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Astaxanthin biosynthesis: A two-step optimization approach and model construction with Response Surface Methodology and Artificial Neural Network

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Abstract

The first part of this research is investigating and comparing yield of a synthetic medium submerged three sugars (glucose, fructose and sucrose) at four different concentrations and solid fermentation systems with wheat bran and lentil waste for biosynthesis of astaxanthin (ASX) pigment by Xanthophyllomyces dendrorhous ATCC 24202 and Sporidiobolus salmonicolor ATCC 24259 microorganisms. The second part is modeling and optimizing the most efficient biosynthesis depending on waste, yeast and production variables consisted of moisture content, pH and temperature using a design matrix. The yields produced by X. dendrorhous were 51.88 µg of ASX/g glucose for the submerged medium with the least glucose, and 210.49 µg of ASX/g glucose for the wheat bran fermentation system. It was understood that the yield values of the submerged systems were lower and there was no requirement for the addition of any supplement to the waste systems. It was found that R^2=0.9869 was the highest value with the maximum predicted ASX amount of 109.23 µg of ASX/g wheat bran with X. dendrorhous using Artificial Neural Network modeling and the moisture content was the most significant production parameter.

Keywords: Astaxanthin biosynthesis, Neural network, Optimization, Response Surface Methodology, Submerged system

Introduction

The toxic and carcinogenic effects of the pigments produced synthetically reveal the importance of natural raw materials and biosynthesis for commercial production (Joshi et al., 2003; Duffosé et al., 2005; Gupta et al., 2011). Gupta et al. (2011) noted a food color market worth an approximate $1.2 billion globally, which has a 31% proportion of natural pigments. The boom in the global market and numerous research work points to the great economic potential for natural pigments, particularly those produced by microorganisms (Joshi et al., 2003, Gupta et al., 2011; Panesar et al., 2015). Among the natural carotenoid pigments, astaxanthin (ASX) has a very attractive commercial appeal due to its antioxidant power, economic value, wide usage, and health benefits for human (Naguib, 2000; Ramírez et al., 2001; Visser et al., 2003; Higuera-Ciapara et al., 2006; Amorim-Carillo et al., 2014; Dong et al., 2016; Niizawa et al., 2018). ASX is considered as the most powerful antioxidant and super-food nutritionally for human health. It is a valuable commercial product due to its marketing price varying from $2500-7000/kg (Panis and Rosales Carreon, 2016). The total value of ASX is predicted to reach to $1.1-1.5 billion in 2020 (Sujarit et al., 2017; Niizawa et al., 2018). ASX produced synthetically has safety and unfavorable (like biological functions) issues for human health. Besides, synthetic ASX has an unsustainable production and causes pollution (Panis and Rosales Carreon, 2016). Microbiologically produced ASX can be described as natural and replaced with the synthetic one. Safety of natural ASX consumption by a human is proven (Panis and Rosales Carreon, 2016; Schewe et al., 2017).

Physico-chemical conditions are very effective in the synthesis of microbial products. In the manufacturing of industri-

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al products, there is a great deal of scientific research on the characteristics and conditions of the production medium. The raw material is a significant parameter for biosynthesis because it directly affects the production efficiency with its variety and nutritive content. It also plays an important role in the process and environmental issues. The variety and quantity of carbon and nitrogen sources of the production medium and water content are the fundamental parameters for the growth and target product synthesis of microorganisms. Additionally, temperature, pH, agitation, and inoculum size are the environmental parameters having an effect on microbial activities. The use of wastes as raw material sources for the production medium has been employed for a very long time in industrial processes worldwide. Compared to the synthetic media, the products synthesized by the utilization of the wastes are qualified and labeled as natural (Nigam and Pandey, 2009).

Sujarit et al. (2017) stated that the biosynthesis conditions have a great effect on ASX accumulation. Therefore, a way of natural characterized, safe and efficient ASX production should be submitted by considering optimization of the process, modeling the system, enhancement of the conditions and utilization of cheap sources (An et al., 2017; Schewe et al., 2017). In addition, optimization based on a design framework is a good way of determining which factor is the most important one. Statistical studies are necessary to be done to discuss and support the significancy of the considerations. Response surface and artificial neural network methodologies are commonly applied tools for modeling, optimization and statistical evaluations in biotechnological practices (Kalil et al., 2000). Production, modeling and optimization applications for enzymes, bio-surfactants, ethanol, gelling agents, pharmaceutical compounds, bio-fuels, pigments, and fermentation medium have been studied recently (Basri et al., 2007; Desai et al., 2008; Kashkouli et al., 2011; Nelofer et al., 2012; Zou et al., 2013; Pilkington et al., 2014; Dikshit and Tallapragada, 2015; Maran and Priya, 2015; Singh et al., 2015; del Rio-Chanona et al., 2016; Sehrawat et al., 2017; Wei et al., 2017; Shafi et al., 2018).

A growth-curve study is generally performed as a preliminary study in microbial processes, which is important for commercially high-grade ASX product in terms of monitoring growth phases of producer microorganisms and determining synthesis period and efficiency. Correlatively, investigation of these terms for ASX producers, *Xanthophyllomyces dendrorhous* ATCC 24202 and *Sporidiobolus salmonicolor* ATCC 24259 yeasts in synthetic media were aimed as the first stage of this study. The conditions of the synthetic media were optimized. In the second stage, wheat bran and lentil waste were used as solid raw materials for ASX biosynthesis. The effects of fermentation temperature, moisture content and pH variables on the biosynthesis were searched within the scope of an experimental design. Modeling and optimization of the biosynthesis were performed by Response Surface Methodology (RSM). The comparison of estimation capabilities of RSM and Artificial Neural Network (ANN) techniques for ASX response were performed. Statistical evaluation of the methodology results were carried out by root mean square error (*RMSE*), mean absolute error (*MAE*), and coefficient of determination (R^2).

