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Kinetic and Stoichiometric Parameters in the Production of Carotenoids by *Sporidiobolus salmonicolor* (CBS 2636) in Synthetic and Agroindustrial Media

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Abstract With the objective of determining the kinetic behavior (growth, substrate, pH, and carotenoid production) and obtain the stoichiometric parameters of the fermentative process by *Sporidiobolus salmonicolor* in synthetic and agroindustrial media, fermentations were carried out in shaken flasks at 25°C, 180 rpm, and initial pH of 4.0 for 120 h in the dark, sampling every 6 h. The maximum concentrations of total carotenoids in synthetic (913 μg/L) and agroindustrial (502 μg/L) media were attained approximately 100 h after the start of the fermentative process. Carotenoid bioproduction is associated with cell growth and the ratio between carotenoid production and cell growth ($Y_{P/X}$) is 176 and 163 μg/g in the synthetic and agroindustrial media, respectively. The pH of the agroindustrial fermentation medium varied from 4.2 to 8.5 during the fermentation. The specific growth rate (μ_X) for *S. salmonicolor* in synthetic and agroindustrial media was 0.07 and 0.04 h^{-1}, respectively. The synthetic medium allowed for greater productivity, obtaining maximum cell productivity (P_x) of 0.08 g L^{-1} h^{-1} and maximum total carotenoid productivity (P_{car}) of 14.2 μg L^{-1} h^{-1}. Knowledge of the kinetics of a fermentative process is of extreme importance when transposing a laboratory experiment to an industrial scale, as well as making a quantitative comparison between different culture conditions.

Keywords *Sporidiobolus salmonicolor* · Synthetic media · Agroindustrial media · Carotenoids · Kinetic parameters · Stoichiometric parameters

Introduction

Interest in carotenoids has increased in the last few years due to an increasing demand for these compounds in the food, pharmaceutical, cosmetic, and animal feed industries. The

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development of biotechnological processes aims at increasing the carotenoid yield and reducing process costs by using low-cost industrial substrates rich in sugars and organic material. In the national context, Brazil is rich in by-products of this nature. In addition, the carotenoids produced are considered to be natural substances; the production can be carried out in a limited space and is not subject to environmental conditions such as the climate, and it is possible to control the cultivation conditions to guarantee production of carotenoids of high importance [1].

The types of carotenoid and relative amounts produced can vary depending on the microorganism, the culture medium, and the operation conditions (temperature, pH, aeration rate, and luminosity). Most of the studies carried out aimed to optimize the culture conditions that directly affected growth of the microorganism and carotenoid production [2–7].

Knowledge of the kinetics of a fermentative process is of extreme importance when transposing a laboratory experiment to an industrial scale, as well as when a quantitative comparison between different culture conditions is required [8–9]. No systematic studies to obtain kinetic and stoichiometric parameters for carotenoid bioproduction by yeasts were found in the literature. Thus, the objective of the present study was to obtain kinetic and stoichiometric parameters for the growth and carotenoid bioproduction by *Sporidiobolus salmonicolor* using synthetic and agroindustrial media.

Material and Methods

Kinetics of the Fermentative Process

The yeast *S. salmonicolor* (CBS 2636) was used in trials to obtain the stoichiometric parameters and determine the growth kinetics, substrate consumption, pH evolution, and carotenoid production using synthetic and agroindustrial media.

The fermentation trials were carried out under the conditions optimized for the synthetic (40 g/L glucose, 10 g/L malt extract, and 14 g/L peptone) and agroindustrial (5-g/L pretreated corn steep liquor, 10-g/L pretreated sugar cane molasses, and 5 g/L yeast hydrolysate—Prodex Lac®) media. The medium (100 mL) was placed in 250-mL conical flasks, sterilized (121°C, 15 min), inoculated (10% *v*/*v*), and incubated in an orbital shaker (Nova Ética, RDB-430) at 25°C and 180 rpm, in the dark, for 120 h. Initial pH was 4.0, and samples were withdrawn every 6 h [10].

