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Research Article
Diversity and Succinic Acid Production of Lactic Acid Bacteria Isolated from Animals, Soils and Tree Barks

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Abstract

Background and Objective: Currently, succinic acid fermentation process has not been successfully commercialized. Key technical problems blocking rapid advances in developing a bioprocess technology for succinic acid fermentation are of low productivity, multiple product formation and inefficient recovery of product from the fermentation system. The aim of this study was to emphasize the need to carry out the isolation, screening and identification of potential bacteria for succinic acid production. **Materials and Methods:** Bacterial strains were isolated from different sources in Thailand. Primary screening on selective medium plate then the fermentation process under the anaerobic condition was performed. The succinic acid was analyzed by TLC method and confirmed by HPLC method. The selected isolates were studied for phenotypic characterization, 16S rRNA gene sequence and phylogenetic analysis. **Results:** One hundred and fifty nine Lactic Acid Bacteria (LAB) were isolated. Only 19 succinic acid producing isolates were divided into three groups: Group I (13 isolates) were identified as *Enterococcus casseliflavus* (Group I-A, 2 isolates), *E. durans* (Group I-B, 1 isolate), *E. faecium* (Group I-C, 2 isolates), *E. hirae* (Group I-D, 7 isolates) and *E. saccharolyticus* (Group I-E, 1 isolate). Group II (2 isolates) were identified as *Lactobacillus fermentum* (Group II-A, 1 isolate) and *L. oris* (Group II-B, 1 isolate). Group III (4 isolates) were identified as *Lactococcus formosensis* (Group III-A, 1 isolate) and *L. garvieae* (Group III-B, 3 isolates). *Enterococcus* strains were capable of producing succinic acid in a range from 23.312-43.482 g L^{-1} while *Lactobacillus* and *Lactococcus* strains produced succinic acid in the range from 12.586-25.247 and 18.847-22.172 g L^{-1}, respectively. The highest production of succinic acid (43.482 g L^{-1}) was obtained from *E. faecium* NS13-dB1. **Conclusion:** Among 19 isolates, the strain *Enterococcus faecium* NS13-dB1 was the most effective for succinic acid production and could be a promising candidate for further applications.

Key words: Lactic acid bacteria, ruminant, faeces, soil, succinic acid, tree bark

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Succinic acid, known as amber acid or butanedioic acid, was a dicarboxylic acid having the molecular formula of $C_4H_6O_4$. It was regarded as a precursor for many industrial chemicals including adipic acid, 1, 4-butanediol, tetrahydrofuran, N-methyl pyrrolidinone, 2-pyrrolidinone, succinate salts and gamma-butyrolactone[1,2]. Besides its application in the agricultural, food and pharmaceutical industries, succinic acid can also be used in the synthesis of biodegradable polymers such as polybutyrate succinate (PBS), polyamides and various green solvents[3].

Succinic acid has been synthesized from petrochemical based maleic acid. However, considering the difficulty in obtaining petroleum resources and the volatility of oil prices, succinic acid fermentation was drawing a great deal of attention in response to the current need to develop sustainable processes using renewable resources[4]. Succinic acid was produced as an intermediate product of the tricarboxylic acid (TCA) cycle and also was a fermentation product of anaerobic metabolism. Thus, it was synthesized in almost all microbial, plant and animal cells. Those organisms suitable for the efficient production of succinic acid can be categorized into either bacteria or fungi[1].

The Gram-positive strains of *Corynebacterium glutamicum*, *Enterococcus faecalis* and *Ruminococcus flavefaciens* have been studied for succinic acid production. Several engineered *C. glutamicum* strains were created by disruption and replacement of genes in optimal culture conditions. The rate of succinic acid production was thus increased seven times while glucose consumption increased fivefold under oxygen deprived conditions[5]. Succinic acids can be produced by Gram-negative strains including *Anaerobiospirillum succiniciproducens*[6], *Actinobacillus succinogenes*[7], *Escherichia coli, Mannheimia succiniciproducens*[8], *Klebsiella pneumoniae* MCM B-325[9]. They have been isolated from various anaerobic environments such as domestic sludge, cattle waste, rice paddy, marine shipworm, dog saliva, rumen and gastrointestines. Only a few species can produce succinic acid with a high yield. Recently, *A. succinogenes*, *A. succiniciproducens* and *M. succiniciproducens* have been considered the best candidates for succinic acid production[1]. This was most likely due to the fact that the rumen was a highly efficient organ providing an ideal environment to produce succinic acid[10]. The rumen was a unique microbial ecosystem found in many species of herbivorous mammals known as ruminants, caused by carbon dioxide, methane and traces of hydrogen production. Moreover, many vitamins and amino acids were abundant in the rumen resulting in minimal requirements to create a medium[4]. Although *A. succiniciproducens* was well known as a good succinic acid producer, the fermentation processes in which this strict anaerobe was involved were more difficult to handle than those using facultative anaerobes[11]. Some facultative anaerobes involved in succinic acid production, such as *Escherichia coli*[12], *A. succinogenes*[7] and *E. faecalis* RKY1[13] have been reported. Among these, *E. faecalis* RKY1 were able to produce succinic acid in high yield if cultured anaerobically with glycerol as a hydrogen donor and fumaric acid as a hydrogen acceptor[14].

