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Shake flask optimization of β-carotene production in *Rhodotorula graminis RC04*

Marielou Ferrao and Sandeep Garg*

Department of Microbiology, Goa University, Taleigao Plateau, Goa – 403 206, India.

The present investigation was focused on the effects and interactions of media components on the production of β-carotene by *Rhodotorula graminis*. Mannitol, potassium nitrate, yeast extract, monopotassium hydrogen phosphate and magnesium sulfate medium components were screened using Plackett Burman Design to identify critical factors. Initial pH of 5.5 of the medium was found to be optimum for β-carotene production. Results suggest that higher amounts of mannitol, yeast extract, potassium nitrate and magnesium sulfate would positively affect both biomass and β-carotene accumulation. Yeast extract was critical for both β-carotene and biomass accumulation. Central composite design was used to study the impact of interaction of mannitol and yeast extract on biomass accumulation and β-carotene production. Concentration of yeast extract was found more significant than mannitol for β-carotene production. From predicted values obtained using fitted binomial equations, it was seen that mannitol should be used in the range of 10 to 20 g/L and yeast extract in the range of 9.5 to 10 g/L to achieve a biomass of 3.8 to 4.3 g/L and a β-carotene content of 190 to 220 µg/L.

Key words: Mannitol, yeast extract, Plackett Burman Design (PBD), central composite design, response surface methodology, yeast, β–carotene.

INTRODUCTION

β-Carotene is an orange yellow pigment of carotenoid family demonstrating antioxidant and anticancer properties. It is commercially used as an additive in food, feed, cosmetic and pharmaceutical products (Soni et al., 2010). *Rhodotorula* yeasts biosynthesize β-carotene in various proportions (Buzzini et al., 2007). The quantity of β-carotene in naturally occurring strains of *Rhodotorula* is very small and its proportion relative to other pigments is also significantly low. There is a need to improve fermentation strategies such that the intracellular accumulation of β-carotene in naturally occurring strains of *Rhodotorula* is feasible on an industrial scale. Optimizations of external and cultural parameters have been investigated for improved β-carotene production by *Rhodotorula* yeasts. Light, temperature, and aeration have been shown to be important factors influencing the quantity of

β-carotene in *Rhodotorula glutinis* (Bhosale and Gadre, 2002; Buzzini and Martini, 1999; Frengova et al., 2003). There are reports available of screening and selection of efficient β-carotene producing wild strains and mutants of *R. glutinis* and on the effect of media constituents on the proportions of β-carotene. A stimulatory effect on the yield of β-carotene from *R. glutinis* has been shown by using glycerol, phenol and glucose as carbon and yeast extract as nitrogen sources, respectively (Bhosale and Gadre, 2001). Among inorganic salts used as sole source of nitrogen, potassium nitrate has been reported as a good source for β-carotene production (Aksu and Eren, 2005; Wang et al., 2007). Komemushi et al. (1994) reported that several divalent cations (Ba, Fe, Mg, Ca, Zn and Co) can act as stimulants for the growth of *R. glutinis.*

Classical methods that use sequential manipulation of a single parameter do not take into consideration the interactions between different factors. Moreover, they are work and time exhaustive. Response surface methodology (RSM) has eliminated these drawbacks. It can also be used to evaluate the relative significance of

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*Corresponding author. E-mail sandeep_garg68@yahoo.co.in or sandeep@unigoa.ac.in. Tel: (O) +91-832-6519280 or (R) +91-832-6630717 or 9422440637

Table 1. Production of biomass and β-carotene by *R. graminis* RC04 in the eight experimental trials as per the PBD: fractional factorial design for 5 variables (Haaland, 1989).

