INTERNATIONAL SYMPOSIUM

"Diversity and Optimum Utilization of Biological Resources in the Torrid and Subtropical Zones" (in honor of Prof. Ayaaki Ishizaki retirement)

Program

12:30 Opening remarks by Seiya Ogata (Kyushu Univ., Japan)

(Chair: Takeshi Kobayashi, Nagoya Univ., Japan)

12:40 Degradation and Utilization of Agricultural Organic Waste by Complex Microbial Communities
Yasuo Igarashi (Univ. Tokyo, Japan)

13:10 Bacterial Strains for the Direct Production of L-Lactic Acid from Cassava and Sago Starch
Sureelak Rodtong (Suranaree Univ. Technol., Thailand)

13:40 Potentials of Sago Starch in the Energy and Lactate Industries
Kopli Bujang (UNIMAS, Malaysia)

14:10 Comparisons of Productivity and Properties of the Starch from Several Tropical Palms
Nadirman Haska (BPPT, Indonesia)

14:40 <coffee break>

(Chair: Ayaaki Ishizaki)

15:10 Differences in Growth and Starch Production among Varieties and Soil Types in Sago Palm
Yoshinori Yamamoto (Kochi Univ., Japan)

15:40 Utilization of Bioresources in Sago Palm Related Activities
Jong Foh Shoon (National Timber & Forest Products, Indonesia)

16:10 Sweet Potato, it's Diversity and Future
Osamu Yamakawa (Kyushu Nat. Agr. Experiment Station, Japan)

16:40 International Collaboration with Southeast-Asian Countries in the Field of Industrial Microbiology and Biotechnology
Toshiomi Yoshida (Osaka Univ., Japan)

17:10 Closing remarks by Ayaaki Ishizaki

18:00 Retirement party (Faculty Club of Kyushu Univ.)

Saturday, June 2, 2001

International Hall of Kyushu University, Hakozaki, Fukuoka

Organized by

Seiya OGATA, Kensuke FURUKAWA, Kenji SONOMOTO & Genta KOBAYASHI

Bacterial Strains for the Direct Production of L-Lactic Acid from Cassava and Sago Starch

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Introduction

Lactic acid is one of typical organic acids produced by microbial fermentation of glucose. The increase in demand of L-lactic acid as a raw material of a biodegradable polymer is going on. Therefore, investigations aiming to obtain efficient lactate production and high productivity have been being attempted by several investigators. The utilization of a cheap and abundant raw material, particularly starchy material, is also of great benefit to the fermentative production of lactic acid. Some bacterial strains have been studied for their lactate production capabilities. Examples are *Lactobacillus amylovorus* (13, 14); *Lactobacillus plantarum* NCIM 2084 (7), metabolically engineered *Escherichia coli* RR1 (2); and *Lactococcus lactis* IO-1 (5, 6). However, potential microorganisms for L-lactic acid production are still desirable.

Lactococcus lactis IO-1, a homolactic fermenting bacterium, has been reported to be able to convert glucose to L-lactate with a conversion rate greater than 90% (5). Unfortunately, it cannot directly utilize starch. The study with aiming to obtain potential microorganisms for homo L-lactic acid production from cassava and sago starch, the major and abundant products in tropical areas, has been carried out at Suranaree University of Technology, Thailand, and is described in this paper. *Lactococcus lactis* IO-1 has been given permission to be used as the reference strain. The positive result could help to minimize the production cost in the hydrolysis step, liquefaction and saccharification of starch to glucose, and to obtain efficient lactate production.

Bacterial strains for L-lactic acid production from cassava and sago starch

1. Selection of bacterial strains for L-lactic acid production from cassava and sago starch

Two isolates of lactic acid bacteria, isolates SUT-1 and SUT-5, were selected for L-lactic acid production from cassava and sago starch. The bacteria were isolated from starch waste samples of the Cassava Starch Production Factory in Nakhon Ratchasima Province, Thailand. The isolates were selected after testing among 167 lactic acid bacterium isolates for their lactic acid production capabilities without gas, carbon dioxide, formation in the glucose medium (5), and for their starch utilization capabilities on the starch agar medium and then in starch broth medium originally adapted from Rogosa SL medium (1), with incubating at 37°C under anaerobic condition for 48 hours. The starch medium composed of (g/l): either cassava starch or sago starch, 10; sodium acetate, 1; pancreatic digest of casein, 5; di-potassium hydrogen phosphate, 6; yeast extract, 5; tri-ammonium citrate, 2; $MgSO_4.7H_2O$, 0.57; $MnSO_4.7H_2O$, 0.12; and $FeSO_4.H_2O$, 0.03, at the initial pH 7.0. Cassava starch was obtained from the Korat Cassava Starch Factory, Nakhon Ratchasima, Thailand. Sago starch (Tepung SAGO, DI-BUNGKUS OLEH, Jalan Jambu, Sibu) was obtained from Associate Professor Sarote Sirisansaneeyakul, Kasetsart University, Thailand. The medium was chosen after investigating suitable media for supporting growth and lactic acid production of bacteria tested.

