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Production of Astaxanthin by *Xanthophyllomyces Dendrorhous* on Fruit Waste Extract and Optimization of Key Parameters using Taguchi Method

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Abstract

Xanthophyllomyces dendrorhous yeasts are capable of synthesizing carotenoid class astaxanthin pigment on agro-industrial wastes. The usefulness of fruit waste extract (FWE) as a sole source of energy for astaxanthin production was done using *X. dendrorhous* (MTCC No: 7536) and the Taguchi method of optimization technique was used to maximize pigment production conditions. Effect of key parameters like pH, temperature, and agitation was studied using L9 orthogonal array by three factor-three level approach and the significant parameters influencing pigment yield were tested out by analysis of variance. Significant parameters affecting pigment yield were suggested by statistical calculations and were tested by a validation test. Identified optimum conditions (pH-5, Temp-20 °C and Agitation-200 rpm) resulted in an increase of pigment production by 10 %. This study shows the use of medium from waste surplus like FWE for pigment production is promising and provides huge scope to be scaled-up for the large scale production.

Key words: carotenoid, astaxanthin, agro-industrial wastes, Taguchi method, optimization

Introduction

Carotenoid class astaxanthin pigment belongs to the group of lipophilic tetraterpenes, it is a combination of eight isoprene units (C_5) and has important application in nutraceutical, cosmetic, food and feed industries due to its high antioxidant activity (Rao et al. 2005; Schmidt et al. 2011; Zhou et al. 2018; Henke and Wendisch 2019). Astaxanthin pigment produced chemically contains only 8 % astaxanthin and costs about $25,000-30,000/Kg (Tinoi et al. 2006). At present, synthetic astaxanthin is the major source of this carotenoid and is widely being used in fish feeds (Lim et al. 2018). However, food and feed additives from biotechnological processes are favored by consumers to that of substances produced by chemical technologies (Schmidt et al. 2011; Henke and Wendisch 2019). Among the microorganisms, *Brevibacterium* (Johnson and An 1991), *Mycobacterium lacticola* (Nelis and Leenheer 1991), *Agrobacterium auratium* (Bon et al. 1997), *Haematococcus pluvalis* (Waldenstedt et al. 2003) and *X.dendrorhous* (Sanpietro and Kula 1998; Tinoi et al. 2006) have the perspective to be used for commercial production of astaxanthin as 80-90 % of the total carotenoids produced by these yeasts accounts for astaxanthin.

Amongst all red-pigmented heterobasidiomycetes yeast, *X.dendrorhous* can produce carotenoids on cheaper substrates like different agro-industrial raw materials with high yields (Frengova and Beshkova 2008) and this yeast strain has the property towards the assimilation and metabolizing mono, di and polysaccharides and also organic acids and alcohols (Schmidt et al. 2011). Till to date, *X.dendrorhous* was in the developmental stage and there is huge attention on this versatile strain to test its ability for significant astaxanthin production on various cheaper substrates (Dufossé 2006; Xie et al. 2014; Stoklosa et al. 2018; Gervasi et al. 2018). Aiming towards cost-cutting approaches, substrates such as waste streams like those from sugar manufacturing processes or the corn wet milling industry, white grape juice, enzymatic eucalyptus wood hydrolyzates (Cruz and Parajo 1998), hemicellulosic hydrolyzates of eucalyptus globules (Parajó et al. 1998), peat hydrolysate (Vázquez and Martin 1998), yucca medium (based on date juice) (Ramírez et al. 2001), corn steep liquor (Kesava et al. 1998), rapeseed meal hydrolysate (Tuan Harith 2019), mesquite pods (Villegas-Méndez et al. 2019) and so forth (Stoklosa et al. 2018) were successfully employed for the production of astaxanthin.

