Proteus vulgaris - Pt Electrode System for Urea to Nitrogen Conversion in Synthetic Urine

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Abstract: One of the most challenging problems when trying to recycle urine for different purposes is the removal of urea. In this project we studied an ureolysis system using the bacterium *Proteus vulgaris* for the transformation of urea to ammonia and its subsequent oxidation to nitrogen at a Pt working electrode. Our system was tested under different pH, microbial reaction times, and urea and bacteria concentrations. Our results indicate that a pH 8 is optimal for the combined *Proteus vulgaris* urease activity and the ammonia oxidation reaction at a Pt electrode. The reaction time and concentration dependence on the ammonia oxidation reaction current densities was also studied. Results showed limited ammonia oxidation under high urea concentrations in ~ 2.5 x 10^9 cfu/mL *Proteus vulgaris* in synthetic urine.

Keywords: ammonia oxidation reaction; Proteus vulgaris; ureolysis; urine; urea

1. Introduction

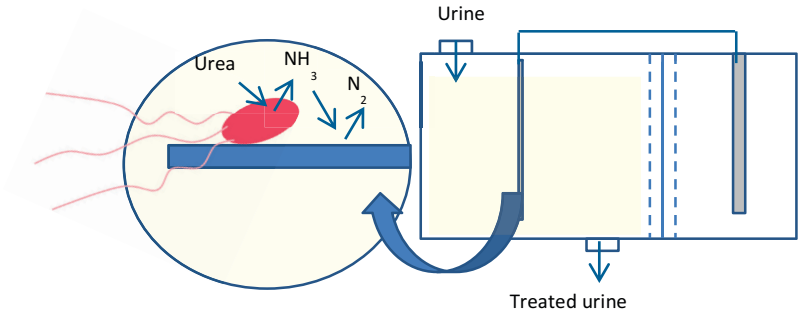
In 2015, the United Nations reported that more than 40% of the global population is affected by water scarcity and that this number will continue to increase. For this reason, water recovery from wastewater is an essential area to study that could be of help to the modern world [1]. One of the major sources of nutrients in wastewaters is urine [2], (80% nitrogen, 50% phosphorus and 9% potassium) [3]. Hence, human waste treatment could benefit tremendously by separating urine in new toilet systems that produce electricity and clean water. This would bring the added advantages of reducing the amount of water used during flushing cycles, allowing for high nutrient recycling, therefore reducing contamination in bodies of water [3, 4]. Apart from urine recycling to obtain water, the removal of urea is important in order to protect the environment. Urea is a key pollutant in agricultural waste due to its stimulation of algal growth, increasing the pH when it is converted to ammonia [5, 6]. Ammonia is hazardous and toxic and can have deadly

consequences when exposed to fauna and flora. It also affects humans when inhaled, since it creates irritation [7]. Another application is in NASA's manned space missions since urine recycling reduces the cost and satisfies the water demand of astronauts [8].

Urine is composed of approximately 95% water, some of the remaining parts include 2% urea. The rest of the components are inorganic salts and organic components that can be removed by reverse osmosis or by membranes. Meanwhile, urea is significantly more difficult to remove because of membrane fouling. Therefore, many systems have been proposed [9] and developed to decompose urea based on electrochemical [10], thermodynamic [11, 12], chemical [7] and biological methods [13-15]. Biological systems have advantages over other processes due to minimal use of energy and a lower operating cost [13, 16, 17]. These systems use microbial enzymes that are more active and stable than plant and animal enzyme systems [18, 19]. A microorganism that produces the enzyme of interest will result in a more self-sustainable system, i.e. compatible with microbial fuel cells (MFC). Prior work has been done for the recovery of nitrogen [20] and electricity generation from urine [21] using MFCs but no work has been done for water recovery and urine treatment. Our group has worked previously on developing a bioelectrochemical method using urease for wastewater treatment [22, 23]. Urease is an enzyme that catalyzes the ureolysis reaction and has been used to remove urea [23, 24]. The overall reaction can be expressed as:

$$NH_2(CO)NH_2 + H_2O \rightarrow 2NH_3 + CO_2 \quad (1)$$

The advantages of using bacteria instead of the enzyme are its robustness, low cost and long-term use [25-27]. Our research has been focused on the development of a robust microbial ureolysis system for recycling of urine for NASA's manned space missions. The use of *Proteus vulgaris* to create an ureolysis system for a urine recycling system is presented here. This bacteria has the ability to produce urease, an enzyme that catalyzes the conversion of urea to ammonia [28]. At the same time, ammonia can be oxidized using platinum electrodes, to eliminate the ammonia and produce an oxidation current that may be used for a self-sustained system. The final objective is to obtain a urea/ammonia-free recycled urine solution that can be later treated by reverse osmosis to eliminate the rest of the components of urine and obtain drinkable water (see Schematic 1). In our system, urine is being fed to the ureolysis device that will treat the liquid to eliminate urea. The red oval represents the bacteria *P. vulgaris* that contains the urease that converts urea to ammonia, while the blue rectangle represents the Pt working electrode where the ammonia oxidation reaction will take place.



Schematic 1. Illustration of the proposed microbial ureolysis system. The urine fed to the system is treated to eliminate the presence of urea. The red oval represents *P. vulgaris* that converts the urea to ammonia and the blue rectangle represents the Pt working electrode where the ammonia oxidation reaction will take place.

2. Experimental

2.1 Materials and equipment

A VMP3 Potentiostat/Galvanostat/EIS from BioLogic U.S.A was used for all the electrochemical analyses. A three-electrode system was used for all the electrochemical experiments. A polycrystalline platinum wire was used as the working electrode, the reference electrode used was RHE, and the counter electrode used was a platinum wire. The synthetic urine contained 8.001 g/L NaCl, 1.641 g/L KCl, 0.234 K_3PO_4, (Sigma Aldrich, USA) 2.632 g/L K_2SO_4, 0.783 g/L $MgSO_4$, 0.661 $KHCO_3$ (Thermo Fisher Scientific). The NH_4Cl and urea, used in the experiments, were purchased from ACROS Organics and Sigma Aldrich, respectively, and used as received.

2.2 Bacteria growth and transfer to synthetic urine

The bacterium used in the experiments was *P. vulgaris* ATCC® 8424™ (purchased from VWR) and maintained on MacConkey agar at 4°C. *P. vulgaris* was grown in 600 mL of Brain Hearth Infusion Broth at 37° C. Figure 1a shows a scanning electron microcopy image of a *P. vulgaris* sample and a picture of the solution used for the ammonia oxidation reaction *via* cyclic voltammetry experiments with a Pt electrode. The typical size of *P. vulgaris* is 3μm long and 1μm wide. All media and solutions were sterilized by using an autoclave. Cells were separated after growth by centrifuging at 3,900 rpm and maintaining a temperature of 4° C for ten minutes. The bacteria colony was then washed twice with 5 mL of sterile saline solution (0.85% NaCl). The saline solution was decanted to obtain a bacteria pellet. Bacteria were kept at 4°C before the experiments. For the preparation of the *P. vulgaris* in synthetic urine solution preparation, the bacteria were suspended in synthetic urine to obtain the same concentration per experiment. The standard plate count method was used to determine the concentration of bacteria suspended in synthetic urine prior to

every experiment. The bacteria concentration, in cfu/mL, for each electrochemical experiment is stated in each section.

