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Characterization of bacterial alginate extracted from biofilm matrix

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ABSTRACT

Alginate has been employed as a model exopolysaccharide for studying biofilm establishment in water purification system. Yet, very few investigations focused on direct alginate extraction from biofilm matrix and characterization. To collect more information on the role of alginate exopolysaccharide in building the biofilm network, bacterial alginate was extracted from the biomass of a lab-scale submerged biofilm reactor, identified and characterized by Raman spectroscopy and MALDI-TOF mass spectrometry. Investigation was as well put on its interaction with Ca^{2+} by the atomic force microscopy. The extracted alginate amounted to (164 $\pm$ 21) mg g VSS^{-1}(as organic content). It was partially *O*-acetylated oligosaccharides blend with the ratio of mannuronic acid residue to guluronic acid residue as 1.19. Strong gel-like film formed by its cross linkage with Ca^{2+}. It may contribute to the formation of biofilm matrix skeleton. Direct exopolysacchairdes extraction and characterization may throw new insight on biofilm building up mechanism.

Keywords: Alginate; Biofilm; Exopolysaccharide; Wastewater treatment

1. Introduction

Biofilms are dense bacterial communities characterized by close association of bacterial cells generally attached to a solid surface and surrounded by a saccharide matrix. Extracellular polysaccharides (EPSs) are the major components of bacterial biofilm matrix. They play an essential role in biofilm structure [1].

To disclose biofilm matrix formation mechanism in water purification system, a few investigations employed alginate as a model EPS [2–4]. Alginate can be produced both by seaweeds and bacteria (algae alginate and bacterial alginate). It is a family of linear EPS consisting of 1,4-1inked uronic acid residues: β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues with random sequences in the molecule (Fig. 1a).

Reasons for taking alginate as a model EPS are as follows: (1) bacteria genera *Pseudomonas* and *Acetobacter* are known to secret bacterial alginate [5]. Their ubiquitous in nature make bacterial alginate as a popular EPS. (2) As one of the ionic polysaccharides, alginate is capable of forming complexes of unique structure with divalent cations (Ca^{2+}, etc.) easily, resulting in a highly compacted gel network [5]. (3) During this gel formation process, alginate has the ability to entrap macromolecules (proteins, DNA, etc.) and bacteria [6], leading to its possibility to be one of the skeleton part of biofilm matrix.

The importance of alginate on biofilm formation has been confirmed by several investigations [7–9]. Yet, it is worth noticing that most of current studies utilized

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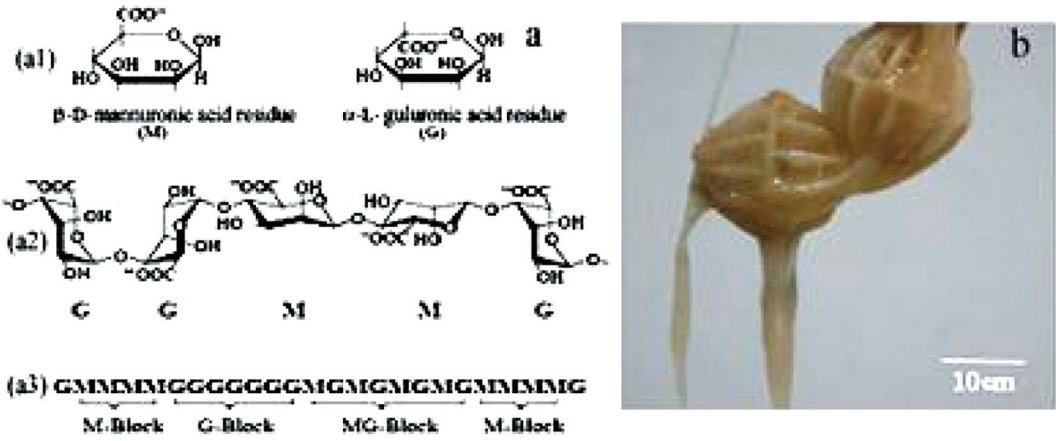

Fig. 1. Alginate structure (after [13]) and the biomass from lab-scale submerged biofilm reactor. (a1) uronic acid residues in alginate molecule (M vs.G); (a2) the alginate polymer; (a3) blocks of the alginate polymer; and (b) biomass of lab-scale submerged biofilm reactor.

algae alginate in their experiments, very few focused on direct alginate extraction from biofilm matrix and characterization. Due to the fact that differences still exist between alginates secreted by algae and bacteria, such as acetylation degree, ratio of mannuronic acid residue to guluronic acid residue (M/G ratio), etc., more information would be collected if direct alginate characterization could be performed. To achieve this purpose, bacterial alginate was extracted from a lab-scale biofilm reactor, identified and characterized in the current research. Interaction between Ca^{2+} and the extracted alginate was investigated as well.

