U (VI) tolerance affects *Shewanella* sp. RCRI7 biological responses: growth, morphology and bioreduction ability

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Abstract

Native *Shewanella* sp. RCRI7 is recently counted as an operative bacterium in the uranium bioreduction. The aim of this study was to investigate the effect of uranium tolerance on the morphology and population of RCRI7 following its potential removal capacity in different time intervals. Accordingly, the bacterial growth and uranium removal kinetic were evaluated in aerobic TSB medium, uranium reducing condition (URC), aerobic uranium containing (AUC) and anaerobic uranium free (AUF) solutions, following evaluations of *omcAB* gene expressions. In addition, spectrophotometry analyses were performed in URC confirming the bioreduction mechanism.

It was found that the bacteria can grow efficiently in the presence of 0.5 mM uranium anaerobically, unlike AUC and AUF solutions during 92 h incubation. The bacteria can kinetically remove a large amount of soluble uranium through adsorption pathway in the first 12 h in URC. This occurs in a circumstance that the removal process keeps on increasing via reduction lane up to 84 h, when the most amount of uranium removal has been indicated. In 92 h, the adsorbed uranium containing unreduced and reduced (U (IV) monomeric), was released to the solution due to either the increased pH or bacterial death. In AUC and AUF, improper conditions lead to reduced bacterial size and increased bacterial aggregations, however, membrane vesicles produced by the bacteria avoid the uranium incrustation in AUC.

1. Introduction

Bacteria have the ability to carry out reduction, oxidation, adsorption on cell surface groups, and precipitation of radionuclides by interacting with ligands such as phosphate (Lloyd and Renshaw 2005; Icenhower et al. 2010; Newsome et al. 2014). The selected *Shewanella* genus is aquatic gram-negative rod-shaped bacteria belongs to the Shewanellaceae family (facultative anaerobic gammaproteobacteria) grown in different environments. These gram-negative bacteria are able to use various organic and inorganic substances, including metals, as electron acceptors in their respiratory pathway which reduce them due to their large number of cytochromes and multiple electron transfer pathways (Pinchuk et al. 2010; Wang et al. 2010; Saffarini et al. 2015). OmcA and OmcB (MtrC), decaheme c-type cytochromes in the *Shewanella* cell surface, are the final reducers in the electron transfer chain playing important roles in the reduction of uranium (VI) (Shi et al. 2007; Kouzuma et al. 2015; Saffarini et al. 2015).

Shewanella RCRI7 is the Iranian native strain isolated and identified in 2011 from Qurugöl Lake near to the city of Tabriz by Dr. Tarhriz. Identification of this bacterium and determination of its phylogenetic tree based on 16s rRNA sequence indicated that this strain is very similar to known strains such as *Shewanella putrefaciens* and *Shewanella xiamenensis*, but most of all it is similar to the *Shewanella oneidensis* MR-1 Strain, a model bacteria in the uranium bioreduction (Tarhriz et al. 2011).

RCRI7 is a new known *Shewanella* strain with little accessible information. Recently, we confirmed that optimum cell growth and uranium removal by *Shewanella* RCRI7 was achieved at 0.5 mM uranium concentration (Abdehvand et al. 2017; Ghasemi et al. 2020). In followings, we were going to consider the

tolerance and removal kinetic of uranium in different time intervals in the mean time of the biological interactions between uranium and bacteria in the bioremediation process. This study –for the first time- attempts to investigate in what way uranium affects bacterial morphology, growth and uranium reduction in different time intervals and which factors cause affect, increase or decrease these properties. This study can reconfirm the suitable and efficient use of RCRI7 strain in the uranium bio-reduction process that provides further studies on the biological and chemical interaction between bacteria and uranium.

2. Materials And Methods

2.1. Preparation of reduction solution

The reduction solution refers to a solution containing all the substances needed for the growth of the bacteria under anaerobic condition while give the bacteria the ability to perform the reduction process. The reduction solution contains vitamins, minerals, soluble salts, electron donor (sodium lactate) and electron acceptor (uranium). Based on the Czerwinski and Polz (2008) experiments, the reduction solution was prepared and oxygen was removed 24 hours before bacterial inoculation (Czerwinski and Polz 2008). Uranium was added to the solution in the form of uranium acetate at 0.5 mM. In the final step, the pH was adjusted to 6.4 with sodium bicarbonate powder and the solutions were divided into determined vials to make them anaerobic.

2.2. Anaerobic atmosphere

The anaerobic condition was carried out with designed glove bag in the dimensions of 30 x 30 x 45 cm by gas replacement method. This was done with gas containing 90% N₂ and 10% CO₂ (Abdehvand et al. 2017; Ghasemi et al. 2020).

2.3. Bacterial cultivation

The stock bacteria (at -80° C) was freshly inoculated into tryptic soy broth (TSB) medium (Merck .Co) incubated at 30° C and 150 rpm, for 24 h. After the incubation, the bacterial red pellet was prepared by centrifuging the bacteria at 4500 rpm for 30 min. After removal of the medium, the bacteria were washed in the sodium bicarbonate solution (~ 22 mM). Following oxygen removal by gas replacement method, the bacterial suspension inoculated into the each reduction solution by needle to reach the final concentration to 10⁹ (optimum final pellet count after centrifuging) (Abdehvand et al. 2017).

