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Optimal Conditions for the Production of Exopolysaccharide by Marine Microorganism *Hahella chejuensis*

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Abstract A marine microorganism, strain 96CJ10356 produced exopolysaccharide, designated as EPS-R. To optimize culture conditions for the production of EPS-R, carbon and nitrogen sources, mineral salts, temperature, and pH were examined. From this study, STN medium for the production of EPS-R was suggested as follows, sucrose 20 g, tryptone 10 g, NaCl 10 g, $MgSO_4$ 5 g, $CaCl_2$ 1 g, KH_2PO_4 76 mg, K_2HPO_4 83 mg, $FeCl_2$ 5 mg, $MnCl_2$ 1 mg, $NaMoO_4$ 1 mg, and $ZnCl_2$ 1 mg per liter at pH 7.0. About 9.23 g/L of EPS-R was obtained from STN medium after cultivation for 120 h at 25°C in a 5-liter jar fermentor with an aeration rate of 0.17 vvm. Apparent viscosity and flocculation activity of the culture broth were increased with the production of EPS-R and the maximal values were 415 cP and 1400 unit/mL against 0.5% activated carbon, respectively

Keywords. exopolysaccharide, marine microorganism, culture conditions, *Hahella chejuensis*

INTRODUCTION

Many microorganisms grow in aggregated form such as biofilm and keep together by extracellular polymeric substance (EPS). Exopolysaccharide, designated as EPS frequently, is the material construction of biofilm matrix, serving as a multipurpose functional element for adhesion, structure, protection, and recognition.

This microbial EPS has been used in a wide range of industries due to its functions such as gel formation, emulsifying, absorption, cohesion, and film formation [1-5]. Furthermore, heavy-metal ion accumulating [6], anti-tumor active [7] and anti-ulcer active [8] EPSs have also been reported. These EPSs have industrial potential as new biomaterials due to these activities.

EPSs from marine microorganisms such as *Zoogloea* sp. [9], *Pseudomonas* sp. [10, 11], *Vibrio fischeri* [12], *Cyanothece* sp. [13], and *Alteromonas macleodii* [14] were reported and their properties were investigated. However, EPSs from marine microorganisms isolated from Korean coastal areas were not studied enough with the exception of EPS from marine bacterium *Zoogloea* sp. KCCM 10036 [15].

We isolated EPS-producing marine bacteria from the coastal regions of Cheju Island. Strain 96CJ10356 was a gram negative, rod-formed bacterium, absolutely demanding 1-3% salt for growth. This strain was identified as *Hahella chejuensis* and the EPS produced by this microroganism was designated as EPS-R.

In the present study, we report on the optimal culture conditions for the production of exopolysaccharide by the marine microorganism *H. chejuensis*.

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MATERIALS AND METHODS

Microorganism

From marine sediment collected in Cheju Island, the strain 96CJ10356 was isolated using ZoBell medium (yeast extract 1 g, peptone 5 g, $FePO_4$ 1 mg, distilled water (DW) 250 mL and aged sea water (ASW) 750 mL at pH 7.0). This strain was named as *Hahella chejuensis* and harbored as KCTC2395. This bacterium produced exopolysaccharide designated as EPS-R. The strain 96CJ10356 was transferred on ZoBell agar plate at 20°C and maintained as glycerol suspension (20%, w/v) at –80°C.

Requirement of Nutrients for the Production of EPS-R

To determine nutritional requirement for the production of EPS-R, several nutrients as carbon, nitrogen, NaCl, $MgSO_4$, $CaCl_2$, and phosphate were tested. The strain 96CJ10356 was cultured in 20 mL of YMG medium as the basal medium (glucose 10 g, peptone 5 g, yeast extract 3 g, malt extract 3 g, DW 500 mL and ASW 500 mL at pH 7.0) for 7 days. The cultivation was carried out on a reciprocal shaker at 25°C and at 120 rpm. To investigate the effect of carbon and nitrogen sources on the production of EPS-R, carbon and nitrogen sources were provided at the concentration of 2 and 0.5%, respectively, instead of carbon and nitrogen sources in YMG medium. Carbon sources tested

