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Biological removal of zinc from automotive hazardous paint sludge: a comparison between an endogenous *Pseudomonas aeruginosa* isolate and *Acidithiobacillus thiooxidans* PTCC1692

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Abstract

Research on the hazardous waste of paint sludge is mostly focused on recovering the sludge and pollution control with methods such as solidification. This research aimed to study the comparison of biological removal of zinc from paint sludge using a native Pseudomonas aeruginosa strain and *Acidithiobacillus thiooxidans* PTCC 1692. Samples of water-based paint sludge were collected under aseptic conditions. Isolation of endogenous strains was performed in Bushnell-Haas (BH) medium from paint sludge, and *A. thiooxidans* PTCC 1692 was cultured in the medium 119. Optimization of the process parameters for the biological removal of zinc was performed using the design of the experimental (DOE) method for both organisms. The results showed that *A. thiooxidans* is capable of biological removal of zinc with a higher pulp density and with a more extended bioleaching period. The results of the chemical analysis show the removal of zinc metal by 57.76% compared to *P. aeruginosa* by 36.39%, and, the removal of other metals such as Cr, Ni, Pb, and Cd were observed in the residual sludge from the process. Energy Disperse X-Ray Analysis (EDX) showed the more favorable effect of this bacterium in reducing heavy metals.

Key words: Automotive industry; Paint Sludge; Bioleaching; *Pseudomonas aeruginosa*; *Acidithiobacillus thiooxidans* PTCC 1692

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1. Introduction

Automobile production in the world is increasing, and among the dangerous wastes caused by this industry is paint sludge (Salihoglu & Salihoglu, 2016). In Iran, about 3 kg of paint sludge is produced for each car (Ghomi Avili et al., 2018), and about 2555-4380 tons/year of paint sludge in factories, which is essential for its recycling and purification due to heavy metals and toxic organic and mineral pollutants (Khezri & et al., 2013).

Sludge treatment is by reducing the content of pollutants through filtration, centrifugation, incineration, and finally disposal in the ground (Arce & et al., 2010). More research focused on patents is the reuse of paint sludge as; sealant (Johnson& Slater, 1990), sealant filler (Weinwurm et al., 1992), production of building materials (St. Louis, 1996), replacement of polymer components (Gerace et al., 1999) Preparation of activated carbon (Kim & et al., 1995, Idris & et al., 2020) Conversion of waste into a reusable paint (Bhatia & et al., 2007), Stabilization and solidification with pretreatment materials such as Lime for decrease pollutant (Arce et al., 2010), replacement concrete in cement (Devi & Gopala Krishnan, 2016) and modified concrete (Dalmazzo et al., 2017). Extraction of valuable substances from organic and inorganic compounds of paint sludge (Burande, 2017) such as; titanium dioxide by flotation and flotation method at the rate of 89.8% (Khezri & et al., 2009), by membrane and electrolysis method until 65.11 and 85.3% respectively (Khezri & et al., 2012), Acidic digestion and centrifuge method until 48.69 and 67.42%, respectively (Khezri & et al., 2013).

Today, special attention has been paid to green technology and the use of bacteria to reduce environmental pollution (Saleh 2016). including biosorption, bioremediation, etc. (Saleh et al., 2018). Among the biological methods to decrease pollution and reuse paint sludge can indication; to preparing compost from melanin resins of paint sludge (Tian & et al., 2018), as well as preparation of compost from water-based paint sludge, in decreasing organic matter, BTEX, and heavy metals such as nickel, and chromium (Salihoglu & et al., 2018). By the vermicomposting process with *Eisenia fetida* worm, chromium metal decreased from 6 to 0.2 mg/kg in paint sludge during 90 days, and subsequently, C/N decreased from 27 units to 14.3 (Ghomi Avili et al., 2018). Vermicomposting process by *Eisenia fetida* with a mixture of biological sludge decreased BTEX to the standard level during 90 days. The present research investigates and compares the bioleaching process in decreasing the pollution of paint sludge rich in heavy metals for disposal in the environment.

