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# Fermentative production of β-carotene from sugarcane bagasse by *Rhodotorula glutinis* CCT-2186

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**Abstract**

β-Carotene is a red-orange pigment that serves as a precursor to important pharmaceutical molecules like vitamin A and retinol, making it highly significant in the industrial sector. Consequently, there is an ongoing quest for more sustainable production methods. In this study, glucose and xylose, two primary sugars derived from sugarcane bagasse (SCB), were utilized as substrates for β-carotene production by *Rhodotorula glutinis* CCT-2186. To achieve this, SCB underwent pretreatment using NaOH, involved different concentrations of total solids (TS) (10%, 15%, and 20%) to remove lignin. Each sample was enzymatically hydrolysed using two substrate loadings (5% and 10%). The pretreated SCB with 10%, 15%, and 20% TS exhibited glucose hydrolysis yields (%wt) of 93.10%, 91.88%, and 90.77%, respectively. The resulting hydrolysate was employed for β-carotene production under batch fermentation. After 72 h of fermentation, the SCB hydrolysate yielded a β-carotene concentration of 118.56±3.01 mg/L. These findings showcase the robustness of *R. glutinis* as a biocatalyst for converting SCB into β-carotene.

**Keywords:** Sugarcane bagasse, Pretreatment, Enzymatic hydrolysis, *Rhodotorula glutinis*, Fermentation, β-carotene.

## 1. Introduction

Lignocellulosic biomass (LCB) is the most abundant organic material on Earth (~200 billion tons). It is mainly comprised of agricultural and forest residues and represents a promising renewable source that can be converted into fermentable sugars for the production of fuels, chemicals, plastics, etc [1]. Sugarcane is one of the main agricultural crops in countries such as Brazil, India, China, and Thailand, among others [2]. According to Food and Agriculture Organization of the United Nations (FAO), the annual sugarcane production was 1,890 million tons in 2020. SCB is mainly composed of cellulose, hemicellulose, and lignin. Cellulose is a linear polymer of glucose units linked by β-1,4-glycosidic bonds and hydrogen bonds that make it difficult to break down. Hemicellulose is a heteropolysaccharide consisting of xylose backbone linking glucose, galactose, arabinose, mannose and acetyl groups. Lignin is a complex polyphenol biomolecule composed of sinapyl alcohol, p-coumaryl alcohol, and coniferyl alcohol. It provides structural and mechanical support to plants and acts as an impermeable barrier that resists biological, mechanical, or chemical decomposition [3].

Due to the recalcitrant nature of SCB, the pretreatment process is considered a fundamental step in the efficient processing of lignocellulosic biomass in biorefineries. The most common pretreatment technologies for lignocellulosic materials include mechanical milling, dilute acid pretreatment, alkaline pretreatment, organosolv, ammonia fiber expansion, oxidation, and biological pretreatment [4]. As a result of these pretreatments, the availability of carbohydrates increases, enabling the action of cellulases to perform enzymatic hydrolysis for the production of cellulosic sugars. The enzymatic hydrolysis of carbohydrate polymers involves the coordinated action of a group of enzymes, known as cellulases, which catalyze the hydrolysis of glycosidic bonds to produce fermentable sugars [5]. In this way, SCB is considered as a potential substrate

for the fermentative production of various value-added products, including carotenoids, which are currently one of the most important molecules not only for their coloring properties but also for their applications in health and nutraceuticals sector [6].

Carotenoids are pigments responsible for a wide range of colors found in plants and microorganisms. They play a crucial role in capturing light and transferring energy during photosynthesis. Additionally, carotenoids act as antioxidants, protecting the photosynthetic system against photooxidative damage and preventing oxidative damage to cells [7,8]. The non-toxic nature of carotenoids makes them excellent natural food additives, and their antioxidant activity adds value when incorporated into food products. β-carotene is a precursor of vitamin A, added to various foods, especially in functional and nutraceutical drinks, and its demand is expected to increase in the future [9]. The global market of carotenoids is expected to be of $2.0 billion by 2026, with a compound annual growth rate of 4.2%. This growth is attributed to the increasing use of natural carotenoids as food dyes and advancements in carotenoid extraction technologies [10].

Recently, carotenoid production using agro-industrial by-products as feedstocks by *Rhodotorula sp* has been studied [11]. The efficient utilization of xylose is essential to improve the profitability of biotechnological processes in biorefineries. Thus, evaluating the use of $C_5$ and $C_6$ sugars for the production of carotenoids by *R. glutinis* is crucial to further improve the titles, yields and rates of this bioprocess. The current study utilized SCB as a feedstock for β-carotene production by the yeast-*Rhodotorula glutinis* CCT-2186. SCB was pretreated with sodium hydroxide using alkaline pretreatment at different solid loadings, followed by enzymatic hydrolysis to obtain C5 and C6 sugars. The resulting enzymatic hydrolysate was then subjected to submerged fermentation to produce β-carotene by *R. glutinis* CCT-2186, in order to evaluate SCB's potential as a renewable carbon source in lignocellulosic biorefineries.

## 2. Material and Methods

### *2.1 Raw material*

Sugarcane bagasse was kindly supplied by Ipiranga Agro-industrial (Descalvado/SP, Brazil). The native bagasse was milled by rotatory knife mill (Marconi MA-680, São Paulo, Brazil). The milled bagasse was then sieved through 20 meshes.

### *2.2 Pretreatment with sodium hydroxide*

Sodium hydroxide pretreatment of SCB was carried out in 250 mL Erlenmeyer flasks. Sodium hydroxide solution (4% w/v) was used for pretreatment using three different solid loadings [10, 15 and 20% (w/v)] at 121°C and 1 atm pressure for 30 min to remove lignin fraction. The alkaline pretreatment method was adopted from Ascencio et al. [12]. After pretreatment, all the biomass was washed to remove solubilized lignin until the pH was neutralized and sun-dried until it reached humidity between 8-10%.

