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Valorization of brown algae (Cystoseira caespitosa) from local region in Algeria for sodium alginate extraction and their application in the immobilization of microbial pectinases

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ABSTRACT/RESUME

Abstract: *Sodium alginate were extracted from brown algae (Cystoseira Caespitosa) collected from Algerian Mediterranean coast. the physicochemical characteristic of sodium alginate showed that the average yield was 12% and the average molecular masse was 9000Da calculated using intrinsic viscosity data. Fourier transform infrared spectroscopy was used to characterize the extracted powder of sodium alginate, COO^- and C-O-C were evident in the finger print region, the ratio of guluronic acid (G) / mannuronic acid (M) was recorded to be 0.91. This polysaccharide were used to immobilize crude pectinolytic enzymes synthesized by a strain of Aspergillus niger sp. The immobilization was carried out by dropwise introduction of alginate-enzyme mixture into a 0.2M $CaCl_2$ solution, by this technique, alginates shrinking in the form of spherical beads. The calcium alginate beads have been analyzed using scanning electron microscopy (SEM), observation under SEM showed that the beads have a rough surface and the enzymes were successfully included in the beads. The immobilization efficiency was about 83.9% and the immobilized enzyme could be reused in 4 cycles keeping 42,14% of its initial activity. The beads were successfully used for apple juice clarification.*

I. Introduction

Pectic substances refer to a complex class of carbohydrate polymer composed of galacturonic acid, a carbohydrate uronic acid linked through t α-1-4-glycosidic linkage and widely found in primary cell walls and middle lamella of higher plants [1]. Pectinases is a generic term used for the complex group of enzymes that catalyze the hydrolysis of pectin breaking α-1-4-glycosidic linkage of galacturonic acid to reduce the viscosity which is responsible to cause turbidity and undesirable cloudiness in the fruit juice. Pectinases are widely used in the food industry for the clarification of fruit and vegetable juices as well as in the wine industries [2]. Despite the high catalytic properties of pectinase, the use of the free enzyme still presents certain problems such as low stability, product recovery and repeated continuous use [3].

Enzymatic immobilization is the only means of support for overcoming these problems, which not only improves the catalytic properties of the enzyme, but allows for continuous reuse of an

expensive enzyme to make it economically viable for industrial applications [4] .

The immobilization of the enzymes is generally carried out by three main methods (i) covalent binding to a support (support), (ii) adsorption of enzyme molecules on a support material and (iii) entrapment or encapsulation of enzyme in polymer matrices. Both covalent bonding and adsorption methods have disadvantages in that they have the potential to affect the substrate binding site of the enzyme and enhance the limitation of diffusion to the enzyme which ultimately results in decreased enzymatic activity [5]. Entrapment is a one-step process in which the chances of lost activity are comparatively low. Different synthetic and non-synthetic polymers such as polyacrylamide, agar and alginate have been used for the entrapment of enzymes as well as cells [6], [7]. The appeal of insoluble calcium alginate beads has been shown to be the most effective approach due to biocompatibility, low cost, availability and resistance to microbial contamination compared to other methods [8]. Calcium alginate beads have been reported to be one of the most potent carriers compared to others for the immobilization of different enzymes [9]. Alginate derived from brown algae is widely used to form gels in which enzymes can be attached by chemical crosslinking agents [10]. Pectinases were immobilized in alginate by single inclusion [11]. However, its residual activity was slightly lower or had a lower stability making it difficult to use on an industrial scale.

The objective of this work is twofold, it consists first in producing microbial free at low cost, then immobilizing them on a support of sodium alginate produced locally from the macroalgae very available
on the Algerian coast. . This experimental approach allows reuse of immobilized pectinases.

II. Material and methods

A. Plant Material Preparation and Extraction of Sodium Alginates.

The algae used are of the genus Cystoseira harvested in January and February 2017 at the beach of Cap Djenat (located in east of Algiers) by manual pulling. The algae samples are cleaned, sorted, washed, dried and then ground to obtain fine powder.

