BIOTECHNOLOGY LETTERS
Volume 18 No.3 (March 1996) pp.235-240
Received as revised 15th January.

PRODUCTION OF BIOSURFACTANTS BY *Cladosporium resinae*

Joaquín M. Muriel[1], José M. Bruque[2], José Manuel Olías[3] and Alfonso Jiménez-Sánchez[1*]

[1] Departamento de Bioquímica y Biología Molecular y Genética, Universidad de Extremadura, E06080 – Badajoz, Spain; [2] Departamento de Física Aplicada, Facultad de Ciencias, Universidad de Extremadura, Badajoz, Spain; [3] Instituto de la Grasa, C.S.I.C., Sevilla, Spain.

SUMMARY

Cladosporium resinae produces extracellular biosurfactants when growing in a hydrocarbon source such as the jet fuel JP8. This production of biosurfactants was observed by the reduction of the surface tension of the aqueous phase of growing medium, and by the increase in emulsion and foaming properties. A partial purification by collapsed foam gave better physical properties by decreasing surface tension and increasing foaming power and stabilization of emulsions. Surface active substances were purified by reversed phase chromatography. Six compounds representing over 75% of fraction containing surface activity were present. This fraction gave an improvement of all surface properties.

INTRODUCTION

Biosurfactants are surface-active substances synthesized by living cells. As with synthetic surfactants, they have the properties of reducing surface tension, stabilizing emulsions, and promoting foaming, but, as an important difference, biosurfactants are generally non-toxic and biodegradable (Cooper and Zajic 1980;Deasi 1987; Haferberg *et al.* 1986; Lang and Wagner1987). In recent years biosurfactants have been increasingly considered by industry because of their better environmental properties over chemical surfactants. Their properties facilitate their use in medicine, cosmetics, detergency and the food industry.

There are a number of microorganisms known to synthesize surface-active substances, most of them bacteria and yeast (Cooper and Zajic 1980; Cooper *et al.* 1979; Deasi 1987; Itoh *et al.* 1971; Kosaric *et al.* 1987). When growing on hydrocarbon

substrates as the only carbon source, these microorganisms synthesize a wide range of chemicals with surface activity from glycolipids and phospholipids to peptolipids and others (Cooper and Zajic 1980;Deasi 1987; Haferberg *et al.* 1986; Inoue and Ito 1982; Itoh *et al.* 1971; Krestschmer *et al.* 1982; Lang and Wagner 1987; Zukerberg *et al.* 1979). These chemicals are apparently synthesized to emulsify the hydrocarbon substrate and facilitate its transport into the cell. This is of particular significance as extracellular hydrocarbon degrading enzymes have not been reported so far.

In this work we show by the first time the production of biosurfactants from the fungus *Cladosporium resinae*. This fungus was known as the "kerosene fungus" because it was frequently isolated from aircraft fuel tanks (Parbery 1971; Parbery and Thistlethwaite 1973), and became a widespread aviation problem when kerosene replaced gasolene as the most widely used aviation fuel. In our lab we have worked with a strain isolated from jet fuel in 1977 by D. G. Parbery (Parbery 1971). We demonstrate the synthesis of surface-active substances by this fungus and show some properties that make them superior to commercial surfactants.

MATERIALS AND METHODS

Organism and culture conditions. Cladosporium resinae strain 77.40 was isolated from jet fuel and obtained by Douglas G. Parbery, University of Melbourne (Australia). Stock cultures of the fungus were maintained on Czapeck-Dox agar (Oxoid) containing 0.1% of yeast extract and transferred at regular intervals. A modified growth medium of Bushnell-Haas (BH medium) (Parbery and Thistlethwaite 1973) was used for the experiments. The composition was $MgSO_4 \cdot 7H_2O$ 0.02g/l, $CaCl_2$ 0.002 g/l, KH_2PO_4 0.1 g/l, $FeCl_3$ 2 drops 1/10 saturated solution, NH_4NO_3 0.95 g/l. Carbon source was added into the medium at 10% (v/v). Cultures were grown in 2,000 ml Erlenmeyer flasks with 1,500 ml of medium and incubated at 25°C without shaking.

