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Screening Identification and Characterization of a Strain of Candida Tropicalis

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Abstract: This study focused on the isolation and characterization of aromatic yeasts from Sauce-Flavor Daqu, a crucial fermentation starter for Chinese Baijiu production. Through systematic screening of 46 microbial isolates, we identified a strain with excellent aroma-producing capabilities. Initial liquid fermentation screening identified 12 strains with distinct aromatic profiles, with subsequent solid-state fermentation experiments confirming strain 12 as the most promising candidate, producing notable wine and floral-fruit aromas. Gas chromatography-mass spectrometry analysis revealed that the fermentation products from strain 12 contained predominantly alcohols (77.89 mg/kg) and esters (3.75 mg/kg), with phenylethanol (43.54 mg/kg) and isoamyl alcohol (29.64 mg/kg) as the major aromatic compounds. Morphological examination and 18S rDNA sequencing identified this strain as Candida tropicalis. Physiological characterization demonstrated that this yeast tolerated pH ranges from 4 to 9, sodium chloride concentrations up to 12%, and ethanol levels up to 7%. The growth curve analysis revealed that the strain entered exponential phase after 4 hours, reached stationary phase at 14-18 hours, and began declining after 18 hours. This study provides valuable insights into aroma-producing yeasts in traditional Chinese baijiu fermentation and identifies a potential candidate for flavor enhancement in fermented foods and beverages.

Keywords: Candida Tropicalis; Aroma Production; Volatile Compounds; Sauce-Flavor Daqu.

1. Introduction

Chinese Baijiu, one of the world's oldest distilled spirits, exhibits diverse flavor profiles classified into several aroma types, among which Jiangxiang (sauce-aroma) type is renowned for its complex bouquet, mellow taste, and long-lasting finish[1]. The distinctive characteristics of Sauce-Flavor Baijiu originate from its unique production process, particularly the microbial communities present in Daqu—the traditional fermentation starter that serves as a crucial saccharifying and fermenting agent[2, 3].

Sauce-Flavor Daqu harbors diverse microbial communities comprising bacteria, yeasts, and molds that collectively contribute to the spirit's characteristic flavor profile[4]. Previous investigations have revealed that bacteria in Sauce-Flavor Daqu primarily include lactic acid bacteria, Bacillus spp., and acetic acid bacteria, which produce key flavor compounds such as ethyl lactate and ethyl acetate[5, 6]. Among fungal communities, various molds including Rhizopus, Aspergillus, and Mucor species facilitate the liquefaction and saccharification of starch[7, 8].

While considerable research has focused on bacterial and mold contributions to Baijiu production, yeasts—particularly non-Saccharomyces species—remain comparatively understudied despite their significant role in alcohol production and ester formation[9-11]. Candida tropicalis, a non-Saccharomyces yeast frequently found in fermentation systems, has demonstrated potential for producing various flavor compounds[12, 13]. Microbial fermentation represents an environmentally friendly approach to produce flavor compounds compared to chemical synthesis or direct extraction from plant materials, offering advantages in sustainability and cost-effectiveness[14, 15]. Therefore, identifying and characterizing aroma-producing microorganisms from Sauce-Flavor Daqu could not only enhance our understanding of Baijiu fermentation mechanisms but also provide potential biotechnological applications in flavor production.

In this study, we isolated and screened microorganisms from Sauce-Flavor Daqu, focusing on identifying strains with superior aroma-producing capabilities. Through systematic evaluation using both liquid and solid-state fermentation approaches, coupled with gas chromatography-mass spectrometry analysis, we identified a promising Candida tropicalis strain and characterized its physiological properties. This research contributes to a deeper understanding of the microbial ecology of Sauce-Flavor Daqu and identifies potential applications for enhancing flavor profiles in fermented products.

2. Materials and Methods

2.1. Sample Collection.

Sauce-Flavor Daqu samples were obtained from a commercial distillery and stored under laboratory conditions until analysis.

2.2. Culture Media

LB: 10.0 g/L NaCl, 10.0 g/L tryptone, 5.0 g/L yeast extract; for solid medium, 20.0 g/L agar was added.

