Screening of carbon and nitrogen sources using mixture analysis designs for carotenoid production by *Blakeslea trispora*

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Abstract The production of many secondary metabolites such as carotenoids is influenced by the type of carbon and nitrogen sources and C:N ratio applied in culture medium. The present study discusses the role of C:N ratio and screening of carbon and nitrogen sources using mixture analysis design in carotenoids production by *Blakeslea trispora*. The C:N ratios of 20, 40, and 60 with six nitrogen sources were evaluated. Results indicated that limitation of nitrogen source (C:N of 60) could improve carotenoids production. Six nitrogen and carbon sources were then screened using mixture analysis design. The most effective nitrogen and carbon sources were soybean powder and glucose, respectively. The productivity of carotenoids (983.8 ± 31.5 mg/L) based on consumed nitrogen and carbon source was 189.10 mg/g soybean powder and 19.66 mg/g glucose. Mixture analysis design indicated single carbon and nitrogen source were more effective than a mixture of sources for carotenoids production.

Keywords Mixture analysis design · Carotenoid · β -carotene · *Blakeslea trispora* · C:N ratio

Introduction

Blakeslea trispora, a species of Mucorales, has been commercially applied for the production of some carotenoids such as β -carotene and lycopene (Chandi and Gill, 2011). This fungus is a valuable template of medium optimization in microbial products and wide range of researches has been accomplished for improvement of β -carotene production in *B. trispora* cultures. Many enhancers and inducers and also different medium components have been studied for increase of β -carotene yield in this fungus (Choudhari and Singhal, 2008; Nanou and Roukas, 2011; Nanou et al., 2012). However, unlike many investigations on the effect of stimulators and inducers, the role of carbon to nitrogen ratios (C:N ratios) and the interactions between different sources of carbon and nitrogen have less been investigated.

Screening of carbon and nitrogen sources, as main compounds in a usual culture medium and their mass balances are the most important issues in the medium improvement. They are linked to biomass and metabolite productivity and influence the secondary metabolism and yield of products (Casas López et al., 2003; Kennedy and Krouse, 1999). Therefore, different strategies are often applied for screening of carbon and nitrogen sources in growth media. Component swapping, one factor at a time, and Plackett–Burman are some popular methods for screening of medium components and have been utilized for medium improvement in *B. trispora* cultures (Choudhari et al., 2008; Papaioannou and Liakopoulou-Kyriakides, 2010). However, the interaction between variables could not be evaluated using these methods. Furthermore, some methods such as Plackett–Burman are suitable for screening of independent factors (Rispoli and Shah, 2007). Mixture analysis design is an alternative method for

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screening and also optimizing of components in culture media. It is a suitable method to evaluate the proportion of ingredients in a medium, while the final concentration of total components is constant. Thus, simultaneous evaluating of different carbon sources or nitrogen sources in culture media will be possible, while the C:N ratio is constant. The other advantage of mixture analysis method is capability to eliminate both neutral- and negative-factors (Par-ekh et al., 2000; Rispoli and Shah, 2007). This method has been applied in extensive researches in food, petroleum, pharmaceutical and microbiology as optimization method (Moreira et al., 2007; Rispoli and Shah, 2007). However, unlike many investigations on medium improvement for the production of β -carotene by *B. trispora* screening of a mixture of carbon and nitrogen sources using experimental design has not been evaluated, yet.

Thus, in the present study, mixture analysis design was applied to screen affecting carbon and nitrogen sources on the production of β -carotene by *B. trispora*. Furthermore, a suitable C:N ratio was investigated to produce the highest amount of β -carotene using the lowest amount of carbon and nitrogen sources.

Materials and methods

Chemicals

β -carotene and lycopene were purchased from Sigma Aldrich (St. Louis, MO, USA). All medium components, chemicals, solvents with analytical grade, and also HPLC grade solvents including methanol and tetrahydrofuran were obtained from Merck (Darmstadt, Germany). Soybean powder was prepared by grinding the whole soybean, variety of Clark which was purchased from Oilseed Research and Development Company (Tehran, Iran). Concentrated corn steep liquor (CSL) was obtained from Zar industrial research and development group (Tehran, Iran).