Both isolates SUT-1 and SUT-5 could produced acid from glucose without gas formation, and were able to utilize cassava and sago starch. (Figure 1, for examples). *Lactococcus lactis* IO-1 was also confirmed as a non-starch-utilizing strain. To obtain the appropriate fermentation medium for the

comparison of lactic acid production capability of selected isolates, some optimum component concentrations of the starch medium were investigated. The batch fermentation was used for all studies of lactic acid production, and performed at 37°C for 24 hours under anaerobic condition and without shaking or agitating. Seed cultures were prepared from late-exponential phase cells adjusting the concentration at approximately 10^6 CFU/ml, and used as 2% (v/v) inocula. Then selected isolates were tested for their lactic acid production capabilities using the optimized medium containing cassava starch as well as sago starch, and also compared with the reference strain, Lactococcus lactis IO-1, using the same basal medium containing glucose. All cultures were cultivated in 125-ml Erlenmeyer flasks with working volume of 50 ml each. Time-courses of the batch fermentation of the selected isolates were recorded. At different fermentation periods, measurements of bacterial growth, pH, and lactic acid concentration were performed. Cell growth was monitored spectrophotometrically at 562 nm absorbance (A_{562}), and also indicated as viable cell concentration as CFU (colony forming units). The pH was measured using pH meter. Lactic acid was determined by High Performance Liquid Chromatography using HPLC System with variable loop autosampler model SpectraSystem AS 3000 (Thermo Separation Products, U.S.A.) and a prepacked column (RT 300-6, 5 polyspher OAHY, Merck) eluted with 0.005 N sulfuric acid.

When the suitable medium for lactic acid production of selected isolates was looked into, the optimum medium was basically concluded. The medium composed of ingredients as follows (g/l): carbon source either glucose or cassava starch/sago starch, 20 or 10; pancreatic digest of casein, 5; K_2HPO_4, 6; yeast extract, 3; tri-ammonium citrate, 1; $MgSO_4.7H_2O$, 0.57; $MnSO_4.7H_2O$, 0.12; and $FeSO_4.H_2O$, 0.03; at the initial pH 7.0. When compared to their lactic acid production capabilities using the optimized medium containing cassava starch, isolates SUT-1 and SUT-5 could produce lactic acid of about 10.5 and 9.4 g/l respectively, which were the maximum amounts achieved when incubated for 24 to 32 hours. When cassava starch was replaced by sago starch, the amounts of produced lactic acid of isolates SUT-1 and SUT-5 were 9.5 and 9.0 g/l which were a little bit lower than that produced in cassava starch medium. While the reference strain, *Lactococcus lactis* IO-1, could produce lactic acid at the concentration about 10 g/l at 24-hour growth in the same basal medium containing glucose. All strains also gave the maximum growth, around 10^9-10^{10} CFU/ml, when cultured for 24 to 32 hours. At the maximum growth and acid production, the pH of culture media dropped to about 4.7 and 4.8 for the media containing cassava starch and sago starch respectively, and 4.2 for the medium with the addition of glucose.

2. Characterization of selected strains of starch-utilizing and homolactic fermenting bacteria

Isolates SUT-1 and SUT-5 were basically characterized according to their morphological and some biochemical characteristics (4, 11). These two isolates were tentatively identified as belonging to the genus *Lactococcus*. They are Gram-positive, spherical to ovoid cells occuring in pairs and chains (Figure 2), and grow in both anaerobic and microaerophilic conditions. They are catalase negative, oxidase negative, and non-motile. The bacteria can grow very well at 37-40°C. They can grow slowly at 45°C but not at 10°C. The two isolates tolerate 6.5% sodium chloride but not to 40% bile. They cannot attack red blood cells. The production of ammonia from arginine is negative, and glucose is fermented to lactic acid without gas formation for all isolates tested. They give their specific fermentation profiles of 18 different carbohydrates. Both isolates SUT-1 and SUT-5 produce acid from fructose, maltose, mannose, melibiose, and raffinose, but not from arabinose, gluconate, mannitol, melezitose, rhamnose, ribose, sorbitol, trehalose, or xylose. The difference between the two isolates is that isolate SUT-1 also produces acid from amygdalin, cellobiose, and esculin, but not from salicin; and isolate SUT-5 produces acid from salicin, but not from amygdalin, cellobiose, and esculin.

