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Characterization, enzymatic and biological properties of a dominant lactic acid bacteria strain of *Lactobacillus sakei* subsp. *sakei* isolated from stinky mandarin fish

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Abstract

Presently in order to excavate suitability of resources of lactic acid bacteria for fermentation of mandarin fish, a strain of lactic acid bacteria (LAB), which was cultivated from stinky mandarin fish via using traditional MRS medium (CMC 1-8), was firstly isolated and purified. Subsequently, it was discovered that the as-harvested LAB was recognized as *Lactobacillus sakei* subsp. *sakei* with assistance of technology of phenotypic identifying and 16S rRNA sequencing. Concurrently, relevant characteristics in aspects of growth curve, acid production capacity, temperature and salt tolerance of *Lactobacillus sakei* subsp. *sakei* were plainly investigated. Furthermore, the preliminary research on protease properties and biological properties was revealed. The results unfolded that the strain could grow and produce acid normally at 37 °C, and deliver a strong tolerance even under 3% NaCl at 12 °C, which could be significant sufficiently to satisfy the low-temperature and salty fermentation of mandarin fish. The best compatible temperature and pH value for protease production were at 70 °C and 9.0, respectively. Resultantly, the main metabolites, containing polyphenols, flavonoids and soluble polypeptides, performed a remarkable effect on scavenging abilities of 2,2'-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), 1,1-diphenyl-2-picrylhydrazine (DPPH) free radical and ferricyanide reducing ability, of which the IC_{50} were 1.05, 6.87, and 4.62 mg/mL, respectively. And, the metabolites displayed partial inhibitory effects on *Escherichia coli*, *Staphylococcus aureus*, *Pseudomonas aeruginosa*, and *Bacillus subtilis*. Therefore, CMC 1-8 can potentially serve as a functional microorganism to directionally ferment mandarin fish.

Keywords: stinky mandarin fish; *Lactobacillus sakei* subsp. *sakei*; protease properties; metabolites; antioxidant activity.

Practical Application: A dominant lactic acid bacteria strain of *Lactobacillus sakei* subsp. *sakei* was isolated from stinky mandarin fish, a Chinese traditional fermented food. This promising strain CMC 1-8 showed strong resistance against low temperature and salt and possessed antioxidant and antimicrobial activities. *Lactobacillus sakei* subsp. *sakei* CMC 1-8 can be used as a potential probiotic starter culture for the fermentation of mandarin fish.

1 Introduction

Mandarin fish (*Siniperca Chuatsi*), as is known to all, is a kind of freshwater fish, which includes major nutritious elements in high protein and low fat. By contrast with mandarin fish, stinky mandarin fish earned its popularity as famous traditional fermented food in China, which can be prepared with high-quality mandarin fish as raw material, low temperature and low salt as fermentation conditions, spices as the added supplement to obtain the "smelly but not stinky" flavor (Yang et al., 2017). Especially, the time-honored stinky mandarin fish is excellently popular among consumers due to its unique regional flavor and rich nutritional value. It was reported that the broadened market developed that total output sale value of stinky mandarin fish in 2021 had exceeded 4 billion CNY. In most cases, natural fermentation still dominated the main method to prepare stinky mandarin fish. Due to huge and complex microbial fermentation system, instability in product quality and flavor turned to be inevitable challenge and limitation to industrialize stinky mandarin fish production. For that reasons, screening the functional microorganisms in the fermentation process is thought of to be the key course to regulate the stinky mandarin fish.

The successful isolation of functional microorganisms from sorts of fermented foods such as fermented sausage (Alkalbani et al., 2019), fermented kimchi (Lee et al., 2011) and fermented wine (Fan et al., 2021) has been achieved. Fortunately, directional inoculation of these isolated functional microorganisms was instrumental in shortening the fermentation cycle, improving product quality and strengthening food flavor (Najjari et al., 2020; Moon et al., 2018). As common bacteria for food fermentation, LAB, which are constituted by *Lactobacillus*, *Leuconostoc*, *Lactococcus* and others, have been confirmed to be closely related to volatile flavor substances and nutrients in most types of fermented foods (Wang et al., 2022). LAB exhibit

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similar antioxidant capacities that can slow lipid peroxidation and prolong food shelf life (Stecchini et al., 2001). Additionally, the LAB-metabolized organic acids and bacteriocin can reduce the pH value of the system and play a so critical role in inhibiting bacteria activities that those metabolites evidently improved food safety (Alvarez-Sieiro et al., 2016). For example, inoculation of *Lactococcus lactis* in stinky mandarin fish inhibited the accumulation of total volatile basic nitrogen (TVB-N) and biogenic amines, improving the production of flavor substances and shortening the fermentation period (Bao et al., 2018).

