

Inulinase Producing Yeast Isolated from Kepok Banana Peels (*Musa acuminata x balbisiana*)

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Abstract. Inulinase enzyme (EC 3.2.1.80) is an enzyme capable of hydrolyzing inulin and is used as a catalyst in the production of High Fructose Syrup (HFS) and fructooligosaccharides (FOS). Inulinase enzymes can be produced by yeasts found in waste such as kepok banana peels (*Musa acuminata x balbisiana*). The aims of this study were to obtain the inulinase enzyme-producing yeast from kepok banana peels and to determine the effect of commercial inulin concentration and incubation time on enzyme activity. This research was conducted experimentally using a factorial completely randomized design with factors being inulin concentration of 1% (K₁), 3% (K₂) and 5% (K₃) and incubation time of 6th (W₁), 12th (W₂), 18th (W₃), and 24th hours (W₄). The results showed that there were 4 isolates of inulinolytic yeasts obtained from kepok banana peels, namely YP1, YP2, YP4, and YP6. However, the most potential isolate to be used in inulinase production was YP2 isolate because it had the highest inulinolytic ratio value of 1.96. The results of inulinase activity showed that only inulin substrate with concentration of 5% (K₃) had a significant or significant effect on inulinase production.

Key words: inulinase, indigenous yeast, *Musa acuminata x balbisiana*.

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INTRODUCTION

Inulinase enzyme (EC 3.2.1.80) is a hydrolytic enzyme belonging to the 32nd group of glycoside hydrolases which can hydrolyze inulin to obtain fructose (Singh and Singh, 2017). Fructose production generally uses starch-containing materials and requires 3 enzymatic steps, namely α -amylase, glucoamylase, and glucose isomerase. However, the three stages only obtained a percentage of fructose reaching 45%, while the use of the inulinase enzyme as a catalyst in producing inulin-based fructose can obtain a fructose percentage of 90-95% (Rawat *et al.*, 2015). This is an advantage that fructose production with the inulinase enzyme is able to produce fructose with a 2-fold greater percentage compared to the 3 enzymatic steps.

Inulinase enzymes are widely needed as catalysts in producing food ingredients such as High Fructose Syrup (HFS) which is a natural calorie sweetener and fructooligosaccharides (FOS) which are potential prebiotics that may be used as functional foods (Singh and Singh, 2019). The requirement for fructose syrup in Indonesia still relies on imports from abroad. This is evidenced by imports of fructose syrup during the 2015-2018 period which grew with an average trend of 18.99%. National fructose syrup

production since 2015-2018 has reached more than 32,000 tons per year, but this production is lower than the average national fructose syrup consumption which reaches 130,000 tons per year (KPPU, 2020). In addition, the use of FOS in Indonesia is relatively limited because it is an imported product whose price is quite high, reaching USD 20/kg (Mamuaja *et al.*, 2018).

Inulinase enzymes can be produced by microorganisms such as yeast. Yeast is a microflora that can be found on the skin of the fruit where the yeast can utilize the substrate on the skin of the fruit for its growth (Aneja *et al.*, 2014). Kepok Banana (*Musa acuminata x balbisiana*) is the fruit of a hybrid cultivar that has intermediate characters between *M. acuminata* and *M. balbisiana* (Sunandar and Kurniasih, 2019). Kepok banana peel have potency to be a substrate that can be grown by yeast. This is because the banana peel is generally not consumed so it will become waste and has the potential to colonize the yeast on the fruit skin. Banana peels are known to have inulin content of around 0.32 - 0.57% of dry weight (Puttha *et al.*, 2019). Inulin is a polymer consisting of fructose units linked by -(2-1)-D-fructosyl fructose bonds with one terminal glucose (Shoib *et al.*, 2016). The inulin contained in bananas can be an inducer for yeast to produce the enzyme inulinase (Saryono *et al.*, 2016).