2. Materials and methods

2.1. Reactor setup

A lab-scale submerged biofilm reactor with a working volume of 10 L, an internal diameter of 30 cm and a filling height of 30 cm was used for conducting the experiment. The reactor was filled with PVC carriers within its working volume, and continuously fed with a synthetic wastewater containing sodium acetate as the sole carbon source (COD loading rate of 6.0 kg m^{-3} d^{-1}) at 25 °C. More details are specified in [10]. About 0.5 L returned activated sludge from Tuandao municipal wastewater treatment plant, Qingdao, China was seeded when the reactor was start-up. The air flow rate in the reactor was 2 L min^{-1}.

2.2. Measurement and analysis

2.2.1. Alginate extraction

The biomass on carriers was collected. The alginate was extracted from the biomass according to [11] with modifications. The biomass was washed twice with deionized water and dried at 105 °C. One gram dried-biomass was extracted by 100 mL 0.2 mol L^{-1} sodium carbonate at 80 °C for 2 h. After filtration, the supernatant pH was adjusted to 2 by adding 0.1 mol L^{-1} H_2SO_4. The precipitation produced was collected by centrifugation (5000 rpm), and dissolved in 0.1 mol L^{-1} NaOH. Sodium alginate in the supernatant was precipitated by 50% (v/v) ethanol, washed twice in 80% (v/v), once in 96% (v/v) ethanol and lyophilized.

Freeze-dried crude sodium alginate was redissolved in phosphate-buffered saline (PBS) supplemented with 5 mM $MgCl_2$ and 1 mM $CaCl_2$, incubated with DNAse I and RNAse A (each at 100 μg/mL) at 37 °C overnight. Proteinase K was added afterwards (100 μg/mL) for 4 h with incubation at 56 °C. Enzymes were inactivated by heating for 30 min at 80 °C. The ethanol precipitation procedure was repeated once.

The purified alginate was chemically identified by FAO methods [12].

2.2.2. Characterization of the extracted alginate

2.2.2.1. FT-Raman spectroscopy

The extracted alginate was characterized by recording its FT-Raman spectra on the Raman spectrometer (Labram Infinity, France; He:Ne laser with excitation wavelength of 632.8 nm).

2.2.2.2. Mass spectrometry

MALDI-TOF MS of the extracted alginate was performed according to [1] with a Bruker Biflex III mass spectrometer (Bruker Daltonics, USA).

