ISOLATION AND IDENTIFICATION OF HEAVY METAL TOLERANT BACTERIA FROM SUGARCANE INDUSTRIAL WASTEWATER

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Abstract. One of the most serious problems of the environment is heavy metal toxicity originating from the industrial activities even in traces which harm the ecosystems. Initially a total of 30 isolates were screened on nutrient agar medium containing heavy metals: zinc, iron, cobalt, cadmium, copper and lead at four different concentrations (25, 50, 75 and 100 µg/mL in their salt form). Three isolates of Bacillus sp. were isolated from effluent industrial wastewater of Abu Kerqas Sugar Factory (27°55’ 28.8” N 30°49’ 00.4” E), identified by using 16S rRNA gene and documented in GenBank as Bacillus spp. SMMAA-1 (LC472522), Bacillus cereus SMMAA-3 (LC472523), Bacillus altitudinis SMMAA-4 (LC472524). The bacterial isolates were selected based on their growth curve; IAA reaction and antibiosis effect were identified based on their morphological, biochemical characterization and utilization of carbohydrates as carbon sources. These isolates were evaluated for their abilities to bioremediate the toxic heavy metals. Results showed that bacteria present resistance to six heavy metals. B. cereus is more effective concerning its removal efficiency percentage for six heavy metals (Zn²⁺, Fe²⁺, Co²⁺, Cd²⁺, Cu²⁺ and Pb²⁺) (94.77%) from Industrial Wastewater than Bacillus spp. (83.19%) and B. altitudinis (83.21%). The highest removal efficiencies by Bacterial isolates were found with Co²⁺, Cd²⁺ and Cu²⁺ and the lowest removal biosorption were recorded with Fe²⁺.

Keywords: Bacillus spp., Bacillus cereus, Bacillus altitudinis, bioremediation, effluent wastewater, 16S rRNA gene, GenBank

Introduction

Many organizations and scientists from various disciplines mentioned that the bioremediation can be used to break down the metal availability below the permissible limit. Most of them are in their advanced stages of developing different protocols and identifying a plethora of bacterial species to solve the issue (Kielak et al., 2017; Sardar et al., 2018). Exceedingly higher concentrations of heavy metals are a menace to human health due to their harmful effects such as genotoxicity towards the DNA and immunotoxicity as they are major irritants to the body. The genomic instability by these metals induces cancer (Leonard et al., 2004).

Heavy metals toxicity is significant environmental pollutants problem of increased significance for ecological, evolutionary, nutritional and environmental reasons (Nagajyoti et al., 2010; Jaishankar et al., 2014). Heavy metals water pollution one of serious important problems in world and Egypt. Heavy metals accumulations were observed in Egyptian
cultivated such as soil, Nile River and air (Abbas and Kamel, 2004). Today, the quality of Nile water is a matter of serious concern due to exposure to increasing at an alarming rate multiple sources of heavy metals pollution including industrial, agricultural and domestic effluents. Effluent industrial wastewater is the main source of Nile pollution by heavy metals. The most commonly found heavy metals in wastewater include arsenic, cobalt, iron, cadmium, chromium, copper, lead, nickel, and zinc, all of which cause risks for human health and the environment (Lambert et al., 2000).

So, quick and easy removal and recovery of heavy metals from industrial wastewater at low cost are needed. Several conventional methods include precipitation, membrane filtration, ion exchange, electrochemical recovery; biological separation and adsorption are used to remove heavy metals from wastewater. Nevertheless, these methods are unfriendly environmental, long processing times and high costs (Carolin et al., 2017). Nowadays, in light of this necessary for attractive alternative strategies low-cost, efficient and environmentally friendly methods instead of traditional to recover heavy metals from wastewater are needed (Moradi et al., 2015). The growing industrialization has spread worldwide and has left persistent toxic heavy metals, like chromium, nickel, lead, zinc, cadmium and copper in our ecosystem. These heavy metals tend to accumulate and deteriorate the environment. This is especially true for developing countries like China and India (Raja et al., 2008; Chauhan et al., 2017).

The bioremediation methods for reducing more of heavy metals in the environment have attracted importance. Living organisms were reported to able to absorb pollutants and remove heavy metals from the environment. Plants, fungi and microorganisms such as yeasts, bacteria, algae, and cyanobacteria are usually used for the bioremediation of heavy metals and recorded to be the best acceptable ones because they are easier to work with (Massoud et al., 2018). Several studies reported that bioremediation is the most effective management tool to manage the polluted environment and recover contaminated environment (Ahemad, 2012). Since heavy metals are ubiquitously present in our environment, microorganisms such as bacteria, yeast or fungi have developed mechanisms to removal contaminated soil and water via heavy metals (Kumar et al., 2011). Bioremediation includes some methods such as phytoremediation, biodegradation, bioventing (Dupont, 1993).

Assessment of microbes for their remediation potential in dealing with industrial pollutants is another point of interest where a bacterium or a fungus produces metabolites as their weapon of degradation (Pathak et al., 2017). Many bacterial strains contain genetic determinants of resistance to heavy metals such as Hg^{2+}, Ag^{2+}, Cu^{2+}, Ni^{2+}, Cd^{2+} and others (Karelova et al., 2011; Chauhan et al., 2017).

