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Levan production using *Bacillus subtilis* natto cells immobilized on alginate

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ABSTRACT

The immobilization of *B. subtilis* natto Takahashi on alginate matrix was attempted for repeated production of levan. Factors that influence the stability of the immobilized-cell beads and the levan production in the process of fermentation were also investigated. The levan production and the stability of immobilized beads were greatly affected by the sucrose concentration, medium pH, metal ions and agitation speed. When the immobilized cells were cultivated in the optimal conditions, high levan production (70.6 g/L) was obtained after 3 d of fermentation and no damaged beads were observed. In the repetitive cultivation, the supplement of organic nitrogen source and control of initial pH was critical for high levan production. Immobilized cells on alginate beads were reused in five successive reaction cycles (each cycle 72 h) without any loss of biocatalytic activity and 9 cycles with 72% residual activity.

1. Introduction

Levan, a β-(2 → 6) fructan biopolymer with occasional β-(2 → 1) branching, was found in many plants and microbial products (Han, 1990). Microbial levan is of commercial importance, which offers a variety of industrial applications in the fields of cosmetics, foods and pharmaceuticals; it can be used as industrial gums, blood plasma extender, sweeteners, hypocholesterolemic agent and antitumor agent (Leibovici & Stark, 1985; Yamamoto et al., 2000). Potential applications of levan have also been proposed as an emulsifier, formulation aid, stabilizer and thickener, surface-finishing agent, encapsulating agent, and carrier for flavor and fragrances (Han, 1990; Jang et al., 2001).

Microbial levans are produced from sucrose-based substrate by transfructosylation reaction of levansucrase (beta-2,6 fructan:D-glucose-fructosyl transferase, EC 2.4.1.10) by a variety of microorganisms (Corrigan & Robyt, 1979; Dedonder, 1966; Hestrin, Avineri-Shapiro, & Aschner, 1943; Kennedy, Stevenson, & White, 1989; Shimamura et al., 1987; Tanaka, Susumu, & Yamamoto, 1979). Although many investigations on the levan production have been reported, all suffer the disadvantages of low yield and contaminating of impure products. In recent years, strategies to improve the yield of levan production by microorganisms attracted greater attention (Melo, Pimmentel, Lopes, & Calazans, 2007; Muro, Rodríguez, Abate, & Siñeriz, 2000; Rhee et al., 2002). In our previous reports (Shih & Yu, 2005; Shih, Yu, Shieh, & Hsieh, 2005) we have found that *Bacillus subtilis* (natto) Takahashi, a commercial natto starter, was able to selectively produce levan in sucrose medium. In addition, it is the most efficient levan producing strain reported to date; it produced the highest amount of levan in less time (21 h) under the same cultivation condition. *B. subtilis* (natto) Takahashi can accumulate 30–50 g/L levan during batch fermentation.

For industrial application, immobilization of biocatalysts such as enzymes or living cells on inert supports is a very appealing approach because it offers several advantages over free-cell fermentation in that it can facilitate product isolation and biocatalyst reutilization. Moreover, immobilized system based on living cells has active metabolic ability to synthesize complicated bioproducts using the multi-enzyme catalysis. In addition, this approach may help to enhance catalytic activity and prolong catalytic life of the biocatalysts (Vignoli, Celligoi, & Silva, 2006). Because of its economical potential, the immobilization of whole growing cells by techniques of encapsulation, entrapment in polymer gels and adhesion onto the surface of carriers has been applied for valuable bioproduct productions (Aykut, Hasirci, & Alaeddinoglu, 1988; Mozes & Rouxhet, 1984). Numerous immobilization supports and matrices have been used for the immobilization of biocatalysts (Furusaki & Seki, 1992). However, because of the mild conditions for immobilization and its simplicity, calcium alginate was the most common immobilization matrix used nowadays and it has been used in various whole-cell immobilizations for bioproduct productions (Gacesa, 1988; Gough, Barron, Zubov, Lozinsky, & McHale, 1998; Prasad & Mishra, 1995).

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Production of levan by immobilization of levansucrase from *Zymomonas mobilis* and *B. subtilis* on different immobilization matrixes using different methods, like ionic binding, covalent binding, cross linking and matrix entrapment have been well documented in the literature (Chambert & Petit-Glatorn, 1993; Iizuka, Yamaguchi, Ono, & Minamiura, 1993; Marx, Koning, & Hartmeier, 1999; Parlot & Mansen, 1984). It was found that levansucrase immobilized on calcium alginate gel strongly increased its polymerase activity, in addition to the highest stability and immobilization efficiency (Esawy, Mahmoudl, & Fattah, 2008). However, investigation of levan production by whole-cell immobilization was scarce (Berkers et al., 2001). In this study, the immobilization of *B. subtilis* natto Takahashi, a commercial natto spore, on alginate matrix was attempted for repeated production of levan. Factors that influence the stability of the immobilized-cell beads and the levan production in the process of fermentation were also investigated.

