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Stimulation of beta-glucan production from *Saccharomyces carlsbergensis* RU01 by tannin

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Abstract

This study aimed to evaluate the tannin-stimulated production of β-glucan from *Saccharomyces carlsbergensis* RU01 in molasses medium. Central composite design was used for the experiment design. The optimum concentrations of molasses (X_1), ammonium sulfate (X_2), and tannin (X_3), which produced the highest biomass, were determined. Optimization analysis revealed that the optimum concentration of substrates for biomass was 3% (w/v) of molasses, 0.1% (w/v) of ammonium sulfate and 0.1% (w/v) of tannin. The maximum biomass production was 2.64 g/L. Meanwhile, the experimental validation was 2.84±0.10 g/L, and the highest β-glucan production was 119.47 mg/g of dry cell weight. Carbohydrate content in yeast cell walls was detected by Congo red staining. The cell wall of yeast grown in the optimized medium with tannin showed higher intensity than that grown in yeast malt medium. These results suggested that tannin addition can enhance β-glucan production with a high β-1,3-glucans content in the cell wall of *S. carlsbergensis* RU01.

Keywords: β-Glucan, Molasses, Stimulation, *Saccharomyces carlsbergensis* RU01, Tannin

1. Introduction

Beta-glucan (β-glucan) is a polysaccharide comprising glucose molecules bound with β-1,3 or β-1,6-glycosidic bonds [1]. It is found in many organisms such as yeast, mushrooms, bacteria, algae, barley, and oat. β-Glucan at a higher concentration can form a gel and is insoluble in water. It has high apparent viscosity and water-holding, oil-binding, and emulsion-stabilizing capacities [2]. These benefits have led to their extensive use in food. For example, Worrasinchai et al. [3] used β-glucan from spent brewer's yeast, which is suitable for people who are concerned about their weight, instead of fat replacer in mayonnaise. β-Glucan properties are considered to play a key role in health promotion, such as in the enhancement of macrophage function and host resistance to many bacterial, viral, fungal, and parasitic infections [4,5]. The yeast cell wall contains about 55%-65% of β-glucan [1,6]. β-Glucan from yeast *Saccharomyces cerevisiae* is a well-known immune modulator with a strong positive influence on the human and animal immune system [7-9]. β-Glucan from yeast can also modulate cytokine secretion. Moreover, β-glucan from yeast decreases cholesterol levels more than that from mushrooms [10]. Yeast cell wall, which is approximately 70 nm thick, accounts for 20% of the cell's weight. Three main groups of polysaccharides form the yeast cell wall, namely, β-glucan (29%-64%), mannans (31%) and chitin (1%-2%), and proteins (13%) and lipids (9%) [11,12].

Additionally, the basal commercial medium is inadequate for industrial fermentation because of its high cost, so low-cost substrate is receiving increased research focus. Molasses is a by-product of sugar industries from

sugarcane and sugar beet. It is a cheap raw material for ethanol production from yeast [13]. The cell wall of yeast is flexible, and it could create new cell walls and adapt according to environmental changes [1]. Ene et al. [14] studied cell-wall elasticity and osmotic-stress resistance. They found that the cell wall of yeast cultured in glucose with 1.0 M NaCl has a longer diameter of the inner β-glucan and chitin layer.

Tannin is a polyphenolic molecule consisting of glucose linked to the 10 gallic acid group [15,16]. It is an antibacterial, antioxidant, and hazardous molecule in living organisms [17]. When yeast is disturbed with interfering chemicals such as tannin, yeast synthesizes thicker cell walls with higher β-glucan content to prevent itself from stress [18].

Therefore, the present work aimed to evaluate the β-glucan production of *S. carlsbergensis* RU01 stimulated by tannin in molasses medium. The influences of various concentrations of molasses, ammonium sulfate, and tannin in medium on yeast biomass production were studied using a statistical experimental design, specifically, central composite design (CCD).

