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An optimization study of carotenoid production by *Rhodotorula glutinis* DBVPG 3853 from substrates containing concentrated rectified grape must as the sole carbohydrate source

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A multivariate statistical approach was employed for the optimization of conditions for carotenoid production by *Rhodotorula glutinis* DBVPG 3853 from a substrate containing concentrated rectified grape must as the sole carbohydrate source. Several experimental parameters (carbohydrate, yeast autolysate and salt concentrations, and pH) were tested at two levels by following a fractional factorial design. Carotenogenesis was most sensitive to both initial pH and yeast autolysate concentration. A Central Composite Design experiment was then performed by obtaining both second-order polynomial models and isoresponse diagrams where initial pH and yeast autolysate concentration were considered as variables. In this way it was possible to determine the conditions (pH = 5.78, yeast autolysate = 4.67 g L^{-1}) which maximize both the concentration of total carotenoids and that of β-carotene (6.9 mg L^{-1} and 1100 μg L^{-1} of culture fluid, respectively, after 120 h of fermentation). *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 41–45.

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Introduction

The pharmaceutical, chemical and food industries have increased their interest in the use of carotenoids mainly as provitamin A (β-carotene) or as natural food and feed colorants [13,18,24,29]. Due to these widespread uses, the microbial production of carotenoids, when compared with extraction from vegetables [7] or chemical synthesis [8], seems to be of paramount interest mainly because of the problems of seasonal and geographic variability in the production and marketing of several of the colorants of plant origin [13], and because of the economic advantages of microbial processes using natural low-cost substrates as carbohydrate sources. Previous studies have shown the possibility of synthesising carotenoids from several by-products and residues of agro-industrial origin [9–11,19,20,26]. Also, recent world market trends might lead to a grape must surplus in certain European wine regions [20,21], suggesting the possibility of using this cheap raw material (otherwise difficult to dispose of) as substrate for microbial fermentations [4,20,21]. Even though the biosynthesis of carotenoids by *Rhodotorula* yeasts is well known [6,14,15,18,19,22,24,27,29], their industrial use is restricted mainly because of poor information on biosynthetic regulating mechanisms [15,18] and the lack of studies aimed both at increasing and maximizing the production of these pigments [13]. In recent years, the possibility of using multivariate statistical analysis in order to obtain a considerable increase in microbial production, through the mathematical modelling of biological processes, has been suggested [2,3].

The main purpose of this work was to perform an optimization study, with respect to several experimental variables, in order to increase production of carotenoids, over previous data [5], on the shake-flask scale, by *Rhodotorula glutinis* DBVPG 3853 from concentrated rectified grape must.

Materials and methods

Microorganism and culture conditions

A strain of *Rhodotorula glutinis* (DBVPG 3853) previously selected as a good carotenoid-producing yeast [5] was maintained on GMY agar [g L^{-1}: glucose 40, KH_2PO_4 8, $MgSO_4 \cdot 7H_2O$ 0.5, yeast autolysate 3.0 (ash 11.5%, P_2O_5 3.2%, total nitrogen 10.5%, soluble nitrogen 4.8%, nicotinic acid 1250 ppm, riboflavin 150 ppm, Oxoid, Basingstoke, UK), agar 15, pH 5.5] by subculturing every second week.

Cells grown for 24 h in 250-ml shaken flasks containing 50 ml of GMY, harvested by centrifugation ($6032 \times g$ for 10 min), washed and resuspended in 10 ml of sterile distilled water ($\approx 10^7$ cells ml^{-1}), were used as inocula (10% v/v) for fermentation studies.

Fermentation conditions

Flasks (250 ml) containing 50 ml of CGM-MY [concentrated grape must, CGM (820 g L^{-1} of total carbohydrates, tc) rectified through appropriate technologies [1,12] 49 ml L^{-1} (corresponding to g L^{-1} 40 of tc), KH_2PO_4 8, $MgSO_4 \cdot 7H_2O$ 0.5, yeast autolysate 3.0, pH 5.5], were

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placed on a heated rotary lab-shaker (160 rpm) model LT-V (Adolf Kuhner AG, Birsfelden, Switzerland) and incubated for 5 days at 30°C. No pH control was used during the processes. Modifications of medium composition according to factorial designs are listed in the Results section.