2.4. Analytical experiments

Uranium toxicity effects were investigated in several time intervals between 0 to 92 h in specific uranium concentration (0.5 mM) in aerobic uranium containing (AUC) and anaerobic uranium containing (uranium

reducing condition (URC)) solutions.

After the incubation time, the results were evaluated in several methods included; 1) spectrophotometry (UV-Visible) 2) Real-Time RT-PCR 3) bacterial counting by Neubauer lam 4) evaluation of uranium removal by Inductively Coupled Plasma Atomic Emission Spectrometry (ICP-AES, Perkin Elmer Optima 2000 DV). Also, the bacterial population in anaerobic uranium free (AUF) solution and aerobic uranium free TSB medium were counted with Neubauer lam. Besides that, the uranium morphological effects were considered by observation of motivation and size changes of the bacteria.

2.4.1. UV-Visible (Spectrophotometry)

Spectrophotometry was used to investigate the production of uraninite in the URC containing 1 mM after one day and two-week incubations. At the end of the incubation times, the whole incubated solution was centrifuged at 10000 rpm for 10 minutes. The precipitate was dissolved in 10 ml of 10 mM citric acid and passed through filters with 0.2 µm pore size (Francis and Dodge 2008). The clear solution was analyzed by the SQ-2800 Single Beam Scanning UV/Visible Spectrophotometer to evaluate the optical absorption of uranium (VI) and (IV).

Uranium (IV) have the peak in the wavelengths of 560 nm and 662 nm while, uranium (VI) has a peak in the 438 nm (Gao and Francis 2008).

2.4.2. Real-Time RT-PCR

In this study, Real-Time RT-PCR was used to evaluate the expression of the *OmcAB* genes in AUC, URC and AUF. At the end of 21 h incubation, the bacteria pellet total RNA extractions were performed using RNeasy® Minikit (Qiagen, Valencia, CA). Then, the remained DNA was digested using DNase I (Thermo Scientific, USA). Isolated RNA was quantified at OD260 and OD280 by NanoDrop™ 2000 spectrophotometer (Thermo Scientific, France). 500 ng RNA was used in each reaction for cDNA synthesis by using Revert-Aid first strand cDNA synthesis kit (Thermo Scientific, USA, #K1621). Specific primers of *omcAB* designed by (Ghasemi et al. 2020) were employed in this study.

Relative expressions were duplicate determined by Real-Time RT-PCR, using a Rotor-Gene® 6000 (Corbett, now available as the Rotor-Gene Q, Qiagen, Germany). Each real-time PCR mixture contained 10 mM cDNA plus RealQ Plus 2× Master Mix Green Without ROX™ (Ampliqon, Denmark) to the final volume of 25 µL. In following of initial denaturations at 95 °C for 15 s, the reactions were cycled as follows: 45 cycles of denaturation at 95 °C for 17 s, annealing at 56 °C for 20 s, and primer extension at 72 °C for 20 s. The melting curves for the amplicons were measured at 95°C for 5 s at the time of completion the run. 16s rRNA was served as the internal control (Yarzabal et al. 2003; Yarzabal et al. 2004) and the reference control -uranium free (AUF) sample- was selected according to the purpose for each experiment.

The fold change in the target genes was determined by the formula $2^{-\Delta\Delta Ct}$. In the present study, the LinRegPCR and REST 2009 were employed to calculation of the gene expressions.

2.4.3. Other Assessments

The bacterial counting was performed using CETI optical microscope and Marienfeld neobar lam. The rates of the cell count changes (growth rate) and uranium removal were calculated with equation 1 and 2 respectively in time intervals. The amount of soluble uranium removal is calculated with equations 3.

Equation 1 (Gr: Growth rate (cell/ml h)):

$$Gr = \frac{\text{Cell count}_f - \text{Cell count}_0}{T_f - T_0}$$

Equation 2 (Rr: Uranium removal rate ($\mu\text{mol/h}$)):

$$Rr = \frac{m_{U_f} - m_{U_0}}{T_f - T_0}$$

Equation 3 (R: Uranium removal (μmol)):

$$R = (C_{U_0} - C_{U_f}) \times \frac{V}{0.238}$$

Cell count₀, Cell count_f, mu₀, mu₁, T₀ and T_f indicate initial cell count, final cell count, initial amount (μm) of soluble uranium, final amount (μm) of soluble uranium, initial time and final time in each time interval, respectively. Cu₀, Cu₁ and V indicate the initial concentration (ppm) of uranium, the final concentration (ppm) of uranium and the solution volume (40 cc), respectively.

2.5. Statistical analysis

Results are reported as the mean of at least two replicates. Statistical data analyses are presented as one-way ANOVA followed by Duncan using SPSS software (version 19.0). Significant levels were defined as $P < 0.05$.

3. Results And Discussion

3.1. Comparison of bacterial growth curve in TSB and URC

According to the Fig. 1, the RCRI7 strain requires more incubation time to enter and exit the stationary phase in the URC solution in compare to the aerobic TSB medium. In TSB, the bacterial populations enter the death phase within 48 to 60 h, while in URC, it happens in 84 h (Fig. 1). These data have been confirmed by the study of Wang et al. (2010) about comparison between the growth process of *S. oneidensis* MR- 1 under aerobic and the fumarate reducing conditions (Wang et al. 2010).

