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Submission date: 30-Apr-2021 10:58AM (UTC+0700)

Submission ID: 1574070544

File name: ction,_characterization,_and_biological_toxicity_of_-glucans.pdf (373.12K)

Word count: 4385

Character count: 22627

Extraction, characterization, and biological toxicity of β -glucans from *Saccharomyces cerevisiae* isolated from ragi

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Ramadhani I, Larissa D, Yuliani Y, Amir M, Kusmiati. 2020. Extraction, characterization, and biological toxicity of β -glucans from *Saccharomyces cerevisiae* isolated from ragi. Journal of Microbial Systematics and Biotechnology 2(2), 35-43

Abstract

β -glucan is a homopolysaccharide with biological activities that are beneficial to health as an immunostimulant, anti-inflammatory, anti-diabetic, anti-cholesterol, and many more. β -glucan extraction results from yeast require characterization related to this bioactive quality, such as β -glucan weight, monomer analysis, functional groups, and cytotoxicity assay. Four *Saccharomyces cerevisiae* isolates were isolated from three local ragi samples, namely the SC-1, SC-2, SC-3, and SAF from instant ragi. This study aimed to obtain the best candidate of *S. cerevisiae* isolates to produce high β -glucan levels and low protein levels and to test the potential for cytotoxicity. The four isolates were rejuvenated on potato dextrose agar (PDA), then inoculated into the liquid glucose yeast peptone (GYP) fermentation medium for six days. *Saccharomyces cerevisiae* cells were extracted by neutralizing acid-base, dried and weighed as a crude β -glucan (mg per 300 mL). The highest yield was SC-2 (818 mg), followed by SC-3 (726 mg), SAF (597 mg), and SC-1 (433 mg). The presence of -OH (alcohol), -C-C-C- (alkane), and -R-O-R- (ether) groups were showed using FTIR characterization. Glucose equivalent β -glucan levels and protein levels were determined using a UV-Vis spectrophotometer. The results showed that β -glucan SC-1 gave the best results with glucose equivalent β -glucan levels of 4,865% and protein levels of 3,804%. The crude β -glucan toxicity test using the brine shrimp lethality test (BSLT) method shows that the β -glucan of the SAF strain has LC₅₀ cytotoxicity of 114.8 ppm followed by β -glucan cytotoxicity from local ragi LC₅₀ was SC-2 (323.5 ppm), SC-1 (331.1 ppm), and SC-3 (354.8 ppm). Therefore, based on the results, SC-1 isolate obtained the highest β -glucan crude and the lowest protein content was SC-2. The β -glucan of SAF extract had the highest toxicity properties based on the IC₅₀ value.

Keywords: Brine Shrimp Lethality Test (BSLT), β -Glucans, FTIR, Glucose, Protein, *S. cerevisiae*

Introduction

Saccharomyces cerevisiae is a single-celled fungi, occur widespread, non-pathogenic, non-toxic, and has long been used in various fermentation processes such as making bread, cassava tapai, sticky tapai, and many more. It can also produce β -glucans, which is a

component of its cell wall. *S. cerevisiae* has a mechanical strength in inner layer of the cell walls, which consist of β 1,3-glucan and chitin from about 50 - 60% of the dry weight of the walls. In the stationary phase, the β 1,3-glucan molecule consists of about 1500 glucose monomers, while the branched β 1,6-glucan contains 130 glucose monomers (Klis *et al.* 2002).

