J Chem Technol Biotechnol 81:1368–1374 (2006)
DOI: 10.1002/jctb.1478

Improving lipase production using a perfluorocarbon as oxygen carrier

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Abstract: Perfluorodecalin, a perfluorocarbon (PFC), was used as an oxygen vector in culture media to enhance lipase productivity by increasing the oxygen availability to *Yarrowia lipolytica*. The effects of different concentrations of PFC were studied in shake-flask yeast cultures at 160 and 250 rpm. Higher specific growth rates of *Y. lipolytica* were found with increasing PFC concentration and agitation rate. Lipase production by *Y. lipolytica* was enhanced 23-fold with the addition of 20% (v/v) PFC at 250 rpm. Moreover, it is shown that using PFC and glucose as substrate is more effective in lipase production than the conventional use of olive oil.

Keywords: lipase; perfluorocarbon; oxygen; *Yarrowia lipolytica*

INTRODUCTION

Lipases (triacylglycerol acylhydrolases, EC 3.1.1.3.) are enzymes that attract the interest of scientists and industrial researchers because they can be exploited for several industrial applications, such as detergents, foods, pharmaceutical and environmental.[1] These enzymes hydrolyse esters, preferentially at the interface between lipids and water in heterogeneous systems, and have also been used for fine organic synthesis.[2] Lipases can be obtained from animals, plants and micro-organisms.[3] Yeasts are interesting lipase producers because they have a short duplication time and many of them have been generally recognised as safe (GRAS), which provides acceptance within the food industry.[4] *Yarrowia lipolytica*, a unique, strictly aerobic yeast with the ability to produce a wide spectrum of products, is considered non-pathogenic and several processes based on this organism have been classified as GRAS by the FDA (Food and Drug Administration).[5] There are a few studies concerning lipase production by *Y. lipolytica*,[6–8] including the use of chemical mutagenesis and genetic engineering approaches to obtain overproducing *Y. lipolytica* mutants, because it naturally and efficiently secretes large amounts of proteins.[9]

Lipase productivity is affected by various environmental factors.[7] The amount of oxygen available to micro-organisms seems to be an important parameter, since many authors have shown the dependence of lipase productivity on system aeration and agitation. Vadehra and Harmon[10] reported that the presence of air was essential for lipase production by *Staphylococcus aureus*. Chen *et al*.[11] obtained higher levels of lipase activity and productivity on raising the system agitation speed. In the same work, dissolved oxygen (DO) increased lipase production only when its concentration was raised from 10 to 20% (air saturation). Higher DO concentrations did not affect enzyme production. Similar results obtained by Elibol and Ozer[12] with *Rhizopus arrhizus* and by Alonso *et al*.[13] with *Y. lipolytica* suggested that lipase production is mainly influenced by the oxygen transfer rate and not by the amount of oxygen present.

Perfluorocarbons (PFCs) are petroleum-based compounds synthesised by replacing hydrogen atoms with fluorine atoms in hydrocarbon molecules. They are both stable and chemically inert owing to the presence of very strong carbon–fluorine bonds. Oxygen solubility in PFCs is 10–20 times higher than that in pure water.[14] The characteristics of PFCs induce a significant increase in the oxygen transfer rate from

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Contract/grant sponsor: GRICES/CAPES; contract/grant number: 102/03
Contract/grant sponsor: FAPERJ
Contract/grant sponsor: CNPq
Contract/grant sponsor: Fundação para a Ciência e a Tecnologia; contract/grant number: POCTI/EQU/44427/2002
(*Received 15 May 2005; revised version received 12 October 2005; accepted 26 October 2005*)
Published online 8 March 2006

the gas phase to micro-organisms. The consequent increase in fermentation yield has been detected for biomass concentration,[15,16] protoplast culture,[17] antibiotic production,[15,18] etc. Furthermore, it has been demonstrated that the inclusion of PFCs or hydrocarbons as a second liquid phase in fermentation media increases the oxygen uptake rate and the oxygen transfer coefficient ($K_L a$).[18–22] Most of these reports mention that $K_L a$ reaches a maximum when the oil concentration is around 20% (v/v), because higher concentrations result in an increase in the viscosity of the system. Another advantage of PFCs is that they are easily recoverable. Elibol and Mavituna[15] reported 95% PFC recovery in actinorhodin production.

