Songklanakarin J. Sci. Technol.
40 (6), 1281-1290, Nov. – Dec. 2018

http://www.sjst.psu.ac.th

Original Article

Optimization of succinic acid production by succinic acid bacteria isolated in Thailand

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Received: 27 December 2016; Revised: 5 June 2017; Accepted: 4 August 2017

Abstract

Two hundred succinic acid-producing bacteria were isolated from cattle dung, soil, bovine rumen, and tree bark collected in Thailand. Eighty-four isolates exhibited a clear zone on the screening plate. The production was analyzed qualitatively by the TLC method and quantitatively by the HPLC method. The succinic acid was in the range of 0.003-0.97 g/g glucose. Only 28 isolates were selected based on morphology, cultural and phenotypic characteristics. The isolates were divided into three groups. Twenty-three isolates in Group I were identified as *Enterococcus*, 2 isolates in Group II were identified as *Lactobacillus*, and 3 isolates in Group III were identified as *Clostridium*. *Enterococcus durans* (isolate NS15-dA1) and *Enterococcus hirae* (isolate NS15-bA2) were selected for further study for succinic acid production. The optimum conditions were 60 g/L of glucose, 30 g/L of yeast extract (NS15-dA1), and 30 g/L of tryptone (NS15-bA2) at pH 7.0 and 37 °C. The highest amounts of succinic acid were obtained from the isolates NS15-dA1 (51.69±0.17 g/L) and NS15-bA2 (53.05±0.35 g/L).

Keywords: *Clostridium*, *Enterococcus*, *Lactobacillus*, succinic acid, 16S rRNA gene sequence

1. Introduction

Succinic acid is a dicarboxylic acid and a common natural organic acid present in humans, animals, plants, and microorganisms (Andersson *et al.*, 2009). It can be used as a precursor to produce many important commodity chemicals, as an ingredient in animal feeds, and to stimulate growth in plants (Wan *et al.*, 2008). Succinic acid can be produced either by a chemical process using petrochemical sources or by a biological process in which bacteria anaerobically produce succinic acid through fermentation. Succinic acid is an intermediate product of the reductive tricarboxylic acid cycle. The biological process is simpler and environmentally friendly (Zeikus, 1980).

Succinic acid has been reported to be produced and accumulated by anaerobic and facultative microorganisms as a product from their metabolic pathways (Song & Lee, 2006). As a result, anaerobic and facultative microorganisms are screened for succinic acid production. The microorganisms include *Succinatimonas hippei* isolated from human feces, which produced 1.94 g/L of succinic acid from 10 g/L of glucose (Morotomi *et al.*, 2010), *Klebsiella pneumoniae* from buffalo rumen fluid, which produced 2.1 g/L of succinic acid from 10 g/L of glucose (Thakker *et al.*, 2006), *Actinobacillus*

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succinogenes from bovine rumen, which produced 35.5 g/L of succinic acid from 50 g/L of glucose (Chen *et al.,* 2011), and *Mannheimia succiniciproducens* from bovine rumen, which produced 13.5 g/L of succinic acid from 20 g/L of glucose (Lee *et al.,* 2002). Most of the known succinic acid-producing bacteria have been screened from the rumen of ruminants. The rumen contains billions of microbes that help with digestion. The ability of bacteria to produce succinic acid can be different depending on certain factors. The environment showed a significant effect on succinic acid production of bacteria including media components. Also, fermentation parameters such as temperature and pH also showed significant effects on succinate production which should be optimized according to the strain. The objectives of this research were to screen and characterize potential succinic acid-producing bacteria from cattle dung, soil, bovine rumen from a slaughterhouse, and tree bark in Thailand. The optimum conditions for succinic acid production from the potential isolates were also studied.

2. Materials and Methods

2.1 Samples and preparation

Samples were collected from various sources that included cattle dung in Surin (SRI-II), Suphanburi (SPI-II), and Nakhonsawan provinces (NS1-12), soil in Suphanburi (SP4-10), bovine rumen from a slaughterhouse in Nakhon sawan (NS13-15), and tree bark (AY1-5) in Ayutthaya, Thailand. Samples from bovine rumen were blended with sterile water (five-fold dilution) to create a suspension before use.

2.2 Screening of succinic acid producing bacteria

Three grams of cattle dung, soil, and tree bark and 50 µL of bovine rumen suspension were enriched in 5 mL medium that contained 20 g/L glucose, 5 g/L polypeptone, 5 g/L K_2HPO_4, 3 g/L yeast extract, 2 g/L NaCl, 2 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2.2H_2O$, 0.4 g/L $MgCl_2.6H_2O$, and 15 g/L $MgCO_3$ (Lee *et al.,* 2002). They were incubated under anaerobic conditions using an anaerobic pack (MGC, Japan) at 37 °C. After 72 h, 50 µL of samples were spread onto a modified Gifu anaerobic medium (GAM; Nissui Pharmaceutical) with an additional 15 g/L of agar. Plates were incubated at 37 °C for 72 h under anaerobic conditions. Single colonies were selected, streaked on GAM agar, and incubated in the same condition as previously stated. Subsequently, single colonies were picked and transferred to a screening medium for succinic acid production. The screening medium contained 20 g/l glucose, 10 g/L polypeptone, 3 g/L K_2HPO_4 5 g/L yeast extract, 1 g/L NaCl, 1 g/L $(NH_4)_2SO_4$, 0.2 g/L $CaCl_2.2H_2O$, 0.2 g/L $MgCl_2.6H_2O$, 15 g/L $MgCO_3$, and 15 g/L of agar (Agarwal *et al.,* 2005). Isolates with succinic acid-producing ability, as evidenced by a clear zone around the colony, were selected. Since the screening medium used $MgCO_3$, the magnesium (Mg^{2+}) reacted with the succinic acid ($C_4H_6O_4$) to produce succinate salt ($MgC_4H_4O_4$), around which a clear zone was observed.

