- Transformation of aromatic pollutants -

Population dynamics of *Ralstonia eutropha* JMP 134 on different substrates

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Abstract

Ralstonia eutropha JMP 134, which is able to grow on phenol is an ubiquitous occurring gram-negative strain of the ß-group of bacteria. It is used as a model organism for the investigation of the growth characteristics of single bacterial strains in mixed populations. With respect to this aim it is exposed to conditions which are similar to natural settings.

Xenobiotica utilizing organisms from natural settings are part of mixed populations whose individuals must be distinguished specifically according to their class, subclass and species in order to get information about their metabolic activity. Group specific and species specific probes for *Ralstonia eutropha* JMP 134 are used.

The individuals were characterized regarding the proliferation activity (measurement of changes in the DNA and rRNA content) and the capacity of assimilation of metabolites via measurement of the membrane potential. The interpretation of the vitality of a bacterial cell by using this method performs data about the actual energetic state of a cell, and contains insofar more information than simple live/dead estimations.

Investigations to the metabolic and generative characteristics of the strain by using different carbon sources (batch cultivation on pyruvate, phenol and acetate) are shown first. Furthermore transient state cultivations in continuous processes were done to investigate the proliferative activity under increasing concentrations of phenol.

Flow cytometry is recommended as an extended monitoring method for optimizing biotechnical decontamination processes of polluted environment using constructed mixed populations.

1. Experiments with mixed populations

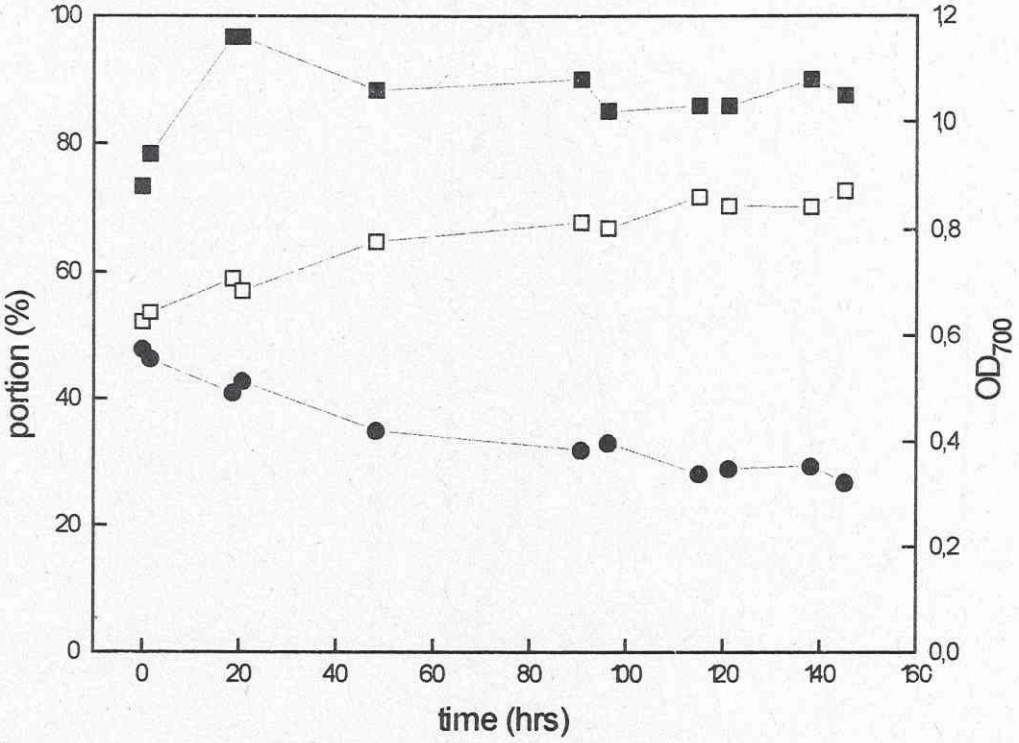


Fig. 1. Continuous cultivation of a mixed population and its optical density (■) consisting of *Ralstonia eutropha* JMP134 (●) and *Acinetobacter calcoaceticus* 69-V (□), which was added at t_0 to the chemostate (D = 0,1 h^{-1}). The population of *Acinetobacter calcoaceticus* 69-V remains in the system while the other population is washed out

The quantity of the membrane potential related fluorescence intensity (mprf) correlates obviously with the synthesis of rRNA and DNA as well as with the growth rates (Figure 2). Five hours later the fluorescence intensity of the membrane potential decreases despite of the increasing OD. The increase of the fluorescence intensity after seven hours is caused by non-specific cellular staining.