Analytical methods

Total carotenoids after extraction [19] were measured spectrophotometrically [9]. The major carotenoids (torularhodin, torulene, β-carotene) were quantified by HPLC [23]. Total carbohydrates were measured by a colorimetric method [16]. Yeast cell dry weight was determined as reported elsewhere [9].

Statistical analysis

All calculations were made by the computer software package Systat, ver. 5.03 [28].

Results

A $2^{(n-k)}$ fractional factorial design was performed to control five variables at two levels (according to the design matrix) with eight experiments ($2^{(5-2)}$): total carbohydrates (tc: 20–60 g L^{-1}), initial pH (pH: 4.5–6.5), yeast autolysate concentration (ya : 1.5–4.5 g L^{-1}), KH_2PO_4 concentration (K: 4.0–12.0 g L^{-1}) and $MgSO_4 \cdot 7H_2O$ concentration (Mg: 0.1–0.9 g L^{-1}) (Table 1). These variables were selected following the results of a previous study [5] where different media were used for carotenogenesis.

The measured responses were: C1 = total carotenoids volumetric concentration (mg of torulene L^{-1} of culture broth); C2 = total carotenoids cellular concentration (μg of torulene g^{-1} of cell dry weight); β-CAR = β-carotene volumetric concentration (μg L^{-1} of culture broth) (Table 1).

The information produced by the fractional factorial design was then converted by use of the Yates algorithm [3,17] into data for evaluating the relative importance of each variable. Initial pH and yeast autolysate concentration appeared to be the most relevant in determining the level of carotenogenesis (Table 2). These two variables were then used to collect further data according to a central composite design (Table 3).

Table 2 Estimated individual effects from the fractional factorial design

Variable	Effects on different responses[a]		
	(C1) 4.05[b]	(C2) 791.64[b]	(β-CAR) 826.2[b]
(tc)	–0.0925	92.375	52.5
(pH)	1.1875	247.275	252.5
(ya)	0.7375	–378.425	502.5
(K)	–0.0725	–28.925	112.5
(Mg)	–0.0625	–86.225	247.5

[a]Estimated effect of the variable variation (range –1 1) on the mean value of the individual response.
[b]Mean value.
Variables: (tc) total carbohydrates g L^{-1}; (pH) initial pH; (ya) yeast autolysate g L^{-1}; (K) KH_2PO_4 g L^{-1}; (Mg) $MgSO_4$ g L^{-1}.
Responses: (C1) total carotenoids mg L^{-1} culture fluid; (C2) total carotenoids μg g^{-1} dry cell weight; (β-CAR) β-carotene μg L^{-1} culture fluid.

Table 3 Results of the central composite design

Exp. No.	(pH)[a]		(ya)[a]		(C1)[b]	(C2)[b]	(β-CAR)[b]
1	–1	4.5	–1	1.5	3.72	442.9	400
2	1	6.5	–1	1.5	0.97	140.6	380
3	–1	4.5	1	4.5	4.59	527.6	1260
4	1	6.5	1	4.5	5.23	758.0	600
5	0	5.5	0	3.0	5.45	789.9	940
6	0	5.5	0	3.0	5.58	744.0	800
7	–1.414	4.09	0	3.0	2.35	559.5	00
8	1.414	6.91	0	3.0	5.16	860.0	1120
9	0	5.5	–1.414	0.88	0.90	157.9	300
10	0	5.5	1.414	5.12	6.85	1037.9	1200
11	0	5.5	0	3.0	5.51	734.7	1180
12	0	5.5	0	3.0	5.56	741.3	920

[a]Variable level; [b]individual response.
Variables: (pH) initial pH; (ya) yeast autolysate g L^{-1}.
Responses: (C1) total carotenoids mg L^{-1} culture fluid; (C2) total carotenoids μg g^{-1} dry cell weight; (β-CAR) β-carotene μg L^{-1} culture fluid.

