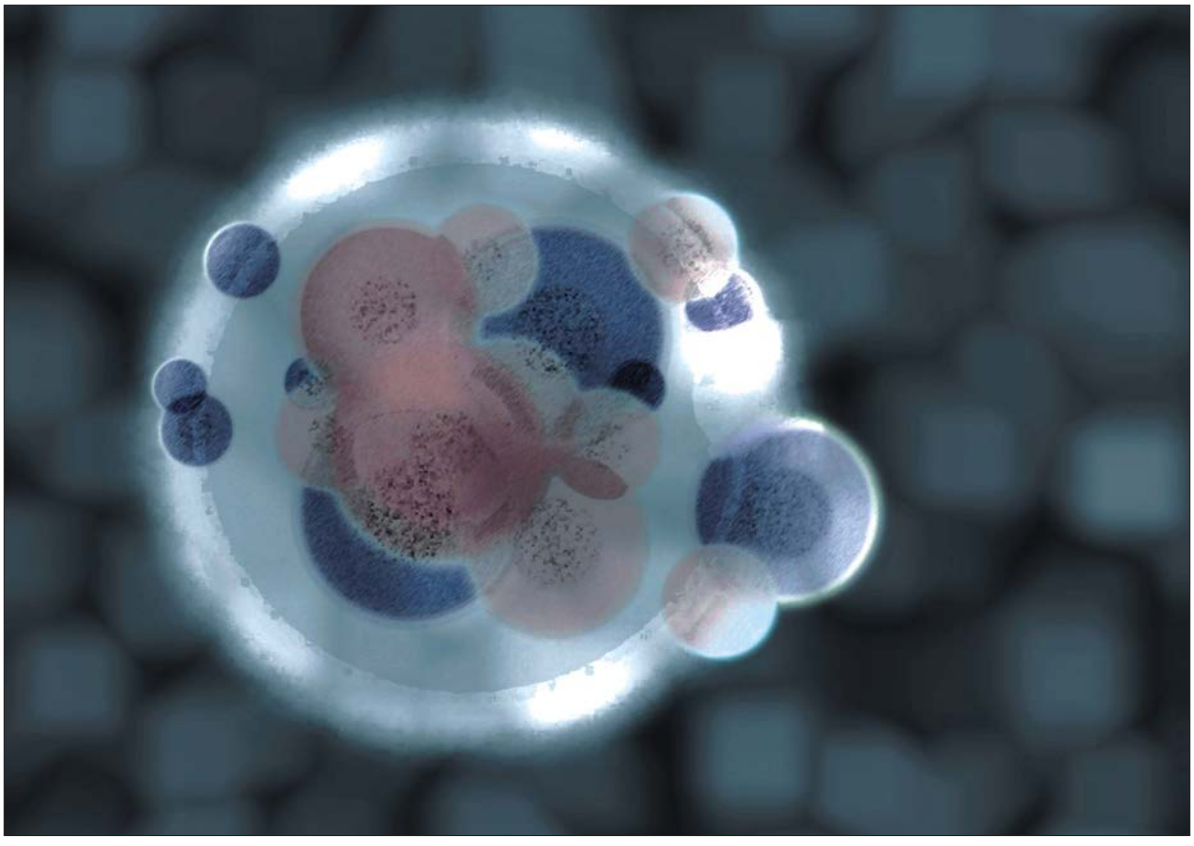

Journal of Biological Sciences

ISSN 1727-3048

science alert

ANSInet
an open access publisher
http://ansinet.com

Journal of Biological Sciences 6 (3): 511-515, 2006
ISSN 1727-3048

Lipopolysaccharide Extracellular Emulsifier Produced by *Penicillium citrinum*

[1]M.M. Camargo de Morais, [1]S.A.F. Ramos, [2]M.C.B. Pimentel,
[2]E.H.M. Melo, [3]M.A. Morais Jr., [4]J.F. Kennedy and [1,2]J.L. Lima Filho
[1]Laboratório de Imunopatologia Keizo Asami, [2]Departamento de Bioquímica, [3]Departamento de Genética,
Universidade Federal de Pernambuco, Cidade Universitária, Recife, PE, Brazil, CEP 50670-901
[4]Chembiotech Laboratories, University of Birmingham Research Park,
Vincent Drive, Birmingham B15 25Q, UK

