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Production of β-carotene by filamentous fungus *Mucor azygosporus* MTCC 414 in synthetic medium by applying response surface methodology

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Abstract

Mucor azygosporus MTCC 414, a homothallic orange colored fungus has been studied for production of β-carotene. Effect of different physico-chemical parameters such as selection of medium, medium pH, incubation temperature, inoculum size as well as carbon and nitrogen sources and role of growth supplements on β-carotene production by *Mucor azygosporus* MTCC 414 in shake flask culture was investigated. The conventional one factor at-a-time method was used for the optimization. Response surface methodology (RSM) using central composite design was further employed to determine the process variables for maximum β-carotene production. The maximum production of β-carotene (3.4 mg/L) from *M. azygosporus* MTCC 414 was obtained experimentally by applying RSM with specific production of 1940 µg/g dcw indicating approximately 4-fold increase than those obtained under conditions before optimization (0.9 mg/L with specific production of 425 µg/g).

Key words: *Mucor azygosporus*; β-carotene; central composite design; response surface methodology; optimization

1. Introduction

Nutraceutical, a combination of words nutrition and pharmaceutical is a food or food product that reportedly provides health and medical benefits including prevention and treatment of chronic diseases. β-carotene is the main source of provitamin A and is widely used as a food colorant and nutraceutical. The global market of β-carotene estimated to surpass $ 1.2 billion in 2015 (Ribeiro *et al.*, 2011). Commercial production of β-carotene from microorganisms competes mainly with synthetic manufacture by chemical procedures. It is produced primarily by filamentous fungi, yeasts and some species of bacteria, algae and lichens (Thakur and Azmi, 2013a). The maximum yield has been obtained by *Blakeslea trispora* was found to be dependent on sexual mating of two compatible strains as well as fermentation cultures become viscous. Therefore, the studies on *Mucor azygosporus* MTCC 414 mucoraceous fungi seems to be important and worth studying.

The fermentation media need to be optimized for efficient utilization of the fermentation technology. Medium optimization by one-factor-at-a-time method involves changing one variable (media components, pH and temperature) while fixing the others at a certain arbitary level. But this conventional approach is very laborious and time consuming. Statistical experimental design is a powerful statistically technique generally employed for medium or process optimization of microbial fermentation. In statistical based approaches, response surface methodology (RSM) has been extensively used and successfully applied for optimization of carotenoid production from *Rhodotorula* sp. (Buzzini, 2000; Bhosale and Gadre, 2001; Vijayalakshmi *et al.*, 2001; Malisorn and Suntornsuk, 2008), *Sporodiobolus salmonicolor* (Valduga *et al.*, 2008) and *Blakeslea trispora* (Choudhari and Singhal, 2008).

The objective of the present study was to apply statistical method to optimize the physico-chemical parameters for the production of β-carotene by culturing *Mucor azygosporus* MTCC 414. Several important factors such as carbon source, nitrogen source, growth supplement, medium pH, incubation temperature, inoculum size and incubation time were studied by one factor-at-a-time and subsequently by RSM.

2. Materials and Methods

2.1 Medium components

All the media components were purchased from Hi-media Limited, Mumbai, India. Standard β-carotene was procured from Sigma-Aldrich Ltd., Mumbai.

2.2 Microorganism and culture conditions

Mucor azygosporus MTCC 414 was used in the production of β-carotene. The culture was maintained on yeast phosphate soluble starch (YpSS) agar slants at 25°C for 5 d. After 5 d, the slants were

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kept at 4°C and thereafter sub-cultured every 30 d.

2.3 Selection of production medium

Since the β-carotene production by using cells of *M. azygosporus* MTCC 414 was found to be associated with mycelial mass, medium producing high mycelial mass with good production of β-carotene was needed to be selected and further optimized. The thirteen media were tested for the production of β-carotene by *M. azygosporus* MTCC 414 (Table 1). The initial pH for all media was adjusted to 5.0. M-8 medium containing (%, w/v) malt extract 0.7, yeast extract 0.05 and peptone 0.25 was selected for further study.