2. Materials and methods

2.1. Reagents, microorganisms and culture medium

B. subtilis (natto) Takahashi was obtained from Gem Cultures (Ft Bragg, CA, USA) or Takahashi Yuzo research facility Japan. Nutrient agar (NA) and nutrient broth (NB) composed of beef extract (3 g/L), peptone (1.5 g/L), and NaCl (5 g/L), pH 7.4 were purchased from DIFCO Laboratories Michigan, USA. Culture medium (SM medium) composed of sucrose (200 g/L), $MgSO_4{\cdot}7H_2O$ (0.5 g/L), $NaH_2PO_4{\cdot}2H_2O$ (3 g/L), $Na_2HPO_4{\cdot}12H_2O$ (3 g/L) were used for levan production. $MgSO_4{\cdot}7H_2O$, $NaH_2PO_4{\cdot}2H_2O$, $Na_2HPO_4{\cdot}12H_2O$, $CaCl_2$, and $AlCl_3{\cdot}6H_2O$ were obtained from Sigma Chemical, USA. All other reagents used were of the highest grade available unless otherwise indicated.

2.2. Immobilization of B. subtilis (natto) Takahashi cells in Ca-alginate gel beads

For immobilization, the powder of *B. subtilis* (natto) Takahashi cells (approximately 3 g of cells, dry wt.) were suspended with 10 mL of sterilized distilled water and mixed thoroughly with 200 mL of 3% (w/v) sterilized sodium alginate solution. After proper mixing, the suspension was dropped by a pipetter into 2% $CaCl_2$ solution. The Ca-alginate gel beads were washed to remove the excess of $CaCl_2$ solution. The mean diameter of beads obtained was about 0.33 mm.

2.3. Conditions for batch and repeated batch fermentation

The immobilized cells were inoculated into 1500 mL of NB composed of beef extract (3 g/L), peptone (1.5 g/L), and NaCl (5 g/L), pH 7.4 in a 2 L sterilized serum flask, and incubated at 37 °C for 24 h with shaking at 100 rpm. After activation, 10 g of the immobilized cells were incubated with 300 mL SM medium into 500 mL Erlenmeyer flasks at 37 °C, pH 5.8 for 80 h at a gentle agitation of 100 rpm. Repeated batch fermentation was conducted in fermentation run up to 80 h/batch. At the end of each fermentation run, the gel beads collected from fermentation broth were washed with sterile water and transferred to the fresh medium. This procedure was continued for runs in this experiment.

2.4. Factors affecting on the levan production and stabilities of alginate-immobilized cells

The immobilized cells were inoculated into NB and incubated with SM medium as described above. In order to determine the effect of sucrose concentration on levan production by Ca-alginate immobilized *B. subtilis* (natto) cells, sucrose concentrations in SM were varied at 0, 50, 100, 150, 200, and 250 g/L. To test the effects of metal ions for enhancing the strength of alginate beads, immobilized cells were incubated with SM (200 g/L sucrose) supplemented with 0.2% of various metal ions (Al^{3+}, Ba^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+}) and cultivated at 37 °C, pH 5.8 for 80 h at a gentle agitation of 100 rpm. The effect of initial pH was studied by conducting fermentation at various initial pH of 3.0, 5.0, 6.0, 7.0, 9.0 and 11.0 adjusted with 0.2 M sodium hydroxide, and cultivated at 37 °C, for 80 h at a gentle agitation of 100 rpm.

2.5. Analytical methods

Levan in the culture supernatant was precipitated using 75 vol% of ethanol and the concentration was determined as fructose units after hydrolysis in 0.1N HCl at 100 °C for 2 h (Viikari & Gisler, 1986). The number-average molecular weight (M_n) of the levan was measured by gel permeation chromatography (GPC) system (Hitachi L6200 series, Japan) on a series of TSK gel G5000PWXL and TSK gel G4000PWXL columns (Toso Haas, Tokyo, Japan) and a refractive index (RI) detector (Bischoff, Model 8110) with de-ionized water as an eluent. The flow rate was set at 1.0 mL/min and the column oven was at 50 °C (Shih & Yu, 2005). ^1H NMR and ^{13}C NMR spectroscopy was performed with a Varain Unity Inova 600 spectrometer. Samples for NMR were dissolved in D_2O solution. For sugar analysis, fermentation samples were filtered through a 0.2 μm filter, the concentration of sugars (sucrose, glucose, and fructose) were measured by HPLC using a Hitachi L6200 system controller equipped with Spherclone 5 μ KS-802 300 × 8.0 mm, a refractive index (RI) detector (Bischoff, Model 8110). The flow rate was set at 0.5 mL/min and the injection volume was 20 μL.

