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*Full Length Research Paper*

# Isolation and screening of arsenic resistant rhizobacteria of *Ludwigia octovalvis*

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***Ludwigia octovalvis*** **was characterized for its potential in arsenic phytoremediation. Epiphyte rhizobacteria from the roots of *L. octovalvis* were isolated in five different arsenic concentrations (4, 20, 40, 60 and 80 mg $kg^{-1}$) and control after single exposure for 35 days. Results show that 109 colonies were isolated which were further grouped into 29 groups of isolated rhizobacteria. After preliminary screening, 17 rhizobacterial isolates were considered to be resistant to arsenic (arsenate or As[V]) and after secondary screening, 12 rhizobacterial isolates showed potential resistance to arsenic. Identification was conducted using biolog GEN III microbial identification system. One of the arsenate potentially resistant rhizobacterial was identified as *Arthrobacter globiformis*. The *k* (growth rate constant) value of *A. globiformis* was 0.112 $h^{-1}$ with *g* (generation time) value of 8.943 h and *ì* (specific growth rate) value of 0.077 $h^{-1}$. Minimal inhibitory concentration (MIC) value of *A. globiformis* on arsenate exposure was 500 mg $L^{-1}$ and has the potential to promote arsenic phytoremediation at contaminated site.**

**Key words:** Phytoremediation, arsenic, isolation, screening, identification, minimal inhibitory concentration (MIC).

## INTRODUCTION

Arsenic is one of the heavy metals that have toxic effects on humans, plants, animals and also microorganisms. Arsenic concentration typically varies from below 10 mg $kg^{-1}$ in non-contaminated soil (Fitz and Wenzel, 2002; Vaughan, 1993; Gonzaga et al., 2006), to as high as 30,000 mg $kg^{-1}$ in contaminated soil. Generally, arsenate [As(V)] is the dominant species in the soil solution under oxidizing conditions, whereas arsenite [As(III)] is the prevailing species under moderately reducing conditions (Quaghebeur and Rengel, 2005). The arsenite is more mobile, highly soluble and more toxic than arsenate (Krumova et al., 2008). In the environment, some arsenic compounds can be easily solubilized in water and taken up by microorganism (Tsai et al., 2009).

Plants can be arsenic hyperaccumulators. It can uptake arsenic and accumulate it in different parts of plant. Plant roots release a wide range of compounds that are involved in attracting beneficial organisms and forming mutualistic associations in the rhizophere (Badri et al., 2009). According to Andrew and Harris (2000) cited by Guo et al. (2010), plant-assosiated bacteria isolated from

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rhizoplane and phylloplane surface are known as epiphyte. According to Wu et al. (2006), accumulation of cadmium by plants could be increased after being inoculated with *Pseudomonas putida* and a noticeable reduction of its phytotoxicity was observed. Plant growth-promoting bacteria (PGPB) or plant growth-promoting rhizobacteria (PGPR) can be applied in agricultural production or for the phytoremediation of pollutants (Compant et al., 2010).

Microbe response to arsenic in nature could occur in different ways such as chelation, compartmentalization, exclusion and immobilization. Each species of bacteria has different response. Arsenic metabolism by prokaryotes such as bacteria can potentially act as an electron donor or acceptor and be part of the electron transport chain. Many bacteria (Gram-negative and -positive) have detoxification mechanisms (Tsai et al., 2009). The mechanism of arsenate resistance is reduced accumulation of arsenate by induced resistant cell (Shukla et al., 2006). Arsenate is toxic to bacteria because it is an analog of phosphate and can inhibit enzymes such as kinases.

In this study, epiphyte rhizobacteria from the roots of *Ludwigia octovalvis* were isolated in different arsenic concentrations. It is a part of a research project that aims to accumulate arsenic using phytoremediation method (Titah et al., 2010). This investigation aims to isolate pure culture rhizobacteria from *L. octovalvis*, and later, the isolated pure culture was further screened for arsenic (in form arsenate) resistance. In addition, identification and determination of growth rate constant and determination of minimal inhibitory concentration (MIC) on arsenate were conducted. The results will then be used for further phytoremediation research. The identified rhizobacteria in this study are potential arsenic resistant rhizobacteria that can help and promote uptake of arsenic by *L. octovalvis*.