Because of its excellent performance, *Lactobacillus sakei*, a type of LAB, had been widely adopted in preparation of starter cultures (Zhang et al., 2022). Boumaiza et al. (2021) and Gao et al. (2014) found that *L. sakei* as the starter culture could tremendously improve fatty acid distribution, reshape sensory properties and decline nitrite and malondialdehyde content in fermented sausages. Fundamentally, the LAB-deduced considerable changes of flavor and nutritional element derived in most case from its metabolic enzymes specificity and activity. However, the study on the activity of its metabolites was indispensable. Owning to different strain sources and survival pressures, subspecies of *L. sakei* widely exist in fermented foods. These subspecies adapt to the current environment through adaptive evolution, and often have better fermentation characteristics.

Up to now, there are few studies either on the isolation of microorganisms from stinky mandarin fish or on evaluation the biological activities of their metabolites. In this paper, a lactic acid bacteria strain CMC 1-8 (*L. sakei* subsp. *sakei*) was isolated from stinky mandarin fish as raw source and then practically purified. Subsequently, its characterization, enzymatic and biological properties were identified and explored with aim to providing theoretical basis for improving and regulating the fermentation technology of mandarin fish.

2 Materials and methods

2.1 Isolation and purification of LAB from stinky mandarin fish

Stinky mandarin fish were provided by Huxingtang Taohua Liushui Food Co., Ltd., Huangshan City, China. MRS medium was purchased from Hangzhou Baisi Biotechnology Co., Ltd. Under aseptic conditions, the back, maw, and tail of the stinky mandarin fish, a total of 20 g of fish meat, was cut into pieces and was added to a conical flask containing 180 mL of sterile physiological saline (containing a certain amount of small glass beads). After shaking and standing, the supernatant was serially diluted by 10 fold with normal saline. 100 μL diluted sample homogenates were drawn and spread on the modified MRS solid medium containing 0.4 g/mL bromocresol purple (Castilho et al., 2015). The plates were placed upside down in an incubator and cultured at 37 °C for 48 h. After the cultivation was completed, a single colony in the yellowing area of the bromocresol purple plate was picked with an inoculation loop, and transferred to the MRS solid medium by the three-area streak method. Cultured at 37 °C for 48 h to further purify the strain.

2.2 Phenotypic identification

The activated bacterial solution was streaked on MRS solid medium, cultured at 37 °C for 24 h, and the colony characteristics were recorded. Single colony was picked for Gram staining, and the color and shape of the strain was observed under a microscope. The microscopic shape of the strain was photographed at a magnification of 5000 times by S-3400N scanning electron microscope.

2.3 Bacterial 16S rRNA gene sequencing and construction of phylogenetic tree

Bacterial genome extraction kit (Tiangen, China) was used to extract, isolate and purify the genomic DNA. Bacterial universal primers (the primers 27F and 1492R) were used for PCR amplification of 16S rRNA sequences (Syafitri et al., 2022). The synthesis of primers and the sequencing of PCR products were entrusted to Shanghai Sunny Biotechnology Co., Ltd. The measured 16S rRNA gene sequences were subjected to Blast alignment and homology analysis in NCBI. The appropriate reference strain sequences were selected, and the phylogenetic tree was constructed according to the Neighbor-Joining method using MEGA 11.0 software. The LAB isolated in this study was identified as *L. sakei* subsp. *sakei*, which has been registered and preserved in the China Center for Type Culture Collection (CCTCC) with the registration number M 2022621.

2.4 Fermentation characteristics test

The cryopreserved strain was inoculated into MRS broth medium for activation twice at 37 °C. The bacterial suspension was adjusted to OD_{600} value equal to 1.0 and then inoculated into MRS broth medium at a volume fraction of 1%. Meanwhile, the liquid medium without inoculation was set as blank control. Within 24 hours, samples were taken every three hours to measure OD_{600} value and pH value (Goswami et al., 2017). The resulting data was plotted as a graph.

Temperature resistance experiment was carried out at 12 °C, while the medium without the inoculated strain was set as blank control. Salt tolerance experiments were carried out in MRS broth medium containing 3% NaCl, with sterile salt-added medium as blank control. Other experimental steps were the same as above.

2.5 Protease properties of CMC 1-8

Preparation of protease solution

CMC 1-8 strain was inoculated into MRS broth medium and fermented at 12 °C for 2 d. Supernatant obtained by centrifugation (10 °C, 8000 r/min, 5 min) of the fermentation broth was slowly added with $(NH_4)_2SO_4$ with a final saturation of 60% to 80% at 4 °C. The sample was salted out at 4 °C for 12 h and centrifuged (4 °C, 8000 r/min, 7 min) to collect precipitate. The precipitate was dissolved with phosphate buffer (10 mmol/L, pH 7.2) to obtain the protease solution.

