Sugarcane Juice Fermentation for Alginate Production by Azotobacter vinelandii

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Abstract—
Alginate production by Azotobacter vinelandii ATCC 9046 using sugarcane juice (approximate content of sucrose 10 g/L) as a main carbon source was investigated by altering pH-controlled values at 5.59, 6.00, 7.00, 8.00, and 8.41 and nitrogen concentrations using 0.02, 0.10, 0.30, 0.50, and 0.58 g/L yeast extract. The maximum biomass concentration was strongly affected by the nitrogen content in the sugarcane juice medium, while both the nitrogen content and the controlled pH of the medium influenced the alginate production ($P<0.05$). However, their interaction effect was not determined for both the biomass and the alginate productions. The highest alginate concentration was achieved under the culture at the pH-controlled value of 6.0 and nitrogen concentration of 0.10 or 0.50 g/L as 6.89±0.98 and 7.29±0.10 g/L, respectively. Moreover, it was found that the molecular weight of the obtained alginate was affected by the initial concentration of nitrogen in the culture. The data from this research suggested that the culture under pH 6.0 and 0.1 g/L nitrogen was suitable for alginate production by A. vinelandii ATCC 9046 using sugarcane juice as a carbon source due to high alginate production.

Index Terms—Sugarcane juice, Alginate, Nitrogen source, pH control, Azotobacter vinelandii.

I. INTRODUCTION

Sugarcane is an economically important crop in the tropical areas, especially in Southeast Asia and some South American countries. The plant was used as a raw material for juice, sugar, and biofuel production [1]. In Thailand, sugarcane was currently cultivated on more than 4.3 million acres and sugarcane plantations also tended to increase. Based on statistical data in the last decade (in the crop year 2008/9-2017/18), the average annual expansion of the cultivation region was 6.09% [2]. Under the situations surplus supply of sugarcane and decline in global sugar price, the biotechnological industry had been developed to alter the utilization of sugarcane, such as bioethanol production [3]. The main compositions of sugarcane juice are sugars (15-18% sucrose and 0.5% reducing sugar), salts, organic acids, and some organic non-sugars [4], sufficient for the microbial growth [5]. The juice was reasonable to be used as a medium for the fermentation process which was operated using sugar as a carbon source.

Alginate is a linear polysaccharide chain; its structure consists of mannuronic acid and guluronic acid [6]. Alginate is naturally occurred from some species of brown algae and synthesized by two bacteria Azotobacter and Pseudomonas [7]. Advantage of bacterial cultivation for alginate synthesis is a possibility to control monomer distribution and molecular mass, which involves its diversity of physicochemical properties [8]. Its specific properties such as swelling ability and the capacity of film-forming support it to be used as a biomaterial in biomedical industries and a stabilizer, emulsifier, and gelling agent in the food industry [7]. Because Pseudomonas genus is an opportunistic pathogen [9], the alginate production using A. vinelandii is therefore preferable to be developed in the biotechnological process. Sugarcane juice was studied as a good carbon source for producing alginate and bacterial growth because of its high in sugar, vitamins, and minerals [10]. In addition, this carbon source is a cheap raw material, therefore, decreasing the cost of production. Trujillo-Roldán et al. [11] demonstrated that the culture of A. vinelandii ATCC 9046 in sugarcane juice obtained the lowest alginate yield due to lower nitrogen concentration, decreasing alginate production. When the culture was grown in sugarcane juice with Burk's medium, the alginate yield was twice enhanced [11]. The aims of this study were to investigate alginate production by A. vinelandii ATCC 9046 using sugarcane juice as a main substrate and to

determine the effect of nitrogen concentrations and pH of culture on the bacterial growth, and alginate quantity and molecular mass.

II. MATERIALS AND METHODS

A. Preparation of inoculum and medium

Azotobacter vinelandii ATCC9046 was studied in this research. The organism was grown in modified Burk's medium with the following composition (g/L): sucrose 20, K_2HPO_4 0.2, KH_2PO_4 0.8, $CaCl_2$ 0.1, $MgSO_4 \cdot 7H_2O$ 0.2, $Na_2MoO_4 \cdot 2H_2O$ 0.01, $FeCl_3 \cdot 6H_2O$ 0.01 and yeast extract 0.5. The culture media was sterilized by autoclaving at 121 °C for 20 min. The inoculum was prepared in 500 mL baffled Erlenmeyer flasks containing 200 ml of the modified Burk's medium and incubated in the water bath shaker (Memmert, WNB 45, Germany) at 130 rpm and 25 °C for 48 h. The bacterial cells were aseptically collected by centrifugation and then re-suspended in 200 ml sterile water.

