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Production of a polypeptide Antimicrobial Compound by Lactic Acid Bacteria Isolated from Rhizospheric Soil of Egyptian Organic Farms

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Abstract

The current study aimed to isolation of lactic acid bacteria (LAB) showing antimicrobial activity mainly against some fungal and bacterial pathogens. A total of 41 LAB strains were isolated from the rhizosphere of healthy and diseased tomato and phaseolus plants using MRS agar medium supplemented with bromocresol green as an indicator of lactic acid production. The isolated bacterial strains were screened for antimicrobial activity against Staphylococcus aureus ATCC 2913 (G +ve), Pseudomonas aeruginosa ATCC 27953 (G -ve) and Mucor racemosus (phytopathogenic fungus). Among the tested LAB isolates, 32 of them showed antimicrobial activity against the tested pathogens with variable potency. However, LAB isolate No.38 exhibited the highest antimicrobial activity against the indicator strains and was the only strain showing antifungal activity against Mucor racemosus. According to 16S rDNA sequence and phylogenetic analysis, strain No.38 showed the highest similarity (98%) with Enterococcus faecium and was designed as Enterococcus faecium MN 38 (accession No.MK280754). Investigation of the effect of various physiological parameters revealed production of the bioactive agents by E. faecium MN 38 at temperature range 30-37 °C, pH range 4-7. In addition, strain MN 38 was able to tolerate NaCl concentration up to 4%. Finally, the precipitation of the bioactive compound from the culture filtrate by 60-80% ammonium sulphate, suggested the proteinaceous nature of the bioactive agent produced by E. faecium MN 38. The results indicated that plant rhizosphere is a promising source of antimicrobial agents producing LAB with potential application in biocontrol of phytopathogens and soil-born pathogenic bacteria and fungi.

Keywords: LAB, Enterococcus faecium, Biocontrol, soil

I. Introduction

Several species of lactic acid bacteria (LAB) have the ability to produce antimicrobial compounds that are able to eliminate a large number of bacterial and fungal pathogens [1,2] . The LAB antimicrobial activity, can be explained by the production of organic acids, hydrogen peroxidase and siderophores. Lactic acid bacteria are commonly found in many habitats including; soil, milk, milk products and considered safe for human consumption. Many species of LAB like species of Lactobacilli, Leuconostoc spp. and Weissella spp. exhibited antagonistic activity against a lot of pathogens. Also, LAB isolated from fresh fruits and vegetables were reported to decrease the infection of phytopathogenic bacteria and fungi namely Xanthomonas campestris, Erwinia carotovora, and Botrytis cinerea. LAB are also promising for fighting soil-borne plant diseases and to enhance the growth of plants [3,4] . The ability of LAB to suppress certain fungi has been shown in the control of post-harvest diseases of fruits and vegetables. In addition, LAB have other properties making them interesting candidates for the biological control of soil-borne pathogens. The technology for mass production of LAB is readily available in the food industry. Management of fungal phytopathogen pre- and post-harvest often includes the use of chemical fungicides. However, increases in fungicide resistance as well as health and environmental concerns associated with the use of harsh chemicals have created the need for effective alternatives.
Lactic Acid Bacteria can play a major role in food industry by acting as safe additives that don't interfere with the food quality or pose any hazards for the consumer. Particularly eliminating food spoilage bacteria like Listeria monocytogenes. Moreover, lactic acid bacteria can produce antimicrobial peptides (bacteriocins) in addition to lactic acid which can combined offer a protection of the food manufacturing and storage processes. The role of bacteriocin produced by LAB in the control of biofilms formed on industrial surfaces has been investigated [4] . On the other hand, the use of natural products for the food products preservation has become a promising tool for maintaining food quality [3,5,6] . Bacteriocins were also used for preventing food contamination without the need for food treatment with excessive heat to destroy pathogens because this process can also lead to the destruction of some important and essential vitamins or other nutritional compounds. The primary role of LAB during the fermentation of food substrates was their ability to offer certain additional food characteristics as well as to prevent pathogenic organisms from infesting this product. [5,7,8,9] . The current study aimed to isolation of lactic acid bacteria (LAB) from rhizosphere of healthy and diseased tomato and phaseolus plants from organic egyptian farms, screening LAB for antimicrobial activity against mainly phytopathogens and other pathogenic bacteria [10,11, 12, 13, 14, 15]

II. Materials and Methods

2.1. Samples collection

Soil samples were collected from rhizosphere of healthy and diseased tomato and phaseolus plants in two organic farms, located in Cairo–Alexandria desert main road (Egypt). The samples were collected from different sites in sterile containers, kept in cold boxes (<10 °C) and transferred to the laboratory within few hours.