Table 1. The composition of STN medium for the production of EPS-R

Sucrose	20 g
Tryptone	10 g
NaCl	10 g
$MgSO_4$	5 g
$CaCl_2$	1 g
KH_2PO_4	0.083 g
K_2HPO_4	0.067 g
$FeCl_3$	0.005 g
$MnCl_2$	0 001 g
Na_2MoO_4	0.001 g
$ZnCl_2$	0 001 g
Distilled water	to 1000 mL
PH	7.0

were glucose, fructose, galactose, maltose, lactose, and soluble starch. Peptone, yeast extract, malt extract, tryptone, soytone, casein, NH_4NO_3, $NaNO_3$, NH_4Cl, and $(NH_4)_2PO_4$ were used as the nitrogen sources. NaCl was provided at the concentration of between 0 to 200 g/L. $MgSO_4$ and $CaCl_2$ were added to the medium in the range of 0 to 20 g/L. Potassium phosphate was added at the concentration of 1 to 50 mM.

Production of EPS-R in a 5-liter Jar Fermentor

To study the production of EPS-R during growth, cultivation in a jar fermentor was carried out with a working volume of 3.0 L (KF 402, KFC, Korea). The culture medium used was STN medium (Table 1). The cells precultured in STN medium were inoculated at the concentration of 1% (v/v). In a jar fermentor, the cells were cultured at 25°C, 300 rpm, and 0.17 vvm

Isolation of EPS-R and Cells from the Culture Broth

One hundred mL of culture broth was mixed with 100 mL of DW and 0 1 g of dried diatomaceous earth (Sigma, St. Louis, USA). After centrifugation (10,000 × *g*, 20 min, 4°C), the precipitate was subsequently washed with DW, dried at 80°C for 3 days and used to determine cell mass. Two volumes of methanol and chloroform were added on the supernatant, and then mixed vigorously. Chloroform phase was removed using a separating funnel. The remaining mixture was mixed with the equal volume of ethanol and then left overnight at 4°C and centrifuged (10,000 × *g*, 20 min, 4°C) The precipitate was dried at 80°C for 3 days to determine the amount of EPS-R (Fig. 1).

Analytical Methods

The amount of total carbohydrate was measured by the method of anthrone-sulfuric acid [16], using glucose as the standard. The apparent viscosity of culture broth was measured with a Viscostar-R (J.P. SELECTA, Spain) fitted with spindle R2, R3, R4, and R5 at 60 rpm at room temperature. For analysis of flocculation activity,

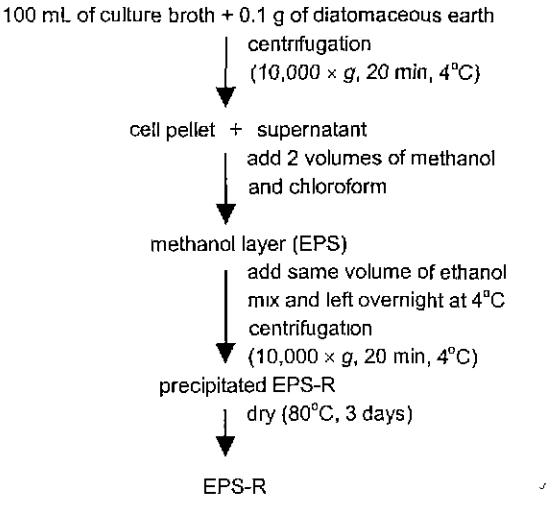

Fig. 1. Separation of EPS-R from the culture broth of the strain 96CJ10356

0.1 mL of cell-free culture broth was added into 10 mL of 0.5% activated carbon containing 0.01% $CaCl_2$, and then mixed with vortexer (IKA MS1, Works Inc. Germany) at the maximal speed for 10 sec. After leaving for 10 min at room temperature, 1 mL of the upper phase was obtained and its absorbance was measured at 550 nm. Flocculation activity was calculated by the method of Nakamura *et al.* [17] as follows:

Flocculation activity (U/mL) =
(A − B) / A × 100 × dilution rate, where
A is absorbance of reference sample and
B is absorbance of reaction mixture.

