Effect of ammonium chloride and disodium hydrogen phosphate with molasses as a substrate for Saccharomyces cerevisiae in the production of β-glucan

Ririn Puspadewi¹, Putranti Adirestuti², Jesiana Agustine Arifin³

¹University of Jenderal Achmad Yani, Jl. Terusan Jenderal Sudirman, Cimahi 40533, West Java, Indonesia

Abstract. Glucans are a type of polysaccharide found in the cell walls of plants, bacteria and yeasts. β-glucans have various biological activities as antitumour, antioxidant, anticholesterol, antiageing and immune system booster known as an immunomodulator. One of the yeasts that can produce β-glucans is Saccharomyces cerevisiae because most of the cell wall structures contain mannoproteins, β-1,3-glucan, and β-1,6-glucan, which function to strengthen cell structure and as a portion reserve food. This study aims to see the effect of adding ammonium chloride and disodium chloride with molasses as a natural substrate for the growth of Saccharomyces cerevisiae in the production of β-glucose. The research stages began with making a growth curve, fermentation process, separation of β-glucan, and qualitative and quantitative analysis. Qualitative analysis showed positive results for β-glucan. In quantitative analysis, the highest amount of cell biomass obtained in Formula 4 = (0.0840 ± 0.0467) %.

1. Introduction

Glucans are a type of polysaccharide with a monomer in D-glucose, which is found in many plants, bacterial and yeast cell walls (1). Polysaccharides, which are composed of monosaccharide monomers, are the primary metabolites in plants. Polysaccharides found in nature can be produced by all types of plants and most microorganisms. Microorganisms that can produce polysaccharides include bacteria, fungi, moulds and algae (2).

β-glucan is a primary metabolite compound that can be isolated from plants and microorganisms. β-glucan is a glucose homopolymer bound by β- (1,3) and β- (1,6) -glucoside bonds found in many cell walls. β-glucan is the main component of polysaccharides found in cell walls (3). Bacteria, mould and yeast can be used to produce β-glucans. Bacteria produce β-glucans through secondary metabolites, while β-glucans in yeast and fungi are present in their cell walls, where their bonds and structures are different. This results in the β-glucan produced having other characteristics. Various extraction and purification techniques for β-glucan are hot extractions, solvent extraction, enzymatic extraction, and alkaline extraction (4).

One of the yeasts that have the potential to produce β-glucans is Saccharomyces cerevisiae because most of the cell wall structures contain most of the mannoproteins, and contain lipids, chitin and
polysaccharides of the types β-1,3-glucan and β-1,6-glucan which function to strengthen the structure cells and as food reserves (5). Besides that, Saccharomyces cerevisiae is a yeast that is easy to obtain and safe to use. A study conducted in 2019 found that the mass of β-glucan produced from Saccharomyces cerevisiae was more significant than other β-glucan-producing microorganisms Xanthomonas campestris and Bacillus natto (2).

A study conducted by Tontowi et al. produced more β-glucan from Saccharomyces cerevisiae; 2% peptone was added, glutamic acid 0.5%, urea 0.2% and diammonium hydrogen phosphate (DHAP) 0.2%.

Phosphate (PO$_{4}^{3-}$) is required as a component of ATP, nucleic acids, and coenzymes such as NAD, NADP, and flavin. Many metabolites, lipids (phospholipids, lipid A), cell wall components (teichoic acid), some capsule polysaccharides, and some proteins have phosphorylated structures. Phosphate is always assimilated as free organic phosphate (P1) (6).

2. Material and Methods

2.1. Making of a Growth Curve

A total of 30 mL of PDB medium and a starter of Saccharomyces cerevisiae, which had been rejuvenated, were put into each of the nine sterilized container bottles. 10% of the Saccharomyces cerevisiae starters were put into each of the nine container bottles containing PDB media. One container bottle was used as a control (without incubation). The container bottle is closed using a cotton plug and covered with aluminum foil, then incubated at 30 °C. Observations were made every 12 hours for four days by weighing the dry cell weight. The weight of dry cells that weighed illustrates the number of yeast cell populations at each growth phase.

The dry cell weight was obtained by filtering the yeast cells using filter paper. Then the yeast cells were dried in an oven at 105 °C to get a fixed weight. Growth curves were made by graphing the relationship between dry cell weight (g) and time (days).

