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Identification and Characteristic Analysis of *Bacillus sonorensis* Strains from Strong-Aroma Daqu

Hongyi Gu, Shuanglian Chen, Jianpeng Zeng, Wenli Quan *, Xun Liu *

School of Bioengineering, Sichuan University of Science & Engineering, Yibin Sichuan, 644000, China
*** Corresponding author:** Wenli Quan and Xun Liu

Abstract: This study investigates the presence and functional attributes of *Bacillus sonorensis* in strong-aroma Daqu, the traditional fermentation starter for Chinese Baijiu production. Through systematic isolation procedures, morphological observation, physiological and biochemical assays, and 16S rDNA sequence homology analysis, we successfully identified a *Bacillus sonorensis* strain, designated as NE-8, from strong-aroma Daqu samples. Environmental tolerance experiments revealed optimal growth conditions at 5% sugar concentration and pH 5.5, with the ability to withstand alcohol concentrations up to 10% vol. Following fermentation on wheat-based solid medium, GC-MS analysis detected significant volatile compounds, including nitrogen-containing compounds (21.504 μg/g), ketones (22.54 μg/g), and alcohols (19.447 μg/g). Notably, the strain produced substantial amounts of 2,3,5,6-tetramethylpyrazine (8.055 μg/g), 2,3-butanediol (10.77 μg/g), and 3-hydroxy-2-butanone (21.57 μg/g). These findings demonstrate that strain NE-8 not only generates diverse volatile flavor compounds that potentially enhance Baijiu quality and sensory characteristics but also exhibits robust environmental resilience suitable for the complex conditions of traditional Baijiu fermentation. This research provides a scientific foundation for the potential application of B. sonorensis in strong-aroma Baijiu production technology.

Keywords: Strong-aroma Baijiu Daqu; *Bacillus sonorensis*; Tolerance Analysis; Volatile Compounds.

1. Introduction

Baijiu, a quintessential Chinese fermented spirit, has garnered global recognition for its illustrious heritage and distinctive organoleptic properties[1,2]. Within the baijiu production process, Daqu functions as an essential fermentation starter, harboring a complex microbial consortium that fundamentally drives the fermentation dynamics[3,4]. Among China's four principal baijiu categories, the strong-aroma variant has achieved widespread consumer acclaim due to its characteristic bouquet and palatability. Within the intricate microbial ecosystem of strong-aroma Daqu, bacterial communities—particularly members of the Bacillus genus—contribute not only to starch hydrolysis but also to the biosynthesis of diverse enzymes and flavor precursors that critically influence the final product quality[5].

Recent advancements in microbiomics and molecular biology have furnished robust methodologies for investigating functional microorganisms in fermented products. The implementation of cutting-edge technologies—including high-throughput sequencing, metagenomics, and metatranscriptomics—has enabled comprehensive characterization of microbial diversity and functionality in traditional fermented foods [6]. *Bacillus sonorensis*, a significant member of the Bacillus genus, was initially isolated and taxonomically classified in 2001 by Palmisano et al. from soil samples collected in Arizona's Sonoran Desert[7]. This microorganism exhibits remarkable physiological attributes, including thermotolerance, xerotolerance, and elevated enzymatic activity, enabling it to maintain metabolic functionality under extreme environmental conditions[8].

Contemporary research, both domestic and international, has identified *Bacillus sonorensis* in various traditional fermented consumables, including Korean soy sauce, Indian rice beverages, and Mexican dairy products, confirming its participation in fermentation processes and its contribution to flavor development[9-11]. Studies have further elucidated potential probiotic properties of this organism, including antimicrobial activity against pathogenic microorganisms, degradation of antinutritional factors, and synthesis of bioactive compounds[12,13]. While this bacterial strain has been detected in Chinese fermented products such as soy sauce[14] and fermented tofu[15], research on its distribution, diversity, and functional characteristics within strong-aroma Daqu remains limited, with its ecological niche and functional significance in the baijiu fermentation ecosystem not yet fully delineated. Considering that *Bacillus sonorensis* strains isolated from diverse ecological niches may exhibit genomic and phenotypic heterogeneity, comprehensive investigation of strains originating from strong-aroma Daqu possesses substantial scientific value and application potential for understanding this bacterium's role in the baijiu fermentation ecosystem.

