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Depolymerisation and biodegradation of a synthetic tanning agent by activated sludges, the bacteria *Arthrobacter globiformis* and *Comamonas testosteroni*, and the fungus *Cunninghamella polymorpha*

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Abstract

Degradation of a synthetic tanning agent CNSF (a condensation product of 2-naphthalenesulfonic acid (2-NSA) and formaldehyde) by four activated sludges, two previously characterised bacterial strains, *Arthrobacter* sp. *2AC* and *Comamonas* sp. *4BC*, and the fungus *Cunninghamella polymorpha*, was studied in batch culture at 25 °C by determining the changes in the concentrations of CNSF and its component monomers and oligomers (n2–n11). The loss of individual oligomers was correlated with the length of the NSA-CH_2 chain. Approximately 25% of the total CNSF was degraded (i.e. mineralised) by the microbes contained in the four activated sludges and by the two bacterial isolates but with different lag phases and at different overall rates. The decline in CNSF concentration was due almost entirely to the biodegradation of the monomers (34.3% of CNSF) and, in particular, 2-NSA (27% of CNSF). There was no change in the n2–n11 components. The growth of *C. polymorpha*, on the other hand, arose from extracellular depolymerisation of CNSF oligomers and the biodegradation of the lower molecular mass products. Between 38% and 42% of total CNSF was degraded by *C. polymorpha* at 25 °C. The order of oligomer degradation was inversely related to degree of polymerisation. Eighty percent and 90% of the n4 and n5 and 100% oligomers n6–n11 were degraded after 120 h. At a higher temperature (37 °C) oligomers n4–n11 were degraded completely after 120 h. A combination of biodegradation (75%) and sorption to fungal biomass (25%) accounted for the measured loss of all oligomers from the solution phase. The CNSF degradation rates and the volume of fungal biomass produced (and therefore the extent of biosorption) were dependent on the presence of a second carbon source (both optimum at glucose 5 g/l). This is the first report that identifies and distinguishes between depolymerisation, sorption and biodegradation processes in the removal of CNSF and its component oligomers. The use of combinations of the depolymerising fungus *C. polymorpha*, and the monomer-degrading bacteria, *Arthrobacter* sp. *2AC* and *Comamonas* sp. *4BC*, have potential for wastewater treatment.

Definitions: Loss – reduction of total CNSF (a condensation product of 2-naphthalenesulfonic acid (2-NSA) and formaldehyde); monomers and oligomers from the aqueous phase as measured by analysis of the supernatant fraction. Loss could be due to sorption to biomass (biosorption), biodegradation and/or abiotic degradation; Biodegradation – microbiologically mediated structural alteration of one or more components of CNSF resulting in the formation of novel metabolites (transformation) and biomass and the evolution of CO_2 (mineralisation); Depolymerisation – the enzymatic breakdown of CNSF to lower oligomers and component monomers

Introduction

A diverse range of compounds is used during the tanning process (Klinkow et al. 1998) and these, together with chemicals already associated with the skins and hides (e.g. insecticides, *p*-dichlorobenzene, benzene hexachloride, sodium silicofluoride, borax) (Brigden et al. 2000), give rise to wastewater which has a high chemical oxygen demand (COD) and contains many potentially toxic chemicals.

Sulfo derivatives of naphthalene, such as naphthalene-2-sulfonic acid (2-NSA), naphthalene-1, 6- naphthalene-2, 6-disulfonic acid (NDSA), are found in tannery wastewater at concentrations of 0.1–30 mg/l (Alonso & Barceló 1999; Song et al. 2003). Naphthalene sulfonates have a low biodegradability (Song et al. 2001; Wittich et al. 1988) and can persist in water for several years (Pitter & Chudoba 1990) where they are both a real and potential hazard because of their direct toxicity to fish and other aquatic organisms (Greim et al. 1994) and their ability to remobilise toxic hydrophobic compounds, such as polychlorinated biphenyls (PCBs) and polycyclic aromatic hydrocarbons (PAHs) (Ruckstuhl et al. 2001). A condensation product of 2-NSA and formaldehyde (known as CNSF) is an important synthetic tanning agent (Alonso & Barceló 1999) as well as a dye intermediate in the textile industry (Piotte et al. 1995). Other uses of CNSF are in cement production, pigment and dyestuff formulation, agrochemicals, haircare and other consumer products, metal industries and mineral processing (Huynh et al. 2001; Piotte et al. 1995; Riediker et al. 2000). Carbon beads in lithium batteries are prepared by the carbonisation of CNSF resin (Yoshio et al. 2003) and CNSF polymers have even been used as antiviral microbiocides to inhibit HIV attachment and to block the fusion of the virus with the host cell membrane (Profy et al. 2000). Total world annual CNSF use is approximately 150,000 tonnes (Ruckstuhl et al. 2002).

The aim of the European Community's Water Framework Directive (OJ EC 2000) was to ensure that member states achieve 'good status' of waters in the Community by reducing point and diffuse sources of pollutants to background levels for natural chemicals and close to zero for synthetic chemicals. In the UK, government legislation is becoming more stringent regarding industrial wastewater treatment and a policy for the zero release of synthetic chemicals has been proposed (Robinson et al. 2002). Efficient microbial degradation during wastewater treatment is, therefore, an essential process in for reducing the release of hazardous chemicals, such as CNSF, into the environment (Larson et al. 1997).

Despite the wide-scale commercial importance of CNSF, there is a surprising paucity of studies into its degradation. Kurozumi & Sugimori (2000) reported the loss (due to biosorption to biomass) of CNSF by the fungus *Cunninghamella polymorpha* in a study primarily concerned with anionic surfactant biodegradation. Ruckstuhl et al. (2002) described CNSF biodegradation in groundwater but focused on the fate of the monomers and dimers rather than the more recalcitrant higher oligomers. They showed that 80% of total monomers and dimers were degraded in 100d but that naphthalene-1,5-disulfonate and CNSF oligomers with a chain length of three or more were persistent. Although it appears that at least some components of CNSF are transformed by a few species of bacteria and fungi (Ruckstuhl et al. 2002; Sastry 1986), it is not known to what extent any decline in CNSF concentration is due to adsorption by the biomass, depolymerisation, transformation or mineralisation.

