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ORIGINAL ARTICLE

Nutrient biomass production from agro-industrial residues using *Yarrowia lipolytica*: screening and optimization of growing conditions

Produção de biomassa nutritiva a partir de resíduos agroindustriais utilizando Yarrowia lipolytica*: triagem e otimização das condições de crescimento*

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Abstract

The possibility of using agro-industrial residues in bioconversion processes advances with the development of biotechnology and the search for processes in which is possible to add commercial value to previously discarded products. These processes should be low cost, easy to control and chemical-free. The yeast *Yarrowia lipolytica* (YL) is widely used to produce lipids, enzymes, citric acid, and proteins, among others. This study aimed to evaluate the capacity of this yeast to use agro-industrial residues as a source of carbon without adding extra carbohydrate sources for the development of cells. The study evaluated the production of proteins and lipids from different carbon sources as well as the optimization of the process (agitation, temperature, and nitrogen source). Indeed, YL produced 22.3% of protein and 9.4% of lipids in dry biomass, a 179% of protein and 660% of lipid increase from raw material, respectively, when using cassava residues as a carbon source. However, lipase production was low, indicating that the strain had priority for cell growth.

Keywords: Yeasts; Biotransformation; Cassava peels; Heterologous protein; Biotechnology; Reuse.

Resumo

A possibilidade de utilização de resíduos agroindustriais em processos de bioconversão tem avançado com o desenvolvimento da biotecnologia e a busca por processos nos quais seja possível agregar valor comercial a produtos anteriormente descartados. Esses processos devem ser de baixo custo, fáceis de controlar e livres de produtos químicos. A levedura *Yarrowia lipolytica* (YL) é amplamente utilizada para a produção de lipídios, enzimas, ácido cítrico, proteínas, entre outros. O objetivo deste estudo foi avaliar a capacidade desta levedura em utilizar resíduos agroindustriais como fonte de

carbono sem adição de fontes extras de carboidratos para o desenvolvimento das células. O estudo avaliou a produção de proteínas e lipídios a partir de diferentes fontes de carbono, bem como a otimização do processo (agitação, temperatura e fonte de nitrogênio). A YL produziu 22,3% de proteína e 9,4% de lipídios a partir da biomassa seca, um aumento de 179% de proteína e 660% de lipídio da matéria-prima, respectivamente, ao utilizar resíduos de mandioca como fonte de carbono. No entanto, a produção de lipase foi baixa, indicando que a cepa tem prioridade para o crescimento celular.

Palavras-chave: Leveduras; Biotransformação; Cascas de mandioca; Proteína heteróloga; Biotecnologia; Reúso.

Highlights

- The aim of this study was to evaluate the capacity of *Yarrowia lipolytica* to use agroindustrial residues as a source of carbon without adding carbohydrate sources for the development of cells for protein and lipids production.

1 Introduction

Along with industrial development comes the search for green processes. There is a need for improving existing technologies as well as creating low cost and eco-friendly alternatives to produce mixtures of industrial interest. Biotechnology has proven to be an alternative and a promising route to produce value-added products through bioconversion processes, which increase the bioavailability of compounds with the use of microorganisms recognized as safe, such as *Yarrowia lipolytica* (YL).

Indeed, *Y. lipolytica* is classified as Generally Recognized as Safe (GRAS) by the Food and Drug Administration (FDA). It is strictly aerobic and considered one of the unconventional yeasts with greater potential in biotechnological applications. This yeast is assumed to be safe for food in human diet by European Food and Safety Authority (EFSA) which also defined YL to be safe as a food supplement for humans (Lopes et al., 2018; Turck et al., 2019). It is able to assimilate hydrocarbons and petroleum compounds (Csutak et al., 2015); glucose, mannitol, acetate, pyruvate, citrate, lactate (Sutherland et al., 2014); and several hydrophobic substrates (Soong et al., 2019). It can be found and isolated in several media, sites contaminated with oil, plants (Ledesma-Amaro et al., 2016); varieties of brie, feta and cheddar cheese and poultry products (Zinjarde et al., 2014); soil and seawater (Sutherland et al., 2014), among others.

YL is one of the most non conventional yeasts used in the industry, it has the capacity to secrete several metabolites of commercial interest (Gottardi et al., 2021) and produces extracellular, intracellular and yeast cell-bound lipase (Yadav et al., 2011). Due to its potential to synthesize lipases, proteases, organic acids (alpha-ketoglutaric acid and citric acid), cytochrome c, among others (Gottardi et al., 2021), it can be isolated from substrates with high lipid content such as dairy products, meat, industrial and sanitary effluents, and brackish environments (Liu et al., 2015; Zhu, Jackson, 2015; Zinjarde et al., 2014).