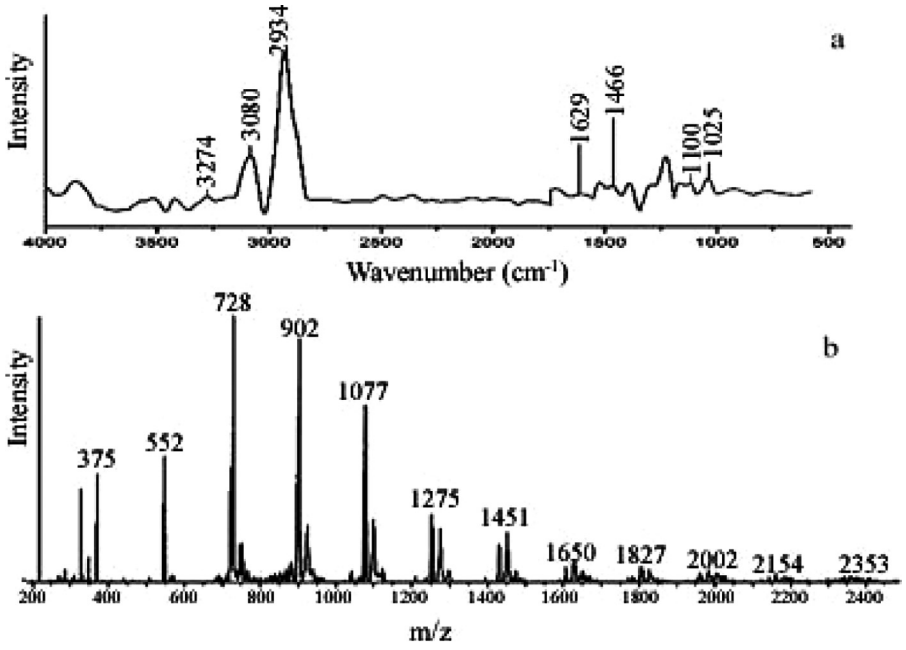

Fig. 2. Raman and MALDI-TOF MS spectra of the extracted alginate. (a) Raman spectrum and (b) MALDI-TOF MS spectrum.

2.2.2.3. Interaction between Ca^{2+} and the extracted alginate

To examine the influence of Ca^{2+} concentrations on the interaction between Ca^{2+} and the extracted alginate, 500 mg L^{-1} extracted sodium alginate solution was extruded through 20 mL syringes into $CaCl_2$ solution with Ca^{2+} concentrations of 20 mg L^{-1}, 40 mg L^{-1}, 100 mg L^{-1}, 500 mg L^{-1}, 1000 mg L^{-1} and 2000 mg L^{-1}, respectively.

Samples at 20 mg L^{-1} were also deposited onto a newly cleaved mica sheet, air dried (1 h) in a dust-free enclosure, imaged by an atomic force microscope (AFM) (SPM-9500J3, SHIMADZU, Japan).

Triplicates were conducted for each experiment above.

3. Results and discussion

3.1. Alginate extraction

Biofilm covered on carriers after 15 days (Fig. 1b). During alginate extraction, soft gel precipitates appeared in the supernatant when its pH reduced to 2. These precipitates were soluble in NaOH, demonstrating reversible sol–gel transition as the pH altered between alkali and acidic condition, which is attributed to the typical characteristics of alginic acid.

After enzymatic purification, the alginate recovered from biofilm matrix amounted to (164 $\pm$ 21) mg g VSS^{-1}(organic content). It displayed positive results to all the FAO alginate identification tests, which confirms that large amount of bacterial alginate existed in the biomass.

3.2. Raman spectrum

Raman spectrum demonstrated those typical peaks of bacterial alginate (Fig. 2a). The peak at 3274 cm^{-1} was attributed to the OH stretching vibration in intramolecular bonding. The strong peak at 2934 cm^{-1} was assigned to $-COCH_3$ group. Peaks at 1629 cm^{-1} and 1466 cm^{-1} were assigned to antisymmetric and symmetric vibrations of ionic COO^- group, respectively. The pattern of absorptions in the 1100–1000 cm^{-1} region was attributed to the COC and CCC bonds of the uronic acid skeleton. In addition, the peak at 3080 cm^{-1} was due to the appearance of C=C, which might be caused by the effect of alginate lyases.

Alginate refers to a family of polysaccharides containing β-D-mannuronic acid (M) and α-L-guluronic acid (G) residues. The proportion of these two residues (M/G ratio) determines the diversity of alginate's physicochemical properties and reactivity [9]. This M/G ratio was able to be obtained by Raman spectroscopy [14,15]: M and G residues manifested their characteristic Raman peaks at 1100 cm^{-1} and 1025 cm^{-1}, respectively. The intensity ratio of these two peaks provided information of M/G ratio. Based on this method, the M/G ratio of the extracted alginate was calculated as 1.19 (Fig. 2a), which indicated that the quantity of M residues existed in this alginate was more than G residues.