The aim of this study was to evaluate the effects of the PFC perfluorodecalin on lipase production by *Y. lipolytica*. No work using PFCs in *Y. lipolytica* lipase production has been previously reported.

EXPERIMENTAL

Materials

Perfluorodecalin, the common brand name of which is Flutec PP6 (F2 Chemicals Ltd., Preston, Lancs, UK). The relevant physical properties of perfluorodecalin at 25 °C and 1.01×10^5 Pa are as follows: density 1.917 g cm^{-3}, vapour pressure 810 Pa and oxygen solubility 127.8 mg dm^{-3}.[23,24] Peptone, yeast extract and glucose were obtained from Merck (Darmstadt, Germany) Oxoid (Basingstoke, Hants, UK) and Isofar (Rio de Janeiro, Brazil), respectively.

Micro-organism

A wild-type strain of *Y. lipolytica* (IMUFRJ 50682) was selected from an estuary in the vicinity of Rio de Janeiro, Brazil.[25]

Culture conditions

For both inoculum and growth conditions, cells were cultivated at 28 °C in a rotary shaker (160 or 250 rpm) in flasks containing YPD medium (w/v: 1% yeast extract, 0.64% peptone (from casein) and 2% glucose). For experiments using PFC, 10 or 20% (v/v) perfluorodecalin was added to the medium. A control experiment was carried out with no PFC. Experiments with olive oil as a carbon source were done as described below, with 1% (v/v) olive oil replacing glucose (YP medium with olive oil). Finally, 20% (v/v) olive oil was employed as an organic phase in place of PFC.

The assays were first performed in 500 cm^3 shake-flasks containing 200 cm^3 of medium (medium volume (V_m)/flask volume (V_f) = 0.4). When the V_m/V_f ratio was reduced, 1 dm^3 flasks were used with the same medium volume ($V_m/V_f = 0.2$).

Analytical methods (in aqueous phase)

Cell growth

Cell growth was followed by optical density measurements at 570 nm converted to mg cm^{-3} using a factor previously established.

Glucose

Extracellular glucose concentration was determined by the glucose oxidase method (Enzymatic Colorimetric Glucose Assay Kit, HUMAN GmbH, Wiesbaden, Germany).

Lipase activity

Lipase activity was determined by a spectrophotometric method as follows. A 0.1 cm^3 aliquot of cultivation medium without cells was added to a solution of 0.504 mmol dm^{-3} *p*-nitrophenyl laurate (*p*-NPL) in 50 mmol dm^{-3} phosphate buffer, pH 7.0. This solution was incubated at 37 °C for 15 min before adding the cultivation medium. The production of *p*-nitrophenol is automatically monitored at 410 nm during the linear period of product accumulation. One unit (U) of lipase activity is defined as the amount of enzyme that produces 1 μmol product min^{-1}.

RESULTS AND DISCUSSION

Effect of PFC on cell growth

The effects of the addition of 10 and 20% (v/v) PFC (perfluorodecalin) were studied in *Y. lipolytica* growth media at two different agitation speeds (160 and 250 rpm). The growth parameters are presented in Table 1.

The results show a significant increase in specific growth rate with PFC concentration at both agitation speeds studied (160 and 250 rpm). Moreover, the increase in agitation speed also leads to faster cellular growth and a stronger contribution of PFC to the increase in specific growth rate. The glucose consumption rate ($-dS/dt$) increases when more PFC is added to the medium (20% v/v) and when the higher agitation speed (250 rpm) is applied. This is in accordance with results of Elibol[16] showing that the use of perfluorodecalin in *Saccharomyces cerevisiae* growth media improves the glucose consumption rate. This can be attributed to the relief of O_2 transfer limitation, the cells being able to easily consume the glucose and the additional oxygen supplied.