B. Preparation of sugarcane juice medium

Sugarcane juice was extracted from Khonkaen 3 sugarcane which was contributed by Mitr phol sugar mill, Suphanburi. The juice was removed from suspended solids by defecation clarification process as follow: Fresh juice was heated to 75 °C, and then adjusted pH to 6.2 using $Ca(OH)_2$. The juice was boiled and removed insoluble solids by a filtration through No. 1 Whatman filter-paper [12]. Then, sucrose concentration in the juice was analyzed using sucrose assay kit (Megazyme, Bray, Ireland). The sugarcane medium was modified from the Burk's medium by replacing sucrose by the prepared sugarcane juice. The content of sugarcane juice was calculated to achieve the final concentration of sucrose as 11.1 g/L. The nitrogen content was varying by adding different content of yeast extract (Himedia, RM027; containing 10.50 % of nitrogen). The pH of the culture was controlled using 0.1 M potassium phosphate buffer at different value as designed.

C. Fermentation

20 ml of this suspension was transferred to 180 ml sugarcane juice medium in 500 ml baffled Erlenmeyer flask (target sucrose concentration at 10 g/L). Thirteen experiments derived according to central composite design (CCD) technique (Table. I) through Minitab statistic software version 18 were operated to study the effect of nitrogen concentration and the pH value of the culture (5.59, 6.00, 7.00, 8.00 and 8.41). The cultures were grown in the water bath shaker at 25 °C. and 130 rpm for 72 h. All the cultures were carried out in duplicate.

Table. I Central composite design for alginate production of A. vinelandii with various nitrogen concentrations and pH-controlled values

Run	Variables	
	Nitrogen (g/L)	pH (-)
1	0.02	7.00
2	0.30	7.00
3	0.30	8.41
4	0.50	8.00
5	0.30	5.59
6	0.30	7.00
7	0.10	6.00
8	0.30	7.00
9	0.50	6.00
10	0.30	7.00
11	0.58	7.00
12	0.10	8.00
13	0.30	7.00

D. Biomass and Alginate Determinations

Biomass and alginate concentrations were determined by the gravimetrical method as follow. 10 mL of the culture sample was mixed with 1 mL of 1 M NaCl and 1 mL of 0.1 Na_4EDTA and then centrifuged at 6000 rpm and 20°C for 40 min to separate bacterial cells. The cell pellet was washed twice by distilled water, re-centrifuged then dried at 80°C for 24 h, and weighted. The supernatant was supplemented with 30 ml of isopropanol to precipitate alginate capsular. The precipitate was collected by filtering through a No.1 Whatman filter-paper, then dried at 80°C for 24 h, and weighted [13], [14].

E. Alginate molecular mass determination

Alginate molecular mass was measured by gel permeation chromatography with a serial set of Ultrahydrogel columns (e2695 and Linear Waters), using a HPLC system with a refractive index detector (Waters, 2414). Elution was performed with 0.05 M $NaHCO_3$ at 30°C at a flow rate of 0.6 ml/min. Pullulans of Aureobasidium pullulans was used for a standard molecular mass (5,000 - 708,000 Da) [14].

