J. Chem. Chem. Eng. 10 (2016) 212-220
doi: 10.17265/1934-7375/2016.05.003

DAVID PUBLISHING

Production of *β*-Carotene by a Newly Isolated *Rhodotorula Glutinis* UCP1555 Strain and Cytotoxic Effect Evaluation

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Abstract: Carotenoids have attracted greater attention due to the beneficial role on human health. It is an essential nutrient and has some biological functions such as species-specific coloration, photoprotection, light absorbing, which is an important component because of its role as precursor of vitamin A. In this study was performed the production of *β*-Carotene by *Rhodotorula glutinis* UCP/WFCC 1555 in presence and absence of blue and white using LED (light-emitting diodes) and evaluation of the cytotoxic effect. The production was investigated in low cost medium constituted by different concentrations of CG (crude glycerin) and CSL (corn steep liquor) from the CCD (Central Composite Design) and the identification and yield of the *β*-Carotene was investigated by chromatographic profile (HPLC). Additionally, the *β*-Carotene produced was tested to evaluate its cytotoxic effect in human tumor cells MCF-7 (breast cancer) and HL-60 (promyelocytic leukemia) and healthy cells of macrophages. The results showed that in the medium composed by 6% glycerin and 0.6% corn steep liquor, in the absence of light, occurred the maximum production of total carotenoids with values of 160 $\mu g \cdot g^{-1}$ and these 100.60 $\mu g \cdot g^{-1}$ correspond to the *β*-Carotene that showed ability in inhibit cell growth in several tumor cells such as MCF-7 cells (breast cancer) and HL-60 (promyelocytic leukemia).

Key words: *β*-Carotene, *Rhodotorula glutinis*, cytotoxic effect, low-cost medium.

1. Introduction

Commercial production of carotenoids from microorganisms competes primarily with the synthetic production by chemical procedures [1, 2]. Although the variety of producing microorganisms is large, only a few are industrially interesting, such as yeasts that beyond of produce carotenoids are natural sources of proteins [3].

The synthesis of carotenoids produced by yeasts of the genus *Rhodotorula* has been investigated indicating high potential for production of pigments [4].

β-Carotene, a yellow-orange pigment of the carotenoid family, has antioxidant properties [5-8]. This pigment has been employed in pharmaceutical industry, in the production of cosmetics, tanning and food industry [9] and has the protective effect in lowering the risk of cancer [10].

Natural pigments have high importance in the market due to the increase in restrictions of synthetic

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dyes [11]. *β*-Carotene had high value in the global market of $233 million dollars in 2010 and is expected to reach $309 million in 2018 with an annual growth rate of 3.6% [12].

The production of natural carotenoids by biotechnological processes offers advantages compared with synthetics due to the rapid growth of microorganisms versus to higher plants. Thus, the production of by bioprocesses involving microorganisms whose growth speed is relatively high, can ensure a productivity to the process which makes it advantageous [13, 14].

Rhodotorula glutinis is strictly aerobic oleaginous yeast that has been used in the production of specific carotenoids as *β*-Carotene (precursor of vitamin A), torulene and torularhodin in process fermentative [15].

The cellular toxicity is a complex event and its expression can manifest itself in a broad spectrum of events that ranging from complex metabolic aberrations to the cell death [16]. Thus, the in vitro cytotoxic evaluation allows to determine the safety substances to be used having as aim to ensure that the product is free of undesirable toxic effects [17].

This study aimed to produce *β*-Carotene by *Rhodotorula glutinis* UCP/WFCC 1555, in economic medium employing biotechnological strategies to biomolecule production of high commercial value and preliminarily elucidate the toxic effects of the *β*-Carotene of *R. glutinis* in the different cell lines.

2. Materials and Methods

2.1 Microorganism

Rhodotorula glutinis (UCP/WFCC 1555) was isolated from semi-arid soil of the State of Pernambuco, Brazil, and is maintained at 4 °C in the medium YMA (Yeast Mold Agar) (0.3% malt extract, 0.3% peptone, 1% glucose and 2% agar). The isolated was registered in the Culture Collection of Catholic University of Pernambuco and in the WFCC (World Federation for Culture Collections).

2.2 Substrates

The substrates used for the production of *β*-Carotene were CG (crude glycerin), obtained from the biodiesel production process, and CSL (corn steep liquor), a byproduct from the corn industry which is rich in amino acids.

