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Isolation and Selection of Non-*Saccharomyces* Yeasts Being Capable of Degrading Citric acid and Evaluation Its Effect on Kiwifruit Wine Fermentation

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Abstract: High citric acid content in kiwifruit wine would lead to bad sensory experience and quality deterioration. It is opportune and crucial to develop an appropriate and feasible method to degrade citric acid for kiwifruit wine. The non-*Saccharomyces* yeasts confirmed to have the ability to degrade citric acid were screened and used in kiwifruit wine fermentation in the study. A representative number of 23 yeasts with a strong citric acid degradation ability was identified by molecular approaches. JT-1-3, identified to be *Pichia fermentans*, was preferred for high citric acid degradation and strong stress resistance in association with RV002 (commercial *Saccharomyces cerevisiae*). Then it was pure-cultured in kiwifruit juice, and the results indicated that citric, malic and tartaric acids declined significantly from 12.30, 3.09 and 0.61 g/L to 11.00, 2.02 and 0.41 g/L after fermentation, respectively, resulting in the significant decrease in total acid in kiwifruit wine. The analytical profiles for amino acids and volatile compounds showed that *Pichia fermentans* JT-1-3 could improve amino acids' proportion and increase the volatile compounds of alcohols, esters and phenols. This work indicated that JT-1-3 has great potential to be applied for fruit wine with high level citric acid.

Keywords: non-*Saccharomyces* yeast; kiwifruit wine; citric acid; deacidification fermentation; GC-MS

1. Introduction

In light of a growing consumer acceptance of kiwifruit wine worldwide, there has been an increased attention expected to obtain wine with high quality and good taste. Significant levels of biologically active components in kiwifruit (Chinese gooseberry) can be transferred into the wine, benefiting human health and reducing the risks of certain diseases such as cancer, cardiovascular diseases and degenerative illnesses [1,2]. The kiwifruit wine was first produced by Graebener in 1894, and Vitkovskij had reported that the wine had similar characteristics to champagne and Riesling wine [3]. The unique aroma also endowed kiwifruit wine with irreplaceable characteristics, and a total of 44 volatiles were reported in kiwifruit wine [4]. However, the retained acid after fermentation breaks the balance of taste, leading to the excessive sour taste and greatly limiting the promotion of the wine products. Soufleros [3] also verified that the produced kiwifruit wine was rich in titratable acidity, and organic acids contributed mostly to the total acidity.

Citric acid has been found to be the main organic acid, and can be as high as 60% of the total acid in kiwifruit wine [3,5–9]. Intaking appropriate dose citric acid is likely to inhibit urinary crystallization

and stone formation by binding to calcium oxalate crystal surface, even ameliorate ketosis and protect against the development of diabetic complications [10–12].

However, long-term consumption or an excessive intake of citric acid may lead to hypocalcemia, owing to promoting calcium excretion and deposition. A high level of citric acid may show strong cytotoxicity, and even inhibit protein synthesis of human gingival fibroblasts [13,14]. The retained high organic acid may further contribute to forming the "fermentation bouquet" [15] and bring to the wine an unacceptable taste, quality deterioration and poor desire to purchase. There is an urgent demand to find a solution for declining the citric acid content in kiwifruit wine.

Biological control has been proposed as a key alternative control method, and some effective microorganisms have already been utilized in the global fruit industry for deacidification. Yeasts are particularly suitable as agents, because they grow rapidly, colonizing fruit surfaces and being tolerant of most agrochemicals [16]. Numerous yeasts such as *Issatchenkia terricola* [17,18] and the *Pichia* genus [19] were verified to own the ability to degrade citric acid. Moreover, the successful control of citric acid content using biological control agents have been realized in hawthorn juice [19] and citric acid carbon source medium [18]. However, few researches have reported the citric acid degradation by specific yeasts in fruit wine. It is further found that some non-*Saccharomyces cerevisiaes*, *C. utilis*, *C. sphaerica*, *H. anomala*, *K. lactis* and *K. marxianus* as Krebs-positive species can use malic acid and other Krebs cycle intermediates for energy supply, but *S. cerevisiae* was short of this ability [20]. Cassio and Leao [21] also found that *C. utilis* possessed tricarboxylate permease to intake citric and isocitric acid. In those living cells, citric acid can be served as an important energy source via the Krebs cycle by carboxylic acid transporters [22]. A high-affinity transporter behaving as a proton symporter was found in *C. utilis* specific for citric acid [21].

The development of yeast strains that can utilize citric acid, and increasing public concern over food safety, are driving a search for safe and healthy citric acid-degradation methods. Therefore, to find out some promising non-*Saccharomyces cerevisiaes* capable of degrading citric acid and implementing its application in kiwifruit wine with high citric acid are of utmost significance, and will be of great interest for researchers and producers. The objective of the study is to identify yeast isolates degrading citric acid and to investigate their efficacy in controlling citric acid content during kiwifruit wine fermentation, further evaluating its effect on the wine quality.

2. Materials and Methods

2.1. Culture Media

Two media were utilized to cultivate yeast strains. YPD medium (1% yeast extract, 2% yeast peptone, 2% glucose, *w/v*) and PDA (Potato Dextrose Agar) medium (20% potato, 2% glucose, 2% agar, *w/v*) both well met the requirements of yeast growth. WL nutrient liquid medium (WLN): (0.5% yeast peptone, 0.4% yeast extract, 0.055% KH_2PO_4, 0.0425% KCl, 0.0125% $CaCl_2$, 0.0125% $MgSO_4$, 0.00025% $FeCl_3$, 0.00025% $MnSO_4$, *w/v*), lacking of a carbon energy source, was modified with different formulae for subsequent specific yeasts isolation and stress resistance analysis. WL citric acid liquid medium (WLC), supplemented with 0.5% citric acid to be unique carbon source in WLN, was used for screening yeasts with the ability to degrade citric acid. WL citric acid solid medium (WLS): WLC plus added 2% agar. This medium was used for yeasts isolation.