3. Results and discussion

3.1. Effects of sucrose concentrations on the levan production by alginate-immobilized cells

The effect of sucrose concentration varied at 0, 50, 100, 150, 200, and 250 g/L on levan production by Ca-alginate immobilized *B. subtilis* (natto) cells were studied. The time course of levan production and sugar concentration during fermentation is shown in Fig. 1. It was previously shown that in the free-cell cultivation, the maximum levan productivity (49.4 g/L) occurred after 21 h cultivation in the medium containing 200 g/L of sucrose, while the yield decreased at higher and lower sucrose concentration (Shih & Yu, 2005). In the immobilized-cell cultivation, there was a lag for the maximum levan production, which appeared after 36 h of cultivation. The levan concentration increased with the increase of sucrose concentration, which reached the highest (86.3 g/L) when the sucrose concentration was 200 g/L. It was previously shown that the highest production of levan obtained by suspended free-cell fermentation for various levan-production strains was 50–60 g/L (about 20–25% yield on available sucrose) for *B. subtilis*, 36 g/L (24% on available sucrose) for *B. polymyxa* (NRRL B-18475), 50 g/L (23% on available sucrose) for *Z. mobilis* and 15 g/L (30% on available sucrose) for *Erwinia herbicola*, respectively (Han & Clarke, 1990; Keith et al., 1991; Shih & Yu, 2005). The highest production of levan, 86.3 g/L (about 43% yield on available sucrose), obtained by fermentation of Ca-alginate immobilized *B. subtilis* (natto) cells was much improved than that obtained by fermentation of suspended free-cell. In addition, the levan-production rate for the suspended free-cell fermentation of *B. subtilis* (natto) was 50–60 g/L/d, which was much higher than those of other levan-producing strains; they are 3.6 g/L/d for *B. polymyxa*, 5.0 g/L/d for

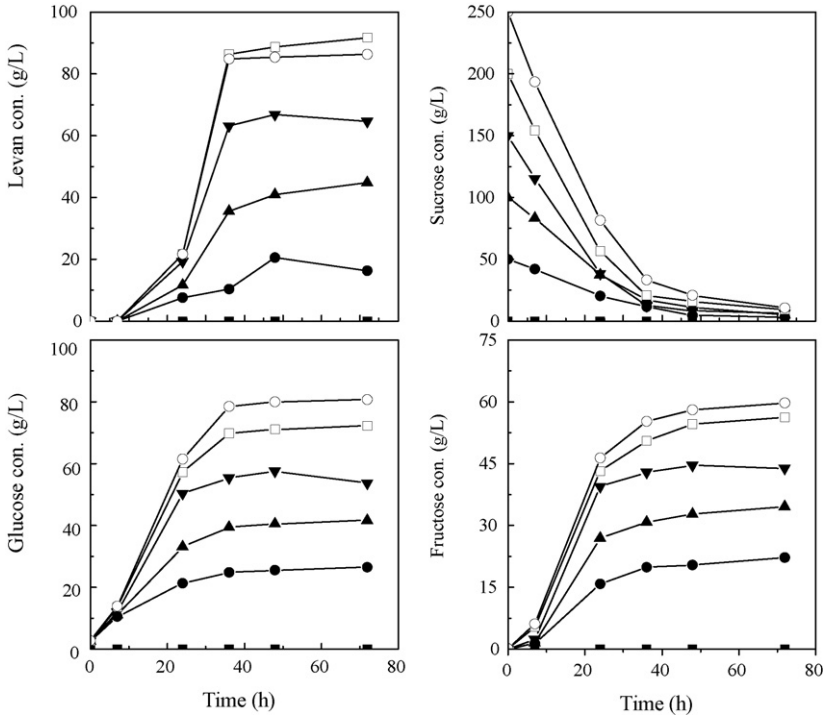

Fig. 1. The time course of levan production and sugar concentration by fermentation of alginate-immobilized *B. subtilis natto* in medium containing various sucrose concentrations. Sucrose concentration (■) 0 g/L; (●) 50 g/L; (▲) 100 g/L; (▼) 150 g/L; (□) 200 g/L; (○) 250 g/L.

E. herbicola and 5–6 g/L/d for *Z. mobilis*, respectively. In contrast, the levan-production rate for the Ca-alginate immobilized *B. subtilis* (natto) was 57.5 g/L/d, which is comparable to that of free-cell fermentation.

