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Feeding strategies for the improved biosynthesis of canthaxanthin from enzymatic hydrolyzed molasses in the fed-batch fermentation of *Dietzia natronolimnaea* HS-1

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HIGHLIGHTS

- The feeding strategies for *D. natronolimnaea* HS-1 fed-batch culture were studied.
- Enzymatic hydrolyzed molasses (EHM) was suitably selected for fed-batch cultivation.
- 14.67 mg/l canthaxanthin (CTX) obtained by fed-batch fermentation of EHM.
- Constant feeding rate strategy was the best strategy to increase the microbial CTX.
- Optimal operating policies for an ideal fed-batch process were determined.

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ABSTRACT

The effect of two enzymatic hydrolyzed molasses (EHM)-feeding strategies including constant-(CFR) and exponential-(EFR) feeding rate on canthaxanthin (CTX) biosynthesis by *Dietzia natronolimnaea* HS-1 fed-batch fermentation was studied. The results showed that the CFR of 7 ml/h with an EHM content of 45 g/l led to the highest values of specific growth rate (0.127 h^{-1}), biomass dry weight (17.66 g/l), total carotenoid (16.31 mg/l) and CTX (14.67 mg/l). A significant decrease in the kinetic growth and production parameters by the increasing EHM concentration from 30 to 60 g/l during EFR fed-batch bioprocess was observed ($p < 0.01$). This study concluded that EHM alone can displace glucose-based medium towards improved CTX biosynthesis from *D. natronolimnaea* HS-1 using a CFR strategy during fed-batch culture.

1. Introduction

Carotenoids as one class of major food micronutrients in human diet are liposoluble tetraterpene-type molecules. These functional compounds are the most extensively distributed group of pigments in nature, displaying orange, red, and yellow colour (Nelis and De Leenheer, 1989; Hannibal et al., 2000; Shi et al., 2002). Although more than 600 various carotenoids are produced by plants, bacteria, fungi and algae, but only a small number of them can be obtained in acceptable amounts through chemical synthesis, microbial fermentation or extraction from their natural sources (da Silva et al., 2012). The carotenoid market is expected to increase to $919 million by 2015 with an average annual growth rate (AAGR) of 2.3%. However, the AAGR for worldwide carotenoids market was estimated to be $766 million in 2007 (Vachali et al., 2012).

Canthaxanthin (CTX; 4,4′-diketo-β-carotene) is an orange-red ketocarotenoid with high antioxidant activity. The production of CTX has increased due to its beneficial health attributes such as anti-inflammatory, anticancer, anti-tumor, anti-dermatosis, and coloring agent (Carpenter et al., 1997; Chan et al., 2009). Compared to the chemical synthesis methods, microbial production of CTX through biotechnological approaches is a paramount evolution due to the problems of geographic and seasonal variability in the production and marketing of some of the CTXs of plant origin such as vegetables and fruits and also the economic benefits using low-cost substrates with agro-industrial origin as carbohydrate sources (Gharibzahedi et al., 2013b). The employed substrates for fermentation processes should be appropriate for the development of microorganisms and products of interest. Besides having a composition capable of meeting the requirements of the microorganism, the substrate should be suitably conditioned in terms of

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temperature and pH and should be rendered aseptic or sterile for optimal efficiency (da Silva et al., 2012). Molasses is a cheap by-product in sugar refinery, which is widely used for the production of carotenoids by some microorganisms. It consists of about 50% (w/w) total sugars (mainly sucrose, glucose, fructose and raffinose), water, crude protein and fat, heavy metals, organic acids, vitamins and others (Angardi and Çalık, 2013).

Dietzia natronolimnaea HS-1 as a biotechnologically important source is able to accumulate high amounts (more than 90%) of CTX. It is Gram positive, catalase positive, and oxidase negative with orange colonies (Khodaiyan et al., 2007). Owing to the fact that simple sugars of glucose and fructose has been shown to enhance CTX production by *D. natronolimnaea* HS-1 (Khodaiyan et al., 2007), the molasses enzymatic hydrolysate was chosen to be applied as an inexpensive and renewable substitute for glucose within the scope of this study.

Fed-batch fermentation is a batch culture continuously or consecutively fed with substrate without any fermentation broth removal. In general, this fermentation mode is superior to batch and continuous processing, and is significantly useful as varying substrate amounts affect the cell biomass and metabolite production (Lee et al., 1999). The design of the feeding strategy and its control in fed batch fermentation are the most influential in determining the productivity and the final titer in carotenoid pigments (Yamane et al., 1997a; Hu et al., 2005). The control of substrate addition is also very important to attain the maximum production of desired products for a fed-batch process in order to avoid underfeeding or overfeeding of the substrate (Hu et al., 2005).

Although many studies concerning the optimization of substrate feeding for fed-batch fermentation process have been performed, but to the best of our knowledge, there is no study on the effect of carbon-substrate feeding strategy for the enhanced microbial CTX. Therefore, the aim of this study was to determine a suitable feeding strategy and an ideal concentration of enzymatic hydrolyzed molasses (EHM) to enhance the cell biomass and CTX synthesis by *D. natronolimnaea* HS-1

2. Methods

2.1. Chemicals and reagents

Hydrochloric (HCl) and sulfuric (H_2SO_4) acids, sodium hydroxide (NaOH), acetonitrile (HPLC grade), methanol (MeOH, HPLC grade), dichloromethane (HPLC grade), 3,5-dinitrosalicylic acid (DNS, 98%) and invertase enzyme were obtained from Merck Chemical Co. (Darmstadt, Germany). Malt extract, peptone, yeast extract, D-glucose, agar and antifoam 204 were purchased from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). The CTX standard was provided by the Bioprocess Engineering Laboratory (BPEL, University of Tehran, Iran). Pure ethanol (99.9%, v/v) was supplied from Bidestan Co. (Qazvin, Iran).

