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Optimization of ultrasounds assisted extraction of polysaccharides from cladodes of *Opuntia ficus-indica* using response surface methodology

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ABSTRACT

In this work, polysaccharides are extracted from cladodes of *Opuntia ficus-indica* using Ultrasounds-Assisted Extraction (UAE). UAE operating conditions are optimized by a Face Centered Central Composite Response Surface Design (FCCRD) with four variables at three levels. The optimal operating conditions are obtained using Response Surface Methodology (RSM) and are the following: Solid-Liquid ratio SL = 1:10 w/v, pH = 2.5, time $t = 20$ min, and temperature $T = 65$ °C. The predicted extraction yield (12.07 ± 1.7 % dw) is in line with the experimental one (11.32 ± 0.25 % dw). The resulting extract is composed mainly of galacturonic acid (25.55 ± 0.30 %), the primary constituent of pectic material, followed by arabinose (14.34 ± 0.01 %) and galactose (13.5 ± 0.22 %). The Esterification Degree (ED) is found 42.84 ± 0.48 %, indicating the extract is Low-Methylated (LM). The FT-IR spectrum demonstrates that the extract has several peaks typical of polysaccharides. Total Phenolic Content (TPC) is equal to 41.33 ± 3.53 $\mu\text{gGAE/g}$ (dw) and the anti-radical ability against the DPPH radical scavenging activity achieves 95.56 % at a concentration equal to 2 mg/mL, demonstrating that the extract is a polysaccharide functionalized with polyphenols. These results can encourage the use of the extract as a new ingredient in nutraceutical cosmetic applications.

1. Introduction

The growth in industrial activities in the food and agricultural sectors is causing a continuous increase in waste production. These wastes could represent an important source of high-added value compounds. Valorization could allow reducing the use of raw materials, decrease the amount of effective waste to be disposed and to incentivize and support the economy of the food and agri-food sector (Bhat et al., 2020).

Opuntia ficus-indica (L.) Mill., 1768) is a crop species native to Mexico (Griffith, 2004) belonging to the Cactaceae family (Silva et al., 2021). Nowadays *Opuntia* is abundantly found in other parts of the world, such as the Mediterranean basin, thanks to its relative ease of vegetative propagation and its ease of growth (Griffith, 2004). The easy spread of *Opuntia* is due to its adaptative ability to difficult conditions (Bayar et al., 2016), which also makes it an excellent supporter in the prevention of soil degradation and desertification (Le Houérou, 2002). Mexico is the first region per hectares of cultivated areas (at least 50.000–70.000 ha) and Italy follows with about 7.000–8.300 ha of intensive plantations, 90% located in Sicily (Di Bella et al., 2022).

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currently, no one has ever thought of evaluating the content of polysaccharides functionalized with polyphenols for *Opuntia cladodes*. The present study therefore aims to fill these gaps.

2. Materials and methods

2.1. Material and sample preparation

All HPLC grade reagents, commercial standards for carbohydrates, TDF-100A kit for total dietary fiber determination, Folin-Ciocalteu reagent, 2,2'-diphenyl-1-picrylhydrazyle (DPPH), and other reagents, were purchased from Merck (Darmstadt, Germany). Ultrapure water (type 1) was obtained from a Direct-Q C9185 water purification system (Merck).

Opuntia cladodes 25–50 cm long were manually harvested from Apulia, in the south of Italy. After a washing phase, the thorns were removed and cladodes were cut into small slices (about 2 × 2 cm). The sample was dried in an air oven at 103 °C until constant weight and subsequently ground. The obtained powder was stored in a tightly closed protected place until further analyses.