Trial	Variables					Response	
	Coded (Actual amount in g/l)						
	Mannitol	Potassium nitrate	Yeast extract	Monopotassium hydrogen phosphate	Magnesium sulfate	Biomass (g/L)	β-Carotene (µg/L)
A	- (2.5)	- (0.8)	- (0.9)	- (0.2)	+ (1.0)	2.19 ± 0.34	29 ± 1
B	- (2.5)	- (0.8)	+ (9.0)	+ (2.0)	- (0.1)	6.53 ± 0.15	122 ± 7
C	- (2.5)	+ (8.0)	- (0.9)	+ (2.0)	- (0.1)	2.500 ± 0.40	31 ± 3
D	- (2.5)	+ (8.0)	+ (9.0)	- (0.2)	+ (1.0)	7.63 ± 0.46	117 ± 3
E	+ (25.0)	- (0.8)	- (0.9)	+ (2.0)	+ (1.0)	1.40 ± 0.30	30 ± 3
F	+ (25.0)	- (0.8)	+ (9.0)	- (0.2)	- (0.1)	7.27 ± 0.47	113 ± 9
G	+ (25.0)	+ (8.0)	- (0.9)	- (0.2)	- (0.1)	5.33 ± 0.40	86 ± 7
H	+ (25.0)	+ (8.0)	+ (9.0)	+ (2.0)	+ (1.0)	14.46 ± 2.77	195 ± 27

+, High setting; -, low setting (showing actual value for each of the five variables).

several variables simultaneously. Medium and process optimization of β-carotene production for *R. glutinis* and *R. gracilis* using response surface methodology has been investigated (Buzzini, 2000; Govindaswamy et al., 1999; Malisorn and Suntorsnuk, 2008).

Rhodotorula graminis is also known as a β-carotene producing yeast. Buzzini et al. (2005) using response surface methodology have reported that trace elements exert a selective influence on the carotenoid profile in *R. graminis*, Al^{3+} and Zn^{2+} had a stimulatory effect on β-carotene production. Macronutrient medium components play important role in biomass and product synthesis of any microbial fermentation. However, no detailed studies are available to see how the macronutrient components will exert effect on β-carotene production and biomass accumulation in *R. graminis*. This investigation is an attempt to see which of the macronutrients are critical and how the interaction of these will impact the production of β-carotene by *R. graminis*.

MATERIALS AND METHODS

Microorganism and cultivation conditions

The strain *R. graminis RC04* was maintained on Yeast Morphology agar (YM, Difco) slants and stored at 4°C. Starter culture was prepared by inoculating loopful of freshly grown culture in 50 ml of YM broth in a 150 ml Erlenmeyer flask. The flasks were incubated at 28°C at 150 rpm for 24 h in dark. Biomass in the flasks reached 0.9 g/L in 24 h.

Screening of significant components using Plackett Burman Design

Screening of medium components was carried out using the Plackett Burman Design to determine their effect and significance on biomass and β-carotene production. Five components of the medium namely: mannitol, potassium nitrate, yeast extract, monopotassium hydrogen phosphate and magnesium sulfate were investigated. The fermentation medium consisted of sodium chloride (1 g /L), calcium chloride (0.25 g/L) and anhydrous sodium sulphate (5.29 g/L). Micronutrients were added from three stock solutions of microelements, amino acids and vitamins.

Microelements (1ml/L) contained boric acid (500 µg/L), copper sulfate (40 µg/L), potassium iodide (100 µg/L), ferric chloride (200 µg/L), manganese sulfate (400 µg/L), sodium molybdate (200 µg/L) and zinc sulfate (400 µg/L). Amino acids (1ml/L) contained L-histidine (10 mg/L), LD-methionine (20 mg/L), and LD-tryptophan (20 mg/L). Vitamins (0.1ml/L) contained biotin (2 µg/L), calcium pantothenate (400 µg/L), folic acid (2 µg/L), inositol (2,000 µg/L), niacin (400 µg/L), PABA (200 µg/L), pyridoxine hydrochloride (400 µg/L), riboflavin (200 µg/L), and thiamine hydrochloride (400 µg/L). Mannitol, potassium nitrate, yeast extract, monopotassium hydrogen phosphate and magnesium sulfate were added at varying concentrations as per the requirement of the experiment as listed in Table 1 (Haaland, 1989). Low and high settings of each of these components were used to prepare a combination of eight trials of the culture medium (Table 1). Fermentation experiments were carried out in 500 ml Erlenmeyer flasks containing 200 ml of fermentation media. Each flask was inoculated with 5% (v/v) of starter culture and incubated at 28°C for 3 days at 150 rpm in the dark. Biomass and β-carotene content was determined. The whole set of experiments was done in triplicate. Regression analysis was performed on the observations along with the matrix of Table 1 to obtain the values of main effects using PASW Statistics 18 software.

