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# Biosorption of lead ions from aqueous solutions by using dead biomass of *Bacillus subtilis*

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**Abstract**
Exaggeration of heavy metal toxicants in aquatic systems arises as an alarming situation and emanating as a serious threat to all life forms. This study examines the potential of *Bacillus subtilis* biomass for Lead metal ions biosorption from contaminated aqueous media. Biosorption efficiency was determined in batch experiments and the metal concentration was analyzed by using (Atomic Absorption Spectrometry). The favorable optimum conditions for maximum removal (83.46 %) of Lead ions from 100 ml solution were obtained at pH 6, biomass dosage of 3 mg/ml, temperature $30^0$C and 20 mg/L of lead concentration at 80 minutes of contact time. The models Langmuir and Freundlich isotherm were determined for Lead metal ions adsorption by *Bacillus subtilis*. The adsorption experimental data was best fitted for Langmuir model ($R^2$ = 0.99892). The result indicates that *Bacillus subtilis* emerging as an efficient sorbent for the remediation of Lead metal ions from aquatic environments.

**Keywords:** heavy metal, *Bacillus subtilis*, biosorption, biosorbent, aquatic environments

## Introduction

Heavy metals are highly toxic and persistent environmental pollutants that lead to the contamination of several aquatic systems. Due to their bio accumulative nature, mobilization of these toxicants has been increased in the atmosphere and may cause disturbance in their biogeochemical cycles and consequently pollute the food chains. "Heavy Metal" are those metallic elements that has comparatively high density and can be toxic even at very less concentrations. Heavy metals cannot be degraded or destroyed (Lenntech, 2004) [1].
Heavy metal is a common term which implements to the group of metals having atomic density more than 4 g/cm$^3$, or 5 times higher than water (Hutton and Symon, 1986) [2]. These inorganic pollutants introduced in the environment mainly by natural sources which include weathering of rocks and volcanic eruptions, and other human-induced sources, which involve mostly agricultural and industrial activities. Most hazardous environmentally relevant heavy metals and metalloids include Cr, Cu, Zn, Ni, Cd, Hg and Pb which are considered as detrimental and silent killers. Prolonged exposure of these metals shows deleterious effects on human beings. Heavy metal contaminated aquatic ecosystems are emerging as a major environmental problem and a matter of public health concern that needs to be resolved in order to meet the forthcoming demands.

Several methods have been developed for the rectification of metal ions from aqueous matrix such as chemical precipitation, filtration, chemical oxidation-reduction, ion-exchange, reverse osmosis (Xia and Liyuan, 2002) [3]. However, several problems may arise by using these conventional methods such as uncertain metal ions expulsion, generation of toxic sludge, difficulty in its removal and demolition (Addour *et al*., 1999) [4].

Consequently, there is a need to explore alternative biological techniques for the remediation and recovery of toxic metal ions from polluted sites and should be able to meet the permissible exposure limits. Biological treatment, considered as a dependable alternative method for the removal of the toxic ions because of its environment friendly an economical nature with a number of other benefits like less dependency on chemicals, immense selectivity and efficiency to remove the toxic metal ions (Matagi *et al*., 1998; Chevalier *et al*., 2000; Mehta and Gaur, 2005) [5, 6, 7].

Bioremediation method basically depends on the use of microorganisms which can efficiently expel toxicants and poisonous heavy metals from contaminated sites. Generally, biological treatment can be done by two different methods-bioaccumulation (an active process) and biosorption (involves passive process) to remediate pollutants from contaminated matrix (Churchill *et al*., 1995; Davis *et al*., 2003) [8, 9]. Biosorption is the process which includes inactive dead biomass of microorganisms for the removal of toxic ions from an aqueous medium by binding biomass passively. Cell wall of non-living microbial biomass is usually used for the process of biosorption of heavy metals (Churchill *et al*., 1995) [8]. Sorption treatment basically involves two phases- Solid phase (contains biomass of selective organisms) and Liquid phase (consists of pollutants like heavy metal ions) (Farooq *et al*., 2010) [10]. Biomass (sorbent) and metal ions (sorbate) mutually showed pronounced affinity for each other which facilitate the attachment or binding of sorbate to sorbent in a passively directed process (Das *et al*., 2008) [11]

Several types of organisms like fungi, bacteria, algae and plants are extensively used for biosorption processes (Aksu and Donmez, 2006) [12]. The potential of these organisms for removal of heavy metal ions, or to promote their transformation to less toxic forms is receiving the attention worldwide, from last few decades. Among all these organisms, bacterial biomass is one of the most potential choice for the removal of heavy metals from aqueous solutions as these organisms not only helps in the detoxification of hazardous components from the environment but are also capable of tolerating unfavorable environmental circumstances as these mechanisms evolved over the years.

