Annals of Agricultural Science (2016) 61(1), 87–96

HOSTED BY

ELSEVIER

Faculty of Agriculture, Ain Shams University

Annals of Agricultural Science

www.elsevier.com/locate/aoas

Optimization of β-carotene production from agro-industrial by-products by *Serratia marcescens* ATCC 27117 using Plackett–Burman design and central composite design

Ahmed A. Abdelhafez [a,*], **Sherif M. Husseiny** [b], **Ali Abdel-Aziz Ali** [c], **Hala M. Sanad** [b]

[a] *Dept. of Agricultural Microbiology, Faculty of Agriculture, Ain Shams University, Egypt*
[b] *Botany Department, Faculty of Women for Art, Science & Education, Ain Shams University, Egypt*
[c] *Dept. of Food Technology, Faculty of Agriculture, Ain Shams University, Egypt*

Received 30 December 2015; accepted 23 January 2016
Available online 26 April 2016

KEYWORDS

β-carotene production;
Optimization;
Agro-industrial by-products;
Serratia marcescens;
Plackett–Burman design;
Central composite design

Abstract β-carotene is a widely known carotenoid molecule, commercially used as food and feed additive, cosmetic and pharmaceutical products. The current study investigates the usability of some agro-industrial by-products for β-carotene production by *Serratia marcescens* and optimizes the production process using Plackett–Burman Design (PBD) and central composite design (CCD).

Rice bran, molasses and sugarcane bagasse were tested for their effects on β-carotene production by *S. marcescens*. Molasses was the high potent source for β-carotene production giving 1.1 mg/L after 2 days of incubation in the dark at 30 °C on a rotary shaker at 150 rpm.

PBD was used to evaluate the effect of lactose, sucrose, beef extract, peptone, NaCl, $MgSO_4$, KH_2PO_4, pH, inoculum size (ml/L) and agitation rate (rpm) on β-carotene production. Sucrose, lactose, peptone, beef extract, pH, inoculum size showed a positive sign of the effect on β-carotene production, while other factors showed a negative sign. The coefficient of determination, R^2, was 0.9829, showing good fitness of the model.

Factors screened by PBD were further optimized using CCD of Response Surface Methodology (RSM). Central composite rotatable design was used to determine the optimum levels of three

* Corresponding author.
E-mail address: aabdelwahab@agr.asu.edu.eg (A.A. Abdelhafez).
Peer review under responsibility of Faculty of Agriculture, Ain-Shams University.

http://dx.doi.org/10.1016/j.aoas.2016.01.005

independent variables (sucrose g/L, peptone g/L and pH) at five different levels, coded as $-\alpha$, -1, 0, 1 and $+\alpha$. Results of CCD were analyzed by standard ANOVA, and the quadratic regression equation was generated. The optimum production medium were composed of 2.5 g/L sucrose, 7.8 g/L peptone and pH 6.7 with a predicted value of 2.51 mg/L and actual value of 2.24 mg/L.

Introduction

In contract to natural pigments, addition of synthetic colorants in food products has safety concerns due to its potentially harmful effects on human health (Velmurugan et al., 2010). On the other hand, naturally produced pigments have been shown as important alternative to synthetic food colorants.

β-carotene, a member of the carotenoids, is a strongly colored red-orange pigment abundant in plants and fruits. It is biosynthesized from geranylgeranyl pyrophosphate.

β-carotene can be obtained by extraction from vegetables, by chemical synthesis or microbial production. The latter, has some advantages due to their natural characteristics, safe use, medicinal properties and production independent of season and geographical conditions (Rashid and Mazumdar, 2014). Moreover, microbial production (by algae, fungi and bacteria) of β-carotene has an economic advantage of using low cost natural substrates used as sources of carbohydrates in microbial processes (Hernández-Almanzaa et al., 2014).

Microbial producers of β-carotene include *Blakeslea trispora* (fungi), *Rhodotorula* spp., and *Saccharomyces cerevisiae* (yeast), *Dunaliella bardawil* (microalgae), and bacteria such as *Serratia marcescens* (Wang et al., 2012).

Several studies showed feasibility for production of carotenoids from low cost substrates, such as rice bran, sugarcane bagasse and corn starch hydrolyzate (Hernández-Almanzaa et al., 2014). Therefore, it is necessary to determine the best culture medium and environmental conditions for microbial fermentation in order to exploit the potential of the selected strain.

In general, natural pigments has an annual growth rate of 5–10%, comprising 31% of the worldwide colorant market, compared to 40% for synthetic colorants (Downham and Collins, 2000; Mapari et al., 2010). Global production of β-carotene reached $ 233 million dollars in 2010, and is expected to reach $ 309 million by 2018 with an annual growth rate of 3.6% (Mata-Gómez et al., 2014).

Therefore, the aim of the current study was set on utilizing cheap agro-industrial by-products as basic materials for production of β-carotene by *S. marcescens*, screening the significant factors affecting β-carotene production process by applying Plackett–Burman design then optimizing the levels of these factors by statistical approach of response surface methodology.

Materials and methods

Microorganisms

S. marcescens ATCC 27117 was obtained from Cairo Microbiological Resources Center (Cairo MIRCEN), Faculty of Agriculture, Ain Shams University, Cairo, Egypt. *S. marcescens* was maintained and sub-cultured on nutrient agar slants at 4 °C until further use.

Production medium

Nutrient broth (composed of (g/L): beef extract (3), peptone (5), pH 7) was used as production medium (Wang et al., 2012) and inoculum preparation. Nutrient agar (NA), containing 20 g/L of agar, was used to maintain *S. marcescens* culture. The pH was adjusted by 1 N NaOH or HCl solution and autoclaved at 121 °C for 20 min.