Table 2. Main effects observed in Plackett Burman Design of components in medium on accumulation of biomass and β-carotene.

Variable	Biomass (g/L)			β-Carotene (µg/L)		
	Effect of variable	Standard error	p-value	Effect of variable	Standard error	p-value
Mannitol	0.0789	0.074	0.367	1.297	0.922	0.254
Potassium nitrate	0.347	0.231	0.230	4.404	2.881	0.224
Yeast extract	0.677	0.206	0.046	11.737	2.561	0.020
Monopotassium phosphate	-0.0103	0.925	0.992	2.448	11.526	0.845
Magnesium sulphate	0.420	1.85	0.833	1.007	23.052	0.968

Influence of initial pH on biomass and β-carotene production

Fermentation medium containing mannitol (25 g/L), potassium nitrate (8 g/L), yeast extract (9 g/L), monopotassium hydrogen phosphate (5 g/L), magnesium sulfate (1 g/L), sodium chloride (1 g/L), calcium chloride (0.25 g/L), and anhydrous sodium sulphate (5.29 g/L) was used. Micronutrients were added from three stock solutions of microelements (1 ml/L), amino acids (1 ml/L) and vitamins (0.1 ml/L). The initial pH was adjusted to obtain range of 3.0 to 8.0 with 0.1 N HCl / 0.1 N KOH. Fermentation experiments were carried out in 500 ml Erlenmeyer flasks containing 200 ml of fermentation media. Each flask was inoculated with 5% (v/v) of starter culture and incubated at 28°C for 3 days at 150 rpm in the dark. Biomass and β-carotene content was determined. The whole set of experiments was done in triplicate.

Effect of interaction of significant components using central composite design (CCD)

Mannitol and yeast extract were used as the significant components in this study. The concentrations of mannitol and yeast extract corresponding to the various coded levels and as per the experimental design are given in Table 3 (Haaland, 1989). Each trial contained different combinations of mannitol and yeast extract. Fermentation medium contained potassium nitrate (8 g/L), monopotassium hydrogen phosphate (5 g/L), magnesium sulfate (1 g/L), sodium chloride (1 g/L), calcium chloride (0.25 g/L), and anhydrous sodium sulphate (5.29 g/L). Microelements (1 ml/L), amino acids (1 ml/L) and vitamins (0.1 ml/L) were added. The pH was adjusted to 5.5. Fermentation experiments were carried out in 500 ml Erlenmeyer flasks containing 200 mL of fermentation media. Each flask was inoculated with 5% (v/v) of starter culture and incubated at 28°C for 3 days at 150 rpm in the dark. Biomass and β-carotene content was determined. Experiment was done in triplicate. Non-linear regression analysis was performed on the observations along with the matrix of Table 3 to obtain equation using PASW Statistics 18 software.

Time course for biomass and β-carotene production in *R. graminis* RC04 in optimized medium

Time course for the production of biomass and b-carotene in *R. graminis* RC04 was carried out using 200 ml optimized medium in 500 ml conical flasks. Composition of fermentation medium was exactly the same as used for CCD analysis using optimized mannitol (15 g/L), yeast extract (10 g/L), and pH was adjusted to 5.5. Each flask was inoculated with 1% (v/v) of starter culture and incubated at 28°C for 4 days at 150 rpm in the dark. Flasks were

withdrawn periodically to monitor biomass and β-carotene production. Experiment was done in duplicate.

Analytical methods

Dry weight estimation

Culture broth (100 ml) was centrifuged at 10,000 × *g* for 20 min. Pellet was obtained and was washed twice with sterile distilled water. Pellet was transferred to pre-weighed aluminium cups and dried at 105°C till constant weight was obtained.

