



Production of caproic acid by *Rummeliibacillus suwonensis* 3B-1 isolated from the pit mud of strong-flavor baijiu



Chaojie Liu^{a,1}, Yuanfen Du^{a,1}, Jia Zheng^b, Zongwei Qiao^b, Huibo Luo^a, Wei Zou^{a,*}

^a College of Bioengineering, Sichuan University of Science & Engineering, Yibin, Sichuan 644005, China

^b Key Laboratory of Wuliangye-flavor Liquor Solid-state Fermentation, China National Light Industry, Wuliangye Group, Yibin, Sichuan 644007, China

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ABSTRACT

Caproic acid is the precursor of ethyl caproate, the main representative flavor substance of strong-flavor baijiu (SFB). Caproic acid-producing bacteria are considered to be the most important type of acid-producing microorganisms in the pit mud of the SFB ecosystem. In this study, the *Rummeliibacillus suwonensis* 3B-1 with a high yield of caproic acid (4.064 g/L) was screened from SFB pit mud. The genome of the *R. suwonensis* 3B-1 was sequenced, the total size was found to be 4117,671 bp and a calculated GC content of 35.86%. The caproic acid biosynthesis pathway was identified and analyzed, and it showed that 3B-1 could not only use ethanol, but it could also use glucose and other carbon sources as substrates to produce caproic acid. According to the genome analysis and with an optimized medium, the optimal conditions for caproic acid production were yeast powder at 3 g/L, sodium acetate at 15 g/L, and 1% biotin at 8 mL/100 mL. The yield of caproic acid reached 4.627 g/L, an increase of 13.9%, which was higher than that of general caproic acid bacteria. This is the first report of the synthesis of caproic acid by *R. suwonensis*. This strain could be used to produce caproic acid, an artificial pit mud preparation, and/or an enhanced inoculum in the production of SFB.

1. Introduction

Caproic acid, also known as lamb's oleic acid, is a colorless or pale-yellow oily liquid that is widely used as a fragrance, food additive, preservative, and antibacterial agent (Cavalcante et al., 2017). Currently, caproic acid is mainly produced via a multi-step chemical conversion of petroleum-based products, which is not only expensive but also environmentally polluting (Lu et al., 2019). Therefore, it is important to find an environmentally friendly method for the synthesis of caproic acid. If caproic acid can be produced by microbial fermentation this method, then it may have great application prospects (Chen et al., 2017). The selection and transformation of a high-producing caproic acid strain is the key to achieving successful microbial production.

Chinese baijiu is a traditional distilled liquor. Based on differences in style, it is divided into three large-flavor types (strong-flavor, sauce-flavor, and light-flavor) and nine small-flavor types (miscellaneous-flavor, feng-flavor, rice-flavor, medicine-flavor, sesame-flavor, special-

flavor, chi-flavor, laobaigan-flavor, and fuyu-flavor) (Zheng and Han, 2016). Strong-flavor baijiu (SFB) accounts for more than 70% of the total amount of baijiu consumed in China because of its fragrant flavor, soft mouthfeel, and long aftertaste (Fan et al., 2021; Xu, 2010). As a characteristic flavor constituent of SFB, ethyl caproate is an important component that imparts an apple flavor to Chinese baijiu, and determines the quality and aroma profiles of SFB (Chen et al., 2014; Xu et al., 2021). Caproic acid-producing bacteria (CPB) are one of the most common among the acid-producing bacteria, and they are intimately associated with the production of ethyl caproate and its precursor caproic acid (Yan et al., 2015).

To date, the types of caproic acid producing bacteria mainly include *Clostridium*, *Bacillus*, *Ruminococcaceae*, and *Caproiciproducens* (Yuan et al., 2022; Zou et al., 2018b; Liu et al., 2017). *Clostridium kluyveri* is a typical gram-positive anaerobic bacterium (Barker and Taha, 1942) that can produce caproic acid using acetate and use ethanol as carbon sources, but it cannot use glucose or other sugars (Spirito et al., 2014). Zhao et al. selected the high-producing caproic acid *Clostridium kluyveri*

Abbreviations: SFB, strong-flavor baijiu; CPB, Caproic acid-producing bacteria; AE medium, acetic acid ethanol modified 1 medium; KAAS, KEGG Automatic Annotation Server.

* Correspondence to: College of Bioengineering, Sichuan University of Science & Engineering, 1 Baita Road, Sanjiang New District, Yibin, Sichuan 644005, China.
E-mail address: weizou1985@163.com (W. Zou).

¹ The two authors contributed equally.

