Batch and Fed-Batch Production of β-carotene by Rhodotorula Toruloides KP324973 Using Corn Steep Liquor As Sole Carbon Source

Alireza Habibi (a.habibi@razi.ac.ir)
Razi University https://orcid.org/0000-0002-4448-2924
Samira Fallahi
Razi University
Saeed Abbasi
Razi University
Rouhallah Sharifi
Razi University

Research Article

Keywords: Carotenogenesis, Fed-batch operation, Optimization, Bubble column reactor

Posted Date: August 4th, 2021

DOI: https://doi.org/10.21203/rs.3.rs-751445/v1

Batch and fed-batch production of *β*-carotene by *Rhodotorula toruloides* KP324973 using corn steep liquor as sole carbon source

Samira Fallahi[1], Alireza Habibi[2,*], Saeed Abbasi[1], Rouhallah Sharifi[1]

[1] Department of Plant Protection, College of Agriculture, Razi University, Kermanshah, Iran

[2] Faculty of Petroleum and Chemical Engineering, Razi University, Kermanshah, Iran.

[*]Corresponding author email: a.habibi@razi.ac.ir. Phone: +989126466594; Fax: +988334343321, https://orcid.org/0000-0002-4448-2924

Abstract

Application of agro-industrial waste in microbial fermentation is interesting in economic and environmental aspects. Carotenoids production by *Rhodotorula toruloides* KP324973 was investigated using corn steep liquor (CSL) as sole carbon source. Haldane model with constants $\mu_{max} = 0.056$ h^{-1}, $K_S = 1.54$ $vv^{-1}\%$, and $K_I = 58.58$ $vv^{-1}\%$ showed best describe of cell growth kinetics on CLS. A same maximum carotenoid production rate (R_p) about 2.23 μg g_{cell}^{-1} h^{-1} was obtained at initial CSL concentration of 5 v/v% after 72 h and 21 h in batch cultivation in shaken flasks and bubble column reactor (BCR), respectively. Further improvement of carotenogenesis was followed by fed-batch cultivation in BCR where the optimal setting of factors at feed flow rate of 5 mL h^{-1}, pH of 5.66, and temperature 14 ˚C gained a highest $R_p = 8.686$ μg g_{cell}^{-1} h^{-1}. Chromatographic analysis showed more than 94% of produced carotenes was *β*-carotene.

Keywords: Carotenogenesis; Fed-batch operation; Optimization; Bubble column reactor.

Introduction

Carotenoids are lipid-soluble pigments which act as membrane-protective antioxidants, precursors of vitamin A, scavenging O_2 and peroxyl radicals [1]. The bioactive molecules decreased the risk of degenerative diseases such as cancer, cardiovascular diseases, macular degeneration and cataract in humans and must be present in human's diet for important biological actions [2]. On the other hand, the natural colorants have decisive influence on the acceptability of food and feed products, and their demands increased in the world at the last decades [3, 4]. The industrial production of famous carotenoids such as *β*-carotene, lycopene, and astaxanthin can be carried out through to i) solid-liquid extraction from plants, and ii) microbiological processes using filamentous fungi, yeasts,

bacteria or microalgae [3, 4]. In comparison with the extraction from vegetables, the microbial production of carotenoids is of paramount interest mainly due to the problems of seasonal and geographic variability in the production and marketing of the colorants extracted from plant origin [4, 5].

Development of the microbiological process is economically depends on minimizing of production cost [5, 6]. Substrate composition, typically carbon source, is among important factor influencing on the operational cost. Many carbohydrates have been utilized as a potential substrate in microbiological carotenoid production process. Raw materials of agro-industrial origin such as corn steep liquor (CSL) shows potential application as a cheap source of nutrients in microbial carotenoid production [7, 8]. CSL is a by-product of corn wet-milling process which contains entirely of the water-soluble components of corn such as amino acids, vitamins and minerals. CSL mainly combined with gluten and fibrous materials and sold as animal feed. Recently, due to the rapid development of the production of cornstarch, substantial amounts of CSL have been produced and some starch manufacturers discharge corn soak water directly into the environment, which has a negative impact on the environment [9]. So, application of CSL as substrate for fermentation processes could be beneficial in terms of environmental protection and also reducing the operational costs of microbial carotenoid production. The main drawback of using CSL in fermentation carotenoid production is the effects of impurities such as heavy metals on performance of carotenogenesing microorganism [3]. Some techniques such as adsorption and acid washing were suggested prior to fermentation for removal of the impurities [6]. However, the pretreatments are usually expensive and led to loss of the major nutrients in CSL.

The fed-batch operation enhances the control of substrate concentration and thus minimizing substrate inhibition [7, 10, 11]. Application of fed-batch operation for carotenoid production at a constant feeding rate is most practical in term of simplicity and it corresponds very well to the nature of carotenoid production in the cells. Because, carotenoids biosynthesis in red yeast cells starts at late logarithmic phase and continuing in the stationary phase, and thus fed-batch mode can provide a reliable nutrient supplement at the stationary phase [8]. For instance, Saenge et al. [12], studied the starting of fed-batch cultivation of *R. glutinis* TISTR 5159 at the middle of the exponential phase (after 12 h initial batch cultivation), with using crude glycerol as substrate for 48 h. They reported an increment of 13% carotenoid production in comparison with the simple batch experiments.

In this study a newly isolated yeast, *Rhodotorula toruloides* KP324973, was used for carotenoids production using CSL as sole carbon source. The experiments were performed at three stages: shaken flasks, batch cultivation in a bubble column reactor (BCR), and fed-batch cultivation in BCR. In the shaken flask experiments, the cell growth kinetics on CSL was determined. Further tests on the effects of initial biomass concentration on the batch

carotenoid production were performed in the BCR. Finally, the effects of feed flow rate, pH, and temperature in the fed-batch mode were statistically evaluated by the response surface methodology (RSM).

Materials and Methods

Chemicals

CSL was obtained from Glucosan Company (Alborz industrial city, Qazvin province, Iran). All ingredients such as KH_2PO_4, K_2HPO_4, $(NH_4)_2SO_4$, $MgSO_4.7H_2O$, and dimethyl sulfoxide (DMSO) were analytical grade and purchased from Merck. The petroleum ether and methanol were respectively purchased from Asia Pajohesh and Kian Kaveh Azma (Tehran, Iran).