Extraction and estimation of β-carotene

Culture broth (100 ml) was centrifuged at 10,000 × *g* for 20 min. Pellet obtained was washed twice with saline and centrifuged again. Pellet was finally suspended in 5 ml normal saline. The suspension was sonicated at 20 kHz for 10 min. Suspension was then mixed vigorously with dimethyl sulfoxide (DMSO), acetone and petroleum ether and kept standing in the dark for 18 h at 4°C. The suspension was repeatedly mixed and kept standing until there was no visible color in the aqueous layer. Organic layer was collected and absorbance was taken at 451 nm and amount of β-carotene was determined using the extinction coefficient 2680 for 1 g/100 ml solution of β-carotene.

RESULTS AND DISCUSSION

Preliminary studies on the effects of different carbon and nitrogen sources on biomass and β-carotene production by *R. graminis* RC04 showed that the highest values of both biomass and β-carotene production were obtained with mannitol as carbon source and yeast extract and potassium nitrate as sources of nitrogen. This study focused on investigating the optimization of biomass and β-carotene production by *R. graminis* RC04 by varying these components along with monopotassium hydrogen phosphate and magnesium sulphate. These five factors were varied at 2 coded levels. The variables were set up for eight experimental runs. The two experimental responses (biomass and β-carotene production) were recorded (Table 1).

Table 2 illustrates the impact that each medium component individually had on biomass and β-carotene

Table 3. Central composite design (CCD) matrix with coded and actual values chosen for mannitol and yeast extract with the experimental and calculated values for biomass and β-carotene production.

Trial	Variable				Value			
	Coded		Actual		Experimental		Calculated	
	Mannitol	Yeast extract	Mannitol (g/L)	Yeast extract (g/L)	Biomass (g/L)	β-Carotene (µg/L)	Biomass (g/L)	β-Carotene (µg/L)
1	+	+	22.5	7.5	2.47 ± 0.35	123 ± 11	2.790	127.514
2	+	-	22.5	2.5	1.02 ± 0.38	67 ± 16	1.186	57.944
3	-	+	7.5	7.5	2.20 ± 0.24	117 ± 20	2.515	137.759
4	-	-	7.5	2.5	0.65 ± 0.08	42 ± 11	0.815	49.1385
5	0	+	15.0	7.5	2.53 ± 0.33	141 ± 15	2.730	145.067
6	0	-	15.0	2.5	1.91 ± 0.33	106 ± 16	1.078	65.972
7	+	0	22.5	5.0	2.42 ± 0.33	134 ± 2	1.744	80.259
8	-	0	7.5	5.0	1.43 ± 0.36	85 ± 17	1.421	80.979
9	- 1.414	0	2.8	5.0	1.55 ± 0.35	88 ± 10	1.194	60.742
10	+ 1.414	0	27.2	5.0	1.51 ± 0.22	30 ± 10	1.718	59.571
11	0	- 1.414	15.0	1.4	0.55 ± 0.09	34 ± 19	0.976	61.957
12	0	+ 1.414	15.0	8.6	3.86 ± 0.43	198 ± 19	3.354	175.854
13	0	0	15.0	5.0	1.51 ± 0.27	86 ± 9	1.660	93.051
14	0	0	15.0	5.0	1.36 ± 0.33	78 ± 9	1.660	93.051
15	0	0	15.0	5.0	1.65 ± 0.14	95 ± 10	1.660	93.051
16	0	0	15.0	5.0	1.37 ± 0.41	65 ± 6	1.660	93.051

production. All variables with the exception of mono-potassium hydrogen phosphate had positive coefficients. From p values < 0.05 it could be seen that effect of yeast extract was critical for biomass and β-carotene production. Mannitol and potas-sium nitrate had a positive impact on biomass accumulation and β-carotene production with similar p values. However, these values are more than 0.05. The mannitol was considered having better impact than potassium nitrate because standard deviation was less for the latter. Other factors were non-significant as having very high p value.

The initial pH of the growth medium has been shown to significantly affect growth and β-carotene production (Kim et al., 2004). Maximum by *R. graminis RC04* when cells were grown in the same medium at a pH of 5.5 and minimum values were recorded when grown at pH 8 (Figure 1).

