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Production of Chitosan Oligosaccharides at High Concentration by Immobilized Chitosanase

Mei MING, Takashi KUROIWA, Sosaku ICHIKAWA*, Seigo SATO and Sukekuni MUKATAKA

Graduate School of Life and Environmental Sciences, University of Tsukuba, 1–1–1 Tennodai, Tsukuba, Ibaraki 305–8572, Japan

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The production of high concentrations of physiologically active pentameric and hexameric chitosan oligosaccharides was investigated. Chitosanase directly immobilized on an agar gel-coated multidisk impeller was used for the hydrolysis of chitosan. As hydrolysis proceeded, the concentration of chitosan was increased from the saturation concentration (20 kg/m^3) to 50 kg/m^3 by stepwise addition of chitosan powder with pH control. The timing of the addition of the chitosan powder was determined by monitoring the change in the torque required to agitate the reaction solution. The target products were produced more efficiently when the chitosan powder was added over a short interval rather than a long interval. When hydrolysis of a 50 kg/m^3 chitosan solution at 50℃ was repeated three times using the same immobilized enzyme, the target products were obtained in high concentrations (20 kg/m^3) from each of the three reactions with no reduction in the activity of the immobilized enzyme.

Keywords: chitosan, chitosan oligosaccharides, immobilized chitosanase, bioreactor

Introduction

The amino polysaccharide chitosan has received much attention as a functional biopolymer for diverse applications in food (Shahidi *et al.,* 1999), pharmaceuticals (Kumar, 2000), and cosmetics (Dodane and Vilivalam, 1998). Evidence is beginning to accumulate that chitosan oligosaccharides possess strong bactericidal and other biological activities (Qin *et al.,* 2002; Kittur *et al.,* 2003; Zheng and Zhu, 2003; Kumar *et al.,* 2004; Kuroiwa *et al.,* 2005). In particular, pentameric and hexameric chitosan oligosaccharides show antibacterial activity (Uchida *et al.,* 1989; Jeon and Kim, 2000; Jeon *et al.,* 2001), antitumor activity (Suzuki *et al.,* 1986; Tokoro *et al.,* 1988), and immunoenhancing effects (Hirano *et al.,* 1991). Therefore, there is much interest in the degradation of chitosan to chitosan oligosaccharides.

Chitosan oligosaccharides are generally produced by partial hydrolysis of chitosan, from which pentamers and hexamers are obtained as reaction intermediates. Hence, strict control of the hydrolysis reaction must be achieved to produce these oligosaccharides efficiently. Conventionally, chitosan oligosaccharides have been produced by acid hydrolysis (Horowitz *et al.,* 1957) at high temperatures under highly acidic conditions. Owing to difficulties in controlling the progress of the reaction, this method produces a large amount of glucosamine (chitosan monomer) and, therefore, low yields of pentamers and hexamers.

Enzymatic hydrolysis has some advantages for production of chitosan oligosaccharides in that some chitosanases can catalyze hydrolysis under mild conditions and do not produce glucosamine (Izume and Ohtakara, 1987; Fukamizo *et al.,* 1994). Furthermore, utilization of immobilized enzyme permits control of the progress of the hydrolysis reaction. High yields of chitosan oligosaccharides can be obtained because the immobilized enzyme can be removed from the reaction mixture when the yield of the target pentamers and hexamers reaches a maximum. Therefore, the use of immobilized enzyme is effective for the production of intermediates of hydrolysis reactions. Immobilization of chitosanase from *Bacillus pumilus* BN-262 on the surface of agar gel particles using the multipoint attachment method has been reported (Ichikawa *et al.,* 2002; Kuroiwa *et al.,* 2002). The immobilized enzyme is highly stable, and chitosan can be hydrolyzed continuously for a month in a packed-bed reactor. A total concentration of 7.0 kg/m^3 of pentamers and hexamers has been produced from a 20-kg/m^3 chitosan solution (Kuroiwa *et al.,* 2003).

