Assessment of the Kinetics of Phenol Bioconversion by *Pseudomonas sp. OX1*

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The present contribution reports on the characterization of the kinetics of phenol conversion by *Pseudomonas. sp. OX1* in a continuously-operated, stirred reactor. The investigation has been carried out in a 2 L reactor, mechanically stirred. The phenol containing solution is delivered at dilution rates (D) varying from 0.025 to 0.5 h^{-1}. The phenol conversion process has been characterized in terms of time resolved measurements the concentration in the liquid phase of phenol, cells, 2-hydroxymuconic semialdehyde, the oxygen uptake rate, the total organic carbon and the total nitrogen. Data were processed to assess the kinetic models - and related parameters – of phenol conversion and cell growth under a wide range of operating conditions, from quasi-batch up to values close to the wash-out conditions. The bacterial maintenance was also assessed for growth on phenol. Kinetic parameters and phenol-to-biomass fractional yield coefficient were significantly different from those estimated during batch cultures. The maximum phenol degradation rate was of about 0.5 g/(L h).

1. Introduction

Phenolic compounds and their derivatives are present in significant amounts in raw wastewaters from different industrial sources. Their concentration must be strictly limited because of the deleterious effect of these molecules on a variety of biochemical functions. Several bacterial strains are able to use aromatic hydrocarbons as a primary source of carbon and energy (Whited and Gibson, 1991). The wide range of aromatic substrates that can be metabolized by these microrganisms constitutes a powerful tool for the bioremediation of environmentally harmful compounds.

Pseudomonas sp. OX1 was isolated from the activated sludge of a wastewater treatment plant, and its ability to mineralize several aromatic hydrocarbons (Baggi et al., 1987) might be relevant for bioremediation strategies. This bacterium grows using as unique carbon and energy sources a large variety of aromatic molecules including phenol, cresols and dimethylphenols, and also non-hydroxylated molecules, even at high concentration (Baggi et al., 1987; Bertoni et al., 1996; Viggiani et al., 2006). Lodato et al. (2007) have showed that this microrganism may convert azo-dyes under anaerobic conditions. In particular, cells may convert any amount of dye if a balanced cycle of anaerobic-aerobic process is carried out. It is likely that metabolites accumulated during the aerobic phase, associated to growth/maintenance of *Pseudomonas sp.* OX1, are

responsible for dye conversion during the subsequent anaerobic phase.

It should be taken into account that phenol degradation by *Pseudomonas* strains is generally limited both by substrate inhibition and by low specific conversion rates (Yang and Humphrey, 1975; Onysko et al., 2002). These issues have stimulated efforts toward adoption of reactor configurations that are specifically suitable for the potential of *P. species* and provide process intensification. In particular, the immobilization of the microrganisms on solid carriers combined with the use of three-phase (gas/liquid/solid) fluidised bed reactors may be adopted for process intensification (Heijnen et al., 1990; Bryers, 2000; Russo, 2008). In any case, design and optimized operations of the bioreactor with free or immobilized cells need a kinetic characterization of the process under operating conditions close to the industrial applications.

A research program is active at the Chemical Engineering and at the Functional and Structural Biology Departments of the University of Naples Federico II aiming at the development of biotechnologies for bioremediation of water effluents with free and immobilized microbial cells. The present contribution is focused on the kinetic characterization of the phenol conversion process by free *Pseudomonas sp.* OX1 in a continuously operated well stirred reactor. The phenol conversion process has been characterized in terms of time resolved measurement of the concentration in the liquid phase of phenol (Ph), cell (X), 2-hydroxymuconic acid semialdehyde (HMAS), oxygen uptake rate (OUR), total organic carbon (TOC) and total nitrogen (TN).

2. Materials and method

2.1 Microrganisms and culture media

Pseudomonas specie OX1 was a gift of Dr. Paola Barbieri (Functional and Structural Biology Department, Università dell'Insubria, Varese, Italy). Stock cultures were maintained, through periodic subculture every week, at 4°C on agar-M9 plates containing 20 mM glucose (Sigma-Aldrich) as carbon and energy sources as reported in Viggiani et al. (2006).

The composition of the liquid medium (pH=6.9) adopted in pre-cultures and cultures is reported by Viggiani et al. (2006). Phenol was used as carbon source.

