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Continuous Degradation of Sodium Alginate in Bioreactor Using Immobilized Alginate Lyase

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The enzymatic degradation of sodium alginate was continuously carried out to effectively produce alginate oligosaccharides using immobilized alginate lyase in a CSTR (continuous stirred tank reactor) system. The alginate lyase was immobilized onto the chitosan beads and the reaction was operated at an initial alginate concentration of 10 g l^{-1} at 35°C and pH 7.0 under the dilution rate of 0.77 to 1.74 h^{-1}. The degradation products mainly consisted of di-, tri-, tetra-, penta-, and hexasaccharides with the highest conversion of 0.34, with the volumetric production rate of the total oligosaccharides dependent on the dilution rate. The production process was mathematically modeled from the basic material balance and the rate equation, and showed agreement between the simulated and experimental results. The present reactor system was found to be effective for obtaining alginate oligosaccharides with a high production rate.

Keywords: sodium alginate, enzymatic degradation, alginate lyase, CSTR system, alginate oligosaccharide

Alginate is one of the constituents of brown algae and consists of domains of polyβ-1,4-D-mannuornate (PM), poly α-1,4-L-guluronate (PG), and the copolymers of β-1,4-D-mannuronate and α-1,4-L-guluronate (Percival & McDowell, 1967). Alginate is industrially produced from seaweed by alkali extraction, and is widely used as a food additive, medicine, cosmetic, etc.

Over the last decade, the oligosaccharides derived from alginate have realized new applications such as the growth promotor of intestinal bacteria (Akiyama *et al.*, 1992), the growth inhibitor of HeLa cells (Yonemoto *et al.*, 1993), and the root elongator of plants (Yonemoto *et al.*, 1993; Natsume *et al.*, 1994; Tomoda *et al.*, 1994). To utilize the alginate oligosaccharides in practice, an effective degradation of alginate is required. However, few reports were found concerning the efficient degradation method of alginate. Murata *et al.* (1992) attempted to continuously degrade sodium alginate using a PFR (plug flow reactor) system with aggregated microbial bacteria, and suggested that the bioreactor system was useful for the continuous production of alginate oligosaccharides. However, the efficiency might not be high enough for practical utilization, presumably because of a nutrition shortage for cell growth.

In the present study, to obtain an efficient enzyme reactor for oligosaccharide production, the continuous degradation of sodium alginate was carried out using an immobilized alginate lyase in the CSTR (continuous stirred tank reactor) system. The enzyme was immobilized onto chitosan beads, and the degradation process was controlled by the dilution rate. The mathematical analysis of the system was also carried out using the basic material balance and rate equation, and the effectiveness of the system was then evaluated.

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Materials and Methods

Materials Sodium alginate (mean molecular weight: 500 kDa) was purchased from Dainihon Seiyaku, Ltd. (Osaka). The ratio of mannuronate residues to guluronate residues (M/G ratio) of the alginate was 0.50 based on the circular dichroism analysis (Morris *et al.*, 1980). Alginate lyase from *Flavobacterium* sp. (Alginate lyase S, activity: 3160 U (g-powder)$^{-1}$) was purchased from Nagase Biochemicals, Ltd. (Osaka). The activity was determined using 0.1% sodium alginate (Nakarai Tesque, Inc., Kyoto) as a substrate. One unit of enzyme activity was defined as an increase of 1.0 in the absorbance of reaction mixtures at 235 nm per min at 35°C and pH 7.0 (Muramatsu *et al.*, 1993). The protein concentration of the enzyme was 71.5 mg (g-powder)$^{-1}$ using the dye-binding method (Sugawara & Soejima, 1977).

Immobilization One hundred wet-g chitosan beads (Chitopearl BCW-3010, Fuji Bouseki Co. Ltd., Tokyo) was soaked in a 20 g l^{-1} alginate lyase solution with agitation for 3 h at 10°C. The enzyme was then physically adsorbed onto the beads. After the adsorption, the chitosan beads with the enzyme were washed using distilled water until the absorbance of the water became less than 0.01. The amount of adsorbed enzyme was evaluated by the initial protein concentration minus the residual protein concentration in solution.

