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Production of Oligosaccharide from Alginate Using *Pseudoalteromonas agarovorans*

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Abstract A marine bacterium was isolated from seawater near the Korean south coast for efficient saccharification from alginate. Based on 16S rDNA sequence, the isolated strain was identified as *Pseudoalteromonas agarovorans*. Various environmental factors affecting saccharification of alginate using *P. agarovorans* CHO-12 have been investigated in flask cultures. The optimum concentration of sugar was obtained at 30 rpm and 29 °C. Among various NaCl concentrations, when NaCl concentration was increased from 10 to 30 g/l, the cell concentration sharply increased, while there is no increase at above 40 g/l. The maximum sugar concentration was obtained at 13.8 when 30 g/l of NaCl was used. Yeast extract and corn steep liquor were the best nitrogen source for efficient saccharification. Especially, the sugar concentration of 14.9 g/l was obtained after 3 days of culture using a mixture of 1.0 g/l of yeast extract and 1.5 g/l of corn steep liquor. Scale up was carried out at 50 l of reactor for 3 days using *P. agarovorans* CHO-12 and *Stenotrophomonas maltophilia* sp. When *S. maltophilia* was used, cell concentration was about twofold higher than that of *P. agarovorans* CHO-12. On the other hand, when *P. agarovorans* CHO-12 was used, the maximum saccharification rate was obtained, 7.5 g/l/day after 2 days of culture, which was about tenfold higher than that of *S. maltophilia.*

Keywords Alginate · Oligosaccharide · Saccharification · *Pseudoalteromonas agarovorans*

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Introduction

Oligosaccharides such as oligomannuronate and oligoguluronate derived from alginate have been investigated as new functional materials such as food, textile, oil, cosmetics, and pharmaceutical industries. In certain plant cultures, the production of pharmaceutically important secondary metabolites has been enhanced by addition of elicitors such as oligosaccharides derived from fungal, plant, and bacterial alginates [1–4]. Certain oligosaccharides have been reported to act as signaling molecules, which can regulate physiological and defense process in some plants, animals, and bacteria. For instance, oligosaccharides derived from alginate have been shown to promote germination and shoot elongation of certain plants [5, 6]. Jeong and Park investigated the growth and secondary metabolite biosynthesis in the culture of *Panax ginseng* hairy root using oligosaccharide [7]. Akimoto et al. reported that the addition of the oligosaccharide to a suspension culture of *Cath. Roseus* L. or *Wasabia japonica* cells promotes the production of antibiotic enzymes [8]. Also, Natsume et al. reported that in barley bioassay, oligosaccharide promoted the root growth [9]. Oligosaccharides were also used as an energy source for efficient penicillin production in *Penicillium chrysogenum* culture. There was approximately 50% maximum increase in penicillin G yield from the biomass in the culture of *P. chrysogenum* P2 and 15% in the culture of *P. chrysogenum NRRL* 1951 when compared to control culture without oligosaccharides addition [10]. In the case of *Bifidobacteria* culture, oligosaccharides accelerated its growth when added as a supplement to the culture medium and the activity of human monocytes to produce cytokinese, which are defense metabolites [4, 11].

However, for producing the oligosaccharides fragments from alginate, various chemicals such as acid and alkali have been used. In addition, technical, environmental, and economic problems arise because of the complexity, chemical use, byproduct treatment, and contamination involved in these saccharification processes using alginate. Matsubara et al. proposed an enzyme reactor for degrading alginate using an immobilized alginate in continuously stirred tank. However, non-degraded alginate remained needed to be removed to obtain an oligosaccharide mixture [12]. In addition, in this case, the purified enzyme is very expensive. We previously isolated *Stenotrophomonas maltophilia* from seawater in Korea and investigated the capability of direct saccharification from alginate and seaweeds to solve the above problems [13].

In this study, we described the isolation of *Pseudoalteromonas agarovorans* CHO-12 capable of saccharifing alginate and its morphological and biochemical characteristics. The optimal culture and medium conditions for saccharification are discussed. In addition, for comparing *P. agarovorans* CHO-12 and *S. maltophilia* on cell concentration, pH, sugar concentration, and sugar production rate, scale up was carried out under the optimized culture and medium conditions.

Materials and Methods

Isolation of Strain and Conditions of Media and Culture

Seawater samples were collected near the south coast of Korea. Two hundred microliters of seawater was thoroughly suspended with 50 mM phosphate buffer (pH 7.0) and subsequently diluted serially in the same buffer. Aliquots were plated on agar media. The plates were incubated at 30 °C for 7 days. Single colonies on the plates were purified by transferring them into new plates and incubated once again under agar media. The purified

colonies were identified by 16S rDNA sequences and used for liquid culture. The agar medium compositions were as follows (g/l): glucose 2.5; alginate, 0.5; peptone, 2.0; K_2HPO_4, 0.3; yeast extract; 0.2; $MgSO_4 \cdot 7H_2O$, 0.5; NaCl, 20, and agar, 15. The medium compositions for the seed culture were as follows (g/l): glucose 2.5; yeast extract 1.0, K_2HPO_4 1.0, $MgSO_4 \cdot 7H_2O$ 0.5, and NaCl 20. For saccharification from alginate, the basal medium compositions for the culture were as follows (g/l): glucose 2.5; alginate, 10–15; peptone 0.5, yeast extract 0.5, K_2HPO_4 0.5, $MgSO_4 \cdot 7H_2O$ 0.5, and NaCl, 20. One loopful of *S. maltophilia* sp and *P. agarovorans* CHO-12 slant culture was inoculated into a 500-ml Erlenmeyer flask 200 ml of working volume containing 50 ml of seed medium. The seed culture was carried out at 30 °C in a rotary shaker at 100 rpm for 1 day. All media were sterilized at 121 °C for 15 min.