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strain through multiple enrichment cultures of the old pit mud, and its highest yield reached 9.77 g/L in 14 days (Zhao et al., 2020). In the early studies, Xue et al. isolated the high-producing caproic acid *Clostridium* sp. W1 from a 500-year-old pit mud. Acetate and ethanol were mainly used as carbon source substrates to produce caproic acid, and glucose was used to produce only a small amount of caproic acid (Xue et al., 1988). The research shows that *Caproiciproducens* and *Clostridium* can produce higher caproic acid and butyric acid content (Liu et al., 2019). *Ruminococcaceae* CPB6 is also an anaerobic bacterium that can produce caproic acid using lactic acid (Zhu et al., 2017). In addition, *Bacillus megaterium*, *Bacillus fusiformis*, and *Bacillus licheniformis* are known to produce caproic acid (Zhao et al., 2012). However, these caproic acid bacteria are relatively specific to the substrate of fermentation, and the yield of caproic acid is generally low. This study selected CPBs from the pit mud of an SFB ecosystem that can use a variety of carbon sources, have good stability, and can produce caproic acid in high yield. The physiological features of the isolated CPBs were studied and the genomes were sequenced and annotated. Genome analysis was used to find the optimal bacterium and fermentation process that could best improve caproic acid production. This study provides a new strain of caproic acid production with easy cultivation and high yield, expands the species of caproic acid bacteria, and can provide some help for the future study of caproic acid bacteria of the same genus.

2. Material and methods

2.1. Isolation and identification of caproic acid producing strains

Samples of fermented pit mud were collected from three different SFB factories in Anhui, Chengdu, and Yibin. The pit mud was stored at 4 °C. To isolate CPBs, BI medium (Popoff, 1984), lactic acid medium, glucose CGM medium, GAB medium, and acetic acid ethanol modified 1 medium (AE medium, see Supplementary Material Table S1) were used in the enrichment and separation of mud cellular strains. The initial pH value of all the used media was adjusted to 7.0, and the media were autoclaved at 121 °C for 20 min. For each pit mud sample 10 g were suspended in 100 mL of sterile peptone physiological salt (PPS) solution, containing 5 g of sterile glass beads (3 mm). The mixture was homogenized for about 20 min by shaking constantly at 180 r/min, and the pit mud samples from Chengdu and Yibin were then heated at 80 °C for 10 min to kill the non-heat-resistant vegetative bacterial cells and thus retain the heat-resistant spores (H. Liu et al., 2020; M.K. Liu et al., 2020). In order to screen more species of caproic acid bacteria, the pit mud samples from Anhui Province were not subjected to any heat treatment. The heat-treated and non-heat-treated suspensions were then inoculated into 100 mL of freshly enriched separation medium and incubated at 35 °C for 6 d. Then 0.2 mL of enrichment liquid was spread on enriched separation medium agar plates, which were incubated for 7 d at 35 °C under anaerobic conditions. The growing strains were isolated and purified at least three times and were inoculated into the fermentation medium and maintained at 35 °C for 10 d. The CPBs were screened by detecting the presence and concentration of caproic acid in the fermentation broth by the copper sulfate detection method (Zhong and Xie, 2004) and gas chromatography (GC) (Yuan et al., 2018). GC conditions were: Agilent DB-WAX column (0.18 mm × 0.18 mm × 20 m); inlet temperature, 250 °C; FID detector temperature, 250 °C; hydrogen (99.999%) as the carrier gas, flow rate of 1 mL/min; split injection method, split ratio 10:1; ramp-up procedure, initial temperature 80 °C, hold for 1 min, ramp up to 200 °C at 20 °C/min, then to 250 °C at 50 °C/min, hold for 5 min. The use of this programmed warming method could improve separation effect and shorten the separation time (Blumberg and Klee, 2001).

The strain with the highest yield of caproic acid was inoculated to the solid fermentation medium by plate partition marking and placed in an anaerobic glove box at 35 °C for 24 h, and the colony morphology was observed and recorded. The morphological characteristics of the cells

were further observed by gram staining. A series of physiological and biochemical tests were carried out on the screened strain according to the *Manual of Bacterial Characteristics and Identification* (Goodfellow et al., 2012). The genome was extracted with the help of a bacterial genome DNA extraction kit (purchased from Tiangen Biochemical Technology (Beijing) Co., Ltd.). The PCR reaction system comprised: 1.0 μL of genomic DNA (20 ng/μL), 5.0 μL of 10 × buffer (containing 2.5 mM Mg²⁺), 1.0 μL of Taq polymerase (5 U/μL), 1.0 μL of dNTP (10 mM), 1.5 μL of 27 F primer (10 μM) (5-AGAGTTGATCCTGGCTCAG-3), 1.5 μL of 1492 R primer (10 μM) (5- CTACGGCTACCTTGTACGA-3), ddH₂O 39.0 μL in a total volume of 50.0 μL. The amplification conditions were: an initial denaturation at 95 °C, 30 s; annealing at 58 °C, 30 s; extension at 72 °C, 90 s; after 35 cycles, a final extension at 72 °C, 7 min. Sequencing was performed by Nanjing Parsenol Gene Technology Co. Ltd., China. The phylogenetic tree was constructed by the neighbor-joining (NJ) method, which was used to identify similar sequences, and then the similar sequences were aligned by Molecular Evolutionary Genetics Analysis (MEGA) 5.0 software (Tamura et al., 2011).

2.2. Growth characteristics of the strain

The selected strain with the highest caproic acid yield was inoculated in the fermentation liquid medium with a 5% inoculation amount and placed in an anaerobic glove box at 35 °C to culture. Samples were taken every 12 h at the beginning of culture, and then every 4 h. OD values were measured at 600 nm, and the growth curve of the strain was plotted.

