Study of beneficial microorganisms in Myanmar's fermented fishery products

Doctoral Dissertation

STUDY OF BENEFICIAL MICROORGANISMS IN MYANMAR'S FERMENTED FISHERY PRODUCTS

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CONTENTS

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ABSTRACT

The objective of this study is to contribute scientifically to Myanmar's modern fisheries industry by solving a series of problems that it may encounter during its economic development. Myanmar's current economic development will inevitably affect its people's lifestyle and natural environment, such as by introducing modern processed foods in place of traditional fermented foods. To preserve and pass on Myanmar's traditional food culture to the next generation, it is important to study and investigate traditional fermentation methods and techniques. In addition, aquatic environments that become polluted as a result of economic development can be remediated through the inherent abilities and functions of microorganisms (so-called bioremediation). On the basis of these social and scientific backgrounds, three scientific topics, namely, diversity of lactic acid bacteria (LAB), production of antibacterial substances as replacements for synthetic food additives, and biodegradation of malachite green (MG), a synthetic dye, were focused on in this thesis.

In the first chapter of this study, a detailed description of the microflora of a traditional fishery product in Myanmar which is fermented with boiled rice was

studied from two viewpoints, namely, the culture-dependent and culture-independent methods. Specifically, isolation and characterization of lactic acid bacteria (LAB) from small freshwater fish fermented with boiled rice, a typical Myanmar fermented product, were performed to contribute to the understanding of its fermentation process. Eight fermented fishery products were used in this study. These isolates were identified as LAB, and they were classified into two groups, homofermentative and heterofermentative isolates, on the basis of their phenotypic characteristics. From the results of the PCR-restriction fragment length polymorphism (RFLP) analysis and the 16S rRNA gene sequencing, most of the isolates were identified as genus *Lactobacillus* species. In addition, some of the isolates were identified as γ-aminobutyric acid (GABA)-producing LAB. Terminal restriction fragment length polymorphism (T-RFLP) analysis was also carried out using DNA samples extracted from these fermented products.

In the second chapter of this study, antibacterial substances produced by bacteria were studied. In consideration of consumers' health, antibacterial substances produced by bacterial isolates from traditional food have been of interest for industrial applications, such as foods preservatives. In this chapter, an antibacterial-substance producing bacteria isolated from Myanmar's fermented

shrimp containing boiled rice were used. This strain was gram-positive-spore forming and rod-shaped bacteria. In addition, according to the 16S rRNA gene sequence result, the isolate was identified as one of the members of the genus *Bacillus* species. The antibacterial substance of the isolate was purified from a culture supernatant using two steps from ion exchange column chromatography and gel filtration column chromatography. Antibiotic activity was measured by the agar well diffusion method. Activity of this substance was observed against only some kinds of gram-positive bacteria, such as *Listeria monocytogenes*. Its activity was not influenced after treatments with several kinds of catabolic enzymes, but was partially reduced after a heat treatment at 121 °C for 15 min. The behavior of the molecular size of this antibacterial-substance differed in the gel filtration chromatography and electrophoresis analyses.

In the third chapter of this study, MG-degradation-microorganisms were studied. The study in this chapter was also performed to contribute to the Thai fisheries industry and fisheries science. MG is a well-known green triphenylmethane dye, and is widely used in various industries and has also been known to have a useful effect, such as anti-fungal and anti-parasitic abilities for the treatment of fish diseases in the aquaculture field. In this chapter, I isolated and examined the

characteristics of MG-biodegradation-microorganisms in order to find applicable microbiological treatments for various environments containing MG. For the screening of the MG-biodegradation-microorganisms, an enrichment culture method was carried out. To contribute to the development of the fisheries science in neighboring countries around Myanmar, various sources in both Myanmar and Thailand were used in this study. Final enrichment broth cultures were streaked on plate agar media containing MG, and colonies which showed decolorization were chosen and isolated as MG-degradation-candidate strains. For the evaluation of taxonomic positions of these isolates, 16S rRNA gene or D1/D2 domain of 26S rRNA gene sequencings were performed. In order to understand the mechanism of decolorization and biodegradation of MG, further studies were carried out. LC liquid chromatography-tandem mass spectrometry (LC/MS/MS) analysis was performed on MG and LMG concentrations in broth media during incubation. In addition, the behaviors of MG and LMG were also analyzed using a UV-VIS spectrophotometer and a proton nuclear magnetic resonance (^{1}H-NMR).

Through the series of studies mentioned above, various kinds of beneficial microorganisms, both prokaryotes and eukaryotes, were isolated from Myanmar's traditional fermented fishery products and were examined in detail from various

viewpoints, namely, bacterial taxonomy and diversity, bioactive compounds for biopreservation, and bioremediation of environmental pollution. Each scientific finding holds promise for studies related to applied microbiology and fisheries sciences, as well as to the fisheries industry itself in Myanmar to contribute to the development of each field.

PREFACE

The objective of this study is to contribute to Myanmar's fishery industry by solving various problems that Myanmar may encounter during its economic development. Currently, Myanmar is one of the largest producers of fishery products in Southeast Asia. Myanmar has about 2,000 km of coastline and expansive inland waterways, most notably the Irrawaddy River. According to the 2008 Food and Agriculture Organization of the United Nations (FAO) statistics, Myanmar ranks tenth in fish catch quantity in the world, third in Southeast Asia, while its fish farming ranks 12th in the world and 5th in Southeast Asia [1].

As its economy grows, the fishery industry is also expected to grow, increasing the importance of fisher science. Although fishery science in Southeast Asia has been developed, Myanmar is still in the burgeoning stage. If you put "Thailand" and "fishery" as key words in the document retrieval system of the Web of Science (Thomson Reuters, http://webofknowledge.com), 233 papers published over the past five years come up. However, in the case of "Myanmar" and "fishery", only 18 papers will be found. Given this situation, in order to expedite the development and advancement of Myanmar's fishery industry, it is imperative to advance Myanmar's fishery science.

Although the targets of creatures in fishery science include diverse and extensive branches such as microbes, fish, shrimp, mollusks, and seaweeds, in this study, I focus on applied microbiology and look into three topics:

1. Microflora of fermented fishery products,
2. Microorganisms that produce antibacterial substances, and
3. Microorganisms that decompose malachite green, a synthetic dye.

In Myanmar, there are various traditionally fermented fishery products, including fish/shrimp fermented with rice, fish/shrimp paste, and fish sauce [2-4]. From these, in this study, I chose fermented fishery products with boiled rice called ngachin and pazunchin-akaungchin to isolate the microorganisms. These fermented fishery products with rice are commonly found in Myanmar markets and family kitchens, and are widely eaten across Southeast Asia [3, 4]. For instance, *pla-som*, *som-fak*, and *pla-ra* are well known in Thailand, and *burong isda* and *burong hipon* in the Philippines, *pa-som* in Cambodia, *shikhe* in Korea and *narezushi* in Japan are well known in their respective countries. Fundamentally, their methods of processing are similar, and are divided into two basic categories: either fish or shrimp materials are salted, followed by fermentation with carbohydrates, namely, boiled rice, roasted rice and rice bran for an appropriate

period. Photographs of the fermented food in Myanmar used in Chapters 1 to 3 of this study are shown in Figure 1; that is, shrimp and fish in *ngachin* and *pazunchin-akaungchin* are shown in Figures 1(A) and 1(B), respectively. Final products are sold in the market either wrapped in banana leaves or by themselves, as shown in Figures 1(C) to 1(F).

On the other hand, fish/shrimp paste, and fish sauce are called *ngapi*/*mhyin ngapi*, and *ngan pyar yae* were used as one of main seasoning in Myanmar. Frankly speaking, they have been used essentially in various dishes and are indispensable to food cultures in Myanmar [5, 6]. Although these fermented products can be seen in daily life in Myanmar, these scientific studies are very scarce, and in my knowledge, there are only a few recent reports concerning such things as the chemical characteristics of fish sauce and lactic acid bacteria in fermented fish/shrimp in rice as recent studies [7-9]. Photographs of these pastes and fish sauces in Myanmar daily markets are also shown in Figures 2(A) to 2(C), and 2(D) to 2(F), respectively.

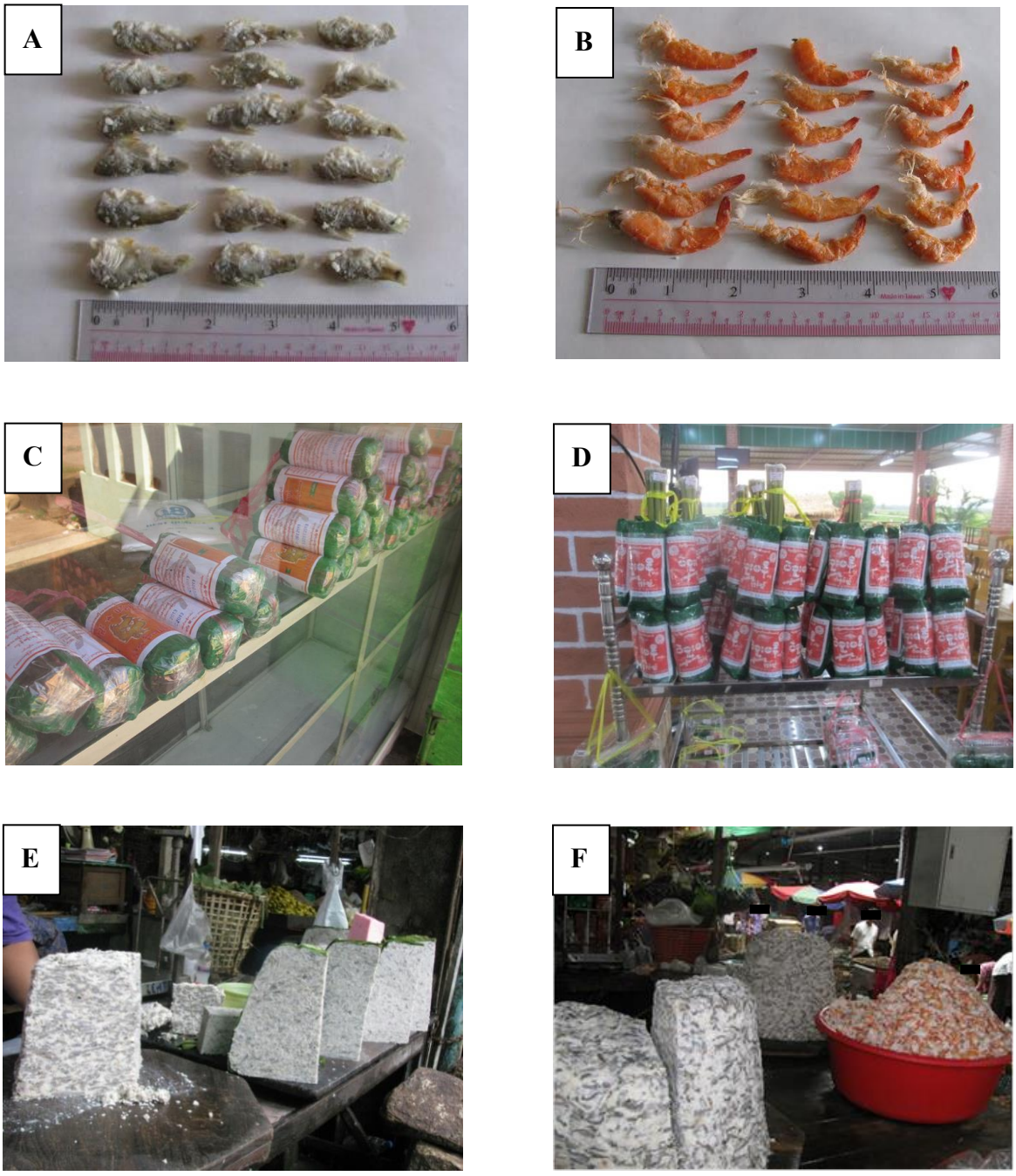

Figure 1 Myanmar fermented fishery products with rice called *ngachin* and *pazunchin-akaungchin*

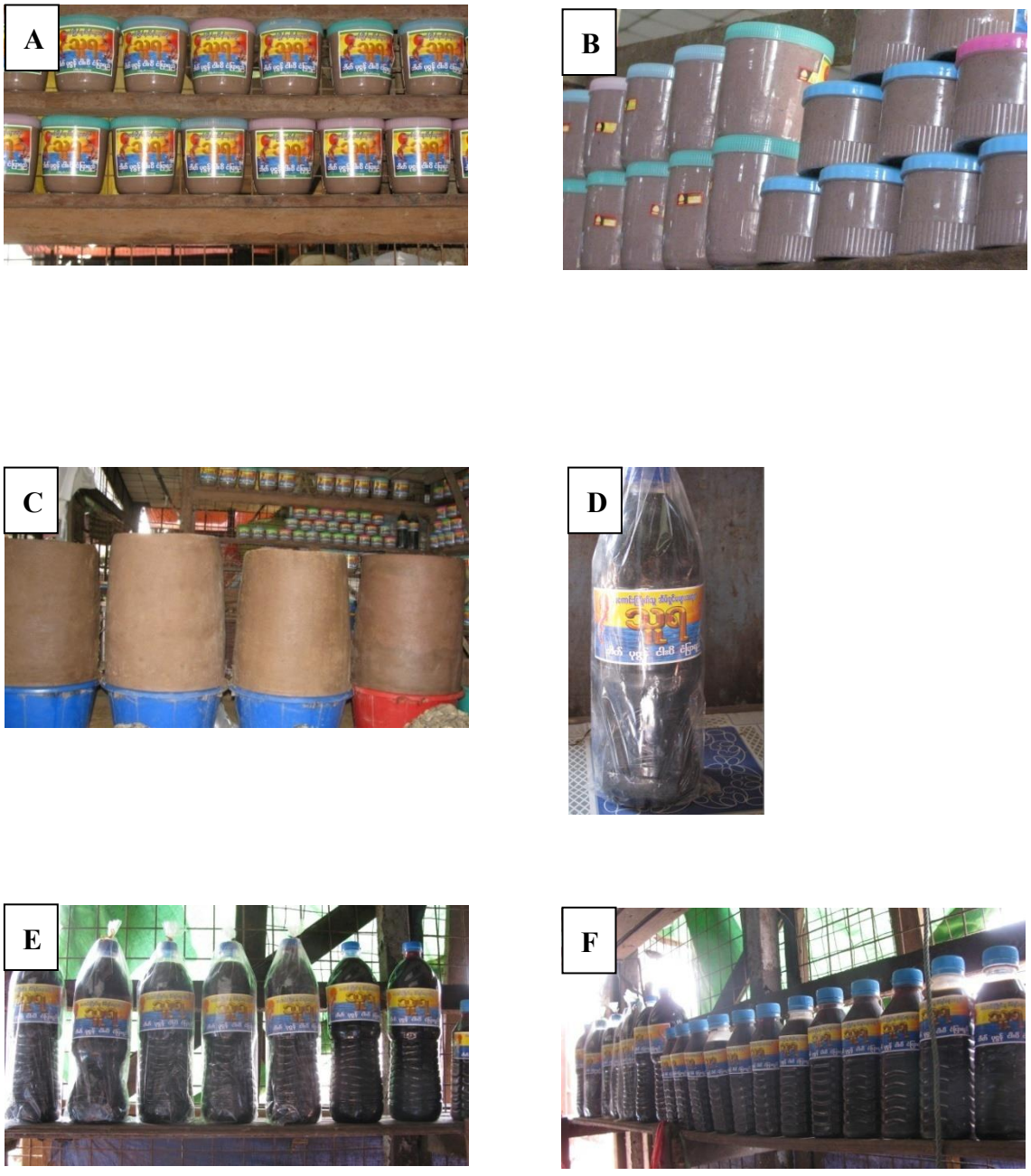

Figure 2 Myanmar fermented fishery products with fish paste called *ngapi* (A to C) and fish sauce called *ngan pyan yea* (D to F)

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CHAPTER I

Characterization of lactic acid bacteria distributed in small fish fermented with boiled rice in Myanmar

Introduction

Fermented fishery products with carbohydrates such as boiled rice are traditional fermented products and are distributed widely in some Southeast Asian countries. They are widely available in urban, rural, and local markets and are consumed daily as popular products in these countries. Although there are various types of fermented fishery products with boiled rice, in some cases, roasted rice or rice bran is also used. Basically, they are processed in two steps: first, fish or shrimp are salted, and then fermented with carbohydrates for an appropriate period [1, 2].

Likewise, in Myanmar, fermented fishery products with boiled rice, which is an essential and indispensable ingredient, are commonly distributed and called different names, such as *ngachin*, *pazun-chin*, *ngagyin-chin*, and *ngaphae-chin*, depending on the fishery product used, such as tinfoil barb *Puntius schwanenfeldii*, speckled prawn *Metapenaeus monoceros*, mrigal *Cirrhinus mrigala*, and featherback *Notopterus notopterus*. Usually, one of these products is served as one of the side dishes in two recipes. They are eaten after frying with adequate

seasonings or directly eaten after mixing well with vegetable oils and various seasonings.

There have been several microbiological studies on these popular fermented fishery products in Southeast Asia, which have been carried out not only with the expectation of them being good sources of various beneficial microorganisms such as lactic acid bacteria (LAB) but also from the viewpoint of enhancing their commercial value. In particular, several extensive studies concerning beneficial bacteria have been carried out on Thai fishery products fermented with rice or rice-related substances, which include LAB characterization in various kinds of products [3, 4], LAB characterization in *pla-som* [5], isolation of LAB from *pla-som*, and its production [6], LAB characterization, and clarification of the role of the substrate used in fermentation of *som-fak* [7], a taxonomic study on novel bacteria such as species of *Lactobacillus*, *Weissella*, and genus of *Piscibacillus* [8, 9], protease production of a halophilic bacterium [10], and bacteriocin-producing bacteria in *pla-ra* [11-13], and *pla-som* [14]. Moreover, fermented fishery products with carbohydrates are also distributed in East Asian countries, and the microbiological diversity of Japanese *narezushi* [15, 16] and a bacterial analysis of Korean *sikhe* [17] were recently demonstrated.

