

5836

GLYCEROL AS A CARBON SOURCE FOR LIPASE PRODUCTION
BY THE FUNGUS RHIZOPUS DELEMAR

Michael J. Haas and David G. Bailey
Eastern Regional Research Center
Agricultural Research Service
U.S. Department of Agriculture
600 E. Mermaid Lane
Philadelphia, PA. 19118

ABSTRACT

The ability of the fungus Rhizopus delemar to synthesize lipase (glycerol ester hydrolase, E.C. 3.1.1.3) when grown on glycerol as the prime carbon source was investigated. Glucose, glycerol and olive oil all supported fungal growth and lipase production. Maximum net and specific lipase activities obtained in glycerol were greater than those obtained in glucose and olive oil, and were reached earlier and maintained longer during growth. In glucose media, lipase activity did not appear until the glucose had been consumed. Differences in lipase activity between glycerol and glucose media were not due to pH-mediated lipase inactivation. In both glucose and glycerol media the appearance of lipolytic activity coincided with the appearance of lipase polypeptide. The data are consistent with the postulate that lipase synthesis is subject to catabolite repression by glucose. These studies identify a simple, single-phase medium for lipase production and establish the utility of glycerol for amplifying enzyme levels.

INTRODUCTION

Lipases (glycerol ester hydrolases, E.C. 3.1.1.3) are enzymes which act at water-oil interfaces, catalyzing hydrolysis of the ester linkages in triacylglycerols (Brockerhoff and Jensen 1974, Borgström and Brockman 1984). Intermediate breakdown products are partially substituted glycerides and fatty acids, with glycerol and fatty acids resulting from total hydrolysis of the substrate. By these reactions, lipases initiate the metabolism of fats and oils in nature.

Lipases are versatile catalysts, and can be used in a number of biotechnological applications involving the hydrolysis, synthesis, or exchange of ester bonds (Ratledge 1984, Harwood 1989, Dordick 1991). These include oleochemical hydrolysis for the production of fatty acids and glycerides (Kimura *et al.* 1983, Hills *et al.* 1990), alteration of the fatty acid content of glycerides (Yokozeki *et al.* 1982, Macrae 1983, Haraldsson and Höskuldsson 1989), synthesis of esters and related compounds (Zaks and Klibanov 1985, Langrand *et al.* 1988, Vorderwülbecke *et al.* 1992), and the enhancement of food flavors (Kilara 1985). Fungal lipases are commonly used in this vigorous area of research.

Due to the biotechnological utility of lipases, and their importance to cellular nutrition, there has been considerable investigation of the factors which regulate their production. Among those parameters influencing lipolytic activity in fungal cultures are the species and individual isolate employed (Sviridenko *et al.* 1979, Baillargeon *et al.* 1989), the complexity and availability of organic nitrogen in the media (Smith and Alford 1966, Sviridenko *et al.* 1979), culture age, extent of agitation (Alford and Smith 1965, Smith and Alford

1966, Chopra and Chander 1983), cellular location of the enzyme [intra- or extracellular] (Akhtar et al. 1974, Nakashima et al. 1988), and the presence of 'inducers'.

A number of simple organic compounds have been reported to stimulate net lipase synthesis in bacteria and fungi. These are usually identical, or related, to the substrates for these enzymes. Among these substances are triglycerides (Yoshida et al. 1968, Akhtar 1979, Sviridenko et al. 1979, Baillargeon et al. 1989, Del Rio et al. 1990), free fatty acids (Yoshida et al. 1968, Ota et al. 1968a, Nakashima et al. 1988), compounds possessing hydrolyzable ester groups (Ota, Miyairi and Yamada 1968, Ota, Suzuki and Yamada 1968), and bile salts (Ota, Suzuki and Yamada 1968). Most typically, triglycerides or fatty acids are employed as the major carbon source in growth media for lipase production.

The inclusion of lipids in growth media introduces several difficulties. Their low water solubilities can limit the availability of carbon and metabolic energy, severely retarding growth. The maintenance of a good emulsion, which optimizes the availability of the substrate during growth, can be difficult. The turbidity of such emulsions can hinder optical and spectrophotometric monitoring of the growth of the culture, and prevent the detection of bacterial contamination of fungal cultures. In addition, triglycerides and free fatty acids can be difficult to remove from crude enzyme preparations and may retard enzyme purification or the determination of lipase activity. The discovery of a soluble substrate which supports vigorous lipase production would overcome these problems, greatly facilitating the production of lipases for basic characterization and biotechnological application.

