Letters in Applied Microbiology 2000, **30**, 183–187

Medium-size droplets of methyl ricinoleate are reduced by cell-surface activity in the γ-decalactone production by *Yarrowia lipolytica*

Y. Waché[1], K. Bergmark[1], J.-L. Courthaudon[2], M. Aguedo[1], J.-M. Nicaud[3] and J.-M. Belin[1]

[1]Laboratoire de Biotechnologie and [2]Laboratoire de Biochimie alimentaire, ENSBANA-Université de Bourgogne, Dijon and [3]Laboratoire de Génétique des Microorganismes, INRA-CNRS, Thiverval-Grignon, France

2256/99: received 6 July 1999, revised 20 October 1999 and accepted 27 October 1999

Y. WACHÉ, K. BERGMARK, J.-L. COURTHAUDON, M. AGUEDO, J.-M. NICAUD AND J.-M. BELIN. 2000. Size of methyl ricinoleate droplets during biotransformation into γ-decalactone by *Yarrowia lipolytica* was measured in both homogenized and non-homogenized media. In non-homogenized but shaken medium, droplets had an average volume surface diameter d_{32} of 2·5 μm whereas it was 0·7 μm in homogenized and shaken medium. But as soon as yeast cells were inoculated, both diameters became similar at about 0·7 μm and did not vary significantly until the end of the culture. The growth of *Y. lipolytica* in both media was very similar except for the lag phase which was lowered in homogenized medium conditions.

INTRODUCTION

Lactones are molecules of interest to the food industry due to their highly aromatic fruity aroma. For example, γ-decalactone which presents a pleasant peachy odour, can be obtained naturally through biotransformation of fatty acids into γ-decalactone by yeasts. Numerous papers and patents are dealing with processes for lactone production (Endrizzi *et al*. 1996) and, in nearly all cases, yeasts are grown in a biphasic medium. Characterization of the medium emulsion is important to understand mass transfer, particularly yeast access to substrate and lactone extraction by the oil phase, two points that are still rather unknown. But very few authors have studied the influence of the substrate dispersion state into the medium (Endrizzi-Joran 1994; Endrizzi *et al*. 1996). Although quite common, the use of homogenization or a surfactant, is not well mastered either (Farbood and Willis 1985; Page and Eilerman 1989). Information concerning the characterization of yeast growth biphasic media can be found in the many studies on single-cell protein production from hydrocarbons and oil biodegradation (Bakhuis and Bos 1969; Erdtsieck and Rietema 1969; Prokop *et al*. 1972; Gutierrez and Erickson 1977; Bruheim *et al*. 1997; Bruheim and Eimhjellen 1998) and in the more recent studies on emulsifiers production by micro-organisms (Cirigliano and Carman 1984; Cirigliano and Carman 1985; Singh and Desai 1989; Iqbal *et al*. 1995; Marin *et al*. 1995; Bredholt *et al*. 1998). Thus it is evident that growth on hydrocarbons occurs directly on droplets as solubility in water is limited (Gutierrez and Erickson 1977). The size of the droplets influences cell growth (Bakhuis and Bos 1969) but, according to Prokop *et al*. (1972), growth is depending on a global equilibrium between the phases, including the inoculum phase. They observed for instance that the interfacial area is greatly affected by inoculation.

Since substrate consumption and trapping of the toxic lactone depend on emulsion characteristics (Souchon *et al*. 1998), we decided to study the droplet size and hence the interfacial area during growth and biotransformation of methyl ricinoleate by *Yarrowia lipolytica*. The investigation of the effect of homogenization on the emulsion showed that the treatment had an effect on droplet size in the medium without cells. But as soon as cells were present, the droplet size in non-homogenized media was reduced to the value obtained with the homogenization treatment and no significant difference was observed during growth. Cells appeared then to be surface active materials, and in their presence, shaking baffled Erlenmeyer flasks is sufficient to disperse the substrate to an average volume surface diameter (d_{32}) under 1 μm.