The quantity of the membrane potential related fluorescence intensity correlates with the synthesis of rRNA and DNA as well as with the growth rates similar to the growth on acetate (Figure 3). After depletion of phenol cell size, rRNA- and DNA content as well as the membrane potential related fluorescence intensity decrease.

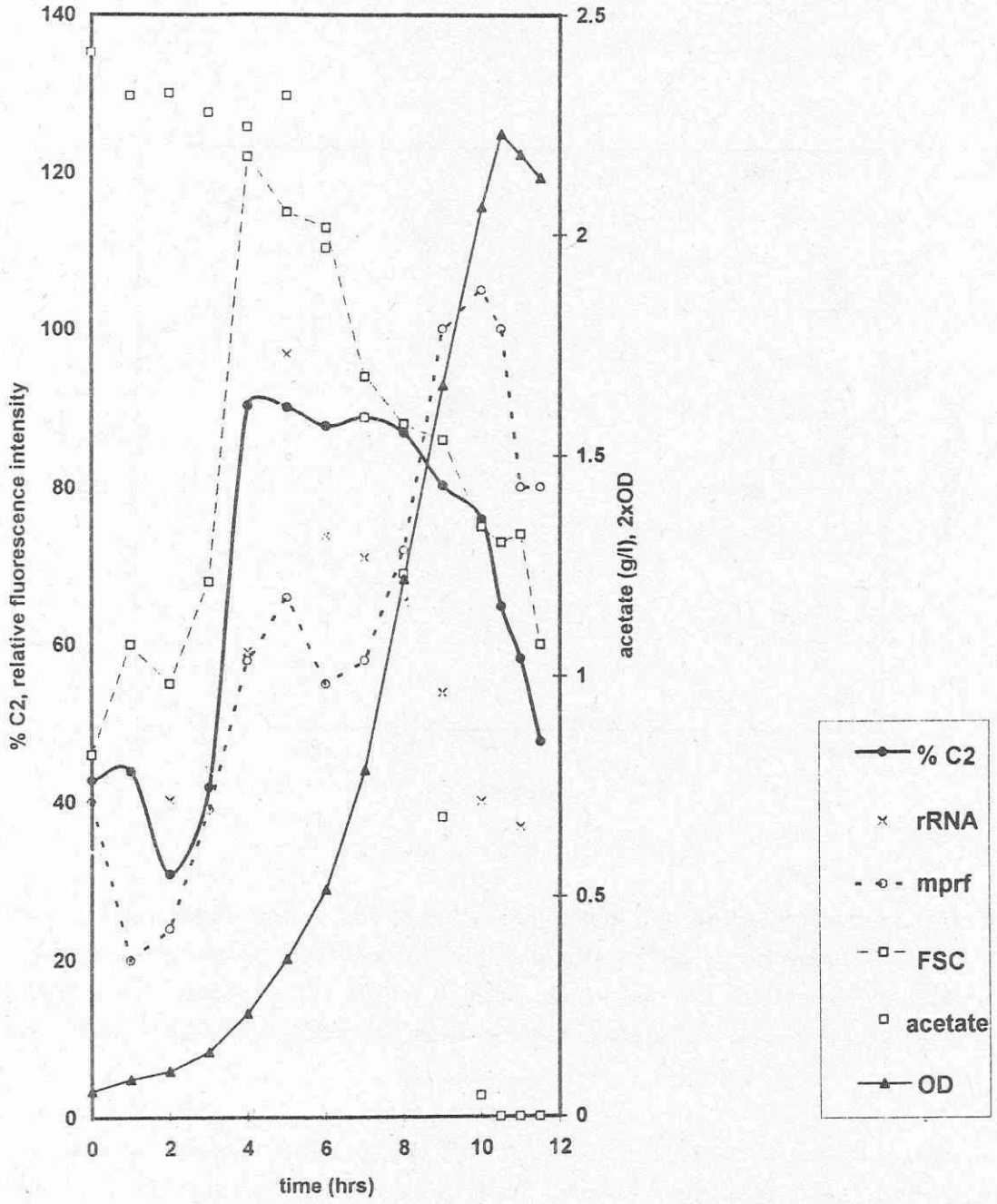
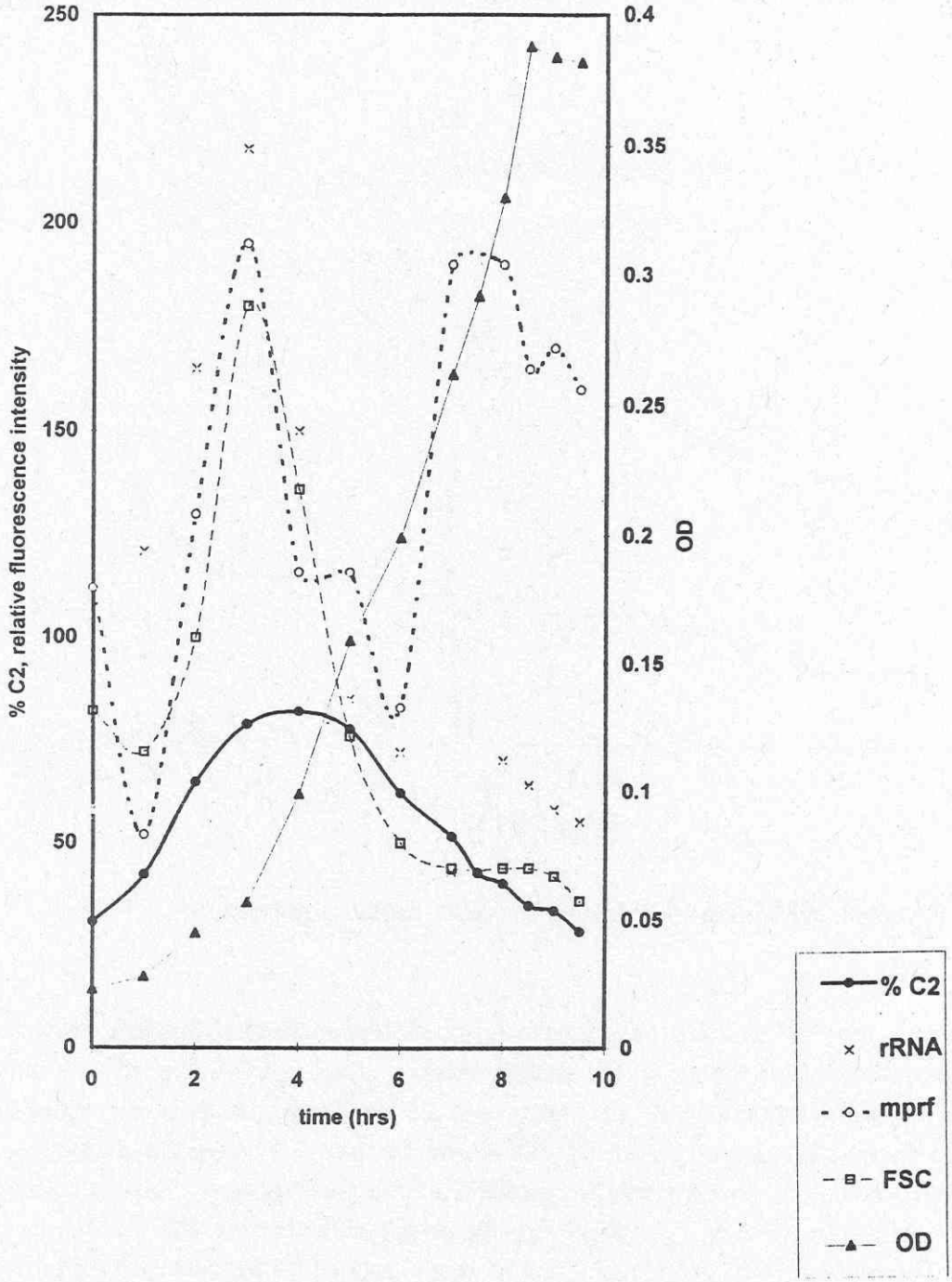


