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Isolation and identification of bacteriocin-producing lactic acid bacteria from plants in Mount Makiling Forest Reserve, Philippines

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ABSTRACT

Sixty-three lactic acid bacteria (LAB) were isolated from trapped or stagnated water in parts of 14 different plant species in Mount Makiling Forest Reserve, Philippines and were screened for bacteriocin production. Thirteen of these isolates were found antagonistic to different indicator bacterial strains through direct assay. However, after screening of the pH-neutralized culture supernatant using 'spot-on-lawn' method against *Enterococcus faecium* 79 as the indicator strain, only one isolate, designated as ML 258, was confirmed to be bacteriocin-producer. Its crude extract of bacteriocin exhibited inhibitory activity against *E. faecium* and *Lactobacillus acidophilus.* The isolate was characterized and found to be gram-positive short rods that occurred in pairs, singly or in chains, catalase negative, microaerophilic, non-motile, non-spore forming and exhibited a heterofermentative type of fermentation. This isolate was obtained from fern (*Microsorum longissimus*) and was identified as *Carnobacterium piscicola* using API 50 CHL kit with good identification of 98%. However, the 16S rRNA gene sequencing and homology search from NCBI database using the BLAST program showed that the isolate is 93-95% similar with *Carnobacterium maltaromaticum*. On the other hand, phylogenetic analysis using MEGA 5 software that constructed a neighbor joining tree thru bootstrap method revealed that this isolate showed more similarity with *Enterococcus* sp.

Keywords: Lactic acid bacteria, bacteriocin, Carnobacterium piscicola, *Carnobacterium maltaromaticum*, Mount Makiling, 16S rRNA

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INTRODUCTION

Lactic acid bacteria (LAB) consist of relatively diverse groups of bacteria, related by a number of typical metabolic and physiological features. In recent taxonomic revisions, this group is composed of at least ten genera, which include *Aerococcus*, *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Vagococcus* (Axelsson 2004, Halász 2009). They are ubiquitous in nature, normally found in man, animal and plant materials (Narvhus & Axelsson 2003). For centuries, they have been used in preparing and improving storage or shelf-life of various foods; but during the ancient times, their role in the preservation of food was not yet understood. Today, they have become so popular and used mainly as starter cultures in a variety of food fermentation that not only preserve but also give the food desirable characteristics such as flavor and texture (Yerlikaya 2014).

LAB have become a topic of many studies and researches because of their many potential applications. In food system, their importance is related to their potential use as a biopreservation tool. This is because many LAB were found to produce various types of antimicrobial compounds which have bactericidal effects and can efficiently inhibit growth of both spoilage and food-borne pathogens such as *Bacillus*, *Enterococcus*, *Listeria*, *Clostridium* and *Staphylococcus*. The mechanisms responsible for this inhibition could be brought about by the formation of organic acids, enzymes, and bacteriocin that contribute to the natural preservation of food (Fijan 2016). Bacteriocins, unlike antibiotics, restrict their activity to strains closely related or strains of the same species (Zacharof & Lovitt 2012). The specificity of the bacteriocin ensures that harmless bacteria do not get killed. Furthermore, bacteriocins are ribosomally synthesized thus easily degraded by proteolytic enzymes, making them safer for human consumption (Woraprayote et al 2016, Yang et al 2014).

Bacteriocin production among LAB species is strain-dependent. In fact, particular LAB strains of several species such as *Lactobacillus*, *Lactococcus*, *Pediococcus*, *Leuconostoc* and *Carnobacterium* were observed to produce bacteriocin (Perez et al 2015). *Lactococcus lactis*, for instance, produces the best known and extensively studied bacteriocin Nisin A, which is approved for use as food additive by the World Health Organization (WHO) and currently being used for food preservation in more than 50 countries. The continuous search is directed towards the goal of discovering new bacteriocin which may be a good candidate for various industrial applications. Pediocin-like bacteriocins or Class IIa bacteriocins, for instance, have been receiving great attention because of their properties that have wide applications particularly in the food industry (Ennahar et al 2000). At present, some bacteriocins are also gaining approval on a per country basis.