## MATERIALS AND METHODS

### Isolation of epiphyte rhizobacteria

Isolation of epiphyte rhizobacteria from *L. octovalvis* roots was carried out after 35 days of arsenic exposure. This method is according to Mittal and Johri (2007), Cakmakci et al. (2007), Abou-Shanab et al. (2005) and Harley and Prescott (2002). Each root of *L. octovalvis* (10 g) with loosely attached soil from different concentration of arsenic spiked sand was suspended in 100 ml sterile distilled water and shaken vigorously at 30°C and 150 rpm for 1 h. After all particles have settled for 1 min, 1 ml of the homogeneous suspension was added to dilution tubes containing 9 ml of sterile saline solution (0.9%) to make a serial dilution ($10^{-1}$ to $10^{-7}$). The suspensions (0.1 ml) were plated onto Trypticase (Tryptic) Soy Agar or TSA (Difco, USA) medium by a serial dilution using spread plate technique. Rhizobacterial isolation was performed on TSA because there were no yeast, peptone and amino acid in this media which can bind with large amounts of divalent Hg, Pb, Ag and Cu ions.

All plates were incubated at 30°C in an incubator (Incucell, Germany), and they were observed for two days. Single colony was picked up and streaked onto a fresh medium to obtain a pure culture. Rhizobacteria isolates were selected to represent distinct types based on differences in colony morphology, including colony form, elevation, diameter, pigment production and motility using hanging drop technique (Prescott et al., 2002; Rathnayake et al., 2009). The general form of the colony and the shape of the edge or margin can be determined through eye vision on the top of the colony. The nature of the colony elevation is apparent when viewed from the side as the plate is held at eye level. Gram-staining and biochemical tests (catalase activity and oxidase activity) of the isolates were examined after 24 or 48 h (depending on the tested isolates) of incubation on TSA agar plates (Drewniak et al., 2008).

### Arsenate screening

The pure culture rhizobacterial isolates were then used for arsenate preliminary screening. The experiment used arsenate concentrations of 200, 250, 350, 500 and 700 mg $L^{-1}$ plus the control plate without arsenate. All the pure culture rhizobacteria were planted on TSA medium plates containing arsenate (sodium arsenate dibasic heptahydrate = $AsHNa_2O_4.7H_20$, Fluka Chemika, Switzerland) using streak plate technique (Malekzadeh et al., 2001). Plates were incubated at 30°C in an incubator and observed until six days.

### Identification of selected rhizobacteria

Rhizobacterial identification was conducted using biolog GEN III microbial identification system (USA) and the reading of ID result used microStation – semi-automated identification system (USA). Biolog GEN III technology has many advantages: no Gram stain needed, no pre test and no follow on test, one ID panel for both Gram-negative and positive aerobic bacteria, and more than 1000 species of aerobic bacteria in database.

### Growth rate of selected species

Growth rate of selected potential bacteria was conducted in TSB (Difco, USA) medium. After being inoculated onto TSA (Difco, USA) medium for 24 h, one colony was inoculated into 50 ml TSB (Difco, USA) medium in 250 ml Erlenmeyer flasks. This culture was incubated in an incubator shaker (Protech, Model SI-100D, Malaysia) at 37°C and 150 rpm. The growth rate was determined from the optical density at 550 nm ($OD_{550}$) using Genesys 10 UV (Thermo Spectronic, USA). Optical density was determined at 0, 2.5, 4.5, 5.5, 6.5, 23.5, 28, 48 and 72 h.

### Arsenate resistance of selected species

The resistance to arsenate was run to select one potential isolated rhizobacteria for phytoremediation processes. The minimal inhibitory concentration (MIC) of arsenate, which is the lowest concentration of arsenate at which it can completely inhibit bacterial growth was also determined. It was conducted in PBS (phosphate buffer saline) medium with a series of concentration of arsenate (control, 250, 500, 750 and 1000 mg $L^{-1}$).

Ingredients of 10 x PBS stock solution according to Harley and Prescott (2002) were 80 g NaCl, 2 g KCl, 11 g $Na_2HPO_4.7\ H_2O$ and 2 g $KH_2PO_4$.

