CHEMICAL ENGINEERING TRANSACTIONS
VOL. 49, 2016
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.aidic.it/cet
Guest Editors: Enrico Bardone, Marco Bravi, Tajalli Keshavarz

ISBN 978-88-95608-40-2; **ISSN** 2283-9216
DOI: 10.3303/CET1649073

The Importance of pH on the Biotransformation of Bromoxynil by *Microbacterium imperiale* CBS 498-74 Resting Cells

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This work enlarges our previous results (Pasquarelli et al., 2015) on the effect of pH on the bioconversion of 3,5-dibromo-4-hydroxybenzonitrile (bromoxynil) using *Microbacterium imperiale* CBS 498-74 resting cells. Their sequential enzymes, nitrile hydratase and amidase, which operate in cascade, transform the nitrile into the corresponding acid, *via* an amide as intermediate. This paper highlights the influence of different pH on the kinetic parameters, V_{max} and K_M, the stability of the enzymes and the completeness of bromoxynil bioconversion in batch reactors. Results from continuous stirred UF-membrane reactors (CSMR) suggest, for real application and high conversion yield (near 75% for more than 180 h continuous process), to operate at pH 6.5 realising the best compromise between enzyme stability, high V_{max}. This even if at pH 7.0 the enzyme half life is higher.

1. Introduction

Bromoxynil, 3,5-dibromo-4-hydroxybenzonitrile, is classified as potentially toxic for human health and extensive use, as pesticide, represents a great concern (Holtze et al., 2008). Sorption studies in soil have shown a medium mobility of bromoxynil and a high mobility of its amide predisposing them to a certain extent for leaching to underlying groundwater.
Numerous strategies have been suggested for pesticide reduction such as bioaugmentation, which introduces or stimulates herbicide-degrading microorganisms in the soil (Schultz-Jensen et al., 2014).
Accordingly, the control under bioreactor operation might be helpful. The literature suggests a biofilm reactor (Müller and Gabriel, 1999) and inoculated biofilters (Albers et al., 2014) able to degrade bromoxynil and its metabolites within a short time.
Many microorganisms are able to transform nitrile compounds through their degradation pathways. *Actinobacteria*, *Agrobacterium radiobacter*, *Aminobacter*, *Fusarium solani*, *Klebsiella pneumonia*, *Pseudomonas*, *Rhizobium*, *Rhodococci*, *Streptomyces felleus*, *Sphingobium chlorophenolicum*, *Variovorax* species and *Microbacterium imperiale* CBS 498-74 (Albers et al., 2014; Holze et al., 2008; Müller and Gabriel, 1999; Schultz-Jensen et al., 2014; Veselá et al., 2012; Frková et al., 2014, Pasquarelli et al., 2015) are able to catalyse the bromoxynil degradation. Some microorganisms start bromoxynil biodegradation, hydrating the bromoxynil nitrile group into its corresponding 3,5-dibromo-4-hydroxybenzamide. This reaction is catalysed by nitrile hydratase (EC 4.2.1.84), NHase. The further hydrolytic reaction into the corresponding 3,5-dibromo-4-hydroxybenzoic acid and ammonia is catalysed by amidase (EC 3.5.1.4), see Figure 1. The lack of amidase in some microorganisms let accumulate the intermediate product, 3,5-dibromo-4-hydroxybenzamide. Some other microorganisms transform in one step, catalysed by nitrilase enzyme (EC 3.5.5.1), the bromoxynil into the corresponding acid (Martínková and Křen, 2010).
Our recent study on bromoxynil biodegradation (Pasquarelli et al., 2015) performed with resting cells of *M. imperiale* CBS 498-74 compared the kinetic behaviour of crude extract preparation and resting cells. Even tough diffusional resistances in resting cells are present, the higher stability at pH 7.0 and the possibility to

