Studies on the structural analysis and physiological properties of exopolysaccharides (EPS) secreted by a newly isolated *Pseudomonas stutzeri* BL58

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Chapter 1

Introduction

1-1. Structural properties and applications of general polysaccharides

Synthetic polymers, such as plastics and elastics, are important materials in our lives and have contributed to the development of novel industrial technologies in many fields, such as medicine [1-3], food [4, 5] and electronics [6, 7] shown in Table 1. Over time, many functional polymers have been developed for a wide range of purposes, but environmental problems have resulted from the large scale distribution and disposal of synthetic polymers that are not biodegradable [8].

Table. 1 Application of various polymers

Polymer used for food and industry as commodity chemicals	
Corn starch and its derivative	Improvement of fluidity
Cellulose and its derivative	Stabilization of suspension
Guar gum and its derivative	Agglomeration of particles
Gum arabic	Coating of material
Alginate	Preparation of emulsion
Pectin	Ion exchange carrier
Carrageenan	Molecular sieve carrier
Carob's gum	Collection of oil
Gatti	Friction resistance reducing agent applied to ship hulls
Polymer used for medicine as fine chemicals	
Cancer inhibitor, Anti-inflammatory, Immune activation, Antidiabetic, Anti-obesity, Anti-oxidative capacity etc.	

To avoid environmental problems, some biodegradable polymers such as polyesters produced by microorganisms are used in the medical and food industries [9-11]. These polymers show many unique physical and chemical properties and therefore, are expected to be useful for application in other fields.

1-2. Types and carbohydrate structure of exopolysaccharides (EPS) for industrial, food and medical uses

Carbohydrate polymers produced by plants, such as starch, cellulose and agar, are also biodegradable polymers [12-14] and many novel derivatives have been developed [15-19]. Xanthan gum, dextrin, alginate, curdlan, scleroglucan gellan and pullulan are biodegradable polymers produced by microorganism [20-23]. In particular, xanthan gum is a representative biodegradable polymer that has been used as a food stabilizer, thickener and gelation agent all over the world [24, 25] as shown in Table 2.

Table. 2 Application of various polymers

Biopolymer	Origin	Composed sugar	Application
Xanthan gum (M.W. 2000K)	*Xanthomonas campestris*	Mannose Glucose Glucuronic acid	Foods (Canning, Retort etc) Fragrance
Gellan gum (M.W. 500K)	*Sphingomonas elodea*	Glucose Glucuronic acid Rhamnose	Foods (Jelly, Jam etc) Fragrance
Welan gum (M.W. 2000K)	*Alcaligenes sp.*	Glucose Glucuronic acid Rhamnose Mannose	Agrochemical Highly fluidized concrete
Pullulan (M.W. 200K)	*Aureobasidium pullulans*	Branched-chain Maltotriose	Foods (Tofu, Soup etc) Medical capsule

1-3. Research of ethanol-assimilating bacterium

The first research using ethanol-assimilating bacteria was initiated by Dr. Sakaguchi et al. in 1932 when he reported the glutamic acid fermentation and production of protease and dextranase. A high incidence of ethanol-assimilating bacteria was observed in carbohydrate-assimilating bacteria. Therefore, it was expected for ethanol-assimilating bacteria to produce various biochemical materials normally

produced by carbohydrate-assimilating bacteria. Screening tests were performed using soil medium including 1.5-30 % of alcohol such as methanol, ethanol, n-propanol and n-butanol, and 27, 36, 23 and 18 strains were isolated for each alcohol respectively. The screening test using ethanol as a sole carbon source was the most effective for the growth of bacteria.

It has been reported that ethanol-assimilating strains are common among *Candida, Pichia, Hansenula, Debaryomyces, Saccharomycces, Torulosis, Endomycosis, Trichosporon* yeast *species* and *Brevibacterium, Corynebacterium, Pseudomonas* bacteria *species*. It is rarely reported for filamentous fungus, basidiomycete, and alga. Also, few studies have been reported on thermophilic, alkalophilic or acidophilic ethanol-assimilating bacteria grown under abnormal environments. However, in general, it is thought that microbes are able to survive under extremely abnormal environments since they produce extracellular polysaccharides and form a biofilm.

1-4. Objective and goal of the study

The objective of my thesis research was to study microbes that assimilate ethanol as a carbon source, grow under abnormal environments such as acidic or alkaline condition and produce extracellular polysaccharides. The optimum culture conditions to produce multifunctional polysaccharides in larger scale were investigated. Moreover, the chemical structure of the exopolysaccharides was analyzed and the characterizations of the exopolysaccharides for some physical and physiological properties in anticipation of possible applications in cosmetics and pharmaceuticals were examined as shown in Figure 1.

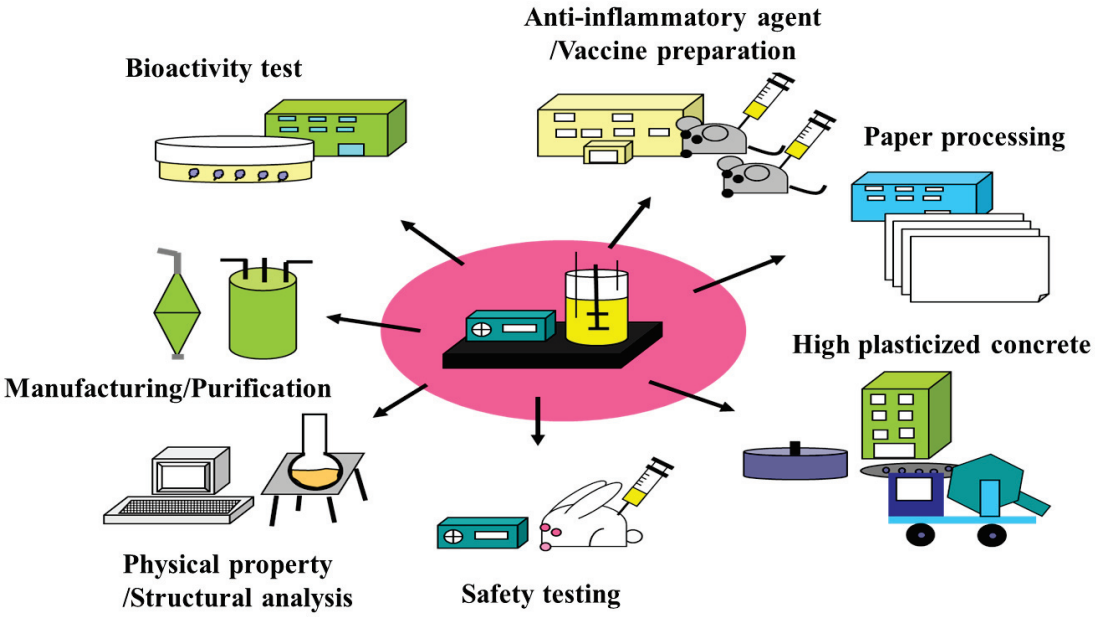


Figure 1 Applications of biodegradable polymers

Chapter 2

Isolation of high exopolysaccharides-producing bacteria BL58 and storage

2-1. Introduction

Soil samples collected from different areas and standard synthetic culture medium for carbohydrate-assimilating bacteria were used to isolate exopolysaccharide producing bacteria. The natural energy source was reduced as much as possible, and ethanol was added as the carbon source. The culture medium was prepared containing ethanol and the other sources, 0.15% NH_4HCO_3, 0.15% $(NH_4)_2SO_4$, 0.15% NH_4NO_3, 0.15% KH_2PO_4, 0.07% K_2HPO_4, 0.03% $MgSO_4 \cdot 7H_2O$, 0.001% $CaCl_2 \cdot 2H_2O$, 0.001% Zn $SO_4 \cdot 7H_2O$, 0.001% $FeSO_4 \cdot 7H_2O$, 0.0001% $MnSO_4 \cdot 7H_2O$ and 0.03% yeast extract, as shown in Table 3. In this chapter, the ethanol-assimilating BL58 strain which highly produce exopolysaccharides under alkaline condition were isolated and identified.

Table. 3 Culture medium for BL58 strain

Ethanol	5 g/L	5 %
NH_4HCO_3	1.5	0.15
$(NH_4)_2SO_4$	1.5	0.15
NH_4NO_3	1.5	0.15
KH_2PO_4	1.5	0.15
K_2HPO_4	0.7	0.07
$MgSO_4 \cdot 7H_2O$	0.3	0.03
$CaCl_4 \cdot 2H_2O$	0.01	0.001
$ZnSO_4 \cdot 7H_2O$	0.01	0.001
$FeSO_4 \cdot 7H_2O$	0.01	0.001
$MnSO_4 \cdot 7H_2O$	0.001	0.0001
Yeast extract	0.3	0.03

2-2. Materials and methods

2-2-1. Preparation of liquid culture medium

Test tubes (φ=18 mm) plugged by cotton were sterilized by dry heat at 180 °C for 2 hours. The pH of the culture medium in Table 3 was adjusted to pH 3, 7 and 10 by 1N-HCl or 5N-NaOH. Autoclave sterilization for the 5 mL of the above medium in test tubes (φ=18 mm) was carried out, and ethanol (final concentration = 5%) was added to the cooled medium under sterile condition.

2-2-2. Preparation of flat plate medium and slant medium

The culture medium in Table 3 was prepared. The pH was adjusted to pH 3, 7 and 10 by 1N-HCl or 5N-NaOH, and the autoclave sterilization was carried out. 1.5% (w/v) of agar medium was prepared and autoclaved. The two medium were mixed (1:1 volume) and ethanol (final concentration = 5%) was added. For flat plate medium, 20 mL of the mixed medium was added into glass plates and solidified after cooling to room temperature. For slant medium 5 mL of the mixed medium was added into the test tubes (φ=18 mm), tilted up to 45 degrees and solidified.

2-2-3. Enrichment culture of ethanol-assimilating microbes

Approximately 0.1 – 0.2 g from solid medium or 100 μL from liquid medium containing ethanol-assimilating microbes cultured from soil was added into another liquid medium. The shaking culture was done at 30 °C for 7 days at pH 3, 7, and 10, and 500 μL of the culture broth were subcultured to flesh liquid medium. This shaking culture was repeated as shown in Figure 2.

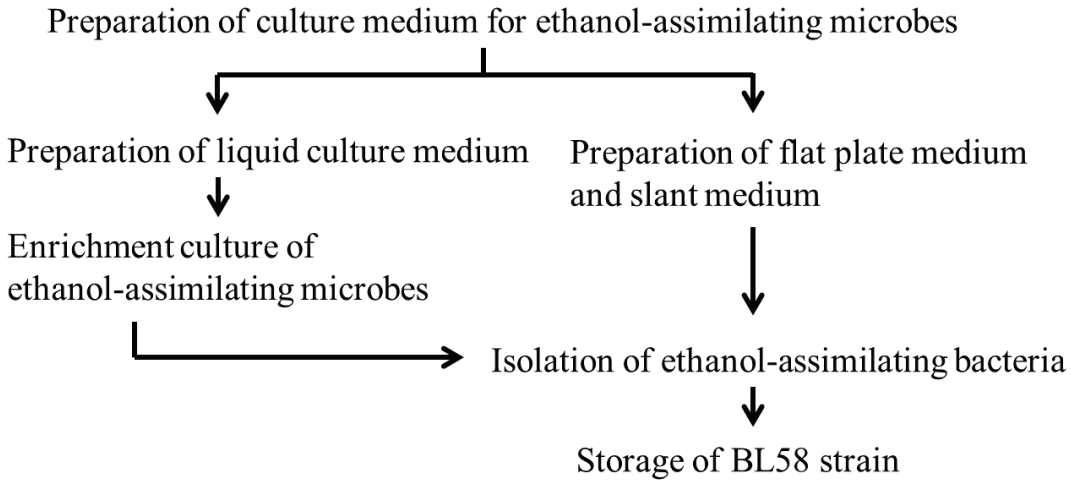


Figure 2 Culture of ethanol-assimilating bacteria and isolation

2-2-4. Isolation of ethanol-assimilating bacteria

After the enrichment culture, bacteria on the inside wall of test tube were collected using a vortex mixer and one inoculation loop of bacteria was inoculated on new flat plate medium. After 3-days cultivation at 30 °C, each colony was inoculated on new flat plate medium. This culture was repeated.

2-2-5 Storage of BL58 strain

The selected target strains through the magnifying glass were inoculated on new slant medium by inoculating loop and the cultivation was done at 30 °C. After 2 days, the test tubes were wrapped by parafilms for prevention of drying and were stored at 4 °C.

2-2-6 Culture of isolated BL58 strain

The BL58 strain isolated and stored was inoculated in new liquid medium including 5% ethanol and adjusting pH to 10. The shaking culture was done at 30 °C for 10 days. The growth of BL58 strain and the total sugar of BL58 polymer were measured.

2-2-7. Measurement of BL58 strain growth

The growth of BL58 strain was measured by spectrophotometer with a test tube holder (HITACHI Model 100-10) at OD 610 nm.

2-2-8. Measurement of total sugar

The total sugar of BL58 polymer was determined by phenol-sulfuric acid method as described below [26]. Firstly standard glucose solution were prepared from a 1% (w/v) stock glucose solution by two serial 10-fold dilutions to prepare the 100 μg/mL of glucose solution that was further diluted to 10 to 90 μg/mL standard glucose solutions.

Standard glucose solutions	1	2	3	4	5	6	7	8	9	10
100 (μg/mL) of glucose sol. (mL)	0	1	2	3	4	5	6	7	8	9
Distilled water (mL)	10	9	8	7	6	5	4	3	2	1

A 0.5 mL aliquot of sample or standard solution was added into Wassermann tube with 0.5 mL of 0.05 g/L Phenol solution and 2.5 mL of concentrated sulfuric acid and mixed. The concentration of each well-mixed sample was measured by spectrophotometer (SIMADZU UV-1200) at OD 490 nm. The total sugar levels of BL58 polymer samples were determined by extrapolation from the standard glucose solution reference curve.

2-2-9. Color reaction tests for sugar

Molisch reaction: 15% α-naphthol-ethanol solution was prepared by dissolving 15 g of naphthol in 100 mL of ethanol. Five drops of the 15% α-naphthol-ethanol was added into 1 mL of BL58 strain culture broth. 2 mL of concentrated sulfuric acid was added gently.

Phenol-sulfuric acid method: phenol solution was prepared by dissolving 5 g of phenol in 100 mL of distilled water, and 0.5 mL of phenol solution and 0.5 mL of BL58 strain culture broth were completely mixed. 2.5 mL of concentrated sulfuric acid was added gently and left to stand.

Anthrone-sulfuric acid method: anthrone solution was prepared by dissolving 200 mg of anthrone in 100 mL of 95% of cold sulfuric acid solution and adding 20 mL of cold distilled water. 3 mL of the anthrone solution and 0.5 mL of cold BL58 strain culture broth were mixed gently in cold water. This solution was heated in boiling water and cooled down using cold water.

2-2-10 Identification of BL58 strain

The isolated BL58 strain was sent to the National Collection of Industrial and Marine Bacteria Ltd (NCIMB) in Scotland and was analyzed.

2-3. Results and discussions

2-3-1. Isolation of ethanol-assimilating bacteria, BL58 strain

Enrichment culture using 176 soil samples collected from different areas were done and many ethanol-assimilating microbes were isolated (Table 4). Many microbes producing amino acids were confirmed and it indicated that fatty acids with structures including double bonds were produced and more analysis to detect the fatty acid will be required. Analysis of D-amino acid and higher amino acid produced by the ethanol-assimilating bacteria to understand the functional properties will be similarly required.

Table. 4 Isolated ethanol-assimilating microbes

Culture condition	pH 3	pH 7	pH 10	Total
Bacteria	19	127	52	198
Yeast	7	19	8	34
Actinomyces	0	42	19	61
Fungus	109	54	6	169
Total	135	242	85	462

In the screening of ethanol-assimilating microbe, more fungi were isolated under acid condition and more bacteria were under alkaline condition. In the condition of neutral pH, no significant difference was confirmed. In this research, it was focused on bacteria grown under alkaline condition at pH 10 and expected for them to produce unique functional products such as exopolysaccharides induced by the growth inhibition.

In preliminary experiments varying the ethanol concentration, the limitation of growth in the medium containing more than 7% ethanol and no growth in the medium containing 10% ethanol were observed.

2-3-2. Production of exopolysaccharides by BL58 strain

The isolated BL58 strain was inoculated in liquid medium including 5% ethanol and adjusting pH to 10 for 9 days. Very high viscous products were produced by the BL58 strain under the abnormal culture condition shown in Figure 3 and 4. The gelation of culture broth was started after 7 days cultivation and it was completed when total sugar concentration reached to around 3.5 g/L.

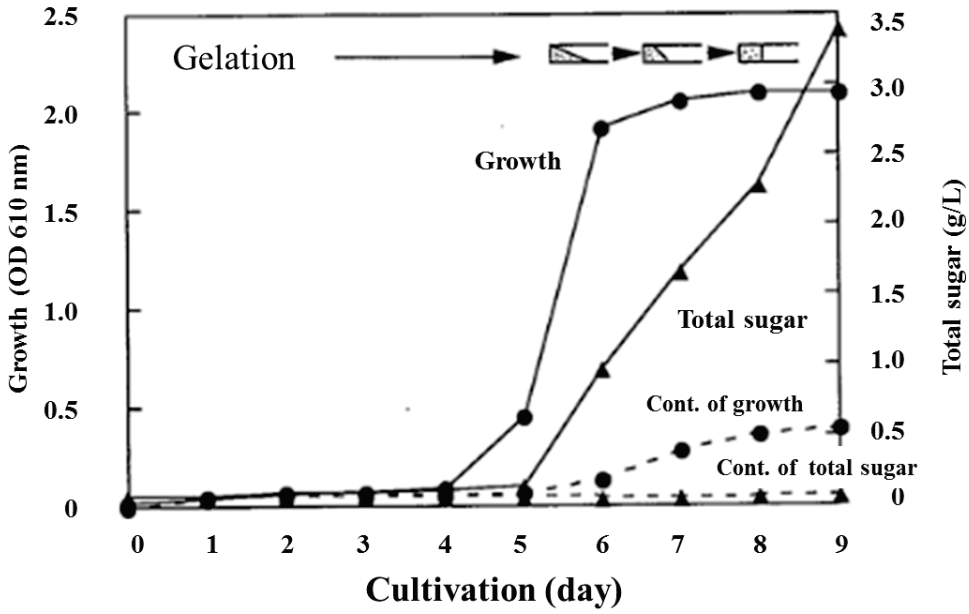


Figure 3 Culture of ethanol-assimilating bacteria, BL58

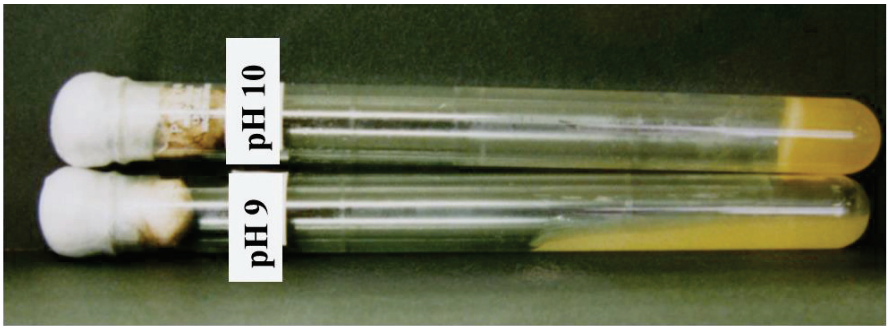


Figure 4 High viscous products produced by BL58 strain

The high viscosity of products suggested that they are polysaccharides. Color reaction tests for sugar such as Molisch, Phenol-sulfuric acid and Anthrone-sulfuric acid methods were performed and all tests showed positive results shown in Figure 5. The products were confirmed as polysaccharides.

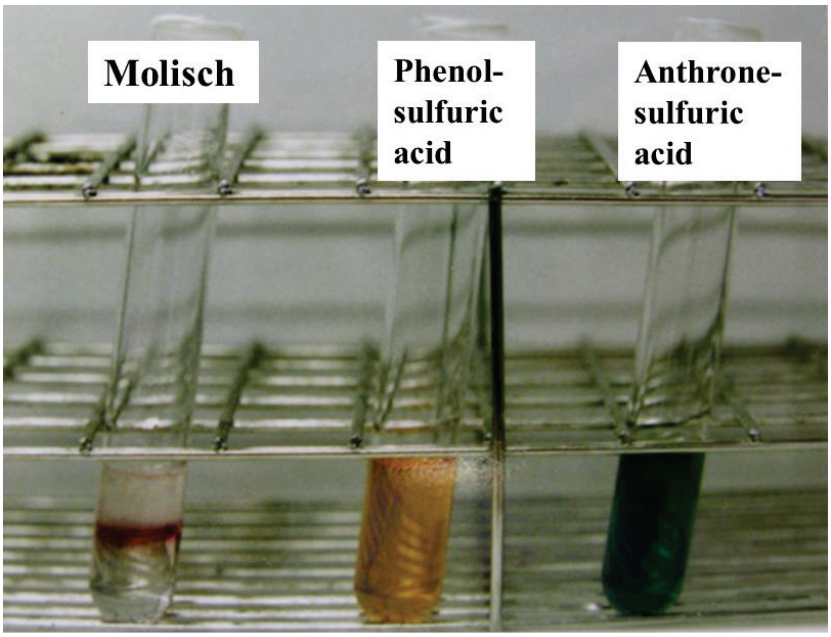


Figure 5 Color reaction tests for sugar

2-3-3. Identification of BL58 strain

The isolated BL58 strain was analyzed by NCIMB and was confirmed from the taxonomical studies that it was Gram-negative, rod-shaped (0.5 to 0.8 by 1.6 to 1.8 μm) and had polar flagella. It grew well in aerobic conditions. Nitrate reduction and indole production were negative, and catalase, oxidase and cytochrome oxidase were positive as shown in the Table 5. The strain was able to utilize glucose, maltose, gluconate, caprate, malate, citrate and starch as carbon sources. The base sequence of 16S rDNA of strain BL58 exhibited 99.5% homology to that of *Pseudomonas stutzeri*. From the results, the isolated strain was identified to be *Pseudomonas stutzeri* BL58. It was not pathogenic bacteria so the polysaccharides produced by *Pseudomonas stutzeri* BL58 could easily to be used for industrial applications. Therefore, a more detailed investigation of the BL58 polymer was conducted.

Table 5 Morphological and physiological characteristics of BL58 strain

Morphology			
Gram stain	Negative	Arginin dihydrolase	-
Spores	Not formed	Urease	-
Motility	Motile with polar flagella	Aesculin hydrolsis	-
Colony	Age 72 hours	Gelatin hydrlysis	-
Morphology	Irregular, undulate	β -garactosidase	-
	edge, cream, rough,	Glucose assimilation	+
	matt opaque,	Arainose assimilation	-
	low convex	Mannose assimilation	-
	2mm in diameter	Monnitol assimilation	-
°C growth	37 ℃ +	N-acetylglucosamine assimilation	-
	41 ℃ +	Maltose assimilation	+
	45 ℃ +	Glucconate assimilation	+
Physiology		Caprate assimilation	+
Catalase	+	Adipate assimilation	-
Oxidasa	+	Malate assimilation	+
NO_3 reduction	-	Citrate assimilation	+
Indole production	-	Phenylacetate assimilation	-
Acid from glucose	-	Cytochrome oxidase	+

Chapter 3

Optimization of culture conditions for BL58 strain

3-1. Introduction

The culture medium for BL58 strain as shown in Table. 3 was re-considered in order to optimize the culture conditions of BL58 strain such as pH, nitrogen and carbon source, ethanol concentration, etc. Based on the alkaline condition of the BL58 strain screening, the optimum pH of culture medium was initially investigated. Secondly, optimum nitrogen and carbon sources were evaluated. For this evaluation, organic nitrogen sources were used since inorganic ammonium salt was volatilized under alkaline condition. Lastly, optimum ethanol concentration considering the volatilization during aeration culture was studied. In this chapter, the culture condition for BL58 strain to produce higher viscosity and higher yield of BL58 polymer was optimized.

