JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Selective Production and Characterization of Levan by *Bacillus subtilis* (Natto) Takahashi

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To meet the industrial need of an efficient microbial method for increased levan production, *Bacillus subtilis* (natto) Takahashi, a commercial natto starter for preparing fermented soybeans (natto), was used to produce levan. After cultivation for 21 h, 40−50 mg of levan mL^{-1} was produced in medium containing 20% (w/w) sucrose, which was ∼50% yield on available fructose. The product consisted of two fractions with different molecular masses (1794 and 11 kDa), which were easily separated by fractionation using an ethanol gradient. The products were well characterized by GPC, ^{13}C NMR, and ^{1}H NMR. The various sugars and concentrations, initial pH, fermentation temperature, and agitation speed affected the levan production by *B. subtilis* (natto) Takahashi. Takahashi strain is the most efficient levan-producing strain among all of the *B. subtilis* strains tested and, as previously reported, it produced the highest yield of levan in the least time (21 h) under the common cultivation condition.

KEYWORDS: Levan; *Bacillus subtilis*; natto; Takahashi; fermentation

INTRODUCTION

Levan is a polymer of fructose linked by β-(2→6) fructofuranosidic bonds present in many plants and microbial products (*1*). Levan offers a variety of industrial applications in the fields of cosmetics, foods and pharmaceuticals; it can be used as an industrial gum, a blood plasma extender, and a sweetener. Potential applications of levan as an emulsifier, a formulation aid, a stabilizer, a thickener, a surface-finishing agent, an encapsulating agent, and a carrier for flavor and fragrances have also been proposed (*1*, *2*). Recently, commercial interest in the production of levan has been intensified. However, use of this biopolymer has yet not been practicable due to a lack of feasible processes for large-scale production.

The microbial levans are produced from a sucrose-based substrate by transfructosylation reaction of levansucrase (β-2,6-fructan:D-glucose-fructosyl transferase, EC 2.4.1.10) by a variety of microorganisms (*3*−*8*). Although many investigations on levan formation have been reported, they suffered the disadvantages of low yields and the contamination of impure products. No work has been done on the industrial mass production of levan by the fermentation processes. To date, only *Bacillus polymyxa* has produced a large quantity of levan at ∼3 times the amount of previously known levan-producing microbes, and it was essentially free from other contaminating polysaccharide byproducts (*9*, *10*). Hence, there is the industrial need of an efficient microbial method for increased levan production.

Bacillus subtilis (natto) Takahashi, a commercial natto starter, is commonly used to prepare fermented soybeans, a product called natto, which has been a traditional Japanese food for more than 1000 years. Recently, we have shown that *B. subtilis* (natto) Takahashi produced a mixture of poly(γ-glutamic acid) and levan when it was grown in a basal medium containing sucrose and L-glutamate (*11*). However, poly(γ-glutamic acid) was mainly produced in medium containing L-glutamic acid without sucrose; in contrast, levan was the only product when the bacteria were cultivated in medium containing 20% (w/w) sucrose without L-glutamate. To further investigate the possibility of the use of *B. subtilis* (natto) Takahashi for the efficient production of the levan product, we studied and describe in this paper the factors affecting the production of levan by this bacterium and the purification and characterization of the products.

MATERIALS AND METHODS

Microorganism and Reagents. *B. subtilis* (natto) Takahashi was obtained from Takahashi Yuzo research facility in Japan. Other bacteria tested for levan-producing abilities were *B. subtilis* (natto) ATCC 7058, *B. subtilis* (natto) ATCC 7059, *B. subtilis* (natto) IFO 13169, and *B. subtilis* (natto) IFO 3335, which were obtained from the Culture Collection and Research Center (CCRC) Taiwan. Reagents for cultivation such as nutrient agar (NA) and nutrient broth (NB) were purchased from Difco Laboratories, Detroit, MI. $MgSO_4 \cdot 7H_2O$, $NaH_2PO_4 \cdot 2H_2O$, and $Na_2HPO_4 \cdot 12H_2O$ were obtained from Sigma Chemical, St. Louis, MO. All other reagents used were of the highest grade available unless indicated otherwise.

