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Research Article

How to link: Plasmid Curing and Lead Tolerance Ability of *Pediococcus pentosaceus*

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ABSTRACT
Pediococcus pentosaceus has a high level of resistance to heavy metals, making it one of the biological alternatives for dealing with heavy metal contamination in the environment. The current study sought to identify the genetic factors responsible for this ability by curing the plasmid of these bacteria using various curing agents (Acridine orange and Sodium Dodecyl Sulfate). The findings demonstrate that both curing agents had perfect curing ability. The bacteria were able to tolerate a wide range of Lead concentrations (50-2000 ppm). This capacity was reduced when the plasmid was removed, but it did not disappear, implying additional resistance genes on the chromosomes. The antibiotic susceptibility observations supported the significance of plasmid genes in lead resistance ability, the findings revealed differences in the pattern of antibiotic resistance between wild and cure plasmid bacteria, the wild one had different antibiotic MIC values for Nitrofurantoin and Trimethoprim/Sulfamethoxazole (<=16 and <=10 μg/mL) respectively), on the other hand for the same antibiotics, the MIC results for plasmid-cured bacteria were 64 and 80 g/mL. Based on the findings, we can conclude that plasmid genes play a significant role in *Pediococcus pentosaceus* to resist lead, and there is a strong correlation between antibiotic resistance and lead resistance.

INTRODUCTION
Heavy metal contamination threatens all living organisms by consuming contaminated food and water. For example, lead (Pb) is one of the most dangerous heavy metals and major environmental pollutants, ranking second and seventh on the list of the most dangerous materials (Jaafar 2020). Bioremediation tactics have recently been employed to eliminate heavy metal contamination in the environment, but the most important ones from these techniques, which rely on bacteria's usage (Jaafar 2020).

Bacteria have used a range of techniques to remove heavy metals, the most well-known of which is the discharge of heavy metals outside the cell. Heavy metal resistance genes have been detected in both plasmids and chromosomes (Marzan et al. 2017). Moreover, according to research, there's a link between antibiotic and heavy metal resistance when they are both present on the same plasmid (Samanta et al. 2012).

Lactic acid bacteria LAB is a category of bacteria that reacts positively to gram stains. They have a rod or coccus shape under the light microscope, and their DNA contains less than 55% mol of G+C (Axelsson 2004). Plasmids are self-replicating genetic materials found in lactic acid bacteria, and their presence has drawn a lot of attention because of the numerous roles they are linked to (Cui et al. 2015). Even though the presence of plasmids in bacteria is not required for their survival at this time, they do provide several benefits, the most notable of which is the ability to withstand various stress conditions, such as antibiotic resistance and heavy metal contamination, which allows the organism that carries it to have a more remarkable ability to survive in the event of competition with other organisms in the same environment (Wegrzyn & Wegrzyn 2002). All these capabilities may be due to the plasmids confer adaptive advantages, improving the growth and behavior of their host cells.

Plasmids can be found in a variety of lactic acid bacteria, including *Bifidobacterium*, *Brevibacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Tetragenococcus*, and *Weissella*. The plasmids in LAB bacteria have different sizes (0.87 kb to more than 250 kb), copy number (from 1 to more than 100 plasmids per cell), and phenotypes conferred to their hosts (Schroeter & Klaenhammer 2009; Mills et al. 2006; Ainsworth et al. 2014; Qin et al. 2012).

Pediococcus sp. mainly, *Pediococcus pentosaceus* and *Pediococcus acidilactici* have different types of plasmids, ranging in size from 1.82–190 kb. Some of these plasmids encrypt a different function, such as the utilization of raffinose and sucrose (Gonzalez & Kunka 1986), resistance toward antibiotics (Tankovic et al. 1993), as well as production of bacteriocin and immunity (Cui et al. 2012). It was also discovered that certain strains had multiple plasmids, allowing the bacteria to ferment sugars such as raffinose, melibiose, and sucrose, and produce bacteriocins (Alegre et al. 2005; Teresa Alegre et al. 2009).

