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Isolation and molecular identification of ethanol-tolerant *Acetobacter* species from *Lansium domesticum* (Dokong) and *Nephelium lappaceum* (Rambutan) vinegar

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ABSTRACT

Aims: Acetic acid bacteria (AAB) are a group of Gram-negative or Gram-variable bacteria that oxidise ethanol during the production of vinegar. The aim of this study was to isolate the AAB from both *Lansium domesticum* (Dokong) and *Nephelium lappaceum* (Rambutan), mother of vinegars (MV) and vinegars, as a potential starter culture for vinegar production.
Methodology and results: The MV and vinegar samples were collected from six to eight weeks of fermented Dokong and Rambutan vinegar from the Food Laboratory of Universiti Malaysia Kelantan (UMK), Jeli. The enriched samples were inoculated on selective Carr and GYC solid media. From the Carr medium, thirty-seven isolates that showed a yellow clear zone and seventy-eight isolates that showed a halo clear zone on the GYC medium were selected. Sixty isolates that produced higher total acidity (>60%) were characterized by Gram staining. Sixteen Gram-negative and fourteen Gram-variable isolates were subjected to 2.0% ethanol Carr medium to select for ethanol tolerance. Five ethanol-tolerant isolates were suitable for 16S rRNA gene sequence analysis and molecular identification because they had 4% to 10% ethanol tolerance level utilisation on Carr solid medium. Based on the morphological and biochemical characteristics, isolates DV1 and RMV30 were Gram-variable. Meanwhile, RMV2, RMV19 and RMV37 were Gram-negative bacteria. RMV2, RMV19, RMV30 and RMV37 isolates were catalase-positive and oxidase negative. Only DV1 was catalase and oxidase positive. From the BLAST analysis, the obtained nucleotide sequences showed 100% homology, with RMV2, identified as *Acetobacter fabarum*, and DV1, RMV19, RMV30 and RMV37 were identified as *A. pasteurianus*.
Conclusion, significance and impact of study: Based on 16S rRNA gene sequences, five isolates were identified as *Acetobacter* species: Four isolates, DV1, RMV19, RMV30 and RMV37 strains, were identified as *A. pasteurianus* and RMV2 was identified as *A. fabarum.* DV1, RMV2, RMV19, RMV30 and RMV37 showed significant differences at (p<0.05) for ethanol utilisation at 4% and the highest toleration up to an ethanol concentration of 10%. The ability to tolerate high ethanol concentration during vinegar fermentation is a desirable in producing high acetic acid for vinegar production.

Keywords: Acetobacter, ethanol-tolerant, 16S rRNA, vinegar

INTRODUCTION

AAB belongs to the Acetobacteraceae family. The main species responsible for producing vinegar belong to the genera such as *Acetobacter* and *Komagataeibacter* because of their high capacity to oxidise ethanol to acetic acid. They have high resistance to acetic acid release into the fermentative medium (Gomes *et al*., 2018). They are a large group of obligatory aerobic Gram-negative or variable bacteria and ubiquitously found in natural habitats, fruits, flowers, fermented food and beverages such as beer and wine that causes acidic and sour taste. They are either ellipsoidal or rod-shaped cells that can occur in single pairs or short chains and can grow and tolerate lower pH values 3-4 conditions (Mamlouk and Gullo, 2013).

They play important roles in food and beverage industries such as vinegar, water kefir, Lambic beer, nata, cocoa and kombucha (De Roos and De Vuyst, 2018). Vinegars are typically used for pickling easily perishable fruits and vegetables and for preparing mayonnaise, salad dressings, mustard, and other food condiments (Budak *et al.*, 2014). Fruit vinegars such as apple and grape vinegar have potential antimicrobial and antioxidants bioactive compounds and were found to be characterized by the type of raw materials (Kelebek *et al.*, 2017). Vinegars are famous for their general health benefit because of their therapeutic properties described

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by many researchers, including antimicrobial, antioxidative, antiobesity, anticarcinogenic, blood sugar control, cardiovascular and antihypertensive effects (Ozturk *et al.*, 2015). For example, cider vinegar has health-beneficial effects on consumers such as having antidiabetic effects and lowering cholesterol blood by inhibiting the oxidation of low-density lipoproteins (Ho *et al.*, 2017).

Vinegar fermentation has two stages of fermentation. The first stage involves breaking down of starch or sugars, such as grains or fruit juices, into alcohol via anaerobic fermentation by yeasts. The second stage involves oxidation of alcohol such as ethanol and sugar alcohol to acetic acid by AAB via aerobic fermentation (Mathew *et al.*, 2019). The acetic acid produced by AAB gives vinegar its characteristic aroma and determines the yield and quality of vinegar. The high ethanol-tolerant (ET) AAB was reported previously for high ethanol vinegar fermentation, using 8% of ethanol percentage (EP), to improve the flavour and quality of watermelon vinegar using isolated ET *A. pasteurianus* (Chen *et al.*, 2017).