The central composite design was used to study the interaction of mannitol and yeast extract at varying concentrations. The details of the design, which includes the coded and actual values of the variables as well as the experimental and cal-culated values for both biomass and β-carotene accumulation, are given in Table 3. These values were fitted in a binomial equation. The values of regression coefficients were calculated and the fitted equations (using actual values of the variables) for predicting biomass and β-carotene production are:

Equation for biomass (g/L) = 0.257 + (0.069*A) - (0.0405*B) - (0.00137*A*A) + (0.039*B*B) - (0.00128*A*B)
Equation for β-carotene production (µg/L) = -4.224 + (7.852*A) - (0.321*B) - (0.221*A*A) + (1.995*B*B) - (0.254*A*B)

Where, A is the mannitol concentration and B is the yeast extracts concentration. For biomass, the linear coefficient of mannitol was biomass and β-carotene production was observed positive where-as the quadratic coefficient was negative. On the
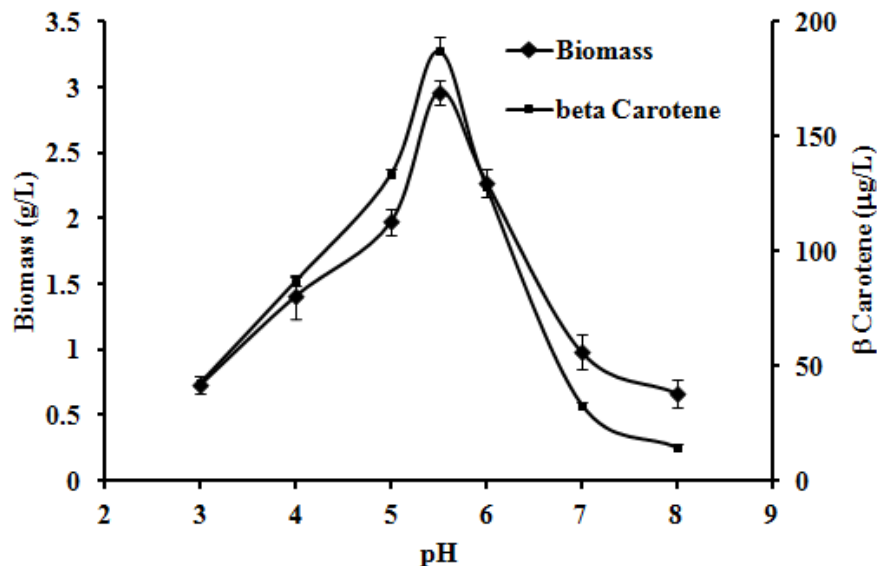
**Figure 1.** The influence of initial pH of medium on biomass and β-carotene production by *Rhodotorula graminis RC04.*
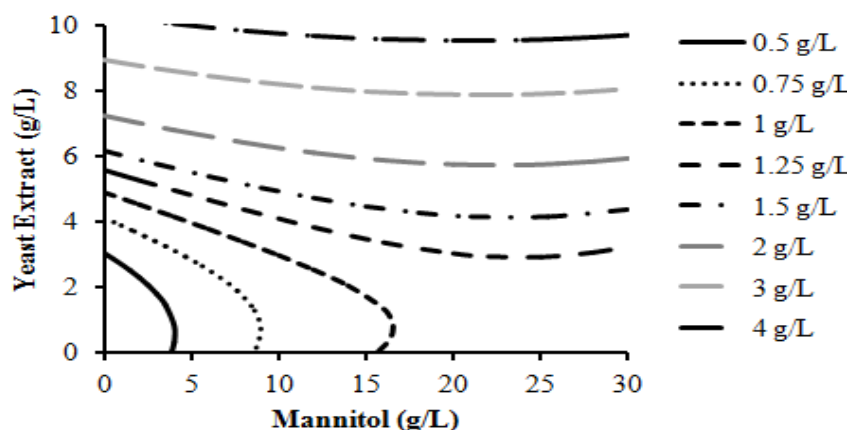
Figure 2. Iso-response plot of biomass production of *Rhodotorula graminis RC04* with varying medium components mannitol and yeast extract as per counter current distribution (CCD).