MRS: 20.0 g/L D-glucose, 10.0 g/L tryptone, 10.0 g/L beef extract, 5.0 g/L yeast extract, 1.0 mL/L Tween-80, 2.0 g/L K_2HPO_4, 2.0 g/L diammonium citrate, 0.25 g/L $MnSO_4 \cdot H_2O$, 0.2 g/L $MgSO_4$.

Liquid fermentation medium: LB medium supplemented with 0.5 g/L K_2HPO_4, 3.0 g/L $(NH_4)_2SO_4$, 60.0 g/L glucose, 0.3 g/L $CaCl_2$, 0.25 g/L $MnSO_4$, and 0.6 g/L $MgSO_4$.

Solid-state fermentation medium: Prepared by soaking sorghum, steaming until partially gelatinized, cooling, and hydrolyzing with amylase.

2.3. Isolation and Screening of Microorganisms.

Daqu samples (5 g) were homogenized in 100 mL sterile water and incubated at 37℃ with shaking (180 rpm) for 24 h. Serial dilutions were prepared and plated on LB agar. After incubation at 37℃ for 24 h, morphologically distinct colonies were purified by successive streak-plating, resulting in 46 isolates.

2.4. Fermentation

2.4.1. Liquid Fermentation

Each isolate was inoculated (4% v/v) into liquid fermentation medium and incubated at 37℃ with shaking (180 rpm) for 48 h. Fermentation broths were evaluated for aroma characteristics, and those exhibiting distinct aromas were selected for volatile compound analysis.

2.4.2. Solid-state Fermentation

Selected isolates were further evaluated using solid-state fermentation. The prepared substrate (70 g) was sterilized (121℃ 20 min) and inoculated with 5 mL of 12 h pre-cultivated culture. Fermentation was conducted at 37°C for 10 days in a constant temperature and humidity incubator. Fermentation products were evaluated for aroma characteristics and volatile compound composition.

2.5. Volatile Compound Analysis

Volatile compounds in liquid fermentation samples were analyzed using gas chromatography-mass spectrometry (GC-MS). Volatile compounds in solid-state fermentation samples were analyzed using headspace solid-phase microextraction (HS-SPME) coupled with GC-MS on an Agilent 7890A-5975B system.

2.6. Identification of Selected Strain

2.6.1. Morphological Observation

Colony morphology was observed after 24 h growth on LB agar at 37°C. Cell morphology was examined under oil immersion (1000×) using Lugol's iodine staining.

2.6.2. Molecular Identification

The 18S rDNA region was amplified using universal fungal primers ITS1 and ITS4. PCR was performed using Prime Star polymerase with the following conditions: initial denaturation at 98°C for 10 min; 35 cycles of 98°C for 10 s, 53°C for 15 s, and 72°C for 40 s; and final extension at 72°C for 5 min. PCR products were verified by agarose gel electrophoresis and sequenced. The obtained sequences were analyzed using BLAST search against the NCBI database, and a phylogenetic tree was constructed using MEGA7.0.26 software.

2.7. Physiological Characterization

2.7.1. Growth Curve

Growth curves were determined by measuring optical density at 600 nm (OD_{600}) every 2 h for 24 h. Pre-cultured cells were inoculated into fresh MRS broth and incubated at 37°C with shaking (180 rpm). Experiments were performed in triplicate.

2.7.2. pH Tolerance

Tolerance to different pH values was determined by inoculating pre-cultured cells (3% v/v) into MRS broth adjusted to pH 3-11 (at intervals of 1 pH unit). After incubation at 37°C with shaking (180 rpm) for 24 h, growth was assessed by measuring OD_{600}. Experiments were performed in triplicate.

2.7.3. Salt Tolerance

Salt tolerance was evaluated by inoculating pre-cultured cells (3% v/v) into MRS broth containing 1-16% (w/v) NaCl. After incubation at 37°C with shaking (180 rpm) for 24 h, growth was assessed by measuring OD_{600}. Experiments were performed in triplicate.

2.7.4. Ethanol Tolerance

Ethanol tolerance was determined by inoculating pre-cultured cells (3% v/v) into MRS broth containing 1-15% (v/v) ethanol. After incubation at 37°C with shaking (180 rpm) for 24 h, growth was assessed by measuring OD_{600}. Experiments were performed in triplicate.