Recently, the isolation of high-tolerant and fast-producing acetic acid AAB has been studied and used for the industrial production of acetic acid (Ouattara *et al.*, 2021). The bioproduction of acetic acid is seen as a potential industry for the development of an eco-friendly fermentation process as an alternative to chemical synthesis. In liquid media fermentation, *Acetobacter* forms a film or pellicle made of cellulose. It is hard to isolate the AAB as AAB and yeasts present in the fermentation broth get entangled in the cellulose pellicle to form a thick mat-like structure called the 'mother of vinegar' (MV). The MV was formed on the surface of vinegar during the fermentation process.

The aim of the study is to isolate the AAB from naturally fermented *Lansium domesticum* (Dokong) and *Nephelium lappaceum* (Rambutan) vinegar and MV of both fruits using selective Carr and GYC media and further enriched in a 2.0% ethanol and yeast extract broth medium to measure its total acidity (TA), total soluble solid content (TSS) and pH. For molecular identification, Gram-negative and Gram-variable isolates with high ethanol tolerance were identified by 16S rRNA gene sequence analysis.

MATERIALS AND METHODS

Sample collection

Four samples of Dokong and Rambutan vinegar with their MV were taken from 6-8 weeks of ten liters of oxidative and traditionally fermented vinegar of Dokong and Rambutan fruits in the Food Laboratory of UMK, Jeli. The samples were labelled as Dokong mother of vinegar (DMV), Dokong vinegar (DV), Rambutan mother of vinegar (RMV) and Rambutan vinegar (RV) and kept refrigerated at 4 °C. An *Acetobacteraceae* strain M1 (Mansor, 2012) given by MARDI (Malaysian Agricultural Research and Development Institute) acted as a control.

Enrichment culture

Yeast extract of 5% broth was prepared and enriched with 2% (v/v) of ethanol. One (1) mL of each sample DMV, DV, RMV and RV was individually inoculated in 200 mL enriched media in a 250 mL of conical flask aseptically. For a control, 1 mL of sterile distilled water was used. Cultures were incubated for three days in a rotary shaker at 30 °C agitated at 150 rpm. The presence of turbidity in the isolated growth was monitored using a spectrophotometer at 600 nm wavelength.

Preparation of Carr and GYC media

To prepare forty Carr solid medium plates in a litre of distilled water, 30 g of yeast extract, 20 g of agar and 0.02 g of Bromocresol green was autoclaved, and 20 mL of ethanol (99.8%) was added subsequently. In a litre of distilled water, 20 g of glucose, 10 g of yeast extract, 20 g of agar and 20 g of calcium carbonate were autoclaved for 40 plates of GYC medium preparation. Autoclaving was at a temperature of 125 °C for 20 min and 15 psi. Both media were poured onto sterilised plates. Serial dilution of each enriched sample was prepared. 100 µL of 10^{-6} sample was inoculated onto Carr and GYC media in triplicates. 100 µL of sample 10^{-8} of serial dilution were inoculated onto Carr and GYC media in triplicates. All the plates were incubated at 30 °C and the growth of colonies was observed after one to three days.

Isolation of pure culture colonies

Selected colonies were first transferred into a partition of either Carr or GYC medium to further observe the yellow and halo clear zones according to their respective media. Pure colonies confirmed to have a prominent yellow clear zone on Carr medium and halo clear zone on GYC medium were then streaked on Carr or GYC medium to obtain pure culture and incubated at 30 °C. Growth of colonies was observed after one to three days. Slant agar was prepared using either Carr or GYC media in Bijou bottles and left to solidify. Pure culture of bacteria was later inoculated in a slant medium accordingly to their previous medium for preservation at 4 °C.

Screening of isolates for ethanol utilisation and Gram staining

Each colony from 115 isolated colonies was inoculated in an enriched 2% (v/v) of ethanol and yeast extract (5%) broth medium for five days at 30 °C in a rotary shaker at 150 rpm. The total acidity (TA), total soluble solid (TSS) and pH were measured by total acidity refractometer, TSS refractometer and pH meter, respectively. Based on TA production (>60.0%), selected sixty isolates were subjected to morphological identification by the Gram staining method as described in Bergey's Manual Determinative Bacteriology. The morphology of isolates was examined under a light microscope at 1000× magnification.

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Screening for ethanol tolerance ability and biochemical characterisation

All sixteen Gram-negative and fourteen Gram-variable isolates were either from Carr or GYC media therefore, they were further observed for their ability to grow in supplemented 2% of ethanol on solid Carr medium. A single pure colony of two to three days of incubation was placed aseptically at the centre of a Petri dish. M1 with five identified ethanol-tolerant isolates were DV1, RMV2, RMV19, RMV30 and RMV37. The five identified isolates were selected for catalase and oxidase tests for biochemical characterization according to the standard guideline of Bergey's Manual Determinative Bacteriology.

