



Research Article

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## First polyphasic identification of clostridium celerecrescens from Luzhou-flavor liquor pit mud

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### ABSTRACT

*In this study, a combination of morphology, physiology, molecular approaches and gas chromatography were utilized to identify the taxonomy of a novel strain isolated from the mud of 180-year old pit. The results demonstrated that the strain, which was nominated strain K-2, utilized D- glucose, mannitol, inulin, L- arabinose, D- sucrose and other carbon sources, possessed hydrolysis activities of urease, esculin and gelatin, the phylogenetic analysis based on the 16S rRNA gene sequence (1346bp) positioned the novel strain among the genus celerecrescens strain in Genus I Clostridium, and the strain demonstrated a highly efficient production of caproic acid (547.26mg / 100mL) and butyric acid capacity (382.45 mg / 100mL), but hardly produced ethyl caproate and ethyl butyrate. This study was the first report of a isolate identified as Clostridium celerecrescens from Luzhou-flavor Liquor Pit Mud, and the strain was endowed with the highly efficient production of caproic acid property.*

**Keywords:** Pit mud, Caproic Acid Bacteria, Luzhou flavor Liquor, polyphasic identification

### INTRODUCTION

At present, there are 12-typed aroma in Chinese liquors, the Luzhou-flavor liquor is the most popular one among them, it accounts for 70% of the annual total output of Chinese liquor. According to the documents reported, there are 674 kinds of flavor compounds in Luzhou-flavor liquor by qualitative detection, and nearly 342 kinds of ingredients by quantitative detection (Chen Y et al,2104; JI Ke-liang et al,2007. The key flavor compounds in Luzhou-flavor liquor are ethyl acetate, ethyl butyrate, ethyl lactate, ethyl caproate, acetic acid, butyric acid and caproic acid, Among them, ethyl caproate serves as the main fragrance, of which the content level and ratio in the liquor determines the type and quality of the liquor(Jia Zheng et al, 2014; Zuobing Xiao et al, 2014).

Ethyl caproate was produced by the esterification reaction of caproic acid with ethanol in the complicated process of grains fermented. It's well known that ethanol is the most abundant product for the grains fermented process in the pit, which is a fermented vessel in the production of strong-flavor liquor, the production level of ethyl caproate depends mostly on the level of caproic acid concentration in the pit and lees. So breeding of high-yielding strains of producing caproic acid to improve the quality of Luzhou-flavor liquor becomes an important issue for brewing institutes and enterprises(Chen Y et al,2104; ZHANG Bin et al,2014; Xie Guo-pai,2011; CUI Hai-hao et al,2014).

The traditional solid-state natural fermentation crafts are applied in the process of Luzhou-flavor liquor making, the pit mud is the paradise for diverse anaerobic microorganisms inhabited and reproduction. Lots of anaerobic organisms, such as methanogenic bacteria, caproic acid bacteria, lactic acid bacteria, sulfate-reducing bacteria, nitrate-reducing bacteria, etc, were screened by the long-term high acidity, high alcohol concentration, long-term anaerobically closed fermentation and domesticated, so have they produced organic acid and aroma to enrich the pit mud. A large body of research documents have shown that the longer the pit mud continuously used, the richer the

microorganisms enriched in it, especially the caproic acid-producing bacteria, the higher the quality of its liquor. This is just the scientific mechanism of a old proverb, which says "thousands years of old cellars and millennium years old of fermented grains. Good liquor depends on old pit mud" (Tao Y et al, 2014; Liang H et al, 2014; Ding X F et al, 2014; WANG Mingyue et al, 2014).

This study intended to screen high efficient caproic acid-producing strain from pit mud by isolation, purification, polyphasic identification and acid-producing assay, to provide excellent starting strains for enterprises of Luzhou-flavor liquor production and further scientific researches.

## EXPERIMENTAL SECTION

### *Materials*

#### **Pit Mud**

The pit mud collected from Wan Bin Distillery Co., Ltd of Luzhou Laojiao Group(a international famous and century-old liquor enterprise located in the south of Sichuan province, China).