Microorganism

The yeast, *R. toruloides,* was previously isolated from a soil sample in Kerend-e Gharb (Kermanshah Province, Iran with latitude and longitude of 38° 3' 39" and 37° 55' 19", respectively) [13]. Genomic DNA of the strain was extracted using the prestandard protocol [14]. The D_1/D_2 domain of the LSN rDNA was amplified using primer NL-1/NL-4 in a T-Personal thermocycler (Biometra, Germany) [15]. The PCR products was purified and sequenced by Tech Dragon, Hong Kong. The target sequence was compared with those available in the GenBank database to find the most similar ones using BLAST online (https://www.ncbi.nlm.nih.gov/Bankit/GenBank). Multiple sequence alignments of the newly generated sequence and the sequences of the valid species, derived from the GenBank were performed with Clustal X software version 2.0.11. A phylogenetic tree was constructed using the neighbor-joining method in MEGA version 5.1. The yeast specie was identified based on the phylogenetic analysis and the topology of the distance tree was tested by resampling data with 1000 bootstraps to provide confidence estimate. The obtained nucleotide sequence was deposited in the NCBI GenBank data base with accession number KP324973. The yeast strain was preserved in a glycerol stock for long-term storage at -20 °C. Two milliliter of the fresh yeast culture mixed with 2 mL of 40% glycerol in a 5 mL cryovial to prepare glycerol stock and stored at -20 °C for future usage. The yeast was cultivated on potato dextrose agar (PDA) for 48 h at 25 °C. The enrichment of the cell was carried out by cultivation on malt extract broth (MEB) for 24 h in a shaking incubator at 30 °C and 180 rpm.

Bubble Column Bioreactor Set up

The BCR consisted of a vertical glass column with an internal diameter of 6 cm and height of 28 cm and a total volume of 800 mL. Aeration to the BCR was supplied by air at constant flow of 15 L h^{-1} and using a gas diffuser at the bottom of BCR. The aeration system was also equipped by an air pump, gas flow meter, and sterile disposable PTFE filter unit with average pore size of 0.22 μm). A controllable thermostat chamber, which contained whole of the BCR system, was used to adjust of cultivation temperature at the desired value. In fed-batch operation mode, the BCR was fed by fresh production medium from a feed tank by an adjustable speed peristaltic pump.

Preparation of Production Medium

In order to prevent of CSL sedimentation, raw CSL solution was filtered by Wathman No. 1 and then separately sterilized in an autoclave at 121 °C for 20 min. Mineral solution included (g L^{-1}): KH_2PO_4, 1.0; K_2HPO_4, 1.0; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 1.0 was separately prepared, at two times concentration, in distilled water and autoclaved. Prior to sterilization, initial pH of solutions were set on desired value using 2 N NaOH solution [16]. For preparation of initial CSL concentration in each experiment, aliquot amount of the sterilized CSL was added to the mineral solution. Methanol (as a progressive agent of carotenogenesis at 2 vv^{-1}%) and soya oil (as anti-foam at 1 vv^{-1}%) were sterilized by a sterile disposable filter unit (0.4 μm) and then added to the mixture [5]. The volume of the medium mixture was reached to final volume by distilled water.

Carotenoids Production Modes

Shaken flask experiments

At these experiments, the effect of initial CSL concentration at 2, 5, 10, 25, 40, 60, and 70 vv^{-1}% were studied by a 90 mL of the production medium with initial pH of 6.5. The media were inoculated by 10 mL of 24 h-MEB grown culture of *R. toruloides* with an optical density of 0.5 (corresponded to the initial biomass of 72 ± 6 mg L^{-1}). The cultures were incubated in a shaking incubator for 120 h at 180 rpm and 25 °C. The experiments were performed at triplicates and mean values ± errors were presented.

Batch production in BCR

The effect of initial biomass concentration was studied at 0.072, 0.230, 0.390, and 0.450 g L^{-1}. The cultivation volume was 550 mL and initial CSL concentration was set on 5 vv^{-1}%. Other operating parameters consisted

initial pH, and temperature were constant at 6.5 and 25 °C, respectively. The experiments were performed at triplicates and mean values ± errors were presented.

Fed-batch production in BCR

At these experiments, 250 mL of the production medium containing 5 v/v% of CSL was inoculated by *R. toruloides* at initial biomass of 0.450 g L^{-1}. The culture was cultivated for 12 h in batch mode (this time is selected on the basis of the data obtained at section 3-2) and after that, a fresh stream of production medium (with CSL at 5 vv^{-1}%) at a constant flow rate was entered to BCR. The final volume of culture similar to batch production was 550 mL. The feed flow rate (X_1) at fed-batch stage, as an operational factor, studied in this work at levels of 1.59, 5.00, 10.00, 15.00, and 18.41 mL h^{-1} which corresponded to the fed-batch time of 16.3, 20, 30, 60, and 188.56 h, respectively. Temperature (X_2) and initial pH (X_3) were other operating parameters studied in the fed-batch production mode. Temperature at levels of 9.91, 14.0, 20.0, 26.0, and 30.09 °C and initial pH at levels of 5.7, 6.0, 6.5, 7.0, and 7.3 were evaluated. The levels of the temperature and initial pH were selected on the previous works [16, 19].

Experimental Design and Statistical Analysis

In this study a rotatable central composite design (RCCD with α = 1.68), as presented in Table 1, was sued for design of the experiments. Each of the independent variables (X_1, X_2, and X_3) were evaluated at five levels and total number of the experiments (N) was nineteen ($N = 2^k + 2k + m$; where, $k = 3$ is the number of factors and $m = 5$ is the repeated experiments at the center point of the design). Pure error was determined from repetitions of center points at Table 1 ($X_1 = 10$ mL h^{-1}, $X_2 = 20.0$ °C, and $X_3 = 6.5$). The actual values of the factors were converted to the coded levels by following expression:

$$X_i = \frac{x_i - [(x_{max} + x_{min})/2]}{(x_{max} - x_{min})} \quad (1)$$

where, X_i, x_i, x_{max} and x_{min} are coded value, actual level, maximum and minimum actual level of each factor, respectively.

The statistical study aimed to create a polynomial model for mathematical description of experimental observations. The following model was used for modeling of response behavior in this work:

$$R_P = \beta_0 + \beta_1 X_1 + \beta_2 X_2 + \beta_3 X_3 + \beta_{12} X_1 X_2 + \beta_{13} X_1 X_3 + \beta_{23} X_2 X_3 + \beta_{11} X_1^2 + \beta_{22} X_2^2 + \beta_{33} X_3^2 \quad (2)$$

Where, R_P as the response measured with a set of regression coefficient: β_0 is intercept and equaled to the mean response value observed at center point of the experiments. β_1, β_2, and β_3 associated with each parameter action.

β_{12}, β_{13}, and β_{23} show parameter interactions, and β_{11}, β_{22}, and β_{33} present the quadratic effects. The best fit values of the regression coefficients were determined by the nonlinear least-squares technique (Levenberg-Marquardt method) and "Goodness of fit" of experimental data to mathematical model was determined by the coefficient of determination (R^2) and standard deviation of residuals (SD) as bellows:

$$R^2 = 1 - \frac{\sum(\text{Experimental value} - \text{Predicted value})^2}{\sum(\text{Experimental value} - \text{Average experimental value})^2} \quad (3)$$

$$SD = \frac{\sum(\text{Experimental value} - \text{Predicted value})^2}{N - 3} \quad (4)$$

The effects of the coefficients in Eq. (2) were statistical evaluated by the analysis of variance (ANOVA) including Fisher test (F-test) and its associated probability (P-value) at a confidence level of 5%. The experimental design, empirical modeling, graphical analysis and also numerical optimization of the variables were performed by "*Design Expert*" software version 7.00.