On the other hand, in the previous study using four types of Myanmar fermented fishery products with boiled rice [18], γ-aminobutyric acid (GABA)-producing LAB were isolated; GABA is a four-carbon, non-protein amino acid that has attracted attention owing to its beneficial effects on human health. The results obtained demonstrated that one of the isolates from fermented small fish called *ngachin*, *L. farciminis*, is a very effective starter culture for GABA accumulation. To comprehend completely the bacterial contribution during the processing of Myanmar fermented fishery products, it is important to clarify the contribution of not only GABA-producing LAB but also other various factors such as those affecting lactic acid fermentation. In addition, detailed elucidation of the fermentation is also necessary to establish the quality and safety of these fermented fishery products. However, despite the popularity of these Myanmar products, reports concerning them are very limited in comparison with those in surrounding countries.

Therefore, in this study, to gain basic insights into bacterial diversity and its function, I isolated and characterized LAB from fermented small fish with boiled rice, which is a typical Myanmar fermented product, and determined the microflora from two aspects, namely, an ordinary culture-dependent method and a culture-

independent method, such as terminal restriction fragment length polymorphism (T-RFLP).

Materials and methods

Fermented fish

Eight fish products fermented with boiled rice (*ngachin*) were purchased from different markets in Yangon, Myanmar from 2010 to 2012. They were transported to our laboratory by air and were stored at 4°C before use.

Determination of fermented fish characteristics

Fermented fish were characterized as described previously [18]. The pH and NaCl concentration of the fermented fish were determined using a pH meter (F-52; Horiba, Kyoto, Japan) and a NaCl meter (C-121; Horiba, Kyoto, Japan), respectively. Lactic acid and GABA were quantified using a high performance liquid chromatograph equipped with a conductivity detector (Organic acid analysis system; Shimadzu, Kyoto, Japan) and an automatic amino acid analyzer (LC-500 automatic amino acid analyzer; JEOL, Tokyo, Japan).

Viable cell count and bacterial isolation from fermented fish

LAB were isolated and enumerated by the ordinary spread plate technique after serial dilution. Each dilution was incubated on de Man, Rogosa, and Shape (MRS) medium (Merck, Darmstadt, Germany) containing 1.5% agar (Ina Food Industry, Nagano, Japan) at 27°C under anaerobic conditions as described previously [18]. Purified isolates were stored at -80°C in MRS broth medium containing 20% glycerol (w/v).

Phenotypical identification of LAB

Isolates were phenotypically identified according to an ordinary routine method using MRS plates and broth medium at 30°C as described previously [18]. Gram staining and microscopic observation of cell form and motility using a light microscope (BX51; Olympus, Tokyo, Japan) were carried out. Growth kinetics under different conditions such as NaCl, pH, and temperature were determined in the MRS broth medium. A carbohydrate fermentation test was carried out using API CH strips with an API CHL medium and APIWEB identification software (Bio Merieux, Lyon, France) following the manufacturer's instructions.

Confirmation of GABA-producing LAB

All of the isolates were grown in 10 ml of 5% monosodium glutamate (MSG; Wako, Osaka, Japan) - MRS broth medium (pH 7.0) containing 0.1 mM pyridoxal-5'-phosphate (PLP; Wako, Osaka, Japan) at 27°C for 7 days. The GABA-producing abilities of the isolates were confirmed after coloring using ninhydrin spray (Wako, Osaka, Japan) after thin-layer chromatography (TLC) (Silica gel 60 F_{254}; Merck, Darmstadt, Germany). In addition, for confirmation of the results of TLC, GABA was quantified using an automatic amino acid analyzer as described above.

Genotypical identification of LAB using 16S rRNA gene

Extracted deoxyribonucleic acid (DNA) samples from each isolate and the following two oligonucleotides primers, namely, 5'-AGA GTT TGA TCC TGG CTC AG-3' (primer 27F) and 5'-GGC TAC CTT GTT ACG ACT T -3' (primer 1492R), were used for gene amplification using Takara rTaq polymerase (Takara Bio, Shiga, Japan) in a DNA thermocycler (ASTEC, Fukuoka, Japan). Amplification was performed under the following temperature profile: 5 min at 94°C; 30 cycles of 1 min at 94°C, 2.5 min at 60°C, and 2.5 min at 72°C; 7 min of final extension at

72°C. For the RFLP analysis, the products were digested with two restriction enzymes, *Mbo* I (GCTC/) and *Afa* I (GT/AC) (Takara Bio), in a water bath for 4 h at 37°C, respectively. For the DNA sequencing, labeled PCR products were prepared using the BigDye Terminator Cycle Sequencing Kit ver. 3.1 (Applied Biosystems, Foster City, CA, USA), and sequencing was carried out using an Applied Biosystems 3730xl DNA analyzer. The obtained results were compiled using the GENETYX computer program (GENETYX, Tokyo, Japan), and the compiled sequences were compared with known sequences using the basic local alignment search tool (BLAST, http://www.ncbi.nlm.nih.gov/BLAST/).

T-RFLP analysis of fermented products

DNAs of fermented products were extracted directly using a kit for extracting DNA (ISOIL, Nippon Gene Co., Ltd., Tokyo, Japan). PCR was performed with the forward primer 27F, which was labeled at the 5' end with the phosphoramidite dye 6-FAM, and the reverse primer 1492R. PCR was carried out using KOD-FX polymerase (Toyobo, Osaka, Japan) in a PC 310 thermal cycler (ASTEC, Fukuoka, Japan). PCR products were purified with Amicon Ultra-100K centrifugal filters (Merck Millipore, Billerica, MA, USA) and digested using the restriction enzyme

Afa I (GT/AC) (Takara Bio) in a water bath for 4 h at 37°C. To determine the length of the terminal restriction fragment, fluorescent PCR products were subjected to capillary electrophoresis using an ABI 310 sequencer running in the GeneScan mode (Applied Biosystems), and GeneScan^{TM} LIZ 1200 (Applied Biosystems) was used as an internal size standard. The fragments, the lengths of which were smaller than 45 bp or the peak heights of which were less than 100 fluorescent units, were excluded from the analysis. In addition, the height of each fragment was divided by the total height of all the fragments.

Results

pH, NaCl, lactic acid and GABA concentrations, and viable cell count of fermented fish

Table 1 shows the basic characteristics of the fermented fish products. Their pHs were 4.5 to 4.9 and their NaCl concentrations were 3.4 to 6.6%. The number of viable cells was in the range from 9.2×10^7 to 2.8×10^9 cells/g. In addition, the lactic acid and GABA concentrations of these products were from 878 to 2587 mg/100 g and from 11.8 to 234.4 mg/100 g, respectively.

General characteristics of isolates

In this study, 46 strains were isolated from the eight fermented products. Among them, only three strains, two from D0 and one from D3, were identified as a yeast-like fungus by microscopic observation. As shown in Table 2, all of the remaining 43 strains were gram-positive, facultative anaerobic, and catalase-negative bacteria. In addition, 40 strains and one strain were clearly rod-shaped and coccoid, respectively, and the remaining two strains were short-rod-shaped. Most isolates did not produce gas from glucose, and only three strains produced gas. Therefore, they were identified as LAB on the basis of their general phenotypic characteristics.

PCR-RFLP analysis and 16S rRNA gene sequencing

Figure 1 shows representative photographs of RFLP results. In addition, Table 2 shows a summary of the bacterial distribution on the basis of the RFLP and DNA sequencing results. The 43 bacterial isolates were classified into six groups on the basis of the results of the analysis using the two restriction enzymes mentioned earlier. Group I consisted of 15 strains and was isolated from D0, D1, D2, and D5 products. In addition, 22 strains that were isolated from D1, D2, D3,

D4, D6, and D7 products belonged to Group II. The remaining six strains belonged to Groups III to VI, and most strains were isolated from D0 product.

16S rRNA gene sequencing results of representative isolates of each group showed high similarity of LAB. Four strains of Group I, namely, D0-1-1, D0-2-2(ii), D1-5-1(i), and D5-5-1-(i), exhibited high similarities to *Lactobacillus plantarum* JCM1149^T (D79210), ranging from 99.27 to 99.87%. Additionally, strains D2-6-1(i), D3-6-2(v), D4-4-1(i), D6-5-1(iii), and D6-5-2(ii) of Group II also exhibited high similarity to *Lactobacillus farciminis* DSM 20185 (AJ417499). Moreover, strain D0-1-2(ii) of Group III, strain D0-1-6(ii) of Group IV, strain D0-1-3(i) of Group V, and strain D4-4-2(v) of Group VI had similar sequences to *Lactobacillus reuteri* NBRC 15892^T (AB680992), *Weissella paramesenteroides* NRIC 1542^T (AB023238), *Pediococcus pentosaceus* NBRC 107768^T (AB682664), and *Lactobacillus futsaii* YM 0097^T (HQ322270), respectively. Their sequence similarities were 99.80, 99.53, 99.87, and 100%, respectively.

GABA-producing bacteria

Eight GABA-producing bacteria were isolated from four fermented products, according to the results of TLC and amino acid analysis using an automatic amino

acid analyzer. Three strains [D0-1-1(s), D0-1-1, and D0-2-2(i)], one strain [D2-6-2(ii)], three strains [(D4-4-1(i), D4-4-1(ii), and D4-4-1(iii)], and a strain [D5-5-2(iv)] were isolated from D0, D2, D4, and D5 samples of different fermented products. According to API identification, five strains, namely, D0-1-1(s), D0-1-1, D0-2-2(i), D2-6-2(ii), and D5-5-2(iv), were identified as *Lactobacillus plantarum* with a high similarity of 99.9%. In addition, they were categorized into the same groups on the basis of the results of RFLP analysis, and strain D0-1-1, which is a representative of these strains, has a high 16S rRNA gene sequence similarity (99.87%) to the type strain of *L. plantarum* JCM 1149^{T} (D79210). Although three other strains [D4-4-1(i), D4-4-1(ii), D4-4-1(iii)] have very similar carbohydrate fermentation profiles, the taxonomic identification of these strains was not successful owing to the ambiguous results of API CHL. However, the 16S rRNA gene sequencing result of strain D4-4-1(i) shows a high similarity (99.80%) to *L. farciminis* DSM 21850 (AJ417499).

T-RFLP analysis

Electropherograms of the T-RFLP analysis of fermented products are shown in Figure 2. Moreover, distributions of dominant terminal restriction fragments (T-

RFs) are summarized as relative abundance (>10%) in Figure 3. Two, 1, 3, 1, 2, 3, 2, and 4 different T-RFs were observed in these samples. T-RF of 486 bp was the most frequent fragment, and T-RFs of similar lengths such as 489 bp were also detected in D6 and D7 samples. Short T-RF of 75 bp was detected in D2 and D5 samples, and a T-RF of similar length (76 bp) was detected in the D2 sample. In addition, long T-RFs of 904, 905, and 908 bp were detected in D4, D0, and D5 samples, respectively. T-RFs of 11 isolates from the plate medium in this study are shown in Table 3. These isolates were divided into three groups according to their T-RF length, namely, long [904, 905, 906, and 909 (912) bp], intermediate (485 and 486 bp), and short (below 80 bp).

Discussion

Until now, the elucidation of the fermentation mechanism of Myanmar fermented fish with boiled rice has not received much attention. To the best of my knowledge, there has been only one report that was focused on GABA-producing LAB and their application to the processing of fermented fish/shrimp with boiled rice [18-20]. Therefore, this report is the first detailed description from the

viewpoint of LAB microflora of these products. According to the result of the characterization of isolates using culture-dependent techniques in this study, the majority (87%) of the isolates found were homofermentative rod-shaped LAB. In contrast, only three strains were heterofermentative short-rod-shaped LAB, and one was a homofermentative coccoid LAB strain. In addition, the viable cell counts of fermented products were as high as 10^7 to 10^9/g, and the pH was 4.7 on average in this study. Therefore, these results indicate that LAB are widely distributed in these fermented products and contribute to the pH decrease owing to lactic acid fermentation, just like in similar fermented fisheries products with boiled rice in other Asian countries. In addition, in this study I isolated three yeast-like fungi from two fermented products. However, due to my focus on LAB, I did not examine their taxonomic characteristics and functional properties. Hence, further studies concerning yeast-like fungi should be helpful for better comprehension of the fermentation mechanism of these products.

To confirm the taxonomic position of isolated LAB, I performed PCR-RFLP and sequencing analyses of the 16S rRNA gene. As a result, I succeeded in obtaining *L. plantarum* group, *L. farciminis*, *L. futsaii*, *L. reuteri, W. paramesenteroides,* and *P. pentosaceus*, most of which were found to be well known in traditionally

fermented fishery product in Southeast Asia. In the case of *L. plantarum* group, from comparison of its sequence with those of the type strains of *Lactobacillus* species in the public DDBJ database, *L. plantarum* JCM 1149^T (D79210) and *L. pentosus* JCM 1558^T (D79211) were shown to be closely related to 1517/1519 (high similarity of 99.87%). Therefore, in this study, I regarded 15 strains belonging to Group I as determined by RFLP analysis as *L. plantarum* group. Previous studies of Thai fishery products fermented with carbohydrates reported that *L. plantarum* group and *L. farciminis* were isolated from fermented products obtained from various markets [3, 4]. In addition, in the studies of a similar fermented fish, *burong isda*, in the Philippines, *L. plantarum* was also isolated as a starch-hydrolyzing LAB species [21]. Although *L. futsaii* was isolated as a new LAB species from Taiwanese fermented mustard products [22], the isolation of this species from Thai *pla-ra* has recently been demonstrated [4]. In addition, a few strains of *W. paramesenteroides* were isolated from Thai *pla-ra* [4] and identified as a bacterial community in fermented *pa-som*, which is a traditional fermented fish product with garlic and rice in Laos [23]. *L. reuteri* is widely recognized as a resident in the human and animal gastrointestinal tracts and was also reported as a producer of inhibitory compounds such as reuterin and reutericyclin [24].

Therefore, I expected that one of isolates, strain D0-1-2(ii), has the ability to produce these inhibitory compounds. However, no bacterial inhibition activity was observed in my preliminary experiment (data not shown).

In this study, GABA-producing *L. farciminis* was isolated from D4 samples, in which the GABA concentration was markedly high and reached 234.4 mg/100 g. With regard to the previous study of GABA-producing *L. farciminis* [18], Su et al. already demonstrated that *L. farciminis* strain D323 is a useful starter culture for GABA accumulation in the trial fermentation of *ngachin*, which is the same type of fermented product used in this study. In that study, compared with 11 fermented fishery products in boiled rice used, the bacterial source of strain D323 contained the highest GABA amount of 193 mg/100 g. Therefore, taken together with these previous results, our results obtained here reconfirmed the relationship between high GABA accumulations in fermented products and the existence of GABA-producing *L. farciminis*.

Unlike the enrichment culture technique used in the previous study [18], in this study, individual colonies appeared on the MRS plate medium in the ordinary plate count technique used to investigate GABA-producing bacteria. In the case of 130 strains isolated by enrichment culture using 11 fermented products, 12 strains

(9.2% of the total number of isolates) showed GABA-producing ability, and these GABA-producing LAB were isolated from five products (45% of the 11 products). Conversely, in this study using 43 strains from eight fermented products, 19% of the total isolates showed GABA-producing ability, and GABA-producing LAB were isolated from four products (50% of the eight products). The differences in screening methods between these two studies were expected to cause some bias in the isolation results. Nevertheless, the probability of isolating GABA-producing LAB seemed to be similar.

In this study, to evaluate the bacterial community in fermented small fish with boiled rice in Myanmar, T-RFLP analysis was also carried out using DNA samples extracted directly from eight fermented products, because various techniques of culture-independent analysis of bacterial communities have been developed recently. In the case of fermented fishery products in Southeast Asia, PCR-denaturing gradient gel electrophoresis (PCR-DGGE) analysis targeting the16S rRNA gene has already performed. Marui et al. [23] examined the fermentation of *pa-som* in Laos and clarified the time-dependent bacterial community changes during the fermentation. Moreover, similar experiments have also been performed

with the Philippine fermented food *burong isda* (fish fermented with rice) and *burong hipon* (shrimp fermented with rice) [25].

On the other hand, T-RFLP analysis is a rapid, sensitive, and high-throughput approach to clarifying the structure of microbial communities [26], and it has been applied to the bacterial community analysis of various types of fermented food such as Tilsit cheese [27], yoghurt and hard cheese [28], and *kimchi* [29]. However, to the best of my knowledge, there have been few reports concerning the bacterial community in fermented fishery products in Southeast Asia examined by T-RFLP analysis. Therefore, to confirm the effectiveness of this technique, I decided to perform this experiment.

Eventually, the results of LAB identification by culture-dependent and culture-independent methods seemed to be similar. The results of T-RFLP analysis in this study suggest that *L. farciminis* or *L. futsaii* is widely distributed in fermented products. Although various T-RF lengths were determined, the major T-RF showed the peak of 486 (484) bp and it was associated with that of *L. farciminis* or *L. futsaii.* In the results obtained by the culture-dependent method, *L. farciminis* was also isolated from most of the fermented samples with a high probability of isolation, except for one sample (D5), whereas *L. futsaii* was isolated from only

two samples; D3 and D4. As mentioned earlier, the distribution of *L. farciminis* has also been shown in several other reports on Thai *pla-chom* and *pla-ra* as the most dominant bacterium, respectively [3, 4]. Information on *L. futsaii* is very limited at present because this species has been proposed recently as a novel species. Nevertheless, this species was already isolated from *pla-ra* and *pla-som* as the second most dominant species [4]. Although T-RFs of 904, 905, and 908 bp were detected in D0, D4, and D5 samples at relative abundance (>10%), as shown in Figure 2, the low height of the corresponding peak was also observed in the electropherograms of six other products. This T-RF corresponded to those of *L. plantarum* group or *W. paramesenteroides.* In addition, the short-length T-RFs of 75 and 76 were detected in D2 and D5 samples, and they were associated with that of *P. pentosaceus.* The major T-RFs of fermented products corresponded with those of the isolated LAB. Therefore, these results indicated that LAB were the dominant constituent microflora in the fermented products used in this study.

In this study, the final fermented products were used for T-RFLP analysis. Therefore, the obtained results were strongly affected by dead bacterial cells. In particular, in the case of the D0 sample, most of the isolates were *L. plantarum* group. In addition, *L. farciminis* and *L. futsaii* could not be isolated from the plate

medium. However, T-RF associated with these two bacterial species could be clearly observed in the electropherogram of D0 products. During fermentation, bacterial flora is generally considered to change owing to a variety of environmental factors, such as pH decrease, exhaustion of the fermentation substrate, and bacterial antagonists. DNA from bacterial cells that have already died during the fermentation can be amplified by PCR.

