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Characterization and fermentation products of *Clostridium butyricum* strains isolated from Thai soils

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ABSTRACT

Five acid forming bacteria, SK3-3, SK3-6B, SK3-7B, SK13-3 and PL20-4S were isolated from soils collected in Samut Songkhram and Phitsanulok provinces. All isolates were Gram-positive, anaerobic, spore-forming, rod-shaped bacteria. The isolates were screened for their end product fermentation and were identified based on their phenotypic characteristics and 16S rRNA gene sequence analyses. They were belonged to the genus *Clostridium* and were closely related to *Clostridium butyricum* DSM 10702^T (99.7-100%) and *Clostridium saccharoperbutylacetonicum* N1-4^T (98.0-98.2%) based on 16S rRNA gene sequence similarity. All 5 isolates were identified as *Clostridium butyricum.* They produced 4.51-8.90 g/L (19.40-54.82% yield) of L-lactic acid with 0.06-0.12 g/L/h productivity, 6.15-7.52 g/L of acetic acid and 24.32-29.67 g/L of ethanol as the end product fermentation.

INTRODUCTION

Clostridium butyricum is a mesophilic, strictly anaerobic endospore-forming Gram-positive rods. They are distributed in soils, sediments, the decaying heartwood of living trees, the stool of healthy children and adults, and soured milk and cheeses (Cato *et al.*, 1986; Meng *et al.*, 1997; Wiegel *et al.*, 2006). They are not uncommonly reported as a human pathogen, however they have been widely used as a probiotic for humans and animals in Asian countries (Seki *et al.*, 2003). These bacteria could inhibit the growth of various enteropathogens (Kuroiwa *et al.*, 1990) and prevent antibiotic-associated diarrhea (Seki *et al.*, 2003). On the other hand, *C. butyricum* strain has been reported to be responsible for enterohemorrhagic *Escherichia coli* and neonatal necrotizing enterocolitis (Howard *et al.*, 1977; Takahashi *et al.*, 2004). In addition, type E botulinum toxin-producing *C. butyricum* has been reported (Aureli *et al.*, 1986). *Clostridium* strains exhibit mixed acid and alcohol fermentations. They form butyric acid, varying concentrations of acetic acid, lactic acid and/or ethanol, propanol or butanol. They produce a main alcohol propanediol when grew on glycerol (Wiegel *et al.*, 2006). In the course of our investigation of acid producing bacteria from soils in Thailand, 5 isolates of Gram-positive, anaerobic, endospore-forming, rod-shaped bacteria were isolated and screened. They were characterized based on the phenotypic characteristics and 16S rRNA gene sequencing including their end product fermentation analysis.

MATERIALS AND METHODS

Sources and Isolation methods

The soils samples were collected from Samut Songkhram and Phitsanulok provinces in Thailand (Table. 1). A 0.25 gram of sample was enriched in 5 ml MRS broth (De Man *et al.*, 1960) and incubated under anaerobic conditions at 37°C for 3 days. The isolates were streaked on MRS agar plate containing $CaCO_3$ (0.5%) and incubated at the same temperature until the colonies developed. They were picked up and purified on MRS or GYP $CaCO_3$ agar plate (Prasirtsak *et al.*, 2013) and then kept on the slant at 4 °C for further study.

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Identification methods

Phenotypic characterization

Phenotypic characteristics such as morphological and cultural of the isolates were observed on the cells grew on GYP $CaCO_3$ agar plate after incubated under anaerobic conditions at 37°C for 3 days.

Cell morphology was observed under a JEOL JSM-5410LV scanning electron microscope. Gram reaction, spore formation, gas formation, catalase activity, nitrate reduction, hydrolysis of arginine and starch, growth at different temperatures (15- 50°C), at different pH values (4-8.5) and in different NaCl concentrations (%,w/v) were performed as described by Tanasupawat *et al.*(1992; 1998). Acid formation from various carbohydrates were determined as described by Tanasupawat *et al.* (1998).

The isomer of lactic acid produced by each strain was analyzed by using high-performance liquid chromatography (Prasirtsak *et al*., 2013).

