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Identification of Gamma-Amino Butyric Acid Isolates Lactic Acid Bacteria Results from The Isolation of Rusip

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Abstract. Gamma-aminobutyric acid (GABA) is a non-protein amino acid produced by glutamate decarboxylation by the enzyme *glutamate decarboxylase* and it is widely distributed in plants, animals and microorganisms. GABA-producing microorganisms including Lactic Acid Bacteria (LAB) which play a role in the fermentation process of food such as rusip. Rusip is a preserved food of marine fish made from raw anchovy of Bangka-Belitung. The purpose of this study was to obtain LAB isolates through the process of isolation from rusip and identify the presence of GABA. Isolation of LAB was carried out by the pour method on MRS agar and NA medium. The growing colonies were then characterized based on observations of colony morphology, Gram staining, catalase test, motility test, and fermentation type test. LAB isolates obtained were selected to obtain isolates which are capable to produce GABA using the Thin Layer Chromatography (TLC) method. The results of isolation from rusip and inoculation on MRS agar medium obtained, three LAB isolates namely RSP-A1, RSP-A2, and RSP-A3. Their characteristics are Gram positive bacteria, negative catalase test, negative motility test, and belong to homofermentative and heterofermentative bacteria groups. They were also able to produce GABA. Research on GABA from isolates isolated from rusip is a new thing in the world of GABA research. This research provides information that is beneficial to produce GABA easily, cheaply, and efficiently.

Key words: *GABA; Lactic Acid Bacteria; Rusip; Isolation*

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INTRODUCTION

Food processing has traditionally been known for a long time, one of them is fermentation. Fermentation is an aerobic or anaerobic process that produces various products involving the activities of microorganisms (Irianto, 2012). One of the processed fermented fish products is rusip. Rusip is a traditional food product from Bangka-Belitung in the form of anchovies processed by fermentation with the addition of certain quantities of salt and palm sugar (Koesoemawardani & Ali, 2016). Rusip is made from anchovy or ikan bilis. Anchovy or ikan bilis are washed, cleaned and drained, then given the treatment of adding salt with a concentration of 25% of the weight of the fish, stirred until evenly distributed and incubated in a jar for 24 hours. Palm sugar water as much as 10% of the weight of the fish is added, then stirred until blended, and closed the jar again. Curing is done at room temperature for 7-14 days (Putri et al, 2014). Fermentation has various benefits, including preserving food products, giving a certain taste and texture to food products (Panda et al, 2011).

Lactic Acid Bacteria (LAB) is the bacteria that contributes greatly to the world of food. LAB can be used to suppress the growth of some spoilage and pathogenic bacteria to increase shelf life and food safety (Muchtadi & Ayustaningwarno, 2010). The characteristics of LAB are their cells react positively to Gram staining, react negatively to catalase test, do not form spore, cocci shape, arranged in pairs or shaped chains, facultative anaerobic, mesophil, and non-motile (Romadhon et al, 2012). LAB is widely used as a functional food and as a natural preservative of a fermented food product (Ibrahim et al, 2015).

Microbial growth is an increase in the number or volume and size of cells (Cowan, 2012). Microbial cell growth usually follows a specific growth pattern so that it will obtain a chart or sigmoid growth curve. Microbial growth curves can be distinguished into 4 main phases namely the lag phase (adaptation phase), exponential phase (logarithmic phase), stationary phase, and decreasing phase (death phase) (Mardalena, 2016). Some studies have shown that LAB can reduce the pathological conditions due to oxidative stress, indicating that LAB has antioxidant activity. One of the metabolites produced by LAB that can act as an antioxidant is gamma-aminobutyric acid (GABA). The function of GABA as an antioxidant is demonstrated by strains of *Lactobacillus plantarum* DM5 (Das & Goyal, 2015).