Accordingly, this investigation aims to isolate and characterize *Bacillus sonorensis* from strong-aroma Daqu, employing multiple identification methodologies including morphological examination, 16S rDNA gene sequencing, and whole-genome analysis to confirm taxonomic identity. Subsequently, through systematic physiological and biochemical characterization, key tolerance parameters—including ethanol tolerance, sugar tolerance, and pH tolerance—will be evaluated. Building upon these foundations, the isolated strain will be inoculated into a wheat-based simulated Daqu medium for solid-state fermentation, emulating traditional brewing methodologies, followed by qualitative and quantitative analysis of volatile flavor compounds in fermentation products using gas chromatography-mass spectrometry (GC-MS). This research will establish a scientific foundation for enhanced understanding of microbial mechanisms in strong-aroma baijiu fermentation, while concurrently providing theoretical

support and microbial resources for scientific optimization of strong-aroma baijiu production techniques and quality enhancement.

2. Materials and Methods

2.1. Samples

Strong-aroma Daqu samples were sourced from premium-grade raw materials obtained from a commercial distillery's Daqu production facility.

2.2. Reagents

All experimental reagents including peptone, NaCl, yeast extract, agar, and glucose were procured from established domestic suppliers.

2.3. Equipment

Analytical instrumentation comprised a TSQ8000 gas chromatography-mass spectrometry system (Agilent, USA), UV-1800 spectrophotometer (Aoyi Instruments, Shanghai), orbital shaking incubators, PCR thermocyclers, and precision electronic balances.

2.4. Culture Media

The study employed specialized media including enrichment and isolation media for strain selection, along with fermentation medium, carbohydrate test media (glucose, sucrose, lactose, starch), Voges-Proskauer test medium, LB broth, and gelatin medium for comprehensive physiological and biochemical characterization of *Bacillus sonorensis*.

2.5. Strain Isolation and Screening

2.5.1. Enrichment Culture

10g of pulverized Daqu was added to 50mL of enrichment medium and cultured at 185r/min for 4h. 10mL was treated in an 80°C water bath for 30min, then 1mL of supernatant was transferred to 50mL of fresh enrichment medium and cultured overnight at 37°C and 180r/min.

2.5.2. *Bacillus sonorensis* Screening

The enriched culture underwent serial dilution (10^{-7}fold) in physiological saline. Aliquots (0.1mL) from 10^{-5}-10^{-7} dilutions were plated on isolation medium and incubated at 37°C for 24h. Colonies exhibiting characteristic morphology (pale yellow, opaque, elevated) were subjected to streak-plate purification, with pure cultures established after three successive transfers.

2.6. Strain Identification

2.6.1. Morphological Characterization

Diluted suspensions of the isolated strain were plated and incubated (37°C, 32h) for colony assessment, followed by Gram and spore staining for microscopic examination of cellular morphology.

2.6.2. Biochemical Profiling

The isolate underwent comprehensive biochemical characterization including catalase activity, anaerobic growth capacity, citrate utilization, carbohydrate fermentation patterns, starch hydrolysis capability, Voges-Proskauer reaction, and gelatin liquefaction assessment.

2.6.3. Molecular Phylogenetic Analysis

Genomic DNA was extracted using a commercial Bacterial DNA Kit. The 16S rDNA region was amplified using universal primers (27F: 5'-AGAGTTTGATCCTGGCTCAG-3', 1492R: 5'-GGTTACCTTGTTACGACTT-3'). Sequencing results were refined, subjected to BLAST analysis against the NCBI database, and integrated into phylogenetic reconstruction using MEGA7.0 software [16,17].

2.7. Strain Tolerance Analysis

2.7.1. Ethanol Tolerance

LB media containing graduated ethanol concentrations (0, 4%, 8%, 10%, and 12% v/v) were inoculated with the target strain (2% inoculum), incubated (37°C, 185r/min, 24h), and growth was quantified spectrophotometrically (OD600) with triplicate measurements for statistical validation.

2.7.2. Acid Tolerance

Growth media adjusted to specific pH values (3.5, 4.0, 4.5, 5.0, 5.5, 6.0) were inoculated and cultured under standardized conditions (37°C, 185r/min, 24h), with growth monitored spectrophotometrically (OD600) in triplicate.