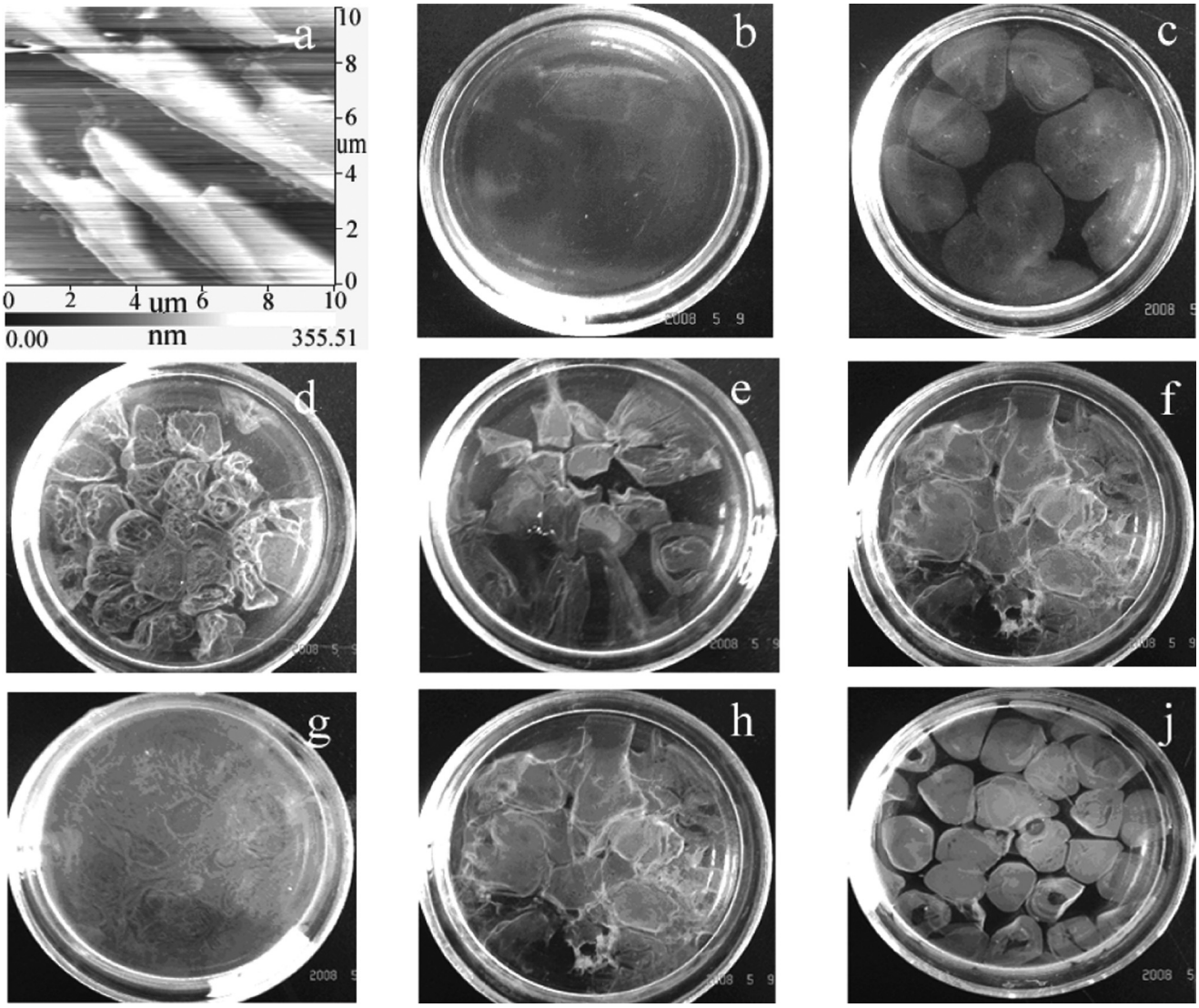

Fig. 3. Interaction between Ca^{2+} and the extracted alginate. (a–f) Alginate concentration 500 mg L^{-1}, Ca^{2+} concentration 20–2000 mg L^{-1}, respectively; (g-h) Ca^{2+} concentration 2000 mg L^{-1}, alginate concentration 250–1000 mg L^{-1}.

3.3. MALDI-TOF Mass spectrum

The extract's molecular weight distribution was determined by MALDI-TOF MS. It was found that the extracted alginate was oligosaccharides blend with low molecular weight and short molecular chain. The most abundant component at m/z 728 (Da) only contained four residues; the highest molecular weight one at m/z 2353 (Da) had 13 residues. Some of these oligosaccharides had acetyl groups, which is one of the characteristics of bacterial alginate.

