(54) **Title:** METHOD FOR TRANSFORMING POLYSACCHARIDES INTO OLIGOSACCHARIDES WITH BIPOLAR MEM-
BRANE ELECTRODIALYSIS

Fig. 9

(57) **Abstract:** In accordance with the present invention, there is provided a new method for producing oligosaccharides using
bipolar membrane electrodialysis (BMED) in an electrodialysis cell. The method allow for the simultaneous transformation of
polysaccharides into oligosaccharides and the demineralization of the resulting oligosaccharides.

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— *with information concerning request for restoration of the right of priority in respect of one or more priority claims*

METHOD FOR TRANSFORMING POLYSACCHARIDES INTO

OLIGOSACCHARIDES WITH BIPOLAR MEMBRANE

ELECTRODIALYSIS

BACKGROUND

Electrodialysis is a technique involving the transport of ions from one solution to another solution through an ion-exchange membrane, under the influence of an applied electrical current. The technique is performed in an electrodialysis cell, usually consisting of a diluate compartment feeding a concentrate compartment separated by an ion-exchange membrane, such as an anion-exchange membrane (AEM), a cation-exchange membrane (CEM) or a bipolar membrane (BP), the latter allowing the dissociation of water molecules at its interface. The compartments, and the membranes defining them, are placed between two electrodes, immersed in a conductive solution, allowing for transmission of electrical current. An usual configuration for an electrodialysis cell is alternating AEM and CEM to form a stacking, or series, of diluate and concentrate compartments.

In an electrodialysis stack, or series, the electrical potential difference in the different compartments induces the migration of negatively-charged anions toward the positively-charged anode. These anions pass through the positively-charged AEM, but are prevented from further migration by the negatively-charged CEM, therefore concentrating in anions the solution contained in the compartment defined by the AEM and the CEM. Similarly, the positively-charged cations migrates toward the negatively-charged cathode, passing through the negatively-charged CEM, and concentrating in cations the solution defined by the CEM and AEM by being prevented from further migration by the positively-charged AEM. As a result of the anions and cations migration, the electrical current flows between the cathode and the anode. Overall, the migration of the ions is necessary to carry

the electrical current across the electrodialysis cell and maintain the solutions in an electrically neutral state.

Polysaccharides are carbohydrate polymers of high molecular weight, containing monosaccharides joined together by glycosidic bonds. Typically, polysaccharides are thus very large molecules, often branched, and are amorphous and insoluble in water. The polymerization of polysaccharides involves the formation of the glycoside bonds between two monosaccharides with the elimination of water. Upon hydrolysis, polysaccharides break down into smaller monosaccharides polymers called oligosaccharides, which usually contains three to nine monosaccharide residues. Oligosaccharides have been found to have a multitude of therapeutic effects, such as for example, modulation of the intestinal flora, stimulation of beneficial short-chain fatty acid production, stimulation of calcium and magnesium absorption, elimination of toxic compounds, modulation of blood sugar, cholesterol and triglycerides levels, etc.

Chitosan is a linear polysaccharide composed of β-1,4 linked D-glucosamine residues extracted from shellfish exoskeletons. This polymer is considered as a totally or partially deacetylated derivative of chitin. Much attention has been paid to convert chitosan to safe and functional chito-oligomers, since these low molecular weight saccharides exhibit biological and pharmaceutical properties, including inhibition of the growth of fungi and bacteria, antitumor and immuno-enhancing effects, and eliciting phytoalexin production in plants.

Several methods have been proposed for preparing oligosaccharides from chitosan, including chemical hydrolysis with inorganic acid (Domard et Cartier, 1989. In Chitin and Chitosan, Skjak-Braek, Anthonsen and Sandford editor, Elsevier, London, pp.383-387) and enzymatic hydrolysis with a polysaccharide-hydrolyzing enzyme, such as amylase for starch, cellulose for cellulose, or chitosanase for chitosan (Boucher et al. Appl. Microbiol. Biotechnol., 38, 188-93, 1992). Preparation of oligosaccharides with polysaccharide-hydrolyzing enzymes is preferred to other methods because it allows production of oligosaccharides having

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a specific degree of polymerization. For example, chitosanase (EC 3.2.1.132) catalyses the hydrolysis of the glycosidic bonds of chitosan, and has been found in bacteria, fungi and plants. The traditional method of producing oligosaccharides with polysaccharide-hydrolyzing enzymes consists in solubilizing the

5   polysaccharides by chemical acidification, then incubating the polysaccharide solution with a polysaccharide-hydrolyzing enzyme, and terminating the enzymatic reaction by heating at 100°C (Fig. 1). However, addition of salts occurring during polysaccharide acidification (resulting from the use of extrinsic chemical acid) results in diminished purity of the oligosaccharides produced.

10   The acid necessary for the first step can be produced *in situ* by electroacidification with bipolar membrane electrodialysis (BMED) instead of the addition of chemical acid (Lin Teng Shee *et al.* J. Agric. Food Chem. 54 (18), 6760-4, 2006). The principle of this technique is based on the dissociation of water molecules, or water splitting, at the interface of bipolar membranes under an

15   electrical field in an electrodialysis system (Mani J., Membr. Sci. 58, 117-38, 1991). Protons and hydroxyl ions produced by water splitting are collected in separate compartments allowing for the acidification and basification of the solutions. Previous studies showed that the use of the alkaline stream can be maximized during the bipolar electrodialysis process. For instance, electrobasification with

20   bipolar membranes has been used at laboratory scale for adjusting the pH of apple juice from 2 to 3.3 (Lam Quoc *et al.*, J. Agric. Food Chem. 48, 2160–6, 2000), and for increasing the pH of passion fruit juice from 2.9 to 4.0 (Vera *et al.*, J. Food Eng. 59, 361-7, 2003), resulting in final products stabilized without the addition of chemical reagents.

25   As for the preparation of chitosan oligomers, chitosan solubilization by acidification of the solution is usually followed by the incubation with chitosanase, and the enzymatic reaction is ultimately terminated by heating the solution at 100 °C during several minutes in order to denature irreversibly the enzyme (Li *et al.*, Polym. Degrad. Stab. 87, 441-8 2005). Enzyme inhibition could also be obtained

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with extreme pH value. The effect of pH on enzyme-catalyzed reactions can be divided in two categories: (a) the effect of pH on the stability of the enzyme, and (b) the effect of pH on the catalytic activity of the enzyme. The stability of the enzyme is related to the integrity of the three-dimensional structure of the active site, which is essential for maintenance of the enzymatic activity. Most enzymes undergo irreversible denaturation in very acidic and very alkaline solutions, due to modifications to their secondary, tertiary, and/or quaternary structures by extreme pH value. The effect of extreme pH values on the catalytic activity of the enzyme is related to the ionization of essential prototropic groups (i.e. capable of ionizing) in the active site of the enzyme. These prototropic groups may be involved in the enzyme-catalyzed reaction by ensuring the maintenance of the native conformation of the active site, the binding of the substrate to enzyme, or the transformation of the substrate into a reaction product.

Therefore, the preparation of oligosaccharides from polysaccharides is a complex technique that traditionally involves the solubilization of the polysaccharides by addition of acid, adjustment of the pH with an alkaline solution to values suitable for hydrolysis by the polysaccharide-hydrolyzing enzyme, addition of the polysaccharide-hydrolyzing enzyme and termination of the enzymatic reaction by thermal denaturation of the enzyme. Besides requiring a lot of manipulations and being costly, such a technique produces oligosaccharides that are contaminated with various minerals and ions originating from the acid used for solubilization and from the alkaline solution used for pH adjustment prior to the enzymatic reaction. Moreover, polysaccharides that were not hydrolyzed, or partly hydrolyzed, by the polysaccharide-hydrolyzing enzyme are also present in the final oligosaccharide product, thus reducing further the purity of the final product.

Therefore, it would be highly desirable to be provided with a method for producing oligosaccharides from polysaccharides that could be applied in large scale or industrial scale, and/or in a continuous manner, and/or that would provide an oligosaccharide preparation having low levels of contaminants. Alternatively, the

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desired method would include a minimal number of manipulation steps and/or would present a reduced cost over the current methods for producing oligosaccharides from polysaccharides.

