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ORIGINAL PAPER

Beneficial effects of enhanced aeration using perfluorodecalin in *Yarrowia lipolytica* cultures for lipase production

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Abstract The inadequate supply of oxygen to biomass is a critical factor to the productivity of most aerobic submerged fermentations. This happens because oxygen is sparingly soluble in the aqueous media. The use of a second liquid phase of perfluorocarbon (PFC), an oxygen-carrying compound, in the culture medium can increase the availability of oxygen to the microorganisms. The effect of perfluorodecalin on *Yarrowia lipolytica* cultures was investigated in shake-flask cultures. It was found that the specific growth rate of *Y. lipolytica*, a strictly aerobic yeast, increases with increasing PFC concentration. Extracellular lipase production was increased with 20% (v/v) of PFC and agitation of 250 rev/min. It was shown that the PFC presence benefitted lipase production and not just its secretion to the extracellular medium.

Keywords Lipase · Oxygen · Perfluorocarbon · *Yarrowia lipolytica*

Introduction

Perfluorocarbons (PFC's) are petroleum-based compounds synthesized by substituting the hydrogen by fluorine atoms at the hydrocarbon molecules. They are both stable and chemically inert due to the presence of the very strong carbon-fluorine bonds. The oxygen solubility in PFC's is 10–20 times higher than that in pure water (Riess and Le Blanc, 1982). Inevitably, the ability of PFC liquids to dissolve respiratory gases has attracted interest from biotechnologists during the past three decades, including investigations on PFC's employment for improving the supply of gases for microorganisms to raise the fermentation yield. Indeed, the results presented in the literature show the success of this approach to increase the oxygen transfer rate in a bioreactor and its productivity (Elibol and Mavituna, 1995; Elibol, 1997: Ju et al., 1991).

Yarrowia lipolytica is one of the most extensively studied ''non-conventional'' yeasts and is currently used as a model for the study of protein secretion, dimorphism, degradation of hydrophobic substrates, and several new fields (Fickers et al., 2004). Being a strictly aerobic yeast, its growth and metabolite secretion are affected by the amount of oxygen available in the culture medium (Alonso et al., 2005; Kamzolova et al., 2003). It has the ability to produce a wide spectrum of products, is considered non-pathogenic, and several processes based on this organism are classified as GRAS by the FDA (US Food and Drug Administration) (Barth and Gaillardin, 1997). One of the most important products secreted by this microorganism is lipase, which is an enzyme that attracts the interest of scientists and industrial researchers because it can be exploited for several applications in the detergent, food, pharmaceutical, and environmental industries (Jaeger and Reetz, 1998).

Lipase productivity is affected by different environmental factors (Corzo and Revah, 1999). The

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carbon source is one of them. Several workers have shown that lipid substrates are the best and glucose might repress enzyme production (Corzo and Revah, 1999; Pereira-Meirelles et al., 1997). Usually the lipase produced by yeasts stays inside the cell (bound to the cell wall) and it is only secreted to the culture medium when the carbon source has been completely consumed, i.e, in the stationary phase of growth (Pereira-Meirelles et al., 2000). The amount of oxygen available to the microorganisms seems to be also an important parameter, since many authors have shown the dependence of lipase productivity on system aeration and agitation (Chen et al., 1999; Elibol and Ozer, 2000; Vadehra and Harmon, 1969). The aim of this work is to evaluate the effects of perfluorodecalin addition on lipase production by *Y. lipolytica*, considering the improvement in oxygen supply.

Materials and methods

Materials

Perfluorodecalin, the common brand name of which is Flutec PP6, was obtained from F2 Chemicals Ltd. (Preston, Lancashire, UK). The relevant physical properties of perfluorodecalin at 25°C and 1 atmosphere are as follows: density 1.917 g/ml, vapor pressure 810 Pa, and oxygen solubility 127.8 mg/l (Dias et al., 2004).