The analysis of CNSF extracted from aqueous environmental samples has been reported previously (Piotte et al. 1995; Ruckstuhl et al. 2001; Wolf et al. 2000). CNSF has been identified as one of the components of the hard (i.e. recalcitrant) COD in tannery wastewater (Song et al. 2003) and recently, the complex chemical structure and composition of CNSF was analysed using ion-pair high pressure liquid chromatography coupled to electrospray ionisation mass spectrometry (Song et al. 2003). The results show that CNSF contains monomers (e.g. 2-NSA, 2-NSA and 2–6 naphthalene disulfonic acid) and oligomers up to 11 NSA units and the method makes it possible to quantify the fate of the individual components. In this paper we report, for the first time, the degradation of CNSF and its component oligomers using activated sludges and defined bacterial or fungal strains.

Materials and methods

Reagents, inocula, microorganisms and growth media

CNSF (a condensation product of naphthalene-sulfonic acid and formaldehyde) was provided by a UK tannery; other chemicals were analytical or equivalent grade from Sigma–Aldrich Co. Ltd (Gillingham, Dorset, UK) unless otherwise indicated. Analysis of the CNSF showed that it is composed of monomers and NSA-CH_2 oligomers up to undemers ($n = 11$). The monomers (34.3%), dimers (14.8%) and trimers (15.3%) are the major components (Song et al. 2003). High performance liquid chromatography (HPLC) grade solvents were purchased from Fisher Scientific Co. Ltd. (UK). HPLC-grade water was prepared by purifying demineralised water in a Milli-Q filtration system (Millipore, Bedford, MA, USA). The ion-pair reagents used to separate polar aromatic sulfonates were triethylamine (TEA) and tetrabutylammonium bromide (TBAB) (Fisher Scientific UK Ltd. Loughborough, UK).

Four sources of non-defined microbial inocula (sewage sludge samples) were used in the experiments and are described in detail elsewhere (Song et al. 2005). All sludges were washed with phosphate buffer solution (PBS, 10mM potassium phosphate, pH7.0), centrifuged (3000 × *g*) and resuspended prior to inoculation of treatment flasks. The two bacteria, *Arthrobacter* sp. 2AC, *Comamonas* sp. 4BC, identified previously as *A. globiformis and C. testosteroni* (Song et al. 2005), were isolated from activated sludges previously exposed to tannery wastewater and maintained on 2-NSA in a mineral salts medium (MSM) which was used also as the growth medium to measure bacterial 2-NSA degradation as described by Song et al. (2005). *Cunninghamella polymorpha* (provided by Dr Sugimori, Fukui National College of Technology, Japan) was maintained on PGY-CNSF (100 mg/l) agar plates (PGYA) at 4 °C and degradation studied using a modified PGY medium (10 g/l glucose; 1 g/l yeast extract; 2.5 g/l peptone; 2.5 g/l $MgSO_4$; 5 g/l KH_2PO_4 and 100–500 mg/l of CNSF) (Sugimori et al. 1999). Cultures of *C. polymorpha* were revived every 4 weeks, subcultured onto PGYA and grown at 37 °C.

Loss of CNSF from the aqueous phase due to activated sludges, Arthrobacter *sp. 2AC and* Comamonas *sp. 4BC, and* C. polymorpha

A 1 ml aliquot of washed sludge or a 48-h culture of *Arthrobacter* sp. 2AC or *Comamonas* sp. 4BC was added to 250-ml Erlenmeyer flasks containing 99 ml MSM plus 100 or 500 mg/l 2-NSA as a sole carbon source. In a second series of experiments aliquots were collected from the flasks inoculated with sludge 24 h after the commencement of CNSF degradation. These CNSF acclimated samples were added to fresh medium containing 500 mg/l CNSF. The flasks were sealed with foam bungs and incubated on an orbital shaker platform at 120 rpm and 25 °C. Samples were taken at designated time intervals and centrifuged (10 min, 13,000 × *g*) and aliquots (2 ml) of the supernatant fraction were stored at 4 °C prior to HPLC and TOC analysis.

To determine the loss of CNSF due to *C. polymorpha* 1 ml of a 48-h culture was added to 100 ml PGY medium plus CNSF (500 mg/l) and incubated at 25 or 37 °C on a rotary shaker at 120 rpm. Non-inoculated medium and medium without CNSF were used as controls. Samples (1 ml) were collected at specified time intervals. Methanol, 10 μl, was added to denature the extracellular enzymes and stop the growth of *C. polymorpha* (Renzi et al. 1997), and thus prevent any further depolymerisation or biodegradation. The samples were centrifuged (10 min, 13,000 × *g*) and the concentration of CNSF in the supernatant fraction determined by HPLC analysis.

Determination of fungal biomass and biosorption of CNSF

To determine fungal biomass, the entire contents of each flask were centrifuged (10 min, 3000 × *g*) after 120 h and the pellet dried at 60 °C for 48 h. The dry weight (DW) of *C. polymorpha* in the control (PGY, no CNSF) after 120 h was 0.20 ± 0.04 g. In the treatment flasks (PGY + CNSF), CNSF associated with the fungal biomass (and not in the supernatant fraction) was extracted from the pellets following centrifugation (10 min, 3000 × *g*) by shaking for 24 h in 50 ml falcon tubes with 25 ml MeOH:H_2O (9:1) + 5 mM triethylamine (TEA) + 5 mM acetic acid (Song et al. 2003). The tubes were centrifuged (13000 × *g*, 5 min), the

supernatant fraction collected and appropriate dilutions of 1 ml samples made before HPLC analysis. Each fungal pellet was extracted three times until the concentration of CNSF removed was below the detection limit (< 0.1 mg/l). Pooled extracts were stored at 4 °C prior to HPLC and TOC analysis. The overall amount of CNSF biodegraded was calculated as the difference between that added at the start of the experiment and that remaining in solution plus that biosorbed after 120 h.