2.2 Apparatus

Figure 1 reports a sketch of the experimental apparatus. Continuous cultures were carried out in a 2 L bench-scale mechanically stirred bioreactor (Biobundle - Applikon) equipped with two 35 mm "Rushton Turbine" stirred on the axel. The temperature of the reactor was controlled by external circulating water in the glass jacket. Oxygen supply to the culture has been ensured by sparging air at the bottom of the reactor and by proper setting of the mixing rate. Dissolved oxygen was determined by a "Clark-type" probe (Mettler-Toledo, INPRO 6050). Air flow was sterilized by means of 0.22 μm filters (Millex FG - Millipore). A water-cooler at the gas outlet acts as humidity trap to minimize water loss by air stripping. Liquid was continuously fed and withdrawn

to/from the reactor by means of a peristaltic pump (Gilson Miniplus 3). Sampling was carried out by means of a submerged pipe equipped with a silicone septum at the top.

The liquid reservoir was a 50 L stainless steel tank equipped with six ports located at the top.

Sterilization of the bioreactor and ancillary apparatus was carried out *in situ* by means of 3 bar saturated steam provided by a steam generator (PG AUTO 9 – Lelit). Sterilization conditions were: 100°C for 40 min.

2.3 Operating Conditions and Procedure

All tests were carried out at 30°C and no pH control was adopted.

Pre-cultures were grown in previously autoclaved 250 mL shake bottles filled with 100 mL of M9 medium and phenol (2 mM). Bottles were incubated for 15 h on a rotary shaker at 125 rpm and 30°C.

The reactor with 1L medium (phenol 190 mg/L) and any ancillary apparatus were steam sterilized for 40 min. Air flow rate and agitation were set at 200 nL/h (3.3 vvm) and 500 rpm, respectively. 100 mL of preculture in late exponential growth phase was inoculated into the reactor and growth was carried out under batch-wise conditions for 1 day. Then the phenol bearing stream was fed continuously at the set flow rate. Drained liquid flow rate was measured by time resolving the weight of the liquid collected at the outlet. Sampling were carried out from bioreactor and reservoir typically at a time interval of about 1h. The phenol conversion process has been characterized in terms of time resolved measurements of pH, X, Ph, HMAS, OUR, TOC and TN in the liquid phase. Typically, runs lasted between 7 and 20 days before the reactor was stopped,

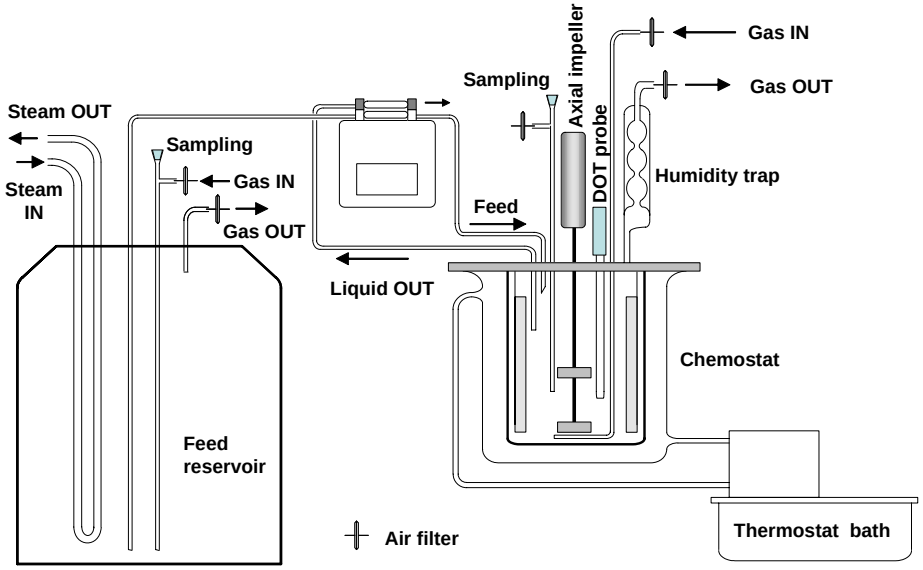

Figure 1 – Experimental apparatus.

cleaned, sterilized and a new run started.