Abstract: A Brazilian strain of *Penicillium citrinum* produced a lipopolysaccharide with emulsifier properties during cultivation on mineral medium, containing ammonium sulfate as nitrogen source, with 1% (v/v) olive oil as the carbon source. The maximal emulsifier production (1.6 U mL^{-1}) was obtained after 60 h of cultivation and the biomass reached 7.5 g L^{-1} at the end of fermentation. The production yield i.e. the amount the carbon source utilized for product synthesis was 54% and the best emulsifying activity was observed for xylene and diesel oil when compared to other carbohydrates tested. The emulsifier was shown to be stable to a wide range of pH and temperature values and was shown to contain D-galactose, D-glucose and D-xylose (8.2:1.0:5.3) with a total carbohydrate content of 43%. The presence of salts stimulated the emulsification activity, suggesting potential for its application in industrial waste or marine remediation.

Key words: *Penicillium citrinum*, citrosan, lipopolysaccharide, emulsifier, surfactant

INTRODUCTION

Emulsifiers are amphyophilic compounds usually obtained from plants or manufactured chemically. However, many microrganisms are now being described as new sources of emulsifying agents. This arises because a number of microrganisms that are able to assimilate hydrocarbons are also capable of emulsifying these hydrocarbons during the substrate degradation process (Gutnick and Rosemberg, 1977; Cirigliano and Carman, 1985; Carrillo *et al*., 1996). Emulsifiers and surfactants of microbial origin have drawn increasing interest because of their biodegradability and potential commercial applications in food, petroleum spill recovery technologies, cosmetic and pharmaceutical industries (Reiger, 1983; Nitschke and Pastore, 2006; Velikonja and Kosaric, 1993; Banat, 1995). This is in contrast to synthetic emulsifiers and surfactants, the production of which is often petrochemical dependent and which are non biodegradable. Most of the microrganisms reported to produce bioemulsifiers and biosurfactants are bacteria and yeasts, although some are filamentous fungi (Muriel *et al*., 1996). The isolation of a *Penicillium citrinum* Brazilian strain has been previously described and this strain is capable of producing extracellular lipase when cultivated in ammonia minimal medium (Pimental *et al*., 1996). The production in batch fermentation of a lipopolysaccharide (called citrosan) with emulsifying properties from this strain of *P. citrinum*, is now reported together with some of its characteristics and emulsification properties.

MATERIALS AND METHODS

Microorganism strain and media: A *Penicillium citrinum* strain (Pimental *et al*., 1994) was grown in MA medium (57 mM $(NH_4)_2SO_4$, 2.6 mM NaCl, 1.1 mM KH_2PO_4, 4.5 mM $MgSO_4$, 5.2 μM $ZnSO_4$, 0.4 μM $FeSO_4$) supplemented with 1% (v/v) either olive oil, hexadecane, glucose or diesel oil as carbon source.

Cultivation conditions: Batch fermentations were performed in a 5L BIOFLO 2000 fermenter (New Brunswick Scientific Co. New Jersey, USA). Pre-inocula consisting of MA medium (100 mL) incubated with 10^5 spores per mL of a culture of *P. citrinum* and cultivated for 5 days at 30°C in a rotatory shaker, were used to inoculate 2.9 L of MA medium. Fermentations were carried out at 30°C, 400 rpm stirring and an aeration rate of 6 volumes of oxygen per volume of medium per min aeration rate. Small scale cultivations in flasks were carried out in MA medium (50 mL) with different carbon sources, using a rotatory shaker at 30°C and 128 rpm.

Corresponding Author: Prof. John F. Kennedy, Chembiotech Laboratories, University of Birmingham Research Park, Vincent Drive, Birmingham B15 25Q, UK

Biomass estimation: Biomass was determined by filtration of sample (10 mL) of culture medium, drying the cell mass for 16 h at 105°C and weighing.

