Preparation of Four Kinds of Fruit Enzymes and Detection of Their Antioxidant Activity

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Abstract [**Objectives**] To prepare four kinds of fruit enzymes from pitaya (dragon fruit), papaya, orange and pineapple, and to detect their physicochemical properties and antioxidant activities. [**Methods**] Using pitaya, papaya, orange and pineapple as raw materials, pitaya enzyme, papaya enzyme, orange enzyme and pineapple enzyme were obtained by natural fermentation. The physical and chemical properties and antioxidant activity of the four fruit enzymes were analyzed, and the dominant strains in papaya ferment were identified. [**Results**] The pH of the four fruit enzymes ranged from 3.32 to 3.59. The sensory evaluation of orange and papaya enzymes was relatively superior; among them, the orange enzyme exhibited the highest hydroxyl radical scavenging rate (95.76%), while the pineapple enzyme had the highest total phenol content (27.21 μg/mL). The papaya enzyme showed the highest values for DPPH, reducing power, and flavonoids, at 70.55, 1.699, and 0.121 6 mg/mL, respectively. Through the comprehensive comparing, the physicochemical properties and antioxidant activity of the papaya enzyme were relatively superior, with its dominant microbial species being *Lactobacillus* and *Saccharomyces cerevisiae*. [**Conclusions**] Papaya enzyme is a kind of functional food with great development potential, and this study can provide reference for the development of fruit enzyme with high added value.

Key words Papaya, Enzyme, Antioxidant activity, Correlation, Dominant strain

0 Introduction

How to eat more scientifically and how to make dietary choices that better promote health have become topics of growing concern in the present society, where material conditions are abundant and technology continues to advance. Fruit consumption has become an integral part of a balanced diet due to its nutritional benefits. Fruits are rich in vitamins, minerals, polyphenols, flavonoids, and other bioactive compounds, along with trace components known for their anti-cancer properties. These nutrients help replenish essential elements required by the body, support cellular renewal, and enhance energy metabolism, thereby contributing to overall health[1]. At present, thanks to favorable climates in tropical and subtropical regions, a wide variety of fruits with distinctive flavors, such as pitaya, papaya, pineapple, and orange, are available[2]. In China, many processed fruit products including preserved fruits, fruit juices, and canned fruits are developed using fruit as the raw material. Given this background, the development of fruit enzymes presents not only high added value but also significant market potential.

Enzymes are highly popular in Japan, Taiwan, the United States, and other regions, where they have established a strong brand presence. In recent years, they have gradually gained popularity in mainland China, with growing applications in the food, agriculture, and beauty industries[3]. The fermentation process of enzymes primarily involves microorganisms such as lactic acid bacteria and yeast. Enzymes are microbial fermented products made from one or several raw materials—including vegetables, fruits, and herbs through the action of various microorganisms. They contain essential nutrients such as minerals, vitamins, enzymes, and secondary metabolites[4]. Due to their rich content of bioactive compounds including enzymes, vitamins, and minerals[5], enzymes are known to offer health benefits such as combating fatigue, eliminating free radicals, delaying aging, lowering blood lipids, and supporting weight loss[6]. Pitaya enzyme is a metabolite produced through the yeast fermentation of pitaya fruit. Thanks to the rich nutritional profile of pitaya, this enzyme can enhance human immunity, offer antioxidant effects, delay aging, prevent diseases, and eliminate excess free radicals[7]. Papaya enzyme, extracted from the stems, leaves, and fruits of the papaya plant, functions effectively in acidic, neutral, and alkaline environments. It aids in digestion and absorption, facilitates detoxification, supports overall health, and contributes to skin beautification[8]. Pineapple enzyme is abundant in dietary fiber and various amino acids. It stimulates the secretion of digestive enzymes within the digestive tract, alleviates symptoms such as abdominal distension and constipation, promotes local blood circulation, helps regulate the body's pH level, and possesses heat-clearing, diuretic, and detoxifying properties. Orange enzyme offers benefits such as antioxidant activity and immune system enhancement. The physicochemical properties, flavor profiles, and antioxidant activities vary significantly across different fruit enzymes. This study aims to compare the active ingredients and antioxidant capacities of enzymes derived from four tropical and subtropical fruits. The physicochemical characteristics and antioxidant activities of these fruit enzymes were analyzed to provide a reference for developing high-value-added fruit enzyme products.

