APPLICATION OF PHOTOSYNTHETIC BACTERIA: BIOCONTROL OF PATHOGENIC ROOT ROT FUNGUS AND OTHER APPLICATIONS

（光合成細菌の応用に関する研究）

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2016

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ABSTRACT

In this study, purple non-sulfur photosynthetic bacteria (PNSB), were isolated and its application in wastewater treatment and biological control of pathogenic root rot fungus (RRF) were investigated. Samples from swine sewage wastewater and lake sediment were applied to isolate PNSB. First, the biological and biochemical characterization of the isolates were investigated. Then, one typical PNSB isolate was applied to treat with swine wastewater, a standard strain, *Rhodopseudomonas palustris* NBRC-100419T was used as a comparison. Furthermore, antagonistic effects of PNSB and other isolated microorganisms on pathogenic RRF was investigated. Finally, the effects of the different microorganisms on the rhizosphere bacterial community shifts were determined by PCR-denaturing gradient gel electrophoresis (DGGE).

PNSB were isolated from swine sewage wastewater and lake sediment. Phylogenetic analysis revealed that photosynthetic bacteria (PSB) isolates were most closely related to *Rhodopseudomonas* species. The results of pigments analyses showed the isolates contain bacteriochlorophyll a, bacteriopheophytin a and carotenoid-like substances. Growth of the isolates under photoautotrophic, photosynthesis heterotrophic, and heterotrophic condition was investigated. Compared with photoautotrophic condition, better growth was observed in all strains under photosynthesis heterotrophic, and heterotrophic condition. Furthermore, compared with NBRC strain, PSB isolates showed better growth in all conditions. Five isolates showed solid-liquid separation ability.

One typical strain, PSB Strain A was selected for the following biological and biochemical study. The effectiveness of utilizing carbon sources was studied by applying variety of organic acid components. The potential of PSB Strain A to treat with wastewater was also examined. The removal effects of volatile fatty acids (VFAs), total organic carbon (TOC) and ammonia nitrogen by applying PSB Strain A and NBRC Strain in swine sewage wastewater were investigated. Compared with standard strain, PSB Strain A showed almost the same removal effects of VFAs and TOC. The results suggest a possibility to treat with swine sewage wastewater with the isolated strain.

A pathogenic RRF was isolated from a sweet potato farm. Antifungal bacteria (AB) were also isolated from the infected farm soil by a plate-spreading method and 7 strains were preliminary chosen as antagonistic candidates. An antagonistic test by using the mycelial disk placement method revealed that one AB strain inhibited the RRF growth alone, which was identified as *Bacillus polyfermenticus* based on phylogeny of 16S ribosomal RNA genes, while two AB strains, identified as *Bacillus aerophilus* need coexistence of PSB Strain A, identified as a purple non-sulfur photosynthetic bacterium, *Rhodopseudomonas faecalis*.

Effect of addition of isolated microorganisms on the rhizosphere microbial communities shifts was determined by PCR-denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal RNA genes (16S rDNA) as well as traditional bacterial counts. All the microcosms showed decreased bacterial counts at day 7. In PCR-DGGE analysis, bacterial community shifts in all microcosms from day 0 to day 7 were minimal. PSB and AUT9 had better survival, which were detected at day 7, while AUT8 was only detected at day 0. In the microcosms amended with PSB, a distinct band was detected. The phylogenetic analysis showed the band related to *Catenulispora yoronensis*, one species of actinobacteria.

INTRODUCTION

Photosynthetic bacteria (PSB) are prokaryotes that are capable of carrying out photosynthesis. They are widely distributed in nature such as soil, lakes, paddy fields, oceans, rivers, and activated sludge (Koblížek et al., 2006; Tanaka et al., 2006). Purple non-sulfur photosynthetic bacteria (PNSB) are one of the most studied PSB, which are metabolically versatile and can live with various modes of energy-generating systems. They have been commonly applied in wastewater treatment, such as industrial waste solutions, municipal wastewater and sewage wastewater (Kobayashi and Tchan, 1973; Zhang et al., 2003; Takeno et al., 2005). Besides, PSB tend to be attractive due to the production of biomass during waste treatments. The substances containing in PSB, such as vitamin B12, ubiquinone (coenzyme Q10) and hydrogen have been studied to apply in medical and industrial fields (Sasaki et al., 2005; Matthew et al., 2008).

Swine sewage wastes contain high concentration of organic components, direct release will lead to environmental problems, such as eutrophication. Many strategies have been developed to treat with sewage wastes, including physical removing, chemical addition, activated sludge (Sneath, 1988; Vanotti and Hunt, 1999; Osada et al., 1991). PNSB have been applied to treat with organic wastewater. One advantage of using these bacteria is that they could grow directly in high organic load wastewater (Sasaki et al., 1991). Besides, swine wastewater generates an offensive odor, one species of PNSB, *Rhodopseudomonas palustris* has been reported to treat with odorous organic acids (Kim et al., 2004). Furthermore, PNSB cells contain various vitamins, amino acids and pigments, which could promote plant growth (Hotta et al., 1997; Han, 1999). Besides, the addition of PNSB improved actinomycetes growth and suppress the growth of pathogenic fungus (Kobayashi, 1984). Recently, molecular biological techniques have been proved to be useful to obtain a better understanding of the role of microbial diversity in ecosystems (Muyzer et al., 1993; Holben and Harris, 1995). The application of DNA extraction methods to environmental samples can eliminate the disadvantage of the traditional cell cultivation, which could obtain only a tiny fraction of the total microbial diversity.

In the present study, application of PNSB was tried as follows: 1) isolation, identification and characterization of PNSB strains from swine sewage wastewater and lake sediment samples; 2) selection of isolates with various organic acids utilization capabilities; 3) investigation of the effectiveness of the selected isolate to treat with sewage swine wastewater; 4) determination of the enhancement effects of isolated bacteria in their antifungal activities on root rot fungi by applying PNSB; 5) elucidation of the bacterial community dynamics by applying isolated bacteria and PNSB in microcosm experiments.

Overall, results of this study will lead to better understanding of applying PNSB in various fields. Especially, the experiment will contribute to understand the role of PNSB that have been used in rhizosphere as a fertilizer. In addition, the study will be vital to clarify the universal applicability of PNSB as a biocontrol agents to pathogenic root rot fungus.

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CHAPTER 1

ISOLATION AND CHARACTERIZATION OF PURPLE NON-SULFUR PHOTOSYNTHETIC BACTERIA

ABSTRACT

Purple non-sulfur photosynthetic bacteria (PNSB), were isolated from swine sewage wastewater and lake sediment. Photosynthetic pigments were extracted from the cells, and analyzed by chromatography. The results showed the main components were carotenoid, bacteriochlorophyll a and bacteriopheophytin a. Ability of the isolates to aggregate and precipitate during their growth, which is defined as solid-liquid separation ability in this study, was investigated by using rice water. Five isolates showed solid-liquid separation ability which signifies their potential as alternative for coagulants in wastewater treatments. PSB Strain A was selected for the following analysis due to its high productivity. Growth of the isolate under anaerobic-light condition with a variety of carbon sources was investigated. Both PSB Strain A and the standard strain showed good growth with acetic acid, propionic acid, and n-butyric acid at a concentration of 20 mM. While at a high concentration of 200 mM, PSB Strain A showed better growth in pyruvate, acetate, propionate, succinate and malate. Phylogenetic analysis revealed that PSB Strain A was most closely related to *Rhodopseudomonas faecalis*.

INTRODUCTION

Unlike oxygenic photosynthesis in eukaryotic and prokaryotic phytoplankton, there is a broad phylogenetic spectrum of anoxygenic (non-evolving oxygen) photosynthetic bacteria (PSB) containing bacteriochlorophyll (BChl). Anoxygenic PSB are divided into four major phyla, purple photosynthetic bacteria, green sulfur bacteria, green non-sulfur photosynthetic bacteria, and heliobacteria (Koblížek *et al*., 2006). Phototrophic bacteria, especially purple non-sulfur bacteria are ubiquitous in fresh and marine water, soil, wastewater, and activated sludge. They are metabolically the most versatile among all procaryotes: anaerobically photoautotrophic and photoheterotrophic in the light and aerobically chemoheterotrophic in the dark (Kim et al., 2004). They contain photosynthetic pigments, bacteriochlorophylls, and carotenoids, and can grow autotrophically with CO_2 as the sole carbon source (Pfennig, 1969). PSB cannot utilize H_2O as the electron donor for the assimilation of carbon dioxide since they due to the lack of photochemical reaction system Ⅱ which exists in plants, eukaryotic algae and cyanobacteria. Instead, photosynthetic bacteria can utilize hydrogen gas, hydrogen sulfide, thiosulfate, low-molecular organic components to conduct anoxygenic photosynthetsis. (Kitamura et al., 1984).

PSB have drawn a lot of attention because of their potential for biofuel, biohydrogen production, wastewater treatment and other extensive uses. The photosynthetic process contains a variety of components, studies have shown that biomass of photosynthetic bacteria is not only rich in high quality protein but also contains significantly large amounts of carotenoid pigments, biological cofactors and vitamins (Kobayashi and Kurata 1978; Vrati and Verma 1983). They could be used as a source of pigmentation, animal feed stock supplements and biofertilizer (Noparatnaraporn and Nagai, 1986; Vrati, 1984; Elbadry et al., 1999).

Purple non-sulfur bacteria have been reported as versatile microorganisms that can utilize energy from sunlight to drive metabolism and cellular growth (Drews, 1996). Species of the genus *Rhodopseudomonas* are the majority of purple non-sulfur bacteria. They have a complete tricarboxylic

acid cycle (TCA cycle) which allows them to grow anaerobically in the light or aerobically in the dark with different carbon sources and electron donors (Pfennig et al., 1974, Kitamura et al., 1984). Various organic compounds, including volatile fatty acids (VFAs) such as acetate, propionate, butyrate are important determinants in the development of phototrophic bacteria (Albers, et al., 1976; Shi, et al., 2006).

In this study, PSB were isolated from swine sewage water and lake sediment. The outstanding characteristics, such as morphological features, photosynthetic pigments and phylogenetic relationships were investigated. Furthermore, a preliminary study was attempted to apply PSB in wastewater treatment by determining solid-liquid separation ability and growth availability in a variety of carbon sources.

MATERIALS AND METHODS

Sample collection

Samples were obtained from swine sewage wastewater of a pig farm (Isa, Kagoshima, Japan), and lake sediment (Kanoya, Kagoshima, Japan) for isolation of PSB. After sampling, all samples were transferred to the laboratory and stored at 10˚C until isolation of microbes. *Rhodopseudomonas palustris* NBRC-100419T was purchased as standard strain from National Institute of Technology and Evaluation (NITE), Japan.

Microorganism isolation and cultivation method

After sampling, PSB was enriched by inoculating the samples into 30 ml of the Basic I medium (Hoshino et al., 1984). Basic I medium was composed of 1ml of the growth factor solution (0.05 g Thiamin-HCl, 0.05 g Nicotinic acid, 0.03 g p-Aminobenzonic acid, 0.01 g Vitamin B12, 0.01 g Pyridoxine-HCl, 0.005 g D-Biotin of 100 ml distilled water), 1ml of the trace element solution (2 g EDTA-2Na, 2 g $FeSO_4 \cdot 7H_2O$, 0.1 g H_3BO_3, 0.1 g $CoCl_2 \cdot 6H_2O$, 0.1 g $ZnCl_2$, 0.1 g $MnCl_2 \cdot 4H_2O$, 0.02

g $Na_2MoO_4 \cdot 2H_2O$ of 100 ml distilled water), including the following reagents (g/l): KH_2PO_4, 0.5; K_2HPO_4, 0.6; $(NH_4)_2SO_4$, 1.0; $MgSO_4 \cdot 7H_2O$, 0.2; NaCl, 0.2; $CaCl \cdot 2H_2O$, 0.05; $Na_2S_2O_3 \cdot 5H_2O$, 0.1; yeast extract, 0.1; malate, 0.5. An aliquot of the enriched culture was streaked on the Basic I double layer agar plates, containing 1.5 % agar for bottom layer and 1.2 % agar for top layer, and cultivated at 30 ˚C for 14 days under illumination of 12/12 light/dark cycles to obtain the colonies. The obtained colonies were transferred onto the Basic I agar slant medium, cultivated under the same condition as above, and stored at 10 ˚C for maintenance. The strains were grown under anaerobic conditions at 30-35℃, illumination of 12/12 light/dark cycles, in Basic I medium for the following analysis.

Cell morphology of PSB isolates

Colonies of PSB isolates were obtained by streaking and cultivating the isolates on the agar plate media. Optical microscopic observation was carried out by applying the liquid culture of the microorganisms. DAPI staining was applied to observe the cells. Firstly, 1ml culture of the isolates were added in 1.5 ml tube, 4',6-diamidino-2-phenylindol (DAPI) solution was added to the tube at a final concentration of 1μg/ml. The tubes were incubated for 5 min in dark condition at room temperature. Then, the mixture was filtered through a 0.2 μm Nuclepore polycarbonate filter (Whatman Ltd., Kent, UK). The cells on the filter were observed with an epi-fluorescence microscope (OLYMPUS BX50F, OLYMPUS, Co., Ltd., Japan) under UV excitation.

Scanning electron microscopy was carried out by applying the liquid culture of the microorganisms. Samples for scanning electron microscopy were prepared as follows: briefly, 40 μl of 25 % glutaraldehyde was added into 1 ml of the culture medium, and the suspension was filtered through a 0.2 μm Nuclepore polycarbonate filter (Whatman Ltd., Kent, UK). Then, a dehydration process was applied using a series of graded ethanol solution (1 ml of 50 to 80 % ethanol, four times; then 1 ml of 99.5 % ethanol twice). The filter was gold-coated with a sputter-coating machine (MSP-Mini Magnetron Sputter, Vacuum Device, Co., Ltd., Ibaragi, Japan) and observed with a scanning

electron microscope, Miniscope TM-1000 (Hitach High Tech, Co., Ltd., Tokyo, Japan).

Identification of PSB isolates

DNA of the isolated PSB Strain A was extracted from the liquid cultures by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was used for PCR amplification of 16S ribosomal RNA genes (16S rDNA) with a universal primer set PrSSU.1F (5'-AGAGTTTGATCATGGCTCAG-3') and PrSSU.1R (5'-GGTTGGATCACCTCCTTA-3'). The PCR reaction mixture was composed of Ex Taq Buffer (Takara Bio, Otsu, Japan), 100 µM of each dNTP, 0.5 µM of each primer, 0.025 units/µl Ex Taq DNA polymerase Hot Start Version (Takara Bio, Otsu, Japan), and one-tenth volume of the bacterial DNA solution. The thermal cycling consisted of an initial denaturation of 1 min at 94˚C, followed by 25 cycles of 30 s of denaturation at 94˚C, 20 s of annealing at 56˚C and 1.5 min of extension at 72˚C, with 7 min of final extension at 72˚C.

The PCR products were electrophoresed by 1.5 % agarose gel in TAE buffer (40 mM Tris-acetate, 1 mM ethylenediaminetetraacetic acid, EDTA) under a constant voltage condition of 100 V with the electrophoresis system GelMate 2000 (Toyobo, Osaka, Japan). The electrophoresed gels were stained with SYBR Gold (Molecular Probes, Life Technologies, Carlsbad, USA) and photographed under a blue transilluminator Safe Imager 2.0 (Invitrogen, Life Technologies, Carlsbad, USA). The obtained bands were cut from the gel and the PCR products were purified from the gel pieces using the MonoFas DNA Purification Kit I (GL Science, Tokyo, Japan).

Nucleotide sequences of the PCR-amplified 16S rDNA were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, USA). The primers used were PrSSU.1F, PrSSU.3F (5'-TGCCAGCAGCCGCGGTA-3'), PrSSU.1R, and PrSSU.2R (5'-TGCCAGCAGCCGCGGTA-3'). The obtained sequences were assembled with the program DNASIS Pro Version 2.7 (Hitachi Solutions, Tokyo, Japan).

Closely related sequences were obtained from the GenBank DNA database by using the basic

local alignment search tool (BLAST) program. Phylogenetic analysis with the neighbor-joining method was conducted using the program MEGA Version 5 (Tamura et al., 2011).

Accession number

The nucleotide sequences of the partial sequence of the 16S rDNA determined in this study have been submitted to the DDBJ database under the accession number LC066638.

