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Production of bioactive compounds by *Rhodotorula glutinis* using milk whey as substrate

Producción de compuestos bioactivos por *Rhodotorula glutinis* empleando suero de leche como sustrato

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Technological innovation: High production of lipids by *Rhodotorula glutinis* P4M422 using whey as substrate.

Industrial Application: The bioactive compounds, *viz*., lipids and carotenoids described in this paper can be used by food, cosmetics, pharmaceutical industries, and for production of renewable energy.

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Resumen

Los compuestos bioactivos son moléculas que poseen propiedades antioxidantes, antiinflamatorias, antimicrobianas y anticancerígenas. Algunos ejemplos de estos compuestos son los carotenoides, las vitaminas, los flavonoides y los ácidos grasos, los cuales pueden encontrarse de forma natural en algunos alimentos y pueden ser producidos por microorganismos. La producción biotecnológica de este tipo de compuestos tiene como ventajas, cortos tiempos de producción, el origen natural de los compuestos sintetizados y la utilización de sustratos de bajo costo. El suero de leche es un residuo que se genera de la producción del queso, el cual llega a representar un problema de contaminación, sin embargo, el lactosuero es rico en azúcares y proteínas, por lo que resulta de interés para el uso como sustrato. El objetivo de este estudio es evaluar el potencial del suero de leche como sustrato de bajo costo para la producción de carotenoides y lípidos por *Rhodotorula glutinis* P4M422. Para lo cual, se utilizó suero de leche obtenido del establecimiento "Los Reyes" (Matamoros, Coahuila), se cuantificó el contenido de grasa, proteína, ceniza y lactosa empleando el analizador de leche Milkoscope Expert Automatic® y se cuantificaron azúcares totales por el método fenol-sulfúrico. Posteriormente, se realizó un diseño factorial para evaluar el efecto del pH (6 y 7) y de la agitación (150 y 180 rpm) sobre la producción de compuestos bioactivos empleando la levadura *R. glutinis* P4M422 y suero de leche como único sustrato. Se realizó una

cinética sobre la producción de biomasa cuantificada por el método de peso seco, el consumo de azúcares por el método de DNS y la producción de lípidos por el método de Blight & Dyer y carotenoides por método espectrofotométrico a 475 nm. Los resultados mostraron la capacidad de crecimiento de *R. glutinis* P4M422 en suero de leche, con una relación 23:1 de C/N como sustrato. La levadura logró acumular 27.48% de lípidos de su peso seco (con lo cual cumple la principal característica para ser clasificada como una levadura oleaginosa) en un medio con pH 7 y, además, alcanzó una producción máxima de carotenoides (1.2176 µg/mL) a pH 6 a 150 rpm a los 13 días de cultivo. Mediante el presente estudió se lograron definir algunos parámetros sobre la producción de lípidos y carotenoides por *R. glutinis* empleando un sustrato de bajo costo, no obstante, es necesario caracterizar los compuestos obtenidos para su posterior aplicación.

Palabras clave: Carotenoides, Compuestos bioactivos, residuo agroindustrial, *Rhodotorula glutinis.*

Abstract

Bioactive compounds are extra-nutritional molecules that can provide health benefits such as, antioxidant, anti-inflammatory, antimicrobial, and anticancer activities. Some examples of these compounds are carotenoids, vitamins, flavonoids, and fatty acids, which can be found naturally in some foods and can be produced by microorganisms. The advantages of biotechnological production of this type of compounds are the short production times, the natural origin of the synthesized compounds and the use of low-cost substrates. Whey is a residue that is generated from the production of cheese, which represents a contamination problem, however, whey is rich in sugars and proteins, so it is of interest for use as a substrate. The objective of this study is to evaluate the potential of whey as a low-cost substrate to produce carotenoids and lipids by *Rhodotorula glutinis* P4M422. For which, whey obtained from the "Los Reyes" establishment (Matamoros, Coahuila) was used, the fat, protein, ash, and lactose content was quantified using the Milkoscope Expert Automatic® milk analyzer and total sugars were quantified by the phenol-sulfuric method. Subsequently, a factorial design was carried out to evaluate the effect of pH (6 and 7) and agitation (150 and 180 rpm) on the production of bioactive compounds using the yeast *R. glutinis* P4M422 and whey as the only substrate. Kinetics were performed on the biomass production quantified by the dry weight method, the sugar consumption by the DNS method and the lipid production by the Blight & Dyer method and carotenoids by spectrophotometric method at 475 nm. It was observed that cheese whey with a C/N ratio of 23:1 supported the growth of *R. glutinis*. Furthermore, the yeast registered a lipids accumulation of 27.48% (on dry weight basis) at pH 7, complying the definition of an oleaginous yeast. In the case of carotenoids, a production of 1.2176 µg/mL was recorded at pH 6 and at 150 rpm. Through this study, it was possible to define some parameters on the production of lipids and carotenoids by *R. glutinis* using a low-cost substrate, however, it is necessary to characterize the compounds obtained for their subsequent application.

