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Optimization of astaxanthin pigment bioprocessing by four different yeast species using wheat wastes

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ABSTRACT

The utilization of wheat wastes in order to produce astaxanthin by pigment producer microorganisms, ATCC 90197 (*Yamadazyma guilliermondii*), ATCC 24060 (*Yarrowia lipolytica*), ATCC 24202 (*Xanthophyllomyces dendrorhous*) and ATCC 24259 (*Sporidiobolus salmonicolor*) using solid state fermentation technique was carried out pursuant to Box-Behnken design (BBD). Incubation temperature, initial pH and moisture content parameters with three levels were generated for each fermentation process of the microorganisms to optimize the astaxanthin bioprocessing. Maximal astaxanthin with the yield of 109.23 μg AX/g WW was produced by ATCC 24202 at the conditions; 20.0 °C temperature, 5.5 pH and 90.0% moisture content. A second optimization for inoculation rate was followed out by steepest ascent method (SAM). It was revealed that one of the major agro-industrial wastes of the whole world, wheat wastes, might be used to produce value-added astaxanthin pigment biotechnologically.

1. Introduction

Food ingredients are used in food products as a necessity of food processing or on the purpose of stabilization, acidification, sweetening, flavor enrichment, jelling, odorization etc. Colorization, using pigments is one of the major food treatments. Pigments have a crucial role in order to consume a food product. Operational, functional and healthy properties of the pigments which based on the origin of them are caused their specificity, importance and usage area.

Pigments in food industry are obtained in two ways, chemically and naturally. Extraction from plants and animals, and biotechnological production are within the scope of natural production; chemical methods are within the synthetic production (Joshi et al., 2003; Nigam and Pandey, 2009; Gupta et al., 2011). Biotechnological methods gain a particular advantage over the others from the point of recovering wastes and producing natural characterized products. Utilizing the wastes as compost, animal feed or combustion material mean direct disposal of the wastes, and represent a major cause for environmental pollution and important loss of biomass which could be used for the production of various metabolites with added commercial value due to their rich content such as fatty acids, proteins, minerals, phenolic compounds etc. Solid State Fermentation (SSF) as may submit a good biotechnological way for recovery of organic wastes (Uyar and Baysal, 2004; Couto and Sanromán, 2006; Yang, 2007). Wheat wastes are indicated as an important by-product of wheat processed industrially (Wang et al., 2010) and present an appropriate solid medium for microorganisms in SSF systems (Nigam and Pandey, 2009).

Astaxanthin (3,3′-dihydroxy-β, β-caroten-4,4′-dione) is an oxygenated carotenoid which presents a wide spectrum from yellow to red and used in areas of aquaculture, cosmetics, pharmaceutical, food, feed and medical. Astaxanthin pigment has several notable health effects as very powerful antioxidant, anti-inflammatory, anticancer, photoprotectant, benefits for eye, heart and skin, helpful for liver functions and immune system (Visser et al., 2003; Guerin et al., 2003; Rodríguez-Sáiz et al., 2010; Ambati et al., 2014). Different kinds of microorganisms are capable of synthesizing astaxanthin pigment such as; a green algae *Haematococcus pluvialis, Brevibacterium* spp. and *Mycobacterium lacticola* bacteria, *Rhodotorula* sp., *Sporidiobolus* sp., *Xanthophyllomyces dendrorhous, Yarrowia lipolytica* yeasts (Johnson and Lewis, 1979; Yamane et al., 1997; Yuanshuai, 2006; Rodríguez-Sáiz et al., 2010; Joshi et al., 2003). Among many microorganisms, *X. dendrorhous* (formerly *Phaffia rhodozyma*) and *H. pluvialis* are the major commercial astaxanthin producing microorganisms (Yuanshuai, 2006).

The aim of the study was to produce astaxanthin pigment from wheat wastes as an agricultural biomass by selected yeasts using SSF technique and optimize the production conditions.

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Table 1
Optimum temperature and pH values for growth of the yeasts.