Agro-industrial by-products

Rice bran, sugar cane molasses and sugar cane bagasse were used for β-carotene production. Rice bran was collected from rice farms in El-Beheira governorate (located in the Nile Delta, north of Cairo). Rice bran was dried at 70 °C for 2 h, cooled and stored in a desiccator for further studies. Sugar cane molasses was obtained from EL-Howamdia Company, Egypt. Sugarcane bagasse was procured from local sugarcane juice vendor. The bagasse was packed in a cloth carrying bag to prevent the entry of flies and other insects and dried in shade. After drying, the pith portion of the bagasse was extracted manually and the outer rind was discarded. The pith was ground to fine powder in a steel kitchen grinder to reach a particle size of 0.2–2 mm.

Chemical analysis of agro-industrial by-products

All agro-industrial by-products were analyzed for N, C, P, protein, ash, fiber, moisture, cellulose, hemicellulose at Arid land Agricultural Research and Services Center, Faculty of Agriculture, Ain Shams University. Cellulose and hemicelluloses contents of agro-industrial by-products were determined using the standard laboratory analytical procedures for biomass analysis provided by the National Renewable Energy Laboratory (NREL, USA) and methods developed by the Association of Official Analytical Chemistry (A.O.A.C., 1995). Three samples were dried at 105 °C to constant weight to determine the biomass moisture content. The ash determination was done by burning the samples at 550 °C in a muffle furnace (Blue M Electric Company, Blue Island, USA) for 3 h, according to the laboratory analytical procedure for determination of ash. Total nitrogen of by-products was determined using the Kjeldahl method (Bradstreet, 1965). Crude protein content was calculated by multiplying total nitrogen percentage by 6.25 and crude fiber was determined by enzymatic-gravimetric method (A.O.A.C., 1995). Organic carbon is measured using a carbonaceous analyzer, which converts organic carbon to carbon dioxide (CO_2) by either

catalytic combustion or wet chemical oxidation. Determination of phosphorus was carried out by semi-automated colorimetric method.

Standard inoculum

One loop of a 24-h-old slant culture grown on NA was mixed with 100 ml of saline solution (0.9%) in 250-ml Erlenmeyer flasks to prepare microbial suspension with 10^8 cfu/ml using optical density at 550 nm for all experiments.

β-carotene production by S. marcescens

For production of β-carotene, all tested by-products were used as the basal production medium giving a final carbon concentration of 1%, therefore, 5.5 g of rice bran in 7 ml distilled water, 2.5 g molasses in 25 ml distilled water or 7.1 g bagasse in 18 ml distilled water were placed, each, in 250-ml Erlenmeyer flask. After sterilization, 5 ml of *S. marcescens* cell suspension was transferred to each of the flasks and nutrient broth was used as control treatment. Flasks were incubated at 30 °C on a rotary shaker operated at 150 rpm in dark place for 5 days. Samples (a whole flask) were removed on a daily basis for biomass and β-carotene determination. All treatments had three replicates and average responses were taken.

Extraction of β-carotene from S. marcescens biomass grown on by-products

For pigment extraction, samples containing waste and cells were mixed with equal volume of 3 M HCl and mixed well with hand shaking. The mixture was then incubated in boiling water for 4 min, quickly chilled for 10 min then centrifuged at 4000 rpm for 5 min. The pellets (cells and waste) were washed twice with distilled water and vortexed well in equal volume of acetone. The above layer containing the pigment was centrifuged at 4000 rpm for 20 min and its supernatant was stored at −20 °C until used for β-carotene analysis (Wang et al., 2012).

Determination of β-carotene

β-carotene was measured by reading the optical density by spectrophotometer at 475 nm (Wang et al., 2012) in each extract. Concentration of β-carotene was calculated by Beer–Lambert law and a standard curve was constructed using β-carotene standard.

Determinations of viable count and biomass

Viable count of *S. marcescens* was carried out on NA medium using plate count technique. Plates were incubated at 30 °C for 48–72 h. Biomass was determined by cell dry weight as follows: one flask was taken and its whole content was centrifuged at 4000 rpm for 5 min. The pellets were dried at 85 °C until reaching constant weight (El-Banna, 2012).

Optimization of β-carotene production

Effect of carbon and nitrogen sources and its concentrations on β-carotene production by S. marcescens from molasses

The influence of supplementing the production medium with various carbon sources at different concentrations on β-carotene production by *S. marcescens* was studied by adding sucrose, lactose and starch at 1.5, 2, 2.5, 3, 3.5 (g/L) to 2.5 g molasses in 25 ml distilled water in 250-ml Erlenmeyer flasks. After sterilization, flasks were inoculated with 5 ml of (standard inoculum) freshly prepared bacteria suspension and incubated at 30 °C on a rotary shaker operated at 150 rpm in dark place for 2 days (Wang et al., 2012). Each treatment has three replicates and average response was taken.

To study the effect of supplementing the production medium with nitrogen sources at different concentrations on β-carotene production by *S. marcescens*, three nitrogen sources were chosen; beef extract at 2.3, 4.6, 7, 9.3, 9.5 g/L, peptone and KNO_3 at 2, 4, 6, 8, 10 g/L, all were added to 2.5 g molasses in 25 ml distilled water into 250-ml Erlenmeyer flasks, then all the procedures were applied as previously mentioned.

Statistical analysis

All data obtained were statistically analyzed (Snedecor and Cochran, 1989) using Costat computer program V 8.303 (2004). LSD at 5% level as significance was used to differentiate between means.

Statistical screening of nutritional and physical parameters by S. marcescens using Plackett–Burman Design (PBD)

Plackett–Burman design was used to screen for significant factors (Table 1). Each independent variable was tested at high (+1) and low (−1) levels. The variables chosen for the present study were: lactose, sucrose, beef extract, peptone, NaCl, $MgSO_4$, KH_2PO_4 (all in g/L), pH, inoculum size (ml/L) and agitation rate (rpm) for molasses as substrate. The inoculated flasks were incubated on a rotary shaker at 30 °C in dark place for 2 days. The experimental Plackett–Burman design was

Table 1 Experimental variable at different levels, estimated effect, for β-carotene production by *S. marcescens* in eleven variable Plackett–Burman design experiments.