β -glucan is a polysaccharide consisting of glucose molecules bound by glycosidic bonds to form the main chain polymer. β -glucan is a functional compound that has many health benefits. The study results reported the benefits of β -glucan in the fields of food, feed, medicine, and cosmetics. Some of health implication involved in colon cancer-fighting activity (Dongowski *et al.* 2002), dietary fiber for better intestinal health (Valle-Jones 1985), increases stool mass and eliminates toxic substances, anti-constipation agents (Tungland 2003), decreases the glycemic index (Jenkins *et al.* 2002), lowers serum cholesterol (Kusmiati & Dhewantara 2016), postprandial leveling glucose levels (Hallfrisch *et al.* 2003) and prevention of coronary heart disease (Ving *et al.* 2002). β -glucans derived from yeast cells can be a modulator of many activities *in vivo* and *in vitro* (Estrada *et al.* 1997). In the field of immunopharmacology, β -glucans shows its anti-tumor effect (Di Luzio *et al.* 1979; Lotzová & Gutterman 1979). The results from Pengkumsri *et al.* (2017) showed that the β -glucan production from yeast strain TISTR5623 with the acid-base neutralization extraction method was obtained a maximum of 3.72 ± 0.31 g/L.

Various regions in Indonesia have different kind of ragi to be applied to a variety of foods. It is assumed from different regions that the diversity of yeast strains and their potential will be different. Therefore, this study aims to obtain *S. cerevisiae* isolates from local yeast, which produce high β -glucan with low protein content, to characterize the functional groups and the toxicity properties of this compounds. To obtain the toxicity activity, the brine shrimp lethality test (BSLT) is used as a method to search for new anticancer compounds through initial testing of the compound's cytotoxic properties. This method is easy to work with, fast, inexpensive, and quite accurate (Meyer *et al.* 1982). On this study, the β -glucan test was carried out in various concentration which show the anticancer activity.

Materials and methods

Materials

The *S. cerevisiae* isolates were used from local ragi, namely SC-1, SC-2, SC-3, and one SAF for instant ragi as a comparison. All isolates were identified and deposited at Research Center for Biotechnology - LIPI. All isolates were examined for morphology using a compound microscope (Olympus BX53, Japan) with 400 \times –1000 \times magnification.

Culture media preparation

Regeneration media

For 20 mL of potato dextrose agar (PDA) were filled in tubes and sterilized using autoclave at 121 $^{\circ}$ C, 15 psi for 15 min. After being sterilized, the tubes were tilted and cooled until solidified.

Fermentation media

Liquid GYP media containing 1% yeast extract, 2% peptone, and 2% glucose (w/v), were dissolved in 800 mL of distilled water and divided into 50 mL to Erlenmeyer flasks. The media was then sterilized by autoclaving at 121 $^{\circ}$ C, 15 psi for 15 min.

Production of β -glucans

One mL of *S. cerevisiae* from fresh culture (2×10^6) was inoculated into 50 mL of liquid GYP media, incubated using an incubator shaker at 30°C for six days.

Extraction of β -glucans (Alkali-acid method)

β -glucan was extracted from the cell walls of *S. cerevisiae* referring to Williams *et al.* (1991) and Hunter *et al.* (2002) methods. Six-days-old of *S. cerevisiae* cell culture has reached the stationary phase and was used to obtain β -glucan. The culture was then centrifuged (Hitachi CT6EL) to 7000 rpm at 30°C for 10 min, removed the supernatant, and the pellet was hydrolyzed with 0.75 M (3%) NaOH in a water bath at 75°C for 6 hours. The hydrolysis result was centrifuged to 6000 rpm at 30°C for 15 min, and then the supernatant was discarded. The precipitate was added with 0.5 M acetic acid, then centrifuged at 6000 rpm for 5 min, then the supernatant was discarded. This acetic acid treatment was carried out for three times. Subsequently, the biomass was rinsed with distilled water and centrifuged at 6000 rpm for 5 min, discarded the supernatant repeated for two times. Next step was to rinse the biomass by 70% ethanol and centrifuged at 6000 rpm for 5 min, and then gently removed the supernatant. Finally, the extract was dried in a vacuum oven at 40°C for 5-6 hours and weighed as a crude β -glucan (mg).

Characterization of crude β -glucans with Fourier Transform Infra-Red (FTIR-8400S)

A total of 2 mg of β -glucan dry extract sample was added with 100 mg of KBr powder, then finely crushed until homogeneous. The mixture was processed by a special printer until transparent pellets were obtained, and the spectrum was recorded at a wavenumber 4000–666 cm^{-1} .