2.3 Culture Conditions

The cultivation of *R. glutinis* was carried out in Erlenmeyer flasks containing 100 mL of Sabouraud medium (glucose 20 g, peptone meat 3 g and distilled water 1,000 mL) and maintained in orbital shaking at 150 rpm and 28 °C, for 24 h. After this period, 5% of Sabouraud broth containing 10^7 cells/mL was used as pre-inoculum.

2.4 Influence of Light

The effect of blue and white LED (light-emitting diodes) was tested on all media of production of total carotenoids in *β*-Carotene as attempt to induce the increased production. The control was incubated in the absence of light.

2.5 Selection of Production Medium

The production of total carotenoids as *β*-Carotene by *R. glutinis* was investigated in different culture media. Initially, it was used the YPD medium (dextrose, peptone and extract yeast) proposed by Martinez et al. [18] and modified by the following composition: 10% glucose, 0.8% peptone and 0.3% yeast extract. This medium was used by Martinez et al. [18] and Dai et al. [19] as standard for growth of *R. glutinis*. Subsequently, replacements of carbon and nitrogen sources were carried out in the modified medium (Table 1).

2.6 Experimental Design

A 2^2 CCD (Central Composite Design) with 4 central points and 4 axial points (Table 2) was carried out to evaluate the influence of concentrations of the chemical composition in the media on the production of biomass and total carotenoids. The data of the

Table 1 Composition of the production media of total carotenoids in β-Carotene by *Rhodotorula glutinis.*

Media production	Chemical composition (%)				
	D-Glucose (%)	Crude glycerin (%)	Yeast extract (%)	Corn steep liquor (%)	Peptone (%)
*YPD	10	-	0.8	-	0.3
Modified (I)	10	-	-	0.8	0.3
Modified (II)	-	10	0.8	-	0.3
Modified (III)	-	10	-	0.8	0.3

*YPD medium modified.

Table 2 Levels of the 2^2 CCD used to production of biomass and total carotenoids.

Factors*	Levels				
	-1.41	-1	0	1	-1.41
Crude glycerin (%)	4.36	6.0	10	14	15.64
Corn steep liquor (%)	0.51	0.6	0.8	1.0	1.08

*Added peptone (0.3%) in all the culture media.

experimental design were analyzed by Statistic software program Windows version 6.0 Statsoft®.

2.7 Determination of Biomass Yield

Cells were collected from the cultivation media by centrifugation at 10.000 g and were washed twice with sterile distilled water. The centrifugation process was repeated and the sample was lyophilized. The yield of biomass was calculated by gravimetry and results were expressed in $g \cdot L^{-1}$ of dry biomass.

2.8 Extraction and Partition of Carotenoids

The total carotenoids were extracted in acetone after cell disruption by maceration method [20]. For this method, 2 g of biomass of *R. glutinis* were transferred to vessels containing acetone (50 mL). The extract was filtered with filter paper (Whatman nº1) and performed the partition in separatory funnel added 40 mL of petroleum ether and 300 mL of distilled water.

After phases separation, was discarded the lower aqueous phase. Washing was repeated three times with distilled water to removal of acetone. The ether phase was collected in volumetric flask of 50 mL, covered with aluminum foil, passing the solution through a glass funnel containing 15 g of anhydrous sodium sulfate to remove residual water. The volume was completed to 25 mL with petroleum ether [21].

2.9 Quantification of Total Carotenoids

The quantification of total carotenoids was carried by spectrophotometry at 450 nm (λ_{max}) in the UV-visible, according to protocol described by Rodriguez-Amaya[20]. The extinction coefficient used was relative to the β-Carotene to petroleum ether $E^{1\%}_{1cm}$ = 2,592. The concentration of total carotenoids was calculated using the formula described by Davies [22]:

$$CT(\frac{\mu g}{100g}) = 100(\frac{A \times V \times 10^4}{E^{1\%}_{1CM} \times m})$$

where, A = absorbance at 450 nm; V = final volume of the sample (mL); m = weight of sample (g) and $E^{1\%}_{1cm}$= extinction coefficient of the β-Carotene in petroleum ether.

2.10 Identification and Quantification of β-Carotene

The identification and quantification of β-Carotene produced by *R. glutinis* were performed in accordance to the chromatographic behavior of the standard beta-carotene (Sigma) by high-performance liquid chromatography-HPLC [23]. This analysis was developed with a column chromatography Sunfire, Waters C_{18}, 4.6 × 250 mm.