GABA is a non-protein amino acid that has four carbon atoms that serves as the primary inhibitor of neurotransmitters in the central nervous system (Indrowati et al, 2015). GABA is a major inhibitor of mammalian neurotransmitters in the central nervous

system (CNS) and regulator of various physiological and psychological processes (Barliana, 2016). GABA can be used to treat severe depression, stress, insomnia, anxiety disorders, and others with elevated GABA levels in the brain (Kim et al, 2010). GABA can be produced from LAB such as *Lactobacillus brevis, L. plantarum, L. delbrueckii, L. paracasei* and *L. lactis.* LAB can produce GABA because of its GAD enzyme activity (Kook & Cho, 2013). The purpose of this study was to obtain LAB isolates through the process of isolation from rusip and identify the presence of GABA. This research can be used as a source of information about LAB isolates from the rusip isolation process to produce GABA so in the future GABA can be produced easily, cheaply and efficiently.

METHODS

The isolation of rusip was done by dilution, by taking aseptically as much as one gram of rusip and put into a sterile container and then adding nine ml of distilled water (10^{-1} dilution), then made a series of dilutions for 10^{-2}, 10^{-3}, and 10^{-4}. The results of the dilution were planted with the pour method on de Man, Rogosa and Sharpe (MRS) agar medium and nutrient agar (NA) medium in the petri dishes, then incubated at 37°C for 48 hours. The growing colony was taken using inoculating loop and was replanted to be purified by the streak method. Purification was carried out on MRS agar + $CaCO_3$ media and incubated again at 37°C for 48 hours.

Characterization of bacterial isolates as a result of isolation from rusip was carried out using five methods namely observation of colony morphology, Gram staining, catalase test, motility test, and fermentation type test. Observation of colony morphology included the shape of bacterial colonies, colony colors, colony edges, and colony elevation.

Gram staining was done by transferring small amount solid pure culture of LAB to a sterile glass objects that had been given a drop of sterile distilled water. Then, the bacteria were fixed above the bunsen flame. Preparations were colored with Gram A for a minute and then washed with flowing water and dried up. Then, colored with Gram B for one minute, washed with flowing water and dried up. They were then colored with Gram C for 30 seconds, washed with flowing water and dried up. The next processes were coloring the preparation with Gram D for 30 seconds, washing with flowing water, and drying up. Observations were then performed using a microscope at a magnification of 1000 times.

Catalase test was done by transferring small amount of solid pure culture of LAB to a objects glass that had been cleaned with alcohol. A drop of 3% H_2O_2 was placed on the object glass. Then, formation of gas bubbles in the preparation was observed. Motility test was done by transferring small amount of solid pure cultures of LAB to the nutrient broth (NB) medium containing 0.5% agar (semi-solid). Then, the culture was incubated for 48 hours at 37ºC. Then, the widespread growth of colonies on medium was observed.

Fermentation type test was done by transferring small amount of solid pure cultures of LAB on MRS broth medium in a test tube that had been given a Durham tube. Then, the culture was incubated for 48 hours at 37ºC. Subsequent observations were conducted to observe the absence of gas trapped in the Durham tube.

RSP-A1, RSP-A2, and RSP-A3 LAB isolates were cultured on 250 mL Erlenmeyer in 100 mL MRS broth medium. Then, the inoculum was incubated for 66 hours at 37ºC. The culture was taken every six hours as much as five ml to measure cell growth directly by the turbidimeter method. Cell growth was determined by measuring optical density (OD) values using a spectrophotometer at λ 600 nm. A small amount of pure cultures of LAB was put into five ml MRS broth medium. Then, the culture was incubated for 24 hours at 37ºC. Then, the inoculum was pipetted as much as 2% (v/v) and put in 100 mL MRS broth medium plus 1% MSG. LAB inoculum was then incubated for 48 hours at 37ºC.

LAB inoculums in MRS broth medium were transferred to each centrifugation bottle. Centrifugation was carried out at 6000 rpm and at 4 ºC for 15 minutes. The resulting supernatant was moved into the eppendorf tube. A line was drawn using a pencil as a starting marker on the Thin Layer Chromatography (TLC) plate measuring 5 x 2.5 cm. TLC plates were made in 4 pieces. The line from the edge of the TLC plate was one cm apart. The initial marker line was marked as many as two points with a distance of one cm using a pencil to spotting the sample. Each dot was marked in the form of numbers 1 to 8. The TLC plate is shown in Figure 1.