2. Materials and methods

2.1 Microorganism

S. carlsbergensis RU01 was obtained from the Department of Biotechnology, Faculty of Science, Ramkhamhaeng University. Working stocks of culture were maintained at -20°C in 20% glycerol cell suspension. Yeast cells for immediate used were stored at 4°C.

2.2 Medium and culture conditions

A fermentation medium used for β-glucan production comprised molasses containing 20% water, 32% sucrose, 14% glucose, 16% fructose, 18% vitamins and minerals [19], ammonium sulfate, and tannin. A loopful of cells from a slant was transferred to 50 mL of yeast malt (YM) broth containing yeast extract 3 g/L, malt extract 3 g/L, peptone 5 g/L, and glucose 10 g/L and then incubated at 30°C and 150 Hz. The seed cultures used for inoculum for all cultivations were grown until the optical density became 1.0±0.1 at 600 nm and then added to 100 mL of the medium in a 250 mL Erlenmeyer flask at 1% (v/v). The culture cultivations were incubated at 30°C and 200 Hz for 36 h (stationary phase) on a rotary shaker. The culture medium was centrifuged at 6,000×g for 15 min at the end of fermentation for dry cell weight (DCW) analysis.

2.3 Experimental design

Response-surface methodology (RSM) is a useful method for the modeling and analysis of all industrial processes. The output is influenced by various input variables, which influence the output. CCD was used to formulate the medium that yielded the highest yeast biomass. CCD is also efficient, simple, economical, and time-saving compared with mixture experiments. The independent selected for the study were molasses (X_1), ammonium sulfate (X_2), and tannin (X_3). The goal was within the range for the maximum biomass. The operation conditions were optimized with Design-Expert 7.0.0 program. For the three factors, this trial was a 2^3 factorial design extended by six axial points coded $\alpha = \pm 1.682$ and six central points, resulting in a total of 20 experiments. The factors and levels corresponding to the CCD are shown in Table 1.

Table 1 Independent variables and levels in the central composite design.

Parameter (g/L)	Code	Levels				
		-α	-1	0	+1	+α
Molasses	X_1	0.32	1.00	2.00	3.00	3.68
Ammonium sulfate	X_2	-0.00	0.02	0.06	0.10	0.13
Tannic acid	X_3	-0.00	0.02	0.06	0.10	0.13

The response variable was fitted by the model expressed as follows:

$$y = \beta_0 + \beta_i \sum_{i=1}^{3} x_i + \beta_{ij} \sum_{i=1}^{2} \sum_{j=i+1}^{3} x_i x_j + \beta_{ii} \sum_{i=1}^{3} x_i^2 \quad (1)$$

where y is the cell biomass, β_0, β_i, β_{ij}, and β_{ii} are the intercept term, linear coefficient, interactive coefficient, and quadratic coefficient, respectively; they are coded as independent variables. Statistical analysis was performed using Design-Expert software (version 7.0).

2.4 Production of β-glucan on optimized molasses medium with tannin supplementation

The comparison of β-glucan production from molasses medium containing 3% (w/v) molasses, 0.1% (w/v) ammonium sulfate supplemented with 0.1% (w/v) tannin (MT) and 96.8% (v/v) water, and M medium without tannin were investigated. The operating conditions were studied in a shake flask within 36 h at pH 4.7, 30°C, and 200 Hz.

2.5 Analytical procedures

DCW was measured after drying the wet cells at 105°C to constant weight [20]. At specified times during fermentation, 1 mL of cell suspension was withdrawn. The samples were centrifuged at 6,000×g at room temperature for 15 min. The supernatant was subjected to the 3,5-Dinitro-2-hydroxybenzoic acid (DNS) method to determine reducing-sugar concentrations [21].