Screening for different ethanol tolerance levels

The DV1, RMV2, RMV19, RMV30, RMV37 and M1 isolates were further screened for different ethanol concentrations 4%, 6%, 8%, 10% and 12% on solid Carr medium. In Sri Lanka, AAB species isolated from various sources were found could tolerate up to 10% of EP (Buddhika *et al.*, 2021). A single pure colony of two to three days of incubation of each isolate was picked up and inoculated at the centre of a Petri dish of solid Carr medium aseptically and incubated at 30 °C for ten days. On each day of incubation, the diameter of the yellow clear zone of the medium was measured. The growth medium without inoculation was kept as a negative control.

Molecular identification of ethanol-tolerant AAB by 16S rRNA gene sequence analysis

Only five bacteria with alcohol utilisation ability ranging from 4% to 10% were sent for molecular identification. The visible pure colonies on cut Carr medium (5 mm × 5 mm) at room temperature were prepared for each ET bacterial strain in triplicates. The control M1 from MARDI was also sent for molecular identification. The primer sequences for the amplification and sequencing of the 16S rRNA primers: forward primers 785f (5'-GGATTAGATACCCTGGTA-3') and reverse primer 907r (5'-CCGTCAATTCMTTTRAGTTT-3') (Mao *et al.*, 2012). PCR amplification for 16S rRNA gene sequences was performed and analysed by 1st Base Laboratory (Selangor, Malaysia). The laboratory provided the service of Bacterial DNA Barcoding, 16S rRNA full length (1500 bp). Then the complete sequence bases of PCR products were analysed by searching the homology alignment for 16S rRNA sequences of all isolates using the NCBI (National Center of Biotechnology Information) nucleotide database.

Phylogenetic analysis

Phylogenetic analysis of the M1, DV1, RMV2, RMV19, RMV30 and RMV37 16S rNA sequence was conducted with MEGA v11.0 software (Tamura *et al.*, 2021) using the neighbour-joining method (Kim *et al.*, 2020). Ten related species with ascension numbers for each AAB strain were selected based on their similar homology sequences generated from NCBI. The 16S rRNA gene sequences in Fasta format were aligned with related species 16S rRNA gene sequences to construct the phylogenetic tree. A bootstrap analysis with a thousand replicates was applied to estimate the confidence values of the tree nodes.

Statistical analysis

The diameter of yellow clear zones was subjected to statistical analysis in ANOVA one-way analytical statistics SPSS version 25. Analyses were conducted in triplicates. The Tukey test evaluated differences among the means and standard deviations at the significance level of $p<0.05$.

RESULTS AND DISCUSSION

Table 1 shows the number of isolates isolated from DMV, DV, RMV and RV. The isolates were observed and an M1 strain from MARDI acted as a positive control. Thirty-seven isolates changed the Carr medium colour from green to yellow, indicating acidic condition as the bromocresol green acted as a pH indicator. Meanwhile, seventy-eight isolates from the GYC medium formed a halo clear zone caused by hydrolysis of $CaCO_3$ by acid released around the spotted bacteria colonies. Carr medium was used because of incorporated ethanol; meanwhile, GYC medium incorporated glucose that provided a different type of carbon source for AAB growth. The changes were observed after 2 to 3 days, as shown in Figure 1. A smaller number of isolates was from Dokong; twenty-four isolates isolated only from DMV GYC medium and seventeen were found in DV compared to forty-seven from RMV and twenty-seven from RV. In a previous study in Thailand, isolation and characterisation of AAB from various fruits showed that no *Acetobacter* species were isolated from longkong (Dokong) fruit, indicating its presence was not dominant in the fruit (Klawpiyapamornkun *et al.*, 2017).

The morphology of colonies was smooth, either circular or irregular in shape and creamy in colour on GYC medium. Meanwhile, on Carr medium, the morphology of colonies was yellowish, irregular, and turned dark blue at the end of incubation. Sixty isolates that produced TA above 60.0% were selected and subjected to Gram staining. In Table 2, the Gram staining result showed twenty-seven isolates were Gram-positive,

Table 1: Number of isolates on Carr and GYC media having yellow and halo clear zones.

