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ORIGINAL ARTICLE

Comamonas sp. halotolerant bacterium from industrial zone of Jovein of Sabzevar introduced as good candidate to remove industrial pollution

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ABSTRACT

Background and Objectives: Heavy metals are considered as high risk biocides due to their harmful effects on human health, the environment and other living organisms. Bacterial strains showing resistance to heavy metals has been used for removing such toxic materials from the environment. In this study we isolated and characterized a heavy metals-resistance halophilic bacterial strains from Kal shoor Jovein of Sabzevar, one of the industrial zone of Khorasan-e- Razavi province in Iran and has naturally saline oils.

Materials and Methods: Strain JC-66 is heavy metals-resistance halophilic bacterial strains isolated from Kal shoor Jovein of Sabzevar. The 16S rDNA gene was sequenced to identify this bacterium. The appropriate conditions for its potency to remove the lead were tested in various temprature, pH and agitation speed. The resistance mechanism of JC-66 to lead were investigated.

Results: JC-66 is a *Comamonas sp.* according to 16S rDNA sequence analysis. Based on minimum inhibitory concentration (MIC) results, the isolated strain has high resistance to the lead metal. The optimal condition for lead removal was exhibited in neutral medium (pH 7) incubation temperature 37 °C, and shaking rate of 180 rpm for JC-66. X-Ray Diffraction results also are indicative of adsorption mechanism to lead metal uptake. Plasmid extraction was performed to confirm the role of plasmids in bacterial resistance to lead.

Conclusion: It can be concluded that the mechanism of resistance to heavy metals in the studied strain, is the result of an expression plasmid, and adsorption. It was concluded that JC-66 is able to be one of the best candidates to remove industrial pollution because it showed high resistance to lead.

Keywords: Heavy metals, Halophilic Bacteria, Lead

INTRODUCTION

The rapid advance in technology, industrial development, mining, industrial and household waste and use of pesticides caused releasing of large amounts of toxic compounds such as heavy metals into water or soil (1). Essential and non-essential heavy metals are highly stable and toxic to living organisms (2). Therefore, water and soil contamination with heavy metals threat the humans health and other living organisms (3). Recovery of heavy metals from industrial effluents to the environment is economically attractive to prevent harmful effects of toxic metals on environment. Conventional methods used for the removal of heavy metals from industrial effluents include chemical and physical separation processes, which are either expensive or inefficient (4). Nowadays, researchers are interested to find efficient

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and cheaper separation methods because the large volume of waste is increasing the contents of heavy metals in our life. Among these methods, biological methods using the living and non-living microorganisms for removing the pollution have attracted more and more attention in recent years (5-7).

Microorganisms play an important role in bio-refining processes which are economical methods in order to reduce the toxicity effects (5-8). Halophilic bacteria can grow at high concentrations of anions and cations and are suitable for bio-refining. These bacteria are not normally able to make some of the heavy metals and need to acquire these elements from environment (9).

Many microorganisms have developed several mechanisms to survive in heavy-metal polluted environments. In bacteria, three resistance mechanisms is evolved to tolerate the heavy metals include: 1-efflux: metal ions move out of the cell actively; 2-cations: especially those that tend to accumulate high sulfur, can form complexes with thiol-containing molecules; 3-the reduced state of metals are turned to oxidated state, that seems to yield materials with lower toxicity (10-12). One of the major resistance mechanisms in bacteria, notably to heavy metal is existence of plasmids which encode efflux pumps. In *Pseudomonas* strains the efflux pumps are involved in chromium, lead, cobalt and zinc resistance. (13-14).

The aim of present study was to characterize a heavy metals-resistance halophilic bacterial strain which isolated from Kal shoor Jovein of Sabzevar. This area is the one of the industrial zone of Khorasan-e- Razavi province in Iran and has naturally saline soils. Various factors on bacterial growth as well as the mechanisms of resistance in the presence of heavy metals are assessed. The result showed that the metal- resistance holophilic bacterium, called JC-66, can grow in Kal shoor Jovein of Sabzevar. JC-66 is able to be one of the best candidates to remove industrial pollution because it showed high resistance to lead.

MATERIALS AND METHODS

Isolation and identification of bacteria: The area of study for this work is one of the industrial zone of Khorasan-e-Razavi provinces in Iran named Kale shoor Jovein which has naturally saline soils. Its geogrephical coordinates are 36 ° 17' 55" North, 56 ° 20' 36" East. Soil samples were collected in order to isolate halophile bacteria.