In the biosorption process, bacterial biosorbents are mainly used for the removal of non-biodegradable toxicants like metal ions and dyes from contaminated sites. However, the process of their characterization, isolation or seclusion, maintenance and sub-culturing on a huge scale is not easy yet they have been considered as one of the most efficient ways for the remediation of toxicants as biosorption can effectively reinstate polluted environments in a cost-effective and eco-friendly approach. Bacteria are proven as a suitable and efficient candidate for detoxifying metal ions by evolving several resistant mechanisms.

Bacteria are the most plentiful and versatile microorganisms. Bacteria are used as bio sorbents due to their very tiny size, ubiquitous nature, and ability to grow under favorable environment. They evolved various tolerant mechanisms against a broad range of varying environmental conditions. Heavy metal uptake has been tested by various bacterial isolates, likely *Bacillus, Micrococcus, Escherichia,* etc. (Duda-Chodak *et al*., 2013) [13]. Moreover, cell walls of bacteria are composed of slime layers of polysaccharide which easily offer carboxyl, amino, phosphate, and sulphate groups for binding of metals (Deng and Wang, 2012; Sag and Kutsal, 2001) [14, 15]. However, extensive diversity in uptake of heavy metal is prevailing among various bacterial species. Bacterial cell wall binds heavy metals on its surface basically involves a two-stage process. In the initial stage, the interconnection between the ions of selected heavy metals and reactive groups takes place on cell surface while the second stage involves deposition of metal ions in elevated concentrations (Vasudevan *et al*., 2001) [16]. The prime attachment site of metal deposition to cell wall is usually glutamic acid carboxyl groups of peptidoglycan.

## Materials and Methods

### The Microorganism

Gram-positive *Bacillus subtilis* (NCIM 2063), was procured from the National Collection of Industrial Microorganisms (NCIM), Pune in a freeze-dried culture, and was stored at -20°C.

### Bacterial Identification and Characterization

The resistant bacterial isolate was identified according to the keys of Bergey's Manual of Systematic Bacteriology in accordance to 2010 edition of Bergey's manual (Holt *et al*., 1986) [17]. These tests including Gram staining, spore staining, motility, and biochemical testes like indole, Voges Proskauer, nitrate reduction, citrate utilization, urease, etc.

### Bacterial Biosorbent (Biomass) Preparation

Nutrient medium was prepared and sterilized. In the nutrient medium bacterial strain was maintained and adequate proportions was used in the experiment. For cultures inoculation standard sterile techniques were applied. A loop of bacterial culture was then streaked on the agar plate to obtained more colonies. Later, they were transferred to nutrient broth and then grown on particular media (LB Medium- for subculturing of Bacillus subtilis). Under a laminar airflow chamber, the media was allowed to cool and then 200µl microbial solution was inoculated in the medium. After inoculation, flasks were kept for incubation in an incubator orbital shaker at 150 rpm at 30°C for two days to obtained the desired biomass. After incubation biomass was harvested by centrifugation at 9000 rpm for ten minutes. The supernatant was then discarded and the cell pellet was re-suspended in distilled water for washing and to make sure that no media left on the cell surface. Cell pellet was centrifuged again and then was first heat-killed, in a hot air oven at 80 °C for 12 hrs to make dead cells. The obtained dead biomass was used for further biosorption studies.

### Preparation of Lead solution

As a source of lead salt of $Pb(NO_3)_2$ (Merck 203580) was used for the preparation of standards.1.5980 gm of Pb $(N0_3)_2$ was dissolved in 1000 ml of distilled water to get 1000 ppm concentrated stock solution. The stock is diluted further to obtained desired metal concentrations (20, 40 60, 80, 100 mg/l).

### Biosorption Studies

In the present study, the biosorptive potential of *Bacillus subtilis* in heavy metal (Pb) uptake was determined under *in-vitro* conditions at various parameters such as pH, biomass dosage, temperature, contact time, and metal concentration. The effect of pH on the sorption of Pb was determined under different pH ranges (1, 3, 4, 5, 6, 7, and 8) and then optimum biomass was dispersed in metal solution of 20 mg/l with the working volume of 100ml. The experiments were conducted at different biomass dosages (1, 2, 3, 4 mg/ml) to analyze the effect of *Bacillus subtilis* biomass on Lead adsorption, under optimum conditions of other parameters. Then, the effect of temperature was examined at different temperature ranges ($25^0$, $30^0$, $35^0$, $40^0$, 45°C), under optimum conditions of other parameters obtained from previous experiments. The effect of contact time was determined for different periods of time (20 to 120 min), by fixing all other parameters constant such as biomass concentration, pH, temperature, and contact time. The effect of initial concentration of Lead was studied by varying the initial concentration in the range (20, 40 60, 80, 100 mg/l), by fixing all the other parameters such as biomass concentration, pH, temperature, and time. All successive biosorption experiments were carried out under optimum conditions obtained from previous work. The experiments were conducted in triplicate in Erlenmeyer flasks containing 100 ml of test solutions in an orbital shaker at 200 rpm. After incubation, the cell pellet of bacterial strain in the solutions were separated by centrifugation process at 9000 rpm for ten minutes. Supernatant was collected and analyzed the residual concentrations of the $Pb^{2+}$ metal ions, using Atomic Absorption Spectrometer, AAnalyst 100, USA.