Analytical Methods

16S rDNA Sequences

The 16S rDNA coding region was amplified by polymerase chain reaction (GenAMP™ polymerase chain reaction (PCR) System 9700), Perkin-Elmer, Boston, MA, USA) using two oligonucleotide primers, 5′-AGAGTTTGATCMTGGCTCAG-3′ and 5′-AAG GAGGTGWTCCARCC-3′. The PCR product was purified using a PCR Preps DNA purification system (Promega Co., Madison, WI, USA), and direct sequencing was performed on ABI PRISM™ 310 genetic analyzer (Perkin-Elmer) and BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer) [14].

Similarity Index Analysis

The 16S rDNA sequence of the isolated strain was compared with several references strains for determination of the similarity index. The accession numbers for the sequences used as reference strains were as follows: *P. agarovorans* AJ417594, *Pseudoalteromonas atlantica* X82134, *Pseudoalteromonas espejiana* X82143, *Pseudoalteromonas carrageenovora* X82136, *Pseudoalteromonas tetraodonis* X82139, *Pseudoalteromonas issachenkonii* AF316144, *Pseudoalteromonas undina* X82140, *Pseudoalteromonas elyakovii* AF082562, *Pseudoalteromonas distincta* AF082564, *Pseudoalteromonas haloplanktis* X67024, *Pseudoalteromonas nigrifaciens* X82146, and *Pseudoalteromonas antartica* X98336. The phylogenetic trees were constructed using the neighbor-joining method and the MEGA3 program with bootstrap values based on 1,000 replications [15].

DNA G+C Concentration

Chromosomal DNA for determination of G+C was extracted from cells and purified. DNA enzymatically degraded into nucleotides. The purified sample was centrifuged at 14,000× 5 min. The supernatant (0.1 ml) was reacted at 100 °C for 5 min. Nuclease (1 unit/μl) was added into the micro tube containing the reaction mixture and reacted at 50 °C for 3 h. Alkaline phosphatase (buffer 10 μl, phosphatase 5 μl) was added into the micro tube containing the reaction mixture and reacted at 37 °C for 12 h. After centrifugation of the nucleotide mixture at 1,400 rpm for 5 min, it was used. The obtained nucleotide mixture was then separated by high performance liquid chromatography using a waters Nova-Pak C_{18} column and eluted by a mixture of 0.2 M $(NH_4)_2H_2PO_4$ and acetonitrile (20:1 *v*/*v*) at a flow rate of 0.5 ml/min and detected by UV absorbance at 270 nm.

Scanning Electron Microscope

The bacteria spread on cover glass were dehydrated by 50~100% of methanol series and then dried with a critical point dryer (HCP-2, Hitachi, Japan). The dried bacteria were platinum coated using Sputter coater (E-1030, Hitachi, Japan), and then observed with a scanning electron microscope (S-4200, Hitachi, Japan).

Sugar and Cell Concentration

Sugar concentration was analyzed by modification of the methods suggested by Lee et al. [16]. Cell concentration was analyzed by measuring optical density with a spectrophotometer using the modified methods suggested by Kim et al. [17].

Results

Morphological, Cultural, and Physiological Characteristics of Strain

To obtain a strain for efficient saccharification from alginate, a number of strains were isolated from seawater sampled at the south coast of Korea. Ten strains were screened for efficient saccharification of alginate. Among these strains, the strain CHO-12 was selected because it exhibited the high saccharification yield. In order to identify the isolated strain CHO-12, its biochemical and physiological characteristics according to Bergey's manual of systematic bacteriology were determined. The results are shown in Table 1. The cell is round-shaped (Fig. 1), Gram-negative, motile, and strictly aerobic. Growth occurs in media containing 1–8% of NaCl. The strain CHO-12 could utilize D-glucose, maltose, lactose, D-mannitol, D-fructose, mannose, and sucrose. Key characteristics such as the absence of nitrate reduction ability to produce catalase, oxidase, and protease indicate that the strain CHO-12 belongs to the *Pseudoalteromonas* genus. Further confirmation of the genus comes from 16s rRNA sequence analysis of the strain CHO-12. The DNA G+C content of the strains CHO-12 was 43.74 mol%, *LL-A_2Pm* (*LL-meso*-diaminopimelic acid) as the diamino acid in the cell wall peptidoglycan contained Q-8 as the major ubiquinone. All these characteristics suggest that the strain CHO-12 is very similar to *P. agarovorans.*

