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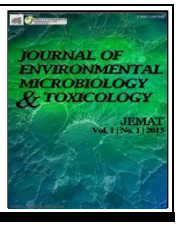


# Isolation and Characterization of a Molybdenum-reducing *Bacillus pumilus* strain lbna

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**Abstract**

The reduction of heavy metals by microorganisms has an important role in biological system and also in the cycling of metals in the environment to remove its toxic effects in soil and wastewater. A molybdenum-reducing bacterium was isolated from soil obtained from Seri Kembangan, Selangor. This isolate is Gram positive bacteria and was identified as bacillus pumilus strain based on 16s rRNA gene sequencing. Molybdenum reduction is optimally supported by glucose at 1.0% (w/v). The optimum phosphate and molybdate concentrations for molybdate reduction in *Bacillus pumilus* strain Lbna was between 2.5 and 5 mM phosphate and 40 mM molybdate. Molybdate reduction is optimum at 37 ºC. The metal ions arsenic, lead, zinc, silver, cadmium, chromium, mercury and copper caused 33.7, 36.0, 51.2, 60.5, 63.9, 91.3, 92.3 and 98.0% inhibition to molybdenum reduction.

## INTRODUCTION

Heavy metals are dangerous pollutants that can be found in the air, water and soil. The contaminations of these heavy metals are serious problems because they cannot be naturally degraded like organic pollutants, and also they accumulate in the environment. The experiment shows that heavy metals can be reduced by many different types of microorganisms; the reduction of this heavy metal by microorganisms has an important role in biological system and also in the cycling of metals in the environment. One of the important heavy metals for biocatalyst activity is molybdenum; this is an essential trace element for all higher organisms because molybdenum enzymes have a number of important roles as molybdenum in nitrate reductase is required by all plants for proper nitrogen assimilation. Nevertheless, this element is harmful at high level because it produces toxic. The first report on microbial reduction of molybdenum to molybdenum blue was mentioned by Levine [1].

There are many studies reported that Molybdenum can be reduced to molybdenum blue by several bacteria such as *Thiobacillus ferroxidans* which has the ability not only to oxidize metal ions but also to reduce them [2]. Another view stated by Campbell *et al.* [3] mentioned that they have discovered a new phenomenon of molybdate reduction to Mo-blue in *Eschericia coli* K12. Also Ghani *et al*, [4] reported that another heterotrophic bacterium, *Enterobacter cloacae* strain 48 (EC 48), could also reduce molybdate (molybdenum 6+) to molybdenum blue (molybdenum 5+) with NADH as an electron donor. The reduction of molybdenum in EC 48 has been demonstrated to be enzymatically-linked by using a modified dialysis tubing method [5].

In 2008 Shukor *et al* [6], they have improve the enzyme assay for the reduction of molybdenum reducing activity using laboratory-prepared phosphomolybdate as the electron acceptor substrate, this substrate is approximately 13 times better than the commercial phosphomolybdate. Shukor *et al* [7] reported that phosphomolybdate as a substrate should be used in the molybdenum reducing enzyme in the heterotrophic microorganisms. Phosphomolybdate has an important intermediary role during molybdate reduction to molybdenum blue [6].

Heavy metal pollutions in Malaysia have grown to a dangerous level. In 2001, a survey was done by the Malaysian Department of Environment and the result showed that 10.4% of

the 420,000 ton of scheduled wastes contained heavy metals including molybdenum. In 2006, a total of 1,064 water quality monitoring stations located within 146 river basins were monitored. Out of these 1,064 monitoring stations, 619 (58%) were found to be cleaned, 359 (34%) slightly polluted and 86 (8%) polluted as characterized by water parameters such as Biochemical Oxygen Demand (BOD), ammoniacal Nitrogen NH3-N and Suspended Solids SS. The majority of molybdenum-reducing bacteria so far are Gram negative bacteria. In this work we report on the isolation of a Gram positive *Bacillus* sp. strain Lbna. This genus offers several advantageous such as extreme environmental tolerance due to its capability to form endospore and a fast doubling time [1] and hence a good bioremediating agent.

## MATERIALS AND METHODOLOGY

### Chemicals

All chemicals used were of analytical grade. Buffers were prepared at the appropriate temperature according to their final use by mixing the appropriate basic and dibasic salts as outlined by Dawson et al. [9].