2.2. Raw material and its pretreatment

Beet molasses was purchased from Marvdasht Sugar Industry (Fars, Iran) during the harvest season of 2012–13. The molasses was diluted to a desired concentration of total sugars (mainly sucrose) in order to discard the insoluble materials. This obtained solution was adjusted to pH 3.0 with 0.5 M H_2SO_4 and allowed to stand for 24 h at room temperature and then centrifuged at 10,000*g* for 10 min. The supernatant was treated using activated carbon without heating to avoid hydrolysis of sucrose and filtered using 0.2 μm filter paper (Sigma–Aldrich Co.) under vacuum and used as fermentation medium (Gharibzahedi et al., 2013a).

2.3. Preparation of the molasses enzymatic hydrolysate

The various ratios of invertase enzyme were preliminary examined to achieve more hydrolysis of sucrose present in beet molasses. An optimal ratio of 0.62% (w/v) for 2 h in a water bath (55 °C) was used. After the enzymatic hydrolysis, pH of the medium was adjusted to 7.0 with 10 M NaOH and the obtained substrate sterilized at 121 °C for 15 min (Goksungur et al., 2002).

2.4. Microorganism and culture medium

The strain of bacterium *D. natronolimnaea* HS-1 (DSM 44860) was obtained from the BPEL. It was kept on yeast/malt (YM) agar plates containing 10 (g/l) D-glucose, 5 (g/l) peptone, 5 (g/l) yeast extract, 3 (g/l) malt extract, 36 ppm Fe^{3+} and 15 (g/l) agar. Every month, single colonies were transferred to a fresh plate, incubated for 4 days, and then maintained under refrigeration condition.

2.5. Inoculum preparation and fed-batch fermentation

After preparing pre-culture in liquid YM medium (i.e., the above formulation without agar added), the inoculum was transferred into 500-ml Erlenmeyer flasks containing 20 g/l the EHM and 10 g/l yeast extract. Finally, the flasks were incubated in an orbital shaking incubator (Model JEIO TEC SI-4000R, Korea) at 180 rpm and 28 ± 2 °C for 6 days used to inoculate the bioreactor. 200 ml of the prepared inoculum into 5 l bioreactor (KF-5L, KBT, South Korea) containing 3 l growth medium containing 8 g/l peptone, 8.2 g/l yeast extract, 25.8 mM sodium citrate, 36 ppm Fe^{3+} and 26.16 g/l EHM was added. The optimum conditions according to the preliminary studies were pH of 7.0; and temperature of 28 °C. The pH-value during the fermentation culture was automatically controlled to remain at 7.0 ± 0.1 using additions of 2 M HCl and 2 M NaOH solutions. The bioreactor was operated isothermally at 28 ± 1 °C with a stirring rate of 130 ± 10 rpm and an aeration rate of 3 ± 1 vvm. Foam was controlled by the addition of 25% antifoam 204.

All fed-batch fermentations were initiated as a batch culture, and the feeding substrate was pumped into the fermenter using a computer coupled peristaltic pump. For the constant feeding rate (CFR) strategy, after 48 h of fermentation at the end of lag-phase, feeding was started to continuously pump concentrated EHM solution at various concentrations (30, 45 and 60 g/l) into the bioreactor at constant speeds of 4, 7 and 10 ml/h. In the exponential feeding rate (EFR) strategy for fed-batch culture, the EHM feeding rate was determined based on Eq. (1), which was derived from a mass balance with the assumption of constant cell yield on the substrate and constant maintenance coefficient throughout fermentation (Angardi and Çalık, 2013). Feeding rate (F, ml/h) at a varied specific growth rate was given by:

$$F = \frac{\mu V_0 X_0}{Y_{X/S}(S_F - S)} \exp(\mu t) \quad (1)$$

where μ is the specific growth rate (h^{-1}), V_0 is the initial culture volume (l), X_0 is the initial cell concentration (g/l), $Y_{X/S}$ is the theoretical cell yield on EHM, S_F is the EHM concentration in the feeding solution (30, 45 and 60 g/l), and t is the culture time.

Samples during certain times were withdrawn from the batch fermenter to evaluate the growth rate and production levels of total carotenoid (TC) and CTX.

2.6. Analytical techniques

For the extraction of carotenoid pigments, aliquots (10 ml) of cultures were taken from bioreactor at the appropriate times

during the fermentation process and the supernatant was collected by centrifuging at 7500*g* for 7.5 min at 4 °C. The cell pellets were then washed twice with normal saline and centrifuged again. These cells were re-suspended three times in 3 ml of pure ethanol by vortexing and centrifuging for 5 min in order to extract the pigment. A water bath (45 ± 1 °C) was also used to completely extract the pigments. The carotenoid extracts subsequently passed through a 0.2 μm hydrophobic fluorophore membrane (Sigma–Aldrich Co., USA) to get culture filtrate (Khodaiyan et al., 2007).