Peptone, yeast extract, and glucose were obtained from Merck, Oxoid, and Isofar, respectively.

Strain, media, and culture conditions

A wild-type strain of *Y. lipolytica* (IMUFRJ 50682) was selected from an estuary in the vicinity of Rio de Janeiro, Brazil (Haegler and Mendonça-Haegler, 1981) and conserved at 4°C on YPD-agar medium. For both inoculum (48 h) and growth conditions, cells were cultivated at 28°C in a rotary shaker (160 and 250 rev/min, respectively), in flasks containing YPD medium (w/v: yeast extract 1%; peptone (from casein), 0.64%; glucose 2%). For experiments using PFC 10% and 20% (v/v) of perfluorodecalin were added to the medium. A control experiment was carried out with no PFC. The assays were first performed in 500 ml shake flasks containing 200 ml medium (Media Volume/Flask Volume, $V_m/V_f = 0.4$). When the Media Volume/Flask Volume ratio was reduced 1-l flasks were used with the same media volume ($V_m/V_f = 0.2$).

Analytical methods

Cell growth

Cell growth was followed by optical density measurements at 570 nm converted to mg d.w./ml using a factor previously established.

Glucose

Extracellular glucose concentration was determined by the glucose oxidase method (Glucose (GO) Assay Kit).

Lipase activity

Lipase activity was performed through a spectrophotometric method (when the method used in the text is not mentioned): 0.1 ml of the cultivation medium without cells was added to a solution of 0.504 mM *p*-nitrophenyl laurate (*p*-NPL) in 50 mM 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 was 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 of product per minute.

To confirm that the enzyme detected by the spectrophotometric method was a lipase and not merely esterase, in some cases it was also performed the titrimetric method (Pereira-Meirelles et al., 1997) using olive oil as substrate. The reaction conditions were: 37°C, pH 7.0, with phosphate buffer 50 mM. One unit (U) of lipolytic activity was defined as the amount of enzyme that produces 1 μmol product per minute under the assay conditions.

Cell free extract preparation

Cells (50 mg/ml) twice washed with distilled water, were resuspended in 0.5 ml of 0.1 M MOPS buffer, pH 7.0. Disruption was obtained using glass beads, with two cycles of vortex-mixing for 1 min and immersion in an iced bath also for 1 min.

K_La measurement

A polarographic oxygen electrode was employed to measure dissolved oxygen concentration in the liquid medium. The mass transfer coefficient (K_La) was determined using the dynamic gassing-out method (Bandyopahyay and Humphrey, 1967).

Results

The effect of PFC on cell growth

The effects of the addition of 10% and 20% (v/v) of PFC (perfluorodecalin) were studied in *Y. lipolytica* growth media in agitation speed of 250 rev/min and the cell growth profiles are presented in Fig. 1.

Comparing these profiles and calculating the growth parameters (Table 1) it is possible to identify that despite the sudden decrease of cell concentration in the initial minutes, the specific growth rate ($1/[x]*d[x]/dt$, where $[x]$ is cell concentration in mg/ml) increased with increasing concentrations of PFC. The glucose consumption rate ($-dS/dt$) also increased when more PFC was added to the media (20% v/v). Elibol (1997) showed that the use of perfluorodecalin in *Saccharomyces cerevisiae* growth media improves the glucose consumption rates.

An oxygen electrode was adapted in the flasks used for *Y. lipolytica* culture permiting the measurement of the oxygen transfer coefficient (K_La) in YPD medium with and without PFC. The results in Table 2 show an increase in the K_La with the addition of 20% (v/v) PFC. This result shows that the PFC presence in an aqueous system raises the oxygen transfer rate. Therefore, the rise in the specific growth rate and glucose consumption can be attributed to the relief of O_2 transfer limitation, the cells being easily able to consume the glucose and the additional oxygen supplied.