Pigments Analyses

Pigments of PSB isolates were extracted by a mixture of acetone and methanol (7:3) from the liquid culture. After maintaining in freezer overnight, extracts were centrifuged for 5 min, at 15,000 rpm, 4 ℃ and the supernatants were used for High Performance Liquid Chromatography (HPLC) analyses as described below. The HPLC system used (HITACHI Advanced HPLC System Manager, Japan) is composed of a main pump (HITACHI L-7100), a detector (HITACHI L-7455). The column oven was HITACHI L-7300, and the column GH-C18 was applied for reverse-phase HPLC. The mobile phase was a mixture of two solvents A and B made of methanol:ammonium acetate 1M (80:20, v/v), and acetonitrile:acetone (60:40, v/v), respectively. The solvent program was defined as follows: a 28 minute linear increase of solvent B from 5% to 100%, a hold at 100% of solvent B for 32 minutes, and a return to initial conditions in 5 minutes. Then, the acetone extracts were injected into the system. Best results were obtained using a flow rate of 0.5 ml/min. Absorbance peaks were graphically recorded at a range of 350 nm to 800 nm that correspond to the short wavelength absorption maxima of each pigment. Peaks were identified according to their absorption spectra measured by means of the detector and stored as matching patterns for further determinations.

Growth ability of PSB isolates

3 PSB isolates were applied to determine their growth ability, NBRC strain was used as the

comparison. Their growth under photoautotrophic, photosynthesis heterotrophic, and heterotrophic condition was investigated. Growth rate of PSB strains was measured by spectrophotometer (HITACHI U-1100, Japan). The cell growth was determined from the attenuance (optical density) of the culture at 660 nm (OD660). For photoautotrophic condition, after adjusting OD660 value to 0.35-0.44, 1 ml of PSB culture was inoculated into 20 ml Basic I medium containing 100 µl 10% sodium ascorbate solution in screw-cap tube. After storing in dark condition for 1 day, photosynthesis culture was carried out under natural light condition at room temperature for 28 days. Culture for each isolate was carried out in triplicate. For photosynthesis heterotrophic condition and heterotrophic condition, 10 ml 802 medium was added into 18 mm diameter tubes with silicon cap. 802 medium was composed of the following reagents (g/L): $MgSO_4 \cdot 7H_2O$, 1; yeast extract, 2; polypeptone, 10. The cultures for photosynthesis heterotrophic condition and heterotrophic condition were incubated for 28 days under natural light condition and dark condition, respectively.

Solid-liquid separation ability

Ability of the isolates to aggregate and precipitate during their growth, which is defined as solid-liquid separation ability in this study. The PSB were incubated as described previously. Rice water was prepared by rinsing rice one time, and the obtained supernatant was mixed with the PSB culture (450 ml rice water mixed with 50 ml PSB culture). After letting the mixture stand for 1 hr, the solid-liquid separation status was observed.

Availability of organic acid by PSB strains

Growth rate of PSB strains was measured by spectrophotometer (HITACHI U-1100, Japan). The cell growth was determined from the attenuance (optical density) of the culture at 660 nm (OD660). After adjusting OD660 value to 0.5-0.6, 1 ml of PSB culture was inoculated into 20 ml Basic I medium in screw-cap tube. Various organic acids were added as the single carbon source in Basic I medium,

such as acetic acid, propionic acid and n-butyric acid which were added at a concentration of 20 mM. Furthermore, acetic acid, propionic acid, n-butyric acid and short-chain fatty acids related with tricarboxylic acid cycle, such as formic acid, citric acid, α-ketoglutaric acid, succinic acid, fumaric acid, malic acid, malonic acid and pyruvic acid were added at a concentration of 200 mM. Except for n-butyric acid, salts of the organic acids were applied in this study. 100 µl of 10 % sodium thioglycolate solution was added as the reductant. Incubation conditions were described previously.

Statistical analysis

Data was subjected to independent t test method to determine significant differences. $p<0.05$ was considered to be statistically significant.

RESULTS

Characterization and identificationof PSB isolates

Colonies of the photosynthetic bacterium (PSB) isolates were observed after 14-days incubation on the Basic I agar plate (data not shown). Scanning electron microscopic observation showed that the PSB cells were rod-shaped with bright spots at both ends of the cells, and the cell length was 3-4 µm (Fig. 1). In pigments analyses, chromatography at 430 nm of PSB Strain A extracts gave rise to multiple peak chromatograms corresponding to the different homologous forms of a unique bacteriochlorophyll (Fig. 2). 4 peaks obtained were eluted at 31.76, 35.17, 37.87, and 40.43 min, respectively. Max Absorption values were obtained near 450 nm to 500 nm. For chromatography at 750 nm of PSB Strain A, 2 peaks obtained were eluted at 29.96 and 34.50 min, respectively. Max Absorption value for 1st peak was obtained at 350 nm, 570 nm, 750 nm, while 350nm, 470 nm, 750 nm for the 2nd peak (Fig. 3).

After 28 days' incubation, compared with photoautotrophic condition, better growth was observed in all strains under photosynthesis heterotrophic, and heterotrophic condition. Compared with

NBRC strain, PSB isolates showed better growth in all conditions (Fig. 4, 5, 6). While no significant differences were found between PSB isolates under photoautotrophic and heterotrophic condition, PSB Strain A showed better growth under photosynthesis heterotrophic condition (Fig. 5). Results of their molecular phylogeny analyses through NCBI Blast showed that PSB isolates were closely related to *Rhodopseudomonas sp.* (Fig. 7), further analyses showed that PSB Strain A was most closely related to *Rhodopseudomonas faecalis* (Fig. 8).

Solid-liquid separation ability and growth of PSB Strains

After 1 hr's observation, five isolates showed solid-liquid separation ability (Table 1). Both PSB Strain A and NBRC strain showed good growth with acetate, propionate, and n-butyric acid at a concentration of 20 mM, no statistically significant differences were found between PSB Strain A and NBRC strain ($p>0.05$) (Fig. 9). Comparatively, PSB Strain A showed better growth with pyruvate, acetate, propionate, succinate and malate at a concentration of 200 mM ($p<0.05$). However, neither PSB Strain A nor NBRC strain showed good growth with n-butyric acid, formate, citrate and α-ketoglutrate (Fig. 10).

DISCUSSION

Purple photosynthetic bacteria are frequently among the predominant populations of microorganisms in ponds, ditches, and other water sources, polluted by sewage or other types of organic matter (Pfennig, 1967). In this study, the isolation and purification of photosynthetic bacteria was conducted by applying the samples from swine sewage wastewater and freshwater lake sediment. Totally, 17 isolates were obtained.

Morphological features showed the isolates were closely related to purple non-sulfur bacteria. The results of pigments analyses showed the isolates contain bacteriochlorophyll a, bacteriopheophytin a and carotenoid-like substances. These pigments were consistent with the pigments contained in the

purple photosynthetic bacteria (Kobayashi and Ohashi, 2006). Thus, the isolates were considered to be a type of photosynthetic bacteria. Identification based on the 16S ribosomal RNA gene (16S rDNA) sequences showed that the isolates were affiliated to the genus *Rhodopseudomonas*.

Organic wastes such as animal slurry contains plant nutrients that are essential for crop production. However, intensive livestock production may lead to a surplus of plant nutrients on farms and, as a consequence, discharge or emission to the environment. In order to ensure that the slurry applied to fields matches the nutrient requirements of the crops, techniques have been developed to reduce the nutrient content of slurry by means of separation, such as chemical additions, membrane separation, and activated sludge (Hjorth et al., 2010; Visvanathan et al., 2000; Nielsen and Keiding, 1998).

Some photosynthetic bacteria have been reported to have flocculating ability which has some advantages for industrial processes because of the simplicity of liquid/ solid separation and the ease of cell mass retention in the reactor (Watanabe et al., 1998). In this study, a preliminary study was conducted to investigate the solid-liquid separation ability of the PSB isolates. Solid-liquid separation activities were observed in 5 isolates mixed with rice water. However, further study should be conducted on the efficiency and mechanism of their solid-liquid separation ability.

One isolate, PSB Strain A was selected to investigate its growth ability and availability of organic acids. Results suggested that PSB Strain A could grow well in a variety of organic acid salts. Better growth was observed with lower concentration of n-butyric acid, indicating that high concentration of organic matters might inhibit the growth of photosynthetic bacteria. Growth availability with different carbon sources of the genera and species of *Rhodopseudomonas* has been reported (Imhoff et al., 2005). Relatively, PSB Strain A could utilize a variety of substrates (Table 2). A long-chain organic acid, n-butyric acid has been reported to be difficult to utilize (Nakajima et al., 1997). However, in this study, PSB Strain A showed growth with n-butyric acid at a concentration of 20 mM. Since acetic acid, propionic acid, and n-butyric acid are the main contents of volatile fatty acids in swine sewage wastewater, the results suggest that PSB Strain A appears to be a promising

practical treatment of swine sewage wastewater.

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FIGURES AND TABLES

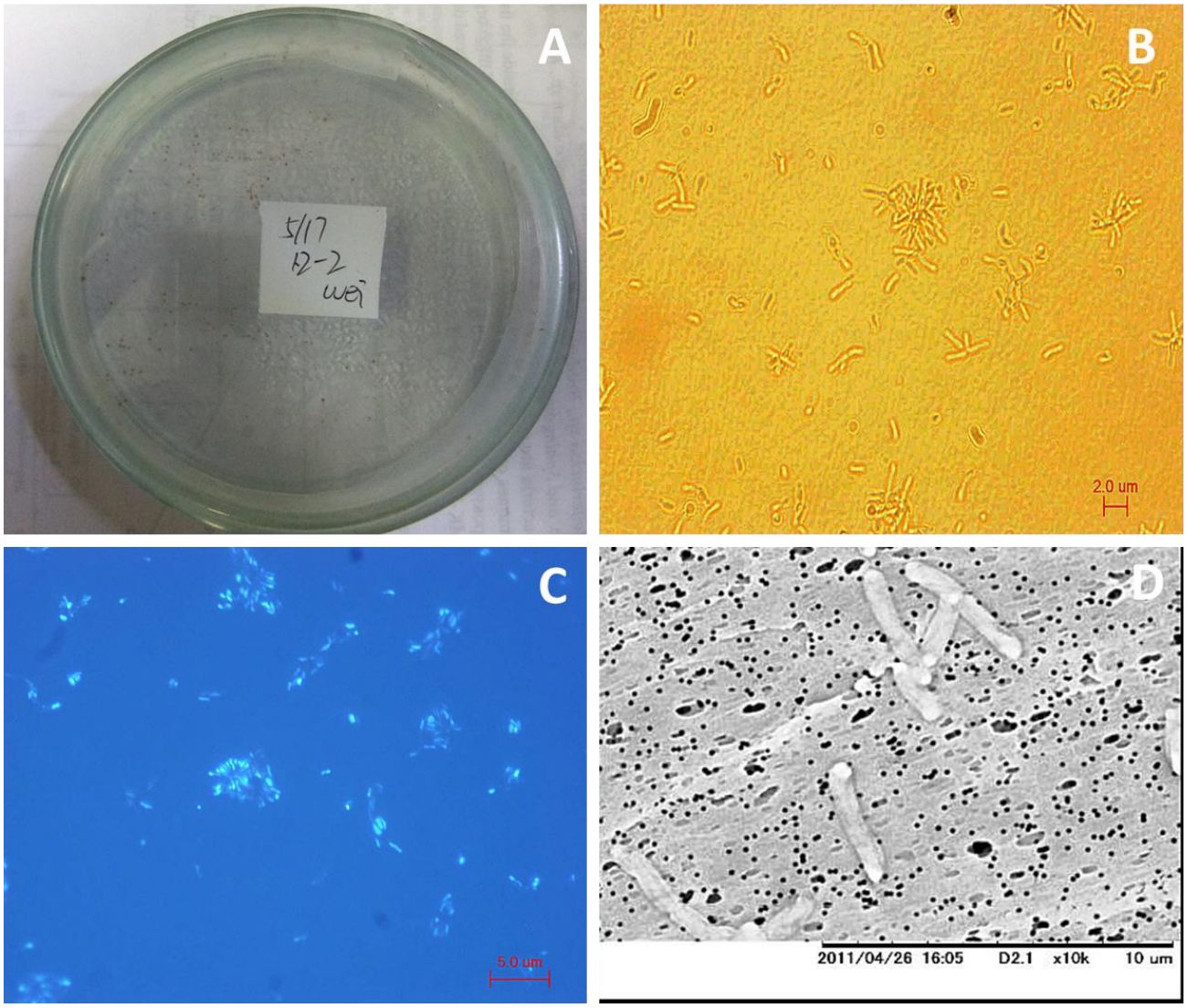

Fig. 1. Photosynthetic bacterium strain A. A: colony form on the Basic I agar plate; B: optical microscopic observation of the cells; C: fluorescent microscopic observation of the cells stained by 4',6-diamidino-2-phenylindol (DAPI); D: scanning electron microscopic observation of the cells.

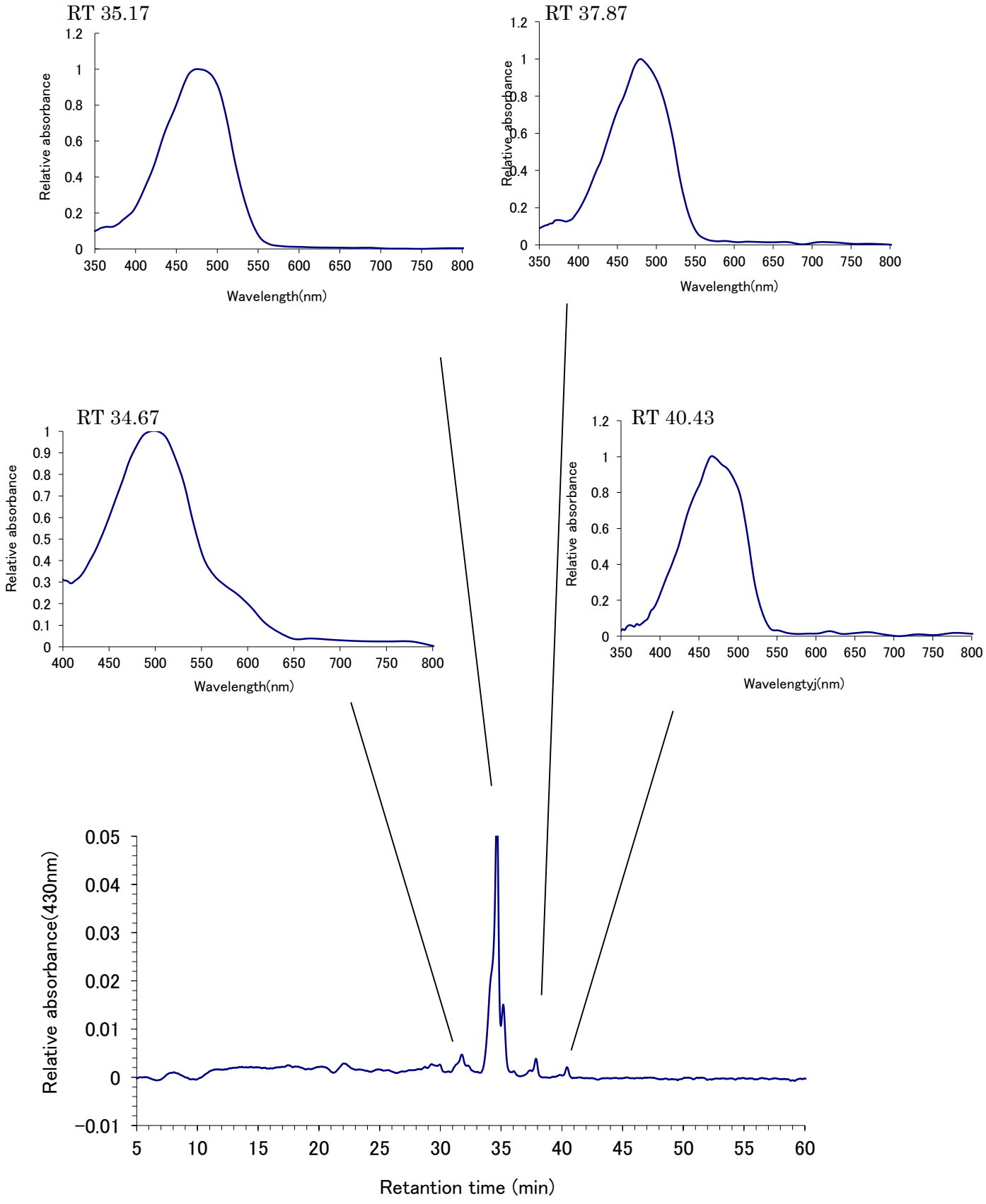

Fig. 2. Chromatogram at 430 nm of the pigments extracted from the photosynthetic bacteria (strain A), RT, Retantion time.

