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# BIODEGRADATION OF NONIONIC SURFACTANTS. I. BIOTRANSFORMATION OF 4-(1-NONYL)PHENOL BY A *Candida maltosa* ISOLATE

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**Abstract**

*Results are reported concerning biodegradation of 4-(1-nonyl)phenol by cultures of a* Candida maltosa *strain isolated from aerobic sludge samples collected at a depuration plant treating wastewaters from a textile industry. The yeast was able to utilize 4-(1-nonyl)phenol as a sole carbon and energy source. Preliminary attempts to draw the actual metabolic pathway evidenced microbial attack on the alkyl chain with the production of 4-acetylphenol. To the best of our knowledge this is the first report describing a microorganism capable of attacking nonylphenol in axenic culture and at the same time allowing for the identification of its degradation products.*

*Keywords:* Biodegradation, *Candida maltosa*, nonionic surfactants, nonylphenol, yeasts.

## INTRODUCTION

Until recently, prokaryotic microorganisms have been attracting the attention of most scientists interested in the exploitation of microbial degradation of xenobiotic compounds. Nevertheless, degradative capabilities of eukaryotic microorganisms, such as yeasts, are well known and increasingly cited in the biodegradation of a large variety of aliphatic and aromatic hydrocarbons (Fedorak *et al.*, 1984; Middelhoven *et al.*, 1992; MacGillivray & Shiaris, 1993).

In particular, the genus *Candida* contains different species able to utilize *n*-alkanes as sole carbon and energy sources and to oxidize different aromatic compounds (Cerniglia & Crow, 1981). *Candida maltosa* can metabolize both *n*-alkane and phenols (Hofmann & Schauer, 1988) as well as monosubstituted phenols, such as monochlorophenol (Polnisch *et al.*, 1992). It may also play an important role in the biodegradation of amphiphilic organic pollutants. Within this class of compounds, alkylphenols represent a typical raw material largely used for the production of nonionic surfactants based on alkylphenol polyethoxylates (APEOs). These last are still manufactured and consumed in large quantities for household and industrial applications. In particular leather and textile industries currently utilize nonylphenol polyethoxylates (NPEOs) in spite of their established environmental harmfulness. Biological treatment of NPEOs containing wastewaters eventually gives rise to mixtures of isomeric nonylphenols (NPs) and other intermediates with one or two residual ethyleneoxy units originating from the microbial attack on the polyoxyethylene chains (Kravetz, 1981).

NPs are rather persistent and extremely toxic to fish (Giger *et al.*, 1984; Yoshimura, 1986) as well as to other marine and freshwater organisms (Weinberger *et al.*, 1987; Granmo *et al.*, 1989). Their bioaccumulation is claimed to occur even in animal tissues (Ekelund *et al.*, 1990). Recalcitrance of NP to ultimate degradation is related to the presence of an aromatic ring substituted with a branched alkyl chain (Sturm, 1973; Kravetz, 1983).

Scarce information is at present available on the possible routes for the complete biodegradation of NPs in APEOs polluted environments (Rudling & Solyom, 1974) and no microorganism capable of attacking NP has been isolated in pure culture and characterized so far. In fact, recent studies on NP degradation have considered solely *consortia* of microorganisms naturally inhabiting freshwater (Sundaram & Szeto, 1981), sewage treatment plants (Kravetz *et al.*, 1982) and seawater and marine sediments (Ekelund *et al.*, 1993).

Our research on microbial degradation of NPs in axenic cultures has been carried out with a yeast closely related to the species *Candida maltosa*. This yeast was isolated from sludge samples collected at a treatment plant of textile industry wastewaters and it was catalogued as LMAR 1 (Vallini *et al.*, submitted).

Commercially available NPs do not have very well defined chemical structures as they are constituted by complex mixtures of isomeric alkylphenols containing also about 10% of unidentified chemical impurities. In order to make available a chemically pure substrate for growth experiments and to simplify the analysis of metabolic products, 4-(1-nonyl)phenol (*p*NP) with linear alkyl chain was synthesised and used as an energy and carbon source in *C. maltosa* cultures.