3-2. Materials and methods

3-2-1. Measurement of BL58 strain growth

The measurement method of BL58 strain growth is described in chapter 2-2-8.

3-2-2. pH measurement

The pH of the culture broth was measured by pH meter (HORIBA Compact pH Meter B-212).

3-2-3. Measurement of total sugar

The measurement method of total sugar is described in chapter 2-2-9.

3-2-4. Measurement of viscosity

The culture broth was diluted 5 fold, and the viscosity of the diluted broth was measured by rotary viscometer (DV-1+, Viscometer, Brook Field Co. Ltd.) at 30 °C. The values for viscosity were expressed in “cp per 1 rpm for 30 s”.

3-2-5. Measurement of ethanol reduction by HPLC

The culture broth was diluted 5 fold, and the cells in the diluted broth were removed by centrifugation at 12,000 rpm for 20 minutes (TOMY MRX-151). The supernatants were analyzed using a HPLC instrument (GILSON MODEL 305) and a glass syringe (HAMILTON 80300) to inject the sample. The conditions for HPLC are listed below.

Column	Shodex KS801
Column size	8 mm x 300 mm
Packing material	Strong-acid cation exchange resin
Temperature	70 °C
Extract	H_2O
Flow rate	0.5 mL/min
Detection	RI
Sample volume	20 μL

3-2-6. Measurement of ethanol reduction by gas chromatography

The culture broth was diluted 5 fold and the cells in the diluted broth were removed by centrifugation at 12,000 rpm for 20 minutes (TOMY MRX-151). The supernatants were analyzed using a gas chromatography instrument (SIMADZU GC-8A, flame ionization detector) and a grass syringe (HAMILTON 80300) for sample injection. The conditions for gas chromatography are listed below.

Column size	2.6 mm x 3 m
Packing material	Porapak TypeQ 50/80 (GL Sciences, Ltd.) + 80/100 (Waters)
Carrier gas	N_2O
Column temperature	From 130 °C to 210 °C, 5 °C/min
Injection temperature	240 °C
Range	10^2
Sample volume	2 μL

3-2-7. Optimization of initial pH in the culture medium

The culture medium shown in Table 3 (without carbon source) was prepared. The pH in the culture medium was adjusted to 9.0, 9.2, 9.4, 9.6, 9.8, 10.0, 10.2, 10.4, 10.6 and 10.8 using 5N-NaOH solution. 5mL of each pH culture medium was added in the test tubes that were plugged by cotton sterilized by dry heat. The autoclave sterilization of

the test tubes was carried out and ethanol (final concentration = 3%) was added to the culture medium under sterile conditions after the culture medium was cooled down. The BL58 strain was inoculated into the medium under sterile conditions and was cultivated at 30 °C with shaking. The growth of BL58 strain and the viscosity of the culture broth were measured every day. After the cultivation for 10 days, the pH and total sugar were measured.

3-2-8. Optimization of nitrogen source

The culture medium shown in Table 3 adding 0.5% each nitrogen source such as peptone, polypeptone, cornsteep liquor, $(NH_4)_2SO_4$, NH_4NO_3, KNO_3 or Urea (without carbon source) was prepared. The pH in the culture medium was adjusted to 10.0 using 5N-NaOH solution. 5mL of each culture medium was added in the test tubes that were plugged by cotton sterilized by dry heat. The autoclave sterilization of the test tubes was carried out and ethanol (final concentration = 3%) was added into the culture medium under sterile conditions after the culture medium was cooled down. The BL58 strain was inoculated into the medium in clean bench and was cultivated at 30 °C with shaking. After the cultivation for 0, 72 and 120 hours, the growth of BL58 strain and the total sugar in the culture broth were measured.

3-2-9. Optimization of carbon source

The culture medium shown in Table 3 (without carbon source) was prepared. The pH in the culture medium was adjusted to 10.0 using 5N-NaOH solution. 5mL of the culture medium was added into the test tubes that were plugged by cotton sterilized by dry heat. The autoclave sterilization of the test tubes was carried out and each carbon source such as methanol, ethanol, propanol, butanol, glycerol (final concentration 0.5%), glucose, fructose, sucrose, maltose, citrate, succinate or fumarate (final concentration 3%) was added into the culture medium in clean bench after the culture medium was cooled down. The BL58 strain was inoculated in the medium under sterile conditions and was cultivated at 30 °C with shaking. The growth of BL58 strain and the viscosity of the culture broth were measured every day. After the cultivation for 10 days, the pH and total sugar were measured.

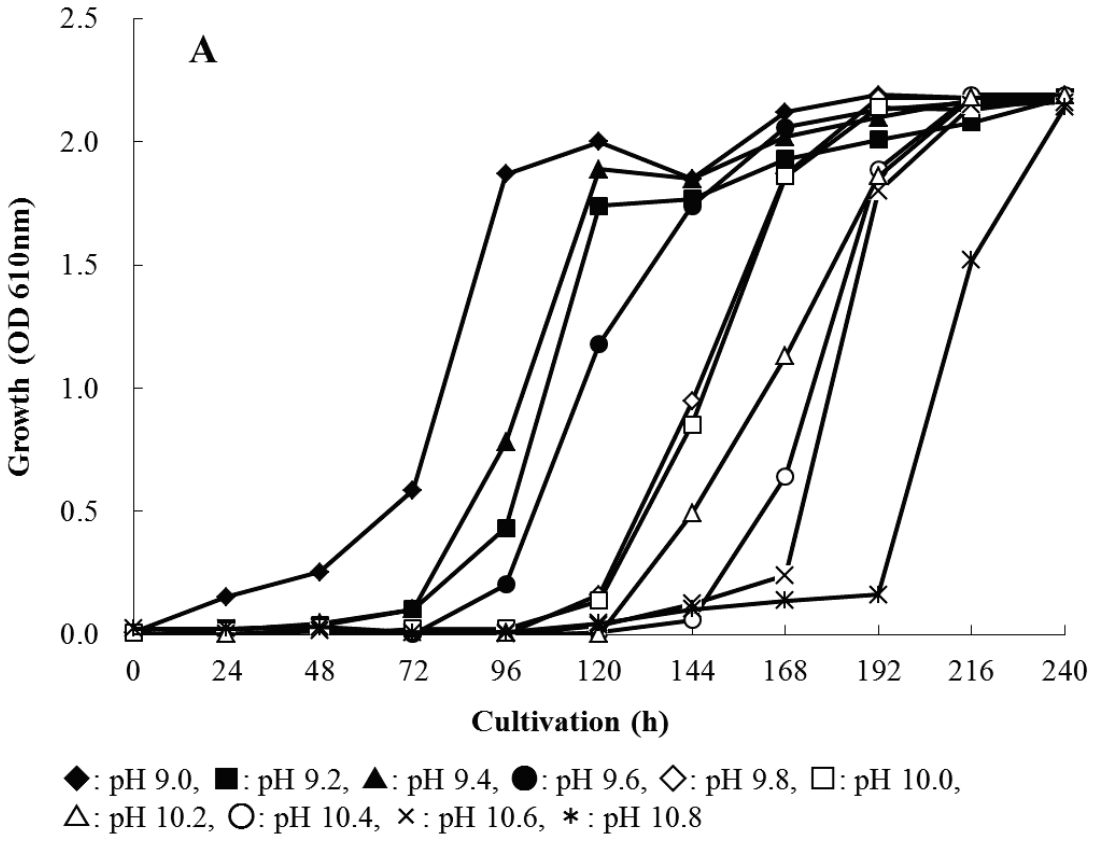
3-2-10. Optimization of ethanol concentration in the culture medium

The culture medium shown in Table 1 (without carbon source) was prepared. The pH in the culture medium was adjusted to 10.0 using 5N-NaOH solution. 5 mL of the culture medium was added in the test tubes that were plugged by cotton sterilized by dry heat. The autoclave sterilization of the test tubes was carried out and ethanol (final concentration 1, 2, 3, 4, 5, 6, 7, 8, 9 and 10%) was added into the culture medium in clean bench after the culture medium was cooled down. The BL58 strain was inoculated in the medium under sterile conditions and was cultivated at 30 °C with shaking. The growth of BL58 strain and the viscosity of the culture broth were measured every day. After the cultivation for 10 days, the pH and total sugar were measured.

3-3. Results and discussions

3-3-1. Optimization of initial pH in the culture medium

Using minimal medium containing 3% ethanol as sole carbon source, initial pH of the culture medium was varied from 7 to 11. The strain exhibited growth in the range of pH 8 to 10, and the gelation of culture broth with accumulation of BL58 polymer was observed at pH 10 (data not shown). A more detailed examination of the growth and polymer production was made from 9 to 10.8 (Figure 6). Although growth was delayed with pH rise, BL58 polymer was accumulated at pH above 9.8, optimally at pH 10.0, as measured by total sugar of cultured broth. After 120 hours cultivation, the accumulation of BL58 polymer reached 4.5 g/L of total sugar at pH 10.0.



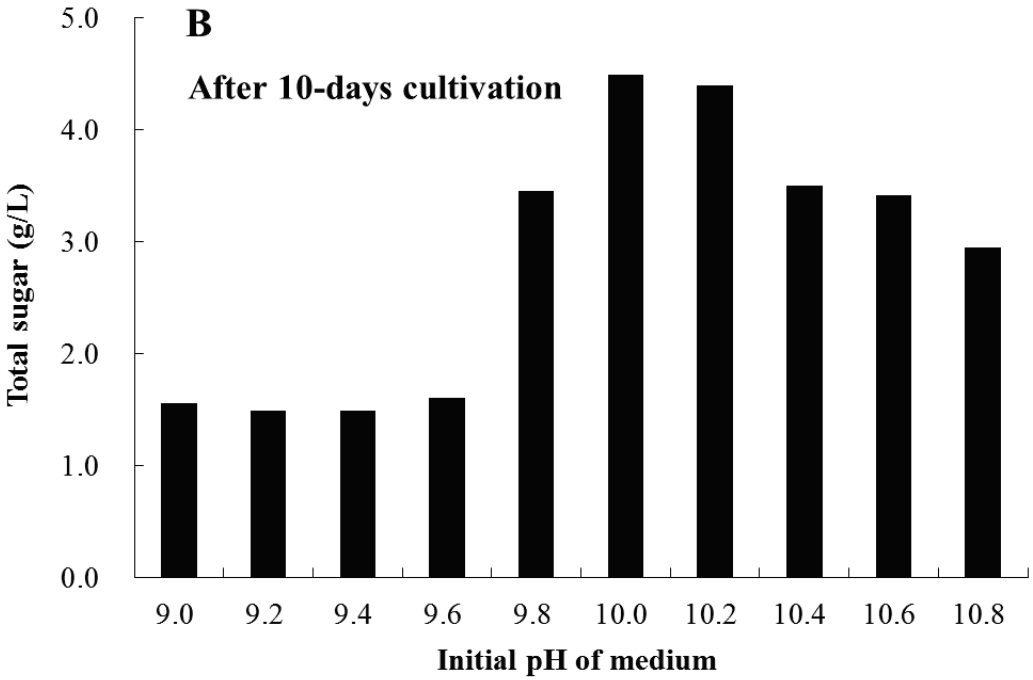
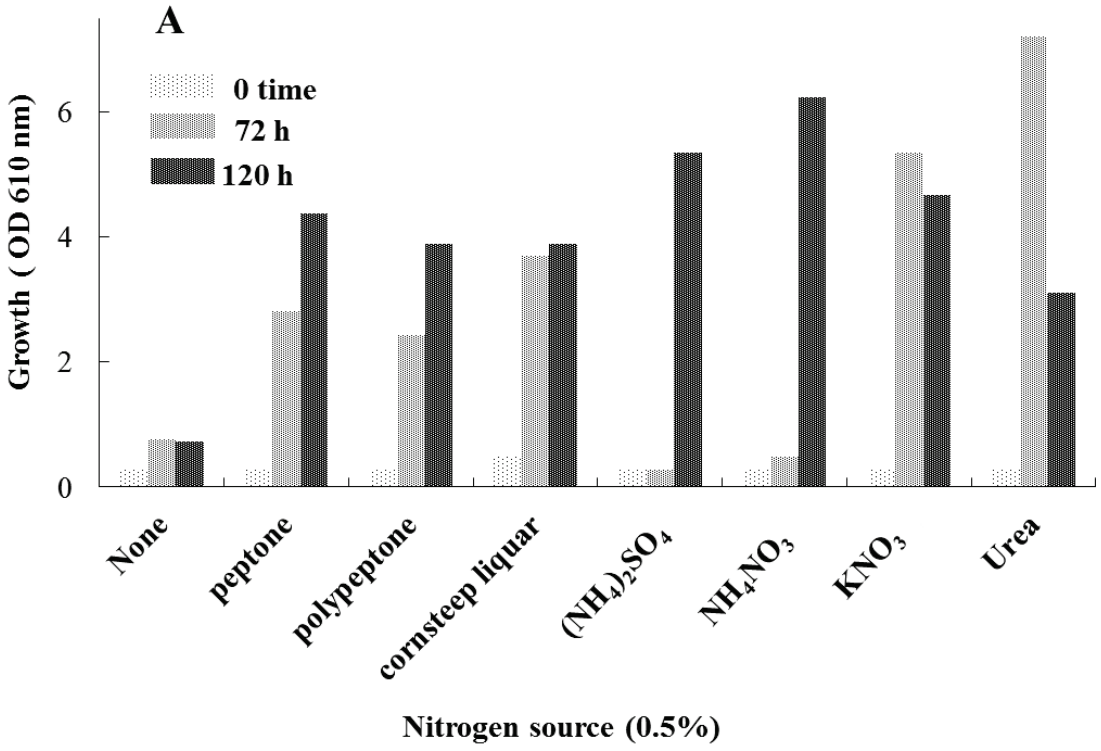


Figure 6 Effect of initial pH on growth and BL 58 polymer production

A) Effect of initial pH on growth of strain BL58, B) Effect of pH on BL58 polymer production. Closed rhombus indicate pH 9.0, close squares pH 9.2, closed triangles pH 9.4, closed circles pH 9.6, open rhombus pH 9.8, open squares pH 10.0, open triangles pH 10.2, open circles pH 10.4, crosses pH 10.6 and asterisks 10.8

3-3-2. Optimization of nitrogen source

The effect of nitrogen source was examined using organic- and inorganic nitrogen (Figure 7). Higher growth (OD 610 nm) was observed with inorganic nitrogen such as $(NH_4)_2SO_4$, NH_4NO_3, KNO_3 or Urea but higher concentration of polymer production was obtained with organic nitrogen such as peptone, polypeptone and cornsteep liquor. Based on the previous consideration, it was confirmed that ammonium nitrogen was evaporated by autoclave. Peptone or polypeptone composed of amino acids is significantly better nitrogen source than ammonium nitrogen.



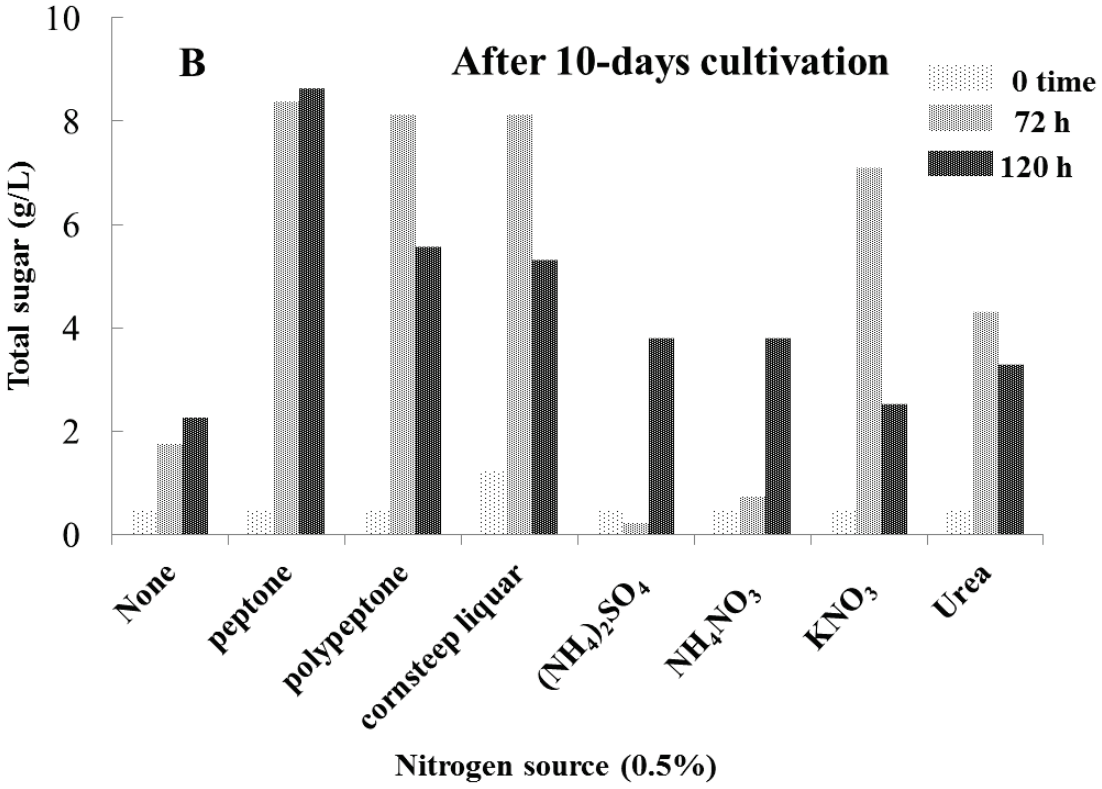
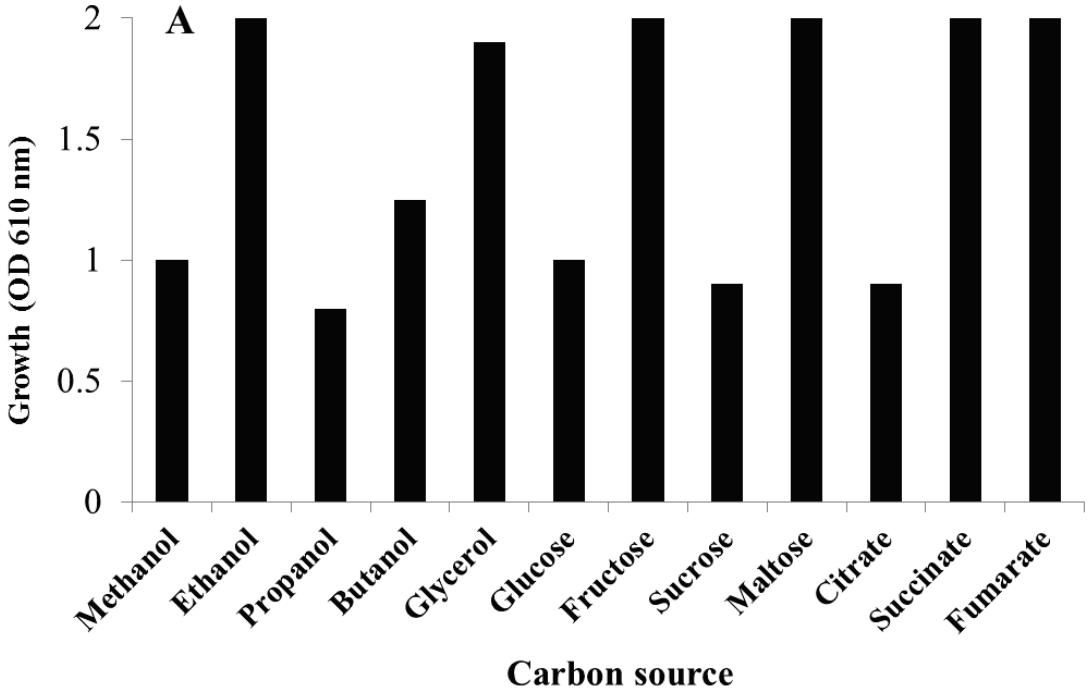


Figure 7 Effect of nitrogen source on growth and BL 58 polymer production

A) Effect of nitrogen source on growth of strain BL58
B) Effect of nitrogen source on BL58 polymer production.

3-3-3. Optimization of carbon source

The effect of carbon source was examined using five alcohols, four sugars and three organic acids (Figure 8). The highest growth (OD 610 nm >2.0) was observed with ethanol and glycerol as alcohols, fructose and maltose as sugars and succinate or fumarate as organic acids in the medium after 24 hours cultivation. The highest concentration of polymer production was obtained with ethanol, glucose and maltose in the medium after 81 hours cultivation. In general, it is known that two molecules of glucose were produced by hydrolyzing maltose and two molecules of ethanol were produced by alcohol fermentation. It was made clear that the main constituent sugar of the BL58 polymer was glucose and the polymer was formed by condensation of sugar constituents. Therefore, ethanol is the optimal carbon source for the BL58 strain.