The composite design was replicated twice, whereas the central point was replicated four times; the latter resulting in a total carotenoid production of 5.52 ± 0.06 mg L^{-1} of culture broth (variation degree ⩽2%). C1, C2 and β-CAR

Table 1 Matrix of fractional factorial design and individual responses

Exp. No.	(tc)[a]		(pH)[a]		(ya)[a]		(K)[a]		(Mg)[a]		(C1)[b]	(C2)[b]	(β-CAR)[b]
1	–1	20	–1	4.5	–1	1.5	1	12.0	1	0.9	3.27	726.7	280
2	1	60	–1	4.5	–1	1.5	–1	4.0	–1	0.1	3.94	1010.3	1190
3	–1	20	1	6.5	–1	1.5	–1	4.0	1	0.9	4.12	1056.4	570
4	1	60	1	6.5	–1	1.5	1	12.0	–1	0.1	3.39	1130.0	260
5	–1	20	–1	4.5	1	4.5	1	12.0	–1	0.1	3.66	488.0	1460
6	1	60	–1	4.5	1	4.5	–1	4.0	1	0.9	2.95	447.0	880
7	–1	20	1	6.5	1	4.5	–1	4.0	–1	0.1	5.33	710.7	890
8	1	60	1	6.5	1	4.5	1	12.0	1	0.9	5.73	764.0	1080

[a]Variable level; [b]individual response.
Variables: (tc) total carbohydrates g L^{-1}; (pH) initial pH; (ya) yeast autolysate g L^{-1}; (K) KH_2PO_4 g L^{-1}; (Mg) $MgSO_4$ g L^{-1}.
Responses: (C1) total carotenoids mg L^{-1} culture fluid; (C2) total carotenoids μg g^{-1} dry cell weight; (β-CAR) β-carotene μg L^{-1} culture fluid.

Table 4 Regression coefficients of the second-order polynomial equations

Coefficient	C1	C2	β-CAR
a_0	5.52[a]	752.47[a]	960.00[a]
a_1	0.23[b]	44.13[b]	112.99[b]
a_2	1.69[a]	243.33[a]	294.10[a]
a_{11}	–0.93[b]	–68.00[b]	–198.75[b]
a_{22}	–0.87[b]	–123.92[b]	–103.75[c]
a_{12}	0.84[b]	133.17[b]	–160.00[b]
r^2	0.88	0.83	0.79
SE	0.23	30.16	42.05
F	11.10[a]	14.57[a]	11.11[a]

(C1) total carotenoids mg L^{-1} culture fluid; (C2) total carotenoids μg g^{-1} dry cell weight; (β-CAR) β-carotene μg L^{-1} culture fluid.
[a]Highly significant ($P < 0.01$); [b]significant ($P < 0.05$); [c]not significant ($P > 0.05$).
SE, standard error.

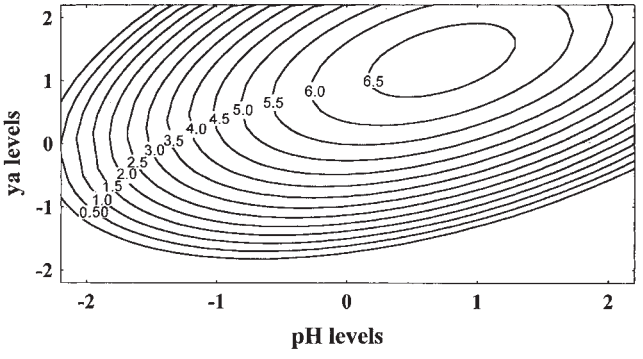

Figure 1 Isoresponse diagrams for response C1 (total carotenoids mg L^{-1} culture fluid) obtained by a central composite design with two variables (pH and ya). Curve numbers indicate response level as a function of initial pH and ya concentration (g L^{-1}).