Protease activity assay

The protease activity was determined according to method (Chimbekujwo et al., 2020) with minor modification. 0.1 mL of diluted protease solution was mixed with 0.1 mL of a 1% casein solution diluted with phosphate buffer (10 mmol/L, pH 7.2), and reacted at 40 °C for 10 min after both were preheated at 40 °C for 2 min. 0.2 mL of 10% trichloroacetic acid was used to stop the reaction. The reaction solution was centrifuged at 12000 r/min for 2 min to obtain supernatant. Then 1 mL of 0.4 mol/L Na_2CO_3 and 0.2 mL of Folin reagent were added into the 0.2 mL supernatant, and the reaction was continued at 40 °C for 20 min. OD value at 680 nm was measured by spectrophotometer. Phosphate buffer (pH 7.2) and substrate reaction was used as the blank control. The protease activity was represented by X and calculated as the follows Formula 1:

$$X = \frac{C \times V \times N}{V_0 \times 10} \quad (1)$$

In the Formula 1: C is the tyrosine concentration of the sample tube, g/mL; V is the total volume of the enzyme reaction, mL; N is the sample dilution ratio; V_0 is the amount of the enzyme involved in the reaction, mL; 10 is the reaction time, min.

The protease activity was defined as: the amount of enzyme that hydrolyzed casein to produce 1 μmol of tyrosine per milliliter of enzyme solution under certain temperature and pH conditions is one activity unit (U).

Preparation of tyrosine standard curve

To prepare 100 μg/mL L-tyrosine standard stock solution, 0.100 g of L-tyrosine standard material pre-dried at 105 °C was accurately weighed, then completely dissolved in 20 mL of 1 mol/L hydrochloric acid, and finally made up to 100 mL with distilled water. L-tyrosine standard stock solutions were diluted with distilled water to 0.0, 10.0, 20.0, 30.0, 40.0, 50.0, 60.0 μg/mL, respectively. 1 mL of 0.4 mol/L Na_2CO_3 and 0.2 mL of Folin reagent were added into the 0.2 mL diluent, and the reaction was continued at 40 °C for 20 min. The L-tyrosine concentrations were taken as the abscissa, the OD values at 680nm wavelength were used as the ordinate, and a standard curve was drawn to obtain a Y=0.037 X+0.0365 (R^2=0.9977) linear regression equation.

Influence of different factors on protease activity

Effects of enzymatic reaction temperature (10 °C, 20 °C, 30 °C, 40 °C, 50 °C, 60 °C, 70 °C, 80 °C), pH value (3.0, 5.0, 7.0, 9.0, 11.0) of phosphate buffer and NaCl concentration (3%, 5%, 7%, 9%, 11%) in phosphate buffer on protease activity were investigated.

2.6 Preparation of CMC 1-8 metabolite

The bacterial suspension after secondary activation was centrifuged. After the supernatant was filtered with a 0.22 μm membrane, the filtrate was extracted with ethyl acetate at a ratio of 1:1.5 (Shan et al., 2017). The organic layer was evaporated and concentrated, then the sample was diluted to different concentrations with dimethyl sulfoxide (DMSO) as the solvent.

2.7 Analysis of the active substances and biological activities of CMC 1-8 metabolite

Total phenol determination

Total phenolic content was determined by the Folin-Ciocalteu colorimetric method (Seon et al., 2021). After 50 μL of metabolites and 50 μL of Folin reagent (0.25 mol/L) were shaken for 3 min, 1 mL of 0.7 mol/L Na_2CO_3 was added to react for 1 h. 200 μL of the reaction solution was taken out and the absorbance was measured at 750 nm. For the blank group, the same amount of deionized water was used to replace the Folin reagent, and the same amount of tannic acid was used to replace the metabolites for the preparation of the standard curve. The standard tannin acid concentration X (50-250 μg/mL) was the abscissa, and the absorbance value Y was the ordinate to prepare the standard curve (Y=0.0019X+0.0164, R^2= 0.9975).

Flavonoid determination

The content of flavonoids was determined by the aluminum salt colorimetric method (Iwansyah et al., 2021). 300 μL of metabolites were mixed with 300 μL of 2% $AlCl_3$ for 1 h. 200 μL of reaction solution was used to detect absorbance at 420 nm wavelength. For the blank group, the same amount of absolute ethanol was used to replace 2% $AlCl_3$, and the same amount of rutin was used to replace the metabolites for the preparation of the standard curve. The standard rutin concentration X (20-100 μg/mL) was the abscissa, and the absorbance value Y was the ordinate to prepare the standard curve (Y=0.0079X+0.0505, R^2= 0.9929).

Soluble peptide determination

The content of soluble peptides was determined by the biuret method (Wu et al., 2021). 100 μL of metabolites, 100 μL of biuret A and 30 μL of biuret B reagent, mixed well, left at room temperature for 0.5 h for color development, and the absorbance was measured at a wavelength of 540 nm. For the blank group, the same amount of deionized water was used to replace biuret reagent, and the same amount of bovine serum albumin (BSA) was used to replace the metabolites for the preparation of the standard curve. The standard curve equation (Y=0.08117X+0.12129, R^2= 0.9919) was drawn with the standard BSA concentration X (0.5-3 mg/mL) as the abscissa and the absorbance value Y as the ordinate.

