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ORIGINAL PAPER

Production and optimization of carotenoid-enriched dried distiller's grains with solubles by *Phaffia rhodozyma* and *Sporobolomyces roseus* fermentation of whole stillage

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Abstract Whole stillage—a co-product of grain-based ethanol—is used as an animal feed in the form of dried distiller's grain with solubles (DDGS). Since animals cannot synthesize carotenoids and animal feed is generally poor in carotenoids, about 30–120 ppm of total carotenoids are added to animal feed to improve animal health, enhance meat color and quality, and increase vitamin A levels in milk and meat. The main objective of this study was to produce carotenoid (astaxanthin and β-carotene)-enriched DDGS by submerged fermentation of whole stillage. Mono- and mixed cultures of red yeasts, *Phaffia rhodozyma* (ATCC 24202) and *Sporobolomyces roseus* (ATCC 28988), were used to produce astaxanthin and β-carotene. Media optimization was carried out in shake flasks using response surface methodology (RSM). Macro ingredients, namely whole stillage, corn steep liquor and glycerol, were fitted to a second-degree polynomial in RSM. Under optimized conditions, astaxanthin and β-carotene yields in mixed culture and *P. rhodozyma* monoculture were 5 and 278, 97, and 275 μg/g, respectively, while *S. roseus* produced 278 μg/g of β-carotene. Since the carotenoid yields are almost twice the quantity used in animal feed, the carotenoid-enriched DDGS has potential application as "value-added animal feed or feed blends."

Keywords Astaxanthin · β-Carotene · Red yeasts · Value addition

Introduction

Dried distiller's grain with solubles (DDGS) is a co-product of grain-based ethanol. Due to a three-fold increase in the number of ethanol plants in the US [37], the production of ethanol co-products has increased, with DDGS production measuring around 10 million metric tonnes [40]. DDGS is used as livestock and poultry feed, since it is rich in fiber, protein, water-soluble vitamins and minerals [11]. During ethanol fermentation of corn, *Saccharomyces cerevisiae* utilizes glucose derived from corn starch, leaving the fiber untouched. In fact, the fiber concentration in DDGS is enhanced by a factor of three compared to corn [11]. Due to its nutrition profile, whole stillage makes an excellent substrate for secondary fermentation and offers unlimited opportunities for value addition.

Astaxanthin and β-carotene are important carotenoids that are added to animal feed as they provide numerous health benefits to animals [6, 20, 31, 40, 44]. Animals are incapable of producing carotenoids, but are able to assimilate the ingested carotenoids [14]. Accordingly, carotenoids are added to animal feeds at dosages ranging from 1 to 120 mg/kg feed [3, 17, 43]. Usually animal feeds are poor in carotenoids [19, 32], and DDGS is no exception. Whole stillage, though abundantly produced, has not been used as a substrate for carotenoid production.

Astaxanthin is commonly produced by red yeast *Xanthophyllomyces dendrorhous* (Ex: *Phaffia rhodozyma*) on various substrates [16] and accounts for 80–90% of its total carotenoids [38]. β-carotene is also produced by *P. rhodozyma*. However, yeasts like *Rhodotorula glutinis*

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and *Sporobolomyces roseus* produce abundant β-carotene [25]. Apart from monoculture fermentation, mixed culture fermentation or co-cultivation of microorganisms has also been employed for enhanced carotenoid production. Dong and Zhao [12] co-cultivated two astaxanthin-over-producing strains, namely *P. rhodozyma* and the micro-alga *Haematococcus pluvialis*, and found that the astaxanthin yield was higher than that in the respective monocultures. Similarly, co-cultivation of an *Aspergillus* sp. or the incorporation of its dried extract (80 μg/ml) into the fermentation of *Phycomyces blakesleeanus* resulted in a fivefold increase of β-carotene [27]. In lieu of co-cultivation, addition of fungal culture filtrates (extracts) has enhanced carotenoid production. *Epicoccum nigrum* extract [13] and extracts from *R. glutinis* and *Rhodotorula rubra* [44] enhanced astaxanthin production of *X. dendrorhus*. Addition of regular yeast extract to the fermentation of *P. rhodozyma* improved astaxanthin production [28, 29]. Though the specific carotenoid triggering mechanism is unknown, it is believed that some of the biochemical intermediates of the co-cultured fungus may serve as precursors and/or elicitors in carotenoid-producing microbes.