In the Philippines, research on bacteriocin is not yet extensively investigated. Only a few had conducted studies on bacteriocin and bacteriocin-producing strains which were mostly isolated from indigenous fermented foods and beverages (Banaay et al 2013) and actual natural environment such as in living plants. A study by Higashikawa et al (2010) suggests that plant-derived LAB are as resistant (or even more resistant) as animal-derived LAB to gastric juices and bile after having found that the strains of *Lactobacillus plantarum* SN13T and SN35N reached the intestine as compared to animal-derived strains. LAB are found to be prolific in plants, but they remain unexploited. Hence, this work attempted to isolate LAB from

indigenous plants in Mt. Makiling, Los Baños, Laguna, Philippines. As far as we know, this is the first attempt in the country to isolate bacteriocinogenic LAB from wild plants.

MATERIALS AND METHODS

Isolation of LAB from Plants in Mount Makiling Forest Reserve

The isolates were obtained from various plants in Mount Makiling Forest Reserve (MMFR) Los Baños, Laguna, Philippines. Trapped or stagnated liquid samples (0.1mL) in stems, leaves, and trunks were transferred to a 9.9mL de Man Rogosa Sharpe (MRS) broth supplemented with 0.01% sodium azide which inhibits yeasts. The samples were then incubated anaerobically in a candle jar at 30°C for 24h in order to minimize growth of aerobic microorganisms and enumerated by serial dilution and pour plating. Each colony of different types was selected and further characterized. Purification of the isolates was performed using MRS medium by successive sub-culturing at 30°C.

Primary and Secondary Screening for Bacteriocinogenic LAB Isolates

Direct assay method or deferred assay was used to initially screen for isolates with the ability to produce bacteriocin-like substances (BLS) (Elegado et al 1997). Each isolate was stab-inoculated to MRS Agar and incubated at 30°C for 24h. When growth became visible, the surface of an agar plate was overlaid with 6mL MRS soft agar medium (0.9% agar) containing 50µL of approximately 10^6 cells of freshly cultured indicator strains of *Leuconostoc mesenteroides*, *Lactobacillus casei*, *Enterococcus faecium*, *Listeria grayi*, *Bacillus subtilis*, *Bacillus coagulans* and *Staphylococcus aureus* and then incubated overnight at 37°C. At the end of the incubation period, the plates were checked for formation of zones of inhibition. Inhibition was scored positive if a clear zone around the colonies of the isolate strains appeared.

For the second screening, only isolates which produced zones of inhibition from direct assay were selected. Isolates were grown in MRS broth and then further subjected for antimicrobial activity screening using the "spot-on-lawn" method as described by Mayr-Harting et al (1972). Cells were separated from the culture broth by centrifugation at 10,000rpm for 20min. The cell-free supernatant was adjusted to pH6.5 using 6N NaOH to exclude the effect of organic acids. The supernatant was boiled for 5min to destroy heat-labile inhibitory enzymes and proteins (eg, proteases), to inactivate cells and preferentially obtain heat-tolerant bacteriocin-like substances. Complete sterilization was done by passing the supernatant through 0.22m membrane filter and diluted serially by two-folds with sterile water. A volume of 10µL was spot inoculated unto a 10mL MRS Agar (1.2%) plate overlaid with 50µL of the selected indicator strain (*Enterococcus faecium* 79) in 6mL soft MRS agar (0.9%) and then incubated at 37°C for 24h.

Confirmatory Assay for Bacteriocin-like Substances

Bacteriocin activity was confirmed and quantified by assaying the supernatant treated with Proteinase K to a final concentration of 1mg mL^{-1} and the untreated

supernatant of the 24h old culture of the bacteriocin-producing isolate against indicator strain *E. faecium* 79 using "spot-on-lawn" method as described previously.

Bacteriocin Assay for Antimicrobial Spectrum

Ammonium sulfate precipitation of the culture supernatant was done to further concentrate the bacteriocin extract that was used for the antimicrobial spectrum assay of the bacteriocin. Isolate ML 258 was grown in 1L modified MRS broth at 37°C for 24h. Cell-free supernatant was obtained by centrifugation at 10,000rpm for 20min at 4°C. Ammonium sulfate was added to the supernatant with slow stirring in an ice bucket to obtain 50% saturation and allowed to stand overnight at 4°C. Protein was precipitated and collected by centrifugation at 10,000rpm for 20min at 4°C and dissolved in a small volume of 5mM phosphate buffer (pH6.5). The dissolved precipitate was filter-sterilized and assayed for bacteriocin activity against the indicator strains mentioned above.