#### **Culture medium**

The culture media for this research were prepared as follows

Enrichment medium: 0.5% sodium acetate 1%, magnesium sulfate 0.02%, ammonium sulfate 0.05%, yeast extract 0.1%, dipotassium hydrogen phosphate 0.04%, pH 6.8 ~ 7.0, 121 °C sterilization 20min, addition with absolute ethanol to 2% before inoculation.

Clostridium strengthened medium: Beef powder 0.5%, pancreatic casein peptone 0.5%, yeast extract 0.015%, 0.025% of dextrose, 0.005% soluble starch, 0.025% of sodium chloride, sodium acetate 0.015%, L-cysteine hydrochloride 0.0025%, agar 6.25 %, heated and stirred were dissolved in 1000ml of distilled water, dispensing, 121 °C autoclaved for 15 minutes, then set aside for use.

Fermentation medium: Sodium acetate 0.5%, yeast extract 0.1%, 0.5% calcium carbonate, 50% of the 1: 3 Grains leachate, sterile ethanol 20mL (before inoculation added separately), pH 7.0 ~ 7.2, 121 °C autoclaving 15 minutes set aside.

#### **Reagents**

Rapid amplification of DNA extraction kit (KG201), Universal DNA purification kit recycling (DP214), DNAMaker (DL2000) were purchased from Tiangen Biotech (Beijing)Co., TLD; Taq enzyme, PCR10 × buffer, dNTP were purchased from TaKaRa Company (Dalian, China) ; caproic acid, n-butyric acid, ethyl caproate, ethyl butyrate standard materials were purchased from Tianjin Institute of Fine Chemicals (Tianjing, China) ; ethanol (HPLC grade) were purchased from Chengdu Kelong Chemical Reagent Factory (Chengdu, China) ; the API 50 CHB test kit (Bio- Merieux, Marcy l'Etoile, France); 16S rDNA bacterial universal primers:

27F: 5'-AGAGTTTGATCTGGCTCAG-3', 1541R: 5'-AAGGAGGTGATCCACCC-3' were Provided by the Shanghai Biological Engineering Co.LTD (Shanghai, China) ; high purity nitrogen, air and hydrogen gas purchased from Luzhou TianYI Gas Limited (Luzhou, China) .

### **Methods**

#### **Preliminary screening**

Weighed 1g pit mud, added into flask containing 100mL sterile water, 180r/min oscillated for 30 min, bathed at 85°C in water pot for 10min, drew 1mL dilution added into anaerobic bottle containing 20mL enriched medium in sterile conditions, cultured for 7d in the vacuum incubator under 35 °C, 0.06MP.

#### **Isolation and Purification**

The methods of isolation and purification for caproic acid-producing bacteria referred to Xue, Z. K (Xue, Z. K, 2014) .

#### **Pretreatment of the fermentation broth**

Drew fermentation broth 460μL and added into 2% standard substance solution of ethyl butyrate, ethyl caproate, butyric acid and caproic acid 10μL respectively, Ultrasonic treatment 10 minutes, adjusted PH to 2.0, centrifuged 10min at 1000g, drew the supernatant 200μL for gas chromatographic analysis.

**Gas Chromatographic Analysis of Fermentation broth**

Gas Chromatographic Column: AT.LZP-930 Dedicated Chromatographic Column for Liquor (25m × 0.32mm, 0.25μm)

Chromatographic conditions: Hydrogen flame ionization detector; The elevated temperature program for GC column: The initial temperature of 65°C, Maintained 5min, Then elevated temperature at a rate of 3.5°C/min, elevated temperature to 150°C and maintained 0min, the injector temperature was 200°C, and the detector temperature of 200°C, The carrier gas (High-purity nitrogen) flow rate: 1.0mL/min, split ratio of 37: 1, the injection volume was 1μL.

Calculation of product concentration in fermentation broth: The method provided by GB/T10345-2007 was adopted to analyse the fermentation products concentration, each sample was repeated for 3 times, and the concentration of the sample was the average value of the three times.

