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Consecutive Purple Sweet Potato Vinegar Fermentation Using Mixed Starter of *Aspergillus* spp, *Saccharomyces Cerevisiae*, and Dried Starter of *Acetobacter pasteurianus*

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ABSTRACT

This study investigated the effect of sorbent material on the viability of dried A. *pasteurianus* starter and the use of defined starter culture of fungi Aspergillus spp, yeast S. cerevisiae, and dried A. *pasteurianus* starter during purple sweet potato vinegar fermentation. Complex carbohydrate in cultivation media protects cells during drying, while rice flour as sorbent material absorbs water to obtain dried starter of A. *pasteurianus*. Increasing the ratio of cultivation media and sorbent material from 1:1 to 1:2 could reduce aw of dried A. *pasteurianus* starter that will prolong storage time. Mixed starter of Aspergillus spp, yeast S. cerevisiae, and dried A. *pasteurianus* starter could convert starch to glucose to EtOH to acetic acid during fermentation. However, media saccharified by A. *usamii* contain lower EtOH and higher acetic acid than media saccharified by A. *shirousamii* ($P<0.05$). It is noteworthy that A. *usamii* could hydrolyze starch and protein in rice flour to prevent nutrient exhaustion during acetic acid production by A. *pasteurianus*. While nutrient exhaustion in media saccharified by A. *shirousamii* leads to the utilization of acetic acid known as over-oxidation.

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1. INTRODUCTION

Traditional cereal vinegar production is a spontaneous mixed cultured process involving many microorganisms that can be classified into fungi, yeasts, and bacteria. Among them, *Acetobacter* and *Aspergillus* are the most important microorganisms during vinegar production (Wang *et al.,* 2016). Traditional Chinese vinegar uses a mixed starter of several fungi and yeast named Qu for simultaneous saccharification and EtOH fermentation while a starter of *Acetobacter* was obtained from previous acetic acid fermentation, known as the back-slopping technique. However, the use of a defined starter is recommended to ensure the quality and uniformity of the fermentation product.

The use of *Aspergillus usamii* and *A. shirousamii* in a mixed starter of fungi and yeast *S. cerevisiae* was able to generate glucose, therefore, enhancing the acid production of *A. pasteurianus*. Meanwhile, a freshly prepared starter of *Acetobacter* was studied by Trinh *et al.* (2016) and Zheng *et al.* (2010). It appeared that glucose content in cultivation media could enhance biomass production, while EtOH could enhance enzyme activity responsible for acid production of *A. pasteurianus*.

The use of starter powder would be a convenient process for vinegar production in terms of the application procedure. Studies showed the use of organic media could prolong the viability of *Acetobacter* (Trinh *et al*., 2016) as well as enhance acid production (Zheng *et al.,* 2010). However, the drying process can decrease cell viability due to the damage to the cell membrane osmotic stress and capillary forces due to the removal of water, and the increase in intracellular pH and salt concentration due to the loss of water (Santivarangkna *et al*., 2007). Moreover, Gram-negative bacteria have the lowest heat tolerance, possibly due to the thin cell wall structure (Fu & Chen, 2011). Several approaches to improve bacteria viability after drying namely low-temperature thermal drying (Trinh *et al.*, 2016) and the use of a protective agent, namely starch (Hoyos-Leyva *et al.,* 2018). Starch namely cassava starch, rice flour, and waxy rice flour has been used in traditional starters in Asian countries (Surono, 2016). The use of starch as sorbent material could help protect *A. pasteurianus* during drying, therefore, maintain its viability after drying.

The development of vinegar expanded on the use of raw materials with high phenolic content such as purple sweet potato due to the interesting color as well as a good source of essential nutrients and phenolic compounds (Kano *et al.,* 2005). Research on starch-based vinegar from purple sweet potato had been done by Chun *et al.* (2014) that obtained total acidity of 4.75% in 27 days while Wu *et al.* (2017) were able to reduce fermentation time to 8 days by using commercial enzymes to hydrolyzed starch into glucose. However, improvement is needed to simplify vinegar production by performing fermentation using a defined starter culture of fungi *Aspergillus* spp, yeast *S. cerevisiae* to convert starch to glucose to EtOH that provides a substrate for dried *A. pasteurianus* starter to produce acetic acid.

