BIODEGRADATION OF AN EMULSIFIED OIL MIXTURE EMPLOYING A VENEZUELAN THERMOPHILIC *ANOXYBACILLUS SP.* STRAIN

Biodegradación de una mezcla de aceites emulsionados utilizando la cepa venezolana termofílica *Anoxybacillus sp.*

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Abstract

Venezuelan thermophilic microorganisms identified as *Anoxybacillus sp.* (DSMZ ID-05-1132) were used to degrade oil mixtures emulsified with different surfactants. An olive oil, triolein and motor oil (1:1:1) mixture, as well as the ingredients separately, emulsified with sodium dodecylsulfate, sodium dodecyl benezenesulfonate, Triton X-100 or Marlox FK-69, were added to mineral liquid medium. Growth rates were faster when the oil mixture was used, instead of the pure oils. Surface tension and chemical oxygen demand (COD) of the culture medium was measured for biodegradation analyses. Results showed that surface tension values remained unaffected during bacterial growth. COD values indicated a diminution of the substances to be oxidized, present in the medium. Surfactants alone were not biodegraded, but stimulated growth when present in the emulsion. The strain apparently degraded exclusively the oil component of the emulsion. The use of these bacteria in the (industrial) cleaning of oily surfaces treated with detergents appears plausible.

Keywords: biodegradation, oils, surfactants, thermophiles.

Resumen

Microorganismos termófilos venezolanos identificados como *Anoxybacillus sp.* (DSMZ ID-05-1132) fueron usados para degradar mezclas de aceites emulsionados con diferentes surfactantes. Una mezcla (1:1:1) de aceite de oliva, trioleína y aceite de motor, así como los components por separado, fueron emulsionados con dodecilsulfato de sodio, dodecilbencensulfonato de sodio, Triton X-100 o Marlox FK-69, y añadidos al medio mineral líquido. El crecimiento bacteriano fue más rápido al usar la mezcla de aceites que los aceites individuales. La demanda química de oxígeno (DQO) y la tensión superficial se midió para evaluar la biodegradación. Los resultados mostraron que la tensión superficial permaneció constante durante el crecimiento bacteriano. La DQO indicó una disminución de las sustancias susceptibles a oxidación presentes en el medio. Los surfactantes individualmente no fueron biodegradados, pero estimularon el crecimiento al estar en la emulsión. La cepa degrada aparente y exclusivamente el componente oleico de la emulsión. El empleo de estas bacterias en la limpieza (industrial) de superficies grasosas en presencia de detergentes parece factible.

Palabras claves: biodegradación, aceites, surfactantes, termófilos.

1 Introduction

Thermophilic organisms are described as those that are able to survive at temperatures between 40 and 70°C (Daniel & Cowan, 2000; Vieille & Zeikus, 2001). The use of these kind of organisms for biotechnological processes are of great importance, for their biochemical pathways can adapt easier to industrial conditions, e.g. high temperatures.

Thermophilic bacteria had found application in the biodegradation of several contaminants, like hydrocarbons, aromatic compounds and other chemical species. The biodegradation of a wide range of hydrocarbons, including aliphatic, aromatic, halogenated and nitrated compounds, has been shown to occur in various extreme habitats. The biodegradation of many components of petroleum hydrocarbons has been reported in a variety of terrestrial and marine ecosystems. Thermophiles, predominantly bacilli, possess a substantial potential for the degradation of environmental pollutants. Some studies have investigated composting as a bioremediation process (Müller *et al.*, 1998; Margesin & Schinner, 2001). Surfactants are normally found in wastewater after their use in domestic and industrial applications, bringing undesirable environmental effects.

Due to their chemical nature, surfactants are a class of xenobiotics that accumulate at interfaces, characteristically present on stones and sediment particles found in river waters (Owen *et al.*, 1997), and are responsible for foaming in drinking water, lowering the water surface tension, emulsifying oil and grease, and destroying some useful bacteria.

In this work, a thermophilic *Anoxybacillus sp.* proceeding from hot water springs Las Trincheras, Venezuela, was used in order to study its possible application for the biodegradation of emulsified oils. The emulsions tested for biodegradation consisted of a mixture of equal proportions of olive oil, triolein and motor oil, using different surfactants.

