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Comparison Of Liquid Luria Broth Media and Molasses Media on Recombinant L-Arabinose Isomerase Enzyme Production From *Geobacillus Stearothermophilus*

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Abstract— **L-Arabinose isomerase (L-AI) is a type of intracellular enzyme catalyzes the reversible isomerization reaction of L-arabinose to L-ribulose, besides that it can convert D-galactose to D-tagatose. One of the bacteria that can produce this enzyme is Geobacillus stearothermophilus. The high price of instant media, which reaches IDR 1,500,000 to IDR 3.000.000,- for every 500 g and the abundance of natural resources that can be used as a medium for the growth of microorganisms encourages researchers to find alternative media from materials that are easy to get and do not require expensive costs. The researchers compared the effectiveness of liquid Luria Broth (LB) media and molasses media for enzyme production. The research involved inoculating bacteria in both media types, followed by subculture and incubation. IPTG was added to induce enzyme production, followed by cell harvesting through centrifugation and sonication. Enzyme activity was evaluated using SDS PAGE and the cysteine carbazole method. Molasses showed promising results due to water solubility, enzyme activity, abundance, and cost-effectiveness. Molasses appears as an alternative with active enzymes, water solubility, abundant availability, and significant cost savings, making it a viable choice for enzyme production.**

Keywords— *L- Arabinose Isomerase, Molasses, Enzyme.*

INTRODUCTION

In the field of microbiology, to grow and study the properties of microorganisms, a medium is needed as a place for the growth of microorganisms. Growth media must meet the nutritional requirements needed by a microorganism [1]. The nutrients needed by microorganisms for their growth include carbon, nitrogen, non-metal elements such as sulfur and phosphorus, metal elements such as Ca, Zn, Na, K, Cu, Mn, Mg and Fe, vitamins, water and energy [2]. Bacteria need food sources containing C, H, O and N which are useful for constructing protoplasm [3]. Carbon is the main substrate for bacterial metabolism, so it can be used as a source of bacterial nutrition. Carbon sources can be obtained from carbohydrates, proteins and fats [4].

L-Arabinose isomerase (L-AI) is a type of intracellular enzyme that catalyzes the reversible isomerization reaction of L-Arabinose to L-ribulose, besides that it can convert D-galactose to D-tagatose [20]. One of the bacteria that can produce this enzyme is Geobacillus stearothermophilus. Based on several previous studies, liquid Luria Broth (LB) media is suitable for growing enzyme production. The high price of instant media, which reaches IDR 1,500,000 to IDR 3,000,000 per 500 g, and the abundance of natural resources that can be used as a medium for the growth of microorganisms, have encouraged researchers to find alternative media using ingredients that are easily available and inexpensive. requires expensive costs. The materials used must contain the nutrients needed for bacterial growth, such as from ingredients that are rich in carbohydrates and proteins [5].

The carbon source commonly used in making growing media is sucrose (granulated sugar) because it is easy to get but is expensive. Another alternative to sucrose is molasses. This material is a by-product of processing sugar cane into granulated sugar. The sugar content in molasses consists of 35% sucrose, 7% glucose, 9% fructose, 4% other carbohydrates [6]. The high total sugar content in molasses has the opportunity to be used as an alternative carbon source which can then be used as a culture medium for the production of the enzyme L-arabinose isomerase [7]. This research was conducted to optimize two types of media, namely liquid Luria Broth (LB) media and Molasses media in their role of being able to produce the L-Arabinose isomerase enzyme derived from *Escherichia Coli* bacteria type *Geobacillus stearothermophilus* and to find alternative media that are more affordable for the production of these enzymes.

METHOD

A. Time and Place

This research was conducted over a span of 6 months, specifically from August to November 2022. This research was carried out at CV. Amalose Tekno Indonesia which is located at Jatijajar Housing Block D3, No 33, Tapos, Depok and at LIPI (Indonesian Research Institute) which is located at Jl. Raya Jakarta - Bogor, No.Km.46, Cibinong, Bogor Regency, West Java.