Samples	Carr	GYC	Total of isolates
DMV	-	24	24
DV	6	11	17
RMV	7	40	47
RV	24	3	27
Total	37	78	115

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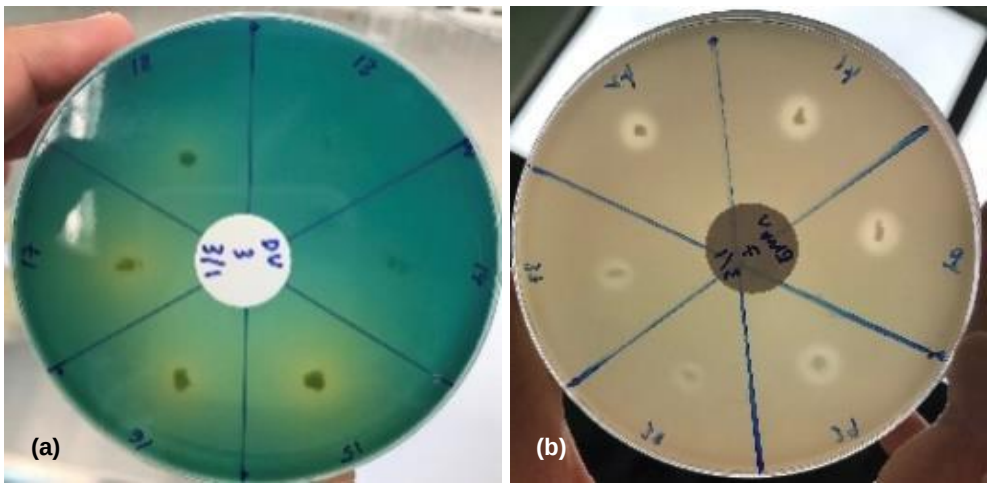

Figure 1: Screening of acetic acid-producing microorganisms on Carr (a) and GYC (b) solid plates. Yellow formation and halo clear zones around the colonies of isolates indicate acid production.

Table 2: Summary of sixty isolates after Gram staining from Carr and GYC media.

Medium	Sample	Gram staining			
		Gram-positive	Gram-negative	Gram-variable	Budding yeast
Carr	DV	5	-	1	-
	RMV	6	3	1	-
	RV	3	3	2	-
	Total	14	6	4	-
GYC	DMV	7	2	4	-
	DV	2	-	1	-
	RMV	2	5	3	-
	RV	2	3	2	3
	Total	13	10	10	3
		27	16	14	3

sixteen isolates were observed as Gram-negative, fourteen isolates were Gram-variable and three budding yeasts. In the previous study, AAB was either Gram-negative or Gram-variable bacteria (Sengun *et al.*, 2022). Therefore, sixteen Gram-negative and fourteen Gram-variable colonies were suspected to be Acetobacteraceae family members. Twenty isolates from all thirty Gram-negative and Gram-variable bacteria were from GYC medium and their ethanol utilisation was yet to confirm. The utilisation of 2% ethanol in solid Carr medium in a Petri dish was observed and compared to control M1. Only five isolates were confirmed to utilise the ethanol and changed the medium colour from green to yellow after 2 to 3 days of incubation at 30 °C. From five ET isolates, only RMV2 from Carr medium and DV1, RMV19, RMV30 and RMV37 from GYC medium. Hence, the GYC medium proved to be a more suitable medium for isolating ET isolates than the Carr medium. Five ET isolates, DV1, RMV2, RMV19, RMV30, RMV37 and control M1, were tested for their ability to grow further at 4%, 6%, 8%, 10% and 12% different EP. The acid production of five isolated ET strains and M1 were compared by observing and measuring the yellow clear zone diameter for ten days incubation period.

Figure 2 presented a graph mean of a clear zone (mm) of M1 and five ET isolates against different ethanol concentrations of 2%, 4%, 6%, 8%, 10% and 12% regardless of days of incubation. The overall range mean of the clear zone at 2% of EP was between 31.03-59.00 mm, at 4% of EP 35.77-77.13 mm, at 6% of EP 37.57-56.47 mm, at 8% of EP 37.20-50.20 and 10% of EP 0.00-47.33 mm. No growth and zero clear zones were observed on 12% of EP for all five ET strains and M1. At 4% EP, it showed the highest overall mean of clear zone values for five ET isolates. Meanwhile, 10% of EP showed the lowest overall mean of clear zone values for all five ET isolates. The graph clearly showed the highest mean of a clear zone for DV1, RMV2, RMV19, RMV30 and RMV37 was best at 4% of EP compared to other 2%, 6%, 8% and 10% EP. M1 showed lower ethanol tolerance level values compared to five ET isolates, and its best was at 8% EP. There was no M1 growth at 10% and 12% EP. In Thailand, the AAB isolates from various kinds of fruits and fermented juices found that isolates were able to tolerate and grow at 4% and 6% of EP (Klawpiyapamornkun *et al.*, 2017). In Sri Lanka, AAB species isolated from various sources were found could tolerate up to 10% of EP (Buddhika *et al.*, 2021). Hence,