Till to date, very limited research has been carried out on fruit wastes and their extracts as a sole source of energy for astaxanthin production using *X.dendrrorhous* (Jirasripongpun et al. 2008; Mata-Gómez et al. 2014). India contributes to 10.9 % of the world's fruit production (FAO 2018) and over the last few years research focus is towards reprocessing and reuse of various fruit wastes to generate nutritive and valuable products (Mahadevaswamy and Venkataraman 1990; Viswanath et al. 1992). Previous findings on fruit wastes signify that they are a rich source of carbohydrates and other minor nutrients to support microbial growth (Mahadevaswamy and Venkataraman 1990; Viswanath et al. 1992). The surplus fruit wastes that are frequently disposed of in the environment after their exhaustive extraction may be possibly used as low-cost substrates for the production of microbial bioactive compounds like pigments. The availability of convenient simple sugars along with macro and micronutrients makes fruit waste as a suitable source for the production of numerous pigments by a variety of microorganisms. The above-ascribed reports used special conditions and/or enriched the

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substrate media for high yield pigments production. In contrast, eying on cheaper and quick synthesis process devoid of providing special conditions, we investigated the capacity of readily prepared fruit waste extract (FWE) as a sole substrate for astaxanthin production by a purchased strain, *X.dendrrorhous*. Therefore, this study outlines the optimum production conditions for carotenoid pigment by *X.dendrrorhous* on FWE using a simple Taguchi method.

Materials and Methods

Chemicals and microorganism

Acetone, ethanol, methanol, ascorbic acid, sodium chloride (NaCl), sodium hydroxide (NaOH), hydrochloric acid (HCL), potassium sodium tartrate and diethyl ether were purchased from Merck, India. The strain *Xanthophyllomyces dendrorhous* (7536) was obtained from MTCC Chandigarh, India. The yeast was maintained on Yeast extract/malt extract (YM) media agar plates at 4 °C and sub-cultured monthly in YM broth media. YM media and agar were procured from HiMedia chemicals, India.

Substrate preparation

Fruit waste is obtained from a juice shop from the local market, Rourkela, Odisha, India, which comprises pineapple waste, orange waste and a minor portion of pomegranate waste. Fruit waste extract (FWE) media is prepared as stated in our earlier report (Tarangini and Mishra 2014) where a mixed waste of 1 kg was boiled for 30 min to extract the soluble sugars by adding 2000 mL of distilled water. The pH of the FEW was observed to be ~7.1. The CHNS (Carbon, Hydrogen, Nitrogen, Sulfur) analysis of the resultant FWE was performed by Vario EL Cube CHNS analyzer and the values are as follows: C (62.35 %), H (4.17 %), N (4.18 %), S (1.08 %).

Culture conditions

The inoculum was cultured in YM broth at 22 °C for 48 h. The yeast cells were collected by centrifugation and washed twice with distilled water. 1 mL of microbial solution (1 % w/v) was inoculated in a 250 ml Erlenmeyer flask having a 50 ml FWE medium. The inoculated cultures were maintained at 22 °C in an incubator shaker at a shaking speed of 150 rpm for pigment production. Here, the YM medium is used as the reference medium for comparison with the FWE medium. Each experiment was repeated thrice to minimize the chance of error.

Pigment production, extraction, and sugar estimation

The pellet of yeast cells obtained after centrifugation was washed twice with distilled water. Pigmented cells were treated with methanol: acetone (1:1) solvents and then centrifuged. This process was repeated until colorless biomass was obtained. The extracted pigment was subjected to phase separation through treatment with equal volumes of petroleum ether and 10% NaCl. The carotenoid was collected from the diethyl ether phase and examined at 474 nm by UV-visible spectrophotometer using an extension coefficient of $A_{1\ cm}^{1\ \%}$= 2,100 (Schroeder and Johnson 1993). Obtained carotenoids were additionally purified using thin layer chromatography using stationary phase - TLC silica gel 60 F_{254} and hexane: methanol (7:3) as a stationary phase and solvent phase respectively. The purified pigment was further analyzed in FTIR to resolve the functional groups.