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1 Materials and methods

1.1 Materials and reagents

1.1.1 Experimental materials. Papaya, pineapple, pitaya, or-

ange and sugar are purchased from Yanling Supermarket of Zhanjiang Lingnan Normal University.

1.1.2 Main drugs and reagents. Trichloroacetic acid, sodium dihydrogen phosphate, anhydrous sodium carbonate, sodium hydroxide, sodium nitrite, analytical pure, Guangdong Guanghua Sci-Tech Co., Ltd.; disodium hydrogen phosphate dodecahydrate, potassium hydrogen phthalate, salicylic acid, ethanol, Xilong Scientific Co., Ltd.; gallic acid, Shanghai Yunguan Mechanical & Electrical Equipment Co., Ltd.; rutin, National Institutes for Food and Drug Control; DPPH, Shanghai Huacheng Industrial Development Co., Ltd.; ferrous sulfate heptahydrate, Guangzhou Chemical Reagent Factory; potassium ferricyanide; Tianjin Damao Chemical Reagent Factory. All the above chemicals were of analytical grade.

Fermentation Mama Fermentation Bucket, Tianlan Shuqing Health Industry Co., Ltd.; T6 Xinyue Visible Spectrophotometer, Beijing Purkinje General Instrument Co., Ltd.; CJ-2D Vertical Laminar Flow Clean Bench, Tianjin Test Instrument Co., Ltd.; H1650 Centrifuge, Hunan Xiangyi Laboratory Instrument Development Co., Ltd.; SPL-250 Biochemical Incubator, Tianjin Labotery Instrument Co., Ltd.; YXQ-LS-75S11 Vertical Pressure Sterilizer, Medical Equipment Factory of Shanghai Boxun Industrial Co., Ltd.

1.2 Methods

1.2.1 Preparation of fruit enzyme. Procedure: Selection of fruit raw materials → Cleaning → Slicing into thin pieces → Loading into the container → Adding purified water → Adding white granulated sugar → Fermentation → Filtration → Finished enzyme product.

Fresh fruits were repeatedly rinsed with an appropriate amount of purified water and air-dried naturally. The fruits were then thinly sliced on a laminar flow clean bench. White granulated sugar was sterilized under ultraviolet light for 30 min. A mixture of 500 g of fruit, 300 g of sugar, and 2 400 mL of water was added to a sterilized Fermentation Mama Fermentation Bucket and stirred thoroughly. Natural fermentation was carried out at room temperature (20 – 25 ℃) for approximately 5 d.

1.2.2 Culture of dominant bacteria in enzyme. Culture medium: MRS broth medium (3 g beef extract, 10 g peptone, 15 g agar powder, 5 g sodium chloride, 1 L distilled water). An appropriate amount of post-fermentation enzyme product was diluted to different concentrations, inoculated onto MRS medium using the spread plate method, and cultured in a constant temperature incubator at approximately 28 ℃ for 48 h after solidification.

Culture medium: YPD medium (20 g peptone, 10 g yeast extract, 20 g glucose, 20 g agar powder, 900 mL distilled water). An appropriate amount of post-fermentation enzyme was transferred to a sterilized beaker, inoculated into the YPD medium using the streak plate method, and then cultured in an inverted position in a constant temperature incubator at approximately 28 ℃ for 48 h.

1.2.3 Sensory evaluation criteria. According to the evaluation standards of the *Edible Plant Enzymes* and the sensory characteristics of liquid enzymes[10], ten participants were invited to score four types of fruit enzymes. The sensory evaluation criteria are shown in Table 1.