The Gel-Permeation chromatograms (GPC) of levan product from Ca-alginate immobilized *B. subtilis* (natto) in fermentation medium containing various sucrose concentrations are shown in Fig. 2. It was shown that two peaks, one has molecular weight greater than 2000 kDa and the other has molecular weight around 6–9 kDa, were always obtained regardless the sucrose concentration used. This is consistent with the previous results obtained for the free-cell fermentation of *B. subtilis* Takahashi in shake flask and fermenter (Shih & Yu, 2005), and for the levansucrase catalyzed formation of levans (Euzenat, Guibert, & Combes, 1997), in that high and low molecular weight levans appeared simultaneously during sucrose consumption. The dual molecular-weight of levan products produced by *B. subtilis* Takahashi in free-cell and immobilized-cell fermentation was rather characteristic; however, the mechanism by which these two different molecular-weight products were formed is still unknown. The potential applications of the high and low molecular weight levans were well documented in the literature and it was shown that levan with different molecular weight was needed for different purposes (Calazans, Lima, de Franca, & Lopes, 2000; Leibovici & Stark, 1985; Schechter & Hestrin, 1963). Therefore, fractionation of levans of low and high molecular weight is necessary. Previously, successful fractionation of levans using an ethanol gradient and membrane ultrafiltration has been demonstrated (Chen, 2009; Shih & Yu, 2005). In addition, the pure levan products produced in this study were also characterized by ^{1}H NMR, ^{13}C NMR. The ^{1}H NMR and ^{13}C NMR spectra show peaks identical to those of authentic samples previously reported (Han, 1990; Shih & Yu, 2005), indicating that the polysaccharide produced was levan type with the linkage of β(2→6) fructofuranoside.

3.2. Effects of metal ions on the levan production and stabilities of alginate immobilized cells

In the previous experiments, it is noticed that the immobilized beads were damaged or disintegrated after 72 h of cultivation. In order to have repetitive use of the immobilized cells, preventing deformation and maintaining the integrity of the beads are critical. It was shown previously that calcium alginate gel can be stabilized with simple treatment with trivalent cations, gel strength can be increased by a factor of two after washing with 0.1 M aluminum nitrate without significant loss of ability for cell immobilization (Rochefort, Rehg, & Chau, 1986). In addition, stronger alginate matrix was demonstrated by adding $CaCO_3$ into fermentation medium that results in higher calcium levels (Morin, Bernier-Cardou, & Champagne, 1992). Furthermore, in the study of the operational stability of the immobilized packed-bed bioreactor for ethanol production, $CaCl_2$ solution (2%) was passed through the column occasionally to prevent disruption and maintain the mechanical structure of Ca-alginate beads before increasing the sugar concentration of the feed (Göksungur & Zorlu, 2001).

The effects of metal ions (Ba^{2+}, Ca^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+}) at 0.2% were studied for enhancing the strength of alginate beads. The results showed that in the medium supplemented with Ba^{2+}, Ca^{2+}, and Mg^{2+}, the maximum levan production reached after 72 h of cultivation; they are 6.7 g/L, 74.4 g/L and 79.0 g/L, respectively. In contrast, in the medium supplemented with Al^{3+}, Fe^{2+} and Fe^{3+}, the levan production was completely inhibited in that no leven production was observed within 80 h of cultivation. However, severely damaged and disintegrated beads were observed after 72 h of fermentation in medium supplemented with Ba^{2+}, Mg^{2+}, Fe^{2+} and Fe^{3+}. Partially damaged and totally intact beads were observed after 72 h of fermentation in medium supplemented with Ca^{2+}, Al^{3+}, respectively. The stabilities of beads enhanced by addition of Al^{3+} in the

