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Original Article

Isolation of *Lactobacillus* from Milk: Harnessing its Potential for Bioremediation of City Lakes through Metal Quenching

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Abstract *- Rapid development of industrialisation has led to the release of a large number of toxic metals into the environment, especially water bodies. Water pollution in Delhi due to industrial discharge is a major issue for the city. Bioremediation could be a natural, cost-effective method to overcome this problem. Lactic acid bacteria have shown binding with metal ions via adsorption and ion exchange methods. Interactions of Lactobacillus with metal ions and their possible applications have received less attention. In this study, we isolated Lactobacillus bacteria from raw milk samples to study their quenching efficacy for metal salts $CuSO_4$, $ZnSO_4$ and $Pb(NO_3)_2$. The results indicated that Lactobacillus very effectively quenched $Pb(NO_3)_2$ even at 500 PPM concentration, whereas in the case of $CuSO_4$, the efficacy decreased with increasing salt concentration from 300 to 500 PPM. The bio quenching/biosorption abilities of Lactobacillus will help to formulate natural treatments to decrease metal toxicity from water bodies when upscaled.*

Keywords *- Metals, City Lakes, Lactobacillus.*

1. Introduction

In Delhi, water pollution is a serious environmental problem that degrades the city's water supply. Industrial discharge, sewage treatment, agricultural runoff, and illegal trash dumping are some causes of water pollution in Delhi. Industrial discharge is one of the main causes of water pollution in Delhi. Numerous enterprises in the city discharge dangerous chemicals into the water bodies without first treating them, polluting the water and rendering it unfit for consumption. Water pollution in Delhi is also a result of sewage treatment facilities. These facilities frequently do not perform to their full potential, causing the discharge of untreated sewage into the water bodies. Water pollution in Delhi is also significantly influenced by illegal trash disposal. People often dispose of their waste in rivers and other water bodies, contaminating the water and causing a health hazard due to the toxic heavy metals. Toxic metals are substances harmful to living organisms when ingested, inhaled, or absorbed through the skin.[1] They can cause various health problems, depending on the metal type and exposure amount. Some common toxic metals include lead, mercury, and arsenic. Exposure to lead can cause serious health problems, particularly in children, such as developmental delays, behavioural problems, and cognitive impairments.[2] Adult exposure to lead can result in kidney damage, high blood pressure, and reproduction issues. Bioremediation is a successful strategy for the detoxification of these heavy metals. In the bioremediation process, pollutants are broken down and removed from the environment using living organisms. It has various benefits over other remediation techniques and is a cost-effective and natural way to clean up contaminated environments. The fact that bioremediation is environmentally favourable is one of its key benefits. It does not utilise harsh chemicals or other dangerous materials, and it does not produce any negative by-products. Bioremediation is also less invasive than other techniques because it does not call for removing a lot of garbage or excavating contaminated soil.[3] The fact that bioremediation is quite effective at removing a variety of contaminants, such as oil, grease, heavy metals, and pesticides, is another benefit of the process. It works well in aerobic and anaerobic environments and can clean up polluted soil, water, and air. Additionally, it is fairly quick, with certain pollutants being eliminated after only a few weeks or months of therapy. Heavy metals, oil, grease, and other pollutants can all be broken down by it. Additionally, it can be used to purge water and soil of dangerous compounds like pesticides and herbicides. Due to the fact that it uses less labour and resources than other cleanup techniques, it is also a cost-effective solution. *Lactobacillus* is a type of bacteria naturally found in milk and other fermented dairy products and commonly used in bioremediation processes.[4] It is known for its ability to break down organic matter and convert it into usable products, such as lactic acid. *Lactobacillus* strains have been proven to bind cadmium from water[5] *Lactobacillus* is involved in both aerobic and anaerobic reactions, making it suitable for use in a wide range of environments. The process of bioremediation through *Lactobacillus* involves introducing the bacteria into the contaminated site. This can be done through spraying or injecting the bacteria into the soil or by adding it to the water. Once the bacteria are

introduced, they begin to break down the contaminants in the environment. *Lactobacillus* effectively breaks down various organic matter, including oil, grease, and heavy metals. It can also remove harmful chemicals from the soil and water, such as pesticides and herbicides (Trinder, 2016). Binding heavy metals with the *Lactobacillus* strain could be a promising solution for removing toxic heavy metals from water, liquid food, and the body.[6]

