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Research Article

Simultaneous alcoholic and acetic acid fermentation in pineapple juice with co-culture of yeast and acetic acid bacteria

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Abstract - Co-culture of yeast and acetic acid bacteria (AAB) is consortium which be useful on food and beverage fermentation. The various fermented beverages; alcoholic and non-alcoholic drinks, are obtained by these microbial communities and their interactions. The activities of co-culture of yeasts and acetic acid bacteria during pineapple juice fermentation, including the growth of starters, physicochemical and key compounds changes during pineapple juice fermentation were investigated. The specific characteristics of initial pineapple juice was appropriate for using as a raw material for single and dual yeast and AAB fermentation with no chaptalization. The adding of AAB could encourage the growth and ethanol production of allochthonous and autochthonous yeasts during pineapple juice fermentation, concurrently, the growth of AAB slowly decreased throughout the fermentation. In order to better understand the relationship between yeast and AAB during pineapple juice fermentation. The influence of pineapple juice properties on the growth of AAB during pineapple fermentation was further investigated.

Keywords: Acetic acid bacteria, co-culture, fermentation, pineapple, yeast

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1. Introduction

Pineapple is an important tropical fruit for Thailand's economy, being one of the leading exporter of processed pineapple products in the world (The Government Public Relations Department, 2023) including pineapple in canned syrup, pineapple juice and pineapple puree etc. The taste of pineapple has unique characteristics and is accepted by consumers because of its sweet and sour taste. It contains vitamins and minerals that are essential for the body, as well as a unique protease enzyme, bromelain. The various researches reported that bromelain in pineapple has been a valuable compound in traditional medicine due to its numerous therapeutic effects, including inflammation, rheumatoid arthritis, osteoarthritis, cardiovascular diseases, skin wounds and burns, perioperative sports injuries and chronic rhinosinusitis (Colletti *et al.*, 2021; Pavan *et al.*, 2012). Recently, bromelain is suggested as an antiviral agent against COVID-19 due to the inhibition of different versions of SARS-CoV-2 (Tallei *et al.*, 2021). Pineapple has an approximate water content of 80-90% and pH value is about 3-4. Freshly squeezed pineapple juice contains about 15-20% of total sugars. Its main sugars and organic acids consist of sucrose, glucose and fructose including citric acid and malic acid. The vitamins and minerals mainly are vitamin C, vitamin A, Magnesium, Phosphorus and Potassium (de Ancos *et al.*, 2017) including nitrogen content of 0.05 % w/v (Chanprasartsuk *et al.*, 2020). With respect to these unique properties of pineapple, it is suitable for use as a medium for microbial fermentation to make various kinds of fermentable food and beverage products which is an alternative value adding of pineapple fruit.

Allochthonous yeast, Saccharomyces, are popular yeasts exploited as starter culture for fermented food and beverages production. It has good fermentation ability and provides consistent fermented beverage and food products. For autochthonous yeast, non-Saccharomyces, are natural fermented yeasts associated with spontaneous fermentations of wine, these yeasts play an important role in the definition of the sensory quality of the final product (Desiderio & Escalante, 2018). Their activities are widely dispersed in these yeasts and can be used to enhance the wine aroma and quality (Ciani *et al.*, 2010). Meyerozyma guilliermondii, a non-Saccharomyces yeast, with high β-glucosidase activity play a vital role in improving the aroma complexity of wines by releasing aroma compounds from glycosidic precursors during fermentation.

Co-culture of yeast and AAB are an effective combination for fermenting foods and beverages. These microbial communities and their interactions produce the numerous fermented beverages including both alcoholic and non-alcoholic drinks. Yeast is able to convert carbohydrates in medium to monosaccharides by periplasmic yeast invertase and ethanol is produced as a result of alcoholic fermentation. AAB oxidize monosaccharides into gluconic acid, and ethanol into acetic acid through oxidative metabolism (He *et al.*, 2022). The previous researches about interaction of this consortium were reported. Kombucha tea, well known fermented beverage, is a fermented sugared tea liquor exploiting main metabolic interaction of a microbial consortium including of yeasts and AAB (Tran *et al.*, 2020; Grassi *et al.*, 2022). Using AAB and yeast strains isolated from a traditional apple recipe for apple cider vinegar fermentation and formulation