Carbon sources tested were jet fuel JP-8, supplied by the Air Force (Base Aérea Militar de Talavera la Real, Badajoz, Spain), kerosene (Fluka Chem.), paraffinic light

Table 1. Growth of *Cladosporium resinae* in the presence of different carbon sources.

Carbon source	Growth (1)	Carbon source	Growth (1)
jet fuel JP-8	++	cracking gasoline	-
diesel oil	+	aromatic light oil	-
kerosene	+	Nigerian crude oil	-
paraffin C10	+	P-900	-
paraffin C14	+	reduced Nigerian crude oil	-
hexadecane	+	paraffin C13	-
P-147	+	aromatic heavy oil SR-10	-
paraffinic light oil	+	paraffinic heavy oil BS-H	-
cracking diesel oil	+	P-120	-

(1) ++ Confluent growth after 5 days; + Confluent growth after 15 days; – No growth

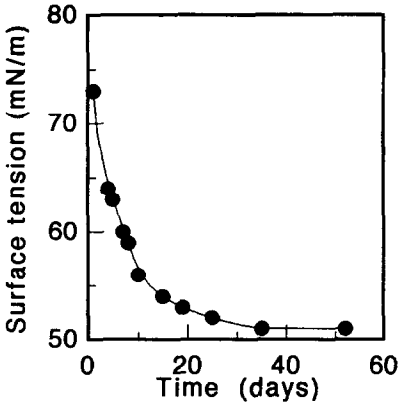

Figure 1. Evolution of surface tension with growth of the fungus.

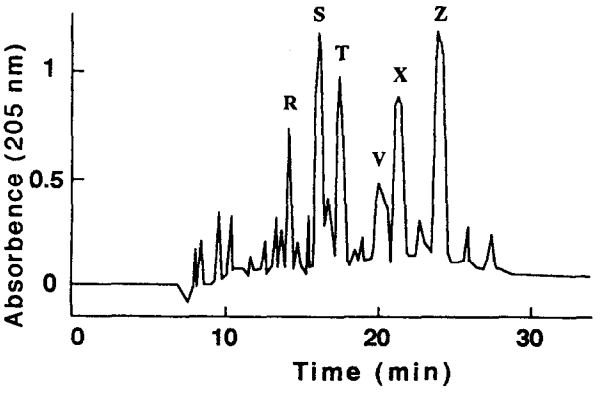

Figure 2. HPLC chromatogram of fraction B

oil, aromatic light oil, Nigerian crude oil, reduced Nigerian crude oil, aromatic heavy oil SR-10, paraffinic heavy oil BS-H (ERTOIL), diesel oil, n-paraffin C10, C13, and C14, mixtures P-120, P-147, and P-900, cracking diesel oil, cracking gasoline (PETRESA), and hexadecane (Merck)

Extraction of surfactants. After removing the fungus by filtration through a Millipore filter of 0.2 μm pore diameter, and decanting fuel, aqueous phase was subjected to foam extraction to concentrate surface active components. Samples were sparged and the overflowing foam was collected and allowed to collapse.

Surface active substances were also purified by chromatography in reversed phase in octadecil (C18) columns (Sep-Pak, Waters). Columns were activated by methanol. Surface active substances were eluted in a fraction by eluting with methanol-water (35:65). Samples obtained were dried with nitrogen, weighed, and redissolved in still water. Samples were analyzed by HPLC in reversed phase C18 (Spherisorb ODS2) using methanol-water (40:60) and acetic acid, 0.02%, in an HPLC Beckman System Gold.

