Characterization and Safety Evaluation of Exopolysaccharide Produced by *Rhodotorula minuta* BIOTECH 2178

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***Abstract*—Yeast exopolysaccharide (EPS) are composed of unique sugar units, which offers an interesting perspective with respect to various food applications. This study aimed to characterize the EPS from *Rhodotorula minuta* BIOTECH 2178. Toxicological assessment of the EPS was investigated to ensure that it is safe for human consumption. The yeast EPS revealed high total sugar composition (59.21-62-47%) and low protein content (17.19%). FTIR spectrum showed prominent functional group such as hydroxyl, carboxyl, beta-linkages, glucose, mannan, and aromatic ring. SEM micrograph of EPS revealed a highly compact with elongated granular structures. Toxicological examination of EPS (50-400mg EPS/kg bodyweight) did not show any symptoms of toxicity and mortality after 14 days administration of aqueous crude EPS. Hematological and histopathological examination in the liver showed no significant alteration between the experimental and control groups. Hence, the highly compact and unique functional properties of *Rhodotorula minuta* EPS showed its potential application in the industry as thickening and stabilizing agent.**

***Index Terms*—yeast exopolysaccharide, *Rhodotorula minuta*, spectroscopy, mice, histopathological analysis**

I. Introduction

The continuous search of new ingredients has been the primary goal of the food industry in order to come up with novel food products. In coping with the growing demand for quality food products, the functionality of polysaccharide is now one of the widely explored food ingredients because of its many prospective applications. Recently, there is a growing interest in the isolation of microbial exopolysaccharide [1], [2]. This compound has been found to have multifarious application in the industry because of its wide diversity in structural and chemical properties. When added to food, EPS can positively improve the rheological properties and sensory qualities of the final product [3]. Moreover, according to several reviews EPS significantly contributes to human health. Fractions of microbial exopolysaccharide (yeast mannan) possess antioxidant, antiviral, antimutagenic activity, function as prebiotic, capable of lowering blood cholesterol, LDL and even sorption of heavy metals [4].

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Considerable number of microorganisms of different taxonomy (bacteria, fungi, yeast) is capable of producing exolpolysaccharide. The yeast and yeast-like fungi which include the genera of *Candida*, *Cryptococcus*, *Pichia*, *Sporobolmyces*, *Trichosporon*, *Lipomyces* and *Rhodotorula* have been described to produce extracellular polysaccharide in the laboratory scale under submerged culture conditions [2], [4], [5]-[8]. The EPS synthesized from yeast can be characterized chemically as β-(1→3)- and β-(1→6)-glucans, mannans, phosphomannans, galactomannans, glucomannans, glucuronoxylomannans and pseudonigeran, while glycoproteins are also produced as additional components of the cell wall [4], [9].

Isolation procedure of EPS produced from yeast offers greater advantage because of its fast and easy separation from the culture medium compared to bacteria. Therefore, it is attractive for large-scale production [10]. However, the information on the chemical and physical properties of yeasts is still scarce. To the best of our knowledge, there are no available reports on synthesis, characterization and toxicological assessment of EPS produced by *Rhodotorula minuta*. Hence, this study focused on the chemical and physical characterization of EPS and safety evaluation using female mice as animal models, produced from locally isolated yeast *Rhodotorula minuta* BIOTECH 2178.

II. Methodology

A. Microorganisms, Media and Growth Conditions

Pure cultures of *Rhodotorula minuta* BIOTECH 2178 was isolated from fresh water Laguna Lake, Philippines and was stored in the Philippine National Collection of Microorganisms located at National Institute of Molecular Biology and Biotechnology (BIOTECH), University of the Philippines Los Baños (UPLB). The yeast strains were maintained on Malt Yeast Agar (MYA) slants and stored at 4℃. The basal medium for EPS production mainly contained (g/L): yeast extract, 18.75; xylose, 6.25; $(NH_4)_2SO_4$, 2.5; KH_2PO_4, 1; $MgSO_4.7H_2O$, 0.5; NaCl, 0.1 and $CaCl_2.2H_2O$, 0.1. The initial pH was adjusted to 5.5, and the medium was sterilized at 121℃ for 15 minutes. All chemicals that were used in this study were of analytical reagent grade [11].

Prior to inoculation, yeast cells were counted using Neubauer hemacytometer. Then, yeast cells (5%v/v) were

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aseptically inoculated into the Erlenmeyer flask (250mL) containing 50mL basal medium and incubated in a rotary shaker (180rpm) at room temperature.