Inspite of these points, succinic acid was used in the agricultural, food and pharmaceutical industries as a key chemical for the preparation of biodegradable polymers. The first step in the fermentative production of succinic acid was the screening of bacterial strains. To date no process or technology has been successfully commercialized to perform this function. The key problem of the fermentation system was the formation of byproducts such as acetic, formic and lactic acids resulting in reduction the succinic acid yield and productivity, while increasing the complexity and cost of succinic acid recovery. Consequently, the objective of this study was to emphasize the need to carry out the isolation, identification and screening of the prominent bacteria with high succinic acid yield and productivity.

MATERIALS AND METHODS

Chemicals, sources and isolation methods: All chemicals were purchased from Merck (Merck KGaA, Darmstadt, Germany). Bacterial strains were isolated from different sources including 12 bovine faeces samples, four buffalo faeces samples and three bovine rumen samples. These samples were collected from Suphanburi, Surin and Nakhonsawan, provinces while soil samples were collected from Suphanburi province. Moreover, tree barks were collected from Ayutthaya and Nakhonpathom provinces in Thailand (Table 1). One gram of each sample was enriched in 5 mL of enrichment broth consisting of 20 g L^{-1} glucose, 5 g L^{-1} polypeptone, 5 g L^{-1} yeast extract, 3 g L^{-1} K_2HPO_4, 2 g L^{-1} NaCl, 2 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $CaCl_2 \cdot 2H_2O$, 0.4 g L^{-1} $MgCl_2 \cdot 6H_2O$ and 15 g L^{-1} $MgCO_3$. They were incubated at 37°C for 48-72 h under anaerobic conditions using an anaerobic pack (MGC, Japan). Positive tubes were subcultured for the enrichment agar plate diluted to 10^{-6} in Phosphate Buffered Saline (PBS) buffer. The diluted cultures (0.1 mL) were spread onto enrichment agar plates and were incubated in anaerobic conditions at 37°C. After 24-48 h, visible colonies were picked and re-streaked on

Table 1: Isolate No., sources, identification and 16S RNA gene sequence similarity (%) of isolates

Isolate No.	Sources	Groups	Identification	Similarity (%)
AY2-bA2	Bark of *Ficus religiosa* L.	I-A	*E. casseliflavus*	99.93
AY2-bB2	Bark of *Ficus religiosa* L.	I-A	*E. casseliflavus*	99.69
NP2-A3	Soil	I-B	*E. durans*	100.00
NS13-dB1	Bovine rumen	I-C	*E. faecium*	99.93
SP8-B4	Soil	I-C	*E. faecium*	100.00
NS13-aB1	Bovine rumen	I-D	*E. hirae*	100.00
NS13-dA1	Bovine rumen	I-D	*E. hirae*	100.00
NS15-aA1	Bovine rumen	I-D	*E. hirae*	100.00
NS15-aA2	Bovine rumen	I-D	*E. hirae*	99.50
SP5-A5	Soil	I-D	*E. hirae*	100.00
SP6-A5	Soil	I-D	*E. hirae*	100.00
SP9-A3	Soil	I-D	*E. hirae*	100.00
BK1-A1	Bark of *Samanae saman*	I-E	*E. saccharolyticus* sub sp.	100.00
NS15-bB2	Bovine rumen	II-A	*L. fermentum*	99.78
NS13-bA1	Bovine rumen	II-B	*L. oris*	99.85
SP14-B2	Dog saliva	III-A	*L. formosensis*	99.93
SP14-A3	Dog saliva	III-B	*L. garvieae*	99.85
SP15-A2	Dog saliva	III-B	*L garvieae*	99.93
SP15-B2	Dog saliva	III-B	*L. garvieae*	99.78

AY: Ayutthaya, BK: Bangkok, NP: Nakhonpathom, NS: Nakhonsawan, SP: Suphanburi and SR: Surin provinces, Thailand

fresh enrichment agar plate and incubated overnight at 37 °C for 24 h under anaerobic conditions.

A single colony extracted from the enrichment agar plates was streaked on screening agar plates consisting of 20 g L^{-1} glucose, 1 g L^{-1} NaCl, 5 g L^{-1} yeast extract, 3 g L^{-1} K_2HPO_4, 1 g L^{-1} $(NH_4)_2SO_4$, 0.2 g L^{-1} $CaCl_2.2H_2O$, 0.2 g L^{-1} $MgCl_2.6H_2O$, 15 g L^{-1} $MgCO_3$ and 15 g L^{-1} agar, pH of the media was adjusted to 6.5 and they were incubated overnight at 37°C under anaerobic conditions. Acid-producing isolates exhibited a clear zone around the colonies were selected and purified. They were maintained on a TSA agar plate or slant which consisted of 17 g L^{-1} pancreatic digest of casein, 3 g L^{-1} soy peptone, 2 g L^{-1} glucose, 5 g L^{-1} NaCl and 2.5 g L^{-1} KH_2PO_4. Then, positive isolates were stored at -70°C or lyophilized for further study.