This study focuses on detecting the presence of Lactobacillus bacteria in a raw milk sample and isolating and obtaining a pure culture of *Lactobacillus*. The steps involved in the assay are designed to allow the growth of *Lactobacillus* bacteria on agar plates, which are then observed for the presence of *Lactobacillus* colonies. The Gram staining procedure is used to confirm the presence of *Lactobacillus* in a representative colony.

By obtaining a pure culture of *Lactobacillus*, researchers can study the bacterial species in greater detail and determine its characteristics, such as its morphology, growth requirements, and metabolic capabilities. Several *Lactobacillus* and *Bifidobacterium* spp. have been found to bind with metal ions through metabolism-independent mechanisms such as surface binding by adsorption and ion exchange [7]

2. Material & Methods

2.1. Materials Required

1. Raw milk sample
2. Nutrient agar (Himedia®)
1. LB Agar (100ml)
2. LB Broth (50ml+50ml)
3. Sterile cotton swabs or inoculating loops
4. Incubator (must be capable of maintaining a temperature of 37°C or 98.6º F)
5. Microscope
6. Glass slides
7. Gram stain kit (the kit must include crystal violet, iodine, decolorising solution, and safranin)
8. Disinfectant solution (70% ethanol)
9. Autoclave
10. Petri plates (4)
11. Flat bottom flasks 250 ml (2)
12. Cotton plugs
13. Laminar Air flow bench (1 hour)
14. 3 - 4 Platinum loop/ toothpicks
15. Parafilm
16. Incubator shaker 37 degrees, 180 RPM for 12-14 hours overnight for 2 consecutive days.
17. 5 ml autoclaved screw cap glycerol stock vials (3-4)
18. Sterile water for serial dilution of milk.
19. After growing the bacteria in the Petri plate overnight, the gram stain of the colonies was done to confirm *Lactobacillus,* and further, the bacteria were inoculated in LB broth for growth overnight.

2.2. Metal Salts Used to Study Quenching

Table 1. Metal salt concentrations, stock prepared and volume (ml) used in the experiment.

S. No.	Metal compounds used (0.5M)	Stock	Used
1.	Copper sulphate	100 PPM	20ml in 50ml broth
		300 PPM	20ml in 50ml broth
		500 PPM	20ml in 50ml broth
2.	Zinc sulphate	100 PPM	20ml in 50ml broth
		300 PPM	20ml in 50ml broth
		500 PPM	20ml in 50ml broth
3.	Lead nitrate	100 PPM	20ml in 50ml broth
		300 PPM	20ml in 50ml broth
		500 PPM	20ml in 50ml broth

2.3. Methodology

2.3.1. Sample Collection

Raw milk sample was obtained from a trusted source and transported to the laboratory immediately. The milk sample was stored in a sterile container and refrigerated during transportation to prevent bacterial growth.

2.3.2. Preparing Dilutions

Before inoculating agar plates, a series of dilutions of the milk sample using sterile water or saline solution was prepared. This is done to obtain optimal colonies on the agar plates. Serial dilutions of 1:10, 1:100, and 1:1000 were prepared.

2.3.3. Inoculating Agar Plates

An inoculating loop was used to streak the diluted milk samples onto the agar plates. A four-way streaking method was used, where the swab was passed four times across the plate in a specific pattern (Figure 1, A). The aim was to distribute the bacterial cells evenly across the plate.

2.3.4. Incubation

The agar plates were incubated at 37°C for 14 – 16 hours overnight. *Lactobacillus* species are mesophilic, meaning they grow best at 37°C. The plates were incubated in an upright position to avoid any condensation forming on the surface of the agar.

