Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 January 2020 doi:10.20944/preprints202001.0043.v1

In-situ sensing of microbial growth phases via two-terminal cyclic voltammetry

Arindam Kushagra[1]*, Diyasa Bazal[1†], Anup Kumar Pradhan[1†], Pratyusha Ghosh[2] and Akshaya Pandey[2]

[1]Amity Institute of Nanotechnology, Amity University Kolkata, India. PIN: 700135
[2]Amity Institute of Biotechnology, Amity University Kolkata, India. PIN: 700135
[†]Diyasa Bazal and Anup Kumar Pradhan contributed equally to this work.
*Correspondence: arindam.kushagra@gmail.com

***Abstract*—Microbial growth has been of prime importance to the researchers in health and biotechnology industries. It has been known to be closely associated to the secretion of extracellular polymeric substances that help in the formation of colonies. Inter-microbial communication happens within such colonies by means of extracellular electron transfer mediated by the aforementioned polymeric substances. Conventionally, different phases of microbial growth are monitored with the aid of a traditional UV-Visible spectrophotometer by measuring the optical density of the liquid medium at 280 nm. In this paper, we have developed an alternative novel way to sense different growth phases employing electrochemical means i.e. two-terminal cyclic voltammetry. This cyclic voltammetry relies on the extracellular electron transfer mechanism taking place via the polymeric substances secreted by the microorganisms, measured by the temporal area changes in the current-voltage hysteresis curves in the inoculated nutrient broth. This work paves a new way to detect the biological activity in the medium, which can be directly correlated to the population of microorganisms. It would be of immense interest to scientists and researchers working in the field of microbiology as well as in development of biosensors, electrochemical sensors etc. which would be helpful in absence of traditional spectrophotometers.**

***Index Terms*—Biosensors, cyclic voltammetry, electrochemical sensors, extracellular electron transfer, extracellular polymeric substances, growth curve, microbial growth phases**

I. Introduction

RESEARCHERS from biotechnology and health industries have long been bothered about the growth of microorganisms for economic and etiological concerns, respectively. Conventionally, the growth phases of microorganisms are determined by measuring the optical absorbance of the liquid nutrient broth media, containing microorganisms at 280 nm. A comprehensive treatise of microbial sensing strategies could be found in **[1]**. Microbial growth is facilitated by the formation of clusters, which is mediated by the secretion and accumulation of extracellular polymeric substances (EPS) for more firmly attached, adhered cells to form the clusters **[2-4]**. Recently, researchers have shown that EPS mediate the microbial extracellular electron transfer (EET) **[5]**. In the said work, the microbial EET is suggested to take place by hopping mechanism. This mechanism is schematically shown in Fig. 1.

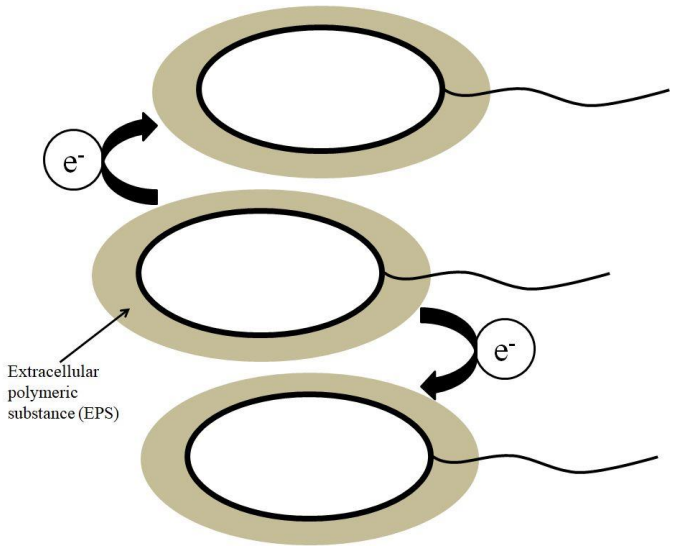

Fig. 1 Schematic representation of the hopping mechanism of charge transfer constituting microbial EET via the EPS, is shown.

