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Acclimated Biomass That Degrades Sulfonated Naphthalene Formaldehyde Condensate

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Abstract: A number of aerobic species were isolated from textile industry activated sludge wastewater. The bacterial consortium was acclimated during seven days before testing its capacity of Sulfonated Naphthalene-Formaldehyde Condensate (SNFC) recalcitrant compound degradation. SNFC's degradation was evaluated by using different techniques including: vapour pressure osmometry, spectroscopy UV-Visible and Chemical Oxygen Demand (COD). The degradation of SNFC by acclimated bacterial consortium was determined by monitoring the decrease of absorbance and of COD at wavelength 288 nm. We were able to deduce that biodegradation of SNFC involves two steps: cleavage of CH_2 bridges and the degradation of the aromatic nuclei. The bacteria species community that was able to degrade SNFC consisted of aerobic Gram-negative rods belonging to the *Pseudomonadaceae* family. The strains were identified as *Bukholderia cepacia*, *Brevundimonas vesicularis*, *Pseudomonas stutzeri*, *Ralostonia picketti*, *Shewanella putrefaciens*, *Sphingomonas paucimobilis* and *Agrobacterium radiobacter*.

Key words: Bioremediation, biomass, heterotrophic bacteria, *Pseudomonadaceae*, sulfonated naphthalene-formaldehyde condensate

INTRODUCTION

Naphthalene sulfonated acid compounds and their formaldehyde condensates (SNFC) were widely used in industrial processes, as tanning agents, mainly in the synthesis of azoic colorants in textile industry (Rivera-Utrilla *et al*., 2002). The sodium salts of condensed products constitute the most important class of synthetic tanning agents, which are largely produced in chemical industry (Redín *et al*., 1999; Alonso *et al*., 2005). Sulfonated Naphthalene Formaldehyde Condensate (SNFC) represents a class of materials able to maintain solid particles in a suspension state inhibiting and preventing their aggregation. SNFC breaks up aggregates into fine particles resulting in a colloidal solution (Ladhari *et al*., 1999). Based on their high aqueous solubility, these compounds are expected to be incompletely eliminated in biological treatment plants and to end up in the aquatic environment (Redín *et al*., 1999; Alonso *et al*., 2002). Aromatic sulfonates were well-studied because of their widespread impact on the environment and their potential health hazard on humans and wildlife. However, the sulfonic naphthalene compounds studied by Rivera-Utrilla *et al*. (2002) presented genotoxic activity at elevated concentrations. The biodegradability of these substances is the result of activate micro-organisms present in the water. The limited biological degradability of sulfonic compounds in environment (Alonso *et al*., 2005) can be enhanced by acclimation of the selective biomass, as indicated by Di Palma *et al*. (2002). For instance, the system of activated sludge involved in textile industrial effluent treatment, was based on the use of micro-organisms, in association, that contribute to select ones which are better adapted against pollution (Huber *et al*., 1998; Wuertz *et al*., 1998; Zhi and Burns, 2005). In this study, we evaluated the degradation of sulfonated naphthalene-formaldehyde condensate through a selective acclimated heterotrophic bacterial biomass by physicochemical parameters and we identified the microbial community that was able to reduce SNFC.

MATERIALS AND METHODS

The dispersant SNFC: The dispersant in this study is Dispergator CC purchased from CIBA, a synthetic

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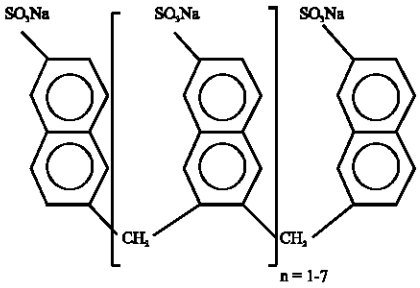

Fig. 1: Chemical structure of Sulfonated Naphthalene Formaldehyde Condensate (SNFC) reconstituted from monomer unit

chemical product, generally used to homogenize dispersed dyes in dyeing baths and to prevent their agglomeration. Concentrated sulphuric acid was added to molten naphthalene, followed by condensation with formaldehyde, neutralization with caustic soda and dilution with water to obtain the final dispersant agent SNFC (Ladhari *et al*., 1999). The final product was composed of numerous ones having different molecular masses. The number-average molecular mass ranges between 242 and 2000 g mol^{-1}. The degrees of condensation were not well known. Two to ten aromatic nuclei were linked (Fig. 1).