Characterization and Identification of LAB Isolates

The isolate, confirmed to produce bacteriocin after the primary and secondary screening, was characterized and identified based on its morphological, cultural and physiological characteristics along with the use of a rapid identification kit and 16S rDNA sequencing. For morphological/cultural and physiological characterization, the isolate was grown in MRS agar and MRS broth. The gram reaction, cell morphology, size and arrangement, spore formation and motility and colonial appearance of the isolate were examined. For the physiological characteristics of the isolate, catalase test, fermentation test (homofermentation or heterofermentation) and oxygen requirement for growth were performed (De Vos et al 2009).

For initial identification, rapid id kit API 50 CHL (BioMerieux, France) was used according to the manufacturer's instructions. API profiles were analyzed using API LAB software (BioMerieux, France). The identity of the bacteriocinogenic LAB isolate was further confirmed through 16S rRNA gene sequencing and through homology search from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program.

DNA Isolation and PCR Amplification

Genomic DNA was isolated using the Cetylmethyl Ammonium Bromide (CTAB) method and the chloroform isoamyl alcohol (24:1) treatment, respectively, as described by Moore (1993). DNA was then precipitated by adding 1/10 volume of sodium acetate (1M, pH4.5) followed by the addition of two volumes of chilled 95% ethanol. The DNA pellets were washed with 70% ethanol and air-dried. It was then dissolved in 50µL of 10mM, pH8 Tris-EDTA (TE) buffer and loaded on 0.8% agarose gel with 1 X TAE buffer to check for its integrity. The nucleic acid in gel was analyzed after electrophoresis at 50V with constant temperature of 40°C using Mupid-2 Mini Gel Electrophoresis Unit (Cosmo Bio Co., Ltd, Japan) and then stained with ethidium bromide (0.5µg mL^{-1}) for 10min and visualized using the UV Transilluminator and Gel Documentation System (Labworks, UVP, Inc., CA, USA).

PCR amplification of the 16S rRNA gene was performed using primers 1101F (5'-AAC GAG CGC AAC CC-3') and 1407R (5'-GAC GGG CGG TGT GTA C-3') (Kawamura et al 1995, Elegado et al 2001). One microliter (1µL) of the isolated DNA was subjected to PCR in a 15µL reaction mixture consisting of 0.5µL of each of the forward and reverse primers and 13µL of the Platinum PCR Supermix High Fidelity (Invitrogen) containing 22U mL^{-1} recombinant Taq DNA polymerase from *Pyrococcus* species GB-D and Platinum Taq Antibody; 66mM Tris-SO_4 (pH8.9); 19.8mM $(NH_4)_2SO_4$; 2.4mM $MgSO_4$; 220µM dNTPs; and stabilizers. Amplification reaction was carried out with primary DNA denaturation step of 94°C for 5min followed by 30 cycles of 1min at 94°C, 20s at 52°C, and 1min and 30s at 72°C. The final extension was done for 7min at 72°C. After amplification, 5µL of PCR products were electrophoresed, stained with ethidium bromide and visualized as above. Homology search from the National Center for Biotechnology Information (NCBI) database using the Basic Local Alignment Search Tool (BLAST) program was done. For sequence assembly and alignment, MEGA 5 software program was used. The sequence information of the isolate was then compared to sequences from different types of bacterial strains held in gene data system and the phylogenetic trees were constructed using the neighbor-joining method. The topologies of trees were evaluated by bootstrap analysis of the sequence data with MEGA 5 software based on 1000 random resamplings.

RESULTS AND DISCUSSION

Isolation of Lactic Acid Bacteria

A total of 63 lactic acid bacteria (LAB) were isolated from different plants located in different altitudes of Mount Makiling Forest Reserve (MMFR). Table 1 shows the sources, the number of isolates in each source and the altitudes from where these isolates were obtained.