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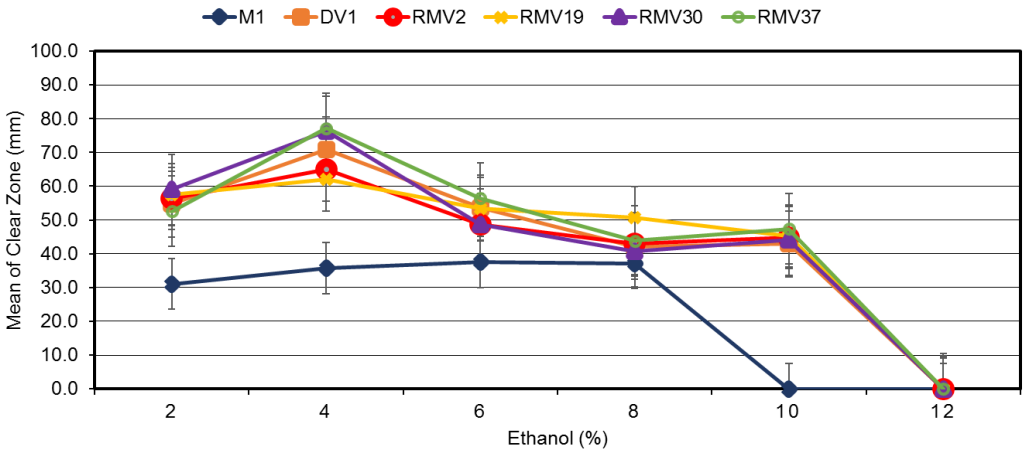

Figure 2: A graph showed the mean of the clear zone of a control M1 and five ethanol-tolerant isolates DV1, RMV2, RMV19, RMV30 and RMV37 at different ethanol concentrations 2%, 4%, 6%, 8%, 10% and 12% regardless days of incubation.

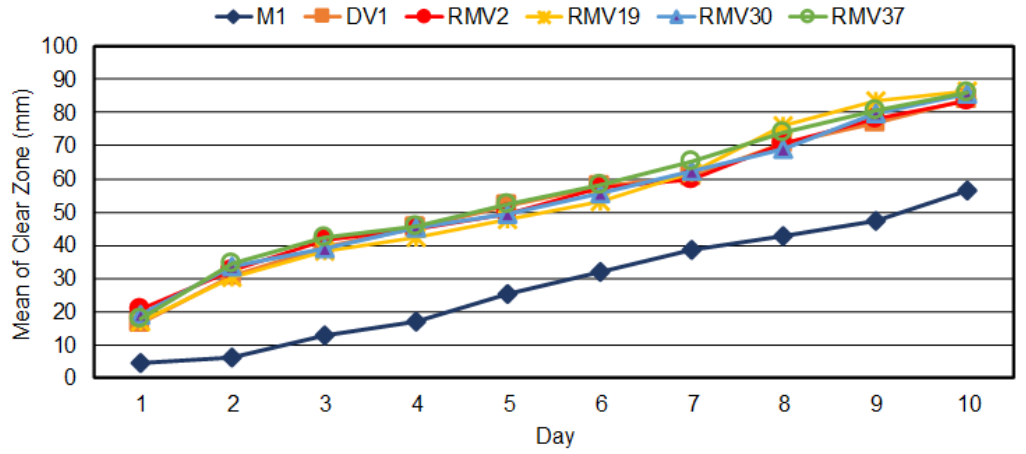

Figure 3: Mean of clear zone (mm) of M1 and five ethanol-tolerant isolates against days of incubation regardless of ethanol percentages 2%, 4%, 6%, 8% and 10%. DV1, RMV2, RMV19, RMV30 and RMV37 showed higher clear zone means than control M1.

the finding of this study showed that the five ET isolates were probably from AAB family members.

Most AAB share a common trait that is able to oxidise ethanol to acetic acid (Diba *et al.*, 2015). Therefore, these ET isolates were further tested for their ability to utilise ethanol as their carbon source. Figure 3 presented the mean of clear zone values in mm for M1, DV1, RMV2, RMV19, RMV30 and RMV37 against days of incubation in Carr medium. The overall range mean of a clear zone for M1 was between 4.67-56.67 mm, for DV1 was between 16.67-84.00 mm, for RMV2 was between 20.53-83.60 mm, RMV19 was between 17.07-86.27, RMV30 was between 19.20-85.47 and RMV37 was between 17.73-85.87 mm. M1 showed a lower overall value compared to the overall values of DV1, RMV2, RMV19, RMV30 and RMV37. Analysis of the Tukey test revealed there was a significant difference mean of the clear zone between five ET strains, DV1, RMV2, RMV19, RMV30 and RMV37 at $p<0.05$ compared to the M1 strain. Thus, the ET isolates in this study were more vigorous in utilising the ethanol incorporated into a solid Carr medium than M1.

Table 3 shows the results of the morphological, physicochemical and biochemical characteristics of five ET isolates DV1, RMV2, RMV19, RMV30 and RMV37, and control M1. Microscopic observation showed that five ET isolates were rod-shaped bacteria. DV1 and RMV30 were Gram-variable bacteria, while RMV2, RMV19 and RMV37 were Gram-negative bacteria. Meanwhile, control M1 was rod-shaped and Gram-negative bacteria. All the bacterial cells were short rod-shaped and occurred in single or short chains. According to previous studies, the *Acetobacter* was either ellipsoidal or rod-shaped, and cells occur singly in short chains and occasionally long chains (Matsushita *et al.*, 2016).

