arrhizus lipases are strictly 1,3-regiospecific, the sn-1 position being largely preferred. The removal of the 2-acyl group is due to the non-catalysed and spontaneous equilibration between the two monoglycerides. Furthermore a study of the behaviour of the lipases in the presence of optically pure sn-1,2-diglyceride leads to the conclusion that the lack of enantioselectivity in organic solvent may be explained by the competition between the hydrolysis and the esterification reactions

Aknowledgements

Thanks are due to the European Community for an ERASMUS support grant to A. H. This work conducted at Nantes University was a part of the Diplomarbeit of A. H. (Heinrich-Heine Universität Düsseldorf; March 1991). Elf Aquitaine (Groupe de Recherche de Lacq) and Amano are gratefully thanked for the generous gifts of the enzymes. We are equally grateful to Mr. I. Jennings for improvements to the English text.

References

- Akesson B. , Gronowitz S. , Herslöf B. , Michelsen P. and Olivecrona T. , *Lipids* , (1983) , **18** , 313.
- Benzonana G. and Esposito S. *Biochim. Biophys. Acta* , (1971) , **231** , 15.
- Bloomer S. , Adlercreutz P. , Mattiasson B. , *J. Am. Oil Chem. Soc.* , (1990) , **67** , 519.
- Ergan F. and Trani M. , *Biotechnology Letters* , (1991) , **13** , 19.
- Heisler A., Rabiller C and Hublin L., Biotechnology Letters, (1991), **13**(5), 327.
- Holmberg K. and Osterberg E., J. Amer. Oil Chem. Soc., (1988), **65**, 1544.
- Holmberg K. , Lassen B. and Stark M. B., *J. Am. Oil Chem. Soc.* , (1989) , **66** , 1796.
- Jensen R. G. , Dejong F.A. and Clark R.M. , *Lipids* , (1983) , **18** , 239.
- Kodali D. R. , Tercyak A. , Fahey D.A. and Small D. M. , *Chemistry and Physics of Lipids* (1990) , **52**, ,163.
- Macrae A.R. , *J. Am.Oil Chem. Soc.*, (1983) , **60** , 243A.
- Rabiller C. and Mazé F. , *Magnetic Resonance in Chemistry* , (1989) , **27** , 582.
- Rabiller C. and Heisler A. , (1991) , unpublished results.
- Ransac S. , Rogalska E. , Gargouri Y. , Deveer A. M. T. J. , Paltauf F. , De Haas G. H. and
- Verger R. , *J. Biol. Chem.* , (1990) , **265** , 20263.
- Rogalska E. , Ransac S. and Verger R. , *J. Biol. Chem.* (1990) , **265** , 20271.
- Schuch R. and Mukherjee K. D. , *J. Agric. Food Chem.* , (1987) , **35** , 1005.
- Schuch R. and Mukherjee K.D. , *Appl. Microbiol. Biotechnol.*, (1989) , **30** , 332.
- Yokozeki K. , Yamanaka S. , Takinami K. , Hirose Y. , Tanaka A. , Sonomoto K. and Fukui S., *European J. Appl. Microbiol. Biotechnol.*, (1982) , **14** , 1.

Biocatalysis in Non-Conventional Media, edited by J. Tramper et al.

Rapid determination, using dielectric spectroscopy, of the toxicity of organic solvents to intact cells

Gary J. Salter & Douglas B. Kell

Department of Biological Sciences, University College of Wales, Aberystwyth, Dyfed SY23 3DA, U.K.

Abstract

Dielectric spectroscopy utilising dual-frequency measurements has been used to study the toxic effects of a number of organic solvents to suspensions of *Saccharomyces cerevisie*. Solvents of a highly apolar nature, such as hexadecane, were identified as being non-cytotoxic, and thus suitable for use with whole-cell systems. A novel approach to aid biotransformations, using ***mixed*** organic solvents, has also been studied. This has revealed that the cytotoxic nature of polar organic solvents, such as octan-1-ol, may be negated by dissolving them first in apolar, non-cytotoxic organic solvents, such as hexadecane, before exposure to the cell suspension.