Pre-adaptation period: A glass flask containing 500 mL of activated sludge, as used in the study of Nicolella *et al*. (2005), was collected from industrial textile wastewater treatment plant and displayed. The specific physicochemical characteristics were described in Table 1. The activated sludge was supplemented with sulfonated naphthalene formaldehyde condensate at 5 g L^{-1} concentration, in order to obtain an acclimated biomass. The flask was incubated in shaker model SI-600 (Lab Companion- Jeio Tech, Korea) at 200 rpm and 30±2°C, for seven days. Bacterial growth was monitored by measuring absorbance at 600 nm (Hedlund *et al*., 1999; Zhao and Owen, 1999).

Enumeration of cultivable bacteria: The heterotrophic cultivable bacteria enumeration was realised as described by Goñi-urriza *et al*. (1999). We used the following solid culture media: Plate Count Agar (PCA) (Bio-Rad-France) for total cultivable bacteria; Trypto-casein Soya Agar (TSA) (Bio-Rad-France) for cultivable heterotrophic bacteria and Mac-Conkey Agar (Sanofi Diagnostics Pasteur-France) for Gram-negative heterotrophic bacteria. All plates were incubated at 30±2°C under aerobic conditions for 48 h. The results were expressed as colony-forming units (cfu) per milliliter of Acclimated Activated Sludge (AAS). The experience was repeated three times in a separate way.

Table 1: Characteristics of activated sludge from a textile wastewater treatment plant

Parameters	Before treatment	After treatment
BOD (mg L^{-1})	1172	328
COD (mg L^{-1})	4016	69
CU Pt-Co	6912	377
pH	11.89	8.2
Temperature (°C)	42°C	34°C

BOD: Biological Oxygen Demand, COD: Chemical Oxygen Demand; CU: Colour Unit

Development of cultivable biomass: All colonies, cultivated in Mac-Conkey agar plates, were inoculated in Luria-Bertoni broth (Wortman and Colwell, 1988) at 30±2°C in an incubator shaker SI-600 at 200 rpm, for 18 h. The Luria-Bertoni broth was centrifuged at 15000 rpm for 1 min at 4°C (Khamlichi *et al*., 1999) and the pellet was washed three times in a Na-K phosphate buffer (50 mM, pH 7.3) (Keck *et al*., 1997). The cells concentration of inoculum was adjusted between 10^9-10^{10} cfu mL^{-1} in mineral solution MS prepared with : 2.1 mM Na_2HPO_4, 0.9 mM KH_2PO_4, 40 mM NH_4NO_3, 10 mM $MgSO_4.7H_2O$, 10 mM $CaCl_2.2H_2O$, 3 mM $NaSiO_3$, 1 mM $MnCl_2$. $4H_2O$, 1 mM H_3BO_3, 1 mM Na_2MoO_4 . $2H_2O$, 5 nM $FeCl_3$. $6H_2O$, 5 nM $CuSO_4$. $5H_2O$, 5 nM $ZnSO_4$. $7H_2O$ and 5 nM $CoSO_4$. $7H_2O$, as indicated by Tarao and Seto (2000). The pH was adjusted to 7.2.

Physicochemical parameters: The biodegradation of the SNFC was carried out and monitored by using physicochemical methods: Vapour Pressure Osmometer (VPO), spectroscopic UV-Visible, Chemical Oxygen Demand (COD) parameters.

VPO analysis: The number-average (M_n) molecular weights determined the biodegradation rate at macromolecular level of the chains determined by using Knauer Vapour Pressure Osmometer (VPO) (Knauer No. 4968, made in West Germany) and polyethylene glycol (PEG) as calibrated solvent (M_n).

