Tandem production of levan and ethanol by microbial fermentation

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Tandem production of levan and ethanol by microbial fermentation using sucrose substrate was investigated. The tandem process involves fermentation of *Bacillus subtilis* (natto) Takahashi in a sucrose medium to produce levan, separation of the levan product from glucose by-product by ultrafiltration and fermentation of the glucose remnant from levan production by *Zymomonas mobilis* to produce ethanol. After cultivation of *B. subtilis* (natto) Takahashi for 48 h, 56.0 ± 0.6 g l^{-1} of levan was produced in a medium containing 250 g l^{-1} sucrose, which was 45 ± 0.5% yield on available fructose. After removing the cells, the fermentation broth was concentrated by ultrafiltration through a membrane of 5 kDa cutoff. The filtrate which contained 179 ± 3 g l^{-1} of hexose was diluted, supplemented with yeast extract (5 g l^{-1}), $(NH_4)_2SO_4$ (1 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}), KH_2PO_4 (1 g l^{-1}) and used for alcohol fermentation by *Z. mobilis*. Incubation of *Z. mobilis* in the medium containing glucose remnant at 30 °C, pH 5.5, 175 rpm for 120 h gave from 21.1 ± 0.3 to 26.5 ± 0.2 g l^{-1} of bio-ethanol depending on the dilution. The tandem process developed in this study is an eco-friendly process in that the sucrose substrate was fully utilized without wasting any by-products in the process; in addition, two invaluable environmentally-friendly biomaterials (levan and ethanol) were produced. Furthermore, the amount of alcohol required for levan recovery could be reduced to a quarter of that generally used in the conventional precipitation.

Introduction

Levan is a polymer of fructose linked by a β-(2→6) fructofuranosidic bond and found in many plants and microbial products.[1] 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 extenders and sweeteners. 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.[1-2] Although many investigations on levan formation have been reported, all suffer the disadvantages of low yield and contamination of impure products. In recent years, strategies to improve the yield of levan production by microorganisms attracted greater attention.[3-5] In our previous reports,[6] we have found that *Bacillus subtilis* (natto) Takahashi, a commercial natto starter, was able to selectively produce levan in a 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 conditions.

As fossil fuel supplies dwindle and environmental awareness has risen, the development of alternative fuels from agricultural wastes and industrial by-products has become more important. This is particularly true due to the growing need for renewable energy sources and bio-ethanol ranks among the top choices at this time. Commercial ethanol fermentation is traditionally produced by *Saccharomyces* strains of yeast primarily fermenting glucose in the batch fermentation.[7-8] However, lately *Zymomonas mobilis* is more often considered as a promising alternative for ethanol production on an industrial scale.[9] *Z. mobilis* is a leading candidate because of its high level of efficiency (accelerated growth rate and increased glucose uptake), high level of ethanol tolerance and its ability to be genetically altered.[8] In addition, *Z. mobilis*, a facultative anaerobic bacterium, does not require oxygen for growth during the fermentation and produces ethanol near to the theoretical yields.[8,10]

Levansucrase (β-2,6-fructan:D-glucose-fructosyl transferase, EC 2.4.1.10) is an enzyme responsible for forming levan from a sucrose-based substrate in a variety of microorganisms.[11-13] This enzyme catalyzed the synthesis of levan from sucrose by transfructosylation reaction while releasing glucose in the menstruum. The glucose by-product generated conceivably is an ideal substrate for bio-ethanol production by yeast and *Z. Mobilis*. The purpose of the present study is to investigate the feasibility of a tandem production of levan and ethanol by microbial fermentation using a sucrose substrate. The tandem process has been successfully developed and is presented, which involves fermentation of *Bacillus subtilis* (natto) Takahashi in a sucrose medium to produce levan, separation of the levan product from the glucose by-product by ultrafiltration and fermentation of the glucose remnant from levan production by *Z. mobilis* to produce ethanol.