Carotenoids Extraction and Separation

Extraction of pigments from the yeast cells was carried out according to the following procedure [17]: The culture was centrifuged at 12000×g for 5 min and the sediment was washed twice with distilled water. Then 10 mL of DMSO was added to the harvested cells and left overnight at room temperature. In the next day, the mixture was centrifuged with 10000×g for 10 min and the red color supernatant phase was collected to another tube. The sediment which had a low pink color was repeatedly suspended in 10 mL of acetone and mixed vigorously for 5 min. The colorful acetone phase was separated by centrifugation and mixed with the colorant DMSO solution in a decanter funnel. Then, 10 mL of petroleum ether and 5 mL of saturated NaCl solution were added to the decanter and the mixture was gently shacked for 5 min. The transferred carotenoids to petroleum ether phase was separated. In order to prevent from carotenoids oxidation, butylated hydroxytoluene (BHT) was add at 20 mg L^{-1} to all solvents during extraction process [5]. Finally, the petroleum ether was evaporated at 50 °C using a rotary vacuum evaporator.

Analytical Methods

Sample was taken from cultures at specified intervals, to measuring biomass formation, glucose utilization, and carotenoids production. All measurements were performed in triplicate and mean values have been reported.

The cell biomass concentration was initially determined spetrophotometically by measuring the absorbance at 600 nm and then converted to the cell dry weight concentration (C_X) using a standard curve. The specific cell growth rate (μ) during logarithmic growth phase was determined as follows [5]:

$$\mu\ (\mathrm{h}^{-1}) = \frac{\ln\left({C_{Xf}}/{C_{Xo}}\right)}{t_l} \tag{5}$$

Where, C_{X0} and C_{Xf} are the cell dry weight concentration at the beginning and final of logarithmic growth, and t_l is the time duration of the logarithmic growth.

In order to determine of reducing sugars, the sample of culture was centrifuged at 12000×g for 5 min and the supernatant was used for reaction with 3. 5 di nitrosalicylic acid according to the prestandard method [18] and the red color developed by the reaction was measured at 540 nm. Glucose at concentrations of 0-1000 mg L^{-1} were prepared as standard reducing sugar solutions.

Identification of carotenoids was accomplished by comparing the reversed phase high performance liquid chromatography (HPLC) retention times with the external standards of β-carotene, torulene, and torularhodine. The HPLC device equipped with NUCLEODUR C18 column (Knauer Co.), and a UV detector. The mobile phase consisting methanol, acetonitrile, and chloroform (47:47:6) with a flow rate at 1.5 mL min^{-1}. Temperature of the HPLC column was set on 30 °C [19].

Quantity of the produced carotenoids concentration (C_P) was determined by measurement of β-carotene absorbance in petroleum ether at 445 nm ($Abs_{445\ nm}$) and using following formula [20]:

$$C_P\ (\mu\mathrm{g\ L}^{-1}) = \frac{10^4 \times Abs_{445\,nm} \times v}{\varepsilon_{1\mathrm{cm}}^{1\%} \times V} \tag{6}$$

Where, V (L) and v (mL) are the volume of culture and petroleum ether solvent, respectively and $\varepsilon_{1\,cm}^{1\%} = 2592$ is the extinction coefficient of β-carotene in petroleum ether [20]. The specific carotenoid production rate (R_p) was determined as follows:

$$R_p\ (\mu\mathrm{g\ g}_{\mathrm{cell}}^{-1}\mathrm{h}^{-1}) = \frac{1}{t} \times \frac{C_{PE} - C_{Pi}}{C_{XE}} \tag{7}$$

Where, t (h) is the cultivation time and the subscripts i and E denote the beginning and end values of concentrations in the fermentation.

Results and Discussion

Shaken Flask Experiments

The main aim of this work is application of CSL as cheap agro-industrial substrate for microbial carotenoids production by *R. toruloides*. The presence of different substances including carbohydrates, fatty acids, proteins, amino acids, and minerals in CSL expected that CSL could provide the most nutrients requirement of cell in carotenogenesis process (The chemical compositions of CSL is presented in Supplementary Material). The results of Fig. 1 show that *R. toruloides* could grow at different initial concentrations of CSL as sole carbon source. A fast growth of test cell was clearly observed from beginning of fermentation when initial CSL concentration was 2, 5, and 10 vv^{-1}% (Fig. 1a, b, and c). In fact, logarithmic growth was taken place from beginning of the cultivation and followed for 48 and 72 h at 5 and 10 vv^{-1}%, respectively. The comparison of the growth curves at Figs. 1c and 1d obviously showed that the logarithmic growth was increased from 96 h to 120 h by increasing of initial CSL concentration from 10 vv^{-1}% to 40 vv^{-1}%. In fact, a lag time was occurred at the beginning of cultivation at higher concentrations than 10 vv^{-1}% and it results the growth curves concaved up especially at initial CSL concentration higher than 40 vv^{-1}% (Fig. 1e, f, and j). At these concentrations, logarithmic growth phase was postponed and initiated after 48 h. After this period, biomass concentration is suddenly decreased due to exhaustion of nutrients from cultures and the cells entered to the death phase. The results of carotenoids production during cultivation on different concentrations of CSL are also presented in Fig. 1. In spite of *R. toruloides* ability in utilizing CSL as carbon source at different initial concentrations, however a satisfactory carotenoid production was only observed at the initial concentrations of 2, 5, and 10 v/v%. The results of Fig. 1 also indicate that carotenogenesis in the cell was carried out simultaneously with logarithmic growth and stopped at the end of the cultivations. The highest concentration of produced carotenoids about 193.4 μg L^{-1} was obtained after 96 h at initial CSL concentration of 10 vv^{-1} %. Similar observation in accumulation of carotenes at late logarithmic phase was previously reported by other researchers in cultivation of *Rhodotorula glutinis* [21].

With using data presented in Fig. 1 and considering Eq. (5), the specific cell growth rate (μ) was determined at different initial CSL concentrations and the results are shown in Fig. 2. The μ value showed an increase with increasing CSL concentrations up to about 0.0425 h^{-1} at initial CSL concentration of 10 vv^{-1}%. Then μ decreased with further increasing of CSL concentration. This pattern clearly shows the growth inhibitory role of the compounds present in CSL which played at high concentrations. The inhibition effects of CSL on biomass formation and carotenoid accumulation in the cells were previously reported by other researchers [7]. In order to modeling of the substrate inhibitory effect, the well-known kinetic model namely Haldane (Eq. 8) was used to treat the experimental data:

$$\mu = \left(\frac{\mu_{\max} C_{Si}}{(K_S + C_{Si})(1 + C_{Si} / K_I)} \right) \quad (8)$$

Where, C_{Si} is initial CSL concentration and the kinetic constants of μ_{max}, K_S, and K_I are the maximum specific growth rate, half-saturation constant, and substrate inhibition constant, respectively.

