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Research article

Production of Herbal Vinegar Using Isolated Microorganisms from Traditional Herbal Vinegar Fermentation

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Abstract

Schizosaccharomyces pombe YM-19 and *Acetobacter pasteurianus* EM2-03 were isolated and identified from the traditional herbal vinegar fermentation process using cultural and molecular techniques. The two microorganisms were prepared to inoculum for an experimental herbal vinegar fermentation, which is called "Loog Plaag Mea." The first starter culture was 10% v/v *S. pombe* YM1-19. Fermentation was carried out under anaerobic conditions at 30°C for 4 days. The alcohol content was 6.18±0.13% v/v and the pH value was 3.52±0.02 on day 4 of the fermentation period. Subsequently, 10% v/v *A. pasteurianus* EM2-03 was added to the fermentation process under aerobic conditions at 30°C for 22 days. The final herbal vinegar product contained 4.91±0.15% v/v acetic acid, and a pH value of 3.04±0.04. Its total phenolic content and IC_{50} value of DPPH radical scavenging were 1,908.38±38.75 µgGAE/mL and 0.017±0.001 µL/mL, respectively. The experimental fermented herbal vinegar had physicochemical properties that very similar to traditional vinegar. As a result, the isolated microorganisms can be used to improve product consistency and quality control in the mass production of vinegar.

1. Introduction

Vinegar is a natural product that is widely used in some countries as a sour seasoning, preservative agent, and healthy drink. According to FOA (Food and Agriculture Organization of the United Nations), vinegar is a liquid fit for human consumption. It is produced from a suitable agricultural raw material using a double fermentation process that involves yeast and acetic acid bacteria for the

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alcoholic and acetic fermentation steps, respectively. Acetic acid and phenolic compounds such as caffeic acid, chlorogenic acid, and gallic acid are found in the vinegar. It has been discovered to have a variety of therapeutic properties that can assist in the treatment of diseases such as diabetes, cardiovascular disease, and cancer [1]. In addition, the bioactive compounds in vinegar were found to have antioxidant capacity and antimicrobial properties [2]. Fermented vinegar has better biological activity than synthetic vinegar as it contains essential amino acids from its raw material [3].

Vinegar can be made from any carbohydrate-rich source including wine, beer, syrup, honey, grapes, apples and hydrolysed starch [4]. The herbal vinegar beverage "Loog Plaag Mae" is a traditional beverage in the northeast of Thailand. It is fermented from a mixture of black pepper (*Piper nigrum* Linn.), bael fruit (*Aegle marmelos*), banana (*Musa sapientum* Linn.) and sucrose. Loog Plaag Mae, used in folk medicine as an elixir, is said to have therapeutic properties such as anti-aging and antioxidant activity. Furthermore, it is said to be able to reduce the effects of diabetes, prevent cardiovascular disease and lower blood pressure.

Microbiological process cause fermentation to occur spontaneously. In this research, a bael fruit, black pepper and ripe banana mixture was fermented for 15 days. Then, fermented banana juice was added and fermentation of all the ingredients for six months at room temperature was allowed to proceed. However, fluctuations in the microbial composition used tend to produce uncontrollable qualities in the final product. Hence, the aim of this research was to identify the isolated yeast and acetic acid bacteria (AAB) from the herbal vinegar fermentation process as described in previous work [5]. For the process control and safety-related characteristics of the herbal vinegar beverage production, the dynamics of microbial growth, chemical properties, and bioactive compounds of the products were analyzed during fermentation.

2. Materials and Methods

2.1 Strain and cultivation conditions

Five isolates of yeast (YM1-12, YM1-19, YM5-02, YM5-03 and YM6-03) and four isolates of acetic acid bacteria (AAB) (EM2-03, EM3-01, EM3-03 and EM6-03) were obtained from our previous study [5] which had been isolated from the herbal vinegar (Loog Plaag Mae) fermentation process. The isolated yeast and AAB were screened for high alcohol production and high acid production, respectively [6]. The isolated yeasts were grown in YPD agar (20 g/L glucose, 20 g/L peptone, 10 g/L yeast extract and 18 g/L agar) whereas AAB were grown in GYC agar (50 g/L glucose, 10 g/L yeast extract, 20 g/L calcium carbonate and 18 g/L agar) at 30˚C, and cultivated after 3 days.

