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Potential Probiotic from Indigenous Indonesian Red Passion Fruit (*Passiflora edulis* Sims)

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ABSTRACT

The present study aimed to characterize the potency of indigenous lactic acid bacteria (LAB) isolated from red passion fruit (Passiflora edulis Sims) as probiotic and evaluate their antibiotic sensitivity. More than 50 suspected LAB was isolated by selective medium of Man Rogosa Sharpe (MRS) agar. Identification of LAB was determined through the morphological, phenotype, and biochemical analysis. Ten isolates (MM1-MM10) were identified as LAB by further analysis of 16s rRNA. However only three isolates (MM1, MM2 and MM3) was indicated having probiotic characteristic; able to survive at low pH media, tolerance to salt and phenol. Three isolates (MM1, MM2, and MM3) were identified 16s rRNa with the results; Bacillus subtillis (MM1), Bacillus wiedmannii (MM2), and Bacillus cereus (MM3). In addition, those isolates also showed resistance against two antibiotics: erythromycin and vancomycin at 5 µg/mL and 2.5 µg/mL, respectively. Both concentrations were higher than minimum inhibitory concentration (MIC). MM1 showed higher susceptibility followed by MM2 and MM3 isolates. Compatibility of isolates (MM1, MM2, and MM3) has been investigated and they are compatible. Thus, red passion fruit can be considered as source of probiotic which resistant to pathogens and antibiotics.

Keywords: Antibiotic, Probiotic, Red passion fruit, Resistance.

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INTROCUCTION

Probiotics are defined as supplementary food products contained living bacteria that beneficialy affect to gastrointestinal (GI) host. It has been reported that probiotic helps to restore function of GI after being infected of GI disorders such as diarrhoea, dysentery and typhus [15]. Probiotics also protect GI from pathogenic bacteria by producing reuterin, bacteriocine, and organic acids (lactic acid and acetic acid) as bioactive compounds that inhibit growth of the bacteria. Indeed, organic acids have an effect on pathogenic bacteria by lowering the pH of GI and exhibit toxic effect on bacterial metabolism (reference). Therefore, tolerant in the pH of the probiotic's growth media has to be evaluated.

Lactic acid bacteria of certain species are non-pathogenic and belong to a group of bacteria that has a generally recognized as safe (GRAS) status which is usually used as a probiotic[4,12].A "characterizing bacterial culture that contains the lactic acid–producing bacteria Lactobacillus bulgaricus and Streptococcus thermophilus" such as yogurt is one of the subject of regulations of FDA[5].The majority of microorganisms used as probiotics are a group of lactic acid bacteria (LAB). The Lactobacillus species are a group of microorganisms that are most often used as probiotics, because of their health potential characteristics as probiotics[15]. The LAB such as Lactobacillus acidophilus, Lactobacillus delbrueckii spp. Bulgaricus and Bifidobacteria are important components of normal microflora in the digestive tract of animals and humans. These bacteria act also as immunemodulators through antimicrobial activity and as a mediator of Th 1-cytokines (IL-12, TNF-α, IFN-γ), anti-inflammatory and oral tolerance activities induced by Th2-cytokines (IL-10 and TGF - β), stimulates local and systemic adaptive immune (IgG and IgA)[6].

Naturally, LAB and probiotics are found in vegetables, fruits, fermented foods[1, 8, & 13] as multistrain[7] and produce synergistic effects that are beneficial to health of the host. Isolation and identification of the LAB from passion fruit and test its activity as an antibacterial have been reported[17].