Microorganisms	ATCC code	Optimum growth conditions	
		T (°C)	pH
Yamadazyma guilliermondii	ATCC 90197	25.0	5.0
Yarrowia lipolytica	ATCC 24060	24.0	5.6
Xanthophyllomyces dendrorhous	ATCC 24202	20.0	4.5
Sporidiobolus salmonicolor	ATCC 24259	18.0	6.0

2. Materials and methods

2.1. Microorganism cultures

Freeze-dried yeasts, ATCC 90197, ATCC 24060, ATCC 24202 and ATCC 24259 were purchased from the American Type Culture Collection (Manassas, USA). Microorganisms were transformed in YM broth and maintained both in YM broth and YM agar. YM medium has the composition: 3 g/L yeast extract (Merck, Germany), 3 g/L malt extract (Merck, Germany), 5 g/L peptone (Merck, Germany), 10 g/L dextrose (Sigma-Aldrich, Germany) and 20 g/L agar (Merck, Germany).

Fresh cultures of the microorganisms were prepared by growing at the optimum temperature values of them for 24–48 h in 10 mL YM broth. Optimum growth conditions of the each yeast with regard to ATCC protocol are shown in Table 1.

A growth curve study was implemented for all the yeasts at the optimum conditions in order to estimate a fermentation period. Pigmentation ability of the yeasts on wheat wastes was investigated during the determined period.

2.2. Sample preparation

Wheat wastes (WW) were supplied from Gaziantep, Turkey and stored at cold storage (+10 °C) in polyethylene packages. It was sieved to size 0.85 mm in order to obtain a uniform material for the fermentation system.

2.3. Experimental design

Different moisture content levels; 60.0%, 70.0%, 75.0%, 80.0% and 90.0% were investigated to use an appropriate moisture content interval, due to significant importance of water amount for microbial growth and taking a homogenous sample from the solid system. It was also investigated that lower and upper limits of each optimum pH value for all the fermentation systems.

BBD was performed for all the yeasts after they succeeded pigmentation on WW. The design was used to evaluate the effects of three independent variables; temperature (x_1), moisture content (x_2) and pH (x_3) which are the most effective parameters for the solid systems and their interactions on the astaxanthin yield response (Bellon-Maurel et al., 2003; Norliza and Ibrahim, 2005). High, middle and low codes of the independent variables are shown in Table 2. There are 17 runs with 5 center points conducted through the design.

2.4. Solid state fermentation

The fermentation was carried out in 250 mL Erlenmeyer flasks containing 100 g total amount of the wastes and water. Amount and pH value of water were adjusted according to the experimental design and mixed with the wastes. The flasks were sterilized by autoclave at 121 °C for 15 min After cooling, they were inoculated with 2 mL fresh culture (10^6 cells/mL) and incubated at the design temperatures during the fermentation period.

Table 2
Levels of the independent variables for Box-Behnken design.

Microorganisms	Coded levels								
	x_1			x_2			x_3		
	−1	0	+1	−1	0	+1	−1	0	+1
ATCC 90197	20.0	25.0	30.0	70.0	80.0	90.0	4.0	5.0	6.0
ATCC 24060	19.0	24.0	29.0	70.0	80.0	90.0	4.6	5.6	6.6
ATCC 24202	15.0	20.0	25.0	70.0	80.0	90.0	3.5	4.5	5.5
ATCC 24259	13.0	18.0	23.0	70.0	80.0	90.0	5.0	6.0	7.0

x_1: temperature, x_2: moisture content, x_3: pH.

2.5. Pigment extraction and spectral analysis

A spectral scanning was performed by spectrophotometer for astaxanthin (AX) standard which was purchased dissolved form in methanol and the wavelength of the maximum pick point was determined. Calibration curve of AX pigment was obtained using absorbance values versus different concentrations of the standard.

5 g fermented content was mixed with 20 mL pure methanol (Sigma-Aldrich, Germany). After the mixture was waited for 2 h, 5 mL of the liquid phase of the mixture was taken and centrifugated at 6000 rpm for 10 min The supernatant was analyzed by double beam UV/VIS Spectrophotometer (Lambda 25 UV/VIS Spectrophotometer, USA) at 474 nm against the pure methanol blank (Babitha et al., 2007). AX concentration was calculated with regard to the equation of AX standard (Chromadex, USA) calibration curve. The results were explained as the mean of triplicate measurements.