Coded factors	Variables	Low level (−1) (g/L)	High level (+1) (g/L)
X1	Sucrose	2 (−1)	3.5 (+1)
X2	Lactose	2 (−1)	3.5 (+1)
X3	Peptone	4 (−1)	8 (+1)
X4	Beef extract	4.6 (−1)	9.3 (+1)
X5	NaCl	0.1 (−1)	1.2 (+1)
X6	$MgSO_4$	0.1 (−1)	1.2 (+1)
X7	KH_2PO_4	0.1 (−1)	1.2 (+1)
X8	pH	4.5 (−1)	7 (+1)
X9	Inoculum size	3 (−1)	7 (+1)
X10	Agitation	100 (−1)	200 (+1)
X11	Dummy 1	−1	+1

Table 2 Plackett–Burman experimental design matrix for screening of important variables for β-carotene production.

Run	A: sucrose (g/L)	B: lactose (g/L)	C: peptone (g/L)	D: beef extract (g/L)	E: NaCl (g/L)	F: $MgSO_4$ (g/L)	G: KH_2PO4 (g/L)	H: pH	J: inoculum size (%)	K: agitation rpm	L: dummy 1
1	3.5	3.5	8	4.6	0.1	0.1	1.2	4.5	7	200	−1
2	3.5	3.5	4	9.3	1.2	1.2	0.1	4.5	3	200	−1
3	3.5	2	4	4.6	1.2	0.1	1.2	7	3	200	1
4	3.5	3.5	4	4.6	0.1	1.2	0.1	7	7	100	1
5	2	3.5	4	9.3	1.2	0.1	1.2	7	7	100	−1
6	2	2	4	9.3	0.1	1.2	1.2	4.5	7	200	1
7	3.5	2	8	9.3	1.2	0.1	0.1	4.5	7	100	1
8	2	3.5	8	9.3	0.1	0.1	0.1	7	3	200	1
9	3.5	2	8	9.3	0.1	1.2	1.2	7	3	100	−1
10	2	2	8	4.6	1.2	1.2	0.1	7	7	200	−1
11	2	3.5	8	4.6	1.2	1.2	1.2	4.5	3	100	1
12	2	2	4	4.6	0.1	0.1	0.1	4.5	3	100	−1

Table 3 Ranges of the independent variables used in RSM.

Variables	$-\alpha$	−1	0	+1	$+\alpha$
Sucrose (g/L)	1.5	2	2.75	3.5	4
Peptone (g/L)	2.6	4	6	8	9.4
pH	3.6	4.5	5.75	7	7.9

Table 4 Central composite design of factors in actual value for optimization of process variables.

Trials	Type	(A) Sucrose (g/L)	(B) Peptone (g/L)	(C) pH
1	Factorial	3.5	8	7.0
2	Center	2.75	6	5.75
3	Factorial	2	4	4.5
4	Center	2.75	6	5.75
5	Factorial	3.5	4	4.5
6	Factorial	2	8	4.5
7	Factorial	3.5	4	7
8	Axial	2.75	6	7.9
9	Axial	4	6	5.75
10	Center	2.75	6	5.75
11	Center	2.75	6	5.75
12	Axial	2.75	2.6	5.75
13	Center	2.75	6	5.75
14	Factorial	2	8	7
15	Factorial	3.5	8	4.5
16	Axial	2.75	9.4	5.75
17	Axial	2.75	6	3.6
18	Axial	1.5	6	5.75
19	Factorial	2	4	7

analyzed using Design Expert 9.0.3.1 software (Stat-Ease Inc. USA).

Twelve assigned variables were screened in 20 experimental designs. All experiments were carried out in duplicate and the average of the β-carotene production was taken as responses (Table 2). From the regression analysis, variables ($p < 0.05$) were found to have significant impact on β-carotene production.

CCD of RSM for optimization of nutritional and physical parameters S. marcescens for β-carotene production

Factors screened by Plackett–Burman design were further optimized using response surface methodology. A three-factor, five-level central composite rotatable design was used to determine the optimum levels of these variables. This central composite design consisted of three groups of design points, including two-level factorial design points, axial or star points and center points. Therefore, three selected independent variables (sucrose g/L, peptone g/L and pH) were studied at five different levels coded as $-\alpha$, −1, 0, 1 and $+\alpha$. The value for alpha (1.68179) is chosen to fulfill the ratability in the design. According to the central composite design matrix, a total of 19 experiments were required, including 8 factorial, 6 axial and 5 center, for estimation of the pure error sum of squares. After inoculation with 5 ml of bacteria suspension, flasks were incubated at 30 °C on a rotary shaker operated at 150 rpm in dark place for 2 days. To identify the significance of the main effects and interactions, ANOVA was performed for each parameter. A p value < 0.05 was

Table 5 Chemical analysis of agro-industrial by-products.

Sample	Chemical composition (% w/w)								
	N	C	Ph.	Protein	Ash	Fiber	Moisture	Cellulose	Hemicellulose
Sugarcane molasses	0.7	40	0.1	4.6	–	–	28	–	–
Sugarcane bagasse	0.22	14	1.1	1.4	1.5	26.1	12	39.2	56.7
Rice bran	2.5	18	1.2	15.63	5.98	4.3	10.7	29.2	23.5

considered to be statistically significant. The levels of factors used for experimental design are given in Table 3 and design of factorial, axial and center points were noted in Table 4. In this experimental design, the statistical software package Design Expert 9.0.3.1 (Stat-Ease, Minneapolis, MN) was used in the design of the experiments, the analysis of the experimental data, and the generation the response surface graphs.

