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# New monolithic enzymatic micro-reactor for the fast production and purification of oligogalacturonides☆

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**Abstract**

Fast production and purification of α-(1,4)-oligogalacturonides was investigated using a new enzymatic reactor composed of a monolithic matrix. Pectin lyase from *Aspergillus japonicus* (Sigma) was immobilized on CIM-disk epoxy monolith. Studies were performed on free pectin lyase and immobilized pectin lyase to compare the optimum temperature, optimum pH, and thermal stability. It was determined that optimum temperature for free pectin lyase and immobilized pectin lyase on monolithic support is 30 °C, and optimum pH is 5. Monolithic CIM-disk chromatography is one of the fastest liquid chromatographic method used for separation and purification of biomolecules due to high mass transfer rate. In this context, online one step production and purification of oligogalacturonides was investigated associating CIM-disk pectin lyase and CIM-disk DEAE. This efficient enzymatic bioreactor production of uronic oligosaccharides from polygalacturonic acid (PGA) constitutes an original fast process to generate bioactive oligouronides.


*Keywords:* CIM-disk; Enzymatic bioreactor; Monolith chromatography; Oligouronides; Pectin lyase

## 1. Introduction

In carbohydrates family, oligosaccharides take part in an extensive scope of biological functions as marker biomolecules [1]. In this context, these compounds are currently used in diverse industrial fields such as pharmaceutics, cosmetic, food industries or agronomy [1–10]. In the past years, anionic oligosaccharides such as oligogalacturonides (OGAs), which are linear molecules of 2 to about 20 α-1,4-D-galactopyranosyluronic acids, were largely described as bioactive and regulatory molecules were able to modify or enhance biological processes from various organisms but more particularly from plants [2]. In fact, it was largely described that addition of OGAs on plant could first, induce the production of specific active oxygen molecules such as oxidative burst and second, act like an elicitor in plant defence system with induction of phytoalexins [3,4].

OGAs can be generated from pectin or polygalacturonic acid (PGA) by action of pectinase, pectate lyase and other polygalacturonases [5]. In agro industry, pectinolytic enzymes are essentially used in fruit juices clarification processing (including decrease of viscosity) to hydrolyze the pectic substances responsible for unattractive suspensions and turbidity in juices [6–8]. Polysaccharide lyases (EC 4.2.2) are enzymes able to cleave the polysaccharide chains via a β-elimination cleavage of glycosidic bond resulting in the formation of a double bond at the newly formed non-reducing end. In this context, it is possible to use pectin lyase to catalyze the production of oligogalacturonides (OGAs).

Nevertheless, low efficiency of processes to produce a large amount of oligogalacturonides is one of the main drawbacks limiting the use of OGAs as bioactive molecules. Consequently, it appears necessary to develop new production strategies.

In the past years, enzymatic micro-reactors have been developed to facilitate routine work in biochemical analysis and in

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biocatalysts and literature concerning enzymes immobilization describes a lot of biotechnology applications such as bioprocessing, affinity chromatography, biotransformation or bioanalysis [9–11].

In biotechnology, reactor with immobilized enzymes present a lot of advantages such as high stability, reusability and the opportunity to work in continuous system for a longer period. Numerous supports have been utilized for the immobilization but particle-based supports are probably the most common. Some studies were already conducted on the immobilization of pectinolytic enzymes [12,13], but the literature concerning pectin lyase immobilization onto matrix is limited as compared to other pectinases and polygalacturonases. Its immobilization and application like bioreactor in continuous industrial processes, therefore, could offer considerable advantage in biotechnology processes [14,15]. However, in this kind of enzymatic reactor, there are large diffusion problems [16], and the mass transfer is primordial to obtain a good reproducibility.

Recently, news monolithic materials such as disks were described in enzyme reactor designing. Monolithic matrices are efficient chromatographic supports where mass transfer is much faster due to the convective flow that consequently becomes a dominant transport mechanism attributable to the almost complete lack of diffusion resistance during the mass transfer. These methacrylate-based monolith matrices, commercialized under the trademark Convective Interaction Media Disk (CIM-disk), are used for the fast bioactive molecules separation [17–21] and represent a perfect model for the immobilization of enzymes and the fast conversion of substrates. In the last decades, different enzymatic bioreactors were proposed using CIM-disk supports [22–27] but never for the production of oligosaccharides.

