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Enhanced production of (1 → 3)-β-D-glucan by a mutant strain of *Agrobacterium* species

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Abstract

We developed a mutant strain, which showed a higher β-glucan production than the parent strain, *Agrobacterium* sp. ATCC 31750. A batch fermentation was carried out in a 300 l stirred tank reactor. A maximum β-glucan concentration of 76 g/l was obtained in 120 h of cultivation with the mutant strain, while the parent strain produced 64 g/l under the same condition. The molecular weight of β-glucan, determined by a high-performance size exclusion chromatography, was about 300,000. From the infrared (IR) and NMR analytical data, the purified glucan was found to exclusively consist of β-D-glucopyranose with 1,3 linkages.

Keywords: β-Glucan; Fermentation; Mutant; *Agrobacterium* sp.

1. Introduction

A variety of β-glucans with the β-1,3 linkages have been known to have immune stimulatory effects. Krestin, a β-glucan extracted from the mycelia of the basidiomycete *Coriolus versicolor*, has a general immune stimulatory effect in humans, which has been used as an anticancer agent [1]. Lentinan, from *Lentinus edodes*, is another β-glucan, which inhibits tumor growth, and increases resistance to infections by bacteria, viruses, and parasites [2]. Schizophyllan, from *Schizophyllum commune*, is another β-glucan with the property of inducing the formation of cytotoxic macrophages and generating antitumor activity in the human body [3]. However, these fungal β-glucans require methods of extraction to obtain comparatively small amounts of pure β-glucans, making the process expensive.

A bacterial β-glucan, composed of β-(1 → 3)-D-glucosidic linkages, is synthesized mostly by *Agrobacterium* species and *Alcaligenes faecalis* under nitrogen-limited conditions [4–6]. This glucan has been given the name 'curdlan', because it forms a curd when heated. This peculiar property has enabled us use as a gelling material to improve the textural quality, water-holding capacity and thermal stability of various foods. in addition, there have been many reports about the biological activity of the bacterial glucan. Jagodzinski et al. [7] reported that curdlan sulfate having a β-(1,3)-glucan backbone showed high anti-AIDS (acquired immunodeficiency syndrome) virus activity with low side effects. Mikio et al. [8] disclosed that a curdlan derivative, modified by reaction with glycidol, developed excellent antiviral activity with extremely low toxicity. Evans et al. [9] reported that both the low toxicity of curdlan and its marked anti-invasion activity on merozoites make it a potential auxiliary treatment for severe malaria. Thus, curdlan and its derivatives have growing potential in the pharmaceutical industry. Moreover, the bacterial production, as opposed to the fungal production, is more advantageous in its purification since it can be readily produced as an exotype of the β-(1,3)-glucan from a large scale submerged bacterial culture.

As previously reported [10–13], there are many crucial factors affecting the production of glucan, including: carbon source, nitrogen limiting timing, phosphate concentration, oxygen supply, and pH. Using the optimal operation strategy, curdlan as high as 64.4 g/l was obtained, with the glucan producing strain of *Agrobacterium* sp. ATCC 31750, in 120 h of batch fermentation [12].

As a part of our efforts for the production of bacterial β-glucan, we have investigated the development of a mutant strain, in an attempt to yield a high production of β-glucan with the strain. Additionally, both the molecular structure and the biological activities of the purified β-glucan are documented.

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2. Materials and methods

2.1. Strains and mutant development

Agrobacterium species ATCC 31750, the parent strain, and *Agrobacterium* sp. R259 Korean culture type collection (KCTC) 1019BP, a mutant derived from the parent strain, were used in this study. Mutant strains, which produce a higher amount of β-glucan than the parent strain, were obtained as follows: ATCC 31750 was grown in a 500 ml baffled flask containing 50 ml of YP medium, with shaking at 30 °C for 17 h. The YP medium contained 20 g/l sucrose, 5 g/l yeast extract, and 5 g/l peptone, at pH 7.0. The cells in 30 ml of the broth were washed with 0.1 M citrate buffer, pH 5.5, and suspended in 25 ml of the buffer containing 1 mg/ml of *N*-methyl-*N*-nitro-nitrosoguanidine (MNNG). The mixture was incubated for 60 min at 30 °C. The cells were then washed with the same citrate buffer, and the cell suspension spread on agar plates containing 0.05 g/l Aniline Blue after appropriate dilution. After incubation for 2 days at 30 °C, colonies, showing as a darker blue than the parent strain, were isolated.