Recently, agro-industries processing capacity has increased and so has the amount of waste created in addition to the operational costs. As a result, biotransformation has advantages such as low cost, ease in applying microorganisms, improved digestibility, and being chemical-free. Several studies have used *Y. lipolytica* to produce biomass, and a few works have added glucose to the mineral medium and thus achieved significant results regarding cell reproduction and consequent bioconversion. Therefore, agro-industrial residues are important as substrates because they supply the carbon and nitrogen required for biofermentation and biomass production (Tanruean et al., 2021).

This work aimed to verify the ability of *Y. lipolytica* to assimilate carbon sources from complex samples using agro-industrial residues as the source of glucose and its secondary metabolites production such as hydrolytic enzymes.

2 Material and methods

2.1 Equipment and reagents

Only high purity reagents and solvents without any prior purification were employed. The *Y. lipolytica* strains QU29, QU31, QU36 and QU69 were kindly provided by Professor Patrícia Valente (Department of Microbiology, Immunology and Parasitology – *Universidade Federal do Rio Grande do Sul* (UFRGS) - Brasil in the state of Rio Grande do Sul (RS) which were isolated from colony cheese in the South region of Brazil. The strain is registered at the Federal University of Minas Gerais (*Universidade Federal de Minas Gerais* (UFMG)) culture collection database as UFMG-CM-Y336. The analyzes were performed in triplicate and the results were associated with the arithmetic mean from the values obtained, at 95% Confidence Interval (CI).

2.2 Substrates

The agro-industrial residues were collected in rural properties near the Federal University of Fronteira Sul (*Universidade Federal da Fronteira Sul* (UFFS)), Laranjeiras do Sul – in the state of Paraná (PR). The residues were dried at 50 °C ± 2 for 24 hours, ground in a hammer mill (Fortinox STAR FT53 - Brazil), sieved through 6-8 mesh sieves, and stored in capped glass bottles at room temperature. The substrates used were cotton seeds, pumpkin peel, banana peel, potato peel, sweet potato peel, orange peel, cassava peel, passion fruit peel, banana stem, oat seeds, corn seeds, and wheat peel and seeds.

2.3 Screening for hydrolytic enzyme expression

2.3.1 Lipases

The different strains of *Y. lipolytica* were inoculated on Tributyrin Agar plates to evaluate the enzyme production capacity. Strains that produced extracellular lipase presented a halo around the colonies (Lawrence et al., 1967).

2.3.2 Proteases

The presence of extracellular proteases was evaluated on Glicose, Yeast extract and Peptone (GYP) Agar supplemented with 2% of casein, incubated at 30 ºC for 5 days. The proteolytic activity was observed by the formation of a halo around the colony (Strauss et al., 2001).

2.3.3 Amylase

Amylolytic activity was determined on GYP Agar supplemented with 2% of starch. The plates were incubated at 30 ºC for five (5) days and after this period the activity was revealed with iodine tincture. The formation of a halo around the colony indicated the presence of amylolytic activity (Strauss et al., 2001).

2.4 Inoculum preparation

The *Y. lipolytica* strains were streaked on GYP Agar as described by Csutak et al. (2015) and incubated at 28 °C ± 2 for 48 hours (Ethik Technology, 4410-5 - Brazil). Then, some colonies were transferred to 10 mL of saline solution in order to achieve an optical density of 0.104 at 500 nm, *i.e.*, 2×10^6 CFU/mL for *Y. lipolytica* cells.

2.5 Fermentation for lipases production

The fermentation was conducted in 125 mL flasks, with 50 mL of medium proposed by Santos Cordeiro et al. with modifications. The medium contained 5% of cassava peels and a mineral supplementation of $NaNO_3$ (1%) (Dynamics), KH_2PO_4 (0.1%) (Impex), $MgSO_4.7H_2O$ (0.05%) (Alphatec), and final pH 7.0. Additionally, 1% of olive oil was used as an inducer of lipase production plus 1 mL of inoculum.

2.6 Obtaining the crude enzymatic extract

After the yeasts had grown in the synthetic medium, the crude extract was obtained by centrifugation at 9500 rpm, at 4 ºC for 10 min. The crude enzymatic extract was stored at 4 ºC until the use in hydrolysis reactions.