Halophytic test. Halophytic test was performed for samples on different concentration of 2, 5, 7, 10, 15, 20, 25, and 30 w/v of NaCl in 100 ml distilled water and incubated at 37 °C for 18-20 hours with constant agitation 170 rpm. For the negative control experiments, sodium chloride-free medium were used. The optical density of bacteria samples was then determined at a wavelength of 600 nm.

Isolation heavy metal resistance bacteria and MIC test: MIC (minimum inhibitory concentration) test was further carried out in order to determine the threshold level of tolerance of heavy metal resistance- strains (15). Initial screening for heavy metal test was performed by adding salts of heavy metals, such as Pb $(NO3)_2$, and $KCrO_4$, in different concentrations including 50-500 mg L^{-1} to the nutrient agar medium, and then incubated at 37 °C for 18-20 hours. The metal-free nutrient broth medium was used as a negative control. The media turbidity was measured using a spectrophotometer (UNICAM 8620) at 600 nm after 24 h of incubation time. The samples with optical density greater than 0.5 at 600nm were identified as a resistant.

Three factors affected on heavy metal removal. In order to select the heavy metal resistance strains, it is necessary to standardize the cultural and physiological conditions of the selected organisms. Among the physico-chemical conditions, temperature, pH level and aeration rate are important parameters on bacterial growth as well as heavy metal removal.

Temperature profile. for determination of optimum temperature to Pb uptake, 20 ml of nutrient broth medium containing 50 ppm of Pb (NO3) with pH 7 for 9 h at various temperatures 25, 37, 45 °C, with constant agitation of 180 rpm. The mediums were removed by centrifugation for 10 min at 12,000 rpm and the supernatant was used for the metal uptake analysis by atomic absorption spectroscopy.

pH profile. pH is assumed to be the limiting factor for bacterial growth. In order to determine the optimum pH for heavy metal removal, the different pH values of nutrient broth mediums containing 50 ppm

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lead nitrate were adjusted in rang 3-9. The Mediums were subsequently incubated for 9 hours at 37 °C at a shaking speed of 180 rpm. The concentration of lead ions remaining in the supernatant was analyzed using atomic absorption spectroscopy.

Aeration profile. To determine the aeration profile, 20 ml of nutrient broth containing 50 ppm lead nitrate were adjusted in rang of 0, 100, 180 and 250 rpm. After 9 hours of incubation at 37 °C the concentration of metal ions remaining in the supernatant was measured using the atomic absorption spectroscopy.

Molecular characterization. JC-66 strain was characterized on the basis of 16S rDNA sequence analysis (16). The 16S rDNA gene was amplified by polymerase chain reaction using universal primers (Forward primer: 5'-AGTTTGATCCTGGCTCAG-3' and Reverse primer: 5'-GGC/TTACCTTGTTACGACTT-3').

The polymerase chain reaction was carried out using optimized program in a volume of 50 µl, in three segments: initial denaturation at 94 °C, for 5 minutes; 30 cycles of denaturation at 94 °C for 45 seconds, annealing at 52 °C for 45 seconds and extension at 72 °C for 90 seconds. The final extension was performed at 72 °C for 5 minutes. Finally, the product of PCR were analyzed and reviewed to determine the nucleotide sequence. Sequencing of amplified DNA, using sequence-searching of DNA, was determined based on the Chain termination method (17-18). The 16S rDNA gene sequence was submitted to NCBI (NCBI accession number: KM873625) (Fig. 1). Using 16S rDNA gene sequences, the strains were identified by BLAST search on NCBI Server (http://blast.ncbi.nlm.nih.gov/Blast.cgi). The sequences of closely related type strains were retrieved for constructing the phylogenetic tree. A phylogenetic tree was drawn from unambiguously aligned nucleotides using the neighbour-joining algorithm with phylodraw (19).

Study of antibiotic resistance. Antibiotics resistance test was performed in the presence of 100 ppm, and 800 ppm of lead, as well, in the presence of chromium (25 ppm) in order to confirm the plasmid role in creating bacterial resistance to heavy metals and antibiotics. The strains were cultured on a nutrient agar plates containing the specific concentration of heavy metal in the presence of our antibiotics including ampicillin (10 µg), tetracycline (30 µg), gentamicin (10 µg), chloramphenicol (30 µg). All the plates were then incubated at 37 °C, for 24 hours. Plates without heavy metal were considered as a negative control.