Antioxidant test

Antioxidant activity was investigated by three indicators: the scavenging ability of ABTS and DPPH free radicals and reducing ability. Three experiments referred to the methods of Kwon et al. (2013) and Wu et al. (2020) with slight modifications. Each experiment was repeated three times and the datum were averaged.

The above sample solutions were diluted to 1, 2, 4, 6, 8 and 10 mg/mL. 50 μL of each diluted sample solution and 100 μL of ABTS solution were mixed and reacted for 5 min in the dark. Their absorbance at 734 nm were determined as A_0. A_1 was the absorbance value measured with an equal volume of distilled

water as the blank group. An equal volume of DMSO was used to replace the sample solution as the control group, and its absorbance value was A_2. Quercetin was used as the positive control. The free radical scavenging rate was calculated as the follows Formula 2:

$$\text{ABTS radical scavenging \%} = \left(1 - \frac{A_0 - A_1}{A_2}\right) \times 100 \quad (2)$$

The above sample solutions were diluted to 1, 2, 4, 6, 8 and 10 mg/mL. 100 μL of each diluted sample solution and 50 μL of DPPH solution were mixed and reacted for 10 min in the dark. Their absorbance at 517 nm were determined as A_0. A_1 was the absorbance value measured with an equal volume of ethanol absolute as the blank group. An equal volume of DMSO was used to replace the sample solution as the control group, and its absorbance value was A_2. Quercetin was used as the positive control. The calculation of DPPH free radical scavenging rate referred to the follows Formula 3:

$$\text{DPPH radical scavenging \%} = \left(1 - \frac{A_0 - A_1}{A_2}\right) \times 100 \quad (3)$$

The reducing ability of metabolite was determined by the potassium ferricyanide method. Sample solutions were diluted to 1, 2, 4, 6, 8 and 10 mg/mL. 100 μL of the diluent was mixed with 100 μL of phosphate buffer solution (pH 6.6) and 100 μL of 1% potassium ferricyanide, respectively, and water bathed at 85 °C for 20 min. After cooling, 100 μL of 10% trichloroacetic acid was added. The solution was centrifuged at 3000 r/min for 5 min. After mixing 100 μL of the supernatant with 100 μL of distilled water and 10 μL of 0.1% $FeCl_3$, let it stand for 10 min, and measure the absorbance at 700 nm. Quercetin was used as the positive control. The sample concentration providing 0.5 of absorbance (IC_{50}) was calculated from the graph of absorbance at 700 nm against sample concentration.

Antimicrobial test

Using filter paper diffusion method (Wu et al., 2020), *E. coli*, *S. aureus*, *B. subtilis*, and *P. aeruginosa* were used as indicator bacteria. The above bacteria were activated and then made into the bacterial suspension (OD_{600}=0.1) with physiological saline. 100 μL of bacterial suspension was spread on luria-bertani (LB) medium. The sample solution was diluted to 2, 5, 10, 15, and 20 mg/mL. 10 μL of them was added to the center of the sterile filter paper on the medium, respectively. After 12 hours of inverted culture at 37 °C, the diameter of the inhibition zone was measured. DMSO and propylparaben (PP) were used as blank control (BK) and positive control, respectively.

2.8 Statistical analysis

The experimental results were expressed as mean ± standard deviation. Origin 8.5 software was used for drawing. Analysis of variance was performed using SPSS 18.0 software, and $P<0.05$ indicated a significant difference.

3 Results and discussion

3.1 Morphological observation, Gram staining and scanning electron microscope observation

Morphological observation of single colony isolated and purified from stinky mandarin fish was shown in Figure 1a. Once ending culture process, it was showed that the colony was round with neat edges, milky white or milky yellow, raised surface, moist and smooth, diameter of 1.8-2.0 mm, accompanied by a sour taste. The strain was named CMC 1-8. The Gram staining and scanning electron microscope observations of the strain were shown in Figure 1b and Figure 1c, respectively. Gram staining was positive, and the cell morphology was short rod-shaped, paired or short-chain without spores.