Optimization of astaxanthin production by *P. rhodozyma* has been achieved by altering physical factors like temperature, aeration, pH, light and media components like C source, C/N ratio, minerals, and nitrogen source. Most optimization studies have relied on powerful statistical designs and response surface methodology [34, 39, 41]. For example, suggested optimum temperatures are 15, 18, 19.7 or 22°C [15, 23, 28, 36, 41], and pH values are 4.0–7.0 [15] or 5.0, 6.0, and 6.9 [28, 36, 41]. Positive influences of organic N sources like yeast extract, beef extract or peptone [1, 15, 36] or inorganic N sources like urea, KNO_3, ammonium salts [1, 15, 30, 33] are well documented. Optimization of whole stillage fermentation to produce carotenoid-enriched DDGS is a necessity for producing cost-effective value-added animal feed.

Since astaxanthin constitutes 80–100% of the total carotenoids in *P. rhodozyma* and the chosen *S. roseus* strain produces β-carotene exclusively, we hypothesized that (1) co-cultivation of these two yeasts would allow the production of carotenoid-enriched DDGS rich in both astaxanthin and β-carotene, and (2) co-cultivation would enhance the carotenoid yields of the respective red yeasts due to stimulatory effects of the co-cultured yeast. Since DDGS is rich in fiber and *P. rhodozyma* is known to degrade corn fiber [20], we chose a strain of *P. rhodozyma* that not only produces astaxanthin but also metabolizes xylose. Specifically, our objectives were: (1) to produce carotenoid-enriched whole stillage by monoculture and mixed culture fermentation of *P. rhodozyma* and *S. roseus*, and (2) optimization of medium ingredients using response surface methodology.

Materials and methods

Microbial cultures

Lyophilized cultures of *Phaffia rhodozyma* (ATCC 24202) and *Sporobolomyces roseus* (ATCC 28988) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), revived on yeast extract malt extract agar (YMA), and incubated at 18°C for 10 days. After revival, cultures were inoculated into yeast extract malt extract broth (YMB) and incubated at 18°C on an orbital shaker at 180 rpm for 5 days. Cultures were then inoculated on YMA slants, incubated for 10 days, and later stored at −80°C for long-term preservation. Additionally, yeast cells from YMB were centrifuged and resuspended in 20% glycerol and stored at −80°C in one ml aliquots. For routine experiments, freshly prepared slants were used. *Phaffia rhodozyma* ATCC 24202 is a known carotenoid producer and has xylose-metabolizing ability [29, 41].

Inoculum generation

From each fungal strain, a loopful of cells from respective slants was inoculated into sterile 100 ml YMB in 500 ml flasks. Flasks were incubated at 18°C and 180 rpm for 72 h. The development of orange and red color in *P. rhodozyma* and *S. roseus* flasks, respectively, indicated good fungal growth. A 10% (v/v) inoculum was used for monoculture fermentation, while 5% of each strain was used in mixed culture fermentation.

Media preparation

Corn whole stillage was procured from Abengoa Bioenergy (Colwich, KS, USA). Apart from whole stillage, the medium consisted of glycerol and corn steep liquor. The supplementation with glycerol and corn steep liquor was considered necessary, as (1) whole stillage is poor in readily utilizable sugars, and the addition of glycerol and corn steep liquor provides readily available carbon and reduces the lag phase, (2) glycerol can act as a carbon source for astaxanthin production by *P. rhodozyma* [24], (3) carotenoid production is increased by the balanced and increased formation of acetyl CoA, pyruvate and glyceraldehyde-3-phosphate, all of which can be produced by the glycolysis of glycerol [7], and (4) glycerol is a cheap and abundantly produced co-product of the biodiesel and soap

Table 1 Macro ingredient variables and their levels tested in a central composite design

Factor	Nutrient (g/l)[a]	Low actual (−1)	Mean (0)	High actual (+1)	+α	−α
A	WSL	150	325	500	619.314	30.68
B	CSL	15	32.5	50	61.93	3.068
C	GLY	30	65	100	6.13	123.86

[a] *WSL* whole stillage, *CSL* corn steep liquor, *GLY* glycerol

industry, and was found to be an effective supplement for β-carotene production by *B. trispora* [26].

Initial medium composition

A liter of the fermentation medium contained 25% whole stillage, 2% corn steep liquor, 5% glycerol and minerals: 1 g KH_2PO_4, 0.5 g $MgSO_4$, 0.5 g $MnSO_4$ and $ZnSO_4$.

Optimized medium

A liter of the fermentation medium contained 15% whole stillage, 1.5% corn steep liquor, 7.7% glycerol and mineral salts (0.6 g KH_2PO4, 0.3 g $MgSO_4$, 0.3 g $MnSO_4$ and 0.7 g $ZnSO_4$).

In both cases, the media pH was about 6.0 before sterilization and was not adjusted any further. Flasks with 50 ml of whole stillage medium were sterilized at 121°C for 30 min.