Key words: Agro-industrial waste, Bioactive compounds, Carotenoids, *Rhodotorula glutinis*.

I. Introduction

Nowadays, there is a growing demand for natural foods that provide high nutritional value, but also, it is sought that these products are obtained employing environmental friendly technologies (Sheffield, 1999; Tilman *et al*., 2011). Bioactive compounds are gaining attention due to their health benefits and use of microorganisms are one of the viable alternatives to produce these compounds. Microbial production of bioactive

compounds have several advantages (it does not depend on the problems of geographic and seasonal variability and natural substrates of low cost can be used in process), over other sources like animals or plant cells (Ribeiro *et al*., 2019; Yen *et al*., 2012). *R. glutinis* is an oleaginous yeast for being able to synthesize more than 20% lipids intis dry weight, and is known for production of carotenoids (Adrio, 2017; Sharma & Ghoshal, 2020) and the antioxidant, anticancer and antimicrobial activities of carotenoids are well documented (Latha & Jeevaratnam, 2010; Schneider et al., 2013). Furthermore, lipids accumulated by *R. glutinis* can be used as a substrate for the production of third generation biodiesel, apart from its use by the food and pharmaceutical industries (Kot *et al.*, 2019). However, the accumulation of lipids and carotenoids are considered as an economic challenge, which highlights the need to evaluate low cost substrates such as agro-industrial wastes (Rakicka *et al*., 2015). Whey is a by-product of the cheese industry and represent an environmental problem when discharged without any treatment (Kanzy *et al*., 2015). Whey contains lactose, protein and vitamins and minerals, which could be sources of carbon and nitrogen for the production of lipids, carotenoids and biomass (Marova *et al*., 2012). This study aimed to evaluate the production of lipids and carotenoids by *R. glutinis* P4M422 with whey as substrate at different pH and agitation conditions.

II. Materials and equipment

All reagents used in this study are analytical grade. The milk analyzer MilkoScope Expert™ Automatic 2017 Model and spectrophotometer Genesys UV-Vis were employed.

III. Experimental methods

Microorganism

R. glutinis P4M422 was obtained from the collection of microorganisms DIA-UAdeC and stored at -20°C until used. Inoculum for the studies was prepared in YM medium with the following composition (g/L): glucose, 10; malt extract, 3; yeast extract, 3 and peptone, 5, and the flasks were incubated at 30 °C and 150 rpm.

Whey preparation and characterization

Whey was obtained from "Los Reyes" company situated in Matamoros, Coahuila and was stored at 4 °C. The content of lipids, proteins, ashes, and lactose content was quantified using the milk analyzer MilkoScope Expert™ Automatic 2017 Model. Subsequently, pH was adjusted to 6 and 7 with NaOH 10 M and sterilized by using autoclave at 121°C for 15 minutes.

Yeast cultivation in flasks

All the studies were performed in Erlenmeyer's flasks containing 50 mL of whey as culture medium, inoculated with $1x10^8$ cells/ mL. The flasks were incubated at 30˚C for 14 days under two agitation conditions, *viz*., 150 and 180 rpm.

