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Synthesis and Characterization of a Novel Extracellular Polysaccharide by *Rhodotorula glutinis*

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Abstract

The aim of this work was to characterize an exopolysaccharide by *Rhodotorula glutinis* KCTC 7989 and to investigate the effect of the culture conditions on the production of this polymer. The extracellular polysaccharide (EPS) produced from this strain was a novel acidic heteropolysaccharide composed of neutral sugars (85%) and uronic acid (15%). The neutral sugar composition was identified by gas chromatography as mannose, fucose, glucose, and galactose in a 6.7:0.2:0.1:0.1 ratio. The molecular weight of purified EPS was estimated to be $1.0–3.8 \times 10^5$ Dalton, and the distribution of the molecular weight was very homogeneous (polydispersity index = 1.32). The EPS solution showed a characteristic of pseudoplastic non-Newtonian fluid at a concentration >2.0% in distilled water. The maximum EPS production was obtained when the strain was grown on glucose (30 g/L). Ammonium sulfate was the best suitable nitrogen source for EPS production. The highest yield of EPS was obtained at a carbon to nitrogen ratio of 15. The EPS synthesis was activated at the acidic range of pH 3.0–5.0 and increased when the pH of the culture broth decreased naturally to <2.0 during the fermentation. When the yeast was grown on glucose (30 g/L) and ammonium sulfate (2 g/L) at 22°C at an initial pH of 4.0, EPS production was maximized (4.0 g/L), and the glucose-based production yield coefficient and carbon-based production yield coefficient were 0.30 g of EPS/g of glucose and 0.34 g (carbon of EPS)/g (carbon of glucose), respectively.

Index Entries: *Rhodotorula glutinis*; exopolysaccharide; extracellular polysaccharide; characterize; uronic acid; molecular weight; culture conditions.

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Introduction

Many microbial polysaccharides and their derivatives have characteristic rheologic and physiologic properties that are different from those of plants, marine algae, natural gums, and synthetic polymers *(1,2)*. They tend to biodegrade more readily and less harmfully to the environment than many chemically synthesized polymers. Some microbial polysaccharides (xanthan, gellan, zooglan, dextran, and curdran) are produced on an industrial scale for applications in food, pharmaceutical and environmental industries, and the oil recovery process *(3,4)*.

Although sugar composition and theoretical synthesis yield are genetically determined, it is possible to influence both by a wide range of environmental parameters, in addition to the effects of the culture medium *(5)*. Most of the previous work on exopolysaccharide production has been focused on the effects of carbon to nitrogen (C:N) ratio, temperature, nutrient limitation, and culture pH, as well as the source and concentration of carbon and nitrogen. It was widely reported that the carbon source affects the properties of the polysaccharide and serves as a precursor for the synthesis of extracellular polysaccharide (EPS) *(6,7)*. The culture pH had a profound influence on cell growth and polysaccharide pullulan production *(8)*. The synthesis of EPS was enhanced in medium containing excess carbohydrate and deficient in other nutrients, particularly nitrogen *(9,10)*.

It is known that *Rhodotorula glutinis* usually produces EPSs. *R. glutinis* produces an exocellular mannan containing 1,3-β- and 1,4-β-D-mannopyranose residues *(11)*. In addition, *R. glutinis* produces a heteropolysaccharide composed of L-fucose and D-galactose in an approximate molar ratio of 1:1 *(12)*. However, there are few reports on the nutritional studies for the production of exopolysaccharide from this strain.

The aim of the present study was to examine culture conditions for enhancing production of exopolysaccharide from *R. glutinis* KCTC 7989 that was newly isolated from soil. This study focused on the effects of culture pH, C:N ratio, and nitrogen sources. The sugar composition, molecular weight, and rheologic properties of this polysaccharide were also investigated.

