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Three phase partitioning of carbohydrate polymers: separation and purification of alginates

A. Sharma, M.N. Gupta*

Department of Chemistry, Indian Institute of Technology, Hauz Khas, New Delhi 110016, India

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Abstract

Three phase partitioning, a technique described for protein purification, has been employed for precipitation and purification of three different commercial preparations of alginates. Three phase partitioning works by the addition of *t*-butanol to aqueous solution of the polymer containing 20–30% ammonium sulphate (w/v). Three phases formed are: upper *t*-butanol layer, interfacial polymer precipitate and lower aqueous phase. In all the three cases, the process optimization was carried out by varying ammonium sulphate concentration, volume of *t*-butanol, alginate concentration and temperature. Fluorescence spectroscopy was used to show that repeated cycles of TPP also resulted in considerable reduction in polyphenol content of a crude alginate preparation.

Keywords: Alginate; Fractionation of water soluble polymer; Three phase partitioning; Smart polymers

1. Introduction

Alginates are composed of linear polymers of 1–4 linked β-D-mannuronic and α-L-guluronic acid residues in varying proportions and sequential arrangements (Skjak-Braek, Murano & Paoletti, 1989). They are isolated from brown seaweed (for example, *Laminaria hyperborea* and *Ascophyllum nodosum*) (Baardseth, 1966) and are also synthesized by bacteria (for example, *Azotobacter* and *Pseudomonas* species) (Gorin & Spencer, 1966; Linker & Jones, 1966). Alginate is a polymer, which becomes reversibly insoluble in the presence of calcium ions (Smidsrod & Skjak-Braek, 1990). The recent work in our laboratory has shown that this property of alginate has been exploited for developing efficient methods for separation of pectinase (Gupta, Guoquiang & Mattiasson, 1993), starch degrading enzymes (Sharma, Sharma & Gupta, 2000a; Teotia, Khare, & Gupta, 2001) and phospholipase D (Sharma, Sharma & Gupta, 2000b). They are also used to thicken solutions, stabilize suspensions and to gel a wide range of mixtures. They have also been used as immobilization matrix for enzymes (Kierstan & Buckle, 1977) and whole cells (Smidsrod & Skjak-Braek, 1990). Alginate has also been found to be a biocompatible substance and has been used for implantation of various tissues and cells (Jork, Thurmer, Cramer, Zimmermann, Gessner, Hamel, Hofmann, Kuttler, Hahn, Josimovie-Alasevic, Fritsch & Zimmermann, 2000; Skjak-Braek et al., 1989). For all such applications especially the last one, it is necessary to employ a purified preparation that is free of extraneous materials such as polyphenols. The existing methods for this purpose tend to be multistep, tedious and often lead to depolymerization. This work describes for the first time the approach for precipitating and purifying alginates by three phase partitioning (TPP). TPP is an elegant emerging technique for concentration and purification of proteins (Sharma & Gupta, 2001; Sharma et al., 2000c). An attractive feature of the technique is that it is easily scalable. The technique consists of addition of *t*-butanol to a salt solution of proteins (Dennison & Lovrein, 1997; Pike & Dennison, 1989). Under suitable conditions, three phases are found. The top most layer consist of *t*-butanol and bottom layer consist of aqueous phase. These two phases are separated by interfacial protein precipitate. In this work, we describe that alginate can also be separated by TPP. It is further shown that the TPP leads to: (i) purification of alginate, which is an important step in its biotechnological and biomedical applications; (ii) three different kinds of alginates precipitate by TPP under different conditions. This shows the possible application of TPP in fractionating such polymers.

2. Materials and methods

Sodium alginate (composed predominantly of mannuronic

* Corresponding author. Tel.: +91-11-659-6599; fax: +91-11-658-1073.
E-mail address: mn_gupta@hotmail.com (M.N. Gupta).