2.2 Yeast strain identification

The YM1-12, YM1-19, YM5-02, YM5-03, and YM6-03 yeast strains were identified based on their morphological and biochemical characteristics according to Kurtzman *et al*. [7]. Molecular techniques were performed according to Murray *et al*. [8].

The ability of five yeast isolates to utilize and grow aerobically on carbon as a sole source of energy was studied on API 20 C AUX (bioMerieux, French). The fermentation abilities of yeast isolates to ferment glucose, galactose, lactose, xylose, mannitol, and raffinose were tested. Their ability to grow at 25, 37, and 40°C on YM agar was determined.

Molecular identification of the purified yeast isolates was done based on sequences of the ITS region. The template was prepared for colony PCR using NaOH and boiling as described previously [8]. Amplification of the ITS region was achieved by forward primer ITS1 (5'-

TCCGTAGGTGAACCTGCGG-3') and reverse primer ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [9]. PCR was conditioned to denaturation at 40°C for 10 s, annealing at 50°C for 30 s and extension at 68°C for 1.5 min. The amplicons were purified with a Gel Mini Purification Kit (Flavogen, Taiwan) and cloned into the TA cloning vector (Yeastern Biotech, Taiwan). The sequencing work was performed by Macrogen, South Korea.

2.3 Acetic acid bacterial strain identification

The morphological and physiological characteristics of the AAB were examined via the morphology of cells and colonies, gram stain, oxidase test, and catalase reaction [9]. 16S rDNA gene sequencing was used to identified the AAB. Genomic DNA preparation was performed using a FavoPrep Plasmid extraction mini kit (Flavogen, Taiwan). The polymerase chain reaction (PCR) amplification was based on the analysis of 16S rDNA. It was monitored with a universal primer consisting of a forward primer 16SF (5'- CCCGGGAACGTATTCACCG-3') and a reverse primer 16SR (5'-GCYTAAYACATGCAAGTCGA-3') from Ruiz *et al*. [10]. PCR was conditioned to denaturation at 94°C for 10 s, annealing at 50°C for 30 s and extension at 68°C for 1.5 min. The amplicons were purified with a Gel Mini Purification Kit (Flavogen, Taiwan) and cloned into the TA cloning vector (Yeastern Biotech, Taiwan). The sequencing work was performed by Macrogen, South Korea.

2.4 Herbal vinegar fermentation

Two-stage fermentation using the identified yeast (*Schizosaccharomyces pombe* YM1-19) and the identified bacteria (*Acetobacter pasteurianus* EM2-03) were prepared as the starter culture for herbal vinegar fermentation.

After mixing 25 g of black pepper, bael fruit and ripe banana with 68 g of sucrose and 450 mL of fermented banana juice, 10% (v/v) *Schizosaccharomyces pombe* YM1-19 (OD_{600} = 0.5) was added for anaerobic fermentation at 30°C for 4 days. Then the fermented mash was pasteurized at 90°C for 15 min. Subsequently, 10% (v/v) of *Acetobacter pasteurianus* EM2-03 (OD_{600} = 0.5) was inoculated. For analysis, the fermentation process was aerobic for 18 days. The fermented mash was then centrifuged at 8,000 rpm at 4°C for 10 min.

2.5 Physicochemical properties

Total sugar concentration was determined using the phenol-sulfuric method [11]. Total acidity was measured by titration with 0.1N NaOH [12] and expressed as acetic acid concentration. The concentration of ethanol was determined by gas chromatography (Shimadzu, GC-2010 Plus, Japan) fitted with a DB-1 column and FID-detector. The cell count of yeast and AAB were determined by the plate count technique. The plates were incubated at 30°C for 3-5 days.