Several investigators have used different bacterial strains like Bacillus sp., Bacillus licheniformis, Bacillus thuringiensis, Pseudomonas sp., Staphylococcus aureus to for the biosorption as well as bioaccumulation of various metal ions like Cr(VI), Ni(II), Zn(II), Pb(II) etc. (Şahin and Ozturk, 2005; Tunali et al., 2006; Zhou et al., 2007; Ziaogva et al., 2007; Wang et al., 2010; Akhter et al., 2017). Thus, the overall objective of the study is to enhance production of bacterial strains with high ability to uptake of heavy metals by different concentrations to helping in the reduction of Industrial wastewater effluent pollution of heavy metal. This emphasizes the importance and needs of carrying out extended testing for the compatibility of biosorption to heavy metals toxicity. It is more effective, cheap than traditional technologies of treating contaminated water with heavy metals, including precipitation, ion exchange or reverse osmosis still generate too large costs.
This study aimed to isolation and identification of bacterial isolates that have ability to heavy metals biosorption and the role of bioremediation as technological method for uptake of six heavy metal ions from industrial wastewater effluent in Egypt to reduce the environmental pollutions.

Materials and methods

This study was conducted at both of Laboratory of Microbiology, Mallawy Agric. Res. Station, Dept. of Agric. Microbiology., Soil, Water and Environmental Research Institute, Agric. Res. Center, Giza, Egypt, and Central Lab., Faculty of Postgraduate Studies for Advanced Sciences, Beni Suef University. Molecular identification of the bacterial isolates was done in Microbiological Laboratory, Faculty of Agriculture, Zhejiang University, Hangzhou City, and East China during the period of 2017-2018.

Industrial wastewater samples collection and preparation

In this study, effluent industrial wastewater samples were collected from the site of the main drain of Abu Kerqas Sugar Factory (27°55’ 28.8” N 30°49’ 00.4” E) (Fig. 1), El-Minia Governorate, March, 2017. Three samples of wastewater each 10 L per sample were collected in plastic jerry cans. Cans were previously sterilized by 75% ethanol, washed three times with wastewater to remove the residual effect of ethanol, and filled with the samples and then transferred immediately to the Microbiology Lab of Mallawy Agric. Res. Station. The three samples were mixed gently to obtain the main sample and kept in the refrigerator (6 ± 2 °C) for the further studies.

![Figure 1. The main drain of Abu Kerqas Sugar Factory (27°55’ 28.8” N30°49’ 00.4” E)](image)

Determination of heavy metal concentrations in the wastewater samples

Samples were taken from the main sample and sent to the Central Lab., Faculty of Postgraduate Studies for Advanced Sciences, Beni Suef University. The concentrations of heavy metals (Zn^{2+}, Fe^{2+}, Co^{2+}, Cd^{2+}, Cu^{2+} and Pb^{2+}) in samples were analyzed and determined by the Atomic Absorption Spectroscopy (Model: Agilent Technologies 200 series AA System) according to the method of EPA (2005).

Bacteria isolation via streak plate technique

Three dilutions (1.00, 0.50 and 0.25) of each of the main sample and collected samples containing heavy metals, was done as described by Azad et al. (2013) to isolate
the desired bacteria that have more tolerance to high concentration of heavy metals. This technique was applied to isolate some bacterial colonies able to grow in the presence of heavy metals. Two loops from the best dilution of the wastewater samples was taken and streaked onto sterile petri plates containing nutrient agar medium (NAM) as recorded by Marzan et al. (2017). Single colonies were selected, picked, purified and inoculated on NAM slant and coded as T1 up to T30. These single colonies were purified on NAM, and preserved on different plates or slants for further experiments.

**Tolerance of bacterial isolates to six heavy metals**

Six heavy metals: zinc (Zn$^{2+}$), iron (Fe$^{2+}$), cobalt (Co$^{2+}$), cadmium (Cd$^{2+}$), copper (Cu$^{2+}$) and lead (Pb$^{2+}$) were used in their salt structures as: ZnSO$_4$, FeSO$_4$$\cdot$7H$_2$O, COC$_{12}$, CdSO$_4$$\cdot$3H$_2$O, CuSO$_4$$\cdot$5H$_2$O and PbSO$_4$. According to the method of Vijayadeep and Sastry (2014) heavy metals salt solutions (25, 50, 75, 100 μg/mL) were prepared in distilled water to obtain concentrations and sterilized by 0.2 μm pore-size Millipore sterile filters. The bacterial isolates (T1, T2 and T3……T30) were checked for the heavy metals tolerance using the agar well diffusion method as reported by Collins et al. (1985) in sterile NAM plates. On incubation of plates at 35-37 °C for 72 h, the inhibition zones (the distance between the end of the zone and the edge of the well, mm) were measured. Isolates showing a clear zone of 1 mm or less was considered as resistance (R) isolate according to Rani and Moreira (2010). The bacterial isolates which appeared resistance to the heavy metal ions at the all tested concentrations were recorded as (T1, T2, T3, T4, T5, T6, T7, T8, T10 and T18).

**Effect of industrial wastewater (IWW) toxicity on each of bacterial growth and indole acetic acid (IAA) production (color change) by the ten bacterial isolates**

The effects of IWW on the growth of the selected isolates (T1, T2, T3, T4, T5, T6, T7, T8, T10 and T18) were determined by growing the bacteria in nutrient broth medium (NBM) supplemented with sterile IWW (SIWW). IWW was sterilized by 0.2 μm pore-size Millipore sterile filters. The cultures of bacterial isolates were grown in the presence of SIWW, and as a blank SIWW were taken out. One loop from each isolate culture was taken and inoculated in 100 mL NBM and incubated under shaking condition (180 rpm) for 24 h at 30 °C as described by Priyadharshini and Kumar (2016). Then, 3.0 mL of the bacterial culture were added to each flask contained 100 mL of SIWW, mixed well and incubated as mentioned before. Bacterial growth was measured using a spectrophotometer (Spectronic 20 BauTchandLomp) at 620 nm as optical density (OD). The recorded data were taken after 1, 2, 3, 4, 5, 6 and 7 days from the incubation.

