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Optimization of culture condition for the gellan production by *Pseudomonas elodea* ATCC 31461

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Abstract

The gellan was produced by *Pseudomonas elodea* under aerobic condition. In this study, the effects of inoculum size, carbon sources and concentration, nitrogen source, and C/N ratio on the cell growth and the production of gellan were evaluated. The maximum growth of *P. elodea* and gellan production was obtained at 5% (v/v) of inoculum size and glucose showed best results among 9 carbon sources tested. The maximum specific yield of 2.22 and productivity of 0.03 g/ℓ/h were obtained at 1.0% (w/v) of glucose. The maximum gellan production was obtained at medium without ammonium nitrate. This indicates that nitrogen limitation is essential for the production of gellan. The highest cell and gellan production were obtained at 20 of C/N ratio.

Key words – fermentation, gellan, optimum culture condition, *Pseudomonas elodea*

Introduction

Currently, one of the exopolysaccharides with great potential for industrial applications is the gellan gum. The gellan gum (PS-60) is a high molecular mass extracellular anionic heteropolysaccharide produced aerobically from *Pseudomonas elodea* ATCC 31461, renamed as *Sphingomonas paucimobilis* [1,11,21].

The gellan consists of linear repeating tetrasaccharide [→3)-β-D-Glc-(1→4)-β-D-GluA-(1→4)-β-D-Glc(1→4)-α-Rha-(1→)] composed of D-glucose (Glc), D-glucuronic acid (GluA), and L-rhamnose (Rha) residues [4,5]. Gellan contains *O*-acetyl group that are readily removed by alkali treatment. Acetyl groups in gellan affect the rheology of gels and the deacetylation of native gellan results in a change from soft, elastic and thermoreversible gel to hard and brittle gel. The native state gellan forms a weak gel in water, however when deacetylated by the treatment of alkali, gellan yields a rigid gel much like agar [3,13,18]. From its novel properties of producing a thermoreversible gel when heated and cooled, gellan gains an importance as a potential agar substitute for tissue culture with optical clarity and gel strength at a given concentration. Besides its application as gelling, thickening, suspending, stabilizing, and emulsifying agent in food systems, gellan has been used in enzyme and cell immobilization and gel electrophoresis. Gellan has also potentials for biomedical applications [2,15, 22,24].

The characteristics and the properties of gellan solutions and gels have been extensively studied and a large number of patents have been registered on food and biological applications of gellan, but the knowledge on

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its production by fermentation is limited. Therefore, the objective of this study is to evaluate the optimum culture condition for gellan production in the flask culture.

Materials and Methods

Microorganism and culture conditions

Pseudomonas elodea ATCC 31461, renamed as *Sphingomonas paucimobilis* was used throughout this study. The chemical composition of basal medium culture as follows per liter of distilled water : Glucose 20 g, K_2HPO_4 0.5 g, $MgSO_4 \cdot 7H2O$ 0.1 g, NH_4NO_3 0.9 g, bactopeptone 0.5 g, mineral salt solution 1 ㎖. The mineral salt solution contained the following composition (㎎/ ℓ, except where stated otherwise): $MnCl_2 \cdot 4H_2O$ 1.8 g/ ℓ, $FeSO_4 \cdot 7H_2O$ 2.487 g/ ℓ, H_3BO_3 0.285 g/ ℓ, $CuCl_2$ 27, $ZnCl_2$ 21, $CoCl_2 \cdot 6H_2O$ 74, $MgMoO_4$ 23, sodium tartrate (dihydrate) 2.1 g/ ℓ. The pH of the medium was adjusted to 6.5-6.8 before the sterilization. The carbon source was autoclaved separately for 15 min at 121℃ and added to the medium under an aseptic condition.

Starter cultures were prepared by transferring cells from agar slants to 5 ㎖ medium in test-tube. These cultures were incubated for 24 h at 30℃ with 200 rpm of agitating speed and used to inoculate for the production of gellan. For the evaluation of the optimal culture conditions, cultures were grown in a 1 ℓ flasks containing 400 ㎖ sterile medium for 72 h under the same conditions used in preparing the starter cultures.

