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Research Article

Bioconversion of plant wastes to β-carotene by *Rhodotorula glutinis* KU550702

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ABSTRACT

Microbial synthesis of β-carotene has gained more interest as an alternative to synthetic β-carotene due to easy extraction and high yield. The vitamin microbial production is mainly dependent on culture conditions and the medium compositions. In this study, the β-carotene production by the *Rhodotorula glutinis* ASU6 (KU550702) was evaluated under different growth conditions and nutrient composition. Different agro-renewable wastes were tested as carbon source for *R. glutinis* to obtain maximum amount of β-carotene. Meanwhile, it is clear that *R. glutinis* could grow well on acid extract of onion peels and produced large amount of β-carotene. Initial statistical screening using a Plackett-Burman design showed temperature, incubation time, fermentation type, non-treated onion waste, KH_2PO_4 and L-asparagine as significantly, influencing β-carotene production. Response surface methodology was applied to determine the mutual interactions between these parameters and optimal levels for β-carotene production. The maximum value of β-carotene production was 204.29 mg/l (7.5-fold) of value observed as central point of the central composite design. All the experimental data are in good agreement with predicted ones, confirming the responsibility of the proposed empirical model in describing β-carotene production by *R. glutinis*. In the whole, the outcomes of this study support the exploitation of onion peels through microbial fermentation for β-carotene production.

Keywords: β-carotene; Agro waste; RSM; *Rhodotorula glutinis*.

1. INTRODUCTION

Carotenoids are the colorful plant pigments of commercial interest that have important biological functions. These pigments act as vitamin A precursors which are necessary for healthy vision and cell growth, and also they have antioxidant properties [1]. Several studies have also suggested that carotenoids provide health benefits in decreasing the risk of many diseases, lowering the risk of cancer and enhancement of immune system function [2]. Cosmetic preparations containing the carotenoids were reported to be efficacious in preventing several kinds of damage resulting from oxidation and exposure to UV light [3]. Carotenoid-containing preparations are also playing an important role as feed additive. Astaxanthin is the

major carotenoid used for pigmentation of fishes and salmons [4]. Beta-carotene, lycopene, and lutein are all different varieties of carotenoids.

Carotenoids production by chemical synthesis or extraction from plants is restricted by low amounts that lead in high production costs. The chemical synthesis of carotenoids cannot meet consumers' desire for natural carotenoids and generated hazardous wastes that can affect the environment. There are some problems regarding carotenoids production from plant origin due to seasonal and geographic variability that cannot be controlled. Thus leads to research towards the microbial production of carotenoids. The microbial production of carotenoids could be a better option about yields and costs, looking for the use of low-cost substrates as agro-industrials wastes [5]. This explains the increasing interest rates in production of microbial carotenoids as substitutes for synthetic carotenoids used as colorants in food [6].

Carotenogenic yeasts are a diverse group of unicellular eukaryotes, occurring in soil, fresh and marine water, on plants, animals and can be also found frequently in man-made habitats such as foods [7]. Due to its ubiquity and world-wide occurrence, these yeasts have been able to colonize a large variety of substrates as a source of nutrients and form space for their growth and metabolism [8]. Carotenogenic yeasts are well known producer of biotechnologically significant carotenoid pigments such as astaxanthin, β-carotene, torulen, torularhodin. The composition and quantity of the carotenoid pigments in numerous natural isolates of the genera *Rhodotorula*/*Rhodosporium* and *Sporobolomyces*/*Sporidiobolus* were studied in detail [9].

Rhodotorula is a basidiomycetous yeast in the fungal family Sporidiobolaceae (Phylum Basidiomycota) [10]. *Rhodotorula* is well recognized as a producer of carotenoid pigments, and has both positive and negative significance in agriculture and food industry [11]. β-carotene, torulene, and torularhodin were produced by *Rhodotorula* in varying proportions [12].

The significant aspect of the fermentation process in microbial vitamin production is the development of a suitable culture medium to obtain the maximum amount of vitamin. Agricultural activities and food industry generate considerable quantities of wastes which are rich in organic matter and could constitute new materials for value added products. The valorization of agro-industrial wastes by the biotechnical processes represents an alternative solution for vitamins production [13]. Lignocellulose waste materials obtained from energy crops, wood and agricultural residues, from food and feed industry represent the most abundant global source of renewable biomass [14]. The conversion of lignocellulose wastes to useful products may ameliorate the problems they cause and will eliminate the environmental pollution.

The onion waste includes onion skins, two outer fleshy scales and roots generated during industrial peeling and undersized malformed or damaged bulbs [15]. Several works had been done on onion wastes to gain knowledge of their dietary fiber component, sulphur content, phenolic content, nutritive mineral elements and fatty acids profile of the oil [15]. Up to 65% or more of onion dry weight may be in the form of non-structural carbohydrates such as glucose, fructose, sucrose and fructooligosaccharides [16]. Also, onion is rich in dietary fiber and flavonoids.

Optimization of media components by the traditional “one variable-at-a-time” strategy is time-consuming and expensive when a large number of variables are to be considered; the most favorable optimum condition may not be found, due to the interactions among various factors that were not considered. One of the most effective techniques to study the process behavior is the factorial designed test with analysis of variance [17, 18]. Statistical methods are increasingly preferred for fermentation optimization because they reduce the total number of experiments needed and provide a better understanding of the interactions among factors on the outcome of the fermentation [19]. The Plackett-Burman design as one of the most important method of factorial design has been used as the experimental design [20]. It provides an efficient way of a large number of variables and identifying the most important ones [21]. The Plackett-Burman design allows screening of the main factors and is quite useful in preliminary studies to fix or eliminates selected variables in further optimization processes such as response surface methodology (RSM). RSM is a collection of statistical techniques for designing experiments, building models, evaluating the effects of factors and searching for the optimum conditions

of factors for desirable responses. It usually involves an experimental design like central composite design (CCD) to suit a second-order polynomial. An equation is employed to explain the test variables, and describes the combined impact of all the test variables within the response [22].