So, this study aims to determine the effects of plasmid-removing compounds on *Pediococcus pentosaceus* resistance to various Lead concentrations. The objectives include isolation of naturally occurring bacteria from fish aquaculture, genetic identification of *Pediococcus pentosaceus*, screening of their Pb heavy metal resistance, comparison of resistance after plasmid curing using two curing methods, and selection of an effective curing agent.

MATERIALS AND METHODS

Isolation and identification of *P. pentosaceus*

Three fishponds on the Al- Garma campus of Basrah University, Marine Science Center, were used to collect water samples using 500 mL glass sterile bottles. The ponds' coordination's are shown in Table 1; in this project, a serial dilution approach was used with sterile physiological saline and plated on MRS Agar; the plates were incubated at 30 °C for 72 to 96 hours. Then pure colonies were identified using some morphological and also employed biochemical characteristics (H2S formation, Catalase, Nitrate reduction, Ure-

ase, Indole Production) (Bergey 1994), VITEK II (Biomerieux, USA), a bacterial identification tool. The 16S rRNA gene was amplified and compared to NCBI reference strains for further emphasis.

Table 1. The Coordination of fish ponds.

Sampling ponds	Latitude	Longitude
1	30° 33' 39.91"N	47°44'28.34"E
2	30° 33' 35.37"N	47°44'25.31"E
3	30° 33' 38.47"N	47°44'30.20"E

Amplification of 16S rRNA gene

16S rRNA gene was targeted for PCR amplification. The following primers were used: Forward- 27F: 5'- AGAGTTTGATCCTGGCTCAG - 3' and reverse – 1492R: 5'- GGTTACCTTGTTACGACTT - 3'. Each PCR reaction tube contained 25 µL mixtures of Bioneer master mix, 2 µl of purified DNA (50 ng/µL), 3 µL of forward and reverse primer (62.5 µmol/L) and H_2O to complete the volume to 50 µl. Thermocycler (3Prime, UK) with the following thermal profile; initial denaturation step at 94°C for 3 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min and extension at 72°C for 1 min, and ending it with a final extension at 72°C for 10 min. To visualize the amplicons, a 1% agarose gel was run (Miyoshi et al. 2005).

The sequencing and analysis of DNA

Eighteen µl of PCR product (forward and reverse) were prepared and sent to MACROGEN/Korea for DNA purification and sequencing. The F and R sequences obtained were first processed with SnapGene software. After sequencing, the 16S rRNA sequences results of the bacteria were revised then aligned with the NCBI data bank to compare with the other resemble bacteria.

Preparation of plasmid cured bacteria

Overnight, *P. pentosaceus* was cultured in nutrient broth. Two tubes (each one contains five mL of nutrient medium) were prepared, one containing 1 mg/mL acridine orange and the other one containing 1 mg/mL sodium dodecyl sulfate (SDS), bacteria were then sub-cultured in the prepared media and incubated at 37°C for four days, and after that 0.5 mL of the growth was cultured on two separate nutrient agar plates, and the colonies that were able to grow were classified as cured bacteria, which succumbed to profile the plasmid to approve the cure of plasmids (GG & OH 2016).

Plasmid profiling

The plasmids were isolated according to the Qiagen kit's instructions, and the loss of the plasmids (plasmid profiling) was confirmed using gel electrophoresis.

The following were the expected outcomes:

A. There are no bands on the gel, indicating that the plasmids were lost, so the plasmid curing technique was successful.
B. The presence of plasmids and failure of the plasmid curing technique is shown by the formation of bands in the gel.

Antibiotic susceptibility test

The VITEK II was used to test bacterial resistance to several antibiotics (for both wild and plasmid cured bacteria). The tests are run likewise on cards which have of diminution of antimicrobials to appoint the breaking point, which mentioned to the minimum inhibitory concentration (MIC) of antibiotics, as well as the Advanced Expert System (AES) was used in conjunction with the VITEK II automated antimicrobial susceptibility test system to ascertain the beta-lactamase in tested bacteria.

