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Ethanol and beta-glucan production from an economically feasible medium prepared from paper napkin hydrolysate

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Abstract

At present, the zero-waste concept is a major concern and a challenging topic. Among several renewable materials that contain high cellulose content, napkin papers are potential sources of ethanol production and other value-added products. This work aimed to study the feasibility of an economic medium formulation from napkin hydrolysate for ethanol production and subsequent beta-glucan extraction from the spent yeasts. The medium costs per gram of ethanol produced from napkins and 3 different nitrogen sources, i.e., yeast malt extract (YM), urea, and ammonium sulfate, were compared. The results revealed that the new medium formulation from napkin-ammonium sulfate is economical at 18% ethanol production over commercial YM medium. Furthermore, the remaining yeast cells from the fermentation process were autolyzed, and beta-glucan was extracted prior to Fourier transform infrared (FTIR) spectroscopy for compositional and structural analyses. The FTIR integration peaks indicated that the *S. cerevisiae* TISTR 5339 cell wall contained beta-1,3-glucan as the main polysaccharide component. Overall, this study was successful in developing a practical, economical medium for the biotransformation of napkins to ethanol and producing value-added beta-glucan from the spent yeast from the ethanol production process; these products can potentially be used in the pharmaceutical or food industries in the future.

Keywords: Paper napkin, Ethanol, Culture medium, Beta-glucan, Spent yeast

1. Introduction

Renewable energy demand is rapidly increasing because many countries are attempting to reduce their petroleum consumption. Gasohol is a type of bioethanol that is commonly blended with petroleum at different concentrations, including 90% petroleum with 10% ethanol (E10) and 15% petroleum with 85% ethanol (E85) [1, 2]. Thus, ethanol production has increased worldwide in the last decade from 17.3 billion liters in 2000 to over 46 billion liters in 2007, and the production of over 125 billion liters is estimated for 2020 [3]. Bioethanol can be produced from many types of renewable materials, such as paper, newspapers and paper napkins [2, 3]. Paper napkins are widespread and abundant in daily use. They have a high cellulose content that can be converted to fermentable sugars for ethanol production. The waste recycling concept is used to consider a low-cost medium for ethanol production using napkins and a cheap nitrogen source. Urea and ammonium sulfate have been used for *Saccharomyces cerevisiae* cultivation [4] to replace commercial nitrogen sources, especially yeast and malt extract (YM) [5-7]. However, the optimal nitrogen concentrations are varied. Urea concentrations of 0.25 g/L [8]

and 2 g/L [9] showed positive effects on the growth of *S. cerevisiae* and ethanol production, while optimal ammonium sulfate concentrations of 0.38 g/L [6] and 2.04 g/L [7] were reported. The differences in optimal nitrogen source concentrations depend on the microbial strain, carbon source concentration and fermentation condition. Therefore, the feasibility of using paper napkin hydrolysate with an inexpensive nitrogen source was studied to formulate an economical medium for ethanol production.

Spent yeast, which is a byproduct of ethanol fermentation, is an important source of beta-glucans. Beta-glucans from the yeast cell wall have been of interest in the pharmaceutical sector and food industry due to their beneficial effects on human health. They function as immune stimulants that protect against infectious diseases, cancer, diabetes and high blood cholesterol levels [10-12]. However, the structural polymers of beta-glucan vary in different yeast cell wall components, contributing to the differences in its biological activities. The yield of beta-glucan production depends on the medium composition and fermentation processing and extraction methods. To the best of our knowledge, the zero-waste concept of recycling paper napkins and using them as a low-cost medium for ethanol production and further recycling of the fermentation byproduct for beta-glucan extraction has not yet been documented.

In the present study, paper napkins were used as the carbon source in the fermentation process to investigate ethanol production, and a cost evaluation of the medium was conducted. Beta-glucan extraction from spent yeasts was also investigated.

2. Materials and methods

2.1 Microorganisms and inoculum preparation

S. cerevisiae TISTR 5339 was inoculated into 100 mL of yeast malt extract (YM) medium containing 3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 10 g/L glucose and incubated on a rotating shaker at 150 rpm and 30°C for 24 h. To increase the cell concentration, the yeast was re-subcultured in YM broth at 150 rpm and 30°C for 18 h. The cells were harvested by centrifugation, resuspended in normal saline solution (0.85% NaCl) and used as an inoculum for ethanol production with a final cell concentration of 10^7 cells/mL.