Fermentation conditions

Submerged fermentations of *P. rhodozyma* and *S. roseus* mono- and mixed cultures were conducted. Flasks were inoculated and incubated at 18°C, 180 rpm for 9 days. Control flasks without inocula were maintained. Samples were harvested on days 5, 7, and 9 of fermentation, centrifuged, and the supernatant was discarded. Pellets were freeze-dried for 24 h and stored at −80°C until further analysis. Two replicates per treatment were employed.

Media optimization

Response surface methodology was used to optimize the major ingredients (i.e., whole stillage, glycerol and corn steep liquor). Design Expert 7.1. 6 (Stat-Ease Inc., Minneapolis, MN, USA) was used to generate experimental designs, estimate the responses of dependent variables, and generate the contour and/or response surface plots.

Response surface methodology

The three independent variables and their levels for a rotatable central composite design (CCD) are given in Table 1. The CCD consisted of six central points and 14 noncentral points. The experiment consisted of 20 runs with no blocking, and the design matrix is provided in Table 2. The relation between coded and actual values is

$$x_i = \frac{X_i - X_0}{\Delta X}, \quad (1)$$

where x_i is the coded value of the independent variable (x_1 = whole stillage, x_2 = corn steep liquor, x_3 = glycerol), X_i is the real value of the independent variable, X_0 is the real value of the independent variable at the center point, and ΔX is the step change value.

The relationship between independent variables and dependent variables was obtained as the sum of the contributions of the three factors through first-order, second-order and interaction terms according to the following quadratic polynomial function:

$$Y = \beta_0 + \sum_{i=1}^{k} \beta_i X_i + \sum_{i=1}^{k} \beta_{ii} X_i X_j + \sum i \sum_{i<k} j \beta_{ij} X_i X_j, \quad (2)$$

where Y is the predicted response, β_i is the linear coefficient, β_{ii} is the squared coefficient, β_{ij} is the interaction coefficient, and k is the number of factors.

Data were square-root transformed prior to analyses, as the min:max ratio was greater than 10. The astaxanthin and β-carotene produced were the two response variables.

Only *P. rhodozyma* monoculture was used for optimization, since (i) it produces both astaxanthin and β-carotene, (ii) astaxanthin is a high-value product, and (iii) optimization of all treatments is laborious due to the volume of experiments.

Validation of optimized conditions

The optimized medium was formulated based on the RSM results and used for validation. Both monoculture and mixed culture fermentations were carried out using the optimized medium. Since both *P. rhodozyma* and *S. roseus* are red yeasts, we assumed that the optimal medium for the former would be applicable for the latter and also for their mixed culture. Three replications were carried out per treatment. Fermentation conditions, sample collection and data analyses were carried out as previously described.

Table 2 Experimental design matrix for macro ingredients and carotenoid yields

Run	A (g/l)	B (g/l)	C (g/l)	Astaxanthin (μg/g)		β-Carotene (μg/g)	
				Actual	Predicted	Actual	Predicted
1	150	50	100	18.15	17.81	196.28	181.81
2	325	61.93	65	45.76	40.10	192.87	140.65
3	30.69	32.5	65	83.95	78.81	296.89	249.30
4	500	15	100	38.23	30.82	192.95	135.91
5	500	15	30	63.94	55.20	66.42	57.21
6	325	32.5	65	56.40	59.14	139.79	160.57
7	500	50	30	39.56	48.20	73.28	88.46
8	325	32.5	65	68.58	59.14	152.93	160.57
9	619.31	32.5	65	48.54	42.29	115.80	91.28
10	325	32.5	65	67.36	59.14	139.18	160.57
11	325	3.07	65	67.64	81.86	136.60	181.80
12	325	32.5	65	58.64	59.14	131.96	160.57
13	150	15	30	79.78	78.17	100.90	88.00
14	325	32.5	123.86	18.30	17.72	102.80	111.62
15	150	15	100	47.50	48.48	288.67	297.78
16	150	50	30	74.10	69.79	102.06	125.94
17	500	50	100	5.17	7.89	37.34	62.19
18	325	32.5	6.14	41.26	42.74	31.87	27.25
19	325	32.5	65	48.94	59.14	154.35	160.57
20	325	32.5	65	47.89	59.14	175.48	160.57

Carotenoid extraction, analyses and identification

A known amount of freeze-dried sample was weighed into a mortar, 0.2 g of acid-washed sand (40–100 mesh size) were added, and carotenoids were extracted by grinding the mixture in dichloromethane solvent. Samples were centrifuged at 5,000 rpm for 5 min and the supernatant was filtered into 1.5 ml HPLC vials using 0.2 μm filters.