2.7 Lipolytic activity quantification

In order to quantify the lipase activity of the crude extract, a reaction medium was prepared containing 10% of olive oil, 5% of gum arabic in a phosphate buffer solution, and pH 7.0. For the reaction, 2 mL of crude extract from the fermentation was added to 20 mL of the reaction medium. The reaction medium was kept at 30 ºC, 150 rpm in an orbital shaker for 35 min. At the end of this period, the reaction was interrupted, adding 10 mL of acetone:ethanol solution, in a 1:1 ratio. The released fatty acid was titrated using a 0.05 mol L^{-1} NaOH solution, considering the definition of lipase activity as 1 µmol of released fatty acid per minute, under the reaction conditions.

2.8 Fermentation for biomass production

Mineral medium as described by Santos et al. (2012) enriched with 1% of sodium nitrate ($NaNO_3$), 0.1% of potassium phosphate monobasic (KH_2PO_4) and 0.05% of magnesium sulfate heptahydrate ($MgSO_4.7H_2O$) was used. Flasks containing 50 mL of modified mineral medium and 5% of carbon source were autoclaved and then 1mL of the previous inoculum was added. Samples were maintained in a water bath for nine days at 30 ºC ± 2, and 100rpm (Dubnoff shaker, Nova Instruments NI 1232 - Brazil).

2.9 Analytical methods

After bioconversion, the biomass was frozen and then freeze-dried (Liotop L101- Liobrás - Brazil). Analyses of protein, total lipids, and fatty acids were performed by gas chromatography in the raw substrate and in the lyophilized biomass.

2.10 Determination of total proteins (Kjeldahl)

The Kjeldahl method was used as described by AOAC (Association of Official Analytical Chemists, 1990) with modifications. This analysis quantifies the nitrogen present in the sample which is then converted into protein through a correction factor, in this case, 6.25 which corresponds to food in general.

2.11 Determination of lipids (Bligh-Dyer)

The analysis was performed as described by Bligh & Dyer (1959) with modifications. Chloroform and methyl alcohol were added to a 2g sample and stirred for thirty-five minutes in a magnetic stirrer. Then, 1.5% of sodium sulfate solution was added for the separation of phases. In another tube, 15mL from the sediment was mixed with 1g of anhydrous sodium sulfate in order to remove any traces of water.

Finally, 5 mL from the last solution was transferred to a pre-weighed beaker, and the solvent present in this sample evaporated at room temperature. From the mass of the residue and the intake mass, the lipid content was calculated.

2.12 Analysis by Gas Chromatography coupled to Mass Spectrometry (GC-MS)

Following the methodology described by Aued-Pimentel et al. (2005), the lipids extracted in the Bligh-Dyer analysis were converted to their Fatty Acid Methyl Esters (FAMEs). Briefly, N-hexane and KOH solution in methanol were added to the lipid extract, then stirred into a saturated sodium chloride solution to separate the phases. The organic phase was collected and then diluted 12.5 times in n-hexane and then it was injected into the chromatograph.

GC-MS analyses of FAMEs were performed on a Shimadzu GCMS-QP2010 Ultra coupled to a GC-2010Plus mass spectrometer (Japan). A fused silica capillary column NST 5 ms (30 m length × 0.25 mm inner diameter, 0.25 µm film thickness) was used to separate FAMEs.

The GC parameters were set to an initial temperature of 110 °C, held for 2 min, ramped to 200 °C at a rate of 10°C/min and held for 10 min. Then, it was ramped to a final temperature of 230 °C at a rate of 5 °C/min and held for 20 min. The value of the detector temperature was fixed at 200 °C and the temperature of the injector was 250 °C. Helium was selected as the carrier gas at a flow rate of 40 cm s^{-1}. The MS was operated in the scan mode, and ions were scanned over an m/z range of 35 to 500. Mass spectrometric detection was performed using Electron Ionization (EI) carried out at 70 eV.

FAMEs were quantified in relation to the peak-area and identified through a mass spectra database (NIST08, NIST08s and NIST11 Library).

3 Results and discussion

3.1 Screening for hidrolases

In order to determine the most suitable strain to optimize lipase production, all the strains were tested: QU29; QU31; QU36; and QU69. All strains tested showed potential for lipase production, given the presence of a halo around the colonies (Figure 1).

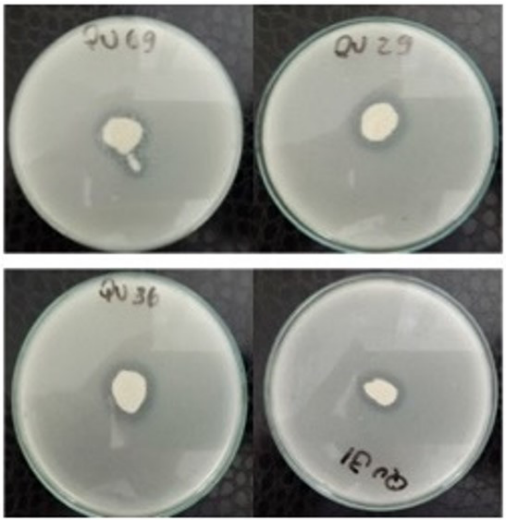

Figure 1. Evaluation of the presence of lipase produced by *Y. lipolytica* strains.