Table 1. Sources and number of isolates obtained from different altitudes of Mount Makiling Forest Reserve

Source	Altitude (feet)	Number of Isolates
Tree trunk (unidentified)	650	4
Sandoricum koetjape	650	5
Costus speciosus	650	4
Decaying log (unidentified)	650	6
Polysticus	980	3
Asplenium nidus cv.	980	3
Microsorum longissimus Fée	980	3
Philodendron scandens subsp. *oxycardium*	1,300	10
Colocasia esculenta Linn.	1,300	5
Musa sp.	2,300	5
Philodendron speciosum	2,600	5
Panicaum maximum	3,280	5
Pandamus odoratissimus	3,280	5
		63

In this study, LAB were found prolific in plants from MMFR. As to our knowledge, this is the first isolation of LAB in this forest reserve. LAB are fastidious and require complex nutritional requirement. However, the presence of these bacteria from stagnant water in different plant parts showed that nutrients are readily available to support growth of LAB in plants. One possible source of nutrients such as carbohydrates, amino acids, fatty acids, salt, vitamins and derivatives of nucleic acids needed by these bacteria comes from the plant itself. Plants are composed of carbohydrates, amino acids, vitamins etc. that could have become available and used as substrate by LAB when the plants were physically cut or scratched by animals and insects. Moreover, abundance of leaf litter from tall trees, decaying fruits and logs, nectar from flowers and remains of insects may have also enabled LAB to thrive in this kind of habitat.

Aside from the available soluble nutrients in plants, other factors attributed to their wide distribution and successful establishment were the conditions in MMFR. The low oxygen tension due to high altitude, the varying temperature, and the humidity of this area were presumed to favor growth of LAB. Furthermore, LAB are also capable of producing metabolites inhibitory to other organisms, thus enabling them to occupy and establish a niche and eliminate competition among the other microflora of the MMFR.

There are also various authors who isolated LAB from plants as reported in several publications. As early as 1968, Mundt and Hammer found different *Lactobacillus* species in plants such as *L. plantarum*, *L. fermentum* and small number of *L. brevis*, *L. casei*, *L. viridescens*, *L. cellobiosus* and *L. salivarius* (Mundt & Hammer 1968). Another lactic acid bacteria, *Lactococcus lactis* ssp*. lactis*, has been found by Salama et al (1995) from trees such as *Lamium purpureum* (red nettle), *Sonchus oleraceus* (common sow thistle), *Rubus discolor* (Himayan blackberry) and *Solanum nigrum* (black nightshade). They also reported presence of *L. lactis* ssp*. lactis* on vegetables such as sweet pea, potato, cucumber, bean, cantaloupe, broccoli and corn.

Primary and Secondary Screening of Bacteriocin-producing LAB

In the primary screening, 13 out of the 63 pure isolates produced zone of inhibition to lawn of various bacterial indicators as presented in Table 2 which is about nineteen percent (19%) of the total isolates obtained. This inhibition was due possibly to organic acids (primarily lactic acid), enzymes, hydrogen peroxide, bacteriocin-like substances, or other metabolic by-products that these LAB may have produced. Martirosyan et al (2004), in their study of sour milk products *Narine*, *Karine* and *Matsun*, revealed that the antimicrobial activity of these milk products against 16 pathogenic microbes, which include *S. aureus*, *E. coli* and *S. flexneri*, was related to the presence of high amount of L-lactic acid and of sodium and calcium salts produced by *L. acidophilus*. Moreover, the lactic acid, in addition to its antimicrobial property due to lowering of the pH, was reported by Alakomi et al (2000) to be capable of permeabilizing gram-negative bacteria (ie, *S. typhimurium, P. aeruginosa, E. coli*) by disrupting their outer membrane and subsequently act as potentiator of the effects of the other antimicrobial substances such as antibiotics, enzymes and bacteriocins.

For the LAB indicator strains (*L. mesenteroides*, *E. faecium* and *L. casei*) used in the experiment, resistance to the effects of lactic acid was already expected, for they can tolerate acidic pH at certain level. Thus, it was presumed that other substances, aside from lactic acid, must be present leading to the inhibition of these indicator organisms. As mentioned earlier, one possible explanation for the inhibition was the presence of bacteriocin-like substances which have antimicrobial activity against gram-positive bacteria and to the closely related species of the producing strain.

For the second screening, the cell-free supernatant of the isolates which produced zone of inhibition during the primary screening was neutralized, filter-sterilized, and boiled for 5min. Boiling of the cell-free supernatant was performed to detect for the presence of bacteriocins belonging to Class II type which are known to be heat stable (Ennahar et al 2000). This was also employed to inactivate the proteolytic enzymes such as proteases released by the isolates in the medium which may degrade the proteinaceous bacteriocins.