2.2. Proximate composition

The proximate composition of cladodes was determined using AOAC official methods (AOAC, 2019). All the analyses were performed in triplicate. Moisture was determined using the AOAC Official Method 925.09 based on sample weight loss after a night passed in the air oven at 103 °C. For ash determination, the gravimetric method was used. Basically, 0.5 g of dry samples were heated in a muffle furnace at 550 °C for 6 h. Lipids were determined using the AOAC Official Method 920.39, where 5 g of sample are treated with petroleum ether in a Soxhlet apparatus. Protein content was determined using the Dumas method, where a CHNS analyzer (Vario MACRO cube, Elementar Italia Srl) was used to determine the % nitrogen. This value was then multiplied by a 6.25 conversion factor to obtain the % protein. Total Dietary Fibers (TDF) were determined using the Total Dietary Fiber Assay Kit TDF-100A which works with a combination of enzymatic and gravimetric methods. The dried sample is firstly treated with stable α -amylase and then digested with protease and amyloglucosidase. The fiber precipitation takes place with the addition of ethanol. Carbohydrates were determined by difference.

2.3. Polysaccharides extraction

The cladodes powder was mixed with acidified water in a beaker at a well-defined Solid-Liquid (SL) ratio and pH. After that, the beaker was placed on a magnetic stirrer equipped with a thermocouple. An ultrasonic tip (VCX750 Ultrasonic Processors – Sonic and Materials Inc), with 40 % amplitude, 750 W power intensity, and 20 kHz frequency, was used to improve the extraction efficiency. The extraction took place at a given temperature and time. After the extraction, the sample was centrifuged with an SL 16R centrifuge (Fisher Scientific Italia) at 4000 rpm for 15 min and then filtered with the aid of a vacuum pump using filter paper Whatman n. 1. Two volumes of 95% ethanol were added to the permeate liquid phase to allow polysaccharides precipitation and the sample was stored at 4 °C overnight to be sure precipitation occurs. The ethanol-added samples were centrifuged again at 4500 rpm for 15 min to make all the extract settle well. The supernatant was removed and the extract was dried at 50 °C until constant weight. The extraction yield Y was calculated as follow:

$$Y(\%) = 100 \times \frac{m_0}{m} \quad \text{Eq. 1}$$

where m_0 is the weight of dried extract (g) and m is the weight of dried cladodes powder (g).

2.4. Experimental design

A face-centered central composite response surface design (FCCRD) was used in this study to investigate the effect and optimize the process variables. Four factors in three levels were tested to obtain the maximum yield in polysaccharides: Solid-Liquid (SL) ratio (1:10–1:40 % w/v), pH (1.5–2.5), extraction temperature (25–75 °C) and sonication time (10–30 min). A total number of 27 experiments, including three replicates at the central point to validate the model, were performed. The experimental data were used to build a mathematical model which expresses the correlation between the four independent variables and the response. To do that, a second-order polynomial equation was used whose generalized form is the following:

$$Y = \beta_0 + \sum_{i=1}^k \beta_i X_i + \sum_{i=1}^k \beta_{ii} X_i^2 + \sum_{i=1}^{k-1} \sum_{j=2(i \neq j)}^k \beta_{ij} X_i X_j \quad \text{Eq. 2}$$

Where Y is the response; β_0 is the model intercept; X_i and X_j are the independent variables (where i and j vary from 1 to k); β_i , β_{ii} , and β_{ij} are respectively the linear, quadratic and second-order terms. k corresponds to the number of independent parameters ($k = 4$).

All data analyses, the model determination, and the selection of the best operational parameters were done with the help of CAT (Chemometric Agile Tool) software (Leardi et al., 2023).

2.5. Model validation

Optimal factor levels were obtained by studying the response surface plots produced by CAT software. After that, three experiments were performed at the optimal levels and the results were compared to the predicted one to validate the model.

Table 3
Experimental results of face centered central composite response surface design.