The extraction of sodium alginates from the algal powder was carried out according to the protocol described by [12]. The previously weighed algal powder was partially depigmented by immersion in chloroform for 24 hours, this operation was followed by leaching in 0.5N sulfuric acid for 6 hours, in this stage of preparation, the insoluble alginate salts located in the cell of algae transforms to insoluble alginic acid, while the calcium, sodium and magnesium ions are released as sulphates and removed by the washings. The alginates are released into the aqueous phase as soluble sodium alginate after carbonation by immersing the leached algal material in a 4% sodium carbonate solution for six hours with gentle agitation at a temperature not exceeding 50 ° c. The carbonation process is followed by flocculation and flotation to separate the sodium alginate solution from the particles of suspended algal material. Finally, the alginates are precipitated by acidic ethanol at pH 2 and the alginate precipitate is recovered by filtration and then dried by lyophilization.

B. physicochemical characterization of alginate

The water content in the sodium alginates was determined by drying portion of 1 g in an oven at 105 ° C to constant weight and the ash content was determined by mineralization of this portion in a muffle furnace at 600 ° C for 6 hours. The average viscometric molecular mass Mv of the alginates was determined by the Mark Houwink equation (equ.1)

$$[\eta] = 1{,}60 \times 10^{-2} Mv^{0{,}748} \qquad (1)$$

Where $[\eta]$ is the intrinsic viscosity. This is the limit of the reduced viscosity of the solution when the concentration tends to 0. It is determined by plotting the reduced viscosity of a series of dilutions of the alginate solution as a function of their respective concentrations.

The sodium alginate powder was also analyzed by infrared absorption spectrophotometry. The spectra (IR) were recorded using a Fourier Transform Infrared Spectrophotometer (FTIR) type Shimadzu 8400.

The absorption range of the wavenumber radiations comprised between 4000 cm -1 and 400 cm -1 makes it possible to reveal the presence of certain characteristic functional groups.

C. Microbial Synthesis of Pectinolytic Enzymes.

The synthesis of pectinolytic enzymes was carried by fermentation according to the technique described by [13]. The fermentation was monitored in 250 ml Erlenmeyer flasks containing the culture medium which is composed of 10 g of citrus peel, 1 g of pectin, 1 ml of $(NH4)_2 SO_4$, 0.1 ml of KH_2PO_4, 1 ml of $FeSO_4$, 0, 01 g of urea and 10 ml of distilled water. After sterilization of flasks at 120 ° C. for 15 minutes, the culture medium was seeded with 1 ml of inoculums of *Aspergillus niger sp.* previously prepared according to [14] . The flasks were incubated at 30 ° C for 10 days; The enzyme was extracted by adding 50 ml of sterile

distilled water to each Erlenmeyer. Flasks were agitated at a speed of 400 rpm / min for 30 minutes, the contents were filtered through a sterile gauze and centrifuged at 10000 rpm for 20 min at 4 ° C. The filtrate obtained was the crude enzymatic extract (EEB) used for the immobilization after its chemical analysis.

The soluble protein in crude enzyme was determined by Lowry method [15] using bovine serum albumin (BSA) as standard and the pectinolytic enzyme activity was determined by using 1% (w/v) citrus pectin as a substrate. One unit (U) of pectinase activity was defined as the amount of enzyme producing 1 µmol galacturonic acid per min at 40°C and pH 4,5. The galacturonic acid content was quantified by dinitrosalicylic acid (DNSA) method.

D. Immobilization of Pectinases by Sodium Alginate.

Five ml of a 0.4% of sodium alginate solution prepared in buffer solution at pH 4,5, was mixed with the same volume of enzyme extract (EEB) and gently stirred several time . The mixture was added dropwise into 0,2M $CaCl_2$ solution using syringe. In the presence of calcium, the alginates shrink as beads. Latter the alginate beads were transferred into another 0.2 M $CaCl_2$ Solution and incubated for 1 hour at 4°C. After this, the alginate beads are recovered by filtration and washed several times until the absorbance of the washings at 280 nm was zero.

E. Characterization of alginate beads

The morphology of the beads was analyzed by scanning electron microscopy (SEM). The amount of enzyme protein loaded into alginate beads was evaluated by subtracting the residual protein content in the solution from the initial protein content. The protein content was quantified by lowry method [15]. The pectinase activity in the beads was determined as it was described previously in paragraph II. 3.

The efficiency of the immobilization (Ei) was determined by equation 2:

$$E_i\% = \frac{A_L - A_S}{A_L} * 100 \qquad (2)$$

Where A_L is the total activity of the crude free enzyme used for immobilization and A_S denotes the total activity in supernatant of the $CaCl_2$ solution.