### Adsorption Isotherms

The Langmuir and Freundlich isotherms were applied to examine the obtained results for the removal of $Pb^{2+}$ by sorption process in order to give a detailed description of the equilibrium between the adsorbed (metal ions adsorbed on the surface of biomass) and remaining quantity of metal in the solution. The Langmuir and Freundlich models equations are given below

Langmuir equation:

$$q_e = q_{max} \frac{K_L C_e}{1+K_L C_e}$$

Where qe is the concentration of metal ion on the sorbent (mg/g), qmax is the maximum amount of metal adsorbed (mg/g), KL is Langmuir constant, and Ce is the equilibrium metal concentration in the solution (mg/l).
Freundlich equation:

$$q_e = K_F (Ce)^{1/n}$$

Where qe is the amount of the sorbate per unit weight of sorbent (mg/g), KF is a Freundlich constant, Ce is the equilibrium metal ion concentration in the solution (mg/l) and 1/n is adsorption intensity.
Prior to data analysis for the Langmuir and Freundlich isotherms, the amount of Lead bound to the adsorbents ($q_e$) and the percentage removal of lead (R) were calculated as follows:

$$q_e = (C_o - C_e) \text{ x } V/M$$

$$R (\%) = [(C_o - C_e) / C_o] \text{ x } 100$$

Where $C_o$ and $C_e$ are the initial and final Lead concentrations (mg/l), respectively, V is the volume of solution (ml), and M is the *Bacillus subtilis* biomass (mg).

## Results and Discussion

In the present study, *Bacillus subtilis* (NCIM 2063) appeared as gram-positive rod-shaped bacteria after gram staining. Results of biochemical analysis of the strain are listed in Table 1

**Table 1:** Biochemical Characterization of *Bacillus subtilis*

| S. No | Biochemical Test | *Bacillus subtilis* |
|---|---|---|
| 1 | Indole | - |
| 2 | V.P Test | + |
| 3 | Methyl Red | - |
| 4 | Catalase | + |
| 5 | Urease | - |
| 6 | Oxidase | - |
| 7 | $H_2S$ | - |
| 8 | Lactase | - |
| 9 | Citrate | + |
| 10 | Glucose | - |

(+) and (-) sign indicate positive and negative reactions, respectively

*Bacillus subtilis* dead biomass was used for the biosorption of $Pb^{2+}$ ions. The parameters influencing the sorption of $Pb^{2+}$ were studied by using *Bacillus subtilis* isolate. The effects of these parameters are given as follows:

### Effect of pH

pH is one of the most significant factors that can influence all most all biological and chemical reactions. Hence, while doing any optimization, this is one of the foremost factors to be considered. Biosorption studies for $Pb^{2+}$ were carried out at pH range of 1 to 8 as shown in (Fig 1). The maximum sorption (83.46%) took place at pH 6. Results depicted that the biosorption of $Pb^{2+}$ was lowest (42.74%) at pH 1.
An increase in biosorption from 78.47% to 83.46% was observed at pH 6 as compared to pH 3. After pH 6 a slight reduction in biosorption was recorded. Metal removal efficiency at pH 7 and 8 was around 81.42 and 81.35%. The observed trend for pH is shown in (Fig 1) from which it can be concluded that highest percent of biosorption by *Bacillus subtilis* occurred at pH 6. Reduction in sorption percentage at higher pH can be associated with decreased solubility of metal complexes which may convolute the biosorption process to some extent (Vijayaraghavan and Yun, 2008) [18]. Similar results were also reported, during the metal ions, sorption by bacterial biomass at pH 3 to 6 and this range is recorded favorable for sorption process, due to the availability of negatively charged carboxyl groups which are responsible for the metal cations binding through ion exchange mechanism (Yang and Volesky 1999, Esposito *et al*., 2002) [19, 20].
Pardo *et al*., (2003) [21], also reported maximum biosorption of Copper and Lead at pH 6 by *Pseudomonas putida*. Similarly, Seki *et al*., (1998) [22] observed a similar trend for the function of pH on lead ions removal by *Rhodobacter sphaeroides*. This variation in sorption of metal ions by microbial biosorbents at various pH could be due to the sensitivity of bacterial cell wall to pH factor. Wang and Chen, (2006) [23] observed similar results for *Bacillus* and *Pseudomonas* species that showed highest capability to adsorb Copper, Cadmium, and Lead at pH 6-7.