The absorbance of the ethanol extracts was determined using a UV/Vis spectrophotometer (Jasco, V-630, Tokyo, Japan) in the spectral region of 300–600 nm with a λ_{max} = 474 nm. The TC content (μg/l) was calculated by using the equation reported by Nasri Nasrabadi and Razavi (2010a):

$$TC = \frac{A_{474} \times V_s \times 10^9}{A^{1\%}_{1\ cm} \times 100} \quad (2)$$

where A_{474}, V_s and $A^{1\%}_{1\ cm}$ are the absorbance maximum of TC in ethanol, the volume of sample solution, and the specific absorption coefficient of TC for a 1% solution in a 1 cm cell (in ethanol, $A^{1\%}_{1\ cm} = 2200$), respectively (Schiedt and Liaaen-Jensen, 1995).

The determination of CTX and other individual carotenoids was carried out according to the modified method of Razavi et al. (2006) using a HPLC system (Knauer Berlin, Germany). This system was including a k-1001 HPLC pump, a k-1001 solvent organizer, an on-line degasser, a dynamic mixing chamber and a UV–Vis detector (K-2600, Knauer, Germany). The separation was performed on a Lichrospher 100 RP-18 silica column (5.0 mm, 250 × 4 mm) at 35 °C. The used isocratic mobile phase was acetonitrile/MeOH/dichloromethane solvent mixture (71:22:7, v/v/v) at a flow rate of 2 ml/min. The volume of solutions injected was 10 μl. A pre-column of the same material was applied to protect the column.

For the evaluation of biomass dry weight (BDW), a concentrated cell suspension was diluted with a suitable amount of the synthetic mineral medium to give an optical density ranging from 0.1 to 1.0 when measured at 600 nm. 10 ml of each dilution was filtered through a dried (at 65 °C for 12 h) and pre-weighed membrane filter (Sigma–Aldrich Co., pore diameter 0.2 μm, USA). All filters were then dried at 105 °C to constant weight (48 h) and placed in a desiccator to cool down. The BDW was calculated as the difference of the filter weight before and after the procedure. Finally, the calibration curve was constructed as the dependence of the optical density on the concentration of BDW (Gharibzahedi et al., 2012).

The Miller method was also applied to assay the reducing sugar concentration using 3,5-dinitrosalisylic acid (DNS) on cell-free supernatants filtered through 0.2 μm filters (Miller, 1959). In brief, 1.0 ml sample was centrifuged at 3500*g* for 5 min, and then 1.0 ml of DNS reagent was added to the supernatant. Samples were heated to boiling for 5 min and placed in an ice-bath. Afterwards, 8.0 ml distillated water was added, and the tubes were shaken for 5 min. Optical density of glucose concentration was measured using a UV–visible spectrophotometer (Jasco, V-630, Tokyo, Japan) at 575 nm.

2.7. Statistical analysis

The experiments were carried out in two replicates and results are presented as the average with standard deviation (SD) represented by error bars in graphs. Analysis of variance (ANOVA) procedure followed by Duncan's test using the SAS statistical