Production of target substances at high concentrations saves time, reduces the cost of condensation of the substances, and increases productivity. A high concentration of product requires a high concentration of the substrate raw material. However, because chitosan solutions are highly viscous even at low concentrations, the use of a high-concentration chitosan solution during hydrolysis would limit the operability of a bioreactor. The saturation concentration of chitosan in 0.1 mol/L acetic acid solution at 35℃ and pH 5–6 is 20 kg/m^3, so chitosan solutions with concentrations higher than 20 kg/m^3 cannot be prepared directly. A method for the production of chitosan oligosaccharides, especially pentamers and hexamers, with immobilized enzyme and with chitosan solutions of greater concentration than 20 kg/m^3 has not yet been developed. In our previous paper (Ming *et al.,* 2006), we described the development of a bioreactor with an agar gel-coated multidisk impeller bearing direct-

* To whom correspondence should be addressed.
E-mail: sosakui@sakura.cc.tsukuba.ac.jp

ly immobilized chitosanase for the production of chitosan oligosaccharides. The chitosanase immobilized on the impeller was highly stable and produced pentamers and hexamers at a concentration of 9.0 kg/m^3 from a 20-kg/m^3 chitosan solution.

In this study, we investigated the use of the bioreactor described above to produce chitosan pentamers and hexamers at a higher concentration from a 50-kg/m^3 chitosan solution. The chitosan concentration was increased by stepwise addition of chitosan powder as the hydrolysis reaction proceeded. We also investigated an effective method for stepwise addition and determined the stability of the immobilized enzyme in the highly viscous solution by re-using the enzyme for repeated hydrolysis reactions.

Materials and Methods

Materials Chitosanase (EC 3.2.1.132) from *Bacillus pumilus* BN-262 was kindly supplied by Meiji Seika Kaisha (Tokyo, Japan). The final products of chitosan degradation by this endo-type enzyme are dimers and trimers of chitosan oligosaccharides (Fukamizo *et al.,* 1994). Chitosan (100% deacetylated, mean molecular weight 370,000) was purchased from Funakoshi (Tokyo, Japan). More details on these enzymes and chitosan are described in our previous paper (Ming *et al.,* 2006). All other chemicals were analytical or extra-pure grade.

Preparation of immobilized-enzyme reactor with agar gel-coated multidisk impeller The multidisk impeller consisted of five disks of stainless steel wire mesh (18 mesh; diameter, 50 mm) each supported by a plastic cross (thickness, 1 mm). The disks were coated with agar gel and the chitosanase was immobilized on the agar-coated disks as reported previously (Ming *et al.,* 2006).

A torque meter (SS-1R, Yamasaki, Kyoto, Japan) was attached to the shaft of the impeller, and a pH probe was inserted into the reactor. The impeller was driven by a variable-speed motor, and the acrylic reactor (diameter, 56 mm; height, 115 mm) was placed in a bath with a thermostat (Fig. 1).

Preparation of chitosan solutions Chitosan solutions with concentrations of 5 and 20 kg/m^3 were prepared as follows. Chitosan powder (1.5 or 6 g) was added to 100 mL of deionized water and dissolved with 70 mL of 1 mol/L lactic acid by stirring. The final pH was adjusted to 5.6 with 5 mol/L NaOH solution, and the volume of the solution was brought to 300 mL to afford either a 5- or 20-kg/m^3 chitosan solution. A 100-mL portion of the solution was used in each hydrolysis experiment to assay the activity of the immobilized enzyme and to produce oligosaccharides.

A 50-kg/m^3 chitosan solution was prepared by adding the chitosan powder directly to the reactor stepwise after the start of hydrolysis of the 20-kg/m^3 chitosan solution. One gram of chitosan powder was added each time. The timing of the addition was determined by monitoring the torque required to agitate the reaction solution. The pH of the starting 20-kg/m^3 chitosan solution was about 3.5 (that is, not adjusted to 5.6). Because the pH of the reaction solution increased with each addition of chitosan powder, 0.2 mL of lactic acid was also added with the powder when the pH of the reaction solution rose above ca. 4. Chitosan powder was added a total of three times, at which point the final chitosan concentration reached 50 kg/m^3.

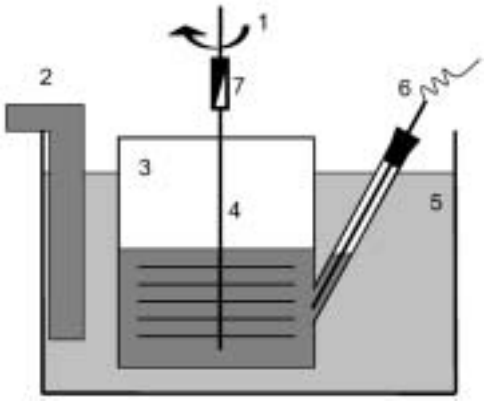

Fig. 1. Reactor equipped with a multidisk agar gel-coated impeller. 1: motor; 2: heater; 3: reactor; 4: impeller; 5: water bath; 6: pH probe; 7 torque meter.