MATERIALS AND METHODS

Strain and culture conditions

The *Y. lipolytica* W29 (ATCC20460; CLIB89) strain was used in this study. This strain produces γ-decalactone from methyl ricinoleate (Pagot *et al*. 1998; Waché *et al*. 1998). It was grown in 500 ml baffled Erlenmeyer flasks containing 200 ml

Correspondence to: Y. Waché, Laboratoire de Biotechnologie ENSBANA, 1, esplanade Erasme, 21000 Dijon, France (e-mail: ywache@u-bourgogne.fr).

of medium: glucose medium (Pagot *et al*. 1998) for preculture and methyl ricinoleate (MR) medium (Waché *et al*. 1998) for growth and lactone production. Flask inoculation consisted of $6{\cdot}5 \times 10^6$ cells ml^{-1} and preculture lasted 18 h until cells were in late log growth phase. Cells were then harvested by centrifugation (10 000 *g*, 5 min) and washed twice with phosphate buffer (50 mmol l^{-1}, pH 7·4).

Homogenization and agitation

Erlenmeyer flasks were shaken at 140 rev min^{-1} on a shaking table (Thermoshake THO5-Gerhardt, Bonn, Germany) during the whole culture. The RM medium could be homogenized after sterilization and before inoculation with a Polytron PT-MR 3000 homogenizer (Kinematica AG, Littau, Switzerland) at 27 000 r.p.m. for 2 min

Emulsion characterization

A Malvern Mastersizer laser diffractometer (Model S2–01; Malvern Instruments, Malvern, UK) was used to determine the droplet size distribution, from which the volume surface average diameter d_{32} (Sauter diameter) and the surface area were derived.

Growth kinetics

Cells grown in MR were counted in a Malassez cell, every 1·5 h for the first 12 h and then at 24 h. Slide Write + (Advanced Graphics Software Inc., Carlsbad, CA, USA) was used to fit curves and to calculate the parameters corresponding with the Gompertz model as modified by Zwietering *et al*. (1990): lag phase λ in h, maximal growth rate μ_{max} in h^{-1} and maximal biomass at the end of the culture Xmax in cell ml^{-1}.

γ-decalactone extraction and analysis

One and a half millilitres of the culture was removed every 1·5 h for cells cultured in MR and centrifuged at 10 000 *g* for 5 min. The supernatant (both aqueous and oil phases) was mixed and acidified to pH 2 with HCl (37%). The internal standard (γ-undecalactone) was added and the mixture was extracted with 1·5 ml diethyl ether in 4 ml glass vials by shaking for 1·5 min. The ether phase was then analysed in a HP6890 gas chromatograph with a HP-INNOWax capillary column (30·0 m × 320 μm × 0·25 μm) using N_2 as a carrier gas at a linear flow rate of 4·3 ml min^{-1}. The split injector (split ratio: 7·1 : 1) temperature was set to 250 °C and the FID detector's to 300 °C. The oven temperature was programmed from 60 to 145 °C at 5 °C min^{-1} and then at 2 °C min^{-1} – 180 °C.

Confocal microscopy

Cells from RM were immobilized on a polylysine-covered slide and observed with a Leica TCS 4D Confocal Laser Scanning Microscope (CLSM; Leica, Heidelberg, Germany).

RESULTS AND DISCUSSION

Effect of homogenization on the methyl ricinoleate droplet size

Homogenization was efficient for the emulsification of the uninoculated MR medium (Table 1): the average Sauter dia-

Table 1 Size of the lipid droplets and interfacial area in the shaken MR medium without cells during a time similar to growth and during growth of *Y. lipolytica*

	Homogenized medium		Non-homogenized medium	
Time of culture (h)	d_{32} diameter (μm)	Interfacial area (m^2 ml^{-1})	d_{32} diameter (μm)	Interfacial area (m^2 ml^{-1})
MR without cells during a time similar to growth				
0	0·76 ± 0·01	8·0 ± 0·2	2·29 ± 1·1	3·1 ± 1·2
7	0·70 ± 0·10	9·3 ± 2·0	2·65 ± 0·8	2·4 ± 0·8
30	0·72 ± 0·20	11·1 ± 2·2	2·44 ± 0·5	2·5 ± 0·5
MR during growth of Y. lipolytica				
0	0·71 ± 0·03	8·5 ± 0·4	0·70 ± 0·03	8·5 ± 0·5
7	0·73 ± 0·06	8·7 ± 0·8	0·66 ± 0·09	9·3 ± 0·8
30	0·70 ± 0·07	9·3 ± 0·9	0·60 ± 0·09	10·4 ± 0·9