The objectives of this study were to investigate the effect of fungus types in a defined starter of *Aspergillus* spp., yeast *S. cerevisiae* and dried *A. pasteurianus* starter on EtOH and acetic acid production during purple sweet potato vinegar fermentation. Different sorbent materials were used as protective agent on *A. pasteurianus* cell during the making of the dried starter. Therefore, the effect of sorbent materials on the viability of dried *A. pasteurianus* starter after drying was investigated.

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2. METHODS

2.1 Sample preparation

The purple sweet potato used in this study was obtained from Talad Thai, Bangkok Thailand. Steamed-cooked purple sweet potato flour prepared followed the method proposed by Cakrawati *et al.* (2021) used for preparing cultivation media of *A. pasteurianus*. Nam Wa banana, cassava starch (fish brand, ETC International Trading Co.Ltd., Bangkruai, Nonthaburi, Thailand), rice flour (Chuanchom, Varavoot Industry Co., LTD., Wiseschaichan, Angthong, Thailand), and waxy rice flour (Chuanchom, Varavoot Industry Co., LTD., Wiseschaichan, Angthong, Thailand) were bought from the local market in Bangkok, Thailand. Cassava starch, rice flour, and waxy rice flour were sterilized at 120 °C for 12 h prior to use.

2.2 Materials

Materials used for microbe's cultivation namely glucose (Lobachemie, Mumbai, Maharastra, India), peptone (bacteriological peptone, RM001, Himedia, Mumbai, Maharashtra, India). Media for microorganism's cultivation including yeast extract (HY-Yest 444, Kerry Ingredients & Flavor, Clackmannanshire, Scotland, UK), malt extract (Himedia, Mumbai, Maharashtra, India), American bacteriological agar (Condalab, Torrejon de Ardoz, Madrid, Spain), bromocresol green ($C_{21}H_{14}Br_4O_5S$, Labchem, Ajax Finechem, Mt. Wellington, Auckland, New Zealand). Chemicals for analysis namely EtOH (Carlo Erba, Val-de-Reuil, Normandy, France), acetic acid glacial (Lobachemie, Mumbai, Maharashtra, India), 4-methyl-2-pentanol ($C_6H_{14}O$, Sigma Aldrich, Saint Louis, Missouri, USA), MeOH (Merck, Darmstadt, Hesse, Germany) were HPLC grade. All other chemicals used were of analytical grade.

2.3 Microorganisms

All microorganisms were obtained from the Thailand Institute of Scientific and Technological Research (TISTR). *Aspergillus usamii* TISTR 3258 and A*spergillus shirousamii* TISTR 3140 were maintained on potato dextrose agar (PDA, Merck, Darmstadt, Hesse, Germany) slant at 4 °C. *Saccharomyces cerevisiae* TISTR 5050 was maintained on yeast extract, malt extract, peptone agar (YM), and stored at 4 °C. *Acetobacter pasteurianus* TISTR 102 was maintained on glucose yeast extract (GYE) agar with $CaCO_3$ and stored at 4 °C.

2.4 Preparation of *Acetobacter pasteurianus* starter dried powder

2.4.1 Preparation of cultivation media containing different carbon sources

Cultivation media containing carbon sources were prepared from glucose, peptone, cooked sweet potato flour (protein 6.82%, carbohydrate 78.37%), peeled banana (protein 0.54%, carbohydrate 23.9%). The glucose-purple sweet potato (GP) media contain glucose as a carbon source while GPE media contain EtOH as a carbon source that was obtained by glucose fermentation using *S. cerevisiae* for 24 h at 30 °C and sterilized at 121 °C for 15 min prior to *A. pasteurianus* inoculation. Both GP and GPE media have a C: N ratio of 4.08: 1. A 25 mL of *A. pasteurianus* starter culture was inoculated in a 175 mL cultivation media (GP or GPE) in a 500 mL Duran bottle and incubated for 48 h at 30 °C.

2.4.2 Effect of starch sorbent materials on the survival of A. pasteurianus starter during drying

Sorbent materials namely cassava starch, rice flour, and waxy rice flour were dried-sterilized at 120 °C for 12 h using a hot air oven (Memmert GmbH + Co.KG, Buchenbach, Baden-Wuerttemberg, Germany). Dried *A. pasteurianus* starter was prepared by mixing

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cultivation media of 48-hour-old *A. pasteurianus* with sorbent material at 1:1 or 1:2 (media to sorbent material) in a sterilized Petri dish glass. The mixtures were dried using a food dehydrator (Lacor 245w, Lacor Menaje Professional, SL., San Juan, Bergama, Spain) at 50 °C for 3 h.