Measurement of chitosanase activity The activity of the immobilized chitosanase was determined according to the method of Uchida and Ohtakara (1988). The concentration of reducing sugar was measured using the modified Schale's method (Imoto and Yagishita, 1971) with D-glucosamine (Sigma, St. Louis, MO, USA) as a reference compound. One unit of chitosanase activity, U, was defined as the amount of enzyme that produced 1 μmol of D-glucosamine equivalent in 1 min. The activity of the immobilized chitosanase is represented as the observed specific activity per unit surface area of the support, U/m^2.

Analysis of chitosan oligosaccharide concentrations The concentrations of chitosan oligosaccharides, from dimers to hexamers, were determined to within $\pm$ 5% by high-performance liquid chromatography (HPLC) using a CAPCELL PAK NH_2 column (Shiseido, Tokyo, Japan). The HPLC operating conditions have been described in the literature (Kuroiwa *et al.,* 2002).

Results and Discussion

Effect of pH on the activity of immobilized chitosanase In this study, the concentration of chitosan was increased by stepwise addition of chitosan powder as the hydrolysis reaction proceeded. When the chitosan powder dissolved in the reaction solution, the pH of the solution increased. Because the solubility of chitosan decreases with increasing pH, keeping the pH of the reaction solution low is preferable for solubilization of the added chitosan powder. However, the activity of the enzyme also depends on pH. Because both the chitosan solubility and the enzyme activity depend on pH, we set about determining how the pH range varied during the hydrolysis reaction.

Ichikawa *et al.* (2002) reported that the optimum pH for

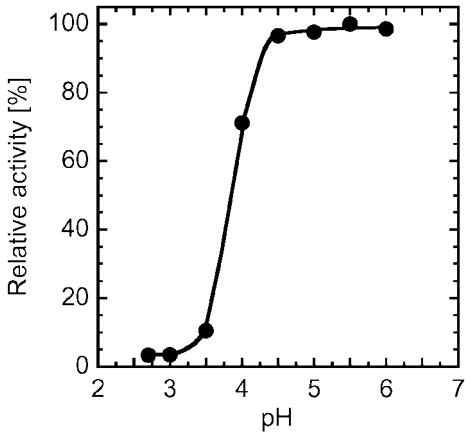

Fig. 2. pH dependence of activity of immobilized chitosanase. Temperature: 35℃; impeller speed: 2 s^{-1}; chitosan solution, 5 kg/m^3.

hydrolysis of chitosan by immobilized chitosanase on agar gel in acetic acid is pH 4–6, whereas the optimum pH for free chitosanase is pH 5.6. In this study, lactic acid was used to dissolve the chitosan, because it has higher chitosan solubility and is odorless. To determine the effect of pH on the activity of immobilized chitosanase for hydrolysis of chitosan dissolved in lactic acid, we investigated the activity of immobilized chitosanase at various pH values (Fig. 2). The results indicated that the activity of the immobilized chitosanase was higher at pH 4–6, as was the case for acetic acid.

To facilitate the dissolution of the chitosan powder, the pH of the reaction solution must be as low as possible in the range over which the hydrolysis reaction can proceed. On the basis of the data shown in Fig. 2, we decided that the pH of the solution could be reduced to 3.5, at which value the activity of the immobilized chitosanase was about one-tenth of the highest activity, at pH 4.5–6. By means of the following experiment, we confirmed that when the pH was raised again, the activity of the immobilized chitosanase could be recovered. The hydrolysis reaction was started at pH 5.6. After 40 min, the pH of the reaction solution was adjusted to 3.3 with lactic acid and kept there for 40 min. Then the pH was increased to 4.5 with 5 mol/L NaOH solution and allowed to remain there for 40 min. During the initial 40-min period, reducing sugar was produced rapidly (1.2×10^{-4} $kg/(m^3 s)$) because of the high activity of the immobilized enzyme at pH 5.6. After the pH was lowered to 3.3, the reaction rate became slower (1.9×10^{-5} $kg/(m^3 s)$) because the pH was not optimum for the activity of immobilized chitosanase. After the pH was increased from 3.3 to 4.5, however, the reaction rate recovered to the same level observed in the initial stage at pH 5.6.