2.6 Total phenolic content

The total phenolic content of the vinegar samples was determined using Folin-Ciocalteu reagent [13]. Briefly, 20 µL of each diluted sample was mixed with 50 µL of Folin-Ciocalteu reagent and 100 µL of 10% w/v Na_2CO_3 and incubated at room temperature for 30 min. The absorbance was measured at 765 nm using a microplate reader (BMG LABtech GmbH, FLUOStar Omega, Germany). The results were expressed as micrograms of gallic acid equivalents in 1 mL of sample (µg GAE/mL).

2.7 Antioxidant capacity

The antioxidant activity of the sample was evaluated using the DPPH (2,2 Diphenyl-1-picrylhydrazyl hydrate) radical assay [14]. Briefly, 50 µL of each sample was mixed with 150 µL of 0.1 mM DPPH radical solution prepared in ethanol and incubated at room temperature in the dark for 30 min. A microplate reader was used to determine the absorbance at 517 nm, and the DPPH radical scavenging capacity (RSC) was calculated using the equation below and reported as inhibitory concentration at 50% scavenging (IC_{50})

$$\%\text{RSC} = \frac{(\text{AC}-\text{AS})}{\text{AC}} \times 100$$

where AC = absorbance of a control, AS = absorbance of sample.

2.8 Statistical analysis

All the analyses were conducted in triplicate, with a completely random design (CRD). The data was analyzed using ANOVA and presented as a mean with standard deviations. Duncan's multiple range test (DMRT) was used to examine significant differences between treatments at a 95% probability level ($P < 0.05$).

3. Results and Discussion

3.1 Isolation and identification of the yeast

The five isolates of yeast were selected from the herbal vinegar fermentation process. High alcohol production, similarity of morphology properties and biochemical test were observed.

The colonies were circular, brownish, glistening, raised, convex, and had an entire margin after 1 month's incubation at 20°C on 5% malt agar. The cells were rod shaped and underwent binary fission, typical properties of the genus *Schizosaccharomyces*, which dominated, as shown in Figure 1. The results of biochemical tests revealed that the five isolates yeast had similar properties, including the ability to use glucose, glycerol, 2-keto-D-gluconate, methyl-D-glucopyranoside, D-maltose, sucrose, and D-raffinose as carbon sources. They fermented D-glucose, sucrose, maltose and raffinose to ethanol and were able to grow at 25, 30 and 37°C.

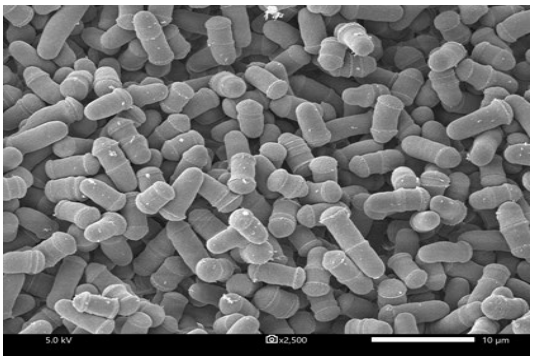

Figure 1. Scanning electron microscope (2,500x) of isolated yeast YM1-19

Based on molecular identification techniques using amplified fragments of ITS region (Figure 2A), five yeast isolates were identified. They were identified as *Schizosaccharomyces pombe* UCDFST: 04-213 (*S. pombe*), which was consistent with the results of morphological and biochemical tests. The similarities (percent identity) and strain types were in the range of 98.42-98.86. (Figure 2B).

