Characterization and Succinic Acid Production of Anaerobic Bacteria *Clostridium* SP17-B1

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***Abstract*—*Clostridium* SP17-B1 isolated from dog saliva was Gram-positive rod-shaped, facultative anaerobe bacteria. It grew in 4% (w/v) NaCl, at a pH range of 5.0–8.0 (optimally at pH 7) and at 30–40 °C (optimally at 37 °C). Major cellular fatty acids were $C_{16:0}$, $C_{17:0}$ cyclo, $C_{16:1}$ w9c, and $C_{18:1}$ w9c of 36.33, 9.67, 13.85 and 10.74% of total component, respectively. The DNA G+C content of the type strain was 40.84 mol%. Based on the result of 16S rRNA gene sequence analysis. It was closely related to *Clostridium amygdalinum* BR-10^T (97.84%), *Clostridium saccharolyticum* WM1^T (97.76%) and *Clostridium celleracrescens* DSM 5628^T (97.69%). There were few researches studied on the production of succinic acid by the genus of *Clostridium. Clostridium* SP17-B1 was able to produce succinic acid 25.07 g/L from 40 g/L of glucose as a substrate. The application of lignocellulose as a carbon source for succinic production from this strain is under studied.**

***Index Terms*—*Clostridium* SP17-B1, *Clostridium amygdalinum*, *Clostridium celleracrescens*, *Clostridium saccharolyticum*, succinic acid**

I. Introduction

Clostridium species were gram positive bacteria, rod-shaped, obligate anaerobes and capable of producing endospores. The discoveries of *Clostridium* species inhabit were in soils and the intestinal tract of animals, including humans. Historically, *Clostridium* sp. was similar to *Bacillus* sp., however C*lostridium* grew only in anaerobic conditions, but *Bacillus* grew well in aerobic conditions. In 1924, Bengtson separated van Ermengem's microorganisms from the *Bacillus* group and assigned them to a new genus, *Clostridium* [1].

The most of *Clostridium* sp. provided few commercial benefits to industry. *Clostridium acetylbutylicum* and *Clostridium histolyticum* were two exceptions. *C. acetylbutylicum* produced the industrial solvents acetone and n-butanol from fermentation of the sugar glucose. *C. histolyticum* was used as a source of the enzyme collagenase, which degrades animal tissue. *C. aerotolerans* DSM 5434 was isolated from the rumen of a sheep, *C. celerecrescens* DSM 5628 was isolated from a methanogenic culture started with a cow manure inoculum, and *C. xylanolyticum* DSM 6555 was isolated from *Pinus patula* chip pile [2], [3]. These strains could transform some cinnamic acid compounds. *C. methoxybenzovorans* DSM 121823^T was isolated from a methanogenic pilot-scale digester fed with olive mill wastewater (Sfax, Tunisia) [4]. It produced H_2, CO_2, formate, acetate and ethanol as the end product. However, the research of *Clostridium* for succinic acid production was neglected. There were few researches that reported the genus of *Clostridium* for succinic acid production such as *C. thermosuccinogenes* [5]) and *C. coccoides* [6].

In this research, we described the morphology, phenotypic and phylogenetic characteristics of the strain SP17-B1 isolated from dog saliva and the ability for succinic acid production.

II. Materials and Methods

A. Microorganism and Isolation of the Strain

The strain SP17-B1 in this study was isolated by Phuengjayaem *et al.* [7]. *C. amygdalinum* DSM 12857, *C. celerecrescens* DSM 5628 and *C. saccharolyticum* DSM 2544 were obtained from the DSMZ (Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany). The Tryptic Soy Broth (TSB) (Difco, France) was used for preparation the inoculum. Then 10 % of inoculum was transferred to Reinforced Clostridial Medium (RCM; Difco, France) and cultivated at 37 ˚C, 200 rpm under the anaerobic condition using Anaero Pack (Mitsubishi Gas Chemical, Japan).

B. Morphological Characterization of the Strain SP17-B1

The morphology of colony including; color, shape, margin, optical property, and elevation were observed on Tryptic Soy Agar plate (TSA) (Difco, France) after cultivation under the anaerobic condition. Cell morphology of the isolate was investigated with a light microscopy (model Nikon YS2-H, Japan) and scanning electron microscopy (model JSM-5410 LV; JEOL, Japan). Sample for scanning electron microscopy was prepared as

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described by Kudo *et al*. [8]. Gram staining was done according to standard procedures [9]. Flagella staining were described by Forbes [10]. The cellular fatty acids were analyzed using GC according to the Microbial Identification System (Sherlock Microbial Identification System (MIDI) [11].