As mentioned earlier, in comparison with the culture-dependent method, the results of T-RFLP analysis did not seem to have major contradictions. Therefore, T-RFLP analysis could be as effective for examining microbiological communities in fermented fishery products with carbohydrates as PCR-DGGE. Hence, for the detailed elucidation of fermentation mechanisms of Myanmar fermented fishery products with carbohydrates, the succession of microbiological communities during the fermentation should be determined using T-RFLP. In addition, in order to improve the precision of the identification of microflora constituting these fermented products, further studies on T-RFLP conditions using different restriction enzymes are also necessary.

In conclusion, this study is the first detailed examination of the microflora of Myanmar traditional fermented fishery products with boiled rice from two

viewpoints, namely, culture-dependent and culture-independent methods. In order to increase the commercial value of Myanmar traditional fermented fishery products, which are essential for people, various aspects, such as quality enhancement, quality control, and hygienic management, should be developed. Therefore, this study is the first step. In order to achieve more, the microbiological characteristics of these fermented products should be fully understood.

Table 1 Chemical properties and distribution of GABA-producing LAB in eight fermented small fish with boiled rice in Myanmar

Sample	NaCl (%)	pH	CFU/g	GABA mg/100g	Lactic acid mg/100g	No. of isolate	No. of GABA producer
D0	6.6±0.0	4.7 ± 0.0	1.1×10^8 /g	56.5 ± 11.1	1652 ±659	11	2
D1	4.6±0.7	4.7 ± 0.1	9.6×10^7 /g	85.1[a]	878[a]	4	0
D2	4.4±1.3	4.6 ± 0.1	2.6×10^9/g	179.0 ± 85.0	1613 ±375	3	2
D3	3.4±0.1	4.9 ± 0.4	2.8×10^9 /g	234.4 ± 115.8	2587 ±643	5	0
D4	3.4±0.0	4.5 ± 0.0	1.4×10^8 /g	206.3 ± 34.3	2051 ±623	5	3
D5	4.4±0.0	4.7 ± 0.0	2.9×10^8 /g	11.8 ± 1.6	1814±637	5	1
D6	5.3±0.4	4.5 ± 0.0	9.2×10^7 /g	16.5 ± 1.7	1665 ±952	5	0
D7	6.3±0.0	4.6 ± 0.1	1.7×10^8 /g	15.1 ± 7.0	1474 ±556	5	0

Data expressed as mean ± standard deviation (SD) from three independent experiments

[a] n = 1

Table 2 Characterization of isolated LAB from eight fermented small fish with boiled rice in Myanmar

PCR-RFLP	Fermented products	Strain			Shape	Homo/Hetero	16S rRNA gene sequencing
I	D0	D0-1-1(s)$^{\$,\#}$	D0-1-2(i)	D0-1-3(ii)	Rod	Homo	*Lactobacillus plantarum* group
		D0-1-1$^{*,\$,\#}$	D0-2-2(i)$^{\$,\#}$	D0-2-2(ii)*			
		D0-2-4(i)					
	D1	D1-5-1(i) *	D1-5-2(v)				
	D2	D2-6-2(ii)$^{\$,\#}$					
	D5	D5-5-1(i)*	D5-5-1(iv)	D5-5-1(v)			
		D5-5-2(iv)$^{\$,\#}$	D5-5-2(v)				
II	D1	D1-5-1 (v)	D1-5-2(iv)		Rod	Homo	*Lactobacillus farciminis*
	D2	D2-6-1(i)*	D2-6-2(v)				
	D3	D3-6-1(iv)	D3-6-2(i)	D3-6-2(ii)			
		D3-6-2(v)*					
	D4	D4-4-1(i)$^{*,\$}$	D4-4-1(ii)$^{\$}$	D4-4-2(i)			
		D4-4-1(iii)$^{\$}$					
	D6	D6-5-1(iii)*	D6-5-1(v)	D6-5-2(i)			
		D6-5-2(ii)*	D6-5-2(v)				
	D7	D7-5-1(ii)	D7-5-1(iii)	D7-5-1(iv)			
		D7-5-2(ii)	D7-5-2(iv)				
III	D0	D0-1-2(ii)*			Rod	Hetero	*Lactobacillus reuteri*
IV	D0	D0-1-6(i)	D0-1-6(ii)*		short-rod	Hetero	*Weissella paramesenteroides*
V	D0	D0-1-3(i)*			Coccoid	Homo	*Pediococcus pentosaceus*
VI	D3	D3-6-1(ii)			Rod	Homo	*Lactobacillus futsaii*
	D4	D4-4-2(v)*					

$^{\$}$ GABA producing LAB

*16S rRNA gene sequencing strain

$^{\#}$Strain D0-1-1(s), D0-1-1, D0-2-2(i),D2-6-2(ii), and D5-5-2(iv) were identified as *L. plantarum* from API results

Table 3 Lengths of 16S rRNA gene terminal restriction fragment of representative isolates from fermented small fish with boiled rice in Myanmar

Isolates	Length
L. plantarum D0-1-1*	905
L. plantarum D5-5-2(iv) *	906
L. plantarum group D1-5-1(i)	904
L. plantarum group D0-1-2(i)	905
L. farciminis D4-4-1(i)*	485
L. farciminis D2-6-1(i)	486
L. farciminis D3-6-2(v)	486
L. futsaii D4-4-2(v)	485
W. paramesenteroides D0-1-6(ii)	909, 912
P. pentosaceus D0-1-3(i)	78, 79
L. reuteri D0-1-2(ii)	48, 49

*GABA producing LAB

The accuracy of terminal fragments was less than 2 bp error for up to 700 bp and less than 5 bp error for up to 1000 bp as previously reported (Moeseneder et al., 2001).

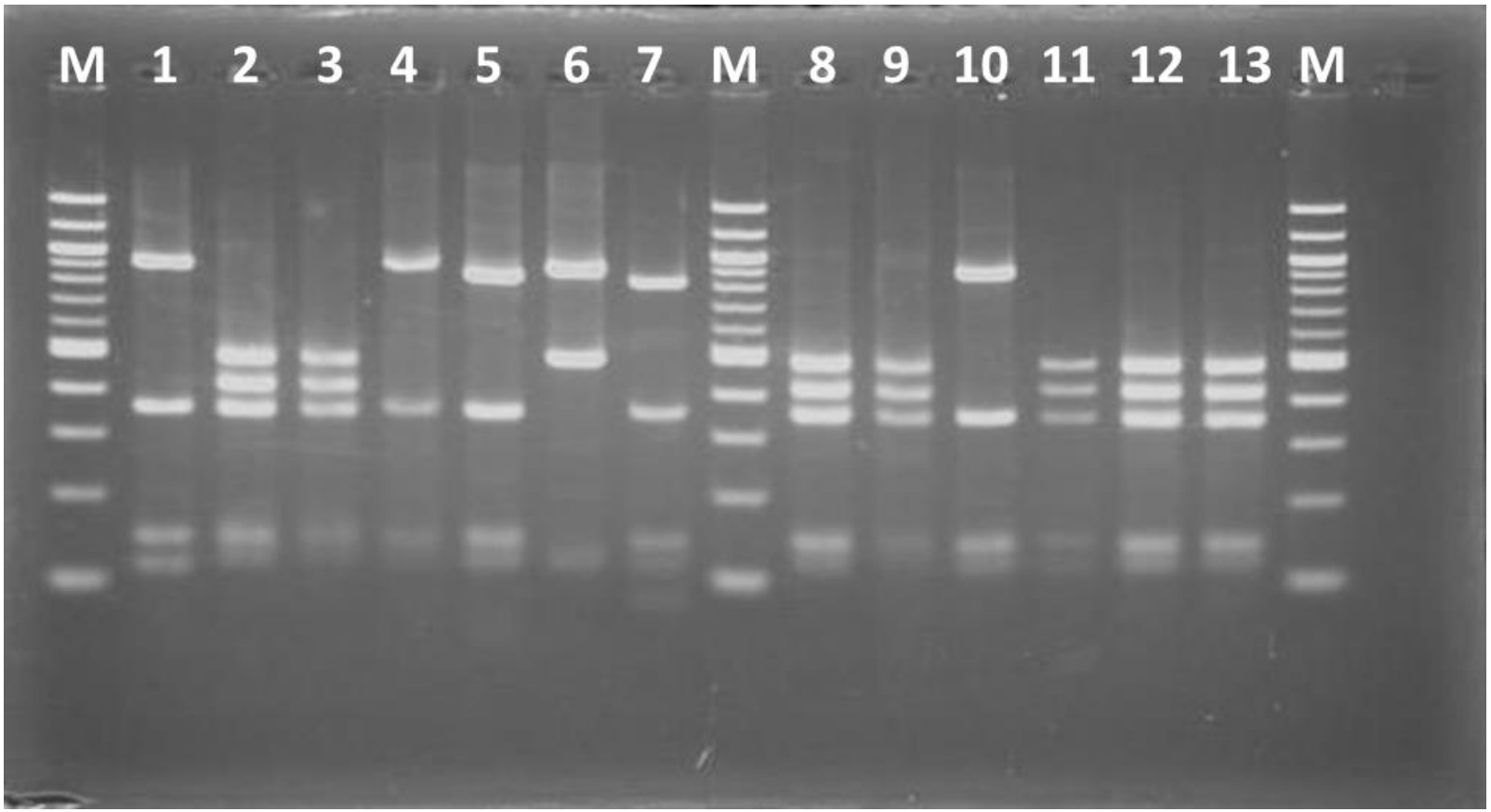

Figure 1 Restriction fragment patterns of *Afa* I-digested 16S rRNA gene from isolated LAB from eight fermented small fish with boiled rice in Myanmar

Lane 1, D1-5-1(i); lane 2, D2-6-1(i); lane 3, D3-6-2(v); lane 4, D0-1-1(s); lane 5, D0-1-2(ii); lane 6, D0-1-6(ii); lane 7, D0-1-3(i); lane 8, D4-4-1(i); lane 9, D4-4-2(v); lane 10, D5-5-1(i); lane 11, D6-5-1(iii); lane 12, D6-5-2(ii); lane 13, D7-5-2(iv)

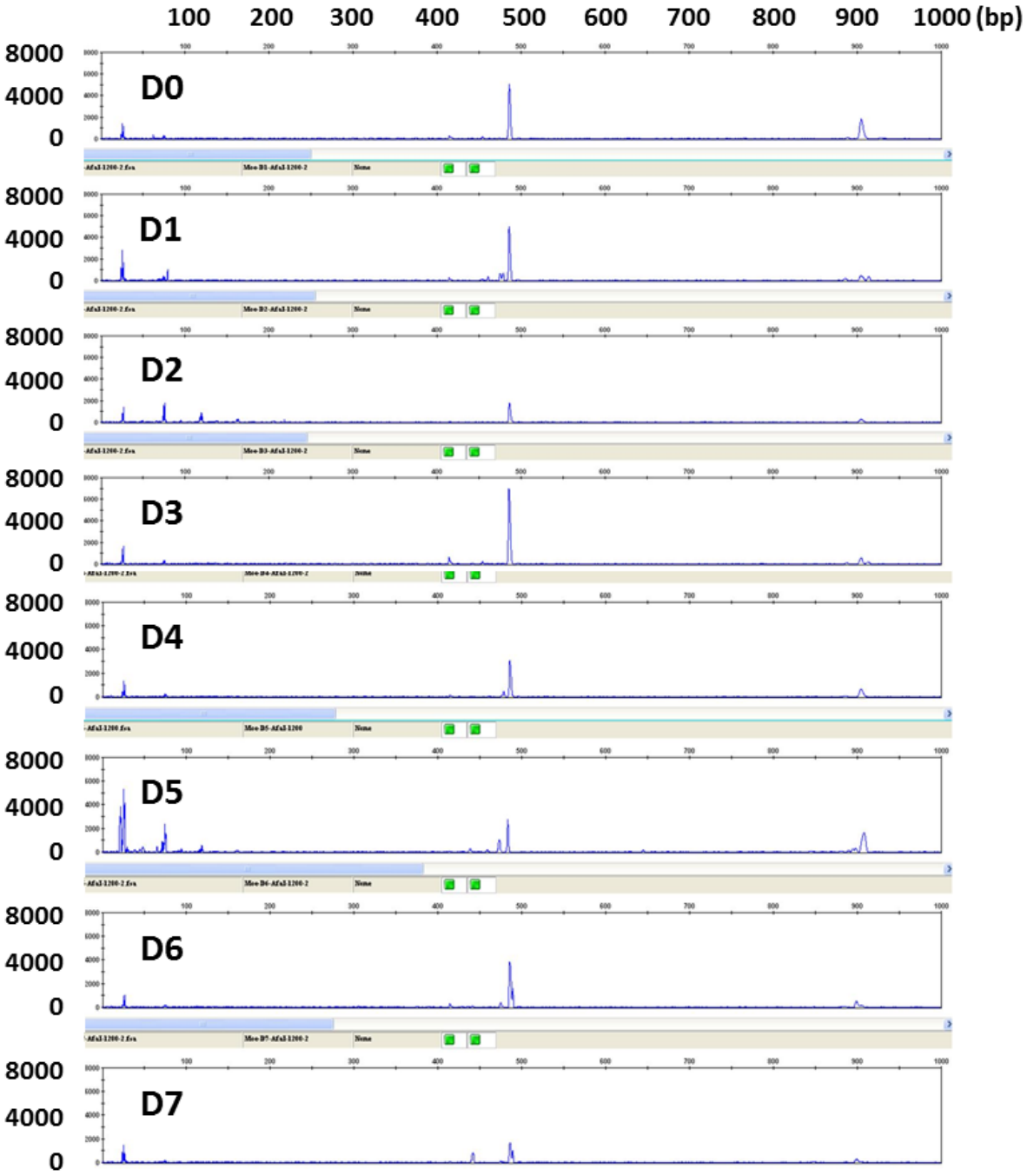

Figure 2 T-RFLP peaks generated by *Afa* I-digestion 16S rRNA gene which was amplified from eight fermented small fish with boiled rice in Myanmar

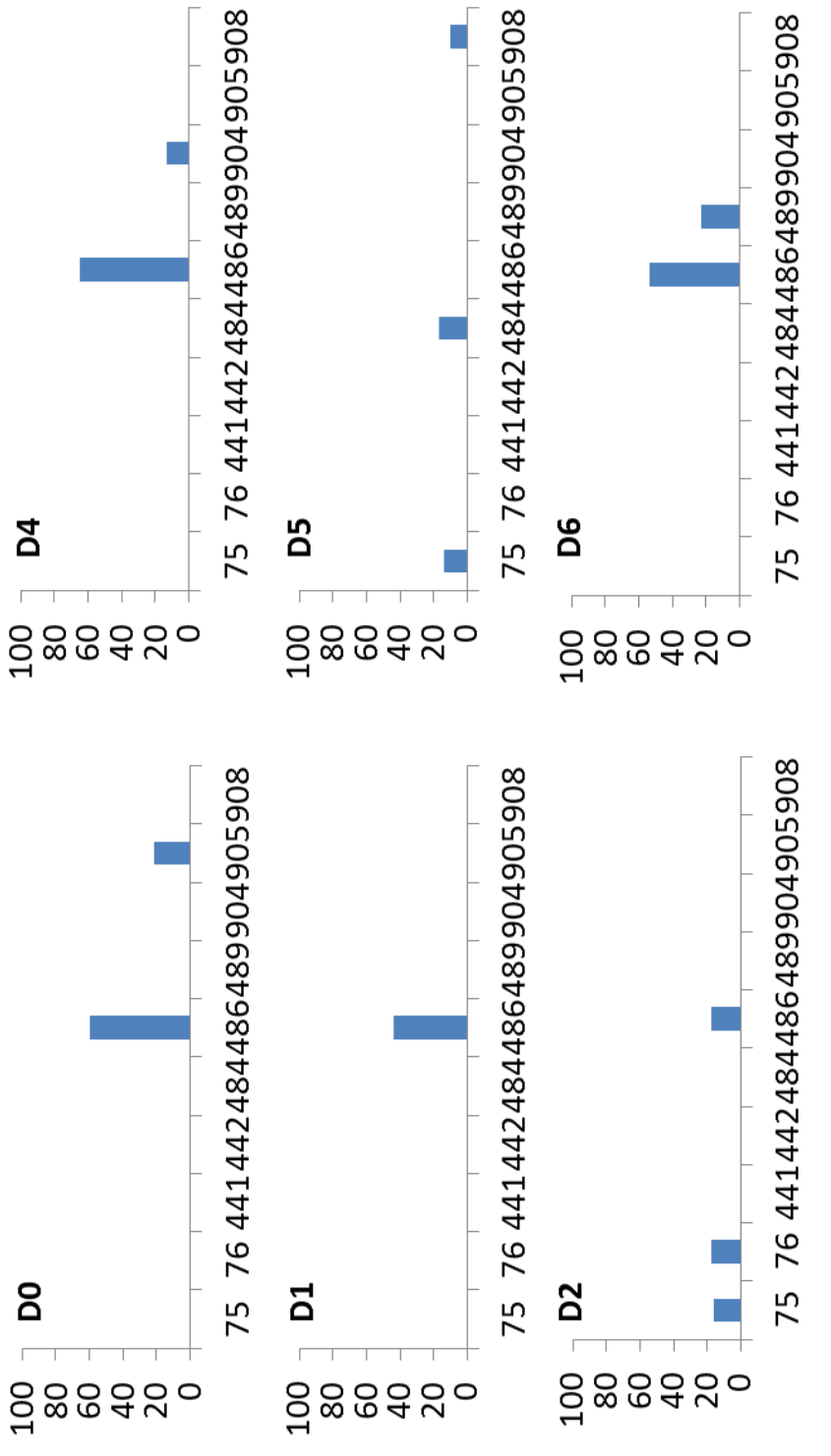

Figure 3 Predominant T-RFLP peaks generated by *Afa* I-digestion 16S rRNA gene which was amplified from eight fermented small fish with boiled rice in Myanmar. The vertical line shows relative abundance of T-RFs and the horizontal line shows fragment length.