The cells were filtered and washed thrice with 0.85% (w/v) NaCl until the supernatant became clear. The mixture was centrifuged at 6,000×g at room temperature for 15 min. The supernatant was discarded, and the sediments were suspended in distilled water. The cells were collected and stored at 4°C until use. β-Glucan was extracted by hot-water and high-pressure method. The cell pellets were heated at 121°C and 15 psi for 15 min, cooled down to room temperature, and centrifuged at 6,000×g at room temperature for 15 min to separate the residual autolyzed cell [22]. The autolyzed yeast cells were dried and stored at 4°C. The total glucans were extracted by adding 0.1 mL of 12 M HCl. The mixture was stirred with a vortex mixer and left at 30°C for 1 h. The solution was diluted to 2 M HCl with water and incubated at 100°C in a boiling water bath for 2 h. Afterwards, 1.0 M NaOH was added to the solution for neutralization. The sediment was removed by centrifugation at 6,000×g and room temperature for 15 min. The amount of β-glucan in the cell-wall fractions was then determined with the enzymatic reaction by using the commercial assay "Enzymatic Yeast β-Glucan Assay Kit" (Megazyme Int., Bray, Ireland).

The specific interaction with β-1,3-D-glucans in yeast cell walls was detected by Congo red staining [23,24]. Cells were centrifuged at 6,000×g for 15 min and then washed thrice with 0.85% (w/v) NaCl. Yeast cells were smeared on the slide, air dried, and fixed onto the slide. A few drops of 0.1% (w/v) Congo red were added onto the smeared slide. It was stained for 15 min, rinsed with water, and air dried. The intensity of the stained cells was investigated with a microscope.

3. Results

3.1 Optimization of fermentation medium by CCD

The optimal concentrations of molasses, ammonium sulfate, and tannin were determined by RSM. The experiment was performed using three independent variables each at two levels as follows: molasses (1% and 3% w/v), ammonium sulfate (0.02% and 0.10% w/v), and tannin (0.02% and 0.10% w/v). A total of 20 sets of experiments corresponding to CCD and biomass yield are shown in Table 2.

The experimental data were made to fit the response function by regression analysis. Equation (2) was derived to represent yeast biomass production (g/L). The independent variables were tested as a function of Y (only significant parameters are shown).

$$Y = -0.55 + 0.46X_1 + 14.68X_2 + 4.06X_3 \quad (2)$$

The regression results and the summary of ANOVA for the selected quadratic equations are shown in Tables 3 and 4, respectively. ANOVA results demonstrated that the models were significant, as evidenced by the low p value ($p<0.05$). According to lack of fit, the model of yeast biomass production was insignificant ($p=0.6788$). The coefficient of determination (R^2) of the biomass production model was 0.8746, indicating that 87.46% of the variation was explained by the model. Moreover, the predicted R^2 of 0.5143 was not as close to the adjusted R^2 of 0.7617 as may normally be expected.

Table 2 Experimental design and results of biomass production.

Experiment	Parameter			Biomass (g/L)
	Molasses (X_1)	Ammonium sulfate (X_2)	Tannin (X_3)	
1	1.00	0.10	0.10	0.97
2	2.00	-0.00	0.60	0.55
3	2.00	0.06	-0.00	0.83
4	2.00	0.13	0.06	1.50
5	3.00	0.10	0.10	2.74
6	1.00	0.02	0.02	0.52
7	2.00	0.06	0.06	1.38
8	2.00	0.06	0.06	1.95
9	2.00	0.06	0.06	1.18
10	1.00	0.10	0.02	0.95
11	3.00	0.02	0.10	2.19
12	2.00	0.06	0.06	2.07
13	3.00	0.10	0.02	2.14
14	3.00	0.02	0.02	1.45
15	2.00	0.06	0.06	1.38
16	1.00	0.02	0.10	1.07
17	0.32	0.06	0.06	0.42
18	2.00	0.06	0.06	1.52
19	3.68	0.06	0.06	2.10
20	2.00	0.06	0.13	2.20

According to the coefficients (Table 3), the positive sign in front of the terms indicated positive on cell biomass. From equation (2), the highest positive value of the molasses (X_1) was 0.46.

Table 3 Regression analysis of biomass.