The production of inulinase enzymes by yeasts can be optimized by regulating good environmental factors for yeast growth to produce inulinase enzymes. Several production optimization factors that can be done to increase inulinase enzyme production are substrate concentration and incubation time. The inulinase enzyme is an enzyme that requires an inducer in the form of inulin to synthesize the enzyme. The addition of inductive compounds with the right concentration can get good results for the production of inulinase enzymes (Saryono *et al.*, 2016). In addition, the incubation time also greatly affects the production of enzymes because the incubation time is closely related to the growth phase of the yeast in producing enzymes. The inulinase enzyme is known as a primary metabolite compound which is produced in a logarithmic or exponential phase. Inulinase enzyme can be synthesized along with cell growth and can increase after 6 to 8 hours (Cazetta *et al.*, 2010). This study aims to explore the potential of indigenous yeasts from kepok banana peels that are able to produce inulinase enzymes and to determine the effect of substrate concentration or inducer in the form of inulin and incubation time to optimize the production of inulinase enzymes from these yeasts.

METHODS

Preparation of Inulinase Selecting Medium (ISM)

The selective medium was prepared by dissolving 1 g of pure inulin (Sigma-Aldrich), 1 g of yeast extract, and 2 g of agar into 100 mL of distilled water. The medium was added 0.05 g of chloramphenicol to prevent bacterial growth and sterilized at 121°C for 15 minutes (2 atm).

Isolation of Inulinolytic Yeast from Banana Peels

A total of 10 g of ripe kepok banana peels were added with 150 mL of distilled water. The mixture was crushed and mashed with a blender, then inoculated into a petri dish containing ISM agar medium. The inoculation results were incubated at 28°C for 24-48 hours. Yeast extract obtained from ISM medium was purified into Yeast Extract Peptone Glucose Agar.

Inulinolytic Activity Screening

Kepok banana peel inulinolytic yeast was inoculated again by spotting into ISM Agar medium and incubated for 24 hours at 28°C. The surface of the agar medium was flooded with 5 mL of 1% Lugol's iodine and waited 10 minutes for the iodine to be absorbed into the medium. The clear

zone indicated that the yeast hydrolyzed inulin from the media extracellularly. The inulinolytic ratio were measured by the following equation. The isolates with the highest inulinolytic ratio were used for the production of inulinase.

$$\text{Inulinolytic Ratio} = \frac{Z}{K}$$

Z = Diameter of the clear zone as a result of hydrolysis of inulin (mm)

K = Diameter of colony (mm)

Preparation of Inulinase Production Medium

The production medium was prepared by dissolving 0,46 g NH₄NO₃, 0,74 g (NH₄)₂HPO₄, 0,2 g K₂HPO₄, 0,1 g MgSO₄.7H₂O, and 0,3 g yeast extract into 200 mL distilled water. The medium was treated with various concentrations of inulin (Beneo Orafiti) of 1%, 3%, and 5% where each treatment medium had a volume of 200 mL. The medium was sterilized at 121°C for 15 minutes (2 atm).

Growth Cells Observation

The selected yeast isolates were inoculated into 50 mL of sterile production medium, and agitated using a rotary shaker at a speed of 120 rpm for 21 hours. A total of 5% (v/v) yeast starter was inoculated into 50 mL sterile production medium with varying concentrations of inulin and then agitated at 120 rpm. Cultures were taken aseptically from samples every 6 hours and their absorbance (optical density) was measured using a spectrophotometer at a wavelength of 520 nm.

Inulinase Production and Activity Determination

Determination of enzyme activity was carried out by dinitrosalicylic acid reduction sugars method (DNS) (Kusmiyati *et al.*, 2020). The measurement of inulinase enzyme activity is formulated by the following equation.

$$\text{Enzyme Activity (IU/mL)} = \frac{(\text{Abs.ES} - \text{Abs.E} - \text{Abs.S}) \text{fructose}}{\text{BM Fructose} \times T} \times P \times 1000$$

Description:

Abs. ES	:	Absorbance of enzyme and substrate
Abs. E	:	Absorbance of enzyme
Abs. S	:	Absorbance of substrate
BM Fructose	:	Molecular mass of fructose (180,1 g/mol)
P	:	Dilution factor (70x)
T	:	Incubation time (30 minutes)

Experimental Design

This research was conducted experimentally using a completely randomized design (CRD) with a factorial pattern. The first factor that was observed was the inulin substrate concentration

which consisted of several levels, namely K_1 (1% (w/v)), K_2 (3% (w/v)), and K_3 (5% (w/v)). The second factor is the incubation time which consists of several levels, namely W_1 (6th hours), W_2 (12th hours), W_3 (18th hours), and W_4 (24th hours). This study was repeated 3 times, data analysis was carried out by ANOVA test with a significance level of 5% ($\alpha = 0.05$). If it is significantly different, then a further test (Duncan's test) is carried out.