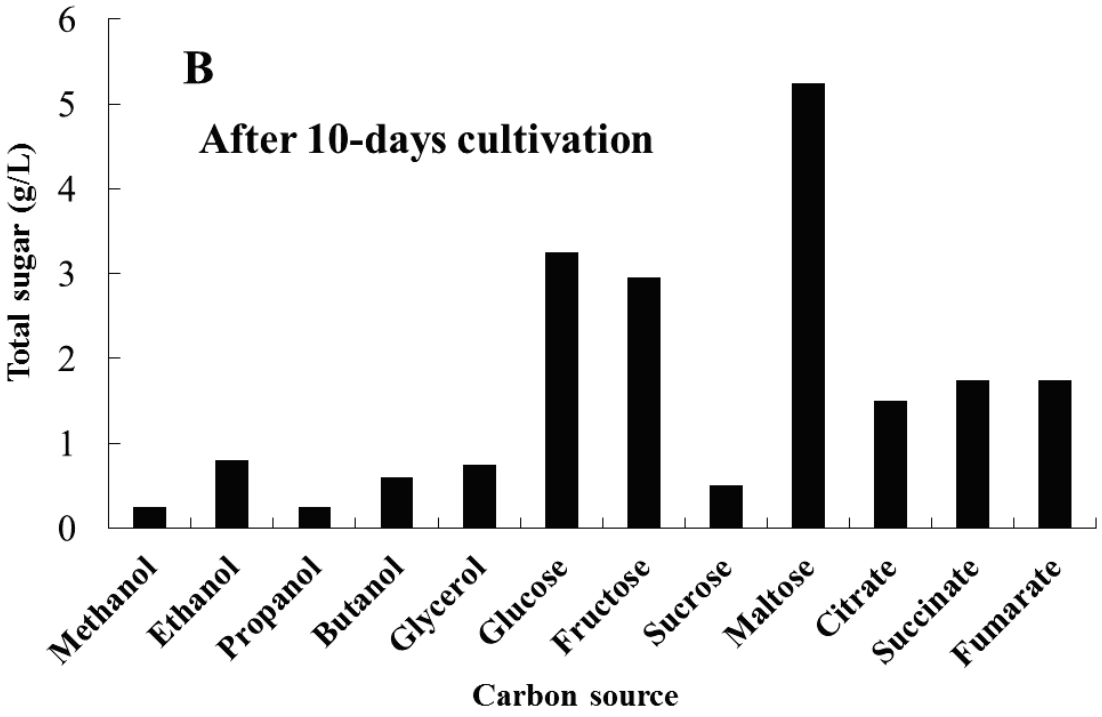
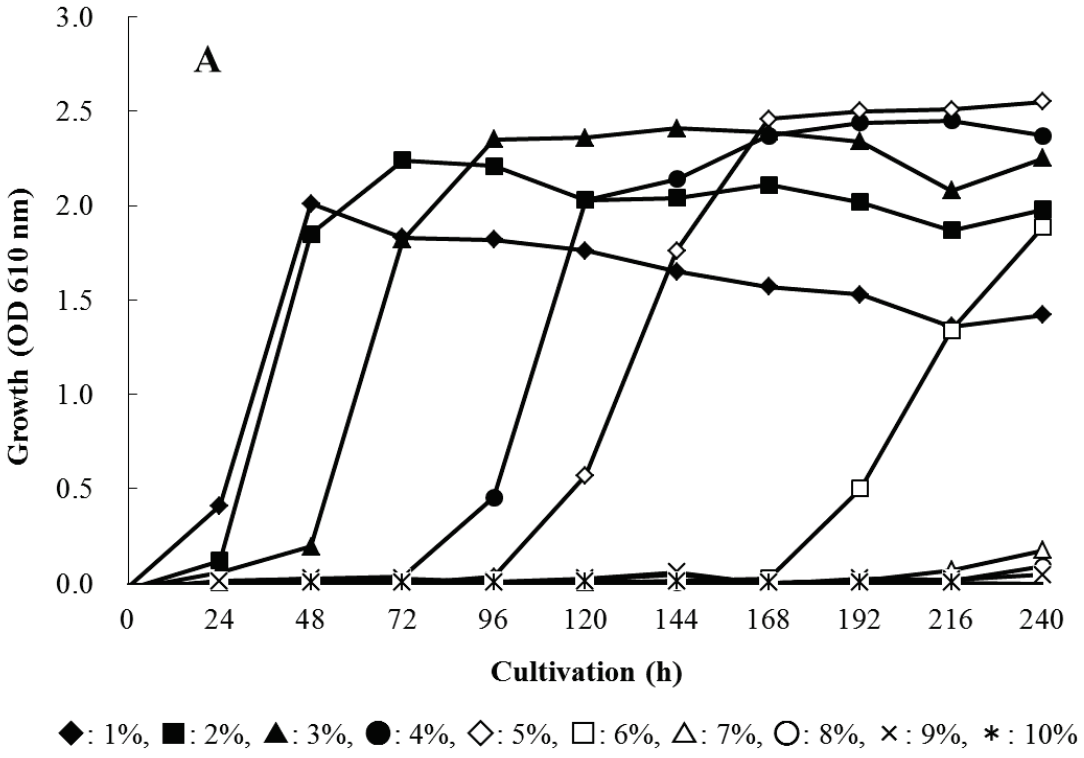


Figure 8 Effect of carbon source on growth and BL58 polymer production

A) Effect of carbon source on growth of strain BL58,
B) Effect of carbon source on BL58 polymer production.

3-3-4. Optimization of ethanol concentration in the culture medium

The effect of ethanol as sole carbon source was examined from 1 to 10% in the culture medium (Figure 9). Up to 6% ethanol, the turbidity derived from growth reached similar levels (OD 610 nm >2.0) independent of the period of lag phase. During the cultivation at pH 10, gelation of cultured broth was observed for the addition of 3% to 5% ethanol, indicating that BL58 polymer was excreted outside of *P. stutzeri* BL58 cells at relatively high concentration of ethanol. The highest concentration of polymer production was obtained with 3-4% ethanol in the medium after 240 hours cultivation.



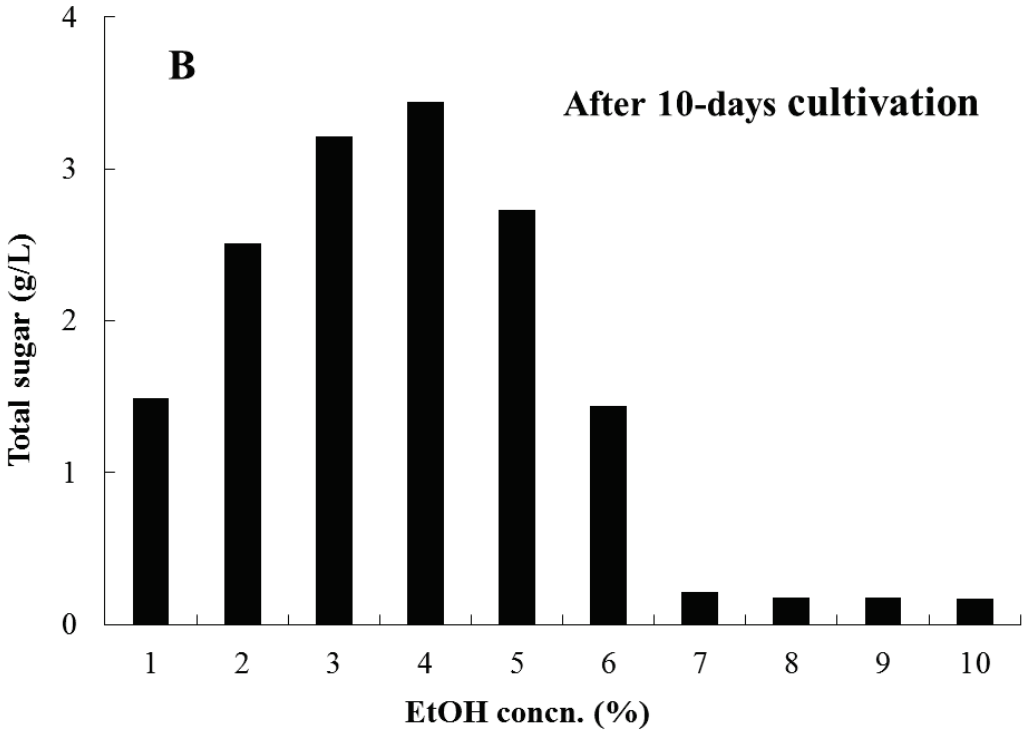


Figure 9 Effect of ethanol concentration on growth and BL58 polymer production

A) Effect of initial pH on growth of strain BL58, B) Effect of initial pH on BL58 polymer production. Closed rhombus indicate 1% ethanol, close squares 2%, closed triangles 3%, closed circles 4%, open rhombus 5%, open squares 6%, open triangles 7%, open circles 8%, cross 9% and asterisks 10%

Exopolysaccharides were secreted by BL58 polymer under alkaline condition as extreme environment. This is similar to the "survival response" when microbes form biofilm under extreme environmental conditions.

It was confirmed that ethanol and organic acid were useful as carbon sources, considering that these carbon sources can be catabolized and converted to sugar through the gluconeogenic metabolic pathway. It was also confirmed that organic nitrogen was effective as a nitrogen source for growth and polymer production. On the other hand, high concentration of ethanol added as a carbon source caused significant delay in the growth and polymer production suggesting that sequential addition of low concentration ethanol would be useful as an industrial process for polymer production. In the next section, conditions of large scale of BL58 polymer are evaluated.

Chapter 4

Batch fermentation for BL58 polymer

4-1. Introduction

In the previous experiments, the production of BL58 polymer was observed and the viscosity of culture broth was increased during shaking cultivation in test tubes. Limited aeration for growth of the BL58 strain was also observed. In this chapter, the large scale fermentation of BL58 polymer using a jar fermenter with aerated mixing was evaluated, along with the sequential addition of ethanol as the sole carbon source. Ethanol was added every 24 hours in order to compensate for the release of volatile ethanol from the culture broth and keep the concentration close to 1%.

4-2. Materials and methods

Two nitrogen sources for the minimal medium, 0.5% inorganic nitrogen salts (NH_4NO_3) and 0.5% peptone for the production of the BL58 polymer were evaluated. Inoculum (50 ml) of BL58 strain were grown for 48 hours with vigorous shaking at 30 °C using the medium supplemented with 3% ethanol as a sole source of carbon. These cultures were inoculated into a 3 L fermenter (New Brunswick Bioflo/Celligen 115, USA) containing 1 L of fresh culture medium supplemented with periodic addition of 1% ethanol every 24 hours. The fermenter temperature was controlled at 30 °C, and the air flow rate was 1.0 L/min with stirring rate of 500 rpm. Sampling was collected every 24 hours, and the growth (OD 610 nm) of BL58 strains, viscosity, pH and total sugar in the culture broth were measured.

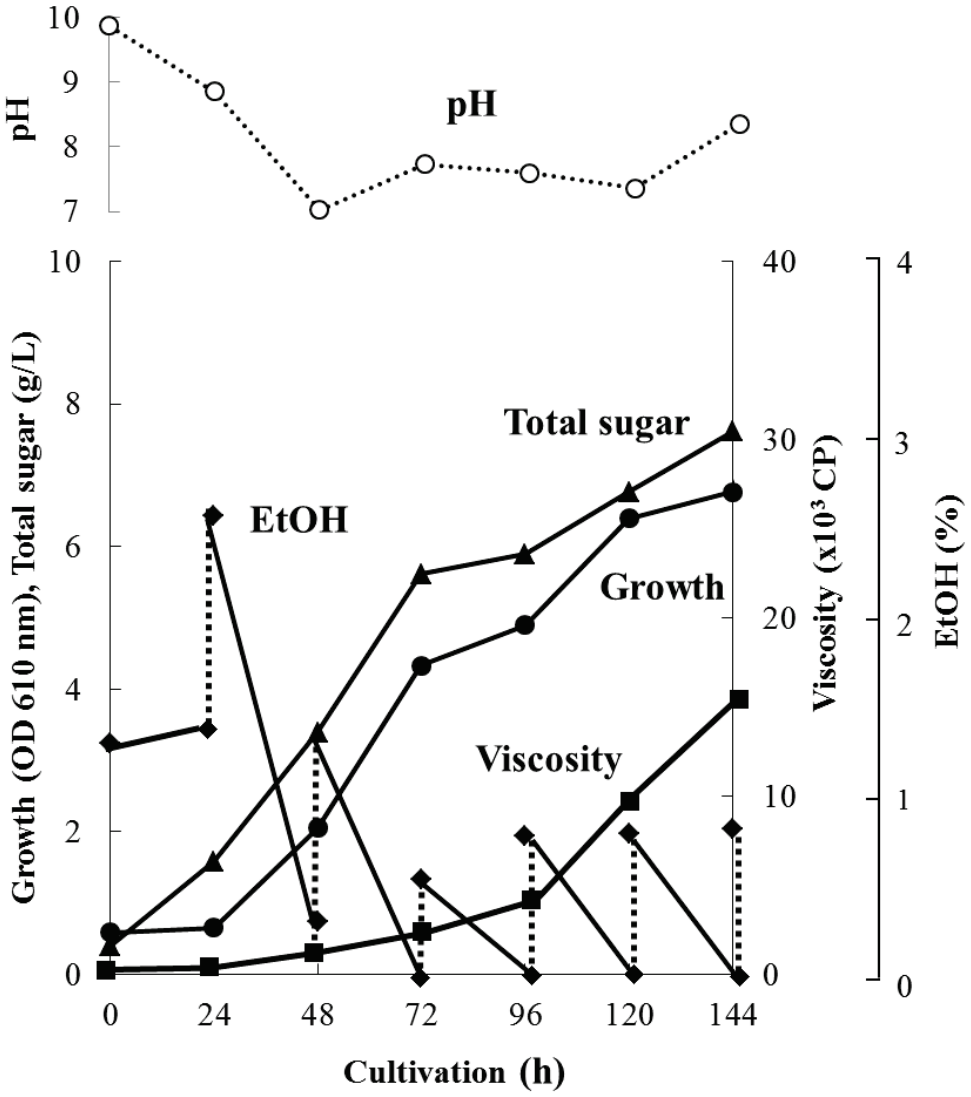
4-3. Results and discussions

In the case of using 0.5% NH_4NO_3 as a nitrogen source, the reduction of added ethanol for growth of the strain and the production of BL58 polymer were observed after the cultivation for 48 hours (Figure 10). Although the amount of added ethanol reached 60 g/L with 144 hours of cultivation, total sugar of the culture broth was still 7 g/L representing about 10 % recovery from ethanol. Since the growth of the strain was enough to produce BL58 polymer, the remainder of utilized ethanol seemed to be catabolized to other metabolites.

However, in the case of using 0.5% peptone as a nitrogen source, the rapid growth of the BL 58 strains was observed and 12.5 g/L of BL58 polymer was produced within 72 hours of cultivation (Figure 11). Although the strain seemed to be able to produce more BL58 polymer, further fermentation was difficult because of gelation of the broth by accumulated polymers. The most efficient fermentation condition for BL58 polymer production was established as one in which ethanol was added sequentially to the medium supplemented with 0.5% peptone as a nitrogen source.

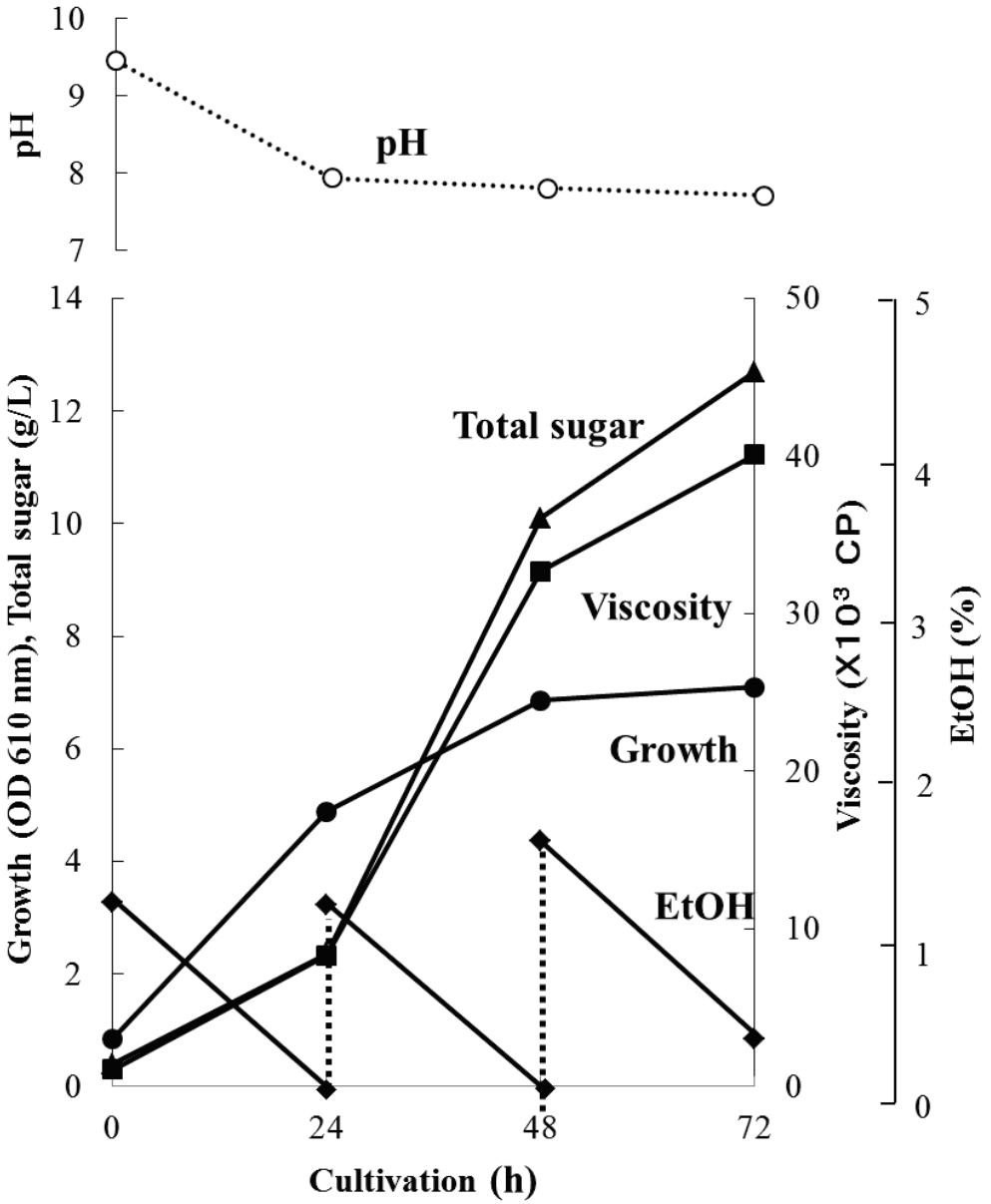
Based on the raw materials in the optimum culture medium containing 0.5% peptone as shown in Table 6, the production cost of BL58 polymer was estimated. Total cost of raw materials for 1 litter of culture medium was 262.44 Japanese yen. From this culture medium, 12.5 g/L of BL58 polymer was produced. Therefore, cost for producing 1 g of BL58 polymer was 21 Japanese yen.

High grade Xanthan gum is a food additive agent that is commercially available, and it costs 15 – 22 yen per gram depending upon amount-purchased. Gellan gum is also sold widely, and it costs 26-30 yen per gram. Therefore, the cost for BL58 polymer is comparable to products sold for various industrial applications.



Closed rhombus indicate ethanol, close squares viscosity, closed triangles total sugar, closed circles growth (OD 610 nm) and open circles culture pH

Figure 10 Time course of BL58 polymer fermentation supplemented with 0.5% NH_4NO_3 as a nitrogen source



Closed rhombus indicate ethanol, close squares viscosity, closed triangles total sugar, closed circles growth (OD 610 nm) and open circles culture pH

Figure 11 Time course of BL58 polymer fermentation supplemented with 0.5% peptone as a nitrogen source

Table. 6 Production cost for BL58 polymer

Raw material	Amount	Cost
Ethanol	30 mL/L	80 Yen/L
Peptone	5.0 g/L	156
KH_2PO_4	1.5	3.75
K_2HPO_4	0.67	2.01
$MgSO_4 \cdot 7H_2O$	0.3	1.38
$CaCl_4 \cdot 2H_2O$	0.01	0.026
$ZnSO_4 \cdot 7H_2O$	0.01	0.0188
$FeSO_4 \cdot 7H_2O$	0.01	0.0196
$MnSO_4 \cdot 7H_2O$	0.001	0.034
Yeast extract	0.3	19.2
	Total cost	262.44 Yen
	Cost / g polymer	21.00 Yen

Chapter 5

Purification of high exopolysaccharides produced by BL58 strain

5-1. Introduction

Typically, microbial exopolysaccharides are easily purified by addition of organic solvent and collection of the precipitate. However, in the preliminary evaluation of BL58 polymer solution, acetone and ethanol were used but no precipitation of polysaccharide was confirmed. This suggested that BL58 polymer might have unique structural properties that had not been previously reported.

For a large scale purification method for the polymer produced at the industrial level, simple and low cost of steps are important and required. In this chapter, the purification methods, in order to remove the cellular material and contaminants and to obtain only target BL58 polymer exopolysaccharides as freeze-dried powder, were evaluated.

5-2. Materials and methods

5-2-1. Removal of bacterial cells

Two methods to remove the BL58 cellular material were evaluated. One was a method to centrifuge the culture broth after bacteriolytic process adjusted pH to 13 using 5N-NaOH solution, and another method was to centrifuge the culture broth after 5 times dilution.

5-2-2. Removal of contaminants

Two methods to remove the contaminants such as proteins, nucleic acids and oligosaccharides were evaluated. One was to centrifuge it after adding 5N-TCA solution to the final concentration of 2.5% and another method was dialysis.

5-2-3. Purification of BL58 polymer

The BL58 cellular material in the culture broth and the contaminants were initially removed shown as above. The residue was lyophilized and the polymer power was obtained (Figure 12).

5-2-4. Water solubility of purified BL58 polymer

1% (w/v) of purified BL58 polymer solution was prepared and was observed every 24 hours. The color of the solution was checked after it was uniformly dissolved and the pH was measured by using pH indicator paper.

5-2-5. Detection of contaminating protein

1% (w/v) of purified BL58 polymer solution was prepared. The Bio-Rad Protein Assay kit (Bio-Rad) was diluted 5-fold and added at 50 times the volume to the BL58 polymer solution. Ninhydrin reagent was prepared, added into the polymer solution and boiled

5-2-6. Use of activated charcoal

The freeze-dry BL58 polymer purified by Method 2 in Figure 12 was used and 0.25% (w/v) was prepared. The activated charcoal was added into 10 mL of 0.25% BL58 polymer solution as shown below and mixed.

0.25% BL58 Polymer solution	Ratio	Activated Charcoal
10 mL	-	0 g
10 mL	1 : 0.1	0.0025 g
10 mL	1 : 0.5	0.0125 g
10 mL	1 : 1	0.025 g
10 mL	1 : 2	0.05 g
10 mL	1 : 5	0.125 g
10 mL	1 : 10	0.25 g

The mixed solution was added into Wassermann tube and was centrifuged at 10,000 rpm for 5 minutes. The odor and absorbance at OD 610 nm of the supernatant were measured.

5-2-7. Use of Hyflo super-cell

The freeze-dry BL58 polymer purified by Method 2 in Figure 12 was used and 0.25% (w/v) was prepared. The Hyflo super-cell, diatomaceous earth, was added into 10 mL of 0.25% BL58 polymer solution as shown below and mixed.

0.25% BL58 Polymer solution	Ratio	Hyflo super-cell
10 mL	-	0 g
10 mL	1 : 0.1	0.0025 g
10 mL	1 : 0.5	0.0125 g
10 mL	1 : 1	0.025 g
10 mL	1 : 2	0.05 g
10 mL	1 : 5	0.125 g
10 mL	1 : 10	0.25 g

The mixed solution was added into Wassermann tube and was centrifuged at 10,000 rpm for 5min. The odor and absorbance at 610 nm of the supernatant were measured.

5-2-8. Use of combination of activated charcoal and Hyflo super-cell

The supernatants collected by the best condition methods described in 5-2-6 (BL58 polymer : activated charcoal = 1 : 2) and 5-2-7 (BL58 polymer : Hyflo super-cell = 1 : 5 or = 1 : 10) were selected. The Hyflo super-cell or activated charcoal was added into 2 mL of the supernatant as shown below and mixed.

Supernatant using activated charcoal	Ratio	Hyflo super-cell
2 mL	-	0 g
2 mL	1 : 10	0.05 g
2 mL	1 : 5	0.025 g

Supernatant using Hyflo super-cell	Ratio	Activated charcoal
2 mL (1:5)	-	0 g
2 mL (1:5)	1 : 2	0.01 g
2 mL (1:10)	-	0 g
2 mL (1:10)	1 : 2	0.01 g

The mixed solution was added into Wassermann tube and was centrifuged at 10,000 rpm for 5 minutes. The odor and absorbance at OD 610 nm of the supernatant were measured.

5-3. Results and discussions

5-3-1. Evaluation of purification method

Two established methods for removing the bacterial cells and the contaminating proteins, nucleic acids, and other cellular materials are shown in the Figure 12.
The properties of BL58 polymer solution using the polymer powder purified by these methods were investigated.