other hand, the interaction of mannitol with yeast extract was positive. The quadratic coefficient of yeast extract for biomass although positive was of a low magnitude indicating that excessive concentrations cannot be used. For β-carotene production, mannitol and yeast extract interacted antagonistically with each other. Thus a medium containing either a combination of low yeast extract and high mannitol concentration or high yeast extract and low mannitol concentration would be ideal for β-carotene production. Even though β-carotene production is known to increase during the stationary phase (Jukyoung et al., 2009), for β-carotene production to occur, a critical amount of biomass is required. This is favored at high mannitol concentration. Thus, a high mannitol and low yeast extract concentration would yield sufficient biomass to yield β-carotene in appreciable titer.

However, the negative quadratic coefficient of mannitol for biomass and the positive but low value for β-carotene production limits the maximum concentration of mannitol that could be used. Beyond these concentrations, both biomass and β-carotene production would be detrimentally affected. The lower nitrogen content of the culture medium mimics the nitrogen depletion that occurs when the culture reaches the stationary phase, triggering β-carotene production. Hence, it appears that the concentration of yeast extract is more critical than mannitol for β-carotene production. Three dimensional graphical representation of the predictive response surface are used to describe the individual and cumulative effect of the two test variables mannitol and yeast extract on the biomass and β-carotene production (Figures 2 and 3).

From predicted values, it is seen that to achieve a
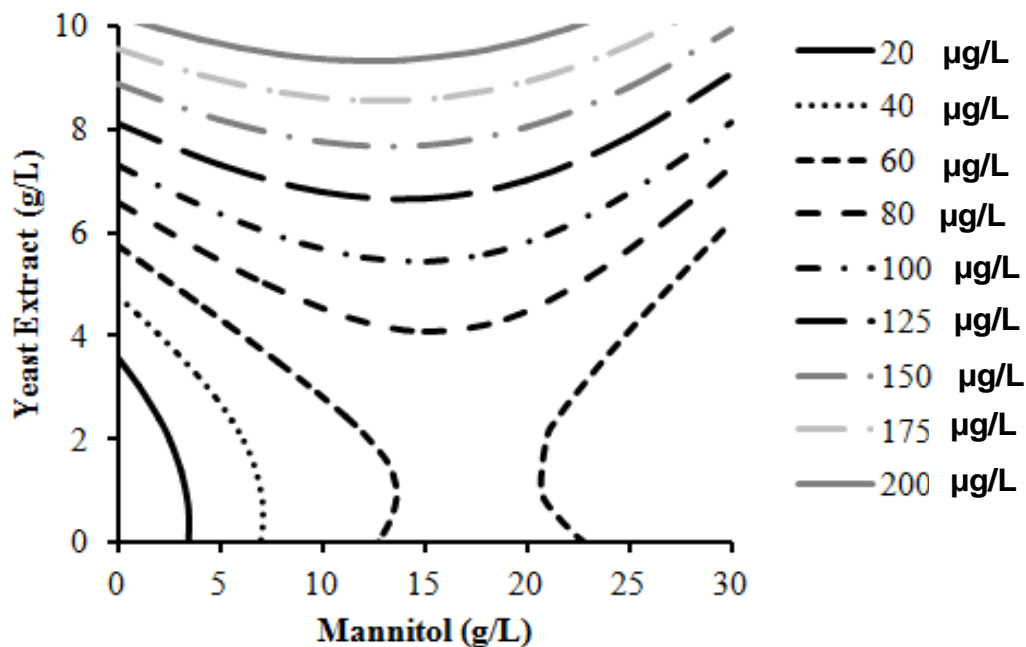
Figure 3. Iso-response plot of β-carotene production in *Rhodotorula graminis RC04* with varying concentrations of mannitol and yeast extract as per CCD.
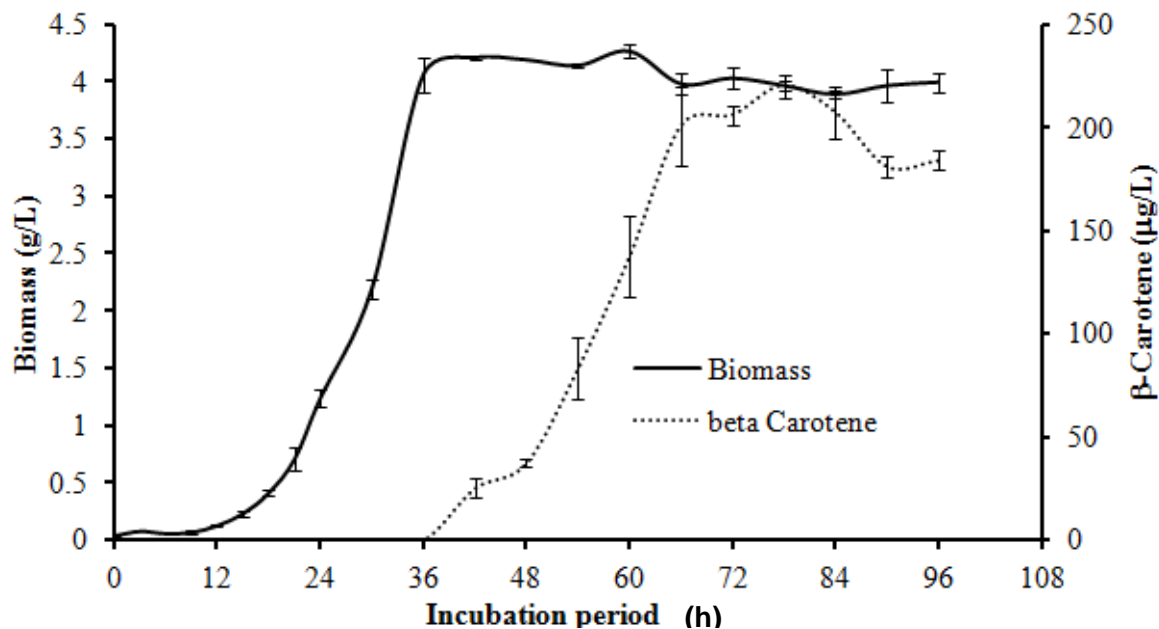
Figure 4. Time course for biomass and β-carotene production in *Rhodotorula graminis RC04* in optimized medium.