The first series measurements of the ΔR Eq. 1 were carried out on PEG with a known average mass (M_n = 400 g mol^{-1}) to calculate the constant K.

$$\Delta R/C = K\,[1/M\,n + A_2C + A_3C^2 + \ldots] \qquad (1)$$

R = Resistance (signal given from apparatus)
C = Concentration of analyzed product
K = Constant determined by calibration
A_i = Viriel constant

The extrapolation of this calibration curve at the origin gives the K/M_n value; knowing that $M_{n\,(PEG)} = 400\ g\ mol^{-1}$, the constant K can then be calculated: K = 3020. Two solutions A and B, were tested, respectively, at 5 and 10 g L^{-1} concentrations, chosen in order to mimic industrial conditions as closely as possible.

Spectroscopic analysis: The spectroscopic analysis was measured with an UV-visible recording spectrophotometer (UV 2401 PC SHIMADZU, Japan). The analysis was achieved in water using a quartz cell at a λ_{max} wavelength (nm). The maximum absorbance in the Ultra-violet spectrum region was 288 nm, adapted to measure the absorbance of free monomer.

Chemical Oxygen Demand (COD): The chemical oxygen demand was carried out with O'Dell (1993) micro method, on samples extracted from biodegradation tests of SNFC at 0.1 and 0.5 g L^{-1} concentrations. While COD was measured every five days.

Enumeration and identification of bacteria: Cultivable Gram-negative bacteria on Mac-conkey plates were counted and growth colonies were isolated and identified from samples that showed a higher reducing rate of SNFC. Colonies were selected according to routine phenotypic tests: morphological characteristics (shape, size, surface texture, colour and opacity), Gram stain, respiration/fermentation, catalase and cytochrome oxidase activities. In order to identify the bacterial species, strain's biochemical profile was completed by using commercial biochemical Api 20NE (bio-Mérieux, 69280 Marcy-l'Etoile/France).

RESULTS AND DISCUSSION

The results during the acclimation period, showed that in samples supplemented with 5 g L^{-1} of SNFC, micro-organisms' growth decreased compared to the control sample (Fig. 2). Therefore, the bacterial communities were disturbed by the discharge of SNFC into the activated sludge. The number of bacteria was enumerated in Plate Count Agar at 57×10^5 cfu mL^{-1} and Gram negative heterotrophic bacteria enumeration was estimated at 47×10^3 cfu mL^{-1}, as shown in Table 2. The average molecular weight of SNFC was measured before and after biological treatment. Before, the SNFC treatment M_n value was calculated at 1680 g mol^{-1}. This result indicates that used SNFC was composed of seven to eight condensed monomers. After 30 days of treatment, M_n values obtained in solutions A (5 g L^{-1}) and B (10 g L^{-1}) were, respectively, 240 and 430 g mol^{-1}. This shows the

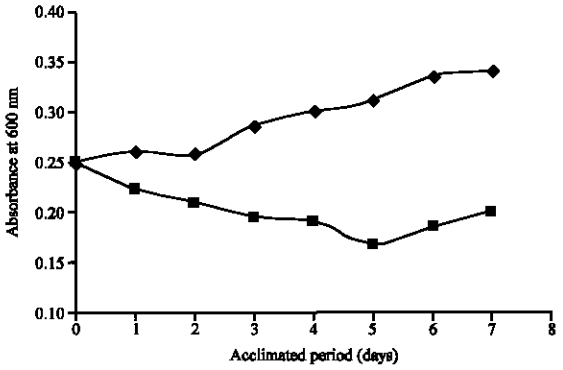

Fig. 2: The effect of SNFC on microbial activated sludge growth during the acclimation period, measured at wavelength 600 nm. (♦): evolution of bacterial growth without SNFC, (■): evolution of bacterial growth supplemented by 5 g L^{-1} of SNFC