2.3 Succinic acid production

All chemicals were purchased from Merck (Merck KGaA, Darmstadt, Germany). The selected isolates were transferred to a succinic acid-production medium containing 60 g/L glucose, 30 g/L yeast extract, 2 g/L urea, 0.07 g/L $MnCl_2$, 4.4 g/L Na_2HPO_4, 3.3 g/L NaH_2PO_4, 1.5 g/L $CaCl_2.2H_2O$, 2 g/L $MgCl_2.6H_2O$, and 30 g/L of $MgCO_3$ (Li *et al.,* 2010). The isolates were incubated at 37 °C for 72 h under anaerobic conditions. After the isolates produced succinic acid, the culture broth was centrifuged at 10,000 rpm for 5 min. The supernatants were analyzed for the presence of succinic acid using thin-layer chromatography (TLC) for the qualitative test and high-performance liquid chromatography (HPLC) for the quantitative test (Agarwal *et al.,* 2005). The solvent system in TLC included ethanol, ammonium hydroxide, and water (20:5:3 v/v). A standard solution (1 mg/mL) of succinic acid was used as a reference. The HPLC system (LC-10AD, Shimadzu Corporation, Japan) was equipped with a cation-exclusion column (Biorad, Aminex HPX-87H, 300x7.8 mm) and maintained at 55 °C in a column oven (Shimadzu-CTO-10A). An eluent of 0.005 M H_2SO_4 was pumped through the system at a flow rate of 0.6 mL/min (Shimadzu-LC-10AD). Succinic acid was detected using a refractive index detector (Shimadzu-RID-10A).

2.4 Phenotypic characteristics determination

All chemicals for phenotypic characteristics were purchased from Wako (Wako, Japan). The morphological and cultural characteristics used in this study included Gram staining, cell morphology, and colony appearance (color, shape, margin, optical property, and elevation). The physiological characteristics included different pH values (3, 5, 7, and 9), temperatures (20-50 °C) and NaCl concentrations (2% and 6% w/v NaCl). The biochemical characteristics determined included catalase, nitrate reduction, gas production, starch hydrolysis, arginine hydrolysis, slime formation, and acid from the carbohydrates of the isolates (Tanasupawat *et al.,* 1998).

2.5 Hierarchical cluster analysis

The isolates were grouped based on their relationships among the phenotypic characteristics (38 characteristics) by cluster analysis. Hierarchical cluster analysis was conducted using SPSS for Windows version 15.0.

2.6 16s rRNA gene sequence and phylogenetic analysis

One isolate from each group with the highest succinic acid production was selected to study 16S rRNA gene sequence. The 16S rRNA gene sequence was amplified by polymerase chain reaction (PCR). The sequences of the primers used for amplification were 20F (5'-AGTTTGATCC TGGCTC-3') and 1530R (5'-AAGGAGGTGATCCAGCC-3'). The PCR product was analyzed with Macrogen® (Korea). Sequence alignment was corrected manually using BioEdit

(version 7.0.2). The sequence database contained over 1000 sequences. The sequence similarities were compared using the database from EzTaxon (www.ezbiocloud.net/eztaxon). Multiple alignments of sequences were determined with CLUSTAL_X (version 1.83). A phylogenetic tree was constructed by the neighbor-joining method with MEGA (version 6.0) (Felsenstein, 1985).

2.7 Optimization of succinic acid production in flask culture

The inoculum (30 mL, Gifu anaerobic medium [GAM; Nissui Pharmaceutical] sterile medium) was incubated at 37 °C under anaerobe conditions for 24 h and then 5% v/v of inoculum (OD_{660} of 3.2) was transferred to a succinic acid production medium in a flask and incubated under the same conditions. The succinic acid production medium contained 60 g/L glucose, 30 g/L yeast extract, 2 g/L urea, 0.07 g/L $MnCl_2$, 4.4 g/L Na_2HPO_4, 3.3 g/L NaH_2PO_4, 1.5 g/L $CaCl_2$. $2H_2O$, 2 g/L $MgCl_2.6H_2O$, and 30 g/L of $MgCO_3$ (Li *et al.,* 2010). Glucose in the succinic acid-production medium was set at three initial concentrations: 30, 60, and 90 g/L. Nine nitrogen sources (yeast extract, peptone, tryptone, urea, KNO_3, NH_4Cl, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, and NH_4NO_3) were varied. The effects of pH in the range of 5.0-8.0 and temperature in the range of 37-39 °C on succinic acid-production were studied.

