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Analytical Methods

Optimisation of xanthan gum production by palm date (*Phoenix dactylifera* L.) juice by-products using response surface methodology

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ABSTRACT

The present study was undertaken investigate and optimise the possibility of xanthan gum production by *Xanthomonas campestris* NRRL B-1459 in batch experiments on date palm juice by-products. Using an experimental Response Surface Methodology complemented with a Central Composite Orthogonal Design, three major independent variables (date juice carbon source, nitrogen source and temperature) were evaluated for their individual and interactive effects on biomass and xanthan gum production. The optimal conditions selected were: 84.68 g/l for carbon source, 2.7 g/l for nitrogen source, and 30.1 °C for temperature. The experimental value obtained for xanthan production under these conditions was about 43.35 g/l, which was close to the 42.96 g/l value predicted by the model. Higher yields of biomass production could be obtained at 46.68 g/l for carbon source, 4.58 g/l for nitrogen source and 30 °C for temperature. The maximum value obtained for biomass production was 3.35 g/l, which was higher than the 2.98 g/l value predicted by the model. The xanthan formed was subjected to HPLC and TLC analyses and its molecular weight as well as pyruvate content were identified. The findings indicated that this polysaccharide contained glucose, glucoronic acid and mannose. Overall, the date palm juice by-products presented in the current study seem to exhibit promising properties that can open new pathways for the production of efficient and cost-effective xanthan gum.

1. Introduction

Palm date is one of the most widely cultivated crops in the Mediterranean region, particularly the Tunisian Sahara, and plays an important role in the life of its people. According to current official estimates, Tunisia is the 10th world producer and the first exporter of dates in value. During the last 4 years, Tunisian palm date production has reached an average of 120.000 tonnes/year. Inopportunely, however, palm date harvesting is often accompanied by substantial fruit losses that occur during the picking, storage and conditioning processes (Besbes et al., 2005). Because of their inadequate texture (too soft), the lost dates, commonly named "date by-products", are not edible and are often discarded. Currently, very little use is made of these by-products and they are, most of the time, used for limited purposes such as animal feed (Besbes et al., 2006).

Only little research has been carried out in regard to the usability of this ill-employed by-product. The currently available studies that have so far been conducted on this issue seem to have focused on the valorization and biologic transformation of this substrate for the production of biomass and various other the biologic transformation especially aiming production of biomass and a variety of other compounds, such as citric acid, oxytetracycline and ethanol (Besbes et al., 2006). Of special interest to the aims of the current study, these by-products seem particularly useful for the production of high value-added components such as xanthan gum.

Xanthan gum was discovered in late 1950s when an American research team, which was conducting extensive search for useful biopolymers, discovered that a bacterium found on cabbage plants, known as *Xanthomonas campestris*, could be fermented to produce a polysaccharide of significant usefulness to the food industry (Kang & Pettit, 1993; Sutherland, Swings, & Civerolo, 1993). In 1969, the Food and Drug Authority (FDA) authorised the use of xanthan gum in food products, marking the introduction of the first industrially produced biopolymer to the food industry. Since then the demand for xanthan gum produced from *X. campestris* has progressively increased, at an annual rate of 5–10%.

Xanthan has a particularly complicated molecular structure. Its main chain consists of glucose molecules connected by β-1,4 glycosidic links and is similar to that of cellulose. Every second glucose unit carries a side chain which is composed of β-D-mannose,

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β-1,4-D-glucoronic acid and α-1,2-D-mannose together with a pyruvic acid unit (Rosalam & England, 2006). Xanthan molecular structure is often reported to be heavily affected by the composition of the production medium. In this respect, several studies have so far focused on a variety of nutrients, particularly the nitrogen and carbon sources, with glucose and sucrose as the most frequently used carbon sources (Garcia-Ochoa & Gomez, 1998; Garcia-Ochoa, Santos, Casas, & Gomez, 2000).

In fact, the cost of the fermentation medium has always been a major concern in the commercial production of xanthan. For this reason, recent research in the field has particularly focused on the search for cheaper natural alternatives for the currently used substrates, namely glucose or sucrose, so as to control the cost of the production process as well as of the final product. The current study is an attempt to contribute to this current search for efficient and cost-effective substrates for xanthan gum production. In this context, the authors postulate that the Allig date palm by-products, which are abundantly available in nature as a waste of palm date harvesting, storing and conditioning processes, can be used as a cheap substrate for xanthan gum production.

Accordingly, the present work was undertaken to explore and further optimise xanthan gum production by *X. campestris* in batch experiments on Allig date palm by-products using response surface methodology. It evaluated three main independent variables, namely carbon sources, nitrogen sources and temperature values, in terms of their individual and combined effects on optimum xanthan and biomass production. The polysaccharide formed was subjected to HPLC and TLC analyses and its molecular weight as well as pyruvate content were identified.

2. Materials and methods

2.1. Samples

The palm date fruit samples used in the current study were second-grade dates (Allig with hard texture) purchased from the industrial plant of the Degach region (South of Tunisia). The physico-chemical composition of the second-grade date was previously analysed in a recent study by the authors (Besbes, Drira, Blecker, Deroanne, & Attia, 2009).