2.2. Fruits and Soil Used for Isolation of Potential Yeast Strains to Degrade Citric Acid

Lemon and orange were chosen for potential yeasts isolation owing to possessing high citric acid content. Lemon fruits (Ziyang, Sichuan Province, China), orange fruits and soil (Zhejiang Citrus Research Institute, Taizhou, Zhejiang Province, China) were harvested and collected into sterile bags immediately in April 2016. The samples were utilized for isolation immediately after transporting to the lab in Huazhong Agriculture University.

2.3. Preliminary Screening and Isolation of Yeasts

The orange and lemon peels were taken into a sterilizing chamber. The peels and soil, weighing 25 g, were placed into separate 500 mL Erlenmeyer flasks containing 225 mL sterile saline and these flasks were shaken at 120 r/min for 20 min at 28 °C in an incubator shaker (IS-RSV1, CRYSTAL). Then a 5 mL aliquot of liquid suspension was added into 100 mL YPD plus added 2 mL streptomycin (1 g/L), and was shaken at 120 r/min for 24 h at 28 °C. A serial dilution with aseptic saline for the 24 h YPD cultivation was conducted to be mixed with WLS adjusted by 0.1 M NaOH to pH 5.5 for preliminary yeast isolation. These obtained plates were incubated at 28 °C for 48 h. Pure cultures of potential target yeasts were selected and identified visually by their typical colony morphologies [23,24].

2.4. Further Screening of Selected Yeasts

The obtained single colonies of selected yeast isolates were inoculated into YPD and cultured at 120 r/min for 24 h at 28 °C. Then 3 mL preculture was centrifugated at 4000 r/min for 5 min to obtain the precipitation. The precipitation was washed by sterile saline twice and then placed into saline for 1 h to consume the retained carbon source. The finally obtained pure precipitation was washed into the 150 mL WLC, and concussion cultured at 120 r/min for 7 d at 28 °C. The initial inoculation was maintained at 10^6 CFU/mL within the OD_{600}, ranging from 0.181 to 0.490. Residual citric acid concentration in WLC was further measured by high performance liquid chromatography (HPLC) [25]. Yeast strains isolated from orange peels were coded as LJD and JP. These yeasts from lemon peels and soil were coded as NP and JT (JST), respectively.

2.5. Yeast Identification

The screened 23 yeasts, which could degrade over 80% citric acid in WLC, were identified by means of polymerase chain reaction–restriction fragment length polymorphism (PCR-RFLP) of 26S rDNA D1/D2 fragment. The yeast cells were collected for DNA extraction according to the GBC Yeast DNA kit (Guangzhou, China). Two sequencing reactions were carried out with forward and reverse primers, NL-1 (5′-GCATATCAATAAGCGGAGGAAAAG-3′) and NL-4 (5′-GGTCCGTGTTTCAAGACGG-3′) [26]. The conditions of PCR amplification were as followed with modifications [26]: initial denaturation at 94 °C for 5 min; 35 cycles of denaturing at 94 °C for 30 s, annealing at 55 °C for 30 s and extension at 72 °C for 1 min; and a final extension at 72 °C for 10 min. The PCR products (2 µL) were separated by gel electrophoresis on 1% (*w*/*v*) agarose gel stained with loading buffer (TsingKe, Wuhan, China). After electrophoresis at 150 V for 20 min, gels were visualized under UV light and photographed by Alpha Innotech (Alpha, San Leandro, CA, USA). Sizes were estimated by comparison against a DNA size marker (1000 bp ladder). The final PCR products were sent to TsingKe (Wuhan, China) for 26S rDNA D1/D2 sequence. The sequences were compared with those available in the GenBank database through the search tool (BLAST). The nucleotide sequences of these yeast isolates were uploaded into the database to get access numbers.

2.6. Stress Resistance Analysis

The 15 selected yeast isolates were grown in WLN with different formulas for stress resistance analysis. Prior to the application of the stress treatment, they were activated in YPD at 28 °C, 120 r/min for 24 h to achieve 10^7 CFU/mL. RV002 (*Saccharomyces cerevisiae*, Angel, Yichang Hubei, China) was used as a reference strain. Incubation was carried out at 28 °C for 48 h and the inoculum amount was 3% (adding 150 µL preculture into 5 mL modified WLN). After incubation, the growth of yeast cells was determined by the measurement of OD_{600} with a UV spectrophotometer (UV-1750, Shimadzu, Tokyo, Japan).

To determine yeast tolerance to glucose permeability, yeast cells were inoculated into WLN with added glucose concentrations of 5%, 10%, 15%, 20%, 25% and 30% (*w*/*v*). For citric acid resistance, yeast cells were inoculated into WLN containing sole citric acid contents 0.50%, 0.75%, 1.00%, 1.25%,

1.50% and 1.75% (*w*/*v*). Ethanol resistance potential was determined by inoculating the yeasts into WLN containing 5% glucose and ethanol 0%, 4%, 8%, 12%, 16% and 20% (*v*/*v*), respectively [27,28]. The SO_2 resistance was evaluated in WLN added with 5% glucose and increasing doses of $K_2S_2O_5$. An arbitrary scale with six levels that corresponded to 80, 120, 160, 200, 240 and 280 mg/L total SO_2 was applied [29]. To induce acid resistance, yeast cells were transferred to WLN containing 5% glucose with pH of 2, 3 and 4 adjusted by 1 M HCL. In all stress cases, a control was carried out with RV002. In each experiment, all of the strains were tested in parallel in order to avoid any modifications.