2.2. Preparation of Molasses

2.2.1 Molasses Purification. The molasses is first dissolved with distilled water and then added with alum as a purifier and shaken for 16 minutes. The molasses solution was heated 90 °C while stir and added activated carbon to the molasses filtrate while shaking. The activated carbon is allowed to settle and is filtered again and then sterilized by autoclaving at 121 °C for 15 minutes. The molasses sample to be analyzed diluted with distilled water (7).

2.2.2 Determination of Glucose Content in Molasses.

This determination is carried out to determine the glucose level in the molasses, which will be used as a substitute for glucose used in the growth medium. The glucose level is used to calculate the amount of glucose equivalent molasses that will be used in various fermentation media of S. cerevisiae. The assay was carried out by using the Phenol-Sulfate Spectrophotometric method UV-Visible at λ 490 nm (8).

2.3. Fermentation process

The variations of the formula are as follows

| Compound                        | Formula         |
|---------------------------------|-----------------|
| Glucose equivalent molasses     | 1 4%            |
| Peptone broth                   | 2 2%            |
| Ammonium chloride               | 3 0,2%          |
| Sodium hydrogen phosphate       | 4 0,02%         |
| Distilled water                 | added 150 mL    |

Table 1. Variation of fermentation media
The fermentation process is carried out by adding 10% of the starter suspension *Saccharomyces cerevisiae* in 150 mL of fermentation medium. The mixture was incubated at 30 °C and fermented for a specific time. The sampling was carried out four times.

A 30 mL culture sample, which is expected to contain β-glucan, was centrifuged at 7000 rpm for 20 minutes. The supernatant was discarded, 5 mL of 2% NaOH was added to the cell biomass pellets, then heated for 5 hours at 90 °C. The cell biomass suspension was again centrifuged at a speed of 5000 rpm for 10 minutes. To the supernatant obtained, 2 M CH3COOH was added dropwise until the pH of the solution was about 6.8-7, after which it was precipitated with three volumes of ethanol. The precipitate formed was separated by centrifugation at a speed of 5000 rpm for 10 minutes. The separated pellets were dried in an oven at 45 °C for ± two days then weighed as the weight of crude β-glucan.

### 2.4 Analysis of glucan compounds

β-glucan is a polysaccharide, so β-glucan must be hydrolyzed first before being tested using the reaction for monosaccharide compounds.

Polysaccharide hydrolysis was carried out by adding 2% sulfuric acid to the sample solution and then heating it in boiling water for 1 hour. The results of hydrolysis were then analyzed using the Molisch and Fehling test.

#### 2.4.1 Qualitative Analysis of Molisch’s Test

The sample was prepared in a test tube then added five drops of Molisch’s solution, then the test tube was tilted, and 2 mL of sulfuric acid was added slowly through the test tube wall. Compounds that have glycosidic bonds will produce a complex purple ring.

#### 2.4.2 Qualitative Analysis of Fehling’s Test

A sample of ± 1 mL was inserted into the test tube and then added Fehling A and Fehling B equally. If there is a reducing sugar, the results obtained from the Fehling test are the presence of green, yellow-orange or brick-red sediment.

#### 2.4.3 Qualitative Analysis with FTIR

± 20 mg of dry fermented β-glucan was prepared mixed with KBr powder then crushed on lumping agate until well blended and taken a little, then put into a disc and pressed with a press holder. After the thin film was formed, the KBr disc was inserted into the KBr disc holder and the sample spectrum was recorded in the range 400-4000 cm-1. The results are compared to the standard Barley spectrum (9).

#### 2.4.4 Quantitative Analysis of β-Glucan Compounds with UV-Vis Spectrophotometry

The β-glucan level is determined as glucose by breaking down (hydrolysis) β-glucan into D-glucose monomer, then the glucose level is determined.