2.2. Culture conditions

YP medium was used to seed the culture. The fermentation medium contained: 140 g sucrose, 4.42 g NH_4Cl, 1.44 g KH_2PO_4, 0.5 g $MgSO_4{\cdot}7H_2O$, and 10 ml of a trace element solution (5 g $FeSO_4{\cdot}7H_2O$, 2 g $MnSO_4{\cdot}H_2O$, 1 g $CoCl_2{\cdot}6H_2O$, 1 g $ZnCl_2$ per litre of 0.1 M HCl) made up to 1 l. For flask cultures, the cells grown at 30 °C for 17 h in 100 ml of the seed medium were inoculated into the fermentation medium containing 0.3% (w/v) calcium carbonate and cultivated at 30 °C. Fermentations were carried out in a 300 l jar fermenter (diameter, 53.5 cm; height, 145.6 cm, KoBiotech Inc., Incheon, Korea). The fermenter was equipped with six blade disk turbine impellers (one 7.9 cm impeller for 5 l fermenter, and two 18 cm impellers and one 23 cm bottom impeller), dissolved oxygen (DO) analyzers and pH controllers. For 300 l jar fermentation, seed culture (1600 ml), cultivated at 30 °C for 17 h in shake flasks, was transferred into the 30 l fermenter containing 14.4 l of the fermentation medium and cultivated for 1 day at pH 7.0. The main culture was started by transferring the cells grown in 30 l fermenter into the 300 l fermenter containing 144 l of the fermentation medium. The culture pH was controlled at 7.0 with 4 M NaOH/KOH at the cell-growing stage. The pH was lowered to 5.5 with 3 M HCl at the time of nitrogen limitation. The aeration rate and the agitation speed were maintained at 0.5 vvm and 200 rpm, respectively, for the whole fermentation period.

2.3. Analytical methods

The concentration of cells and glucan were determined by measuring the dry weight. A suitably diluted sample was centrifuged at 8000 g at 4 °C for 30 min. The pellet consisting of cells and glucan was washed with 0.01 M HCl, and harvested by centrifugation. The glucan was kept soluble by the addition of 0.5 M NaOH over 1 h period. Cells were separated by centrifugation at 8000 g for 30 min. The glucan present in the supenatant phase was precipitated under acidic conditions by the addition of an appropriate volume of 2.0 M HCl. Both cells and glucan were washed and freeze-dried to a constant weight. For the analysis of sucrose, samples were hydrolyzed at 100 °C for 15 min following the addition of 10 μl of 1 M HCl to 1 ml of samples. Their concentrations were determined by the dinitrosalicylic acid method [14]. Ammonia was determined by the indophenol method [15].

2.4. Analyses of molecular structure

The molecular weight was determined by size exclusion high-performance liquid chromatography (HPLC). The chromatographic system consisted of an HPLC pump (Waters 501, Miliford, MA), a differential refractometer (Waters 410), and a data module (Waters 746). The chromatograms were obtained with two series of PLgel MIXED-B columns (300 × 7.5 mm i.d., 8 μm-particle size, 300 Å pore size, Polymer Laboratories Inc., Amherst, MA). The mobile phase of dimethyl sulfoxide was pumped at a flow rate of 1.0 ml/min. The system was operated at 80 °C. Dextrans (MW; 15,000–20,000; 60,000–90,000; 143,000; 580,000; and 2000,000), purchased from Sigma, were used as standard samples. The composition of glucan was determined by the analysis of the monosaccharide using gas chromatography–mass spectrometry (GC–MS). The glucan was hydrolyzed with 2.5 M trifluoroacetic acid, which derived the monosaccharides into their alditol acetate forms. Alditol acetates were recovered with CH_2Cl_2 and analyzed by GC–MS. The GC–MS system analyses were performed on a Hewlett-Packard 5890 Series II gas chromatograph (Hewlett-Packard, Avondale, USA) with a JMS-AX 505 WA mass spectrometer using a DB-23 capillary column (30 m × 0.215 mm i.d. × 0.25 μm, J&W Scientific Inc., CA) with He carrier gas. The column temperature was 220 °C at an isocratic mode. Myo-inositol was used as the internal standard. Infrared spectra were obtained on a Matton fourier transform-infrared (FT-IR) spectrophotometer (Thermomattson, Madison, WI) employing potassium bromide (KBr) discs. ^{13}C NMR data were obtained on a JEOL JNM-LA 400 spectrometer (Jeol Co. Ltd., Tokyo, Japan) operating at 100 MHz. The samples of the purified β-glucan were dissolved in dimethyl sulfoxide.