Effect of addition timing in stepwise addition of chitosan powder on the production of pentamers and hexamers In a heterogeneous reaction system such as an immobilized enzyme reaction, the intensity of agitation is an important determinant of the reaction rate and mass transfer rate, especially in viscous solutions. The rates of both reaction and mass transfer affect the yield of chitosan oligosaccharides to a remarkable extent (Kuroiwa *et al.,* 2002). In our previous paper (Ming *et al.,* 2006), we reported the effect of impeller speed on the yield of chitosan oligosaccharides in the hydrolysis of 5- and 20 -kg/m^3 chitosan solutions using the same reactor as was used in this study. No significant increase in yield was obtained when the impeller speed was increased from 2 to 4 s^{-1}, although at slower impeller speeds the yield of target products did increase with increasing impeller speed (up to 2 s^{-1}). Because the chitosan solution used in this study was more concentrated and more viscous than the 20-kg/m^3 solution, the impeller speed was set at 4 s^{-1}. Impeller speeds higher than 4 s^{-1} caused the solution to rise along the impeller shaft, and then agitation of the solution became difficult.

Chitosan solution is highly viscous even at low concentrations, and the saturation concentration is about 20 kg/m^3 at pH 5–6 and 35℃ in lactic acid solution. Therefore, preparing a solution with a concentration exceeding 20 kg/m^3 in one step is difficult. In this study, stepwise addition of chitosan powder enabled the production of a solution of higher concentration, and the pH of the reaction solution was temporarily lowered to facilitate the dissolution of the added chitosan powder. The timing of powder addition was determined from the change in the torque necessary for agitating the reaction solution, because the torque reflects the viscosity change in the reaction solution: the viscosity increases as the chitosan powder dissolves, and then decreases as the chitosan is hydrolyzed.

To determine the appropriate timing for powder addition, we added chitosan powder consecutively by one of two methods: a long-interval method and a short-interval method. In the long-interval method, the powder was added when the torque decreased to half of the maximum value observed after the previous addition (Fig. 3a). In the short-interval method, the new powder was added when the torque reached its maximum after the previous addition (Fig. 3b). At the addition stage, the pH was adjusted to ca. 4 with lactic acid. When the final chitosan concentration reached 50 kg/m^3, the pH was adjusted to 4.5–5.0.

The temporal variation in the concentration of chitosan oligosaccharides produced by the two methods of stepwise addition is shown in Fig. 4. The maximum concentrations of the target pentamers and hexamers were 11 kg/m^3 for the long-interval method and 15 kg/m^3 for the short-interval method. In the long-interval method (Fig. 4a), a broad peak with lower pentamer and hexamer concentrations was obtained. In contrast, in the short-interval method, a narrower peak with a higher total concentration of the target products was obtained (Fig. 4 b). These results suggest that the molecular weight distribution of the substrate is important for production of the target products at a higher concentration. That is, because pentameric and hexameric chitosan oligosaccharides are intermediates of chitosan hydrolysis, the presence of substrates and products with widely different