2.4 Optimization of production medium using one-factor-at-a-time method

2.4.1 Effect of medium pH

The initial pH of the selected medium (M-8) was varied from 4-10 and was inoculated with 50µL of inoculum containing 12×10^3 spores in order to find out the optimum initial pH for the production of β-carotene by *M. azygosporus* MTCC 414.

2.4.2 Effect of incubation temperature

Most favorable temperature for β-carotene production was obtained by incubating the production medium (pH-5.5) at different temperatures (20-45°C) for 5 d and the mycelial mass obtained was used for β-carotene estimation.

2.4.3 Effect of inoculum size

The different inoculum sizes ranging from 25 - 150µL (containing 6×10^3 – 36×10^3 spores) were used to inoculate the M - 8 production medium. The inoculated medium was incubated at 30°C and β-carotene production was estimated after 5 d of incubation.

2.4.4 Effect of carbon source

In order to find out the most suitable carbon source for the growth and production of β-carotene by M. azygosporus MTCC 414 the production medium containing (%, w/v) malt extract 0.7, yeast extract 0.05 and peptone 0.25 was supplemented with different carbon sources, *viz.*, sucrose, lactose, glucose, galactose, glycerol, fructose, maltose, mannitol, xylose and starch at concentration of 1.0% (w/v) and incubation was continued for 4 d at 30°C.

2.4.5 Effect of nitrogen sources and growth supplements

To study the effect of nitrogen source on the production of β-carotene by *M. azygosporus* MTCC 414, different organic nitrogen sources such as peptone, tryptone, casein and gelatin (1%, w/v) were used in the production medium (pH-5.5) containing (%, w/v) starch 0.75, malt extract 0.7 and yeast extract 0.05. The effect of added nutrients such as growth supplements (malt extract, beef extract, soya peptone), on the production of β-carotene by *M. azygosporus* MTCC 414 was observed by adding them at 1% (w/v) in the production medium.

2.5 Optimization of screened components by RSM

Once the critical variables were screened, RSM was performed to optimize the screened medium components for enhanced β-carotene production using the central composite design uniform precision (CCD). Five variables were selected and full factorial central composite factorial design was used $2^5 = 32$ a total of 50 experiments were performed (Tables 2, 3). The relation between the coded values and actual values has been described as the following equation for statistical calculations;

$$X_i = \frac{Xi - Xcp}{\text{delta } X_i} \quad i = 1, 2, 3 \ldots\ldots\ldots k$$

where x_i is dimensionless value of an independent variable,
Xi real value of an independent variable,
Xcp, real value of an independent variable at the center point
Dx_i is the step change of real value of the value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i.

The β-carotene content has been taken as the response. The experiments were carried out in triplicate, which was necessary to estimate the variability of measurement. Replicates at the center of the domain in three blocks permit the checking of the absence of the bias between several sets of experiments. The relationship of the independent variables and the response was calculated by the second order polynomial.

$$Y = \beta o + \beta ii + \beta ij/k$$

where Y is the predicted response, βo a constant,

βi the linear coefficient,

βii the squared coefficient

βij the cross product coefficient, k is the number of factors.

The second order polynomial coefficients were calculated using the software package design expert version 8.0.5 to estimate the response of the dependent variable as well as to draw the response surface plots. The analysis of variance (ANOVA) was used to analyze the data.

2.6 Estimation of biomass and β-carotene

The fungal biomass of *M. azygosporus* MTCC 414 grown in the culture broth and produced β-carotene was extracted with hexane and ethyl acetate (Thakur and Azmi, 2013b). The content of β-carotene was determined spectrophotometrically with UV-Vis spectrophotometer and HPLC (Azmi *et al.*, 2011).

