U (VI) tolerance affects *Shewanella sp*. RCR17 biological responses: growth, morphology and bioreduction ability

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Abstract
Native *Shewanella sp*. RCR17 is recently counted as an operative bacterium in the uranium bio-reduction. The aim of this study was to investigate the effects of uranium tolerance on the morphology and population of RCR17, following its potential removal capacity in different time intervals. In this research, 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) solution, following evaluations of *omcAB* gene expressions. In addition, spectrophotometry analyses were performed in URC confirming the bio-reduction mechanism. It was found that the bacteria can grow efficiently in the presence of 0.5 mM uranium anaerobically, unlike AUC and AUF solutions. Since the bacterium’s adsorption capacity is quickly saturated, it can be deduced that uranium reduction should be dominant as incubation times proceed up to 84 h in URC. In 92 h incubation, the adsorbed uranium containing unreduced and reduced (U (IV) monomeric), was released to the solution due to either increased pH or bacterial death. In AUC and AUF, improper conditions lead to the reduced bacterial size (coccus-shape formation) and increased bacterial aggregations; however, membrane vesicles produced by the bacteria avoid the uranium incrustation in AUC. In overall, this study implies that *Shewanella sp*. RCR17 are well tolerated by uranium under anaerobic conditions and the amount of regenerated uranium increases over time in the reduced form.

Keywords Anaerobic respiration · Bioremediation · Growth curve · Toxicity

Introduction
Bacteria have the ability to carry out reduction, oxidation, adsorption on the 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). Members of the genus *Shewanella* are facultative anaerobic, Gram stain-negative, rod-shaped bacteria isolated from a variety of environments including soil, fresh water and marine sediments (Pinchuk et al. 2010; Wang et al. 2010; Saffarini et al. 2015). They are well known for their ability to use a wide range of electron donors and acceptors, as metal reduction is their most important feature (Saffarini et al. 2015). OmcA and OmcB (MtrC), deca-heme c-type cytochromes in the *Shewanella* cell surface, are known as 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). In uranium reduction process, the bacteria transfer electron from electron donor (such as acetate, lactate, etc.) to uranium (VI) converting to uranium (IV), which is less toxic and mobile (Saffarini et al. 2015; Ghasemi et al. 2020).

*Shewanella* strain RCR17 was first isolated from a Quagol Lake, (located near Tabriz, Iran) water sample. As reported in Tarhiz et al., (2011), strain RCR17 was phylogenetically most closely related to *Shewanella putrefaciens* strain Hac411T (X81623), *Shewanella xiamenensis* S4T (FJ589031), and *Shewanella oneidensis* SCH0402T.
(AE014299), with a 16S rRNA sequence similarity of ≥ 99.1% (Tarhriz et al. 2011).

RCR17 is a new known Shewanella strain with little accessible information. Recently, in the studies by Ghasemi et al. (2020) and Abdehvand et al. (2017), the optimum cell growth and uranium removal by Shewanella RCR17 was achieved at 0.5 mM uranium concentration (Abdehvand et al. 2017; Ghasemi et al. 2020). In followings, this research intends to consider the growth of the strain RCR17 and kinetics of the soluble uranium removal, evaluated in the aerobic and anaerobic media. The purpose of this study was to examine the effect of soluble uranium on the cell morphology, growth and the kinetics of the soluble uranium reduction through strain RCR17. This study can re-confirm the suitable and efficient operations of RCR17 strain in the uranium bio-reduction process that provides further studies on the biological and chemical interactions between bacteria and uranium.

**Materials and methods**

**Preparation of reduction solution**

The reduction solution contained all the materials and elements to support the growth of the strain RCR17 under anaerobic conditions. The reducing medium contained sodium lactate (10 mM) along with the following salts (g L⁻¹): NH₄Cl (0.25), KCl (0.5), CaCl₂·2H₂O (0.15), NaCl (1.0), MgCl₂·6H₂O (0.62), l-aminobenzoic acid (0.05), thiamine–HCl (0.02), pyridoxine–HCl (0.1), cyanocobalamin (0.001) and trace minerals including (mg L⁻¹): MnCl₂·4H₂O (0.1), CoCl₂·6H₂O (0.12), ZnCl₂ (0.07), H₃BO₃ (0.06), NiCl₂·6H₂O (0.025), CuCl₂·2H₂O (0.015), Na₂MoO₄·2H₂O (0.025), FeCl₂·4H₂O (1.5). 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 aliquoted into 40 cc portions in vials with a determined vials to make them anaerobic (Abdehvand et al. 2017; Ghasemi et al. 2020).