This resulted in an empirical model. For four variable systems, the model Eq. (1) is as follows:

$$Y = \beta_0 + \beta_1 A + \beta_2 B + \beta_3 C + \beta_{11} A^2 + \beta_{22} B^2 + \beta_{33} C^2 + \beta_{12} AB + \beta_{13} AC + \beta_{23} BC \quad (1)$$

where y is the measured response, β_0 is the intercept term, β_1, β_2, β_3 are linear coefficients, β_{11}, β_{22}, β_{33} are quadratic coefficients, β_{12}, β_{13}, β_{23} are interaction coefficients and A, B and C are coded independent variables. The fit of the regression model attained was determined by the coefficient of determination (R-Squared), and the adjusted coefficient (Adjusted R-Squared). Appropriate model significance was determined by Fischer's F-test. The three dimensional graphical representation and their respective contour plots were determined by the interaction of dependent variable and the independent variables (Ghosh et al., 2012).

Results and discussion

Chemical composition of agro-industrial by-products

The chemical compositions of the three different agro-industrial by-products investigated in this work are shown in (Table 5). The highest fractions of cellulose (39.2%), and hemicellulose (56.7%) were recorded in sugarcane bagasse and rice bran, respectively. The highest moisture content was in molasses (28%) and the lowest was in rice bran (10.7%). Rice bran contained the highest nitrogen content (2.5%) and protein (15.63% w/w), while molasses contained the highest carbon content (40%). Sugarcane bagasse contained the lowest nitrogen and carbon content (0.22% and 14%, respectively), while having the highest fiber content (26.1%).

Screening of agro-industrial by-products for β-carotene production by S. marcescens

Rice bran, sugar cane molasses, and sugar cane bagasse were used as cheap substrate and rich with nutrients for β-carotene production by *S. marcescens* ATCC 27117.

Pigment production by *S. marcescens* was observed in Table 6. The high concentration of β-carotene was produced after two days with molasses (1.1 mg/L) and on day 3 with both rice bran and nutrient broth medium giving 0.62 mg/kg and 0.91 mg/L, respectively. Similar studies showed that natural carotenoid-producing bacteria, such as *Brevibacterium* sp. (Hsieh et al., 1974), *Micrococcus roseus* (Berry, 1981), *Mycobacterium* sp. (David, 1974), and *Flavobacterium* sp. produce β-carotene as a minor product only (Masetto et al., 2001). In another study, maximum concentration of β-carotene (360.2 mg/L) was obtained by *B. trispora* in culture grown in molasses solution containing 5% (w/v) sugar supplemented with linoleic acid (37.59 g/L), kerosene (39.11 g/L), and antioxidant (1.0 g/L) (Goksungur et al., 2004).

Optimization of β-carotene production

The selected isolate which produces high concentration of β-carotene, was used for this experiment with the best by-product, which was run in triplicate.

Table 6 Screening of agro-industrial by-products for β-carotene production by *S. marcescens*, compared with nutrient broth medium.

Incubation (days)			By-products					
	NA medium		Rice bran		Molasses		Bagasse	
	β-carotene (mg/L)	DW (g/L)	β-carotene (mg/kg)	DW (g/kg)	β-carotene (mg/L)	DW (g/L)	β-carotene (mg/kg)	Dry weight (g/kg)
1	0.58^{c}	4.62^{d}	0.46^{d}	18^{e}	0.60^{cd}	9^{cd}	0.16^{d}	6^{c}
2	0.66^{b}	7.02^{c}	0.54^{b}	30^{c}	$\mathbf{1.1}^{a}$	20^{a}	0.28^{b}	10^{b}
3	$\mathbf{0.91}^{a}$	9.06^{a}	$\mathbf{0.62}^{a}$	60^{a}	0.82^{b}	14^{b}	$\mathbf{0.40}^{a}$	17^{a}
4	0.63^{b}	7.22^{b}	0.50^{c}	48^{b}	0.71^{bc}	11^{bc}	0.23^{c}	11^{b}
5	0.51^{d}	4.51^{e}	0.38^{e}	22^{d}	0.51^{d}	7^{d}	0.13^{d}	8^{bc}

Means of triplicate samples value with different significance according to the statistical analysis Duncan's multiple range test ($p \leqslant 0.05$).
Means within each column marked with similar letters are not significantly different ($p < 0.05$).
Values shown in bold illustrate the highest records of the measured parameter.

Table 7 Screening of different carbon sources and concentrations for β-carotene production and biomass by *S. marcescens* incubated for 2 days at 30 °C.

Sucrose			Lactose			Starch		
Concen. (g/L)	β-carotene (mg/L)	DW (g/L)	Concen. (g/L)	β-carotene (mg/L)	DW (g/L)	Concen. (g/L)	β-carotene (mg/L)	DW (g/L)
1.5	1.3^{c}	16^{c}	1.5	1.0^{c}	17^{bc}	1.5	0.8^{bc}	11^{c}
2.0	1.7^{ab}	21^{ab}	2.0	1.3^{b}	18^{abc}	2.0	1.0^{ab}	14^{abc}
2.5	**1.9**^{a}	**24**^{a}	2.5	**1.6**^{a}	**21**^{a}	2.5	**1.2**^{a}	**16**^{a}
3.0	1.8^{a}	19^{bc}	3.0	1.52^{ab}	19^{ab}	3.0	0.9^{abc}	15^{ab}
3.5	1.4^{bc}	17^{c}	3.5	1.23^{bc}	15^{c}	3.5	0.6^{c}	12^{bc}

Means of triplicate samples value with different significance according to the statistical analysis Duncan's multiple range test ($p \leqslant 0.05$).
Means within each column marked with similar letters are not significantly different ($p < 0.05$).
Values shown in bold illustrate the highest records of the measured parameter.