In case of effect of IWW on IAA produced by the bacterial isolates under investigation, the ten bacterial isolates were grown overnight in sterilized NB medium at 30 °C for 24 h, and then 2.0 mL from each isolate were taken and inoculated with 5 mL SIWW to determine the degree of IAA by change of color visually as described by Glickmann and Dessaux (1995).

**Methods to determine the antibiosis activities of the ten bacterial isolates**

The antagonism of the bacterial isolates against F. solani, S. rolfsii and E. coli was performed using well diffusion method of Nedialkova and Naidenova (2005). The filtrate of E. coli was dropped (1-2 mL) in prepared holes of the NAM inoculated with
ten bacterial isolates and incubated at 30 °C for 24-48 h. Concerning to the antagonistic effect against F. solani and S. rolfsii, disc from each fungus growing on potato dextrose agar medium (PDA) was taken, and inoculated with the tested bacterial isolates. Three petri dishes per each of isolate and pathogen were used as replicates. The plates were incubated at 28 °C for 6 days, and the antagonistic activities were then recorded.

**Biological and molecular identification of the selected bacterial isolates**

The three bacterial isolates (T1, T3, and T4) which selected based on their tolerance to heavy metals; growth curve, IAA production and antagonistic effects were identified according to the methods described by Juni (1986) reported in Bergey’s Manual of Systematic Bacteriology (1986, 2012).

Using 16S rRNA gene the identification of the three bacterial isolates was confirmed. NAM slants of the isolates were sent to Microbiology Laboratory, Faculty of Agriculture, Zhejiang University, Hangzhou City and East China. The genomic DNA of isolates was extracted using a Gene JET extraction kit (Thermo K0721) according to the Manufacturer’s instructions. 16S rRNA region was amplified with the following bacterial primers (F: 5’AGA GTT TGA TCC TGG CTC AG3’ and R: 5’GGT TAC CTT GGT ACG ACTT3’). Thermal cycling consisted of initial denaturation at 94 °C for 2-5 min, followed by a cycle of denaturation at 95°C for 30 s, annealing at 55-60 °C for 1 min and elongation at 72 °C for 60 s, and finally, at 72 °C for 5-10 min for completion. The PCR amplification was performed using Gene JET Gel Extraction (ThermoK0701). Sequencing was performed using the ABI PRISM BigDyeTM Terminator Cycle Sequencing Kits, ABI PRISM 3730XL Analyzer (96 capillary type) sequencer (Applied Biosystems), MJ Research PTC-225 Peltier Thermal Cycler, DNA polymerase (FS enzyme) (Applied Biosystems). The DNA sequences of the PCR product of the 16S rRNA gene of the three bacterial isolates were through the BLAST P program which available on the National Center for Biotechnology Information website http://blast.ncbi.nlm.nih.gov/Blast.cgi? PROGRAM=blast p and PAGE – TYPE=BLAST Search and LINK – LOC=blast home (Altschul et al., 1990).

**Selected bacterial isolates (T1) Bacillus spp., (T3) B. cereus and (T4) B. altitudinis effects**

The effects of the selected isolates (Bacillus spp., B. cereus and B. altitudinis) were tested for their ability to alleviate or eliminate the heavy metal concentrations for industrial wastewater effluent.

1 ml from each bacterial isolate that growing in nutrient broth medium was inoculated to flasks contained 100 ml of sterilized industrial wastewater (SIWW). The flasks were incubated in a shaker for 24 h at 30 ± 2 °C under static conditions as mentioned by Priyadharshini and Kumar (2016). The flasks were filtered (Whatman filter paper No.1) then the samples were received to the Central Lab., Faculty of Postgraduate Studies for Advanced Sciences, Beni Suef University to determine the heavy metals concentrations for each sample. The percentage of removal heavy metals (Zn²⁺, Fe²⁺, Co²⁺, Cd²⁺, Cu²⁺ and Pb²⁺) was calculated as *Equation 1* and that recoded by Bakar et al. (2013) as follows:

\[
\text{Removal efficiency\%} = \frac{\text{Initial metal conc.} - \text{Final metal conc.}}{\text{Initial metal conc.}} \times 100 \quad (\text{Eq.1})
\]
**Statistical analysis**

The data were prepared by mean ± standard deviation (n = 3) and carried out as a randomized complete design (Snedecor and Cochran, 1980) using LSD test to compare means of treatments in investigation. Statistical significance was defined as P < 0.05. Mean values of three replicates followed by the same letters in each column are not significantly different (P > 0.05) (Duncan multiple range test).

**Results and discussion**

This study was designed to isolate some promising bacteria able to tolerate the high levels of heavy metals in EIWW in a trail to decrease or remove the heavy metals present in the EIWW. Therefore, determination of heavy metals concentration in EIWW samples was conducted for the six heavy metal ions (Zn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Cu$^{2+}$ and Pb$^{2+}$) that threatening the environments, human, plants and animals.

**Heavy metal concentrations in the EIWW samples**

Data illustrated in Table 1, showed that the concentrations of the six selected heavy metal ions in EIWW samples. The concentrations were ranged from 6 ppm (Zn$^{2+}$) to 1.4 ppm (Co$^{2+}$). It was noted that all tested heavy metals presented in high concentration compared to permissible limits excepted for Fe$^{2+}$ ion which was the lowest one (2.1 ppm) compared to permissible rate (3.0 ppm). Also, the concentrations of Co$^{2+}$, Cd$^{2+}$ and Pb$^{2+}$ heavy metals were more folds than the guidelines and standers of WHO, therefore, these ions were considered as the highest toxic ions. This notes in harmony with that reported by Rajendran and Gunasekaran (2007) and Murthy et al. (2012).