The use of mixed organic solvents with immobilised cell systems was shown to be possible by the growth of *Lactobacillus brevis*, (immobilised within hollow ceramic microspheres), in the presence of 5% (v/v) octan-1-ol dissolved within 5% (v/v) hexadecane, added to the media. Dispersion of these immiscible solvents was improved by the addition of 5% (v/v) ethanol and 0.05% (v/v) tween 80. Cell growth occurred (as measured by dielectric spectroscopy) over a 70- hour period.

1. INTRODUCTION

There is much current interest in biotransformations using or aided by organic solvents (e.g. [1-3]). The organic solvent may itself be of interest as a substrate, or it may be needed to shift the equilibrium composition in a favourable direction. In addition, many substrates are insoluble in aqueous media, and this will tend strongly to limit the rate of the biotransformation.

While the use of organic solvents with enzymes is becoming more common, their use with intact, viable cells has received much less attention, and examples of their use with immobilised cell reactors are few [4-6]. Many of the advantages conferred by the immobilisation of cells for "normal" aqueous biotransformations also hold true for systems using organic solvents.

It has been demonstrated that the passive electrical properties of cellular suspensions at radio frequencies (described in considerable detail elsewhere [7- 14]) reflect the state (intactness) of the cellular membrane. As the primary, cytotoxic site of action by organic solvents is the cell membrane, one may expect that the passive electrical properties of the cell suspension could be used to assess the level of cell viability when challenged with organic solvents, *in situ* and in real-time [15]. Using this method to determine the suitability or otherwise of organic solvents, and mixtures of organic solvents, for use with intact viable cells, we show herein that it was possible to develop a system where organic solvents could be used within an immobilised cell reactor.

2. MATERIALS AND METHODS

Microorganisms

All work involving the growth of cells immobilised within columns was carried out using *Lactobacillus brevis* NCIB 947. General studies using the Biomass Monitor to determine solvent toxicity were carried out using a strain of *Saccharomyces cerevisiae* in pressed form, obtained locally.

Solvent toxicity

All measurements were carried out using a Bugmeter™ Biomass Monitor (Aber Instruments, Aberystwyth Science Park, Cefn Llan, Aberystwyth, SY23 3AH, Dyfed, UK). Solvent toxicity, determined as the loss of integrity of cellular membranes, was studied using a standard Biomass Monitor electrode inserted laterally into the base of a 100 ml polypropylene container and sealed with epoxy resin. A tight-fitting lid was placed on the container, onto which a large centrally positioned baffle was positioned. Agitation was achieved by the use of magnetic follower inserted into the container, the arrangement being placed on a stirrer. All sample volumes were 50 ml.

Cells of *S. cerevisiae* at a known concentration were suspended in 20 mM KH_2PO_4, to which one or more organic solvents were added. When two organic solvents were used the more polar of the two was first dissolved within the less polar solvent before addition to the cell suspension.

The organic solvents were dispersed by the addition of 5% ethanol (Aldrich) and 0.05% Tween 80 (polyoxyethylene sorbitan mono-oleate, Sigma); the system was emulsified by agitation (see above).

The effects of various solvents and solvent combinations were followed over time with the Biomass Monitor, using external control and two-frequency measurements (0.3 and 9.5 MHz).

Method of use of the Biomass Monitor

A "low" and a "high" frequency were selected (typically 0.3 and 9.5 MHz), and readings were taken at both frequencies throughout the experiment. The 'real' or delta capacitance was obtained by the subtraction of the capacitance obtained at the high frequency, from that at the low frequency.

External control of the Biomass Monitor was achieved by the use of an IBM-type personal computer and a Blackstar 2308 Interface (ADC). The relevant software was written by Dr.G.H. Markx. The data were stored in a file and analysed in a spreadsheet.