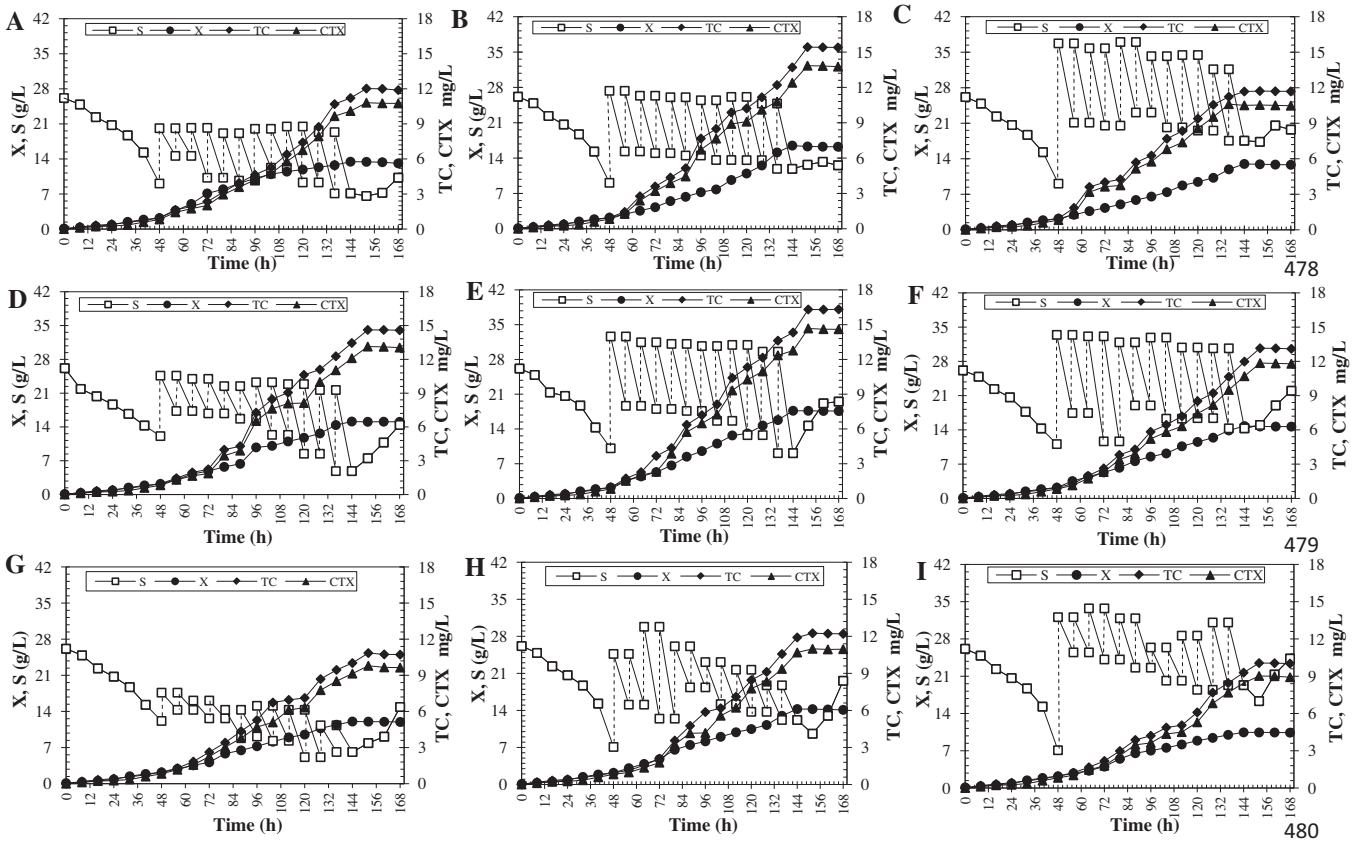

Fig. 1. Variation in carbon substrate (S), BDW (X), TC and CTX concentrations with cultivation time at CFRs of 4 (A–C), 7 (D–F) and 10 (G–I) ml/h with the different EHM concentrations (30 g/l (A, D and G); 45 g/l (B, E and H); 60 g/l (C, F and I)) in a fed-batch bioreactor (Starting point: 48 h of batch fermentation time, feeding EHM concentration: 26.16 g/L).

computer package (SAS 9.2 software Institute, Inc., Gary, NC, USA) was applied to determine the significant difference ($p < 0.05$) between treatment means.

3. Results and discussion

Fed-batch process is usually applied in industrial fermentations, for instance, in the production of baker's yeast, some organic acids, amino acids, vitamins, enzymes, antibiotics, microbial cells, growth hormones and other compounds (Lee et al., 1999). Abadias et al. (2003) demonstrated that fed-batch process using an optimum feeding strategy can highly decrease substrate inhibition or Crabtree effect maintaining a better oxygen level and realize maximum metabolite productivity. Unlike CTX, several studies have examined the fed-batch production of microbial astaxanthin (ASX) using different feeding strategies (Yamane et al., 1997a; Hu et al., 2005). Two CFR and EFR strategies were thus evaluated to determine the most suitable of nutrients flow for an enhanced cell growth and production of target metabolites. Cultures growth was started in a batch mode with optimum amount of carbon substrate (26.16 g/l) for 48 h and then the fed-batch culture was started by feeding the fresh fermentation medium with 30, 45 and 60 g/l EHM. Since the use of high concentrations of carbon substrate inhibit cell growth, the process was started with smaller amounts of EHM so that the CTX formation was maximized by increasing the biomass level. Hu et al. (2005) and Yamane et al. (1997b) have also reported that the semi-continuous production of ASX by the red yeast *Phaffia rhodozyma* (*Xanthophyllomyces dendrorhous*) was enhanced with an optimum and high C/N ratio.

Time-course profile of CTX, TC and cell mass production and also EHM consumption by *D. natronolimnaea* HS-1 in a fed-batch

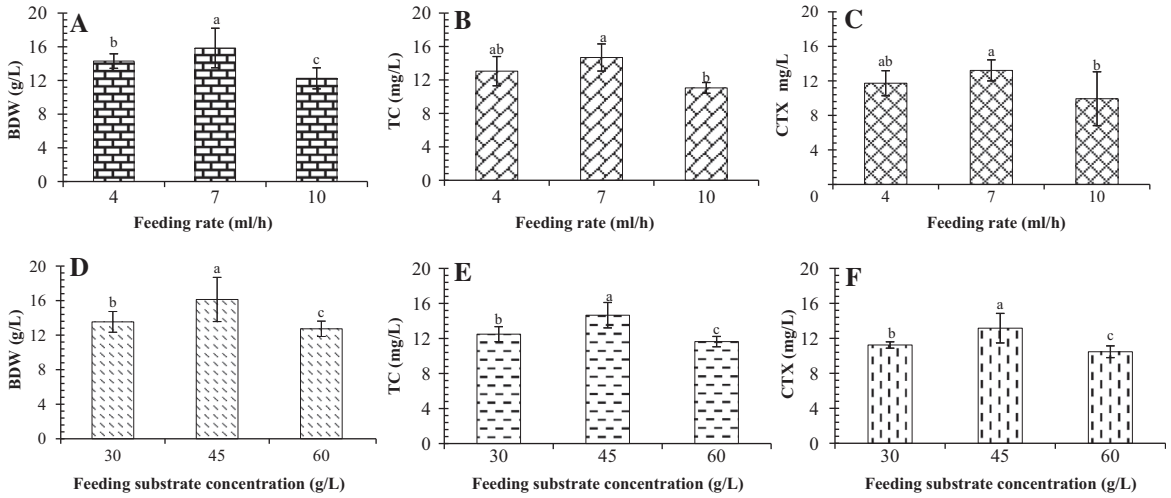

Fig. 2. Effect of the different levels of CFR (A–C) and feeding EHM concentration (D–F) on the BDW (A and D), TC (B and C), and CTX (C and F) produced by *D. natronolimnaea* HS-1 in a 152 h fed-batch bioprocess. Different letters (a–c) indicate statistical differences ($p < 0.05$) among each treatment among the different treatments.