The manuscript encompasses three objectives: firstly, isolation of the most powerful yeast strains for β-carotene production and selection the most suitable carbon sources from the tested wastes materials. A second equal aim was employed to screen the fermentation parameters that influenced β-carotene production using Plackett-Burman design. Finally, the optimization of the adequate variables was standardized using RSM.

2. MATERIALS AND METHODS

2.1. Raw materials

Ten different plant wastes (wheat straw, rice straw, maize wastes, onion wastes, date palm wastes, peanut fruit wastes, peanut leaf wastes, sugarcane wastes, potato peels and mango peels) were screened as carbon source utilized by the yeast cells for β-carotene production. The wastes were washed to remove dust, separated, dried, ground and sieved through 1-mm mesh screen. The wastes were prepared (40 g/l) by two methods. Firstly known as aqueous extracts which was prepared by heating waste (at 80 °C for 2 h with continuous stirring) with distilled water at a ratio of two parts of water to one part of waste (by weight). The mixture was filtrate then centrifuged at 20,000 x g for 10 min. to remove the cellulosic debris while the supernatant was used essentially as a carbon source [23]. Secondly known as acid extracts which the wastes were degraded to convert cellulose content into more available sugars by chemical treatments. Fifty ml of 10 % (w/v) hydrochloric acid was added to each waste (4 g) in a 250 mL conical flask. The solution was placed in water bath at 100°C for one h. After being allowed to cool, it was filtered through Whatman filter paper. Then the solution/ broth was completed to 100 mL with distilled water. The broth was adjusted to pH 4.5 with 2.5 M sodium hydroxide [24, 25]. The chemical analysis of selected plant waste (onion waste) was performed at Faculty of Agriculture, Assiut University. The carbohydrate content of the onion waste was determined according to Fales [26] and Schlegel [27].

2.2. Yeast isolation and identification

Different yeast isolates were isolated from maize, Egyptian clover, lemon, zucchini, banana, and spinach plants grown in different localities in Assiut Governorate. The isolates were maintained on yeast malt agar (YME) slants at 4 ºC until used. All yeast isolates were screened for their ability to produce β-carotene production on the glucose fermented medium. Morphological and physiological characteristics (such as surface characteristics, presences of pseudohyphae, ascopore formation and vegetative reproduction) of the highest β-carotene isolates were determined [28]. Also, the highest β-carotene isolates were chosen for molecular identification according to the method described by Harju et al. [29]. The sequence obtained of the yeast isolates were used for a BLAST search in the EMBL/GenBank database (http://www.ncbi.nlm.nih.gov/BLAST/). Yeasts sequences were further aligned and compared with published yeast sequences using the taxonomy browser of the National Center for Biotechnology Information (NCBI; Bethesda, MD, USA) and GenBank.

2.3. Screening for β-carotene accumulation by yeast using different wastes

Modified glucose asparagine yeast medium (GAY) was used as fermented medium for β-carotene production containing (g/l): glucose, 30.0; yeast extract, 1.5; KH_2PO_4, 1.5; L-asparagine, 0.12; $MgSO_4 \cdot 7H_2O$, 0.5 in 1000 ml of distilled water (pH 5.4). Different waste materials as carbon sources were added into the production glucose-free medium. Chloramphenicol (250 mg/ml) was sterilized separately by membrane filtration, using a membrane of pore size 0.22 mm and added as bacteriostatic agent to sterilize medium. Yeast inoculum was prepared by scraping 24-h old yeast culture grown on YME plats into 250 ml Erlenmeyer flasks containing 50 ml of YME medium. After the flasks incubation for 18 h at 28°C ± 1 until a final concentration approximately 2×106 cells/ml using a direct count method. One ml

of yeast spores was inoculated to 250 ml Erlenmeyer flasks containing 50 ml sterile medium. Fermentation was carried out at 28 ± 1 °C on a rotary shaker (150 rpm) for 72 h. Biomass was collected by centrifugation at 4000 xg rpm for 15 min at room temperature and washed thoroughly with distilled water (twice) [30].

2.4. Plackett-Burman design

Plackett-Burman design was employed to screen the fermentation parameters that influenced β-carotene production with respect to their main effect and not the interaction effects between various constituents of the medium [31, 32]. The Plackett-Burman design for screening factors under investigation as well as the levels of each factor is shown in Table 1. The total number of trials to be carried out according to Plackett-Burman design is k + 1 where k is the number of variables. Each independent variable was tested at two levels, high and low, which were denoted by (+) and (−), respectively. In the present study, the eleven assigned variables were screened in twelve experiments (with one dummy variable). The number of positive signs and negative signs per trails are (k + 1)/2 and (k – 1)/2, respectively. In each column and row should contain equal number of negative and positive signs. All experiments were carried out in duplicate and the average of β-carotene yield was taken as the response.

The coefficients for the variables were determined by:

$$A_i = \frac{1}{N} \sum_{i=0}^{N} x_i \, \mathrm{k}_i \quad (1)$$

Where Ai = coefficient values, Xi = experimental yield, Ki = coded value of each variable corresponding to the respective experimental yield Xi and N = number of experiments and the predicted data is given by:

$$Y_t = \sum_{i=0}^{N} A_i \, \mathrm{k}_i \quad (2)$$

For i = 0, a dummy level of +1 was used and the coefficient obtained was called A0. The standard error was determined as the sum of the squares of the difference between the experimental and predicted yield for each run. The estimated error is given by:

$$S_b = \sqrt{\frac{S_e^2}{N}} \quad (3)$$

The student's t-test was performed to determine the significance of each variable employed (t-value = coefficient/Sb). The statistics significance was evaluated using student's t-test and $P < 0.05$ was taken as significant. The program Statistical QI Macros SPC software was used to analyze the experimental PB design.

Table 1. Plackett–Burman design for screening of variables for β-carotene production by *Rhodotorula glutinis* ASU6(KU550702).