High-performance liquid chromatography (HPLC) was used to quantify carotenoids. Astaxanthin and β-carotene standards were obtained from Sigma–Aldrich (St Louis, MO, USA). A Shimadzu HPLC equipped with an LC-20AB pump, a SIL-20AC autosampler, an SPD-M20A PDA detector and a CTO-20A column oven was used. A Phenomenex Prodigy C_{18} column (150 mm length and 4.6 mm internal diameter) along with a C_{18} guard column were used to separate carotenoids. Acetonitrile:methanol (80:20) were used as the mobile phase. Flow rate was maintained at 2.0 ml/min and the column was maintained at 40°C. About 20 μl of the sample were injected using the autosampler. HPLC data were acquired using Lab Solutions software. Carotenoid yield was expressed as μg/g of freeze-dried whole stillage sample instead of yield per gram of yeast dry weight, as it was impossible to separate yeast cells from the whole stillage solids. Total carotenoids were calculated as the sum of astaxanthin and β-carotene yields.

Identification of purified carotenoids was carried out by MALDI/TOF MS (using a Bruker Ultraflex II TOF/TOF mass spectrometer) and proton NMR (on a Varian Inova, 400 MHz).

Statistical analyses

Data before and after validation were analyzed using SAS (version 9.1.3). PROC GLM was used to compare multiple treatments, and (when necessary) pair-wise comparisons were made using Tukey–Kramer at $P = 0.05$. Optimization data were analyzed by Design Expert 7.1.6.

Results

Both *P. rhodozyma* monoculture and mixed culture fermentations produced whole stillage enriched in astaxanthin and β-carotene, while *S. roseus* monoculture produced only β-carotene-enriched whole stillage.

Production profile of astaxanthin and β-carotene before optimization

The ANOVA statistics for the astaxanthin production profile are provided in Table 3. The astaxanthin yields on days 5, 7, and 9 of fermentation by *P. rhodozyma* and

Table 3 Carotenoid yields (μg/g of freeze-dried whole stillage) from unoptimized medium

Carotenoids[a]	Treatment[b]	Day 5	Day 7	Day 9
Astaxanthin	Mx	11.26 ± 0.8^{B}	12.59 ± 0.49^{B}	17.41 ± 0.17^{A}
	PR	25.95 ± 2.9	31.21 ± 0.99	35.73 ± 1.64
	SR	–	–	–
β-Carotene	Mx	135.58 ± 5.12^{B}	135.92 ± 3.74^{B}	187.89 ± 0.6^{A}
	PR	76.28 ± 8.95	89.92 ± 4.48	104.72 ± 4.96
	SR	149.97 ± 1.34^{C}	192.72 ± 4.98^{B}	232.99 ± 2.55^{A}
Total	Mx	146.84^{B}	148.51^{B}	205.3^{A}
	PR	102.33	121.13	140.45
	SR	–	–	–

[a] Means and standard errors are provided; significance was set at $P \leq 0.05$. ANOVA: astaxanthin: Mx-F = 33.0, $P = 0.0091$; PR-F = 5.89, $P = 0.0914$; β-carotene: Mx-F = 66.85, $P = 0.0033$; PR-F = 4.86, $P = 0.1145$; SR-F = 155.67, $P = 0.0009$; total carotenoids: Mx-F = 89.73, $P = 0.0021$; PR-F = 5.11, $P = 0.1082$; significantly different treatments across days do not share a letter (uppercase)

[b] *Mx* mixed culture, *PR P. rhodozyma*, *SR S. roseus*

mixed culture, respectively, showed an increasing trend. Astaxanthin yield in *P. rhodozyma* fermentation did not vary over time (Table 3), but the mixed culture fermentation yield on day 9 was significantly greater than those on days 5 and 7 (Table 3).

The ANOVA statistics for the β-carotene production profile are provided in Table 3. β-Carotene yields in all three treatments showed an increasing trend: *S. roseus* yields on days 5, 7, and 9 were significantly different from each other, *P. rhodozyma* did not vary significantly, and the yield from the mixed culture fermentation was the greatest on day 9 and significantly different from those on days 5 and 7 (Table 3).

Optimization

Response surface methodology

A central composite design of 20 experiments was carried out to evaluate the effect of three independent macro ingredients on astaxanthin and β-carotene production. A second-order polynomial equation was used to correlate the independent variables with astaxanthin and β-carotene production, respectively. The actual and predicted values of the response variables are provided in Table 2.

Table 4 provides the ANOVA for astaxanthin production. The model was significant, with an F value of 26.02. The coefficient estimates and their corresponding P values suggest that all of the variables and the interaction of glycerol and corn steep liquor are significant. The different variables were correlated with astaxanthin production by multiple regression according to Eq. 2. The final equation in coded terms is given below:

$$\text{Sqrt(Astaxanthin)} = 7.69 - 0.71 \times A - 0.81 \times B - 1.50 \times C - 0.56 \times B \times C - 1.30 \times C^2. \quad (3)$$

The R^2 value for Eq. 2 was 0.91, indicating that 91% of the variation in astaxanthin production is explained by the quadratic polynomial.