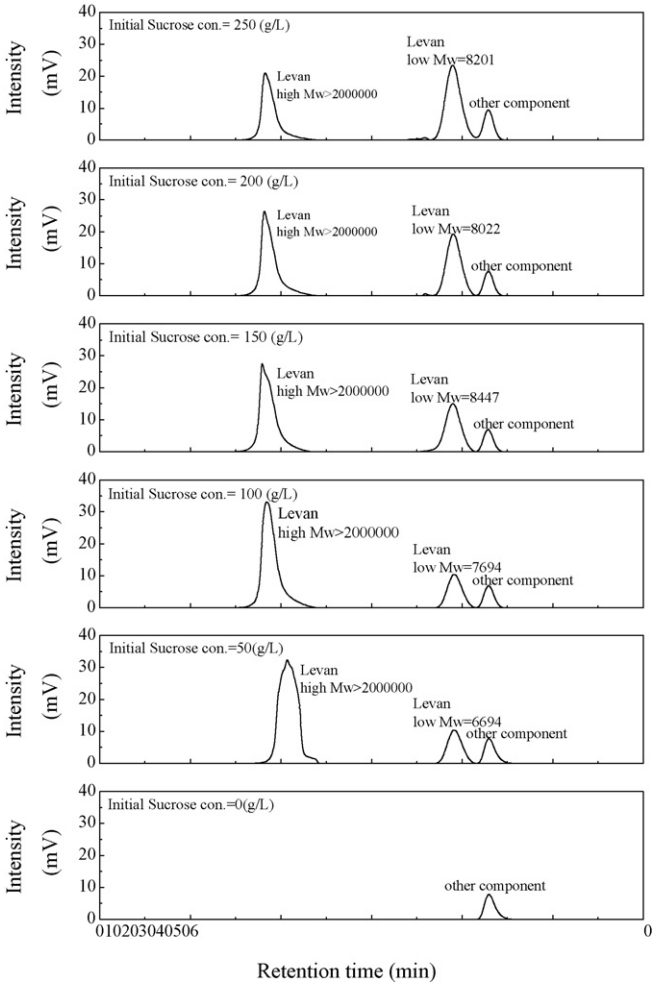

Fig. 2. GPC chromatogram of levan products from Ca-alginate immobilized *B. subtilis* (natto) in fermentation medium containing various sucrose concentrations.

medium were significant, although the levan production was completely diminished for the reason still unknown.

To further study the effects Al^{3+}on the stability and levan production of immobilized cells, various concentrations of Al^{3+} (0%, 0.01%, 0.03%, 0.05%, 0.1% and 0.2%) were supplemented with SM medium in addition of 0.2% of Ca^{2+}. Fig. 3 shows the levan production under the influence of various Al^{3+} concentrations. The results showed that when the Al^{3+} concentration was less than 0.1%, the maximum levan production reached after 72 h of cultivation; they

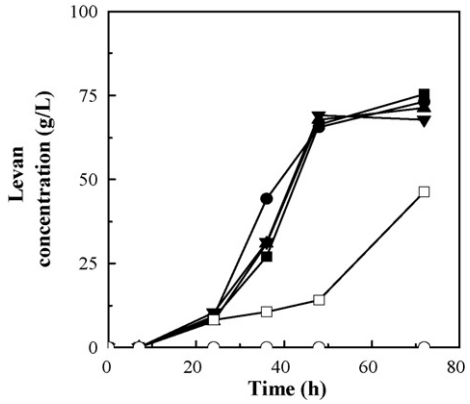

Fig. 3. The levan production by Alginate-immobilized *B. subtilis* (natto) in fermentation medium supplemented with various concentration of Al^{3+}. Al^{3+}concentration (■) 0%; (●) 0.01%; (▲) 0.03%; (▼) 0.05%; (□) 0.1%; (○) 0.2%.

are 75.4 g/L, 73.1 g/L, 71.3 g/L and 67.7 g/L when Al^{3+} concentration was 0%, 0.01%, 0.03% and 0.05%, respectively. When the Al^{3+} concentration was 0.1%, the levan production was partially inhibited and the maximum levan production after 72 h of cultivation was 46.3 g/L. In contrast, the levan production was completely inhibited when Al^{3+} concentration was higher than 0.2%, in that no levan production was ever observed within 80 h of cultivation. On the other hand, severely damaged and disintegrated beads were observed after 72 h of fermentation when Al^{3+} concentration was less than 0.1%; however, totally intact beads were observed after 72 h of fermentation when the Al^{3+} concentration beyond 0.1% was added. The supplement of Al^{3+} concentration higher than 0.1% has greatly enhanced the stability of beads, which was compromised by the reduction of levan production. It was also noticed that the addition of metal ions has made no difference to the molecular weight of levan products, in that two peaks, one has molecular weight greater than 2000 kDa and the other has molecular weight around 6–9 kDa, were always obtained regardless the metal ion used, a result similar to what was described in the previous section.

3.3. Effects of initial pH and agitation speed on the levan production by alginate immobilized cells

The effect of various initial pH on the levan production of the immobilized *B. subtilis* (natto) Takahashi during the batch fermentation is illustrated in Fig. 4(A). The results showed that the levan production by alginate-immobilized *B. subtilis* (natto) was high in a neutral environment with an initial pH in the region of 5.0–7.0, generally 65–80 g/L of levan was produced after 72 h of cultivation.

