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Agro-industrial wastes for the synthesis of carotenoids by *Xanthophyllomyces dendrorhous*: Mesquite pods-based medium design and optimization

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HIGHLIGHTS

- A wastes-based media for carotenoids biosynthesis was designed and optimized.
- Taguchi method was used for optimizing the component levels in the designed media.
- A strategy to define the impact of interactions among factors was proposed.
- Process performance was transferred to a more favorable zone of the solution space.
- Carotenoids production with improved media was nearly 40% higher than the control.

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ABSTRACT

Utilization of agro-industrial wastes as cheap nutrient sources for the microbial production of added-value compounds in combination with a statistical approach integrated into earlier process development is a route to ensure a competitive and sustainable bioprocess. In this context, a wastes-based medium was designed and optimized for the biotechnological production of carotenoids by *Xanthophyllomyces dendrorhous*. The work involved different stages such as waste screening (corncob, cotton husk, mesquite pods, corn steep liquor, residual brewery yeast, and urea), optimization of medium components (Taguchi method), data analysis, and validation of optimum settings. Mesquite pods and corn steep liquor were the selected wastes. The relative influence of factors and individual effects of media components were identified. Mesquite extract was the factor with the highest influence on the total production of carotenoids, while corn steep liquor impacted the yield (carotenoids/biomass). A strategy to define the possible impact of interactions among factors (main disadvantage of saturated designs) was proposed and it was demonstrated that the process performance can be improved with minimum experimental requirements. Optimum designed medium contained (g/L), mesquite pods extract (20), corn steep liquor (3) and yeast extract (3) which allowed to surpass by 40% the carotenoids production using the control medium.

1. Introduction

Carotenoids are organic compounds that provide yellow to red pigmentation in nature, generally grouped as carotenes or xanthophylls. These pigments have antioxidant activity in cells, and some are vitamin A precursors; as a result, they can reduce the risk of degenerative diseases such as cancer, hypertension, and atherosclerosis [1,2]. Besides, carotenoids have a wide application in the industry as a food colorant, animal feeding (i.e., salmon, shrimp, poultry) and nutritional supplement [3,4]. Therefore, the carotenoids market is projected to reach $ 1.8 billion USD by the year 2020 [5]. Within carotenoids, astaxanthin is a xanthophyll reported to possess anti-inflammatory and antioxidant properties, which grant an added value in food and pharmaceutical industries [6]. As the most natural pigments, astaxanthin obtained by chemical synthesis has more commercialization between industries due to lower production cost [7]; nonetheless, long term usage of synthetic compounds has concerns regarding negative effects on health. Owing to the situation, the biotechnological processes offer a

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natural and ecological alternative source of metabolites [8]. There are many reported microbial sources of astaxanthin, but the major pigment exploited sources for commercial production are the microalgae *Haematococcus pluvialis* and the yeast *Xanthophyllomyces dendrorhous* since they have the highest yields in nature [9,10]. *X. dendrorhous* appears a better source for a competitive industrial process due to its high growth rate in shorter cycles, meaning a reduction time for astaxanthin production [11]. On the other hand, the large-scale production of astaxanthin from microalgae demands strict control and maintenance, and it requires a more extended production period [11,12]. Even though microbial production of bio-compounds has a better acceptance over the years, still it is not a quite profitable process mainly due to the high production costs. Therefore, several studies have the principal goal of getting affordable microbial pigments for commercial competition [13]. Given that raw materials used as substrates represent from 30 to 70% of a bioprocess cost, the strategy of using agroindustry wastes as inexpensive nutrient sources for microorganisms can lower the production costs and at the same time mitigating the disposal problem of agricultural activities [14]. Certainly, the above brings out the concept of biorefinery, which has been presented as an alternative for producing not only biofuels but also added-value compounds such as carotenoids [15]. The trend is evident; there is a need for sustainable and eco-friendly processes, which has also spread towards the production of microbial colorants [16,17]. In the same way, different agro-food residuals from cassava, corn, coconut, mussel, and rice had been used as low-cost astaxanthin sources in *X. dendrorhous* with successful production yields [18]. From this perspective, there are raw materials that can be explored as substrates in microbial carotenoids production. For instance, seeds from *Prosopis* spp (mesquite pods) have application as feedstock, but also they have been used as the carbon source in fermentations due to their high sugar concentration (> 30% dry weight based) [19,20]. Additionally, it has been reported that this type of strain is capable of metabolizing hydrolysates-based media; therefore, hydrolysates from cellulosic raw materials particularly corncob and cotton husk have the potential to be utilized as raw materials [21]. On the other hand, corn steep liquor has been described as a promising low-cost nitrogen source in biosurfactants production [22] as well as residual brewery yeast that contains additional nutritious supplements for the microorganism [23]. Another material with the potential to reduce the process costs is the urea which is an accessible, low-cost nitrogen source. The selection and application of a new raw material (especially an agroindustrial waste) and its biotransformation into high value-added compounds requires the evaluation of various aspects. For instance, regional and seasonal availability of these alternative raw materials must be considered in order for the process to be competitive and sustainable. Government legislations should also be considered since in some regions, incentives are offered for the use of certain raw materials or taxes are imposed in others. Moreover, the bioprocess requirements are critical; if the product is directly synthesized by the catabolism of a specific carbon source, then this factor will drive the selection of the raw materials for the culture medium formulation. Lastly, proper optimization of the formulation of the agroindustrial wastes-based media will ensure the synthesis of high yields, thus reduced production costs. Within the known optimization methodologies, the Taguchi method has gain popularity especially at industrial scale level because it involves minimum experimental trials for process improvement [24]. This methodology involves using orthogonal arrays with many advantages such as the integrated search for an optimum, consistency of performance, and it does not require the fit between a model and the data [25,26]. However, one of the drawbacks of the Taguchi method is that when a saturated design is used, the effect of the interactions is not considered. Therefore, an essential step in the Taguchi method is a confirmation of the optimum settings, principally when the optimum is different from the runs included in the orthogonal array [26]. In this context, the objective of this work was to design and optimize a waste-based medium for the biotechnological production of carotenoids by *Xanthophyllomyces dendrorhous* following the Taguchi methodology.