Variable	Unit	Level		
		Low (-)	Central point (0)	High (+)
A: Initial pH		4	5	6
B: Temperature	C°	25	30	35
C: Incubation time	h	48	72	96
D: Inoculums size	%	1	2	3
E: Fermentation type		Static	Shaking	Shaking
F: Onion waste (acid extract)	%	0	0	+
G: Onion waste (aqueous extract)	gl^{-1}	30	40	50
H: Yeast extract	gl^{-1}	0.5	1.5	2.5
I: KH_2PO_4 (g/l)	gl^{-1}	0.5	1.5	2.5
J: $MgSO_4 \cdot 7H_2O$	gl^{-1}	0.1	0.5	1
K: L-asparagine	gl^{-1}	0	0.12	0.5

2.5. RSM experimental design

Response surface methodology (RSM) was used to determine the optimum levels and the interaction amongst the significant screened variables for enhanced β-carotene production using the central composite design (CCD) [33]. The variables and its levels chosen were set based on the result of PB analysis, of which five variables were selected (temperature (X1), incubation time (X2), onion waste (X3), KH_2PO_4 (X4) and L-asparagine (X5). Each variable was evaluated at three coded levels (-1, 0, 1) as detailed in Table 2. The variables were set up for 56 experiments consisting of 54 experimental runs and 2 additional runs at the center point level. All the variables were taken at a central coded value (considered as zero).

The variables are coded for statistical calculation according to the following equation:

$$xi = Xi - X0/\delta X \quad (4)$$

Where xi is the dimensionless value of the independent variable; Xi is the real value of that independent variable; X0 is the real value of that independent variable at the center point; δX is the step change of real value of the variable i corresponding to a variation of a unit for the dimensionless value of the variable i.

The role of each five variables, their interaction and the response to obtain the predicated β-carotene yields is explained by the following fitting quadratic polynomial equation:

$$Y = \beta 0 + \sum \beta i\, xi + \sum \beta ii\, xi2 + \sum \beta ijxij \quad (5)$$

Where Y is predicted response; β0 is a constant; βi is linear effect; βii is squared effect; βijis the cross product effect; xi and xj the levels of the independent variables. This regression equation was optimized for optimal values using Sigma XL (Version 6.12). Statistical analysis of the model was performed to evaluate the analysis of variance (ANOVA) and the quality of fit for the regression model equation was expressed as R2. Response surface plots were developed to indicate the optimum level using the fitted polynomial equations obtained by putting one of the independent variables at a constant value and changing the levels of the other four variables. The software Sigma XL (Version 6.12) was used in this investigation.

Table 2. Independent variables and their levels for central composite design (CCD).

Levels of variable			Variable	Symbol
+1	**0**	**-1**		
35	30	25	Temperature (°C)	X_1
96	72	48	Incubation time (h)	X_2
50	40	30	Onion waste (acid extract) (g/l)	X_3
2.5	1.5	0.5	KH_2PO_4 (g/l)	X_4
1	0.55	0.1	L-asparagine (g/l)	X_5

2.6. β-carotene extraction and biomass estimation

Fermentation broth was sampled for analysis of yeast growth and β-carotene yield. Yeast cells were disintegrated using a mechanical disruption by shaking in a grinding mortar using liquid nitrogen until complete cell breakage occurred, then extracted using petroleum ether: acetone (1:1). Repeated extractions of β-carotene pigment were carried out with the above solvent until a colorless residue was obtained. The cell mass residue obtained after solvent extraction of carotenoids pigments was centrifuged at 4000 x g for 15 min, washed thoroughly with distilled water (twice), and dried at 40 °C overnight until getting constant dry weight [34].

2.7. Analytical analysis

The standard method of estimation of β-carotene is spectrophotometric [35]. Carotenoid concentrations in the pigmented layer were quantified spectrophotometerically at optical density of 450 nm. Standard graph was plotted for the concentrations varying from 2 to 12 µg/ml [36]. Also samples were analysis in Pharmaceutical

services center, Assiut University, Egypt. The color layer of organic phase containing β-carotene was separated, filtered through a 0.22 µm membrane and analyzed by HPLC (AYounglin Autochro-3000). The HPLC analysis was performed on a reversed phase C-18 column (Vensil XBP, 250 mm×4.6 mm, 5 µm, Waters, Aela Technologies, USA) with a detection wavelength at 450 nm using Young Lin UV/Vis detector (model UV730D) with dual wavelength detection (LTD, Korea). A mobile phase was composed of methanol and acetonitrile (90:10, v/v, Merck, Darmstadt, Germany) with a flow rate of 1.5 ml min^{-1}. β-carotene was used as an external standard eluted from the column at a retention time of 7.26 min. All data acquisition were performed on Young Lin Autochro-3000 software [37].

3. RESULTS

3.1. Isolation and identification of yeast strain

Twenty yeast isolates were recovered from different parts of maize, Egyptian clover, lemon, zucchini, banana, and spinach plants. The purified isolates were screened for their ability to produced β-carotene on fermented medium (Table 3). Only two isolates were selected for further characteristics based on the highest β-carotene production. The selected yeast isolates were used for molecular identification using phylogenetic analysis of 18S rRNA gene sequences. The sequence of approximately 577 base pairs of yeast isolate ASU 6 has sequence with 99% similarity to *Rhodotorula* glutinis AUMC 7774 (JQ425397). Also, the sequence of approximately 581 base pairs of yeast isolate ASU7 has sequence with 99% similarity to *Rhodotorula mucilaginosa* (KP223715). So, the yeast isolates were identified as *Rhodotorula* glutinis ASU6 (KU550702) and *Rhodotorula mucilaginosa* ASU7 (KU550703). Morphological and physiological characteristics of *R. glutinis* ASU6 (KU550702) and R. *mucilaginosa* ASU7 (KU550703) were determined and illustrated in Table 4. Only *R. glutinis* ASU6 (KU550702) was selected based on its highest β-carotene production (42.17 ± 0.4 mg/l) for further experiments (Fig. 1).

Table 3. Screening for β-carotene production on glucose fermented medium using different yeast isolates.