Analytical methods

The biomass was quantified by the dry cell weight (DCW) method. For the extraction of carotenoids, the grown biomass was separated by centrifuging the cultures at 10,000 rpm for 10 minutes. The obtained biomass was dried, and the carotenoids were extracted by using DMSO: acetone mixture (2:1) at 60ºC and constant agitation. The extract was quantified by using a spectrophotometer (Genesys UV-Vis) at 475 nm. The total lipids content of the biomass was determined by the gravimetric method of Bligh and Dyer (Blight & Dyer, 1959). The concentration of total and reducing sugars was measured by phenol-sulfuric acid (Dubois *et al*., 1956) and dinitrosalicylic acid (DNS) methods (Miller, 1959), respectively.

Statistical analysis

All analyzes were performed in triplicate and Statgraphics® software was used for the statistical analysis of the data obtained.

IV. Results and discussion

Physicochemical characterization of whey

Physicochemical characteristics of whey are described in Table 1. Lactose was the principal sugar present in the whey and observed that its composition was like the previous report by Kanzy *et al*. (2015). The whey obtained from the "Los Reyes" showed a pH of 3.54, and is classified as acid whey (Zall, 1992), however, the optimal range for the growth of *R. glutinis* as well as the production of the metabolites of interest is between 5 and 7, therefore pH was adjusted before the fermentation process. The protein content was 3.01%, which was lower than the values reported in the literature, normally in the range of 10-12% (Solak & Akin, 2012). This concentration of the protein in whey used in the present study could be due to the composition of the milk used for the cheese preparation, type of cheese and processes such as several treatments before the casein coagulation employed (Jelen, 2011). The main proteins present in whey are colloidal casein, serum casein, β-lactoglobulin, and α-lactoalbumin (Kassem, 2018).

Table 1. Physicochemical characteristics of whey.

Parameter	Mean % ± standard deviation
Fat	1.17 ±0.038
Lactose	4.49 ± 0.016
Proteins	3.01 ± 0.009
Minerals	0.67 ± 0
Reducing sugars	18.82 ± 3.4 g/L
pH	3.54 ± 0.04

4.1 Biomass production

The highest biomass yield was observed in whey with an initial pH of 6.0 and at an agitation of 180 rpm, which recorded 23.6 g/L (Figure 1). The biomass yield at pH 6.0 and 7.0 and at 150 rpm was 20.06 g/L and 16.23 g/L, respectively.

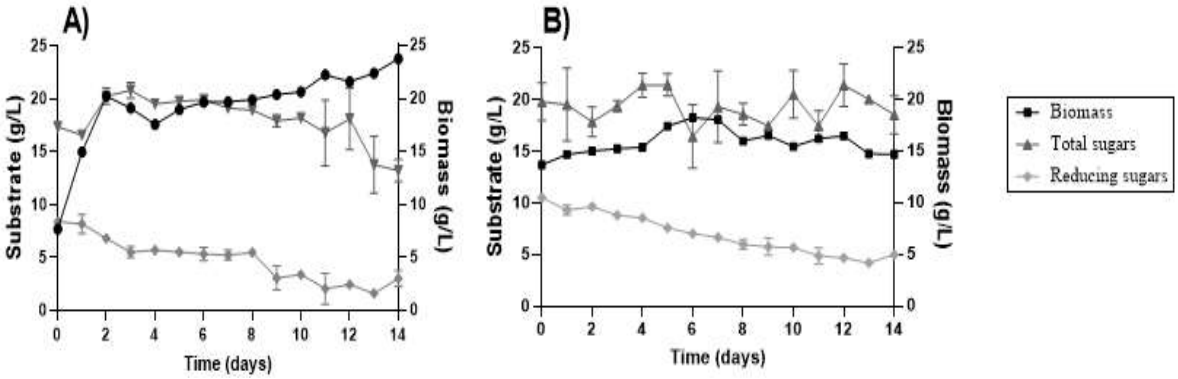


Figure 1. Kinetics of biomass production and substrate consumption by *Rhodotorula glutinis* at different pH and agitation conditions A) pH 6 and 180 rpm. B) pH 7 and 180 rpm (p=0.0001).