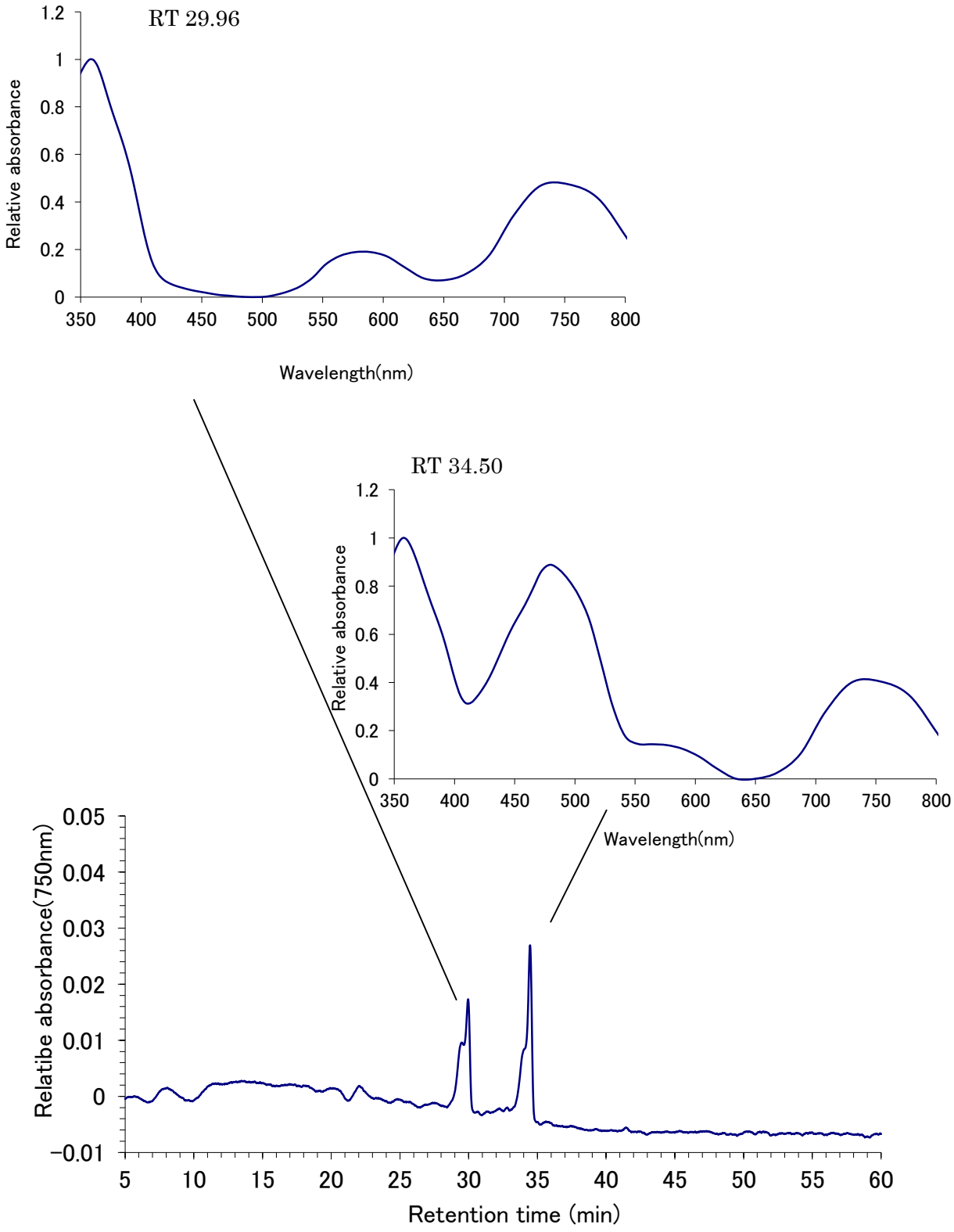

Fig. 3. Chromatogram at 750 nm of the pigments extracted from the photosynthetic bacteria (strain A), RT, Retetion time.

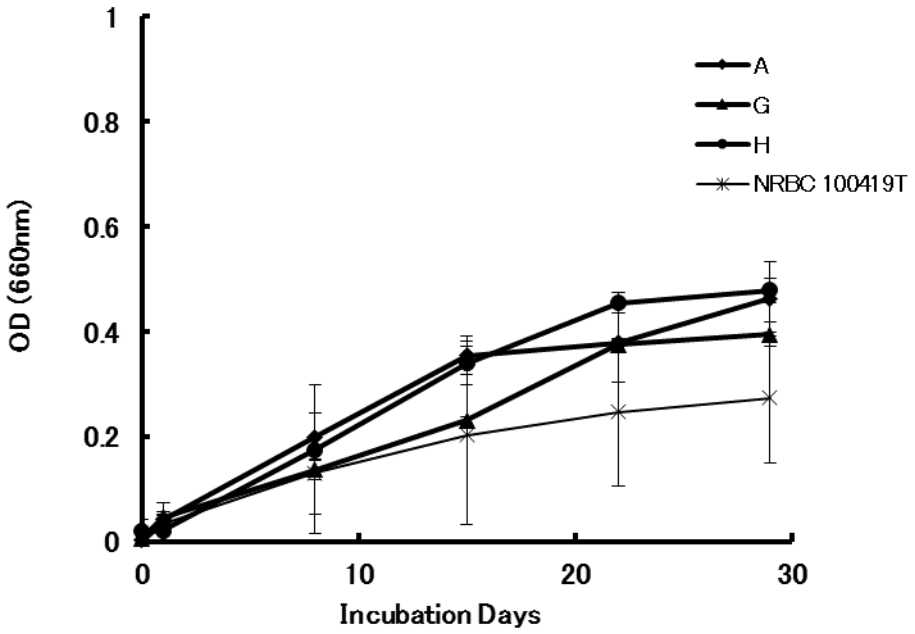

Fig. 4. The change of the growth rate of PSB isolates and type strain *Rhodopseudomonas palustris* (NBRC- 100419^{T}) under photoautotrophic condition in Basic I medium (n = 3, ± standard deviation). A: PSB Strain A; G: PSB Strain G; H: PSB Strain H. OD: optical density.

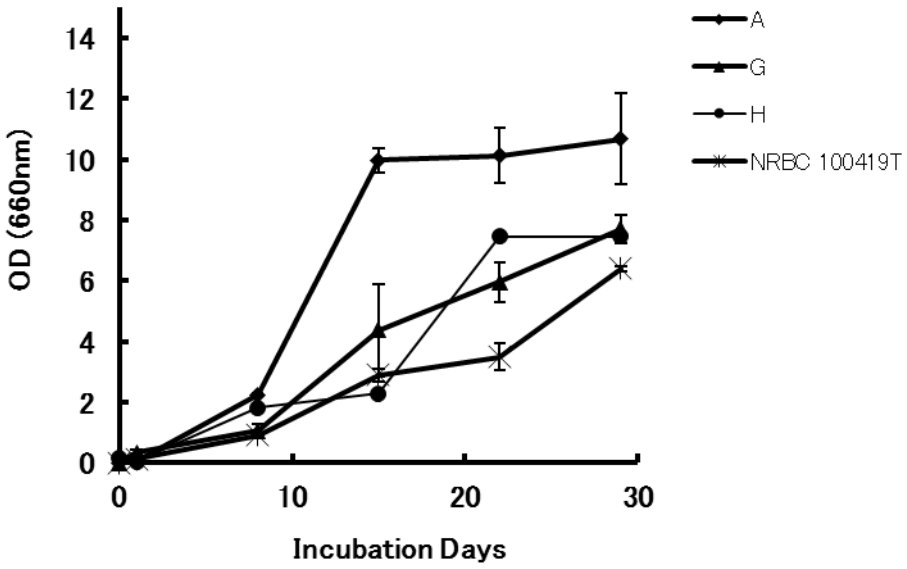

Fig. 5. The change of the growth rate of PSB isolates and type strain *Rhodopseudomonas palustris* (NBRC- 100419^{T}) under photosynthesis heterotrophic condition in Basic I medium (n = 3, ± standard deviation). A: PSB Strain A; G: PSB Strain G; H: PSB Strain H. OD: optical density.

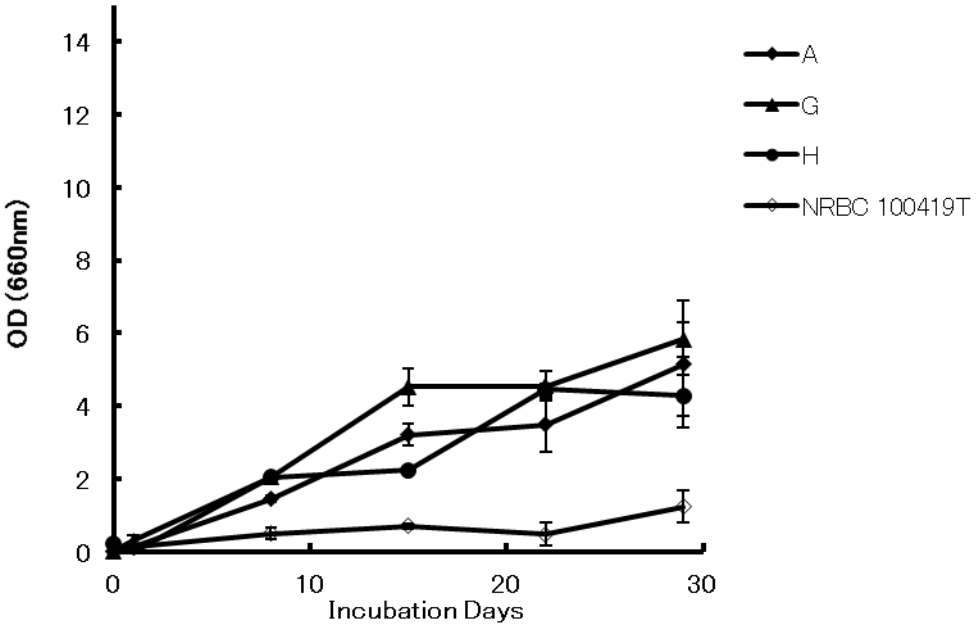

Fig. 6. The change of the growth rate of PSB isolates and type strain *Rhodopseudomonas palustris* (NBRC- 100419^{T}) under heterotrophic condition in Basic I medium (n = 3, ± standard deviation). A: PSB Strain A; G: PSB Strain G; H: PSB Strain H. OD: optical density.

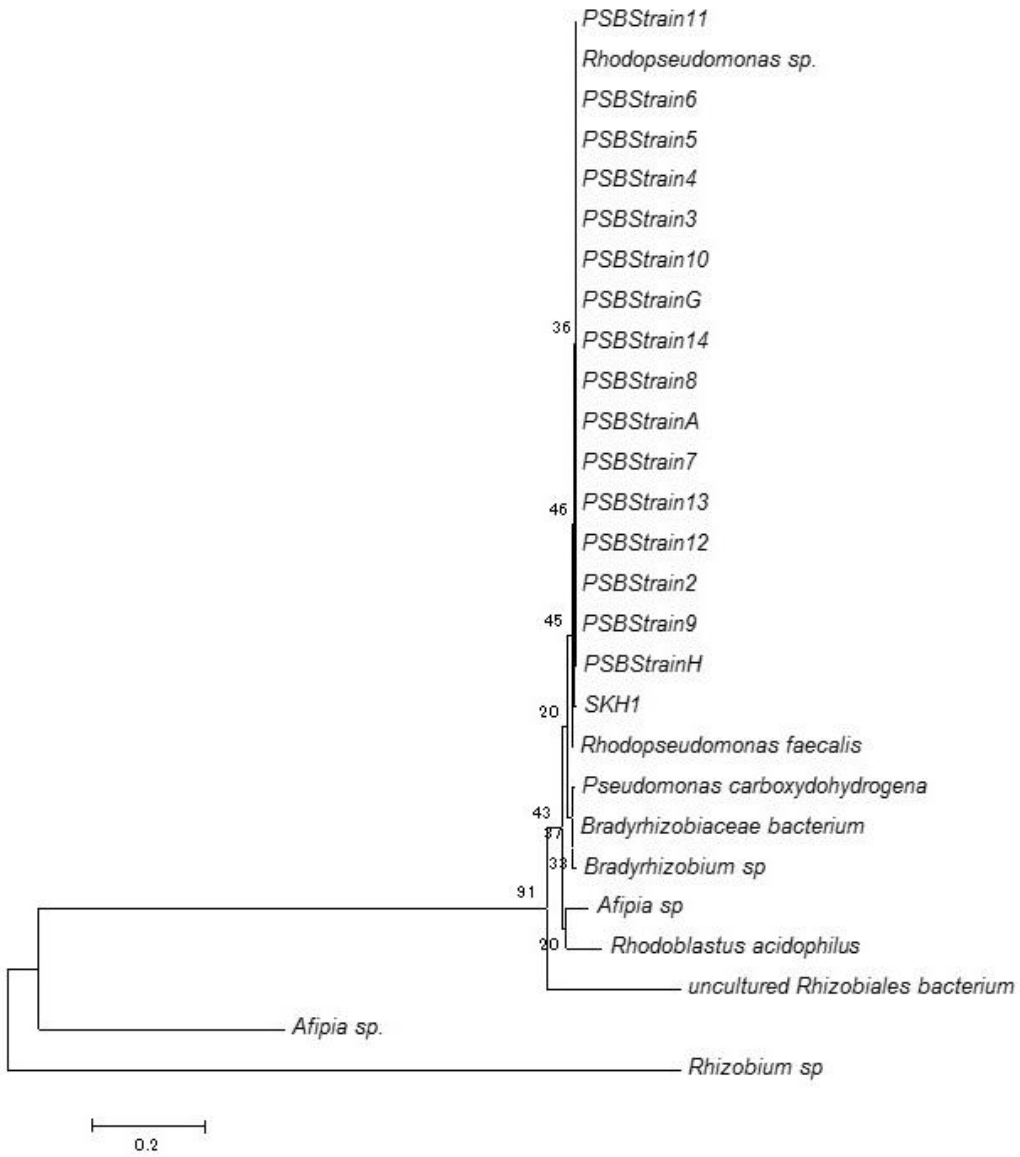

Fig. 7. Unrooted phylogenetic tree of the photosynthetic bacterial isolates based on the partial 16S rDNA sequences. Bootstrap values are shown at the branching points. Scale bar, genetic distance.

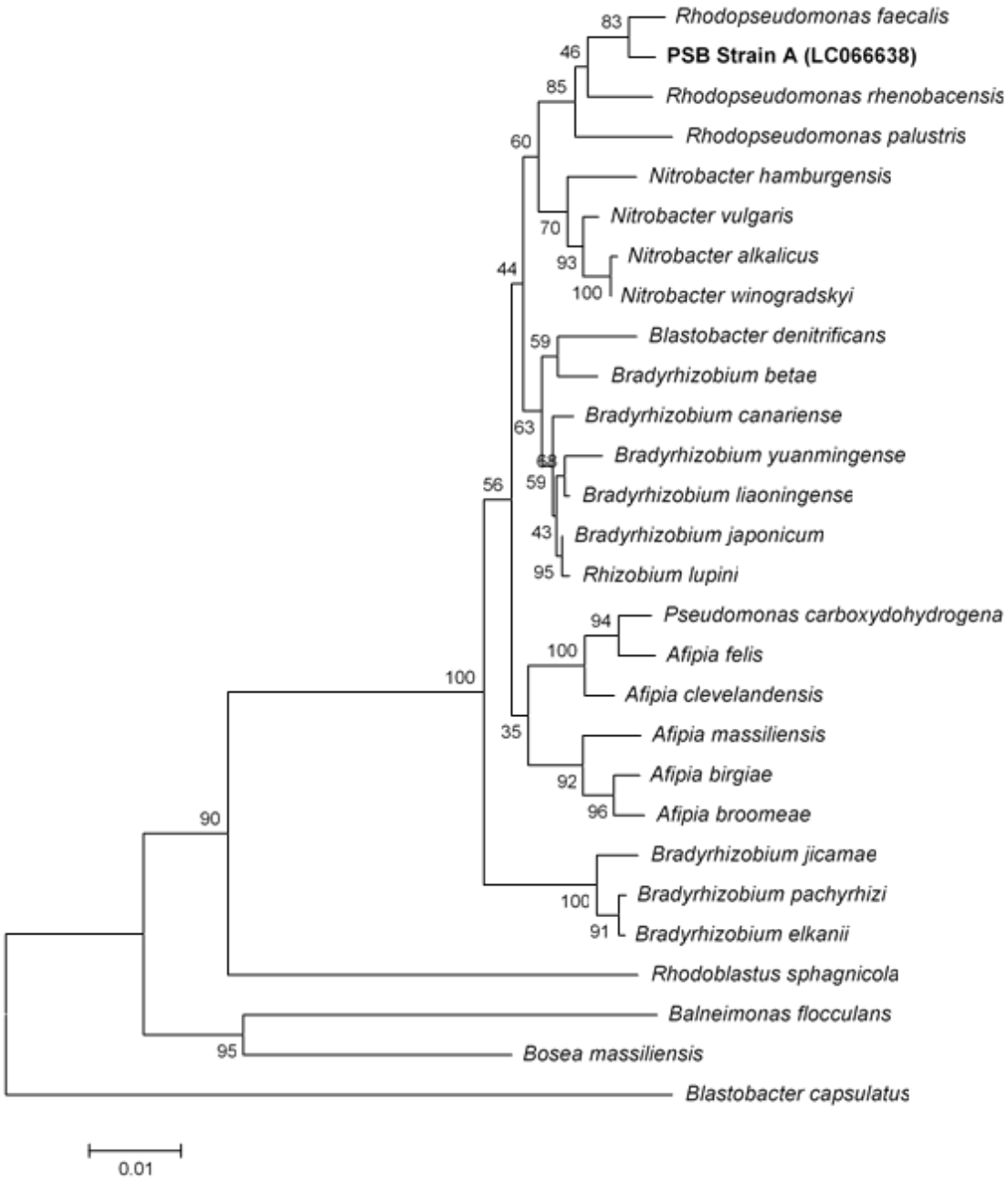

Fig. 8. Unrooted phylogenetic tree of PSB Strain A based on the 16S rDNA sequences. Bootstrap values are shown at the branching points. Scale bar, genetic distance. Nucleotide sequence information of the photosynthetic bacterial isolate has been registered in DNA Data Bank of Japan (DDBJ), accession number: LC066638.