2.7.3. Osmotic Tolerance

LB media supplemented with increasing glucose concentrations (0%, 5%, 10%, 15%, 20%, 25% w/v) were inoculated, incubated under controlled conditions (37°C, 185r/min, 24h), and growth was quantified (OD600) with triplicate measurements.

2.8. Volatile Metabolite Analysis

Precisely 4.0g of fermented wheat medium was transferred to a 15mL headspace vial, subjected to thermal equilibration (60°C, 15min) followed by headspace extraction (30min). Volatile compounds were analyzed by GC-MS using 2-ethylbutyric acid as internal standard. Chromatographic separation was performed on a DB-WAX capillary column (60m×250μm, 0.25μm film thickness) using manual injection (inlet: 230°C) and a multi-ramp temperature program (40°C for 3min, 5°C/min to 120°C, 7°C/min to 230°C, hold 10min). Mass spectrometric detection employed the following parameters: mass range 20-500u, ionization energy 70eV, with source and interface temperatures maintained at 230°C.

3. Results and Discussion

3.1. Colony and Morphological Characteristics

Strain NE-8, isolated from strong-aroma Daqu, exhibited characteristic morphology consistent with *Bacillus sonorensis*: opaque, cream-white colonies with irregular margins, rough and desiccated surfaces, and flat growth pattern (Fig. 1). Microscopic examination following Gram staining confirmed the isolate as Gram-positive with ellipsoidal rod-shaped cellular morphology (Fig. 2).

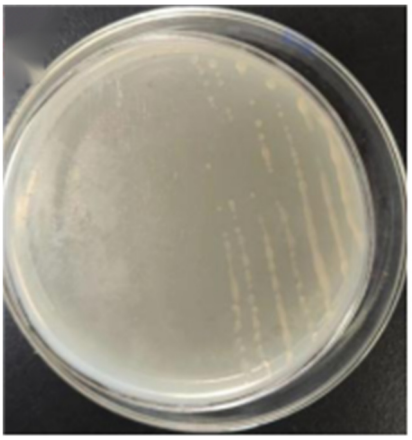

Fig 1. Morphology of strain NE-8

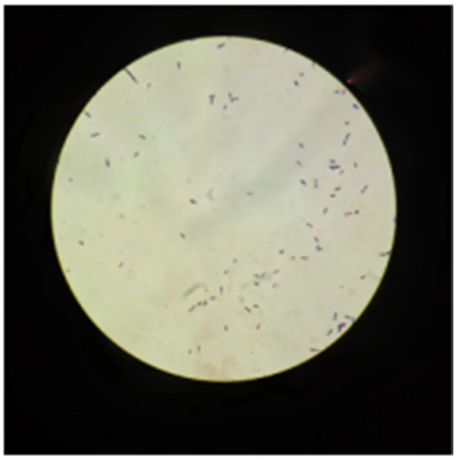

Fig 2. Gram staining of strain NE-8

3.2. Physiological and Biochemical Identification

The physiological and biochemical test results for strain NE-8 (Table 1) showed that, except for the negative glucose gas production test, all other indicators—including spore staining, glucose acid production, anaerobic growth, V-P test, nitrate reduction, starch hydrolysis, catalase, and gelatin hydrolysis tests—were positive. According to Bergey's Manual of Determinative Bacteriology, the strain was preliminarily identified as belonging to the Bacillus genus.

Table 1. Identification Results of Physiological and Biochemical Experiments

Experimental Test	Gram Staining	Spore Staining	Glucose Gas Production Test	Glucose Acid Production Test	Anaerobic Growth Test	V-P Test	Nitrate Reduction Test	Starch Hydrolysis Test	Catalase Test	Gelatin Hydrolysis Test
Result	+	+	-	+	+	+	+	+	+	+

3.3. Molecular Identification

Genomic analysis through 16S rDNA gene sequencing and subsequent phylogenetic reconstruction using MEGA7.0 software positioned strain NE-8 in closest proximity to *Bacillus sonorensis* reference strains (Fig. 3). Integration of this molecular evidence with morphological and biochemical data provided definitive identification of strain NE-8 as *Bacillus sonorensis*.

