Combined Theoretical and Experimental Approaches for Bacterial Aggregation Studies Towards the Improvement of Bioremediation Processes

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Introduction

The formation of clusters that persist in suspension has already been described as a third microbial lifestyle. *Pseudomonas extremaustralis* 2E-UNGS is a native microorganism from the highly contaminated Reconquista River, Buenos Aires, Argentina. This strain is able to aggregate, develop biofilms and biosorb metals, secreting biosurfactants and exopolymeric substances, properties that contribute to its application in the design of sustainable environmental biotechnologies. The goal of this work was to study the kinetics of this microorganism cluster formation by a combination of experimental and theoretical modelling approaches to apply in the design of improved biotreatments, especially of electroplating effluents.

Experimental procedure

- **Microorganism:** *Pseudomonas extremaustralis* 2E-UNGS (NCBI GenBank CP091043.1)
- **Culture media (g/l):**
  - Mineral broth (M9): 1M, 0.100 mL; glycerol, 20 (2.0%) Na_2HPO_4, 6.0; KH_2PO_4, 3.0; NH_4Cl, 1.0; NaCl, 0.5; $MgSO_4 \cdot 7H_2O$ 1M, 0.800 mL; $CaCl_2$
  - Enriched broth (PY): bactopeptone, 2.5; yeast extract, 1.25; glucose, 5.0 (0.5%) o 50.0 (5.0%)
  - Nutrient Broth (NB): meat peptone, 5.0; meat extract, 3.0.
  - Dilution 1:2 of Nutrient Broth (NB-½)
- **Incubation:** thermostatic bath at 32 ºC under agitation

Aggregate formation kinetics with 6-well plate:

Inoculum culture
- **15 ml media** (M9-2.0%glycerol, PY-0.5%glucosa or PY-5.0%glucosa)

24 h

Culture
- 10% inoculum in 50 mL of the same fresh medium
- Same incubation conditions

Sampling
- times (h): 0, 4, 8
- 1 ml for OD_{600nm} and pH
- 3.0 ml for digital image study (for duplicate)

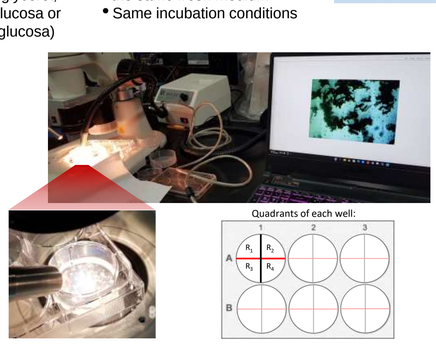
Aggregate formation kinetics with confocal Petri-dish:

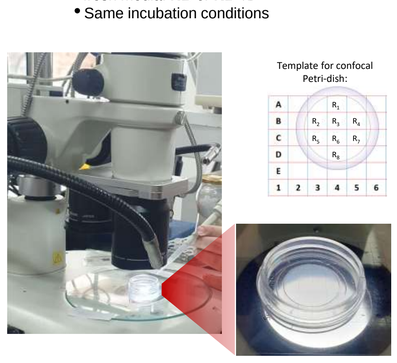
Inoculum culture
- **15 ml NB medium**

24 h

Culture (duplicate, A and B)
- 10% inoculum in 50 mL of fresh media: **NB** or **NB-½**
- Same incubation conditions

Sampling
- times (h): from 0 to 24
- 1 ml for OD_{600nm} and pH
- 1.0 ml for digital image study





Acquisition of 2D-Digital images:

✓ Stereomicroscope "NIKON – SMZ 1270".

✓ 6-well cell culture plates were illuminated with overhead light sources.

✓ Confocal Petri-dishes were illuminated from above and below the sample.

