

# Isolation, Purification and Identification of a Novel HX-Producing Strain and Optimization of its Fermentation Medium

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## Abstract

Hexanoic acid (HX) is a crucial flavor compound and precursor of ethyl caproate (EA), which determines the quality of Chinese Luzhou-flavor liquor (CLFL). The isolation, purification, identification, and optimization of fermentation conditions of HX-producing bacteria are essential for industrial CLFL production. In this study, one strain of HX-producing bacterium was isolated from six candidate bacterial strains and identified as *Clostridium sartagoneforme*. Then, the growth characteristics and HX production of *Clostridium sartagoneforme* were investigated. Sodium acetate medium was identified as the optimal fermentation medium from four candidate media. *Clostridium sartagoneforme* yielded  $800.85 \pm 12.87$  mg/100 mL HX in sodium acetate medium. Then, to further optimize the formula of the fermentation medium, the carbon and nitrogen sources and inorganic salt component of the fermentation medium were investigated using HX yields as an optimization index. Optimization was performed with a single-factor experiment and the Taguchi design method. The single-factor experiment showed that the highest HX outputs were obtained when the sodium acetate medium contained 2.5 g/L yeast extract, 1.8 g/L KCl, 20 g/L sodium acetate, 15 mL/L ethanol, and 1.5 g/L glucose. In the orthogonal experiment designed using the Taguchi design method, HX yields reached  $2018.29 \pm 46.37$  mg/100 mL in sodium acetate medium that contained 3.5 g/L yeast extract, 1.8 g/L KCl, 25 g/L sodium acetate, and 15 mL/L ethanol.

**Keywords:** Isolation; Identification; Hexanoic acid-producing strain; Fermentation medium; Optimization

## Introduction

Hexanoic acid (HX) is the precursor of ethyl caproate (EA), the main flavor component in Chinese Luzhou-flavor liquor (CLFL) according to the National Standard GB/T 10781.1-2006. EA is produced during the late fermentation stage of CLFL production in a mud pit, a fermentation container, by spirit reaction with HX. EA yields are determined by HX quantity from excessive spirits in lees, or fermented grains. The improvement of HX yields in CLFL production is a crucial technical problem for CLFL industries. Numerous methods have been developed to increase HX output in traditional liquor production [1].

One of the simplest and most widely adopted methods for improving CLFL quality is prolonged fermentation time. The fermentation time for regular liquor production is 45 days from lees entry to lees exit. To improve HX yield, the fermentation time for a pit in good fermentation condition is extended to 60, 90, or even 180 days. The relative HX amount, EA content, and CLFL quality increase as fermentation time increases. Another widely adopted method for improving CLFL production is multiple fermentation rounds (double fermentation). In this method, lees are retained in the bottom of the fermentation pit and are mixed with new fermentation starter powders (e.g., daqu in Chinese) for another one or two fermentation periods. After two or three fermentation periods, the amounts of HX and other organic acids increase and the microbial biochemical reaction of spirits with HX or other organic acids is enhanced, thus increasing total acid and ester contents and improving liquor flavor. The traditional methods to improve CLFL quality, however, decrease production efficiency and increase production cost [2].

For several centuries, the attempts of CLFL makers to economically improve liquor quality have been unsuccessful. A breakthrough in CLFL production finally occurred in the 1960s when EA was identified as the main flavor component of CLFL. The isolation, purification, and identification of HX-producing bacteria and the optimization of fermentation conditions are now routine work for the CLFL industry and researchers [3].

In the past decades, several HX-producing microbes have been isolated and identified from CLFL pit mud. The HX production of these bacterial strains outside of fermentation pits, however, are limited at 700 mg/mL HX [4].

In this study, we aimed to isolate and identify highly effective HX-producing bacteria. We also aimed to optimize fermentation conditions for the application of HX-producing bacteria in practical production.

## Materials and Methods

### Materials

**Pit mud:** The pit mud used in the present study was collected from the four corners and center of the bottom of a 180-year-old pit in Luzhou Laojiao Group Luzhou Laojiao Group Co. Ltd, a famous CLFL producer in Southern Sichuan, PRC.

**Reagent:** Yeast extracts, KCl, glucose, sodium acetate, and absolute ethanol were purchased from Kelong Reagent Factory (China, Chengdu). The standard material 12-ethyl butyrate was purchased from Aladdin Reagent Co., Ltd (China, Shanghai).

### Culture medium

**Isolation and enrichment culture medium:** The culture medium contained the following components: 5g sodium acetate; 1g yeast extracts; 10 mL 4% potassium dihydrogen phosphate; 5

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mL 2% magnesium sulfate; 20 mL 0.5% calcium sulfate; 10 mL 5% ammonium sulfate; and 1 mL 5 mg/L biotin. The culture medium was brought to 1000 mL with tap water and its pH was adjusted to 6.0–6.5. Then, 2% agar was added to solidify the medium before sterilization at 121°C for 30 min in an autoclave. Prior to inoculation, 20 mL absolute ethanol was added to the medium.

**Fermentation medium:** Four fermentation media were selected for screening HX-producing bacteria.

## Methods

**Isolation of HX-producing bacteria from pit mud:** ⇒ Pit mud ⇒ Preparation of seed solution ⇒ Enrichment culture Plate separation ⇒ Slope inoculation ⇒ Primary screening ⇒ Rescreening Gas ⇒ chromatography (GC) analysis ⇒ Strain preservation.