2.2 Ethanol fermentation and analysis

The paper napkins (Tesco, Thailand) used as a substrate in the experiments were purchased from a supermarket in Khon Kaen Province, Thailand. The substrate was subjected to enzymatic hydrolysis using commercial Cellic CTec2 (Novozymes, Denmark). Hydrolysis was performed by adding 5.0 g of paper napkins in 100 mL of sodium citrate buffer (50 mM, pH 5.0) with a total cellulase activity of 70 U. Hydrolysis was conducted at 50°C at 150 rpm for 48 h. The hydrolysate was collected and stored in a bottle at -20°C before use.

Separated hydrolysis and fermentation (SHF) was performed in 250 ml Erlenmeyer flasks containing 100 mL of fermentation medium (3 g/L yeast extract, 3 g/L malt extract, 5 g/L peptone, and 50 g/L reducing sugar napkin hydrolysate) with 10% (v/v) inoculum. Cultivations were performed in duplicate at 30°C and 150 rpm for 24 h. The samples were centrifuged at 12,000 rpm (16K 230 V, EU; Bio-Rad Laboratories) for 5 min. The supernatants were further analyzed for ethanol concentration, reducing sugar concentration and viable cell counting.

Simultaneous saccharification and fermentation (SSF) was conducted by adding 5.0 g of paper napkins to 100 mL of glucose-free YM medium with a total cellulase activity of 70 U and 10% (v/v) inoculum. The same conditions were used as in previous experiments. The ethanol concentration and reducing sugar concentrations were determined at 24 h.

Nitrogen source replacement was designed to investigate the effect of the two main nitrogen sources-urea (A: 0.2, 0.4, 0.6, 0.8, and 1.0 g/L) and ammonium sulfate (B: 0.3, 0.9, 1.2, 1.8 and 2.4 g/L)-at five different levels. The replacement was performed using 50 g/L reducing sugars from napkin hydrolysate as carbon sources for ethanol fermentation. The incubation was performed at 30°C and 150 rpm for 24 h. Analysis of variance (ANOVA) was carried out to estimate statistically significant parameters.

The analytical methods were carried out as follows:

The ethanol concentration (*P*, g/L) was analyzed by gas chromatography (Shimadzu GC-14B, Japan; solid phase: polyethylene glycol (PEG-20 M); carrier gas: nitrogen; 150 °C isothermal packed column; injection temperature: 180°C; flame ionization detector temperature: 250°C; C-R7 Ae plus Chromatopac Data Processor), and 2-propanol was used as an internal standard [13]. The reducing sugars were analyzed by the dinitrosalicylic acid (DNS) method [14]. Yeast cell viability was determined directly using a hemocytometer with a methylene blue staining technique.

2.3 Cost evaluation

In this study, variable operation costs, including raw materials, chemicals, and enzymes, were applied for cost evaluation, which was based on the total medium (yeast extract, malt extract, peptone, glucose, urea, ammonium sulfate, enzyme and paper napkins) costs. The cost of all medium components was considered from the price of all purchases from distributors in Thailand. However, the costs of any equipment, installation, and government subsidies were not included. Wang et al. [15] applied the formulation for economic analysis based on total installed equipment cost as follows:

$$\text{Cost per g of ethanol (USD)} = \frac{\text{Total cost (USD)}}{\text{Ethanol production (g)}}$$

$$\text{Total cost (USD)} = \sum(\text{ Composition cost per unit} \times \text{number of units used })$$

2.4 Extraction of beta-glucan from yeast cells

The suspensions of yeast cells (1% v/v) in distilled water pH 5.0 were incubated at 50 °C and 150 rpm for 24 h. The autolysis reaction was terminated by incubating the samples at 80°C for 15 min. The yeast cells were collected at 4 °C by centrifugation at 12000 rpm for 5 min. The pellets were dried at 60°C and stored at 4°C until they were ready for use. Beta-glucan was extracted by adding 5-fold 1.0 M NaOH to yeast cells, and the samples were incubated at 80°C for 2 h. The cell pellet was harvested by centrifugation at 12000 rpm for 10 min at 4 °C and suspended in 3-fold distilled water. The yeast cells were separated and dissolved in 5-fold 0.1 M CH_3COOH, incubated at 80 °C for 2 h and centrifuged at 12000 rpm for 10 min at 4 °C. The pellets were washed with distilled water 3 times and dried in an oven at 60 °C under an air atmosphere. The obtained glucan pellets were stored at 4°C until further analyses were performed [16-17].