Heavy metal tolerance assays

Preparation of heavy metals concentrations

To make stock solutions from lead, the exact weight of Pb $(NO_3)_2$ was dissolved in sterile deionized distilled water. Different concentrations of Pb (II) were then prepared using an appropriate serial dilution of these stock solutions (Jaafar 2020).

The Minimum Inhibitory Concentration of wild and plasmid cured *P. pentosaceus*

The MIC test was usually used to determine the lowest concentration of lead required to suppress *P.pentosaceus* growth. The following is how the test was carried out; on a Nutrient agar plate, 0.1 mL of a pure broth culture of *P. pentosaceus* bacteria grown overnight at 37°C in MRS broth (MRS, Hi medium) was spread aseptically, then, in the center of the plates, 6mm diameter paper discs saturated with various concentrations of lead (25, 50, 100, 250, 500, 1000, 1500, 1800, and 2000 ppm) were placed. The plates (including the control) were then incubated for 24 hours at 37 °C, the diameter of the zone around the disc was calculated (Raghad et al. 2016). This experiment was done in triplicate; acridine orange-cured bacteria, SDS-cured bacteria, and wild-type bacteria.

RESULTS AND DISCUSSION

Isolation and identification of *P. pentosaceus*

For further diagnosis, we used an isolate that gave positive gram stain and negative catalase reactions in the current study. The bacteria was initially identified as *P.pentosaceus* based on its morphological, cultural, and biochemical properties (Table 2). The colony characteristics of these bacteria were investigated by handling the colony aseptically and transferring it to the selective medium (MRS) to monitor the isolates' growth patterns. Initially, the colonies were thought to belong to the genus *P. pentosaceus* because they were

creamy white, circular, low convex, and had a complete rim (Table 2).

Table 2. *P. pentosaceus* morphological feature

Biochemical tests	
H2S formation	-
Catalase	-
Nitrate reduction	-
Urease	-
Indole Production	+
Colony	**Morphology**
Arranging	Round
Shape of cell	Round
Motility	Non motile
Color of colony	White Creamy
Gram stain	Positive
Outward	Mucoid

Genetic identification

The agarose gel electrophoresis was carried out for five replicates to check the PCR results of the amplified 16S rRNA gene. The bands of about 1450bp were observed on the gel, as shown in (Figure 1).

The amplified 16S rRNA gene sequencing results were revised and analyzed using the Basic Local Alignment Search Tool (BLAST) to search for a similar sequence in the national center for biotechnology information database. The result was diagnosed as *P. pentosaceus*.

