LIPIDS AND β-CAROTENE PRODUCTION BY *Rhodotorula glutinis* USING CARBON SOURCES DERIVED FROM NaOH-ASSISTED ORGANOSOLV PRETREATMENT OF TOBACCO STEM

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ABSTRACT

Tobacco cultivation poses environmental challenges, since its residues offer potential in biorefinery processes. Glycerol, a biodiesel by-product, aids in valorization through alkaline pretreatment, benefiting enzymatic saccharification. *Rhodotorula glutinis* utilizes these substrates, accumulating lipids up to 40-70% dry biomass weight and synthesizing industrially relevant compounds. Utilizing *R. glutinis* cultures with tobacco stem hydrolysate and glycerol signifies a significant advance. This study focuses on alkaline organosolv pretreatment of tobacco stem, enzymatic hydrolysis, and *R. glutinis* fermentation, utilizing recovered glycerol. Results show promising yields in cell mass, lipids, and β-carotene, highlighting tobacco residues' potential within biorefinery frameworks.

Keywords: Single Cell Oil; postbiotics; Alkaline pretreatment; bioprocesses.

1 INTRODUCTION

Tobacco is a prominent non-food agricultural crop globally, but its cultivation poses significant environmental challenges, such as substantial CO_2 emissions and waste generation [1]. The application of these residues into biorefinery processes emerges as a promising solution to mitigate such impacts and produce valuable compounds [1]. Organosolv pretreatment assisted by NaOH can be used to the fractionation of this biomass with the aim to apply the obtained monomers from the remained cellulose and hemicellulose (after an enzymatic hydrolysis) in a bioprocess to produce different bioproducts[2]. This kind of pretreatment improve enzymatic saccharification [2] that can be used as a carbon source for oleaginous microorganisms like *Rhodotorula glutinis*. *R. glutinis* can accumulate lipids up to 40-70% of the dry biomass weight, besides synthesizing other industrially relevant compounds such as β-carotene[3]. The utilization of *R. glutinis* cultures with tobacco stem hydrolysate and glycerol, a byproduct from pretreatment represents a significant advancement in valorizing the tobacco production chain, highlighting a sustainable approach to address environmental concerns and exploit commercial opportunities. According to the consulted literature, this is the first time that *R. glutinis* has been tested with these carbon sources (recovered glycerol and enzymatic hydrolysate of tobacco stem), in this context, this work aimed to perform NaOH-assisted organosolv pretreatment of tobacco stem, followed by enzymatic hydrolysis and cultivation of *R. glutinis* for the production of lipids and β-catotene.

2 MATERIAL & METHODS

Tobacco stem was ground to a particle size of 30 to 40 mesh and it was then subjected to a process for extractive compounds solubilization using an 8:2 ethanol/water solution at 5% (w/v) solids, at 2000 rpm, 60 °C, and 1 h in a jacketed extraction tank. Subsequently, the remaining solids were dried in an air-circulating oven at 60 °C to attain constant weight. Next, this biomass underwent a NaOH-assisted organosolv pretreatment with 15% (w/v) solids, 3% (w/v) NaOH, and 50% (v/v) glycerol, in an autoclave at 120 °C for 1 h[4]. After pretreatment, the suspension was diluted with distilled water in a 1:8 (v/v) ratio for the separation of the liquid phase[4] through filtration. The solid phase was washed to neutrality and dried in an air-circulating oven at 105 °C for 24 h, while the liquid phase was designated for glycerol recovery.

The liquid fraction from the pretreatment was subjected to lignin recovery by reducing the pH to 1-2 using a 10 M sulfuric acid solution [4,5], and separated by centrifugation. The remaining liquid had the pH readjusted to neutrality using 10 M NaOH. Finally, it was detoxified using powdered activated carbon at 10% (w/v) at 28 °C for 1 h [6], and then separated from the activated carbon by ultracentrifugation at 30000 xg for 1 h to obtain the concentrated glycerol solution. The solid fraction from the pretreatment was subjected to enzymatic hydrolysis in 250 ml Erlenmeyer flasks incubated in an orbital shaker at 200 rpm and 50 °C for 72 h, using 10% (w/v) SL, 50 mM sodium citrate buffer (pH 4.8), and an enzyme (CelliCtec2®-Novozymes) loading of 12 FPU/g (biomass) [2]. Reducing sugars (RS) obtained from the enzymatic hydrolysis were analyzed by High-Performance Liquid Chromatography (HPLC).

