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Article

Characterization of polished rice washing water fermented with milk and its inhibition activity toward tomato bacterial canker causative agent *Clavibacter michiganensis* subsp. *michiganensis*

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Abbreviations: CMM, *Clavibacter michiganensis* subsp. *michiganensis*; PS medium, potato semi-synthetic medium; RWWM, polished rice washing water fermented with cow's milk; RWW, polished rice washing water; PET, polyethylene terephthalate; SD, standard deviation.

Polished rice (seihaku-mai in Japanese) washing water (RWW) exerts a heavy load on gray water treatment. Some farmers ferment RWW with milk and use it for managing healthy plant growth (Fukushima, 2010). In this study, we prepared RWW fermented with cow's milk (RWWM) and found that it generated flammable gas and protein debris, lowered pH at the early stage of fermentation, and produced lactic acid. RWWM also inhibited the growth of *Clavibacter michiganensis* subsp. *michiganensis* (CMM), a causative agent of tomato bacterial canker, but not the growth of *Bacillus subtilis*, *Staphylococcus aureus,* or *Escherichia coli*. Thus, we assumed that lactic acid bacteria are involved in the antibacterial activity of RWWM and that some metabolites in RWWM inhibit the growth of CMM.

Key words: fermented polished rice washing water, *Clavibacter michiganensis*, characterization, growth inhibition

Introduction

In Japan, polished rice (seihaku-mai in Japanese) is washed several times with water to remove sticky bran before cooking. This polished rice washing water (RWW) is usually thrown away, thus exerting a heavy load on gray water treatment (1,2). Farmers often use homemade fermented organic materials for the management of healthy plant growth (3). An example is polished rice washing water fermented with cow's milk (RWWM), which was found to protect plants against southern blight caused by *Sclerotium rolfsii* (4,5). Fukushima (4) stated that lactic acid bacteria might be related to the "anti-disease" effect of RWWM. If this were true, the use of RWWM would contribute to both reducing the load on gray water treatment and managing healthy plant growth. However, RWWM has not been characterized so far. We attempted to characterize RWWM and estimate its antimicrobial activity. We describe herein some properties of RWWM and its inhibition activity toward *Clavibacter michiganensis* subsp. *michiganensis* (CMM). CMM is known to be a causative agent of

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tomato bacterial canker (6,7).

Materials and Methods

Materials

The cultivars of rice (*Oryza sativa* subsp. *japonica*) used in this study were mostly "Koshihikari" and "Kinuhikari" produced in Kyoto and Shiga Prefectures and obtained from local markets. *C. michiganensis* subsp. *michiganensis* (CMM) strain 05M1-2 (a gift from Dr. Akira Kawaguchi) was cultured in potato semi-synthetic (PS) liquid medium (7). *Bacillus subtilis* strain 168 (a gift from Dr. Yasutaro Fujita) was cultured in Luria Bertani (LB) medium. *Escherichia coli* AB1157 (ATCC 29055) and *Staphylococcus aureus* (ATCC 31890) were purchased from the American Type Culture Collection, USA and cultured in LB medium and Staphylococcus medium No. 110, respectively. Cow's milk was obtained from local markets.

Preparation of RWWM

RWWM was prepared essentially according to the method described by Fukushima (4). Polished rice (400 g) was washed four times with 400 mL of tap water. After washing, the four portions of washing water were combined and water was added to make 1600 mL (RWW). RWW was poured into a 2000 mL PET bottle together with 400 mL of commercially available cow's milk. Then, the bottle was capped and incubated statically at 25ºC in the dark for a specified period. Due to the generation of gas, the PET bottle was decapped quickly once a day until the tenth day after the start of incubation. Preparation of RWWM on the one-quarter scale was also conducted in a 500 mL PET bottle. We designated the polished rice washing water fermented with milk as RWWM.

Measurement of inhibition activity of RWWM toward CMM growth

CMM was pre-cultured in PS liquid medium at 30ºC for 24 hr with reciprocal shaking at 150 rpm. CMM growth in the 24-hr pre-culture was at the early stationary phase and cell density was approximately 2 x 10^8 cell/mL. RWWM cultured for 14-60 days was used, unless otherwise noted. RWWM used for measurement of inhibition activity was sterilized by passing through a filter (0.2 μm, Advantec 25AS020AS).