Source	Coef	SE Coef	F value	*p* value
Intercept	-0.55	0.13	7.75	0.0018
Molasses (X_1)	0.46	0.086	44.03	<0.0001
Ammonium sulfate (X_2)	16.48	0.086	7.20	0.0230
Tannin (X_3)	4.06	0.086	12.73	0.0051
X_1X_2	3.03	0.11	1.01	0.3377
X_1X_3	2.57	0.11	0.73	0.4142
X_2X_3	-59.56	0.11	0.55	0.4756
X_1^2	-0.06	0.084	0.49	0.5987
X_2^2	-101.11	0.084	2.85	0.1221
X_3^2	22.09	0.084	0.14	0.7198

Table 4 ANOVA for the selected model of cell dry-weight productions.

Source	Degree of Freedom	Sum of Square	Mean of Square	F value	*p* value
Regression	9	0.79	0.12		
Lack of Fit	5	0.46	0.08	0.65	0.6788
Pure error	4	0.62	0.12		
Total	19				

The result revealed that molasses was the dominant factor affecting biomass production (p<0.05). Additionally, tannin (X_3) also significantly affected β-glucan production because of the positive coefficient of X_3 (4.06). The effects of molasses, ammonium sulfate, and tannin on biomass production are shown in Figures 1-3. The maximum biomass production was achieved when the initial concentrations of molasses, ammonium sulfate, and tannin were 3% (w/v), 0.1% (w/v), and 0.1% (w/v), respectively. The maximum value of cell mass predicted using RSM was 2.64 g/L after 36 h of cultivation. The experimental validation of the model produced 2.84±0.10 g/L of biomass by using a shake flask was studied. The experimental value well agreed with the predicted value. Therefore, model equation (2) could be used to predict biomass production.

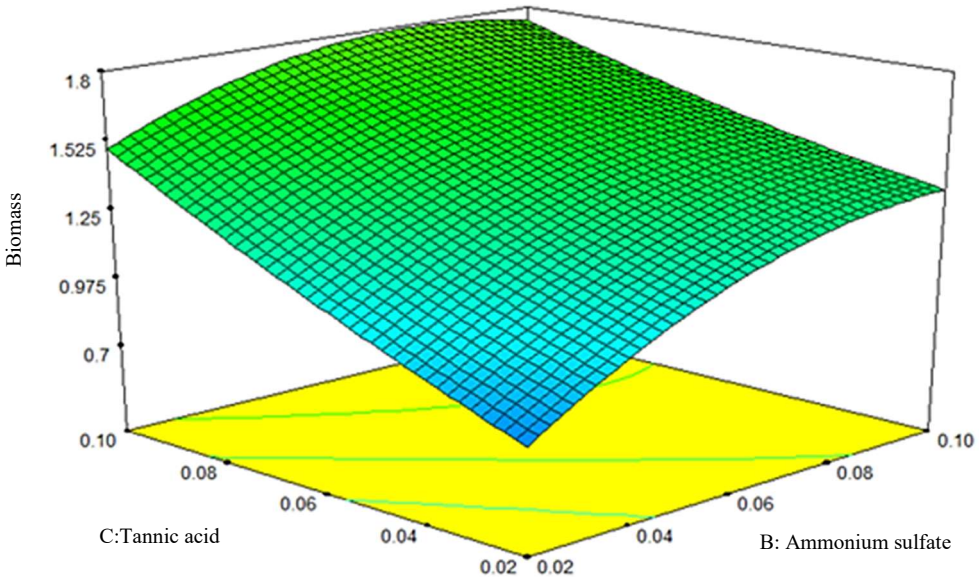

Figure 1 Response-surface plot of the effect of ammonium sulfate (X_2) and tannin (X_3) on biomass production at 3% molasses.