It can be deduced that RCRI7 is capable of using uranium as an electron acceptor under anaerobic condition (URC) where uranium proper amount can be associated with the cell growth. The bacteria used

uranium for bacterial energy supply (see following sections) leading to the delayed bacterial death phase. However, due to the cost that uranium detoxification imposes to the growing cells in URC, the biomass yield from bacterial growth in the presence of uranium is generally lower than expected growth in normal condition (Sanford et al. 2007).

3.2. Bacterial growth curve responses in URC, AUC and AUF

Unlike URC samples, the bacterial population in AUC with the same amount of uranium, as well as AUF samples had generally declining trends as time goes on ($P < 0.05$) (Fig. 2). In AUF samples, lactate and hydrogen are present in the solution as electron donors, while due to the lack of electron acceptor, the bacterial population decreases over time. This result reveals how much the changes in the bacterial population in anaerobic conditions depend on the presence of uranium as electron acceptor in the environment (Fig. 2).

Decreased bacterial count in the AUC samples (Fig. 2), can indicate the toxic effect of uranium in the presence of oxygen, which would also be an evidence of uranium reduction leading to increase the population in the anaerobic condition. In approval, decreased expression of the genes (*omcAB*) involved in the reduction pathway (Fig. 3) (which leads to the decreased uranium (VI) reduction) in comparison to URC, would cause the toxic effect of unreduced uranium at the bacterial population in the aerobic condition (Fig. 2). Comparison of the bacterial population changes in aerobic and anaerobic conditions pointed out that although, uranium is capable of contributing to the bacterial growth in anaerobic condition (URC), but in the presence of oxygen (AUC), it is only a toxic substance that reduces the bacterial population (Fig. 2). Since in the presence of oxygen, the cells are not willing transfer electrons to the uranium, the non-reduced uranium (VI) has a high toxic effect on the bacteria (Wall and Krumholz 2006; Reguera 2012) that acts as a lethal substance resulting the bacterial population reduction. This process gradually reduces the bacterial count by increasing the incubation time, so the population decreases 3.3 times in compare to the initial count at the end of 91 h ($P < 0.05$) (Fig. 2). In these samples, the sizes of the bacteria also decreased as the incubation time increased (Table 1).

3.3. The expression of the *OmcAB* genes in URC, AUF and AUC

Despite the decrease in the bacterial population in AUF, the rate of *omcAB* gene expressions are higher than URC and AUC (Fig. 3). As the bacteria have been trying to respire in the absence of oxygen and other electron acceptors, the expression of the genes associated with the respiratory pathway have also been increased. Under this condition, it is speculated that the expression of the genes related to the other respiratory pathways have also been deduced. Berchinger et al. (2016) also found that the expression of *mtrA*, *omcB*, and *omcA* and the genes involved in the maturation of their products, including *ccmH*, *ccmF*,

and *ccmG*, increased in *S. oneidensis* MR-1 in the electron acceptor limitation condition (Barchinger et al. 2016).

Gene expressions in URC were lower than AUF and less in AUC. In AUC, in the presence of oxygen, the bacteria decreased their anaerobic gene expressions. In URC, although gene expressions are higher than AUC due to anaerobic condition, but it is lower than AUF. This indicates that the availability of the electron acceptor has reduced the bacterial demand to increase the expression of the genes needed for respiration in anaerobic solution. Various studies confirm the lower expression of *omcAB* genes under aerobic conditions rather than anaerobic conditions (Reyes et al. 2010; Jin et al. 2013; Kasai et al. 2015).

3.4. Morphological changes in URC, AUF and AUC

Morphological observations indicated that in the URC solution, unlike AUF and AUC samples, the bacteria had a normal size and motility under 84 h. They had just a slightly reduction in size and particular aggregations in the environment after 92 h incubation (Table 1).

In AUF solution, as the incubation time increases, the size of the bacteria gradually decreases, however the number of bacterial aggregations upsurges (Table 1). Decreasing the size of the bacteria is likely to be caused by a reductive division and dwarfing of the bacteria. This phenomenon is caused by improper conditions, because the reductive division increases the cell surface-to-volume ratio, and dwarfing is a type of self-digestion which caused by the degradation of intracellular material, especially the cytoplasm and external membrane (Astudillo and Acevedo 2008; Prabhakaran et al. 2016). Also, several researches have indicated that under stress conditions, the bacteria generally tend to gathering together to form biofilm (Lai et al. 2009; Prabhakaran et al. 2016; Manobala et al. 2019). Biofilm increases the bacterial resistance to the stress condition by 1000 times as compared to the planktonic state (Prabhakaran et al. 2016).

Microscopic observation of uranium-containing aerobic samples (AUC) indicated that (Table 1), the bacteria stayed inactive and aggregated with scattered particles after 12 h of inoculation, implying the bacterial cell damage (Table 1). Besides that, the bacterial population in the aerobic environment gradually decreased together with decreasing the mobility of the bacteria and their size (some were seen as cocci in the solution) (Table 1). Also, as stated above reductive division and dwarfing maybe carried out as a defensive reaction against heavy metals because of the improper condition, which the cells are trying to increase their surface-to-volume ratio for food intake and better respiration (Astudillo and Acevedo 2008; Prabhakaran et al. 2016). Also, some studies have shown that at high concentrations of bicarbonate, the bacteria became coccus form to reducing the toxic effects of uranium.