2.3 Electrochemical experiments

Cyclic voltammetry was done in 0.5M H_2SO_4, using the hydrogen adsorption/desorption, to determine the Pt electrode active surface area for the proper normalization of the ammonia oxidation currents in each ureolysis experiment. The system was continuously purged with N_2 to eliminate oxygen in the cell in order to avoid oxygen adsorption or oxygen reduction at the catalytic surface. For the blank experiment, the ammonia oxidation reaction was done in 0.1M urea in synthetic urine at a scan rate of 10 mV/s with an initial potential of 0.4 V vs. RHE and a final potential of 1.0 V vs. RHE. Then, cyclic voltammetry was repeated after suspending the *P. vulgaris* in 0.1M urea in synthetic urine and incubated at room temperature for 10 minutes unless otherwise stated. A schematic representation of the electrochemical cell with *P. vulgaris* setup is shown in Figure 1c.

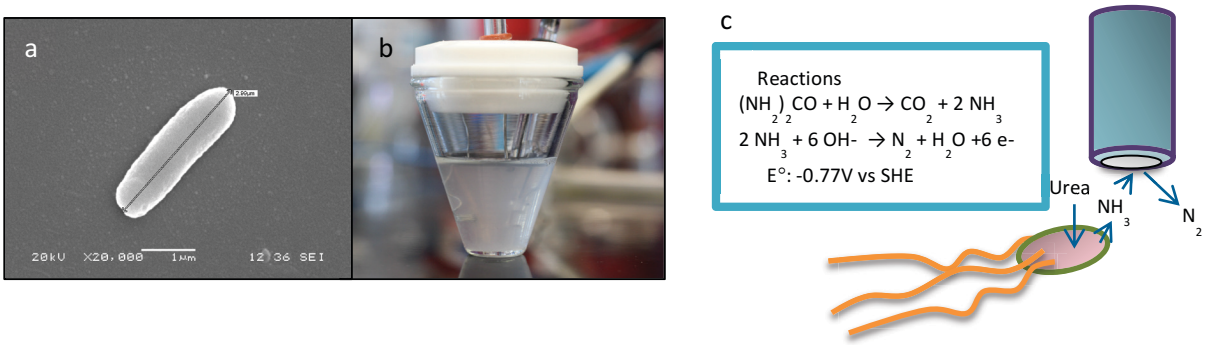


Figure 1. (a) Scanning electron microscopy image of a *P. vulgaris* at x20,000 magnification, (b) picture of the electrochemical three-electrode setup used for the experiments, containing the bacteria in synthetic urine and (c) a schematic of the bioelectrochemical process occurring at the Pt working electrode. In the image, blue cylinder represents the Pt working electrode and the oval shape represents *P. vulgaris*.

The bacterially formed ammonia from the microbial solution can be oxidized at a Pt electrode according to the following reaction:

$$2NH_3 + 6OH^- \rightarrow N_2 + 6H_2O + 6e^- \quad \text{at a standard potential of -0.77 V vs. SHE} \quad (2)$$

The ammonia oxidation process can thus remove ammonia and effectively remediate water and produce energy in a fuel cell format.

4. Results and Discussion

4.1 Ureolysis system pH response in synthetic urine

Proteus vulgaris was first transferred to synthetic urine in order to find the optimal conditions for the conversion of urea and the ammonia oxidation reaction to occur, since both processes require different optimal pH values. A control cyclic voltammetry experiment was carried out using a solution containing both synthetic urine and bacteria. These experiments gave us information regarding how the biofilm affects the double layer capacitance of the electrochemical system. After this, we added 0.1 M urea in synthetic urine to *P. vulgaris*, and waited for 10 minutes for the urea to be converted into ammonia by the urease enzyme present in *P. vulgaris*. The solution was then purged with N_2 for 15 minutes and cyclic voltammetry was performed to detect the ammonia available in solution. This was done by measuring the ammonia oxidation reaction peak current density by cyclic voltammetry, as observed in Figure 2b. This shows that the conversion of urea to ammonia in synthetic urine only occurs in the presence of *P. vulgaris*.