The seed liquid was inoculated in the fermentation liquid medium at 5%, and the effects of initial pH (1, 2, 3, 4, 5, 6, 7, 8, 9), ethanol concentration (v/v) (0%, 2%, 4%, 6%, 8%), and temperature (29 °C, 33 °C, 35 °C, 37 °C, 40 °C) on the growth of the strain were observed.

2.3. Data processing method

Origin (<https://www.originlab.com/>) was used to draw graphs and histograms, Minitab 17 (<https://www.minitab.com/zh-cn/>) was used to perform variance analysis and draw the related graphs, and Adobe Illustrator 2020 (<https://www.adobe.com/cn/products/illustrator>) was used to draw related graphs.

2.4. Genome sequencing, assembly, and annotation

The isolated strain was inoculated in the liquid fermentation medium and incubated at 35 °C until OD600 reached 0.5–1 (logarithmic growth phase). Then the bacteria were collected and centrifuged and precooled PBS was used to resuspend the bacteria three times. After centrifugation, the bacteria were kept in the ultra-low temperature refrigerator at –80 °C for subsequent genome extraction and sequencing.

Sequencing of the whole genome of *R. suwonensis* 3B-1 was completed by Shanghai Parsenol Biotechnology Co., Ltd., mainly using the whole-genome shotgun (WGS) strategy to construct the library of different inserted fragments. Using next-generation sequencing (NGS) and the Illumina Novaseq Sequencing platform, double-end petroleum-end (PE) sequencing was performed on these libraries. After quality inspection, library construction, and computer sequencing of the sample, the base was read to obtain the computer data, and high-quality data were obtained by data filtering.

A5-miseq (Coil et al., 2014) and SPAdes (Bankevich et al., 2012) were used to assemble the sequencing data of the removed joint sequence from scratch to construct the contig and scaffold. The assembly results obtained were evaluated and compared, and the SPAdes results were finally selected, and Pilon (Walker et al., 2014) software was used for base correction. Genemark was used to predict protein-coding genes in the bacterial genome (John et al., 2001). The tRNA genes in the whole genome were predicted by tRNAscan-SE (Lowe and Eddy, 1997). Barrnap was used to predict the rRNA genes, while the rest of the non-coding

RNA was predicted by comparison with the Rfam database (Kalvari et al., 2018). Annotation was performed using the National Center for Biotechnology Information (NCBI) Prokaryotic Genome Annotation Pipeline (Tatusova et al., 2016). KO and pathway annotation of the protein-coding genes was mainly completed by the KEGG Automatic Annotation Server (KAAS) (Yuki et al., 2007).

2.5. Optimization of the 3B-1 fermentation process for caproic acid production based on the genomic analysis

Acetic acid ethanol fermentation medium contained (per 1 L): 15 g of sodium acetate, absolute ethanol (filtered and sterilized) 20 mL, 0.2 g of magnesium sulfate heptahydrate, calcium carbonate (sterilized separately, added after sterilization) 10 g, sulfuric acid ammonium 0.5 g, dipotassium hydrogen phosphate trihydrate 0.04 g, yeast extract powder 10 g, para-aminobenzoic acid 0.0625 g/100 mL, L-cysteine hydrochloride 0.5 g, at pH 7.0, 121 °C, sterilized for 20 min.

The candidates chosen for optimization came from the metabolic pathway annotation by KAAS. The selected nitrogen sources were ammonia, ammonium chloride, yeast powder, peptone, and urea; carbon sources included glucose, sodium lactate, sodium acetate, and sodium succinate. Due to the incomplete biotin pathway, 1% biotin was added.

3. Results and discussion

3.1. Screening and isolation of CPBs from the pit mud of the SFB ecosystem

A total of 16 CPBs with different caproic acid-producing capabilities were isolated from different pit mud samples (Table 1 or Fig. 1). The agarose gel electrophoresis results for the PCR products of the 16S rDNA of the strains are shown in Fig. 2. Most of these are from *Rummeliibacillus suwonensis* and *Clostridium butyricum* with a relatively high capacity of caproic acid synthesis. Among them, strain 3B-1 isolated from the pit mud of YB produced the highest caproic acid yield (4.064 g/L) (Table 1). *Clostridium kluveri* N6 isolated from the pit muds of SFB produced up to 3.05 g/L of caproic acid (Hu et al., 2015). Zhao et al. screened three high-producing caproic acid bacteria from the high-quality pit mud of SFB. They were identified as *Bacillus fusiformis* A17, *Bacillus licheniformis* a57, and *Bacillus megaterium* C78. The maximum caproic acid production can reach 2.137 g/L (Zhao et al., 2012). Since the caproic acid production of this strain is higher than that of most other caproic acid bacteria, it was selected for subsequent experiments. It was further identified as *Rummeliibacillus suwonensis* according to the phylogenetic