Please cite this article as: Pasquarelli F., Chuchat N., Spera A., Cantarella L., Cantarella M., 2016, The importance of ph on the biotransformation of bromoxynil by microbacterium imperiale cbs 498-74 resting cells, Chemical Engineering Transactions, 49, 433-438 DOI: 10.3303/CET1649073

drive near completeness the reaction to the less toxic product were highlighted and indicated the resting cells as a good candidate for real application. The toxicity tests, EC50, indicated the following series 3,5-dibromo-4-hydroxybenzonitrile, 5.0 ± 2 μM, > 3,5-dibromo-4-hydroxybenzamide, 7.9 ± 0.1 μM, > 3,5-dibromo-4-hydroxybenzoic acid, 42.0 ± 2 μM (Veselá et al., 2012). Resting cells of *M. imperiale* CBS 498-74 have been used for the hydration of a large number of nitriles to their corresponding acid and ammonia, *via* an amide as intermediate (Cantarella et al., 2008, 2011, 2013, 2014).
This second paper focuses only on *M. imperiale* resting cells enhancing the investigation on the pH effect on bromoxynil biodegradation. The kinetic parameters and the enzymatic stability were assessed in the whole pH-range. Both batch and continuously stirred UF-membrane bioreactors (CSMR) were operated either in differential or integral mode to assess optimal conditions for total conversion of 3,5-dibromo-4-hydroxybenzonitrile. Interestingly, the dependence from pH of both kinetic parameters and stability allows to achieve, at pH 6.5 with a higher rate, a high conversion of bromozynil in long-term experiments (> 140 h).

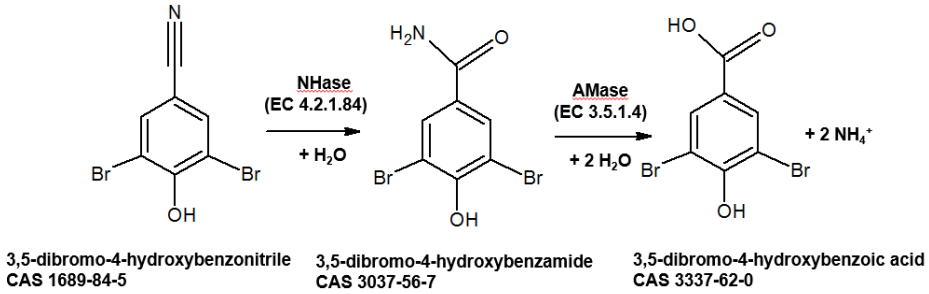

Figure 1: Bromoxynil biodegradation via a two-step catalized pathway from M. imperiale CBS 498-74.

2. Materials and methods

2.1 Microorganism

Microbacterium imperiale CBS 498-74 was cultured as reported elsewhere (Cantarella et al., 2002, 2008). The buffered suspention of resting cells was freshly prepared for each experiment in duplicate; the relative standard deviations calculated were < 5 %. 1.0 unit of OD_{610} corresponds to 0.26 mg_{DCW} mL^{-1}, 0.76 U_{NHase} mg_{DCW}^{-1} and 0.09 U_{AMase} mg_{DCW}^{-1} (DCW correspond to Dry Cell Weight).

2.2 Product analysis

HPLC analyses of substrate and products were performed on an HPLC Waters Alliance 2695 separation module (Waters Corp., Milford, MA, USA) equipped with a UV detector (Waters Corp.) and using a Waters Spherisorb column, 5 μm ODS2, 4.0 x 250 mm, maintained at 35 °C. The flow-rate of mobile phase, an aqueous solution of 25 % acetonitrile and 0.1 % H_3PO_4, was 1.0 mL min^{-1}; the injection volume of samples was 20 μL. The substrate and products were identified through their retention time, comparing them to calibration curves, obtained with external standards. Software Empower Waters analysed the data and quantified the compounds by integrating peak areas. All samples were analysed at least in two replicates.

2.3 Activity assay

One unit of NHase activity, U_{NHase}, was defined as the amount of enzyme that catalyses 1 μmol of acrylonitrile per min at 20 °C under stirring (250 rpm) with a proper amount of whole cells (5 or 10 mg_{DCW}) re-suspended in 2 mL of Na-phosphate buffer, 50 mM, pH 7.0, with acrylonitrile (100 mM), respectively. After 15 min incubation, the reaction was immediately quenched by addition of HCl (1 mL, 0.5 M) and centrifuged (10 min at 10,000 rpm). In the same way was defined and measured the U_{AMase} with acetamide (100 mM) as substrate. The products were evaluated through spectrophotometer method at 235 nm or HPLC analyses.

2.4 Batch Reactor

Bromoxynil conversion was investigated in jacketed batch reactor. A magnetic stirrer assured a 250 rpm stirring. The temperature control was ensured by external circulating water. The reaction was started adding in the buffered substrate, the amount of resting cells.