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## MATERIALS AND METHODS

### 4-(1-Nonanoyl)phenol

The procedure described by Ralston *et al.* (1940) for the synthesis of *p*NP was adopted (Scheme 1). A mixture of 69·6 g (0·39 mol) of nonanoyl chloride, prepared immediately before use according to the protocol by Sulzbacher and Bergmann (1948), 100 ml of 1,1,2,2-tetrachloroethane and 61·9 g (0·46 mol) of aluminum trichloride was heated at 50°C under stirring. In a separate flask, 58·2 g (0·44 mol) of aluminum trichloride were slowly added to a solution of 40·9 g of phenol (0·43 mol) in 100 ml of tetrachloroethane and the resulting mixture was heated at 50°C under stirring until the evolution of hydrogen chloride had subsided. The solution of nonanoyl chloride–aluminum trichloride complex was then added dropwise to the phenol–aluminum trichloride complex and the resulting mixture was heated at 50°C for 6 h. The reaction mixture was hydrolyzed, the solvent removed by steam distillation, and the residue was extracted with diethyl ether. The ether solution was extracted with 3% aqueous sodium hydroxide and water. The aqueous phase was acidified with HCl, and finally extracted with diethyl ether. The extract, after removal of the volatile products under vacuum, gave a solid residue that was purified by crystallization from a 5 : 1 petroleum–ether-carbon tetrachloride mixture. 37·4 g (37%) of 4-(1-nonanoyl)phenol were obtained as white needles with m.p. 51°C.

The structure of 4-(1-nonanoyl)phenol was assessed and confirmed by proton nuclear magnetic resonance ($^1$H-NMR), Fourier transform-infrared (FT-IR) spectroscopy and mass spectroscopy (MS).

### 4-(1-Nonyl)phenol (*p*NP) (Coulthard *et al.*, 1930; Martin, 1942)

A mixture of 26·4 g (0·4 mol) of powdered zinc, 3·52 g (0·013 mol) of mercuric chloride, 3 ml of concentrated hydrochloric acid and 35 ml of water was stirred at room temperature for 10 min. The aqueous phase was decanted off, then 59 ml of 1 : 1 HCl, 7 ml of ethanol and 13·0 g of 4-(1-nonanoyl) phenol (0·06 mol) were added in that order. The mixture was heated at reflux for 20 h, washed with 5% aqueous $NaHCO_3$, after cooling at room temperature, then extracted with diethyl ether. The collected extract was dried over anhydrous sodium sulphate, and after removal of the solvent the residue was recrystallized from petroleum ether to yield 16·7 g (83%) of 4-(1-nonyl)phenol as a yellowish solid with m.p. 37–38°C (purity by GLC >99%).

The structure of 4-(1-nonyl)phenol was assessed and confirmed by proton nuclear magnetic resonance ($^1$H-NMR), Fourier transform-infrared (FT-IR) spectroscopy and mass spectroscopy (MS).

### Yeast strain and culture conditions

*Candida maltosa* strain LMAR 1 was isolated during previous research carried out on the microbial degradation of alkylphenol polyethoxylates nonionic surfactants (Vallini *et al.*, submitted.). The strain was maintained at 4°C on Malt agar (Difco) slants. Before biodegradation testing, the yeast was grown on Malt broth (Difco) and cells were harvested by centrifugation.

Degradation experiments were carried out on yeast nitrogen base (YNB) broth (Difco) supplemented with *p*NP as the sole carbon and energy source, at a concentration of 100 mg litre$^{-1}$. In order to attain a better dispersion of insoluble *p*NP in liquid cultures, emulsions in distilled water were aseptically prepared by sonication with a Branson 250 sonicator (horn frequency 19·85–20·05 kHz, 65 W for 10 min every other 10 s). Emulsions were transferred into 300 ml Erlenmeyer flasks containing 100 ml of sterile YNB broth and inoculated with yeast cells. Cultures were incubated at 28°C, in the dark, on a rotatory shaker (130 rpm) for 21 days. Control experiments were also carried out in the absence of either yeast inoculum or *p*NP.