**Table 1. Heavy metals concentrations in the main EIWW sample collected from Abu Kerqas Sugar Factory, El-Minia, Egypt, compared to permissible rate (ppm) according the guidelines and standers of WHO**

| Metals | Conc. (ppm) | Permissible rate (ppm) | References |
|--------|-------------|------------------------|------------|
| Zn$^{2+}$ | 6.0 | 5.000 | WHO (1984) |
| Fe$^{2+}$ | 2.1 | 3.000 | WHO (2006) |
| Co$^{2+}$ | 1.4 | 0.010 | WHO (2003) |
| Cd$^{2+}$ | 1.8 | 0.005 | WHO (1984) |
| Cu$^{2+}$ | 1.5 | 1.000 | WHO (1984, 2003) |
| Pb$^{2+}$ | 1.9 | 0.050 | WHO (1984, 2003) |

WHo = World Health Organization

**Bacteria isolation via streak plate technique using NA media supplemented with different dilutions of EIWW**

Data represented in Figure 2 show the presence of single colonies exhibited Bacillus-like species cultural properties grown on NAM inoculated with different dilutions of EIWW. The lowest concentration (0.25 mL) from (EIWW) have purified single separated colonies compared that of 0.50 and 1.00 mL. The last two dilutions showed crowded, compacted and irregular colonies on the NAM which are not easily
distinguished. Therefore, it has been observed that of the dilution of 0.25 mL was considered the best dilution to obtain the single separated colonies which were easily selected, picked, purified and easily distinguished. A number of 30 isolates were picked, purified and coded symbols of T1, T2, T3 and T4 …to T30.

Figure 2. Cultural properties of the isolated bacteria grown on different dilutions of EIWW

Tolerance of bacterial isolates to six heavy metals

The bacterial isolates were coded as T1 up to T30, and evaluated for their abilities to tolerant a number of six heavy metals (Zn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Cu$^{2+}$ and Pb$^{2+}$) at four concentrations (25, 50, 75 and 100 µg/mL) as shown in Table 2. Based on the diameter of inhibition zones, the isolates were classified as sensitive or tolerant to each heavy metal ion and its concentration. The isolates were varied in their abilities to tolerant the heavy metal concentrations. The isolates that able to grow at concentrations of metal ions at 25, 50, 75 and 100 (µg/mL) were considered as tolerant as they showed a clear inhibition zone of 1 mm (Fig. 3) or less according to (Rani et al., 2010). Data in Table 2, showed that T10 isolate appeared tolerance to Cd$^{2+}$, Cu$^{2+}$ and Pb$^{2+}$, while T18 isolate were tolerant to Zn$^{2+}$, Fe$^{2+}$ and Co$^{2+}$. Results also showed that the highest tolerant bacterial isolates to the four concentrations of the six for heavy metal ions were T1, T2, T3, T4, T5, T6, T7, and T8. On the other direction, T23 isolate appeared sensitivity to all tested heavy metal ions except for with Pb, as it was tolerant (Table 2).

Figure 3. Tolerance of the bacterial isolate to heavy metals [resistant (a) and clear of inhibition zone as sensitive (b)]

At the lowest concentration (25 µg/mL) of all six heavy metal ions, all bacterial isolates were tolerant except for the heavy metal ion (Pb$^{2+}$). These results are in agreed with that reported by Malik and Jaiswal (2000), who showed that acceptable
concentration of metal ions, which could be used for distinguishing metal tolerant and metal-sensitive bacteria, strains able to grow at concentrations of metal ions at and above 1.0 mM were considered resistant. Based on the experimental findings, the heavy metal-tolerant bacteria could be selected as especially promising microorganisms for bioremediation application of heavy metals polluted places.

**Effect of EIWW toxicity on each of bacterial growth and indole acetic acid (IAA) produced by bacterial isolates**

Effects of SIWW on bacterial growth, representing in each of growth curve [expressed as optical density (OD) (λ = 620 nm) as recommended by Priyadharshini and Kumar (2016), and bacterial growth periods [1, 2, 3, 4, 5, 6 and 7 days] of ten isolates (T1, T2, T3, T4, T5, T6, T7, T8, T10 and T18) was carried out.