### Isolation of Molybdate-Reducing Bacterium phenotype identification

Soil samples were taken (5 cm deep from topsoil) from Seri Kembangan, Selangor, Malaysia. Five grams of soil sample were suspended in 45 ml of 0.9% saline solution. A suitable serial dilution aliquot (0.1 ml) of soil suspension was spread plated onto an agar of low phosphate (2.9 mM phosphate) media (pH 7.0) containing glucose (1%), $(NH_4)_2SO_4$ (0.3%), $MgSO_4.7H_2O$ (0.05%), NaCl (0.5%), yeast extract (0.0.5%), $Na_2MoO_4.2H_2O$ (0.242%) and $Na_2HPO_4$ (0.05%) [4]. Glucose was autoclaved separately. Growth in liquid media uses the same media as in the solid media above. Several blue colonies appeared after an overnight incubation at room temperature. One single colony exhibiting the strongest blue intensity observable by eye was inoculated into 50 ml of low phosphate media and incubated at 30 $^oC$ for 24 hours. The production of molybdenum blue from the media was measured at 865 nm. Identification at species level was performed by using molecular phylogenetics studies**.**

### The 16s rDNA Gene Sequencing and Phylogenetic Analysis

Genomic DNA was extracted from bacterial colonies by alkaline lysis. PCR amplification was performed using a thermal cycler (Biometra, Gottingen, Germany). The PCR mixture contained 0.5 pM of each primer, 200 µM of each deoxynucleotide triphosphate,10x reaction buffer, 2.5 U of Taq DNA polymerase (Promega) to achieve a final volume of 50 □1. The 16s rDNA gene from the genomic DNA was amplified by PCR using the following primers; 5'-AGAGTTTGATCATGGCTCAG-3' and 5'-ACGGTTACCTTGTTACGACTT-3'corresponding to the forward and reverse primers of 16s rDNA respectively [10]. PCR was performed under the following conditions: initial denaturation at 94 °C for 3 min; 25 cycles of 94 °C for 1 min, 50 °C for 1 min, and 72 °C for 2 min; and a final extension at 72 °C for 10 min. Cycle sequencing was subsequently performed with the Big Dye terminator kit (Perkin-Elmer Applied Biosystems) as recommended by the manufacturer. Sequence data were initially recorded and edited using CHROMAS Version 1.45. The resultant 1448 bases were compared with the GenBank database using the Blast server at NCBI (http://www.ncbi.nlm.nih.gov/BLAST/). This analysis showed this sequence to be closely related to *rrs* from Gammaproteobacteria. The 16s rRNA ribosomal gene sequences for this isolate has been deposited in GenBank under the following accession number EU851976

### Phylogenetic Analysis

A multiple alignment of 19 16s rRNA gene sequences that closely matches Strain Dr.Y6 was retrieved from GenBank and was aligned using ClustalW [18] with the PHYLIP output option. The alignment was checked by eye for any obvious misalignments. Alignment positions with gaps were excluded from the calculations. A phylogenetic tree was constructed by using PHYLIP, version 3.573 [J. Q. Felsenstein, PHYLIP—phylogeny inference package, version 3.573, Department of Genetics, University of Washington, Seattle, WA (http://evolution.genetics.washington.edu/phylip.html], with *Serratia marcescens FM163485* as the outgroup in the cladogram. Evolutionary distance matrices for the neighbor-joining/ UPGMA method were computed using the DNADIST algorithm program. The program reads in nucleotide sequences and writes an output file containing the distance matrix. The model of nucleotide substitution is from Jukes and Cantor, [11]. Phylogenetic tree (**Figure 1**) was inferred by using the neighbor-joining method of Saitou and Nei, [12]. With each algorithm, confidence levels for individual branches within the tree were checked by repeating the PHYLIP analysis with 1,000 bootstraps [12] by the SEQBOOT program in the PHYLIP package. Majority rule (50%) consensus trees were constructed for the Appl Biochem Biotechnol (2008) 149:33–43 35 topologies found using a family of consensus tree methods called the Ml methods [13] using the CONSENSE program, and the tree was viewed using Tree View [14].