C. Physiological and Biochemical Tests of the Strain SP17-B1

The determination of optimal temperature and pH were studied. The strain SP17-B1 was cultivated in a RCM medium with initial pH values in the range 5.0-9.0 and incubated at 20-60 ºC. The following carbohydrates were tested as carbon sources including; D-glucose, D-mannitol, D-lactose, D-saccharose (sucrose), D-maltose, salicin, D-xylose, L-arabinose, glycerol, D-cellobiose, D-mannose, D-melezitose, D-raffinose, D-sorbitol, L-rhamnose and D-trehalose. In addition to the hydrolysis reaction of gelatin (bovine origin) and esculin ferric citrate were tested by API 20A biochemical kits (Identification system for Anaerobic bacteria; BioMe´rieux; France) after cultivation this strain in the medium of 20A medium for 24-48 h. To test for the presence of catalase, cell material was exposed to 10% H_2O_2. The activity of enzymes including; alkaline phosphatase, esterase (C 4), esterase lipase (C 8), lipase (C 14), leucine arylamidase, valine arylamidase, cystine arylamidase, trypsin, α-chymotrypsin, acid phosphatase, naphthol-AS-BI-phosphohydrolase, α-galactosidase, β-galactosidase, β-glucuronidase, α-glucosidase, β-glucosidase, N-acetyl-β-glucosaminidase, α-mannosidase and α-fucosidase were determined after growth on TSA (Difco, France) at 37 ºC for 48 h using the API ZYM systems (System for the research of enzymatic activity; BioMe´rieux; France), according to the manufacturer's instructions.

D. The 16S rRNA Gene of the Strain SP17-B1

The nucleotide sequence of 16S rRNA gene was done by PCR with two primers (20F: 5'-AGTTTGATCCTGGCTC-3' and 1530R: 5'-AAGGAGGTGATCCAGCC3'). The PCR products were DNA sequenced (Macrogen) using universal primers [12]. The 16S rRNA sequences were analyzed using the BioEdit software version 7.0.2 and BLAST analysis was performed using the EzTaxon database [13]. A phylogenetic tree was constructed by maximum likelihood method using the program MEGA version 6 [14]. A bootstrap analysis of Felsenstein [15] was performed to determine confidence values of individual branches in the phylogenetic tree with 1000 replications.

E. Isolation of Genomic DNA

Bacterial strains were cultivated under the anaerobic condition in RCM broth on a rotary shaker at 200 rpm at 37 ºC for 48 h under the anaerobic condition. Then bacterial cells were harvested by refrigerated centrifuge at 12,000 ×g (model 6500, Kubota, Japan). Wet biomass was washed with a solution containing 0.15 M NaCl and 0.1 M EDTA, pH 8.0, and transferred to fresh buffer and added 10 mg/ml egg white lysozyme (Sigma, Germany) for the bacterial cell wall digested then incubation at 37 °C for 2 h and subsequent addition of 1% SDS and buffer B 0.15 M of Tris-HCl. Precipitate the DNA helix by added absolute ethanol then wash DNA by 70% EtOH and 95% EtOH, respectively. The DNA were dissolved in 0.1×SSC and stores at 4 °C. Further purification was carried out according to the method of Saito and Miura [16].

F. DNA G+C Content Determination

The percentage of guanine-cytosine content was determined by HPLC [17]. The G+C content (mol%) was calculated by following (1):

$$\frac{\left[\frac{G_S}{G_r}+\frac{C_S}{C_r}\right]}{\left[\frac{A_S}{A_r}+\frac{G_S}{G_r}+\frac{T_S}{T_r}+\frac{C_S}{C_r}\right]} \times 100 \quad (1)$$

where A_s is the peak area of adenine of sample, A_r is the peak area of adenine of reference, C_s is the peak area of cytosine of sample, C_r is the peak area of cytosine of reference, G_s is the peak area of guanine of sample, G_r is the peak area of guanine of reference, T_s is the peak area of thymine of sample and T_r is the peak area of thymine of reference.

G. DNA–DNA Hybridizations

DNA–DNA hybridization tests were performed as described by Spierings *et al*. [18].