### *2.3 Biomass composition analysis of native and pretreated SCB*

In order to determine the chemical composition of SCB (hemicellulose, cellulose, lignin, ashes and extractives), the National Renewable Energy Laboratory Colorado, USA protocols were followed as reported elsewhere [13,14]. Briefly, 0.3 g of dry weight of each biomass was weighed in test tubes by triplicate. Subsequently, 3 mL of sulfuric acid (72% w/v) were added in the flasks. The tubes were placed in a thermostatic bath at 30 °C for 1 hour. After the thermostatic bath, 84 mL of distilled water were added to each sample, pouring the respective solutions into 250 mL-Erlenmeyer flasks. The flasks were autoclaved at 1 atm and 120 °C for 1 hour. Then, samples were filtered employing kitasato flasks and crucibles filters (pore size of 16 - 40 μm). Samples were prepared for HPLC analysis to quantify the content of monosaccharides (glucose, xylose, arabinose) for cellulose and hemicellulose fractions. For soluble lignin, a 1:5 dilution (filtered

solution: distilled water) was made and analyzed for spectrophotometry at of 240 nm. Insoluble lignin was determined by gravimetry.

*2.4 Enzymatic hydrolysis*

After pretreatment, each pretreated biomass using 3 different total solids loadings *viz*. 10, 15 and 20% of TS (total solids) were subjected to enzymatic saccharification. The enzymatic hydrolysis experiments were performed in 125 mL flasks. For the enzymatic saccharification at 5% and 10%TS, 2.5 g of biomass was added in 50 mL and 25 mL of sodium citrate buffer (pH 4.8, 50 mM), respectively. Cellic CTec 2 (Commercial cellulase) from Novozyme Inc., Curitiba, Brazil, was added to flasks with a loading of 10 FPU/g of dry biomass in both 5 and 10% TS concentrate on and sealed with aluminum foil. Flasks were then placed in an incubator shaker (New Brunswick Scientific, Innova 4000, USA) at 50±0.5 °C at 200 rpm. Liquid samples (enzymatic hydrolysates) were collected at 0, 12, 24, 48 and 72 h of incubation and analyzed for the released sugars analysis.

Conversion of cellulose and hemicellulose during enzymatic hydrolysis was taken into consideration to calculate the hydrolysis yield [15]. It was calculated as follows:

Conversion of Cellulose (%)

$$= \frac{\text{Resleased glucose (g)}}{\text{Presence of the cellulose in pretreated bagasse (g)} \times 1.11} \times 100$$

Hemicellulose conversion (%)

$$= \frac{\text{Resleased xylose (g)}}{\text{Presence of hemicellulose in pretreated bagasse (g)} \times 1.14} \times 100$$

*2.5 Microorganism and its cultivation*

*Rhodotorula glutinis* CCT-2186 was procured from André Tosello Tropical Culture Collection (Campinas, SP, Brazil). For the preparation of the inoculum, *R. gluitinis* CCT-2186 was grown in the medium containing yeast extract (10 g/L), peptone (20 g/L), and glucose (20 g/L). The medium was prepared in 100 mL Erlenmeyer flasks and cells were cultivated for 48 h at 30 °C and 150 rpm in an incubator shaker (New Brunswick Scientific, Innova 4000, USA). The inoculum was prepared containing 0.2 mg/mL yeast cells.

For the production of β-carotene, synthetic media was prepared by adding of 10 g/L asparagine, 4 g/L peptone, 0.52 g/L $KH_2PO_4$ and 0.52 g/L $MgSO_4$. Growth conditions were used as mentioned by Mussagy et al. [16]. The production of the β-carotene was performed by submerged fermentation and fermentative tests were performed to determine the effect of carbon sources (glucose and xylose). The study was performed in 125 mL Erlenmeyer flasks using 50 mL of medium having different concentrations of glucose and xylose as shown in Table 1. Pre-culture (5 % v/v) was added in the flasks and incubated at 30 °C with an agitation of 170 rpm for 72 h. For the quantification of carotenoids, the cells were recovered by centrifugation at 2500 × g for 10 min at 4 °C. The recovered cell pellets were used for the quantification of cell biomass and β-carotene production. The cultivation assays using shake flasks were carried out in triplicates.

*2.6 Hydrolysate fermentation*

The enzymatic hydrolysate of SCB was used to evaluate its potential as a primary carbon source for the production of β-carotene. Peptone (4 g/L), was added in the enzymatic hydrolysate, as the

as the only nitrogen source. Each experiment was performed in triplicate and performed in 125 mL flasks. The temperature and agitation speed were controlled to 30±0.5 °C and 170 rpm, using an incubator shaker (New Brunswick Scientific, Innova 4000, USA), respectively. As a response variable, β-carotene production was analyzed at 72 h of cultivation.

### *2.7 Analysis*

#### *2.7.1 Quantification of sugars.*

The sugar monomers were quantified by HPLC in an Agilent 1200 chromatograph (CA, USA). The device was equipped with an isocratic pump and a Bio Rad Aminex HPX-87H column (Hercules, CA, USA), 0.01 N of $H_2SO_4$ used as eluent at 45 °C, injection volume of 20 µL, and flow rate of 0.6 mL $min^{-1}$ during 25 minutes [12]. The samples were filtered by Sep Pak C18 filter (Waters Corporation, Milford, MA, USA). The total reducing sugars were also analyzed by the spectrophotometric method [17].

#### *2.7.2 Determination of dry cell weight*

Briefly, the cells from fermented broth were harvested by centrifugation (2500 × g for 15 min at 4 °C) and washed twice with the same volume of distilled water, dried at 50° C for 48 h. Dried samples were weighed in an analytical balance for determination of dry cell weight [18].

**Table 1** Carbon sources concentrations studied for β-carotene production by *R. gluitnis* CCT-2186

| **Carbon source** | **Concentration (**g/L**)** | | | |
|---|---|---|---|---|
| **Glucose** | 10 | 20 | 30 | 40 |
| **Xylose** | 10 | 20 | 30 | 40 |
| **Glucose + Xylose** | 2+8 | 4+16 | 6+24 | 8+32 |

*2.7.3 Recovery and analysis of carotenoids*

β-carotene recovery was carried out following a method developed in the Laboratory of Bioprocesses and Sustainable Products (LBios), of the Biotechnology Department, at the University of São Paulo. Briefly, after dry cell weight determination, cell pellets were transfer to 50 mL falcon and mixed with NaOH solution and were taken to a thermostatic bath. After chemical treatment, the samples were centrifuged at 2500 × g at 4 °C for 15 min and the pellet was washed twice with distilled water and then placed in the freezer for 24 h to be frozen. The freezer pellets were resuspended in a methanol/acetone solution and disrupted by sonication (Hielshcer Ultrasound Technology, UP400St Powerful ultrasonicator) for 10 min (20 seconds ON/5 seconds OFF). After the recovery of carotenoids, the samples were re-centrifuged at 2500 × g at 4 °C for the analysis of supernatant for β-carotene amounts. The visible-light adsorption spectra from 380 to 600 nm was used and compared with the carotenoids calibration curve at 450 nm using pure β-carotene (standard). It is evident that carotenoids are light-sensitive. During the assays, the falcon tubes were wrapped with aluminum foil to avoid the oxidation, photodegradation and isomerization of carotenoids.