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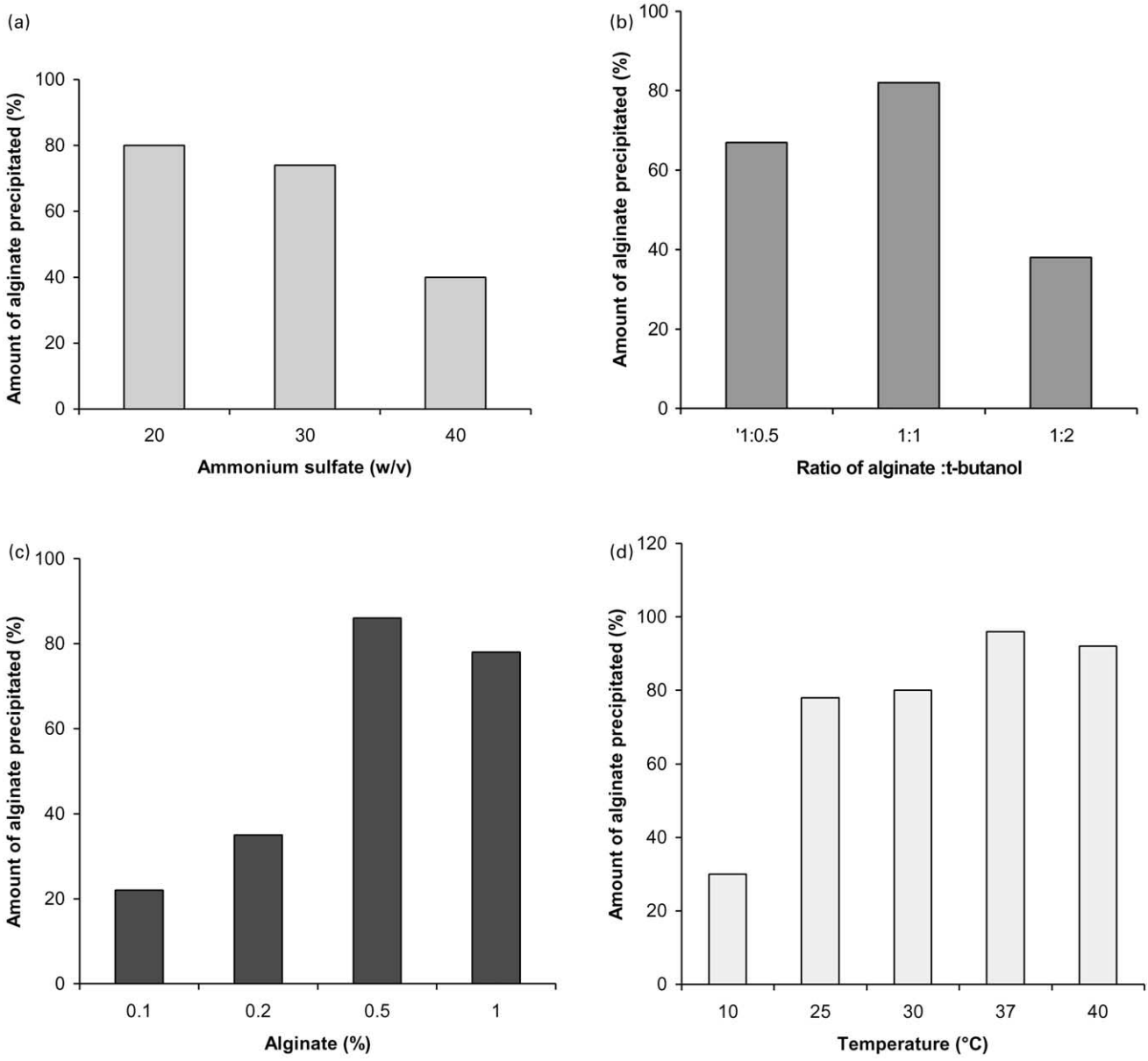
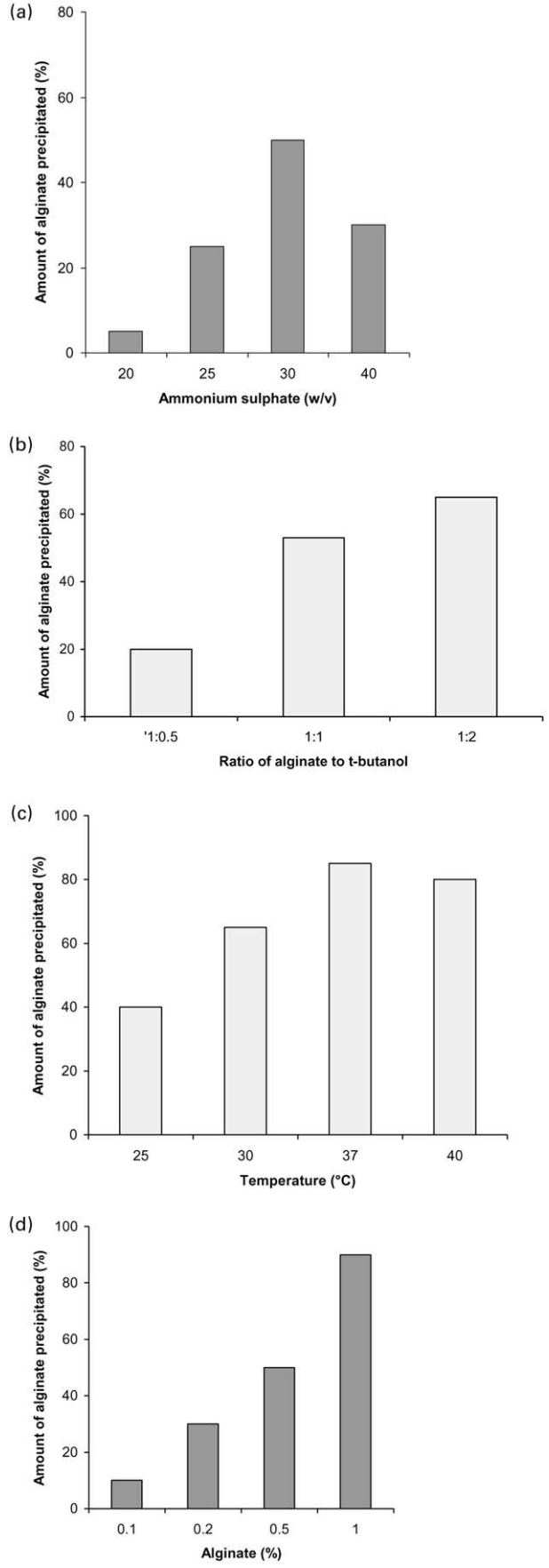


