GROWTH AND PRODUCTION OF IMMOBILISED LIPASE FROM *Rhizopus delemar* CULTIVATED IN SSF ON A SYNTHETIC RESIN (AMBERLITE)

Pierre Christen[1] & Sergio Revah[2]

[1] Laboratoire de Biotechnologie, Centre ORSTOM, BP 5045; 34032 Montpellier Cedex 1, France
[2] Universidad Autonoma Metropolitana, Unidad Iztapalapa, Departamento de Ingenieria, Av. Michoacan y Purisima, Col. Vicentina, Apdo. Postal 55-535. Mexico, D.F., C.P. 09340

Abstract

In this work, a study of lipase production by *Rhizopus delemar* grown on a polymeric resin (Amberlite IRA 900) added with a medium previously optimised is presented. In the first part, the activity of several commercial preparations and the *R. delemar* lipase produced in liquid was compared. It also appeared that the resin can adsorb more than 24% of the lipase produced per g Amberlite. Desorption realised using 5g NaCl / g Amberlite at pH 5 allowed to recover 35 % of the adsorbed lipase. It was also shown that the resin displayed a thermo-protective effect since no loss in activity was observed when the adsorbed enzyme was heated at 80 °C for 24 hours. In solid state fermentation (SSF), the fungus was shown to produce high amounts of enzyme (93 U / g dry Amberlite against only 14 U /ml in submerged culture) when dextrin was used as carbon source after only 24 hours (against 48 hours in liquid culture). Significative activity was also detected with maltose and more surprisingly with glucose (68 and 57 U / g dry Amberlite respectively). The strong inhibitory effect of glucose observed in liquid culture was reduced in SSF.

Introduction

Lipases are widely used enzymes that can be obtained from animals, plants and micro-organisms. Microbial lipases have been used in the food industry, mainly in dairy products, and are also important in detergents, pharmaceutical, cosmetics and leather processing (Seitz, 1973). The enzyme modified cheeses (EMC) are also an interesting application involving lipases (Revah and Lebeault, 1989). New trends in that field are directed toward the use of immobilised lipases in organic solvent for ester synthesis, triglycerides hydrolysis, flavouring compounds synthesis (Christen and López-Munguía, 1994).

Solid state fermentation can be a suitable method for producing enzymes such as pectinases, amylases, or cellulases (Lonsane and Ghildyal, 1992), but few papers have dealt with lipases. Nevertheless, Yamada (1977) reported that, in Japan, most of the microbial lipases comes from *Aspergillus* strain cultivated in liquid culture (LC) and SSF. More recently, Rivera Muñoz *et al.* (1991), using *Penicillium candidum* grown on wheat bran, found that SSF has many advantages against LC for lipase production. To understand better fungal growth, inert supports impregnated with a nutritive solution have been reported. The aim of this work was to study the growth and

the lipase production by *Rhizopus delemar* on Amberlite, a well studied anionic resin (Auria *et al.*, 1990; Christen *et al.*, 1994). This include, characterisation of the lipolytic activity in LC, SSF experiments and interaction support/enzyme.

Micro-organisms and culture media

Two *Rhizopus delemar* strains were tested: CDBB H313 (CINVESTAV-MEXICO) and NRRL 1472. They were periodically transferred on Potato Dextrose Agar (PDA) slants and stored at 4°C. Spores were produced in Erlenmeyer flasks on PDA at 29°C during 6 days. The nutritive medium previously optimised by Martinez Cruz *et al.* (1993) was used both in LC and SSF. LC were made in 250 ml Erlenmeyer flasks placed on a rotatory shaker. Initial conditions were : temperature, 29°C; pH, 6; inoculum size, $1x10^7$ spores/ml; agitation speed, 180 rpm. In solid state cultures, an anionic resin (Amberlite IRA-900, Rohm & Haas) was used and prepared according to Christen *et al.* (1993). Nutritive medium was added to the dried support to complete 58% final water content, the maximum absorption capacity of the resin. The cultures were carried out in small columns placed in temperature controlled bath. Initial conditions were: temperature, 29°C; pH, 6; inoculum size, $1x10^7$ spores/g Initial Dry Matter (IDM) and aeration rate, 0.5 l/h.g IDM.

Analytical procedures

In LC, growth was followed by the evolution in dry weight. In SSF, respirometry was used which allowed to calculate CO2 production rate (CDPR) as previously described (Christen *et al.*, 1993).Water activity, moisture content and pH were also determined at the end of the fermentation.