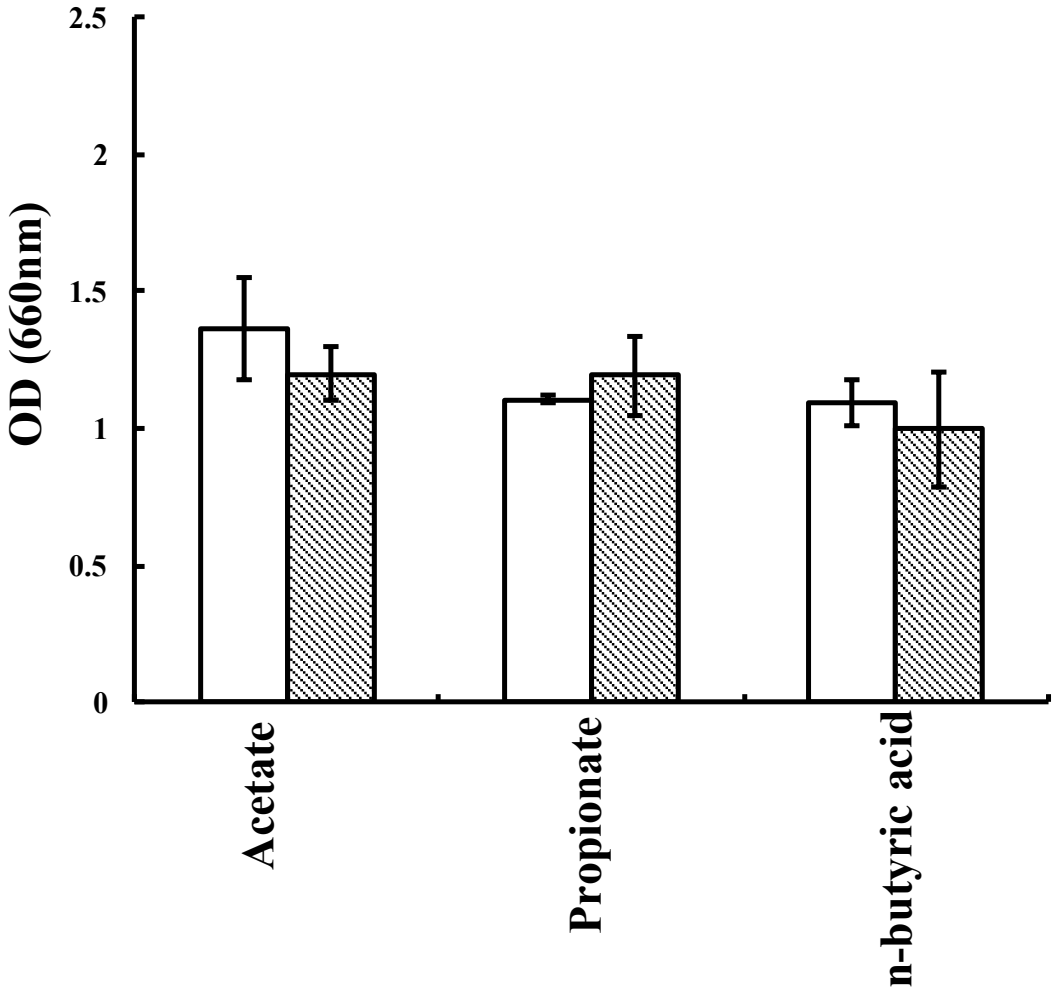

Fig. 9. Growth of strain A (□) and the type strain *Rhodopseudomonas palustris* (NBRC- 100419^{T})(▨) in an artificial medium added with several organic acid (20 mM) as sole carbon source (n = 3, ± standard deviation).

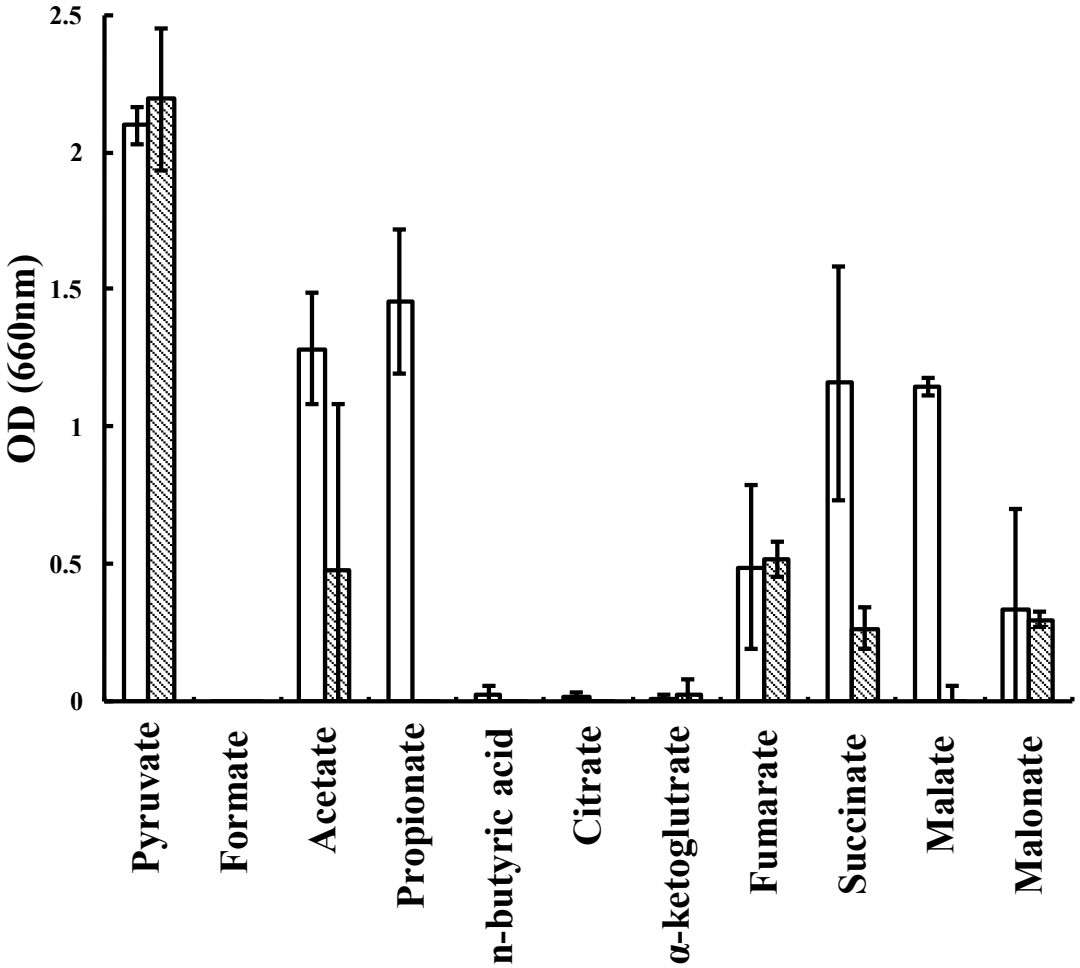

Fig. 10. Growth of strain A(□) and the type strain *Rhodopseudomonas palustris* (NBRC-100419^{T})(▨) in an artificial medium added with several organic acid (200 mM) as sole carbon source (n = 3, ± standard deviation).

Table 1 Solid-liquid separation ability of PSB isolates.

Organism	Solid-liquid Separation Ability
PSB Strain 2	-
PSB Strain 3	-
PSB Strain 4	-
PSB Strain 5	-
PSB Strain 6	-
PSB Strain 7	-
PSB Strain 8	+
PSB Strain 9	-
PSB Strain 10	+
PSB Strain 11	-
PSB Strain 12	+
PSB Strain 13	-
PSB Strain 14	-
PSB Strain A	+
PSB Strain G	+
PSB Strain H	-

[a]Symbols: +, positive in solid-liquid separation availability; -, negative in solid-liquid separation availability.

Table 2. Comparision of growth availability of PSB with other *Rhodopseudomonas* species in several organic acids.

Organism	Carbon Source											References
	Acetate	Butyrate/ Butyric acid	Citrate	Formate	Fumarate	Malate	Ketoglutrate	Malonate	Propionate	Pyruvate	Succinate	
PSB A strain	+	+	-	-	+	+	-	+	+	+	+	This study
R. palustris NBRC	+	+	-	-	+	-	-	+	+	+	+	This study
R. palustris	+	+	+/-	+	+	+	nd	+	+	+	+	Imhoff J.F. et al.
R. rhenobacensis	+	+	-	+	+	+	nd	nd	-	+	+	Imhoff J.F. et al.
R. julia	+	+	-	+	+	+	nd	-	+	+	+	Imhoff J.F. et al.
R. cryptolactis	+	nd	nd	nd	nd	+	nd	nd	nd	+	+	Imhoff J.F. et al.

[a]Symbols: +, positive in growth availability; -, negative in growth availability; +/-, variable in different strains; nd, not determined.

CHAPTER 2

SWINE WASTEWATER TREATMENT BY APPLYING PHOTOSYNTHETIC BACTERIA STRAIN A

ABSTRACT

A photosynthetic bacterial strain, PSB Strain A was selected for the treatment of swine sewage wastewater. Growth rate of PSB Strain A under anaerobic-light condition in the wastewater was investigated, a standard strain, *Rhodopseudomonas palustris* NBRC-100419T was applied as the comparison. After 216-h incubation, the concentration of VFAs, which were acetic acid and propionic acid, decreased from 158.0 mM to 120.2±2.9 mM, and 14.9 mM to 9.3±0.9 mM, respectively. After 330-h incubation, the concentrations of TOC and ammonia nitrogen dropped from 4508.0 mg/L to 3104.0±451.5 mg/L, and 629.7 mg/L to 424.1±7.4 mg/L, respectively. The isolated PSB Strain A showed almost the same efficiency compared with standard strain on removal effects of VFAs and TOC. The results suggest a possibility to treat with swine sewage wastewater with the isolated strain.

INTRODUCTION

Water pollution by organic matter discharged from industrial and domestic premises has become an environmental problem. Intensive farming has increased the problem of disposal of farm waste, especially for the large volumes of pig or cattle waste. Swine wastewater contains large amount of organic matters and high concentration of ammonia, which is toxic to fish and other aquatic organisms (Obaja et al., 2003). Besides, swine wastes generate an offensive odor composed of volatile fatty acids (VFAs), which makes them difficult to use them as a manure (Lindall et al., 1974). Directly discharging these wastewaters might cause eutrophication and could be a potential risk to public health (Bernet et al., 2000). Many strategies have been developed to treat this kind of wastewater, such as gravity sedimentation, precipitation, and activated sludge (Liao et al., 1993; Martinez, et al., 1995; Guo et al., 2013).

Biodegradation has been for many years the method used for treatment of domestic waste, and aerobic treatment has been extensively studied for nitrification and denitrification of wastewaters (Silverstein et al., 1983; Jern, 1987). Comparatively, anaerobic digestion is widely used to remove organic matter from high strength wastewaters because of its relatively low sludge production and energy needs (Hobson, 1973; Barber et al., 1999).

Purple non-sulfur bacteria (PNSB) are widely distributed in nature especially in water source polluted by organic matter. They play a major role in purifying the environment because they combine photosynthesis with the ability to photometabolize many organic substances (Kobayashi 1982). Since the 1970s, bioremediation of wastewater with high concentration of organic matter has been investigated by applying PNSB (Kobayashi and Tchan, 1973, Kitamura et al., 1980). One advantage of using these bacteria in bioremediation of agroindustrial wastewaters is that they can grow directly in high organic load wastewater. Removal of VFAs and ammonia has also been reported by using PNSB (Lee et al., 1992, Zhan et al., 2013,). Besides, one advantage using these bacteria is that a variety of bacterial biomass could be produced during the process. Yielded biomass could be used as diet for aquaculture, animal feed stock supplements and biofertilizer (Kobayashi and Kurata, 1978,

Noparatnaraporn et al., 1983).

Species of the genus *Rhodopseudomonas* are the majority of purple non-sulfur bacteria. They have a complete tricarboxylic acid cycle (TCA cycle) which allows them to grow anaerobically in the light or aerobically in the dark with different carbon sources and electron donors (Pfennig et al., 1974, Kitamura et al., 1984). Genus *Rhodopseudomonas* has been demonstrated to be the dominant species in microbial mats in a swine wastewater ditch (Okubo et al., 2006). Furthermore, the study has been carried out to investigate the ability to treat with sewage wastewater by applying *Rhodopseudomonas* strain (Nagadomi et al., 2000).

In this study, a preliminary study was attempted to investigate the possibility of using PNSB in the treatment of swine sewage wastewater. A PNSB strain was isolated from swine sewage wastewater. An in vitro experiment was conducted to monitor the microbial growth in swine sewage wastewater. Besides, the effect on the microbial degradation of VFAs, TOC and ammonia in swine wastewater was evaluated by applying the PNSB culture.

MATERIALS AND METHODS

Sampling and microorganisms

Swine sewage wastewater was sampled from a pig farm (Isa, Kagoshima, Japan), PSB Strain A was deposited in the Microbiology Laboratory, Faculty of Fisheries, Kagoshima University and stored at 10˚C for maintenance. For experimental application, the PSB were transferred into 30 ml of the Basic I liquid medium. After incubation at 25˚C under a dark condition for 24 h, the culture was incubated at 30˚C for 14 d under a condition of 12/12 light/dark cycles. *Rhodopseudomonas palustris* NBRC-100419T was purchased as standard strain from NITE, Japan.

Swine sewage waste water pretreatment

After sampling, swine sewage wastewater was centrifuged at 3,000 rpm for 15 min to obtain

the water layer fraction. Undiluted water layer fraction and its 2-time dilution were applied in the following experiments.

Incubation experiment in swine sewage wastewater

Pretreated wastewater and its 2-time dilution were sterilized by autoclaving. After adjusting OD660 value to 1.0, 1 ml of PSB culture was inoculated into 20 ml sterilized undiluted wastewater or its 2-time dilution. 100μl of 10% sodium thioglycolate solution was added as the reductant. Incubation conditions were described previously.

Growth rate, VFAs and TOC analyses

Growth rate of PSB strains in pre-treated wastewater was measured by OD660 as described earlier. The supernatant of culture medium was were taken and centrifuged for 10 min (15,000 rpm) at 20℃ to ensure complete separation of oil from the aqueous component. Thereafter, the aqueous subsamples were diluted and The concentrations of VFAs (acetic acid and propionic acid) was detected at 210 nm with 5 mM $HClO_4$ as a mobile phase in HPLC analysis (Column: SHIMADZU, CTO-10A, detector: UV). Total organic carbon (TOC) was tested by TOC Analyzer (TOC-V, SHIMADZU, Japan).

Ammonia nitrogen, nitrite nitrogen and nitrate nitrogen analyses

After centrifugation (15,000 rpm, 10 min, 20 ℃), the supernatant of culture medium was used for the following analysis. The concentration of ammonia nitrogen was analyzed by indophenol method as described by Dora (1976). 10 ml samples were transferred into acid-treated tubes, 0.5 ml phenol alcohol solution (5 g phenol in 50 ml 95 % ethyl alcohol) was added. The tubes were vortexed well for 30 seconds. Then, 0.5 ml sodium nitroprusside solution (5 g sodium nitroprusside in 200 distilled water), 1 ml oxidizing reagents (solution A: 40 g trisodium citrate dehydrate, 2 g NaOH in 200 ml distilled water; solution B: 1.5 N sodium hypochlorite solution; mixed before use in ratio of A:

B= 4:1, v/v) were added into samples and vortexed well in 30 seconds, respectively. The tubes were covered with aluminum foil and placed over one hour in the dark. The attenuance (optical density) of the culture were measured at 640 nm (OD640). Nitrite nitrogen and nitrate nitrogen were analyzed by Auto Analyzer (BLTEC, SWAAT, Japan) while pH was tested using pH test paper.