2.5 Continuous UF-membrane reactor (CSMR)

Continuous long-term experiments in CSMR were performed in a Amicon stirred cell module (Model 8010, Cat. No. 5121; Amicon, USA) fitted with a 10,000 MWCO UF-membrane FS81PP (DDS Danish separation system, DK), used only once avoiding interferences linked to membrane fouling. The reactor, magnetically stirred (250 rpm) for minimizing the concentration polarization phenomenon was filled with the appropriate

amount of resting cells. The substrate solution was fed using a BIORAD ECONO pump (USA), set at the suitable flow-rate. The module was thermostated in a water bath which temperature was controlled (± 0.1 °C). A fraction collector collected the reactor permeate, which was HPLC analysed.

3. Results and discussion

3.1 Effect of pH on the bromoxynil degradation Kinetics in batch reactor

Long-term experiments were performed in batch reactor to follow bromoxynil biotransformation at pH 6.0, 6.5 and 7.0. Product and substrate concentration are reported in Figure 2 where the left column refers to 150 U_{NHase} mL^{-1} and the right one to 300 U_{NHase} mL^{-1} of catalyst (see other details in the figure caption).

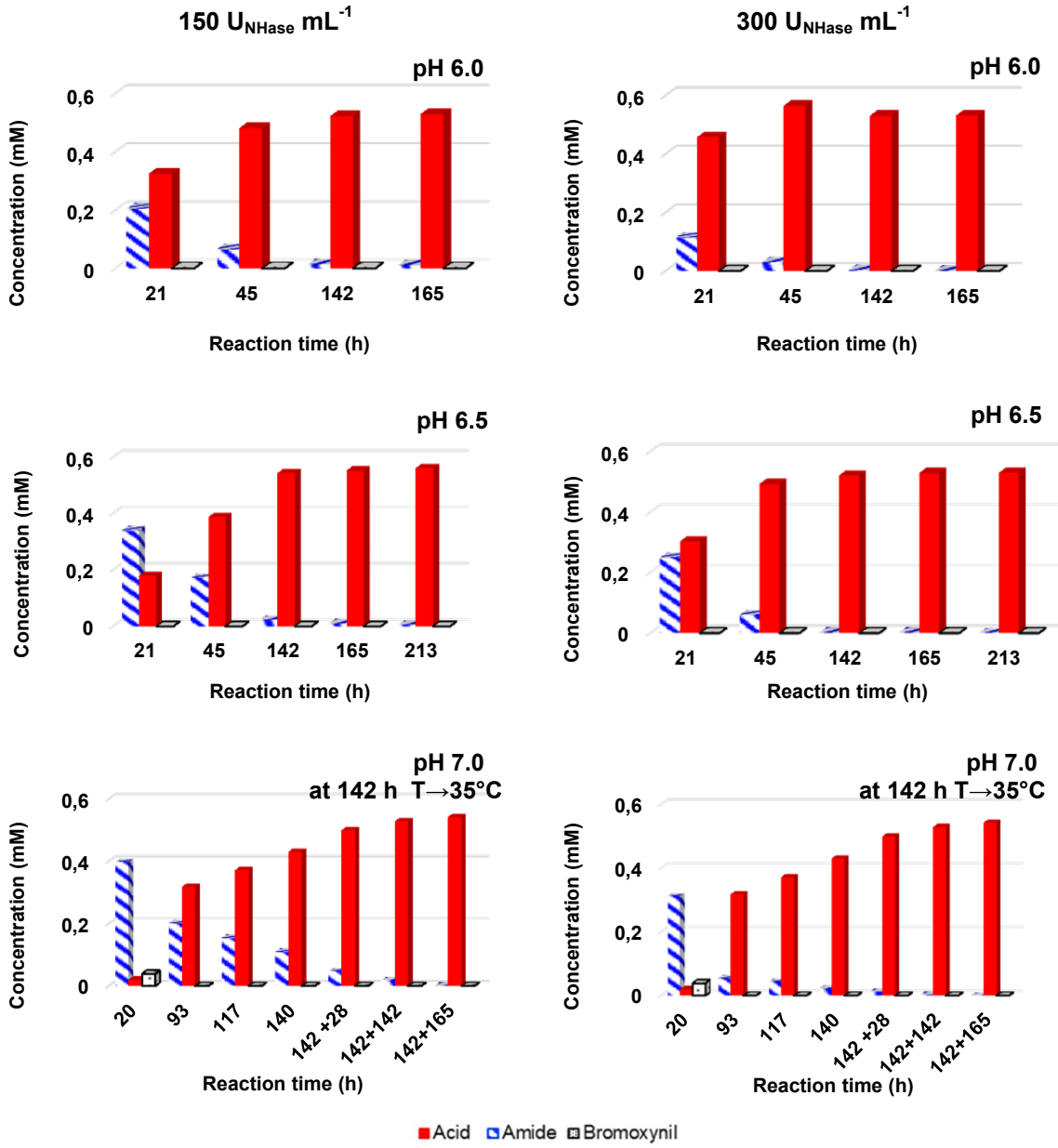

Figure 2: Substrate and product concentration vs. reaction time at different pH and resting cell loading 150 U_{NHase} mL^{-1} (left column), and 300 U_{NHase} mL^{-1} (right column). Reaction conditions: 20 °C, 250 rpm, 100 mL of 0.2 mM bromoxynil in 50 mM Na-phosphate buffer. Standard deviations of the product evaluation were < 5 %.

At pH 6.0 rather rapidly bromoxynil is totally converted into products and higher the cell load lower the time for reaching 100 % conversion. At pH 6.5 and 7.0, this latter pH has been generally used for other nitrile