Red passion fruit (Passiflora edulis Sims.) is one of traditionally fruits contain varieties nutrition and a unique fruit because of their varietas morphologically. There are green, yellow, orange, red and pink varietas of passion fruits, by which traditionally syrup was produced with a typical sour taste and smell. Previous studies reported that pulp of the passion fruits fermented in Man Rogosa Sharpe Agar (MRS) broth media contain LAB[14] and exhibited inhibitory activitiy against pathogenic bacteria[10]. The recent study carried out isolation, identification and characterization of probiotic properties of the LAB isolated from red passion fruit. The probiotics characterization was performed accordance with WHO[4], such as tolerant in acid pH (2.5 and 3), salt NaCl (1%, 4%, and 8%), and phenol. The probiotics shoud be resistance against vancomycin and erythromycin, the drug of choice antibiotics for Methicillin Resistance Staphylococcus aureus and or other Muti Drug Resistance (MDR) bacteria.

MATERIALS AND METHODS

The material or reagent used is red passion fruit pulp (Passiflora edulis Sims.) Obtained from the Krembung area (Sidoarjo-East Java) that has been identified by Herbarium Malangensis (University of Malang), a multistrain probiotic isolate from Passiflora edulis Sims., Media de Men Men Rogrosa Sharpe Broth (MRS-B), de Men Rogrosa Sharpe Agar (MRS-Agar), triple sugar iron agar (TSA) media, Sulfid Indol Motility

(SIM) media, MR-VP media (methyl red-voges proskaurt), media simmons citrate, kovac reagents, methyl red indicators, gram staining test materials (violet crystals, iodine lugol, safranin, 95% alcohol, and aquades), H2O2, indole reagents.
The research instruments used in this study include TC plate 24 well (SPL), glassware, filter paper, laminar air flow (SV 1200 SG), Biosafety cabinet (Sanyo), Hight speed micro refrigerated centrifuge or cold centrifuge (Tomy), microscope (Olympus), incubator (memmert), vortex (Phoenix RS-VA 10), kits for isolation and identification of bacteria genotypically (16s rRNA) (Qiagen).

Isolation of lactic acid bacteria

Isolation of LAB from red passion fruit was done by weighing 5 grams of red passion fruit pulp, dissolved in 45 mL of sterile solution of 0.9% NaCl, thenincubated in rotary shaker 150 rpm at 30°C for 24 hours. A serial dilution was made by transfering 1 mL ofthe pulp suspension into 9 mL of NaCl 0.9% to obtain 10-1 dilution. Furthermore, the dilution was continued up to 10-10. 1 mL of each dilution series (10-1 to 10-10) was transferred into sterile petri dish, added by MRS-agar media melted at 45-50°C swirred homogenously, then incubated at 37°C for 24 - 48 hours. Morphological of the bacteria were observed through its colony. Bacterial colonies suspected of being LAB were isolated and streaked on the MRS slant agar media, then incubated 24 hours at 37°C. The stock culture was used on further identification analysis of LAB.

Biochemical identification of lactic acid bacteria

Biochemical identification on the LAB isolate was performed according previous study[1,16].

The catalase tests

The catalase test was carried out by inoculating isolates on TSA media then incubated for 24 hours at 37°C. Furthermore, the isolate was dripped with hydrogen peroxide. Catalase test was done to demonstrate the ability of organisms to produce catalase enzymes that convert hydrograph peroxide into water and oxygen. Positive results were expressed in the presence of air bubbles.

The sulfide, indol, motility (sim) test

The LAB isolates were inserted into the SIM media using sterile Öse, then incubated for 24 hours at 37°C. Positive motility test was characterized by spreadingthe bacterial colony. The Indol test was carried out by adding Kovac reagents to isolates that had been incubated for 24 hours on SIM media. Positive indole test was characterized by the formation of red colourin the top layer of the media.

Methyl red vogesproskauer (MR-VP) test

The isolate was inoculated on MR-VP media, incubated at 37°C for 24 hours, and then methyl red reagent was added. A positive test was characterized by a change in the media to red colour, which indicated that acids were formed.

Triple sugar iron agar (TSIA) test

The isolate was inoculated on TSIA media, and then incubated at 37°C for 24 hours.

Simmons citrate test

The isolate was inoculated on Simmons citrate media and then incubated at 37°C for 24 hours. A positive test was indicated by changing the media to blue colour.