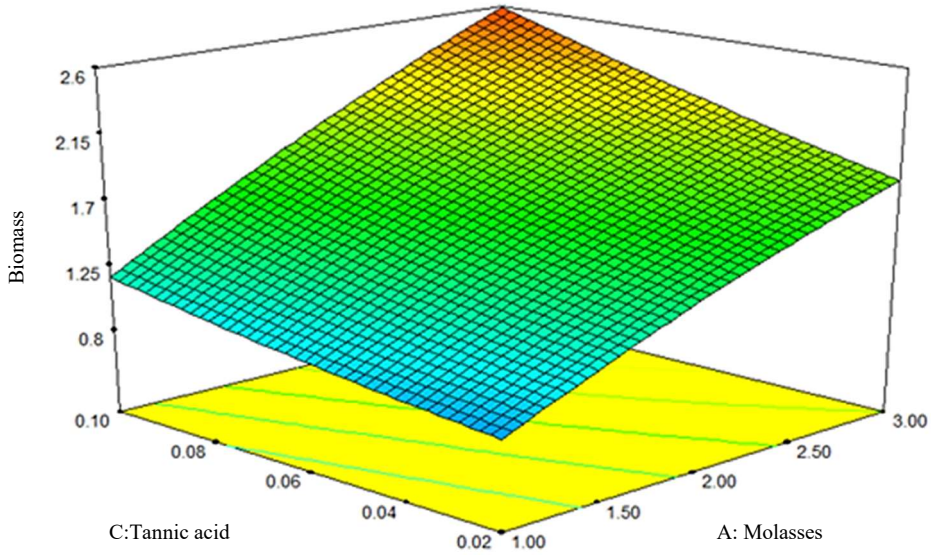

Figure 2 Response-surface plot of the effect of molasses (X_1) and tannin (X_3) on biomass production at 0.1% ammonium sulfate.

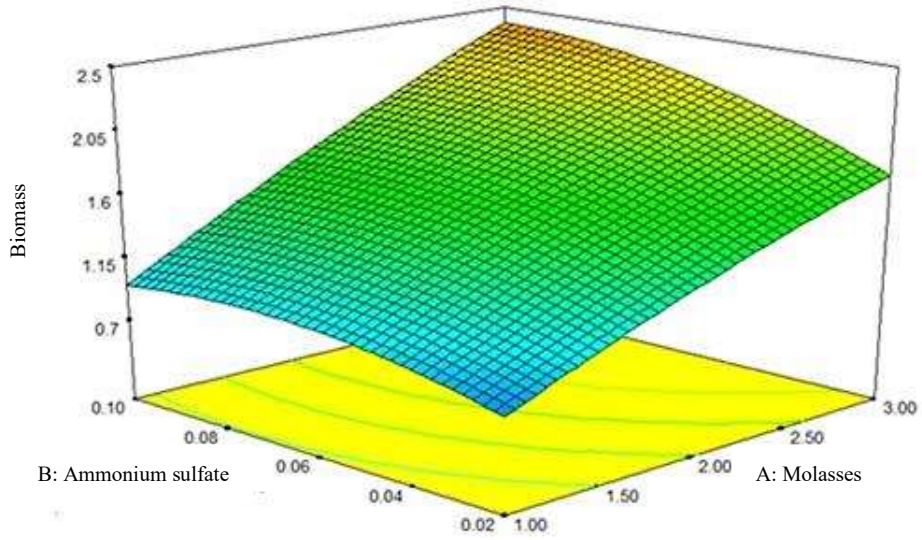

Figure 3 Response-surface plot of the effect of molasses (X_1) and ammonium sulfate (X_2) on biomass production at 0.1% tannin.

3.2 Production of β-glucan from molasses medium and molasses medium with tannin supplementation

Yeast cells *S. carlsbergensis* RU01 were stained with Congo red after incubation for 36 h to detect specific interaction with β-1,3-D-glucans in yeast cell wall. YM medium was used to prove the morphology of the normal yeast cell, whereas M medium supplemented with tannin was used to study the effect on yeast cell morphology. The result in Figure 4 showed that yeast cells cultured in MT (Figure 4B) had more intense red color than yeast cells cultured in YM medium (Figure 4A). The result showed the effect of molasses and Tannic to change the morphology of yeast cells.