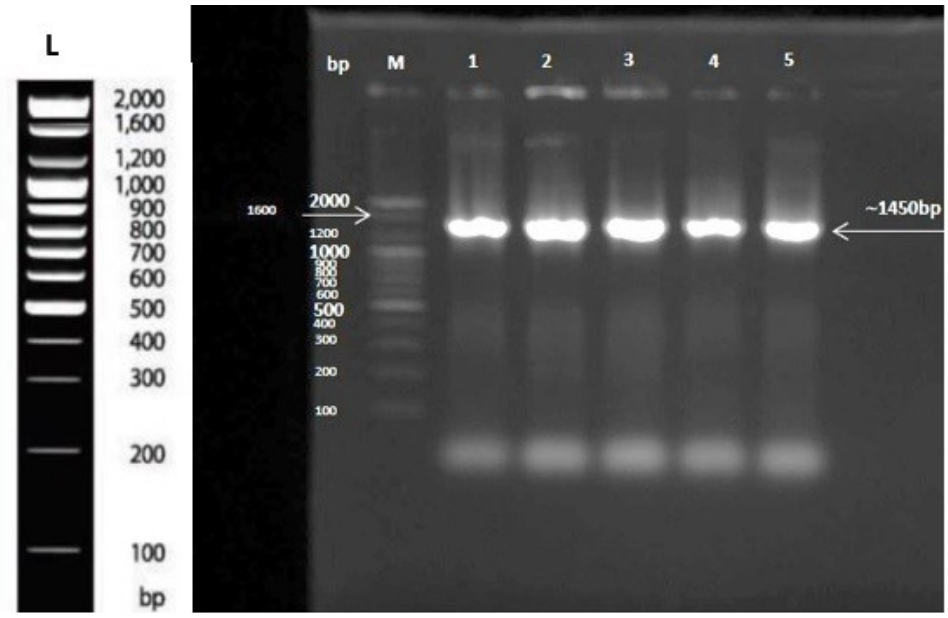

Figure 1. The analysis of 1% agarose gel electrophoresis of extracted DNA from pure bacterial colonies amplified by universal 16S rRNA primer: M: 100bp marker; 1, 2, 3, 4, 5: five replicates of isolated bacteria; standard 100bp ladder.

Plasmid profiling of wild and cured bacteria

Acridine orange and SDS have indicated a positive curing results, and that agree with (Sulochana M.B., Arunasri R 2014; Haque 2017). The current work focuses on the relationship between *P. pentosaceus*' ability to tolerate different concentrations of Lead and the loss of the plasmids and identifying an effective curing agent.

In contrast to the wild type, which exhibited a single band in lane (1), the two materials employed to remove plasmids from P. pentosaceus had a good effect, as no bands formed during the gel electrophoresis process in both lanes (2) and (3) (Figure 2), the single band in lane (1) representing a size of about 10kbp appeared as a result of the plasmid extraction from it, and this proves the success of extraction process. Species *of Pediococci*, mostly *P. pentosaceus* and *P. acidilactici*, have plasmids, extending in size from 1.82–190 kbp encode different functions (Cui et al. 2015).

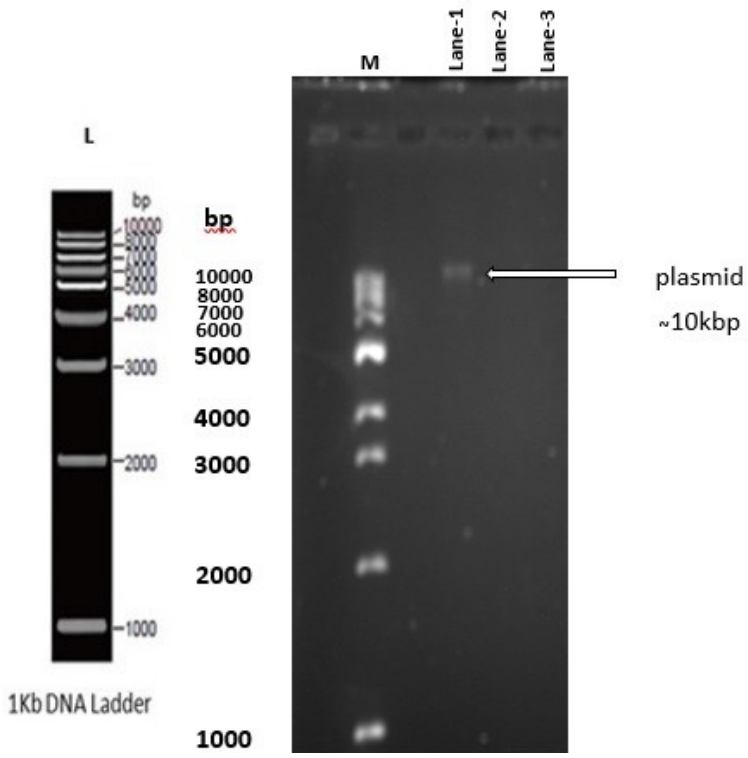

Figure 2. Left (standard 1kb DNA ladder); Right (M:1Kb DNA Ladder, lane-1: plasmid profile of wild-type *P. pentosaceus*, lane-2: plasmid profile of cured-type bacteria with 1mg/mL of SDS, lane-2: plasmid profile of cured-type bacteria with 1mg/mL of acridine orange.