Exp No	SL (g/mL) X_1	pH X_2	t (min) X_3	T ($^{\circ}$ C) X_4	Experimental Yield %
1	10	1.5	10	25	10.14
2	40	1.5	10	25	3.22
3	10	2.5	10	25	9.76
4	40	2.5	10	25	6.40
5	10	1.5	30	25	9.40
6	40	1.5	30	25	3.57
7	10	2.5	30	25	8.81
8	40	2.5	30	25	7.61
9	10	1.5	10	75	10.59
10	40	1.5	10	75	4.11
11	10	2.5	10	75	10.47
12	40	2.5	10	75	9.70
13	10	1.5	30	75	11.23
14	40	1.5	30	75	6.83
15	10	2.5	30	75	11.96
16	40	2.5	30	75	8.54
17	10	2	20	50	12.89
18	40	2	20	50	7.19
19	25	1.5	20	50	8.68
20	25	2.5	20	50	9.52
21	25	2	10	50	7.20
22	25	2	30	50	9.06
23	25	2	20	25	8.29
24	25	2	20	75	8.33
25	25	2	20	50	8.07
26	25	2	20	50	7.61
27	25	2	20	50	8.36

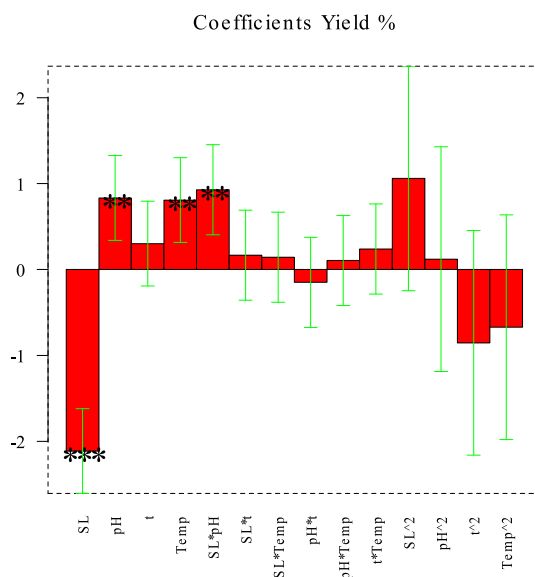


Fig. 1. Coefficients graph. The green lines represent the error. * visually indicates the significance of the coefficients (p-value): *** = $p < 0.01$; ** = $p < 0.001$.

3.2. Polysaccharides extraction

3.2.1. Experimental design and model building

The UAE experimental results are shown in [Table 3](#). These results are processed with the multiple regression analysis using the CAT software ([Leardi et al., 2023](#)) in order to obtain a model expressed as a second-order polynomial equation. The resulting model is the following:

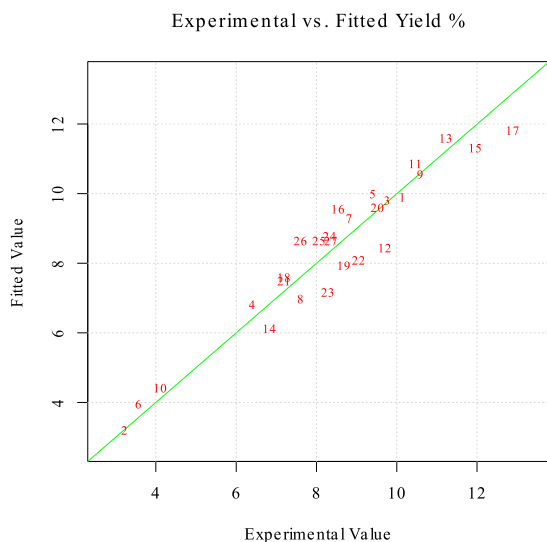


Fig. 2. Experimental vs Fitted yield. The red values represent the experimental data while the green line represents the fitted values obtained through the model.