B. Tools and Materials

The equipment utilized for this research includes a Laminar Air Flow (LAF), Scales, Autoclaves, Hot plates, Ph meters, Incubators, PCR, Centrifugators, Shakers, SDS-PAGE, Spectophotometers, Electrophoresis, Vortex, spindown, Durant bottles, test tubes, petri dish, Erlenmeyer, Falcon, microtube, PCR tube, sonication tool, micropipette and tip, spatula, stirrer, tube needle, L stirrer, microplate, tweezers, gloves, Plastic Wrap, Aluminum Foil.

The material used in this research is the host or bacteria producing the L-arabinose isomerase enzyme E. coli BL21-pET21b-LAI strain which is available at the Carbohydrate Bioengineering Research Group (CBRG) Laboratory of the LIPI Cibinong Biotechnology Research Center.

Other chemical materials that are used in production and optimization include LB (Luria Broth), Molasses concentration 0.25%, agarose, distilled water, spirit, 70% alcohol, ampicillin, NZY Taq 11 2α green master mix, primer forward, primer reverse, dH2O nuclease free , plasmid E. coli BL21-pET21b-LAI, glycerol, Buffer TAE 1x, staining, marker SMOBIO PM 2700, Marker SMOBIO DM 3100, isopropylthiogalactoside (IPTG), Phosphate Buffer pH 7 50 mM , Tris HCL Buffer 10 mM pH 8 protein loading , sodium dedosyl sulfate (SDS), ammonium persulfate (APS), acrylamide, N,N,N-tetramethylethylenediamine (TEMED), electrophoresis buffer, protein marker, commasie blue, cysteine, carbazole, absolute ethanol, H2SO4 , HCL 1 N, fructose , galactose 800Mm, Tris HCL pH 8, and ice cubes.

C. Research Method

The research was conducted in seven stages, namely rejuvenation of bacteria, PCR electrophoresis, and production of enzymes consisting of culture, subculture, harvesting and sonication of enzymes, SDS PAGE, and assay of enzyme activity using cysteine carbazole.

Rejuvenation (streak) Bacteria

Luria Broth medium (LB) agar of 32 g/L was prepared. Furthermore, the LB agar media is heated with a hotplate until it melts, after it melts, wait until the media is not too hot. Then 20 mL of LB agar medium was poured into a sterile petri dish, after the LB agar had hardened, 20 µL of ampicillin was added and leveled with a triangular stirring rod that had been dipped in alcohol and had been heated with a Bunsen burner. Furthermore, the ampicillin was leveled until it absorbed into the LB agar medium. Next, colonies originating from the E. coli BL21-pET21b-LAI masterplate were taken using a loop needle, then streaked on the prepared media. Next, close the petri dish and cover the edges with plastic wrap. After finishing the streak results put into the incubator overnight with a temperature of 37^{o}C.

PCR electrophoresis

Agarose gel was prepared with a composition of 0.5 g / 50 mL of TAE buffer, then the agar was heated to boiling and waited until lukewarm then added 1.25 µL of staining and stirred using a stirrer. Once it has been mixed and warm, the agar that has been made is poured into an electrophoresis mold with a comb attached. Then wait until it hardens, then the mold is placed in the electrophoresis apparatus and soaked in 400 mL of TAE 1x buffer. Then running the electrophoresis begins with a period of 20

minutes with a voltage of 100 V. After running, the results of the electrophoresis are viewed using Ultra Violet (UV) light.

Culture

Liquid LB media was prepared with a concentration of 25 g/L and Mollases media with the concentration of 0.25% was autoclaved beforehand. Next, 2 test tubes were prepared, namely as negative control and positive colonies. After the liquid LB media was warm, the media was poured into 2 test tubes with 5 mL each. Then the tube marked as a positive colony was added with 5 µL of ampicillin. After adding ampicillin, the masterplate was prepared, then the colonies on the masterplate were taken using a micropipette tip and put into a tube which had been marked as a positive colony. Next, the two reaction tubes were put into a shaker at a speed of 160 rpm and a temperature of 37ºC overnight (16-18 hours).