Microbial growth was monitored during incubation time by means of CFU (Colony Forming Units) determinations on Malt agar plates. The influence of *p*NP on microbial growth was evaluated by cultivating LMAR 1 strain on YNB broth supplemented with glucose (10 g litre$^{-1}$) in the presence of 50 and 200 mg litre$^{-1}$ of *p*NP. In these cultures cell growth was monitored with time by means of optical density determinations at 660 nm with a Beckman DU-7 spectrophotometer.

### Extraction of *p*NP and degradation products from yeast cultures

A current liquid–liquid partitioning methodology was applied for the extraction of *p*NP and its degradation products from the liquid cultures. Different amounts of aqueous solutions, acidified to pH 2·5 with concentrated HCl, were filtered on Whatman No. 1 filter paper and then extracted with corresponding volumes of ethyl acetate in a separating funnel. Ethyl acetate extracts were then dried on anhydrous sodium sulphate and evaporated in a rotatory evaporator under vacuum.

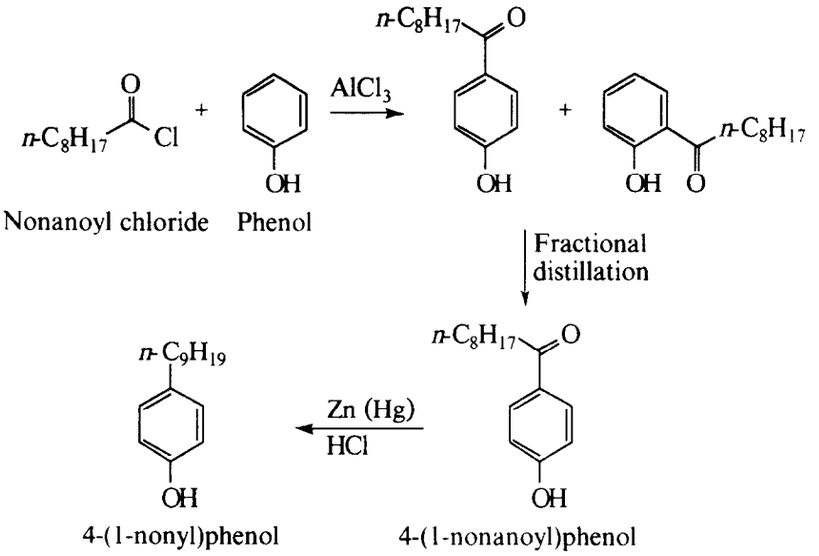
A similar procedure was used to extract the cell pellets collected on filter paper once dried under vacuum. The extracts from culture solution and cell pellet were collected, concentrated under vacuum to about 0·2 ml and then diluted to exactly 1·0 ml with diethyl ether.

### NMR analysis

NMR spectra were obtained with a Varian Gemini 200 spectrometer on sample solutions in perdeuterated solvent in 5-mm diameter tubes. $^1$H-NMR spectra were recorded at 200 MHz. Spectral conditions were as follows: size, 11 968 points; spectral width, 3 kHz; pulse, 30°; acquisition time, 2 s; number of scans, 1.

### Gas–liquid chromatography (GLC)

GLC analyses were performed on 0·8 $\mu$l diethyl ether solution of ethyl acetate extracts by using a Perkin–Elmer 8700 gas chromatograph equipped with flame ionization detector (FID). Analytical conditions were




**Scheme 1.** Synthesis of linear 4-(1-nonyl)phenol.

as follows: Db-1 glass column (length 15 m, i.d. 0·53 mm, film thickness 1·5 $\mu$m) from J&W Scientific (Folsom, CA, USA), helium as gas carrier at 30 ml min$^{-1}$ flow rate, injector temperature 280°C, FID temperature 280°C and oven temperature raised from 70 to 250°C at 5°C min$^{-1}$. Integration of peak areas was performed by a Hewlett Packard 3396 integrator.