B. Isolation of Crude Exopolysaccharide by R. Minuta BIOTECH 2178

After 4 days fermentation, the culture broth was centrifuged at 5000rpm for 5 minutes at 5 ℃ to separate the yeast cells. The exopolysaccharide was precipitated in the cell free supernatant with two volumes of cold 96% ethanol then stored at 4 ℃ for 24 hours. The precipitated EPS was separated by centrifugation at 5000 rpm for 15 minutes at 12 ℃. The supernatant was discarded and the precipitate was immediately stored in the freezer. Frozen EPS was lyophilized (Leybold-Heracus, Germany) until a constant weight was observed. A precision analytical balance was used to verify the quantity of EPS obtained (grams EPS per liter of culture medium).

C. Estimation of Total Carbohydrate and Protein Content

The concentration of EPS (total sugar content) was estimated by phenol-sulphuric acid method [12]. The absorbance of the samples was determined at 490nm and calibrated using glucose as a standard. The determination of crude protein of the EPS was determined using the standard Kjeldahl methodology [13]. Each sample was measured and analyzed in triplicates.

D. Scanning Electron Microscopic (SEM) Analysis

Surface morphology of the EPS was determined using Scanning Electron Microscopic (SEM) analysis. SEM sample preparation involved fixation of lyophilized EPS in 2.5% glutaraldehyde and five times washing in phosphate buffer (pH 7.2). Post fixation of EPS included addition of 1%OsO_4 then washing again with phosphate buffer (repeated 5 times). Dehydration of fixed EPS involved soaking in a series of analytical grade ethanol in different concentration (50-100%) at 4 ℃. Then it was soaked in 100% ethanol at room temperature. After soaking in ethanol, it was soaked in 50% and 100% isoamyl acetate. Critical point drying was done using CO_2 (Hitachi HCP-2 instrument) for two hours. Dehydrated powder was gold sputtered using a sputtercoater device (Ion sputter JFC 1100 JEOL) and the microstructure was visualized under SEM (JEOL –JSM 5310) with an accelerating voltage of 20kV at different magnification (100X-7, 500X).

E. Fourier Transform Infrared Spectroscopy (FTIR)

A quantity of 50mg lyophilized EPS was taken mixed with 150mg of KBR powder and ground well to fine mixture. The mixture was pressed to a disc using a hydraulic press. The disc was subjected to FTIR spectral measurement using Shimadzu IR Prestige-21 equipped with diffuse reflectance Accessory. It was scanned in a frequency range of 4000-500/cm.

F. Test Material Preparation for Toxicological Examination of Crude EPS

The optimized crude EPS from *Rhodotorula minuta* BIOTECH 2178 was used as a test material for acute toxicity studies. EPS was dissolved in a sterile isotonic saline solution at a concentration of 5g/L.

G. Determination of Acute Toxicity Test

After acclimatization, the female adult mice weighing 20-25 grams were subjected to acute toxicity test. The mice were randomly selected and divided into 4 equal groups (5 mice each) and treated as follows: group 1 was the control given 0.5ml saline solution; group 2 treated with 50mg EPS/kg body weight; group 3 treated with 225mg EPS/kg body weight; and group 4 treated with 400mg EPS/kg body weight. The doses were set on the basis of the recently recorded body weight of each individual animal. Then it was suspended in sterile saline solution with a constant volume of 0.5ml per mice. They were individually caged and treated for 14 successive days by oral gavage. The mice were observed for signs of toxicity and possible death 14, 24 and 72 hours after administration of EPS. The daily weight and food intake were equally monitored and from the data obtained, LD_{50} was determined.

H. Biochemical Analyses

Biochemical tests were determined before oral administration of EPS and at the end of the administration period in all treated animals. Blood samples (placed in heparinized tubes) were withdrawn in the orbital sinus from each mouse. Blood plasma was separated by centrifugation 3000 x g for 10 minutes and immediately processed for biochemical parameters: Alanine Transaminase (ALT) and creatinine levels.

I. Histopathological Analyses

Liver organs were removed from all experimental animals following euthanasia and fixed in 10% formaldehyde. After 48 hours, the liver organs were sliced in its desired section and were transferred in fresh formaldehyde. These were sectioned using a microtome and stained with Hematoxylin-Eosin (H.E.).

III. Results and Discussion

Rhodotorula minuta BIOTECH 2178 given an optimal fermentation conditions can produce 2.1 grams lyophilized EPS powder per liter culture broth showing a powdery off-white characteristic (Fig. 1). Total sugar estimation of the optimized crude EPS is 59.21-62.47%. It showed high total sugar composition similar to other studies. The optimized EPS from *A. pullulan* showed varying sugar composition ranging from 59% to 85% in ethanol precipitated crude EPS [14]. The protein content of crude exopolysaccharide was measured as Kjeldahl nitrogen. The optimum crude EPS produced by *Rhodotorula minuta* was found to be 17.19%. It contains low protein considering that this crude EPS did not undergo several purification processes. [15] Nichols *et al.* (2005) isolated crude EPS from deep-sea marine bacterium containing high protein (40-50%). Furthermore, Tunier *et al.* (1999) isolated EPS from *L. cremoris* wherein it passed through microfiltration and diafiltration [16]. After these tedious and expensive purification steps,

the lyophilized powder contained 63% EPS and 18% protein, almost similar to the result of the present study.