RESULTS AND DISCUSSION

Isolation of Inulinolytic Yeast from Kepok Banana Peels

Based on the isolation results obtained, it was found that 4 potential yeast isolates from kepok banana peels were able to grow on a medium containing inulin, namely YP1, YP2, YP4, and YP6 yeasts. The yield of isolates that tend to be low was also found in a study conducted by Risky *et al.* (2019) which found 4 yeast isolates from cherry fruit that were able to grow on inulin medium. The limited number of isolates in this study was caused by the use of selective inulin medium which tended to only obtain yeast isolates from kepok banana peels that were able to grow using a single carbon source of inulin. This is in accordance with Gavrailov and Ivanova (2016) who stated that the use of inulin as a carbon source in the medium only found colonies that had inulinase activity. The isolated yeast morphology can be observed in

Figure 1 and Table 1.

The four yeast isolates had several colony character similarities such as the entire edge and white color. This is in accordance with Kurtzman *et al.* (2011) who stated that most of the yeasts produced growth with white to brownish-cream colonies. In addition, the four isolates reproduced asexually by forming multilateral budding (Figure 1). Multilateral budding is a type of asexual reproduction characterized by the formation of buds in various parts of a yeast cell (Kurtzman *et al.*, 2011).

Research conducted by Muhibuddin *et al.* (2019) found *Candida* sp. on the skin of banana kepok with bone-white colonies, thick, curved elevation, and oval-shaped cells. In addition, *Pichia* sp. with white, textured colonies, curved elevations, and round cells. According to Soare Vladu *et al.* (2021), *Pichia* spp. and *Candida* spp. has a high potential for inulinase production with high yield and activity. Research conducted by Erfianti *et al.* (2021) found inulinolytic yeasts from cherry fruit that had similarity with *Candida rugosa* which had white colonies, smooth surfaces, butyrous, entire, and had round cell shapes.

In addition, research conducted by Brooks (2008) found *Debaryomyces hansenii* from ripe banana peels. According to Kocková-Kratochvílová *et al.* (1978), the genus *Debaryomyces* has a globose or round cell shape.