RESULTS AND DISCUSSION

Effect of Carbon and Nitrogen Sources on the Production of EPS-R

Glucose, galactose, fructose, lactose, sucrose, and starch were used to determine the effect of carbon source on the production of EPS-R. The highest yield of EPS-R from the strain 96CJ10356 was observed when sucrose was supplied as the carbon source with a yield of EPS-R ($Y_{P/S}$) of 0.41 (Fig. 2). In other reports, glucose reported to produce the highest yield of EPSs using such strains as *Lactobacillus casei* CG 11 [18] and *Sphingomonas pancimobilis* [19]. As shown in Fig. 3, the productivity of EPS-R was higher grown in organic nitrogen compounds compared to in inorganic compounds Among the nitrogen sources tested, tryptone provided the highest production level of the EPS-R. However, in case of *Sphingomonas pancimobilis*, soytone provided the highest productivity of EPSs [19]. Production of EPS-R was examined in various ratios of carbon and nitrogen sources (C/N ratio), in which 2% of sucrose was supplied and the concentration of tryp-

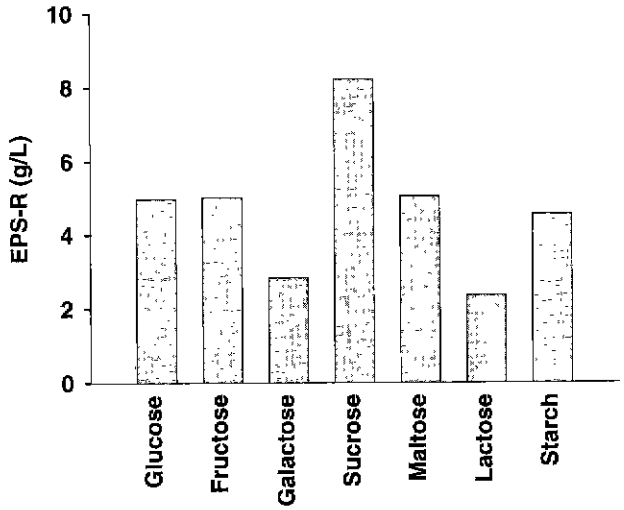

Fig. 2 The effect of carbon sources on the production of EPS-R by the strain 96CJ10356. The strain was cultured for 7 days at 25°C and 120 rpm in YMG medium (pH 7.0) containing 2% of each carbon source.

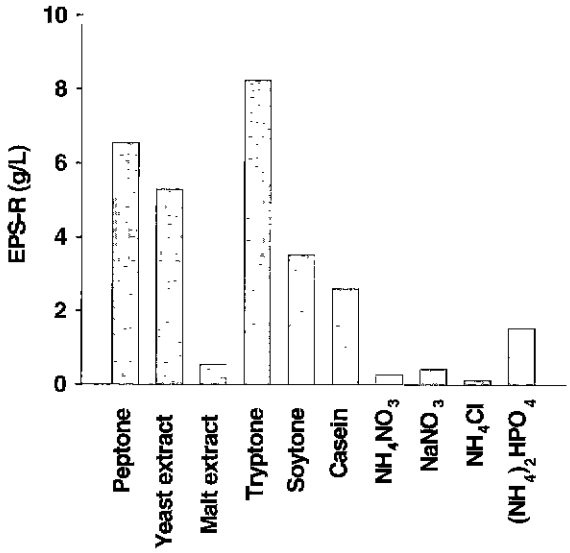

Fig. 3 The effect of nitrogen sources on the production of EPS-R by the strain 96CJ10356 The strain was cultured for 7 days at 25°C and 120 rpm in YMG medium(pH 7 0) with 0.5% of each nitrogen source.