3. Results and Discussion

3.1 Optimization using one factor-at-a-time

The effect of composition of different growth media on growth of *M. azygosporus* MTCC 414 and β-carotene production was determined after completion of incubation period of 5 d (Table 1). During the course of fermentation, the medium components not only act as major constituents for building of cellular material but also an important energy source. The culture of *M. azygosporus* MTCC 414 was grown in thirteen different media reported for production of β-carotene. The results suggest that the medium M-8 (pH 5.0) was the best for the production of β-carotene by *M. azygosporus* MTCC 414. However, no growth was obtained in media M-5. The optimization of microbial growth and the production of β-carotene was studied by various workers. Libkind and Broock, (2006) have reported that MYP medium having same composition as that of M-8 medium, showed a higher colony colour intensity in case of potagonian yeast of *Cryptococcus sp.* which may be due to higher carbon availability and therefore different C/N ratios.

The cells of *M. azygosporus* MTCC 414 grew well at a wide range of pH. A higher yield of β-carotene (598 µg/g dcw) and biomass (2.4 g/L) was obtained at pH 5.5 (Figure 1). The final pH of the broth was found to increase with increase in initial pH of the production medium. Dispersed growth as well as less β-carotene production was observed at pH beyond 6.0. However, no growth was obtained at both the extreme values, *i.e.*, pH 4.0 and 8.0. Similar results have been obtained in case of β-carotene production using *B. trispora* and optimum pH was found to be 6.0 (Chaudhari and Singhal, 2008). Similarly, Malisorn and Suntornsuk (2008) have reported decrease in growth and β-carotene production at both extreme pH 4.0 and pH 8.0 in case of *R. glutinis* DM28 in fermented radish brine. The results obtained by Valduga *et al.*, (2011) were also in accordance with the present study where maximum level of carotenoids (1019 µg/L) by the cultivation of *S. salmonicolor* CBS 2636 in bioreactor was obtained at initial acidic pH 4.0. An initial pH of 5.0 was found to be optimal for the β-carotene production by *R. lactosa* (Martelli *et al.*, 1992).

The incubation temperature was reported to control the concentration of enzymes involved in carotenoid production and changes in enzyme concentration ultimately control the carotenoid levels in microorganisms (Hayman *et al.*, 1995). The maximum β-carotene production (750 µg/g dcw) with 2.4g/L biomass was observed at 30^0C (Figure 2). However, on further increase in the temperature there was decrease in the mycelial mass as well as β-carotene production. Moreover, no growth was observed at temperature beyond 45^0C. The increase in final pH was found to be associated with the growth. The maximum production of β-carotene was obtained when the incubation temperatures were set at 30^0C for *R. glutinis* DM28 (Buzzini, 2000; Malisorn and Suntornsuk, 2008) and 25^0C for *B. trispora* (Mantzouridou *et al.*, 2002; Roukas *et al.*, 2003; Chaudhary and Singhal, 2008), *Cryptococcus sp.* (Libkind and Broock, 2006), *S. salmonicolor* (Valduga *et al.*, 2011).

The inoculum size is very important factor and results (Figure 3) revealed that maximum β-carotene production (792 µg/g dcw) with significant biomass (2.8 g/L) was obtained when 50 µL inoculum containing 12×10^3 spores has been used to inoculate the production medium. These results are in accordance with Chaudhary and Singhal, (2008) who have reported the optimum spore size to be 10^6 spores/ mL whereas, Malisorn and Suntornsuk, (2008) have obtained maximum β-carotene production when the medium was inoculated with 5×10^6 CFU of yeast starter *R. glutinis* DM28.

During the course of microbial fermentations, the carbon source not only acts as a major constituent for building of cellular material, but also as an important energy source. The results exhibit that the maximum β-carotene production (1095 µg/g dcw) with mycelial mass 3.2 g/L was obtained when starch was added to the medium (Figure 4). The glucose has been used as main carbon source in the optimization of the β-carotene production with synthetic medium by *B. trispora* (Mantzouridou *et al.*, 2002). The highest yield of biomass (4.01 g/L) and carotenoid content (0.1%) by *Micrococcus* sp. was obtained when medium was supplemented with fructose (Attri and Joshi, 2005). The maximum β-carotene (1166 µg/g dcw) was produced at 0.75% (w/v) of starch. The further increase in starch leads to decrease in β-carotene production as well as mycelial mass owing to osmotic effects.