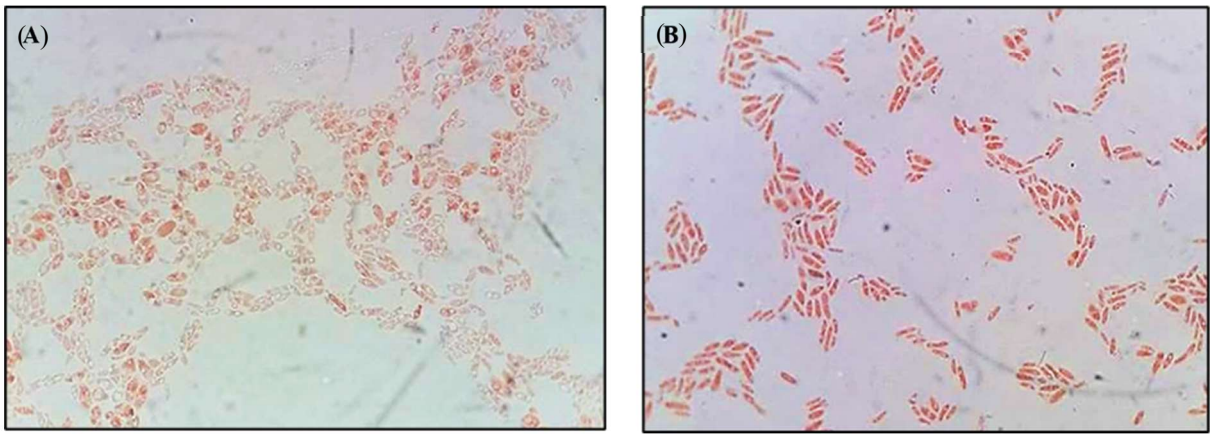

Figure 4 Congo red staining of *S. carlsbergensis* RU01 after 36 h fermentation: (A) YM medium and (B) MT medium.

β-Glucan production from molasses medium supplemented with 0.1% (w/v) tannin (MT) was compared with that from molasses medium without tannin (M). The biomass concentration, concentration of β-glucan, and reducing sugar were determined, as shown in Figures 5-7. A biomass concentration of 2.53 g/L was obtained from M medium after 36 h of cultivation, whereas the biomass concentration obtained from MT medium was 2.84 g/L (Figure 5). The concentration of β-glucan in MT medium was higher than that in M medium (Figure 6). The concentration of β-glucan at 36 h obtained from M and MT media were 86.16 and 119.47 mg/g DCW, respectively. At 12 h, the reducing-sugar utilization in MT medium was slightly higher than that in M medium and did not differ after 24 h (Figure 7).

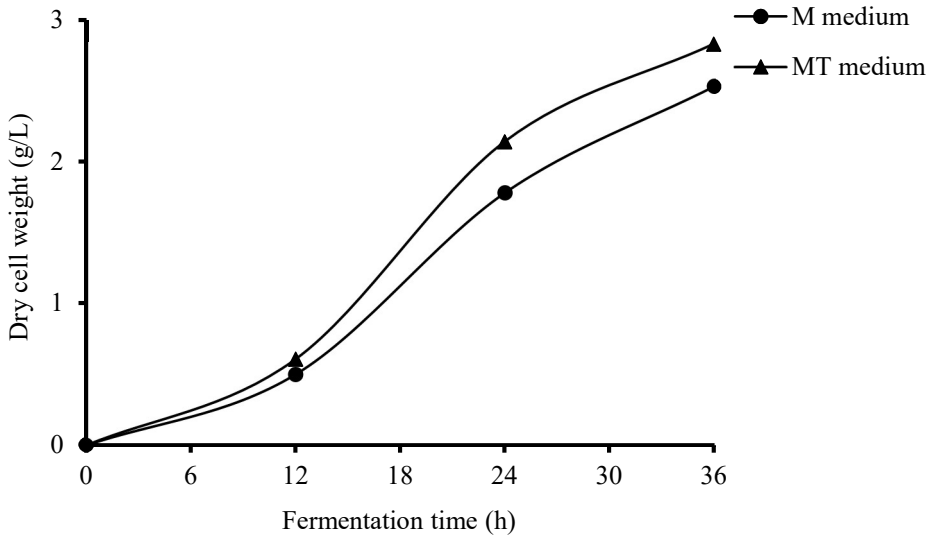

Figure 5 Dry cell-weight production of *S. carlsbergensis* RU01.