The agroindustrial substrates were pretreated with acids according to the methodology described by Valduga et al. [11]. The corn steep liquor and sugar cane molasses were used at a concentration of 100 g/L. Initially, the pH values of the molasses and corn steep liquor were adjusted to 3.0 with 1 N sulfuric acid and 1 N phosphoric acid, respectively. The substrates remained in this condition for 24 h at 24°C and were then centrifuged (Eppendorf 5403) at 5,000 rpm for 15 min. The pH of the supernatant was adjusted to 4.0 for the molasses and to 5.5 for the corn steep liquor, using a 2-N NaOH solution (Vetec).

The biomass was determined directly using a gravimetric method, after drying in an oven (Fanem, SE-320) at 105°C to constant weight. Substrate consumption was measured from the glucose content present in the fermentation broth, which was determined by the enzymatic method—Glucose PAP, liquiform (LABTEST, Diagnóstica, 2005). The pH values were determined using a pH meter (DMPH-2, Digimed), and the carotenoid production was estimated from the maximum absorbance at 448 nm in a spectrophotometer (Agilent 8553) using the equation described by Davies [12], after recovering the

carotenoids using liquid nitrogen combined with dimethyl sulfoxide (Nuclear) for cell rupture and extraction with a (7:3) mixture of acetone (Quimex)–methanol.

Stoichiometric Parameters

Conversion Factors

The conversion factor for the substrate in the product, $Y_{P/S}$ (microgram carotenoids per gram glucose), was expressed by Eq. 1 [9].

$$Y_{P/S} = r_X/r_S \tag{1}$$

The conversion factor for the substrate in the biomass, $Y_{X/S}$ (gram cells per gram substrate), was expressed by Eq. 2 [9].

$$Y_{X/S} = r_X/r_S \tag{2}$$

The ratio between carotenoid production and cell production, $Y_{P/X}$ (microgram carotenoids per gram cells), was expressed by Eq. 3 [9].

$$Y_{P/X} = r_P/r_X \tag{3}$$

Where: r_x=the cell growth rate (g/L h); r_s=the substrate consumption rate (g/L h); r_p=the carotenoid production rate (g/L h).

Productivity

The instantaneous productivity in cells and carotenoids in a batch system at constant volume is defined as the r_x or r_p rate, respectively.

Instantaneous and Specific Rates

The rates of microbial growth (r_x), product formation (r_p), and substrate consumption (r_s) can be determined by the mass balance for each component at a given time. as presented in Eqs. 4, 5, and 6, respectively [9].

$$r_X = \mathrm{d}X/\mathrm{d}t \tag{4}$$

$$r_P = \mathrm{d}P/\mathrm{d}t \tag{5}$$

$$r_S = -\mathrm{d}S/\mathrm{d}t \tag{6}$$

The specific rates for growth (μ_x), product formation (μ_p), and substrate consumption (μ_s) can be obtained dividing the instantaneous rates by the cell concentration, as expressed by Eqs. 7, 8, and 9, respectively [9].

$$\mu_x = r_X/X \tag{7}$$

$$\mu_p = r_P/X \tag{8}$$

$$\mu_s = r_s/X \tag{9}$$

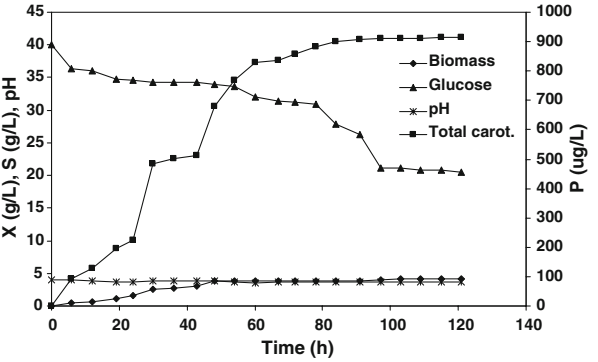

Fig. 1 Kinetics of *S. salmonicolor* (CBS 2636) growth, substrate consumption, carotenoid production, and pH variation in the synthetic medium (40 g/L glucose, 10 g/L malt extract, and 14 g/L peptone), at 25°C and 180 rpm

Carotenoid Analysis

The extract with the carotenoids was lyophilized (Edwards) and redissolved in 3 mL of acetone (Merck). Carotenoid separation and quantifications were carried out by an high-performance liquid chromatography (HPLC) system (Agilent 1100 Series), consisting of a quaternary pump, automatic injector (20-μL loop), degasser, column heater, an diode array detector, and a data acquisition system.