2.2. Enrichment and isolation of lactic acid bacteria

Isolation of LAB was carried out by the accumulation method, previously reported by Chen et al [16] with some modification. Briefly, in order to enrich the LAB, one gram of rhizospheric soil samples were aseptically transferred into sterile 15 mL falcon tubes containing 5 mL of MRS broth[17] and incubated at 30 °C for 72 h. After the incubation period, samples were serially diluted in 0.75% (w/v) NaCl solution

up to 10^{-8} dilution. Aliquots of 0.1 mL were plated in duplicate on the surface of MRS agar medium supplemented with 0.0025% (w/v) of bromocresol green (MP Biomedicals) and 0.01% (w/v) cycloheximide in order to inhibit fungal growth. The plates were incubated at 30 °C for several days. Different colonies of acid-producing bacteria, determined by a yellow zone around the colonies, were picked and sub cultured several times in fresh MRS agar plates until single homogeneous colonies were obtained; and glycerol stocks of each strain were prepared and stored at -80 °C. MRS broth consisted of: glucose (20 g/L), digested of casein (10 g/L), meat extract (10 g/L), sodium acetate (5 g/L), yeast extract (4 g/L), dipotassium phosphate (2 g/L), tri-ammonium citrate (2 g/L), Tween 80 (1.08 mL/L), magnesium sulphate heptahydrate (2 g/L) and manganese sulphate tetrahydrate and (0.05 g/L).

2.3. Screening of LAB for antimicrobial activity (bioassay)

The antimicrobial activity of the isolated LAB isolates was assayed using the standard agar well diffusion method. Briefly, each LAB isolate was inoculated separately into 50 mL of MRS broth [17] and incubated at 37 °C for 48 h. Then, the cultures were centrifuged aseptically at 6000 rpm for 10 min and the cell free supernatants were collected. The indicator pathogens, including *Staphylococcus aureus* ATCC 2913 (G +ve), *Pseudomonas aeruginosa* ATCC 27953 (G -ve) and *Mucor racemosus* (phytopathogenic fungus), were grown on fresh Nutrient agar for bacteria and potato-dextrose agar for fungi overnight at 37 °C. After the incubation period, the cells were collected using sterile loop and suspended in sterile saline solution (0.9% NaCl) to be equivalent to 0.5 McFarland standards. The cells suspensions were inoculated into the surface of nutrient agar plates, using sterile cotton swabs. Thereafter, about 1 cm diameter wells were prepared, using suitable sterile Cork borer, in the agar media containing each indicator pathogen separately and 0.1 ml of cell-free culture filtrate of each LAB isolates was loaded into the agar wells. The plates were incubated at 37 °C for 24 h and monitored for any inhibition zones around the wells. The LAB strain showing the highest antimicrobial activity against the indicator pathogens, was selected for further investigations.

2.4. Bacterial identification

2.4.1. Morphological and biochemical tests

The colony properties of each LAB isolate were recorded. Classification of the isolates as Gram positive or Gram negative was done by Gram stain reaction. For Gram staining the Color Gram 2 kit of bioMérieux (Marcy l'Etoile, France) was used. In addition, catalase test was carried out by re-suspension of a loopful of the bacterial cells in drops of 10% hydrogen peroxide and monitored for the development of effervescence.