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CHAPTER II

Production of an antibacterial substance by *Bacillus mojavensis* strain F412 isolated from a Myanmar shrimp product fermented with boiled rice

Introduction

The control of both the spoilage of food and growth of harmful food-borne bacteria is very important for maintaining food hygiene [1, 2]. Although a variety of antibacterial substances have already been found, those produced by fermentation bacteria in traditional food have been very attractive for food preservation. Because consumers generally regard their own health as important, the utilization of chemical additives has been increasingly discouraged [3]. In addition, microorganisms distributed in traditional fermented food are considered safe owing to the long history of consumption and familiarity of the people with such food [4].

Considering the above, there have been many studies on natural substances produced by bacteria present in food [1, 5, 6]. In Southeast Asian countries, there have also been several studies on antibacterial substances produced from fermented fishery products [7-12], because these traditional products are expected to be significant biological sources owing to their diversity [13, 14]. Among them, particularly in the case of various types of Thai fermented fishery product,

antilisterial lactic acid bacteria [7] and bacteriocin-producing bacteria in *pla-ra* [8-10], *pla-som* [11], and *kapi* [12] were already reported.

On the other hand, in Myanmar, there are various fermented fishery products, namely, fish fermented with rice, fish paste, and fish sauce, as well as in surrounding countries [13, 14]. To the best of my knowledge, to date, there has been no report on antibacterial-substance-producing bacteria isolated from Myanmar fermented fisheries products. Even in the case of agriculture fermented products, there is only one report on antibacterial activity in an alcohol starter culture [15]. Hence, Myanmar fermented fishery products are valuable as source materials for the screening of novel or beneficial microorganisms, and in this work, I performed a detailed study of antibacterial-substance-producing bacteria from a traditional Myanmar fermented food.

Materials and methods

Bacterial strain

Strain F412 was isolated from a Myanmar shrimp product fermented with boiled rice called *pazunchin-akaungchin*. This product is prepared using the

speckled prawn *Metapenaeus monoceros*, and was purchased from a grocery market in Yangon City, Myanmar, in 2011. For the isolation of antibacterial substances, the double-layer plate technique was used. Serially diluted fermented products were spread onto trypticase soy agar (TSA) plate medium (Becton, Dickinson and Company, Franklin Lakes, NJ, USA) containing 5% NaCl and then incubated anaerobically at 27°C using an AnaeroPack system (Mitsubishi Gas Chemical, Tokyo, Japan). After incubation for eleven days, the upper layer of tryptic soy broth (TSB) medium (Becton, Dickinson and Company) containing 0.8% agar (Ina Food Industry, Nagano, Japan) and *Listeria innocua* ATCC 33090^T cells, which were precultured in TSB medium at 27°C overnight, was laid over colonies on the TSA plate medium. After aerobic incubation for one day, the colony that inhibited the growth of *L. inocua* was isolated and named strain F412.

Bacterial identification

Basic phenotypic characterization of strain F412 was performed by conventional and routine methods using TSA plates or TSB medium at 27°C. The carbohydrate fermentation test was carried out using API CH strips, API CHB medium, and APIWEB identification software (Bio Merieux, Lyon, France) in

accordance with the manufacturer's instructions. For the sequencing of the 16S rRNA gene, extracted DNA samples were amplified using 5'-AGA GTT TGA TCC TGG CTC AG-3' (forward primer) and 5'-GGC TAC CTT GTT ACG ACT T-3' (reverse primer) [16]. In addition, for the amplification of *gyrA*, two primers, CAG TCA GGA AAT GCG TAC GTC CTT (forward primer) and CAA GGT AAT GCT CCA GGC ATT GCT (reverse primer), were used [17]. PCR products were prepared using a DNA thermal cycler (Gene Atlas 482; ASTEC, Fukuoka, Japan), purified using a commercial spin column (Amicon Ultra-100K centrifugal filters; Merck Millipore, Billerica, MA, USA), and then sequenced using an ABI 3730xl DNA analyzer (Applied Biosystems, Foster City, CA, USA).

Measurement of antibacterial activity

L. monocytogenes IID 577 (IID: Laboratory Culture Collection, Institute of Medical Science, University of Tokyo, Tokyo, Japan) was used as the indicator strain for the preparation steps and for the evaluation of the properties of the antibacterial substance as follows. Antibacterial activity was evaluated by the double-layer plate technique and agar well diffusion method following the modified techniques described in a previous study [18]. TSA plate medium and TSB

medium containing 0.8% agar were used for the lower and upper layers, respectively, in a plastic petri dish (90 mm diameter). Samples of 100 µl volume after twofold serial dilution were added to agar wells of 8 mm diameter and incubated aerobically at 27°C overnight. One arbitrary unit (AU) was defined as the reciprocal of the final dilution that could cause a growth inhibition zone.

Preparation of antibacterial substance

Strain F412 was cultured in 500 ml baffled flasks containing 100 ml of TSB medium with shaking (120 rpm). After incubation for 3 days at 27°C, the cells were centrifuged at 8,000 rpm (10,600 × *g*) and their supernatants were used for the purification of the antibacterial substance. The antibacterial substance from the isolate was precipitated with ammonium sulfate at 90% saturation, and the precipitate was dialyzed; then, the culture supernatant was subjected to two steps of column chromatography. Firstly, anion exchange chromatography was carried out using a DEAE sepharose fast-flow column (GE Healthcare UK, Buckinghamshire, UK, 1.6 × 38 cm), and samples were eluted using 0.05 M Tris-HCl buffer (pH 7.0) with a linear gradient of NaCl (0 to 1.0 M). After that, active fractions were pooled and precipitated by ultrafiltration, and then the samples were

subjected to Sephacryl S-300 gel filtration chromatography (GE Healthcare UK) (1.6 × 97 cm) with elution using 0.1 M NaCl-0.05 M Tris-HCl (pH 7.0). A gel filtration standard (Gel Filtration Calibration Kit; GE Healthcare UK) was used for column calibration. Active fractions eluted by gel filtration chromatography were pooled and stored at -20°C until use for the experiments for the characterization of the antibacterial substance.

Effect of enzymatic treatment on antibacterial substance

A solution of the antibacterial substance with a final activity of 100 AU ml^{-1} was prepared and used for the following experiment. To evaluate the effect of enzymatic treatment, seven commercially available enzymes were used, namely, protease (*Streptomyces griseus*; Wako, Tokyo, Japan), proteinase K (*Tritirachium album*; Merck, Darmstadt, Germany), trypsin (bovine pancreas; Wako), papain (*Carica papaya*; Wako), pepsin (porcine gastric mucosa; Wako), catalase (bovine liver; Sigma-Aldrich, St. Louis, Mo, USA), and lysozyme (chicken egg liver; Sigma-Aldrich). Each enzyme solution was added to the antibacterial substance solution at a final concentration of 1 mg ml^{-1}, and then allowed to react at 37°C for 2 h. Remaining activity was determined by the double-layer plate technique.

Effect of pH and heat treatments on antibacterial substance

The thermal stability of the antibacterial substance solution with a final activity of 100 AU ml^{-1} was tested at 60°C for 30 min, 100°C for 15 min, 100°C for 30 min, and 121°C for 15 min. pH stability was tested at pHs 3.0, 5.0, 7.0, 9.0, and 11.0 at 4°C overnight and then neutralized. pH was adjusted using small volumes of NaOH and HCl solutions. After these treatments, remaining activity was determined by the double-layer plate technique.

Spectrum of antibacterial substance

Eighteen strains shown in Table 1 were used as indicators for the determination of the activity spectrum of the antibacterial substance. Antibacterial activity was determined after 1 day of incubation at 27°C using the double-layer plate technique and the antibacterial substance solution with a final activity of 100 AU ml^{-1} as described above. In the case of *Vibrio alginolyticus*, alkaline peptone water (Nissui, Tokyo) containing 2% NaCl was used as the basal medium for the lower and upper layers.

Effect of antibacterial substance on growth of *L. monocytogenes*

L. monocytogenes IID 577 cells were precultured in TSB medium for 1 day at 27˚C. Then, 45 µl of the preculture was inoculated into 4.5 ml of TSB medium, and 500 µl of partially purified products (300 AU ml^{-1}) or 0.1 M NaCl-0.05 M Tris-HCl (pH 7.0) was immediately added. After incubation at 27˚C for 24, 48 and 72 h, viable cell count in the broth medium was determined by the spread plate method on Listeria Selective Agar (Oxford Formulation) (Oxoid Limited, Basingstoke, Hampshire, UK).

In addition, to confirm the mechanism underlying the effect of the antibacterial substance, 180 µl of broth culture, to which *L. monocytogenes* IID 577 cells were inoculated, was incubated in the wells of a 96-well plate. After 16 h, 20 µl of partially purified products (300 AU ml^{-1}) or 0.1 M NaCl-0.05 M Tris-HCl buffer (pH 7.0) was added. During the incubation at 27˚C for a total of 27 h, the optical density (O.D.) of the broth medium at 630 nm was determined using a plate reader (Model 550; Bio-Rad, Hercules, California, USA).

Electrophoresis

Partially purified products were subjected to sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS–PAGE) using two systems, namely, glycine-SDS-PAGE in 10% gel [19] and tricine-SDS-PAGE in 15% gel [20]. Rainbow molecular weight markers (GE Healthcare UK) were used as standards.

To evaluate the antibacterial activity after SDS-PAGE, the gel was cut into three portions. One was put on a double-layer TSA plate medium containing *L. monocytogenes* IID 577, followed by incubation at 27°C overnight to measure antibacterial activity. The remaining gels were subjected to Coomassie Brilliant Blue (CBB) staining (EzStain Aqua; ATTO, Tokyo, Japan) and Periodic Acid-Schiff (PAS) staining (Pierce Glycoprotein Staining Kit; Thermo Fisher Scientific Inc., Rockford, IL, USA).

Nucleotide sequence accession number

The 16S rRNA gene and *gyrA* sequences of strain F412 obtained in this study have been deposited in DDBJ under accession nos. LC020019 and LC020020, respectively.

Results

Taxonomic position of antibacterial-substance-producing bacteria

Strain F412 was a gram-positive, spore-forming, motile, facultative anaerobic, and rod-shaped bacterium. It was able to grow at 10 to 50°C, 0 to 15% NaCl, and pHs 5.0 to 9.0. On the basis of the API identification system, it was identified as *Bacillus subtilis* or *Bacillus amyloliquefaciens* with a high identity of 96%. In terms of the 16S rRNA gene sequence, it has a high similarity with members of the genus *Bacillus* such as *B. subtilis* NBRC 13719^{T} (AB271744), *B. amyloliquefaciens* NBRC 15535^{T} (AB325583), and *B. mojavensis* NBRC 15718^{T} (AB363735), with sequence similarities of 99.53% (1469/1476), 99.25% (1465/1476), and 99.73% (1472/1476), respectively. In addition, the similarities of the *gyrA* sequence of strain F412 to those of *B. mojavensis* strain B0031 (AY599914) and *B. mojavensis* ^{T} (EU138598) were 99.34% (904/910) and 96.92% (882/910), respectively. On the other hand, the gene sequence similarities to those of *B. subtilis*^{T} (EU138592) and *B. amyloliquefaciens* ^{T} (EU138596) were 82.86% (754/910) and 83.96% (764/910), respectively.

Behaviors of antibacterial substance on column chromatography

The antibacterial substance was purified from a culture supernatant using two steps of column chromatography. In the case of the initial DEAE Sepharose column chromatography, the sample with antibacterial activity was retained and eluted into one fraction. As shown in Figure 1, in the case of the secondary gel filtration chromatography using Sephacryl S-300, the sample with antibacterial activity was eluted into one fraction again and the peak activity corresponded to an absorbance of 280 nm. On the basis of the standard molecular weight marker, the sample with the highest antibacterial activity was estimated to have a molecular weight of approximately 97 kDa.

Polyacrylamide gel electrophoresis

To separate the antibacterial substance, polyacrylamide gel electrophoresis was performed using two types of electrophoretic system, namely, glycine-SDS-PAGE and tricine-SDS-PAGE. After electrophoresis, Tris-glycine polyacrylamide gels were incubated on the double-layer plate medium. After overnight incubation, a clear zone caused by the growth inhibition of *L. monocytogenes* was observed near the bottom edge of the gel side, and the molecular weight of the antibacterial

substance was estimated to be less than 24 kDa. On the other hand, in the case of tricine-polyacrylamide gels, the growth inhibition zone (halo) was observed on the double-layer plate medium, and the molecular weight of the antibacterial substance was estimated to be between 3.5 and 8.5 kDa, as shown in Figure 2. In addition, on the CBB-stained and PAS-stained gels, blue and blue-purple bands, respectively, were observed at the position corresponding to the growth inhibition zone.

Characteristics of antibacterial substance

The effects of the representative enzymes on the activity of the antibacterial substance are shown in Figure 3. The antibacterial activity was not affected by treatments with most catabolic enzymes. It was decreased partially by lysozyme treatment only. The inhibition spectra of the substance against various bacterial strains are shown in Table 1. This substance showed activity against gram-positive bacteria except strain F412, which is the strain producing the substance. In contrast, the growth of gram-negative bacteria was not inhibited. The effects of heat and pH treatments on the antibacterial activity of the substance are shown in Table 2. The remaining antibacterial activity gradually decreased with increasing

treatment temperature but partially remained even after heat treatment for 15 min at 121°C.

Effect of antibacterial substance on growth of *L. monocytogenes*

Figure 4 shows the effect of the antibacterial substance on the viable cell count of *L. monocytogenes* in the broth medium. Before the addition of the antibacterial substance, the viable cell count of *L. monocytogenes* was 1.1×10^7 cfu ml^{-1}. After the addition of the antibacterial substance, a significant difference in the growth of *L. monocytogenes* was observed between the broth culture containing the antibacterial substance and the reference broth culture. In the case of the broth culture containing the antibacterial substance, the viable cell counts of *L. monocytogenes* were 1.5×10^7, 9.4×10^6, and 1.7×10^6 cfu ml^{-1}, whereas in the case of the reference broth, the viable cell counts were 9.6×10^8, and 1.9×10^8, and 1.4×10^8 cfu ml^{-1} after 24, 48, and 72 h of incubation, respectively.

Figure 5 shows the effect of the antibacterial substance on the O.D. of *L. monocytogenes* in broth culture. After the addition of the antibacterial substance, the O.D. of the broth culture of *L. monocytogenes* decreased gradually and finally

reached the initial value. In contract, in the case of the addition of Tris-HCl buffer instead of the antibacterial substance, the O.D. was 0.29 after 27 h of incubation.

Discussion

Strain F412 was identified as a member of the genus *Bacillus* owing to their high similarity in the 16S rRNA gene sequence. Although 16S rRNA gene sequencing is a useful identification tool for bacterial classification, in this study, I was not able to distinguish the taxonomic position of this isolate and closely related species on the basis of analytical results. Therefore, to confirm the taxonomic position of strain F412 in detail, I performed *gyrA* sequencing analysis, the result of which indicated that strain F412 can be identified as *B. mojavensis* owing to their high similarity. This identification result also corresponded to the phenotypic characterization of strain F412, because it has phenotypic characteristics common to the members of the genus *Bacillus*, such as being a spore-forming, gram-positive, catalase-positive, and aerobic rod-shaped bacterium.

Although there are many types of beneficial bacterium used in traditional fermentation technology, some *Bacillus* species have been known as highly

important as, for example, producers of enzymes [21], vitamin B_{12} [22], and peptides such as the so-called bacteriocins [23]. In addition, *B. mojavensis* was initially reported as isolates from desert soil. However, recent studies have demonstrated the relationship between *B. mojavensis* and foodborne illnesses, and stable toxigenic factors of some isolates, such as the heat-stable toxin amylosin, have been reported [24, 25]. However, as mentioned earlier, strain F412 was isolated from a traditionally fermented shrimp product, which has a long history of domestic consumption. Although strain F412 can be considered as safe for human health at present, if this antibacterial substance will be used in the future as food preservation on the industrial scale, further studies are also necessary to evaluate harmful factors, such as poisoning toxins, surfactant activities, and enterotoxic activities according to established criteria such as those of the European Food Safety Authority [23, 26].

Although the substance from strain F412 showed activity against gram-positive bacteria, including food-borne bacteria such as *L. monocytogenes* and *Staphylococcus aureus*, the growth of strain F412 was not inhibited. This finding suggests that strain F412 might have immunity to the self-produced antibacterial substance. In addition, the growth of *L. monocytogenes*, which is responsible for

listeriosis, could be markedly inhibited in broth culture for 3 days. Therefore, this finding suggests that this antibacterial substance might be an attractive natural food additive for the control of food-borne bacteria. Additionally, in this study, a decrease in the O.D. of the bacterial culture was observed after the addition of this substance. Therefore, this observation suggests that this substance might have bacteriolytic activity against *L. monocytogenes*.

The antibacterial activity of this substance differed in the gel filtration and electrophoresis analyses. This finding suggests that this antibacterial substance has a unique behavior, because the results of these two analyses indicated different molecular weights. At present, the reason for this is unknown; however, it can be speculated that it is due to the aggregation between the antibacterial substance and certain additional substances by intermolecular electrostatic interaction. Consequently, because of the denaturing effect of SDS, the antibacterial substance might be separated into two moieties in SDS–PAGE. In this study, the antibacterial activity of this substance was almost unaffected after treatments with several catabolic enzymes, which might not readily react with the antibacterial substance under the aggregation condition.

Moreover, electrophoresis analysis results suggest that the antibacterial substance from strain F412 might be a glycopeptide with an estimated molecular weight between 3.5 and 8.5 kDa because the bacterial growth inhibition zone on the electrophoresis gel could be stained with CBB and PAS reagents. *Bacillus* species as well as lactic acid bacteria have been known as bacteriocin producers, and the glycoprotein related to bacteriocin, thermocin 10, which is produced by *B. stearothermophilus* NU-10, was already reported as a bacteriocin that is stable against high temperature and proteolytic enzymes [27]. In addition, recently, several studies on the glycosylation of bacteriocins, which are produced by gram-positive bacteria including *Bacillus* species, have been carried out, and the putative structures of glycopeptide bacteriocins have been clarified. Sublancin, which is produced by *B. subtilis* strain 168 [28], has an S-linked glycopeptide containing a glucose linked to the Cys residue [29]. In addition, glycocin F, which is a bacteriocin produced by the lactic acid bacterium *Lactobacillus plantarum* strain KW30 [30], has a *β*-O-linkage between an N-acetylglucosamine and the Ser residue, and an S-linkage between an N-acetylhexosamine and the C-terminal Cys residue [31]. In this study, strain F412 was identified as *B. mojavensis,* and this taxonomic position is very close to that of *B. subtilis* owing to their highly

similar 16S rRNA gene sequences. Therefore, glycopeptide bacteriocins of strain F412 should be focused on as a subject of considerable interest in future studies.