In addition in AUC samples, the supernatant was red and clear in the samples incubated above 12 h after centrifugation (Table 1). The *Shewanella* bacteria are generally red in color due to the presence of many heme containing cytochromes (Fu et al. 2018). So, it can be deduced that in the supernatant, there are some substances, lighter than bacteria which could not be deposited after centrifugation. The red color of

the supernatant indicates that the cytochromes may be dispersed in the solution, independently or together with a part of the cell membrane. Various studies show that the bacteria can produce membrane vesicles (MVs) and disperse them into the environment. MVs originating from Gram-negative bacteria are spherical structures compositionally similar to the outer membrane from which they are formed (Shao et al. 2014). During the formation of vesicles, the part of the outer membrane surrounds the part of the periplasmic contents and separates it from the main cell, without losing outer membrane integrity (Kulp and Kuehn 2010; Schwechheimer et al. 2013; Shao et al. 2014).

Bacteria apply MVs to facilitate a multitude of biological functions, including the secretion of soluble and insoluble molecules like enzymes, nutrient acquisition, biofilm formation, survival under environmental stress, and pathogenesis (Kulp and Kuehn 2010; Shao et al. 2014; Bitto and Kaparakis-Liaskos 2017). The formation of membrane vesicles by *Shewanella* species in the presence of uranium has been reported in various studies (Gorby et al. 2008; Shao et al. 2014). A study by Shao et al. (2014) suggested that in *Shewanella oneidensis* MR-1, vesicles are produced to allow the removal of surface-accumulated uranium, due to the maintaining of the cells viability and avoiding uranium incrustation (Shao et al. 2014). They also stated that the increased expression of the genes encoding spheroplast protein precursor and outer membrane proteins, was due to the supply of the membrane needed for MVs formation (Shao et al. 2014). In the study of Gorby et al. (2008), it was also stated that MVs produced by *Shewanella putrefaciens* CN32 in the Fe (III) reducing condition, act as shuttles that reduce Fe (III), U (VI), and Tc (VII) by the electrons retained in their unoxidized cytochromes. The events occur under anaerobic conditions which using H₂ as the electron donor. They also found that MVs were produced in uranium containing aerobic solution, in which c type cytochromes were one of the most abundant membrane proteins (Gorby et al. 2008).

Therefore, the red color of supernatant can be explained by the presence of cytochromes in the membrane of vesicles. Less expression of *omcAB* means that bacteria didn't apply anaerobic electron transfer chain (Fig. 3) but other c type cytochromes probably were produced in large amounts. Under aerobic condition, electrons do not enter the anaerobic electron transfer chain (Han et al. 2016), so these vesicles are not able to reduce uranium. Furthermore, observing the absence of uranium removal in the aerobic solutions with more than 12 h incubation (refer to the subject #3.6), can possibly confirm the avoiding of uranium incrustation. So, this is a defense strategy to limit uranium toxicity. Biological identifying of these vesicles produced by RCRI7 bacteria requires further analysis in the future studies.

3.5. Time dependent uranium (VI) removal in URC

Examination of uranium removal in the present study indicates that RCRI7 strain is able to remove uranium from the solution at the same time as its anaerobic growth (Fig. 1, 2 & 4). This removal is either due to adsorption or reduction pathways. Differentiation of the adsorption and reduction processes by present instruments is impossible and requires more accuracy in the bacterial growth and uranium

removal processes. Hence, based on the results (Fig. 1 & 2; 4 & 5) a schematic diagram was drawn (Fig. 6) which suggests the changes of adsorption and reduction during the experiment.

Based on the Fig. 4 and 5, the uranium removal rate is maximum (Fig. 5-B) while, the specific removal amount is minimum in 0 to 12 h time interval (Fig. 4). This means that uranium removal was carried out mainly at first 12 h whereas; the remnant is slightly removed later. Because the bacterial population did not increase significantly ($P > 0.05$) (Fig. 2), it can be deduced that the adsorption is the major factor in the uranium removal in this period of time (Fig. 6) (Xie et al. 2018), so it has not been used more to increase the bacterial population. Between 12 and 21 h, the removal rate decreased as compared to the previous times ($P < 0.05$) but was still positive and higher than later time intervals ($P < 0.05$) (Fig. 5-B). The rate of population growth during this period is maximum (Fig. 5-A), this means that the highest growth and consequently the highest uranium consumption and reduction has occurred during this period. So, it can be deduced that in this period of time, the major removal of uranium in the solution has been undertaken by the reduction process (Fig. 6), although the amount of removal was lower than the removal of initial period (Fig. 4). At 21 to 84 h, with a decrease in nutrients (including uranium required for cellular respiration), the population count is almost constant (Fig. 1 & 2) and the population growth rate gradually decreased (Fig. 5-A), while uranium removal increased slightly (Fig. 4) with no change in the removal rate ($P > 0.05$) (Fig. 5-B). Regarding that the uranium adsorption capacity by the bacteria at the specific pH and specific bicarbonate concentration is constant (Finneran et al. 2002; Xie et al. 2018), these issues indicate that, during this period, in addition to not increasing the amount of uranium adsorbed onto the bacterial surface, the reduction was occurring in a small amount in a constant rate. In addition, the amount of reduced uranium increased slightly to the maximum value at the end of 84 h (Fig. 6). This reduction process is performed on the unadsorbed uranium, which increases the removal of uranium in the solution (Fig. 4), and also on the adsorbed uranium on bacterial cell surface (Xie et al. 2018).