Materials and Methods

Wastes and microorganism cultures

Wheat bran and lentil waste were sourced from Gaziantep, Turkey and kept in cold storage (+10 °C) in polyethylene packages. They were sieved to a size of 0.85 mm in order to obtain a uniform material. Freeze-dried forms of ATCC 24202 (*Xanthophyllomyces dendrorhous*) and ATCC 24259 (*Sporidiobolus salmonicolor*) were purchased from the American Type Culture Collection (Manassas, USA). The microorganisms were maintained in yeast malt extract broth (YMB) that has the following composition: 3 g/L of yeast extract (Merck, Germany), 3 of g/L malt extract (Merck, Germany), 5 g/L of peptone (Merck, Germany), 10 g/L of dextrose (Sigma-Aldrich, Germany). The growth of the yeasts with a 2% (v/v) inoculum size was performed in 20 °C-4.5 pH and 18 °C-6.0 pH conditions which are the optimum growth conditions of ATCC 24202 and ATCC 24259, respectively according to the ATCC protocol.

Growth curve determination

Growth parameters of the yeasts were determined to observe the phases of the growth and the fermentation period. *Optical density* (O.D.) was measured by a double-beam UV/VIS spectrophotometer (Lambda 25 UV/VIS spectrophotometer, USA) at 540 nm. Additionally, *viable cell* was determined by plate counting method and *biomass* was weighted as dry cell. Each measurement (Lopes et al., 2007; Aber et al., 2012) was carried out daily for 20 days. Experimental data of the growth parameters were plotted versus time. Natural logarithm of optical density value In [O.D.] versus fermentation period for each yeast was modeled by SigmaPlot Version 11.0 (Systat Software GmbH, Erkrath, Germany) program using Gompertz-4 Parameter equation:

$$y = y_0 + a^x \exp\left(-\exp\left(-\frac{x - x_0}{b}\right)\right)$$

Where y is response, x is time, a and b are coefficients.

Astaxanthin pigment analysis

Astaxanthin analysis was performed for the synthetic media content, and the forms of un-fermented and fermented contents of the wastes according to Babitha et al. (2007). A mixture of the sample and methanol (Sigma-Aldrich, Germany) at 1:4 ratio was centrifuged at 6000 rpm for 10 minutes, and the supernatant was analyzed at 474 nm using the UV/VIS spectrophotometer. The results were recorded as the mean of triplicate measurements.

Yeast growth and productivity in synthetic media

Synthetic media were prepared using no sugar (only containing nitrogenous compounds) and three different sugar types (glucose, fructose and sucrose) at 4 different concentrations (5 g/L, 10 g/L, 20 g/L and 40 g/L) measure the growth and product formation capabilities of the yeasts by keeping the amount of malt extract, yeast extract and peptone ingredients constant. Optical density values of the media during

the fermentation period and the ASX pigment produced in the synthetic media were measured at the last day of the fermentation period.

Experimental design and model construction of the fermentation systems

An experimental design was generated for each combination of yeast and waste based on the optimal growth conditions of the yeasts using Box-Behnken design (BBD) with the independent variables of temperature (x_1), moisture content (x_2) and pH (x_3). Levels coded as high (+), middle (0) and low (−) are given in Table 1. Seventeen experiments were conducted for each combination to produce astaxanthin (response). The experimental data were analyzed using RSM (Design-Expert Version 7.1.5, Minneapolis, USA) and ANN (MATLAB Version 7.10, USA) methodologies.

Table 1. 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 24202	15	20	25	70	80	90	3.5	4.5	5.5
ATCC 24259	13	18	23	70	80	90	5.0	6.0	7.0

The ndividual and interaction effects of the process variables on the response are demonstrated in the equation below which is a second-order polynomial equation quadratic model:

$$y = \beta_0 + \sum_{j=1}^{k} \beta_j x_j + \sum_{j=1}^{k} \beta_{jj} x^2{}_j + \sum_{i=1}^{j-1} \sum_{j=2}^{k} \beta_{ij} x_i x_j$$

Where y is the predicted response, β_0 is constant, β_j, β_{ij} and β_{jj} are the regression coefficients and x_j and x_i are the levels of the independent variables.

RSM requires a mathematical model such as quadratic for the data prediction. However, ANN uses vectors based on a function for this purpose. This specification provides the main advantage of ANN methodology (Baş and Boyacı, 2007; Singh et al., 2015). Non-linear mapping was performed by using the process parameters as the input variables and the response as the output variable. A Gausiian function with 0.75 spreadability was applied, which uses the equation below to estimate the data with a 3 input layer, one hidden layer with 17 nodes and 1 output layer (3-17-1) topology.

$$a_{hk} = \exp\left(- \frac{\| x_h - x_k \|^2}{\sigma_h{}^2} \right)$$

Where a_{hk} is basis function or activation of h-th unit in the hidden layer; x_h is unit center or n-dimensional position of the centre of h-th (n as input number); x_k mean or center of the function, σ_h is standard deviation or local scaling constant.

The prediction capability of the methodologies was investigated by *RMSE (root mean square error)*, *MAE (mean absolute error)*, and R^2 statistical measurements. R^2 values for RSM came out from the design program and for ANN were calculated by regression analysis tool in MS Excel program.

$$RMSE = \sqrt{\frac{1}{N} \sum_{i=1}^{N} (MR_{pre,i} - MR_{\exp,i})^2}$$

$$MAE = \frac{1}{N} \sum_{i=1}^{N} (MR_{pre,i} - MR_{\exp,i})$$

Where n is number of data, $R_{pre,i}$ is predicted model value, and $R_{exp,i}$ is experimental value.