Materials and Methods

Microorganism and Culture Conditions

R. glutinis KCTC (Korean Collection for Type Cultures) 7989 was isolated from soil and identified by morphologic characteristics, biochemical properties, and carbon assimilation test *(13)*. The cells were maintained on yeast malt agar plates at 4°C and transferred monthly. The inoculum of *R. glutinis* was grown in 250-mL Erlenmeyer flasks containing 50 mL of the culture medium with 10 g/L of glucose, 3.0 g/L of yeast extract, 3.0 g/L of malt extract, and 5.0 g/L of peptone at 22°C for 42 h. The basal EPS production medium contained 30 g/L of glucose, 2.0 g/L of $(NH_4)_2SO_4$, 1.0 g/L of

KH_2PO_4, 0.5 g/L of $MgSO_4$, 0.1 g/L of $CaCl_2$, and 0.1 g/L of NaCl unless stated otherwise. The 100-mL portion of the basal medium in 500-mL Erlenmeyer flasks was inoculated with 5% of a 42-h-old preculture and incubated on a reciprocating shaker at 150 rpm for 168 h at 22°C. Various nitrogen sources were added at a concentration of 2 g/L to the basal EPS production medium containing 30 g/L of glucose to evaluate the effect of nitrogen sources on cell growth and EPS production. The C:N ratio of the medium was adjusted by altering the initial ammonium sulfate concentration (0.5–5.0 g/L) and using a fixed initial concentration of 30 g/L of glucose. The initial pH in the medium was adjusted to a desired value using 1 *N* HCl or 1 *N* NaOH. A jar fermentor KF-5L (Korea Fermentor, Inchon, Korea) fitted with a 5-L vessel (working volume of 2 L with 5% inoculum) was used for batch cultivation. The fermentation pH was not adjusted and the temperature was maintained at 22°C. The agitation speed was 400 rpm and the aeration rate was controlled at 1 vvm. For the pH-controlled experiment, the pH in the fermentor was maintained at the initial value (pH 4.0) by adding sterilized 2.0 *M* NaOH solution. Samples were withdrawn periodically to determine cell growth, residual substrate, and product yield. The results represent the mean of triplicate measurements.

Isolation and Purification of EPS

The broth was diluted with 2 vol of distilled water and centrifuged at 10,000*g* for 20 min. The sediment was removed and the supernatant was added to 2 vol of cold ethanol. The mixture was vigorously shaken in a vortex mixer for 3 min and settled for 30 min. The resultant precipitate was recovered on a preweighed Whatman GF/C filter disk, which was then dried in a vacuum oven at 60°C. When a constant weight was reached, the precipitate was taken out of the oven and reweighed. The EPS concentration in the supernatant was then calculated. For further purification, the precipitated EPS was suspended in distilled water, and sodium azide (0.01% [w/v]) was added to prevent bacterial growth. The slurry was dialyzed at 4°C for 2 d against 5 L of distilled water. Finally, the EPS obtained was freeze-dried and used for further analysis.

Analytical Methods

Cell growth was estimated by using a calibration curve relating absorbance at 660 nm and dry cell weight. The infrared (IR) spectrum was obtained on a Shimadzu FTIR-8101 spectrophotometer. Elemental analysis was carried out using an elemental analyzer (EA 1108 CHNS/O MODE; Fisions, Milan, Italy). The dinitrosalicylic acid method was applied for the quantification of reducing sugars using glucose as a standard *(14)*. Ammonium concentration was quantified by the Berthelot method *(15)*. Total sugar and uronic acids were determined by the phenol–sulfuric acid method and carbazole–sulfuric acid method using glucose and glucuronic acid as reference compounds, respectively *(16,17)*. Protein content was

determined by the Bradford *(18)* method with bovine serum albumin as a standard.

For the analysis of monosaccharide composition in EPS, the purified EPS (100 mg) was hydrolyzed using 5 mL of 4 *M* trifluoroacetic acid in a sealed vial (Teflon-lined cap) at 100°C for l h. After hydrolysis, the solution was washed five times with methanol (5 mL) to eliminate the acid and dried using a rotary evaporator under reduced pressure. Monosaccharides in the hydrolysate were converted to the corresponding alditol acetates and analyzed by gas chromatography mass spectroscopy (GC-MS) with myoinositol as an internal standard *(19)*. GC-MS analysis was performed on a Hewlett-Packard 6890 series chromatograph fitted with an HP 5973 mass selective detector and an HP-5MS capillary column (30 m × 0.25 μm) packed with crosslinked 5% pH ME siloxane. The column temperature was maintained at 190°C for 4 min and then raised at a rate of 4°C/min up to 230°C.

The molecular weight of the purified EPS was determined by gel permeation chromatography. A TSK-GEL column ($G5000PW_{XL}$; TOSOH, Tokyo, Japan) and a refractive index detector (HP 1047A; Hewlett-Packard) were used for the operation. For elution, 0.1 *M* $NaNO_3$ was used at a flow rate of 0.8 mL/min. Several dextran standards (mol wt range: 50,000–750,000 Dalton) (Fluka, Switzerland) were used for the molecular weight calibration.