Table 2. Inhibition of various indicator organisms by lactic acid bacteria isolates from plants of Makiling Forest Reserve

Isolate Code	*L. mesenteroides*	*E. faecium*	*L. grayi*	*L. casei*	*B.subtilis*	*B.coagulans*	*S. aureus*
1	-	-	-	-	++	++	-
19	++	++	-	-	-	-	-
20	-	+	-	-	-	+	+
23	-	++	+	+	-	-	-
35	++	++	++	++	++	++	++
39	-	-	+	-	-	-	-
51	-	-	+	-	-	-	-
72	+	+	++	+	-	-	-
80	++	++	++	+	-	-	-
501	+	++	++	-	-	-	-
258	-	+	++	-	++	++	++
530	-	+	+	-	++	+	+
532	-	-	-	-	++	++	-

Legend: + = <2.5mm diameter zone of inhibition; ++ = >2.5mm diameter zone of inhibition

Among the isolates with activity against the different indicators, only the isolate from a fern designated as ML 258 gave a positive result for bacteriocin-like substance (BLS). This bacteriocin-like substance was observed when the neutralized, filter-sterilized and boiled cell-free supernatant was spotted on a lawn of *E. faecium* 79 as described earlier. However, it is possible that the other isolates produced BLS not belonging to class II which were heat sensitive; hence, these were destroyed during the heating process. Moreover, factors such as method of detection, selection of indicator organisms and media may have also influenced the results of the assay. Furthermore, the choice of indicator organism used in the screening is also extremely important because if the selected indicator is resistant to the bacteriocin being tested, no antimicrobial activity will be detected.

To confirm whether the BLS produced by the selected isolate was really bacteriocin, cell-free supernatant without and with added enzyme Proteinase K was spotted on lawn of the same isolate and of *E. faecium* as the indicator organisms, respectively. This was performed to determine if the BLS has activity against its producer and if it was degraded by the enzyme.

Confirmatory Test for Bacteriocin

The results obtained confirmed that the substance with antimicrobial activity present in the supernatant was indeed bacteriocin. In the supernatant treated with proteolytic enzyme Proteinase K, loss of activity against *E. faecium* was observed. Likewise, no activity was detected when the supernatant was spotted on lawn of the producing isolate. Both observations met the criteria of Konisky (1982) that led to the conclusion that there are only two true requisites for a substance to be called bacteriocin. One criterion is that bacteriocin should be proteinaceous in nature while the other was the lack of lethality to the cells that produce them.

Antimicrobial Spectrum of Ammonium Sulfate Precipitate

The antimicrobial spectrum of the ammonium sulfate precipitate of the crude extract of bacteriocin is presented in Table 3. The precipitate of crude extract of bacteriocin exhibited a very narrow range of inhibitory activity against the selected Gram-negative (10 strains) and Gram-positive (10 strains) bacterial species. Among the 20 species tested, only *Enterococcus faecium* and *Lactobacillus acidophilus* showed strong sensitivity to the bacteriocin with zones of inhibition of 20mm and 16.5mm, respectively. No antagonism, however, was observed for the Gram-negative and some Gram-positive bacterial species used as test organisms. It was expected that Gram-negative bacteria would not be inhibited since bacteriocins produced by LAB are generally active only towards Gram-positive bacteria (Klaenhammer 1988). The narrow spectrum of bacteriocin activity obtained from the isolate ML 258 is common among *Carnobacterium* as reported by different authors. Schillinger et al (1993) characterized the bacteriocin of *C. piscicola* LV 61 and found to have activity against *Carnobacterium*, *Enterococcus* and *Listeria*. Piscicocin produced by *C. piscicola* CS526, on the other hand, was also found by Yamazaki et al (2005) to inhibit strains of *Enterococcus* and *Listeria* but with antagonism as well to strains of *Pediococcus* and *Leuconostoc*. It is surprising, however, that the crude extract of bacteriocin produced by ML 258

isolate did not have any activity against *Listeria monocytogenes,* a bacterium known to be sensitive to bacteriocins produced by *Carnobacterium* species as reported in several literatures.

The absence of the activity of the isolate's bacteriocin against this species of bacterium could not be elucidated. It was first suspected that the gene responsible for bacteriocin production is plasmid-borne and the subsequent transfer of the culture may have resulted in loss of the plasmid. Another possibility is the resistance of the particular strain of *L. monocytogenes* used as bacteriocin activity is also known as strain dependent. However, when the precipitate was spotted on lawn of *E. faecium*, large zone of inhibition was detected. Hence, this only confirmed that the bacteriocin was still present but with no antimicrobial activity against *L. monocytogenes*, although inhibition was observed in direct or deferred assay which may be due to the presence of lactic acid or enzymes produced by this isolate and not due to bacteriocin.

