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Journal of Molecular Catalysis B: Enzymatic 5 (1998) 403–409

JOURNAL OF MOLECULAR CATALYSIS B: ENZYMATIC

Degradation of morpholine and thiomorpholine by an environmental *Mycobacterium* involves a cytochrome *P*450. Direct evidence of intermediates by in situ ^{1}H NMR

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Received 26 September 1997; accepted 24 November 1997

Abstract

A strain of *Mycobacterium* sp. RP1 was isolated from a contaminated activated sludge. It was capable of utilizing morpholine, a waste of chemical industry, as sole source of carbon, nitrogen and energy. The kinetic of biodegradation of morpholine was followed directly on the incubation medium using in situ ^{1}H NMR. This technic allowed to identify two intermediates of the degradative pathway: glycolate and 2-(2-aminoethoxy)acetate. The inhibitory effects of metyrapone on the degradative abilities of the strain RP1 indicated the involvement of a cytochrome *P*450. This observation was confirmed by spectrophotometric analysis and ^{1}H NMR. Metyrapol, a known metabolite of metyrapone, was also found to be an inhibitor. The same study of degradation of thiomorpholine showed the formation of sulfoxide, which confirmed the presence of a cytochrome *P*450.

Keywords: Degradation; Morpholine; Thiomorpholine; In situ ^{1}H NMR; Metyrapone; Cytochrome *P*450

1. Introduction

Morpholine (C_4H_9NO) is a simple heterocyclic compound with a great industrial importance. It is used as anticorrosive agent and as chemical intermediate (catalyst, solvent, antioxidant) in the production of various pharmaceuticals and pesticides. Its high solubility in water and its high potential for *N*-nitrosation make this xenobiotic of special interest from an environmental point of view [1,2]. This chemical had been considered as persistent for many years. Knapp et al. [3] first discovered two strains of *Mycobacterium chelonae* (Mor D and Mor G) able to utilize morpholine as main source of carbon, nitrogen and energy. A few years later, Dmitrenko et al. [4] isolated a strain of *Arthrobacter*, while Cech et al. [5] isolated a strain of *M. aurum* MO1 presenting degradative properties of morpholine. The group of Knapp studied other strains of *Mycobacterium* sp. isolated from activated sludges [6,7].

A few studies were carried out in order to understand the biodegradation process of mor-

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1381-1177/98/$19.00
PII: S1381-1177(98)00019-8

pholine and its regulation. Swain et al. [8] proposed an hypothetical pathway for the degradation of morpholine by *M. chelonae* (Fig. 1), in which the latter stages of catabolism gave two C2 units products: glycolate and ethanolamine.

However, up to date no tool for direct detection of intermediates, or even morpholine, was available. Only indirect strategies were developed such as COD (chemical oxygen demand), OD (optical density) or NH_3 measurements, growth on intermediates, in vitro enzyme assays... Consequently, only hypothetical pathways were proposed and limited interpretations of various experiments were made.

In this work, we describe the degradation of morpholine by a strain of *Mycobacterium* sp. RP1 that was isolated from an activated sludge. For this purpose, in situ ^{1}H NMR spectroscopy was used. This technique allowed us to evidence directly intermediates and also to quantify the degradation of morpholine. Experiments performed with inhibitors and spectrophotometric analysis demonstrated the implication of a cytochrome *P*450 in this degradation. The results were used to propose a pathway for morpholine degradation by this strain. The degradation of thiomorpholine, a sulfur analogue of morpholine, was also studied.