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10.1021/jf058084o CCC: $30.25
Published on Web 09/20/2005

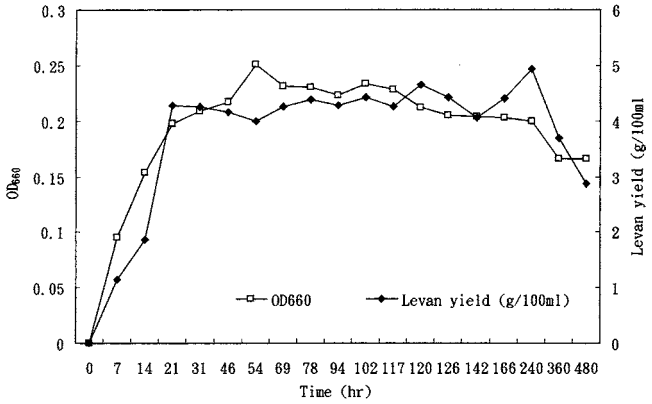

Figure 1. Time course of cell growth and levan production by *B. subtilis* (natto).

Media and Culture Conditions. *B. subtilis* (natto) Takahashi was first cultured on NA (Difco Laboratoies) containing agar (15 g/L), beef extract (3 g/L), and peptone (5 g/L) at 37 °C, pH 7.4, overnight. The colonies were inoculated into 5 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 30 mL test tube, and incubated at 37 °C for 48 h with shaking at 150 rpm. After incubation, the bacteria were inoculated (5%, v/v) into 100 mL of a 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), and $Na_2HPO_4 \cdot 12H_2O$ (3 g/L) in a 250 mL flask and then were incubated at 37 °C, pH 7.0, with shaking at 150 rpm for 21 h. The culture medium was centrifuged to remove the bacterial cells, and then the levan was harvested by precipitation with the addition of cold ethanol from the culture broth, followed by dialysis through a membrane with 10 kDa cutoff. The products were characterized by ^{1}H NMR, ^{13}C NMR, and gel permeation chromatography (GPC).

To study the effects of various sugars and the concentrations on the levan productivity, sucrose in medium was substituted by glucose, fructose, lactose, and maltose. The concentrations of sucrose were varied at 0, 20, 50, 70, 100, and 200 g/L. To study the optimal condition for levan production, the initial pH was varied from 4.5 to 8 at 0.5 intervals, the temperature was varied from 25 to 40 °C, and the shaking speed was at 0, 150, 175, and 200 rpm.

Analytical Methods. The number-average molecular weight (M_n) of the levan was measured by GPC using a Hitachi L6200 system controller equipped with Shodex KB800 series columns (two KB80M, one KB802.5) and a refractive index (RI) detector (Bischoff, model 8110). Dextran standards (Phenomenex, Torrance, CA; M_w, 7.20, 16.23, 35.60, 74.30, 170.00, 535.00, 1580.00, and 2754.00 kDa) were used to construct a calibration curve. The eluant flow rate of the deionized water was 1 mL min^{-1}, and the column was kept at 50 °C. The total carbohydrate contents of the products were determined according to the phenol−sulfuric acid method (*12*) and were expressed as the amount of glucose equivalent. ^{1}H NMR and ^{13}C NMR spectroscopy was performed with a Varain Unity Inova 600 spectrometer. Samples for NMR were dissolved in D_2O solution. Optical rotation was measured on a polarimeter (type AA-10, Optical Activity Ltd.,) with a sodium lamp and a 100 mm length sample tube.