**Bacteria count of the fermentation broth**

Take 15-day fermentation broth, counting the bacterial number of the liquor under oil microscope for 100 squares, the mean of per square was obtained by the total number of the 100-square divided 100, the number of bacteria per cubic millimeter was calculated by the mean of 1 square divided 1/4000, each sample was repeated for 3 times.

**Identification of the caproic acid bacteria**

In present study, the caproic acid- producing bacteria were taxonomically identified by the indices of morphology, physiology and biochemistry, and 16SrRNA gene sequence alignment.

**Morphological Observation**

The cell and colony characteristics of strain K-2, which cultured in the clostridium strengthened medium for 3 days, were observed under the 100-fold oil microscope.

**Physiological and Biochemical Identification**

The main indicators of physiological and biochemical identification of bacteria, referring to "Bergey's Manual of Systematic Bacteriology: Volume 3: The Firmicutes" (VosP *et al*, 2009).

**Identification of Molecular Biology**

Extraction of total DNA of strain K-2: Picked up a single colony, which was identified to be high efficient production of caproic acid by GC, and cultured in a vacuum incubator at 34 °C for 7 days, and collected the bacteria from the fermentation broth, and extracted the total DNA by rapid DNA extraction and amplification Kit (KG201) in the instruction of the manual provided by the Manufacturer.

PCR amplification and sequencing: the template of 16sRNA gene PCR amplification was the total DNA from the high efficient fermentation strains, and the primers showed as follows: 27F: 5'-AGAGTTTGATCCTGGCTCAG-3', 1541R: 5'-AAGGAGGTGATCCACCC-3', the optional system of PCR amplification was a reaction mixture of 10ng DNA template, 1μL 1×10Taq reaction buffer, 4pmol forward primer and reverse primer respectively, 4μmol dNTP, 0.5units of Taq polymerase, the total volume of 10μL. The optimized reaction program was initially at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, at 52°C for 1 min, at 72 °C for 30 second, and then held at 72 °C for 10 min, and finally kept at 4 °C. PCR product was subjected to agarose gel electrophoresis (1%) to confirm the amplified 16sRNA gene, then the PCR products were purified using the Universal DNA purification kit (Tiangen Biotech) following the manufacturer's protocol, and the purified PCR products were directly sequenced by Sangon Biotech (Shanghai) Co., Ltd (Shanghai, China).

Sequencing analysis and constructing molecular phylogenetic tree: The sequencing results were corrected artificially with reference to the positive and negative sequencing map by Chromas software, the similarities of neighboring species were obtained by blasting the sequencing results in the NCBI database bank by BLAT tool in NCBI. Clustalw was used to align 16S rRNA gene sequence of the strain of K-2 with neighboring species, and a phylogenetic tree was constructed by using the neighbor joining and maximum likelihood methods (a 1,000 iterations; at least 80% bootstrap support) in MEGA5.0.

**Data Processing**

The test data was processed by excel and SPSS17 soft package

## RESULTS AND ANALYSIS

## Gas chromatographic analysis

The fermentation broth of single colonies of K-2 for 15-day fermented was detected by gas chromatography under the chromatographic conditions of item 1.2.5 in this study, and the results were shown in Figure 1.

