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RESEARCH ARTICLE

Isolation and identification of *Shenghua* microorganisms in fermented chili pepper from Southwest China

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Abstract: Microorganisms causing the phenomenon of *Shenghua* were isolated, purified and identified from fermented chili pepper by potato glucose media (PDA) and nutrient agar media (NA) via streak plate method. The purified strains were validated by tie-back test and identified by phenotype and genotype methods. Results showed that two strains were isolated from the fermented chili with *Shenghua*. It was identified as *Pichia kudriavzevii* that causing *Shenghua* by morphological and molecular biology from PDA media while the strain isolated from the NA media that could not cause *Shenghua* was identified as gram-positive bacterium by microscopic and colony morphology.

Keywords: fermented chilli pepper, *Shenghua*, *Pichia kudriavzevii*, Guizhou

1 Introduction

Chili pepper, (also known as *Capsicum annuum* L., pepper, chili) belongs to genus *Capsicum*, Solanaceae which is a one of most commonly consumed and economically important vegetable crop[1]. The output of pepper in Chinese is up to 40 million tons, which is the largest production and consumption country in the world in 2018. Chili pepper is the most popular and emblematic Chinese condiment especially in Southwest in China and represents an alternative source of vitamins and nutraceuticals such as ascorbate, carotenoids, capsaicin,tocopherols, flavonoids, and capsaicinoids[2–4]. The content of vitamin C is considered much higher than potatoes and tomato vegetables[5]. There are several studies on the antioxidant activity of pepper or Capsaicin based on DPPH, -NO and ·OH[6,7]. Moreover, Capsaicin a kind of carotenoid in pepper was regarded as an ideal food colorant which was an essential spicy food in many areas of China, especially in Sichuan, Guizhou, Yunnan and other Southwest China[8].

Benefit from unique climate and soil of Guizhou, pepper has been developed prominently in Guizhou, especially in Zunyi. Pepper has become an important characteristic cash crop in Zunyi, Southwest China and developed into one of the largest pepper trading market in China, which was known as Chinese Pepper City[9]. There are many kinds of processed pepper products, such as paste pepper powder, flavor oil pepper, pickled pepper and fermented chili pepper. Fermented chili pepper becaused of bright color, unique flavor and sour is favored by people and showed broad market prospect.

Fermented chili pepper exhibited effect of sweating, dehumidifying and appetizing. The *Lactobacillus* in fermented food could also improve the intestinalflora of human[10]. However, in the later stage of fermentation of chili pepper, white film occured that commonly known as *Shenghua*. *Shenghua* is the formation of a layer of white film on the surface of fermented chili pepper. Once the *Shenghua* appeared on the surface, the white film spreaded quickly into patches or fragmen[11], which seriously affects the sensory quality. The surface of fermented chili pepper will form a white patchy or fragmented flower-like film. The fermented chili pepper may begin to rot, the broth may become turbid and then the taste and quality will be affected. Moreover, the phenomenon of *Shenghua* due to potential hidden danger limited the production and preservation of fermented chili pepper. At present, there is less report on the cause of *Shenghua* of fermented chili pepper.

Thus, this paper is to investigate the cause of *Shenghua* which will provide theoretical basis for prevention, production and solving the problem.

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2 Materials and methods

2.1 Fermented chili pepper

The fermented chili pepper sample was home-made, whose surface was completely covered with white film.

2.2 Isolation and Purification of Microorganisms

White film on the surface of *Shenghua* chilli pepper were selected and diluted 10 times to 10^{-3} in 9 mL aseptic water after shaking. 1 ml gradient suspensions of each concentration were coated on PDA and NA plate. PDA medium was cultured in aerobic medium at 28°C for 24-36 h and NA medium was at 36°C for 36-48 hours. Single colonies with different morphological characteristics were selected from plate and purified several times. Then the purified strain was applied to further study and then stored at 4°C[12].

2.3 Tie-back test

The purified strains were cultured in suspension with 0 mL, 1 mL, 2 mL and 4 mL bacterial suspension in sterilized chili pepper without any food preservatives at 26°C. The phenomenon of *Shenghua* was observed every day.

2.4 Identification of microorganisms

The isolated strains were identified by Gram staining and DNA sequences. Genomic DNA was extracted by Genomic DNA Extraction Kit (Solarbio-D2300-100T) and amplified using universal primers (ITS1:5'-TCCGTAGGTGAACCTGCGG-3'; ITS4:5'-TCCTCCGCTTATT-GATATGC-3'). The PCR (20 μL) procedure was followed by: initial pre-denaturing at 94°C for 3 min, which preceded 30 cycles of denaturing at 94°C for 30 s, annealing at 55°C for 30 s, and extension at 72°C for 60 s, with a final extension at 72°C for 10 min. The PCR products were sent to Beijing Boyoushn Beijing Bioulab Technology Co., Ltd. Blast software in GenBank (https://blast.ncbi.nlm.nih.gov/Blast.cgi) was applied for homologous analysis. Phylogenetic tree by the neighbor-joining method was constructed by Mega 5.1.