(Mathew *et al.*, 2019). Ko *et al.* (2018) investigated the behavior in co-culture of the alcoholic fermentation yeast (beer yeast) and acetic acid bacteria in glucose-containing broth. The interplay between yeast and AAB could produce the complexity of metabolites during fermentation (Tran *et al.*, 2020). The aim of this study was to investigate activities of co-culture of yeast and AAB in pineapple juice fermentation. The microbial dynamics in terms of populations and their impact on the chemical composition of pineapple juice were investigated.

2. Materials and methods

2.1 Microorganism and starter cultures preparation

A commercial yeast strain, Saccharomyces cerevisiae TISTR 5169 obtained from Department of Food Science, Faculty of Science, Burapha University. An indigenous non-Saccharomyces yeast, Meyerozyma guilliermondii isolate, isolated from natural fermentation of Pattawia pineapple juice was used as autochthonous yeast. Its strain was identified by the morphological examination and molecular methods. The diagnostic methods for examination was performed by the sequence analysis of the 26S rDNA D1/D2 of rDNA and primers as according to Kurtzman and Robnett (1998). These yeasts were cultivated on malt extract agar (MEA) at 30 °C for 48 hours. For yeast inoculum preparation, pineapple juice was pasteurized at 72 °C for 1 minute, then cooling immediately. These yeasts were separately cultivated in 100 mL of pasteurized pineapple juice at 200 rpm for 24 hours. Acetobacter aceti TISTR 428 obtained from Department of Food Technology, Faculty of Science, Chulalongkorn University was cultivated on acetic acid ethanol (AE) agar at 30 °C for 72 hours. It was inoculated in 60 mL of sterile AE broth, then incubated at 200 rpm for 48 hours. The suspension was centrifuged at 4,000 rpm for 10 minutes, then the pellet was conducted by washing 3 times and resuspended with 10 mL of 0.1% w/v sterile normal saline for AAB inoculum. All experiments were aseptically conducted.

2.2 Fermentation of pineapple juice

The Pattawia pineapple juice (Ananus comosus (L.) Merr.) were obtained by Siam Foods Products Public Company (Limited) located in Chonburi province, eastern Thailand. The juice was filtered through a sterile cheesecloth, then the chemical characteristics; total soluble solid (TSS; °brix), pH, total titratable acidity (%TTA as citric acid) and nitrogen content (%w/v) of juice were determined. Then, potassium metabisulphite ($K_2S_2O_5$) was added to decontaminate pineapple juice with final concentration in the juice of 100 mg/L. The prepared starter cultures in 2.1 were separately inoculated into pineapple must at initial population of 6 log cfu/mL (Chanprasartsuk *et al.*, 2012) as in Table 1. The inoculated juices were incubated with static condition at ambient temperature (30-32 °C) for 12 days. The cultured juices were collected every 4 days of fermentation for microbiological determination and physicochemical analysis; total soluble solid (TSS; °brix), pH, total titratable acidity (%TTA as citric acid) and ethanol content.

Table 1. Single and co-culture inoculation of pineapple juice fermentation

No.	Culture inoculums
1	Single culture of *S. cerevisiae* TISTR 5169
2	Single culture of *M. guilliermondii* isolate
3	Co-culture of *S. cerevisiae* TISTR 5169 and *A. aceti* TISTR 428
4	Co-culture of *M. guilliermondii* isolate and *A. aceti* TISTR 428

2.3 Microbiological and physicochemical analysis

Yeasts and AAB population were determined by cultivation on MEA and AE agar, respectively, at 30 ^{o}C for 72 hours. The analysis of TSS was performed by refractometer, pH was determined using a pH meter and TTA was proceeded by titration with 0.1N NaoH were performed throughout the fermentation (Chanprasartsuk *et al.*, 2020; Gullo *et al.*, 2006).