2. Material and methods

2.1. Raw material and media

Corncob, cotton husk, mesquite pods and residual brewery yeast (RBY) were collected from local farmers (Torreon, Coahuila). Corn steep liquor (CSL) and urea (UR) were provided by the Department of Chemical Engineering (Saltillo, Coahuila). The solid raw materials were milled using a cutting mill (Retsch SM 100, Germany) and dried at 60 °C in a convection oven (CEB-2600, México). Fermentable sugars from corncob and cotton husk were obtained by acid hydrolysis pre-treatment with sulfuric acid (1.5% v/v, 121 °C, 15 psi, 60 min) and detoxified with activated charcoal [27]. While for mesquite pods, fermentable sugars were extracted in water at 45 °C for 60 min by constant stirring. Yeast malt (YM) medium (g/L; glucose, 10; peptone, 5; yeast extract, 3 and malt extract, 3) was used for yeast growth.

2.2. Microorganism and inoculum preparation

Xanthophyllomyces dendrorhous ATCC 24202 was acquired from the American Type Collection (ATCC, Beltsville, USA). The strain was loop inoculated in a 125 mL Erlenmeyer flask containing 25 mL of YM medium for 48 h in a rotatory shaker (Inova 94, New Brunswick Scientific, USA) at 200 rpm and 20 °C. The inoculum size was 10^6 spores/mL defined by Neubauer chamber.

2.3. Waste based-medium design and optimization

Medium design and optimization involved different stages (Fig. 1): i) waste screening for selecting carbon and nitrogen sources to replace the original components of control media, ii) optimization of culture waste based-medium components with the Taguchi method; iii) data analysis to define the relative influence of factors, their individual effects, and optimum settings; iv) validation trial (confirmation test) of optimum settings.

2.3.1. Wastes screening and culture conditions

The screening experiments consisted of glucose (carbon source) and peptone (nitrogen source) substitution in the YM medium. Corncob hydrolysate (CCH), cotton husk hydrolysate (CHH) and mesquite pods extract (MPE) were used as raw materials for carbon source substitutes where total sugar content was adjusted to 10 g/L. Similarly, the nitrogen source was replaced by RBY, CSL, and UR at the same level of peptone composition in the YM medium (5 g/L). All screened media was adjusted to a pH of 5.5. Experiments were conducted in a working volume of 25 mL and incubated (120 h, 200 rpm, 20 °C) in an orbital shaker (Inova 94, New Brunswick Scientific, USA). YM medium was used as the control, and the experiments were performed in triplicate.

2.3.2. Optimization of waste-based medium components

Once the production media was selected, the Taguchi method was applied to identify the potential effect of the media components on the yeast growth and synthesis of astaxanthin. Choosing the most suitable design involves first defining the number of factors. The medium is composed of four factors (Waste carbon source, A; waste nitrogen source, B; yeast extract, C; malt extract, D). For proper optimization, at least three levels are required; thus, an L9 array is suitable for analyzing four factors at three different levels. The experimental matrix is shown in Table 1. Studied levels were designated by coded values (-1, 0 and 1) indicating the low, intermediate, and high levels of each. The actual levels were selected based on the results of the screening study; thus, these are detailed in the results section. Process conditions were the same used for the residues screening. Evaluated responses were