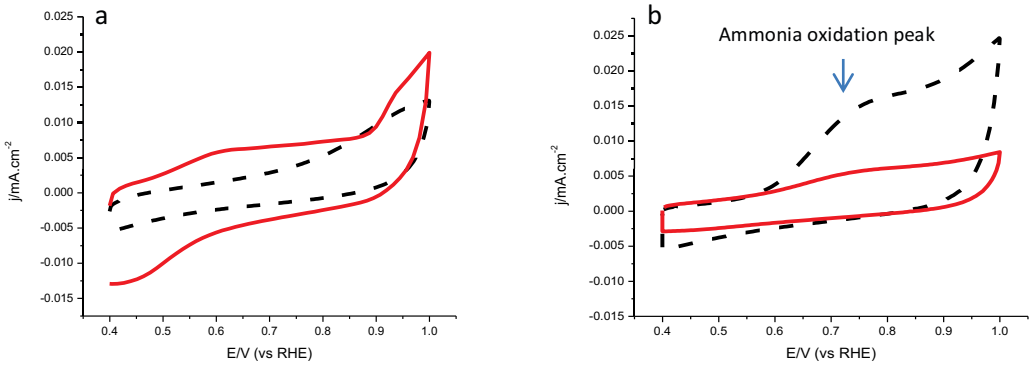


Figure 2. Cyclic voltammograms of a polycrystalline Pt working electrode in synthetic urine using a potential scan rate of 10 mV/s between 0.4 and 1.0 vs. RHE, (a) without (—) and with 0.1 M urea (---) at pH 8.00. The cyclic voltammetry was done after 10 minutes of incubation at room temperature. These cyclic voltammograms served as blank measurements, since no bacteria were detected in the medium. (b) Cyclic voltammograms in synthetic urine at pH 8.00 with 3.5 x 10^9 cfu/mL of *P. vulgaris* and without (—) and with (---) 0.1 M urea. The ammonia oxidation peak current densities, from the urea, can be observed at the blue arrow.

Synthetic urine, with different pH levels, was used to optimize the ureolysis system. Since the optimum pH for urease activity is 7.4 and the pKa of ammonia is 9.25, we aimed at finding an optimal pH value for the ureolysis system where neither of the two reactions was compromised. Since the oxidation current is related to the concentration of urea converted to ammonia, we could compare the available ammonia by cyclic voltammetry of the ammonia oxidation reaction as a function of the solution alkalinity. Since pH was the only parameter changed, we were able to observe that at pH 9 the ammonia oxidation reaction peak current density was optimum, 1.8 mA/cm^2 (see Figure 3). Although pH 7.5 is closer to the optimum pH for the urease enzyme activity, and since at that pH most of the ammonia is in the form of the ammonium cation, the ammonia oxidation reaction peak current density is not optimal, 1.1 mA/cm^2. On the other hand, at pH 10, the oxidation current is also low, 0.9 mA/cm^2. This may be because at this pH the urease activity may be too low to convert urea to ammonia efficiently. Hence, since our focus is to convert urea to ammonia, we used pH 8 for the remainder of the experiments presented here. This pH gave an ammonia oxidation reaction peak current density of 1.1 mA/cm^2.