**Preparation of seed solution:** A total of 1 g pit mud was added to 9 mL liquid isolation medium in a stoppered test tube. The tube was then placed in a 90°C water bath for 10 min. After cooling, 150 µL absolute ethanol was added to the tube. The seed solution was then cultured at 34°C and 0.08 MPa in a vacuum incubator until gas was produced.

**Enrichment culture:** A total of 1 mL HX-producing seed solution was pipetted and added to 9 mL liquid isolation culture medium in a stoppered tube. The tube was then placed in a 90°C water bath for 10 min. After cooling to 70°C, 150 µL absolute ethanol was added to the enrichment culture. The culture was then incubated under the above-mentioned conditions for 15 days.

**Plate separation:** The tube of enrichment culture that produced the highest amount of gas was selected. A total of 10 µL enriched culture medium was drawn from the selected tube, spread on solid culture medium, and incubated at 90°C in an incubator for 10 min. Subsequently, 2% (V/V) absolute ethanol was added to the incubated culture medium until the culture medium cooled down to 70°C. The inoculated medium was incubated under the above conditions until the colonies of HX-producing bacteria appeared [5-9].

**Slant culture:** Colonies that possessed a regular morphology were selected and transferred to the surfaces of slant media and cultured in the above-mentioned conditions until the colonies of HX-producing bacteria appeared.

**Preliminary screening:** Colonies with entire edges and slightly raised and smoothly rounded tops were selected. A loopful of bacteria was picked up, placed in a drop of water on a slide, and stained by Gram staining. The stained bacteria were observed under microscopy. Motile, Gram-positive, rod-shaped bacterial cells with ellipsoid spores were selected for future screening.

**Rescreening:** Individual colonies from six selected clones were picked and inoculated into 5 mL isolation culture medium under the above conditions for 6 days. The bacteria were then transferred to 50 mL of fermentation culture medium under the above conditions for 15 days. GC was used to investigate 15-day-old fermentation broths to select the most efficient strain of HX-producing bacteria based on HX yields.

**Pretreatment of fermented broth:** The pH of 1 mL fermented esterifying liquid was adjusted to pH 3 with 1 M hydrochloric acid solution. The amount of hydrochloric acid solution required to adjust the solution pH to 3 was recorded.

A total of 10 µL 2-ethyl butyrate, the internal standard solution, was added to 490 µL fermented esterifying liquid. The mixture was

vortexed vigorously. Then, the mixture was acidified to pH 3 with 1 M hydrochloric acid solution at half the volume of the 1 mL esterifying liquid. The acidified mixture was vortexed vigorously and then centrifuged for 10 minutes at 12,000 r/min. The supernatant was drawn and filtered using a 0.22 µm filter and stored at 4°C for future use.

**Conditions of GC analysis:** Chromatographic column: LZP-930 Liquor special analysis column (purchased from the Chinese Academy of Sciences, Lanzhou Institute of Chemical Physics).

Chromatographic conditions:

Detector temperature: 200°C

Vaporizer temperature: 200°C

Injection mode: Split ratio: 50:1

Column length: 25.0 mm

Inner diameter: 0.32 mm

Coating thickness: 1 µm

Programmed temperature: 65°C ⇒ 5 min ⇒ 3.5°C/min ⇒ 145°C

Duration of Analysis: 28 min.

## Identification

**Molecular identification:** Molecular biological identification was conducted based on the study of Xue [4].

**Characteristics of growth and HX-production:** Thirty 15 mL anaerobic tubes were filled with 15 mL fermentation culture medium each and inoculated with the seed liquid of HX-producing bacteria at 10% proportion. The inoculated media were cultured in a vacuum incubator at 34°C and 0.08 MPa. Three anaerobic tubes were selected every 2 days for bacterial counts and HX analysis.

**Strain preservation screening fermentation culture media for high yields of HX:** Four fermentation culture media, as described by Xue [4] were screened for high HX output.

## Optimization of fermentation culture media

**Screening of carbon source, nitrogen source, and inorganic salt:** A single-factor experiment was used to change only one nutrient for the fermentation culture medium, e.g., the sodium acetate medium mentioned in section 1.2.3 of the present paper. The amounts of carbon sources (glucose, starch, lactose, and sucrose), nitrogen sources (yeast extract, beef extract, peptone, ammonium chloride, ammonium sulfate, and urea), and inorganic salts (KCl, K<sub>2</sub>HPO<sub>4</sub>, KH<sub>2</sub>PO<sub>4</sub>, MgSO<sub>4</sub>, MnSO<sub>4</sub>, CaCO<sub>3</sub>) were the same as those in the screened fermentation medium.

**Single-factor experiment:** A single-factor experiment was performed to determine the dosage range of the main components of fermentation culture medium A e.g., glucose, yeast extracts (used instead of beef extract due to cost), KCl, sodium acetate, and alcohol. The experimental concentration gradient was composed of two to three concentration levels that increased or decreased the concentrations of the components of the sodium acetate culture medium.

**Optimization of fermentation culture medium by Taguchi design method:** Based on the single-factor experiment, four ingredients yeast extract, sodium acetate, ethanol and KCl of the fermentation culture medium at three levels (Table 1) were selected for further optimization. A scheme for the orthogonal test (Table 2), which includes three factors and three levels, was designed using the Taguchi design method in Minitab 17.

Level	Factor			
	Yeast extract	KCl	Sodium acetate	Ethanol
1	1.5	1.2	15	10
2	2.5	1.8	20	15
3	3.5	2.4	25	20

Table 1: Factors and levels.