Probiotics characterization

1. ***Survival in acid test***
   The LAB isolates of 24 hours in MRS-Broth was inoculated in MRS-Broth as a control (1), in MRS-Brothjusted pH at 2.5 (2) and pH 3 (3) respectively. After incubating at 37°C for 120 minutes, then the cultures were inoculated in MRS-Agar, incubated at 37°C for 48 hours[8]. The colonies growth was observed.
2. **NaCl tolerance test**
   1 mL of LAB isolates of 24 hours at MRS-Broth was transfered into MRS-Broth (+ 1% NaCl), MRS-Broth (+ 4% NaCl), MRS-Broth (+ 6% NaCl), and MRS-Broth (+ 6.5% NaCl) respectively. After incubating for at 37oC for 24 hours, then the cultureswas inoculated on the MRS-Agar, and then incubated at 37°C for 48 hours. The colonies growth was observed[15].
3. **Phenol resistance test**
   One mL of the LAB isolates of 24 hours at MRS-Broth, was transfered into 5% of phenol solution then cultured on MRS-Agar, incubated for 48 hours at 37°C[1].

Molecular identification of 16s rRNA

Molecular identification of 16s rRNA to find out BAL strains were carried out by means of a colony polymerase chain reaction (PCR) by using primer 16s rRNA forward and reverse.

Susceptibility test against erythromycin and vancomycin

The antibiotics sensitivity test was performed by preparing the antibiotic test solutions of erythromycin and vancomycin each above the MIC of 5 ppm. Each antibiotic solution was mixed with MRS-Agar media melted 45-50°C in a sterile petri dish, after solidifying the agar, one ose of the LAB colony was streaked (1 cm) on the surface of the antibiotic containing agar media, and then incubated for 48 hours at 37°C. The colony growth was evaluated[9].

Compatibility test of three isolates (MM 1, MM 2, and MM 3)

Compatibility test of three BAL isolates of Passiflora edulis Sims which had probiotic characteristics using direct tests method by growing three BAL isolates (mixed cultured) on 4 mL of 10% skim milk, measuring the pH of the system, then incubated at 37°C for 24 hours, and measuring pH after incubation. The increase in acidity (increasingly acidic pH) in the growth media is a parameter of good interaction between the isolate mixture or the compatibility of the mixed culture[7].

RESULTS AND DISCUSSION

Based on the results of LAB isolation from red passion fruit pulp, three isolates of LAB candidates were obtained based on their morphological form (Fig. 1), Three isolates were chosen by those criteria; small, medium-sized colonies, convex elevation, flat edges, sparkling surfaces, milky white colour)[1]. Identification of phenotypic LAB isolated from red passion fruit was carried out by biochemical characteristic testing in accordance with Bergey's Manual of Determinative Bacteriology (Table 1). All isolates were fulfilled the characteristic as probiotic. They survived at low pH (2.5 and 3) and tolerated in both solution NaCL (1%, 4% and 8%) and 5% phenol (Table 2).
Molecular identification of 16s rRNA to find out BAL

strains were carried out by means of a colony polymerase chain reaction (PCR) by using primer 16s rRNA forward and reverse. Visualization performed by electrophoresis using 1.4% agarose with a voltage of 100v for 20 minutes. Predictable positive results BAL strain carrying the gtf gene is to produce an amplicon in size approximately 700pb (Fig. 2). The sequences obtained were then carried out blasts using NCBI blasts (https: //blast.ncbi.nlm.nih.gov/Blast.cgi). Blast results can be seen in Table 3, phylogenic tree of MM1 (Fig. 3), phylogenic tree of MM2 (Fig. 4), and phylogenic tree of MM3 (Fig. 5).

One of the most expected characteristics of a microorganism that can be considered potential as a probiotic is the ability to survive when the probiotic is given together with antibiotics. MM2 and MM3 isolates showed insensitive on both antibiotics. It was indicated by visible growth of their colonies in media containing the antibiotics at 5 ppm of erythromycin and 2.5 ppm of vancomycin (Fig. 6). Antibiotic susceptibility test of MM1, MM2, and MM3 against erythromycine at 5 ppm showed that survival of MM1 isolate was stronger than two other isolates, even MM1 almost sensitive againts the antibiotic (Fig. 7). All isolate was inoculated by streaking on the MRS media in sterile petri disk using negative control.