Fig. 1. Optimization of the conditions for precipitation of protanal SD-H by TPP. (a) The alginate solution (2 ml, 1%, w/v) was added to varying amount of ammonium sulphate (w/v), mixed with 2 ml of *t*-butanol (v/v). The interfacial precipitate was collected after keeping these systems at 25°C for 1 h. (b) The alginate solution (2 ml, 1%, w/v) was added to ammonium sulphate (20%, w/v), TPP was carried out at 25°C by varying the ratio of polymer solution to *t*-butanol (v/v). (c) The ammonium sulphate (20%, w/v) was added to varying amount of alginate solutions (0.1, 0.2, 0.5 and 1%, w/v). The ratio of alginate solutions to *t*-butanol was 1:1 (v/v) in all the cases. The three phases formed after keeping these mixtures at 25°C for 1 h were collected. (d) The alginate solution (2 ml, 0.5%, w/v) was added to ammonium sulphate (20%, w/v), mixed with 2 ml of *t*-butanol. TPP was carried out at different temperatures. In all the cases, the precipitated solution was suspended in distilled water.The amount of alginate precipitated was estimated as described in Section 2. Each set of above experiment was carried out in duplicate which varied within ±5%.

acid residues) was purchased from Sigma (St. Louis, MO, USA). Protanal LF 10/60 (sodium alginate from brown seed, having high guluronic acid content (65–75%) and protanal ester SD-H (propylene glycol alginate, degree of esterification = 40–60%)) were products of Protan A/S (Drammen, Norway). Crude (sodium alginate) was purchased from Central Drug House (Mumbai, India). To avoid confusion, the various alginate preparations will be called Sigma alginate, Protanal LF 10/60, Protanal ester SD-H and crude alginate, respectively, in the following text. All other chemicals were of analytical grade.

3. Preparation of alginate solutions

Alginate (2%, w/v) was dissolved in distilled water. The solution was stored at 4°C and diluted with appropriate amount of distilled water for further use.



4. Three phase partitioning of alginate solutions

Alginate solutions (2 ml) (Protanal ester SD-H, Protanal LF 10/60 and Sigma alginate) (various concentration, pH 7.0) were mixed with varying concentrations of ammonium sulphate (w/v). Appropriate amount of *t*-butanol (v/v) was added to this solution, vortexed and incubated at suitable temperature for 1 h. Three phases were formed. The top layer of *t*-butanol was separated from lower aqueous layer by an interfacial precipitate of the polymer. The precipitated alginate in the middle layer was dissolved in 2 ml of distilled water. The amount of alginate precipitated was estimated by phenol sulfuric acid test (Hirs, 1967). The amount of alginate precipitated was calculated by taking the starting amount of alginate as 100%.

5. Results and discussion

Fig. 1(a) shows the amount of Protanal SD-H precipitated (%), with varying amount of ammonium sulphate during TPP. During this process 1:1 ratio of *t*-butanol to polymer solution at 25°C was used. As 20% (w/v) ammonium sulphate gave the best results, the ratio of alginate to *t*-butanol (v/v) was varied using 20% ammonium sulphate (w/v) at 25°C. This showed that 1:1 ratio of alginate to *t*-butanol is the best ratio to operate (Fig. 1(b)). In these experiments, 1% alginate had been used. Fig. 1(c) showed that alginate concentration is also critically important and 86% precipitation of the polymer could be achieved with 0.5% alginate using 20% ammonium sulphate (w/v), 1:1 ratio and at 25°C. Changing the temperature to 37°C improved the extent of precipitation (Fig. 1(d)). Thus, by using 20% ammonium sulphate, 1:1 ratio and 0.5% alginate, 95% alginate could be precipitated from its solution in distilled water.

Protanal LF 10/60 alginate behaved in a similar fashion when subjected to TPP. Fig. 2(a)–(d) shows the effect of varying ammonium sulphate concentration, ratio of alginate solution to *t*-butanol, temperature and alginate concentration

Fig. 2. Optimization of conditions for precipitation of Protanal LF 10/60 by TPP. (a) The alginate solution (2 ml, 0.5%, w/v) was added to varying amount of ammonium sulphate solutions (w/v), mixed with *t*-butanol in the ratio of 1:1 (v/v). The interfacial precipitate was collected after keeping this system at 25°C for 1 h. (b) The alginate solution (2 ml, 0.5%, w/v) was added to ammonium sulphate (30%, w/v), TPP was carried out at 25°C by varying polymer to *t*-butanol ratio (v/v). (c) The alginate solution (2 ml, 0.5%, w/v) was added to ammonium sulphate (30%, w/v), mixed with 4 ml of *t*-butanol. TPP was carried out at different temperatures. (d) The ammonium sulphate (30%, w/v) was added to the varying amount of alginate solutions (0.1, 0.2, 0.5 and 1%, w/v). The ratio of alginate solution to *t*-butanol was 1:2 (v/v) in all the cases. The three phases formed after incubation of mixtures at 37°C for 1 h were collected. The amount of alginate precipitated in all the above cases was estimated as described in Section 2. Each set of above experiment was carried out in duplicate which varied within ±5%.