2.8 Analytical methods

2.8.1 Cell growth

Cell growth was monitored by measuring the absorbance at 660 nm (OD_{660}) using a spectrophotometer. HCl (0.5 M) was added to the samples (2:1 v/v) in order to dissolve $MgCO_3$ to form soluble magnesium chloride and carbon dioxide (Lin *et al.,* 2008). Cell dry weight (CDW) was calculated from a standard curve that related the OD_{660} to CDW.

2.8.2 Reducing sugars

The culture broth was centrifuged at 10,000 rpm for 5 min. The supernatants of 50 µL were transferred to micro-tubes and 150 µL of dinitrosalicylic acid reagent was added. After that the mixture was boiled using a water bath for 5 min to a red-brown color. The mixture was cooled to room temperature and 1 mL of distilled water was added. Absorbance was recorded with a spectrophotometer at 540 nm against the blank (Miller, 1959).

2.8.3 Succinic acid

The culture broth was centrifuged at 10,000 rpm for 5 min and the supernatants were filtered with a 0.45 µm cellulose membrane. Succinic acid was determined using HPLC with a refractive index detector. The analysis was performed using a Bio-Rad Aminex-87H column. The analysis conditions were a sample volume of 20 µL, 0.005 N H_2SO_4 as a mobile phase, a flow rate of 0.6 mL/min, and a column temperature at 45 °C (Agarwal *et al.,* 2005).

3. Results and Discussions

3.1 Screening of succinic acid producing bacteria

A total of 200 bacteria were isolated: cattle dung (78); soil (49); bovine rumen (28); and tree bark (45). They were screened for succinic acid-production ability on a screening agar plate. Eighty-four isolates produced succinic acid on agar plates as evidenced by a clear zone around the colonies.

3.2 Succinic acid production

The 84 isolates were further analyzed with TLC. Standard succinic acid showed a clear yellow spot with R_f of 0.56 (data not shown). The positive isolates such as AY5-bA2, AY5-bB1, AY5-bB2, and AY5-bB4 showed an R_f of 0.56. All 84 isolates were confirmed to have succinic acid-producing ability using HPLC. Succinic acid yields were in the range of 0.003-0.97 g/g glucose.

3.3 Phenotypic characteristics determination

The morphological and cultural characteristics of the 84 isolates with succinic acid production ability were observed (Table 1). All isolates were Gram-positive. Seventy-four (No. 1-74) of the 84 isolates were facultative anaerobic, cocci in chains, with circular, entire, opaque colonies. Only 10 (No. 75-84) were rods, including eight isolates that were strict anaerobes and two isolates that were facultative anaerobes. The facultative anaerobic bacteria were able to grow in a GAM liquid tube under aerobic conditions. Strictly anaerobic bacteria were only able to grow near the bottom of the tube, where oxygen could not penetrate the medium. The 84 isolates could be divided into 5 categories: category I (30 isolates: No. 1-30); category II (26 isolates: No. 31-56); category III (18 isolates: No. 57-74); category IV (2 isolates: No. 75-76); and category V (8 isolates: No. 77-84).

In this study, most isolates were facultative anaerobes, since the sources of the isolates were exposed to air; strict anaerobes die in the presence of oxygen. Many studies reported that bacteria with succinic acid producing ability, such as *Actinobacillus succinogenes* (Chen *et al.,* 2011), *Mannheimia succiniciproducens* (Lee *et al.,* 2002), *Klebsiella pneumoniae* (Thakker *et al.,* 2006), *Basfia succiniciproducens* (Kuhnert *et al.,* 2010), and *Phascolarctobacterium succinatutens* (Watanabe *et al.,* 2012) were facultative anaerobes. Only 8 isolates were strict anaerobes. Other studies reported that bacteria with succinic acid-producing ability, such as *Anaerobiospirillum succiniciproducens* (Davis *et al.,* 1976) and *Succinatimonas hippei* (Morotomi *et al.,* 2010), were strictly anaerobic.

Isolate NS15-dA1 (Group IB) produced the highest succinic acid of 0.97 g/g glucose from all isolates which were screened from bovine rumen. Similary, *Actinobacillus succinogenes* was screened from bovine rumen which produced a succinic acid yield of 0.83 g/g glucose (Guettler *et al.,* 1999) and *Anaerobiospirilum succiniciproducens* was screened from bovine rumen which produced a succinic acid yield of 0.86 g/g glucose (Lee *et al.,* 1999).

Table 1. Morphological and cultural characteristics and succinic acid yield (g/g glucose) of 84 isolates.