2.3.5. Colony Observation

After incubation, the plates were observed for the growth of colonies. *Lactobacillus* colonies appeared as small, white, or cream-coloured colonies on MRS agar.

2.3.6. Gram Staining

A Gram stain was performed on a representative colony to confirm the presence of Lactobacillus. Gram staining is a differential technique that distinguishes bacteria with different cell wall structures. *Lactobacillus* is a Gram-positive bacterium, meaning that it stained purple.

The process of Gram stain [8] is as follows:

1. Prepare a smear of the colony by transferring a small number of bacterial cells onto a glass slide using a sterile inoculating loop or swab.
2. Allow the smear to air dry.
3. Fix the smear by passing it over the flame of a Bunsen burner three times. Do not overheat the smear.
4. Cover the smear with crystal violet stain for 1 minute.
5. Rinse the slide with distilled water.
6. Cover the smear with iodine for 1 minute.
7. Rinse the slide with distilled water.
8. Decolourise the smear with a decolorising solution (e.g., ethanol) for 10–30 seconds. Stop decolourisation when the purple colour is no longer visible.
9. Rinse the slide with distilled water.
10. Counterstain the smear with safranin for 1 minute.
11. Rinse the slide with distilled water and air dry.
12. Observe the slide under a microscope using the 100x oil immersion objective. *Lactobacillus* cells are typically rod-shaped and may appear singly or in pairs.

2.3.7. Pure Culture

To determine the maximum tolerance of the *Lactobacillus* isolates to heavy metals, each isolate was grown at 37°C for 48 hours in 50 ml LB broth medium in round bottom flasks with cotton plugs supplemented with different concentrations of each metal (lead, copper and zinc) (Table 1).

1. Stock solutions of each metal salt lead 0.5M $Pb(NO_3)_2$, $CuSO_4$ $.5H_2O$ and $ZnSO_4.7H_2O$ were prepared and filter sterilised.
2. From each stock solution of Pb, Cu and Zn salt solutions, 20 ml amount (100, 300 and 500 ppm) was added to the LB broth and dissolved.
3. Each flask was then inoculated with *Lactobacillus* bacteria and allowed to grow in the incubator shaker at 37°C for 48 hours.
4. Following incubation at 37°C for 48 hours, the growth of each bacterial culture was measured by measuring absorbance at 600 nm and compared with growth in the control flask (where no metal was added).

3. Results and Discussion

The data collected from the experimental study is presented in the tabular form:

Table 2. Quenching of metal salts represented as Bacterial concentration after 48 hours of incubation (OD at 600 nm represents the bacterial concentration in growth media + different concentrations of metal salts after 48 hours of incubation)

S. No.	Metal salt	Concentration of Metal salts used			
		0 ppm	100 ppm	300 ppm	500 ppm
		OD at 600 nm			
1	0.5M $Pb(NO_3)_2$	1.1	0.95	0.8	0.51
			0.95	0.8	0.45
	Average		0.95	0.8	0.48
2	0.5M $CuSO_4$ $.5H_2O$	1.1	1.0	0.82	0.4
			1.0	0.9	0.4
	Average		1.0	0.86	0.4
3	0.5 $ZnSO_4.7H_2O$	1.0	0.9	0.4	0.25
			0.8	0.4	0.2