The dried starter powder was equilibrated for 24 h at room temperature and relative humidity. The viability of *A. pasteurianus* was enumerated in Carr media using bromocresol green as an indicator for the acid-producing colony (Maal & Shafiee, 2010). The growth of *A. pasteurianus* was reported as cfu/g.

Water activity (a_w) of dried starter was measured using a water activity meter (Aqualab 4TE, Meter Group, Inc., Pullman, Washington, USA) by placing a dried *A. pasteurianus* starter powder into a plastic cup. Moisture content was determined by oven drying using the temperature of 105 °C.

The starch sorbent material and the mixing ratio were selected by considering the highest viability of *A. pasteurianus* and the lowest a_w.

2.5. Effect of fungal species on SSCF during acid production by *Acetobacter pasteurianus* TISTR 102 dried powder

2.5.1. Preparation of amylolytic fungal and yeast inocula

A. usamii TISTR 3258 and *A. shirousamii* TISTR 3140 were grown on potato dextrose agar slant (PDA) for 3 days at 30 °C in an incubator (B50, Memmert GmbH +Co.KG, Buechenbach, Baden-Württemberg. Germany). *Aspergillus* inoculum was prepared by scraping the spore and mycelium of a 3-day-old *Aspergillus* spp. into a 5 mL sterilized 0.85% NaCl. The 5 mL of spore suspension was added to 10 g of tray-dried cooked purple sweet potato flour and incubated for 3 days at 30 °C, designated *Aspergillus* koji of 15 g.

Baker's yeast *S. cerevisiae* TISTR 5050 was grown on YM agar for 3 days at 30 °C and scraped into 5 mL of sterilized GEP4.0 media.

2.5.2 Preparation of production media before acetic acid fermentation

Drum-dried purple sweet potato flour was prepared by steaming purple sweet potato at 100 °C for 1 h using a gas-stove cooker then cooled down by soaking in water until reached room temperature. The sweet potato was peeled then 1 kg of sweet potato was mixed with 400 mL water and mashed using cutter mixers (Robot-Coupe R2, Robot-Coupe®, Vincennes, France).

The sweet potato was dried using a locally made drum drier with a dimension drum diameter of 21.5 cm, length of 45 cm, the speed at 0.87 rpm, and the gap between the drums was 0.15 mm. Fermentation media contained 60 g/L drum-dried steam-cooked purple sweet potato flour and 16 g/L glucose was sterilized at 121 °C for 15 min. Amylolytic fungal and yeast inocula were prepared as described in section 2.5.1 above to contain a C: N ratio of 19.67 :1 and incubated at 30 °C for 24 h in the 250 mL sterilized fermentation media in a 1000 mL Duran bottle to saccharify sweet potato starch and convert glucose to EtOH.

2.5.3. Efficacy of *A. pasteurianus* dried powder starter on acetic acid production

A dried *A. pasteurianus* starter was prepared as described in section 2.4.2. Rice flour was used as sorbent material at a ratio of cultivation media to sorbent material of 1: 2. A hundred g of dried *A. pasteurianus* starter was inoculated in a production media prepared as described in section 2.5.2 above to obtain 10^8 cfu/mL of *A. pasteurianus*.

Two hundred and fifty mL of the production media containing a mixture of *A. usamii* and *S. cerevisiae* or *A. shirousamii* and *S. cerevisiae* to produce glucose and EtOH after 24 h

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incubation at 30 °C were inoculated with the dried *A. pasteurianus* starter powder without sterilization. The total volume was adjusted to 300 mL using 50 mL of sterilized distilled water. The fermentation was carried out in a 1000 mL Duran bottle under the static condition to avoid the oxidation of anthocyanins. The SSCF process was carried out for 20 days at 30 °C in an incubator.

Ten mL of sample were taken on days 1, 7, 14, and 20, centrifuged at 3500*g* for 15 min using an Ohaus benchtop centrifuge (Frontier^{TM}5000, Ohaus, Melbourne, Victoria, Australia). The supernatant was separated and kept at -18 °C before analysis namely glucose, monomer anthocyanin, EtOH, and acetic acid.

2.5.4. Evaluations

2.5.4.1. Analysis of glucose

Glucose content was analyzed using GOPOD-Format (Megazyme International Ireland, Wicklow, Ireland) following the method described by Hall and Keuler (2009). A GOPOD reagent of 3 mL was added to 0.1 mL of supernatant or standard solution containing D-glucose followed by incubation for 20 min at 50 °C. The absorbance was measured at 510 using a Genesys 10 UV spectrophotometer (Thermo Fischer Scientific, Waltham, Massachusetts, USA) against the reagent blank and reported as g/L.