Table 4 Astaxanthin and β-carotene responses from RSM: ANOVA for response-surface reduced quadratic model

Carotenoid	Source	Coefficient estimate	SE	Sum of squares	*df*	*F* value	*P*-value[a] Prob > *F*
Astaxanthin	Model or intercept	7.69	0.17	44.16	5	26.02	**<0.0001**
	A	−0.71	0.16	6.81	1	20.05	**0.0006**
	B	−0.81	0.16	8.90	1	26.23	**0.0002**
	C	−1.50	0.19	20.81	1	61.31	**<0.0001**
	BC	−0.56	0.21	2.54	1	7.49	**0.0169**
	C^2	−1.30	0.20	14.35	1	42.28	**<0.0001**
β-Carotene	Model or intercept	12.67	0.40	149.39	6	12.53	**<0.0001**
	A	−1.85	0.38	46.93	1	23.61	**0.0003**
	B	−0.48	0.38	3.18	1	1.60	0.2280
	C	1.59	0.38	34.48	1	17.35	**0.0011**
	AC	−0.95	0.50	7.14	1	3.60	0.0804
	BC	−1.40	0.50	15.75	1	7.93	**0.0146**
	C^2	−1.69	0.37	41.89	1	21.07	**0.0005**

[a] Significant P values (<0.05) are shown in boldface

The response surface and contour plots for astaxanthin production were generated. At the optimal point for the corn steep liquor, three-dimensional plots of two factors, whole stillage and glycerol, versus astaxanthin production were drawn along with the corresponding contour plot (Fig. 1). Based on Eq. 3, and confirmed by the contour plot, all three variables negatively influenced the astaxanthin production, indicating that lower concentrations of these ingredients in the medium would result in a higher production of astaxanthin. According to the contour plot, the mean astaxanthin production was 78 μg/g of the freeze-dried whole stillage (Fig. 1).

ANOVA results for β-carotene are shown in Table 4. The significance of the model is indicated by $F = 12.53$. The estimates for the coefficients and the corresponding P values are provided in Table 4. The final equation in coded terms after multiple regression analysis was

$$\text{Sqrt}(\beta\text{-carotene}) = 12.67 - 1.85 \times A - 0.48 \times B + 1.59C - 0.95 \times A \times C - 1.40 \times B \times C - 1.69 \times C^2. \quad (4)$$

The goodness of fit for Eq. 3 is given by the coefficient of determination, R^2, which was found to be 0.85, indicating that 85% of the variability in β-carotene production is explained by the model.

Just as done for astaxanthin production, response surface and contour plots were generated for β-carotene (Fig. 2). From Eq. 4 and the contour plot, β-carotene production was negatively influenced by whole stillage and positively by glycerol. Corn steep liquor negatively influenced β-carotene production, although it was not statistically significant. However, the interaction of glycerol and corn steep liquor had a significant effect. The mean β-carotene production as seen in the contour plot was 257 μg/g of freeze-dried whole stillage (Fig. 2). Overall, the optimal medium constituents were 150 g/l of whole stillage, 15 g/l of corn steep liquor, and 7.7 g/l of glycerol.

Minerals like Zn, Mn, Mg, and K were optimized using a mixture design. Even though the minerals had a positive