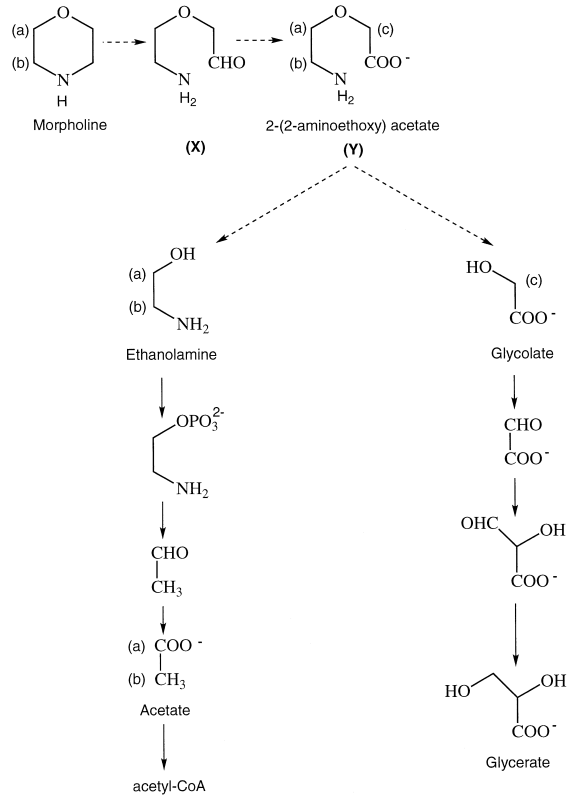

Fig. 1. Hypothetical biodegradative pathway of morpholine in *M. chelonae* by Swain et al. [8].

2. Results

2.1. Identification of a morpholine-degrading bacterium

Strain RP1 was isolated, by enrichment culture using morpholine as sole source of carbon and energy, from an activated sludge collected in a chemical waste water treatment plant. It formed red convex, slightly mucoid colonies on solid medium. RP1 was gram-positive, partially acid fast, catalase positive and oxidase negative. The analysis by the Institut Pasteur (Paris) assigned strain RP1 to the genus *Mycobacterium.* Experiments are in progress to determine the 16*S* rRNA sequence of this microorganism and to establish its phylogenetic relationship to other mycobacteria.

2.2. Incubation of RP1 resting cells with morpholine

Mycobacterium sp. RP1 was grown on Trypcase-soy broth (bioMérieux, France) at 30°C and 200 rpm and harvested after 48 h of culture. Cells were centrifuged for 15 min at 5°C; the supernatant was eliminated, the pellet was washed twice with Knapp buffer (KH_2PO_4 1 g/l, K_2HPO_4 1 g/l, $FeCl_3$ 4 mg/l, $MgSO_4 \cdot 4H_2O$ 40 mg/l, pH 6.6) and finally resuspended in this buffer (5 g of wet cells in 50 ml buffer). The resting cells were incubated with 10 mM of morpholine in Erlenmeyer flasks at 30°C under agitation (200 rpm). Samples were

taken (1 ml) every hour for 12 h and prepared for NMR analysis as follows: incubation samples were centrifuged (12,000 *g* during 5 min), the supernatant (540 μl) was supplemented with 60 μl of tetradeuterated trimethylsilylpropionate sodium, $TSPd_4$ (usually 10 mM in D_2O) and adjusted to pH 10 with 4 N NaOH. D_2O was used for locking and shimming. $TSPd_4$ constituted a reference for chemical shift (0 ppm) and quantification.

2.3. In situ NMR spectra

^1H NMR spectra were performed at 300.13 MHz on a 300MSL Bruker spectrometer at 21°C using 5-mm diameter tubes; water was suppressed by saturation. For each sample, recording time was about 20 min in order to detect minor compounds (150 scans, 90° pulse 3.7 μs, relaxation delay 6 s, acquisition time 1.3 s, 8 K data point, saturation time 2 s).

An example of kinetic of morpholine degradation is presented in Fig. 2. Only spectra recorded at time 0 h, 10 h and 20 h are shown. In spectrum at time zero, three main signals are visible: the singlet at 0 ppm belongs to CH_3 of $TSPd_4$; two pseudotriplets at 2.88 and 3.72 ppm correspond to CH_2(b) and CH_2(a) of morpholine (see Fig. 1). The singlet corresponding to the NH of morpholine was not detected because of the quadrupolar moment of ^{14}N. At time 10 h, the signals of morpholine were decreasing, while a singlet at 3.95 ppm was increasing. This signal was assigned to glycolic acid as evi-