Group	Yeast extract	KCl	Sodium acetate	Ethanol
		g/L		mL/L
1	1.5	1.2	15	10
2	1.5	1.8	20	15
3	1.5	2.4	25	20
4	2.5	1.2	20	20
5	2.5	1.8	25	10
6	2.5	2.4	15	15
7	3.5	1.2	25	15
8	3.5	1.8	15	20
9	3.5	2.4	20	10

Table 2: Orthogonal test for HX yields.

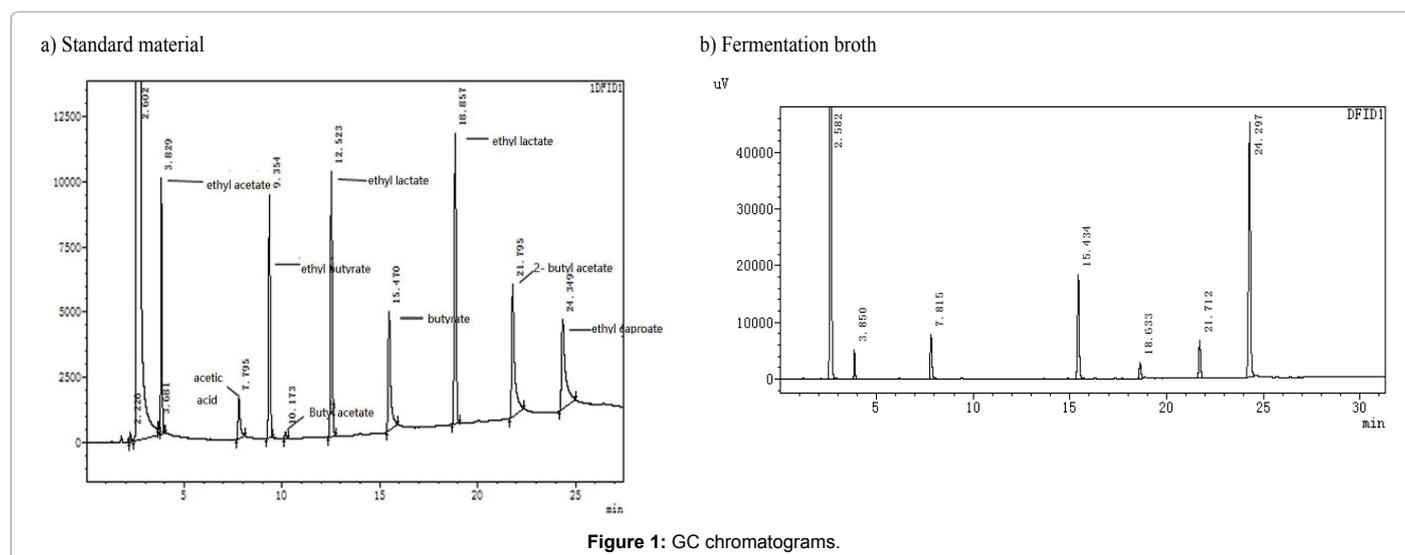


Figure 1: GC chromatograms.

The total cell number, which is represented by the optical density (OD) of 10 day fermentation broth, was detected by a spectrophotometer. The caproic acid content of the 15 day fermentation broth was detected using GC.

#### Validation experiments

Validation experiments were performed based on the optimal formula in section 1.2.4.3.

**Data processing:** Data were processed using Excel 2007 (Microsoft Excel, USA), Minitab 17 (PA, USA), and SPSS19 (Chicago, USA).

## Results and Discussion

### GC analysis of fermented broth

The HX chromatograms of the standard material and fermentation broth.

The chromatographic peak in Figure 1 demonstrated that the GC conditions in the present study were suitable for HX detection.

The HX yields of six clones in 15 day fermented broth are shown

in Table 3. The HX yields of six clones were compared with one-way ANOVA in SPSS 17. The results indicated that the HX yields of clone 5 in 15-day fermented broth was significantly higher than those of clones 1, 2, 3, 4, and 6 ( $\text{Sig}_{\text{low-tailed}}=0,0,0,0<0.01$ ). Therefore, clone 5 was selected for investigation.

### Morphological characterization of clone 5

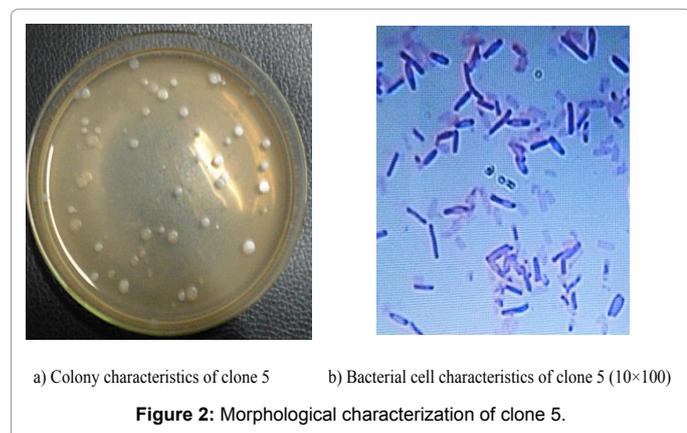
The results for the morphological characterization of clone 5 are (Figure 2).