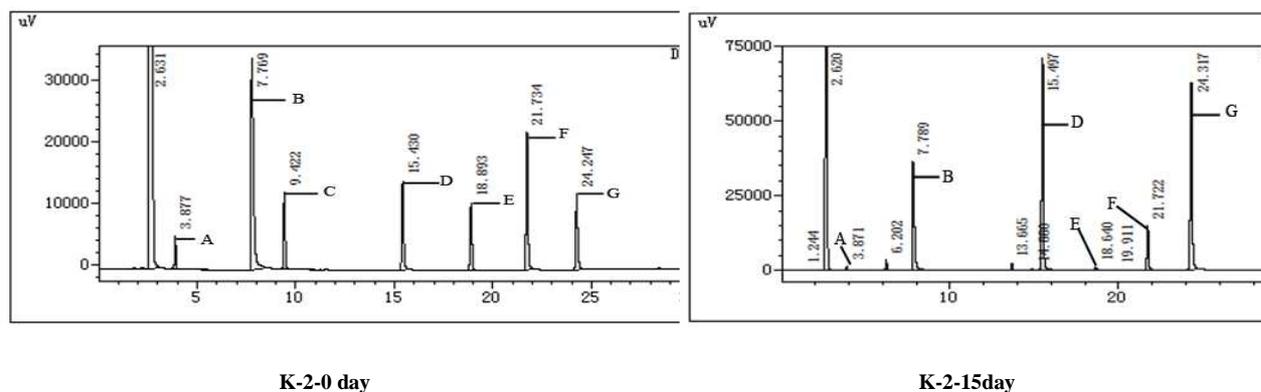


Fig 1 the GC-Analysis of fermentation broth of K-2(A, B, C, D, E, F, and G stand for the Internal standard substance amyl acetate, acetic acid, Ethyl butyrate, butyric acid, ethyl caproate, Internal standard substance 2-ethyl butyrate, and caproic acid respectively )

the results in figure 1 demonstrated that whether the 0-day culture or the 15- day fermentation broth existed no substances to interfere with the detection of internal standard substances by gas chromatography, the retention time of each detection components are: amyl acetate (A) is about 3.7min, so is acetic acid (B) about 7.7min, ethyl butyrate (C) is about 9.4min, butyric acid (D) is about 15.4min, ethyl caproate (E) is about 24.7min, 2-ethyl butyrate (F) is about 21.7min, caproic acid (G) is about 24.7min.

Metabolite concentrations were calculated from the peak area of metabolites through the formula provided by GB/T 10345-2007, the results showed in tab 1.

Table 1 the results of Metabolites of K-2 strain tested by gas chromatography

products fermenting time	Butyric acid (mg/100mL)			Caproic acid (mg/100mL)			Ethyl butyrate (mg/100mL)			Ethyl caproate (mg/100mL)		
	Detection value	Mean±sd	P	Detection value	Mean±sd	P	Detection value	Mean±sd	P	Detection value	Mean±sd	P
15-day fermentation broth	339.33 392.573 415.48	382.45 ±39.07	P    0.004	516.53 558.93 566.33	547.26 ±26.87	P    0.001	3.26 4.57 0	2.61 ±2.35	P    0.20	32.07 27.38 0	19.82 ±17.32	P    0.19
0-day fermentation broth	10.45 8.45 8.74	7.88 ±2.23	< 0.01	14.95 10.52 11.15	12.54 ±2.1	< 0.01	0 0 0	0±0	> 0.05	0 0.31 0	0.10 ±0.18	> 0.05

The data of metabolites concentration of K-2, which fermented 0 and 15 days, was compared by paired-samples Test in spss software package, the results were showed as follows:

1.The metabolite concentration of ethyl butyrate and ethyl caproate of strain K-2 had no significant difference ( $P = 0.2, 0.19 > 0.05$ ) between fermenting 0 days and 15 days, the results demonstrated that K-2 strain are capable of producing none of the two esters, the two esters in the fermentation broth may be regarded as the natural products of the interaction of ethanol with butyrate acid and caproic acid respectively.

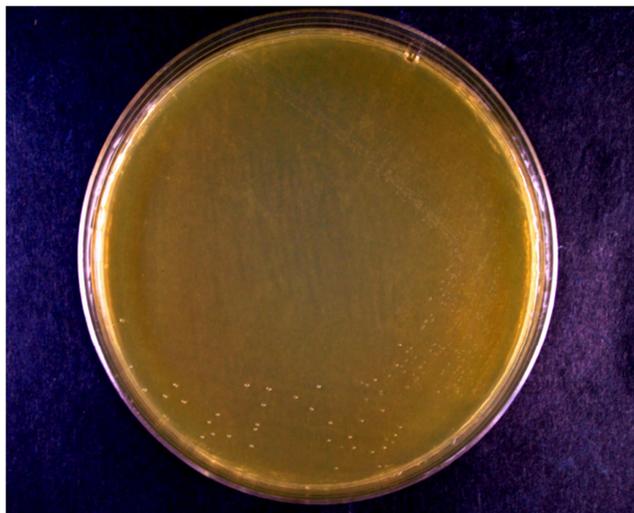
2. the concentration of butyric acid of strain K-2 was significantly different ( $P = 0.004 < 0.01$ ) between 0 days and 15 days in the fermentation broth, so was the caproic acid concentration. All the results demonstrated that the K-2 strain can produce butyrate and caproic acid efficiently.