meter of oil droplets could be reduced from 2·3 μm with simple agitation to 0·7 μm after a 27 000 r.p.m. homogenization. These values were stable for 30 h in the culture conditions. The energy supplied to the system by homogenization was durably lowering the droplet size by a factor of 3·6 compared with simply shaken baffled flasks where oil droplets still had a d_{32} over 2 μm although Tween 80® was used as an emulsifier. On the contrary, with Tween 40® as an emulsifier, no effect of homogenization was detectable and the droplets diameter was over 2 μm (results not shown).

Effect of homogenization on the droplet size during growth

In homogenized medium, droplets had the same diameter after cell inoculation and then during the whole growth corresponding with an interfacial area of approximately 9 m^2 ml^{-1} of oil (Table 1). Droplets in non-homogenized medium became three to four times smaller after inoculation, reaching the same values as the homogenized medium droplets. In those conditions, the homogenization had no effect as if a powerful surface active agent had been added to the medium so that simple baffled Erlenmeyer flask agitation gave the same droplet size as a 2-min 27 000 r.p.m. homogenization. This surface active action was detectable at the first measurement, 15 min after yeast inoculation.

Y. lipolytica is able to produce an emulsifier called liposan when grown on hexadecane (Cirigliano and Carman 1984; Cirigliano and Carman 1985) and methyl ricinoleate metabolism may also result in emulsifying compounds production. But those products appear in the medium after several hours of culture (Prokop *et al*. 1972). In our case, the surface active effect is noticed from the beginning of the culture as if it was depending on the very presence of the cells. Such a phenomena has already been observed by Prokop *et al*. (1972) who noted that cell presence at the oil drop's surface tends to lower the density difference between the two phases. This oil–cell adhesion can be explained by the cell surface properties resulting from cell wall hydrophobicity (Klotz 1989). Bredholt *et al*. (1998) also reported the emulsion stabilization by cell adhesion and believe it is caused by the release of hydrophobic cell surface discarded by the cells. This phenomena has been studied in *Candida tropicalis* where a mannan-fatty acid complex is produced during growth in alkane after a few hours of culture. This lipopolysaccharide dramatically enhances alkane adhesion to the cell compared with cells precultured in glucose especially in the case of poorly emulsified hydrocarbons (Käppeli and Fiechter 1976; Käppeli *et al*. 1978). However, in our study, the preculture substrate was glucose and cells were thus not in the most hydrophobic conditions. Moreover, the surface active action was immediate and not significantly enhanced during culture, contradicting Gutierrez and Erickson (1977) and Prokop *et al*. (1972) who observed a growing interfacial area *vs* time. Droplets have been reduced to the same size as homogenized medium droplets which is much smaller than yeast size (Fig. 1) and should facilitate substrate transfer to cells (Bakhuis and Bos 1969). This occurred without time for hydrophobic adaptation.

Effect of droplet size on growth and lactone production

Y. lipolytica's growth was investigated in both homogenized and non-homogenized media. Before inoculation, the interfacial area between the MR substrate and the aqueous phase was three to four times larger in the homogenized medium. However, just after inoculation both interfacial area became similar (8·5 m^2 ml^{-1}) (Table 2). The calculated Gompertz growth parameters were, excepted for lag phase, very similar in both conditions: the maximal growth rate was around 0·13 and 0·14 h^{-1} and the maximal biomass at the end of the culture was about $28{\cdot}5 \times 10^6$ cell ml^{-1}. But the lag phases were different: 60% longer for the non-homogenized medium