BRIEF SUMMARY

The method for producing oligosaccharides by bipolar membrane electrodialysis according to the present invention involves the acidification of a polysaccharide solution to solubilize polysaccharides, the incubation with a polysaccharide-hydrolyzing enzyme for hydrolysis of the polysaccharides, the termination of the hydrolysis reaction by a basification of the solution, and a demineralization to remove minerals from the oligosaccharide solution. Preferably, the acidification, basification and demineralization steps all take place in a single electrodialysis cell.

One aspect of the present invention is to provide a method for producing oligosaccharides in an electrodialysis cell comprising an acidification compartment, a basification compartment, and a demineralization compartment, the method comprising the steps of:

circulating a polysaccharide solution into the acidification compartment of the electrodialysis cell to acidify said polysaccharide solution, thereby producing an acidified solution;

incubating the acidified solution of step a) with a polysaccharide-hydrolyzing enzyme to induce the hydrolysis of the polysaccharides into oligosaccharides, thereby producing a hydrolyzed solution;

circulating the hydrolyzed solution of step b) into the basification compartment of the electrodialysis cell to basify said hydrolyzed solution, thereby producing a basified solution;

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circulating the basified solution of step c) into the demineralization compartment of the electrodialysis cell to demineralize said basified solution, thereby producing a demineralized solution; and

collecting the demineralized solution of step d), wherein said demineralized solution comprises oligosaccharides.

The method may further comprises the step of isolating oligosaccharides from the demineralized solution of step e). The pH of the acidified solution of step a) can be adjusted to a value of 4.5 to 6.5 prior to incubation with the polysaccharide-hydrolyzing enzyme. The adjustment of the pH can be performed at least partly by adding a portion of a previously produced basified solution of step c) to the acidified solution of step a).

At least one of the hydrolyzed solution of step b) and the basified solution of step c) may contains non-hydrolyzed polysaccharides that can be collected from either solutions and reintroduced in the polysaccharide solution of step a).

Step a), step c) and step d) of the method can be performed simultaneously. Step b) and step e) of the method can also be performed simultaneously to step a), step b) and step d).

The polysaccharide can be chitosan and the polysaccharide-hydrolyzing enzyme can be a chitosanase.

Another aspect of the present invention is to provide an electrodialysis cell comprising a cathode, a first bipolar membrane, an anion-exchange membrane, a cation-exchange membrane, a second bipolar membrane and an anode, with the first bipolar membrane and the anion-exchange membrane defining an acidification compartment, the anion-exchange membrane and the cation-exchange membrane defining a demineralization compartment, and the cation-exchange membrane and the second bipolar membrane defining a basification compartment. The electrodialysis cell can be used for the production of oligosaccharides.

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Another aspect of the present invention is to provide an electrodialysis cell comprising a cathode, an acidification compartment, a demineralization compartment, a basification compartment and an anode, with the acidification compartment being defined by a first bipolar membrane and an anion-exchange

5   membrane, the demineralization compartment being defined by the anion-exchange membrane and a cation-exchange membrane, and the basification compartment being defined by the cation-exchange membrane and a second bipolar membrane. The electrodialysis cell can be used for the production of oligosaccharides.

10   Another aspect of the present invention is to provide a bipolar membrane electrodialysis system comprising an electrodialysis cell, a first reservoir unit connectable to the acidification compartment of the electrodialysis cell as a closed circuit, a second reservoir unit connectable to the basification compartment of the electrodialysis cell as a closed circuit, and a third reservoir unit connectable to the

15   demineralization compartment of the electrodialysis cell as a closed circuit, with the first, second and third reservoir units comprising pumping means for circulating a fluid into the compartments of the electrodialysis cell. The first, second and third reservoir units can be interchangeable. The system can further comprise a fourth reservoir unit connectable to an electrode rinse solution compartment of the

20   electrodialysis cell as a closed circuit.

BRIEF DESCRIPTION OF THE DRAWINGS

Having thus generally described the nature of the invention, reference will now be made to the accompanying drawings, showing by way of illustration, a

25   preferred embodiment thereof, and in which:

Fig. 1 illustrates the production of oligosaccharides by enzymatic hydrolysis with a conventional method.

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Fig. 2 illustrates an electrodialysis cell in accordance with the present invention. AEM : anion-exchange membrane, CEM : cation-exchange membrane, BP : bipolar membrane, A⁻ : anions, C⁺ : cations.

Fig. 3 illustrates the complete scheme for chitosan hydrolysis into oligomers
5   using a three-compartment Bipolar Membrane Electrodialysis system.

Fig. 4 illustrates a scheme of the circulation in the three different compartments of the electrodialysis cell. AEM : anion-exchange membrane, CEM : cation-exchange membrane, BP : bipolar membrane, A⁻ : anions, C⁺ : cations.

Fig. 5 illustrates an electrobasification cell with a basified compartment (1),
10   an acidified compartment (2) and a NaCl electrolyte (3); AEM : anion-exchange membrane, CEM : cation-exchange membrane, BP : bipolar membrane, A⁻ : anions, C⁺ : cations.

Fig. 6 illustrates the residual chitosanase activity after enzyme storage in buffers at various pH and duration. Activity was tested at 37 °C during 10 min.

15   Fig. 7 illustrates the pH profile during electrobasification of a chitosan hydrolyzate. Incubation of 5 U chitosanase with 1 L chitosan 0.5 % during 1 hour at pH 5.5 and 25 °C.

Fig. 8 illustrates the reducing sugars content of chitosan hydrolyzate after pH adjustment by electrobasification. (*) Samples at t= 4 h remained at pH 5.5
20   during 1 h.

Fig. 9 illustrates the three-compartment bipolar membrane electrodialysis cell configuration used for production of chito-oligomers with an electroacidified compartment (1), an electrobasified compartment (2) and a demineralized compartment (3); BPM : bipolar membrane, AEM : anion-exchange membrane,
25   CEM : cation-exchange membrane.

Fig. 10 illustrates a general scheme for chitosan transformation into oligomers using a bipolar membrane electrodialysis (BMED) system.

Fig. 11 comprises Fig. 11a, 11b and 11c, and illustrates the evolution of pH during chito-oligomers preparation by BMED or chemical method during chitosan acidification (Fig. 11a), chitosanase basification (Fig. 11b) and chito-oligomers demineralization (Fig. 11c).

Fig. 12 comprises Fig. 12a and 12b, and illustrates the evolution of ash levels during chito-oligomers preparation by the BMED method (Fig. 12a) and by the chemical method (Fig. 12b). "R^2" is the correlation coefficient and "a" is the slope coefficient for linear regression.

Fig. 13 illustrates the evolution of soluble chitosan as a function of time using a BMED system.

Fig. 14 illustrates the results of a thin layer chromatography of (1) standard of dimer, trimer and tetramer, (2) standard of glucosamine monomer, (3) chito-oligomers solution demineralized by BMED, and (4) original chitosan.

DETAILED DESCRIPTION

In accordance with the present invention, there is provided a new method for producing oligosaccharides from polysaccharides using bipolar membrane electrodialysis (BMED) in an electrodialysis cell. The method allows for the simultaneous transformation of polysaccharides into oligosaccharides and the demineralization of the resulting oligosaccharides.

The electrodialysis cell according to the present invention comprises two electrodes, an anode and a cathode, separated by preferably four membranes, arranged according to Fig. 2. The electrodes are disposed opposite to each other at either sides of the cell in order to allow for a flow of current between the cathode

and the anode. The membranes closest to each of the electrodes are bipolar membranes (BP), and are separated by an anion-exchange membrane (AEM) and a cation-exchange membrane (CEM), closer to the anode and the cathode respectively. The four membranes define three different compartments within the

5   cell: a BP-AEM compartment (acidification compartment), a AEM-CEM compartment (demineralization compartment), and a CEM-BP compartment (basification compartment), as illustrated from left to right on Fig. 2. Preferably, the electrodialysis cell also comprises an electrode rinse solution compartments for circulating electrode rinse solution for each of the electrodes, as it is known in the

10   art of electrodialysis cells.