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Experimental

Microorganisms and reagents

B. subtilis (natto) Takahashi was obtained from Gem Cultures (Ft Bragg, CA, USA) or Takahashi Yuzo research facility Japan. The strain of *Zymomonas mobilis* BCRC 10808 used for ethanol production was also obtained from Bioresource Collection and Research Center (BCRC, Hsin-Chu, Taiwan). The *Z. mobilis* cultures were maintained as stab cultures in YG agar medium (yeast extract 0.5%, glucose 2% and agar 2%) at 4 °C. Reagents for cultivation such as nutrient agar (NA), nutrient broth (NB) were purchased from DIFCO Laboratories Michigan, USA. $MgSO_4 \cdot 7H_2O$, $NaH_2PO_4 \cdot 2H_2O$, $Na_2HPO_4 \cdot 12H_2O$ were obtained from Sigma Chemical, USA. All other reagents used were of the highest grade available unless otherwise indicated.

Fermentation for levan production

B. subtilis (natto) Takahashi was cultured following the protocol developed previously.[6] It was first cultured on NA (Difico Laboratoies) containing agar (15 g l^{-1}), beef extract (3 g l^{-1}), peptone (5 g l^{-1}) at 37 °C, pH 7.4 overnight. The colonies were inoculated into 5 ml of NB composed of beef extract (3 g l^{-1}), peptone (1.5 g l^{-1}), and NaCl (5 g l^{-1}), pH 7.4 in a 30 ml test tube, and incubated at 37 °C for 24 h with shaking at 150 rpm. After incubation, the 2nd activation was carried out by transferring 5 ml of the broth into 500 ml of NB in 1 litre flask which was incubated at the same conditions for 24 h. For the levan production, the bacteria in NB were inoculated (10%, v/v) into 2 litres of a medium composed of sucrose (250 g l^{-1}), $MgSO_4 \cdot 7H_2O$ (0.5 g l^{-1}), $NaH_2PO_4 \cdot 2H_2O$ (3 g l^{-1}), $Na_2HPO_4 \cdot 12H_2O$ (3 g l^{-1}) in a 5 litre fermenter, and then were incubated at 37 °C, pH 6.0 with agitation speed at 175 rpm for 48 h. Experiments were carried out in duplicate, and the results were averaged.

Separation of biopolymer and unfermented sugar molecules by ultrafiltration

After the growth, the culture was centrifuged at 2400 × g to remove bacterial cells. The unfermented sugars and any fermentation products with smaller molecular weight were separated from large molecular-weight levan product by ultrafiltration. The cell-free supernatant (18,000 ml) of the culture was cycled though a Tami ultrafiltration membrane system (the molecular weight cut-off (MWCO); 5 kDa, Quebec, Canada). During the separation, the product is fractionated in two phases: the concentrated retentate, which contains levan (56.0 ± 0.6 g l^{-1}); the filtrate, which contains glucose, fructose and unreacted sucrose. The levan in the concentrated retentate was harvested by precipitation by addition of 75 vol% cold ethanol, followed by dialysis through a membrane with 10 kDa cutoff. The levan products were characterized by C^{13}-NMR and gel permeation chromatography (GPC). The filtrate containing glucose (114 ± 1 g l^{-1}), fructose (65 ± 2 g l^{-1}) and unreacted sucrose (10.1 ± 0.4 g l^{-1}) was used for ethanol fermentation as described below. It was found that dilution of filtrate for the fermentation was necessary (detailed in the Results and discussion section); therefore, two-fold and four-fold dilutions of the filtrate were made.