Table 1 Sensory evaluation criteria of enzymes

Evaluation indicators	Scoring criteria	Score points
Appearance	Clear, free of suspended solids and impurities	8 – 10
	A small amount of suspended solids and impurities	4 – 7
	Turbid with a large amount of suspended solids and impurities visible	1 – 3
Color	Good color, transparent	8 – 10
	General color, a little dull	4 – 7
	Bad color, dull	1 – 3
Smell	Aromatic, fruity	8 – 10
	General smell, too strong alcoholic smell	4 – 7
	Pungent smell	1 – 3
Mouthfeel	Good and smooth	8 – 10
	General and a little astringent	4 – 7
	Bad and not smooth	1 – 3
Taste	No strange taste, pleasant flavor	8 – 10
	No strange taste, general flavor	4 – 7
	Strange taste, bad flavor	1 – 3

1.2.4 Prepared the standard buffer solution of pH 4 and pH 6.68, took a proper amount of four kinds of fruit enzymes which have been fermented for 5 d, used the pH meter to calibrate them twice and then measured them directly, and took the average value after three times of measurement.

1.2.5 Determination of total phenols. The total phenols was determined using Folin-Ciocalteu colorimetric method[11]. With 10 mL reaction system, took 0, 1, 2, 3, 4, 5 mL of 12 μg/mL gallic acid standard solution into 5 colorimetric tubes, respectively, and then added appropriate amount of distilled water into the colorimetric tubes to 5. Then added 2.5 mL of 10% Folin phenol reagent and 2 mL of 7.5 mg/mL sodium carbonate solution, kept the temperature in a water bath at 45 ℃ for 15 min, mixed well, placed it in the dark for 60 sec, measured the absorbance at the wavelength of 765 nm, and plotted a standard curve as $y = 0.006\ 74x - 0.003$, $R^2 = 0.998$.

Sample determination: took 5.5 mL of fruit enzyme solution, repeated the following operations, substituted the measured absorbance of the sample into the standard curve as $y = 0.006\ 74x - 0.003$, $R^2 = 0.998$, to obtain the total phenol content of different samples, and took the average value of three determinations.

1.2.6 Determination of flavonoids. Using 9 mL reaction system, successively measured 0 – 5 mL of 0.15 mg/mL rutin standard solution into 6 colorimetric tubes, then added 30% ethanol solution into the 5 mL colorimetric tubes, and added 0.50 mL of 5% sodium nitrite into the colorimetric tubes, shook well, and placed at room temperature for 6 min. Then added 0.50 mL of 10% aluminum nitrate, and placed at room temperature for about 6 min. Finally, added 3.0 mL of 4% sodium hydroxide solution, mixed well, and placed at room temperature for 15 min. Measured the absorbance value (A) at 510 nm[12], to obtain the standard curve

$y = 7.039x - 0.025\ 1$, $R^2 = 0.997\ 7$.

Sample determination: took 5 mL of fruit enzyme solution, repeated the following operations, substituted the measured absorbance of the sample into the standard curve as $y = 7.039x - 0.025\ 1$, $R^2 = 0.997\ 7$, to obtain the flavonoids content of different samples, and took the average value of three determinations.

1.2.7 Determination of hydroxyl radical scavenging rate. The hydroxyl radical scavenging rate was determined using salicylic acid method. Using 15 mL reaction system, added 1.0 mL of 9 mmol/L ferrous sulfate solution, 1.0 mL of 9 mmol/L ethanol-salicylic acid solution into three colorimetric tubes, then added 5.0 mL of sample and appropriate amount of distilled water, and then added 1 mL of 8.8 mmol/L H_2O_2 solution, shook well. After 15 min of water bath at 37 ℃, the absorbance was measured at 510 nm[13], and the hydroxyl radical scavenging rate was calculated by the following formula:

Hydroxyl radical scavenging rate (%) $= A_0 - (A_x - A_{X0})/A_0$

1.2.8 Determination of reducing power. Took 2 mL of fruit enzyme into a 20 mL colorimetric tube, added 2.5 mL of phosphate buffer solution with pH of 6.6 and 2.5 mL of 1% potassium ferricyanide solution, gently mixed them, put them into a water bath at 50 ℃ for 20 min, then added 2.5 mL of 10% trichloroacetic acid solution, and mixed them. Centrifuged at 3 000 r/min for 10 min, then took 2.5 mL of centrifuged supernatant, added 2.5 mL of 0.1% ferric chloride solution and 2.5 mL of distilled water, measured the absorbance at 700 nm, and took the average value after three measurements[14].