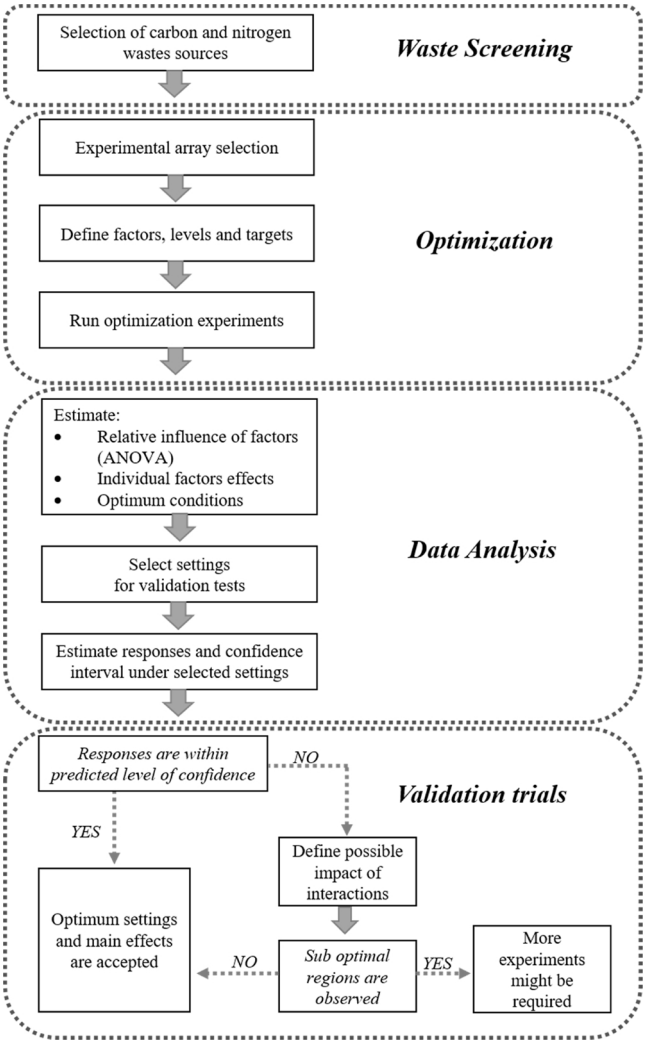

Fig. 1. Schematic flow diagram for waste based-medium design and optimization.

Table 1
Experimental Matrix L9 (Four factors, three levels).

Run	Factors			
	Carbon source A	Nitrogen Source B	Yeast Extract C	Malt Extract D
1	−1	−1	−1	−1
2	−1	0	0	0
3	−1	1	1	1
4	0	−1	0	1
5	0	0	1	−1
6	0	1	−1	0
7	1	−1	1	0
8	1	0	−1	1
9	1	1	0	−1

Levels are described in the results section.

carotenoids production (P, μg/L), biomass (X, g/L) and carotenoids yield on biomass (Yx, μg/g). The optimization target was to simultaneously increase only P and Yx.

2.3.3. Data analysis

Factorial ANOVA design ($p < 0.05$) was used to analyze all responses for all the low-cost medium (screening). The Post-host Tukey test was used for means comparison. For the Taguchi trials, the responses were analyzed first with a two-way Analysis of Variance (ANOVA). If all interactions were assumed negligible, then the level for each factor that maximizes the response was chosen independently, and this level was the one with the subset of data giving the higher average response [33]:

$$il_j = il|_{Max(\bar{R}_{i,j})} \quad (1)$$

where il_j is the selected level of the factor j and $il|_{Max(\bar{R}_{i,j})}$ the setting i of factor j that presented the highest average of each response at each factor among the evaluated levels ($\bar{R}_{i,j}$).

The estimate responses (R_{est}) were obtained using marginal means addition [25]:

$$R_{est} = \bar{R} + \sum_{j=1}^{n_f} (\bar{R}_{il_j} - \bar{R}) \quad (2)$$

where $\bar{R}$ is the average of all replicates obtained at all the nine runs (grand average), n_f is the number of factors, $\bar{R}_{il_j}$ is the average of the replicates obtained with factor j at the chosen setting il_j. The confidence interval of the predictions for 90% confidence level was calculated as previously reported [26]. Statistical analyses were made with STATISTICA 9. (StatSoft, Inc., 2005, USA) and Excel (Microsoft, 2010, USA).

2.3.4. Validation trials

When an optimization methodology is applied where the entire solution space is not tested, it is suggested to run a validation test with the optimum conditions selected mainly if these settings are not among the nine combinations initially tested (Table 1, Matrix L9). Therefore, selected conditions for validation trials will depend on the data analysis of the resulted L9 data. It has been suggested to define the possible impact of interactions with a further analysis of the data of the nine runs of de L9 design as if it was the result of a full factorial design where only two factors are significant, the other two are not, and their previous estimated effect is due to the components of the interactions between the other factors mixed in that column [25]. For each pair of factors (A–B, A–C, A–D, B–C, B–D, C–D), a surface model is used to estimate the response performance at the conditions selected for the validation trials:

$$R_{est,m,n} = a_{0,m,n} + a_{1,m,n} il_m + a_{2,m,n} il_n + a_{3,m,n} il_m^2 + a_{4,m,n} il_n^2 + a_{5,m,n} il_m il_n + a_{6,m,n} il_m^2 il_n + a_{7,m,n} il_m il_n^2 + a_{8,m,n} il_m^2 il_n^2 \quad (3)$$

where $R_{est,m,n}$ is the estimated response obtained by assigning the results exclusively to the main and interactive effects of factors m and n (assuming the other factors to be negligible), il_m and il_n are the selecting settings used for the validation trials for factors m and n, respectively, and the coefficients a are obtained from the analysis of the L9 data as a full factorial design of factors m and n. The model not only considers the linear by linear interactions but also the linear by quadratic and quadratic by quadratic components.