**Anaerobic atmosphere**

Anaerobic conditions were established in the modified glove bag with the dimensions of 30 x 30 x 45 cm based on the Vinyl Glove Box model, Coy Laboratory Products Factory, USA by gassing with a mixture of 90% N₂ and 10% CO₂ (Haas and Northup 2004; Mullen 2007; Czerwinski and Polz 2008; Abdehvand et al. 2017). Then, the vials lids were crimped and H₂ gas injected into the solution by a continuous hydrogen generator model WM.H2.180 to remove the remained oxygen (Abdehvand et al. 2017). Injected hydrogen can also be used as an electron donor for bacteria along with sodium lactate (Lovley et al. 1991; Bencheikh-Latmani et al. 2005; Mullen 2007; Jiang et al. 2018). The vials were inoculated 24 h after the establishment of anaerobic conditions.

**Bacterial cultivation**

The stock bacteria (at –80 °C) were freshly inoculated into tryptic soy broth (TSB) medium (Merck.Co) incubating at 30 °C and 150 rpm, for 24 h. After the pre-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) (Ghasemi et al. 2020). 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 (as optimum final pellet count after centrifuging) (Abdehvand et al. 2017).

**Analysis of uranium effects**

The effect of uranium (0.5 mM) on the cell growth and morphology, gene expression and the reduction process in soluble uranium were evaluated under aerobic uranium-containing (AUC), uranium-reducing conditions (URC) and anaerobic uranium-free (AUF) conditions.

Over the course of an incubation time of 92 h, samples were periodically taken for analysis: (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). Additionally, the bacterial populations 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 bacterial motility and size changes of the bacteria through microscopical observations.

**Uraninite production confirmation**

Spectrophotometry was applied to investigate the presentation 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 10,000 rpm for 10 min. The precipitate was dissolved in 10 ml of 10 mM citric acid and passed through filters (0.2 μm pore size) (Francis and Dodge 2008; Abdehvand et al. 2017). 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) represents the peak in the
wavelengths of 560 and 662 nm while, uranium (VI) has a peak in the 438 nm (Gao and Francis 2008; Abdehvand et al. 2017).

Expression of OmcAB genes

The expression of the OmcAB genes under AUC, URC and AUF conditions was examined using Real-Time RT-PCR based on the comparative Ct experiment. 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 digested by DNase I (Thermo Scientific, USA). Isolated RNA was quantified at OD260 and the ratio of RNA to protein of the samples was evaluated based on OD260/ OD280 to investigate the purity of RNA by NanoDrop™ 2000 spectrophotometer (Thermo Scientific, France). 500 ng RNA was prepared in each reaction for cDNA synthesis using Revert-Aid first-strand cDNA synthesis kit (Thermo Scientific, USA, #K1621).

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. Specific primers of 16 s rRNA and omcAB (designed by primer3plus online software, used in our previous study (Ghasemi et al. 2020)) are shown in Table 1. In following of initial denaturation 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 of the run. 16 s rRNA served as the internal control (Yarzábal 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 calculate the gene expressions.

Other assessments

The bacterial counting was performed using CETI optical microscope and Marienfeld Neubauer lam (Abdehvand et al. 2017; Ghasemi et al. 2020). The rates of cell growth and the uranium removal, were calculated with Eqs. 1 and 2, respectively, in different time intervals. The amount of soluble uranium removal is calculated with Eq. 3.

\[
\begin{align*}
Gr &= \text{Growthrate} \left( \frac{\text{cell}}{\text{ml h}} \right) \quad : \quad Gr = \frac{\text{Cellcount}_f - \text{Cellcount}_0}{T_f - T_0}, \\
Rr &= \text{Uraniumremovalrate} \left( \frac{\text{μmol}}{\text{h}} \right) \quad : \quad Rr = \frac{m_{U_f} - m_{U_0}}{T_f - T_0}, \\
R &= \text{Uraniumremoval(μmol)} \quad : \quad R = \left( C_{U_0} - C_{U_f} \right) \times \frac{V}{0.238}.
\end{align*}
\]

Cell count$_0$, Cell count$_f$, m$_{U_0}$, m$_{U_f}$, T$_0$ 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. C$_{U_0}$, C$_{U_f}$ and V indicate the initial concentration (ppm) of uranium, the final concentration (ppm) of uranium and the solution volume (40 cc), respectively.

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 $P < 0.05$.