Genotypic characterization

The 16S rRNA gene was PCR amplified using primers 27F (5'-AGAGTTTGATCMTGGCTCAG-3'), 518F (5'-CCAGCAGCCGCGGTAATACG-3'), 800R (5'-TACCAGGGTATCTAATCC-3') and 1492R (5'-TACGGYTACCTTGTTACGACTT-3'). The amplified 16S rRNA gene sequence was analyzed by Macrogen®, Korea. Sequence alignment was employed using the BLAST software from the Gen Bank. Multiple alignments of the sequences determined were performed with a program CLUSTAL_X (version 1.83; Thompson *et al*., 1997).

Gaps and ambiguous bases were eliminated prior to construction of a phylogenetic tree. A phylogenetic tree was constructed by the neighbour-joining method (Saitou and Nei, 1987) with the program MEGA version 5.05 (Tamura *et al.*, 2011). The confidence values of individual branches in the phylogenetic tree were determined by using the bootstrap analysis of Felsenstein (1985) based on 1000 replications.

Determination of end product fermentation

The isolates grew on GYP $CaCO_3$ slant at 37°C for 2 days were transferred to GYP preculture medium with glucose 10 g/L at 37°C for 2 days. After that the preculture broth was transferred into the fermentation medium containing glucose 120 g/L and incubated at the same temperature for 3 days. At the end of fermentation, the supernatant was collected for the analysis of lactic acid and the remained glucose using high-performance liquid chromatography (HPLC; Biorad, Aminex HPX-87H ion exclusion organic acid column, 300mm x 7.8mm) maintained at 45°C in a column oven (Shimadzu-CTO-6A). An eluent, 0.005 M H_2SO_4, was pumped through the system at the flow rate of 0.6 ml/min (Shimadzu-LC-10Avp). A refractive index detector (Shimadzu-RID-10A) was used to detect the organic compound details.

RESULTS AND DISCUSSION

Four isolates, SK3-3, SK3-6B, SK3-7B and SK13-3 were isolated from soils collected in Samut Songkhram and a isolate PL20-4S was isolated from soil collected in Phitsanulok province, Thailand (Table. 1).

Table. 1: Isolate number, location, sequence similarity (%) and closest species.

Isolate no.	Province	Similarity (%)	Closest species
SK3-3	Samut Songkhram	99.8	*C. butyricum* DSM 10702^T
SK3-6B	Samut Songkhram	99.9	*C. butyricum* DSM 10702^T
SK3-7B	Samut Songkhram	99.9	*C. butyricum* DSM 10702^T
SK13-3	Samut Songkhram	100	*C. butyricum* DSM 10702^T
PL20-4S	Phitsanulok	99.7	*C. butyricum* DSM 10702^T

Fig. 1: Colonial appearance of *C. butyricum* SK13-3 grew on GYP $CaCO_3$ agar.

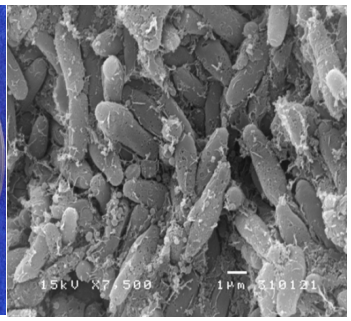

Fig. 2: Scanning electronmicrograph of *C. butyricum* SK13-3 grew on GYP $CaCO_3$ agar.

Colonial appearances of them are round, and have an undulate margin and are slightly convex, opaque and greyish-white smooth (Figure 1). Cells are straight rods with round ends and occur singly or in pairs. Spores are oval, central to subterminal and usually do not swell the cells (Figure 2). They were anaerobic Gram-positive rod-shaped bacteria. They fermented glucose to L-lactic acid heterofermentatively but produced no gas from glucose.

They showed positive reaction for starch hydrolysis but showed negative reaction for catalase, nitrate reduction and arginine hydrolysis. They grew at pH 5.5-8.5, in 0-3% NaCl, and at 20-40 °C. They produced acid from D-glucose, D-cellobiose and D-fructose. Some isolates produced acid from L-arabinose, lactose, D-maltose, D-mannitol, D-mannose, D-melibiose, sucrose, sorbitol, sorbose, D-trehalose and D-xylose the same as reported by Cato *et al*. (1986). They produced no acid from inulin, raffinose and dextran. Their variable characteristics are shown in Table 2.