Under the action of the electrical current, and with the three compartment loaded with salt solution for example, the solution in the acidification compartment undergoes an electroacidification, the basification compartment undergoes an electrobasification, and the demineralization compartment is depleted of its

15   charged ions. Preferably, the electrical current is inferior to the limiting current density, which corresponds to the maximal current density to achieve the electrode reaction before hydrogen or other extraneous ions are discharged simultaneously. Therefore, the electrical current in an electrodialysis cell according to the present invention is of about 0.1 to 20 mA/cm^2, preferably of about 1 to 15 mA/cm^2, more

20   preferably of about 5 to 15 mA/cm^2, and even more preferably of about 10 mA/cm^2, so that it is inferior to the limiting current density.

Polysaccharides are first suspended in an aqueous solution, by means known in the art. An homogenous suspension is preferred, with a concentration of about 1 to 25 g/L, preferably of about 2 to 20 g/L, and more preferably of about 5 to

25   10 g/L. The suspension of polysaccharides in water for example is a suspension having a pH value of about 7. Since polysaccharides are insoluble in aqueous media at neutral or basic pH, solubilization is usually achieved by adding acid to the polysaccharide solution, in order to acidify it. Lowering of the pH to acidic values of about 1 to 6, preferably of about 2 to 5, and more preferably of about 2 to

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4, induces the solubilization of the polysaccharides, relaxing the polysaccharide structure so that enzymatic sites targeted by the polysaccharide-hydrolyzing enzyme is exposed and available for the enzyme. For example, chitosan powder suspended in water forms a solution having a pH of about 6 to 7. Complete

5   solubilization is achieved by adjusting the pH of the chitosan solution to about 2 to 5, preferably to 2.5.

In accordance with the present invention, the acidification of the polysaccharide solution is achieved by electroacidification in the acidification compartment of the electrodialysis cell (Fig. 3). Under the influence of the electrical

10   current, the acidification compartment is enriched in anions from the AEM that induce a decrease of the pH of the polysaccharide solution in the compartment, thus acidifying the polysaccharide solution and producing an acidified solution. It is of note that either a polysaccharide solution or a polysaccharide powder can be added to the acidification compartment, the polysaccharides in powder form

15   dissolving into the solution contained in the acidification compartment after addition.

Once the polysaccharides are solubilized, a polysaccharide-hydrolyzing enzyme is added and mixed to the acidified solution. The polysaccharide-hydrolyzing enzyme is preferably added in sufficient concentration for hydrolysis to

20   occur. It is worth noting that if the acidified solution containing the polysaccharide-hydrolyzing enzyme may be incubated according to techniques known in the art and with respect to the enzyme specifications before being submitted to the following steps of the method. Alternatively, the polysaccharide-hydrolyzing enzyme may be added and mixed to the polysaccharides solution (or the

25   polysaccharides may be added and mixed to a polysaccharide-hydrolyzing enzyme solution) before introducing the solution into the acidification compartment. Further, the polysaccharide-hydrolyzing enzyme may be added and mixed with the polysaccharides solution during the acidification step, or during the basification step.

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Polysaccharide-hydrolyzing enzymes work best at various optimal pH values, generally from pH 4 to 7. It is of note that various polysaccharide-hydrolyzing enzymes have various optimal pH values. For example, chitosanase, catalyzing the hydrolysis of chitosan, works at an optimal pH of 5.5. Therefore,

5   since the acidified solution has very acidic pH, such as 2.5 for example, the pH of the acidified solution is not optimized for the hydrolysis reaction. Thus, an adjustment of the pH is needed in order to optimize hydrolysis. While this can be done by adding an alkaline powder or solution to the acidified solution, the preferred way in accordance with the present invention is to take a portion of the

10   basified solution obtained from the next step and adjust the pH of the acidified solution to a suitable pH for the polysaccharide-hydrolyzing enzyme to exert its hydrolysis effect on the polysaccharides, as will be discussed later. This allows for the basification of the solution without introducing external ions to the solution.

The polysaccharide-hydrolyzing enzyme is added to the solution at a

15   concentration ranging from about 0.1 to 10 unit, preferably of about 0.5 to 5 unit, per gram of polysaccharides. The concentration of enzymes, as well as its activity, must be sufficient to allow for the most efficient hydrolysis of polysaccharides into oligosaccharides. As a non-limitative example, a concentration of 1 unit of chitosanase is usually recommended by manufacturers for hydrolyzing 1 gram of

20   chitosan when incubated for 12 hours at 25 °C.

Once the enzymatic reaction has occurred, it is necessary to terminate it to ensure that no glucosamine monomers are present in the hydrolyzed solution.

According to the present invention, termination of the enzymatic reaction is achieved through electrobasification of the hydrolyzed solution. The

25   electrobasification compartment preferably has a pH of about 7 to 14, more preferably of about 8 to 13, even more preferably of about 8 to 12, and yet more preferably of about 9 to 11. The alkalinity of the basification compartment reduces the stability of the polysaccharide-hydrolyzing enzyme three-dimensional structure, leading to its denaturation, and thus toward termination of the enzymatic reaction.

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In addition, the catalytic activity of the enzyme was found to be greatly reduced in extreme alkaline condition, contributing to the termination of the reaction. Moreover, since polysaccharides precipitate at alkaline pH, the transition from acidic pH to alkaline pH induces a "desolubilization", or precipitation, of the polysaccharides that did not undergo enzymatic hydrolysis, and these polysaccharides are therefore not available anymore as substrate, which also contributes to the termination of the enzymatic reaction. The termination of the enzymatic reaction according to the present invention thus occurs on three different aspects; denaturation of the enzyme; inhibition of its catalytic activity; and withdrawal of the substrate. In opposition, the traditional thermal denaturation of the enzyme only terminates the enzymatic reaction by denaturing the enzyme.

Termination of the enzymatic reaction by electrobasification provides the possibility of treating the resulting basified solution by means known in the art to selectively precipitate the enzyme before the basified solution is transferred into the demineralization compartment. Since the basic pH did not irremediably denature the enzyme, the pH of the enzyme can be lowered, by resuspension into an aqueous solution at about optimal pH for the enzyme for example, to reverse enzyme denaturation. Then, the enzyme can be re-used with a fresh acidified solution produced by the acidification step. Alternatively, the enzyme can also be precipitated from the demineralizing solution for reversing its denaturation.

In accordance with the present invention, the basification of the polysaccharide solution is achieved by electrobasification in the basification compartment of the electrodialysis cell (Fig. 3). Under the influence of the electrical current, the basification compartment is enriched in cations from the CEM that induce an increase of the pH of the hydrolyzed solution in the basification compartment, thus basifying the hydrolyzed solution to produce a basified solution.

Following the basification step, the polysaccharides that did not undergo enzymatic hydrolysis and that were precipitated by the basification step can be precipitated by techniques known in the art and reintroduced into the acidification

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compartment. This ensures that all polysaccharides present in the original polysaccharide solution are ultimately transformed into oligosaccharides, and this method therefore virtually eliminates the waste of polysaccharides.

Further, and as mentioned previously, portions of the basified solution can
5   be taken out and used to adjust the pH of the acidified solution in order for it to reach a pH value optimal for the hydrolysis reaction to occur, before addition of the polysaccharide-hydrolyzing enzyme. Similarly, portions of the acidified solution can also be used in the same manner to lower the pH of the polysaccharide solution acidified solution, particularly for fine adjustments for reaching an exact pH value.
10   This prevents having recourse to external sources of acids or bases to adjust pH, and therefore, reduces the introduction of external ion sources into the system.

Once the enzymatic reaction has been terminated, the basified solution mostly contains oligosaccharides that can be collected by precipitation or other methods known in the art. However, the acidification and basification steps
15   introduce ions in the solution, either from internal or external sources, that can be viewed as contaminants according to the projected use of the oligosaccharides. Moreover, additional minerals originating either from the polysaccharides preparation prior to the solubilization or from the solubilizing solution can be present in the basified solution. Therefore, a demineralization of the basified
20   solution is greatly desired in order to increase the purity grade of the collected oligosaccharides.