2. Materials and Methods

2.1 Composition of paint sludge

Five kilograms of water-based paint sludge from the automotive industry was prepared then air-dried and screened by sieves of 1, 2, and 3 mm model ELE International- England, UK. Heavy metals before and after the bioleaching process determined by the EPA 3050B method as described: 1 gr of waste was digested with Merck acids, kept in HNO_3 (65%) with a ratio of 1:1 at 90-95 °C for 10 to 15 minutes, then left to cool down, and 5 mL of concentrated HNO_3 was added and refluxed for 30 minutes; again was cooled down, followed by adding 2mL of water and 3mL of H_2O_2 (30%); then, 10mL of HCL (37%) was added to the digest reflux sample for 15 minutes. The digested solution was centrifuged at 2000 rpm and filtered through a 0.45 µm filter and diluted to a final volume of 100 mL (Navarro et al., 2011; United State Environmental Protection Agency (USEPA) 2010). The liquid obtained from the digestion method was measured by the absorption spectroscopy model (Spectra Varian AA, 220FS, Australia) and ICP to measure the amount of zinc metal.

2.2 Microorganisms

A sampling of water-based paint sludge was done under aseptic conditions in a Falcon tube. Two strains of microorganism (a) Endogenous *P. aeruginosa* bacterium endogenous isolated in Bushnell- Haas (BH) medium from paint sludge (Honarjooy & et al., 2020), then maintained in nutrient broth medium, and (b) a live culture prepared of *A. thiooxidans* bacterium (PTCC1692) from (IROST), in the recommended culture medium 119 with the following composition:

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NH_4Cl, 0.1 gr; KH_2PO_4, 3gr; $MgCl_2.6\ H_2O$, $0._1$ gr; CaCl2.2 H2O, 0.14 gr; and powdered sulfur, 10 gr. All materials, except sulfur, were dissolved in 1000 mL of distilled water, and after the pH was adjusted to 4.2, they were autoclaved. Sulfur was poured into tubes with screw caps and sterilized by autoclaving at 121°C for 15 minutes. Before use, sulfur was placed on the surface of a sterile liquid medium under aseptic conditions was used for the bioleaching process (Mostafavi & et al.,2018).

2.3 Experimental methods

Design of experiments; to determine the optimal variables of the experiments, the common method of response surface methodology (RSM) and central composite design of (CCD) was used, and in this method, 4 parameters of particle size, shaker speed, temperature, and pH were checked. Based on the definition of the parameters of the Design of Experiment software 11, processes were executed on three levels and one block from the following path in the software, and 30 test series including (16 factorial tests, 8 axial points, and 6 central points) were designed to optimize the process. The particle size variable was defined at three levels of 1, 2 and 3 mm and the temperature variable was 37, 34.5, and 32 °C, as well as the shaker diameter of 120, 150, 180 jets for both types of microorganisms. According to the past research texts, according to the appropriate growth range for each microorganism, different pH was chosen so that *P. aeruginosa* was defined in the range of 3, 5, 7 and *A. thiooxidans* in 2.2, 2.3, 2.4. Adaptation of bacteria to metals; by the serial culture of bacteria. First, a pure culture of microorganisms is provided in 100 ml of the culture medium, which has the minimum acceptable primary bacterial cell density for the bioleaching process of about 107-109 per milliliter, then adding a small amount of sterile waste in 90 ml of fresh culture medium along with 10 ml of previously adapted culture medium and checking the growth process of the microorganism by periodically counting the number of bacteria, counting by the Olympus model microscope, model BH-2 and reaching the number of 107-109 bacteria again. The next step; serial culture starts by adding 90 ml of fresh culture medium to 10 ml of the previous culture medium along with adding more grams of waste, each step takes time which is repeated by this serial process when counting the number of bacteria is fixed. That is, it has reached the stationary phase compromise has occurred, so it has returned to the dilution of the previous stage, which is the tolerable toxicity concentration for the bacteria (Heydarian & et al., 2018). This serial cultivation process for *P. aeruginosa* bacteria started after 10 hours with the number of 7.5*108 bacteria and a pulp density of 1 gr and ended at 7 gr/l. The same process for *A. thiooxidans* bacteria after 8 days and with the number of bacteria 108 started and ended with a pulp density of 10 g/l.