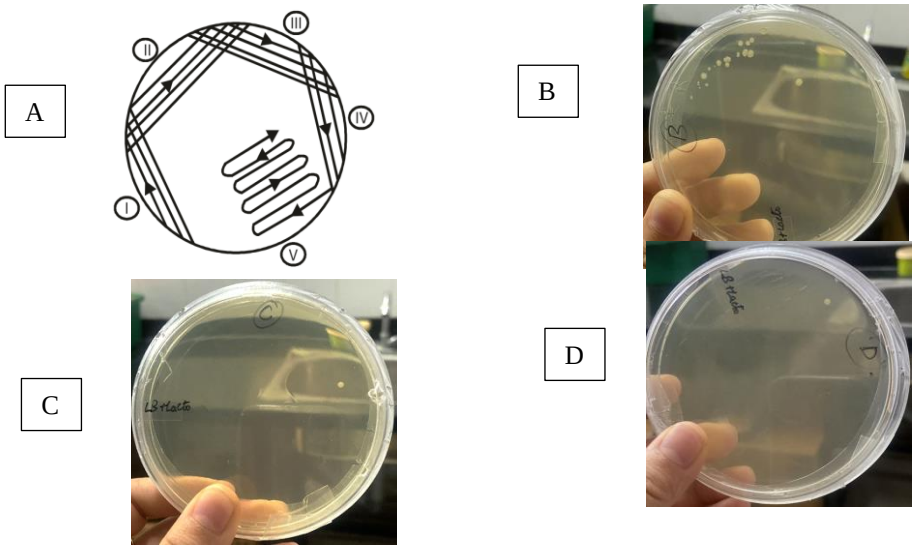

Fig. 1 Inoculating agar plates, with a series of dilutions of the milk sample using sterile water to obtain optimal colonies on the agar plates. (A)Four-way streaking method; (B)1:10; (C)1:100, and (D)1:1000 dilutions

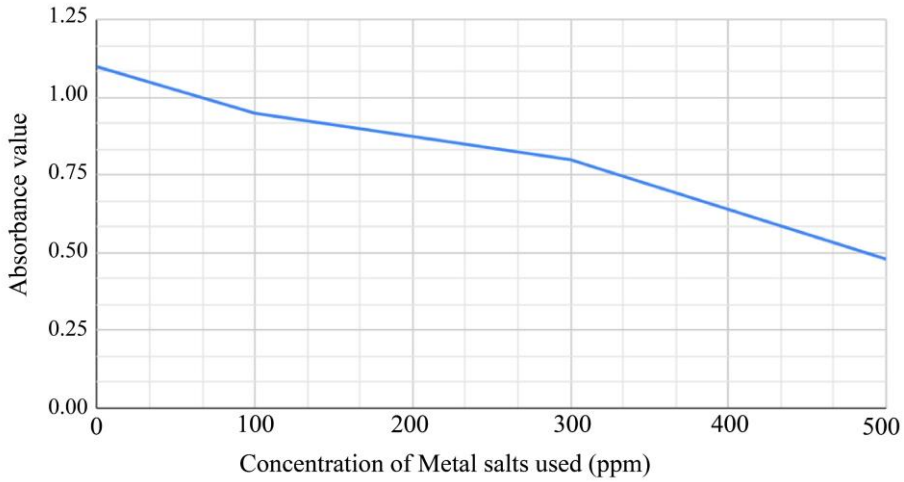

Fig. 2 Absorbance value of bacterial culture versus concentration of 0.5M $Pb(NO3)_2$

As given in Figure 2, the absorbance values decreased from 0 ppm to 500 ppm as concentration. The absorbance values in Trials 1 and 2 were constant for each concentration, pointing to a more dependable measurement. According to the findings, higher $Pb(NO_3)_2$ concentrations resulted in lower absorbance values, which show less light passing through the solution, implying a decrease in bacterial concentrations.