The apparent viscosity of EPS solution was measured by a Brookfield viscometer (LVDVII+) fitted with a small sample adapter, SSA-8R (Brookfield, Stoughton, MA) at a temperature of 25°C.

Results and Discussion

Sugar Composition and Molecular Weight of EPS

The elemental analysis showed that the purified EPS contained 44.2% C and 7.9% H and had a repeating unit empirical formula of $C_{3.7}H_{7.8}O_{2.9}$. Neither nitrogen nor sulfate was found. Protein was not detected. The IR spectra exhibited a broad O-H stretching absorption band around 3483 cm^{-1}, a minor C-H stretching band at 2928 cm^{-1}, and a strong C-O stretching band at 1074 cm^{-1}. The spectra also displayed bands of carboxyl group at 1643 cm^{-1} and near 1400 cm^{-1} (Fig. 1). The EPS was identified as an acidic heteropolysaccharide composed of neutral sugars (85%) and uronic acid (15%) by colorimetric analysis of total sugar and uronic acids. The neutral sugar composition of EPS was identified as mannose, fucose, glucose, and galactose in an approximate molar ratio of 6.7:0.2:0.1:0.1. The molecular weight of purified EPS was estimated to be 1.0–3.8 × 10^5 Dalton, and the distribution of molecular weight was very homogeneous (the polydispersity index was 1.32).

The EPS produced from *R. glutinis* KCTC 7989 was identified as a uronic acid/mannose-rich exopolymer that has a quite different sugar composition from those reported earlier *(11,12,20)*. The Fourier transform (FT)-IR spectrum implies the presence of carboxylate ions that have two

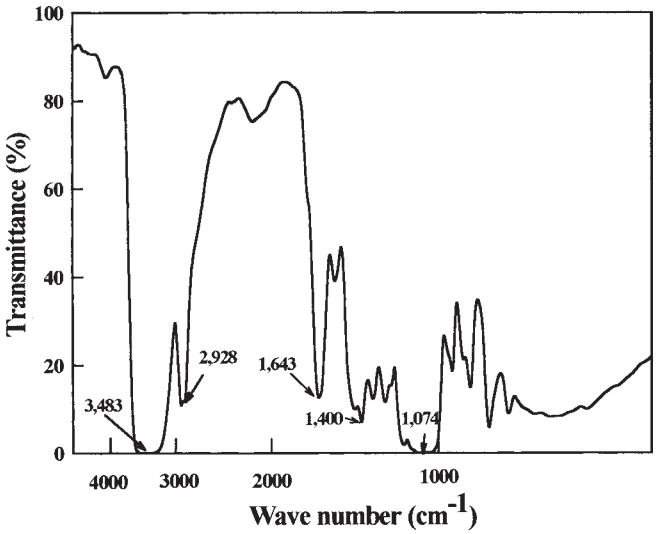

Fig. 1. FT-IR spectra of purified EPS.

stretching bands: a strong asymmetrical band near 1650 cm^{-1}, and a weaker symmetrical band near 1400 cm^{-1}. It has not been reported that polysaccharides produced by *R. glutinis* contain uronic acids. Thus, this is probably a novel EPS. Uronic acids confer a net negative charge to the polymer and also play an important role in the binding capacities of the polymer. The acidic polysaccharides containing uronic acid have been expected to have some heavy metal binding capability and have potential for applications in the fields of biodetoxification and wastewater treatment *(21,22)*. The yeast mannan has been extensively studied regarding its fibrinolytic activity and enzyme activation in blood *(23,24)*. Therefore, this polymer obtained in our study can be utilized for environmental and pharmaceutical applications.

EPS Concentration and Apparent Viscosity

The apparent viscosity of aqueous EPS solutions increased with increasing EPS concentration. The EPS solution showed a characteristic of pseudoplastic non-Newtonian fluid at a concentration >2.0% in distilled water (Fig. 2). However, the degree of pseudoplasticity—the effect of shear rate on the viscosity—was much less than that of other polysaccharides *(25,26)*. The effect of shear rate on viscosity of the EPS solutions was markedly decreased with decreasing EPS concentration, and the EPS solution showed nearly Newtonian-fluid characteristics.