Apparatus A schematic diagram of the CSTR system is shown in Fig. 1. The continuous operation was carried out by the chemostat method. Feed solution (10 g l^{-1} sodium alginate) was supplied to a reactor tank (MDL-300, Marubishi Co., Ltd., Tokyo) with 200 wet-g immobilized enzyme from a feed tank. The enzyme reaction occurred in the reactor tank with agitation (200 rpm), and the degradation products

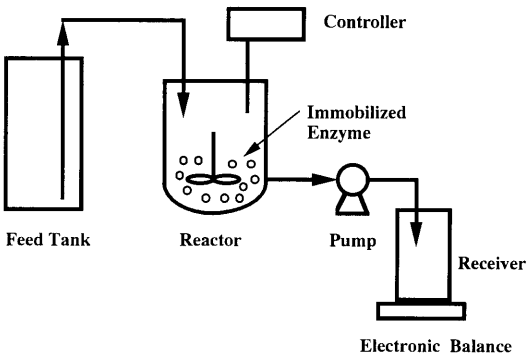

Fig. 1. A schematic diagram of CSTR system with immobilized alginate lyase.

were removed by pumping (Iwaki Magnetic Pump Ex, Iwaki Co., Ltd., Tokyo). The volume of the reaction mixture was kept constant by adjusting the effluent which was measured by an electronic balance. The temperature in the reactor was controlled at 35°C. The total working volume of the CSTR was 0.816 *l* (0.80 and 0.016 *l* of reactor tank and tubes, respectively).

Chemical analysis Standard alginate degradation products were prepared by the enzymatic degradation of 10 l^{-1} sodium alginate solution (corresponding to 3.93 gC l^{-1} based on carbon concentration, pH 7.0) at 35°C for 5 min. The products were fractionated by gel chromatography using a Bio-Gel P-4 (45–90 μm, Bio-Rad Lab., Richmond, Va.) column (XK 26/100, 186 cm, Pharmacia Fine Chem., Uppsala, Sweden) in which the mobile phase was 50 mM phosphate buffer (pH 7.0) according to the method of Muramatsu *et al.* (1993). The degradation products in the fractions were pooled, concentrated using a rotary evaporator, and freeze dried. A small quantity of water was added to each freeze-dried product and cooled in ice water. After removing the sodium phosphate crystals deposited in the solution, each product was also fractionated by gel chromatography using a Bio-Gel P-2 (45–90 μm) column (XK 26/100, 93 cm) in which the mobile phase was water in order to remove the residual sodium phosphate. Powdered standard products, alginate oligosaccharides (di-, tri-, tetra-, penta-, and hexasaccharides), were obtained by freeze-drying after the concentration of each solution using a rotary evaporator (Muramatsu *et al.*, 1993). Alginate oligosaccharides greater than the heptamer were ignored because these oligosaccharides were scarcely produced during degradation.

The concentration of each degradation product was determined using a high performance gel chromatograph with an RI detector (Waters Associates, Ltd., Milford, Mass.) and a gel filtration column (Superdex Peptide HR 10/30, Pharmacia Biotech, Uppsala, Sweden) in which the mobile phase was 50 mM phosphate buffer (pH 7.0), the flow rate was 0.4 ml min^{-1}, and the temperature was 40°C. Each concentration was converted to units of carbon concentration (gC l^{-1}).

The concentration of total uronate was measured using the *m*-phenylphenol method (List *et al.*, 1985): the sample solution, concentrated sulfuric acid with 0.0125 M sodium tetraborate, and a 0.15% solution of *m*-hydroxydiphenyl in 0.5% NaOH were mixed, and the absorbances were measured at 520 nm using glucuronic acid (Aldrich Chemical Co., Inc., Milwaukee, Wis.) as the standard. The concentration of total uronate was converted to units of carbon concentration. The carbon concentration of total uronate must be kept constant during the degradation while that of each product changes, which was also experimentally confirmed. For simplicity, the substrate concentration during degradation was assumed to be equal to the total uronate concentration minus the product (the sum of di-, tri-, tetra-, penta-, and hexasaccharides) concentration.