Figure 1. The TLC plate

A total of 10 µl of standard GABA solution was deposited or dropped on an aluminum TLC plate (Silica gel F254, Merck, Indonesia) at point number 1. Then, 1% MSG, the LAB isolate supernatant of RSP-A1, RSP-A2, and RSP-A3 sequentially dropped on the TLC plate. Then, TLC was run for 15 minutes. TLC was carried out using an eluent consisting of a mixture of n-butanol, acetic acid, and distilled water in a ratio of 5: 3: 2. The TLC plate was sprayed using a 0.5% ninhydrin solution and then heated at a temperature of 60ºC for 10 minutes. Spot GABA can be easily confirmed by comparing the spots obtained from the results of the study with the spots from standard GABA solution.

RESULTS AND DISCUSSION

Bacterial inoculation of isolates isolated from rusip was performed on NA and MRS agar medium. Four isolates namely RSP-A1, RSP-A2, RSP-A3, and RSP-A4 were obtained from the isolation on MRS agar medium. Results of the isolation of rusip in the MRS agar medium is presented in Figure 2. The bacterial isolates were then observed for the colony morphology, including the form, elevation, edge, and color of the colony. Result of observation of colony morphology is shown in Table 1.

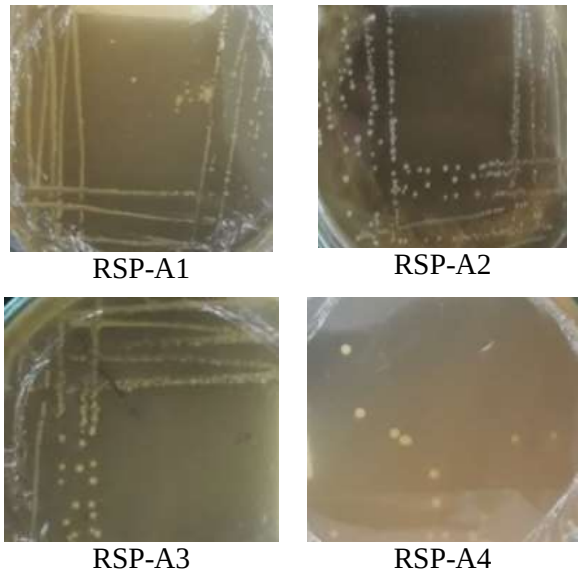

Figure 2. Bacterial colonies isolated from rusip in the MRS agar medium after 48 hours of incubation at 37^{o}C

Morphological observations of bacterial colonies showed that bacterial colonies have a circular form with convex elevation, and entire edges. The colors of the colony are yellowish white and milky white. It is assumed that all isolates are LAB because they have LAB morphological characteristics and are able to form clear zones on the medium. This is in accordance with the opinion of Ibrahim et al (2015) which stated that LAB has the characteristics of a milky white or yellowish white color, round form, flat edge, smooth glossy surface, and convex elevation. This is also consistent with Putri & Kusdiyantini research (2018) which stated that LAB has a round shape, flat-edged, convex elevation, and white or cream colored colony with a smooth glossy colony surface.

Table 1. Morphological characteristics of bacterial colonies isolated from rusip on MRS agar medium

Isolates Code	Morphological Characteristics			
	Form	Elevation	Edge	Color
RSP-A1	Circular	Convex	Entire	Yellowish White
RSP-A2	Circular	Convex	Entire	Milky White
RSP-A3	Circular	Convex	Entire	Milky White
RSP-A4	Circular	Convex	Entire	Milky White

The result of isolation in NA medium obtained four isolates of bacteria that were coded as RSP-B1, RSP-B2, RSP-B3, and RSP-B4. Isolation on NA medium was done to filter out bacteria that can not grow on MRS agar medium. The results of the inoculation of bacteria isolated from rusip in NA medium is presented in Figure 3.