Effect of Initial Glucose Concentrations on Cell Growth and EPS Production

When the initial glucose concentration was varied from 30 to 100 g/L, it was utilized up to 70 g/L. When the glucose concentration was higher than 70 g/L, the residual glucose was detected in the broth (Table 1). Both

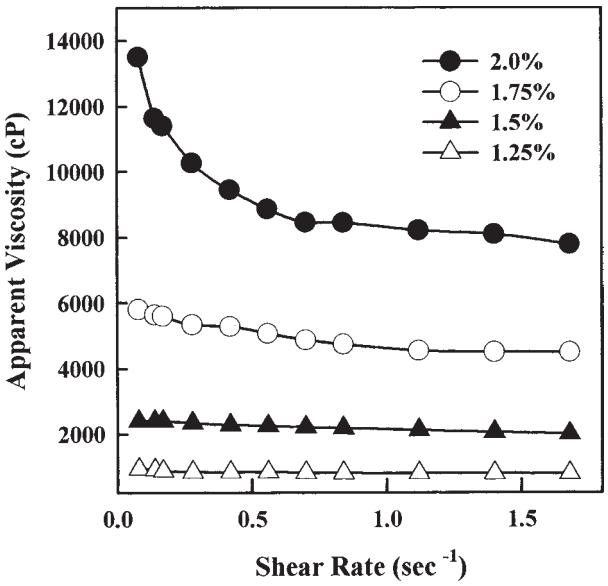

Fig. 2. Viscosity vs shear rate for various concentrations (w/v) of EPS.

Table 1
Effects of Initial Glucose Concentration on Cell Growth and EPS Production

Initial glucose concentration (g/L)	Residual glucose concentration (g/L)	Dry cell weight (g/L)	EPS production (g/L)	Specific EPS production (g/g of dry cell weight)	EPS yield (g/g of initial glucose)
30	0	6.1	3.8	0.62	0.13
50	0	7.5	5.4	0.72	0.11
70	1.7	7.8	5.9	0.75	0.08
100	24.7	8.0	6.2	0.78	0.06

EPS production and cell growth increased with increasing glucose concentration, but the yield of EPS produced to initial substrate concentration (g of EPS/g of initial glucose) decreased. Increasing glucose concentration caused an increase in the viscosity of culture broth owing to the increase in accumulated EPS. The increase in viscosity of culture broth leads to oxygen limitation, imperfect mixing, and inhibits the mass transfer between the cells and nutrients, which causes a decrease in the production yield of polysaccharides.

Effect of Nitrogen Sources on Cell Growth and EPS Production

Ammonium salts such as ammonium sulfate, ammonium chloride, and ammonium nitrate promoted EPS production. Nitrate salts and organic

Table 2
Effects of Nitrogen Sources on Cell Growth and EPS Production

Nitrogen source	Dry cell weight (g/L)	EPS production (g/L)[a]	Final pH
$(NH_4)_2SO_4$	6.1	3.8	1.9
NH_4Cl	4.7	2.5	1.9
NH_4NO_3	5.1	2.1	1.9
KNO_3	6.1	ND	3.5
$NaNO_3$	2.1	ND	3.3
Yeast extract	8.7	ND	2.8
Peptone	7.4	ND	3.1
Tryptone	8.6	ND	2.8

[a]ND, not detectable.

nitrogen sources were totally ineffective, even though the cells grew well on them (Table 2). The largest amounts of EPS were obtained with ammonium sulfate, followed by ammonium chloride and ammonium nitrate.