Identification of isolates

Phenotypic characterization: The morphological and cultural characteristics including Gram reaction, spore formation, cell morphology and colonial appearance of the isolates were determined on the cells grown on a Gifu anaerobic medium (Nissui Pharmaceutical Company, Tokyo, Japan) agar plate after incubation under anaerobic conditions at 37°C for 18-24 h. The physiological characteristics included different pH values (3.5-9), temperatures (20-50°C) and NaCl concentrations (2 and 6% w/v NaCl). The biochemical characteristics determined included catalase activity, nitrate reduction, gas production, starch hydrolysis, arginine hydrolysis, slime formation and acid formation from various carbohydrates were tested as described by Tanasupawat *et al.*[15,16].

16S rRNA gene sequence and phylogenetic analysis: The 16S rRNA gene of isolates was PCR amplified using primers, 27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and 518F (5′-CCAGCAGCCGCGGTAATACG-3′), 800R (5′TACCAGGGTAT CTAATCC′3) and 1492R (5′TACGGYTACCTTGT-TACGACTT′3). The amplified 16S ribosomal RNA gene sequences were analyzed with Macrogen®, from Korea. Sequence alignment was determined by the EzTaxon database. Multiple alignments of sequences were performed by the program BioEdit version 7.0.2. A phylogenetic tree was constructed by the neighbor-joining method using the program MEGA, version 6[17]. A bootstrap analysis of Felsenstein[18] was performed to determine confidence values of individual branches in the phylogenetic tree with 1000 replications.

Growth and succinic acid fermentation of isolates: The ability of isolates to produce succinic acid was investigated by anaerobic fermentation in a medium consisting of 30.0 g L^{-1} yeast extract, 2.0 g L^{-1} urea, 2 g L^{-1} $MgCl_2.6H_2O$, 1.5 g L^{-1} $CaCl_2$, 0.07 g L^{-1} $MnCl_2$, 4.4 g L^{-1} Na_2HPO_4, 3.3 g L^{-1} NaH_2PO_4, 30 g L^{-1} $MgCO_3$ and the pH was adjusted to 7[19]. Glucose was separately sterilized at 121°C for 15 min and added to the medium to maintain the initial concentration of 60.0 g L^{-1}. About 0.3 µg L^{-1} of biotin and 0.2 µg L^{-1} of thiamin were prepared by sterile membrane filtration (0.22 µm nylon, Millipore Express, Ireland) and were added. The cultivation medium was inoculated with 10% seed inoculum (TSB medium) and incubated at 37°C, 200 rpm for 48 h under anaerobic conditions.

Analytical method: The culture broth used for succinic acid determination was prepared by centrifuging at 10,000 rpm for

10 min at 4°C. The supernatants were initially analyzed for the presence of succinic acid using thin layer chromatography and the succinic acid was confirmed using High Performance Liquid Chromatography (HPLC).

Cell concentration: The insoluble $MgCO_3$ in the samples was removed by adding 0.2 M of HCl. Then the cell concentration was measured as the amount of absorbance at a 660 nm wavelength using a spectrophotometer (UV160, Shimadzu Corporation, Japan).

Glucose concentration: Sugar concentration was measured with the DNS (3, 5-dinitrosalicylic acid colorimetric) method[20]. The absorbance at a 540 nm wavelength was measured using a spectrophotometer.

Thin Layer Chromatography (TLC): Thin layer chromatography is commonly applied as an inexpensive, efficient and fast method for primary detection of succinic acid[21]. The test samples (10 µL) and 2 g L^{-1} of standard succinic acid were spotted onto silica gel TLC plates (Silica gel 60 F254, Merck, Darmstadt, Germany) and resolved using a solvent system comprising ethanol, ammonium hydroxide and water (20:5:3) for 30 min. The air dried plates were sprayed with green bromocresol (0.04% w/v in ethanol) and heated at 160°C for 5 min to reveal the organic acid spots.

High-performance liquid chromatography (HPLC): Fermentation products (succinic, acetic and formic acid) were analyzed with HPLC (LC-6A, Shimadzu Corporation, Japan). Twenty microliters of sample were filtered (0.45 Am, 13 mm membrane disc filters) and loaded on HPLC using a system equipped with a cation-exclusion column (Aminex HPX-87H; 300 mm 7.8 mm; Bio-Rad Chemical) and a refractive index detector (Shimadzu Model RID-6A). The mobile phase was 5 mM of H_2SO_4 solution at a flow rate of 0.6 mL min^{-1} with the column operated at 55°C.

Statistical analysis: The experiments were tested in triplicate and the related data were expressed as averages values. Statistical Package for the Social Sciences (SPSS) version 15 for Windows was used to analysis the data.

RESULTS

Screening and isolation of microorganisms: One hundred and fifty nine isolates from various sources in Thailand were screened for succinic acid production under anaerobic conditions. One hundred and forty one isolates exhibited a clear zone on the screening medium. From these, one hundred and twenty one isolates were found by TLC analysis to produce succinic acid. Subsequently, secondary screening analysis using HPLC, found 73 isolates capable of producing succinic acid and 19 isolates producing succinic acid in excess of 20% (g g^{-1} glucose) yield. The potential 19 isolates were selected for further study.