2.4 Analysis

Analysis of culture samples was carried out after centrifugation at 11,000 rpm for 10 min. The solid phase was characterized for biomass concentration. The cell density was measured as optical density at 600 nm (Cary 50 -Varian). Calibration tests indicated that the optical density is proportional to *P. specie* dry mass and under the operating conditions tested it resulted 1.5 OD_{600}=1 g_{DM}/L. Elemental analysis of dry biomass was carried out by a C/H/N 600 LECO analyser. Liquid phase of the sampled cultures was characterised in terms of concentration of soluble components. pH was measured by a pHmeter (Hanna Instruments). Phenol and HMAS concentration were measured by UV-VIS absorption at 271 and 380 nm respectively (ε_{271}=1.65 $mM^{-1}cm^{-1}$, ε_{380}=29 $\mu M^{-1}cm^{-1}$). TOC and TN were measured by means of a TOC/TN analyzer (V_{CSH} – Shimadzu). OUR was measured by means of a respirometric assay according to the APHA Standard Methods (1992) in a 10 mL bottle equipped with a DOT probe.

3. Kinetics and Stoichiometry Model

The growth stoichiometry of an aerobic microbial culture can be described by means of a non-structured model:

$$\frac{1}{Y_{X/TOC}}TOC+\frac{1}{Y_{X/O2}}O_2+\frac{1}{Y_{X/TN}}TN+...\rightarrow X+.... \quad (1)$$

where $Y_{X/TOC}$, $Y_{X/O2}$, $Y_{X/TN}$ are the fractional yield coefficient of biomass with respect to TOC, O_2 and TN. Similar fractional yields can be defined referring to the elemental biomass composition ($Y^{C}_{X/TOC}$, $Y^{O}_{X/O2}$ and $Y^{N}_{X/TN}$). The fate of each substrate may be distinguished in two paths: the consumption of substrate for growth and for maintenance. In agreement with the model proposed by Pirt (1965) and with reference to a generic substrate S it results:

$$\frac{1}{Y_{X/S}}=\frac{1}{Y^{*}_{X/S}}+\frac{m_S}{\mu} \quad (2)$$

where $Y^{*}_{X/S}$ is the theoretical maximum fractional yield and m_S the maintenance coefficient.

The material balance referred to cell, phenol and oxygen and extended to the reaction volume under steady state conditions are:

$$\mu=D \quad (3a)$$

$$\frac{\mu}{Y^{*}_{X/Ph}}X^{OUT}+m_{Ph}X^{OUT}=D\left(Ph^{IN}-Ph^{OUT}\right) \quad (3b)$$

$$OUR = K_L a_L \left(O_2^{eq} - O_2^{OUT} \right) \tag{3c}$$

The model eq.s 3 relies on the following assumptions: i) the maintenance contribute to the phenol consumption (see eq. 2) was taken into account; ii) convective flow of oxygen in the liquid streams was neglected; iii) the contribute of gas convective flow to the phenol balance was neglected (Viggiani et al., 2006).

The growth kinetics of free *P. sp. OX1* in batch culture by adopting phenol as carbon source was characterized by Viggiani et al. (2006). In particular, the kinetics was described by the Haldane model:

$$\mu = \mu^M \frac{Ph}{K_{Ph} + Ph + \frac{Ph^2}{K_I}} \tag{4}$$

Viggiani et al. (2006) reported values of $\mu^M = 0.71\ h^{-1}$, $K_{Ph}=0.31$ g/L, $K_I=0.13$ g/L. The kinetics was such that the maximum of $\mu=0.17\ h^{-1}$ is at Ph=0.2 g/L. Moreover, the fractional yield coefficient $Y_{X/Ph}$ resulted 0.82 for Ph<400 mg/L and decreased to zero for Ph approaching 600 mg/L.

4. Results and Discussions

The reactor system has been characterized for what concerning the residence time distribution of the liquid phase and the gas-liquid mass transport phenomena under operating conditions of interest for the present investigations (Chianese, 2007). In particular, the reactor behaves as a CSTR even over a time scale one order smaller than that characteristic of the conversion process, 6 h (inverse of the expected maximum value of $\mu=0.17\ h^{-1}$).