Clone 5 possesses the following characteristics:

1. Colony characteristics: round or roughly round; white or yellowish; opaque or translucent; relatively uniform edges; smooth surface; colony diameter 1.5-2 mm;
2. Bacterial cell morphology: long rods;
3. Spore morphology: ellipsoidal;
4. Spore-bearing position: mostly located in the center of the cell with a small number of cells at one end;

Clones	1		2		3		4		5		6	
HX yields (mg/100 mL)	189.56	198.99 ± 11	234.76	263.1 ± 26.29	206.33	260.3 ± 47.52	365.89	383.64 ± 16.4	585.78	609.22 ± 20.46	167.34	174.43 ± 7.11
	196.35		286.69		295.89		386.78		623.45		181.55	
	211.07		267.84		278.67		398.24		618.44		174.39	

Table 3: HX yields of six clones.



5. Gram staining: G<sup>+</sup>.

### Molecular identification

The gel electrophoresis results of 16SrDNA amplified by PCR were listed in Figure 3, the sequencing results for 16SrDNA fragments is listed in Figure 4 and the evolutionary tree constructed by Mega 7 was shown in Figure 4.

The sequence of the 16srDNA primer amplification product in Figure 3 was 1427-bp long, BLAST results in NCBI ([https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE\\_TYPE=BlastSearch&LINK\\_LOC=blasthome](https://blast.ncbi.nlm.nih.gov/Blast.cgi?PROGRAM=blastn&PAGE_TYPE=BlastSearch&LINK_LOC=blasthome)) revealed that clone 5 had a 100% sequence similarity to uncultured bacterium *claab17d12*, uncultured bacterium clone *aaa62b11* ≥ 99.5% to *Clostridium sartagoneforme*. A phylogenetic tree was constructed by selecting 100 strains of uncultured and cultural bacterial strains with similarity ≥ 90% and the sequence of each strain to be tested. The strain of clone 5 was located in the same branch with the uncultured bacterium *claab17d12* the similarity was 100% (Figure 4). According to the phylogenetic tree similarity combined with morphological characteristics, cultural characteristics, the strain was identified as *Clostridium sartagoneforme*, which is a cultural bacterium.

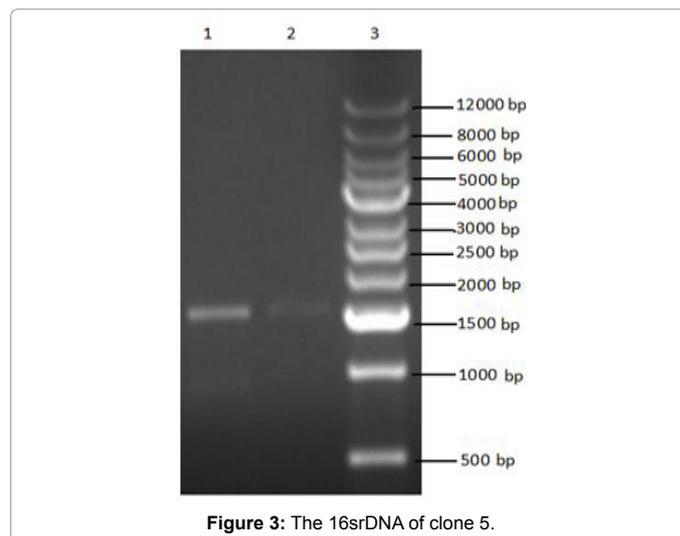
This is the first study to discover that *C. sartagoneforme* produces HX in CSFL pit mud.

### Growth curve and HX-production

The results for HX-producing bacterial counts and HX contents are shown in Figure 5.

By analyzing the curves in Figure 5, the following conclusions were drawn:

1. Based on the bacterial growth curve, the initial lag period lasted for 4 days. The total number of bacterial cells (TNBC) negligibly increased during this period. The logarithmic growth phase occurred during the sixth to eighth days of fermentation. The TNBC increased the fastest during this period. The stationary phase occurred on the eighth to tenth days of fermentation. The TNBC negligibly increased during this period. Bacterial decay occurred from the 10th day to the end of fermentation. The highest TNBC was  $53.72 \pm 1.32 (\times 10^7 \text{ cfu/mL})$  and was observed on the 10<sup>th</sup> day.



2. Given that the fastest increase in HX production occurred during the eight to the 60<sup>th</sup> day of fermentation, the optimal fermentation period for HX production is 16 days.

### Screening of HX-producing fermentation culture medium

The results of GC analysis for HX yield in the four candidate fermentation culture media are shown in Table 4.

The results in Table 4 revealed that the highest HX yield was in fermentation culture medium A. Further analysis by one-way ANOVA indicated that HX yield in fermentation culture medium A was significantly higher than those in fermentation culture media B, C, and D. Moreover, HX yield in fermentation culture medium A was higher than those in fermentation culture media B, C, and D by 4.07, 3.90, and 2.04 times, respectively.

### Screening for carbon source, nitrogen source, and inorganic salt

HX yields in fermentation liquids with various carbon sources, nitrogen sources, and inorganic salts are shown in Figure 6. The following conclusions were drawn from the screening results for the optimal carbon source, nitrogen source, and inorganic salt for fermentation culture medium A:

1. Among the four carbon sources, the highest HX output 9 (Figure 6A) was achieved with 1 g/L glucose as the carbon source. ANOVA analysis revealed that HX output with glucose as the carbon source was significantly higher ( $P < 0.01$ ) than those with lactose, sucrose, or starch as carbon sources.