The yield of immobilization was determined by equation 3:

$$Y_i\% = \frac{A_{IE}}{A_{IT}} * 100 \qquad (3)$$

where Y_i is the yield of immobilization (%), A_{IE} is the total experimental activity of the immobilized enzyme (calculated considering the total mass of immobilized enzyme) and A_{IT} is the total theoretical activity of the immobilized enzyme ($A_{IT} = A_L - A_S$).

The operational stability (OS) of the immobilized enzyme was determined after successing batch cycles of defined amount of the immobilized extract on the hydrolysis of citric pectin. The reaction time for each batch cycle was 5 min. The activity of the first cycle was considered as 100%. After each cycle, the bead containing pectinases were removed from medium and washed with distilled water and sodium acetate buffer.

F. Clarification of an Apple Juice.

The juice was extracted from apples purchased from local market using a household juice extractor, the juice was centrifuged at 3000rpm for 15 min and then filtered. The transmittance of the filtrate was measured at 660 nm using a visible UV spectrophotometer type Jasco V 530. The juice was heated at 50 °C and introduced into a column sizing 100 mm in height and 10 mm in diameter, using a peristaltic pump at a constant flow rate of 0.75 ml / min. The column was filled with calcium alginate beads. At the outlet of the column, fractions of juice were collected. each fraction was centrifuged and analyzed by reading its transmittance at 660 nm.

III. Results and discussion.

III. 1. Characterization of Plant Material

The macroscopic analysis of harvested brown algae has shown that it is *Cystoseira caspitosa* which belongs to the family of *fucal cystoseiraceae* of the class of *pheophyceae* which are *ochrophytes*. They vary in size from 8 cm to 12 cm, the outer morphology observed from a magnifying glass has shown that the species is composed of several axes, the thallus is branched and thorny, carrying an encrusting base and a short trunk carrying primary and secondary branches, the ultimate branches have the shape of thorns, (fig.1).

The moisture content in the algae reported by Bouchaud et al., **[16]** is 11% whereas that found in brown algae in this study varies between 10 and 35%. This difference in value in moisture is due to the storage conditions and the species of algae used. The average ash content was found to be 24.15%, this content is in the range reported by several authors **[17]** who found values that typically ranged from 8% to 40%.

Figure1. *Brown algae used; a: before harvest; b: after harvest.*

III. 2. Characterization of Sodium Alginates

The sodium alginates are obtained in the form of a white powder which tends towards brown (fig. 2). This color is relatively lighter compared to the color of alginates extracted from *Sargassum duplicatum* by Indriani et al., **[18]**.

Figure 2*. Sodium Alginate Powder Obtained*

The Extraction and dehydration methods influence the color of alginates, which may contain traces of colored pigments. The extraction yield obtained is 16%; according to Romo et al.,**[19]** and Loareg et al., **[20]** the alginate content in brown algae varies from 10 to 45% (relatively to dry matter). This difference varies according to the species considered, the physiological state of the algae, the period of harvest, also the age of the plant, or the extraction methods and the solutions used for their precipitation. The moisture content in the alginates was 4%, this value depends on the intensity and duration of drying and the storage conditions of the material. The ash content is 36%, a value close to those found by several authors who have worked on different varieties of brown algae. Values of 30% and 30.6% were reported by Sari-Chmayssen et al., **[12]** and Bi et al., **[21],** respectively, whose work focused on *Sagassum terranium*.

The average viscosimetric molecular mass of alginates recorded is 9000 Da, this value is low compared to that found by several authors, **[22]**, **[21]**. Indeed, the average molecular mass of commercial alginates varies between 32,000 and 200,000 Da. According to Cho et al., **[23],** the conventional process for extracting alginates makes it possible to extract only the water-soluble alginates, which corresponds to a yield of less than 10%. In addition, these water-soluble alginates have rheological qualities much lower than the alginates resulting from an alkaline extraction, in terms of ability to form gels and viscosity in aqueous solution. These differences can be explained by a large difference in mean molecular weights: 800 kDa for alginates from alkaline extraction and 12-45 kDa for alginates that have been pretreated with strong acids.