Figure 2 shows relevant data of a representative run carried out in the bioreactor. The reactor was operated under batch conditions for one day, then the phenol bearing stream (Ph^0 = 450 mg/L) was fed to the reactor at volumetric flow rate Q=55 mL/h ($D=0.055\ h^{-1}$). The process was characterized in terms of soluble species concentration and of OUR until steady state conditions were established, and then the liquid volumetric flow rate was stepwise increased. The vertical dashed lines mark the times at which the dilution rate was changed. The pH was about 6.9 throughout the run, without any pH control. The concentration of HMAS (data not reported) was characterized by a sharp increase after one day of continuous operation up to value of about 16 μM and a decay to negligible values within two days. Data measured during the run reported in Fig. 2 were worked out according to the model described in section 3 (eq.s 1, 2 and 3) and value of the results are reported in Fig. 3 as a function of D (run 1).

Data of phenol-to-biomass fractional yield coefficient ($Y_{X/Ph}$) and phenol in the reactor are reported in Figure 3 as a function of the dilution rate and parametric in the phenol

concentration in the fed stream, ranging between 0.44 and 1.0 g/L. It is noteworthy that data did not depend on the run and on the culture age (the time measured since the reactor inoculation). Therefore, the possible effects of wall growth of cells may be ruled out.

The agreement of the $Y_{X/Ph}$ vs D data with the Pirt model (eq. 2) appears satisfactory. Data regression yields: the theoretical maximum fractional yield $Y^*_{X/Ph}$ =1.31 g_{DM}/g and the phenol maintenance coefficient m_{Ph}=0.0034 g/(g_{DM}·h).

Figure 3 shows that the phenol conversion was about complete for D smaller than 0.45 h^{-1} and that wash-out conditions establish at D between 0.45 and 0.6 h^{-1}. Moreover, it should be mentioned that the culture age of the test at D=0.6 h^{-1} was of about one week. These observations further support the negligible role of the cell wall growth on the performance of the reactor.

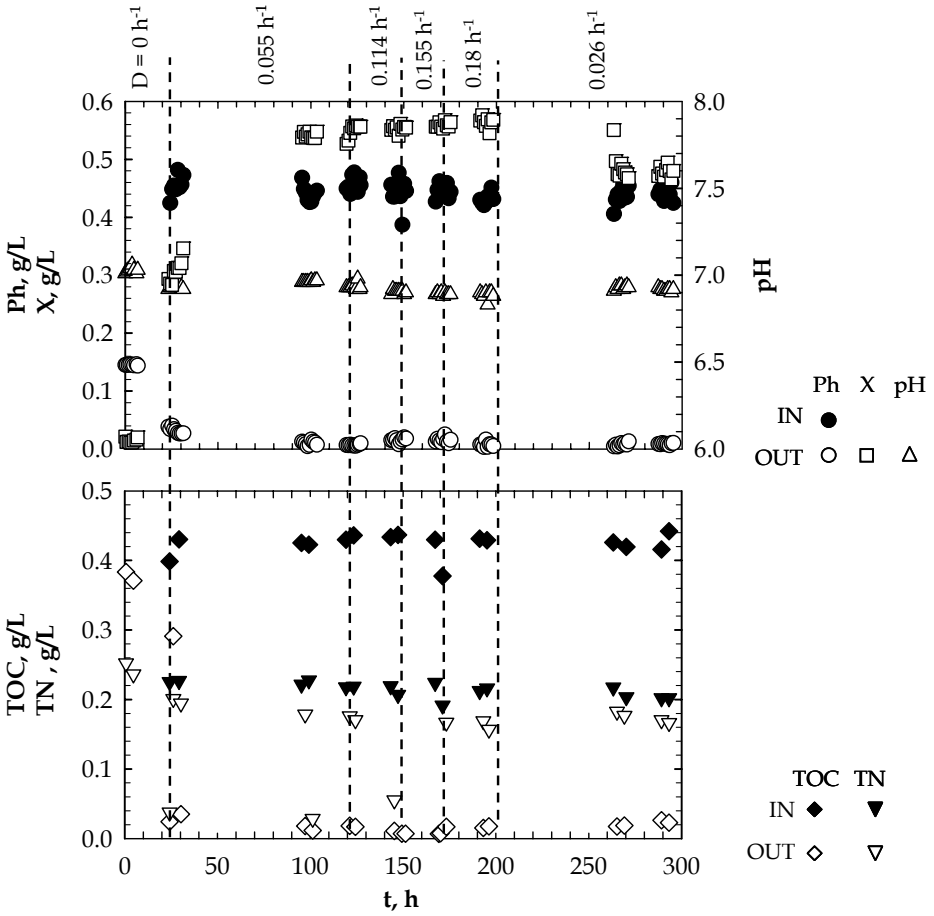

Figure 2 – Relevant data measured during a continuous culture of Pseudomonas sp. OX1. Medium: M9 added of phenol (carbon source).