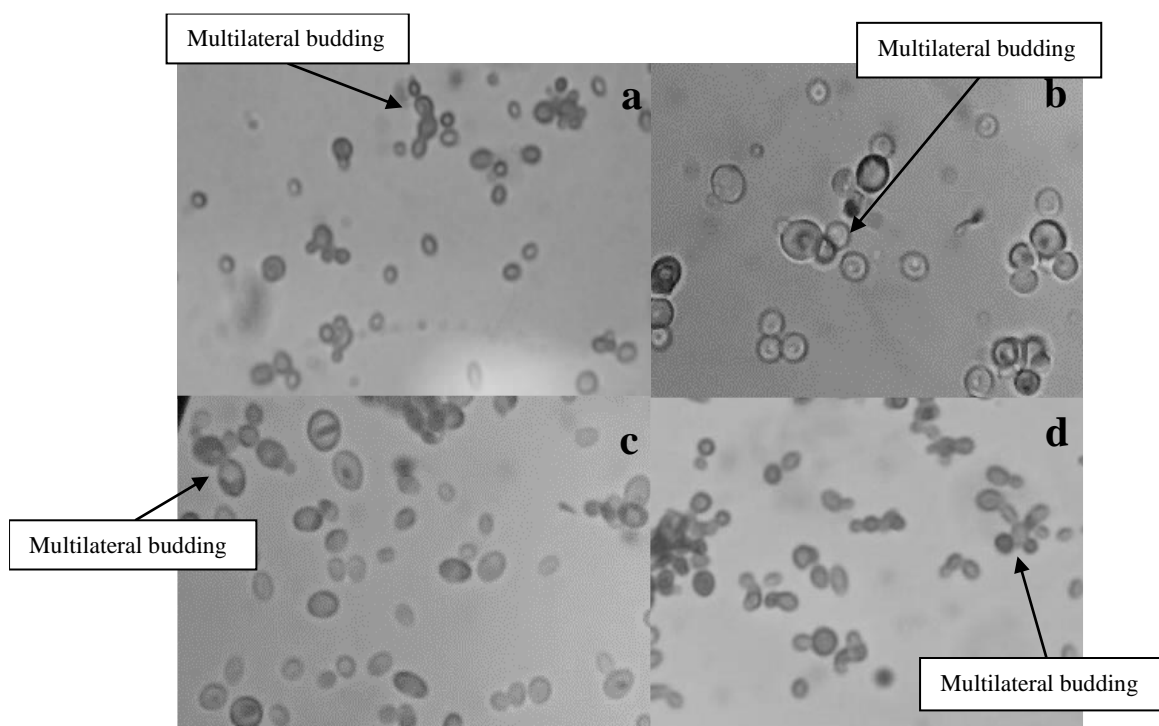


Figure 1. Inulinolytic Yeast Isolate Morphology of Kepok Banana Peels (1000x Magnification). (a) YP1 isolate, (b) YP2 isolate, (c) YP4 isolate, (d) YP6 isolate

Table 1. Morphology of Inulinolytic Yeast Isolated from Kepok Banana Peels

Characteristics	YP1	YP2	YP4	YP6
Edge of colony	Entire	Entire	Entire	Entire
Surface	Glisten	Smooth	Very Glisten	Glisten
Color	White	White	White	White
Elevation	Raised	Flat	Raised	Raised
Colony Shape	Punctiform	Round	Punctiform	Punctiform
Reproduction	Multilateral budding	Multilateral budding	Multilateral budding	Multilateral budding
Cell shape	Subglobose to Ovoid	Globose	Ovoid	Ovoid to Ellipsoid

Kurtzman and Fell (1998) stated that some species of the genus *Debaryomyces* have white colonies and entire edges. According to Rawat *et al.* (2017), *Debaryomyces hansenii* is known to have the ability to produce inulinase. Research conducted by Nakase (2006) stated that *Kluyveromyces marxianus* was found on banana peels. According to Kurtzman and Fell (1998), the genus *Kluyveromyces* has ovoid to ellipsoid-shaped cells. Some species of this genus have glossy white and butyrous colonies. Singh and Singh (2017) stated that *Kluyveromyces* spp. is known to be a yeast that produces the enzyme inulinase.

The presence of yeast on the kepok banana peel indicated that the banana peel is a good habitat for yeast. According to Gana *et al.* (2014), the presence of yeast on the banana surface also comes from several vectors such as insects, dust, and other airborne particles attached to the banana surface. In addition, the utilization of overripe kepok banana peels as isolate source was also a trigger for yeast growth. According to Umeh *et al.* (2017), Banana ripeness is caused by the presence of ethylene gas which is able to stimulate microorganisms to produce enzymes to degrade carbohydrates in bananas into simple sugars and degrade pectin which causes banana peels to ripen and soften. Reginio *et al.* (2020) added that some of the sugars known to be detected in ripe kepok bananas are sucrose, glucose, and fructose. According to Endoh *et al.* (2021), several sugars such as sucrose, glucose, and fructose can be used as a carbon source for yeast growth.