Calibration [16] was achieved by the use of a dilution series of the relevant microorganism (*L. brevis* or *S. cerevisiae*) suspended in 20 mM KH_2PO_4 or growth medium. Capacitance and/or conductance readings were taken over a range of known cell concentrations (determined by dry weight and/or wet weight and/or optical density; for immobilised biomass cells were lysed by boiling in 0.2 M NaOH for 10 min and assayed for protein by a modification of the Folin method). Through a calibration curve the capacitance was then directly related to biomass cocentration, resulting in the following calibrations: *S. cerevisiae*, 1 pF = 1.75 g/l dry weight of cells; *L. brevis*, 1 pF = 4.40 g/l dry weight of cells. These values were used throughout.

Growth of immobilised cells

Columns containing gold Biomass Monitor electrodes and packed with hollow ceramic microspheres as a cell support were prepared in a similar manner to that used previously [16]. Cells of *L. brevis* were then immobilised by pumping a cell suspension through the column from the top at a flow rate exceeding 3 column volumes/minute. The column was then washed by passing 10 column volumes of sterile, cell free buffer through the column, at a rate comparable to that above. Cells entrapped within a column were then grown by passing a suitable liquid medium (see below) through the column from the bottom. Cell growth was followed by the Biomass Monitor using two-frequency (0.3 and 9.5 MHz) measurements. A constant temperature of 30°C was maintained by working within a drying oven with a temperature controller (RS components) fitted.

A commercially available medium, MRS (Merck), was used, and was autoclaved at 121°C for 30 min prior to inoculation.

3. RESULTS AND DISCUSSION

Preliminary experiments demonstrated a need to disperse the organic solvents through the aqueous phase as quickly as possible to attain the maximum cytotoxicity in a reasonable time. (It thus follows that the converse is also true, demonstrating that the expression of the toxic effects of many immiscible organic solvents is strongly diffusion-limited.) This would seem to be achieved most easily by the addition of a detergent, the detergent chosen being Tween 80 (polyoxyethylene sorbitan mono-oleate). We also added ethanol, in an attempt to make the dispersion of the organic phase more stable. Indeed, overall mixing was improved (as observed visually), and the rate of agitation could be reduced considerably (not shown). Comparison of data showing the toxicity of organic solvents used alone with those when Tween 80 and ethanol were added showed that the addition of Tween 80 and ethanol had little effect on the *final* toxicity for the organic solvents studied herein. The *speed of action*, however, was altered considerably, with a given extent of cell death occurring after a shorter exposure to the test solvent (data not shown). Thus 5% ethanol and 0.05% Tween 80 (both v/v) were used in the experiments displayed.

It is known that organic solvents with very low polarities or a high log P value (log P = octanol-water partition coefficient of the solvent) tend not to be cytotoxic [17-19], as the solvent is highly insoluble in water, and thus cannot gain access to the apolar regions of enzymes and membranes via which they would be able to effect denaturation. Further, less polar solvents cannot disturb the water layer found around biomolecules, and which is essential to their activity. Toxicity studies on a range of organic solvents confirmed this: as judged by dielectric measurements, polar organic solvents (as illustrated by octan-1-ol, Fig 1) are much more cytotoxic than are those that are relatively apolar (as illustrated by hexadecane, Fig 2). This poses an interesting dilemma, however: highly apolar organic solvents that are most amenable for use with viable cells are not those capable of best solvating many compounds of interest in biotransformations [17-19]. In addition, the correlation between cytotoxicity and log P is anyway less than perfect, and Schneider [20], for instance, when considering the choice of individual organic solvents, has argued that other properties such as H-bonding are of importance.

We consider that a better solution to this problem lies in the use of ***mixed organic solvents***. It is well known from work with chromatographic methods such as TLC and HPLC that mixtures of solvents can have a much greater range of properties than can single solvents [21]. In the present case, it is to be expected that mixtures of a highly apolar solvent (such as hexadecane) with a more polar one which is itself poorly water-soluble will combine the desirable properties of each: the more polar, but still organic, solvent will allow the solvation of (or be used as) the