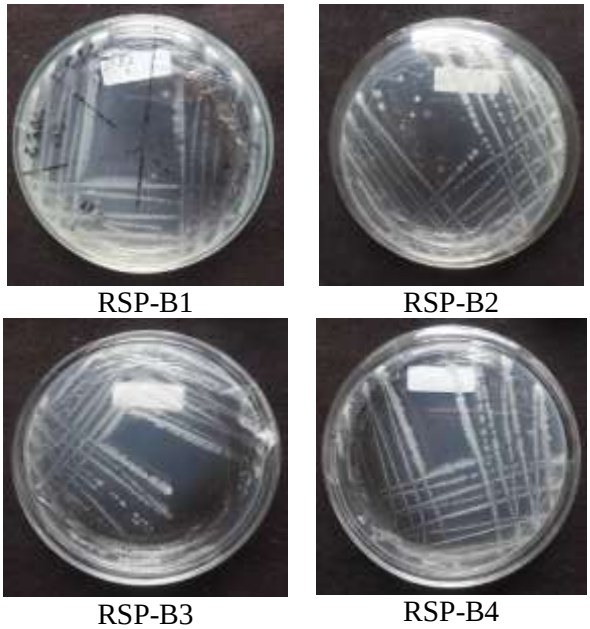

Figure 3. Bacterial colonies isolated from rusip in the NA medium after 48 hours of incubation at 37^{o}C

The bacterial isolates were then observed for the colony morphology, including the form, elevation, edge, and color of the colony. The result of observation of colony morphology is shown in Table 2.

Morphological observations of bacterial colony macroscopically showed that bacterial colonies have amoeboid, irregular, circular, and curled forms. The

bacterial colonies have convex elevation with white color and undulate edge. It is suspected that those bacteria are not a LAB, because the four isolates have wavy edges. This is in accordance with the opinion of Yolanda & Meitiniarti (2017) which stated that LAB has the characteristics of a milky white or murky white color, round form, flat edge, convex elevation, and smooth glossy surface. The results of Gram staining of bacteria isolated from rusip on MRS agar and NA medium are shown in Table 3.

Table 2. Morphological characteristics of bacterial colonies isolated from rusip in NA medium

Isolates Code	Morphological Characteristics			
	Form	Elevation	Edge	Color
RSP-B1	Amoeboid	Convex	Undulate	White
RSP-B2	Irreguler	Convex	Undulate	White
RSP-B3	Circular	Convex	Undulate	White
RSP-B4	Curled	Convex	Undulate	White

Table 3. Gram staining of bacteria isolated from rusip that grown on MRS agar and NA medium

MRS Agar Medium			NA Medium		
Isolates Code	Gram	Cell Shape	Isolates Code	Gram	Cell Shape
RSP-A1	(+)	Bacillus	RSP-B1	(-)	Coccus
RSP-A2	(+)	Bacillus	RSP-B2	(-)	Coccus
RSP-A3	(+)	Bacillus	RSP-B3	(-)	Coccus
RSP-A4	(-)	Bacillus	RSP-B4	(-)	Coccus

The results of Gram staining of isolates grown on MRS agar medium showed that all bacteria were in bacillus shape with three purple-colored isolates and one red-colored isolate. It is assumed that RSP-A1, RSP-A2, and RSP-A3 isolates are classified as LAB because the Gram staining results are Gram positive, while RSP-A4 isolate is not considered as LAB because Gram staining results are Gram negative. Mumtianah et al (2014) stated that LAB are Gram-positive with round or rod shaped, do not form spores, negative in catalase test, and microaerophilic groups. This is also consistent with Putri et al research (2014) which stated that Gram staining of bacteria isolated from rusip grown on MRS agar medium shows Gram positive bacteria in the form of bacillus and coccus cells. The results of Gram staining on bacteria grown on MRS agar medium are shown in Figure 4.

The results of Gram staining showed that all isolates grown on NA medium were red (Gram negative) and in the form of coccus cells. These four isolates were not LAB because they were included in the group of Gram negative bacteria. Yolanda & Meitiniarti (2017) stated that LAB are Gram-positive with round or rod shaped, do not form spores, and negative in catalase test. Gram staining of bacteria grown on NA medium is shown in Figure 5.