The initial screening revealed that all the strains had lipolytic activity, so a quantitative analyzes were performed to identify the most promising strain.

The *Y. lipolytica*'s potential for producing amylase, that is, an enzyme that degrades starch into simpler sugars, was tested (Gęsicka et al., 2020). None of the strains tested showed any amylolytic potential, as there was no halo around the colonies.

The screening for protease was performed as it was already identified in *Y. lipolytica* strains (Suzzi et al., 2001). However, all the strains were negative for protease production.

It is possible that the lack of protease activity is due to the five day-period applied for the test, seeing that positive results for hydrolytic activities were possible after longer periods, seven or eight days of fermentation (Suzzi et al., 2001) and (Carrazco-Palafox et al., 2018). The proteolytic activity was expected, considering that the *Y. lipolytica* strains tested were isolated from cheese, where protease plays an important role in improving aromas.

As all strains showed potential for lipase production, submerged fermentation and lipolytic activity dosage were performed. Submerged fermentation was executed using crushed cassava husk and urea as a carbon and nitrogen source, respectively. The four strains of *Y. lipolytica* tested in the screening were used in the fermentation, in triplicate, and the crude enzymatic stratum was used to determine the activity of each strain (Table 1). Putti Paludo et al. (2018), using different culture media, obtained strains of *Y. lipolytica* with lipolytic activity ranging between 0.87 and 2.23 U mL^{-1}.

Table 1. Lipolytic activity dosage for diferente *Y. lipolytic* strains.

	Y.lipolytica			
	QU29	**QU31**	**QU36**	**QU69**
Lipase activity (U mL^{-1} ± sd)[a]	1.18 ± 0.48	1.51 ± 0.15	1.10 ± 0.25	0.52 ± 0.15

[a]Data expressed as mean ± confidence interval (CI) at 95%.

Despite the very promising results above, the QU69 strain was chosen for presenting more consistent results regarding growth (production of lipids) and reproduction (production of heterologous proteins).

3.2 Production of proteins

Some carbon sources such as cotton seeds, potato peels, orange peels, passion fruit peels, and wheat chaff and seeds were not able to produce proteins, as it revealed a negative variation, which means the initial protein amount was consumed during the process (Table 2). These results showed the difficulty of diffusion of the mineral medium through the carbon source and the yeast cells, even under agitation. Therefore, these substrates were not suitable for bioconversion by *Y. lipolytica* QU69 under the conditions tested in this experiment.

Pumpkin peel, banana peel, banana stem and oat seeds residues were considered suitable for bioconversion processes since they reached values ranging from 5.3% to 16.1%. Therefore, only substrates displaying protein increases above 100% were evaluated. The low protein yield might be due to the complex matrix of the carbon source and the ineptness of the yeast to process that. Another possibility might be related to the conditions of the experiment, like time and temperature, preventing the reaction. A complete analysis of the carbon composition would help elucidate this problem, as well as work on the optimization of the experimental procedures.

Table 2. Protein value in raw material and in frozen dried biomass after bioconversion depending on the substrate used using *Y. lipolytica* QU69.

Carbon sources	Protein in raw material[a] (%)	Protein in frozen dried biomass[a,b] (%)	Protein Variation (%)
Banana peels	5.4 ± 0.5	9.4 ± 1.2	74
Banana stem	4.3 ± 0.6	5.3 ± 1.2	22
Cassava peels	8.0 ± 0.8	22.3 ± 0.9	179
Corn seeds	10.2 ± 0.4	26.8 ± 5.4	163
Cotton seeds	26.4 ± 1.7	22.2 ± 2.5	-16
Oat seeds	13.6 ± 0.4	16.1 ± 1.2	18
Orange peels	6.8 ± 0.5	5.8 ± 1.8	-15
Passion fruit peels	5.8 ± 0.4	2.3 ± 0.8	-60
Potato peels	14.1 ± 1.2	10.9 ± 1.2	-23
Pumpkin peels	11.4 ± 0.5	13.0 ± 3.7	14
Sweet potato peels	3.6 ± 0.6	15.9 ± 0.8	342
Wheat chaff and seeds	18.7 ± 1.7	12.6 ± 0.8	-33

[a]Data expressed as mean± confidence interval (CI) at 95%. [b]Data expressed with the subtraction of 6.6 corresponding to the contribution of the sodium nitrate in the result.