Subculture

The tools and materials are prepared beforehand, then the liquid LB is heated with a hotplate, after it is lukewarm the liquid LB media is poured into an Erlenmeyer as much as 50 mL. Furthermore, 500 µL of culture results were taken with a micropipette and added to an Erlenmeyer containing liquid LB. Then the Erlenmeyer which had been added with the results of the subculture was put into a shaker with 160 rpm at 37oC for 3-4 hours. After 3-4 hours the Erlenmeyer is taken from the shaker, then 50 µL of IPTG with a concentration of 0.1 M is added with the tip inserted into the Erlenmeyer containing liquid LB. Furthermore, the Erlenmeyer that had been added with IPTG was put into a 160 rpm shaker at 37ºC overnight (16-18 hours). This was also done on molasses media with a concentration of 0.25%.

Harvest and Enzyme Sonication

The tools and materials were prepared, then 50 mL of the subculture results were transferred to the tube and then centrifuged at 5,000 rpm for 5 minutes at 24ºC. Furthermore, after obtaining the precipitate in the form of pellets, the supernatant was discarded and the pellet was concentrated 5 times using 50 mM Phosphate buffer pH 7, which was 10 mL. Next, after concentrating, sonication was carried out with an amplitude of 60 and a cycle of 0.5 for 3.5 minutes. After the total sonication results were obtained, 2 mL was transferred into microtube 2. Then the remainder was centrifuged again at 10,000 rpm for 10 minutes at 4ºC. Next, the supernatant was transferred to a 15 mL falcon and the pellet was resuspended using 8 mL of 50 mM pH 7 Phosphate buffer.

SDS PAGE

To perform SDS-PAGE, tools and materials were prepared, then 10 µL of the enzyme was put into the PCR tube, then mixed with 10 µL of protein loading dye and heated at 100ºC for 5 minutes. Next, the SDS-PAGE components are prepared. Once the gel has been created, the impression comb is placed to create a well in which to place the sample. Next, wait for the gel to harden. After hardening, the gel was placed in the electrophoresis device in a standing position and soaked using 1x TAE buffer. Then 8 µL of marker and sample were added to each well. After all the samples have been inserted, electrophoresis is then run for a period of 60 minutes. Once finished, the gel is soaked in commasie blue solution for approximately 60 minutes or overnight.

L-Arabinose Isomerase (L-AI) Enzyme Activity Test

Enzymes can work optimally under certain conditions, the characteristics of enzymes such as pH, temperature, the influence of metal ions, heat stability, and others greatly affect the performance of enzymes [1]. Apart from that, the enzyme activity test requires molecules that will work with the enzyme, which are called substrates. The substrate will later bind to the enzyme. The substrate used in the L-AI activity test was galactose, while the buffer used in the L-AI enzyme activity test was phosphate buffer pH 7. A blank solution, substrate blank, enzyme blank, and reaction mixture (enzyme+substrate) were prepared. After all the mixtures have been made, then incubated with the optimum temperature treatment (60ºC for 60 minutes). Furthermore, the enzyme is inactivated and the results of the enzymatic reaction are put into the freezer.

Cysteine Carbazole

First, a cysteine carbazole solution was prepared. Each mixture (buffer blank, substrate blank, enzyme blank, and reaction mixture) was aliquoted in 20 µl into a 2 mL microtube and 480 µl of Cysteine Carbazole solution was added. Then 20 µl of fructose standard was made and µl of Cysteine Carbazole solution was added. Then each sample and fructose standard were incubated at 60ºC for 30 minutes. After that, the samples and fructose standards were cooled in the refrigerator for 10 minutes. Next, activity measurements were carried

out using a spectrophotometer with a wavelength of 560 nm.