2.5.4.2. Total monomer anthocyanin

A pH- differential method described by Lee *et al.* (2005) was used to determine monomer anthocyanin in fermentation media. Two buffer systems: 0.025 M potassium chloride buffer, pH 1.0, and 0.4 M sodium acetate buffer, pH 4.5 was used. An extract of 0.1 mL was mixed with either 0.9 mL of 0.025 M potassium chloride buffer, pH 1.0, or 0.4 M sodium acetate buffer, pH 4. The absorbance was read at 510 nm and 700 nm using Genesys 10 UV and expressed as mg C3G/L.

2.5.4.3. EtOH, and acetic acid using HS-SPME-GC-FID

EtOH and acetic acid content were analyzed by the head space-SPME method using gas chromatography with a flame ionization detector (GC-FID) (Nam *et al.*, 2019). The sample was weighted 0.5 g and diluted to 50 mL. Five mL of the diluted sample that spiked with 5 µL (50 ppb) of 4-methyl-2 pentanol as internal standard was added to a 20 mL vial and incubated at 50 °C for 10 min. The SPME fiber was manually inserted through the Teflon septum into the headspace of the vial. Analysis of the sample was performed using Carboxen/PDMS fiber (Supelco, Bellefonte, Pennsylvania, USA) coating exposed at 50 °C for 30 min. The SPME coating fiber containing the headspace volatile compounds was introduced into the GC injection port at 250 °C and kept for 10 min for desorption. An Agilent 6890N gas chromatography (GC), equipped with a split/splitless injector and flame ionization detector (FID) (Agilent Technology, Santa Clara, California USA). The injection port was lined with a 0.75 mm i.d. splitless glass liner. The desorbed compounds were separated in capillary column DB-FFAP (30 m x 0.25 mm x 0.25 mm; Agilent Technology, Santa Clara, California, USA). The oven temperature was initially programmed at 35 °C for 5 min, then was increased to 225 °C at 4 °C/min and held at this temperature for 10 min (Noomsiri & Lorjaroenphon, 2018). Standard calibration curves were constructed by exposing the fiber to a mixture of acetic acid concentrations ranging from 80 to 700 mg/L and EtOH ranges from 50 to 700 mg/L as external standard. Estimated concentrations of EtOH and acetic acid in samples were calculated based on a ratio of the peak area of known concentration of external standard and internal standard

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compare to a ratio of the peak area of acetic acid and EtOH in samples and internal standard. Results were expressed as g EtOH/L and g acetic acid/L.

2.6. Statistical analysis

Treatments and analyses were performed in duplicate, and results were presented as mean ± standard deviation. Data were analyzed by ANOVA and Duncan's multiple range tests using SPSS ver. 12 program (SPSS Inc., Chicago, IL, USA) for the determination of significant differences at $P < 0.05$.

3. RESULTS AND DISCUSSION

3.1. Effect of types of carbon source and sorbent materials on the viability of *A. pasteurianus*

The growth of *A. pasteurianus* in different carbon sources, namely GP and GPE was 10^8 cfu/g. However, the numbers of *A. pasteurianus* after drying in the presence of starch sorbent at the ratio of cultivation media: sorbent material as 1: 1 at 50 °C for 3 h varied from 10^7 to 10^8 cfu/g. The results showed that rice flour sorbent had the highest number of *A. pasteurianus* at 10^8 cfu/g; while cassava starch sorbent resulted in the lowest number of *A. pasteurianus* at 10^7 cfu/g (**Figure 1**).