Phylogenic Analysis of the 16S rDNA Sequence

The 16SrDNA of the strain CHO-12 was amplified by PCR. The lengths of the almost complete 16 rRNA gene sequences of strain CHO-12 were 1,455 bp. The 16s rRNA gene sequences of the related taxa were obtained from Gene Bank. Phylogenetic analysis based on 16 rRNA gene sequences indicated that the strain CHO-12 is similar to the type of strains belonging to *Pseudoalteromonas* species, indicating that the strain CHO-12 is a member of the genus *Pseudoalteromonas* (Fig. 2). The strain CHO-12 was found to form a coherent cluster with the type strain of *P. agarovorans* (99.4%), *P. atlantica* X82134 (97.3%), *P. espejiana* X82143 (97.1%), *P. carrageenovora* X82136 (96.8%), *P. tetraodonis* X82139 (96.8%), *P. issachenkonii* AF316144 (95.0%), *P. undina X82140* (96.7%), *P. elyakovii* AF082562 (98.3%), *P. distincta* AF082564 (98.1%), *P. haloplanktis* X67024 (97.1%), *P. nigrifaciens* X82146 (97.6%), and *P. antartica* X98336 (94.3%). Therefore, the strain CHO-12 was determined to be *P. agarovorans* CHO-12, a taxon that is physiologically, chemotaxonomically, and phylogenetically different from the related strains.

Table 1 Biochemical and physiological characteristics of strain CHO-12.

Characteristics	Strains CHO-12
Morphological	
Form	Round
Gram staining	Gram-negative
Flagella	Monotrichus
Pigmentation	–
Growth at/in:	
4 °C	–
5–38 °C	+
40 °C	–
1–8% of NaCl	+
0.25% of NaCl	–
pH range of growth	6–10
Nitrate reduction	–
Production of	
Amylase	+
Alginase	+
Agarase	+
Chitinase	+
DNase	+
Oxidase	+
Catalase	+
Protease	+
Utilization of	
Maltose	+
D-Glucose	+
D-Fructose	+
Mannose	+
Sucrose	+
Glycerol	+
DNA G+C content (mol%)	43.74

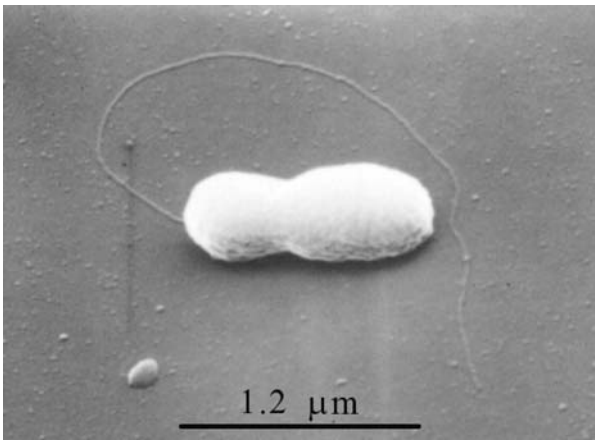

Fig. 1 Morphology of *P. agarovorans* CHO-12 observed by using scanning electron micrograph