The inulinolytic yeast isolate that has been found was then selected to be used in the production of the inulinase enzyme based on the highest inulinolytic ratio. The inulinolytic ratio is the ratio between the diameter of the clear zone and the diameter of the colony (Silvera *et al.*, 2018). The clear zone formed around the colony after administration of Lugol indicated that the yeast was able to produce extracellular inulinase. According to Guttikonda *et al.* (2017), the method of adding

Lugol's iodine specifically binds to inulin to form a brownish color, while the presence of a clear zone indicates that there is no inulin around the colony because it has been hydrolyzed into simpler sugars such as inulooligosaccharides, fructose, and glucose. The results of the average inulinolytic ratio of the four isolates are shown by Table 2. According to Sunarti *et al.* (2022), inulinase activity is classified into three categories consisting of weak activity with a ratio of <1, moderate or moderate activity with a ratio of <1-2, and high activity with a ratio of >2. Based on these results, the four yeast isolates showed moderate activity. However, YP2 isolate showed the highest activity ratio value compared to other isolates. Therefore, YP2 isolate was used as the selected isolate for the production of inulinase enzyme.

Table 2. Inulinolytic Ratio of Yeast isolated from Banana Peels

Isolate	Inulinolytic Ratio		
	Replication 1	Replication 2	Average
YP1	1.42	1.17	1.30
YP2	1.85	2.06	1.96
YP4	1.59	1.28	1.44
YP6	1.53	1.19	1.36

Growth Profile of Selected Yeast

YP2 growth in several mediums with different inulin concentrations is shown in Figure 2. The graph of the yeast growth curve on the three mediums shows that at 0 hours, the OD value was still relatively low. However, the graph does not show the lag phase because the use of 5% starter inoculated into the production medium has reached the logarithmic phase so there is no adaptation phase during enzyme production. This is in accordance with Mahazar *et al.* (2017) who stated that the starter or inoculum is an important factor in optimizing the production of a microorganism where a healthy inoculum will reduce the length of the lag phase. In addition, Rolfe *et al.* (2012) also stated that the use of starter or inoculum aims to

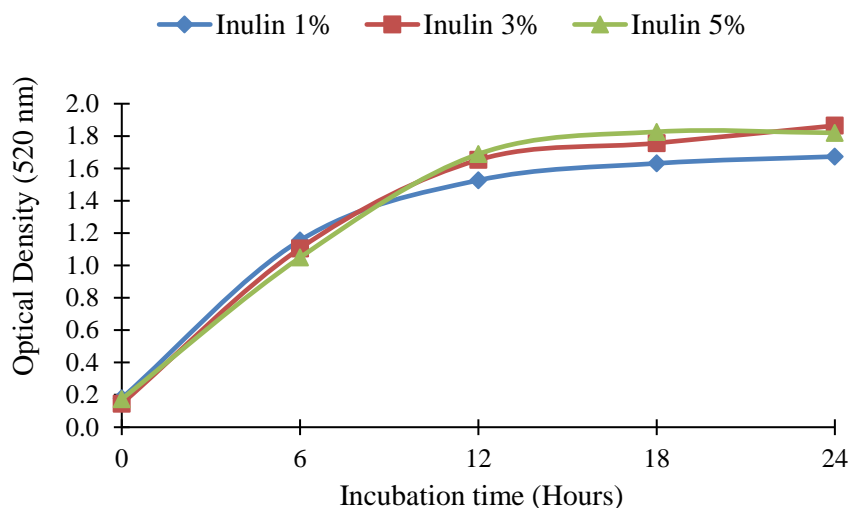


Figure 2. YP2 Yeast Growth Curve Graph in Several Inulin Concentration Treatments

minimize variation in experimental culture conditions and ensure reproducible population lag times.

YP2 yeast growth in all three mediums at 6 hours increased significantly (Figure 2). This is in accordance with Cazetta *et al.* (2010) who stated that yeast cell growth increased along with inulinase synthesis from the 6th hour to the 8th hour. The increase in OD value was due to the yeast entering the logarithmic phase and inulin was effectively utilized as a carbon source and hydrolyzed by the inulinase enzyme produced by the yeast to obtain a simple sugar in the form of fructose which was needed to obtain energy for the growth of the yeast. This is in accordance with Olivares-Marin (2018) who stated that the carbon source during the logarithmic or exponential phase is utilized by yeast to form energy in the form of ATP which is essential for yeast growth. In addition, Wang *et al.* (2019) stated that the carbon source is used as a substrate in cell metabolism, where the carbon source will be used as a component of the cell.