RESULTS AND DISCUSSION

Production of levan by *B. subtilis* (Natto) Takahashi. *B. subtilis* (natto) Takahashi produced a large quantity of extracellular polysaccharide when it was grown on media containing sucrose. The levan production was noted after a few hours of cell growth and reached the maximum after cell growth reached the stationary phase (**Figure 1**). The levan production by *B. subtilis* (natto) Takahashi depended on the variety and the concentration of the sugar substrates used (**Table 1**). A large amount of the levan was produced when the bacteria were cultivated using sucrose, but the yields varied with the sucrose concentration. The maximum levan productivity (49.4 g/L) was obtained on the medium containing 200 g/L of sucrose, whereas the yield decreased at the higher or the lower sucrose concentrations. A small amount of polysaccharide (alcohol precipitate) was also produced when the bacteria were grown on lactose and maltose, but no polysaccharide was produced using glucose or fructose. The sugar and concentration-dependent yields were consistent with those of *Bacillus polymyxa* as previously reported (*9*). The factors affecting optimal production of levan by the Takahashi strain, such as pH, temperature, and agitation speed, were studied, and the results (data not shown) showed that the optimum pH for cell growth and levan production was pH 6, the suitable temperature range for growth and levan production was from 25 to 40 °C, and the suitable shaking speed was from 150 to 200 ppm; however, the still culture significantly reduced the productivity of levan to half of the highest yield.

Table 1. Effect of Sugar on Levan Production by *B. subtilis* (Natto) Takahashi

sugar	g/L	levan[a] (g/L)
control	0	0
sucrose	20	2.5
sucrose	50	9.1
sucrose	70	14.3
sucrose	100	21.4
sucrose	200	49.4
sucrose	300	30.6
glucose	50	ND[b]
fructose	50	ND[b]
lactose	50	1.0[c]
maltose	50	1.5[c]

[a] Average of triplicates. [b] Not detectable. [c] Alcohol precipitate without identification.

Under the optimal culture condition, a 21 h cultivation time was usually needed for the maximum yield. In contrast, the levan production by *B. polymyxa*, the most productive strain for levan known to date, was only noted after a few days of cell growth and the level reached the maximum after at least a 10 day cultivation time (*9*, *10*). In a typical fermentation, 4−5 g of levan had been produced in medium containing 20% (w/w) sucrose, which was ~50% yield on available fructose. The levan yield by the Takahashi strain was comparable to that by *B. polymyxa*; however, the fermentation time needed was much less.

Levan was harvested by precipitation from the culture broth by the addition of cold ethanol. However, the yield and the quality of the products were varied with the amount of alcohol added; the maximum yield of levan was precipitated when the medium-to-alcohol ratio was 1: 4. Further increase in the alcohol content resulted in hardening of the levan; the more the alcohol added, the less fluid the levan was.

To compare the levan-producing ability, the other *Bacillus subtilis* (natto) species such as *B. subtilis* (natto) ATCC 7058, *B. subtilis* (natto) ATCC 7059, *B. subtilis* (natto) IFO 13169, and *B. subtilis* (natto) IFO 3335 were cultivated under the same conditions as those used for *B. subtilis* (natto) Takahashi. During the same 21 h cultivation time, all of the tested bacteria produced small amounts of levan (**Table 2**); however, prolonged cultivation time resulted in increased levan production by these bacteria. After 10 days of cultivation, all tested bacteria produced similar levels of levan at a range of 42−52 g/L. We realized that every individual strain might behave differently in various conditions. However, in this study we did not intend to optimize the cultivation conditions of levan production for every individual strain except that of *B. subtilis* (natto) Takahashi.