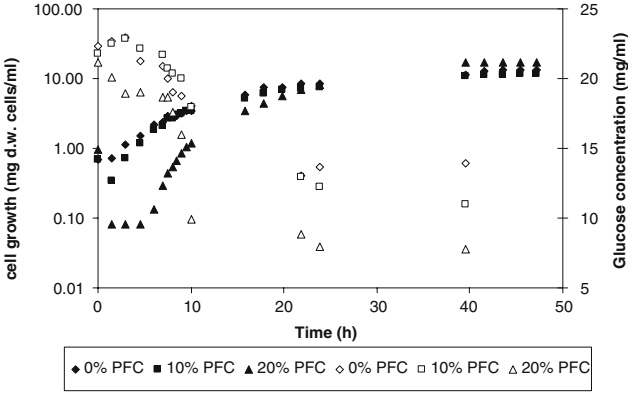

Fig. 1 The influence of PFC in cell growth (closed symbols) and glucose consumption (open symbols) of *Y. lipolytica* cultivated in 500 ml flasks with 200 ml of medium ('100-y' % YPD medium + 'y' % PFC) agitated at 250 rev/min. The experiments were performed in triplicate and the characteristic profiles are presented

Table 1 The influence of PFC and agitation speed in the growth parameters of *Yarrowia lipolytica*

PFC concentration (%)	μ (h^{-1})	t_d (h)	$-dS/dt$
0	0.17 ± 0.01	4.00 ± 0.11	0.31 ± 0.01
10	0.25 ± 0.01	2.75 ± 0.07	0.34 ± 0.01
20	0.53 ± 0.01	1.31 ± 0.02	0.39 ± 0.03

Table 2 Values of K_La for Erlenmeyer flasks with YPD medium and PFC agitated at 250 rev/min in different V_m/V_f (Medium Volume/Flask Volume) ratio

PFC (%)	K_La (h^{-1})	
	$V_m/V_f = 0.4$	$V_m/V_f = 0.2$
0	1.7 ± 0.2	2.8 ± 0.3
20	4.3 ± 0.3	7.6 ± 0.3

The sudden reduction in cell growth concentration in the initial minutes that can be observed in Fig. 1 was due to the migration of cells to the organic PFC phase. Since the cell concentration was measured just in the aqueous phase, when the cells migrate to the PFC phase as the agitation starts, a reduction in cell concentration in the aqueous medium is observed. This phenomenon was studied by Amaral et al. (submitted for publication) and it seems that there is a high affinity of this particular strain of *Y. lipolytica* to organic non-polar compounds, such as the PFC or saturated hydrocarbons, due to some peculiarities of its cell wall (the presence of glycoproteins that show a hydrophobic behaviour). The consequence of this fact is an underestimation of cell concentration in the medium.

In order to investigate this underestimation, several cell suspensions were prepared in 0.1 M phosphate buffer, pH 7.0, with different cell concentration and 20% (v/v) PFC. The system was agitated at 250 rev/min and samples were taken along time from the aqueous phase. Table 3 shows the results after the equilibrium was achieved in the system. It is possible to notice that the percentage of cells in the PFC phase diminishes with increasingly cell concentration, which shows a limit to solubility. Therefore, with the progression of

Table 3 The percentage of cells in PFC phase of a cell suspension (0.1 M phosphate buffer, pH 7.0) with 20% (v/v) PFC agitated at 250 rev/min for 120 min

Cell concentration (mg/ml)	Percentage of cells in the PFC[a]
5.8	49.1
2.8	62.7
1.2	84.2
0.5	98.9

[a] Percentage of cells in the PFC = (initial aqueous cell concentration – final aqueous cell concentration)/(initial aqueous cell concentration)*100

the fermentation, this sub-estimation becomes less important.