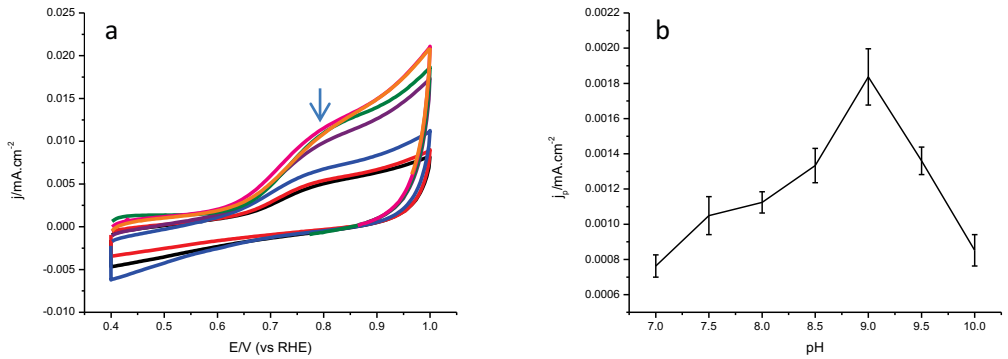


Figure 3. (a) Cyclic voltammograms of a Pt electrode in ~1.9 x 10^9 cfu/mL *Proteus vulgaris* in 10 mL of synthetic urine with 0.1M urea, at different pH: 7 (black), 7.5 (red), 8 (blue), 8.5 (purple), 9 (green), 9.5 (pink) and 10 (orange). The measurement was done after 10 minutes of the addition of the 10 mL to the bacteria pellet. A vortex was used to dissolve the pellet. The cyclic voltammetry scan rate was 10 mV/s. (b) Ammonia oxidation reaction peak current density as a function of pH in synthetic urine solutions. The ammonia oxidation peak current densities, at different pHs, can be observed at the blue arrow.

4.2 Concentration effect of ureolysis system variables

Once the pH of the ureolysis system was optimized, additional parameters were studied. To obtain the results as similar to the desired practical system as possible, the effect of the urea concentration in the ureolysis system was performed by suspending the bacteria pellet in the synthetic urine solution with urea. The bacteria were allowed to act for ten minutes before purging with nitrogen for 15 minutes and running the cyclic voltammetry for the

ammonia oxidation reaction. Figure 4 shows the urea concentration effect on the cyclic voltammograms of ammonia oxidation in presence of ~2.5 x 10^9 cfu/mL *P. vulgaris.* The cyclic voltammograms of polycrystalline Pt electrodes in *Proteus vulgaris,* in synthetic urine at pH 8.00, were recorded at urea concentrations of 0.01, 0.05, 0.1, 0.22 and 0.5 M. The ammonia oxidation peak current densities were measured from cyclic voltammograms done at a potential scan rate of 10 mV/s. With a bacteria concentration of ~2.5 x 10^9 cfu/mL, most of the urea was not converted to ammonia at high concentrations of urea. At these high concentrations lower ammonia oxidation reaction peak current densities were observed, between 0.5 to 0.3 mA/cm^2 for 0.2M and 0.5M in urea, respectively. For comparison, at lower urea concentration the current density reached 3.0 mA/cm^2, at 0.1M in urea. This may be occurring since at high urea concentrations, the urea may adsorb on the electrocatalytic active platinum surfaces, resulting in low ammonia oxidation reaction currents. The absorption of urea has been widely studied on Pt electrodes by Climent et al. [29, 30] and others [31-33]. These studies have been done mainly in 0.5 M H_2SO_4 and 0.1 M $HClO_4$ and urea surface coverage of 0.45 has been found on Pt (111) surfaces [34]. The mechanism proposed is an ion pairing interaction between co-adsorbed urea molecules and HSO_4^- ions on the Pt surface. In our case, we are under alkaline conditions and the adsorption mechanism may be different. Nevertheless, urine has a high concentration of anions such as sulfates and phosphates, that if used with real urine samples, may be an additional interference. As observed, at higher urea concentrations, the ammonia oxidation peak current densities were lower with small changes in magnitude. This tendency is observed at urea concentrations higher than 0.1 M, at which the maximum current density was observed, 3.0 mA/cm^2. Below this concentration value, enough enzymatic reaction time is available for urea to convert to ammonia without poisoning the platinum catalytic surface sites. Since the concentration of urea in real urine is approximately 0.22 M, the possible blockage of the catalytic sites at higher urea concentration should not be a problem for the proposed design, since at the operational concentration the ammonia oxidation reaction peak current was observed.