3. the comparison of the concentrations of butyric acid with caproic acid on 0-day and 15-day fermentation broth. There was no significant difference between the 0-day concentrations of butyric acid and caproic acid in fermentation broth ( $P = 0.059 > 0.05$ ), however there were extremely significant difference between the 15-day concentrations of butyric acid and caproic acid in fermentation broth ( $P = 0.002 < 0.01$ ). The results showed that the K-2 strain produces much more caproic acid than that of butyric acid for 15- day fermentation broth.

In summary, the analytical results of metabolites in fermentation broth in this study indicates that the K-2 strain has high efficient production of butyric acid and caproic acid capacity, and the yielding capacity of caproic acid is extremely higher than that of butyric acid, but the strain hardly produced ethyl hexanoate and ethyl butyrate.

### Morphological observation

Observing the colony Morphology of strain K-2 cultured on clostridium strengthened medium at 37 °C for 3 days, the strain K-2 was rod-shaped bacteria, Gram-positive,  $0.6 \times 2.54.5\mu\text{m}$ , single or chain arrangement, and its colonies were white, nearly transparent, droplet-shaped, round, shiny.



Colony morphology



Cell body morphology

Fig2 the clone and cell body morphology of strain k-2

### Physiological and biochemical identification

Physiological and biochemical identification were conducted by the API 50 CHB test kit (Bio- Merieux, Marcy l'Etoile, France), the results were shown in tab 2.

Analyzing data in Table 2, Conclusion can be reached that K-2 strain was consistent with Clostridium (Clostridium) in C source assimilation and hydrolase activity, K-2 strain should belong to the genus Clostridium on the genus level identification.

Tab2. the results of physiological and biochemical identification of strain K-2

substrate	result	substrate	result	Substrate	result	substrate	result
D- glucose	+	Mannitol	+	D- lactose	-	D- sucrose	+
D- maltose	+	Arginine double hydrolase	-	D- xylose	+	D- arabinose	+
Glycerin	+	D- cellobiose Disaccharide	-	D- mannose	+	Caproate-producing	+
D- raffinose	+	Sorbitol	-	L- rhamnose	-	d- trehalose	+
Indole test	-	Urease	+	Gelatin test	+	Esculin hydrolysis	+
D- Ribose	+	Inositol	-	Glycogen	-	D- arabitol	-
$\beta$ -methyl-D-xylosidic	-	Inulin	+	D- lyxose	-	2,5 diketo -D -gluconate	-
Nitrate reduction	-	D-sorbitol	-	N-acetylglucosamine	-	Salicin	-
melibiose	-	D- melizitose	-	Xylitol	-	D- tagatose	-
L-arabitol	-	Laetrile	-	D-gentibiose	-	D- fucose	-
potassium gluconate	-	L- arabinose	+	Adon alcohol	-	D-fructose	+
Dulcitol	-	$\alpha$ - methyl -D- mannoside	-	Arbutin	+	starch	-
D-Toulon sugar	-	L- fucose	-	D-galactosidase	-	Butyric acid producing	+

### Identification and phylogenetic analysis of 16SrDNA sequence

The 16SrDNA was amplified from total DNA of strain K-2, while the amplified products was sequenced after purified, the result was shown in figure3.