2.6. Modeling and optimization of SSF

Optimal conditions of the fermentation systems were determined by Response Surface Methodology (RSM) (Design Expert 7.1.6 Version) which is defined as a collection of statistical and mathematical methods. A second-order polynomial (quadratic) model generated by RSM is presented as;

$$y = \beta_o + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_{12} x_1 x_2 + \beta_{13} x_1 x_3 + \beta_{23} x_2 x_3 + \beta_{11} x_{11} + \beta_{22} x_{22} + \beta_{33} x_{33} \quad (1)$$

where y is response or dependent variable; β_o is model constant; β_1, β_2, β_3 are linear coefficients; β_{12}, β_{13}, β_{23} are cross product coefficients (present the interactions between the variables); β_{11}, β_{22}, β_{33} are quadratic coefficients (Montgomery, 2001).

The data were evaluated by regression and variance analyses (ANOVA). In addition, MS Excel 2010 and SigmaPlot Version 11 were used to validate the optimization and the modeling respectively.

2.7. Optimization of inoculation rate

Five different inoculation rates (1.0%, 1.5%, 2.0%, 3.0% and 4.0%) were studied. Viable cell (VC) of the fermented medium by plate counting method and pigment analysis were performed at the end of the fermentation period for each inoculation rate. The results were modeled by SigmaPlot Version 11 with the equation [f=a*exp(−0,5*(ln(x/x0)/b)2)/x)] and optimized by SAM and MS

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Table 3
Box-Behnken design for production of astaxanthin by four microorganisms.

Run	x_1	x_2	x_3	Astaxanthin yield (μg AX/g WW)			
				ATCC 90197	ATCC 24260	ATCC 24202	ATCC 24259
1	+1	−1	0	14.35 ± 0.61	12.20 ± 0.67	26.82 ± 2.76	16.76 ± 1.79
2	0	0	0	20.33 ± 0.26	11.56 ± 0.20	79.64 ± 5.17	37.26 ± 0.87
3	−1	−1	0	18.96 ± 1.36	15.49 ± 0.23	34.98 ± 2.90	17.21 ± 0.48
4	0	0	0	22.29 ± 0.04	10.45 ± 0.16	86.80 ± 8.65	36.62 ± 1.78
5	−1	0	−1	14.26 ± 0.89	15.80 ± 0.33	95.00 ± 2.45	30.50 ± 0.16
6	0	+1	−1	15.67 ± 0.72	17.47 ± 3.24	84.46 ± 0.00	35.32 ± 1.19
7	+1	0	−1	9.61 ± 0.87	9.08 ± 0.28	72.84 ± 1.26	20.76 ± 0.71
8	−1	+1	0	16.19 ± 1.57	13.28 ± 1.11	66.31 ± 1.75	51.56 ± 2.18
9	0	0	0	16.53 ± 0.45	9.76 ± 0.57	60.85 ± 1.55	32.78 ± 0.92
10	+1	0	+1	11.16 ± 0.75	16.57 ± 0.70	70.76 ± 4.53	35.37 ± 0.38
11	+1	+1	0	9.79 ± 0.67	4.92 ± 0.31	60.84 ± 2.20	60.54 ± 1.83
12	0	0	0	15.43 ± 0.42	9.74 ± 0.58	88.99 ± 0.17	21.42 ± 0.56
13	0	0	0	17.47 ± 0.17	12.18 ± 0.48	87.70 ± 8.20	19.90 ± 0.62
14	−1	0	+1	13.13 ± 0.73	17.28 ± 1.53	55.90 ± 1.21	41.81 ± 2.47
15	0	−1	−1	15.46 ± 0.21	10.48 ± 0.50	28.96 ± 2.89	11.60 ± 0.20
16	0	−1	+1	16.65 ± 0.38	9.34 ± 0.33	33.40 ± 5.27	11.49 ± 0.32
17	0	+1	+1	15.21 ± 0.18	10.17 ± 0.39	109.23 ± 12.08	51.83 ± 1.68

μg AX/g WW: μg astaxanthin/g wheat wastes.