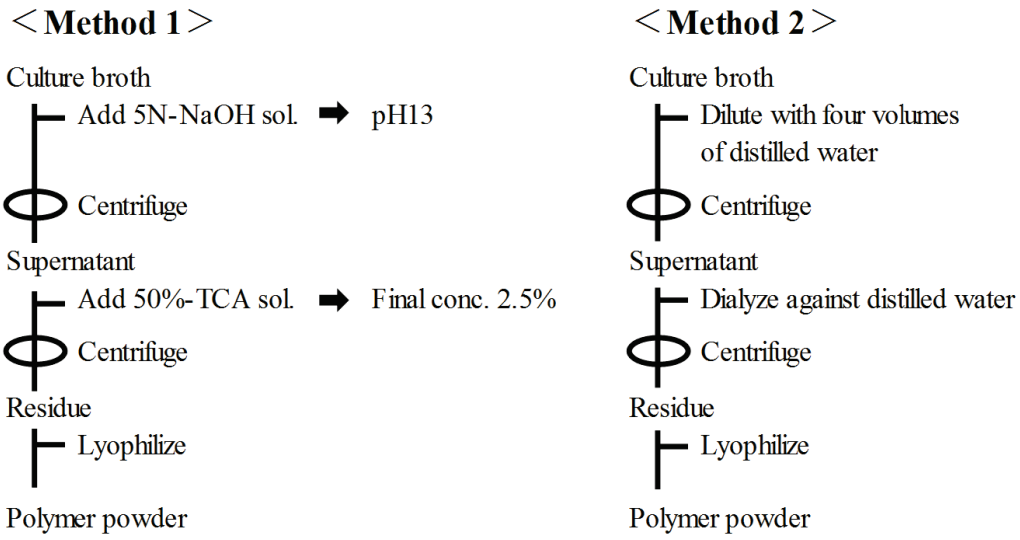


Figure 12 Purification methods of BL58 polymer

5-3-2. Water solubility of purified BL58 polymer and the contaminating protein

The results on the water solubility of purified polymer were shown in the Table 7. The polymer purified by the Method 1 was possible to be pulverized and included no contaminating protein but it was lacking in water solubility and was acidic in solution. The polymer purified by the Method 2 was highly soluble in water and was neutral in pH, but it was not possible to be pulverized and contained some contaminating proteins.

The two established purification methods were not capable of obtaining both high water soluble and pure polymer. The purified BL58 polymer still contained odor,

coloring and protein contamination. Therefore, further studies were needed to find a better purification method for the BL58 polymer.

Table 7 Properties of purified BL58 polymer from method 1 and 2

	Purified by Method 1	Purified by Method 2
Polymer after freeze-dry	Possible to be pulverized	Impossible to be pulverized
Polymer solution		
Water-soluble	-	+
pH	Acid	Neutral
color	White	Pale yellow
Bio-Rad Protein Kit	Positive	Positive
Ninhydrin reaction	Negative	Positive

5-3-3. Effect of activated charcoal

The measurement results are shown in the Table 8-1. The odor was removed by all concentrations of activated charcoal. The absorbance at OD 610 nm was not high (ranged from 0.357 to 0.426). The coloring, odor and some bacteria were removed by adding the activated charcoal.

5-3-4. Effect of Hyflo super-cell

The measurement results are shown in the Table 8-2. The odor was removed by all concentrations of Hyflo super-cell, similar to the activated charcoal. The absorbance at OD 610 nm was not high (ranged from 0.345 to 0.445). The coloring, odor and some bacteria were removed by adding the hyflo super-cell.

5-3-5. Effect of combination of charcoal activated and hyflo super-cell

The measurement results are shown in the Table 8-3. The odor was already removed in the first step using charcoal activated or hyflo super-cell. The absorbance at OD 610 nm after using both treatments was much lower than the use of only one. Therefore, the steps of purification method using both of activated charcoal and Hyflo super-cell was superior. In addition, the recovery of the BL58 polymer was obtained at high efficiency. In this present study, two purification methods were established as shown in Figure 13.

Table 8-1 Effect of addition of activated charcoal on purification of BL58 polymer

BL58 polymer : Activated Charcoal	Odor	OD 610 nm
1 : 0	no	0.417
1 : 0.1	no	0.426
1 : 0.5	no	0.407
1 : 1	no	0.372
∗ 1 : 2	no	0.357
1 : 5	no	0.367
1 : 10	no	0.390

Table 8-2 Effect of addition of Hyflo super-cell on purification of BL58 polymer

BL58 polymer : Hyflo super-cell	Odor	OD 610 nm
1 : 0	no	0.430
1 : 0.1	no	0.426
1 : 0.5	no	0.445
1 : 1	no	0.426
1 : 2	no	0.397
∗ 1 : 5	no	0.370
∗ 1 : 10	no	0.345

Table 8-3 Effect of addition of absorbents on purification of BL58 polymer

BL58 polymer : Activated charcoal	BL58 polymer : Hyflo super-cell	Odor	OD 610 nm
1 : 2	1 : 0	no	0.318
1 : 2	1 : 5	no	0.158
∗ 1 : 2	1 : 10	no	0.130

BL58 polymer : Hyflo super-cell	BL58 polymer : Activated charcoal	Odor	OD 610 nm
1 : 5	1 : 0	no	0.192
∗ 1 : 5	1 : 2	no	0.100
∗ 1 : 10	1 : 0	no	0.136
1 : 10	1 : 2	no	0.142

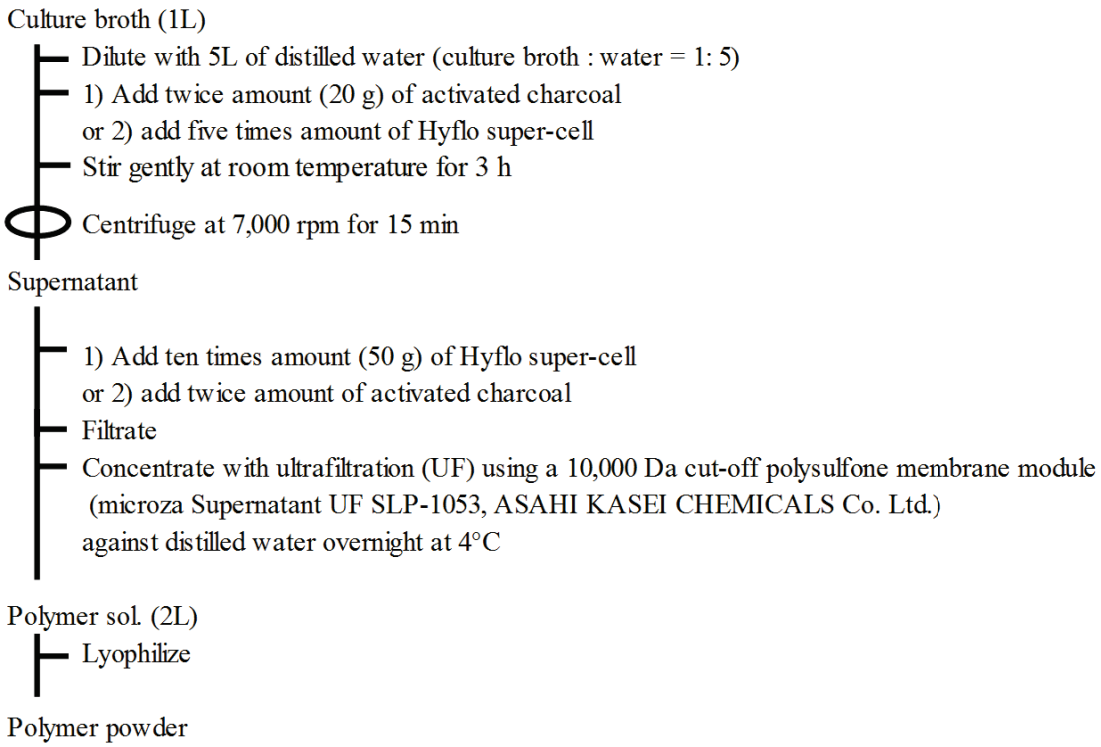


Figure 13 Improved purification methods of BL58 polymer

Chapter 6

Physical properties of BL58 polymer

6-1. Introduction

An effective purification method for the BL58 polymer was developed by combining adsorption with activated charcoal, filtration with Hyflo super-cell and dialysis with UF membrane as described in Figure 13. Using this method, BL58 polymer was purified from 1 L of the 72 h culture broth from the jar-fermenter, and isolated as lyophilized powder.

Microbial polysaccharides such as xanthan gum, gellan and pullulan etc. shown in the Table. 2 are known as thickener used for various foods. Therefore, in this chapter, the physical properties of BL58 polymer for food industry and industrial applications are investigated.

6-2. Materials and methods

6-2-1. Molecular mass determination

Three kinds of 1% (w/v) dextran solution, blue dextran (average molecular weight: 2,000 kDa), high dextran (average molecular weight: 170-200 kDa) and low dextran (average molecular weight: 50-70 kDa), were prepared and 10uL of each dextran solution was injected into HPLC system. The conditions of the HPLC are listed below.

Column	Shodex Asahipak G3000PW
Column size	7.5 mm × 300 mm
Packing material	Strong-acid cation exchange resin
Temperature	RT
Extract	H_2O
Flow rate	0.5 mL/min
Detection	RI
Sample volume	10 μL

A calibration curve of the semi-logarithmic plot (x axis: retention time, y axis: molecular weight) was made using the data from the Dextran reference materials. 0.5% (w/v) of BL58 polymer was prepared and 10 μL of it was similarly injected into the

HPLC system. The molecular weight of BL58 polymer was calculated based the retention time compared to the calibration curve.

6-2-2. pH measurement

The measurement method of pH is described in chapter 3-2-2.

6-2-3. Measurement of total sugar

The measurement method of total sugar is described in chapter 2-2-9.

6-2-4. Measurement of viscosity

The measurement method of viscosity is described in chapter 3-2-4.

6-3. Results and discussions

The summary of the physical properties are shown in the Table 9. The purified polymer was white, odorless-powder and the purity (total sugar content) was estimated to be 96.6% by the phenol-sulfate method. The purified polymer had a mean molecular weight of approximately 1,800 kDa and its viscosity and pH of the 1% solution were 160,000-250,000 cp and 6.3, respectively. The number of viable bacteria in the 1% solution was <10 cells/ml. The weight loss of the polymer after desiccation at 105 ºC for 4 hours was <10%. Concentration of Pb and AS_2O_3 were <10 μg/g and <4 μg/g, respectively.

Table 9 Physical properties of BL58 polymer

Powder properties	White, Odorless
Purity	96.6%
Molecular weight (ave.)	1,800kDa
Viscosity (1% sol.)	160,000-250,000 cp
pH (1% sol.)	6.3
Number of viable bacteria (1% sol.)	< 10 cells/ml
Loss on drying (105°C, 4hrs)	< 10%
Concentration of Pb	< 10 μg/g
Concentration of AS_2O_3	< 4 μg/g

Chapter 7

Rheological properties of BL58 polymer

7-1. Introduction

In the previous chapter, the physical properties were analyzed. In order to understand the rheological property of BL58 polymer, the effects of BL58 polymer concentration, temperature and pH on BL58 polymer solution viscosity were analyzed. In this chapter, the rheological properties of BL58 polymer were investigated.

7-2. Materials and methods

7-2-1. Effect of BL58 polymer concentration

0 – 1.5% (w/v) of BL58 polymer solution was prepared, heated up by microwave and stored in the refrigerator overnight. After uniformly dissolved polymer solution was confirmed, the viscosity was measured by rotary viscometer (DV-1+, Viscometer, Brook Field Co. Ltd.) at 30 °C and the values were expressed in "cp per 1 rpm for 2 min"

7-2-2. Effect of temperature on BL58 polymer solution

1% (w/v) of BL58 polymer solution was prepared, heated up by microwave and stored in the refrigerator overnight. After uniformly dissolved polymer solution was confirmed, the solution was incubated from 90 °C to 0 °C and the viscosity was measured by rotary viscometer (DV-1+, Viscometer, Brook Field Co. Ltd.) at 30 °C and the values were expressed in "cp per 1 rpm for 2 min"

7-2-3. Effect of pH on BL58 polymer solution

2% (w/v) of BL58 polymer solution was prepared, mixed with each buffer solution (Citrate-HCl, Na-phosphate and Tris-NaOH) at a rate of 1:1, heated up by microwave and stored in the refrigerator overnight. After uniformly dissolved polymer solution was confirmed, the solution was incubated at 30 °C for 10 minutes and the viscosity was measured by rotary viscometer (DV-1+, Viscometer, Brook Field Co. Ltd.) at 30 °C and the values were expressed in "cp per 1 rpm for 2 min".

7-3. Results and discussions

7-3-1. Effect of BL58 polymer concentration

Effect of concentration on viscosity of the polymer solution was shown in Figure 14-1. The viscosity of the polymer solution logarithmically increased above 0.6% (w/v) and the solution formed viscoelastic gel. The viscosity of 1% (w/v) of the polymer solution was estimated to be 280,000 cp and it was 10 times higher value than 1% (w/v) of xanthan gum solution.

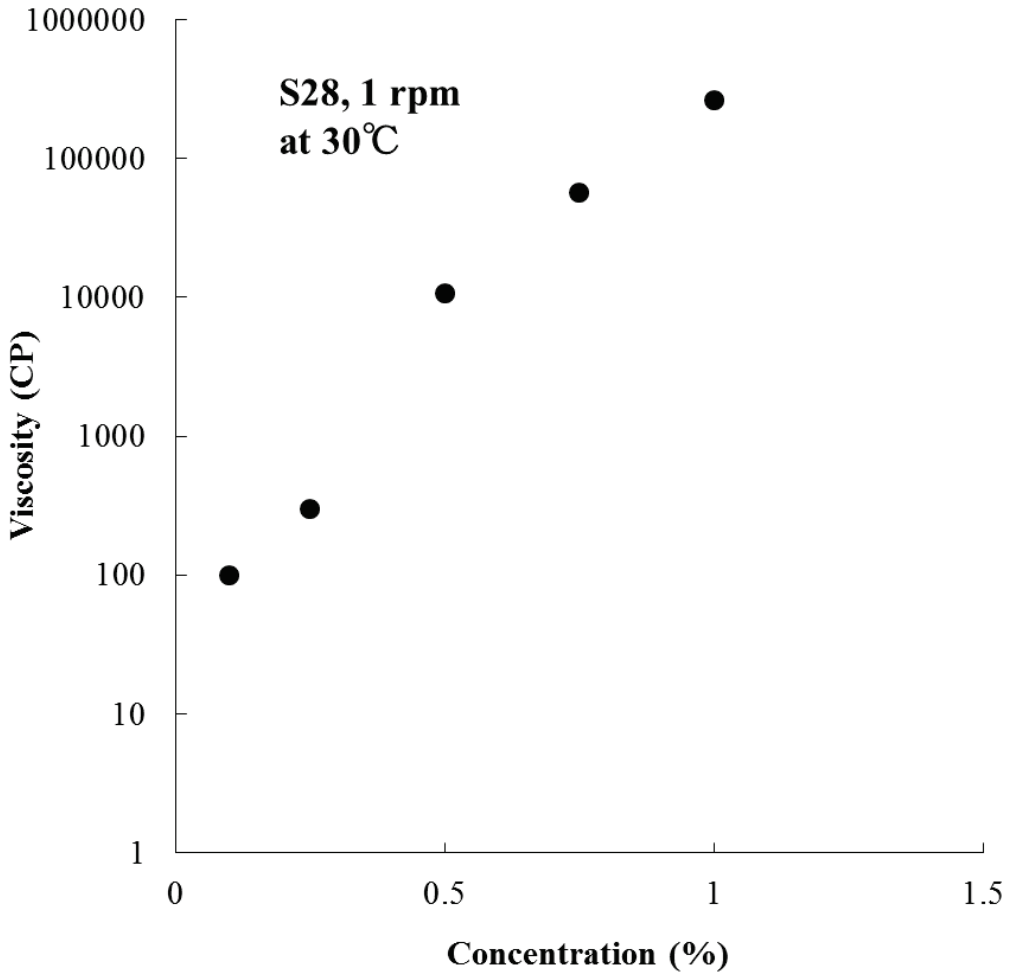


Figure 14-1 Effect of BL58 polymer concentrations

7-3-2. Effect of temperature on BL58 polymer solution

Effect of temperature on viscosity of the 1% polymer solution was shown in Figure 14-2. The viscosity of the polymer decreased with increased temperature and reached a constant value (ca. 1,000 cp) at 40 to 80 ºC. Further decrease was observed over 80 ºC, suggesting that conformation of the polymer might be temporary destroyed. Cooling of the polymer solution resulted in the logarithmical increase of viscosity (especially, under 40 ºC) and formation of the viscoelastic gel was observed, suggesting that conformation of the polymer might be more strongly recovered by repeated heating-cooling cycle.

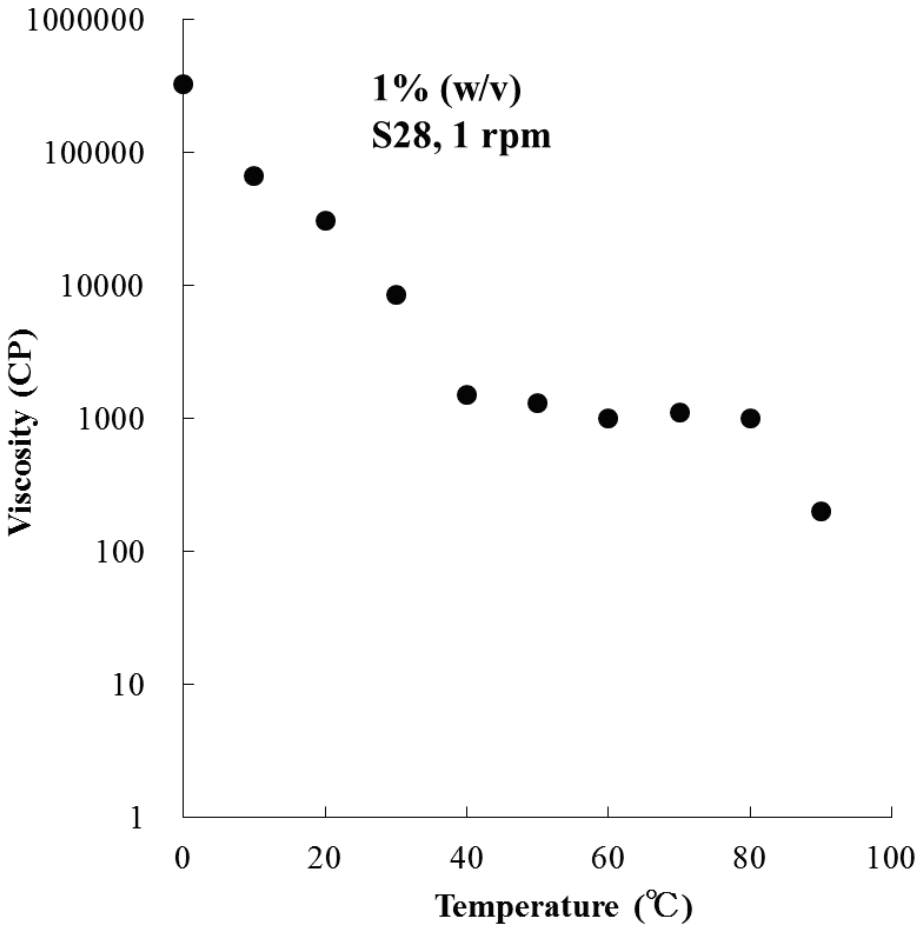


Figure 14-2 Effect of temperature on 1% (w/v) solution

7-3-3. Effect of pH on BL58 polymer solution

Effect of pH on viscosity of the 1% polymer solution was shown in Figure 14-3. Very low viscosity was observed at pH 3 but formation of viscoelastic gel was observed from pH 5 to 12. This formulation of viscoelastic gel might be caused by binding sodium ion from NaOH to constituent sugar of BL58 polymer.

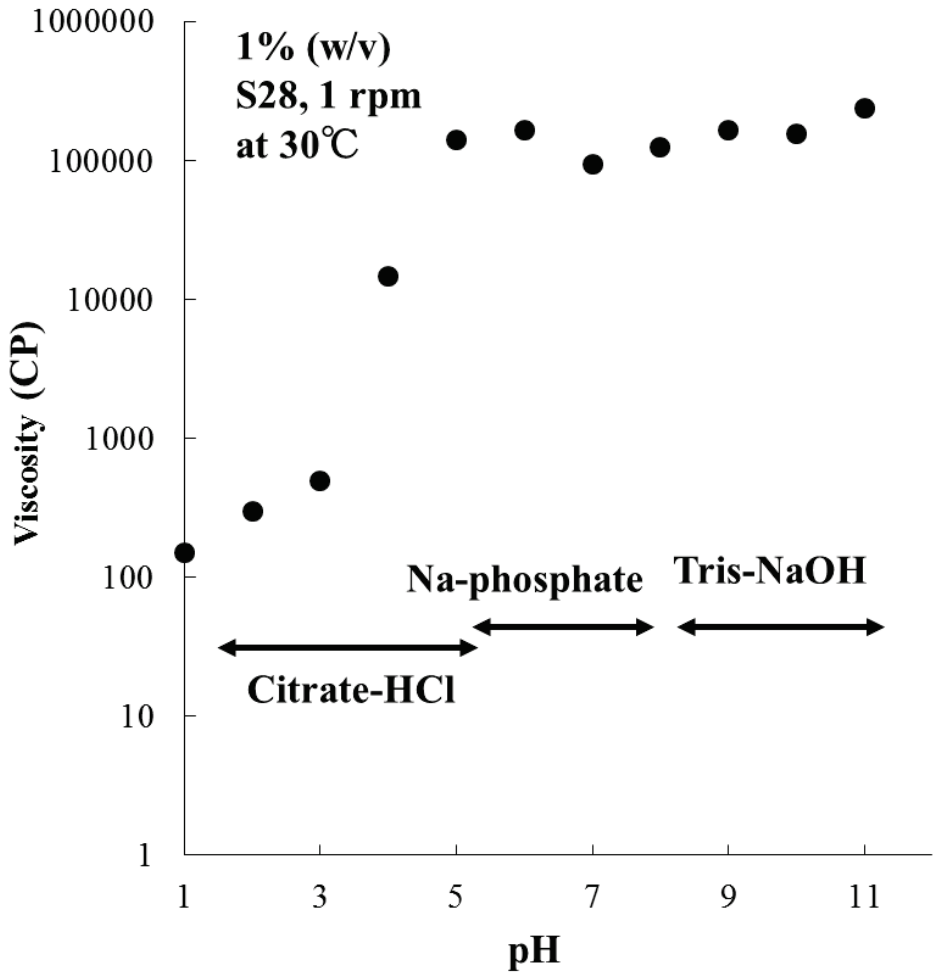


Figure 14-3 Effect of pH on 1% (w/v) solution

The rheological study of BL58 polymer solution discovered that 1% (w/v) of the polymer solution was higher viscosity with 280,000 cp that was 10 times higher than the same concentration of xanthan gum solution that is one of the most familiar microbial polysaccharides and is widely used. The 1% (w/v) polymer solution was more

viscoelastic at less than 40 ºC and at pH 5 through 12. Thus, the rheological property of the BL 58 polymer was very unique and suggested that this behavior was due to its unique carbohydrate structure.

Chapter 8

Structure analysis of BL58 polymer

8-1. Introduction

It is important to clarify the safety evaluation and correlation with physical properties based on the structure of BL58 polymer in order to develop the application methods using the unique specificity of gel formulation and the physiological activity. In this chapter, the structure of BL58 polymer was analyzed.