The 3,5-dinitrosalicyclic acid (DNS) method of miller (Miller 1959) was used to estimate the reducing sugars in the FWE medium. DNS reagent is prepared by dissolving 10 gm of DNS in 2 N NaOH solution of 200 mL. About 1 ml of the sample was centrifuged at 3,500 X *g* for 5 min, 1 ml of DNS reagent was added to the supernatant and then boiled for 5 min. 0.3 ml of 40 % potassium sodium tartrate was added and after that, the sample was placed in the ice bath for rapid cooling. Now, distilled water was added to the samples and vortexed for 5 min. The optical density was measured at 575 nm. Known amounts of glucose solutions are used as controls to determine reducing sugars in FWE.

Taguchi approach and statistical analysis

For the investigation, a simple Taguchi orthogonal array design was selected where there are three factors and three levels. From the reported studies, pH (4-6), temperature (15-25 °C) and agitation (100-300 rpm) in the specified range are significant in obtaining higher amounts of astaxanthin production by *X.dendrorhous* (Domíguez-Bocanegra and Torres-Muñoz 2004; Zheng et al. 2006; Grazma-Michalowska and Stachowiak 2010). In this study, the Taguchi approach was used for the optimization of pigment production conditions. The optimization of the ascribed factors in three different levels is illustrated in Table 1. To perform the Taguchi technique, 9 different experiments using the L9 orthogonal array was run as shown in Table 2. Based on the primary results, a verification test was performed to check the optimum condition for higher pigment yield and analysis of variance (ANOVA) for the obtained results was inspected. Design of experiments, ANOVA and the optimization of the process was done using MINITAB- 14 software.

Table 1. Factors and their levels studied by the Taguchi method.

Factor	*Level 1*	*Level 2*	*Level 3*
pH	4	5	6
Temperature (°C)	15	20	25
Agitation (rpm)	100	200	300

Free radical scavenging activity

The effect of astaxanthin from *X.dendrorhous* on DPPH free radical was studied as per the reported method (Grazma-Michalowska and Stachowiak 2010) with some modifications. Initially, 2 mg/ml pigment suspension was prepared in varying volumes of ethanol (10 to 60 µl). To this suspension, 1 ml of DPPH solution containing 0.1 mM DPPH and 99.5 % ethanol (99.5 %) was added. This mixture was thoroughly mixed and allowed to stand for 40 min at room temperature in the dark. Ascorbic acid is used as a positive control and the pigment solution without DPPH serves as a negative control. The radical scavenging activity was

measured as a decrease in the UV-visible absorbance spectrum of DPPH at 517 nm wavelength and was calculated using the following formula:

$$\% \text{ Scavenging activity} = \frac{(Ac - As)}{(Ac)} \times 100$$

Where A_c, A_s are absorbance values from control and sample.

Analytical methods

UV-visible spectra and radical scavenging activity were measured using a UV-visible spectrophotometer (Shimadzu, UV-3600). Morphological characteristics of the used microorganism was studied by using scanning electron microscope (SEM, JEOL JSM 6480 LV), active functional groups of the produced pigment was identified through Fourier infrared spectroscopy (FTIR, Bruker, USA) supported with a horizontal attenuated total reflectance (ATR) device with zinc selenide (ZnSe) crystal.

