Electrostimulation of heterotrophic biofilm originated from *Enterobacter dissolvens*

**Peng She (Guest researcher)**

Department of Biotechnology, Delft University of Technology, Delft, the Netherlands

Institute of Biochemical Engineering, Tsinghua University, Beijing, China

E-mail: P.She@tnw.tudelft.nl; Shepeng00@mails.tsinghua.edu.cn
Abstract: The use of biofilms for water and wastewater treatment dates back to over a century. However, only in recent five years the application of biofilm in bioremediation process targeting xenobiotic compounds has become a focus of interest. However, relatively low efficiency of traditional bioremediation techniques is a crucial problem and could turn to be a bottleneck in case of urgency. In this regard, to activate environmental microorganisms by weak electric current, which is designated as the concept of “electrostimulation”, is promising to provide an effective approach to accelerate the biodegradation process.

In present research, heterotrophic biofilm originated from an indigenous phenanthrene-degrading strain, Enterobacter dissolvens, was developed by a Roto Torque reactor. The biofilm structure was imaged by fluorescent microscopy using BacLight Live/ Dead stain, and the biodiversity of the mixed culture was characterized by Denaturing Gradient Gel Electrophoresis (DGGE) analysis. After exposure to external electric field, the changes in respiratory activity of biofilm floc was measured by a Clark-type oxygen microsensor (Tip dia.: 25 um). An increase around 50% of the original respiratory activity can be obtained after 15-min exposure to AC current (50 Hz, 10 mA). These results were in accordance with the electrostimulation experiments conducted in cell suspension, which is characterized by oxygen uptake rate (OUR).

Preliminary research confirmed the feasibility of utilizing electric current to activate the biofilm system. Further efforts will be focused on exploring the practical application of electrostimulation effects on biofilm reactor of flow-cell type, and elucidating the mechanism of activity increases from the monitoring of intracellular metabolic intermediates, e.g., NADH and ATP.
Bacterium, medium, and culture condition

- Indigenous bacterium screened by using phenanthrene as the sole carbon source.

- Identified as **Enterobacter dissolvens** based on 16s rDNA gene sequence:

  AGCGGTCGATTACCGGTTAGCTCCGGAAAGGCCGCTCAAGGCAACACCTCAAATCGACATCGGGACTACCAG
  GGTGTCTCTGAATTCATCTCCCTTTTGGTCCCCAGTTTCAGCTGACGTGACGCTACCGTTGCTCGGTATTTCTG
  GATCTCCTACGCATTTTCACCGCTACACCTGAAATCTACCCCCCTCTCCTACAAGACTCTAGCCTGCCAGTTTCGAATGCAGTTCCCAGGT
  TGAGCCCGGGGATTTCAATCCGACTTGACAGACCGCTCGTGCCTTTAAGGCCAGTAATTCCGATTAACGCTTGCACCCTCCGT
  ATTACCGCGTGCTGGCAGGCGGATTTCAATCCGACTTGACAGACCGCTCGTGCCTTTAAGGCCAGTAATTCCGATTAACGCTTGCACCCTCCGT
  CCCGCTGAAAAGTAATTCATTACAACCCGAAGGCCTTCTTCATACCCGCCATCTGACACCGCTGCCATGCTGCGCTTTACGCCCAGTAATTCCGATTAACGCTTGCACCCTCCGT
  CCCACTGCTGCTCC

- **Bacillus**, gram negative, phylogenetically closed to **E.coli**.

- **Medium**: basal mineral medium (**K2HPO4** (0.8), **KH2PO4** (0.2), **NH4NO3** (0.8), **MgSO4** (0.25), **FeSO4.7H2O** (0.09), and **CaCl2** (0.032)) with glucose as the sole carbon source.
Electrostimulation of immobilized cells in agar gels (as model biofilms)

Objectives

To investigate the intrinsic effects of weak electric current on cell respiratory activity when the influences by electrokinetics and electrode reaction were excluded.

Procedures

1. Immobilization of pre-cultured cells in agar gel

   - Cell age: 14h (in the early stationary phase, to prevent gel deformation due to cell growth and obtain cells with relatively high activity)
   
   - Cell harvest: Centrifugation (3000 rpm, 10 min, room temp.); rinsed by phosphate buffer for two times; resuspended in fresh mineral medium.
   
   - Immobilization: by mixing cell suspension with agar-containing medium at 50°C, and solidified on plate at room temperature. Final agar concentration: 1%; final glucose concentration: 1%; final cell density (40 mg wet biomass/ g gel)

2. Microsensor measurements of oxygen profiles in agar gel

   - Gel cutting – 20mm (l) X 5mm (w) X 5mm (h)
   
   - DO profiling on gel slice in the absence of electric field (cold test)
   
   - Implementation of electric field for 5 min at a voltage gradient of 5V/ cm or 10V/ cm
   
   - Cut off electric field, then immediately measure the DO profile at the same location on gel as the cold test
Results

1. **Oxygen profiles in the absence of electric current (cold test)**

   Oxygen profiles measured at different locations on the surface of cell-free agar gel

   Oxygen profiles measured at different locations on the surface of agar gel (cell & glucose containing)

2. **Oxygen profiles after the exposure to electric current**
   - As a function of frequency (voltage gradient: 5V/cm, including the case of DC current)
Figure 4 Changes in oxygen profile as a function of current frequency
- As a function of amplitude (voltage gradient: 5V/cm and 10V/cm, including the case of DC current)

![Graphs showing oxygen profile changes with different voltage gradients](image)

*Figure 5 Changes in oxygen profile as a function of voltage gradient (as an index to current amplitude)*

**Conclusions**

1. Resistance in oxygen diffusion was relatively small in cell-free agar gel (Figure 2).

2. The presence of immobilized cell leads to a site-nonspecific oxygen profile in agar gel, attributed to its homogeneity (Figure 3).