8-2. Materials and methods

8-2-1. Hydrolysis of BL58 polymer

1% (w/v) of BL58 polymer solution was prepared and mixed with an equal volume of 4N trifluoroacetic acid solution. Hydrolysis of the polymer was carried out at 100 ºC for 12 hours. The hydrolysate was lyophilized and dissolved in distilled water. The components of hydrolysates were analyzed qualitatively with TLC silica gel 60 plates (Merk, aluminum sheet, 20 x 20 cm) using ethyl acetate / acetic acid / water (2/1/1 (v/v/v)) as the developing solvent. The developed products were detected by heating at 105 ºC after spraying plates with 50% sulfuric acid. Fine crystal cellulose TLC plates (Funacel SF, 20 x 20 cm) were also used with phenol / ammonia / water (160/1/40 (v/v/v)) as the developing solvent, and detection was carried out by heating at 105 ºC after spraying with diphenylamine-aniline solution. Quantitative analysis by HPLC was carried out under the following conditions.

System	GILSON MODEL 305
Column	Shodex KS801
Column size	8 mm × 300 mm
Packing material	Strong-acid cation exchange resin
Temperature	70 ºC
Extract	H_2O
Flow rate	0.5 mL/min
Detection	RI
Sample volume	20 μL

8-2-2. Alditol-acetate analysis of the hydrolysates of BL58 polymer

In order to investigate the constituent sugars of the BL58 polymer, alditol acetates of the hydrolysate were prepared following the method as shown in Figure 15. Purified BL58 polymer (3 mg) was hydrolyzed with 2N trifluoroacetic acid solution for 12 hours at 100 ºC and the hydrolysate was lyophilized. The hydrolysate was dissolved in 150 μL of water and reduced with 100 mg of sodium borohydride ($NaBH_4$) for 12 hours at 30 ºC. After 12 hours, excess sodium borohydride was discharged with acetic acid, and the reaction mixture was concentrated under reduced pressure. The residues were dissolved in 1 mL of methanol/acetate (20/1 (v/v)), and concentrated under reduced pressure twice. To the residues, 1.5 mL of acetic anhydride was added and incubated at 80 ºC for 12 hours. The alditol acetates were extracted with dichloromethane (CH_2Cl_2) and dried by evaporation. The alditol acetates were re-dissolved in dichloromethane and analyzed by GC-MS under the following conditions.

System	Perkin Elmer Q-MASS910
Column	DB17 capillary column
Carrier gas	Helium
Column Temperature	120 ºC for 2 min → 10 ºC/min to 190 ºC, → 2 ºC/min to 210 ºC → 10 ºC/min to 280 ºC for 2 min
Injection Temperature	280 ºC
Sample volume	1 μL

8-2-3. Methylation of BL58 polymer

The purified BL58 polymer was methylated by the method of Hakomori [27]. The BL58 polymer or its partial hydrolysate (10 mg) was dried under reduced pressure at 37 ºC for 24 hours. Two mL of DMSO was added to the dried material, and a homogeneous suspension was obtained by ultrasonication (1 min x 5 times) under ice-cooled conditions. To the suspension, 0.5 mL of methyl sulfinyl carbanion dissolved by hot water was added under a nitrogen atmosphere and the reaction mixture was stirred for 4 hours at room temperature. Methyl iodide (CH_3I, 1 mL) was added and the mixture was stirred for another 1.5 h under ice-cooled conditions. After excess methyl iodide was removed, the reaction mixture was dialyzed against distilled water for 48 h.

The dialyzed sample was concentrated to dryness under reduced pressure, and used as the methylated derivative.

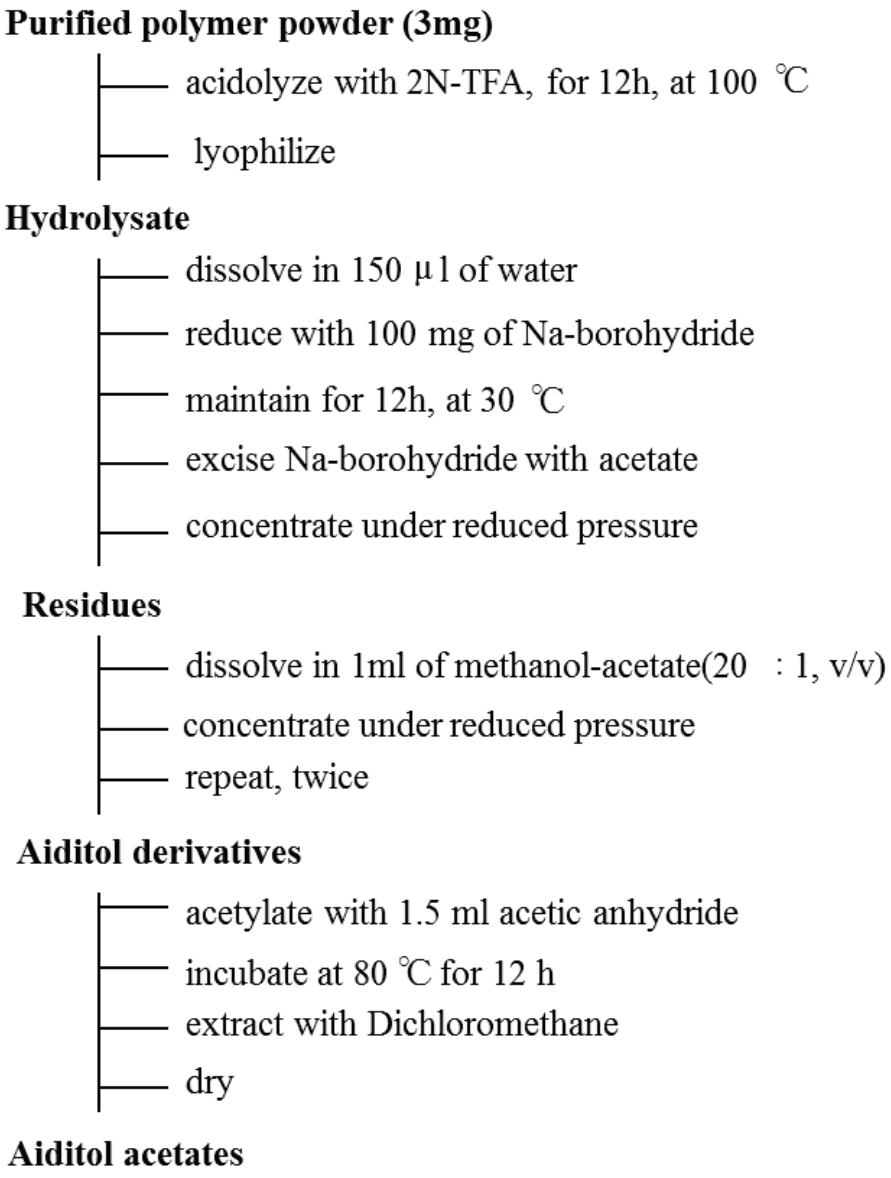


Figure 15 Preparation method for alditol acetates of the hydrolysate

8-2-4. Preparation of methylated alditol-acetate derivative

The dried residue of the methylated derivative prepared from 10 mg of BL58 polymer was dissolved by dichloromethane (CH_2Cl_2). The solution was transferred into a sealed bottle and was concentrated / dried under a nitrogen atmosphere. 0.3 mL of 90% formic acid (CH_2O_2) was added and the solution was heated at 100ºC. After 12 hours, distilled water was added and concentration was repeated under reduced pressure to remove the residual formic acid and the dried material was obtained. The dried material was added into 0.2 mL of distilled water and 0.2 mL of 4N trifluoroacetic acid solution and the methylated polymer was completely hydrolyzed at 100 ºC for 7 hours. The reaction mixture was repeatedly concentrated under reduced pressure by the addition of methanol-water to remove excess trifluoroacetic acid and the dried material was obtained.

The dried material was dissolved in 1mL of distilled water and one or two drops of 2 N aqueous ammonium solutions was added for neutralization. Sodium borodeuteride ($NaBD_4$, 50 mg) was added to the solution and stirred for 15 hours at room temperature. After generated hydrogen gas in the solution was confirmed, Amberlite IR120 (H^+) was added to the reaction mixture to remove excess sodium borodeuteride. After no generated hydrogen was confirmed, the reaction mixture was filtered and the filtrate was concentrated under reduced pressure.

After the residue was dried under reduced pressure for 48 hours, the dried residue was acetylated with 0.2 mL of acetic anhydride and 0.2 mL of pyridine at room temperature for 15 hours. The reaction mixture of the acetylated residue was repeatedly concentrated under reduced pressure by the addition of methanol. The methylated alditol-acetate was dissolved in 0.1 ml of chloroform (CH_3Cl) and was applied for GC-MS analysis under the same conditions as the alditol-acetate analysis.

8-2-5. NMR spectroscopy

^{1}H-NMR data for the BL58 polymer was recorded on a JEOL JNM-EPC500 FT-NMR spectrometer in 0.6% D_2O solution (w/v) at 60 ºC. Spectra were externally referenced to 3-trimethylsilylpropanoate. ^{1}H-NMR, ^{13}C-NMR, H-H COSY, HMQC, HMBC, DEPT and NOESY data for compound X were recorded on the same spectrometer in D_2O solution at room temperature. In all cases, chemical shifts are reported in ppm relative to sodium 3-(trimethylsilyl)-1-propane-sulfonate, which was used as an internal standard.

Data for the purified peak 1 in Figure 16, designated as compound X (3-*O*-[(R)-1-carboxyethyl]-L-rhamnopyranose (intramolecular ester form)): ^{1}H-NMR (D_2O, sodium 3-(trimethylsilyl)-1-propanesulfonate): δ 1.26 (α-form, 3H, d, $J_{5,6}$=6.42, H6), 1.28 (β-form, 3H, d, $J_{5,6}$=5.96, H6),1.39 (α-form, 3H, d, $J_{CH3\text{-}CH<,\ CH3\text{-}CH<}$=6.87, CH_3-CH<), 1.39 (β-form, 3H, d, $J_{CH3\text{-}CH<,\ CH3\text{-}CH<}$=6.87, CH_3-CH<), 3.38 (β-form, 1H, dd, $J_{4,5}$=9.17, $J_{5,6}$=5.96,H5), 3.42 (β-form, 1H, dd, $J_{2,3}$=2.75, $J_{3,4}$=8.76, H3), 3.45 (β-form, 1H, dd, $J_{3,4}$=8.76, $J_{4,5}$=9.17, H4), 3.52 (α-form, 1H, dd, $J_{3,4}$=9.62, $J_{4,5}$=9.17, H4), 3.58 (α-form, 1H, dd, $J_{2,3}$=3.21, $J_{3,4}$=9.62, H3), 3.86 (α-form, 1H, dq, $J_{4,5}$=9.17, $J_{5,6}$=6.42, H5), 4.04 (α-form, 1H, dd, $J_{1,2}$=1.83, $J_{2,3}$=3.21, H2), 4.07 (β-form, H2, 1H, dd, (broad), $J_{1,2}$=0.92, $J_{2,3}$=2.75, H2), 4.13 (α-form, 1H, q, $J_{CH3\text{-}CH<,\ CH3\text{-}CH<}$=6.87, CH_3-CH<), 4.15 (β-form, 1H, q, $J_{CH3\text{-}CH<,\ CH3\text{-}CH<}$=6.87, CH_3-CH<), 4.82 (β-form, H1, 1H, d, $J_{1,2}$=0.92, H1), 5.12 (α-form, 1H, d, $J_{1,2}$=1.83, H1); ^{13}C-NMR (D_2O, sodium 3-(trimethylsilyl)-1-propane-sulfonate): δ 19.57 (C6, α-form or β-form), 19.60 (C6, α-form or β-form), 21.49 (CH_3-CH< , α-form or β-form), 70.72 (C2, α-form), 71.02 (C2, β-form), 71.14 (C5, α-form), 73.36 (C4, β-form), 73.67 (C4, α-form), 74.70 (C5, β-form), 77.84 (CH_3-CH<, α-form or β-form), 78.05 (CH_3-CH<, α-form or β-form), 80.81 (C3, α-form), 83.29 (C3, β-form), 96.22 (C1, β-form), 96.64 (C1, α-form), 183.94 (>C=O, α-form or β-form).

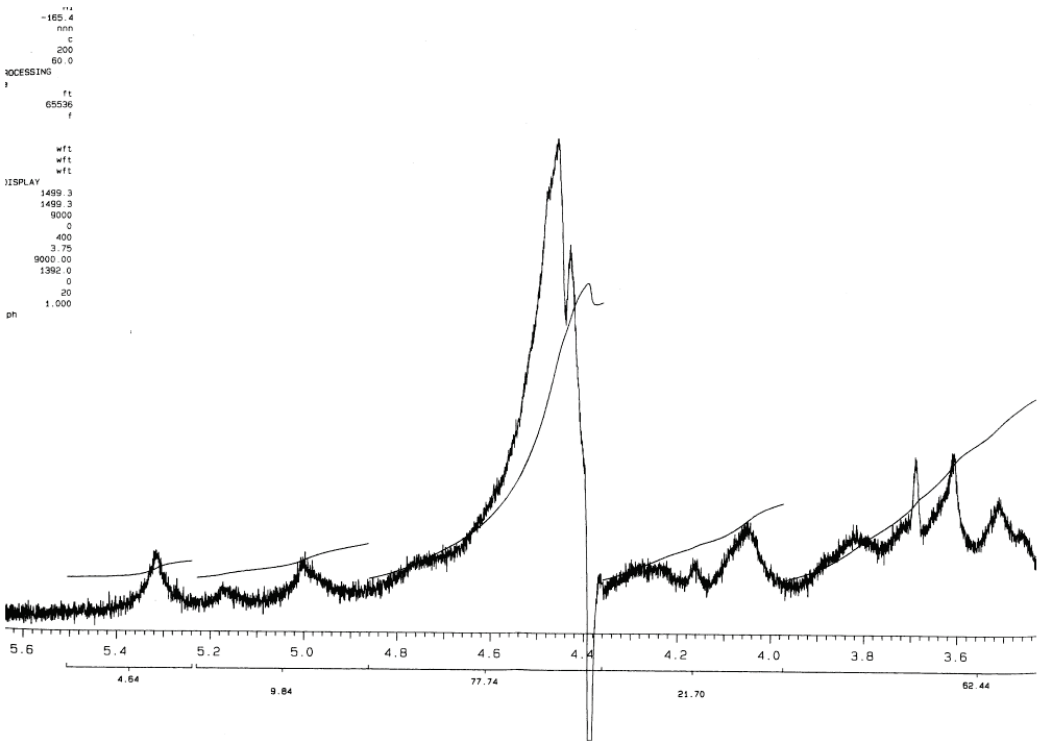


Figure 16 600-MHz ^{1}H-NMR spectrum of native BL58 polymer at 60℃

8-2-6. CI-GCMS analysis

CI-GCMS analyses were carried out under the following conditions.

System	JEOL SUM300
Column	HP-1 capillary column
Column size	30 mm × 320 μm Thickness: 0.25 μm
Carrier gas	Helium
Reagent gas	Iso-butane
Column Temperature	60 ºC for 1 min → 10 ºC/min to 160 ºC, → 2 ºC/min to 180 ºC → 10 ºC/min to 230 ºC for 4 min
Injection Temperature	200 ºC

8-3. Results and discussions

8-3-1. NMR analysis of BL58 polymer

^{1}H-NMR analysis of BL58 polymer was carried out and the spectrum was compared to those of the poly- and oligosaccharides produced by *P. stutzeri* ATCC 17588 and *Pseudomonas sp.* OX1 (previously known as *P. stutzeri* OX1) [28-31], which were previously reported by Osman et al. and Leone et al., respectively [28,29]. Three broad peaks were observed at 5.32, 5.16 and 4.98 ppm, suggesting that these are anomeric protons. The peaks around 3.2 and 4.4 ppm are thought to be protons of the pyranose or furanose rings (data not shown). Osman et al. reported that signals for three anomeric protons were observed at 4.95, 4.75 and 4.45 ppm in the ^{1}H-NMR analysis of the native polysaccharide from *P. stutzeri* ATCC19588 [28] (Figure 16). These results indicated that the BL58 polymer has a different structure from the already known polymers.

^{1}H-NMR analysis of partially hydrolyzed BL58 polymer was carried out. Two peaks were observed at 5.3 and 5.0 ppm and were not reported on anomeric protons of oligosaccharides produced by *P. stutzeri* ATCC 17588 (Figure 17). These results also indicated that the BL58 polymer is a novel polymer.

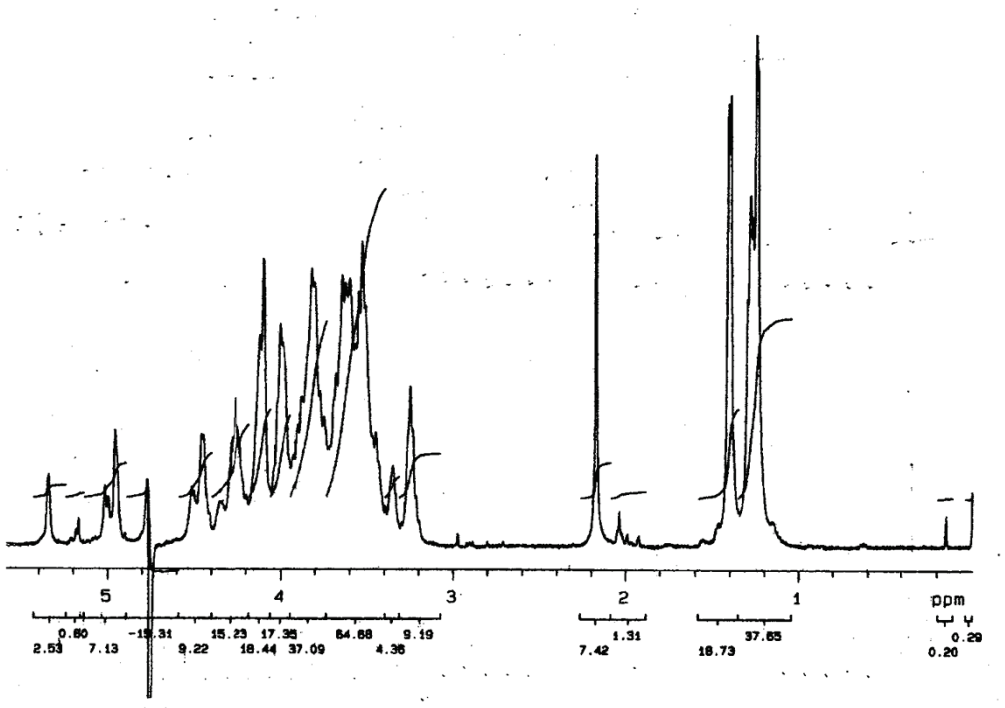


Figure 17 ^{1}H-NMR spectrum of partially hydrolyzed BL58 polymer

8-3-2. Acid hydrolysis and alditol-acetate analysis of BL58 polymer

Acid hydrolysis and alditol-acetate analysis were attempted to investigate the constituent sugars of the BL58 polymer. TLC analysis of the completely hydrolyzed products showed that glucose, mannose and rhamnose were the main components of the BL58 polymer. Alditol-acetate analysis supported the results of the GC-MS analysis and the ratio of these sugars was estimated as D-rhamnitol hexaacetate (peak 1): D-mannitol hexaacetate (peak 2): D-glucitol hexaacetate (peak 3) were equaled to 0.07: 0.6: 1.0 (Figure 18). In addition to the peaks of the three monosaccharides, an unknown peak was observed in the alditol-acetate analysis.

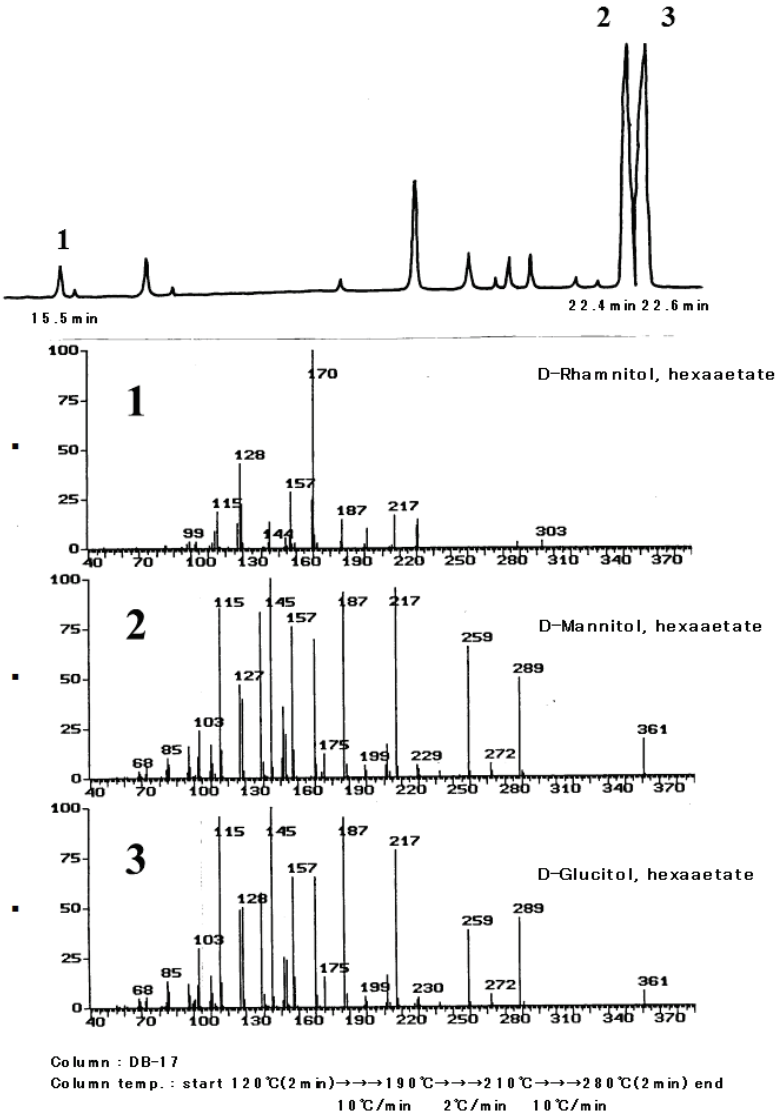


Figure 18 GC-MS analysis of alditol acetates of BL58 polymer

8-3-3. Alditol-acetate analysis of methylated BL58 polymer

To obtain information about the bonding between these constituent sugars, alditol-acetate analysis of methylated BL58 polymer and its hydrolyzates was attempted by using GC-MS [32]. The GC-MS spectra of alditol-acetate analysis of methylated non-hydrolyzed (MW: ca.1,800,000) and partially-hydrolyzed (MW: ca. 180,000) BL58 polymers are shown in Figure 19. In both spectra, four peaks (peaks 1, 2, 3 and 4 in Figure 19) were observed at the same retention times. In the spectrum of the partially hydrolyzed products, one new peak (peak 5 in Figure 19) increased remarkably with the decrease of peak 4. These results indicated that the polymer has a branched chain or substituted group of organic acid and the peak 5 was produced as a new peak after these branched chain and substituted group were hydrolyzed. Each peak was assigned based on the analysis of the mass spectra and comparison of retention times.