Considering the appearance of C=C in Raman spectrum, it could be predicted that those alginate oligosaccharides within the biofilm matrix were the product of alginate lyases' performance. Alginate lyases can be secreted by a number of bacteria species, they usually cut alginate molecules by β-elimination, produce C=C between C_4 and C_5 of mannuronic acid residues [16]. Similar result was also reported by [1] that the biofilm matrix of *Pseudomonas sp*. OX1 grown on phenol was mainly constituted by alginate oligosaccharides. So far, EPS with higher molecular weight (>10,000 Da) has been considered as the model substance for biofilm formation. However, the current research indicated that, oligosaccharides with molecular weight less than 2500 Da may also play a part in biofilm formation.

3.4. Alginate–Ca^{2+} interaction

During the investigation on alginate–Ca^{2+} interaction, it was observed that as the soluble sodium

alginate drops (500 mg L^{-1}, 0.5 mm in diameter) contacted with $CaCl_2$ solutions, they spread on the surface and form white gel-like film quickly. These alginate–Ca film inclined to be stronger and extended to a larger area as Ca^{2+} concentration rose from 20 mg L^{-1} to 2000 mg L^{-1} (Fig. 3a–f). The mean size of those films at Ca^{2+} 20 mg L^{-1} was 10 μm in length, 2 μm in width and 0.2 μm in thickness by AFM observation (Fig. 3a). In comparison, the films' length and width enlarged to more than 30 mm and 20 mm, respectively, with the same drops of sodium alginate at Ca^{2+} 2000 mg L^{-1} (Fig. 3f).

Alginate concentration was also an important factor that determined gel-like film formation. Under sufficient Ca^{2+} (2000 mg L^{-1}), as alginate concentration increased from 250 mg L^{-1} to 1000 mg L^{-1}, gel-like film with tremendously improved strength could be seen (Fig. 3h–i).

Alginate consists of M and G residues. Due to their particular shapes and modes of linkage, respectively, three types of blocks as G block, M block and MG block, which are substantially different, can be formed. They distribute randomly in alginate chains (Fig. 1a3). Specifically, the G-block is buckled (Fig. 1a2). If two G-blocks are aligned side by side, diamond shaped holes result. Their dimensions are ideal for the cooperative binding of divalent cations, typically Ca^{2+}. This is the so-called ''egg-box'' model [13].

Under fixed concentration of alginate, the more the Ca^{2+} ions are, the more alginate chains they will cross-link, and the stronger and larger the alginate–Ca^{2+} film will be (Fig. 4). Vice versa, if the concentration of Ca^{2+} is fixed, the higher the concentration of alginate is, the more the alginate chains exist, and the easier the Ca^{2+}-G block complex can be formed. Therefore, although the alginate within biofilm matrix is oligosaccharides blend with low molecular weights, it is still able to build gel-like film with Ca^{2+}. This gel-like film could be an important part of biofilm skeleton.

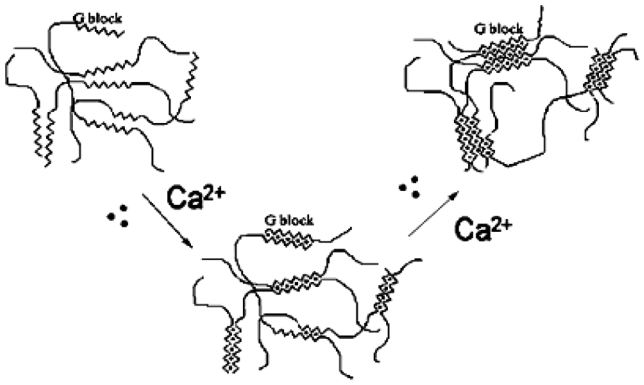

Fig.4. ''Egg-box'' model of alginate–Ca^{2+} interaction (after [13]).

In addition, the extracted alginate has more M residues than G residues according to the result of Raman spectroscopy, which indicated that there were large quantities of M-blocks. As the M-block has flat ribbon-like shape [17], it may contribute to the stretching out of the alginate–Ca film on the surface.

4. Summary

Bacterial alginate exopolysaccharide was successfully extracted from lab-cultured biofilm matrix. Alginate yield after enzymatic purification amounted to (164 ± 21) mg g VSS^{-1} (as organic content). It was partially *O*-acetylated oligosaccharides blend with mannuronic acid residue to guluronic acid residue ratio of 1.19. By cross linkage with Ca^{2+}, this extracted alginate was capable of forming gel-like film, which demonstrates that low molecular weight alginate oligosaccharides also greatly contribute to the formation of biofilm matrix. Direct alginate extraction and characterization supplies important information for the role of bacterial exopolysaccharides in building up biofilm matrix.