Analytical methods

Samples collected at various interval from shaking flasks were heated to 95℃ for 15 min for the deacetylation of gellan. The pH was adjusted to 10 by the addition of 1N NaOH and then neutralized with 1N HCl. The pretreated broth was centrifuged at 12,000 rpm for 20 min to separate the cells. To determine the cell growth, cell were washed with distilled water and dried at 105℃ until the weight was constant. The supernatant fluids were mixed with two volumes of 95% (v/v) isopropanol and held at 4℃ for 24 h to precipitate the crude gellan, and the gellan precipitate was separated by the centrifugation at 8,000 rpm for 30 min and dried at 105℃ until the weight was constant.

After the removal of gellan from the supernatant of culture broth by isopropanol precipitation, the isopropanol was removed by a rotary evaporator at 50℃ and the volume was adjusted to 10 ㎖ with distilled water, which was used for the measurement of the residual sugar. The phenol-sulfuric acid method was used for measuring the residual sugar [4].

The viscosity of culture broth was measured using a Brookfield viscometer, model DV-Ⅲ (Brookfield Engineering Laboratories, Stoughton, MA, USA) at 1.40 sec^{-1} of shear rate with the experimentation time of 5 min. The viscometer was attached to a circulating water bath maintaining the temperature at 30℃.

Results and discussion

Effect of inoculum size

The increase in the amount of inoculum size minimizes the length of the lag phase and generates the maximum biomass from the production fermenter in a short operation time, thus, increases vessel productivity. The normal inoculum size for microbial cell culture is between 3 and 10% (v/v) of the medium volume [7]. Therefore, inoculum size for the production of gellan was varied from 2 to 15% (v/v).

The maximum growth of *Pseudomonas elodea* and gellan production was obtained at 5% of inoculum size (Fig. 1). The specific yield of gellan at this inoculum size was 1.73 and that of gellan decreased with inoculum size higher than 5%. When inoculum size was higher than 5%, the substrates were utilized for the cell growth rather than the production of gellan by excessive increase in cell density. Therefore it was not effective for the production of gellan at this condition. Also, the space for

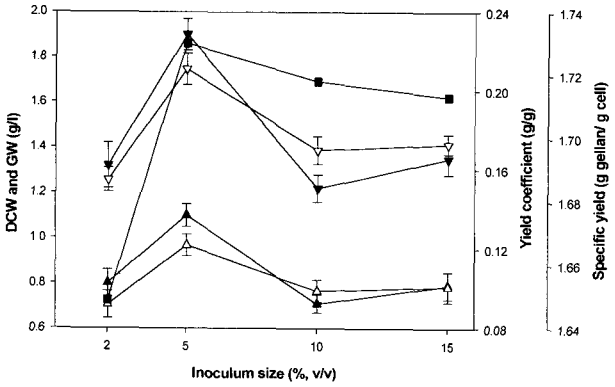

Fig. 1. Effect of inoculum sizes for gellan production by *P. elodea* in shaking flasks culture at 2% glucose, 30℃, 200 rpm.
▲: dry cell weight (g/ ℓ, DCW), ▼: gellan weight (g/ ℓ,GW), △: yield coefficient (g cell/ g utilized substrate) of cell mass, ▽: yield coefficient (g gellan/ g utilized substrate) of gellan, ■: specific yield (g gellan/ g cell).

cell growth was not sufficient, cell density was decrease. This is generally similar to those used for other polysaccharides production, such as reports on the production of pullulan, and α-glucan formed by *Aureobasidium pullulans*, indicating the inoculum size of 5 to 10%. However, Madi compared inoculum levels of 1, 5 and 10% and found that 1% was optimal for high yield of pullulan [16]. As inoculum size increased, the yield of polysaccharide decreased. Conversely, biomass concentration was highest with a 10% inoculum and declined accordingly with 5 and 1% in inoculum size. In this study, both highest cell density and gellan production were observed at 5% inoculum. The possible reason for this is that the production mode of gellan was growth associated type.