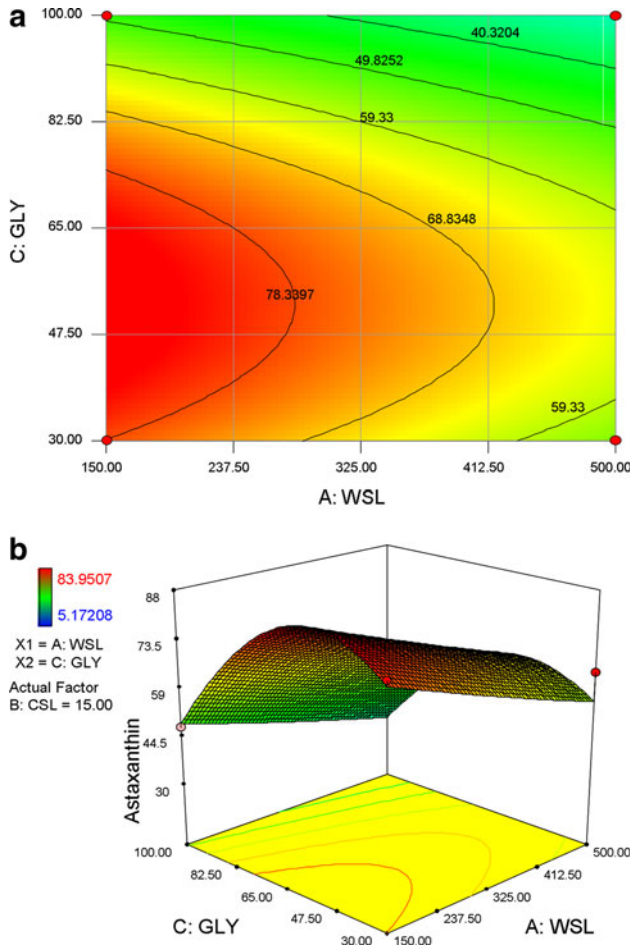

Fig. 1 **a** Response surface and **b** contour plots for astaxanthin using the macro ingredients of whole stillage, corn steep liquor and glycerol

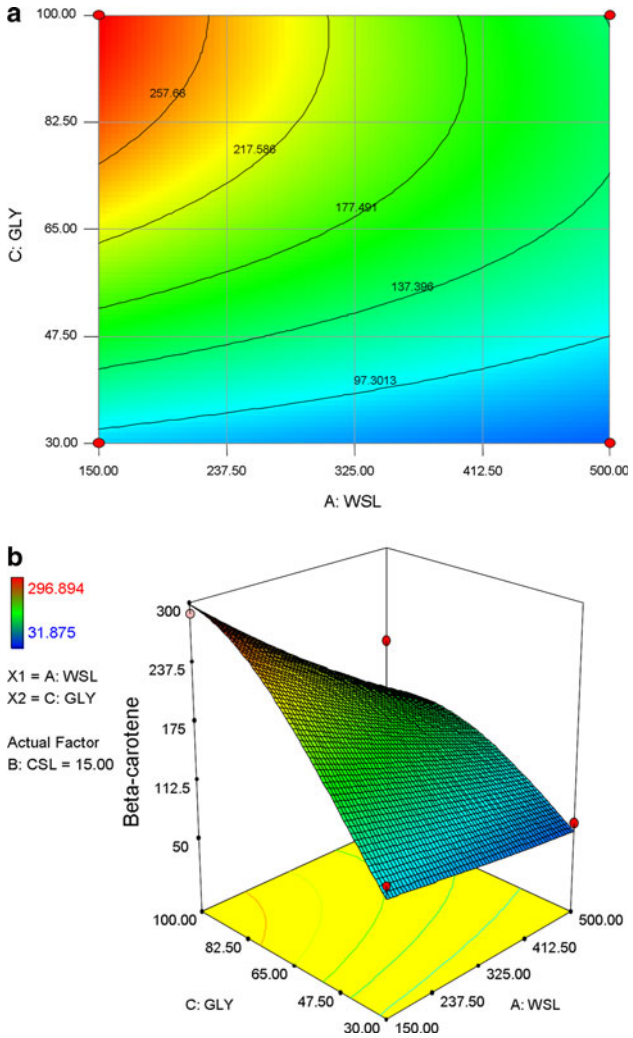

Fig. 2 **a** Response surface and **b** contour plots for β-carotene using the macro ingredients of whole stillage, corn steep liquor and glycerol

influence on carotenoid production, the results are not provided here as they were not statistically significant. Therefore, with the exception of Zn, we chose to add the minimum concentrations of all minerals: 0.6 g/l of K, 0.3 g/l Mg and Mn, and 0.7 g/l of Zn.

Validation

The astaxanthin and β-carotene yields from mono- and mixed culture fermentations of optimized medium are provided in Table 5. The astaxanthin and β-carotene yields from *P. rhodozyma* on day 7 were 67 and 265 μg/g, respectively, and both were comparable to the predicted values of 78 and 257 μg/g, respectively, obtained from the contour plots of macro ingredients.

Media optimization improved *P. rhodozyma* astaxanthin yield by 119% and β-carotene yield by 197% on day 7 (Table 5). Astaxanthin yield in *P. rhodozyma* increased by 177% on day 9, confirming the enhanced astaxanthin production in the late log phase or exponential phase. Although the optimized conditions of *P. rhodozyma* were applied to the *S. roseus* monoculture and mixed culture fermentations, we observed only a marginal increase in carotenoid production, except in the astaxanthin yield of the mixed culture, where a yield reduction of 71% was observed. This indicates that *S. roseus* monoculture and mixed culture fermentations require separate optimization studies.

The HPLC detection of astaxanthin and β-carotene in whole stillage was further confirmed by MALDI/TOF-MS and NMR (see the "Electronic supplementary information").

Discussion

In an effort to sustain the bioethanol industry, we have outlined the value addition of corn whole stillage to produce premium animal feed. This study demonstrated the successful production of visually appealing carotenoid-enriched whole stillage that is rich in both astaxanthin and β-carotene (Fig. 3). Since the carotenoid levels in carotenoid-enriched whole stillage were in the range that is generally used in animal feed, carotenoid-enriched DDGS has a potential application as a "value-added animal feed."