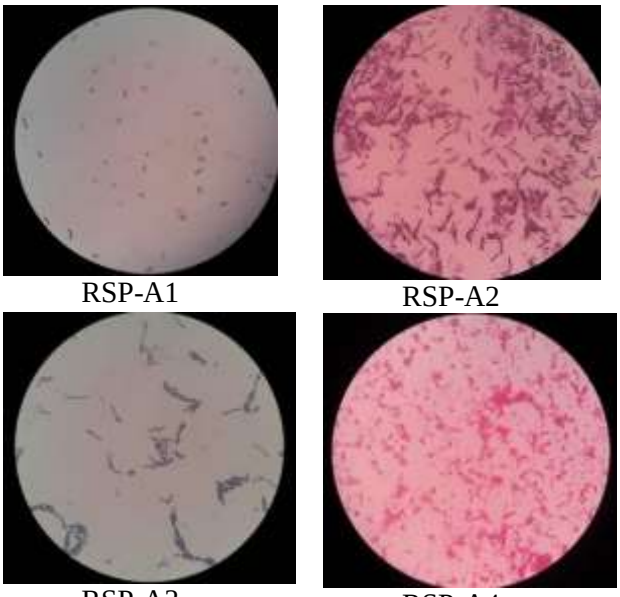

Figure 4. Staining of bacteria isolated from rusip that grown on MRS agar medium

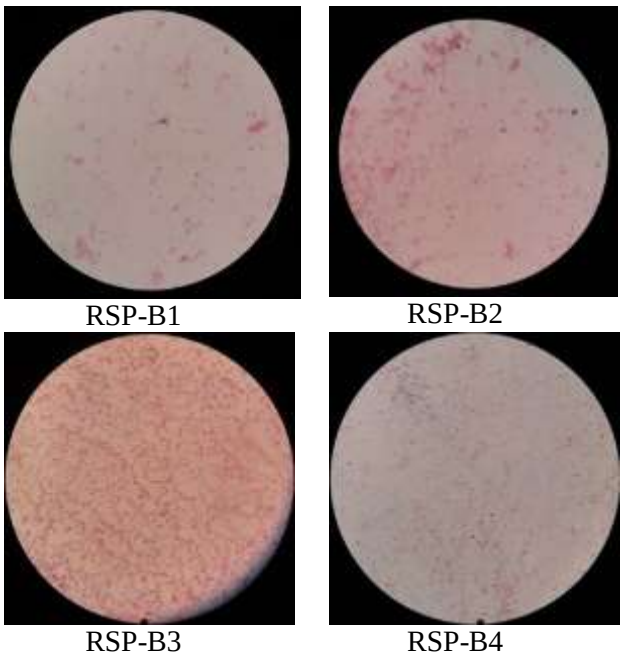

Figure 5. Staining of bacteria isolated from rusip that grown on NA medium

Catalase test is a test conducted to determine the presence of the catalase enzyme in an isolate. The nature of the reaction in the catalase test is determined by the appearance of gas bubbles which give an indication of the release of O_2 gas. Catalase test on isolates RSP-A1, RSP-A2, and RSP-A3 showed negative results in which gas bubbles were not formed on the objects glass containing bacteria when dripped with 3% H_2O_2. This shows that the bacteria do not

produce the catalase enzyme which can convert hydrogen peroxide into water and oxygen. Therefore, isolates RSP-A1, RSP-A2, and RSP-A3 are suspected to be LAB. Amaliah et al (2018) stated that the catalase test is a biochemical test that shows the activity of bacteria that produce the catalase enzyme. LAB are negative for the catalase test because they do not have this enzyme. This is consistent with Putri et al research (2014) which showed that the results of catalase tests on LAB isolates are negative. Catalase test on RSP-A4 isolates showed positive results in which gas bubbles were formed in the objects glass containing bacteria when dripped with 3% H_2O_2. This shows that the bacteria produce the catalase enzyme. Therefore, RSP-A4 isolate was thought not to be LAB. The results of the isolates catalase test are shown in Table 4.