The protein concentration was measured using the dye-binding method: the sample solution and Coomassie brilliant blue G 250 (Fluka Chemie, Buchs, Switzerland) were mixed, and the absorbances were measured at 595 nm using bovine serum albumin (Sigma Co., Ltd., St. Louis, Mo.) as the standard.

Batch experiments The feed solution (10 g l^{-1} sodium alginate solution) was prepared by dissolving sodium alginate in hot water at 70–80°C, and the pH of the solution was adjusted to 7.0 with 1 N NaOH. The batch experiment was carried out under the conditions of 10 g l^{-1} sodium alginate solution, 250 wet-g l^{-1} chitosan beads with immobilized enzyme, 35°C, and pH 7.0. After the reaction, the concentration of the total degradation products was measured using high performance gel chromatography.

The inhibitory effect of the initial alginate oligosaccharide (the sum of the di-, tri-, tetra-, penta-, and hexasaccharides) contained in the feed solution on the alginate oligosaccharide formation during the enzymatic reaction was measured at the initial degradation product concentration of 0.8–3.0 gC l^{-1} by the same method as above for 1 h. The concentration ratios of each oligosaccharide to the total oligosaccharide in the feed solution were 0.05, 0.37, 0.39, 0.13, and 0.05 for the di-, tri-, tetra-, penta-, and hexasaccharides, respectively. The oligosaccharide concentration produced in the reaction was determined by subtracting the initial concentration from the final concentration.

Continuous experiments Alginate solution in the feed tank was first supplied to the reactor tank, then the immobilized enzymes with chitosan beads were added, and a batch operation was carried out at 35°C for 15 min to obtain the best conversion. After the batch operation, a continuous operation was started at a dilution rate of 1.74, 1.25, or 0.77 h^{-1} in runs 1, 2, and 3, respectively, to determine the appropriate experimental conditions of about 3 h. Thereafter, the continuous operation was also carried out for 60 h under the same conditions as run 3. The changes in the concentrations of the substrate and product with time were measured.

Results and Discussion

Standard alginate oligosaccharides The enzymatic degradation product of the sodium alginate solution was fractionated into dimer to hexamer oligosaccharides (data not shown). The evaluation of the molecular size of these oligosaccharides was made by the relationship between the molecular weight of each oligosaccharide and their elution volume during gel chromatography, which showed a good correlation. The alginate oligosaccharides obtained in the

present method were used as the high performance gel chromatography analysis standard.

Immobilization of enzyme The amount of enzyme immobilized onto the chitosan beads was calculated to be 0.358 mg (wet-g beads)$^{-1}$ based on the protein concentration. This suggests that 50% of the enzyme was adsorbed into the beads. The activity of the immobilized enzyme was compared with that of the free enzyme, and found to be reduced to 12.9%. This is due to the steric interaction between the enzyme and the beads (Woodward, 1985).

Batch experiments The alginate degradation product in the batch experiment contained di-, tri-, tetra-, penta-, and hexasaccharides. Figure 2 shows the time course of the degradation product formation from sodium alginate. Each conversion, defined by the concentration ratio of the feed to each oligosaccharide, gradually increased with time, and reached 0.02, 0.14, 0.15, 0.05, and 0.02 for the di-, tri-, tetra-, penta-, and hexasaccharides, respectively, at 60 min. The values further increased slightly after 60 min on the basis of the carbon concentration. This suggests that the degradation of sodium alginate in the present study proceeds by degrees, and that a long reaction period may be required to have sufficient conversion. The high viscosity of the feed solution, which arises from the complicated chemical structure of sodium alginate, may limit the enzymatic degradation of the sodium alginate (Iwasaki *et al.*, 1996).