Media and culture conditions for alcohol production

Inocula were prepared for *Zymomonas mobilis* BCRC 10808 (Bioresource Collection and Research Center, Hsin-Chu, Taiwan) by transferring a loopfull of cells from the agar plate to NB composed of beef extract (3 g l^{-1}), peptone (1.5 g l^{-1}), and NaCl (5 g l^{-1}), pH 7.4 in a 30 ml test tube, and incubated at 30 °C for 48 h with shaking at 150 rpm. After being incubated in NB for 48 h, the bacteria were inoculated (10% v/v) into a medium, which was made up with 300 ml of the aforementioned filtrate, supplementing with 5 g l^{-1} yeast extract, 1 g l^{-1} $(NH_4)_2SO_4$, 0.5 g l^{-1} $MgSO_4 \cdot 7H_2O$ and 1 g l^{-1} KH_2PO_4. The pH was adjusted 5.5. The culture was maintained at 30 °C, 175 rpm for 120 h. Experiments were carried out in duplicate, and the results were averaged.

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 0.1 N HCl at 100 °C for 2 h.[14] The number-average molecular weight (M_n) of the levan was measured by gel permeation chromatography (GPC) (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^{-1} and the column oven was at 50 °C.[6] H^1-NMR and C^{13}-NMR spectroscopy was performed with a Varain Unity Inova 600 spectrometer. Samples for NMR were dissolved in D_2O solution. For sugar and ethanol analysis, fermentation samples were filtered through a 0.2 μm filter, the concentration of sugars (sucrose, glucose, fructose) and ethanol 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).

Results and discussion

Levan production

We previously showed that the *B. subtilis* (natto) Takahashi produced a large quantity of extracellular polysaccharide when it was grown in a medium containing sucrose in shake-flask experiments,[6] the levan production was noted after a few hours of cell growth and reached a maximum after cell growth reached the stationary phase. Factors such as sucrose concentration, pH, temperature and agitation speed affected the optimal production of levan by the Takahashi strain; the optimal sucrose concentrations for cell growth and levan production were 200–250 g l^{-1}, the optimum pH was 6, the suitable temperature and agitation speed ranged from 25–40 °C and 150–200 rpm, respectively. Under the optimal culture conditions, usually a 21 h cultivation time was needed for a maximum yield in that 40–50 mg ml^{-1} of levan (about 50% yield on available fructose) was produced in 21 h by *B. subtilis* Takahashi. The *B. subtilis* Takahashi strain is a more efficient levan-producing strain than *Bacillus polymyxa*, a strain known to produce a comparable amount of levan in 10 days under the optimal conditions.[15]

In the present study, the levan production was carried out in a 5 litre fermenter using the optimal conditions developed previously in the shake-flask experiments.[6] The time course for the appearance and disappearance of levan, glucose, sucrose and fructose during the fermentation is shown in Fig. 1. The highest production of levan was obtained after cultivation of *B. subtilis* (natto) Takahashi for 24 h; 56.0 ± 0.6 g l^{-1} of levan was produced (22 ± 0.2% yield on available sucrose and 45 ± 0.5% yield on available fructose) and the production rate was approximately 56.0 ± 0.6 g $l^{-1}d^{-1}$. In contrast, the highest production of levan obtained for *Bacillus polymyxa* (NRRL B-18475), *Zymomonas mobilis* and *Erwinia herbicola* was 36 g l^{-1} (24% on available sucrose), 50 g l^{-1} (23% on available sucrose) and 15 g l^{-1} (30% on available sucrose), respectively. However, the production rates were much lower than that of *B. subtilis* (natto) Takahashi; they are 3.6 g $l^{-1}d^{-1}$ for *B. polymyxa*, 5.0 g l^{-1} d^{-1} for *E. herbicola* and 5–6 g l^{-1} d^{-1} for *Z. mobilis*, respectively.[15-16] In addition to the production of the desired levan product, high amounts of glucose (114 ± 1 g l^{-1}) and fructose (65 ± 2 g l^{-1}) by-products and a small amount of unreacted starting sucrose (10.1 ± 0.4 g l^{-1}) were also produced in the broth after 24 h of fermentation. The sugar by-products were separated from levan biopolymer by ultrafiltration and used for ethanol fermentation as described in the next sections.