The extraction efficiency of CNSF sorbed to *C. polymorpha* was measured using heat-killed biomass (Liao & Tseng 1996; Yadav & Reddy 1993). Five-day cultures grown in PGY medium without addition of CNSF were centrifuged (10 min, 3000 × *g*) and the pellets heat-killed by autoclaving at 121 °C for 15 min (Gouri et al. 1998; Tsang et al. 1999). A known weight of the biomass was added to flasks containing 100 ml CNSF (500 mg/l) and shaken for 120 h at 120 rpm at 37 °C. The concentration of CNSF remaining in the liquid phase and that associated with the biomass was determined as above.

Abiological loss (i.e. in the absence of *C. polymorpha*) of CNSF was measured using a sterile non-inoculated solution of CNSF (500 mg/l) with 500 μM 10 mg/l H_2O_2 and 100 mg/l $FeCl_2$. $4H_2O_2$ (Kerem et al. 1998).

Total, intracellular and extracellular CNSF depolymerising enzymes

In order to confirm that depolymerisation of the CNSF oligomers by *C. polymorpha* took place extracellularly rather than intracellularly three crude enzyme extracts were prepared: total, intracellular and extracellular (Wildeman et al. 2001). Total enzyme (a mixture of intra- and extracellular) was prepared as follows. A 5-d *C. polymorpha* culture (2.0 ml) was transferred into a bead beating phial and Tris/HCl buffer (1 ml, 10 mM, pH 9.0) and glass beads (0.5 g, 0.05–0.10 mm) were added. The cell suspension was subjected to five cycles of bead beating (45 s, 2000 rpm, 30 s rest) followed by centrifugation (10 min, 13,000 × *g*). The supernatant fraction contained a mixture of intracellular and extracellular enzymes. Intracellular enzymes were derived from 5-d *C. polymorpha* cultures by bead beating the resuspended pellet after discarding the supernatant fraction. Extracellular enzymes were measured in the supernatant fraction following filtration (Whatman No1 paper) of 5d *C. polymorpha* cultures and centrifugation (as above) (Kotterman et al. 1998). Each of three enzyme fractions, 1 ml, was added to 100 ml of a PGY medium containing 500 mg/l CNSF. After incubation for 120 h at 37 °C, 1 ml samples were collected and analysed for CNSF monomers and oligomers.

CNSF analysis and TOC and COD measurements

The procedures for analysing CNSF and its components have been described fully elsewhere (Song et al. 2003). In summary, reverse-phase ion-pair chromatography (IPC) was carried out using tetrabutylammonium bromide (TBAB). HPLC-IPC-TBAB analysis was conducted with an HPLC system (Kontron instruments, Watford, UK) equipped with an UV Diode Array Detector (UV-DAD) 440, Autosampler 465, gradient pump and SP 4600 Data Integrator. Data storage and quantification was carried out using Kontron software (KromaSystem 2000, Kontron instruments, Watford, UK). Chromatographic separation and quantification was performed on an Econosi C18 column (250 mm × 4.6 mm 5 μ; Alltech Associate Applied Science LTD, UK) using a binary gradient consisting of (A) water and (B) acetonitrile, both with 0.01 M TBAB and acetic acid (1% v/v) at a column temperature of 20 ± 1 °C. The elution gradient composition was: 0 min 40% B, 35 min 65% B, 45–46 min 80% B, 47 min 40% B with 5 min for equilibration. UV-DAD spectrum was set at the range of 200–380 nm and a flow rate was 1 ml/min. TOC was measured by high temperature catalytic combustion using a DC-190 Total Organic Carbon Analyzer (Rosemount Dohrmann Analytical Inc., Santa Clara, CA) as described previously (Song et al. 2005).

Results and discussion

Loss of CNSF due to four activated sludges, Arthrobacter *sp. 2AC and* Comamonas *sp. 4BC*

Loss of CNSF using the non-defined inocula from the four activated sludges was assessed by changes in the TOC and the concentration of the total CNSF in the supernatant fraction (Figure 1). The initial TOC values in the MSM growth medium were 276 mg/l (500 mg/l CNSF) or 55 mg/l

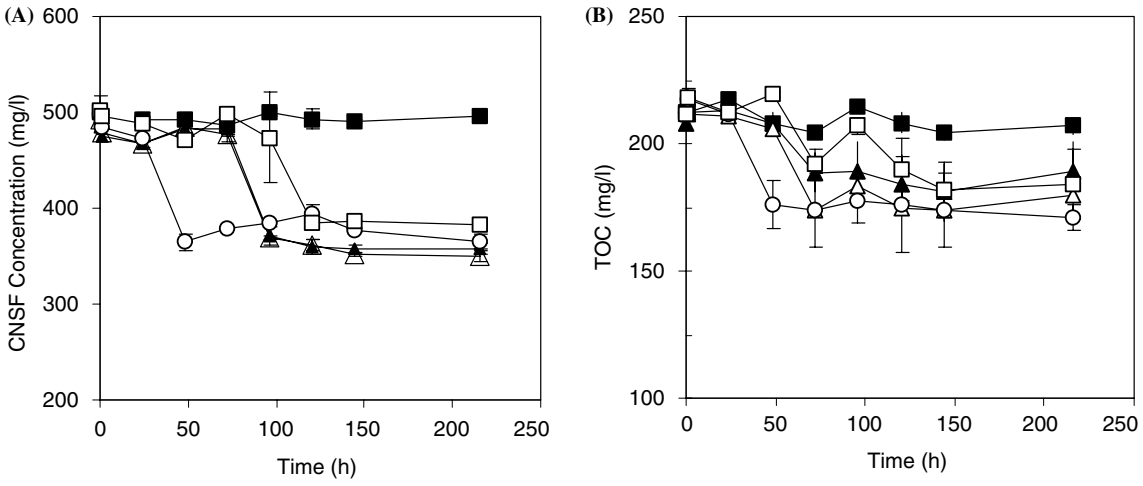

Figure 1. Removal of CNSF by four native sludges (■ – control, ▲ – S1, Δ – S2, ○ – S3 and □ – S4): (A) HPLC measurement; (B) TOC removal. The data are means ± standard deviation for triplicate incubations. When the error bar is not visible it is within the data point).