Isolates SK3-3, SK3-6B and SK3-7B showed almost the same phenotypic characteristics but they were different from isolates SK13-3 and PL20-4S (Table 2). These isolates were belonged to the genus *Clostridium* based on their phenotypic characteristics (Wiegel *et al.*, 2006). They were closely related to

Clostridium butyricum DSM 10702^T with 99.7-100% and *Clostridium saccharoperbutylacetonicum* N1-4^T (98.0-98.2%) based on 16S rRNA gene sequence similarity (Figure 3).

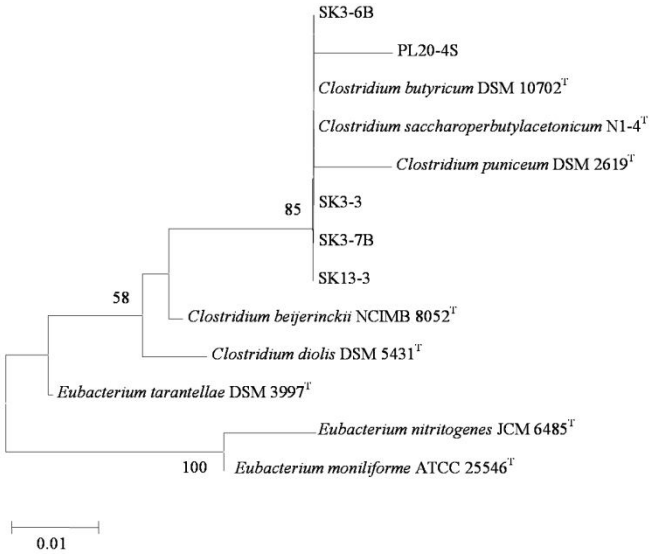

Fig. 3: Phylogenetic tree constructed using the neighbour-joining method showing the position of *C. butyricum* isolates and related species based on 16S rRNA gene sequences.

Table. 2: Phenotypic characteristics of isolates.

Characteristics	SK3-3	SK3-6B	SK3-7B	SK13-3	PL20-4S
Cell form	Rods	Rods	Rods	Rods	Rods
Growth temp. (°C)	20-40	15-45	20-45	20-45	15-45
Growth in NaCl (%)	0-5	0-5	0-7	0-3	0-3
Growth at pH	5.5-8.5	5.5-8.5	5.5-8.5	5.5-8.5	5.5-8.5
Acid from					
L-Arabinose	+	-	-	-	-
D-Galactose	-	+	+	-	-
Lactose	+	+	+	-	-
D-Maltose	+	+	+	-	+
D-Mannitol	+	-	-	-	-
D-Mannose	-	+	+	+	+
D-Melibiose	-	+	+	-	-
D-Sorbitol	+	-	-	-	+
Sorbose	-	-	+	-	-
Sucrose	+	+	+	-	-
D-Trehalose	+	+	+	+	-
D-Xylose	+	-	-	-	-

Table 3: Lactic acid, acetic acid, ethanol and remained glucose of isolates.

	Concentration (g/L)				Lactic acid	
Strain no.	Remained glucose	Final lactic acid	Acetic acid	Ethanol	Productivity (g/L.h)	Yield (%)
SK3-3	95.41	5.31	6.67	28.34	0.07	21.60
SK3-6B	111.78	4.51	6.64	29.67	0.06	54.82
SK3-7B	95.36	4.78	7.52	24.32	0.07	19.40
SK13-3	88.78	8.90	6.85	25.47	0.12	28.51
PL20-4S	93.75	5.59	6.15	25.35	0.08	21.30

Therefore, they were identified as *Clostridium butyricum* (Wiegel *et al.*, 2006). The identification of *C. butyricum* strains has been reported using the PCR primer designed from the unique inserted sequence in type B strain to differentiate probiotic strains at the biovar level (Nakanishi *et al.*, 2005). However, in this study we identified them using the phenotypic characteristics and 16S rRNA gene sequence analysis.