1.2.9 Determination of DPPH radical scavenging rate. Took 2 mL of fruit enzyme solution diluted by 10 times, measured 2 mL of 0.2 mmol/L DPPH ethanol solution with a pipette, shook and placed in the dark for 30 min, determined the absorbance A_1 at 517 nm, and measured 2.0 mL of absolute ethanol instead of DPPH ethanol solution to determine the absorbance A_2. Used 2 mL of absolute ethyl alcohol to replace the sample to measure its absorbance A_0, measured three times and took the average value[15], and the DPPH radical scavenging rate was calculated by the following formula:

DPPH radical scavenging rate (%) $= A_0 - (A_1 - A_2)/A_0$

1.3 Morphological identification of dominant strains in enzyme from papaya (i) Colony morphology. Take the MRS broth medium and YPD medium inoculated and cultured using the method in Section **1.2.2**, observed the characteristics of the colonies on the plate with naked eyes, and recorded the size, edge, shape, texture and color of the colonies, respectively[16]. (ii) Cell morphology. A drop of distilled water was placed on a glass slide. Using a repeatedly flame-sterilized and cooled inoculation loop, a small amount of the cultured yeast from the inoculated YPD medium was sampled and evenly spread on the slide. Observation was then conducted under a microscope with an objective lens (40×). For bacterial observation, a drop of saline solution was added to another glass slide. Using a flame-sterilized and cooled inoculation loop, a small amount of the cultured bacteria from the inoculated MRS broth medium was sampled, subjected to Gram staining, and observed under a microscope with an objective lens (100×). The morphology and size were recorded[17].

1.4 Data processing Excel 2010 was used for data processing and drawing, SPSS software was used for data variance analysis and difference significance comparison, Pearson correlation coefficient method was used for data correlation analysis, and the significance level was $P < 0.05$.

2 Results and analysis

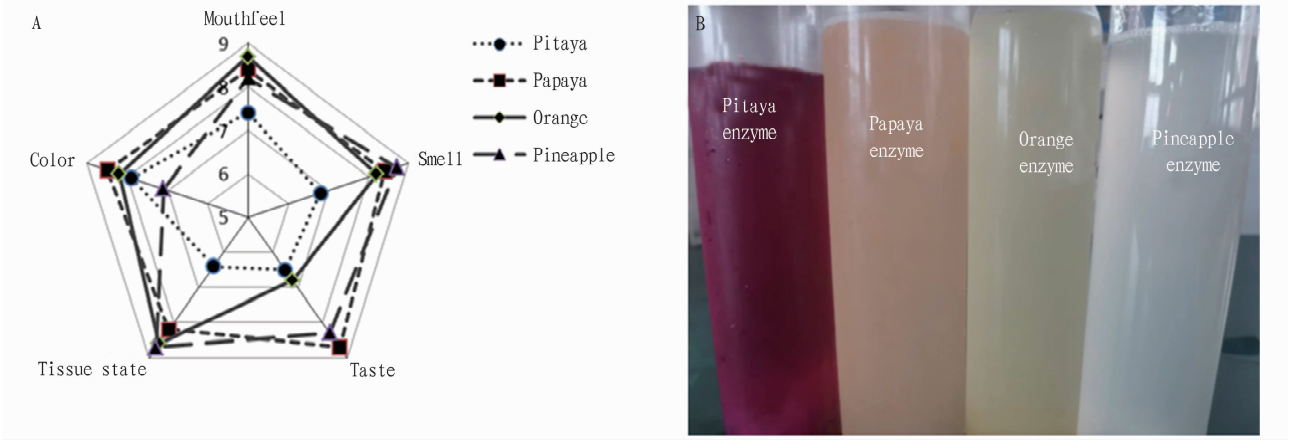
2.1 Sensory evaluation of four fruit enzymes As can be seen from Fig. 1, in terms of taste, the orange enzyme beverage scored higher than those made from papaya, orange, and pineapple, with a score of 8.7. The papaya enzyme beverage exhibited good color, clarity, no off-flavors, a well-balanced flavor, and a pleasant taste, with all scores above 8 points. The orange enzyme beverage had a smooth taste, was clear and transparent without suspended solids or impurities, and all indicators scored above 8.2 points except for its taste, which was relatively low at 6.8 points. The pitaya enzyme beverage showed a small amount of suspended solids and impurities, no off-flavors but an average overall flavor, with scores across indicators ranging from 6.4 to 7.9 points, resulting in a relatively poor overall evaluation. The pineapple enzyme beverage had an aromatic scent with a fruity fragrance, was clear and transparent without impurities, and all indicators scored above 8.2 points except for its color, which was relatively low at 7.1 points. Thus, it can be concluded that the sensory evaluations of the pineapple and papaya enzyme beverages were relatively superior.