Table 4
Experimental and predicted values of the optimized conditions.

Optimized conditions	ATCC 90197		ATCC 24060		ATCC 24202		ATCC 24259	
	Exp	Pred	Exp	Pred	Exp	Pred	Exp	Pred
Temperature	25.0	23.83	24.0	19.0	20.0	21.48	23.0	23.0
M. content	80.0	70.0	90.0	90.0	90.0	86.92	90.0	90.0
pH	5.0	5.07	4.60	4.60	5.5	5.5	6.0	7.0
Yield (μg/g)	22.29	19.76	17.47	20.06	109.23	95.35	60.54	62.74

Exp: Experimental. Pred: Predicted.

Table 5
Quantitative model assessment tools.

Tools	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259
Model	+	−	+	+
Lack of fit	−	+	−	−
Standard deviation	0.002364	0.003431	0.016	0.007477
Mean	0.015	0.012	0.067	0.031
C.V.%	15.31	28.35	23.30	23.86
R^2	0.79	0.57	0.83	0.89
Adjusted R^2	0.51	0.01	0.60	0.74

(+): $\alpha < 0.1$; significant. (−): $\alpha < 0.1$; not significant. Significant result is not good for 'lack of fit'.

Excel 2010 solver tool.

2.8. Antioxidant capacity by DPPH radical scavenging activity assay

Extracts of both fermented and un-fermented content were prepared for DDPH (1,1-diphenyl-2-picrylhydrazyl) analysis. 5 g content was filled up to 50 mL with pure methanol (Sigma-Aldrich, Germany). The mixture was shaken (Innova 40R New Brunswick Scientific, USA) at 250 rpm and 30 °C for 2 h. The extract was waited for 30 min and then filtered. The filtrate was centrifugated at 6000 rpm for 10 min.

The mixture of 3 mL of 60 μM DPPH radical (Sigma-Aldrich, Germany) in methanol and 250 μL extract were left at dark and room temperature for the reaction. Control sample was prepared with distilled water instead of the extract. Remaining purple color was measured at 517 nm after 25 min (Kwon et al., 2006). The scavenging activity (SA) of the DPPH radical (or inhibition of DPPH radical) was calculated by the following equation:

$$SA\% = \frac{(\text{absorbance of control} - \text{absorbance of extract})}{\text{absorbance of control}} \times 100 \quad (2)$$

3. Results and discussion

The SSF systems with four yeasts ATCC 90197, ATCC 24060, ATCC 24202 and ATCC 24259 were achieved according to the design matrixes (Table 3). The fermentation periods were settled 12 days for ATCC 24202 and ATCC 24259, 15 days for ATCC 90197 and ATCC 24060.

The parameters were optimized based on the maximal yield for each yeast as shown in Table 4. 109.23 μg AX/g WW as the maximum yield by ATCC 24202 and wheat wastes fermentation was obtained at the conditions; 20.0 °C temperature, 5.5 pH and 90.0% moisture content. Johnson and Lewis (1979) reported that higher astaxanthin amount was obtained at 4.5 pH than the amount at 5.5 pH. However, Yuanshuai (2006) indicated that 5.0 pH is selected in most recent studies and a temperature range, 15–20 °C is recommended and for astaxanthin production. Ananda et al. (2011) stated that 66.75 μg astaxanthin per gram wheat bran by ATCC 24202 was reached at the day 11th of the process.

The relation between the experimental and the predicted values were stated by statistical evaluation in Table 5. It is seen that the selected model is adequate at the [$\alpha < 0.1$] probability for 'model' and 'lack of fit' tools for all the yeasts, except ATCC 24060. R^2 and adjusted R^2 values of ATCC 24259; 0.89 and 0.74 respectively presented more satisfied results. Low standard deviation and coefficient of the variance (C.V.%) values are better results for precision and reliability of the experimental data (Montgomery, 2001; Zou et al., 2013). ATCC 90197 demonstrated the lowest standard deviation and C.V.% values.