**Table 2. Tolerance of bacterial isolates to six heavy metals expressed as zone of inhibition**

| ICs | Zn\(^2\) | Fe\(^2\) | Co\(^2\) |
|-----|----------|----------|----------|
|     | 25       | 50       | 75       | 100      | 25       | 50       | 75       | 100      | 25       | 50       | 75       | 100      |
| T1  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T2  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T3  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T4  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T5  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T6  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T7  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T8  | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T9  | R        | R        | R        | 3.5 (S)  | 3.7 (S)  | 3.5 (S)  | 3.9 (S)  | R        | R        | R        | R        | R        |
| T10 | R        | R        | R        | 3.5 (S)  | 4 (S)    | 3.5 (S)  | 3.7 (S)  | R        | R        | R        | R        | R        |
| T11 | R        | R        | 1 (S)    | 1 (S)    | R        | R        | 0.5 (S)  | 1 (S)    | 1 (S)    | 1 (S)    | 1 (S)    | 1 (S)    |
| T12 | R        | R        | 0.5 (S)  | R        | R        | R        | 1 (S)    | R        | R        | R        | R        | R        |
| T13 | 2 (S)    | 2 (S)    | 2 (S)    | 2.6 (S)  | 1.6 (S)  | 1.6 (S)  | 1.6 (S)  | 1.6 (S)  | R        | R        | R        | R        |
| T14 | R        | R        | R        | 1.4 (S)  | 2.6 (S)  | 2.6 (S)  | 2.6 (S)  | 1.6 (S)  | 1.6 (S)  | 1.6 (S)  | 1.6 (S)  | 1.6 (S)  |
| T15 | R        | 2 (S)    | 1.3 (S)  | 1.9 (S)  | 1.8 (S)  | 2.1 (S)  | 1.8 (S)  | 2.5 (S)  | R        | 2 (S)    | 1 (S)    | 1.3 (S)  |
| T16 | R        | 0.9 (R)  | 1.2 (S)  | 1.3 (S)  | 2.2 (S)  | 3.1 (S)  | 2.3 (S)  | R        | 0.9 (S)  | 0.9 (S)  | 1 (S)    | 1.1 (S)  |
| T17 | R        | R        | R        | R        | R        | R        | R        | R        | R        | 1.2 (S)  | 1.2 (S)  | 1.2 (S)  |
| T18 | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        | R        |
| T19 | 1 (S)    | 1 (S)    | 1 (S)    | R        | R        | 0.7 (S)  | 0.7 (S)  | 1.9 (S)  | 1.9 (S)  | 1.9 (S)  | 1.9 (S)  | 1.9 (S)  |
| T20 | R        | R        | 1 (S)    | 2 (S)    | 1.3 (S)  | 1.7 (S)  | 1.1 (S)  | 2.5 (S)  | 1.5 (S)  | 1.5 (S)  | 1.9 (S)  | 2 (S)    |
| T21 | R        | R        | 0.9 (S)  | 1.8 (S)  | R        | R        | 0.5 (S)  | 0.5 (S)  | R        | R        | 1 (S)    | 1 (S)    |
| T22 | R        | R        | R        | 0.9 (S)  | R        | R        | R        | 1.6 (S)  | R        | R        | R        | 1 (S)    |
| T23 | 1.9 (S)  | 1.9 (S)  | 2 (S)    | 2 (S)    | 1.2 (S)  | 1.2 (S)  | 1.5 (S)  | 1.7 (S)  | 1.2 (S)  | 1.2 (S)  | 1.8 (S)  | 1.8 (S)  |
| T24 | R        | R        | R        | R        | R        | 2.6 (S)  | 3.6 (S)  | 3.6 (S)  | 3.6 (S)  | 3.6 (S)  | 3.6 (S)  | 3.6 (S)  |
| T25 | R        | R        | 1.9 (S)  | 1.9 (S)  | R        | R        | 0.5 (R)  | 0.5 (R)  | 1.8 (S)  | 1.8 (S)  | 2.8 (S)  | 2.8 (S)  |
| T26 | R        | R        | R        | R        | R        | 1.7 (S)  | 1.7 (S)  | R        | R        | 1.5 (S)  | 1.5 (S)  | 1.5 (S)  |
| T27 | R        | R        | 2.8 (S)  | R        | R        | R        | R        | R        | R        | 1.9 (S)  | 1.9 (S)  | 1.9 (S)  |
| T28 | R        | R        | R        | 3.8 (S)  | R        | R        | 0.1 (R)  | 0.1 (R)  | R        | R        | 3.9 (S)  | 3.9 (S)  |
| T29 | R        | R        | R        | 2 (S)    | R        | R        | 1.6 (S)  | 1.6 (S)  | R        | R        | 0.5 (S)  | 0.5 (S)  |
| T30 | R        | R        | R        | 3.2 (S)  | R        | R        | 1 (S)    | 1 (S)    | R        | R        | R        | 2.6 (S)  |
Results in Figures 4 and 5 revealed that the lag phase growth started in the 1st day of bacterial isolates (T1, T2, T3, T4 and T5) and in the 2nd day for the isolates (T6, T7, T8, T10 and T18). Four recognizable phases are seen when the increase in cell number was determined in relation to time by lag phase, log phase, stationary phase and decline phase (Priyadharshini and Kumar, 2016).

The lag phase continuously in the growth for the 3rd day and the highest OD (0.61 nm) was recorded with isolates T10 and T18. Regarding to the log phase, the three isolates coded T1, T3 and T4 registered the highest values (0.41, 0.40 and 0.46 nm) respectively. The stationary phase was observed in the 5th day and the high value was recorded for T3 isolate (0.42 nm) and T6 isolate (0.41 nm). From the 6th day all the tested bacterial isolates started in the decline phase. The obtained data indicated that the selected bacterial isolates (T1, T3 and T4) were continuously to the growth from the 1st day until the 5th day and the optimum growth period was registered in the 4th day. These
results considering that the selected bacterial isolates (T1, T3 and T4) are promising bacteria for up taking the heavy metal ions from EIWW.

Effect of EIWW toxicity on the abilities of the selected bacterial isolates to produce IAA was studied. Results in Table 3 (illustrated by Fig. 6) showed that the selected bacterial isolates were varied in their production of IAA affected by EIWW.

![Figure 4](image1.png)

**Figure 4.** Optical density ($\lambda = 620$ nm) of ten selected bacterial isolates (T1, T2, T3, T4, T5, T6, T7, T8, T10 and T18) as affected by growth periods

![Figure 5](image2.png)

**Figure 5.** Optical density ($\lambda = 620$ nm) of three selected bacterial isolates (T1, T3, and T4) as affected by growth periods
These results are harmony with that reported by Özdal et al. (2016) who reported that the production of IAA dependence on the Bacillus spp. isolates and fermentation time. Isolate (T2) gave the highest degree of color change that evidence on its high ability for the consumption of L-tryptophan and IAA production, while, the T1, T3, T4, T5 and T6 isolates gave the moderate degree of color change. The lowest degree for color change was noticed by T7, T8, T10 and T18 isolates. From these results three bacterial isolates coded T1, T3 and T4 were selected and subjected to biological and molecular identification.