2.4.2. Molecular identification of LAB

The selected bioactive metabolite producing LAB strain was identified using 16S rRNA gene sequence analysis as per the standard protocols. The bacterial isolate was grown overnight in 5 mL MRS broth. Thereafter, the cell biomass was collected by centrifugation at 10000 rpm for 10 min and the cells pellet was washed twice with distilled water. The total bacterial DNA was extracted using DNeasy Blood & Tissue Kits (Qiagen, USA) according to the manufacturer's instructions. Eubacterial-specific forward primer: 16F27 (5′-AGA GTT TGA TCC TGG CTC AG-3′), and reverse primer: 16R1525 (5′-AAG GAG GTG ATC CAG CCG CA-3′) were used to amplify 16S rRNA gene [18]. PCR amplification was performed in a final reaction volume of 50 µL. The reaction mixture contained 2 × 25 µL GoTaq® Green Master Mix (Promega, USA), 1 µL forward primer (10 µM), 1 µL reverse primer (10 µM) 5

µL DNA template (200 ng) and 18 µL nuclease-free water. The PCR reaction run for 35 cycles in a DNA thermal cycler, under the following thermal profile: Initial denaturation at 95 °C for 5 min, denaturation at 95 °C for 1 min, primers annealing at 52 °C for 1 min, and extension at 72 °C for 1.5 min. The final cycle included extension for 10 min at 72 °C to ensure full extension of the products. PCR products were ran on agarose gel electrophoresis, and then the PCR product was purified from the gel using a QIAquick gel extraction kit (Qiagen, USA). The purified 16S-rDNA was sequenced using an automated sequencer, and the obtained sequence was aligned with reference 16S-rDNA sequences available in NCBI homepage (National Center for Biotechnology Information) using the BLAST algorithm.

2.5. Fermentation parameters affecting the antimicrobial compound production by *Enterococcus facium*

Different fermentation parameters of the growth of *Enterococcus facium* and it's antimicrobial compound production were tested. The medium used was MRS broth (50ml MRS broth in 250ml conical flasks), inoculated by 5ml cell suspension of *Enterococcus facium*. The tested fermentation parameters include; incubation temperature (25-45 ºC), pH (4-9), aeration (100-150 rpm in a shaking incubator), incubation period (48-96 h) and medium salinity (1-7 % added NaCl).

2.6. Production, extraction and partial purification of the antimicrobial compound

Production: *Enterococcus facium* MN 38 was propagated when 1L MRS broth inoculated with 10 ml bacterial culture and incubated for 72 h in a shaking incubator (120 rpm) at 37°C.

Extraction: A cell-free filtrate was obtained by centrifuging (10,000 rpm for 20 min. at 4°C, followed by filtration of the supernatant through a 0.2 um pore size cellulose acetate filter. Various organic solvents including iso–amylalcohol, chloroform, n-propanol, hexane, Diethyl ether, petroleum ether were added to purify antimicrobial substance in 1:1 ratio. The organic phase was concentrated to dryness under vacuum by using a rotary evaporator.

Partial purification (Precipitation): The precipitation process of the peptide antibiotic was carried out using ammonium sulfate. Culture supernatant was treated with solid ammonium sulphate to 0, 20, 40 and 60% saturation. The mixtures were stirred for 2 h at 4°C and later centrifuged at 9,000 rpm for 5 min at 4°C. The pellet was re-suspended in 25 ml of 0.05 M potassium phosphate buffer pH 7.0. Dialysis: was carried out against the same buffer for 18 h in spectrapor dialysis tubing. The heat deactivated compound (120 °C for 5 minutes) was used as a control. Bioasssay of the antimicrobial compound activity was Carried out for the different precipitated protein fractions.

2.7. Verification of the antimicrobial activity of the different protein fractions

Each separated protein fraction from the previously described partial purification was subjected to bioassay using *Staph. aureus* as an indicator strain.

III. Results & Discussion

3.1. Sample collection, enrichment and isolation of LAB

Enrichment and isolation of LAB from rhizospheric soil samples collected from healthy and diseased tomato and phaseolus plants from organic farms; was carried out by the accumulation method on MRS agar as described above. The enrichment and isolation process resulted in the isolation of 41 LAB bacterial colonies strains. Only 9 colonies showed a yellow zone around their growth indicating lactic acid production. All isolates showed whitish colonies and the microscopic examination revealed that all strains were coccoid or cocco-bacelloid in cell shape. Gram staining procedures indicated that 4 strains were G –ve, 37 strains were G +ve and all were catalase negative (**Table1**).