Characterization of Levan Produced by *B. subtilis* (Natto) Takahashi. The levan produced by *B. subtilis* (natto) Takahashi

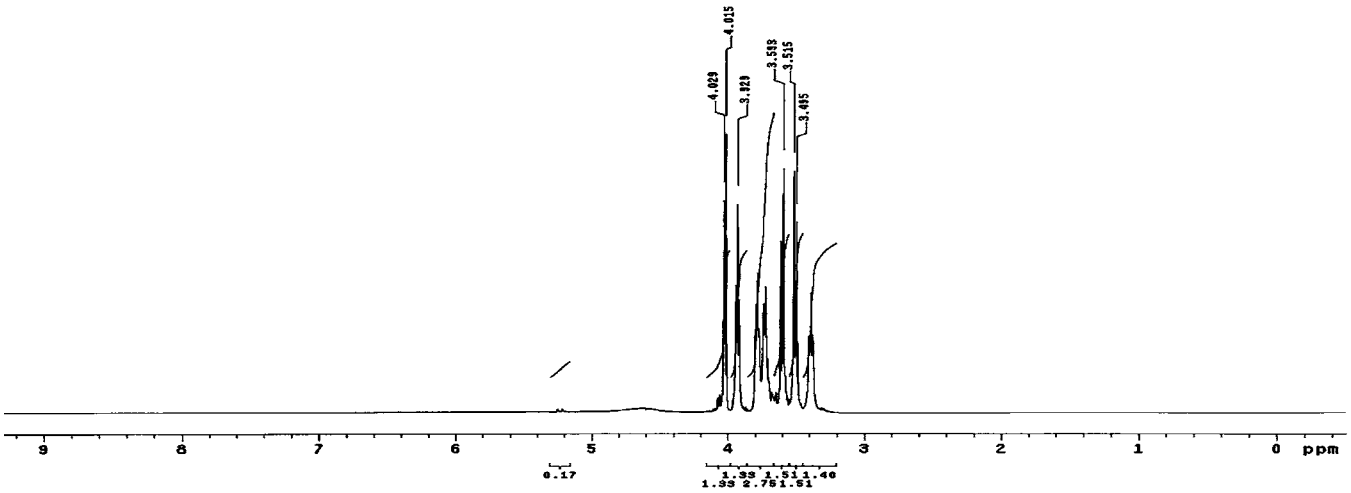

Figure 2. ^{1}H NMR of the levan produced by *B. subtilis* (natto) Takahashi.

Table 2. Levan Production by Various Microorganisms

	levan[a] (g/L)		
bacteria	21 h	120 h	240 h
B. subtilis (natto) Takahashi	48.4	49.0	49.9
B. subtilis (natto) ATCC 7058	3.6	13.8	46.9
B. subtilis (natto) ATCC 7059	20.2	46.1	52.2
B. subtilis (natto) IFO 3335	9.1	16.7	45.3
B. subtilis (natto) IFO 13169	4.1	11.6	42.4

[a] Average of triplicates.

Table 3. Chemical Shifts of ^{13}C NMR Spectra of Levans Produced by *B. polymyxa* and *B. subtilis* (Natto) Takahashi

	chemical shifts (ppm)	
carbon atom	levan of *B. polymyxa*[a]	levan of *B. subtilis* (natto)
C-1	60.7	60.1
C-2	104.2	104.4
C-3	77.0	76.5
C-4	75.7	75.4
C-5	80.5	80.5
C-6	63.6	63.6

[a] Assignment cited from Han and Clarke (*10*).

was readily soluble in water at room temperature, but it is nonhydroscopic. It was very susceptible to hydrolysis in boiling 0.5% oxalic acid. It consisted of 99% fructose, as revealed by the HPLC analysis of the acid hydrolysate, and had a specific optical rotation of −42.0. The above properties were similar to those of levan produced by *B. polymyxa* (*9*, *10*). The ^{1}H NMR spectrum (**Figure 2**) shows seven protons between 3.4 and 4.2 ppm, and the ^{13}C NMR spectrum (not shown) shows six main resonances at 60.1, 63.6, 75.4, 76.5, 80.5, and 104.4 ppm, which are almost identical with peak positions (60.7, 63.6, 75.7, 77.0, 80.5, and 60.7 ppm) for levan produced by *B. polymyxa* as previously reported (**Table 3**), indicating that the polysaccharide produced by *B. subtilis* (natto) Takahashi was levan type with the linkage of β(2→6)fructofuranoside (**Figure 3**).