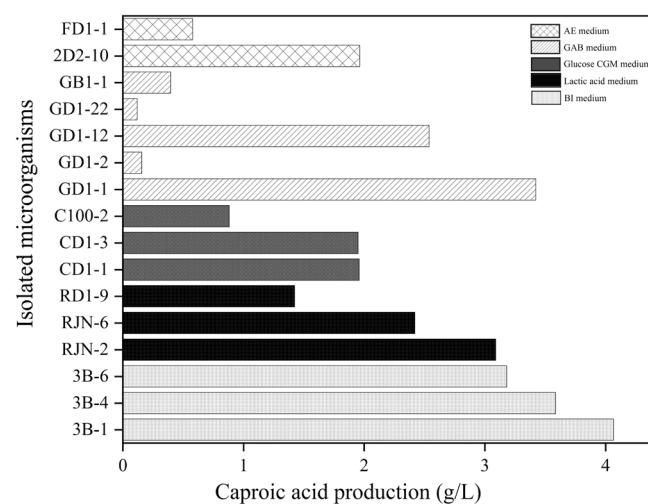


Fig. 1. Caproic acid yields of isolated microorganisms under different media.

tree based on 16S rDNA as shown in Fig. 3. GenBank accession no. PRJNA687825. This work is the first to study the production of caproic acid by *R. suwonensis*. *R. suwonensis* has not been reported as a caproic acid-producing strain from the SFB ecosystem before (Yuan et al., 2022). A previous study showed that *Rummeliibacillus* was positively correlated with ethyl caproate, ethyl butyrate, and isovaleric acid (H. Liu et al., 2020; M.K. Liu et al., 2020). In addition, *Rummeliibacillus* and caproic acid content were also positively correlated (H. Liu et al., 2020; M.K. Liu et al., 2020). Therefore, the study of the strain on caproic acid is meaningful.

3.2. Morphological, phenotypic, and biochemical characteristics of strain 3B-1

Cells of strain 3B-1 were facultative anaerobic, Gram-positive, and terminal spore forming. The colony was large, more regular, and round, and opaque with a light yellow and milky white middle bulge (Fig. 3). The growth curve of this strain is shown in the Supplementary Material (Fig. S1). The suitable growth temperature range of the strain was 33–37 °C (optimum, 35 °C), at pH 1–10, the optimum pH being 7, and the ethanol concentration was 5%. Resistance to caproic acid concentration was up to 30 g/L. In terms of biological characteristics, the strain was cultured in a sodium acetate enriched and separated solid medium at 35 °C for 3 d. Compared with strains in the BI medium, the culture characteristics, morphological characteristics, physiological and

Table 1
Identification, separation, and caproic acid-producing strains from Chinese strong-flavor baijiu pit mud.

Strain number	Enrichment separation medium	Identification results	Related GenBank sequence/Identities	Caproic acid production (g/L)	Pit mud source ^a
3B-1	BI medium	<i>Rummeliibacillus suwonensis</i>	NR_109749.1/99.44%	4.064	YB
3B-4	BI medium	<i>Rummeliibacillus suwonensis</i>	NR_109749.1/99.58%	3.587	YB
3B-6	BI medium	<i>Rummeliibacillus suwonensis</i>	NR_109749.1/99.51%	3.18	YB
2D2-10	AE medium	<i>Clostridium butyricum</i>	NR_113244.1/99.15%	1.964	D1
RJN-2	Lactic acid medium	<i>Rummeliibacillus suwonensis</i>	NR_109749.1/99.58%	3.09	JN
RJN-6	Lactic acid medium	<i>Clostridium butyricum</i>	NR_113244.1/99.15%	2.42	JN
RD1-9	Lactic acid medium	<i>Staphylococcus saprophyticus</i>	NR_074999.2/99.79%	1.423	D1
CD1-2	Glucose CGM medium	<i>Clostridium butyricum</i>	NR_113244.1/99.23%	1.96	D1
CD1-3	Glucose CGM medium	<i>Clostridium butyricum</i>	NR_113244.1/99.08%	1.95	D1
C100-2	Glucose CGM medium	<i>Bacillus fumarioli</i>	NR_114086.1/97.77%	0.882	YB
GD1-1	GAB medium	<i>Clostridium butyricum</i>	NR_113244.1/99.08%	3.422	D1
GD1-12	GAB medium	<i>Clostridium butyricum</i>	NR_113244.1/99.01%	2.539	D1
GD1-2	GAB medium	<i>Enterococcus faecium</i>	NR_114742.1/99.86%	0.156	D1
GD1-22	GAB medium	<i>Enterococcus faecium</i>	NR_114742.1/99.79%	0.118	D1
GB1-1	GAB medium	<i>Enterococcus faecium</i>	NR_114742.1/99.86%	0.396	B1
FD1-1	AE medium	<i>Clostridium butyricum</i>	NR_113244.1/99.23%	0.579	D1

^a : YB: A baijiu factory in Yibin, Sichuan, China; D1 and B1: The bottom and cellar walls of a fermentation pit of a baijiu factory in Anhui, China, respectively; JN: A baijiu factory in Chengdu, Sichuan, China.



Fig. 2. Agarose gel electrophoresis result of PCR products of 16 S rDNA of 16 strains.