*2.7.4 Data analysis*

The production data of carotenoids were analyzed statistically using Statistica (StaSoft, Inc., Oklahoma, USA). The numerical data were presented as the mean value. The experimental values were verified for the significant differences through a one-way analysis of variance (ANOVA) using Tukey's post hoc test with a level of significance at $p<0.05$.

## 3. Results and Discussion

### *3.1 Chemical characterization of natural and dilute acid pretreated SCB.*

The chemical composition of the native SCB and biomass subjected to alkaline pretreatment (sodium hydroxide pretreatment), known as holocellulose, was analyzed. Table 2 presents the quantities of carbohydrates (cellulose and hemicellulose), lignin, ash, and extractives in both the native and pretreated SCB samples. The native SCB exhibited average contents of 43.05±0.14% cellulose, 28.48±0.23% hemicellulose, 23.01±0.74% lignin, and 0.62±0.05% ash. These values align with previous findings. For instance, Pereira et al. [19] reported cellulose, hemicellulose, lignin, and ash contents of 38.47%, 28.86%, 13.45%, and 0.79%, respectively. Moreover, Philippini et al. [20] investigated the impact of pretreatment on five different hybrid sugarcane bagasse species. The chemical characterization results for all species demonstrated cellulose contents of 38-42%, hemicellulose contents of 23-25%, and lignin contents of 33-34%. It has been documented that variations in the chemical composition of SCB may be influenced by factors such as crop variety and climatic conditions (location, crop growth and temperature) [21].

After pretreating SCB with sodium hydroxide, hemicellulose was removed mainly from the biomass. Alkaline reagents degrade uronic ester side chains and glycosidic bonds, causing structural modifications in lignin, cellulose swelling and de-crystallization, as well as hemicellulose solvation [22]. The chemical composition of the holocellulose fraction of SCB (cellulose and hemicellulose) is presented in Table 2. This lignocellulosic composition aligns with data reported by Ascencio et al. [12], who registered 50.16% cellulose, 32.22% hemicellulose, and 8.91% lignin using a 1% (w/v) NaOH solution and 10% (w/v) total solids in the pretreatment step. In another study conducted by Maryana et al. [23], SCB exhibited cellulose, hemicellulose, lignin,

and ash contents of 62.16±0.87%, 18.62±1.65%, and 7.16±0.32%, respectively, following alkaline pretreatment with a 1N sodium hydroxide solution at 121 °C for 30 minutes.

The holocellulose obtained after pretreatment using 10% total solids (TS) exhibited a lignin removal efficiency of 83.57%, while retaining 91.51% of cellulose and 47.42% of hemicellulose. Sodium hydroxide acts specifically on the linkages in lignin and glycosidic bonds of carbohydrates, resulting in the disruption of the lignin structure and subsequently reducing the degree of polymerization and crystallinity [12,24]. For the holocellulose derived from the pretreatment of SCB using 15% TS, a delignification rate of 81.97% was observed, with cellulose and hemicellulose retentions of 92.34% and 61.36%, respectively. Finally, the holocellulose obtained from the pretreatment with 20% TS exhibited a delignification efficiency of 80.27%, while retaining 92.34% cellulose and 61.36% hemicellulose in the pretreated biomass.

**Table 2** Chemical composition (% dry weight) of native SCB and after pretreatment with sodium hydroxide (holocellulose) at different solid loadings, and respective lignin removal (% g/g).

| **Biomass** | **Cellulose** | **Hemicellulose** | **Lignin** | **Ash** | **Extractives** | **Total** | **Lignin Removed (%)** |
|---|---|---|---|---|---|---|---|
| SCB native | 43.05±0.14 | 28.48±0.23 | 23.01±0.74 | 0.62±0.05 | 3.36±0.12 | 98.51±0.89 | - |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Holocellulose (10% TS) | 72.5±0.76 | 24.85±0.23 | 6.96±0.16 | 0.73±0.02 | ND | 104.92±1.09 | 80.26[a] |
| Holocellulose (15% TS) | 69.74±0.10 | 27.36±0.21 | 7.36±0.48 | 0.76±0.03 | ND | 105.08±1.63 | 81.96[b] |
| Holocellulose (20% TS) | 66.16±0.66 | 29.08±0.77 | 7.56±0.61 | 0.75±0.04 | ND | 103.42±1.72 | 83.56[c] |

ND: No detected. [a-c]Different letters indicate significant difference according to Tukey HSD test ($p<0.05$).

Partial removal of hemicellulose is associated with the recovery of reducing sugars, primarily 5-carbon sugars such as xylose and arabinose [24]. The cellulose retention rate was 92.34% under identical conditions, indicating that increased biomass concentration during the pretreatment stage leads to greater carbohydrate retention in the fiber. However, these findings suggest that an increase in the concentration of total solids (% w/v) results in a decrease in lignin removal efficiency. The highest recorded lignin removal occurred at a lower total solid load (10% w/v), indicating that a smaller amount of biomass is likely to provide a larger contact surface area and enhanced reactivity with the NaOH solution, resulting in higher lignin solubility in the aqueous medium. A Tukey HSD test conducted among the different treatments (10%, 15%, and 20% total solids) showed a significant difference ($p<0.05$) in lignin removal. Therefore, alkaline pretreatment with lower solid loads effectively removes a majority of the lignin, weakening the lignocellulosic biomass and facilitating the availability of cellulose for hydrolysis, leading to the release of substantial amounts of sugars from the biomass.

