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Microbial lipid produced by *Yarrowia lipolytica* QU21 using industrial waste: A potential feedstock for biodiesel production

Jandora Severo Poli [a,b,*], Mirra Angelina Neres da Silva [c], Ezequias P. Siqueira [d], Vânya M.D. Pasa [c], Carlos Augusto Rosa [b], Patricia Valente [a]

[a] Department of Microbiology, Immunology and Parasitology, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500 sala 154, CEP 90050-170 Porto Alegre, RS, Brazil
[b] Departamento de Microbiologia, ICB, C.P. 486, Universidade Federal de Minas Gerais, CEP 31270-901 Belo Horizonte, MG, Brazil
[c] Fuel Laboratory, Chemistry Department, Universidade Federal de Minas Gerais, Av. Antônio Carlos 6627, CEP 31270-901 Belo Horizonte, MG, Brazil
[d] Centro de Pesquisas René Rachou, Av. Augusto de Lima 1715, CEP 30190-002 Belo Horizonte, MG, Brazil

HIGHLIGHTS

- The first study using brewery waste for lipid production by *Yarrowia lipolytica*.
- Combined industrial wastes may be used for lipid production by *Y. lipolytica* QU21.
- Fresh yeast extract combined with crude glycerol promoted linolenic acid production.

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ABSTRACT

This study aimed to evaluate the effect of medium composition and culture conditions on lipid content, fatty acid profile and biomass production by the yeast *Yarrowia lipolytica* QU21. Lipid production by the yeast growing on glycerol/$(NH_4)_2SO_4$ (10%/0.1%) reached 1.48 g/L (30.1% according to total cell dry weight). When glycerol was replaced by crude glycerol (industrial waste), the lipid yield was 1.27 g/L, with no significant difference. Some particular fatty acids were found when crude glycerol was combined with fresh yeast extract (FYE, brewery waste), as linolenic acid (C18:3n3), eicosadienoic acid (C20:2), eicosatrienoic acid (C20:3n3) and eicosapentaenoic acid (C20:5n3). In addition, the FYE promoted an increase of more than 300% on polyunsaturated fatty acid content (PUFA), which is an undesirable feature for biodiesel production. The fatty acid composition of the oil produced by *Y. lipolytica* QU21 growing on crude glycerol/$(NH_4)_2SO_4$ presented a potential use as biodiesel feedstock, with low PUFA content.

1. Introduction

Concerns about the climate change have attracted attention of many researchers, and boosted recent investigations on biofuels as a renewable, environmentally friendly alternative to fossil fuels. In 2011, the United States jumped from the 4th to 1st place with 63 thousand barrels of biodiesel per day (TBBD) while Germany and Brazil produced 52 and 46 TBBD, respectively. From 2010 to 2011, Argentina increased its total production from 36 to 47 TBBD and has remained on 3rd place in world's largest producer rank (Index Mundi, 2013). The most common biofuel in Europe is the biodiesel. The blend of biodiesel to diesel changes according to each country. Currently, the blend commercializedin Brazil is called B5, i.e., 5% of biodiesel blended to diesel, and the country has the capacity to produce B10 without need of new biorefineries. Nevertheless, the projected increase of the blend to 20% (B20), planned for 2020, requires a total production of 14.3 million litters, which means an extra 9.2 million litters to the current production capacity (FGV, 2010).

The primary feedstock for biodiesel production is vegetable oil as rapeseed and canola oil in Europe and soybean oil in Brazil and North America. With the increasing incentives for biofuels production in the last years, there is a crescent demand for land areas destined to biofuel production, which results in competition with existing food plantations and a consequent increase of food prices.

The crude glycerol is the main by product of the biodiesel production chain. The volume of crude glycerol has increased considerably with the recent growth of biodiesel production. For each ton of biodiesel produced it is estimated 100 Kg of crude glycerol is

* Corresponding author at: Department of Microbiology, Immunology and Parasitology, Universidade Federal do Rio Grande do Sul, Rua Sarmento Leite 500 sala 154, CEP 90050-170 Porto Alegre, RS, Brazil. Tel.: +55 5133084196.
E-mail addresses: 00162027@ufrgs.br, jandora.poli@gmail.com (J.S. Poli).

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generated (Bowker et al., 2008). Thereby, questions regarding the utilization of such byproduct arise, due to the concerns with their inadequate disposal into the environment.

Fermentation employing oleaginous microorganisms (Papanikolaou, 2011) (2nd generation (2G) biodiesel production) is a very promising alternative to overcome the critical bottlenecks of 1st generation (1G) biodiesel production. Yeasts are a promising source of microbial oil (Papanikolaou and Aggelis, 2011), since some strains can accumulate up to 70% of their dry weight in lipids (Angerbauer et al., 2008). Moreover, the production of microbial oil is of particular interest due to the capacity of various microorganisms to synthesize lipids of medical and dietetical interest, like the polyunsaturated γ-linolenic fatty acid, among others (Papanikolaou and Aggelis, 2011). Examples of oleaginous yeasts (and their carbon sources for lipid production) include the species: *Yarrowia lipolytica* (animal fats and industrial lipids/glycerol), *Rhodotorula glutinis* (glucose), *Rhodosporidium toruloides* (glucose and xylose), *Lipomyces starkeyi* (xylose, ethanol and L-arabinose) and *Cryptococcus curvatus* (culture media containing oils) (Ageitos et al., 2011). *Y. lipolytica* has the advantage to be considered as GRAS (generally regarded as safe) (Groenewald et al., 2013), and some strains are capable of accumulating lipids using crude glycerol (Juszczyk et al., 2013; Cheirsilp and Louhasakul, 2013; Rywinska et al., 2013), as well as sugarcane bagasse hydrolysate (Tsigie et al., 2011) and rice bran hydrolysate (Tsigie et al., 2012) as carbon sources. For these reasons, it is considered a robust and promising microorganism to work with.