## RESULTS AND DISCUSSION

Linear 4-(1-nonyl)phenol (*p*NP), synthesized according to Scheme 1, was utilized as the sole carbon and energy source in growth experiments carried out with *C. maltosa* strain LMAR 1.

LMAR 1 strain was able to grow in the presence of 100 mg litre$^{-1}$ *p*NP. During the first week of incubation, pseudomycelium was produced (Fig. 1(a)); afterwards, budding cells massively coating *p*NP crystals (Fig. 1(b)) were observed. This transition from yeast-like cells (phase Y) to pseudohyphae forms (phase M) is well known for many species belonging to the genus *Candida* (Kockova-Kratochvilova, 1990). The transition begins with the production of a germ tube which can be stimulated by either raising the incubation temperature (Soll & Bedell, 1978) or addition of surface-active compounds, such as alkylphenol polyethoxylates (Sullivan & Shepherd, 1982). The surface-active action exerted by *p*NP, although to a moderate extent, was likely to induce cell transition in LMAR 1 cultures. Reversion to the phase Y after the first week of incubation may be attributed to the alkylphenol degradation by the yeast.

CFU determination of cultures of strain LMAR 1 supplemented with 100 mg litre$^{-1}$ of *p*NP clearly showed growth throughout the incubation time (from $4·5 \times 10^6$ cells ml$^{-1}$ at the start to $5·6 \times 10^8$ cells ml$^{-1}$ after 21 days), while no significant increase of the number of cells was detected in unfed controls (from $1·7 \times 10^6$ cells ml$^{-1}$ the start to $3·8 \times 10^6$ cells ml$^{-1}$ after 21 days). The limited increase of cell numbers in controls has to be attributed to the utilization of reserve material present in the inoculum.

To investigate the influence of pNP on cell growth in the presence of easily assimilated carbon sources, *C.*

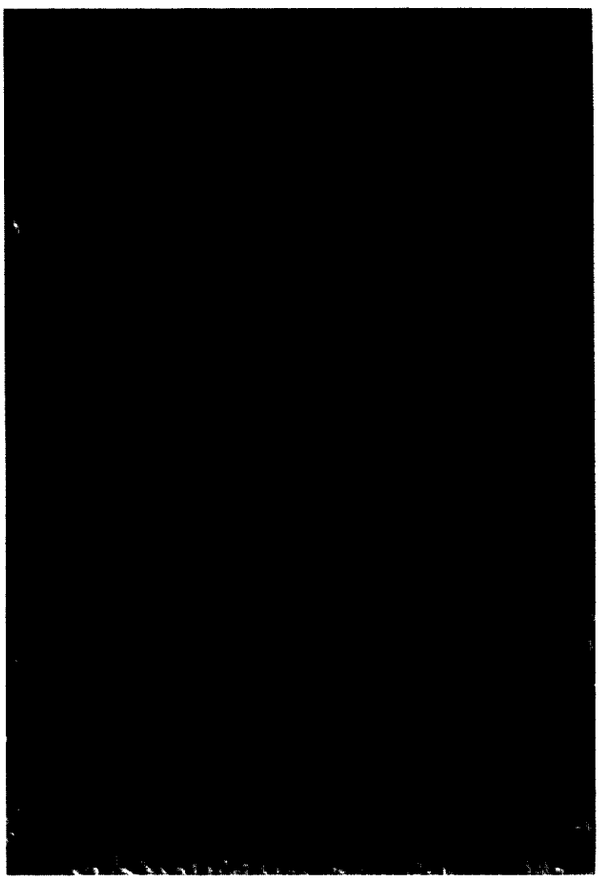


**Fig. 1.** Interference contrast micrographs of *C. maltosa* LMAR 1 growing on *p*NP: (a) pseudomycelium formed at the third day of incubation; (b) yeast-like cells coating *p*NP beads after fifteen days of incubation. Arrows point to *p*NP crystals.