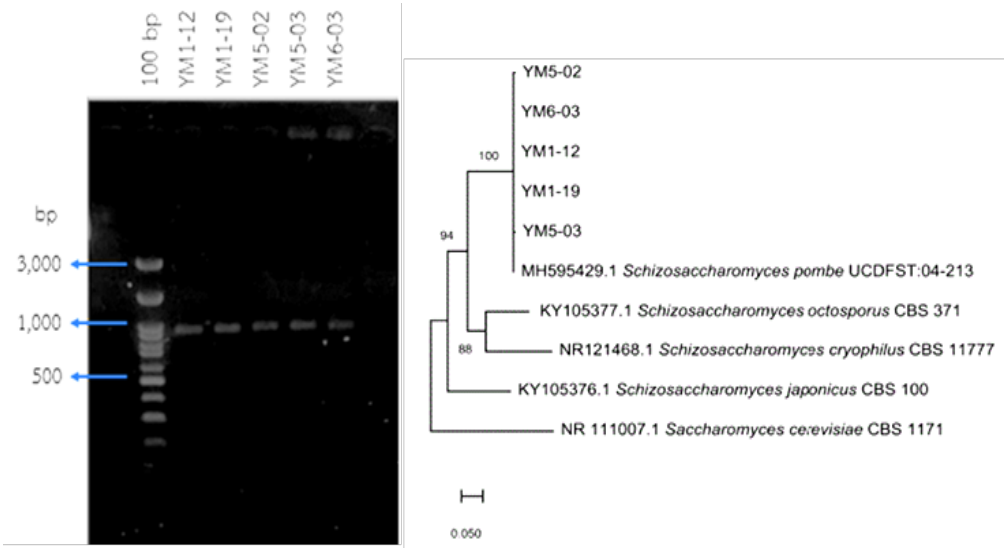

Figure 2. (A) Gel electrophoresis of the PCR product in the internal transcribed spacer region (ITS region) of yeast isolates, and (B) The chart shows the genetic correlation of yeast isolates, Neighbor-Joining with MEGAX program.

Schizosaccharomyces yeast can tolerate high osmotic pressure. It can grow at 50% w/v of concentrated glucose and 10% v/v of ethanol concentration. In previous studies, it was reported that *S. pombe* was isolated from fruit and beverages such as papaya, grapes, sugar cane, kombucha, beer, and could be isolated from Shanxi aged vinegar in China as well as fresh coconut sugar in Thailand [15, 16]. *Schizosaccharomyces pombe* had an ethanol fermentation capacity of 15% v/v, which was comparable to *Saccharomyces cerevisiae* [13, 17]. Benito *et al*. [17] indicated that *S. pombe* produced a lower alcohol content than *S. cerevisiae* (0.23% v/v) whereas Jayarman *et al*. [18] fermented different types of fruit wines with *S. pombe*, which was isolated from ripe papaya, and found that grape wine, broth wine, and jaggery wine gave low alcohol content of 2.57%, 3.29%, and 6.22%, respectively.

3.2 Isolation and identification of acetic acid bacteria

Four isolates of bacteria (EM2-03, EM3-01, EM3-03 and E6-03) belonging to the AAB were gram-negative, rod shaped (Figure 3), catalase positive, and oxidase negative. Their colonies appeared small, circular, pale and showed a clear zone on GYC agar after incubation at 30°C for 3 days. All isolates showed similar properties in biochemical test results to *Acetobacter pasteurianus* [19]. They overoxidized ethanol and had positive growth on glucose, xylose, and ethanol. Moreover, they were unable to form cellulose and water-soluble brown pigments.