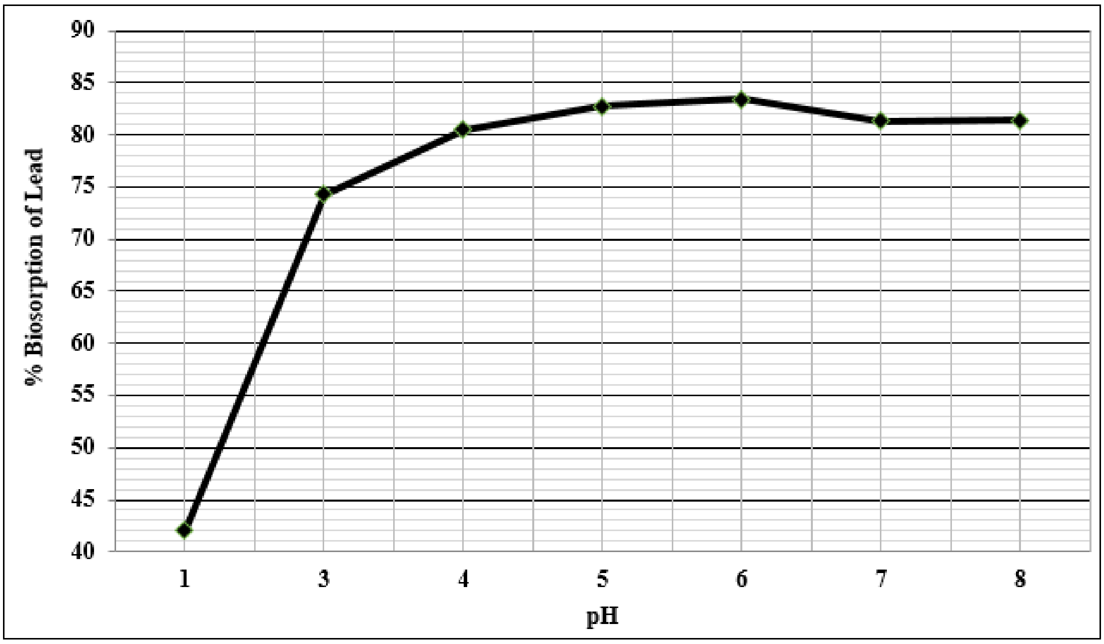


**Fig 1:** Effect of pH on Biosorption of $Pb^{2+}$ onto *Bacillus subtilis* biomass

**Effect of Biomass Concentration**

One of the major cost factors in large-scale application of the biosorption methods is "the amount of biomass" required for the expulsion of toxic heavy metal ions. The influence of biomass dose on the biosorption percentage of Lead is given in (Fig 2). The biomass dosage was taken in a range of 1, 2, 3, 4 mg/ml to achieve the maximum biosorption efficiency of the sorbent for Lead. Results showed that 3 mg/ml biomass dosage was found adequate for maximum biosorption of $Pb^{2+}$ ions under favorable experimental conditions. It is apparent from (Fig 2) that sorption percentage increased from 1 mg/ml to 3mg/ml biomass dosage but further increases in the dose of biomass did not affect the sorption rate significantly and biosorption was found nearly constant. This reduction might be due to the unavailability of binding sites for the metal ions or because of the blockage of binding sites by excessive biomass. Therefore, 3mg/ml biosorbent dose was found optimum for the biosorption by *Bacillus subtilis*.

It can be concluded that biosorbent dosage can influence sorption process to some extent. Usually, with increase in the concentration of biomass, the amount of solute sorbed also increases, due to the expansion of sorbent surface area which results in increased number of binding sites (Esposito *et al*., 2001) [24]. Same finding has been inferred for Pb metal ions adsorption by using *Spirulina maxima* biomass from aqueous solutions at different biosorbents dosage (Gong et al., 2005) [25]. Sometimes when the concentration of biomass increases beyond the optimum range it may decrease the growth of organisms and sorption rate of heavy metal ions. It has been reported that high concentration of biosorbents can cause cell agglomeration and contraction in the intercellular distance (Pons and Fuste, 1993) [26]. Itoh *et al*., (1975) [27] concluded that when the intercellular distance is more at less sorbent concentration, metal uptake was higher and this condition assures optimum electrostatic interaction between cells.