Table 8 Screening of different nitrogen sources with different concentration for β-carotene production and biomass by *S. marcescens* incubated for 2 days at 30 °C.

Beef extract			Peptone			Potassium nitrate		
Concen. (g/L)	β-carotene (mg/L)	DW (g/L)	Concen. (g/L)	β-carotene (mg/L)	DW (g/L)	Concen. (g/L)	β-carotene (mg/L)	DW (g/L)
2.3	1.3^{b}	16^{bc}	2	1.2^{c}	15^{b}	2	0.7^{c}	8^{c}
4.6	1.8^{a}	19^{ab}	4	1.3^{bc}	17^{b}	4	0.8^{bc}	10^{abc}
7.0	**1.9**^{a}	**21**^{a}	6	1.5^{abc}	21^{a}	6	**1.2**^{a}	**13**^{a}
9.3	1.6^{ab}	20^{a}	8	**1.8**^{a}	**23**^{a}	8	1.1^{ab}	12^{ab}
9.5	1.44^{b}	14^{c}	10	1.6^{ab}	16^{b}	10	0.8^{bc}	9^{bc}

Means of triplicate samples value with different significance according to the statistical analysis Duncan's multiple range test ($p \leqslant 0.05$).
Means within each column marked with similar letters are not significantly different ($p < 0.05$).
Values shown in bold illustrate the highest records of the measured parameter.

Table 9 Plackett–Burman experimental design of 12 runs for 11 variables with observed and predicted values of β-carotene production by *S. marcescens*.

Run	Observed value of β-carotene (mg/kg)	Predicted value of β-carotene (mg/kg)
1	0.93	0.90
2	0.86	0.86
3	1.47	1.56
4	2.21	2.16
5	2.42	2.29
6	2.02	2.07
7	1.94	2.06
8	1.72	1.63
9	1.12	1.23
10	2.45	2.46
11	1.00	0.92
12	2.32	2.33

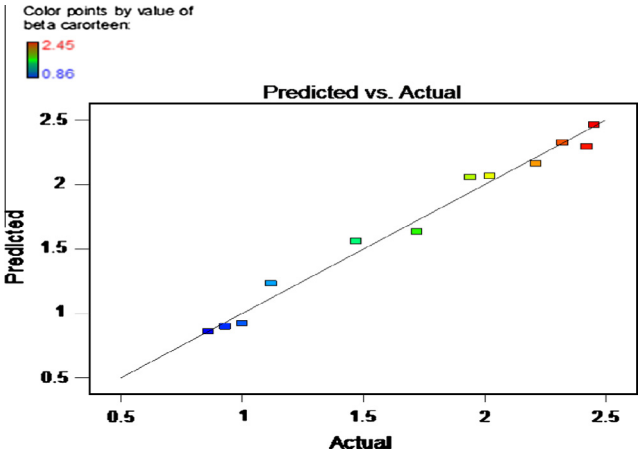

Fig. 1 Predicted response vs. actual value for β-carotene production from *S. marcescens*.

Effect of carbon and nitrogen sources and concentrations on β-carotene production by S. marcescens from molasses

Different sources and concentrations of carbon and nitrogen were tested to maximize β-carotene production by *S. marcescens*. The amount of carbon and nitrogen compounds were added to the production medium to give a final concentration of 1% C and 1% N, to eliminate errors due to differences in carbon and nitrogen concentration in each source. Results shown in Tables 7 and 8 indicated that maximum production of β-carotene by *S. marcescens* on molasses were obtained in the presence of sucrose at 2.5, lactose at 2.5, beef extract at 7 and peptone at 8 (g/L) giving 1.9, 1.6, 1.9 and 1.8 mg/L molasses, respectively. Bhosale and Bernstein (2004) reported that the maximum β-carotene level was 7.85 mg/L culture, representing 80% (w/w) of the total carotenoid produced by *Flavobacterium multivorum* with optimum medium contained 4000 and 4070 mg/L urea and sodium carbonate, respectively.

Statistical screening of physical and nutritional factors for β-carotene production by S. marcescens using Plackett–Burman design

The PB results, shown in Table 9 and Fig. 1, indicated that there was a variation in pigment production in the twelve trials

Table 10 Estimated effects, linear regression coefficients, corresponding *F*-values and *p*-values for β-carotene production by *S. marcescens* in Plackett–Burman design experiment.

Variable	Effect	Coefficient	*F*-value	*p*-Values
Intercept	–	1.71	–	–
Model	–	–	47.93	0.0003
A-sucrose	0.42	0.21	38.72	0.0016
B-lactose	3.333E−003	–	–	–
C-peptone	0.19	0.093	7.53	0.0406
D-beef extract	0.073	0.037	1.16	0.3303
E-NaCl	−0.037	–	–	–
F-$MgSO_4$	−0.12	−0.062	3.29	0.1296
G-KH_2PO_4	−0.013	–	–	–
H-pH	1.04	0.52	235.16	< 0.0001
J-inoculum size	0.090	0.045	1.75	0.2432
K-agitation	−0.080	–	–	–
L-dummy1	0.12	–	–	–

ranged from 0.86 to 2.45 mg/kg β-carotene production. Among the 6 factors, sucrose, lactose, peptone, beef extract, pH, inoculum size showed a positive sign of the effect on β-carotene production, and all other factors showed a negative sign of the effect. When the sign of the effect (Table 10) of the tested variable is positive, the β-carotene production is greater at a high level of the parameter, and when it becomes negative, the β-carotene production is greater at a low level of the parameter (Kiruthika et al., 2011). The coefficient of determination, R^2, was found to be 0.9829, showing good fitness of the model. The adequacy of the model was calculated, and the variables exhibiting statistically significant effects were screened using ANOVA. Factors with *p* value (Table 10) lower than 0.05 were considered to have significant effects on the production of β-carotene, and were therefore selected for further optimization studies using CCD. Value of pH, with a *p* value of (< 0.0001), was considered as the most significant factor, followed by sucrose (0.0016) and peptone (0.0406). Plackett–Burman design experiments on production of β-carotene from *S. marcescens* ATCC 27117 on molasses indicated that the most important parameters were sucrose, peptone and

Table 11 Central composite design (CCD) of factors for optimization of 19 trials for 3 variables with observed and predicted value of β-carotene production by *S. marcescens*.