Effect of C:N Ratio on Cell Growth and EPS Production

The effect of nitrogen limitation has been strongly emphasized in that it influences the synthesis of polysaccharide *(27)*. The highest yield of EPS (3.8 g/L) was obtained at a C:N ratio of 15. When the initial concentration of ammonium sulfate was >2 g/L (C:N < 15), the excess amounts of ammonium sulfate remained in the broth and EPS production was negatively affected. In addition, when the C:N ratio was increased above 15, cell growth and EPS formation were rapidly inhibited again. The strain *R. glutinis* KCTC 7989 did not produce EPS at a high C:N ratio of 60, whereas the synthesis of extracellular lipid was observed. It has been reported that *R. glutinis* KCTC 7989 produced extracellular lipid mainly composed of palmitoleic acid, linoleic acid, and arachidic acid when the optimum C:N ratio for the lipid synthesis was 50 *(13,28)*. Accordingly, it might be assumed that when the C:N ratio is much higher, the metabolic pathways would be changed to lipid production, thereby causing EPS not to be produced. These results suggest that the greatest yield of EPS could be obtained under optimally designed nitrogen-limited conditions. As shown in Table 2, organic nitrogen sources strongly improved cell growth while EPS tended not to be produced. In the case of using organic nutrients containing small portions of nitrogen, the C:N ratio was markedly increased above the optimum value (C:N = 15), thereby causing EPS production to be inhibited. The coaddition of yeast extract and ammonium sulfate showed similar effects. The additional supply of yeast extract to the medium containing ammonium sulfate resulted in an increase in C:N ratio, and the specific EPS production decreased while the cell growth increased (Table 3). It has been reported that the addition of mixed nitrogen sources, amino acids, and vitamins to an optimized ammonium sulfate content contributed to an

Table 3
Effects of Coaddition of Yeast Extract and Ammonium Sulfate on Cell Growth and EPS Production

$(NH_4)_2SO_4$ (g/L)	Yeast extract (g/L)	Dry cell weight (g/L)	EPS production (g/L)	Specific EPS production (g/g of dry cell weight)
2	0	6.1	3.8	0.62
2	1	7.6	3.5	0.46
2	2	8.8	3.0	0.34

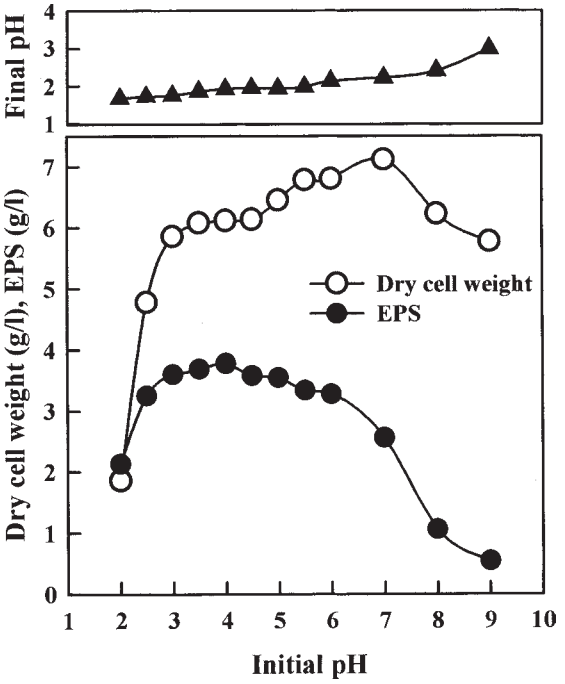

Fig. 3. Effects of initial pH on cell growth and EPS production.

increase in cell growth rather than EPS production, probably owing to the increase in C:N ratio *(6,10)*.

Effect of pH on Cell Growth and EPS Production

The optimum initial pH for EPS production was observed between pH 3.0 and 5.0. At higher pH values, such as pH 8.0 and 9.0, the cells barely produced any EPS. EPS synthesis was activated at the acidic range of pH 3.0–5.0, even though the strain could grow at a wide range of pH (Fig. 3). The pH of the culture medium decreased from its initial value of pH 2.0–9.0 to the self-stabilized final value of 1.6–2.9. It could be presumed that the utilization of ammonium ion as a nitrogen source pro-