2.4. Analytical methods

Biomass was recovered by centrifugation for 5 min at 12,000 rpm (Sigma-18KS, Germany) and washed twice with distilled water. The washed biomass was dried for 12 h at 60 °C. For total carotenoids quantification, fresh recovered cells were used for cell disruption using the Dimethyl sulfoxide (DMSO) method. For carotenoid extraction, 0.2 mL of 0.01 M sodium phosphate and 2 mL of hexane: ethyl acetate 1:1 were added to the disrupted cells and vortex agitated for 30–40 s. The samples were then centrifugated for 5 min at 12,000 rpm and 4 °C to separate the organic phase. Finally, carotenoids concentration has been successfully quantified spectrophotometrically [6,11,28–30] by simply measuring the optical density at 480 nm (Unico UV 2150, USA). Total carotenoids yield was calculated using Eq. (4):

Table 2
Results of carotenoids and biomass production using agroindustrial wastes as carbon and nitrogen sources.

Agro-industrial waste substrate		Results		
Carbon Source	Nitrogen Source	P, μg/L	Yx, μg/g	X, g/L
CHH	CSL	654.26 ± 88.41[b c]	467.33 ± 63.15[a]	1.4 ± 0[b]
	RBY	573.64 ± 95.47[c]	132.38 ± 22.03[d]	4.33 ± 0.61[a b]
	UR	0	0	0
MPE	CSL	1072.87 ± 190.94[a]	259.56 ± 46.2[b c]	4.13 ± 0.23[a b]
	RBY	1131.78 ± 169.07[a]	171.48 ± 25.62[d]	6.60 ± 0.53[a]
	UR	0	0	0
CCH	CSL	846.51 ± 37.21[a b c]	309.70 ± 13.61[b]	2.73 ± 0.31[b]
	RBY	930.23 ± 85.26[a b]	196.53 ± 18.01[c d]	4.73 ± 0.12[a b]
	UR	0	0	0
YM Medium		1502.58 ± 253.87	315.43 ± 30.13	4.71 ± 0.34

Marked differences (superscript letters a,b,c,d) are significant at $p < 0.05$; CCH: corncob hydrolysate; CHH: cotton husk hydrolysate; CSL: corn steep liquor; RBY: residual brewery yeast; UR: urea; P: carotenoids production; Yx: carotenoids yield per dry cell weight; X: biomass.

$$Yx = \frac{v^*A^*10^6}{E^{1\%}{}^*100^*m_s} \quad (4)$$

where *Yx* represents the carotenoids yield (μg/g DW); *A* the optical density (OD_{480nm}); *v* the volume of solvent used (mL); m_s the dry cell mass (g) and E^1 the specific absorptivity of solvent (2150) [31,32].

3. Results and discussion

3.1. Agro-industrial waste substrate screening

The results on the total production of carotenoids (P), the yield of carotenoids per biomass (Yx) and biomass production (X) when agro-industrial waste substrates substituted the carbon source (CS) and nitrogen source (NS) are presented in Table 2. According to the ANOVA, both carbon and nitrogen sources presented a significant effect ($p < 0.05$) on all the evaluated responses. Although the microorganism was able to grow and produce carotenoids among the evaluated carbon sources, significative differences ($p < 0.05$) can be observed when hydrolysates and MPE were used as substrates. Higher yeast growth was observed with MPE (47% and 30% approximately) than with hydrolysates (CHH and CCH, respectively). The difference can be attributed to the carbohydrate composition in the agroindustrial wastes; sucrose, glucose, and fructose have been previously reported as main sugars in MPE [34] while glucose and xylose as hydrolysates main products [35]. It has been suggested that this strain can efficiently metabolize carbohydrates such as sucrose, glucose, and fructose [36,37]. However, other studies have reported the ability of *Xanthophyllomyces dendrorhous* of utilizing xylose or hydrolysates [38,39]. It could also be possible that even though the hydrolysates were detoxified, by-products of the hydrolysis such as furfural, hydroxymethyl furfural or acetic acid are remaining and affecting the microorganism growth. In terms of total carotenoids, the highest production of total carotenoids among the evaluated agroindustrial wastes was obtained using MPE as the carbon source and CSL as the nitrogen source, showing no significant difference ($p < 0.05$) with the media formulated with CSL and RBY. On the other hand, in terms of carotenoids per biomass, the cotton husk hydrolysate showed a high yield compared with the other agro-residues. Nonetheless, its biomass content was very low; therefore, the total production of carotenoids was low as well. In contrast, urea utilization as nitrogen source did not promote growth nor carotenoids production. Other studies have reported that inorganic nitrogen in medium decreased cellular growth; yet, also promoted astaxanthin yield [40,41]. Also, the ability of *Xanthophyllomyces dendrorhous* to express urease activity for catalyzing the hydrolysis of urea to free ammonia has been elucidated [42]. In this study, however, complete inhibition of growth and production were obtained using urea; it is possible that the combination with the carbon sources suppressed yeast propagation. In any case, as the objective of this study was to achieve a higher production of total carotenoids, the medium formulated with MPE and CSL was selected for further optimization studies to obtain a similar or higher production level than the one obtained with control YM medium (P = 1502.58 ± 253.87 μg/L, Yx = 315.43 ± 30.13 μg/g).