H. Succinic Acid Production by the Strain SP17-B1

The inoculum consisted of 50 ml of 3% tryptic soy broth (TSB) medium (Difco, France). The inoculated culture was cultivated at 37°C at 200 rpm under anaerobic conditions for 24 h. The fermentation was conducted in a 250 ml flask with 50 ml of the production medium containing (g/L) yeast extract, 30; urea, 2.0, $MgCl_2.6H_2O$, 2; $CaCl_2$, 1.5; $MnCl_2$, 0.07; Na_2HPO_4, 4.4; NaH_2PO_4, 3.3; $MgCO_3$, 30 and the pH was adjusted to 7 [19]. Biotin 0.3 µg/L and thiamin 0.2 µg/L were added after being sterilized by membrane filtration (0.22 µm, Millipore Express, Ireland). Glucose was separately sterilized at 115°C for 20 minutes and added to the medium to maintain the initial concentration of 60 g/L glucose. It was incubated at 37°C with agitation at 200 rpm for 48 h with 10% seed inoculum. Organic acid (succinic, lactic, formic and acetic acid) were analyzed by HPLC as described by Phuengjayaem *et al.* [7].

III. Results and Discussions

A. Morphological Characterization of Strain SP17-B1

Colonies of the Strain SP17-B1 on TSA agar plate were cream, convex and opaque with entire margins and reached 1-2 mm in diameter after cultivation for 48 h at 37 °C under the anaerobic condition. It was rod-shaped under light electron microscope. Cells were slightly thickened in the middle and occurred singly. Spores were oval at the middle to the end of side similar to bowling pin (Fig. 1) and whole-cell of SP17-B1 was 0.3-0.6×2.9-3.5 µm and the spore was 1.1-1.3×2.0-2.5 in size. In the early-exponential phase of growth, cells were slightly motile by means of peritrichous flagella, but older cells lost their motility.

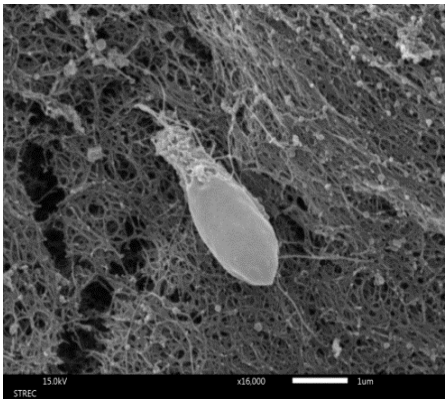

Figure 1. Morphology of the strain SP17-B1 under the Scanning Electron Microscope (SEM) with 15,000x.

B. Physiological and Biochemical Tests

Physical and biological characterization of the strain SP17-B1 compared with type strains including *C. amygdalinum* DSM 12857, *C. celerecrescens* DSM 5628 and *C. saccharolyticum* DSM 2544. These results illustrated that none of the strains hydrolyzed nitrate, esculin ferric citrate and urease [3], [20], [21], while only the strain SP17-B1 gave positive result of arginine hydrolysis. Both strains of the strain SP17-B1 and *C. amygdalinum* DSM 12857 capable hydrolyzed starch.

The results of carbohydrates utilization were shown that all strain could utilize D-glucose, D-lactose, D-saccharose (sucrose), D-maltose, salicin, L-arabinose, D-cellobiose, D-mannose and D-trehalose while the ability of other carbohydrates utilization were varied (Table I).

TABLE I. CHARACTERISTICS USEFUL FOR DISTINGUISHING STRAIN SP17-B1 AND RELATED SPECIES OF THE GENUS CLOSTRIDIUM SP

Characteristics	**1**	**2**	**3**	**4**
Cell form	Rods	Rods	Rods	Rods
Arginine hydrolysis	-	-	No	No
Starch hydrolysis	+	+	-	-
Catalase	+	-	-	-
Indole formation	-	+	-	+
Gelatin hydrolysis	-	-	-	+
Optimum pH	5.0-8.0 (optimum 7.0)	6.5-8.0 (optimum 7.0)	6.0-8.8 (optimum 7.5)	7-8 (optimum 7.0)
Temperatue	30-40 ◦C	20-60 ◦C	17-43	30-37
Optimum temperature	37 ◦C	45 ◦C	37 ◦C	37 ◦C
Utilization of:				
D-mannitol	+	+	+	+
D-xylose	+	+	+	+
glycerol	+	-	+ (w)	+
D-melezitose	+	+	-	+
D-raffinose	-	+	-	+
D-sorbitol	+	+ (w)	+	+
L-rhamnose	+ (w)	+	+	+
API zym				
Esterase Lipase (C 8)	++	+(w)	-	-
Lipase (C 14)	-	-	+(w)	-
Leucine arylamidase	++	-	+(w)	+(w)
Valine arylamidase	+(w)	-	-	-
Cystine arylamidase	-	-	-	-
Trypsin	+	+(w)	-	+(w)
α-chymotrypsin	+	-	+(w)	-
α-galactosidase	-	-	-	++
β-galactosidase	++	-	-	-
β-glucuronidase	-	+	-	-
β-glucosidase	++	++	-	-
N-acetyl-β-glucosaminidase	-	+(w)	+(w)	-
DNA G+C content (mol%)	38.65	32	45	43.9
Genome		No	4,662,87 -4,662,871	5,170,849 - 5,170,849

Strains: 1, SP17-B1; 2, *C. amygdalinum* DSM 12857 [21] ; 3, *C. saccharolyticum* DSM 2544 [20]; 4, *C. celerecrescens* DSM 5628 [3]; All data were obtained from this study. +, Positive reaction; w, weakly; -, negative reaction.