In accordance with the present invention, the demineralization occurs in the demineralization compartment of the electrodialysis cell (Fig. 3). Under the influence of the electrical current, the negatively charged ions migrates from the
25   demineralization compartment toward the anode, through the AEM, while the positively charged ions pass through the CEM to move toward the cathode. As a result, the concentration of charged ions, including minerals, is greatly reduced, and the purity of the oligosaccharides collected at the exit of the demineralization compartment is increased.

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In an aspect of the present invention, the transformation of polysaccharides into oligosaccharides occurs in three steps. The acidification of a polysaccharide solution solubilizes the polysaccharides and allows the enzymatic reaction with polysaccharide-hydrolyzing enzyme to occur, the basification of the reaction solution terminates the enzymatic reaction, and the demineralization of the oligosaccharide solution increases the purity of the collected oligosaccharides.

It will be understood that the present invention can be applied either to anionic or cationic polysaccharides, with the disposition of the membranes within the electrodialysis cell and the sequence of the acidification and basification steps being adjusted based on the utilization of either anionic or cationic polysaccharides.

The demineralization compartment further plays a role in the continuous use of the electrodialysis cell. Its location between the acidification compartment and the basification compartment ensures that no clogging of the separating membranes occur. If the acidification and the basification compartments were to be side by side, the membrane separating the two compartments would eventually become basic on the surface of its side exposed to the acidification compartment, because of the proximity of the basification step. Polysaccharides precipitating at basic pH, this would lead to the precipitation of the polysaccharides circulating in the acidification compartment that would come in contact with the membrane surface. Not only would this decrease the quantity of solubilized polysaccharides produced by the acidification step, but this would also induce clogging of the membrane. The membrane would therefore need frequent washing or replacements in order to keep an adequate production rate. The presence of the demineralization compartment between the acidification and basification compartments therefore act as a buffer between the two environments having opposed pH values.

In accordance with the present invention, all of these three steps take place in the same electrodialysis cell, as illustrated in Fig. 2. Preferably, after a

polysaccharide solution has circulated into the acidification compartment, the resulting acidified solution is positioned, either by hand or by an automated mechanism, for circulation into the basification compartment, while a fresh polysaccharide solution is introduced into the acidification compartment. Then,

5   after basification has been completed, the resulting basified solution is positioned for circulation into the demineralization compartment, the acidified solution is transferred into the basification compartment, and another fresh polysaccharide solution is positioned to be circulated into the acidification compartment.

The circulation of the solutions into the compartments can be achieved by

10 any means known in the art, such as with a system of pumps and reservoirs for example. The solutions are circulated in the compartments in closed circuits, as illustrated on Fig. 4. It will be understood that any configuration of the circulation system allowing a sequential passage of a solution from the acidification compartment to the basification compartment and then to the demineralization

15 compartment can be used.

In accordance with an aspect of the present invention, all three compartments are used simultaneously. The simultaneous use of the three compartments allows for a synergistic effect of the demineralization compartment on the other two compartments. By being separated from the acidification

20 compartment with an anion-exchange membrane, the demineralization of the oligosaccharide solution, under the influence of the electrical current, expulses the negatively charged ions through the AEM bordering the left side of the demineralization compartment of Fig. 2, towards the anode, into the acidification compartment. Therefore, the acidification is performed without the need of adding

25 an extrinsic acid, by anions originating from the basified solution circulating into the demineralization compartment. Similarly, the basified solution circulating into the demineralization compartment also expulses positively-charged ions through the CEM bordering the right side of the demineralization compartment of Fig. 2, towards the cathode, into the basification compartment. The basification thus also

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occurs from within the electrodialysis cell, without the need of adding an alkaline solution to the acidified solution circulating into the basification compartment. Therefore, a continuous circulation in the three compartment is preferred, thus allowing for a continuous use of the electrodialysis cell for the production of

5   oligosaccharides from polysaccharides.

The simultaneous use of the three compartments also provides a maximization of the electrical current. While using the three compartments sequentially or separately would require a different electrical current for each of the three steps, the methods and systems according to the present invention rather

10   maximize the use of every electron generated by the electrical current by making each electron performing three actions (basification, demineralization and acidification). This result in the need of a lower electrical current usage and contributes to a lesser production cost over existing methods and systems for the production of oligosaccharides from polysaccharides.

15   When the system is first started by introducing a polysaccharide solution into the acidification compartment, the other two components are preferably filled with a solution containing salts and/or minerals, in order to take advantage of the synergistic effect of the demineralization compartment on the acidification step. Then, when the acidified solution is transferred into the basification compartment

20   and a second polysaccharide solution is introduced into the acidification compartment, only the demineralization compartment is to be filled with a solution containing salts and/or minerals. The introduction of a third polysaccharide solution into the acidification compartment, after the acidified and basified solutions have respectively been transferred into the basification and demineralization

25   compartments, allow for a continuous use of the system and thus, a continuous production of oligosaccharides.

Therefore, the system and method presented herein are self-sufficient and provide all of the reaction products needed for the transformation of

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polysaccharides into oligosaccharides, exception made of the original polysaccharide powder and the polysaccharide-hydrolyzing enzyme.

The present invention will be more readily understood by referring to the following examples which are given to illustrate the invention rather than to limit its
5　scope.

EXAMPLE I : Effect of bipolar membrane electrobasification on chitosanase activity during chitosan hydrolysis

The effect of pH on the stability of chitosanase and on the catalytic activity
10　of chitosanase during chitosan hydrolysis was studied. The stability of chitosanase was evaluated by measuring the activity left after storing the enzyme in buffers of different pH values after different application times. The catalytic activity of chitosanase was determined by measuring the catalytic activity of the enzyme after pH adjustment of a chitosan / chitosanase hydrolysis solution using alkali from a
15　bipolar membrane electrodialysis system.

For chitosanase stability, low molecular weight chitosan (approximately 100 kDa with 96% degree of deacetylation) and chitosanase (9.6 U/mL, isolated and purified from *Streptomyces* sp. N174) were used. Chitosanase stability was evaluated by storing the enzyme (100 µL) in buffers (900 µL) at various pH values
20　(2, 3, 4, 5.5, 7, 8, 9, 10, 12) for various application times (0, 1, 7, 24 and 48 h) at room temperature (25 °C). The pH values for buffers were chosen so as to cover the pH optimum for chitosanase activity (pH 5.5) as well as extreme acidic and alkaline pH values. Buffers were prepared with reagent grade chemicals, following the composition presented in Table 1.

Table I : Buffer composition for determination of chitosanase stability

pH value	Buffer composition
pH 2	50 ml KCl 0.2M + 13 ml HCl 0.2M
pH 3	80 ml glycine 0.1M + 20ml HCl 0.1M
pH 4	20 ml sodium acetate 0.1M + 80 ml acetic acid 0.1M
pH 5.5	80 ml sodium acetate 0.1M + 20 ml acetic acid 0.1M
pH 7	50 ml KH$_2$PO$_4$ 0.1 M + 30 ml NaOH 0.1 M
pH 8	50 ml Tris 0.1M + 30ml HCl 0.1M
pH 9	50 ml Tris + 6 ml HCl 0.1M
pH 10	50 ml sodium bicarbonate 0.05 M + 10 ml NaOH 0.1 M
pH 12	25 ml KCl 0.2 M + 6 ml NaOH 0.2 M

For each combination of pH and buffer application time, residual activity of chitosanase was measured by incubating the enzymatic preparation (100 μL) in a 0.1 % (w/v) chitosan solution (200 μL) with a pH 5.5 buffer (700 μL). Incubation was carried-out at 37 °C for 10 minutes. The enzymatic reaction produces chitosan oligomers, that are reducing sugars. The concentration of reducing sugars produced during incubation was measured in triplicate using a colorimetric method as follow : an aliquot of sample (100 μL) was added to a test tube containing 1 mL of neocuproine reagent, 1 mL of copper-glycine oxidant, and 900 μL of distilled water. Coloration was developed by heating in a water bath at 100 °C for 15 min, before cooling under tap water, and dilution of the colored product in 10 mL of distilled water. After centrifugation at 10,000 rpm for 10 minutes, the supernatant was collected and the absorbance was measured at 455 nm with a HP 8453 spectrophotometer. Comparison was made with a blank solution prepared by the same procedure with distilled water replacing the chitosan hydrolyzate solution.