Figure 1. Lyophilized crude EPS powder.

The FTIR spectrum of crude EPS (Fig. 2) displayed a broad intense peak at around 3219.19 cm^{-1} which showed similar type of spectra found in various studies [17], [18]. This band represented the stretching vibration of the hydroxyl groups in the carbohydrate ring. Occurrence of a large number of hydroxyl groups increases their affinity for binding water molecules, which is responsible for the solubility of the EPS. An asymmetrical stretching peak at 1664.57 cm^{-1} suggests the presence of carboxyl groups, a strong indication that the samples were indeed exopolysaccharide [19]. Specifically, the peaks 1427.32 cm^{-1} ascertain the presence of polysaccharides C-O-C that was a typical structure for glucose in pyranose form.

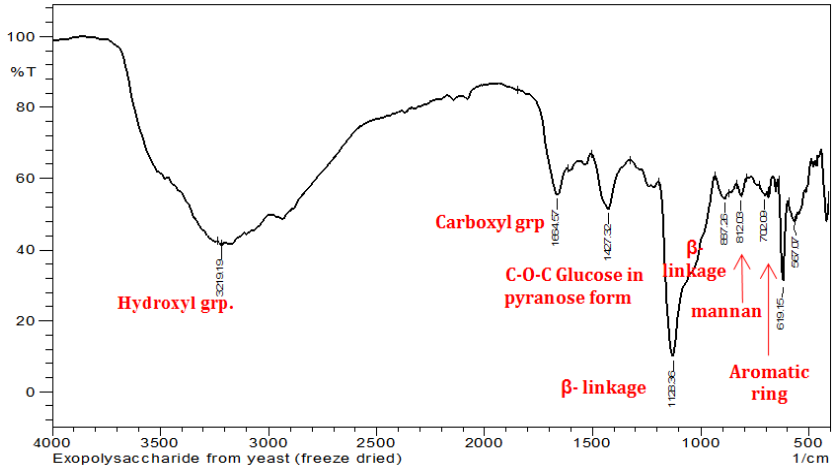

Figure 2. FTIR spectrum of crude EPS from *Rhodotorula minuta* BIOTECH 2178.

Distinctive absorption band maxima for sugar in mid-infra red region were mainly mannan detected at 812.03 cm^{-1} while 887.26 cm^{-1}, and 1128.36 cm^{-1} may be taken as evidence for the presence of beta- glycosidic linkages. According to Lal and Sharma (2009) these peaks indicated the presence of mannans and *beta*-glucans in *R. minuta* exopolysaccharide [20]. *Rhodotorula spp.* is widely known to produce exocellular mannans [4]. Mannan from *Rhodotorula acheniorum* (92.8%) has been tested in food application because of their good water-binding capacity and intrinsic dynamic viscosity [21]. Moreover, *Rhodotorula glutinis* mannan was found to be useful in the diagnosis of leptospirosis and may also contain anti-tumor properties [4], [11]. The peak 702 cm^{-1} indicates aromatic ring with bend hydrogen. Thus, FTIR spectrum analysis showed that the EPS produced was generally composed of a unique structure of polysaccharide containing functional groups wherein other unidentified peaks (619.15 cm^{-1} and 567.07 cm^{-1}) only proves its complexity.

The determination of the structural characteristics of EPS through Scanning Electron Microscopy (SEM) is also important to determine the textural and morphological properties of the polysaccharide produced. The 2000X magnification of the control and the crude EPS appeared to have a porous structure (Fig. 3). Interestingly, the crude EPS showed several canals or crevices. Upon 7500X magnification, its porosity exhibits a grain-like (rice grains) elongated structural units having an average dimension of 0.2-1µm long and 0.1-0.5µm thickness. Owing its smaller pore size distribution, the crude EPS enables the polymer to hold water wherein according to [22] Majumder and Goyal (2009), it can be used as texturizing agent in the food industry. In addition, the grain-like structure may also be responsible for the compactness of the polysaccharide and provide stability of the gel structure when subjected to external forces and maintenance during storage. Furthermore, the crevices found in the EPS may also aid in fast adsorption and interaction of water molecules into the polysaccharide. Thus, the microstructure of the crude EPS indicates its potential use as a texturizer, thickener, viscofier and stabilizing agent for novel food products.