The *R. glutinis CCT 2182* strain, stored in a cryotube at -80°C, was reactivated in YPD medium (pre-inoculum) [7,8], composed of yeast extract (3 g/L), peptone (5 g/L), and glucose (10 g/L). Cultures in the pre-inoculum were incubated for 24 h at 200 rpm, 30°C, and pH 5.5, before being transferred to the inoculum stage. In this phase, the culture medium included the pre-inoculum (10% of the total volume), sugarcane molasses (20 g/L), yeast extract (5 g/L), ampicillin (0.05 g/L), $(NH_4)_2SO_4$ (1 g/L), KH_2PO_4 (1 g/L), $Na_2HPO_4{\cdot}12H_2O$ (1 g/L), $MgSO_4{\cdot}7H_2O$ (2 g/L), NaCl (1 g/L), $CaCl_2$ (0.02 g/L), and $FeCl_3{\cdot}6H_2O$ (0.01 g/L) [7,8]. Inoculum proceeded in an orbital shaker at 200 rpm, 30°C, and pH 5.5 for 40 hours [8]. After 24 h from the start of inoculation,

addition of a pulse of 10 g/L of molasses and 2.5 g/L of yeast extract was made to increase cell concentration. Next, the inoculum was centrifuged at 5000 xg, the supernatant discarded, and the cell pellet washed and resuspended in 0.1% peptone water, yielding a cell concentration of 75 g/L.

Different proportions of detoxified tobacco hydrolysate and recovery glycerol were evaluated as culture media, containing a carbon source of 30 g/L. The enzymatic hydrolysate: glycerol proportions tested were 2:1, 1:1, 1:2, 1:0, and 0:1. The suspension of 4 g/L of yeasts were inoculated in culture media added to 1.3 g/L of urea, 0.1 g/L of ampicillin, and the same mineral concentration as the inoculum media [7,8]. The assays were performed in 250 ml Erlenmeyer flasks placed in an orbital shaker incubator at 150 rpm, 30°C, and pH 5.5 for 72 h [7,8]. Samples were collected to evaluate the profile of sugars, glycerol, and nitrogen over time, as well as cell growth. The concentration of glucose, xylose, and glycerol was determined by HPLC, and cell growth was measured by the gravimetric method. After 72 h, the suspension obtained from the five-cultivation conditions were centrifuged at 5000xg, the supernatant was discarded, and the cell pellet was lyophilized and reserved for β-carotene and total lipid analysis according to the methodology described by [9].

