Lipase production by *Rhizopus delemar* grown on a synthetic support in solid state fermentation

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SUMMARY

Lipase production by *Rhizopus delemar* has been studied by growing on a polymeric resin (Amberlite IRA 900), after absorbing a medium previously optimized in solid state fermentation (SSF) and also in submerged fermentation (SmF). The activity of *R. delemar* lipase, produced in liquid culture (LC), could be adsorbed to an extent of 24% on 1 g Amberlite. Desorption, carried out using 5 g NaCl / g Amberlite at pH 5, allowed to recover 35% of the adsorbed lipase. Data indicated that the resin displayed a thermo-protective effect, since only little loss in activity was observed, when the adsorbed enzyme was heated at 80°C for 24 hours. In solid state fermentation (SSF), the fungus produced high amounts of enzyme (93 U/g dry Amberlite at 24 hours, against 14.1 U/ml at 48 hours in submerged culture), when dextrin was used as a carbon source. Significant activity was also detected with maltose and 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.

Keywords: Solid state fermentation, submerged fermentation, lipase production, *Rhizopus delemar*, synthetic support, Amberlite IRA 900, comparative titres, thermo-protective effect, glucose inhibitory effect.

RESUME

Production de lipases par *Rhizopus delemar* cultivé sur un support synthétique en fermentation solide.

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La production de lipases, par *Rhizopus delemar* cultivé sur un support synthétique (Amberlite IRA 900) imprégné d'une solution nutritive optimisée, a été étudiée d'abord en fermentation en milieu solide (FMS) et ensuite en fermentation submergée (SmF). L'activité lipase de *R.delemar*, produite en SmF, a pu être absorbée à 24% pour 1 g d'Amberlite. La desorption, réalisée en utilisant 5 g de NaCl/ g d'Amberlite à un pH 5, a permis de récupérer 35% de la lipase absorbée. Les résultats indiquent que la résine montre un effet thermo-protecteur vis-à-vis des lipases, étant donné qu'une faible perte d'activité enzymatique a été observée lorque l'enzyme a été absorbée et portée à 80°C pendant 24 h. En milieu solide (FMS) en présence de la dextrine comme source de carbone, le champignon filamenteux produit des quantités élevées d'enzymes (93 U/g poids sec d'Amberlite à 24 h, au lieu de 14,1 U/ml à 48h en fermentation submergée (SmF). Des activités importantes ont été détectées avec du maltose ou avec du glucose (68 et 57 U/g poids sec d'Amberlite, respectivement). L'hinibition forte provoquée par le glucose a été uniquement observée pour les cultures liquides alors que cette inhibition été nettement réduite en FMS.

Mots Clés : Fermentation en milieu solide, Fermentation submergée, Production de lipases, *Rhizopus delemar*, Support synthétique, Amberlite IRA 900, Production comparée liquide-solide, Effet thermo protecteur, Inhibition par le glucose.

INTRODUCTION

Lipases are the widely used enzymes, which can be obtained from animals, plants and microorganisms. Microbial lipases are used in the food industry, mainly in dairy products, and are also important to detergents, pharmaceutical, cosmetics and leather processing industries (Seitz, 1973). The enzyme modified cheeses (EMC) are also an interesting application involving lipases (Revah and Lebeault, 1989). New trends are directed towards the use of immobilized lipases in organic solvent for ester

synthesis, triglycerides hydrolysis and 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 originate from *Aspergillus* strain cultivated in SmF and SSF. More recently, Rivera Muñoz *et al,* (1991), using *Penicillium candidum* grown on wheat bran, found that SSF has many advantages over the SmF for lipase production.

To better understand the fungal growth, inert supports, impregnated with a nutritive solution, have been employed (Auria *et al,* 1990; Christen *et al,* 1994). The aim of this work was to study the growth of and the lipase production by *Rhizopus delemar* on Amberlite, an anionic resin. Data on characterization of the lipolytic activity in SmF and SSF experiments and interaction between support/enzyme are also presented.