No.	Sources	Provinces	Isolates	Morphological and cultural characteristics	Yield of Succinic acid (g/g glu)
1	Cattle dung	Surin	SRI-B4	Facultative anaerobic cocci in chains, colonies were grey-white, circular, entire, opaque and flat	0.12
2		Suphanburi	SPI-B2		0.78
3			SPI-B3		0.03
4			SPI-B5		0.02
5			SP4-A1		0.68
6			SP4-B2		0.47
7			SP5-A6		0.85
8			SP5-B1		0.44
9			SP5-B4		0.78
10			SP5-B5		0.63
11			SP8-A4		0.79
12			SP8-B1		0.83
13			SP9-A2		0.80
14			SP9-B1		0.32
15			SP10-B4		0.88
16		Nakhonsawan	NS1-A2		0.82
17			NS2-A3		0.79
18			NS3-B2		0.40
19			NS5-A3		0.05
20			NS7-B2		0.25
21	Bovine rumen	Nakhonsawan	NS13-aA1		0.43
22			NS14-aA2		0.87
23			NS15-aB2		0.91
24			NS13-cB2		0.66
25			NS14-dB1		0.92
26	Tree bark	Ayutthaya	AY1-aA1		0.08
27			AY2-aA1		0.86
28			AY4-aA1		0.81
29			AY5-aB1		0.83
30			AY1-bB3		0.09
Total 30 isolates					0.02-0.92
31	Cattle dung	Surin	SRI-B3	Facultative anaerobic cocci in chains, colonies were grey-white, circular, entire, opaque and raised	0.31
32		Suphanburi	SP4-A2		0.77
33			SP4-A3		0.12
34			SP4-B4		0.61
35			SP4-B5		0.68
36			SP7-B3		0.04
37			SP8-A1		0.68
38			SP9-B3		0.65
39			SP10-B1		0.28
40		Nakhonsawan	NS2-A1		0.72
41			NS2-B3		0.85
42			NS3-A1		0.79
43			NS5-B1		0.13
44			NS6-A1		0.21
45			NS6-B4		0.38
46			NS10-A1		0.30
47			NS11-A2		0.25
48			NS12-B1		0.03
49	Bovine rumen	Nakhonsawan	NS13-aA2		0.26
50			NS14-aB3		0.15
51			NS13-bB1		0.56
52			NS15-bB1		0.42
53			NS14-cB1		0.28
54			NS15-dA1		0.95
55	Tree bark	Ayutthaya	AY1-bB4		0.09
56			AY3-aA1		0.04
Total 26 isolates					0.03-0.95
57	Soil	Suphanburi	SP4-B3	Facultative anaerobic cocci in chains, colonies were white, circular, entire, opaque and raised	0.23
58			SP5-A4		0.24
59			SP6-B6		0.17
60			SP7-B1		0.39
61	Cattle dung	Nakhonsawan	NS2-A2		0.20
62			NS7-A1		0.01
63			NS8-B2		0.07
64			NS10-B1		0.42
65			NS12-B4		0.18
66			NS12-B5		0.22

Table 1. Continued.

No.	Sources	Provinces	Isolates	Morphological and cultural characteristics	Yield of Succinic acid (g/g glu)
67	Bovine rumen	Nakhonsawan	NS13-aA2	Facultative anaerobic cocci in chains, colonies were white, circular, entire, opaque and raised	0.46
68			NS14-aB4		0.56
69			NS15-bA2		0.97
70			NS15-cA1		0.52
71			NS15-dB2		0.49
72	Tree bark	Ayutthaya	AY1-bA2		0.63
73			AY3-bA1		0.64
74			AY4-bB2		0.18
Total 18 isolates					0.01-0.97
75	Tree bark	Ayutthaya	AY5-bA2	Facultative anaerobic rods, colonies were white, circular, entire, opaque and convex	0.14
76			AY5-bB6		0.12
Total 2 isolates					0.12-0.14
77	Cattle dung	Surin	SRI-B1	Strictly anaerobic rods and were spore-forming, colonies were white-yellow, circular, irregular, opaque and umbonate	0.01
78			SRI-B5		0.009
79		Nakhonsawan	NS9-B2		0.009
80	Tree bark	Ayutthaya	AY2-aB1		0.003
81			AY3-bB2		0.008
82			AY4-bA2		0.007
83			AY5-bB3		0.03
84			AY5-bB4		0.05
Total 8 isolates					0.003-0.05

3.4 Hierarchical cluster analysis

Thus 28 isolates out of 84 isolates (Table 2) were used for further study of their physiological and biochemical characteristics and for a hierarchical cluster analysis. Twenty-eight isolates were divided into 3 groups based on phenotypic characteristics as shown in the dendrogram (Figure 1). Group IA (from SP10-B4 to NS14-dB1), Group IB (from NS2-B3 to NS2-A1), and Group IC (from NS15-bA2 to AY3-bA1) were cocci in chains. Group II (AY5-bA2 and AY5-bB6) and Group III (AY5-bB3, AY5-bB4, and SRI-B1) were rods. The phenotypic characteristics of the three groups are shown in Table 3. All three groups (28 isolates) were able to grow at 20-37 ℃, pH of 5-7, in 2% and 6% NaCl (Table 3). No growth was observed at pH 3. The results of phenotypic characterization showed different reactions (Table 3).