## RESULTS AND DISCUSSION

### Identification of the isolate

The phylogenetic tree was constructed using PHYLIP [15] as shown in figure 1. A moderate bootstrap value (67.2%) is seen when strain Lbna is associated to bacillus pumilis strain XJU-7. The phylogenetic relationship between the species is moderately strong (Figure 1). Thus, this bacterium is assigned as *bacillus pumilus strain Lbna*, this strain is able to reduce molybdenum to molybdenum blue under aerobic condition. There are many other species from the previous works showed the ability to reduce molybdenum to molybdenum blue, most of these species are from the family of enterobacteriaceae [3,4,5,6].

### The effects of carbon sources on molybdate reduction

Different carbon sources such as lactose, glucose, mannitol, mannose, maltose, sucrose, and starch were used at an initial concentration of 1.0% (w/v) in low phosphate media containing 10 mM molybdate and 2.9 mM phosphate to study their effect on the molybdate reduction of bacterium. The results (Figure 2) showed that glucose was the best carbon source for bacteria growth followed by sucrose and maltose after 24 hours of incubation, all of these sources supported the reduction of the bacteria while the others supported the growth only. The Optimum concentration of glucose for molybdate reduction was 1.0% (w/v) after 24 h of static incubation. In a previous study, Campbell *et al.* [3] also reported that the best carbon source that supported the reduction for *E. coli* K12 is glucose. However, Ghani *et al.* [4] and Shukor *et al.* [6] mentioned that sucrose is the best carbon source

that supports the reduction of the bacteria. Therefore, molybdate reduction by the bacterial cell will be increased in a medium containing suitable carbon source. NADH is a byproduct of glycolysis which the cellular molybdate reduction depends on, thus more glucose will provide more NADH Shukor *et al.* [6]. The Optimum concentration of glucose for molybdate reduction was 1.0% (w/v) after 24 h of static incubation.

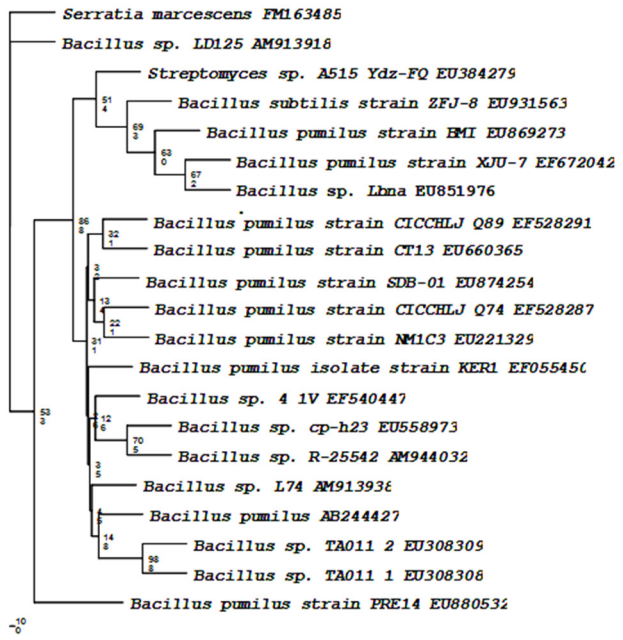


**Figure 1:** Neighbor-joining method showing phylogenetic relationship between strain Lbna and other related reference microorganisms based on the 16s rRNA gene sequence analysis. Species names are followed by accession number of their 16s rRNA sequences. The numbers at branching point refer to bootstrap value, based on 1000 re-samplings. *Serratia marcescens* strain FM163485 is the outgroup.