The residue of sweet potato peel resulted in the highest protein gain (342%). It varied from 3.6% to 15.9%, after a nine-day fermentation period. Drzymała et al. (2020) claimed that raw materials with higher sugar content in their compositions had higher biomass content in fermented and higher protein content in dry biomass; the rye straw had 28.1 g of sugar per 100 g of sample, seeing that it produced 35 g L^{-1} of biomass and 30.5 g of protein per 100 g of dry biomass; and oat bran which had 66.2 g of sugar per 100 g of sample produced 9.35 g L^{-1} of biomass and 44.45 g of protein per 100 g of dry biomass. As the source of carbon used had naturally significant amounts of sugar, it contributed to the greater cellular development and consequent greater production of protein.

Cassava peels and corn seeds resulted in 179% and 163% protein gain and final protein contents were 22.3% and 26.8%, respectively. Both were considered the most promising samples because of the protein content produced. Since corn is widely used in human and animal feeding, it is not considered a residue and therefore only cassava peels were evaluated as a carbon source. In addition, unfortunately, there is currently no application able to add value to cassava peels.

Vuong et al. (2021) used cassava-based as a carbon source, *Y. lipolytica* and *Trichoderma harzianun* for solid-state fermentation and obtained a 27% and 17% increase in the crude protein, respectively. Vendruscolo et al. (2009) used apple pomace as a carbon source for semi-solid fermentation with *Gongronella butleri* CCT 4274 for seven days and it resulted in up to 15.2% of soluble proteins. Sodium nitrate was the source of nitrogen in the supplementation medium.

3.3 Production of microbial lipids

In preliminary tests, the biomass produced by *Y. lipolytica* QU69 from banana peel presented lower results when using an ultrasonic bath, stirring with glass beads or an association of both methods to disrupt the yeast cell wall (1.3% ± 0.3, 1.9% ± 0.51 and 2.0% ± 0.24, respectively) when compared to solvent extraction (5.4%

± 0.61). For this reason, the cell lysis technique was not employed to quantify lipids in biomass. The biomass produced was analyzed for lipid content and the results are shown in Table 3.

Poli et al. (2013) tested different techniques in association with the Bligh-Dyer methodology that could most efficiently disrupt *Y. lipolytica* QU21 cell wall and extract the intracellular oil. Lysis with vortex and glass beads and ultrasonic bath and glass beads were tested and the results were superior when compared to the extraction with solvents alone.

Table 3. Lipidic value in raw material and in frozen dried biomass after bioconversion depending on the substrate used.

Carbon sources	Lipid in raw material[a] (%)	Lipid in frozen dried biomass[a] (%)	Lipid Variation (%)
Cotton seeds	19.6 ± 1.5	8.8 ± 0.3	-55
Banana peels	5.6 ± 0.7	5.4 ± 0.6	-4,28
Potato peels	1.0 ± 0.2	2.0 ± 0.1	92
Sweet potato peels	2.5 ± 0.8	2.6 ± 0.6	1,57
Orange peels	4.0 ± 0.2	10.1 ± 0.9	158
Cassava peels	1.2 ± 0.4	9.4 ± 1.8	660
Passion fruit peels	1.6 ± 0.2	1.5 ± 0.0	-6
Banana stem	2.6 ± 0.5	5.5 ± 0.5	110
Oat seeds	6.0 ± 0.3	8.4 ± 0.9	39
Corn seeds	5.4 ± 1.6	4.2 ± 0.5	-22
Wheat peel and seeds	2.3 ± 0.3	2.1 ± 0.7	-8,70

[a]Data expressed as a mean ± confidence interval (CI) of 95%.

Cotton and corn seed, banana peel, sweet potato peel, passion fruit peel, and wheat seeds and chaff yielded negatively regarding lipid gain indicating not being suitable sources for bioconversion and lipid production with *Y. lipolytica* QU69 under the conditions applied in this experiment. Poli et al. (2014) and Christophe et al. (2012) reported that nitrogen supplemented media promote the production of proteins because it stimulates cell reproduction whereas in media without nitrogen, higher lipid production occurs because yeasts produce oil to protect and keep their cells viable in the poor environment.

Potato peels, orange peels, and banana stem produced lipids after bioconversion and so it is possible to infer that the yeast had difficulty adapting and consequently produced oil to protect the cells and keep them active.