The test tube for assay contained 5 mL of PS liquid medium, 50 μL of pre-cultured CMM, and 500 μL of filter-sterilized RWWM. Sterilized water was added to the control test tube instead of RWWM. After the test tubes were incubated at 30ºC for 24 hr in the dark with reciprocal shaking at 150 rpm, the turbidity at 660 nm was measured. Measurement was routinely conducted in triplicate and expressed as growth rate relative to control in %.

The agar cup method was used to estimate the inhibition activity of RWWM. Hot PS agar medium was poured into a sterile Petri dish (9 cm diameter) and solidified. Then, sterilized penicillin cups were put concentrically on the agar medium. PS agar medium (15 mL) containing CMM was layered onto the solidified PS agar medium in the Petri dish, cooled, and solidified. Then, the penicillin cups were removed gently. The filter-sterilized RWWM (150 μL) was pipetted into each well. Water was used as control. After the Petri dishes were incubated at 30ºC for 48 hr, a clear zone along the well was observed.

Determination of lactic acids

D-Lactic acid and L-lactic acid were determined according to the instructions attached to the F-kit for the determination of D-lactic acid/L-lactic acid (Roche Diagnostics GmbH).

Measurement of whiteness value

Rice whiteness value was measured with a rice whiteness tester (Model C-300-3, Kett Electric Laboratory) and expressed as whiteness value in %.

Results and Discussion

Characterization of RWWM

Whiteness of polished rice and wash-free rice (musen-mai in Japanese) is approximately 40% and more than 45%, respectively (8). Therefore, sticky

rice bran is produced while polished rice is refined to wash-free rice. Whiteness of polished rice used in this experiment was 42.0 ± 0.4% (mean ± SD, n=4). Thus, it is suggested that RWW contains sticky rice bran.

RWW was mixed well with milk in the PET bottle to obtain a milky white homogeneous emulsion. When the emulsion was incubated statically at 25ºC in the dark, white milk curd appeared gradually both on top and at the bottom of the PET bottle (Fig. 1). In addition, the cloudiness of the emulsion was decreased and its color gradually turned pale yellow. One year later, most of the supernatants of RWWM were clear and had a pale yellow color. During the first two weeks, PET bottle containing RWWM continued to expand because of gas generation. We confirmed the

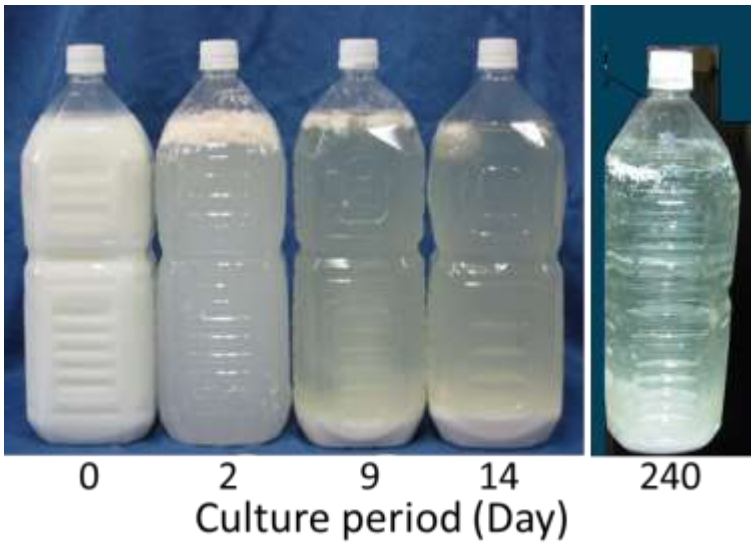

Fig. 1 Visual changes of RWWM
RWWM in PET bottles was incubated at 25ºC in the dark after RWW was mixed well with milk.