Fig. 2. Growth on acetate (batch-cultivation)

To reach steady state conditions chemostate cultivation must be performed (Figure 4). In the experiment shown the method of transient state cultivation is used. Via a successive increase in the nutrient flow stream in small periods quasi-stationary states are achieved. The conditions of constant growth allows firstly, a more distinct estimation of the generation time and secondly, an investigation of the changing growth characteristics of *Ralstonia eutropha* JMP 134 under known and controlled conditions. The effects of increasing concentrations of phenol on the proliferative activity of the bacterial cells were investigated.



measurement of the DNA: via counting the amount of cells with two chromosomes

measurement of the rRNA: via binding of oligonucleotide probes directed to a group (ß) and strain specific level

measurement of the membrane potential: see techniques

measurement of the FSC: via small angle scatter (3-9°)

measurement of acetate and phenol: via HPLC

Fig. 3. Growth on phenol (batch cultivation)

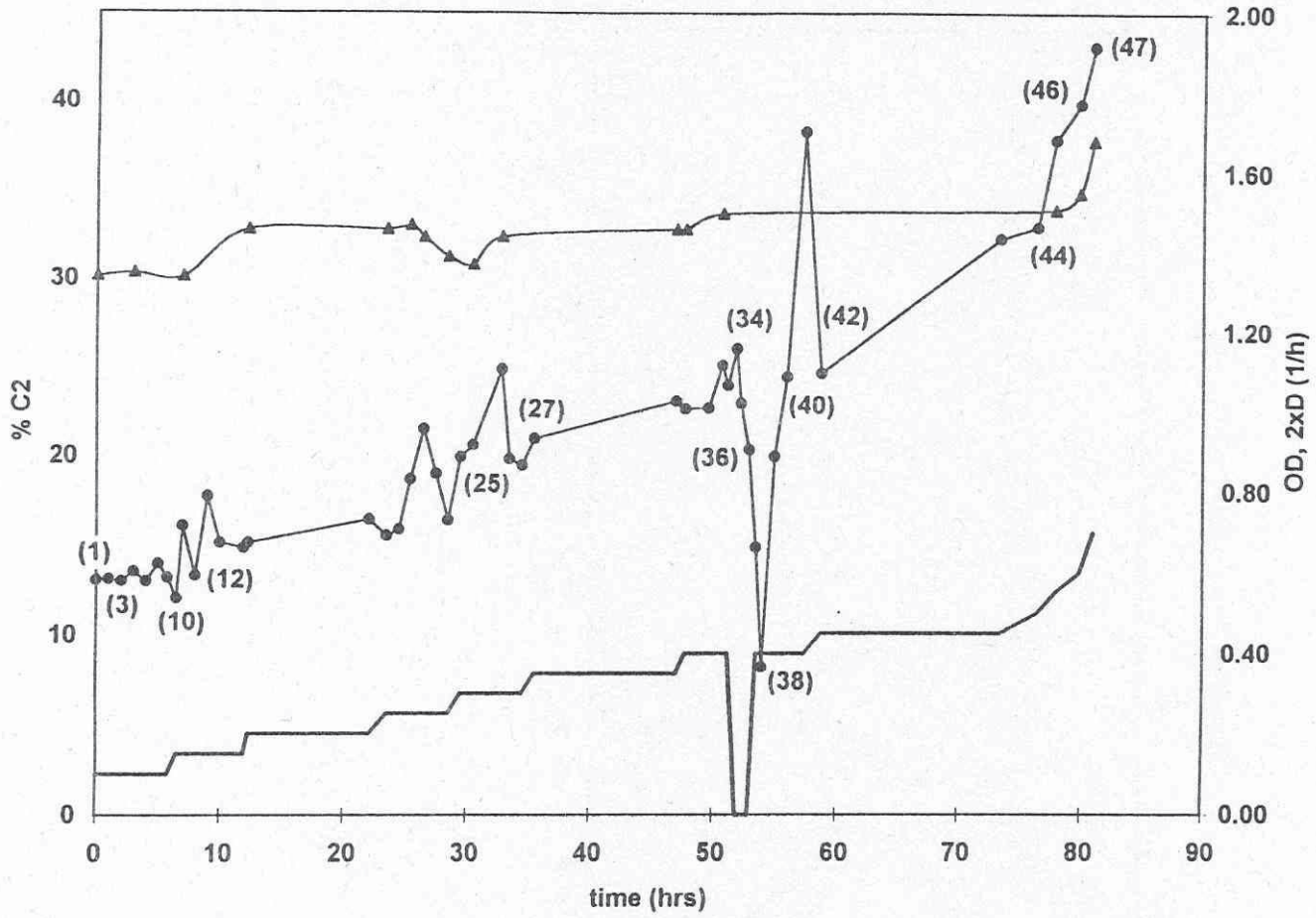


Fig. 4. Transient state cultivation of *Ralstonia eutropha* JMP134 on phenol

The typical course of bacterial cells grown on phenol by increasing nutrient flow rates. The given dilution rates correspond to the growth rates μ, whereby the actual concentrations of phenol are known to be very small. The increase in the proliferation activity correlates with an increase in the dilution rate very well. By this reason we used it as a reliable parameter for 'vitality' of the population, demonstrated as % cells with two chromosomes. The estimation of both the size of the cells and the optical density is not able to give the same value of information. The measurement of the membrane potential related fluorescence intensity is not workable for the control of such industrial decontamination processes because of logistical reasons. The degree of sensitivity of the method is proved by incorporating a carbon exhaustion phase. The state of vitality could be estimated via measurement of cell division and following replication by application of new substrate.

2. Techniques

Single bacterial cells are characterized by using the dye 3,3'-dihexyloxacarbocyanine, which is known to show the quantity of the actual membrane potential. $DiOC_6(3)$ is bound to the inner