3 Results and discussion

3.1 Colony Characteristics

Two strains (W, N) were isolated from *Shenghua* chilli pepper. The colony on PDA medium was large and thick, wet, smooth, opaque and viscous. The colony color was milky white, easy to pick out, the colony bulged and had round edge, strong wine flavor, and could grow under aerobic and anaerobic conditions (Figure 1a). While, the colony on NA medium was small, wet, smooth, opaque, light yellow colour, easy to pick, slightly raised, glossy, spherical, growing only under aerobic conditions (Figure 1b).

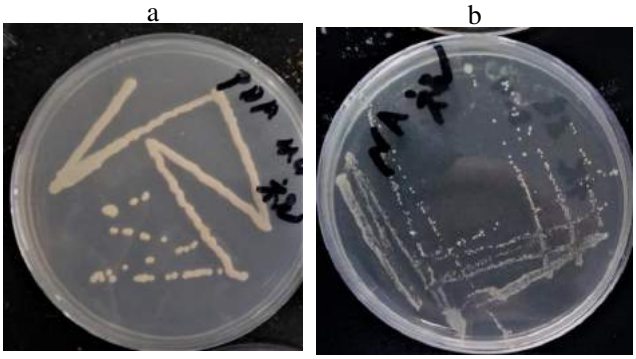

Figure 1. The colony on PDA medium and NA medium. **a**: strain W; **b**: strain N

3.2 Gram staining of microscopic examination

W isolated from PDA medium were observed under 40×microscope (Figure 2a), they were ellipse. N were observed under 100× oil microscopy (Figure 2b) after Gram staining. They were Gram-positive and globular.

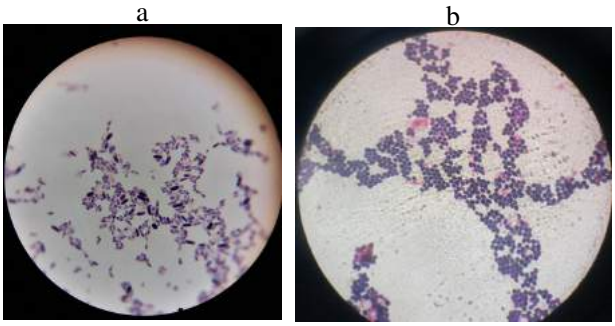

Figure 2. Gram staining of W and N. **a**: strain W; **b**: strain N

3.3 Tie-back test

Shenghua phenomenon occured to some extent on the 10th day of incubation after adding suspension of W. Besides, The more serious the phenomenon was, the higher the concentrations of bacterial suspension were (Figure 3). In Figure 3, a is the blank control, b is the result of 1 mL inoculated suspension, c is the result of 2 mL inoculated suspension, and d is the result of 4 mL inoculated suspension. Nevertheless, the phenomenon of *Shenghua* did not appeared by adding the suspension of N (Figure 4). In Figure 4, a is the blank control, b is the result of 1

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mL inoculated suspension, c is the result of 2 mL inoculated suspension, and d is the result of 4 mL inoculated suspension.

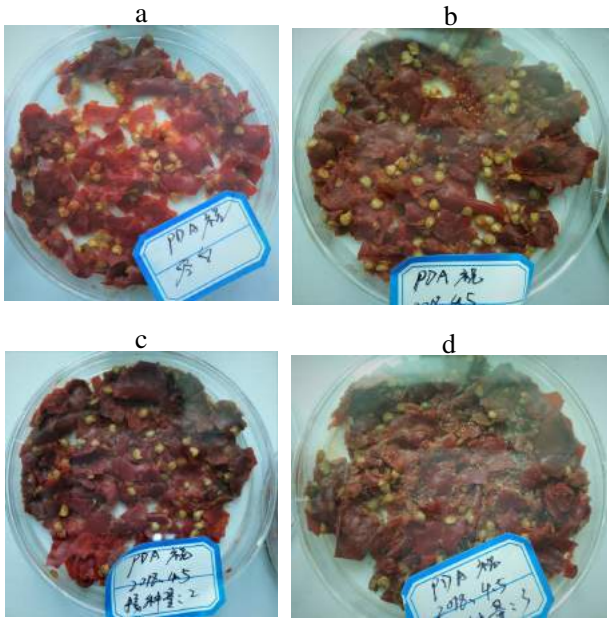

Figure 3. The results of the return experiment (inoculated by W)