*3.2 Enzymatic saccharification.*

After pretreatment, each holocellulose sample underwent enzymatic hydrolysis using 5% and 10% total solids (TS) for saccharification. Previous studies have shown that enzymatic hydrolysis of lignocellulosic materials (cellulose and hemicellulose) primarily converts them into pentose and hexose sugars [25]. High-performance liquid chromatography (HPLC) analysis of the resulting hydrolysate from sugarcane bagasse (SCBH) revealed that xylose was the main product of hemicellulose conversion, while glucose was the primary product of cellulose conversion. Table 3 provides the cellulose hydrolysis yield (%wt) in terms of glucose yield and hemicellulose hydrolysis yield (%wt) in terms of xylose yield for each pretreated holocellulose sample using 5% and 10% TS in the enzymatic hydrolysis process.

**Table 3** Enzymatic hydrolysis with 5 and 10% of TS showing the yield (wt%) for glucose and xylose analysis by HPLC, from different pretreatment conditions.

| Pretreated biomass | % TS (w/v) in enzymatic hydrolysis | Conversion | Hydrolysis yield (%wt) | | | | |
|---|---|---|---|---|---|---|---|
| | | | 0 h | 12 h | 24 h | 48 h | 72 h |
| Holocellulose (10%) | 5% | Cellulose | 3.89±0.20 | 68.65±4.88 | 75.86±2.53 | 82.64±1.32 | 93.10±3.14 |
| | | Hemicellulose | 1.51±0.14 | 58.93±2.61 | 59.44±1.72 | 68.86±6.23 | 74.32±3.51 |
| | 10% | Cellulose | 3.85±0.15 | 68.36±0.71 | 73.54±4.71 | 84.47±2.81 | 89.97±4.26 |
| | | Hemicellulose | 3.36±0.29 | 51.40±0.65 | 52.16±2.71 | 60.41±1.83 | 65.77±5.69 |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| Holocellulose (15%) | 5% | Cellulose | 3.82±0.12 | 74.23±3.22 | 82.39±6.98 | 86.17±2.38 | 91.88±6.57 |
| | | Hemicellulose | 1.66±0.20 | 55.06±1.97 | 58.83±3.89 | 63.31±2.65 | 75.88±5.84 |
| | 10% | Cellulose | 4.07±0.35 | 62.45±6.05 | 77.00±2.17 | 81.81±7.24 | 89.60±5.36 |
| | | Hemicellulose | 4.41±0.38 | 45.84±4.07 | 54.49±1.48 | 58.35±1.04 | 67.07±4.56 |
| Holocellulose (20%) | 5% | Cellulose | 4.44±0.28 | 71.97±4.91 | 85.88±1.79 | 88.84±2.3 | 90.77±6.32 |
| | | Hemicellulose | 2.76±0.36 | 52.71±1.65 | 63.41±2.04 | 75.85±2.51 | 78.07±3.51 |
| | 10% | Cellulose | 4.21±0.37 | 65.52±0.91 | 69.58±2.71 | 83.68±3.08 | 87.47±4.87 |
| | | Hemicellulose | 5.12±0.33 | 49.54±0.53 | 50.74±2.19 | 66.52±2.93 | 65.08±4.12 |

Fig. 1 and Fig. 2 depict the saccharification process using 5% and 10% TS from each pretreated biomass sample, respectively. Fig. 1A illustrates the glucose recovery during enzymatic hydrolysis of the extracted SCB samples, while Fig. 1B presents the enzymatic hydrolysis profile for xylose after 72 h. The sample pretreated with 10% TS (w/v) and hydrolyzed with 5% TS (w/v) in the enzymatic hydrolysis process exhibited the highest glucose recovery, achieving a concentration of 37.46±0.45 g/L of glucose and a yield of 93.10%. This can be attributed to the low solids load used in both the pretreatment (10%) and enzymatic hydrolysis (5%), allowing for sufficient biomass availability for reaction with sodium hydroxide and cellulolytic enzymes. As a result, this approach facilitated effective lignin removal and subsequent optimal conversion of sugars during enzymatic hydrolysis. In contrast, the holocellulose sample treated with 20% TS (w/v) in the pretreatment step and 5% TS (w/v) in enzymatic hydrolysis achieved a maximum xylose recovery of 12.94±0.23 g/L of xylose, corresponding to a yield of 78.07%. This can be attributed to the pretreatment of holocellulose with 20% total solids, which led to the highest retention of hemicellulose (61.36%) compared to samples pretreated with 10% and 15% (54.17% and 47.42% retention, respectively). The increased retention of hemicellulose facilitated a greater release of xylose during the process.

Figure 2 depicts the glucose profile (Figure 2A) and xylose profile (Figure 2B) obtained during enzymatic hydrolysis of each pretreated biomass with a 10% TS concentration. Generally, the enzymatic hydrolysis samples with 10% TS did not exhibit the highest yields. However, the difference compared to samples with 5% TS was minimal, resulting in a maximum sugar conversion yield of 89.97% for cellulose and 67.07% for hemicellulose.

Recent studies on pretreatment technologies have demonstrated that holocellulose obtained after pretreatment with 1% (w/v) NaOH for 30 minutes at 121 °C resulted in the recovery of 32.82 g/L of total reducing sugars after 72 h of reaction, using a 10% TS pretreated biomass concentration

[12]. Enzymatic hydrolysis using 5% TS yielded 19.08 g/L of glucose and 13.36 g/L of xylose. Conversely, Liu et al. [26] utilized an alkaline pretreatment (0.5 M NaOH, 80 °C, and 2 h of reaction time) for the enzymatic digestibility of SCB, resulting in the recovery of 12.9 g/L of glucose and 4.72 g/L of xylose after 72 h of incubation. Furthermore, the holocellulose sample treated with 20% TS (w/v) during the pretreatment step and 5% TS (w/v) during enzymatic hydrolysis achieved a maximum xylose recovery of 12.94±0.23 g/L, corresponding to a yield of 78.07% (wt%).

These data suggest that increasing the charge of pre-treated biomass from 5% to 10% in enzymatic hydrolysis results in a decrease in the recovery yield of glucose and xylose, in relation to higher concentrations of total solids. The best yields were obtained with lower TS load, indicating that a lower amount of biomass provides higher moisture content and availability for cellulolytic enzymes, which are dependent on fiber disruption based on the type of treatment performed. However, it is important to mention that several factors can affect the accessibility of enzymes to cellulose, including pore size and volume, particle size, specific surface area, lignin content, hemicellulose content, acetyl group presence, crystallinity of cellulose, and degree of polymerization [27].