Peak 1 had a retention time at 15.35 min and mass peaks at 144, 129, 118, 101, 72, 59 and 43 (Figure 20) and was charactered as a fragmentation pattern of Hexulose terminal but not assigned. Therefore, Peak 1 was resulted in an unknown material. Peaks 2 had a retention time at 16.62 min and mass peaks at 233, 162, 129, 118, 102, 87 and 43 and was assigned as a fragmentation pattern of 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-glucitol (Figure 21). Peak 2 was confirmed as a much higher peak in the spectrums of both methylated non-hydrolyzed and partially-hydrolyzed BL58 polymers. Therefore, these results indicate that the polymer has 1,4-linked glucose units as the main chain. Peak 3 had a retention time at 18.69 min and mass peaks at 305, 118, and 43 and was assigned to 1,3,4,5-tetra-*O*-acetyl-2,6-di-*O*-methyl-glucitol (Figures 22). Peak 3 also confirmed in the spectrums of both methylated non-hydrolyzed and partially-hydrolyzed BL58 polymers. Therefore, these results indicated that the polymer has a 1,4-linked glucose units and branched chain or substituted group at the 3 position of glucose. Peak 4 has a retention peak at 19.55 min and mass peaks at 362, 289, 259, 217, 187, 170, 115, 103 and 86 but it was not assigned from the mass spectrum analysis (Figure 23). However, the compounds in peaks 4 and 5 are expected to be structurally related, because peak 5 increased with the decrease of peak 4 after acid hydrolysis of the BL58 polymer. Peak 5 has a retention time at 16.32 min and mass peaks at 233, 162, 129, 118, 102, 87 and 43 and was assigned to 1,4,5-tri-*O*-acetyl-2,3,6-tri-*O*-methyl-mannitol (Figure 24). These results suggest that the mannose unit is also one of the constituents of the main chain of the BL58 polymer and branched chains or substituted groups are present at the 2,3,6-positions of the mannose units.

The chemical structure of peak 1 was not assigned on the basis of the mass spectrum analysis (Figure 20). Therefore, peak 1 (designated as the compound X) was

chromatographically isolated from the partial hydrolysate of the BL58 polymer, and compound X was analyzed by ^{1}H- and ^{13}C, 2D-NMR and EI- and CI-MS spectroscopy. To estimate coupling constants of the signals in the 1H-NMR spectrum, a decoupling technique was applied in the analysis. In the ^{1}H-NMR spectrum of compound X (Figure 25), two doublet signals (5.12 and 4.82 ppm) were observed. Coupling constants (1.83 Hz for 5.12 ppm, 0.92 Hz for 4.82 ppm) indicate that signals at 5.12 and 4.82 ppm are assigned to α and β anomeric protons, respectively, at the 1 position of the sugar ring. Based on the information from 2D-NMR (HH-COSY, HMQC and HMBC) spectra (data not shown), the signals at 4.04 (dd), 3.86 (dq), 3.58 (dd), 3.52 (dd) and 1.26 ppm (d) in the ^{1}H-NMR spectrum were assigned to the H2, H5, H3, H4 and H6 protons of the α-form. The doublet signal at 1.39 ppm and quartet at 4.13 ppm were assigned to the three protons of CH3-CH< and one proton of CH3-CH<. Similarly, the signals of the β-form appeared at 1.28 (H6, d), 3.38 (H5, dd), 3.42 (H3, dd), 3.45 (H4, dd), 4.07 (H2, broad dd), 1.39 (CH_3-CH<, d) and 4.15 ppm (CH_3- CH<, q). The ^{13}C-NMR spectrum also supports the assignment of the ^{1}H-NMR spectrum (data is summarized in the Experimental section). Another important finding from the completely decoupled ^{13}C-NMR and DEPT spectra is the presence of >C=O structure (183.9 ppm). In the NOESY spectrum, correlation between the proton of CH_3CH< and H3 was observed (Figure 26), suggesting that the intermolecular ester of compound X is the R form [33]. From the NMR analyses, compound X is strongly suggested to be an intramolecular ester form of the rhamnose derivative, 3-*O*-[(R)-1-carboxyethyl]-L-rhamnopyranose. To confirm this structure, CI-GCMS analyses of the acetylated form of the compound were carried out by using iso-butane as a reaction regent. In the CI mass spectrum of acetate, two main peaks were observed and these can be tentatively be assigned to be α and β anomers. The $[M+1]^{+}$ peak at 303 was observed in the MS spectra of both anomer peaks (Figures 27A and 27B). Furthermore, CI-GCMS analysis was performed for the acetylated alditol (compound X reduced by NaBD4) (Figure 27C). Significant peaks at m/z 348 ($[M+1]^{+}$) and at m/z 390 ($[M+43]^{+}$) were observed. These CI-GCMS data supported the molecular weight (302) of the proposed chemical structure, the intramolecular ester form of 3-*O*-[(R)-1-carboxyethyl]-L-rhamnopyranose.

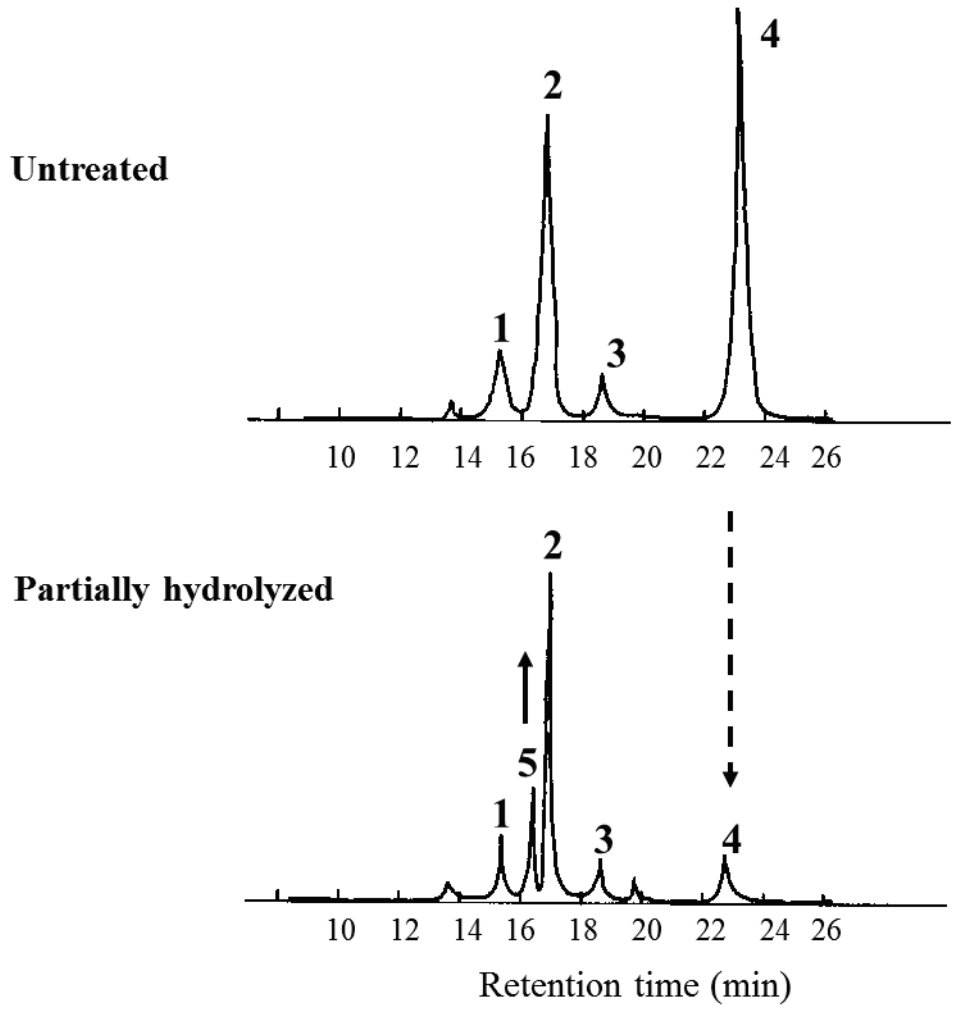


Figure 19 GC-MS analysis of partially hydrolyzed BL58 polymer

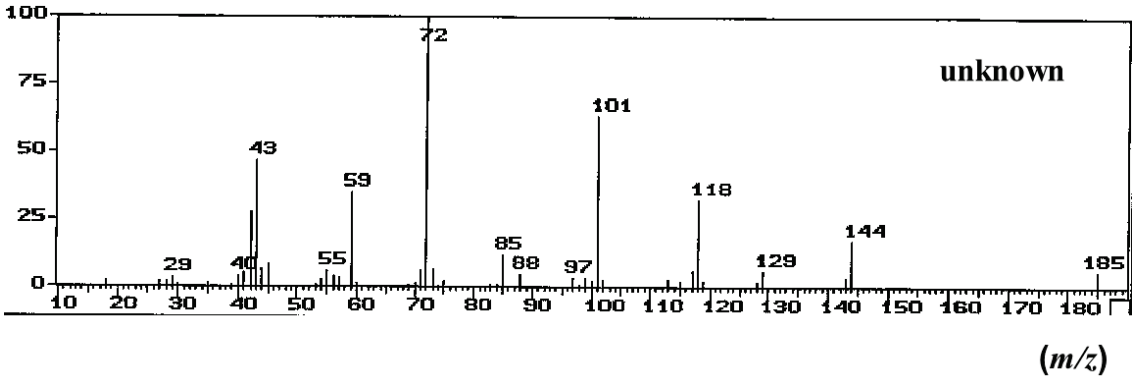
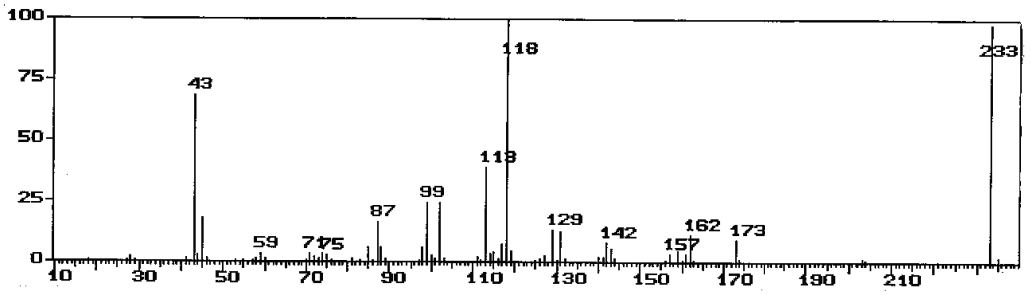
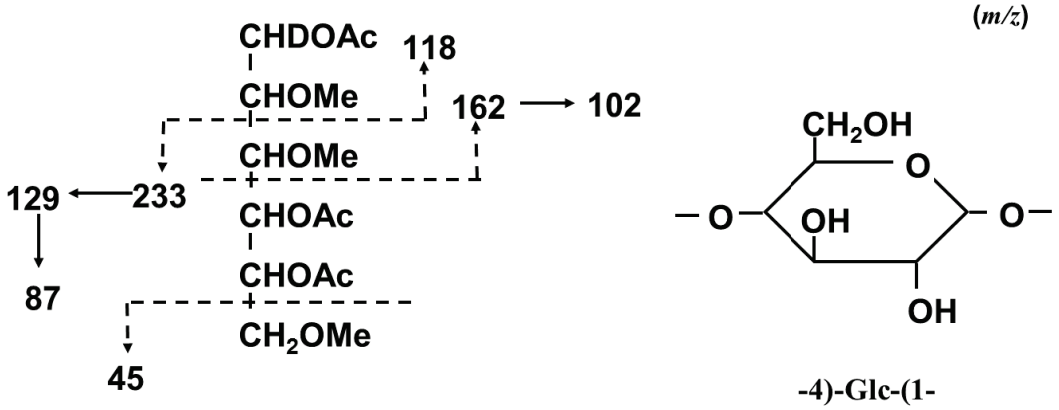


Figure 20 Mass spectra of peak 1

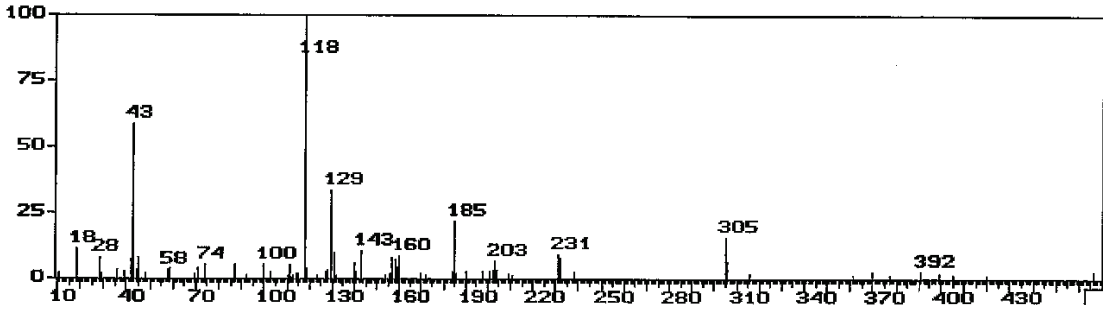


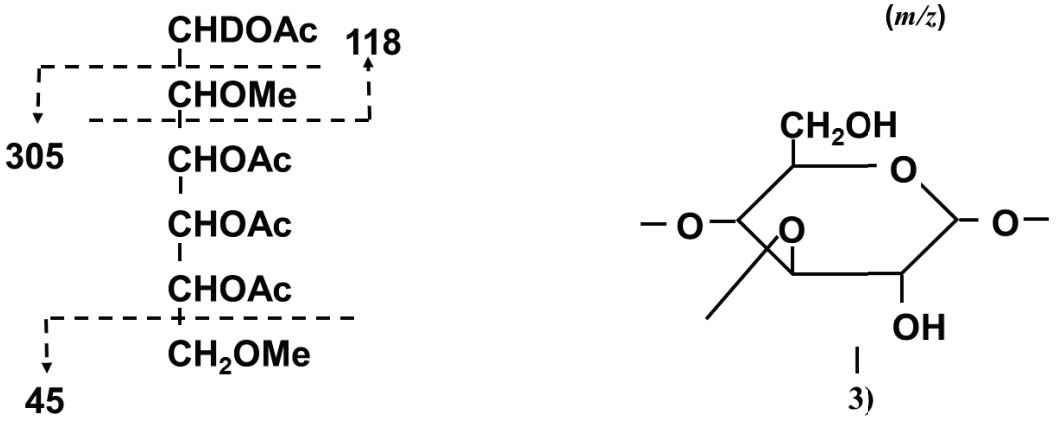


1,4,5-Tri-*O*-Ac-2,3,6-tri-*O*-Me-hexitol

-4)-Glc-(1-

Figure 21 Mass spectra of peak 2

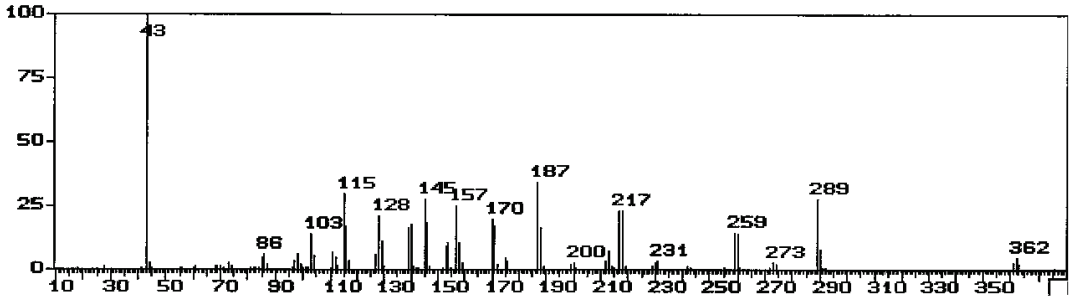


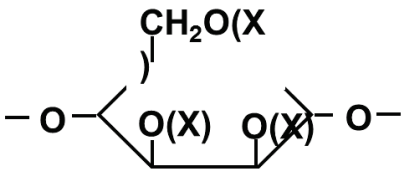


1,3,4,5-Tetra-*O*-Ac-2,6-di-*O*-Me-Glucitol

-4)-Glc*p*-(1-

Figure 22 Mass spectra of peak 3

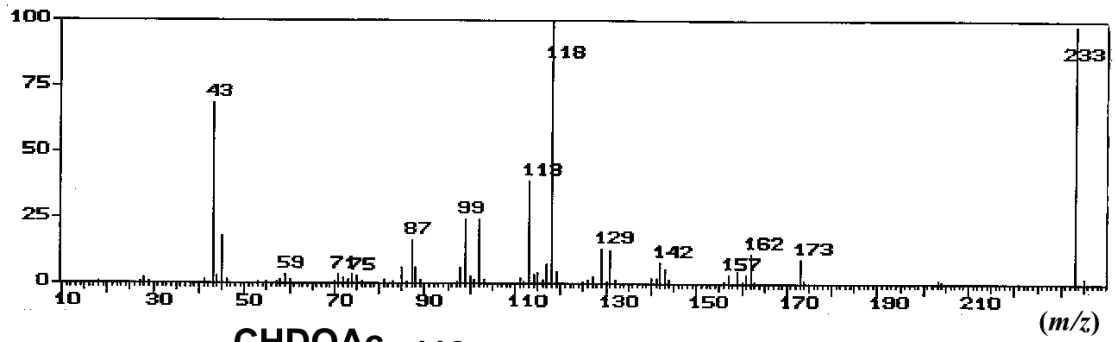


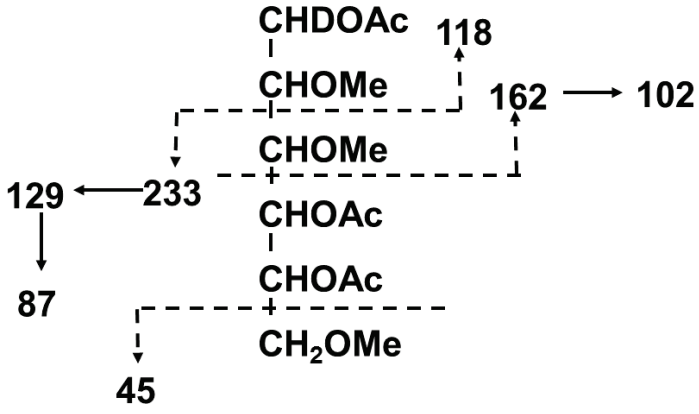


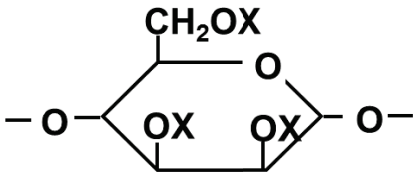
X: Carboxyethyl (?)

-4)-Man-(1-
(2, 3,or 6-substituted)

Figure 23 Mass spectra of peak 4







-4)-Man-(1-
(2, 3,or 6-substituted)

1,4,5-Tri-*O*-Ac-2,3,6-tri-*O*-Me-Mannitol

Figure 24 Mass spectra of peak 5

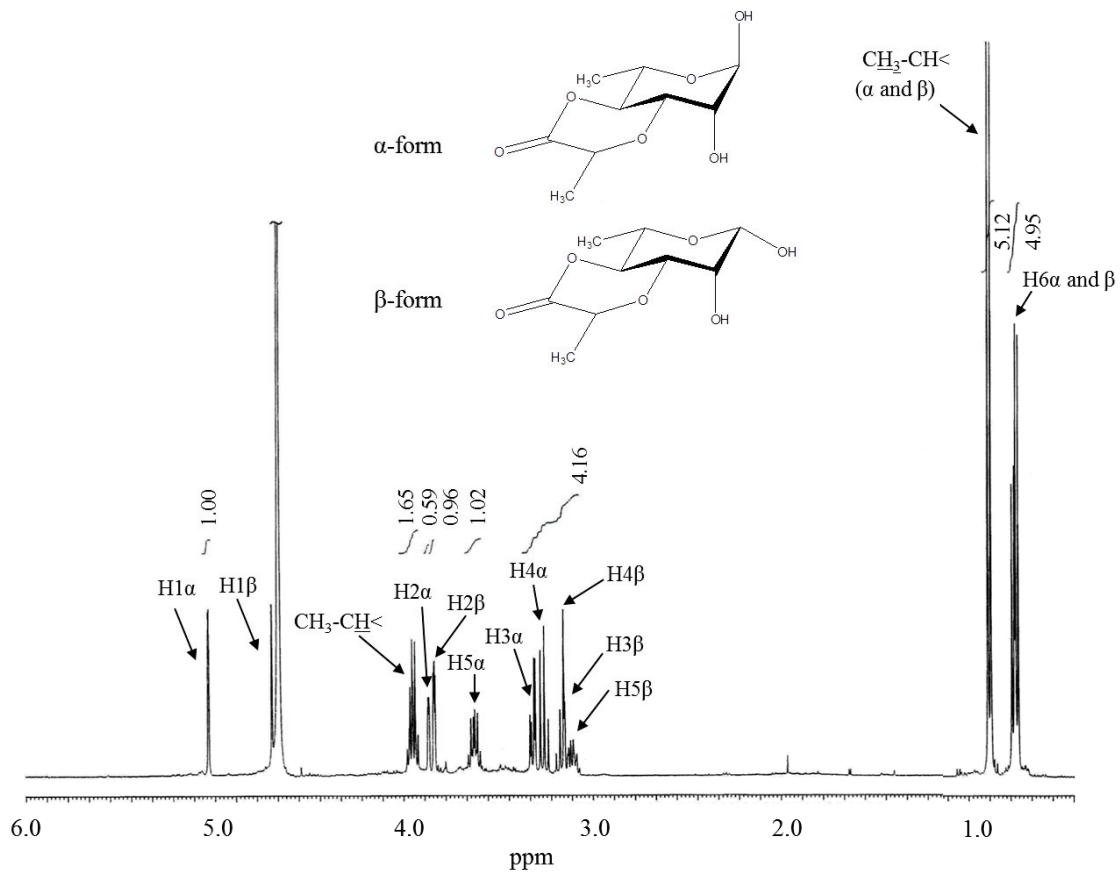


Figure 25 ^{1}H-NMR spectrum of peak 1 (compound X)

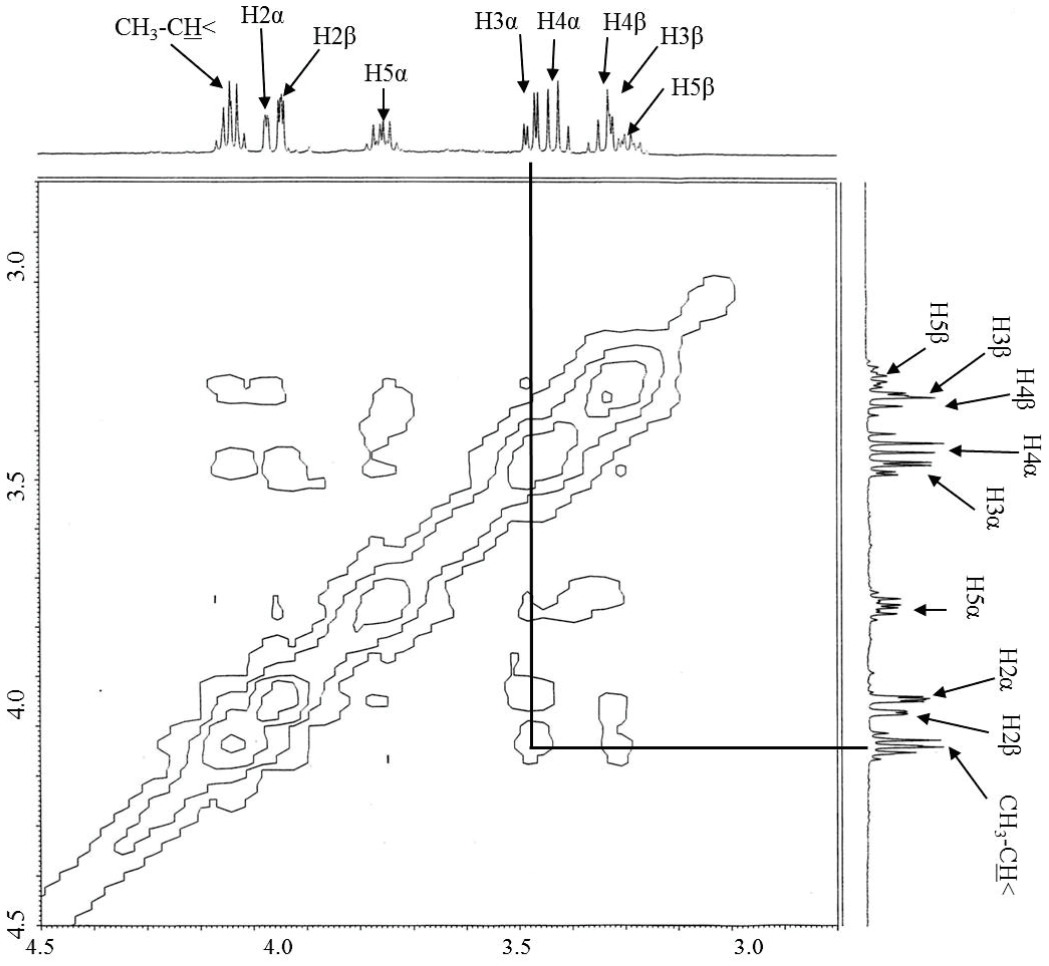


Figure 26 NOESY spectrum of peak 1 (compound X)