The MIC determination was carried out in 250 ml Erlenmeyer flask with 50 ml working volume. The cultures were incubated in an incubator shaker (Protech, Model SI-100D, Malaysia) at 37°C and 150 rpm. Bacterial growth was determined using an absorbance

550 nm at 0, 3, 6, 19 and 24 h.

## RESULTS AND DISCUSSION

### Isolation of epiphyte rhizobacteria

Based on an observation for 48 h, 83 to 100% of rhizobacterial colonies could not grow in high dilution ($10^{-6}$ and $10^{-7}$). A total of 109 colonies were isolated from roots of *L. octovalvis* on plates of control and different concentrations of arsenic grown on TSA medium. All pure cultures were stained using Gram-staining and further characterized using morphological (shape, diameter, elevation, margin, pigment production and motility), and biochemical tests (catalase activity and oxidase activity). Gram-positive and negative isolates varied in morphology from rods, coccobacill to filamentous types.

According to Prescott et al. (2002), bacterial colony characteristics on agar media were described using the following term: form/shape, elevation, margin, appearance, pigmentation (color) and texture. Based on the results, all pure culture bacteria were divided into 29 groups of the same characterization (Table 1).Figure 1 shows percentages of morphological observation. A lot of colony shapes were circular (90%), other shapes were irregular (7%) and rhizoid (3%). Some kinds of elevation were flat (34%), convex (31%), raised (14%), crateriform (14%) and umbonate (7%). Bacterial margin were undulate (38%), entire (34%), erose (14%), lobate (10%) and curled (3%). The appearances of all colonies were shiny (62%) (majority) and dull (38%). Diversed colors of isolated bacteria were white (66%), yellow (17%), pink and orange (7%), and cream (3%). Most of the bacterial textures were smooth (83%) and about 17% had rough texture.

There were 8, 6, 6, 11, 12 and 10 eight different groups of isolated rhizobacterial derived from the control, 4, 20, 40, 60 and 80 mg $kg^{-1}$ arsenate, respectively. All these rhizobacterial groups could be found both in the control and the exposed arsenate concentration.

### Preliminary screening of pure culture rhizobacteria to arsenate

Qualitative scoring for the results of the preliminary screening was conducted to determine rhizobacterial isolates that were potentially resistant to arsenic. This qualitative scoring was based on the growth of bacteria and the color change when it was compared with the color of colony without arsenic. There were 17 rhizobacterial isolates potentially resistant to arsenate until 300 mg $L^{-1}$. After secondary screening, some of them showed extreme tolerance arsenate. They were further reduced to 12 rhizobacterial isolates that were potentially resistant to arsenate in 500 and 700 mg $L^{-1}$.It was reported that the most resistant bacteria tolerant to arsenate at concentrations up to 500 mM (37,500 mg $L^{-1}$, approaching the solubility limit of As(V) in Luria Broth medium) was isolated from ancient gold mine in Poland (Drewniak et al., 2008). Basic detoxification mechanism in Gram-negative and positive bacteria employ a similar arsenic mechanism based on the *ars* operon (typically *ars*RDABG) encoded either on the chromosome or on plasmids (Tsai et al., 2009). Despite its toxicity, a number of bacteria are capable of using either the oxidized form of inorganic arsenic (arsenate) or the reduced form (arsenite) in their metabolism, and even more microorganisms are capable of resisting arsenic toxicity though the *ars* genetic system (Jackson et al., 2005).

### Identification, growth rate, and minimal inhibitory concentration (MIC)

One of the potential rhizobacteria with code B (Table 1) showed resistance to arsenate. This rhizobacteria was identified using biolog GEN III microbial identification system. Based on the result, rhizobacteria code B was identified as *Arthrobacter globiformis*. The growth rate, exponential, log or logarithmic growth phase within 3 h and stationary phase after 28 h is shown in Figure 2. *A. globiformis* was Gram positive bacteria, where cells in young cultures were irregular rods with measurements of 0.8 - 1.2 x 1.0 - 8.0 ìm, aerobic, chemoorganotropic, usually grow on simple media plus biotin with an oxidative metabolism, and positive catalase (Holt et al., 2000).