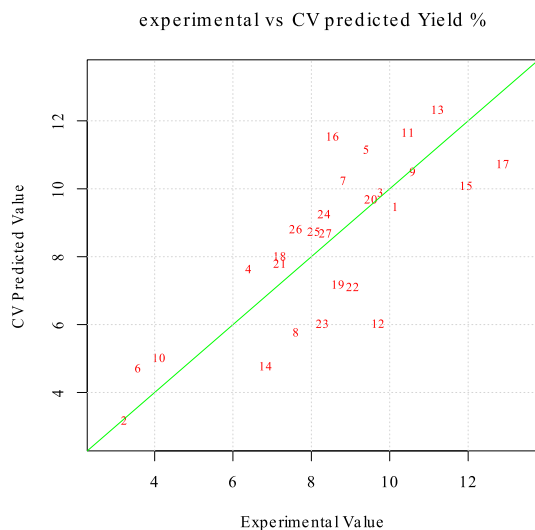


Fig. 3. Experimental data vs Cross-Validated (CV) predicted data. The red values represent the experimental data while the green line represents CV predicted values.

$$\begin{aligned}
 Y(\%) = & 8.658 + (-2.114 * X_1) + (0.833 * X_2) + (0.300 * X_3) + (0.808 * X_4) + (0.929 * X_1 * X_2) + (0.167 * X_1 * X_3) + (0.142 * X_1 \\
 & * X_4) + (-0.149 * X_2 * X_3) + (0.104 * X_2 * X_4) + (0.239 * X_3 * X_4) + (1.060 * X_1^2) + (0.120 * X_2^2) + (-0.854 * X_3^2) \\
 & + (-0.672 * X_4^2)
 \end{aligned}$$

Eq. 5

As can be seen from the equation, the parameter that most influences the process is the solid-to-liquid ratio, followed by pH and temperature. It seems that the sonication time has not a great influence on the tested interval. From the equation, it can be concluded also that the process is influenced by the correlation between the SL ratio and pH. The coefficient graph in Fig. 1 shows graphically the effect of each single variable and their interactions on the system response. As expected, the most important parameter is the SL ratio with an inversely proportional correlation with the response. All the other relevant parameters are directly proportional to the response.

The explained variance of the model is equal to 82.63 %. This value expresses the accuracy with which the model describes the