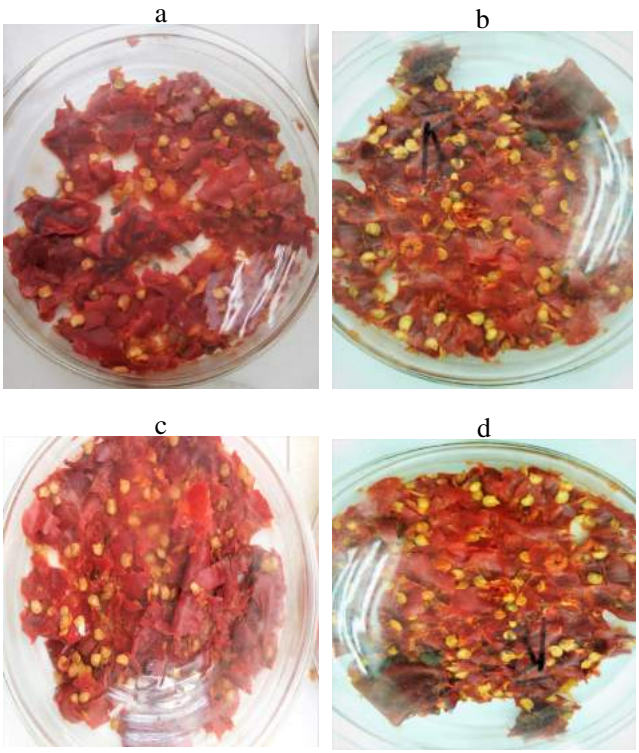

Figure 4. The results of the return experiment (inoculated by N)

3.4 Identification of *Shenghua* Strain

The total DNA of isotaled strains were extracted from strain W and then ITS sequence was amplified by PCR. Gel electrophoresis showed that there was a bright single band at 500-600 bp, which was consistent with the theoretical value (Figure 5). Strain W was identified as *Pichia kudriavzevii* with similarity 99% based on the sequences compared with those in NCBI database by BLAST online. The phylogenetic tree was constructed by Mega 5.1 software (Figure 6). According to the colony characteristics and microscopic structure, W was identified as *Pichia kurdiensis*.

4 Conclusions

Two suspected strains were isolated from *Shenghua* fermented chili pepper. Strain W isolated from PDA medium was proved to be the microorganism causing *Shenghua*. It was identified as *Pichia kudriavzevii* by phenotypic and genotypic characteristics. Another strain isolated from NA medium could not lead to *Shenghua* belongs to Gram-positive coccus Gram staining.

Conflicts of interest

The authors declare there was no conflict of interest.

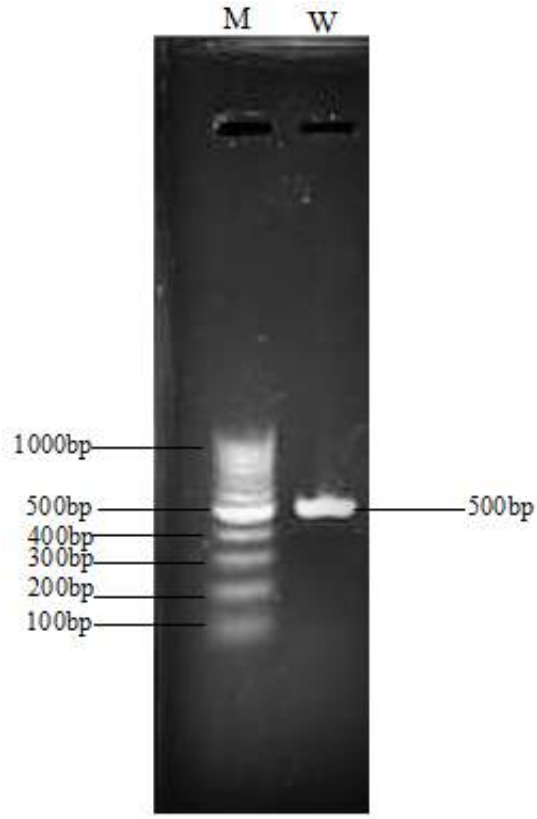

Figure 5. Gel electrophoresis of purified PCR products. M, 100 bp DNA Marker; W, *Shenghua* strain

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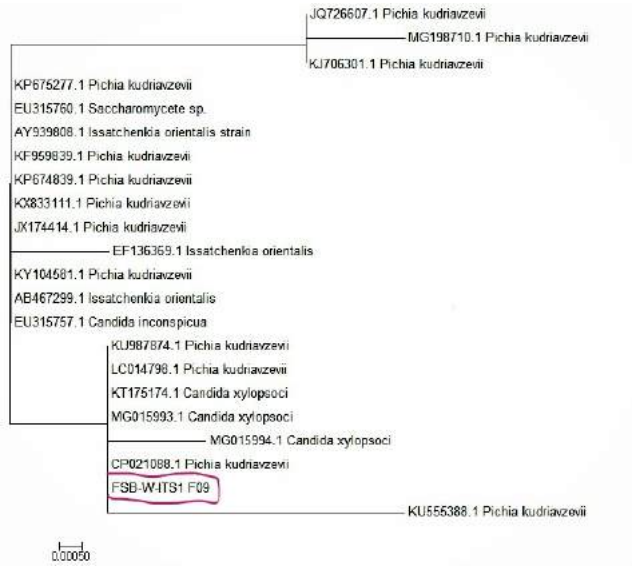

Figure 6. Phylogenetic tree of W based on ITS sequences

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