Figure 3 shows the inhibitory effect of the initial alginate oligosaccharide on the alginate oligosaccharide formation during the enzymatic degradation of sodium alginate. With increasing concentration of the degradation products in the feed solution, the produced degradation product linearly decreased. Takeshita (1994) examined the inhibitory effect of the guluronic oligosaccharide on the enzymatic degradation of polyguluronate, and kinetically showed that two kinds of guluronic oligosaccharides or more inhibit the enzymatic reaction. In the present reaction, certain degradation products are also considered to inhibit the degradation reaction. Further investigation is needed to elucidate the details of the inhibition mechanism.

Continuous experiments The changes in concentrations of product for runs 1, 2, and 3 with time are shown in Fig. 4. The concentration is expressed as carbon concentration, and is normalized by the substrate concentration in the feed solution. In each run, the stable states during product formation were observed after 30–40 min. From the feed rate and working volume, the mean residence times were calcu-

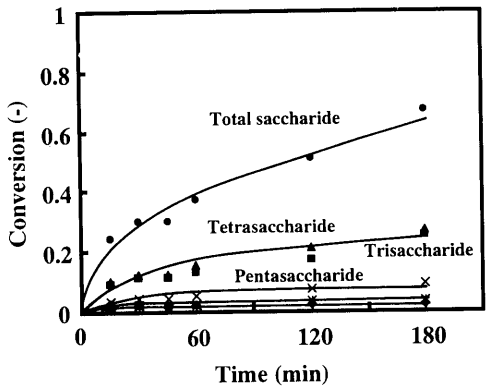

Fig. 2. Course of the alginate oligosaccharide formation in the batch experiment. Sodium alginate concentration, 10 g l^{-1}; temperature, 35°C; pH 7.0. ●, total saccharide; ◆, disaccharide; ■, trisaccharide; ▲, tetrasaccharide; ×, pentasaccharide; ∗, hexasaccharide.

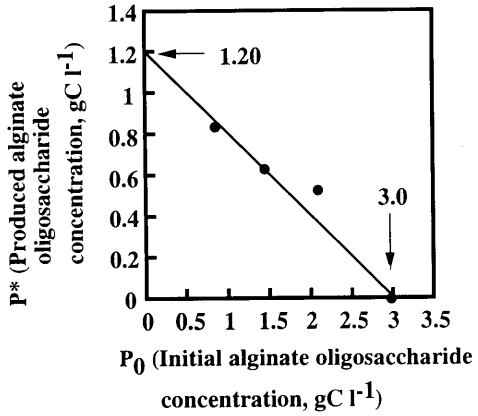

Fig. 3. The inhibitory effect of initial alginate oligosaccharide concentration on the alginate oligosaccharide formation in the enzymatic degradation reaction in the batch experiment under the conditions of 10 g l^{-1} sodium alginate, 35°C, and pH 7.0. Solid line refers to the simulation result from Eq. (5). See text.