RESULT AND DISCUSSIONS

Rejuvenation of Bacteri

Figure 1. Results of streaks of E. coli BL21-pET21b-LAI

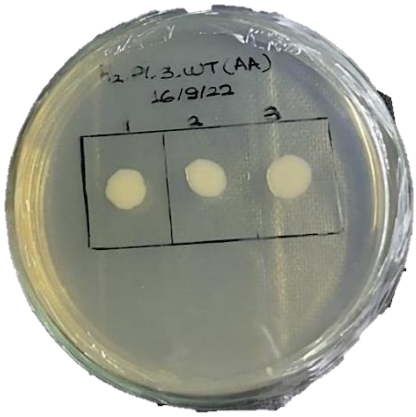


Figure 2. Results of E. coli BL21-pET21b-LAI master plate after incubation overnight in an incubator at 37^{o} C.

Bacterial rejuvenation needs to be done with the aim that the bacteria can start their metabolism again after storage. Rejuvenation of bacteria is carried out by taking a pure culture of the original bacterial strain with a loop needle, then streaking it onto the surface of LB agar media [4]. The streak plate or scratch plate method is carried out on bacterial colonies with the aim of separating the colonies so that a pure culture can be obtained. Maintenance of bacterial isolates needs to be carried out periodically so that the metabolic work of the bacteria can still work properly [5]. Based on the research carried out, streak results were obtained from the rejuvenation of the E. coli strain BL21-pET21b- LAI (Figure 1), a number of 3 single colonies in the streak results were obtained to be transferred to the Master plate (Figure 2) and used as a source for enzyme production.

PCR Electrophoresis

The electrophoresis method is by injecting DNA into an agarose gel, then fusing the gel with electricity. The results obtained are that small DNA strands will move more quickly and large strands between the gel will show positive results [6]. Based on the results of the research carried out in this activity, it was found that all three colonies tested positive (Figure 3). The results of the transformation of pET21b-LAI gene from Geobacillus stearothermophilus to E. coli BL21(DE3) obtained as many as 3 colonies growing in the dishes. Colonies that grow in colony PCR to determine the success of the transformation process. A total of 3 selected colonies were then PCR tested, the result was that all positive colonies carried the plasmid pET21b-LAI gene from Geobacillus stearothermophilus with the appearance of a band at 1512 bp in size.

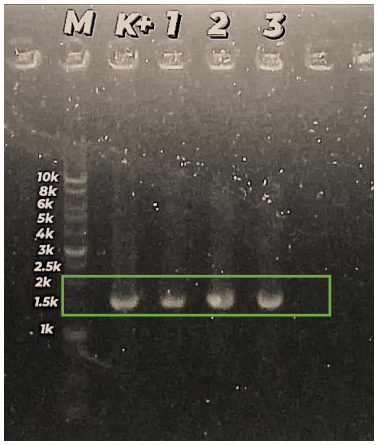


Figure 3. PCR results from E. coli BL21-pET21b-LAI colonies. M: marker, K+: positive control in the form of a plasmid originating from a positive colony of E. coli BL21-pET21b-LAI which is available in the positive Carbohydrate Bioengineering Research Group (CBRG) laboratory, 1: Colony 1, 2: colony 2, 3: colony 3.

Culture

To produce enzymes, it is necessary to carry out a microbial culture first. After the colony results have been obtained, then we can carry out a culture from one of the positive colonies. Microbial culture is a method for multiplying microbial organisms by allowing the microbes to reproduce in a predetermined culture medium. In this study, culture was carried out in two media treatments, namely liquid LB (Luria Broth) media and molasses media with a concentration of 0.25% which was poured into a test tube. The microbes cultured in the enzyme production process this time came from the positive streak results of the E. coli BL21-pET21b-

LAI strain. Successful culture results are indicated by the media turning cloudy.