Each of the three isolates was inoculated to contact each other. if there is a clear zone in the intersecting area then it is not compatible. Based on the results of compatibility tests that have been done show that the intersection area of the three isolates has no clear zone (Fig. 8).

In general, probiotics must be surviving in the low enviromental pH and with stand gastric acidity, tolerant togeneral and bile salts in the digestive tract, and to be able to ferment oligosaccharides and provide clinical benefit assistance[4]. Corcoran et al. (2005) reported Lactobacillus rhamnosus GG survival in simulated gastric acid liquid pH 2. The L. rhamnosus GG survival in acidic conditions occurred only in the presence of sugars that it could metabolize efficiently. Therefore, tolerance to low pH is very important to evaluate the ability of probiotics in carbohydrate metabolism as an energy source for growth. The MRS media used in this study is composed of selective nutrition for LAB, but not all the LAB was survival in low pH condition. Optimization of carbon sources might be needed to improve the probiotics survival and growth in low pH conditions.

This study concerned with empowerment of local natural potency and exploration of probiotics from passion fruits, in which antibacterial activities substanes has been reported. The uniqueness of the passion fruit pulp that has many varieties and is rich in nutrients turns out to contain many lactic acid bacteria that are characteristic of probiotics that are actively resistant to the antibiotics like erythromycin and vancomicin. Both of these antibiotics are the drugs of choice for bacteria that cause infections which are clinically difficult to overcome, because of their resistance character.

Evaluation of the probiotics susceptibility that have the ability to produce various active compounds, especially as an antimicrobial, has been widely reported. The results of the study of Nurrosyidah et al. (2019) and Hamzah et al. (2019) have proven that passion fruit pulp has the potential to be developed as a source of antibacterial compounds and LAB with their metabolites as anti microorganims. Resistance of MM1, MM2, and MM3 against erythromycin and vancomycin was the important issues for developing the probiotic as supplement or complementary antibiotic drug therapy. Resistance to vancomycin by the Lactobacillus strain has been associated with the presence of D-Ala-D-lactate in its peptidoglycan and not the normal D-Ala-D-Ala dipeptide, which is the target of these antibiotics[11]. Bio-molecular identification of LAB isolates using melecular approaching 16 SrRNA is needed to determine the potential newly strains. Characterization of probiotic properties in term of resistancy to bile salt should be evaluation accordance with WHO[4]. Evaluation of suceptibility of the three probiotic isolates against vancomycin and erythromycin was done by twotimes replication and performed by the same condition.It was found that the different response among the isolates against erythromycin and vancomycin at 5 ppm and 2.5 ppm respectively might be caused by the different strain. Therefore, analysis of genomic profile will be expected to solve the problems. The methods should also be developed quantatively by serial or micro dilution.

In the future studies it will be very useful to investigate the susceptibility test on all probiotic isolates, followed by a compatibility test[7]. Therefore, multi-strain probiotics can be developed to increase the synergy of its activity as an antibacterial.

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TABLES AND FIGURES

Table 1. Biochemical Test Results Based on The Bergey Of Determinative Bacteriology Manual

Biochemical Test	**MM1**	**MM2**	**MM3**	**MM4**	**MM5**	**MM6**	**MM7**	**MM8**	**MM9**	**MM10**
Gram staining	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +	Gram +
Shape	Basil	Basil	Basil	Basil	Basil	Cocci	Basil	Basil	Basil	Basil
Catalase Test	-	-	-	-	-	-	-	-	-	-
SIM Test	-	-	-	-	-	-	-	-	-	-
MR-VP Test	+	+	+	+	+	+	+	+	+	+
Simmons Citrate Test	+	+	+	+	+	+	+	+	+	+
TSIA Test	+	+	+	+	+	+	+	+	+	+