2. Among the six nitrogen sources, the highest HX output (Figure 6B) was achieved with 2.5 g/L beef extract as then nitrogen source. ANOVA analysis revealed that HX output in the fermentation liquid with beef extract as the nitrogen source was significantly higher than those with ( $P = 0.048$ ), ammonium sulfate, ammonium chloride, and urea ( $P < 0.01$ ) as nitrogen sources. HX yields with yeast or beef extracts

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TAAACATGCAAGTCGAGCGAGGAGATTCCCTTCGGGGATGAACCTAGCGGCGGACGGGTGAGTAACACGTGGGCAAC
CTGCCATTATAGAGGGGAATAGCCTTCCGAAAGGAAGATTAATACCGCATAACATTACATTTTCGCATGAAGAAGTAATT
AAAGGAGTAATCCGCTATAAGATGGGCCCGCGGCATTAGCTAGTTGGTGAGGTAACGGCTACCAAGGCGACGATG
CGTAGCCGACCTGAGAGGGTGATCGGCCACATTGGGACTGAGACACGGCCAGACTCTACGGGAGGCAGCAGTGG
GGAATATTGCACAATGGGGGAAACCCTGATGCAGCAACGCCGCTGAGTGATGAAGGTCTTCGGATCGTAAAGCTCTG
TCTTCAGGGACGATAATGACGGTACCTGAGGAGGAAGCCACGGCTAACTACGTGCCAGCAGCCGCGTAATACGTAGG
TGGCGAGCGTTGTCGGATTTACTGGGCGTAAAGGGAGCGTAGGCGGATTTTAAAGTGAGATGTGAAATACCCGGGCT
CAACTTGGGTGCTGCATTTCAAACCTGGAAGTCTAGAGTGACAGGAGAGAGTGGAATTCTAGTGTAGCGGTGAAA
TGCGTAGAGATTAGGAAGAACCAGTGGCGAAGGCGACTCTCTGGACTGTAACCTGACGCTGAGGCTCGAAAGCGTG
GGGAGCAAACAGGATTAGATACCTGTAGTCCACGCCGTAACGATGAATACTAGGTGTAGGGGTTGTCATGACCTC
TGTGCCCGCCGAAACGCATTAAGTATTCGCCCTGGGGAGTACGGTCGCAAGATTAAGTAAAGGAAATTGACGGGG
GCCCCACAAGCAGCGGAGCATGTGGTTAATTTGAAGCAACGCGAAGAACCTTACCTAGACTTGACATCTCCTGAAT
TACTCTTAATCGAGGAAGTCCCTTCGGGGACAGGAAGACAGGTGGTGCATGGTTGTCGT-
CAGCTCGTGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCCTATTGTTAGTTGCTACCATTAAGTTGAGC
ACTCTAGCAAGACTGCCCGGTTAACCAGGAGGAAGTGGGGATGACGTCAAATCATCATGCCCTTATGTCTAGGGC
TACACACGTGCTACAATGGCAAGTACAAAAAGATGCAATACCGCAAGGTGGAGCTAAACTTAAAACTTGTCTCAGTT
CGGATTGTAGGCTGAAACTCGCTACATGAAGCTGGAGTTGCTAGTAATCGCGAATCAGAATGTCGCGGTGAATACGTT
CCCCGGCCTTGTACACACCGCCGTCACACCATGAGAGTTGGCAATACCCAAAGTTCGTGAGCTAACCCGTAAGGGA
GGCAGCGACCTAAGGTAGGGTCAGCGATTGGGGTGAAGTCAACA
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Figure 4: 16srDNA gene sequence.

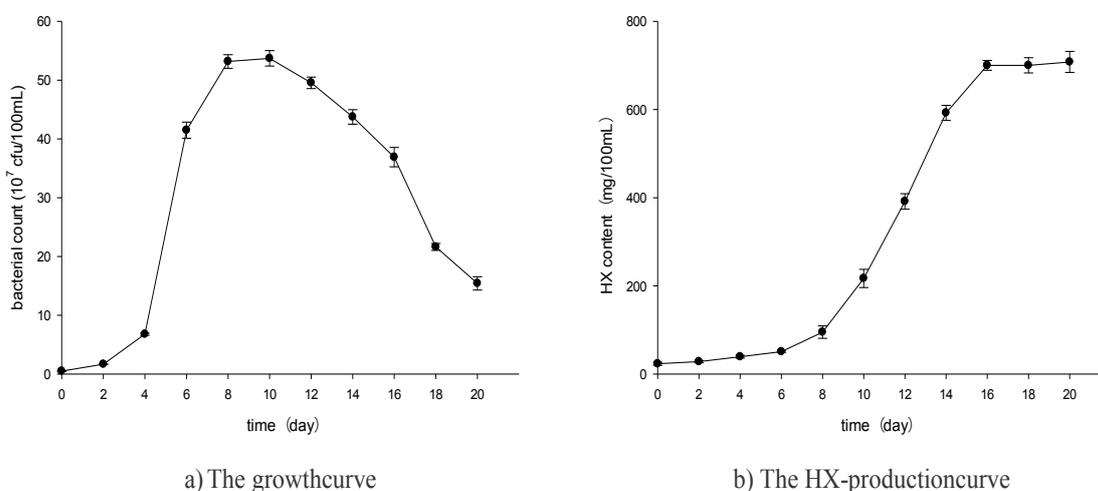


Figure 5: The growth and HX-production curves.

Medium	A		B		C		D	
HX yields (mg/100 mL)	788.726	800.85 ± 12.87	189.110	196.87 ± 7.69	194.150	205.54 ± 12.32	374.65	391.99 ± 15.34
	814.352		204.496		203.847		397.54	
	799.46		196.991		218.617		403.79	

Table 4: X yields in four candidate fermentation culture media.

as nitrogen sources were not significantly different ( $P=0.23$ ). Therefore, yeast extract was used as the nitrogen source for further optimization because of economic reasons.