Statistical analysis

Data was subjected to independent t test method to determine significant differences. $p<0.05$ was considered to be statistically significant.

RESULTS

Contents of swine sewage wastewater after pre-treatment

The main contents of pre-treated swine sewage wastewater were shown in Table 1. The concentrations of TOC and ammonia nitrogen were 9150.0 mg/L and 1386.0 mg/L, respectively, which are typical features of organic wastewater. Analysis of VFAs showed that the concentrations of acetic acid and propionic acid were 270.5 mM and 27.0 mM, respectively.

PSB growth in swine sewage wastewater

Fig. 1 shows the growth curve of PSB strains in swine sewage wastewater. Both PSB Strain A and NBRC strain showed low growth rate in undiluted wastewater. Comparatively, PSB Strain A and NBRC strain entered logarithmic growth phase after 48-h incubation in 2-time diluted wastewater. After 330-h incubation, the biomass reached maximum level. PSB Strain A showed lower cell concentration compared with NBRC strain.

Reduction of VFAs contents

Table 2 shows the results of VFAs reduction. Due to the low yield in undiluted swine sewage

wastewater, no reduction of VFAs was observed with both two strains. While in 2-time diluted swine sewage wastewater, the concentration of acetic acid decreased from 158.0 mM to 120.2±2.9 mM and 130.9±1.5 mM after 216 h incubation with PSB Strain A and NBRC strain, respectively. While propionic acid decreased from 14.9 mM to 9.3±0.9 mM, and 10.9±1.6 mM after 216 h incubation with PSB Strain A and NBRC strain, respectively.

Reduction of TOC

Fig. 2 shows the time course of TOC concentration. No significant reduction was observed in undiluted swine sewage wastewater. While in 2-time diluted swine sewage wastewater, TOC concentration showed same trend with both PSB Strain A and NBRC strain, which decreased slowly at the beginning, and then decreased rapidly after 145-h incubation. After 330-h incubation, the concentration dropped from 4508.0 mg/L to 3104.0±451.5 mg/L, and 3733.5±205.3 mg/L with PSB Strain A and NBRC strain, respectively. No statistically significant differences were found between PSB Strain A and NBRC strain ($p>0.05$).

Reduction of ammonia nitrogen

Fig. 3 shows the time course of ammonia nitrogen concentration. The initial concentration was 1609.5 mg/L. After 330-h incubation with PSB Strain A and NBRC strain, ammonia nitrogen was reduced to 1388.3±138.4 mg/L, and 1427.7±125.6 mg/L, respectively. While in 2-time diluted swine sewage wastewater, the concentration was reduced from 629.7 mg/L to 424.1±7.4 mg/L, and 376.8±82.4 mg/L after 330-h incubation with PSB Strain A and NBRC strain, respectively. No statistically significant differences were found between PSB Strain A and NBRC strain ($p>0.05$).

DISSCUSSION

It has been reported that photosynthetic bacteria contributed to the purification of polluted

water in nature (Kobayashi et al., 1966). Since 1970s, the treatment method has been published for industrial wastewater (Kobayashi et al., 1973; Speece, 1983; Kantachote et al., 2005). From then, photosynthetic bacteria has been studied to treat with wastewater containing high concentration of organic components, such as food processing wastewater, sewage wastewater (Takeno et al., 2005; Chitapornpan et al., 2013). *Rhodopseudomonas palustris* was one of the most effective PSB strains which have been studied to treat with wastewater containing high concentration of organic matters (Getha et al., 1998; Kim et al., 2004). In this study, a photosynthetic bacterial PSB Strain A was isolated, characterized and applied to treat swine sewage wastewater, *Rhodopseudomonas palustris* NBRC-100419T was purchased as standard strain in this study.

Generally, VFAs in swine sewage wastewater was mainly acetic acid, propionic acid and n-butyric acid (Jun, 2000). In this study, the analyses results of swine sewage wastewater indicated that 82 % of TOC was VFAs, the concentration of n-butyric acid was below the detection limit. Similarly, NO3-N and NO2-N were also below the detection limit. Thus, the nitrogen content was mainly ammonia nitrogen. The pH of swine sewage wastewater was 8.2.

Together with high growth rate of PSB Strain A and NBRC strain, reduction of VFA and TOC contents was found in 2-time diluted swine sewage wastewater. While in undiluted swine sewage wastewater, no significant reduction was observed due to poor growth of the two strains. Similar with the results of growth in organic acids before, undiluted swine sewage wastewater containing high concentration of organic components might not be fit for PSB strains. It has been reported that the high concentration of organic matters as well as high pH might inhibit the growth of microorganisms (Cristina et al., 2008, Suehara et al., 2005). In this study, pH was the same as 8.2 in both undiluted and 2-time diluted swine sewage wastewater. Thus, it was considered that the main reason for the inhibition of the PSB growth was probably the high concentration of organic matters.

Compared with the standard strain, PSB Strain A showed better efficiency in the reduction of acetic acid, propionic acid. Time course of TOC could be divided into two phases, which was coincident with the growth curve (Figs. 1 and 2). It has been reported that high cell yield of purple

non-sulfur bacteria could be obtained under anaerobic-light condition with organic matters. In this photosynthetic process, production of carbon dioxide was not observed (Hoshino et al., 1984). Thus, the influence of carbon dioxide on TOC was not discussed in this study. While under aerobic-dark condition, oxygen was utilized as the electron acceptor in respiration (Izu et al., 2001; Ono-Izu et al., 2004). Due to low efficiency of anaerobic metabolism, organic matters were assimilated as the electron donor to produce ATP. Although 10 % sodium thioglycolate solution was added as reductant in this study, with the existence of the remaining oxygen, aerobic metabolism was performed at the beginning. After oxygen was consumed, anaerobic metabolism was considered to start along with the consumption of organic matters.

Among the numerous influences on anaerobic digestion, the ammonia concentration of some substrates seems to play an important role. Ammonia is produced by the biological degradation of the nitrogenous matter, mostly in the form of proteins and urea (Kayhanian, 1999). It has often been regarded as the main reason for digester failures as well as for the inability to treat certain substrates anaerobically (Braun et al., 1981). Ammonium ion (NH_4^+) and free ammonia (FA) (NH_3) are the two principal forms of inorganic ammonia nitrogen in aqueous solution. Generally, high nitrogen concentration was found in swine sewage wastewater (Cristina et al., 2008). High concentration of ammonia nitrogen has been suggested to be the main cause of inhibition since it is freely membrane-permeable (De Baere et al., 1984; Hansen et al., 1998). Analysis of wastewater used in this study also showed the same findings (Table 1). In this study, high concentration of 1,386 mg/L ammonia was found in swine sewage wastewater. By applying PSB Strain A and NBRC strain, the concentrations of ammonia nitrogen dropped from 629.7 mg/L to 424.1±7.4 mg/L, and 629.7 mg/L to 376.8±82.4 mg/L, respectively. The inefficient reduction might be related to the high concentration of ammonia nitrogen. It has been reported that under anaerobic conditions with infrared irradiation the enriched PNSB was able to remove 99.6% NH^{4+}-N from primary settled domestic wastewater (Hülsen et al., 2014). In this study, regardless of the growth rate, reduction of ammonia nitrogen was found in both undiluted and 2-time diluted swine sewage wastewater. Comparatively, the removal rates were not so high, which

were 24.31% in undiluted swine sewage wastewater and 33.82% in 2-time diluted wastewater.

Compared with *Rhodopseudomonas palustris* NBRC-100419T, the isolated PSB Strain A showed better growth in utilizing a variety of organic acids. During the treatment with swine sewage wastewater, PSB Strain A also showed almost the same efficiency in the reduction of VFAs and TOC compared with NBRC strain. The results indicated that PSB Strain A was efficient in treating wastewater containing high concentration of organic matters. Furthermore, yielded PSB cells contain a number of valuable components. Thus, it appeared promising for swine sewage wastewater treatment to obtain a zero-emission. Further study should concentrate on the optimization of PSB growth efficiency to establish a practical treatment system.

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FIGURES AND TABLES

Table 1. Chemical composition of the pretreated pig farm effluents.

Components		Concentration
Total organic carbon	(mg /L)	9150.0
Acetate	(mM)	270.5
Propionate	(mM)	27.0
NH_4^+-N	(mg /L)	1386.0
pH		8.2

Table 2. Change in volatile fatty acid 〔VFAs (acetate and propionate)〕 concentrations and reduction rate by strain A and the type strain *Rhodopseudomonas palustris* (NBRC- 100419^T) with 2-time diluted pig farm effluents.

	VFA concentration (mM)			
	0 h		216 h	
VFA	A	*R. palustris* (NBRC-100419^T)	A	*R. palustris* (NBRC-100419^T)
Acetate	158.0	158.0	120.2±2.9	130.9±1.5
Propionate	14.9	14.9	9.3±0.9	10.9±1.6

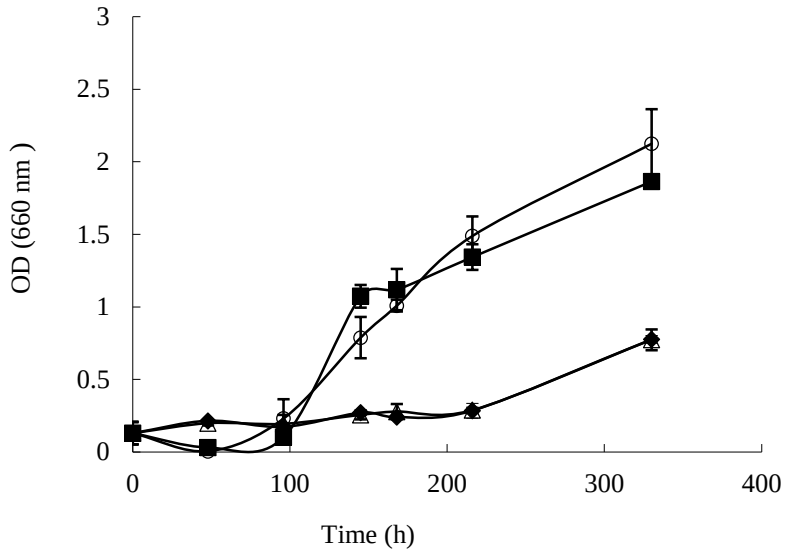

Fig. 1. The change of the growth rate of strain A and type strain *Rhodopseudomonas palustris* (NBRC-100419^{T}) with undiluted and 2×diluted pig farm effluents (n = 3, ± standard deviation), ◆: strain A in undiluted effluents, △: strain NBRC in undiluted effluents, ■: strain A in 2× diluted effluents, ○: strain NBRC in 2×diluted effluents. .

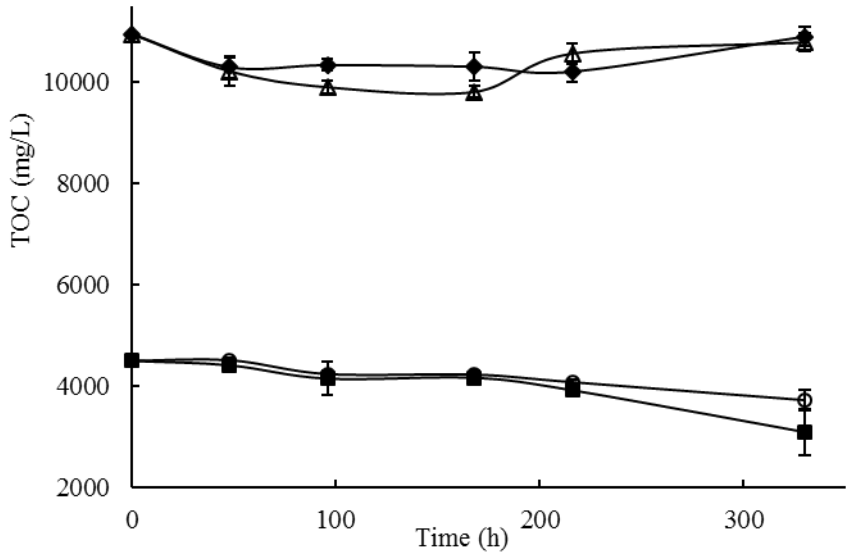

Fig. 2. The change of total organic carbon (TOC) by strain A and type strain *Rhodopseudomonas palustris* (NBRC- 100419^{T}) with 2×diluted pig farm effluents (n = 3, ± standard deviation) , ◆: strain A in undiluted effluents, △: strain NBRC in undiluted effluents, ■: strain A in 2× diluted effluents, ○: strain NBRC in 2×diluted effluents.

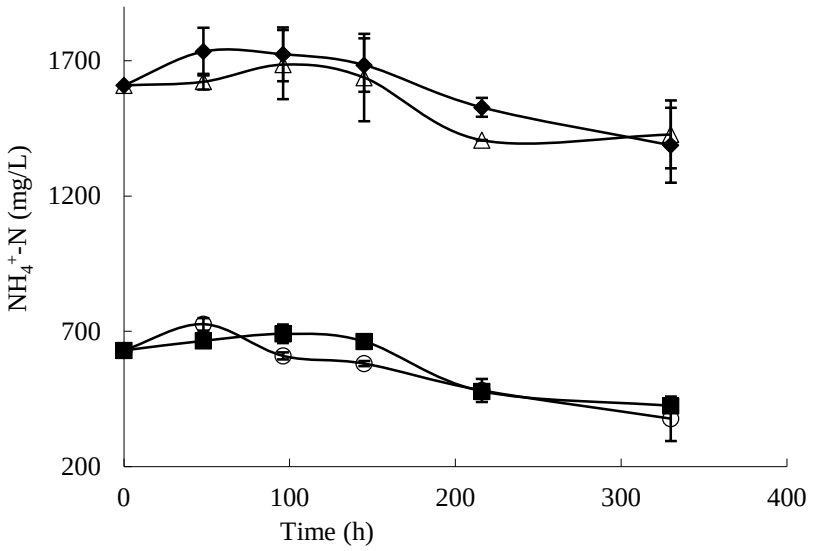

Fig. 3. The change of NH_4^+-N concentration by strain A and type strain *Rhodopseudomonas palustris* (NBRC- 100419^T) with undiluted and 2×diluted pig farm effluents (n = 3, ± standard deviation) , ◆: strain A in undiluted effluents, △: strain NBRC in undiluted effluents, ■: strain A in 2×diluted effluents, ○: strain NBRC in 2×diluted effluents.

CHAPTER 3

ENHANCEMENT OF ISOLATED BACTERIA IN THEIR ANTIFUNGAL ACTIVITIES ON ROOT ROT FUNGI BY APPLYING PHOTOSYNTHETIC BACTERIA

ABSTRACT

Antagonistic effect of microorganisms on a pathogenic root rot fungus (RRF) was investigated. A photosynthetic bacteria strain deposited in the Microbiology Laboratory, Faculty of Fisheries, Kagoshima University was used in this study. Antifungal bacteria (AB) in RRF-contaminated sweet potato farms were isolated, and seven strains were initially chosen as antagonistic candidates. An antagonistic test by using the mycelial disk placement method revealed that one AB strain alone inhibited the RRF growth. This AB strain was identified as *Bacillus polyfermenticus* based on phylogeny of 16S ribosomal RNA genes. However, two AB strains (*Bacillus aerophilus*) need coexistence with photosynthetic bacterial strain A (a purple non-sulfur photosynthetic bacterium *Rhodopseudomonas faecalis*) to display their antagonistic activity. The results suggest a possibility of the isolates as potential agents for the biological control of infection of agricultural products in fields with RRF.

INTRODUCTION

In a previous study, a purple non-sulfur photosynthetic bacterial strain was isolated from swine sewage wastewater. Effects on wastewater treatment were investigated by applying this strain. Phylogenetic results showed that it was closely related to *Rhodopseudomonas faecalis*. On the other hand, purple non-sulfur photosynthetic bacteria have been reported as versatile microorganisms that promote growth and enhance yield of plants (Kobayashi et al., 1971, Elbadry et al., 1999). Furthermore, applying photosynthetic bacteria as organic fertilizers might improve the growth of microorganisms which produce antibiotics, consequently suppressing the growth of pathogenic fungi (Kitamura et al., 1984).

Diverse plant diseases are caused by phytopathogenic filamentous fungi. Among them, the violet root rot disease, caused by different species of the genus *Helicobasidium*, has prevailed around the world (Nakamura et al., 2004). Different strategies have been tested to reduce the impact, including soil cultivation, invasion prevention, chemical and biological control, and use of resistant rootstock (Coffey and Guillemet, 1987; Cotterill, 1993; Erwin and Ribeir, 1996). Biological control appears promising due to its friendliness to the environment (Pratibha et al., 2012).