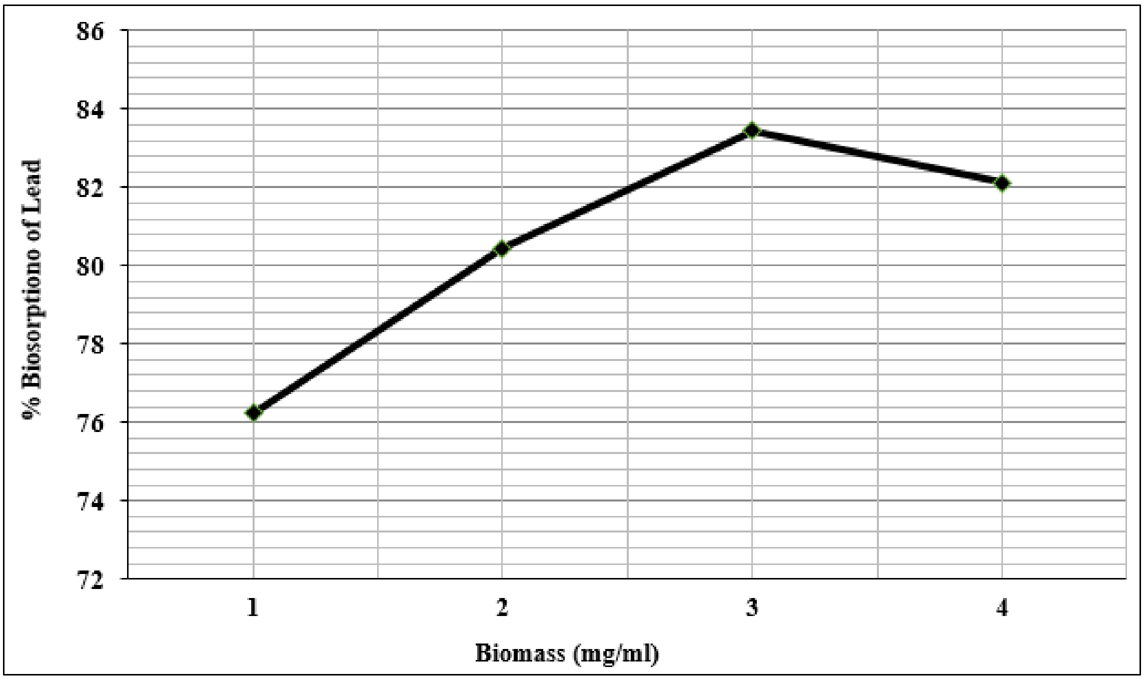


**Fig 2:** Effect of biomass dosages on Biosorption of $Pb^{2+}$ onto *Bacillus subtilis* biomass

**Effect of Temperature**

Temperature is considered as a crucial parameter in the adsorption process. In the present study, inactive biomass of *Bacillus subtilis* at varying ranges of temperature was used and it was found that $30^0$C was the most favorable temperature for maximum sorption in comparison to other ranges. Effect of temperature is given in (Fig 3). Highest biosorption (almost 83.46%) was occurred at $30^0$C. From the experiments, it has been revealed that percentage of sorption was slightly increased with an increase in the temperature up to $30^0$C. It is shown in the figure that sorption decreases slightly with increasing further ranges of temperature. No significant impact of temperature on biosorption performances was observed at 25-$45^0$C range. From the results, it was evident that $30^0$C temperature was optimum for Lead sorption by using *Bacillus subtilis.*

Beyond the optimum range of temperature ($30^0$C), sorption rate was slightly decreased this may be due to the cells shrinkage at higher and lower ranges of temperatures which decreases the surface area for the contact (Vijayaraghavan and Yun, 2008) [18]. (Aksu, 1992) [28] also reported that temperature does not influence greatly the sorption processes at 20-$35^0$C range. Temperature influences sorption rate only to some extent within the range of 20 to $35^0$C (Vegliò and Beolchini, 1997) [29]. Moreover, at relatively higher temperatures physical disruption of the sorbent can be expected. In some sorption processes, due to their exothermic nature with an increase in temperature, the sorption capacity of the biomass gets reduced (Mameri *et al*., 1999; Suhasini *et al*., 1999) [30, 31]. It is always feasible to conduct biosorption at room temperature, as this environment is quite easy to replicate.

