



Isolation and identification of a caproic acid-producing K-1 strain from Luzhou-flavor Liquor Pit Mud

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ABSTRACT

In the present study, a bacterial strain producing caproic acid, which was isolated and purified from Luzhou-flavor Liquor Pit Mud, and characterized by morphological, physiological and molecular biological methods. Results showed that the strain had the typical characteristics of Bacillus clone, acid-producing capacity as strong as 4.1g/L. Analysis of its 16SrRNA gene in Ribosomal Database through online tools supported that the strain was Lysinibacillus sp in genus Lysinibacillus. The present study was the first report that Lysinibacillus sp has capacity for producing caproic acid.

Key words: Pit mud, Caproic acid-producing strain, Luzhou-flavor Liquor, Identification

INTRODUCTION

“Thousand years of old cellar, and Million years of distiller's grains, Good liquor depends on the old pits”, the old liquor-making proverb in China reveals the brewing mechanism of high quality Luzhou-flavor liquor, which is the most common and best-selling liquor, accounts for 70 % of the total output of Chinese liquors at present in China(Zheng et al, 2011). Modern scientific research discovered that aged pit mud is the basis of Luzhou flavor Daqu liquor brewing and an organic carrier for pits microbes inhabiting was involved. The main microorganisms in pit mud are anaerobic heterotrophic bacteria such as caproic acid bacteria, methanogenus, lactobacillus and so on; among them, the most important of which is the caproic acid bacteria (Ding et al 2014;Hu et al 2014;XU et al,2011;ZHANG et al,2006). In the fermentation process, caproic acid bacteria utilized the fermentation products of ethanol, acetic acid, butyric acid, etc, to produce caproic acid through complicated metabolic process, and then ethyl caproate. It is well-known that the ethyl caproate is the main fragrant substance in Luzhou flavor Daqu liquor, the quantity of microbial flora of the caproic acid bacteria and its growth directly affects quality of fermentation in the process of Luzhou-flavor daqu liquor. Therefore, the bacteria have become a hot topic of Luzhou-flavor liquor fermentation industry(Ke et al,2013;MIAO et al 2012; YAO et al,2010;Zhao et al 2012).

At present study, a strain efficient to yield caproic acid was isolated and purified from mud of a hundred-year pit; the morphological, physiological and molecular biological properties were utilized to identify the strains isolated, providing basic materials for the further research on the fermentation mechanism of Luzhou-flavor liquor.

EXPERIMENTAL SECTION

Materials

Pit mud

Collected from 100-year pits in Luzhou Laojiao Co., Ltd., a famous liquor-making enterprise for Luzhou-flavor liquor, located in Luzhou city, the southeast of Sichuan province, PRC.

Culture medium

The culture medium for present study was prepared as follows:

Enriched medium: sodium acetate 0.5%, magnesium sulfate 0.02%, ammonium sulfate 0.05%, yeast extract 0.1%, dipotassium phosphate 0.04%, pH 6.8~7.0, 121°C sterilization 20 min, addition of 2% aseptic ethanol absolute before inoculation.

Isolation medium: added 2% agar into enriched media to a solid medium, which was used for the isolation and culture of caproic acid bacteria.

Fermentation medium: sodium acetate 0.5%, magnesium sulfate 0.02%, ammonium sulfate 0.05%, yeast extract 0.5%, dipotassium phosphate 0.04%, pH 6.8~7.0, 121°C sterilization 20 min, addition of 2% aseptic ethanol absolute before inoculation.

Reagents

Centrifugal columnar bacterial genomic DNA extraction kit (Tiangen Biochem (Beijing) Co., Ltd.) ; agarose, DreamTaq-TM DNA Polymerase (MBI) , SanPrep column DNA gel extraction kit, DNTP, DNA Ladder Mix maker, DNA Ladder Mix maker (Sangon Biotech (Shanghai) Co., Ltd) ; Hexanoic acid (Chengdu Kelong Chemical Reagent Factory); All other reagents were of analytical grade.

16S rDNA bacterial universal primers:

Forward Primer: CAGAGTTTGATCCTGGCT

Reverse Primer: AGGAGGTGATCCAGCCGCA

Primer sequences were synthesized by Sangon Biotech (Shanghai) Co., Ltd.