Table 1: Isolation of lactic acid bacteria (LAB) from rhizosphere of healthy and diseased tomato and Phaseolus plants

Property		Number of isolates
Lactic acid producing strains	+ve	9
	-ve	32
Gram staining	+ve	37
	-ve	4
Catalase reaction	+ve	0
	-ve	41

3.2. Screening of LAB for antimicrobial activity (bioassay):

Results shown in table 2 and Figure 1, clearly demonstrate that LAB strain No. 38 was the best antimicrobial compound producing bacterial isolate showing inhibition zones 1.4, 1.7, 2.5 cm against *S. aureus, P. aeruginosae* and *M. racemosus* respectively.

Table 2 Screening of antimicrobial activity of lactic acid bacteria

Isolate No.	Indicator strains	Inhibition zone (cm)
2	*Staph.aureus*	-
	P. aeruginosa	1.6
	M. racemosus	1.3
9	*Staph.aureus*	1.3
	P. aeruginosa	1.2
	M. racemosus	-
13	*Staph.aureus*	1.4
	P. aeruginosa	1.6
	M. racemosus	-
27	*Staph.aureus*	-
	P. aeruginosa	1.8
	M. racemosus	2.4
28	*Staph.aureus*	-
	P. aeruginosa	1.7
	M. racemosus	2.2
30	*Staph.aureus*	-
	P. aeruginosa	1.8
	M. racemosus	2.3
38	*Staph.aureus*	**1.4**
	P. aeruginosa	**1.7**
	M. racemosus	**2.5**
40	*Staph.aureus*	-
	P. aeruginosa	1.7
	M. racemosus	2.0
41	*Staph.aureus*	-
	P. aeruginosa	1.4

	M. racemosus	1.8

C *B* *A*

Figure 1: *Antimicrobial activity of LAB strain No.38 against (**A**) P. aeruginosa, (**B**) Staph. aureus and **(C)** M. racemosus. LAB No.38 is represented by No.5 well of the Petri-dish photo C.*

3.3. Identification of LAB isolate No.38

LAB strain No.38 was G +ve, whitish creamy in color, had round, smooth colonies (figure 2), catalase negative and it was the best antimicrobial compound producing bacterial strain. It was identified using 16S rDNA gene sequence analysis. The bacterial isolate was grown overnight and the total DNA was extracted using DNA extraction kits. Eubacterial-specific forward and reverse primers were used to amplify the 16S rDNA. PCR product analysed in agarose gel electrophoresis, purified from the gel and sequenced. According to the sequence and phylogenetic analysis, strain No.38 showed highest similarity (98%) with *Enterococcus faecium* and thus was designed as *Enterococcus faecium* strain MN 38. The 16S rDNA sequence was deposited in the GeneBank under accession No.MK280754.

Fig. 2: *LAB isolate No.38 grown on MRS agar at 37 °C*

In a similar research, A total of 119 lactic acid bacteria (LAB) were isolated from rhizosphere samples of olive trees and desert truffles by culture-dependent method and evaluated for different biotechnological properties, using the variability of the intergenic spacer 16S-23S and16s rRNA genes equences, the isolates were identified as the GENERA *Lactococcus, Pediococcus, Lactobacillus*, *Weissella* and *Enterococcus sp.*. All the strains showed proteolytic activity with variable rates 42% were EPS producers, while only 10% showed the ability to grow in 9% NaCl [21]. LAB strain No.38 showing the highest antimicrobial activity was selected for further investigation. LAB strain No.38 showed a white round colony with smooth surface (**Figure 1**).

3.4. Effect of some fermentation parameters on the production of antimicrobial compound by *Enterococcus facium* MN 38

The effect of different conditions on growth and antimicrobial activity of *Enterococcus faecium* strain MN 38 was investigated (**Table 2**). This process was carried out by the application of the bioassay described earlier against *Staph. aureus* as an indicator strain. The results revealed that the antimicrobial compound was produced by *E. facium* in temperatures ranging from 30-37 °C with an optimum production at 37 ºC and pH range from 5 to 7. For NaCl concentration; the antimicrobial compound production was observed in NaCL concentrations range 1-4%. The cells were Gram +ve cocci. Strain No.38 was able to grow at 25 to 40°C, with maximum growth at 37 C. It was able to grow in a pH range from 5 to 7. In addition, strain No.38 tolerated NaCl concentration up to 4%, exhibiting maximum growth and activity at 2%.