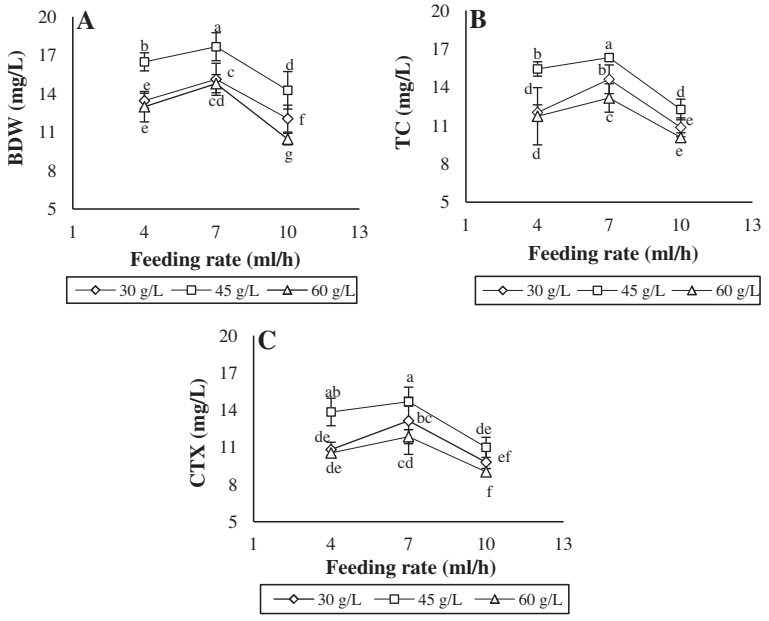

Fig. 3. Interaction effect of the feeding EHM concentration and feeding rate on the BDW (A), TC (B), and CTX (C) produced by *D. natronolimnaea* HS-1 in a 152 h fed-batch bioprocess. Means with the same letter (a–g) are not significantly different at $p < 0.05$.

Table 1
Maximum specific growth rate and coefficients of productivity and yield of cell biomass and carotenoid pigments biosynthesized by *D. natronolimnaea* HS-1 under fed-batch cultivation with the different concentrations of EHM in CFRs.

Growth and production factors[a,b]	Feeding EHM substrate concentration (g/l)								
	30			45			60		
Constant feeding rate (CFR, ml/h)	4	7	10	4	7	10	4	7	10
Maximum specific growth rate (μ_{max}, h^{-1})	0.109 ± 0.006^{d}	0.121 ± 0.004^{a,b}	0.092 ± 0.005^{f}	0.115 ± 0.024^{c}	0.127 ± 0.007^{a}	0.101 ± 0.024^{e}	0.106 ± 0.003^{d}	0.117 ± 0.024^{b}	0.082 ± 0.024^{g}
Total carotenoid production yield ($Y_{P\text{-}TC/S}$, mg/g)	2.44 ± 0.56^{c}	2.97 ± 0.08^{b}	1.71 ± 0.13^{e}	2.53 ± 0.77^{c}	3.40 ± 0.21^{a}	1.93 ± 0.08^{d}	1.87 ± 0.24^{d}	1.43 ± 0.13^{f}	1.10 ± 0.44^{g}
Canthaxanthin production yield ($Y_{P\text{-}CTX/S}$, mg/g)	2.20 ± 0.47^{d}	2.89 ± 0.37^{b}	1.55 ± 0.74^{f}	2.46 ± 0.09^{c}	3.07 ± 0.09^{a}	1.74 ± 0.61^{e}	1.69 ± 0.19^{e}	1.29 ± 0.05^{g}	0.98 ± 0.31^{h}
Biomass production yield ($Y_{X/S}$, g/g)	2.55 ± 0.34^{b,c}	2.41 ± 0.11^{c}	1.81 ± 0.26^{f}	2.76 ± 0.04^{b}	3.53 ± 0.53^{a}	2.16 ± 0.66^{d}	1.93 ± 0.31^{e}	1.53 ± 0.04^{g}	1.07 ± 0.25^{gh}
Biomass volume productivity (Q_X, g/lh)	0.123 ± 0.002^{c}	0.124 ± 0.032^{c}	0.113 ± 0.029^{d}	0.155 ± 0.011^{bc}	0.170 ± 0.008^{a}	0.136 ± 0.012^{b}	0.118 ± 0.025^{cd}	0.140 ± 0.022^{b}	0.097 ± 0.004^{e}
Total carotenoid volume productivity (Q_{TC}, mg/lh)	0.118 ± 0.004^{de}	0.151 ± 0.047^{b}	0.108 ± 0.007^{f}	0.153 ± 0.016^{b}	0.163 ± 0.007^{a}	0.122 ± 0.026^{d}	0.114 ± 0.007^{e}	0.131 ± 0.045^{c}	0.099 ± 0.024^{g}
Canthaxanthin volume productivity (Q_{CTX}, mg/lh)	0.106 ± 0.009^{d}	0.146 ± 0.014^{a}	0.097 ± 0.025^{e}	0.138 ± 0.004^{b}	0.147 ± 0.014^{a}	0.110 ± 0.003^{cd}	0.103 ± 0.032^{d}	0.1180 ± 0.006^{c}	0.089 ± 0.018^{f}