The best fit values of kinetic constants in Eq. (8) were determined at μ_{max} = 0.056 h^{-1}, K_S = 1.54 vv^{-1}%, and K_I = 58.58 vv^{-1}%. A comparison between the experimental data and prediction by the Haldane model is presented in Fig. 2. The results show that growth kinetic of *R. toruloides* on CSL was well described by Haldane model (The R^2 and *SD* were 0.9139 and 0.0025, respectively). Dependency of R_P on initial CSL concentration is also presented at Fig. 2 where the specific carotenoids production rate has relatively similar trend to the specific cell growth rate and reaches to a highest value about 2.233 μg.g_{cell}^{-1} h^{-1} at initial CSL concentration of 10 vv^{-1}%. This observation agreed with previous works showed a linear relationship between the R_P and μ and it indicating carotenoids are associated growth products [22]. Further increase of initial CSL concentration from 10 vv^{-1}% to 40 vv^{-1}% resulted in decrease of R_P from 2.233 μg g_{cell}^{-1} h^{-1} to 0.524 μg g_{cell}^{-1} h^{-1} due to the inhibitory effects of CSL. In fact, CSL contains several necessary substances (amino acids, vitamins, and trace elements) important for growth of microorganism and stimulating carotenoid biosynthesis however, higher concentration of these nutrient has inhibitory role on the enzymes involved in carotenogenesis process [23]. The R_P value was decreased to 0.5 μg g_{cell}^{-1} h^{-1} when initial CSL concentration was higher than 40 vv^{-1}%. The yield of carotenoid to substrate ($Y_{P/S}$) is among important factors in economic aspect of the process. As be seen in Fig 2, the $Y_{P/S}$ was reached to the highest value about 35.1 μg g^{-1} when the initial CSL was 5 vv^{-1}%. Even higher concentration of CSL resulted to decrease of $Y_{P/S}$. This behavior is mainly due to the inhibitory effects of some metals such as Fe^{2+} and Mn^{2+} on carotenoid synthesis in the red yeast cells [3]. The cell growth and carotenoids production on CSL in shaken flasks experiments showed potential of the substance as feedstock of *R. toruloides* cultivation. Further studies on using of CLS were performed at a BCR.

Batch Production in BCR

Application of BCR in this study led to an earlier initiation of pigment production in *R. toruloides* cells and also faster consumption of TRS from medium in comparison with shaken flask experiment at the same cultivation conditions (C_{Si} = 5 vv^{-1}% and C_{Xi} = 0.072 g L^{-1}). The cells reached to the late logarithmic growth after 21 h in BCR, however this time was corresponded to 72 h in the shaken flask experiments. In fact, aeration is among influential factors on providing an oxic environment for stimulating of cells for carotenoids production [19, 23].

The further analysis showed 82.2% of TRS was utilized after 21 h in BCR where R_P was 2.207 μg g_{cell}^{-1} h^{-1}. Initial biomass concentration is another critical factor in design of the microbiological processes. In this study, the nutrient of medium was consumed faster at higher biomass concentrations. It can be seen at Fig. 3 that the logarithmic growth time was decreased from 21 h to 12 h with increasing of the initial biomass concentration from 0.072 to 0.450 g L^{-1}. During of cultivation with initial biomass concentration of 0.450 mg L^{-1}, 88.4% of TRS was exhausted from culture in BCR after 12 h and only TRS about 0.8 g L^{-1} was remained in the culture.

The carotenoid to substrate yield is also increased by increasing of initial biomass concentration and reached to 46 μg g^{-1} when initial biomass concentration was 0.450 mg L^{-1} and the R_P was enhanced to 5.979 μg g_{cell}^{-1} h^{-1} which was 2 times higher than the result obtained at initial biomass concentration of 0.072 g L^{-1}. The increasing of R_P with decreasing of the production time at Fig. 3 is obviously shown. It indicating the importance of pigment oxidation during fermentation. Cell membrane autolysis and chemical oxidation reactions of carotenoids over a more extended time were already reported by other researchers previously [19, 24]. Therefore, determination of the late logarithmic time is crucial for achievement to a high R_P value in a microbial carotenogenesis process. At batch cultivation, due to a rapid exhaustion of nutrient after the logarithmic growth the cells was suddenly entered to the dead phase, without a noticeable stationary phase (See Fig. 1). So, fed-batch cultivation with a constant feeding rate is most practical approach to control of substrate concentration which was important in this study in minimizing substrate inhibition and as well as following carotenogenesis process by active cells after the logarithmic phase in batch cultivation.

Fed-Batch Production in BCR

In fed-batch operation, the feeding of culture with an appropriate rate of fresh nutrient solution provides a suitable condition for following carotenogenesis process after the logarithmic growth. The experimental R_P values obtained by fed-batch cultivation in BCR is presented in Table 1. The results show that R_P was changed from 0.536 μg $g_{cell}^{-1}h^{-1}$ in experiment run number five to the highest value equaled 8.475 μg $g_{cell}^{-1}h^{-1}$ in experiment run number fifteen. Notice that the R_P in most fed-batch cultivations was lower than that obtained by batch fermentation (5.979 μg g_{cell}^{-1} h^{-1}). It obviously indicates the importance of feeding rate in the fed-batch carotenoids production. On the basis of the non-linear regression analysis, the variations of R_P with coded levels of test factors was mathematically modeled by RSM at following expression:

$$R_P\ (\mu g\ g_{cell}^{-1}\ h^{-1}) = 2.55 - 1.79\ X_1 - 0.57\ X_2 - 0.38\ X_3 + 1.28\ X_1X_2 + 0.46\ X_1X_3 + 0.67\ X_2X_3 + 0.93\ X_1^2 - 0.20\ X_3^2 \quad (9)$$

where, the ANOVA presented in Table 2 shows β_{22} was non-significant statistically at 5% confidence level and thus omitted from the final developed model. The "lack of fit" was not statistically significant by the model and also the comparative high value of $R^2 = 0.9897$ confirmed the agreement between the experimental results and the predicted values by the quadratic model (see Table 1). Confidence distribution (CD), adjusted R^2, the predicted residual error sum of squares (PRESS), and adequate precision are respectively at 0.29, 0.9814, 4.50, and 40.927 for the Eq. (9). The close values of R^2 and adjusted R^2 shows all remianed coeffients in Eq. (9) are statistically significant in predicting of R_P in this work [5, 23].

The relative contribution of each factor on R_P can be directly detected by the respective coefficient in Eq. (9) [5]. The greatest coefficient is β_1 = -1.79 which indicating the fed-batch time was most influential factors on carotenogenesis in fed-batch cultivation at BCR. The negative signs of β_1, β_2, and β_3 indicate the R_P was decreased by increasing the factors. In fact, increase of fed-batch time result in more oxidation of produced pigments in the culture. Increase of temperature increased simultaneously the growth rate and as well as pigments. Negative sign of β_2 reveals pigment oxidation progressed higher than the carotenoid production when the temperature was increased from 14 ^{o}C to 26 ^{o}C in *R. toruloides* cells. Also, increasing of pH from 6.0 to 7.0 decreased R_P mainly due to the morphological changes and altering the permeation of cell membrane [25].

Three dimensional response surface diagrams are useful tools for illustration of interactions in RSM. Fig. 4a shows the interaction of feed flow rate and temperature on R_P where the variation of R_P on feed flow rate is obviously depends on the level of cultivation temperature. The R_P plot in Fig. 4a shows a curvature by variation of feed flow rate indicates a nonlinear dependency of R_P to this factor (β_{11} is significant in Eq. 9). However, temperature has a linear behavior on R_P. Relevant to the findings, β_{22} is not significant in Eq. 9.