The strain characterization of isolates SUT-1 and SUT-5 using nucleic acid methodology was also investigated. The randomly amplified polymorphic DNA (RAPD) technique was applied using

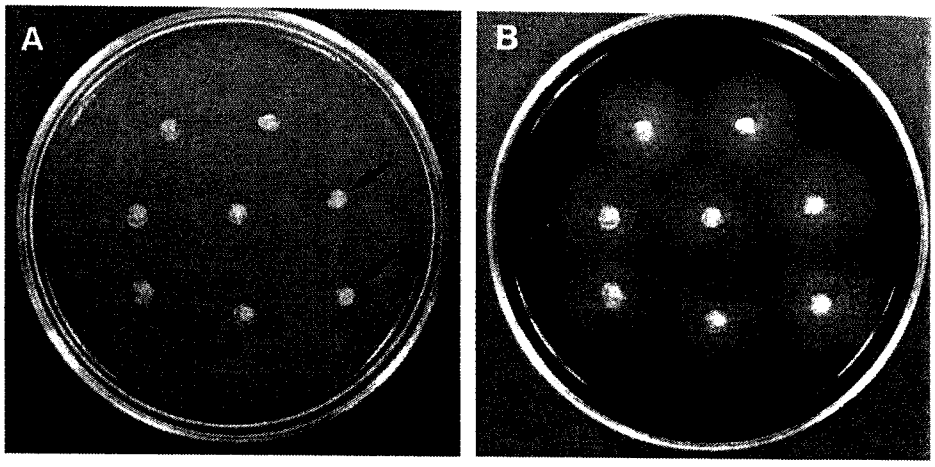

Figure 1. Growth and amylase expression of selected bacterium isolates: (A) growth on the agar medium containin 1% cassava starch, and (B) the addition of iodine solution (8) onto the agar surface of (A). Dark zone revealed the reaction of iodine and starch, and clear zones surrounding bacterial colonies revealed th expression of bacterial amylases. Arrows indicated isolate SUT-5 colonies.

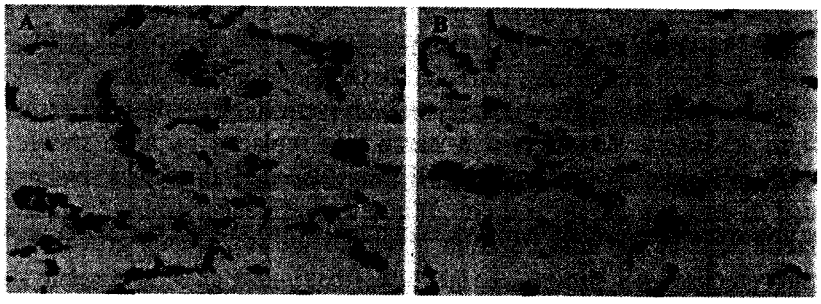

Figure 2. Gram stain of bacterial cells, bright-field microscopy (X1000): (A) isolate SUT-1, and (B) isolate SUT-5.

primer P3 (5′-CTGCTGGGAC-3′) and polymerase chain reaction amplification performed as describe by Tailliez *et al.* (10). RAPD patterns showed that the isolates SUT-1 and SUT-5 represent differen strains, and also they revealed different patterns from *Lactococcus lactis* IO-1 (Figure 3).

In addition, the nucleic acid technique was applied to investigated the gene coding for bacteriocii of isolates SUT-1 and SUT-5. Since nisin has been reported as the best known bacteriocin produce by several strains of *Lactococcus lactis* (9), therefore, the gene coding for bacteriocin of the tw isolates as well as *Lactococcus lactis* IO-1 was attempted to amplify using Primer 3 (5′ CCATGTCTGAACTAACA-3′) and Primer 4 (5′-CGCGAGCATAATAACGGCT-3′), which were designe from *Lactococcus lactis* F15876 DNA sequence containing the precursor nisin structural gene, a described by Rodriguez *et al.* (9). The approximate 350-bp amplification product was obtained fron all genomic DNA tested (Figure 4).