It can be observed that pH 6.0 and 180 rpm showed a significant effect (p=0.0001) on biomass yield on dry weight basis. Earlier (Kot *et al*., 2019) reported that pH between 5.0 and 7.0 favored the growth of this oleaginous yeast. In addition, Mantzouridou *et al*. (2002) observed that agitation created shear forces, and influenced the cell growth, its morphology and metabolite formation. Kanzy *et al*. (2015) reported the highest concentration of biomass (12.93 g/L) at 120 h using ricotta cheese whey as substrate. On the other hand, Kot *et al*. (2017) evaluated potato wastewater as a substrate for the growth of *R. glutinis*, and reported a biomass production of 18 and 17.2 g/L at

pH 6 and pH 7 respectively. In the present study, the growth gradually increased with time and recorded a maximum growth on 13th day (Figure 1). It has been reported that *Rhodotorula* species such as *R. lava* can assimilate lactose as a carbon source (Hasegawa, 1959) and the β-galactosidase activity has been reported between pH 6.5-7.0 (Santos *et al*., 1998).

4.2 Production of carotenoids

The production of biomass and carotenoids at 150 and 180 rpm agitation conditions are shown in Figure 2. The highest accumulation of carotenoids in the cells (23.25 µg/g) was obtained on 13th day at pH 6.0 and at 150 rpm, which coincided with the highest biomass yield (23.76 g/L) obtained on the same day and conditions. Earlier, El-salam *et al*. (2014) reported a carotenoids production of 474.76 µg/g at 144 hours in a co-culture of *R. glutinis* and *Lactobacillus casei sub sp. casei* NRRL B-441 with cheese whey as substrate. Carotenoids are secondary metabolites, which are produced in the stationary phase in response to different stressors such as light, aeration, pH (Gong *et al*., 2019; Kot *et al.*, 2019). In this study, agitation had a significant effect on carotenoid synthesis (p=0.0003) and was found that 150 rpm resulted in higher yield of carotenoids. It has been reported that pH influenced the carotenogenesis process by regulating the concentration and activity of the enzymes that catalyze the reactions of mevalonate (MVA) pathway (Hayman et *al*., 1974), however, in the present study the evaluated pH does not present a significant effect on the carotenoids production.

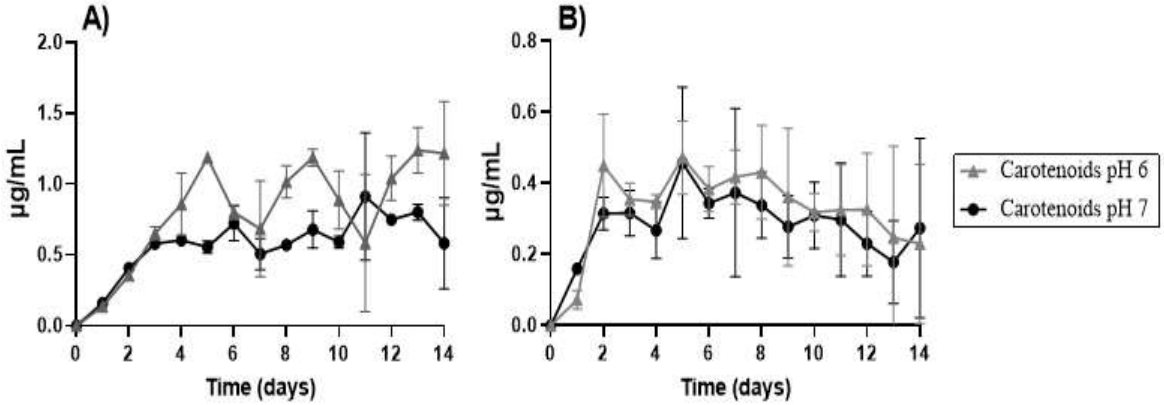


Figure 2. Kinetics of carotenoids production by *Rhodotorula glutinis* using milk whey as substrate. A) 150 rpm. B) 180 rpm (p=0.0003).