Glucose analysis by phenol-sulfate method

Two milligrams of crude β -glucan of each *S. cerevisiae* isolates were weighed and then diluted with 2 mL NaOH 0.5 N. A total of 0.25 mL of the test solution was added, then added with distilled water to a total volume of 0.5 mL to the test tube. After that, 0.25 mL of 5% phenol and 1.25 mL of concentrated H_2SO_4 were added to a test tube. The test tube was shaken and left for 1 min before boiled at 100°C for 15 min. After cooling, the absorption value of the solution was measured by a UV-2401 PC (UV-Vis spectrophotometer, Shimadzu, Kyoto, Japan) at 490 nm. Glucose equivalent β -glucan content (%) was calculated by following formula:

$$\text{Glucose equivalent } \beta\text{-glucan content (\%)} = \frac{C \times V_f \times D_f \times 100\%}{DW}$$

(C = sample concentration (mg/L); V_f = final volume (L); D_f = dilution factor; DW = β -glucan dry weight (mg))

Protein analysis with the Lowry method

Firstly, 0.25 mL of the test solution that has been made before, was diluted by adding, distilled water to 0.5 mL, then it was added 0.25 mL of NaOH 1 N for boiling process for 20 min. After cooling, the solution was added by 1.25 mL of reagent D solution (50 mL Na_2CO_3 5%, 1 mL $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ 1%, and 1 mL potassium tartrate 2%). The mixture was shaken for 10 min until homogeneous, then 0.25 mL of Folin-Ciocalteu was added until constructing a blue color and left for 30 min. Finally, the absorption of this solution was measured using a UV-2401 PC (UV-Vis spectrophotometer, Shimadzu, Kyoto, Japan) at 750 nm.

Toxicity tests with the ⁹ Brine Shrimp Lethality Test (BSLT)

Toxicity tests with the brine shrimp lethality test (BSLT) were carried out using the method of Sarah *et al.* (2017). A total of 20 mg egg of *Artemia salina* L. hatched in a container containing artificial seawater (38 g NaCl non-iodine per L distilled water) and irradiated with fluorescent lamp. After 24 hours, nauplii of *A. salina* L. were transferred to another container, and 24 hours later, the nauplii or larvae can be used as tested animals. The concentration of the test solution used was 400, 200, 100, 50, and 10 ppm in triplicate. Ten tails of nauplii was stored to the vial tube which contains 3 mL of test solution and 2 mL of artificial seawater. Observations were performed after 24 hours by counting the number of larvae mortality. The percentage of death (% mortality) of *A. salina* larvae was calculated by the following formula:

$$\% \text{ Mortality} = \frac{\sum \text{larvae mortality}}{\sum \text{total larvae}} \times 100 \%$$

Furthermore, a linear regression analysis was performed to construct a relationship model between the dependent variable and independent variables. Therefore, a linear regression equation is obtained.

$$y = ax + b$$

(y = probit value *A. salina* mortality and x = sponges extract concentration)

³ The lethality concentration (LC₅₀) was calculated using probit analysis. The LC₅₀ (median lethal concentration) values were calculated using the regression line obtained by plotting the concentration against the death percentage on a probit scale. Calculating of LC₅₀ using regression equation ($y = a + bx$) with log D as the x-axis and y-axis as a percent of mortality. An active or toxic substance expressed when the LC₅₀ value <1000 mg/mL.

Results and discussion

Morphological identification

The morphological characteristics of four isolates from local ragi have the same characteristics: colonies on PDA are light cream, smooth and with an entire margin. The cells are globose to elongate (2.5–5.5 × 6.0–9.0 μm) and occur singly or in pairs (Figure 1). Based on the morphological features, these isolates belong to the genus *Saccharomyces* (Naseeb *et al.* 2017).