2.2 Detection of physical and chemical properties of four fruit enzymes

2.2.1 pH. During the fermentation of fruit enzyme, because the growth of microorganisms requires maintaining a proper pH, the growth and reproduction of microorganisms and the production of metabolites are closely related to pH. The smaller the pH, the more acidic the fruit enzyme. It can be seen from Fig. 2 that the enzyme pH of the four fruits is 3.32 – 3.59, which meets the requirements of liquid edible plant enzyme pH (pH≤4.5), among which the pH of papaya is the highest (3.59), and there is no difference with the pH of orange ($P > 0.05$). The order of enzyme pH of the four fruits was papaya > orange > pitaya > pineapple.

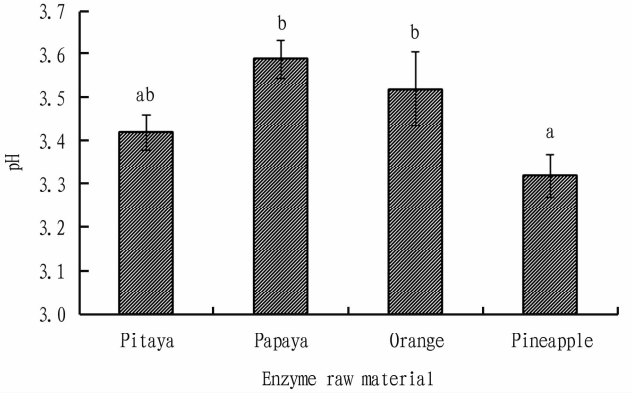
2.2.2 Total phenols. The total phenol in fruit enzyme is a kind of compound with health care effect, which has strong antioxidant activity, and its antioxidant function can prevent many coronary heart diseases, cancers and aging[18]. It can be seen from Fig. 3 that the content of pineapple enzyme is the highest (27.21 μg/mL), followed by papaya enzyme, and its total phenol content is (24.91 μg/mL), which is significantly different from the other three fruit enzymes ($P < 0.05$). The total phenol content of four kinds of fruit enzyme was in the order of pineapple > papaya > orange > pi-

taya, and the total phenol content of pitaya enzyme was relatively low (13.38 μg/mL), which was 49.17% of pineapple enzyme. It can be seen that the content of total phenols varies significantly with different fruit raw materials.



NOTE A. Sensory evaluation radar chart; B. Four fruit enzyme liquid.

Fig. 1 Sensory evaluation chart of four fruit enzymes



NOTE Different letters indicate significant differences ($P<0.05$). The same below.