Results

Growth curve study

The optical density, viable cell and biomass data obtained in the synthetic YMB medium are presented in Figure 1 for ATCC 24202 and ATCC 24259 yeasts. Based on the reasonable progress of the growth parameters, the fermentation period was determined to be ten days. Figure 2 shows the growth curves of the yeasts modeled with the data of In [O.D.] versus fermentation period. The results of the statistical evaluation of the modeling are given in Table 2. The R-squared values are quite high, 0.97 for ATCC 24202 and 0.96 for ATCC 24259, meaning a good fitness of the regression model.

Table 2. Modeling report of the growth curve determination

Sources	ATCC 24202	ATCC 24259
a	2.969	4282.1518
b	0.7998	2.4397
x_0	0.6075	–17.8474
y_0	14.4159	–4264.3628
R^2	0.9721	0.9618
Adj. R^2	0.9581	0.9427
Normality test*	0.3128	0.1285

*: passed at $p<0.05$

Effect of sugar types and concentrations

The media prepared with glucose (G), fructose (F) and sucrose (S) sugars at four different concentrations were investigated to see the effects of the sugar type and the concentration on the growth and product formation abilities of the selected yeasts. The optical density values (including viable cells, dead cells, and metabolites) of the yeasts are shown in Figure 3 and Figure 4. The optical density changes observed between 100 and 150 hours are striking for both yeasts. A decrease in cell concentration was seen between about 100 to 125 hours for ATCC 24202 yeast. There seems to be a few non-significant data points such as the increase in S5 after 150 hours in Figure 3, which is distinct because of the optical values including the viable cells, dead cells and metabolites. It can be stated that glucose at any concentration presents a high cell concentration for ATCC 24202 yeast. The O.D. values of ATCC 24259 yeast showed significant changes from 75 hours at any concentration of glucose and sucrose sugars (Figure 4). The cell concentrations at low fructose concentrations (5 and 10 g/L) were quite high for ATCC 24259 yeast. It is thought

that it took time for ATCC 24259 yeast to adapt to the F20 medium whereas there was no active growth on the F40 medium. ATCC 24202 yeast at no sugar medium showed a curve similar to the G40's and the growth trend of ATCC 24259 yeast with 'no sugar' medium resembles the trend obtained from the media of glucose and sucrose sugars in Figure 5.

It is understood that as the sugar concentration of the media increases, the amount of ASX decreases for both yeast as seen in Table 3. The ASX amount produced by ATCC 24202 in any medium is higher than that produced by ATCC 24259. Maximum amounts of 51.88 µg ASX/g glucose and 17.98 µg ASX/g fructose were measured for ATCC 24202 and ATCC 24259, respectively. It is seen that the amount of ASX is less in the absence of sugar than in other media for both yeasts. When the relationship between cell concentration and product formation is evaluated (Table 3), it could be concluded that there is no connection between them.

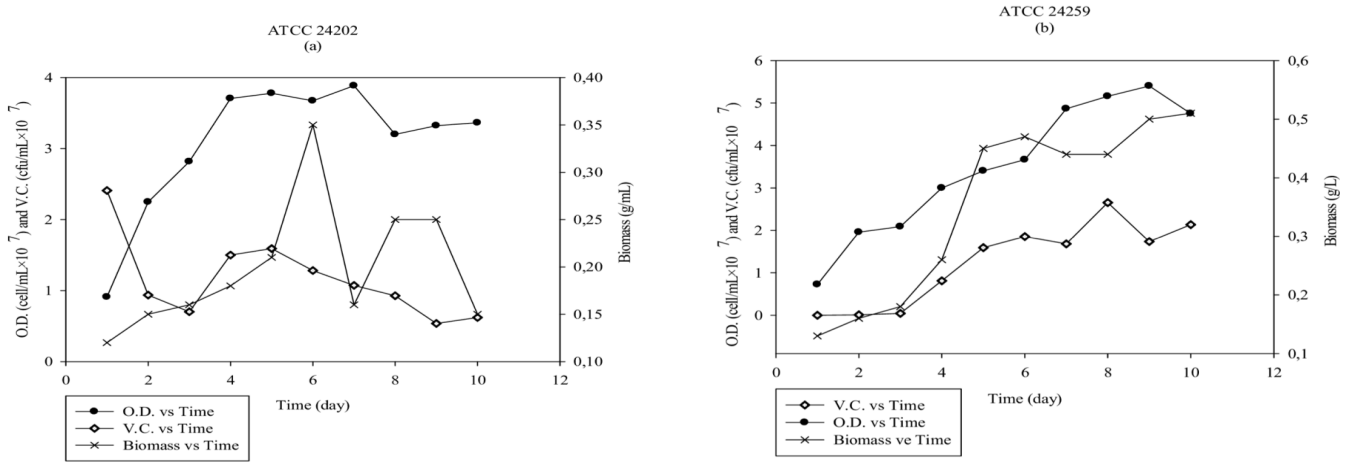

Figure 1. The growth parameters plotted for X. dendrorhous ATCC 24202 (a) and S. salmonicolor ATCC 24259 (b)