Rhizopus delemar produces both intracellular and extracellular lipases (Akhtar et al. 1974, Iwai and Tsujisaka 1974). The extracellular enzymes of this, and other species of Rhizopus, have been used in a number of applications for the modification of triglycerides and the synthesis of useful esters (Macrae 1983, Nicotra et al. 1989, Yadwad et al. 1991, Hayes and Gulari 1992). We have investigated the ability of glycerol, a water soluble product of the action of lipases on triglycerides, to support lipase production by this organism.

MATERIALS AND METHODS

Culture conditions. Rhizopus delemar (presently designated Rhizopus oryzae), ATCC 34612, was obtained from the American Type Culture Collection (Rockville, MD)¹. Progeny of individual spores were propagated for use as inocula. Spore suspensions were prepared by inoculating malt extract agar (per liter: 20 gm malt extract [Difco Laboratories, Detroit, MI], 20 gm glucose, 1 gm Bacto Peptone [Difco], 20 gm agar) and incubating at 30 C until black spores were visible. A sterile glass rod was then used to tease the spores off the mycelial pad into M9 salts solution (Maniatis et al. 1982). Spore densities of approximately 10^6 per ml were obtained in this manner.

Basal growth medium was adapted from Westergaard and Mitchell (1947) and contained 3.7 mM monobasic potassium phosphate, 0.136 mM calcium chloride, 0.86 mM sodium chloride, 1

mM magnesium sulfate, 25 mM potassium nitrate, 37 mM ammonium chloride, 0.5% (w/v) Casamino Acids (Difco), 5 ug biotin per liter, and trace elements (King 1974). After this medium was sterilized, the carbon sources under study (glucose, glycerol, triglyceride) were added as sterile stock solutions. Media (1L) in 2L Erlenmeyer flasks were inoculated with 0.1% volume of spore suspension and shaken at 150 rpm on a New Brunswick Scientific (Edison, NJ) Model G10 rotary shaker at 30 C.

Analytical methods. For the determination of extracellular lipase activities and protein concentrations, 5 ml samples were withdrawn from growing cultures, filtered through glass wool and frozen. Lipolytic activity was determined with a continuous titrating pH-stat method using 0.1 N NaOH as titrant (Junge 1984). A unit (U) of activity releases one μ mole of free fatty acid from emulsified olive oil per min at 26 C, pH 7.5. Protein concentrations were determined with the Bio Rad protein assay (Bio-Rad Laboratories, Richmond, CA). Lipase specific activities are expressed as units of lipolytic activity per μ g of extracellular protein.

Glycerol was measured via a spectrophotometric assay based on the glycerol-dependent consumption of NADH by lactate dehydrogenase in the presence of glycerokinase, pyruvate kinase, phosphoenolpyruvate and adenosine triphosphate (Sigma Chemical Co., St. Louis, MO, cat. no. 334-B). Glucose were determined by monitoring the glucose-dependent oxidation of o-dianisidine by peroxidase in the presence of glucose oxidase (Sigma, cat. no. 510-DA).

The ability of an organic compound to support microbial growth, and possibly lipase production as well, is a function of the amount of carbon in that molecule. Therefore it could be

misleading to express the concentrations of molecules of different sizes on the basis of their molar concentrations. Concentrations will be expressed here in terms of the amount of "glycerol equivalents" of carbon provided. Thus, a 1M glycerol solution is one molar in glycerol equivalents while a 1 M glucose solution is two molar in glycerol equivalents.

Determination of lipase stability *R. delemar* was grown for 30 h in glycerol media as described above. Aliquots (10 mL) were adjusted to desired pH values with 0.1 N HCl or 0.1 N NaOH and incubated at room temperature for 1 h. Lipase activities were then determined titrimetrically as described above. Enzyme activities were expressed relative to those of control samples whose pH had not been adjusted, after correction for the dilution which occurred during pH adjustment.