Methods**Enrichment culture**

1 gram pit mud sample was weighed, and added into flask containing sterile water 100 mL, the flask was shaking 180 r/min for 30 min, the sample was dispersed in sterile water well-distributed, and then bathed in 90 °C water for 10 min to remove non-spore bacteria, the flask was shaking to avoid local heating constantly, then 1 mL sample solution was drawn and added into 20 mL enriched culture, added 6 mL liquid paraffin to seal the liquid medium to isolate of the outside air, Cultured for 7 days under the condition of constant temperature of 35 °C, gas production in the test-tube was observed.

Clones isolated

Enrichment medium producing gas was treated in 90 °C water for 10 min again, then 5 µL enrichment culture broth was drawn to spread plate containing isolation medium, the plate was cultured for 7 days after added sterile liquid paraffin covering, the colony growth was observed.

Preliminary screening

Single colonies were picked and inoculated into test tubes containing 10 mL fermentation medium for static culture 7 days at 30 °C, select the tubes producing gas more for rescreening.

Rescreening

Suspension culture of the Preliminary screening bacteria was inoculated into 250 mL fermentation medium, stationarily cultured for 7 days, caproic acid content was test and calculated number of bacteria by hemocytometer.

Detection of caproic acid content**Qualitative Analysis**

4 mL fermented fluid was drawn, 2% copper acetate solution 1 mL was added, and then 1 mL diethyl ether was added, eddied for 1 min and then stood for 10 min, observed ether layer color after standing.

Quantitative Analysis

Preparation of dilution liquid

0.4% sodium acetate; 0.1% yeast extract; 0.05% ammonium sulfate; 0.04% dipotassium hydrogen phosphate; 0.02% magnesium sulfate; 1% ethanol.

Preparation of stock solution

116.16 mg caproic acid was weighed, 10 mL diluents was added to dilute the caproic acid, PH of the mixture was adjusted to 6.8, then the mixture was shaken to uniform.

Drawing the standard curve

15 mL mixture, which contained caproic acid concentration in 0.5808 mg/mL, 0.2902 mg / mL, 0.1451 mg/mL, 0.07255 mg/mL respectively, was prepared by adding certain amount of diluents and stock solution mentioned above, 5% solution of copper acetate (2 mL), diethyl ether (5 mL) into the test tube with stopper, stood layered after vortex 1 min, the ether layer was drawn and measured OD values at 660 nm wave length, standard curve was drawn with caproic acid concentration as the abscissa, OD value for the vertical.

Detection caproic acid content of the fermented liquid

The PH value of the caproic acid bacteria fermented liquid was adjusted to 6.8. the solution for detection was added 7 mL diluent, 5 mL ether, 2 mL 5% acetic acid copper solution, 1 mL caproic acid fermentation broth respectively, eddied for 1 min and stood for 10 min, draw

supernatant for detection; the blank control mixture was prepared by adding 8 mL dilution,

5 mL ether, 2 mL 5% solution of copper acetate respectively, eddied for 1 min and stood for 10 min, drawn Supernatant for detection. 5 replicates for each sample, obtain the caproic acid content of the tested sample according to the standard curve.

Cloning and Analysis of coproic acid bacteria 16S rRNA gene

The Genomic DNA of coproic acid bacteria was extracted according to the instructions of Centrifugal columnar bacterial genomic DNA extraction kit Tiangen Biochem (Beijing) Co., Ltd.) .

PCR amplification

PCR amplification of the 16S rRNA gene was performed using the universal primer mentioned above in reagents, the reaction system and condition were listed in table 1 and 2 respectively.

Tab 1 PCR reaction system in 16SrRNA gene amplification

reagent	Volume (μL)
Template (genomic DNA)	0.5
10 \times Buffer (with Mg^{2+})	2.5
dNTP (each 2.5mM)	1
DreamTaq-TM DNA Polymerase	0.2
F (10 μM)	0.5
R (10 μM)	0.5
Add double-distilled H_2O up to	2.5

Tab 2 PCR reaction conditions in 16SrRNA gene amplification

temperature	time	procedure
94 $^{\circ}\text{C}$	4min	pre-degenerative
94 $^{\circ}\text{C}$	45sec	
55 $^{\circ}\text{C}$	45sec	30cycle
72 $^{\circ}\text{C}$	1min	
72 $^{\circ}\text{C}$	10min	extension
4 $^{\circ}\text{C}$	∞	termination reaction

Gel electrophoresis

The PCR products were examined by electrophoresis on 1% agarose gels for 20min at 150 V voltage and 100mA current.

Purification and recovery of DNA

The desired DNA electrophoresis bands of PCR product were recovered, purified according to the manual of SanPrep column DNA gel extraction kit (Sangon Biotech (Shanghai) Co., Ltd), Purified PCR products were sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing.