biotransformation (Cantarella et al., 2014), the total bromoxynil bioconversion requires a longer reaction time. The ratio acid to amide is greatly dependent from the pH medium and at the lower pH the reaction is driven to the total conversion into 3,5-dibromo-4-hydroxybenzoic acid, the less toxic compound. At pH 7.0, to attain the same results, a further step at 35 °C, which activated, in other nitrile bioconversion, the AMase activity, was imperative. From these results it is clear that pH 6.0 requires 142 h and 150 U_{NHase} mL^{-1}; pH 6.5 requires same reaction time but double enzyme; at pH 7.0 it is necessary a double step at higher temperature and at double enzyme concentration. So far, these results weaken the previous one (Pasquarelli et al., 2015).

3.2 Effect of pH on the bromoxynil degradation kinetics

Bromoxynil (1 mM) biodegradation was investigated at different pH, at 20 °C, for 18 h reaction time, and in the presence of resting cells equivalent to 100 U_{NHase} mL^{-1}. Figure 3A shows the typical bell-curved behaviour obtained for the reaction rate *vs.* pH; the optimal pH being around 6.0, with phosphate buffer (Pasquarelli et al., 2015). Therefore, the kinetic parameters were evaluated in the substrate range 0.1 to 4 mM and at pH 6.0, 6.5 and 7.0. As shown in Figure 3B the v_{max} increases at values of pH lower than pH 7.0, and, at pH 6.0, its value approximates that obtained with a crude extract in the absence of possible mass-transfer phenomena due to the cell membrane (data not shown). The medium pH is less effective on the substrate affinity as the K_M remains roughly constant. In all kinetic assays (reaction rate *vs.* substrate concentration) the reaction appears substrate inhibited. The results achieved at pH 6.0 and illustrated in Figure 2 found their rationale in the difference in the kinetic parameters shown in Figure 3 B.