3.2 Bacterial 16S rRNA gene sequencing and construction of phylogenetic tree

We downloaded the gene sequences of the eight strains and the sequencing results were indexed to the GeneBank database. According to the phylogenetic tree (Figure 2), CMC 1-8 was in the same evolutionary branch as *L. sakei* subsp. *sakei* DSM 20017 (NR 042443.1). It was indicated that the two were most closely related. For further verification, the sequences of the two were aligned with BLAST on the NCBI website. The result

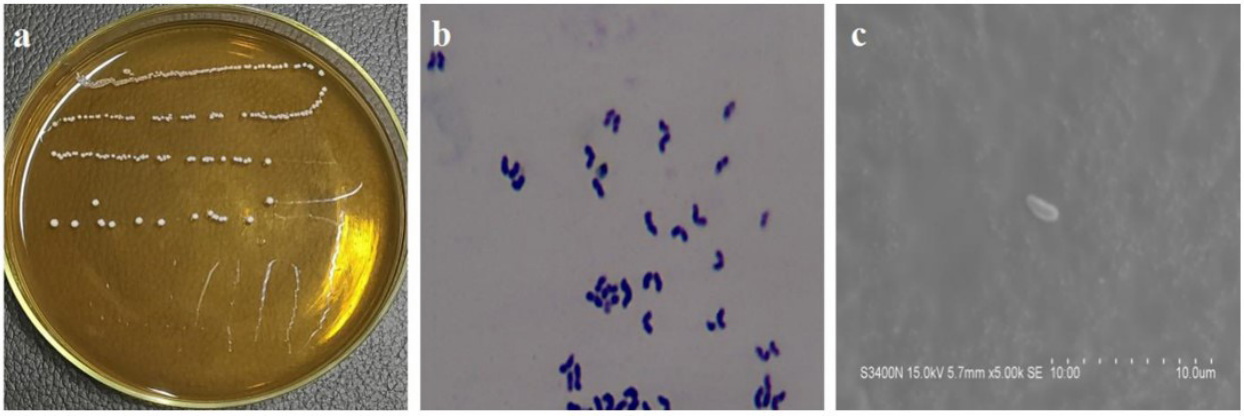

Figure 1. Colony morphology (a), Gram staining cell morphology (b) and scanning electron micrograph (c) of strain CMC 1-8.

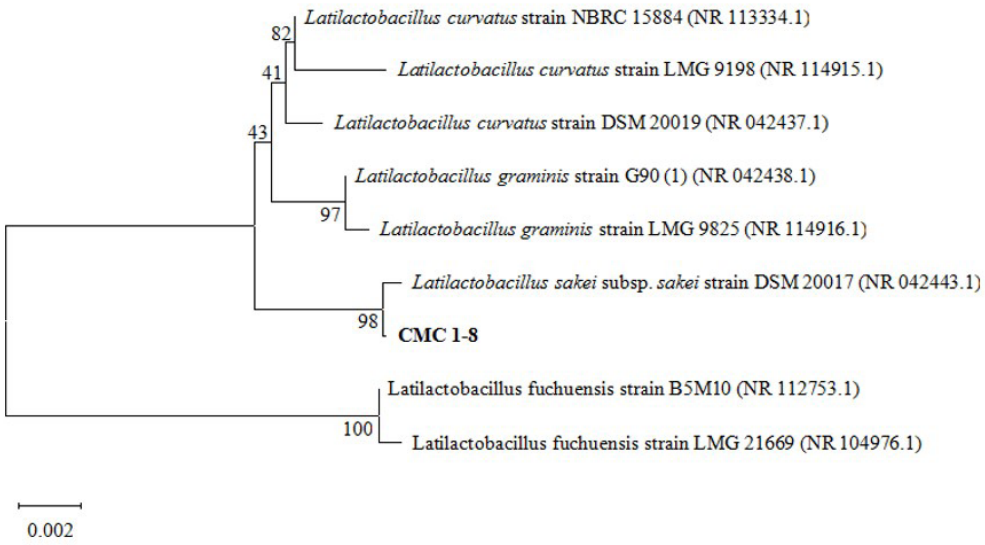

Figure 2. Phylogenetic tree of CMC 1-8 based on 16S rRNA gene sequence.

showed that the homology was up to 98%. According to the conformation mentioned above, it was proved that CMC 1-8 was *L. sakei* subsp. *sakei*. Dai et al. (2013) isolated 39 strains of *L. sakei* from stinky mandarin fish, which were the dominant species in fermentation process.

3.3 Growth curve and acid production capacity

To investigate the changes of bacterial density and acid-produced capacity of bacteria during the cultivated procedure, the OD_{600}-related growth curve and pH curve of CMC 1-8 had been exhibited in Figure 3. During the logarithmic growth period from 3 to 9 h in Figure 3a, the cell density ascended dramatically. At the same time, the acid production efficiency approached the highest counts. After 9 h, the growth of the bacteria gradually came into a stable phase, while the rate of acid production decreased weakly. As was displayed in Figure 3b, the ambient pH dropped to 5.1 at 24 h from 6.5 at starting moment, which foresaw the increase of environmental acidity and fundamentally stemmed from lactic acid metabolism of *L. sakei* and as-producted short-chain acids such as acetic acid, butyric acid, and propionic acid (Li et al., 2022). To sum up, CMC 1-8 could acidize fermentation environment, which was beneficial to fermenting mandarin fish.