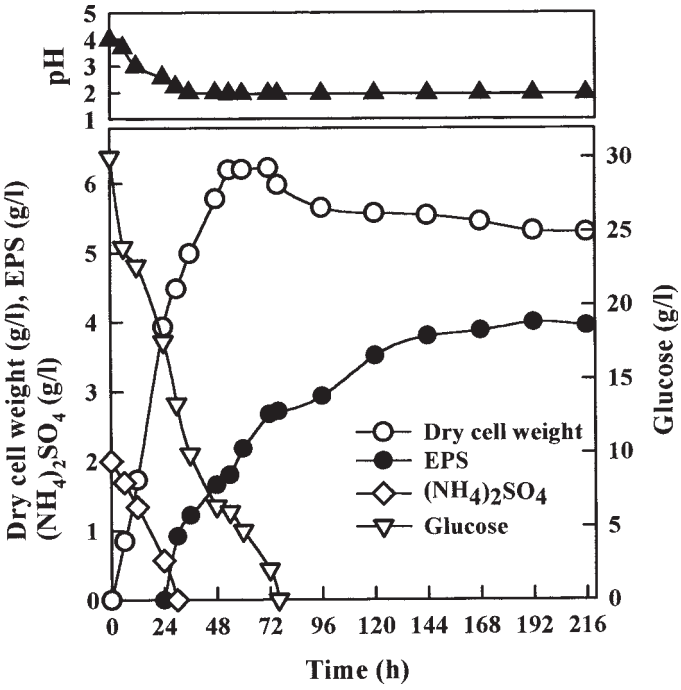

Fig. 4. Time courses of EPS production, dry cell weight, glucose and ammonium sulfate concentration, and pH profile during growth of *R. glutinis* KCTC 7989 under optimum conditions for EPS production.

duced an expulsion of protons from the cells, causing the medium to become acidic. When the pH was controlled (pH 4.0), the cell growth increased vigorously to 9.1 g/L within 48 h, resulting in a 49% increase in biomass concentration as compared with that of the pH-uncontrolled experiment. Interestingly, no EPS was produced in the pH-controlled experiment. This was probably owing to the carbon source being used mostly for cell growth rather than the synthesis of EPS. This suggests that the culture pH should be allowed to decrease naturally during the fermentation. The influence of culture pH was also revealed in the experiments using different nitrogen sources, as shown in Table 2. The final pH of the broth dropped below 2.0 in the presence of ammonium salts. By contrast, when sodium nitrate, potassium nitrate, yeast extract, peptone, and tryptone were used, the pH remained between 2.7 and 3.5, and EPS was not synthesized. From all the experiments, we found that the EPS production was obtained when the pH of the broth was decreased to <2.0.

Time Course of Cell Growth and EPS Production

The yeast *R. glutinis* KCTC 7989 was cultivated using the optimized production medium in a 5-L jar (Fig. 4). The carbon source was totally utilized within 72 h of fermentation, and the growth reached a maximum of 6.1 g/L. The pH of the culture decreased from its initial value (pH 4.0) to

the self-stabilized final value (pH 1.9) within the first 48 h, after which it remained almost constant. EPS formation was detected after about 30 h of cultivation, after the ammonium sulfate was entirely consumed. The maximum EPS production using the optimized culture condition was 4.0 g/L.

EPS was produced during both exponential and stationary phases of growth. The increase in EPS concentration paralleled the cell growth and continued to increase after the onset of the stationary phase. About 67% of the total EPS was synthesized during the exponential phase of growth, and the rest of the EPS was produced after the cessation of growth. Such increases in EPS synthesis on cessation of growth could be owing to the release of isoprenoid cofactors, used preferentially for the synthesis of peptidoglycans, lipopolysaccharides, and exopolysaccharides during cell growth *(1)*. The biomass yield was approx 0.31 g of dry cell weight/g of glucose in the absence of EPS production (within 24 h of cultivation). This indicated that about 69% of the total carbon source was available for the production of EPS. The glucose-based production yield coefficient and carbon-based production yield coefficient were calculated to be 0.30 g of EPS/g of glucose and 0.34 g (carbon of EPS)/g (carbon of glucose), respectively. Only a small portion (34%) of the initial carbon was converted into EPS. This was consistent with the observation that the decrease in glucose concentration affected the cell growth rather than the EPS production during the growth cycle.

Conclusion

The EPS produced from *R. glutinis* KCTC 7989 was a novel uronic acid/mannose-rich acidic heteropolysaccharide composed of neutral sugars (85%) and uronic acid (15%). The neutral sugar composition of this polymer was identified as mannose, fucose, glucose, and galactose in a 6.7:0.2:0.1:0.1 ratio. The molecular weight of purified EPS was estimated to be 1.0–3.8 $\times$ 10^5 Dalton, and the EPS solution showed a characteristic of pseudoplastic non-Newtonian fluid at a concentration >2.0% in distilled water. The highest yield of EPS was obtained at a C:N ratio of 15 and at an acidic range of pH 3.0–5.0. When the yeast was grown on glucose (30 g/L) and ammonium sulfate (2 g/L) at 22°C at an initial pH of 4.0, EPS production was maximized (4.0 g/L).

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