2.5 Beta-glucan analysis by Fourier transform infrared (FTIR) spectroscopy

An attenuated total reflection (ATR) accessory (Specac, UK) fitted with a diamond crystal was employed to collect FTIR spectroscopic images. This ATR accessory was placed in an imaging chamber (IMAC) that was attached to an FTIR spectrometer (Equinox 55, Bruker, UK). The imaging data were recorded using a focal plane array (FPA) detector and OPUS software. FTIR spectra were recorded in a single experiment, resulting in an image size of approximately 638 × 525 μm^2 [17-19].

2.6 Statistical analysis

All the measurements were performed in triplicate using IBM SPSS Statistics 20 software for the analysis of variance (ANOVA) with 95% confidence intervals.

3. Results and discussion

3.1 Ethanol production from paper napkin hydrolysate

The fermentation of paper napkin hydrolysate by SHF resulted in an ethanol concentration of 22.84 g/L, while an ethanol concentration of 20.89 g/L was obtained from the SSF of paper napkins at 24 h of cultivation (Table 1). SHF was selected as a more efficient process than SSF for the following reasons: (1) small pieces of papers that were difficult to separate from the spent yeasts after fermentation remained after the SSF process, (2) significantly higher ethanol production was obtained from the SHF process and (3) there was less paper contamination during the extraction of beta-glucan from the spent yeast in subsequent experiments.

Another study of enzymatic saccharification of newspaper and its use for ethanol fermentation by *S. cerevisiae* was reported by Kuhad et al. [20]. Ethanol was produced at a concentration of only 5.64 g/L due to the lower sugar concentration in the newspaper hydrolysate (14.64 g/L), and an increase in sugar content to 38.21 g/L by fed-batch fermentation provided ethanol at a concentration of 14.77 g/L. Marques et al. [21] used recycled paper sludge for ethanol fermentation by *Pichia stipitis* CBS 5773. They found that 19.6 g/L ethanol was obtained from SHF after 179 h of cultivation, while the SSF process was completed after 48 h of incubation and produced 18.6 g/L ethanol. Thus, ethanol production from paper napkin hydrolysate was more effective than production from newspaper and recycled paper sludge substrates. However, there were concerns over not only the replacement of the carbon source but also the nitrogen source, which is another factor that could lower the ethanol production cost.

Table 1 Ethanol production from paper napkins by *S. cerevisiae* TISTR 5339 via SHF and SSF for 24 h.

Medium	P (g/L)	Q_p (g/L/h)	Cell number (cells/mL)
napkin hydrolysate (SHF)	22.84 ± 0.44[a]	0.95 ± 0.20 [a]	6.0×10^{7} [a]
napkin paper (SSF)	20.89 ± 0.18[b]	0.92 ± 0.10 [a]	3.5×10^{7} [b]

3.2 Effects of nitrogen sources on ethanol production

During fermentation, the factors affecting the growth of yeast cells and ethanol production are the carbon sources and nitrogen sources. Urea is an essential and inexpensive nitrogen source for yeast growth. *S. cerevisiae* can convert urea to ammonia for new complex nitrogenous molecule synthesis [4]. Ethanol production from napkin hydrolysate at different urea concentrations is shown in Figure 1. After 24 h of cultivation, an increased urea concentration from 0.2 to 1.0 g/L did not show a significant difference in ethanol production. Thus, 0.2 g/L urea was considered the optimum concentration because it was the lowest concentration that could save on the cost and provide 10.54 g/L ethanol.