The increasing trend of uranium removal over time from anaerobic solution has been observed in many studies (Truex et al. 1997; Liu et al. 2002a; Liu et al. 2002b; Gu et al. 2005). Increasing of reduced uranium, in addition to the population balance, can be related to the amount of bacterial access to uranium. It is important to know that in URC, the bioavailability of uranium to the bacteria is one of the most important factors involved in the reduction process (Belli et al. 2015; Kulkarni et al. 2016; Xie et al. 2018). Unavailable uranium will be slightly accessible for bacteria over time, so they use that to save their lives and maintain their population balance during the stationary phase.

Between 84 and 92 h, as the bacterial count declines (Fig. 1 & 2), the uranium removal also decreases (Fig. 4). This likely indicates the dependence of uranium removal on the bacterial population. Decrease in bacterial population relative to stationary phase ($P < 0.05$) (Fig. 1 & 2), decrease in the uranium removal rate ($P < 0.05$) (Fig. 5-B) and decrease in removed uranium ($P < 0.05$) (Fig. 4) in this period indicates a decline in the amount of uranium adsorbed onto the bacterial surface as a result of bacterial destruction (Fig. 6). Adsorbed uranium on the cell surface includes unreduced (U (VI)) and monomeric reduced (U (IV)) uranium, which can be separated from the cell surface and redissolved by changing conditions

(Alessi et al. 2012; Newsome et al. 2015; Seder-Colomina et al. 2018). The reduced amorphous uranium called monomeric U (IV) differs from crystalline uraninite and can bind to the cell surface ligands such as phosphate and carboxyl. Adsorbed U (VI) and monomeric U (IV) can be separated from the cells by increasing the pH and the concentration of (bi) carbonate in the environment (Alessi et al. 2012; Newsome et al. 2015). After the separation of monomeric U (IV) from the cell surface, either it will remain as U (IV) in the bond with carbonate (Alessi et al. 2012) or will be reoxidized and dissolved as U (VI) to bind with carbonate (Seder-Colomina et al. 2018). It seems to be more likely to dissolve as uranium (VI) because reduced uranium tends to be oxidized in aqueous environments and at alkaline pH. Therefore, the releasing of uranium into the solution in 92 hours is because of the releasing of uranium (VI) and monomeric uranium (IV) attached to the cell wall either due to cell destruction in the death phase, or increased pH after lactate consumption. Uraninite is more stable and more difficult to be redissolved in the solution (Alessi et al. 2012). Time dependent reduction and adsorption changes are shown in Fig. 6 and Table 2.

3.6. Time dependent U (IV) production in URC

Anaerobic samples were examined after one-day, and two-week incubations at 1 mM uranium containing solutions by spectrophotometry analysis. Fig. 7-A indicates that in the cell free solution after two weeks incubation, the only observed peak was in the 400 to 700 nm which belongs to U (VI). The spectrum of the sample incubated in URC for one day shows the peak of U (VI) with some protrusions at 560 and 662 nm, which can indicate the presence of a small amount of reduced uranium in the solution. Also, the absorption around 600 nm can be due to the presence of bacteria in the environment (McBirney et al. 2016), as the residual peaks are not observed around 600 nm (Fig. 7-C) after two weeks of the incubation and bacterial destruction in the environment (Fig. 2). Fig. 7 indicates that a large amount of the uranium (VI) reduced to uranium (IV) after two weeks of incubation, as compared to the cell free solution.

According to Fig. 4, the highest uranium removal rate was in the first 24 hours of incubation. Therefore, it can be found that most of the removed uranium in the first 24 hours belongs to the uranium adsorbed on the cell wall, either as U (VI) or U (IV) monomeric, could be easily returned to the solution during the sample preparation for analysis. Evidence suggests that by increasing the incubation time, monomeric uranium (IV) can take on a crystalline structure and produce nanouraninite particles (Newsome et al. 2015). Some studies propose that uraninite is more resistant to re-oxidation (Alessi et al. 2012), but others suggest that the re-oxidation time of uraninite is not different from the monomeric one (Newsome et al. 2015). By comparing the peak of uranium (IV) in Fig. 7-B and 8-C, the resistance of uranium to oxidation has been increased, so it can be deduced that the produced uraninite is probably more resistant to re-oxidation.

According to Fig. 6, the maximum rate of reduction is between 12 and 21 hours, and the highest amount of reduced uranium is produced at the end of 84 hours in the environment. Therefore, in addition to increasing the resistance of uraninite particles that have formed over time, the increase in reduced

uranium in the stationary phase can be another reason for the rise of peak U (IV) and shortening of peak U (VI) in Fig. 7-C.

3.6. Time dependent uranium (VI) removal in AUC

Examination of the soluble uranium removal under aerobic condition (Fig. 8) indicates that only in the first 12 h, when more bacteria were present in the solution, uranium is removed. In followings, there is no uranium removal with increasing incubation time over 12 h, implying the recent removed uranium have also returned to the solution (Fig. 8). This is in contrast to what was observed in the URC samples.

In the early hours, when there were more live and active bacteria in the solution, uranium adsorbed on their cell surfaces. Then, as stated above by MVs production in the times above 12 h, the uranium removed from the cells and there was no uranium attached to the bacteria. Also by the addition of HNO₃ in the ICP sample preparation, the uranium possibly separates from the MVs or MVs destructed resulting in the uranium releasing into the solution.