Effect on types of carbon sources

The productivity of a fermentation process is often determined by the choice of carbon source, particularly if the product results from the direct dissimilation of it. A carbon source is the basic energy source and constituent for the cell growth and exopolysaccharide production. To find a suitable carbon source for the production of gellan, the *P. elodea* was cultivated in the medium containing various carbon sources (10 g/ ℓ). Among these carbon sources, the maximum production of gellan was 1.52 g/ ℓ with glucose (Fig. 2). The glucose was the most effective carbon source for production of gellan. The fact that glucose was suitable carbon source for gellan production was reported elsewhere [11,25,27,29]. However, Souw *et al.* founded that sucrose was best carbon source for xanthan production by *Xanthomonas campestris* and Yim *et al.* reported starch to be suitable for pullulan production [23,28]. The culture broths from glucose, lactose and maltose became viscous during the fermentations. Fig. 3 shows the viscosity of broth at various carbon sources. The viscosities of culture broths from those carbon sources were 6911, 6623 and 6515 at 1.40 sec^{-1} of shear rate, respectively. The yield of gellan from glucuronic acid was similar to those from lactose and maltose. However, the viscosity of gellan from glucuronic acid was low. This was probably due to the production of low molecular weight of gellan from glucuronic acid as a carbon source. The constitutive sugars such as glucuronic acid and rhamnose showed low yield of gellan and low viscosity. This indicates that the constituting sugars are processed from the metabolic

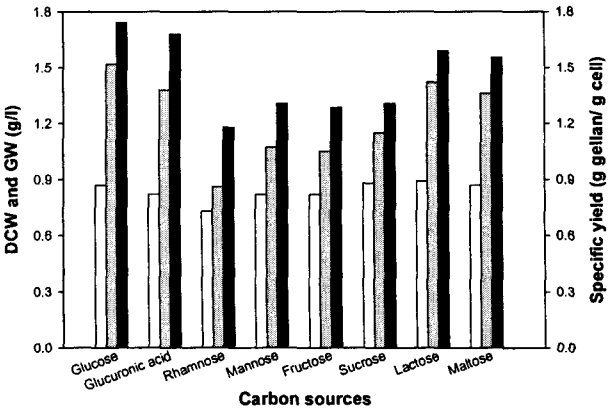

Fig. 2. Effect of carbon sources for gellan production by *P. elodea* in shaking flasks at 5% inoculum size, 2% carbon source concentration. 30℃, 200 rpm.
□: dry cell weight (g/ ℓ), □: gellan weight (g/ ℓ), ■: specific yield (g gellan/ g cell).

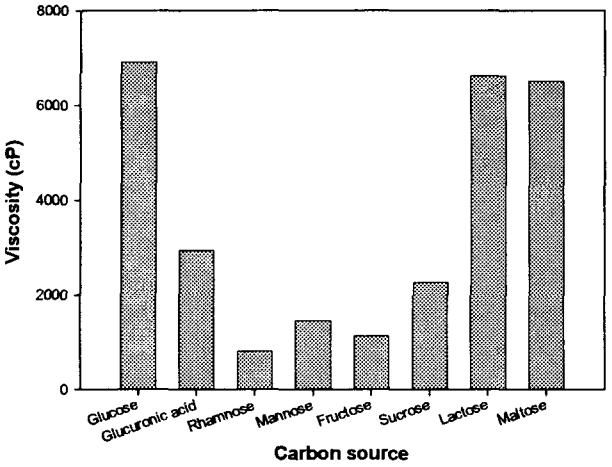

Fig. 3. Viscosity of broth for gellan production by *P. elodea* in shaking flasks culture. Viscosity was measured at 1.40 sec^{-1} of shear rate.