Antibiotic susceptibility in the wild, and plasmid curing

P. pentosaceus

Table 3 demonstrates the susceptibility pattern of *P. pentosaceus* (wild and plasmid cured) to various antibiotics, based on the MIC value, which indicates whether the bacteria are sensitive, mild opponents, or opponents to antimicrobials. The current study's findings show that the wild kind of bacteria tested was responsive to a wide range of antibiotics with varying MIC values. The plasmid curing bacteria by both of acridine orange and SDS

Table 3. The results of the antibiotic sensitivity pattern to the various antibiotics using VITEK II.

	Wild		Plasmid Cured by SDS		Plasmid Cured by acridine orange	
Antimicrobial	MIC	interpretations	MIC	interpretations	MIC	interpretations
Beta-lactamase	POS	+	NEG	-	NEG	-
Levofloxacin	<=0.12	S	<=0.12	S	<=0.12	S
Erythromycin	1	I	2	I	4	I
Linezolid	1	I	1	S	1	S
Teicoplanin	<=0.5	S	2	S	2	S
Vancomycin	<=0.5	S	4	S	4	S
Tetracycline	<=1	S	<=1	S	<=1	S
Tigecycline	<=0.12	S	<=0.12	S	<=0.12	S
Nitrofurantoin	<=16	S	64	I	64	I
Trimethoprim/ Sulfamethoxazole	<=10	R	80	R	80	*R

*=AES modified, POS: positive, NEG: negative, S: sensitive, I: intermediate, R: resistant.

agent have differed sensitivity manner, they were resistant to some type of antibiotic (Nitrofurantoin, Trimethoprim/Sulfamethoxazole), with MIC value, 64 and 80 µg/mL respectively. The fluctuated result may be due to the elimination of plasmids harboring antibiotic resistance genes.

Beta-lactams are the most widely used group of antimicrobial agents in human bacterial disease treatment; a high rate of mutations characterizes BL-encoding genes. Moreover, these genes are usually found in mobile genetic elements (plasmids, transposons), which facilitates their rapid spread among infectious bacteria, as well as bacteria inhabiting the natural environment (water, soil) (Rozwandowicz et al. 2018).

As shown in Table 3, beta-lactamase was indicated as a positive result in the wild *P. pentosaceus*; while this enzyme wasn't detected in both cured bacteria, and that may be due to the loos of plasmid carrying the beta-lactamase coding genes, these results demonstrate the loss of Plasmids during the curing process.

MIC of wild and plasmid cured *P. pentosaceus*

The MIC of (1000, 1500, and 1800) ppm was the clearest different inhibition results so that we add it in the (Figure 3) which shows the effect of the Minimum inhibitory concentration of Pb on the *p. pentosaceus*, the wide diameter of the inhibition zone related to the wild bacteria comparing to the narrow zone of the cured one with SDS and acridine orange, so that the removal of plasmids has a detrimental effect, resulting in reduced bacterial resistance to Pb concentrations. On the other hand, the bacterial resistance did not decline permanently, and that may be due to the chromosome-borne genes responsible for metals resistance as well as these harboring on plasmids, and that agree with (Carattoli 2003) that suggested that these genes can exchange between plasmid (s) and the bacterial chromosome.