Table 3. Antimicrobial spectrum of ammonium sulfate precipitate of isolate ML 258

Source	Scientific Name	Presence of Inhibition Zone	
		Direct Assay	$(NH_4)_2SO_4$ precipitate
IBS, UPLB	*Bacillus cereus*	+	-
BIOTECH, UPLB	*Bacillus subtilis*	+	-
IBS, UPLB	*Enterobacter cloacae*	-	-
IBS, UPLB	*Enterobacter sakazeki*	+	-
IBS, UPLB	*Enterococcus faecium*	+	+++
IBS, UPLB	*Esherichia coli*	+	-
BIOTECH, UPLB	*Lactobacillus acidophilus*	+	+++
BIOTECH, UPLB	*Lactobacillus casei*	-	-
IBS, UPLB	*Lactobacillus paracasei*	+	-
IBS, UPLB	*Lactobacillus plantarum*	+	-
IBS, UPLB	*Lactobacillus rhamnosus*	+	-
BIOTECH, UPLB	*Leuconostoc mesenteroides*	+	-
BIOTECH, UPLB	*Listeria monocytogenes*	+	-
BIOTECH, UPLB	*Listeria grayi*	+	-
BIOTECH, UPLB	*Micrococcus luteus*	+	-
BIOTECH, UPLB	*Pediococcus pentosaceus*	-	-
BIOTECH, UPLB	*Pediococcus acidilactici*	+	-
IBS, UPLB	*Pediococcus cerevisiae*	+	-
IBS, UPLB	*Pseudomonas aeruginosa*	+	-
BIOTECH, UPLB	*Salmonella typhimurium*	+	-
BIOTECH, UPLB	*Staphylococcus aureus*	+	-

Legend: + = <2.5mm; ++ = >2.5-10.0mm; +++ = >10.0mm diameter zones of inhibition

Characterization and Identification of LAB Isolate

Morphological / cultural and physiological characteristics of the isolate. ML 258 isolate was gram-positive short rods which occur singly, in pairs or in chains. Cells were non-motile and non-spore forming. In MRS agar plate, colonies were circular with shiny and smooth surface, convex with entire margin, yellowish in color and approximately 1mm in diameter. Physiologically, ML 258 isolate was negative for catalase, microaerophilic and has a heterofermentative type of fermentation.

Identification using rapid test kit. The pure isolate of ML 258, using the API 50 CHL kits, was identified as *Carnobacterium piscicola* with a good identification remark of 97.7% on the basis of the carbohydrates it can metabolize. The positive results, as indicated by the change in color of the indicator due to decrease in pH, revealed the biochemical profile of the isolate which was used as reference by the software with database provided by the manufacturer in identifying the isolate. Twenty out of 49 carbohydrates were metabolized by the isolate which include glycerol, ribose, galactose, glucose, fructose, mannose, lactose, and mannitol (Table 4). These results, together with the morphological and physiological characteristics, were used in the identification of the isolate.

16S rRNA sequencing and homology analysis. Results of the PCR amplification of the fragment of the 16S rRNA genes of ML 258 showed a 300bp PCR product which was amplified using the primers 1101F and 1407R as shown in Figure1.

Table 4. Acid production of isolate ML 258 from fermentation of different carbohydrates