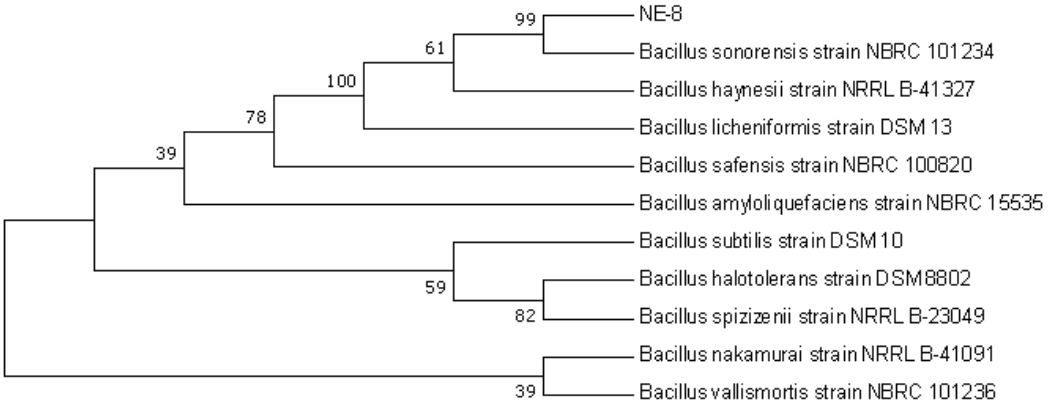

Fig 3. NE-8 phylogenetic tree

3.4. Physiological Tolerance Parameters

3.4.1. Ethanol Tolerance

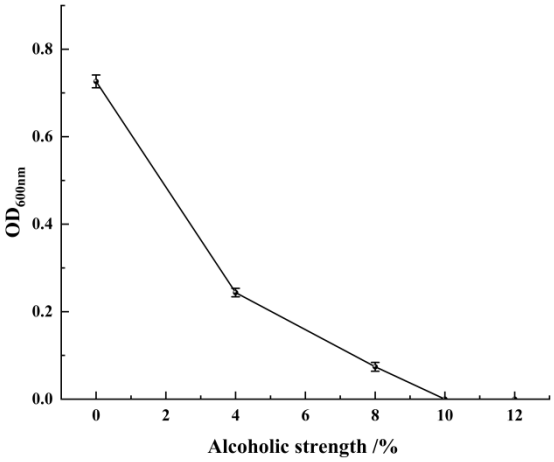

Fig 4. Effects of different alcohol contents on the growth of NE-8

Growth kinetics assessment under increasing ethanol concentrations demonstrated progressive inhibition of strain NE-8 proliferation (Fig. 4). At 10% vol ethanol, cellular growth was effectively arrested (OD600 approaching negligible values), establishing the strain's tolerance threshold below this concentration—a characteristic relevant to fermentation environments.

3.4.2. Osmotic Tolerance

The strain's response to varying glucose concentrations followed a bell-shaped curve (Fig. 5), with optimal growth observed at 5% glucose. Sub-optimal growth at concentrations below 5% reflected carbon limitation, while concentrations exceeding 20% imposed osmotic stress that significantly restricted cellular proliferation.

3.4.3. pH Tolerance

Strain NE-8 exhibited pH-dependent growth with enhanced proliferation correlating with increasing pH values within the tested range (Fig. 6). Marked growth inhibition occurred at pH values below 4.0, while robust growth was observed at pH 6.0, indicating adaptation to mildly acidic

conditions—a physiological attribute consistent with the fermentative habitat from which it was isolated.