Only one isolate from each group with the highest succinic acid production was selected to study the 16S rRNA gene sequence. Isolates NS14-dB1 (Group IA) and NS15-dA1 (Group IB) produced large amounts of succinic acid (0.92 and 0.95 g/g glucose, respectively). Isolate NS15-bA2 (Group IC) produced the highest amount of succinic acid (0.97 g/g glucose). Isolate AY5-bA2 (Group II) produced only small amounts of succinic acid (0.14 g/g glucose), while isolate AY5-bB4 (Group III) produced very little succinic acid (0.05 g/g glucose).

3.5 16s rRNA gene sequence and phylogenetic analysis

On the basis of 16S rRNA sequence analyses (Figure 2), isolate NS14-dB1 (Group IA) and isolate NS15-dA1 (Group IB) were closely related to *Enterococcus durans* CECT411^T (100% similarity). They were deposited in the DDBJ database under accession number LC271164. Isolate

Table 2. Summary of 28 isolates selected based on morphology, cultural characteristics and yield of succinic acid concentration.

Sources	Provinces	Category	Isolate no.	Yield of Succinic acid (g/g glu)
Cattle dung	Surin	V	SRI-B1	0.01
	Suphanburi	I	SPI-B2	0.78
	Nakhonsawan	I	NS1-A2	0.82
			NS2-A3	0.79
		II	NS2-A1	0.72
			NS2-B3	0.85
			NS3-A1	0.79
Soil	Suphanburi	I	SP5-A6	0.85
			SP5-B4	0.78
			SP8-A4	0.79
			SP8-B1	0.83
			SP9-A2	0.80
			SP10-B4	0.88
		II	SP4-B5	0.68
Bovine rumen	Nakhonsawan	I	NS14-aA2	0.87
			NS15-aB2	0.91
			NS14-dB1	0.92
		III	NS15-bA2	0.72
		II	NS15-dA1	0.97
Tree bark	Ayutthaya	I	AY2-aA1	0.86
			AY4-aA1	0.81
			AY5-aB1	0.83
		III	AY1-bA2	0.63
			AY3-bA1	0.64
		VI	AY5-bA2	0.14
			AY5-bB6	0.12
		V	AY5-bB3	0.03
			AY5-bB4	0.05
Total			28 isolates	0.01-0.97

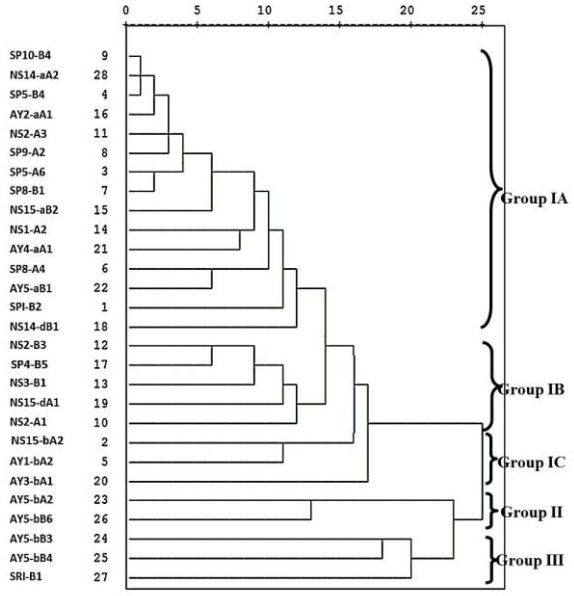

Figure 1. Dendrogram of the hierarchical cluster of the 28 selected isolates (produced using SPSS).

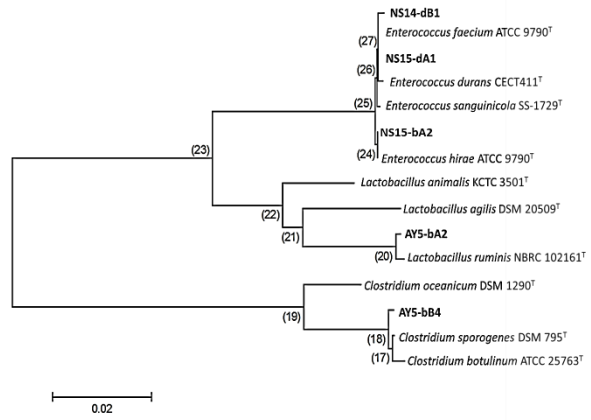

Figure 2. Neighbour-joining-tree showing the phylogenetic position of 5 representative isolates of each group based on 16S rRNA gene sequence. Bar = 0.02 nucleotide substitution per site.

NS15-bA2 (Group IC), isolate AY5-bA2 (Group II), and isolate AY5-bB4 (Group III) were closely related to *Enterococcus hirae* ATCC 9790^T (100% similarity), *Lactobacillus ruminis* NBRC 102161^T (99.71% similarity), and *Clostridium sporogenes* DSM 795^T (99.78% similarity), respectively. They were deposited in the DDBJ database under the following accession numbers LC271163, LC271158, and LC271159, respectively.

The results of the physiological and biochemical characteristics depended on the genus. Within the genus *Enterococcus* (Group IA, IB, and IC), each species showed different reactions of acid from carbohydrates while other biochemical tests were similar.

Isolate NS14-dB1 (Group IA) and isolate NS15-dA1 (Group IB) were identified as *Enterococcus durans* while NS15-bA2 (Group IC) was identified as *Enterococcus hirae*.