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The standard curve was prepared using D-glucosamine. Results for chitosanase stability were expressed in percentage of residual activity as a function of pH values and application times, considering a 100% activity for chitosanase at initial time (t=0).

5   The catalytic activity of chitosanase was evaluated in presence of chitosan substrate at various pH from pH 5.5 to alkaline pH (pH > 11). The pH values were adjusted with a bipolar membrane electrodialysis system. The configuration of the electrobasification cell consisted of two BP membranes (BP-1 bipolar membranes), three CEM (CMX-SB cation-exchange membranes) and one AEM (AMX-SB anion-
10   exchange membrane) (Fig. 5). The arrangement defined three flow circuits : the chitosan / chitosanase hydrolyzate solution (1 L) circulated in the basified compartment ; a KCl solution (2 g/L, 6 L) circulated in the acidified compartment ; and a NaCl (20 g/L, 6 L) used for electrode rinse. The module used was a MP type cell (100 cm^2 of effective surface) equipped with a Dimensionally Stable Anode
15   (DSA®), and a 316 SS cathode from ElectroCell AB (Täby, Sweden). The flow rate was maintained at 3 L/min in the three compartments.

          The chitosan / chitosanase hydrolyzate solution had an initial pH of 5.5. The solution was prepared by incubating 5 units of chitosanase in 1 liter of chitosan solution 0.5 % (w/v) and addition of HCl 37% to obtain pH 5.5. Incubation was
20   performed under agitation at room temperature (25 °C) for 1 hour. After incubation, the pH of the chitosan hydrolyzate was adjusted by basifying the solution with a bipolar membranes electrodialysis system using a current density of 4 mA/cm^2 or 1 mA/cm^2. The current density was lowered at 1 mA/cm^2 for pH ranging from 6 to 10 in order to allow sampling of chitosan hydrolyzate when pH increases rapidly. The
25   values of current densities used in this experiment were inferior to the limiting current density (5 mA/cm^2) in order to prevent water splitting at the interface of monopolar membranes. Enzyme-catalysis reactions of the chitosan / chitosanase system were determined by dosing the reducing sugars on electrobasified samples (10 mL) collected at pH 5.5, 6.0, 7.0, 9.0, 10.0, 11.0 at different times (t = 0, 1 or 3

h) at 25 °C. At t = 3 h, the pH of samples were readjusted to pH 5.5 with HCl for 1 hour in order to evaluate residual activity of chitosanase. All samples were boiled at 100 °C for 5 minutes on collection to terminate the enzymatic reaction prior to determine their content of reducing sugars by the colorimetric method described previously. The pH of the chitosan hydrolyzate solution was measured with a pH meter equipped with an automatic temperature compensated electrode.

Effect of pH on chitosanase stability

The effects of pH and application times on chitosanase stability were determined by storing the enzyme into various buffers from pH 2 to 12, for a duration of 1 to 48 h at 25 °C. The residual activity of chitosanase was measured for each combination of pH and application time in the same conditions (pH 5.5, 37 °C). Statistical analysis showed that application time and pH had a significant effect on chitosanase stability. Chitosanase from *Streptomyces sp. N174* was stable in the pH range from 3 to 8 for at least 7 h (Fig. 6). Outside of the pH stability range, we found that chitosanase lost about 50 % of its activity after 1 h at pH values higher than 9, while the loss of activity could reach 90 % at pH 2 after 1 h (Fig. 6). This loss in stability is caused by the denaturing effect of pH on the three dimensional structure of the chitosanase protein. We noted that the denaturation of chitosanase from *Streptomyces sp. N174* required highly acidic pH (pH < 3), but only moderate basic pH (pH > 9). This can be explained by the stabilization of chitosanase by positive charges in acidic medium, such that denaturation requires lots of protons to destabilize the protein structure. Inversely, at moderate basic pH values, chitosanase is negatively charged, resulting in a less stable form of the protein, thus confirming that denaturation of chitosanase occurs preferentially in alkaline pH conditions rather than in acidic medium.

Application times also presented a significant effect on chitosanase stability. A 80% loss of activity was observed after 7 h at pH values higher than 9, while the decrease in stability was of about 20 % at pH values between 3 to 8 after 24 h (Fig. 6). While the effect of pH is usually involved in such a stability decrease,

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environmental conditions such as ionic strength may also be involved in the loss of activity of an enzyme.

Effect of electrobasification on the catalytic activity of chitosanase

After determining that chitosanase activity decreased in moderately alkaline pH ranges, we evaluated the effect of electrobasification on the catalytic activity of chitosanase in presence of chitosan substrate. In this experiment, the alkali produced by in the basified compartment was used to terminate the on-going chitosanase-based hydrolysis of chitosan (Fig. 4).

Electrobasification consisted of circulating the chitosan hydrolyzate solution in the basified compartment where hydroxyl ions are produced at the anionic interface of bipolar membranes (Fig. 5). As the concentration of hydroxyl ions increased in the solution, the pH increased as a function of electrical charge (Fig. 7). The number of charge (C) was calculated from the product of time (in seconds) and electrical intensity (in A). The total number of charges used in the process was 1934 ± 43 C, corresponding to a duration of 90 minutes at two current intensity (1 or 4 mA/cm^2). The evolution of pH during electrobasification of chitosan hydrolyzate was mainly due to the presence of chitosan polymer and its oligomers. Starting with a protonated chitosan hydrolyzate solution at pH 5.5, electrobasification enabled deprotonation of the chitosan amino groups. Complete deprotonation of chitosan products resulted in chitosan insolubilization for pH > 7. However, chitosan oligomers having biological properties should remain soluble even at alkaline pH values, due to their low molecular weight.

The catalytic activity of chitosanase after electrobasification was evaluated by measuring the content in reducing sugars liberated after reaching different pH values by electrobasification. Prior to pH adjustment, partial hydrolysis of chitosan by chitosanase was carried-out in batch mode in a reaction tank under agitation and resulted in a chitosan hydrolyzate containing 0.16 ± 0.02 µmol /mL of total reducing sugars (Fig. 8). Considering that part of the total reducing sugars was due

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to chitosan polymers (0.08 ± 0.02 μmol /mL), the overall rate of enzymatic reaction was 0.08 $μmol.mL^{-1}.h^{-1}$ before pH adjustment, as determined by the amount of reducing sugars liberated in one hour. After pH adjustment of the chitosan hydrolyzate solution by electrobasification, the samples collected at the different

5   pH were allowed to stay at 25 °C during 1, 3 and 4 hours and their content in reducing sugars was determined for each combination of pH and time. Chitosanase was still active at pH 5.5 after 4 hours, since production of reducing sugars increased linearly (R^2= 0.998) with a rate of 0.07 $μmol.mL^{-1}.h^{-1}$ (Fig. 8). Production of reducing sugars decreased by almost 2-fold at pH 6.0, with a rate of

10   0.03 $μmol.mL^{-1}.h^{-1}$. At pH higher than pH 7, chitosanase was not active anymore, since the amount of reducing sugars did not change significantly as a function of time. These results showed that the enzyme-catalysis reaction rate decreased with the increase of pH values above pH 5.5, until complete inhibition of the reaction occurred for pH values higher than 7. In the pH region from 5.5 to 7, the decrease

15   of reaction rate can be explained by progressive insolubilization of chitosan substrate.

In addition, we tested chitosanase stability after 3 hours of exposition at the different pH, by readjusting the pH of basified samples at pH 5.5, and by measuring their reducing sugars content 1 hour later (t = 4 h). Only samples with

20   pH 5.5 and pH 6 still had a chitosanase activity, whereas samples with pH > 7 did not recover any chitosanolytic capacity (Fig. 8). We observed that denaturation occurred at pH as low as pH 7 in electrodialysis, whereas chitosanase was denatured at pH higher than 9 when stored in buffers. The difference in pH denaturation can be explained by a higher temperature in electrobasification with

25   temperatures that could locally reach 35 °C due to heating by centrifugal pumps and joule effect.