Fig. 2 Comparison of pH level of four fruit enzymes

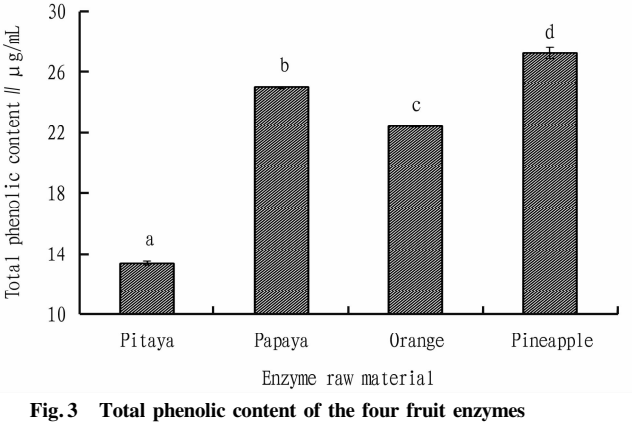


Fig. 3 Total phenolic content of the four fruit enzymes

2.2.3 Flavones. Flavonoids in fruit enzyme have good antioxidant activity, have good scavenging effect on oxygen free radicals in human body, and have the effects of lowering blood pressure, regulating blood sugar, clearing intestines and detoxifying[19]. The flavonoid contents of the four fruit enzymes are shown in Fig. 4. It can be seen from Fig. 4 that the flavonoid content of papaya is the highest (0.121 6 mg/mL), which is significantly different from that of the other three fruit enzymes ($P<0.05$), and the flavonoid content of pitaya enzyme is the lowest (0.032 6 mg/mL). The flavonoid content of enzyme in the four fruits was in the order of papaya > orange > pineapple > pitaya. Therefore, the flavonoid content in different fruit enzymes was different, and the flavonoid content of papaya enzyme was the best among the four fruit enzymes.

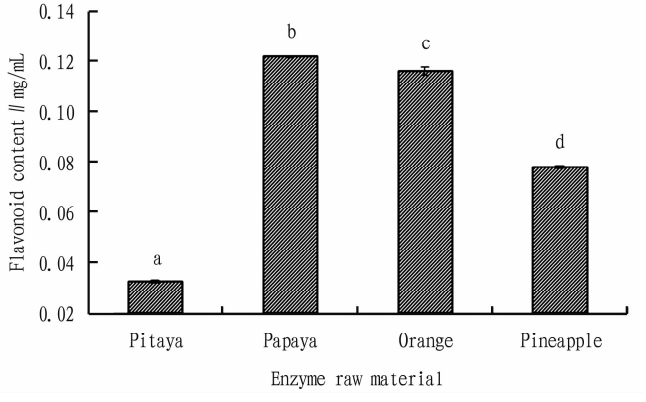


Fig. 4 Flavonoid content of the four fruit enzymes

2.3 Comparison of antioxidant activity of four fruit enzymes

2.3.1 Hydroxyl radical scavenging. Hydroxyl radical is one of the strong oxidants in aqueous solution because of its high ability to get electrons, that is, oxidation ability[20]. If the strong free radicals in the human body are in an excessive state for a long time and can not be removed in time, it will accelerate the aging of normal cells in the human body, and even form tumors and cancers. Fruit enzyme can scavenge all kinds of free radicals to a certain extent. It can be seen from Fig. 5 that there is no significant difference between orange enzyme and pineapple enzyme in scavenging hydroxyl free radical ($P>0.05$), and orange enzyme has the strongest ability to scavenge hydroxyl free radical (95.76%), while pineapple

and papaya enzyme have the strongest ability to scavenge hydroxyl free radical (95.76%). The hydroxyl radical scavenging activity of enzyme was 93.59%, 79.48% and 44.78%, respectively. Therefore, because different fruits contain different nutrients, their ability to scavenge hydroxyl radicals is also different.

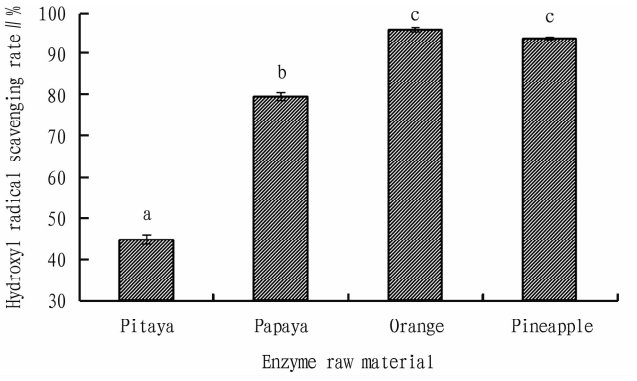


Fig. 5 Hydroxyl radical scavenging rate of four fruit enzymes

2.3.2 Reducing power. In the determination of reducing power, the higher the absorbance value, the stronger the reducing power and the better the oxidation resistance[21]. It can be seen from Fig. 6 that the reducing power of papaya enzyme is the highest (1.699), which is significantly different from the other three fruit enzymes ($P<0.05$). The reducing power of the four fruit enzymes is papaya > pitaya > pineapple > orange, and the reducing power of orange enzyme is the lowest (1.3). It can be seen that the reducing power of different fruit enzymes is significantly different, and the papaya enzyme is the best among the four fruit enzymes.