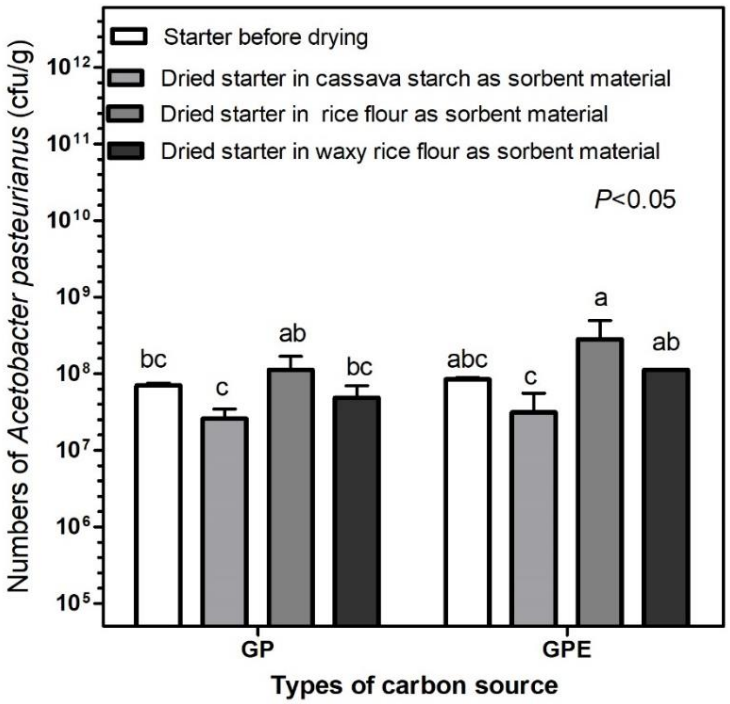

Figure 1. Numbers of *A. pasteurianus* before and after drying in different sorbent materials. The cultivation media was mixed with sorbent material at a ratio of 1: 1 (vol/wt). Bars represent standard deviation.

The viability of *A. pasteurianus* decreased during drying due to cell damage. Sorbent materials helped sorb the water, increased total solid before drying, and shortened the drying time. The moisture content of dried *A. pasteurianus* starter powder range from 32-34%, while water activity (a_w) ranges from 0.98 to 0.99 (**Table 1**). The water activity in dried *A. pasteurianus* starter was 0.98, which was not low enough to inhibit microbial spoilage during the storage of dried powder. Therefore, the ratio of cultivation media to sorbent material was increased to reduce initial moisture content and subsequent a_w.

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The cultivation media GPE was chosen to mix with rice flour sorbent at the ratio of media to rice flour of 1: 2 to decrease initial moisture content. The dried powder obtained after drying at 50 °C for 3 h resulted in the viability of *A. pasteurianus* of 10^6 and the final a_w as 0.55.

The use of steam-cooked purple sweet potato in cultivation media demonstrated protection to *A. pasteurianus* cells during drying since steaming increased soluble starch (Nevara *et al*., 2019). Moreover, Tantratian and Pradeamchai (2020) reported soluble starch demonstrated better protection to *Lactobacillus plantarum* during drying and storage.

Table 1. Moisture content and water activity of dry starter prepared in different C-source and sorbent materials. The cultivation media was mixed with sorbent material at the ratio of 1: 1 (vol/wt).

Types of sorbent materials	Types of carbon source			
	GP (glucose-starch)		GPE (glucose-starch-EtOH)	
	Moisture content (%)	Water activity (a_w)	Moisture content (%)	Water activity (a_w)
Cassava starch	34.68±0.13	0.986±0.003	34.62±1.20	0.9867±0.006
Rice flour	32.78±2.23	0.984±0.003	34.41±1.78	0.9892±0.002
Waxy rice flour	32.61±2.68	0.983±0.001	33.02±4.36	0.9900±0.001

Means (n =2) ± standard deviation.

Sorbent material can effectively sorb water to obtain a dry starter. In addition, the presence of protein in rice flour and waxy rice flour provided peptide and amino acid for the growth, compared to cassava starch, enhancing bacterial capability to withstand the sub-lethal effect of increasing osmotic pressure due to declined a_w.

3.2. Effect of Aspergillus species during successive saccharification and co-fermentation (SSCF) with yeast on EtOH production and acetic acid production using *A. pasteurianus* starter dried powder

Purple sweet potato flour prepared by drum-drying contained cyanidin-based anthocyanins. Therefore, the cultivation media had color changes during the fermentation process (**Figure 2**). At the beginning of the fermentation, the color of the cultivation media was purple. However, after 24 h, EtOH concentration increased, leading to the changes of the anthocyanin color into purplish-red color. Moreover, the changes in EtOH and acetic acid concentration during fermentation also caused the changes in anthocyanin color. On the 20th day, when EtOH concentration decreased and acetic acid concentration increased purple sweet potato vinegar showed bright red color. It should be noted that the fermentation process was allowed to proceed until the 20th day under static conditions to reduce the oxidation and bleaching of cyanidin-based anthocyanin while enhancing acetic acid production.