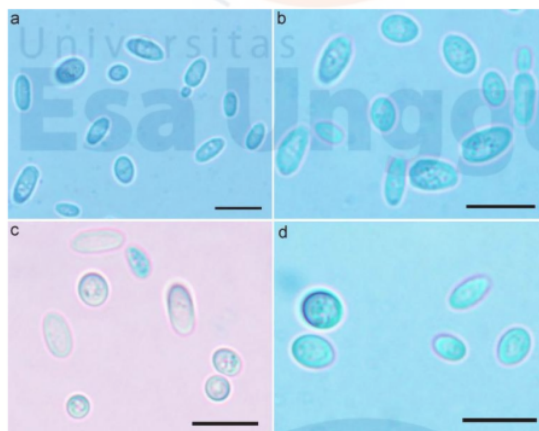


Figure 1. The ¹⁹ microscopic morphology of four isolates from local ragi on PDA after 3 days at 25 °C. a) SC-1, b) SC-2, c) SC-3, d) SAF. Scale bars: 10 μm

The dry weight of crude β -glucan

The dried extract of each *S. cerevisiae* isolate was weighed to constant weight as dry weight of crude β -glucan (Figure 2).

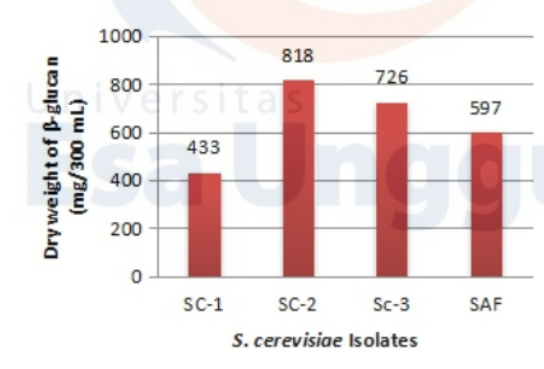


Figure 2. Average dry weight (n = 4) of crude β -glucan of four *S. cerevisiae* isolates (mg/300 mL culture).

The highest yield of crude β -glucan per 300 mL of medium was obtained by isolate *S. cerevisiae* SC-2 at 818 mg, while the lowest was obtained by isolate *S. cerevisiae* SC-1 at 433 mg. One-way ANOVA results of the effect of different *S. cerevisiae* isolates on the acquisition of β -glucan dry weight showed a significant difference (F count > F table). The difference in β -glucan production in the four *S. cerevisiae* isolates could be due to differences in their genetic characteristics. In other case, the difference in *S. cerevisiae* genetic characteristics can affect acetate yield (Curiel *et al.* 2016). Bzducha-Wróbel *et al.* (2018), showed that modification of the cell wall structure of *S. cerevisiae* strains can increase β -glucan synthesis.

Characterization of crude β -Glucans with Fourier Transform Infra-Red (FTIR)

Characterization of crude β -glucan was derived from four *S. cerevisiae* isolates, namely SC-1, SC-2, SC-3, and SAF, using FTIR. This aims to know the existence of a functional group that matches the structure of the β -glucan compound. FTIR results show that the crude β -glucan from four *S. cerevisiae* isolates has a functional group by the structure of the β -glucan (standard) compound consisting of an -OH group (alcohol), a -CC group (alkane), and a ROR group (ether) (Gonzaga *et al.* 2013). The results of the crude β -glucan functional group analysis by FTIR are shown in Table 1.

Table 1. Analysis of functional groups of β -glucans crude from four isolates of *S. cerevisiae* by FTIR

Functional groups	Wavenumber literature (cm ⁻¹) ¹⁷	β -Glucan standard	Wavenumber sample (cm ⁻¹)			
			β -glucan samples			
			SC-1	SC-2	SC-3	SAF
stretch -OH	3000-3700	3173.65	3445.63	3428.27	3457.42	3167.14
stretch-C-C-C	1680-1600	1608.52	1630.47	1648.26	1618.12	1623.28
		1668.31	1662.43	1645.32	1634.22	
stretch-R-O-R	1050-1260	1075.24	1073.26	1103.10	1072.02	1156.12
		1155.28	1152.32	1157.36	1154.22	
		1228.57				