3. Among the inorganic salt sources, the highest HX output (Figure 6C) was achieved with 1.8 g/L KCL. ANOVA analysis revealed that the HX output with KCL was significantly higher than all the others ( $P<0.01$ ).

Therefore, the optimal carbon source, nitrogen source, and inorganic salt for HX production were glucose, beef extract, and KCL.

### Single-factor experiment

The HX contents in the 15-day fermentation liquid of the single-factor experiment are shown in Figure 7.

The optimal concentration for each ingredient of the fermentation

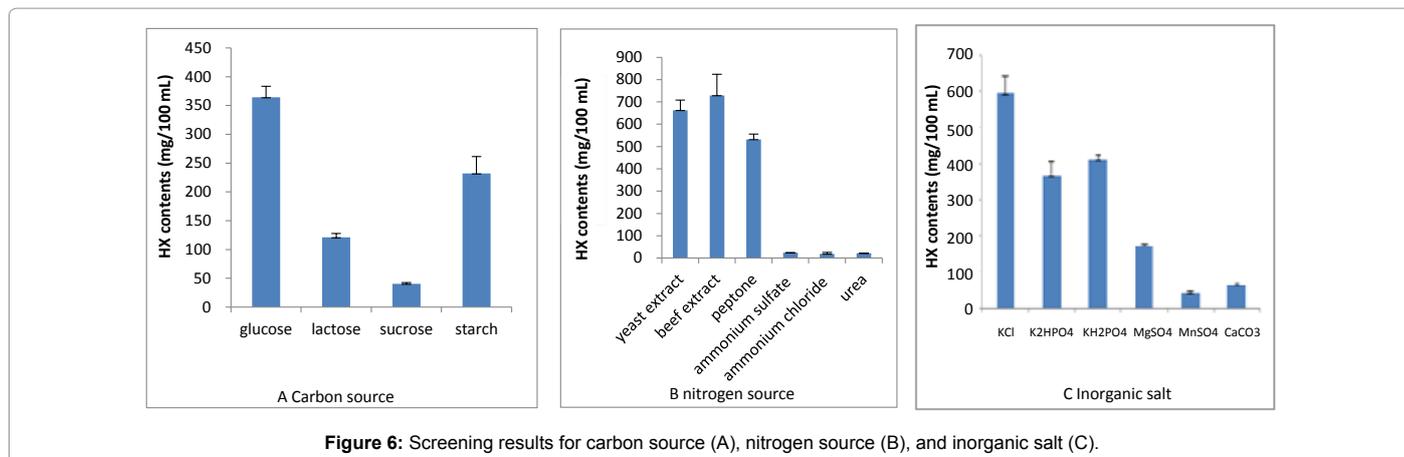


Figure 6: Screening results for carbon source (A), nitrogen source (B), and inorganic salt (C).