It has been suggested that both type and concentration of the nitrogen source may be important in regulating key enzyme systems involved in nitrogen assimilation, thereby affecting the pool of intracellular metabolites (Auer and Seviour, 1990). Among the other sources evaluated, casein gave good growth (6.2 g/L) but less β-carotene production (129 µg/g dcw) production which may be due to hard pellet formation of the mycelial biomass and further the less extraction of β-carotene from the pellet. A high level β-carotene production (1250 µg/g dcw) was obtained when tryptone was used as nitrogen source (Figure 6). On optimization of concentration of tryptone maximum β-carotene production (1260 µg/g dcw) was achieved at 0.75% (w/v) of tryptone (Figure 5). Tryptone was reported as the most suitable nitrogen source for *B. trispora* by Filotheou *et al.* (2010), which strengthen the results obtained in the present study.

Among growth supplements malt extract was selected for increased production of β-carotene. Maximum amount of β-carotene (1280 µg/g dcw) was obtained (Figure 6) when malt extract was added to the production medium followed by control (1255 µg/g dcw). The mycelial mass was found to be maximum (7.2 g/L) in case of soybean meal followed by malt extract (6.4g/L) which may be due to the less solubility of soybean meal in the production medium. It has been demonstrated determined that the malt extract had significant influence on the specific production of carotenoids (287µg/g) by *S. salmonicolor* CBS 2636 in shake flasks (Valduga *et al.*, 2009) is in consistence with the current study. The maximum β-carotene production (1290 µg/g dcw) was obtained at 0.75% (w/v) malt extract.

3.2 Optimization of β-carotene production by RSM

In order to examine the combined effect of five different independent variables (medium pH, incubation temperature, malt extract, starch and tryptone concentration) on β-carotene production by *M. azygosporus* MTCC 414, a central composite design of 50 experiments were performed. Second order polynomial equation was used to correlate the independent process variables, with β-carotene production. The second order polynomial coefficient for each term of the equation determined through multiple regression analysis using the Design Expert. The designs of experiments and respective experimental yields have been given in Table 2 and the coded values of independent variables have been shown in Table 3. The results were analyzed by using ANOVA (Analysis of Variance), suitable for analysis of the designed experiment. The results are shown in Table 4. The model F-value is calculated as ratio of mean square regression and mean square residual. Model p-value (prob>F) was found to be very low (<0.0001), which signifies the relevance of the model. The p values were used as a tool to check the significance of each of the coefficients, which in turn are necessary to understand the pattern of the mutual interactions between the test variables. The smaller the magnitude of the p, the more significant will be the corresponding coefficient. Values of p less than 0.05 generally indicate model terms are significant.

The coefficient estimates and the corresponding p-values suggest that among the test variables used in the study, E (tryptone), AB (temperature x pH), AD (temperature x malt extract), BC (pH x starch), BE (pH x tryptone) and CD (starch x malt extract) were the significant model terms. Temperature, malt extract, starch and tryptone ($p<0.0001$) has the largest effect on α-carotene production followed by starch and malt extract ($p<0.005$). The fit of the model was also expressed by the coefficient of determination R^2, which was found to be 0.83, indicating that 83% of the variability in the response could be explained by the model. The closer the R^2 value

is to 1, the better the model is fit to experimental data, the less is the distance between the predicted and the observed values.

The 3D plots of two factors versus β-carotene were drawn and the corresponding contour plot was obtained by keeping another variable at its optimal level and from the bump of 3-D plot or the central point of its respective contour plot, the optimal composition of medium components was identified (Figure 7). The optimal concentration condition for the five components or variables as obtained from the maximum point of the model were as follows: incubation temperature 25^{0}C, medium pH 6.0, starch concentration 7.5g/L, malt extract concentration 7.5g/L and tryptone concentration 7.5g/L. By substituting the levels of factors into the regression equation, the maximum predictable response for β-carotene by *M. azygosporus* MTCC 414 was calculated and was experimentally verified. The maximum production of β-carotene from *M. azygosporus* MTCC 414 obtained experimentally using the optimized medium was 3.4 mg/L with specific production of 1940 µg/g dcw, indicating approximately 4.0 fold higher than those under initial condition before optimization (0.9 mg/L with specific production of 425 µg/g).