**The Effects of Molybdate and phosphate Concentrations on Molybdate Reduction**

The ratio of molybdate to phosphate is more important in molybdate reduction than the actual concentration of molybdate or phosphate Campbell et al. [3]. When molybdenum reduced to molybdenum blue under acidic environmental, it will binds with phosphate ion to form Mo-blue complex [16,17]. Phosphate in molybdate reduction was studied using phosphate concentration from 1 to 100 mM by fixing the molybdate concentration at 10 mM. The effects of molybdate in molybdate reduction were studied using molybdate (sodium molybdate) concentration ranging from 10 to 100 mM while fixing phosphate concentration at 2.5 mM. The optimum phosphate concentration for molybdate reduction in *Bacillus pumilus strain Lbna* was between 2.5 and 5 mM. The reduction decreased rapidly at higher concentration of phosphate and was totally inhibited at 100 mM phosphate (Figure 3). The effect of molybdate concentration on molybdate reduction showed that molybdate reduction linearly increased from 0 to 40 mM and started to decrease at higher molybdate concentrations (Figure 4). The optimum concentration for molybdate reduction was at 40 mM. Ghani et al [4] demonstrated that phosphate concentration inhibits the molybdate reduction if the concentration is higher than 0.5mM. The inhibition of molybdate reduction by phosphate has been maintaining in neutral pH. At this pH, the formation and stability of phosphomolybdate has been effected [17]. The ratio of molybdate to phosphate is more important in molybdate reduction than the actual concentration of molybdate or phosphate Campbell et al. [3]. The phosphate concentrations at greater than 0.5 mM will inhibit molybdate reduction Ghani *et al.* [4]. When molybdenum is reduced to molybdenum blue under acidic condition, it will binds with phosphate ion to form Mo-blue complex [16,17].

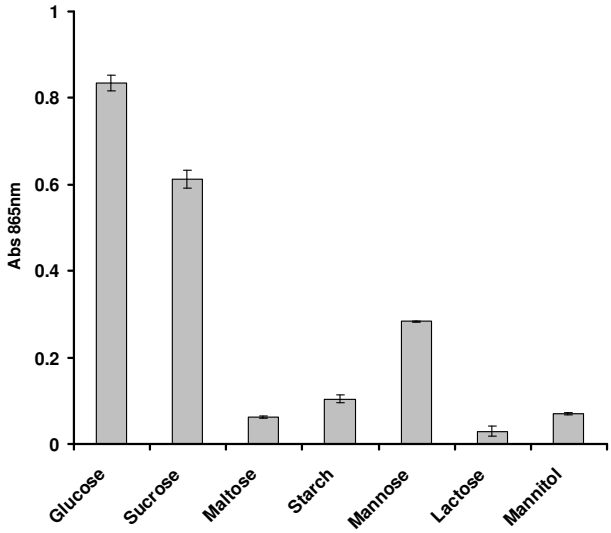


**Figure 2:** The effect of carbon sources on molybdate reduction by *bacillus pumilus* strain *Lbna*. Error bars represent the standard error of the mean between three replicates.

**Optimization of temperature**

The most important environmental factor for life is temperature, as it affects most of the biochemical reaction. The effect of temperature on the molybdate reduction of bacterium was studied at temperature ranging from 10 ºC to 60ºC. Figure 5 show's that the bacterium grow at a temperature range of 25-45 ºC with an optimum at 37 ºC. After 45 ºC, the bacteria growth dropped rapidly and almost no growth occurred at temperatures 50°C and above. This shows that the strain Lbna is a mesophilic bacterium which is A group of bacteria that grow and thrive in a moderate temperature ranging between 20-45 ºC. The previous study on mesophilic mo-reducing bacterium reported that the optimum temperature for the growth of EC 48 is 30ºC Ghani et al, [4], while in Campbell et.,al, [3] mentioned that the optimum temperature for *E. coli strain K12* is ranging between 30 to 36ºC. Whereas Shukor et al, [8] reported that the optimum temperature for *S. marcescens* strain Dr.Y6 is 35ºC.

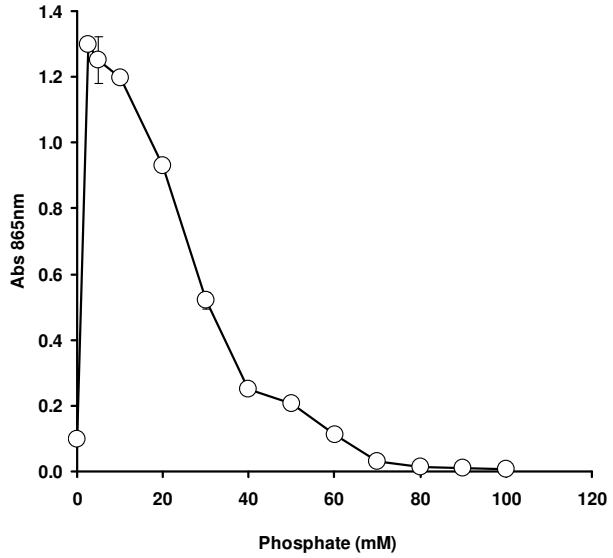


**Figure 3:** The effect of phosphate concentration on molybdate reduction by *bacillus pumilus* strain *Lbna*.. Error bars represent the standard error of the mean between three replicates.