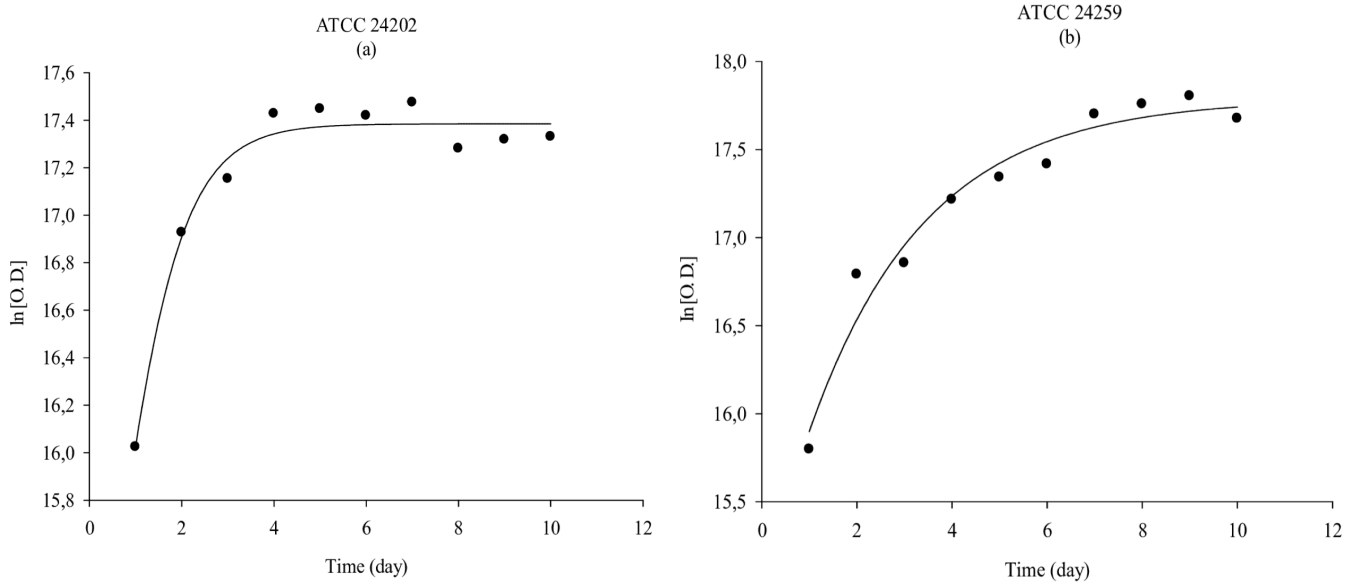

Figure 2. Modeled growth curves of for *X. dendrorhous* ATCC 24202 (a) and *S. salmonicolor* ATCC 24259 (b). The lines and dots indicate the model regression of estimated and experimental data, respectively.

Table 3. Astaxanthin yield and cell concentration in synthetic media

Sugar concentrations[a]	Astaxanthin concentration (mg ASX/L YMB)[b]		Astaxanthin amount (µg ASX/g sugar)[c]		Cell concentration (cell/mL×10^7)[d]	
	ATCC 24202	ATCC 24259	ATCC 24202	ATCC 24259	ATCC 24202	ATCC 24259
G5	2.08 ± 0.02	0.51 ± 0.02	51.88 ± 0.57	12.72 ± 0.44	1.42	2.57
G10	1.96 ± 0.02	0.6 ± 0.01	24.44 ± 0.22	7.53 ± 0.11	1.14	3.05
G20	1.87 ± 0.02	0.81 ± 0.0	11.71 ± 0.13	5.04 ± 0.02	3.72	2.69
G40	1.76 ± 0.02	0.75 ± 0.02	5.5 ± 0.05	2.34 ± 0.06	0.93	2.52
F5	1.88 ± 0.02	0.72 ± 0.01	46.93 ± 0.59	17.98 ± 0.23	1.24	3.32
F10	2.08 ± 0.03	0.68 ± 0.04	26.02 ± 0.34	8.49 ± 0.50	2.35	2.23
F20	2.42 ± 0.02	1.38 ± 0.02	15.13 ± 0.11	8.61 ± 0.16	1.69	2.94
F40	0.7 ± 0.02	1.19 ± 0.01	2.2 ± 0.07	3.73 ± 0.04	1.23	5.67
S 5	1.91 ± 0.02	0.34 ± 0.01	47.67 ± 0.49	8.39 ± 0.28	2.34	2.07
S10	1.9 ± 0.01	0.41 ± 0.02	23.71 ± 0.07	5.1 ± 0.29	1.46	4.26
S20	2.72 ± 0.01	0.37 ± 0.01	16.98 ± 0.05	2.33 ± 0.09	0.94	1.12
S40	1.82 ± 0.02	0.33 ± 0.02	5.7 ± 0.06	1.03 ± 0.05	1.8	2.03
No sugar	1.6 ± 0.01	0.27 ± 0.02	-	-	1.92	1.91

[a]: G: glucose, F: fructose, S: sucrose
[b]: Astaxanthin concentration: milligram astaxanthin/Liter yeast malt extract broth as mean value of duplicate results
[c]: Astaxanthin amount: microgram astaxanthin/gram sugar as mean value of duplicate results
[d]: Optical density values at 540 nm