In this present work, CIM monolithic disk were used as a support for the immobilization of pectin lyase (PL). The aim of this paper is to consider the possible implication and development of enzymatic micro-reactor in biotechnology for the fast online production and purification of bioactive oligogalacturonides using monolithic micro-reactor with pectin lyase immobilized onto CIM epoxy disk supports.

## 2. Materials and methods

### 2.1. Pectin lyase immobilization onto CIM epoxy disk

Solution of 2 mg/mL of pectin lyase *Aspergillus japonicus* (1.1 U/mg) (from Sigma, St. Louis, MO, USA) was prepared by dissolving lyophilized pectin lyase in 0.5 M phosphate buffer, pH 7.8. Support for the enzyme immobilization was CIM epoxy disk (BIA Separations, Ljubljana, Slovenia) with an I.D. of 12 mm, a thickness of 3 mm and a volume of 0.34 mL. For immobilization procedure, enzyme solution was pumped through the CIM epoxy disk during 24 h at 4 °C. After immobilization was completed, enzyme-modified CIM-disk was thoroughly washed with deionized water and finally with the working buffer at room temperature. The amount of pectin lyase coupled to the CIM-disk was determined by the material balance (amount of enzyme in supernatant before and after immobilization) measured by Bradford assay.

### 2.2. Protein determination

Protein amount was determined using Coomassie-protein assay (Bradford, 1976) as described in Microassay Biorad instruction manual using bovine serum albumin as a standard.

### 2.3. Enzyme activity assay

Pectin lyase activity was measured by monitoring the increase of absorbance at 235 nm using a spectrophotometer. Reaction mixture was composed of 5 mL of 0.2% (w/v) PGA solution in 50 mM potassium acetate buffer pH 5. For free enzyme, 1.2 mg of pectin lyase preparation was incubated during 48 h. After incubation, enzymatic β-elimination was stopped by dipping the reaction medium into 95 °C water bath during 5 min. Mixture of oligomers was then centrifuged at 15,000 × *g* for 20 min at 20 °C. Concerning immobilized process, the PGA solution was pumped on closed circuit through the CIM epoxy-pectin lyase disk during 48 h. Different samples were recovered during the processes in order to measure the absorbance at 235 nm.

One unit (U) of enzyme activity was defined as the enzyme amount required to catalyze an increase of 1 in absorbance at 235 nm/min.

### 2.4. Biochemical characterization of pectin lyase activity

Optimum temperature for pectin lyase was estimated by performing the standard assay within the temperature range of 20–60 °C. The optimal pH for pectin lyase activity was evaluated by varying the pH of the reaction mixture between 3 and 9 by using three different buffers at 50 mM: potassium acetate–HCl (pH 3–4), acetic acid–potassium acetate (pH 4–7) and Tris–HCl (pH 7–9).

### 2.5. Online production and purification of oligouronides

Online production and purification of oligouronides was performed at 30 °C using pressure liquid chromatography with a Waters 600E System controller pump (Waters). The detection was performed by using a Waters 2487 Dual Wavelength detector.

One chromatographic module CIM-disk epoxy-pectin lyase coupled with CIM-disk DEAE was performed using the gradient HPLC system equipped with UV detector for analysis of unsaturated oligogalacturonan (ΔOGA) at 235 nm. Twenty microliters of PGA solution (0.2 g/L in acetate buffer (pH 5; 50 mM) was injected at the flow rate of 4 mL/min. Elution was done using NaCl buffer gradient (0–1 M in the same acetate buffer). Fractions of 0.5 mL were collected for colorimetric assay.

### 2.6. Oligogalacturonide assays

Concentration of oligogalacturonides was determined by colorimetric assay according to the procedure of Van den Hoogen et al. [28] where uronic acids were quantified at 490 nm

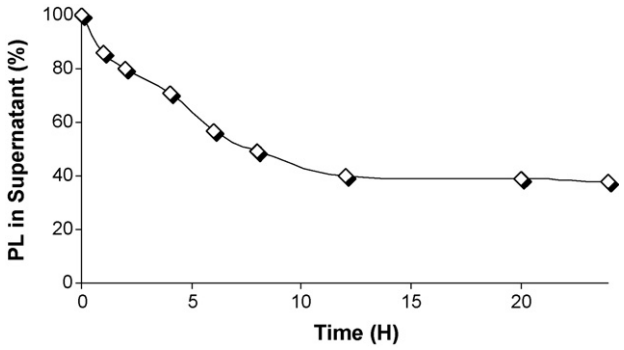


Fig. 1. Evolution and quantification of pectin lyase immobilized on CIM-disk epoxy during 24 h of incubation in 0.5 M phosphate buffer, pH 7.8. Amount of pectin lyase coupled to CIM support was determined by the material balance (the amount of enzyme in the supernatant before and after the immobilization) measured using Bradford assay.