2.4 Ethanol, sugars and organic acids contents analysis

The fermented pineapple juice samples were centrifuged by Eppendrof 5453 Minispin Plus centrifuge (Eppendrof, Germany) at 5,000 rpm (1,957 xg) for 15 min to separate yeast cell, pulp and other substances. The juices were then filtered through a 0.45 micron syringe filter to separate small particles in samples. The filtrates were poured into a vial, which was capped and put in an autosampler tray for injection.

Ethanol, sugars and organic acids concentration in fermented pineapple juices were analysed by HPLC instrument coupled with Diode Array Detector (DAD) and Refractive Index Detector (RID) (Agilent 1260 Infinity II LC system, Agilent Technologies, Inc., USA.) using the method of Chanprasartsuk and Prakitchaiwattana (2022). The analytical column (HPX-87H, 300x7.8 mm ion exclusion column, Bio-Rad, USA.) was run at 25^{o}C using orthophosphoric acid in water (0.06%) as mobile phase at a flow rate of 0.5 mL/min. The detection of ethanol and sugars were performed by RID and organic acids were performed by DAD at 210 nm. The data were analysed by Agilent ChemStation software (Agilent Technologies, Inc., USA.). The method was calibrated using ethanol, mixture of sugars; glucose, fructose and sucrose, and mixture of organic acids; citric, malic, lactic and acetic acid, as standard since they are the main sugars and organic acids found in pineapple juice.

2.5 Statistical analysis

One-way single factor analysis of variance and Tukey's range test were used to determine significant differences between means using Minitab statistical software version 18 (Minitab, LLC., USA). Significant differences were considered at p value of less than 0.05. All experiments were carried out in triplicate.

3. Results and discussions

3.1 Pineapple juice properties

The physicochemical characteristics of pineapple juice was demonstrated in Table 1. The juice consisted of 10.1 °brix TSS and 9.13 g/ 100 mL total sugars content. Its proportion of sucrose: glucose: fructose of pineapple juice was 1.7: 1.1: 1.0, which sucrose was the main sugar in pineapple juice, followed by glucose and fructose, with glucose at a slightly higher content than fructose. The juice contained 0.57 % TTA as citric acid with pH value of 3.67, citric acid was the main organic acid, followed by malic acid of ratio 2:1. The amount of lactic acid and acetic acid in pineapple juice were also observed. According to these characteristics of pineapple juice, it was appropriate for using as a raw material for single and dual yeast and AAB fermentation with no sugar adding (Ribéreau-Gayon *et al.*, 2006).

3.2 Fermentation activities of starters in pineapple juice

The kinetic changes of microbiological and physicochemical properties of pineapple juice during fermentation were indicated in Figure 1. Since there was no significantly change after day 8, the results have been demonstrated during 0-8 day of fermentation. Single yeast starter culture fermentation as Figure 1a and 1b, *S. cerevisiae* TISTR 5169 yeast grew stably from 6.0 log cfu/mL to maximum in day 4 of fermentation, whilst the population of *M. guilliermondii* isolate was maximum at day 8 of fermentation. Their TSS were apparently decreased from 10.1 to approximately 3.5 - 4.0 °brix corresponding to the rising of ethanol content, and the pH and TTA was insignificant different all the time of fermentation.

Table 2. Physicochemical analysis of pineapple juice

Pineapple juice characteristics	Values ± SD
Total soluble solid (° brix)	10.1 ± 0.1
pH	3.67 ± 0.00
Total titratable acidity (% as citric acid)	0.57 ± 0.00
Nitrogen content (% w/v)	0.06 ± 0.00
Sugars content (g/100 mL)	
Sucrose	4.00 ± 0.01
Glucose	2.73 ± 0.02
Fructose	2.40 ± 0.01
Organic acids content (g/L)	
Citric acid	6.22 ± 0.01
Malic acid	3.40 ± 0.03
Lactic acid	0.52 ± 0.02
Acetic acid	0.31 ± 0.01