Glucose equivalent β -Glucan content

The glucose level in the crude β -glucan extract is essential to be determined as because the monomer of β -Glucans, namely d-glucose, binds to form polymers with β 1,3 and 1,6 glycosidic bonds (Ruiz-Herrera & Ortiz-Castellanos 2019). As a result, the greater the glucose level, the higher the β -glucan was obtained. Our results of the analysis suggested that of the highest glucose equivalent β -glucan content were obtained by *S. cerevisiae* SC-1 isolates, however the lowest value was obtained when weighing the crude SC-2 extract. This result might related to the level of purity of β -glucan obtained since the high yield of crude extract weighing does not necessarily result in high glucose levels. This is because the cell wall of *S. cerevisiae* consists of 50% compounds 1,3 β -Glucans, 10% 1,6 β -Glucans, 40% mannoproteins, and 1- 3% chitin (Lipke & Ovalle 1998). This situation might be strongly linked to β -glucan with other compounds as proteoglycans, glucomannan, and others. The glucose equivalent β -Glucan levels are listed in Table 2.

Table 2. Glucose equivalent β -glucan content of four *S. cerevisiae* isolates using UV-Vis spectrophotometer λ 490 nm

<i>S. cerevisiae</i>	SC-1	SC-2	SC-3	SAF
Glucose equivalent β -glucan content (%)*	4.8652 ^b	1.2754 ^a	1.5945 ^a	1.9322 ^a

* average of four replications

** average numbers followed by the same letter are not significantly different

One-way ANOVA test results of different treatment of *S. cerevisiae* isolates on glucose equivalent β -glucan levels provided results $F_{count} > F_{table}$, this shows that there was a significant difference due to different *S. cerevisiae* isolates to glucose equivalent β -glucan levels. Tukey's test showed a significant difference ($\alpha = 0.05 \geq \text{Sig}$). They were using different *S. cerevisiae* isolates. *S. cerevisiae* SC-1 produced β -Glucans, significantly different from *S. cerevisiae* SC-2, SC-3, and SAF to glucose equivalent β -glucan levels.

Determination of protein levels

The protein content was determined because protein and β -glucan are components of the cell wall of *S. cerevisiae*, which are bound as peptidoglycans and mannoproteins (Klis *et al.* 2002). Analysis using the Lowry method with a standard solution of bovine serum albumin (BSA). The results of the one-way ANOVA test effect of different *S. cerevisiae* isolates showed that $F_{count} > F_{table}$, so there was a significant difference in the effect of different *S. cerevisiae* isolates on β -Glucan protein levels. Tukey's test showed a significant difference in protein levels in different *S. cerevisiae* isolates ($\alpha = 0.05 \geq \text{Sig}$). Protein levels in β -glucan *S. cerevisiae* SC-2 and SC-3 did not show any significant differences, whereas in β -glucan *S. cerevisiae* SC-1 and SAF showed significant differences (Table 3).

Table 3. Protein content in β -Glucans of four *S. cerevisiae* isolates

<i>S. cerevisiae</i>	SC-1	SC-2	SC-3	SAF
Protein Content (%)*	3.8039 ^c	0.6426 ^a	0.9782 ^a	2.3436 ^b

* average result of four replications

** average numbers followed by the same letter are not significantly different

The difference in protein content in crude β -glucan from each sample is thought to be due to the different types of isolates from different regions. Environmental factors also influence the formation of *S. cerevisiae* cell wall proteins such as growth media (nutrition), chemical factors (Fe^{3+} , Cu^{2+} , Mo^{2+} , Mn^{2+} , Zn^{2+} cations, and physical factors, namely pH, temperature, agitation (Sofyan *et al.* 2015).