3. Results and Discussion

3.1. Isolation and Screening of Flavor-Producing Strains

From the Sauce-Flavor Daqu sample, 46 microbial strains were isolated and purified. These isolates were subjected to liquid fermentation screening to evaluate their aroma-producing capabilities. Sensory evaluation of the fermentation broths revealed that 12 out of 46 strains (26.1%) produced distinctive aromas, while the remaining strains primarily produced undesirable sour, rancid, or earthy odors. The aroma-producing strains exhibited diverse fermentation broth colors and aroma profiles (Table 1). Three strains (A8, A12, and A2) produced milky-white fermentation broths, while the remaining nine strains (B1, B6, C1, C5, C7, 8, 12, 13, and 14) produced light yellow fermentation broths. Among these, strain A2 produced distinctive acidic and caramel notes, strains A8 and A12 exhibited acidic and fruity aromas, while strains B1, B6, C5, and C7 primarily produced acidic notes. Notably, strains C1, 8, 12, 13, and 14 exhibited wine-like aromas with varying intensities, accompanied by floral and fruity notes in strains 8, 12, 13, and 14. Strain 12 demonstrated the most pronounced wine aroma combined with floral-fruity notes, making it particularly promising for further investigation.

GC-MS analysis of the volatile compounds in the liquid fermentation broths confirmed the distinct aroma profiles observed in sensory evaluation (Fig. 1). The analysis revealed significant differences in the volatile compound composition among the strains. Strain A2 primarily produced phenolic and acidic compounds, corresponding to its caramel-like and acidic aroma profile. In contrast, strains 12 and 13 predominantly produced alcohols, esters, and minor amounts of acids, which contributed to their wine-like and fruity-floral aromas. The major volatile compounds detected in strain 12's fermentation broth included phenylethanol, isoamyl alcohol, ethyl acetate, and isoamyl acetate, which are known to contribute rose-like and fruity notes to fermented beverages.A comprehensive analysis of the volatile profiles revealed interesting patterns among the strains. Notably, strains 8 and 12 showed similar volatile compound compositions, suggesting they might be related species, while strains 13 and 14 also displayed similar profiles. Strains with acidic aromas (B1, B6, C5, and C7) contained lower concentrations of volatile compounds compared to the wine-aromatic strains, explaining their less complex aroma profiles. Based on the sensory evaluation and volatile compound analysis, strains A2, 12, and 13 were selected for further investigation using solid-state fermentation to evaluate their performance under conditions more closely resembling traditional Baijiu production.

Table 1. Sensory evaluation of fermentation broth with obvious aroma

Strain	Broth color	Sensory evaluation
A2	Milky white	Acidic aroma, caramel notes
A8	Milky white	Acidic aroma, fruity notes
A12	Milky white	Acidic aroma, fruity notes
B1	Light yellow	Acidic aroma
B6	Light yellow	Acidic aroma
C1	Light yellow	Mild wine aroma
C5	Light yellow	Acidic aroma
C7	Light yellow	Acidic aroma
8	Light yellow	Mild wine aroma, floral-fruity notes
12	Light yellow	Wine aroma, floral-fruity notes
13	Light yellow	Mild wine aroma, floral-fruity notes
14	Light yellow	Mild wine aroma, floral-fruity notes
A2	Milky white	Acidic aroma, caramel notes