MATERIALS AND METHODS

MICROORGANISMS AND CULTURE MEDIA

Two *Rhizopus delemar* strains were tested: CDBB H313 (CINVESTAV-MEXICO) and NRRL 1472. These 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 SmF and SSF. SmF was studied in 250 ml Erlenmeyer flasks placed on a rotary shaker. Initial conditions were: temperature, 29°C; pH, 6; inoculum size, $1x10^7$ spores/ml; and agitation speed, 180 rpm. In solid state cultures, an anionic resin (Amberlite IRA-900, Rohm and Haas) was used and prepared according to Christen *et al* (1993). Nutritive medium was added to the dried support to achieve 58% final water content, the maximum absortion capacity of the resin. The cultures were carried out in small columns placed in a 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 SmF, growth was followed by dry weight determination. In SSF, respirometry was used to calculate CO_2 production rate, 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 by the method used by Nahas (1988) with some modifications. The substrate was a 5% tributyrin emulsion, prepared in a 1% Tween solution in 2.5 M tris-maleate buffer (pH = 6) by homogenizing in an Ultraturax apparatus (8000 rpm during 2 min). The reaction mixture contained 18 ml substrate and 12 ml enzyme extract. In the case of adsorbed lipase, 1 g Amberlite was added to the reaction medium. The reaction was carried out using 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 reaction. One unit (U) was defined as the amount of enzyme releasing one mmol of free fatty acid per min.

ADSORPTION/DESORPTION STUDY

R. delemar lipase adsortion study was carried out using entire, (average diam : 0.53 mm), or ground (average diam : 0.10 mm) Amberlite. Amounts varying from 0.5 to 6 g Amberlite were contacted with 50 ml enzymatic extract in 250 ml Erlenmeyer flasks placed on a rotary shaker (150 rpm) for 24 h. Temperature was 29°C and pH was adjusted to 6. Desorption study was carried out using 2 g Amberlite in 50 ml of sodium chloride solution concentrations ranging from 10 to 120 g/l and at different pH. Conditions were similar to those of the adsorption studies. Results are reported as : units absorbed, units expressed by the resin, total units desorbed and units desorbed as the % of the total expressed activity.

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 containing emulsified tributyrin (1%).

The diameter of the clearing zone around the colony, corresponding to the hydrolysis of the substrate, was measured after 3 days. The CDBB H313 strain gave an average diam of 2.75 mm calculated from one hundred colonies, against 2.28 mm for the NRRL 1472 strain. The former strain was used in all further experiments.

GROWTH AND LIPASE PRODUCTION KINETICS IN SMF

SmF was used to produce the enzymatic extract needed for the adsorption/desorption experiments (Fig. 1). It can be seen that maximum activity (14.1 U/ml) corresponds to maximum growth (12.3 mg/ml). These values were reached within 2 days and are similar to those obtained by Martinez Cruz *et al,* (1993). Centrifugation at 5000 rpm for 5 min resulted in a loss of more than 50% lipolytic activity, probably due to proteolytic activity and/or denaturation of the protein. This was not observed when Amberlite (2 g/50 ml) was present in the medium (Angeles, 1995). The extract was more stable at pH 5, while the optimum activity was obtained at 6.5 (Fig. 2).

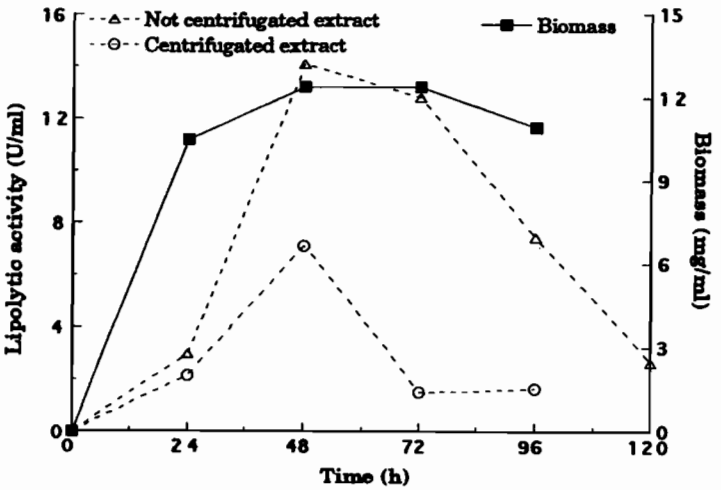

Fig. 1 : *R. delemar* lipase production kinetic in LC effect of centrifugation on lipolytic activity.