**Table 3. Degree of color changes of bacterial isolates (T1, T2, T3, T4, T5, T6, T7, T8, T10 and T18) to producing IAA on NBM containing SIWW, and L-tryptophan (0.1%, v/w)**

| Bacterial isolates code | IAA visual reaction degrees |
|-------------------------|----------------------------|
| T1                      | Moderate                   |
| T2                      | High                       |
| T3                      | Moderate                   |
| T4                      | Moderate                   |
| T5                      | Moderate                   |
| T6                      | Moderate                   |
| T7                      | Low                        |
| T8                      | Low                        |
| T10                     | Low                        |
| T18                     | Low                        |

**Determination of antibiotic activities of the ten bacterial isolates**

Data presented in Table 4 and Figure 7 revealed that the all tested bacterial isolates gave antibiotic reaction against *E. coli* when growing on NAM. Similar results were obtained by Yilmaz et al. (2006), who mentioned that *B. cereus* has inhibitory affect both against Gram-positive and Gram-negative bacteria. Negative reaction was recorded with T2 and T6 isolates against *F. solani* and *S. relfsii*. The rest of bacterial isolates, T1, T3, T4, T5, T7, T8, T10 and T18 have positive reaction against the three tested pathogens (*F. solani*, *S. relfsii* and *E. coli*).

These results are in harmony with that of Kim et al. (2015), who showed that the Bacillus spp. had broad-spectrum antifungal activity against *F. solani* and *F. oxysporum*. Results of Ghai et al. (2007) also supported this study, as they recorded that the more strains of Bacillus spp. had antagonistic activities against some fungal
pathogens, e.g. *Clerotium rolfsii*, *Fusarium oxysporum* and *Rhizoctonia solani*. Based on these results it has been observed that, three isolates coded T1, T3 and T4 were selected and subjected to biological and molecular identification.

**Table 4. Antibiosis activities of bacterial isolates (T1, T2, T3, T4, T5, T6, T7, T8, T10 and T18) against *F. solani*, *S. rolfsii* and *E. coli***

| Bacterial isolates code | *F. solani* | *S. rolfsii* | *E. coli* |
|-------------------------|-------------|--------------|-----------|
| T1                      | +           | +            | +         |
| T2                      | -           | -            | +         |
| T3                      | +           | +            | +         |
| T4                      | +           | +            | +         |
| T5                      | +           | +            | +         |
| T6                      | -           | -            | +         |
| T7                      | +           | +            | +         |
| T8                      | +           | +            | +         |
| T10                     | +           | +            | +         |
| T18                     | +           | +            | +         |

+: Antibiosis, -: Non antibiosis

**Figure 7. Antibiosis activities of abacterial isolate against *F. solani* (A), *S. rolfsii* (C) and *E. coli* (E) compared to the growth alone to every one (B, D and F), respectively**

**Biological identification of the selected bacterial isolates**

The bacterial isolates which were selected based on their growth curve; IAA reaction (color change) and antibiosis effect were identified according to (Juní 1986) mentioned in Bergey’s Manual of Systematic Bacteriology (1986, 2012) and Barrow and Felltham (1993).

Morphological, biochemical characterization and utilization of carbohydrates as carbon sources via the bacterial isolates which coded with T1, T3 and T4 are presented in **Table 5**. On NAM, all 48 h-old cultures of tested isolates were positive to Gram stain, motile, rod shaped with white colony for T3, T4 isolates and yellowish colony for T1
isolate. Furthermore, all bacterial isolates were positive effect on both catalase and Indole tests. The coded isolates T1 and T3 were negative with oxidase, Methyl-red and citrate tests. All isolates utilized sucrose and lactose as carbon sources, while, T1 failed to utilize glucose, maltose and xylose. From the obtained data and classifying bacterial isolates in accordance to Claus and Berkeley (1986) that identified the coded (T1, T3 and T4) as Bacillus spp., Bacillus cereus and Bacillus altitudinis, respectively.

Table 5. Morphological and biochemical characterization, utilization of carbohydrate test of Bacterial isolates (Claus and Berkeley, 1986; Barrow and Felltham, 1993)

| Characters                     | T1       | T3       | T4       |
|--------------------------------|----------|----------|----------|
| Colony color on agar           | Yellowish| White    | White    |
| Gram stain                     | Positive | Positive | Positive |
| Cell shape                     | Rod      | Rod      | Rod      |
| Motility                       | Motile   | Motile   | Motile   |
| **Morphological characteristics** |          |          |          |
| Oxidase                        | +        | +        | +        |
| Catalase                       | +        | +        | +        |
| Indole                         | +        | +        | +        |
| Methyl-Red                     | -        | +        | +        |
| Citrate                        | -        | +        | +        |
| **Biochemical test results**   |          |          |          |
| Glucose                        | -        | +        | +        |
| Sucrose                        | +        | +        | +        |
| Maltose                        | -        | +        | +        |
| Xylose                         | -        | +        | +        |
| Lactose                        | +        | +        | +        |
| **Utilization of carbohydrate**|          |          |          |
| **Nomenclatures**              | Bacillus spp. | Bacillus cereus | Bacillus altitudinis |

+: Positive, -: Negative

**Molecular identification of the selected bacterial isolates**

Biological identification of the three bacterial isolates alone was not completely enough, and these tests consume a lot of time and chemicals. Due to the advanced technology such as 16SrRNA gene, primers had been developed by investigators to target specifically the 16S rRNA sequence of the bacteria. In this search, the three bacterial isolates obtained from EIWW samples were also identified using primers targeting their 16S rRNA sequence (Jeffrey, 2008). The nucleotide sequences of 16S rRNA gene were partially determined using the DNA template of the three bacterial isolates (T1, T3, and T4). Results showed that partial sequences of 985, 997 and 983 nts were obtained for the three isolates, respectively. These sequences were compared with four universal bacterial isolates as mentioned in Table 5. These bacteria were classified as Bacillus sp. SMMAA-1, Bacillus cereus SMMAA-3 and Bacillus altitudinis SMMAA-4 and documented in GenBank under the accession numbers of LC472522, LC472523 and LC472524, respectively. Results in Table 6 showed that the percent
identities between the three bacterial strains and those similar strains recorded in GenBank ranged from 84.85% to 100.00%. Phylogenetic trees of the three bacterial strains compared to that similar strain in GenBank confirmed the biological identification of these strains as illustrated in Figure 8.