The effect of PFC in lipase production

The influence of V_m/V_f ratio

Lipase production in the culture medium of *Y. lipolytica* was detected during time by measuring lipase activity of the cell-free extract. To study the influence of PFC, 10% and 20% (v/v) of PFC were added to the culture medium (200 ml of liquid medium in 500 ml flask) and the system was agitated at 250 rev/min. A control assay (0% PFC) was also carried at these conditions. The highest lipase activity values of each culture condition detected along the 50 h of fermentation was divided by the respective batch time resulting in the productivity of this enzyme at the conditions studied as shown in Table 4. It is possible to notice that the addition of 10% (v/v) of PFC had a positive effect in lipase productivity and, with 20% (v/v) of PFC, lipase productivity increased more than 10 times. It is possible that the raise in the K_La of the system have induced the rise in lipase production as it has been proved that this enzyme production depends on the oxygen available in the medium (Chen et al., 1999; Elibol and Ozer, 2000; Vadehra and Harmon, 1969).

The reduction in Medium Volume/Flask Volume (V_m/V_f) ratio modifies the oxygenation of shake flasks significantly because it increases the gas-liquid interfacial area. The results presented in Table 2 confirm this modification in the oxygenation profile by the raise in the K_La of the systems with the reduction of V_m/V_f. Therefore, to study this effect, assays were also carried with 200 ml medium in 1-l flasks ($V_m/V_f = 0.2$) under 250 rev/min agitation speed with the addition of 20% (v/v) PFC in comparison to control (without PFC). The results presented in Table 4 show that the reduction of V_m/V_f ratio raised lipase productivity even without PFC addition. In the presence of 20% (v/v) PFC the reduction of V_m/V_f ratio promotes a 4-fold rise in the productivity of the enzyme. Besides, the benefit of PFC presence with V_m/V_f (0.2) is larger than with V_m/V_f (0.4). The lipase productivity in the first condition (V_m/V_f 0,2) raised 23 times with the addition of 20% (v/v) of PFC in comparison with its respective control (without PFC, $V_m/V_f = 0.2$) and for the V_m/V_f ratio of 0,4 the addition of the same PFC concentration raised the lipase productivity only 11-fold in comparison to its respective control.

Table 4 The influence of PFC and V_m/V_f (Medium Volume/Flask Volume) ratio in lipase productivity by *Y. lipolytica*

PFC concentration (%)	Lipase productivity (U/l.h)	
	$V_m/V_f = 0.4$	$V_m/V_f = 0.2$
0	4.4 ± 0.1	8.2 ± 0.7
10	6.3 ± 0.1	-
20	48.5 ± 1.0	190.4 ± 2.1

Esterases are enzymes that can also able to catalyse the production of *p*-nitophenol by *p*-NFL which is the reaction that takes place in the spectrophotometric method to measure lipase activity. Therefore, one specific method to measure lipase activity, the titrimetric method (Pereira-Meirelles et al., 1997), was performed to guarantee that the results presented so far were not overestimated. Figures 2 and 3 shows the lipase activity profile from the culture medium of *Y. lipolytica* grown in YPD medium with and without PFC, respectively. It is possible to notice the similarity in the profiles obtained from both lipase activity

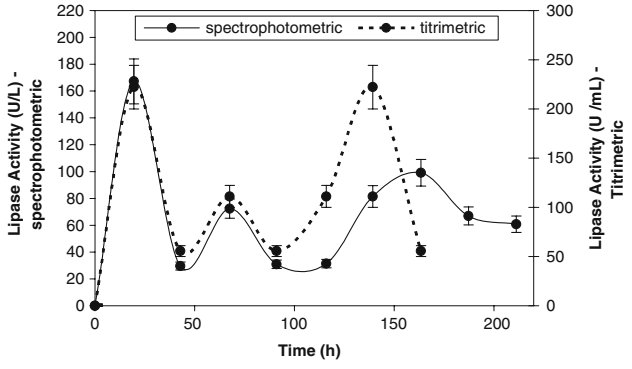

Fig. 2 Lipase activity in the growth medium (1-l flasks with 200 ml of medium) of *Y. lipolytica* as a function of time. Comparison between two lipase activity measurements, spectrophotometric and titrimetric