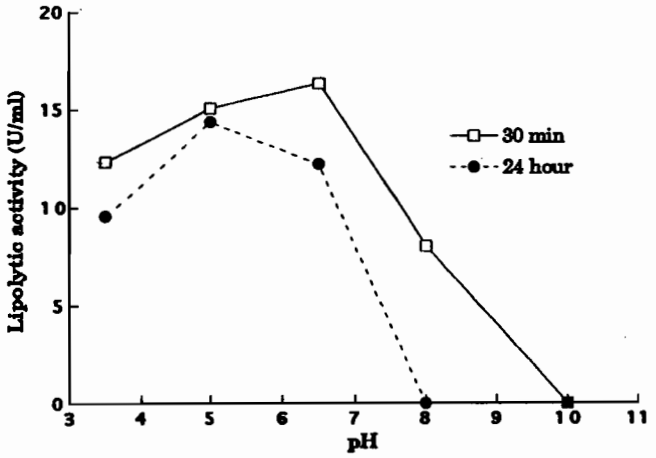

Fig 2. : Extract stability at different pH.

GROWTH AND LIPASE PRODUCTION IN SSF

Glucose is known to repress lipase production in SmF (Haas and Bailey, 1993). One of the particular aims of the experiments in SSF was to see if this catabolite repression could be partially or totally overcome. Three carbon sources (glucose, maltose and dextrin, each at (20 g/l) were used in the SSF experiments.

Results are presented in Figs. 3 and 4. The growth, followed by CO_2 production rate, was maximum between 15-20 h, a very short time in SSF, and did not display significant differences between the 3 substrates (Figs. 3 and 4). Maximum lipase production was found at 24 h, corresponding to the lower pH in the medium. Best production was found with dextrin (95.6 U/g IDM), against 68.2 U/g IDM with maltose and 57.7 U/g IDM with glucose (Fig. 4). The equivalent in U/ml reactor (Table 1) shows that SSF gave a higher productivity with the same substrate (dextrin) than in SmF. This activity decreased after in all 3 cases.

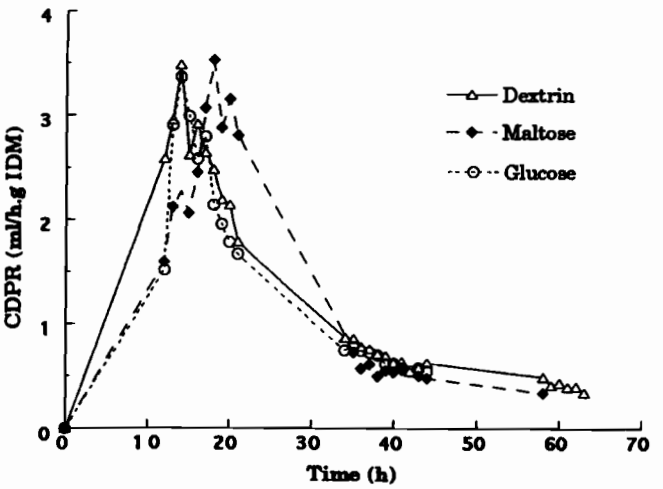

Fig. 3 : Respirometric activity of *R. delemar* grown in SSF with 3 different carbon sources.

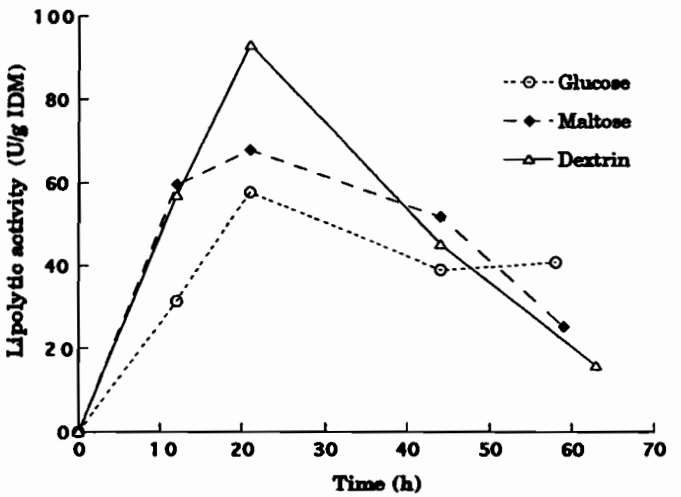

Fig. 4 : Lipolytic activity for *R. delemar* grown in SSF with 3 different carbon sources

Table 1. Comparative data of SmF and SSF for lipase production.