The performance of the bioreactor in terms of amount of phenol degraded for unit of time and volume was estimated by working out data of liquid flow rate and phenol conversion measured during the tests. It results that the maximum throughput of the bioreactor was about 0.50 g_{Ph}/(L h) at a phenol concentration in the feed stream of about 1.0 g/L. The performance resulted quite high when compared with data available in literature (Allsop et al., 1992; Feitkenhauer et al., 2003).

The comparison of the kinetic parameters and fractional yield coefficient, $Y_{X/S}$, estimated in the present work with those estimated in batch culture experiments (Viggiani et al., 2006) is now in order Under continuous culture conditions the maximum growth rate results about 3 times that estimated by means of batch cultures (0.6 h^{-1} vs 0.17 h^{-1}). The $Y_{X/S}$ in continuous culture conditions is 1.5 times that estimated in batch culture conditions. The difference between the two sets of data suggests that the growth behaviour of *P. sp. OX1* strongly depends on the culture modality, similarly to previous investigation carried out with other bacteria (Strobel, 1995; Feitkenhauer et al., 2003). The higher value of $Y_{X/S}$ measured in continuous culture should indicate that phenol was degraded very efficiently by *P. sp. OX1*. Partial accumulation of metabolites in the liquid phase during the first transient period points out that the pathways to complete the phenol conversion may be kinetically disadvantaged with respect to the initial phenol degradation pathway. The observed behaviour may be due to the time-scale of the metabolic mechanism and in particular to the comparison of the cell relaxation time with the time scales of the reactor (Bailey and Ollis, 1986). In batch conditions the environment of cell growth changes continuously with the time (nutrient depletion, biomass increase etc.). In a continuous culture growth conditions remain stable in the time. Therefore, it is evident that for some bacteria the batch cultures may not be considered representative of quasi-steady states and non structured models can not be adopted.

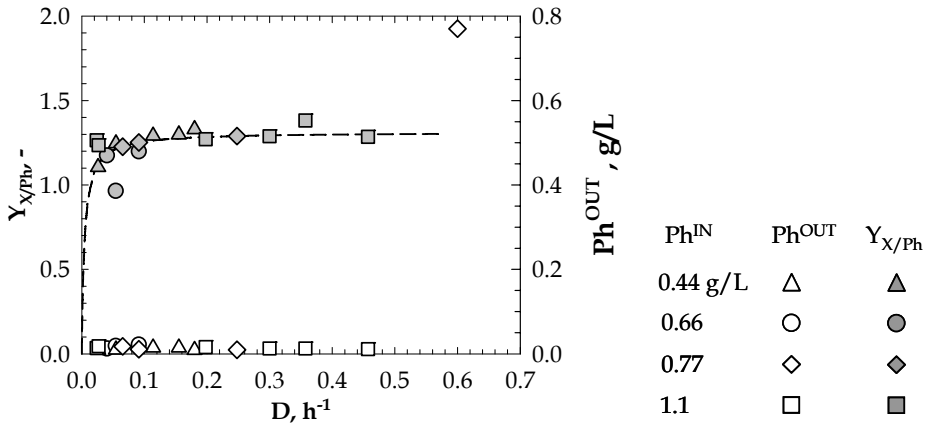

Figure 3 - Phenol to biomass fractional yield and phenol concentration in the reactor as a function of the dilution rate.

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Nomenclature

D	Dilution rate	Ph	Phenol concentration
HMAS	2-hydroxymuconic acid semialdehyde	TN	Total nitrogen carbon
		TOC	Total organic carbon
K_La_L	Mass transfer coefficient	X	Biomass concentration
m	maintenance coefficient	Y	Fractional yield coefficient
O_2	Oxygen concentration		
OUR	Oxygen uptake rate	μ	Biomass specific growth rate

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