(100 mg/l CNSF). The four sludges showed marked differences in the length of the lag period prior to the initiation of CNSF loss. Sludge 3 gave the shortest lag phase (24 h) and most rapid rate of compound removal (27% in the next 24 h). However, there was no further loss after 48 h. CNSF removal by sludges 1 and 2 began after a 72 h lag phase but the subsequent rate and total amount removed during the next 24 h (25.5 ± 0.5%) was comparable to sludge 3. The loss of CNSF brought about by sludge 4 began after a 96 h lag phase and 28.6% was degraded within the next 24 h. No further loss occurred after 120 h. On average, CNSF reduction by sludges 1, 2 and 3 was 26.9 ± 1.5% and TOC reduction was 15 ± 4%. Sludge 4 (an activated sludge from a domestic sewage works receiving tannery effluent) reduced the CNSF concentration by a similar amount but the TOC by only 6 ± 2%. In all four cases the discrepancy between the loss of CNSF and the reduction in TOC may be due to the generation of recalcitrant metabolites from CNSF degradation which will contribute to the TOC measurement (Song et al. 2005). HPLC analysis showed that there was no 2-NSA (originally 27% of CNSF) in any of the inoculated flasks at the end of the experiment (120 h) suggesting that the measured loss of CNSF was due largely to the biodegradation of the 2-NSA component. There was no reduction in NDSA (originally 4% of CNSF) which has been reported previously as highly recalcitrant (Cook et al. 1999). Inocula collected from the flasks 24 h after the commencement of CNSF degradation were added to fresh media containing CNSF (results not shown). All four acclimated sludges reduced the lag phase (S1 = 24 h, S2 = 24 h, S3 = 4 h and S4 = 72 h) but this did not increase significantly the total amount of CNSF lost in the subsequent 24 h.

Two bacterial strains, *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC, shown previously to be capable of utilising 2-NSA as the sole source of carbon and energy (Song et al. 2005) were inoculated into the CNSF medium. The decline in CNSF concentration is shown in Figures 2 and 3B. Both strains exhibited similar lag phases (10 h). All the loss of CNSF occurred between 10 and 40 h. HPLC analysis showed that, for both bacteria (as with the sludge inocula), the entire reduction in CNSF concentration could be accounted for by the biodegradation of 2-NSA (Figure 3A and B). Once again, there was no reduction in the concentration of 1-NSA, NDSA or any of the higher sulfonated oligomers (n = 2–11).

Loss of CNSF and its components due to C. polymorpha

The fungus *C. polymorpha* has been reported previously to remove CNSF contained in textile

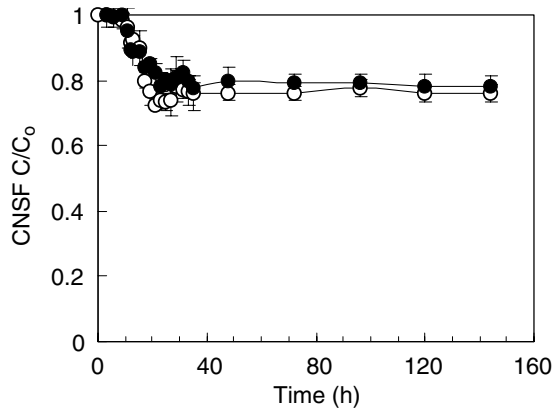

Figure 2. Biodegradation of 500 mg/l CNSF in MSM by *Arthrobacter* sp. 2AC (○) and *Comamonas* sp. 4BC (•). C/Co is the ratio of remaining CNSF concentration to initial CNSF concentration (the data are means ± standard deviation for triplicate incubations. When the error bar is not visible it is within the data point).

wastewater but through sorption to biomass (Kurozumi & Sugimori 2000; Sugimori et al. 1999). We conducted a preliminary study to discover whether biosorption was contributing to the loss measured in the supernatant fraction or if depolymerisation and biodegradation were the major routes of loss. In contrast to the bacteria, when *C. polymorpha* was used all the oligomers were reduced and some ($n = 4$–11) disappeared entirely (Figure 3C). In fact, only monomers, dimers and trimers remained in the supernatant phase after 120 h. In addition, some peaks (at RT 6–8 min) indicate the appearance of novel monomers and these are assumed to have arisen from oligomer degradation.

One of the major constraints to the mineralisation of the oligomers of CNSF is likely to be their molecular mass which will restrict the transport into the cell and, therefore, metabolism. This is a common feature of many macromolecular 'potential' substrates (e.g. cellulose, starch) (Burns 1982; Luxhøi et al. 2002; Nazhad et al. 1995) but may be overcome due to the activities of extracellular depolymerases (Reid 1995; Scherer et al. 1999). If depolymerisation of CNSF could be achieved by a specialist microorganism or group of microorganisms, then uptake and metabolism of the low number oligomers and monomers might be a function of a wide variety of bacteria. However, neither the mixed cultures contained in the acclimated sewage sludge nor the two 2-NSA degrading bacteria were able to depolymerise CNSF oligomers.