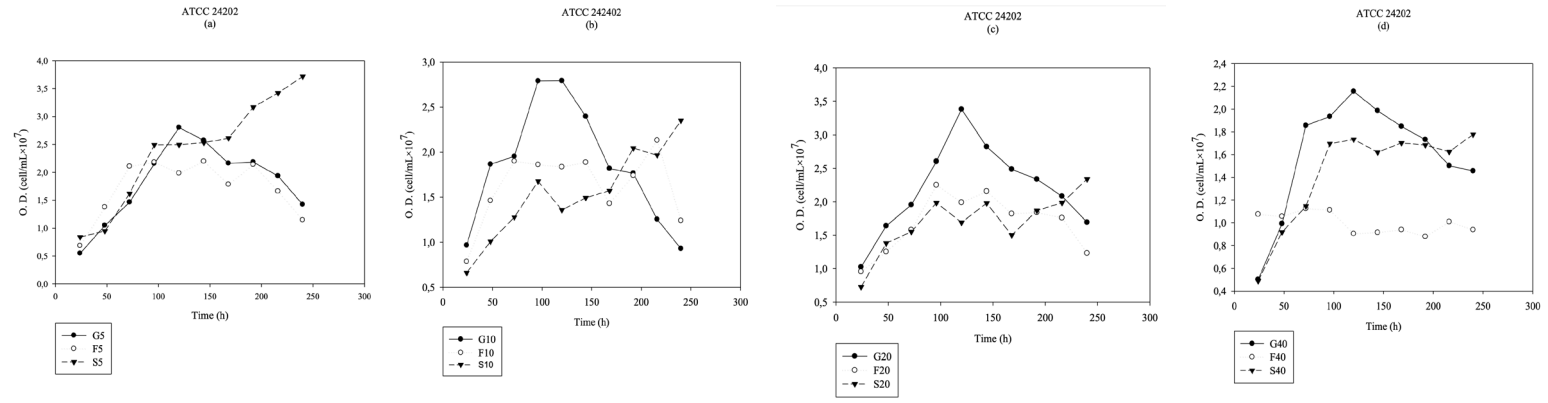

Figure 3. Time versus optical density values for *X. dendrorhous* ATCC 24202 at sugar concentrations of; a: 5 g/L, b: 10 g/L, c: 20 g/L, c: 40 g/L

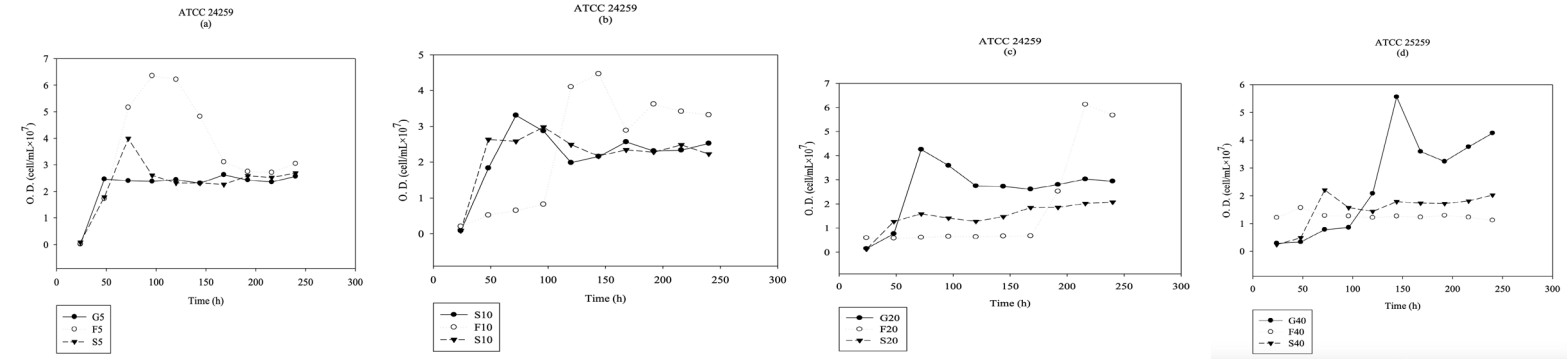

Figure 4. Time versus optical density values for *S. salmonicolor* ATCC 24259 at sugar concentrations of; a: 5 g/L, b: 10 g/L, c: 20 g/L, c: 40 g/L

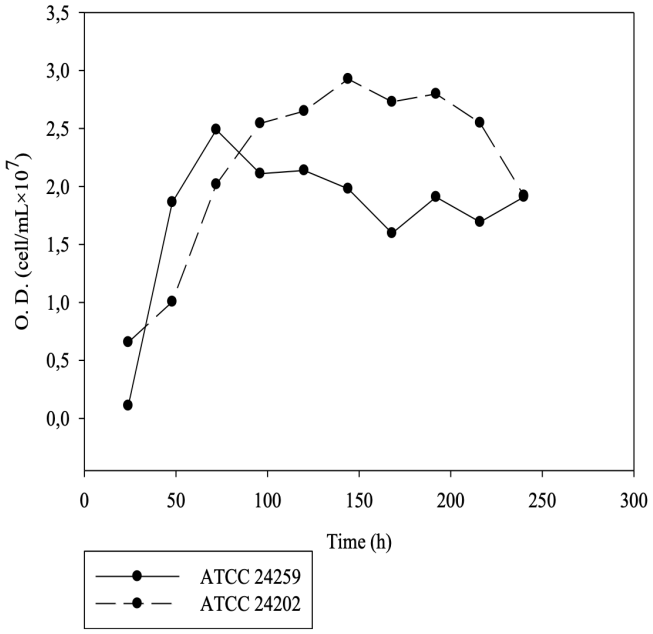

Figure 5. Time versus optical density values at no sugar medium for *X. dendrorhous* ATCC 24202 and *S. salmonicolor* ATCC 24259

Modeling and optimization study

The production of the ASX pigment from the wheat bran and lentil waste was achieved in the optimum growth conditions of the yeasts (Table 4). Based on the sugar content of the wastes, the wheat bran provided the highest ASX yield (201.49 µg ASX/g glucose) for both yeasts. After considering the ASX yield obtained from the wastes, an optimization study for both yeasts was conducted by generated BBD experimental design. The ASX yield of both yeasts can be seen in Table 5. The optimized conditions were determined depending on the actual maximum yield. For the fermentation system of the wheat bran and ATCC 24259 yeast, the maximum yield of 60.54 µg ASX/gm was obtained under the conditions of 23 °C, 90% and pH of 6.0. The maximum yield of 109.23 µg ASX/gm was synthesized from the wheat bran fermentation medium with ATCC 24202 yeast at the conditions of 20 °C, 90% and pH of 5.5, which was the data from previously published work elsewhere and used to compare with the other fermentation systems in this study. The wheat bran and ATCC 24202 yeast system again maximized the yield. Furthermore, 100.25 µg of ASX/gm was a quite high amount of ASX produced from the fermentation system of lentil waste and ATCC 24202.