Isolate No.	Plant	β-carotene (mg/l)	Dry mass (g/l)
ASU 1	Egyptian clover	1.33 ± 0.16	0.44 ± 0.025
ASU 2	Spinach	0	0.19 ± 0.05
ASU 3	Banana	0	0.17 ± 0.02
ASU 4	Egyptian clover	0.39 ± 0.05	0.26 ± 0.02
ASU 5	Maize	8.0 ± 0.28	0.73 ± 0.06
ASU 6	Egyptian clover	42.17 ± 0.4	1.80 ± 0.09
ASU 7	Maize	40.89 ± 0.28	2.10 ± 0.048
ASU 8	Egyptian clover	20.11 ± 0.51	1.39 ± 0.029
ASU 9	Spinach	10.72 ± 0.35	1.48 ± 0.034
ASU 10	Zucchini	4.67 ± 0.44	1.09 ± 0.019
ASU 11	Maize	36.72 ± 0.35	2.27 ± 0.1
ASU 12	Spinach	18.50 ± 0.31	1.13 ± 0.014
ASU 13	Lemon	7.33 ± 0.36	0.91 ± 0.015
ASU 14	Maize	6.22 ± 0.12	0.79 ± 0.08
ASU 15	Maize	4.61 ± 0.18	0.74 ± 0.016
ASU 16	Lemon	1.22 ± 0.2	0.48 ± 0.012
ASU 17	Banana	17.61 ± 0.58	1.57 ± 0.014
ASU 18	Egyptian clover	0.56 ± 0.09	0.95 ± 0.09
ASU 19	Zucchini	0	0.70 ± 0.019
ASU 20	Spinach	0	0.97 ±0.03

Table 4. Phenotypic characterization of the selected yeast strains.

Parameter	*R. glutinis* ASU6 (KU550702)	*R. mucilaginosa* ASU7 (KU550703)
Shape	Ovoid	Rounded
Color	Orange-red	Red
Texture	Mucoid	
Surface	Smooth,glossy	Smooth
Margin	Entire	Entire
Budding	Multilateral	Multilateral
Formation of: Pseudohyphae	-	+
Formation of: Mycelium	-	-
Formation of: Arthroconidia	-	-
Formation of: Ascospore	-	-
Formation of: Ballistoconidia	-	-
Formation of: Basidiocarps, Teliospores, Basidia	-	-
Formation of: Chlamydospore	-	-
Formation of: Endospore	-	-
Glucose fermentation	-	-
Starch formation	-	-
Carbon source utilization: L-Arabinose	-	+
D-Fructose	+	+
D- Glucose	+	+
D-Galactose	+	+
Glycerol	+	+
Sucrose	+	+
Lactose	-	-
Maltose	+	+
Protease enzyme	-	+
Lipase enzyme	+	+
Cellulase enzyme	+	+
Gelatine hydrolysis	+	-
Growth at 4°C	-	-
Growth at 20°C	+	+
Growth at 35°C	+	+
Growth at 37°C	+	+

3.2. Effect of aqueous and acid extracts of different plant residues on *R. glutinis* ASU6 growth and β-carotene production

The effect of aqueous and acid extracts of different plant residues on β-carotene produced by *R. glutinis* ASU6 (KU550702) was illustrated in Fig. 2A, B. Ten plant wastes were tested as a carbon source for the growth of *R. glutinis* ASU6 and β-carotene production. The results indicated that acid extracted wastes promote fungal growth and produce a higher amount of β-carotene than aqueous extraction. Remarkably, yeast growth and β-carotene production were largely impressed by the type of plant waste. It is clear that an acid extract of onion waste represents the most efficient carbon source for β-carotene production (27.4 mg/l) followed respectively by potato peels (19.44 mg/l), peanut peels (18.44 mg/l) and wheat straw (14.88 mg/l). It is worthy to mention that date palm wastes, mango peels, peanut leaf wastes, rice straw and sugarcane wastes contribute

low production of β-carotene matching 1.56, 2.44, 0.72, 4.3, 4, and 1.83 mg/l β-carotene (aqueous extract) and 2, 5.61, 1.33, 5.06 and 8.94 mg/l β-carotene (acid extract), respectively. It is interesting to note that onion peels support better growth and β-carotene yield compared to other plant wastes tested. This could be ascribed to the enrichment of onion peels with minerals and carbohydrates (Table 5). The current study clearly proved that *R. glutinis* ASU6 (KU550702) could grow well on onion wastes and produced a large amount of β-carotene.

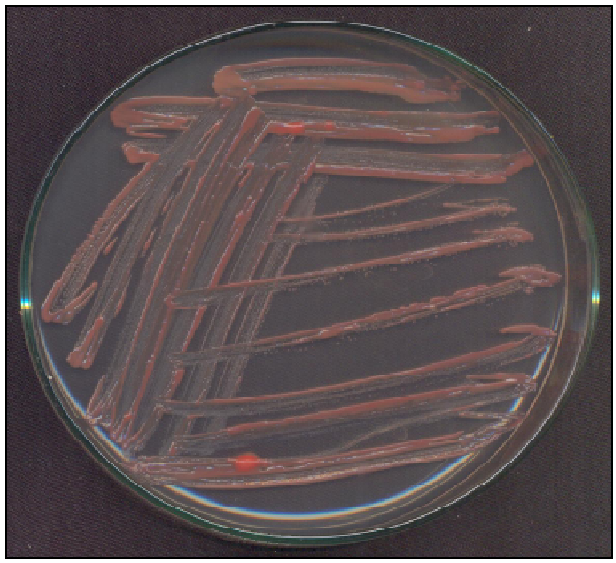

Figure 1. Growth of *R. glutinis* ASU6 (KU550702) on YME medium.