The model coefficients (Table 6) and response surface plots (Figs. 1–4) depicted that the effect of each variable and the interactions of them on the response. Temperature was more effective and significant parameter for ATCC 90197 and ATCC 24060 fermentation systems. Temperature levels close to the optimum growth temperature values of ATCC 90197, ATCC 24060 and ATCC

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Table 6
Coefficients of the model equations of each optimized fermentation system.

Coded coefficients	ATCC 90197	ATCC 24060	ATCC 24202	ATCC 24259
β_0	+0.018	+0.011	+0.081	+0.030
β_1	−0.002203	−0.002384	−0.002617	−0.0009544
β_2	−0.00107	−0.000208	+0.025	+0.018
β_3	+0.0001416	+0.00006739	−0.001497	+0.005289
β_{12}	−0.0004495	−0.001267	+0.0006725	+0.002355
β_{13}	+0.0006686	+0.001504	+0.009256	+0.0008.253
β_{23}	−0.0004117	−0.001543	+0.00508	+0.004154
β_{11}	−0.003648	+0.001776	−0.012	+0.005737
β_{22}	+0.0000586	−0.001041	−0.022	+0.001185
β_{33}	−0.002723	+0.002169	+0.004801	−0.00322

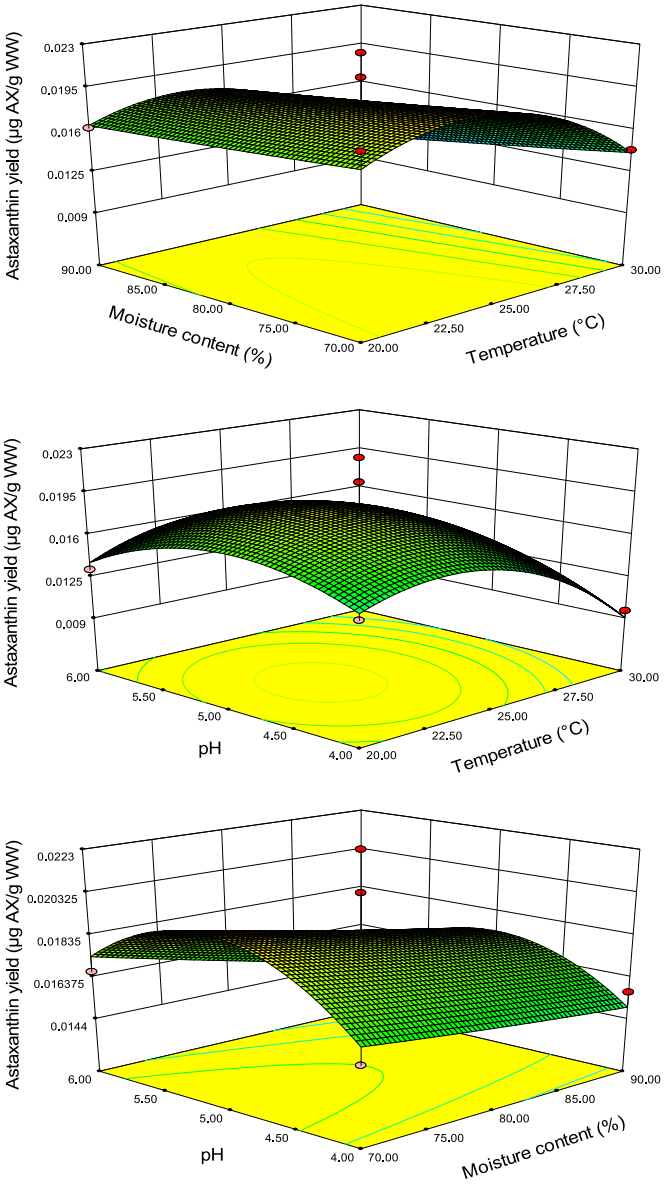

Fig. 1. 3D plots showing the interaction effects of the independent variables on the astaxanthin yield for ATCC 90197 and wheat wastes fermentation.