To determine the appropriate and optimal time of the bioleaching process, a) *P. aeruginosa* bacteria, 100 ml of nutrient broth with 10% (v/v) compatible bacteria as inoculation, in a 500 ml Erlenmeyer flask (Pangayao & et al., 2018), together with 7 gr of paint sludge was incubated for 14 days at 80 rpm, then the biological leaching process was optimized in 8 days (Honarjooy & et al., 2021) (b) A. thiooxidans in 100 ml medium 119 with 10% (v/v) of compatible bacteria. The pulp was inoculated and incubated for 40 days with 140 revolutions per minute, in the same way, during the control of the parameters, the time of the 30days paint sludge bioleaching process was optimized (Honarjooy & et al., 2021). In order to compensate for evaporation losses, deionized water were added to the flask were added. Every two days, the number of pH changes in the measurement process, as well as the number of bacterial cells were counted on the Neubauer Slide, and a sample was taken to measure the metal was analyzed. Bioleaching experiments were run, based on the design parameters of the 30 samples, they were passed through the Wattman filter (NO:4), and were centrifuged for 20 minutes to remove the bacteria for 20 minutes. The fluid inside the Falcon Tube was measured in terms of metal concentration (Bahaloo-Horeh & Mousavi., 2017). Experiments were performed in three repetitions with a control non sample. Equation 1 was used to calculate the percentage of heavy metal removal in the leach.

Table1: Results of ANOVA analysis of paint sludge bioleaching by *P. aeruginosa* and *A. thiooxidans*

	P.aeruginosa			*A. thiooxidans*		
Parameter	F- value	P- value		F- value	P- value	
Model	39/59	< 0.0001	significant	15/55	< 0.0001	significant
A	26/17	0/0001		13/96	0/0011	
B	244/56	< 0.0001		13/13	0/0014	
C	71/90	< 0.0001		12/02	0/0021	
D	83/68	< 0.0001		13/08	0/0014	
AB	11/67	0/0038		-	-	
AC	0/3236	0/5779		18/31	0/0003	
BC	0/8177	0/3801		-	-	
BD	34/48	< 0.0001		-	-	
CD	27/92	< 0.0001		-	-	
A2	7/41	0/0488		22/78	< 0.0001	
B2	10/19	0/0061		-	-	
Residual	1/88			7/39		
R2	0/9736			0/8022		
R2 Adjusted	0/9460			0/750		
R2 predicate	0/9141			0/688		
C.V	5/73%			13/13%		
Lack of fit	0/1341		non-significant	0/867		non-significant
SD	1/37			2/72		
Pure error	0.856			8/25		

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Table2: The amount and percentage of removal of heavy metals in the primary paint sludge and the residue on the filter after the biological leaching process by microorganisms with chemical digestion by method 3050B and comparison with the sludge disposal standard

Parameters	Sludge disposal standard mg/kg	Heavy metal in primary paint sludge mg/kg	Heavy metal in paint sludge after bioleaching by *A. thiooxidans* mg/kg	Heavy metal in paint sludge after bioleaching by *P. aeruginosa* mg/kg	Removal percentage of heavy metals by *A. thiooxidans*	Removal percentage of heavy metals by *P. aeruginosa*
Cr	1200	290	56	100	80/68%	65/51%
Zn	2800	63860	26970	40620	57/76%	36/39%
Ni	420	160	<5	<5	96/78%	96/87%
Cd	39	20	<5	<5	75%	75%
Pb	300	230	4	13	98/26%	94/34%