For simultaneous fermentation of *S. cerevisiae* TISTR 5169 and *A. aceti* TISTR 428 (Figure 1c), yeast propagated from 6.0 to 8.3 log cfu/mL in the first four days, then slightly decreased to 7.6 log cfu/mL in day 8 of fermentation. The generally decreasing of AAB population was observed throughout the fermentation

from 5.8 to 4.4 log cfu/mL. The TSS was certainly decreased from 10.1 to 3.4 in the first four days, then slightly increased to 3.6 in day 8 of fermentation according with its alcohol content which rose to 4.16 %v/v in day 4, then climb to 4.40% v/v in day 8 of fermentation. Co-culture of *M. guilliermondii* isolate and *A. aceti* TISTR 428 fermentation (Figure 1d), yeast population grew from 6.2 to maximum at 8.3 CFU/mL in day 4, then slightly decreased for the next four days to 7.9 log cfu/mL. AAB population lightly decreased to 5.8 log cfu/mL in day 4, then obviously dropped to 4.0 log cfu/mL for the next four days. The TSS was apparently decreased from 10.1 to 3.7, then slightly dropped to 3.6 for the next four days. Its alcohol content rose to 4.01 %v/v in day 4, then slightly climb to 4.08 % v/v in day 8. The insignificant different pH and TTA were observed throughout co-culture fermentation.

The yeast cells of simultaneous fermentation were higher than that observed for single culture fermentation in day 8, suggesting that the presence of *A. aceti* TISTR 428 influenced the growth of yeast

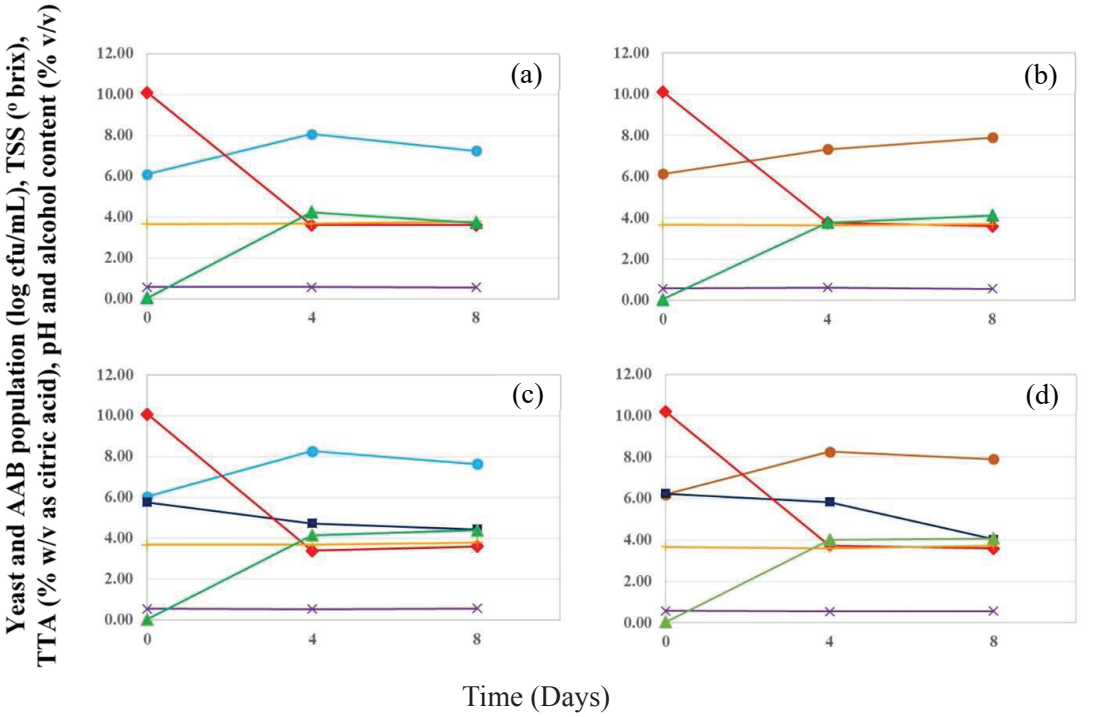

Figure 1. Changes of yeast and acetic acid bacteria (AAB) populations, TSS, pH, TTA and alcohol content of inoculated pineapple juice during fermentation; single *S. cerevisiae* TISTR 5169 (a), single *M. guilliermondii* isolate (b), mixed *S. cerevisiae* TISTR 5169 and *A. aceti* TISTR 428 (c), mixed *M. guilliermondii* isolate and *A. aceti* TISTR 428 (d), *S. cerevisiae* TISTR 5169 (•), *M. guilliermondii* isolate (•), *A. aceti* TISTR 428 (■), TSS (♦), pH (+), TTA (x), alcohol content (▲)