The OD value of YP2 yeast grown on a medium with 1% and 3% commercial inulin concentration (Beneo Orafiti) at 12 to 24 hours increased. The average OD value of YP2 yeast grown in 5% inulin medium reached its maximum value at 18 hours and decreased at 24 hours (Figure 2). The decrease in the OD value indicated that the yeast growing on the medium had undergone a stationary phase. This phenomenon could be caused by limited nutrients in the medium as well as the accumulation of byproducts that are not required for the growth of high amounts of yeast during the stationary phase so that stress is formed on the media. This is in

accordance with Cao *et al.* (2020) who stated that the cell density in the stationary phase reached a maximum and no growth was observed. According to Wijanarka and Sarsa (2019), the stationary phase occurs at an incubation time between 24-30 hours which is characterized by a decrease in the number of cells due to reduced nutrients contained in the medium.

Inulinase Activity of Selected Yeast

The treatment factors used in testing the production of the inulinase enzyme were inulin substrate concentration and incubation time. The activity of the inulinase enzyme based on these two factors is shown in Figure 3. Based on the figure, there are differences in the value of inulinase activity at each concentration of inulin. The highest average inulinase activity value obtained from the 5% (w/v) inulin concentration treatment was 1,779 IU/mL at the 12th and 18th hours (K₃W₂ and K₃W₃), while the highest inulinase activity was at 3% medium (w/v) was 1.315 IU/mL at the 6th hour (K₂W₁) and the highest inulinase activity in 1% medium (w/v) was 1.211 IU/mL at the 24th hour (K₁W₄).

Analysis of variance (ANOVA) showed that the concentration of inulin as an independent variable had a significant effect on the production of the inulinase enzyme. Duncan's test was also carried out to determine the best treatment that gave the most significant effect on the inulinase activity listed in Table 3. The table shows that the use of inulin at a concentration of 5% (K₃) had a significant effect on the production of the inulinase enzyme compared to the other two treatments. Based on the result, it can be said that increasing the

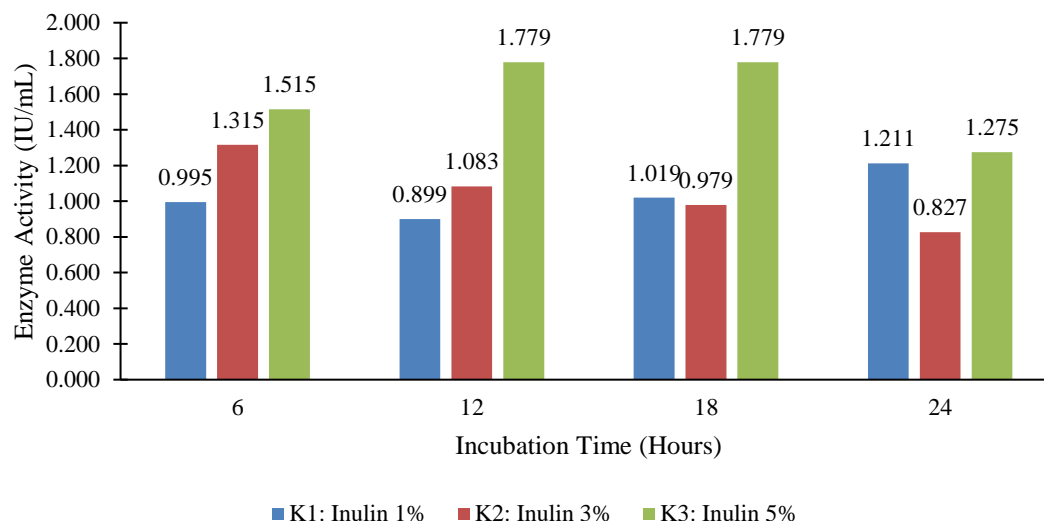


Figure 3. Histogram of Average value of Inulinase activity by YP2 with varying inulin concentration and incubation time

concentration of inulin until it reaches the optimum can increase the activity of the inulinase enzyme where the enzyme synthesis process really requires an inducer in the form of inulin. This is in accordance with Chesini (2013) who stated that inulin is the best carbon source and a specific inducer in the synthesis of the inulinase enzyme, which is then secreted out of the cell to hydrolyze inulin and produce fructose. Saryono *et al.* (2016) also stated that the use of inductive compounds at accurate concentrations would provide significant results in producing the induced enzymes.