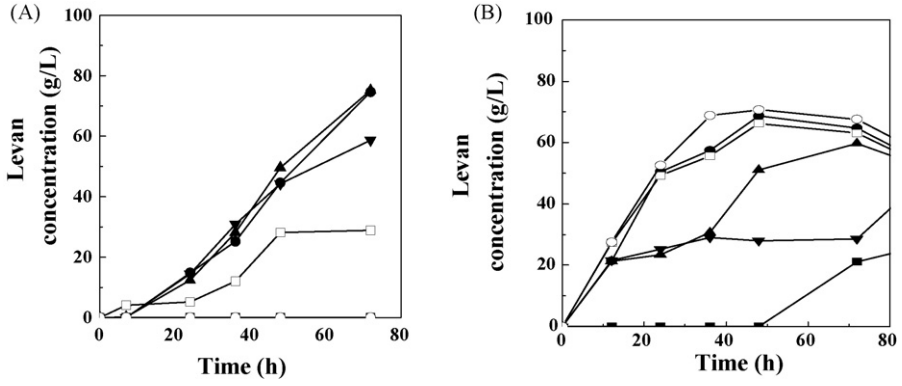

Fig. 4. The time course of levan production by alginate-immobilized *B. subtilis* natto at (A). Various initial pH (B) various shaking speeds. Initial pH (■) 3.0; (●) 5.0; (▲) 6.0; (▼) 7.0; (□) 9.0; (○) 11.0. Shaking speed (rpm) (■) 0; (□) 100; (●) 125; (○) 150; (▲)175; (▼) 225.

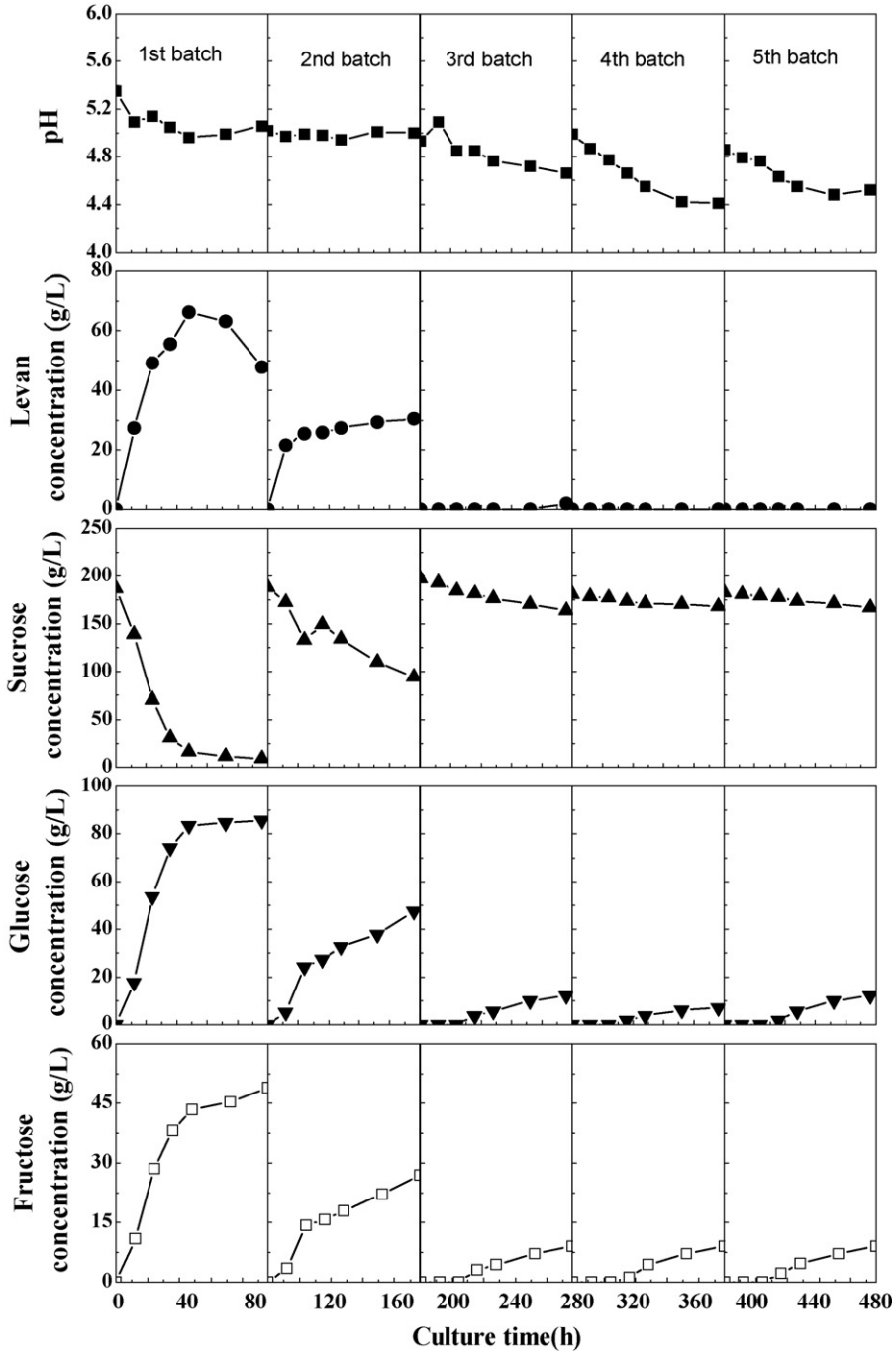

Fig. 5. The time course of levan production by immobilized cells during repeat-batch fermentation without pH control.