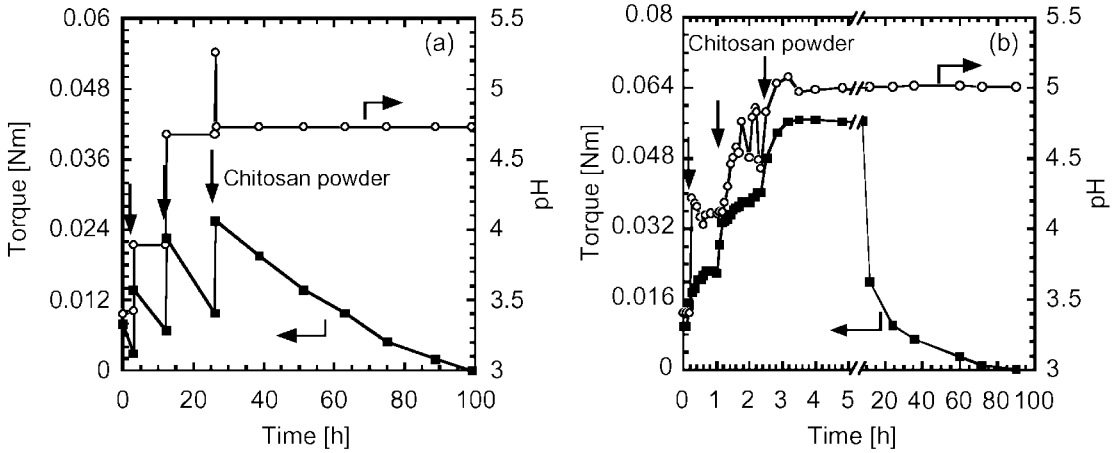

Fig. 3. Changes in pH (circles) and torque (squares) during chitosan hydrolysis by immobilized chitosanase. The chitosan concentration was increased from 20 kg/m^3 to 50 kg/m^3 by stepwise addition of chitosan powder three times by long-interval (a) and short-interval (b) methods. Arrows show the addition of chitosan powder. Observed specific activity: 307 U/m^2; temperature: 35℃; impeller speed: 4 s^{-1}.

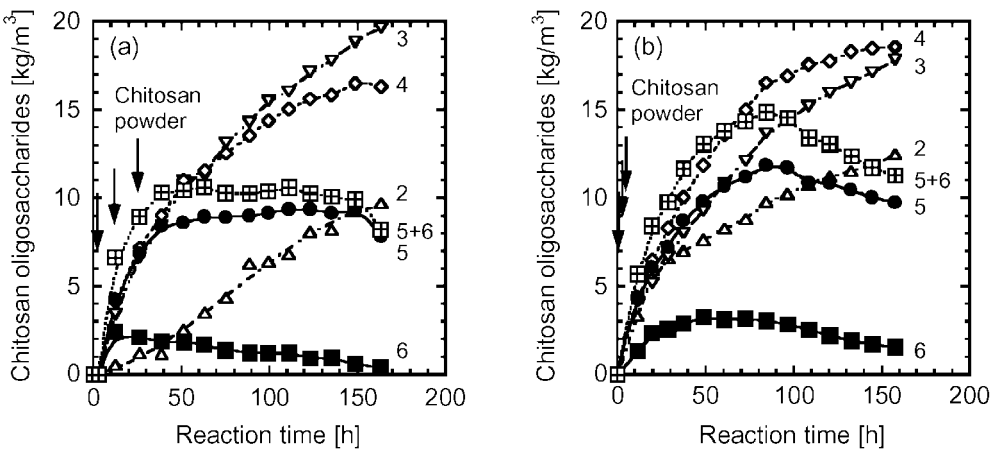

Fig. 4. Time courses of chitosan oligosaccharide concentrations during hydrolysis of a 50-kg/m^3 chitosan solution by chitosanase immobilized on an agar gel-coated multidisk impeller. Chitosan powder was added three times by (a) long-interval and (b) short-interval methods. The numbers next to the symbols correspond to the degree of polymerization of oligosaccharides. Observed specific activity: 307 U/m^2; temperature: 35℃; impeller speed: 4 s^{-1}.

hydrolysis histories lowers the yield of the target products. In the long-interval method, the distribution of molecular weights of chitosan in the reaction solution is wide, owing to delayed addition of native chitosan powder during the hydrolysis reaction. In contrast, in the short-interval method, the molecular weight distribution in the reaction mixture would be relatively narrow because the addition of chitosan powder was finished at a fairly early stage of the hydrolysis reaction. Therefore, the peak concentration of the intermediate products became sharp, and a higher maximum concentration was obtained with the short-interval method.

The effects of the addition method on the maximum concentration of pentamers and hexamers, $C_{(5+6)\max}$, obtained with immobilized chitosanases of different activities are summarized in Table 1. The value of $C_{(5+6)\max}$ for the short-interval method was higher than that for the long-interval method for each immobilized chitosanase of different activity. From the viewpoint of the yield of the target products, the short-interval method was more favorable than the long-interval method for the production of chitosan pentamers and hexamers.