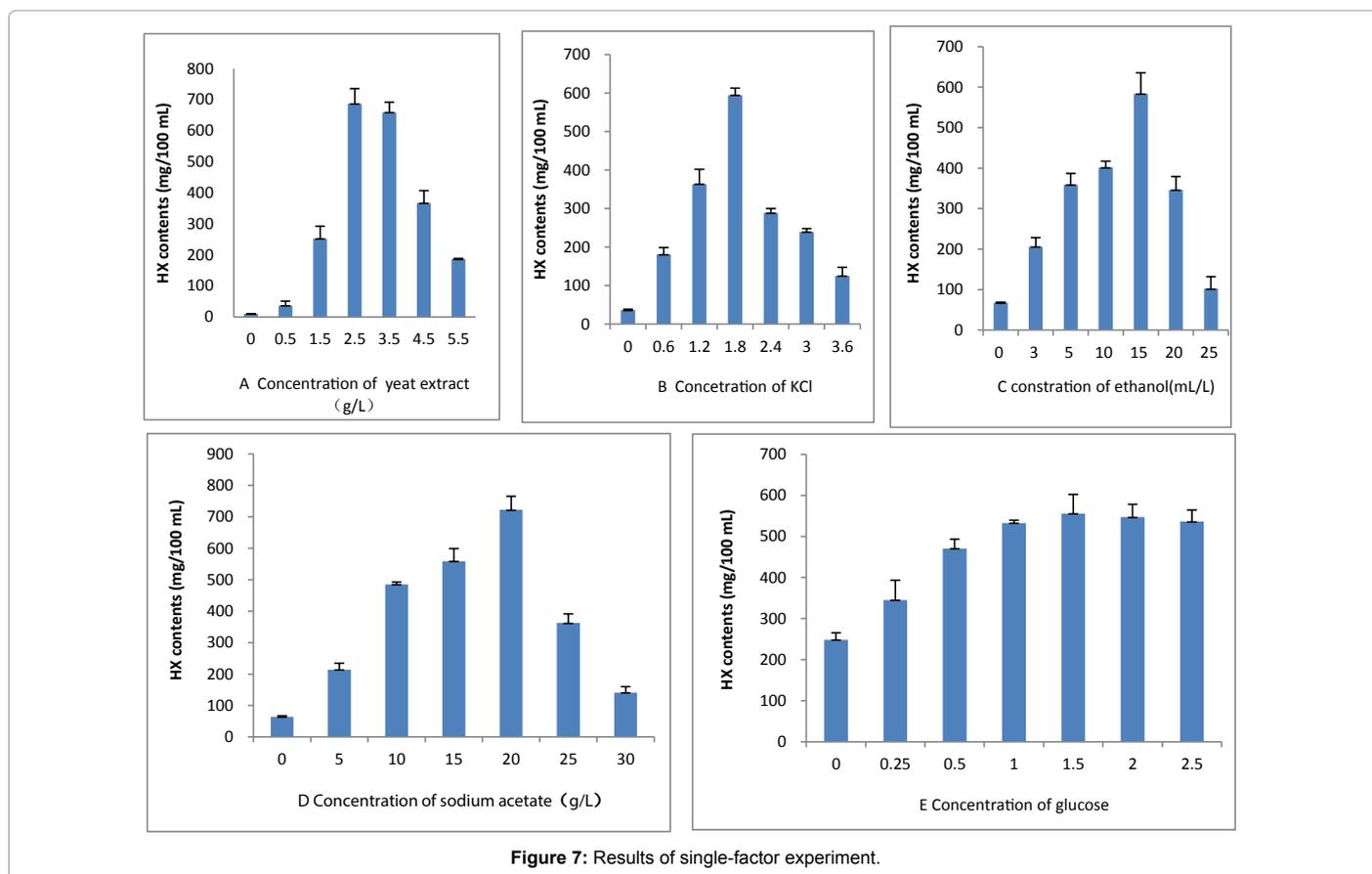


Figure 7: Results of single-factor experiment.

culture medium in HX production with strain K3 was 2.5 g/L yeast extract, 1.8 g/L KCl, 15 mL/L ethanol, 20 g/L sodium acetate, and 1.5 g/L glucose. The influence of each individual factor was calculated by subtracting the zero concentration from the highest HX production. Comparing the effects of individual factors revealed that sodium acetate was the most effective factor of HX production, whereas glucose was the most ineffective. Therefore, glucose was excluded from further optimization.

### Total cell number of fermentation broth by Taguchi design method

A spectrophotometer was used to detect the total cell number,

which was represented by OD, in 10-day fermentation broth that was optimized via the Taguchi design method. The results are shown in Figure 8.

The OD in Figure 8 demonstrated that group 1 had the highest cell number among all experimental groups. Comparing the OD in all the experimental groups revealed that the OD values of groups 1, 2, 6 and 9 were not significantly different ( $P > 0.05$ ). The OD values of these groups, however, are significantly higher than those of other groups ( $P < 0.05$ ).

### Optimization of HX production by Taguchi design method

The GC detection results of the 16 day fermentation liquid are (Table 5).

Minitab 17 (Minitab Inc.; State College, PA) software was used to analyze the experimental results in Table 5 to obtain the response means of HX content, response means of SN ratios, mean main effect diagram, and the SN ratio of the main effect diagram. The location, dispersion, and adjustment factors were analytically determined to obtain the optimal factor and level combination for the fermentation medium [5]. The results were showed in (Tables 6 and 7), (Figures 9 and 10).

To identify the factors that significantly influence the mean value, the mean variance of the experimental results was analyzed further. The results are shown in Table 8 analysis of variance of mean value.

Based on the analysis of response for the means of HX content response means for signal-to-noise ratios (SNR); main effect diagram for means of HX content (Figure 9); main effects diagram for SN ratios of each factor (Figure 10) results of variance analysis, and with caproic acid yields as the index, yeast extract, KCl, sodium acetate, and ethanol all significantly influence HX content. The relative importance of these factors was as follows: sodium acetate>KCl>ethanol>yeast extract. The optimal factor and level combination for HX output was level 3 yeast extract, level 2 KCl, level 3 sodium acetate, and level 2 ethanol. Specifically, the optimal formula for maximum HX output was 3.5 g/L yeast extract, 1.8 g/L KCl, 25 g/L sodium acetate, and 15 mL/L ethanol.

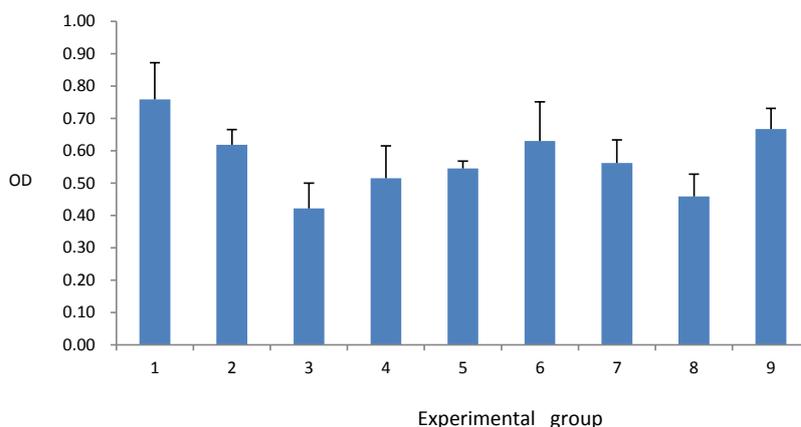


Figure 8: OD(optical density) of 10-day fermentation broth

Test number	Factors				HX content (mg/100 mL)			Y (mg/100 mL)
	Yeast extract (g/L)	KCl (g/L)	Sodium acetate (g/L)	Ethanol (mL/L)	1	2	3	
1	1.5	1.2	15	10	782	795	820	799
2	1.5	1.8	20	15	1050	1026	1050	1042
3	1.5	2.4	25	20	838	870	888	865.33
4	2.5	1.2	20	20	717	730	697	714.67
5	2.5	1.8	25	10	1169	1190	1269	1209.33
6	2.5	2.4	15	15	671	680	691	680.67
7	3.5	1.2	25	15	1283	1295	1383	1320.33
8	3.5	1.8	15	20	828	830	858	838.67
9	3.5	2.4	20	10	759	770	780	769.67

Table 5: The HX content of the Taguchi design.