3.2. Waste-based medium optimization

The concentration levels of the four components of previously selected media were optimized following the Taguchi methodology (Table 1). The concentration levels for MPE (Factor A) were 10, 15 and 20 g/L (total sugar content), due to complete consumption of the substrate (10 g/L) by the microorganism during the screening phase. Regarding the concentration of CSL (Factor B), the levels were set as 3, 5 and 7 g/L; these levels were chosen based on the concentration used during the screening studies (5 g/L). As to the yeast and malt extract (Factors C and D, respectively), the studied levels were 0, 1.5, and 3 g/L. The levels of these two components were selected in order to test if the formulated media could contain a lower concentration than the utilized in the control media (YM, 3 g/L) or to evaluate if it could be possible to leave these two components out from the designed media in order to reduce costs. Results for each run of the matrix L9 are presented in Table 3. It can be seen that the production of carotenoids varied from 1035.66 to 2347.29 μg/L (run 1 and 9, respectively), the yield on biomass from 186.05 to 293.41 μg/g (run 2 and 9, respectively) and the biomass from 3.60 to 8.07 g/L (run 1 and 8, respectively). These results indirectly indicate that the factors under study are affecting the responses. However, it is not possible to withdrawn conclusions from a set of data like the one presented in Table 3. For this purpose, the ANOVA is required, which is shown in Fig. 2 as the relative influence of the factors on the responses. The low percentage of the error for P and B means that the four factors under study are

Table 3
Taguchi L9 average results of P, Yx, and X.

Run	Results		
	P, μg/L	Yx, μg/g	X, g/L
1	1035.66 ± 23.41	287.68 ± 6.50	3.60 ± 0.
2	1153.49 ± 161.39	186.05 ± 26.03	5.93 ± 0.61
3	1568.99 ± 61.94	261.50 ± 61.94	6.00 ± 0.53
4	1714.73 ± 215.43	273.63 ± 34.38	6.27 ± 0.12
5	1534.88 ± 283.84	239.83 ± 44.35	6.40 ± 0.35
6	1497.67 ± 109.67	209.95 ± 15.37	7.13 ± 0.42
7	2235.66 ± 140.87	289.09 ± 18.22	7.73 ± 0.58
8	1767.44 ± 140.46	219.10 ± 17.41	8.07 ± 0.58
9	2347.29 ± 248.97	293.41 ± 31.12	8.00 ± 1.06

P: carotenoids production; Yx: carotenoids yield per dry cell weight; X: biomass.

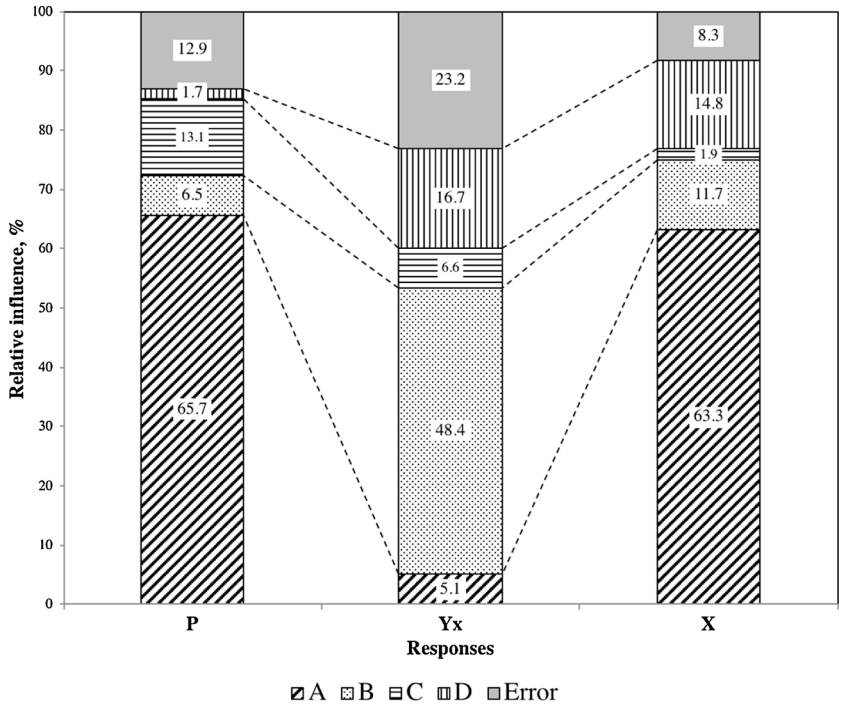

Fig. 2. Relative influence of the factors (carbon source, A; nitrogen source, B; yeast extract, C; malt extract D) on the evaluated responses (carotenoids production, P; carotenoids yield, Yx; biomass, X).