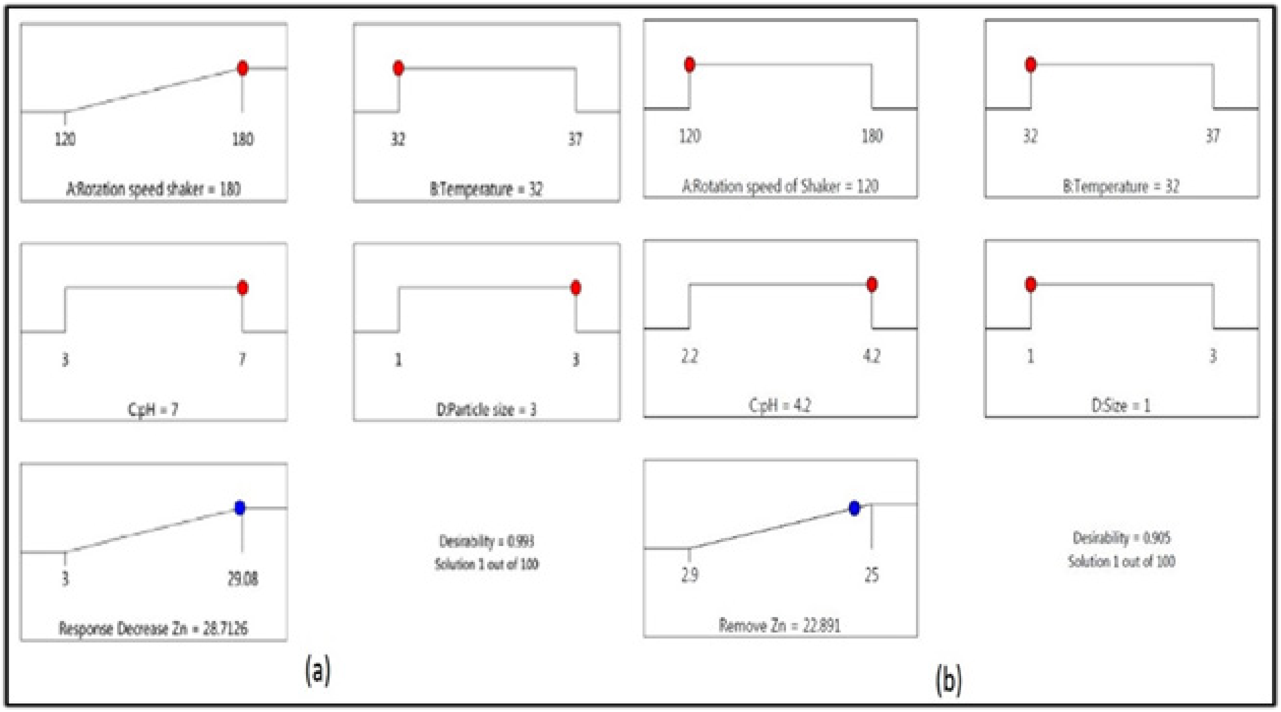

Figure 1: Desirability and numerical optimization ramp of variables (a) *P. aeruginosa* and (b) *A.thiooxidans* by adjusting the software for maximum response

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Bioleaching = [(Cs× Vs) / (Cf× Mf)] ×100 (Equation 1)

Where Cs is the concentration of metal in bioleaching liquid (mg/l), Vs is the volume of bioleaching solution (l), Cf is the amount of metal in the paint sludge waste (mg/kg), and Mf is the weight of the paint sludge waste is in (kg). The sludge remaining on the filter was digested by the 3050B method and its heavy metals were measured according to equation 2; the metal removal percent in the solid part of the residue was reported, Where Ci is the metal concentration in the primary paint sludge before digestion - after the leaching process Cf, the metal concentration in the secondary paint sludge after digestion - residue in (mg/kg) (Xue & et al., 2018).

Absorption =[(Ci-Cf)/ (Ci)] × 100 (Equation 2)