Identification of isolates: The 19 isolates were divided into three groups based on phenotypic characteristics including cell form, cell arrangements, Gram-reaction, catalase production, gas production, arginine hydrolysis and growth environments (temperatures, pH and osmotic pressure). These isolates belonged to the genera *Enterococcus*, *Lactococcus* and *Lactobacillus* based on 16S rRNA gene sequencing and phylogenetic analysis (Table 1).

All isolates were facultative anaerobic and did not produce gas from glucose with the exception of isolates in group II-A as shown in Table 2. They did not reduce nitrate concentration except for the isolates in group I-A. Similarly, none of the isolates hydrolyzed starch or produced slime. Isolates in group I (except for group I-E isolate BK1-A1), group II and III were able to hydrolyze arginine. All isolates in group I grew at 20°C except for groups I-B (isolate NP2-A3) and I-E (isolate BK1-A1) could not grow at 40-50°C. Isolates in group II and III grew at 20°C but did not grow at 40° or 50°C. Most isolates could grow at pH in the range 5-9 and in 6% NaCl. The ability of group I, II and III isolates in sugar fermentation were varied.

Isolates were divided into 3 groups. Group I consisted of thirteen isolates of five sub-groups (Fig. 1, Table 1).

Isolates of Group I-A: They were AY2-bA2 and AY2-bB2. Cells were cocci in chains or lenticular form and colonies were white, circular and translucent (0.5-1.0 mm in diameter). The 16S rRNA gene sequences determined in this study was deposited in the DNA Data Bank of Japan (DDBJ) database under the following accession numbers: AY2-bA2, LC122272 and AY2-bB2, LC120365. They were closely related to *Enterococcus casseliflavus* ATCC 49996^T with 99.93% (1383 bps) and displayed 99.69% (1295 bps) sequence similarity, respectively. Therefore, they were identified as *Enterococcus casseliflavus*[22].

Isolate of Group I-B: Group I-B consisted of only one isolate: NP2-A3. Cell was cocci in chains and colony was white, punciform and opaque (0.3-0.5 mm in diameter). The DDBJ accession number for the 16S rRNA gene sequence of isolate NP2-A3 is LC122273. It was closely

Table 2: Phenotypic characteristics of isolates

	Group I					Group II		Group III	
Characteristic	A	B	C	D	E	A	B	A	B
No. of isolate	2	1	2	7	1	1	1	1	3
Cell form	Cocci in chains					Rods		Cocci in chains	
Arginine hydrolysis	+	+	+	+	-	-	+	+	+
Gas from glucose	-	-	-	-	-	+	-	-	-
Nitrate reduction	+	-	-	-	-	-	-	-	-
Growth in 6% NaCl	+(w)	+	+	+	+	+	-	+	+
Growth at pH 3.5	-	-	-	-	-	+	-	-	-
pH 5.0	+	+(w)	+	+	-	+	+	-	+
pH 9.0	+	+	+	+	+	-	+	+	+
20°C	+	+	+	+	+(w)	+	+(w)	+	+
40°C	+	-	+	+	-	-	-	-	-
50°C	+(w)	-	+(w)	+(w)	-	-	-	-	-
Acid from:									
D-amygdalin	- (4)	+	+	+	-	-	+	-	+
L-arabinose	- (3)	- (1)	+	+(w)	-	+	-	-	-
Cellobiose	- (4)	+	+	+	-	-	+	-	+
D-fructose	- (2)	+	+	+	+	+	+	+	+
D-galactose	- (3)	+	+	+	-	-	+	-	+
Gluconate	- (4)	+	+	-	-	+	-	+	+
Glucose	+	+	+	+	+	+	+	-	+
Lactose	+ (5)	+	-	+	-	+	-	-	-
Maltose	+	+	+	+	-	+	-	-	+
D-mannitol	+	+	+	+(w)	-	-	+(w)	+	+
D-mannose	- (3)	+	+	+	-	+	-	+	+
Melibiose	+	+	+	+	-	+	+	-	+
∞-methyl-D- glucoside	- (3)	- (3)	-	-	-	-	-	-	-
Raffinose	- (4)	+	-	-	-	+	+(w)	-	-
Rhamnose	- (2)	-	+(w)	-	-	-	+(w)	-	-
Ribose	- (2)	-	+	+	-	+	+	+	+
Salicin	- (2)	-	+	+	-	-	-	+	+
Sorbitol	-	-	-	-	-	-	+(w)	-	-
Sucrose	+	+	+	+	+	+	-	+	+
Trehalose	-	+	+	+	-	-	-	-	+
D-xylose	+	- (2)	+	-	-	+	+	-	-

+: Positive reaction, w: Weakly positive, -: Negative reaction. Numbers in parentheses indicate the number of the isolates that showed positive, weakly positive or negative reaction

related to *Enterococcus durans* CECT411^T with 100% (1285 bps) sequence similarity. Therefore, it was identified as *Enterococcus durans*[23].

Isolates of Group I-C: Group I-C consisted of two isolates NS13-dB1 and SP8-B4. Cells were cocci in chains and cocci in pairs. Colonies were yellow, punciform and translucent (0.3-0.5 mm in diameter). The 16S rRNA gene sequences determined in this study were deposited in the DDBJ database under the following accession numbers: NS13-dB1, LC122274 and SP8-B4, LC122275. They were closely related to *Enterococcus faecium* ATCC19434^T with 99.93% (1376 bps) and 100% (1376 bps) sequence similarity, respectively. Therefore, they were identified as *Enterococcus faecium*[22].