2.5. Interferon-γ (INF-γ) secretion

The leukocyte-rich fraction, from peripheral blood mononuclear cells of a healthy donor, was obtained by density gradient centrifugation, following the addition of Ficoll solution (Sigma), and washed with phosphate buffered saline, pH 7.4. The resultant fraction was treated with red

blood cells (RBC) lysis buffer (pH 7.4) to remove the RBC, and was resuspended in RPMI 1640 medium (GibcoBRC, Grand Island). Mononuclear cells were distributed onto 96 well tissue culture plate at a concentration of 5×10^6 per well, and incubated for 24 h in a humidified atmosphere of 5% CO_2 in the glucan containing medium. The concentration of INF-γ was measured by an enzyme-linked immunosorbent assay method using OptEIA^{TM} human INF-γ kits (Pharmingen, CA).

3. Results

3.1. Mutant development

More than 2000 mutants were derived from the parent strain. Some strains appeared white, while others appeared as different intensities of blue. Based on the studies of Naganishi et al. [16], we knew that only β-glucan producing colonies appeared blue on the Aniline Blue agar plates. Therefore, we only chose colonies covered with blue slime. Preliminary test results indicated that the color intensity of the colonies have a positive relationship to the concentration of β-glucan. To select stable strains, which have least possibility of occurring revertants, we transferred strains from plate to plate eight times. Seven strains showing up as a darker blue were chosen, and their β-glucan production evaluated. To compare the mutants with the parent strain for β-glucan production, flask cultures were carried out. The experiments were carried out in triplicate. As shown in Table 1, β-glucan production from one of the mutants, R259, was 31.5 g/l in 3 day cultivation, which was 23% greater than from the parent strain. The production yield of the mutant strain was as high as 0.59 in relation to g β-glucan/g sucrose, while that of parent strain was 0.45. This indicates that with the mutant strain, sucrose is more efficiently converted into β-glucan. Strain R259 was deposited in the Korean culture type collection.

Table 1
Comparison of the parent strain and mutants for color intensity, β-glucan concentration, and production yield from sucrose

Strain	Color intensity	β-glucan concentration (g/l)	Production yield (g β-glucan/g sucrose)
Parent strain	+	25.6	0.45
R59	++	27.5	0.50
R111	++	28.8	0.51
R165	+++	29.0	0.53
R259	+++	31.5	0.59
R1711	++	27.8	0.47
R1885	++	26.2	0.51
R2241	+++	30.4	0.54

A two-step culture technique was employed. Cells were cultivated in the YP medium for 17 h at 30 °C. Then, cells (50 mg, dry weight) harvested by centrifugation at 5000 g for 15 min were suspended in 100 ml of the nitrogen-free medium. Further cultivation was performed for 72 h at 30 °C. Data are means of three independent determinations within deviation of not more than 10%.