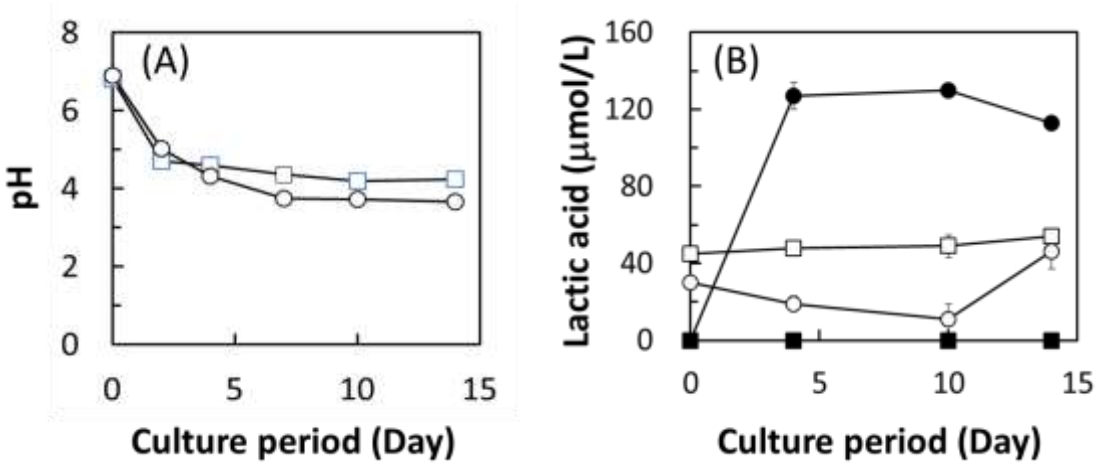

Fig. 2 Changes of pH (A) and lactic acid contents (B) during the incubation of RWWM and RWW
RWWM and RWW in PET bottles were incubated at 25ºC in the dark. pH was measured with a pH meter. Lactic acid contents were measured with a commercially available kit as described in Materials and Methods. Data are expressed as means ± SD (n=3). (A): RWWM (○), RWW alone (□). (B): L-Lactic acid (○) and D-lactic acid (●) in RWWM, L-lactic acid (□) and D-lactic acid (■) in RWW.

generation of flammable gas, probably methane, by burning. The pH change during the first two weeks of incubation is shown in Fig. 2A. pH of RWWM and RWW gradually decreased from the original pH 6.8-7.0 during incubation. One week after the start of incubation, pH of RWWM decreased to 3.75 whereas that of RWW, to 4.36. On day 14, pH of RWWM was 3.66 whereas that of RWW was 4.25 (Fig. 2A). Even after the prolonged incubation period, pH of RWWM was maintained around 3.3-3.7, whereas that of RWW did not reach values below 4.0 (Table 1).

Table 1 Effects of RWWM and RWW on CMM growth

Tested material			Relative growth of CMM
Material	Culture period (day)	pH	(%)
Experiment 1			
Water alone	0	nm	100 ± 3
RWWM (RWW + milk)	10	3.72 ± 0.06	2 ± 1
RWWM (RWW + milk)	14	3.66 ± 0.04	6 ± 1
RWWM (RWW + milk)	32	3.49 ± 0.01	3 ± 1
RWWM (RWW + milk)	55	nm	1 ± 1
Experiment 2			
RWWM (RWW + milk)	100	nm	1 ± 1
RWWM (RWW + milk)	200	3.68 ± 0.13	3 ± 1
Experiment 3			
Water alone	14	nm	100 ± 2
RWW + water	14	4.25 ± 0.02	86 ± 2
RWW + water	60	5.85 ± 0.01	83 ± 3
Water + milk	14	5.86 ± 0.02	109 ± 19
RWWM (RWW + milk)	14	3.66 ± 0.04	5 ± 1
Experiment 4			
Water alone	14	nm	100 ± 3
Autoclaved RWW + milk	14	4.97 ± 0.04	101 ± 13
RWW + autoclaved milk	14	4.61 ± 0.18	5 ± 2
RWWM (RWW+ milk)	14	3.66 ± 0.04	5 ± 1

Tested materials including RWWM were cultured in 2 L PET bottles at 25ºC in the dark for the indicated periods, and pH of the tested materials was measured in triplicate at the end of the culture. The assay for inhibition activity toward CMM growth was conducted by using the liquid culture method as described in Materials and Methods. Data are expressed as means ± SD (n=3). nm, not measured.

It is speculated that lactic acid bacteria are related to RWWM (4). Thus, we determined the lactic acid contents of RWWM and RWW for 14 days. Total lactic acid content in RWWM increased, with the increase in D-lactic acid content contributing to the total increase (Fig. 2B). In contrast, neither D-lactic acid nor L-lactic acid content increased in RWW, resulting in the unchanged lactic acid content (Fig. 2B). For RWWM incubated for 180 days, the contents of D-lactic acid and L-lactic acid were 31.9±3.2 and 26.5±1.6 mmol/L, respectively, and pH was 3.48. Total lactic acid content of RWWM on day 180 was approximately 300 times higher than that on day 14. The increase in D-lactic acid content