RESULTS AND DISCUSSION

The morphological properties



LJ-1 Clones morphology

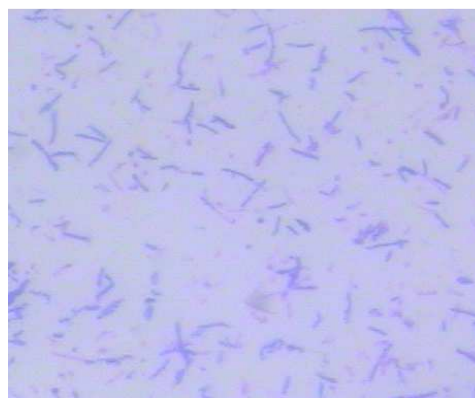
LJ-1 cellular morphology (10 \times 40)

Fig1. Morphological characteristics of caproic acid bacteria strain K-1

The screened strain of caproic acid bacteria from pit mud in the present study showed consistent colony characteristics, those are ivory-white, round, wavy edge, the colony characteristics of the bacteria were white, round, wavy edge, diameter 2.5-3.5mm, protruding into a table-like surface or smooth shape; the morphology of the bacterial cell were rod-shaped bacteria, slightly curved, one end of enlargement, single, in pairs or short chains, ends rounded or slightly pointed, $0.5-0.9 \times 1.4-2.5\mu\text{m}$, Gram-positive. the bacterium was named the strain K-1 in present study.

The physiological properties

The growing and acid-producing properties of the screened caproic acid bacteria was detected by the means of Hemocytometer Measurement, copper acetate chromogenic assay in conjunction with the quantitative colorimetric assay, the results were shown in Table 3.

Tab3 Quantitative assay of producing caporic acid of strain K-1

Test items	Groups					mean
	1	2	3	4	5	
Bacteria number ($\times 10^7$ cfu/mL)	4.5	3.6	4.2	4.6	5.2	4.42 \pm 0.58
Caproic acid conten (mg/100mL)	346.4	418.6	486.4	315.8	493.5	412.14 \pm 80.28

Analysis of the data in the table3 showed properties as follows: (1) slow growth. The number of bacteria of the strain K-1 was merely 4.42×10^7 (cfu / mL) for 7days growth period, indicating the strain had a slow growing characteristics;(2)capacity for caproic acid producing. The yielding of caproic acid was up to 412.14 mg/100mL for 7 days fermentation period, indicating the present strain had the ability to produce caproic acid efficiently.

PCR amplification of 16SrRNA gene

Under the set PCR conditions in present experiment, the DNA bands of about 1400bp appeared, indicating that the 16SrRNA gene amplified successfully, the last results need be confirmed by sequencing

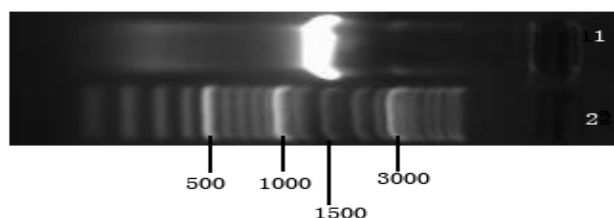


Fig2 the agarose gel electrophoresis of 16SrRNA gene in K-1 strain (Lane 1、 2 PCR products,Lane3 DNA marker)

Sequencing identification of 16SrRNA gene

The PCR product of 16SrRNA gene in K-1 strain was sent to Sangon Biotech (Shanghai) Co., Ltd for sequencing, The result was as follow:

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GGGTGCTATACATGSCAAGTCGAGCGAACAGAAAAGGAGCTTGTCTCCTTTGACGTTAGCGGCGGACGGGTGAGTAACACGTGGGCAACCTACCTATA
GTTTGGGATAACTCCGGGAAAACCGGGGCTAATAACCGAATAATCTCTTTACTTTCATGCGGAAAGACTGAAAGACGGCATCTCGCTGTGCTATAGGATG
GGCCCGCGCGCATTAGCTAGTTGGTGAGGTAAACGGCTCACCAAGCGACGATGCGTAGCCGACCTGAGAGGGTATCGGCCACACTGGGACTGAG
ACACGGCCCAGACTCTACGGGAGGCAGCAGTAGGGAATCTTCCACAATGGGGCGAAAGCCTGATGAGCAACGCGCGTGAGTGAAAGGTTTTTC
GGATCGTAAAACCTGTGTTGTAAGGGAAGAACAAGTACAGTAGTAACTGGCTGTACCTTGACGGTACCTTATTAGAAAGCCACGGGCTAACTACGTGCCA
GCAGCCGCGTAATACGTAGGTGGCAAGCGTTGTCGGGAATTTATGGCGTAAAGCGCGCGCAGGCGGTCTTTAAGTCTGATGTGAAAGCCCACGG
CTCAACCGTGGAGGGTCACTGGAAACTGGGGGACTTGAGTG CAGAAAGGAAAGTGAAATCCAAGTGAGCGGTGAAATGCGTAGAGATTGGGA
GGAACACCAGTGGCGAAGCGACTTTCTGTCTGTAACGTAGCGCTGAGGCGCGAAAGCGTGGGAGCAAACAGGATTAGATACCTGGTGTAGTCCAC
GCCGTAAACGATGAGTGTAAAGTGTAGGGGGTTTCCGCCCTTAGTGTCTGAGCTAACGCATTAAGCACTCCGCTGGGGGAGTACGGTCCGCAAGACT
GAAACTCAAAGGAATTGAGCGGGGCCCGCACAAAGCGGTGGAGCATGTGTTTAATTGAAAGCAACGCGAAGAACCCTTACCAGGTTCTGACATCCCGT
TGACCACTGTAGAGATATAGTTTCCCTTCGGGGGCAACGCTGACAGGTGTTGTCATGTTGTCGTGAGCTGTGTGAGATGTTGGGTTAAGTCCC
GCAACGAGCGCAACCTTGTACTTAGTTCCATCATTTAGTTGGCACTCTAAGGTGACTGCGGTGACAAACCGGAGGAAGGTGGGGATGACGTCA
AATCATCATGCCCTTATGACCTGGCTACACACGTGCTACAATGGACGATACAAACGGTTGCCAACTCGCGAGAGGGAGCTAATCCGATAAAGTGTT
CTCAGTTCCGATTGTAGGCTGCAACTCGCCTACATGAAGCCGGAATCGCTAGTAATCGCGATCAGCATGCCGCGGTGAATACGTTCCCGGGCCTTGTA
CACACGCCCCGTACACCACGAGAGTTTGTAAACCCGAAGTCGGTGAGGTAACCTTTGGAGCCAGCCGCGGAAAGGT
```

Fig3 the sequencing result of 16SrRNA gene in K-1 strain

Blast16SrRNA gene sequence of K-1 strain in Ribosomal Database (<http://rdp.cme.msu.edu/index.jsp>), the results were shown as follows:

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+ rootrank Root (0/20/1354916) (selected/match/total RDP sequences)
+ domain Bacteria (0/20/1321669)
+ phylum Firmicutes (0/20/432732)
+ class Bacilli (0/20/261033)
+ order Bacillales (0/20/166283)
+ family Planococcaceae (0/20/4078)
+ genus Lysinibacillus (0/20/1061)
  S000434843 not_calculated 0.983 1391 Bacillus sp. ZYM; AY043358
  S000444645 not_calculated 0.982 1385 Bacillus sp. LY; AY787805
  S000859427 not_calculated 0.984 1351 Bacillus sp. ge04; EF503613
  S000859428 not_calculated 0.984 1351 Bacillus sp. ge05; EF503614
  S000981370 not_calculated 0.984 1211 Bacillus sp. TTV-7; EU363693
  S001575652 not_calculated 0.994 1374 Lysinibacillus sp. 210_19; GQ199721
  S002231972 not_calculated 0.993 1303 Lysinibacillus sp. RSNPB26; HM588166
  S002917307 not_calculated 0.983 1270 Lysinibacillus sp. DT12; JN208190
  S002952831 not_calculated 0.994 1374 Lysinibacillus xylanilyticus; M-SZ30; JF731236
  S002956201 not_calculated 0.983 1314 Lysinibacillus sp. IARI-B-3; JN411377
  S003261959 not_calculated 0.987 1395 Lysinibacillus sphaericus; NBRC 3341; AB680068
  S003261994 not_calculated 0.987 1395 Lysinibacillus sphaericus; NBRC 3526; AB680103
  S003281684 not_calculated 0.983 1353 bacterium NLAE-zl-P8; JQ606849
  S003285844 not_calculated 0.988 1302 Bacillaceae bacterium SE6; JQ714089
  S003299606 not_calculated 0.986 1364 Lysinibacillus fusiformis; R3; JQ991002
  S003315456 not_calculated 0.982 1367 Lysinibacillus sphaericus; BL7; JQ923445
  S003714984 not_calculated 0.987 1425 Lysinibacillus sp. S51-22; KC160856
  S003754414 not_calculated 0.987 1432 Lysinibacillus fusiformis; 44A; KC329831
  S003804087 not_calculated 0.997 1338 Lysinibacillus sp. Y20; KC708570
  S004046236 not_calculated 0.984 1341 Lysinibacillus sp. S-2; KF033126

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Fig4 the blasting results of 16SrRNA gene in K-1 strain

the blasting results demonstrated that the 16SrDNA gene in K-1 strain was almost identical to that of Lysinibacillus sp Y20 (Sequence No S003804087), the identity was up to 0.997, indicating that the screened K-1 strain is Lysinibacillus sp Y20.

DISCUSSION