In this study, I was unable to evaluate completely the characteristics of the antibacterial substance produced by strain F412. I already tried N-amino acid sequencing analysis of this substance; however, I was unable to transfer electrophoretically the representative bands obtained by SDS-PAGE onto a PVDF membrane for amino acid sequencing analysis. In addition, it is also necessary to obtain other lines of evidence aside from PAS staining results to confirm that this substance is a glycopeptide; therefore, the detailed characteristics of this substance are now being determined. However, as mentioned earlier, although many studies on antibacterial substances produced by fermentation bacteria distributed in traditional fishery food products in Southeast Asia have already been carried out, no information on the varieties of traditional Myanmar fermented fishery products from the microbiological viewpoint is available. Therefore, to the best of my knowledge, this is the first detailed study showing an antibacterial substance produced by a bacterial isolate from Myanmar fermented fishery products.

Table 1 Activity of partially purified antibacterial substance from strain F412 against various bacterial strains

Indicator strains		Diameter of inhibition zone (mm)	S.D
Bacillus subtilis	IAM 12118^T	2.53	0.56
Bacillus licheniformis	IAM 13417	2.50	0.18
Brevibacterium halotolerans	JCM 12400	3.44	0.21
Enterococcus faecalis	JCM 5803^T	8.97	0.08
Lactobacillus paraplantrum	JCM 12533	8.65	0.33
Lactobacillus pentosus	JCM 1558^T	6.47	0.37
Listeria innocua	ATCC 33090^T	7.79	0.15
Listeria monocytogenes	IID 577	10.23	0.93
Listeria monocytogenes	IID 578	10.40	0.23
Listeria monocytogenes	IID 580	11.30	0.29
Staphylococcus aureus	IAM 12082	10.37	0.06
Aeromonas hydrophila	IAM 12337	-	-
Enterobacter cloacae	IAM 12349^T	-	-
Escherichia coli	JCM 1649^T	-	-
Pseudomonas aeruginosa	IAM 1514^T	-	-
Serratia marcescens	IAM 12142^T	-	-
Vibrio alginolyticus	NBRC 15630^T	-	-
F412		-	-

Data are expressed as mean ± standard deviation (SD) from three independent experiments. Diameter of inhibition zone excluding diameter of well "-" means no inhibition of the growth of indicator strains. Reference strains were obtained from five institutes as follows: JCM (Japan Collections of Microorganisms), NBRC (NITE Biological Resource Center), ATCC (American Type Culture Collection), IID (Laboratory Culture Collection, Institute of Medical Science, University of Tokyo, Tokyo, Japan), IAM Culture Collections (Institute of Applied Microbiology, Culture Collection, The University of Tokyo, Tokyo, Japan. (Collection transferred to JCM). Diameter of inhibition zone was measured excluding diameter of well.

Table 2 Effects of heat and pH treatments on partially purified antibacterial substance from strain F412

Condition	Residual relative specific activity
Heat;	
No heating	100
60°C, 30 min	103
100°C, 15 min	67
100°C, 30 min	61
121°C, 15 min	44
pH;	
3.0	84
5.0	98
7.0 (No adjustment)	100
9.0	93
11.0	69

Data are expressed as mean from three independent experiments. Relative specific activity is the ratio of the activity of the antibacterial substance to that with no treatment (× 100).

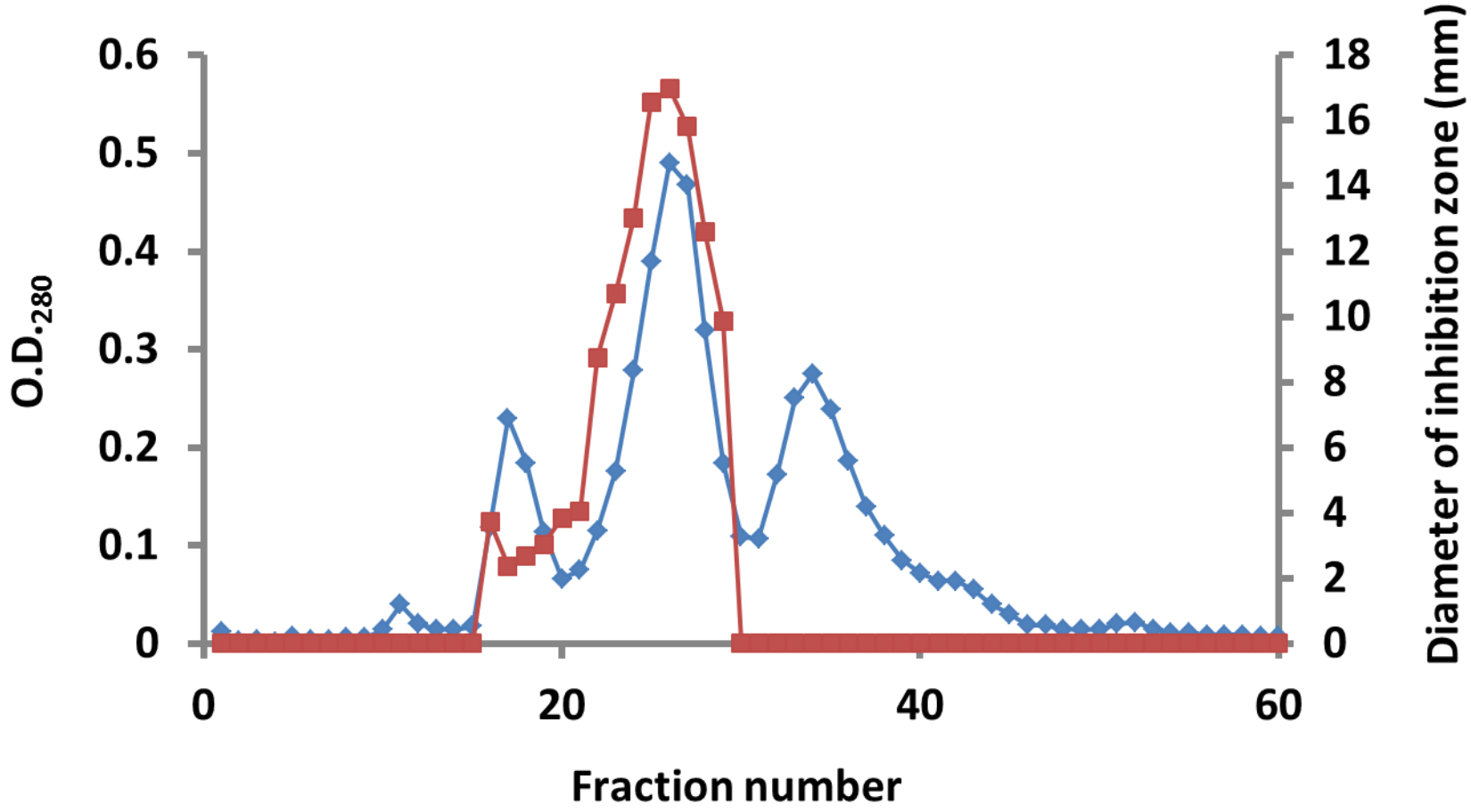


Figure 1 Gel filtration chromatography of active fraction from DEAE sepharose fast-flow column. Flow rate, 0.5 ml/min; fraction time, 10 min/fr; sample volume, 7 ml; absorbance of each fraction at 280 nm (◆), diameter of inhibition zone of each fraction against *L. monocytogenes*, excluding diameter of well (■). The diameter of inhibition zone was measured excluding diameter of well.

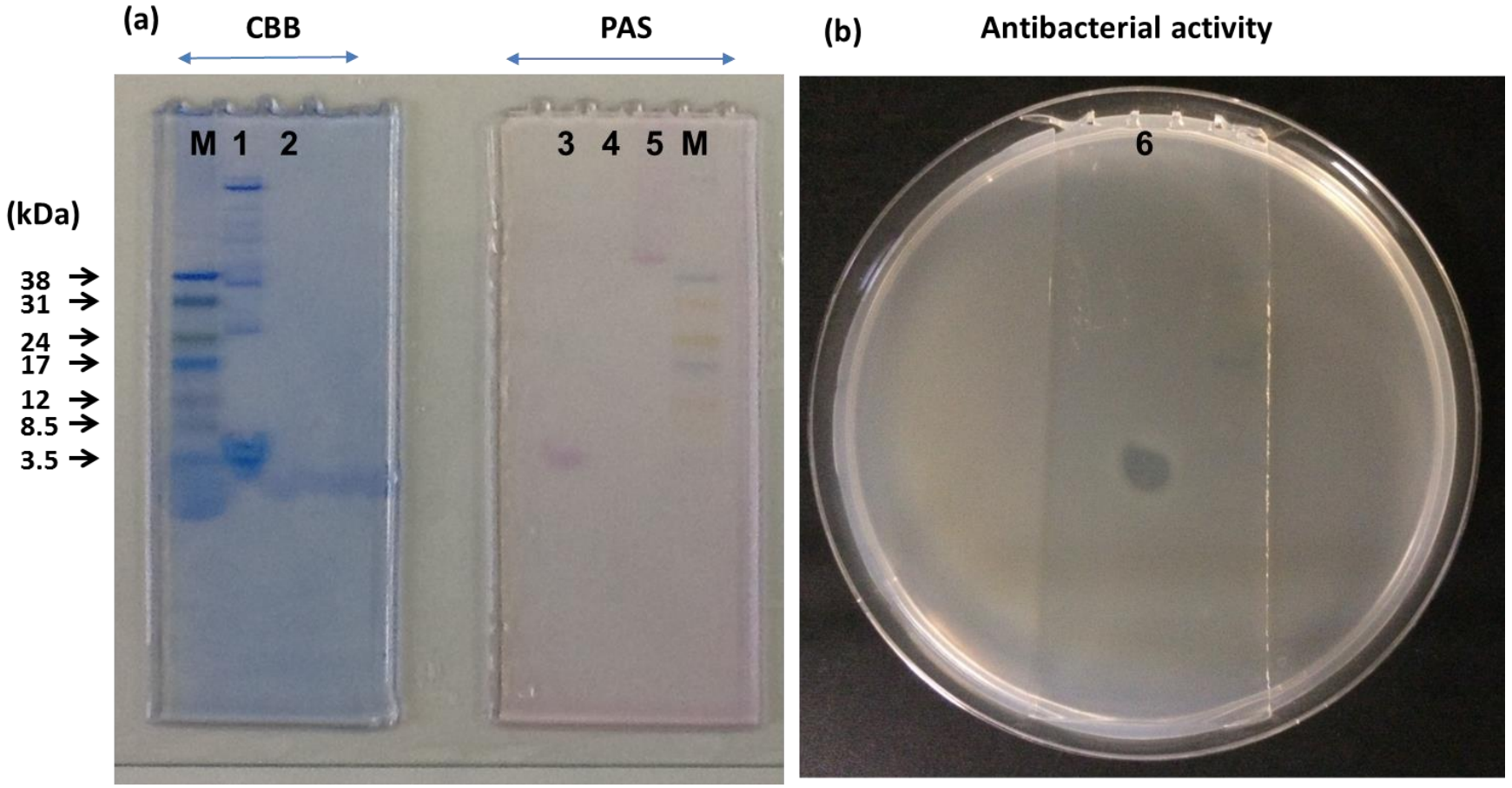
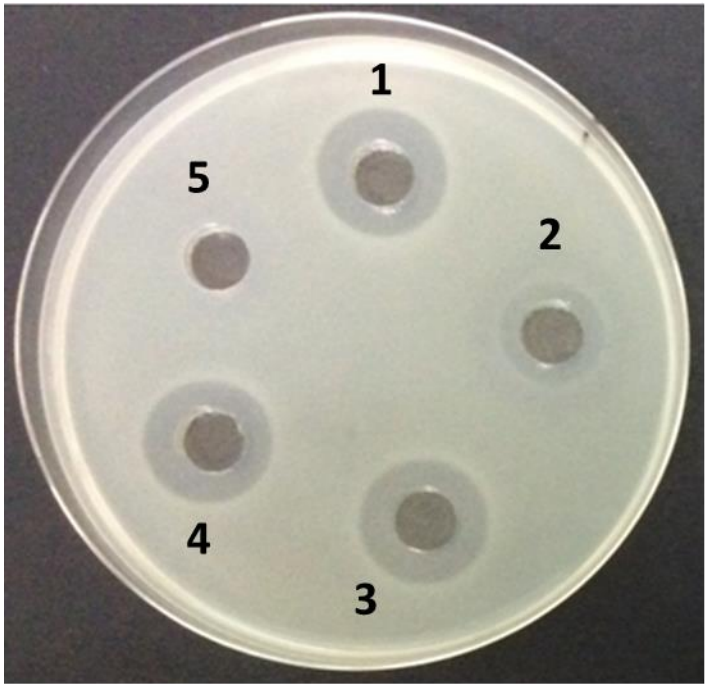
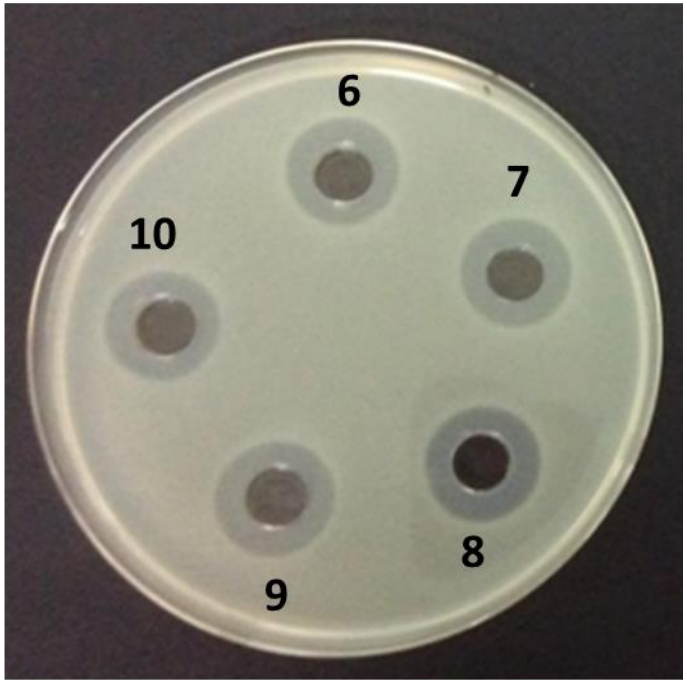


Figure 2 Tricine SDS-PAGE photograph of partially purified antibacterial substance from strain F412. (a) Electrophoresis was carried out on 15% Tricine SDS-PAGE gel under nonreducing conditions. They were stained with CBB or PAS. (b) Electrophoresis was carried out on 15% Tricine SDS-PAGE and then the gel was set on the plate containing *L. monocytogenes*. The inhibition zone was observed after an overnight incubation at 27˚C.
M: Molecular weight marker. Lanes 1, 3, 6: Partially purified antibacterial substance. Lanes 2, 4: Negative control (distilled water). Lane 5: Positive control (horseradish peroxidase).





1: No treatment
2: Lysozyme
3: Catalase
4: Proteinase K
5: Water (Negative control)

6: No treatment
7: Papain
8: Pepsin
9: Protease
10: Trypsin

Figure 3 Effect of enzymatic treatments on the partially purified antibacterial substance from strain F412.

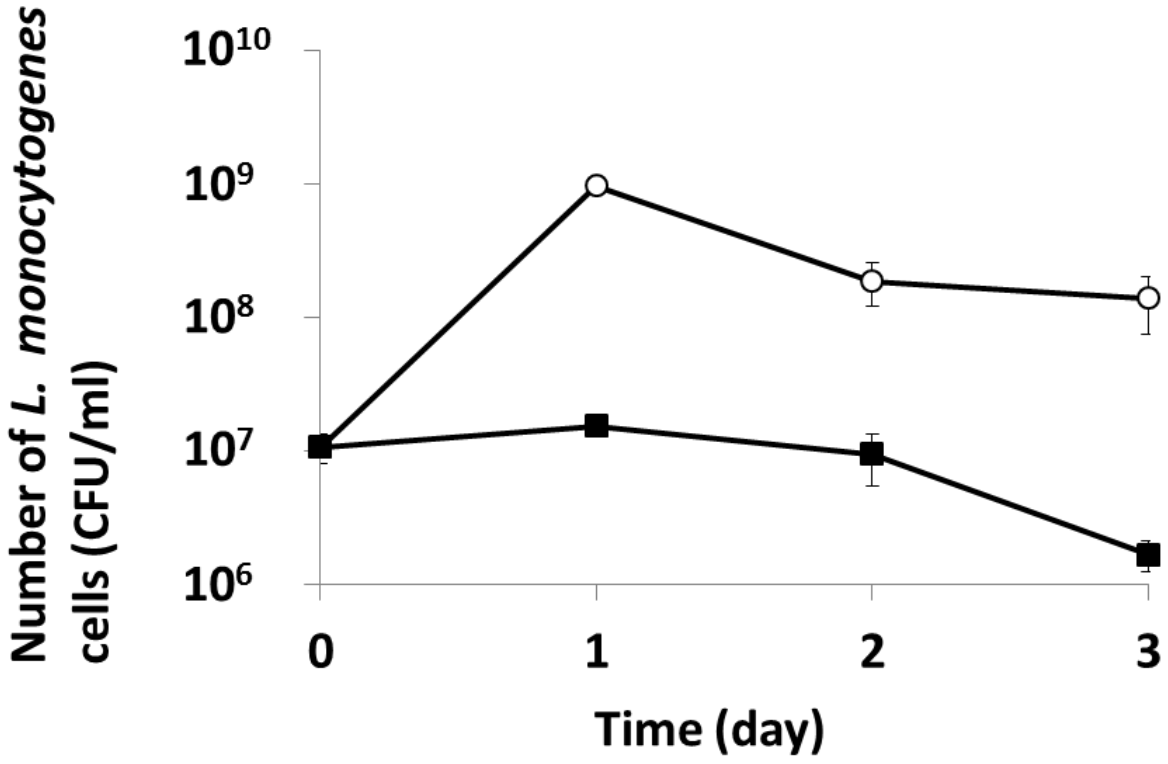


Figure 4 Effect of partially purified antibacterial substance from strain F412 on viable cell count of *L. monocytogenes.* Data are expressed as mean ± standard deviation (SD) from three independent experiments. Tris buffer added (□), antibacterial substance added (■).