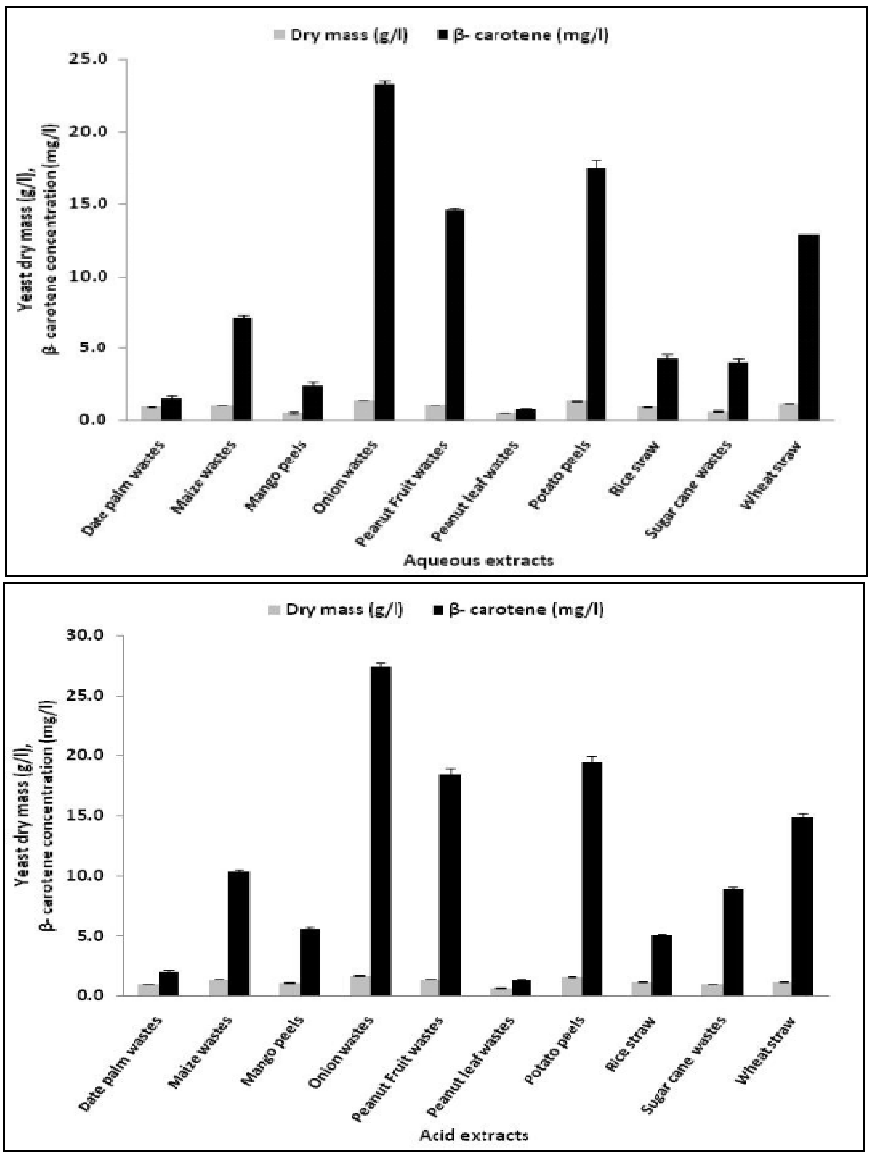

Figure 2. Effect of different waste materials on β-carotene production and biomass of *Rhodotorula glutinis* ASU6 (KU550702), (A) Non-treated waste materials, (B) Acid-treated waste materials.

Table 5. Chemical analysis of onion waste extract (OWE).

Parameter	Unit	Value
C/N Ratio		15
Nitrogen (N)	ppm	35
Phosphorus (P)	ppm	9.68
Potassium (K)	ppm	132
Sodium (Na)	ppm	369
pH		5.5
E.C.	ppm	1049.6
Total carbohydrates	mg/g dry weight	553.74

3.3. Plackett-Burman design

Plackett-Burman design was then employed to screen which variables have significant effects on β-carotene production. Eleven variables, including medium components and culture conditions were investigated (Table 6). All variables were taken at a central-coded value of zero, were screened in Plackett-Burman experiments. The results of the PB experimental design for 12 trials with two levels of each variable and the corresponding β-carotene concentrations were presented in table 6. The data showed that there were wide variations in β-carotene production from (4.5 to 37.3 mg/l) and also the dry mass (0.03 to 1.7 g/l).The maximum β-carotene production rate was achieved in the following conditions: temperature 35˚C, pH 6, incubation period 48 h, inoculums size 3%, acid extract onion waste material, KH_2PO_4 0.5 g/l, $MgSO_4$ 1 g/l, yeast extract 0.5 g/l and shaking rate at 150 rpm.

The experimental results proved that varying the initial cultivation pH of the medium between pH 4 and 6 had not significant effect on β-carotene production, while temperature had a significant effect on β-carotene production. Fermentation type showing increasing significant effect on vitamin production which indicates that equal aeration is very momentous in the fermentation process and those carotenogenesis is an aerobic process.

The effects of the variables on the response and significant levels were illustrated in Table 7. Based on the statistical analysis, some factors imposed the greatest impacts on the production of β-carotene by *R. glutinis* ASU6 (KU550702). These factors were identified as (B) temperature, (C) incubation time, (E) fermentation type, (F) acid onion waste extract, (I) KH_2PO_4 and (K) L-asparagine. These variables were selected for further investigation to optimize their levels by RSM.

3.4. Optimization of medium components and culture conditions by RSM

The variables showing significant effects in the Plackett-Burman design were selected and further optimized using CCD. Also, the levels of the factors chosen were based on data of the Plackett-Burman design. A complete five factor-three-level factorial design were used to optimize the effective parameters on β-carotene production.

The model gave the following regression equation for the β-carotene production RSM Regression Model: β-carotene (Y) (mg/l) = (127.04) + (-9.87) * X1 + (-12.9) * X2 + (4.03) * X3 + (3.79) * X4 + (-0. 98) * X5 + (-6.84) * X1X2 + (-0.30) * X1X3 + (5.95) * X1X4 + (2.71) * X1X5 + (-2.73) * X2X3 + (-6.84) * X2X4 + (9.96) * X2X5 + (0.4) * X3X4 + (-10.14) * X3X5 + (-17.46) * X4X5 + (-18.88) * X1X1 + (7.55) * X2X2 + (-33.31) * X3X3 + (1.27) * X4X4 + (50.98) * X5X5. (6)

Where, Y a function of temperature (X1), incubation time (X2), onion waste (X3), KH_2PO_4 (X4) and L-asparagine (X5).