2.7. Kiwifruit Juice Fermentation Experiment

2.7.1. Kiwifruit Juice Pre-Treatment

The eating-ripe kiwifruits (*Actinidia deliciosa*) (Zhouzhi County, Shanxi Province, China) were subjected to washing under running water. Subsequently, the kiwifruits were peeled and broken into pieces. Pectinase (30,000 U/g, Aladdin, Shanghai, China) was further added according to the manufacturer's suggestion as 1 g/kg kiwifruit. The kiwifruits with pectinase were kept at 30 °C for 3 h, and then pressed to filter through four layers of medical gauze. The finally obtained kiwifruit juice was pasteurized at 80 °C for 15 min.

2.7.2. Fermentation Deacidification Experiment

The final selected yeast *Pichia fermentans* JT-1-3 was utilized to start the fermentation in pure culture. Fermentations were carried out in 250 mL Erlenmeyer flasks containing 150 mL juice. The flasks were inoculated with 24 h precultures grown in YPD at 28 °C, 120 r/min and the initial inoculation was counted to be 10^6 CFU/mL. After inoculation, the juice was placed at 28 °C and fermented for 16 d with a speed of 80 r/min (the progress was monitored until citric acid varied within ±0.1 g, 80 r/min was chosen to avoid the too quick release of alcohol and volatile components). Kiwifruit juice without inoculation was set as the control. All the fermentations were carried out as independent experiments, each performed in triplicate.

2.8. Quality Analysis

2.8.1. Determination of Total Acidity, Total Sugars, pH and SSC (Soluble Solids Content)

The total acidity of kiwifruit juice and wine was assessed by titration with NaOH (0.1 M) to pH 8.2, and expressed as tartaric acid (%) according to GB/T 15038-2006. The total sugars were determined using a glucose titration method based on GB/T 15038-2006. The pH value was measured using a digital pH meter (PB-10, Sartorius, Goettingen, Germany). The soluble solids content (SSC) was measured by a portable refractometer (LYT-330, Shanghai, China).

2.8.2. Determination of Organic Acid

The citric, tartaric and malic acids' content in kiwifruit juice and wine were measured by high performance liquid chromatography (HPLC) (e2695, Waters, Milford, MA, USA) [25]. The samples were centrifuged at 8000 r/min for 15 min, twice, and the obtained supernatant was filtered into chromatographic sampling vials by a 0.22 μm water phase filter before chromatographic separations. The separations were accomplished with an Agilent Zorbax SB-Aq C18 column (4.6 mm × 250 mm, 5 μm particle size). Commercial organic acids (Aladdin, Shanghai, China) were used as the markers for quantification. The flavor intensity for organic acids in wine was further calculated as followed: R = S/T, where S was the measured values of organic acids and T represented the flavor thresholds of corresponding organic acids.

2.8.3. Determination of Total Flavonoids and Total Phenolics

The content of total flavonoids and phenolics in juice and wine was measured as previously reported by Zhong [25]. The estimation was carried out in triplicate and the results were averaged.

2.8.4. Determination of Ethanol

Chromatography experiments were carried out using an Agilent 6890 gas chromatograph (Palo Alto, CA, USA) equipped with an EPC split–splitless injector system and a flame ionization detector. The injector temperature and detector temperature were kept at 280 and 220 °C, respectively. The split ratio and split flow were set as 20.0 and 40 mL/min, respectively. Separation was carried out on a DB-FFAP (Agilent 122-3232, 30.0 m × 250 μm × 0.25 μm) capillary column in the constant flow mode. The oven temperature was programmed as follows: initial 50 °C, increasing to 100 °C at the rate of 1 °C/min, from 100 °C (held for 2 min) to 240 °C (held for 2 min) at the rate of 70 °C/min. The total time for one GC run was 56 min. Hydrogen gas was generated for FID at a flow of 40 mL/min. The injection volume was 5 μL.

2.8.5. Determination of Amino Acid Content

The kiwifruit juice and wine samples were sent to SGS (Shanghai, China) for amino acid determination by HPLC [30]. 16 kinds of amino acids were measured. Essential amino acid (EAA) includes Threonine, Valine, Methionine, Isoleucine, Leucine, Phenylalanine, Lysine, and total amino acid (TAA) is further summed. E/T values are calculated as follows:

$$\frac{\mathrm{E}}{\mathrm{T}} = \frac{\text{EAA content}}{\text{TAA content}} \times 100\% \quad (1)$$

2.8.6. Determination of Volatile Compounds

The manual SPME device equipped with a 50/30 μm DVB/CAR/PDMS fiber (Supelco, Bellfonte, PA, USA) was used for volatile compounds' extraction. The kiwifruit juice (5 mL) and wine (5 mL) were placed into 10 mL vials. 2.60 g NaCl and 20 μL cyclohexanone (0.946 mg/mL) used as an internal standard were further added. The vial was sealed up and equilibrated at 50 °C water bath for 10 min, then the volatile compounds were headspace extracted by fiber for 40 min. After extraction, the fiber was inserted into the injection to desorb the analytes for 5 min.

Gas chromatograph mass spectrometry (GC-MS) was carried out in triplicate using Gas chromatography 7820 and the quadrupole mass selective detector 5977A (Agilent Technologies, Palo Alto, CA, USA). The mass spectral ionization temperature was set at 230 °C, and the mass spectrometer was operated in the electron impact ionization mode at a voltage of 70 eV. Mass spectra were taken over the *m*/*z* range 30–550 AMU/sec.

The separation was accomplished on DB-5 column (30 m × 0.25 mm × 0.25 μm). The injector temperature was 250 °C. The temperature program was set as follows: the column was heated from 40 °C (held for 3 min) to 160 °C (held for 2 min) at 3 °C/min, and finally increased to 220 °C (held for 3 min) at a rate of 8 °C/min. Volatile components were identified in MS libraries (NIST 14). An internal standard method was used to quantify the content of identification compounds.