An increasing interest has emerged with respect to the importance of microbial diversity in soil habitats. The extent of the diversity of microorganisms in soil is seen to be critical to the maintenance of soil health and quality, as a wide range of microorganisms is involved in important soil functions (Garbeva et al., 2004). Numerous antagonistic microorganisms isolated from different soils and host plants have been found to produce antibiotics in vitro (Raaijmakers et al., 2002). They include many representative genera, such as the genera *Bacillus* and *Trichoderma* which are among the most studied (Weller, 1988, Handelsman, 1996). Antibiotic production by marine photosynthetic bacteria has also been reported (Burgess et al., 1991). On the other hand, using a combination of biocontrol species could be one approach to improve the performance of a biological control. Duffy et al. (1996) suggested that mixture of *Trichoderma* and fluorescent *Pseudomonads*, compared to individual agents, resulted in a substantially better disease control and. for control of take-all on wheat. Currently, there

is limited research focused on antifungal effects of photosynthetic bacteria, especially in combination with other antagonistic bacterial strains.

In Kagoshima Prefecture, the amount of sweet potato harvested ranks first in Japan. But during production, plant diseases have brought problems such as farmland desertion, low product quality, agricultural yield decrease, and most diseases are caused by pathogenic fungus. Among them, the violet root rot caused by *Helicobasidium mompa*, is an important root rot disease of sweet potato. Crop rotation and fungicide have been applied but could not provide a consistent control on a commercial scale. The isolated photosynthetic bacteria strain has been applied in the farm as fertilizer, and somehow suppressed the growth of violet RRF, but the scientific mechanism remained unknown. In addition, antifungal fungus growth-inhibiting rhizobacteria was also isolated from the infected farm. This study was initiated because of a lack of information about the ability of PSB and its combination with other strains to suppress violet RRF in Kagoshima Prefecture, Japan. Thus, the aim of this study is to establish a practical method for the biological control of the violet root rot disease affecting the farming of sweet potato.

MATERIALS AND METHODS

Sample collection

Soil samples and sweet potato samples were collected from an infected sweet potato farm land (Kanoya City, Kagoshima Prefecture, Japan) in February 2012. After sampling, all samples were transferred to the laboratory and stored at 10˚C until isolation of microbes.

Isolation, cultivation and identification of microorganisms

A photosynthetic bacteria strain (PSB Strain A) was deposited in the Microbiology Laboratory, Faculty of Fisheries, Kagoshima University and stored at 10˚C for maintenance. For experimental application, the PSB were transferred into 30 ml of the Basic I liquid medium (Kitamura et al., 1984).

After incubation at 25˚C under a dark condition for 24 h, the culture was incubated at 30˚C for 14 d under a condition of 12/12 light/dark cycles.

A pathogenic RRF was isolated by an agar plate dilution method using the oatmeal medium (Nakamura, 2009). Mycelia on the surface of the rhizomes were cut off and transferred into 1 ml of water. After shaking vigorously, a 10-fold serial dilution was applied, and 100 µl of the dilution solution was added to the agar medium. The plates were cultivated at 25˚C for 7 day, and the obtained colonies were isolated.

Bacteria were isolated from the infected farm soil by a plate-spreading method using the nutrient broth (NB) plate medium (1% Polypepton, 0.5% meat extract, 0.2% NaCl, 1.5% agar, pH 7.0-7.2). One gram of the soil samples was suspended into 1 ml of water, and the suspension was serially diluted 10-fold. A hundred microliter of the dilution was spread onto the agar plates and cultivated at 25˚C for 7 day to isolate the colonies. Representative colonies of RRF and bacteria strains were re-streaked and cultivated on new agar plates. All the isolates were stored at 10˚C for further analysis.

Colonies of the microorganisms were obtained by streaking and cultivating the isolates on the agar plate media. Optical microscopic observation was carried out by applying the liquid culture of the microorganisms while observation by DAPI staining was also conducted.

DNA of the isolated microorganisms was extracted from the liquid cultures by using the DNeasy Plant Mini Kit (Qiagen, Hilden, Germany). The extracted DNA was used for PCR amplification of 16S ribosomal RNA genes (16S rDNA) with a universal primer set PrSSU.1F (5’-AGAGTTTGATCATGGCTCAG-3’) and PrSSU.1R (5’-GGTTGGATCACCTCCTTA-3’). The PCR reaction mixture was composed by Ex Taq Buffer (Takara Bio, Otsu, Japan), 100 µM of each dNTP, 0.5 µM of each primer, 0.025 units/µl Ex Taq DNA polymerase Hot Start Version (Takara Bio, Otsu, Japan), and one-tenth volume of the bacterial DNA solution. The thermal cycling consisted of an initial denaturation of 1 min at 94˚C, followed by 25 cycles of 30 s of denaturation at 94˚C, 20 s of annealing at 56˚C and 1.5 min of extension at 72˚C, with 7 min of final extension at 72˚C.

The PCR products were electrophoresed by 1.5% agarose gel in TAE buffer (40 mMTris-

acetate, 1 mMethylenediaminetetraacetic acid) under a constant voltage condition of 100 V with the electrophoresis system GelMate 2000 (Toyobo, Osaka, Japan). The electrophoresed gels were stained with SYBR Gold (Molecular Probes, Life Technologies, Carlsbad, USA) and photographed under a blue transilluminator Safe Imager 2.0 (Invitrogen, Life Technologies, Carlsbad, USA). The obtained bands were cut from the gel and the PCR products were purified from the gel pieces using the MonoFas DNA Purification Kit I (GL Science, Tokyo, Japan).

Nucleotide sequences of the PCR-amplified 16S rDNA were determined with the BigDye Terminator v3.1 Cycle Sequencing Kit (Life Technologies, Carlsbad, USA). The primers used were PrSSU.1F, PrSSU.3F (5'-TGCCAGCAGCCGCGGTA-3'), PrSSU.1R, and PrSSU.2R (5'-TGCCAGCAGCCGCGGTA-3'). The obtained sequences were assembled with the program DNASIS Pro Version 2.7 (Hitachi Solutions, Tokyo, Japan).

Closely related sequences were obtained from the GenBank DNA database by using the basic local alignment search tool (BLAST) program. Phylogenetic analysis with the neighbor-joining method was conducted using the program MEGA Version 5 (Tamura et al., 2011).

Accession number

The nucleotide sequences of the partial sequence of the 16S rDNA determined in this study have been submitted to the DDBJ database under the accession number LC092191, LC092192, LC092193.

Antagonistic test of microorganisms to pathogenic root rot fungus

Preliminary test

Selection of the isolated PSB and AB was conducted according to the method by Cassandra et al. (2004). A hundred microliter of the RRF culture, cultivated in the oatmeal liquid medium at 25˚C for 14 day, was inoculated on the oatmeal agar medium. Sterilized filter disks with a diameter of 5 mm,

perforated from Advantec No. 2 filter paper (Toyo Roshi Kaisha, Co., Ltd., Tokyo, Japan), were placed on the agar plates. Isolated AB strains were incubated in NB medium at 25 ˚C for 3 day, while PSB was incubated in Basic I liquid mediumat 30˚C for 14 day, then 10 µl of the microbial culture was spotted into the disks. The test plates were incubated at 25˚C and the inhibition zones around the filter disks were observed after 14-day cultivation.

Antagonistic test using mycelial disk placement method

Potential candidates antagonistic to the RRF which were screened by the preliminary test were further tested using a modified mycelial disk placement (MDP) method (Shimane and Takahashi, 1993). Sterilized filter disks with a diameter of 5 mm were placed on each oatmeal agar plate and 10 µl of the culture of the antagonists was spotted. To investigate the synergistic effects, both AB cultures and their mixture with PSB were applied. Mycelial disks with a diameter of 5 mm were cut from the surface of the RRF-cultivated agar plates and overlaid on the filter disks. The plates were incubated at 25˚C and the antagonistic activity was evaluated on the basis of suppression of the mycelial growth.

RESULTS

Isolation and identification of the microorganisms

Isolation of microorganisms was conducted using infected soil and sweet potato samples. After the 7-day incubation on the agar plates, many colonies were observed on the agar plates. The typical colonies were picked up, and finally one RRF infecting the rhizomes of sweet potato samples and 11 bacteria strains inhabiting the soil of sweet potato fields were isolated from the rhizome and the soil samples, respectively. The bacteria isolates showed similar cell morphology, the cells were short rod-shaped with similar sizes (Fig. 1). On the other hand, RRF showed morphological features of the violet root rot fungal species, *Helicobasidium mompa*, i.e., mycelia with light red color were extended over

the agar plates (Fig. 2a), and mycelial structure was observed microscopically (Fig. 2b).

Phylogenetic analysis of the AB isolates revealed that three strains, AUT-3, 8, and 9 were all the members of the genus Bacillus (Fig. 3). In addition, AUT-3 was closely related to *Bacillus polyfermenticus*, while AUT-8 and 9, which had almost the same 16S ribosomal RNA gene sequences, were relatives of *Bacillus aerophilus*.

Preliminary test of antagonistic activity to pathogenic root rot fungus

Antagonistic activity of the isolated PSB and AB to RRF was compared by inoculating their liquid culture onto the paper disks placed on the fungus-cultivated agar plates. The AB strains AUT-3, 5, 7, 8, 9, 10, and 11 showed antagonism in 14 day (data not shown). Thus, these strains were selected for further analysis of antagonism. Although PSB strain showed limited growth-inhibiting zones of RRF in 7 day, PSB was applied in the following experiment to investigate the synergistic effects on antifungal activities.

Antagonistic test of the bacterial isolates by using the mycelial disk placement method

The results of antagonistic test using MDP method are shown in Figs.4 and 5. After 5 day cultivation, the test plates to which the AB strains AUT-5, 7, and 10 were inoculated showed the RRF growth, although RRF also grew on AUT-8, 9, and 11 in 10 day (Figs.4d and 4e). On the other hand, AUT-3 completely impeded the RRF growth (Fig. 4e, 2-AUT3; Fig. 5c, left), suggesting high antagonistic effect. Interestingly, the PSB strain synergistically suppressed the RRF growth: although the AB strains AUT-8 and 9 were little antagonistic (Fig. 4e, 5-AUT8 and 6-AUT9; Figs. 5a and 5b, left). However, addition of PSB led to the complete growth inhibition of RRF (Fig. 4e, 12-PSB+AUT8 and 13-PSB-AUT9; Figs.5a and 5b, right).

DISCUSSION

Numerous species of soil bacteria which flourish in the rhizosphere of plants, but which may grow in, on, or around plant tissues, stimulate plant growth by a plethora of mechanisms. These bacteria are collectively known as PGPR (plant growth promoting rhizobacteria). The search for PGPR and investigation of their modes of action are increasing at a rapid pace as efforts are made to exploit them commercially as biofertilizers (Vessey, 2003). Generally, PGPR could be divided into two types: those that form a symbiotic relationship, which involves formation of specialized structures or nodules on host plant roots, and those are free-living in the soils (Kloepper et al., 1989; Frommel et al., 1991). The genus Bacillus has been extensively studied, due to their enhancement of plant growth, such as seedling segment elongation, coleoptiles bending, and biocontrol of plant disease (Hemming, 1990; Idris et al., 2004; Compant et al., 2005). Photosynthetic bacteria has been also widely used to improve plant growth as nitrogen source and biofertilizer (Maudinas et al., 1981; Lee et al., 2008). In this study, the antagonistic microorganisms were isolated from infected sweet potato farm, the phylogenetic analyses showed the isolates was closely related to the genus *Bacillus*, known as antagonists to plant pathogens. The results in previous study showed that PSB Strain A was most closely related to *Rhodopseudomonas faecalis*, one of the purple non-sulfur photosynthetic bacteria.

Numerous studies have been conducted on plant disease such as damping off, root rots and wilts (Khan et al., 1997; Ryder et al., 1999; Fravel et al., 2003). Recently, interest in biocontrol has increased to suppress the disease fuelled by public concerns over use of chemicals in the environment in general (Whipps, 2001). A range of different bacterial genera and species have been studies as biocontrol agents, such as *Pseudomonas*, *Bacillus*, and *Streptomyces* (Uthede et al., 1999; Warren and Bennett, 1999; Singh et al., 1999). In this study, we succeeded to isolate several bacterial strains which showed antagonistic effects on a root rot fungus, *Helicobasidium mompa*. Though AUT3 could suppress root rot fungus (RRF) growth alone, application of a single biocontrol strain has limited tolerance to changes in environmental conditions (Weller et al., 1994).

On the other hand, disease control has been achieved using the combination of

Pseudomonas, Bacillus and *Trichoderma* strains or other mixing of biocontrol species (Kamal et al., 2009; Latha et al., 2011). Although PSB did not show any antagonistic activity by itself in this study, the results suggested that PSB might be able to intensify the inhibitory effect on the RRF growth. Furthermore, PSB contain a number of enzymatic conversions that may yield economically valuable products and improve the growth of photosynthetic plant (Sasaki et al., 1987; Fuller, 1995). Thus, combining biocontrol strains should be suggested to enhance the level and consistency of antagonistic effects. Further studies should be conducted to elucidate the mechanism and optimize the antagonistic effects for practical use.

This study clarified the antagonistic characteristics of the AB as potential agents for the biological control of the pathogenic root rot fungus. The synergistic antifungal activities of AB and PSB suggest that applying a variety of antibiotics and other useful microorganisms to treat root rot fungus should be considered. With the presence of PSB, the secretion of antibiotics by *Bacillus* species was somehow intensified. Chemical and biological analyses should be conducted to investigate the mechanism of this cooperation. Furthermore, the antibiotics should be identified using chemical techniques, such as chromatography. The findings of this study would be very significant from the viewpoint of biological prevention and control against the root rot disease of cultivated sweet potato.

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FIGURE AND TABLES

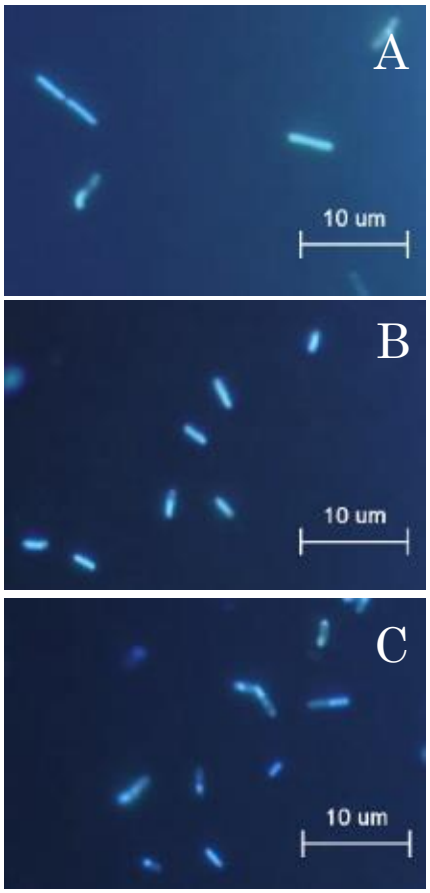

Fig. 1. Microscopic observation of antifungal bacteria by DAPI staining. A, AUT-3; B, AUT-8; C, AUT-9, scale bar, 10 μm.

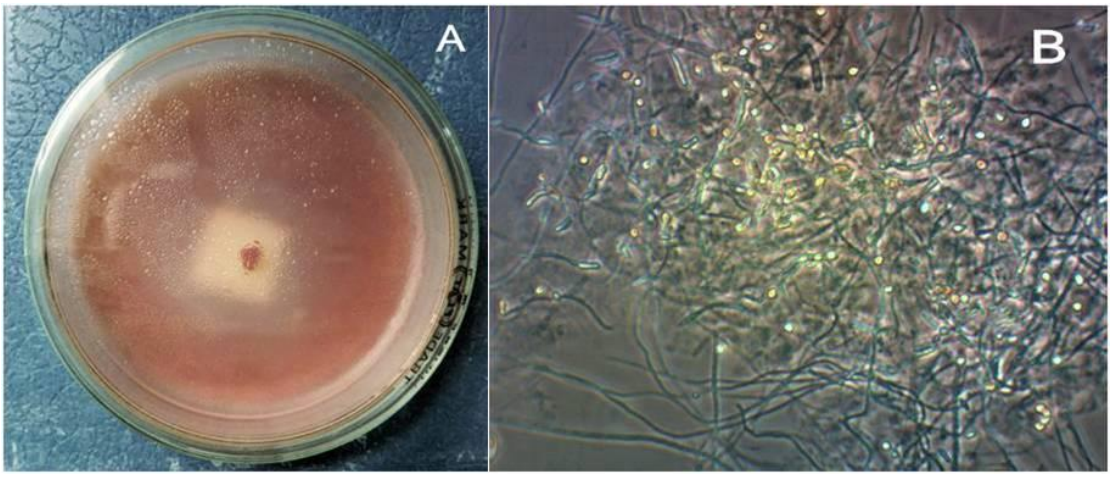

Fig. 2. Root rot fungus. A, colony form on the oatmeal agar plate; B, optical microscopic observation of the mycelial structure.

