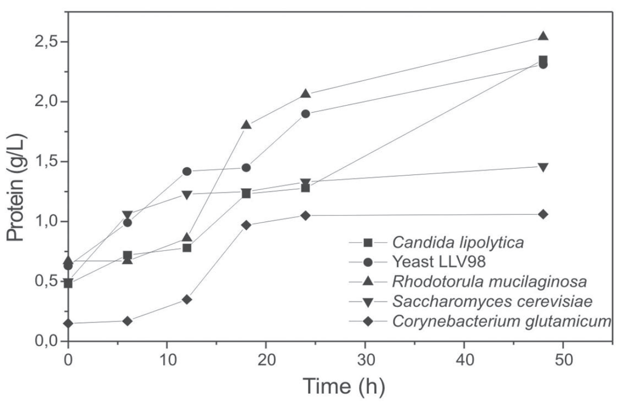

Figure 4. Protein productin (g/L) in 25% molasses and 75% vinasse after 48 hours.

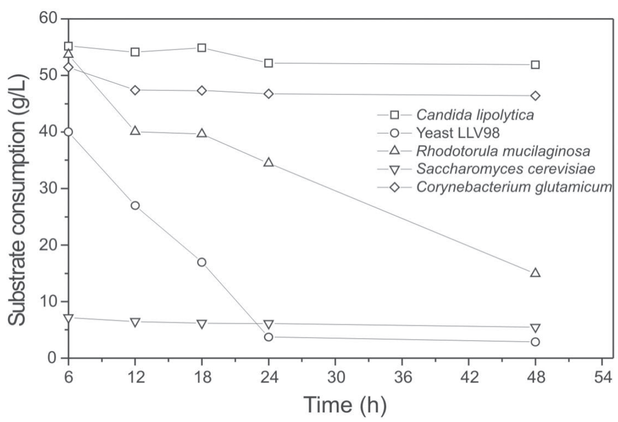

Figure 5. Substrate consumption (g/L) in 25% molasses and 75% vinasse after 48 hours.

The total lipid production by *C. glutamicum* was 14,4%; both *R. mucilaginosa* and yeast LLV98 produced about 10%, while *C. lipolytica* and *S. cerevisiae* achieved little more than 8%. This lipid content was lower than that obtained in molasses plus vinasse at 50%-50%, except to yeast LLV98 that obtained slight higher values. It seems that this vinasse concentration caused a decrease in the lipid synthesis, so that its availability for biomass production increased. The decrease in lipid synthesis did not occur for yeast LLV98 because it was already adapted to the vinasse.

The highest lipids yields, 0.78g/L and 0.73 g/L, in such culture condition, were obtained by *R. mucilaginosa* and yeast LLV98, respectively. *Candida lipolytica* and *C. glutamicum* produced 0.50 g/L while *S. cerevisiae*, 0.34 g/L (Fig. 6).

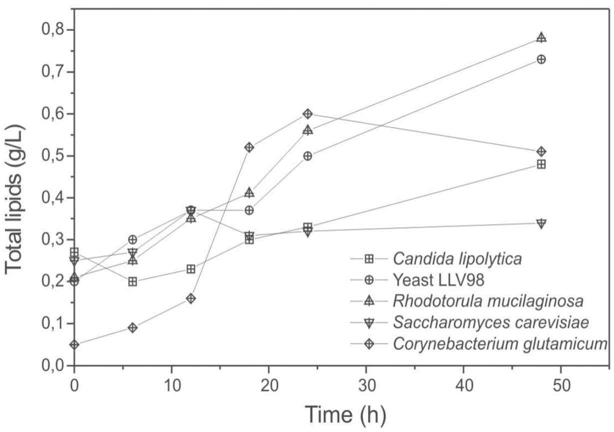

Figure 6. Total lipid production (g/L) in 25% molasses and 75% vinasse after 48 hours.

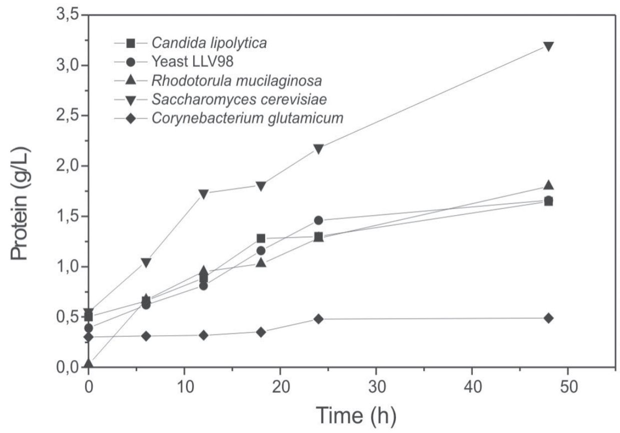

Figure 7. Protein production (g/L) in 75% molasses and 25% vinasse after 48 hours.

and yeast LLV98 produced about 1.65 g/L and *C. glutamicum* only 0.50 g/L.