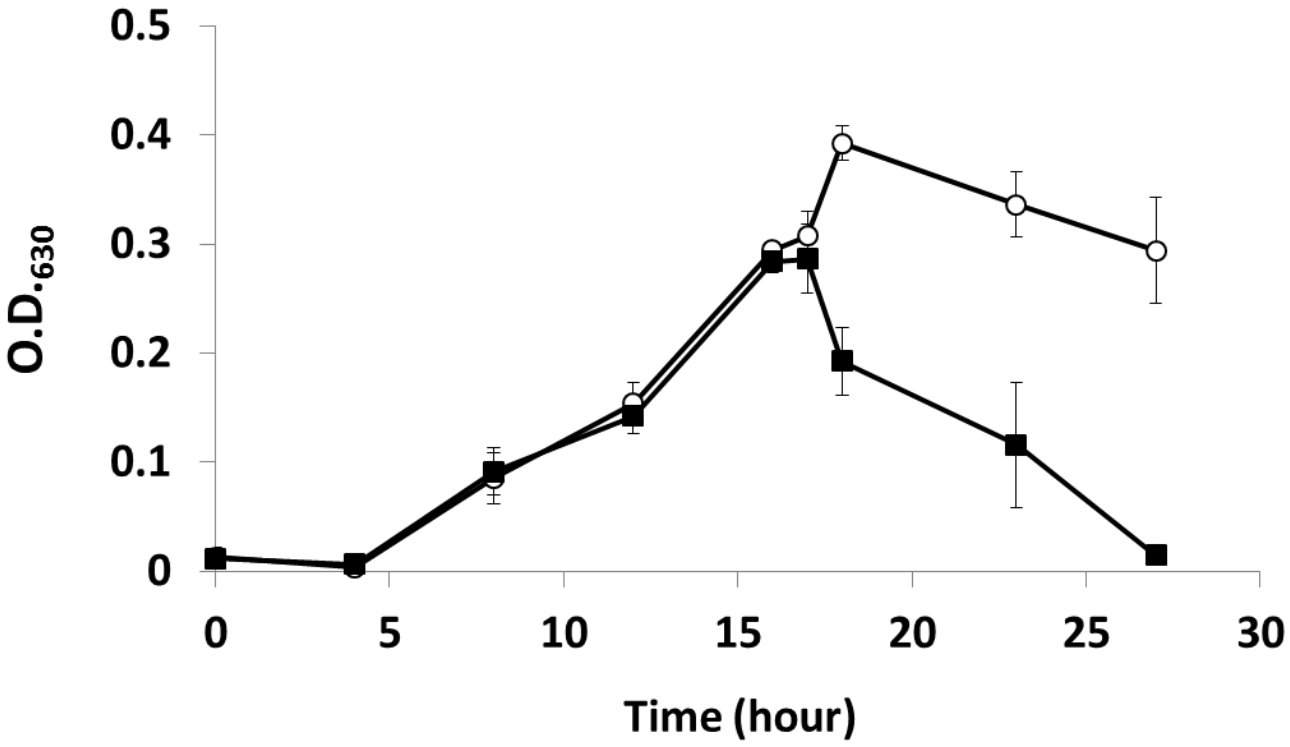


Figure 5 Effect of partially purified antibacterial substance from strain F412 on O.D. of *L. monocytogenes* broth culture. Data are expressed as mean ± standard deviation (SD) from five independent experiments. Tris buffer added at 16 h (□), antibacterial substance added at 16 h (■).

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CHAPTER III

Isolation and characterization of malachite green-removing yeast from a traditional fermented fishery product

Introduction

Synthetic dyes have been widely used in various industries owing to their usability, and simultaneously, the biodegradation of these dyes using microorganisms has also been focused on to deal with environmental pollutants [1]. Among them, malachite green (MG), which is a well-known green aromatic compound, has been known as not only a usable triphenylmethane dye for color dyeing but also an effective drug owing to its antifungal and antiparasitic abilities for the treatment of fish diseases, namely, white spot disease and saprolegniasis in aquaculture. However, recently, attention has been focused on the risks of residual MG and leucomalachite green (LMG), the reduced form of MG, in fish farmed for food purposes, because several reports concerning their toxic effects as well as carcinogenic effects have been published [2, 3]. Therefore, in many countries, the administration of MG to farmed fish for food purposes has already been prohibited, and MG- and LMG-containing fishery products have also been strictly regulated. In Japan, these compounds have also been strictly regulated since 2005 in accordance with the Pharmaceutical Affairs Law [4].

On the basis of the background mentioned above, numerous studies concerning MG-degrading microorganisms have been reported. For example, the decolorization ability under various experimental conditions [5-7] and the presumed degradation pathway involving complicated and various degradation steps and various intermediates, such as aromatic compounds that have one to three benzene rings, have already been determined by liquid chromatography-mass spectrometry (LC/MS) and gas chromatography-mass spectrometry (GC/MS) [5, 6, 8]. In addition, the taxonomic positions of these isolates are widely distributed in procaryotes and eucaryotes, and recently, *Micrococcus* sp. [8], *Deinococcus radiodurans* [7], *Pseudomonas* sp. [5], *Pseudomonas aeruginosa* [6], *Penicillium ochrochloron* [9], *Penicillium pinophilum* and *Myrothecium roridum* [10], and *Kocuria rosea* [11] have been demonstrated.

To study the degradation and decolorization of MG by microorganisms in a culture medium, several analytical methods, such as photospectrometry [5, 6, 8, 11], thin-layer chromatography (TLC) [6, 11], LC/MS [5, 8], liquid chromatography-tandem mass spectrometry (LC/MS/MS) [10], GC/MS [5-8], Fourier transform infrared spectroscopy (FTIR) [6, 8, 11], and nuclear magnetic resonance spectroscopy (NMR) [12, 13] have been used. Among these methods, LC/MS/MS

is considered to be the most sensitive and accurate method for quantifying MG and LMG. However, in the routine analysis of residual MG and LMG in fishery products, the chromatographic conditions have to be optimized for these two molecules, because the molecules that cannot be retained in the column and cannot elute from the column under optimized conditions might be excluded. On the other hand, NMR, which is a method for elucidating the carbon-proton framework of a molecule [14] to determine the structure of organic compounds [15], has been applied to the analysis of metabolites in a biological sample [16-18]. In the metabolite analysis of a complex biological sample, ^{1}H-NMR is a universal and quantitative analytical technique in which we can observe and quantify all the protons (hydrogen atoms) belonging to all the metabolites in a sample tube at once [19]. However, previous studies on the microbiological decomposition of MG using ^{1}H-NMR were quite limited. To the best of my knowledge, confirmation of the degradation of MG in the output of a bioreactor using immobilized cells [13] and the finding of didesmethyl-MG from a culture of the filamentous fungus *Acremonium kiliense* [12] are the only two examples in which MG biodegradation was analyzed by NMR. On the basis of these social backgrounds, in this study, I screened MG-degrading microorganisms to develop new bioresources for

bioremediation. In addition, the MG-degrading ability of the isolate was determined by ^{1}H-NMR to detect all expected and unexpected degradation products, and by LC-MS/MS to detect the expected LMG with high sensitivity, respectively**.**

Materials and methods

Sample

Sediment samples were collected from different tilapia (*Oreochromis niloticus*) ponds (pond-1 and pond-2) and their irrigation channels in Yangon, Myanmar in 2012. In addition, sediment and water samples were also collected from different tilapia ponds (pond-A and pond-B) and their irrigation channels in Suphanburi, Thailand in 2013. Fermented small fish (tinfoil barb *Puntius schwanenfeldii*) with boiled rice was purchased from a grocery market in Yangon, Myanmar in 2013.

Chemicals

Three types of triphenylmethane compound were used in this study. MG oxalate (MG oxalate) was purchased from Wako Pure Chemical Industries, Ltd.

(Tokyo, Japan). LMG and malachite green carbinol base (MGC) were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Screening of MG-degrading microorganisms

For the isolation of MG-degrading microorganisms, an enrichment culture method was carried out. 1/10-MB [1/10 strength of Marine Broth 2216 (Becton, Dickinson and Company (BD), Franklin Lakes, NJ, USA)] containing MG at a final concentration of 100 mg of MG oxalate per l was used for the isolation. Five grams of each collected sample (5ml: in the case of a water sample) was inoculated into 100ml of 1/10-MB containing MG under aseptic conditions, followed by the enrichment culture experiment performed at 27°C under static conditions. In the case of screening from samples in Myanmar, enrichment culture under shaking conditions (120 rpm) was also performed. After several enrichment cultures, the final culture products were streaked and incubated on 1/10-MA [1/10 strength of Marine Agar 2216 plate medium (BD)] containing MG at a final concentration of 100 mg of MG oxalate per l. After an appropriate incubation period at 27°C, colonies that formed a white zone were isolated and considered as MG-degrading candidates.

Identification of MG-degrading candidate strains

To evaluate the taxonomic positions of 14 bacterial strains, 16S rRNA gene sequencings were performed as described previously [20]. In addition, for the identification of a yeast strain designated M3, the sequencing of the D1/D2 domains of the 26S rRNA and 18S rRNA genes was performed. The domains were amplified using the primer sets [26S-NL-1 (5′- GCA TAT CAA TAA GCG GAG GAA AAG -3′) and 26S-NL-4 (5′- GGT CCG TGT TTC AGA CGG -3′)] [21] and [18S-NS1 (5′- CCA GTA GTC ATA TGC TTG TC -3′) and 18S-R-EF3 (5′- TCC TCT AAA TGA CCA AGT TTG -3′)] [22], respectively. The amplified PCR products were purified using a commercial spin column and directly sequenced using the BigDye Terminator v3.1 Cycle Sequencing kit and an ABI 3730xl capillary DNA sequencer (Applied Biosystems, Foster City, CA, USA) according to manufacturer's instructions.

Phenotypic characterization of strain M3

Microscopic observation of the cells was carried out using a light microscope (BX51; Olympus, Tokyo, Japan). Growth performance under various conditions,

such as NaCl concentration, pH, and temperature, was determined in YM broth medium (BD).

Cultivation of strain M3 in MG-containing media

Strain M3 was precultured on 1/10-MA slant media at 27 °C for three to four days. These colonies on the media were suspended in 1 ml of 0.85% saline (NaCl) solution. After that, 0.1 ml of this suspension was inoculated into 20 ml of 1/10-MB containing MG.

Sample preparation for LC-MS/MS and NMR analyses of MG-containing media

To evaluate the decomposition and decolorization of MG, samples were taken at weekly intervals from the broth containing MG. Before analysis, the samples were mixed well with an equal volume of acetonitrile (Wako) and then filtered directly through a 0.22-μm-pore-size mixed cellulose ester filter (ADVANTEC, Tokyo, Japan).

Viable cell count and UV-visible analysis of MG-containing media

The number of viable cells was enumerated by plate count techniques using potato dextrose agar (PDA) plate medium (Merck, Darmstadt, Germany). The MG decolorization was measured using a UV-VIS spectrophotometer (UV-2400PC; Shimadzu, Kyoto, Japan) at 618 nm.

LC/MS/MS analysis of MG-containing media

To quantify the MG and LMG concentrations of the broth media, mass spectrometry (LC/MS/MS) analysis was performed. An LC/MS/MS system (API4000, AB SCIEX, MA, USA) equipped with an ODS column (Inertsil ODS-SP 2.1x100 mm, GL Science, Tokyo, Japan) was used for the analysis, and samples were eluted with a linear gradient of 10 to 90% acetonitrile. Samples were injected directly after adequate dilutions using acetonitrile. Compounds containing the stable isotopes MG-d5 and LMG-d6 (Hayashi Pure Chemical, Osaka, Japan) were used as internal standards. The precursor ions of MG and LMG, m/z 329.2 and m/z 331.2, respectively, were selected for fragmentation. The fragment ions m/z 313.2 and m/z 208.1 were used for quantification, respectively. The other

fragment ions, m/z 239.1 for MG and m/z 223.1 for LMG, were used to confirm the identity of each peak corresponding to each compound.

Biosorption of MG to cells of strain M3

Strain M3 was cultured in 200 ml of 1/10-MB at 27 ^{o}C for 4 days. The cells were harvested from a total of 1 l of broth medium by centrifugation at 8,000 rpm (10,600 x *g*). They were washed twice using sterilized physiological saline solution and divided into two portions. They were suspended in the sterilized physiological saline solution, and then one portion was sterilized by heat treatment at 121 °C for 15 min. 1 ml of the sterilized or nonsterilized suspension, which contained approximately 0.05 g of wet cells, and 1 ml of 1/5-MB containing MG oxalate were mixed well at a final concentration of 100 mg of MG oxalate per l. They were incubated at 27 °C for 24 h with shaking at 120 rpm. During incubation, decolorization was observed with the naked eye.

^{1}H-NMR analysis of broth culture of strain M3

Each extracted sample was lyophilized and solubilized in 500 µl of methanol-d4 (Acros Organics, Geel, Belgium) for ^{1}H-NMR spectroscopy. 0.24 µmol of 4,4-

dimethyl-4-silapentane-1-sulfonic acid-d6 (DSS, Wako) was added to each ^{1}H-NMR sample as reference for zero ppm concentration. One-dimensional (1D) ^{1}H-NMR spectra using the zg30 pulse program were obtained for these samples using a Bruker Avance III 600 MHz instrument equipped with a SampleCase™ auto sample changer (Bruker Biospin, MS, USA). A total of 1024 scans were accumulated for each sample. The order of the samples was randomized.

Result

Isolation of MG-degrading candidate bacteria

A total of 14 strains that showed decolorization of green color around colonies on the plate medium containing MG were isolated as candidate MG-degrading strains. As shown in Table 1, six strains, namely, 13CS-B, 13CS-D1, 13CS-D2, 13CS-E, 14C-2, and 15C-1, were isolated from three different sediment samples obtained from a fish farm and its related channel in Myanmar. In addition, strain M3 was isolated from a traditional fermented food, namely, small fish with boiled rice called *ngachin*. Moreover, four strains (T8-2, T8-3, T11, and T12-1) and three

strains (T9-1, T9-2, and T10-2) were isolated from the sediment and water samples, respectively, which were obtained from a fish farm in Thailand.

Taxonomical identification of isolates from sediment and water samples

From the results of the 16S rRNA gene sequencing, the isolates from two sampling sites were divided into five taxonomical groups. As shown in Table 1, the sequences of five strains (13CS-D1, 13CS-D2, 13CS-E, T9-1, and T11) were similar to each other, and highly similar to that of *Enterobacter cloacae* subsp. *cloacae* DSM 30054^T (HE978272) (between 99.00 and 99.73% similarities). Strains 13CS-B and T-8-2 showed high similarities (99.32% and 99.25%, respectively) to *Pseudomonas monteili* CIP 104883 (AB021409). Strains T-9-2 and T-10-2 showed a high similarity (99.53%) to *Stenotrophomonas maltophilia* IAM 12423 (AB294553). In the case of the remaining 4 strains, strains 14C-2, 15C-1, T-8-3, and T-12-1 showed high sequence similarities of 98.51%, 99.49%, 97.86%, and 99.26% to *Inquilinus limosus* strain AU476^T (AY043374), *Rhizobium multihospitium* CCBAU83401^T (EF035074), *Enterobacter oryzendophyticus* strain REICA 142^T, and *Pseudomonas nitroreducens* IAM 1439^T (AM088473), respectively.

Taxonomical identification of strain M3 from a fermented fishery product

The D1/D2 domain of the 26S rRNA sequence of strain M3 matched that of *D. nepalensis* JCM2095^T (JN940507) with 100% similarity (612/612). In addition, strain M3 had round cells and showed budding under light microscopy observation. Moreover, it could grow on the plate medium only aerobically, and at 10 to 37°C, 0 to 20% (w/v) NaCl, and pH 3.0 to 10.0.

Growth and decolorization of MG during incubation of strain M3

In this study, strain M3 was used for further experimentation to evaluate MG behavior during incubation, because isolates from a source which have eating experiences were expected to be advantageous to the actual application of bioremediation. Figure 1 shows changes in the color of the broth medium within 8 weeks of incubation of strain M3. Marked decolorization was observed after 2 weeks of incubation, and the initial absorbance (618 nm) of 39.9 decreased to 0.05 after 8 weeks of incubation.

Figures 2 and 3 show changes in the number of viable cells of strain M3 and pH of the broth culture, respectively. The initial viable cell count was 7.3 x 10^4 /ml,

which then increased to 9.0 x 10^5 / ml after 3 weeks of incubation. Additionally, the pH of the broth medium gradually increased from 5.5 to 6.5.

MG and LMG analyses by LC/MS/MS during incubation of strain M3

Figure 4 shows changes in the concentrations of MG and LMG in the broth culture. Initially, the concentrations of MG and LMG were 85.3 mg/l and below 0.4 mg/l, respectively. During the incubation of strain M3, the MG concentration gradually decreased and reached 8.7 and <0.4 mg/l after 4 and 8 weeks, respectively. Conversely, the LMG concentration gradually increased and reached 29.6 and 33.6 mg/l after 4 and 8 weeks of incubation, respectively.