In the enzyme activity by API ZYM showed that alkaline phosphatase, Esterase (C 4), acid phosphatase, naphthol-AS-BI-phosphohydrolase and α-glucosidase were positive in all strains. Likewise, cystine arylamidase, α-mannosidase and α-fucosidase were negative in all strains. The different results of the enzyme activities were shown in Table I.

C. DNA G+C Content Determination

The DNA G+C content of the strain SP17-B1 was 40.84 mol%, which was in the range of species of the genus *Clostridium* with validly published names (Table 1).

D. The 16S rRNA Gene of the Isolate

Almost complete 16S rRNA gene sequences the strain SP17-B1 (1398 bp) was the highest closely related to *Clostridium amygdalinum* BR-10^T (97.84%), *Clostridium saccharolyticum* WM1^T (97.76%), *Clostridium celleracrescens* DSM 5628^T (97.69%).

The phylogenetic trees based on 16S rRNA gene sequences using maximum-likelihood algorithms (Fig. 2) and neighbour-joining phylogenetic tree showed that the strain SP17-B1 form a tight cluster with the type strains of the genus *Clostridium*.

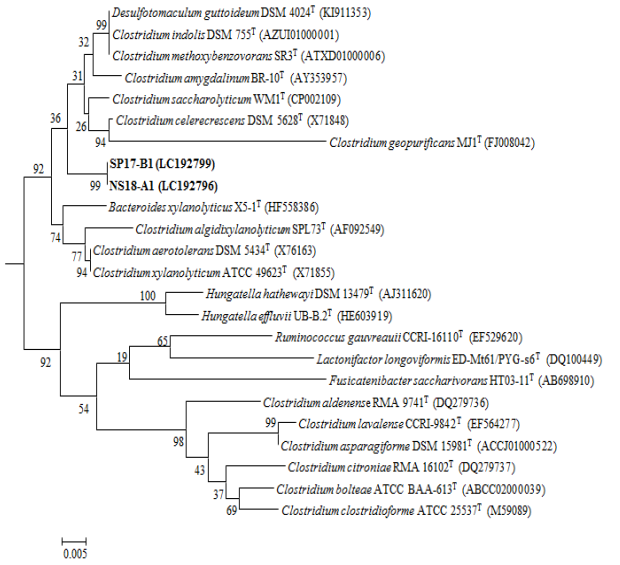

Figure 2. Maximum Likelihood phylogenetic tree base on 16S rRNA gene sequences showing the relationship between the strain SP17-B1 and other related strains. Bootstrap values were expressed as percentages of 1000 replication.

E. Fatty Acid Determination

The dominant components of the strain SP17-B1 was $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{17:0}$ cyclo, $C_{18:0}$, $C_{19:0}$ cyclo w8c, $C_{16:1}$ w9c, and $C_{18:1}$ w9c of 3.24, 8.23, 36.33, 9.67, 2.21, 3.39, 13.85, and 10.74% of total component, respectively. All the type strains showed the same fatty acid profiles, the distinctive fatty acids were $C_{12:0}$, $C_{14:0}$, $C_{16:0}$, $C_{18:0}$ and $C_{18:1}$ w9c. The major fatty acid profile of the strain SP17-B1 closely related to *C. amygdalinum* DSM 12857. While particular differences in relative numbers of $C_{15:0}$ 2OH, $C_{17:0}$ cyclo, $C_{17:0}$ 2OH, $C_{17:0}$ anteiso, $C_{19:0}$ cyclo w8c, $C_{16:1}$ w9c, iso-$C_{15:0}$, Summed in feature 3, Summed in feature 7, and Summed in feature 8 could be observed (data not shown).

Especially, $C_{17:0}$ cyclo and $C_{16:1}$ w9c of the strain were 10 fold higher than those of *C. amygdalinum* DSM 12857. On the other hand, $C_{17:0}$ 2OH, summed in feature 3 and Summed in feature 8 were not observed in the strain SP17-B1 but high content in *C. amygdalinum* DSM 12857 (data not shown).