Based on the experimental response, the values of β-carotene produced by *R. glutinis* ASU6 (KU550702) ranged from 85.71 to 204.29 mg/l. All the experimental data were located proximity to the predicted values of RSM model as shown in Fig. 3. These support that RSM model is sufficient to explain the data variations and to describe the actual relationships of variables to obtain the maximum β-carotene production. By using multiple regression analysis of the experimental data in Table 8, the coefficients of variables X1, X2, X3 and X4 were

found to be statistically significant at 95% confidence level. However, the variable X5 and interaction term X1X3, X3X4 and X4X4 of the model were found to be not significant of which the probability value higher than (0.05).

The statistical model was checked by F-test, and the analysis of variance (ANOVA) for the response surface quadratic model is summarized in Table 9. The adjusted R2 value was 98.53 %. The Model F-value of 94.34 implies that model is highly significant with very low probability value ($P < 0.0001$). Also, the lack-of-fit is not significant relative to the pure error ($P < 0.0001$). The R2 value, being a measure of the goodness of the match of the model, indicated that the 99.07 % of the entire variation was explained by the model.

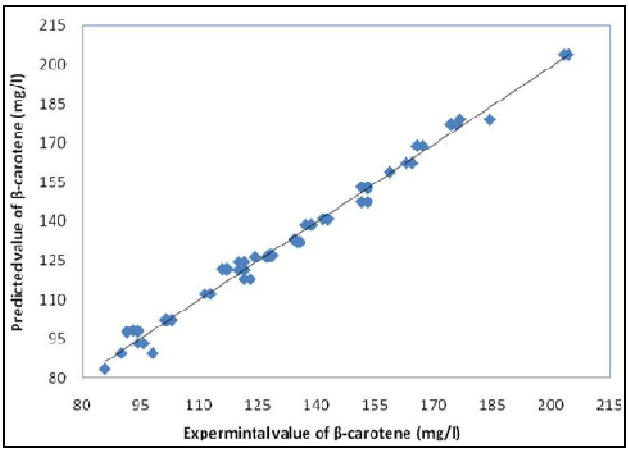

Figure 3. Comparison between β-carotene (mg/l) experimental and predicted values of the RSM model.

Table 6. Experimental design of Plackett-Burman with β-carotene production as response.

Trials	1	2	3	4	5	6	7	8	9	10	11	12
Initial pH	+	-	+	-	-	-	+	+	+	-	+	-
Temperature	+	+	-	+	-	-	-	+	+	+	-	-
Incubation time	-	+	+	-	+	-	-	-	+	+	+	-
Inoculums size	+	-	+	+	-	+	-	-	-	+	+	-
Fermentation type	+	+	-	+	+	-	+	-	-	-	+	-
Acid extract	+	+	+	-	+	+	-	+	-	-	-	-
Aqueous extract	-	+	+	+	-	+	+	-	+	-	-	-
Yeast extract	-	-	+	+	+	-	+	+	-	+	-	-
KH_2PO_4	-	-	-	+	+	+	-	+	+	-	+	-
$MgSO_4 \cdot 7H_2O$	+	-	-	-	+	+	+	-	+	+	-	-
L-asparagine	-	+	-	-	-	+	+	+	-	+	+	-
β-carotene (mg/l)	**37.29**	**32.80**	**4.50**	**29.25**	**11.80**	**4.65**	**17.63**	**25.25**	**14.25**	**25.97**	**12.75**	**9.08**
Dry mass (g/l)	**1.71**	**1.59**	**0.03**	**1.00**	**0.27**	**0.09**	**0.39**	**0.57**	**0.38**	**0.65**	**0.2**	**0.17**

Table 7. Statistical analysis of Plackett-Burman design for β-carotene production by *R. glutinis*.

Variable	Degree of freedom	β-carotene (mg/l)	
		% increase (+) or decrease (-)	Significance level
A - Initial pH	1	-0.31	0.98 NS
B - Temperature	1	17.4	< 0.05*
C - Incubation time	1	-3.51	< 0.05*
D - Inoculums size	1	0.60	0.73 NS
E - Fermentation type	1	9.64	< 0.05*
F - Onion waste: Acid extract	1	-3.18	< 0.05*
G - Onion waste: Aqueous extract	1	1.23	0.35 NS
H - Yeast extract	1	0.59	0.75 NS
I - KH_2PO_4 (g/l)	1	-4.89	< 0.05*
J - $MgSO_4.7H_2O$	1	-0.34	0.63 NS
K - L-asparagine	1	2.148	< 0.05*
Model	12	12.51	0.00 *

* Significant at $p \leq 0.05$, NS, non-significant at $p \geq 0.05$.

Table 8. Estimated regression coefficient, t-test and P values for optimization of β-carotene using of the central-composite design.

Factor	Coefficient	t value	P value
Constant	127.044	142.72	0.0000*
X_1: **Temperature**	-9.873	-16.389	0.0000*
X_2: **Incubation time**	-12.905	-21.422	0.0000*
X_3: **Onion waste**	4.0316	6.693	0.0000*
X_4: **KH_2PO_4**	3.794	6.297	0.0000*
X_5: **L-asparagine**	-0.984	-1.633	0.1113 N
$X_1 X_2$	-6.839	-10.704	0.0000*
$X_1 X_3$	-0.304	-0.475	0.6375 N
$X_1 X_4$	5.9463	9.306	0.0000*
$X_1 X_5$	2.7141	4.248	0.0002*
$X_2 X_3$	-2.732	-4.276	0.0001*
$X_2 X_4$	-6.839	-10.704	0.0000*
$X_2 X_5$	9.964	15.595	0.0000*
$X_3 X_4$	0.411	0.643	0.5247 N
$X_3 X_5$	-10.143	-15.874	0.0000
$X_4 X_5$	-17.464	-27.332	0.0000
$X_1 X_1$	-18.877	-11.559	0.0000
$X_2 X_2$	7.551	4.624	0.0000
$X_3 X_3$	-33.306	-20.394	0.0000
$X_4 X_4$	1.2654	0.774850	0.4436 N
$X_5 X_5$	50.979	31.217	0.0000

t - student's test, p - corresponding level of significance,* significant at $p \leq 0.05$, N, non-significant at $p \geq 0.05$

Table 9. Analysis of variance (ANOVA) for the selected quadratic model.

