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Experimental eradication of heavy metal ions via bacteriocin TXJ for Lactobacillus salivarius strain from greek yogurt

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***Abstract*---**Pollution in general is constantly increasing being polluted by environmental pollution with heavy metals that may be located in air, soil, water, or even food. In spite of being bacteria in general are harmful to humans, they are sometimes considered very valuable and safety, especially in biological treatment like probiotic bacteria group. *Lactobacillus salivarius* one of the maximum essential sorts of microorganism which are harmless to human and that may be play very capability position to cast off and removal heavy metals in different media. The main object in this study is extract bacteriocin TXJ for *Lactobacillus salivarius* isolated from Greek yogurt, which performs very important function by experimentally in biological reaction to the different heavy metal ions and reduce their concentrations and hopefully to using in the near future for treatment and reduce the pollution in the environment. This project there were used three various concentrations 20, 40, 60 PPM for five main heavy metals (Cu, Cd, Pb, Cr and Zn), which were added to extracted bacteriocin. Atomic absorption technique was used for determine the levels of heavy metal. Furthermore, the experiment was recorded based on the comparison for before and after adding bacteriocin protein at the lab. Astonishing, the results showed that bacteriocin TXJ has a high ability in the process for removing harmful all heavy metals that used for this study. The highest percentage level removal of the heavy metal was Iron (Fe) and Zinc (Zn) for 10, 20, and 40 ppm

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at almost 70- 95%. In contrast, in terms of Chromium (Cr) and Copper (Cu), the percentage level removal was between 35-68%. While Cadmium (Cd) level was removed by less than 32%.

***Keywords*---**lactobacillus, bacteriocin TXJ, atomic absorption, heavy metals.

Introduction

Heavy metals are considered a real threat to the environment, as they have a negative impact on public health. Heavy metals are known as metallic traces that have a comparatively high concentration contrast to water (Fergusson, 1990). Recently, there has been raising environmental and international public health attention related to ecosystem contamination by many kinds of elements (Bradl, 2005; Duffus & chemistry, 2002). Addition, human being exposure has increased clearly as a consequence of the repeated use in different aspects like agricultural and industrial purposes. There are many sources of environmental contamination that can be happened to heavy metal pollution like metal erosion, atmospheric precipitation, soil abrasion of metal ions and releasing, evaporation, and residue for all heavy metals through the water resources to topsoil and reaching the groundwater (Nriagu, 1989). Also, Natural phenomena like mounts weathering and volcanic explosions have also been identified to dramatically engage to increase the percentage of heavy metals accumulations (Shallari, Schwartz, Hasko, & Morel, 1998). As well as, Industrial provenances such as metal treatment in mines, using charcoal in power stations, petroleum burning, nuclear power plants and Electronic units, plastics industries , textile factories, wood, and wood-processing plants (Arruti, Fernández-Olmo, & Irabien, 2010; Sträter, Westbeld, Klemm, & Research, 2010).

Bacteriocins extracted from Lactobacillus spp. is known as polypeptides protein that probability acts as an antibacterial and other activity (Guo et al., 2020). The utilization of bacteriocins has several features related to their quality effectiveness, low toxicity and non-specific residue pointing(Barbosa, Caetano, & Mendo, 2015; de Lima, de Moura Fernandes, & Cardarelli, 2017). *Lactobacillus salivarius* is one of bacterium that produces many different kinds of bacteriocins, it is probiotic Gram-positive, and usually present at the community bacteria of dairy products, fermented foods and beverages (Mokoena, Mutanda, Olaniran, & Research, 2016). The characterization of bacteriocin has possessed the ability to interact directly with heavy metals and decreased their level as a precipitate (Riley, 2009).

Materials and Methods

Isolation and Identification of bacterial

Samples have been isolated from Greek yogurt (goat's milk) after cultured on MRS specific Lactobacillus ssp. Then, the bacterial cell has confirmed that gram positive by using Gram stain kit. Also, the micro-organism was analyzed biochemical methods called VITEK2 technique and it was identified *Lactobacillus*

salivarius. Finally, the molecular tool (PCR) was used to confirm and determine the eight housekeeping genes (*clpX, dnaK, groEL, murC, pepX, pyrG,rpoB, and uvrC)* of bacteria and defining it definitively that *Lactobacillus salivarius* by appeared all PCR fragments genes on gel at similar molecular weight of the origin theoretically.