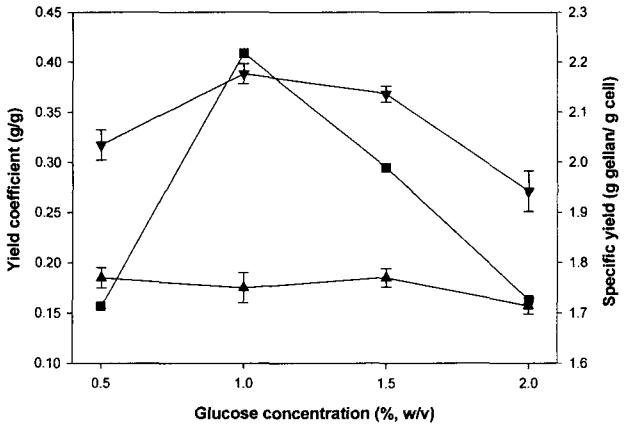

Fig. 4. Effect of glucose concentration for gellan production by *P. elodea* in shaking flask culture at 5% inoculum size, 30℃, 200 rpm.
▲: yield coefficient (g cell/ g utilized substrate) of cell mass, ▼: yield coefficient (g gellan/ g utilized substrate) of gellan, ■: specific yield (g gellan/ g cell).

pathway starting from glucose, suggested by Ligio and Correia [14].

Effect of glucose concentration

The optimum concentration of carbon source was evaluated for the gellan production because broad ranges of optimal concentration of carbon source have been reported depending on the microorganisms. Generally, optimal carbon source concentration was 1~8% (w/v) in the production of polysaccharide by microorganism. In the case of xanthan gum production, glucose or sucrose was used within 4% for the production of polysaccharide, and zooglan was produced at less than 2.5% of glucose. Most of media used in gellan production were low concentration of carbon source less than 2% [5-6,9-10, 14,19,23].

As shown in Fig. 4, the maximum specific yield of 2.22 and productivity of 0.03 g/ ℓ /h were obtained at 1.0% of glucose concentration. When the glucose concentration was higher than 1.0%, the specific yield decreased. Also, high glucose utilization (52% in 72 h) was obtained at glucose concentration of 1.0% compared with 37.3% and 35.0% at glucose concentration of 1.5 and 2.0%, respectively. The cell growth was increased with the increasing glucose concentration, however, a glucose concentration higher than 1.0% inhibited the production of gellan. With 2% glucose concentration in the medium, gellan production was declined probably due to substrate inhibition from the high concentration of glucose.

Effect ammonium nitrate with bactopeptone

Medium constituents other than carbon source also affect the production of gellan. Nitrogen can be an important factor for the regulation of gellan synthesis. The nitrogen sources are used in amount ranging from about 0.05% to 0.20% by the weight of the aqueous medium. Ammonium nitrate is most commonly used as the nitrogen source to sustain gellan production by *P. elodea* [9-11,17]. The ability of this strain to utilize alternate nitrogen sources to synthesize the exopolysaccharide has not been fully examined.

The nitrogen sources in the basal medium for the cell growth and the production of gellan were 0.05% (w/v) bactopeptone and 0.09% (w/v) ammonium nitrate [11]. Therefore, the effect of ammonium nitrate concentration on cell growth and gellan production was investigated in medium that contained 1.0% glucose and 0.05% bacto

peptone. As a result, the highest production of gellan was obtained from the medium without ammonium nitrate (Fig. 5). Also, when the effect of bactopeptone was investigated in medium with 0.09% ammonium nitrate, the production of gellan in all concentration bactopeptone was very low (data not shown). It indicates that nitrogen limitation is essential for the production of gellan. This result agrees with those of West *et al.* [26].