2.9. Statistical Analysis

All experiments were conducted in triplicate, and the data were analyzed using GraphPad Prism software (V.5.0, GraphPad Inc., La Jolla, CA, USA), OriginPro software (V.8.5, Southampton, MA, USA), and SPSS Statistics software (V.17.0, SPSS Inc., Chicago, IL, USA). Duncan test and independent sample T test were used as significance analysis.

3. Results

3.1. Isolation and Screening of Deacidification Yeast

A total of 100 yeast isolates based on morphological observation [23,24] were picked out from WLS (WL citric acid solid medium) with the ability to degrade citric acid. A representative number of 23 yeast isolates, which could degrade over 80% citric acid in WLC (WL citric acid liquid medium),

were further picked out and identified to be non-*Saccharomyces cerevisiaes* by the 26S rDNA D1/D2 sequence (Table 1).

Table 1. Yeast species isolated form lemon, orange, soil and their ability to degrade citric acid in WLC.

Strain Number	Putative Species	Residual Citric Acid Concentration (g/L)	GenBank No.
LJD-7-2	*Candida natalensis*	0.13 ± 0.10b	MN736495
NP-19-2	*Candida xylopsoci*	0.16 ± 0.02b	MN736497
JP-4-2	*Meyerozyma guilliermondii*	0.17 ± 0.13b	MN736499
NP-1-1	*Meyerozymaguilliermondii*	0.18 ± 0.21b	MN736500
JST-18-3	*Pichia guilliermondii*	0.20 ± 0.05b	MN653214
JT-1-3	*Pichia fermentans*	0.22 ± 0.34b	MN314412
NP-5-5	*Meyerozyma guilliermondii*	0.24 ± 0.21b	MN736501
JT-3-5	*Pichia fermentans*	0.25 ± 0.36b	MN650654
JP-17-2	*Pichia fermentans*	0.35 ± 0.22b	MN736502
JT-12-2	*Saturnispora silvae*	0.37 ± 0.30b	MN650662
JP-17-1	*Pichia fermentans*	0.37 ± 0.20b	MN736503
JST-7-1	*Debaryomyces hansenii*	0.39 ± 0.44b	MN704642
NP-10-12	*Meyerozyma guilliermondii*	0.45 ± 0.00b	MN736522
JT-20-3	*Pichia fermentans*	0.46 ± 0.20b	MN653215
JP-11-3	*Pichia fermentans*	0.47 ± 0.34b	MN736541
JST-8-2	*Pichia fermentans*	0.49 ± 0.40b	MN704646
JT-1-5	*Pichia fermentans*	0.55 ± 0.21b	MN704563
JST-9-1	*Saturnispora silvae*	0.61 ± 0.08b	MN704647
NP-8-5	*Pichia fermentans*	0.64 ± 0.00b	MN736543
JT-6-2	*Pichia fermentans*	0.67 ± 0.06b	MN704574
JP-1-11	*Pichia fermentans*	0.76 ± 0.12b	MN736544
JT-14-1	*Pichia fermentans*	0.81 ± 0.19b	MN704650
JT-20-4	*Pichia fermentans*	0.84 ± 0.00b	MN736545
NP-7-5	*Saccharomyces cerevisiae*	4.98 ± 0.00a	MN736547

Values are mean ± standard deviation (SD). Different lower letters (a, b) illustrate the significant differences ($p < 0.05$) in the same column.

The residual citric acid concentration in WLC ranged from 0.13 to 0.84 g/L with an average of 0.43 g/L, and showed insignificant difference among the 23 non-*Saccharomyces cerevisiaes*. These non-*Saccharomyces cerevisiaes* belonged to five species, including 14 strains of *Pichia* sp., four strains of *Meyerozyma* sp., two strains of *Candida* sp., two strains of *Saturnispora* sp., and one strain of *Debaryomyces* sp (Table 1). One isolate identified to be *Saccharomyces cerevisiae* was also listed in Table 1, and it was verified to be unable to degrade citric acid. The top highest 15 yeast strains in citric acid degradation capacity were taken for further research.

3.2. Stress Resistance Analysis

A further screening was carried out with the 15 yeast isolates to test their ability to survive subjected to several stress conditions associated with wine fermentation in comparison with commercial *S. cerevisiae* RV002. The following characteristics were chosen for further investigations: (i) osmotic pressure tolerance (growth in the presence of 5% to 30% glucose); (ii) citric acid pressure tolerance (growth capacity up to 0.5% to 1.75% citric acid); (iii) alcohol accumulation tolerance (ability to grow in expose to 0% to 20% ethanol); (iv) SO_2 pressure tolerance (80 to 280 mg/L SO_2); (v) growth capacity in a typical pH range (pH 2.0 to 4.0).

The data concluded from Table S1 indicated that the yeasts showed wide biodiversity under different stress conditions. It was obvious to find that these selected non-*Saccharomyces cerevisiaes* all showed good tolerance in a hypertonic environment, and they even behaved well at 300 g/L glucose (Table S1). It was mentioned that RV002 always maintained high level of growth exposed to osmotic pressure. These yeast isolates, except for JST-7-1, all showed high tolerance to citric acid, showing

irregular fluctuations under gradient citric acid (Table S1). The ethanol stress results indicated that JP-17-1, NP-19-2 showed stronger resistance than other yeasts which were significantly different, comparable to that of *S. cerevisiae* (Table S1).The variable concentration of SO_2 did not cause any obvious promotion or inhibition to the growth of these yeasts, and JT-1-3 showed the highest resistance within the arbitrary concentration from 80 to 280 mg/L, even stronger than RV002 (Table S1). Low pH might inhibit the growth of non-*Saccharomyces cerevisiaes*, and the best growth of some yeasts, including RV002, was interesting to be found in the medium at pH 3.0 (Table S1).