Isolates of Group I-D: They were NS13-aB1, NS13-dA1, NS15-aA1, NS15-aA2, SP5-A5, SP6-A5 and SP9-A3. Cells were cocci in chains and cocci in pairs. Colonies were white, round and opaque (0.5-1.0 mm in diameter). The 16S rRNA gene sequences determined in this study were deposited in the DDBJ database under the following accession numbers: NS13-aB1, LC122276; NS13-dA1, LC122277; NS15-aA1, LC122278; NS15-aA2, LC122279; SP5-A5, LC122280; SP6-A5, LC122281 and SP9-A3, LC122282. They were closely related to *Enterococcus hirae* ATCC 9790^T with 100% sequence similarity (1349, 1419, 1333, 1283, 1284 and 1284 bps) except NS15-aA2 which had 99.50% sequence similarity (1387 bps). Therefore, they were identified as *Enterococcus hirae*[23].

Isolate of Group I-E: It was BK1-A1. Cell was cocci in chains or lenticular form. Colony was white, round and opaque (0.5-0.7 mm in diameter). The DDBJ accession number for the 16S rRNA gene sequence of isolate BK1-A1 is LC122283. It was closely related to *Enterococcus saccharolyticus* subsp.

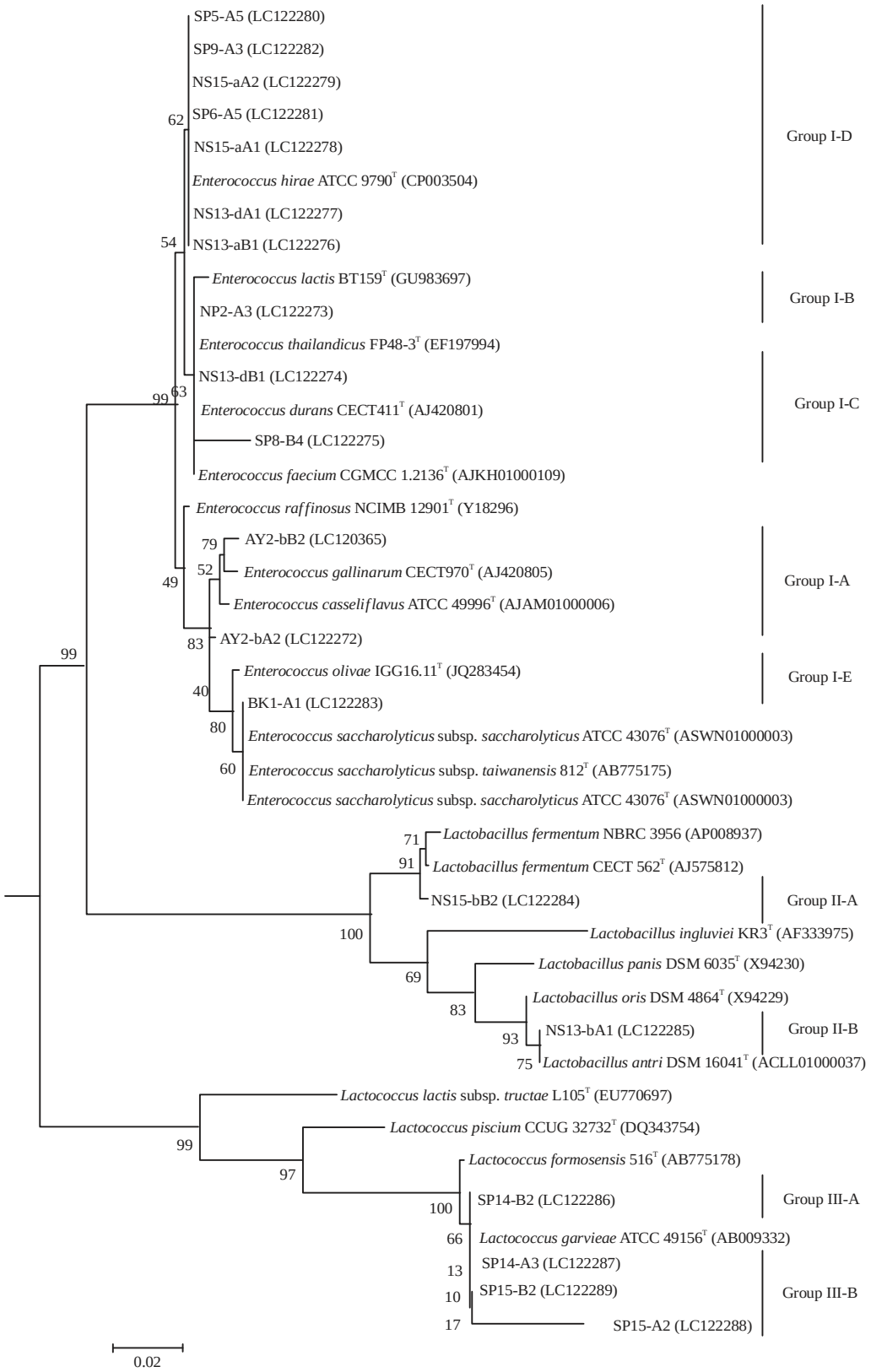

Fig. 1: Neighbor-joining phylogenetic tree of isolates in group I to III based on 16S rRNA gene sequences

Saccharolyticus ATCC 43076 with 100% sequence similarity (1364 bps). Therefore, it was identified as *Enterococcus saccharolyticus*[24].