Digital image analysis for aggregate formation kinetics with the NB media – FIJI®

Culture media: Nutrient broth (NB y NB-½)

Image acquisition: 0.300 ml of sampling in Petri-dish confocal (in duplicate for each sampling)

Sampling times (h):
- cell culture: 0, 2, 4, 5, 7, 9, 23 and 24
- cell free control: 5 (t_3) and 24 (t_7)

For the 8 regions (R_i)

Control of 5 h (t_3)

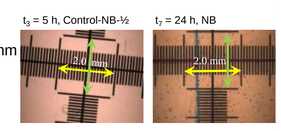
Control of 24 h (t_7)

Average of the Maximums of Control t_3 and t_7

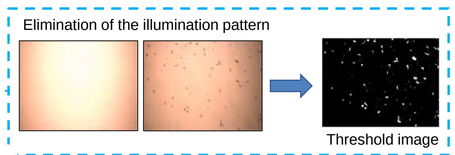
Displayed ROI (Region Of Interest) as an overlay of the image original

Sampling → Scaling-up → Lighting pattern → Lighting pattern subtraction → "Analyze Particles"

Average (10 images):
NB:(1518 ± 6) pixels = 2.00 mm
NB-½: (1538 ± 2) pixels = 2.00 mm
Image size:
- 3264 pixels x 2472 pixels
- NB: 4.30 mm x 3.26 mm
- NB-½: 4.12 mm x 3.13 mm

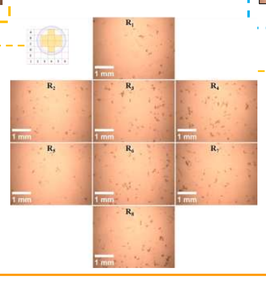


Elimination of the illumination pattern

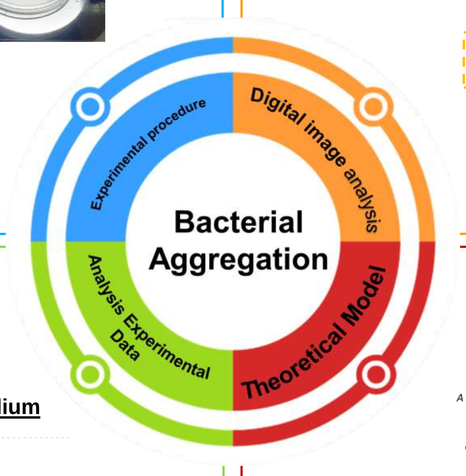


Threshold image

Grid with 5 mm x 6 mm spacing between rows and columns.



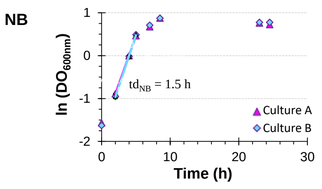
Distribution of the 8 non-overlapping images corresponding to t_5 = 9 h of incubation for an NB culture maintaining the R_i position of the template for the confocal Petri-dish methodology.

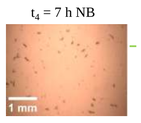


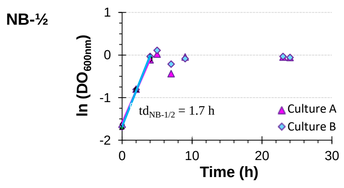
Experimental data analysis

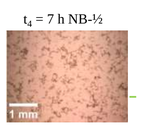
Growth curves:

Recorded over exponential and stationary phases for NB y NB-½



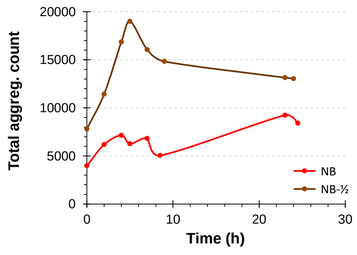






OD_{600nm} becomes a relative parameter of bacterial growth.