3 RESULTS & DISCUSSION

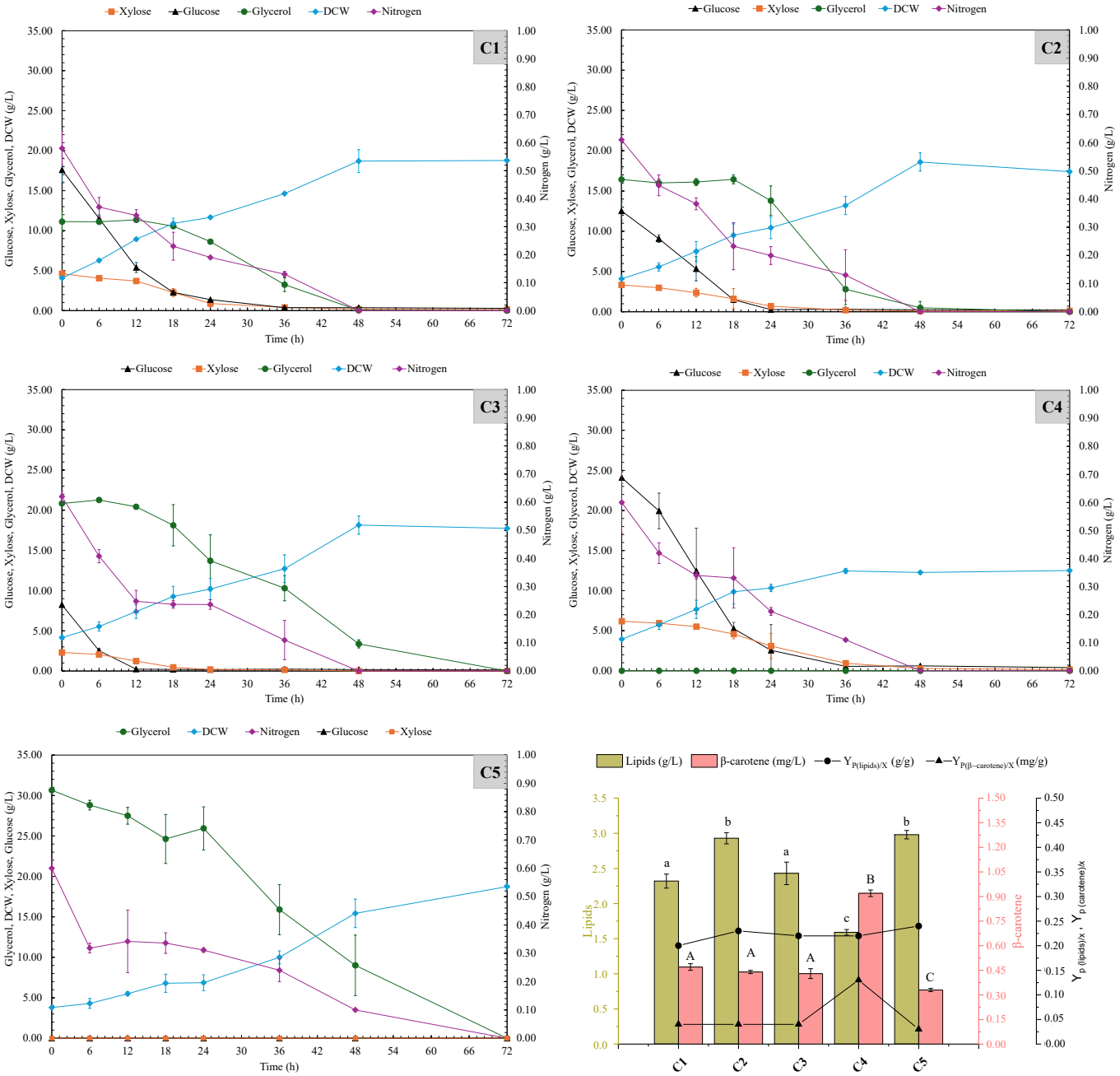

Figure 1. Fermentative kinetics of *R. glutinis* under different cultivation conditions, varying the proportion of carbon source (hydrolysate: glycerol) for the production of lipids and β-carotene.

In Figure 1, the kinetic behavior of *R. glutinis* at different proportions of carbon sources (enzymatic hydrolysate and glycerol) derived from pretreatment and enzymatic hydrolysis of tobacco stem (C1, C2, C3, C4, and C5) can be observed. *R. glutinis* was able to consume the available carbon sources for its growth and production of lipids and β-carotene in all cultures conducted up to 72 h. However, glycerol only began to be consumed after glucose and xylose limitation, indicating that in the presence of these sugars, the cell prioritizes their utilization before turning to glycerol consumption (Figure 1 – C1, C2, and C3). Lipid production for all the cultures

was, respectively, 2.56, 2.66, 2.33, 1.49, and 2.88 g/L, while for β-carotene, the corresponding values were 0.44, 0.47, 0.43, 0.92, and 0.33 mg/L. Lipid and β-carotene concentrations at the end of the cultivations were influenced by the proportion of the carbon source in the medium and the cultivation time. For example, in the C4 cultivation (Figure 1), composed exclusively of hydrolysate (glucose + xylose), cells consumed sugars within the first 36 hours, resulting in a significant increase in carotenoid content. The preference of *R. glutinis* for carotenoid production in the presence of sugars rather than glycerol is inherently linked to its metabolic adaptation to environmental conditions and substrate availability. These factors directly influence the biochemical and metabolic processes involved in the synthesis of these antioxidant pigments [9]. On the other hand, in the C5 cultivation, composed solely of glycerol, a high lipid concentration was recorded, but with a low β-carotene concentration. These findings corroborate with the results of Santos Ribeiro et al., [9], who cultivated *R. glutinis* with 30 g/L of RT at different C/N ratios (26.5, 18.2, and 31.6), achieving, in 120 h of cultivation, lipid concentrations of 1.59, 1.19, and 1.34 g/L and corresponding β-carotene concentrations of 0.88, 0.57, and 0.60 mg/L.