Central composite design (CCD) has widely been used response surface design when the experimental region is defined by the upper and lower limits of each factor and not extended beyond them (Neter *et al.*, 1996). A combination of factors which generate certain optimal response could be identified by this approach. Malisorn and Suntornsuk (2008) reported 15% increase in the production of β-carotene from *R. glutinis* DM 28 by using fermented radish brine and applying RSM. However, 1.4-fold increase in the production of β-carotene by *B. trispora* has been observed by applying RSM (Chaudhari and Singhal, 2008). In an interesting study on the production of spores by *B. trispora* (Roukas *et al.*, 2011), 95% more spores were obtained by the use of RSM.

4. Conclusion

A central composite design of 50 experiments were performed to examine the combined effect of five different independent variables (pH, temperature, malt extract, starch and tryptone concentration) on â-carotene production by *M. azygosporus* MTCC 414. The results were analyzed by using ANOVA as well as 3D surface plots. The optimal concentration for the five components as obtained from the maximum point of the model were calculated to be as 25^{0}C, 6.0, 7.5 g/L, 7.5 g/L and 7.5 g/L for temperature, pH, starch concentration, malt extract concentration and tryptone concentration, respectively. The maximum production of β-carotene obtained experimentally using the optimized medium was 3.4 mg/L with specific production of 1940 µg/g dcw indicating approximately 4-fold higher yield than those obtained under initial condition before optimization.

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Conflict of interest

We declare that we have no conflict of interest.

Table 1: The composition of the medium used for the growth and production of β-carotene from *M. azygosporus* MTCC 414

Medium	Composition of medium (%, w/v)	Biomass (g/L)	β-carotene (µg/g dcw)	Final pH	Reference
M1	Malt extract 5.0, yeast extract 0.5 and dextrose 1.0	9.8	55	6.2	Papp *et al.*, 2009
M2	Potato dextrose broth	3.0	173	6.8	Goksungur *et al.*, 2002
M3	Glucose 4.0, KH_2PO_4 0.8, $MgSO_4.7H_2O$ 0.05, yeast extract 0.3	4.8	98	5.8	Buzzini, 2000
M4	Lactose 4.5, $(NH_4)_2SO_4$ 0.6, KH_2PO_4 0.55, Na_2HPO_4 0.3, $MgSO_4.7H_2O$ 0.05, yeast extract 0.5	3.8	142	6.4	Simova *et al.*, 2003
M5	Glucose 0.5, yeast extract 0.05, $(NH_4)_2SO_4$ 0.015, glutamic acid 0.015	-	-	7.0	Papp *et al.*, 2009
M6	Yeast extract 0.3, malt extract 0.3, peptone 0.5, dextrose 1.0	5.6	162	6.9	Libkind and Broock, 2006
M7	Glucose 1.0, $(NH_4)_2SO_4$ 0.2, KH_2PO_4 0.2, $MgSO_4$ 0.05, $CaCl_2.2H_2O$ 0.01, yeast extract 0.1	2.6	253	7.2	Libkind and Broock, 2006
M8	Malt extract 0.7, yeast extract 0.05, peptone 0.25	2.6	453	6.4	Libkind and Broock, 2006
M9	Yeast extract 0.25, peptone 0.5, dextrose 0.1	2.2	368	6.9	Attri and Joshi, 2005
M10	Malt extract 2.0, glucose 2.0, peptone 0.1	4.4	57	7.5	Valduga *et al.*, 2009
M11	Yeast extract 4.0, soluble starch 1.0 K_2HPO_4 1.0, $MgSO_4.7H_2O$ 1.0	4.0	42	7.6	Mantzouridou *et al.*, 2002
M12	Glucose 3.0, corn steep liquor 0.5, casein acid hydrolysate 0.2, yeast extract 0.1, L-asparaginase 0.2, KH_2PO_4 1.0, $MgSO_4.7H_2O$ 0.05, Tween 80 0.1, Span 20 0.10, Thiamine HCl 0.5	4.8	75	7.5	Mantzouridou *et al.*, 2002
M13	Glucose 1.0, yeast extract 0.5, K_2HPO_4 0.15, NaH_2PO_4 0.085, Mineral solution 1mL	5.0	46	8.4	Silva *et al.*, 2004