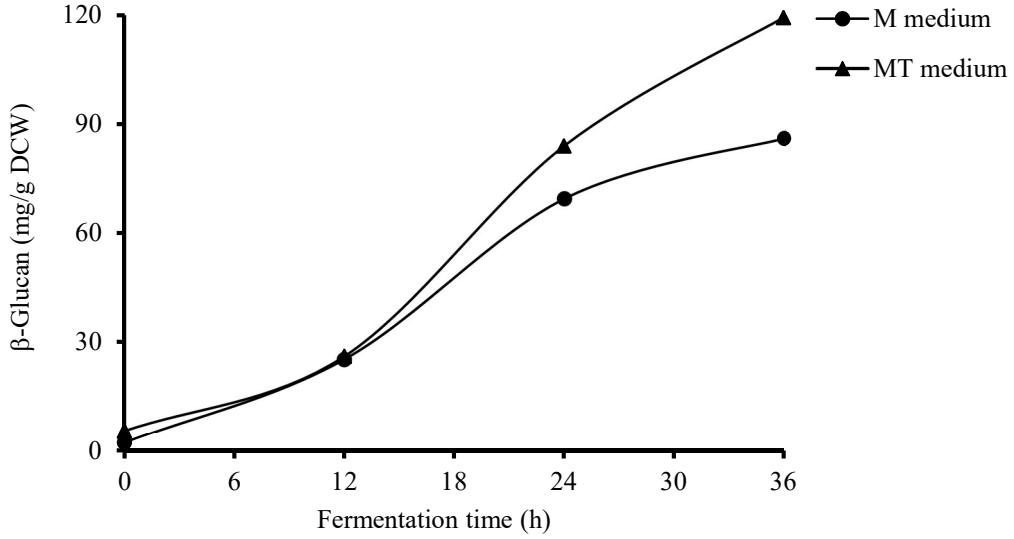

Figure 6 β-Glucan production of *S. carlsbergensis* RU01.

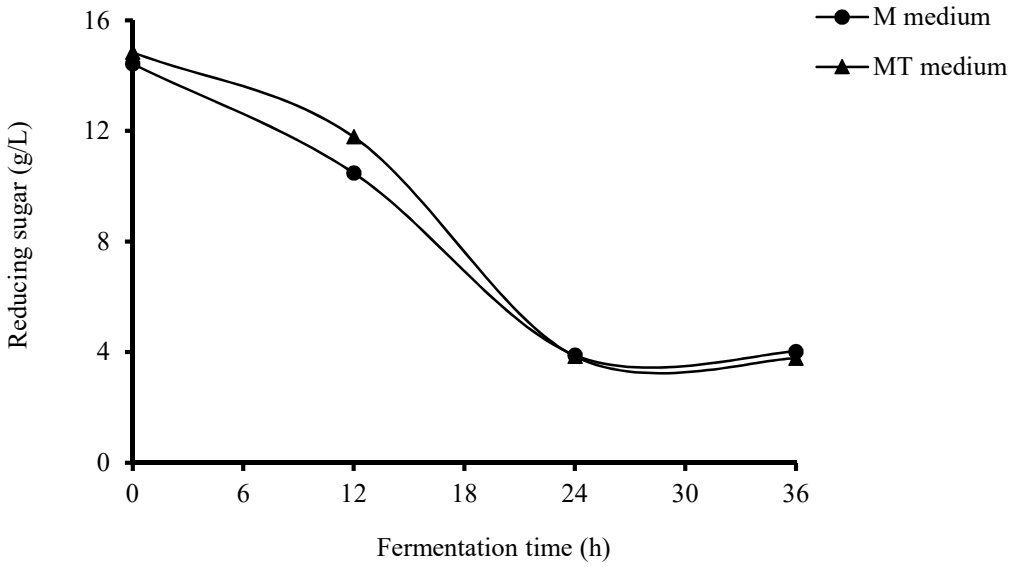

Figure 7 Reducing-sugar utilization of *S. carlsbergensis* RU01.