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Table 3: Type of medium, Gram staining, total acidity (TA), pH and total soluble solid (TSS), catalase and oxidase. Results of a control M1 and five ethanol-tolerant bacteria DV1, RMV2, RMV19, RMV30 and RMV37.

	Medium	Gram stain	TA (%)	pH	Total soluble solid (°Brix)	Catalase test	Oxidase test
M1	Agar	G-ve	62.30 ± 0.58^{a}	3.35 ± 0.05^{bc}	10.73 ± 0.06^{bc}	+	-
DV1	GYC	G-var	98.30 ± 0.15^{e}	3.25 ± 0.05^{abc}	10.83 ± 0.06^{c}	++	+
RMV2	Carr	G-ve	64.00 ± 1.00^{a}	3.40 ± 0.10^{c}	10.10 ± 0.10^{a}	+	-
RMV19	GYC	G-ve	81.80 ± 0.76^{b}	3.18 ± 0.08^{ab}	10.65 ± 0.05^{b}	++	-
RMV30	GYC	G-var	93.10 ± 0.90^{d}	3.35 ± 0.05^{bc}	10.83 ± 0.06^{c}	++	-
RMV37	GYC	G-ve	84.80 ± 0.76^{c}	3.08 ± 0.08^{a}	11.07 ± 0.06^{d}	++	-

Note: G-ve = Gram-negative, G-var = Gram-variable, + = positive, ++ = strong positive.
Values are expressed as mean ± SD of triplicates measurements. ^{abcde} = different superscripts signify significant mean differences at $p<0.05$ between a Control M1 and five ethanol-tolerant isolates DV1, RMV2, RMV19, RMV30 and RMV37.

The overall TA range values of five ET isolates and M1 were between 62.00-98.30%. TA was significantly different ($p<0.05$) within the five ET isolates. TA of DV1 and RMV30 have the highest values of 98.30% and 93.10%, respectively. RMV37 was 84.80%, RMV19 was 81.80%. Meanwhile, RMV2 was 64.00% and M1 was 62.30% showing among the lowest TA values. The overall pH range values of five ET isolates and M1 were between 3.08-3.40%. There was no significant difference ($p<0.05$) in pH between five ET isolates and M1. A low pH range of 3.0 indicated that the broth culture was acidic, and this showed the relation between pH as an indicator and acids produced after incubation. Acid presence had a direct relationship with pH. Its high tolerance to acidic conditions suggested that the colonies' growth was potential AAB strains. The AAB are known for their capability of converting ethanol into acetic acid, and high acidic conditions at the final stages of vinegar fermentation showed their presence in the vinegar (Luzón-Quintana *et al.*, 2021). The overall range of TSS values of five ET isolates and M1 was between 10.10-11.07°Brix. The amount of soluble solids values showed no significant difference ($p<0.05$) between the five ET isolates and M1. The five ET isolates had similar TSS, and pH values characteristics compared to M1. However, the ET isolates showed higher TA values than M1 and showed their characteristic of producing more acid compared to M1. Thus, this showed that the five ET isolates confirmed their characteristic of AAB preferring ethanol in producing acetic acid.

According to the result shown in Table 3, all five ET isolates were catalase positive. RMV2 showed less catalase reaction strength than DV1, RMV19, RMV30 and RMV37, which showed stronger catalase reactions. RMV2, RMV19, RMV30 and RMV37 were catalase-positive and oxidase negative. These ET isolates were catalase-positive and oxidase negative and were agreeable to the research findings of *Acetobacter* genus biochemical characteristics (Outtara *et al.*, 2021). However, DV1 was positive for both catalase and oxidase tests. Hence, it was differed from the other four ET isolates. The only ET isolate used the cytochrome system, which was usually only present in aerobic organisms capable of utilising oxygen as the final hydrogen receptor. The metabolism's end product was either water or hydrogen peroxide which was later broken down by the catalase enzyme in the bacterial cells (Chavan *et al.*, 2022).

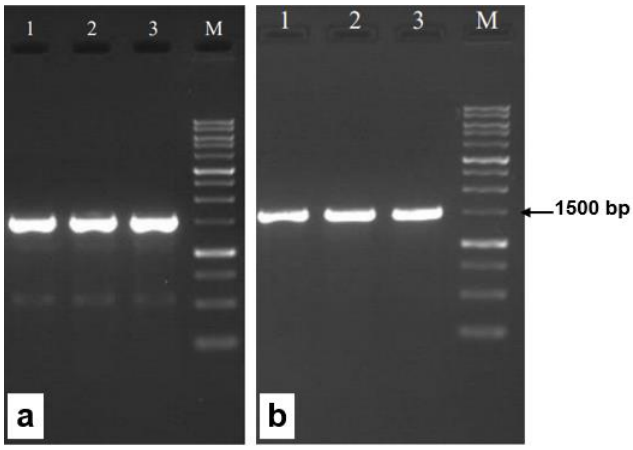

Figure 4: PCR product of amplified 16S rRNA (a) three isolates; M: Ladder, 1: RMV2, 2: RMV30, 3: RMV37 and (b) a control and two isolates; M: Ladder, 1: M1, 2: DV1, 3: RMV19.