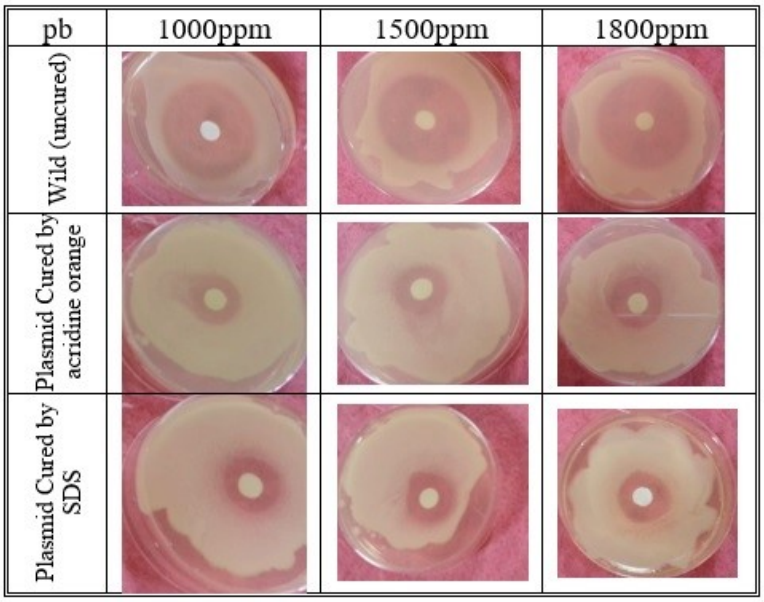

Figure 3. Selected MIC effect of (1000, 1500, 1800) ppm on the wild and cured *P. pentosaceus*

Figure 4 illustrates that both materials (acridine orange and SDS) employed in the plasmid curing technique had a good effect; the effect was nearly similar, also the figure demonstrated that the diameter of the inhibition zone increased gradually as a result of an increase in the Pb concentration, and that due to the increment the harsh and toxic effect of the heavy metal on the bacterial metabolic activity (Sobolev & Begonia 2008).

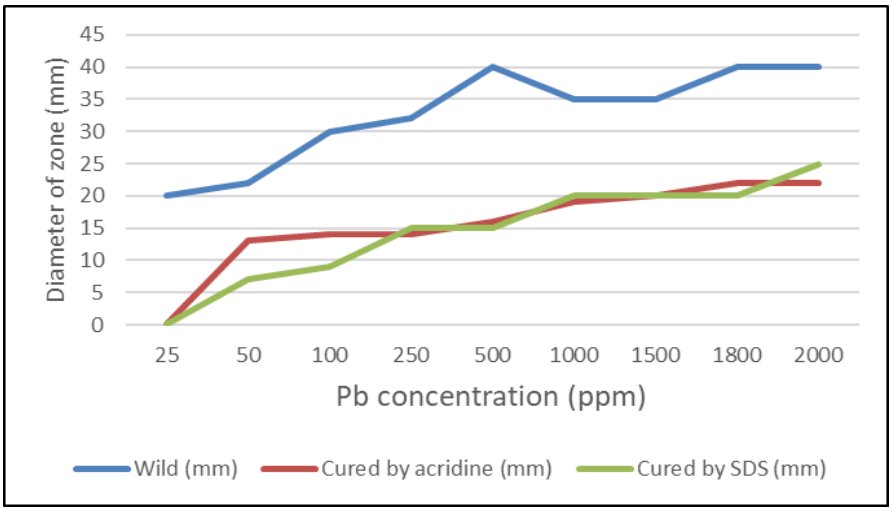

Figure 4. Concentrations of Pb and the diameter of inhibition zones in wild and cured *P. pentosaceus*

CONCLUSION

Based on the MIC value, the fluctuated result may be due to the elimination of plasmids harboring antibiotic resistance genes. Beta-lactams are the most widely used group of antimicrobial agents in human bacterial disease treatment, Beta-lactamase was indicated as a positive result in the wild *P. pentosaceus*; while this enzyme wasn't detected in both cured bacteria, and that

may be due to the loos of plasmid carrying the beta-lactamase coding genes.

The removal of plasmids leads to reduced bacterial resistance to Pb concentrations. However, the bacterial resistance did not decline permanently, and that may be due to the chromosome-borne genes responsible for metals resistance as well as these harboring on plasmids; on the other hand, both acridine orange and SDS have a positive effect.

AUTHORS CONTRIBUTION

All authors contributed to the data conception, design, analysis, and interpretation, as well as authoring or critically editing the article for key intellectual content.

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CONFLICT OF INTEREST

On behalf of all authors, the corresponding author confirms that there have been no involvements that could raise questions about bias in the work reported or in the conclusions, implications.

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