Isolates SK3-3, SK3-6B, SK3-7B, SK13-3 and PL20-4S produced small amount of L-lactic acid, 4.51-8.90 g/L (19.40-54.82% yield) and 0.06-0.12 g/L/h productivity; 6.15-7.52 g/L of acetic acid and 24.32-29.67 g/L of ethanol (Table 3; Figures 4 and 5).

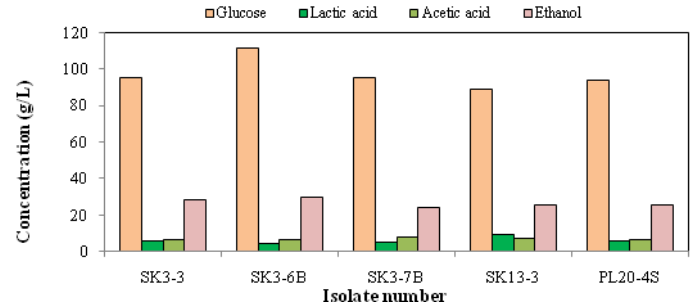

Fig. 4: Fermentation products; lactic acid, acetic acid, ethanol and remained glucose **(**g/L**)** of isolates.

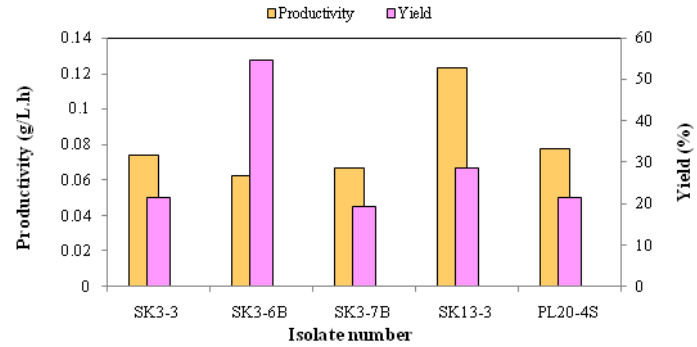

Fig. 5: Yields of lactic acid and productivities of isolates. Each experiment was performed at 37 °C, 72 h in GYP broth containing 120 g/L glucose.

In the previous reports, *C. butyricum* strains were isolated from soils, fresh water and marine sediments, wood, animal and human feces, clinical specimens, soured milk, and cheeses (Cato *et al*., 1986; Wiegel *et al.*, 2006). They produced butyric acid, acetic acid and formic acid, and sometime lactic acid and succinic acid, including butanol and ethanol when peptone-yeast extract-glucose (PYG) broth was used for the cultivation (Cato *et al*., 1986). *C. butyricum* strains are involved in organic acid fermentation mainly butyric acid that was used in perfumes, as a food additive, and as an intermediate in alternative fuels (Zigova *et al.*, 1999). In addition, in making alternative fuels from biomass feedstocks, the production of butyric acid is a key intermediate in the two-step production of butanol (Du *et al*., 2012). The production of 1,3-propanediol (1,3-PD) by several groups of bacteria including *C. butyricum* strains has been reported for a long time (Abbad-Andaloussi *et al*., 1995). Some *C. butyricum* strains are used as probiotics as beneficial bacteria whereas a few strains have been reported to be pathogenic (Seki *et al.*, 2003). In Thailand, we are the first research group to report *C. butyricum* strains isolated from soils and their fermentation products, however our bacterial strains are needed for further study on their application on butyric acid fermentation and probiotic activity.

CONCLUSION

In conclusion, we found five anaerobic Gram-positive, endospore forming, rod-shaped bacteria that produced L-lactic acid, acetic acid and ethanol as the end products. They were isolated from soils collected in Samut Songkhram and Phitsanulok provinces, Thailand and they were identified as *C. butyricum* based on their phenotypic characteristics and 16S rRNA gene sequence similarity including their fermentation products. The isolates produced 4.51-8.90 g/L and 0.06-0.12 g/L/h of productivity of L-lactic acid, 6.15-7.52 g/L of acetic acid and 24.32-29.67 g/L of ethanol.

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