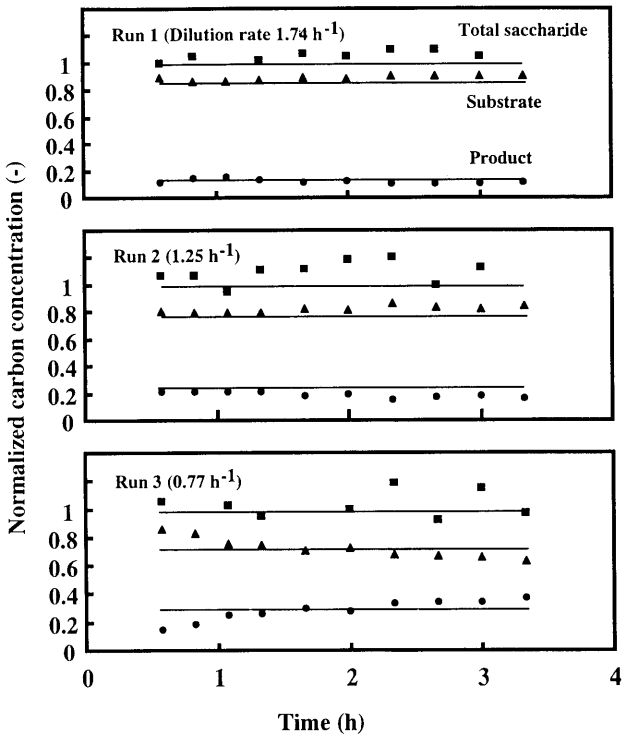

Fig. 4. Changes in concentrations of products from continuous degradation of 10 g l^{-1} sodium alginate solution with time for runs 1, 2, and 3. ●, normalized product (the sum of di-, tri-, tetra-, penta-, hexa-, and heptasaccharides) concentration; ▲, normalized substrate concentration; ■, normalized total alginate saccharide concentration. Solid lines refer to the simulation results from Eqs. (10), (11), (12), and (13). See text.

lated to be 34.5, 48.0, and 77.9 min for runs 1, 2, and 3, respectively. The product concentration was in the range of 0.10–0.34 in all runs. The conversion of sodium alginate to alginate oligosaccharides (the sum of the di-, tri-, tetra-, penta-, and hexasaccharides) was determined to be 0.10, 0.20, and 0.34 at the stable state in runs 1, 2, and 3, respectively. As the mean residence time increased, conversion increased from 0.10 to 0.34. The highest conversion to alginate oligomer [0.34] was observed at the mean residence time of 77.9 min, which was evidently similar to the batch experiment [*ca.* 0.40].

Figure 5 shows the changes in concentrations of the substrate and product during the continuous operation for 60 h with time. The stable concentration of 0.35 was obtained after 3 h for the mean residence time of 77.9 min. This suggests that the present CSTR system is stable for the production of alginate oligosaccharides for the 60 h operation.

Figure 6 shows the relationship between the production rate (normalized production rate of total degradation product below the hexasaccharide, h^{-1}, calculated from the material balance) and dilution rate (h^{-1}) in runs 1, 2, and 3. The production rate was dependent on the dilution rate. This suggests that the present reaction may be limited by the substrate supply.

In general, to examine the sodium alginate degradation with immobilized enzyme, the activity of the enzyme and the inner diffusion of sodium alginate within the beads must be considered. Moreover, the changes in sodium alginate, degradation products, and enzyme concentration need to be taken into account. A very complicated model is required for analyzing these mass transfer-catalytic reactions. For the practical operation of such a reactor system, a simplified expression is desirable. In the present experiment, the dilution rate may be the limiting factor for controlling the degradation of sodium alginate.

Simulation The production rate (r) of alginate oligosaccharides in the batch experiment is reported to be expressed by the Michaelis-Menten type equation with inhibitory effect (Takeshita, 1994). In the present batch experiment, the r might be approximately assumed to be as follows from Fig. 3.

$$r=r_m\{S/(S+K_m)\}\{(P_c-P)/P_c\}, \quad (1)$$

where S is the substrate concentration in the reaction mixture (gC l^{-1}), P is the product (the sum of the di-, tri-, tetra-, penta-, and hexasaccharides) concentration (gC l^{-1}), r_m is the maximum production rate (gC l^{-1} hl^{-1}), K_m is the constant (gC l^{-1}), and P_c is the critical product concentration (gC l^{-1}).

Normalizing each parameter in Eq. (1) by the initial substrate concentration (S_0), the following equation is obtained.

$$C_r=C_{rm}\{C_s/(C_s+K_s)\}\{(C_{pc}-C_p)/C_{pc}\}, \quad (2)$$

where C_r is the normalized production rate (h^{-1}, r/S_0), C_{rm} is the normalized maximum production rate (h^{-1}), K_s is the constant (−), C_s is the normalized substrate concentration (−, $=S/S_0$), C_p is the normalized product concentration (−, P/S_0), and C_{pc} is the normalized critical product concentration (−).