24202 induced high AX production. Moisture content was the most effective parameter for ATCC 24202 fermentation system. The AX yield increased when the level of the moisture content increased. High moisture content and pH levels influenced the AX

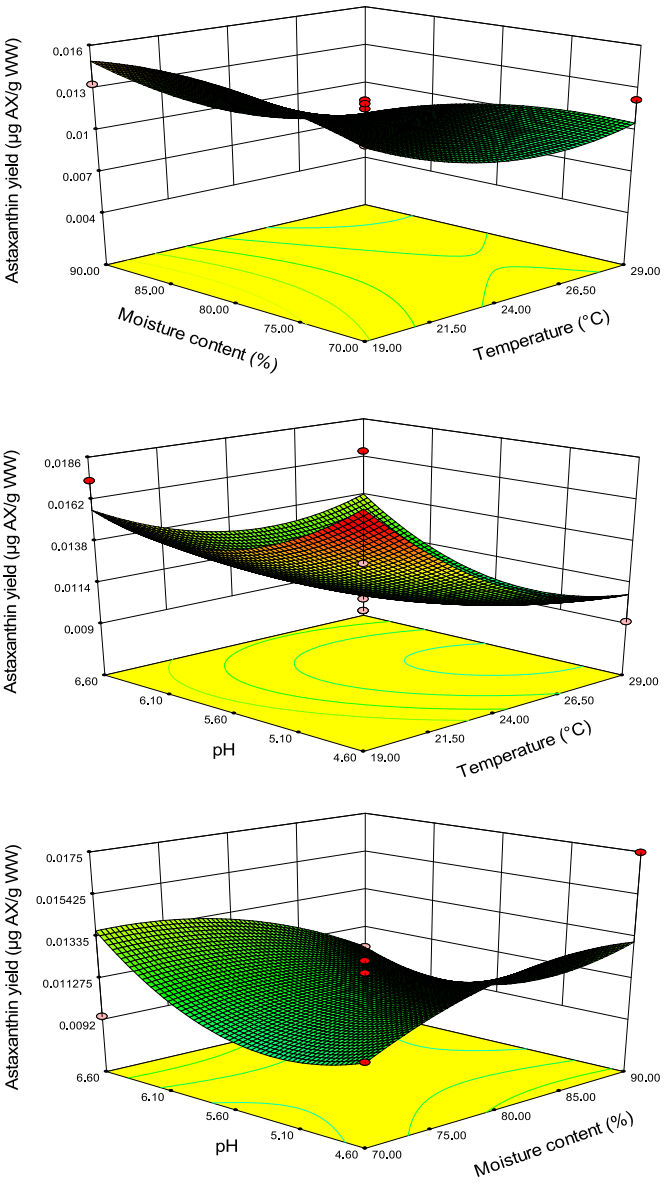

Fig. 2. 3D plots showing the interaction effects of the independent variables on the astaxanthin yield for ATCC 24060 and wheat wastes fermentation.

yield positively for ATCC 24259 fermentation. When the interactions of temperature and moisture content, and also moisture content and pH increased, the yield increased for ATCC 24259 and ATCC 24202 fermentation systems. All the yeasts were affected by increasing of the interaction of temperature and pH parameters.

noculation rate study was carried out for ATCC 24202 yeast at the optimized conditions by BBD. AX yield and VC number with regard to different inoculation rates changed in a same pattern (Fig. 5). Generally it is expected to observe a parallel increasing in product yield and inoculation rate. An increasing was determined till 2% rate, but there was decline after this value. The chosen model in SigmaPlot showed a quite high R^2 value (0.93) for the AX yield. Optimization results obtained by SAM method and MS Excel solver tool (goal seek) referred similar inoculation rate as seen in Table 7. Hence, the inoculation rate used for the fermentation systems (2%) was confirmed.