The mobile phase was composed mobile phase consisting of methanol: ethyl acetate: acetonitrile (50:40:10), with flow 0.6 $mL \cdot min.^{-1}$, run time 7.5

minutes and 30 °C oven temperature. Detection was performed using chromatograms read in wavelength of 450 nm. The solvents used were chromatographic grade and all the samples were filtered through a polyethylene membrane with pore 0.22 µm.

The identification of *β*-Carotene in the sample was performed by comparing the retention time obtained with the standard *β*-Carotene and the quantification was performed after construction of the standard curve with six different concentrations (0.05-0.5 µg/mL) using the peak area for the calculation of the concentration [20].

2.11 Evaluation of the Cytotoxic Activity of β-Carotene

The cytotoxic activity was performed using the reduction method MTT ([3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] [24, 25]. The J744.A1 cells (murine macrophage) were maintained in DMEM culture medium and the human tumor cell HL-60 (human promyelocytic leukemia) and MCF-7 (breast cancer) were maintained in RPMI culture medium. The medium was supplemented with 10% fetal bovine serum and 1% antibiotic solution (penicillin and streptomycin). The cells were maintained in incubator at 37 °C in atmosphere enriched with 5% CO_2. The MCF-7 (10^5 cells/mL), HL-60 (3×10^5 cells/mL) and J744.A1 (1.75×10^5 cells/mL) cells were plated in 96 well plates and incubated for 24 h. Subsequently, the *β*-Carotene extract produced by *R. glutinis* was dissolved in DMSO (1%) and added to the wells in final concentration of 25 µg/mL.

The *β*-Carotene (SIGMA) at a concentration of 25 µg was used as standard. After 72 h of re-incubation was added 25 µL of MTT (5 mg/mL) and after of 3 h incubation, the cultivation medium with MTT was aspirated and 100 µL DMSO was added to each well. The absorbance was measured in reader of microplate at a wavelength of 560 nm. The experiments were analyzed with the program Graph Pad Prism 5.0. Each sample was tested in triplicate. A scale of intensity was used to evaluate the cytotoxic effect of the tested samples of *β*-Carotene: absence activity (1 to 20% inhibition), with little activity (cell growth inhibition varying from 20 to 50%), with moderate activity (cell growth inhibition varying from 50 to 70%) and much activity (growth inhibition varying from 70 to 100%) [26].

3. Results and Discussion

3.1 Production of Total Carotenoid by Rhodotorula Glutinis under Influence of Blue and White LEDs (Light- Emitting Diodes) and in the Absence of Light

R. glutinis is grown in absence of light (control) in YPD medium containing glucose as sole carbon source and produced 123.12 $\mu g \cdot g^{-1}$ of total carotenoids. However, the higher production of the total carotenoids (127.86 $\mu g \cdot g^{-1}$) occurred in modified medium (III) consists of crude glycerin (10%), corn steep liquor (0.8%) and peptone (0.3%), after 96 h of cultivation under the influence of blue LED (Fig. 1). Lababpour et al. [27] and Tatsch [28] affirmed that the light can be used as a factor influencing the production of carotenoids.

3.2 Effect of the Concentrations of Culture Medium Components

Based on the initial results of higher productivity in total carotenoids induced by blue LED (light-emitting diodes), the modified medium (III) was used for further investigations.

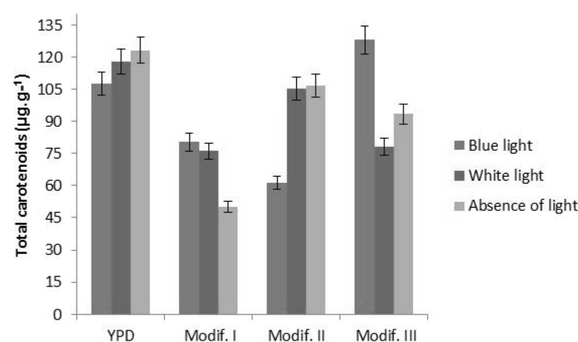

Fig. 1 Total carotenoid produced by *Rhodotorula glutinis* in YPD media and modified media I, II and III.

A 2^2 CCD was carried out to analyze the effect of the concentrations of culture medium components on the biomass production (g·L^{-1}) and total carotenoids (µg·g^{-1} of dry biomass) as response variables.

The results showed that the condition 1 of the CCD allowed the increase of the production of total carotenoids (156.65 µg·g^{-1}) induced by blue LED (Table 3). In this condition, the culture medium was composed by minimal levels of CG (6%) and CSL (0.6%).