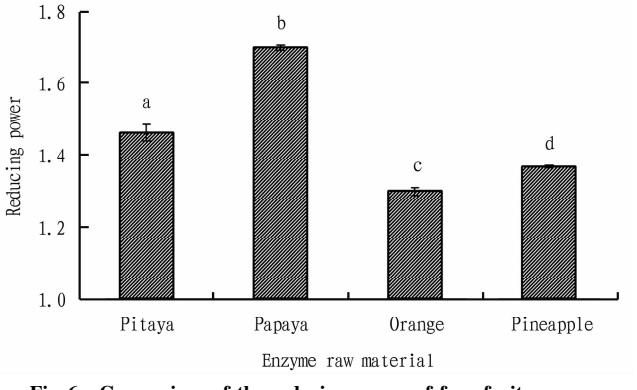


Fig. 6 Comparison of the reducing power of four fruit enzymes

2.3.3 DPPH. As shown in Fig. 7, there is no significant difference in DPPH free radical scavenging capacity between papaya enzyme and pitaya enzyme ($P<0.05$), and papaya enzyme has the strongest DPPH free radical scavenging capacity (70.55%). The DPPH radical scavenging activity of enzyme from pitaya, orange and pineapple was 69.34%, 59.46% and 62.95%, respectively. It can be seen that the DPPH radical scavenging ability of fruit enzymes from different raw materials is different, and their oxidation ability is also different. Among the four enzymes, the DPPH radical scavenging ability of papaya is relatively strong, and its antioxidant activity is the strongest.

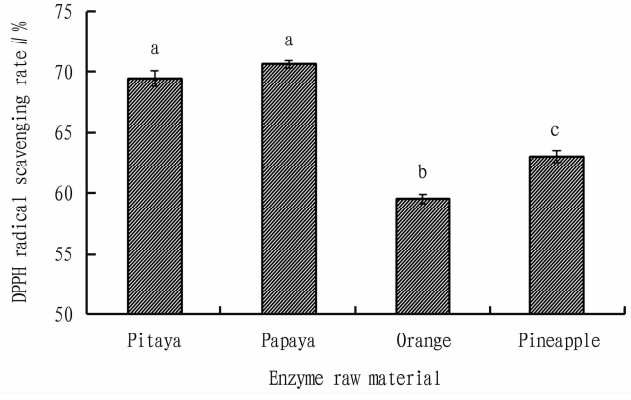


Fig. 7 Comparison of DPPH free radical scavenging rate of four fruit enzymes

2.4 Correlation analysis To verify the correlation between the physical and chemical properties of the four fruit enzymes and their antioxidant activity, the correlation between pH, total phenols, flavonoids and hydroxyl radical scavenging, reducing power, DPPH of the four fruit enzymes was analyzed by Pearson correlation coefficient method. It can be seen from Table 2 that the ability to scavenge hydroxyl free radicals in fruit enzyme has a significant correlation with the content of flavonoids and total phenols ($P<0.01$), and has a significant positive correlation with DPPH ($P<0.05$), but has a weak correlation with pH; while the reducing power and DPPH have little correlation with pH, flavonoids and total phenols. Therefore, the scavenging of hydroxyl radicals by fruit enzyme was closely related to the content of total phenols and flavonoids.

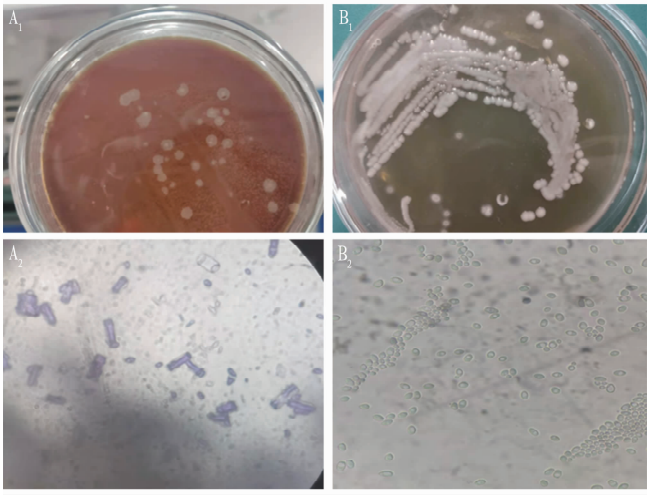
Table 2 Correlation of physicochemical properties and antioxidant activity of the four fruit enzymes