Microorganisms

B. trispora DSMZ 2387 (plus mating type) and DSMZ 2388 (minus mating type) were obtained from Persian Type Culture Collection (PTCC). For sporulation, strains were incubated on malt extract agar at 28 °C for 7 days. The spore suspensions used as inoculums were prepared by washing and scrapping the slants surfaces using 10 mL of sterile aqueous solution of glycerol [10% (v/v)].

Seed culture preparation

An amount of 200 mL of culture medium containing 20 mL/L CSL, 40 g/L glucose, and 1 mL soybean oil with adjusted pH of 7 was placed into 1000 mL Erlenmeyer flask and then autoclaved. Each flask was inoculated with approximately 10^6 spores of each fungus, separately and incubated in a shaker incubator (Clim-O-Shaker, Adolf Kühner, Birsfelden, Switzerland) for 48 h at 28 °C and 160 rpm.

Evaluation of suitable C:N ratio

Since the level of nitrogen in culture medium is a determinative factor for the production of secondary metabolites, suitable ratio of carbon to nitrogen in the medium was evaluated. Therefore, a simple basal medium including KH_2PO_4 : 1.2 g/L and MgSO_4 : 0.4 g/L was prepared and 1 mL of trace elements solution including (mg/L) H_3BO_3 (2860), $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$ (1810), ZnCl_2 (222), $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ (390), $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ (79), $\text{Co}(\text{NO}_3)_2 \cdot 6\text{H}_2\text{O}$ (49), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (1000), and $\text{FeCl}_3 \cdot 6\text{H}_2\text{O}$ (1000) was added to the culture media after sterilization.

For the nitrogen sources, three inorganic and three organic components including di-ammonium sulphate, di-ammonium phosphate, ammonium acetate, soybean powder, yeast extract, and CSL was selected. For inorganic components the percentage of nitrogen was calculated according to the molecular weights and for organic nitrogen sources it was obtained based on information of manufacturers. Three different C:N ratios of 60, 40, and 20 were calculated for each nitrogen sources while the concentration of glucose was constant in 50 g/L. Then, calculated concentrations of pure nitrogen sources were added to the basal medium, separately, while the content of glucose as the only carbon source was fixed on 50 g/L.

Evaluation of the role of BHT and span 20 on the carotenoids production using different nitrogen sources

According to the results of C:N ratio, a ratio of 60 was selected. Butylated hydroxytoluene (BHT) and span 20 are two popular components with capability of increasing carotenoids production in *B. trispora*. Although, the role of these two components has well been known on carotenogenesis, further investigation was necessary to find the effect of these ingredients in accompany with different nitrogen sources on the carotenoids production. Thus, BHT (4.4 g/L) and span 20 (0.2%) were added to culture media with six different nitrogen sources while the C:N of culture media was adjusted on 60. The concentration of BHT and

span 20 was chosen according to our previous experiences in the laboratory.

Experimental design for screening of nitrogen sources using mixture analysis

For screening of nitrogen sources a simplex lattice design in degree 2 with center point and axial points was selected. According to this method six levels of each nitrogen source will be checked in culture medium in the presence of other nitrogen sources, while the final concentration of nitrogen in culture medium is constant. Thus, in each trial the C:N ratio is constant on 60 and by increasing the concentration of any nitrogen source in culture medium the levels of other nitrogen sources are decreased, deliberately. This design allows us to evaluate the effect of different sources of nitrogen and their negative or positive interactions, in a mixture. The experimental design of mixture analysis for screening of nitrogen sources is indicated in Table 1.

Experimental design for screening of carbon sources using mixture analysis

Based on screening of nitrogen sources, soybean powder was selected as the most effective nitrogen source and six popular carbon sources were screened using mixture analysis. Glucose, soluble starch, maltodextrin, glycerol, soybean oil, and sodium acetate were carbon sources which added to the basal medium. Soybean powder was added to the all culture media as fixed nitrogen source and different carbon sources were substituted with glucose while C:N ratio fixed on 60 (Table 2). The characteristics of design were similar to that of explained for screening of nitrogen sources.

At the time of each experiment, the desired concentrations of components based on the experiments were added to the basal medium into 250 mL Erlenmeyer flasks. Flasks were capped by cotton and autoclaved for 10 min at 121 °C. Each Erlenmeyer flask was then inoculated with 10 mL of seed cultures (5 mL 2388 strain and 5 mL 2387 strain) and incubated in shaker incubator (Clim-O-Shaker, Adolf Kühner) with 200 rpm at 28 °C for 120 h.