Bacteriocin production

Twenty three grams of Nutrient broth had been weighed and dissolved in a single liter of distilled water, then sterilized with Autoclave and left to cool. Then the sterile liquid turned into inoculated with Lactobacillus microorganism developing on MRS agar medium and incubated in an incubator 28-30°C for 72 hours till the protein commenced to seem and grow. Then the bacterial culture was centrifuged at (5000) rpm for (15) seconds, then The obtained filtrate was sterilized using microfilters with a diameter of 0.20µl, then it was cultured on the nutrient agar media and incubated under the same conditions above to ensure its purity and free from any bacterial contamination (Abo-Amer, 2007). The bacterial filtrate was dried by placing it in glass Petri dishes by the electric oven at 35-40°C until it dried well, thus the dry powder of the filtrate of Lactobacillus sp. Which was preserved in tubes with a capacity of one cm^3 by freezing until it is used in the study.

Table1. The primers sequences for housekeeping genes for Lactobacillus salivarius that used in this study

	The name of primer	Sequencing
1	*clpX/F*	CGCACGGAAGCAGAAAC
2	*clpX/R*	GAGTCGGTCCCAAACCC
3	*dnaK/F*	GACAACGGTCCGCTCCACT
4	*dnaK/R*	TCGGCTTCTTCCTTCTTCTTCT
5	*groEL/F*	CCGACAACGACAAGATGG
6	*groEL/R*	CCAAGGCAGGGATAACG
7	*murC/F*	TTTGAAGCCGACGAATACC
8	*murC/R*	CGATGTCCTCGCTACCC
9	*pepX/F*	AAAGAAGACGAGCAACCAACC
10	*pepX/R*	CGGAGTCCTTAGTCCCGATT
11	*pyrG/F*	TCATTGGGTCGGCTGTT
12	*pyrG/R*	GGTCCATCCCTTGCTTTTG
13	*rpoB/F*	GAAGTTCCGCCGCTCTA
14	*rpoB/R*	GGTCCCATCTGGCATGTAC
15	*uvrC/F*	TCGTCACCTCCTCCAATAA
16	*uvrC/R*	TGGTTCGGTAATCCCTCC

Dodecyl Sulphate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

The bacteriocin protein extraction process was identified and diagnosed from the bacterial culture, and the molecular weight of bacteriocin protein was determined and compared with the molecular weights according to biochemical analyzes and NCBI website, Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

(SDS-PAGE) has been applied and used for this purpose. Then, using Phyre2 website to figure out the structural three dimension of protein after determine the precise molecular weight and protein sequences (Albanna & Alomari, 2020).

High Performance Liquid Chromatography (HPLC)

In order to determine the molecular mass and bacteriocin TXJ, HPLC were Identified the sharp peak through the high affinity of mobile phase water: methanol (20:80), flow rate 1ml/ min , detector 280 nm, column C18 and the injection 20µl, then the final calculate of protein concentration after compared to the standard level. Notably, the protein identification via HPLC was followed and according to the Shimadzu company protocol.

Determination the heavy metals levels

The determination of the heavy metals level and the samples were carried out by Analytik Jena GmbH - novAA 350 - Flame Atomic Absorption Spectrometer and the wavelength of each Cu, Cd, Pb, Cr and Zn were 580, 228.8, 283.3, 373 and 213.8 nm respectively. In order to examination bacteriocin TXJ activity, three different concentration prepares 20, 40 , 60 ppm each of Cu, Cd, Pb, Cr and Zn and adding 20 ml bacteriocin for each heavy metal. For quantifying, standard curve have created for each heavy metal started from concentrations (0.5, 1, 3, 5, 10, 20, 40 ppm), take into consideration the wavelength for Cu: 580nm, Cd: 228.8nm, Pb:283.3nm, Cr:373nm and Zn: 213.9nm. According to industrial protocol, the results of the sample were blotted into the standard curve and get it precise concentration.