Table 1. *Physicochemical Characteristics of Sodium Alginates.*

Parameter	**Value**
Moisture (%)	4
Ash(%)	36
Viscosimetric molecular mass Mv (Da)	9000
Yield(%)	16 %

III. 3. Infrared Spectrophotometry of Sodium Alginates

The Fourier Transform Infrared (FTIR) spectrum of alginate extracted from brown algae (Figure 3) contains a broad absorption band, the maximum of which is observed at 3433 cm-1, this band is characteristic of hydroxyl group (OH), a weaker signal at 2924 cm-1 is attributed to the stretching vibrations of the C-H bonds The presence of two strong peaks at 1637 cm-1 and 1418 cm-1 is assigned to asymmetric and symmetric stretching vibrations of carboxyl groups of alginate (COO). The anomeric region of Carbohydrate, between 950 and 750 cm-1, is the most discussed in the literature **[24]**. In the 948 cm-1 region, the C-O stretching vibrations of the uronic acid residues appear while under our experimental conditions a peak at 993 cm-1 is observed in our spectrum. The guluronic units give a band at approximately 1030 cm-1, while the mannuronic units originate a band at 1093 cm-1 **[25]**. According to other authors **[26]**, the characteristic peak of the mannuronic group appears at 1125 cm -1 and that of the guluronic group appears at 1030 cm -1. Indeed, in this work a broad band appears between 1020 and 1239 cm-1, it is characteristic of the CO-bonding osidic while the peaks which appear at 1122.41cm-1 and 1113.81cm-1 can be assigned to mannuronic and guluronic acid respectively.

The physicochemical properties of alginate solutions and gels in aqueous media depend on their structure and the proportion of mannuronic

residues with respect to guluronic residues (ratio noted M / G) as well as the number of and the length of the blocks MM, GG and MG. The most commonly used technique for calculating this ratio is the nuclear magnetic resonance (NMR). Several authors also try to estimate this ratio by means of infrared spectroscopy **[26]**. In the same way we estimate the ratio M / G by calculating the ratio of the absorbances at the frequencies 1122.41 cm-1 and 1113.81 cm-1 corresponding to the mannuronic and guluronic acid respectively. This ratio is found to be equal to 0.97, this value is close to 0.99 found by Bekattmania et al., **[26]** during infrared spectroscopic analysis of *Dictyopteris* species from Morocco.

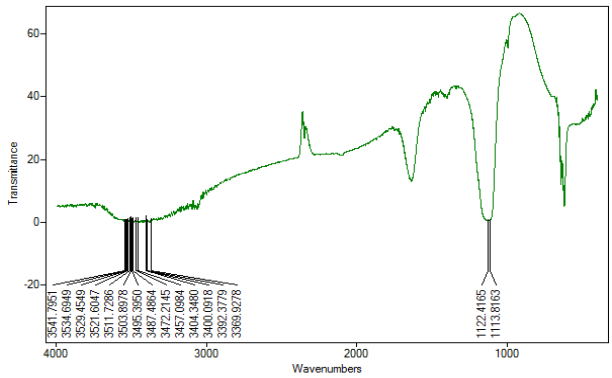

Figure 3. *FTIR spectrum of alginate powder extracted from algae.*

III. 4. Characterization of the Enzymatic Extract Brut (EEB)

After 10 days of incubation of the Erlenmeyer flasks at 30 ° C., the surface of the culture medium was completely covered by the characteristic black spores of the microorganism. The extraction of pectinases gave an EEB characterized by a soluble protein level equal to 63.82 mg / ml and an enzymatic activity equal to 395 U / ml which remains high compared to that observed by Suresh et al., **[27]**. Indeed, these authors used the DNSA method, and reported values between 22.12 and 162.5 U / ml for exo-PG activity produced by *Aspergillus carbonarius*. Otherwise, the exo-PG activity produced by *Aspergillus niger* was142 U / ml whereas that produced by *Aspergillus oryzae* was40 U / ml **[28].**

III.5. Study of Immobilization of Pectinolytic Enzymes by Calcium Alginate.

The enzyme immobilization technique gave spherical beads 0.5 cm in diameter (FIG.4). The soluble protein level in the alginate beads was in the order of 11750 µg / mg. A pectinolytic activity in these beads confirms the entrapment of the enzymes by the calcium alginate polysaccharide. In fact, a value of 392.23 U / mg of polygalacturonase was obtained, a value close to the pectinolytic activity of free enzymes in the EEB.