As the Fig. 3 indicates after 21 hours of incubation, the expression level of *omcAB* genes in the presence of uranium in aerobic samples is lower than the anaerobic samples. This means that in aerobic condition, the bacteria can not apply reduction process in the uranium removal. So in addition to less soluble uranium removal, unreduced U (VI) toxic effect reduces the bacterial population (Fig. 2).

4. Conclusion

Shewanella RCRI7 is a facultative anaerobic bacterium possessed the ability to grow in the anaerobic solution containing 0.5 mM uranium (URC) with less population and longer stationary phase in compare to the TSB medium growth. However this strain can't be alive in AUF and AUC solutions. Also, the gene expressions in URC were lower than AUF and less in AUC. In URC, the maximum uranium removal and adsorption rates are in the first 12 h. But uranium reduction mostly carried out in 12-24 h and then continues at a slight rate up to the 84 h, with the highest amount of reduced uranium/removed uranium. In 92 h, the bacterial population decreased and environmental pH increased, so adsorbed U (VI) and monomeric U (IV) released into the solution. Bacterial aggregation, coccus shape formation and the membrane vesicles production are the main self-protection mechanisms that RCRI7 employed in this study in the presence of uranium.

Declarations

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References

- Abdehvand AZ, Keshtkar A, Fatemi F, Tarhriz V, Hejazi MS (2017) Removal of U (VI) from aqueous solutions using *Shewanella* sp. RCRI7, isolated from Qurugöl Lake in Iran. *Radiochimica Acta* 105:109-120
- Alessi DS et al. (2012) Quantitative separation of monomeric U (IV) from UO₂ in products of U (VI) reduction. *Environmental science & technology* 46:6150-6157
- Astudillo C, Acevedo F (2008) Adaptation of *Sulfolobus metallicus* to high pulp densities in the biooxidation of a flotation gold concentrate. *Hydrometallurgy* 92:11-15
- Barchinger SE et al. (2016) Regulation of gene expression in *Shewanella oneidensis* MR-1 during electron acceptor limitation and bacterial nanowire formation. *Appl. Environ. Microbiol.* 82:5428-5443
- Belli KM, DiChristina TJ, Van Cappellen P, Taillefert M (2015) Effects of aqueous uranyl speciation on the kinetics of microbial uranium reduction. *Geochimica et Cosmochimica Acta* 157:109-124
- Bitto NJ, Kaparakis-Liaskos M (2017) The therapeutic benefit of bacterial membrane vesicles. *International journal of molecular sciences* 18:1287
- Czerwinski KR, Polz MF (2008) Uranium enrichment using microorganisms. In. Google Patents
- Finneran KT, Housewright ME, Lovley DR (2002) Multiple influences of nitrate on uranium solubility during bioremediation of uranium-contaminated subsurface sediments. *Environmental microbiology* 4:510-516
- Francis A, Dodge C (2008) Bioreduction of uranium (VI) complexed with citric acid by *Clostridia* affects its structure and solubility. *Environmental science & technology* 42:8277-8282
- Fu H, Liu L, Dong Z, Guo S, Gao H (2018) Dissociation between iron and heme biosyntheses is largely accountable for respiration defects of *Shewanella oneidensis* fur mutants. *Appl. Environ. Microbiol.* 84:e00039-00018
- Gao W, Francis AJ (2008) Reduction of uranium (VI) to uranium (IV) by *Clostridia*. *Appl. Environ. Microbiol.* 74:4580-4584
- Ghasemi R, Fatemi F, Mir-Derikvand M, Zarei M (2020) Evaluation of mtr cluster expression in *Shewanella* RCRI7 during uranium removal. *Archives of Microbiology* 202:2711-2726 doi: 10.1007/s00203-020-01981-1
- Gorby Y, McLean J, Korenevsky A, Rosso K, EL-NAGGAR MY, Beveridge TJ (2008) Redox-reactive membrane vesicles produced by *Shewanella*. *Geobiology* 6:232-241