An environment, which is too acidic and alkaline, is not conducive for levan production. The levan concentration was only 22.4 g/L when the initial pH was 9.0, it was completely diminished when the initial pH was 3 and 11, and this is probably due to the fact that the extreme initial pH brought too much stress on the organism metabolic abilities (Göksungur & Guvenc, 1997). These results are similar to what was observed for free-cell fermentation previously reported (Chen, 2009), although immobilized cells are often proved to be more stable to changes in their surroundings than free cells (Göksungur & Guvenc, 1997). When the initial pH was in the region of 3.0–6.0, the beads remained intact; beyond that range, severely damaged and disintegrated beads were observed after 72 h of fermentation.

In Fig. 4(B), the effect of various shaking speeds on the levan production of the immobilized *B. subtilis* (natto) Takahashi during the batch fermentation is illustrated. The results showed that the levan production by alginate-immobilized *B. subtilis* (natto) was higher in shake culture than in static culture; the maximum levan production was 66.2 g/L, 68.7 g/L, 70.6 g/L in 48 h and 59.7 g/L in 72 h for shaking speed at 100 rpm, 125 rpm, 150 rpm and 175 rpm, respectively; in contrast, the maximum levan production lagged behind and only 28.8 g/L was obtained in static culture. It should be noticed that the beads remained intact during the process of cultivation when the shaking speed was in the region of 0–150 rpm, while severely damaged beads were observed at 225 rpm. It was again observed that the variation of initial pH and agitation

speed had no influence on the dual molecular weight of levan products.

3.4. Repeated batch fermentation of alginate immobilized cells

The importance of cell immobilization on solid materials lies in the fact that the biocatalysts thus generated can be easily separated from the reaction mixture for reuse if the immobilized cells are sufficiently stable. In this work, repeated batch cultivation was conducted in the optimal medium contents (sucrose: 200 g/L, $MgSO_4 \cdot 7H_2O$: 0.5 g/L, $NaH_2PO_4 \cdot 2H_2O$: 3 g/L, $Na_2HPO_4 \cdot 12H_2O$: 3 g/L, $CaCl_2$ 0.2%, $AlCl_3 \cdot 6H_2O$ 0.1%) and the optimal culture condition (pH 5.6–5.8, 37%, 150 rpm) obtained from the above studies, and the results are shown in Fig. 5. It was seen in the first batch of fermentation that the pH of the medium was lower than the initial pH while the sucrose was consumed in the process of cultivation, which was accompanied by the production of levan and the accumulation of glucose; the maximum levan production (66.2 g/L) was observed after 48 h of fermentation. In the second batch of fermentation, the initial pH dropped immediately as soon as the beads recovered from the first batch were transferred into the 2nd batch medium, this is probably due to the fact that low pH medium at the end of first fermentation was partly carried over by the beads into the 2nd batch medium, that led to the drop of initial pH. The same phenomena were seen in every batch of the repeated fermentation. It was also observed that the sucrose consumption accompanied by the production of levan and the accumulation of glucose in the process of 2nd batch cultivation was dramatically reduced; the maximum levan production observed was only 30.4 g/L. Furthermore, the sucrose consumption accompanied by the production of levan and the accumulation of glucose was completely diminished from the 3rd batch of fermentation afterward. The drop in the 2nd batch and the completely diminished from the 3rd batch of levan production and sucrose consumption is likely to be attributed to the lack of nitrogen supply in the medium which composed only of sucrose and salts (sucrose: 200 g/L, $MgSO_4 \cdot 7H_2O$: 0.5 g/L, $NaH_2PO_4 \cdot 2H_2O$:3 g/L, $Na_2HPO_4 \cdot 12H_2O$:3 g/L, $CaCl_2$ 0.2%, $AlCl_3 \cdot 6H_2O$ 0.1%). The limited nitrogen supply in the 1st batch of fermentation is likely to be contributed from the carry-over of the immobilized beads which were pre-incubated in nutrient broth (NB) composed of beef extract (3 g/L), peptone (1.5 g/L), and NaCl (5 g/L) before they were transferred into levan-production medium. This is consistent with the facts that limited nitrogen is a critical factor for high polysaccharide production in that high polysaccharide was obtained when bacterial cells were supplemented with an abundant carbon source and a limited nitrogen source (Lee & Lee, 2001; Saudagar & Singhal, 2004). Therefore, it is conceivable that with the control of the initial pH and the supplement of needed nitrogen will revive levan production. In fact, as seen in Fig. 6A and B, the levan production revived to a small extent when the initial pH was controlled at 5.6–5.8 for every batch after the immobilized beads were transferred from the previous batch. In addition, the levan production has resumed to a full extent when NB powder was supplemented in the concentration of 0.8 g/L to the medium of each batch from the 2nd batch afterward. Immobilized cells on alginate beads were reused in five successive reaction cycles (each cycle 72 h) without any loss of biocatalytic activity (Fig. 6C) and 9 cycles with 72% residual activity (data not shown).