The sugar consumption was high only to *S. cerevisiae* and yeast LLV98 (more than 80%); on the other hand, it was low to *C. lipolytica* (about 30%), reaching approximately 10% to both *R. mucilaginosa* and *C. glutamicum* (Figure 8).

Total lipids contents ranged from 10.69 to 19.62%. These values were slightly lower than those obtained in 50% molasses and 50% vinasse. On the other hand, these values were higher than those obtained in experiments using 25% molasses and 75% vinasse by *C. lipolytica*, *R. mucilaginosa*, *S. cerevisiae* and *C. glutamicum.* The highest total lipid contents, 0.86 g/L and 0.76

Table 3 - Biomass yield (g/L), total lipid yield (%) and substrate consumption (%) in molasses 75% and vinasse 25% after 48 hours.

Microorganisms	Biomass (g/L)	Substrate consumption(%)	Total lipids (%)
Candida lipolytica	4.14	30.53	12.48
Yeast LLV98	4.96	81.40	10.86
Rhodotorula mucilaginosa	5.34	10.16	14.25
Saccharomyces cerevisiae	7.20	95.09	12.48
Corynebacterium glutamicum	2.78	11.00	19.68

When comparing the protein productions, one can observe that *S. cerevisiae* showed the best results in this matter. On the other hand, *C. glutamicum* showed the lowest protein production in all culture media. In general, the yeast LLV98 did not reach satisfactory results, except at 25% molasses and 75% vinasse where this microorganism showed a protein production only lower than *S. cerevisiae.*

Regarding the lipid production percentages, the culture medium using 50% molasses and 50% vinasse was the best condition for all microorganisms, except yeast LLV98 that was not influenced by culture mediums showing

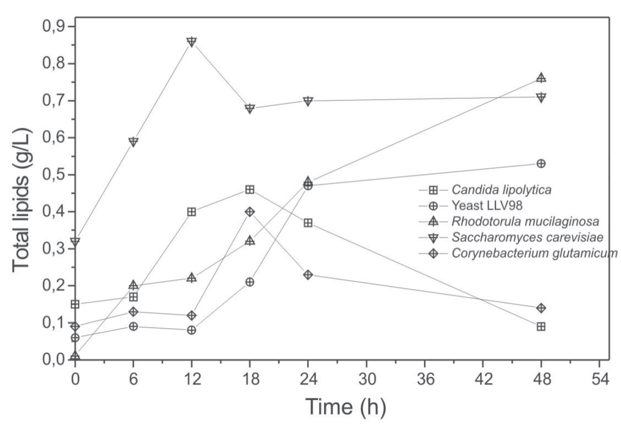

Figure 8. Substrate consumption (g/L) in 75% molasses and 25% vinasse after 48 hours.

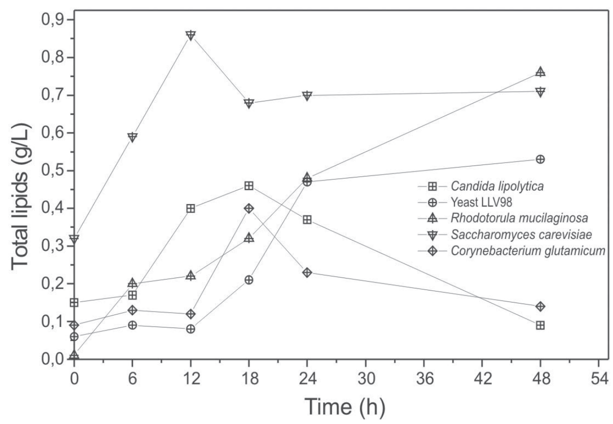

Figure 9. Total lipid productions (g/L) in 75% molasses and 25% vinasse after 48 hours

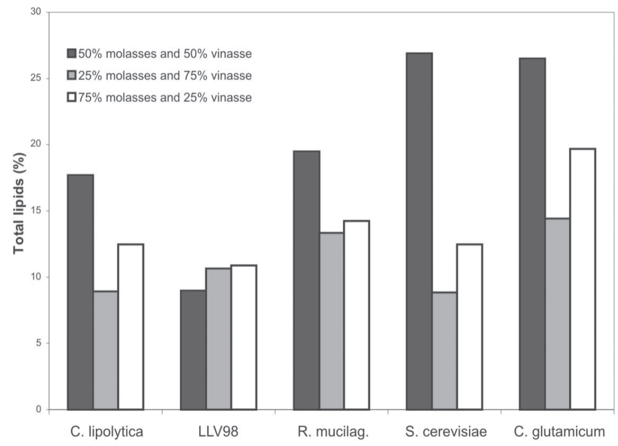

Figure 10. Total lipid productions (%) in the different molasses and vinasse ratio.

similar productions in all molasses and vinasse ratios (Figure 10). The 25% molasses and 75% vinasse was the worst culture medium for lipid productions**.** According to Ziino et al. (1999), the microbial composition is strongly affected by the cultivations conditions, medium composition being the most important. Generally, the poorer the carbon source the faster the growth rate and the lower the biomass lipid content, because the metabolic activities in such conditions are oriented preferably towards protein rather than lipid synthesis.

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