Lipolytic activity was assayed with the method used by Nahas (1988) with some modification. The substrate was a 5 % tributyrin emulsion prepared in a 1 % Tween solution in 2.5 M tris-maleate buffer (pH = 6) by homogenising with an Ultraturax apparatus (8000 rpm during 2 mn). The reaction mixture contained 18 ml substrate and 12 ml extract solution. In the case of adsorbed lipase, one gram of Amberlite was added to the reaction medium. The determination was achieved with a Mettler DL 21 pH stat , at 37°C and pH adjusted to 6. The butyric acid released was titrated with 5 mM NaOH solution during 5 min. One unit (U) was defined as the amount of enzyme releasing one mmol of free fatty acid per minute.

Adsorption/desorption study

R. delemar lipase adsortion study was achieved using entire, (average diameter : 0.53 mm), or ground Amberlite (average diameter: 0.10 mm). Amounts varying from 0.5 g to 6 g of Amberlite were contacted with 50 ml of enzymatic extract in 250 ml Erlenmeyer flasks placed in a rotatory shaker (150 rpm) during 24 h. Temperature was 29°C and pH adjusted to 6.

Desorption study was achieved using 2g of Amberlite in 50ml of chloride sodium concentrations ranging from 10 to 120 g NaCl /l and at different pH. Conditions were similar to those of the adsorption studies.

Results are reported as :

. **Uads** is defined as U0-Ures, where U0 was the initial and Ures the residual ac-

tivity of the extract after the adsorption experiment.

. **Uexp** which is measured and represents the activity shown by the Amberlite after the adsorption experiment.

. **Udes tot** : 100 x (Udes/Uads) where Udes represents the desorbed lipase in the medium and is assayed as described previously. It is expressed as percentage of total adsorbed lipase.

. **Udes rel** : 100 x (Udes/Uexp). It is expressed as percentage of total ex-pressed lipase.

Results and Discussion

Evaluation of the R. delemar strains

Lipolytic activity of the 2 strains was evaluated according to Corzo (1993) by growing the mould in Petri dishes on PDA added with emulsified tributyrin (1%). The diameter of the halo around the colony, corresponding to the hydrolysis of the substrate was measured after 3 days. The CDBB H313 strain gave an average diameter, calculated from one hundred colonies, of 2.75 mm against 2.28 mm for the NRRL 1472 strain. The former was then used in all further experiments.

Growth and lipase production kinetics in LC

LC was used to produce the enzymatic extract needed to the adsorption/desorption experiments. Results are plotted in figure 1. It can be seen that maximum activity (14.07 U/ml) corresponds to maximum growth (12.3 mg/ml). These values were reached within 2 days and correspond to those obtained by Martinez Cruz *et al.* (1993). It was shown that a centrifugation at 5000 rpm during 5 mn was not recommended because a loss of more than 50% in lipolytic activity was observed, probably due to cell bound protein. The important decrease in lipolytic activity observed after 2 days may be due to proteolytic activity and/or denaturation of the protein. This was not observed when Amberlite (2g/50ml) was present in the medium (Angeles, 1995). In figure 2, it can be observed that the extract was more stable at pH 5 while the optimum activity was obtained at 6.5.

Growth and lipase production in SSF

Glucose is known to be a repressor of lipase production in LC Haas and Bailey (1993). One of the particular goal of the experiments in SSF was to see if this catabolic repression could be partially or totally overcome. Three carbon sources (20 g/l) were used in the SSF experiments: glucose, maltose and dextrin.

Results are presented in figures 3 and 4. The growth, followed by CDPR, did not display significative differences between the 3 substrates. Maxima of this parameter were between 15 and 20 hours - a very short time in SSF - and reached values of 3.5 ml/h.g IDM.. Maximum lipase production was found after 24 hours, just after the maximum of CDPR, corresponding to the lower pH in the medium. Best production was found for dextrin (95.6 U/g IDM) against 68.2 U/g IDM for maltose and 57.7 U/g IDM for glucose (See Figure 9). The equivalence in U/ml reactor is given in table 1 and

it is shown that SSF gave a higher productivity with the same substrate (dextrin) than LC. This activity decrease after this maxima for the 3 cases.