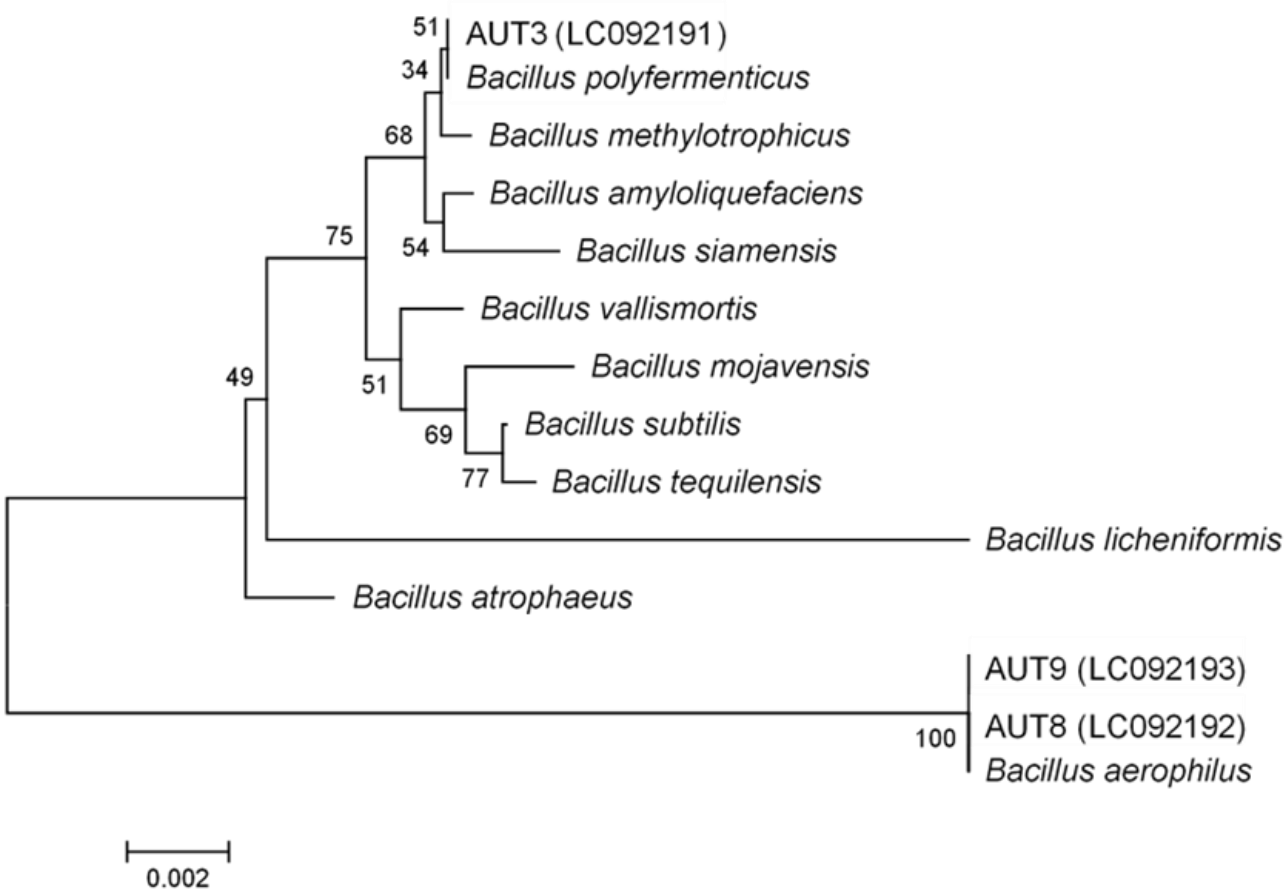

Fig. 3. Unrooted phylogenetic tree of the antifungal bacterial isolates based on the 16S rDNA sequences. Bootstrap values are shown at the branching points. Scale bar, genetic distance.

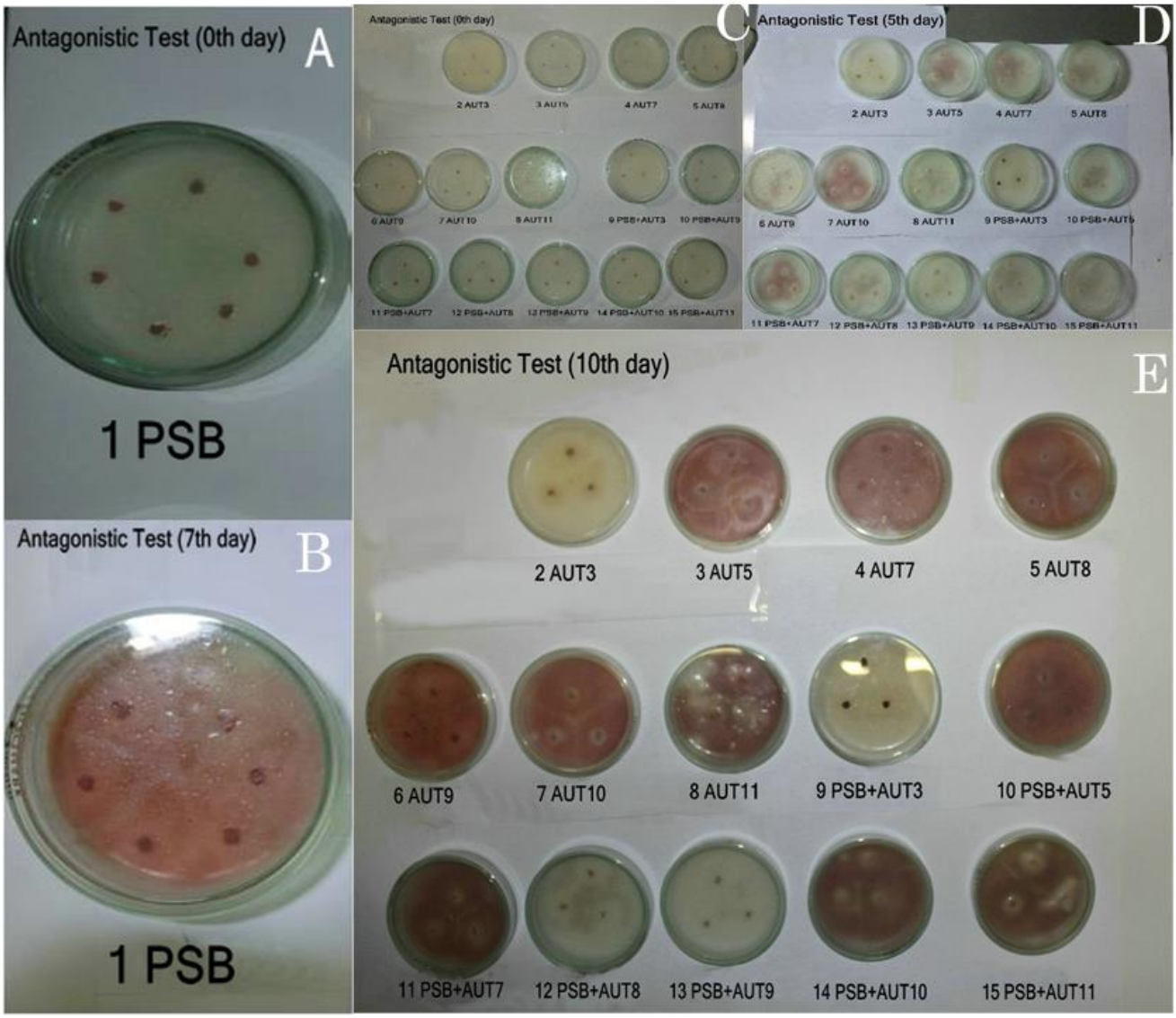

Fig. 4. Antagonistic test using the mycelial disk placement method. The AB and PSB culture was added to the paper disks on the agar plate media, and the agar pieces containing the root rot fungus were overlaid. A, B, PSB Strain A; C, D, and E, the antifungal bacterial strains alone and combined with PSB after 0, 5, and 10 day cultivation, respectively.

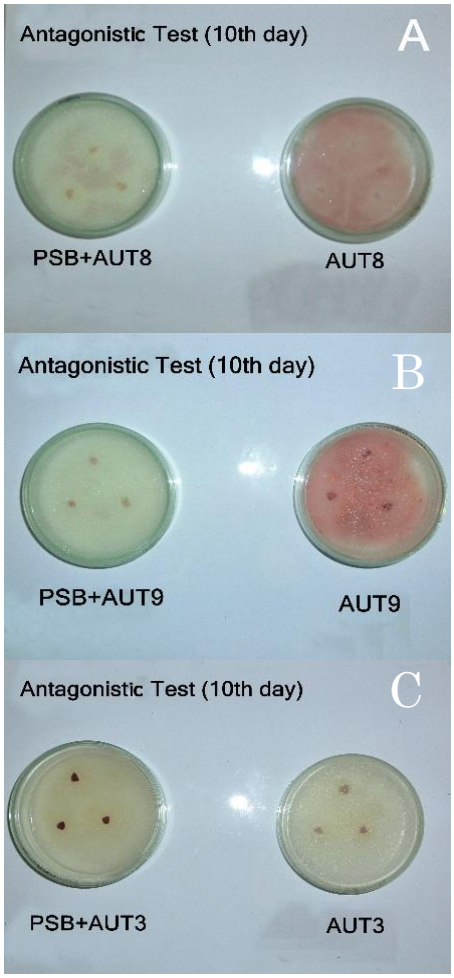

Fig. 5. Synergistic effect of the photosynthetic bacterium on antagonistic activity of the antifungal bacteria. The synergistic effect was estimated by the mycelial disk placement test. A, the antifungal bacterial strain AUT-8; B, AUT-9; C, AUT-3. Left, with the photosynthetic bacterium; right, without the photosynthetic bacterium.

TABLE 1. Antagonistic effects of microorganisms on root rot fungi using mycelial disk placement method.

Treatment No.	Microorganisms	Antagonistic Effect*
1	PSB	-
2	AUT3	+
3	AUT5	-
4	AUT7	-
5	AUT8	-
6	AUT9	-
7	AUT10	-
8	AUT11	-
9	PSB + AUT3	+
10	PSB + AUT5	-
11	PSB + AUT7	-
12	PSB + AUT8	+
13	PSB + AUT9	+
14	PSB + AUT10	-
15	PSB + AUT11	-

* Antagonistic effect was evaluated from the formation of the blank area through a modifi mycelial disk placement (MDP) method.

CHAPTER 4

APPLICATION OF ISOLATED BACTERIA AND PHOTOSYNTHETIC BACTERIA IN THE RHIZOSPHERE OF SWEET POTATOS AND THEIR EFFECT ON THE BACTERIAL COMMUNITY

ABSTRACT

In this study, effect of addition of isolated microorganisms to the rhizosphere microbial communities shifts was determined by PCR-denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal RNA genes (16S rDNA) as well as traditional bacterial counts. After 7 days, all the microcosms showed decreased bacteria counts of PSB. In PCR-DGGE analysis, bacterial community shifts in all microcosms from day 0 to day 7 were minimal. PSB and AUT9 had better survival, which were detected at day 7, while AUT8 was only detected at day 0. In the microcosms amended with PSB, a distinct band was detected. The phylogenetic analysis showed the band was related to *Catenulispora yoronensis*, one species of actinobacteria.

INTRODUCTION

It has been well known that plant growth could be enhanced by some plant growth promoting bacteria (PGPB), and the most widely studied group of PGPB are plant growth promoting rhizobacteria (PGPR) colonizing the root surface and the closely adhering soil interface and the rhizosphere (Kloepper et al., 1999; Gray and Smith, 2005). There has been many studies describing the potential to use PGPR as agents stimulating plant growth and managing soil and plant health (Gaffney et al., 1994; Hallman et al., 1997; Welbaum et al., 2004).

PGPB can also provide systemic resistance against a broad spectrum of plant pathogens. After application of certain bacteria, diseases of fungal, bacterial, and viral origin have been reduced (Kerry, 2000; Sturz et al., 2000; Ramamoorthy et al., 2001). *Bacillus* species have been studied for biocontrol of some fungal diseases, such as *Botrytis*, *Fusarium*, and *Rhizoctonia* (Tourél et al., 1995; Benhamou et al., 1996; Yu et al., 2002).

Introduction of specific bacteria into soils has been performed in agricultural practice for decades. The release of microorganisms has been successfully conducted by direct inoculation of the culture or using carriers (Chanway and Nelson, 1990; Dalia et al., 2013). Detection of the inoculated microbial agents is essential to study the maintenance of sufficient activity of the inoculant population. Numerous strategies have been developed for the detection and isolation of bacteria from environmental samples. Conventional methods are designed for enumeration of culturable bacteria obtained after growth on a suitable medium (Roszak and Colwell, 1987; Postma et al., 1988). However, it is now well known among microbiologists that only a small fraction of all bacteria could be isolated and characterized. The application of molecular biological techniques to detect and identify microorganisms by certain molecular markers, such as 16S rRNA or its encoding gene, is now more and more frequently used to explore the microbial diversity and to analyse the structure of microbial communities(Muyzer and Smalla, 1998). Recently, another genetic fingerprinting technique, denaturing gradient gel electrophoresis (DGGE) of PCR amplified ribosomal DNA fragments has been introduced into microbial ecology (Muyzer et al., 1993).

In this study, microorganisms were isolated and their effect of addition of bacterial cell consortium to the rhizosphere microbial communities was determined. Furthermore, the study also aims to determine the synergistic effect of the isolated bacteria when applied in free form. Soil samples were obtained from a sweet potato farm (Chiran, Kagoshima, Japan). After applying the isolated microorganisms, environmental DNA in the soil at the defined time intervals was extracted and subjected to PCR-DGGE. Prominent bands were also excised and sequenced to determine their taxonomy.

MATERIALS AND METHODS

Sample collection

Soil samples were collected from an infected sweet potato farm land (Chiran, Kagoshima Prefecture, Japan) in September 2015. PSB Strain A and two *Bacillus* strains were deposited in the Microbiology Laboratory, Faculty of Fisheries, Kagoshima University and stored at 10˚C for maintenance. After sampling, all samples were transferred to the laboratory and stored at 10˚C.

Microcosm set-up

The soil samples were sieved (0.5 cm-mesh) after sampling to remove rocks, grasses and other plant debris. Sieved soil was placed in 50 ml semi-closed tubes to allow aerobic conditions. For each treatment, 10 g soil and 2 ml cell suspension containing different microorganisms were added. After the microcosm assembly, the tubes were kept at room temperature. The contents of the treatments are shown in Table 1. Bacterial cells were added at 10^8 cells/g for PSB cell treatments and AUT cell treatments. At days 0, 7, 14, 1 g soil from each treatment were sampled for DNA extraction and bacterial counts.

Determination of cultivable PSB counts

Total cultivable bacterial counts of PSB in each treatment were determined by conventional plate count technique. 0.1 g of the soils collected from each treatment was suspended in 1 ml distilled water. The soil suspensions were then serially diluted and 0.1 ml of each dilution was spread on nutrient broth agar (1% Polypepton, 0.5% meat extract, 0.2% NaCl, 1.5% agar, pH 7.0-7.2). The plates were then incubated at 25 ℃ and bacterial counts were determined after 3-5 days of incubation.

DNA extraction

Environmental DNA was extracted from soil samples obtained from the different microcosms. DNA was extracted using PowerSoil DNA Isolation Kit (MOBIO Laboratories, Inc., Carlsbad, CA, USA) following the manufacturer's instructions. Presence of the extracted DNA was then confirmed by agarose gel electrophoresis using 4 µl DNA samples.

PCR amplification of 16S rDNA

The 16S ribosomal RNA gene (16S rDNA) was amplified using PCR primers 341F-GC containing a 40-bp GC-clamp to enhance separation in DGGE (clamp sequence in italics, 5'-CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCGCCTACGGGAGGCAGCAG-3') (Muyzer et al., 1996) and 907r (5'-CCGTCAATTCCTTTGAGTTT-3') (Yu and Morrison, 2004). The 20 μl PCR reaction mixture contained ExTaq buffer, 100 μM each of dNTP mixture, 0.5 μM of the forward and reverse primers, and 0.025 units μl-1 of ExTaq DNA polymerase (Hot Start Version, Takara Bio, Otsu, Japan). Amplification program used was touch-down PCR with thermal cycling conditions as follows: initial denaturation of 95℃ for 1min, 19 cycles of denaturation at 95 ℃ for 1 min, annealing at 62 ℃ for 1 min with a decreasing temperature of -0.8 ℃ at every cycle, and extension at 72 ℃ for 1 min, followed by 9 cycles of 95 ℃ for 1 min, 52 ℃ for 1 min and 72 ℃ for 1 min, and a final extension of 72 ℃ for 10 min. PCR amplifications were performed with the

ASTEC PC320 thermal cycler (ASTEC, Fukuoka, Japan). The PCR products were visualized by agarose gel electrophoresis with staining of Gel RedTM (Biotium Inc., Hayward, CA, USA).