In a previous study, Hernández-Almanza *et al*. (2014) reported that addition of a metabolic regulator and moisture control are significant factors for the production of carotenoids by *R. glutinis* in solid-state fermentation. Kanzy *et al*. (2015) observed a carotenoids production of 438 µg/g by *R. glutinis* in a medium containing whey under optimized conditions and the carotenoids present were torularhodine and β-carotene. But, Pi *et al*. (2018) reported the main carotenoids synthesized by *R. glutinis* in a synthetic medium were β-carotene, torularhodine and torulene.

4.3 Lipid production

The highest percentage of lipids was recorded at pH 7.0 and at 180 rpm (27.48%). The statistical analysis showed that pH has a significative effect in lipid production by *R. glutinis* P4M422, once pH is an important factor in lipid synthesis. On the other hand, *Rhodotorula glutinis* is an aerobic yeast, agitation could accelerate the transfer of nutrients from whey to cells for cell growth and lipidic production for its cellular maintenance, however, the conditions evaluated (150 and 180 rpm) in

this study not showed a significant effect. *R. glutinis* P4M422 used in this study complied the requirement of an oleaginous yeast, as they recorded more than 20% of lipids accumulation. In general, most of the microorganisms need to synthesize primary lipids for their cell membrane and its growth and maintenance; and hence high lipid percentages were observed during the first days of growth, reflecting its relation as a parameter indicating biomass growth (Figure 3).

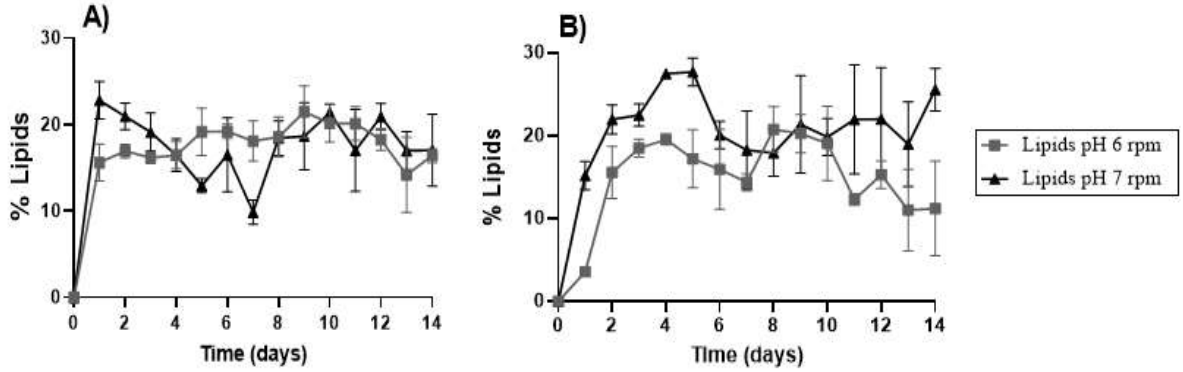


Figure 3. Kinetics lipids production by *Rhodotorula glutinis* using milk whey as substrate. A) 150 rpm. B) 180 rpm.

It is known that a high C/N ratio influences lipid production, as one of the principal enzymes of the oleaginous microorganism is isocitrate dehydrogenase (IDH) and their activity during the tricarboxylic acid cycle is affected in response to the high C:N ratio. Acetyl-CoA is the principal precursor related to the formation of lipids and carotenoids. C/N ratio of the whey used in this study was 23:1 and hence, the synthesis of lipids was favored over the accumulation of carotenoids (Somashekar & Joseph, 2000). Ribeiro *et al*. (2019) using cassava wastewater as a substrate for the production of fatty acids found that the principal lipids to be produced were saturated acids: oleic and linoleic acid. On the other hand, Gong *et al*. (2019) in a synthetic medium with glucose and using irradiation as stress obtained unsaturated fatty acids (mainly C16: 1, C18: 2 and C18: 3).

V. Conclusions

It can be concluded from this study that cheese whey is a potential low-cost substrate for growth of *Rhodotorula glutinis* P4M422. Results also confirmed its potential for accumulation of lipids and production of carotenoids by *Rhodotorula glutinis* P4M422. It was observed that pH and agitation are stressing factors, which influenced the production and accumulation of these bioactive compounds by the yeast used in this study with cheese whey as a substrate.

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