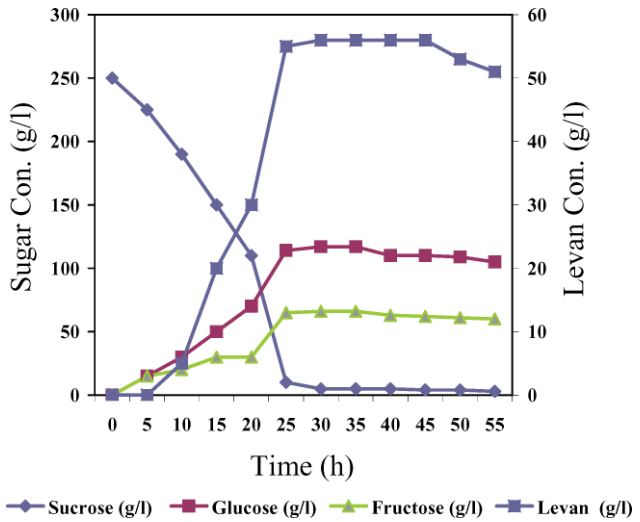

Fig. 1 Time course of levan production by fermentation of *Bacillus subtilis* natto in 10 litre fermenter.

Separation of biopolymer by ultrafiltration and biopolymer recovery

The sequences for separation of fructan biopolymer levan and unfermented sugar molecules in the fermentation broth are depicted in the flow chart shown in the Fig. 2. Cells were removed from the fermentation broth by centrifugation at 2400 × g, or it was passed through a Tami filtration system with a membrane of 0.45 μm pore size. Microscopic examination of the supernatant after centrifugation or after microfiltration determined it to be cell free. The cell-free solution, a volume of 18,000 ml, was further cycled though the Tami ultrafiltration system with the membrane having a molecular weight cut-off (MWCO) of 5,000 (Fig. 2, Route 1). The concentrated retentate, which was

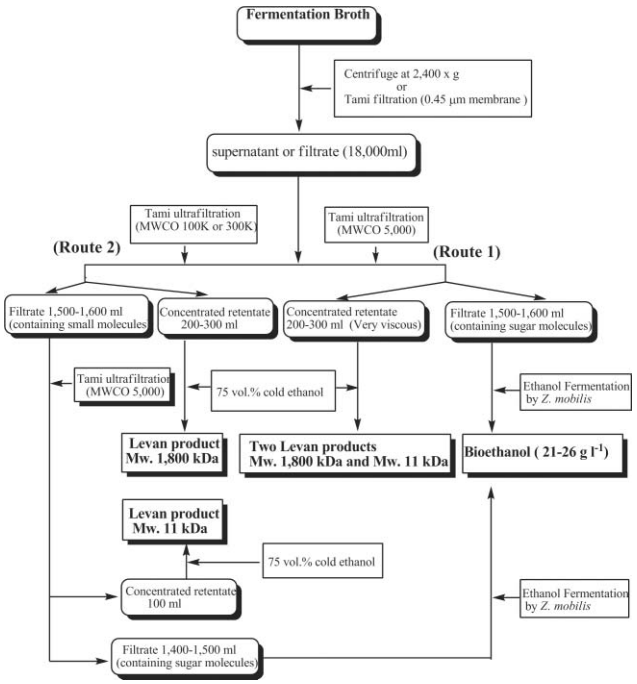

Fig. 2 Scheme of the sequences for separation of fructan levan and unfermented sugar molecules in the fermentation broth.

very viscous and usually had volumes of 200–300 ml, required addition of 75 vol% cold ethanol (aqueous solution to alcohol ratio was 1 : 4) to give high yield with a total recovery of levan. The product thus obtained consisted of high and low molecular weight levans as described below. The filtrate containing glucose (114 ± 1 g l^{-1}), fructose (65 ± 2 g l^{-1}) and unreacted sucrose (10.1 ± 0.4 g l^{-1}) was used for ethanol fermentation.