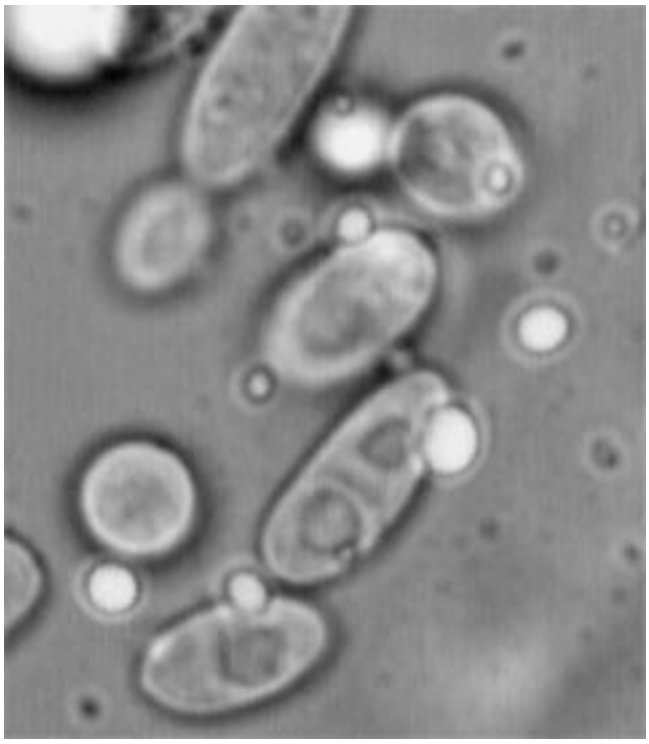

Fig. 1 View in CLSM of *Y. lipolytica* cells after 30 h of culture in methyl ricinoleate (objective 63/1·4 and 100/1·3). On the surface of the cells appear small fatty globules

Table 2 Effect of homogenization on growth and lactone productivity in the 12 first hours of culture by *Y. lipolytica*

	Homogenized medium	Non-homogenized medium
Lag phase λ (h)	$1{\cdot}11 \pm 0{\cdot}02$	$1{\cdot}79 \pm 0{\cdot}08$
μ_{max} (h^{-1})	$0{\cdot}13 \pm 0{\cdot}02$	$0{\cdot}14 \pm 0{\cdot}02$
Xmax (cell ml^{-1})	$27{\cdot}8 \times 10^6$	$29{\cdot}7 \times 10^6$
γ-decalactone productivity ($mg\,l^{-1}$)	61 ± 2	60 ± 2

despite the similar interfacial area from the inoculation time.

For the lactone productivity, no significant difference was detectable and the productivity in the first 12 h of culture was around $5\,mg\,l^{-1}\,h^{-1}$.

Many authors have observed an effect of globule size on the growth of micro-organisms (Bakhuis and Bos 1969). In our study, cell addition resulted in a similar droplet size so that we were not expecting differences in the growth parameters. Indeed, the lag phase duration was increased for the non-homogenized medium showing that cell adaptation was longer in those conditions as if the reduction of the interfacial tension between oil and aqueous phase and the resulting optimization of the droplet size was an active phenomenon. It could, for instance, result from a release of hydrophobic materials, present also in glucose grown cells for *Y. lipolytica*, that the cell would have to counteract.

This alkane-assimilating yeast thus appears to have good surface active characteristics which help the emulsification of nonaqueous substrates. The study of this property is of interest not only because of its ability to lower the homogenization treatment or save chemical surfactants in growth media in the different technological processes using this yeast, including oily effluents degradation, but also to elucidate our understanding of the transport of hydrophobic compounds across the membrane and cell wall.

REFERENCES

Bakhuis, E. and Bos, P. (1969) Correlation between growth of *Candida lipolytica* and size of droplets in the hydrocarbon containing medium. *Antonie Van Leeuwenhoek* **35,** F47–F48.

Bredholt, H., Josefsen, K., Vatland, A., Bruheim, P. and Eimjhellen, K. (1998) Emulsification of crude oil by an alkane-oxidizing *Rhodococcus* species isolated from seawater. *Canadian Journal of Microbiology* **44,** 330–340.

Bruheim, P., Bredholt, H. and Eimhjellen, K. (1997) Bacterial degradation of emulsified crude oil and the effect of various surfactants. *Canadian Journal of Microbiology* **43,** 17–22.

Bruheim, P. and Eimhjellen, K. (1998) Chemically emulsified crude oil as substrate for bacterial oxidation: differences in species response. *Canadian Journal of Microbiology* **44,** 195–199.