Analytical techniques

After 120 h the culture media were filtered through a Whatman No. 1 filter paper and dry biomass was determined using freeze drying (Lyovac GT3, Leybold Heraeus, Cologne, Germany) the solids.

For extraction of carotenoids 0.2 g of wet biomass was ground with mortar and pestle in presence of glass powder (Sigma-Aldrich) until a homogenized mixture was obtained. The mixture was transferred to a Falcon tube and

the mortar rinsed four times with 10 mL of methanol and pooled. Then, 10 mL hexane was added to the solution, shaken for 5 min, centrifuged at $6000 \times g$ for 15 min and supernatant was separated. Extraction was repeated with the residue two times and hexane phase was separated by addition of 25 mL distilled water and centrifugation at $3000 \times g$ for 5 min. The absorbance of the hexane solution was recorded at 450 nm using UV–Vis spectrophotometer (Unicam 8620, Thermo Spectronic, Cambridge, UK) (Kim et al., 1996). A serial dilution of β -carotene between 1 and 12 $\mu\text{g/mL}$ was applied as standard curve (Choudhari et al., 2008).

To evaluate the effect of different carbon and nitrogen sources on the percentage of β -carotene, γ carotene, and lycopene, 5 μL of extracted carotenoids in tetrahydrofuran was injected to a Knauer high performance liquid chromatography (HPLC) (Knauer, Berlin, Germany) system with column temperature of 40 ± 2 °C. The isocratic program applied to elute carotenoids was methanol (100%) (Craft, 1992). β -carotene and lycopene were detected at 450 and 475 nm, respectively. The concentration of γ carotene was estimated as explained earlier (Nanou and Roukas, 2011; Takaichi, 2000).

Antioxidant activity of total carotenoids in the selected medium was also evaluated using 2,2-diphenyl-1-picrylhydrazyl (DPPH) method (Lira et al., 2017). Briefly, fresh biomass of *B. trispora* washed two times with distilled water and 0.1 g of the wet biomass was extracted with 10 mL ethanol after grinding by mortar and pestle, as described earlier. The ethanolic solution was then centrifuged (Beckman, GS-6, Beckman Coulter Inc, Fullerton, CA, USA) at 10,000 rpm and 4 °C for 10 min. The extraction was repeated with pellet two times until a colorless precipitate was seen after centrifugation. For determination of antioxidant activity 100 μL ethanolic solution of the extracted carotenoids was mixed with 100 μL ethanolic solution of 0.15 mM DPPH and incubated at room temperature for 30 min in the dark. The absorbance of the solution was then recorded at 517 nm using an Epoch microplate reader (Biotek Instruments, Winooski, USA). The scavenging activity was calculated according to the following equation:

$$\text{Scavenging activity (\%)} = 100 \times (1 - (\text{Sample} - \text{blank}) / \text{Control})$$

The absorbance of the blank was obtained from a solution of 100 μL of carotenoids extract and 100 μL of ethanol. The absorbance of the control was also obtained from a mixture of 100 μL DPPH and 100 μL of a serial dilution of ethanolic solutions of ascorbic acid with concentrations of 0.5–100 $\mu\text{g/mL}$.

Table 1 Experimental design for screening of nitrogen sources using mixture design