The condition 8 of the CCD resulted in higher biomass yield (4.98 g·L^{-1}), in medium with high level of CSL (1.082%) and intermediate levels of CG (10%). Peptone (0.3%) was constant in all the conditions of CCD (Table 3).

Total carotenoids, thereby as relative amounts of pigment, vary according to the species of *Rhodotorula* and different conditions of the medium [29]. In this context, the total carotenoid content produced in this study were higher than those produced by *R. lactosa* (105.8 µg·g^{-1}) and *R. minuta* (103.7 µg·g^{-1}) in medium containing glucose as only carbon source [30] and similar to the results obtained by Squina and Mercadante [31] that resulted in 123.5 µg·g^{-1} of total carotenoids by *Rhodotorula rubra.*

The analysis of variance (ANOVA) of the data from 2^2 CCD determined the independent factors that had the greatest influence on the production of carotenoids. Statistical analysis shows that small values of p. associated to the higher values of F validate statistical model effects once and demonstrate that effects were higher than the standard error (Table 4).

The evaluation of glycerin concentrations and corn steep liquor on the L.T (linear term) and Q.T (quadratic term), thereby as their interactions are represented by diagram with 95% of confidence (Fig. 2).

Table 3 Results of the CCD for biomass production and total carotenoids by *Rhodotorula glutinis* induced by blue LED.

Conditions	Glycerin (%)	Corn steep liquor (%)	Total carotenoids (µg·g^{-1})	Biomass (g·L^{-1})
1	6.00	0.60	156.65	4.20
2	6.00	1.00	87.63	2.95
3	14.00	0.60	50.01	3.88
4	14.00	1.00	120.62	3.66
5	4.36	0.80	100.75	3.01
6	15.64	0.80	62.38	2.97
7	10.00	0.51	111.23	3.61
8	10.00	1.08	128.06	4.98
9	10.00	0.80	124.32	4.00
10	10.00	0.80	127.10	3.94
11	10.00	0.80	123.86	3.61
12	10.00	0.80	123.41	3.20

*Added peptone (0.3%) in all conditions.

Table 4 ANOVA (analysis of variance) of the significant factors.

Variable response	Independent variables	Sum of squares	Degree of freedom	Mean of the squares	F-Valor	Confidence level (p)
T.C (Total carotenoids)	Glycerin$_{(L.T)}$	1,065.42	1	1,065.42	604.82	0.000147
	Glycerin$_{(Q.T)}$	1,902.88	1	1,902.88	1,080.2	0.000062
	Corn steep liquor$_{(L.T)}$	114.87	1	114.877	65.214	0.003967
	Con steep liquor $_{(Q.T)}$	25.87	1	25.873	14.688	0.0311309
	Assoc. $_{Glic.(L.T)/Milhoc.(L.T)}$	2,585.72	1	2,585.72	1,467.8	0.0000039
	Adjustment of error	11.32	3	3.775	2.143	0.273716
	pure error	5.28	3	1.762		
	Total sum of squares	5,699.30	11			