2 Materials and methods

2.1 Chemicals

Peptone was purchased from ROTH, yeast extract from MERCK, and agar and gellan (Phytagel™) were from SIGMA. Sodium dodecyl benezenesulfonate (DBS), SDS, and Triton X-100 were purchased from FLUKA. Marlox FK-69 was from CONDEA Chemie GmbH, Hamburg, Germany. Motor oil was type super HD II (15W-40). All other materials used were of high purity scientific grade.

2.2 Bacterial growth conditions

Venezuelan thermophilic bacteria were isolated from water samples taken from the hot water springs Las Trincheras, at 65°C, and cultured first in complex medium [5 g/L peptone, 3 g/L yeast extract, 4 g/L KH_2PO_4, 3.6 g/L Na_2HPO_4, 0.25% ($^w/_v$) SDS and 1% ($^v/_v$) oil mixture (olive oil-triolein-motor oil 1:1:1)], for biomass production. For colony selection, a sample of the culture was grown in Petri dishes with selective medium [1 g/L NH_4Cl; 0.5 g/L NaCl; 4 g/L KH_2PO_4, 3.6 g/L Na_2HPO_4, 1% ($^v/_v$) oil mixture] supplemented with the respective detergent [0.25% ($^w/_v$) SDS, 0.15% ($^w/_v$) DBS, 0.01% ($^v/_v$) Triton X-100, or 0.01% ($^v/_v$) Marlox FK-69] and with either agar (25 g/L) or gellan (10 g/L). Detergent concentrations were chosen according to critical micelle concentration (CMC) values. After morphological studies and partial 16S rDNA gene sequencing, the bacterial strain was identified as an *Anoxybacillus sp.* (DSMZ ID 05-1132). Growth measurements in liquid selective medium were followed by optical density at 600 nm and correlated with a calibration curve to obtain the cell number per mL. Cell numbers were counted in an electronic microscope using a Neubauer chamber (Zeiss, Oberkochen, Germany).

2.3 Surface tension measurements

The SITA-online t60-2 tensiometer was used (SITA Messtechnik GmbH, Dresden, Germany), which worked according to the bubble technique and registered temperature, bubble frequency and bubble half-life. The surface tension values (σ) were measured for each of the surfactants at different concentrations in order to performe a calibration curve, and afterwards the CMC was determined. Estimation of CMC was taken as the point in which further increases in surfactant concentration no longer decreased the surface tension value. The valid range of σ to be used was taken as the range of detergent concentration before the slope change. From the data obtained, a linear regression analysis was applied in order to determine the slopes of each of the curves obtained for the surfactants, by plotting the values of σ versus the inverse of detergent concentration. Intersection with the ordinate was defined as σ_w, water surface tension at 22°C.

2.4 COD measurements

In order to determine the total amount of substances capable of being oxidized, present in the supernatant of centrifuged (800 × *g*, 5 min) culture samples, the COD values were measured using the Dr. Lang COD determination kit N°014, and the Dr. Lang photometer CADAS 2000. The procedure was done according to the specifications of the manufacturer (Hach Lange, Düsseldorf, Germany).

3 Results and discussion

Anoxybacillus sp. isolated from the hot water springs Las Trincheras, Venezuela, were grown on selective medium cotaining 1% of the oil mixture emulsified separately with SDS, DBS, Triton X-100 or Marlox FK-69 (Fig. 1). Control experiments done with no oil content in medium confirmed that bacteria were not able to grow in the presence of detergents alone in 48 h (data not shown). Growth was faster when the oil mixture was used than when the pure oils were used separately.

Aliquots at given times were taken and the surface tension was measured. No profound changes in the sigma values were obtained (data not shown). This was indicative that the detergent concentration remained essentially the same throughout the time of bacterial growth. Thus, it can be thought that growth is basically due to the selective degradation of oil as if it were the only C-source present in medium. In order to transform the values of sigma (mN/m) to detergent concentration units, calibration curves with the respective surfactants were done (Table 1). Once the CMC for each detergent was determined, it was chosen to work with concentrations slightly above of the CMC value that could assure emulsification of the oil. This was done in order to detect any minor difference in the sigma value, which would imply a reduction in detergent concentration.

COD values from the bacterial growth were determined (Fig. 1). As it could be predicted, there is a decrease in the COD values when bacteria grow in the different media containing the emulsified oil mixture. Control experiments without oil were carried out and the value subtracted from the emulsified samples.