Fermentation	LC	SSF		
Carbon source	Dextrin	Glucose	Maltose	Dextrin
Time max. production (h)	48	18	18	15
CDPR max (ml/h.g IDM)	-	2.8	3.5	3.48
R.Q. (Range)	-	1-1.3	1-1.3	1-1.4
pH max. production	-	5.7	4.9	5.6
Aw	-	0.998	0.999	0.994
Lipolytic Act. (U/g IDM)	-	57.7	68.2	95.6
Lipolytic Act. (U/ml)	14.08	10.21	11.33	15.66

Table 1. Comparative data of LC and SSF lipase production.

In table 1 are summarised the most important data about LC and SSF for lipase production by *R. delemar*. It can be seen that the enzyme is produced faster and with a better productivity in SSF. Moreover, Amberlite is an adequate support for this purpose: it provided a good stability for pH, moisture content (which remained stable) and Aw, all three being key parameters in SSF. The best carbon source was dextrin as already observed in LC (Martinez Cruz *et al.*, 1993), but in SSF, the catabolic repression due to glucose was not as important as expected (only a decrease of 40% against dextrin). R.Q. observed are typical of the oxidative use of the carbon sources.

R. delemar lipase adsorption/desorption study

. Adsorption study

Results for lipase adsorption on entire and ground Amberlite are represented in figures 5 and 6. It can be seen on figure 3 that the amount of lipase adsorbed per g of Amberlite decreased for higher amounts of resin, while the contrary trend is observed for the residual activity. Only 3% of the adsorbed enzyme was active when 0.5 g of resin where used, but this increased to 26% for 6 g. The important losses observed in expressed activity may be due to partial denaturation of the protein, inactivation of the active sites due to the anionic properties of the support or partial diffusion of the protein inside the resin.

In the case of ground Amberlite (Cf figure 6), as low as 0.5 g were sufficient to adsorb all the lipase present in the reaction medium. In the same way than for entire Amberlite, the relation between expressed and adsorbed lipase increased from 12.4% to 34% with the increase of amount of resin in the medium. Nevertheless, the values for adsorbed and expressed activities are higher than for entire Amberlite, probably due to the increase in the contact area in that case. It may also be explained by a decrease in the limitation of diffusion.

Figure 1 : R. delemar lipase production kinetic in LC
Effect of centrifugation on lipolytic activity

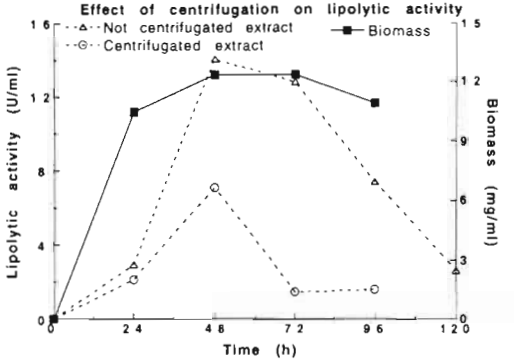


Figure 2 : Extract stability at different pH

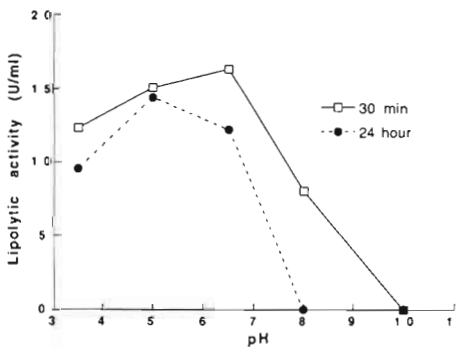


Figure 3 : Respirometric activity of R. delemar grown in SSF with 3 different carbon sources

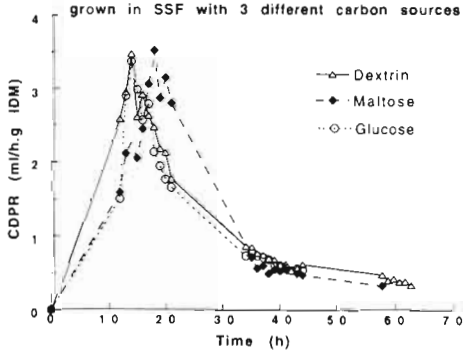


Figure 4 : Lipolytic activity for R. delemar grown in SSF with 3 different carbon sources