The highest R_P value was obtained at a low flow rate (1.59 mL h^{-1}) when the temperature was kept constant at low level (9.9 ^{o}C). It is because carotenoid as secondary metabolite is produced at the end of logarithmic growth and continued at stationary phase. Decreasing of feed flow rate, results to sustain the concentration of nutrient at the minimum aliquot for longer time in the fed-batch production. So, decreasing of temperature at a long time production prevented the oxidation of produced carotenoids. The dependency of R_P on simultaneous variation of feed flow rate and initial pH is shown in Fig. 4b. It can be seen that increasing of initial pH from 6.0 to 7.3 results to decrease of R_P at all feed flow rates while a little acidic environment (initial pH 5.66) shifted R_P to the highest value especially at feed flow rate of 1.59 mL h^{-1}. In fact, acidic medium increases the environmental stress on the cells and thus results to enhance the accumulation of carotenoids, as a protective agent, in the cell wall. Fig. 4c also shows obviously the interaction of two important factors in biological systems *i.e,* temperature and initial pH.

Increase of pH from 6.0 to 7.34 raised up the R_P from 1.6 to 2.6 μg g_{cell}^{-1} h^{-1} when temperature was 30.1 °C while the same variation of pH, negatively affected R_P at 9.9 °C. Temperature and pH affected on the cell's behavior and also its metabolite production. Changes of pH effects on transportation of nutrients and also waste materials from the cell membrane. However, the role of temperature on the biological process is more complicated because it effects on dissolved oxygen in the medium, transportation of nutrient, and also oxidation of produced carotenoids in the BCR. In this study, decrease of temperature shifted the R_P to higher values when initial pH was 5.66 while decreasing of temperature resulted to decrease of R_P at initial pH of 7.34. Previous findings showed pH=6 at 28 °C was the best condition for carotenoids production by *Sporidiobolus pararoseus* [25].

In this study, the optimal setting of test factors was determined by the desirability function available in Design Expert software version 7. The optimization criterion was maximal R_P value with considering the operational factors at the studied ranges of Table 1. Desirability is theoretically changed from zero (a completely undesirable situation to gain criterion) to one (a highly desirable condition to gain criterion). The dependency of desirability value on test factors shown that the maximum R_P was only obtained at initial pH of 5.66 whoever at this initial pH, the dependency of desirability on feed flow rate and temperature is illustrated at Fig. 5. The Fig. 5 shows high sensitivity of desirability on variations of feed flow rate and temperature where the highest desirability 1.0 only obtained at the red zone of Fig. 5. An optimal setting of the test factors at the feed flow rate of 5 mL h^{-1}, temperature of 14 and initial pH of 5.66 was experimentally examined and resulted to a R_P about 8.686 μg g_{cell}^{-1} h^{-1}. The quality of carotenoids produced by *R. toruloides* in this conditions was evaluated by HPLC analysis and the results indicating two identifying carotenoid picks at 7.00 and 18.37 min which corresponded to totorulene and *β*-carotene standard solutions, respectively. On the basis of the under curve area, *β*-carotene was more than 94% of the produced carotenes by *R. toruloides* in BCR.

A comparison of works reported in carotenoids production from CSL at fed-batch cultivation are presented in Table 4.

A highest R_P about 16.0 μg g_{cell}^{-1} h^{-1} was also reported in cultivation a mutant strain of *Phaffia rhodozyma* where relatively high concentration of initial CSL (90 g L^{-1}) was used as carbon source [6]. Fed-batch production by *Sporidiobolus salmonicolor* using 40 g L^{-1} of CSL after 106 h obtained R_P about 8.5 μg g_{cell}^{-1} h^{-1} [7]. Carotenoid production of *Phaffia rhodozyma* in fed-batch cultures under constant feeding, provided R_P about 3.747 μg g_{cell}^{-1} h^{-1}after 98 h [10]. Fed-batch fermentation was also studied Han et al. [25] as a strategy for enhancing specific carotenoid production by *Sporidiobolus pararoseus*, and best R_P equaled to 9.265 μg g_{cell}^{-1} h^{-1} was obtained at initial pH 6, temperature 28 °C, air flow rate of 0.14 $vv^{-1}min^{-1,}$ agitation speed of 90 rpm after 60 h. In comparison

with these significant works, fed-batch cultivation of *R. toruloides* on 6.7 g L^{-1} of CSL provided a relevant R_P about 8.475 μg g_{cell}^{-1} h^{-1} which showed potential of the newly isolated yeast strain in microbial carotenoid production processes.

Conclusion

The CSL, cheap agro-industrial waste, as sole carbon source was successfully used for carotenoids biosynthesis by a newly isolate red yeast, *R. toruloides* KP324973 in stages of shaken flasks, batch, and fed-batch productions. At batch studies, inoculation with high initial biomass gained higher specific carotenoid production due to a rapid logarithmic growth, and thus prevention the cell membrane autolysis and chemical oxidation reactions of carotenoids over a more extended time in BCR. Fed-batch cultivation at optimal conditions provided a reliable nutrients for continuing the carotenogenesis in cells after initial batch cultivation. Minimizing of the substrate inhibition is another advantage of fed-batch fermentation. Carotenoids production enhanced by 42% in the fed-batch cultivation when compared with the batch operation.

Declarations: The authors declare no conflict of interest for review and publication of manuscript.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors.

Ethics Approval: This article does not contain any studies with human participants performed by any of the authors.

Authors' Contributions: This work was carried out in collaboration between all authors. **Samira Fallahi** gathered the initial data and produced the initial draft. **Alireza Habibi** anchored the field study, interpreted the data, and wrote the manuscript. **Saeed Abbasi** managed the literature searches and helped to revised of the manuscript**. Rouhallah Sharifi** performed the isolation and characterization of bacterial species and carried out preliminary data analysis.

References

1. Krinsky, N. Y., (2001). Carotenoid antioxidants. *Nutrition*, *17*, 815–817.

2. El-Agamey, A., Lowe, G., M., McGarvey, D., J., Mortensen, A., Phillip, D., M., Truscott, T., G., Young, A., J., (2004). Carotenoid radical chemistry and antioxidant/pro-oxidant properties. *Archives of Biochemistry and Biophysics, 430*, 37-48.

3. Frengova, G., I., Beshkova, D., M., (2009). Carotenoids from *Rhodotorula* and *Phaffia*: yeasts of biotechnological importance. *Journal of Industrial Microbiology and Biotechnology*, *l36*, 163–180.

4. Mata-Gómez, L., C., Montañez, J., C., Méndez-Zavala, A., Aguilar, C., N., (2014). Biotechnological production of carotenoids by yeasts: an overview. *Microbial Cell Factories, 13*, 1-11.

5. Alipour, S., Habibi, A., Taavoni, S., Varmira, K., (2017). *β*-carotene production from soap stock by loofa-immobilized *Rhodotorula rubra* in an airlift photobioreactor. *Process Biochemistry, 54*, 9-19.