Cassava peels revealed outstanding results for both protein and lipid production and therefore a great source for the bioconversion process. In addition, cassava peels demonstrated high solubility in the medium, which contributed to greater interaction between the medium, the carbon source, and the yeast cells. According to Faria et al. (2011), the ash content presented in these residues was up to 5%, and that contributed to the yeast uptake of macro and micronutrients.

3.3.1 Profile analysis of fatty acids produced

After quantifying lipids, they were esterified according to the previously described methodology and analyzed for the fatty acid profile.

FAMEs chromatographic peak areas were analyzed and identified by similarity using the NIST08, NIST08s, and NIST11 libraries as a reference. Only compounds with a similarity above 80% were considered. Table 4 shows the fatty acids corresponding to the methyl esters identified. The results were expressed as a percentage of the peak area in relation to the total amount of FAMEs detected in the sample. Fatty acids with peak areas below 10% were not described and are presented as "other fatty acids".

Among those described as "other fatty acids" were paullinic acid (C20:1 n-7), peak area ranging from 4.3 to 7.1%, palmitoleic acid (C16: 1 n-7 cis) peak area ranging from 1.5% to 6.7% and pentadecanoic acid (C15: 0t) peak area ranging from 1.1% to 9.2%. Capric acid (C10: 0t) was detected only in the residue (6.5%) and in the biomass (3.4%) from the fermentation with sweet potato peels. The heneicosanoic acid (C21: 0t) was present in the oat seeds biomass with a peak area of 1.6%.

The following long chain fatty acids were produced during the bioconversion process: palmitic acid (C16:0t) concentrations ranging from 3.6 to 74.4%, found only in the banana peels biomass at a concentration of 13.9%; elaidic acid (C18:1n9t) found in most biomass analyzed (11.1 to 47.2%); banana peels and banana stem; and linoleic acid (C18:2n9,12 cis) found in all biomasses (17.2 to 53.5%) except in banana stem.

Table 4. Fatty acid profile produced (%) from *Y. lipolytica* QU69 fermentation on different carbon sources.

Wheat chaff and seeds	Corn seeds	Oat seeds	Banana stem	Passion fruit peels	Cassava peels	Orange peels	Sweet potato peels	Potato peels	Banana peels	Cotton seeds	Origem	Carbon sources
-	-	-	-	-	-	-	12.4	-	-	-	Raw	Trans -Cinnamic Acid
-	-	-	-	-	-	-	9.2	-	-	-	Biomass	
-	-	-	-	-	-	-	28.1	-	-	-	Raw	Lauric acid C12:0 t
-	-	-	-	-	-	-	22.8	-	-	-	Biomass	
-	-	-	-	-	-	-	-	-	-	0.8	Raw	Myristic acid C14:0 t
-	-	-	-	-	-	2.7	2.7	-	-	2.0	Biomass	
24.2	14.8	40.2	74.4	61.2	44.1		15.6	-	-	3.6	Raw	Palmitic acid C16:0 t
40.0	32.4	25.4	-	26.1	26.8	55.9	18.1	53.1	42.0	31.0	Biomass	
-	-	-	-	-	-	26.0	-	36.6	21.5	23.6	Raw	Margaric Acid C17:0 t
-	-	-	-	-	-	-	-	-	-	-	Biomass	
-	-	-	-	-	-	-	-	-	-	-	Raw	Heptadecanoic acid C17:1 n-7t
-	-	-	-	-	-	-	-	-	13.8	-	Biomass	
1.0	2.8	-	7.3	-	-	4.6	2.8	8.3	10.7	-	Raw	Stearic acid C18:0 t
3.1	-	1.2	-	-	1.9	-	2.6	5.5	2.6	3.0	Biomass	
-	-	-	-	-	-	-	-	-	-	-	Raw	Elaidic acid C18:1 n-6t
-	-	-	-	-	-	-	-	-	-	-	Biomass	
-	-	-	-	-	-	-	-	-	11.3	-	Raw	Vaccenic acid C18:1 n-7t
-	-	-	-	-	23.7	-	-	-			Biomass	
19.1	37.9	7.1	-	-	47.7	-	-	-	24.6	15.9	Raw	Elaidic acid C18:1 n-9t
11.1	47.2	43.2	-	47.0	12.4	43.7	13.8	11.8	-	23.0	Biomass	
54.6	44.6	4.6	13.3	-	7.3	35.6	29.1	40.8	36.5	53.0	Raw	Linoleic acid C18:2 n-9-12c
31.0	20.5	17.2	-	24.5	19.2	30.2	24.5	21.2	19.7	36.2	Biomass	
-	-	-	-	38.7	-	-	5.5	12.5	-	-	Raw	Linoleic acid C18:3 -9,12,15c
-	-	-	-	-	-	-	-	-	-	-	Biomass	
-	-	10.5	-	-	-	3.9	6.5	1.8	3.7	0.6	Raw	Other fatty acids
1.5	-	10.8	-	2.5	13.4	2.4	3.4	8.4	-	6.1	Biomass	