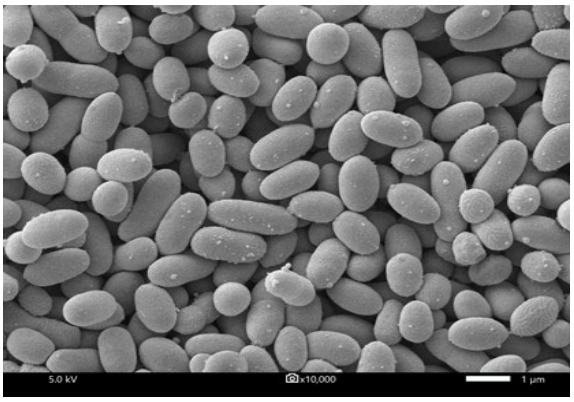

Figure 3. Scanning electron microscope (10,000x) of isolated bacteria EM2-03

The four high-acid production strains were further described based on the identification results. PCR amplification was performed utilizing universal 16S rDNA PCR primers and the genomes as templates, and the gel electrophoresis result indicated a cleat band at approximately 1,500 bp (Figure 4A). Sequence alignment was performed using BLAST (National Center for Biotechnology Information). The 16S rDNA of all isolates was found to be 99.90-100.00% homologous with *Acetobacter pasteurianus*.

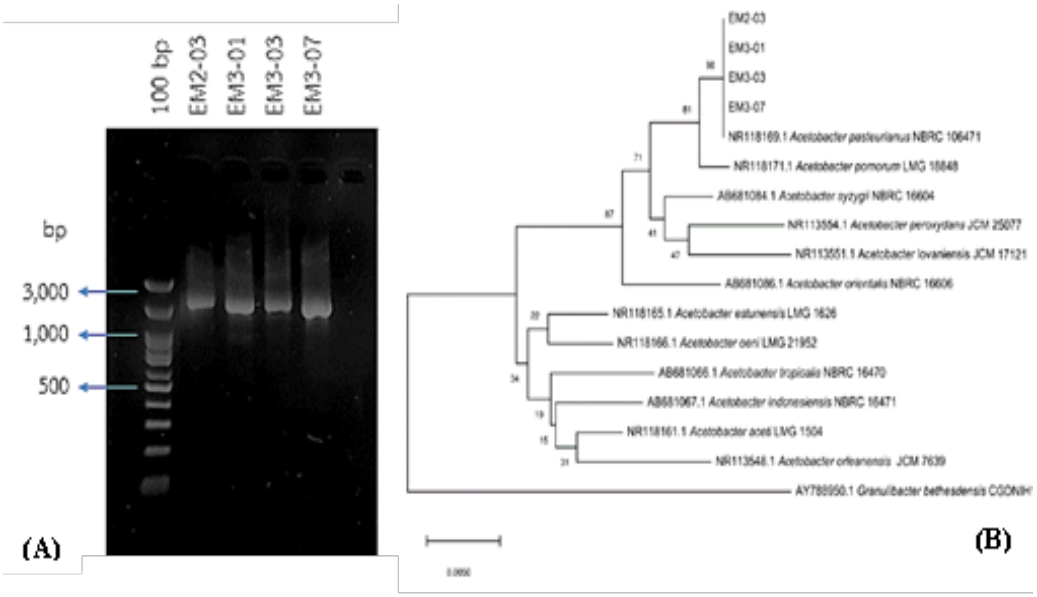

Figure 4. (A) Gel electrophoresis of the PCR product 16S rDNA region of bacterial isolates, and (B) The chart shows the genetic correlation of four isolated AAB: EM2-03, EM3-01, EM3-03 and EM3-07 created with the method Neighbor-Joining with MEGAX program.

The phylogenetic tree of all bacterial isolates and *A. pasteurianus* was clustered in the same branch with a bootstrap support rating of 98%, followed by *A. pomorum* with 81%, based on the 16S rDNA sequencing. To generate the phylogenetic tree, *Granulibacter bethesdensis* was used as

an out-group (Figure 4B). They were identified as *A. pasteurianus* based on their morphological and biochemical characteristics. It has been reported that *A. pasteurianus* was isolated from fermented foods such as vinegar, wort wine, and fruits such as red grapes, coconut water, and cocoa [20, 21].