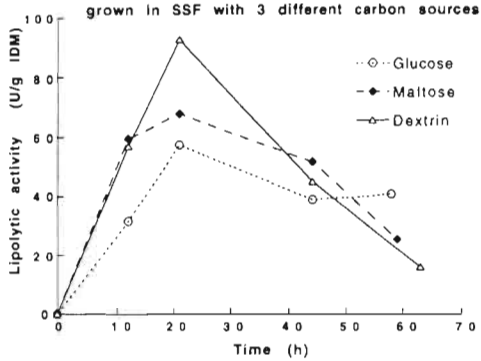


Figure 5 : R. delemar lipase adsorption on Amberlite

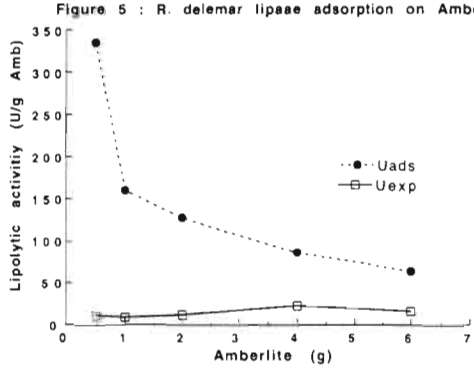


Figure 6 : R delemar lipase adsorption on ground Amberlite

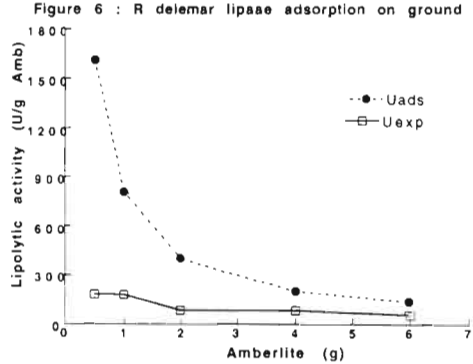


Figure 7 : R. delemar lipase adaorption kinetic on ground and entire Amberlite

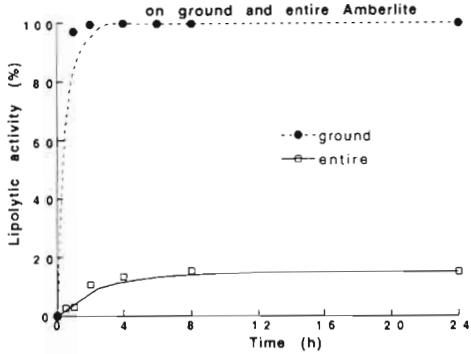


Figure 8 : influence of [NaCl] on lipase desorption (pH=6).

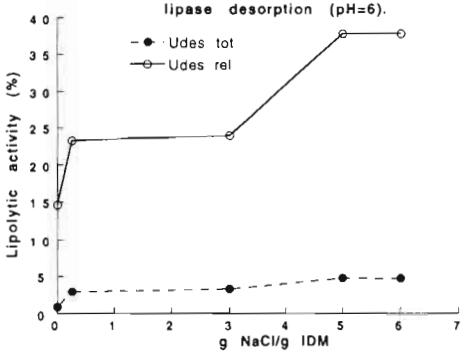
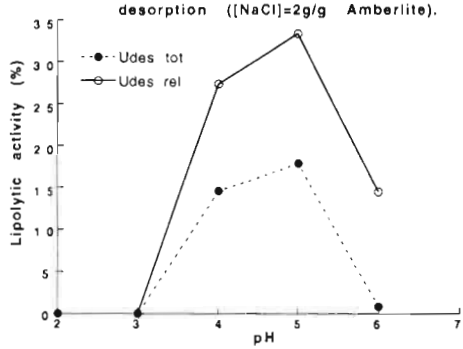


Figure 9 : Influence of pH on lipase desorption ([NaCl]=2g/g Amberlite).



In figure 7, it can be seen that all the lipase present in the medium is adsorbed on ground Amberlite within about 2 hours while, entire Amberlite was saturated after 8 hours, with only a little more than 20% of the lipase present in the reactive medium. These experiments showed that all the lipase was adsorbed on Amberlite in maximum 8 hours. The amount of adsorbed lipase and the adsorption dynamics depend strongly on the size of the resin. This confirmed the hypothesis presented above.

Moreover, the adsorption on Amberlite displayed a thermo protective effect since no loss in activity was observed after that a sample of adsorbed lipase stayed 24 hours at 80°C (Angeles, 1995). The adsorption of the produced enzyme on Amberlite during growth on SSF may serve as a method to concentrate it simultaneously.