PFC was recovered from the medium by centrifugation (3000 × *g*) followed by decantation, and it was

Table 1. Influence of PFC and agitation speed on growth parameters of *Yarrowia lipolytica* and its lipase productivity when cultivated in 500 cm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium + *x*% PFC). Values are mean ± standard deviation

	μ (h^{-1})	$-dS/dt$ (mg cm^{-3} h^{-1})	Lipase productivity (U dm^{-3} h^{-1})
160 rpm			
0% PFC	0.13 ± 0.01	0.18 ± 0.06	8.4 ± 0.3
10% PFC	0.16 ± 0.02	0.17 ± 0.02	6.3 ± 0.1
20% PFC	0.23 ± 0.01	0.26 ± 0.01	6.3 ± 0.2
250 rpm			
0% PFC	0.17 ± 0.01	0.31 ± 0.01	4.4 ± 0.1
10% PFC	0.25 ± 0.01	0.34 ± 0.01	6.3 ± 0.1
20% PFC	0.53 ± 0.01	0.39 ± 0.03	48.5 ± 1.0

possible to reuse around 93% of PFC in the next experiment. Similar values were reported by Elibol and Mavituna.[15] Therefore, despite being an expensive reagent, PFC is not necessarily an over-cost of the process as it can be reused in several batches.

Effect of PFC on lipase production

Figure 1 depicts the lipase production profile from flasks agitated at 160 rpm. The addition of PFC in this condition did not improve lipase activity. All assays (0, 10 and 20% v/v PFC) showed maximum lipase activity at the same time (around 20 h), and those with PFC presented lower lipase activities.

Different results were obtained when the 250 rpm agitation speed was applied, as shown in Fig. 2. Apparently, the use of 10% (v/v) PFC at 250 rpm did not affect lipase production; however, maximum lipase activity was obtained earlier than in the medium with no PFC, increasing the enzyme productivity as reported in Table 1.

The most remarkable result in the presence of 20% (v/v) PFC is that the production of lipase does not stop after 20 h as in the previous cases, but lipase activity levels continue to grow even after 50 h, by which time lipase activity and productivity are an order of magnitude higher than in previous assays.

It is shown that PFC had a significant effect on lipase production at 250 rpm. It is believed that, at the lower agitation speed (160 rpm), PFC provides a small increase in the amount of oxygen transferred to the aqueous phase at the beginning of growth, when there is a low cell density, leading to a higher specific growth rate. However, as the oxygen requirement increases, the PFC contribution becomes insignificant, because, as Ju *et al.*[26] emphasised in their work, the application of low shear disperses PFC only partially into droplets of very large size that tend to coalesce, reducing the PFC–medium interfacial area and consequently the oxygen transfer rate. Elibol[18] showed that in a 1 dm^3 bioreactor with 0.5 vvm (volume of gas per volume of aerated liquid per minute, min^{-1}) airflow rate and 15% (v/v) PFC the mass transfer coefficient (K_La) was higher than in the control only when the agitation speed was above 500 rpm. Therefore there must be a certain agitation speed for each system that limits the benefit of PFC to oxygen transfer.

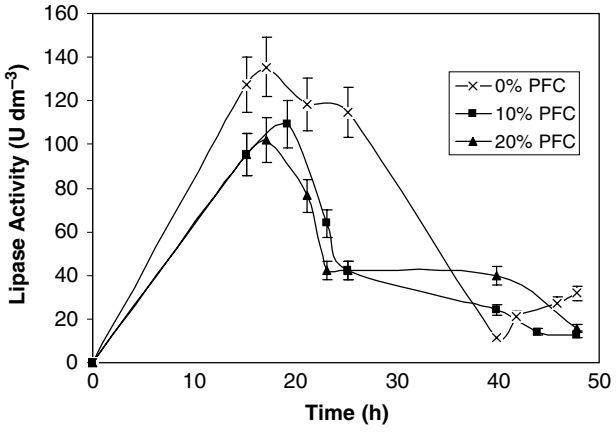

Figure 1. Effect of PFC (% v/v) on lipase production by *Yarrowia lipolytica* in 500 cm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium + *x*% PFC) agitated at 160 rpm. Error bars represent standard deviation.