*3.3 Carotenoids production by R. glutinis CCT-2186.*

The potential of *R. glutinis* for carotenoids production was evaluated using a basal medium containing commercial glucose, xylose and a mixture of both, at different concentrations as a carbon source. For the fermentations using both carbon sources, same glucose-xylose ratio that SCBH presented (2 g/L of xylose and 8 g/L of glucose) was maintained in synthetic media. Table 4 shows the cells dry weight (CDW) production of *R. glutinis* growing on different concentrations

of glucose and xylose in the culture media. It was observed that biomass production increases when the initial concentration of sugars is higher. When 10 g/L of glucose were used, a biomass production of 5.36±0.06 g/L was observed, while for the medium with xylose and the mixture of glucose and xylose, under the same concentration, CDW production of 5.09±0.07 and 5.25±0.08 g/L, were found. respectively. A study by Mussagy et al. [18] using an initial concentration of glucose of 10 g/L with a supplementation of $NH_4NO_3$ (4 g/L) and asparagine (10 g/L) as nitrogen sources, showed a similar biomass production (5.48 g/L), after 72 h of fermentation. CDW production did not present a significant difference employing glucose or xylose as a carbon source with the initial concentration of 10 g/L, demonstrating the ability of *R. glutinis* CCT-2186 to metabolize xylose also as a single carbon source. Instead, Pinheiro et al. [28] studied carotenoid production using xylose as the sole substrate by *Rhodotorula* sp. Total carotenoid and lipid yields on cell mass for the whole batch cultivation were 0.85 and 0.3 g/g CDW, respectively, after 7 days of cultivation using an initial xylose concentration of 70 g/L.

**Table 4** Cell biomass production after 72 h of fermentation by *R. glutinis* CCT-2186 using different concentrations of glucose and xylose.

| Experiment | Glucose (g/L) | Xylose (g/L) | Cells dry weight (CDW) production (g/L) |
|---|---|---|---|
| 1 | 10 | 0 | 5.36±0.07 |

| | | | |
|---|---|---|---|
| 2 | 20 | 0 | 8.13±0.38 |
| 3 | 30 | 0 | 8.17±0.07 |
| 4 | 40 | 0 | 8.17±0.09 |
| 5 | 0 | 10 | 5.09±0.07 |
| 6 | 0 | 20 | 6.19±0.01 |
| 7 | 0 | 30 | 6.15±0.32 |
| 8 | 0 | 40 | 5.93±0.33 |
| 9 | 8 | 2 | 5.25±0.08 |
| 10 | 16 | 4 | 7.52±0.26 |
| 11 | 24 | 6 | 8.67±0.17 |
| 12 | 32 | 8 | 8.54±0.11 |

On the other hand, an increase in cell biomass production was observed as the concentration of the carbon source was enhanced. In the case of glucose, no significant difference in cell mass was noticed at different concentrations of 20, 30, and 40 g/L, with a maximum production of 8.17±0.07 g/L of CDW in Experiment 4. When the microorganism was grown on xylose as the sole carbon source, a CDW production of 6.19±0.01 g/L was observed (Experiment 6). During co-fermentation of glucose and xylose, the maximum CDW production of 8.67±0.17 g/L was found in Experiment 11, where the total sugar concentration was 30 g/L (24 g/L glucose and 6 g/L xylose).

These biomass productions suggest that *R. glutinis* maintains its cell growth at high glucose concentrations, as well as during co-fermentation, metabolizing both C5 and C6 sugars, at concentrations up to 40 g/L of total sugars. The production of carotenoids is largely influenced by different growth conditions, particularly temperature and pH. The type and concentration of carbon and nitrogen sources have a significant influence, which may vary depending on the yeast strain used [29]. For instance, Tkáčová et al. [30] performed a kinetic analysis to study cell growth and β-carotene synthesis by *R. glutinis*. The study showed an overproduction of carotenoids caused by glucose depletion, leading to the accumulation of other intracellular products such as lipids and polyhydroxyalkanoic acids. They observed that after glucose consumption, the growth rate became slow, with the accumulation of palmitic and oleic acids resulting from the β-oxidation of triacylglycerols. The acetyl-CoA formed entered the mevalonate/isoprenoid/carotenoid pathways, particularly for β-carotene biosynthesis, establishing the metabolic association between glucose depletion, utilization of storage fatty acids, and overproduction of carotenoids in the form of β-carotene by *R. glutinis*. The increase in carotenoid synthesis is directly related to stress in the biological tissues of cells, which leads to a biochemical response, increasing enzymatic activity and carotenoid production under these unfavorable conditions [31]. Therefore, the selection of

appropriate carbon and nitrogen constituents in the production medium is one of the most important tasks for the production of β-carotene.

Figure 3 depicts the biomass production (g/L) and β-carotene (mg/L) using glucose as the sole carbon source at various concentrations. As previously discussed, higher glucose concentration corresponds to increased biomass production. This phenomenon has been previously reported, where high concentrations of carbon sources assist in the production of cellular biomass and lipid synthesis by the yeast *R. glutinis* [32]. Conversely, carotenoid production increases at lower concentrations of carbon sources. However, the behavior of carotenoid synthesis differs at high glucose levels. Carotenoid synthesis is lower when initial concentrations of 20, 30, and 40 g/L of total sugars or glucose are used. The maximum production of carotenoids is achieved using 10 g/L of glucose, reaching a concentration of 128.43±2.95 mg/L of β-carotene. Maldonade et al. [33] conducted a statistical optimization of cellular biomass and carotenoid production by the yeast *R. mucilaginosa*, analyzing different carbon and nitrogen sources in medium composition. They reported a maximum production of 122 mg/g of cell grown on medium (10 g/L of glucose and 5 g/L of yeast extract).

Figure 4 illustrates the production of biomass and β-carotene using glucose, xylose, and a composite medium (glucose and xylose), having 10 g/L of total sugars, supplemented with peptone (4 g/L) and asparagine (10 g/L) as nitrogen sources. According to the Tukey HSD test conducted among the different carbon sources, biomass production showed no significant difference ($p<0.05$), indicating that *R. glutinis* can generate cellular biomass from both C5 and C6 sugars, producing 8.36 g/L of cellular biomass using glucose. On the other hand, carotenoid synthesis exhibited a significant difference when xylose was a principal carbon source, resulting in a lower β-carotene production (119.75±2.88 mg/L). However, the metabolism of xylose in *Rhodotorula*

sp. is not yet fully understood, with only a few studies focusing on this aspect [28]. Conversely, the use of glucose and the combination of glucose and xylose as carbon sources did not show significant differences, yielding a maximum recovery of β-carotene of 128.43±2.95 mg/L. This finding is highly desirable for the utilization of SCBH (rich in glucose and xylose) as the sole carbon source.