Total aggregate count of Culture A for each medium

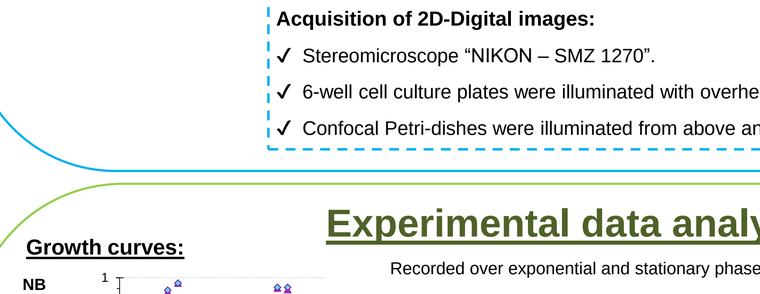


NB-½ favoured bacterial growth by forming aggregates.

Number of clusters as a function of area:

For both, the aggregate areas were predominantly around 0.1x10^{-4} mm^2.

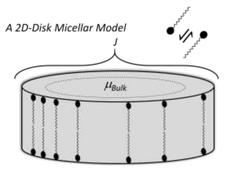
Number of clusters as a function of time for highest 10 bins:



In this case, for the highest 10 bins the corresponding to (0.0-0.1) x10^{-4} mm^2 and (0.1-0.2) x10^{-4} mm^2 represented more than the 50% of the particles (blue and violet line).

2D - Micellar model

Study of the energetic of the bacterial aggregation



Assumptions
- stationary regime
- total bacteria volume fraction is much smaller than one
- the total number of aggregates N is constant

Chemical Potential $\mu_j = \mu_j^0 + \ln \rho_j$ with $\mu_j^0 = j\mu_{bulk} + \alpha\sqrt{j}$, $\rho_j = \frac{j}{N}$ and ρ_j the density of aggregates j

Bacteria aggregation/fragmentation based on an On-Off Model $\dot{\rho}_j = -\rho_j k_{on} + \rho_{j+1} k_{off}$ on $\Longleftrightarrow$ off (or $j \Longleftrightarrow j+1$)

Equilibrium $\mu_j = j\mu_1 \longrightarrow \frac{k_{on}}{k_{off}} = \exp(\Delta U^{eq}) = (\rho_1 \exp(\beta\alpha)) \exp(-\beta\alpha(\sqrt{j+1} - \sqrt{j}))$

Out of Equilibrium (active state) $\mu_j \neq j\mu_1 \longrightarrow \frac{k_{on}}{k_{off}} = \exp(\Delta U^{act}) = (1 + \frac{1}{j})^{\Gamma}$

Difference of the energy cost of the transition $j \rightarrow j+1$ between Cultures 1 and 2

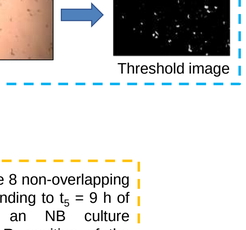
$$\Delta U_{tot,j} = (\Delta U_j^{act} - \Delta U_j^{eq})_2 - (\Delta U_j^{act} - \Delta U_j^{eq})_1$$

$$\Delta U_{tot,j} = (\Gamma_2 - \Gamma_1)\ln(1 + \frac{1}{j}) + \Delta(1 - \sqrt{j+1} + \sqrt{j})$$

with $\Delta = \alpha_1 - \alpha_2$

The Γ exponents for both cultures display a non monotonic dependence on the sampling time

Γ of the Power-Law distribution is obtained from experimental data



$\Delta U_{tot,j}$ is negative for all sampling times except for t = 4 h

Aggregation $j \rightarrow j+1$ is a more energetic costly process for the Culture 1 (NB) for most of the sampling times.

Conclusions

✓ Diluting the nutrient concentration, the number of flocs of *P. extremaustralis* 2E-UNGS increased significantly confirming that NB-½ stimulated aggregation.

✓ The proposed model based on micelles can describe the behaviour of aggregates from the point of view of energy cost after comparing the histograms of their areas between both kinetics studied.

Acknowledgments

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