**Table 6.** Sequences producing significant alignments of the three bacterial strains compared to those similar strains in GenBank with E-value (0.0)

| Description | Query cover (%) | Identities (%) | Accession |
|-------------|-----------------|----------------|-----------|
| **T1 isolate (LC472522)** | | | |
| *Bacillus altitudinis* strain P-10 chromosome, complete genome | 91 | 84.97 | CP024204.1 |
| *Bacillus aerophilus* strain 232 chromosome, complete genome | 91 | 84.85 | CP026008.1 |
| *Bacillus altitudinis* strain SGAir0031 chromosome, complete genome | 91 | 84.85 | CP022319.2 |
| *Bacillus cellulasensis* strain GLB197, complete genome | 91 | 84.85 | CP018574.1 |
| **T3 isolate (LC472523)** | | | |
| *Bacillus cereus* strain ATCC 14579 16S ribosomal RNA (rrnA), partial sequence | 98 | 100.00 | NR_074540.1 |
| *Bacillus thuringiensis* 16S rRNA gene and 16S-23S IGS, strain SBS-BT6 | 98 | 99.73 | AM779002.1 |
| *Bacillus mycoides* 16S rRNA gene, strain MWS5303-1-4 | 98 | 99.46 | Z84591.1 |
| *Bacillus thuringiensis* 16S rRNA gene and 16S-23S IGS, strain SBS-BT3 | 98 | 99.46 | AM778999.1 |
| **T4 isolate (LC472524)** | | | |
| *Bacillus altitudinis* strain P-10 chromosome, complete genome | 99 | 99.08 | CP024204.1 |
| *Bacillus cellulasensis* strain GLB197, complete genome | 99 | 98.98 | CP018574.1 |
| *Bacillus aerophilus* strain 232 chromosome, complete genome | 99 | 99.08 | CP026008.1 |
| *Bacillus altitudinis* strain SGAir0031 chromosome, complete genome | 99 | 99.08 | CP022319.2 |

**Figure 8.** Phylogenetic trees of the three bacterial strains compared to those similar strains in GenBank
Role of selected bacterial isolates (Bacillus spp., B. cereus and B. altitudinis) on the biosorption of six heavy metals (Zn$^{2+}$, Fe$^{2+}$, Co$^{2+}$, Cd$^{2+}$, Cu$^{2+}$ and Pb$^{2+}$) from industrial wastewater (IW)

Biosorption efficiency of heavy metals from SIWW by Bacillus spp., B. cereus and B. altitudinis is depicted in Table 7 and Figure 9. Data showed that the initial values of six heavy metals (ppm) were founded by diversity in IWW and wide ranged from 1.4 ppm to 6 ppm. The maximum conc. was found with Zn$^{2+}$ (6 ppm) while, the minimum conc. was found with Co$^{2+}$ (1.4 ppm). The values of final metal conc. of heavy metals in SIWW were reduced as result of biosorption occurred by bacteria and ranged from 0.109 ppm to 0.409 ppm compared to the initial metal conc. (2.450 ppm). The ability of bacterial isolates tested (Bacillus spp., B. cereus and B. altitudinis) for biosorption of the six heavy metals were differed among them.

**Table 7.** Initial and residual values of heavy metals (ppm) in IWW as affected by SIWW inoculated with the selected bacteria (Bacillus spp., B. cereus and B. altitudinis)

| Heavy metals | Initial metal conc.(ppm) | Residual values of heavy metals (ppm) | Mean |
|--------------|--------------------------|---------------------------------------|------|
|              | SIWW + Bacillus spp.     | SIWW + B. cereus                      | SIWW + B. altitudinis |      |
| Zn           | 6.000                    | 0.551                                 | 0.016 | 0.490 | 0.352 |
| Fe           | 2.100                    | 1.782                                 | 0.552 | 1.782 | 1.372 |
| Co           | 1.400                    | 0.002                                 | 0.004 | 0.008 | 0.004 |
| Cd           | 1.800                    | 0.017                                 | 0.016 | 0.019 | 0.017 |
| Cu           | 1.500                    | 0.017                                 | 0.004 | 0.014 | 0.011 |
| Pb           | 1.900                    | 0.087                                 | 0.063 | 0.097 | 0.082 |
| Mean         | 2.450                    | 0.409                                 | 0.109 | 0.401 |      |

IW = Industrial Wastewater, SIWW = Sterile Industrial Wastewater

**Figure 9.** Average of the mean values (A) of the heavy metals removal % of IWW as affected by SIWW inoculated with the selected bacteria (Bacillus spp., B. cereus and B. altitudinis). Values having the same alphabetical letter within each column are not significantly different at the 0.05 level according to (Duncan multiple range test).

Data exhibited that B. cereus was more effected than B. altitudinis and Bacillus spp. for biosorption final metal conc. (0.109, 0.401 and 0.409 ppm) respectively. Also, the data indicated that (Co$^{2+}$) ion was more biosorption by the three bacterial isolates and gave the
little values compared to (Fe\(^{2+}\)) which gave the highest values in IWW. The ions (Co\(^{2+}\) and Cu\(^{2+}\)) recorded the highest biosorption by the three bacterial isolates followed by the (Zn\(^{2+}\), Pb\(^{2+}\) and Cd\(^{2+}\)) but the lowest biosorption recorded with Fe\(^{2+}\) metal.