The changes in anthocyanin concentration were also observed during fermentation in both media saccharified by *A. usamii* and *A. shirousamii* (P<0.05) shown in Figure 3(A). On day 0, anthocyanin content in media was 4.85±0.79 mg C3G/L then increased to 8.57±0.54 mg C3G/L when EtOH concentration increased on day 1 (**Figure 3A**). Anthocyanin concentration was 4.74±0.65 mg C3G/L on day 20th in both media.

Different *Aspergillus* species did not significantly affect glucose content (**Figure 3B**). The glucose concentration in the media was 15.29±0.65 g/L. Glucose drastically decreased on day 1 to 0.33±0.01 g/L in both media. However, glucose concentration varied in both media and ranged from 0.29±0.02 g/L to 0.62±0.36 g/L on day 20.

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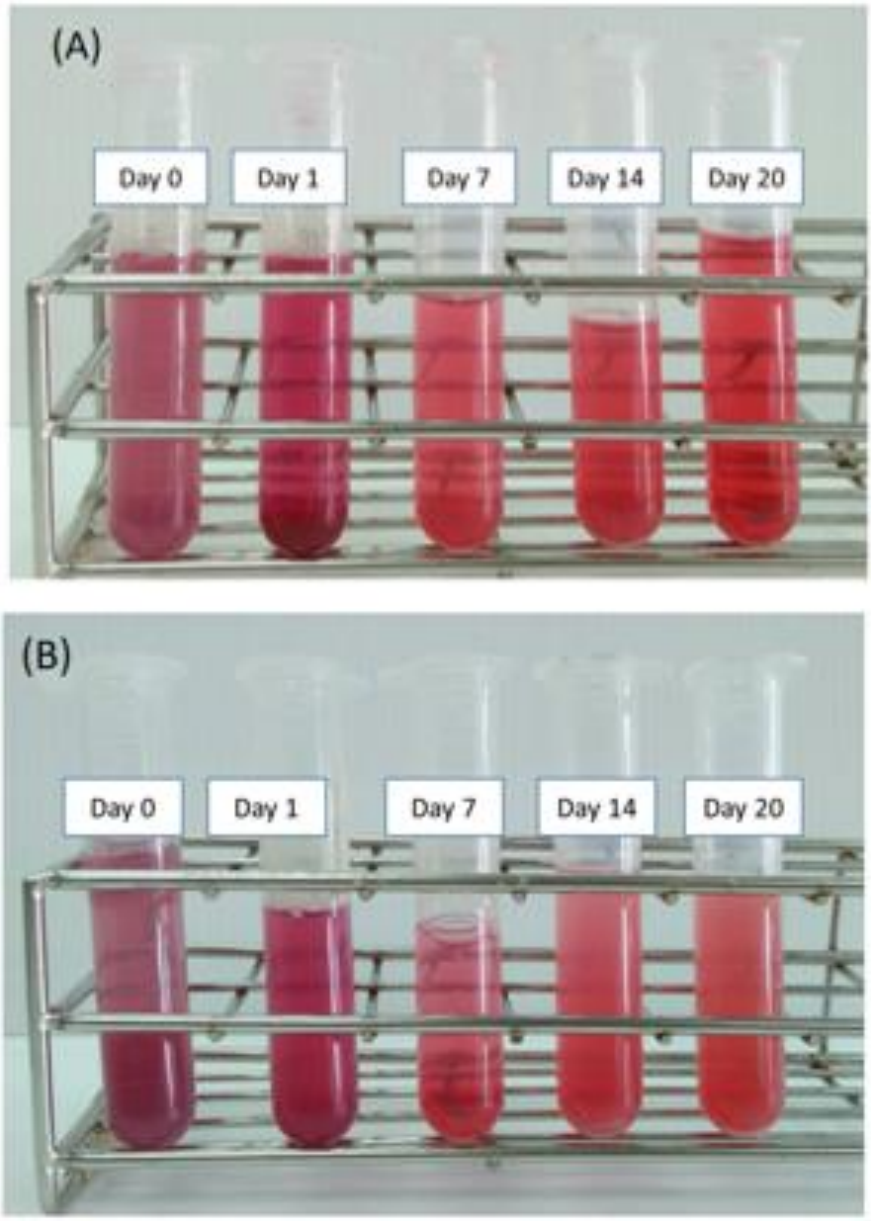

Figure 2. Changes in color during purple sweet potato vinegar fermentation using (A) *A. usamii*, *S. cerevisiae,* and dried *A. pasteurianus* starter; (B) *A. shirousamii*, *S. cerevisiae* and dried *A. pasteurianus* starter.