After being sorted, the dates were pitted and the fleshes were washed and air dried over twelve hours before grinding. The date juice was prepared by adding water to the date paste at a ratio of (3:1, v/w) as described by Youssif, Al-Shaawan, Mininah, and El-Taisan (1987) and Youssif, Abou Ali, and Abou Idreese (1990). The date paste–water mixture that was obtained was gently boiled for 15 min with continuous stirring and then centrifuged for 45 min at 10,000 rpm. About 2.5 l of date juice were obtained from 1 kg of date. The soluble solids content (Degree Brix) of the date juice was about 20 Degree Brix. It was determined at 25 °C using a refractometer (type NAR-1T).

2.2. Microorganism, culture media and inoculum preparation

A wild-type strain of *X. campestris* was used throughout this study. The preparation of the inoculum was performed by the transfer of the microorganism from the stock solution to the Yeast Mold agar plates (YM agar) and its subsequent incubation for 48 h at 30 °C. A loopful of cells from the (YM) plates was then transferred to a 100 ml conical flask containing 25 ml of the sterile YM medium and incubated for 24 h at 30 °C and 180 rpm. This was ultimately used as the inoculum. Fermentation was carried out in 250-ml Erlenmeyer flasks, each of which contained 50 ml of the sterile production medium. The medium was inoculated for 24 h with 5% (v/v) of the *X. campestris* culture.

The medium used for the growth and maintenance (YM agar) contained (g/l): glucose, 46.6; peptone, 5; yeast extract, 3; and agar, 17 (pH 7). Bacterial cells in agar slants were incubated for 48 h at 30 °C and then stored at 4 °C for further use.

The medium used for xanthan gum production contained (g/l): date juice: D-glucose, 84.68; KH_2PO_4, 5; $MgSO_4$, 0.2; $(NH_4)_2SO_4$, 2.7; citric acid, 2; H_3BO_3, 0.006, $ZnCl_2$, 0.006; $FeCl_3$, 0.0024 and $CaCO_3$, 0.02. The pH was measured at 20 °C using an MP 220 pH meter (Mettler-Toledo GmbH, Schwerzenbach, Switzerland) and adjusted to 7 by the addition of HCl (1 M). The two solutions were sterilized for 20 min at 121 °C.

2.3. Analytical methods

2.3.1. Determination of dry cell weight (DCW)

The cells were collected after centrifugation for 30 min at 4 °C and 12,000 rpm and the supernatant was discarded. The biomass was subsequently washed twice with alcohol to remove traces of xanthan before being subjected to another centrifugation for 10 min at 9000 rpm. The cells were then dried in a hot air oven for 3 h at 105 °C (Memmert, Germany) and weighed.

2.3.2. Xanthan gum production and concentration

Xanthan production was made through aerobic fermentation in batch in an orbital shaker (Certomat H/HK) set at 30 °C and 180 rpm for 72 h. The fermented broth was centrifuged for 30 min at 12,000 rpm to remove bacterial cells (Sorvall Instruments model RC-5C) at 4 °C.

The cell-free supernatant (10 ml) that was obtained through the procedures described above was then added to three volumes of ice cold ethyl alcohol, and the mixture was kept at 4 °C for 12 h to precipitate xanthan gum. Afterwards, the precipitate was recovered by centrifugation for 30 min at 4 °C and 10,000 rpm. The xanthan gum separated by centrifugation was then washed with ethyl alcohol and dried in a hot air oven for 24 h at 40 °C. The production of the biopolymers of this strain was evaluated by measuring the weight of the dry product per liter of fermented broth and the average was expressed in g/l.

2.3.3. Determination of glucose concentration

Fifteen milliliters broth samples were centrifuged for 10 min at 12,000 rpm, and the supernatant was used for the determination of glucose concentration. This was performed using the dinitrosalicylic acid method of Miller (1959).

2.3.4. Determination of pyruvate content

The percentage of pyruvate covalently attached to the polysaccharide was determined by a lactate dehydrogenase enzymic assay (Pyruvate kit from Sigma Diagnostics) after the hydrolysis of the xanthan samples in 0.1 M HCl for 4 h at 100 °C (Cheetman & Punruckvong, 1985).

2.3.5. Determination of xanthan molecular weight

Xanthan molecular weight was determined by HPLC on a ZORBAX BIO SERIES GF-450 Size Exclusion Column (3 × 100 cm), using 0.1 M NaCl as eluent at a flow rate of 2 ml/min. The detector used was a High Sensitive Refractive Index Detector, Model ERC-7515 A (ERC Inc., Japan). The calibration of the column was done with known concentrations of available commercial xanthan gum (SIGMA). An aliquot of 50 μl was injected to the column after filtration through 0.45 μm Millipore filter at room temperature.

Before estimating the pyruvate content and molecular weights, the xanthan samples were subjected to alcohol precipitation using ethyl alcohol (1:3, v/v) and a 20% (w/v) KCl solution to a final concentration of 5%. Precipitation was repeated three times and the polysaccharide was then lyophilised.