The scavenging activity of AX produced by ATCC 24202 at the conditions put forward by BBD and SAM and un-fermented WW was measured using DPPH radical. 66.39% and 96.71% SA of AX for un-fermented and fermented WW respectively were attained. The

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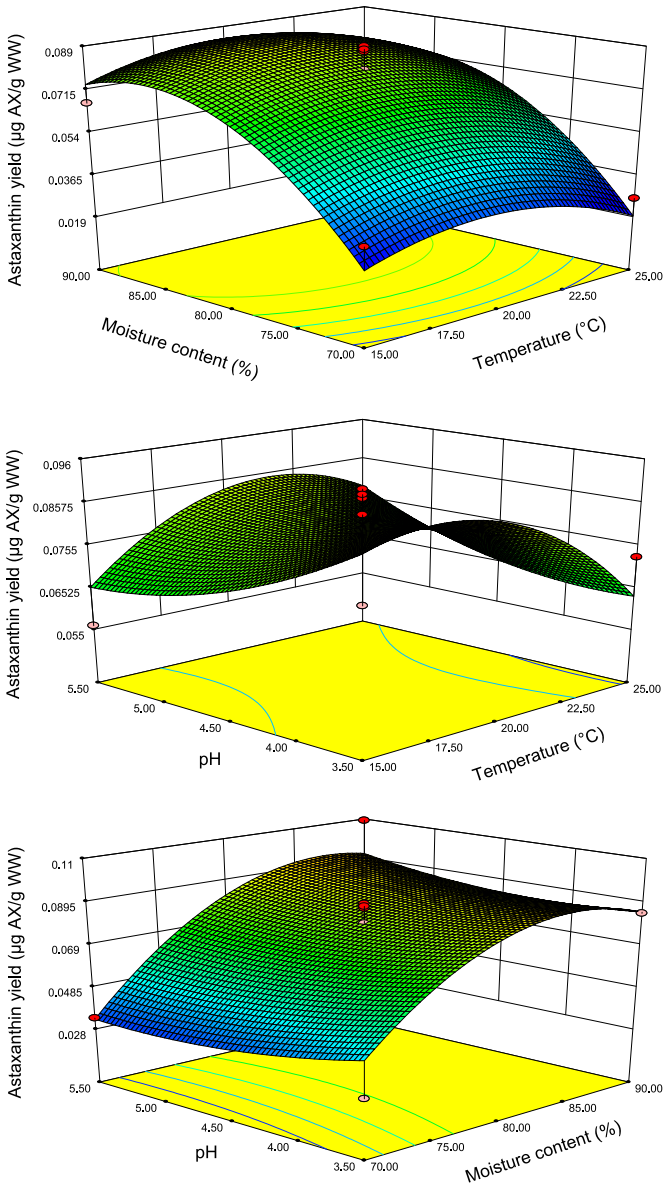

Fig. 3. 3D plots showing the interaction effects of the independent variables on the astaxanthin yield for ATCC 24202 and wheat wastes fermentation.