The preliminary identifications according to ethanol tolerance range from 4% to 10% and morphological, physicochemical and biochemical tests brought about the possibility of selected ET isolates belonging to the *Acetobacter* genus. The 16S rRNA gene of isolates was successfully amplified by polymerase chain reaction (PCR). After amplified, five isolates and control M1 presented a clear band and Figures 4(a) and 4(b). Thus, these were complementary to the size of 16S rRNA (~1500 bp).

Results obtained after sequencing made it possible to establish the consecutive alignment of the nucleotide sequences 16S rRNA of five ET isolates and M1. A comparison of the nucleotide sequences of isolates showed a rate of homology of 100%. Percent identity reflected the highest percent of relatedness to the closely related species registered in the *Acetobacter (A.)* and *Komagataeibacter* (*K.*) genera, as presented in Table 4. The sequence of DV1, RMV19, RMV30 and RMV37 genes of isolated ET bacteria showed 100% similarity to

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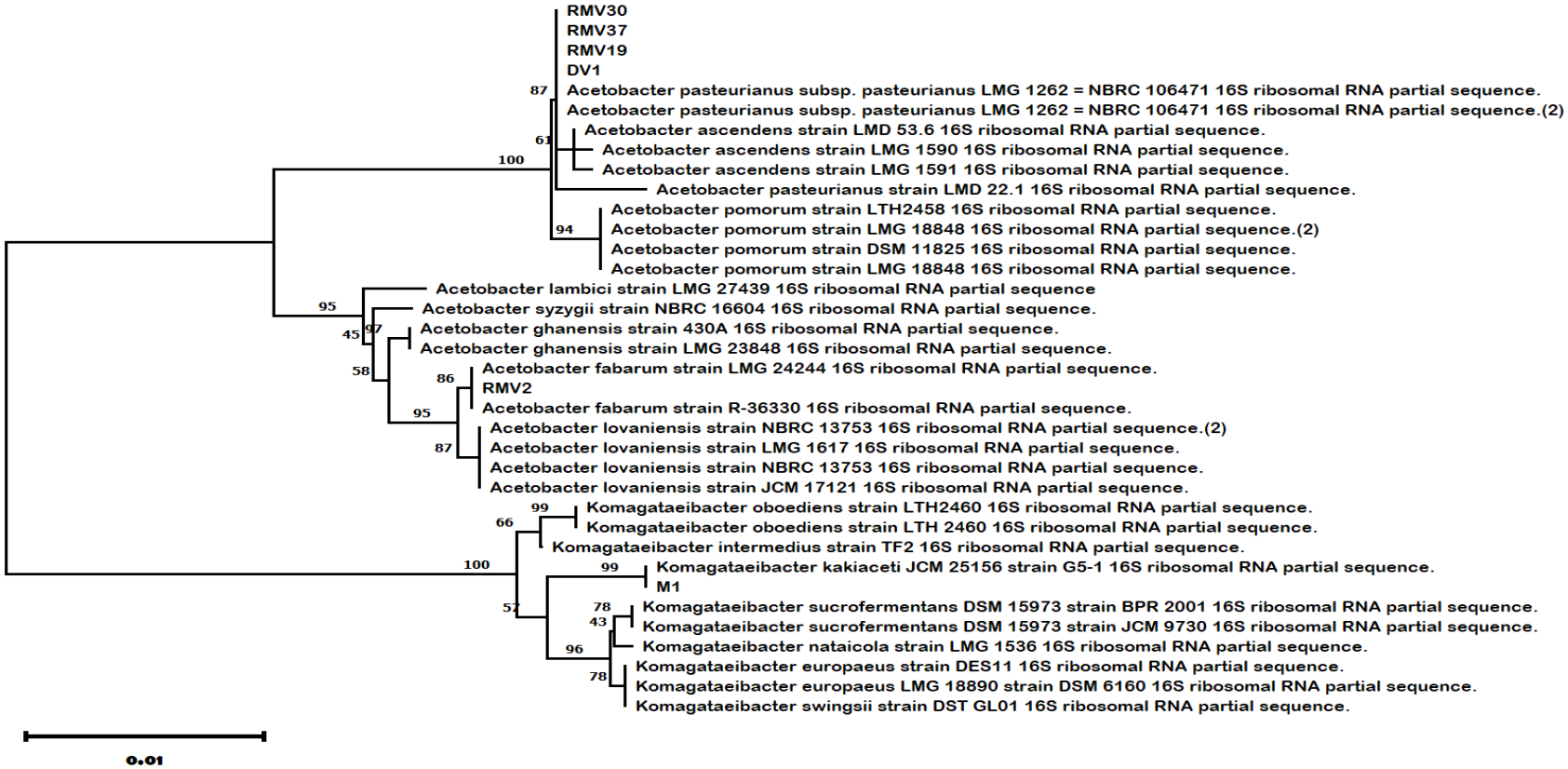