Results and discussion

Comparison of bacterial growth curve in TSB and URC

As reported in Fig. 1, strain RCR17 required more time to reach the stationary phase under URC conditions when compared to the aerobic TSB. In TSB medium, the strain

| Gene   | Primer sequence | Tm (˚C) | GC%  | Product size |
|--------|-----------------|---------|------|--------------|
| 16s rRNA* | F: 5′-CCTAC CGGAGGCACGAG-3′ | 59.6 | 70.59 | 165 bp |
|        | R: 5′-CGTGC TTCTTCTGTTGATTACAG-3′ | 64.7 | 52.17 | |
| mtrC   | F: 5′-AACGCGCATGTTGATGTGATG-3′ | 60.15 | 45.5 | 119 bp |
|        | R: 5′-TTAATGCCTGACCCCTGTTTACGGATTACAG-3′ | 59.06 | 45.5 | |
| omcA   | F: 5′-TTTCTCTGTATGCAATGCAC-3′ | 57 | 42.9 | 121 bp |
|        | R: 5′-GCCTACATCCAGACCCACA-3′ | 59 | 47.6 | |
RCR17 reached the phase of decline in 48 h while the phase of decline was reached in 84 h in URC.

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 fumarate-reducing conditions (Wang et al. 2010).

It can be deduced that RCR17 is capable of consuming uranium as an electron acceptor under the anaerobic condition (URC), where uranium proper amount can be associated with the cell growth. The uranium was used 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 total cell yield from the bacterial growth in the presence of uranium is generally lower than the expected growth in the normal condition (Sanford et al. 2007).

### 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 were presented in the solution as the electron donors. While due to the lack of electron acceptor (uranium), the bacterial populations decreased over time. This result reveals how much the changes in the bacterial population in the anaerobic conditions depend on the presence of the uranium as electron acceptor in the environment (Fig. 2).

Decreased bacterial count in the AUC samples (Fig. 2), indicated the toxic effect of uranium in the presence of oxygen, which would also be an evidence of uranium reduction leading to the population increasing 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), would cause the toxic effect of the unreduced uranium at the bacterial populations in the aerobic condition as compare to URC (Fig. 2).

Comparison of the bacterial population changes in the aerobic and anaerobic conditions pointed out, although uranium is capable of contributing to bacterial growth in the anaerobic condition (URC), in the presence of oxygen (AUC) strain RCR17 is not able to reduce soluble uranium (VI) into a non-toxic form of uranium. This procedure results no cell growth and cell number reduction (Fig. 2). Since in the presence of oxygen, the cells are not willing to transfer the electrons to the uranium, the non-reduced uranium (VI) assumes a high toxic effect on the bacteria as a lethal substance (Wall and Krumholz 2006; Reguera 2012). This process gradually declines the bacterial count by increasing the incubation time, and 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 2).

**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 is 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 has also been increased. Under this condition, it is speculated that the expressions of the genes related to the other respiratory pathways have also been reduced. Berchner 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, the bacteria decreased the anaerobic gene expressions in the presence of oxygen. In URC, although gene expressions are higher than AUC due to anaerobic condition, but it is lower than AUF. This fact
implies that the availability of the electron acceptor has reduced the bacterial demand to increase the expression of the impressive genes in the anaerobic respiration. Various studies confirm the lower expression of \textit{omcAB} genes under aerobic rather than anaerobic conditions (Reyes et al. 2010; Jin et al. 2013; Kasai et al. 2015).

### Morphological alteration 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; however, there was

| 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 had a 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\textsubscript{2} |
|        |                    | Red supernatant after centrifuging | Production of MVs, due to the avoiding of uranium incrustation |
a slight reduction in the cell size and particular environmental aggregations after 92 h (Table 2).

In AUF solution, as the incubation time increases, the size of the bacteria gradually decreased; however, the number of the bacterial aggregations increased (Table 2). Decreasing the size of the bacteria is likely to be caused by a reductive division and dwarfing of the bacteria. This phenomenon occurs in conditions, since the reductive division increases the cell surface-to-volume ratio. Besides that, the dwarfing is a type of self-digestion, caused by the degradation of intracellular material, especially the cytoplasm and external membrane (Astudillo and Acevedo 2008; Prabhakaran et al. 2016). Furthermore, previously published results have reported that under stress conditions, bacteria will clump together forming a biofilm (Lai et al. 2009; Prabhakaran et al. 2016; Manobala et al. 2019). Biofilm formation 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 2), the bacteria became inactive and aggregated with scattered particles after 12 h of inoculation, implying the bacterial cell damages (Table 2). The bacterial population in the aerobic environment gradually decreased together with decreasing the mobility of the bacteria and the size (some were seen as cocci in the solution) (Table 2). Moreover, as stated above, reductive division and dwarfing shape may be carried out as a defensive reaction against heavy metals, in response to the improper condition. This issue declares the fact that the cells are trying to increase their surface-to-volume ratio for nutrition intake and better respiration. As well, some studies have shown that at high concentrations of bicarbonate, the bacteria presented coccus form to reduce the toxic effects of uranium (Astudillo and Acevedo 2008; Prabhakaran et al. 2016).