Carbon source	SmF Dextrin	SSF Glucose	SSF Maltose	SSF Dextrin
T max (h) *	48	18	18	15
CO_2 production rate, (ml/h.g IDM)	-	2.8	3.5	3.5
R.Q. (range)	-	1-1.3	1-1.3	1-1.4
pH max *	-	5.7	4.9	5.6
Final Aw	-	0.998	0.999	0.994
Lip. Act. (U/g IDM)	-	57.7	68.2	95.6
Vol. Lip. Act. (U/ml)	14.1	10.2	11.3	15.7

* Refers to time and pH of maximum lipase production.

Data summarized in Table 1 show that the enzyme is produced faster and with a better productivity in SSF. Moreover, Amberlite is an adequate support for this purpose, as it provided a good stability for pH, moisture content and Aw, all of these being key parameters in SSF. The best carbon source was dextrin as in SmF (Martinez Cruz *et al*, 1993), but in SSF, the catabolite repression due to glucose was not as strong as in SmF (only 40% decrease against dextrin). Respiratory quotients (RQ) observed are typical of the oxidative use of the carbon sources.

R. DELEMAR LIPASE ADSORPTION/DESORPTION STUDY

Adsorption study

Data (Fig. 3) showed that the amount of lipase adsorbed per g Amberlite decreased for higher amounts of resin, while an opposite trend is observed for the residual activity. Only 3% of the adsorbed enzyme was active when 0.5 g resin was used, but this increased to 26% for 6 g. The losses observed in terms of the expressed activity may be due to the 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.

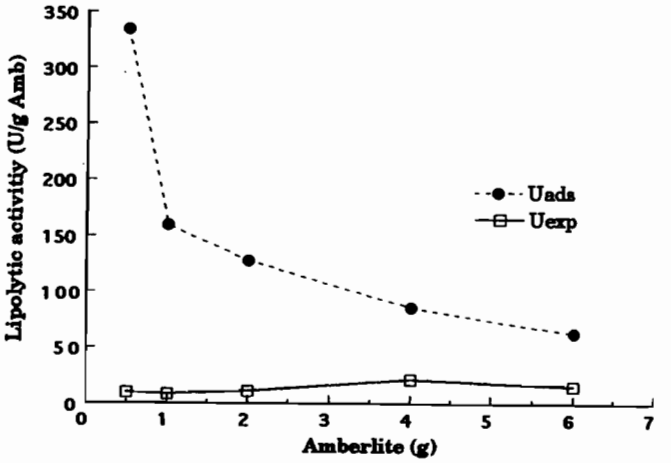

Fig. 5 : *R. delemar* lipase adsorption on Amberlite.

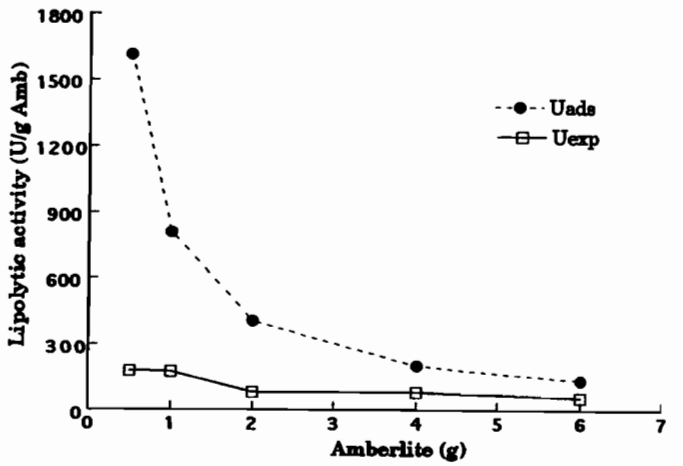

Fig. 6 : *R. delemar* lipase adsorption on ground Amberlite.

In the case of ground Amberlite (Fig. 6), as low as 0.5 g support was sufficient to adsorb all the lipase present in the reaction medium. 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 those for entire Amberlite, probably due to the increase in the contact area of the resin and decrease in the limitation of diffusion.

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 about 20% of the lipase present in the reactive medium (Fig. 7). These experiments showed that all the lipase was adsorbed on Amberlite in a maximum of 8 hours. The amount of adsorbed lipase and the adsorption dynamics depend strongly on the size of the resin. Moreover, the adsorption on Amberlite displayed a thermo-protective effect, since no loss in activity was observed, after a sample of adsorbed lipase was kept for 24 hours at 80°C (Angeles, 1995). The adsorption of the enzyme on Amberlite during growth on SSF may also serve as a method to concentrate it simultaneously.