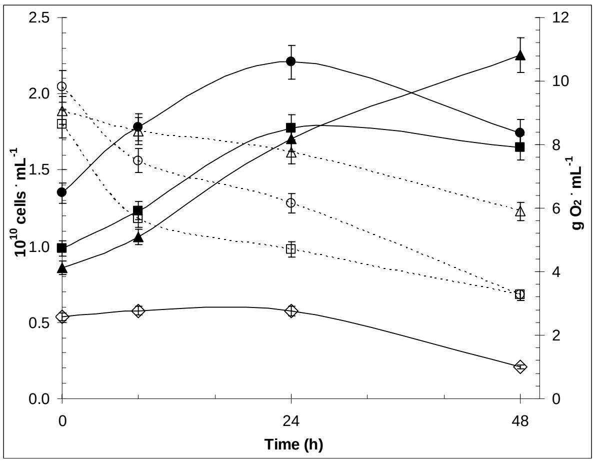

Fig. 1 Growth measurements of thermophiles in the presence of different detergents (left axis) and COD values (right axis). Bacteria samples isolated from the thermal water fountain Las Trincheras (Venezuela), were grown in liquid mineral medium in the presence of 1% of an (1:1:1) oil mixture of olive oil-triolein-motor oil. Oils were emulsified with 0.20 % ($^{w}/_{v}$) SDS [cells per mL (—■—); g O_2 per mL (- -□- -)]; 0.10 % ($^{w}/_{v}$) DBS [cells per mL (—●—), g O_2 per mL (- -○- -)], 0.01% ($^{v}/_{v}$) Triton X-100 [cells per mL (—▲—); g O_2 per mL (- -△- -)] or 0.01% ($^{v}/_{v}$) Marlox FK-69 (—◇—). Controls with only detergents were performed. The negative control contained no emulsion. COD for the Marlox FK-69 sample was not measured.

Table 1 Experimental CMC values of several detergents and minimal σ value

Detergent	MW ($g{\cdot}mol^{-1}$)	MW micelle (kDa)	CMC (theory) (mM)	CMC (theory) (% $^{w}/_{v}$)	CMC[a] (experimental) (% $^{w}/_{v}$)	Slope values for linear regression[a,b] $y = a{\cdot}x + b$ (± 1)	Min. σ value[a,c] (mN/m) (± 1)
SDS	288	18	6.0 - 8.3	0.17- 0.23	0.25 ± 0.05	-155	40
DBS[d]	349	-	1.2	0.04	0.15 ± 0.05	-144	51
Triton X-100	628	90	0.24	0.015	0.010 ± 0.005	-2905	45
Marlox FK-69	-	-	-	-	0.007 ± 0.001	-3764	47

[a] All values reported as mean values ($n = 3$).
[b] slope =a = $(\sigma-\sigma_w)\cdot(D)^{-1}$ [$(mN{\cdot}m^{-1})$/ (% $^{w}/_{v}$)]; $y = \sigma$ = surface tension ($mN{\cdot}m^{-1}$); b=σ_w = 71.5 ± 0.5 $mN{\cdot}m^{-1}$ (water surface tension at 22°C); $x = [D]^{-1}$ = [1/detergent concentration (% $^{w}/_{v}$)].
[c] Refers to the minimal valid σ value (at 22°C), where the linear regression can be used.
[d] According to previous reports (Magdy, 2000).

Care was taken in order to minimize side effects over the COD measurements by centrifuging all samples and taking the supernatant for the COD assay. However, centrifugation produced a contrary effect, in that the emulsion was broken and two phases were observed in the supernatant, which had to be homogenized by thoroughly mixing. In this way, oil biodegradation was proven, for there was a reduction in the COD values of all samples.

Venezuelan thermophilic *Anoxybacillus sp.* was able to biodegrade an oil mixture emulsified with different types of surfactants. Curiously, bacteria were able to specifically select the oil component of the emulsion and use it as sole carbon source, leaving the surfactant unaffected.

The thermophilic bacteria coming from the thermal water fountains Las Trincheras (Venezuela) were studied to evaluate their adaptation to a mineral liquid medium containing the emulsified oil. It was possible to observe bacterial growth in mineral medium supplemented with emulsified oil in 48 h. Each of the detergents employed had a different effect over the growth rate. Triton X-100 had apparently the best growth stimulating effect over the culture (Fig. 1). SDS and DBS also stimulated bacterial growth in a high proportion. Controls done without detergents showed that the lag phase was significantly shorter in the presence of detergents (data not shown). Due to the absence of surfactants, bacterial cells must obligatory segregate lipases, in order to access the C-source and solubilize it. In the presence of surfactants, oils are emulsified and solubilized, decreasing the need of lipolytic enzymes. Thus, cellular growth is facilitated. Further experiments are being done to prove lipase activity.