3.4 Temperature tolerance and salt tolerance

Stinky mandarin fish is one of the low-salt (salt concentration <5%) and low-temperature (10-15 °C) fermented fish products (Li et al., 2013). Therefore, the salt and temperature tolerance had been explored during the process of growing CMC 1-8 under the condition at 12 °C and 3% NaCl, respectively. It was observed that CMC 1-8 performed a better growth viability at 12 °C (OD_{600} at 24 h was 1.5) than at 37 °C (OD_{600} at 24 h was around 1.4). Despite in 3% salt concentration, the logarithmic growth period of CMC 1-8 could end even early at 6 h, and its stable growth retained within 6-24 h. In this circumstance, the highest OD_{600} value still

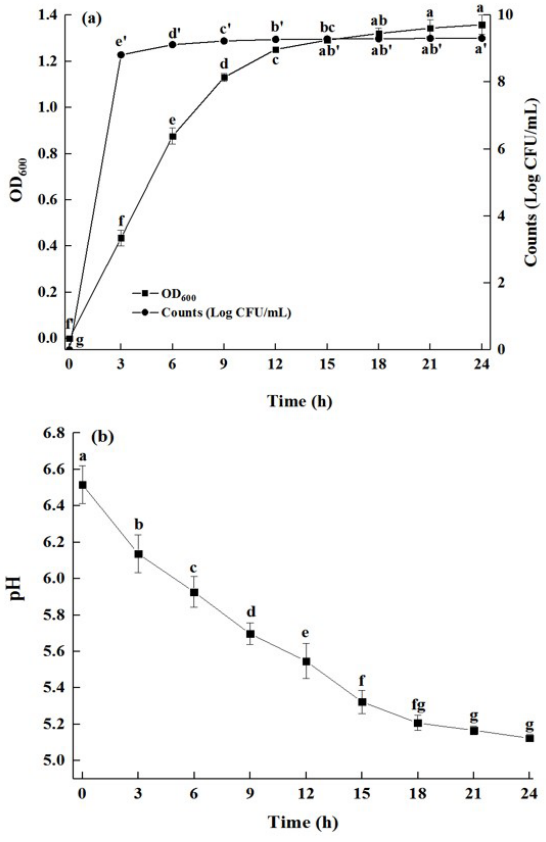

Figure 3. Growth curve and pH curve of CMC 1-8.

could reach 0.9-1, which demonstrated that the cell concurrently absented obvious attenuation phenomenon. The above results issued that CMC 1-8 could adapt to the fermentation environment of mandarin fish and locate the potential position to be focusedly developed as the starter culture (Figure 4).

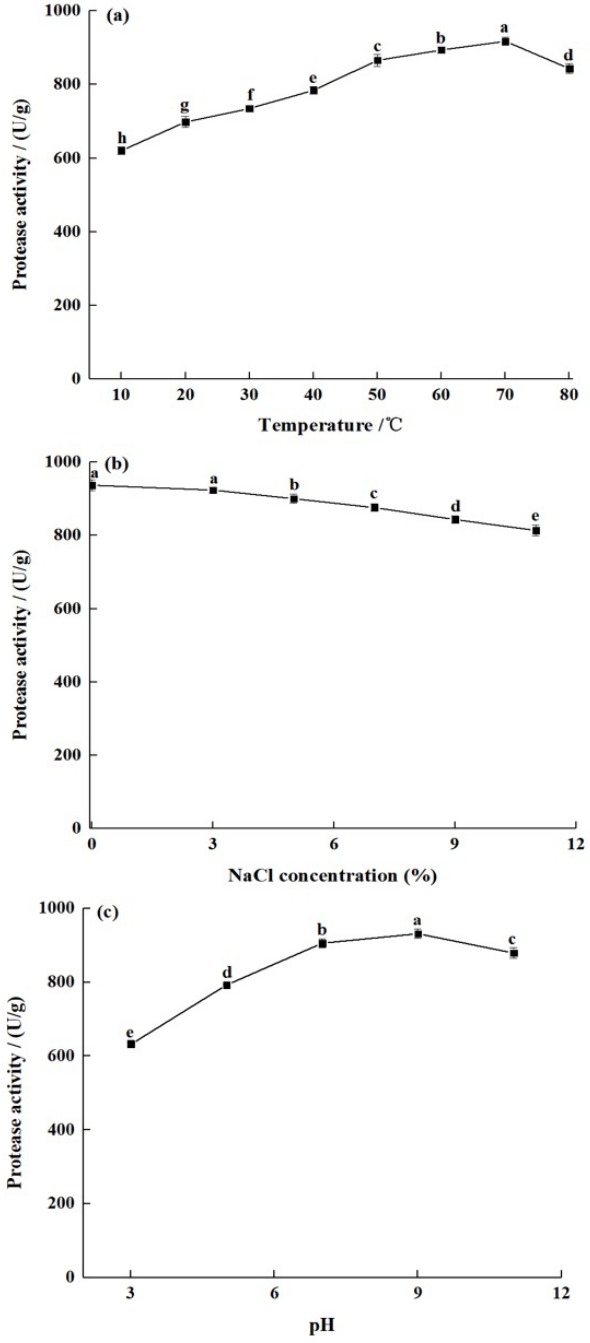

Figure 5. Effect of temperature (a), NaCl concentration (b), pH (c) on protease activity.