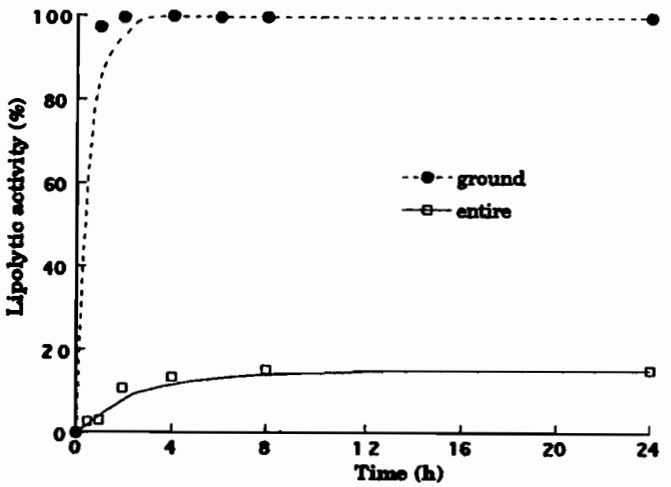

Fig. 7 : *R. delemar* lipase adsorption kinetic on ground and entire Amberlite.

Desorption study

To study the recovery of the adsorbed *R. delemar* lipase on Amberlite, the entire particles were used at 29°C for 24 hours (150 rpm). The effect of NaCl concentration and pH was explored (Figs. 8 and 9). Addition of NaCl, previously used by Corzo (1993) for lipase desorption, allowed a 38% of desorption at 100 g NaCl/L level and an optimum pH of 5.

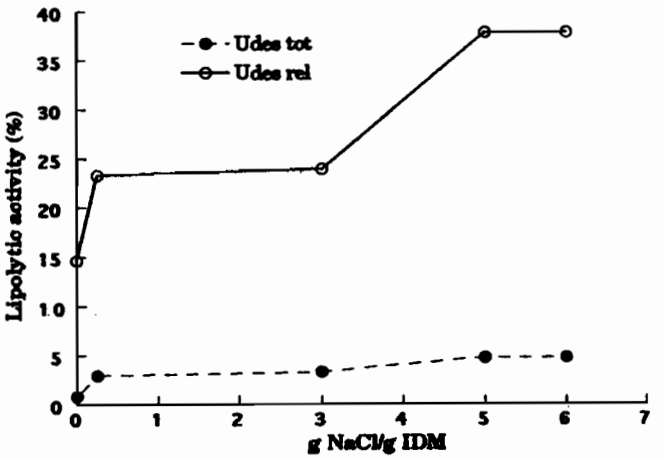

Fig. 8 : Influence of (NaCl) on lipase desorption (pH=6).

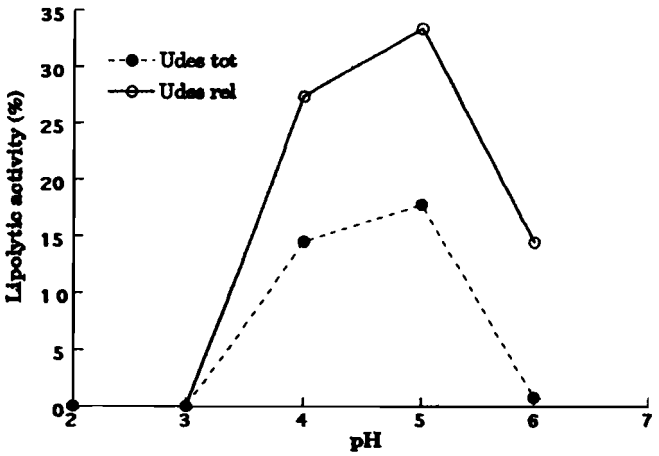

Fig. 9 : Influence of pH on lipase desorption (NaCl) = 2g/g Amberlite).

CONCLUSION

R delemar CDBB H313 strain was a better lipase producer than NRRL 1472. In SmF, the negative effect of centrifugation on lipase recovery was observed. It was established that the enzyme was more time stable at pH 5.0, while its optimum activity was at pH 6.0. 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, while lower glucose repression was observed than in SmF. 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 enzyme adsorbed Amberlite (defined as initial adsorbed and the residual activity after desorption) and actual active lipase on Amberlite (only 26% and 34% for entire and ground support). The recovery of the adsorbed enzyme was lower (only 38% with 2 g NaCl/g IDM at pH 5.0). It will be preferable to use the enzyme

adsorbed on Amberlite than 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 immobilization in SSF.

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