[a] Values represent as mean ± standard deviation.
[b] Values in the same rows followed by different letters (a–h) are significantly different ($p < 0.05$).

process under the CFR (4, 7 and 10 ml/h) strategy with different EHM concentrations are shown in Fig. 1. As depicted in these figures, the BDW, CTX and TC levels were decreased by increasing feeding rate from 7 to 10 ml/h. However, an increase in feeding rate from 7 to 10 ml/h led to a significant decrease in the values of growth and production parameters. Fig. 2A–C also illustrates that feeding rate had a significant effect on the studied variables ($p < 0.05$). The values of cell biomass at CFRs of 4, 7 and 10 ml/h were 14.85, 15.85 and 12.25 g/l, respectively. The production of TC and CTX at the same feeding rates was 13.05 and 11.73, 14.69 and 13.21, and 11.05 and 9.90 mg/l, respectively. Therefore, the increase of CFR from 4 to 7 ml/h led to increase in the BDW, TC and CTX production by *D. natronolimnaea* HS-1. Meanwhile, increase of CFR up to 10 ml/h, significantly decreased the production parameters. The resulted findings could be attributed to the substrate consumption rates (Kim et al., 2009a,b). EHM concentration in the culture medium at the ideal feeding rate of 7 ml/h was insignificantly higher than at 4 ml/h feeding rate, which was low and not inhibitory to the cell growth. At lower feeding rate (4 ml/h), the consumption rate of simple sugars in the fed-batch culture exceeded the feeding rate, leading to a rapid drop in carbon substrate concentration and to EHM depletion in the culture at 24 h. As the feeding rate surpassed the optimum level (10 ml/h), the feeding rate exceeded the consumption rate, leading to a rapid EHM accumulation in the culture medium (Fig. 1). This EHM accumulation had a considerable effect on growth inhibition resulting in lower BDW, TC and CTX production. Our findings were in accord with those reported by Xu et al. (2011). They by investigating the optimal fed-batch operation for efficient beauvericin production by *Fusarium redolens* Dzf2 growth found that the glucose feeding rate of 1.8 g/h can more lead to increase the production parameters than 0.9 and 3.6 g/h CFRs.

The ANOVA analysis showed that CFR had more effect than EHM concentration on the growth and production parameters under fed-batch fermentation ($p < 0.01$). Thus, the existence of a suitable feeding rate can maintain the nutrients content, especially carbon substrate, at an optimum level, increasing yield and productivity of the fermentation process (Yu et al., 2012). Similar results were obtained by Bhosale and Gadre (2002) who showed the feeding rate compared to feeding carbon-substrate concentration is stronger factor to significantly enhance the cell growth and β-carotene from *Rhodotorula glutinis* mutant.

Fig. 2D–F revealed that the selected carbon concentration (45 g/l) in the medium concentration for used carbon substrate could yield the highest values of BDW, TC and CTX. However, a considerable decrease in these parameters was observed by increasing EHM concentration from 45 to 60 g/L ($p < 0.001$). The amount of produced BDW at CFRs with 30, 45 and 60 g/l EHM were 13.54, 16.13 and 12.73 g/l, respectively. An increase in carbon substrate from 30 to 45 g/l caused the increase in TC and CTX levels from 12.49 to 14.66 and 11.24 to 13.16 mg/l, respectively. However, an increase in EHM content to 60 g/l led to a significant decrease in the TC (11.64 mg/l) and CTX (10.47 mg/l) values ($p < 0.0001$). Therefore, the existence of 45 g/l EHM in the CFR was optimum.

Fig. 3 also shows the interaction effect of EHM and CFR concentration on the investigated parameters. Some kinetic parameters of cell growth and carotenoid production in fed-batch cultivations with different levels of CFR and EHM are shown in Table 1. As can be seen in Fig. 3 and Table 1, the combination of 45 g/l EHM and 7 ml/h feeding rate can lead to the highest values of BDW (17.66 g/l), TC (16.31 mg/l), CTX (14.67 mg/l), biomass yield ($Y_{X/S}$, 3.53 g/g), TC yield ($Y_{P\text{-}TC/S}$, 3.40 mg/g), CTX yield ($Y_{P\text{-}CTX/S}$, 3.07 mg/g), and volume productivities of biomass (Q_X, 0.170 g/lh), TC (Q_{TC}, 0.163 mg/lh) and CTX (Q_{CTX}, 0.147 mg/lh) by *D. natronolimnaea* HS-1 in a fed-batch bioreactor. Conversely, the lowest amounts of

BDW (10.45 g/l), TC (10.06 mg/l), CTX (9.02 mg/l), $Y_{X/S}$ (1.07 g/g), $Y_{P\text{-}TC/S}$ (1.10 mg/g), $Y_{P\text{-}CTX/S}$ (0.98 mg/g), Q_X (0.097 g/lh), Q_{TC} (0.099 mg/lh) and Q_{CTX} (0.089 mg/lh) were obtained using the highest used CFR (10 ml/h) and EHM concentration (60 g/l). The maximum specific growth rate (μ_{max}) ranged from 0.082 (10 ml/h feeding rate and 60 g/l EHM) to 0.127 h^{-1} (7 ml/h feeding rate and 45 g/l EHM).