Surface activity measurements. Surface tension was measured with a Lauda Autotensiometer at 20°C. The reciprocal of the critical micelle concentration (cmc^{-1}), as an indirect measure of surfactant concentration, was determined by measuring surface tension of sequential dilutions of samples. Emulsification activity was measured by vortexing 4 ml of sample containing 1 ml of kerosene (Fluka) for two minutes. The resultant uniform oil in water (O/W) microemulsion was allowed to sit for 10 min after which its absorbance through 1 cm pathlength was measured at 540 nm. Decay constant (K_d) was calculated from the slope of the line of the log of absorbance. Bushnell-Haas modified medium and kerosene were used as blank respectively. To determine the water in oil (W/O) emulsification power, equal volumes of sample and kerosene were mixed by vortexing and the percentage of the volume occupied by the W/O emulsion was measured after 2 hours and expressed as the percent of total volume (Parra *et al.* 1990). The amount of foaming was determined by measuring foam heights at intervals after samples were sprinkled with air. The glass tube used was 3.7 cm x 30 cm. It was usually necessary to dilute samples to obtain a manageable foam height.

Table 2. Surface tension and emulsification activity of surfactants produced by *Cladosporium resinae*. Fraction B was used at 28 mg/ml, SDS was 2.31 mg/ml (1 c.m.c.), and Triton X-100 was 0.18 mg/ml (1 c.m.c.).

	Surface tension (mN/m)	Emulsion O/W 1 h (1)	Emulsion O/W Kd	Emulsion W/O (%)
Medium (no growth)	73	0.02	0.00	0
Medium (after growth)	50	0.18	-3.68	18
Collapsed foam	40	0.25	-2.87	52
Fraction B	35	1.15	-3.46	37
SDS	33	0.15	-3.75	55
Triton X-100	32	0.70	-2.68	41

(1) Oil in water emulsion measured as the absorbance at 540 nm after 1 h.

RESULTS AND DISCUSSION

C. resinae was grown in BH medium containing an upper phase of carbon source (Table 1). Jet fuel JP-8 gave the best growth and was consequently used in all cultures. Static cultures were used as aeration by shaking or air bubbling gave less growth.

During growth, surface tension of the aqueous phase was determined on 20 ml samples (Fig. 1). The reduction of the tension from 72 mN/m to 50 mN/m after 25 days showed the production of an extracellular surfactant, which we called Cladosan.

Surfactants produced by *C. resinae* were partially purified by recovering collapsed foam from sparged growth medium. This extraction gave a surface tension of 40 mN/m. Collapsed foam was subjected to organic extraction with chloroform-methanol (2:1) and the chloroform phase concentrated by evaporation and dissolved in water. This solution gave a surface tension of 28 mN/m.

An extraction and partial purification was obtained by reversed phase chromatography using C18 columns. The compounds were extracted with methanol-water. Three fractions were obtained after eluting with methanol-water 8:92, fraction A; 35:65, fraction B; and 55:45, fraction C. Only fraction B decreased surface tension to 35 mN/m. This surface active fraction gave 156 mg of dried weight per liter of growth medium. Analysis by HPLC showed it to be a mixture of six compounds corresponding to 75% of total fraction (Fig. 2). These compounds have been purified and analyzed and their chemical composition will be published elsewhere.

All purifications gave stable foaming (Fig. 3). Values of foaming obtained for collapsed foam, diluted 16 fold, and that of fraction B, at 0.25 mg/ml, were similar to those obtained with SDS at 64 fold dilution of its cmc.

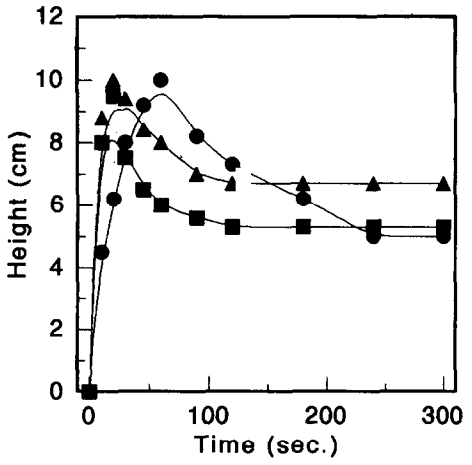

Figure 3. Foaming power of Cladosan measured by foam height of sparged grown medium diluted 8 times (●), collapsed foam diluted 16 times (■), and fraction B (0.25 mg/ml) (▲).