Table 3. Phenotypic characterization of the three groups (28 isolates).

Characteristics	Group I			Group II	Group III
	Group IA	Group IB	Group IC		
Number of isolates	15	5	3	2	3
Cell form	Cocci in chains			Rods	
Growth at 20 °C	+	+	+	+	+
Growth at 30 °C	+	+	+	+	+
Growth at 35 °C	+	+	+	+	+
Growth at 37 °C	+	+	+	+	+
Growth at 45 °C	+	+ (-2)	+(-1)	+	+
Growth at 50 °C	+	-	-	-	-(+1)
Growth at pH 3.0	-	-	-	-	-
Growth at pH 5.0	+	+	+	+	+
Growth at pH 7.0	+	+	+	+	+
Growth at pH 9.0	+	+	+	+	-
Growth in 2% NaCl	+	+	+	+	+
Growth in 6% NaC	+	+	+	+	+
Catalase	-	-	-	-	+
CO_2 production	-	-	-	-	+
Arginine hydrolysis	+	+	+	-	-
Nitrate reduction	-	-	-	-	+
Acid from:					
D-Amygdalin	+ (-5)	+ (-1)	+	+	-
L-Arabinose	+	-	-	-	-
Cellobiose	+	+	+	+	-
Gluconate	+ (-7)	- (+1)	-	+	+
Lactose	+ (-6)	+	+	-	-
D-Mannitol	+	-	-	-	-
D-Mannose	+ (-1)	+	+	+	+
α-Methyl-D-glucoside	-	- (+2)	-	-	+
Raffinose	-	-	+	+	-
Rhamnose	-	-	-	-	-
Salicin	+	+	+	+	-
Sorbitol	- (+2)	-	-	-	-
Sucrose	+	+	+	+	-
Trehalose	+ (-2)	+ (-2)	+	-	-
D-xylose	+	-	-	-	-

These two species have no reports of succinic acid production. But other species have reports of succinic acid production. Kang *et al.* (2000) reported that *Enterococcus faecalis* was able to produce succinic acid from glycerol (0.81g/g glycerol). Also, *Enterococcus flavescens* was able to produce succinic acid from glucose with a succinic acid yield of 0.14 g/g glucose (Agarwal *et al.*, 2007). Isolate AY5-bA2 (Group II) was identified as *Lactobacillus ruminis*. Kaneuchi *et al.* (1988) reported that *Lactobacillus* strains produced succinic acid in the range of 0.02-0.56 g/g glucose. Isolate AY5-bB4 (Group III) was identified as *Clostridium sporogenes*. However, there have been no reports of succinic acid production in this genus. Thus isolate NS15-dA1 (Group IB, *Enterococcus durans*) and isolate NS15-bA2 (Group IC, *Enterococcus hirae*) were selected for their potential use for producing succinic acid because they could produce high amounts of succinic acid and there are no previous reports of succinic acid production.

3.6 Optimization of succinic acid production in flask Culture

3.6.1 Effect of glucose concentration

At the initial glucose concentration of 30 g/L, glucose was completely consumed within 12 h (Figure 3). Similarly, when 60 g/L of glucose was used, glucose was completely consumed within 24 h. However, the initial glucose concentration of 90 g/L resulted in excessive carbon sources. At 24 h, the highest succinic acid concentrations of 50.01±0.51 and 52.47±0.11 g/L were obtained from isolate NS15-dA1 and isolate NS15-bA2, respectively (Figure 4). Similar results were reported by Gonzáleza *et al.* (2008) who observed that 54.7 g/L of glucose was the optimum carbon source which resulted in a high succinic acid concentration of 33.80 g/L by *Actinobacillus succinogenes* ZT-130. Chen *et al.* (2011) reported that a high glucose concentration was due to the osmotic effects in the succinic acid fermentation. Cell growth and production concentration were also inhibited by high glucose concentration probably due to substrate inhibition, which is a common issue in fermentation (Kotzamanidis *et al.*, 2002). Therefore, the initial 60 g/L of glucose and 24 h of cultivation time were chosen for further study.

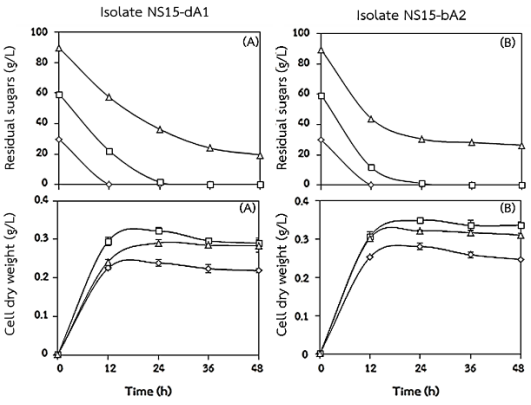

Figure 3. Time courses of residual sugars (A) and cell dry weight (B) when cultivated isolate NS15-dA1 and NS15-bA2 using glucose in the range 30-90 g/L as a carbon source.
(◇: 30 g/L; □: 60 g/L; △: 90 g/L)