Table 2
Biochemical characteristics of *Agrobacterium* sp. R259

Characteristics	Reaction	Characteristics	Reaction
Oxidase	+	Mannose assimilation	+
NO_3 production	−	*N*-Acetyl-glucosamine assimilation	+
Indole production	−	Maltose assimilation	+
Glucose acidification	−	Gluconate assimilation	−
Arginine dihydrolase	−	Caprate assimilation	−
Urease	+	Adipate assimilation	−
Esculin hydrolysis (β-glucosidase)	+	Malate assimilation	+
Gelatinase	−	Citrate assimilation	−
Beta galactosidase	+	Phenyl-acetate assimilation	−
Glucose assimilation	+		
Arabinose assimilation	+		

Biochemical tests were carried out by using API 20NE (BioMerieux, USA) assay kit.

The morphological properties of the mutant strain were different from those of the parent in that they appeared as smooth and glittering on the agar plate after incubation at 30 °C for 1 day, while the parent strain appeared winkled and rough. Results of biochemical tests are summarized in Table 2.

3.2. Production of β-glucan

A high production of β-glucan was attempted by employing the optimal operation strategy applied to the parent strain [6,10–12]. A typical batch fermentation profile for the 300 l stirred tank reactor is shown in Fig. 1. The initial concentrations of sucrose and ammonium were 140 and 1.5 g/l, respectively. When the ammonium was exhausted in the culture broth, the cell concentration had reached 12 g/l, resulting in yield of 8.0 g cells/g ammonium. At the cell-growing stage, the pH was controlled at 7.0 with 4 M NaOH/KOH, which was lowered to 5.5, by addition of 3 M HCl, at the time of nitrogen limitation. β-Glucan production began from the onset of nitrogen exhaustion. A maximum concentration of 76 g/l was obtained in 120 h of cultivation, while the parent strain produced 64 g/l under the same conditions [12]. The production yield was 0.55 g glucan/g sucrose.

3.3. Structure analysis

The molecular weight of β-glucan, as determined by size exclusion high-performance liquid chromatography, was about 300,000. The purified β-glucan was found to consist exclusively of glucose from the analysis of the monosaccharide. To find the configuration of glucose, infrared (IR) spectroscopy was carried out. As shown in Fig. 2, the IR spectrum shows an absorption band at 890 cm^{-1}, indicating that D-glucopyranose has a β-configuration. It was concluded that no α-configuration exists since there was no characteristic absorption band at 840 cm^{-1}. Fig. 3 shows the ^{13}C NMR spectrum of the bacterial β-glucan. Correlations

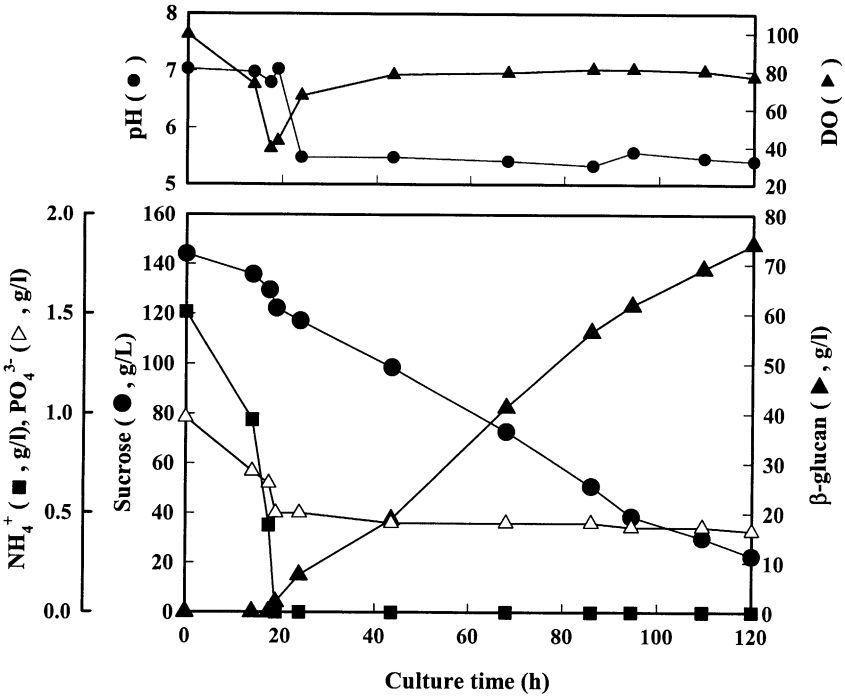

Fig. 1. Time profiles of concentrations of β-glucan, sucrose, ammonium, phosphate, dissolved oxygen level, and pH. Jar fermentation was carried out in a 300 l jar fermenter.