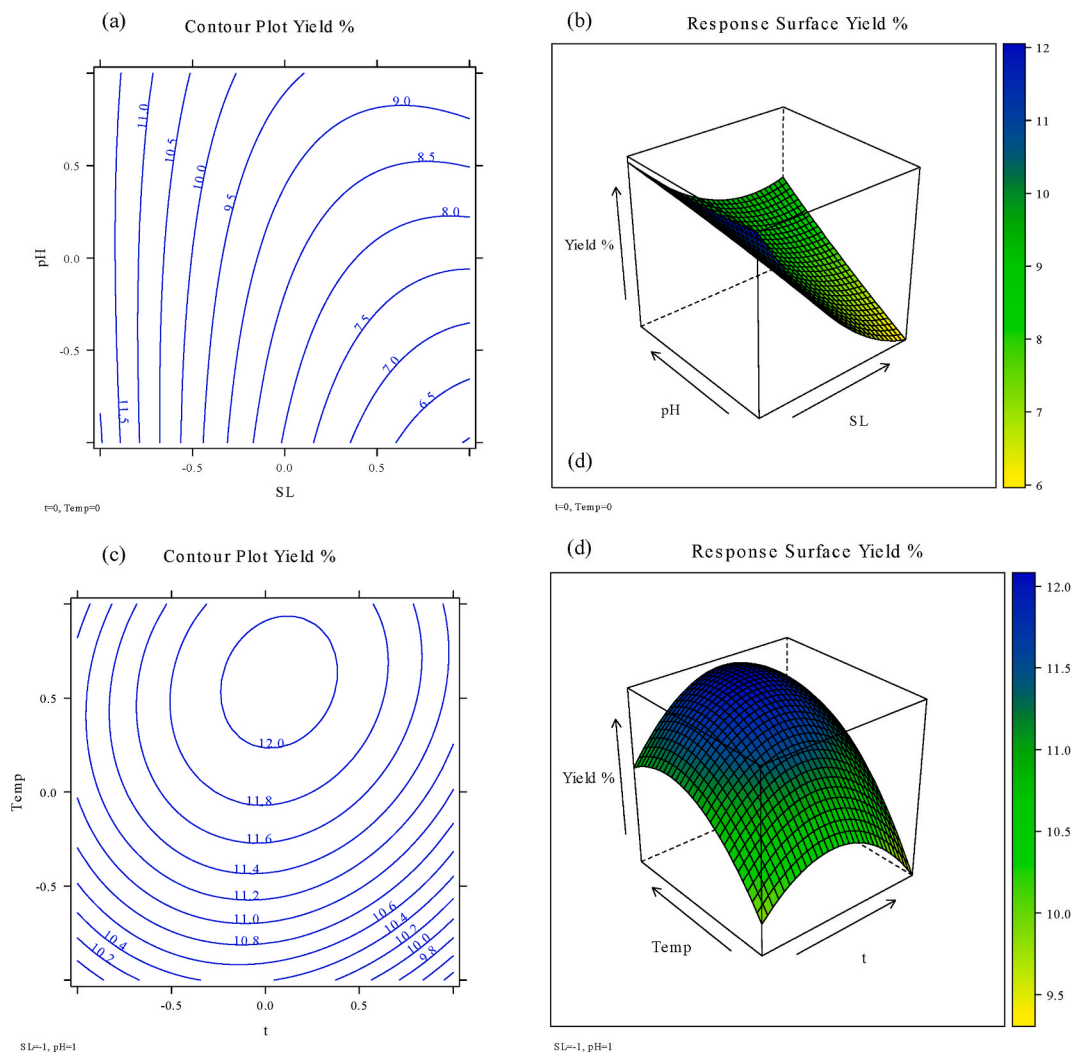


Fig. 4. Response Surfaces. (a) and (b) shows the correlation between pH and SL ratio in 2D and 3D respectively, keeping t and T at their middle values. (c) and (d) shows the correlation between t and T in 2D and 3D respectively, keeping the SL ratio at its lowest value and pH at its highest value.

variability of the data.

The model accuracy is graphically represented in Fig. 2, where experimental data are very close to the line representing the fitted values.

In Fig. 3 the experimental vs Cross Validation (CV) predicted data are reported. The CV is a procedure that allows to obtain important information regarding the stability and generalizability of the regression model. The CV procedure splits randomly the data into two sets: training data for developing the model and validation data for evaluating the model's predictive ability. To evaluate the consistency of the model, the fit of the model to the training dataset can be compared with the refit of the model to the validation dataset. The software also reports the mean squared error of CV (RMSECV), which is equal to 1.553. In the figure the experimental values are close to the green line representing the CV predicted values. All these observations lead to the conclusion that the model is stable and generalizable.

3.2.2. Response surfaces

Response Surface Methodology (RSM) allows to describe the interaction between data to obtain statistical forecasts. It is applied to investigate the influence of each variable on the response of the system. As already mentioned, the adopted design is the FCCRD, with four variables in three levels (-1, 0, 1). The response surfaces allow us to visualize the first-order interaction between two variables, keeping the others constant.

Fig. 4 shows the 2D-RSM and 3D-RSM diagrams that demonstrate what has already been concluded in the previous paragraph. In Fig. 4 (a) and (b) the response of the system to variations in the SL ratio and pH, keeping t and T at their middle values, is illustrated. The SL ratio is confirmed as the most important parameter. At tested conditions, its variation leads from extraction yields of a minimum of 6 % (when SL is equal to 1) to yields of a maximum of 12 %, where SL is equal to -1. The inverse proportionality between working