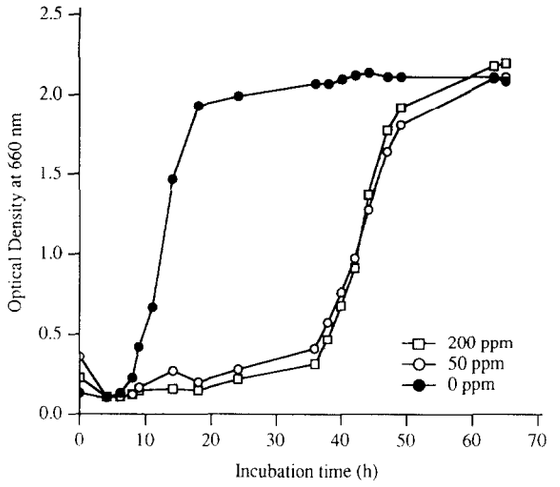



**Fig. 2.** Growth rate of *C. maltosa* LMAR 1 strain in YNB batch cultures supplemented with glucose (10 g litre $^{-1}$) in the presence of 0 (–●–), 50 (–○–) and 200 (–□–) mg litre $^{-1}$ *p*NP.

*maltosa* LMAR 1 was cultivated on YNB broth supplemented with glucose (10 g litre $^{-1}$) and different amounts (0, 50, and 200 mg litre $^{-1}$) of 4-(1-nonyl)phenol. In *p*NP-containing cultures, cell growth dynamics showed a longer lag phase than the corresponding controls (Fig. 2). However the same optical density was eventually reached independent of the initial *p*NP concentration. The prolonged induction time (36 h) observed in the presence of *p*NP suggested a possible toxic effect of 4-(1-nonyl)phenol on *C. maltosa*.

In order to verify whether cell growth in yeast cultures containing *p*NP as the sole carbon source corresponded to an effective biodegradation of *p*NP, replicated cultures were extracted with ethyl acetate at different incubation times. GLC analyses of the extracts showed an extensive attack of the alkylphenol

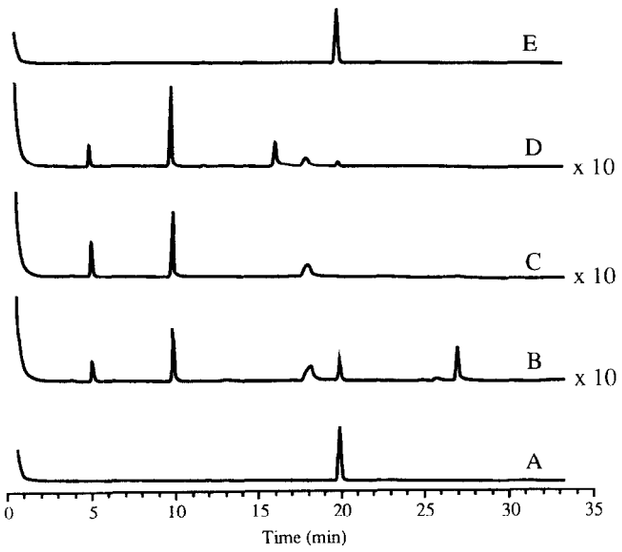



**Fig. 3.** GLC traces of ethyl acetate extracts of *C. maltosa* LMAR 1 cultures in the presence of 100 mg litre $^{-1}$ *p*NP after (A) 0, (B) 7, (C) 14 and (D) 21 days of incubation. Trace (E) refers to uninoculated control after 21 days of incubation.