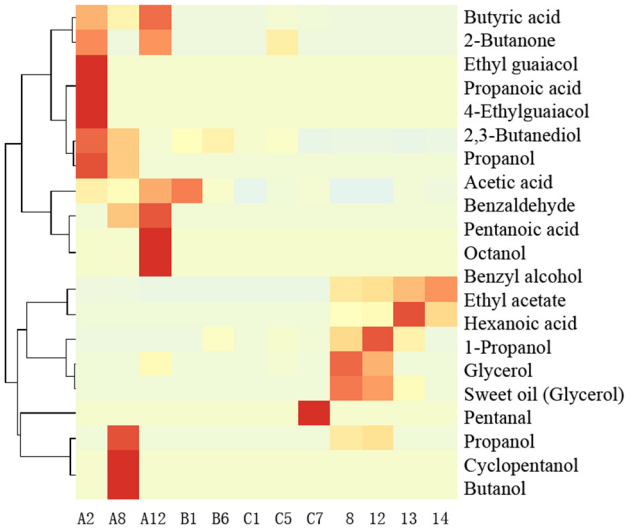

Fig 1. Heatmap analysis of aroma composition of fermentation broth

3.2. Solid-state Fermentation Performance

Based on the liquid fermentation results, strains A2, 12, and 13 were selected for solid-state fermentation using sorghum as substrate. After 10 days of fermentation, strain 12 produced the most desirable aroma profile, characterized by prominent wine-like and fruity-floral notes. In contrast, the fermentation products of strains A2 and 13 displayed predominantly sorghum-like aromas, indicating limited aroma production under solid-state conditions. HS-SPME-GC-MS analysis of strain 12's solid-state fermentation product revealed a complex volatile compound profile dominated by alcohols (77.89 mg/kg) and esters (3.75 mg/kg), with smaller amounts of acids (1.15 mg/kg), aldehydes/ketones (0.72 mg/kg), and phenols (0.10 mg/kg) (Table 2). A detailed examination of the individual compounds showed that higher alcohols were the most abundant aroma constituents, with phenylethanol (43.54 mg/kg) and isoamyl alcohol (29.64 mg/kg) representing approximately 94% of the total alcohol content. Phenylethanol, which imparts a characteristic rose-like aroma, was the predominant volatile compound in the fermentation product. Isoamyl alcohol, contributing apple-like notes, was the second most abundant compound. Other alcohols detected in lower concentrations included 2,3-butanediol (2.41 mg/kg) and isobutanol (2.31 mg/kg), which contribute to the overall complexity of the aroma profile. The ester fraction, though present in lower concentrations than alcohols, played a significant role in the overall aroma profile. Ethyl acetate (1.73 mg/kg) and isoamyl acetate (1.37 mg/kg) were the major esters identified, comprising approximately 83% of the total ester content. These compounds are known to impart fruity notes reminiscent of apple and pear. Minor esters included ethyl valerate and ethyl hexanoate (both 0.32 mg/kg), which contribute to the fruity character with pineapple and apple-like notes, respectively. Organic acids constituted a smaller portion of the volatile profile, with acetic acid (0.48 mg/kg), pentanoic acid (0.47 mg/kg), and hexanoic acid (0.21 mg/kg) detected. Although present in relatively low concentrations, these acids contribute to the complexity of the aroma by providing subtle sour notes that balance the sweet and fruity character of alcohols and esters. The aldehyde and ketone fraction included isovaleraldehyde (0.37 mg/kg), acetoin (0.23 mg/kg), pentanal (0.11 mg/kg), and acetaldehyde (0.01 mg/kg). These compounds, particularly acetoin, contribute buttery and creamy notes that enhance the complexity of the aroma profile. Phenolic compounds, represented by phenol (0.10 mg/kg), were detected in trace amounts. This volatile compound profile aligned well with the sensory evaluation results, confirming that strain 12 produced distinctive wine-like and fruity-floral aromas during solid-state fermentation. The predominance of phenylethanol and isoamyl alcohol, combined with fruity esters and balanced by subtle acidity, creates a complex and pleasant aroma profile similar to that found in traditional Sauce-Flavor Baijiu. The significant production of these valuable aroma compounds under solid-state conditions highlights the potential of strain 12 for applications in traditional Baijiu fermentation and other fermented food products.

3.3. Identification and Characterization of Strain 12

3.3.1. Morphological and Molecular Identification

Strain 12 formed small, round, smooth, slightly raised, white colonies on LB agar plates after 24 h incubation at 37°C (Fig. 2, left). Microscopic examination of the cells using Lugol's iodine staining revealed oval-shaped cells typical of yeast morphology (Fig. 2, right). The morphological characteristics observed are consistent with those of Candida species.