Currently, caproic acid bacteria generally refers to a class of microorganisms which can ferment grains to produce secondary metabolites-caproic acid in the fermentation process, there are extensive microbial species consisting with the definition, but mainly bacteria. The first caproic acid producing bacteria is Clostridium Kuyveri which was named by H Barker in 1942. This bacterium belongs to Phylum Firmicutes, class Bacilli, order Bacillales, Bacillaceae, Clostridium, the cell of which is rod-shaped bacteria, $0.9 - 1.1 \times 3 - 11 \mu\text{m}$, Gram-positive, mobile, grow in high concentration yeast extractat, ferment ethanol to caproic acid, and which is the only bacteria in genus clostridium that can accumulate caproic acid (Paul et al, 2009; Thauerk et al 1968; Jia et al, 2013; Seedorf et al, 2008; Ding et al, 2010).

The study of caproic acid bacteria in Chia rose in 1964 in the duration of ethyl caproate to be discovered the pit bottom fragrant substance in Maotai liquor (WU et al, 2007), from then on the different research units and liquor-making companies had isolated many strains of bacteria with capacity of producing caproic acid for research and production (WU et al, 2007; MIAO et al 2012; YAO et al, 2010; Zhao et al 2012;), Among these strains, the most famous one is Inner Mongolia light industry 30# (Inner 30#), which was isolated and purified from pit mud of Wuliangye Group Co., Ltd in 1975 by Light Industry Research Institute of Inner Mongoli, the Inner 30# was identified as Clostridium Kuyveri (WU et al, 2007), the strain, which was preserved in China center of industrial culture collection (<http://www.china-cicc.org/>) as the strain No 8022, was one of the most studied strain on morphology physiology and function until now, However, the molecular biological characteristics need to be further investigated

In the present study, so as to understand the taxonomic status of K-1 caproic acid bacteria, the Morphological and physiological characteristics were studied. the results showed that there was no much difference with the ones isolated from pit mud earlier in Morphology (MIAO et al 2012; YAO et al, 2010; Zhao et al 2012), but it had higher acid-producing efficiency (412.14mg/100mL) closer to YAO et al (YAO et al, 2010), obviously higher than those of MIAO et al and Zhao et al (MIAO et al 2012; Zhao et al 2012).

To further explore the taxonomic status of K-1 caproic acid bacteria, the 16S rRNA gene in the strain was cloned and analyzed. 16S rRNA is a kind of ribosomal RNA in prokaryotic microorganism, approximately 1.5 kbp fragment size. Composed by a number of arms, some base pairing, and the rest to form a ring, and can be divided into four domains, the sequence of it is highly conserved. Known as a "bacterial fossils," the microbe species identification based on the 16S rRNA gene sequence analysis had become a routine method for bacterial classification (Ding et al, 2014; Dougal et al, 2014; Hu et al, 2014; TANG et al, 2011). The cloned 16S rRNA gene was blasted in the Ribosomal Database Online (<http://rdp.cme.msu.edu/index.jsp>), the results showed that the strain isolated in present study was identical to *Lysinibacillus* sp Y20 in 16S rRNA gene, indicating this strain belongs to Phylum Firmicutes, class Bacilli, order Bacillales, Bacillaceae, *Lysinibacillus*. another strain, which has caproic acid producing characteristics, have been identified as *Lysinibacillus fusiformis* in this genus [7], this study is the second caproic acid producing strain from the genus *Lysinibacillus*, The strain was also first reported to have caproic acid producing properties at home and abroad.

CONCLUSION

In short, the results of present study showed that a new caproic acid-producing strain named *Lysinibacillus* sp Y20 was identified for the first time by mean of morphology, physiology and molecular ecological methods, so present discovery broadened the scope of caproic acid-producing bacteria and provided a new and high-yielding strain for the research and production of Luzhou-flavor liquor.

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