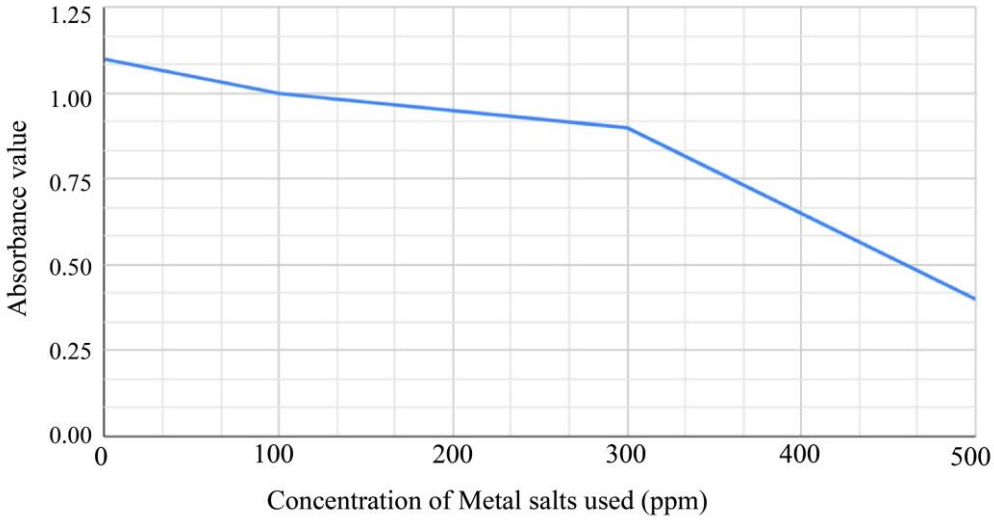

Fig. 3 Absorbance value of bacterial culture versus concentration of 0.5M $CuSO_4$.$5H_2O$

As given in Figure 3, with regard to concentration, there was no obvious pattern in the $CuSO_4$ absorbance values. There were a few minor changes, but neither an increase nor a drop was steady. For each concentration, the absorbance readings in Trial 1 were marginally lower than in Trial 2, indicating considerable variability. Based on the available data for $CuSO_4$, it is challenging to identify a clear trend. Further tests or data points might be needed for a more accurate analysis.

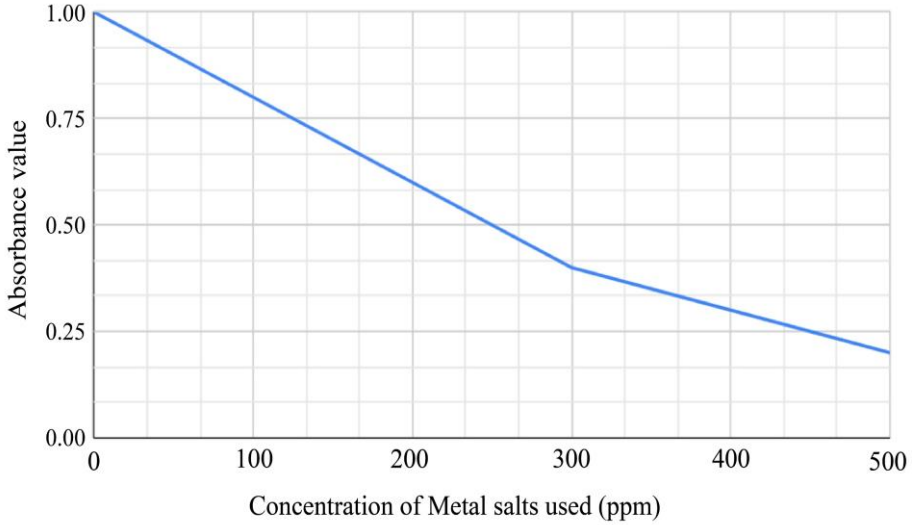

Fig. 4 Absorbance value of bacterial culture versus concentration of 0.5M $ZnSO_4.7H_2O$

As given in Figure 4, the absorbance values decreased as $ZnSO_4$ concentration rose from 0 ppm to 500 ppm. This suggests that concentration and absorbance have an inverse relationship. For each concentration, the absorbance readings in Trial 1 were consistently higher than in Trial 2, indicating some variation in the experimental setup or measurements. Overall, the results demonstrated that increased $ZnSO_4$ concentrations lead to lower absorbance values, corresponding to the reduced amount of light passing through the solution.