F. DNA–DNA Hybridizations

The level of DNA–DNA relatedness values between the strain SP17-B1 with each other. The identical level of DNA–DNA relatedness values between the strain SP17-B1 and *C.* amygdalinum DSM 12857 (36.14%), *C. saccharolyticum* DSM 2544 (40.71%) and *C. celerecrescens* DSM 5628 (35.83%) gave low levels of DNA–DNA relatedness.

These values were lower than the 70% cut off level for assigning strains to the same species [22] and indicated that strains SP17-B1 may be a member of novel species.

The ultimate of these results, the strain SP17-B1 could be different from C. amygdalinum DSM 12857, C. saccharolyticum DSM 2544 and C. celleracrescens DSM 5628 in terms of their phenotypic, biochemical and growth characteristics, enzyme activity as well as the genotypic data described above. In addition, based on the BLAST analysis of 16S rRNA sequence and DNA–DNA hybridization, the strain SP17-B1 was distinct from species of the genus Clostridium with validly published names. Therefore, Clostridium SP17-B1 should be deeply study for a novel species of the genus Clostridium.

G. Description of Clostridium SP17-B1

Clostridium SP17-B1 was isolated from dog saliva. It was anaerobic, Gram-positive rod shaped. Spore forming was oval at the middle to the end of side similar to bowling pin, which was motile with peritrichous flagella but older cells lost their motility. Whole-cell of SP17-B1 was 0.3-0.6×2.9-3.5 µm and the spore was 1.1-1.3×2.0-2.5 in size after incubation at 37 °C on RCM medium for 48 h. The early stage of growth, cream color, circular (0.5–1.0 mm in diameter), convex and translucent colonies were distinguished in 24 h after that cell had cream, circular (1.0–2.0 mm in diameter), convex and opaque with entire margins after incubation at 37 °C on RCM medium for 48 h. The hydrolyzed nitrate, esculin ferric citrate, urease, arginine hydrolysis, indole formation, gelatin hydrolysis and catalase were negative while it was positive result of starch hydrolysis similar to type strain *Clostridium amygdalinum* DSM 12857. Growth was observed at a pH in the range 5.0–9.0, 30-45 °C and in 4% (w/v) NaCl. The optimum for cell growth was pH 7.0, 37 °C but it was not growth at pH lower that 4.0, 50 °C and in 6% (w/v) NaCl. It was ability to utilize carbohydrates including; D-glucose, D-lactose, D-saccharose (sucrose), D-maltose, salicin, L-arabinose, D-cellobiose, D-mannose, D-raffinose and D-trehalose but not D-mannitol, D-xylose, glycerol, D-melezitose, D-sorbitol and L-rhamnose. In case the enzyme activity tested by API ZYM, alkaline phosphatase, esterase (C 4), esterase Lipase (C 8), leucine arylamidase, valine arylamidase (weak), α-chymotrypsin, acid

phosphatase, naphthol-AS-BI-phosphohydrolase, β-galactosidase, α-glucosidase and β-glucosidase were positive. Lipase (C 14), Cystine arylamidase, Trypsin, α-galactosidase, β-glucuronidase, N-acetyl-β-glucosaminidase and α-fucosidase were negative. Major cellular fatty acids were $C_{16:0}$, $C_{17:0}$ cyclo, $C_{16:1}$ w9c, and $C_{18:1}$ w9c. The DNA G+C content of the type strain was 40.84 mol%.

H. Succinic Acid Production by Clostridium SP17-B1

Clostridium SP17-B1 was a facultative bacterium that can grow well under anaerobic condition and survive in limit aeration condition. When *Clostridium* SP17-B1 was cultured under anaerobic conditions after 24 h, succinic acid (25.07 g/L) was produced as a major product. Formic (9.75 g/L) with a small amount of acetic acid (4.20 g/L) were also produced. Maximum succinic acid of 12.44, 17.99 and 24.18 g/L were obtained from 20, 30 and 40 g/L of glucose as a carbon source, respectively, at 24 h of cultivation time. Likewise, glucose was completely consumed in 24 h. The maximum cell growth (OD660) of 6.462, 7.182 and 4.404 were obtained from glucose of 20, 30 and 40 g/L at 36 h of cultivation time (data not shown).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

Dr. Siriluk Teeradakorn analyzed the data and wrote the paper; Miss Sukanya Phuengjayaem conducted the research; Professor Somboon Tanasupawat gave valuable suggestion and comment; all authors had approved the final version.

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