III. RESULTS AND DISCUSSION

A. Biomass production

The biomass concentration during the cultivation shown in Fig. I presented that the bacterial growth started without a lag phase in all the cultures. The curves were clearly separated into high and low biomass productions. Even if the pH values of the culture were different, the low level of biomass production was found when the concentration of nitrogen was low (0.02 and 0.10 g/L). The biomass concentrations in the cultures with 0.10 and 0.02 g/L nitrogen exponentially increased for 12 h, and then they were constant till the end of fermentation. In contrast to that in 0.30, 0.50, 0.58 g/L nitrogen conditions, the biomass continuously increased and maintained constant when the bacterial growth phase ended at 30 and 48 h. Based on the results, it was clear that the nitrogen concentration influenced the bacterial growth stronger than pH value of the culture. Increasing nitrogen concentration up to 0.30 g/L led to increase the bacterial growth, beyond this concentration the bacterial growth did not improve. It indicated a saturation of supplementing nitrogen concentration [15]. In the conditions with similar nitrogen concentration but different pH of culture, the slight differences in maximum biomass concentration were obtained as shown in the cultures with pH 6.00 and pH 8.00. Moreover, in the culture grown under 0.30 g/L nitrogen, changes in pH-controlled values affected the highest cell biomass content. The lowest maximum biomass concentration was obtained as 1.07±0.12 g/L under the cultures at pH 7.00 and 0.02 g/L nitrogen and the highest biomass concentration was obtained as 5.88±0.42 g/L under the culture at pH7.00 and 0.30 g/L nitrogen, which was similar to the maximum biomass concentration obtaining at pH 8.00 and 0.50 g/L nitrogen, pH 5.59 and 0.30 g/L nitrogen, pH 6.00 and 0.50 g/L nitrogen, and pH 7.00 and 0.58 g/L nitrogen as 5.72±0.87, 5.41±0.21, 5.74±0.63, and 5.40±0.59 g/L nitrogen, respectively. The statistical analysis showed the bacterial growth was individually affected by pH-controlled value and nitrogen concentration ($P<0.05$), but there was no significant interaction effect ($P>0.05$) as shown in Table. II The microorganism required nitrogen for proteins, amino acids, DNA, and RNA synthesis [16]. Although, Azotobacter species were tolerant to a wide range of pH. Several studies [17], [18] found the optimum pH for bacterial growth of A. vinelandii was natural pH as 6.5-7.5 because it affected a substrate consumption and a nutrient requirement of microbial cell [19].

B. Alginate Production

The alginate production of A. vinelandii in sugarcane juice media was evaluated as shown in Fig. II Regardless of the pH-controlled value, the alginate production under 0.02 and 0.10 g/L nitrogen in the growth phase was about 65%, while it was about 82-92% under fermentation

using 0.30, 0.50, and 0.58 g/L nitrogen of the media. It indicated the alginate 6.00 obtained the maximum alginate concentration larger the media. Similarly, the maximum alginate concentrations at 0.30 g/L were significantly different when the cultures grown at different pH values due to a sensitivity of alginate synthesis to pH of substrate [20]. Also, the enzymes responsible for the bacterial alginate synthesis were affected by external pH [20].

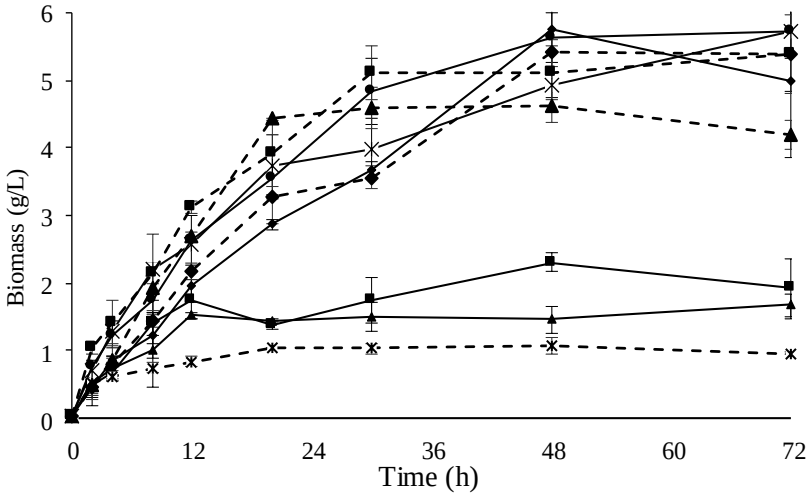

Fig. I Bacterial growth during alginate production by A.vinelandii ATCC 9046. Biomass concentrations at pH 6.0, 0.1 g/L N (—■—); pH 8.0, 0.1 g/L N (—▲—); pH 6.0, 0.5g/L N (—◆—); pH 8.0, 0.5 g/L N (—✳—); pH 7.0 0.02 g/L N (· -✳- -); pH 7.0, 0.58 g/L N (· -■- -); pH 5.59, 0.3 g/L N (· -◆- -); pH 8.41, 0.3 g/L N (· -▲- -) and pH 7.0, 0.3 g/L N (—●—)