Source	Degree of freedom	Sum of squares	Mean square	F-value	P-value
Model	20	48486	2424.3	185.56	<0.0001
Error	35	457.25	13.064		
Lack of Fit	6	369.82	61.637	20.444	<0.0001
Pure Error	29	87.431	3.015		
Total (Model + Error)	55	48943	889.87		

In order to determine the optimal levels of each variable for maximum β-carotene production, the three-dimensional plots were constructed by plotting the response against each of the two independent variables, while maintaining the third variable at fixed (zero) level. The response surface plots of the effects of temperature (X1), incubation time (X2), onion peels (X3), KH_2PO_4 (X4) and L-asparagine (X5) on β-carotene production were illustrated in Fig. 4 (A-H). The patterns of the response surface plots indicate the nature and extent of the interactions. Additionally, from the bump of the three-dimensional plot, the optimal composition of the medium components was identified. Meanwhile, the optimal concentrations for enhancement the production of β-carotene by *R. glutinis* ASU6 (KU550702)were: onion waste 50 (g/l); yeast extract 1.5 (g/l); KH_2PO_4 2.5 (g/l); $MgSO_4 \cdot 7\ H_2O$ 0.5 (g/l), L-asparagine 0.1 (g/l), incubation for 48h. at 350˚C.

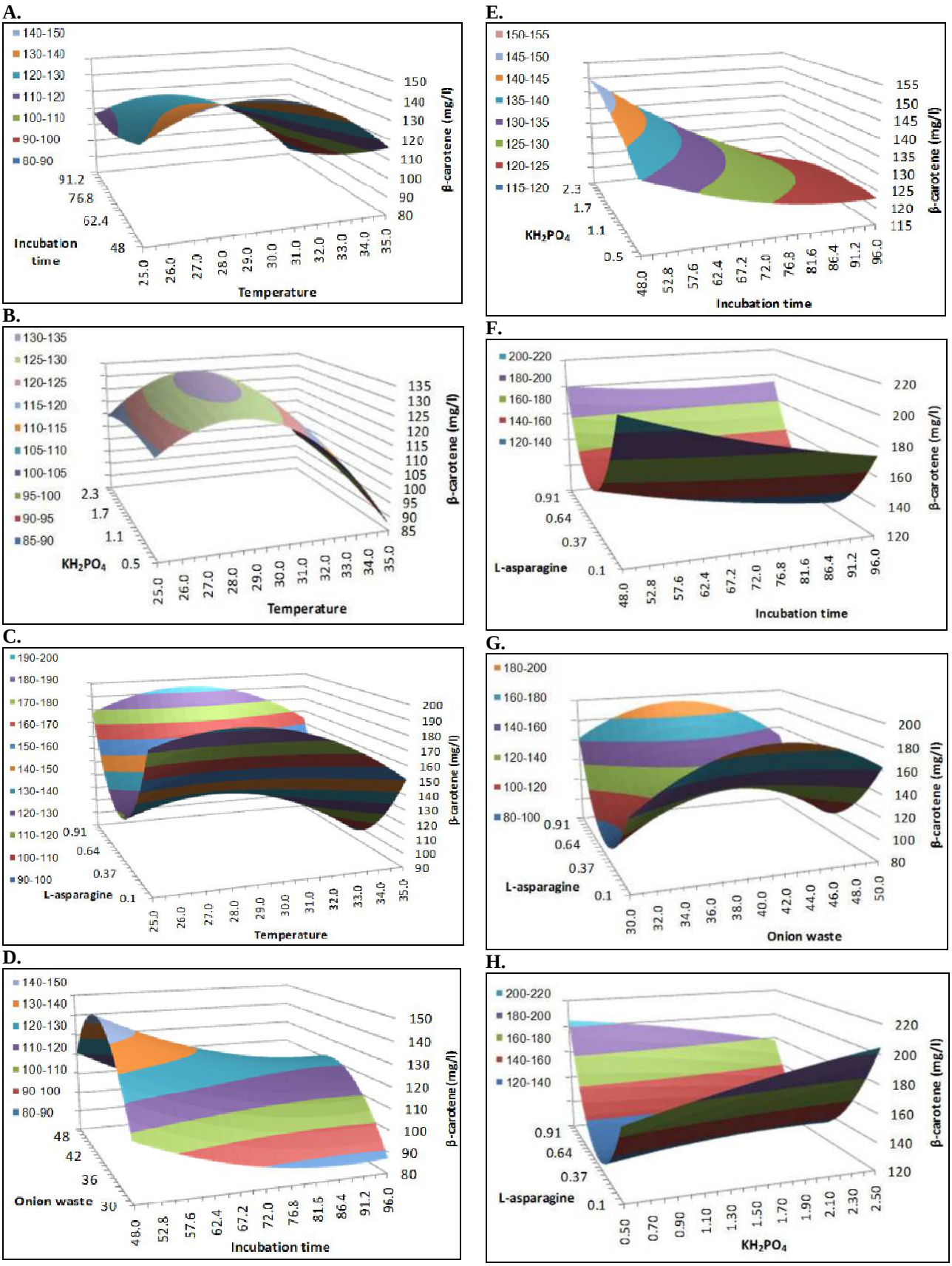

Figure 4. Response surface plots of β-carotene production by *Rhodotorula glutinis* (KU550702) showing the effect of two variables (other variables were kept at zero in coded unit): significant interaction between (A) Temperature and Incubation time (B) Temperature and KH_2PO_4, (C) Temperature and L-asparagine, (D) Incubation time and Onion waste, (E) Incubation time and KH_2PO_4, (F) Incubation time and L-asparagine, (G) Onion waste and L-asparagine, (H) KH_2PO_4 and L-asparagine.