Group II consisted of two isolates of two sub-groups (Fig. 1, Table 1).

Isolate of Group II-A: Group II-A was NS15-bB2. Cell was rod-shaped. Colony was white, punciform and opaque (0.3-0.5 mm in diameter). The DDBJ accession number for the 16S rRNA gene sequence of isolate NS15-bB2 is LC122284. It was closely related to *Lactobacillus fermentum* NBRC 3956^T with 99.78% sequence similarity (1391 bps). Therefore, it was identified as *Lactobacillus fermentum*[25].

Isolate of Group II-B: It was NS13-bA1. Cell was rod-shaped. Colony was yellow, irregular and opaque (0.6-0.9 mm in diameter). The DDBJ accession number for the 16S rRNA gene sequence of isolate NS13-bA1 is LC122285. It was closely related to *Lactobacillus oris* DSM 4864^T with 99.85% sequence similarity (1359 bps). Therefore, it was identified as *Lactobacillus oris*[26].

Group III consisted of four isolates of two sub-groups.

Isolate of Group III-A: Group III-A was SP14-B2. Cell was short and rod-shaped and colony was white, circular and opaque (0.5-1.0 mm in diameter). The DDBJ accession number for the 16S rRNA gene sequence of isolate SP14-B2 is LC122286. It was closely related to *Lactococcus formosensis* 516^T with 99.93% (1398 bps) sequence similarity. Therefore, it was identified as *Lactococcus formosensis*[27].

Isolate of Group III-B: They were SP14-A3, SP15-A2 and SP15-B2. Cells were short and rod-shaped with white, circular and translucent colonies (1.0-1.5 mm in diameter). The 16S rRNA gene sequences determined in this study were deposited in the DDBJ database under the following accession numbers: SP14-A3, LC122287, SP15-A2, LC122288 and SP15-B2, LC122289. They were closely related to *Lactococcus garvieae* ATCC 49156^T with 99.85% (1368 bps), 99.93% (1343 bps) and 99.78% (1360 bps) sequence similarity, respectively. Therefore, they were identified as *Lactococcus garvieae*[28].

From these results, the 9 potential isolates, namely, AY2-bB2 (Group I-A), NP2-A3 (Group I-B), NS13-dB1 (Group I-C), NS15-aA1 (Group I-D), BK1-A1 (Group I-E), NS15-bB2 (Group II-A), NS13-bA1 (Group II-B), SP14-B2 (Group III-A) and SP14-A3 (Group III-B) were selected as representative of each group for further study of succinic acid production.

Determination of cell growth and succinic acid production: Representative isolates from three groups were cultivated (Fig. 2). The growth of isolate BK1-A1 (Group I-E) increased rapidly and reached a maximum cell growth (OD660) of 31.500 after 48 h followed by isolate NS13-bA1 (Group II-B), SP14-B2 (Group III-A) and NS13-dB1 (Group I-C) which achieved a maximum cell growth of 26.40, 24.660 and 21.280, respectively. The rest of the isolates (Group I-A, I-B, I-D, II-A and II-B) showed a similar growth rate compared to group I-C as shown in Fig. 2.

Remarkably, the growth lag phase in fermentation of groups II and III were 12 h longer than the other isolates. While a 3 h lag phase was observed when group I (except Group I-A) was cultivated (Fig. 2).

The residual glucose concentration during anaerobic fermentation showed that group I isolates exhibited a sharp decrease in the amount of glucose consumed. Group I-C utilized the initial glucose from 58.028 g L^{-1} decreased to 0.755 g L^{-1} and showed no distinct change after 12 h. The residual glucose from succinic acid fermentation by group I-D and I-E were 1.352 and 1.132 g L^{-1}, respectively, after 12 h of cultivation time. Similarly, the cell growth of Group I showed no significant change after 12-48 h. This may be cell growth was inhibited by the organic acids produced from that bacterial strain. The residual glucose in fermentation culture of group II and III isolates decreased slightly after 12 h of cultivation time.

A maximum amount 43.482 g L^{-1} of succinic acid was obtained from isolate NS13-dB1 (*E. faecium*) with a yield of 0.749 g g^{-1} glucose and a productivity of 0.906 g L^{-1} h^{-1} after 48 h of cultivation time (Table 3). Minor products; acetic and formic acids were 0.144 and 0.290 g L^{-1}, respectively.

DISCUSSION

The potential succinic acid producer; *Enterococcus faecium* NS13-dB1; reported in this study was compared with the other studies (Table 4)[4,7,11,29-31]. A maximum of succinic acid of 43.482 g L^{-1} with a yield of 0.749 g g^{-1} glucose and productivity of 0.906 g L^{-1} h^{-1} were obtained from isolate *E. faecium* NS13-dB1 after 48 h of cultivation time. From the other reports, *Enterococcus faecalis* RKY1[10,11] and *Actinobacillus succinogenes* NJ 113[7] gave a high yield of succinic acid 0.900 and 0.904 g g^{-1} substrate, respectively. However, the isolate from this study gave a yield 1.6-3.6 times higher than that of *Enterococcus flavescens*[31], *Corynebacterium crenatum*[30] and *C. glutamicum* R[4] (Table 4).