The reduction of CNSF (500 mg/l) by *C. polymorpha* at 25 and 37 °C was assessed in more detail by measuring the changes in the concentrations of individual oligomers (n1–n11) both in the supernatant fraction and those sorbed to biomass (Figure 7). The degree (n) of polymerisation decreased progressively with time (also see Figure 3C). At 25 °C CNSF declined by 40 ± 2% within 120 h. Figure 4 shows that the reduction in the concentration of the individual oligomers was inversely correlated ($r = 0.97$) to their degree of polymerisation. For example, the half-lives of n11, n10 and n9 were 20, 22 and 25 h while those for n5, n4 and n3 were 30, 45 and 72 h. This shows that the bonds linking the higher oligomers were cleaved by the depolymerases more rapidly than those connecting the lower oligomers. This is in agreement with what is known as the 'distance principle' (Perales et al. 1999; Swisher 1987), which states that the degradation of longer ($n > 5$) linear alkylbenzenesulfonates (LAS) homologues begins more rapidly and proceeds at a faster rate than it does for the shorter LAS. However, it should be noted that the principle is based on degradation studies of LAS and other surfactants that contain long linear alkyl chains and can only be applied to molecules that contain such structures (e.g. different LAS isomers and homologs). The concentrations of all the oligomers (n2–n11) decreased during the course of the experiment. Oligomers n6–n11 (34.5% of CNSF) were 100% degraded after 72 h. Only monomers (104.1 ± 2.1% of initial monomer concentration), dimers (79.1 ± 3.2%), and trimers (61.5 ± 1.2%), tetramers (20.6 ± 2.3%) and pentamers (7.1 ± 0.5%) remained after 120 h. These five accounted for 60 ± 3% of the initial CNSF concentration and 100% of the final CNSF concentration.

A significant ($p = 0.001$) increase in the concentration of monomers occurred between 20 and 40 h. This must be because the depolymerisation of oligomers proceeds at a rate greater than the mineralisation of the initial or newly generated monomers. HPLC analysis confirms that depolymerisation has taken place and that there has been an increase in the amount and diversity of monomers (Figure 3C). It was difficult to actually quantify the depolymerisation due to the mixture of component oligomers and because the

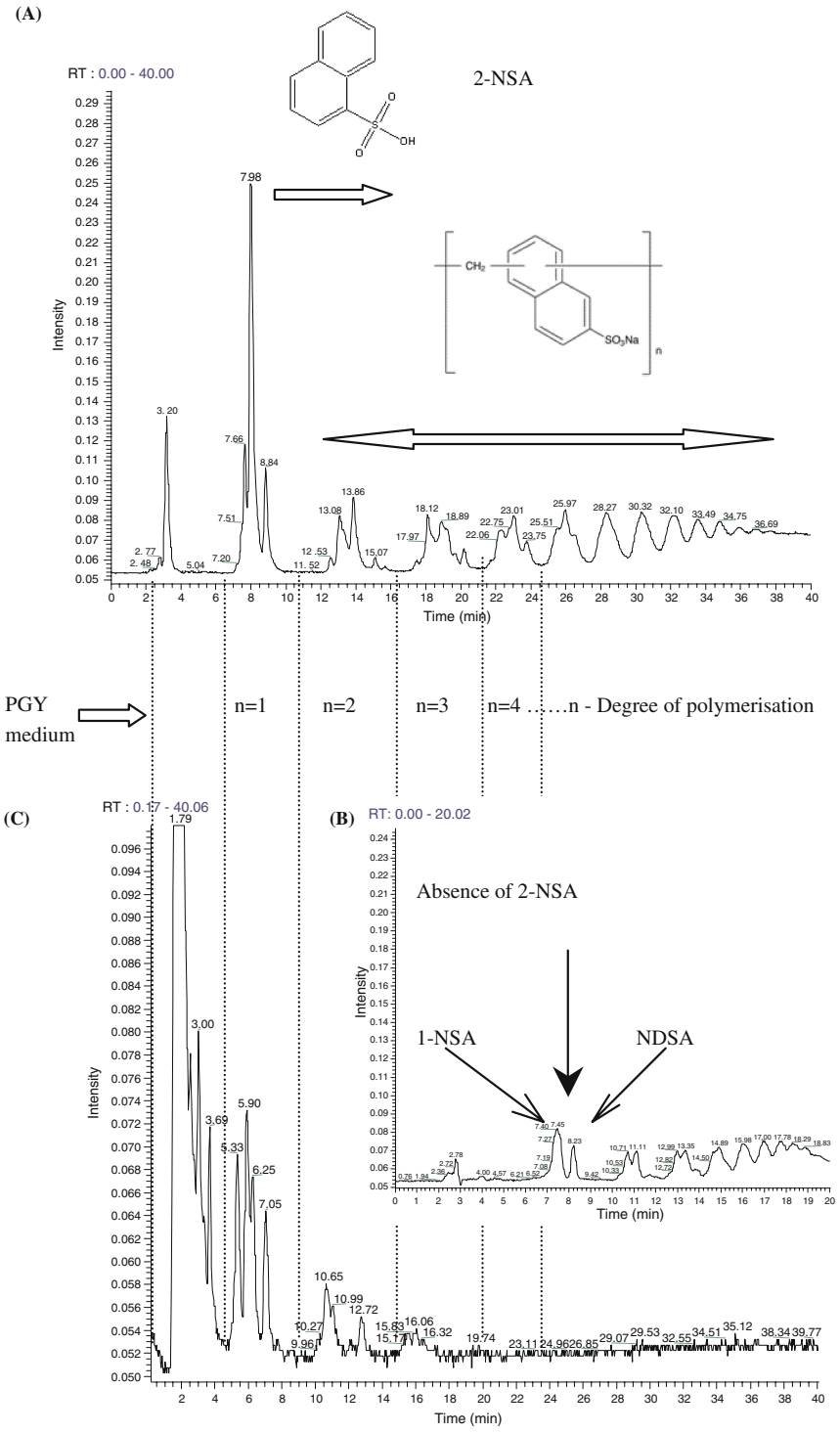

Figure 3. HPLC-UV gradient-elution chromatogram of CNSF before and after biodegradation by bacteria and the fungus: (a) chromatogram of CNSF; (b) chromatogram of CNSF after growth of *Arthrobacter* sp. 2AC or *Comamonas* sp. 4BC and (c) chromatogram of CNSF after growth of *C. polymorpha*.