The PCA of the data derived from stress resistance properties was utilized to further reveal the diversity among these yeasts (Figure 1). PC1, PC2 and PC3 comprised 41.802%, 28.114% and 11.206%, respectively, totally enough to explain most information. Their loadings in PCA were shown in Figure S1. As shown in Figure S1, the all-resistance parameters were in the forward direction in PC1 loading. As for PC2 and PC3, citric acid and SO_2 resistance contributed mostly to the forward direction, but ethanol resistance was both in the negative direction. JT-1-3, JP-4-2, JP-11-3, NP-1-1, JT-3-5 were all positioned in the forward direction of PC1, PC2 and PC3, showing the high resistance to these stress conditions, especially high citric acid and SO_2 resistance, combined with relatively low ethanol resistance (Figure 1). Among that, JT-1-3 stands out especially, owing to its highest SO_2 resistance properties. NP-19-2 and RV002 were in the lower quadrant for the strong ability to bear high alcohol (Figure 1). Furthermore, the screened non-*Saccharomyces cerevisiaes* and commercial *S. cerevisiae* RV002 were easily distinguished in Figure 1. JT-1-3 showed the greatest potential to survive under these stress environments, or even more hostile ones than other non-*Saccharomyces cerevisiaes*, and it was selected as the optimal yeast strain for further research.

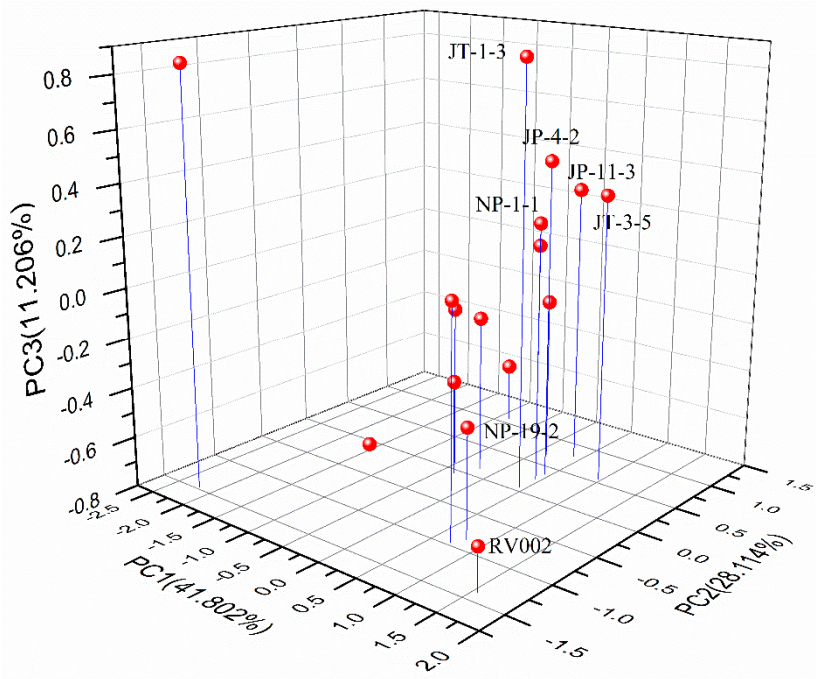

Figure 1. Principal Component Analysis (PCA) of the resistance properties of pure cultures of the screened non-*Saccharomyces cerevisiaes* and commercial *S. cerevisiae* RV002.

3.3. Analytical Profiles of Kiwifruit Juice and Wine

3.3.1. Changes in Properties in Kiwifruit Juice and Wine

The selected *Pichia fermentans* JT-1-3 was used in pure cultures for kiwifruit wine fermentation to further verify its ability to degrade citric acid and study its effect on the wine. It was found that JT-1-3 was present to produce limited quantities of ethanol, being only 4.23% ± 0.45%. The changes in the properties of kiwifruit juice and wine were listed in Table 2. The obvious decline in SSC and

total sugars coincided with the great consumption of sugars by JT-1-3. Before fermentation, citric acid occupied 74.13% of the total acid, and the proportion for malic and tartaric acids was limited to 18.41% and 3.65% (Table 2). After fermentation, a significant decline was found in the three organic acids, contributing to the significant decline in total acid. Finally, citric, malic and tartaric acids declined to 69.49%, 12.76% and 2.59% of the total acid in kiwifruit wine, respectively.

Table 2. Properties of kiwifruit juice and wine.

	Kiwifruit Juice	Kiwifruit Wine
pH	3.41 ± 0.02a	3.45 ± 0.05a
SSC (%)	13.0 ± 0.0	6.0 ± 0.0
Total sugars (g/100 g)	11.56 ± 0.58a	4.33 ± 0.34b
Acids (g/L)		
Tartaric acid	0.61 ± 0.03a	0.41 ± 0.04b
Malic acid	3.09 ± 0.18a	2.02 ± 0.12b
Citric acid	12.30 ± 0.09a	11.00 ± 0.10b
Total acid	16.80 ± 0.04a	15.83 ± 0.60b
Active ingredients		
VC (mg/100 mL)	4.85 ± 0.96a	5.53 ± 1.41a
Total flavonoids (mg/mL)	0.078 ± 0.002a	0.088 ± 0.001a
Total phenol (mg/L)	109.89 ± 0.29a	60.46 ± 3.49b

Values are expressed as the mean ± SD of three independent experiments (n = 3). Mean values in the same row with different lowercase letters (a,b) are significantly different ($p < 0.05$).