Table 4. Characterization of catalase test, motility test, and fermentation type test results of bacteria isolated from rusip that grown on MRS agar medium

BAL Isolate	Catalase Test	Motility Test	Type Fermentation Test
RSP-A1	-	-	Homofermentative
RSP-A2	-	-	Homofermentative
RSP-A3	-	-	Heterofermentative
RSP-A4	+	-	-

Motility test is a test carried out to determine the ability of a bacteria to move on the semi-solid medium. Bacteria are said to be motile when their growth spreads on upright agar and grows around the site of inoculation. Bacteria are said to be non-motile when their growth is only centered on the site of inoculation and does not spread from the initial stab line. The motility test of the four bacterial isolates that were isolated from rusip showed negative or non-motile results. Elvira et al (2016) stated that the motility test aims to determine the motility or movement of a bacterium. This is consistent with Putri et al research (2014) which showed that the results of motility tests on LAB isolates are negative or non-motile. The results of the motility test of bacteria isolated from rusip are shown in Table 4.

Fermentation type test is a test conducted to determine the type of fermentation of LAB. Fermentation type test of RSP-A1 and RSP-A2 isolates showed that there was no gas trapped in the Durham tube. This shows that RSP-A1 and RSP-A2 isolates belong to a group of homofermentative LAB that only produce lactic acid from the fermentation process. Fermentation type test of RSP-A3 isolates showed that there was gas trapped in the Durham tube. This shows that RSP-A3 isolates belong to a group of heterofermentative LAB that produce lactic acid and other organic acids such as acetic acid, CO_2 gas, and ethanol from the fermentation process. This is in accordance with Romadhon et al research (2012) which showed that LAB isolated from shrimp intestines consist of homofermentative and heterofermentative bacteria. The results of the fermentation type test of bacteria isolated from rusip are shown in Table 4.

Making a growth curve aims to determine the length of the fermentation process needed to produce metabolite compounds. LAB growth curves of isolates RSP-A1, RSP-A2 and RSP-A3 are shown in Figure 6.

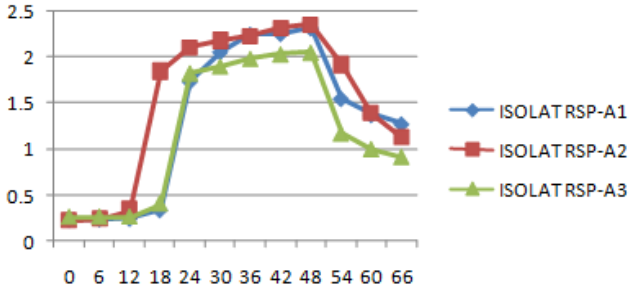

Figure 6. LAB growth curves of RSP-A1, RSP-A2 and RSP-A3 isolates

The lag phase of RSP-A1 and RSP-A3 isolates lasts for 12 hours, while the lag phase of RSP-A2 isolates lasts for 6 hours. In this phase, the bacterial cells are adapting to the substrate and producing metabolic enzymes. Sharah et al (2015) stated that microbes that are transferred into a medium will undergo an adaptation phase to adapt to the surrounding environmental conditions. The lag phase of the bacteria depends on the composition of the media, pH, temperature, aeration, and the physiological properties of microorganisms on the previous media.

The logarithmic phase of RSP-A1 isolates occurred at the 18-36 hours of incubation. The logarithmic phase of RSP-A2 isolates occurred at the 12-24 hours of incubation. The logarithmic phase of RSP-A3 isolates occurred at the 18-24 hours of incubation. In this phase, the bacterial cells are conducting metabolism by utilizing the available substrate. Sharah et al (2015) stated that in the logarithmic phase the bacterial cells will divide actively by utilizing the nutrients contained in the growth media used. The logarithmic phase of a bacterial cell is strongly influenced by temperature, pH, agitation speed, and dissolved oxygen level.

The stationary phase of RSP-A1 isolates occurred at the 36-48 hours. The stationary phase of RSP-A2 and RSP-A3 isolates occurred at the 24-48 hours. In this phase, the number of cell populations is fixed because the number of cells growing is the same as the number of cells that die. Mardalena (2016) stated

that the stationary phase occurs when the rate of bacterial growth is equal to the rate of death, so that the overall bacterial count will remain.