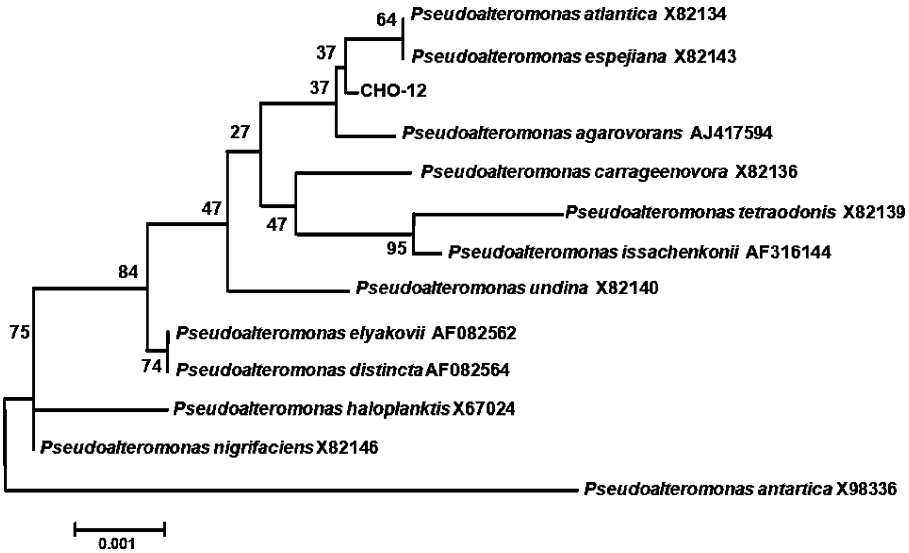

Fig. 2 Phylogenetic tree base on 16S rDNA sequences of strains CHO-12

Optimum Culture Conditions for Saccharification

To investigate the effect of agitation rate on pH, cell concentration, and sugar concentration, batch cultures at 5, 30, 60, 120, and 180 rpm were carried out in flasks containing basal medium for 3 days. The results are shown in Fig. 3. When agitation rate increased from 5 to 30 rpm, cell concentrations after 24 h of culture ranged from 0.32 to 0.38 OD at 660 nm. However, they increased from 0.45 to 0.70 OD at 660 nm when agitation rate increased from 60 to 120 rpm. The pH ranged from 6.7 to 7.1 after 12 h of culture except for the culture at 180 rpm. Sugar concentrations increased with the increase of culture time except for the culture at above 120 rpm. However, the maximum saccharification was obtained at 30 rpm after 3 days of culture. To investigate the effect of preculture time on the concentration of cell and sugar and pH, batch culture was performed at 30 rpm for 3 days at various inoculation times in media containing 10 g/l of alginate. In order to investigate the effect of temperature on cell concentration, saccharification, and pH, batch culture was carried out after 1 day of seed culture at 30 rpm for 3 days. Culture temperatures varied within the range of 27–33 °C at intervals of 2 °C. The results are shown in Fig. 4. Cell concentrations generally were increased when culture temperature decreased from 33 to 27 °C. Especially, cell concentrations in the culture between 29 and 31 °C were within the range of 0.26 and 0.33 OD at 660 nm after 1 day of culture. In the case of cultures at 27 °C, however, cell concentrations ranged from 0.35 to 0.4 OD at 660 nm after 1 day of cultured and from 0.22 to 0.25 OD at 660 nm for the cultures at 33 °C. The pH ranged from 6.8 to 7.0 at 29–31 °C of culture but decreased from 7.5 to 6.8 after 1 day of culture at 33 °C and then increased to 7.2 after 2 days of culture. Especially, when culture was carried out at 27 °C, pH sharply decreased from 7.5 to 6.2 after 1 day and then increased to 6.6. The concentrations of sugar were increased with increasing the culture time. Especially, when culture temperature increased from 27 to 29 °C, the concentration of sugar increased from 10.2 to 12.6 g/l after 3 days of culture. On the other hand, it decreased when the culture was carried out at above 31 °C. This indicates that saccharification was significantly affected by culture

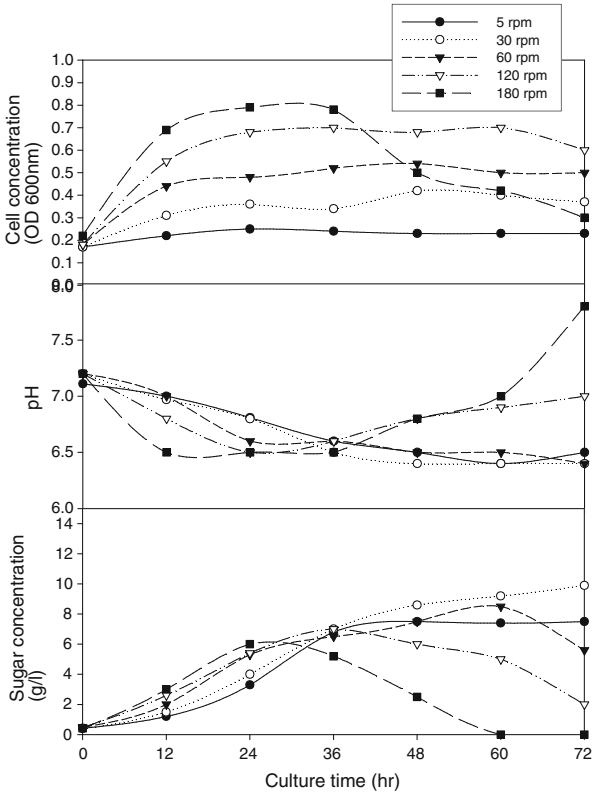

Fig. 3 Effect of agitation rate on cell concentration, pH, and sugar concentration

temperature in the culture of *P. agarovorans* CHO-12. In order to investigate the effect of the initial pH of the medium for saccharification from alginate, the batch culture was carried out with initial pH ranging from 6.0 to 10.0 in flasks containing basal medium at 29 °C after 1 day of seed culture and 30 rpm for 3 days. The results are shown in Fig. 5. Cell concentrations were generally affected by the pH of the medium. Especially, when pH increased from 6.0 to 10, cell concentration was increased. When the cultures are carried out at the pH 8.0 and 9.0, cell concentrations ranged from 0.26 to 0.30 OD at 660 nm from 1 day of culture to the end of culture. However, in the case of pH 10.0, the range was 0.36 at 660 nm after 1 day of culture and from 0.38 to 0.40 OD at 660 nm throughout the entire culture time. The pHs of culture broth generally decreased with the culture time except for pH 6.0. The optimum pH for efficient saccharification ranged between 8.0 and 9.0. The maximum concentration of sugar was obtained at pH 8.0, 13.0 g/l after 3 days of culture in the culture *P. agarovorans* CHO-12.

Optimum Medium Conditions for Saccharification

Using optimum culture conditions, to investigate the effect of NaCl concentration on the cell concentration, saccharification and pH, 10, 20, 30, 40, and 50 g/l of NaCl were used. Batch culture was carried out in 500-ml flasks containing 200 ml of basal medium at 29 °C after 1 day of seed culture and 30 rpm for 3 days. The results are shown in Fig. 6. Cell concentrations ranged from 0.27 to 0.33 OD at 660 nm when 30 to 50 g/l of NaCl were used. However, below 20 g/l of NaCl, they decreased. The pHs of the culture broth was