6. Kesava, S., An, G., H., Kim, C., H., Rhee, S., K., Choi, E., S., (1998). An industrial medium for improved production of carotenoids from a mutant strain of *Phaffia rhodozyma*, Bioprocess Engineering, 19, 165–170.

7. Colet, R., Luccio, M., D., Valduga, E., (2015). Fed-batch production of carotenoids by *Sporidiobolussalmonicolor* (CBS 2636): kinetic and stoichiometric parameters. *Eurpian Food Research and Technology, 240*, 173–182.

8. Su-Jin, K., Kim, G., J., Park, D., H., Ryu, Y., W., (2003). High-level production of astaxanthin by fed-batch culture of mutant strain *Phaffia rhodozyma* AJ-6-1. *Journal of Microbiology and Biotechnology*, *13*(2), 175-181.

9. Silva, A., C., Guimaraes, P., M., R., Teixeira, J., A., Domingues, L., (2010). Fermentation of deproteinized cheese whey powder solutions to ethanol by engineered *Saccharomyces cerevisiae*: Effect of supplementation with corn steep liquor and repeated-batch operation with biomass recycling by flocculation. *Journal of Industrial Microbiology and Biotechnology*, *37*(9), 973-982.

10. Ho, K.,P., Tam, C.,Y., Zhou, B., (1999). Growth and carotenoid production of *Phaffia Rhodozyma* in fed-batch cultures with different feeding methods. *Biotechnology Letters, 21*, 175–178.

11. Luna-Flores, C., H., Ramírez-Cordova, J.,J., Pelayo-Ortiz, C., Femat, R., Herrera-López, E.,J. (2010). Batch and fed-batch modeling of carotenoids production by *Xanthophyllomycesdendrorhous* using *Yucca fillifera* date juice as substrate. *Biochemical Engineering Journal*, *53*, 131-136.

12. Saenge, C., Cheirsilp, B., Suksaroge, T., T., Bourtoom, T., (2001). Potential use of oleaginous red yeast *Rhodotorula glutinis* for the bioconversion of crude glycerol from biodiesel plant to lipids andcarotenoids. *Process Biochemistry, 446*, 210–218.

13. Jamali, S., Gharaei, M., Abbasi, S., (2016). Identification of yeast species from uncultivated soils by sequence analysis of the hypervariable D1/D2 domain of LSU-rDNA gene in Kermanshah province. Iran. *Mycologia Iranica, 3*, 87-98.

14. Suh, S.,O., Zhang, N., Nguyen, N., Gross, S., Blackwell, M., (2008). Lab manual for yeast study. Mycology lab Louisiana State University, USA.

15. Kurtzman, C.,P., Robnett, C., J. (1998). Identification and phylogeny of ascomycetous yeasts from analysis of nuclear large subunit (26S) ribosomal DNA partial sequences. *Antonie van Leeuwenhoek*, *73*, 331–371.

16. Varmira, K., Habibi, A., Bahramian, E., Jamshidpour, S., (2016). Progressive agents for improvement of carotenogenesis in *Rhodotorula Rubra*. *Journal of Advances in Food Science and Technology, 3*(2), 70-78.

17. Park, P., K., Kim, E., Y., Chu, K., H., (2007). Chemical disruption of yeast cells for the isolation of carotenoid pigments. *Separation and Purification Technology, l53*, 148-152.

18. Miller, G., L., (1959). Use of dinitrosalicylic acid reagent for determination of reducing sugar. *Analytical Chemistry*, *31*(3), 426-428.

19. Varmira, K., Habibi, A., Moradi, S., Bahramian, E., (2016). Statistical optimization of airlift photobioreactor for high concentrationproduction of torularhodin pigment. *Biocatalysis and Agricultural Biotechnology*, *8*, 197–203.

20. de Carvalho, L., M., J., Gomes, P., B., de Oliveira Godoy, R., L., Pacheco, S., do Monte, P., H., F., de Carvalho, J., L., V., Nutti, M., R., Lima Neves, A., C., L., Vieira, A., C., R., A., Ramos, S., R., R., R., (2012). Total carotenoid content, α-carotene and β-carotene, of landrace pumpkins (*Cucurbita moschata* Duch): a preliminary study, *Food Research International*, *47*(2) 337–340.

21. Taskin, M., Sisman, T., Erdal, S., Kurbanoglu, E., B., (2011). Use of waste chicken feathers as peptone for production of carotenoids in submerged culture of *Rhodotorula glutinis* MT-5. *Eurpian Food Research and Technology,* 233, 657–665.

22. Taavoni, S., Habibi, A., Varmira, K., Alipour, S., (2018). Kinetics of continuous production of *β*-carotene in an airlift bioreactor. *Asia-Pacific Journal of Chemical Engineering*, *13*(1), e2160.

23. Mantzouridou, F., Roukasa, T., Kotzekidoua, P., Liakopoulou, M., (2002). Optimization of *β*-carotene production from synthetic medium by *Blakeslea trispora*: A mathematical modeling, *Applied Biochemistry and Biotechnology*, *101*, 153-175.

24. Varzakakou, M., Roukas, T., Papaioannou, E., Kotzekidou, P., Liakopoulou-Kyriakides, M., (2011). Autolysis of *Blakeslea trispora* during carotene production from cheese whey in an airlift reactor. *Preparative Biochemistry and Biotechnology*, *41*(1), 7–21.

25. Han, M., He, Q., Zhang, W., G., (2012). Carotenoids production in different culture conditions by *Sporidiobolus pararoseus*. *Preparative Biochemistry and Biotechnology*, 42(4), 293–303.

Caption of Figures

Fig. 1 Temporal features of C_X and C_P during cultivation of *R. toruloides* at different initial CSL concentrations ($vv^{-1}\%$): 2 (a), 5 (b), 10 (c), 25 (d), 40 (e), 60 (f), and 70 (j).

Fig. 2 The dependency of the μ and R_P on initial CSL concentration (S_i). Comparison of experimental μ and predicted values by Haldane model is also presented.

Fig. 3 Effect of initial biomass concentration on batch carotenoids production in the BCR.

Fig. 4 Dependency of R_P in fed-batch carotenoids production on: X_1 and X_2 (a), X_1 and X_3 (b), and X_2 and X_3 (c).