Results and Discussion

The results of isolation and identification of *Lactobacillus salivarius* from Geek yogurt that has confirmed Gram positive bacteria under the microscope, sixty three positives biochemical analysis via VITEK2 technique and eight essential housekeeping genes have approved by polymer chain reaction (figure 1). The results was matched compared to the previous studies for conversional isolation and identification bacteria (Bin Masalam et al., 2018). Also, for VITEK 2 results, the biochemical analysis was similar responded according to the bioMérieux company consequence (Mahmood, Hameed, & Sciences, 2018). Furthermore, in terms of housekeeping genes, the eight DNA fragments have similar compared to recent study (Xu et al., 2022).

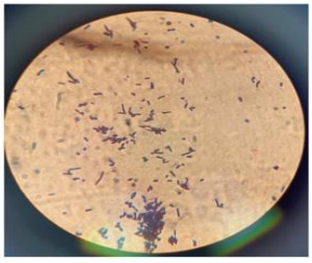
In contrast, after production adequate volume of bacteriocin, it was estimated the protein mass by SDS-PAGE gel and the molecular weight was 10 kDa (A-figure 2). Then identify the kind of protein via High Performance Liquid Chromatography (HPLC) through the using suitable mobile phase ratio (20:80 water and methanol), and the protein called bacteriocin TXJ after compared to the standard item according to Shimadzu co. protocol (B-figure 2).

Furthermore, the bacteriocin TXJ has evaluated the interaction directly of heavy metals by adding 20, 40, and 60 ppm each of Cu, Cd, Pb, Cr and Zn, and then each concentration added 20 ml of bacteriocin liquid separately, and their

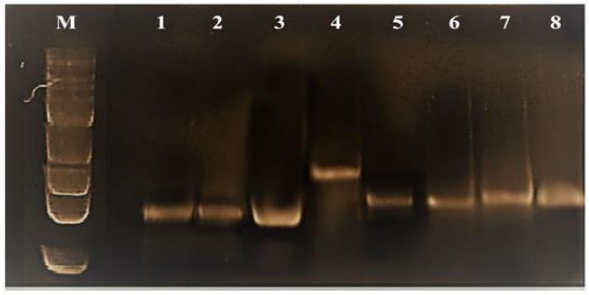
determined by Flame Atomic Absorption. This experiment compared the values of heavy metals before and after added the standards into the bacteriocin. The highest significantly percentage of reduction was Zn between (92.99 – 97.92%). Also, in term of Fe was (68.45-84.80%), while in Cu and Cr were (34.98-68.16%). In contrast, the dramatically percentage of reduction was (27.63-32.53%) as showed table 2 & figure 3. During the experiment, the standard curves of each (Cu, Cd, Pb, Cr and Zn) have figured out in order to plotting the results values and determination precisely (figure 4).

Taking together, the removal activity of heavy metals have gave results astonishing in particular in Zn and Fe. On the other hand, possible to using the bacterial biomass extraction by design a special station to eradicate these heavy metals to keep the surrounding environment free from the harmful effects in spite of a little decrease levels happened such as Cd element.

A. VITEK2 tool for sixty three biochemical analysises for *Lactobacillus salivarius*



B. Gram stain of *Lactobacillus salivarius*,the bacilli showed Gram-positive under the 100X microscope objective



C. DNA PCR fragments for eight housekeeping genes for *Lactobacillus salivarius* included 1-8 (*clpX, dnaK, groEL, murC, pepX, pyrG, rpoB, and uvrC),* while the M is 1KB ladder for comparison.