- Gu B, Yan H, Zhou P, Watson DB, Park M, Istok J (2005) Natural humics impact uranium bioreduction and oxidation. *Environmental science & technology* 39:5268-5275
- Han R et al. (2016) Effects of incubation conditions on Cr (VI) reduction by c-type cytochromes in intact *Shewanella oneidensis* MR-1 cells. *Frontiers in microbiology* 7:746
- Icenhower JP, Qafoku NP, Zachara JM, Martin WJ (2010) The biogeochemistry of technetium: a review of the behavior of an artificial element in the natural environment. *American Journal of Science* 310:721-752
- Jin M et al. (2013) Unique organizational and functional features of the cytochrome c maturation system in *Shewanella oneidensis*. *PLoS One* 8:e75610
- Kasai T, Kouzuma A, Nojiri H, Watanabe K (2015) Transcriptional mechanisms for differential expression of outer membrane cytochrome genes *omcA* and *mtrC* in *Shewanella oneidensis* MR-1. *BMC microbiology* 15:68
- Kouzuma A, Kasai T, Hirose A, Watanabe K (2015) Catabolic and regulatory systems in *Shewanella oneidensis* MR-1 involved in electricity generation in microbial fuel cells. *Frontiers in microbiology* 6:609
- Kulkarni S, Misra CS, Gupta A, Ballal A, Apte SK (2016) Interaction of uranium with bacterial cell surfaces: Inferences from phosphatase-mediated uranium precipitation. *Appl. Environ. Microbiol.* 82:4965-4974
- Kulp A, Kuehn MJ (2010) Biological functions and biogenesis of secreted bacterial outer membrane vesicles. *Annual review of microbiology* 64:163-184
- Lai S, Tremblay J, Déziel E (2009) Swarming motility: a multicellular behaviour conferring antimicrobial resistance. *Environmental microbiology* 11:126-136
- Liu C, Gorby YA, Zachara JM, Fredrickson JK, Brown CF (2002a) Reduction kinetics of Fe (III), Co (III), U (VI), Cr (VI), and Tc (VII) in cultures of dissimilatory metal-reducing bacteria. *Biotechnology and bioengineering* 80:637-649
- Liu C, Zachara JM, Fredrickson JK, Kennedy DW, Dohnalkova A (2002b) Modeling the inhibition of the bacterial reduction of U (VI) by β -MnO₂ (s). *Environmental science & technology* 36:1452-1459
- Lloyd JR, Renshaw JC (2005) Bioremediation of radioactive waste: radionuclide–microbe interactions in laboratory and field-scale studies. *Current opinion in biotechnology* 16:254-260
- Manobala T, Shukla SK, Rao TS, Kumar MD (2019) A new uranium bioremediation approach using radio-tolerant *Deinococcus radiodurans* biofilm. *Journal of biosciences* 44:122
- McBirney SE, Trinh K, Wong-Berlinger A, Armani AM (2016) Wavelength-normalized spectroscopic analysis of *Staphylococcus aureus* and *Pseudomonas aeruginosa* growth rates. *Biomedical optics express* 7:4034-4042

- Newsome L, Morris K, Lloyd JR (2014) The biogeochemistry and bioremediation of uranium and other priority radionuclides. *Chemical Geology* 363:164-184
- Newsome L, Morris K, Shaw S, Trivedi D, Lloyd JR (2015) The stability of microbially reduced U (IV); impact of residual electron donor and sediment ageing. *Chemical Geology* 409:125-135
- Pinchuk GE et al. (2010) Constraint-based model of *Shewanella oneidensis* MR-1 metabolism: a tool for data analysis and hypothesis generation. *PLoS computational biology* 6
- Prabhakaran P, Ashraf MA, Aqma WS (2016) Microbial stress response to heavy metals in the environment. *Rsc Advances* 6:109862-109877
- Reguera G (2012) Electron transfer at the cell–uranium interface in *Geobacter* spp. In: Portland Press Ltd.
- Reyes C, Murphy JN, Saltikov CW (2010) Mutational and gene expression analysis of *mtrDEF*, *omcA* and *mtrCAB* during arsenate and iron reduction in *Shewanella* sp. ANA-3. *Environmental microbiology* 12:1878-1888
- Saffarini D, Brockman K, Beliaev A, Bouhenni R, Shirodkar S (2015) *Shewanella Oneidensis* and Extracellular Electron Transfer to Metal Oxides. In: *Bacteria-Metal Interactions*. Springer, pp 21-40
- Sanford RA et al. (2007) Hexavalent uranium supports growth of *Anaeromyxobacter dehalogenans* and *Geobacter* spp. with lower than predicted biomass yields. *Environmental microbiology* 9:2885-2893
- Schwechheimer C, Sullivan CJ, Kuehn MJ (2013) Envelope control of outer membrane vesicle production in Gram-negative bacteria. *Biochemistry* 52:3031-3040
- Seder-Colomina M et al. (2018) Carbonate facilitated mobilization of uranium from lacustrine sediments under anoxic conditions. *Environmental science & technology* 52:9615-9624
- Shao PP, Comolli LR, Bernier-Latmani R (2014) Membrane vesicles as a novel strategy for shedding encrusted cell surfaces. *Minerals* 4:74-88
- Shi L, Squier TC, Zachara JM, Fredrickson JK (2007) Respiration of metal (hydr) oxides by *Shewanella* and *Geobacter*: a key role for multihaem c-type cytochromes. *Molecular microbiology* 65:12-20
- Tarhriz V, Mohammadzadeh F, Hejazi MS, Nematzadeh G, Rahimi E (2011) Isolation and characterization of some aquatic bacteria from Qurugol Lake in Azerbaijan under aerobic conditions. *Advances in Environmental Biology*:3173-3179
- Truex MJ, Peyton BM, Valentine NB, Gorby YA (1997) Kinetics of U (VI) reduction by a dissimilatory Fe (III)-reducing bacterium under non-growth conditions. *Biotechnology and bioengineering* 55:490-496
- Wall JD, Krumholz LR (2006) Uranium reduction. *Annu. Rev. Microbiol.* 60:149-166

Wang H, Hollywood K, Jarvis RM, Lloyd JR, Goodacre R (2010) Phenotypic characterization of *Shewanella oneidensis* MR-1 under aerobic and anaerobic growth conditions by using Fourier transform infrared spectroscopy and high-performance liquid chromatography analyses. *Appl. Environ. Microbiol.* 76:6266-6276

Xie J, Wang J, Lin J, Zhou X (2018) The dynamic role of pH in microbial reduction of uranium (VI) in the presence of bicarbonate. *Environmental pollution* 242:659-666

Yarzabal A, Appia-Ayme C, Ratouchniak J, Bonnefoy V (2004) Regulation of the expression of the *Acidithiobacillus ferrooxidans* rus operon encoding two cytochromes c, a cytochrome oxidase and rusticyanin. *Microbiology* 150:2113-2123

Yarzabal A, Duquesne K, Bonnefoy V (2003) Rusticyanin gene expression of *Acidithiobacillus ferrooxidans* ATCC 33020 in sulfur-and in ferrous iron media. *Hydrometallurgy* 71:107-114

Tables

Table 1: Morphology of *Shewanella* RCRI7 after incubation in URC (0.5 mM U), AUC (0.5 mM U) (B) and AUF (C) (Image magnification = 100).