. Desorption study

To study the recovery of the adsorbed *R. delemar* lipase on Amberlite, the entire particle was used. The influence of NaCl concentration and pH were explored (See figures 8 and 9). These experiments were realised at 29°C; agitation, 150 rpm during 24 hours. Addition of NaCl, previously used by Corzo (1993), for lipase desorption allowed a 38 % of desorption at 100 g NaCl/l at an optimum pH of 5.

Conclusions

In this study:

. *R delemar* CDBB H313 strain was selected for its better lipase production.

. In LC, the negative effect of centrifugation on lipase recovery, was demonstrated. It was established that the enzyme was more time stable at pH 5, while its optimum activity was at pH 6.

. In SSF, the mould showed a good capacity to grow on Amberlite with various carbon sources (dextrin, maltose and glucose). Best lipase production was found with dextrin (as in LC) while lower glucose repression was observed than in LC.

. In sorption/desorption experiences, it was evidenced that entire Amberlite was saturated with 24 % of the lipase while ground Amberlite was able to adsorb all the lipase present in the medium (about 700 U). There is an important difference between the "adsorbed" Amberlite (defined as initial rested from residual activity) and actual active lipase on Amberlite (only 26% and 34% for entire and ground support). The desorption experiments showed that the recovery of the adsorbed enzyme was uneasy (only 38% with 2 gNacl/g IDM and pH 5). It will be preferable to use the enzyme adsorbed on Amberlite than to try to desorb it. Furthermore, such lipase displayed a good thermostability. The use of Amberlite as a support opens interesting possibilities to study simultaneous enzyme production and separation in SSF.

References

Angeles, N. 1995. Producción de lipasa por *Rhizopus delemar* sobre una resina sintética (Amberlita IRA 900). Licence thesis, UNAM, México. In press.

Auria, R., Hernandez, S., Raimbault, M., Revah, S. 1990. Ion exchange resin: a model support for solid state growth fermentation of *Aspergillus niger*. *Biotechnol. Techniques*, 4(6) : 391-396.

Christen, P., Auria, R., Vega, C., Villegas, E., Revah, S. 1993. Growth of *Candida utilis* in solid state fermentation. *Biotech. Adv.* 11 : 549-557.

Christen, P., Auria, R., Marcos, R., Villegas, E., Revah, S. 1994. Growth of *Candida utilis* on Amberlite with glucose and ethanol as sole carbon sources. *Adv. Bioprocess Eng.*, E. Galindo and O.T. Ramirez (Eds), KJuwer Acad. Pub. : 87-93.

Christen, P., Lopez Munguía, A. 1994. Enzymes and food flavor - A review. *Food Biotechnol.*, 8(2&3) : 167-190.

Corzo, G. 1993. Estudio comparativo de la producción de lipasas por *Yarrowia lipolytica* en sistemas líquido y sólido. Master thesis, UNAM, México, 94 Pp.

Haas, M.J., Bailey, D.G. 1993. Glycerol as carbon source for lipase production by the fungus *Rhizopus delemar*. *Food Biotechnol.*, 7(1) : 49-73.

Lonsane, B.K., Ghildyal, N.P. 1992. Exoenzymes. *In*: Solid substrate cultivation, H.W. Doelle, D.A. Mitchell, C.E. Rolz (Eds), 191-209, Elsevier Appl. Sci., London.

Martinez Cruz, P., Christen, P., Farres, A. 1993. Medium optimization by a fractional factorial design for lipase production by *Rhizopus delemar*. *J. Ferment. Bioeng.* 76(2) : 94-97.

Nahas, E. 1988. Control of lipase production by *Rhizopus oligosporus* under various growth conditions. *J. Gen. Microbiol.* 134 : 227-233.

Revah, S., Lebeault, J.M. 1989. Accelerated production of blue cheese flavors by fermentation on granular curds with lipase addition. *Lait*, 69 : 281-289.

Rivera-Muñoz, G., Tinoco-Valencia, J.R., Sanchez, S., Farres, A. 1991. Production of microbial lipases in a solid state fermentation system. *Biotechnol. Lett.*, 13(4) : 277-280.

Seitz, E.W. 1973. Industrial applications of microbial lipases: A review. *J. Am. Oil Chem. Soc.*, 51 : 12-16.

Yamada, K. 1977. Recent advances in industrial fermentation in Japan. *Biotechnol. Bioeng.*, 19 : 1563-1621.