with carbon chemical shifts at 103.1, 72.8, 86.2, 68.4, 76.3, and 60.9 ppm are assigned as C-1, C-2, C-3, C-4, C-5, and C-6, respectively, which represent the (1 → 3)-β-D-glucan backbone in the polymer chain. From the NMR spectrum, it can also be concluded that the (β-glucan has a linear (1 → 3)-linkages, since evidence of other linkages was not seen in the spectrum.

3.4. Immune stimulatory effect of β-glucan

Interferon-γ, mainly produced by T cells and natural killer cells, is well known to have broad effects on the immune system. INF-γ is a potent activator of major functions of macrophages, including: antigen presentation, tumor cell cytotoxicity, intracellular killing of pathogens, cytokine

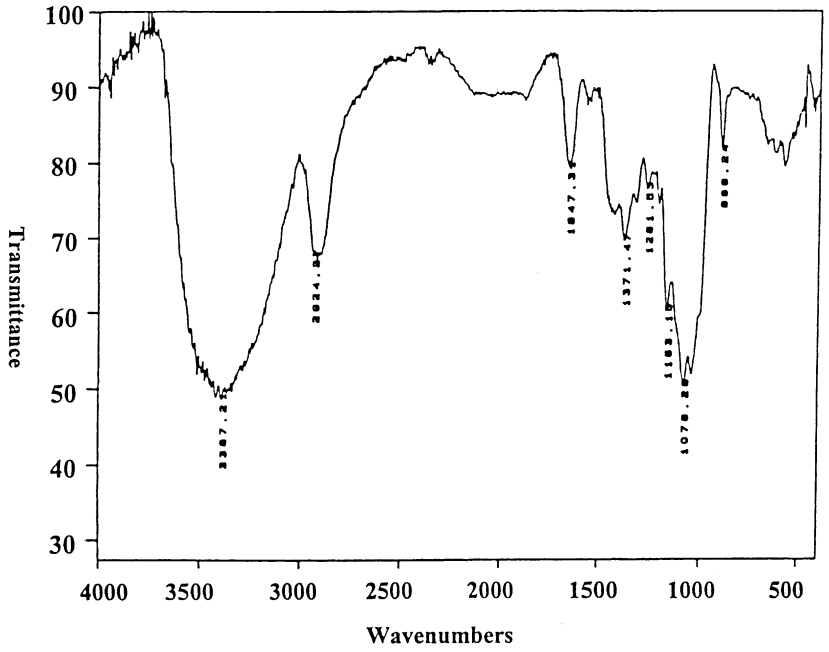

Fig. 2. FT-IR spectrum of purified β-glucan from *Agrobacterium* sp. R259 KCTC 1019BP.

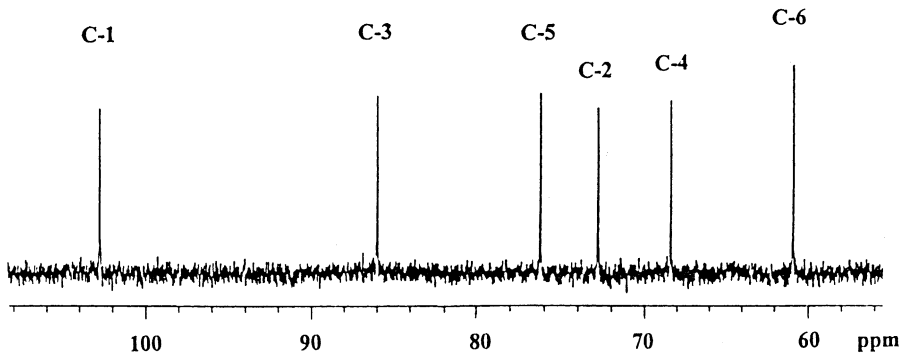

Fig. 3. ^{13}C NMR spectrum of the purified β-glucan from *Agrobacterium* sp. R259 KCTC 1019BP.