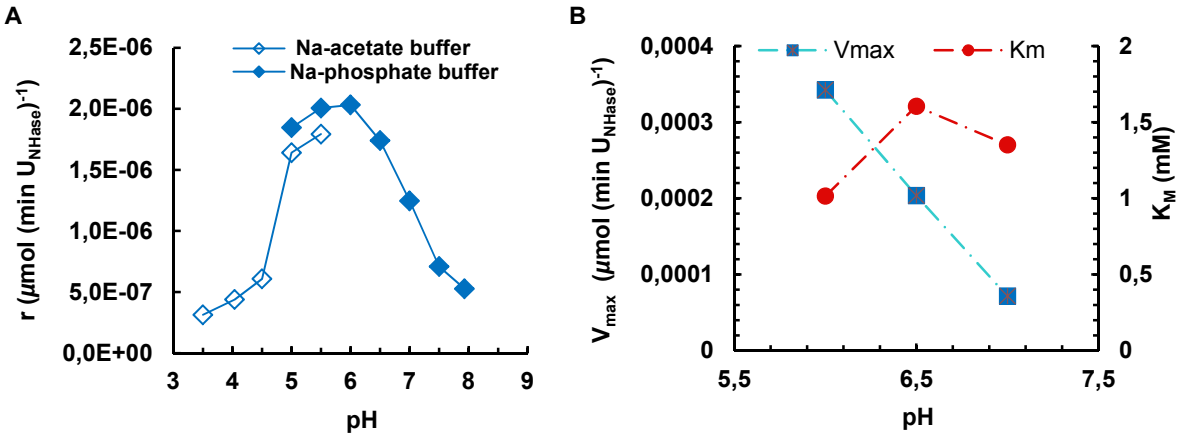

Figure 3: A-The effect of pH on the reaction rate. Reaction conditions : resting cells, 100 U_{NHase} mL^{-1}, bromoxynil 1 mM in Na-acetate and Na-phosphate buffer. B-Kinetic parameters at different pH and bromoxynil concentration ranging from 0.1 to 4 mM. Standard deviations of the product evaluation were always < 5 %.

3.3 Effect of pH on stability test in Continuous Stirred Membrane-UF Reactor

As known enzyme stability could be greatly dependent from the pH of the reaction medium. The pH-range (5.5 to 7.0) was explored by means of long-term experiments performed in continuous stirred UF-membrane bioreactors (CSMR) loaded with resting cell (150 U_{NHase} mL^{-1}) and fed with the substrate solution, 0.5 mM bromoxynil, prepared in 50 mM Na-acetate buffer at pH 5.5 and Na-phosphate buffer at higher pH-values.

The evaluation of the concentration of reaction products, collected every each hour, allowed the calculation of the specific reaction rate of NHase activity. A first-order deactivation mechanism, described by the second part of Eq(1), induced by pH was evidenced in Figure 4. Indeed, at steady state regime a linear response of the time course of the reaction rate in a semi-log plot is obtained.

$$r_t = \frac{Q \times [P]}{U_{NHase}} = r_0 e^{-k_d t} \qquad (1)$$

Where Q is the flow-rate (mL h^{-1}) and [P] is the concentration of amide formed (amide plus acid, both HPLC-detected) (mM). k_d (h^{-1}) is the kinetic deactivation constant and r_0, as µmol (min U_{NHase})$^{-1}$, is the initial specific reaction rate.

The straight line interpolating the data at steady state allows the evaluation of initial reaction rate and inactivation constant, r_0- and k_d-values, respectively, applying the logarithmic form of the second part of Eq(1). Table 1 summarises these parameters together with their regression coefficient, R^2, and the consequent half-life.

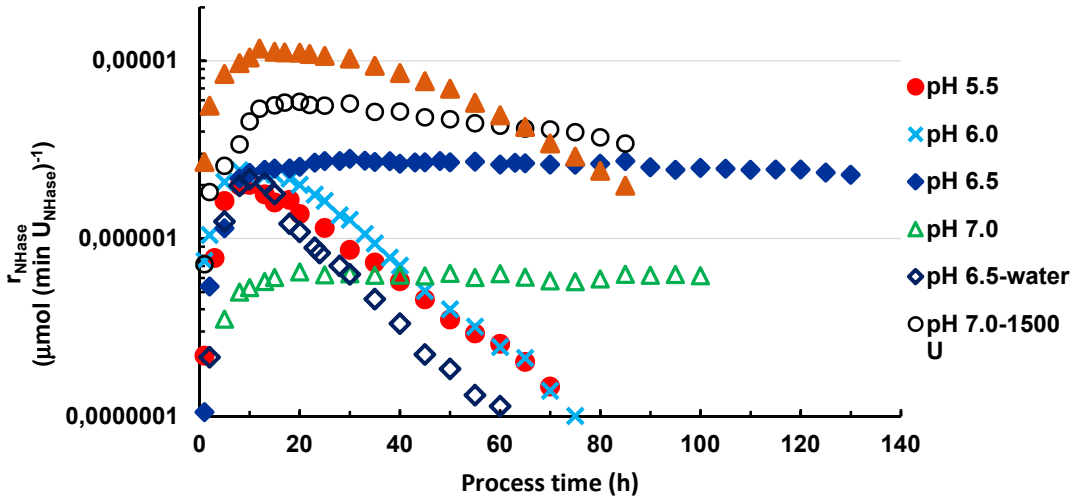

Figure 4: Biodegradation of 0.5 mM Bromoxynil at different pH. Time course of reaction rate in CSMR operated at 20 °C, 250 rpm and 11,000 U_{NHase}, unless differently indicated. Standard deviations of the product evaluation were always < 5 %.