Fig. 5 Desirability for achievement of maximum R_p value as function of X_1 and X_2 (X_3 was 5.66)

Figures

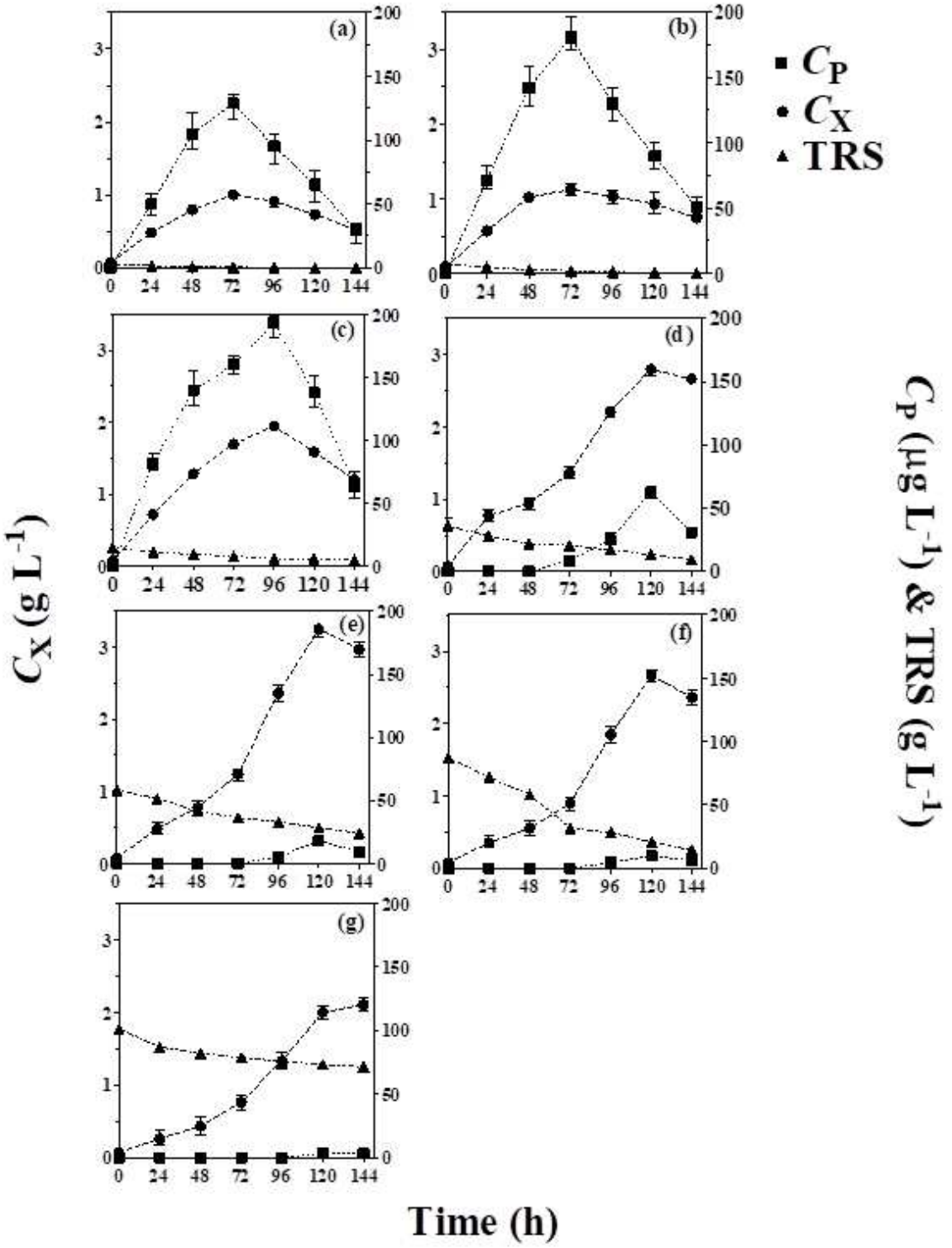

Figure 1

Temporal features of CX and CP during cultivation of R. toruloides at different initial CSL concentrations (vv-1%): 2 (a), 5 (b), 10 (c), 25 (d), 40 (e), 60 (f), and 70 (j).

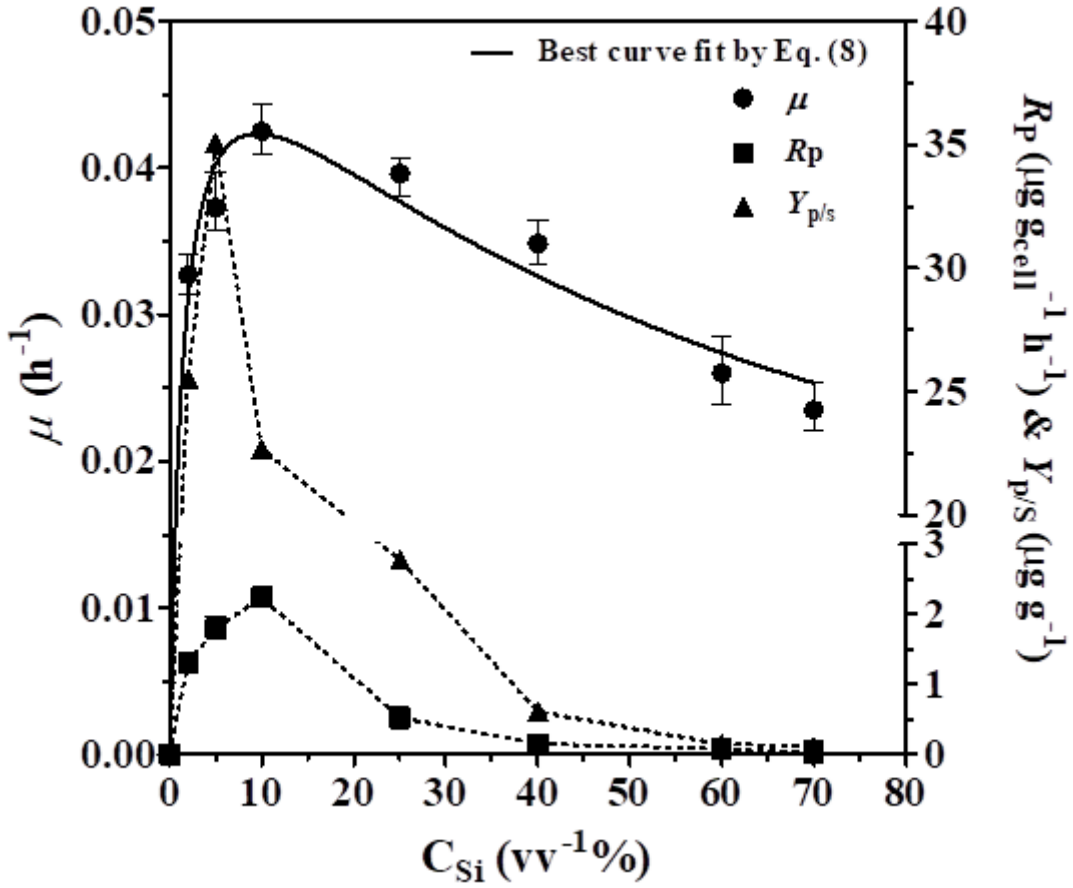

Figure 2

The dependency of the μ and RP on initial CSL concentration (Si). Comparison of experimental μ and predicted values by Haldane model is also presented.