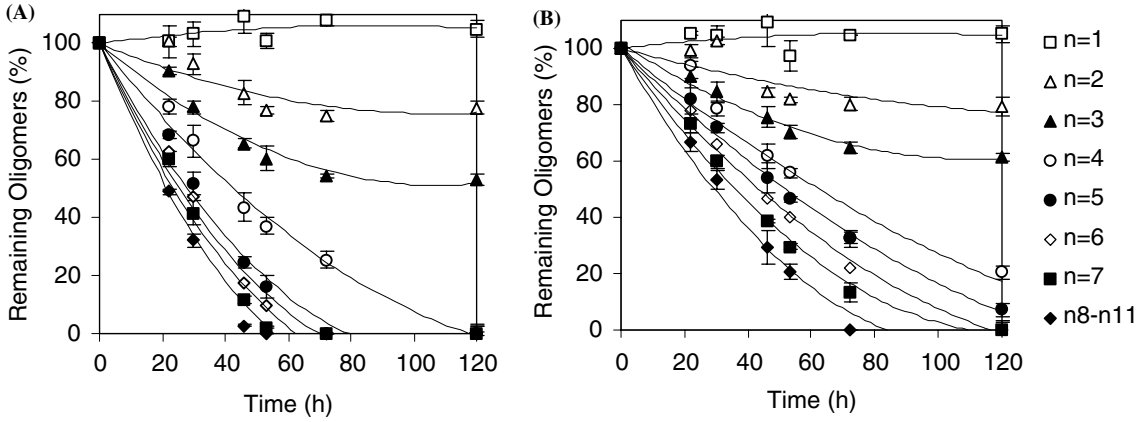

Figure 4. Loss curves of individual CNSF oligomers due to the fungus *C. polymorpha* at (A) 37 °C and (B) 25 °C.

standards for many the oligomers are unavailable. The increase of the monomers in the supernatant fraction (4.1%) was considerably less that the loss of CNSF (40%). However, our preliminary results from the *C. polymorpha* growth experiment did not show that any mineralisation of naphthalene-2-sulfonate had taken place. This suggests that some of the monomers are adsorbed to the biomass and this is discussed in the section on adsorption and biodegradation. Similar results were obtained at 37 °C (4.4% increase in monomers, 45 ± 2% decline in CNSF concentration at a greater rate). Only monomers (104.4 ± 2.5% of initial monomer concentration), dimers (77.8 ± 6.3%) and trimers (53.0 ± 1.1%) remained after 120 h and these three accounted for 55 ± 4% of the initial CNSF concentration and 100% of the final CNSF concentration.

The results of CNSF degradation by the various crude enzyme extracts show that 46.2 ± 1.3 % of the CNSF was degraded after 120 h in the presence of the extracellular fraction, presumably containing a number of depolymerases. This is almost the same level (49.7 ± 0.6%) as achieved by the growing cultures. There was little degradation (3.1 ± 2.9%) when the intracellular cell extract was used. The results confirm that the depolymerisation of CNSF is primarily a function of extracellular enzymes.

Effect of initial concentration of CNSF on losses of monomers and oligomers due to C. polymorpha

The effect of CNSF concentration on the growth (measured as increase in biomass) of *C. polymorpha* and the losses of individual monomers and oligomers was studied. Cultures were grown in PGY medium containing 50–500 mg/l CNSF at 37 °C for 120 h. The results (Table 1) show that, although the percent declines in n1–n3 were reduced at the higher CNSF concentrations (e.g. n3 50 mg/l = 100%; 500 mg/l = 47%), 100% of the n4–n11 oligomers were removed at all concentrations.

Increases in initial CNSF concentration lead to increases in the fungal biomass produced (Table 1). The biomass produced when the initial CNSF concentration was 500 mg/l was 4.05 ± 0.06 g/l and was approximately double that produced in the control flasks (glucose, no CNSF). Biomass at CNSF 100 mg/l was 3.31 ± 0.09 g/l. Initial CNSF concentrations also affected the size and number of the fungal pellets. Colony (i.e. fungal pellet) size in the control was 13–17 mm after 120 h and there were less than 10 colonies per 100 ml. A greater number (ca. 50) of smaller (7.4–8.4 mm) colonies were formed at a CNSF concentration of 100 mg/l. There were many more (> 100/100 ml) colonies at CNSF 500 mg/l but the average diameter was 5 mm. Therefore, colony size decreased as initial CNSF concentration increased. The results are in contrast to those of Sinha et al. (2001) who reported that the colonies of *Paecilomyces japonica* increased in size as the initial substrate concentration (sucrose) increased. Of course, here we used different substrates, fungus and growth conditions. At the higher concentrations, the rate of loss of CNSF and some of its oligomers was reduced (Table 1). Large colonies may experience substrate, enzyme

Table 1. Effects of initial CNSF concentrations on degradation and fungal biomass production

Initial CNSF Conc. (mg/l)	Loss of individual oligomers (%)				Loss of total CNSF (%)	Biomass DW (g/l culture)	Size of fungal pellets (mm)
	$n = 1$	$n = 2$	$n = 3$	$n > 3$			
Control (0)	–	–	–	–	–	2.19 ± 0.03	15.1 ± 1.9
50	5.4 ± 4.8	25.2 ± 1.5	100.0	100.0	56.5 ± 3.4	2.83 ± 0.21	10.3 ± 1.0
100	3.5 ± 7.8	24.3 ± 1.3	95.5 ± 1.2	100.0	55.0 ± 5.1	3.31 ± 0.09	7.9 ± 0.5
250	−2.1 ± 6.8[a]	22.2 ± 1.7	76.9 ± 1.3	100.0	49.9 ± 6.5	3.75 ± 0.15	6.8 ± 1.7
500	−4.9 ± 8.8	22.2 ± 2.2	47.0 ± 1.9	100.0	44.7 ± 6.1	4.05 ± 0.06	4.9 ± 0.9

[a]A negative value indicates that concentration increased. Values presented are means ± standard deviations for experimental flasks (triplicate).

or O_2 diffusional limitations so only peripheral cells may function efficiently (Smith et al. 1990; Van Suijdam & Metz 1981).