Results and Discussion

X. dendrorhous are popular among pigment-producing yeasts and astaxanthin produced by them are having significant commercial scope in the fields of cosmetics, pharmaceuticals, and agriculture (Frengova and Beshkova 2008; Schmidt et al. 2011; Mata-Gómez et al. 2014). Astaxanthin production from cheaper substrates stood in focus and optimization of the process parameters for the pigment production are extensively studied to emphasize its production in commercial-scale (Vázquez and Martin 1998; Parajó et al. 1998; Kesava et al. 1998; Cruz and Parajo 1998; Ramírez et al. 2001; Dufossé 2006; Stoklosa et al. 2018; Gervasi et al. 2018). Substrate utilization/growth and pigment production by this yeast were significantly influenced by important factors like pH, temperature and agitation (Vázquez and Martin 1998; Parajó et al. 1998; Kesava et al. 1998; Cruz and Parajo 1998; Ramírez et al. 2001; Dufossé 2006; Stoklosa et al. 2018; Gervasi et al. 2018). Hence, these factors played an imperative role in the cost-effectiveness of pigment production.

Experimental design by Taguchi method

The principal objective of this study was to optimize the production of carotenoid class pigment, astaxanthin by *X. dendrorhous* on FWE using a Taguchi method which is a fractional factorial experimental design. Unlike traditional one variable at a time examination, multivariate analysis like the Taguchi method has advantages of saving chemicals, experimentation time, qualitative and quantitative parameter inclusion, etc. In this study, the effects of key parameters like pH, temperature, and agitation were studied and the results of experiments (Table 2) display that the maximum average yield of pigment is 1.252 mg/g biomass.

Table 2. Levels of three different factors applied in each of nine trials, with observed results. The tabulated output values are average values after replicating thrice.

pH	Temperature (^{O}C)	Agitation (rpm)	Astaxanthin (mg/g)	Biomass (g/L)
4	15	100	0.402	0.321
4	20	200	0.556	0.548
4	25	300	0.525	0.620
5	15	200	1.240	1.133
5	20	300	1.252	1.340
5	25	100	0.910	0.728
6	15	300	0.978	1.280
6	20	100	0.880	0.704
6	25	200	1.211	1.103

Fig. 1 depicts the main effect of each of the key factors, which means the average results obtained for each factor. To screen significant factors; the ANOVA with F test was employed for the simulation reflecting the data as shown in Table S1. The F value of 23.71 from ANOVA states that pH has a significant effect on pigment production while the parameter temperature showed a negligible effect on pigment yield. Considering the yield contributions from Fig. 1, the optimum experimental condition was selected from the attained result with conditions as follows: pH 5, temp 20 ^{o}C and agitation 200 rpm.

Table S1. Analysis of variance of the main effects of factors

Analysis of Variance for Means						
Source	**DF**	**Seq SS**	**Adj SS**	**Adj MS**	**F**	**P**
pH	2	0.7009	0.7009	0.3504	23.71	0.04
Temperature	2	0.0007	0.0007	0.0003	0.03	0.974
Rpm	2	0.1160	0.1160	0.0580	3.93	0.203
Residual Error	2	0.0295	0.0295	0.0147		
Total	8	0.8474				

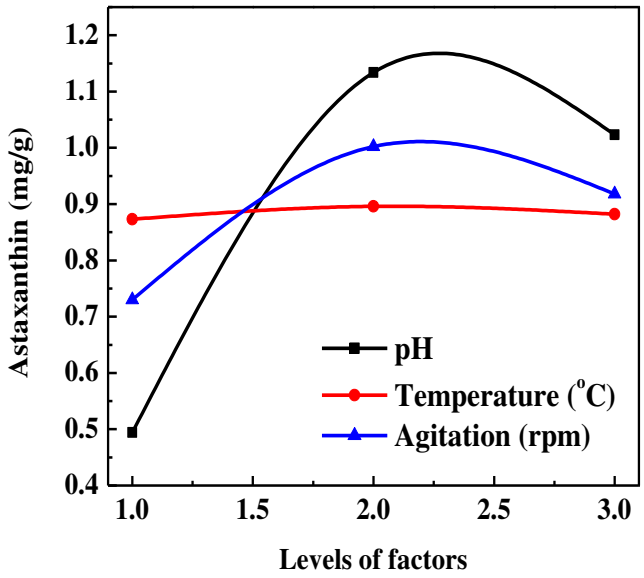

Figure 1. Main effects of factors or average of obtained results as mg/g biomass in which each factor is at a given level. For the description of 'levels' refer to Table 1.