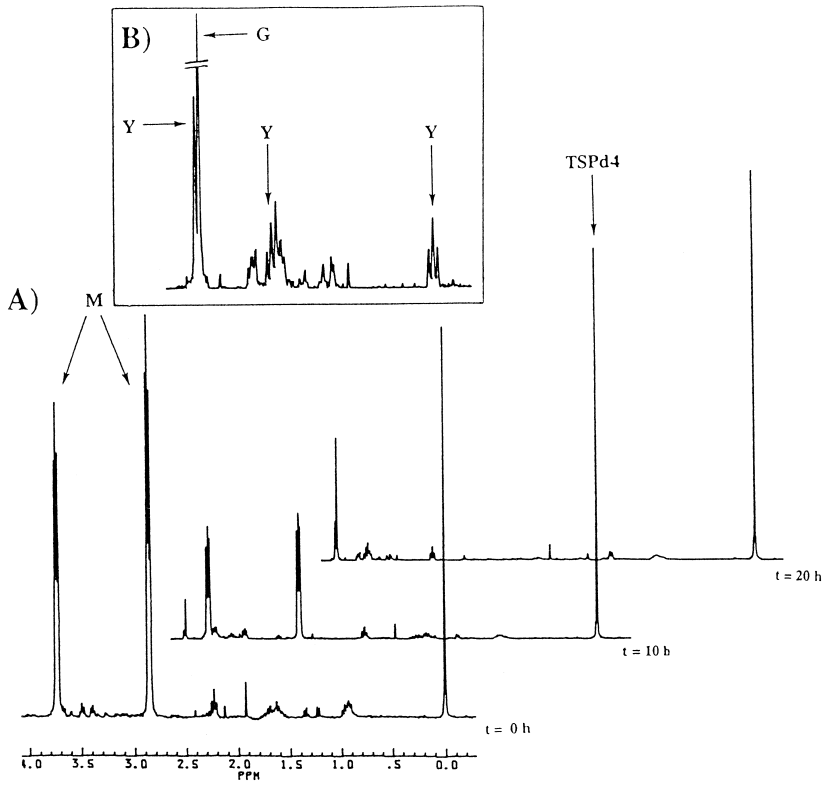

Fig. 2. (A) Kinetics of morpholine degradation by *Mycobacterium* sp. strain RP1. Resting cells (5 g wet cells in 50 ml of Knapp buffer) were incubated with 10 mM of morpholine at 30°C under agitation (200 rpm). Samples (1 ml) were collected every hour; after centrifugation, the supernatants of these samples were analyzed by ^1H NMR spectroscopy at 300.13 MHz. Tetradeuterated trimethylsilylpropionate sodium ($TSPd_4$) is used as reference for chemical shift and quantification. (B) Expanded scale from 2.60 to 4.00 ppm of the 20 h spectrum. **M**: morpholine; **Y**: 2-(2-aminoethoxy)acetate; **G**: glycolate.

denced by addition of commercial compound in the sample. Three new signals are also present, resonating respectively at 3.96 ppm (singlet), 3.67 ppm (pseudotriplet) and 3.15 ppm (pseudotriplet). These signals correspond to those of 2-(2-aminoethoxy)acetate. This last compound was synthesized in our laboratory in three steps from 2-(2-aminoethoxy)ethanol: protection of the amino group with Boc_2O, oxidation of the alcohol into acid and deprotection with a 3 N solution of HCl in ethyl acetate.

2.4. Inhibition of morpholine degradation by selective inhibitors

Degradation of saturated heterocycle ring is likely to begin by the breakage of a bond between the heteroatom and an adjacent carbon atom. It was demonstrated that xenobiotic compounds bearing amine and ether functional groups could serve as substrates for flavin-containing monooxygenase or cytochrome *P*450 [9–11]. From previous work [3,12], it was shown that morpholine degradation was associated with oxygen consumption. According to these results and the chemical structure of morpholine, it was possible that the enzyme responsible for the ring cleavage was a monooxygenase.

In order to check the involvement of such enzymes in the first steps of morpholine degradation by *Mycobacterium* sp. strain RP1, the influence of selected inhibitors was tested on the degradative ability of this strain. Metyrapone (2-methyl-1,2-di-3-pyridil-1-propanone) was chosen as a specific cytochrome *P*450 inhibitor [13,14] and methimazole (2-mercapto-1-methylimidazole) as a competitive inhibitor of flavin-containing monooxygenase [15].

First of all, the effects of both these inhibitors were tested on the growth of *Mycobacterium* sp. strain RP1. The addition of metyrapone in the medium containing morpholine as sole source of carbon, nitrogen and energy led to an inhibition of the growth (the stationary phase was reached after 150 h instead of 60 h) (data not shown). No effect was observed when methimazole was added. Metyrapone did not affect the viability of *Mycobacterium* sp. strain RP1 since the addition of this chemical to succinate-containing medium did not prevent cell growth. These results suggest that a cytochrome *P*450, and not a flavin-containing monooxygenase, is