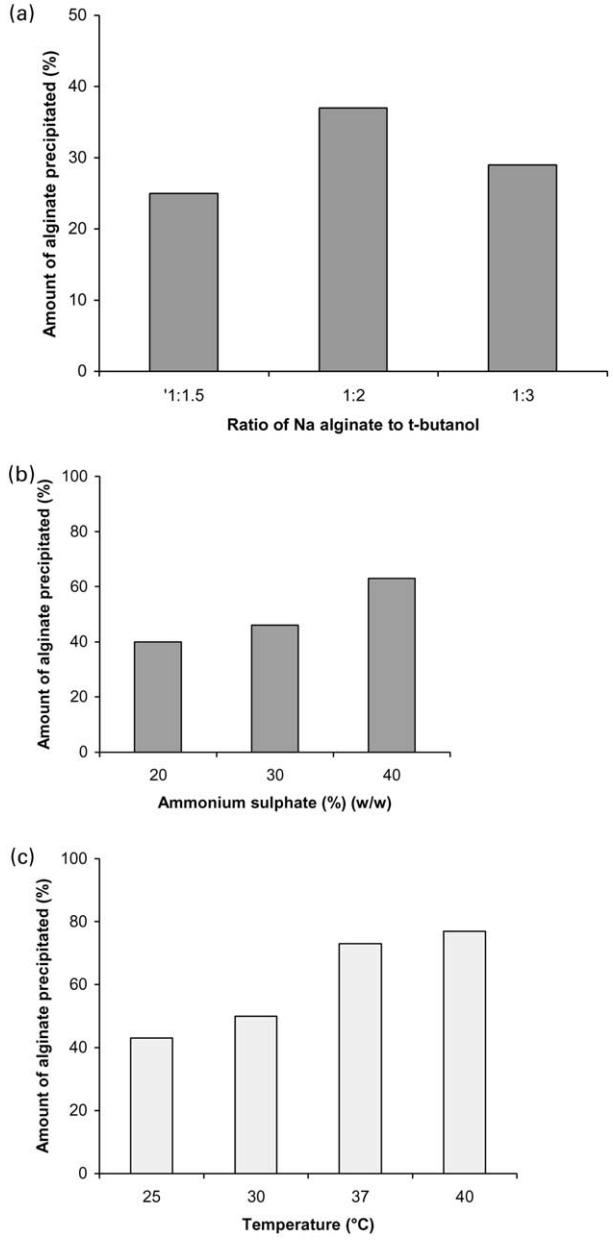


Fig. 3. Optimization of conditions for precipitation of Sigma alginate by TPP. (a) The alginate solution (2 ml, 2%, w/v) was added to ammonium sulphate (30% w/v), TPP was carried out at 25°C by varying polymer to *t*-butanol ratio (v/v). (b) The alginate solution (2 ml, 2%, w/v) was added to varying amount of ammonium sulphate (w/v) and mixed with 4 ml of *t*-butanol. The interfacial precipitate was collected after keeping these system at 25°C for 1 h. (c) The alginate solution (2 ml, 2%, w/v) was added to 30% ammonium sulphate (w/v) and mixed with 4 ml of *t*-butanol. TPP was carried out at different temperatures. Each set of above experiment was carried out in duplicate which varied within range of ±5%.

on the precipitation of alginate (details explained in legends). It was found that ammonium sulphate (30%, w/v), 1:2 ratio of alginate to *t*-butanol (v/v), ammonium sulphate (30%, w/v) and 1% alginate concentration gave the best result leading to 95% alginate precipitation in the interfacial precipitate. However, Sigma alginate when