Similar results regarding the fatty acid profile were reported by Mattana et al. (2014) when using 16 strains of *Y. lipolytica* for lipid production. They concluded that organic nitrogen sources were more efficient for biomass production and lipid yield and that after a three-day fermentation period there was no significant increase in the lipid content. The supplementation medium may also interfere in lipid production.

Some fatty acids were present in the carbon source and were consumed by the yeast during the bioconversion process (Table 3). (Darvishi et al., 2019) (Fabiszewska et al., 2021) reported a decrease in lipid content after a long cultivation period, suggesting that oleaginous microorganisms use accumulated lipids during cell growth after a decrease in glucose during the stationary phase.

Considering that in this work the process lasted nine days, the cells may have produced a greater quantity and variety of fatty acids, but these were consumed as a result of the long fermentation time.

Banana stem biomass resulted in a 110% of lipid increase (Table 2). Although, no fatty acids were detected when analyzing the sample (Table 3) indicating that when lipid analysis was performed lipophilic compounds with affinity for the solvent were extracted, but not fatty acids.

The oil produced by oleaginous yeasts can be used to produce biodiesel. Poli et al. (2013) claimed that the oil sample should present a large amount of saturated and/or unsaturated long chain fatty acids to be considered a suitable raw material for biodiesel. Regarding the results of *Y. lipolytica* QU69, the heterogeneity of long chain fatty acids could be considered proper for producing biodiesel. However, more analysis must be conducted concerning the medium, the temperature, and the stirring in order to improve the amount of lipid produced.

3.4 Optimization of the protein production process

Cassava peel residue as a carbon source in bioconversion with *Y. lipolytica* QU69 displayed an increase of 179% and 660% in protein and lipid content respectively (Table 2 and 3), corresponding to a final amount of 22.3% for proteins and 9.4% for lipids.

In order to improve bioconversion aiming protein production, tests with cassava peels as a carbon source were carried out. Additional tests with sodium nitrate ($NaNO_3$), ammonium sulfate ($(NH_4)_2SO_4$) and urea (CH_4N_2O), at different temperatures (20, 30 and 40 °C ± 2) as well as using and not using shaking water bath were performed. Finally, it was analyzed the time required for maximum protein production to occur. These results are presented in Figure 2.

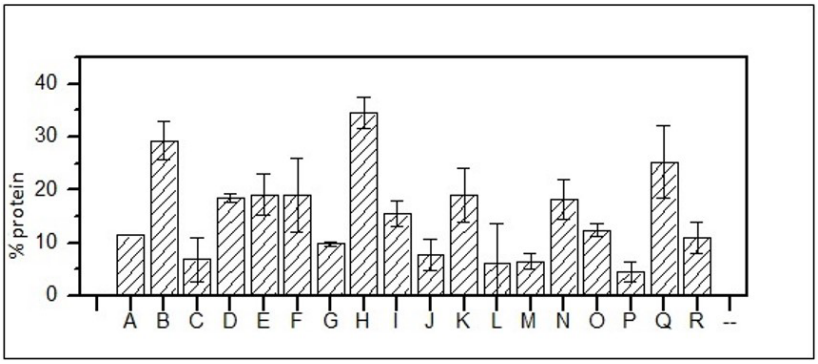

Figure 2. Protein production enhancement by *Y. lipolytica* QU69 in relation to nitrogen source, temperature and shaking water bath. Data expressed by subtracting sodium nitrate (NaNO3) (6.6), ammonium sulfate ((NH4)2SO4) (19.0) and urea (CH4N2O) (37.0) that are related to the nitrogen added to the medium. A - NaNO3, at 20°C, with shaking water bath; B - CH4N2O, at 20°C, with shaking water bath; C – (NH4)2SO4, at 20°C, with shaking water bath…; R - (NH4)2SO4, at 40°C, without shaking water bath.

Processes performed in a shaking water bath at 100 rpm resulted in a greater increase in protein content regardless of the supplementation medium and the temperature used. In a shaking water bath, the average protein content was 18.2%, while in a regular water bath, 12.3%. Indeed, *Y. lipolytica* is a strictly aerobic microorganism, therefore, it requires the presence of oxygen to adapt and grow, which could be enhanced by the shaking water bath.