Variables	Di-ammonium sulphate	Ammonium acetate	Di-ammonium phosphate	Soybean powder	Yeast extract	CSL	Total carotenoid (mg/L) ^a
Minimum	0	0	0	0	0	0	
Maximum (g/L)	1.57	1.83	1.57	5.2	3.3	4.7	
Variables	Di-ammonium sulphate	Ammonium acetate	Di-ammonium phosphate	Soybean powder	Yeast extract	CSL	Total carotenoid (mg/L) ^a
Ratio of components (%)							
50	0	0	0	0	0	50	212.0 ± 7.9
0	0	0	100	0	0	0	69.4 ± 5.8
0	50	0	0	50	0	0	312.9 ± 11.6
0	0	0	0	50	0	50	245.7 ± 11.1
58.3	8.3	8.3	8.3	8.3	8.3	8.3	270.6 ± 9.0
8.3	8.3	8.3	58.3	8.3	8.3	8.3	140.1 ± 14.5
0	0	0	0	0	100	0	152.2 ± 6.3
0	0	0	50	50	0	0	94.2 ± 5.9
50	0	0	0	0	50	0	91.1 ± 7.6
0	0	0	50	0	0	50	250.0 ± 17.7
0	0	0	0	0	0	100	563.1 ± 17.9
50	50	0	0	0	0	0	188.0 ± 14.8
8.3	8.3	8.3	8.3	8.3	8.3	58.3	309.3 ± 21.0
0	100	0	0	0	0	0	189.1 ± 12.3
50	0	50	0	0	0	0	100.3 ± 9.6
0	0	0	0	100	0	0	909.1 ± 61.9
50	0	0	0	50	0	0	360.0 ± 10.1
8.3	8.3	8.3	8.3	8.3	58.3	8.3	271.4 ± 9.5
0	0	0	0	50	50	0	270.3 ± 12.7
0	50	0	0	0	50	0	288.4 ± 6.1
0	0	0	0	0	50	50	209.0 ± 8.1
8.3	58.3	8.3	8.3	8.3	8.3	8.3	281.9 ± 13.3
100	0	0	0	0	0	0	90.1 ± 9.0
0	50	50	0	0	0	0	437.5 ± 15.8
0	0	50	0	0	50	0	203.6 ± 12.1
16.6	16.6	16.6	16.6	16.6	16.6	16.6	225.2 ± 18.4
8.3	8.3	8.3	8.3	58.3	8.3	8.3	196.8 ± 16.0
0	50	0	0	0	0	50	174.6 ± 8.9

^aEach value is expressed as mean ± standard deviation of three replicates

Statistical analysis

Total carotenoids was applied as dependent variable while different nitrogen and carbon sources were independent variables in mixture analysis designs. Regression for mixture with Alpha = 0.05 was applied to evaluate the significance of interactions. Two way and one way ANOVAs were used for finding the best C:N ratio. To produce confidence interval for the differences between means, Tukey's multiple range tests at $p < 0.05$ were applied.

Data were analyzed using Minitab V.16 (Minitab Inc., State College, PA, USA) software.

Results and discussion

Total carotenoids and dry biomass produced by *B. trispora* in six different nitrogen sources with different C:N ratios are shown in Fig. 1(A). Both independent variables including the kind of nitrogen source and C:N ratio and

Table 2 Experimental design for screening of carbon sources using mixture design

Variables	Glucose	Sodium acetate	Soluble starch	Maltodextrin	Glycerol	Soy bean oil	Total carotenoid (mg/L) ^a
Minimum	0	0	0	0	0	0	
Maximum (g/L)	50	113	45	45	51	28.5	
Variables	Glucose	Sodium acetate	Soluble starch	Maltodextrin	Glycerol	Soy bean oil	Total carotenoid (mg/L) ^a
Ratio of components (%)							
0	0	0	0	0	50	50	280.2 ± 12.1
50	50	0	0	0	0	0	45.5 ± 3.9
0	0	0	100	0	0	0	216.6 ± 8.3
0	0	0	50	50	0	0	173.0 ± 11.9
50	0	0	0	0	0	50	82.4 ± 7.4
58.3	8.3	8.3	8.3	8.3	8.3	8.3	365.6 ± 16.7
0	0	0	50	0	0	50	125.5 ± 8.4
0	0	0	0	0	0	100	99.4 ± 8.6
0	0	50	50	0	0	0	213.4 ± 16.6
0	50	0	0	0	50	0	19.3 ± 1.6
8.3	8.3	8.3	8.3	8.3	58.3	8.3	292.2 ± 14.0
0	50	0	50	0	0	0	10.2 ± 0.9
50	0	0	50	0	0	0	207.5 ± 12.2
0	0	50	0	0	0	50	74.9 ± 6.1
50	0	50	0	0	0	0	246.3 ± 13.6
0	0	100	0	0	0	0	215.3 ± 12.7
50	0	0	0	0	50	0	450.7 ± 19.8
100	0	0	0	0	0	0	724.6 ± 26.1
8.3	8.3	8.3	8.3	8.3	8.3	58.3	237.4 ± 17.9
16.6	16.6	16.6	16.6	16.6	16.6	16.6	150.4 ± 11.0
0	50	0	0	0	0	50	80.5 ± 7.3
8.3	8.3	8.3	58.3	8.3	8.3	8.3	187.6 ± 12.6
0	0	50	0	50	0	0	23.6 ± 2.1
0	50	50	0	0	0	0	181.6 ± 8.5
0	0	0	0	100	0	0	58.6 ± 4.8
8.3	58.3	8.3	8.3	8.3	8.3	8.3	33.6 ± 3.1
8.3	8.3	58.3	8.3	8.3	8.3	8.3	217.0 ± 9.7
0	100	0	0	0	0	0	15.0 ± 1.4