Molecular Weight and Fractionation of Levan Products. The levan product, after dialysis through a membrane with a 10 kDa cutoff, gave two sharp and clear peaks on the GPC chromatogram (**Figure 4**); one has a molecular mass of 1794 kDa; the other has a molecular mass of 11 kDa. In contrast, the levan product produced by *B. polymyxa* gave only a single, sharp and clear peak just below 2×10^3 kDa on Sephacryl S-500 after dialysis through a membrane with a 12 kDa cutoff (*9*, *10*).

The two products of different molecular weights of levan were easily fractionated by precipitation with increasing ethanol concentration. When the medium-to-alcohol ratio was less than 1:2, the high molecular weight product was precipitated completely. After the first-stage separation, ethanol was further added to the supernatant until the medium-to-alcohol ratio reached 1:4 to completely precipitate the low molecular weight product. The two products were confirmed by GPC (**Figure 5**), ^{13}C NMR, and ^{1}H NMR. The ^{13}C NMR and ^{1}H NMR spectra of the high molecular weight product were completely identical to those of the low molecular weight product.

It was further noted that the product distribution was affected by the fermentation time (**Figure 6**). At initial time (0 h), no levan was formed and only a sucrose peak showed on the GPC chromatogram (**Figure 6A**). After 7 h, the high molecular mass product (1794 kDa) predominated (**Figure 6B**). However, as the fermentation proceeded further, the amount of high molecular weight product declined and that of low molecular weight product increased. After 21 h, the amounts of the two products were comparable, with the amount of the low molecular weight product slightly higher than that of the high molecular weight product (**Figure 6C**). After cultivation for 10 days, the low molecular mass product (11 kDa) became the major product (**Figure 6D**). The dual molecular weights in the product were rather characteristic, and the molecular weight shift with the cultivation time was very intriguing. It was previously reported that when incubated with sucrose, levansucrase of *B. subtilis* catalyzed the formation of high and low molecular weight levans (*13*). Euzenat et al. (*14*) investigated the conditions of high and low molecular weight levan production by levansucrase of *B. subtilis* C4 using various temperatures and sucrose concentrations; their results indicated that the high and low molecular weight levans appeared simultaneously during sucrose consumption, whereas the low molecular weight levans increased throughout the experiment, an observation similar to what was found in our experiments. These observations demonstrated that the low molecular weight levans observed in the reaction were not produced only at the end of the run, when the sucrose concentration became too low to be efficiently transformed by the enzyme. Although the data were not shown, it was also noted that the similar duality of molecular distributions was also observed in the levan products produced by the other *B. subtilis* (natto) species used in this study, indicating that the levasucrases of these species were likely to act the same way as that of *B. subtilis* C4 in terms of levan production. However, the mech-

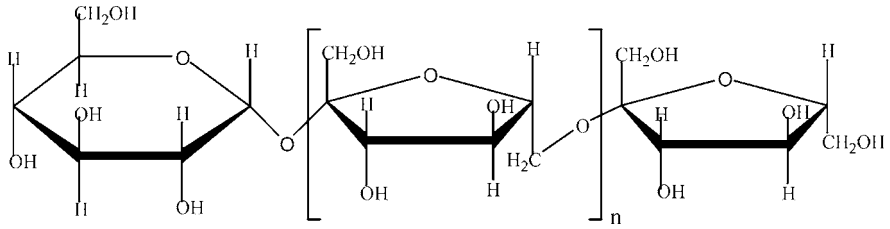

Figure 3. Chemical structure of levan (G1-2F6-2F6-2F6-2F).