Extraction of plasmid DNA. Plasmid extraction was carried out with a plasmid purification kit according to the protocol provided by the supplier (Sinaclone, Iran). Plasmid extraction of bacteria that grow in the absence or presence of metals was performed to confirm the role of plasmids in bacterial resistance to lead.

X-ray diffraction study. The strains were cultured in the presence of 200 ppm lead and incubated at 37 ˚C for 24 h in 170 rpm. The suspensions were then centrifuged at 5,000 rpm for 15 min. The supernatant was discarded and the pellet was analyzed using X-ray diffraction in Tarbiat Modares University. The diffraction spectra were recorded using monochromatic copper anode tube with the diffractometer in the range of 2 θ: 10 -89.93.

RESULTS AND DISCUSSION

Isolation and identification of bacteria: Different bacterial strains were collected from Kal Shoor, in Jovein abroad of Sabzevar samples. The heavy metal tolerance property of the halotolerance strains were assessed by their ability to grow in the presence of sodium chloride and heavy metals. The strain called JC-66, a Gram-negative and rod shaped, was selected.

Identification of the bacteria: Multiple alignment and phylogenetic tree showed that JC-66 with 99 % sequence similarity with the 16S rDNA of *Comamonas sp.* (Fig.1). The sequence was submitted to GeneBank (accession number: KM873625).

Determination of the strains and type of halophytic. The halophytic test was performed to determine the ability of the strains to grown in the presence 0-10% NaCl but grows optimally at 0-5% NaCl. (8). The result confirmed that JC-66 is a halotolerance bacterium.

MIC Test. Agar dilution method was used to deter-

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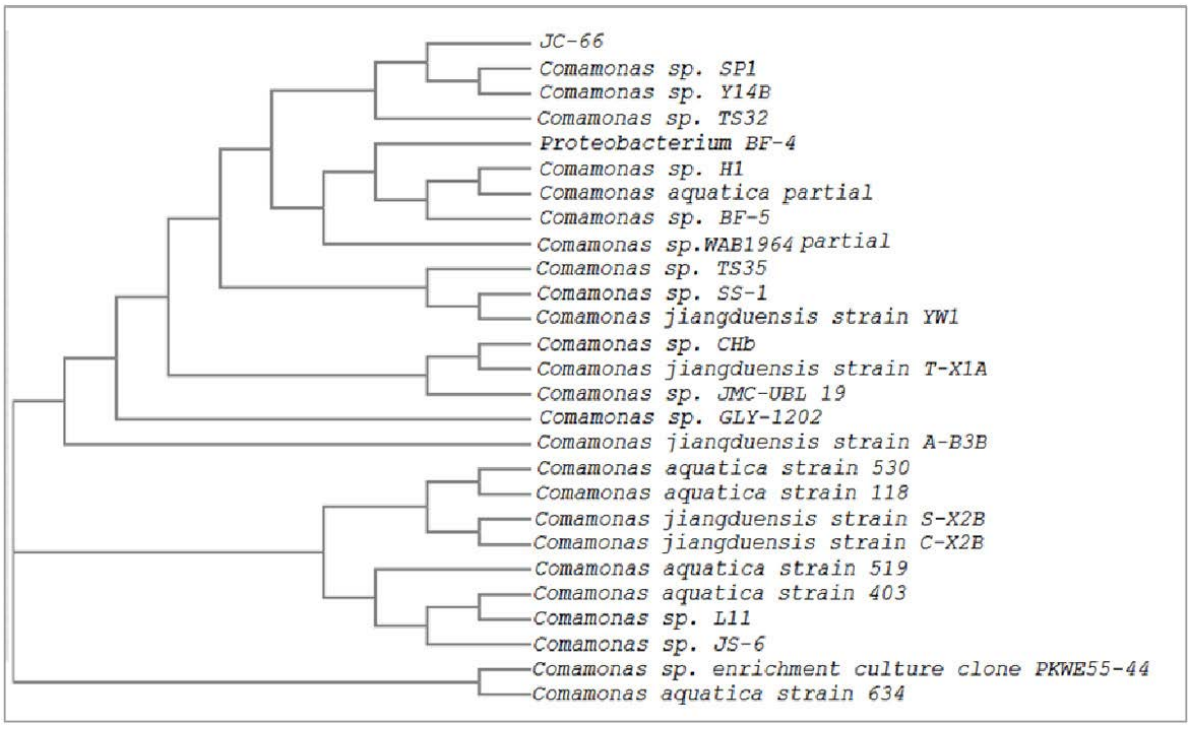

Fig.1. The phylogenetic tree of JC-66 on the base of 16s rDNA sequence.

mine the MIC of isolated bacteria against heavy metals. It has been shown that Gram-negative bacteria have the ability to grow in the presence of heavy metals (20). TheJC-66 showed high resistance to lead (up to 40000 ppm) whereas it was resistance to Cd and Cr at low concentrations (200 and 50 ppm, respectively). Lead is highly toxic and has a profound effect on microorganisms' growth.