Run	Observed value of β-carotene (mg/L)	Predicted value of β-carotene (mg/L)
1	2.00	2.11
2	2.46	2.29
3	0.87	0.76
4	2.00	2.29
5	0.60	0.31
6	1.00	1.14
7	1.20	1.06
8	1.90	1.82
9	0.90	1.02
10	2.26	2.29
11	2.40	2.29
12	0.50	0.90
13	2.35	2.29
14	1.80	2.09
15	0.80	0.93
16	2.51	2.11
17	0.32	0.40
18	1.50	1.38
19	1.40	1.27

Table 12 Quadratic regression coefficients and corresponding *F*-values and *p*-values for β-carotene production by *S. marcescens* by the RSM–CCD design experiment.

Source	Coefficient	*F*-values	*p*-Values
Intercept	2.29	–	–
Model	–	11.66	0.0006
A-sucrose	−0.11	1.90	0.2012
B-peptone	0.36	20.96	0.0013
C-pH	0.42	29.12	0.0004
AB	0.059	0.33	0.5810
AC	0.059	0.33	0.5810
BC	0.11	1.12	0.3168
A^2	−0.39	24.27	0.0008
B^2	−0.28	12.63	0.0062
C^2	−0.42	28.42	0.0005

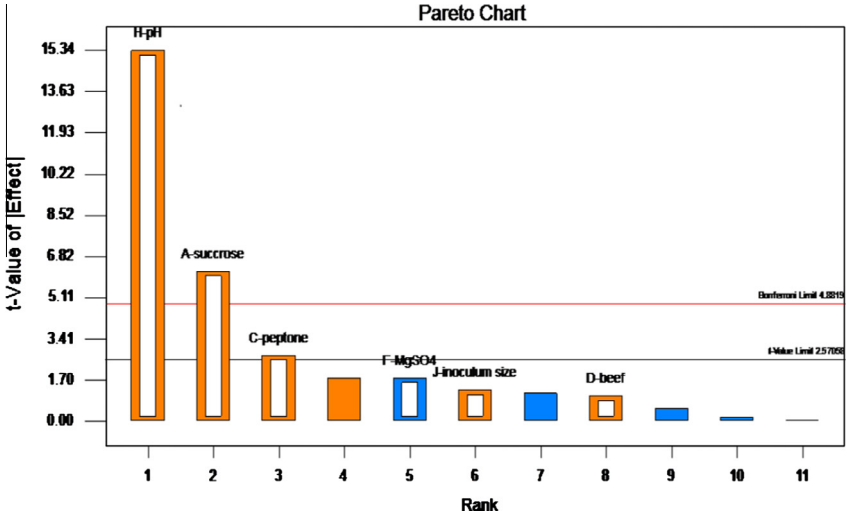

Fig. 2 Pareto chart showing the effect of medium components on β-carotene production from *S. marcescens*.