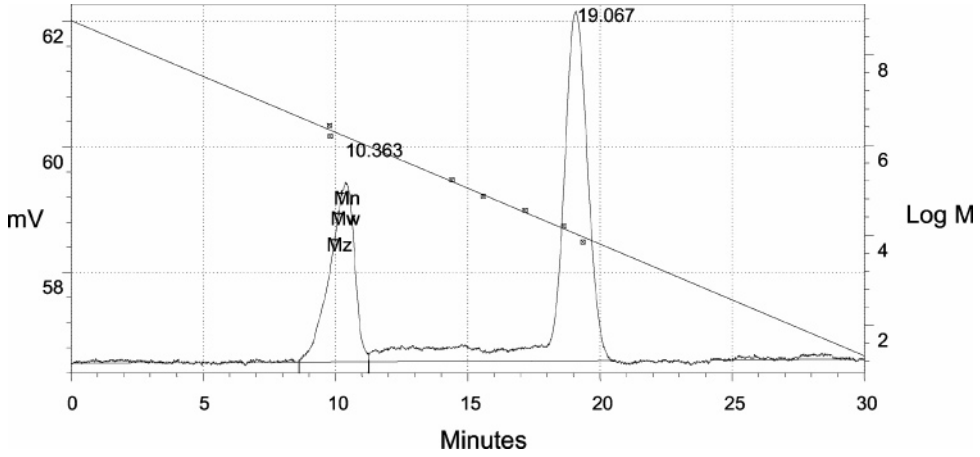

Figure 4. GPC chromatogram of levan produced by *B. subtilis* (natto) Takahashi.