The levan was harvested by precipitation from the culture broth by addition of cold ethanol and the pure levan products were characterized by H^1-NMR, C^{13}-NMR and gel permeation chromatography (GPC). The C^{13}-NMR spectra (Fig. 3) shows six main resonances at 59.9, 63.5, 75.2, 76.3, 80.3 and 104.3 ppm, and the GPC chromatogram (Fig. 4) showed two peaks; one has a molecular weight at 1,800 kDa and the other has a molecular weight at 11 kDa. Both the C^{13}-NMR spectrum and GPC chromatogram were identical with those of levan produced previously in the shake flask experiments.[6] In contrast, GPC analysis of the levan by the bacterium *Erwinia herbicola* indicated a molecular weight of approximately 1.1 × 10^6–1.6 × 10^6 Da, and levan from *B. polymyxa* gave a molecular

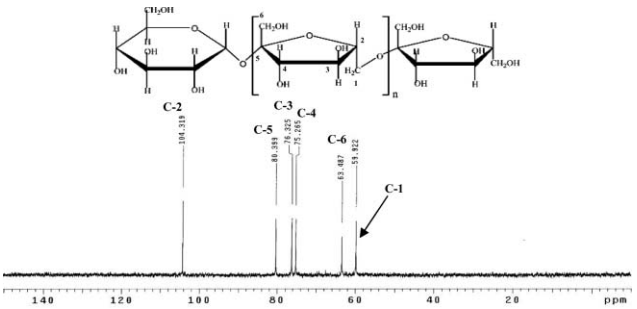

Fig. 3 C^{13}-NMR chromatogram of the levan produced by *B. subtilis* (natto) Takahashi.

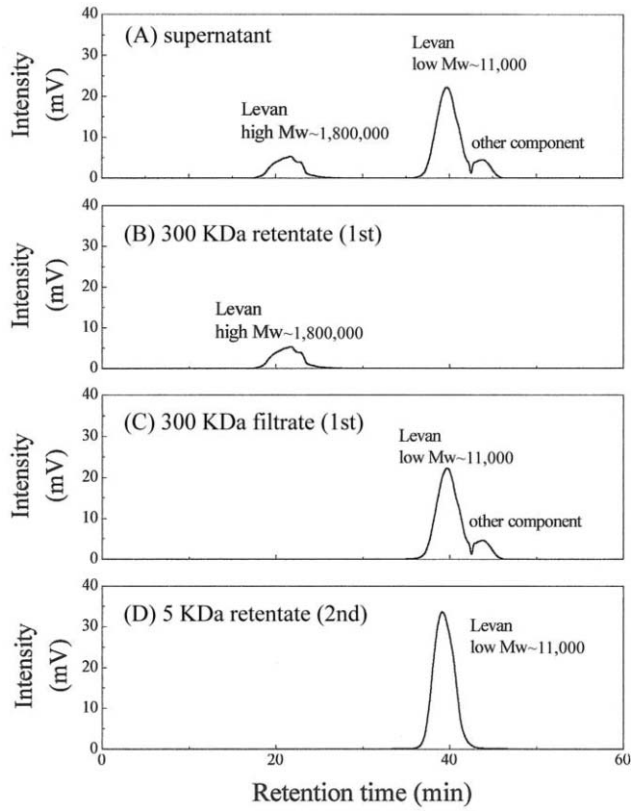

Fig. 4 GPC chromatograms of different fractions at various stages of ultrafiltration. (A) Fermentation broth after removal of cells. (B) The first concentrated retentate after filtration through a membrane of 300,000 MWCO. (C) The first filtrate after filtration through a membrane of 300,000 MWCO. (D) The second concentrated retentate after filtration through a membrane of 5,000 MWCO.

weight of 2.0×10^6 Da when it was evaluated on the same GPC system.[15]