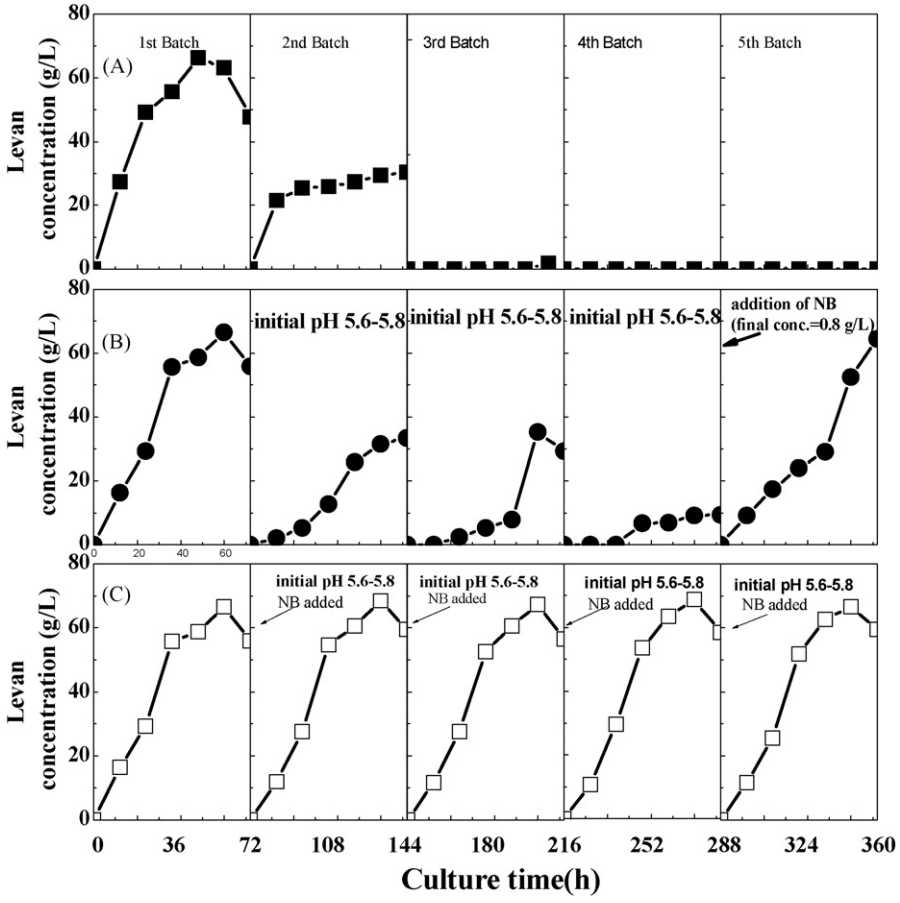

Fig. 6. Levan production by immobilized cells in repeat-batch fermentation. (A) Without initial pH control and no addition of nitrogen source; (B) with initial pH control and no addition of nitrogen source; (C) with initial pH control and addition of nitrogen source.

4. Conclusions

In this study, successful immobilization of *B. subtilis* natto Takahashi, a commercial natto spore, on alginate matrix for repeated production of levan was demonstrated. The levan production and the stability of immobilized beads were greatly affected by the sucrose concentration, medium pH, metal ions and agitation speed. Using immobilized *B. subtilis* (natto) and cultivation in the optimal medium (sucrose: 200 g/L, $MgSO_4 \cdot 7H_2O$: 0.5 g/L, $NaH_2PO_4 \cdot 2H_2O$: 3 g/L, $Na_2HPO_4 \cdot 12H_2O$:3 g/L, $CaCl_2$ 0.2%, $AlCl_3 \cdot 6H_2O$ 0.1%) at optimal pH (5.6–5.8), temperature (37 °C) and agitation speed (150 rpm), high levan production (70.6 g/L) was obtained after 3 d of fermentation, which is significantly higher than by fermentation using free cell. In addition, no damaged and disintegrated beads were observed in the fermentation process. In the repetitive cultivation, the supplement of organic nitrogen source and control of initial pH were critical for high levan production. Immobilized cells on alginate beads were reused in five successive reaction cycles (each cycle 72 h) without any loss of biocatalytic activity and 9 cycles with 72% residual activity. The immobilized *B. subtilis* natto Takahashi has the advantages of high levan production even after long term and repetitive fermentation.

Acknowledgements

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