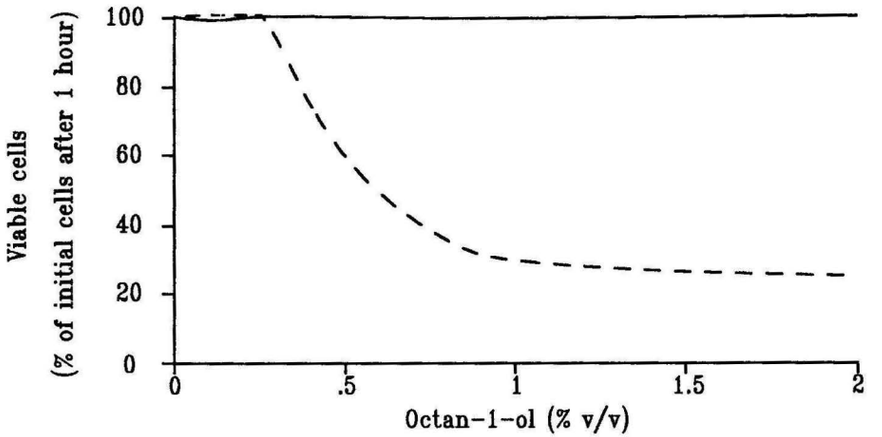

Figure 1: Effect of octan-1-ol on the capacitance of a cell suspension of *S. cerevisiae* using tween 80 and ethanol as cosolvents. *S. cerevisiae* in pressed form was suspended in a 20 mM solution of KH_2PO_4 to give a cell concentration of 90 g/l (wet weight of cells) in the final 50 ml sample. The cell suspension was placed in a 100 ml plastic container, fitted with a standard Biomass Monitor 4- terminal gold electrode (see Methods) inserted laterally. Agitation was provided by a magnetic follower, the formation of a central vortex being prevented by a large, centrally positioned baffle. Solvent dispersion was promoted by the addition of 5% (v/v) ethanol and 0.05% (v/v) tween 80 to the cell suspension, the final volume of the mixture being maintained at 50 ml. Cell viability was monitored via dielectric spectroscopy at two frequencies (0.3 and 9.5 MHz). Broken line: toxicity of octan-1-ol, continuous line: toxicity of octan-1-ol dissolved in 10% (v/v) hexadecane (final concentration) prior to addition to the cell suspension.

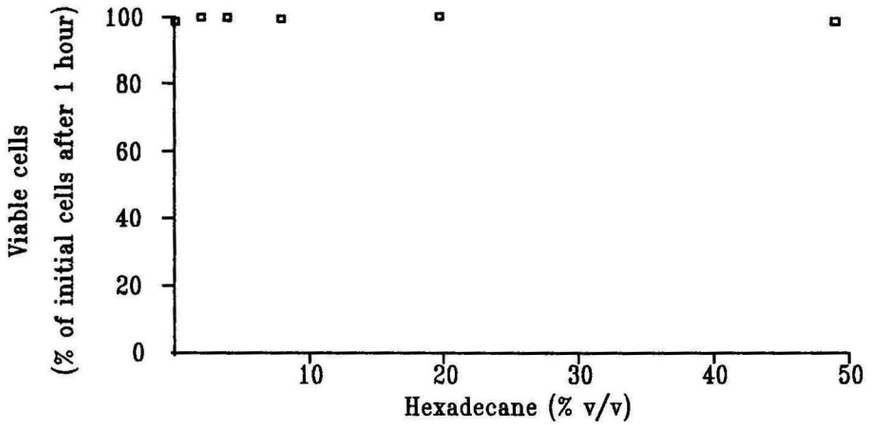

Figure 2: Effect of hexadecane on the capacitance of a cell suspension of *S. cerevisiae*, using tween 80 and ethanol as cosolvents. Other conditions as for fig 1.