The dual molecular-weight of levan products produced by *B. subtilis* Takahashi in the shake flask and fermenter was rather characteristic; however, the mechanism by which these two different molecular-weight products were formed is still unknown. Fractionation of levans of low and high molecular weight is necessary, because levans with different molecular weights are needed for different purposes. The potential applications of the high and low molecular weight levans were well documented in the literature.[17-19] Microbial levans of high molecular weight ($>10^7$ kDa) display a direct effect on tumor cells due to a modification in the cell membrane and cell permeability,[18] as well as radioprotective and antibacterial activities.[20] Levans of low molecular weight (< 100 kDa) have a great potential as a substitute for blood plasma volume extenders.[19] In pharmaceutical applications, it is known that the low molecular-weight, less branched levan usually has a low viscosity, and can be used as a tablet binder in immediate-release dosage forms, while levans of medium and high viscosity grade are used in controlled-release matrix formulations.[21] We have previously demonstrated successfully the fractionation of levans by precipitation using an ethanol gradient.[6] However, attempts were made to carry out the fractionation of levans by ultrafiltration through ceramic membranes before their precipitation by alcohol.

First of all, the concentrated retentate obtained above (Fig. 2, Route 1) was attempted to be cycled through a membrane of 100,000 and 300,000 MWCO to fraction the low and high molecular weight levans, before their precipitation by alcohol. However, no success was achieved without performing pre-dilution because the highly viscous nature of the concentrated retentate to be filtered created great difficulty in the process of ultrafiltration. Ironically, the dilution resulted in increased usage of alcohol in the later precipitation. To overcome the problem, an alternative and greener route for the ultrafiltration was taken (Fig. 2, Route 2). The cell-free solution, a volume of 18,000 ml, was first cycled though the Tami system with a membrane of 100,000 or 300,000 MWCO. The first concentrated retentate, 200–300 ml, required addition of 75 vol% cold ethanol to give high yield with the total recovery of high molecular weight levan (90 ± 2 g). The first filtrate was further cycled through a membrane of 5,000 MWCO. The second concentrated retentate, 100 ml, was harvested with the total recovery of low molecular weight levan (27 ± 1 g) by the addition of 75 vol% cold ethanol. The GPC chromatogram of the low and high molecular levans is shown in Fig. 4. The second filtrate containing glucose, fructose and unreacted sucrose was used for later ethanol fermentation.

The most widely used method for the recovery of levan from cell-free broth is alcohol precipitation. An alcohol concentration of 75–80% (v/v) was usually required for the levan recovery.[6,15] To reduce the amount of alcohol required for the recovery of levan by precipitation, concentration of levan in the cell-free culture broth was necessary. It is advantageous to concentrate cell-free culture broth containing levan during the recovery process, which is more important when large-scale and greener production is taken into account. This study has demonstrated the fact that the concentration process by ultrafiltration dramatically reduced the amount of alcohol used for the total recovery of levan in the process; in addition, it has potential for industrial-scale application. Thus, it is an essential unit operation for recovery of levan.

Fermentation for ethanol production by *Z. mobilis*

The filtrate after ultrafiltration usually contained high concentrations of glucose (114.0 ± 1.0 g l^{-1}) and fructose (65.0 ± 2.0 g l^{-1}), in addition to a trace of unreacted sucrose (10.1 ± 0.4 g l^{-1}), which is suitable for fermentation into ethanol by *Z. mobilis*. *Z. mobilis* is a gram-negative bacterium that catabolizes only D-fructose, D-glucose and sucrose as carbon and energy sources into ethanol by the Entner-Doudoroff pathway (E-D pathway). The E-D pathway catabolizes glucose to pyruvate using a set of enzymes different from those in glycolysis and the pentose phosphate pathway used by most bacteria.[22] *Z. mobilis* is also an osmo- and ethanol-tolerant bacterium that can grow in an environment of high osmotic pressure, and has a high sugar tolerance (up to 400 g L^{-1}) and resistance to high ethanol concentrations (up to 12%).[23] In addition, it has shown higher specific rates of glucose uptake that correspondingly guarantees its higher ethanol productivity, generally 3–5 fold higher than that of *S. cerevisae*.[24] It was chosen for the ethanol fermentation in the preset study. The time course for the consumption of glucose, fructose and sucrose, and the production of ethanol are shown in Fig. 5. It was found that dilution of filtrate for the fermentation