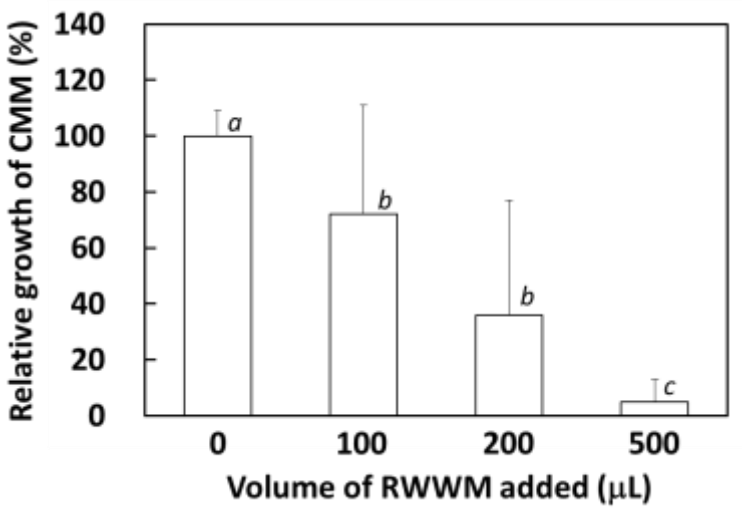

Fig. 3 Effects of RWWM added on CMM growth
CMM cultured in PS liquid medium at 30ºC for 24 hr with reciprocal shaking at 150 rpm was used. One turbidity unit at 660 nm, attained approximately 24 hr after the start of culture, corresponded to 2 x 10^8 cells/mL under the culture condition used. The test tube for assay consisted of 5 mL of PS liquid medium, 50 μL of CMM, and an aliquot volume of RWWM. In the control test tube, water was added instead of RWWM. After the test tubes were incubated for 24 hr at 30ºC with reciprocal shaking at 150 rpm, turbidity at 660 nm was measured. Data are expressed as means ± SD (n=4-10). Different letters attached to the bars in the panel mean significant difference at $P < 0.05$ by the Student's t-test.

suggests the involvement of lactic acid bacteria in RWWM. The isolation and identification of lactic acid bacteria in RWWM are in progress.

Inhibition of CMM growth by RWWM

It is assumed that lactic acid bacteria are present in RWWM, as described above. Lactic acid bacteria usually produce antibacterial substances called bacteriocin (9). Many bacteriocins produced by lactic acid bacteria are generally effective against Gram-positive bacteria (10-12). To evaluate the antimicrobial activity of RWWM, CMM was chosen because it is a Gram-positive phytopathogenic bacterium that causes tomato bacterial canker and its growth was inhibited by RWWM in a preliminary experiment.

We first tried to establish an assay for the inhibition activity of RWWM toward CMM growth. As shown in Fig. 3, the addition of 500 μL of RWWM inhibited CMM growth almost completely. The addition of 100-200 μL of RWWM inhibited CMM growth significantly compared to no RWWM addition, although no significant difference was observed between the addition of 100 μL and 200 μL. Therefore, we employed the reaction mixture consisting of 5 mL of PS liquid medium, 50 μL of pre-cultured CMM, and 500 μL of filter-sterilized RWWM, as described in Materials and Methods. Inhibition of CMM growth was also confirmed by the agar cup method. CMM inhibition zones were observed around wells 1 and 3 filled with RWWM, but not around control wells 2 and 4 filled with water (Fig. 4).

CMM growth inhibition was first observed approximately 10 days after the start of culture of RWWM (*Experiment 1* in Table 1). The inhibition activity of RWWM was detected even after 200 days (*Experiment 2* in Table 1). Seventy-six of the 91 lots of RWWM (culture period, 14 to 60 days) showed more than 50% inhibition of CMM growth under the assay condition described in Materials and Methods. Sixty-three lots showed more than 90% inhibition.

Fig. 4 Inhibition of CMM growth by RWWM
Inhibitory effect of RWWM was estimated by the agar cup method as described in Materials and Methods. Wells 1 and 3 were filled with RWWM and wells 2 and 4, with water as control. The Petri dish was incubated at 30ºC for 48 hr in the dark. Growth inhibition was estimated from the inhibition zone around the well.