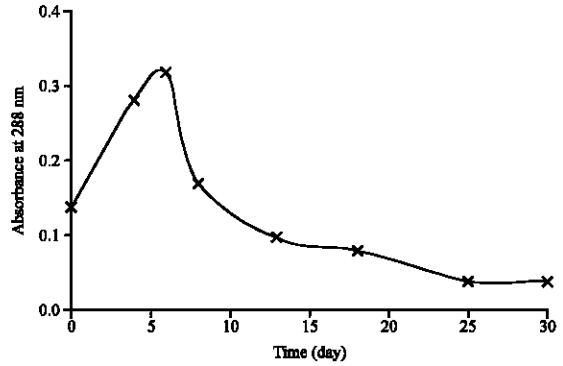

Fig. 3: Evolution of the absorbance at 288 nm of SNFC (×) at 0.1 g L^{-1} during 30 days under aerobic treatment

Table 2: Enumeration of cultivable bacteria from activated sludge supplemented with 5 g L^{-1} of SNFC after acclimation period during seven days

Cultivable bacteria on	Average No. cfu mL^{-1} at 30°C during 48 h
Plate Count Agar	57×10^5
Tryptocasein Soja Agar	35×10^4
Mac-Conkey Agar	47×10^3

cfu mL^{-1}: Colony forming unit per milliliter

bio-transformation of the SNFC into dimmers and monomers structures. This phenomenon was more pronounced in case of a less concentrated sample in SNFC. Indeed, at a SNFC concentration of 0.1 g L^{-1}, the absorbance measurements, at wavelength 288 nm, showed an increase up to the 6th day, followed by a decrease until the 30th day (Fig. 3). The same results were obtained with 0.5 g L^{-1} of SNFC (data not shown). The increase of absorbance at 288 nm during the six first days may be explained by the cleavage of methylene bridges which causes an increase of the monomer number (Miller, 1985). Whereas, the decrease of absorbance, observed

Table 3: Physiological characteristics and API 20NE phenotypical profile of strains

	Strains						
Tests	S1	S2	S3	S4	S5	S6	S7
Gram stain and morphology	Gram-negative rods						
Oxidase activity	+						
Catalase activity	+						
Motility	Mobile						
Respiration/Fermentation	AS/NF						
Growth at 30°C	+						
Reduction of nitrates to nitrites	-	-	-	+	-	-	+
Reduction of nitrates to nitrogen	+	+	+	-	-	-	
Indole production	-	-	-	-	-	-	-
Fermentation of glucose	-	-	-	-	-	-	-
Arginin	-	-	-	-	-	-	+
Urease	+	-	-	+	-	+	-
Hydrolysis of esculin	+	-	+	+	+	+	+
Hydrolysis of gelatin	-	-	-	+	-	-	-
Production of β-galactosidase	+	-	-	-	+	+	+
Assimilation of							
Glucose	+	+	+	-	+	+	+
Arabinose	+	-	+	-	-	+	+
Mannose	+	-	-	-	-	+	+
Mannitol	+	+	-	-	-	+	-
N-acetyl-glucosamine	+	-	-	+	-	+	+
Maltose	+	-	-	-	-	+	-
Gluconate	+	+	+	+	-	+	+
Caprate	+	+	+	+	-	-	+
Adipate	+	+	+	-	-	-	-
Malate	+	+	+	+	-	+	+
Citrate	+	+	+	-	-	-	-
Phenyl-acetate	+	-	-	-	-	-	+

S1: *Bukholderia cepacia*; S2: *Pseudomonas stutzeri*; S3: *Ralostonia picketti*; S4: *Shewanella putrefaciens*; S5: *Brevundimonas vesicularis*; S6: *Agrobacterium radiobacter*; S7: *Sphingomonas paucimobilis*; +: Positive reaction; -: Negative reaction; AS: strict aerobic; NF: Non-Fermentative glucose