3. In case of DC current, faster consumption rate of oxygen than the control run was observed (Figure
4). However, significant increase in gel temperature was recorded by glass-term thermometer (>10 °C), indicating the rise in cell respiratory activity was most likely to be due to the heat generated by electric current.

4. In case of AC current, no difference in gel temperature was recorded by glass-term thermometer. Slight increase in the consumption rate of oxygen was obtained when 50-hz AC current was used. At higher frequency from 500 hz to 500000 hz, no apparent change in oxygen profile was obtained (Figure 4). The reason why this difference occurred is still unclear, and AC current at 50 hz was used in the following experiments on biofilm floc.

5. The increase in voltage gradient has no significant influence on cell respiratory activity in case of AC current (figure 5). In case of DC current, higher activity was obtained, however, accompanied with more rise in temperature.

6. One question remains. How to effectively characterize the temperature rise after the implementation of electric current? The size and sensitivity of ordinary thermometer impeded its application in precise measurement. A temperature microsensor will be much more fitting to this process.
Electrostimulation of biofilm originated from Enterobacter dissolvens

**Experimental**

1. Incubation of biofilm by Roto torque reactor

   - Starting of biofilm cultivation: 24-hours batch cultivation after inoculation, then switch to continuous mode.
   - Continues cultivation: 16 days fed with 20-times diluted medium used for batch culture.
   - Rotational speed of the reactor: 150 rpm
   - Sampling time (days after the start of continues mode): 3, 10, 16

   *Ananlysis:*
   - DGGE analysis of the 16 s rDNA fragments (forward primer 341F-GC, reverse primer 907R). Urea-formamide (UF) gradient: 0 – 80%.
   - Cell viability: Live/ Dead staining.
Results

1. Formation of biofilm on polycarbonate slides

- Biofilm on polycarbonate slide periodically taken out of the Roto Torque Reactor

<table>
<thead>
<tr>
<th>Incubation period (day)</th>
<th>Biofilm formation on polycarbonate slide</th>
<th>DGGE analysis of the biofilm after 16 days incubation</th>
</tr>
</thead>
<tbody>
<tr>
<td>3</td>
<td><img src="image1" alt="Biofilm Image" /></td>
<td><img src="image2" alt="DGGE Image" /> 1. from the biofilm layer</td>
</tr>
<tr>
<td>10</td>
<td><img src="image3" alt="Biofilm Image" /></td>
<td></td>
</tr>
<tr>
<td>16</td>
<td><img src="image4" alt="Biofilm Image" /></td>
<td><img src="image5" alt="DGGE Image" /> 2. from the pure culture of E. dissolvens</td>
</tr>
</tbody>
</table>

- **Key learning:** Flocculent biofilm was formed by Enterobacter dissolvens, similar to the known case of E.coli. DGGE analysis indicates Enterobacter dissolvens was the unique species presented in the biofilm sampling after 16-days cultivation, as only one band corresponding to Enterobacter dissolvens was observed on gel.

2. Preparation and characterization of the biofilm floc used in microsensor measurement

The biofilm layer on polycarbonate slide was too thin (< 1 mm) to be directly subjected to microsensor measurements. Thus the biofilm layer was collected on glass slide and exposed to open environment for two weeks. Finally a floc with height at 2 mm was obtained (figure 7), and characterized by DGGE and Live/Dead staining analysis on biodiversity and cell viability, respectively.
**Key learning:** Two-weeks stewing led to the presence of 6 – 7 bacterial species in the community of tested biofilm floc (figure 9). The viability of the cells in biofilm floc still maintained (figure 10).

3. **Oxygen profiles in the absence of electric field (cold test)**

   - Checking the reproducibility of oxygen profiling by random measurements within the labeled area by white line (figure 10)
Key learning: Homogeneous distribution of the oxygen profiles in the biofilm floc was observed, which confirmed the reliability of microsensor measurement in this case (figure 11).

4. Oxygen profiles after the exposure to electric current

Parameters: 50 Hz, 10V, 15 min (treating period), no detectable change in temperature during the experiments.
Key learnings:

- Approximate calculation of increases in OUR: \( \frac{700-450}{450} \times 100\% = 55.6\% \) (figure 12), much higher than the results obtained in the cell-immobilized agar gel. The only difference is in agar gel cells were mounted by the cross-linked structure of agar molecules, thus the effects of electrokinetics were excluded.

- After exposure to AC current, oxygen profile gradually retrieved to the original level (Figure 13), indicating the stimulation effect, either intrinsically induced by AC current or as a consequence of electrokinetics, is not permanent but a temporary phenomenon.

Conclusions & Prospects

- The exposure of biofilms to external AC current, including model system (agar gel) and real system (biofilm originated from Enterobacter dissolvens), can lead to an increasing respiratory activity of cells. More apparent phenomenon was obtained in the case of biofilm floc, indicating the contribution of electrokinetics (inferred to be the vibration of cells in the presence of AC current) to activity promotion cannot be neglected.

- In traditional research on biofilm behavior in the presence of electric current, the bactericidal effects of DC current were the main emphases. Present research preliminarily evaluated the feasibility of utilizing external AC current to enhance the cell respiratory activity. The positive results indicate it's promising to extend present study to relative bioconversion processes based on aerobic biofilm techniques, to invert the enhanced respiratory activity to higher biodegrading efficiency.