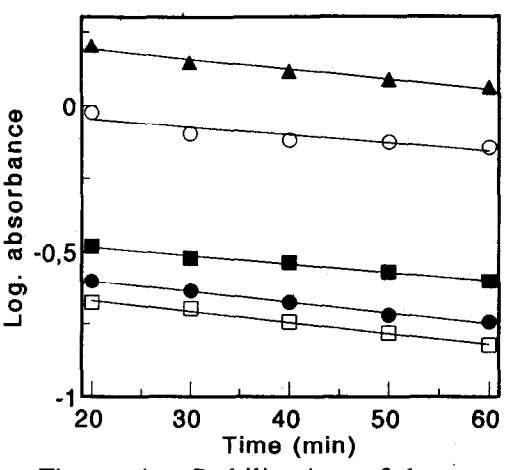

Figure 4. Stabilization of kerosene emulsion in the presence of Cladosan from cell free culture medium (●), or collapsed foam (■), or fraction B (▲), versus SDS (□) and Triton X-100 (○).

Emulsification properties was measured by either O/W and W/O emulsion (Table 2 and Fig. 4). These measurements were compared with commercial lauryl sulfate (SDS) and Triton X-100 (Sigma) used at their critical micellar concentration (1 cmc). Data show that surfactants synthesized by *C. resinae* can be considered among the best commercial surfactants.

Accordingly with results we obtain a mixture of surfactants with better O/W emulsion property than tested commercial surfactants when using fraction B, and better W/O emulsion in the collapsed foam. The higher O/W emulsion after purification by reversed phase chromatography points out that the composition of the surface active products have changed during purification. This increment attests that fraction B could be a mixture of surfactants with a high hydrophilic balance.

Acknowledgments

We are grateful to D. G. Parbery for making available to us his isolation of the fungus. This research was supported by grants CNA90 y CNA93/06 from Central Nuclear de Almaraz, and PB91-0559 from DGICYT, Spain.

References

Cooper, D.G. and Zajic, J.E. (1980). *Adv. Appl. Microbiol.* 26, 229-253.

Cooper, D.G., Zajic, J.E. and Gerson, D.F. (1979). *Appl. Environ. Microbiol.* 37, 4-10.

Desai, J.D. (1987). J. Scient. Ind. Res. 46, 440-449.
Haferberg, D., Hommel, R., Claus, R. and Kleber, H.P. (1986). *Adv. Biochem. Eng. Biotechnol.* 33, 53-93.
Inoue, S. and Ito, S. (1982). *Biotechnol. Letters* 4, 3-8.
Itoh, S., Honda, H., Tomita, F. and Suzuki, T. (1971). *J. Antibiotics* 24, 855-859.
Kosaric, N., Cairns, W.L. and Gray, N.C. (1987). *In* Marcel and Dekker (ed.), Inc New York.
Krestschmer, A., Bock, H. and Wagner, F. (1982). *Appl. Environ. Microbiol.* 44, 864-870
Lang, S. and Wagner, F. (1987). *Surfactant. Sci. Ser.* 25, 21-45.
Parbery, D.G. (1971). *Material und Organismen* 6, 161-208.
Parbery, D.G. and Thistlethwaite, P.S. (1973). *Int. Biodetn. Bull.* 9, 11-16.
Parra, J.L., Pastor, J., Comelles, F., Manresa, M.A. and Bosch, M.P. (1990). *Tenside Surf. Det.* 27,302-306.
Zukerberg, A., Diver, A., Peeri, Z., Gutnick, D.L. and Rosenberg, E. (1979). *Appl. Environ. Microbiol.* 37, 414-420.