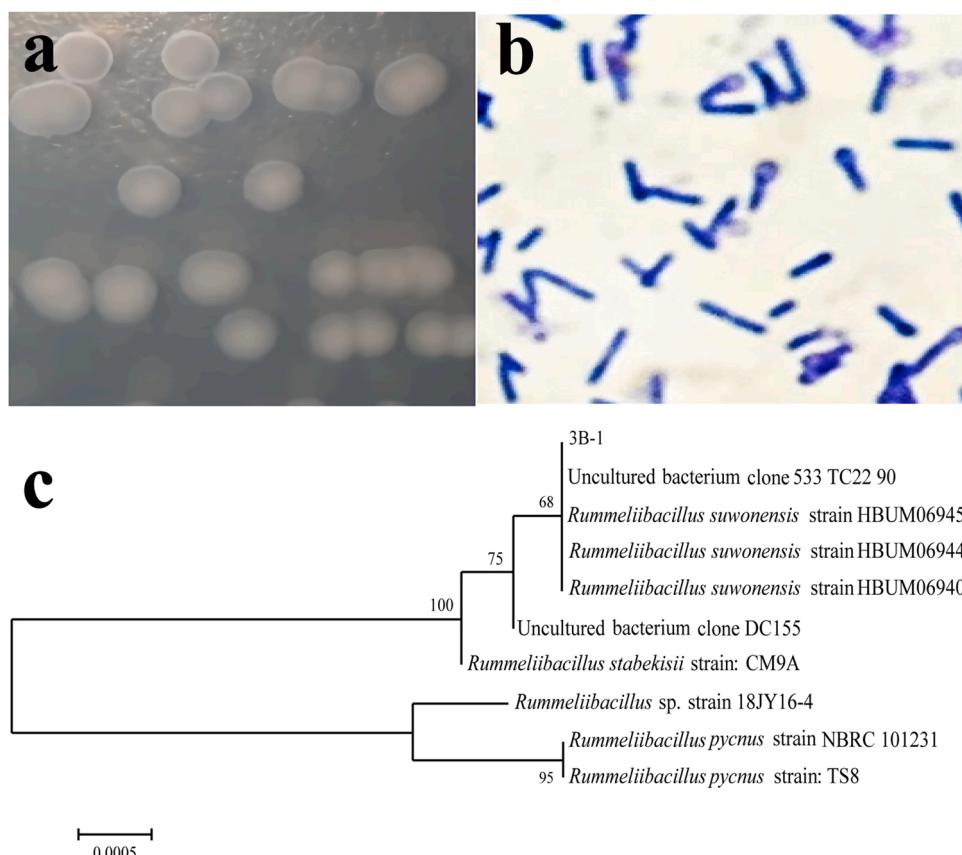


Fig. 3. Microscopic morphology and Colony morphology (a, b) under anaerobic environment and phylogenetic tree of 16S rDNA of strain 3B-1(c).

biochemical characteristics and other biological characteristics of 3B-1 had no significant changes. The results for the phenotypic characteristics of strain 3B-1 are listed in Table 2. Physiological and biochemical results showed that 3B-1 was negative in terms of carbohydrate metabolism experiments, methyl red experiments, and V-P experiments. The

indole and nitrate reduction experiments results were positive. In addition, 3B-1 could assimilate 14 carbon sources except xylitol and L-sorbose (Table 2).

3.3. Genome sequencing and analysis of *Rummeliibacillus suwonensis* 3B-1

The genomes of caproic acid bacteria *Caproicacidobacter* *galactitolivorans* BS-1 T, *Ruminococcaceae* bacterium CPB6, and *Clostridium kluyveri* JZZ have been sequenced (Bengelsdorf et al., 2019; Tao et al., 2017; Wang et al., 2018). At present, there are relatively few sequences of caproic acid bacteria. Therefore, it is necessary to sequence the genome of this strain so that we can fully understand the physiological and metabolic characteristics of this strain.

3.3.1. Genome assembly and annotation

A total of 8463,888 raw reads with 99.43% high quality reads were acquired from the Illumina Novaseq platform (Table 3). The draft genome sequence for the *R. suwonensis* 3B-1 strain comprises 83

Table 2

Differential characteristics of strain 3B-1 results.

Characteristics	Results ^a	Characteristics	Results
Methyl red	-	Gelatin	+
V-P	-	L-rhamnose	+
Indole production test	+	Sucrose	+
Nitrate reduction	+	Xylitol	-
D-Glucose	+	D-sorbitol	+
D-lactose	+	Starch	+
D-galactose	+	Cellobiose	+
D-xylose	+	L-sorbose	-
D-mannitol	+	Mycose	+
L-gulalose	+	Glycerin	+

^a + : indicates positive reaction; - indicates negative reaction.

Table 3
Genome characteristics of *R. suwonensis* 3B-1.

General features	Number
Reads Num	8463,888
Total base	1269,583,200
GC Content	35.86%
Q20_rate	98.33%
Q30_rate	94.60%
HQ Reads	8415,326
HQ Reads %	99.43
HQ Data (bp)	1254,410,538
HQ Dat%	98.80
Total sequence number of contigs	94
Contigs N50	106,437
Scaffold N90	29,079
ORFs	3891
rRNAs Copy Number	6
tRNA Copy Number	73
ncRNA Copy Number	339

scaffolds with a total size of 4117,671 bp and a calculated GC content of 35.86%. The maximum scaffold size was 355,227 bp in length and the N50 scaffold size was 119,142 bp. In the genome sequence of *R. suwonensis* 3B-1, 3217 genes were annotated with COG function, accounting for 82.68% of the sequenced genome sequence. Here, 1969 genes of *R. suwonensis* 3B-1 were KEGG annotated, accounting for 50.60% of the total genes. In terms of metabolism, carbohydrate metabolism accounted for 6.72%, energy metabolism 3.85%, lipid metabolism 1.98%, nucleotide metabolism 2.49%, amino acid metabolism 7.10%, and metabolism of cofactors and vitamins 4.50% (Fig. 4).