Table 2. Strain 12 solid fermentation products are mainly composed of flavor substances

Compound class	Compound name	Content (mg/kg)	Total (mg/kg)
Alcohols	Isobutanol	2.31	77.89
	Isoamyl alcohol	29.64	
Esters	Ethyl acetate	1.73	3.75
	Isoamyl acetate	1.37	
	Ethyl valerate	0.32	
	Ethyl hexanoate	0.32	
Acids	Acetic acid	0.48	1.15
	Pentanoic acid	0.47	
	Hexanoic acid	0.21	
Aldehydes/Ketones	Acetaldehyde	0.01	0.72
	Pentanal	0.11	
	Isovaleraldehyde	0.37	
	Acetoin	0.23	
Phenols	Phenol	0.10	0.10

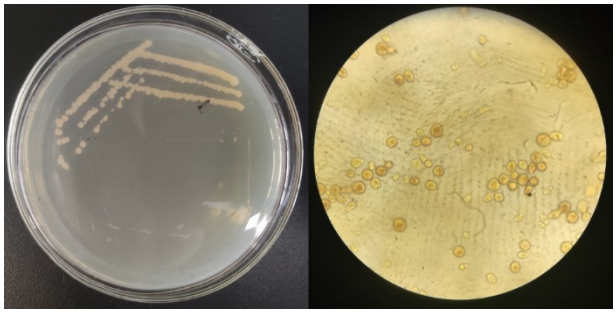

Fig 2. Strain 12 colony morphology (left) and cell morphology (right)

For molecular identification, the 18S rDNA region of strain 12 was amplified by PCR using universal fungal primers ITS1 and ITS4. Gel electrophoresis of the PCR products revealed a clear band of approximately 600 bp (Fig. 3), confirming successful amplification of the target region. Sequencing of this PCR product and subsequent analysis using BLAST against the NCBI database identified strain 12 as Candida tropicalis.

Phylogenetic analysis was performed to determine the taxonomic position of strain 12 within the Candida genus. As shown in Fig. 4, strain 12 clustered with the Candida tropicalis ATCC 750 reference strain with a high bootstrap value of 99%, providing strong support for the identification. The phylogenetic tree also demonstrated clear separation between strain 12 and other Candida species, including C. sojae, C. sanyaensis, C. albicans, and others. The evolutionary distance scale (0.020) indicates the number of base substitutions per site, demonstrating the close genetic relationship between strain 12 and the reference C. tropicalis strain. This molecular evidence, combined with the morphological characteristics, conclusively identifies strain 12 as Candida tropicalis.