Dried β-glucan derived from the fermentation process is weighed carefully, then each added 4 mL of 1 N NaOH until dissolved. The volume of the test solution used is adjusted so that the absorption on the UV-Visible spectrophotometer is always between 0.2-0.8; the dilution factor (Fp) of the control solution and the test solution may differ from one another. Each test solution is taken specific volume and added with distilled water so that the total volume is 1.0 mL. The dilution results are added with 0.5 mL of 5% phenol, shaken homogeneously, plus 2.5 mL of concentrated H2SO4, left to stand for 10 minutes. The absorption of each reaction product was measured using a UV-Visible spectrophotometer at λ 490 nm. The absorption measurement results are extrapolated to the standard glucose regression line equation to obtain the glucose levels in the measured test solution. We can determine the dry weight of crude β-glucan. We can be determined, the glucose equivalent β-glucan content in each test solution (8).
3. Results and Discussion

The growth curve of *Saccharomyces cerevisiae* isolate was made using the indirect method, namely by measuring the dry cell weight. This method is used because yeast is difficult to suspend and cannot be homogeneous. The growth curve was made for four days with a sampling time of every 12 hours.

![Growth curve of *Saccharomyces cerevisiae*](image)

**Figure 1. Growth curve of *Saccharomyces cerevisiae***

Based on Figure 1, it can be seen that the yeast growth of *Saccharomyces cerevisiae* experiences a lag/adaptation phase for up to 36 hours. In the lag phase, *Saccharomyces cerevisiae* cells are introduced to their new growth medium, and this is the time required for them to adapt (10).

The next phase is the logarithmic/exponential phase. During this phase, the cells are in an equilibrium state. Saccharomyces cerevisiae cells are newly synthesized, and their mass increases exponentially. This continues until one or more nutrients in the medium have been depleted, or toxic metabolite products are collected and inhibit growth (6,10). In Saccharomyces cerevisiae, the log phase occurred at 36 to 60 hours.

After the log phase, a stationary phase occurs. This stationary phase in *Saccharomyces cerevisiae* occurred at 60 to 82 hours. The stationary phase is the phase in which cells have stopped growing. Growth stops due to the depletion of nutrients or the accumulation of toxic products. In this phase, cell loss occurs through death, which is offset by forming new cells' through cell growth and division (6).

The purpose of making a growth curve is to estimate the right time to obtain β-glucan with maximum results. From the growth curve of Saccharomyces cerevisiae, the time for sampling was determined at 24, 60, 72 and 90 hours.

β-glucan is a polysaccharide which, in this study, was obtained from *Saccharomyces cerevisiae*. These polysaccharides need to be hydrolyzed to become monosaccharides and be identified in qualitative testing. The method for hydrolysis used is acid hydrolysis. Acid hydrolysis is also known as non-enzymatic hydrolysis. This hydrolysis method uses HCl as a strong acid as a catalyst. A high temperature of 140-160 °C is required in acid hydrolysis. This method has several drawbacks, including the need for equipment resistant to corrosion, which causes degradation of carbohydrates and recombination of degradation products that can affect colour and taste (11).
Table 2. Result of the Molisch test

| Sample                  | Molisch test            | Result |
|-------------------------|-------------------------|--------|
| Starch 1%               | No purple ring was perfomed | Negative |
| Hydrolized of starch 1% | Purple ring was perfomed  | Positive |
| Formula 1               | Purple ring was perfomed  | Positive |
| Formula 2               | Purple ring was perfomed  | Positive |
| Formula 3               | Purple ring was perfomed  | Positive |
| Formula 4               | Purple ring was perfomed  | Positive |

Table 3. Result of the Fehling test

| Sample                  | Fehling test            | Result |
|-------------------------|-------------------------|--------|
| Starch 1%               | No rust sediment was perfomed | Negative |
| Hydrolized of starch 1% | rust sediment was perfomed | Positive |
| Formula 1               | rust sediment was perfomed | Positive |
| Formula 2               | rust sediment was perfomed | Positive |
| Formula 3               | rust sediment was perfomed | Positive |
| Formula 4               | rust sediment was perfomed | Positive |

The results obtained from the separation are not pure β-glucan, so there are still other mixtures such as mannoproteins, which are components of Saccharomyces cerevisiae's cell wall, which are still bound to β-glucans. The results of the analysis by FTIR, there are still peaks indicating the presence of these mannoproteins. The infrared spectrum is divided into two regions, namely the functional group area (4000-1300 cm\(^{-1}\)) and the fingerprint area (1300-650 cm\(^{-1}\)). In the analysis result spectrum, it was found that the O-H strain was at wave number 3400-3200 cm\(^{-1}\) with medium intensity. A wavenumber 3000-2850 cm\(^{-1}\), a stretch is found, which indicates the presence of a CH group in the compound and at wave number 1651 cm\(^{-1}\) there is acylamino which indicates the presence of mannoproteins. Then at wave number 1300-1000 cm\(^{-1}\), there is a peak indicating the presence of a CO group is thought to be the ether group of the glycosidic bonds on the polysaccharide, and among them, there is a wave number at 1130 cm\(^{-1}\), which indicates the characteristic absorption peak of the piranoid ring. The spectrum results, it can be predicted that the compound is a polysaccharide, which is expected to be β-glucan, and there is a mixture of mannoproteins (12,13).