Effect of glucose concentration on the loss of CNSF

The loss of CNSF oligomers was dependent on the addition of a second carbon source (Figure 5). In the absence of glucose, the loss of CNSF at all concentrations was < 10% after 120 h. The addition of 2.5 g/l glucose to the medium increased the loss of CNSF to 28 ± 2%. This was further increased to approximately 50% with 5 g/l glucose but was not further enhanced at higher glucose concentrations. CNSF removal (Figure 5) was not directly related to biomass production, as the ratio of reduction to biomass (% CNSF/g biomass) declined even with the addition of more glucose. Together with the consideration of the effect of initial CNSF concentrations on biomass production and fungal pellet size and number, this suggests that CNSF loss is due mainly to fungal metabolism and not to biosorption. Direct extraction of CNSF from biomass confirmed this. CNSF oligomers $n > 3$ were 100% degraded at a glucose concentration of 5 g/l. Furthermore, the fungal biomass produced

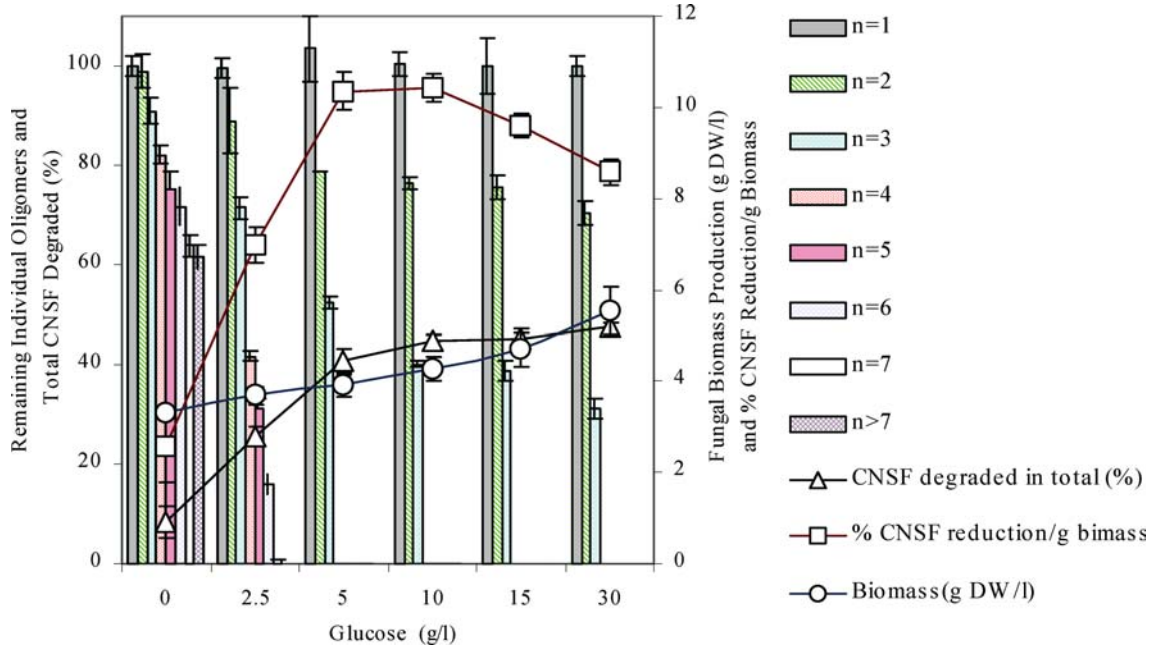

Figure 5. Effect of glucose concentration on the loss of CNSF oligomers by *C. polymorpha*.

increased only a small amount on the addition of glucose and the rate and amount of CNSF loss from the supernatant fraction did not change at all. This suggests that glucose acts as a cosubstrate but not the primary growth substrate. Glucose at 5 g/l gave the highest ratio of CNSF loss to the amount of biomass production (0.10/g dry biomass). This is an important series of relationship to consider when optimising biotreatment plant design when attempting to minimise biomass production, maximise CNSF loss and ultimately reduce discharge costs. Possible functions of glucose in CNSF degradation (in addition to acting as a cosubstrate) may include the generation by glucose oxidases of H_2O_2 (Reid 1995; Swamy & Ramsay 1999) required for extracellular peroxidase activity (Kirby et al. 1995).

Interpretation of loss of CNSF: adsorption and biodegradation

To interpret the loss of CNSF and its oligomers from measurements of the supernatant phase concentration, it is necessary to measure sorption to fungal biomass. This enables the losses to be allocated to degradation and/or biosorption. Heat-killed *C. polymorpha* biomass was used to determine the sorption and recovery rates of CNSF and its oligomers.

After 120 h, the fungal pellet and associated CNSF and oligomers were extracted. Table 2 shows that 10.0 ± 3.6% of the total CNSF was sorbed strongly to fungal biomass. The higher oligomers were sorbed to a greater extent than the lower oligomers (e.g. heptamers 23.4%, monomers 2.6%). Sayadi et al. (2000) claimed that high molecular weight polyphenols (e.g. *p*-coumaric acid) adsorb strongly to the mycelia of *P. chrysosporium* by hydrogen bonding between polyphenolics and proteins or by coagulation. To evaluate the extraction recovery rates, the above values were compared with the results calculated by subtracting the total CNSF and component oligomers in the liquid phase from the initial value. The results show that extraction rates ranged from 87.5% to 100% for individual CNSF oligomers, and overall were 96.0 ± 1.8% of the sorbed CNSF.

The calculated distribution of CNSF and its component oligomers 120 h after inoculation with *C. polymorpha* is shown in Table 2. Fifty-five percent of the CNSF added remained in the solution and this was composed of monomers (104% of original concentration), dimers (77.8 ± 6.3), trimers (53.0 ± 1.1) and tetramers (1.1 ± 0.5%). No oligomers with $n > 4$ were detected in the solution phase. This means that 45% of the total CNSF added was either mineralised or sorbed to biomass.