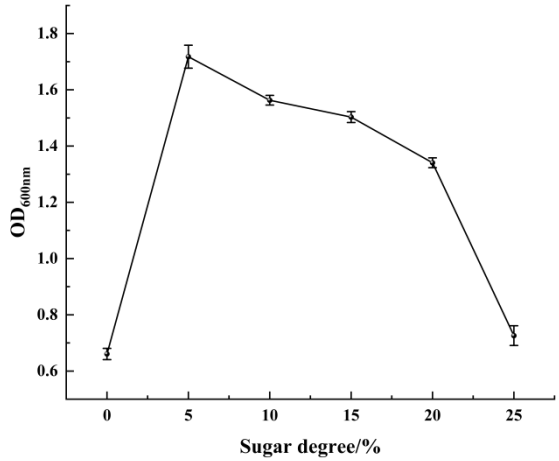

Fig 5. Effects of different glucose concentrations on NE-8 growth

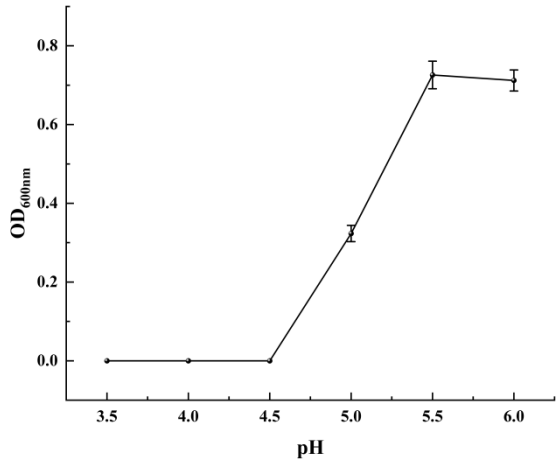

Fig 6. Effects of different initial pH on NE-8 growth

3.5. Volatile Metabolite Profile

Solid-state fermentation of wheat substrate inoculated with strain NE-8 (Fig. 7) yielded a complex array of volatile compounds as characterized by GC-MS analysis, predominantly comprising nitrogenous compounds, alcohols, and ketones.

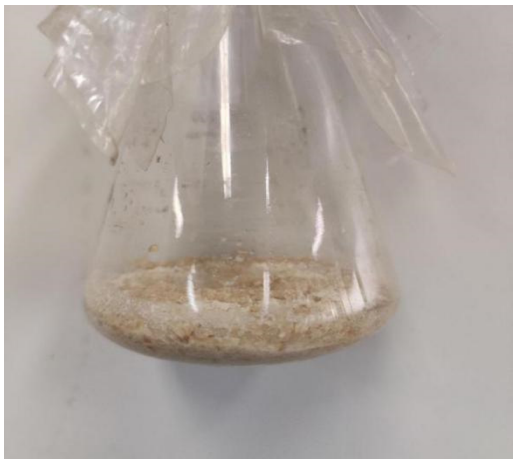

Fig 7. NE-8 fermentation process

Nitrogen-containing compounds constituted a significant fraction (21.504 μg/g) of the volatile profile (Table 2), with predominant pyrazine derivatives including 2,3,5,6-tetramethylpyrazine (8.055 μg/g), 2,3,5-trimethylpyrazine (7.597 μg/g), and 2,5-dimethylpyrazine (5.402 μg/g). These compounds contribute distinctive organoleptic properties—reminiscent of almond, roasted, and meaty notes—that enhance the flavor complexity of distilled spirits while potentially offering physiological benefits including vasodilation and circulatory enhancement.

Table 2. Nitrogen-containing compounds in solid-state fermentation

number	Flavor compounds		content (ug/g)	amount to (ug/g)	Method of identification	retention time
1	nitrogenous compounds	2,5-dimethylpyrazine	5.402	21.504	MS RI	11.29
2		2,3,5-trimethylpyrazine	7.597		MS RI	12.68
3		2,3,5,6-tetramethylpyrazine	8.055		MS RI	13.83

Alcoholic components totaled 19.447 μg/g (Table 3), with 2,3-butanediol predominating (10.77 μg/g), followed by phenylethyl alcohol (2.698 μg/g) and 1-octanol (1.952 μg/g). These compounds impart smooth, subtly sweet sensory characteristics and serve as crucial ester precursors—thereby significantly influencing the distinctive style of strong-aroma baijiu.

Table 3. Alcohol content in solid-state fermentation

number	Flavor compounds		content (ug/g)	amount to (ug/g)	Method of identification	retention time
1	alcohols	isoamyl alcohol	0.9414	19.447	MS RI	9.3
2		n-hexyl alcohol	1.126		MS RI	11.78
3		2,3-butanediol	10.77		MS RI	14.77
4		1-Octanol	1.952		MS RI	14.95
5		phenethyl alcohol	2.698		MS RI	18.85
6		1,2:5,6-diglycosyl alcohol	1.960		MS RI	15.85

Ketonic compounds reached 22.54 μg/g (Table 4), dominated by 3-hydroxy-2-butanone (acetoin) at 21.57 μg/g. This metabolite contributes distinctive buttery and lipid-like aromatic notes that enhance the compositional complexity and sensory integration of the spirit.