dominating these responses while the obtained error for Yx could mean that some interactions between factors might be affecting the process and which were not considered. It is noted that the most significant factor affecting the total production of carotenoids was the carbon source with a contribution of 65.7%, while it was nitrogen source (48.4%) for carotenoids yield per unit of biomass. On the other hand, the malt extract was not significant for P and B, whereas the yeast extract and carbon source did not present a significant effect on the carotenoids yield. These results differed from those obtained with a medium formulated with sweet sorghum medium [21], where the concentration of yeast extract has a significant effect on both P and Yx. The mean effect plots showing the effects of the individual factors are obtained from calculating the average performance at each level, and each factor (Fig. 3). The interpretation of these plots is as follows: a horizontal line indicates that the factor has no effect at studied levels; if the line is different to a horizontal line, then the factor present an effect on the response at the studied levels. The larger the line deviates from the middle horizontal line (grand average), the larger is the effect. Regarding the effect of carbon source on P, it can be seen that the production of carotenoids increased by increasing the concentration level of mesquite extract. This tendency of total carotenoids accumulation due to high concentrations of sugar in the fermentation media has been reported in wild type strains as well as overproducing strains where the sugar is utilized for biomass production and when sugar is consumed, carotenoids synthesis begins [28,43,44]. Accordingly, carotenoids accumulation in *X. dendrorhous* has been partially associated with cellular growth [6,45]. In regard to Yx and the effect of the most significant factor affecting this response (CSL, factor B); it can be observed that a higher yield was obtained when a minimum concentration of nitrogen source was used, and a minimum yield was obtained at the intermediate level. This effect might be related as well with the nitrogen/carbon ratio used in each trial or due to the interactions between factors. It has been previously reported that high C/N ratio (> 15:1) increased the carotenoid yield due to the nitrogen limitation [43]. A similar trend of the effect of nitrogen limitation on carotenoids yield was observed in saccharide fluid of cassava, sugar cane juice, and sweet sorghum juice media [21,46,47]. Regarding the effect of yeast extract, it can be seen that a higher Yx and P are obtained at the maximum level, although the increment between the intermediate and maximum level is not as high for both responses. This means that this component cannot be fully eliminated from the media components, either a lower or similar composition than the control media must be utilized in order to maintain high production of carotenoids. An optimization report indicated that yeast extract in the production media is one of the most relevant factors in the carotenoids production but must be complemented with other minerals in order to achieve higher carotenoid yield [48]. In regard to the malt extract, results indicated that this component could be completely eliminated from the formulated media, and it will not negatively impact the process. In this case, it is possible that the raw materials acted as a substitute for the malt extract nutrients. In literature, there is no mention of the individual effect of this component in the carotenoids production [40,48]. In any case, if all interactions are assumed negligible, the optimum levels can be chosen independently, and they will be the level with the subset of data giving the higher average response. The optimum conditions were as follows: P (A = 1, B = 1, C = 1, D = 1) and Yx (A = 1, B = -1, C = 1, D = -1).

3.3. Validation trials

It can be noted that the optimal levels for maximizing P conflicted with those for Yx. However, optimum conditions for maximizing Yx were chosen for further experimental validation trials due to the effect of malt extract is negligible for P and the total production of carotenoids is very similar using 5 or 7 g/L of CSL. Therefore, it was proposed to run the experimental validation trials with the conditions that result in a higher Yx (Trial 1). Since the optimum levels were estimated from nine combinations of settings of a total of eighty-one possible combinations (four factors, three levels), there is a high probability for the estimations being erroneous in at least one run of the eighty-one combinations of settings if the interactive effects are significant [33]. In order to have confidence not only in the optimum settings but also in the identified main effects, an extra validation with different settings than the optimum is proposed. In this study, the second set of conditions for the experimental validation trials were those where a low P

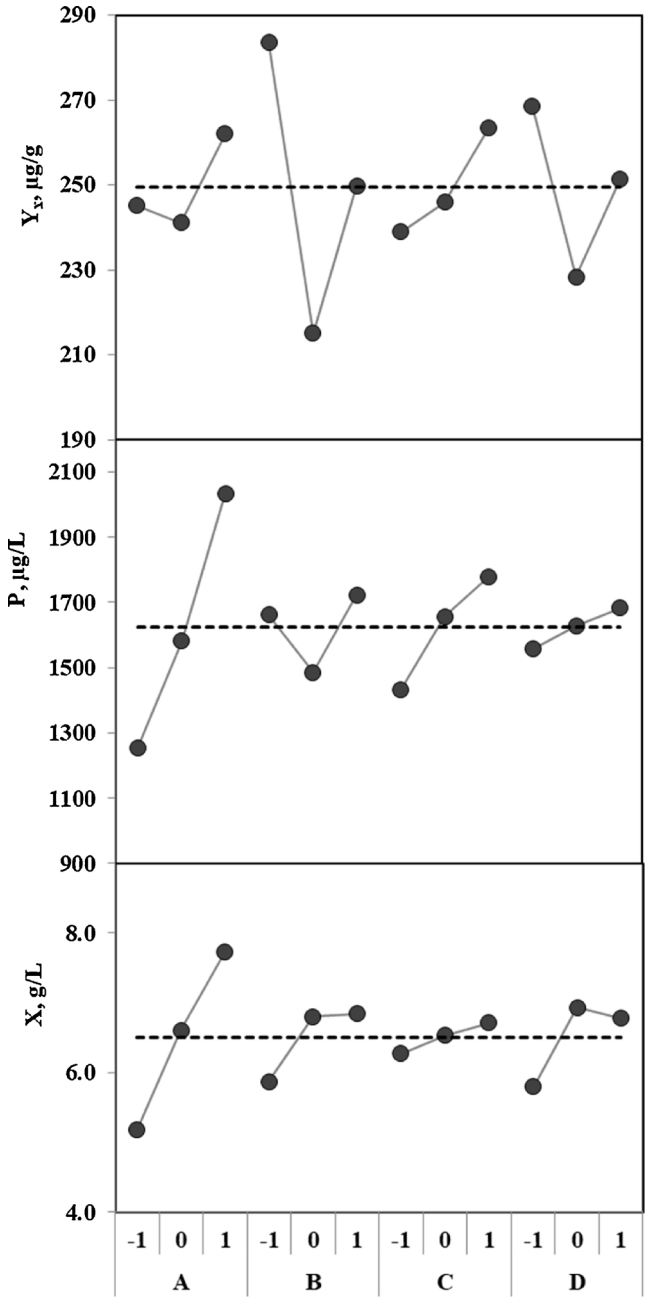

Fig. 3. Individual factors performance at different levels for carotenoids production (P), carotenoids yield (Yx), and biomass (X).