*3.4 Carotenoids production from SCBH*

The fermentation tests revealed that the selected sugar concentration in the synthetic medium (10 g/L of total sugars) resulted in similar β-carotene production when SCBH was used as a primary carbon source (Table 5). Cell biomass production was also comparable when *R. glutinis* was grown on synthetic medium containing glucose and SCBH, with a maximum CDW production of 5.80±0.06 g/L achieved. It is worth mentioning that the fermentation using SCBH only required supplementation with peptone (4 g/L). This implies that SCBH does not require additional salt or nitrogen sources to achieve optimal cell growth and β-carotene production, similar to concentrations obtained using complex media with synthetic sources. Palm oil has been studied as a principal carbon source for carotenoid and lipid production by other strains of *R. glutinis* CCT 2186 [34]. The results obtained showed higher cell mass of 28.8 g/L. However, the β-carotene recovery was low at 0.35 mg/g biomass, using a composite medium consisted of 25 g/L of palm oil. This study shows that while palm oil can enhance biomass growth as a supplemental carbon source, it does not contribute to increased β-carotene production.

In other studies, *Rhodotorula* sp. were used for carotenoid production using hydrolysates from lignocellulosic material; however, the amount obtained was low. Liu et al. [35] reported a carotenoids production by the yeast *R. toruloides*, growing on wheat straw hydrolysate in

submerged fermentation for 120 h, achieving a maximum concentration of total carotenoids of 24.58 mg/L. Tea residues have also been analyzed as a carbon source for carotenoid production by the yeast *R. toruloides* [36]. The tea residue hydrolysate was obtained through pretreatment with dilute sulfuric acid and showed high concentrations of xylose and arabinose, establishing a precedent for the consumption of pentoses from lignocellulosic hydrolysates by *Rhodotorula* sp. The tea hydrolysate, supplemented with peptone (10 g/L), yielded 10.75 ±0.65 of cell biomass and a β-carotene recovery of 16.83±0.08 mg/g of biomass after 120 h of fermentation.

Deeba et al. [37] studies solid-state cultivation (SSC) for the production of xylooligosaccharides, carotenoids, biodiesel, and animal feed. Enzymatically hydrolyzed solid-state-cultivated biomass when supplemented with 5% (v/v) molasses, showed a maximum production of 210.4 mg/L of β-carotene by *Rhodotorula pacifica* INDKK after 192 h of fermentation. However, several processing steps still require optimization to achieve industrially competitive yields and productivity for β-carotene production. Additional investigations are needed to study processing steps such as extraction, recovery, and purification to efficiently separate lipids and carotenoids. Therefore, research studies focusing on efficient downstream recovery methods to obtain high productivity of β-carotene at an industrial scale are necessary.

**Table 5** Impact of different carbon sources (at 10 g/L) on biomass and β-carotene production by *R. glutinis*.

| Carbon source tested | Glucose (g/L) | Xylose (g/L) | Yeast cells production (g/L) | β-carotene (mg/L) |
|---|---|---|---|---|
| | Variables | | Responses | |
| Glucose | 10 | - | 5.361±0.069 | 128.43±2.95 |
| Xylose | - | 10 | 5.098±0.073 | 119.75±2.88 |
| Glucose + Xylose | 8 | 2 | 5.256±0.082 | 124.29±3.67 |
| SCBH | 6.5 | 3.4 | 5.803±0.066 | 118.56±3.01 |

## 4. Conclusion

The sodium hydroxide pretreatment with high solids loadings effectively removed lignin, leading to a significant increase in the recovery of second-generation (2G) sugars via enzymatic hydrolysis of sugarcane bagasse (SCB). This research demonstrates that the fermentable 2G sugars obtained from SCB can be used as a readily available raw material for carotenoid production by *R. glutinis*, eliminating the necessity for asparagine supplementation as a nitrogen source. Subsequent investigations will evaluate the potential of rice bran hydrolysate and soybean hydrolysate as sustainable and renewable nitrogen sources, in combination with sugarcane bagasse hydrolysate, with the aim of creating an efficient and sustainable fermentation medium for β-carotene production in lignocellulosic biorefineries.

**Author Contributions** Erick Díaz-Ruiz completed the experiments, collected the data and contributed in manuscript writing; Thércia R. Balbino contributed in planning of the study and helped in analysis of the fermentation samples for caretenoids; Júlio C. dos Santos helped in constructive discussion on experiments and data analysis; Vinod Kumar contributed in manuscript writing and constructive discussion; Silvio S. da Silva provided some infrastructure for performance of experiments and helped in manuscript editing; Anuj K. Chandel planned the study, and provided basic infrastructure, data analysis, checked the manuscript. All authors read and approved the final manuscript.

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**Data Availability** Data available on request from the authors.

**Declarations:**

**Ethics Approval** Not applicable.

**Consent for Publication** Not applicable.

**Competing Interests** There are no conflicts of interest to declare.

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**Figure 1** Enzymatic hydrolysis profile (5% of TS) of different pretreated biomasses, for glucose (A) and xylose (B) recovered. - The SCB was pretreated at 10(■), 15(●) and 20(▲) %(m/m) of TS.