Influence of PFC concentration

Experiments were performed at five different PFC concentrations varying from zero (control) to 50% (v/v). Figure 3 shows the maximum lipase activity (%) obtained in the culture medium for each PFC concentration relative to the highest value achieved (100%). It is clear that the best PFC concentration for lipase production by *Y. lipolytica* is around 20% (v/v). This may be due to the fact that this PFC concentration maximises the K_La of the system. PFC benefits the oxygen transfer rate until a certain concentration, beyond which it modifies the rheological behaviour of the medium, increasing its viscosity and consequently affecting oxygen transfer. There are many reports in the literature[22,27,28] on the dependence of K_La of different kinds of systems on

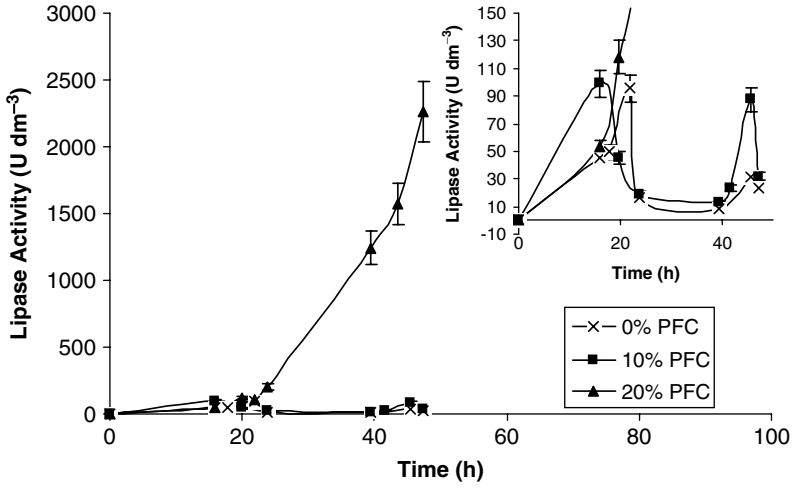

Figure 2. Effect of PFC (% v/v) on lipase production by *Yarrowia lipolytica* in 500 cm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium + *x*% PFC) agitated at 250 rpm. Error bars represent standard deviation.

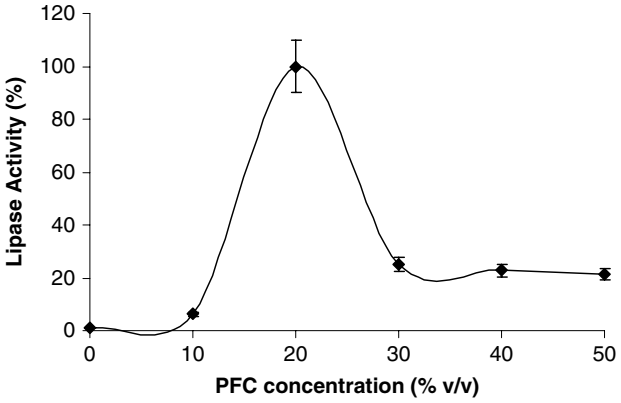

Figure 3. Effect of PFC on lipase production by *Yarrowia lipolytica* in 1 dm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium + *x*% PFC) agitated at 250 rpm. Error bars represent standard deviation.