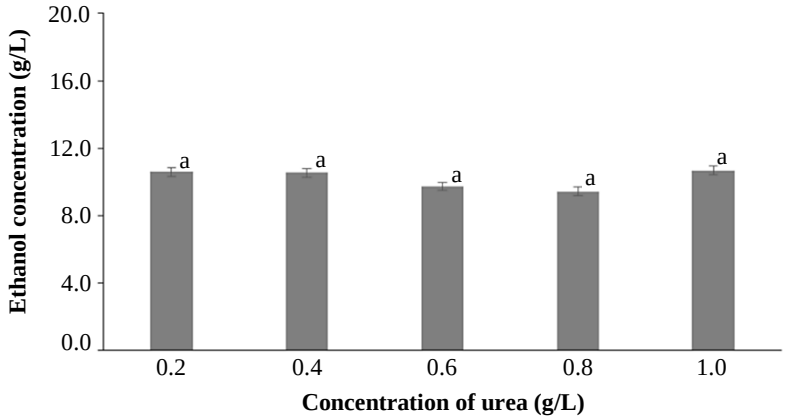

Figure 1 Ethanol production by *S. cerevisiae* TISTR 5339 at 30°C for 24 h using medium composed of napkin hydrolysate supplemented with different urea concentrations. (The results were expressed as the mean ± SD).

S. cerevisiae can utilize ammonium ions by converting them to amino acids, glutamate, and glutamine [22]. By varying the concentration of ammonium sulfate as a nitrogen source from 0.3 to 2.4 g/L for fermentation, ethanol was produced, as shown in Figure 2. The ethanol production was found to be insignificant when either 0.9 or 1.8 g/L ammonium sulfate supplement was used. Thus, 0.9 g/L ammonium sulfate with 15.50 g/L ethanol was considered the optimum concentration based on cost savings. High concentrations of ammonium sulfate caused the fermentation medium to be more acidic, causing inhibition of yeast growth and ethanol production [23]. Excess ammonium ions could also induce the yeast cell death pathway by shortening the chronological life of the cells. Controlling the pH throughout the entire fermentation period might improve the ethanol yield and fermentation efficiency [6].

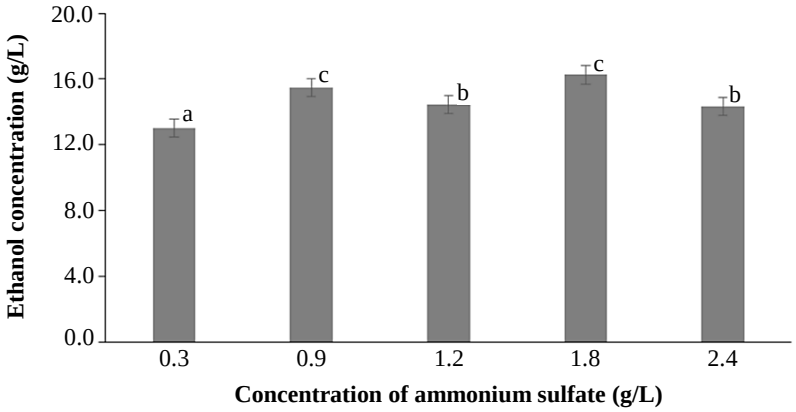

Figure 2 Ethanol production by *S. cerevisiae* TISTR 5339 at 30°C for 24 h using medium composed of napkin hydrolysate supplemented with different ammonium sulfate concentrations. (The results were expressed as the mean ± SD).

3.3 Cost evaluation of ethanol production from raw materials

The medium composition affects the cost of ethanol production. To formulate the economical medium from the paper napkin hydrolysate, the ethanol obtained from various media of paper napkin hydrolysate with yeast-malt extract (NK-YM), paper napkin hydrolysate with urea (NK-U), and paper napkin hydrolysate with ammonium sulfate (NK-AS) were compared. As shown in Figure 3, the maximum ethanol concentration of 22.84 g/L was obtained from NK-YM, followed by NK-AS (15.50 g/L) and NK-U (10.60 g/L).