Figure 1. Stages of Isolation and Identification for *Lactobacillus salivarius* included: Gram stain, VITEK2 and PCR techniques

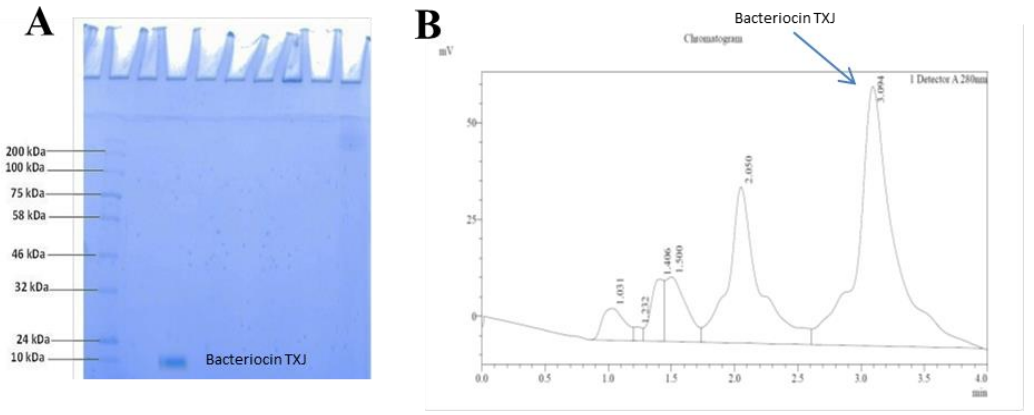


Figure2. (A) SDS-PAGE gel for determine bacteriocin in general stained with Coomassie blue at lane 2, and compared to protein marker at 10 kDa. (B) High Performance Liquid Chromatography (HPLC) for identified the bacteriocin TXJ at retention time almost 3 min, the peak come up at the height 67.082 mV and the detector was 280 nm according to Shimadzu co. protocol.

Table 2. The bacteriocin TXJ examination by added three various concentrations for (Fe, Cr, Cd, Zn, and Cu), and the percentage of reduction for each heavy metal. Note all the experiments have tested via Flame Atomic Absorption technique

Heavy metals	Concentrations	Before Incubation	After Incubation	Reduction	Reduction percentage %
Fe	20ppm	17.383	5.4852	11.8978	68.45
	40ppm	28.1295	4.4441	23.6854	84.20
	60ppm	28.811	4.3508	24.4602	84.90
Cr	20ppm	19.6926	12.8039	6.8887	34.98
	40ppm	22.5163	8.6589	13.8574	61.54
	60ppm	32.9331	11.5017	21.4314	65.08
Cd	20ppm	18.9779	12.8039	6.174	32.53
	40ppm	23.7635	16.6749	7.0886	29.83
	60ppm	26.7055	19.3269	7.3786	27.63
Zn	20ppm	19.6465	0.7735	18.873	96.06
	40ppm	23.5632	1.6514	21.9118	92.99
	60ppm	24.5611	0.5118	24.0493	97.92
Cu	20ppm	18.9237	6.521	12.4027	65.54
	40ppm	38.7791	12.3473	26.4318	68.16
	60ppm	59.2007	24.7765	34.4242	58.15

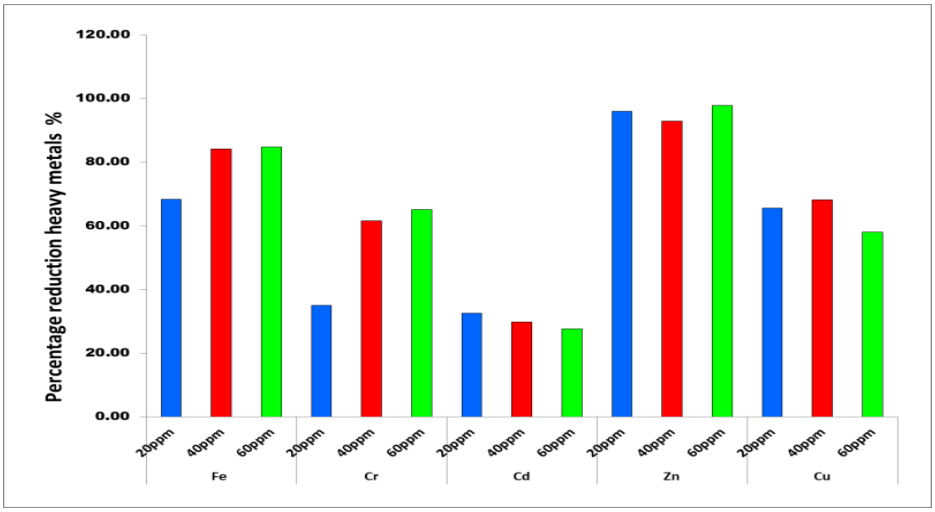


Figure 3. Comparison ability of bacteriocin TXJ reduction for three different concentrations of heavy metals (Fe, Cr, Cd, Zn and Cu). The highest percentage was Zn, while the lowest percentage was Cd.