From Table 2, it can be seen that the monomers and oligomers are sorbed to biomass to different extents, e.g. monomers 5.1%, dimers 7.6%, trimers 22.0% and tetramers 29.3% of original concentration. Higher concentrations of the n3 and n4 oligomers were extracted from live biomass than from heat-killed biomass (+9.9% and +13.2%, respectively). This, together with the increase in the percentage of monomers in solution (4.4%) plus those adsorbed to the biomass (5.1%), confirms that depolymerisation of higher oligomers has taken place. Higher concentrations of lower oligomers (i.e. n3, n4) associated with the biomass, in contrast to that of higher oligomers, suggests that adsorbed lower oligomers may be less bioavailable compared to both adsorbed higher oligomers and all oligomers in the solution phase. After correction for sorption, it is apparent that approximately 75% of the measured CNSF loss that takes place over 120 h (i.e. 33.3% of initial CNSF) is due to fungal metabolism. Adsorption to fungal biomass accounts for the remaining 25%. Table 2 shows the corrected degradation of dimers, trimers and tetramers by *C. polymorpha* and there were 14.6%, 26.3% and 71.2%, respectively. The degradation of the higher oligomers ranged from 85.6% (n5) to 100% (n7–n11).

The reduction of CNSF in the solution phase is due to a combination of fungal metabolism and biosorption with the former playing the dominant part. For example, 21.0 ± 3.6% of hexamers and 23.4 ± 2.5% of heptamers were recovered from dead biomass, whereas in experiments in which CNSF was inoculated with live *C. polymorpha*, no hexamers or heptamers were detected after 120 h in either the biomass or the solution phase. The possibility that CNSF loss was due to non-biological oxidation can be excluded as none of the individual oligomers showed any change in HPLC spectra after 120 h incubation with H_2O_2 (Kerem et al. 1998).

The results also suggest that the depolymerisation of higher oligomers occurred through cleavage along the formaldehyde chain backbone resulting in the monomer which is clearly visible in

Table 2. Mass balance evaluation of individual CNSF oligomers (500 mg/l) in batch CNSF degradation for 120 h by *C. polymorpha* – distribution of loss between biosorption and biodegradation [a]

CNSF and mass balance		Individual CNSF oligomers (%)								Total
		$n = 1$	$n = 2$	$n = 3$	$n = 4$	$n = 5$	$n = 6$	$n = 7$	$n = 8$–11	
Initial CNSF	Percent of individual oligomers	34.3	14.9	15.3	12.1	8.8	5.9	3.6	5.1	100.0
Distribution of CNSF in the presence of fungal biomass	In solution	97.4 ± 3.7	95.0 ± 1.8	90.1 ± 1.5	86.8 ± 4.7	81.7 ± 4.4	79.0 ± 3.6	76.6 ±2.5	74.0 ± 1.6	91.0 ± 3.6
	Biosorbed [b]	2.6 ± 0.3	5.0 ± 0.3	9.8 ± 0.8	12.5 ± 1.0	17.8 ± 1.2	20.6 ± 0.3	23.0 ± 1.0	25.1.0 ± 0.6	9.9 ± 0.5
Biodegradation mass balance	Oligomers remaining in solution	104.4 ± 2.5	77.8 ± 6.3	53.0 ± 1.1	1.1 ± 0.5	0	0	0	0	55.4 ± 1.0
	Oligomers extracted from biomass [c]	5.1 ± 0.1	7.6 ± 1.5	22.0 ± 2.5	29.3 ± 2.5	14.4 ± 3.6	7.3 ± 2.0	0	0	11.5 ± 2.6
	Total mass of biological removal	−9.4 ± 3.9 [d]	14.6 ± 1.5	26.3 ± 1.1	71.2 ± 2.4	85.6 ± 0.9	92.7 ± 0.7	0	0	33.3 ± 1.5

[a] Values presented are means ± standard deviations for experimental flasks (triplicate). [b] Recovery rates for all monomers and oligomers were > 97% and not significant different. [c] Extracted oligomers percentage were corrected for the recovery rates for the individual oligomers. [d] Negative number indicates that concentration has increased due to the depolymerisation of oligomers by *C. polymorpha*.

the chromatograph. The formaldehyde residues remaining after depolymerisation of CNSF would be released into solution or cause cross-linking between adjacent cell wall hydroxyl groups and glucose (Rowell 1995). The accumulation of formaldehyde released from the CNSF may inhibit fungal growth (Garrido et al. 1995) and explain why no further CNSF degradation occurred after 120 h. The incomplete degradation of CNSF could be also due to the need for a community of microbes to the process and/or the limited bioavailability of biosorbed lower oligomers (Guerin & Boyd 1997).

Results presented here show that the removal of CNSF was not increased by cell extract compared to that of whole cell pellets and that no intermediates were detected. It is, therefore, suggested that cell uptake is not the rate-limiting step during the reduction of CNSF oligomers.

Conclusions

We have demonstrated the extensive biodegradation of all components of CNSF by the fungus *Cunninghamella polymorpha*. In contrast, the absence of depolymerisation was a major limiting factor in bacterial degradation of CNSF. The bacteria did not take up the oligomeric components nor depolymerise them, and activated sludge and defined bacterial isolates were only able to degrade the existing monomer component (2-NSA). *C. polymorpha*, on the other hand, depolymerised CNSF extensively prior to metabolism and also removed CNSF and metabolites from the aqueous phase by sorption to the biomass. Complete loss of CNSF oligomers (n4–n11) took place at a faster rate when compared with the lower oligomers (n1–n3) and was dependent on the presence of a second carbon source (glucose). Biodegradation was the main factor in reducing the concentration of the higher oligomers (n4–n11). On the other hand, biosorption played an important part in removal of the dimers and the trimers (but not monomers) from solution. The monomers (i.e. 2-NSA) present in CNSF and those generated during fungal depolymerisation of the higher oligomers are readily degraded by *Arthrobacter* sp. *2AC* and *Comamonas* sp. *4BC*. It is proposed that a combination of *C. polymorpha* and *Arthrobacter* sp. *2AC* or *Comamonas* sp. *4BC* has potential in the treatment of tannery wastewater. Two-phase degradation of the synthetic tanning agent in tannery wastewater using a submerged hollow-fibre membrane bioreactor containing immobilised *C. polymorpha* followed by a GAC biofilm reactor containing *Arthrobacter* sp. *2AC* and *Comamonas* sp. *4BC* has been developed and this will be reported in a subsequent paper.

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