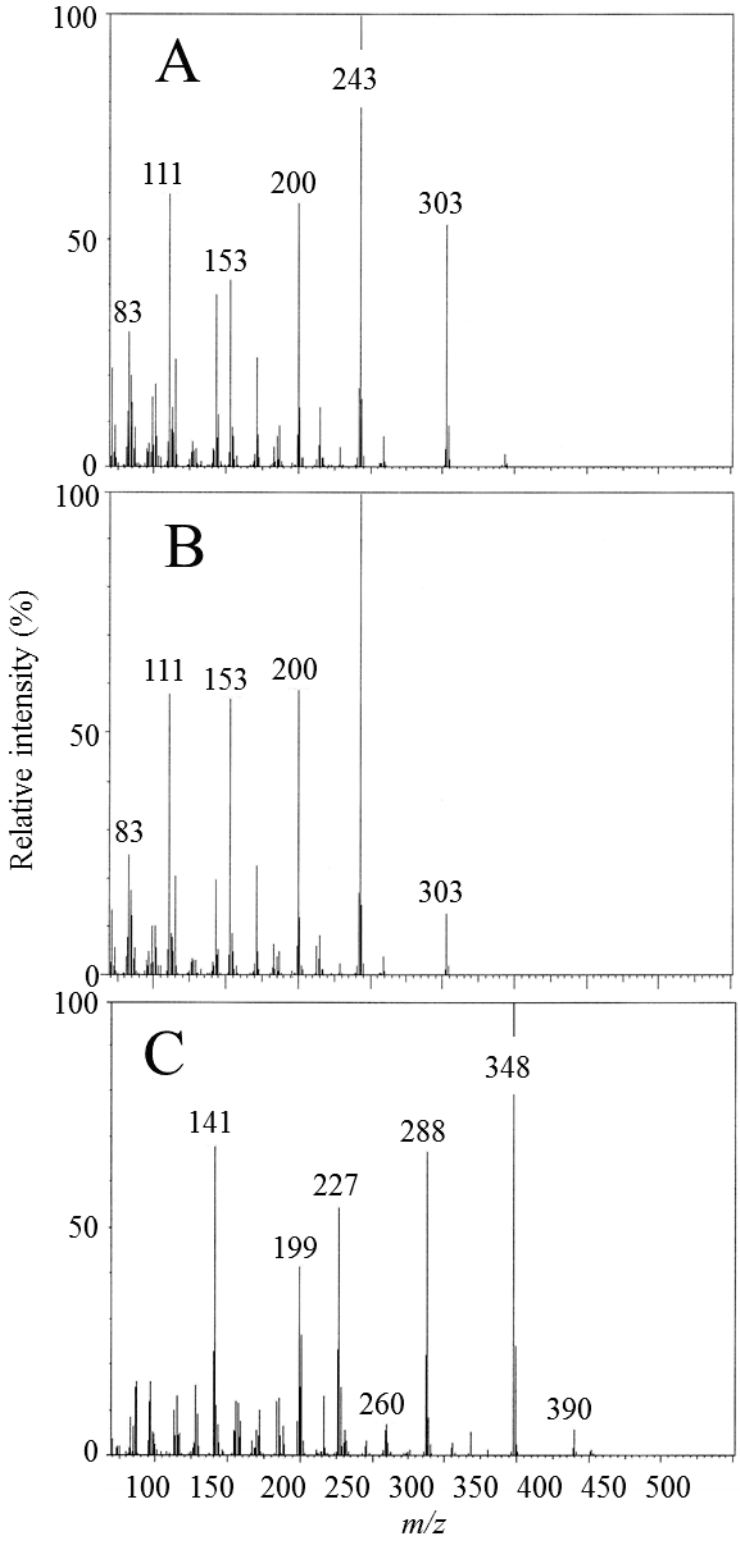


Figure 27 CI-GCMS spectrum of peak 1 (compound X)

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8-3-4. Proposed structure of the BL58 polymer

The results from all analyses in this chapter indicate that the BL58 polymer is comprised of four types of monosaccharide units: a "-4)-glucose-(1-"unit, a "-4)-mannose-(1-"unit, a "-4)-glucose-(1-"unit with a branch at the 3 position and 3-*O*-carboxyethyl rhamnose unit (Figure 28). Most likely, compound X is an intramolecular ester form of 3-*O*-[(R)-1- carboxyethyl]-L-rhamnopyranose, which is supposed to be a secondary metabolite produced from 3-*O*-carboxyethyl rhamnose during the hydrolysis of the BL58 polymer. The final proposed structure is shown in Figure 29. Previously, Osman et al. and Maaleja et al. reported that a major structural motif of the polysaccharides produced by *P. stutezeri* ATCC17588 and AS22 was assigned as follows: it is composed of a main chain consisting -4)-β-glucose-(1- and -3,4)-mannose-(1-residues, in which a considerable part of the -3,4)-mannose-(1-resides is branched at C-4 position with 3-*O*-carboxyethyl rhamnose and with an acetyl group on *O*-2 [33,34]. The proposed structure of BL58 polymer differed from the previous *P. stutzeri* polysaccharides in that 3-*O*-carboxyethyl rhamnose was branched at 3 position of glucose unit. Therefore, the BL58 polymer is novel microbial polysaccharide.

However, because the spectral data was not assigned completely, further work is needed to determine the carbohydrate structure of highly polymerized BL58 polymer. Physiological properties of BL58 polymer Maaleja et al. reported that the polysaccharides from *P. stutzeri* AS22 strains exhibited the physiological functions, such as antioxidant activity and dermal wound healing [35]. In addition, the rheological properties such as a pseudo-plastic behavior, high elasticity, good mechanical strength and stability with high-absorption ability, were expected applications as food and cosmetic additives [36]. Consequently, we evaluated further physiological properties of BL58 polymer, which may have similar rheological properties to the AS22 strain polysaccharide, starting with toxicity testing using several safety tests.

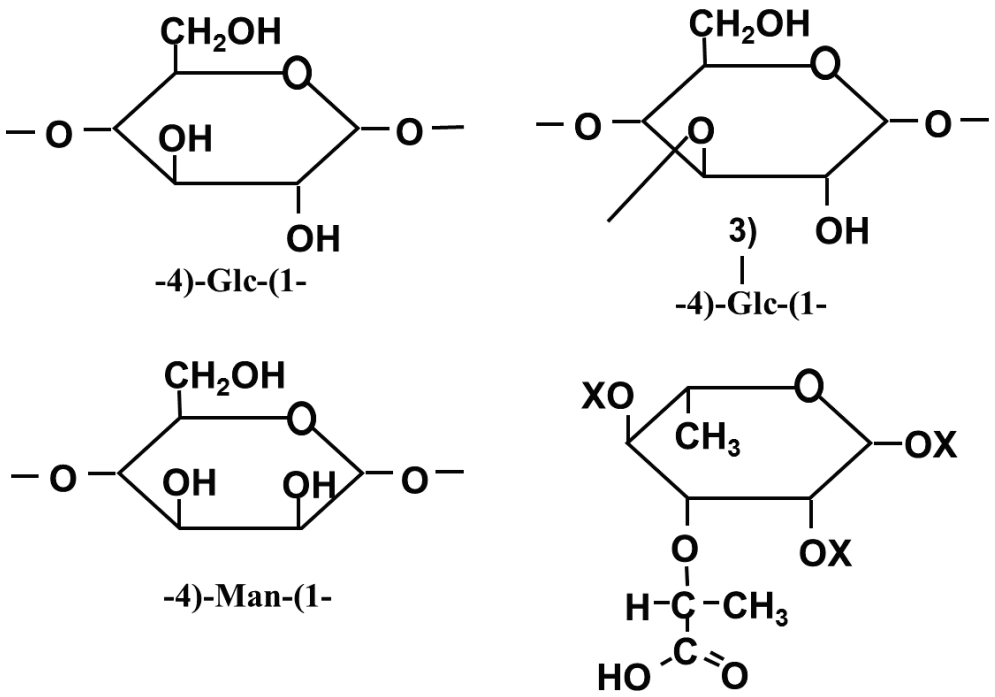


Figure 28 Monosaccharide units of BL58 polymer produced by *P. stutzeri* BL58

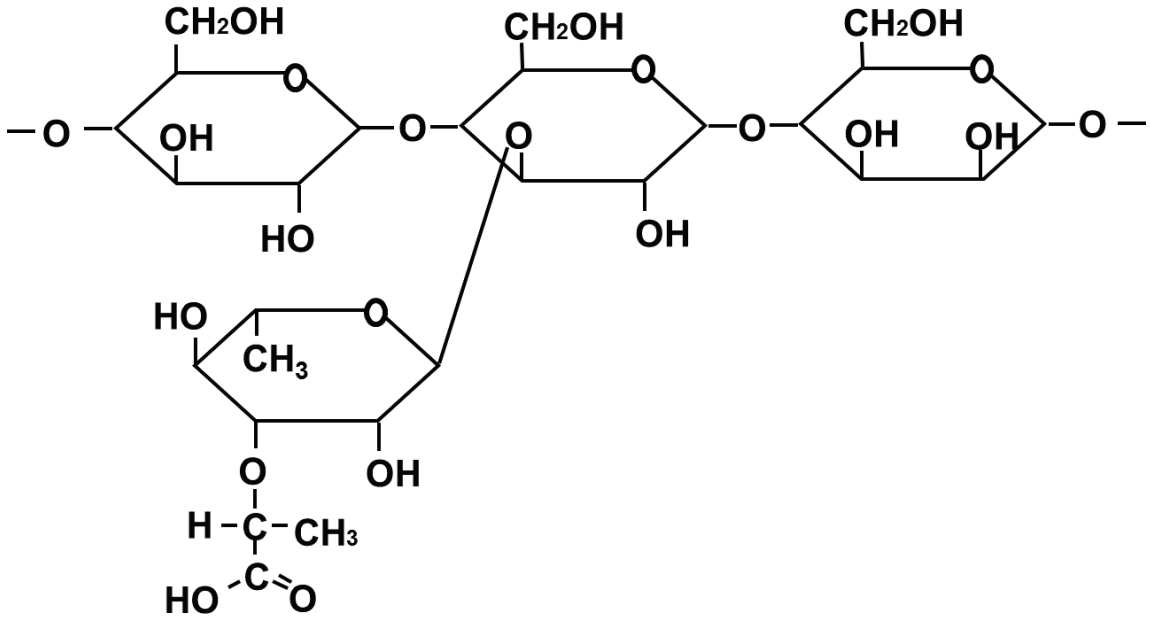


Figure 29 Proposed structure of BL58 polymer produced by *P. stutzeri* BL58

Chapter 9

Physiological study

9-1. Introduction

In the previous chapter, the structure of BL58 polymer was analyzed and indicated that the BL58 polymer is comprised of four types of monosaccharide units including specific structure that 3-*O*-carboxyethyl rhamnose was branched at 3 position of glucose unit. It contributed to the rheological properties such as a pseudo-plastic behavior, high elasticity, good mechanical strength and stability with high-absorption ability that was expected to apply to food and cosmetic additives [36]. In this chapter, further safety evaluation of BL58 polymer as cosmetic materials and bioactivity test as pharmaceutical materials, starting with toxicity testing were evaluated.

9-2. Materials and methods

9-2-1. Acute toxicity test

Acute toxicity test was performed according to the method as previously described with some modifications [37]. BL58 polymer and xanthan gum (Sigma) (250 mg/kg, 500 mg/kg and 1000 mg/kg as 1% of aqueous solution) were orally administrated to 4 weeks old SPE male mice (Japan SLC, Inc.) using animal feeding needles. The weight of the mice was 15-17 g. Before the administration was started, mice were normally bred for 4 days. From the evening of the day before administration, mice were completely deprived of food except water for 15 hours. Mice were divided into 4 (high, middle, low and none using pure water) per subgroup. The average weight of the food-deprived mice was adjusted to be approximately 20 g in each subgroup. Mice were fed at 23 ± 3 ºC room temperature, at 55 ± 5% relative humidity, 13 times/hour of air-ventilation and 12 hours/day of artificial lightning in barrier breeding room. The solid feedstuff MF (Oriental yeast Co. Ltd.) was used after γ ray irradiation. The drinking-water was filtrated by RO membrane and fed freely to the mice. Examination was performed for all mice that were observed for 2 weeks following the administration of test polymers. The weight of mice was measured on days 0, 2, 3, 6, 9 and 13. Mice on days 14 were sacrificed by cervical dislocation, and megascopic pathological check for internal organs was conducted in detail.

9-2-2. Eye irritancy test

Eye irritation test was performed according to the Draise rabbit eye test [38]. One hundred μL of 1% aqueous solution of BL58 polymer was instilled into the conjunctival sac of right eye of three 10 weeks old Japanese white male rabbits. The left eye without instillation was used as controls. Anterior region of the right eye (cornea, iris and conjunctiva) was megascopically and ophtalmoscopically checked at 1, 24, 48 and 72 hours after the instillation and compared with those of the left eye used as controls. At 24 hours after the instillation, abnormalities of cornea were examined by using the FLUORES Ocular Examination Test Paper and the eye irritancy was evaluated according to the Draize standard.

9-2-3. Primary rabbit skin irritation test

Three Japanese white male rabbit (10 weeks old) were used for rabbit skin irritation test [39]. Fur on neck of the rabbit was removed and cotton pad with 1% of BL58 polymer was applied occlusively to the naked skin of the rabbit. The irritancy for rabbit skin was evaluated according to the Draize standard at 24, 48 and 72 hours after the application.

9-2-4. Primary human skin irritation test

Finn chamber patch including 1% of BL58 polymer solution was applied to inside of upper arm of 45 healthy adults (men, 16; women, 29) for 24 hours. At 0.5 (as 24 hour-assessment) and 24 hours (as 48 hour-assessment) after the above test was finished, skin irritancy was megascopically checked [39]. The symptom was assessed in 6 levels, (-): no reaction, (±): light erythematous, (+): erythematous, (2+): papula, (3+): erythematous, infiltratous, papula, and emphlysis, (4+): bullosum. The skin irritation index for methyl paraben was used as a low irritancy control when substituted for the BL58 polymer.

9-2-5. Micronucleus test (Clastogenicity test)

ICR male mice (6 weeks old, SPF) were used for micronucleus test [40]. Mice were divided into subgroups contained 5 mice each and BL58 polymer (0.25, 0.5 and 1.0 g/kg as 1% aqueous solution) were orally administrated once a day for two days (total two times). At 24 hours after the final administration, mice were killed and myeloid cells were collected from the femur. Smears of myeloid were immobilized with

methanol for 3 minutes and finally stained with Giemsa-stain solution for 35 minutes. The number of micronucleated polychromatophilic erythrocytes on the smears was examined under the microscope. The number of orthochromatic erythrocytes in the same scope field was also examined.

9-2-6. Rec assay

BL58 polymer was autoclaved at 121 ºC for 5 minutes and suspended in DMSO to obtain 250 mg/ml of solution as the final concentration. This suspension was successively diluted to 1.22-5000 mg/disk (total 7 concentrations) and used for the *Bacillus subtilis* H17 Rec^+ and M45 Rec^- assay [41]. Eight mm in diameter of filter-paper disks impregnated 20 μL of BL58 polymer were applied in the middle of the streak on the plate and the incubation was carried out at 37 ºC for 24 hours. The result was judged as positive when the inhibition zone against H17 Rec^+ was 0-1 mm and the difference in zones of H17 Rec^+ and M45 Rec^- was more than equal to 3 mm.

9-2-7. Anti-bacterial and anti-virus test

Escherichia coli K12 was incubated in NB culture overnight and was transplanted to the freshly prepared culture containing 0-2000 μg/ml of BL58 polymer. Absorbance at OD 600 nm was measured to quantify turbidity after the overnight incubation. Human influenza virus (H1N1, H3N2) was infected to MDCK cells, and the infectious inhibitory action of BL58 polymer (15.6-2000 μg/mL) was checked measuring the lactate dehydrogenase activity in the culture medium [42].

9-2-8. Skin sensitization test

Male guinea pigs were used for the skin sensitization test. Guinea pigs were divided into subgroups contained 5 mice each. A filter paper including emulsion of 2% of DNCB and 0.1% or 0.5% of BL58 polymer was applied to the skin of the guinea pig three times a week. At 2 weeks after final induction, further filter paper including emulsion of 0.25% of DNCB and 0.1% or 0.5% of BL58 polymer was applied to skin. Saline was used as a control in place of BL58 polymer.

9-2-9. Antigenicity test

The antigenicity of BL58 polymer was investigated by active systemic anaphylaxis (ASA) assay using guinea pigs. Egg albumin (EA) was used as a positive control. BL58 polymer (1 mg/kg) was injected for the induction to both subgroups contained guinea pigs weakly sensitized by 1 mg/kg and highly sensitized by 5 mg/kg of BL58 polymer.

9-2-10. Anti-proliferation test of carcinoma cells

0.5% of BL58 polymer aqueous solution was autoclaved and the undissolved precipitate was removed by centrifugation. The supernatant was used for the repression test of human acute promyelocytic leukemia cells (HL60: JCRB0085 purchased from JCRB Cell Bank), human monocytoid leukemia cells (U-937: JCRB9021), human liver carcinoma cells (HepG2: JCRB1054) and human normal pulmonic cells (MRC5: JCRB9008). Cells (0.5-1.0 × 10^4 cells/well) were added to 100 μL of culture medium in 96-well plate. Ten % of BL58 polymer was added to the culture media (RPMI-1640 or DMEM+FBS 10%). Three concentration of polymer (50, 5 and 0.5 mg%) in the medium were prepared, and cells were cultivated in these preparations at 37ºC for 72 hours. The growth and viability of cells was quantitated by the MTT assay, measuring tetrazorium dye reduction by monitoring the absorbance at OD 550 nm [43].

9-3. Results and discussions

9-3-1. Acute toxicity test

Mice were orally administrated 250 mg/kg, 500 mg/kg and 1000 mg/kg of BL58 polymer or xanthan gum. Death of individual mice was not observed in any cases at 2 weeks after the administration of these polysaccharides. The weight of individual mouse steadily increased during experiments. Remarkable pathological change of internal organs was not observed except for slight decolorization of liver. The slight decolorization of liver is assumed to be due to the bleeding when mouse was killed by cervical dislocation because same result was observed in subgroup administrated distilled water. These results suggest that BL58 polymer has no acute toxicity.

9-3-2. Eye irritancy test

The eye irritancy was examined according to Draize standard by two test methods. One was the anterior eye region test (cornea, iris, and conjunctiva) of the rabbit's right eye that was checked at 1, 24, 48 and 72 hours after 1% solution of BL58 polymer macroscopically and ophtalmoscopically and compared with those of the left eye used as a control. Another was the test for abnormalities of cornea that was examined at 24 hours after the instillation by using the FLUORES Ocular Examination Test Paper. No change or irritancy was observed in any cases during observation period and the score based on the Draize criterion was zero. "Non-irritation was judged by the classification of eye irritation according to Draize Kay and Calanda.

9-3-3. Primary rabbit skin irritation test

The irritancy for rabbit skin was evaluated according to the Draize standard at 24, 48 and 72 hours after the BL58 polymer was administrated to the naked skin. There are no changes of rabbit skin or irritancy observed in any cases during the observation period.

9-3-4. Primary human skin irritation test

Finn chamber patch including 1% of BL58 polymer solution was applied to inside of upper arm of 45 healthy adults (men, 16; women, 29) for 24 hours. Four subjects became erythematous within 30 min after the patch test was finished, and three of them were very light symptoms. After 48 hours, all subjects showed no symptom. Skin irritation index of BL58 polymer was 5.6, indicating that the polymer has no irritancy for human. In control experiments, skin irritation index of 0.1% of methyl paraben, which is generally recognized by the FDA as safe for use as preservative in food and cosmetics, was 6.7.

9-3-5. Micronucleus test (Clastogenicity test)

After the oral administration of BL58 polymer (0.25, 0.5, or 1.0 g/kg) to the ICR male mouse, no increase of micronucleated polychromatophilic erythrocytes was observed (1 to 2 polychromatophilic erythrocytes per 1000 orthochromatic erythrocytes). In the case of administration of mitomycin (2 mg/kg) as positive control, the increase in number of micronucleated polychromatophilic erythrocytes was statistically significant

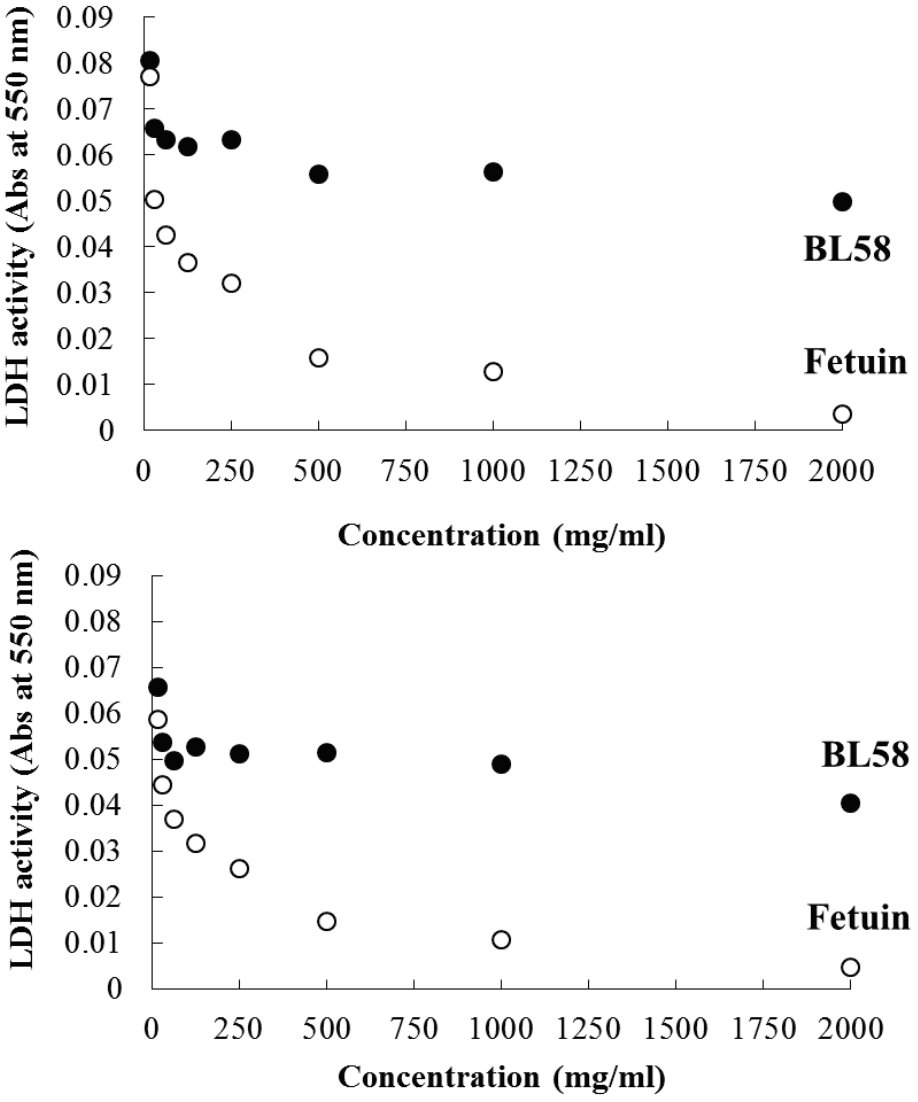
(10 of polychromatophilic erythrocytes per 1000 orthochromatic erythrocytes). These results indicated that BL58 polymer has no clastogenicity or genotoxicity.