Emulsifier production and emulsifying activity measurement: Emulsifier production was measured by monitoring spectrophotometrically the filtered culture medium against a medium blank at 202 nm as a means of measurement of light dispersion. This value was selected by scanning the last sample of the first fermentation in the range of 189 to 910, a peak of absorbance being observed at 202 nm. Emulsification activity during microbial growth in the fermenter was monitored by determination of the degree of dispersion in xylene or other hydrocarbons, according to Cirigliano and Carman (1985). One unit of emulsification activity was defined as that amount of bioemulsifier that effected an emulsion with absorbance value of 1.0 at 540 nm. The emulsification index (E) was calculated according to Abu-Ruwaida *et al.* (1991).

Bioemulsifier extraction: Emulsifying agent was extracted from the filtered culture medium by precipitation with cold ethanol (ethanol:medium ratio 4:1) at -20°C for 16 h. The precipitated material was recovered by centrifugation at 4000×g for 15 min and air drying the pellet. The resulting white powder was soluble in water, buffers or salt solutions.

Compositional analysis: The protein content of the bioemulsifier preparation was measured according to Lowry *et al.* (1951). Carbohydrate content was estimated by the phenol-sulphuric method described by Chaplin and Kennedy (1994). The free lipid content was determined by extracting bioemulsifier (0.25 g) twice with diethyl ether (4.0 mL). The pooled ether extract was dried using warm air and weighed.

Filtered culture medium was hydrolysed with 4 M HCl for 16 h at 100°C in sealed tubes. The hydrolysates were resolved on silica gel G plates (MERCK). For lipid detection, the plates were pre-saturated with silver nitrate and the chromatogram was run using chloroform as solvent. Development was made with 0.15% w/v 2,7-dichlorofluorescein in ethanol and visualisation under UV light. Hydrolysis to liberate the carbohydrate as monosaccharides showed that maximal release occurred using 2 M trifluoroacetic acid (10 mg bioemulsifier per mL) in sealed tubes at 121°C for 1 h. HPLC was performed on a Dionex analyser with a PAD detector and a Carbopak PAI column.

Effect of different salts, pH values and temperatures on the emulsifying activity: The effect of the presence of salts on the emulsifying agent was determined by dissolving the precipitated powder (0.1% final concentration) in 10 mM $CaCl_2$ or 1000 mM NaCl. The emulsification index was calculated and compared to that of the same solutions in water. The stability of the emulsifying agent at different pH values was estimated by dissolving the emulsifier (0.3% w/v final concentration) in 0.1 M sodium acetate buffer pH 3.0; 0.1 M citrate-phosphate buffer pH 5.0; 0.1 M tris-maleate buffer pH 7.0 and 0.1 M borate buffer pH 10.0). After 1 h of incubation with reciprocal agitation, the emulsifying activity was measured as described. The emulsification index was compared to that of the corresponding solution in water. The determination of the stability of the emulsifying agent at different temperatures was carried out by incubating the emulsifying agent at 0.3% w/v in water for 30 min at 25-120°C before measuring the emulsifying activity.

RESULTS AND DISCUSSION

Emulsifier production: *P. citrinum* strain used in this study was originally isolated as an olive oil contaminant and its extracellular lipase has been characterised (Pimental *et al.*, 1994). The time course of bioemulsifier production during growth in a 5 L fermenter in MA medium containing 1% olive oil as carbon source.

Figure 1 showed that bioemulsifier production was growth-linked and started at about 48 h of cultivation and reaching maximum levels at 72 h of cultivation. Bioemulsifying activity, however, reached the highest level (1.6 U mL^{-1}) at 60 h of cultivation, thereafter remaining constant. This behaviour could be explained by the fact that bioemulsifier is being produced to facilitate the carbon source assimilation. Similar results have also been observed with *Pseudomonas marginalis* (Burd and Ward, 1997), *Rodococcus* sp. 51T7 (Espuny *et al.*, 1996) and *Rhodotorula glutinins* IIP-30 (Johnson *et al.*, 1992). The bioemulsifier production yield (product in g/carbon source in g) was 0.539 (w/w), indicating that under these conditions about 54% of the carbon source was utilised for bioemulsifier biosynthesis. This yield, higher than the value of 38% reported for *Pseudomonas aeroginosa* 44T1 using 2% olive oil (Robert *et al.*, 1989), was closer to that obtained by Jarman and Pace (1984) with a continuous culture of *Xanthomonas campestris* to produce xanthan gum (60-70%).