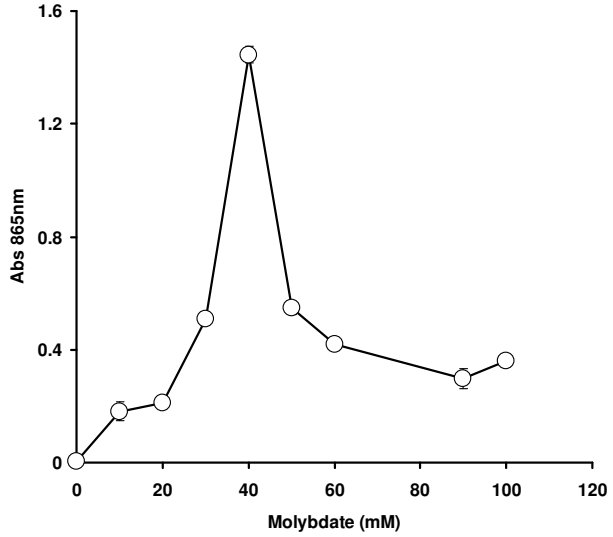


**Figure 4:** The effect of molybdate concentration on molybdate reduction by *bacillus pumilus* strain *Lbna*.. Error bars represent the standard error of the mean between three replicates.

**Optimization of pH**

Figure 6 shows that, the high level of Mo-blue complex production was between pH 7 and 8. The pH of the medium affected the reduction of molybdenum. Most of the best pH for best reduction of bacteria is ranging between pH 6-8. Strain Lbna grew well in molybdenum medium in pH ranging between 7.0-8.0 in phosphate and Tris-HCL buffer where the maximum reduction of molybdenum was observed at pH 7.5 (Figure 6).This mean that this strain is neutrophile bacterium. Previous study shows that, the high level of molybdenum production was between pH 6 and 7, as abserved on *E. coli* K-12 Campbell et al, [3], *Enterobacter clocae* strain 48 proposed by Ghani et al, [4] and the same goes to *S. marcescens* strain Dr. Y6 mentioned by Shukor et al, (2008). While Sugio et al, [2] has reported that *thiobacillus ferreoxidans* could reduce molybdenum at pH 3.0. The decrease of the production of molybdenum blue at basic pH was referred to the destraction of stability of the plasma membrance, inhibiting the enzyme and transport protein of the bacteria.

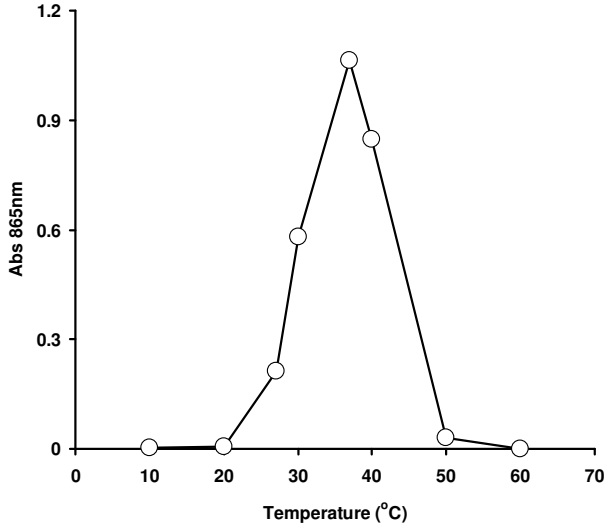


**Figure 5:** The effect of temperature on molybdenum reduction by *bacillus pumilus* strain *Lbna*. Error bars represent the standard error of the mean between three replicates