The flavor intensity for the three kinds of organic acids (malic, citric and tartaric acids) in wine were further displayed (Figure 2). Citric acid owned significantly higher flavor intensity than the other two acids in kiwifruit wine, contributing mostly to the mild and refreshing acid taste. It was also observed that the V_C content was both in a low level in kiwifruit juice and wine (Table 2). No significant effect was found on the total flavonoid content, but significant lower total phenol values may have implications during fermentation (Table 2). The results above verified that JT-1-3 did degrade citric acid during fermentation, which contributed mostly to the sour taste in kiwifruit wine, and brought not much side effects on those biologically active components.

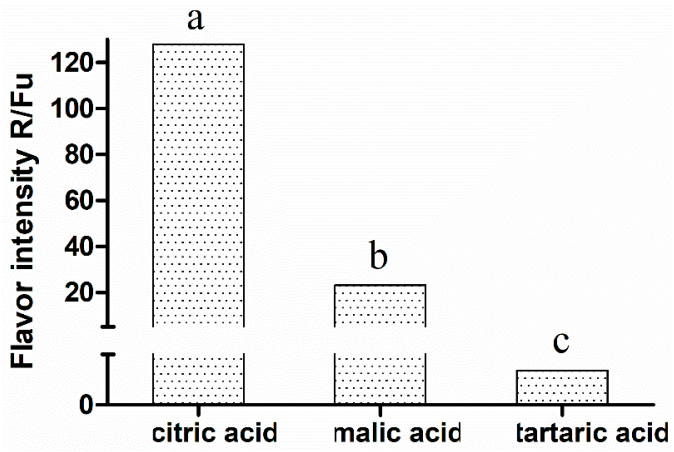

Figure 2. The flavor intensity of three kinds of organic acids (citric, malic and tartaric acids) in kiwifruit wine. Different lowercase letters (a,b,c) illustrate the significant differences ($p < 0.05$).

3.3.2. Changes in Amino Acids Profiles in Kiwifruit Juice and Wine

The changes in amino acid profile in kiwifruit juice and wine were listed in Table 3. Glutamic acid (Glu) was the main amino acid in both kiwifruit juice and wine (Table 3). Only an increase in Proline (Pro) was found after fermentation, while almost all of the other amino acids decreased

or remained stable in wine. In kiwifruit wine, EAA showed a slight decline, but TAA decreased significantly, indicating that *Pichia fermentans* JT-1-3 could consume more nonessential amino acids. This was accordant with the significant increase in E/T values. The results verified that JT-1-3 would improve the amino acid composition in wine.

Table 3. The content of 16 kinds of amino acid in kiwifruit juice and wine.

Amino Acids Content (g/100 g)	Kiwifruit Juice	Kiwifruit Wine
Aspartic acid	0.07 ± 0.00a	0.06 ± 0.01a
Threonine	0.03 ± 0.00	0.03 ± 0.00
Serine	0.03 ± 0.00	0.02 ± 0.00
Glutamic acid	0.21 ± 0.00a	0.16 ± 0.00a
Glycine	0.03 ± 0.00	0.03 ± 0.00
Alanine	0.03 ± 0.00	0.03 ± 0.00
Valine	0.03 ± 0.00	0.03 ± 0.00
Methionine	n.d.	n.d.
Isoleucine	0.03 ± 0.00	0.03 ± 0.00
Leucine	0.03 ± 0.00	0.02 ± 0.00
Tyrosine	0.02 ± 0.00	0.02 ± 0.00
Phenylalanine	0.02 ± 0.00	0.02 ± 0.00
Lysine	0.03 ± 0.00	0.03 ± 0.00
Histidine	0.02 ± 0.01a	0.01 ± 0.00a
Arginine	0.07 ± 0.00	0.05 ± 0.00
Proline	0.02 ± 0.00	0.03 ± 0.00
EAA	0.17 ± 0.00	0.16 ± 0.00
TAA	0.66 ± 0.01a	0.57 ± 0.01b
E/T (%)	25.76 ± 0.39b	28.32 ± 0.25a

n.d.: Not detected. Values are expressed as the mean ± SD. Mean values in the same row with different lowercase letters (a, b) are significantly different ($p < 0.05$).

3.3.3. Changes in Volatile Components

The changes in volatile profile before and after fermentation were listed in Table 4. In the analysis of the volatile components present in kiwifruit juice, a total of 21 compounds were identified, including ten aldehydes, three ketones, three acids, two alcohols, two esters and one phenol (Table 4). Benzaldehyde, followed by 2,4-dimethyl-benzaldehyde, (E)-2-decenal and (Z)-2-hexenol, were the major components found in the greatest proportions before fermentation. At the end of fermentation, a total of 26 compounds were identified, including seven esters, six alcohols, six aldehydes, three acids, two phenols, one ketone and D-limonene (Table 4). In the aroma profile of kiwifruit wine, phenylethyl alcohol was the one found in the greatest concentrations, representing 45.21% of all volatiles. Other predominant components were 4-ethyl-2-methoxy-phenol, 3-methyl-1-butanol and 9-Octadecenoic acid ethyl ester, representing 11.64%, 7.88% and 6.15%, respectively. It was obvious to find the increase in alcohols, esters and phenols, and a slight decrease in aldehydes after fermentation (Table 4).

For kiwifruit wine, it was interesting to find the significant increase for phenylethyl alcohol and five newly identified alcohols. Whereas (Z)-2-hexenol, the main alcohol compound in juice, was not detected in wine. The esters compounds in kiwifruit juice and wine were totally different, and there were seven new esters being detected in wine; nevertheless, the only two esters existing in juice were not both detected in wine. Among these esters, (E)-9-octadecenoic acid ethyl ester, ethyl-9-hexadecenoate and hexadecanoic acid, ethyl ester were the esters found in highest concentrations in wine. The loss detection of six volatile compounds in wine resulted in the slight decrease in aldehydes. Benzaldehyde was the main aldehyde in kiwifruit juice, which decreased significantly after fermentation, and another main aldehyde 2-undecenal was produced during fermentation. No promotion or inhibition effects were found in ketones, and D-limonene was further only found in kiwifruit wine. The results above clearly indicated that JT-1-3 could mostly release the aroma compounds to enrich the aroma profile, especially the alcohols, esters and phenols compounds.