Additional volatile constituents included phenolic compounds (4.537 μg/g, Table 5), organic acids (4.003 μg/g), and esters (2.796 μg/g) as detailed in Table 6. Despite their relatively modest concentrations, these compounds provide critical aromatic elements that contribute to the distinctive

sensory signature of strong-aroma baijiu.

Table 4. Ketone content in solid-state fermentation

number	Flavor compounds		content (ug/g)	amount to (ug/g)	Method of identification	retention time
1	Ketone	acetoin	21.57	22.54	MS RI	10.7
2		Oxetanyl-2-keto	0.9704		MS RI	28.86

Table 5. Phenolic content in solid-state fermentation

number	Flavor compounds		content (ug/g)	amount to (ug/g)	Method of identification	retention time
1	Phenols	4-tert-butylphenol	3.301	4.537	MS RI	21.27
2		guaiacol	1.236		MS RI	18.34

Table 6. Content of other flavor compounds in solid-state fermentation

number	Flavor compounds		content (ug/g)	amount to (ug/g)	Method of identification	retention time
1	Acids	acetic acid	2.394	4.003	MS RI	13.42
2		3-methylvaleric acid	1.609		MS RI	16.31
3	Esters	Butyl nitrite	1.553	2.796	MS RI	27.68
4		Hydroxylated nonadecanoic acid ester	1.243		MS RI	14.33
5	Other	Hydrazine monohydrate	4.187	8.861	MS RI	3.9
6		2-chloro-2-nitropropane	2.703		MS RI	16.05
7		carbon dioxide	1.971		MS RIa	1.64

3.6. Discussion

The successful isolation and characterization of *Bacillus sonorensis* strain NE-8 from strong-aroma Daqu extends previous findings of this species in fermented foods and provides new insights into the microbial contributors to Baijiu production. The strain's physiological characteristics—tolerance to ethanol (up to 8% vol), optimal growth at pH 5.5, and preference for 15% glucose concentration—align well with the environmental conditions of traditional Baijiu fermentation processes. These tolerance parameters suggest that strain NE-8 is most active during the early to middle stages of fermentation before ethanol concentrations reach inhibitory levels.

The volatile metabolite profile produced by strain NE-8 reveals significant contributions to flavor development in strong-aroma Baijiu. The substantial production of pyrazine derivatives, particularly 2,3,5,6-tetramethylpyrazine (8.055 μg/g), contributes characteristic nutty and roasted aromas essential to premium Baijiu [18,19]. Similarly, the high concentrations of 3-hydroxy-2-butanone (acetoin, 21.57 μg/g) and 2,3-butanediol (10.77 μg/g) enhance the spirit's sensory complexity with buttery and smooth notes [20,21].

The distinctive metabolic profile of strain NE-8 suggests specialized adaptation to strong-aroma Daqu fermentation conditions. While other Bacillus species produce similar compounds [22,23], the quantitative profile observed for strain NE-8 indicates a particularly significant role in flavor development. The strain's robust environmental tolerance combined with its capacity to generate desirable flavor compounds suggests potential applications as a starter culture component for enhancing or standardizing the sensory characteristics of strong-aroma Baijiu.

Future research should focus on genomic analysis to elucidate the genetic mechanisms underlying the strain's metabolic capabilities and investigate its interactions with other microorganisms within the complex ecosystem of Daqu. Such studies would clarify the strain's ecological role and potential applications in improving Baijiu quality or standardizing production processes.

4. Conclusion

This investigation has successfully isolated and characterized *Bacillus sonorensis* strain NE-8 from strong-aroma Daqu. Physiological assessment established optimal growth parameters at 10% vol ethanol concentration, pH 6.0, and 20% glucose concentration. Volatile metabolite analysis via GC-MS demonstrated the strain's capacity to generate diverse aromatic compounds, including nitrogen-containing derivatives (21.504 μg/g), ketones (22.54 μg/g), and alcohols (19.447 μg/g). Notably high concentrations of 2,3,5,6-tetramethylpyrazine (8.055 μg/g), 2,3-butanediol (10.77 μg/g), and 3-hydroxy-2-butanone (21.57 μg/g) were detected. These compounds not only enhance the organoleptic properties of fermented products but potentially offer functional health benefits, thereby establishing a scientific foundation for the application of this strain in strong-aroma baijiu production.

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