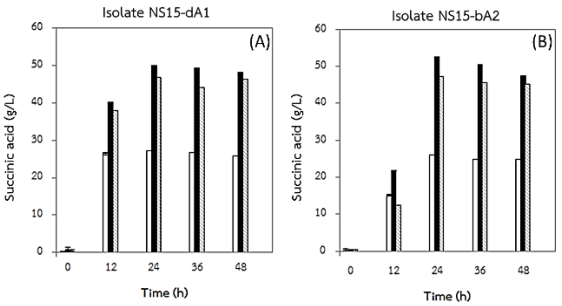

Figure 4. Succinic acid production by isolates NS15-dA1 (A) and NS15-bA2 (B) using glucose in the range 30-90 g/L as a carbon source.
(□: 30 g/L; ■: 60 g/L; ▨: 90 g/L)

3.6.2 Effect of different nitrogen sources

Among the nitrogen sources of yeast extract, peptone, tryptone, urea, KNO_3, $NHCl_4$, $(NH_4)_2SO_4$, $(NH_4)_2HPO_4$, and NH_4NO_3, yeast extract yielded the highest production of succinic acid at 49.96±0.02 g/L and cell growth of 0.29±0.00 g/L by isolate NS15-dA1 (Figure 5A). That was optimum for both cell growth as well as succinic acid production. Yeast extract, apart from acting as source of nitrogen, also supplies vitamins and trace metals. Therefore, it affected the growth of the organism and thus increased succinic acid production. Furthermore, Kang *et al.* (2000) reported that *Enterococcus faecalis* RKY1 used yeast extract as a nitrogen source and produced the highest concentration succinic acid at 27 g/L. Lee *et al.* (2002) observed that *M. succiniciproducens* MBEL55E could produce a high level of succinic acid (14 g/L) using yeast extract as the source of nitrogen.

In the case of isolate NS15-bA2, tryptone was found to be the best source of nitrogen. Moreover tryptone is optimum for both cell growth of 0.37±0.01 g/L as well as succinic acid production of 53.89±0.05g/L (Figure 5B). Tryptone provides nitrogen, amino acids, and vitamins for the growing bacteria. Furthermore, Isar *et al.* (2006) reported that tryptone was the best nitrogen source which resulted in the production of 2.0 g/L of succinic acid by *Bacteroides fragilis*. Similarly, among the various organic sources of nitrogen tested by Agarwal *et al.* (2007), tryptone maximally enhanced the production of both succinic acid (3.8 g/L) as well the enzyme activity of phosphoenolpyruvate carboxykinase by *Enterococcus flavescens*. Therefore, yeast extract was chosen for further study of isolate NS15-dA1 and tryptone was chosen for further study of isolate NS15-bA2.

3.6.3 Effect of initial pH

The effects of different levels of initial pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5, and 8.0 on succinic acid production are shown in Figure 6. The initial pH of 7.0 was found to be the optimum initial pH that resulted in the highest production of succinic acid of 50.70±0.47 g/L and cell growth of 0.31±0.00 g/L by NS15-dA1 (Figure 6A). Similarly, the initial pH of 7.0 was found to be the optimum initial pH that resulted in the highest production of succinic acid and cell growth of 53.21±0.20 g/L and 0.34±0.01 g/L, respectively, by NS15-bA2 (Figure 6B).

Wee *et al.* (2002) reported that pH 7-8 is the optimum pH for succinic acid production by *Enterococcus faecalis*. Ryu *et al.* (1999) also reported that at pH 7 *Enterococcus faecalis* could produce the maximum concentration of succinic acid of 65.90 g/L. Moreover Lee *et al.* (2009) observed that *M. succiniciproducens* MBEL55E grew well in the pH range of 6.0-7.5. The most probable reason could be that the activity of the enzyme responsible for succinic acid production was maximally induced within the given pH range. Therefore, pH 7.0 was chosen for further studies of isolates NS15-dA1 and NS15-bA2.

3.6.4 Effect of temperature

The effects of different temperatures of 35, 37, and 39 °C on succinic acid production are shown in Figure 7. The optimum temperature was found to be 37 °C and the highest succinic acid production of 51.69±0.17 g/L and cell growth of 0.31±0.00 g/L were obtained from isolate NS15-dA1 (Figure 7A). Similarly, 37 °C was found to be the optimum temperature for the highest succinic acid production and cell growth of 53.05±0.35 g/L and 0.346±0.02 g/L, respectively, from isolate NS15-bA2 (Figure 7B).

The results of the effects of temperature were similar to the phenotypic characterizations of isolates NS15-dA1 and NS15-bA2 which showed good cell growth in the range of 20-45 °C. Isar *et al.* (2006) reported that 37±2 °C was the optimal temperature for succinic acid production from *Bacteroides fragilis*. Macy *et al.* (1978) observed that 37±1 °C was the most suitable temperature of succinic acid production form *Bacteroides fragilis*. Lee *et al.* (1999) reported that 37±1 °C was the optimal temperature for growth and succinic acid production by *Anaerobiospirillum succiniciproducens*. Huh *et al.* (2004) also reported that *Mannheimia succiniciproducens* produced maximum succinic acid at 37 °C. The probable reason is that 37 °C may be the optimal temperature for enzyme activity. Therefore, 37 °C was chosen for further studies of isolates NS15-dA1 and NS15-bA2.

