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Isolation and Identification of a New *Clostridium Butyricum* XYB11Strain Producing 1, 3-Propandiol from Soil

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Abstract. 1, 3-propanediol (1,3-PD) is an important chemical material, which is wildly used in many fields. *Clostridium butyricum*is key strain which can transform glycerol into 1,3-PD. In this study, a new strain producing 1,3-PD was isolated from soil. On the basis of morphology, biochemical-physiological characteristics, this strain was preliminarily identified as *C. butyricum*. Then, the molecular method was used in the further identification. The 16S rDNA sequence analysis showed that this strain performed 99 % homology with *C. butyricum*. So this strain was identified as *C. butyricum* finally, and named as *C. butyricum* XYB11. This strain will give more choice for the strains usage in the biosynthesis of 1,3-PD.

Introduction

1, 3-propanediol (1, 3-PD) is a widely-used organic synthetic compound, which can be utilized as antifreeze, detergent, preservative, emulsifier, flavoring agent in industry and food fields. Because of the more extensively application, 1,3-PD is considered as the most potential chemical in the 21th century. So much focus has been attracted into the study of 1, 3-PD production.

So far, there are many studies on the microbial fermentation of 1,3-PD. The metabolic pathway, by which glycerol is transferred to 1,3-PD, exists in many microorganisms such as *Klebsiella pheumoniae, Clostridium butyricum, Lactobacillus reuteri* and *Citrobacter freundii*. Comparing with, other strains, *C. butyricum* obviously possess more potential for high substrate tolerance, less by-products and other advantages[1]. In addition, in most microorganisms, glycerol is metabolized via a reductive branch which leads to 1,3-PD formation under anaerobic conditions. In this branch, glycerol is converted to 3-hydroxypropionaldehyde (3-HPA)by glycerol dehydratase (GDHt). Afterwards, 3-HPA is reduced to 1,3-PD by NADH-dependent 1,3-PD dehydrogenase. GDHt can dominate the decomposition of glycerol, whose role is very vital in this reductive branch. GDHt is coenzyme B12-dependent in most bacteria, while in *C. butyricum*, GDHt is B_{12}-independent. So, the costly coenzyme B_{12} is avoided, which reduces the production cost of 1,3-PD vastly. More important, *C. butyricum*as one of the most important probioticsis very helpful for human beings.

According to the study of Yang Jinmei[2], the growth of cancer cell would be repressed by the composition of *C. butyricum* or metabolites produced in a certain environment. It was reported that *C. butyricum*has already been used to cure some indigestion sufferers, and it was made into a kind of microecological modulator in Japan last century.With all the biological function and intricate characteristics of microecology, *C. butyricum* has gain more attention by the researchers from many fields. Due to above advantages, *C. butyricum* is a good candidate strain for the biosynthesis of 1, 3-PD [3].

In this study, a stain of *C. butyricum* was isolated from soil sample, and was identified by morphological, physiological and biochemical characteristics and 16S rDNA sequencing.

Materials and Methods

Media and Materials

Reinforced Clostridium Medium (RCM) contained the following components per liter of distilled water: Yeast extract powder3g , Beef extract10 g, Tryptone 10g, Glucose 10g, starch 1g, NaCl 5g, sodium acetate 3g, cysteine hydrochloride 0.15g, ager 15g(if solid medium is necessary). TSN medium contained the following components per liter of distilled water: Tryptone 10g, Yeast extract 3g, sodium sulfite 10g, iron(III) citrate tribasic hydrate 0.5g, Novobiocin sodium salt 0.02g, polymyxin B Sulfate 0.05g.Medium of gelatin liquefaction contained the following components per liter of distilled water: peptone 5g, gelatin 100-150g. (The initial pH at 7.2-7.4 regulated by NaOH). Medium for fermentation of carbohydrate contained the following components per liter of distilled water: Trypticase 10g, Carbohydrate 5g, Sodium Chloride 5g, Phenol red 0.189mg. Fermentation of 1,3-PD was used 200 ml RCM medium adding with 8 g glycerol in 500 ml flask for culture 24 h at 37 ^{o}C. Soil samples was collected from the campus of Jiangsu University, China.

Enrichment and Isolation

90 ml of sterilized distilled water containing 10g soil sample was stirred until homogenous, then heated in a water bath at 80℃ for 30 min. The mixture was put to 50 ml RCM medium and incubated under anaerobic condition for 24h. Each culture was transfer into TSN medium for another 24h. Then the culture was gradient diluted and spread to the RCM medium for extra 24h. Typical milky white colonies with similar characteristics described in Bergey's Manual of Determinative Bacteriology were re-streaked on RCM for aerobic and anaerobic parallel culture for 48 h. The cultures which had grown in anaerobic condition were used for following procedures and preservation [4].

Morphology Physiology and Biochemistry

Microscopic morphology was observed using light microscope. Gram staining, gelatin hydrolysis test and carbohydrate fermentation test were conducted according to Bergey's Manual of Determinative Bacteriology.