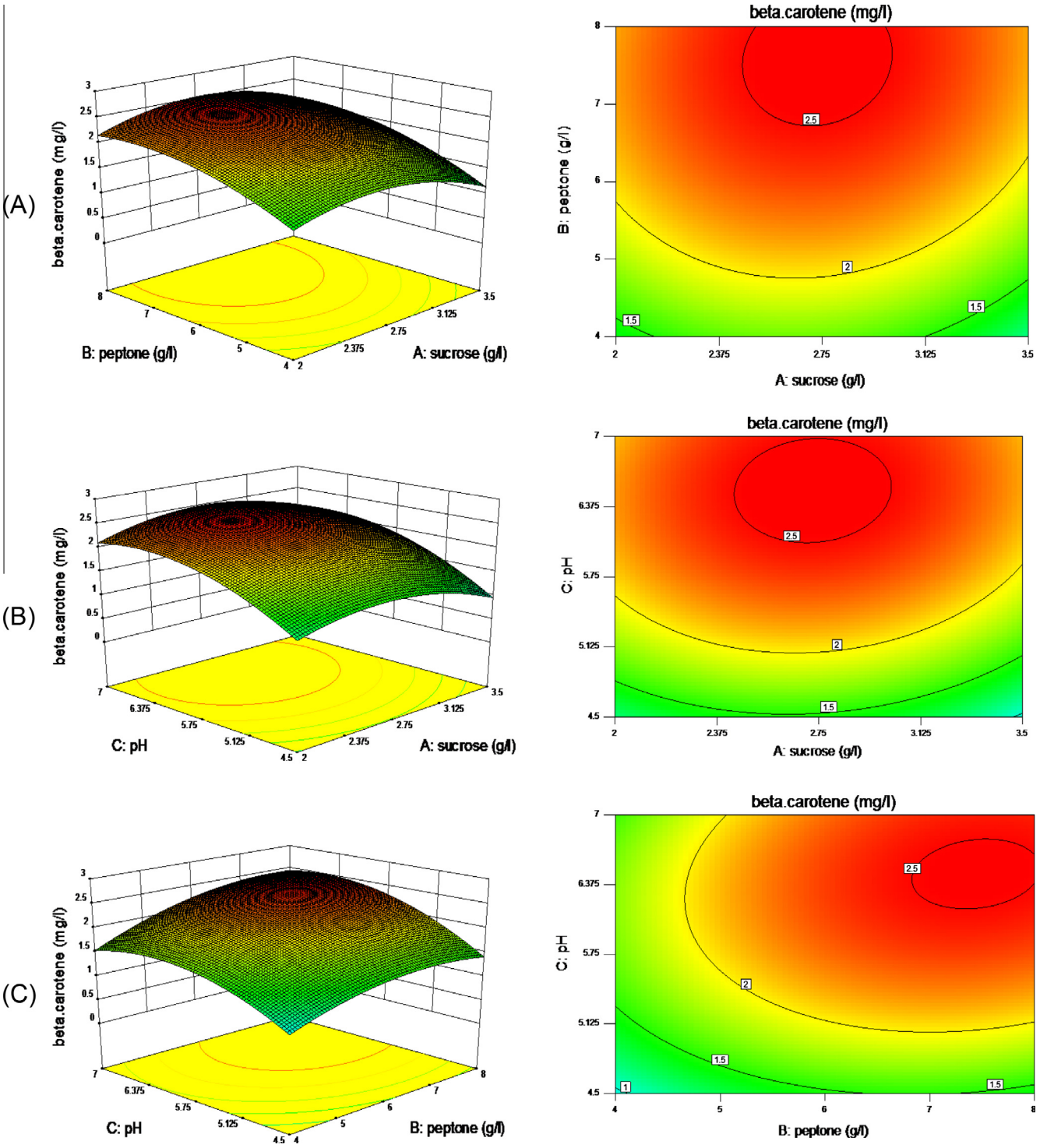

Fig. 3 3D surface and contour plots for β-carotene production at varying concentrations of: (a) sucrose and peptone, (b) sucrose and pH and (c) peptone and pH.

pH as shown in the Pareto charts (Fig. 2). The Model *F*-value of 47.93 implies the model is significant. There is only a 0.03% chance that an *F*-value this large could occur due to noise. Values of "Prob > F" less than 0.0500 indicate model terms are significant. In this case A, C, H are significant model terms. Values greater than 0.1000 indicate the model terms are not significant. The "Pred *R*-Squared" of 0.9016 is in reasonable agreement with the "Adj *R*-Squared" of 0.9624; (i.e. the difference is less than 0.2) indicating a good agreement between the experimental and predicted values on β-carotene production. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 17.814 indicates an adequate signal. This model can be used to navigate the design space.

Central composite design of RSM for optimization of nutritional and physical parameters by S. marcescens for β-carotene production

Based on CCD experiment, the effects of three independent variables; sucrose, peptone and pH, on β-carotene production are shown in (Table 11). The predicted and observed responses were reported. The results obtained from CCD were then analyzed by standard analysis of variance (ANOVA), and the quadratic regression equation was applied for prediction of β-carotene production. Based on the full quadratic model application, (Table 12) it appeared that quadratic effect of pH value (p value = 0.0005), peptone (p value = 0.0062) and sucrose concentration (p value = 0.0008) should to be significant on the model. The linear effect for peptone and pH were still considered as an important factor in this model ($p < 0.05$) than sucrose concentration (p value = 0.2012). The polynomial model for β-carotene production (Y) was regressed by only considering the significant terms ($p < 0.05$) as shown in the following Eq. (2):

$$Y = 2.29 + 0.36B + 0.42C - 0.39A^2 - 0.28B^2 - 0.42C^2 \quad (2)$$

where Y is the predicted β-carotene production yield, B is peptone concentration, C is pH value. A^2, B^2 and C^2 are quadratic coefficients of sucrose concentration, peptone concentration and pH. The regression equation obtained from analysis of variance (ANOVA) with the R^2 value (multiple coefficients of determination) of 0.9210 revealed that the model should to be fitness. The Model F-value of 11.66 implies the model is significant. There is only a 0.06% chance that an F-value this large could occur due to noise. The "Lack of Fit F-value" of 3.88 implies the Lack of Fit is not significant relative to the pure error. There is a 10.66% chance that a "Lack of Fit F-value" this large could occur due to noise. Non-significant lack of fit is good. "Adeq Precision" measures the signal to noise ratio. A ratio greater than 4 is desirable. Your ratio of 9.429 indicates an adequate signal. This model can be used to navigate the design space. This model can be used to navigate the design space. ANOVA analysis also confirmed a satisfactory adjustment of the reduced quadratic model to the experimental data. The three-dimensional response surface graphs were plotted to illustrate the interaction between the parameters and the optimum level of tested components on β-carotene production. The optimum conditions were Sucrose (2.5 g/L), peptone (7.8 g/L) at pH 6.7 with a maximum predicted β-carotene production of 2.51 mg/L molasses and actual 2.24 mg/L. Fig. 3 shows 3D surface and the contour plots of β-carotene production for each pair of factors by keeping the other two factors constant at its middle level.

Wang et al. (2012) noticed that the cultivation conditions for β-carotene production by *S. marcescens* RB3 were optimized as 2.0% lactose, 2.0% peptone, 0.3% beef extract, 1.0% NaCl supplemented with 0.05% Fe^{2+}, pH 6.0 and 30 °C. Under the optimal conditions, the yield of β-carotene was 2.45 μg/ml. Zhai et al. (2014) produced 1.19 mg/g dry biomass of carotene by *Arthrobacter globiformis* from medium containing (g/L) sugarcane molasses, 40.0 and corn steep liquor, 50.0, incubated at 100 rpm, 30 °C, and with pH 7.5.

Krishna (2008) reported that *S. marcescens* at 37 °C did not show any pigment production in nutrient broth and the culture broth was white in color, and the level of pigment production over a range of pH from 5.0 to 9.0 and maximal pigment were recorded at pH 6.0 in spite of a decline in pigment production along with increase in pH. Highly acidic (2.0–4.0) and alkaline (10.0–13.0) m media did not support pigment production. Korumilli and Mishra (2014) reported that the maximum pigment production by *Bacillus clausii* was achieved being 107 ± 1.2 mg/3 g of rice powder by Taguchi method of optimization with conditions set at pH 7 and 35 °C temperature.