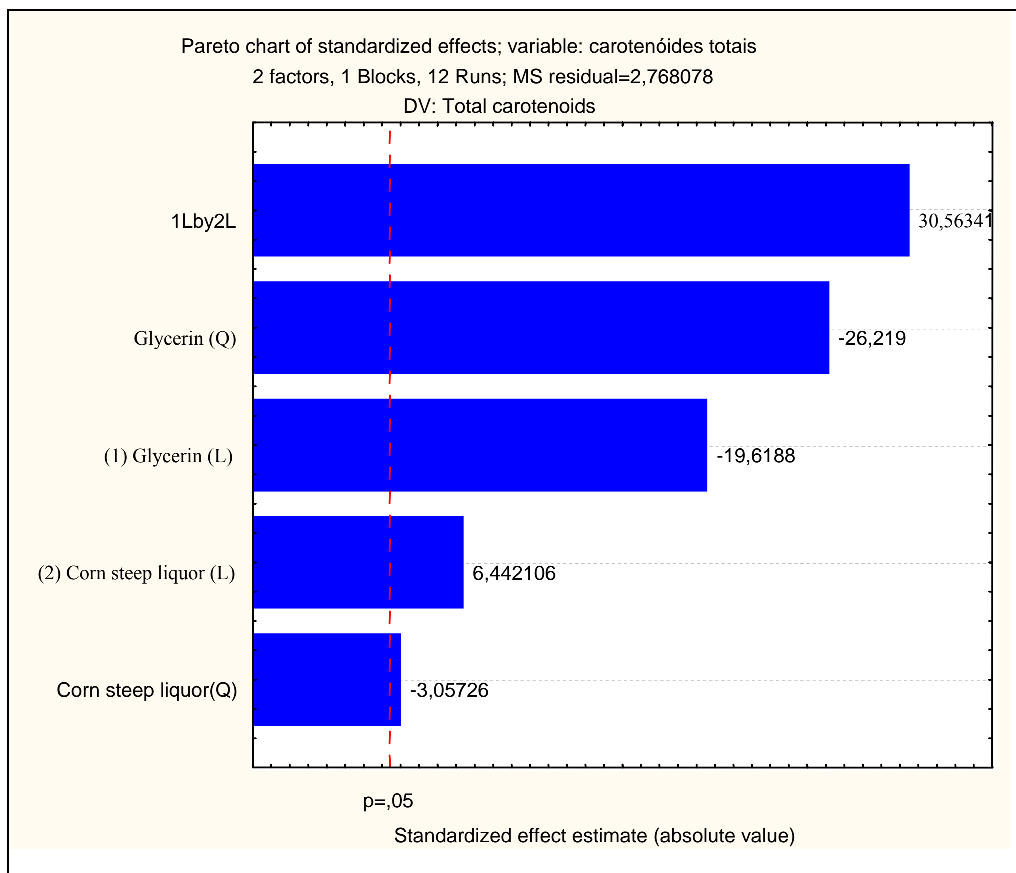

Fig. 2 Pareto diagram for studies of the effects of the independent variables glycerin and corn steep liquor on the production of carotenoids by *Rhodotorula glutinis.*

According to Pareto diagram the association between glycerin and corn steep liquor in linear contributions presented greater influence in the production of total carotenoids. This indicated the reach of optimal concentrations to the culture medium under on conditions tested in this work.

Moreover, all independent variables were significantly represented with values above the line p.

3.3 Identification of the β-Carotene by Rhodotorula Glutinis by HPLC

The identification and yield of the production of *β*-Carotene was investigated by chromatographic profile (HPLC) using as standard the *β*-Carotene (Sigma) (Fig. 3A). Fig. 3B shows the chromatographic profile (HPLC) of the *β*-Carotene produced by *R. glutinis* in medium consisting of 6%, glycerin, 0.6% CSL and 0.3% peptone in presence of blue light and the Fig. 3C shows the identification of *β*-Carotene produced by *R. glutinis* in the same medium in the absence of blue light.

The *β*-Carotene standard was identified by RT (retention time) = 8.850 min. Similar results were found in the *β*-Carotene extracted from biomass of *R. glutinis* (RT = 8.851 min.) produced in medium consisting of 6% glycerin, 0.6% CSL and 0.3% peptone in presence of blue light.

In the same medium in the absence of blue light the retention time found was similar to the standard (RT = 8.844 min), confirming the presence of *β*-Carotene in sample.

The yield of *β*-Carotene in medium containing blue light was 82.02 µg/g and in medium with absence of blue light was 100.60 µg/g of *β*-Carotene. These results show that the higher yields of *β*-Carotene were obtained after removal of blue light of the production process ensuring a low-cost production.