Incubation time and interaction between inulin concentration and incubation time have no significant effect with inulinase production in ANOVA test. It also showed with inulinase activity average value in 6th hours until 24th which tends to be stagnant (Figure 4).

Table 3. Duncan’s Test Results based on Inulin Concentration

Inulin Concentration	Inulinase Activity Value (IU/mL)
K ₁ (1%)	1.031 ^a
K ₂ (3%)	1.051 ^a
K ₃ (5%)	1.587 ^b

The average value of inulinase activity at each incubation time in Figure 4 is in contrast to changes in the optical density value from the 6th hour to the 24th hour which is increasing in Figure 2. It was occurred caused by reducing sugar accumulation such as fructose that is produced from hydrolytic activity. Fructose could be utilized as a carbon source to gain the energy for yeast growth so that the number of cells increased. This is in accordance with Endoh *et al.* (2021)

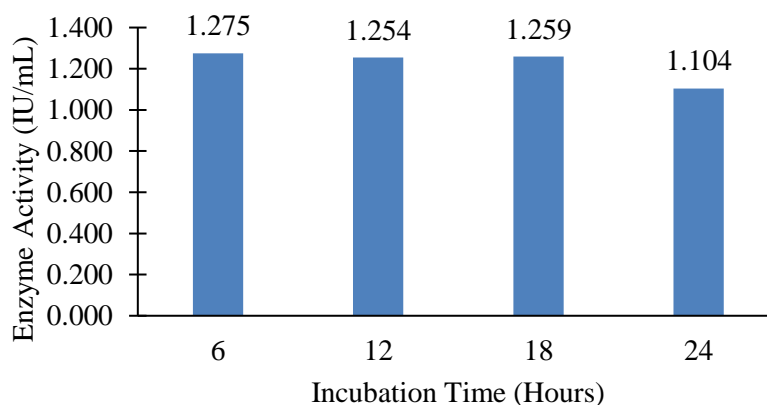


Figure 4. Histogram of Inulinase Activity Average Value of YP2 in varying incubation time

which stated that fructose is a carbon source that is easy to be metabolized by yeast.

Moreover, fructose accumulation caused catabolite repression during the logarithmic phase. Catabolite repression is a phenomenon where expression of the gene encodes enzyme synthesis that is required for carbon source metabolism was inhibited caused by simple carbon source availability that easily metabolized (Fitrania *et al.*, 2018). A signal produced by the accumulation of fructose will inhibit gene expression that has the potential to change enzyme conformation and affect enzyme activity. (Singh *et al.*, 2018). In accordance to Corrado *et al.* (2021), inulinase produced by inulin induction also sensitive against catabolite repression by reducing sugar. Fructose accumulation in high level in medium could inhibit inulinase production.

This research was conducted for the first time regarding the exploration of yeast from kepok banana peels in producing the inulinase enzyme. In previous studies, yeasts derived from banana peels were explored to produce bioethanol and several enzymes such as amylase, lipase, and proteases (Brooks, 2008 and Gana *et al.*, 2014). This research contributes to the development of enzyme production as a catalyst in producing several food products such as HFS and FOS.

CONCLUSION

Based on the results, there were 4 isolates of inulinolytic yeasts obtained from kepok banana peels, namely YP1, YP2, YP4, and YP6. The most potential isolate to be used in inulinase production was YP2 with the highest ratio inulinolytic of 1.96. In addition, the substrate concentration that has a significant effect on inulinase production with Orafiti's optimal inulin concentration for inulinase production is 5% (K_3). However, incubation time and its interaction with substrate concentration did not significantly affect inulinase production. This research needs to be continued by identifying the species of inulinolytic yeast contained in the kepok banana peel with the molecular approach and carrying out another optimization factor in inulinase production.

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