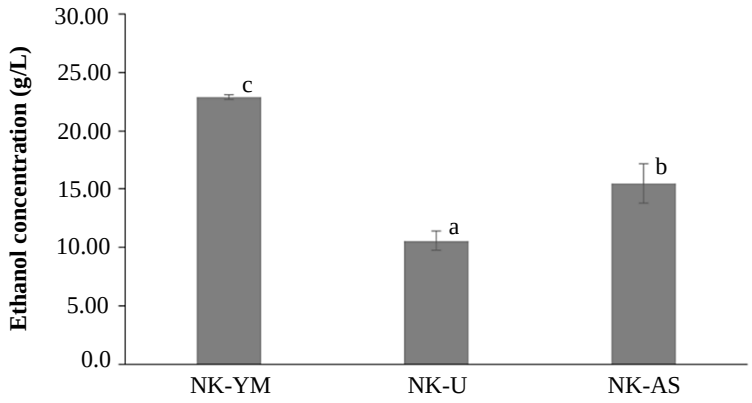

Figure 3 Ethanol production from different nitrogen sources, NK-YM (napkin with YM medium), NK-U (napkin with urea), and NK-AS (napkin with ammonium sulfate), using *S. cerevisiae* TISTR 5339 at 30°C for 24 h. (The results were expressed as the mean ± SD).

Kwiatkowski et al. [24] reported that the increased ethanol production cost from 0.235 USD to 0.365 USD per liter resulted from the price of corn increasing from 0.071 USD to 0.125 USD per kilogram. Thus, the ethanol production cost was focused on medium composition; the summation of each component expense was calculated as shown in Table 2. The costs of NK-YM, NK-U and NK-NH media were 2.62, 1.40 and 1.41 USD, respectively. The cost analysis results based on the medium expense per gram of ethanol produced were 0.11, 0.13, and 0.09 USD/g-ethanol, respectively (Table 3). Thus, the use of a medium comprising napkin hydrolysate and ammonium sulfate for the fermentation process was effective for ethanol production with 18% lower operation cost, which would be in line with an economic reduction policy and address environmental concerns.

Table 2 Total cost calculation of different media for ethanol production from modified carbon and nitrogen sources.

Medium Preparation					
Medium	Compositions	Composition cost per gram (USD/g)	Amount (g/L)	Cost (USD)	Total cost (USD)
NK-YM	Yeast Extract	0.09	3.0	0.26	2.62
	Malt Extract	0.17	3.0	0.51	
	Peptone	0.09	5.0	0.44	
	Enzyme	0.07	20.0	1.40	
NK-U	Urea	0.02	0.2	0.004	1.40
	Enzyme	0.07	20.0	1.40	
NK-AS	Ammonium sulfate	0.01	0.9	0.01	1.41
	Enzyme	0.07	20.0	1.40	

Table 3 Cost evaluation of ethanol production from different carbon and nitrogen sources in the fermentation.

Medium	Ethanol production (g/L)	Total cost (USD)	Cost per gram of ethanol (USD/g ethanol)
NK-YM	22.84	2.62	0.11
NK-Urea	10.60	1.40	0.13
NK-Ammonium sulfate	15.50	1.41	0.09

3.4 Beta-glucan extraction from the yeast cell wall

The spectral bands of beta-glucan extracts were determined by FTIR, and the band area of the beta-glucan spectrum was compared with that in published references [25, 26]. The peaks in the wavenumber range from 800 to 1200 cm^{-1} were related to the stretching of C-O and C-O-C bonds of the polysaccharides. Galichet et al. [25] reported that the peaks in the FTIR spectra of *S. cerevisiae* observed at 1077 cm^{-1}, 1103 cm^{-1} and 1141 cm^{-1} revealed the presence of beta-1,3-glucan. According to Pengkumsri et al. [16], beta-1,3-glucans extracted from various strains of *S. cerevisiae* appear at wavenumbers of 1048 cm^{-1}, 1108 cm^{-1} and 1138 cm^{-1}. Compared with the known spectra from previous publications, the main spectra from the cell wall extract contain peaks at 1048 cm^{-1}, 1108 cm^{-1} and 1138 cm^{-1} due to the glycosidic bond absorptions in the linear structure of beta-glucan, which contains 1-3 beta glycosidic bonds (Figure 4). Therefore, *S. cerevisiae* TISTR 5339 and the yeast cell wall solution contained beta-1,3-glucan as the main polysaccharide component, while proteins and lipids were also present but in smaller amounts.