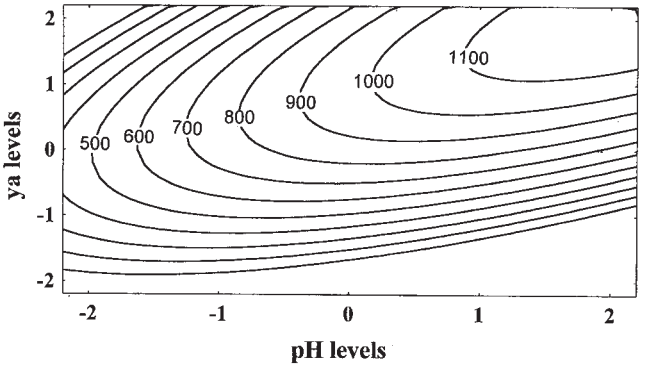

Figure 2 Isoresponse diagrams for response C2 (total carotenoids μg g^{-1} dry cell weight) obtained by a central composite design with two variables (pH and ya). Curve numbers indicate response level as a function of initial pH and ya concentration (g L^{-1}).

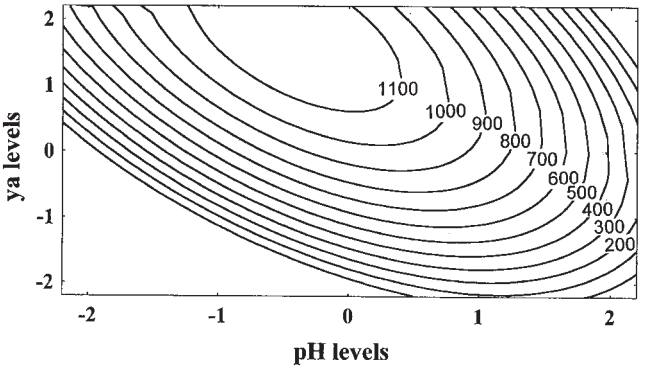

Figure 3 Isoresponse diagrams for response β-CAR (β-carotene μg L^{-1} culture fluid) obtained by a central composite design with two variables (pH and ya). Curve numbers indicate response level as a function of initial pH and ya concentration (g L^{-1}).

Table 5 Fermentation parameters of carotenoid production by *Rhodotorula glutinis* DBVPG 3853 under optimized conditions

Parameter	Days				
	1	2	3	4	5
Total carotenoids					
volumetric production (mg L^{-1})	0.73 ± 0.05	3.19 ± 0.1	4.88 ± 0.2	6.15 ± 0.4	6.97 ± 0.4
produced carotenoids (%)	10.4	45.7	70.1	88.4	100.0
volumetric production rate (mg L^{-1} day^{-1})	0.73	2.46	1.69	1.27	0.82
cellular production rate (μg g^{-1} cell dry weight day^{-1})	221	1295	3380	4233	2733
yield (μg g^{-1} sugar day^{-1})	60.3	543.4	528.1	1587	1025
Cell growth					
produced dry cell weight (%)	52.2 ± 2.1	83.7 ± 3.5	90.5 ± 5.2	95.1 ± 4.8	100.0
dry cell weight production rate (g L^{-1} day^{-1})	3.3	1.9	0.5	0.3	0.3
specific growth rate (g dry cell weight g^{-1} sugar day^{-1})	0.27	0.40	0.16	0.37	0.37
Total carbohydrates					
converted carbohydrates (%)	40.4 ± 3.7	56.1 ± 6.8	66.7 ± 5.4	69.2 ± 6.1	71.9 ± 7.1
carbohydrate utilization rate (g L^{-1} day^{-1})	12.1	4.7	3.2	0.8	0.8
pH	5.77	5.91	6.02	5.99	6.13

responses (Table 3) were then converted in a second-order polynomial equation [30]:

$$y = a_0 + a_1x_1 + a_2x_2 + a_{12}x_1x_2 + a_{11}x_1^2 + a_{22}x_2^2$$

where y = response, x_1 and x_2 = the considered variables, and a_{ij} a generic coefficient.

The equation coefficients and the ANOVA for all responses examined are given in Table 4. The levels of significance of the coefficients were evaluated and classified as reported elsewhere [25]. Isoresponse diagrams, calculated from the polynomial equations [2], were plotted as a function of initial pH and yeast autolysate concentration (Figures 1, 2 and 3).