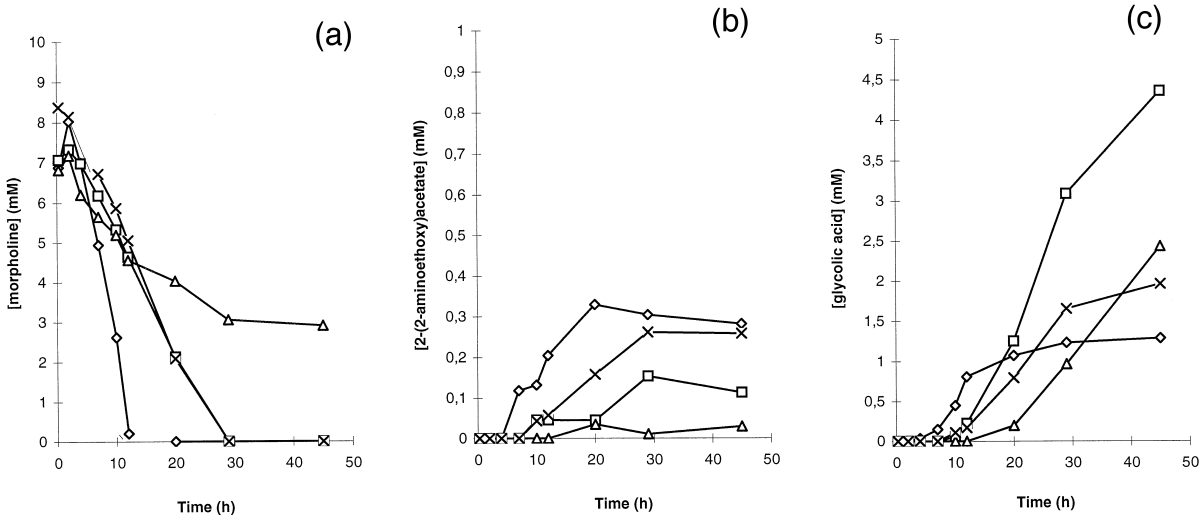

Fig. 3. Incubation of *Mycobacterium* sp. RP1 cells (100 g/l) with morpholine (10 mM) in the presence of 5 mM (□) or 10 mM (△) of metyrapone, of 5 mM of metyrapol (×) or in the absence of inhibitors (◇). Time course of the concentrations of morpholine (a), 2-(2-aminoethoxy)acetate (b) and glycolate (c).

involved in the oxidative catabolism of this amine.

To have direct evidence of the inhibitory effect of metyrapone, experiments were carried out with different concentrations of this compound and analyzed by ^{1}H NMR. In the flasks containing the cells (100 g/l) were added metyrapone at different concentrations (5 and 10 mM) and morpholine (10 mM). The kinetics of morpholine degradation are reported in Fig. 3.

The addition of metyrapone led to an inhibition of the degradative reactions of morpholine that is concentration dependent. When the concentration of metyrapone was increased, the following effects were observed: (i) the rates of morpholine degradation (Fig. 3a) and 2-(2-aminoethoxy)acetate formation (Fig. 3b) were decreased; (ii) the appearance of the intermediates was delayed (Fig. 3b,c); (iii) the final concentration of 2-(2-aminoethoxy)acetate was lowered, while that of glycolate was increased.

On the NMR spectra, several new signals appeared very quickly, which were assigned to metyrapol, a known metabolite of metyrapone [16,17]. Its concentration increased at the same time that metyrapone concentration decreased. The question is to know which product is the real inhibitor. Experiments were carried out with 5 mM of metyrapol and analyzed by ^{1}H NMR. The effects observed on the morpholine degradation were very similar to those obtained in the presence of a same concentration of metyrapone (Fig. 3a). The only difference concerned the