($A_{490\,nm}$) after reaction with *m*-hydroxybiphenyl (mHBP). The results were expressed in D-galacturonic acid μg/mL equivalent.

## 3. Results and discussion

As it was already described in detail in literature [29], different factors play important roles in the efficiency of the enzymatic reactor and as a general rule, performance of enzymatic reactor depends on the total of immobilized enzyme and the hydrodynamic properties of the matrix. In order to develop a new enzymatic reactor for the production of bioactive oligogalacturonides, monolithic CIM-disk epoxy matrix was investigated for immobilization of pectin lyase.

### 3.1. Immobilization of pectin lyase

Since enzymes are expensive, the reuse of the catalyst is essential for many processes and provides better environment for enzyme activity and then improve the product purity. For this reason, the development of enzyme reactor is decisive. Immobilization of pectin lyase on monolithic CIM-disk has been done to study the fast enzymatic degradation of polygalacturonan into oligogalacturonides (OGAs). This pectinolytic enzyme immobilization was carried out in a flow-through mode closed circuit according to the immobilization procedure of Vodopivec et al. [27] where it has been shown that the enzyme coupling yield was much better. Moreover, epoxy-activated monolithic CIM-disks have been confirmed as excellent supports for immobilization of protein ligand [27].

For enzyme immobilization, a total amount of 2 mg of pectin lyase was pumped through the CIM epoxy disk for 24 h at 4 °C. During the process (Fig. 1) the amount of pectin lyase coupled to the CIM support was determined by the material balance (the amount of enzyme in the supernatant before and after the immobilization) measured by Bradford assay. Consequently, the immobilization yield resulted in 60% and the binding capacity of the epoxy CIM-disk was then estimated around 1.2 mg of pectin lyase per CIM-disk.

### 3.2. Biochemical properties

Similar to every enzyme, pectin lyase can be more or less active according to the conditions in which it is used. Its activity will mainly be influenced by pH and temperature. These specific parameters have been tested for both free and immobilized enzyme to compare them.

#### 3.2.1. Effect of buffer's pH on pectin lyase activity

The pH of the buffer can interfere both on free and immobilized enzyme activity because it can modify the charges of the different amino acids of the protein and consequently, modify the interactions between the enzyme and its substrate. Moreover, in extreme conditions, i.e. at extreme pH, the protein can be denatured.

The effect of pH on the activities of free and immobilized pectin lyase preparations for PGA degradation was studied using three different buffers at 50 mM: potassium acetate–HCl (pH 3–4), acetic acid–potassium acetate (pH 4–7) and Tris–HCl (pH 7–9). As shown in Fig. 2a the optimum pH values for free and immobilized enzymes are the same that is to say pH 5.0. Nevertheless, the results suggest that the immobilization process did not affect the pH stability of the pectin lyase significantly.

#### 3.2.2. Influence of temperature on reaction rate

Temperature is one of the main parameters, which can interfere with the stability of the enzymatic system. When the temperature rises, more collisions between enzyme and substrate occur, and consequently, the enzymatic activity changes.