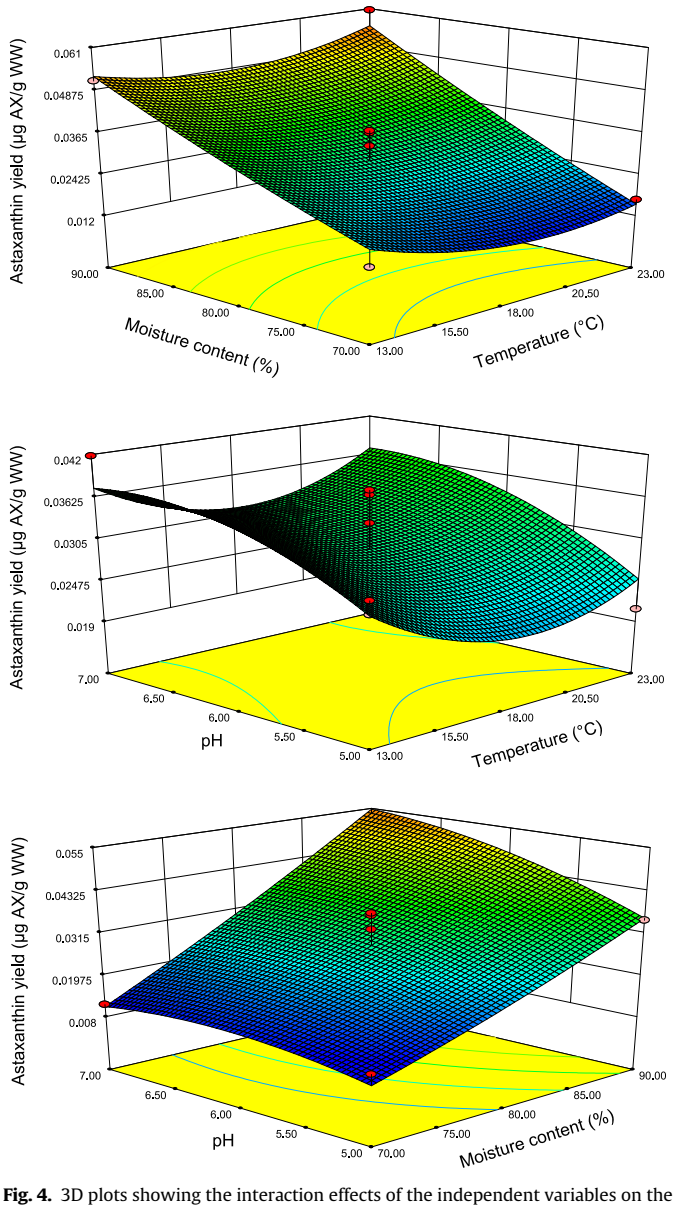

Fig. 4. 3D plots showing the interaction effects of the independent variables on the astaxanthin yield for ATCC 24259 and wheat wastes fermentation.

difference between the results revealed that the antioxidant capacity of AX produced. Gramza-Michałowska and Stachowiak (2010) stated that 90–95% antioxidant potential resulted by DPPH analysis of *X. dendrorhous* extracts.

4. Conclusion

This study has disclosed the bioprocessing of the WW as economic and environmentally; developing SSF system for agro-industrial wastes by different microorganisms; modeling and optimizing the fermentation parameters and inoculation rate; determining the significant parameters of each fermentation system; producing AX carotenoid as an important and powerful antioxidant bio-product. BBD introduces the effectiveness for multi-processing and main experiment points, thus it is a useful way to reach data with less number of experiments at short time.

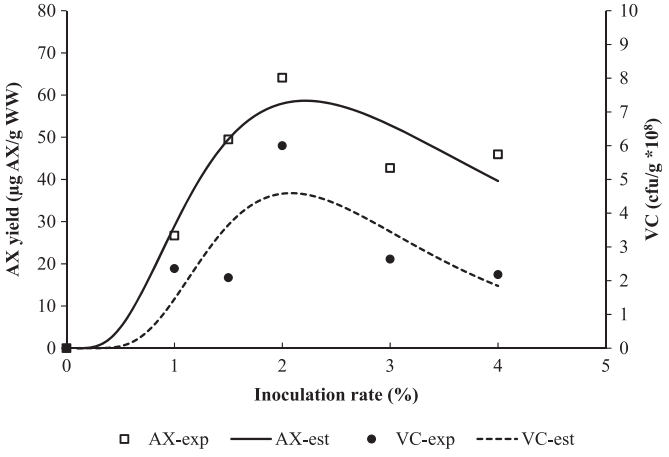

Fig. 5. Experimental data points (AX-exp, VC-exp) and estimated model (AX-est, VC-est) graphs of the relation between the inoculation rate and astaxanthin yield and viable cell.

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Table 7
Modeling and optimization results of inoculation rate.

Responses	Modeling				Optimization	
	a	b	x_0	R^2	SAM	Goal seek
Yield (μg AX/g WW)	162.33	0.6684	3.46	0.93	2.2132	2.2134
VC (cfu/g*10^8)	10.75	0.4835	2.63	0.68	2.0863	2.0889

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