Table 1. Parameters of the cultivation conditions conducted.

Variables	Fermentative assays				
	C1	C2	C3	C4	C5
Glycerol:Hydrolyzed	**1:2**	**1:1**	**2:1**	**0:1**	**1:0**
Glucose (g/L)	17.59 ± 1.50	12.52 ± 1.14	8.25 ± 0.07	24.09 ± 0.73	0.00 ± 0.00
Xylose (g/L)	4.61 ± 0.38	3.33 ± 0.25	2.30 ± 0.04	6.19 ± 0.25	0.00 ± 0.00
Glycerol (g/L)	11.13 ± 1.28	16.44 ± 1.39	20.84 ± 0.04	0.00 ± 0.00	30.65 ± 0.08
$Y_{X/S}$ (g/g)	0.37 ± 0.03	0.36 ± 0.06	0.35 ± 0.03	0.25 ± 0.03	0.40 ± 0.03
$Y_{X/N}$ (g/g)	20.62 ± 0.57	17.84 ± 1.41	17.10 ± 0.81	11.40 ± 0.32	20.13 ± 0.03
$Y_{P/S\ lipids}$ (g/g)	0.07 ± 0.01	0.08 ± 0.01	0.07 ± 0.00	0.05 ± 0.00	0.09 ± 0.00
$Y_{P/X\ lipids}$ (g/g)	0.20 ± 0.01	0.24 ± 0.01	0.22 ± 0.00	0.22 ± 0.00	0.24 ± 0.01
$Y_{P/S\ carotene}$ (mg/g)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.03 ± 0.00	0.01 ± 0.00
$Y_{P/X\ carotene}$ (mg/g)	0.04 ± 0.00	0.04 ± 0.00	0.04 ± 0.00	0.13 ± 0.00	0.03 ± 0.00
Q_X (g/L/h)	0.25 ± 0.01	0.23 ± 0.02	0.15 ± 0.01	0.19 ± 0.01	0.17 ± 0.01
$Q_{P\ lipids}$ (g/L.h)	0.03 ± 0.00	0.04 ± 0.00	0.03 ± 0.00	0.02 ± 0.00	0.04 ± 0.01
$Q_{P\ carotene}$ (mg/L.h)	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.01 ± 0.00	0.00 ± 0.00
Lipids (g/g)	0.16 ± 0.00	0.14 ± 0.00	0.14 ± 0.01	0.13 ± 0.01	0.16 ± 0.00
β-carotene (mg/g)	2.33 ± 0.24	2.74 ± 0.10	2.59 ± 0.08	7.50 ± 0.14	1.75 ± 0.39
Protein (%)	28.56 ± 0.36	27.48 ± 0.13	25.07 ± 1.10	26.34 ± 0.85	23.67 ± 2.28

Table 1 presents the fermentative parameters for all cultivation conditions conducted in this study. It can be observed that Yp/x remained around 0.2 for all cultivation conditions, indicating that the cell was able to store 20% of lipids during the cultivation period. Additionally, glycerol played a crucial role in achieving higher substrate to cell conversions (Yx/s), exceeding 0.35 g/g. The results demonstrate that the cell was capable of growing on different carbon sources, achieving significant yields in cell mass, lipids, and β-carotene similar to those reported in the literature. The findings from this study pave the way for the exploration of tobacco residues in generating important biomolecules within the biorefinery concept.

3 CONCLUSIONS

By the results obtained in this study it was possible highlight the potential of tobacco stem into lipids and β-carotene production by *R. glutinis* through the application of carbon sources from NaOH-assisted organosolv pretreatment . Under the tested conditions, the highest lipid accumulation of 20% and β-carotene production of up to 0.92 mg/L was attained by C4 (0:1). This sustainable approach addresses environmental concerns and offers commercial prospects in the biorefinery, laying the groundwork for further exploration of tobacco residues in biomolecule generation.

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