Antibiotic susceptibility test. Existence of heavy metals in the environment of bacteria could affect their susceptibility to antibiotics. In most cases, there is a positive correlation between the antibiotic resistance and metal tolerance. It is possible that the metal resistance genes and antibiotic resistance genes are located on the same plasmid. The measurement of halo diameter around discs of antibiotics was affirmed the pattern to various antibiotics resistance (21). The results showed the lead metal has effect on antibiotic resistancy in this strain (Table 1). Accordingly, JC-66 is resistance to ampicilin and also in the high amount of lead maintained its resistancy. It seems that exposure to bacteria in the environment containing the metal causing the expression of resistance to metals. The expression of these genes could be associated with antibiotic resistance gene on a plasmid (22-23). These results confirmed that the combined expressions of heavy metal tolerance and antibiotic resistance may

Table. 1. The antibioibittic susceptibility test results for JC-66 strain in media containing heavy metal and free-heavy metal media.

Strain JC-66	Tetracycline 30	Gentamicin 10	Chloramphenicol 30	Ampicillin 10
control	Resistance	Susceptible	Resistance	**Resistance**
25 ppm Cr	Susceptible	Susceptible	Intermediate	**Susceptible**
100 ppm Pb	Resistance	Susceptible	Resistance	**Resistance**
800 ppm Pb	Intermediate	Susceptible	Intermediate	**Resistance**

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not be a chance phenomenon rather these are the results of selection by heavy metals present in an environment.

Factors affecting on heavy metal removal. Adsorption and enzymatic conversion are two known mechanisms which bacteria implement to remove the metals from the environment. Studies have shown that lead is possibly removed from the environment, through adsorption and intracellular accumulation. To obtain initial conditions for maximum removal of heavy metals and using it in bio-refining, the continuing influence of different conditions on metal uptake with the purpose of optimizing initial conditions, was studied. The effect of various parameters including temperature, pH and aeration on the lead uptake was investigated through the remaining amount of lead in the environment.

Effect of temperature on the lead uptake. As shown in Fig. 5 the maximum uptake of lead in JC-66 strains is occurred at 37 ˚C. After 9 hours of incubation, the 50 ppm lead is not detectable in media (Fig. 2). JC-66 strain was reached to maximum growth at 37 ˚C caused increase lead uptake. It is apparent that by increasing the temperature (25 ˚C to 37 ˚C) metal-binding sites increase. In contrast, metal uptake decreased at 45 ˚C due to either reducing bacterial growth or changes that may occur on the bacterial membrane at high temperatures (24).

Effect of pH on the rate of harvest. The results of various pH on lead metal uptake by JC-66 showed that the minimum and maximum uptake occurred at pH 3, and pH 7, respectively (Fig. 3). This may be related to the fact that pH of media affects the accessibility of metal ions and metal-binding sites such as functional groups (carboxyl, phosphate, hydroxyl) on the bacterial surface. The lead uptake capability of JC-66 strain was reached to maximum level at pH 7 because of the bacterial growth rate increasing. It seems that the speed of the action of the metal on the landing sites increase in the pH 7, the probability of the formation of insoluble compounds can penetrate the cell wall, and in turn affects the metal uptake mechanism (24-25).

Effect of aeration on the removal of metal. The results showed that the maximum lead uptake by the JC-66 strain occurred at the 180 rpm agitation speed. Also, lead absorption at zero speed for JC-66 is at least, by increasing the rate of aeration the harvest speed increases. Ultimately at speeds over 180 rpm, the harvest is downward (Fig. 4). By increasing air speed, all metal-binding sites located on the bacterial surface are accessible and therefore the number of metal could attach to the surface of bacteria increases