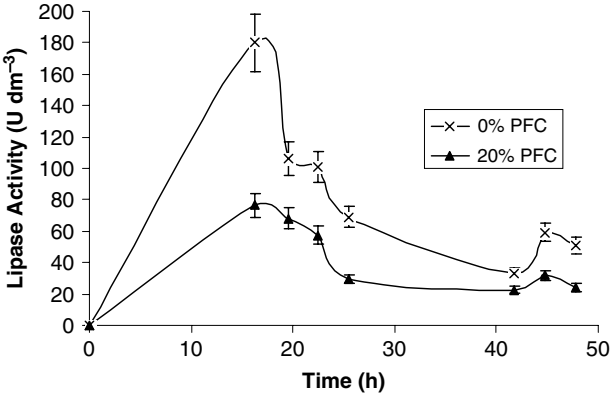

Figure 4. Effect of PFC (% v/v) on lipase production by *Yarrowia lipolytica* in 1 dm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium + *x*% PFC) agitated at 160 rpm. Error bars represent standard deviation.

PFC concentration, and all of them show the optimum at approximately 20% (v/v) PFC.

Reduction in V_m/V_f ratio

The reduction in V_m/V_f ratio modifies the oxygenation of shake-flasks significantly because it increases the gas–liquid interfacial area. Therefore, to study this effect, assays were carried with 200 cm^3 of medium in 1 dm^3 flasks under 160 and 250 rpm agitation speeds with the addition of 20% (v/v) PFC in comparison with the control (without PFC). The results are presented in Figs 4 and 5.

It was found that the reduction in V_m/V_f decreases lipase production at 160 rpm. For $V_m/V_f = 0.4$ this agitation speed reduces lipase production by 30% when compared with the control experiment, while for $V_m/V_f = 0.2$ at the same agitation speed the reduction was about 60%.

The lipase activity profiles obtained at 250 rpm and $V_m/V_f = 0.2$, reported in Fig. 5, were very similar to those obtained with the same agitation speed and $V_m/V_f = 0.4$, reported in Fig. 2. Comparing the two control assays (0% v/v PFC, $V_m/V_f = 0.4$ and 0% v/v PFC, $V_m/V_f = 0.2$), it noticeable that the increase in superficial area achieved by reducing the V_m/V_f ratio has benefited lipase production, as a higher enzyme activity was obtained at the lower V_m/V_f ratio. Moreover, the addition of 20% (v/v) PFC to the assays with the lower V_m/V_f ratio (0.2) induced an increase in lipase productivity almost three times higher than that detected with $V_m/V_f = 0.4$.

When studying the effect of oxygen transfer on lipase production by *Acinetobacter radioresistens*, Chen *et al*.[11] achieved a twofold increase in lipase productivity on increasing the agitation speed or aeration of the system. A much higher increase in lipase production (sevenfold) was obtained by Fickers *et al*.[9] when using a new overproducing mutant of *Y. lipolytica*. In the present work the addition of 20% (v/v) PFC has caused a 23-fold increase in lipase productivity from *Y. lipolytica* at 250 rpm and $V_m/V_f = 0.2$ in comparison with the corresponding control (0% v/v PFC, 250 rpm and $V_m/V_f = 0.2$).

Effect of PFC in presence of olive oil

It has been reported in the literature that *Y. lipolytica* produces higher levels of lipase when cultivated in

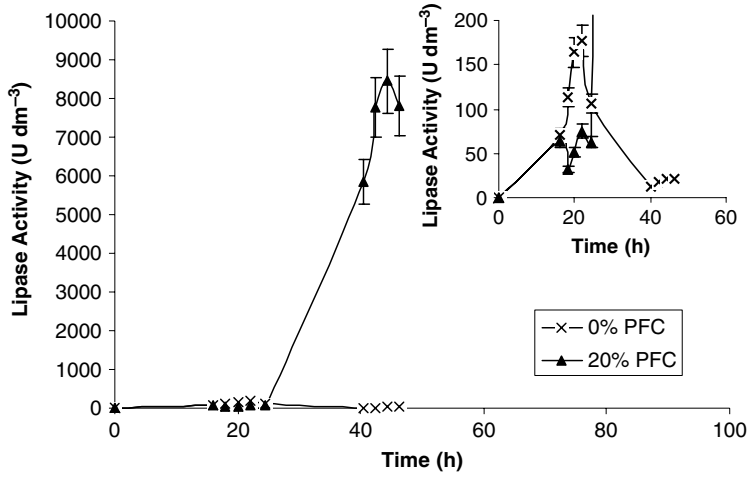

Figure 5. Effect of PFC (% v/v) on lipase production by *Yarrowia lipolytica* in 1 dm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium + *x*% PFC) agitated at 250 rpm. Error bars represent standard deviation.