Based on the shake-flask cultures using different substrates, the highest emulsifying activity (0.21 U mL^{-1}) production was observed using olive oil 1% (v/v) (Fig. 2). *P. citrinum* was however able to grow with hexadecane, glucose and diesel oil as carbon sources but these materials were not good inducers of bioemulsifier activity.

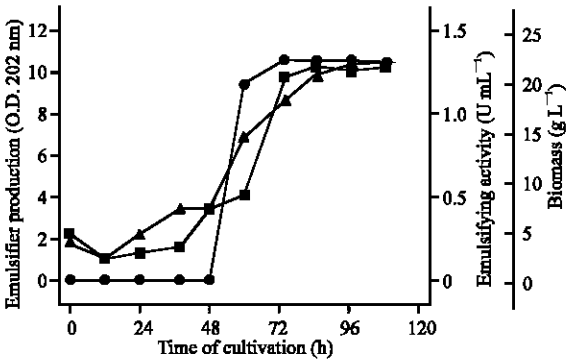

Fig. 1: Time course of production by *P. citrinum*, cultivated in a fermenter with 1% olive oil as substrate, of emulsifying activity (λ), emulsifier production (γ) and biomass (σ)

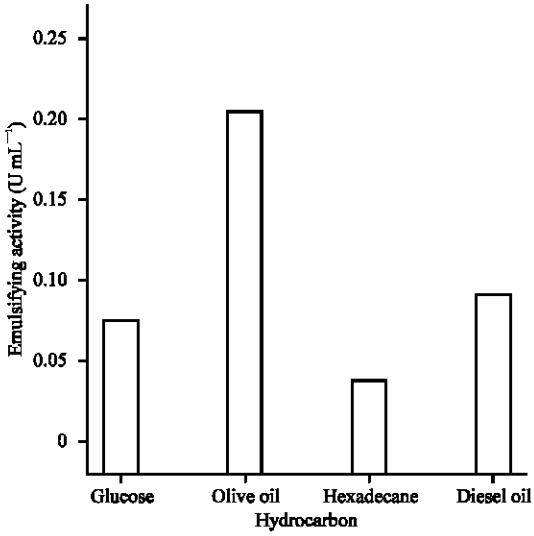

Fig. 2: Production by *P. citrinum* of emulsifying activity as a function of carbon sources

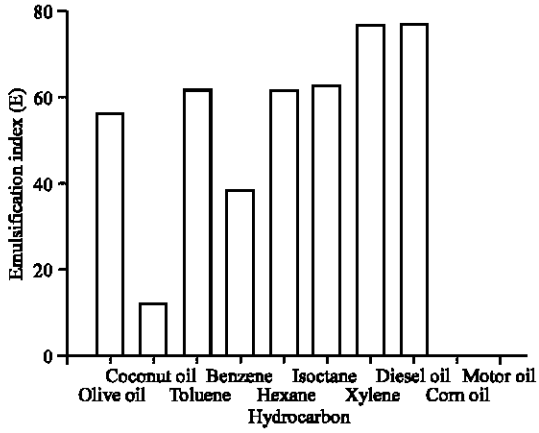

Fig. 3: Emulsifying activity of the filtered culture medium with various hydrocarbons and oils

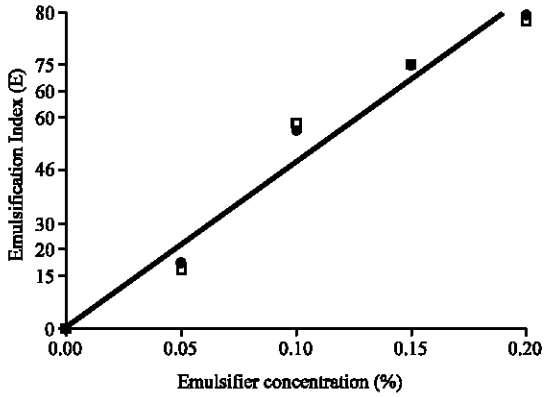

Fig. 4: Effect of bioemulsifier concentration on the emulsifying activity in toluene and xylene (λ)

Compared to cultivation in the fermenter, production activity in shake flasks was lower, probably due to an aeration-dependence, since the production occurs in the exponential phase of growth.