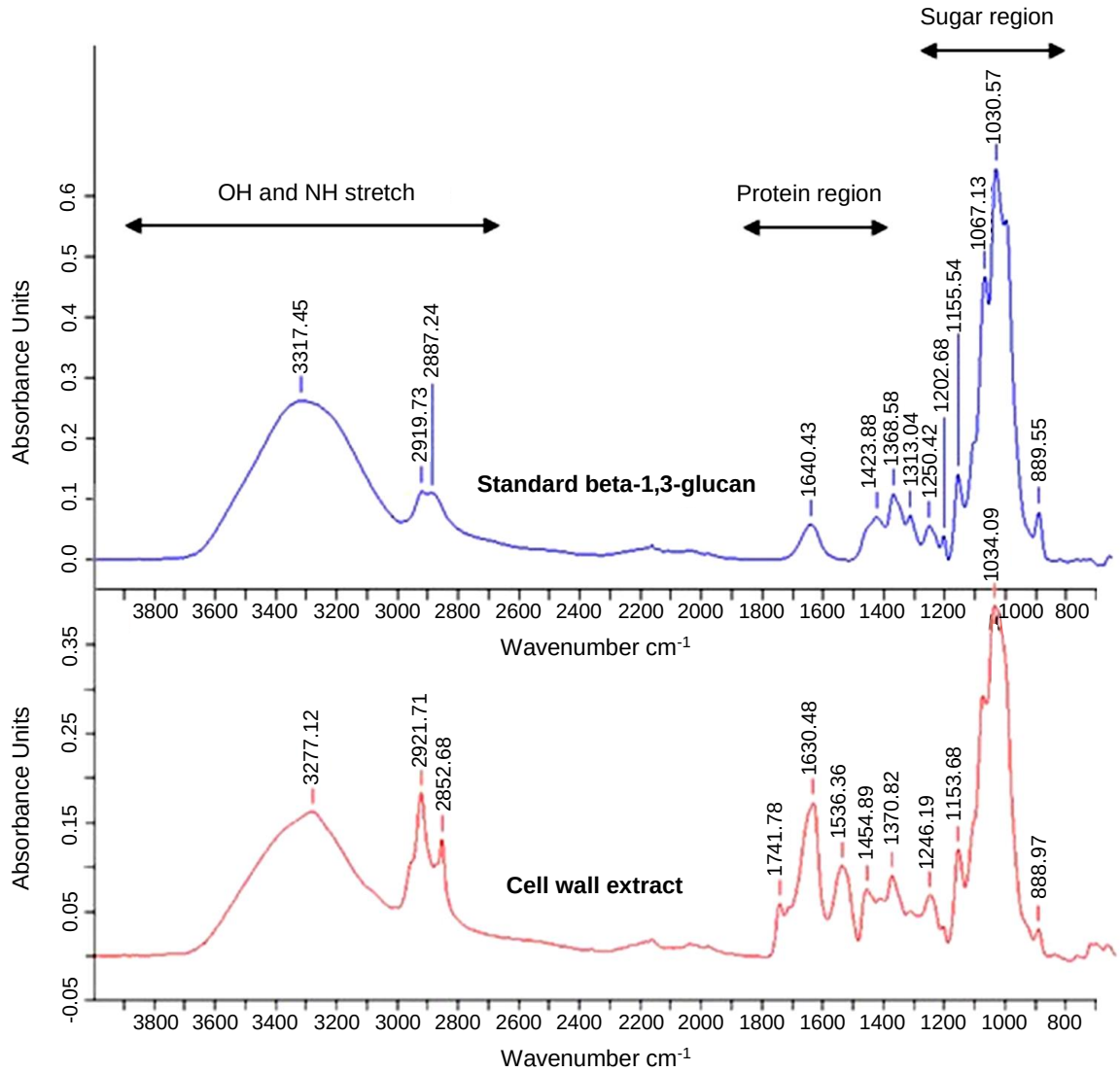

Figure 4 FTIR spectra of standard beta-1,3-glucan and yeast cell wall extract.

4. Conclusion

The feasibility of converting paper napkins to ethanol using appropriate inexpensive nitrogen sources was examined. The cost analyses based on cultivation medium price per gram ethanol obtained indicated that ethanol production from napkins is economical when the cultivation media is modified to napkin-ammonium sulfate. The beta-glucan extract from spent yeast cells of the fermentation process showed a high content of beta-1,3-glucan.

Therefore, this study successfully developed a practical economic medium for ethanol production and produced beta-1,3-glucan from spent yeasts.

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