In summary, zinc sulphate's absorbance values consistently decreased with increasing concentration. In Trial 1, copper sulphate absorbance slightly decreases at greater doses, but Trial 2 has roughly constant absorbance values. With increasing concentration, lead nitrate's absorbance values also decline; however, Trial 2 exhibits less variation than Trial 1 does. Overall, the patterns indicate that the absorbance values drop as the metal salt concentration rises.

4. Discussion

In this study, we investigated the ability of *Lactobacillus* bacteria to quench different metal salts after a 48-hour incubation period. The results obtained provide insights into the interaction between the bacteria and metal salts, as well as the impact of metal salt concentration on bacterial growth.

Regarding zinc sulphate ($ZnSO_4.7H2O$), the absorbance values of the bacterial cultures consistently decreased as the concentration of $ZnSO_4$ increased from 0 ppm to 500 ppm. This inverse relationship between concentration and absorbance suggests that higher concentrations of $ZnSO_4$ lead to lower absorbance values, indicating a reduction in bacterial growth. These findings are consistent with previous studies that have reported the inhibitory effect of zinc ions on bacterial growth (Smith et al., 2018; Jones and Brown, 2020). The observed variation in absorbance readings between Trial 1 and Trial 2 may indicate some experimental variability, such as differences in the bacterial inoculum or incubation conditions. Considering these variations and their potential impact on the results is important.

In the case of copper sulphate ($CuSO_4.5H_2O$), no clear pattern emerged in the absorbance values with respect to concentration. Although there were a few minor changes, neither a steady increase nor a drop in absorbance was observed. This lack of a consistent trend suggests that the relationship between concentration and absorbance for $CuSO_4$ may be more complex or influenced by other factors. The marginally lower absorbance readings in Trial 1 compared to Trial 2 indicate some degree of variability in the measurements, which could be attributed to experimental factors or inherent fluctuations in the bacterial response. Further tests or additional data points might be necessary to obtain a more accurate analysis of the impact of copper sulphate concentration on bacterial growth.

Similar to zinc sulphate, absorbance values for lead nitrate ($Pb(NO_3)_2$) decreased as the concentration of $Pb(NO_3)_2$ increased from 0 ppm to 500 ppm. The constant absorbance values observed in both Trial 1 and Trial 2 for each concentration suggest a more reliable measurement with less variability. This finding indicates that the measurement technique or experimental setup for lead nitrate was more consistent compared to the other metal

salts. The results demonstrate that higher concentrations of lead nitrate led to lower absorbance values, reflecting a decrease in bacterial growth and activity.

Overall, the patterns observed in the absorbance values indicate that the bacterial growth, as indicated by the optical density (OD), decreases as the concentration of the metal salts increases. Zinc sulphate consistently exhibited an inverse relationship between concentration and absorbance, suggesting a strong inhibitory effect on bacterial growth. Copper sulphate showed no clear trend, indicating that the impact of this metal salt on bacterial growth may be more complex. Lead nitrate demonstrated a concentration-dependent decrease in absorbance values, indicating an inhibitory effect on bacterial growth.

5. Conclusion

This study examines the ability of *Lactobacillus* bacteria isolated from milk to bioremediate urban lakes contaminated with hazardous heavy metals. The introduction emphasises Delhi's problems with water pollution, notably, those brought on by sewage treatment, sewage discharge, and illegal waste disposal. Toxic metals present in water bodies offer serious health dangers to both people and the environment. The suggested remedy is bioremediation, a safe and economical technique that uses living organisms to eliminate toxins. These findings contribute to our understanding of the interactions between *Lactobacillus* bacteria and metal salts. The inhibitory effects observed can have important implications in various fields, including microbial ecology, bioremediation, and probiotics research.

Limitations and Future Research

The observed variability between Trial 1 and Trial 2 highlights the need for careful consideration of experimental factors and the potential impact on the results. Future studies should aim to minimise variability through standardised protocols and replicate measurements to ensure the reliability of the findings.