During the logarithmic growth phase, a bacterial culture mimics a first-order chemical reaction; the rate of increase of cells is proportional to the number of bacteria present at that time. The constant of proportionally, *k*, is an index of the growth rate and is known as growth rate constant. The formulas shown were used to calculate *k* (Prescott et al., 2002):

$$k = \frac{\log X_n - \log X_0}{0.301(t_n - t_0)} \quad (1)$$

$$g = \frac{1}{k} \quad (2)$$

$$\mu = 0.693k = \frac{0.693}{g} \quad (3)$$

Where, *k* is the growth rate constant $X_n$ is the OD at $t_n$; $X_0$ is the OD at *t0*; $t_n$ is the time at *n*; $t_0$ is the time at 0; *g* is the generation time and *ì* is the specific growth rate. Based on the equation, *k* for *A. globiformis* was determined as 0.112 generation $h^{-1}$ with *g* (generation time) of 8.943 h and *ì* (specific growth rate) of 0.077 $h^{-1}$. Generation time (8.943 h) was still within the generation

**Table 1.** Morphological and biochemical characteristics of rhizobacterial isolates.

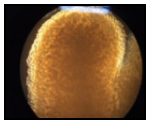
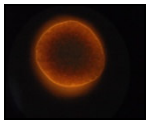
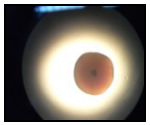
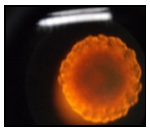
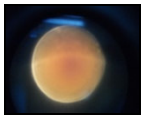
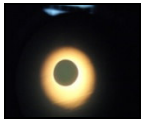
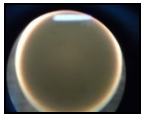
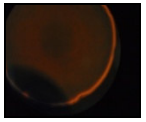
| S/N | Bacterial code | Gram-staining | Single colony x40 | Morphology | | | | | | | Biochemical | | As |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | Color | Shape | Φ (mm) | Elevation | Margin | Texture | Motility | Oxidase | Catalase | ($mgkg^{-1}$) |
| 1 | A | + |  | White | Circular | 3 | Raised | Undulate | Rough, dull | - | + | + | 0 |
| 2 | B | + |  | White | Circular | 3 | Flat | Erose | Smooth, dull | - | + | + | 0, 40 |
| 3 | C | - |  | White | Circular | 1 | Convex | Entire | Smooth, dull | - | - | + | 0 |
| 4 | D | - |  | White | Circular | 1 | Flat | Erose | Smooth, dull | - | + | + | 0 |
| 5 | E | - |  | Yellow light | Circular | 0.25 | Flat | Erose | Smooth, shiny | + | + | - | 0, 20, 40 |
| 6 | F | + |  | Pink | Circular | 0.5 | Flat | Entire | Smooth, shiny | - | + | - | 0 |
| 7 | G | - |  | Cream | Circular | 0.5-1 | Convex | Undulate | Smooth, shiny | - | - | + | 0, 4, 40, 60 |
| 8 | H | - |  | Orange | Circular | 1 | Convex | Entire | Smooth, shiny | + | + | + | 60, 80 |
| 9 | I | - |  | White | Circular | 2 | Flat | Entire | Smooth, shiny | + | + | + | 0, 60 |

**Table 1.** Contd.

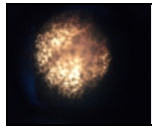
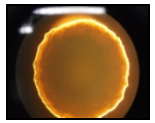
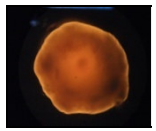
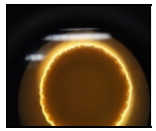
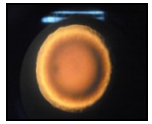
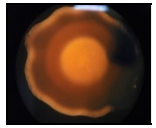
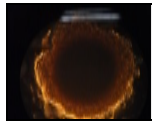
| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 10 | J | + |  | White | Rhizoid | - | Flat | Lobate | Rough, dull | + | - | + | 4, 20, 40, 60, 80 |
| 11 | K | + |  | Yellow | Circular | 3 | Flat | Undulate | Rough, dull | - | - | + | 4, 40, 60, 80 |
| 12 | L | - |  | White | Circular | 1 | Flat | Undulate | Smooth, shiny | - | - | + | 4, 40, 60, 80 |
| 13 | M | - |  | Yellow light | Circular | 3 | Convex | Entire | Smooth, dull | - | - | + | 4, 20 |
| 14 | N | + |  | Orange | Circular | 1 | Convex | Entire | Smooth, shiny | - | - | + | 4, 40 |
| 15 | O | - |  | Pink | Circular | 3 | Crateriform | Undulate | Smooth, shiny | + | - | + | 60, 80 |
| 16 | P | - |  | Yellow | Irregular | - | Raised | Curled | Smooth, dull | - | + | + | 20 |