Table S2. Optimum conditions suggested by statistical analysis after performing the experiments.

Factor	Level description	Level	Contribution in 'mg/g'
pH	5	2	1.134–0.883 = 0.251
Temperature (ºC)	20	2	0.896 –0.883 = 0.013
Agitation (rpm)	200	2	1.00 –0.883 = 0.117

Validation of the model

Fig. 1 and Table S2 show the expected conditions for maximum pigment production. Statistical calculations suggested that if the conditions were chosen as given in Table S2, the pigment production should reach 1.264 mg/g biomass. Furthermore, after performing the investigation with yeast cells in the FWE medium at the said condition, the produced astaxanthin yield was 1.40 ± 0.14 mg/g. The difference between the predicted and actual results was ~ 10 % and is regarded as acceptable and promising. The morphology of the *X. dendrorhous* cells and the color change due to astaxanthin production in FWE was shown in Fig. 2.

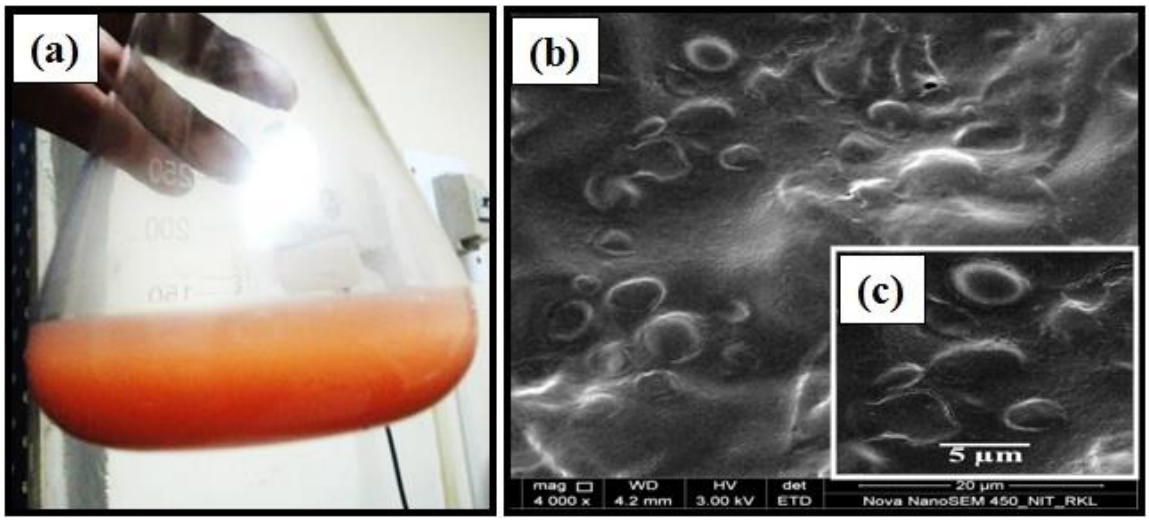

Figure 2. (a) Pigmentation of FWE by yeast cells of *X. dendrorhous* at optimum conditions. (b) and (c) are lower and higher magnification SEM images of the yeast cells from the medium.

Pigment yield, biomass trend and glucose utilization

Table S2 shows the optimum condition for astaxanthin pigment production. Growth kinetics, pigment production, cell mass, and substrate utilization has been studied at the optimum experimental condition. Fig. 3a shows that both biomass and astaxanthin production enhanced over time as total soluble sugars content decreased. The maximum levels of biomass (~22.4 g/L) and astaxanthin (~31.5 mg/L) are seen at 84 and 96 h, whereas the carbon source i.e. soluble sugars were depleted and reached a constant level from 96 to 144 h (Fig. 3a).