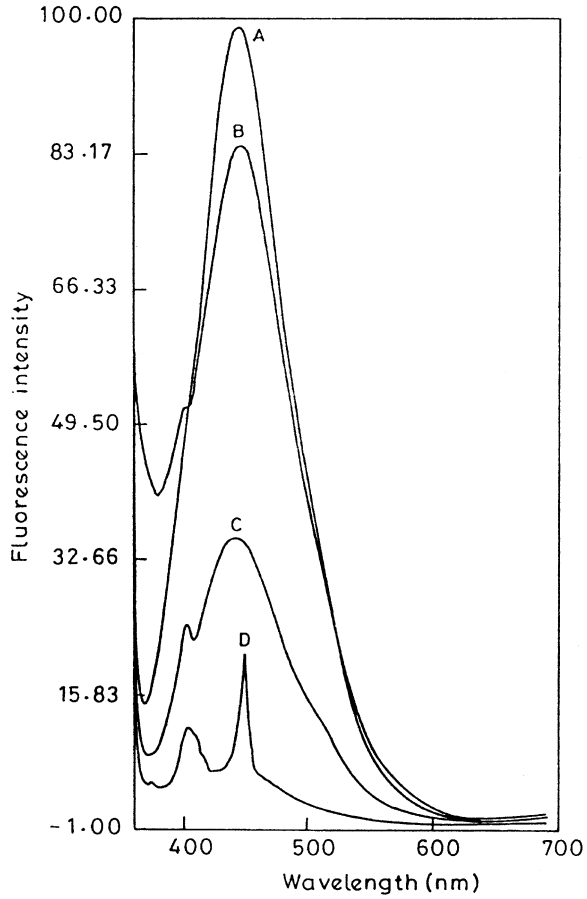


Fig. 4. Fluorescence spectra of crude sodium alginate. Emission spectrum was recorded on Shimadzu RF-5000 spectrofluorometer at an excitation wavelength of 366 nm. The first TPP of crude alginate was carried out by adding ammonium sulphate (30%, w/v) to 2 ml of alginate (2%, w/v), mixed with 4 ml of *t*-butanol. The mixture was then kept at 37°C for 1 h. For second and third cycle of TPP, the interfacial precipitate was dissolved in 2 ml of distilled water. (A) spectra of crude alginate before TPP; (B) after first TPP; (C, D) after second and third TPP, respectively.

subjected to optimized conditions resulted only in 70% alginate precipitation (Fig. 3(a)–(c)).

The brown algae that are the sources of alginates contain many polyphenolic compounds and immunogenic materials such as proteins, complex carbohydrates and nucleotides (Pedersen, 1984). Martinsen, Skjak-Braek and Smidsrod (1989) have described various procedures for obtaining alginates free from polyphenols. For example, selectively removing the algal tissue rich in polyphenol at the time of extraction and crosslinking remaining polyphenols with formaldehyde prior to extraction. Alternatively, extracted alginates are treated with hydrogen peroxide or sodium hypochlorite followed by dialysis. However, such procedures are known to depolymerize alginates. Thus, it was of interest to monitor the effect of TPP on polyphenol content of alginate. For this purpose, a much cruder sodium alginate (2%, w/v) was used as starting material. The polyphenol content was monitored by fluorescence emission spectra wherein emission

around 450 nm region was taken as indicator of polyphenol content (Jork et al., 2000). It can be seen (Fig. 4) that repeated TPP cycles considerably reduced the polyphenol content. The yield at the end of third TPP was about 50% of the original alginate.

The physiochemical principles behind the technique even in case of proteins are not clear (Singh, Gourinath, Sharma, Singh, Roy, Gupta & Betzel, 2001) but it seems to involve kosmotrophy, electrostatic forces, conformational tightening and protein hydration shifts (Dennison & Lovrein, 1997; Lovrein, Goldensoph, Anderson & Odegaard, 1987). Other reversibly soluble–insoluble polymers have also found considerable application in biotechnology (Galaev, Gupta & Mattiasson, 1996). Further work is being carried out to explore whether TPP can work as a general method for their isolation, fractionation and purification. Thus, TPP constitutes a gentle and scalable approach to obtaining alginate which can be used for biotechnological and biomedical applications.

Acknowledgements

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