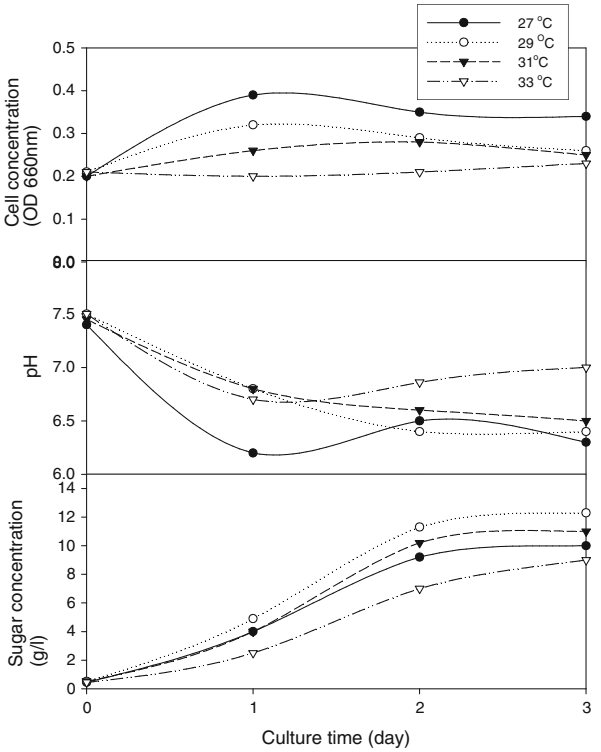

Fig. 4 Effect of culture temperature on cell concentration, pH, and sugar concentration

also affected by NaCl concentrations. The pH of the culture broth generally decreased with the increase of the culture time. When 30 to 50 g/l of NaCl concentration were used, pH ranged from 6.4 to 6.7 after 1 day of culture. However, in the case of 10 to 20 g/l of NaCl, they ranged from 7.2 to 7.6 after 1 day of culture. Sugar concentrations increased with the increase of the culture time. Especially, when 30 g/l of NaCl was used, the maximum sugar concentration was 13.8 g/l. To determine the optimal mixing ratio of yeast extract and corn steep liquor to produce a high level of sugar, batch culture was carried out at 29 °C in flasks containing basal medium at 30 rpm for 3 days. Altogether, 36 kinds of nitrogen sources were tested. The results are shown in Fig. 7. When the yeast extract concentration increased from 0.5 to 2.0 g/l, sugar concentration increased from 3.1 to 7.0 g/l, but it did not improve at the levels above 2.5 g/l. Cell concentrations also increased with the increase of yeast extract concentration up to 2.0 g/l. On the other hand, corn steep liquor concentration affected cell concentration insignificantly (data not shown). When corn steep liquor concentration increased from 0.5 to 1.5 g/l, sugar concentration increased from 3.4 to 6.0 g/l, but it did not improve at the level above 2.0 g/l. The optimal mixing ratio was 1.0 g/l of yeast extract and 1.5 g/l of corn steep liquor, which gave sugar concentration of 14.9 g/l.

Scale Up of Saccharification

Using the optimized culture and medium conditions obtained above, for comparing the *P. agarovorans* CHO-12 and *S. maltophilia* sp. on cell concentration, pH, sugar concentration, and sugar production rate, batch culture was carried out in a 50-l reactor containing 40 l of working volume with 15 g/l of alginate for 3 days. Cell concentration, pH, sugar