Among the factors that are known to promote lipid accumulation in the oleaginous microorganisms, the high carbon/nitrogen (C/N) ratio is considered the most important. In most of the performed studies, the nitrogen limitation is the easiest condition to control the carbon/nitrogen (C/N) ratio in order to induce the lipid accumulation in microorganisms (Beopoulos et al., 2009; Papanikolaou and Aggelis, 2011). Moreover, the modification of culture conditions affects the lipid composition (Chen et al., 2013; Sitepu et al., 2013), which may determine the oil application, e.g., biodiesel production or nutritional use. The aim of the present work is to evaluate the effect of medium composition and culture conditions on lipid content, fatty acid profile and biomass production by the yeast *Y. lipolytica* QU21.

2. Methods

2.1. Yeast strain and culture conditions

The yeast strain *Y. lipolytica* QU21 (Poli et al., 2013) was maintained at 4 °C. This strain was pre-grown on YEPD agar (%, yeast extract 1, peptone 2, glucose 2, agar 2) at 28 °C for 24 h. The cells (an equivalent of 0.03 g dry biomass) were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of liquid media comprised of (%): carbon source (CS) 10, nitrogen source (NS) 0.1 and incubated in a rotating shaker at 150 rpm, 28 °C for 4 days, unless otherwise stated. All liquid media were enriched with KH_2PO_4 0.1% and $MgCl_2{\cdot}6H_2O$ 0.05%. Liquid cultures were centrifuged at 3000×*g* for 10 min to remove the supernatant, the cell pellets were washed twice with 0.15 M potassium chloride (KCl) in 0.01 M phosphate buffered saline (PBSKCl, pH 7.0), and used for dry biomass determination (Section 2.6) and lipid extraction (Section 2.7).

2.2. Effect of carbon and nitrogen sources on yeast growth and lipid production

Pure glucose and glycerol (grade reagents) were chosen to evaluate the effect of carbon source (CS) on growth, lipid accumulation and lipid profile of *Y. lipolytica* QU21. The fermentation was carried out using each sugar as the single CS, and $(NH_4)_2SO_4$ (0.1%) as the nitrogen source (NS). The culture conditions were as described in Section 2.1.

The influence of organic NS (yeast extract, tryptone and urea) and inorganic NS [$(NH_4)_2SO_4$ and NH_4NO_3] were also evaluated using the best CS from the previous experiment at a concentration of 10%. The fermentation was performed with each component as a sole NS in a concentration of 0.1%. The culture conditions were as described in Section 2.1.

2.3. Evaluation of culture conditions: aeration, agitation speed and their effect on yeast growth and lipid production

With the culture media obtained in Section 2.2, experiments were conducted to evaluate the culture conditions on the fermentation process. The effect of aeration on biomass, lipid production and fatty acid composition was evaluated by modifying the volume of liquid media (100 and 75 mL), while keeping the size of the Erlenmeyer flask (250 mL) the same (the ratio between the volume of the Erlenmeyer flask and the volume of culture medium were 2.5 and 3.33, respectively) (Silva et al., 2010). The aeration was not measured. The influence of agitation speed (150 and 200 rpm) was also investigated.

2.4. Evaluation of C/N ratio on yeast growth and lipid production

With the best results obtained in Sections 2.2 and 2.3, the effect of C/N ratio was evaluated on the yeast growth, lipid production and fatty acid profile. The five treatments were designed by varying the glycerol or $(NH_4)_2SO_4$ contents according to the specifications listed on Table 1. For the calculation of C/N ratio, carbon content in glycerol of 39.1% and nitrogen content in $(NH_4)_2SO_4$ of 21.2% were assumed. The culture conditions were as described in Section 2.1, except for the liquid media volume that was 75 mL.

2.5. Industrial wastes as substrates for growth

Two residual industrial wastes were investigated as substrates for lipid production. The crude glycerol (CrGly) (glycerol content 82.73% w/w) obtained from a biodiesel industry as the sole carbon source was used in a concentration of 10%. Impurities of crude glycerol were sulphated ash (6.3%), NaCl (5.15%), chloride (3.13%), sodium (2.02%), methanol (0.008%), pH 6.0. The nitrogen sources used were 0.1% $(NH_4)_2SO_4$ or fresh yeast extract (FYE) (obtained from a brewery) at 50% (FYE_{50}) or 100% (FYE_{100}). The FYE was prepared as described by Silva and Almeida (2006). All liquid media were enriched with 0.1% KH_2PO_4 and 0.05% $MgCl_2{\cdot}6H_2O$. The culture conditions were as described in Section 2.1, except for the liquid media volume that was 75 mL.

2.6. Determination of dry biomass and glycerol

Cell pellets obtained from 30 to 35 mL of submerged cultures (described in Section 2.1) were kept at 65 °C to constant weight. Glycerol was determined in filtered aliquots of the culture medium

Table 1
Content of glycerol and $(NH_4)_2SO_4$ on liquid media and the final C/N ratio for each assay.

Treatment	Glycerol (%)	$(NH_4)_2SO_4$ (%)	C/N ratio
C/N 18	10	1	18.45
C/N 36	10	0.5	36.89
C/N 184	10	0.1	184.5
C/N 276	15	0.1	276.7
C/N 368	20	0.1	368.9