Table 2. Probiotic Characteristic Test Results

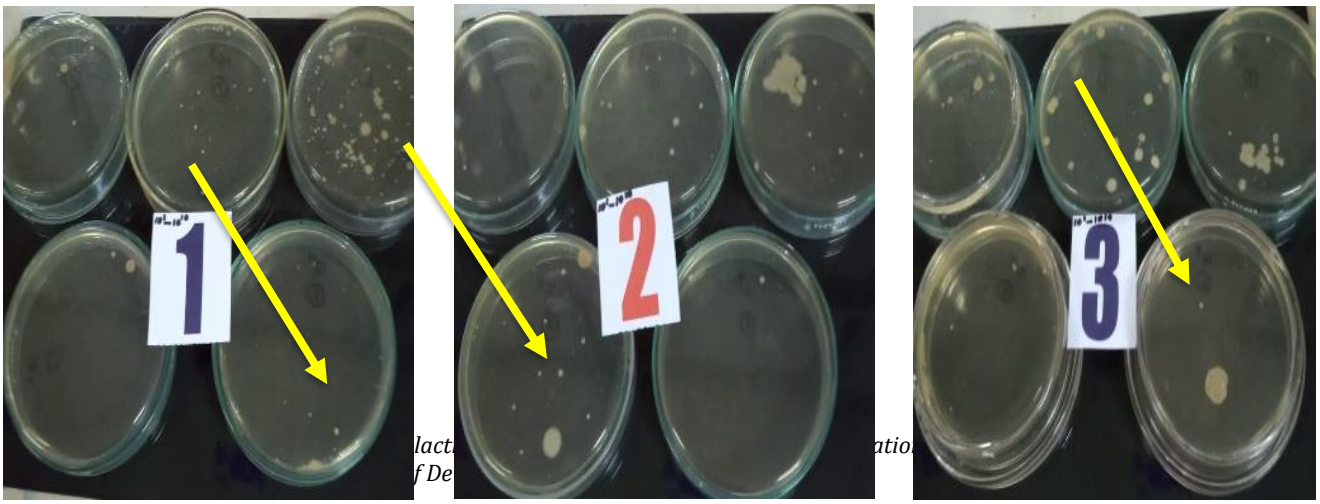
Isolates	**Acid Tolerance Test**		**NaCl Tolerance Test**			**Phenol Resistance Test**
	2,5	**3**	**1%**	**4%**	**8%**	
MM 1	R	R	R	R	R	R
MM 2	R	R	R	R	R	R
MM 3	R	R	R	R	R	R
MM 4	S	R	R	R	R	S
MM 5	S	S	S	S	S	S
MM 6	S	S	S	S	S	S

MM 7	R	R	R	R	R	S
MM 8	S	R	R	R	R	S
MM 9	R	R	R	R	R	S
MM 10	R	R	R	R	R	S

*) S = Sensitive
R = Resistance

Table 3. Identification 16s Rrna Test Results

Sample	Homolog (% Identity)	Identity
MM1	97,44	*Bacillus subtilis* strain IAM 12118
MM2	85,80%	*Bacillus wiedmannii* strain FSL W8-0169
MM3	100	*Bacillus cereus* ATCC 14579