	Carbohydrates	Code	Results		Carbohydrates	Code	Results
0	Control	Control	-	25	Esculine	ESC	+
1	Glycerol	GLY	+	26	Salicine	SAL	+
2	Erythritol	ERY	-	27	Cellobiose	CEL	+
3	D-arabinose	DARA	-	28	Maltose	MAL	+
4	L-arabinose	LARA	-	29	Lactose	LAC	+
5	Ribose	RIB	+	30	Melibiose	MEL	-
6	D-xylose	D-XYL	-	31	Saccharose	S	+
7	L-xylose	L-XYL	-	32	Trehalose	TRE	+
8	Adonitol	ADO	-	33	Inuline	INU	-
9	b-Methyl-xyloside	BMDX	-	34	Melezitose	MLZ	-
10	Galactose	GAL	+	35	D-raffinose	RAF	-
11	D-glucose	GLU	+	36	Amidon	S	-
12	D-fructose	FRU	+	37	Glycogene	GLYG	-
13	D-mannose	MNE	+	38	Xylitol	XLT	-
14	L-sorbose	SBE	-	39	b-Gentibiose	GEN	+
15	Rhamnose	RHA	-	40	D-Turanose	DTUR	-
16	Dulcitol	DUL	-	41	D-Lyxoe	DLYX	-
17	Inositol	INO	-	42	D-Tagatose	DTAG	-
18	Mannitol	MAN	+	43	D-Fucose	DFUC	-
19	Sorbitol	SOR	-	44	L-Fucose	LFUC	-
20	a-Methyl-D-Mannose	AMDM	+	45	D-Arabitol	DAR	-
21	a-Methyl-D-Glucoside	AMDG	-	46	L-Arabitol	LAR	-
22	N-acetyl glucosamine	NAG	+	47	Gluconate	GNT	+
23	Amygdaline	AMY	+	48	2-keto-gluconate	2KG	-
24	Arbutine	ARB	+	49	5-keto-gluconate	5KG	-

Legend: (+) – positive result; (-) – negative result

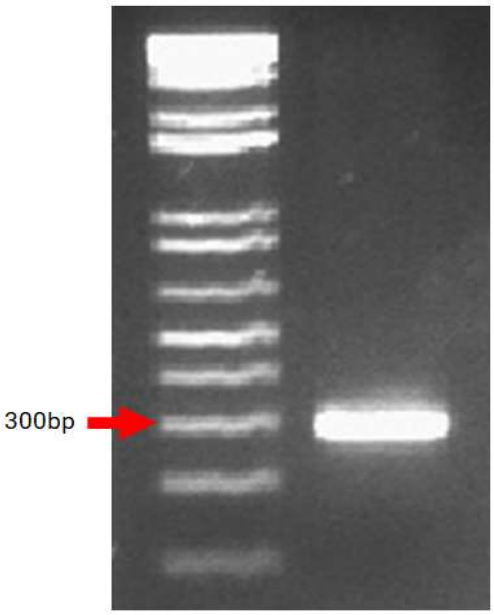

Figure 1. PCR product of ML 258 amplified using the primers 1101F and 1407R (lane 2). Lane 1 is 100bp ladder

By sequence analysis, BLAST hits were recorded for the forward and reverse primers used. The result demonstrates that the sequence is most similar to *Carnobacterium maltaromaticum* with a maximum identification of 95% and 93% for forward and reverse primers, respectively. However, according to Roselló and Amman (2001), standardization of a species corresponds with approximately 97% rDNA similarity although they have also cautioned that a species should not be solely distinguished based on rRNA or rDNA sequence information. On the other hand, Zoetendal et al (2004) discussed that if the sequence similarity is anywhere between 95 and 99% it is considered as the same species. On the contrary, according to Tajima et al (1999) there are no exact 16S rDNA similarity limits for defining specific taxa such as genus and species; in general, species definition requires sequence similarities greater than 98%.

Tajima et al (1999) cited that phylogenetic clustering of bacterial groups, rather than similarity value, should be used as a guide for defining bacterial taxa. For phylogenetic analysis of isolate ML 258, a total of 25 sequences were considered for phylogenetic analysis to clarify their taxonomic position based on neighbor-joining methods. Twenty-four conserved 16S ribosomal DNA sequences of known microorganisms were downloaded using BLAST to perform sequence comparisons. Multiple sequence alignment of the known microorganisms and of the unknown sequences was carried out using the ClustalW alignment function of MEGA and phylogenetic tree was constructed using the neighbor-joining function of the same software. Results revealed as shown in Figure 2 that isolate ML 258 (IS258-1407) was more similar to the genus *Enterococcus* sp.