Table 2: Central composite rotable design matrix of independent variables and their corresponding experimental and predicted yields of β-carotene

Run	Temp	pH	Starch conc (g/L)	Malt extract (g/L)	Tryptone (g/L)	β-carotene (mg/L) Actual	β-carotene (mg/L) Predicted
1	35	6	5	10	5	1.39	1.34
2	30	5.5	7.5	7.5	7.5	1.76	1.82
3	41	5.5	7.5	7.5	7.5	1.05	1.13
4	25	6	10	10	5	2.10	2.08
5	35	5	5	5	5	0.76	0.86
6	25	5	10	10	5	1.12	1.08
7	35	5	5	10	5	1.21	1.25
8	30	5.5	7.5	7.5	7.5	2.06	1.96
9	35	5	10	10	10	1.67	1.58
10	30	5.5	7.5	1.55	7.5	0.95	0.93
11	30	5.5	13.45	7.5	7.5	1.59	1.60
12	18.11	5.5	7.5	7.5	7.5	1.31	1.43
13	25	6	5	10	10	1.80	1.78
14	35	6	5	5	10	0.79	0.88
15	25	6	10	5	5	2.30	2.41
16	30	5.5	7.5	7.5	7.5	1.97	1.99
17	30	5.5	7.5	7.5	7.5	1.83	1.69
18	25	6	5	5	10	1.89	1.87
19	30	4.31	7.5	7.5	7.5	0.85	0.92
20	25	5	5	5	5	1.51	1.58
21	35	6	10	10	10	0.96	1.00
22	25	5	10	5	10	0.89	0.93
23	25	5	10	10	10	0.82	0.83
24	35	5	10	5	5	1.16	1.24
25	30	5.5	7.5	13.45	7.5	1.20	1.58
26	35	6	5	5	5	0.75	0.64
27	35	6	10	10	5	1.14	1.04
28	30	5.5	7.5	7.5	1.55	0.56	0.58
29	25	5	5	10	10	1.69	1.57
30	35	6	10	5	10	0.34	0.38
31	35	5	5	5	10	1.52	1.64
32	30	5.5	7.5	7.5	7.5	0.84	0.93
33	25	5	10	5	5	2.34	2.24
34	25	6	10	5	10	1.99	1.95
35	35	5	10	10	5	1.21	1.24
36	25	5	5	10	5	1.81	1.64
37	35	6	10	5	5	0.78	0.76
38	30	5.5	1.55	7.5	7.5	0.72	0.60
39	30	5.5	7.5	7.5	7.5	1.71	1.71
40	30	5.5	7.5	7.5	7.5	1.76	1.62
41	30	5.5	10	10	10	0.83	0.80
42	35	6	5	10	10	0.051	0.054
43	25	6	7.5	7.5	7.5	3.44	3.34
44	25	6	5	5	5	1.85	1.94
45	25	6	5	10	5	1.72	1.94
46	35	5	10	5	10	1.76	1.94
47	30	6.69	7.5	7.5	7.5	1.74	1.94
48	25	5	5	5	10	1.69	1.94
49	35	5	5	10	10	1.70	1.94
50	30	5.5	7.5	7.5	13.45	1.72	1.94

Table 3: Coded values of independent variables

Independent variable	Coded values (– α)	– 1	0	+1	(+α)
Incubation temperature	21.554	25	30	35	33.44
Medium pH	4.3108	5.0	5.5	6.0	6.689
Starch concentration	1.5539	5.0	7.5	10.0	13.446
Malt extract concentration	1.5539	5.0	7.5	10.0	13.446
Tryptone concentration	1.5539	5.0	7.5	10.0	13.446

Table 4: Analysis of variance (ANOVA) for the experimental results of the central composite design