3. Study on lactate dehydrogenase genes of selected bacterium strains

Lactate dehydrogenase is a key enzyme in lactic acid fermentation by most lactic acid bacteria Most bacterial species possess only one lactate dehydrogenase gene (3). The DNA sequence analysis of the gene encoding lactate dehydrogenase is an important prerequisite to the genetic manipulation and improvement of starch utilization and lactic acid production strains. The lactate dehydrogenase

gene polymorphism is also useful for lactococcal classifications (12). Genes coding for lactate dehydrogenase of isolates SUT-1 and SUT-5 as well as *Lactococcus lactis* IO-1 were amplified from genomic DNA in polymerase chain reactions using four primers designed from sequences complementary to conserved sequences at the extreme ends of the coding region of L-lactate dehydrogenase gene according to Urbach *et al.* (12). The primers were LDHF1 (5'-ATGGCTGATAAACAACGTA-3'), LDHF2 (5'-GTTGCTGCTAACCCAGTTGA-3'), LDHR1 (5'-TTAGTTTTTAACTGCAGAAG-3'), and LDHR2 (5'-GTCAAGATGTCAACTGGGTT-3'). All of three bacterial strains produced results of approximate 670-bp, 1,000-bp, and 400-bp amplification products with specific primers LDHF2/LDHR1, LDHF1/LDHR1, and LDHF1/LDHR2 respectively (Figure 5). The sequence analysis of some of these amplified fragments will be in our futher investigation.

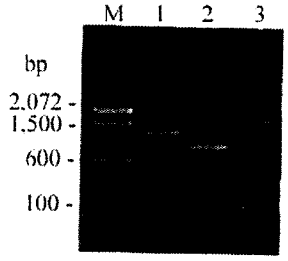

Figure 3. RAPD patterns of three bacterial strains using primer P3. Lanes: M, 100bp DNA ladder (GIBCOBRL) as a molecular weight marker; 1, *Lactococcus lactis* IO-1; 2, isolate SUT-1; and 3, isolate SUT-5.

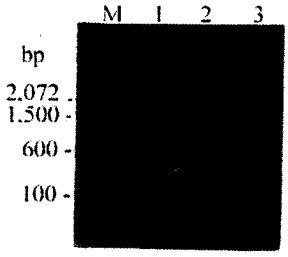

Figure 4. Agarose gel electrophoresis of PCR fragments generated with specific primers: Primer 3 and 4, of bacteriocin gene (9). Lanes: M, 100bp DNA ladder (GIBCOBRL) as a molecular weight marker; 1, *Lactococcus lactis* IO-1; 2, isolate SUT-1; and 3, isolate SUT-5.

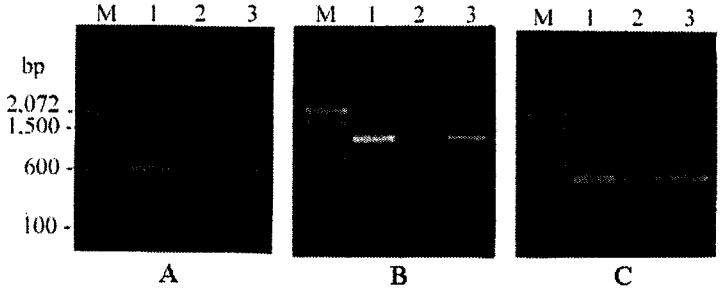

Figure 5. Agarose gel electrophoresis of PCR fragments generated with specific primers: (A) LDHF2/LDHR1, (B) LDHF1/LDHR1, and (C) LDHF1/LDHR2, of L-lactate dehydrogenase gene. Lanes: M, 100bp DNA ladder (GIBCOBRL) as a molecular weight marker; 1, *Lactococcus lactis* IO-1; 2, isolate SUT-1; and 3, isolate SUT-5.

Conclusion

Two bacterial strains, isolates SUT-1 and SUT-5, isolated from starch waste samples in Nakhon Ratchasima Province, Thailand, could actively convert cassava and sago polysaccharides to lactic acid. The two isolates were confirmed as homofermentative microorganisms. When compared to their lactic acid production capabilities using the investigated medium containing cassava starch, isolates SUT-1 and SUT-5 could produce lactic acid of about 10.5 and 9.4 g/l respectively, which were the

maximum amounts achieved at 24- to 32-hour cultivation. When cassava starch was replaced by sag starch, the amounts of produced lactic acid of isolates SUT-1 and SUT-5 were 9.5 and 9.0 g/ respectively. Isolates SUT-1 and SUT-5 were tentatively identified as belonging to the genu *Lactococcus* according to their morphological and some biochemical characteristics. The randoml amplified polymorphic DNA (RAPD) technique was also applied for strain characterization. RAPI patterns reveal that the isolates SUT-1 and SUT-5 are different strains. Genes coding for bacteriocii and L-lactate dehydrogenase of isolates SUT-1 and SUT-5 as well as *Lactococcus lactis* IO-1, th reference strain, could be amplified for further investigation.

Acknowledgements

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