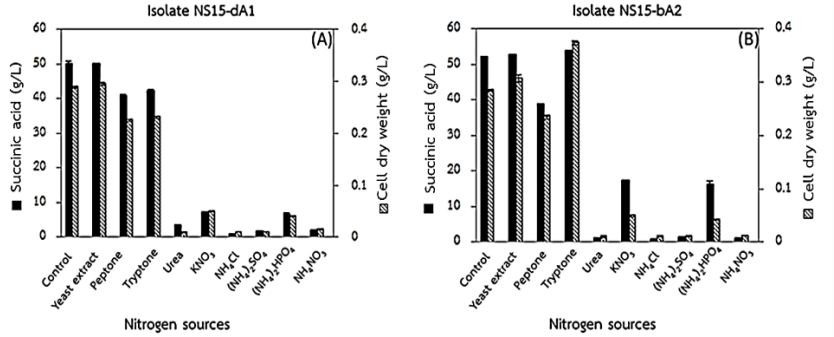

Figure 5. Effect of different nitrogen sources on succinic acid production and cell dry weight by isolates NS15-dA1 and NS15-bA2.

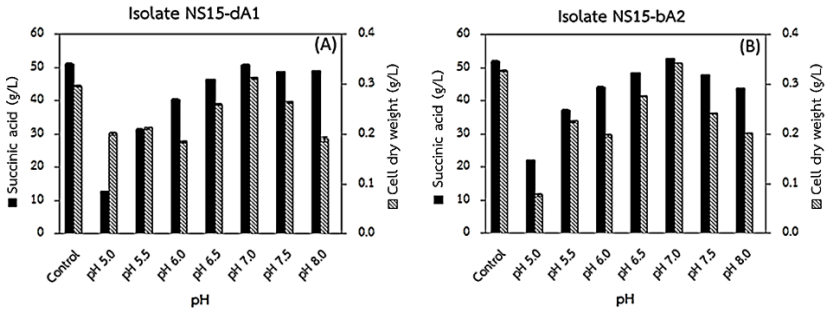

Figure 6. Effect of initial pH on succinic acid production and cell dry weight by isolates NS15-dA1 and NS15-bA2.

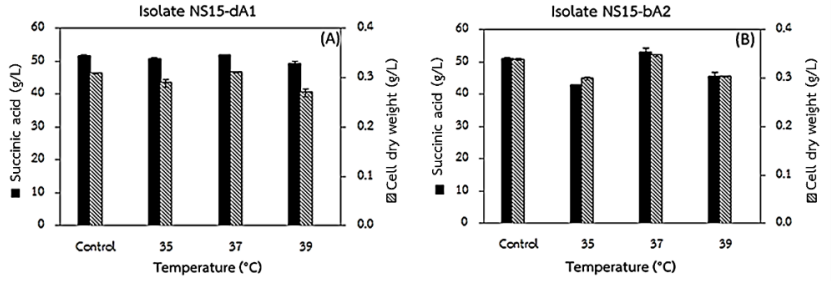

Figure 7. Effects of temperature on succinic acid production and cell dry weight by isolates NS15-dA1 and NS15-bA2.

4. Conclusions

Of 200 bacterial isolates from various sources, 84 isolates were gram positive and produced succinic acid (in the range of 0.003-0.97 g/g glucose). However, only 28 out of 84 isolates were selected to study the physiological and biochemical characteristics. Twenty-eight isolates were divided into three groups based on phenotypic characteristics. Groups IA, IB, and IC were cocci in chains, while Groups II and III were rods. Five isolates from each group, including NS14-dB1 (Group IA), NS15-dA1 (Group IB), NS15-bA2 (Group IC), AY5-bA2 (Group II), and AY5-bB4 (Group III), produced the highest amounts succinic acid. Therefore, they were selected for further study using 16s rRNA gene sequence analysis. Isolates NS14-dB1 (Group IA) and NS15-dA1 (Group IB) were identified as *Enterococcus durans* and NS15-bA2 (Group IC) was *Enterococcus hirae*. The isolate AY5-bA2 (Group II) was identified as *Lactobacillus ruminis* and AY5-bB4 (Group III) was *Clostridium sporogenes*. Isolates NS15-dA1 and NS15-bA2 were selected to study the optimization of succinic acid production. The optimum conditions for succinic acid production by isolates NS15-dA1 and NS15-bA2 were 60 g/L of glucose as a carbon source, 30 g/L of yeast extract (for isolate NS15-dA1), and 30 g/L of tryptone (for isolate NS15-bA2) as nitrogen sources at the pH of 7.0 and at 37 °C. The highest concentrations obtained for succinic acid were 51.69±0.17 g/L and 53.05±0.35 g/L from isolates NS15-dA1 and NS15-bA2, respectively.

Acknowledgements

The authors acknowledge with thanks the financial support from the Annual Government Statement of Expenditure (2016) (GRB_ BSS_102_59_61_09) of Thailand.

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