Table 1 The kinetics of ethanol production by *Z. mobilis* in the batch mode

	2x dilution			4x dilution		
Kinetic parameters	Sucrose	Glucose	Fructose	Sucrose	Glucose	Fructose
Initial Con. (g l^{-1})	5.0	57.0	32.5	2.50	28.5	16.3
Final Con. (g l^{-1})	3.90	0.34	2.20	1.64	1.02	1.64
Sugar Consumption (%)	22.0	99.4	93.7	34.4	96.4	83.8
Maximum ethanol Con. (g l^{-1})	26.5 ± 0.2			21.1 ± 0.3		
Fermentation time/h	120			120		
[a] $Y_{E/S}$ (g g^{-1})	0.30			0.51		
[a] *YE* (%)	59.8			100		
[a] Q_p (g g^{-1} h^{-1})	0.15			0.26		

$Y_{E/S}$: Yield coefficient for ethanol from monosaccharide (g g^{-1}); *YE*: Yield efficiency (g ethanol produced per g monosaccharide utilized/theoretical yield of ethanol (*i.e.* 0.5 1)).; Q_p: specific ethanol production rate (g g^{-1} h^{-1}).[a] The sucrose consumption was scarce, its contribution to ethanol production was omitted, only the contribution from glucose and fructose was taken into account.

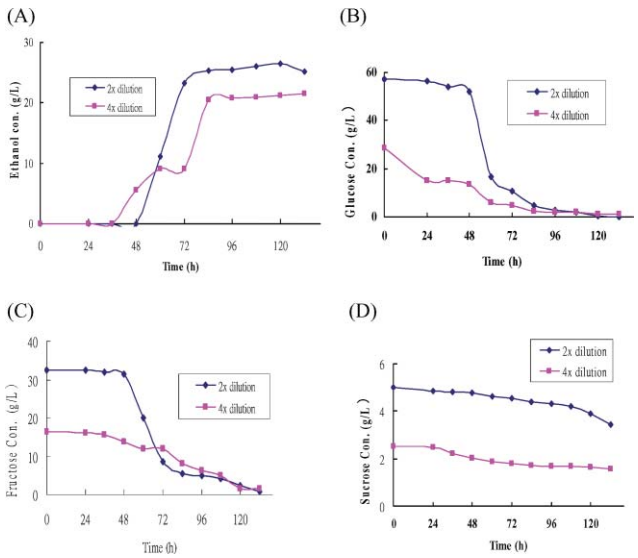

Fig. 5 The time course for the production of ethanol (A), the consumption of glucose (B), the consumption of fructose (C), the consumption of sucrose (D) during the fermentation by *Z. mobilis*.

was necessary due to the fact that no ethanol was produced even after 120 h of fermentation when no dilution was made to the filtrate (data not shown). When two-fold or four-fold dilutions were made so that the sugar concentrations in the filtrate were 50% or 25% of the original values, ethanol production became apparent after nearly 40 h of fermentation; at the end, 26.5 ± 0.2 g l^{-1} and 21.1 ± 0.3 g l^{-1} of ethanol was produced respectively after 120 h of fermentation. *Z. mobilis* is a highly osmo-tolerant bacterium and it can efficiently convert high concentrations of sugar to ethanol at levels between 50 and 250 g l^{-1}. However, an increase in glucose concentration from 150 g l^{-1} to 250 g l^{-1} results in a significant decrease in the specific growth rate, cell yield, and ethanol yield. In addition, higher glucose concentrations also increase the total time of fermentation and lead to incomplete utilization of glucose.[24] The best glucose concentration for high ethanol yield efficiency using *Z. mobilis* ATCC 10988 has been suggested to be 50–150 g l^{-1}.[25-26] The total sugar (glucose, fructose and sucrose) concentration in the filtrate after 2-fold and 4-fold dilution was estimated to be near 100 and 50 g l^{-1}, respectively. Thus, it is within the range of the best sugar concentrations for optimal ethanol production previously suggested.[24-26]