Emulsifier composition: The bioemulsifier was shown to be constituted basically of carbohydrates (43%) and lipids (51%) and a small content of protein (6%). Similar component proportions were reported by Pruthi and Cameotra (1997) for a biosurfactant with emulsification properties isolated from *Serratia marcescens*. HPLC analysis of the hydrolysate of the bioemulsifier showed that the carbohydrates (43%) contained galactose, glucose and xylose in the ratio of 8.2:1.0:5.3 (w/w/w).

Emulsifier activity: Bioemulsifier activity was tested with different hydrocarbons and oils at 42°C because some vegetable oils tested are solid at room temperature (Fig. 3). The emulsions showed good stability during the whole period of observation (20 days) and highest emulsion values were obtained with xylene and diesel oil. Citrosan was not effective in emulsifying coconut oil, corn oil or motor oil, suggesting that citrosan has a high emulsification specificity. This phenomenon has been observed by Abu-Ruwaida *et al.* (1991), Kaplan and Rosenberg (1982) for other surface active compounds and can be a useful tool for distinct applications for petroleum and environmental remediation agencies. After extracting from the culture medium, citrosan was submitted to different conditions of pH, temperature and ions to elucidate some of the emulsifier's properties (Table 1). The emulsifier showed acceptably good stability up to 100°C, with 70% of its initial activity remaining after 30 min. Nevertheless, temperatures above 100°C had a deleterious effect on the emulsifying activity. The pH did not affect significantly the

Table 1: Thermal, ionic concentrations and pH stabilities of citrosan produced by *P. citrinum*

Factor	Value	Initial emulsifying activity (%)
pH	3.0	88.0
	5.0	100.0
	7.0	95.0
	10.0	100.0
Temperature (°C)	25.0	100.0
	50.0	94.0
	70.0	88.0
	100.0	72.0
	120.0	44.0
Ionic concentration (mM)	10	150.0
	1000	132.0

the emulsifying activity, with a slight loss in activity occurring when a pH as low as 3.0 was reached. These results are very similar to those reported for *Sphingomonas paucimobilis*, *Bacillus subtilis* and *Rodococcus* species (Abu-Ruwaida *et al*., 1991; Ashtaputre and Shah, 1995; Kim *et al*., 1997).

Due to the fact that sodium salt is the main component in sea water and that calcium salt is commonly present in industrial water (Ilori *et al*., 2005), citrosan was tested in both sodium and calcium salt solutions. Both salt solutions caused an increase in emulsifying activity; a stimulatory effect also observed for the *Rodococcus* biosurfactant (Abu-Ruwaida *et al*., 1991). Since the bioemulsifier produced by *Penicillium citrinum* is not strongly affected by changes in pH, temperature and salinity of the medium, it holds good potential for use in a variety of situations.

The relationship between the emulsifier concentration and emulsifying activity (Fig. 4) was approximately linear with toluene as growth carbon source. This is similar to that obtained by Kim *et al*. (2005), who described a linear correlation between emulsifier concentration and emulsifying activity for *Bacillus subtilis* emulsifier.

ACKNOWLEDGMENTS

This study was supported by CAPES, FACEPE, FINEP, CNPq, PADCT, BNB and The British Council. The authors thank to Dr. Nicácio H. da Silva for his help with the citrosan extraction and to Dr. William. M. Ledingham for his helpful contributions to the manuscript preparation.

REFERENCES

Abu-Ruwaida, A.S., I.M. Banat, S. Haditirto, A. Salem and M. Kadri, 1991. Nutritional requirements and growth characteristics of a biosurfactant-producing *Rhodococcus* bacterium. Acta Biotechnol., 11: 315-324.

Ashtaputre, A.A. and A.K. Shah, 1995. Emulsifying property of a viscous exopolysaccharide from *Sphingomonas paucimobilis*. World J. Microb. Biotechnol., 11: 219-222.

Banat, I.M., 1995. Characterisation of biosurfactants and their use in pollution removal-state of the art (review). Acta Biotechnol., 15: 251-267.

Burd, G. and O.P. Ward, 1997. Energy-dependent accumulation of particulate biosurfactant by *Pseudomonas marginalis*. Can. J. Microbiol., 43: 391-394.