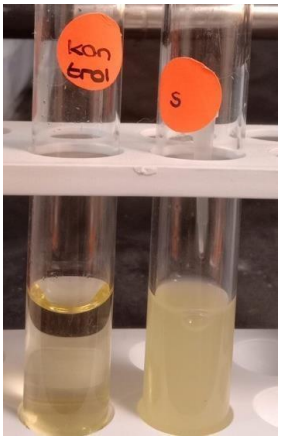


Figure 4. Results of E. coli BL21-pET21b-LAI culture in a test tube containing 5 mL of liquid LB media.

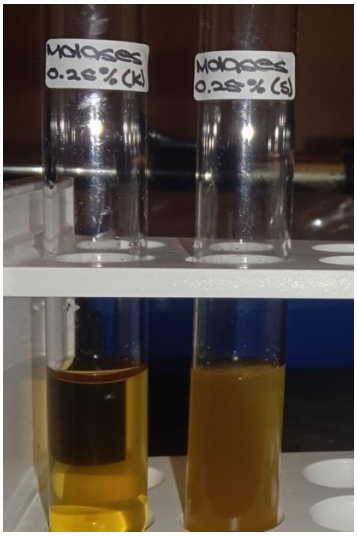


Figure 5. Results of E. coli BL21-pET21b-LAI culture in a test tube containing 5 mL of 0.25% Molasses media.

Based on the results of the culture carried out using liquid LB media or Molasses with a concentration of 0.25% and put into a Shaker Incubator at a temperature of 37oC and left overnight (16-18 hours), it can be seen that there is a difference between the two test tubes that grew bacteria and those that did not, namely in tube K (negative control) liquid LB media remained clear in color, whereas in the positive control tube (L-AI) there was a change in color to become more cloudy (Figure 4). Likewise in Molasses media, tube K (negative control) remained clear in color, while in the positive control tube (L-AI) there was a change in color to become more cloudy (Figure 5). This indicates that the culture has been successful.

Subculture

In studies conducted with culture results using Liquid LB media will be transferred to new liquid LB media in Erlenmeyer. Meanwhile, culture results using Molasses media with a concentration of 0.25% will be transferred to new Molasses media with a concentration of 0.25% in Erlenmeyer. After the transfer has been carried out, the subculture is put back into the Shaker Incubator with 160 rpm at 37oC for 3-4 hours, then IPTG is added to the subculture. Based on the results obtained in this study, it can be seen that there was a change in color to become cloudy in the subculture results using either Liquid LB or Molasses media after adding IPTG (Figure 6) and (Figure 7). The added IPTG functions to express protein from microbes. Isopropyl β-D-1 (IPTG) is an induction reagent commonly used for the production of recombinant proteins [10]. The mechanism of induction of IPTG is that IPTG will bind to the lac repressor protein, this bond will cause a change in the structure of the lac repressor protein. This change in the structure of the lac repressor protein will cause the protein to detach from the lac operator sequence.

Figure 6. Results of subculture of E. coli BL21-pET21b-LAI with Liquid Lb media after adding IPTG and placing it in a shaker incubator at 160 rpm at a temperature of 37ºC for 3-4 hours**.**

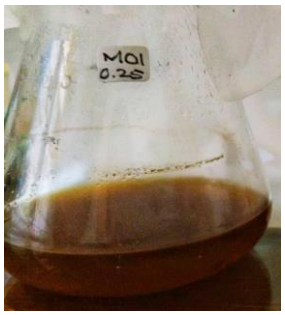


Figure 7. Results of subculture of E. coli BL21-pET21b-LAI with Molasses media with a concentration of 1% after adding IPTG and putting it in a shaker incubator at 160 rpm at a temperature of 37ºC for 3-4 hours.

Harvest and Sonicate the Enzyme

Activities carried out after harvesting and sonication, obtained samples in the form of total enzymes, supernatant and pellets. The sonication results can be seen in the results of the supernatant after sonication which is clear (Figure 8) and (Figure 9). This indicates that the cell wall has been completely broken down and has completely settled on the pellet after the second centrifugation at 10,000 rpm 10oC for 10 minutes. In the production of enzymes, the sample used is the supernatant. It can be seen that the pellet sample has the clearest color, with the results obtained it is estimated that the cell wall has completely broken down and there is no more activity in the pellet. Furthermore, to ensure the activity of each sample, it is necessary to test it with SDS-PAGE and test the enzyme activity.