Conclusion

The pilot study targeted at the capability that high percentage removal of most various heavy metals by bacteriocin TXJ extracted from probiotic bacteria *Lactobacillus salivarius*, and the final outcome was satisfied scientifically and safety. In terms of Zinc (Zn) and Iron (Fe) were 70-90% removals. While, Chromium (Cr) and Copper (Cu) were 35-68%. In contrast, the lowest percentage of removal was Cadmium (Cd) was 32%. Also, The main reason for this study is to find a biological remedy to getting rid of these minerals that are relatively quite difficult, in particular, there are many methods of removal accompanied by harmful side effects on the public health and the environment. Overall, the high significance of an efficient Bacteriocin TXJ extracted from probiotic bacteria was shown to capture a good reduction in a wide variety of heavy metals like (Zn, Fe, Cu, and Cr), but in Cd, the results found the percentage of the reduction was less than expected and could be considered potentially unstable to eradicate fully by bacteriocinTXJ.

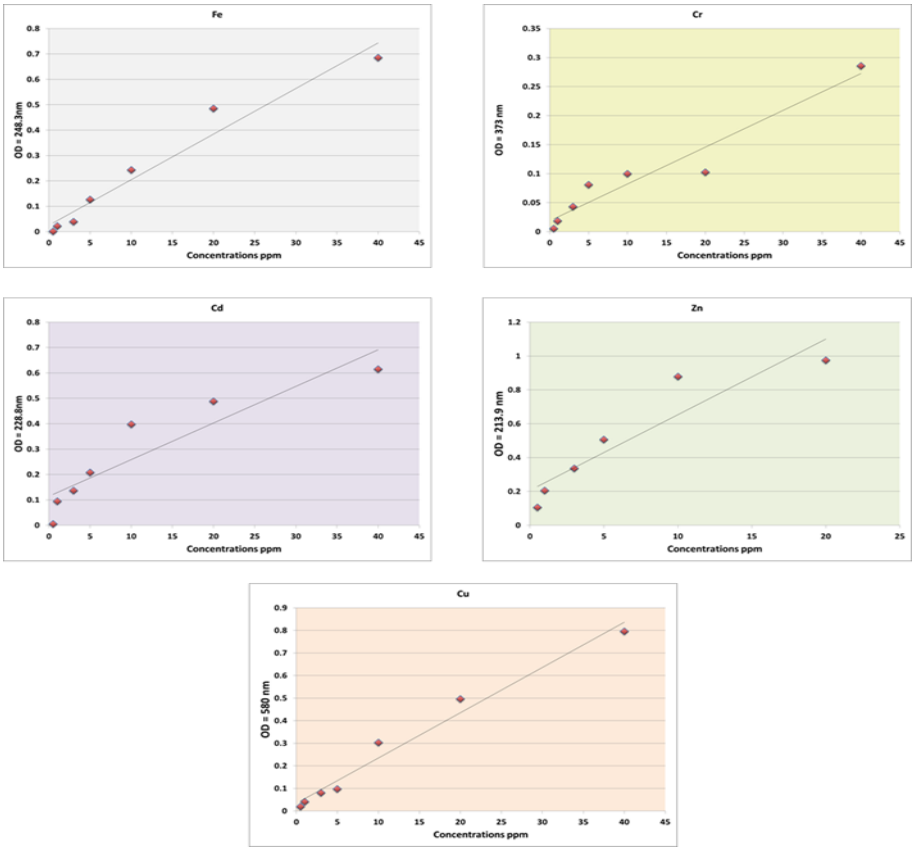


Figure 4. Standard curves for Fe, Cr, Cd, Zn, and Cu elements starting from (0.5, 1, 3, 5, 10, 20, 40ppm), in order to plot and measure the unknown sample at the Flame Atomic Absorption Spectrometer.

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