The carotenoids were fractionated in a C_{18} column (Agilent, 5 μm, 4.6×250 mm) and the eluted compounds were analyzed at 470 nm. Mobile phase was tetrahydrofuran (Merck)–water–methanol (Merck) in a ternary gradient, keeping the ratio 15:4:81 for 40 min and then changing the solvent ratio to 35:0:65, which was kept until the end of analysis (70 min). Flow rate was 0.5 mL/min and injection volume 20 μL [13]. The solvents were of HPLC grade and were filtered in 0.45-μm polyvinylidene fluoride membranes (Millipore) before use. Samples were also filtered before injection.

Identification and quantification of β-carotene was carried out by injection of authentic standard solutions of this carotenoid (100 μg/L β-carotene, Sigma).

Results and Discussion

Stoichiometric Parameters

Figures 1 and 2 show the growth kinetics, substrate consumption, pH evolution, and carotenoid consumption for the synthetic and agroindustrial media, respectively.

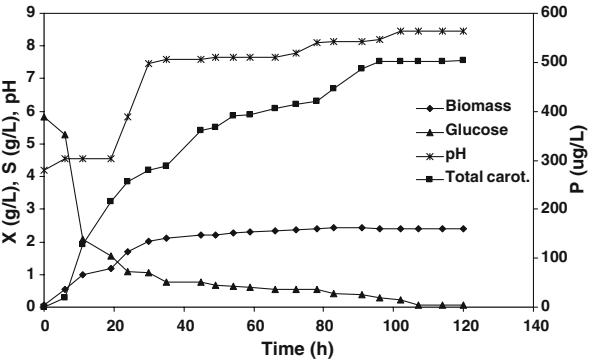

Fig. 2 Kinetics of *S. salmonicolor* (CBS 2636) growth, substrate consumption, carotenoid production, and pH variation in the agroindustrial medium (5-g/L pretreated corn steep liquor, 10-g/L pretreated sugar cane molasses, and 5 g/L yeast hydrolysate—Prodex Lac®), at 25°C and 180 rpm

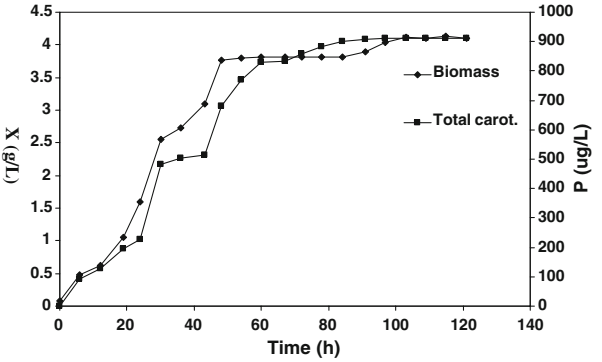

Fig. 3 Kinetics of *S. salmonicolor* (CBS 2636) growth, showing the association of the carotenoid production with cell growth in the synthetic medium (40 g/L glucose, 10 g/L malt extract, and 14 g/L peptone)

As shown in Figs. 1 and 2, the maximum total carotenoid values in the synthetic (913 μg/L) and agroindustrial (502 μg/L) media were attained approximately 100 h after the start of the fermentative process. At this point, the glucose concentration observed was about 20 g/L in the synthetic medium and 0.2 g/L in the agroindustrial medium.