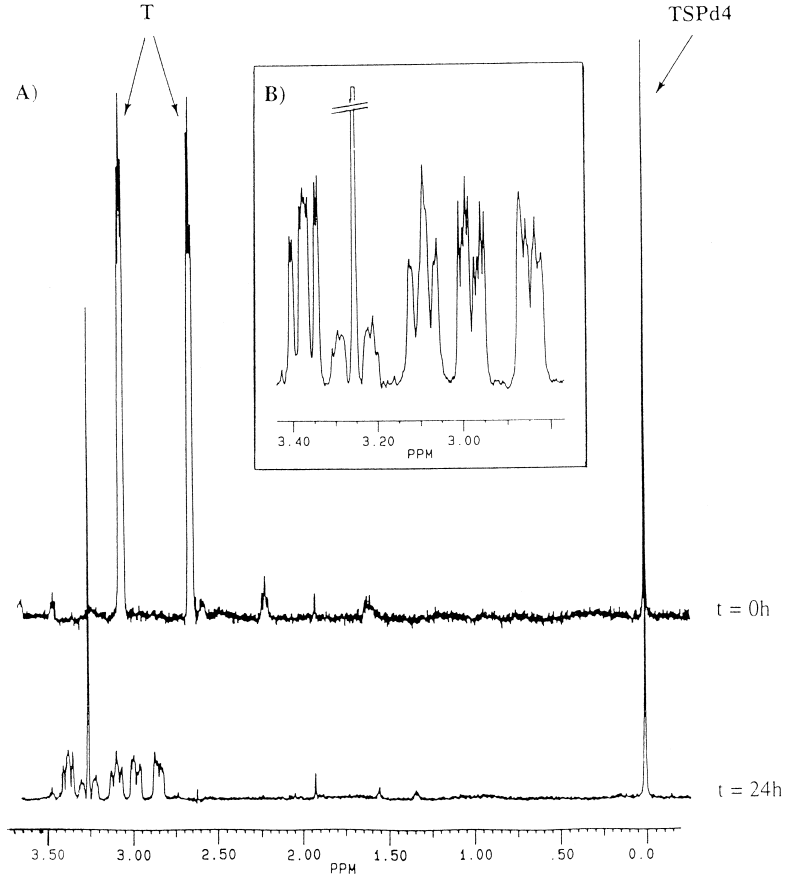

Fig. 4. (A) Kinetics of thiomorpholine degradation by *Mycobacterium* sp. strain RP1. Same conditions used as those described previously. (B) Expanded scale from 2.80 to 3.40 ppm of the 24-h spectrum.

final concentration of glycolate, which was lowered in the presence of metyrapol compared with metyrapone (Fig. 3c). This phenomenon has not been explained yet.

These results confirm the presence of an activity due to a cytochrome *P*450 in the degradative pathway of morpholine. In addition, the effects of metyrapone and of metyrapol on 2-(2-aminoethoxy)acetate formation indicate that the oxidation leading to the opening of the heterocycle takes place during the early events of morpholine degradation.

2.5. Spectrophotometric evidence of the induction of a cytochrome P450

The spectrum of the co-treated vs. non-treated reduced extract of morpholine-grown bacteria exhibited a typical peak at 449 nm. This result demonstrates the presence of a soluble cytochrome *P*450 in this cell extract. Such monooxygenase was not detected, or could be present at a low level, in the protein extracts of succinate- or acetate-grown bacteria (data not shown). This indicated that the presence of a soluble cytochrome *P*450 in *Mycobacterium* sp. strain RP1 was induced by the growth on morpholine.

2.6. Degradation of thiomorpholine by Mycobacterium sp. strain RP1

In order to give more evidence on the biodegradative process, the degradation of thiomorpholine, a sulfur analogue of morpholine, was studied. *Mycobacterium* sp. strain RP1 was not able to grow on this compound but it induced the production of a cytochrome *P*450. Thus, the accumulation of a metabolite oxidized by the cytochrome *P*450 was expected.

The experiments were carried out under the same conditions as previously. In Fig. 4, the spectra at time 0 h and 24 h, obtained during the biodegradation of thiomorpholine are collected. In the spectrum at time zero, the two pseudotriplets at 2.66 and 3.08 ppm belong to thiomorpholine. At time 24 h, thiomorpholine has completely disappeared while new signals were present: four multiplets which seem to belong to the same molecule because of their simultaneous evolution. As the presence of a cytochrome *P*450 has been shown previously, an oxidation reaction of the sulfur could be expected. This hypothesis was checked by synthetizing the corresponding sulfoxide. The synthesized sulfoxide was then added in a sample of the incubation medium. The resonances were perfectly overlapping with those of the metabolite. A singlet was also detected at 3.25 ppm which has not been assigned yet; it could correspond to dithioglycolate.

3. Discussion and conclusion

An actinomycete that grew on morpholine as sole source of carbon, nitrogen and energy was isolated from an activated sludge. This microorganism was identified as *Mycobacterium* sp. RP1.

^{1}H NMR spectroscopy, performed directly on incubation medium was shown to be a performant tool to study the degradation of morpholine by this strain. It allowed us to identify unambiguously two intermediates: 2-(2-aminoethoxy)acetate and glycolate. The biodegradative pathway of morpholine observed seems to be very similar to that obtained very recently with *M. aurum* MO1 [18]. These two strains cleave the C–N bond of the morpholine ring.