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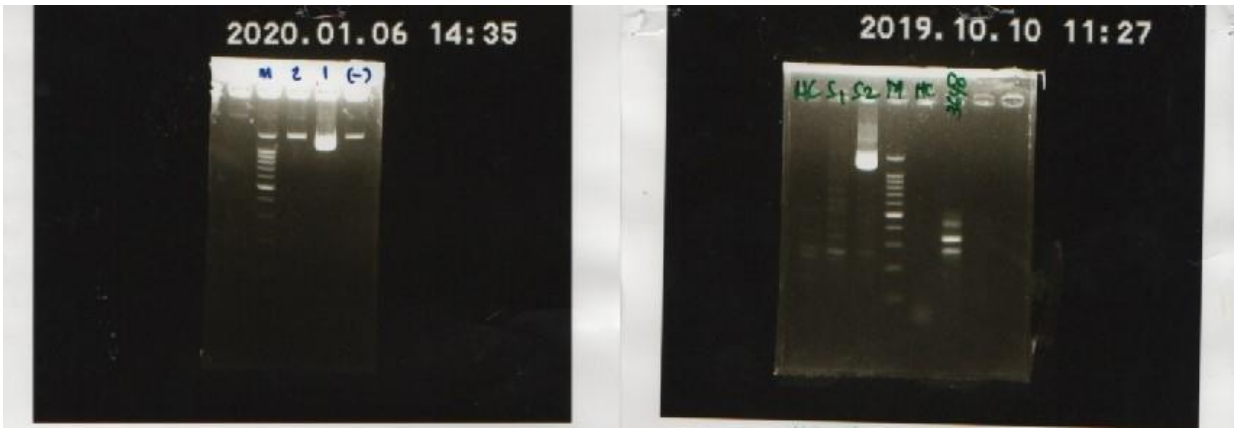

Figure 2. Polymerase Chain Reaction (PCR) Results of Isolates MM1(1), MM2 (2), and MM3 (M) (after amplification, the amplicon of three isolates (MM1, MM2, and MM3) are above 700)