Conclusion

In this study, β-carotene was produced by *S. marcescens* grown on molasses as a low-cost substrate with other nutrients and compared with rice bran and bagasse. Different chemical and physical factors were screened by Plackett–Burman design for β-carotene production. RSM was used to determine the effects of sucrose, peptone, and pH on β-carotene production. The model generated in this study by RSM satisfied all the necessary arguments for its use in the optimization. This article provides a detailed study that used statistical analysis to determine the optimum levels and interactions among the above mentioned parameters in β-carotene production from *S. marcescens*.

Acknowledgments

The authors wish to thank the "Microbial Inoculants Activity", Faculty of Agriculture, Ain Shams Univ., for providing all facilities and open their laboratories to run the experiments and analysis.

References

A.O.A.C., 1995. Official Methods of Analysis of the Association of Official Analytical Chemistry, 16th ed. AOAC International, Washington, USA, Pages: 1141.

Berry, A., 1981. Inhibition of carotenoid synthesis in *Micrococcus roseus*. Can. J. Microbiol. 27 (4), 421–425.

Bhosale, P., Bernstein, P.S., 2004. β-carotene production by *Flavobacterium multivorum* in the presence of inorganic salts and urea. J. Ind. Microbiol. Biotechnol. 31 (12), 565–571. http://dx.doi.org/10.1007/s10295-004-0187-9.

Bradstreet, R.B., 1965. The Kjeldahl Method for Organic Nitrogen. Academic Press, New York.

David, H.L., 1974. Biogenesis of β-carotene in *Mycobacterium kansasii*. J. Bacteriol. 119 (2), 527–533.

Downham, A., Collins, P., 2000. Colouring our foods in the last and next millennium. Int. J. Food Sci. Technol. 35 (1), 5–22.

El-Banna, A.A., 2012. Some factors affecting the production of carotenoids by Rhodotorula glutinis var. glutinis. Food Nutr. Sci. 03 (January), 64–71. http://dx.doi.org/10.4236/fns.2012.31011.

Ghosh, S., Chakraborty, R., Raychaudhuri, U., 2012. Optimizing process conditions for palm (*Borassus flabellifer*) wine fermentation using response surface methodology. Int. Food Res. J. 19 (4), 1633–1639.

Goksungur, Y., Mantzouridou, F., Roukas, T., Kotzekidou, P., 2004. Production of beta-carotene from beet molasses by *Blakeslea trispora* in stirred-tank and bubble column reactors: development of a mathematical modeling. Appl. Biochem. Biotechnol. 112 (1), 37–54.

Hernández-Almanzaa, A., Montañez-Sáenz, J., Martínez-vila, C., Rodriguez-Herrera, Raūl, Aguilar, C.N., 2014. Carotenoid production by *Rhodotorula glutinis* YB-252 in solid-state fermentation. Food Biosci. 7, 31–36.

Hsieh, L.K., Lee, T., Simpson, K.L., 1974. Biosynthesis of carotenoids in *Brevibacterium* sp. KY-4313. J. Bacteriol. 118 (2), 385–393.

Kiruthika, P., Nisshanthini, S.D., Saraswathi, A., Rajendiran, R., 2011. Application of statistical design to the optimization of culture medium for biomass production by *Exiguobacterium* sp. HM 119395. Int. J. Adv. Biotechnol. Res. 2 (4), 422–430.

Korumilli, T., Mishra, S., 2014. Carotenoid production by *Bacillus clausii* using rice powder as the sole substrate: pigment analyses and optimization of key production parameters. J. Biochem. Technol. 5 (4), 788–794.

Krishna, J.G., 2008. Pigment production by marine *Serratia* sp. BTW J8. Ph.D. Thesis. Department of Biotechnology, Cochin University of Science and Technology, Cochin, Kerala, India.

Mapari, S.A.S., Thrane, U., Meyer, A.S., 2010. Fungal polyketide azaphilone pigments as future natural food colorants? Trends Biotechnol. 28 (6), 300–307.

Masetto, A., Flores-cotera, L.B., Diaz, C., Langley, E., Sanchez, S., 2001. Application of a complete factorial design for the production of zeaxanthin by *Flavobacterium* sp. J. Biosci. Bioeng. 92 (1), 55–58.

Mata-Gómez, L.C., Montañez, J.C., Méndez-Zavala, A., Aguilar, C. N., 2014. Biotechnological production of carotenoids by yeasts: an overview. Microb. Cell Fact. 13 (12). http://dx.doi.org/10.1186/1475-2859-13-12.

Rashid, M., Mazumdar, R.M., 2014. Anti-bacterial activity of pigments isolated from pigment-forming soil bacteria. Br. J. Pharm. Res. 4 (8), 880–894.

Snedecor, G.W., Cochran, W.G., 1989. Statistical Methods, eighth ed. Iowa State University Press, Ames.

Velmurugan, P., Kamala-Kannan, S., Balachandar, V., Lakshmanaperumalsamy, P., Chae, J.C., Oh, B.T., 2010. Natural pigment extraction from five filamentous fungi for industrial applications and dyeing of leather. Carbohydr. Polym. 79 (2), 262–268.

Wang, B., Lin, L., Lu, L., Chen, W., 2012. Optimization of β-carotene production by a newly isolated *Serratia marcescens* strain. Electron. J. Biotechnol. 15 (6). http://dx.doi.org/10.2225/fulltext-4.

Zhai, Y., Han, M., Zhang, W., Qian, H., 2014. Carotene production from agro-industrial by-products by *Arthrobacter globiformis* in shake-flask culture. Prep. Biochem. Biotechnol. 44 (4), 355–369. http://dx.doi.org/10.1080/10826068.2013.829498.