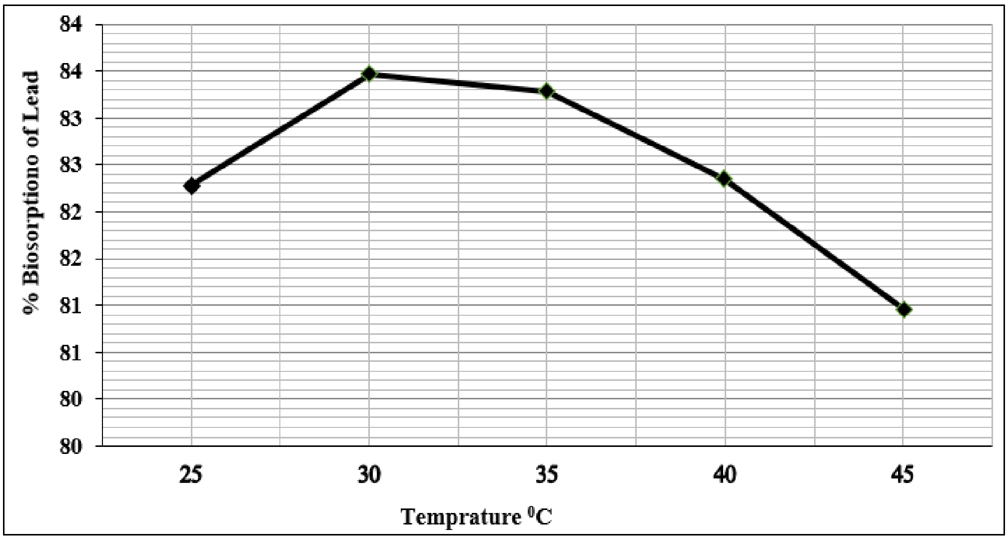


**Fig 3:** Effect of temperature on Biosorption of $Pb^{2+}$ onto *Bacillus subtilis* biomass.

**Effect of Contact Time**

Contact time is also a key factor that to be considered for optimization. The adequate amount of biomass was dispersed in the solution and equilibrium time at which maximum sorption took place was determined.

Lead adsorption experiments were conducted at different time intervals with an optimum adsorbent dose of 3 mg/ml at pH 6 and 30$^0$C. The results are presented in (Fig 4), which indicate that for Lead, maximum sorption occurred at 80 min and further increase in time did not affect the sorption rate and it was found almost constant. It has been concluded that 80 min time was found sufficient for Lead ions adsorption. Once the equilibrium is reached, the biosorption of lead ions did not change significantly. This was considered as the equilibrium stage for biosorption of lead ions by *Bacillus subtilis* biomass.

Studies related to minimum time required for maximum sorption of heavy metal ions reported by many authors (Chen et al., 2005; Tsezos and Volesky, 1982) [32, 33]. Similar findings were also observed by Zoubolis *et al.*, (2004) [34] and Gabr *et al*., (2008) [35] that the equilibrium time required for the biosorption process is important for a high biosorption rate for metal ions.

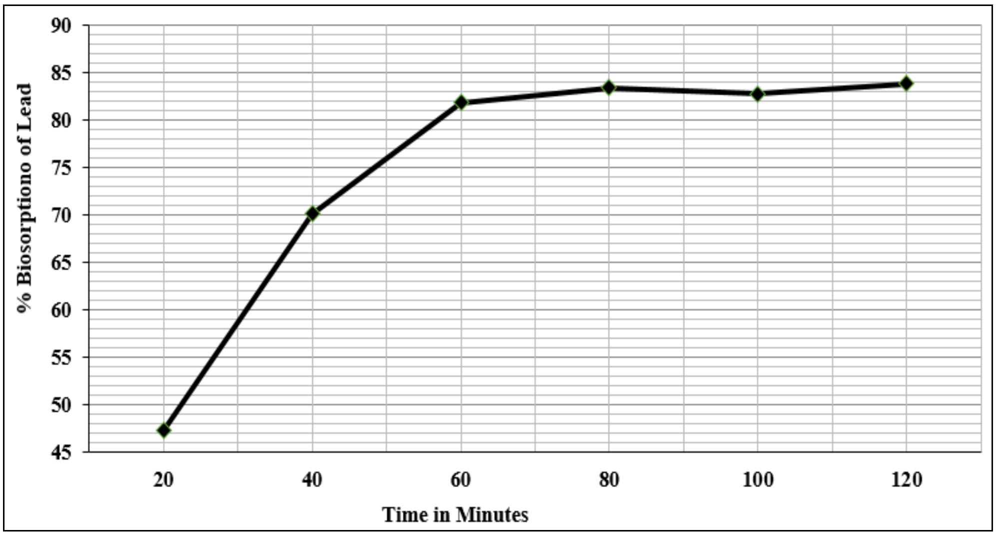


**Fig 4:** Effect of Contact Time on Biosorption of $Pb^{2+}$ onto *Bacillus subtilis* biomass

**Effect of Initial Metal Concentration on Biosorption**

Initial metal concentration in the solution is considered a significant parameter affecting metal adsorption. The effect of initial metal ion concentration on metal sorption by *Bacillus subtilis* biomass was evaluated as shown in (Fig 5). Results showed that the maximum biosorption percentage (around 83.46 %) was observed at low initial metal concentration of 20mg/L and the minimum biosorption was observed (around 70.02 %) at 100 mg/L. It was observed that with increasing metal concentration, percentage of biosorption tend to decrease. This indicates that biosorption rate decreases with an increasing concentration of metal. At low initial metal ion concentration, maximum adsorption of metal was recovered and the decrease in the biosorption rate might be due to the unavailability of free sites for sorption of metals.