and a low Yx are obtained (Trial 2). The idea of performing a validation trial with the best and worst conditions is to challenge the ability of Eq. (2) to predict an outcome at two sets of conditions with opposite performance. If the results of the experimental validations trials are within the confidence interval of the expected results (Eq. (2)), then, one can trust in the optimum selected settings and the indentified main effects. However, if the results are not within the expected results, then it is possible that the interactions are signigicant. Table 4 shows the conditions selected for Trial 1 and Trial 2, the contribution of each factor, minimum and maximum expected results predicted by marginal means addition (confidence interval, Eq. (2)) and obtained experimental results for both Trials for the target responses. Experimental results of P, for both trials, were within the confidence interval of expected results, only some few replicates for Trial 2 were outside and not too far from the estimation with the marginal means addition. Thus, the assumption of the Taguchi method that all interactions are negligible was accepted for this response as well as optimum settings and main effects. Therefore not further data analyses were done for P (See Fig. 1). On the other hand, regarding the results for Yx, the obtained results for both validation trials were outside the confidence interval, and results were significantly lower than the expected. As stated before, one of the major drawbacks of using the Taguchi method is that all interactions among the evaluated factors are assumed negligible, which is the reason why is highly recommended to validate the process under the optimum conditions found. If the results are within the confidence interval, then one can control the process within an optimum range with reliability on the predictions. However, if this is not the case, it is possible that the effect of an interaction is mixed with the effect of a factor assigned to another column. Therefore, the errors in the predictions could be attributed to possible interactions among the evaluated factors. The possible impact of interactions was assessed as described in the materials and methods Section (2.3.4 Validation trials). It is important to mention that an interaction between factors will not be identified by looking at the goodness of fit (R^2), a possible interaction will be identified if the prediction by the surface model with any pair of factors (Eq. (3)) is closer to the obtained response during the experimental validation trials than the obtained with the marginal means addition (Eq. (2)). The fittings of the surface model (Eq. (3)) for the six pair of factors (A–B, A–C, A–D, B–C, B–D, C–D) are not shown but most of the experimental results obtained for Yx were out of the confidence interval of the predicted responses, despite the higher correlation coefficient obtained for all the surface models (R^2 close to 0.90). Only the predictions for the model that considers the effect of factor C and D were better than the obtained with the marginal means addition. Estimations with the surface model (Eq. (3)) considering C and D were 239.82 ± 35.80 µg/g (Conditions Trial 1) and 209.95 ± 35.80 µg/g (Conditions Trial 2); hence, most of the experimental batches with conditions used for Trial 1 were within the confidence interval of the surface model, while for Trial 2 only some of the experimental batches were within this interval. However, although not all replicates of the experimental results were within the error of the estimations of the surface model for Trial 2, the

Table 4
Contribution of factors over the responses, expected results within a confidence interval and experimental results at selected settings.

Factors	Trial 1 Contribution			Trial 2 Contribution		
	Levels	P, µg/L	Yx, µg/g	Levels	P, µg/L	Yx, µg/g
A	1	411.60	12.62	−1	−370.66	−4.34
B	−1	38.64	34.05	0	−138.10	−34.42
C	1	156.47	14.06	−1	−189.78	−10.50
D	−1	−65.92	19.05	0	5.57	−21.05
Expected minimum		1989.79	305.48		756.03	155.39
Expected maximum		2338.54	352.91		1104.78	202.81
Experimental result		2058.91 ± 214.02	215.97 ± 22.44		1195 ± 98.66	149 ± 12.33

A = mesquite pods extract; B = corn steep liquor; C = yeast extract; D = malt extract. P: carotenoids production, Yx: carotenoids yield per dry cell weight.

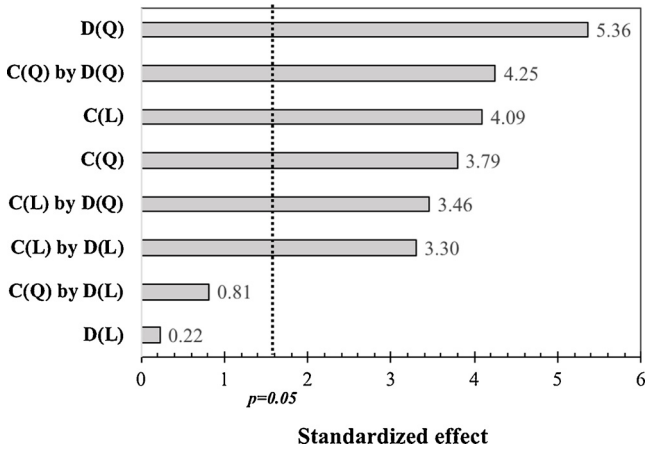

Fig. 4. Pareto chart of the linear, quadratic and interactive effects between yeast and malt extract. (linear effect, L; Quadratic effect, Q).