Source	Sum of Squares	df	Mean Square	F Value	p-value Prob > F
Model	14.72	20	0.74	7.21	< 0.0001
A-Temperature	0.16	1	0.16	1.54	0.2241
B-pH	0.30	1	0.30	2.97	0.0952
C-Starch	0.049	1	0.049	0.48	0.4923
D-Malt extract	0.016	1	0.016	0.15	0.6991
E-Tryptone	1.38	1	1.38	13.52	0.0010
AB	0.46	1	0.46	4.51	0.0423
AC	0.12	1	0.12	1.22	0.2777
AD	2.54	1	2.54	24.90	< 0.0001
AE	0.17	1	0.17	1.70	0.2020
BC	0.74	1	0.74	7.23	0.0118
BD	0.11	1	0.11	1.08	0.3069
BE	0.63	1	0.63	6.20	0.0188
CD	0.94	1	0.94	9.19	0.0051
CE	0.090	1	0.090	0.88	0.3548
DE	0.24	1	0.24	2.40	0.1322
A^2	0.041	1	0.041	0.40	0.5303
B^2	0.44	1	0.44	4.27	0.0479
C^2	2.76	1	2.76	27.04	< 0.0001
D^2	0.13	1	0.13	1.30	0.2644
E^2	4.28	1	4.28	41.94	< 0.0001
Residual	2.96	29	0.10		
Lack of Fit	0.42	22	0.019	0.052	1.0000
Pure Error	2.55	7	0.36		
Cor Total	17.68	49			

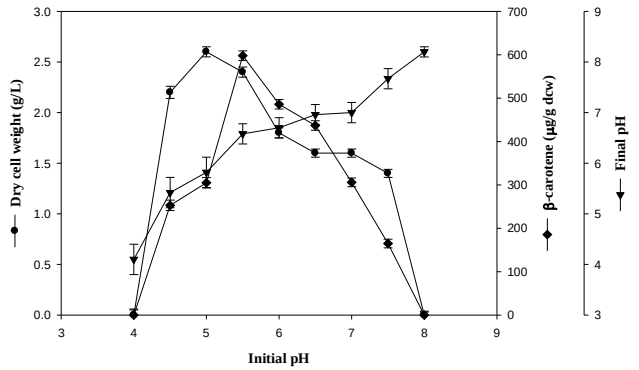

Figure 1: Effect of pH on growth and production of β-carotene by M. azygosporus MTCC 414

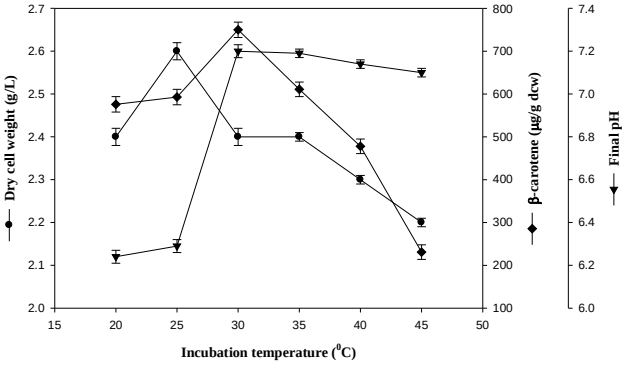

Figure 2: Effect of incubation temperature on growth and β-carotene production by M. azygosporus MTCC 414

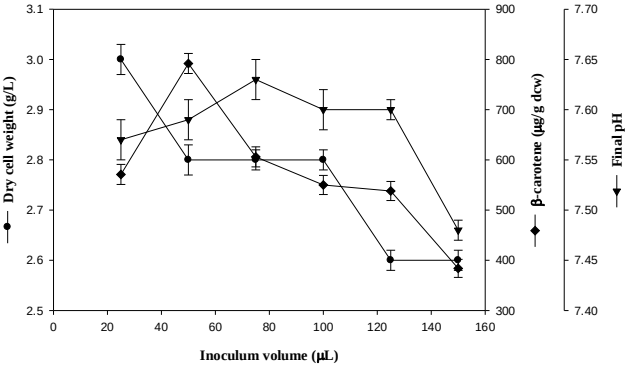

Figure 3: Effect of inoculum size on growth and production of β-carotene by M. azygosporus MTCC 414