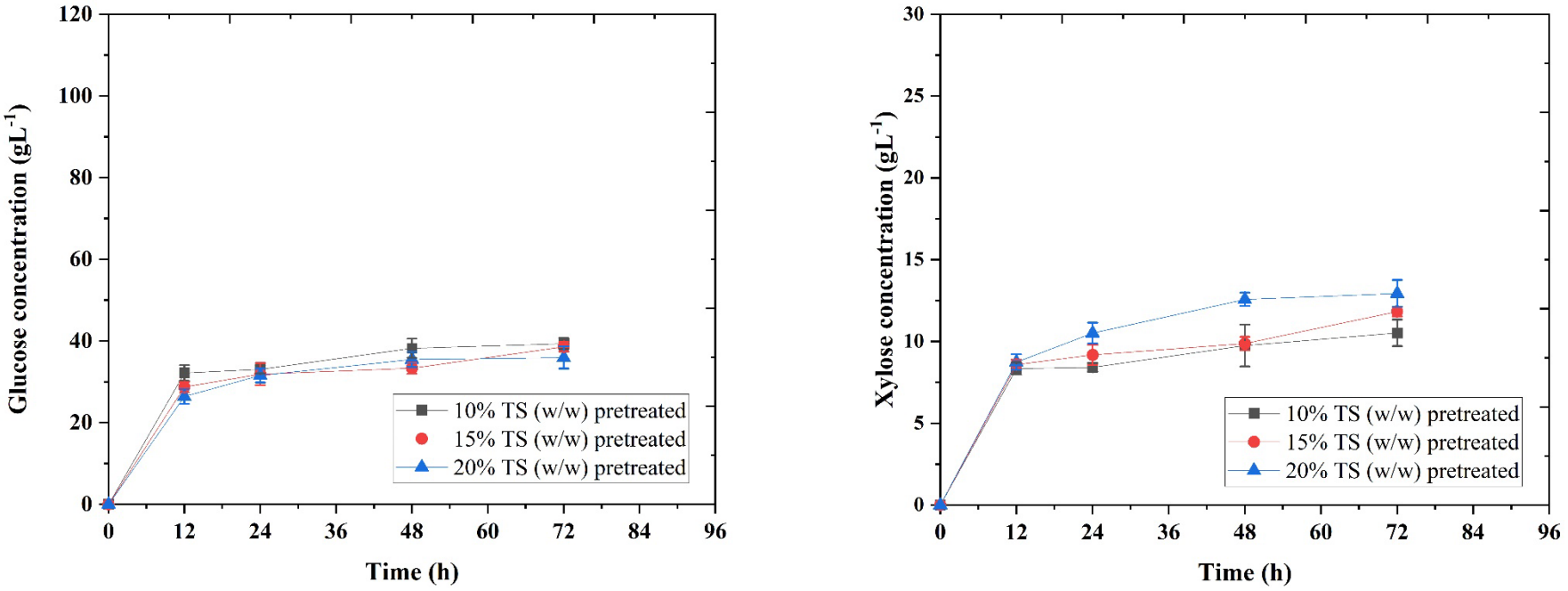


**Figure 2** Enzymatic hydrolysis profile (10% of TS) of different pretreated biomasses, for glucose (A) and xylose (B) recovered. - The SCB was pretreated at 10(■), 15(●) and 20(▲) %(m/m) of TS.

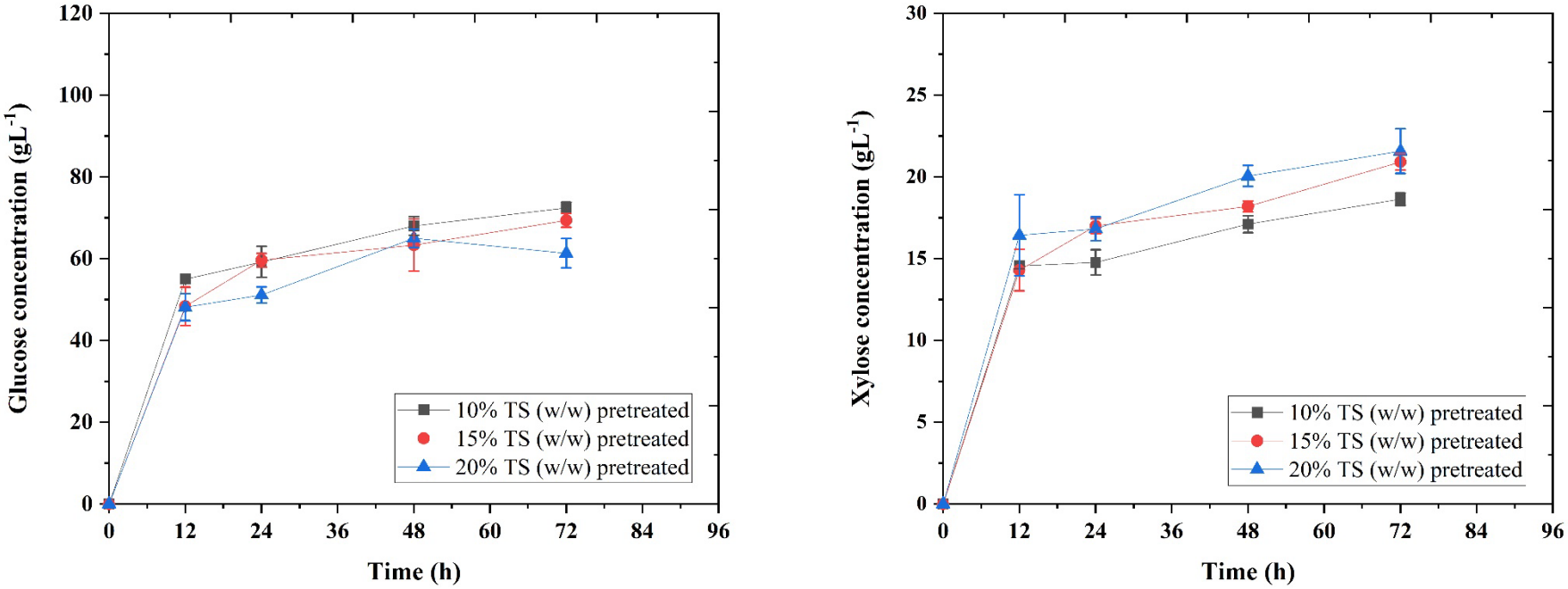


**Figure 3** β-carotene and biomass production using different carbon source concentrations after 72 h of fermentation by *R. glutinis* CCT-2186.
[a-e]Different letters indicate significant difference according to Tukey HSD test ($p<0.05$).