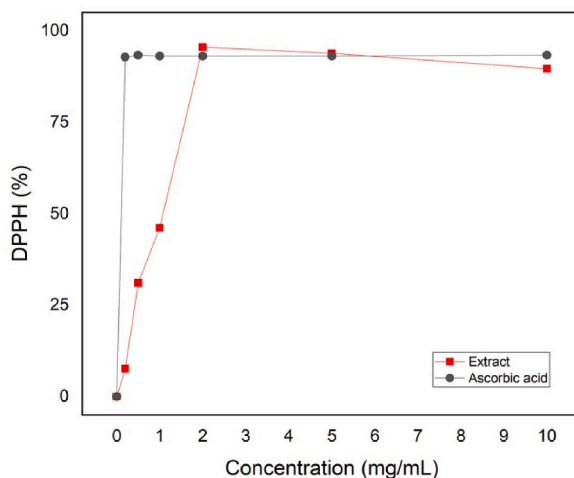


Fig. 5. DPPH anti-radical activity of the extract and the reference ascorbic acid.

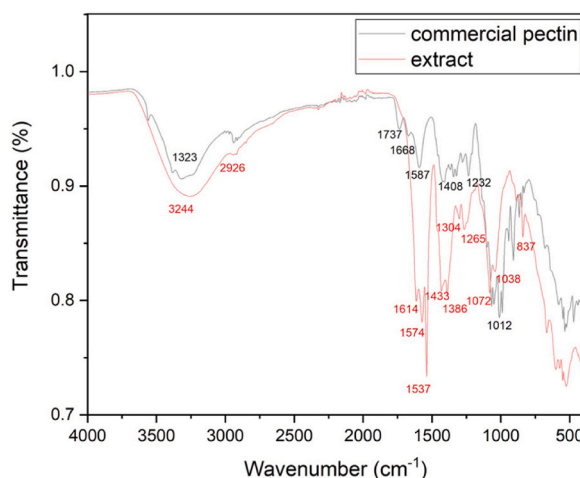


Fig. 6. FTIR spectra of extract and commercial pectin.

was performed.

Employing the free radical 2,2-diphenyl-1-picrylhydrazyl (DPPH) is a common method to determine the antioxidant ability of phenolic compounds contained in natural tissues and food sources. The antiradical activity of antioxidants is determined using DPPH free radical, which has an absorption band at 517 nm in the UV-Vis spectrum. The absorbance decrease is directly proportional to the antioxidant ability and to the concentration of the added compound (Brglez Mojzer et al., 2016). The DPPH radical assay was done using ascorbic acid as a standard antioxidant. According to Fig. 5, the anti-radical ability increases with the concentration up to 95.56% at a concentration of 2 mg/mL. This considerable result could be due to the high TPC, the degree of methylation, and the high galacturonic acid content of the extract (Bayar et al., 2018). Moreover, it can be concluded that the extraction conditions do not alter the antioxidant ability of polyphenols in the extract. The extract can therefore be defined as a heteropolysaccharide functionalized with polyphenols. In recent years, interactions between polyphenols and polysaccharides have attracted interest since they play a role in the physiological properties, bioavailability, and stability of compounds (Guo et al., 2022).

3.3.4. FTIR analyses

FTIR spectra of the extract are compared with the spectra of commercial pectin in Fig. 6. Both samples show a peak in the region between 3550 and 3200 cm^{-1} which is due to the O-H stretching frequency (Pasandide et al., 2017). Between 3000 and 2840 cm^{-1} there is the C-H stretching band, in which both samples have a peak at 2926 cm^{-1} , typical of sugars (Niu et al., 2021), that indicates the symmetric stretching vibration of the C-H group of the methyl ester of galacturonic acid (Bayar et al., 2016). Wavenumbers lower than 1800 cm^{-1} are characteristic of the fingerprint region while the carbohydrates fingerprint is in the region between 1200 and 800 cm^{-1} (Bayar et al., 2016). More differences between the extract and pectin can be seen in these areas. Commercial pectin has a peak at 1737 cm^{-1} related to the vibration of the esterified carboxyl group C=O (Méndez et al., 2021). The extract does not have this peak maybe