^{1}H-NMR spectra of MG, MGC and LMG

Figure 5 shows the ^{1}H-NMR spectra of MG, MGC, and LMG directly solubilized in methanol-d_4, and the spectra of these compounds extracted from the broth medium for the cultivation of strain M3. MG which was directly solubilized in methanol-d_4 showed aromatic signals, three doublet signals at 7.05 (4H), 7.37 (2H), and 7.44 ppm (4H), and two triplet signals at 7.74 (1H) and 7.60 (2H) ppm. LMG which was directly solubilized in methanol-d_4 showed three doublet signals at

6.70 (4H), 6.91 (4H), and 7.06 (2H) ppm and two triplet signals at 7.13 (1H) and 7.22 (2H) ppm. MGC which was directly solubilized in methanol-d_4 showed two doublet signals at 6.70 (4H) and 7.04 (4H) ppm, and overlapped signals (5H) at 7.19–7.27 ppm. These set of signals are necessary and sufficient to explain the structure of each compound. However, the signal pattern of MG changed when MG was added to the broth medium and extracted by adding acetonitrile. Doublet signals at 6.70, 7.18, and 7.39 ppm, a triplet signal at 7.23 ppm, a quartet signal at 7.13 ppm, and a multiplet signal at 7.32 ppm were observed in the extracted MG sample as additions to the set of signals of intact MG. This spectrum was identical to that of MGC that was extracted from the medium. On the other hand, the LMG extracted from the broth showed the same set of signals as the LMG that was directly solubilized in methanol-d_4. To quantify MG and LMG in the culture broth media of strain M3, integrated values of signals specific for MG (a triplet at 7.74 ppm) and LMG (a doublet at 6.91 ppm) were used in subsequent experiments. Other signals of LMG overlap between MG and LMG.

Changes in ^{1}H-NMR spectrum of the extract during incubation of strain M3

Figure 6 shows the ^{1}H-NMR spectra of the MG-containing medium during the incubation of strain M3. It can be clearly observed that the triplet at 7.74 ppm of MG decreased as the doublet at 6.91 ppm of LMG increased; in addition, the triplet at 7.74 ppm of MG was not detected after 8 weeks of incubation. The concentrations of LMG and MG in the broth medium were calculated by comparing integration of the signals of each compound to that of the signals of standard compounds with known concentration. The concentration of LMG reached 25.1 mg/l, and MG was not detected after 8 weeks of incubation.

Biosorption of MG to cells of strain M3

No decolorization of MG was observed in the mixture of the MG solution and stabilized cells. On the other hand, decolorization was markedly observed in the mixture of the MG solution and nonstabilized cells by the naked eye, and these mixtures became colorless within 24 h.

Discussion

In this study, I isolated thirteen MG-degrading candidate strains from all the ponds and canals in selected fish farms. Therefore, this result indicated that MG-degrading candidates are widely distributed in fish farms. In addition, it was considered that most strains, namely, 13CS-B, 13CS-D1, 13CS-D2, 13CS-E, 14C-2, T-8-2, T-9-1, T-9-2, T-10-2, and T-11 were not suitable for industrial applications owing to the potential clinical and pathogenic characteristics of each isolate. For example, as in Table 1, *E. cloacae* has been found to be a clinically important pathogen [23]. In addition, *P. monteilii* was isolated from clinical specimens [24], and *I. limosus* and *S. maltophilia* were also demonstrated as being multidrug-resistant species [25] as well as causing nosocomial infection [26].

On the other hand, the remaining 4 strains that were isolated as MG-degrading candidates were divided into bacterial and yeast groups. Among them, 3 bacterial strains were identified to be closely related to *R. multihospitium*, *E. oryzendophyticus*, and *P. nitroreducensa,* which were demonstrated to have beneficial effects, such as root nodule symbiosis [27], plant growth promotion [28], and production of polyhydroxyalkanoates [29], respectively. Moreover, strain M3

isolated from a traditionally fermented fish product, which has a long history of domestic consumption, was identified as *D. nepalensis.* Several studies on the industrial application of *D. nepalensis* were previously reported, such as in fermentable sugars [30], pectin lyase and pectate lyase [31], and arabitol and ethanol [32]. Hence, considering risk management of human health in future applications and previous achievements, in this study, I focused on strain M3 as a representative strain for the further experiment to evaluate MG degradation using LC/MS/MS and ^{1}H-NMR.

As mentioned earlier, for application to biological treatment to remove triphenylmethane dyes including MG, numerous dye-degrading microorganisms from various sources have already been reported. In these previous studies on MG biodegradation, decolorization of the green color of MG is frequently used as this is the simplest indicator of MG degradation. However, MG has been reported to change to a compound with a similar structure, such as LMG, which is a colorless substance. The microbiological reduction of MG to LMG has been frequently observed [33]. In addition, MG was reported to show equilibrium with the colorless anomer MGC depending on the environmental pH [34]. Therefore,

the evaluation based on the decolorization of the incubation medium is insufficient for the selection of MG-degrading microorganisms.

The LC/MS/MS analysis results showed the disappearance of MG and the increase in the amount of LMG. In addition, this result suggested that approximately 60% of the initial MG disappeared. Figure 7 shows the relationship among MG, LMG, and MGC. NMR data showed that MG and MGC produce an anomeric equilibrium mixture. Similarly to the LC/MS/MS analysis results, the ^{1}H-NMR analysis results indicated a decrease in the amount of MG depending on the incubation time of strain M3. Consequently, after 8 weeks of incubation, the NMR spectra of the sample degraded by strain M3 and the reference broth medium containing LMG were almost identical. Therefore, these results suggested that strain M3 changed MG into LMG; in addition, considering the results of LC-MS/MS analysis, approximately 60% of the initial MG was metabolized to compounds other than LMG. Therefore, these results showed that ^{1}H-NMR is suitable for monitoring the behavior of MG during microbiological incubation. In addition, as mentioned earlier, in the previous studies on the screening of MG-degrading microorganisms, as well as in my study, various and numerous strains that cause MG decolorization have been isolated. In order to isolate MG-degrading

microorganisms, the evaluation of degradation is indispensable. Therefore, ^{1}H-NMR analysis might be a helpful tool for confirming the result of the quantification of MG and LMG using LC/MS/MS from a holistic viewpoint.

As mentioned earlier, in previous studies, the MG degradation pathways of microorganisms and the intermediates of MG were already examined. These pathways were demonstrated to be very complicated, and various degradation steps such as oxidation, reduction, hydrolysis, methylation, and ring cleavage were reported. In the case of *Micrococcus* sp. strain BD15, which was isolated from sewage, 4-(dimethylamino) benzophenone, Michler's ketone, 4-(methylamino) benzophenone, 4-aminobenzophenone, 4-methylaminobenzoic acid, 4-hydroxyl-*N*,*N*-dimethylaniline, *N*,*N*-dimethylaniline, hydroxyl-4-(dimethylamino), benzophenone, and 4-hydroxyl-aniline were identified as intermediates of MG degradation [8]. In addition, in the case of *Pseudomonas aeruginosa* NCIM 2074, 4-[4-aminophenyl) (phenyl) methyl] *N*,*N*-dimethylaniline, 4-dimethylamino benzophenone, aniline, and benzophenone were also detected [6].

However, in this study, ^{1}H-NMR analysis showed that the above-mentioned aromatic compounds, which have one to three benzene rings and are produced

through MG degradation, were not detected at the end of strain M3 incubation as a major component (Figure 6). The reasons why these compounds were not detected are unclear at present; however, some possibilities were speculated as follows. For example, considering the effect of a solvent's extraction ability from a culture medium, other solvents might be suitable. In addition, owing to the small amounts of various intermediates except LMG, these intermediates might not be detected on the ^{1}H-NMR spectrum; carefully observed NMR spectrums after incubation for 1 week, there were minor signals at 6.83 ppm, 7.50 ppm and 7.56 ppm, for instance. To evaluate these minor signals, further NMR and mass spectral analysis are necessary after purifying the culture medium using chromatographic methods.

Moreover, the biosorption of MG to microbial cells was also expected. In a previous report, the biosorption of MG to yeast cells was already shown, and decolorization on immobilized dead yeast cells was demonstrated [35]. Therefore, to confirm the biosorption of MG to the cells of strain M3, I performed an experiment using heat-killed cells. However, heat-killed cells were not effective in the decolorization of MG. Therefore, this result suggested that the biosorption of MG to the cells of strain M3 is not significant. However, because there are many

types of colorless compound such as LMG and other intermediates, their biosorption to the cells of strain M3 remains to be established.

In conclusion, strain M3 was isolated from a fermented food which has been eaten traditionally; therefore, this strain was expected to be advantageous to the actual application of bioremediation. In addition, this study shows the first detailed description of ^{1}H-NMR analysis for monitoring the trends of MG during microbiological incubation. Further studies are under way to clarify the detailed MG-removing mechanism of strain M3 and the decolorization mechanisms of the other candidate strains.

Table 1 MG-degrading candidates isolated from various samples collected in Myanmar (A) and Thailand (B)

(A)

Strain	Source	16S rRNA gene sequence
13CS-B	Canal	*Pseudomonas monteilii*
13CS-D1	Canal	*Enterobacter cloacae* subsp. *cloacae*
13CS-D2	Canal	*Enterobacter cloacae* subsp. *cloacae*
13CS-E	Canal	*Enterobacter cloacae* subsp. *cloacae*
14C-2	Tilapia pond-1	*Inquilinus limosus*
15C-1	Tilapia pond-2	*Rhizobium multihospitium*
M3	Fermented small fish with boiled rice	*Debaryomyces nepalensis**

Strain 14C-2 and 15C-1 were isolated under shaking conditions.
* From the results of the sequencing of the D1/D2 domain of the 26S rRNA gene

(B)

Strain	Source	16S rRNA gene sequence
T-8-2	Tilapia pond-A**	*Pseudomonas monteilii*
T-8-3	Tilapia pond-A**	*Enterobacter oryzendophyticus*
T9-1	Tilapia pond-A*	*Enterobacter cloacae* subsp. *cloacae*
T9-2	Tilapia pond-A*	*Stenotrophomonas maltophilia*
T10-2	Tilapia pond-B*	*Stenotrophomonas maltophilia*
T11	Tilapia pond-B**	*Enterobacter cloacae* subsp. *cloacae*
T12-1	Canal**	*Pseudomonas nitroreducens*

*Sediment, **Water

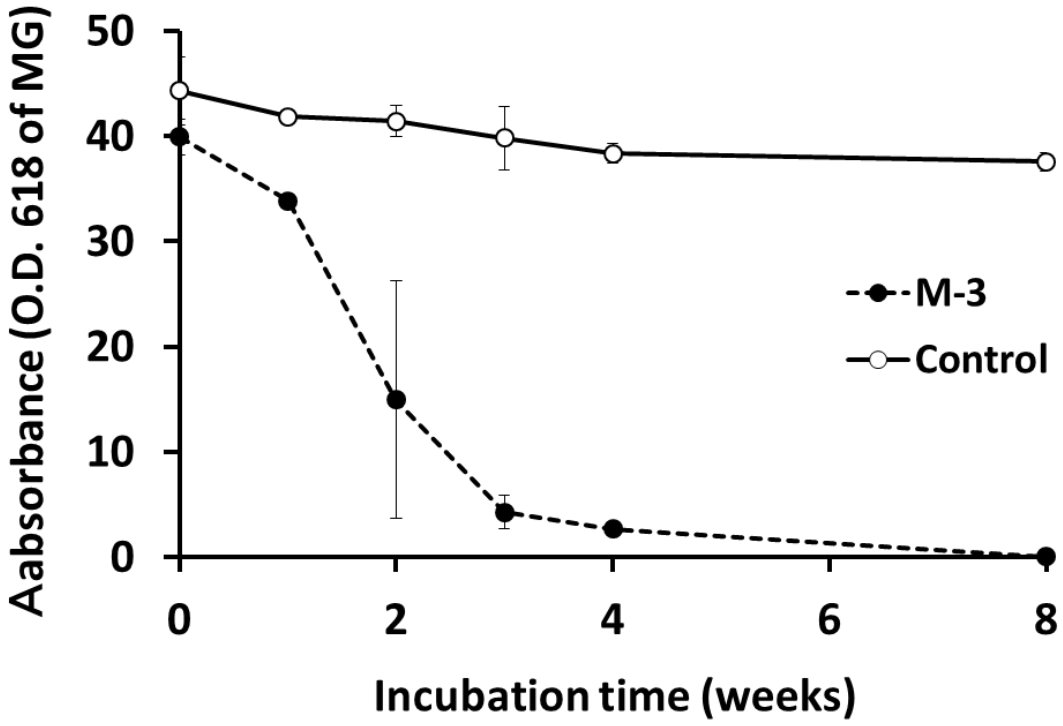

Figure 1 Changes in MG concentration during the incubation of strain M3 at 27 °C. Data are expressed as mean ± standard deviation (SD) from three independent experiments.

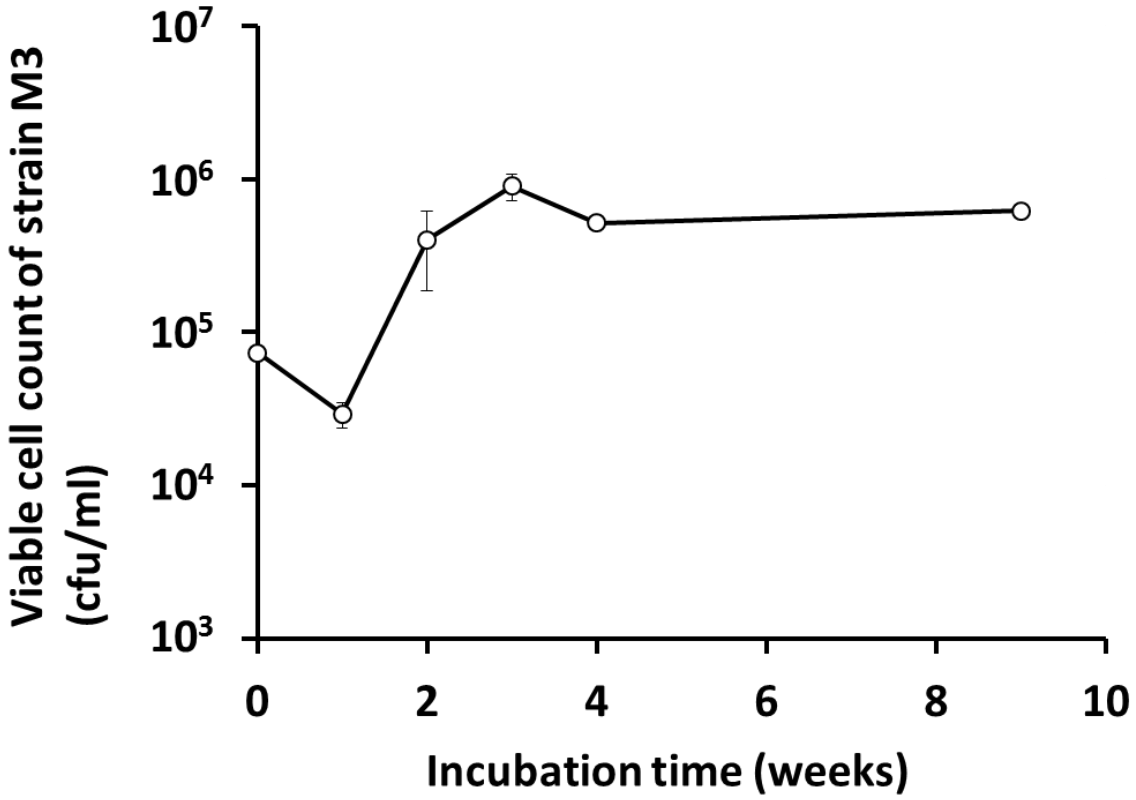

Figure 2 Changes in the viable cell count of strain M3 during incubation at 27°C. Data are expressed as mean ± standard deviation (SD) from three independent experiments.

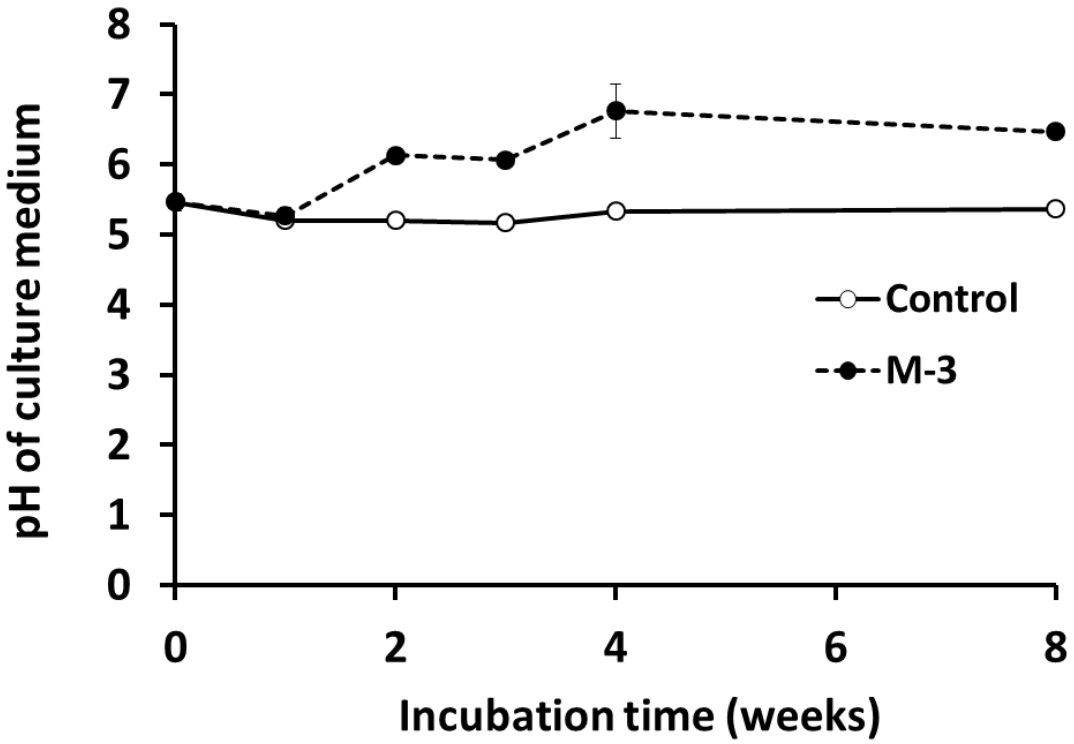

Figure 3 pH change of culture medium during the incubation of strain M3 at 27°C. Data are expressed as mean ± standard deviation (SD) from three independent experiments.