Level	Factors			
	Yeast extract	KCl	Sodium acetate	Ethanol
1	902.1	944.7	772.8	926.0
2	868.2	1030.0	842.1	1014.3
3	976.2	771.9	1131.7	806.2
Delta	108.0	258.1	358.9	208.1
Rank	4	2	1	3

Table 6: Response means for HX content.

Level	Factors			
	Yeast extract	KCl	Sodium acetate	Ethanol
1	59.05	59.18	57.73	59.14
2	58.46	60.16	58.39	59.81
3	59.54	57.71	60.94	58.10
Delta	1.07	2.45	3.21	1.71
Rank	4	2	1	3

Table 7: Response means for signal-to-noise ratios.

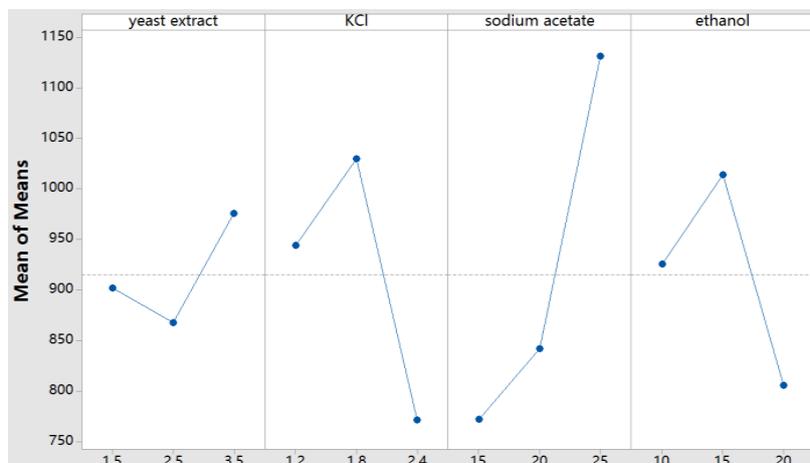


Figure 9: Main effects diagram of each factor for HX content.

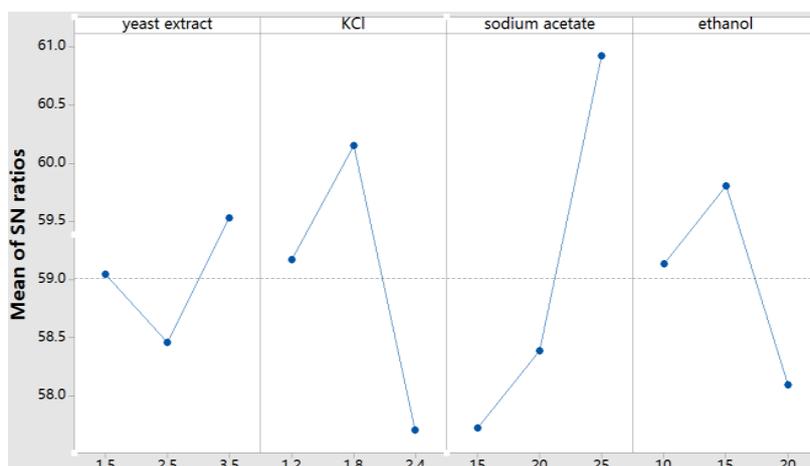


Figure 10: Main effects diagram of each factor for SN ratios.

Source	DF	AdjSS	AdjMS	F-value	P-value
Yeast extract	2	47115	23557	233.07	0
KCl	2	288283	144142	1426.10	0
Sodium acetate	2	592738	296369	2932.20	0
Ethanol	2	173352	86676	857.55	0
Error	18	1819	101		
Total	26	1103307			

Table 8: Analysis of variance of mean value.

The predicted HX yield using this formula was 2112.346 mg/100 mL.

### Validation experiment

The mean of HX yields in the validation experiment was 2018.29 ± 46.37 mg/100 mL and the SNR was 8.78dB. The results demonstrated that the HX yields in the validation experiment were close to the predicted HX yield, thus indicating that this optimization scheme can achieve the desired effect and has good stability [10-12].

### Conclusion

In the present study, an efficient HX-producing strain was isolated from 280 year old pit mud. This strain yielded 609.22 ± 20.46 mg/100 mL HX. Morphological identification indicated that the strain

possessed the typical characteristics of HX-producing strains, such as G<sup>+</sup> and spore formation [12-14]. Bioinformatics analysis revealed that the strain shared 99.1% sequence similarity with *C. sartagoneforme*. Meanwhile, the four candidate fermentation culture media A, B, C and D were tested on the basis of maximum caproic acid yield. Single-factor experiments were performed based on the selected fermentation culture medium A. The results of the single-factor tests showed that the maximum HX yields in 15-day-old fermentation broth were obtained with culture medium A that contained 2.5 g/L yeast extract, 1.8 g/L KCl, 20 g/L sodium acetate, and 15 mL/L ethanol. A further orthogonal experiment was designed using Taguchi methods in Minitab 17. The results demonstrated that the optimum formula for maximum HX output was 3.5 g/L yeast extract, 1.8 g/L KCl, 25 g/L sodium acetate, and

15 mL/L ethanol. The validation experiment had a maximum HX yield of  $2018.29 \pm 46.37$  mg/100 mL.

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