4. DISCUSSION

Yeast growth and β-carotene production by *Rhodotorula* glutinis ASU6 (KU550702) were largely impressed by the type of plant waste. Acid extract of onion waste represents the most efficient carbon source for β-carotene production (27.4 mg/l). Pretreatment is a mandatory step before bioprocessing the lignocelluloses for further purposes in order to increase the digestibility of lignocelluloses structure, by increasing the bioavailability of the carbohydrates (cellulose and hemicellulose) for subsequent bioprocesses [38]. The liquid fraction of biomass from the pretreatment, which contains carbohydrates in polymeric and monomeric forms originating from the breakdown of hemicelluloses, is considered the most optimal solution. Nevertheless, due to the rich carbohydrate composition, this waste stream has the potential to be utilized as well as a nutritional source for cultivating red yeast strains, resulting in the production of different groups of metabolites [39].

The presence of a suitable carbon source is important for carotenoid biosynthesis. *Rhodotorula* species have potential commercial value as a dietary source of natural carotenoids; however, the high cost of production limits the use of these yeasts. Different agro-industrial raw materials that cause rigorous environmental problems may be possibly used as low-cost carbohydrate sources, with the perspective of minimizing production cost and environmental problems [40, 41]. Various natural substrates were tested as carbon sources for carotenoid production, such as: grape must [42]; hydrolyzed mung bean waste flour [43]; sugarcane and sugar-beet molasses [44, 45]; corn syrup [46]; milk whey [41].

Rhodotorula glutinis could produce 70 mg/l β-carotene after 48-h fermentation [47], 201 µg/l after 24-h fermentation on radish brine pH 6 [37]. *Sporobolomyces roseus* was produced 1.23-1.56 mg/g of β-carotene on pretreated wheat straw [39]. *Candida* utilis was produced 0.4 mg per g (dry weight) of cells [48]. *Saccharomyces cerevisiae* recorded the highest β-carotene yield (50.39 mg/l) on fermentation medium supplemented with glucose (1.40 mg/l/h) [49] and the yeast dry weight was 5.9 mg/g. The carotenoid-producing yeast *Xanthophyllomyces dendrorhous* was introduced and over expressed in *S. cerevisiae* [50]. The current study clearly proved that *R. glutinis* ASU6 (KU550702) could grow well on onion wastes and produced a large amount of β-carotene.

In this study, Plackett-Burman design was then employed to screen which variables have significant effects on β-carotene production. Wide variations in β-carotene production from (4.5 to 37.3 mg/l) and also the dry mass (0.03 to 1.7 g/l). The maximum β-carotene production rate was achieved in the following conditions: temperature 35˚C, pH 6, incubation period 48 h, inoculums size 3%, acid extract onion waste material, KH_2PO_4 0.5 g/l, $MgSO_4$ 1 g/l, yeast extract 0.5 g/l and shaking rate at 150 rpm. Carotenoid production depends on differences between strains of the same species and is strongly influenced by the cultivation conditions [41]. The red yeast is capable to grow under a wide range of initial pH conditions from 2.5 to 9.5 and over a wide range of temperatures from 5 to 26°C [51, 52]. Temperature is another important factor affecting the performance of cells and product formation. The effect of temperature depends on the species specificity of the microorganism and often manifests itself in quantity variations of synthesized carotenoids. It was reported that lower temperatures (25°C) seemed to favor synthesis of β-carotene and torulene, whereas higher temperatures (35°C) positively influenced torularhodin synthesis by *R. glutinis* [41, 42]. Fermentation type showing increasing significant effect on vitamin production which indicates that equal aeration is very momentous in the fermentation process and those carotenogenesis is an aerobic process. The effect of aeration is dependent on the species of the microorganism. The reported optimal values of air flow rate and agitation are in the range 0.5-1.9 l/min and 180-900 rpm for carotenogenesis in *Rhodotorula*. The aeration influenced not only the amount of carotenoids produced, but also the composition of individual pigments making up the total carotenoids. At higher aeration, the concentration of total carotenoids increased relative to the biomass and fatty acids in *R. glutinis*. In contrast, *Sporobolomyces roseus* responds to enhance aeration by a shift from the predominant β-carotene to torulene and torularhodin [41, 53].

In the present, variables showing significant effects in the Plackett-Burman design were selected

and further optimized using CCD. The values of β-carotene produced by *R. glutinis*ASU6 (KU550 702) ranged from 85.71 to 204.29 mg/l. The optimal concentrations for enhancement the production of β-carotene by *R. glutinis* ASU6 (KU550702) were: onion waste 50 (g/l); yeast extract 1.5 (g/l); KH_2PO_4 2.5 (g/l); $MgSO_4 \cdot 7\ H_2O$ 0.5 (g/l), L-asparagine 0.1 (g/l), incubation for 48 h at 35˚C. It was demonstrated the powerful advantage of RSM for the optimization of medium components and culture conditions to achieve vitamin production from microorganisms [54]. A face-centered central composite design was applied to optimize a cultivation condition for improved β-carotene production by *Rhodotorula* glutinis DM28 fermented radish brine as a sole substrate, yielded 2.7 g/l biomass and the maximum β-carotene of 201 µg/l after 24-h [37].

5. CONCLUSIONS

The current study proved that the possibility of utilizing onion peels as an economical source for yeast β-carotene production. The major objective of this research was the development of a statistical approach to modeling and optimization of conditions and medium composition for producing β-carotene. After two optimization stages the production increased with a 7.5-fold increase in β-carotene production (204.29 mg/l) compared to the production of the original level (27.4 mg/l) when using: onion peels 50 (g/l); yeast extract 1.5 (g/l); KH_2PO_4 2.5 (g/l); $MgSO_4 \cdot 7\ H_2O$ 0.5 (g/l), L-asparagine 0.1 (g/l), incubation for 48h at 35˚C which indicated that using statistical experimental designs is very effective in increasing production.

AUTHORS' CONTRIBUTION

MMKB: Supervision, revision and editing. MHA: Supervision, revision and editing. FMM: Participate in research design, revision and editing. NAN: Participate in research design, revision and editing. GAM: Designed, recorded the experimental data and wrote the research. The final manuscript has been read and approved by all authors.

TRANSPARENCY DECLARATION

The authors declare no conflicts of interest.

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