From the figure, the time course of the biomass growth displayed the characteristic exponential and stationary phases and reached a maximum level after about 84 h. Furthermore, it was noted that the pigment production was not constant at the stationary phase of biomass growth and went on increasing up to 108 h. The observed behavior infers the ability of the used strain to give significant amounts of astaxanthin even in the stationary phase, which is a beneficial effect in the used conditions and substrate, FWE.

From this result, it is clear that the astaxanthin production was partially dependent on biomass growth and still occurred even after growth and sugar diminution. Such activity is certainly related to the fact that *X.dendrorhous* excretes and stocks some extracellular carbon intermediates, which stimulate late carotenogenesis (Johnson and An 1991). The spectral property of the produced astaxanthin in FEW media was shown in Fig. 3b. The absorption maximum from the UV-Visible spectrum was identified to be at 474 nm in methanol and was in good agreement with the reported value (Buchwald and Jencks 1968).

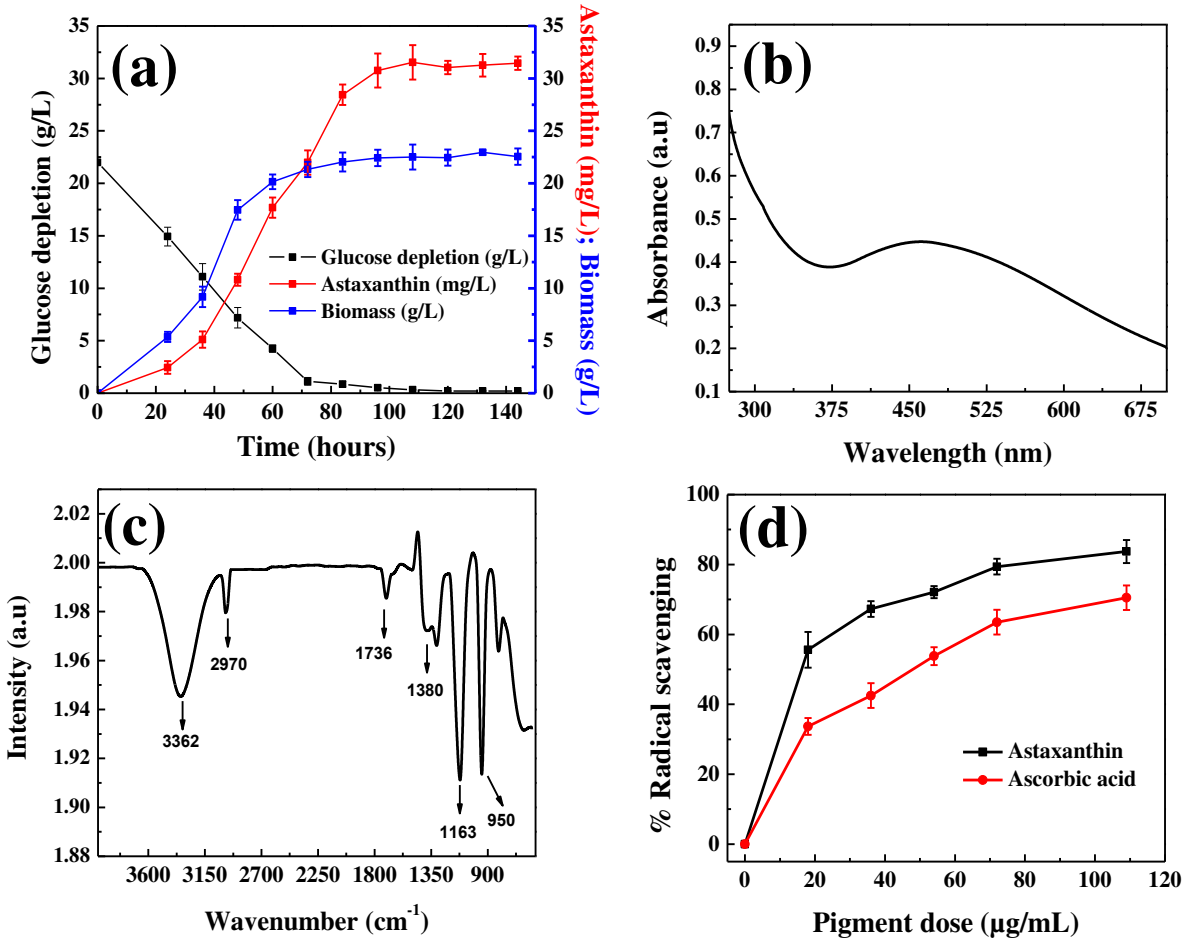

Figure 3. (a) Time course of the growth and production of astaxanthin by *X.dendrorhous* in FWE. The experiment was carried out at optimum conditions i.e. pH (5), temperature (20 ºC) and agitation (200 rpm). (b) UV-Visible and FTIR spectrum (c) of the produced pigment in methanol. (d) Antioxidant activity (DPPH radical scavenging) of astaxanthin and ascorbic acid.

FTIR analysis

The purified pigment from the TLC plate was dissolved in ethanol and examined for FTIR spectral analysis and the results are shown in (Fig. 3c). According to Coates et al., (Coates 2006) the peak descriptions of the FTIR spectra can be illustrated as follows: The peak at 3362 cm^{-1} shows the presence of the O-H hydrogen bond and the peak at 2970 cm^{-1} is due to methine C-H stretch. A peak at

1736 shows the presence of a C=O bond in the molecule and the peak at 1380 cm^{-1} is due to the methyl C-H asymmetric band. The peak at 1163 cm^{-1} is for C-O stretch and the peak at 950 cm^{-1} stands for the skeletal vibrations. The spectral behavior of the pigment strongly resembles the structure of astaxanthin which is predominantly obtained by *X.dendrorhous* (Chen et al. 2007).