Wee *et al.*[11] reported *Enterococcus faecalis* RKY1 was able to produce succinic acid with a high yield when anaerobically culture with glycerol as a hydrogen donor and fumaric acid as

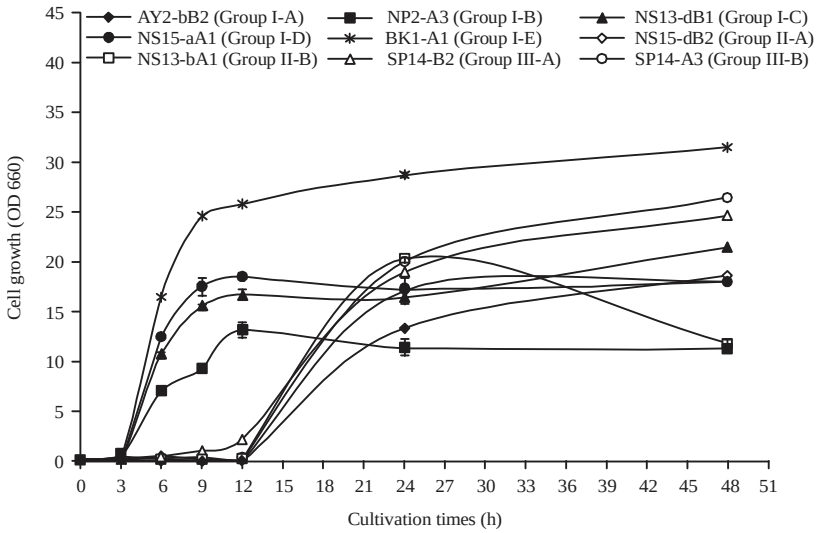

Fig. 2: Time course of cell growth of the representative isolate of each group under anaerobic condition

Table 3: Succinic acid production of 19 isolates obtained from this study

Isolate No.	Group	Initial glucose (g L^{-1})[a]	Residual glucose (g L^{-1})[a]	Glucose utilization (%)[a, b]	Succinic (g L^{-1})[a, c]	Formic (g L^{-1})[a]	Acetic (g L^{-1})[a]	Succinic acid yield (g g^{-1} glucose)[a]	Succinic acid productivity (g L^{-1} h^{-1})[a]
AY2-bA2	I-A	58.023±0.083	0.117±0.059	99.748±0.071	38.667±0.457	2.028±0.002	0.634±0.130	0.665±0.007	0.806±0.010
AY2-bB2	I-A	57.084±0.127	0.179±0.077	99.685±0.002	30.121±0.478	1.540±0.004	0.542±0.184	0.526±0.008	0.627±0.010
NP2-A3	I-B	57.242±0.010	0.014±0.007	99.830±0.205	38.857±0.459	1.194±0.103	0.332±0.237	0.678±0.007	0.810±0.010
NS13-dB1	I-C	58.028±0.033	0.046±0.040	99.936±0.021	43.482±0.514	0.290±0.019	0.144±0.120	0.749±0.009	0.906±0.011
SP8-B4	I-C	58.431±0.145	0.205±0.033	99.671±0.031	26.893±0.000	0.089±0.033	0.257±0.065	0.459±0.000	0.560±0.000
NS13-aB1	I-D	58.743±0.173	0.245±0.076	99.752±0.240	38.973±0.461	0.211±0.125	0.773±0.174	0.662±0.009	0.812±0.010
NS13-dA1	I-D	58.431±0.102	0.144±0.022	99.865±0.157	42.172±0.499	0.099±0.030	0.172±0.060	0.721±0.010	0.879±0.010
NS15-aA1	I-D	56.424±0.068	0.096±0.045	99.811±0.027	36.252±0.429	0.450±0.071	0.252±0.045	0.641±0.007	0.755±0.009
NS15-aA2	I-D	58.413±0.118	0.167±0.092	99.682±0.046	40.405±0.478	0.288±0.087	0.291±0.070	0.690±0.008	0.842±0.010
SP5-A5	I-D	58.312±0..142	0.201±0.018	99.745±0.127	41.249±0.000	0.398±0.121	1.630±0.065	0.706±0.001	0.859±0.000
SP6-A5	I-D	58.431±0.101	0.143±0.047	99.755±0.001	41.050±0.000	0.377±0.115	7.129±0.018	0.701±0.000	0.855±0.000
SP9-A3	I-D	58.531±0.120	0.170±0.098	99.646±0.090	36.987±0.000	5.163±0.759	5.717±0.018	0.630±0.001	0.771±0.000
BK1-A1	I-E	57.713±0.025	0.036±0.016	99.824±0.160	23.312±0.276	1.053±0.069	0.399±0.059	0.403±0.004	0.486±0.006
NS15-bB2	II-A	57.525±0.025	0.035±0.021	99.795±0.204	12.586±0.149	0.659±0.099	0.413±0.078	0.218±0.002	0.262±0.003
NS13-bA1	II-B	56.235±0.014	0.020±0.027	99.855±0.154	25.247±0.298	0.319±0.076	0.260±0.134	0.448±0.005	0.526±0.006
SP14-B2	III-A	58.751±0.028	0.039±0.035	99.822±0.157	21.789±0.494	0.566±0.013	0.273±0.025	0.370±0.007	0.453±0.010
SP14-A3	III-B	57.179±0.030	0.042±0.003	99.933±0.009	22.172±0.499	1.562±0.072	0.529±0.014	0.388±0.009	0.462±0.010
SP15-A2	III-B	57.987±0.115	0.163±0.044	99.828±0.155	18.847±0.459	1.400±0.085	0.307±0.107	0.324±0.009	0.393±0.010
SP15-B2	III-B	57.798±0.110	0.156±0.030	99.848±0.166	19.119±0.462	0.210±0.018	0.263±0.000	0.330±0.009	0.3985±0.010