Table 4. Aroma volatile compounds (μg/mL) of kiwifruit juice and wine detected by gas chromatograph mass spectrometry (GC-MS).

Compound Name	Kiwifruit Juice	Kiwifruit Wine
Alcohols		
(Z)-2-Hexen-1-ol	2.141 ± 0.107	n.d.
3-methyl-1-Butanol	n.d.	26.849 ± 0.856
2-methyl-1-Butanol	n.d.	14.355 ± 0.792
2,3-Butanediol	n.d.	1.134 ± 0.000
1-Hexanol	n.d.	2.677 ± 0.513
Phenylethyl Alcohol	1.672 ± 0.427b	154.082 ± 11.247a
(Z)-3,7-dimethyl-3,6-Octadien-1-ol	n.d.	3.020 ± 0.000
Subtotal	3.813	202.117
Aldehydes		
Furfural	1.645 ± 1.236	n.d.
€-2-Heptenal	0.456 ± 0.000	n.d.
Benzaldehyde	8.672 ± 0.083a	3.183 ± 0.335b
(E,E)-2,4-Heptadienal	1.272 ± 0.025	n.d.
Benzeneacetaldehyde	0.761 ± 0.085a	1.912 ± 0.041a
€-2-Octenal	1.394 ± 0.769	n.d.
Nonanal	1.728 ± 0.009	n.d.
4-ethyl-Benzaldehyde	n.d.	1.464 ± 0.000
Decanal	0.429 ± 0.007a	0.833 ± 0.103a
2,4-dimethyl-Benzaldehyde	5.369 ± 0.222	n.d.
€-2-Decenal	2.870 ± 0.071a	2.983 ± 0.320a
2-Undecenal	n.d.	3.104 ± 0.000
Subtotal	24.596	13.479
Ketones		
3,5-Octadien-2-one	0.745 ± 0.000	n.d.
(E)-1-(2,6,6-trimethyl-1,3-cyclohexadien-1-yl)-2-Buten-1-one	0.323 ± 0.014	n.d.
(E)-6,10-dimethyl-5,9-Undecadien-2-one	0.405 ± 0.024	n.d.
5-hexyldihydro-2(3H)-Furanone	n.d.	1.437 ± 0.027
Subtotal	1.473	1.437
Esters		
Propanoic acid, 2-methyl-, 3-hydroxy-2,2,4-trimethylpentyl ester	0.357 ± 0.013	n.d.
1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester	0.394 ± 0.013	n.d.
Decanoic acid, ethyl ester	n.d.	0.897 ± 0.058
Dodecanoic acid, ethyl ester	n.d.	2.105 ± 0.334
Tetradecanoic acid, ethyl ester	n.d.	1.109 ± 0.155
Ethyl 9-hexadecenoate	n.d.	20.050 ± 2.586
Hexadecanoic acid, ethyl ester	n.d.	17.555 ± 1.772
Linoleic acid ethyl ester	n.d.	9.533 ± 0.717
(E)-9-Octadecenoic acid ethyl ester	n.d.	20.949 ± 0.997
Subtotal	0.751	72.198
Acids		
n-Hexadecanoic acid	0.506 ± 0.128a	0.894 ± 0.122a
Oleic Acid	1.794 ± 1.328a	0.859 ± 0.08a
(E)-9-Octadecenoic acid	n.d.	1.078 ± 0.114
Octadecanoic acid	0.616 ± 0.099	n.d.
Subtotal	2.916	2.831
Phenols		
4-ethyl-Phenol	n.d.	7.852 ± 0.466
4-ethyl-2-methoxy-Phenol	1.078 ± 0.000b	39.663 ± 3.220a
Subtotal	1.078	47.515
Alkene		
D-Limonene	n.d.	1.224 ± 1.040
Subtotal	0.000	1.224
Total	34.627	340.801

n.d.: Not detected. Values are expressed as the mean ± SD. Mean values in the same row with different lowercase letters (a,b) are significantly different ($p < 0.05$).

4. Discussion

The aim of the present study was to utilize the biological method by screening the yeasts showing good performance in degrading citric acid during fermentation, and evaluate its potential to positively

improve the wine without being detrimental for *S. cerevisiae*. To obtain fruit wine with low level citric acid is not only for improving the wine taste, but also for benefiting human health. Citrate is the most abundant organic ion found in urine [31], and 11.9 mM of citric acid is enough to cause extracellular acidosis, further doing harm to human tissues and periodontal wounds [14]. Some non-*Saccharomyces* owned the ability to metabolize citric acid for containing carboxylic acid transporters [20]. In the present study, five yeast species including *Candida* sp., *Meyerozyma* sp., *Saturnispora* sp., *Debaryomyces* sp. and *Pichia* sp., were all verified to have the ability to degrade citric acid.

Non-Saccharomyces yeasts are always considered to improve the wine bouquet with low tolerance properties [32]. The high alcohol content is highly toxic to yeast growth [27], and induces water stress in yeast cells [33]. Some certain non-*Saccharomyces*, such as *H. guilliermondii* and *C. stellate*, could tolerate a higher ethanol content than previously reported [34,35]. Active oxygenation was found to increase cell viability to ethanol stress in comparsion to growth under anaerobiosis [22]. Yeast cells also experience hypertonic stress mainly caused by high sugars, leading water flowing out from the cells and reducing water availability [36]. Elevated glucose is likely to provoke stuck fermentations (Ivorra et al., 1999; Munoz and Ingledew, 1989) and causes imbalance of intracellular and extracellular osmotic pressure, even cell death. Indeed, the glucose in many fruit genera was far less than 300 g/L, and kiwifruit only owned around 5 g/100 g glucose [37]. Fortunately, the screened yeasts in this study all survived well in the elevated glucose environment. Furthermore, the accumulated organic acids lowered the pH and posed a threat for the yeasts' survival during fermentation. In this report, it was interesting to find that *Pichia fermentans* JT-1-3 showed the strongest SO_2 resistance among these yeasts. *P. fermentans* could show the same level of tolerance to sulfur dioxide as *S. cerevisiae* in the present of 0.1 g/L SO_2, and can be applied as a good fermentation starter [32,38].