Sample	Time of incubation	Observation	Reason
URC	12-84 h	Normal in size and motility	Normal growth in the presence of uranium in the reducing condition
	84-92 h	Slightly reduced in size and a had few aggregations	Lack of nutrient in environment and bacterial death phase
AUF	12-92 h	Destructed bacteria, very small sizes, low motility and a lot of aggregations	Reductive division, dwarfing and biofilm formation have been applied as the defense strategies due to lack of electron acceptor
AUC	12-92 h	Destructed bacteria, small sizes, low motility and a lot of aggregations	Reductive division, dwarfing and biofilm formation have been applied as the defense strategies due to uranium toxicity effect in the presence of O ₂
		Red supernatant after centrifuging	Production of MVs, due to the avoiding of uranium incrustation

Table 2: Suggested uranium adsorption and reduction changes during 92 h incubation in URC containing 0.5 mM uranium.

Time interval	U (VI) removal rate	U (VI) adsorption rate	U (VI) reduction rate	Removed U (VI)	Adsorbed U (VI) / Removed U (VI)	Reduced U (VI) / Removed U (VI)
0-12 H	Max	Max	Very low	High	Max	Min
12-21 H	High	Highly decrease	Max	Increase	Decrease	Increase
21-36 H	Low	×	Decrease	Increase	Decrease	Increase
36-48 H	Low	×	Low	Increase	Decrease	Increase
48-60 H	Low	×	Low	Increase	Decrease	Increase
60-72 H	Low	×	Low	Increase	Decrease	Increase
72-84 H	Low	×	Low	Max	Decrease	Max
84-92 H	Negative	Negative	Negative	Decrease	Min	Decrease

Figures

Figure 1

Comparison of the bacterial growth curve in TSB aerobic medium with URC (0.5 Mm). Significant differences between the samples in 6, 12, 24, 36, 48, 60, 66, 84 and 90 h in TSB medium with other samples in this treatment are shown with a, b, c, d, e, f, g, h and i, respectively and significant differences between the samples 0, 12, 21, 36, 48, 60, 72, 84 and 92 h in URC with other samples in this treatment are shown with j, k, l, m, n, o, p, q, and r, respectively ($P < 0.05$).

Figure 2

Variations in *Shewanella* RCRI7 population during incubation at 30° C in URC (0.5 Mm), AUC (0.5 mM) and AUF solutions. Significant differences between the samples 0, 12, 21, 36, 48, 60, 72, 84 and 92 h in URC with other samples in this treatment are shown with a, b, c, d, e, f, g, h and i, respectively, significant differences between the samples 0, 12, 24, 36, 48, 60, 72, 84 and 91 h in AUC with other samples in this treatment are shown with j, k, l, m, n, o, P, q, and r, respectively, and significant differences between 0 h in AUF with other samples in this treatment is shown with s ($P < 0.05$). Other samples have not significant differences ($P > 0.05$).

Figure 3

Comparison of *omcA* and *omcB* expressions in AUF, AUC and URC after 21 h of incubation at 30 ° C. Significant differences between the samples AUF, AUC and URC with the other samples for the same gene are shown with a, b and c, respectively ($P < 0.05$).

Figure 4

The amount of removed uranium in URC (0.5 Mm) incubated at 30° C. Significant differences between the samples 12, 24, 36, 48, 60, 72, 84 and 92 h with other samples, are shown with a, b, c, d, e, f, g and h, respectively ($P < 0.05$).

Figure 5

The rates of bacterial growth and uranium removal in URC (0.5 Mm) during 12 to 92 h of incubation at 30° C. A: The changes of bacterial growth rate in specified time intervals B: The changes of uranium removal rate in specified time intervals. Significant differences between the samples 12, 21, 36, 48, 60, 72, 84 and 92 h with other samples are shown with a, b, c, d, e, f, g and h, respectively ($P < 0.05$).

Figure 6

Suggested schematic diagram of the synergy in the reduction and adsorption processes in uranium removal during the 92 h incubation period. This diagram is based on the interpretation of the results of changes in bacterial population, uranium removal, bacterial growth rate and uranium removal rate at specified intervals. In this diagram, the maximum possible value for each examined characteristic is 100 and the minimum is zero.

Figure 7

Uranium (IV) production after one day and two week incubations at 1 mM uranium containing solutions at 30 °C. A: Spectrum of the cell free sample after two weeks of incubation in URC B: Spectrum of the cell containing sample after one day of incubation in URC C: Spectrum of the cell containing sample after two weeks of incubation in URC.

Figure 8

The amount of removed uranium in AUC (0.5 Mm) incubated at 30° C. Significant differences between the samples 12, 24, 36, 48, 60, 72, 84 and 91 h with other samples are shown with a, b, c, d, e, f, g and h, respectively ($P < 0.05$).