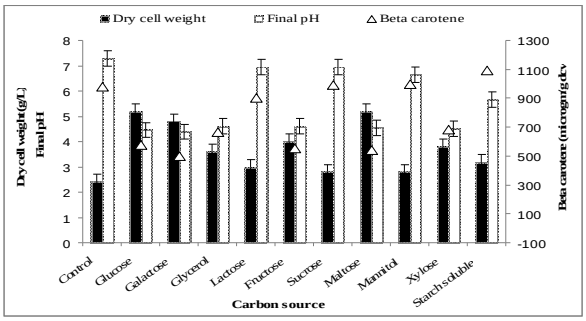

Figure 4: Effect of carbon source on growth and β-carotene production by M. azygosporus MTCC 414

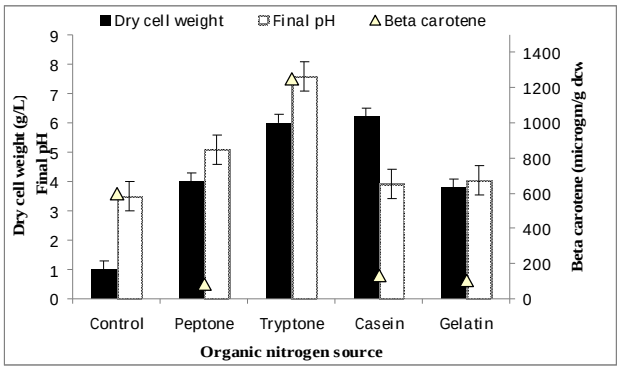

Figure 5: Effect of different organic nitrogen source on growth and β-carotene production by M. azygosporus MTCC 414

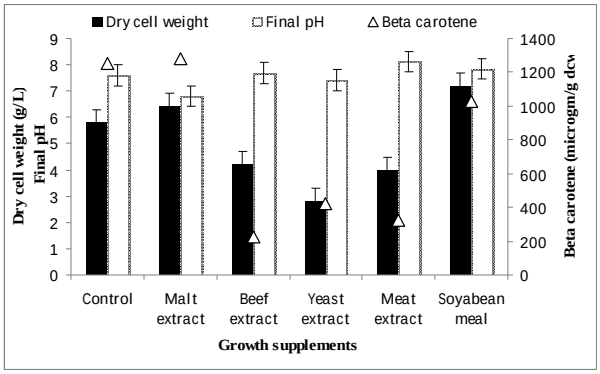

Figure 6: Effect of different growth supplements on growth and β-carotene production by M. azygosporus MTCC 414

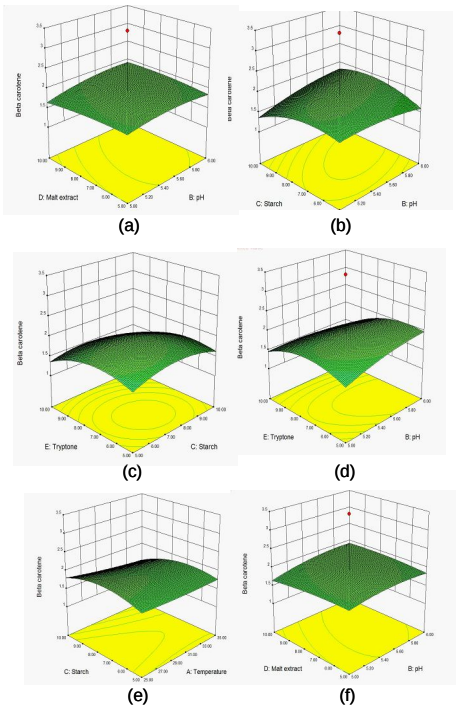

Figure 7: Response surface plots for the yield of β-carotene, changing components were malt extract and pH (a), starch and pH (b), tryptone and starch (c), tryptone and pH (d), starch and temperature (e), malt extract and pH (f).

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