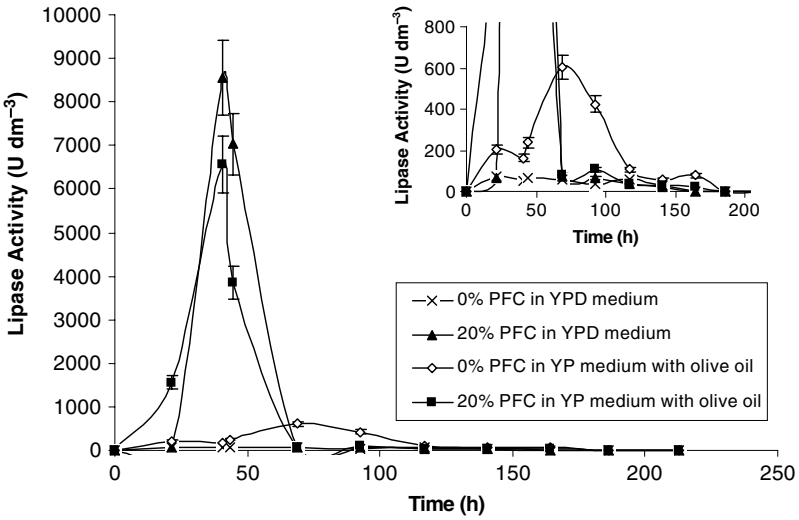

Figure 6. Effect of PFC (% v/v) on lipase production by *Yarrowia lipolytica* in 1 dm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium or YP medium (with 1% v/v olive oil) + *x*% PFC) agitated at 250 rpm. Error bars represent standard deviation.

media containing olive oil or long-chain triacylglycerides as carbon source.[4,7] Experiments were thus conducted using media with glucose replaced by olive oil, under conditions that enabled maximum lipase productivity ($V_m/V_f = 0.2$, 250 rpm agitation speed and 20% v/v PFC). Figure 6 depicts the lipase activity profiles from the assays with two different carbon sources (glucose and olive oil), both with and without PFC.

The results show that olive oil is the best carbon source for lipase production when no PFC is added to the medium, confirming results previously reported in the literature.[4,7] As olive oil is the substrate for lipase biocatalysis, its presence in the medium is expected to induce lipase production. In fact, lipase from *Y. lipolytica* is encoded by the gene LIP2, and its native promoter is induced by fatty acid and oil.[9] However, in the presence of PFC, higher lipase activity was obtained when glucose was employed as carbon source. Both PFC assays (with glucose and olive oil) presented maximum lipase activity at the same time. The experiment with glucose provided higher lipase productivity (210.6 U dm^{-3} h^{-1}) than that with olive oil as carbon source (161.7 U dm^{-3} h^{-1}). This result shows that PFC addition is more effective than the induction achieved by the presence of olive oil, which is considered the specific substrate for this enzyme.

The relevance of oxygen availability in lipase production is well known[10–13]. Positive effects of the presence of fatty oils in the productivity of this enzyme have also been described.[4,7,29] It was expected that PFC would stimulate extracellular lipase production by increasing the oxygen transfer rate.[21] Nevertheless, Fickers *et al.*[30] have reported the induction of LIP2 gene in a *Y. lipolytica* mutant in the presence of glucose, showing that lipase production is uncoupled from catabolite repression. The low level of lipase activity when glucose is the carbon source might be related to the absence of an external stimulus for its production/secretion, which is achieved when PFC is added to the culture medium.