In hopping, the electron transfer takes place between the localized states in the EPS and strong electronic vibrational interactions with full vibrational relaxation, given by the rate constant

Preprints (www.preprints.org) | NOT PEER-REVIEWED | Posted: 5 January 2020 doi:10.20944/preprints202001.0043.v1

$$\omega(s^{-1}) = \kappa_0 e^{-\beta\Delta R}\frac{\omega_{eff}}{2\pi}e^{-\frac{(\lambda+\Delta G^0)^2}{4\lambda k_B T}} \quad (1)$$

where $\frac{\omega_{eff}}{2\pi}$ is the effective vibrational frequency of nuclear modes (~10^{12} s^{-1}). Activation free energy is given by nuclear reorganization free energy, λ, and the reaction free energy, ΔG^0. $\kappa(=\kappa_0 e^{-\beta\Delta R})$is the electronic transmission coefficient determined by the hop step size, ΔR, and the decay factor, β. Such charge transfer mechanisms, with the magnitudes of current as direct indicators of the microbial population in the medium, could be used as indicators of different phases of growth.

In this paper, we have reported the development of a novel sensing mechanism to detect different phases of microbial growth by measuring the two-terminal cyclic current-voltage (I-V) characteristics at different time intervals, as discussed further in the paper. This work would bypass the need of sophisticated instruments required for the detection of microbial growth phases like well-plate readers **[6, 7]**, UV-Visible spectrophotometers **[8]**, microplate readers, metabolite screening **[9]** etc. and would enable researchers working in the interdisciplinary domains requiring to monitor microbial growth **[10]**.

.

II. MATERIALS AND METHODS

A. Preparation of microbial sample

One gram of garden-variety soil sample (collected from the neighborhood) was thoroughly mixed with 100 ml of distilled water by vigorous stirring. The soil-water sample was then allowed to settle overnight. The supernatant water part of the mixture was taken further as microbial sample inoculum.

B. Chemicals

1.32 grams of nutrient broth powder (Himedia, MM244) was dissolved in 100 ml of distilled water. This broth was divided into four parts. Out of these, two parts were inoculated with 0.5 ml of microbial sample each (preparation discussed earlier) and remaining two parts were kept as control samples after further addition of 0.5 ml distilled water each. The final samples were kept for electrical and optical measurements, classified as under:

2×Experimental (**E**):	0.5 ml of soil water supernatant + 1.5 ml of nutrient broth medium
2×Control (**C**):	0.5 ml of distilled water + 1.5 ml of nutrient broth medium

C. Electrical measurements

The measurements were done with the aid of makeshift devices using eppendorf tubes and metallic all pins (shown in Fig. 2) using a Keysight B2901A Precision Source/Measure Unit. Data acquisition was done using QuickIV.4.1.1821.3680 open source GUI. Following settings were kept for the measurements: Mode: Voltage, Shape: DC, Source Delay: 0, Sweep: Linear Double, Start: -5V, Stop: +5V, Step: 0.204V, Compliance: 1A, Range limit: 1mA, Measurement delay: 0.5s. 10 cycles of measurements were done at each time interval. All the remaining parameters were kept in their default settings. The experiment was conducted for 55 hours.

D. Optical measurements

The optical densities of experimental and control samples were measured at 280 nm, using Eppendorf Biospectrometer (Basic). The experiment was conducted for 55 hours.

E. Data Analysis

Post data-acquisition analysis was done using Origin 8 Pro® graph plotting software. Mathematical area was calculated using the software for all the 10 cycles of voltage sweeps done. The mathematical area is defined as the sum of areas of the curve above and below the mean axis as it is. This means that such an area would indicate the positive value for the parts of the curve above the mean axis and negative values for the parts of the curve that lie below the mean axis [**11**].

III. RESULTS AND DISCUSSION

As discussed in the Materials and Methods section, two sets of control and experimental samples were used for this study. One set was used for measurement of optical density (@ 280nm) using spectrophotometer to determine the microbial growth phases. The other set was used to measure the electrical behavior of the samples. Distinct colors of control and experimental samples could be seen, thus giving an indication about the contaminated and clean sample, as shown in Fig. 2.