The optimal conditions which maximize both C1 and C2 ranged from 6.35 to 6.75 and from 4.65 to 5.70 g L^{-1} for pH and ya, respectively (Figures 1 and 2), whereas the optimal conditions which maximize both C1 and β-CAR ranged from 5.67 to 5.90 and from 4.15 to 5.20 g L^{-1} for pH and ya, respectively (Figures 2 and 3). No interval range was found to simultaneously maximize all three responses (Figures 1, 2 and 3).

In order to confirm the validity of these findings, the time course of total carotenoid and β-carotene production (responses C1 and β-CAR) was followed for a time span of 120 h in GCM-MY medium at pH and ya levels fixed within the above reported intervals (5.78 and 4.67 g L^{-1}, respectively). The results of triplicate experiments are reported in Table 5.

Maximum carotenogenesis was observed within 24–72 h (maximum volumetric production rate within 24 and 48 h) whereas the highest dry cell weight production was measured in the course of the first 48 h (Table 5).

The ratio among the major carotenoid pigments was also quantified. At the steady-state conditions torularhodin was the major pigment synthesised (73% of total carotenoids) while torulene and β-carotene showed significantly ($P < 0.01$) different concentrations (7 and 17.9%, respectively).

Discussion

The main conclusion that can be made by observing data of fractional factorial design is the great importance of the initial pH and of the concentration of the yeast autolysate on carotenogenesis.

All linear and quadratic coefficients of polynomial models, obtained as a result of the central composite design (pH and ya as variables) showed statistically significant values (at least to the 95% confidence level), with the only exception of a_{22} for β-CAR (Table 4). Both linear terms a_1 and a_2 positively affected all examined responses; on the contrary, terms a_{11} and a_{22} showed negative values (Table 4). A positive influence was estimated only in the first two responses (C1 and C2) for the last term a_{12} (Table 4).

The polynomial models and isoresponse diagrams of C1, C2 and β-CAR were considerably different. The possibility of pin-pointing the optimal conditions to simultaneously maximize both total carotenoids and β-carotene volumetric concentration would be very interesting, as the great economic importance of the latter compound, currently used as natural yellow food colorant as well as provitamin A in pharmaceutical and food industries, is well known [18,24].

The concentration of both total carotenoids and β-carotene in the cultural broth was about 116 and 200% respectively of that previously obtained [5]. Furthermore, these productions were considerably higher than those observed for *Rhodotorula* yeasts either in other raw materials of agro-industrial origin [9,19,26] or in more conventional substrates [6,15,22,27].