Fig. 4 exhibits time-course profile of BDW, TC, and CTX production and EHM consumption by *D. natronolimnaea* HS-1 in a fed-batch fermenter system with EFR strategy at different concentrations of EHM. As clearly illustrated in this figure, by increasing EHM content in the EFR the production values of BDW, TC and CTX were gradually decreased. The maximum values of produced BDW using 30, 45 and 60 g/l EHM in fed-batch cultivation with EFR strategy were 14.15, 13.56 and 12.45 g/l, respectively ($p > 0.05$). The produced TC and CTX levels at similar concentration ranges of EHM were 11.97 and 10.78, 11.54 and 10.38, and 11.29 and 10.16 mg/l, respectively ($p < 0.05$). The highest μ_{max} (0.111 h^{-1}) was obtained at 30 g/l EHM, while an increase in the concentration of carbon substrate from 30 to 60 g/l led to a significant decrease in μ_{max} value ($p < 0.01$). The results also showed that the highest $Y_{X/S}$ (2.53 g/g), $Y_{P\text{-}TC/S}$ (2.30 mg/g), $Y_{P\text{-}CTX/S}$ (2.08 mg/g), Q_X (0.125 g/lh), Q_{TC} (0.114 mg/lh) and Q_{CTX} (0.103 mg/lh) were found for an EFR containing 30 g/l EHM ($p < 0.05$; Table 2).

Reduction in cell growth and metabolite production using the EFR strategy during the growth phase might be due to the high concentration of carbon substrate in the medium and increasing the ratio of substrate feeding rate to its consumption rate (Fig. 4). Thus, the lowest concentration of carbon substrate used in EFR strategy led to the highest values of cell growth and production kinetic parameters. Further increase in EHM amount can cause an imbalance in consumption of this substrate and followed by high density of the additional EHM in medium inhibits the *D. natronolimnaea* HS-1 cell growth and biosynthesized CTX. It is evident that the higher concentration of carbon-substrate (45 g/l) can improve the BDW, TC and CTX during a CFR-fed batch process. The low BDW at higher levels of carbon source (60 g/l) can be due to the osmotic stress and/or substrate inhibition effect in culture media (Gharibzahedi et al., 2013a). Park et al. (2008) showed that the decline in the cell growth and increase in lag phase duration of β-Ionone resistant mutant of *X. dendrorhous* was caused by the substrate inhibition. The high access of cells to oxygen can stimulate the aerobic metabolism, resulting in higher ATP availability for BDW production. Thus, the existence of high viscosity at high concentrations of EHM can also limit oxygen transfer and decrease yield of the synthesized biomass and CTX (Gharibzahedi et al., 2012). Avci et al. (2013) also demonstrated that the by-products formed during the anaerobic fermentation performed by *Klebsiella pneumonia* can reduce the 1,3-propanediol production via inhibition of the enzymes activity involved in its biosynthesis pathway.

The statistical analysis of the results demonstrated that fed-batch cultivation with a CFR strategy is much better than the EFR strategy ($p < 0.01$). Moriel et al. (2005) by comparing the production of ASX by the *P. rhodozyma* ATCC 24202 using two CFR and EFR strategies of diluted sugar cane juice reported a reduction in the cellular ASX concentration (303.34 mg/g biomass) and biomass concentration (18.75 g/l) in pulsed fed-batch processes. They showed that the continuous fed-batch processes led to a