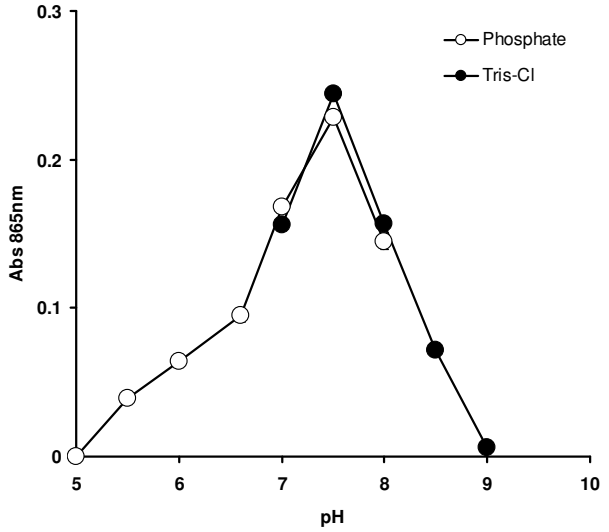


**Figure 6:** The effect of pH on molybdenum reduction by *bacillus pumilus* strain *Lbna*. Error bars represent the standard error of the mean between three replicates.

**Absorption spectra of molybdenum blue**

Figure 6 shows the absorption spectra of molybdenum blue produced by *bacillus pumilus* strain *Lbna* after 24 hour of incubation. The result has demonstrated that the absorbance peaks for the molybdenum blue produced by the bacterium was at

865nm with a shoulder peak at 700nm. The molybdenum blue produced by this strain shows the same characteristics absorption profile to that of EC 48 Shukor et al. [17] with a maximum peak of 865 nm and a shoulder peak at 700 this unique fingerprint was found to increase proportionately to the blue intensity as molybdate reduction progresses. Figure 5 shows the absorption spectra of molybdenum blue produced by *bacillus pumilus* strain Lbna after 24 hour of incubation. The result has demonstrated that the absorbance peaks for the molybdenum blue produced by the bacterium was at 865nm with a shoulder peak at 700nm. The previous study of S. marcescens strain Dr.Y6 is also closely similar with a peak maximum at 865 nm and a shoulder at 700 nm [8]. In the phosphate determination method the spectra are similar to the Mo-blue produced by the ascorbic acid-reduced phosphomolybdate with a peak maximum at 880 nm and a shoulder at 700 nm [17]. According to this suggest the increasing of the absorption spectra of molybdate reduction in strain Lbna is an increase in the reduction of phosphomolybdate, all of the results including this work indicated that phosphomolybdate plays a central role in molybdenum reduction in the heterotrophic bacteria.

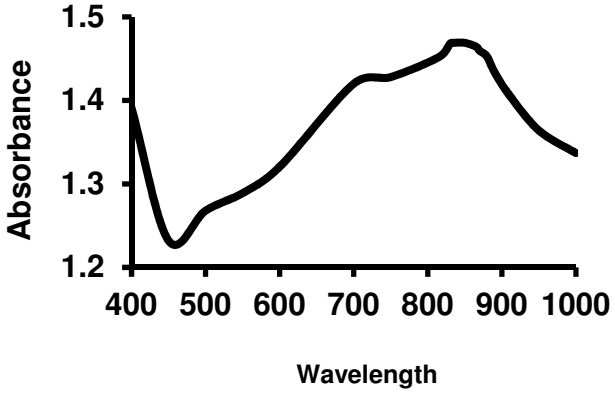


**Figure 6:** The absorption spectra of molybdenum blue produced by *bacillus pumilus* strain *Lbna* after 24 hour of incubation.

**Molybdenum Reduction Study**

Molybdate Reduction was studied by analyzing the molybdate reduction curve of a microbial culture. The Purpose of this study was to observe the maximum time for the maximum molybdenum blue production by certain bacterium. The reduction of molybdenum study was assayed using LPM according to Ghani *et al*., [4]. As early studied by Ghani *et al.*, [4], the incubation of EC48 gave highest molybdenum blue produced at 28 hours incubation time. The reduction plot showed that the reaction starts at 12 hours incubation. Four different strains were used in comparing the incubation time towards the reduction of molybdate. Those strains were Bacterium strain Dr Y13, *Klebsiella oxitoca* Dr Y14, *Acinetobacter sp*. strain Dr Y12 and *Serratia marcescens* strain Dr Y10. Each of every strain gave different period of time with *Acinetobacter sp*. strain Dr Y12 was the fastest (20 hours) and *Klebsialla oxitoca* strain Dr Y14 was the last one(>40hours)[8]. This comparison showed that even though the reaction reported to be similar, but the reaction period was different on different strain. The result of this study shows that there was no increasing in absorbance until 12 hours of incubation. After 12 hours incubation, the absorbance has been increased with the maximum absorbance at 30 hours incubation (**Figure 7**). Previous study reported that, most of the bacterium optimum growth was at 24 hours incubation. The reduction of molybdate was at stationary phase after 30 hours incubation. This indicates that the production rate of bacterium is similar with the death rate after 24 hours.