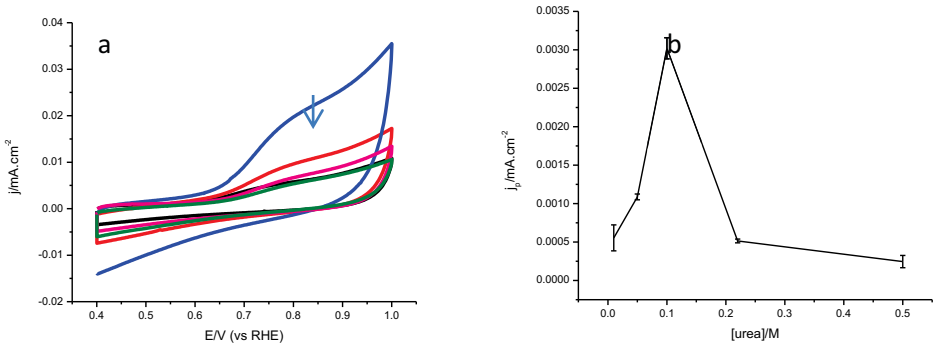


Figure 4. Urea concentration effect, on the ammonia oxidation reaction peak current density, in presence of *P. vulgaris*. (a) Cyclic voltammograms of polycrystalline Pt electrodes in ~2.5 x 10^9 cfu/mL *P. vulgaris* in synthetic urine at pH 8.00 at different urea concentrations; 0.01 (black), 0.05 (red), 0.1 (blue), 0.22 (pink), and 0.5 M (green). (b) Ammonia oxidation reaction peak current densities vs. urea concentration in synthetic urine solutions at pH 8. The peak current densities were measured from the cyclic voltammetry data performed at a scan rate of 10 mV/s. The ammonia oxidation peak current densities at different urea concentrations can be observed at the blue arrow.

The effect of the bacteria concentration on the urea conversion to ammonia was studied as well. Three *P. vulgaris* concentrations were used; 1.9, 2.9, and 3.5 x 10^9 cfu/mL (see Figure 5). The bacterial conversion of urea to ammonia was followed by cyclic voltammetry of the ammonia oxidation reaction at a Pt electrode. For this experimental study, 5 mL of 0.1 M urea in synthetic urine at pH 8.00 was added to the bacteria pellet. After 10 minutes of the addition, cyclic voltammograms were done between 0.4V and 1.0V vs. RHE at a scan rate of 10 mV/s. Bacteria concentrations of 1.87x10^9 cfu/mL, 2.88x10^9 cfu/mL, and 3.5x10^9 cfu/mL were used. Here, cfu is colony-forming units. The ammonia oxidation peak current density measured, at different bacteria concentrations, can be observed at Figure 5b. When the bacterial concentration was changed from 1.9 to 2.9 x 10^9 cfu/mL, the ammonia oxidation peak current density almost doubled its value, from 0.3 mA/cm^2 to 0.6 mA/cm^2. Increasing the bacteria concentration to 3.5 x 10^9 cfu/mL showed a larger ammonia oxidation peak current density (3.2 mA/cm^2), resulting in a faster urea to ammonia conversion. These results suggest that a change in the concentration of bacteria has a significant effect on the amount of ammonia available for the ammonia oxidation reaction. This result is in accordance with what was expected from an ureolysis system, since higher concentrations of bacteria are able to convert urea to ammonia at a higher rate.