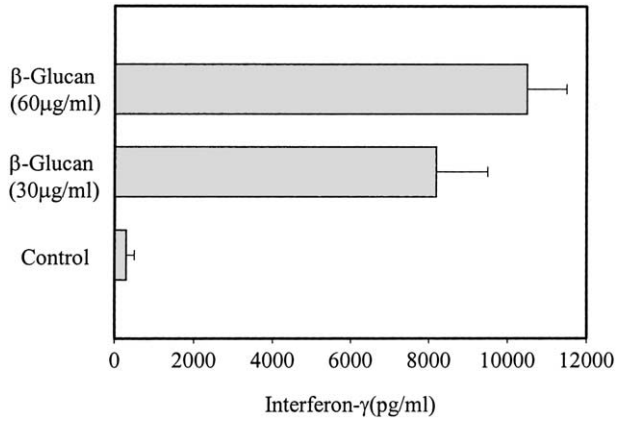

Fig. 4. The effect of β-glucan on interferon-γ secretion from peripheral blood mononuclear cells.

production and phagocytosis. Thus, to find the potential of β-glucan as an immune stimulator, we investigated the secretion of INF-γ from peripheral blood mononuclear cells. The levels of INF-γ were significantly increased from β-glucan-stimulated cells (Fig. 4). From the result, it seems likely that the β-glucan, from *Agrohacterium* sp. R259, activates leukocytes and induces the production of cytokines.

4. Discussion

In this study, we have developed mutant strains which produce β-glucan at a high yield. Many researchers have investigated the bacterial production of β-glucan; the highest productivity reported being 0.5 g/l/h, which was obtained from the batch culture of *Agrobacterium* sp. ATCC 31750 under optimal conditions [12]. Using the mutant strain developed in this study, we increased the productivity to 0.63 g/l/h under the same conditions. The production yield from sucrose was also enhanced by 20% compared to that of the parent strain. The recovery process was based on the conformational transition that occurred when the concentration of alkali exceeded 0.2 M. Under alkaline conditions, the biomass can be separated from the dissolved glucan, which remains in the supenatant. Neutralization of the alkaline supenatant resulted in the formation of an insoluble gel from the polymer, which can be recovered by centrifugation, with the polymer subsequently being washed free of contaminating salts. Procedures for preserving β-glucan in a dry state include spray drying. Using this simple procedure, we produced β-glucan that was 99.5% pure with a 89% yield. From an economic point of view, the bacterial production of β-glucan is much more advantageous than the fungal process, provided the biological activities are comparable.

Differences in biological activities of β-glucans seem to be dependent upon the degree of branching, molecular conformation, and their molecular weight. Ssaki et al. [17] examined the effect of chain length on the antitumor activity using β-glucans of different molecular weights, obtained from the acid-hydrolysis of glucan. The results show that insoluble β-glucans with a number-average degree of polymerization (DPn) greater than 50 exhibit strong antitumor activity [17]. The bacterial β-glucan obtained in this study was found to have a molecular weight of 300,000 and consisted exclusively of the 1,3 linkage of glucose residue. From the result shown in Fig. 4, it is clear that this bacterial β-glucan activates cells to enhance INF-γ secretion. To conclude, since this polymer can be economically produced in large quantities and show biological activity as an immune stimulator, it has a potentially large market in both the nutraceutical and the pharmaceutical industries.

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References

[1] J.K. Kamisato, M. Nowakowski, Morphological and biochemical alterations of macrophages produced by a glucan, PSK, Immunopharmacology 16 (1988) 88–96.