This work demonstrated two effects of pH on chitosanase activity. First, extreme pH values such as pH < 3 and pH > 8 affected the stability of chitosanase after only 1 hour. The loss of activity was due to denaturation of the protein at

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extreme pH. Secondly, the catalytic activity of chitosanase during chitosan hydrolysis decreased after pH adjustment by electrobasification. The reaction rate decreased by 50 % from pH 5.5 to pH 6, whereas the reaction was completely inhibited at pH > 7. The decrease of reaction rate was due to chitosan substrate

5   insolubilization and chitosanase denaturation at alkaline pH values. Adjusting the pH at 7 would be enough to terminate the reaction without denaturing chitosanase; in this case, the enzyme could be recovered for further chitosan hydrolysis. Otherwise, if chitosanase has to become completely inactive in the end product containing chitosan oligomers, adjusting the pH at a value higher than 9 would be

10   necessary for destruction of the enzyme structure. Electrobasification with bipolar membrane is therefore interesting for chitosanase inhibition instead of thermal denaturation, because the effect of alkaline pH on precipitation of high molecular weight chitosan would allow to recover chitosan oligomers of low molecular weight in the supernatant without adding any chemicals in the reaction mixture. Moreover,

15   electrobasification of chitosanase can be carried-out simultaneously with electroacidification of chitosan in a single-step process. Indeed, the conventional method of production of chitosan oligomers includes two separate steps: chitosan has to be solubilized in a preliminary step and chitosanase has to be inactivated in a final step (Fig. 1). Electrobasification allows for the chitosan suspension to be

20   converted into its soluble form after circulating in the acidified compartment, while chitosanase can be inactivated after circulating in the basified compartment. In this electrochemical method, external acid and heating equipment are replaced by acid and base generated in situ respectively, which results in a safer process with ecological benefits.

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EXAMPLE II : Three-step chitosan transformation into oligomers using an
integrated bipolar membrane electrodialysis system

The aim of this study was to test a complete process for chitosan
transformation into chito-oligomers using a three-compartment BMED configuration
(bipolar / anionic / cationic / bipolar).

Chitosan (96 % deacetylation) had a moisture content of 7.1 ± 0.1 % (w/w
on wet basis), and an ash content of 0.40 ± 0.01 % (w/w on dry basis). According
to the company, chitosanase, isolated and purified from *Streptomyces sp. N174*,
had an enzyme activity of 9.6 U/mL.

The BMED configuration consisted in a three-compartment electrodialysis
cell (Fig. 9). The ionic membranes were Neosepta BP-1 bipolar membranes (BP),
AMX-SB anionic membranes (AEM) and CMX-SB cationic membranes (CEM) from
Tokuyama Soda Ltd., and purchased from Ameridia (Somerset, NJ, USA). The
electrodialysis stack was composed of two elementary cells. The electrode rinse
was a NaCl solution (20 g/L, 6 L). The other reservoir tanks were filled with 1.5 L of
the following solutions: 1) a chitosan suspension in the acidified compartment, 2)
an enzymatic solution of chitosanase and chitosan oligomers in the basified
compartment, and 3) a solution of chito-oligomers in the diluate compartment (Fig.
9). BMED consisted of applying a constant current density of 10 mA/cm^2 during 60
min. The flow rates were maintained constant at 4 L/min.

The production of chito-oligomers using BMED started with chitosan
solubilization, continued with chitosanase inactivation and ended with oligomers
demineralization (Fig. 10). However, before performing the three BMED steps
simultaneously, preliminary chitosan acidification and chito-oligomers basification
were realized to provide the solutions for all compartments. The specific procedure
for each BMED compartment is described hereafter.

Acidification. Chitosan was solubilized in the acidified compartment by a
stepwise feeding mode. Six aliquots of 1.15 g chitosan powder were added every

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10 min in 1.5 L of 0.05 M NaCl solution. At the end of acidification process, the cumulative addition of chitosan corresponded to a content of 4.6 g/L.

Enzymatic hydrolysis. Chitosan solution recovered from electroacidification had a pH of 2 and was adjusted at pH 5.5 using the base produced by BMED.
5   Then, the solution was collected in a beaker and incubated with chitosanase during 12 h at 25°C under agitation, using a ratio of 1 unit of chitosanase for 1 g of chitosan.

Basification. Enzymatic reaction of chitosan with chitosanase was terminated by circulating the enzymatic solution in the basified compartment. The
10   initial pH of 5.5 was adjusted at alkaline value up to pH 11.

Demineralization. The previous alkaline chito-oligomers solution was demineralized in the diluate compartment. Samples of each compartment (15 mL) were collected every 10 min from 0 to 60 min for chemical analysis.

Physico-chemical parameters were monitored during the BMED process by
15   measuring the pH of the solutions in the three compartment reservoirs Chemical analysis of the samples consisted of determining their chitosan content, reducing sugars content and ash level. Finally, chito-oligomers were characterized qualitatively by thin-layer chromatography in the final product.

The pH of the solutions in the three compartments was measured with a pH
20   meter (SP 20 model, ThermoOrion, purchased from VWR International, Montreal, Canada) equipped with an automatic temperature compensated electrode (N° 14002-778).

The soluble chitosan content was measured on samples collected in the acidified compartment using the colorimetric method described previously. The
25   reducing sugars content was measured on chitosan hydrolyzate samples using the colorimetric method described previously.

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The ash content, corresponding to minerals contained in the solution after heating of the solution, was measured by washing the crucibles beforehand in 3N nitric acid, rinsed with deionized water, drying at 100 °C and cooling in a desiccator. About 5 g of chitosan solution samples were dried in an oven and washed at 500 °C for a minimum of 18 h. The samples were cooled in a desiccator and weighed. The following formula was used for determining the ash content: Ash (% w/w) = [(weight of residue, g) / (sample weight, g)] x 100.

Chitosan oligomers were qualitatively analyzed by thin layer chromatography (TLC) using a silica gel plate (Kieselgel 60 F254, Merck). After the oligomers were developed with a solvent system of n-propanol-30% ammonia water-acetonitrile (2:2:1, vol/vol), the detection was performed by spraying 0.1% ninhydrine into n-butanol, and by baking the plate in an oven at 110°C for 10 min. Standards used were D-glucosamine (Sigma, Ontario,Canada), and dimers, trimers and tetramers of glucosamine (ISM Biopolymer, Granby, Canada).

Chito-oligomers were also prepared using the traditional chemical method. Chemical method was carried-out by adding $5,4.10^{-2}$ mol/L of HCl or NaOH under agitation in a glass beaker. The concentration of chemical reagents to be added was determined by titration of acid and base produced by the BMED system at specific conditions (current density of 10 mA/cm^2 and two elementary cells). Acidification of chitosan was achieved by the addition of 112 µL of 12.1 M HCl every minute during 60 min to a 0.05 M NaCl solution (1.5 L), while chitosan was added stepwise as in the BMED method. Following the chitosan hydrolysis with chitosanase, chemical basification of chito-oligomers was carried-out by addition of 135 µL of 10 M NaOH every minute during 60 min to the chitosan hydrolyzate (1.5 L). Measurements of pH and ash levels were done on samples for comparison of chemical and BMED method.

Chitosan acidification

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Chitosan was solubilized by acidification using either the BMED system or a chemical acid (HCl). The pH of the resulting solution was measured along the process and expressed as a function of acidification time (Fig. 11a). The kinetic of chitosan acidification was similar for BMED or chemical method, with a pH decrease of 4.3 units in average, from pH 6.3 to 2.0 due to H^+ protons from BMED water dissociation or HCl acid. Calculations made using chitosan pK_a value of 6.5 showed that the fraction of protonated chitosan is 99.9 % at pH 2.0. These conditions of extreme acidic pH values allowing full protonation of chitosan amine groups resulted in the conversion of chitosan base into its polyelectrolyte soluble form.