DPPH radical scavenging activity

DPPH is a stable free radical that shows maximum absorbance at 517 nm in the UV-visible spectrum. When DPPH radicals come across a proton-donating substrate such as an antioxidant, the radicals would be scavenged and the resultant absorbance would be reduced (Scalzo 2008). The decrease in absorbance is taken as a measure for radical-scavenging activity. The DPPH radical-scavenging activity was investigated at different concentrations from 0 to 109 µg/ml of the produced astaxanthin and the results are shown in Fig. 3d.

After 40 min of incubation, it could be observed that the radical scavenging activity increased swiftly with an increase in astaxanthin dose when compared to ascorbic acid (control) and reached up to ~85 % at the dose of 109 µg/ml. However, it is also noted that ascorbic acid facilitated in achieving only ~70 % under the same conditions. Even though astaxanthin and ascorbic acid both showing an increase in % radical scavenging, it is noteworthy that the former was higher than the latter at any moment within the studied pigment dose (Fig. 3d).

In summary, the key factors that independently influence biomass growth and pigment production were identified by the Taguchi technique. At identified key parameters, FWE appears to be a promising substrate since a remarkable astaxanthin production (1.4 ± 0.14 mg/g of biomass) was obtained when compared to the YM medium (i.e. 1.66 ± 0.21 mg/g biomass) at the same culture conditions. YM medium is an expensive source for astaxanthin production with C to N proportion of 1.0 and 0.3 %, while FWE is an inexpensive alternative with C to N proportion found to be 1.0 and 0.066 % (observed by CHNS analysis). This work shows that the FWE medium plays a key role in astaxanthin production by *X.dendrorhous* and it is worth noting that, in our case, we achieved increased astaxanthin production with free radical scavenging activity without any substrate supplements and/or special conditions. Free radical scavenging activity of the obtained pigment gave ~15 % better activity than ascorbic acid and encourages its applicability as a constitutional ingredient in cosmetics.

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