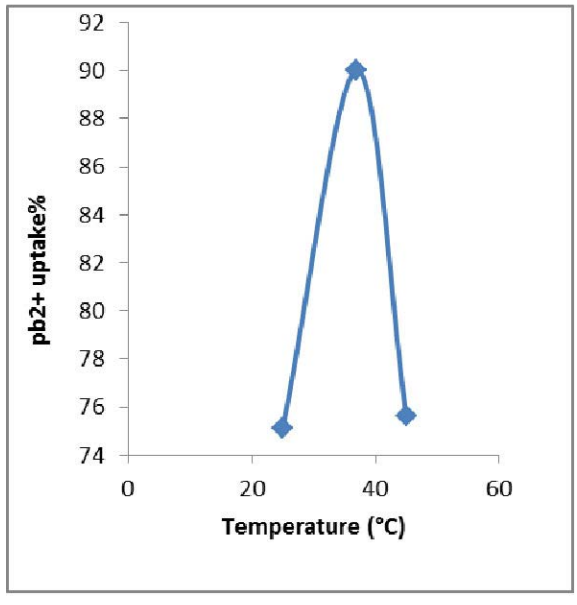

Fig. 2. Temperature profile of JC-66 showed the effect of temperature on the lead uptake.

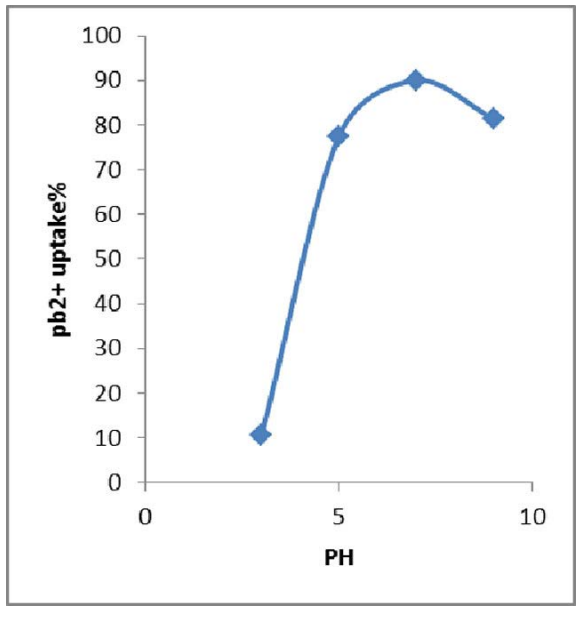

Fig. 3. The effect pH on metal removal in JC-66 strain

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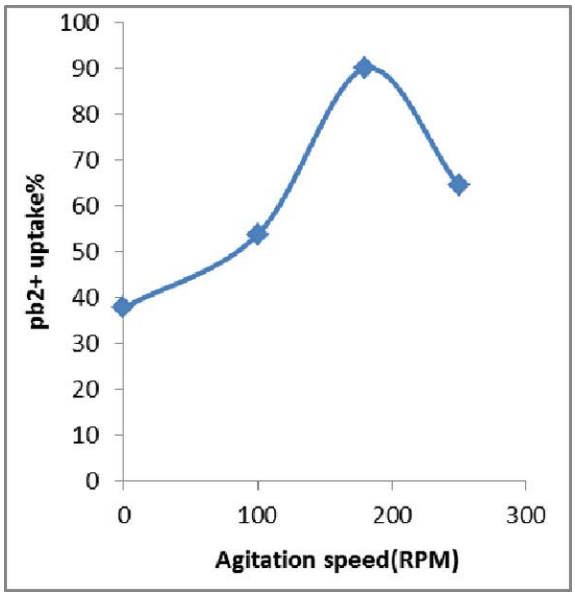

Fig. 4. The aeration profile of JC-66 showed the aeration effect on lead removal.

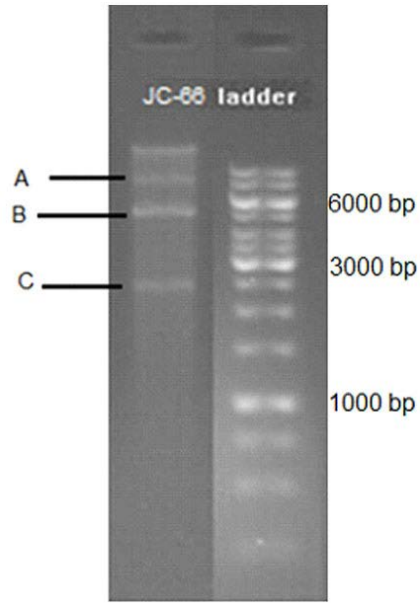

Fig. 5. Extraction of plasmid DNA from JC-66 strain confirmed the genetic factor in the mechanisms of lead resistance. (A: Nicked plasmid, B: Linear plasmid and C: Super coil plasmid)

whilst above the optimum speed the attached metals reduce due to turbulence in the environment.