Astaxanthin

Wild-type strains of *P. rhodozyma* typically yield around 200–300 μg/g of yeast of astaxanthin [22]. The maximum astaxanthin yield by *P. rhodozyma* upon optimization was 97 μg/g of freeze-dried sample. Compared to published estimates, our astaxanthin yield may appear low. However, it should be emphasized that our yield was underestimated, as it was calculated per gram of freeze-dried whole stillage and not per gram of yeast cells, as done in most studies. Frengova and Beshkova [16] have reviewed the astaxanthin yields of *P. rhodozyma* on both synthetic media and agricultural substrates; the yields have been highly variable, ranging from 174 μg per gram of yeast cells on *Eucalyptus* hydrolysates [8] to 7,200 μg/g on hydrolyzed corn syrup [21], with intermittent production on various substrates. This variability in yield may be due to the inherent variability in the *P. rhodozyma* strains used and/or the carbon source in the media [29]. Hayman et al. [18] evaluated six co-products of corn wet-milling for astaxanthin production by *P. rhodozyma* and found that thin stillage and corn condensed distiller's solubles (CCDS) supported maximum yields of 4.1 and 3.1 μg/ml, respectively. The evaluated co-products are rich in corn fiber, arabinoxylan, a complex crosslinked structure not easily degraded by enzymes. Their study clearly demonstrated the ability of *P. rhodozyma* to degrade corn fiber without any pre-treatment of the

Table 5 Validation of optimization: carotenoid yields (μg/g of freeze-dried whole stillage) from optimized medium

Carotenoids[a]	Treatment[b]	Day 5	Day 7	Day 9
Astaxanthin	Mx	5.91 ± 0.93 (−54%)[c]	5.076 ± 0.33 (−58%)	5.08 ± 0.31 (−71%)
	PR	47.86 ± 2.07^{C} (88%)	67.77 ± 4.22^{B} (116%)	97.71 ± 1.59^{A} (177%)
	SR	–	–	–
β-Carotene	Mx	212.47 ± 8.04^{B} (57%)	244.96 ± 15.01^{AB} (80%)	278.86 ± 9.65^{A} (48%)
	PR	241.83 ± 2.97 (217%)	265.77 ± 23.63 (197%)	275.20 ± 16.38 (164%)
	SR	243.39 ± 6.28 (63%)	237.52 ± 9.95 (23%)	278.58 ± 28.00 (20%)
Total	Mx	218.38 ± 8.32^{B} (48%)	250.03 ± 15.34^{AB} (68%)	283.94 ± 9.36^{A} (38%)
	PR	289.69 ± 4.89^{B} (183%)	333.53 ± 27.65^{AB} (175%)	372.91 ± 15.63^{A} (165%)
	SR	–	–	–

[a] Means and standard errors are provided; significance was set at $P \leq 0.05$. ANOVA: astaxanthin: Mx-F = 0.64, $P = 0.5578$; PR-F = 76.6, $P = <0.0001$; β-carotene: Mx-F = 8.63, $P = 0.0172$; PR-F = 1.06, $P = 0.4025$, SR-F = 1.60, $P = 0.2768$; total carotenoids: Mx-F = 8.22, $P = 0.0191$; PR-F = 5.04, $P = 0.052$; significantly different treatments across days do not share a letter (uppercase)

[b] *Mx* mixed culture, *PR P. rhodozyma*, *SR S. roseus*

[c] % in parentheses is the percentage increase (or decrease) in the yield compared to that from unoptimized medium (Table 3)

Fig. 3 **a** Freeze-dried whole stillage. **b** Freeze-dried carotenoid-enriched whole stillage from mixed culture fermentation. Similar products are available from *Phaffia rhodozyma* and *S. roseus*

substrates. Ngheim et al. [29] also evaluated corn fiber for astaxanthin production by *P. rhodozyma*, but the corn fiber was pre-treated with enzymatic degradation to yield the respective sugars. Incidentally, arabinose gave the highest astaxanthin yield.

β-Carotene

The *Sporobolomyces roseus* strain used in this study predominantly produced β-carotene, and the maximum yield was about 278 μg/g of freeze-dried whole stillage. The β-carotene yield from *S. roseus* on yeast extract-based synthetic medium has typically ranged from as low as 11.8 μg per gram of yeast cells [5] to 230 μg/g [45], with intermittent production rates of 101 μg/g [9] and 118 μg/l on YMB [25].