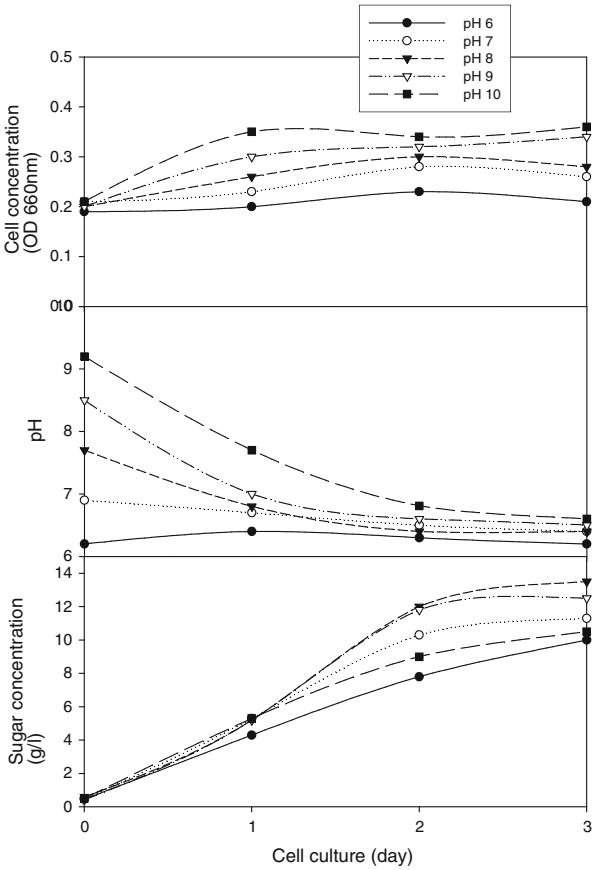

Fig. 5 Effect of initial pH on cell concentration, pH, and sugar concentration

concentration, and sugar production rate are shown in Fig. 8. When culture using *S. maltophilia* sp. was carried out, cell concentrations were increased with the increase of culture time up to 3 days of culture. Especially, cell concentration was 0.42 OD at 660 nm after 1 day of culture and then increased to 6.2 OD at 660 nm after 2 days of culture. On the other hand, when a culture using *P. agarovorans* CHO-12 was carried out, it was 0.30 OD at 660 nm after 1 day of culture and then did not increase further throughout the entire culture time. pH decreased from 7.6 to 7.2 when culture using *S. maltophilia* was increased to 1 day and remained the same throughout the entire culture time. On the other hand, when the culture using *P. agarovorans* CHO-12 was carried out, pH decreased to 6.7 after 1 day of culture and ranged from 6.7 to 6.9 during all culture. When the culture using *P. agarovorans* CHO-12 was carried out, sugar concentration was 15.0 g/l after 2 days of culture and then decreased, which was about tenfold higher than that of *S. maltophilia*. In the case of sugar production rate, the maximum rate of sugar production was obtained after 2 days of culture.

Discussion

Alginate is a linear polysaccharide composed of (1-4)-linked beta-D-mannuronate and alpha-L-guluronate. These uronic acids are arranged in block structures, which can be

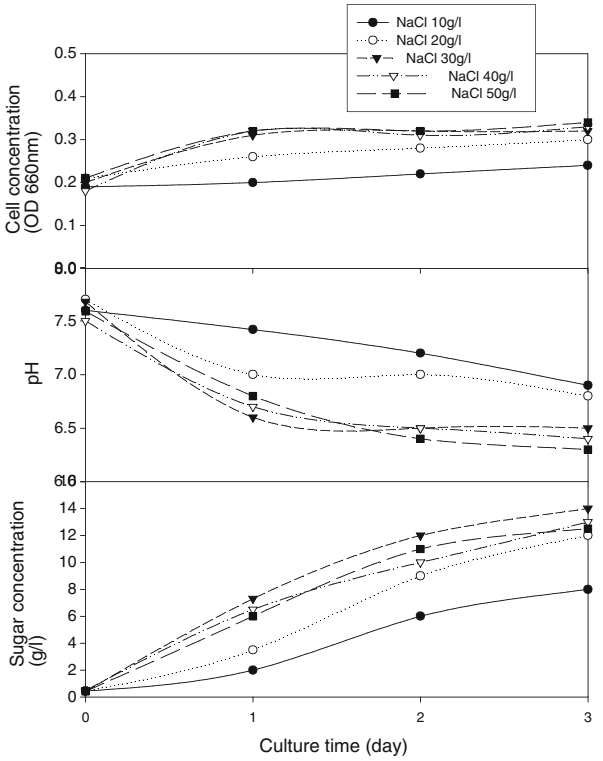

Fig. 6 Effect of NaCl concentration on cell concentration, pH, and sugar concentration

homopolymeric or heteropolymeric, i.e., essentially random sequences and all three types of block may be present within a single alginate molecule [1, 2]. In addition, alginate is a gelling polysaccharide present in cell walls and intracellular material of the brown seaweeds, making almost 30% of the dry weight of these plants. Approximately 22,000 tones/annum of alginate are extracted mainly from three of the 265 known genera of brown algae. The saccharification of alginate by a beta-elimination mechanism using alginate lyase between monomers is carried out. A double bond is formed between the C4 and C5 carbons of the six-

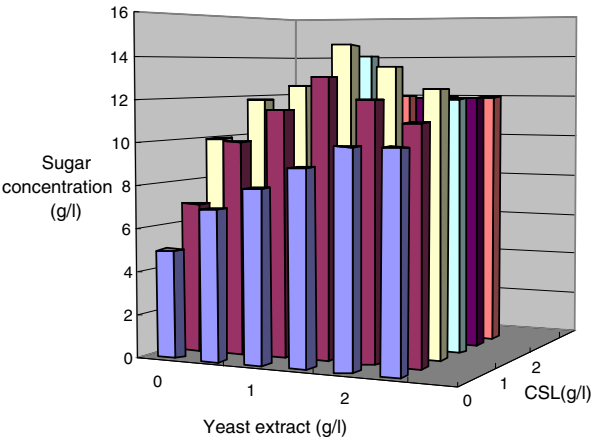

Fig. 7 Effect of mixture ratio of yeast extract and corn steep liquor on sugar concentration