3.5 Protease properties of CMC 1-8

Temperature, NaCl concentration in phosphate buffer, and pH of phosphate buffer were pivotally with regard to protease activity (Figure 5). In the range of 10-80 °C in Figure 5a, 70 °C was the optimum temperature because protease activity peaked to 917.37 U/g. On relationship of NaCl concentration with protease activity, as NaCl concentration rose from 0% to 11% in Figure 5b, the protease activity decreased by 13.20%, interpreting a positive inhibitory tendency. Hence, the low-salt fermentation of mandarin fish favored maintenance of CMC 1-8 protease activity. As shown in Figure 5c, the pH value performed the greatest effect on the protease activity. When the pH value was 3, protease activity was lowest. It was noticeable that protease activity was the highest value as the pH value was 9. The protease activities differed by 299.36 U/g from each other. Accordingly, the protease of CMC 1-8 tended to exert its effect in a weak alkaline environment. In recent years, LAB have been widely employed as probiotics in functional fermented foods and beverages. LAB-improved protease production is devoted to formation of nutrition and flavor of fermented product (Ashaolu, 2020). *L. sakei* had the function of hydrolyzing protein and producing flavor amino acids during fermentation of mandarin fish (Zhao et al., 2020). We confirmed that *L. sakei* subsp. *sakei* CMC 1-8 could indeed drain highly active protease. Although the fermentation conditions of stinky mandarin fish were not the optimal conditions for protease formation, it could still ensure that its activity was beyond 600 U/g.

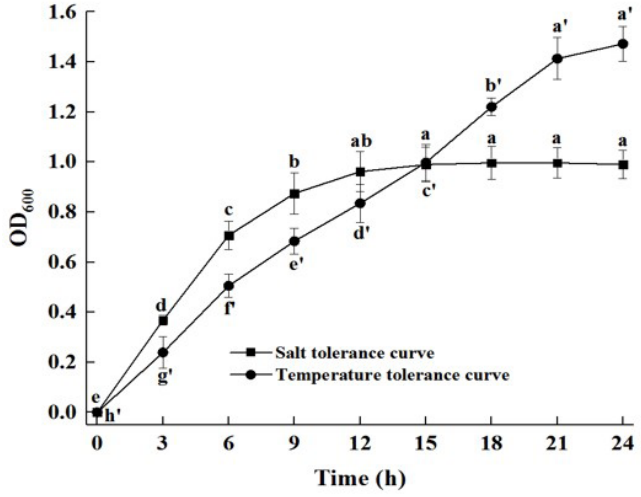

Figure 4. The salt tolerance curve and temperature tolerance curve of CMC 1-8.

3.6 Active substances and antioxidant activities of metabolites

Polyphenols and flavonoids are important biologically active substances, which have been widely applied in the fields of food, pharmacy, medical treatment, and cosmetics (Tresserra-Rimbau et al., 2018). However, how to collect these natural products must confront the limitations in complicated processes and low yields. The construction of microbial cell factories and utilization of synthetic biology techniques to achieve mass production of secondary metabolites such as phenols and flavonoids were deemed to be green and efficient biological methods (Liu et al., 2017). CMC 1-8 was a potential chassis strain, which fulfilled the ability of secondary metabolism to produce phenols and flavonoids (Table 1). The secondary metabolites of CMC 1-8 were rich in soluble polypeptides, which again supported the strain to produce high active protease. As a culture starter, CMC 1-8 was expected to improve the decomposition of crude protein in mandarin fish to gain peptides and amino acids that were more beneficial to digestion, nutrition and flavor.

Table 1. Active substances and antioxidant activities of CMC 1-8 metabolite.

Objects	Total phenolics (mg/g)	Total flavonoids (mg/g)	Soluble polypeptide (mg/g)	IC_{50} (mg/mL)		
				ABTS	DPPH	Reducing power
CMC 1-8	18.27 ± 1.03	4.21 ± 0.93	201.51 ± 11.71	1.05 ± 0.02	6.87 ± 0.25	4.62 ± 0.06
Quercetin	-	-	-	0.017 ± 0.002	0.013 ± 0.001	0.043 ± 0.007

Table 2. Diameter of inhibitory zone of CMC 1-8 against four common bacterial strains.