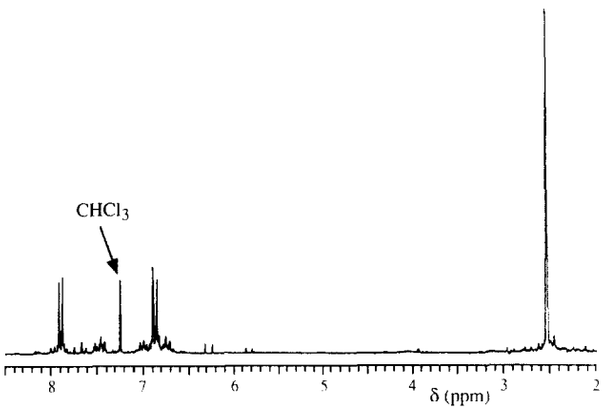



**Fig. 4.** $^{1}$H-NMR spectrum of extracts of *C. maltosa* LMAR 1 cultures supplemented with *p*NP after 21 days of incubation.

as unambiguously indicated by the almost complete disappearance of the *p*NP signal after 7 days of incubation (Fig. 3). The remarkable decrease of *p*NP peak was accompanied by the appearance of at least four new peaks, totaling 40% of the overall initial *p*NP, that can be attributed to *p*NP degradation products.

The attack on *p*NP by strain LMAR 1 seems to proceed relatively fast. Actually, significant changes occurred during the first week of incubation, whereas after this only slight variations, with the presence of different signals and peak relative intensities, could be detected in the GLC chromatograms (Fig. 3). Degradation rate slowed down after 7 days, probably because of the production of toxic intermediates. Also the inability of the yeast to utilize most of the formed metabolites can be proposed as a possible explanation.

No significant abiotic degradation was observed in uninoculated sterile controls. In fact GLC analyses of ethyl acetate extracts did not reveal any reduction of *p*NP initial concentration or the formation of by-products within 21 days of incubation (Fig. 3).

$^{1}$H-NMR spectra of ethyl acetate extracts of 21-day cultures exhibited typical signals at 2·55 ppm and at 6·85 and 7·9 ppm (Fig. 4) corresponding to acetyl group and para disubstituted phenyl ring, respectively. On the other hand no significant signal relevant to long aliphatic chains was detectable. Accordingly, the most prominent peak present in GLC traces at about 10 min retention time was identified as 4-acetylphenol. This attribution was supported by co-elution with a standard sample of 4-acetylphenol.

These results indicate that a complete *p*NP bioconversion occurred in cultures of *C. maltosa* LMAR 1. The concurrent significant growth of the yeast suggests that this compound is at least partially utilized as a carbon and energy source.

Taking into account that *C. maltosa* is able to grow on phenol (Hofmann & Krüger, 1985) and substituted phenols (Polnisch *et al.*, 1992), LMAR 1 strain could be expected to attack *p*NP by an initial oxidative cleavage of the aromatic ring. However, the presence of

4-acetylphenol as the most prominent degradation product in the culture broth, may imply that an oxidative degradation of the *p*NP alkyl chain takes place prior to benzene ring cleavage. β-Oxidation, followed by a decarboxylation reaction, occurring either enzymatically or during extracts work-up, may be claimed as a consistent step of the biodegradation process. This is also in agreement with the well known assimilation pattern of medium–long *n*-alkanes by *C. maltosa* (Blasig *et al.*, 1984).

## CONCLUSIONS

The present investigation demonstrates that 4-(1-nonyl)phenol (*p*NP), a biodegradation-refractory intermediate occurring in the biological degradation of nonionic surfactants (APEOs), can be degraded by a *Candida maltosa* isolate. To the best of our knowledge this is the first report on the attack of this substrate-type by a single microbial strain. Results highlight also the possible involvement of yeasts in the abatement of environmentally important pollutants, such as nonionic surfactants and metabolites derived therefrom.

GLC analysis of the culture medium revealed the almost complete biotransformation of the *p*NP and the contemporary formation of several different degradation products among which 4-acetylphenol appears to be the major component. Research is in progress to identify the chemical structure of all metabolites and to get further substantiation on the degradative pathway. Comparison with *C. maltosa* type strain and other *Candida* species is also under investigation.

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