Molecular Biology Identification

The colony PCR was carried out in a mixture containing: 12.5 μL 2× taq master

mix(dye plus), 1 μL each of forward and reverse primers (final concentration 0.1 mM) ,2 μL template strain culture and deionized water to a final volume of 25 μL. This mixture containing all of the above was then transferred to a thermocycler (T100 Thermal Cycler, BIO-RAD). The universal primer pair contains: upstream primer (named 27F), 5'- AGAGTTTGATCCTGGCTCAG -3'; downstream primer (named 1492R), 5'- TACGGCTACCTTGTTACGACTT -3'. The reaction cycle for PCR consisted of an initial denaturation at 95 ^{o}C for 5 min, 34 cycles of denaturation at 94 ^{o}C for 30 s, annealing at 60 ^{o}C for 30 s, and extension at 72 ^{o}C for 60 s. The final extension was conducted at 72 ^{o}C for 10 min and then infinitely hold at 12 ^{o}C [5].The amplified DNA fragment waschecked by electrophoresis in1% agarose gel. DL 5000 DNA marker was used in the electrophoresis. The PCR product was sequenced by Synbio Biotechnologies Co. Ltd. (China). Then BLAST was used in the identificationbase on the 16S rDNA sequencing. Using the neighbor-joining method through MEGA 5.0 software, phylogenetic tree was built and bootstrap analysis was performed for 500 replicates to estimate the reliability of the tree topologies.

Capability of Producing 1, 3-PD

Products 1, 3-PD was detected by high-performance liquid chromatography (HPLC) system with an Ultimate XB-NH_2 HPLC Column (Welch, China).

Results and Discussions

Morphology physiology and biochemistry characteristics

In the preliminary screening, water bathing was to remove the microoganismwithout spore, and antibiotic was used to wipe out the Gram-negative strains. After preliminary screening, 13 strains was obtained by morphology characteristics and 10 stains among them were excluded by the gram staining and gelatin hydrolysis. Thereafter, 1 strain named XYB11 was completely matched the characteristics of the Bergey's Manual of Determinative Bacteriology through the test of carbohydrate fermentation (Table 1). Furthermore, after 36 hours of culture, XYB11 strain could grow and forminto 1-3 mm diameter milky white colony with a small serrated edge (Fig. 1). In addition, this grampositive strain is rod-shaped under the microscope and capable of producing 1,3-PD. All the above characteristics of this strain were consistent with those of *C. butyricum.* So it was identified as *C. butyricum* preliminarily.

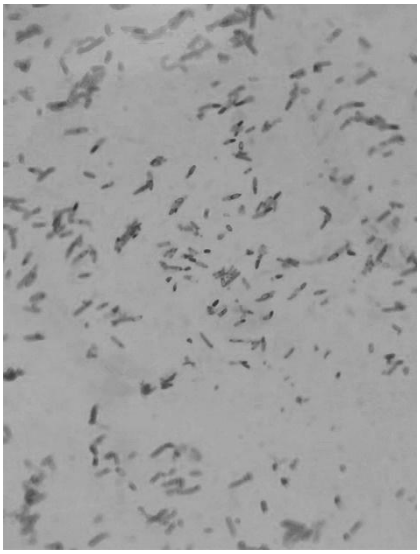

Figure.1 Colony morphology of strain XYB11

Table 1. Physiology and biochemistry characteristics of XYB11

Test	result
Gram staining	positive
Gelatin hydrolysis	negative
Starch	positive
Glycerol	positive
Melibiose	positive
Melezitose	negative

Molecular Biology Characteristics

In order to further identify XYB11strain, the 16s rDNA sequence was determined through molecular method. A fragment about 1.5 Kbwas obtained through colony PCR (Fig. 2). The BLASTresult showed that the 16s rDNA of XYB11strainshared 99% identity to that of *C. butyricum* BOH3. Thephylogenetic tree onstructed by neighbor-joining method also showed that this strain is *C. butyricum*(Fig.3). So this stain was named as *C. butyricum*XYB11 finally.

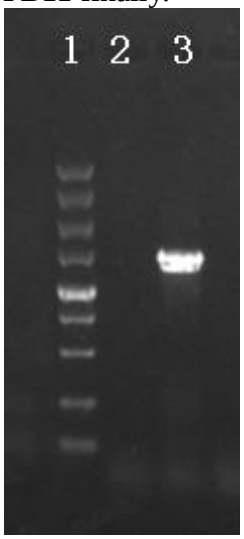

Figure.2PCR product of 16S rDNA

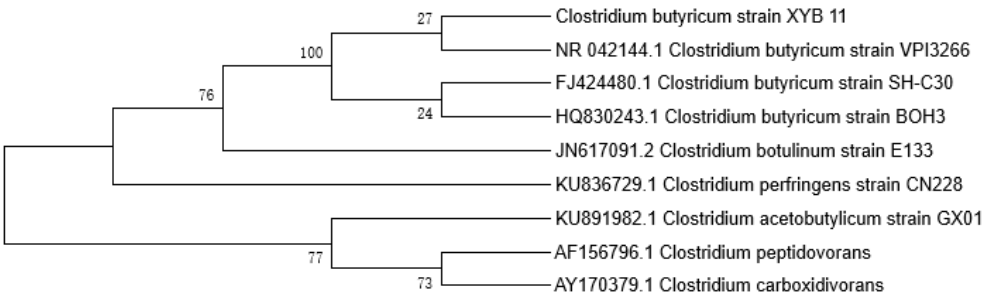

Figure. 3 The Phylogenetic tree analysis of XYB11

Capability of Producing 1,3-PD

RCM medium with 30 g glycerol was used for the 1,3-PD fermentation. After 24 h fermentation,1.3 g of 1,3-PD was produced by *C. butyricum*XYB11, and the yield of 1,3-PD from glycerol was 0.16g/g.The isolated strain of *C. butyricum*XYB11 had the capacity of producing 1,3-PD.

Conclusion

Via preliminary screening based on morphology, physiology and biochemistry, a gram-negative strain named XYB11 with rod-shape was obtained from the soil sample. Subsequently, this strain was further identified as *C. butyricum* XYB11 by 16S rDNA sequencing and BLAST.This strain had the capability of producing 1, 3-PD.

Acknowledgment

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