**Table 1.** Contd.

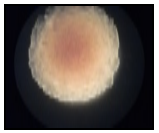
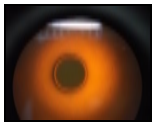
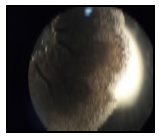
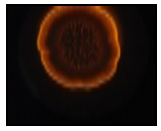
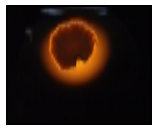
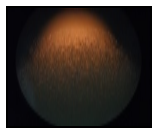
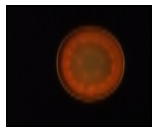
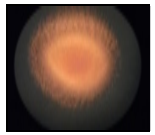
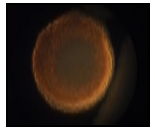
| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 17 | Q | + |  | White | Circular | 2 | Umbonate | Undulate | Rough, dull | - | - | + | 20, 60, 80 |
| 18 | R | + |  | Yellow | Circular | 1 | Crateriform | Lobate | Rough, dull | - | - | + | 20, 40 |
| 19 | S | - |  | White | Irregular | - | Umbonate | Lobate | Smooth, shiny, slimy | + | + | + | 40 |
| 20 | T | - |  | White | Circular | 0.5-3 | Raised | Undulate | Smooth, shiny | - | - | + | 40 |
| 21 | U | - |  | White | Circular | 5 | Raised | Undulate | Smooth, shiny | + | + | + | 40 |
| 22 | V | + |  | White | Circular | 1 | Crateriform | Undulate | Smooth, shiny | + | - | + | 60 |
| 23 | W | - |  | White | Circular | 6 | Flat | Undulate | Smooth, dull | - | + | + | 60 |
| 24 | X | - |  | White | Circular | 2 | Convex | Entire | Smooth, shiny | - | + | - | 80 |

**Table 1.** Contd.

| | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 25 | Y | - |  | White light | Circular | 2 | Flat | Entire | Smooth, shiny | - | - | - | 80 |
| 26 | Z | + |  | White | Circular | 1 | Convex | Undulate | Smooth, shiny | - | + | + | 60 |
| 27 | AA | + |  | White | Circular | 4 | Crateriform | Erose | Smooth, shiny | - | - | + | 60 |
| 28 | BB | + |  | White | Circular | 1 | Convex | Entire | Smooth, shiny | - | - | - | 80 |
| 29 | CC | - |  | White | Circular | 1 | Convex | Entire | Smooth, shiny | + | + | - | 80 |

time typically for bacteria because the typical generation time ranges from 20 min to 20 h, depending on the bacterial species/strain and the conditions during which log-phase occur (Abedon, 1999).

Based on the results from the MIC determination as shown in Figure 3, MIC value of *A. globiformis* on arsenate exposure was 500 mg $L^{-1}$, indicating that *A. globiformis* still survived under arsenate concentration of 500 mg $L^{-1}$. This shows that *A. globiformis* possibly used arsenic in their growth and can be the candidate for accumulation or reducing arsenic in phytoremediation of arsenic contaminated soil. According to Krumova et al. (2008), *Pseudomonas stutzeri* and *Pseudomonas aeruginosa* show MIC value of arsenate 11 mM (825 mg $L^{-1}$) and *Pseudomonas* sp. show MIC of arsenate 10 mM (750 mg $L^{-1}$). Further investigation will be conducted to evaluate or determine the arsenic biosorption by *A. globiformis* in order to further clarify whether the arsenic is being adsorbed to *A. globiformis*.