In Eqs. (1) and (2), the K_m becomes equal to K_sS_0 when the K_s is independent of S. The value of K_sS_0 is dependent of S_0. In the present experiment, the relation $C_s \gg K_s$ was valid from the values of S_0[3.93 gC l^{-1}], K_s[1.5–6×10^{-3} (Muramatsu *et al.*, 1993)], and C_s[0.6–1.0]. The C_r value is then represented as follows.

$$C_r=C_{rm}\{(C_{pc}-C_p)/C_{pc}\}. \quad (3)$$

From integration of Eq. (3), the following equation is obtained.

$$(C_p-C_{pc})/(C_{p0}-C_{pc})=\exp\{-(C_{rm}/C_{pc})t\}, \quad (4)$$

where C_{p0} is the normalized initial product concentration (−).

The normalized concentration of the product produced by the reaction (C_p*) is expressed as follows.

$$C_p^*=C_p-C_{p0}=[1-\exp\{-(C_{rm}/C_{pc})t\}](C_{pc}-C_{p0}) \quad (5)$$

The values of C_{pc} and C_{rm} were determined to be 0.76 (−) and 0.39 (h^{-1}) from Fig. 3, where the calculated line denoted by the solid line agreed well with the experimental data.

In the continuous experiments, the solution in the reactor tank was fully mixed. The system, therefore, was simulated using the CSTR model.

The material balance for the substrate and product is given

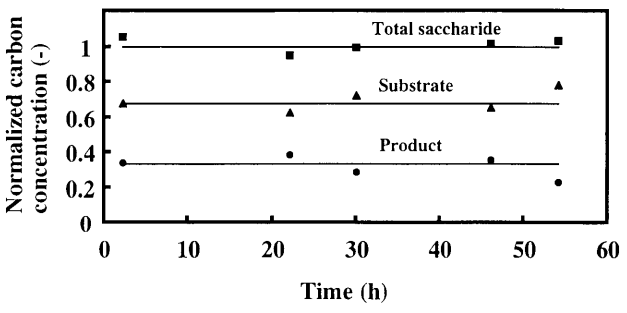

Fig. 5. Changes in concentrations of products from continuous degradation of 10 g l^{-1} sodium alginate solution with time for 60 h. Symbols are the same as those in Fig. 4. Solid lines refer the simulation result from Eqs. (10), (11), (12), and (13). See text.

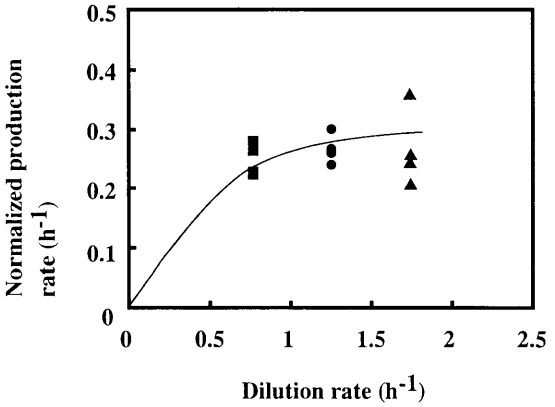

Fig. 6. Relationship between the normalized production rate and dilution rate in runs 1 (▲), 2 (●), and 3 (■). Solid line refers to the simulation result from Eq. (12). See text.

Table 1. Chemical composion of products during the continuous degradation of sodium alginate.

Degradation products	Feed solution (g l^{-1})	Concentration (g l^{-1})		
		Run 1	Run 2	Run 3
Disaccharide	ND[a]	0.024	0.034	0.098
Trisaccharide	ND	0.204	0.386	1.238
Tetrasaccharide	ND	0.436	0.668	1.398
Pentasaccharide	ND	0.208	0.308	0.526
Hexasaccharide	ND	0.168	0.218	0.234

[a]Not determined.
Each concentration is estimated on the basis of the saturated sodium glucuronate. The values for the products are averages obtained under a stable effluent in the CSTR system with immobilized enzyme.

Table 2. Comparision of productivities of different reactor systems for the degradation of sodium alginate.