Bacterial cell required maintenance energy to control pH when it was exposed to pH beyond the optimum range resulting in reducing the energy available for alginate production [17,21]. The results of this study indicated that the optimum pH for alginate production from sugarcane juice medium was in the rage of 6.00 – 7.00, which was supported by the observation in the studies of Vermani et al. [19] and Saeed et al. [17], finding that the optimum pH for producing alginate was neutral. It was consistent with the work of Auhim and Hassan [22] indicated that the maximum alginate yield from A. vinelandii was obtained under the fermentation with the initial pH 6.5 [22]. However, David [23] produced alginate by the cultivation of A. chroococcum and observed the maximum alginate yield obtaining the culture grown at a range of pH 7.00 and 8.00. A similar maximum alginate concentration was obtained at different nitrogen concentrations of 0.10 and 0.50 g/L although production in this study was associated with the growing bacteria. it might be related to supplement nitrogen source in the culture medium. Zapata-Vélez et al. [15] showed that a large fraction of alginate production in the diazotrophic culture was obtained in the stationary growth phase, while the highest amount of polymer production in a culture grown by adding nitrogen source happened in the exponential growth phase. As shown in Fig. II, Alginate production was altered sorely depending on pH-controlled value more than nitrogen content. The culture grown under a pH 6.00 obtained the maximum alginate concentration larger the media. Similarly, the maximum alginate concentrations at 0.30 g/L were significantly different when the cultures grown at different pH values due to a sensitivity of alginate synthesis to pH of substrate [20]. Also, the enzymes responsible for the bacterial alginate synthesis were affected by external pH [20]. A similar maximum alginate concentration was obtained at different nitrogen concentrations of 0.10 and 0.50 g/L although the cultures grown under the same pH-controlled value. In the cultures were operated using pH-controlled of 7.00, the alginate production was affected by nitrogen concentration. An increase in nitrogen content from 0.02 to 0.30 g/L caused improving alginate production. Increasing nitrogen concentration to 0.58 g/L reduced the

alginate production, demonstrating a saturation of nitrogen content for alginate synthesis condition. The highest amount alginates were found using pH-controlled 6.00 both 0.10 and 0.50 g/L nitrogen with values of 6.89±0.98 and 7.29±0.10 g/L, respectively. Lower alginate production was obtained under high pH-controlled 8.00 in both 0.10 and 0.50 g/L nitrogen media (2.67±0.05 and 2.29±0.14 g/L) and pH-controlled at 8.41 in the 0.30 g/L nitrogen (2.91±0.10 g/L). Complementing highest nitrogen content (0.58 g/L) obtained lower alginate production (2.59±0.26 g/L). It was contrast to the research of Zapata-Vélez et al. [17] reporting that an increase in nitrogen sources both inorganic and organic nitrogen enhanced biomass and alginate productions.

Table. II Statistical analysis of alginate production of A. vinelandii

Factor	P-value		
	$Biomass_{max}$	$Alginate_{max}$	MMW
Nitrogen concentration	<0.0001*	<0.0001*	<0.0001*
pH-controlled value	0.0285	<0.0001*	<0.0001*
Interaction	0.3525	0.2745	<0.0001*

* Statistically significant difference (P<0.05)

However, in the case of peptone, reduction of carbon/nitrogen ratio from 29/1 and 19/1 in the medium observed no differences in alginate production, showing a saturation trend in the supplement nitrogen amount [15]. Although a change in yeast extract concentration from 1.0 to 3.0 g/L led to a bit drop in alginate production with a value of 3.10 and 2.80 g/L. It might be possible that the number of other nutrients had a relation with the amount of nitrogen to play a role in polymer production by bacteria. This study found that an increase in nitrogen concentration from 0.30 to 0.58 g/L resulted in decreasing alginate production.