## Conclusions

A total of 109 epiphyte rhizobacteria were isolated from roots of *L. octovalvis* at five different arsenic concentrations which later could be characterized to 29 groups. After secondary screening, 12 rhizo-

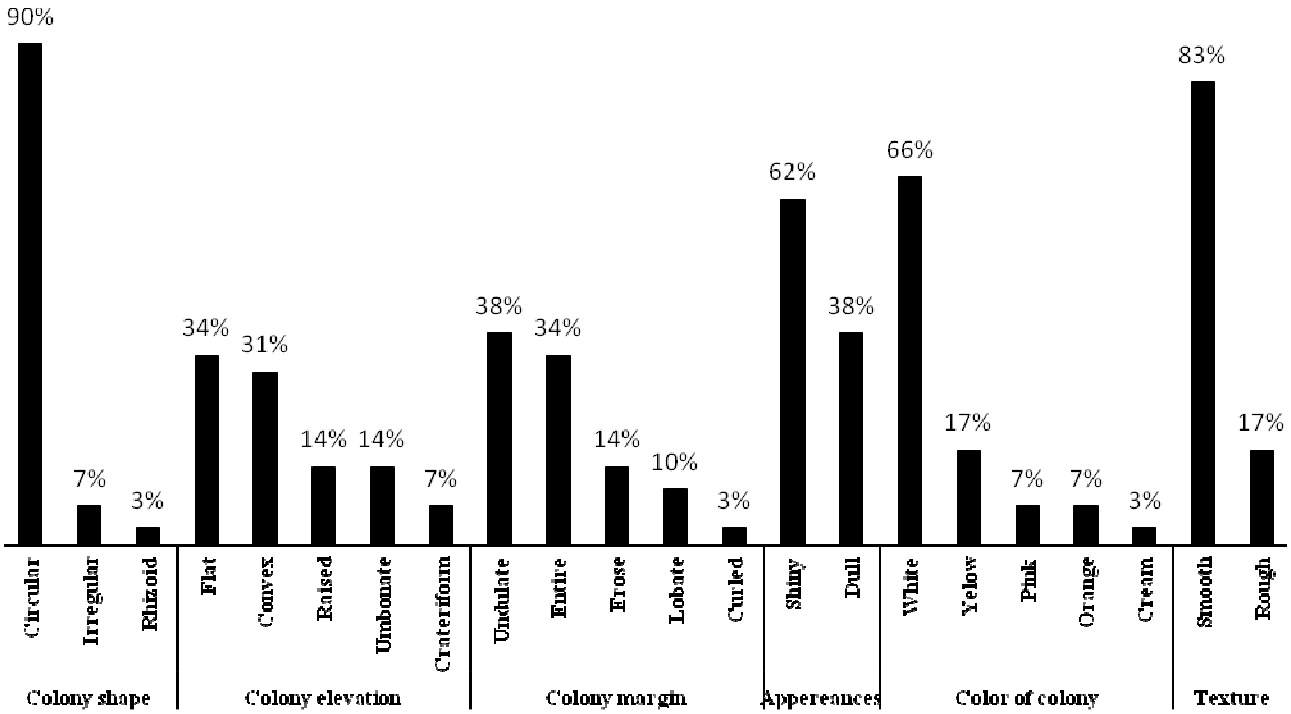



**Figure 1.** Percentages of morphological observation.

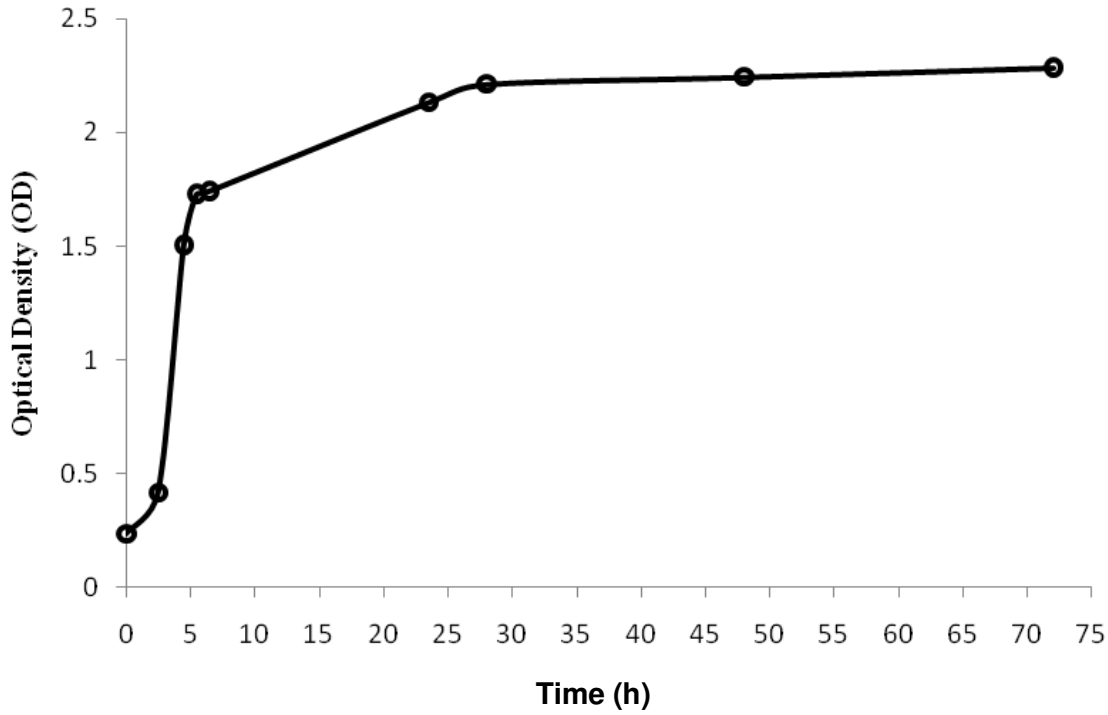



**Figure 2.** Growth rate of *A. globiformis* in TSB medium.