Table 4. Astaxanthin yield produced in waste medium at optimum yeast growth conditions

Medium	Sugar content (g glu/gm)[a]	Astaxanthin amount (µg ASX/gm)[b]		Astaxanthin amount (µg ASX/g glu)[c]	
		ATCC 24202	ATCC 24259	ATCC 24202	ATCC 24259
Wheat bran	0.44 ± 0.01	87.83 ± 5.67	35.55 ± 1.19	201.49 ± 13.02	81.56 ± 2.72
Lentil waste	0.75 ± 0.0	59.33 ± 4.57	21.70 ± 0.51	79.02 ± 6.09	28.90 ± 0.67

[a]: Sugar content: gram glucose/gram waste as mean value of duplicate results
[b]: Astaxanthin amount: microgram astaxanthin/gram material as mean value of triplicate results
[c]: Astaxanthin amount: microgram astaxanthin/gram glucose as mean value of triplicate results
±: standard deviation

When the yields are compared in Table 4 and Table 5, it is seen that the product yield increased while the temperature and moisture content levels increased at the optimum pH value in the fermentation systems of ATCC 24202 yeast. The highest ASX yield was obtained at a lower temperature and moisture content value than the optimum ones at a constant pH value for the fermentation system of wheat bran and ATCC 24259. Increasing values of pH and moisture content parameters induced product formation for the fermentation system of lentil waste and ATCC 24259.

The ANOVA results and model coefficients for all the fermentation systems are shown in Table 6. The significant status (+) of 'model' is good, but not the same for 'lack of fit', which rates how the model chosen at the determined probability represents a fit between the experimental and predicted data. The quadratic model was fitted to the experimental data of all fermentation systems at $p<0.1$ with a minimum 0.82 correlation for the findings that shows the proportion of the total variation of the response that fitted the model. The effects of the independent variables depending on the model coefficients are also seen in Table 6. They are supportive outcomes of the model for the fitness.

Table 5. BBD design matrix and experimental astaxanthin data of two fermentation systems

Run	x_1	x_2	x_3	Astaxanthin amount (µg ASX/gm)			
				Wheat bran		Lentil waste	
				ATCC 24202	ATCC 24259	ATCC 24202	ATCC 24259
1	-1	0	-1	95.0 ± 2.5	30.5 ± 0.16	37.40 ± 1.0	14.75 ± 0.34
2	-1	0	1	55.9 ± 1.2	41.81 ± 2.47	31.50 ± 1.1	47.73 ± 6.29
3	-1	-1	0	34.98 ± 2.9	17.21 ± 0.48	11.53 ± 1.0	14.16 ± 0.74
4	-1	1	0	66.31 ± 1.8	51.56 ± 2.18	38.02 ± 3.1	36.85 ± 1.46
5	0	-1	-1	28.96 ± 2.9	11.6 ± 0.2	19.17 ± 0.0	9.44 ± 1.13
6	0	0	0	79.64 ± 5.2	37.26 ± 0.87	49.44 ± 0.0	20.06 ± 0.56
7	1	0	1	70.76 ± 4.5	35.37 ± 0.38	51.35 ± 5.60	16.22 ± 1.24
8	0	0	0	86.8 ± 8.7	36.62 ± 1.78	59.59 ± 3.1	14.05 ± 0.42
9	0	0	0	60.85 ± 1.6	32.78 ± 0.92	57.28 ± 8.6	25.3 ± 0.28
10	1	1	0	60.84 ± 2.2	60.54 ± 1.83	90.51 ± 1.0	36.11 ± 3.13
11	1	0	-1	72.84 ± 1.3	20.76 ± 0.71	64.22 ± 2.6	15.65 ± 0.45
12	0	-1	1	33.4 ± 5.3	11.49 ± 0.32	70.20 ± 52.4	16.04 ± 1.16
13	0	1	1	109.23 ± 12.1	51.83 ± 1.68	100.25 ± 0.0	35.82 ± 1.28
14	0	1	-1	84.46 ± 0.0	35.32 ± 1.19	75.15 ± 0.0	58.15 ± 1.41
15	0	0	0	88.99 ± 0.2	21.42 ± 0.56	61.12 ± 2.1	20.89 ± 0.56
16	1	-1	0	26.82 ± 2.8	16.76 ± 1.79	16.70 ± 0.8	13.05 ± 0.04
17	0	0	0	87.7 ± 8.2	19.9 ± 0.62	64.72 ± 5.4	20.54 ± 0.63