There are some limitations when bacteria are used to biodegrade oils. Usually, substrates have low solubility values, reducing the bioavailability to the degrading bacteria. Addition of surfactants contributes to substrate solubility, however this may have positive, negative or no effect over thermophiles growth. Accordingly, characterization of these variables is important in order to profit from bacteria for cleaning oil wastes (Van Hamme & Ward, 1999).

According to their chemical properties, detergents must be accurately selected for better biodegradation results. The most relevant characteristics are the net charge, the hydrophile-lipophile balance (HLB) and the CMC (Van Hamme & Ward, 1999). Some characteristics of the detergents used in this study are summarized in Table 1. Theoretical and experimental data were compared. Unfortunately, it was not possible to obtain the theoretical CMC value for Marlox FK-69. This is a not very common surfactant, chemically characterized as a fatty alcohol with an ethoxylate propoxylate adduct. In any case, it was possible to observe that appropriate surfactant selection is important, and factors as molecular structure, HLB and concentration must be taken into consideration, as already reported (Van Hamme & Ward, 1999).

Previous studies done with biodegradation of polycyclic aromatic hydrocarbons (PAH) show that surfactant characteristics must be chosen in such a way that first, they do not inhibit PAH biodegradation and second, the surfactant should be slowly degradable in order to achieve effective bioremediation (Chen *et al*., 2001).

It has been reported (Konopka *et al*., 1996), that linear alkylbenzene sulfonate (LAS) with concentrations over 45 mg·L^{-1} (0.0045% w/v) resulted inhibitory for bacterial growth, when they were tested in a biomass recycle reactor. This value results extremely small in comparison with concentrations used in this study (between 0.01 and 0.2 % w/v). Yet, they were not working with thermophiles. Our results show that 0.01% (w/v) Triton X-100, a non-ionic surfactant, is the most appropriate selection to improve oil biodegradation by *Anoxybacillus sp.* It is interesting to notice that in none of the cases studied, detergent consumption was observed. The surface tension measurements are very accurate and efforts were done to work in an appropriate concentration range in order to visualize a change in the surface tension values.

COD results (Fig. 1) indicated that substances present in medium were oxidized. Taking into account that a reduction in detergent concentration would have been detected in surface tension measurements, it was possible to assume that a reduction in COD is due solely to a consumption of oil as C-source for biomass production. Yet care must be taken in order to treat samples conveniently. Otherwise, errors affecting COD lectures might appear, like the presence of excreted biomolecules into the medium during bacterial growth, emulsion break-up, sample heterogeneity, etc.

An application field for this type of bacteria can be found in the biotreatment of greasy wastewater coming from food industry (Reimann *et al.*, 2002). The scarce solubility of fats in water makes them harder to biodegrade. Nevertheless, if temperature can be raised, there is an increase in fat solubility, and thermophilic organisms, like the one studied here, could be used to treat this kind of wastewater.

A still not discussed point in this study is the biochemical pathway present in the bacterium that enables it to degrade oil selectively. The normal mechanism involved in the uptake of oil molecules has to do with the presence of oxygenases, which “activate” the substrate, converting the oil molecule into an alcohol. Further in the pathway, this alcohol is converted into an aldehyde by means of an alcohol dehydrogenase. At the final oxidation step, fatty acids are obtained, which are introduced into the β-oxidation cycle (Owen *et al.*, 1997; Van Beilen *et al.*, 2003). Other enzymes involved in detergent biodegradation are the alkylsulfatases, which catalyze the hydrolytic cleavage of the ester bond to liberate inorganic sulfate (Kahnert & Kertesz, 2000; Ellis *et al.*, 2002). It still has to be experimentally proven if these enzymatic systems are also present in the thermophiles studied.

4 Conclusions

It was possible to work with an *Anoxybacillus sp.* that selectively biodegraded oil as carbon source in the presence of detergent molecules that were forming an emulsion. The bacteria must have a predominant biochemical route in which they selectively choose oils when these are emulsified with different detergents. The results obtained in this study are also of importance in the cleaning industry. It could be possible to utilize these bacteria in cleaning processes of metallic parts, improving the elimination of oil spots, which are generally treated only with detergents. In this way, bacteria would help the cleaning process of (metallic) surfaces without lowering detergent concentration, and the detergent can be used later on in the same process for further cleaning.

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