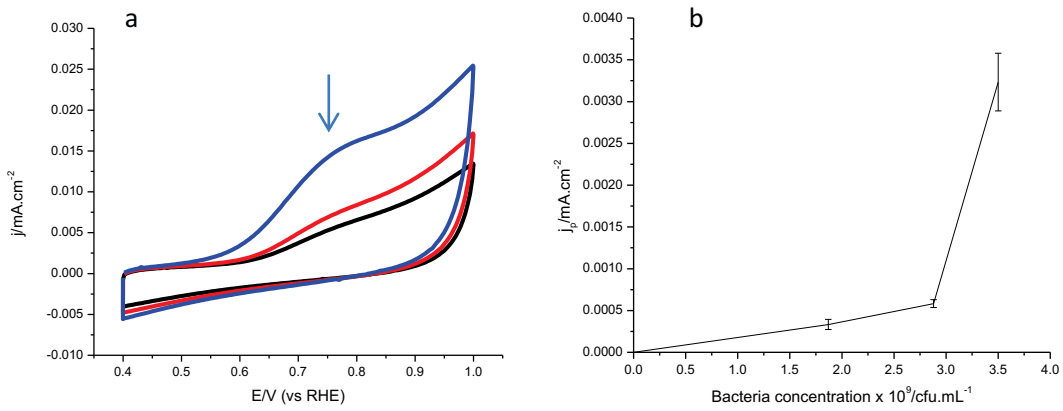


Figure 5. Bacteria concentration effects on the ammonia oxidation reaction in a *P. vulgaris* ureolysis system. (a) Cyclic voltammetry measurements and (b) ammonia oxidation peak current densities vs. *P. vulgaris* concentrations in colony-forming units per mL (cfu/mL); 1.87 x10^9 (black), 2.88 x 10^9 (red), and 3.5 x 10^9 cfu/mL (blue). The synthetic urine solution was at pH 8.00. The ammonia oxidation peak current densities can be observed at the blue arrow.

4.3 Time dependence of urea removal and current production by the ureolysis system.

For further application of the ureolysis system, a time dependent urea removal reaction was done with *P. vulgaris* in synthetic urine with urea. Moreover, since bacteria are continuously converting urea to ammonia over time and, therefore, increasing the solution pH, we wanted to study the effect it may have on the ammonia oxidation reaction. For this study, the bacteria were allowed to react, at different time intervals, with 0.1M urea in synthetic urine at pH 8.00 and 9.5 x 10^8 cfu/mL in *P. vulgaris*. The cyclic voltammetry was done using a Pt working electrode and a RHE reference electrode at a potential scan rate of 10 mV/s. The potential window was between 0.4V and 1.0V vs. RHE. The graphs, of ammonia oxidation current density vs. time of bioelectrochemical reaction, were plotted selecting the maximum ammonia oxidation reaction peak current densities from each cyclic voltammogram. The ammonia oxidation peak current densities can be observed at the blue arrow. An increase on the ammonia oxidation peak current density, up to 2.2 mA/$cm^{2,}$ was observed after allowing 24 hours of bacterial reaction to convert all the available urea to ammonia. Since the solution pH increases slightly with the ammonia production, more ammonia is available for oxidation than at lower pH. This may be seen in the peak current density increase. With time, the change in ammonia oxidation peak current densities became steadier as the limit of available urea was reached. The ammonia oxidation peak

current density reached a plateau after 60 min of bacterial reaction, at 1.6 mA/cm^2, with a slight increase to 2.2mA/cm^2 after 24 hours of biochemical reaction.