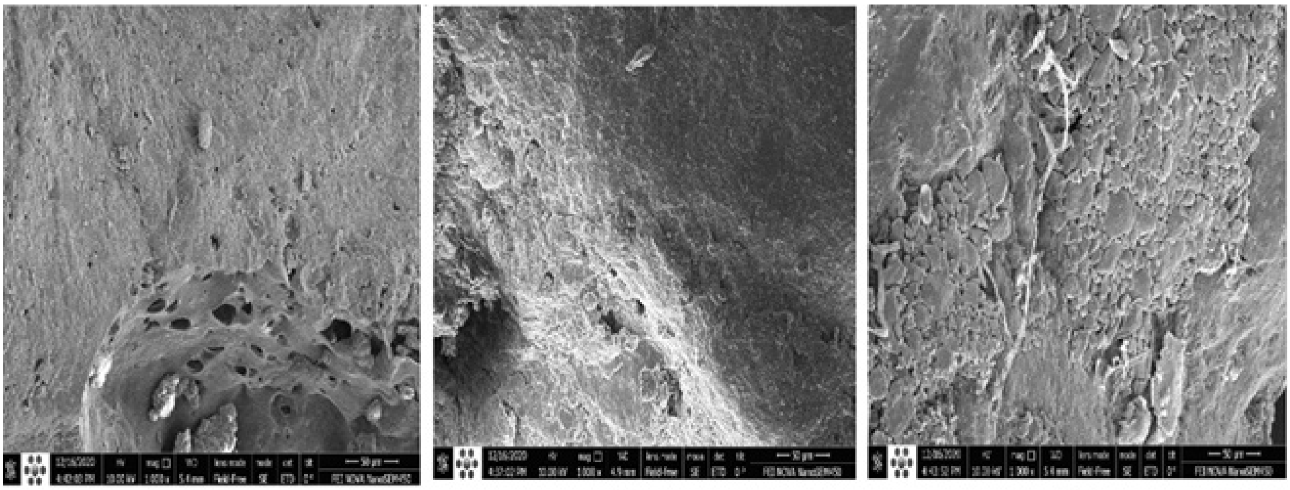
3.3 Results and Discussion

The experiments were performed according to thirty runs of software plan and the results of the atomic absorption of the bioleaching obtained from the process both of them microorganisms as shown in ANOVA Table 1.

ANOVA table examines the significance of linear effects, quadratic regression model coefficients, and interaction effects for each response at 5% to 1% confidence levels. Term R2 is used to evaluate the quality of the data, which is the ratio of the forecast variance to the total variance (Khayati & et al., 2014) of 0.8 (Montgomery, 2017). When R2 is close to one, it confirms the satisfactory agreement between the experimental data and the quadratic model (Arshadi & Mousavi., 2015). Based on this, the model is acceptable and R2) between 0.8-0.9 confirms the model's validity. p-Value less than 0.5 indicates that the variables of temperature, particle size, rotation shaker, and pH are significant and the results of interaction of parameters AB, AC, BD, and BC, in the performance of *P. aeruginosa* bacteria are higher F-Value is shown the importance of parameters, for *A. thiooxidans* the rotation shaker (A2) increases the bacterial performance in removing zinc metal, and for *P. aeruginosa*, temperature (B) is considered the most important parameter of the model in removing Zinc. Lack of fit is a comparison between the net error and the residual error, and the values of 0.86 and 0.13 in the model indicate the non-significant and appropriateness of the model. The desirability is shown in Fig1(a). Indicated rotation shaker 180 rpm, temperature 32 °C, pH 7, and particle size 3 mm, 27.81% leaching of zinc metal from paint sludge. The purpose of the experiment design is to determine the prediction conditions for the maximum response, and the desirability functions are used to find the optimal prediction conditions (El-Naggar & Rabei, 2020). The finding of bioleaching on coal ash by other species of P. aeruginosa showed that at 37 °C, 90 rpm, in more than 15 days, the percentage of zinc metal removal was 10.63 and the pH value in the nutrient broth medium changed from 8.2 to 8.86. In another research, 41% removal in six glucose percentages was observed in nutrient broth culture medium by pulp density 1:100 or (1%), temperature 37 °C, and rotation shaker 32 rpm (Shabani & et al., 2019). In the metallurgical bioleaching process with *(P. fluorescens)* at a temperature of 32 °C for four days at a pH of 7 and a pulp density of 1, a four percent removal in crystalline slag sludge and 3 percent in amorphous slag sludge was observed (Potysz & et al., 2016). It seems that endogenous mesophilic microorganisms *(P. aeruginosa)* have optimal activity at a temperature of 32 °C, and at this temperature and initial pH of 7, it shows the production of organic acids and better growth. Fig 1 (b) for A. thiooxidans with a rotation shaker of 120 rpm, a temperature of 32 °C, pH 2.4, and particle size of 1 mm, the amount of zinc removal is 22.89%. In similar research on sewage sludge under desirable conditions (2% waste, 5 gr of sulfur, 10% of bacterial inoculum and the rotation shaker is 150 rpm) through direct and indirect mechanisms 96.5% of Zinc (Wen & et al., 2012), and from the sludge of petrochemical facilities Metallurgical leach residues under desirable conditions (sulfur concentration 25.1 g/l, pulp density 21.5 g/l, and initial pH 3.3) more than 75% of zinc metal removed (Sethurajan & et al., 2017). It has been proven that the presence of a suitable pH in the culture environment can increase the activity of microbes in the leaching process and affect the dissolution of metals and the stability of metal ions in the liquid phase (Asghari & et al., 2013) 5/4-5 in general for *A. thiooxidans* is favorable, and the initial pH of 4 is favorable for the bacterial washing of metals from anaerobic sludge, using iron and acid-oxidizing bacteria (Gu & et al., 2018). This finding is with the results of our study in the Optimization of zinc metal removal corresponding to pH 4.2. Kumar investigated the metals present in contaminated soils in the initial pH range of 3 to 7, the results showed that chromium and lead are transferred more at pH 5, while zinc, copper, cadmium, and generally most metals are most likely at higher initial pH are removed. This showed that the increase in pH and its tendency towards neutral in contaminated soil does not affect the biological leaching system of *A. thiooxidans* and this bacterium can still use sulfur (Kumar & Nagendran, 2007). The zinc biodegradation of sewage sludge showed that the highest removal of heavy metals was obtained at pH 6 and the lowest at pH 2 (Wen & et al., 2012), confirming the low efficiency of zinc removal from paint sludge at low pH. The removal efficiency of zinc metal was higher in the smaller size of colored sludge particles, which shows that reducing the particle size increases the surface area and also the contact surface between the washing agents and the solid particles (Deveci & et al., 2003). Temperature increases the efficiency of the biodegradation process. In other words, increasing the temperature increases acidity and bacterial activity. Lin, in the biological study of sewage sludge in the temperature range of 30-10 °C using *A. thiooxidans*, showed that the optimal temperature (28.9 °C) led to the removal of zinc up to 78.42%. According to the standard range required for sludge treatment and reducing energy consumption, the temperature of 20 °C also showed good results and within five days, zinc metal was removed up to 77.7%, and the amount of zinc metal in the output sludge was within the standard range. (Lin & et al., 2010) Although the best removal of zinc metal was observed at 32°C, the temperature range in this study was set at 32-37°C to reduce energy loss. It has been found that microorganisms, as shown in previous studies, show optimal activity at this temperature, however, optimal conditions at 25 °C for the primary cultivation of *A. thiooxidans* according to the IROST guidelines was determined. The results of the chemical digestion of 3050B on the primary sludge and the residual sludge resulting from the process in the optimal sample resulting from the performance of both bacteria during three repetitions in Table 2 show high amounts of heavy metals including zinc and titanium in the initial content of the sludge. Also, the results indicate the performance *A. thiooxidans* bacteria were better in removing the target metal (zinc) as well as other metals. However, zinc metal has not yet reached the standard amount of sludge removal, but other metals (chromium, cadmium, lead) had a very high reduction from the lowest level of 65 to the highest level of 98%.