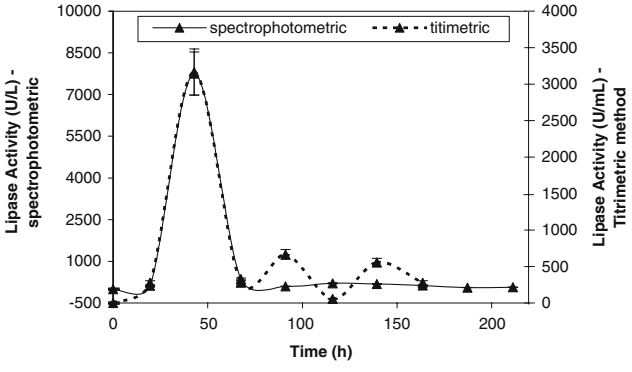

Fig. 3 Lipase activity in the growth medium (1-l flasks with 160 ml of YPD medium + 40 ml PFC) of *Y. lipolytica* as a function of time. Comparison between two lipase activity measurements, spectrophotometric and titrimetric

measurements, which confirms the results for lipase activity herein presented.

The dispersion of PFC before inoculation

The dispersion of PFC by the agitation of the system before the inoculation of cells is a strategy performed by some authors (Elibol and Mavituna, 1999: Elibol, 1997) to make sure that the PFC is homogenously dispersed throughout the fermentation. Therefore, to investigate whether this approach had an influence in extracellular lipase production 20% (v/v) of PFC was added to the medium before the inoculation and agitated at 400 rev/min during 15 min. The results were compared to a control (0% PFC) and to the lipase production in the medium were 20% (v/v) of PFC was added just after the inoculation. These experiments were carried out with a $V_m/V_f = 0.2$. By the profiles of lipase activity presented in Fig. 4, one can notice that the dispersion of PFC did not significantly affect lipase production. For that reason, further experiments were performed by adding the PFC just after the inoculation.

The influence of PFC in lipase secretion

Some investigators have shown that the secretion of intracellular lipase is induced by low levels of lipid substrate in the medium (Pereira-Meirelles et al., 2000). In the present work the carbon source was not a lipid, however, significant values of extracellular lipase were also detected only in stationary phase (after 40 h of fermentation), when the glucose concentration decreased, as Figs. 2–4 depict.

In order to investigate the effect of the PFC in lipase excretion, intracellular lipase activity was measured in the cell-free extract samples during the fermentation of *Y. lipolytica* in 1-l flasks with 200 ml of medium with and without 20% (v/v) PFC. The profiles are shown in Fig. 5 and it is possible to identify that lipase production inside the cell is always higher in the presence of PFC. It can be observed in Fig. 5 that there is no significant reduction in lipase activity levels, and in the case of the presence of PFC there is a rise in those levels in the initial hours of fermentation. This result shows that the presence of PFC in the culture medium induces the rise in lipase production and not only its secretion for the extracellular medium.

The presence of an emulsifier

The addition of an emulsifier in the medium where the PFC is present as an oxygen vector is often used to avoid the coalescence of the PFC droplets that may lead to increase of particle size and decrease of contact area between PFC and aqueous phase. Elibol (1999) has shown that the addition of PFC emulsified with pluronic in the culture medium raised the K_La of a bioreactor in comparison to the use of pure PFC. Therefore, an emulsifier, Span 20, was added to the medium at a concentration of 1% (w/v) and the effect of its presence in lipase production during time is depicted in Fig. 6. The use of the emulsifier without the PFC caused no increase in lipase activity as compared to the control (0% PFC and no Span). Although the presence of the PFC emulsified with span increased lipase production in relation to the control, the addition of pure PFC in the medium was better for lipase productivity. The emulsifier did not affect the yeast growth (data not shown). So, it might have inhibited lipase excretion to the medium or affected the oxygen transfer from the PFC to the aqueous phase. Clearly,