9-3-6. Rec assay

The growth inhibition zone for both *Bacillus subtillis* H17 Rec^+ and M45 Rec^- by the BL58 polymer treatment (1.22 to 5000 μg/disk) was not observed. Therefore, BL58 polymer had no damaging effect on DNA.

9-3-7. Anti-bacterial and anti-virus test

Escherichia coli K12 was incubated in NB culture overnight, and inoculated into the freshly prepared culture containing 0 to 2,000 μg/mL of BL58 polymer. When the growth at OD 600 nm was measured after the overnight incubation, more than 500 μg/ml of BL58 polymer exhibited anti-bacterial effect against *Escherichia coli* cells. For the anti-virus test, Human influenza virus (H1N1, H3N2) was infected to MDCK cells and lactate dehydrogenase activity in the culture medium was assayed as a measure of infected cell lysis and, leakage of lactate dehydrogenase into the culture medium (Figure 30). BL58 polymer exhibited concentration-dependent inhibition of viral infection, but the activity of the polymer was lower than that of positive control, fetuin.



A) H1N1 type, 2) H3N2 type
Closed circles indicate BL58 polymer and open circles fetuin

Figure 30 Anti-virus test of BL58 polymer produced by *P. stutzeri* BL58

10-3-8. Skin sensitization test

The skin sensitization was further evoked by the application of the emulsion of 0.25% of DNCB and 0.1% or 0.5% of BL58 polymer to the skin at 2 weeks after final induction by emulsion of 2% of DNCB and 0.1% or 0.5% of BL58 polymer. Saline was used as a control instead of BL58 polymer. At 24 and 48 hours, BL58 polymer has no repression effect on skin sensitization.

9-3-9. Antigenicity test

Since BL58 polymer has no repression effect on skin sensitization, in order to investigate antigenicity of BL58 polymer, active systemic anaphylaxis (ASA) response for guinea pig was investigated. Both test groups sensitized with 1 and 5 mg/kg of BL58 polymer developed ASA response such as scratching nose, decrease of tension level, dyspnea, emiction, defecation and collapse was delayed to 3-4 hours after the injection of 1 mg/kg of BL58 polymer. Two dead animals were observed in weakly sensitized subgroup. In the positive control experiments using 2 mg/kg of egg albumin, ASA response was immediate after the induction injection. Decrease of tension level, twitch and collapse were still continued after 4-5 hours. In the positive control, one dead animal was observed. BL58 polymer showed ASA response and delay compared with the positive control with egg albumin, suggesting that BL58 polymer has weak antigenicity. From these results obtained here, a certain level of safety of the BL58 polymer was confirmed.

9-3-10. Anti-proliferation test of carcinoma cells

9-3-10 (a) Anti-proliferative activity on human acute promyelocytic leukemia cells (HL60) and human monocytoid leukemia cells (U-937)

Effect of BL58 polymer on growth number of human acute promyelocytic leukemia cells (HL60) was examined. Suppression of the cell growth was not observed up to the addition of 50 mg% of BL58 polymer (Figure 31-a), and the cells almost survived after 72 hours (Figure 31-b) and kept proliferating. On the other hand, survival of human monocytoid leukemia cells (U-937) decreased to 70% by the addition of more than 5 mg% of the polymer (Figure 32-a). Addition of 50 mg% of the polymer resulted in further decrease to 10%, and destruction of the cells was observed. The situations of the cell growth before and after addition of BL 58 polymer were shown in the Figure 32-b; the cells were lysed after the addition, and the number of live cells was decreased. HL60

and U-937 are classified as the same leukemia cell types; however, susceptibility to the BL58 polymer was clearly different. The reason is still unclear; however, addition of the polymer might affect signaling related to proliferation, differentiations, or apoptosis (programmed cell death) of these cells.

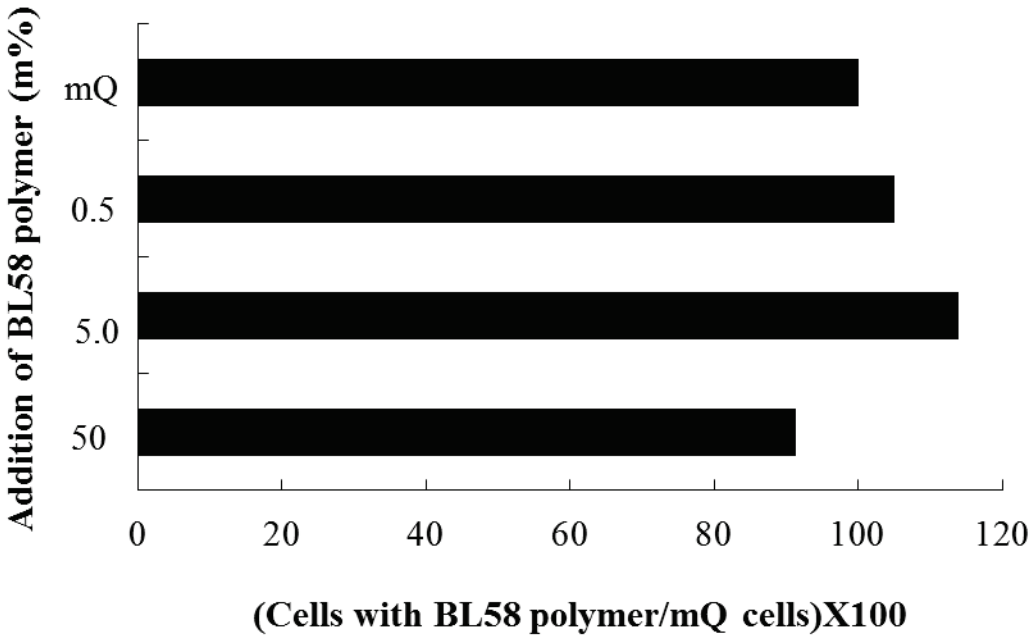


Figure 31-a Change of HL60 cell number after 72 hours cultivation

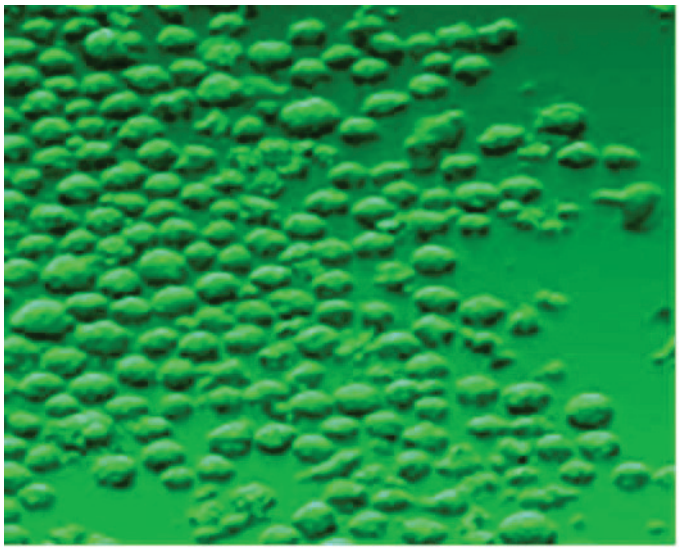


Figure 31-b HL60 cells after 72 hours cultivation

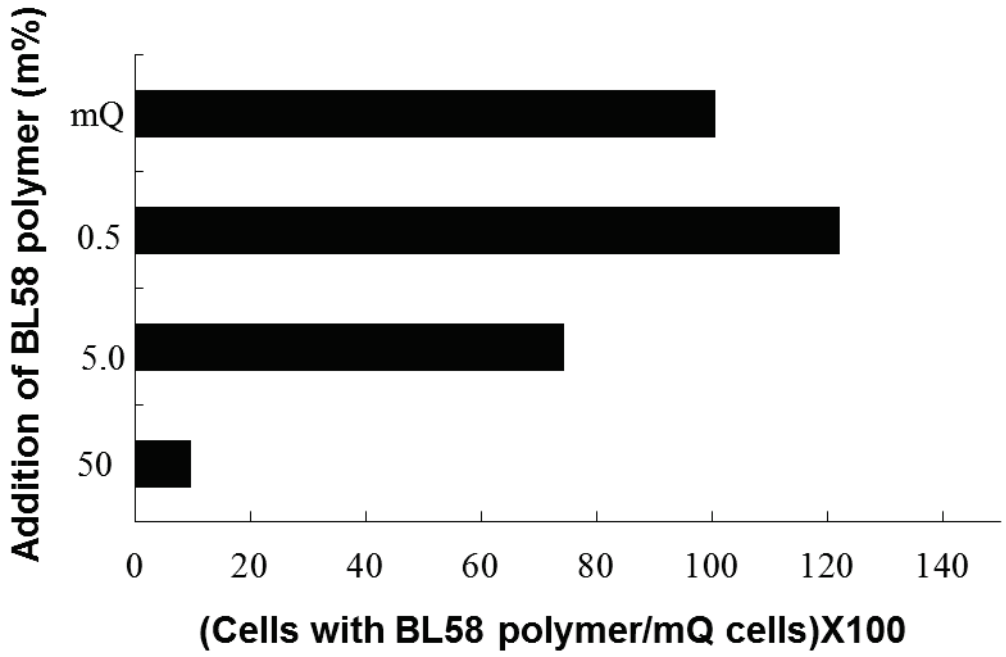
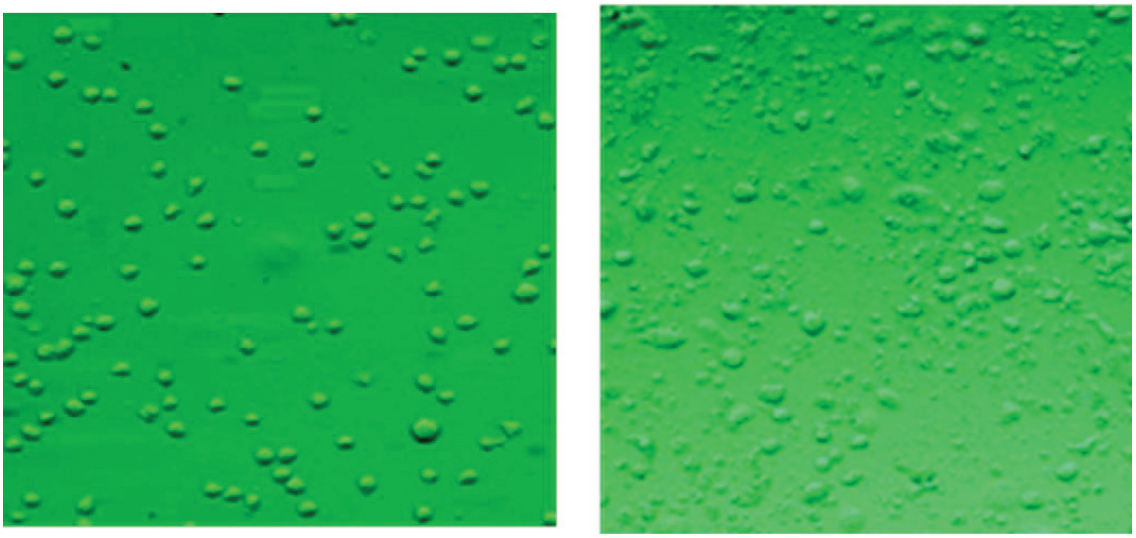


Figure 32-a Change of U937 cell number after 72 hours cultivation



Before addition **After addition**

Figure 32-b U937 cells after 72 hours cultivation

9-3-10 (b). Anti-proliferative activity on human liver carcinoma cells (HepG2) and human normal pulmonic cells (MRC5)

Effect of BL58 polymer on the number of human liver carcinoma cells (HepG2) was shown in Figure 33-a. The ratio of surviving cells was not changed up to the addition of 5 mg% polymer however addition of 50 mg% of polymer resulted in decrease to 65%. This value is same as the number of new-onset cells and the destruction of HepG2 was not observed after the addition of the polymer suggesting repression of cell proliferation. HepG2 cells were very strongly bounded on the plate and there were no dead cells (Figure 33-b). These results for HepG2 cells were very similar to those for human normal pulmonic cells (MRC5). Addition of 50 mg% of polymer resulted in decrease to 58% (Figure 34), suggesting repression of cell proliferation for this non-transformed cell line as well. From the results obtained here, the BL58 polymer exhibited interesting physiological properties, suggesting potential applications in the pharmaceutical field. We are currently studying the mechanism of proliferation repression for the human cell lines tested here.

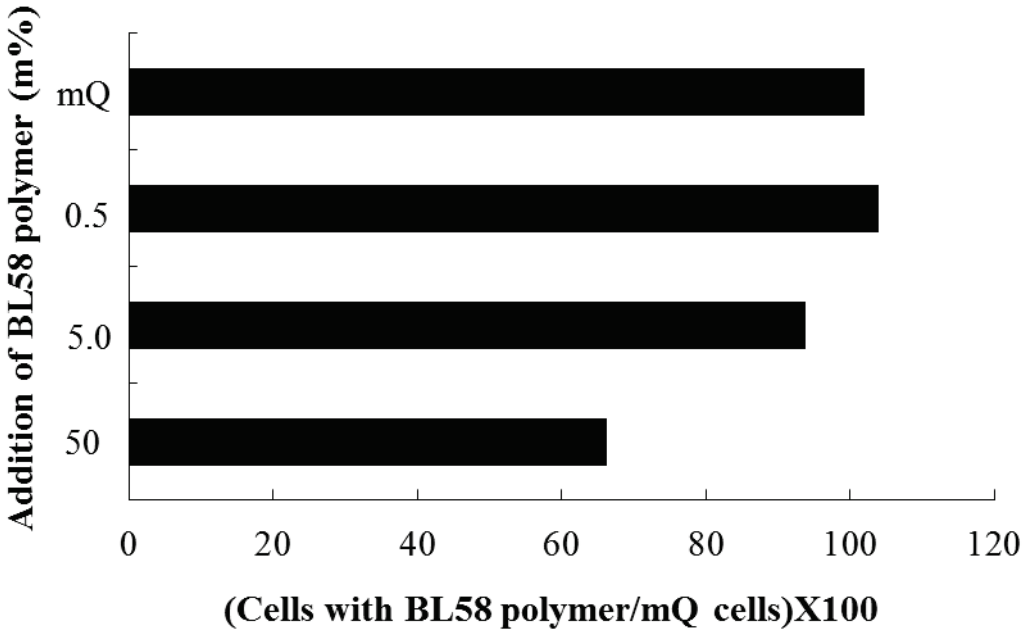


Figure 33-a Change of HepG2 cell number after 72 hours cultivation

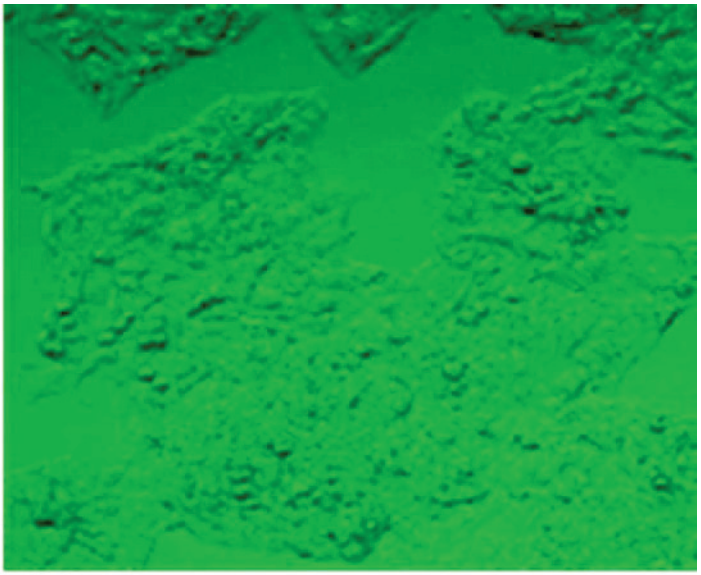


Figure 33-b HepG2 cells after 72 hours cultivation

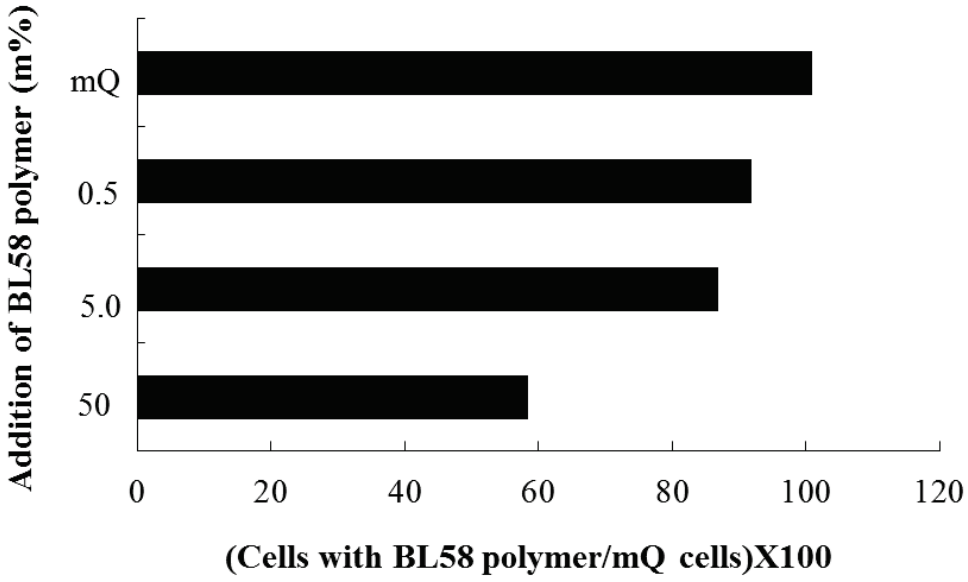


Figure 34 Change of MRC5 cell number after 72 hours cultivation

Chapter 10

Summary and conclusions

The microbial polysaccharides have been studied because of their beneficial bioactive functions as well as their unique rheological properties. Using a severe growth condition, 5% ethanol under alkaline conditions of pH 10, we isolated a *P. stutzeri* BL58 strain capable of producing large amounts of a novel exopolysaccharide which was called BL58 polymer, and we developed an effective purification method for the polymer, not using organic solvent. Typically, microbial polymers are precipitated with an organic solvent, but the highly branched BL58 polymer having a molecular weight of 1,800 kDa was swollen by the addition of ethanol or acetone, so solvent precipitation could not be used. The adsorption of organic solvent into BL58 polymer may be a useful property for industrial chemistry applications in the future.

The rheology of BL58 aqueous solution exhibited a transition from high viscosity solution to viscoelastic gel dependent on the temperature and the pH, suggesting that this behavior is due to its unique carbohydrate structure. Although the constituent sugars of BL58 polymer were the same as those of already reported for *P. stutzeri* polysaccharides by Maaleja et al. [34, 36], the estimated structure of BL58 polymer differed from the previously reported *P. stutzeri* polysaccharides in that 3-*O*-carboxyethyl rhamnose was branched at the 3 position of the glucose rather than the mannose residues. These results suggested that *Pseudomonas* strains synthesize the exopolysaccharides via a common pathway, but the expression of enzymes involved in glycogenesis might be regulated at different levels in each strain and under different culture conditions. The different conditions could control molecular size, ratio of constituent sugars and carbohydrate structure of *P. stutzeri* exopolysaccharides.

Anticipating usage as a base material for cosmetics and pharmaceuticals, we evaluated BL58 polymer in preliminary safety and physiological tests. The acute toxicity, eye irritation, skin irritation, and mutagenicity were negative and the polymer exhibited both antibacterial and antiviral activities. The anti-bacterial activity may be due to the divalent cation chelating activity of polymer observed by viscosity increase and gelation in the presence of Mg^{+} and Ca^{2+}, cations important for cell growth (data not shown). Anti-viral activity has been reported in the past for exopolysaccharides [44]; however the mechanisms are not clearly understood. Perhaps, the most intriguing observation was the anti-proliferative effect on leukemia cells, HL60 and U-927 that were affected at different levels of the polymer. Since both HL60 and U-937 are

classified as having monocytic cell lineage, the difference in susceptibility to polymer inhibition may reflect physiological differences in the two leukemia cell lines. We will attempt to clarify the mechanism for anti-proliferation activity using several tumor cell lines in the future.

In order to use the exopolysaccharides for industry, foods, cosmetics and pharmaceuticals, the condition of large scale fermentation was studied and low cost methods for purification and preparation were established. The structural analysis, rheological properties, stability test, physiological properties were analyzed.

The *Pseudomonas stutzeri* BL58 strain was highly safe, and the developed production and purification methods for the BL58 polymer are inexpensive. Therefore, the possibility of application to various industries is very high.

The BL58 polymer was novel, and it is anticipated that it could be used for as a new thickener of foods and cosmetics due to its non-toxic nature and its physical properties. Another possible use is in the pharmaceutical industry as an anti-inflammatory agent and, perhaps, a therapeutic agent for Alzheimer treatment due to its unique bioactivity.
Dr. Taniguchi at Faculty of Medicine Tottori University evaluated the BL58 polymer for its effect on Alzheimer's disease, and she suggested that BL58 polymer reduces cytotoxicity produced by oxidative stress, acts upon the extension of neurite, and selectively inhibits the production of Aβ1-42, amyloid protein.

More analysis and evaluation will be required to demonstrate efficacy in these areas; however, presuming that the high molecular weight of branched polysaccharide plays a role in intercellular recognition and infection, the analysis of intracellular synthetic metabolic pathway and cell surface secretion mechanisms will make it possible to synthesize various polymer derivatives and expand the use of this unique polymer.

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Publication list

1) Hiroyuki Yamada, Shinichi Yoshida, Koji Nozaki, Tomoyuki Doi, Hiroko Nakamura, and Hideshi Yanase. (2016) Structural Analysis and Physiological Properties of Exopolysaccharides Secreted by a Newly Isolated *Pseudomonas stutzeri* BL58 under Alkaline Conditions. J Biotechnol Biomater 6: 4

Conference presentations

1) ○Hiroyuki Yamada, Hideshi Yanase and Kenji Okamoto (September 22, 2017) Characterization of polysaccharides secreted by *Pseudomonas stutzeri* under alkaline condition. Kansai branch of the Society of Biotechnology, Japan (JSBBA). A-p10
2) ○Hiroyuki Yamada, Hideshi Yanase and Kenji Okamoto (December 1, 2018) Structural and physiological characterizations of polysaccharides secreted by *Pseudomonas stutzeri* under alkaline condition. Nishi-Nihon Branch of the Society for Biotechnology, Japan. C-1

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