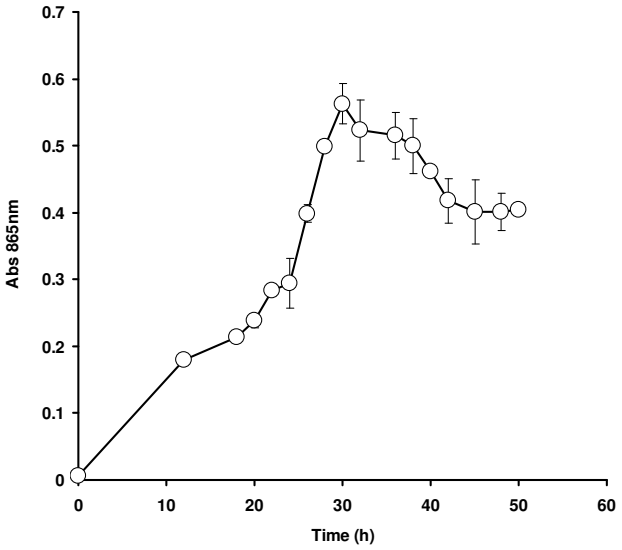


**Figure 7:** The study of molybdate reduction through time (Hours). Error bars represent mean ± standard error (n=3).

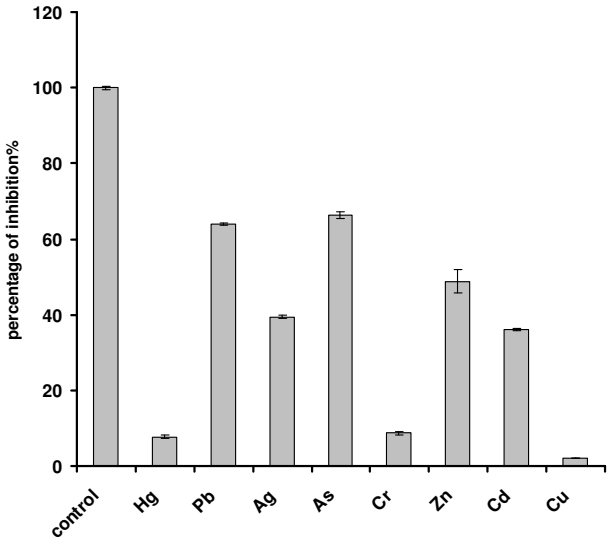


**Figure 8:** Heavy metal caused inhibition to molybdenum reduction.

**Effect of Heavy Metals on Molybdenum-reducing enzyme**

The results showed that molybdenum reduction in *Bacillus pumilus* strain Lbna were inhibited by all heavy metals used. The metal ions arsenic, lead, zinc, silver, cadmium, chromium, mercury and copper caused 33.7, 36.0, 51.2, 60.5, 63.9, 91.3, 92.3 and 98.0% inhibition to molybdenum reduction (Figure 8). The lowest percentage of Molybdenum activity obtained from incubation of enzymes with Copper which was only 1.434% thus has the highest inhibition factor towards enzymes activity. All

heavy metals are tested at the standard 1 mg/L. Shukor et al., [18] also reported that copper can be hindered mo-reducing enzyme from *S. marcescens* with $IC_{50}$ values of 0.099 ± 0.013 mg/L. Inhibition mechanism of copper is still not clear, while mercury and lead may be prevent most sulfhydryl group at the active sites. The inhibition by toxic metal ions in this study is seen in all of the Mo-reducing bacteria [5, 8, 17, 19-22, 23-27] and in other heavy-metal reducing bacteria as well [28-30]. The inhibition of metal-reducing activity by heavy metals generally indicates an enzymatic origin and not abiotic [31-33].

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