According to the analysis of the 3B-1 KEGG metabolic pathway, this strain has a complete synthesis pathway for 20 amino acids, as well as thiamine, folic acid, riboflavin, vitamin B6, niacin, pantothenic acid, and CoA. However, biotin was unable to be synthesized by this strain due to the absence of biotin amidase and other biotin-related enzymes. The metabolic pathway of biotin is shown in the Supplementary Material (Fig. S2). Therefore, the addition of biotin is one of the strategies needed to optimize the product yield in the later stage. The *R. suwonensis*

3B-1 genome was deposited in the NCBI GenBank database under the accession number PRJNA687825.

3.3.2. Caproic acid-producing metabolic pathway analysis

The metabolic pathway from substrates to caproic acid was identified on the basis of the KAAS annotation and caproic acid biosynthesis pathway of *Clostridium kluyveri* (Zou et al., 2018a). The metabolic pathway of strain 3B-1 for the production of caproic acid and some other by-products is shown in Fig. 5. First, by synthesizing butanoyl-CoA, butanoyl-CoA and acetyl-CoA generate 3-oxo-hexanoyl-CoA and CoA under the action of acetyl-CoA C-acetyltransferase, and 3-oxo-hexanoyl-CoA generates (S)-3-hydroxy-hexanoyl-CoA under the action of 3-hydroxyacyl-CoA dehydrogenase. (2E)-hexenoyl-CoA is generated by enoyl-CoA hydratase, and (2E)-hexenoyl-CoA produces hexanoyl-CoA under the action of butyryl-CoA dehydrogenase. Hexanoyl-CoA is finally generated by the medium-chain acyl-[acyl-carrier-protein] hydrolase to produce caproic acid. The key genes and enzymes involved in the 3B-1 caproic acid synthesis pathway are shown in the Supplementary Material (Table S2).

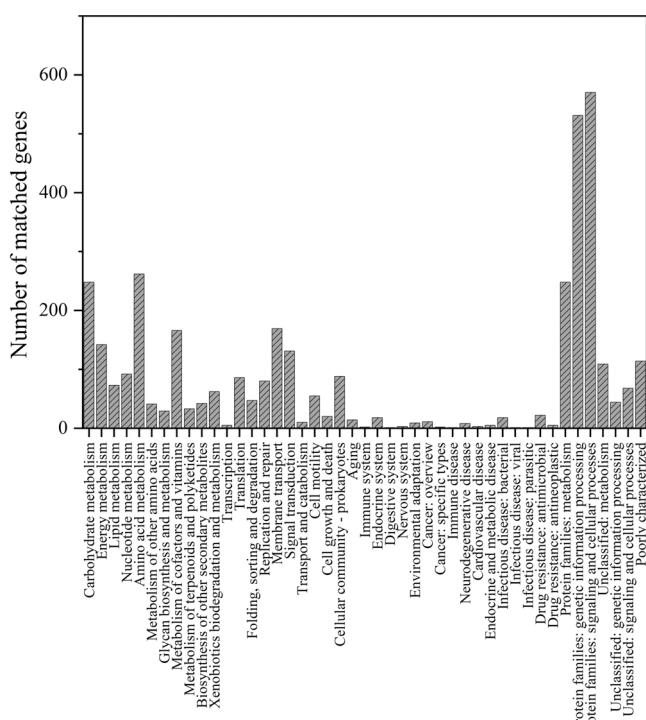
Analysis of the metabolic pathway diagram showed that the formic acid production pathway of this bacterium is incomplete. In addition, there are complete synthesis pathways of acetic acid, propionic acid, butyric acid, caproic acid, and butanediol. According to the analysis of the ABC transport protein, this bacterium has biotin, phosphate, part of the amino acid and branched chain amino acid, nucleoside, part of the metal ion, and other transporters.

In other aspects, although there is no complete nitrate utilization pathway among the metabolic pathways, there is a complete intracellular transport complex of nitrate (*nrtD* and *cynD*). Moreover, the two-component systematic pathway analysis showed that this bacterium has the ability to self-regulate oxygen via the genes *resE* and *resD*; hence, it is speculated that this may be a facultative aerobic bacterium (Her and Kim, 2013). Compared with other caproic acid bacteria, *R. suwonensis* has a wide range of available carbon sources and relatively short fermentation cycle, and it can grow under aerobic or anaerobic conditions.