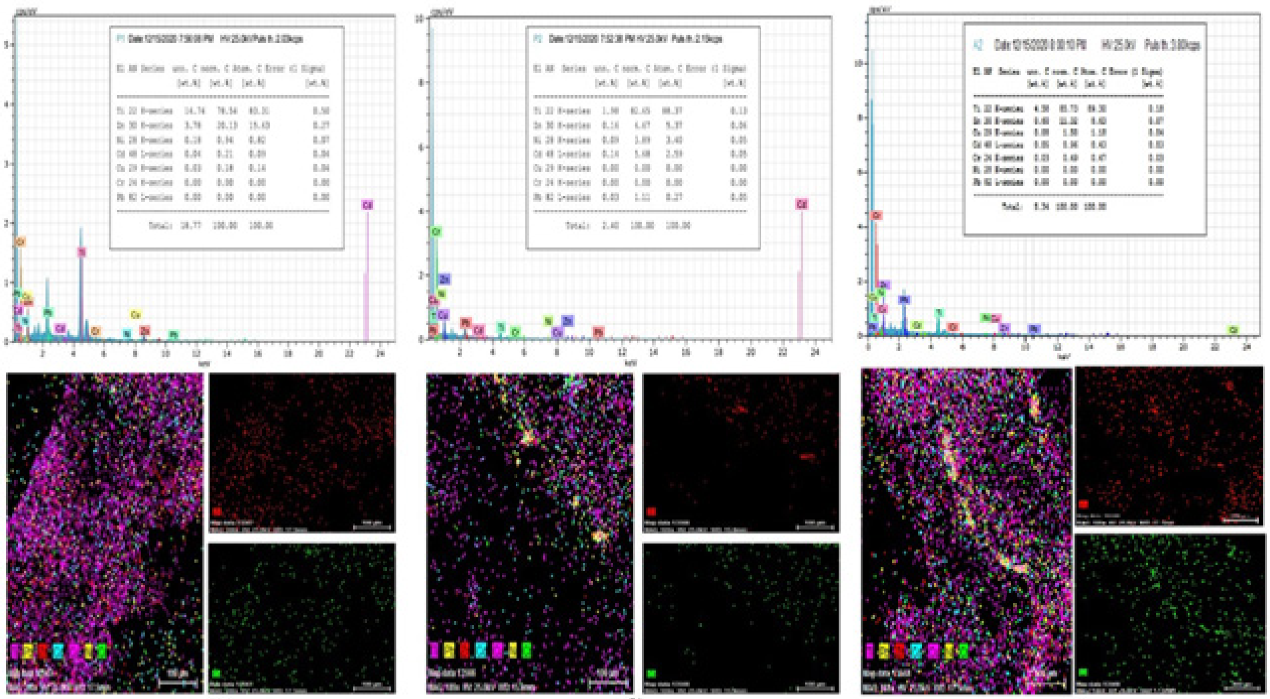
In examining the morphological changes that occurred on the residue surfaces during this process with the SEM microscopic images shown in Fig 2, the texture of the paint sludge and the size of the particles in Fig (a) shows that the primary paint sludge has a cohesive texture and no pores, while in Fig (b) and (c), the fine slim texture contains fine-grained slime particles with more pores probably, as a result, the bioleaching process is. Probably due to the effect of the bioleaching process, which is more porous caused by *A. thiooxidans.*