Figure 8. Yields of E. coli BL21-pET21b-LAI on Liquid LB media before and after sonication.

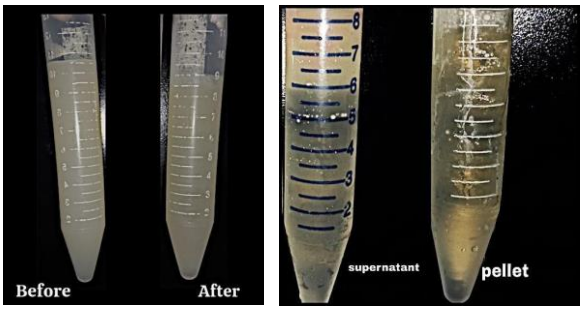


Figure 9. Yields of E. coli BL21-pET21b-LAI on molasses media before and after sonication.

SDS PAGE

IPTG that has been added for protein expression needs to be visualized using SDS-PAGE. The working principle of SDS-PAGE is to involve initial denaturation of protein components with an anionic detergent which also binds the protein, with this, all proteins will have a negative charge proportional to the mass of the protein molecule. Furthermore, electrophoresis was carried out through a porous gel matrix acrylamide which could separate proteins based on molecular mass. If the size of the molecule is smaller it will move faster in the gel, whereas if the size of the molecule is larger it will move slowly resulting in a band close to the well in the gel [12]. The results of SDS-PAGE show that E. coli BL21-pET21b-LAI has successfully expressed the enzyme, namely L-arabinose isomerase, in the form of a fusion protein with the addition of IPTG as an expression inducer for E. coli BL21-pET21b-LAI (Figure 10).

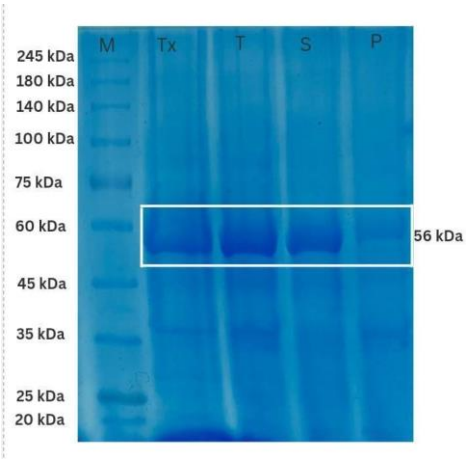


Figure 10. SDS-PAGE results of L-AI enzyme in liquid LB media M: marker, Tx: Total without sonication, T: Total, S: Supernatant, P: Pellet.

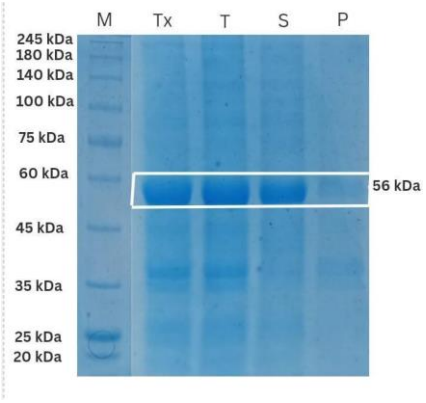


Figure 11. DS-PAGE results of L-AI enzyme in molasses media M: marker, Tx: Total without sonication, T: Total, S: Supernatant, P: Pellet.

Based on the resulting SDS-PAGE data, the total and supernatant have the same band thickness, whereas for bands in thin pellets, activity testing needs to be carried out to ensure whether each enzyme (Tx, T, S, P) still has activity or not. The thick band on the SDS-PAGE results indicates a lot of dissolved protein. Expression of the recombinant protein targeting L-arabinose isomerase (L-AI) in E. coli BL21(DE3) host cells which insert the pET21b plasmid carrying the gene from Geobacillus stearothermophilus produces a protein

with a size of 56 kDa [6] (Figure 10) and (Figure 11).