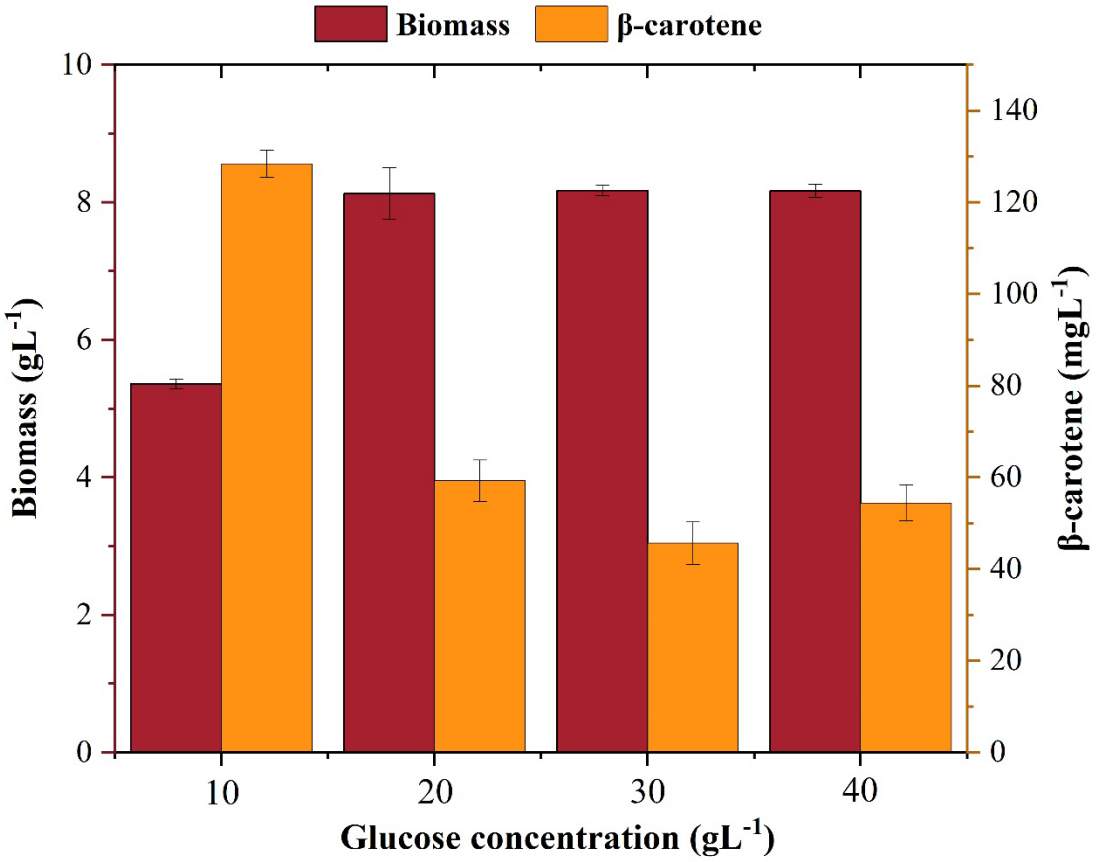
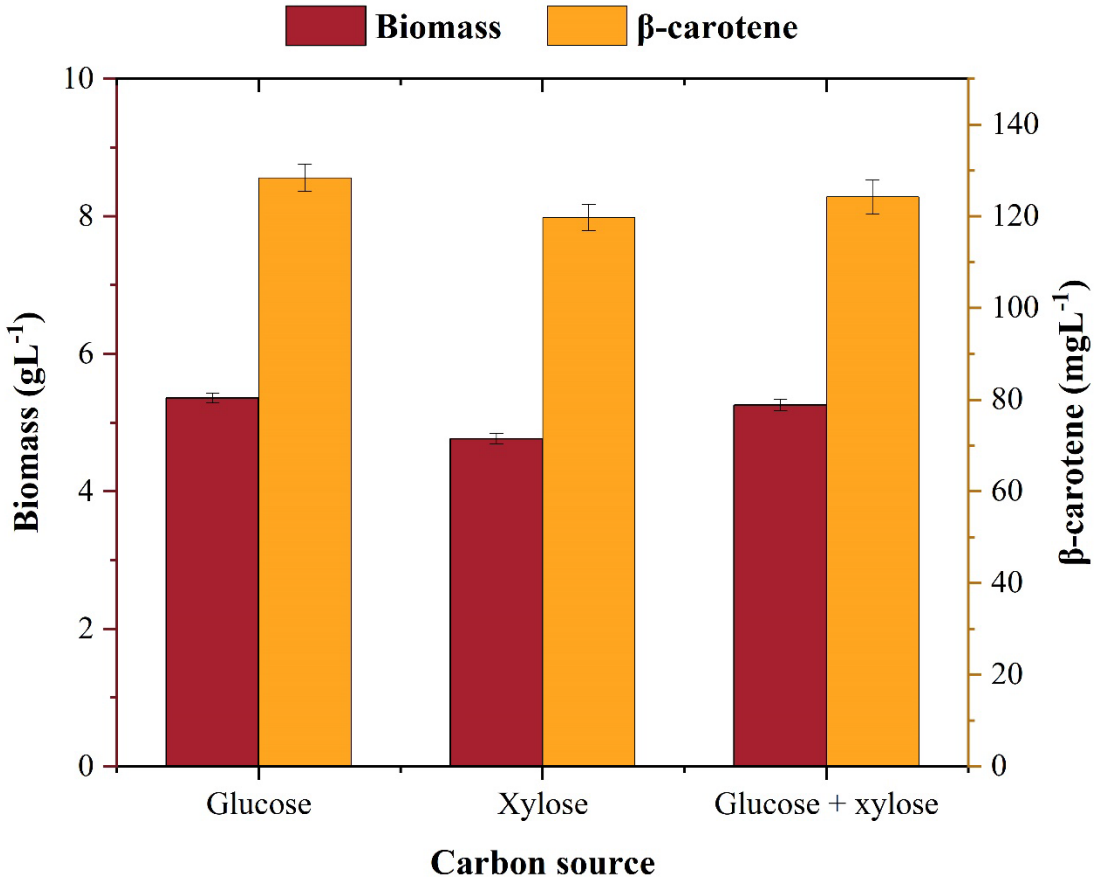


**Figure 4** β-carotene and Biomass production by *R. glutinis* CCT-2186 using glucose, xylose, and mixture of glucose and xylose, at initial concentration at 10 g/L after 72 h of fermentation.
[a-c]Different letters indicate significant difference according to Tukey HSD.



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# Fermentative production of -Carotene from sugarcane bagasse hydrolysate by Rhodotorula glutinis CCT-2186

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