tone was varied. As shown in Fig. 4, when the C/N ratio was higher than 3 . 1, the yield ($Y_{P/S}$) and the productivity (dried EPS-R weight/dried cell weight) were decreased, and when the C/N ratio was higher than 10, the yield and productivity of EPS-R was decreased drastically. Compared to the C/N ratio of 10 to 40 for EPS production from *Xanthomonas camperstris* [20], the optimal C/N ratio for the production of EPS-R by the strain 96CJ10356 was 2: 1 (Fig. 3), showing similar tendency in the ratio by *Xanthomonas* sp [21] and *Azotobactor* sp. [22], which produce more EPS in the carbon-limited condition Therefore, it appears that carbon source,

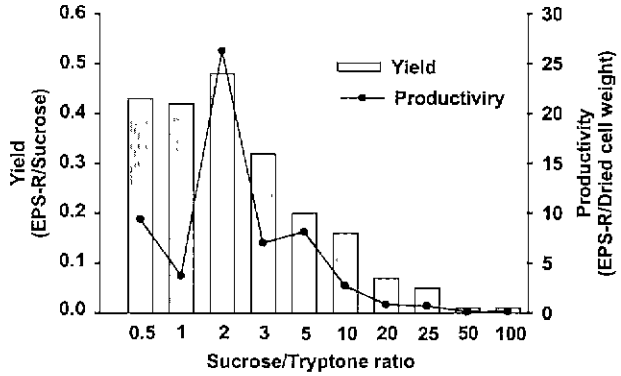

Fig 4. Effect of carbon/nitrogen ratios on the production of EPS-R by the strain 96CJ10356 The strain was cultured for 7 days at 25°C and 120 rpm in YMG medium (pH 7.0) containing 2% sucrose as carbon source and tryptone as nitrogen source. Yield, dried EPS-R weight/sucrose weight(20 g), Productivity, dried EPS-R weight/dried cell weight

nitrogen source, and C/N ratio for EPS production would depend on microbial species and sorts of EPS.

To determine the effect of NaCl on the production of EPS-R, various concentrations of NaCl ranging from 0 to 20% (w/v) were added to YMG medium. 1% of NaCl provided the highest yield of EPS-R as shown in Fig. 5a. The effects of $MgSO_4$ and $CaCl_2$ were investigated (Fig. 5(b), (c)). 0.5% of $MgSO_4$ and 0.01% of $CaCl_2$ resulted in the highest amount of EPS-R. The strain 96CJ110356 required NaCl, $MgSO_4$, and $CaCl_2$ strictly for the cell growth.

Phosphate was reported to participate in the synthesis of polysaccharide with the formation of carbohydrate-phosphate form [23]. In this study, the production of exopolysaccharide was increased with the addition of phosphate. The yield of EPS-R was almost the same at various concentrations of phosphate ranging 0 1 to 50 mM, but the highest productivity (dried EPS-R weight/ dried cell weight) was measured when 1 mM of phosphate was added .

Effect of Temperature and pH on the Production of EPS-R

The highest productivity of EPS-R was obtained at 20 to 25°C. At above 30°C, the productivity of EPS-R was reduced, contrast to an increase in the productivity of red pigments (data not shown). Furthermore, more red pigments were obtained with inorganic nitrogen compounds in the medium (data not shown), compared to organic nitrogen sources, where the higher productivity of EPS-R was observed. From these results, we considered that the production of EPS-R is related with the production of pigments. The high productivity of EPS-R was yielded at pH 6 to 8. Especially, the highest productivity was acquired at pH 7 (9.19 g/L) with the yield ratio ($Y_{P/S}$) of 4.6. The yield of EPS-R was decreased under pH 5 and above pH 9.