Figure 5: A phylogenetic tree showing relationships between control M1, five ethanol-tolerant isolates DV1, RMV2, RMV19, RMV30 and RMV37 and related species. The tree was constructed from an alignment of a full-length sequence of 16S rRNA from various species using the neighbour-joining method. The number on the nodes corresponds to the bootstrap percentages based on 1,000 pseudoreplicates. The bar (——) denotes the relative branch length.

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Table 4: Bacteria identification based on NCBI BLAST results for the 16S rRNA gene sequences of a control M1 and five ethanol-tolerant isolates DV1, RMV2, RMV19, RMV30 and RMV37.

	Closest species based on 16S rRNA sequence	Accession number	Identity %
M1	*Komagataeibacter kakiaceti*	NR113301	100
DV1	*Acetobacter pasteurianus*	NR117258	100
RMV2	*Acetobacter fabarum*	NR042678	100
RMV19	*Acetobacter pasteurianus*	NR117258	100
RMV30	*Acetobacter pasteurianus*	NR117258	100
RMV37	*Acetobacter pasteurianus*	NR117258	100

A. pasteurianus gene sequence in the NCBI database. Meanwhile, the sequence of the RMV2 gene of the isolated ET bacterium showed 100% similarity to *A. fabarum* gene sequence in the database. The sequence of the control M1 gene showed 100% similarity to the *K. kakiaceti* gene sequence in the database. Hence, *Acetobacter* and *Komagataeibacter* were from Acetobacteraceae family, commonly found in fermented vinegars (Saichana *et al.*, 2015). This finding highlighted that the *Acetobacter* genus was involved in fermenting of fruit Dokong and Rambutan vinegars. *Acetobacter pasteurianus* genus is also known as vinegar bacteria (Sengun *et al.*, 2022). Therefore, the presence of RMV19, RMV30 and RMV37 *A. pasteurianus* in MV of Rambutan showed this genus was commonly found and active in Rambutan vinegar fermentation. They were resistant to the acidic environment as low as pH 3.08.

The phylogenetic tree illustrated in Figure 5 used the gene sequences that had the nearest similarity alignments of the registered bacterial 16S rRNA nucleotide sequences from the database. It showed all the ET strains isolated were in a Acetobacteraceae family. Four ET strains DV1, RMV19, RMV30 and RMV37 were located within *A. pasteurianus* group, RMV2 was identified as *A. fabarum* and M1 was identified as *K. kakiaceti*. From previous studies, *A. pasteurianus* was first isolated by Hansen in 1889 from a beer. Meanwhile, as stated by Matsushita *et al*. (2016), *A. fabarum* was isolated by Cleenwerck in 2008 from a Ghanian cocoa bean heap fermentation and *K. kakiaceti* was isolated by Iino *et al*. in 2012 from kaki vinegar in Japan.

The Neighbour-joining method was to see the relatedness of the 16S rRNA genetic sequences. DV1, RMV19, RMV30 and RMV37 ET isolates were resolved together as *A. pasteurianus* strains with 87% bootstrap support. Meanwhile, RMV2 isolate was separated from other isolates with 86% bootstrap support *A. fabarum* and M1 as a *K. kakiaceti* with 99% bootstrap support. Hence, five ET acetic acid bacteria isolated from Dokong and Rambutan vinegar were taxonomically assigned to the family Acetobacteraceae members. Four ET DV1, RMV19, RMV30 and RMV37 were identified as *A. pasteurianus* and RMV2 was identified as *A. fabarum*.

CONCLUSION

In this study, AAB were isolated from MV of Dokong and Rambutan vinegar. The enrichment using ethanol medium, GYC and Carr solid media had proven effective for selecting high ethanol tolerance level AAB from fruit vinegars. The identified RMV2 (*A. fabarum*), DV1 (*A. pasteurianus*), RMV19 (*A. pasteurianus*), RMV30 (*A. pasteurianus*) and RMV37 (*A. pasteurianus*) had 100% percentage identity of respected *Acetobacter* sp. They were able to utilise the ethanol until 10%, and at best, 4% of ethanol concentration. The AAB ability to tolerate high ethanol levels is an essential factor in producing high acetic acid in vinegar fermentation. In future work, these strains will be used as a potential starter culture using Dokong and Rambutan substrates, and their percentage of acetic acid production will be quantified using the HPLC method.

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