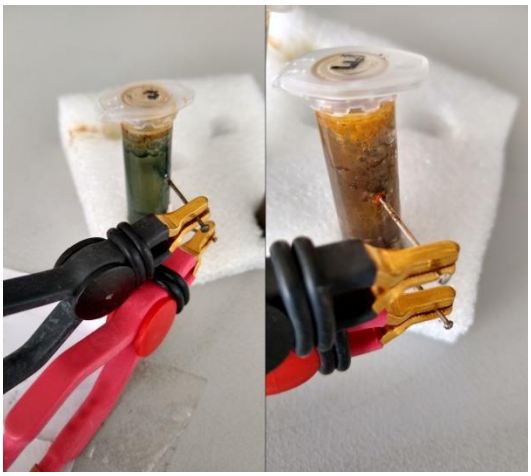

Fig. 2 Distinct colors can be seen in control (labeled as **C**) and experimental (labeled as **E**) samples, after 48 hours of measurements. Description of these samples is given in Materials and Methods section.

As discussed earlier, the voltage sweeps were conducted for 10 cycles to get an averaged trend of the electrical behavior of the growing microbes in the nutrient broth. Representative current-voltage trace curves taken after 4, 28 and 51 hours of inoculation, respectively, are shown in Fig. 3.

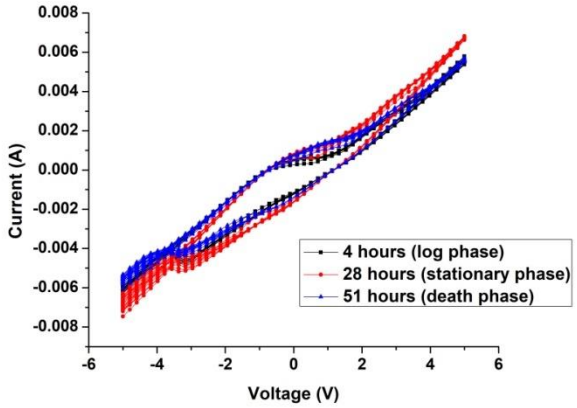

Fig. 3 Representative current-voltage curves indicating various stages of microbial growth, taken after 4, 28 and 51 hours of inoculation have been shown.

It is evident from the curves in Fig. 3 that the time derivatives of current at zero voltage values are different for different phases of microbial growth. The maximum slope is seen for the stationary phase (red curve) and almost same slopes are obtained for the log (black curve) and death (blue curve) phases, thus leading to maximum area for stationary phase in comparison to the log and death phases. The mathematical area of these I-V curves (plotted against time elapsed) depict an exactly similar trend as seen in the A_{280}-time curve, shown in Fig. 4.

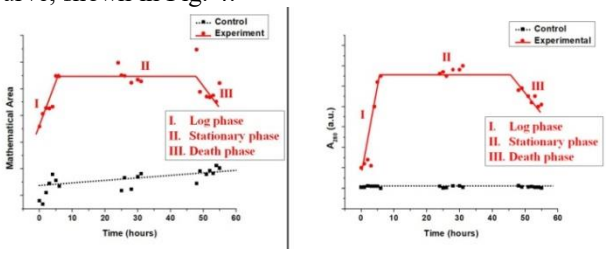

Fig. 4 Comparison of temporal behaviors of mathematical areas (left) and A_{280} (right) values, show exactly similar trends.

With reference to the left panel of Fig. 4, the production of EPS with microbial growth leads to an increase in the quantity of charge transfer media, thus leading to an increase in the corresponding electrical power consumption. This electrical power consumption is denoted by the calculated mathematical area of the current-voltage curves at every time interval. This explains the increasing trend of area in the phase I. When the microbes reach the stationary phase of their growth, their numbers stop increasing. This leads to the approximately same amount of electrical power consumed leading to the constant trend of area in phase II. In the final phase of their life cycle i.e. death phase, the number of live microbes starts decreasing. This would reduce the overall metabolic activities happening in the nutrient broth and thus quantitatively decreasing the EET due to such activities. This leads to the decreasing trend of area in phase III.