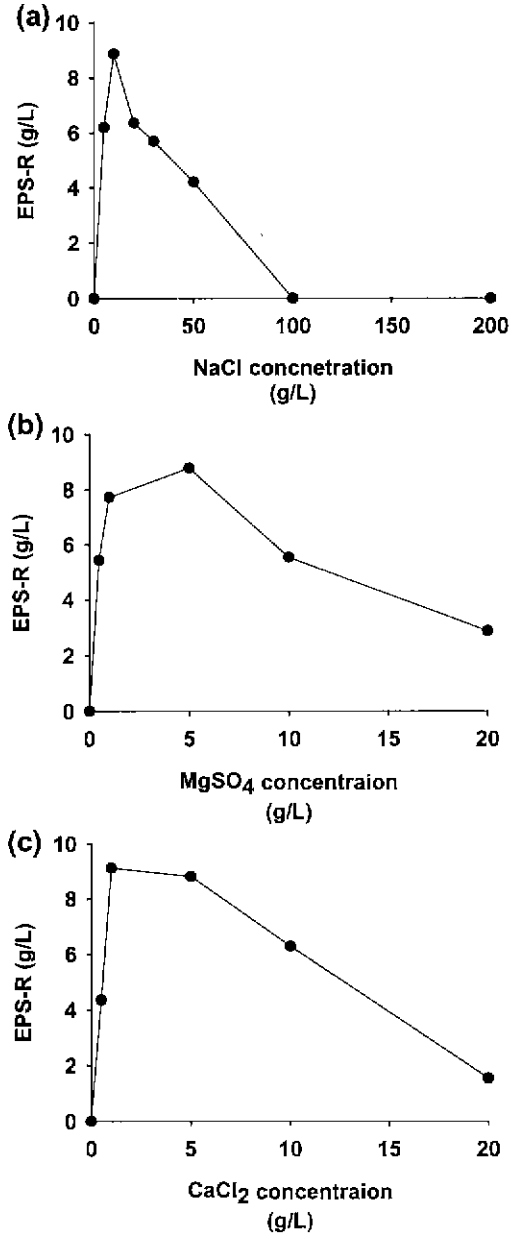

Fig. 5. Effect of mineral salts on the production of EPS-R by the strain 96CJ10356. The strain was cultured for 7 days at 25°C and 120 rpm in YMG medium (pH 7.0) containing 2% of sucrose as carbon source and 1% of tryptone as nitrogen source. (a) NaCl, (b) $MgSO_4$, (c) $CaCl_2$.

The Production of EPS-R in a 5-liter Jar Fermentor

Cell growth, EPS-R production, apparent viscosity, and flocculation activity were measured in a 5-liter jar fermentor with 3 liters of STN medium (Fig. 6). The results of the current study showed that the EPS-R production was associated with cell growth. Cell mass reached to 2 g/L after 24 h, and then slightly increased.

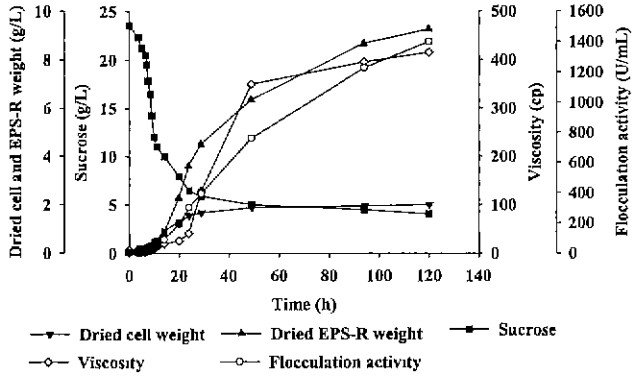

Fig. 6. Time course of the EPS-R production by the strain 96CJ10356. The strain was cultured in STN medium (pH 7.0) for 120 h at 25°C, 300 rpm and 0.17 vvm.

The production of EPS-R was increased with the increasing cultivation time and reached to 9.23 g/L after 120 h. Apparent viscosity and flocculation activity of the culture broth were increased with cell growth and production of the EPS-R, and the maximal values were reached to 415 cP and 1400 units/mL against 0.5% activated carbon, respectively.

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