As compared to free enzyme, the optimum temperature of immobilized pectin lyase is similar and was observed at 30 °C

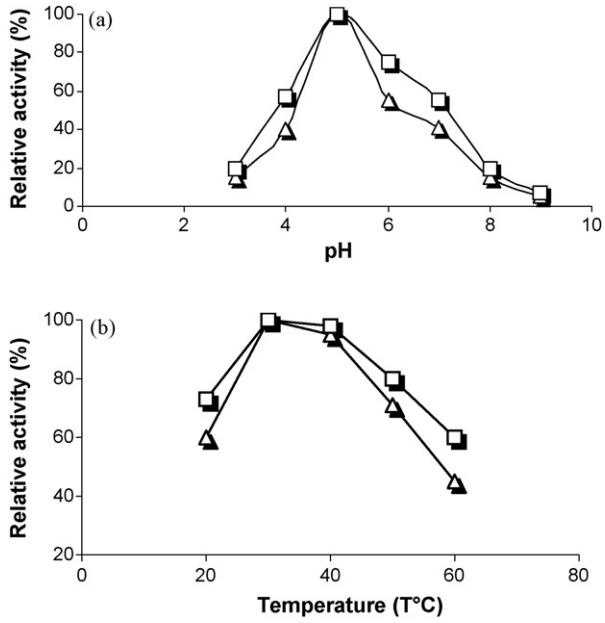


Fig. 2. pH optimum (a) and temperature optimum (b) for pectin lyase activity. Free (△) and immobilized (□). Relative enzyme activity at pH 5.0 and the relative enzyme activity at $T = 30$ °C is taken as 100% corresponding to 1.1 U/mg protein. Experiments were run in duplicate and the difference in individual sets of readings was less than 5%.

(Fig. 2b). In fact, the formation of bond, covalent and non-covalent between the support and the enzyme reduces the degree of freedom of the molecular structure of the protein and then protects it from denaturation caused by the high temperatures.

Note that the loss of the activity of immobilized pectin lyase was lower than that of the free pectin lyase with 10% difference at extremes temperatures.

Therefore, this enzymatic reactor has a protecting effect at the high temperatures at which enzyme deactivation takes place.

### 3.2.3. Reusability

An enzymatic reactor has to provide stable conditions to several uses in continuous cycles to be appropriate to industrial scale up. In order to estimate the reproducibility and the reusability of the immobilized pectin lyase on CIM-disk, the support was washed with buffer solution after use and a fresh aliquot of PGA substrate was injected to measure the enzymatic activity. This procedure was repeated 10 times.

It was observed that the CIM-disk support provides high operational stability towards recycling in batch hydrolysis of PGA. In fact, no significant decrease in the efficiency of immobilized pectin lyase (5%) was observed after running 10 batches through the CIM-disk system.

Consequently, the immobilized pectin lyase can be reused 10 times without any loss of activity. The above results show that the strategy of immobilization of pectin lyase on CIM-disk can be successfully used in the context of designing reusable, efficient and stable biocatalysts for bioconversions of PGA to bioactive oligogalacturonides.

### 3.3. Batch reaction

Time course cleavage of PGA was performed at 30 °C and pH 5 for the free and immobilized processes (Fig. 3) using the same amount of pectin lyase (1.2 mg). As shown in Fig. 3a it is important to mention the maximum conversion capacity of PGA at around 4 h for free process and around 8 h for the immobilized process. Moreover, the immobilized pectin lyase results in better cleavage of PGA (hydrolysis of PGA to unsaturated OGAs) as shown by a greater initial rate and

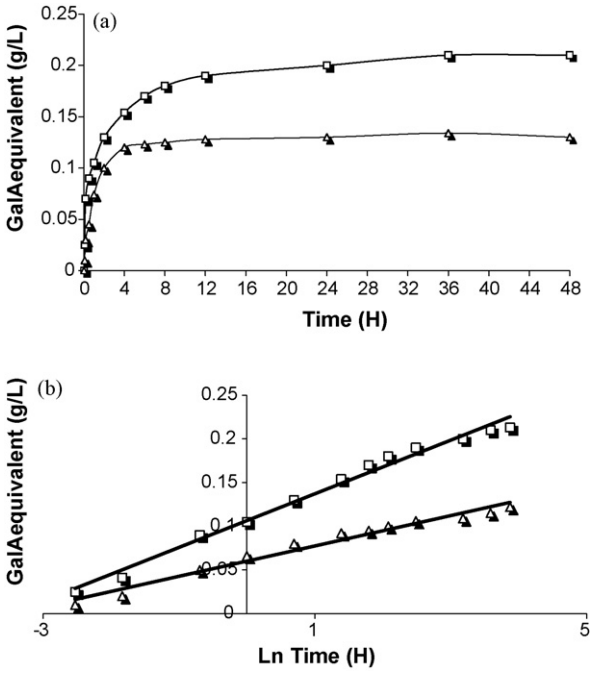


Fig. 3. Analysis of the production of OGAs during the time course enzymatic cleavage of PGA (a) and logarithmic relation between time course degradation of PGA and oligosaccharide concentration (b) with free (△) and immobilized (□) pectin lyase process performed at 30 °C and pH 5. Amount of OGA was determined by colorimetric assay with *m*-hydroxybiphenyl (mHBP). Results were expressed in D-galacturonic acid (GalA) equivalent g/L.