Astaxanthin usually accounts for 80–90% [38] or even 100% [38] of the total carotenoids of *P. rhodozyma*. However, under microaerophilic conditions, β-carotene is accumulated at the expense of astaxanthin [22, 35]. In this study, β-carotene production by *P. rhodozyma* accounted for 75% of its total carotenoids, indicating that the medium was probably microaerophilic. The macro ingredients probably increased the medium's viscosity, leading to lesser diffusion of oxygen. This is indirectly supported by the RSM model, which suggested that decreasing the concentration of macro ingredients leads to higher carotenoid production. In mixed culture fermentation, the β-carotene yield was comparable to that of *P. rhodozyma* and *S. roseus*, and was not cumulative compared to those of the two strains.

Total carotenoids

Astaxanthin and β-carotene constitute the total carotenoid pool in this study. However, *S. roseus* produces other carotenoids such as torulene and torularhodin [9, 10]. The total carotenoid content of *S. roseus* on synthetic medium has ranged from 82.3 μg/g (22.9 μg/g of torularhodin and 33.2 μg/g of torulene; [5]) to 237 μg/g (10 μg/g of torularhodin and 71 μg/g of torulene; [25]). Similarly, *P. rhodozyma* is also known to produce torulene and torularhodin ([16] and references therein). While we did not evaluate the carotenoid-enriched whole stillage for these additional carotenoids, it is likely that they are produced by both the *P. rhodozyma* and the *S. roseus* strains. The total carotenoid content in our value-added DDGS will be further enhanced if these carotenoids are accounted for. Usually, about 30–120 μg/g of total carotenoids is added to aquaculture feed [42]. In our study, both mixed culture and *P. rhodozyma* monoculture fermentations were able to provide the prescribed amount of total carotenoids before optimization, and nearly 2.5 times this amount after optimization.

Optimization

Overall, the optimization studies indicate that, in shake flasks, lower concentrations of whole stillage, glycerol and corn steep liquor improve the carotenoid yield. The optimized medium had 40% less whole stillage, 25% less corn steep liquor and 54% more glycerol. Our results indirectly confirm that carotenoid production, especially astaxanthin production, is influenced by aeration. As the medium viscosity increases, the amount of dissolved oxygen is reduced, severely affecting astaxanthin production. We increased the glycerol concentration as it positively influenced the β-carotene production. It is likely that reducing the amount of glycerol would further increase astaxanthin production, but it could also negatively influence β-carotene production.

We did not optimize other factors like aeration, temperature, pH, inoculum size or N source. We identified the best conditions based on well-documented studies. For example, we chose 18°C, pH 6.0, and 10% inoculum. Since whole stillage is a fermented product with residual yeast, we did not add yeast extract or any other N source.

Also, the procured whole-stillage sample had a pH of 6.0, and we did not alter it as it was well within the documented range. Further media optimization by lowering glycerol concentration, including factors like aeration/dissolved oxygen and temperature in the statistical design, and improving the strains or using overproducing strains can enhance the astaxanthin yield in *P. rhodozyma* monoculture.

Potential applications

According to the global market for carotenoids [4], the worldwide market value of all commercially used carotenoids is set to exceed $1 billion, to which astaxanthin and β-carotene contribute $257 and $254 million, respectively. The feed industry has a huge demand for astaxanthin due to its pigment and antioxidant properties, and for β-carotene, due mostly to its pigment properties. Since DDGS is predominantly sold as livestock and poultry feed, carotenoid-enriched DDGS can not only provide value-added animal feed but can also improve the market base of DDGS. Additionally, the use of biological astaxanthin in feeds has more advantages than synthetic astaxanthin. Firstly, biological astaxanthin produces similar effects to synthetic astaxanthin but at only half the concentration. For example, An et al. [2] showed that synthetic astaxanthin at 45 mg/kg feed and biological astaxanthin at 22.5 mg/kg feed provide similar levels of pigmentation in egg-laying hens. Secondly, biological astaxanthin is also associated with higher lipid synthesis in yeasts, thereby allowing greater absorption of carotenoids [2]. Aquaculture, especially salmonid and crustacean aquaculture, are dependent on astaxanthin to provide the visually appealing and characteristic pink color, and it is the principal market driver for astaxanthin [42]. Astaxanthin is the most expensive ingredient in salmonid feed [22]. Since DDGS is being explored as aquaculture feed [11], carotenoid-enriched DDGS could prove to be "cost-effective, naturally pigmented" aquaculture feed.

Conclusions

Carotenoid-enriched whole stillage is a unique product that is not only visually appealing but also provides astaxanthin and β-carotene, the predominant carotenoids in animal feed. Depending on the type of carotenoids required in the feed, mono- or mixed culture fermentation can be employed. The carotenoid-enriched DDGS can be used in livestock and poultry feed, but it can also capture the aquacultural feed base due to its inherent need for carotenoid pigments.

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