3.4. Optimization of the fermentation process for caproic acid production based on genome annotation

Based on genome annotation, the nitrogen source, carbon source, and biotin addition amount were optimized in turn for the production of caproic acid by strain 3B-1. First, the nitrogen source was optimized, and on this basis, the carbon source was next optimized, and finally the amount of biotin added was optimized. One source of nitrogen and the carbon sources added according to the content of the acetic acid ethanol fermentation medium, in a fluid volume of 80%, at an initial pH of 7, and according to the seed liquid 10% inoculation in acetic acid ethanol fermentation liquid medium, incubation was conducted at 39 °C for 10 d. With the caproic acid content in the fermented liquid as an index, the best nitrogen source, carbon source, and biotin addition amount were selected. As shown in Fig. 6, the best nitrogen source for caproic acid production of strain 3B-1 was yeast powder, the best carbon source was sodium acetate, and the best 1% biotin supplemental amount was 8 mL/100 mL. The yield of caproic acid reached 4.627 g/L, an increase of 13.9% upon optimization.

Caproic acid producing bacteria can be used as a compound microbial agent, as well as used in pit maintenance and pit protection to prevent pit aging. Its metabolites can be directly applied to the production technology reserve of strong-flavor pit mud (Li et al., 2019). The research shows that technology of microbial co-cultivation can be used to explore the effect of co-cultivation with other functional bacteria, such as methanogens (Yan and Dong, 2018), which is an effective strategy to increase the production of caproic acid.



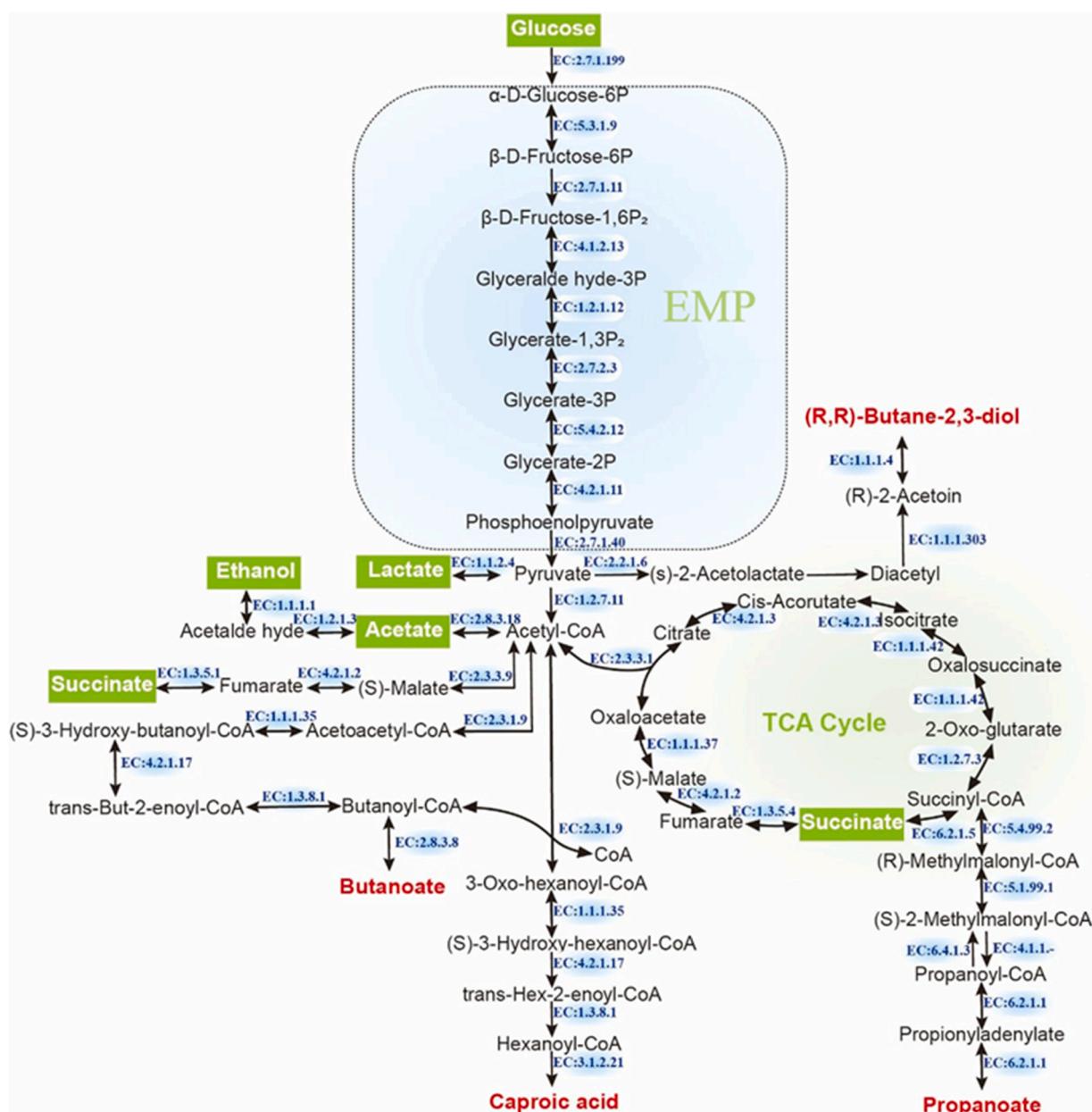


Fig. 5. Pathway of caproic acid synthesis of *R. suwonensis* 3B-1.