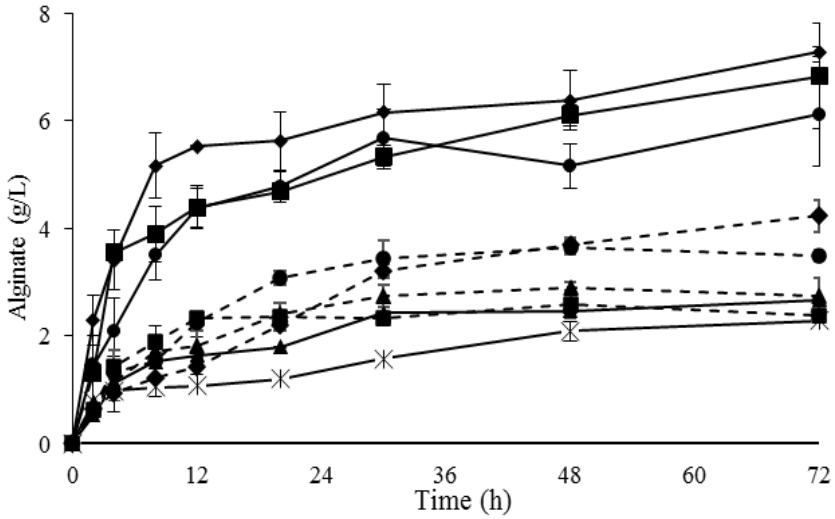

Fig. II Alginate production during alginate production by A.vinelandii. (a) alginate concentrations at pH 6.0, 0.1 g/L N (—■—); pH 8.0, 0.1 g/L N (—▲—); pH 6.0, 0.5g/L N (—◆—); pH 8.0, 0.5 g/L N

(—⁎—); pH 7.0 0.02 g/L N (· -⁎- ·); pH 7.0, 0.58 g/L N (· -■- ·); pH 5.59, 0.3 g/L N (· -◆- ·); pH 8.41, 0.3 g/L N (· -▲- ·) and pH 7.0, 0.3 g/L N (—●—)

Table. III Maximum biomass and alginate concentrations in the cultures of A. vinelandii

Variables		$Biomass_{max}$	$Alginate_{max}$
Nitrogen (g/L)	pH (-)	(g/L)	(g/L)
0.02	7.00	$1.07\pm0.12^{a,AB}$	$3.64\pm0.13^{bc,B}$
0.30	7.00	$5.88\pm0.42^{b,AB}$	$6.11\pm0.97^{a,B}$
0.30	8.41	$4.63\pm0.27^{b,AB}$	$2.91\pm0.10^{a,CD}$
0.50	8.00	$5.72\pm0.87^{b,A}$	$2.29\pm0.14^{ab,D}$
0.30	5.59	$5.41\pm0.21^{b,B}$	$4.29\pm0.29^{a,BC}$
0.10	6.00	$2.31\pm0.14^{a,A}$	$6.89\pm0.98^{ab,A}$
0.50	6.00	$5.74\pm0.63^{b,A}$	$7.29\pm0.10^{ab,A}$
0.58	7.00	$5.40\pm0.59^{b,AB}$	$2.59\pm0.26^{c,CD}$
0.10	8.00	$1.68\pm0.17^{a,A}$	$2.67\pm0.05^{ab,D}$

The different superscript letters in the same column showed significant differences ($P<0.05$; Tukey test) between the maximum biomass and alginate concentration at different nitrogen concentration ($^{a-c}$) and pH-controlled ($^{A-D}$).

The results in this study indicated that pH-controlled value and nitrogen concentration in culture medium were important roles for alginate production by bacteria. Based on the statistical analysis, showing that pH-controlled value and nitrogen concentration had an individual effect on alginate production ($P<0.05$). However, the interaction effect was not found ($P>0.05$) as shown in Table. II The maximum alginate production was obtained from the culture with the initial pH 6.00 and supplemented nitrogen 0.50 g/L, which was not significantly different to supplemented nitrogen at 0.10 g/L as presented in Table. III The molecular weight of the obtained sodium alginate was measured as 76 kDa, close to the molecular weight of algae alginate 76 – 154 kDa, which obtained from Macrocystis pyrifera [24]. However, the molecular weight of alginate obtained from the culture with pH 6.00 (same pH as above) and supplemented with N 0.10 g/L (lower than the above condition) was up to 1,355 kDa. It was clearly that the molecular weight of alginate was affected by the nitrogen content in the media. It was like the result of [15], [25] investigating that alginate produced at nitrogen concentration above 0.10 g/L obtained the molecular weight lower than 85 kDa under the controlled-pH conditions and below 0.10 g/L obtained the molecular weight of alginate up to 1000 kDa.

CONCLUSION

Nitrogen concentration and pH-controlled value of the sugarcane juice medium had an impact on alginate production of A. vinelandii ATCC 9046. Beyond nitrogen concentration of 0.30 g/L observed higher the bacterial growth. The highest maximum concentration was obtained under the culture with the pH-controlled value of 6.00 and the initial nitrogen concentration 0.50 or 0.10 g/L. However, the optimum culture condition was the pH-controlled value of 6.00 and 0.10 g/L nitrogen due to the higher molecular weight. The pH-controlled value and nitrogen concentration

individually affected the alginate quantity and the bacterial growth ($P<0.05$).

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