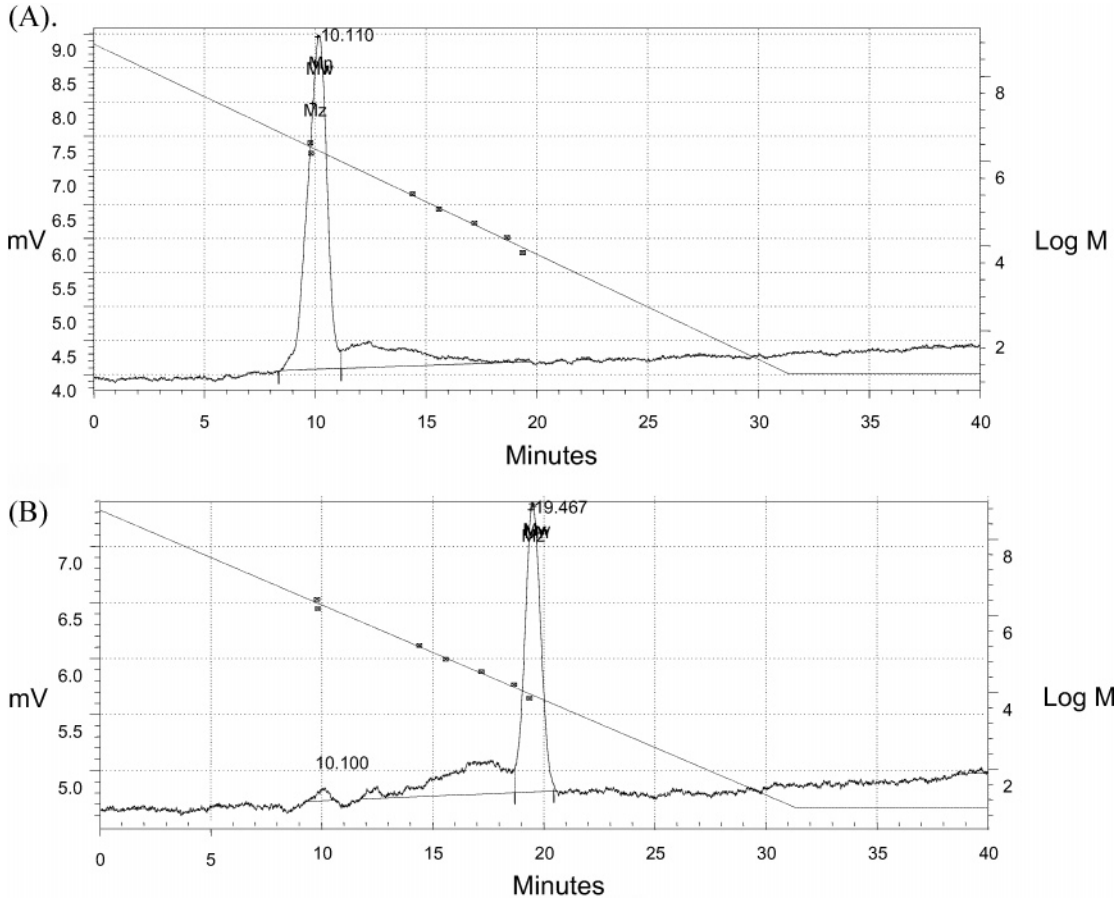

Figure 5. GPC chromatogram of levans produced by *B. subtilis* (natto) Takahashi after fractionation by stepwise alcohol precipitation: (**A**) media-to-alcohol ratio was less than 1:2; (**B**) media-to-alcohol ratio was 1:4.

anism by which the high and low molecular weight products were formed is yet to be determined.

The use of microbial levans has yet not been practicable due to a lack of feasible processes for large-scale production. In this study, a large amount of levan was produced in a relatively short time by *B. subtilis* (natto) Takahashi, and the levan products were easily purified and free from other contaminating polysaccharide byproducts. This is the most efficient production

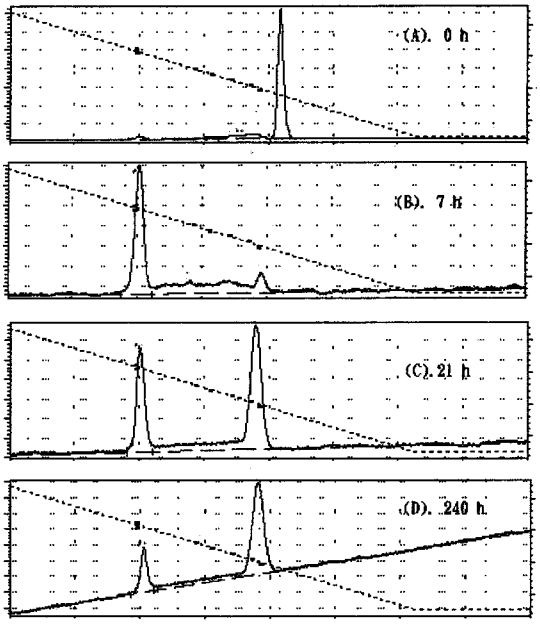

Figure 6. Effect of cultivation time on the molecular weight of levans produced by *B. subtilis* (natto) Takahashi.

of levans by microbial fermentation described to date, suggesting that the Takahashi strain is an ideal candidate that can be used for further process development in the mass production of levan for industrial applications. It is likely that the low production cost of levan will permit a much increased use of levan soon.

The potential applications of the high and low molecular weight levans are well documented in the literature (*15*−*17*). It was reported that the levans with molecular masses higher than 10^7 kDa were effective for a direct effect on tumor cells, which is related to a modification in the cell membrane, including changes in cell permeability (*15*, *16*); the effect was lost when the polymer degraded. Levan is similar to bacterial dextran in physicochemical properties; it is not toxic, and it is not antigenic in small molecular weight; in addition, it is slowly eliminated from the body when injected into the bloodstream (*17*). Therefore, levan with a molecular mass below 100 kDa has potential as a blood plasma volume extender (*17*, *18*). In pharmaceutical applications, it is known that the low molecular weight, less branched levan usually provides a low viscosity and can be used as a tablet binder in immediate-release dosage forms, whereas levans of medium- and high-viscosity grade are used in controlled-release matrix formulations (*19*). It is undoubted that levans with different molecular weights are needed for different purposes. The facts that the Takahashi strain produced the low and high molecular weight levans simultaneously and that the products of the two different molecular weights were easily separated by fractionation using an ethanol gradient make the versatile applications of levans more feasible.

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Received for review April 15, 2005. Revised manuscript received July 21, 2005. Accepted July 22, 2005.

JF058084O