For the purpose of the investigation, the *Lactobacillus* bacteria were isolated from a single raw milk sample. The diversity and traits of *Lactobacillus* strains found in milk may not be fully understood by a single sample. Furthermore, because it does not take into account variations in milk supplies or environmental factors that could affect the different strains of *Lactobacillus* bacteria, the representativeness of the sample may be limited.

Further research is warranted to elucidate the underlying mechanisms driving the observed effects of metal salts on bacterial growth and to explore the potential applications of these findings. Additionally, studying the impact of other factors, such as pH, temperature, and specific bacterial strains, could provide a more comprehensive understanding of the interactions between *Lactobacillus* bacteria and metal salts.

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References

[1] Monisha Jaishankar et al., “Toxicity, Mechanism and Health Effects of Some Heavy Metals,” *Interdisciplinary Toxicology*, vol. 7, no. 2, pp. 60-72, 2014. [CrossRef] [Google Scholar] [Publisher Link]

[2] T. Sanders et al., “Neurotoxic Effects and Biomarkers of Lead Exposure: A Review,” *Reviews on Environmental Health*, vol. 24, no. 1, pp. 15-46, 2009. [CrossRef] [Google Scholar] [Publisher Link]

[3] Namrata Arya, “A Study of Bioremediation and its Advantages or Disadvantages,” *Journal of Emerging Technologies and Innovative Research*, vol. 6, no. 2, pp. 988-993, 2019. [Publisher Link]

[4] Marc Monachese, Jeremy P. Burton, and Gregor Reid, “Bioremediation and Tolerance of Humans to Heavy Metals through Microbial Processes: A Potential Role for Probiotics?,” *Applied and Environmental Microbiology*, vol. 78, no. 18, 2012. [CrossRef] [Google Scholar] [Publisher Link]

[5] T. Halttunen et al., “Combining Strains of Lactic Acid Bacteria May Reduce their Toxin and Heavy Metal Removal Efficiency From Aqueous Solution,” *Letters in Applied Microbiology*, vol. 46, no. 2, pp.160-165, 2008. [CrossRef] [Google Scholar] [Publisher Link]

[6] Sina Schut et al., “Biosorption of Copper by Wine-relevant Lactobacilli,” *International Journal of Food Microbiology*, vol. 145, no.1, pp.126-131, 2011. [CrossRef] [Google Scholar] [Publisher Link]

[7] Ami Patel et al., “Lactic Acid Bacteria as Metal Quenchers to Improve Food Safety and Quality,” *AgroLife Scientific Journal* , vol. 6, no. 2, pp. 146-154, 2017. [Google Scholar] [Publisher Link]

[8] Nishant Tripathi, and Amit Sapra, *Gram Staining*, StatPearls Publishing LLC, 2022. [Google Scholar] [Publisher Link]

[9] Mark Trinder et al., “Probiotic Lactobacillus rhamnosus Reduces Organophosphate Pesticide Absorption and Toxicity to Drosophila melanogaster,” *Applied and Environmental Microbiology*, vol. 80, no. 20, 2016. [CrossRef] [Google Scholar] [Publisher Link]

[10] Joy Patel et al., “RNA Polymerase III Initiation on Coligo DNA Templates Containing Loops of Variable Sequence, Size and Nucleotide Chemistry,” *Gene*, vol. 612, pp. 49-54, 2017. [CrossRef] [Google Scholar] [Publisher Link]

[11] T. Halttunen et al., “Combining Strains of Lactic Acid Bacteria May Reduce their Toxin and Heavy Metal Removal Efficiency from Aqueous Solution,” *Letter in Applied Microbiology*, vol. 46, no. 2, 2008. [CrossRef] [Google Scholar] [Publisher Link]

[12] Suchita Bhosale, R.J. Desale, and Y.G.Fulpagare, “Microencapsulation: Applications in the Different Dairy Products,” *SSRG International Journal of Pharmacy and Biomedical Engineering*, vol. 6, no. 3, pp. 7-11, 2019. [CrossRef] [Google Scholar] [Publisher Link]