lipid bilayer because of its lipophilic character and cationic charge. By this reason the dye functions for detection of living cells in contrast to dead cell dyes like from the oxonol class or dyes used for estimating enzyme activities.

The following parameters should be settled to establish the method for characterization of the physiological state of *Ralstonia eutropha* JMP 134:

1. Staining method for estimation of the membrane potential:
   The optical density of the bacterial culture is estimated by 700 nm. Without delay cells were centrifuged (3 min, 6000g) and adjusted to 3 x 10^8 cells/ml using imidazole-HCl-buffer (20 mM, pH 7.0). Following 5µl $DiOC_6(3)$ (0.12 µM) were applied and measured flow-cytometrically.

2. The disadvantage of the non-specific staining of the intracellularly lipophilic compound within the bacterial cell is observed in the case of to much concentrations of the dye as well as in the case of staining highly stationary phase cells. By these reasons hyperpolarization and depolarization of the membrane potential is provoked (via gramicidin and valinomycin), to prove the specificity of the dye binding.

3. The alteration in substrate uptake capability via measurement of the membrane potential is determined in dependence of both the kind of carbon source and the growth rate.

3. Conclusions

Using flow cytometry *Ralstonia eutropha* JMP 134 was characterized concerning proliferation activity (by measuring of the cellular DNA content), activity of the protein synthesis (by measuring the rRNA content) and capability of substance uptake (by measuring of the membrane potential). Information about changing cell sizes were obtained by measuring of the light scatter.

The analysis of these characteristics qualified for estimation of the temporary physiological state of a single cell within a bacterial culture. The physiological states could be observed in mixed cultures operating in-situ hybridization techniques.

In the future the application of the method should enable for process optimization during industrially performed decontamination of polluted soils running artificially mixed populations.

References

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