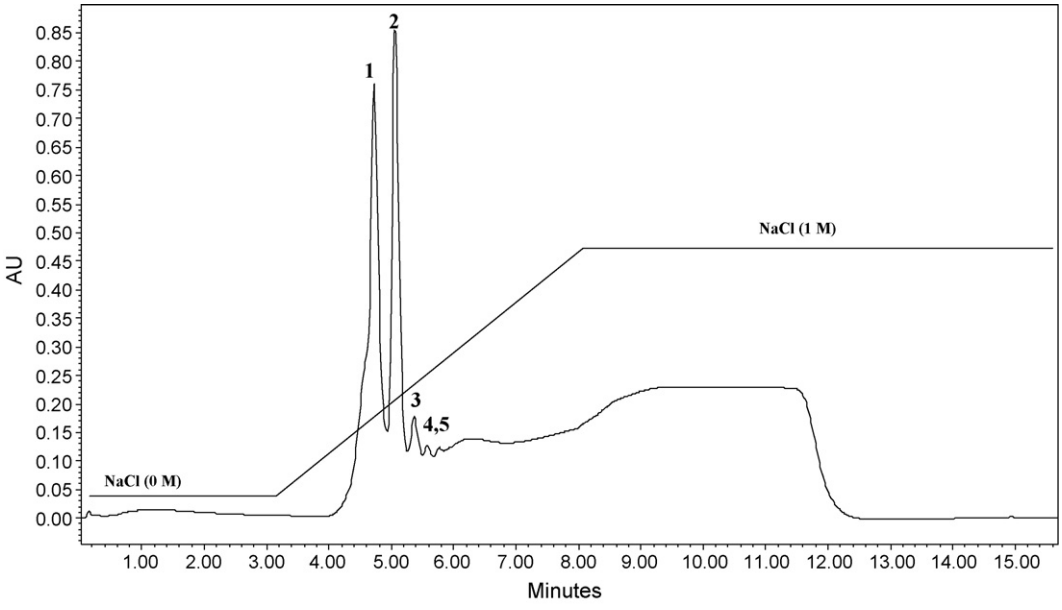


Fig. 4. Analysis of the oligogalacturonides (OGAs) mixtures using CIM-disk DEAE. Twenty microliters of OGA solution (acetate buffer, pH 5, 50 mM) is injected at the flow rate of 4 mL/min. Elution was done using NaCl buffer gradient (0–1 M in the same acetate buffer) and detected by UV at 235 nm. Numbers above eluted compounds refer to their degree of polymerization (dp) by comparison with standards.