The pH of the fermentation medium containing synthetic substrates (glucose, malt extract, and peptone) only showed a slight variation during the fermentative process (120 h), decreasing from 4.0 to 3.7 (Fig. 1). However, when using agroindustrial substrates (corn steep liquor, sugar cane molasses, and yeast hydrolysate) for carotenoid bioproduction (Fig. 2), the pH_{final} of the fermentation medium increased considerably (~8.5), evolving progressively from 4.2 to 7.5 in the first 30 h, followed by a slower increase to values of 7.65 in the following 36 h. After 96-h fermentation, the pH rose to 8.46, where it remained up to the end of the fermentative process (120 h). These changes in pH may be related to proteolysis of the microorganism, a natural phenomenon that occurs after complete substrate exhaustion as a consequence of amino acid degradation, with the formation of ammoniacal compounds that cause the pH to rise, principally when the strain cannot use another carbon source. This can also be explained from the fact that the yeast hydrolysate concentration had a significantly positive effect ($p<0.05$), indicating that, in the range studied, an increase in the concentration of this component in the medium led to an increase in pH value during cultivation and that this was also significantly influenced by the positive effects of the interaction between the concentrations of molasses and yeast hydrolysate [11]. Mantzouridou et al. [14] showed an increase in the fermentation pH from 5.0 to 7.5 during

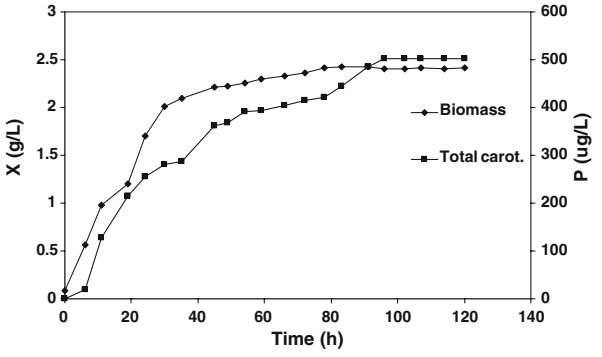

Fig. 4 Kinetics of *S. salmonicolor* (CBS 2636) growth, showing the association of the carotenoid production with cell growth in the agroindustrial medium (5-g/L pretreated corn steep liquor, 10-g/L pretreated sugar cane molasses, and 5 g/L yeast hydrolysate—Prodex Lac®)

Table 1 Kinetic and stoichiometric parameters for the carotenoid production by *S. salmonicolor* (CBS 2636) in synthetic (40 g/L glucose, 10 g/L malt extract, and 14 g/L peptone) and agroindustrial (5-g/L pretreated corn steep liquor, 10-g/L pretreated sugar cane molasses, and 5 g/L yeast hydrolysate—Prodex Lac®) media.

Parameter	Synthetic medium			Agroindustrial medium		
Fermentation phases	Phase 1 (0–48 h)	Phase 2 (48–78 h)	Phase 3 (78–121 h)	Phase 1 (0–30 h)	Phase 2 (30–78 h)	Phase 3 (78–120 h)
P_x (g/L h)	0.08	0.01	0.01	0.06	0.01	0
P_{CAROT} (μg/L h)	14.2	10.7	0.7	10.4	2.5	1.9
$Y_{P/S}$ (μg/g)	206.8	39.3	1.38	53.5	378.6	3.0
$Y_{X/S}$ (g/g)	1.20	0.03	0.03	0.30	0.91	0.06
$Y_{P/X}$ (μg/g)	176.1	319.57	35.1	163.0	295.8	-
μ_X (h^{-1})	0.073	0.001	0.001	0.041	0.036	0

Phase 1 linear, *Phase 2* deceleration, *Phase 3* stationary

19 days of cultivation, when producing carotenoids from *Blakeslea trispora* in corn steep liquor, olive oil, soybean oil, and supplements (yeast extract, casein hydrolysate, asparagines, KH_2PO_4, $MgSO_4$, and thiamine). The authors also attributed this increase to deamination of amino acids from the corn steep liquor.

According to Frengova et al. [15], the biosynthesis of carotenoids causes pH changes in the fermentation medium as a consequence of yeast growth. In general, the pH of a fermentation medium decreases in the first 72 h of fermentation and then rises during the intense carotenogenesis phase. After this point, the pH remains constant, indicating the end of the fermentative process.