Repeated production of chitosan pentamers and hexamers at high concentration To verify the stability and the re-usability of the bioreactor used in this study, we investigated repeated batch production of chitosan pentamers and hexamers using the same immobilized enzyme (three times). Generally, as the temperature increases, the viscosity of a solution decreases and the solubility of a solid substance increases. From the viewpoint of chitosan viscosity, solubility, and hydrolysis, a higher temperature is preferable to a higher concentration of chitosan. However, the activity and stability of enzymes also depend on temperature. The activity of chitosanase

Table 1. Comparison of maximum concentrations of pentamers and hexamers produced by long-interval and short-interval stepwise addition of chitosan powder.

Observed specific activity [U/m^2]	$C_{(5\text{-}6)max}$ [kg/m^3]	
	Long interval	Short interval
820	4.4	8.3
307	10.6	14.9

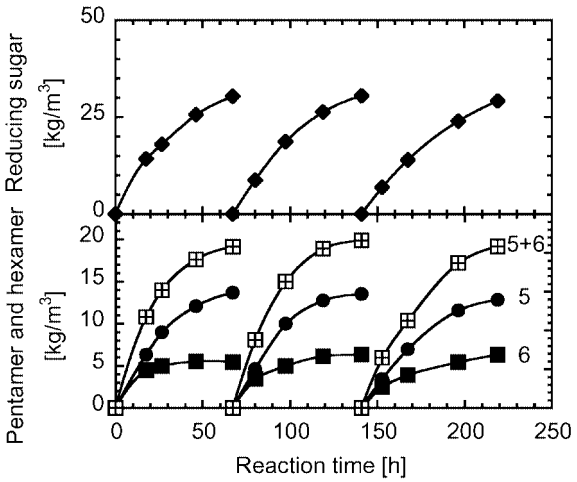

Fig. 5. Repeated hydrolysis of chitosan to produce chitosan pentamers and hexamers at high concentration using the same immobilized chitosanase. Observed specific activity: 209 U/m^2; temperature: 50℃; impeller speed: 4 s^{-1}.

immobilized on agar gel by the multipoint attachment method is almost completely preserved even after incubation for 250 h at 50℃ (Ichikawa *et al.,* 2002), whereas the activity decreases to about 75% of the initial value after incubation for 200 h at 55℃ (data not shown). On the basis of these data, we set the reaction temperature at 50℃. The substrate concentration was increased to 50 kg/m^3 by the short-interval stepwise addition method (Fig. 3b). When the concentration of pentamers and hexamers reached a plateau, the impeller and the reactor were washed, and then the next reaction was started. As shown in Fig. 5, the time courses of the concentrations of reducing sugars and the target oligosaccharides were almost identical in the three batch reactions. This result shows that the chitosanase immobilized on the agar gel-coated multidisk impeller was highly stable during the reaction at 50℃ in a highly viscous solution. The maximum concentration of pentamers and hexamers was about 20 kg/m^3 (a 40% yield with respect to the amount of substrate used). To our knowledge, there have been no previous reports of the production of pentamers and hexamers at concentrations higher than 9.5 kg/m^3 using free enzyme (Ming *et al.,* 2006). Thus, the bioreactor presented here has great advantages in terms of both stability and yield of the target products at high concentrations. The reactor should be useful for the practical production of physiologically active chitosan oligosaccharides. We believe that the production method developed in this study will find application in industry.

Conclusion

Chitosan pentamers and hexamers were produced at high concentration in a bioreactor using chitosanase immobilized on an agar gel-coated multidisk impeller. The optimum pH for the immobilized chitosanase was 4.5–6.0, and over this pH range, the highest activity could be recovered even after exposure of the enzyme to a lower-pH environment (3.3–4.0). The concentration of chitosan could be increased to 50 kg/m^3 by stepwise addition of chitosan powder into the reaction mixture, with control of the reaction pH. The timing of the addition of chitosan powder significantly affected the yield of the target products. Long- and short-interval methods were tested, and the latter was more favorable for efficient production of pentamers and hexamers. Finally, batch reactions with 50-kg/m^3 chitosan solutions were carried out three times using the same immobilized enzyme. No decrease in activity was observed over the course of the repeated reactions. The maximum concentration of pentamers and hexamers was 20 kg/m^3 (a 40% yield with respect to the amount of substrate used) in each batch reaction. This concentration is substantially higher than that obtained with previous methods (Ming *et al.,* 2006).

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