growth. Notably, *A. aceti* TISTR 428 could improve the growth of *S. cerevisiae* TISTR 5169 and *M. guilliermondii* isolate, meanwhile its growth decreased throughout the fermentation which were differed from the previous report that AAB are remarkably resistant to the membrane-permeable toxic compounds ethanol and acetic acid at a low pH (Mullins *et al.*, 2008), and there was positive impact of the addition of fermented medium on AAB growth (Liu *et al.*, 1996). These results might be explained by the interaction of nutrients starvation and proteolytic activity of bromelain in pineapple juice. Although the mechanism behind the antimicrobial activity of bromelain is not well known yet, it is believed that bromelain may inhibit bacterial growth by hydrolyzing some peptide bonds in the bacterial cell wall (George *et al.*, 2012). When bromelain digests the surface proteins, the cell wall is damaged, allowing the cell to leak, swell, and open (Mamo & Assefa, 2019). Furthermore, bromelain also inhibits the growth of some bacteria by preventing bacterial adhesion to specific glycoprotein receptors on the surface (Mameli *et al.*, 2020; Mamo & Assefa, 2019). Thus, bacterial sensitivity to protease activity depends on the specific peptidoglycan structure of cell wall which may vary with its physiological state (Vollmer *et al.* 2008). The previous research reported that bromelain shows antimicrobial activity against both Gram-positive and Gram-negative bacteria (Ajibade *et al.*, 2015).

The higher average ethanol content of simultaneous fermentations of yeast and AAB, than those of single yeast culture was observed. Adding of AAB inoculum with *S. cerevisiae* TISTR 5169 could significantly stimulate the production of ethanol content from 3.72 to 4.40 %v/v in the day 8 of fermentation, whereas co-inoculation of AAB with *M. guilliermondii* isolate could raise ethanol content from 3.78 to 4.01 %v/v in the first four day of fermentation. These results were coincided with reports of Jayabalan *et al.* (2007) and Kumar and Joshi (2016) that AAB could convert glucose to gluconic acid, then into glucuronic acid which in turn stimulates the growth of yeast to produce ethanol. The levels of the last TSS were nearly the same in the four batches of pineapple juice. Similarly, there was no significant difference in contents of pH and TTA among four batches of pineapple juice during the late of fermentation.

3.3 Changes of key compounds and metabolites associated to fermentation property

Based on the sugars and organic acids content analysis (Figure 2), sucrose and glucose, were rapidly uptaken during solo yeast fermentation (Figure 2, a1 and a2). *S. cerevisiae* TISTR 5169 and *M. guilliermondii* isolate finished all sucrose and glucose in the first four day of fermentation. The slower fructose assimilation rate of *M. guilliermondii* isolate than that of *S. cerevisiae* TISTR 5169 was observed. Fructose content of single yeast fermentation was significantly decreased from 2.35 - 2.40 to 0.15 - 0.75 g/100 mL, respectively in the first four days, then slightly dropped up to day 8 of fermentation. The similarity of sugars consumption for simultaneous *S. cerevisiae* TISTR 5169 and *A. aceti* TISTR 428 fermentation with that of single *S. cerevisiae* TISTR 5169 was observed (Figure 2, a3). For the concurrent fermentation of *M. guilliermondii* isolate and

A. aceti TISTR 428 (Figure 2, a4), notably, their sugars assimilation was slower than that of single *M. guilliermondii* isolate. Sucrose and glucose were all taken up in day 8 of fermentation, while fructose was consumed from 2.42 to 1.20 g/100 mL in the first four days then slightly decreased to 0.16 g/100 mL in day 8 of fermentation.