Figure 4. *Formation of alginate / pectinolytic enzyme beads.*

immobilization efficiency and yield were 83.98% and 116.5% respectively. In previous work of Bustamante et al., **[29]**, the immobilization efficiency of pectinases on a matrix composed of alginate / gelatin / calcium oxalate was 61.7% with the yield was 127%. Regarding the operational stability of the immobilized enzymes at each reaction cycle, a new amount of substrate was added for a new batch reaction until a residual activity of less than 50% was obtained, the same reactions conditions were used for a batches. The immobilized pectinase retained more than 54.82% residual activity on its second cycle and 42.14% during the fourth cycle of use (fig.5). In the work of Bustamante et al., **[29]**, the immobilized derivative obtained using AGOCa as the immobilization support showed the best results, 42.38% of residual activity after 10 cycles of use. Relative activities of 46.33 and 44.89% were obtained after 8 and 7 cycles respectively for the alginate/oxalate and alginate/Calcium matrices. The loss of activity of entrapped enzyme is ma be due to leakage of enzyme from calcium alginate beads as results of washing of beads at the end of each cycle, in addition to possible conformational changes and mechanical damage after repeated cycles **[30].**

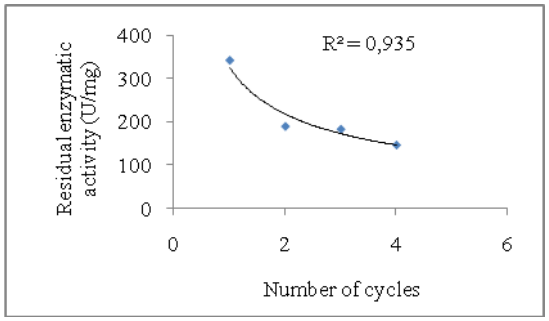

Figure 5. *Operational stability of immobilized enzymes*

III. 6. Morphological Study of Calcium Alginate beads

The pores of the calcium alginate beads were clearly observed in the calcium alginate grain micrographs at different magnification scales (Figure 6 (a), (b)). In photos (c) and (d) of Figure 4, the pores were coated with dense enzyme particles.

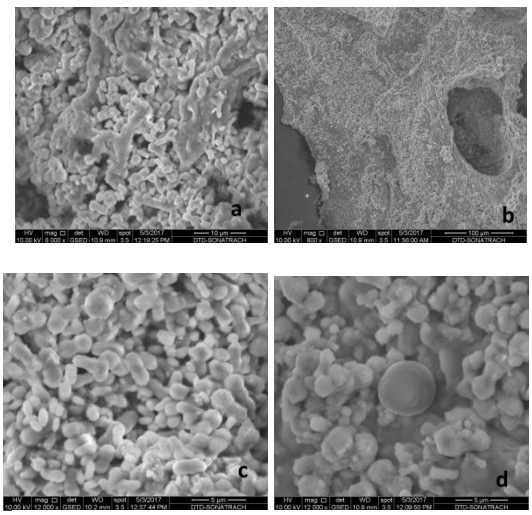

Figure 6*. Scanning electron microscopic analysis of immobilized enzyme alginate*

III. 7. Clarification of apple juice by immobilized enzymes

The transmittance at 660 nm of cloud apple juice increased from 2% to 88.6% in the clarified juice obtained at the outlet of the column.

This difference is due to the enzymatic hydrolysis of the pectins responsible of the cloud in the juice (FIG 7). These results are similar to those obtained by Hachemi et al., **[14]** during the clarification. of an apple juice by the free pectinases synthesized by the same microorganism which was cultivated on orange peels. Preliminary work on clarification of apple juice by immobilized pectinases reported a 45% transmittance in clarified juice **[31].**

Figure 7. *Clarification of apple juice by the immobilized pectinolytic enzymes: (a) raw juice, (b) clarified juice.*

These particles are similar to those observed by Rehman et al., **[30]**. The structural morphology of the calcium alginate beads is maintained after immobilization and the immobilized pectinases appear as black objects in the pores of the calcium alginates.

IV. Conclusion

This study allowed us to exploit the brown algae of a coastal region located east of Algiers in Algeria for the production of sodium alginates. This biomaterial has been found suitable for the immobilization of pectinolytic enzymes by simple inclusion. The immobilized enzymes could be used more than four times for the enzymatic hydrolysis of pectic disorders. The enzymatic activity of the free pectinases synthesized by the microorganism was mostly preserved after their immobilization by the alginates and the immobilization efficiency was 83.89%. Moreover, the scanning electron microscopic analysis of the alginate beads after immobilization clearly showed the dense particles of enzymes that cover the pore of sodium alginates.

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