Figure 2. Infrared spectrum of crude β-glucan

The results obtained from the fermentation process in Formula 1 with a substrate consisting of 4% molasses, 2% peptone broth and distilled water up to 150 mL showed that the maximum β-glucan content was obtained at the 60th hour, namely 0.0673% with the amount of β of 0.3586 mg of cell biomass weight is 543.60 mg (Fig.3 and 4).
Formula 2 with a substrate consisting of 4% molasses, 2% peptone broth, 0.2% NH4Cl and distilled water up to 150 mL, the maximum β-glucan content at the 96th hour is 0.0647% with the amount of β-glucan, and cell biomass is 0.3748 mg and 589.20 mg.

Formula 3 with a substrate with a composition of 4% molasses, 2% peptone broth, 0.02% Na2HPO4 and distilled water up to 150 mL produces maximum β-glucan at the 72nd hour with levels of 0.0432% and a weight of 0.2754 mg of cell biomass weight 667.10 mg.

Formula 4 consists of a substrate containing 4% molasses, 2% peptone broth, 0.2% NH4Cl, 0.02% Na2HPO4 and distilled water up to 150 mL showing the maximum β-glucan content at the 72nd hour, namely 0.0840% with the amount of 0.4394 mg of cell biomass is 523.25 mg.

The maximum weight of β-glucan is found in Formula 4 (Fig.3 and 4), namely 0.4394 ± 0.2445 mg with a β-glucan level of 0.0840 ± 0.0467 mg after fermentation for 72 hours. The addition of nitrogen to the substrate causes the growth of Saccharomyces cerevisiae to be longer. When the peptone broth runs out during growth, additional NH4Cl can be used as a reserve for protein formation during the synthesis of Saccharomyces cerevisiae cell walls (14).

Microorganisms can use nitrogen from both organic and inorganic sources. Ammonia and ammonia salts (such as ammonium sulphate or ammonium nitrate) are sources of inorganic nitrogen salts that can provide an acidic or alkaline environment, type of salt. When ammonia ions are consumed, ammonium sulphate will produce an acidic atmosphere, while ammonium nitrate will provide an alkaline atmosphere. Amino acids, protein or urea serve as a source of organic nitrogen. Microorganisms are capable of overgrowing rapidly in organic nitrogen, and some require absolute nitrogen requirements. Using pure amino acids is very expensive. Therefore precursors are used, such as methionine and threonine are taken from soybean hydrolyzate(15).

In the substrate, there is the addition of inorganic nitrogen, namely ammonium chloride (NH4Cl). Nitrogen is used for protein synthesis in cells. The protein that is formed can be an enzyme that plays a role in the formation of β-glucans. The decrease in the substrate's protein content indicated that the N source in the media was utilized by S. cerevisiae. NH4Cl can be used as a source of additional nitrogen to increase the amount of S. cerevisiae that grows during the fermentation process after running out of peptone broth in the substrate (16,17).

In addition to nitrogen, a phosphate source is also added to the substrate, namely disodium hydrogen phosphate (Na2HPO4). The presence of phosphate in the substrate will make S. cerevisiae cells start to grow and work much faster. S. cerevisiae cells take up phosphate as ATP and convert it to the phosphate's polymerized form, which is often found in cell mitochondria (18).
Figure 3. Weigh of β-glucan

![Graph showing weight of β-glucan over time](image)

Figure 4. Levels of β-glucan

![Graph showing levels of β-glucan over time](image)

4. Conclusion

The addition of ammonium chloride and Disodium hydrogen phosphate affected beta-glucan levels after 72 hours of fermentation in Formula 4 = (0.0840 ± 0.0467) %.

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