[a]Standard deviations was calculated from three independent samples. [b]Glucose utilization was defined as the percentage of glucose concentration utilized by the bacteria in initial glucose, [c]Succinic acid was obtained from cells grown in anaerobic conditions for 48 h

a hydrogen acceptor. It gave the maximum succinic acid of 72 g L^{-1} with a yield of 0.900 g g^{-1} fumarate and productivity of 17.100 g L^{-1} h^{-1} in continuous fermentation with initial fumarate concentration of 80 g L^{-1}. However, fumarate was inapplicable because of the expensive carbon source. To solve this problem, Moon *et al.*[10] used a combined fungal-bacterial two-step process with *Rhizophus* sp. and *E. faecalis* RKY1, in the first step, the fungus produced fumarate which was then transferred to a second reactor where *E. faecalis* RKY1 efficiently converted it to succinic acid. The high yield of 0.950 g g^{-1} substrate and the productivity of 2.2 g L^{-1} h^{-1} were obtained. However, this process was not practical due to the application of the two difference microorganisms.

Results from the above study could be concluded that Enterococci capable to produce succinic acid because they efficiency converted fumarate to succinate[10,11,14].

This study of succinic acid from *E. faecium* NS13-dB1 was lower than *E. faecalis* RKY1[10,11]. It might be the limitation of fumarate during the succinic acid production process. The further study will be required to optimize the fermentation process by integrating nutrient sources and cultivation conditions.

Table 4: Comparison of bacterial strains with succinic acid ability

Strains	Succinic acid ($g L^{-1}$)	Substrate		Time (h)	Productivity ($g L^{-1} h^{-1}$)	Succinic acid yield ($g g^{-1}$)	Byproduct	References
L. reuteri M9	1.46	Fumarate	(2 g L^{-1})	48	0.030	0.730	ND	Kaneuchi *et al.*[29]
A. succinogenes NJ 113	45.20	Glucose	(30 g L^{-1})	70	0.646	0.904	2.9 g L^{-1} of ace	Xi *et al.*[7]
C. glutamicum R	1.87	Glucose	(9.04 g L^{-1})	3	0.623	0.206	6.27 g L^{-1} of Lac and 0.18 g L^{-1} of ace	Raja and Dhanasekar[4]
C. crenatum	9.32	Glucose	(48.5 g L^{-1})	10	0.932	0.190	26.9 g L^{-1} of Lac and 0.03 g L^{-1} of ace	Chen *et al.*[30]
E. faecalis RKY1	72.00	Fumarate	(80 g L^{-1})	-[a]	17.100	0.900	ND	Wee *et al.*[11]
E. faecalis RKY1+*Rhizopus* sp.	42.00	Glucose	(50 g L^{-1})	-[a]	2.200	0.950	ND	Moon *et al.*[10]
		Rice bran	(5 g L^{-1})					
E. flavescens	14.25	Sucrose	(30 g L^{-1})	30	0.475	0.475	ND	Agarwal *et al.*[31]
E. faecium NS13-dB1	43.119	Glucose	(60 g L^{-1})	48	0.906	0.749	0.290 g L^{-1} of for and 0.144 g L^{-1} of ace	Present study

*Continuous, ND: Not detected, For: Formic acid, Ace: Acetic acid, Lac: Lactic acid, Mal: Malic acid, Pry: Pyruvic acid, Pro: Propionic acid and EtOH: Ethanol

CONCLUSION

From this study, 159 bacterial isolates from various sources were obtained. Nineteen isolates were Gram positive and produced succinic acid (in the range of 12.586-43.482 g L^{-1}). They were divided into three groups based on physiological, biochemical and phenotypic characteristics. Group I were identified as genus *Enterococcus.* Group II were identified as genus *Lactobacillus.* Group III were identified as genus *Lactococcus.* Among these isolates, *Enterococcus faecium* NS13-dB1 was the potent strain with high ability of succinic acid production (43.482 g L^{-1}) and has not been reported elsewhere.

SIGNIFICANCE STATEMENTS

- The novelty of this study is the high ability of succinic acid producing, *Enterococcus faecium*, was obtained which has not been reported elsewhere, whereas most of the succinic acid producing microorganisms were Gram-negative bacteria
- The isolation, screening and identification of succinic acid producing bacteria were concerned emphasizing to accomplish

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