Operating conditions	Reactor system			
	AB PFR	IE CSTR		
		Run 1	Run 2	Run 3
Temperature (°C)	30	35	35	35
pH	7.2	7.0	7.0	7.0
Substrate (g l^{-1})	2	10	10	10
Productivities (g l^{-1} h^{-1})	0.026–0.071	1.7	2.5	2.6
Conversion	0.013–0.038	0.10	0.20	0.34
References	Murata *et al.* (1992)	This study		

AB: aggregated bacteria, IE: immobilized enzyme, PFR: plug flow reactor, CSTR: continuous stirred tank reactor. Each conversion shows the concentration ratios of substrate to total oligosaccharide (the sum of di-, tri-, tetra-, penta-, and hexasaccharides).

as follows.

$$V(dS/dt)=F(S_0-S)-rV, \quad (6)$$

$$V(dP/dt)=rV-FP, \quad (7)$$

where V is the total working volume of the reactor (0.816 l), F is the feed rate (1 h^{-1}), and t is time (h).

Normalizing each parameter in Eqs. (6) and (7) by the initial substrate concentration (S_0), the following equations are obtained.

$$dC_s/dt=C_{fv}-C_r-C_{fv}C_s, \quad (8)$$

$$dC_p/dt=C_r-C_{fv}C_p, \quad (9)$$

where C_{fv} is the dilution rate (h^{-1}, $=F/V$).

From the experimental data in runs 1–3 shown in Fig. 4, the concentrations of C_s and C_p can be assumed to be approximately constant during the reaction. The values of dC_s/dt and dC_p/dt in Eqs. (8) and (9) are regarded to be zero, and the following equations are given.

$$C_s=(C_{fv}-C_r)/C_{fv}, \quad (10)$$

$$C_p=C_r/C_{fv}. \quad (11)$$

From Eqs. (3) and (11), C_r is given by

$$C_r=C_{rm}C_{fv}C_{pc}/(C_{rm}+C_{pc}C_{fv}). \quad (12)$$

Figure 6 shows the dependence of C_r on C_{fv}, where the simulation curve agreed with the experimental data.

The normalized total uronate concentration in the reaction mixture (C_t) is expressed as the sum of C_p and C_s.

$$C_t=C_p+C_s. \quad (13)$$

The initial experimental conditions of C_p and C_s were 0.10 and 0.90 (run 1), 0.22 and 0.78 (run 2), and 0.28 and 0.72 (run 3), respectively. The solid lines in Figs. 4 and 5 show the simulated results for C_p, C_s, and C_t. The simulated lines agreed well with the experimental results in the three different experiments (Fig. 4) and 60 h operation (Fig. 5). This suggests that the continuous degradation of sodium alginate can be predicted using the mathematical model described above.

Chemical composition The degradation products in the present study result in the elimination of sodium alginate, and are a mixture of oligomers which consists of α-L-guluronate and β-D-mannuronate. Table 1 shows the chemical composition of the products under the stable states in runs 1, 2 and 3. With decreasing dilution rate, the total oligosaccharide concentration increased. The concentration ratios of each oligosaccharide to total saccharide in run 3 (mean residence time 77.9 min) were almost the same as those in the 60 min batch experiment, being 0.01, 0.12, 0.14, 0.05, and 0.02 for the di-, tri-, tetra-, penta-, and hexasaccharides, respectively.

Productivity Table 2 summarizes the productivities of the different reactor systems. Murata *et al.* (1992) reported the productivity of 0.026–0.071 g l^{-1} h^{-1} for the PFR system with aggregated microbial bacteria. In the present study, the productivities of 1.7–2.6 g l^{-1} h^{-1} were obtained in runs 1–3. The volumetric productivities obtained in our study were significantly higher than those of Murata *et al.* (1992). This is due to the high concentrations of the substrate and enzyme. From these, the enzymatic degradation of sodium alginate by the CSTR system was found to have a higher productivity than the other reactor system. However, the conversion of 0.10–0.34 in our study might not be efficient from a practical point of view. Further investigation into the conversion is needed to improve the system for the effective degradation of sodium alginate.

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