Table 2: Effect of the different physiological parameters on growth and antimicrobial activity Enterococcus faecium strain MN 38.

Parameter	**Inhibition zone (cm)**	
Temperature (C)	25	-
	30	1.2
	37	1.5
	45	-
pH	4	-
	5	1.2
	6	1.2
	7	1.4
	8	-
	9	-
Aeration (rpm)	100	-
	120	1.5
	150	-
NaCl Concentration (%)	1	1.4
	2	1.4
	3	1.2
	4	1.2
	5	-
	6	-
	7	-
Incubation period (h)	48	-
	96	1.5
	120	-

3.5. Production and extraction of Antibacterial Substance with Organic Solvents:

1 liter of MRS broth inoculated with 10 ml bacterial culture and incubated for 72 h in a shaking incubator (120 rpm) at 37°C.

Various organic solvents were tested for the extraction of peptide antibiotic produced by *Enterococcus facium* MN 38 from the cell free supernatant of the fermentation medium. It was observed that extraction with polar solvents such as hexane, di-ethyl ether and petroleum ether did not result in the removal of the antibacterial substance produced at the aqueous phase to the organic phase, while chloroform extraction completely destroyed the antibacterial substance activity. However, when different alcohols such as n-propanol and Iso-amyl alcohol were used in the extraction procedure,

antibacterial substance was removed from the aqueous phase and recovered from the organic phase (Table 3).

Table 3: Extraction of antimicrobial compound by organic solvents

Organic solvent	Mean diameter of inhibition zone (cm)	
	Organig phase	Aqueous phase
Iso-amyl alcohol	0.9	0.9
Chloroform	0	0
n-propanol	0.8	0
Hexane	0	0
di-ethylether	0	1.2
Petroleum ether	0	0

3.6. Precipitation of antimicrobial compound by Ammonium Sulfate and test for antimicrobial activity:

The cell free filtrate of as *Enterococcus faecium* strain MN 38 was subjected to fractional precipitation by ammonium sulphate and antimicrobial activity of all fractions against the indicator pathogens. As shown in **Table 4**, the fraction precipitated at 60-80% ammonium sulphate saturation exhibited antimicrobial activity against all of the indicator pathogens as verified by bioassay. The precipitation of the antimicrobial metabolite from the cell free culture filtrate by ammonium sulphate suggested its proteinaceous nature. Therefore, the bioactive protein/polypeptides produced by *Enterococcus faecium* strain MN 38 could be bacteriocin polypeptide since it was previously reported different bacteriocins production by other *Enterococcus faecium* strains. In a similar research done by Burianek and Yousef [14], bacteriocin was precipitated in the fraction 0-40% ammonium sulphate saturation.

Table 4: Ammonium sulphate precipitation of bacteriocin

Amm. Sulphate concentration (%)	Total protein (mg)	Antimicrobial activity		
		p. aeruginosa	*S. aureus*	*M. racemosus*
Crude culture filtrate	47.5	+	+	+
0-40	9.5	-	-	-
40-60	20.3	-	-	-
60-80	17.7	+	+	+

+/- referring to the presence/absence of inhibition zone due to presence/absence of a proteinaceous antimicrobial compounds

IV. Conclusion

A total number of 32 LAB strains showing antimicrobial activity were isolated from rhizosphere of healthy and diseased tomato and phaseolus plants. Among which LAB isolate No.38, identified as identified as *Enterococcus faecium* strain MN 38, exhibited the highest antimicrobial activity against the indicator pathogens and it was the only isolate showing antifungal activity against the phytopathogen, *M. racemosus*. The bioactive metabolite produced by *Enterococcus faecium* strain MN 38 was found to be protein in nature. The results indicated a high rate of antimicrobial activity among the rhizosphere-derived LAB strains, suggesting that rhizosphere may be a promising source for various bioactive metabolite for potential biotechnological applications including biocontrol of phytopathogens, post-

harvest crops deleterious and soil-born pathogenic bacteria and fungi. Further investigations to elucidate the nature of inhibitory action will be in the next report.

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