[2] S.C. Jong, J.M. Birmingham, Medicinal and therapeutic value of the Shiitake mushroom, Adv. Appl. Microbiol. 39 (1993) 153–184.
[3] Y. Numasaki, M. Kikuchi, Y. Sugiyama, Y. Ohba, A glucan Sizofiran: T cell adjuvant property and antitumor and cytotoxic macrophage inducing activities, Oyo Yakuri Pharmacometrics 39 (1990) 39–48.
[4] T. Harada, K. Fujimori, S. Hirose, M. Masada, Growth and β-glucan 10C3K production by a mutant of *Alcaligenes faecalis* var. *myxogenes* in defined medium, Agric. Biol. Chem. 30 (1966) 764–769.
[5] H.G. Lawford, J.D. Rousseau, Effect of oxygen on the rate of β-(1,3)-glucan microbial exopolysaccharide production, Biotechnol. Lett. 11 (1989) 125–130.
[6] I.Y. Lee, W.T. Seo, G.J. Kim, M.K. Kim, C.S. Park, Y.H. Park, Production of curdlan using sucrose and sugar cane molasses by two-step fed-batch cultivation of *Agrobacteriurn* species, J. Ind. Microbiol. Biotechnol. 18 (1997) 255–259.
[7] P.P. Jagodzinski, R. Wiaderkiewicz, G. Kurzawski, M. Kloczewiak, H. Nakashima, E. Hyjek, N. Yamamoto, T. Uryu, Y. Kaneko, M.R. Posner, D. Kozbor, Mechanism of the inhibitory effect of curdlan sulfate on HIV-1 infection in vitro, Virology 202 (1994) 735–745.
[8] K. Mikio, O. Yoshiro, O. Hirotomo, K. Yoshikazu, I. Hiroshi, M. Hisashi, K. Takeshi, Water soluble β-(1,3)-glucan derivative and antiviral agent containing the derivative, Jpn. Patent 07228601 (1995).
[9] S.G. Evans, D. Morrison, Y. Kaneko, I. Havlik, The effect of curdlan sulfate on development in vitro of *Plasmodium falciparum*, Trans. R. Soc. Trop. Med. Hyg. 92 (1998) 87–89.
[10] M.K. Kim, I.Y. Lee, J.H. Lee, K.T. Kim, Y.H. Rhee, Y.H. Park, Residual phosphate concentration under nitrogen-limiting conditions regulates curdlan production in *Agrobacterium* species, J. Ind. Microbiol. Biotechnol. 25 (2000) 180–183.
[11] J.H. Lee, I.Y. Lee, M.K. Kim, Y.H. Park, Optimal pH control of batch processes for production of curdlan by *Agrobacterium* species, J. Ind. Microbiol. Biotechnol. 23 (1999) 143–148.
[12] I.Y. Lee, M.K. Kim, J.H. Lee, W.T. Seo, J.K. Jung, H.W. Lee, Y.H. Park, Influence of agitation speed on production of curdlan by *Agrobacterium* species, Bioprocess Eng. 20 (1999) 283–287.
[13] I.Y. Lee, Curdlan, Biopolymers, in: A. Steinbuchel, E.J. Vandamme, S. de Baets (Eds.), Polysaccharides I: Polysaccharides from Prokaryotes, vol. 5, Wiley–VCH, Weinhein, 2000, pp. 135–158.
[14] G.L. Miller, Use of dinitrosalicylic acid reagent for determination of reducing sugar, Anal. Chem. 31 (1972) 426–428.
[15] F. Srienc, B. Arnold, J.E. Bailey, Characterization of intracellular accumulation of poly-β-hydroxybutyrate (PHB) in individual cells of *Alcaligenes eutrophus* H16 by flow cytometry, Biotechnol. Bioeng. 26 (1984) 982–987.
[16] I. Naganishi, K. Kimura, S. Kusui, E. Yamazaki, Complex formation of gel-forming bacterial (1 → 3)-β-D-glucan (curdlan type polysaccharide) with dyes in aqueous solution, Carbohydr. Res. 32 (1974) 47–52.
[17] T. Ssaki, N. Abiko, Y. Sugino, K. Nitta, Dependence on chain length of antitumor activity of (1 → 3)-β-D-glucan from *Alcaligenes faecalis* var. *myxogenes*, IFO 13140, and its acid-degraded products, Cancer Res. 38 (1978) 379–383.