Table 2 Effect of RWWM on growth of various bacteria

Bacterium species	Medium	Tested material	Relative growth rate (%)
CMM	PS	Water	100 ± 3
	PS	RWWM	3 ± 1
Bacillus subtilis	LB	Water	100 ± 1
	LB	RWWM	96 ± 5
Staphylococcus aureus	St. #110	Water	100 ± 1
	St. #110	RWWM	99 ± 2
Escherichia coli	LB	Water	100 ± 2
	LB	RWWM	96 ± 1

The assay for the inhibition activity of RWWM was conducted by using the liquid culture method as described in Materials and Methods with the exception that CMM was substituted by other bacterium. Data are expressed as means ± SD (n=3).

RWWM consists of RWW and milk. When RWW was cultured with water, only low inhibition (less than 20%) was observed (*Experiment 3* in Table 1). Culture of milk alone showed no inhibition of CMM growth. Therefore, both RWW and milk are necessary to produce the inhibition activity toward CMM growth. Combination experiments of autoclaved and non-autoclaved materials revealed that non-autoclaved, RWW was necessary to produce the inhibition activity (*Experiment 4* in Table 1). Autoclaved milk was as effective as non-autoclaved milk. These results suggest that RWW is the main source of microorganisms and milk supplies the nutrients required for the growth of microorganisms.

Then, the effect of RWWM on the growth of other bacteria was examined. The growth of *B. subtilis* and *S. aureus*, both Gram-positive bacteria, was not inhibited (Table 2). The growth of *Escherichia coli*, a Gram-negative bacterium, was not inhibited as well. Thus, the inhibition activity of RWWM was specific to CMM among the bacteria examined. Nisin, which is used as an additive for food preservation, is produced by *Lactococcus lactis* (9,12). Nisin inhibits the growth of some but not all Gram-positive bacteria. These results also suggest that lactic acid bacteria are involved in RWWM.

It is also speculated that the low pH of RWWM is a cause of the inhibition of CMM growth because the pH of RWWM is usually around 3.5-3.8. Therefore, the effect of medium pH on CMM growth was examined. Instead of RWWM, water was added to PS media adjusted to pH 5.0 and 6.8, and this was followed by the addition of CMM. RWWM and CMM were added to the standard PS medium. The pH of the assay tubes containing CMM in PS medium adjusted to pH 5.0 and 6.8 was 5.2 and 7.0, respectively, before the incubation for assay. After the incubation for 24 hr, the pH of the assay tubes was 5.6 and 6.7, respectively (Table 3). The growth rate of CMM in the medium with pH 5.0 was 82% compared to the 100% growth rate in the medium with pH 6.8. pH of the standard assay tube containing CMM and RWWM was 5.4 and 5.6 before and after the assay, respectively (Table 3). However, CMM growth in the standard assay tube was inhibited almost completely. Effect of lactic acid addition on CMM growth was also examined. pH of the assay tube containing PS medium and CMM was 5.9 when lactic acid was added to the tube (final concentration: 0.3 mM), but CMM growth was not inhibited (data not shown). From these results, we conclude that the low pH of the assay tube was not the cause of the inhibition of CMM growth. We assume that the presence of bacteriocin-like compounds is responsible for the inhibition of CMM growth and are now trying to isolate and identify them.

Fukushima (4) described that RWWM reduced damage in green onion caused by southern blight disease. It is well known that southern blight disease is caused by *Sclerotium rolfsii*, which produces many sclerotia under stress conditions. Sclerotia are alive for a few years in soil.
RWWM inhibited neither mycelial growth nor sclerotia germination of *S. rolfsii* (data not shown). This suggests that RWWM does not act directly on mycelia or sclerotia of *S. rolfsii*.

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Table 3 Effect of medium pH on CMM growth

Medium	Tested material	pH		Relative growth of CMM (%)
		before assay	after assay	
PS medium (pH 6.8)	Water	7.0	6.7	100 ± 1
PS medium (pH 5.0)	Water	5.2	5.6	82 ± 7
PS medium (pH 6.8)	RWWM	5.4	5.6	1 ± 1

PS media adjusted to pH 6.8 and 5.0 before autoclaving were prepared. Water (500 μL) and CMM (50 μL) were added to each PS medium and pH was measured and designated as “pH before assay”. After 24 hr incubation, pH and turbidity of the assay test tubes were measured and designated as “pH after assay” and “relative growth of CMM”. RWWM used in this experiment was a 44-day culture with pH of 3.6. Data for relative growth of CMM are expressed as means ± SD (n=3).

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