Basification of chitosan hydrolyzate solution

Enzymatic reaction of chitosan with chitosanase was terminated by basifying the solution using the BMED or chemical method. Evolution of pH as a function of basification time is presented in Fig. 11b. The increase of pH from 5.5 to 12 was due to hydroxyl ions from BMED or NaOH base. The kinetic of basification was characterized by a sigmoidal curve, which is typical of a titration curve of chitosan by a strong base. Indeed, chitosan and its depolymerized products can be considered as weak acids due to their protonated amine groups. As the basification progresses, the amine groups are deprotonated, until all NH_3^+ groups are neutralized, corresponding to the equivalence point on the sigmoidal curve. Although the equivalence point was the same for the BMED and the chemical method, the increase of pH was faster for the BMED. This can be explained by the lower concentration in chitosan material in the BMED system because of chitosan precipitation during the pH adjustment of the solution from pH 2 to pH 5.5 by BMED. The loss of chitosan material could be prevented by adding chitosan powder to the acidic solution (pH 2) until reaching pH 5.5, then finishing the pH adjustment of the chitosan hydrolyzate by basification with BMED.

Mineralization of the basified solution was measured by total ash measurement for the BMED (Fig. 12a) and chemical method (Fig. 12b) as a

function of time. The ash content increased by 66 % in average for BMED and chemical method. Concerning the chemical method, the mineralization was due to Na^+ cations from NaOH. As for the BMED method, the cationization was done by Na^+ and K^+ from NaCl and KCl salts, which were added initially to conduct

5   electricity. Moreover, the mineral contents were similar at the beginning of the basification process (0.4 % in average).

Demineralization of chitosan hydrolyzate solution

        After basification of the chitosan hydrolyzate, the solution was demineralized in the diluate compartment of the BMED system (Fig. 9). The pH of the solution

10   was measured as a function of electrodialysis time. The pH value decreased by 4 % from pH 11.9 to pH 11.4 (Fig. 11c). The decrease of pH can be explained by partial dealkalinisation of the solution, since part of hydroxyl ions left the diluate compartment to migrate to the adjacent acidified compartment by crossing the anionic membrane (Fig. 9).

15   The demineralization level in the diluate compartment was measured by ash determination. The ash level decreased linearly as a function of electrodialysis time (Fig. 12a). The demineralization of 53% led to a final ash content of 0.26 % (w/w on wet basis), which is close to the initial ash content before the BMED treatment. This result suggests that, after the initial mineral addition to start the BMED

20   system, the three-compartment electrodialysis does not require further salt addition, as the salts necessary for conducting the electricity were provided by the desalting compartment.

Performance of BMED

        The main advantage of the BMED over the chemical method is the

25   possibility to remove the salts that were added during acidification and basification, so that at the end of a one hour treatment, the ash content returns to its initial value (close to 0.26 %). However, the production of chito-oligomers purified by electrodialysis with bipolar membrane is effective only if the following conditions

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are respected: 1) a high chitosan solubilization rate during acidification; 2) a complete inhibition of enzymatic reaction; 3) an effective demineralization; 4) a good membrane integrity; and 5) a low energy consumption.

Chitosan solubilization rate

5   Fig. 13 demonstrates that complete solubilization of chitosan was achieved using the BMED system, as shown by the linear increase of soluble chitosan content as a function of time to reach a final solubilization rate of 4.6 g/L. Since the final product contained an excess of protons, an additional amount of chitosan can be solubilized, which would allow to adjust the pH to the optimum pH of the
10   enzyme. This operation should be carried-out outside of the cell in order to prevent chitosan overloading in the electrodialysis system.

Inhibition of enzymatic reaction

          Complete inhibition of the enzymatic reaction was ensured by the extreme basic pH of solution (pH > 11). As shown hereinabove, inhibition of reaction occurs
15   at pH 7, but higher pH are required if denaturation of the enzyme is required. It was previously shown that alkaline pH values have also an effect on chitosan, by precipitating the high molecular weight chitosan, while the low molecular weight material remains soluble. Thin-layer analysis of the final chito-oligomers solution confirmed the presence of low molecular weight chitosan as dimers, trimers and
20   tetramers, with some other oligomers (Fig. 14).

Demineralization rate

          Increase of minerals in chito-oligomers was due to initial salt addition and to successive acidification and basification of the solution using BMED. The diluate compartment allowed the demineralization of the chito-oligomers solution up to 53
25   %. The final ash content (0.26 % (w/w) on wet basis) is more than twice inferior in comparison with the chemical method. If further demineralization is required, one

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easy option consists in setting up additional elementary cells in order to increase the surface exchange area.

The global process of chitosan hydrolysis into oligomers using the BMED system is presented on Fig. 3.

5   In the first step, the acidification compartment was used to solubilize the chitosan powder. The resulting chitosan solution (pH 2.5) was taken out of the BMED system and the solution was adjusted at the pH of incubation of chitosanase by adding chitosan powder. Then, the chitosan solution was incubated with chitosanase under controlled conditions of pH and temperature.

10   In the second step, the basification compartment was used to terminate the chitosan hydrolysis by elevating the pH of the solution (pH > 7). In the same time, another amount of chitosan was solubilized in the acidified compartment. The resulting acidified solution was treated like previously by adjusting the pH and incubating with chitosanase. As for the basified solution, a centrifugation step

15   allowed for the separation of insoluble chitosan and soluble chito-oligomers.

In the third step, the three compartments were running simultaneously. The acidification and basification compartments were used for chitosan solubilization and chitosanase inactivation respectively. Meanwhile, the diluate compartment situated was used for demineralization of the basified solution to produce purified

20   chitosan oligomers.

This work showed that BMED can perform three operations in a single operating unit, including chitosan solubilization, chitosanase inactivation and chito-oligomers demineralization. This innovative "3-in-1" process for chitosan transformation into oligomers has great potential for industrial application, since it

25   is convenient, ecological and produces oligomers with a lower mineral content in comparison with the conventional method.

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While the invention has been described with particular reference to the illustrated embodiment, it will be understood that numerous modifications thereto will appear to those skilled in the art. Accordingly, the above description and accompanying drawings should be taken as illustrative of the invention and not in a

5　　limiting sense.

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WHAT IS CLAIMED IS:

1.   A method for producing oligosaccharides in an electrodialysis cell, said electrodialysis cell comprising an acidification compartment, a basification compartment, and a demineralization compartment, the method comprising the steps of:

    a)   circulating a polysaccharide solution into the acidification compartment of the electrodialysis cell to acidify said polysaccharide solution, thereby producing an acidified solution;

    b)   incubating the acidified solution of step a) with a polysaccharide-hydrolyzing enzyme to induce the hydrolysis of the polysaccharides into oligosaccharides, thereby producing a hydrolyzed solution;

    c)   circulating the hydrolyzed solution of step b) into the basification compartment of the electrodialysis cell to basify said hydrolyzed solution, thereby producing a basified solution;

    d)   circulating the basified solution of step c) into the demineralization compartment of the electrodialysis cell to demineralize said basified solution, thereby producing a demineralized solution; and

    e)   collecting the demineralized solution of step d), wherein said demineralized solution comprises oligosaccharides.

2.   The method of claim 1 further comprising the step of isolating oligosaccharides from the demineralized solution of step e).

3.   The method of claim 1, wherein the pH of the acidified solution of step a) is adjusted to a value of 4.5 to 6.5 prior to incubation with the polysaccharide-hydrolyzing enzyme.

4.   The method of claim 3, wherein the adjustment of the pH is performed at least partly by adding a portion of a previously produced basified solution of step c) to the acidified solution of step a).

5.   The method of claim 1, wherein at least one of the hydrolyzed solution of step b) and the basified solution of step c) contains non-hydrolyzed polysaccharides, and wherein said non-hydrolyzed polysaccharides are collected from said at least one of the hydrolyzed solution of step b) and the basified solution of step c) and reintroduced in the polysaccharide solution of step a).

6.   The method of claim 1, wherein step a), step c) and step d) are performed simultaneously.

7.   The method of claim 1, wherein step a), step b), step c), step d) and step e) are performed simultaneously.

8.   The method of claim 1, wherein the polysaccharide is chitosan and the polysaccharide-hydrolyzing enzyme is a chitosanase.

9.   An electrodialysis cell comprising

        a cathode;

        a first bipolar membrane;

        an anion-exchange membrane;

a cation-exchange membrane;

a second bipolar membrane; and

an anode,

wherein the first bipolar membrane and the anion-exchange membrane defining an acidification compartment, wherein the anion-exchange membrane and the cation-exchange membrane defining a demineralization compartment, and wherein the cation-exchange membrane and the second bipolar membrane defining a basification compartment.