Carrillo, P.G., C. Mardaraz, S.I. Pitta-Alvarez and A.M. Giulietti, 1996. Isolation and selection of biosurfactant-producing bacteria. World J. Microbiol. Biotechnol., 12: 82-84.

Chaplin, M.C. and J.F. Kennedy, 1994. Carbohydrate Analysis. A Practical Approach. 2nd Edn., Oxford:IRL Press, pp: 1-36.

Cirigliano, M.C. and G. Carman, 1985. Purification and characterisation of Liposan, an emulsifier from *Candida lipolytica*. Applied Environ. Microb., 50: 846-850.

Espuny, M.J., S. Egido, I. Rodón, A. Manresa and M.E. Mercadé, 1996. Nutritional requirements of a biosurfactant producing strain *Rodococcus* sp. 51T7. Biotechnol. Lett., 18: 521-526.

Gutnick, D.L. and E. Rosemberg, 1977. Oil tankers and pollution: A microbiological approach. Ann. Rev. Microbiol., 31: 379-396.

Ilori, M.O., C.J. Amobi and A.C. Odocha, 2005. Factors affecting biosurfactant production by oil degrading *Aeromonas* spp. isolated from a tropical environment. Chemosphere, 61: 985-992.

Jarman, T.R. and G.W. Pace, 1984. Energy-requirements for microbial exopolysaccharide synthesis. Arch. Microbiol., 137: 231-235.

Johnson, V., M. Singh, V.S. Saini, D.K. Adhikari, V. Sista and N.K. Yadav, 1992. Emulsifier production by an oleaginous yeast *Rhodotorula glutinis* HP-30. Biotechnol. Lett., 14 :487-490.

Kaplan, N. and E. Rosemberg, 1982. Exopolysaccharide distribution of and bioemulsifier production by *Acinetobacter calcoaceticus* BD4 and BD413. Applied Environ. Microbiol., 44: 1335-1341.

Kim, H.S., B.D. Yoon, C.H. Lee, H.H. Suh, H.M. Oh, T. Katsuragi and Y. Tani, 1997. Production and properties of a lipopeptide biosurfactant from *Bacillus subtilis* C9. J. Ferm. Bioeng., 84: 41-46.

Lowry, O.H., N.J. Rosenbrough, A.L. Farr and R.J. Randall, 1951. Protein measurement with the folin phenol reagent. J. Biol. Chem., 193: 265-275.

Muriel, J.M., J.M. Bruque, J.M. Olías and A. Jiménez-Sanchez, 1996. Production of biosurfactants by *Clostridium resinae*. Biotechnol. Lett., 18: 235-240.

Nitschke, M. and M.G. Pastore, 2006. Production and properties of a surfactant obtained from Bacillus subtilis grown on cassava wastewater. Bioresource Technol., 97: 336-341.

Pimentel, M.C.B., N. Krieger, L.C.C.B. Coelho, J.O. Fontana, E.H.M. Melo, W.M. Ledingham and J.L. Lima Filho, 1994. Lipase from a Brazilian strain of *Penicillium citrinum*. Applied Biochem. Biotechnol., 49: 59-74 .

Pimentel, M.C.B., E.H.M. Melo, J.L. Lima Filho and N. Durán, 1996. Production of lipase free of citrinin by *Penicillium citrinum*. Mycopathologia, 133: 119-121.

Pruthi, V. and S.S. Cameotra, 1997. Rapid identification of biosurfactant-producing bacterial strains using a cell surface hydrophobicity technique. Biotechnol. Technol., 11: 671-674.

Reiger, M., 1983. Emulsions. In: Theory and Practice of Industrial Pharmacy. Lachman, L., H.A. Lieberman and J.K. Kanig (Eds.). New Delhi: Versege Publishing House, pp: 184-214.

Robert, M., M.E. Mercadé, M.P. Bosch, J.L. Parra, M.J. Espuny, M.A. Manresa and J. Guinea, 1989. Effect of the carbon sourceon biosurfactant production by *Pseudomonas aeroginosa* 44TI. Biotechnol. Lett., 11: 871-874.

Velikonja, J. and N. Kosaric, 1993. Biosurfactant in Food Applications. In: Biosurfactants-products, Properties and Applications. Kosaric, N. (Ed.). New York: Marcel Dekker, pp: 419-466.