Figures 1 and 2 also show that the growth was not exponential. The rate of growth was constant for the first 54 h in the synthetic medium and for 30 h in the agroindustrial medium. The growth rate subsequently moves to deceleration and stationary phases. The rates of growth were 0.08 and 0.06 g L^{-1} h^{-1}, respectively, for the industrial and synthetic media, respectively.

Carotenoid bioproduction was associated with cell growth (Figs. 3 and 4). The maximum cell (4.1 and 2.4 g/L) and carotenoid (913 and 502 μg/L) concentrations occur simultaneously in the synthetic and in the agroindustrial media. Tinoi et al. [6] showed a similar behavior when carotenoid was produced by *Rhodotorula glutinis* in an agro-industrial-medium-paralleled cell growth, the maximum cell and pigment concentrations occurring simultaneously.

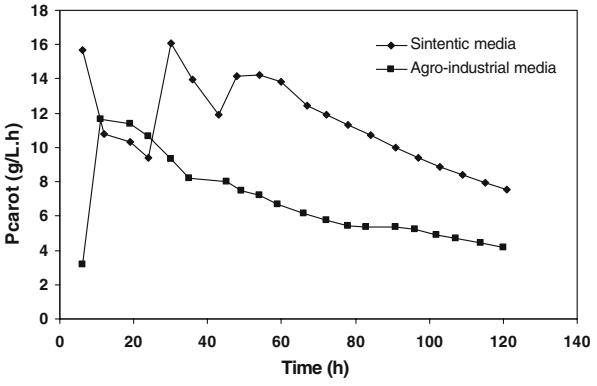

Fig. 5 Comparison of carotenoid productivity between the synthetic (40 g/L glucose, 10 g/L malt extract, and 14 g/L peptone) and agroindustrial (5-g/L pretreated corn steep liquor, 10 g/L pretreated sugar cane molasses, and 5 g/L yeast hydrolysate—Prodex Lac®) media

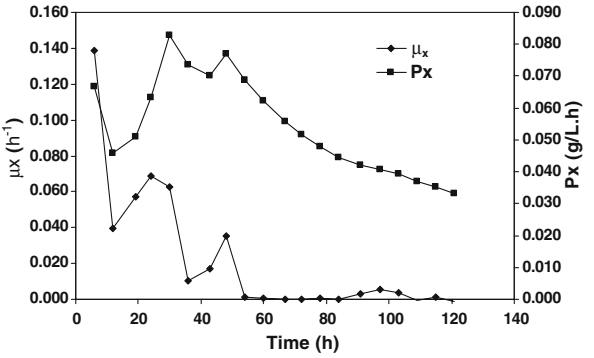

Fig. 6 Evolution of the specific growth rate and cell productivity in the synthetic medium (40 g/L glucose, 10 g/L malt extract, and 14 g/L peptone)

The ratio between carotenoid production and cell growth ($Y_{P/X}$) was 176.1 μg/g in the synthetic medium. This result is close to that obtained in the agroindustrial medium, 163.0 μg/g (Table 1). These values were higher than those obtained by Davoli et al. [7], who produced carotenoids (109 μg/g) using *Sporobolomyces roseus* (D99040) in a synthetic medium contained in straight-type flasks.

Figure 5 shows the carotenoid productivity throughout fermentation. It can be seen that the synthetic medium yielded higher productivities. It seems possible that the pretreatment of the agroindustrial substrates with acids (sulfuric or phosphoric) could have removed nutrients essential for this production or, otherwise, that the treatment used may not have completely removed all the inhibitors present in the medium [11]. Another probable cause of the lower productivity when using agroindustrial substrates would be the metabolism of nitrogen compounds, producing large amounts of ammonia compounds into the medium. This hypothesis confirms the results for pH, which presented a considerable increase during fermentation when using industrial media, contrary to the fermentation in the synthetic medium.