^aEach value is expressed as mean ± standard deviation of three replicates

also their interaction were effective on the total carotenoids as dependent variable, significantly ($p \leq 0.05$, data analyzed using two-way analysis of variance). Furthermore, the kind of nitrogen source was more effective than C:N ratio on the carotenoids production (F ratio of 701.65 opposite to 346.5 for nitrogen source and C:N ratio, respectively). One way analysis of the kind of nitrogen source indicated that soybean powder had the highest efficiency on the production of carotenoids. This is the first time that soybean powder is introduced as nitrogen source for the production of carotenoids by *B. trispora*. This property may be due to high levels of L-asparagine in

soybean seed. Asparagine constitutes up to 50% of total free amino acids in the developing cotyledon in soybean and receiving of nitrogen by seed embryo is through assimilation of glutamine and asparagine. Therefore, soybean could be considered as enriched source of L-asparagine (Pandurangan et al., 2012; Rainbird et al., 1984). In a research, Choudhari and Singhal (2008) evaluated the role of different nitrogen sources on the production of β -carotene by *B. trispora* and obtained low efficiency for soybean meal. Soybean meal is by product of oil extraction companies from soybean and the quality and composition

Fig. 1 Production of carotenoids in mg/L (A) and mg/g (B) with six nitrogen sources based on C:N ratio. Di-ammonium sulphate (AS), ammonium acetate (AA), di-ammonium phosphate (AP), soybean powder (Soy), yeast extract (YE), corn steep liquor (CSL)

of amino acids in soybean powder may be different from soybean meal.

Significant differences between C:N ratios of 60, 40, and 20 were also obtained using one way analysis ($p = 0.021$). Overall, the C:N ratio of 60 produced adequate biomass content and the highest concentration of carotenoids. In the secondary metabolism, limitation of carbon, nitrogen, phosphate, and other components of culture media could act as physiological stresses and increase productivity of secondary metabolites (Ruiz et al., 2010). In the present study a limitation on nitrogen source in culture medium was an effective factor for increasing the productivity of carotenoids as secondary metabolite, while excess nitrogen levels produced higher biomass with simultaneous decrease of carotenoids when organic nitrogen sources were used [Fig. 1(A)]. Calculation the yield of carotenoids production based on biomass indicated that limitation of nitrogen source improves accumulation of carotenoids inside the cells [Fig. 1(B)]. This can clearly be seen with organic

nitrogen sources, especially. For instance, when soybean powder was applied as nitrogen source, accumulation of carotenoids increased from 2.10 ± 0.30 to 9.60 ± 0.38 mg/g biomass while the C:N ratio was changed from 20:1 to 60:1. Thus, it can be concluded that limitation of nitrogen sources in culture medium could act as strategy to increase yield of the carotenoids production while produced biomass is constant or even less [Fig. 1(B)].

Many researchers have produced high levels of carotenoids by *B. trispora* while high levels of nitrogen components have been utilized in culture media (He et al., 2017; Nanou and Roukas, 2016). It may be due to application of many inducers and enhancers in culture media which affect on metabolic pathways. In the present study addition of BHT and span 20 increased significantly both biomass and carotenoids in culture media. BHT and span 20 improved carotenoid production with all six nitrogen sources of di-ammonium sulphate (4.4 time), ammonium