Regarding the temperature during the bioconversion process, it did not influence the final protein content, considering the great variation of the results. The results seem to rely greater on the source of nitrogen and the agitation than on temperature.

Urea as a source of nitrogen for fermentation yielded higher results regardless of temperature and agitation applied, that was above 19% of protein. On the other hand, sodium nitrate and ammonium sulfate were the lowest. This is possibly due to the better interaction between the yeast and urea, a molecular nitrogen source.

Gao & Shi (2013) and Mattanna et al. (2014) mentioned that yeasts might absorb organic sources of nitrogen more easily. Hence, *Y. lipolytica* QU69 could easily use urea as a substrate. On the contrary, ammonium sulfate and sodium nitrate are inorganic sources and therefore the yeast faced difficulties in absorbing them which resulted in lower protein production.

Concerning protein, the best results were obtained when using a shaking water bath at 100 rpm, urea as a nitrogen source, at 40 ºC. There was a 331% increase in the initial protein content, reaching 34.5%, and therefore it is considered as the ideal condition for protein production among those tested.

Vendruscolo et al. (2009) obtained similar results concerning ammonium sulfate, sodium nitrate, and urea when using apple pomace as a carbon source and *G. butleri* CCT 4274 as a bioconverter. It could be found a final protein content of 19.63%, corresponding to an increase of 230% in relation to the initial content.

Then, the best time to reach the peak of protein production was evaluated. A new fermentation was conducted based on the parameters previously mentioned. Samples were collected every 24 hours for 11 days, and frozen dried to determine nitrogen and subsequent conversion to proteins. Figure 3 shows the protein production profile over the days.

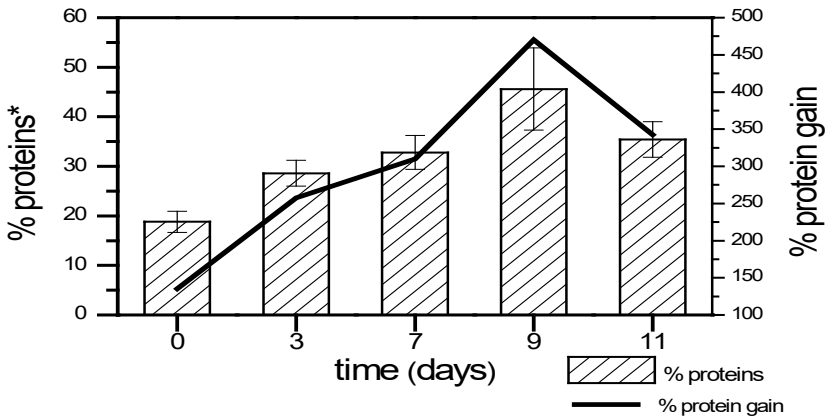

Figure 3. Protein production profile by*Y. lipolytica* QU69 after 11 days and protein gain. * Data expressed subtracting 37.0 for urea, corresponding to the contribution on nitrogen to the medium.

At the beginning of the process, the growth rate was stable between day zero and one and protein increase was around 110%, what was expected since microorganisms need some time to adapt to the medium in which they are inoculated.

From the third day of the experiment, there was an important increase in protein of 255%. Considering the bioconversion process on an industrial scale, it could be interrupted on the third day, since production reached

28.2%. Considering the costs with energy and equipment, conducting the process for a longer period would become economically unfeasible.

After seven days, the production reached 32.8%, corresponding to a protein increase of 310%.

On the ninth day, the sample presented the highest protein content (45.6%), corresponding to an increase of 470% in the initial protein content of the carbon source (8%). It was associated with the peak of protein production and after that, production remained stable around 38% until eleventh day.

Fermentations were conducted for nine days according to Santos et al. (2012) who used strains of *Rhizopus arrhizus* var. *arrhizus*. Nine days can also be considered ideal to reach the peak of protein production by *Y. lipolytica* QU69, but as mentioned above, the process could end on the third day when a significant gain in protein is achieved.

4 Conclusion

The strains tested revealed a weak lipolytic potential and an absence of protease and amylase production.

The experiments demonstrated that *Y. lipolytica* QU69 can be used in bioconversion processes, and that it showed high performance in the production of proteins with different carbon sources. The use of agro-industrial waste as a carbon source is a viable and valuable alternative other than improperly disposing of it in the environment.

The best results for protein production were obtained with cassava peels as a carbon source, and fermentation in a shaking water bath at 100 rpm, and urea, at 40 ºC. Since the biomass has a high protein value, it can be used in animal feed and therefore decrease production costs.

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