Effect of C/N ratio

Based on previous results on nitrogen limitation, the production of gellan was examined in various ratios of carbon source and nitrogen source (C/N ratio), in which 1% of glucose was supplied and the concentration of bactopeptone was varied. As shown in Fig. 6, the maximum gellan production of 1.66 g/ ℓ and gellan yield coefficient of 0.24 were obtained at 20 of C/N ratio. Also, maximum specific yield of 1.50 was obtained at this C/N ratio. Gellan production was decreased by the medium with lower or higher than C/N ratio of 20. When the C/N ratio was higher than 20, the cell mass

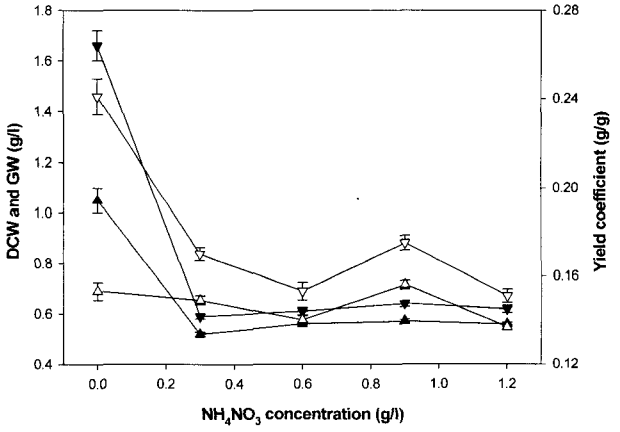

Fig. 5. Effect of ammonium nitrate concentration with 0.05% of bactopeptone for gellan production by *P. elodea* in shaking flask culture at 5% inoculum size, 1% glucose, 30℃, 200 rpm.
▲: dry cell weight (g/ ℓ), ▼: gellan weight (g/ ℓ), △: yield coefficient (g cell/g utilized substrate) of cell mass, ▽: yield coefficient (g gellan/ g utilized substrate) of gellan.

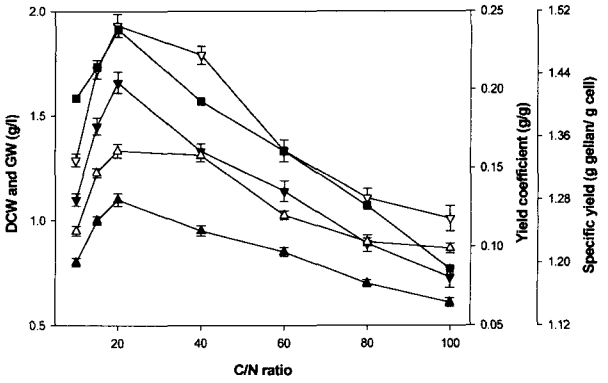

Fig. 6. Effect of C/N ratio for gellan production by *P. elodea* in shaking flask culture at 5% inoculum size, 1% glucose, 30℃, 200 rpm.
▲: dry cell weight (g/ ℓ), ▼: gellan weight (g/ ℓ), △: yield coefficient (g cell/ g utilized substrate) of cell mass, ▽: yield coefficient (g gellan/ g utilized substrate) of gellan.

and production of gellan were decreased drastically. Kang and Cottrell reported that excess nitrogen in medium reduced conversion of carbon source to extracellular polysaccharide although nitrogen was necessary for cell growth and polysaccharide synthesis [12]. Williams and Wimpenny reported that the reduction of nitrogen level favored the polysaccharide production [27]. In this study, the optimum C/N ratio was 20 and farther reduction in nitrogen decreased cell growth and gellan production due to the retardation of cell growth.

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초록 : *Pseudomonas elodea* ATCC 31461에 의한 gellan 생산의 최적 배양조건

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Pseudomonas elodea ATCC 31461에 의해 생산되는 미생물 다당류인 gellan의 최적 조건을 확립하기 위해 flask상에서 inoculum size, 탄소원 종류 및 농도, 질소원 종류, C/N ratio등을 조사하였다. Gellan 생산을 위한 최적 inoculum size는 5%(v/v)로 나타났으며, 1.0%(w/v)의 glucose에서 2.22의 최대 specific yield 와 0.03 g/ℓ의 생산성을 얻을 수 있었다. 또한 ammonium nitrate가 없을 때 최대 생산을 얻을 수 있어, gellan 생산을 위해서 nitrogen limitation이 필요함을 알 수 있었다. Glucose와 bactopeptone을 이용하여 C/N ratio를 조사한 결과 20에서 gellan 생산이 가장 높게 나왔다.