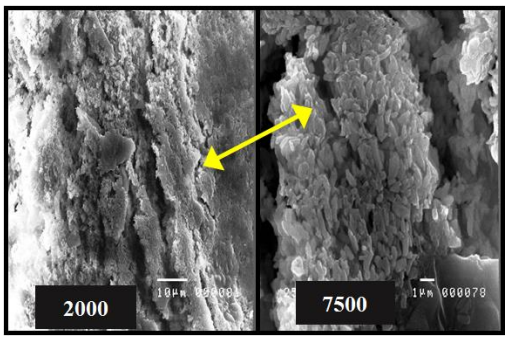

Figure 3. Scanning electron microscopy of lyophilized crude EPS powder. Yellow arrow indicates the crevices in EPS surface.

From the result of the acute toxicity study of crude EPS from *Rhodotorula minuta* BIOTECH 2178 in female mice, no signs of toxicity and mortality was recorded in

any of the test groups after 14, 24, 72 hours and up to 14 days treatment period. Blood chemistry analysis showed creatinine levels of mice treated with crude EPS showed no significant difference on the negative control (Table I). Moreover, all mean creatinine levels are within the normal range taken before (0 day) and after (14 days) administration of EPS. Creatinine is a breakdown product of creatinine phosphate released from the skeletal system filtered by the glomerulus in the kidney [23]. It is generally used to estimate how well the kidney is filtering blood and usually used as a screening test for early kidney impairment. Hepatic enzymes such as alanine aminotransferases (ALT) are a reliable marker of hepatocellular injury or necrosis. Results showed no significant difference observed in ALT levels from day 0 to day 14 and were within the normal range (25-76IU/L).

TABLE I. MEAN CREATININE AND ALT OF THE NEGATIVE CONTROL AND EPS-TREATED FEMALE ICR MICE

Treatment	Creatinine (mg/dL)		ALT (U/L)	
	0 day	14 days	0 day	14 days
T_0 (0.5ml saline solution)	0.24 ±0.01	0.25 ±0.02	28.87 ±0.37	28.89 ±0.35
T_1 (50mgEPS/kg Body weight)*	0.27 ±0.02	0.29 ±0.01	29.53 ±0.86	29.62 ±0.72
T_2 (225mg/kg body weight)	0.26 ±0.01	0.29 ±0.01	29.30 ±0.52	29.37 ±0.48
T_3 (400mgEPS/kg bodyweight)	0.26 ±0.01	0.28 ±0.02	28.8 ±0.89	28.82 ±0.88

ALT – L-alanine: 2-oxoglutarate aminotransferase.
Each value is a mean average of five mice. No significant difference between control and *R. minuta* crude EPS-fed groups $p>0.05$ level of significance. *EPS powder were dissolved in 0.5ml saline solution

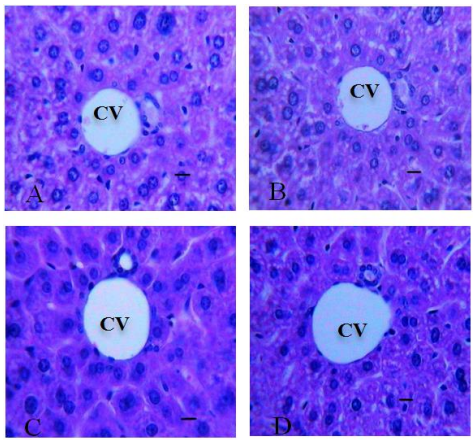

Figure 4. Liver section of female ICR mice showing normal strands of hepatocytes and Central Vein (CV) (H&E x 400): a - negative control; b - treated with 50mg EPS/kg body weight; c - treated with 225mg EPS/kg body weight; d - treated with 400mg EPS/kg body weight

The liver is a large, complex organ that is well designed for various metabolic functions playing a key role in detoxification, regulation and maintaining homeostasis [24]. Macroscopic examination of liver organs of the animals treated with crude EPS from *Rhodotorula minuta* BIOTECH 2178 showed no changes in color compared to the control (Fig. 4). The control and treated group showed normal hepatic architecture showing intact hepatic cells, nucleus, sinusoidal spaces and a central vein.

IV. CONCLUSIONS

This tentative characterization of crude exopolysaccharide produced by *Rhodotorula minuta* BIOTECH 2178 has indicated a unique chemical and physical properties. It may be considered as a highly promising compound applied in various industries as texturizer, thickening and stabilizing agent. The compact microstructure of the EPS suggested its potential application as biofilms. Moreover, safety evaluation of crude EPS demonstrated that it did not cause hematological and histopathological alteration of the liver in female mice. Implying that the EPS produced is safe when applied to food. These findings suggest the necessity of further exploration of EPS such as potential development of novel products and future studies that would direct its potential application in the pharmaceutical industries.

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