bacterial isolate were potentially resistant to arsenate. One of them was identified as *A. globiformis* which possibly used arsenic in their growth mechanism. The *k* value for *A. globiformis* was 0.112 $h^{-1}$ with *g* value of 8.943 h, *μ* of0.077 $h^{-1}$. MIC of arsenate exposure for *A. globiformis* was determined to be 500 mg $L^{-1}$.

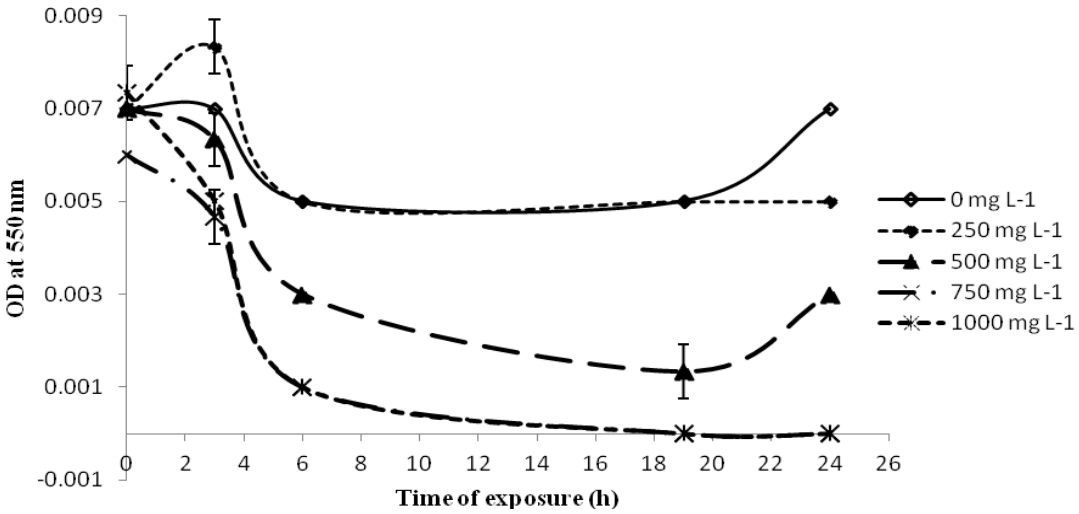



**Figure 3.** Graph of growth rate of *A. globiformis* on arsenate exposure.

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