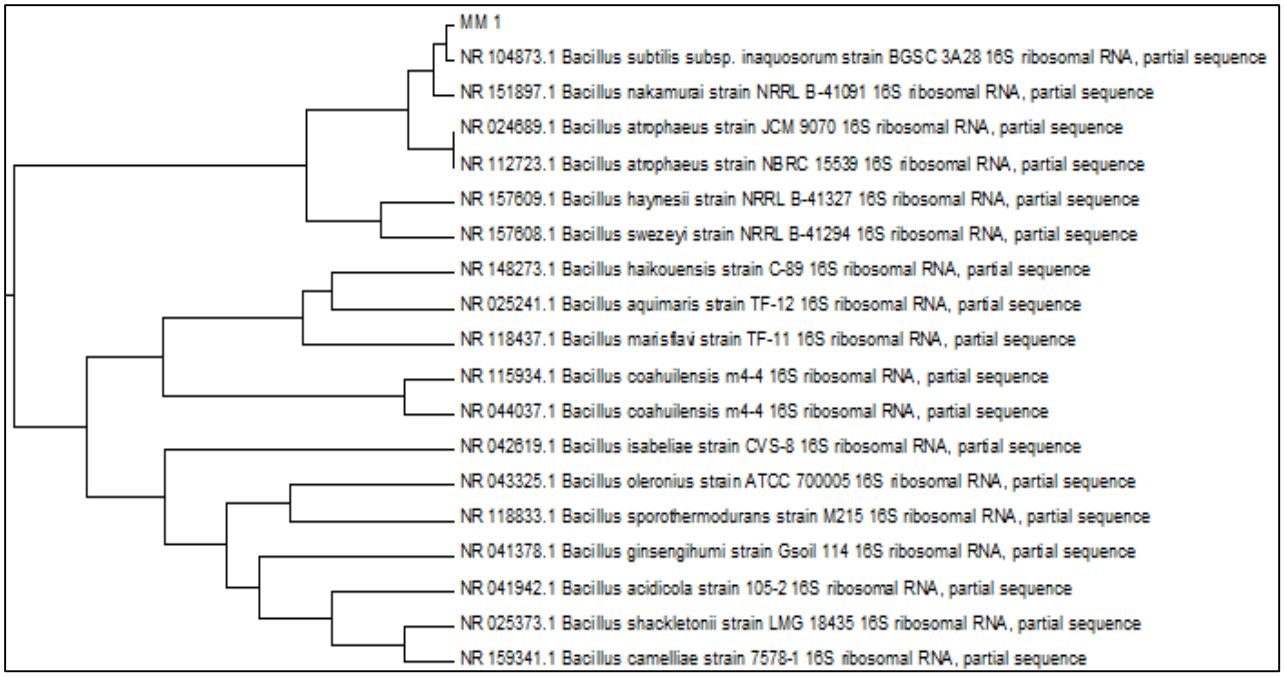

Figure 3. Phylogenic tree of MM1

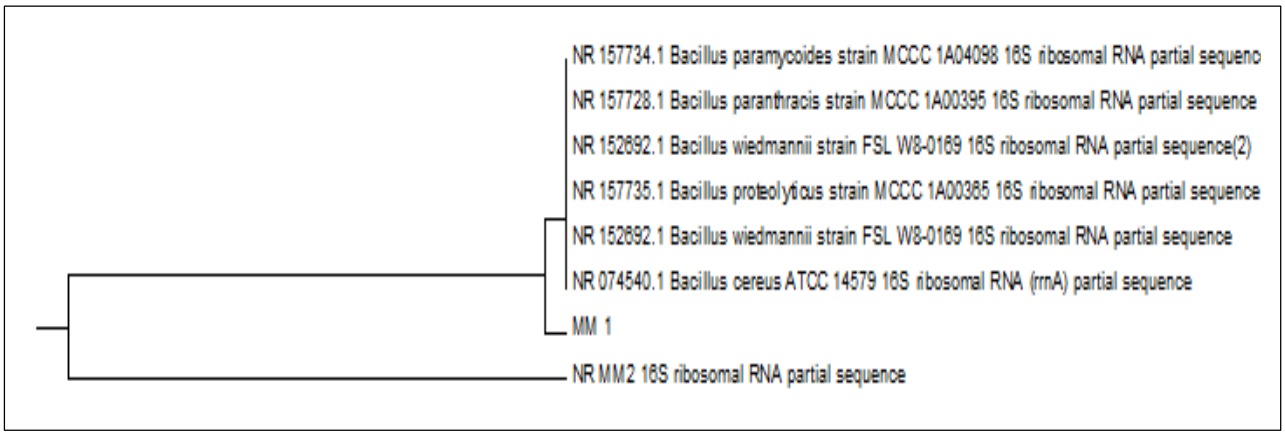

Figure 5. Phylogenic tree of MM3

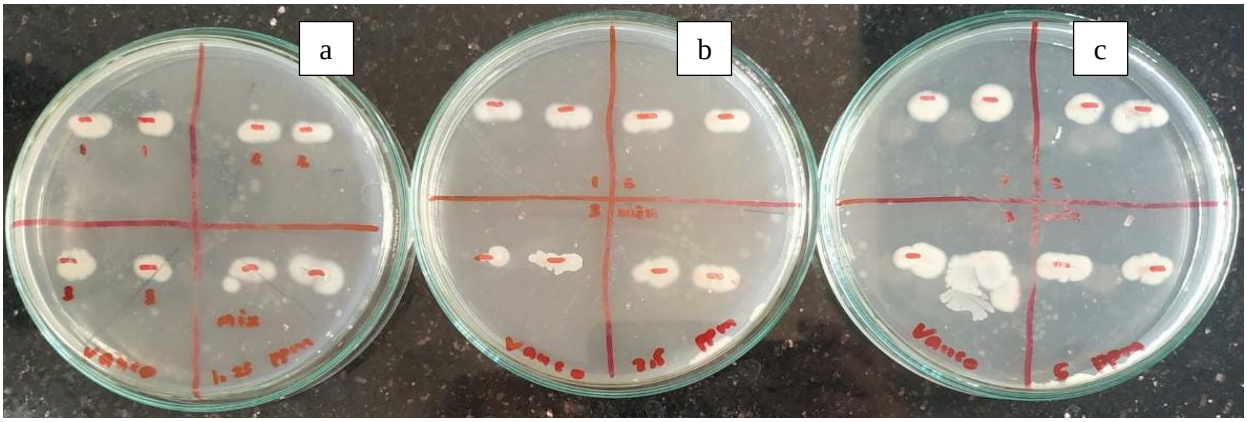

Figure 6. Susceptibility against vancomycin (a) 1,25 ppm, (b) 2,5 ppm, (c) 5 ppm