Cystein Carbazole

Testing the activity of the L-arabinose isomerase enzyme can be carried out using the cysteine-carbazole method, the principle of the cysteine carbazole method is the color change of the test solution before and after adding the sample. The color change that occurred in the test solution became purple after adding cysteine carbazole due to the breaking of the double bond in the O group belonging to the ketohexose sugar by the sulfur contained in the H2SO4 solution and cysteine which later reacted with the carbazole. So that if the sugar from ketohexose is present in the sample solution, the color will be more concentrated, this color change will ultimately affect the absorption value. When the sample is measured with a spectrophotometer with a wavelength of 560 nm [8].

Staining treatment with cysteine carbazole solution will produce a purple color if the sample contains tagatose formed. The color change to purple is due to the presence of a solution of cysteine carbazole sulfuric acid, whereas tagatose is a kentose sugar which will turn purple when reacted with cysteine carbazole [8]. The darker the purple color in the carbazole cysteine product, the L-AI enzymatic activity indicates the higher the tagatose level, the more tagatose in the sample, the intensity of the purple color will increase, so that the absorbance at 560 wavelength will be higher [8] .

Based on the results of staining using cysteine carbazole, it can be seen that the total and supernatant samples in both liquid LB media (Figure 12) and molasses media (Figure 13) experienced a color change to purple. This indicates that the L-arabinose isomerase enzyme successfully produces tagatose. The results of cysteine staining with these two media produced color differences in the total sample and supernatant that were not very significant, so it was necessary to re-analyze the absorbance of cysteine carbazole staining using a spectrophotometer. In measuring arbobancy in samples, fructose serves as a standard for the product that has been formed. The results of cysteine carbazole (Figure 12) and (Figure 13) show that in each pellet sample there is no color change to purple, this indicates that the pellet probably no longer has activity.

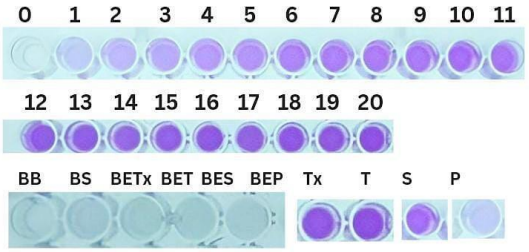


Figure 12. Results of cysteine carbazole in liquid LB media ket: 0-20: fructose standard. BB: Blank buffer, BS: Blank Substrate, BET: Blank Total Enzyme, BES: Blank Enzyme Supernatant, BET: Blank Total Enzyme, Tx: Total without sonication T: Total, S: Supernatant, P: Pellet.

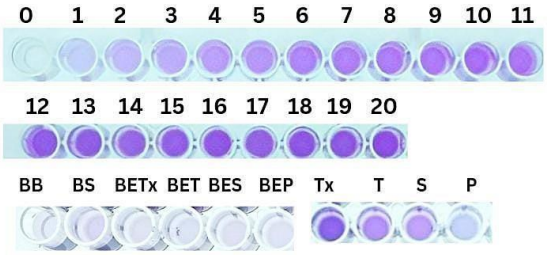
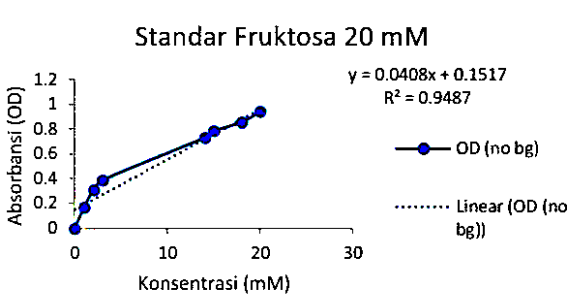


Figure 13. Results of cysteine carbazole in liquid LB media ket: 0-20: fructose standard.