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References

1 Amerine MA and WV Cruess. 1960. Winery by-products. In: The Technology of Wine Making (Amerine MA and WV Cruess, eds), pp 577–606, AVI Publishing Company, Westport, Connecticut.
2 Box G and NR Draper. 1987. Empirical Model-building and Response Surfaces. John Wiley & Sons, New York.
3 Box G, WG Hunter and JS Hunter. 1978. Statistics for Experimenters. John Wiley & Sons, New York.
4 Buzzini P, M Gobbetti, J Rossi and M Ribaldi. 1993. Utilization of grape must and concentrated rectified grape must to produce gluconic acid by *Aspergillus niger* in batch fermentations. Biotechnol Lett 15: 151–156.
5 Buzzini P and A Martini. 1999. Production of carotenoids by *Rhodotorula glutinis* DBVPG 3853 cultured in raw materials of agro-industrial origin. Biores Technol 71: 41–44.
6 Costa I, HL Martelli, IM da Silva and D Pomeroy. 1987. Production of β-carotene by a *Rhodotorula* strain. Biotechnol Lett 9: 373–375.
7 Coulson J. 1980. Miscellaneous naturally occurring colouring materials for foodstuff. In: Developments in Food Colour (Walford J, ed), pp 189–218, Applied Science Publishers, London.
8 Counsell JN. 1980. Some synthetic carotenoids as food colours. In: Developments in Food Colour (Walford, J, ed), pp 151–187, Applied Science Publishers, London.
9 Frengova G, E Simova, K Pavlova, D Beshkova and D Grigorova. 1994. Formation of carotenoids by *Rhodotorula glutinis* in whey ultrafiltrate. Biotechnol Bioeng 44: 888–894.
10 Frengova G, ED Simova and DM Beshkova. 1995. Effect of temperature changes on the production of yeast pigments co-cultivated with lactic acid bacteria in whey ultrafiltrate. Biotechnol Lett 17: 1001–1006.
11 Frengova G, ED Simova and DM Pavlova. 1997. Caroteno-protein and exopolysaccharide production by co-cultures of *Rhodotorula glutinis* and *Lactobacillus helveticus*. J Ind Microbiol Biotechnol 18: 272–277.
12 Giuliano R and ML Stein. 1989. Prodotti enologici. In: Quaderni di Chimica degli Alimenti (Giuliano R and ML Stein, eds), pp 13–20, Editore Bulzoni, Rome.
13 de Haan A, RM Burke and JAM de Bont. 1991. Microbial production of food colorants. Med Fac Landbouww Rijksuniv Gent 56: 1655–1660.
14 Hari RPK, T Patel and A Martin. 1992. A new strain of *Rhodotorula rubra* isolated from yogurt. J Ind Microbiol 11: 43–51.
15 Hayman EP, H Yokoyama, CO Chichester and KL Simpson. 1974. Carotenoid biosynthesis in *Rhodotorula glutinis*. J Bacteriol 120: 1339–1343.
16 Herbert D, PJ Phipps and RE Strange. 1971. Chemical analysis of microbial cells. In: Methods in Microbiology (Norris JR and DW Ribbons, eds), pp 209–344, Academic Press, London.
17 Himmelblau DM. 1970. Process Analysis by Statistical Methods. John Wiley & Sons, New York.
18 Johnson EA and WA Schroeder. 1996. Microbial carotenoids. In: Advances in Biochemical Engineering and Biotechnology (Fiechter A, ed), pp 119–178, Springer-Verlag, Berlin.

19 Martin AM, L Chun and TR Patel. 1993. Growth parameters for the yeast *Rhodotorula rubra* grown in peat extracts. J Ferm Bioeng 76: 321–325.
20 Meyer PS and JC du Preez. 1994. Astaxanthin production by a *Phaffia rhodozyma* mutant on grape juice. World J Microbiol Biotechnol 10: 178–183.
21 Moresi M and G Marchionni. 1982. Preliminary study on the operating variables of SCP production from grape must. Eur J Appl Microbiol Biotechnol 16: 204–207.
22 Nakayama T, G Mackinney and HJ Phaff. 1954. Carotenoids in asporogenous yeasts. Antonie van Leeuwenhoek 20: 217–228.
23 Nam HS, SY Cho and JS Rhee. 1988. High-performance liquid chromatographic analysis of major carotenoids from *Rhodotorula glutinis*. J Chromatogr 448: 445–447.
24 Nelis AH and AP de Leenheer. 1991. Microbial sources of carotenoid pigments used in foods and feeds. J Appl Bacteriol 70: 181–191.
25 Parente E, M Petruccioli, M Moresi and F Federici. 1992. Produzione di acido fumarico da sorgo zuccherino. Ann Microbiol Enzimol 42: 111–120.
26 Shih CT and YD Hang. 1995. Production of carotenoids by *Rhodotorula rubra* from sauerkraut brine. Lebensm Wiss u Technol 29: 570–572.
27 Sympson KI, TOM Nakayama and CO Chichester. 1964. Biosynthesis of yeast carotenoids. J Bacteriol 88: 1688–1694.
28 Systat® 1991. User's guide. Ver. 5.03, Evanstone, IL, USA.
29 Vandamme EJ. 1992. Production of vitamins, coenzymes and related biochemicals by biotechnological processes. J Chem Tech Biotechnol 53: 313–327.
30 Wold S, C Albano, WJ Dunn, U Edlund, K Ebensen, P Geladi, S Hellberg, E Johansson, W Lindberg and M Sjostrom. 1984. Multivariate data analysis in chemistry. In: Chemometrics (Kowalski BR, ed), pp 17–96, Reidel, Dordrecht, The Netherlands.