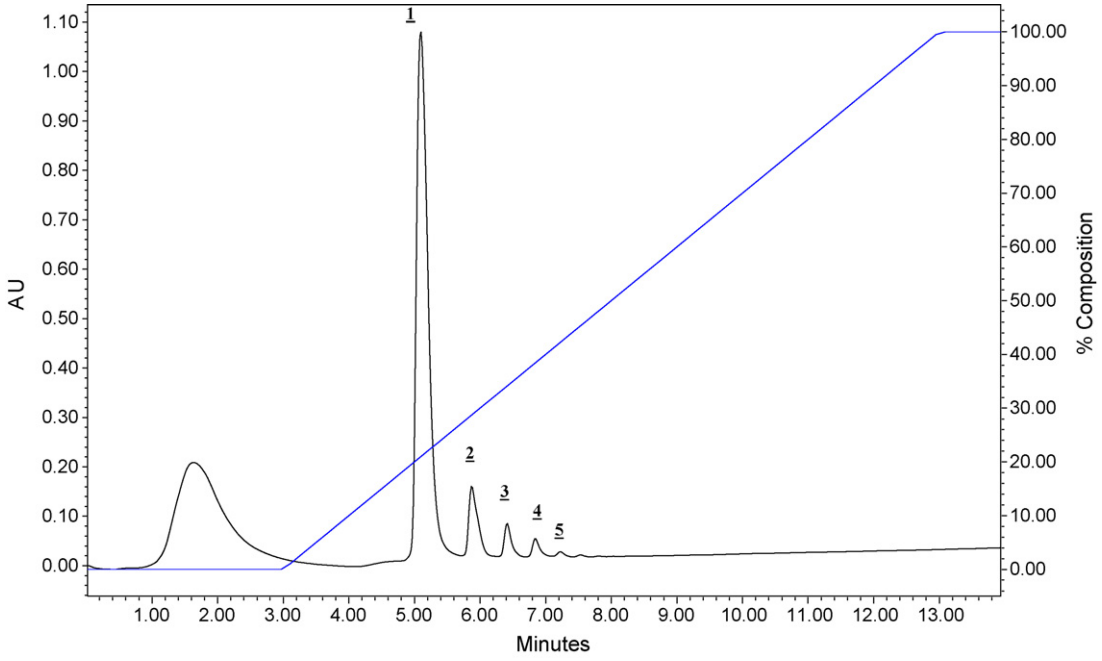


Fig. 5. Online process analysis for the production and purification of oligogalacturonides (OGAs) using housing monolithic with two coupled CIM-disk: CIM-disk epoxy-pectin lyase and CIM-disk DEAE. Twenty microliters of PGA solution (acetate buffer, pH 5, 50 mM) is injected at the flow rate of 4 mL/min. Elution was done using NaCl buffer gradient (0–1 M in the same acetate buffer) and detected by UV at 235 nm. Numbers above eluted compounds refer to their degree of polymerization (dp) by comparison with standards.

a greater PGA conversion by comparison to the free enzyme process.

In fact, we obtained 0.2 and 0.12 g/L of oligogalacturonides for the immobilized pectin lyase and for the free pectin lyase, respectively. That is to say, we obtained around twice as much oligosaccharide concentration in the case of immobilized pectin lyase. After that, the composition of oligogalacturonides was analyzed by CIM-disk DEAE (Fig. 4).

In this chromatogram we can observe that for 1–24 h of process, similar chromatograms were obtained with the presence of oligogalacturonides with degree of polymerization (dp) up to 5. The similar response is maintained for about 10 injections.

### 3.4. Online process

Online production and purification of OGAs was investigated using monolith module with CIM-disk epoxy-pectin lyase and CIM-disk DEAE. As we have shown firstly, the efficiency to produce oligogalacturonides by this micro-reactor and secondly, their fast purification using CIM-disk DEAE, it was logical in this context to envisage the online one step production and purification of oligogalacturonides associating the CIM-disk pectin lyase and CIM-disk DEAE in the same CIM housing. For that, PGA solution (0.2 g/L in potassium acetate buffer pH 5, 50 mM) was injected on CIM-disk system and elution was done using NaCl buffer gradient (0–1 M in the same acetate buffer) at the flow rate of 4 mL/min.

As shown in Fig. 5, it is interesting to comment on the fast production and purification of OGAs using this micro-reactor. In fact, after less than 8 min OGAs with dp up to 5 are produced and purified in one step. Consequently, during this online process, a PGA conversion to oligogalacturonide of degree of polymerization up to 5 (Fig. 5) was obtained at 37.5% efficiency. Note that after 10 injections, we obtained the same result. Therefore, immobilized enzyme CIM-disk coupled with CIM-disk DEAE offers a simple and fast method for promising preparative isolation of anionic oligosaccharides.

## 4. Conclusion

Monolithic CIM-disk is an appropriate polymeric support for immobilization of enzymes with high efficiency. This study provides facts that the immobilization of pectin lyase on epoxy activated CIM-disk can be of potential interest for the fast production and purification of oligogalacturonides. The immobilized pectin lyase on the epoxy CIM-disk is stable; exhibits good reproducibility; provides optimum conditions for enzymatic reactions similar to those of free enzymes and seems still active after different runs. Consequently, immobilization of pectin lyase on CIM-disk can be of potential interest for the fast production and purification of oligogalacturonides. This model is a very promising miniaturized system for screening and developing preparative scale method. Nevertheless, supplementary studies are still needed to improve the efficiency of this system for practical and industrial scale-up applications.

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