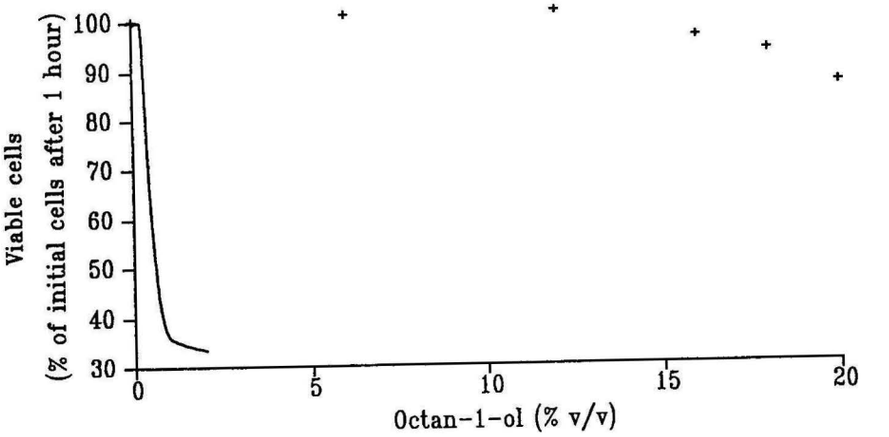

Figure 3: A comparison of the toxicity towards *S. cerevisiae* of octan-1-ol with and without 10% (v/v) hexadecane. Different volumes of octan-1-ol (plus cosolvents, as in fig 1) either dissolved in 10% (v/v) hexadecane (squares), or alone (continuous line) were added to each cell suspension, the final volume of the mixture being maintained at 50 ml.

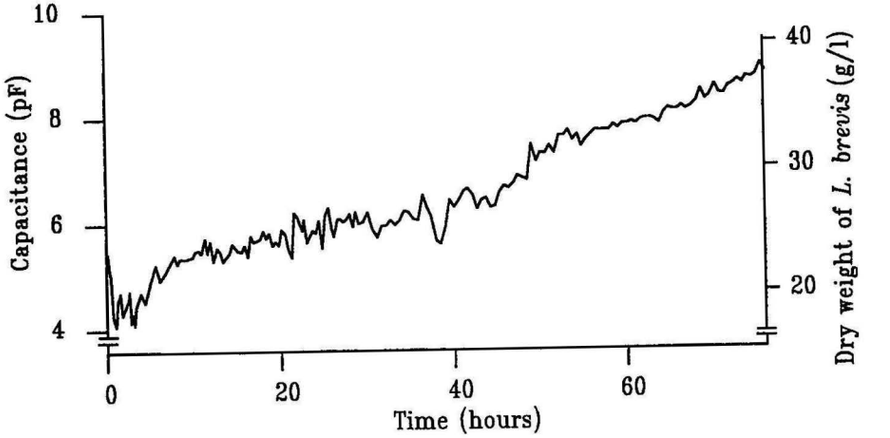

Figure 4: Growth curve for *L. brevis* immobilised on ceramic particles and grown in the presence of octan-1-ol and hexadecane. A column was formed from a standard 20 ml syringe barrel (containing a 4-terminal gold electrode; see Methods) with 7 g of ceramic particles [16]. *L. brevis* NCIB 947 was grown anaerobically on an orbital shaker in 100 ml of medium at 30°C, and immobilised cells grown as described in the Methods section.

substrate of interest, whilst both substrate and the more polar organic solvent will themselves be dissolved in the (generally) more biocompatible, highly apolar solvent.

In order to test this contention the highly cytotoxic (relatively polar) solvent octan-1-ol was dissolved in hexadecane, with the result that the toxic effects of octan-1-ol were removed at every concentration of octan-1-ol tested (Fig 1). In an effort to determine the concentration of octan-1-ol that a 10% (v/v) of hexadecane would render non-cytotoxic, it was found that even in the presence of 20% (v/v) of octan-1-ol viability was maintained at over 80% after 60 minutes (Fig. 3).

To determine whether the toxicity of octan-1-ol had really been negated, or merely delayed, we set up a column of growing cells, with octan-1-ol and hexadecane added to the growth medium. It is known that Gram-positive organisms are much more susceptible to the effects of organic solvents than are Gram- negative ones [22]. Thus in order to make the test more stringent a Gram-positive organism, *L. brevis*, was chosen. Preliminary experiments showed that, as with yeast, octan-1-ol was highly cytotoxic to this organism. A column of immobilised *L. brevis* was produced as described in Methods [16], MRS growth medium was then supplied containing 5% (v/v) octanol and 5% (v/v) hexadecane dispersed by the addition of tween 80 and ethanol, the medium reservoir being kept agitated at all times. Biomass within the column was measured by dielectric spectroscopy using dual frequency measurements (0.3 MHz and 9.5 MHz) under external control.