10.   An electrodialysis cell comprising

a cathode;

an acidification compartment;

a demineralization compartment;

a basification compartment; and

an anode,

wherein the acidification compartment is defined by a first bipolar membrane and an anion-exchange membrane, wherein the demineralization compartment is defined by the anion-exchange membrane and a cation-exchange membrane, and wherein the basification compartment is defined by the cation-exchange membrane and a second bipolar membrane.

11. Use of the electrodialysis cell of any of claims 9 or 10 for the production of oligosaccharides.

12. A bipolar membrane electrodialysis system comprising

the electrodialysis cell of any of claims 9 or 10;

a first reservoir unit connectable to the acidification compartment of the electrodialysis cell as a closed circuit;

a second reservoir unit connectable to the basification compartment of the electrodialysis cell as a closed circuit; and

a third reservoir unit connectable to the demineralization compartment of the electrodialysis cell as a closed circuit,

wherein the first, second and third reservoir units comprises pumping means for circulating a fluid into the compartments of the electrodialysis cell.

13. The bipolar membrane electrodialysis system of claim 12, wherein the first, second and third reservoir units are interchangeable.

14. The bipolar membrane electrodialysis system of claim 12, wherein the system further comprises a fourth reservoir unit connectable to an electrode rinse solution compartment of the electrodialysis cell as a closed circuit.

Fig. 1 (prior art)

Fig. 2

Fig. 3

Fig. 4

Fig. 5

Fig. 6

Fig. 7

Fig. 8

Fig. 9

Chitosan

| 1st BMED step |

Solubilization
by acidification

Chitosanase →

Enzymatic
hydrolysis

| 2nd BMED step |

Reaction ending
by basification

| 3rd BMED step |

Demineralization

Purified chito-oligomers
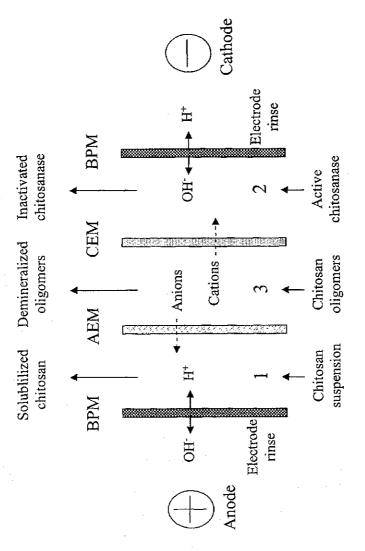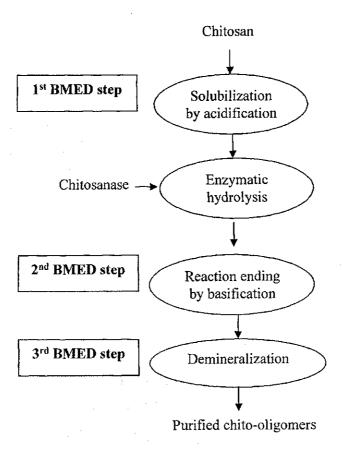
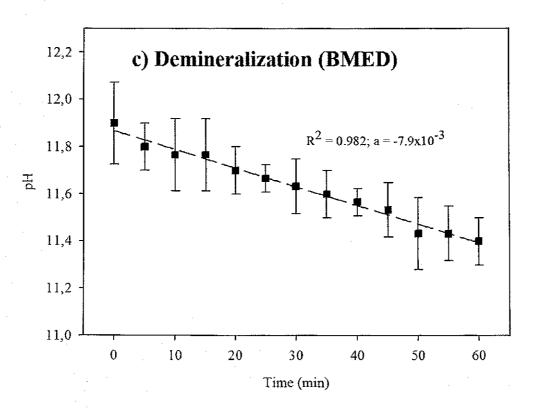# Fig. 10

Fig. 11

Fig. 11 (cont.)

c) Demineralization (BMED)

$R^2 = 0.982; a = -7.9 \times 10^{-3}$

Fig. 11 (cont.)

Fig. 12

Fig. 13

Fig. 14

A. CLASSIFICATION OF SUBJECT MATTER

IPC: *C07H 3/06* (2006.01), *B01D 61/42* (2006.01), *B01D 61/52* (2006.01), *C08B 37/08* (2006.01),
C12P 19/00 (2006.01), *C25B 9/00* (2006.01)
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC: *C07H 3/06* (2006.01), *B01D 61/42* (2006.01), *B01D 61/52* (2006.01), *C08B 37/08* (2006.01),
C12P 19/00 (2006.01), *C25B 9/00* (2006.01)

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic database(s) consulted during the international search (name of database(s) and, where practicable, search terms used)
STN, Intellect, Questel Orbit (Fampat), Delphion
Keywords: oligosaccharide, polysaccharide, enzyme, chitosan, hydrolysis, electrodialysis, membrane, cathode, anode, demineralization

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 5,904,823 (SHARIFIAN, H. et al.) 18 May 1999 (18-05-1999) see figure 11	9, 10 and 12
A	JP 2003-088394 (TOMOKICHI, S. et al.) 25 March 2003 (25-03-2003)	1-14
A	JP 2001-017977 (HIROSHI, O. et al.) 23 January 2001 (23-01-2001)	1-14

[ ] Further documents are listed in the continuation of Box C.   [X]   See patent family annex.

*	Special categories of cited documents :	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance		
"E"	earlier application or patent but published on or after the international filing date	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"O"	document referring to an oral disclosure, use, exhibition or other means	"&"	document member of the same patent family
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search	Date of mailing of the international search report
04 December 2008 (04.12.2008)	19 January 2009 (19-01-2009)
Name and mailing address of the ISA/CA Canadian Intellectual Property Office Place du Portage I, C114 - 1st Floor, Box PCT 50 Victoria Street Gatineau, Quebec K1A 0C9 Facsimile No.: 001-819-953-2476	Authorized officer Erin Todd 819- 934-6733

Box No. II	**Observations where certain claims were found unsearchable (Continuation of item 2 of the first sheet)**

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons :

1. [ ] Claim Nos. :
>   because they relate to subject matter not required to be searched by this Authority, namely :

2. [ ] Claim Nos. :
>   because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically :

3. [ ] Claim Nos. :
>   because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box No. III	**Observations where unity of invention is lacking (Continuation of item 3 of first sheet)**

This International Searching Authority found multiple inventions in this international application, as follows :

See supplemental Box

1. [X] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying additional fees, this Authority did not invite payment of additional fees.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claim Nos. :

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim Nos. :

Remark on Protest   [ ] The additional search fees were accompanied by the applicant's protest and, where applicable, the payment of a protest fee.

[ ] The additional search fees were accompanied by the applicant's protest but the applicable protest fee was not paid within the time limit specified in the invitation.

[X] No protest accompanied the payment of additional search fees.

Continued from Box III

Group A - Claims 1-8 are directed to a method for producing oligosaccharides by enzyme hydrolysis of polysaccharides in an electrodialysis cell, said cell comprising an acidification compartment, a basification compartment and a demineralization compartment.

Group B - Claims 9-14 are directed to an electrodialysis cell (claims 9 and 10), the use of the electrodialysis cell to produce oligosaccharides (claim 11) and a bipolar membrane electrodialysis system comprising the cell of claim 9 or 10 (claims 12-14).

The claims must be limited to one inventive concept as set out in Rule 13 of the PCT. The claims of group A and group B do not currently define any linking inventive features. The method for producing oligosaccharides defined in claim 1 can be accomplished with any cell that comprises an acidification compartment, a basification compartment and a demineralization compartment. The cells and system of claims 9, 10 and 12 could be used for many different uses and are are not limited to their use in preparing the oligosaccharides of claim 1.

Patent Document Cited in Search Report	Publication Date	Patent Family Member(s)	Publication Date
US 5904823A	18-05-1999	EP 0870532A2	14-10-1998
		EP 0870532A3	11-11-1998
		JP 11001788A	06-01-1999
JP 2003088394A	25-03-2003	None	
JP 2001017977A	23-01-2001	None	