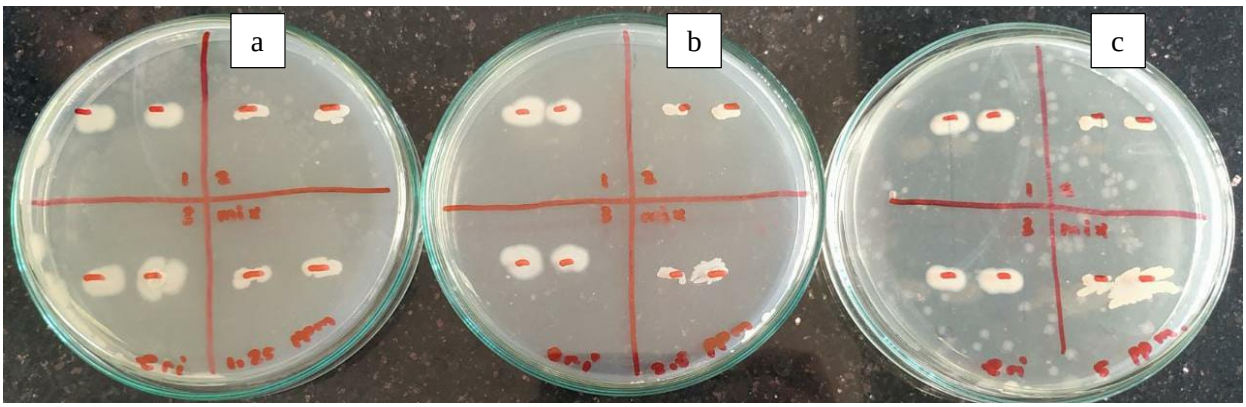

Figure 7. Susceptibility against erithromycin (a) 1,25 ppm, (b) 2,5 ppm, (c) 5 ppm

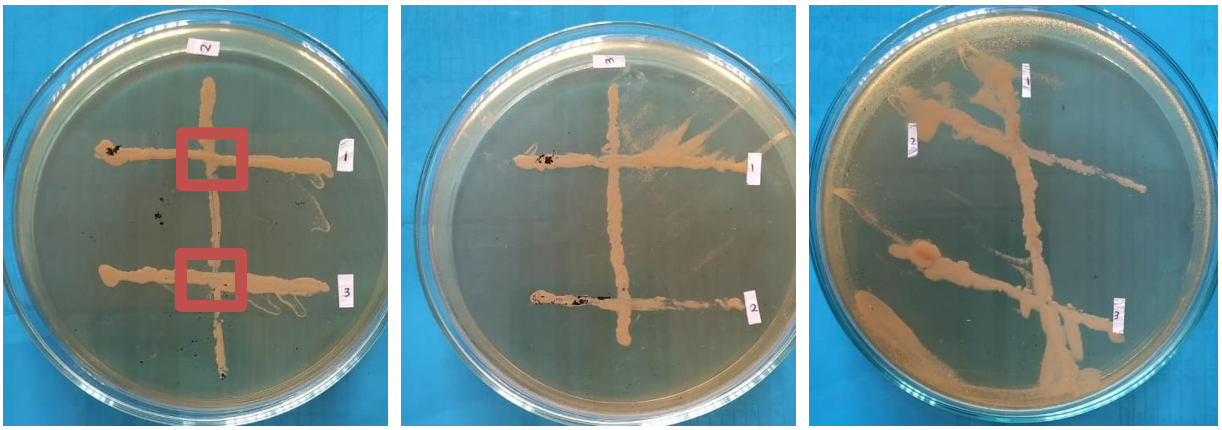

Figure 8. Compatibility test result between isolates (MM1, MM2, and MM3)