From the obtained result data revealed that all bacterial isolates play an important role in detoxification of heavy metals founded in the IWW of Abu-kerqas sugar factory-El-Minia, Egypt. The all studied bacteria were more effective for the biosorption of the heavy metal contents in SIWW. Co\(^{2+}\) ion was more absorbed by Bacillus spp. (0.002 ppm) followed by B. cereus (0.004 ppm) and B. altitudinis (0.008 ppm).

The results presented in Table 8 and Figure 9 show the biosorption ability of B. cereus is significantly effective for removal efficiency percentage of six heavy metals (Zn\(^{2+}\), Fe\(^{2+}\), Co\(^{2+}\), Cd\(^{2+}\), Cu\(^{2+}\) and Pb\(^{2+}\)) (94.77%) from IWW. However, the removal efficiency % of it is heavy metals using Bacillus spp. and B. altitudinis recorded 83.19% and 83.21% with insignificant difference between them. This indicted that B. cereus has a higher capability of biosorption potentiality of heavy metals compared to the others. Similar results are recorded by Huang et al. (2013).

Regarding to the effects of the different heavy metals tested for the removal efficiency %, the results in Table 8 and Figure 10 show significant effects. Available data revealed that higher removal efficiencies of 99.66% for Co\(^{2+}\), 99.22% for Cu\(^{2+}\) and 99.03% for Cd\(^{2+}\) and lower removal efficiencies 34.66% for Fe\(^{2+}\) were recorded as Mullen et al. (1989).

Table 8. Removal efficiency % of heavy metals from IWWas affected by SIWW inoculated with the selected bacteria (Bacillus spp., B. cereus and B. altitudinis)

| Treatments                  | Removal efficiency % of heavy metals | Mean of A |
|-----------------------------|-------------------------------------|-----------|
|                            | Zn\(^{2+}\) | Fe\(^{2+}\) | Co\(^{2+}\) | Cd\(^{2+}\) | Cu\(^{2+}\) | Pb\(^{2+}\) |
| Bacillus spp. + SIWW        | 90.83 d    | 15.14 f    | 99.86 a    | 99.06 a    | 98.87 a    | 95.42 bc    | 83.19 b    |
| B. cereus + SIWW            | 99.73 a    | 73.71 e    | 99.71 a    | 99.11 a    | 99.73 a    | 96.68 b    | 94.77 a    |
| B. altitudinis + SIWW       | 91.83 c    | 15.14 f    | 99.42 a    | 98.94 a    | 99.06 a    | 94.89 bc    | 83.21 b    |
| Mean of B                   | 94.13 b    | 34.66 c    | 99.66 a    | 99.03 a    | 99.22 a    | 95.66 b    |             |

Mean values of three replicates followed by the same letters in each column are not significantly different (P > 0.05) (Duncan multiple range test). LSD at (0.05%) for A = 0.0715, B = 1.012, AB = 1.753

Figure 10. Average of the mean values (B) of the different six heavy metals removal % from IWW. Values having the same alphabetical letter within each column are not significantly different at the 0.05 level according to (Duncan multiple range test)
Also, similar data in Table 8 and Figure 11 revealed that a significantly higher removal efficiencies for Co\textsuperscript{2+}, Cd\textsuperscript{2+} and Cu\textsuperscript{2+} when combined with bacterial isolates (Bacillus spp., B. cereus and B. altitudinis) without significant differences among them. The highest residual values and the lowest removal biosorption by bacterial isolates were recorded with Fe\textsuperscript{2+} similar results are recorded by Tuzen et al. (2007).

![Figure 11. The interaction (AB) effect between the treatments (SIWW + Bacillus spp., SIWW + B. cereus, SIWW + B. altitudinis) and six heavy metals removal % of IWW. Values having the same alphabetical letter within each column are not significantly different at the 0.05 level according to (Duncan multiple range test). IWW = Industrial Wastewater, SIWW = Sterile Industrial Wastewater](image)

**Conclusion**

The isolation and identification of tolerant bacteria against six heavy metals (Zn\textsuperscript{2+}, Fe\textsuperscript{2+}, Co\textsuperscript{2+}, Cd\textsuperscript{2+}, Cu\textsuperscript{2+} and Pb\textsuperscript{2+}) give the new technology of bioremediation method. Among 30 isolates of Bacillus spp., 3 isolates exhibited more resistance effect against 6 heavy metals. These isolates were identified by morphological, biochemical characterization, molecular identification by using 16S rRNA gene and documented in GenBank as Bacillus sp. SMMAA-1 (LC472522), Bacillus cereus SMMAA-3 (LC472523), and Bacillus altitudinis SMMAA-4 (LC472524). These isolates are promising for further studies and can be used in bioremediation. The findings showed that bacteria present resistance of six heavy metals. B. cereus is more effective for removal efficiency percentage of six heavy metals (Zn\textsuperscript{2+}, Fe\textsuperscript{2+}, Co\textsuperscript{2+}, Cd\textsuperscript{2+}, Cu\textsuperscript{2+} and Pb\textsuperscript{2+}) (94.77\%) from IWW than Bacillus spp. (83.19\%) and B. altitudinis (83.21\%). The highest removal efficiencies by Bacterial isolates were found with Co\textsuperscript{2+}, Cd\textsuperscript{2+} and Cu\textsuperscript{2+} and the lowest removal biosorption were recorded with Fe\textsuperscript{2+}. Data recommended to using of Bacillus cereus in bioremediation of heavy metals from industrial wastewater effluent especially with Co\textsuperscript{2+}, Cd\textsuperscript{2+} and Cu\textsuperscript{2+} metals.

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