Objects	Concentration (mg/mL)	Inhibition zone diameters (mm)			
		E. coli	*S. aureus*	*B. subtilis*	*P. aeruginosa*
BK		-	-	-	-
CMC 1-8	2	8.13 ± 0.50^{e}	8.27 ± 0.50^{d}	8.17 ± 0.59^{d}	8.37 ± 0.25^{d}
	5	9.10 ± 0.56^{d}	8.93 ± 0.71^{d}	8.80 ± 0.40^{d}	8.70 ± 0.40^{d}
	10	10.37 ± 0.38^{c}	10.13 ± 0.42^{c}	9.97 ± 0.51^{c}	9.83 ± 0.42^{c}
	15	10.80 ± 0.35^{bc}	10.77 ± 0.50^{bc}	10.77 ± 0.76^{c}	10.37 ± 0.60^{bc}
	20	11.27 ± 0.21^{b}	11.70 ± 0.44^{b}	12.03 ± 0.35^{b}	10.63 ± 0.12^{b}
PP	2.0	14.60 ± 0.66^{a}	14.40 ± 0.56^{a}	14.43 ± 0.96^{a}	14.37 ± 0.31^{a}

Different letters in the same row indicated that the data were significantly different ($P<0.05$).

In Table 1, it was of worthiness to conceive that the free radical scavenging ability toward ABTS was positively correlated with the concentration of metabolites. When the metabolite concentration was 1.05 mg/mL, the ABTS free radical could reach 50% scavenging rate. Very strikingly, as the concentration was 4 mg/mL, the scavenging rate is as high as 98.55 ± 0.68%, which was consistent with the result of former report of Han et al. (2017).

The ability to scavenge DPPH free radical is one of the most common indicators in the detection of antioxidant activity of lactic acid bacteria (Kang et al., 2019; Li et al., 2012). Compared with the free radical scavenging ability of ABTS, the scavenging ability of CMC 1-8 metabolites to DPPH free radical was slightly weaker (Table 1). When the concentration of metabolites was arranged in 1-10 mg/mL, the scavenging rate was among 12.57-63.54%. Kamiloğlu (2022) found that supernatant free cells of two strains of *L. sakei* derived from Turkish dry-fermented sausage had a DPPH free radical scavenging rate of 20-25%. Oh & Jung (2015) articulated that a strain of *L. sakei* isolated from the Korean fermented beverage *Omegisool* owned the scavenging rate between 30-45% in the culture supernatant. Herein, our resulted data of scavenging rate supported that antioxidant activity of as-isolated lactic acid bacteria was similar with the reported ones. Compared with the capacity of scavenging ABTS free radical, CMC 1-8 metabolites possessed an only a bit inferior activity to scavenge DPPH free radical.

Antioxidants can reduce Fe^{3+} in potassium ferricyanide to Fe^{2+}. Potassium ferrocyanide further coupled with $FeCl_3$ to form Prussian blue ($Fe_4[Fe(CN)_6]_3$), of which the maximum absorption wavelength centered at 700 nm. The intensity of absorbance is usually analyzed to reflect reducing power. Different concentrations of metabolites took a visible effects on reducing ability ($P<0.05$) (Table 1). Referred to prevenient article (Bajpai et al., 2016), exo-polysaccharides as one of metabolites secreted by *L. sakei* may be devoted to enhancement of reducing ability.

3.7 Antimicrobial activity of metabolites

The strong bacteriostatic action is the synergistic effect of bacteriocin, organic acids (mainly lactic acid), H_2O_2 and other antibacterial substances produced by lactic acid bacteria (Budde et al., 2003; Gao et al., 2015). The metabolites of CMC 1-8 were made up by a broad-spectrum group of antimicrobial substances, which implemented inhibitory effects on both Gram-negative and Gram-positive bacteria (Table 2). The bacteriocin sakacin C2, which inhibited Gram-positive and Gram-negative bacteria, was purified from the metabolites of *L. sakei* (Gao et al., 2010). It was speculated that CMC 1-8 may also contain such bacteriocin or other bacteriocins.

4 Conclusions

In this study, a strain of *L. sakei* subsp. *sakei* was isolated and purified from stinky mandarin fish using traditional microbial culture methods and 16S rRNA sequencing. Then, the regulations of its growth and acid production were explored. Temperature and salt tolerance test showed that this strain was suitable for the fermentation technology of mandarin fish. The protease activity of CMC 1-8 was above 600 U/g. The best temperature of the protease was 70 °C and the optimum pH was 9.0, and the enzyme activity decreased with the increase of NaCl concentration. The fermentation metabolites, which included polyphenols, flavonoids and soluble polypeptides, can be corroborated to unfold excellent abilities to scavenge ABTS and DPPH free radicals, and reducing power. Also, these metabolites can distinctly inhibit the activity of *E. coli*, *S. aureus*, *B. subtilis* and *P. aeruginosa*. The as-exploring results indicated that *L. sakei* subsp. *sakei* had taken over great potential as the starter culture for directional inoculation of stinky mandarin fish. The further work is to investigate the effect of inoculation with this strain on the fermented flavor, quality and nutrition of stinky mandarin fish as well as the identification and purification of active substances in metabolites.

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