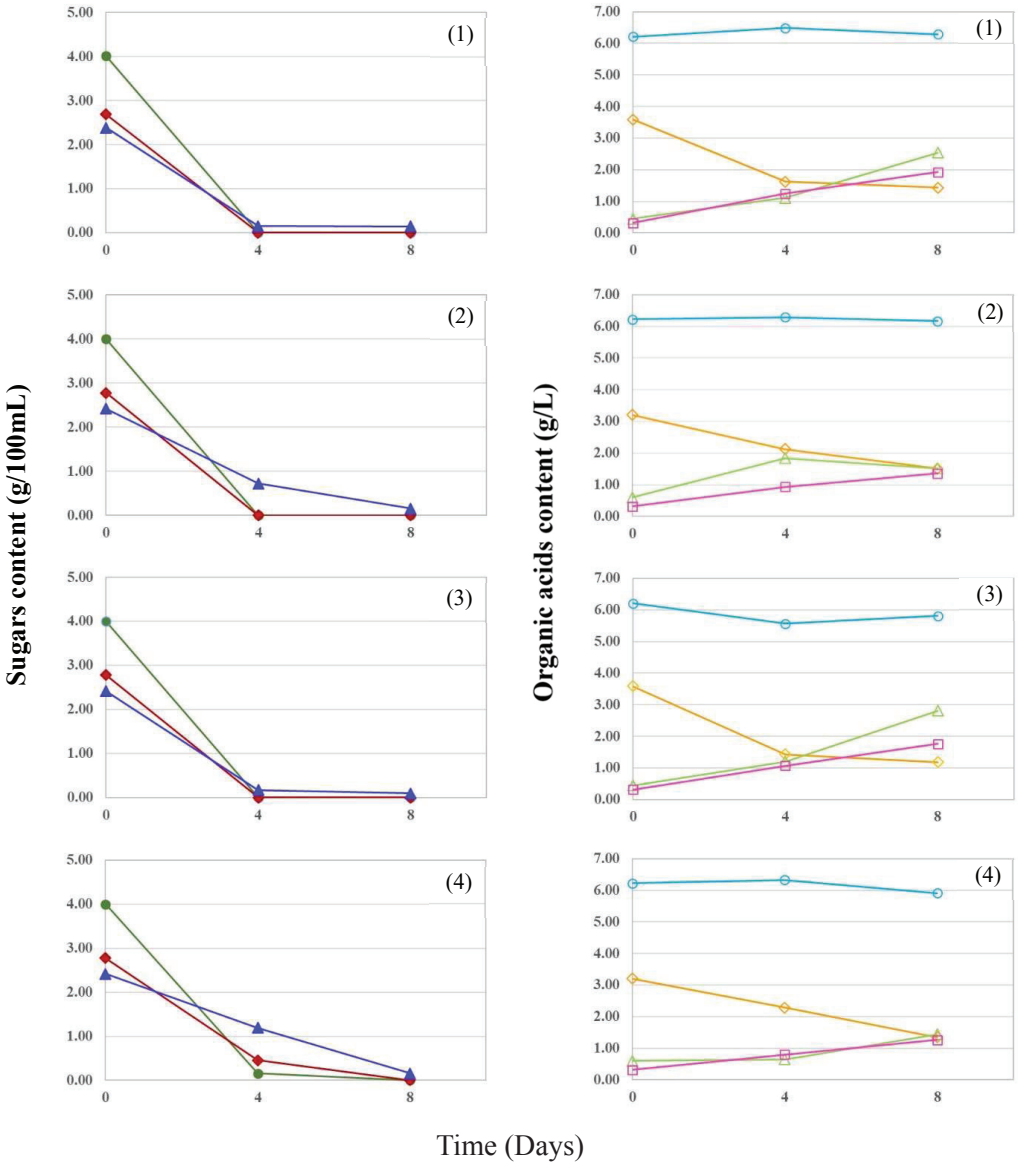

Figure 2. Changes of sugars (a) and organic acids (b) contents of inoculated pineapple juice during fermentation with single and co-culture of yeast and acetic acid bacteria; single *S. cerevisiae* TISTR 5169 (a1 and b1), single *M. guilliermondii* isolate (a2 and b2), mixed *S. cerevisiae* TISTR 5169 and *A. aceti* TISTR 428 (a3 and b3), mixed *M. guilliermondii* isolate and *A. aceti* TISTR 428 (a4 and b4), sucrose (●), glucose (♦), fructose (▲), citric acid (○), malic acid (◊), lactic acid (△), acetic acid (□)

Sucrose, the main sugar of pineapple juice, is a disaccharide converted to glucose and fructose acting as nutrient for yeast and AAB growth during fermentation. Both fermentative yeasts, *S. cerevisiae* TISTR 5169 and *M. guilliermondii* isolate, possess a highly expressing periplasmic invertase enzyme, which rapidly converts sucrose to glucose and fructose. The slower fructose assimilation rate of *M. guilliermondii* isolate found in present study corresponding to the previous report that this yeast strain performed a higher conversion of glucose to ethanol than fructose preceded by the highest conversion of sucrose during fermentation of sucrose, glucose, and fructose mixtures (Khattab & Kodaki, 2016). The adding of acetic acid bacteria higher influenced the sugar consumption rate of *M. guilliermondii* isolate those of *S. cerevisiae* TISTR 5169. The interaction between different yeast and AAB species may stimulate or interfere with the growth of other species, and their metabolic characteristics can influence the chemical composition of the product (Villarreal-Soto *et al.*, 2018).

The changes of organic acids during the fermentation were illustrated in Figure 2, b1-b4. The citric acid content, a main organic acids of pineapple juice, of 5.80 - 6.50 g/L was observed throughout all fermentation. Decreasing of malic acid content of pineapple juice inoculating with *S. cerevisiae* TISTR 5169 was remarkably faster than those of *M. guilliermondii* isolate in the first four days of fermentation. Lactic acid of the juices fermented with *S. cerevisiae* TISTR 5169 