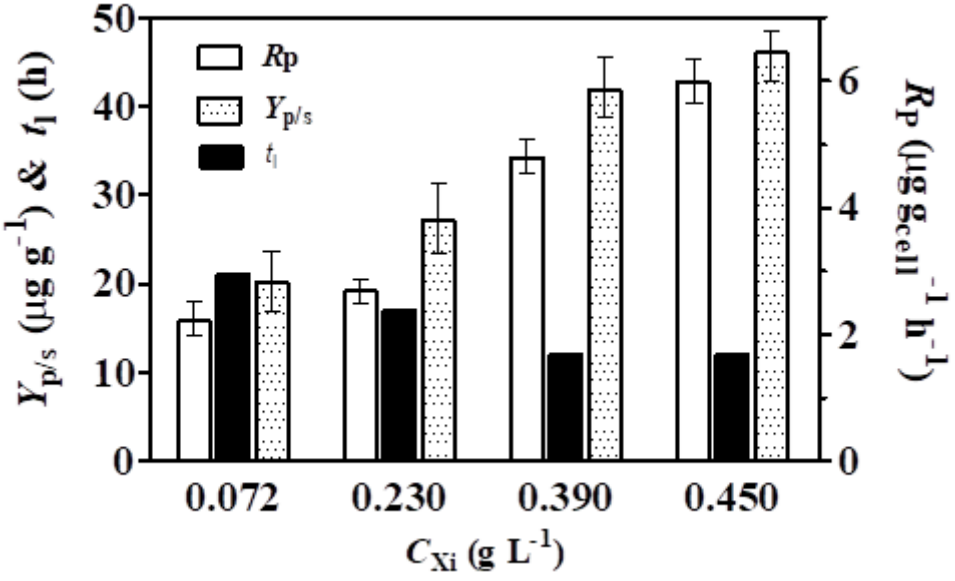

Figure 3

Effect of initial biomass concentration on batch carotenoids production in the BCR.

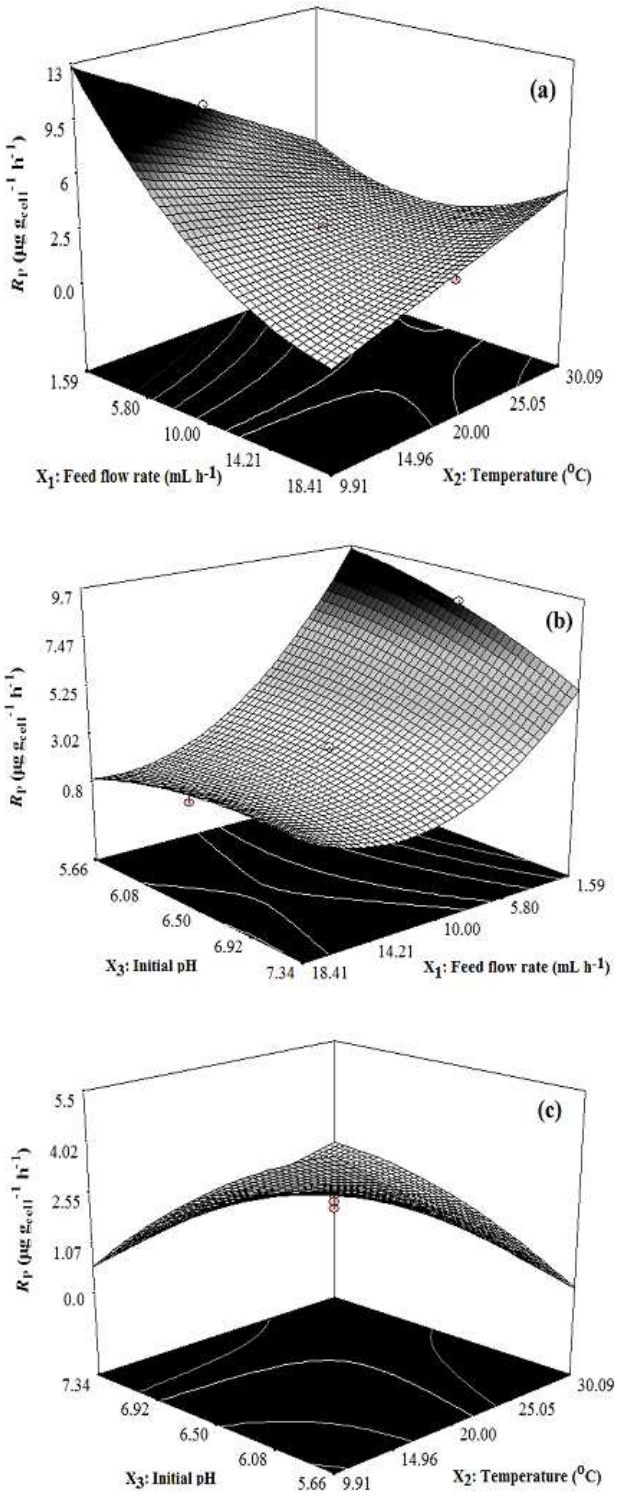

Figure 4

Dependency of RP in fed-batch carotenoids production on: X1 and X2 (a), X1 and X3 (b), and X2 and X3 (c).

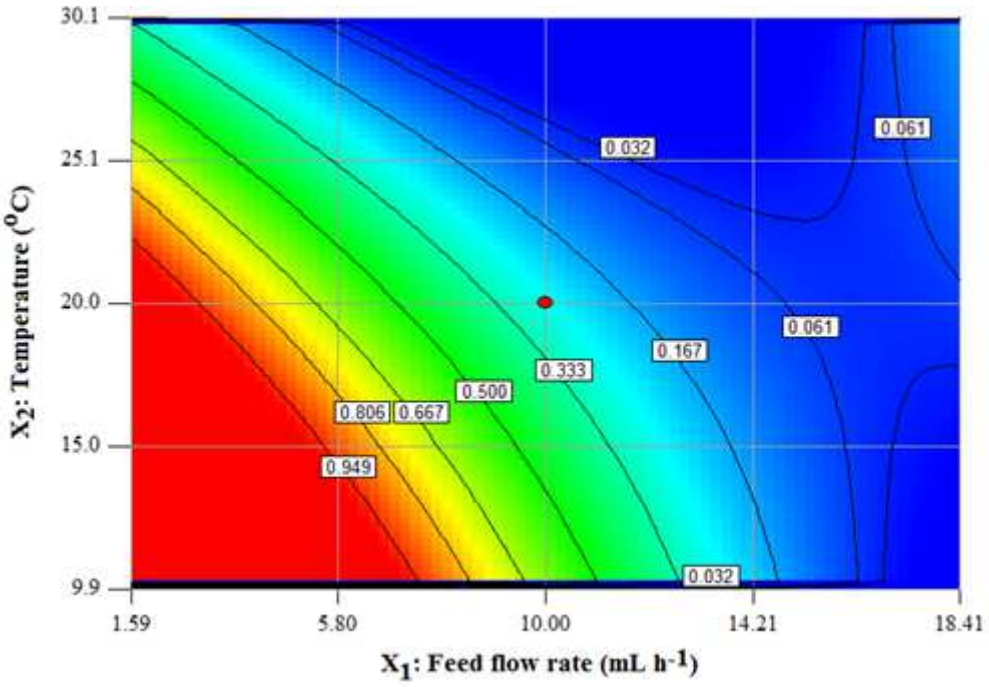

Figure 5

Desirability for achievement of maximum Rp value as function of X1 and X2 (X3 was 5.66)

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Supplimentraryinformation.docx