As may be seen (Fig 4), the cells were effectively shielded from the toxic effects of 5% (v/v) octan-1-ol, even under these stringent conditions. Slow cell growth was seen to occur within the column for over 70 hours.

We conclude from the above work that mixed organic solvents do offer the opportunity to carry out previously difficult biotransformations using viable cells in a continuous process. In addition, feed concentrations of substances that are poorly soluble or cytotoxic may be increased dramatically to allow higher process efficiencies. It would seem probable that such an approach might also be applied with advantage to enzymic reactions carried out in low-water systems.

Acknowledgments

We thank the Biotechnology Directorate of the Science and Engineering Research Council, U.K., for financial support.

References

1. C. Laane, J. Tramper and M.D. Lilly, Eds, *Biocatalysis in organic media.* (Elsevier, Amsterdam, 1987).
2. F.H. Arnold, *Trends Biotechnol.*, **8,** 244-249 (1990).
3. P.J. Halling, *Biotechnol. Bioeng.*, **35,** 691-701 (1990).
4. J. Frenz, C. Largeau and E. Casadevall, *Enzyme Microb. Technol.*, **11,** 717-724 (1989).
5. K. Sode, I. Karube, R. Araki and Y. Mikami, *Biotechnol. Bioeng.*, **33,** 1191-1195 (1989).
6. M.D. Hocknull and M.D. Lilly, *Appl. Microbiol. Biotechnol.*, **33,** 148-153 (1990).
7. R. Pethig, *Dielectric and Electronic Properties of Biological Materials.* (Wiley, Chichester, 1979).
8. C.M. Harris and D.B. Kell, *Biosensors*, **1,** 17-84 (1985).
9. D.B. Kell, in *Biosensors: fundamentals and applications.*, A.P.F. Turner, I. Karube and G.S. Wilson, Eds. (Oxford University Press, Oxford, 1987). p. 427.
10. C.M. Harris, R.W. Todd, S.W. Bungard, R.W. Lovitt, J.G. Morris and D.B. Kell, *Enzyme Microb. Technol.*, **9,** 181-186 (1987).
11. R. Pethig and D.B. Kell, *Phys. Med. Biol.*, **32,** 933-970 (1987).
12. C.L. Davey and D.B. Kell, in *Emerging Electromagnetic Medicine.*, O'Connor, M., Bentall, R.H.C. and J.B. Monahan, Eds. (Springer-Verlag, Heidelberg, 1990). p. 19.
13. D.B. Kell, G.H. Markx, C.L. Davey and R.W. Todd, *Trends Anal. Chem.*, **9,** (6), 190-194 (1990).
14. D.B. Kell and C.L. Davey, in *Biosensors: a Practical Approach.*, A.E.G. Cass, Ed. (Oxford University Press, Oxford, 1990). p. 125.
15. N.G. Stoicheva, C.L. Davey, G.H. Markx and D.B. Kell, *Biocatalysis.*, **2,** 245-255 (1989).
16. G.J. Salter, D.B. Kell, L.A. Ash, J.M. Adams, A.J. Brown and R. James, *Enzyme Microb. Technol.*, **12,** 419-430 (1990).
17. C. Laane, S. Boeren, K. Vos and C. Veeger, *Biotechnol. Bioeng.*, **30,** 81-87 (1987)
18. S.J. Osborne, J. Leaver, M.K. Turner and P. Dunnill, *Enzyme Microbial Technol.*, **12,** 281-291 (1990).
19. P.J. Halling, *Biotechnol. Adv.*, **5,** 47-84 (1987).
20. L.V. Schneider, *Biotechnol. Bioeng.* **37,** 627-638 (1991).
21. J.C. Berridge *Techniques for the automated optimization of HPLC separations.* Wiley, Chichester, 1985.
22. A.J. Harrop, M.D. Hocknull and M.D. Lilly, *Biotechnol. Lett.*, **11,** 807-810 (1989).