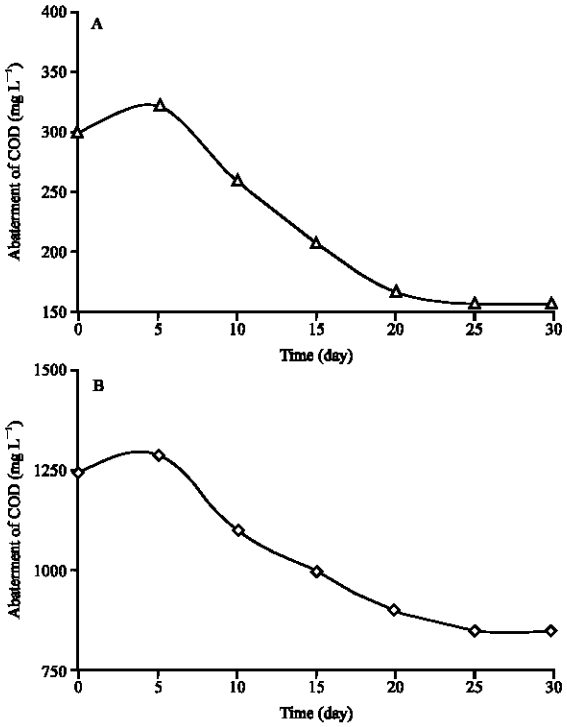

Fig. 4: Evolution of COD versus time of SNFC, (A) of 0.1 g L^{-1} (Δ) and (B) of 0.5 g L^{-1} (◇), during 30 days under aerobic treatment

after six days, may be explained by the ring cleavage of the aromatic compounds. This may be due to a bacterial attack leading to the opening of the naphthalene rings, source of the reduction of their number. Some authors, Lendenmam and Spain (1996) and Ramesha *et al.* (2005), reported that ring cleavage was a key reaction in microbial degradation of aromatic compounds. The result of biochemical identification indicated in Table 3 showed that major active bacteria, present in the various samples were Gram-negative bacilli, responded positively to the oxidase test. These natural isolated strains belong to the *Pseudomonas* and *Agrobacterium* genera. The species identified were: *Bukholderia cepacia*, *Sphingomonas paucimobilis*, *Brevundimonas vesicularis*, *Pseudomonas stutzeri*, *Ralostonia picketti*, *Shewanella putrefaciens* and *Agrobacterium radiobacter*. Moreover, many studies have shown that biodegradation of sulfonated naphthalene was enabled by several species of bacteria belonging to the *Pseudomonadaceae* family (Pandey and Rakesh, 2002; Halden *et al.*, 1999; Vidal *et al.*, 1993). Although, Lee and Clark (1993) identified a natural isolate *Pseudomonas maltophilia* which was originally selected for growth with benzene sulfonic acid as sole carbon and energy source. Furthermore, Zhi and Burns (2005) indicated that degradation of a synthetic tanning agent CNSF (a condensation product of 2-naphthalenesulfonic acid (2-NSA) and formaldehyde) may be possible with a high rate by associated *Arthrobacter* sp. 2AC and *Comamonas* sp. 4BC and the fungus *Cunninghamella polymorpha*. While, COD reduction % of SNFC by acclimated bacterial consortium at 0.5 and 0.1 g L^{-1} were 34 and 46%, respectively within 30 days (Fig. 4A, B). The efficiency of removal COD was obtained with five times less concentration than 0.5 g L^{-1} of SNFC. This result may be attributed to the toxicity effect of SNFC on heterotrophic biomass. Moreover, the reduction of COD becomes almost stable from the twentieth day of treatment; probably due to the diminution of the biological activity without nutrient source. Bioremediation against recalcitrant pollutants could be enhanced by adding microbial material that performs appropriate metabolism functions (Ramesha *et al.*, 2005). Thus, it was important to investigate the mechanisms that lead micro-organisms to adapt and therefore increase their rate of biodegradability (Klatt and LaPara, 2003) against chemicals compounds, in aims to protect environment. However, Anjaneyulu *et al.* (2005) showed that biological treatment have an advantage compared to certain

physico-chemical treatment methods is that over 70% of the organic material present measured by the COD test may be converted to biosolids.

CONCLUSION

The present study indicated the potential of new acclimated microbial consortium to degrade Sulfonated Naphthalene-Formaldehyde Condensate (SNFC). Indeed, the use of microbial biomass for degradation of textile industry effluents is becoming a promising alternative in which some bacterial are used to enhance or to replace present treatment processes.

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