The kinetic parameters for the fermentation are shown in Table 1. It is known that the yield of ethanol for fermenting is 0.511 grams per gram of hexose or pentose; thus, it is possible to estimate the theoretical ethanol yield from sugar consumption. In 2-fold dilution, the glucose, fructose and sucrose concentrations present in the filtrate used for fermentation were 57.0, 32.5 and 5.0 g l^{-1}, and the residual sugar concentration after 120 h fermentation was 0.34, 2.20 and 3.90 for glucose, fructose and sucrose, respectively. Since the sucrose consumption was scarce, its contribution to ethanol production was omitted, only the contribution from glucose and fructose was taken into account. Accordingly, the yield coefficient for ethanol from monosaccharide ($Y_{E/S}$) was 0.30 g g^{-1}, the yield efficiency (YE) was 59.8% and the specific ethanol production rate (Q_p) was 0.15 g g^{-1} h^{-1}. In contrast, the glucose, fructose and sucrose concentrations present in the filtrate were 28.5, 16.3 and 2.50 g l^{-1} in 4-fold dilution; the residual sugar concentrations after 120 h fermentation were 1.02, 1.64 and 1.64 for glucose, fructose and sucrose, respectively. When the sucrose contribution was omitted because of its scarcity and only the contribution from glucose and fructose was taken into account, the $Y_{E/S}$, YE and Q_p was near to 0.51 g g^{-1}, 100% and 0.26 g g^{-1} h^{-1}, respectively. The results showed that both glucose and fructose were almost completely consumed in both 2-fold and 4-fold dilution strategies indicating that glucose and fructose were utilized efficiently by *Z. mobilis* ATCC 10988. However, the values of $Y_{E/S}$, YE and Q_p indicated that ethanol production is more efficient in 4-fold dilution (total sugar concentration ~50 g l^{-1}) than in 2-fold dilution (total sugar concentration ~100 g l^{-1}); thus, 4-fold dilution is suggested. The high YE obtained in 4-fold dilution is consistent with the implication that glucose is metabolized by *Z. mobilis* to ethanol *via* the E-D pathway with a yield close to the theoretical maximum.[24] The sugar conversion for glucose, fructose and sucrose was 99.4%, 93.7%, 22% in 2-fold dilution and 96.4%, 83.8%, and 34.4% in 4-fold dilution, respectively. The sugar consumption by *Z. mobilis* for glucose is apparently greater than that for fructose and sucrose. Studies for the determining fermentation pattern of *Z. mobilis* strains on different substrates have been performed that resulted in a conclusion that ethanol yield obtained from fructose or sucrose in batch fermentation is

generally lower than that from glucose.[27] However, comparative studies regarding glucose, fructose and sucrose consumption by *Z. mobilis* in the same experimental conditions were not seen previously.

Conclusions

A process for tandem production of levan and ethanol by microbial fermentation using a sucrose substrate has been successfully demonstrated. Efficient production of levan by fermentation of *B. subtilis* (natto) Takahashi in a sucrose medium carried out in a 5 litre fermenter using the optimal conditions developed previously in the shake-flask experiments resulted in a high production of levan. Concentration of a cell-free culture broth containing levan by ultrafiltration was found to be an effective method for total recovery of levan because the amount of solvent used for precipitation could be dramatically reduced. In addition, the fact that the Takahashi strain produced the low and high molecular weight levans simultaneously and the products of the two different molecular-weights were easily separated by fractionation using ultrafiltration make the versatile applications of levans more feasible. The remnant from levan production collected after ultrafiltration can be directly applied for fermentation by *Z. mobilis* to be converted into ethanol efficiently. The process developed in this study not only has potential in industrial-scale applications, it is also an eco-friendly and greener production process in that the sucrose substrate was fully utilized without wasting any by-products, in addition to the production of two invaluable environmentally-friendly biomaterials (levan and ethanol) and the dramatic reduction of the use of organic solvents for levan recovery.

Acknowledgements

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