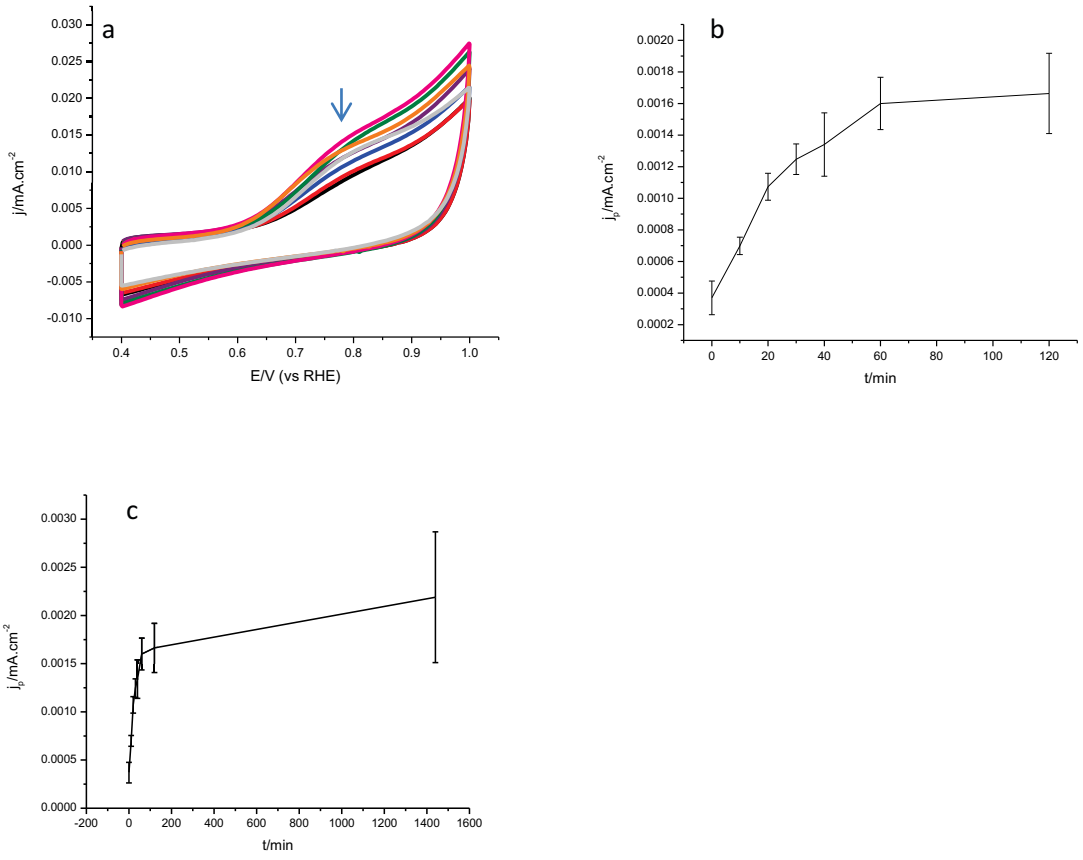


Figure 6. (a) Cyclic voltammetry of the ammonia oxidation reaction at different bacteria exposure times in 0.1M urea in synthetic urine at pH 8.00. The bacteria concentration was 9.5 x 10^8 cfu/mL *P. vulgaris*. The time intervals were *0 (black), 10 (red), 20 (blue), 30 (purple), 40 (green), 60 (pink), 120 min (orange) and 24 hrs (grey)*. Plots of maximum ammonia oxidation reaction peak current densities vs. bacterial reaction time intervals for a total time of exposure of (b) 120 min and (c) 24 hrs. The cyclic voltammetry was done at a potential scan rate of 10 mV/s. The graphs were recorded selecting the maximum ammonia oxidation reaction peak current densities from each cyclic voltammogram. The ammonia oxidation peak current densities can be observed at the blue arrow.

5. Conclusions

This work demonstrates the possibility of *P. vulgaris* being used for an ureolysis system for water recovery from synthetic urine. The urea in urine solution was converted to ammonia by the bacterial urease. The ammonia oxidation reaction was detected by cyclic voltammetry. The optimal conditions for both processes, the urea to ammonia microbial conversion and ammonia oxidation electrochemical reaction, were determined. High concentrations of urea in the synthetic urine system may reduce the oxidation efficiency Pt electrodes by poisoning the surface by urea adsorption [29]. The bacteria concentration in the ureolysis system is crucial to avoid this ammonia oxidation reaction efficiency problem. For the average urea concentration in urine of 0.22 M, the current ammonia oxidation peak obtained may be improved by increasing the exposure time of the bacteria with the urine before the ammonia oxidation, or by increasing the bacteria concentration in order to increase the amount of urea converted to ammonia in an interval of time. Further improvements are needed to maximize the ammonia oxidation current densities as well as the optimal *P. vulgaris* concentration needed in real urine in order to make a self-sustainable ureolysis system.

6. Acknowledgements

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7. References

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