When solution contains low metal concentration, available ions could interact with the binding sites and as a result, rate of sorption is likely to increase. At high metal concentrations, minimum adsorption was occurred which may be due to the available saturated adsorption sites. Same findings have been shown by many authors (Kadukova and Vircikova, 2005; Lu *et al.*, 2006; Pandiyan and Mahendradas, 2011) [36, 37, 38]. For *Bacillus* species, maximum removal was seen at 20 mg/L for Copper ions and it sequentially decreased up to 100 mg/L. Similar result was seen for *Klebsiella aerogenes* in which the sorption rate for metal ions gradually decreased with increasing initial metal concentration. Species of *Pseudomonas* isolate showed the maximum growth at 60 mg/L Cadmium metal concentration. Similar results were also reported by Norberg, (1984), it was reported that highest adsorption of Cadmium ion occurred at 60 mg/L by *Zoogloea ramigera* dead cells (Norberg, 1984) [39]. In case of *Micrococcus* species, maximal growth was observed at 40 mg/L Lead in synthetic aqueous media (Kiran *et al*., 2005) [40]. Rani and Haripriya, (2003) [41] explained that at lower metal ion dose, metal adsorption by sorbents is not fully utilized. Various earlier studies revealed that *Bacillus* species have the ability for removal of Zinc, Copper and Lead (Salehizadesh and Shojaosadati, 2003) [42].

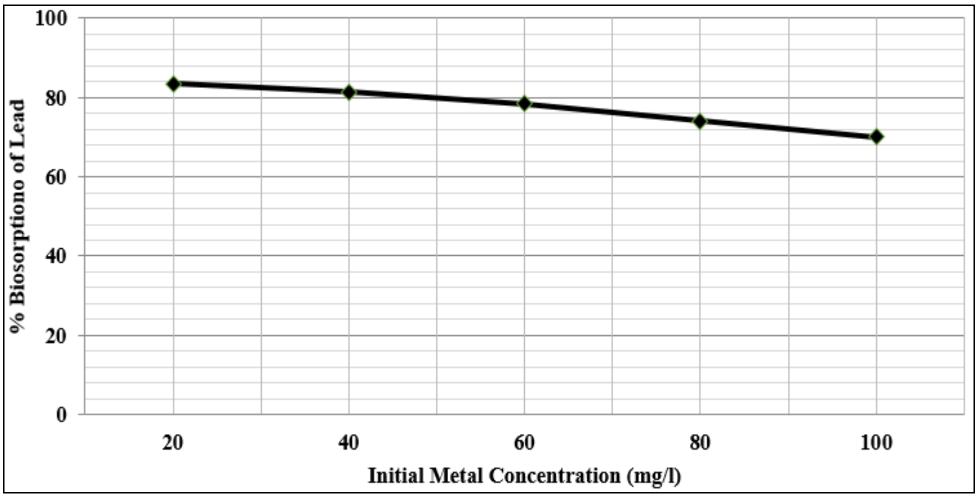


**Fig 5:** Effect of initial metal concentration on Biosorption of $Pb^{2+}$ onto *Bacillus subtilis* biomass

**Adsorption Isotherm of Lead**

Adsorption isotherms, specified by definite constants whose values determine the surface properties and affinity for the sorbent. In the solution, adequate biomass was dissolved in the selected concentration at varying ranges from 20 mg/l to 100 mg/l for Lead metal ions. Adsorption isotherms equilibrium data was obtained at optimum conditions at pH 6, biomass dosage of 3 mg/ml, temperature $30^0$C and time period of 80 min. Langmuir isotherm model linear plots were given in (Fig 6 a) and Freundlich isotherm model plots are shown in (Fig 6 b). These isotherms depicted the relationship between the sorbed amounts of $Pb^{2+}$ ions by *Bacillus subtilis* and the residual concentration of $Pb^{2+}$ions in the solution. While Comparing the $R^2$ of Freundlich model (0.98403) with the Langmuir model (0.99892), it can be concluded that the Langmuir isotherm model was best suited according to equilibrium data. The present study confirmed that the Langmuir model provides a better fit data than the Freundlich model for the sorption of Lead ions by using selected adsorbent. It was also apparent from the study that at low metal concentrations, adsorption sites adsorb the available metals more easily. Moreover, at maximal concentrations, the metal requires to diffuse into the surface of biosorbent by a process called intra particular diffusion and comparatively, diffusion of hydrolyzed ions occurs with slower rate (Sang *et al*., 2009) [43]. The overall observation from both postulated Langmuir and Freundlich models for metals biosorption was studied by other authors (Pardo *et al*., 2003, Sar *et al*., 1999) [21, 44]. Mohammad *et al*., (2012) [45] reported that the *Bacillus thuringiensis* biomass efficiently removed the Cu, Ni, Pb, Cd, and Cr metal ions from aqueous systems. Su *et al*., (2014) [46] used *Bacillus catenulatus* for biosorption of Cd and the biosorption equilibrium data were best fitted for the Langmuir adsorption model.