4. Discussion

β-Glucan is a polymer with multiple functional and bioactive properties related to hypertension, immune, and obesity. It can be produced from yeasts, fungi, and cereal. β-Glucan from *S. carlsbergensis* RU01 is an optional source for revealing its health-promoting properties [25].

The concentrations of carbon and nitrogen or C/N ratio are important factors affecting metabolite production from microorganisms. Molasses are used as a substrate for yeast growth. It contains 65%-75% of sugars, primarily sucrose. Sucrose is hydrolyzed by yeast into glucose and fructose, which are transported to and incorporated into yeast metabolism as carbon sources. However, high concentrations of molasses affect osmotic pressure in cells; consequently, yeast cell growth decreased. Ammonium sulfate was added to molasses substrate as a nitrogen source for the growth of yeast cells. The growth of *S. carlsbergensis* RU01 was due to the presence of sucrose, which is the major carbon source in molasses [26]. This study predicted the optimal concentration and yield of β-glucan by RSM using CCD. We found that M medium supplemented with 0.1% (w/v) tannin gave the highest DCW at 2.84 g/L, whereas M medium without tannin had a DCW of 2.53 g/L. In a previous report, the cell walls of yeast are contained an insoluble 1, 3-carbon backbone with elongated 1,6-

carbon branches [27]. The effects of β-glucan solubility and physiological impact depend on a different molecular backbone, level of branching, and molecular weight. In the current study, β-glucan in the yeast cell wall was examined. The hot-water and high-pressure methods were used to extract overall glucan in yeast cells [22]. Moreover, tannin is a polyphenolic molecule comprising glucose linked to the 10-gallic acid group. It is antibacterial activity against the living organism. In this research, tannin at low concentrations was found to stimulate yeast growth. The yeast cells must be able to protect and maintain the critical features of the internal homeostasis from the external conditions. They prevent themselves from stress by synthesizing thicker cell walls with higher carbohydrate content [18]. As a result, tannin affected β-glucan production of *S. carlsbergensis* RU01. The concentration of β-glucan obtained from MT was higher than that from the control by about 1.39-folds. Yeast also increased the composition of carbohydrates such as mannan and β-glucan in cell wall, which protected them from the tannin in the medium [28]. The hydroxyl group of tannin can interact with structures and biopolymers such as some proteins, digestive enzymes, and chemical properties [29]. The result of Congo red staining showed that *S. carlsbergensis* RU01 obtained from MT medium had a more intense red color than that from YM medium without tannin. The morphology of yeast cells was changed. Moreover, Congo red was used to investigate the specific interaction with β-1, 3-D-glucans in the cell wall of *S. carlsbergensis* RU01. This finding implied that yeast disturbed by tannin could synthesize thicker cell walls with higher β-glucan content to protect itself from the stress [18,23,24]. Thus, tannin could stimulate β-glucan production in yeast cell walls.

5. Conclusion

Molasses and ammonium sulfate could serve as substrate for *S. carlsbergensis* RU01 growth, and tannin could stimulate increased β-glucan production. The optimization of medium from RSM and CCD indicated that the maximum biomass concentration of *S. carlsbergensis* RU01 was 2.64 g/L, while 2.84 g/L for experimental validation. The experimental value well agreed with the predicted one. The highest β-glucan concentration of 119.47 mg/g DCW was observed in MT. Cell walls in MT had more intense color with Congo red than those in YM medium. Overall, this study showed that tannin can stimulate β-glucan production in the yeast cell wall of *S. carlsbergensis* RU01 by promoting β-1,3 glucan synthesis.

6. Acknowledgements

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