IV. Conclusions

In this paper, we have developed a novel way to sense different phases of microbial growth in nutrient broth by doing *in situ* two-terminal cyclic voltammetry. The mathematical area calculated from the cyclic voltammetry hysteresis curves depicted various stages of microbial growth. These mathematical areas when plotted against time showed trends that were in perfect agreement with the optical density data (i.e. absorbance @ 280nm). Thus, researchers equipped with this novel method would be able to detect different microbial growth stages in absence of a conventional spectrophotometer. This work would find applications in development of novel electrochemical sensors for various biological and chemical purposes.

Author Contributions

A.K. and P.G. conceived and designed the experiments. A.K., D.B., A.K.P. and A.P. performed the experiments. A.K., D.B. and A.K.P. analyzed the data. A.K. and P.G. wrote the manuscript. All authors have read and agreed to the current version of the manuscript.

Funding

No funding has been availed for this research work.

Acknowledgements

The authors wish to acknowledge Dr. R.R. Bhattacharjee, Head, Amity Institute of Nanotechnology, Amity University Kolkata, for his insights and helpful discussions during the course of this work.

Conflicts of Interest

The authors declare no conflicts of interest.

References

1. Abbasian, F.; Ghafar-Zadeh, E.; Magierowski, S. (2018) Microbiological Sensing Technologies: A Review. Bioengineering 5, 20. doi: https://doi.org/10.3390/bioengineering5010020

2. Janissen, R., Murillo, D., Niza, B. et al. (2015) Spatiotemporal distribution of different extracellular polymeric substances and filamentation mediate Xylella fastidiosa adhesion and biofilm formation. Sci Rep, 5, 9856. doi: https://doi.org/10.1038/srep09856

3. Decho, A.W. and Gutierrez, T. (2017) Microbial Extracellular Polymeric Substances (EPSs) in Ocean

Systems. Front. Microbiol., 8, 922. doi: https://doi.org/10.3389/fmicb.2017.00922

4. Guo Y-S, Furrer J.M., Kadilak A.L., Hinestroza H.F., Gage D.J., Cho Y.K. and Shor L.M. (2018) Bacterial Extracellular Polymeric Substances Amplify Water Content Variability at the Pore Scale. Front. Environ. Sci. 6, 93. doi: https://doi.org/10.3389/fenvs.2018.00093

5. Y. Xiao, E. Zhang, J. Zhang, Y. Dai, Z. Yang, H. E. M. Christensen, J. Ulstrup, F. Zhao (2017) Extracellular polymeric substances are transient media for microbial extracellular electron transfer. Sci. Adv. 3, e1700623. doi: https://doi.org/10.1126/sciadv.1700623

6. Hazan, R., Que, Y., Maura, D. and Rahme L.G. (2012) A method for high throughput determination of viable bacteria cell counts in 96-well plates. BMC Microbiol 12, 259. doi: https://doi.org/10.1186/1471-2180-12-259

7. Stevenson, K., McVey, A., Clark, I., Swain, P.S. and Pilizota, T. (2016) General calibration of microbial growth in microplate readers. Sci Rep 6, 38828. doi: https://doi.org/10.1038/srep38828

8. Maia, M.R.G., Marques, S., Cabrita, A.R.J., Wallace, R.J., Thompson, G., Fonseca, A.J.M. and Oliveira, H.M. (2016) Simple and Versatile Turbidimetric Monitoring of Bacterial Growth in Liquid Cultures Using a Customized 3D Printed Culture Tube Holder and a Miniaturized Spectrophotometer: Application to Facultative and Strictly Anaerobic Bacteria. Front. Microbiol. 7, 1381. doi: https://doi.org/10.3389/fmicb.2016.01381

9. Robador, A., LaRowe, D.E., Finkel, S.E., Amend, J.P. and Nealson, K.H. (2018) Changes in Microbial Energy Metabolism Measured by Nanocalorimetry during Growth Phase Transitions. Front. Microbiol. 9, 109. doi: https://doi.org10.3389/fmicb.2018.00109

10. Mathijssen, A.J.T.M., Culver, J., Bhamla, M.S. and Prakash, M. (2019) Collective intercellular communication through ultra-fast hydrodynamic trigger waves, Nature 571, 560–564. doi: https://doi.org/10.1038/s41586-019-1387-9

11. https://www.originlab.com/doc/Origin-Help/Math-Integrate, accessed on 04th January, 2020.