3.3 Herbal vinegar fermentation

The herbal vinegar fermentation process is a derived microbiological transformation with a two-stage fermentation process that involves yeast alcoholic fermentation of sugars into ethanol and acetic acid bacteria oxidation of ethanol into acetic acid [22, 23]. In the first stage, *Schizosaccharomyces pombe* YM1-19 was added to the fermentation process. The alcohol content gradually increased and reached its maximum on day 4 (6.18±0.13% v/v). The total sugar content decreased from 724.64±11.38 g/L to 432.33±7.31 g/L. The results are shown in Figure 5. The amount of *S. pombe* YM1-19 increased from 6.28±0.01 LogCFU/mL to 8.98±0.09 LogCFU/mL on day 4. The fermentation process was stopped by pasteurizing the fermented mash at 90°C for 15 min. Then 10% v/v *Acetobacter pasteurianus* EM2-03 was added under aerobic conditions.

During the 14 days of fermentation period, the total acid content increased rapidly from 1.85±0.06% w/v to 3.78±0.07% w/v. Consistent with the reduction of the alcohol content, the amount of AAB increased to 8.40±0.15 LogCFU/mL on day 8 after that decreased throughout the fermentation period. According to Lee *et al*. [6], *A. pasteurianus* is able to grow in a medium with a high acetic acid concentration of 3-5% v/v, whereas 7% v/v acetic acid concentration inhibits the growth. Total sugar concentration slightly decreased on day 4 from 423.3±7.31 g/L to 396.22±8.00 g/L. The AAB showed the ability to use alcohol as a carbon source rather than sugar [24]. This result was consistent with Arifuzzaman *et al*. [20] and Wu *et al*. [9], who reported that *A. pasteurianus* could not use sucrose as a carbon source for acetic acid production. However, acetic acid was produced from glucose [12, 19] resulting in a slight decreased in the total sugar content.

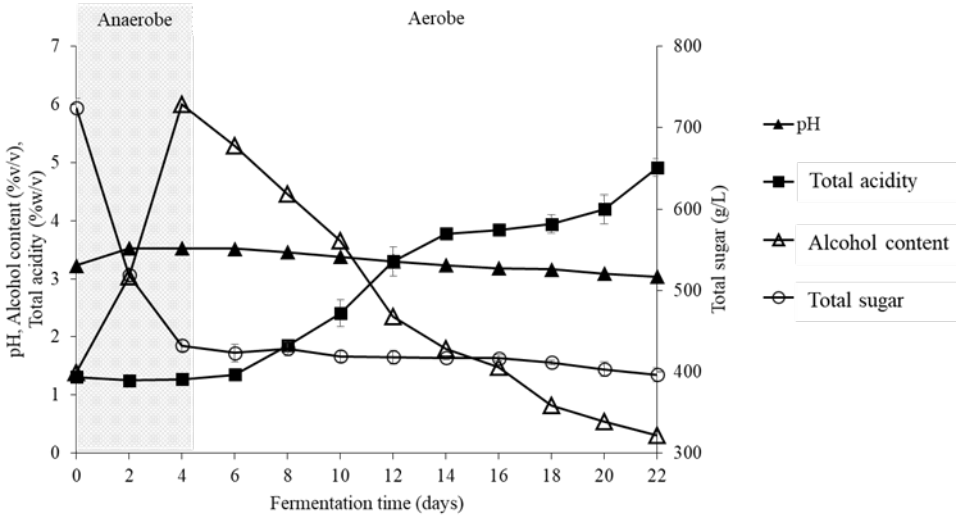

Figure 5. Physiochemical properties of herbal vinegar fermentation

For phenolic compound, there was a rapid increase on day two from 477.53±41.85 µgGAE/mL to 1,718.29±80.71 µgGAE/mL as a result of the maceration of plant herbs in the

fermentation medium, which was rich with bioactive compounds such as coumarins, marmelosin and tannin from bael fruit [25] and piperine from black pepper [26]. Then, phenolic compounds slightly increased throughout the fermentation period, resulting in a decrease in the IC_{50} values of DPPH during fermentation time from 0.102±0.007 µL/mL to 0.017±0.001 µL/mL as shown in Figure 6.