The death phase of RSP-A1, RSP-A2, and RSP-A3 isolates began at 48 hours. In this phase, the growth rate eventually decreases due to reduced nutrient levels and the accumulation of toxic products disrupting the process of cell division. Mardalena (2016) stated that the decline phase is characterized by an increase in the death rate that exceeds the growth rate so that it will cause a decrease in the growth curve.

Results of TLC analysis of RSP-A1, RSP-A2, and RSP-A3 isolates showed that GABA was detected in the presence of red spots. The Rf value of the standard GABA solution is 0.48. The Rf value of 1% MSG is 0.342. The Rf value of the RSP-A1 + 1% MSG isolate supernatant was 0.49. The Rf value of RSP-A1 non MSG isolate supernatant was 0.49. The Rf value of RSP-A2 + 1% MSG isolate supernatant was 0.48. The Rf value of the non-MSG RSP-A2 isolate supernatant was 0.48. The Rf value of RSP-A3 + 1% MSG isolate supernatant was 0.47. The Rf value of RSP-A3 non MSG isolate supernatant was 0.47. The function of MSG is as a substrate for the production of GABA by LAB. The isolates tested have an Rf value close to the Rf value of a standard GABA solution so that it can be concluded that the isolate is capable of producing GABA compounds. This is consistent with the results of research from Handayani et al (2016) which stated that the Rf value of the GABA standard is 0.48. MSG is as a substrate for the production of GABA. Indrowati et al (2015) stated that based on the results of observations of the TLC GABA chromatogram profile on ethanol extracts of *Artocarpus altilis* leaves, GABA were detected in samples with spot appearance at an average Rf of 0.49. Kook & Cho (2013) stated that TLC (Thin Layer Chromatography) and RP-HPLC (Reverse Phage High Performance Layer Chromatography) can be used for quantitative and quantitative analysis of GABA. The selection results of GABA-producing isolates are shown in Figure 7.

Research on GABA from bacteria isolated from rusip is a new thing in the world of GABA research. Research on rusip is usually limited to the process of isolation, characterization, and proximate analysis. While research on GABA is usually only limited to isolates isolated from plants. LAB isolates isolated from rusip are able to produce GABA maximally in the stationary phase. Information obtained from this research can help to produce GABA easily, cheaply, quickly, and efficiently.

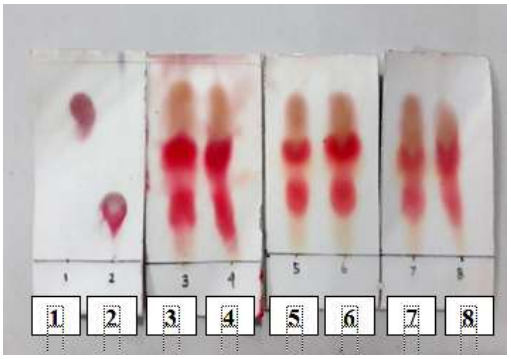

Figure 7. TLC analysis of LAB isolates cultured for 48 hours. Explanation: 1 = Standard GABA Solution; 2 = MSG 1%; 3 = RSP-A1 + MSG 1%; 4 = RSP-A1 Non MSG; 5 = RSP-A2 + MSG 1%; 6 = RSP-A2 Non MSG; 7 = RSP-A3 + MSG 1%; 8 = RSP-A3 Non MSG

CONCLUSION

Based on the research that has been done, it can be concluded that there are three LAB isolates isolated from rusip that can be grown on MRS agar medium, namely, RSP-A1, RSP-A2, and RSP-A3 isolates. All LAB isolates have milky white or yellowish-white colonies, round shape, flat edge, smooth glossy surface, and convex elevation. The bacteria are included in Gram-positive bacteria with negative catalase test, negative motility test, and have homofermentative and heterofermentative fermentation types. LAB isolates of RSP-A1, RSP-A2, and RSP-A3 are able to produce GABA compounds.

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