Figure 4 compares the long-term experiments performed, at different pH, with a huge amount of resting cells, in the attempt, to achieve a complete biodegradation of bromoxynil. The runs at pH 5.5 and 6.0, which were performed because of favoured ratio acid/amide in batch reactor, unfortunately lose their activity rather rapidly. The bioreactor operated at pH 5.5 and 6.0 reached a high conversion, 73 % after 10 h operation. But after 30 h, the substrate conversion dropped around 25% (see Table 1 for details) and after 75 h of process time the enzyme activity ceased; the reaction products were no more detectable. Unfortunately, due to the scarce stability at pH 5.5, the high acid to amide ratio obtained (Pasquarelli et al. 2014) could not be realised in continuous bioreactors.
The CSMR operated at buffered pH 6.5 (11,000 U_{NHase}) reached at steady state a high substrate conversion, 78.9 % that remained constant for, at least, the following 150 h. The worst performances were obtained in a reactor whose pH, 6.5 was obtained in distilled water, not buffered, that could represent an economical process choice. Instead, the reactor operated at pH 7.0 reached a lower substrate conversion, 14.65 %, and the reaction rate remained constant until the end of the experiment. The ratio between the r_0 of these last runs is of one order magnitude different. The two runs were also performed at a lower amount of enzyme, hence operating the bioreactor differentially. The inactivation constant is of course higher (14x at pH 6.5 and 16x at pH 7.0) but the r_0 confirmed the data obtained in batch reactor; 2.8 fold increase at pH 6.5. Noteworthy is the fact that both reactors operated at pH 7.0 reached the same conversion after 30 h process, but the specific reaction rate is an order of magnitude higher at lower enzyme load; the hypothesis that part of the enzyme is excluded from the kinetics at pH 7.0 is sound. On the other hand, the higher half-life, at higher enzyme load, suggests that the inactivated enzyme is riplaced by the one exluded from the kinetics and thus still active.

Table 1: 0.5 mM Bromoxynil biodegradation in CSMRs performed at 20 °C, 250 rpm.

pH	U_{NHase}	% Conversion (30 h)	% Conversion (70 h)	r_0 (µmol (min*U_{NHase})$^{-1}$)	k_d (h^{-1})	R^2	Half–life (h)
5.5	11,000	24.15	4.85	0.000003	0.044	0.996	15.75
6.0	11,000	26.00	2.48	0.000006	0.054	0.998	12.84
6.5	11,000	78.90	74.77	0.000006	0.002	0.886	346.57
7.0	11,000	14.65	14.11	0.0000006	0.0005	0.144	1,386.29
6.5	1,000	19.00	6.73	0.00002	0.028	0.989	24.75
7.0	1,500	14.77	10.20	0.000007	0.008	0.976	86.64

As shown in Table 1 pH affects the half–life ($t½ = \ln 2/k_d$) of the enzyme, dramatically. The reactors operated, in integral mode, at pH 6.5 and 7.0 appear possible candidate for a full-scale process, after an accurate economical evaluation. However, a better combination between reaction rate and stability at pH 6.5 reinforces this latter. In these reactors the amount of acid was barely detectable but this bottleneck could easily be overcome operating with series-arranged bioreactors, optimised for AMase activity (Cantarella et al., 2011).

4. Conclusions

This paper investigates on the effect of pH on bromoxynil biodegradation performed with *M. imperiale* resting cells and highlights a higher activity, at pH 6.0 and 6.5, than pH 7.0. The evaluation of the kinetic parameters showed the highest V_{max} at pH 6.0. The enzymatic stability was assessed, in the pH-range, 5.5 – 7.0, by means of continuous stirred UF-membrane bioreactors, operated at 20 °C. The lowest inactivation constant, allowing the higher enzyme half-life, was that at pH 7.0, justifying our previous data (Pasquarelli et al., 2015). However, the kinetic parameters at pH 6.5 certainly balance the higher stability at pH 7.0, and in long-term runs (> 140 h) a conversion of bromozynil (in amide) higher than 75 % was achieved at a reaction rate, which was an order of magnitude higher than that at pH 7.0. Hence, the favourable combination of higher reaction rate and conversion reached at pH 6.5, supports this one for real application. This study also suggests adopting series-arranged bioreactors, optimised for amide biotransformation by AMase activity.

Acknowledgments

The authors acknowledge the University of L'Aquila Research Fund, and, the Gate mobility Program for the PhD scholarship of Miss N.Chuchat, from Thammasat University, Thailand, as Erasmus Mundus student.

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