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AGGAAGTTTTCGGACGGAATTGAAATTGACTGAGCGGCGGGACGGGTGAGTAACCGGTGGGTAAACC
TGCCTCATACAGGGGGATAACAGTTGGAAACCGACTGCTAATACCSCATAAAGCACACAGTSCCGCA
TGGTACGGTGTGAAAAACTCCGGTGGTATGAGATGGACCCGCTCTGATTAGGTAGTGGTGGG
TAACGGCCACCAAGCCGACGATCAGTAGCCGACTGAGAGGGTGACCGGCCACATTGGGACTGA
GACACGGCCCAAACCTCTACGGGAGGCAGCAGTGGGGAAATATGGACAATGGGGGAAACCCTGAT
CCAGCGACGCCCGTGGTGAAGAAGTATTCGGTATGTAAGCTCTATCAGCAGGGGAAGAAAAT
GACGGTACTGACTAAGAAGCCCGCTAATACGTGCCAGCAGCCGCGGTAATACGTAGGGGGC
AAGCGTTATCCGGATTTACTGGGTGTAAAGGGAGCGTAGACGGCACTGCAAGTCTGGAGTGAAG
CCCGGGGCTCAACCCCGGGACTGCTTGGAACTGTGGTGTAGAGTGCAGGAGAGGTAAGTGGGA
ATTCTAGTGTAGCGGTGAAATGCGTAGATATAGGAGGAACACCAGTGGCGAAGCCGGCTTACT
GGACTGTAACGTGACGTTGAGGCTCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTC
CACGCCGTAAACGATGAATACTAGGTGTTGGGGAGCAAAGCTCTTCGGTGC CGCCGCTAACGCCAA
TAAGTATTCCACTGGGGAGTACGTTCGCAAGAATGAAACTCAAAGGAATTGACGGGGACCCGCA
CAAGCGGTGGAGCATGTGGTTAATTCGAAGCAACCGAAGAACCCTTACCAAGTCTTGACATCGG
AATGACCGGGATGTAACGATCCCTTCCCTTCGGGGCATTCAGACAGGTGGTGCATGTTGTCTGT
CAGCTCGTGTCTGAGATGTTGGGTAAAGTCCCGCAACGAGCGCAACCCCTTATCCTTAGTAGCCA
GCAAGTCAAGTTGGGCACTCTGGGGAGACTGCCAGGGATAACCTGGAGGAAGGTGGGGATGACGT
CAAATCATCATGCCCTTATGATTTGGGCTACACACGTGCTACAATGGCGTAAACAAAGGGAAAGC
AAAGGAGCGATCTGGAGCAAACCCAAAATAACGTCTCAGTTCGGATTGTAGTCTGCAACTCGA
CTACATGAAGCTGGAATCGCTAGTAATCGCGGATCAGAATGCCGCGGTGAATACGTTCCCGGGTCT
TTGTACACACCGCCCGTCAACCATGGGAGTTGGTAACGCCCGAAG-

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Fig3 the sequencing result of 16SrDNA of strain K-2

On the basis of the distance matrix, the phylogenetic tree on the 16SrDNA sequence of strain K-2 and related species was constructed by neighbor-joining methods using MEGA software version 5. The bootstrap was performed with 1000 replicates, and the phylogenetic tree was shown in figure 4.