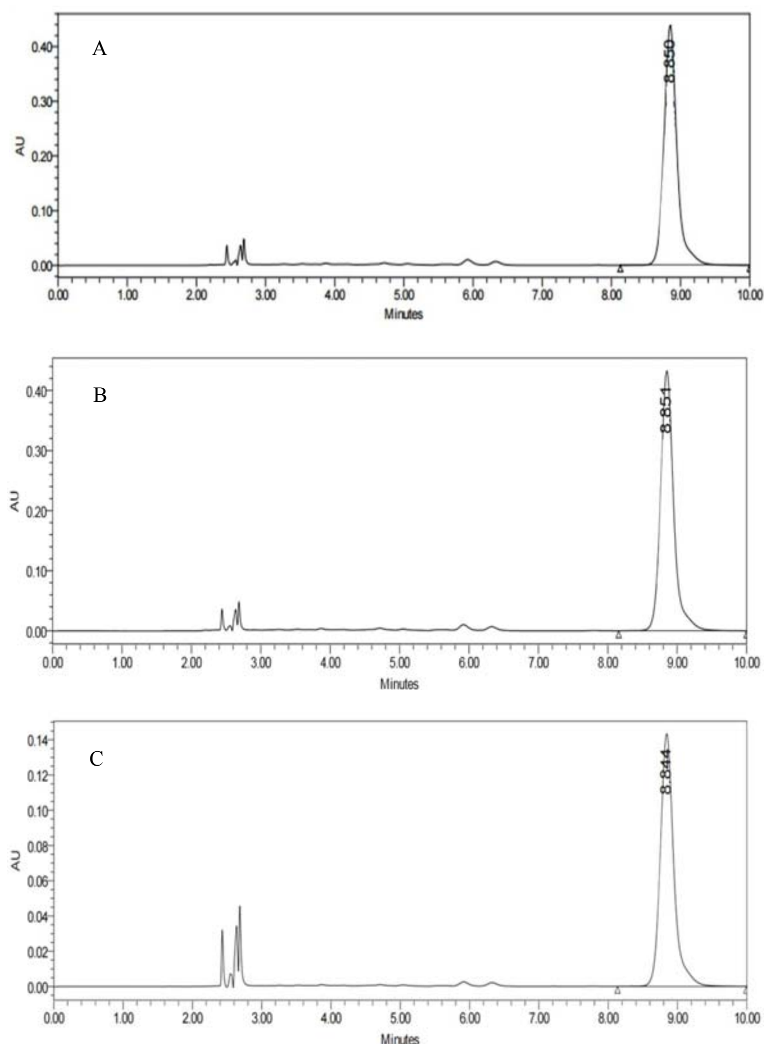

Fig. 3 Chromatogram of *β*-Carotene produced by *Rhodotorula glutinis*: (A) Standard *β*-Carotene (Sigma); (B) *β*-Carotene produced in medium consisting of 6% glycerin, 0.6% corn steep liquor and 0.3% peptone in blue light and (C) *β*-Carotene produced in medium consisting of 6% glycerin and 0.6% corn steep liquor and 0.3% peptone, in the absence of blue light.

The production of *β*-Carotene by *R. glutinis* in this study was similar to that obtained by Neto [32], which worked with a strain of *R. glutinis* that was able of *β*-Carotene produce in medium containing cashew juice as carbon source. The production ranged from 5.0 to 120 mg/g of *β*-Carotene.

3.4 β-Carotene Cytotoxicity

The cytotoxic activity of carotenoids on many cancer cell lines has been attributed to their prooxidant activity through the generation of ROS [33]. In this context, in this work the *β*-Carotene produced by *R. glutinis* in medium consisting of 6% glycerin and 0.6% CSL and 0.3% peptone, was tested to evaluate its cytotoxic activity in human tumor cells MCF-7 (breast cancer) and HL-60 (promyelocytic leukemia) and healthy cells of macrophages (J774.A1) (Table 5).

The results demonstrated that the sample of *β*-Carotene of *R. glutinis* inhibited cell growth of MCF-7 (cells of breast cancer) and J774.A1 (cells of macrophages) in 27.93% and 21.72%, respectively, resulting in low cytotoxic activity. However, the cytotoxic activity of *β*-Carotene of *R. glutinis* was moderate against the HL-60 cell (promyelocytic leukemia). *β*-Carotene has the potential to inhibit cell growth of various tumor cells such as melanoma [17],

Table 5 Evaluation of the cytotoxic effect of *β*-Carotene produced by *Rhodotorula glutinis.*

Test product	% Inhibition of cell growth					
	MCF-7	S.E	HL-60	S.E	J774.A1	S.E
β-Carotene sample	27.93	1.42	61.17	0.00	21.72	1.43
β-Carotene standard	25.49	1.75	35.86	0.38	23.53	2.34

*Standard error—S.E

prostate [3], colon [11], lung, breast and cancer of the oral mucosa [2]. In this context, the *β*-Carotene produced by *R. glutinis* under the conditions studied in this work demonstrated to be a potent carotenoid effective in inhibiting the cellular growth of tumor cells such as MCF-7 cells (breast cancer) and HL-60 (promyelocytic leukemia).

Acknowledgments

This work was financially supported by CAPES (Coordenação de Aperfeiçoamento de Pessoal de Nível Superior), CETENE (Centro de Tecnologias Estratégicas do Nordeste) and Corn Products (Cabo de Santo Agostinho-PE, Brazil) kindly provided the substrate of corn steep liquor. The authors are also grateful to the NPCIAMB (Nucleus of Research in Environmental Sciences and Biotechnology), Catholic University of Pernambuco (Recife-PE, Brazil) for the use of its laboratories.

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