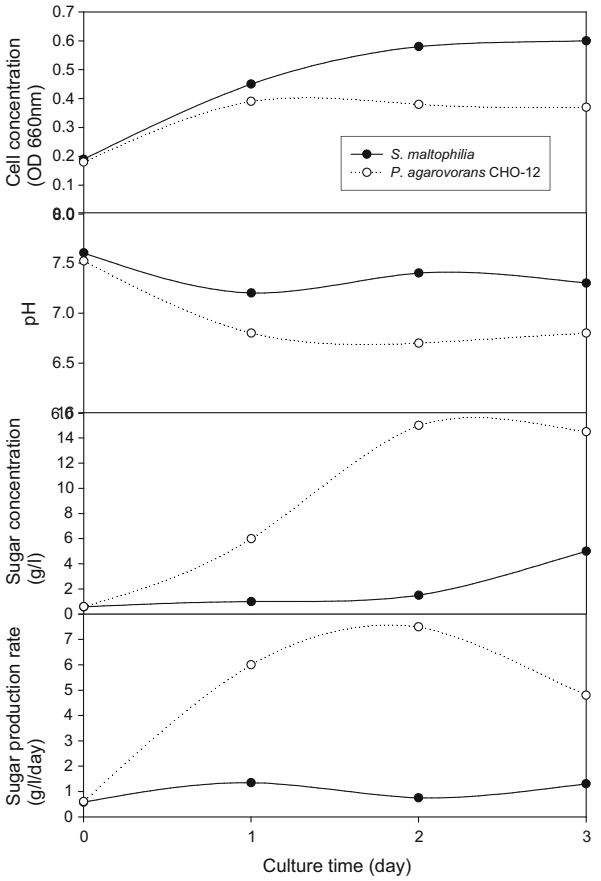

Fig. 8 Comparison of saccharification from alginate in the culture of *P. agarovorans* CHO-12 and *S. maltophilia*

membered ring from which the 4-*O*-glycosidic bond is eliminated, depolymerizing alginate and simultaneously yielding a product containing 4-deoxy-L-erythro-hex-4-enopyranosyluronic acid [18]. Gacesa [2, 19] proposed a three-step reaction to depolymerize alginate. The three steps include (a) removal of the negative charge on the carboxyl anion; (b) a general base-catalyzed abstraction of the proton on C5, where one residue may be required as the proton abstractor and another as the proton donor; and (c) a transfer of electrons from the carboxyl group to form a double bond between C4 and C5, resulting in the beta elimination of the 4-Oglycosidic bond. In the proposed mechanism for epimerase, the replacement of the proton at C5 takes place in step (c).

Alginate and its oligosaccharides are used for a variety of applications by the food, textile, oil, cosmetics, and pharmaceutical industries. Most oligosaccharides are made of chemicals and purified enzyme. However, studies on saccharification of alginate using microorganisms are still few in number. To produce oligosaccharides from alginate in practice, an efficient saccharification method is required. As a preliminary step, we isolated a marine bacterium classified as *S. maltophilia* and investigated how various environment conditions affect efficient saccharification from alginate or seaweeds to solve these problems. Recently, we also screened the strain for the efficient saccharification from alginate. The isolated strain grows at 5–38 °C with optimal temperature from 20–33 °C with no growth at all below 4 or above 40 °C. Nitrate reduction is negative. The strain is

taxonomically very close to *P. agarovorans*, which produces catalase, oxidase, DNase, protease, amylase, alginase, agarase, and chitinase. Based on 16S rDNA sequence it was identified as *P. agarovorans* and designated as *P. agarovorans* CHO-12. In addition, this strain showed sensitivity to carbenicillin (30 μg), tetracycline (30 μg), ampicillin (20 μg), and streptomycin (30 μg), respectively (data not shown). The major cellular fatty acids in strain CHO-12 include: $C_{12:0}$ 3OH (5.11%), $C_{16:0}$ (18.93%), and $C_{17:1}$ 8c (12.09%; data not shown).

In order to obtain the optimum culture conditions for the efficient saccharification using *P. agarovorans* CHO-12, first, agitation rate, culture temperature, and pH of the medium have been investigated. The agitation rate is an important factor for saccharification from alginate. Cell concentrations are strongly affected by agitation rates. When agitation rates were increased, cell concentrations sharply increased compared to the initial culture. Especially, in the case of 180 rpm, the 0.80 OD at 660 nm after 24 h of culture was obtained and then decreased by 0.3 OD at 660 nm after 72 h of culture. pH sharply decreased to 6.45 after 12 h of culture and starts to increase from 48 h of culture and increased by 7.8 after 72 h of culture. However, the maximum sugar concentration was obtained when culture was carried out at 30 rpm. This result is about twofold higher than that of 5 rpm. Especially, at 180 rpm, sugar concentration reached the peak after 24 h of culture, but it remarkably decreased after 48 h of culture. Finally, the produced sugar was completely consumed after 60 h of culture. This result indicates that the sugar production from alginate was strongly affected by the cell growth of *P. agarovorans* CHO-12 during culture at different agitation rates. The cultures were carried out at various preculture periods. The pH was similar to that of other preculture time irrespective of culture time and ranged from 6.7 to 7.2 from 1 day of culture to the end of culture. On the other hand, cell concentrations depend on preculture time. Especially, when preculture was carried out for 24–36 h, the maximum concentration of cell was obtained. However, below 12 and above 48 h of preculture, they were decreased. After 24 h of preculture, maximum concentration of sugar was obtained (data not shown). Saccharification, pH, and cell concentration were significantly affected by culture temperature in *P. agarovorans* CHO-12. Especially, when the culture was carried out at 27 °C, the maximum cell concentration was obtained, which was about twofold higher than that under 33 °C. On the other hand, in the case of 29 °C of culture, the maximum concentration of sugar was obtained. The similar phenomenon was also observed in *Alteromonas* sp. strain H-4, *Azotobacter chroococcum*, and *Azotobacter vinelandii* [20, 21], *Enterobacter cloacae* M-1 [22] producing alginate lyases. However, in case of the *Beneckea pelogia*, it was 25 °C [23]. In case of the *Pseudomonas* marine N-9 and *Pseudomonas alginovora* [24, 25], the optimal temperature was found to be in the range of 45 to 50 °C. Also, in case of *Sphingomonas* sp., the optimum temperature was 70 °C [26]. In general, cells can only grow within a certain pH range and metabolite formation is also often affected by pH. *P. agarovorans* CHO-12 grows at the boundary range of pH 5.0–10. Especially, cell concentrations increased with the increase of pH. Thus, far most marine bacteria have been reported to grow at pH 6.0–7.0. However, the bacterial strain 2–40, which was isolated by Andrykovitch and Marx [225] and preliminary identified as a number of genus *Alteromonas*, grows at pH 5.0–8.0 with the optimum pH 6.0. On the other hand, in the case of *P. agarovorans* CHO-12, the pH for efficient saccharification from alginate ranged between 8.0 and 9.0. These results indicate that *P. agarovorans* CHO-12 is alkali-tolerant.