Use of olive oil as an organic phase

Since the solubility of oxygen in olive oil is close to its solubility in PFCs[31] and since the use of olive oil as carbon source seems to lead to enhanced lipase production, we attempted to use olive oil as organic phase, expecting to reap the benefits of a compound that could simultaneously act as oxygen vector and favourable carbon source. Olive oil in the same concentration previously used for PFC (20% v/v) was employed as an organic phase in place of PFC. The results obtained are present in Fig 7. It is possible to observe that, in both assays (20% v/v perfluorodecalin and 20% v/v olive oil), maximum lipase activity was reached at the same time, i.e. after about 45 h of the process. However, the value of maximum activity achieved was approximately 12 times higher for PFC (around 8600 U dm^{-3}) than for olive oil (around 680 U dm^{-3}), leading to 14-fold increase in lipase productivity when perfluorodecalin was applied. These results clearly show that high oxygen solubility is not enough to produce a good oxygen vector. As discussed by Dumont and Delmas,[32] mass transfer enhancement in oil-in-water systems is dependent on other factors than just gas solubility, and in the case of high-viscosity oils the effect of high gas solubility will be limited owing to low gas diffusivity.

CONCLUSION

Perfluorodecalin addition to culture media benefited *Y. lipolytica* growth rate and its extracellular enzyme production. Lipase productivity increased 11-fold with the addition of 20% (v/v) PFC when the system was agitated at 250 rpm with a V_m/V_f ratio equal to 0.4. This effect was further magnified when the V_m/V_f ratio was reduced to 0.2. This condition provided a 23-fold enhancement of enzyme productivity with the addition

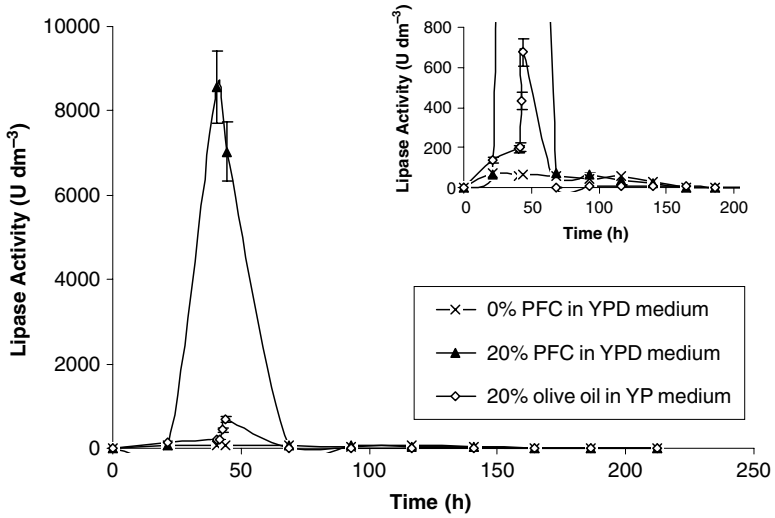

Figure 7. Comparison of effects of PFC (% v/v) and olive oil (% v/v) as oxygen vectors on lipase production by *Yarrowia lipolytica* in 1 dm^3 flasks with 200 cm^3 of medium ((100 − *x*)% YPD medium + *x*% PFC or 80% YP medium + 20% olive oil) agitated at 250 rpm. Error bars represent standard deviation.

of 20% (v/v) PFC in relation to the control system without PFC. Moreover, the presence of olive oil, a difficult substrate to work with, became unnecessary with PFC addition, as lipase production in YPD medium in the presence of PFC was notably higher than in YP medium with olive oil. The results achieved using olive oil as organic phase to improve oxygen supply permitted us to identify that the bottleneck in the aeration of biological systems lies in the mass transfer rate and not in oxygen solubility.

ACKNOWLEDGEMENTS

The authors are grateful for financial support from GRICES/CAPES (102/03), FAPERJ, CNPq and Fundação para a Ciência e a Tecnologia (POCTI/EQU/44427/2002).

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