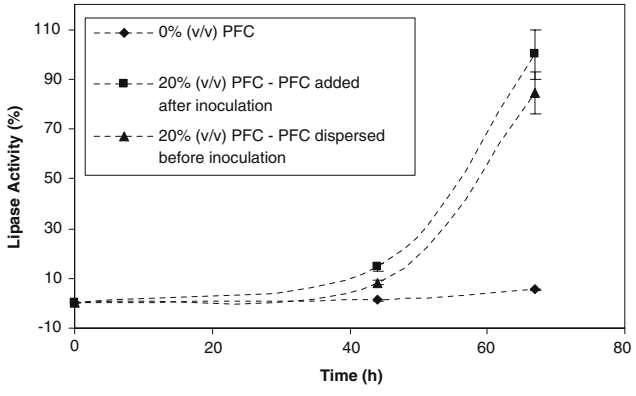

Fig. 4 Lipase activity in the growth medium (1-l flasks with 200 ml of medium ('100-y' % YPD medium + 'y' % PFC)) of *Y. lipolytica* as a function of time

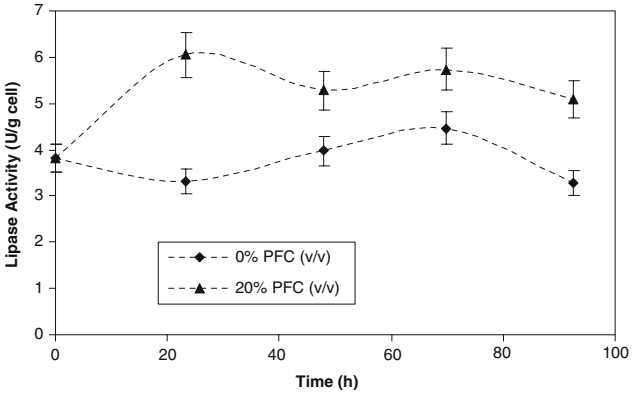

Fig. 5 Intracellular lipase activity in *Y. lipolytica* cells grown in YPD medium (1-l flasks with 200 ml of medium ('100-y' % YPD medium + 'y' % PFC)) as a function of time

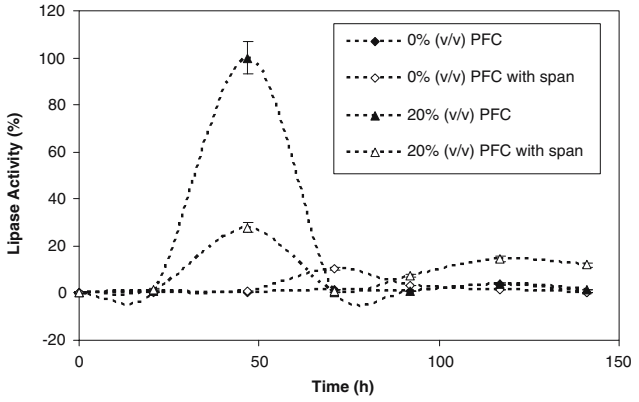

Fig. 6 The influence of the presence of an emulsifier in the medium with and without PFC in lipase production by *Y. lipolytica* in YPD medium (1-l flasks with 200°ml of liquid medium ('100-y' % YPD medium + 'y' % PFC))

additional research is needed to fully understand these results.

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 systems were agitated at 250 rev/min in a V_m/V_f ratio equal to 0.4. This effect was even magnified with the reduction on Medium Volume/Flask Volume ratio to 0.2. This condition provided an enhancement in the enzyme productivity of 23 times with the addition of 20% (v/v) PFC in relation to its control without PFC. Perfluorodecalin has induced lipase production and not only its secretion. Moreover, the presence of an emulsifier in the medium did not further enhance the effect of PFC in lipase production.

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