4. Conclusions

In this study, 16 strains of caproic acid-producing bacteria were screened in different substrate screening media from the pit mud of an SFB ecosystem, among which strain 3B-1 produced the highest yield of caproic acid (4.064 g/L). Combined with genome annotation, the preliminary optimization of the production of caproic acid by *R. suwonensis* has achieved a yield of 4.627 g/L, which is higher than most CPB. In addition, compared with other caproic acid bacteria, *R. suwonensis* can use more carbon sources and is easier to culture, and so it is easier to produce caproic acid. The bacteria can be mainly used to produce caproic acid, SFB fermentation enhancer, and an artificial pit mud preparation. In the future, the yield of caproic acid of the strain can be further improved by means of mutagenesis and metabolic engineering.

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CRediT authorship contribution statement

Chaojie Liu: Methodology, Formal analysis, Visualization, Writing – original draft preparation, Writing – review & editing. **Yuanfeng Du:** Methodology, Investigation, Data curation, Writing – original draft preparation. **Jia Zheng:** Validation. **Zongwei Qiao:** Investigation. **Huibo Luo:** Supervision, Project administration. **Wei Zou:** Conceptualization, Formal analysis, Supervision, Methodology, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence

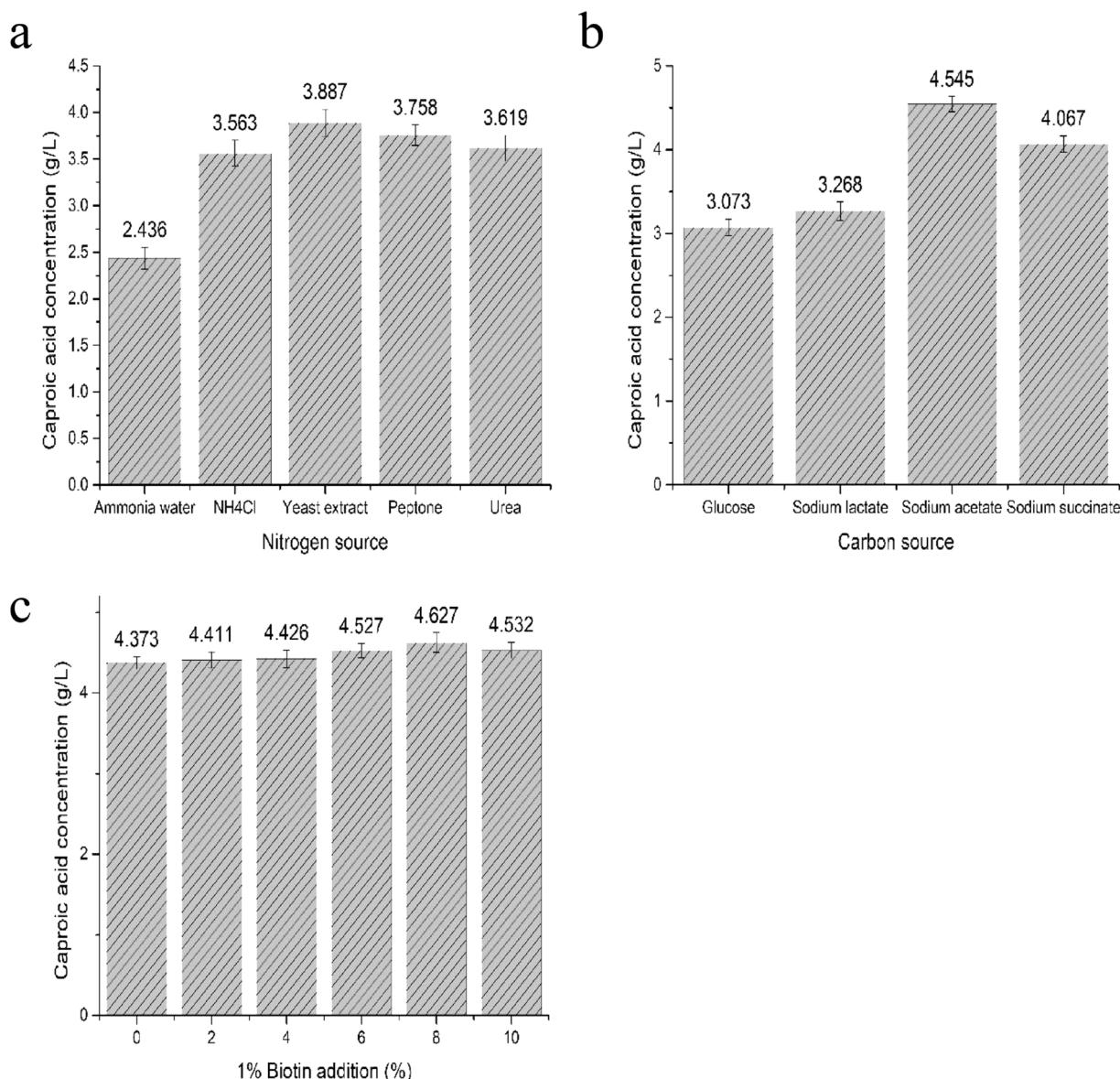


Fig. 6. Effect of different nitrogen sources (a), carbon source (b) and different 1% biotin addition amount (c) on caproic acid production of.

the work reported in this paper.

Data availability

The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.jbiotec.2022.08.017](https://doi.org/10.1016/j.jbiotec.2022.08.017).

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