Using optimum culture conditions, to obtain the optimum media conditions for efficient saccharification from alginate, NaCl, K_2HPO_4, $MgSO_4 \cdot 7H_2O$, and nitrogen source were investigated. Sodium ions are essential for growth of *P. agarovorans* CHO-12. NaCl

concentrations in the saccharification process using seawater also are an important factor for efficient preparation of the medium. The results of the effect of NaCl concentration reveals that the marine bacterium grows only slightly better when about 2.0% of NaCl was added to the growth medium. It is commonly assumed that marine bacteria, since they live in the sea, are salt-tolerant organisms. Most alginate lyase-producing marine bacteria were isolated from marine sources and show specific requirements for NaCl for their growth. In case of *P. agarovorans* CHO-12, when NaCl concentration was increased from 10 to 30 g/l, cell concentration increased as well. However, it did not increase at the level above 40 g/l. Among various NaCl concentrations, the sugar concentration increased with the increase of NaCl by 30 g/l. However, when culture was carried out below 10 g/l of NaCl, sugar concentration remarkably decreased and in case of below 2.5 g/l, there is almost no sugar saccharified from alginate (data not shown). This indicates that saccharification form alginate is strongly affected by NaCl concentration in the culture *P. agarovorans* CHO-12.

Among various minerals, K_2HPO_4 and $MgSO_4 \cdot 7H_2O$ were the best ones for efficient saccharification (data not shown). These mineral ions are recognized as favorable bio-elements for cell growth. Cell concentrations were similar regardless of K_2HPO_4 concentrations. pHs also did not depend on K_2HPO_4 concentrations. However, in case of saccharification among various concentrations of K_2HPO_4, when 0.5 to 1.0 of K_2HPO_4 were used, maximum sugar concentration was obtained. In case of $MgSO_4 \cdot 7H_2O$, cell concentrations were also similar regardless of $MgSO_4 \cdot 7H_2O$ concentrations. pHs were also similar irrespective of $MgSO_4 \cdot 7H_2O$ concentrations. However, in case of saccharification, when 1.0 to 2.0 g/l of $MgSO_4 \cdot 7H_2O$ were used, maximum sugar concentration was obtained (data not shown). The nitrogen source is an important factor that affects cell growth and saccharification. To determine the best nitrogen source on saccharification from alginate, organic nitrogen sources, such as peptone, yeast extract, corn steep liquor, and malt extract, and inorganic nitrogen sources, such as urea, and ammonium sulfate were used. Among various nitrogen sources, yeast extract and corn steep liquor were found to be the best nitrogen source for efficient saccharification (data not shown). Especially, when the mixture of yeast extract and corn steep liquor was used, sugar concentration was higher than that of the sole yeast extract or corn steep liquor. When inorganic nitrogen sources were used, cell concentration and pH were similar to those of other organic nitrogen sources (data not shown). However, sugar concentrations decreased compared to that of organic nitrogen sources. Especially, aspartic acid, glutamate, lysine, glycine, and arginine were consumed to a much greater extent than other amino acids when organic nitrogen sources were used but decreased when a mixture of organic and inorganic nitrogen source was used (data not shown). This indicates that saccharification is affected by aspartic acid, glutamate, lysine, glycine, and arginine in the culture *P. agarovorans* CHO-12.

We previously isolated *S. maltophilia* from seawater in Korea and investigated the capability of direct saccharification from alginate and seaweeds [13]. Using the optimized culture and medium conditions, scale up for comparing the *P. agarovorans* CHO-12 and *S. maltophilia* on cell concentration and saccharification was carried out. When *S. maltophilia* was used, cell concentration was increased with the increase of culture time. It was about twofold higher than that of *P. agarovorans* CHO-12 after 2 days of culture. Sugar concentration was also increased with the increase of the culture time by 2 days. The maximum saccharification rate was obtained after 2 days of culture of *P. agarovorans* CHO-12 and then decreased from 3 days of culture. It was 7.5 g/l/day after 2 day of culture, which was about tenfold higher than that of *S. maltophilia.* In the case of saccharification yield, it was 100% after 2 days of culture, which was threefold higher than that of *S. maltophilia* after 3 days of culture.

We are recently researching the new saccharification process from various seaweeds and investigating the production feasibility of antibiotic, ethanol, lactic acid, and acetic acid from oligosaccharides fragments saccharified from alginate or seaweeds using various microorganisms. If oligosaccharides fragments saccharified from seaweeds could be used, the raw material costs would be reduced and the main components contained in seaweeds could be used.

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