Denaturing gradient gel electrophoresis and direct nucleotide sequencing of 16S rDNA

Amplified 16S rDNA fragments were run in denaturing gradient gel electrophoresis (DGGE) with the D-Code System (Bio-Rad, Hercules, CA, USA). DGGE was performed in a 6% polyacrylamide gel with denaturing gradient of 25-55% (where 100% denaturing gels contain 7 M urea and 40% formamide in 0.5X TAE buffer). Electrophoresis was run at a constant voltage of 60V for 16 h at 60 ℃. Gels were stained with SYBR Gold (Invitrogen, Carlsbad, CA, USA) and viewed with Safe Imager^{TM} 2.0 blue light transilluminator (Invitrogen, Carlsbad, CA, USA).

The DGGE bands were excised from the gels using 200 µl pipette tips. Excised gels were suspended in 100 µl TE buffer (10 mM Tris-HCl, pH 8.0, 1 mM ethylenediamine tetraacetic acid) in 1.5 ml microtubes and placed in 20 ℃ overnight to elute the amplified DNA. The eluted DNA fragments were re-amplified and subjected to DGGE with the same conditions as described above to confirm the recovery based on comparison with the first DGGE. The resultant single bands were re-excised, recovered, and re-amplified in the reaction volume of 50 µl. The PCR products were then applied onto 1.5% agarose gel electrophoresis at 100V. The bands were excised using a sterilized surgical blade and purified using the MonoFas DNA Purification Kit I (GL Science, Tokyo, Japan) with an elution volume of 30 µl. Thermal cycle nucleotide sequencing of the PCR-amplified 16S rDNA was run using the ABI PRISM BigDye Terminator Cycle Sequencing Kit Ver. 3.1 (Applied Biosystems, Carlsbad, CA, USA) with ≤5 ng template DNA and a -341F primer without a GC clamp. The products were purified using the BigDye XTerminator Kit (Applied Biosystems, Carlsbad, CA, USA) and analyzed by the ABI PRISM 3500xl Genetic Analyzer (Applied Biosystems, Carlsbad, CA, USA).

Phylogenetic and statistical analysis

Closely related sequences were obtained from the GenBank DNA database by using the basic local alignment search tool (BLAST) program. Phylogenetic analysis with the neighbor-joining method was conducted using the program MEGA Version 5 (Tamura et al., 2011).

RESULTS

Changes in the bacterial counts of PSB

Bacterial counts of PSB in the different microcosms are shown in Fig. 1. In the PSB-only treatment, bacterial counts started with 1.2×10^8 CFU/g at day 0. While in the treatment mixed with AUT3 and AUT9, the bacterial counts started with 4.7×10^7 CFU/g and 6.1×10^7 CFU/g respectively at day 0. All the microcosms showed decreased bacterial counts at day 7.

Temporal changes of the bacterial population structures and survival of the amended bacterial strains in the microcosms

The denaturating gradient gel electrophoresis (DGGE) profiles in Fig. 2 show the temporal changes in the bacterial community structures of the different microcosms amended with or without microbial agents. In all microcosms, bacterial community shifts from day 0 to day 7 were minimal. The microcosm of control showed almost unchanging bacterial community structures throughout the experimental period. The bacterial consortium added to the microcosms showed different responses. PSB and AUT9 had better survival, which were both detected at day 7 (band 1*, 3* in Fig 2). AUT8 was only detected at day 0 (band 2* in Fig. 2). In the microcosms amended with PSB, a distinct band was detected. The band related to *Catenulispora yoronensis* (Table 2), one species of actinobacteria was found at day 7.

DISCUSSION

A variety of bacteria have been used in soil inoculations intended to improve the supply of nutrients to crop plants. However, failures in releasing microorganisms into soils have been reported as well (Van Elsas and Heijnen, 1990; Akkermans, 1994). A key factor involved in the lack of success has been the rapid decline of the size of populations of active cells, to levels ineffective to achieve the objective, following introduction into soil (Van Veen et al., 1997). In the absence of plant roots, introduced bacteria is typically followed by a rapid decline in its population and, often, its elimination from the soil ecosystem (Acea et al., 1988). In this study, the bacterial counts of PSB decreased significantly after 7-day incubation in the microcosms. Survival of the augmented strains were confirmed by PCR-DGGE analysis. Corresponding with the result of bacterial counts, PSB was detected at day 7. While AUT3 strain could also be detected, AUT9 showed poor survival after 7-day incubation.

One notable species whose DGGE band was unique in the treatment of PSB was *Catenulispora yoronensis*, one species of actinobacteria which appear globally distributed. They have been reported to produce antibiotics and other bioactive microbial metabolites (Busti et al., 2006; Tamura et al., 2007). The addition of PSB improved actinobacteria growth and suppress the growth of pathogenic fungus (Kobayashi, 1984). The bands were only detected in the microcosms added with PSB cells in the present study. This signifies that the appearance of actinobacteria in the microcosms is attributed to the added PSB strain.

The PCR-DGGE analysis combined with traditional bacteria counts in the present study was applied to investigate the shift in bacterial communities by the addition of different bacterial agents. Sequencing of the excised bands revealed presence of amended strains in the microcosms. One species of actinobacteria was dectected only with the presence of PSB, suggesting the influence of PSB in the bacterial communities in these microcosms. Although underlying mechanisms need to be elucidated in the following study.

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FIGURE AND TABLES

Table 1. Matrix of the microcosm design

Treatments	Factors*			
	Distilled water (ml)	PSB (ml)	AUT3 (ml)	AUT9 (ml)
Control	1	0	0	0
1	0	1	0	0
2	0	0	1	0
3	0	0	0	1
4	0	0.5	0.5	0
5	0	0.5	0	0.5

*The concentration of microorganisms was adjusted to 10^8 cells/g

Table 2. Closest relatives of 16S rDNA sequences obtained from the excised DGGE bands of the microcosm added with the bacterial agents based on BLAST homology search.

Band No.	Closest relatives in database	Genebank Acc. No.	Homology	Taxonomic groups
1*	PSB Strain A (Rhodopseudomonas palustris)	KT180201	100%	Alphaproteobacteria
2*	AUT3 (Bacillus methylotrophicus)	KT720125	100%	Firmicutes
3*	AUT9 (Bacillus aerophilus)	KT719996	100%	Firmicutes
4	Catenulispora yoronensis	NR041613	97%	Actinobacteria

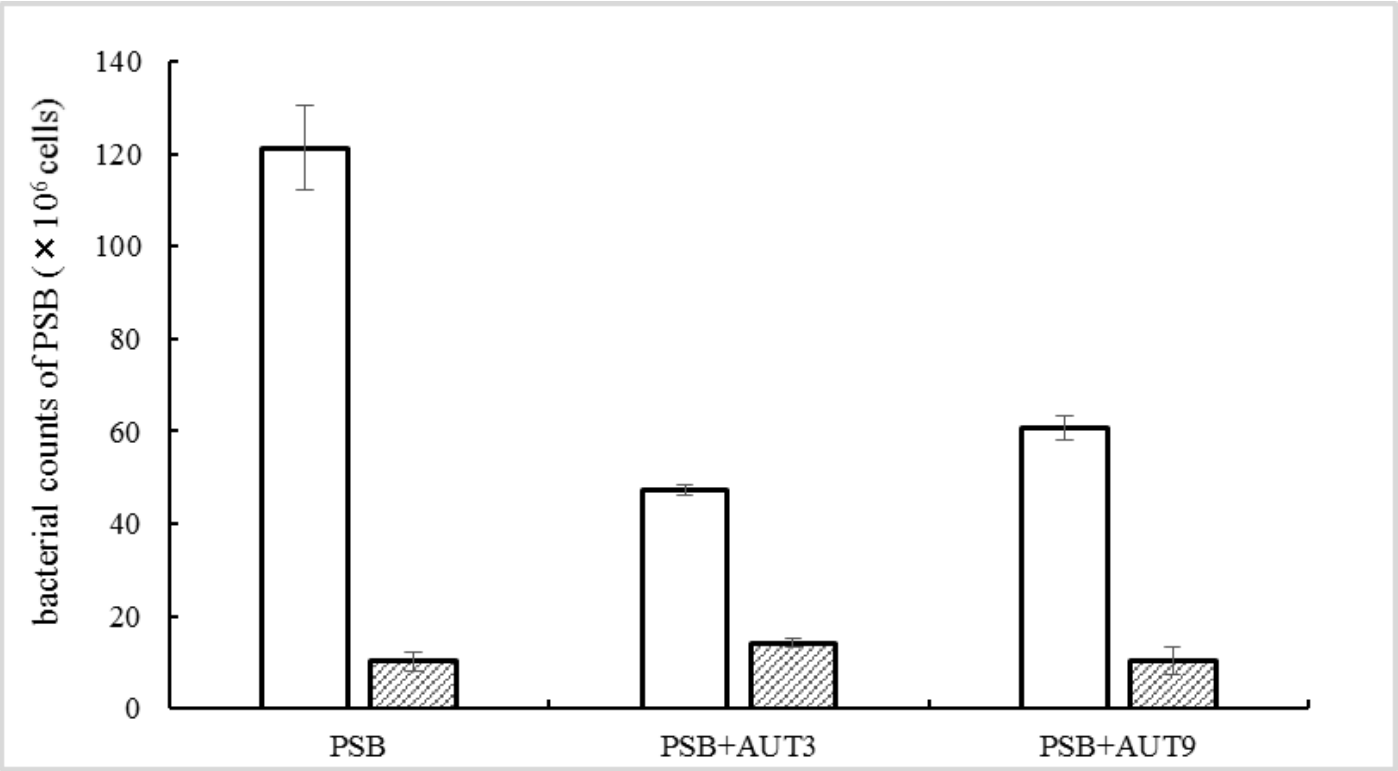

Fig. 1. Changes in the total cultivable bacterial counts of PSB in the different microcosms after 7 days of incubation (day 0:□, day 7: ▨n = 3 ± standard deviation).

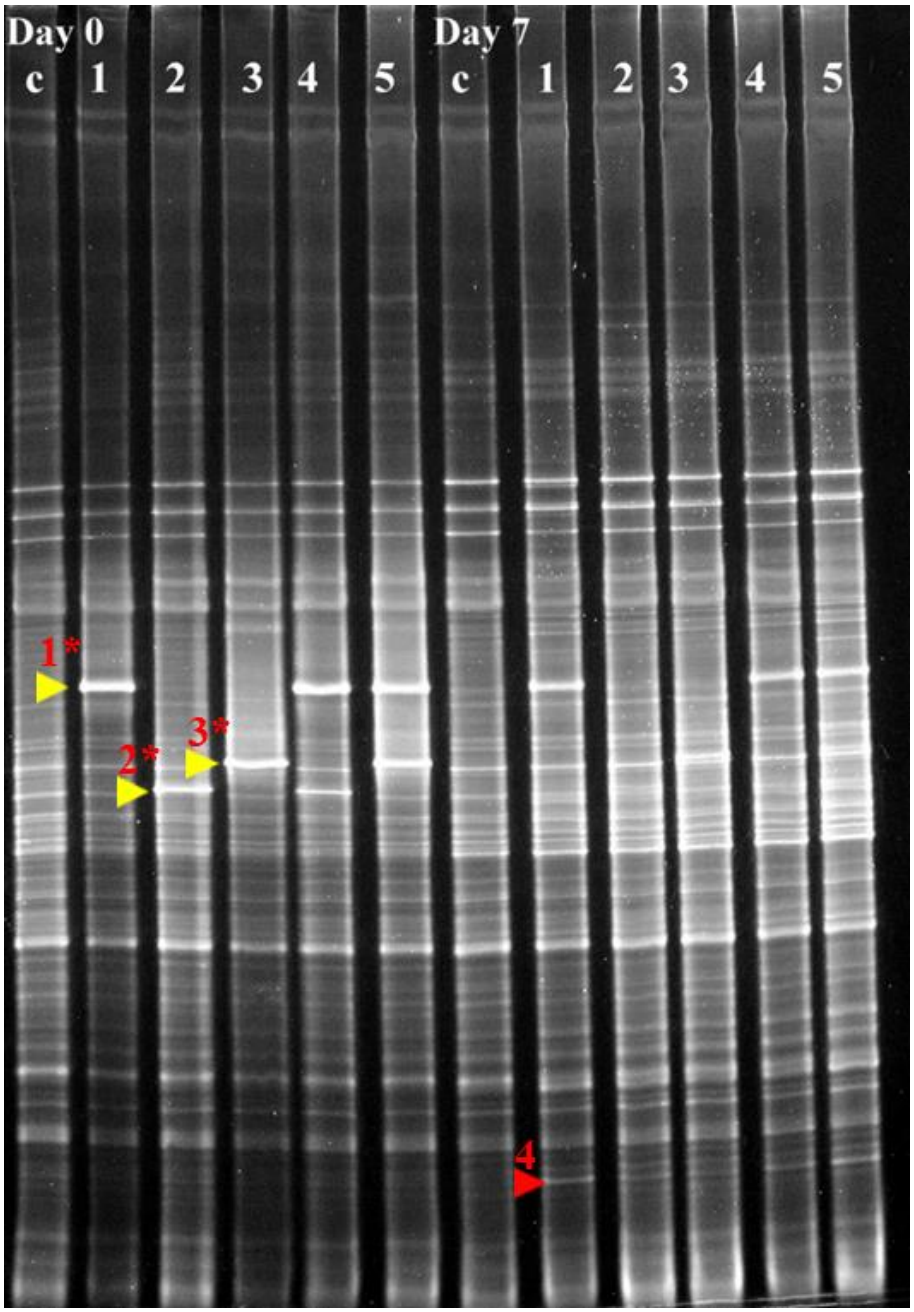

Fig. 2. DGGE analysis of the amplified 16S rDNA fragments from soil samples of the microcosms. The labels above the lane indicate the treatment number. The bands subjected to nucleotide sequencing were indicated by the yellow (added strains) and red (indigenous strains) arrowheads.

GENERAL CONCLUSION

The application of purple non-sulfur bacteria, one typical photosynthetic bacterium (PSB) was discussed in the present study. The isolation and identification of the bacterial strains revealed that the isolates were closely related to *Rhodopseudomonas* species. The cell morphology and pigment analyses also showed the typical characteristics of purple non-sulfur bacteria. One typical strain, PSB Strain A was selected for the following biological and biochemical study, a standard strain, *Rhodopseudomonas palustris* NBRC-100419T was applied as a comparison. Evaluation of its carbon-utilizing capability showed that the isolate could utilize variety of organic acids. Compared with standar strain, PSB Strain A showed better growth in pyruvate, acetate, propionate, succinate and malate at a concentration of 200 mM. During the treatment with swine sewage wastewater, PSB Strain A also showed almost the same efficiency in the reduction of VFAs and TOC compared with NBRC strain. The results indicated that PSB Strain A was efficient in treating wastewater containing high concentration of organic matters.

Furthermore, in the antagonistic test against a pathogenic root rot fungus (RRF), PSB Strain A was revealed to enhance the antagonistic activity of isolated *Bacillus* species. With the presence of PSB, the secretion of antibiotics by *Bacillus* species was somehow intensified. To clarify the mechanism of this cooperation, PCR-denaturing gradient gel electrophoresis (DGGE) of 16S ribosomal RNA genes (16S rDNA) was applied to determine the effect of addition of isolated microorganisms to the rhizosphere microbial communities shift. Sequencing of the excised DGGE bands revealed the presence of amended strains in the microcosms. *Catenulispora yoronensis*, one species of actinobacteria, was dectected only with the presence of PSB, suggesting the influence of PSB in the bacterial communities in these microcosms.

ACHKOWNEDGEMENTS

My research and academic years in Japan would not have been possible without the following. I acknowledge with gratitude, my debt of thanks to:

My thesis panel and advisers: Dr. Hiroto Maeda, Dr. Takeshi Yoshikawa, Dr. Masao Sakai, Dr. Shusaku Kadowaki, and Dr. Yuto Kamei for the guidance, encouragement and their wisdom.

The United Graduate School of Agricultural Sciences (Rendai) student support program for the research and travel funds.

The KUFF Laboratory of Microbiology, Dr. Suguru Okunishi, Dr. Sheila Mae Salarda Santander, Dr. Sharon Notato Nuňal , Mr. Shigeaki Sakaguchi, and all my past and present labmates for all the help and assistance and fun.

The faculty and administrative staff of the Faculty of Fisheries, Rendai, and International Students Office for their day to day guidance.

Kagoshima City, the people of Kagoshima, Kagoshima University International Residence Hall and the Kagoshima University Foreign Students Association (KUFSA) for providing me a warm home away from home.

The Chinese and Filipino communities in Kagoshima especially the Chinese and Filipino students for being my family in Japan. My foreign students and Japanese friends in Kagoshima for making my student life more interesting.

My family and friends in China for the all the support, encouragement and love.

WHY, 2016