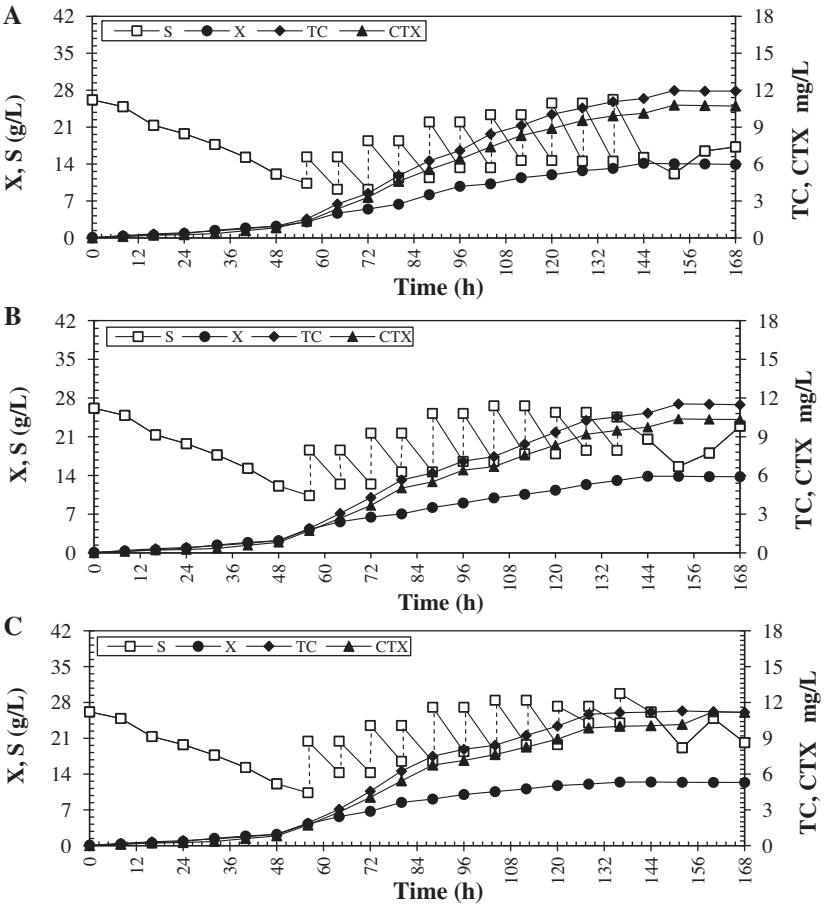

Fig. 4. Variation in carbon substrate (S), BDW (X), TC and CTX concentrations with cultivation time at EFR with the different EHM concentrations (A), 30 g/l; (B), 45 g/l; (C), 60 g/l in a fed-batch bioreactor.

Table 2
Maximum specific growth rate and coefficients of productivity and yield of cell biomass and carotenoid pigments biosynthesized by *D. natronolimnaea* HS-1 under fed-batch cultivation with an EFR strategy at the different concentrations of EHM.

Growth and production factors[a,b]	Feeding EHM substrate concentration (g/l)		
	30	45	60
Maximum specific growth rate (μ_{max}, h^{-1})	0.111 ± 0.011[a]	0.109 ± 0.012[a]	0.095 ± 0.005[b]
Total carotenoid production yield ($Y_{P\text{-}TC/S}$, mg/g)	2.30 ± 0.15[a]	1.63 ± 0.07[b]	1.29 ± 0.04[c]
Canthaxanthin production yield ($Y_{P\text{-}CTX/S}$, mg/g)	2.08 ± 0.02[a]	1.46 ± 0.04[b]	1.16 ± 0.04[c]
Biomass production yield ($Y_{X/S}$, g/g)	2.53 ± 0.18[a]	1.75 ± 0.08[b]	1.23 ± 0.05[c]
Biomass volume productivity (Q_X, g/lh)	0.125 ± 0.003[a]	0.114 ± 0.004[b]	0.099 ± 0.001[c]
Total carotenoid volume productivity (Q_{TC}, mg/lh)	0.114 ± 0.002[a]	0.107 ± 0.002[b]	0.104 ± 0.004[b]
Canthaxanthin volume productivity (Q_{CTX}, mg/lh)	0.103 ± 0.003[a]	0.096 ± 0.006[b]	0.094 ± 0.001[b]

[a] Values represent as mean ± standard deviation.
[b] Values in the same rows followed by different letters (a–h) are significantly different ($p < 0.05$).

considerable increase in the biomass productivity without any losses in ASX production by the studied yeast (Moriel et al., 2005). A same reduction in the cellular ASX concentration, using pulsed fed-batch processes and the same substrates was observed by Reynders et al. (1997). However, Hu et al. (2005) found a significant enhancement (54.9%) in ASX production by *X. dendrorhous* after 132 h fermentation in pulse fed-batch process as compared with batch process.

In this study, the highest values of BDW, TC and CTX produced by *D. natronolimnaea* HS-1 were obtained for the performed experiment with a CFR of 7 ml/h containing 45 g/l EHM. Nasri Nasrabadi and Razavi (2010a,b) previously showed a considerable increase in the TC and CTX production by this bacterium in a fed-batch bioreactor from waste molasses by optimization of tricarboxylic acid cycle intermediates and trace elements. However, the produced amounts of BDW, TC and CTX in the present study respectively were 22.8%, 17.8% and 12.1% more than the report of Nasri Nasrabadi and Razavi (2010b). This fact may be attributed to the more bioavailability to simple sugars of glucose and fructose with enzymatic hydrolysis of molasses and also discrepancy strategy in feeding rate of carbon substrate. A similar result has also been found for the production of β-carotene by recombinant *Escherichia coli* (Kim et al., 2009a,b), zeaxanthin by *Flavobacterium multivorum* (Bhosale et al., 2004), ASX by *Haematococcus pluvialis* (Zhang et al., 1999), and lutein by the green microalga *Chlorella* (Shi et al., 2002; Wu et al., 2007). In general, the fed-batch process is a preferred operational mode due to the substantial enhancement in CTX production from *D. natronolimnaea* HS-1 by overcoming substrate inhibition and catabolite repression compared to the batch process (Khodaiyan et al., 2007; Gharibzahedi et al., 2012). From an economic point of view, the CFR-fed batch mode by feeding 45 g/l EHM as a cheap raw material could significantly facilitate low-cost industrial CTX production. Thus it is reasonable that much energy consumption and labor would be saved when an equal amount of CTX is produced by fed-batch mode compared to batch culture.

4. Conclusion

A fed-batch fermentation process with two strategies of EFR and CFR for CTX production from cheap raw material (EHM) was developed using the strain *D. natronolimnaea* HS-1. The fed-batch fermentation with CFR strategy was better than EFR strategy to increase the growth and production parameters. A maximum CTX production of 14.67 mg/l was obtained at 152 h when the amount of added EHM was 45 g/l using a CFR of 7 ml/h. The proposed efficient CFR-fed batch fermentation for CTX production by the studied strain is potentially competitive for industrial production from EHM in terms of CTX productivity and yield.

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