and *M. guilliermondii* isolate steadily increased from initial contents of 0.40 - 0.60 to 2.50 - 2.80 g/L (Figure 2, b1 and b3), and 1.40 - 1.50 g/L (Figure 2, b2 and b4), respectively. The rising of acetic acid content was detected throughout all batch of fermentation, notably, adding of acetic acid bacteria resulted in lower acetic acid content in the day 8 (1.25 - 1.80 g/L, Figure 2, b3 and b4) when compared with those of single yeast fermentation (1.35 - 1.95 g/L, Figure 2, b1 and b2).

The citric acid content remained approximately of 6.00 g/L as the initial level of pineapple juice, it also is an organic acid of tricarboxylic cycle found in microbial cells. The depletion of malic acid along with increasing of lactic acid content suggesting that fermentative yeasts might convert L-malic acid into L-lactic acid catalyzing by malolactic enzymes (MLE) leading to a reduction in acidity and the production of aroma and flavor compounds (Ferreira & Mendes-Faia, 2020). In term of acetic acid, it is a physiological product of yeast fermentation formed via the enzymatic reactions of citrate lyase, aldehyde dehydrogenase, and acetyl kinase (Jost & Piendi, 1975). The concentration of acetic acid produced during alcoholic fermentation may vary with the species and strain of yeast, the composition of the juice and the physical factors. (Shang *et al.*, 2016).

4. Conclusion

The adding of AAB could significantly promote the growth and ethanol production of allochthonous and autochthonous yeasts during pineapple juice fermentation, concurrently, the growth of AAB decreased throughout the fermentation. These results revealed the interaction between yeast and AAB during fresh crushed pineapple juice fermentation which could be used as the guildline to control the fermentation better as well as increase the alcohol content during

fermentation. In order to better understand the relationship between yeast and AAB during in pineapple juice fermentation, the influence of pineapple juice properties on the growth of AAB during pineapple fermentation was further investigated.

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