biomass of 3.8 to 4.3 (g/L) and a β- carotene content of 190 to 220 (µg/L), mannitol should be used in the range of 10 to 20 g/L and yeast extract in the range of 9.5 to 10 g/L. Based on R^2 values, the used model shows goodness of fit. Results of growth kinetics of *R. graminis RC04* in the optimized medium show that maximum growth was achieved in 56 h after the inoculum was added to the culture while maximum β-carotene content was achieved at 78 h. *R. graminis RC04* began to grow after 6 h and grew exponentially up to 36 h. β-carotene accumulation started at 42 h and increased rapidly. Approximately, 220 µg/L of β-carotene was achieved at the end of the stationary phase (Figure 4). It is clear then that β-carotene accumulation is not growth associated in

R. graminis RC04.

Conclusion

Higher concentrations of mannitol, yeast extract, potassium nitrate and magnesium sulfate positively affect both biomass and β-carotene accumulation in *R. graminis RC04*. Mannitol had a significant positive impact on biomass accumulation and yeast extract had a significant positive impact on β-carotene production. Results of application of central composite design indicate that the concentration of yeast extract is more critical than mannitol for β-carotene production. From predicted values obtained using fitted binomial equations, it was seen that mannitol should be used in the range of 10 to 20 g/L and yeast extract in the range of 9.5 to 10 g/L to achieve an optimum biomass of 3.8 to 4.3 g/L and an optimum β-carotene content of 190 to 220 µg/L. This report therefore presents the optimized medium using statistical experiment design for higher volumetric production of β-carotene from *R. graminis RC04* and has potential for exploitation by biotechnological industries.

ACKNOWLEDGMENTS

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