Figure 4 shows EtOH and acid production during vinegar fermentation. EtOH and acetic acid concentration in media saccharified by A*. usamii* and *A. shirousamii* were not different from day 1 to day 14. However, EtOH concentration was significantly lower in media saccharified by *A. usamii* that reach 5.46±1.77 g/L on day 20 (P<0.05) while EtOH was 14.36±1.49 g/L in media saccharified by *A. shirousamii* (Fig. 4A). In contrast, acetic acid concentration was significantly higher (P<0.05) in media saccharified by *A. usamii* that reach 77.6±0.52 g/L on day 20 while acetic acid concentration was 55.2±0.82 g/L in media saccharified by *A. shirousamii* (**Figure 4B**).

Acetic acid production in media saccharified by *A. shirousamii* reached a plateau after 14 days (**Figure 4B**). This might be due to nutrient exhaustion that led to the utilization of acetic acid known as over-oxidation (Saeki *et al.*, 1997). Meanwhile, acetic acid concentration in media saccharified by *A. usamii* was still increased until day 20. Shochu koji fungi *A. usamii* produced raw starch digestive enzymes. Hongsprabhas and Buckle (1989) reported that *A.*

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usamii hydrolyzed starch and protein from raw cassava flour. *A. usamii* hydrolyzed raw starch and native protein in rice flour sorbent material, providing additional carbon and nitrogen sources for acetic acid production by *A. pasteurianus*.

The low EtOH concentration in media saccharified by *A. usamii* could be explained by the possibility that*A. usamii* utilize EtOH for energy by the TCA pathway. Vinayavekhin *et al*. (2020) reported *Aspergillus niger*, a close relative to *A. usamii*, produced alcohol dehydrogenase to convert EtOH into acetate.

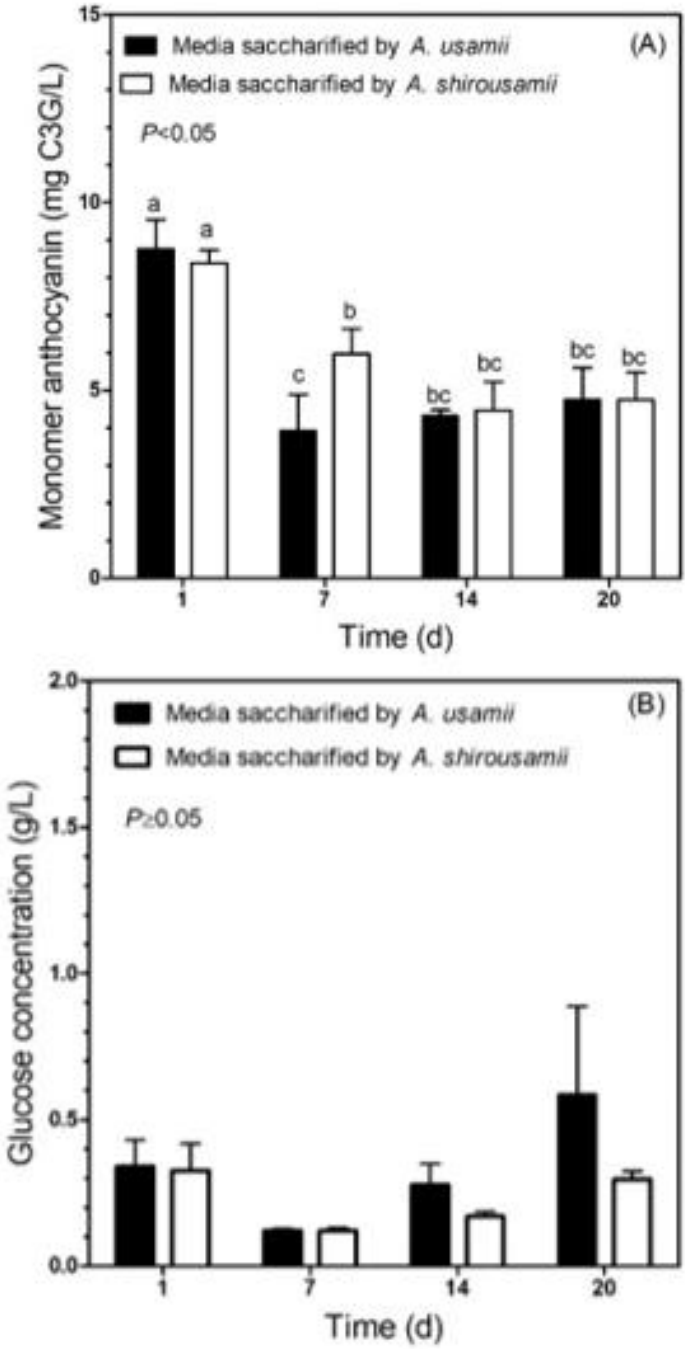

Figure 3. Effect of *Aspergillus* species on (A) monomeric anthocyanin and (B) glucose concentration throughout acetic acid fermentation. The purple sweet potato media was saccharified by *A. usamii* or *A. shirousamii*. Bars represent standard deviation.