±: standard deviation

Table 6. ANOVA results and equation coefficients for each fermentation system

Tools	p<	Wheat bran		Lentil waste	
		ATCC 24202	ATCC 24259	ATCC 24202	ATCC 24259
Model	0.1	+	+	+	+
	0.05	–0.0507	+0.0136	+0.0206	–0.0523
	0.01	–	–	–	–
Lack of fit	0.1	–	–	+	+
	0.05	–0.1701	–0.6817	+0.0183	+0.026
	0.01	–	–	–	–
Std. deviation		15.67	7.48	13.61	8.8
Mean		67.26	31.34	52.83	24.4
C.V.%		23.3	23.86	25.76	35.86
PRESS		19561.22	2233.4	18867.08	7642.66
R^2		0.8246	0.8858	0.8696	0.82
Adj. R^2		0.5991	0.7389	0.7019	0.59
Coefficients (coded values)					
β_0		80.8	29.6	58.43	20.17
β_1		–2.62	–0.1	13.04	–4.06
β_2		24.59	17.77	23.29	14.28
β_3		–1.5	5.29	7.17	2.23
β_{12}		0.67	2.35	11.83	0.09
β_{13}		9.26	0.83	–1.74	–8.1
β_{23}		5.08	4.15	–6.48	–7.23
β_{11}		–11.97	5.74	–19.66	–0.7
β_{22}		–21.5848	1.1846	0.419	5.5748
β_{33}		4.8008	–3.2201	7.3457	4.1216

The results of the response data predictions of RSM and ANN for each experimental run are presented in Table 7. The lower RSME and MAE values and higher R^2 results revealed that the prediction capability of ANN methodology was better than RSM (Table 8). This result was depicted in Figure 6 and Figure 7 by visualizing the data distribution. The fitted lines obtained from the observations and the model calculations supported a good fit with ANN methodology. The fermentation systems of ATCC 24259 yeast indicated more successful predictions of the ASX yield depending on the correlation and error calculations.

Table 7. Predicted astaxanthin data of the methodologies for each fermentation system

Run	Wheat bran				Lentil waste			
	ATCC 24202		ATCC 24259		ATCC 24202		ATCC 24259	
	RSM	ANN	RSM	ANN	RSM	ANN	RSM	ANN
1	86.99	95.0	28.6	30.5	24.17	37.4	17.32	14.75
2	65.49	55.9	37.53	41.81	41.99	31.5	37.97	47.73
3	25.94	34.98	22.05	17.21	14.69	11.53	14.91	14.16
4	73.77	66.31	52.89	51.56	37.61	38.02	43.29	36.85
5	46.0	28.96	8.65	11.6	29.25	19.17	6.12	9.44
6	80.8	80.8	29.6	29.6	58.43	58.43	20.17	20.17
7	78.77	70.76	37.27	35.37	64.58	51.35	13.66	16.22
8	80.8	80.8	29.6	29.6	58.43	58.43	20.17	20.17
9	80.8	80.8	29.6	29.6	58.43	58.43	20.17	20.17
10	69.88	60.84	55.69	60.54	87.35	90.51	35.36	36.11
11	63.25	72.84	25.04	20.76	53.73	64.22	25.41	15.65
12	32.85	33.4	10.92	11.49	56.56	70.2	25.04	16.04
13	92.18	109.23	54.78	51.83	90.17	100.25	39.14	35.82
14	85.02	84.46	35.89	35.32	88.80	75.15	49.15	58.15
15	80.8	80.8	29.6	29.6	58.43	58.43	20.17	20.17
16	19.36	26.82	15.43	16.76	17.11	16.7	6.61	13.05
17	80.8	80.8	29.6	29.6	58.43	58.43	20.17	20.17

Table 8. Statistical evaluation results of RSM and ANN methodologies

Sources	Wheat bran				Lentil waste			
	ATCC 24202		ATCC 24259		ATCC 24202		ATCC 24259	
	RSM	ANN	RSM	ANN	RSM	ANN	RSM	ANN
RMSE	10.0551	5.6881	4.7979	4.0517	8.7337	2.7684	5.6143	1.9469
MAE	8.5645	2.4826	3.969	2.1026	7.1955	1.1929	4.4768	0.7325
R^2	0.8246	0.9439	0.8858	0.9185	0.8696	0.9869	0.8228	0.9787

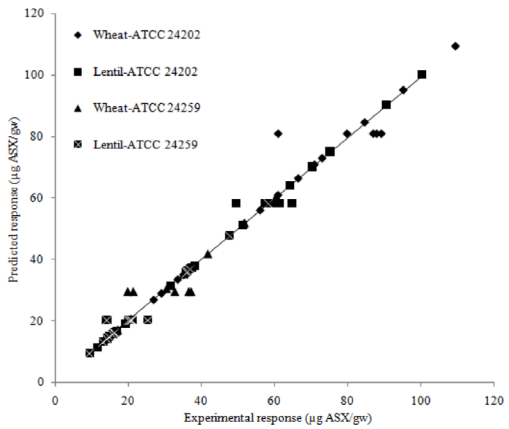

Figure 6. The fitness plot of the ANN model for all fermentation systems

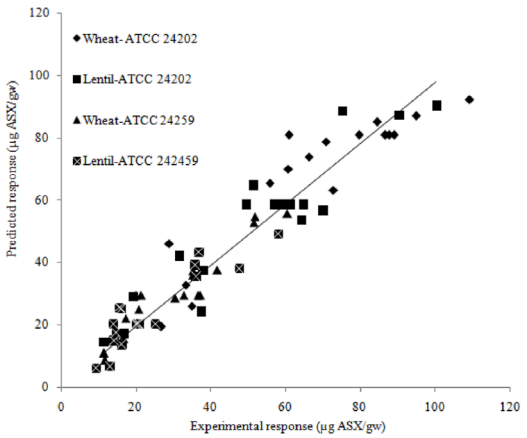

Figure 7. The fitness plot of the RSM model for all fermentation systems

Discussion

Cell growth is an exquisite and complex process which is realized by microorganisms utilizing elements such as carbon, nitrogen and oxygen to grow and produce metabolites. The microbiological growth depends on consuming substrates in the medium which is important for the targeted product formation. In the synthetic medium, the constituents are well known so that the growth may be followed easily. The period, interactions and product formation may also be easy to determine (Bailey and Ollis, 1986). The growths of the yeasts were exhibited in Figure 1 to observe the changes of viability and weight of the cells that are the fundamental items for cell kinetics (Bailey and Ollis, 1986).