acetate (4.1 time), di-ammonium phosphate (1.7 time), soybean powder (6.7 time), yeast extract (2.1 time), and CSL (8.2 time) in comparison with results obtained without BHT and span 20. The highest level of carotenoids production was 798.9 ± 51.3 mg/L which was obtained with soybean powder as nitrogen source. The yield of carotenoids production was 35.34 ± 2.25 mg/g biomass which was 3.6 times higher than that of obtained without BHT and span 20 [Fig. 1(B)]. BHT and span 20 are well known components to increase mass transfer and oxidative stress (Nanou and Roukas, 2016). In fact, reactive oxygen species (ROS) such as superoxide (O_2^-) and hydroxyl radicals ($HO^\cdot$) which are formed during fermentation are responsible of many destructive damages in the cell. Carotenoids produced by *B. trispora* are capable of removing oxygen radicals from the cell. Thus, by increasing oxidative stresses inside the cell carotenogenesis will probably increase (Roukas, 2015). In the present research, during a preliminary study, a series of span 20 and BHT concentrations were added to the culture media, separately and also in combination together (data were not shown here). Results indicated that a combination of span 20 and BHT with described concentrations was the most effective. As discussed earlier, this could be due to increase of mass transfer and oxidative stress caused by span 20 and BHT, respectively (Roukas, 2015).

Results for screening of nitrogen source using mixture analysis design

The most effective nitrogen source screened by mixture analysis design (Table 1) was soybean powder. Regression analysis of results and interactions between nitrogen sources have been shown in Table 3 which included four significant interactions ($p \leq 0.05$). However, in mixture analysis due to strong correlation between all components regular *t* test is not applied to evaluate the significance of each factor and a special plot named response trace plot is usually applied to find the behavior of each component inside the mixture (Cornell, 2002). Response trace plot (Cox direction) of six nitrogen components is indicated in Fig. 2(A). In this plot, the trends of soybean powder and CSL have high efficiency for carotenoids production in comparison with other nitrogen sources and soybean powder was the most efficient independent variable. A significant model was observed in ANOVA table of mixture design (Table 3). The results for the regression model were as follow:

Table 3 Regression information and ANOVA table of mixture design for the production of carotenoids using six different nitrogen sources ($R^2 = 93.6$)

Term	Coefficient	<i>p</i> Value
Model	*	0.016
Linear	*	0.001
Quadratic	*	0.039
Di-ammonium sulphate (X_1)	96	*
Ammonium acetate (X_2)	183	*
Di-ammonium phosphate (X_3)	53	*
Soybean powder (X_4)	876	*
Yeast extract (X_5)	154	*
CSL (X_6)	559	*
$X_1 \times X_2$	319	0.446
$X_1 \times X_3$	188	0.649
$X_1 \times X_4$	− 487	0.258
$X_1 \times X_5$	21	0.960
$X_1 \times X_6$	− 328	0.434
$X_2 \times X_3$	1315	0.013
$X_2 \times X_4$	− 897	0.058
$X_2 \times X_5$	589	0.180
$X_2 \times X_6$	− 699	0.120
$X_3 \times X_4$	− 1552	0.006
$X_3 \times X_5$	468	0.275
$X_3 \times X_6$	− 178	0.666
$X_4 \times X_5$	− 978	0.042
$X_4 \times X_6$	− 1909	0.002
$X_5 \times X_6$	− 472	0.271

$$Y = 96X_1 + 183X_2 + 53X_3 + 876X_4 + 154X_5 + 559X_6 + 319(X_1 \times X_2) + 188(X_1 \times X_3) - 478(X_1 \times X_4) + 21(X_1 \times X_5) - 328(X_1 \times X_6) + 1315(X_2 \times X_3) - 897(X_2 \times X_4) + 589(X_2 \times X_5) - 699(X_2 \times X_6) - 1552(X_3 \times X_4) + 468(X_3 \times X_5) - 178(X_3 \times X_6) - 978(X_4 \times X_5) - 1909(X_4 \times X_6) - 472(X_5 \times X_6)$$

To obtain the best proportion of nitrogen source the optimization tool in software was applied. Soybean powder with 100% concentration was the most efficient nitrogen source in optimization plot and application of single source of nitrogen was more effective than a mixture of nitrogen sources. This may be due to the application of a high efficient nitrogen source (soybean powder) in culture media as described earlier.