Investigate the mechanism of resistance of strains in the presence of heavy metals. Extraction of plasmid DNA: Genetic factors play an important role in determining the rate of bacterial resistance to heavy metals. One of the mechanisms of resistance to an essential metal can be chromosomal and also one of the excretion mechanisms of toxic metal ions can be plasmid (21,22). Agarose gel electrophoresis was performed to confirm the presence of plasmid in lead presence (Fig. 5). It should be noticed that any plasmid was not obtained in lead absence (data not showed). Then, plasmid extraction in lead presence confirmed that genetic factors play important role to lead uptake in JC-66.

XRD. The chemical nature of metal attached to the surface of bacterial cells detected using X-ray diffraction. After comparing the spectrum with the standard diffraction patterns, the International Centre ICCD, the results confirmed that the Pb_2O_3, NH_4NO_3 and $Pb3(NO_3)(OH)5$ crystals formed on the surface of JC-66 strain. The XRD spectrum shows a sharp and high-intensity peak in the 2Θ: 37.055, which are related to the Pb_2O_3 crystal (Fig. 6). It suggest that the presence of NH4NO3 and Pb3(NO3)(OH)5 crystals on surface of bacteria is related to lead nitrate $Pb(NO_3)_2$ which added to the media (26, 27).

CONCLUSION

It is concluded that the JC-66 strain is a halotolerant bacterium that the results of the MIC confirm its high resistance to lead. The evidence showed two mechanisms to remove the lead by JC-66 strain, plasmids and adsorption. It seems that the plasmid system increase the efflux pump expression in membrane of bacteria. Then, leads ions enter into the cell via efflux pumps, which are known to the mainstream. The XRD results also suggest the adsorption mechanism for its strain resistancy. Also, there was a connection between metal tolerance and antibiotic resistance in the JC-66 strain. It seems that the increased heavy metals in the environment leads to increased resistance of microorganisms to the heavy metal in the environment.

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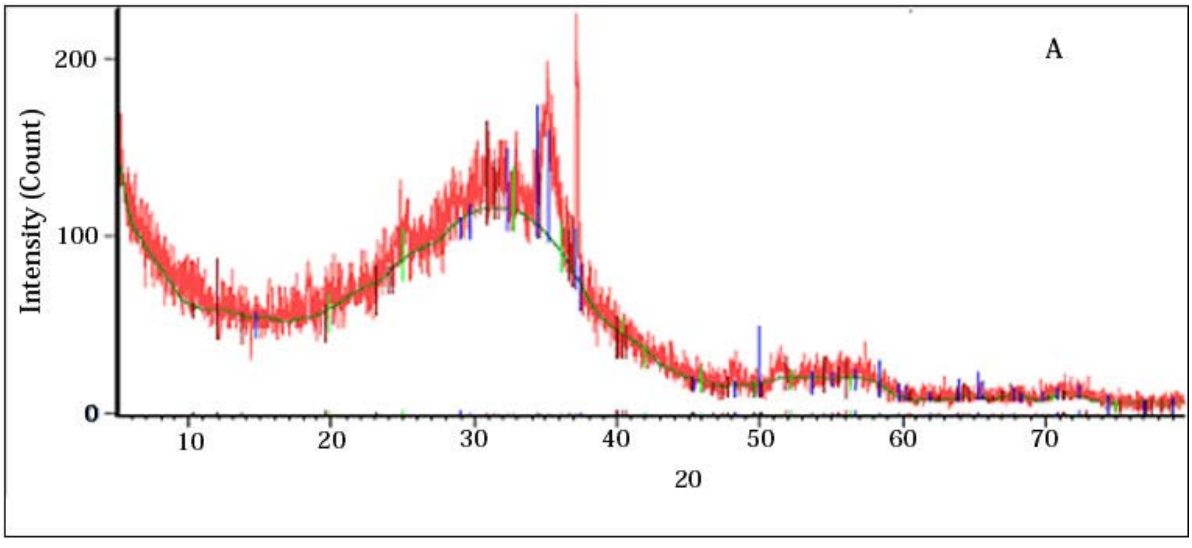

Fig. 6. The XRD spectrum of JC-66 strain showed that the Pb2O3, NH4NO3 and Pb3(NO3)(OH)5 crystals formed on the surface of JC-66 strain.

92 from the Hakim Sabzevari University.

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