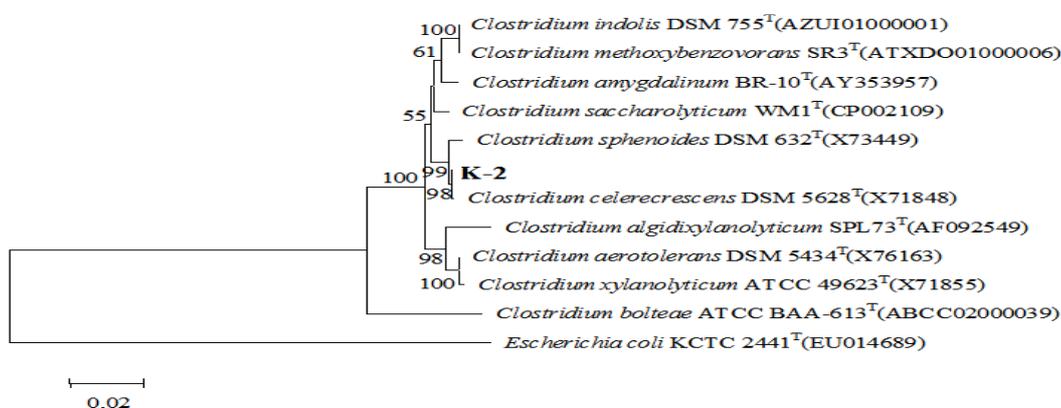


Fig4 the phylogenetic tree of 16SrDNA sequence of K-2 strain and its neighboring species (phylogenetic tree node displays only numerical Bootstrap values greater than 50%, on the subject of "T" indicates the type strain)

It can infer from figure 4 that strain K-2 and *Clostridium celerecrescens* DSM 5628T located on the same branch inferred from Fig. 4, Bootstrap support value was 98%, all the results demonstrated that strain K-2 was *Clostridium celerecrescens*.

## DISCUSSION

The caproic acid bacteria or caproic acid bacteria is a class of microorganisms which produced secondary metabolites-caproic acid that can react with ethanol to generate ethyl caproate (the main aroma substances in strong-flavor liquor) in the process of strong-flavor liquor making. The type strain was *Clostridium Kluyveri* (strain ATCC 8527 / DSM 555 / NCIMB 10680), the strain was isolated from Sludge for the first time in 1936 by H.A. BARKER, and nominated *Clostridium Kuyveri* (Barker H A et al, 1942; George M. Garrity et al, 2005), The strain belongs to Phylum Firmicutes, Bacilli, order Bacillales, Bacillaceae, *Clostridium* in the taxonomy. At present, strains producing caproic acid like CICC8022 also include species, such as *Clostridium scatologenes*, *Clostridium* sp. BS-1 in *Clostridium*, as well as other species, like *Rhodospirillum rubrum*, *Eubacterium limosum*, *Megasphaera elsdenii*, *Bacillus Megaterium*, *Bacillus fusiformis* etc (Holdeman LV et al, 1977; Kohlmiller Jr E F et al, 1951; Genthner B R et al, 1981, Marounek M et al, 1989; Jeon B S et al, 2010; ZHAO Hui et al, 2012).

In order to improve the quality of strong-flavor liquor, since the 1960s of the last century, more and more caproic acid bacteria were isolated from mud of old cellar pit, currently, the hexoic acid strains preserved in China Center of Industrial Culture Collection (Abbreviated as CICC) has reached more than 30 strains, in which CICC8022 (Inner Mongolia # 30) is the most fully studied one in physiology, fermentation conditions and acid-producing capacity, but it needs further studies in the taxonomy (SHEN Yi Fang, 1998; ] Zhou Henggang, 1997; WU Yan-yong, 2007)

In this study, the hexoic bacterium isolated and identified from the mud of 180-year old cellar pit in Wan Bin Distillery Co., Ltd of Luzhou Laojiao Group was consistent with *Clostridium kluyveri* in characteristics of

morphology and physiology,(Barker H A et al,1942)Comparison of caproic acid producing ability of strain K-2 to the other strains were listed in Table 3.

Tab 3 the comparison of capacity of producing caproic acid in several strains

metabolites strain	Caproic acid (mg/100mL)	Butyric acid (mg/100mL)	documents
Clostridium Lushun	308.4	/	(WU Yan-yong,2007)
SJ - 20	438	/	(ZHANG Bin et al , 2014)
4 #	297.6	/	(MIAO Zijian,2014)
<i>Clostridium</i> sp. BS-1	299		(Jeon B S et al, 2010)
C78			
( <i>Bcillus fusiformis</i> )	213.69	30.37	(ZHAO Hui et al, 2012)
<i>Megasphaera elsdenii</i>			
NCIMB 702410	469	/	(Choi K,et al,2013)
<i>C.kluyveri</i> 3231B	1280	/	(Weimer P J et al,2012)
Inner Mongolia 30 #	700	/	(Luo Wei et al,2002)
k-2			
( <i>Clostridium celerecrescens</i> )	547.26	382.45	This study