Acknowledgements

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References

[1] S. Leone, A. Molinaro, F. Alfieri, V. Cafaro, R. Lanzetta, A.D. Donato and M. Parrilli, The biofilm matrix of *Pseudomonas* sp. OX1 grown on phenol is mainly constituted by alginate oligosaccharides, Carbohydr. Res., 341 (2006) 2456-2461.
[2] Y. Ye, P. Le Clech, V. Chen, A.G. Fance and B. Jefferson, Fouling mechanisms of alginate solutions as model extracellular polymeric substances, Desalination, 175 (2005) 7-20.
[3] Y. Ye, P. Le Clech, V. Chen and A.G. Fane, Evolution of fouling during cross-flow filtration of model EPS solution, J. Membr. Sci., 264 (2005) 190-199.
[4] Y. Ye, V. Chen and A.G. Fane, Modeling long-term subcritical filtration of model EPS solutions, Desalination, 191 (2006) 318-327.
[5] U. Remminghorst and B.H.A. Rehm, Bacterial alginates: from biosynthesis to applications, Biotechnol. Lett., 28 (2006) 1701-1712.
[6] Y.S. Khotimchenko, V.V. Kovalev, O.V. Savchenko and O.A. Ziganshina, Physical-chemical properties, physiological activity, and usage of alginates, the polysaccharides of brown algae, Russ. J. Marine Biol., 27 (2001) S53-S64.
[7] S. Lee and M. Elimelech, Relating organic fouling to reverse osmosis membranes to intermolecular adhesion forces, Environ. Sci. Technol., 40 (2006) 980-987.
[8] K. Katsoufidou, S.G. Yiantsions and A.J. Karabelas, A study of ultrafiltration membrane fouling by humic acids and flux recovery by backwashing: experiments and modeling, J. Membr. Sci., 266 (2005) 40-50.

[9] J.P. Maleriat, P. Jaouen, N. Rossignol, J.P. Schlumpf and F. Quemeneur, Influence of alginates adsorption on properties of ultrafiltration and microfiltration organic membranes, Rev. Sci. Eau, 13(3) (2000) 269-287.
[10] J.H. Tay, Q.S. Liu and Y. Liu, Microscopic observation of aerobic granulation in sequential aerobic sludge blanket reactor, J Appl. Microbiol., 91 (2001) 168-175.
[11] D.J. Mchugh, A Guide to the Seaweed Industry, FAO Fisheries Technical Paper 441, FAO of the United Nations, Rome, 2003.
[12] FAO (Ed.), Compendium of Food Additive Specifications: Addendum 5. FAO Food and Nutrition Paper-52 add.5, Joint FAO/WHO Expert Committee on Food Additives 49th session, Rome, 1997.
[13] I. Donali and S. Paoletti, Material properties of alginates, in: B.H.A. Rehm (Ed.), Alginates: Biology and Applications, Springer-Verlag, Berlin, Heidelberg, 2009, pp. 1-54.
[14] T. Salomonsen, H.M. Jensen, D. Stenbæk and S.B. Engelsen, Chemometric prediction of alginate monomer composition. A comparative spectroscopic study using IR, Raman, NIR and NMR, Carbohydr. Polym., 72(4) (2008) 730-739.
[15] L. Pereira, A. Sousa, H. Coelho, A.M. Amado and P.J.A. Ribeiro-Claro, Use of FTIR, FT-Raman and 13C NMR spectroscopy for identification of some seaweed phycocolloids, Biomol. Eng., 20 (2003) 223-227.
[16] T.Y. Wong, L.A. Preston and N.L. Schiller, Alginate lyase: review of major sources and enzyme characteristics, structure-function analysis, biological roles, and applications, Annu. Rev. Microbiol., 54 (2000) 289-340.
[17] T.A. Davis, F. Llanes, B. Volesky and A. Mucci, Metal selectivity of Sargassum spp. and their alginates in relation to their α-L-guluronic acid content and conformation. Environ. Sci. Technol., 37 (2003) 261-267.