It is a well-known phenomenon for the growth of microorganisms that a carbon source is essential, glucose is particularly fundamental and the concentration of the source determines the growth phases and period, metabolite production and limitations in growth. Besides, glucose and fructose are directly utilized by yeasts in their metabolic mechanisms. Sucrose may then be metabolized (Carlson, 1987). The concentration of fermentable sugars is a significant parameter for the growth rate of the microorganisms (Arroyo-López et al., 2009). The product formation of the yeasts, according to the sugar type and concentration -as given in Table 3- showed that it is not necessary to supply more substrate to the microorganism in order to reach a high production level and promote cell growth, which is an explanation supported by Bailey and Ollis (1986). The highest ASX produced by *X. dendrorhous* resulted from having glucose as the sugar type and at its lowest concentration. Meyer and du Preez (1994), Ramírez et al (2000) and Stoklasa et al. (2018) reported that high sugar concentration caused reduction of ASX synthesis. Johnson and Lewis (1979) studied different sugar types, including glucose and sucrose, to produce ASX with *X. dendrorhous* (formerly *P. rhodozyma*), and indicated that the concentration of ASX was higher in the sucrose medium than in the glucose medium. Guo et al. (2010) studied with different strains of *X. dendrorhous* and found that ASX yield is higher in a sucrose medium than glucose or fructose. The increasing sugar concentration revealed a substrate limitation on ASX production for both yeasts in the study, whereas there was no such effect for cell concentration. It has been reported that high glucose concentration inhibits the pigment production for *X. dendrorhous* by Hu et al. (2005). The 'no sugar' medium showed that other nutrients, particularly nitrogen, are required for product formation. The nitrogen source and its concentration are important parameters for ASX production and yield (Ramírez et al 2000; Ni et al., 2007; Guo et al., 2010).

It is very important that products with commercial importance -from cheap and highly nutritious substrates- can be manufactured on an industrial scale. Besides, the optimization of the process is required not only for synthetic media but also in media consisting of this kind of substrate in terms of raw material and environmental conditions due to the high product on goal (Stoklasa et al., 2018). Haard (1988) reported that he managed to produce much more ASX utilizing molasses with *X. dendrorhous* than the synthetic media prepared from glucose, sucrose and fructose sugars. However, a parallel result with the synthetic media was obtained where lower sugar content provided the maximum ASX yield (Table 3). Ni et al. (2007) stated that the optimization of the fermentation conditions for ASX manufacturing is very important. In previous studies, the maximum ASX amount from ATCC 24202 was reached at 19.7 °C and a pH of 6.0 using a synthetic medium within the scope of an experimental design generated by Ramírez et al. (2001). Ananda and Vadlani (2011) studied wheat bran to produce ASX using ATCC 24202. At the end of an 11 day fermentation period, they managed to produce 66.75 µg of ASX/g of substrate from wheat bran.

The yeast *S. salmonicolor* has been mainly studied for carotenoid production by Valduga et al. (2008; 2009): the maximum total carotenoids in the synthetic (913 µg/L) and agro-industrial (502 µg/L) media were produced by the yeast in approximately 100 hours; and the maximum concentration of 1.019 µg/L for the total carotenoids was obtained using the same yeast in a synthetic medium containing 40 g/L glucose at 25 °C, and an initial pH of 4.0 after the optimization with response surface methodology.

Pérez-Guerra et al. (2003) and Mitchell et al. (2004) emphasized that moisture content is the most critical parameter for solid-state fermentation systems. In parallel, the moisture content parameter is the most effective parameter for ASX production in all the fermentation systems studied in this work (Table 6). The interactions of the moisture content with the pH and temperature induced a higher ASX yield. Moreover, the interaction with high moisture content engendered an increase in the yield, whether or not the other parameter had a high or low value. The water content of the synthetic YMB media was higher than the waste media when prepared with 90% water. However, the amount of ASX produced in those waste media was higher (Table 4). It is thought that the nutrient content of the wastes has an enhancing effect on the yield whether or not the other parameters changed. Improving effects of medium ingredients such as vitamins and trace elements on ASX synthesis were reported by Shewe et al. (2017). The increasing temperature affected only the pigment production of *X. dendrorhous* in the lentil fermentation medium positively. In the wheat bran media fermented by the yeasts, the increasing temperature introduced favorable interactions with the pH and moisture content for the ASX production. The effect of pH on the ASX biosynthesis was positive for *X. dendrorhous* and wheat bran fermentation system. However, Shewe et al. (2017) reached the result of induced ASX production by lowering pH.

The ANN-based modeling approach was better in fitting the inputs/variables to the response in comparison to RSM. More accurate results in estimations using the ANN methodology can be understood by using the statistical (Table 8) and parity plots (Figure 6 and 7) for ASX production. It could be stated that ANN is a good and powerful tool for the modeling of the non-linearity of bioprocesses, whereas RSM is the most used method for the optimization of fermentation conditions. This statement is also supported by Shafi et al. (2018).

The media, including high levels and a 'no sugar' had an

adverse effect on ASX production in the submerged fermentation systems. The investigation of whether *S. salmonicolor* has potential as an ASX producer on an industrial scale resulted in the success of *X. dendrorhous*. The experimental design results showed that the solid fermentation system of the wheat bran and *X. dendrorhous* produced the highest ASX amount at the highest moisture content level. The lentil waste medium provided a quite high productivity and demonstrated that it could be a good resource for biosynthesis. The Artificial Neural Network was statistically determined to be the more effective and accurate modeling for astaxanthin biosynthesis from solid waste materials.

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