Many researches have reported the significant variations in the mixed fermentations of *S. cerevisiae* and non-*Saccharomyces*, but few have reported the effect of pure cultured non-*Saccharomyces* on the wine, not to mention the application in degrading citric acid during fermentation. The interactions between *Saccharomyces* and non-*Saccharomyces* yeasts in spontaneous fermentations could modulate the expression of some oenological traits and influence the growth and death kinetics in non-*Saccharomyces* yeasts [39,40]. Thus, the obtained *Pichia fermentans* JT-1-3 was applied in the pure culture of kiwifruit wine to study its potential effect.

It was interesting to study the effect of *Pichia fermentans* JT-1-3 on the wine. Apart from citric acid, it was surprising to find the high deacidification rate for malic acid. The significant decline in malic acid might show that *Pichia fermentans* also could conduct malo-ethanolic fermentation (MEF). The produced ethanol and increasing pH [41] were also helpful by reducing the organic acids' flavor intensity. The significant loss of phenolic compounds was found, and this was likely caused by aerobic culture conditions and the degradation ability of JT-1-3. Chtourou [42] reported that some specific yeasts could grow on phenol as the unique source of carbon in batch or continuous culture, and that the isolated yeast *Trichosporon cutaneum* could accomplish the phenol biodegradation within 48 h. Adav [43] also reported that aerobic conditions would help specific yeasts degrade phenols.

Free amino acids are contributed to be the major nitrogen content in kiwifruit exceeding protein and help the digestibility for humans [44]. Arg, Glu and Ser are considered to present benefits for human health [30], but the content of the three amino acids all declined after fermentation. Glu is considered to be the dominating amino acid in kiwifruit [6], which occupied 31.82% of TAA in kiwifruit juice and 28.07% in wine. Ser is primarily synthesized from 3-phosphoglycerate, and the pool of Ser molecules is affected by reversible cleavage by serine hydroxymethyltransferase in *P. pastoris* [45].

Some studies have indicated that most of the yeasts belonging to the genera *Hanseniaspora*, *Pichia*, *Saccharomycodes* and *Zygosaccharomyces* might enhance the wine aroma by releasing aromatic compound [34]. *Pichia fermentans* has been isolated from wine frequently, but its individual effect on aroma fraction is not clear. Viana et al. [46] found that the genus *Pichia* showed high production of ethyl acetate and acetate esters. Shiota [47] first reported the volatile compounds of kiwifruit and identified ethyl butanoate, 2-hexenal, 2-henenol, but only 2-hexenal was detected in this study. (Z)-

and (E)-3-henenol are considered to mainly contribute the "old cut grass" aroma in kiwifruit juice by Young [48], but only (Z)-2-hexenol was found in kiwifruit juice in the present study. A relatively high content of methanol is always found in kiwifruit wine, owing to the use of pectinase [3], but it was lucky that methanol was not detected in the present study.

Some aldehydes, alcohols and esters as lipid degradation products were also found in the volatiles of kiwifruit wine. Hexanal was previously reported as the important odorant of kiwifruit [49,50], but it was not detected. A similar result was obtained by Paterson [51], who reported that esters were the major odorous compounds in kiwifruit. Some esters containing the unique flavor characteristic, such as methyl butyrate, ethyl laurate and ethyl butyrate were found in kiwifruit wine [52]. C6 aldehydes and alcohols were considered to be responsible for the fresh, grassy, green sense [50]. (E)-2-hexenal was quantified to be the major compound in the commercial kiwifruit essence, whereas this was not detected in both juice and wine samples. (E,E)-2,4-Heptadienal was identified in the fresh kiwifruit puree and 3-methylbutanal, as one of the major aldehydes, was identified in the kiwifruit essence at level traces, but the former was only detected in kiwifruit juice, and 3-methyl-1-Butanol was only in the kiwifruit wine. *P. fermentans* could not even produce much in the way of volatile acids.

5. Conclusions

In conclusion, *Pichia fermentans* JT-1-3 was screened to be the final yeast strain for showing strong citric acid degradation ability and stress resistance properties. *Pichia fermentans* JT-1-3 does degrade citric acid, while pure-cultured in kiwifruit juice for fermentation and effectively improve the wine taste by reducing the flavor intensity of organic acids. Moreover, JT-1-3 could improve the amino acid composition and mostly enrich the aroma profile of kiwifruit wine.

Supplementary Materials: The following are available online at http://www.mdpi.com/2311-5637/6/1/25/s1, Figure S1: The loading for PC1, PC2 and PC3, Table S1: The growth of the screened non-*Saccharomyces cerevisiaes* and commercial *S. cerevisiae* RV002 at the end of incubation exposing to different glucose, citric acid, alcohol, SO_2 concentration and pH stress treatments, Table S2: Flavor threshold and flavor description of organic acids (malic, citric and tartaric acids) [53–57].

Author Contributions: W.Z. carried out the experiments and analyzed the results. He wrote the manuscript and takes responsibility for the interpretation of the data. T.C. helped conduct the yeast isolation experiments. H.Y. reviewed the manuscript. E.L. led the research program and supervised the experiments. All authors have read and agreed to the published version of the manuscript.

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