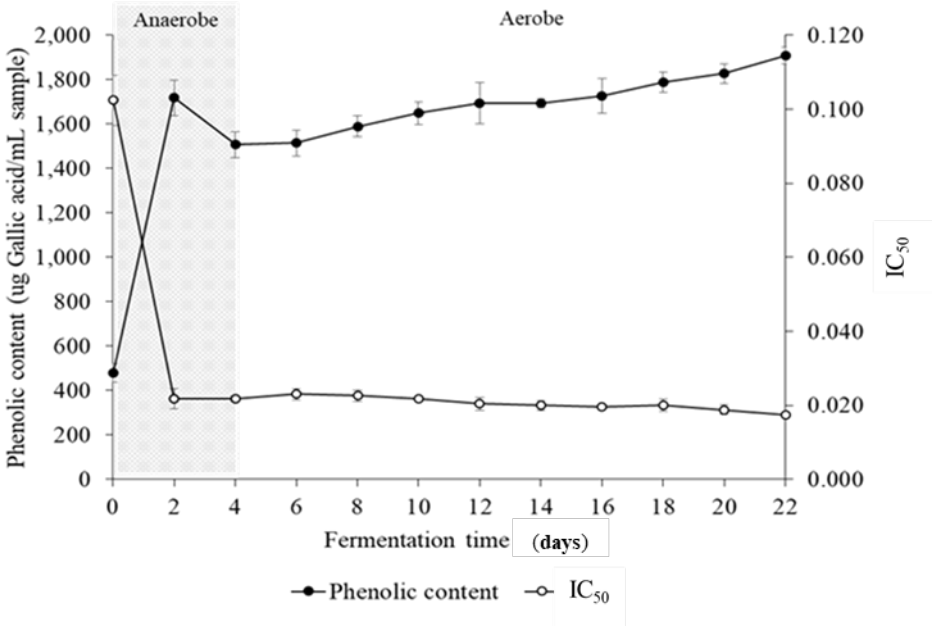

Figure 6. Total phenolic content and inhibitory concentration at 50% scavenging (IC50) of DPPH in fermented vinegar during a fermentation process

The chemical characteristics of the vinegar were similar to those of traditional vinegar and were not significantly different ($P > 0.05$). However, the alcohol content (0.30%±0.06 v/v) was lower than traditional vinegar fermentation. It is healthier for consumers and ferments in the short period of time (22 days), as shown in Table 1.

Table 1. Chemical quality and antioxidant activity of vinegar using isolate yeast and acetic acid bacteria in comparison with the traditional vinegar product

Properties	Vinegar	
	Two-stage fermentation	**Traditional fermentation**
pH	3.04±0.01[a]	3.03±0.01[a]
Total acid (%w/v)	4.91±0.15[a]	5.05±0.04[a]
Total sugar (g/L)	396.22±8.88[a]	334.26±7.91[a]
Alcohol (%v/v)	0.30±0.06[b]	1.54±0.05[a]
Phenolic content (µg GAE/mL)	1,908.38±38.75[b]	2,410.71±11.87[a]
IC_{50} (µL/mL)	0.017±0.001[a]	0.016±0.001[b]
Fermentation time (days)	22	180

Values with different letters in the same row are significantly different at the 0.05 level (2-tailed)

4. Conclusions

The results of this research showed that *Schizosaccharomyces pombe* YM1-19 and *Acetobacter pasteurianus* EM2-03 could be isolated from the herbal vinegar fermentation process and that they were potential starters for vinegar production. The yeast and acetic acid bacteria were able to accelerate the fermentation process and were used to produce vinegar with desirable qualities, reduced alcohol content, and shorter fermentation time. These microorganisms can be utilized in the industrial mass-production of vinegar with shorter fermentation time and product consistency.

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