Metyrapone and not methimazole inhibited the growth of *Mycobacterium* sp. RP1 on morpholine, strongly suggesting that a cytochrome *P*450 is implicated in the degradation of this compound. In parallel experiments, we have directly shown that morpholine is a substrate for the cytochrome *P*450: when metyrapone was added in morpholine-containing mineral salts medium, the analysis by ^{1}H NMR indicated that the degradation of morpholine was inhibited and the kinetics of formation of 2-(2-aminoethoxy)acetate were made slower. The keto-re-

duction of metyrapone into metyrapol was also observed. Assays of the morpholine degradation in the presence of metyrapol showed a very similar inhibitory activity.

The presence of a soluble heme-containing monooxygenase was also confirmed by the CO-difference spectrum of cell extracts of strain RP1 grown on liquid mineral salts medium amended with morpholine. To our knowledge, the implication of cytochrome *P*450 in degradation mediated by mycobacteria has only been demonstrated for halogenated phenols. These monooxygenases were membrane-associated [19].

The study of the degradation of a sulfur analogue of morpholine by the same strain has also shown the key role of the cytochrome *P*450. The formation of sulfoxide was evidenced by ^{1}H NMR.

In conclusion, this work, using in situ ^{1}H NMR technique, is pioneer in the direct quantification and identification of degradative pathways. Assays of degradation in the presence of specific inhibitors and spectrophotometric analyses have shown the involvement of a cytochrome *P*450. It attacks morpholine at the C–N position and oxidizes the sulfur of thiomorpholine into sulfoxide. This study underlines the importance of mycobacteria in the degradation of xenobiotic compounds.

References

[1] K. Suzuki, T. Mitsuoka, I.A.R.C Sci. Publications 57 (1984) 275.
[2] H. Enzmann, H. Zerban, A. Kopp-Schneider, E. Loser, P. Bannasch, Carcinogenesis 16 (1995) 1513.
[3] J.S. Knapp, A.G. Callely, J. Mainprize, J. Appl. Bacteriol. 52 (1982) 5.
[4] G.N. Dmitrenko, V.M. Udod, P.I. Gvozdyak, Khim. Teknol. Vody 7 (1985) 71.
[5] J.S. Cech, P. Hartman, M. Slosarek, J. Chudoba, Appl. Environ. Microbiol. 54 (1988) 619.
[6] J.S. Knapp, J. Whytell, Environ. Pollut. 68 (1990) 67.
[7] V.R. Brown, J.S. Knapp, J. Heritage, J. Appl. Bacteriol. 69 (1990) 54.
[8] A. Swain, K.V. Waterhouse, W.A. Venables, A.G. Callely, S.E. Lowe, Appl. Microbiol. Biotechnol. 35 (1991) 110.
[9] F.P. Guengerich, Biochem. Mol. Biol. 25 (1990) 97.
[10] S.F. Sariaslani, Adv. Appl. Microbiol. 36 (1991) 133.
[11] G.F. White, N.J. Russel, E.C. Tidswell, Microbiol. Rev. 60 (1996) 216.
[12] N. Mazure, N. Truffaut, Can. J. Microbiol. 40 (1994) 761.
[13] B. Testa, P. Jenner, Drug Metab. 12 (1981) 1.
[14] S. Nagamine, F. Horisaka, Y. Fukuyama, K. Maetani, R. Matsuzawa, S. Iwakawa, S. Asada, Biol. Pharm. Bull. 20 (1997) 188.
[15] I. Tomasi, I. Artaud, Y. Bertheau, D. Mansuy, J. Bacteriol. 177 (1995) 307.
[16] L.A. Damani, P.A. Crooks, D.A. Cowan, Drug Metab. Disp. 9 (1981) 270.
[17] S. Pospisil, P. Sedmera, V. Havlicek, J. Tax, Appl. Environ. Microbiol. 60 (1994) 1561.
[18] B. Combourieu, P. Besse, M. Sancelme, H. Veschambre, A.M. Delort, P. Poupin, N. Truffaut, Appl. Environ. Microbiol. 64 (1998) 153.
[19] J.S. Uotila, M.S. Salkinoja-Salonen, J.H.A. Apajalahti, Biodegradation 2 (1991) 25.