Fig. 2 Response trace plot (Cox direction) of six nitrogen (A) and six carbon (B) compounds

Results for screening of carbon source using mixture analysis design

Regression analyses of the mixture design and interactions have been indicated in Table 4. The results for the regression model were as follow:

$$\begin{aligned}
 Y = & 713X_1 - 11X_2 + 201X_3 + 198X_4 + 54X_5 \\
 & + 93X_6 - 1156(X_1 \times X_2) - 729(X_1 \times X_3) - 895(X_1 \times X_4) \\
 & + 422(X_1 \times X_5) - 1137(X_1 \times X_6) \\
 & + 423(X_2 \times X_3) - 294(X_2 \times X_4) + 87(X_2 \times X_5) \\
 & + 247(X_2 \times X_6) \\
 & + 143(X_3 \times X_4) - 273(X_3 \times X_5) - 153(X_3 \times X_6) \\
 & + 314(X_4 \times X_5) + 39(X_4 \times X_6) + 1001(X_5 \times X_6)
 \end{aligned}$$

However, strong tendency for consumption of glucose as carbon source [Fig. 2(B)] was observed in response trace plot (Cox direction) and glucose with 100% was the most effective carbon source in optimization plot.

Glucose has been applied as carbon source for the production of carotenoids in *B. trispora* by many investigators (Choudhari et al., 2008; He et al., 2017; Nanou and Roukas, 2011). However, in many researches glucose has been used in accompany with other carbon sources. Nanou and Roukas (2011) utilized glucose as main carbon source in accompany with 20% linoleic acid. Papaioannou and Liakopoulou-Kyriakides (2010) evaluated the efficiency of many substrates including lactose and starch on the production of β -carotene and indicated that both lactose and

Table 4 Regression information and ANOVA table of mixture design for the production of carotenoids using six different carbon sources ($R^2 = 93.1$)

Term	Coefficient	<i>p</i> Value
Model	*	0.021
Linear	*	0.003
Quadratic	*	0.107
Glucose (X_1)	713	*
Sodium acetate (X_2)	− 11	*
Soluble starch (X_3)	201	*
Maltodextrin (X_4)	198	*
Glycerol (X_5)	54	*
Soybean oil (X_6)	93	*
$X_1 \times X_2$	− 1156	0.019
$X_1 \times X_3$	− 729	0.096
$X_1 \times X_4$	− 895	0.050
$X_1 \times X_5$	422	0.302
$X_1 \times X_6$	− 1137	0.020
$X_2 \times X_3$	403	0.323
$X_2 \times X_4$	− 294	0.464
$X_2 \times X_5$	87	0.826
$X_2 \times X_6$	247	0.536
$X_3 \times X_4$	143	0.717
$X_3 \times X_5$	− 273	0.495
$X_3 \times X_6$	− 153	0.699
$X_4 \times X_5$	314	0.434
$X_4 \times X_6$	39	0.920
$X_5 \times X_6$	1001	0.033

starch are more efficient than glucose as carbon source. They also indicated that a mixture of glucose and different vegetable oils are more efficient than glucose alone. In the present study, glucose was the most effective carbon source and the other carbon sources as single carbon source or even in a mixture were not as efficient as glucose. Choudhari and Singhal (2008) evaluated the effect of different carbon sources by one factor at the time method and indicated that glucose is more effective than other carbon sources. However, investigating the interaction and efficiency of a mixture of carbon sources was not possible by their method.

The role of different concentrations of glucose (40, 50, and 60 g/L) was also evaluated when the level of soybean powder was constant on 5.2 g/L. However, high levels of glucose were not more effective on carotenoids production when data analyzed. The highest level of carotenoids was obtained when glucose concentration was 50 g/L (983.8 ± 31.5 mg/L). This may be due to glucose repression as mentioned by Choudhari and Singhal (2008). Many microorganisms switch off a large number of genes

when high contents of glucose are applied in their culture media. High levels of glucose repress respiration and mitochondrial activities, decrease the level of ATP during oxidative growth and repress the production of secondary metabolites (Adnan et al., 2018; Ronne, 1995; Sandmann, 2015). However, this phenomenon has not well been studied in *B. trispora* and further investigations are necessary to find the role of glucose repression on carotenogenesis.