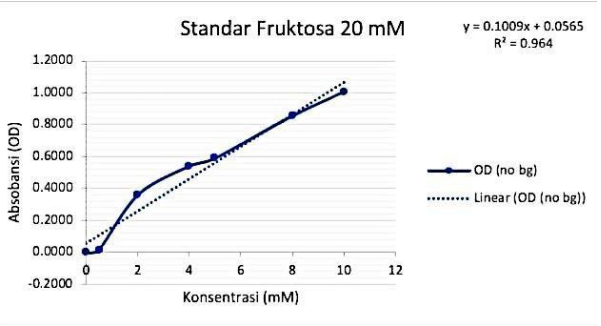
The results of staining with carbazole cysteine were then measured using a spectrophotomer to measure the absorbance of the color with a wavelength of 560 nm. After measuring the activity of the enzyme using 20 mM fructose, the standard curve was obtained for liquid LB media (Curve 1) with a linear regression of R2: 0.9487, while for the standard curve for molasses media (Curve 2) with a linear regression of R2: 0.964 . This fructose standard is then used to determine the concentration of the enzyme sample in the molasses and liquid LB media, the standard of fructose greatly influences the results of calculating enzyme activity units because from this standard a formula will be obtained to calculate the concentration of the sample.



Curve 1. Standard Fructose 20 mM used in samples by incubation in liquid LB medium.

Table 1. Results of L-AI Enzyme Activity Units in liquid LB media.

Fraksi	UA/mL Enzim
T x	*0.2961*
T	*0.1700*
S	0.1450
P	0.0743



Curve 2. Standard Fructose 20 mM used in samples by incubation in molasses medium.

Table 2. Results of L-AI Enzyme Activity Units in Molasses media.

Fraksi	UA/mL Enzim
T x	0.6392
T	0.5676
S	0,5671
P	0.1959

The results obtained showed that in liquid LB media (Table 6) the yield of total enzyme units was greater than in molasses media (Table 7), but the difference between the two was not very significant. Total enzyme is a combination of supernatant and pellet enzymes, the total is high at the optimization temperature because the pellet does not show any activity. The activity contained in the pellets can reduce the value of the total. Based on the total enzyme results obtained by liquid LB media, it was 0.6392 UA/mL, while the total enzyme results obtained by molasses media were 0.2961 UA/mL. The supernatant yield obtained by liquid LB media was 0.5671 UA/mL, while the total enzyme yield obtained by molasses media was 0.1700 UA/mL. The pellet yield obtained by liquid LB media was 0.1959 UA/mL, while the total enzyme yield obtained by molasses media was 0.0743 UA/mL.

The presence of activity in the pellet is a mixture of bacterial membranes and inclusion bodies which have no activity at 60oC. This can happen because there is a possibility that some parts of the enzymes that form the inclusion bodies join the pellet. Inclusion bodies are target proteins in the form of aggregates that are insoluble. Inclusion bodies themselves have very low activity, possibly even no activity [10]. There are several factors that can influence the activity of enzymes, which can be classified as physico-chemical (pH, temperature, ionic strength, activity, water, etc.). Chemical (activators, inhibitors, stabilizers etc.). And for physics namely (pressure, shear force, friction etc.) [10].

CONCLUSION

The L-arabinose isomerase enzyme expressed in liquid LB media has a higher and more optimal enzyme activity unit value than in molasses media. Molasses has great potential to be used as an alternative medium for the production of the L-arabinose isomerase enzyme. With only 0.25% molasses used, the L-arabinose isomerase enzyme produced reached 0.2961 U/mL or 50% compared to liquid LB media of 0.6392 U/mL. Meanwhile, in terms of costs, there are savings of up to 7 times. In conclusion, molasses has great potential to be used as an alternative medium, because the enzymes produced are active and water soluble, the availability of molasses is abundant, and it is easy to obtain.

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