In addition in AUC samples, a red and apparent supernatant was observed in the samples incubated above 12 h after centrifugation (Table 2). The Shewanella bacteria are generally red in color due to the presence of numerous heme-containing cytochromes (Fu et al. 2018). The red color of the supernatant indicates that the damaged cytochromes (in the cell membrane or cytoplasm) may be released into the solution. Various studies declared that the bacteria can produce membrane vesicles (MV) 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 the vesicles, the part of the outer membrane surrounds the part of the periplasmic contents and separates the vesicle from the main cell, without losing outer membrane integrity (Kulp and Kuehn 2010; Schwebelheimer 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 also 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 which reduce Fe (III), U (VI), and Tc (VII) by the electrons retained in their oxidized cytochromes. The events occur under anaerobic conditions through applying 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 the supernatant can be explained by the presence of numerous cytochromes in the membrane of vesicles. Less expression of omcAB indicates the lack of active anaerobic electron transfer chain (Fig. 3); however, other c-type cytochromes were probably produced in the large amounts. Under aerobic condition, electrons do not enter the anaerobic electron transfer chain (Han et al. 2016), so the membrane vesicles are not capable to reduce uranium. Furthermore, observing the absence of the 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 to limit the uranium toxicity which is a potent defense strategy. Identifying the biological characters of these vesicles produced by RCR17 bacteria requires more investigations in future studies.

**Time-dependent uranium (VI) removal in URC**

Uranium removal was defined as total eliminated uranium containing reduced and adsorbed uranium to the bacterial cell wall. Examination of uranium removal in the present study found that RCR17 strain is able to remove uranium from the solution during anaerobic growth (Figs. 1, 2 and 4). Therefore, 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 (Figs. 1, 2, 4 and 5), a schematic diagram is presented (Fig. 6) which suggests the changes of adsorption and reduction during the experiment.

Based on the Figs. 4 and 5, the uranium removal rate is maximum in 0 to 12 h (Fig. 5B) while, the specific removal amount is minimum (Fig. 4). This means that the uranium removal was carried out mainly at first 12 h, whereas the remnant uranium is slightly removed later. Since the bacterial population did not increase significantly (Figs. 1, 2) (Xie et al. 2018), it can be deduced that the adsorption is the major factor in the uranium removal (Fig. 6). The removal rate was decreased at 12 to 21 h, in comparison to the previous phases (P < 0.05) but has still been progressive and also higher than the later time intervals (P < 0.05) (Fig. 5B). The rate of the population growth during this time is maximum (Fig. 5A), notifying that the highest growth and consequently the peak uranium consumption (reduction) have been occurred during this period. Consequently, it can be deduced that the major removal of the uranium in the solution has been undertaken by the reduction process in this period of time (Fig. 6), although the represented amount of removal was lower than initial phase (Fig. 4). At 21 h to 84 h, along with a decrease in the nutrients (including uranium required for cellular respiration), the population count is almost constant (Fig. 5A), while the population growth rate was gradually decreased (Fig. 5A). In the meantime, the uranium removal was increased slightly (Fig. 4) with no change in the removal rate (P > 0.05) (Fig. 5B). Regarding that the uranium adsorption capacity by the bacteria at the specific pH and specific bicarbonate concentration is constant (Finnean et al. 2002; Xie et al. 2018), it can be deduced that, during this period, in addition to not increasing the amount of uranium adsorbed onto the bacterial surface, the reduction was also occurring in a small amount with a constant rate. Furthermore, the amount of reduced uranium was increased slightly to the maximum value at the end of 84 h (Fig. 6). This reduction process is performed on the unab sorbed uranium, which increases the removal of uranium in the solution (Fig. 4), as well as the adsorbed uranium on the 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, b; Gu et al. 2005). Despite the constancy of the bacterial count, increasing of reduced uranium, could be related to the amount of the bacterial access to the uranium. It should be noted 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 could be slightly accessible for the bacteria over time, so the bacteria utilize it to survive and maintain their population balance during the stationary phase.