Toxicity test of the extract of β -glucan *S. cerevisiae* strains with BSLT

Brine shrimp lethality test (BSLT) using larva of *A. salina* L. is a very useful monitory instrument for cytotoxicity of natural products such as yeast extracts. The biocompound from some extracts may contain active compounds that can be toxic to human health. Hence it was important to determine the extracts' LC_{50} values that showed positive activity using the BSLT in this study. The results of toxicity tests with this method showed a correlation with power cytotoxic anticancer compounds. This method is easy to be performed, inexpensive, fast, and accurate (Sarah *et al.* 2017).

In addition, the extract has the ability or biological activity when generating high mortality based on its LC_{50} value which concentration of the extract can show 50% lethal of the tested organisms, such as the larvae of *A. salina*. The lower value of the LC_{50} , the greater ability of its toxicity. An extract is considered highly toxic when the LC_{50} value of below 30 ppm, is considered toxic if it has a 30-1000 ppm LC_{50} and considered not toxic if LC_{50} values above 1000 ppm. Cytotoxicity assay has to be conducted to analyze its bioproperties before it is used for humans. The smaller value of LC_{50} the compounds are more toxic and more potential as an anticancer compound. Simple cytotoxicity assay was carried out using *A. salina* to obtain LC_{50} . If the LC_{50} value is more than 1000 ppm, it means that the materials tested were not toxic (Meyer *et al.* 1982). Table 4 showed the result of the percentage of mortality of *A. salina* L. against extract of β -glucan *S. cerevisiae* (SC-1, SC-2, SC-3, and SAF) by the BSLT method and LC_{50} results were shown in Table 4.

Table 4. Test calculations result of BSLT extract of β -glucan *S. cerevisiae* (SC-1, SC-2, SC-3, and SAF).

Samples	Dose (ppm)	Log D (x)	Dead	Life	Accumulated dead	Accumulated life	% mortality (y)	LC_{50} (ppm)
SC-1	400	5	27	3	92	3	96.84 %	331.1
	200	4	25	5	65	8	89.04 %	
	100	3	20	10	40	18	68.96 %	
	50	2	12	18	20	36	35.71 %	
	10	1	8	22	8	58	12.12 %	
SC-2	400	5	25	5	92	5	94.84 %	323.5
	200	4	24	6	67	11	85.89 %	
	100	3	20	10	43	21	67.18 %	
	50	2	13	17	23	38	37.70 %	
	10	1	10	20	10	58	14.70 %	
SC-3	400	5	26	4	92	4	95.83 %	354.8
	200	4	25	5	66	9	88.00 %	
	100	3	22	8	41	17	70.68 %	
	50	2	13	17	19	34	35.84 %	
	10	1	6	24	6	58	9.37 %	
SAF	400	5	30	0	106	0	100.00 %	114.8
	200	4	27	3	76	3	96.20 %	
	100	3	26	4	49	7	87.50 %	
	50	2	15	15	23	22	51.11 %	
	10	1	8	22	8	44	15.38 %	

*average result of three replications.

The most toxic extracts can be seen from the ability to cause test animals' death larger with increasingly lower concentrations. Based on the IC₅₀ value, the most toxic extract is an extract of β -glucan *S. cerevisiae* SAF with 114.8 ppm, followed by SC-2 extract was 323.5 ppm, SC-1 extract was 331.1 ppm, and SC-3 extract was 354.8 ppm.

Conclusion

Of the four strains, the highest glucose levels (monomer β -glucan) were obtained in *S. cerevisiae* SC-1 and the lowest protein content was *S. cerevisiae* SC-2. Characterization of the β -glucan showed that the functional groups contained an -OH group (alcohol), a -C-C-C-group (alkane), and an R-O-R (ether) group. The β -glucan of SAF extract had the highest toxicity properties compared to local ragi samples.

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Conflict of interest

The authors state no conflict of interest from this manuscript.

Author contributions

All authors have reviewed the final version of the manuscript and approved it for publication. IR, MA, and K designed the study, analyzed the data and wrote the paper. DL and YY performed research and collected the data. All authors are the main contributor to this manuscript.

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