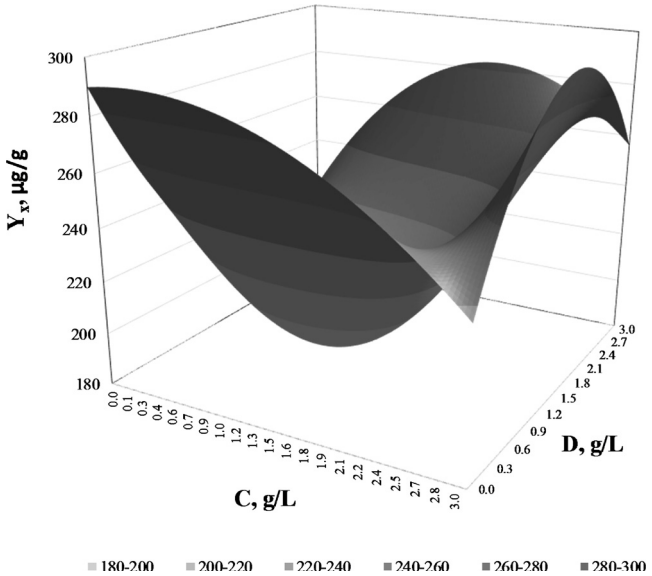

Fig. 5. Surface response plot comparing Yx as function of yeast (C) and malt extract (D).

estimations for Trial 1 are highly better than the ones obtained with marginal means addition (Eq. (2)). Therefore, an ANOVA was performed for the Yx model considering only factors C & D and all of the possible interactions. Results are presented in Fig. 4. It can be observed that several interactive effects were significant: the quadratic by quadratic effects, the linear effect of factor C by the quadratic effect of factor D and the linear by linear effects. The surface model is shown in Fig. 5, where all the possible interactive effects can be observed. According to Taguchi analysis (Eqs. (1) and (2)), the optimum region for Yx can be found in C = 1 and D = -1. Also, the Taguchi analysis showed a linear increment of Yx by increasing the concentration of C, while a minimum Yx was obtained at the intermediate setting of D (Fig. 3). The surface model, on the contrary, shows that the effect of D determined with Taguchi analysis was only observed when the settings of C are selected at an intermediate level. In the same way, the effect of C determined with the Taguchi analysis was only observed when the settings of D are selected at an intermediate level. Moreover, if a minimum level is selected for factor D, then factor C presents a quadratic effect on Yx, and the yield decreased using settings above 0.9 g/L of factor C. This interaction between factors could be the reason why a much lower result for Yx was obtained during the experimental validation trials. Three suboptimal regions can be observed in the surface model at i) C = -0.4/D = -1, ii) C = 1/D = 0, and iii) C = 0/D = 1; however, if regions i and iii are selected then it will compromise the results for the total production of carotenoids. The only worth region would be the region ii; however, studies have shown that there could be possible interactive effects among the nitrogen sources used to formulate the culture media [41]. The nitrogen content in malt extract (Factor D), can vary significantly depending on the supplier and the raw material used for its obtaining. Therefore, the formulated media without this component prevents any variability due to the possible interactive effects with the rest of the media components. Thus, the conditions used for Trial 1 sufficed the purpose of improving the process performance and no further experiments were performed. The obtained total production of carotenoids with optimum conditions was nearly 40% higher than the control medium and 90% higher than the unoptimized conditions using MPE and CSL. Thus, it is safe to assume that the process performance was transferred to a more favorable region of the solution space by analyzing only a small fraction (13.5%) of the total set of possible conditions for the number of factors and levels analyzed. Accordingly, obtained productions levels with the optimized media are similar to carotenoids accumulation using cassava residue (P = 2.98 mg/L) and enzymatic eucalyptus wood hydrolysates (P = 2.14 mg/L) [38,49]. Most of the studies performed with the wild type strain of *X. dendrohous* using low-cost substrates report carotenoids production in range with the obtained in this study; nonetheless, there are recombinant strains that can achieve higher values [10].

4. Conclusions

This work presents a feasible route to follow for using agroindustry wastes as raw materials for the biosynthesis of high value-added compounds, such as carotenoids. A media was designed and optimized with mesquite pods extract and corn steep liquor as main components. According to the Taguchi method, mesquite extract concentration was the factor with the highest influence on the total production of carotenoids, while corn steep liquor was on the yield (astaxanthin/biomass). If further improvement is desired, it will be worth evaluating a higher concentration of the mesquite pods extract since it is the factor dominating the process performance and the production of carotenoids was directly proportional to the evaluated levels. The yeast and malt extract were original components of the control media; thus, the possible identified interaction between them could be further analyzed first in the control medium if it is desired to use this medium as a base to study other wastes. Optimum designed wastes-medium contained (g/L), mesquite pods extract (20), corn steep liquor (3) and yeast extract (3); which allowed to surpass by 40% the carotenoids production using the control medium and by 90% the un-optimized waste medium. Thus, it was demonstrated that the process performance was improved by applying a statistical design that has maximum efficiency with minimum experimental requirements such as the Taguchi method. Also, the follow-up strategy to define the possible impact of interactions and overcoming one of the main disadvantages when a saturated design is used was suggested with promising results for its application in the optimization of other processes.

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