The cell growth rates obtained in the linear phase for the media under consideration were 0.08 and 0.06 h^{-1}, respectively, which, as shown in Figs. 6 and 7, means that the specific growth rates decreased in these intervals. These results suggest that glucose was not the limiting substrate since the behavior was the same in the synthetic medium, and the limitation accentuated with time. Thus, two distinct phases were observed, the linear growth phase and the stationary phase, where the rate was near 0. A well-defined deceleration phase was also observed in the agroindustrial medium.

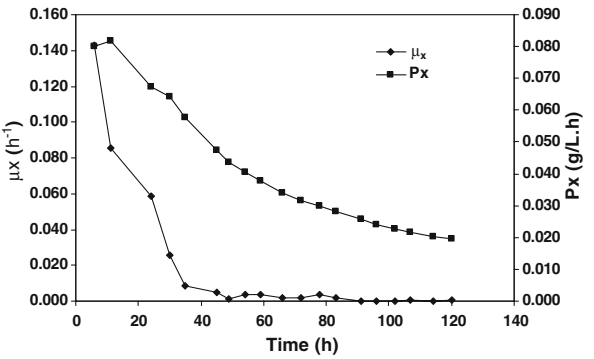

Fig. 7 Evolution of the specific growth rate and cell productivity in the agroindustrial medium (40 g/L glucose, 10 g/L malt extract, and 14 g/L peptone)

Table 2 Partial characterization of carotenoids produced by *S. salmonicolor*.

Peak number	Carotenoid	t_R [b] (min)	Percentage
1[a]	β-carotene	38.23	100.00
2	NI	17.28	2.54
3	NI	34.13	41.8
4	NI	35.82	22.71
5	β-carotene	38.09	32.31
6	NI	45.55	0.64

NI not identified

[a] Standard of β-carotene

[b] Retention time

Table 1 shows the main kinetic parameters for *S. salmonicolor*, calculated for the three fermentation phases observed (linear, deceleration, and stationary) in the synthetic and agroindustrial media. Under these conditions, the maximum specific growth rate (μ_{max}) for *S. salmonicolor* was 0.07 and 0.04 h^{-1}, respectively. The maximum cell productivity (P_x) was 0.08 and 0.06 g L^{-1} h^{-1}, and total carotenoid productivity was 14.2 and 10.4 μg L^{-1} h^{-1} for synthetic and agroindustrial media, respectively.

As shown in Table 1, the conversion factor, $Y_{X/S}$, was 1.2 g/g in the synthetic medium, which is a very high value suggesting that another substrate was also consumed, possibly the nitrogen sources used, such as malt extract and peptone. A much lower value (0.30 g/g) was obtained in the agroindustrial medium, suggesting a preferential consumption of the glucose present in the medium in this case.

In the synthetic medium, the conversion factor $Y_{X/S}$ decreased considerably after 54 h and then remained constant at 0.03 g/g. This indicates that the substrate was consumed with little cell production, which could be an indication of some bioconversion to other products or a marked change in the metabolism. This sharp drop was only found in the agroindustrial medium after 78 h when the cells stopped growing completely.

The same behavior was observed for the conversion factor $Y_{P/S}$ in the synthetic medium. A sharp drop in this yield factor was observed, indicating that the rate of production dropped quicker than the substrate consumption rate. A decrease in this factor in the agroindustrial medium was observed, but it was preceded by an increase between 30 and 78 h of fermentation. In this interval, the results suggest that the rate of production decreases slower than the substrate consumption rate.

Carotenoid Analysis

The HPLC results are presented in Table 2. The major carotenoid produced by this strain was identified as β-carotene (peak #5), which corresponds to 32% of total carotenoid of the samples. Other carotenoids were not identified due to the lack of commercial chromatographic standards.

Maldonado et al. [13] studied the composition of carotenoids produced by Brazilian yeast strains, showing that the genus *Sporobolomyces* produced 237 μg/L of total carotenoids. The major compounds that were produced by this strain were β-carotene (118 μg/L), torulene (71 μg/L), γ-carotene (20 μg/L), β-zeacarotene (18 μg/L), and torulahodin (10 μg/L).

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