Between 84 and 92 h, as the bacterial counts declined (Figs. 1 and 2), the uranium removal diminished (Fig. 4). This likely indicates the dependence of uranium removal on the bacterial population growth. In this period, decreasing in three indicators, such as bacterial population (relative to stationary phase (P < 0.05)) (Figs. 1 and 2), uranium removal rate (P < 0.05) (Fig. 5B) and removed uranium.
Fig. 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 ($P < 0.05$).

Fig. 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.
amount ($P < 0.05$) (Fig. 4), indicates a decline in the quantity of uranium adsorbed onto the bacterial surface as a result of the bacterial destruction (Fig. 6).

It should be implied that, the adsorbed uranium on the cell surface includes unreduced (U (VI)) and monomeric reduced (U (IV)) uranium, which could release from the cell surface and re-dissolved through changing conditions in the incubating solution (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 which 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 remaining as U (IV) in the bond with carbonate (Alessi et al. 2012) or will be re-oxidized 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 the aqueous environments at alkaline pH. Therefore, the releasing of the uranium into the solution in 92 h is due to 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. In fact, uraninite is more stable and more tough to be re-dissolved in the solution (Alessi et al. 2012). Time-dependent reduction and adsorption changes are shown in Fig. 6 and Table 3.

It is finally proposed that in URC, the primary mechanism for removal of soluble uranium was adsorption to the cell wall, which occurred during the first 12 h of incubation. The process was followed by maximum reduction of uranium during 12–24 h. In 92 h, the changing in the bacterial population and environmental pH leads to the releasing of the adsorbed U (VI) and monomeric U (IV) into the solution.

Table 3 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                      |

Fig. 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.
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. Figure 7A indicates that in the cell-free solution, the only observed peak was in the 438 nm which belongs to U (VI). The spectrum of the samples incubated in URC (Fig. 7B, C) showed the peak of U (IV) at 560 and 662 nm, which indicated the presence of reduced uranium in the solutions.

The light absorption spectrum seen around 600 nm (Fig. 7B), can be due to the presence of the bacteria in the environment, whereas the indicated peaks are not observed after two weeks of the incubation (Fig. 7C) due to the probable environmental bacterial destruction at this time (McBirney et al. 2016).

The highest uranium removal rate noticed in the first 24 h of incubation (Fig. 4), suggesting the fact that the most removed uranium in the first 24 h refers to the adsorbed uranium on the cell wall, either as U (VI) or U (IV) monomeric, which could be easily returned to the solution during the analysis sample preparation as confirmed by differences in the sharp peaks in Fig. 7B, C. Evidence proposed that increasing the incubation time (Fig. 7C), forms the monomeric uranium (IV) on a crystalline structure which leads to produce nano-uraninite particles (Newsome et al. 2015), that is probably more resistant to re-oxidation as confirmed by another study (Alessi et al. 2012).

The maximum rate of reduction is between 12 and 21 h with the highest amount of reduced uranium produced at the end of 84 h (Fig. 6). Therefore, in addition to increasing the resistance ofuraninite particles formed over time, the increase in the reduced uranium in the stationary phase can be another reason for the specified peaks of U (IV) and U (VI) (Fig. 7C).

Time-dependent uranium (VI) removal in AUC

Examination of the soluble uranium removal under aerobic condition (Fig. 8) indicated that the uranium is removed only in the first 12 h, once more bacteria were presented in the solution. In followings, there is no uranium removal with increasing the incubation time over 12 h, implying that the recent removed uranium has also returned to the solution (Fig. 8). This is in contrast to the observation in the URC samples.

Initially, in viable cells, the soluble uranium adsorbed to the cell surface and then, as noted above, the bound uranium was released by the formation of membrane vesicles (MV) after 12 h of cell growth. Also, by the addition of HNO₃ in the ICP sample preparation, the uranium possibly separated from the destructed MVs or MVs resulting in the uranium releasing into the solution.

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

Conclusion

Shewanella RCRI7 grows functionally in URC as compare to the TSB growth medium; however, this strain cannot survive in AUF and AUC solutions due to the bacterial survival compatibility. Bacterial aggregation, coccus-shape formation, and the membrane vesicle production are the main self-protection mechanisms, employed by Shewanella RCRI7 in the presence of uranium leading the bacteria to survive. As a final point, Shewanella RCRI7 proficiently tolerates the uranium in the anaerobic
conditions along with increasing uranium reduction through the time.

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