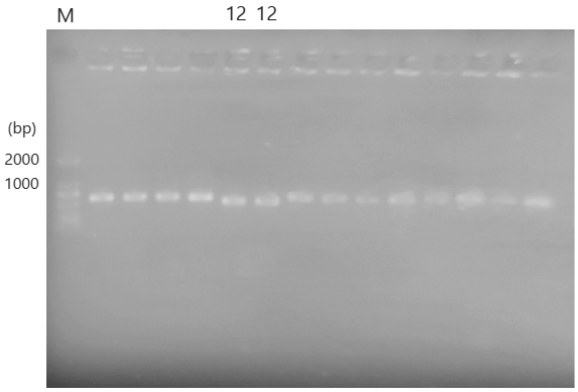

Fig 3. Gel electrophoresis results

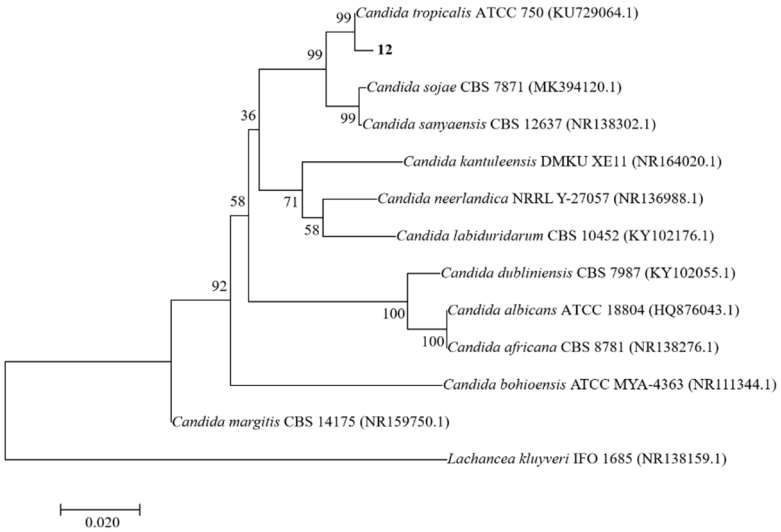

Fig 4. Strain 12 phylogenetic tree

3.3.2. Growth Characteristics

The growth characteristics of C. tropicalis strain 12 were determined by measuring the optical density at 600 nm (OD_{600}) over a 24-hour period (Fig. 5a). The growth curve exhibited distinct phases typical of microbial growth. The lag phase lasted approximately 4 hours, during which cells adapted to the new environment with minimal growth observed. Between 4 and 14 hours, the strain entered exponential phase, characterized by rapid cell multiplication and a steep increase in OD_{600} values from 0.4 to 1.9. The maximum OD_{600} value (approximately 1.9) was reached at 16 hours, indicating the peak of cellular density. The stationary phase occurred between 14 and 18 hours, with OD_{600} values remaining relatively stable. After 18 hours, the culture entered a gradual decline phase, with OD_{600} values decreasing to approximately 1.7 by 24 hours. This growth pattern is consistent with typical yeast growth kinetics and provides valuable information for optimizing fermentation conditions for aroma production.

3.3.3. Stress Tolerance

The physiological characteristics of C. tropicalis strain 12 under various stress conditions were investigated to understand its adaptability to fermentation environments (Fig. 5b-d).

pH tolerance tests demonstrated that strain 12 could grow across a pH range of 4-9, with negligible growth observed at pH 3, 10, and 11 (Fig. 5b). Optimal growth occurred at pH 5, with an OD_{600} value of approximately 4.1, followed by pH 6 . Growth gradually decreased at pH values above 6, with OD_{600} values of 3.7, 3.5, and 2.7 at pH 7, 8, and 9, respectively. This broad pH tolerance, particularly in the slightly acidic range, is advantageous for fermentation processes where pH fluctuations commonly occur during microbial metabolism.

Salt tolerance experiments revealed that strain 12 could tolerate NaCl concentrations up to 12% (w/v), beyond which growth was severely inhibited (Fig. 5c). Maximum growth was observed at 6% NaCl, with a gradual decrease in growth as salt concentration increased to 8% , 10% , and 12% . At 14% and 16% NaCl, growth was minimal , indicating these concentrations exceeded the strain's osmoregulatory capabilities. This relatively high salt tolerance suggests potential applications in fermentation processes where moderate salt concentrations may be present.

Ethanol tolerance studies showed that C. tropicalis strain 12 could grow in media containing up to 7% (v/v) ethanol (Fig. 5d). The highest growth was observed at 1% ethanol , with decreasing growth at 3% and 5% . At 7% ethanol, growth was significantly reduced , while at concentrations of 9% and above, growth was virtually absent. This moderate ethanol tolerance is consistent with the strain's potential role in Baijiu fermentation, where ethanol concentrations gradually increase during the fermentation process and typically reach 7-12% before distillation.

These physiological characteristics collectively indicate that C. tropicalis strain 12 is well-adapted to the variable conditions encountered during traditional Baijiu fermentation, including fluctuating pH, osmotic pressure, and increasing ethanol concentrations.