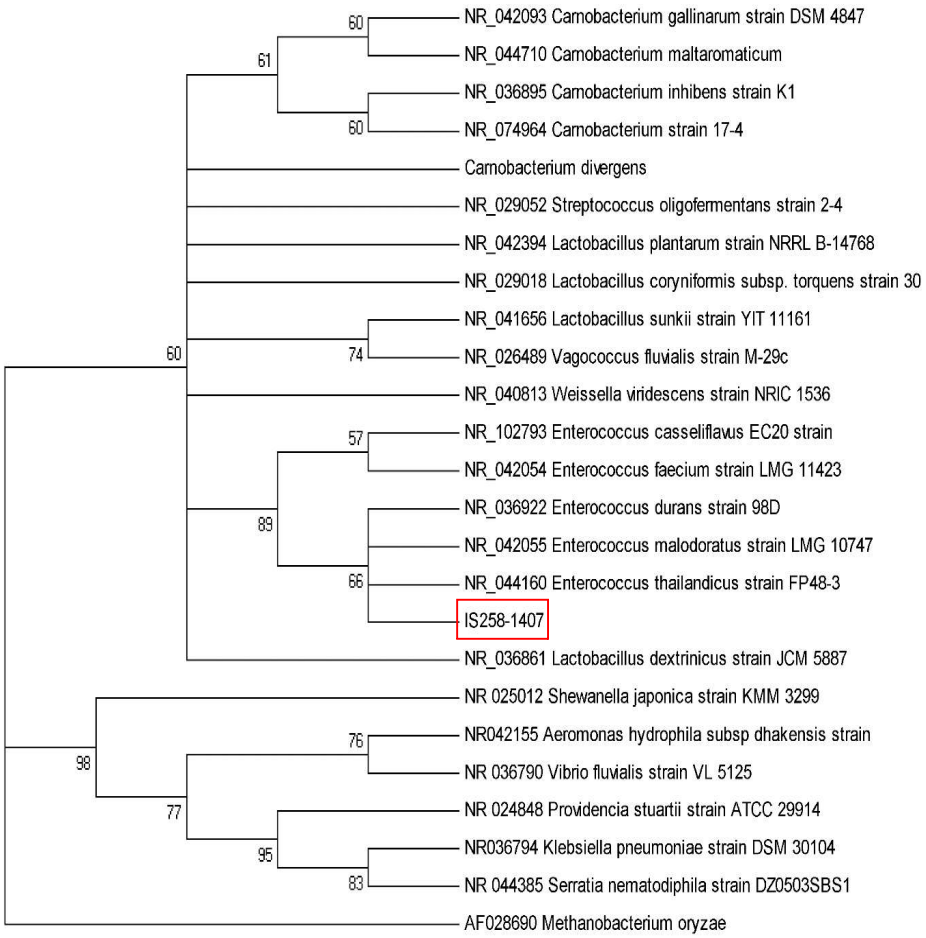

Figure 2. Molecular Phylogenetic analysis by Maximum Likelihood method

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura et al 1993) The bootstrap consensus tree inferred from 1000 replicates (Felsenstein J 1985) is taken to represent the evolutionary history of the taxa analyzed (Felsenstein J 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein J 1985). Initial tree(s) for the heuristic search were obtained automatically as follows. When the number of common sites was <100 or less than one fourth of the total number of sites, the maximum parsimony method was used; otherwise BIONJ method with MCL distance matrix was used. The analysis involved 25 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions with less than 80% site coverage were eliminated. That is, fewer than 20% alignment gaps, missing data, and ambiguous bases were allowed at any position. There were a total of 200 positions in the final dataset. Evolutionary analyses were conducted in MEGA5 (Tamura et al 2011).

SUMMARY AND CONCLUSION

Lactic acid bacteria were isolated from plants in different altitudes of Mount Makiling Forest Reserve, Los Banos, Laguna, Philippines (MMFR), 13 of which showed antagonism versus *L. mesenteroides, E. faecium, L. grayi, B. subtilis, B. coagulans* and *S. aureus* through direct assay. However, only one isolate from a fern, designated as ML 258, was found to have bacteriocin-like activity against the indicator strain *E. faecium* 79. ML 258 was morphologically, culturally and physiologically typical of lactic acid bacteria. Using the API 50 CHL kit, it was identified as *Carnobacterium piscicola* (98%), but 16S rRNA gene sequencing and homology search from NCBI gene bank using the BLAST program revealed 93-95% similarity to *C. maltaromaticum*. Moreover, after phylogenetic analysis, the isolate was found more similar with *Enterococcus* sp. although its morphology showed otherwise. Full characterization of the bacteriocin after thorough purification should be the next steps to further elucidate this bacteriocinogenic lactic acid bacteria. This work also proposes that for a thorough identification of isolates, further molecular characterization like DNA-DNA hybridization and DNA fingerprinting using housekeeping genes and other conserved genes are needed.

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