Antioxidant activity of carotenoids is the main property of these components. Antioxidant activity of extracted carotenoids from the biomass of *B. trispora* was $31.9 \pm 4.7\%$ in comparison with the same concentration of ascorbic acid solution. Unfortunately, a few investigations have evaluated the antioxidant activity of carotenoids in microorganisms. Lira et al. (2017) evaluated antioxidant activity of total carotenoids extracted from shrimp and obtained 64.2–68.9% antioxidant activity which is more than two times higher than that of obtained in the present study. However, the main carotenoid of shrimp was astaxanthin which is more efficient than other carotenoids such as β -carotene. Overall, it could be concluded that extracted carotenoids from *B. trispora* could be considered as efficient antioxidant component.

Carbon and nitrogen are usually the main components of culture media and thus, assign considerable part of culture media price. Therefore, consumption of the lowest levels of these substrates for commercial production of different metabolites is important. The highest concentration of carotenoids obtained in the present study was 983.8 mg/L which is 19.66 mg/g glucose as carbon source. The productivity of carotenoids based on soybean powder as nitrogen source was also 189.10 mg/g soybean powder which is a considerable amount in comparison with many investigations (Choudhari et al., 2008; Nanou et al., 2012; Papaioannou and Liakopoulou-Kyriakides, 2010). Nanou and Roukas (2011) obtained 3680 mg/L β -carotene in *B. trispora* cultures in a complex medium. They consumed 80 g CSL as main nitrogen source in culture medium. Therefore, their productivity based on CSL was almost 96 mg/g CSL which is approximately half of the productivity in the present study based on consumed nitrogen source. Varzakakou and Roukas (2009) also investigated the production of β -carotene by *B. trispora* in a complex medium. Their productivity was almost 880 mg/L which is less than that of obtained in this study. Nanou and Roukas (2016) produced 2021 mg/L carotenoids in a culture medium included of 80 g/L CSL as nitrogen source. This content of carotenoids is almost 2 times higher than that of obtained in the present study. However, they have utilized over than 8 times nitrogen components in comparison with the present study. Choudhari et al. (2008) produced 828 mg/L β -carotene in culture medium with moderately

low levels of nitrogen components. They added remarkable amount of Vitamin A acetate (1000 mg/L) to the culture medium which is an expensive component. Yan et al. (2013) investigated the production of β -carotene in a complex medium and produced 669 mg/L β -carotene in optimum condition in shake flask. With considering glucose as main carbon source in their investigation, the productivity of β -carotene based on carbon source was 9.3 mg/g glucose which is approximately two times less than that of obtained in this study. Overall, in the present study the production of high concentrations of β -carotene in moderately simple culture media with low levels of nitrogen was possible. However, the main goal of the present study was screening of carbon and nitrogen sources. Thus, simultaneous optimization of carbon and nitrogen sources beside of BHT, span 20, and other effective factors is necessary to obtain final C:N ratio and produce higher concentrations of β -carotene using low levels of glucose and soybean powder. This could be accomplished using Taghuchi, RSM, fuzzy logic method, and etc. (Kennedy and Krouse, 1999).

HPLC analysis

HPLC analysis of the extracted carotenoids revealed three main peaks with carotenoid like spectra. An adequate separation of lycopene, β -carotene, and γ -carotene was obtained using this method and the retention times were 16.12, 19.51, and 21.98 min for lycopene, β -carotene, and γ -carotene, respectively. The concentration of lycopene, β -carotene, and γ -carotene changed between 0.4–1.9, 81.8–89.2, and 10.3–17.4% during consumption of six different nitrogen sources, respectively. The highest percentage of β -carotene (89.2%) obtained when di-ammonium phosphate consumed as nitrogen source while the lowest percentage of β -carotene (81.8%) obtained using soybean powder.

The percentage of lycopene, β -carotene, and γ carotene changed between 0.9–1.8, 75.9–89.4, and 10.5–22.5% during consumption of six different carbon sources, respectively. The fungus produced the highest level of β -carotene (93.7%) when soybean oil was consumed as carbon source while the lowest level of β -carotene (75.9%) was produced by utilizing glucose. Varzakakou and Roukas (2009) indicated that the level and concentration of vegetable oils was effective on the percentage of different carotenoids produced by *B. trispora*. In the present study carbon source was an effective factor on the percentage of different carotenoids and especially β -carotene. The levels of lycopene did not change considerably during utilizing of different carbon sources.

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Compliance with ethical standards

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