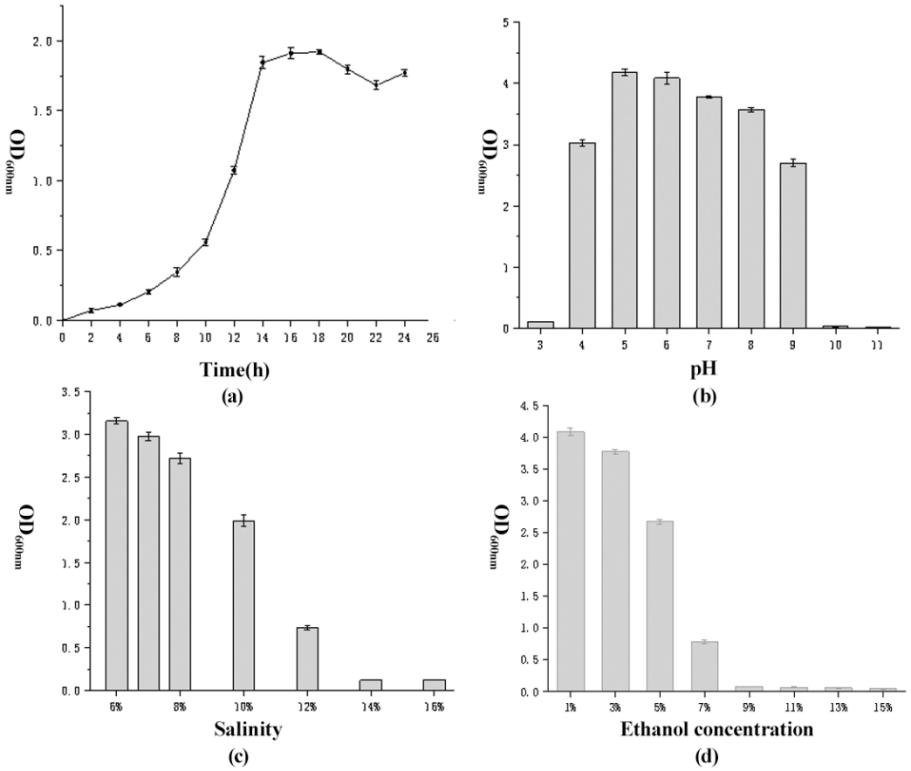

Fig 5. Physiological characteristics of strain 12. (a) Growth curve; (b) Acid tolerance; (c) Salt tolerance; (d) Ethanol tolerance

3.4. Discussion

The isolation and characterization of C. tropicalis strain 12 from Sauce-Flavor Daqu provides valuable insights into the microbial contributions to the complex aroma profile of Sauce-Flavor Baijiu. The strain's ability to produce significant amounts of phenylethanol and isoamyl alcohol during solid-state fermentation is particularly noteworthy, as these compounds are known to contribute positively to the aroma profiles of fermented beverages[16-18]. Phenylethanol, with its rose-like aroma, is a highly valued flavor compound in the food and beverage industry, while isoamyl alcohol contributes fruity notes that enhance the complexity of fermented products[19].

The physiological characteristics of C. tropicalis strain 12, including its broad pH tolerance, moderate salt tolerance, and ability to withstand ethanol concentrations up to 7%, suggest that this strain is well-adapted to the traditional Baijiu fermentation environment. These properties would enable the strain to survive and contribute to aroma development throughout the fermentation process, even as conditions change due to metabolic activities of the microbial community. Previous studies have demonstrated the important roles of non-Saccharomyces yeasts in flavor formation during alcoholic fermentation[20-22].The identification of strains with desirable aroma-producing capabilities, such as C. tropicalis strain 12, offers potential applications in the improvement of traditional fermented products and the development of novel flavor-enhancement strategies. Future research could explore the optimization of culture conditions to maximize aroma production, the genetic basis of flavor compound synthesis in this strain, and potential applications in other fermentation systems.

4. Conclusion

This study successfully isolated and characterized an aroma-producing Candida tropicalis strain from Sauce-Flavor Daqu. Through systematic screening using both liquid and solid-state fermentation approaches, strain 12 demonstrated superior aroma-producing capabilities, generating distinctive wine-like and fruity-floral notes. Chemical analysis of the volatile compounds revealed that this strain predominantly produced alcohols (particularly phenylethanol and isoamyl alcohol) and esters during fermentation, which contributed to its desirable aroma profile. Molecular identification confirmed the strain as Candida tropicalis, while physiological characterization demonstrated its adaptability to various environmental conditions, including pH ranges from 4 to 9, salt concentrations up to 12%, and ethanol levels up to 7%. These characteristics suggest that the strain is well-suited to the complex and dynamic environment of traditional Baijiu fermentation. Our findings provide insights into the microbial contributions to the distinctive aroma profile of Sauce-Flavor Baijiu and identify a promising candidate for potential applications in flavor enhancement of fermented foods and beverages. Future research should focus on optimizing culture conditions for enhanced aroma production, elucidating the metabolic pathways involved in flavor compound synthesis, and exploring practical applications of this strain in industrial fermentation processes. The isolation and characterization of indigenous microorganisms from traditional fermentation starters not only contributes to the preservation of traditional brewing knowledge but also offers opportunities for innovation in the development of improved fermentation processes and novel flavor-enhancing strategies.

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