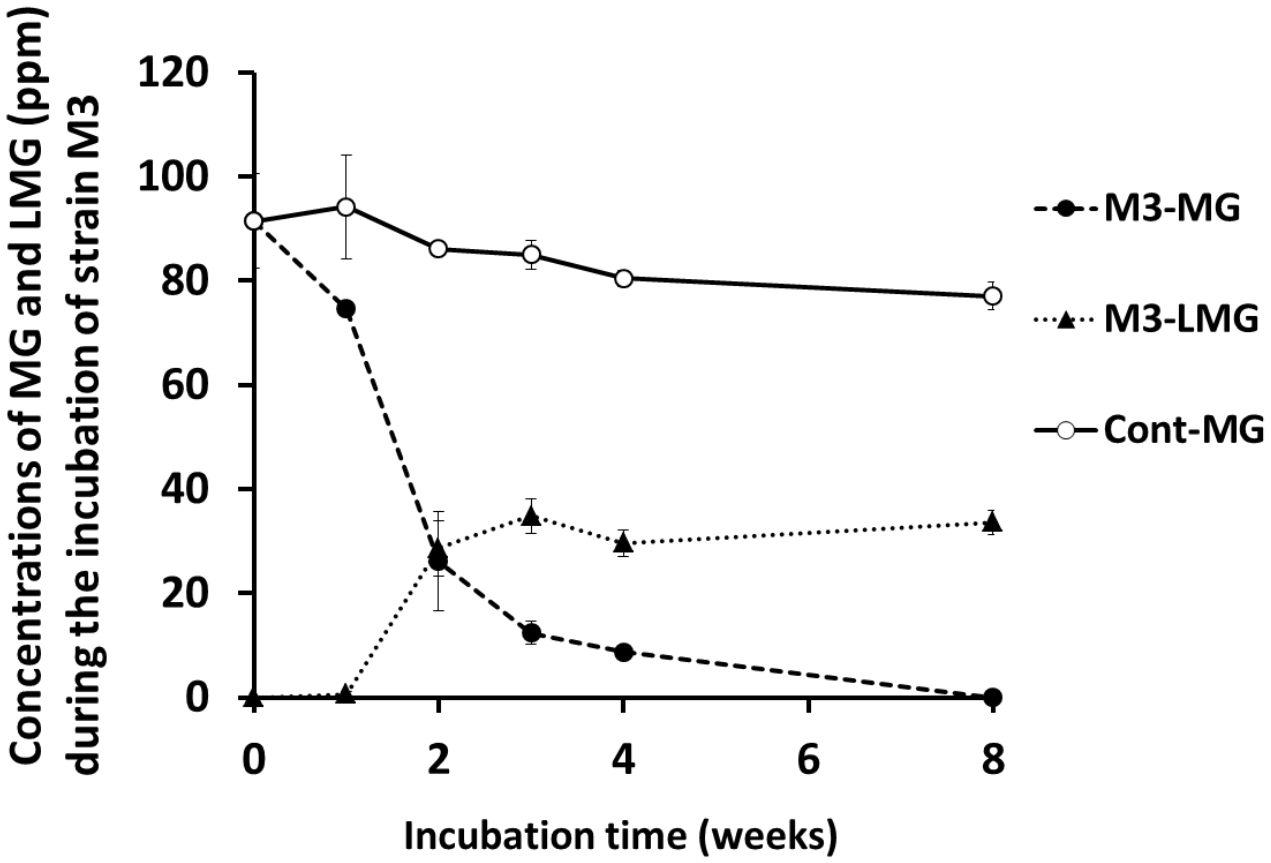

Figure 4 Changes in concentrations of MG and LMG during the incubation of strain M3 at 27°C. Data are expressed as mean ± standard deviation (SD) from two independent experiments.

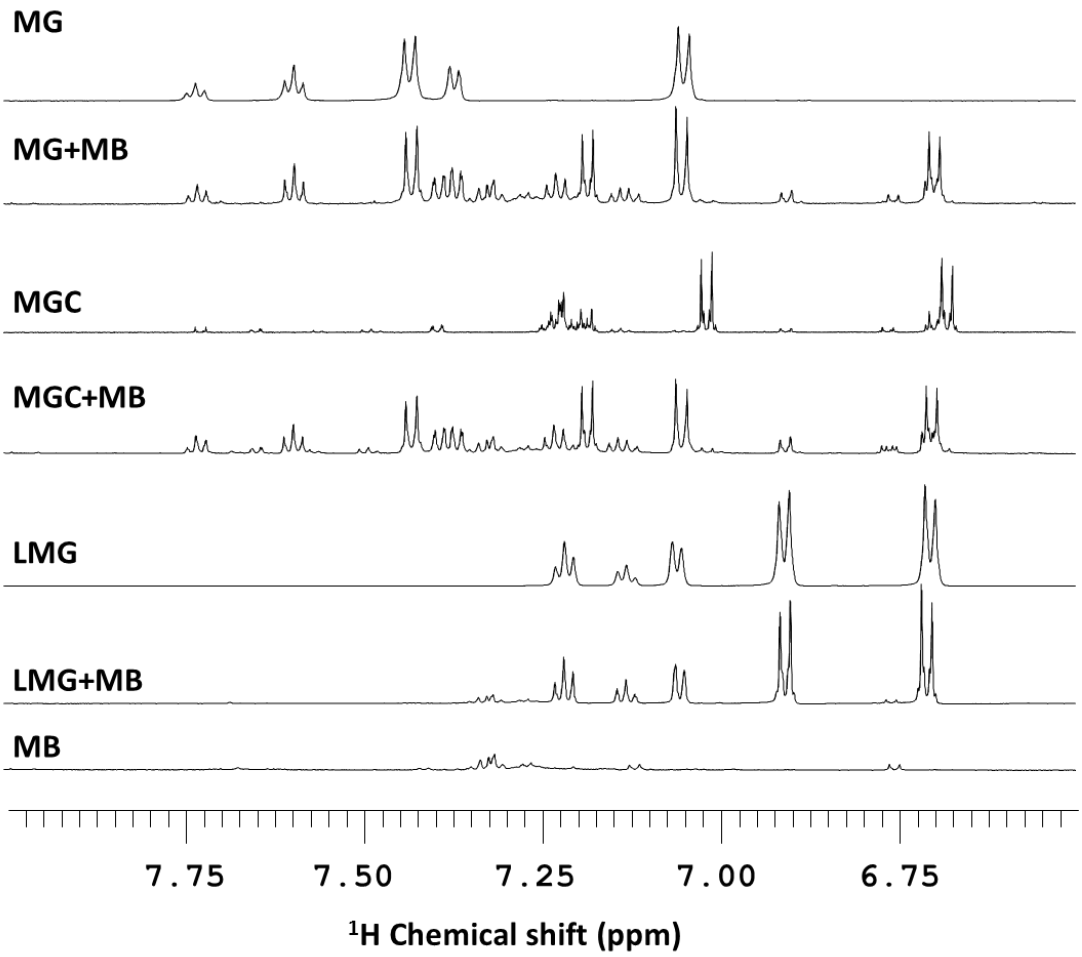

Figure 5 ^{1}H-NMR spectra of MG, LMG, and MGC directly solubilized in MeOH-d_4, and spectra of these compounds extracted from the broth medium for the cultivation of strain M3.

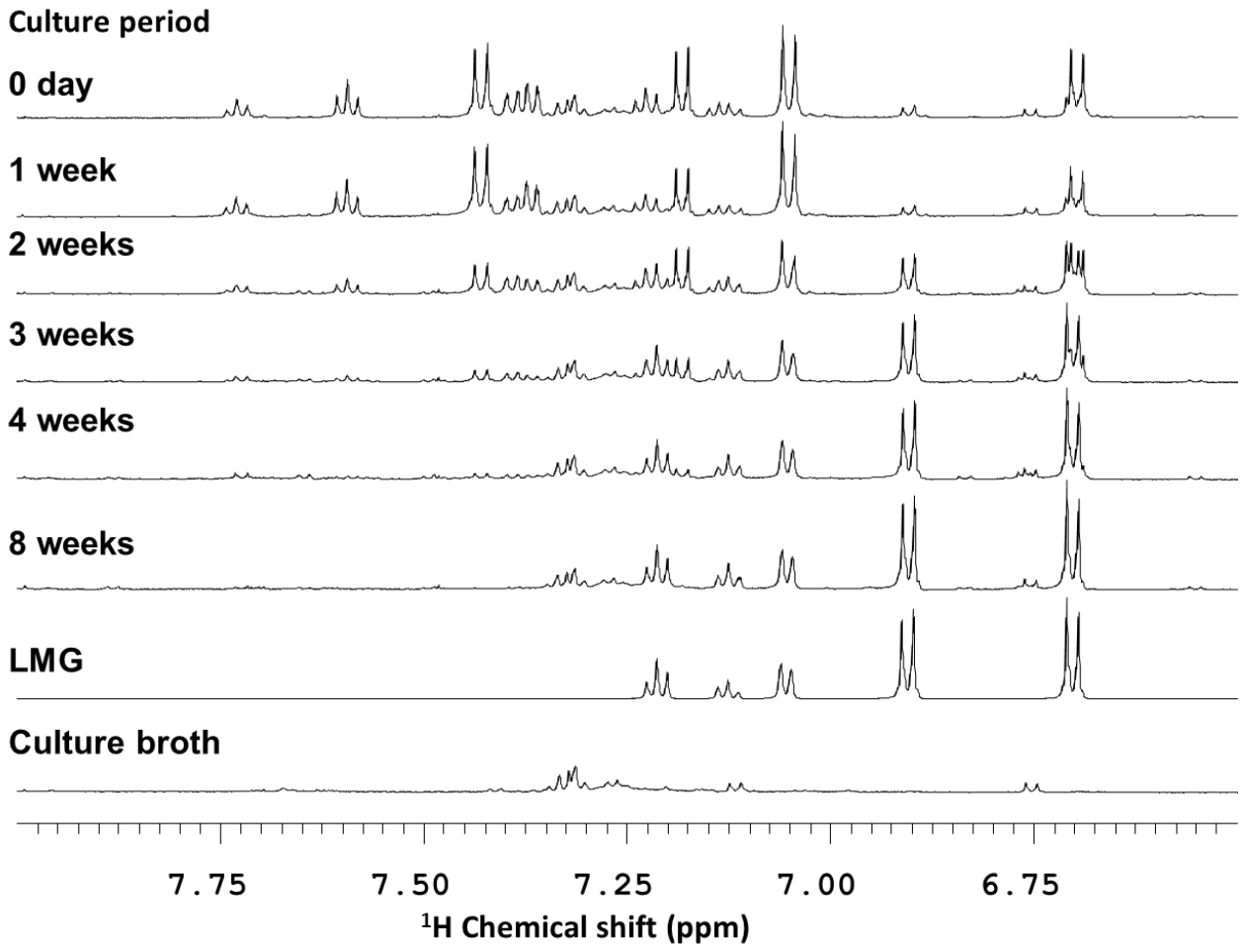

Figure 6 ^{1}H-NMR spectra of MG-containing medium during incubation of strain M3.

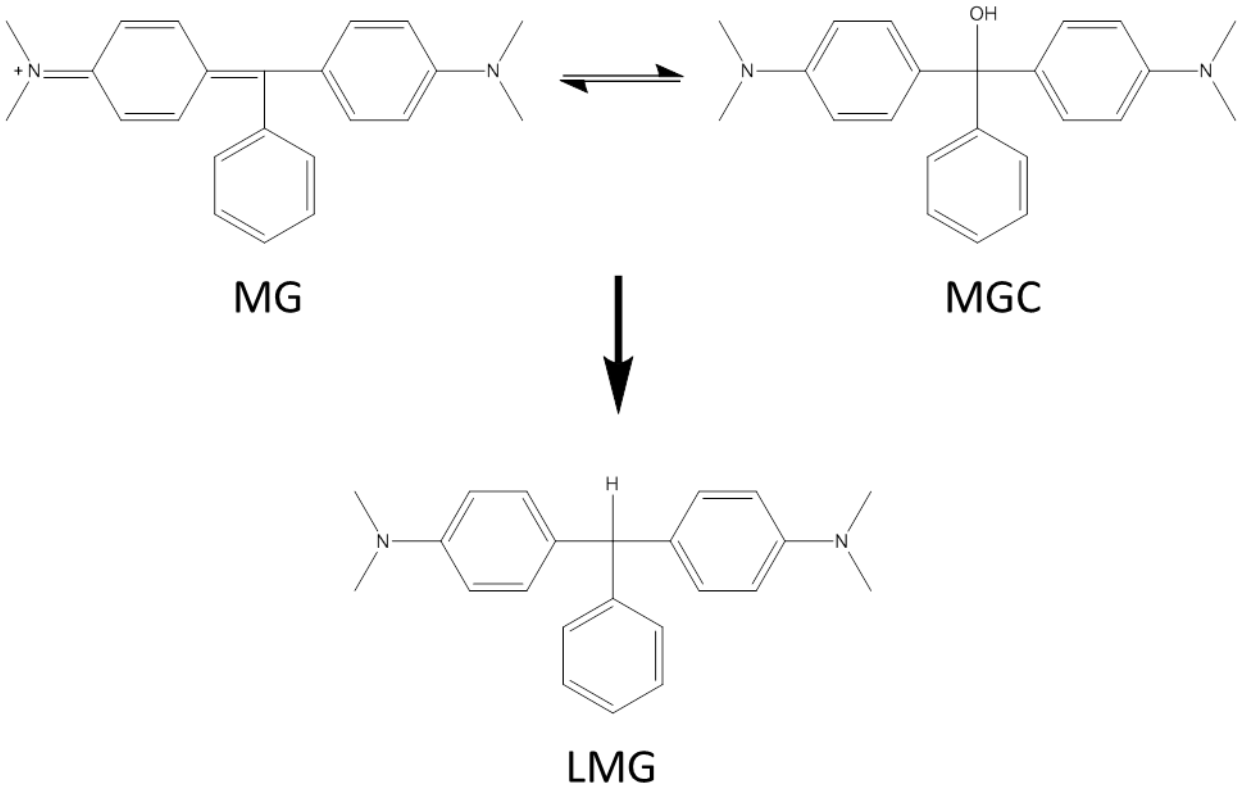

Figure 7 Mutual relationship of MG, LMG, and MGC.

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General conclusion

This study was performed to contribute to the development of Myanmar's fisheries industry. For this purpose, three scientific topics concerning microbiology were focused on in this thesis. Nowadays, fishery products are widely used in Southeast Asian countries. Recently, Myanmar fisheries have devised short- and long-term plans for fisheries, including plans for issues such as food safety, HACCP and the conservation of resources in fisheries. It is clear that reliable, accurate and precise scientific information is needed by the fishery industry, as well as planning to realize the sustainable fisheries.

In the first part of this thesis, the diversity of lactic acid bacteria (LAB) in Myanmar traditional fermented products was examined. This study is a detailed description of the microflora of a traditional fishery product in Myanmar which is fermented with boiled rice. I approached this analysis from two viewpoints; namely, the culture-dependent and culture-independent methods. In Southeast Asia, there are various types of traditional fermented fishery products. In this study, I isolated and characterized lactic acid bacteria (LAB) from small freshwater fish (tinfoil barb) fermented with boiled rice, a typical Myanmar fermented product, to contribute to the understanding of its fermentation process. Eight fermented fishery products were purchased from different markets in Yangon. Forty-three of

the 46 isolates were identified as LAB, and they were classified into two groups, 40 homofermentative and three heterofermentative isolates, on the basis of their phenotypic characteristics. From the results of PCR-restriction fragment length polymorphism (RFLP) analysis and 16S rRNA gene sequencing, these isolates were identified as *Lactobacillus plantarum*-group, *Lactobacillus farciminis*, *Lactobacillus futsaii*, *Lactobacillus reuteri, Weissella paramesenteroides,* and *Pediococcus pentosaceus.* In addition, *L. plantarum* and *L. farciminis* were identified as γ-aminobutyric acid (GABA)-producing LAB. Terminal restriction fragment length polymorphism (T-RFLP) analysis was also carried out using DNA samples extracted from these fermented products. In comparison with culture-dependent methods, the results of T-RFLP analysis did not seem to have major contradictions.

In the second part of this thesis, an antibacterial-substance-producing bacterium was examined. Strain F412 used in this part was isolated from a traditional Myanmar shrimp product fermented with boiled rice. It was a gram-positive, spore-forming, and rod-shaped bacterium, and identified as *Bacillus mojavensis* on the basis of the *gyrA* sequence. The antibacterial substance of this strain was partially purified from a culture supernatant using two steps of column

chromatography. This substance was found to be widely effective against gram-positive bacteria, including *Listeria monocytogenes*. The antibacterial activity of this substance was not susceptible to treatments with several proteolytic enzymes. The antibacterial activity gradually decreased with increasing treatment temperature, but it remained even after heating for 15 min at 121°C. This antibacterial substance showed different molecular weights, as shown by the results of gel filtration and electrophoresis analyses. Staining results after electrophoresis suggest that the antibacterial substance might be a glycopeptide with an estimated molecular weight between 3.5 and 8.5 kDa. From the decrease in optical density of a culture of the *L. monocytogenes* treated with this antibacterial substance, it was suggested that this substance might have bacteriolytic activity.

In the third part of this thesis, Malachite green (MG) was focused on from the viewpoint of application to bioremediation. The screening of malachite green (MG)-degrading microorganisms was carried out using various sources, namely, fish farms and a traditional fermented fishery product in Myanmar and Thailand. The enrichment culture method was performed using MG-containing broth media, and colonies that showed the decolorization of MG on plate media were isolated

as MG-degrading candidates. From the results of the sequencing of the D1/D2 domain of the 26S rRNA gene, strain M3, a representative strain of MG-degrading candidates was identified as a halotolerant yeast, namely, *Debaryomyces nepalensis*. Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis indicated that during the incubation of this strain, the MG concentration gradually decreased and eventually reached undetectable levels. Conversely, the concentration of leucomalachite green (LMG) increased, and the final LMG amount in the broth culture was estimated to be approximately 40% of the initial MG amount. In addition, results of proton nuclear magnetic resonance spectroscopy (^{1}H-NMR) analysis also showed that MG and the tautomer of MG or other aromatic decomposition products of MG were not detected as a major component at the end of incubation. These results suggested that strain M3 removed MG and changed approximately 40% to LMG and 60% to some metabolites other than LMG.