The data in table 3 demonstrated that the caproic acid producing capacity of strain k-2 is lower than those of *C.kluyveri* 3231B(Weimer P J et al,2012), Inner Mongolia 30 # (Luo Wei et al,2002), but is apparently greater than those of *Clostridium* Lushun(WU Yan-yong,2007), SJ - 20(ZHANG Bin et al , 2014), 4 # (MIAO Zijian,2014) , *Clostridium* sp. BS-1 (Jeon B S et al, 2010) , C78 (Jeon B S et al, 2010) ,and NCIMB 702410(Choi K,et al,2013). So the strain k-2 isolated and identified in this study is a high yielding caproic acid.

In order to obtain the taxonomic characteristics of K-2 strain, the multiphasically taxonomic technology, which combines morphological, physiological, biochemical and molecular biology, was adopted , the results showed that strain K-2 belongs to Phylum BXIII Firmicutes, classI clostridia, OrderI clostridiales, FamilyI clostidiaceae, *Clostridium*, species *Clostridium celerecrescens*.

*Clostridium* (GenusI *Clostridium*) are a class of rot /anaerobic, rod-shaped form,and endospores of Gram-positive bacteria, mainly distributing in soil, water, sediments, anaerobic body or deepened tissue,and rich in organic matter environment; its vegetative and secretion of extracellular enzymes have been broadly used to degrade substrates, including agricultural or municipal waste, natural and man-made organic poisons, such as simple or complex carbon-containing compound, and no tolerance phenomenon of degradation of the substrate or products inhibition, more over it has a variety of metabolic pathways to produce extremely diverse of metabolites, such as butyric acid, acetic acid, lactic acid, caproic acid, butanol, acetone, ethanol, gaseous matter (CO<sub>2</sub>products such as H<sub>2</sub>), malodorous compounds (such as cresol), isobutyric acid, indole, indole acid, phenyl acetate, valeric acid, isovaleric acid and antibiotics Closthionide,etc. Therefore, *clostridium* is a class of microorganisms which have great value to environment protection, agricultural production and bio-engineering applications (Tracy BP et al,2012), there are 220 species in *Clostridium* at present, the most famous strains of which *Clostridium* are *Clostridium kluyveri* and *Clostridium butyricum* (Barker H A et al, 1942; George M.Garrity et al, 2005; Holdeman LV et al, 1977) , the caproic acid bacteria isolated from pit mud of old cellar pit were almost all identified as *Clostridium kluyveri* in last century, but other caproic acid-producing strain including *Clostridium scatologenes*, *Clostridium* sp. BS-1,*Clostridium difficile*, *Clostridium scatologenes*, *Clostridium butyricum*, etc had constantly been discovered (Madan E et al, 1988; Kridelbaugh D et al, 2009; Butel MJ et al, 1995) . The strainK-2isolated and identified in present study is strictly anaerobic, rod-shaped Gram-positive bacteria, the type strain of *Clostridium celerecrescens* was isolated and denominated from cellulose-rich medium for producing Methane in1989 for the first time by Palop, M.Land collaborator, the strain was widely used to produce hydrogen ethanol, organic acid derivatives ,electric fuel, Cinnamic acid derivatives used as agents from biomass in fermentation industry(Palop, M.L et al, 1989; Bouvet P et al, 2012; Honkalas V S et al, 2015; Mischnik A et al,2011; Monroy O A et al, 2011).

## CONCLUSION

In conclusion, *Clostridium celerecrescens*, which was identified to have the capacity of highly efficient production of caproic acid (547.26mg / 100mL) and butyric acid (382.45 mg / 100mL), was isolated and identified from old cellar pit mud for the first time at home and abroad by using the combination of morphological,

physiological, biochemical methods and 16sRNA gene sequence analysis, The study provided a theoretical and practical basis for the application of the strain in liquor production and further scientific researches.

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