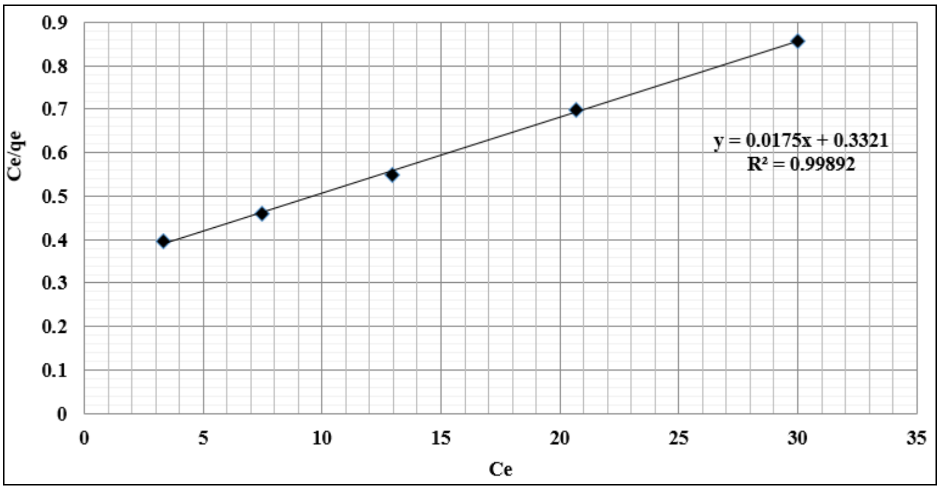


**Fig 6(a):** Langmuir Isotherm for $Pb^{2+}$ adsorption by *Bacillus subtilis* Biomass

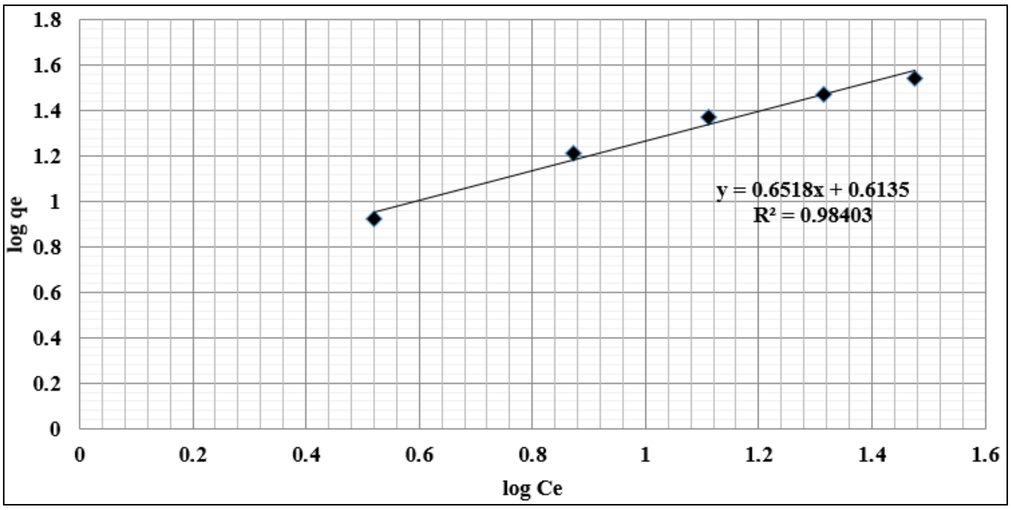


**Fig 6 (b):** Freundlich Isotherm for $Pb^{2+}$ adsorption by *Bacillus subtilis* Biomass

## Conclusion

Present study imparts significant information regarding the suitable biosorbent *Bacillus subtilis* for lead ions expulsion from aqueous solutions. The batch biosorption study showed that the adsorption process depends on various parameters like pH of the solution, biosorbent dosage, temperature, contact time and the metal concentration of $Pb^{2+}$ ions. Optimum conditions for the remediation of Lead by the bacterial biosorbent (*Bacillus subtilis)* were obtained at pH 6, 3 mg/ml of biomass dose, 30 °C temperature, 80 min of contact time and 20 mg/l of Lead metal concentration. The adsorption data best fitted for the Langmuir isotherm model. From the study, it can be concluded that *Bacillus subtilis* can be used as an effective biosorbent for removal of Lead ions from the aquatic environment.

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