Cirigliano, M.C. and Carman, G.M. (1984) Isolation of a bioemulsifier from *Candida lipolytica*. *Applied and Environmental Microbiology* **48,** 747–750.

Cirigliano, M.C. and Carman, G.M. (1985) Purification and characterization of Liposan, a bioemulsifier from *Candida lipolytica*. *Applied and Environmental Microbiology* **50,** 846–850.

Endrizzi, A., Pagot, Y., Le Clainche, A., Nicaud, J.-M. and Belin, J.-M. (1996) Production of lactones and peroxisomal beta-oxidation in yeasts. *Critical Reviews in Biotechnology* **16,** 301–329.

Endrizzi-Joran, A. (1994) Biotransformation de ricinoléate de méthyle en gamma-décalactone par des levures. Dijon: Thèse de l'Université de Bourgogne Dijon.

Erdtsieck, B. and Rietema, K. (1969) Interface area limitation in the growth of yeast on liquid n-alkanes. *Antonie Van Leeuwenhoek* **35,** F19.

Farbood, M. and Willis, B. (1985) *Production of Gamma-Decalactone*. Patent WO 83/01072.

Gutierrez, J.R. and Erickson, L.E. (1977) Hydrocarbon uptake in hydrocarbon fermentations. *Biotechnology and Bioengineering* **XIX,** 1331–1349.

Iqbal, S., Khalid, Z.M. and Malik, K.A. (1995) Enhanced biodegradation and emulsification of crude oil and hyperproduction of biosurfactant by a gamma ray-induced mutant of Pseudomonas aeruginosa. *Letters in Applied Microbiology* **21,** 176–179.

Käppeli, O. and Fiechter, A. (1976) The mode of interaction between the substrate and cell surface of the hydrocarbon-utilizing yeast *Candida tropicalis*. *Biotechnology and bioengineering* XVIII. 967–974.

Käppeli, O., Müller, M. and Fiechter, A. (1978) Chemical and structural alterations at the cell surface of *Candida tropicalis*, induced by hydrocarbon substrate. *Journal of Bacteriology* **133,** 952–958.

Klotz, S.A. (1989) Surface-active properties of *Candida albicans*. *Applied and Environmental Microbiology* **55,** 2119–2122.

Marin, M.M., Pedregosa, A.M., Ortiz, M.L. and Laborda, F. (1995) Study of hydrocarbon-utilizing and emulsifier-producing *Acinetobacter calcoaceticus* strain isolated from heating oil. *Microbiologia* **11,** 447–454.

Page, G.V. and Eilerman, R.G. (1989) Process for the preparation of gamma and delta lactones. Patent WO 89/12104.

Pagot, Y., Le Clainche, A., Nicaud, J.-M., Waché, Y. and Belin, J.-M. (1998) Peroxisomal Beta-oxidation activities and gamma-decalactone production by the yeast *Yarrowia lipolytica*. *Applied and Microbiological Biotechnology* **49,** 295–300.

Prokop, A., Ludvik, M. and Erickson, L.E. (1972) Growth models of cultures with two liquid phases. VIII. Experimental observations on droplet size and interfacial area. *Biotechnology and Bioengineering* **XIV,** 587–608.

Singh, M. and Desai, J.D. (1989) Hydrocarbon emulsification by *Candida tropicalis* and *Debaryomyces polymorphus*. *Indian Journal of Experimental Biology* **27,** 224–226.

Souchon, I., Spinnler, H.E., Dufossé, L. and Voilley, A. (1998) Trapping of gamma-decalactone by adsorption on hydrophobic sorbents: application to the bioconversion of methyl ricinoleate by the yeast *Sporidiobolus salmonicolor*. *Biotechnology Techniques* **12,** 109–113.

Waché, Y., Pagot, Y., Nicaud, J.M. and Belin, J.M. (1998) Acyl-CoA oxidase, a key step for lactone production by *Yarrowia lipolytica*. *Journal of Molecular Catalysis B: Enzymatic* **149,** 165–169.

Zwietering, M.H., Jongenburger, I., Rombouts, F.M. and Van't Riet, K. (1990) Modelling of the bacterial growth curve. *Applied and Environmental Microbiology* **56,** 1875–1881.