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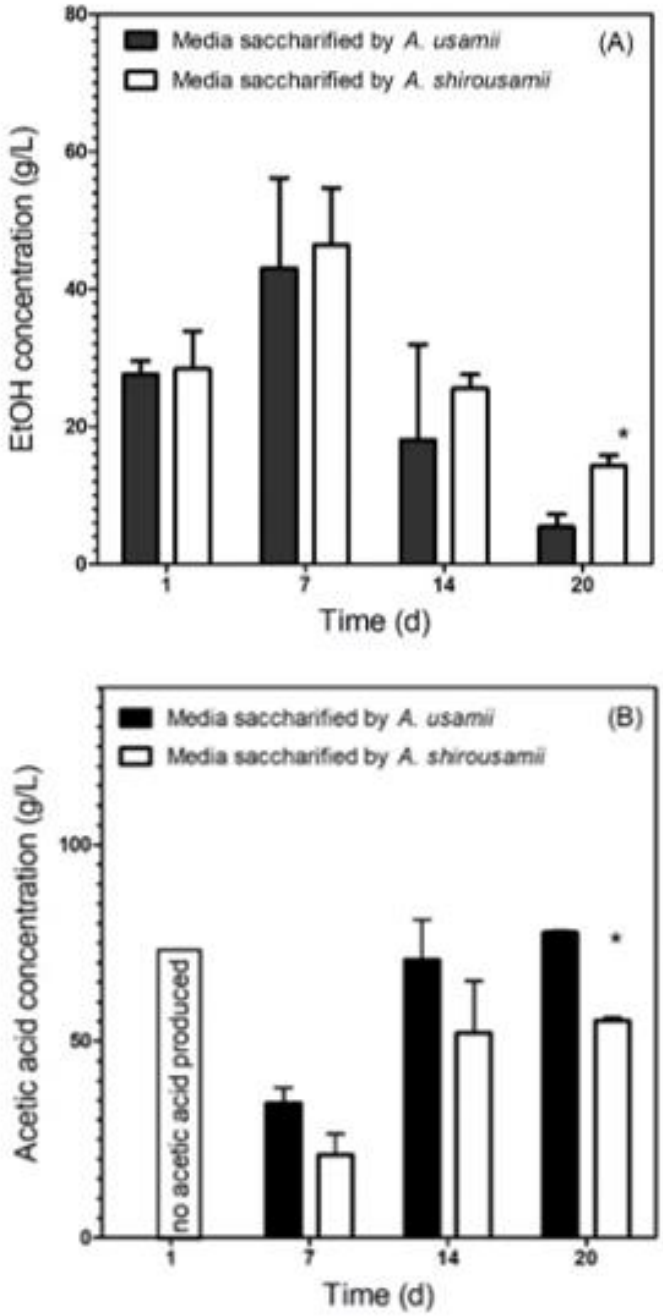

Figure 4. Effect of *Aspergillus* species on (A) EtOH concentration and; (B) acetic acid concentration throughout acetic acid fermentation. The purple sweet potato media was saccharified by *A. usamii* or *A. shirousamii*. Bars represent standard deviation.

4. CONCLUSION

In summary, purple sweet potato vinegar production can be performed consecutively using mixed starter fungi Aspergillus, yeast S. cerevisiae, and dried A. pasteurianus starter that simultaneously converts starch to glucose to EtOH to acetic acid. Rice flour as sorbent material help protect A. pasteurianus during drying and also provide carbon and nitrogen source that enhance acetic acid production in media saccharified by A. usamii. This study highlighted the possibility of using a defined culture starter of fungus, yeast, and acetic acid bacteria for simplified vinegar production.

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6. AUTHORS' NOTE

The authors declare that there is no conflict of interest regarding the publication of this article. Authors confirmed that the paper was free of plagiarism.

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