For initial identification and to provide quantitative information about the composition of paint sludge, SEM-EDX analysis was performed, which (as a percentage of the weight of the components) was shown in the set of Fig No. 3. Energy Dispersive X-Ray Analysis Microanalysis is an elemental analysis technique associated with an electron microscope that determines the energy of X-rays and the energy difference between two shells and the atomic structure of the emitting element, allowing the elemental composition of the sample to be measured (Goldstein & et al., 2007). Fig (a) shows the composition of the total elements in the paint sludge to the extent of 18.77% before the biological leaching process. The reduction of colored spots in mapping, in Fig (b) and (c) shows the removal of heavy metals after the process, which is not only observed in zinc metal as a parameter exceeding the standard but also in titanium and cadmium, which shows



(a) (b) (c)

Figure 2: Morphological changes of the surfaces with 1000 magnification and 50 scale (a) before, (b) after the leaching process by *P. aeruginosa*, and (c) after the leaching process by *A.thiooxidans*.



(a) (b) (C)

Figure 3: EDX analysis and Mapping in (a) before the dye sludge bio-leaching process, (b) after the bioleaching process with *P.aeruginosa*, (c) after the bio-leaching process with *A.thiooxidans* from the residual sludge sample on the filter.

the effectiveness of the bioleaching characterized by a decrease in the density of colored dots.

Fig (a) shows the high amount of zinc metal in 1 Kev peak, which confirms the high amount of zinc and titanium metal during digestion with method B 3050. Titanium metal creates the highest peak at 4.5 Kev. These results are according to the XRD analysis (Khezri & et al., 2012); where titanium is the dominant metal present in paint sludge. In EDX analysis of paint residue sludge after the bioleaching process with P. aeruginosa, zinc metal decreased from 15.63 atomic percent to 5.37 percent in Fig (b) and During the same process; zinc reached 8.63 by A. thiooxidans in Fig (c).

4. Conclusion

Considering the good efficiency of the bioleaching process in removing the target pollutant; Zinc metal, which is one of the highest elements in paint sludge, and at the same time other heavy metals up to the permitted standards for sludge disposal, and obtaining favorable results for both microorganisms at a lower temperature, and a lower rotation shaker in the application of *A. thiooxidans* as seems to be with the aim of Preventing the loss of energy, checking at a lower temperature and rotation shaker to reduce the pollution of this waste before disposal in the environment, and burning this waste in a kiln to form ash, and resulting from its energy, in order to recover precious metals and reduce pollution could be done in small-scale (semi-industrial) ash disposal.

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