	pH	Total phenols	Flavonoids	Hydroxyl radical scavenging capacity	Reducing power	DPPH
pH	1.000					
Total phenols	−0.010	1.000				
Flavonoids	0.532	0.713**	1.000			
Hydroxyl radical scavenging capacity	0.043	0.883**	0.766**	1.000		
Reducing power	0.419	0.044	0.205	−0.318	1.000	
DPPH	0.253	−0.354	−0.282	−0.705*	0.863**	1.000

NOTE **: at 0.01 level (two-tailed), the correlation is significant; *: at 0.05 level (two-tailed), the correlation is significant.

2.5 Morphological identification of dominant strains in papaya enzyme The colony morphology of the dominant bacteria in the papaya enzyme is shown as A_1 and B_1 in Fig. 8. The A_1 colony is round and white in the nutrient agar, with a bulge in the mid-

dle, which is easy to be picked up, and its diameter is about 3 mm, while the surface of the B_1 colony is relatively smooth, showing milky white, which is easy to be picked up; The cell morphology of the dominant bacteria is shown as A_2 and B_2 in Fig. 8 under the microscope. A_2 cells are rod-shaped under the microscope and appear purple after Gram staining, so they are Gram-positive bacilli. B_2 cells are oval under the microscope, with vacuoles and nuclei, and their reproductive mode is budding. Therefore, it is inferred that *Lactobacillus* and *Saccharomyces cerevisiae* are the dominant bacteria in papaya enzyme, which is consistent with the research results of Du Liping *et al.* [22].



NOTE A_1: *Lactobacillus colony*; B_1: *S. cerevisiae* colony; A_2: *Lactobacillus* cells; B_2: *S. cerevisiae* cell.

Fig. 8 Morphological observation of the dominant bacteria of papaya enzyme

3 Conclusions and discussion

Through analyzing and comparing the sensory characteristics, physicochemical properties and antioxidant activities of pitaya enzyme, papaya enzyme, pineapple enzyme and orange enzyme, it was found that the physicochemical properties and antioxidant activities of different fruit enzymes were different. The pH of four kinds of fruit enzyme was 3.32 – 3.59, which was in line with the requirements of edible plant enzyme pH, in which orange enzyme had the highest scavenging hydroxyl radical (95.76%), and pineapple enzyme had the highest total phenol (27.21 μg/mL). Papaya enzyme has a relatively high content of total flavonoids, and its reducing power, DPPH scavenging ability and sensory evaluation are the best, and its dominant strains are *Lactobacillus* and *S. cerevisiae*. Therefore, papaya enzyme is a functional food with great potential for development, and its functional evaluation and other active ingredients need to be further studied.

According to the experimental data, enzymes are primarily produced through fermentation by microorganisms such as yeast, mold, and lactic acid bacteria. For instance, Dong Jie *et al.* [23] employed lactic acid bacteria and yeast to ferment golden jujube (*Ziziphus jujuba*) paste enzyme at temperatures between 30 ℃ and 37 ℃. Similarly, Sun Daqing *et al.* [24] reported that amylase activity in millet enzyme was highest at 32 ℃. In contrast, the fermentation temperature used in this experiment for fruit enzyme ranged from 18 ℃ to 25 ℃, which falls outside the optimal temperature range for microbial activity. This suboptimal temperature may have prevented the microorganisms from functioning effectively, thereby influencing the experimental outcomes. Therefore, it is necessary to conduct a single-factor optimization experiment on temperature to determine the most suitable fermentation conditions. Additionally, due to the limited number of fermentation vessels, the enzymes were tested sequentially after fermentation rather than simultaneously. Variations in the preparation of chemicals and reagents might also have introduced errors. Whether these factors significantly affected the results requires further verification.

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(From page 36)

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