Immunological methods. The *R. delemar* extracellular lipase was purified to homogeneity according to the method of Haas *et al.* (1992). Polyclonal antibodies to this enzyme were elicited in rabbits. Samples of cell-free *R. delemar* growth media to be examined for the presence of lipase cross-reacting material were subjected to polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (Laemmli, 1970). The resulting separated proteins were transferred to 0.45 μ m nitrocellulose membranes (Schleicher and Schuell, Keene, NH) using a Bio-Rad Laboratories Trans-Blot according to the manufacturers' directions. The method of Winston *et al.* (1987) was then used to detect lipase immunologically: The membranes were probed with rabbit anti-lipase and subsequently treated with goat anti-rabbit antibodies and alkaline phosphatase-conjugated *Staphylococcus aureus* Protein A (Sigma Chemical Co.). Sites of immunological cross-reaction were visualized by incubating the

membranes with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma).

RESULTS

Initial experiments indicated that under the conditions employed here maximum growth and lipase activity were obtained at a glycerol concentration of 30 mM (data not shown). Therefore, this concentration was employed in subsequent experiments. Glucose was added to growth media to a concentration of 15mM (30 mM glycerol equivalents).

The time courses of lipase and protein production were determined during growth in glycerol- and glucose-fortified media, both carbon sources being present at concentrations of 30 mM glycerol equivalents. Protein accumulated in both media throughout the course of incubation, reaching between 25 and 45 µg/mL by the end of the incubation period (data not shown). Extracellular protein concentrations in glycerol exceeded those in glucose by about 1.5 fold throughout the experiment.

Figure 1 illustrates lipase production profiles typical of those observed in over 20 independent experiments. During growth on glycerol there was a relatively early initiation of net lipase synthesis, with enzyme activity becoming detectable about 15 hours (h) after inoculation (Fig. 1). At this time the cultures were young, with the foci of fungal mycelia being small but visible. Lipase activity rose very rapidly thereafter in glycerol, reached a maximum of approximately 9 U/ml about 30 h after inoculation, and remained stable for about the next 60 h. After this broad plateau the lipase levels diminished, consistent with the visual observation that the cultures were quite senescent at these advanced ages.

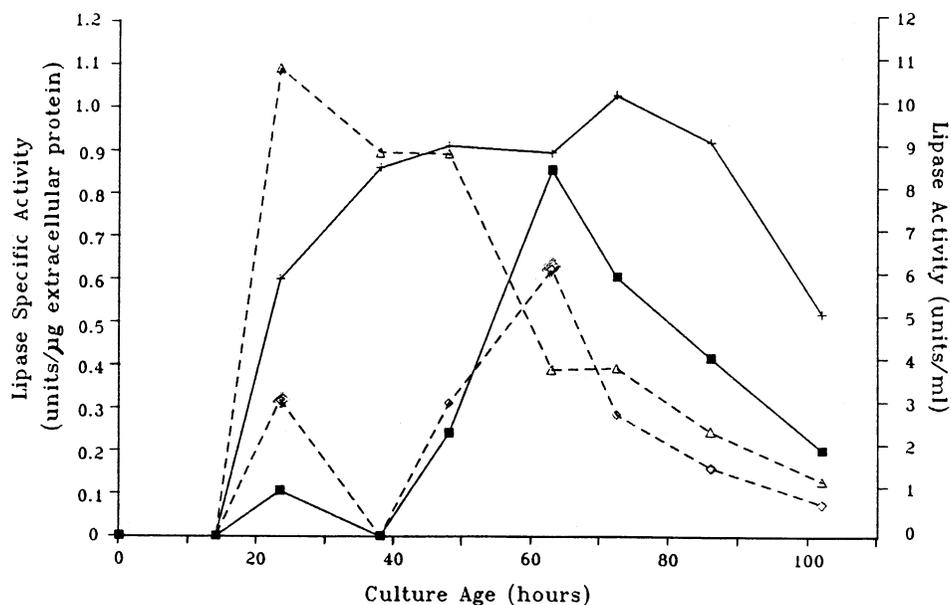


FIGURE 1

The time course of lipase production in media containing 30 mM glycerol equivalents of glycerol or glucose. Data shown are the averages of duplicate determinations. Symbols: +, total activity, glycerol media; ■, total activity, glucose media; Δ, specific activity, glycerol media; ◊, specific activity, glucose media.

In glucose media, only small amounts of lipolytic activity were detected until nearly 48 h after inoculation. Enzyme activity then rose rapidly to a sharp peak at approximately 60 h of growth and declined quickly. Total lipolytic activity in glycerol exceeded that seen in glucose media at all times during growth.

The lipase specific activity in glycerol also equaled or exceeded that obtained in glucose at nearly all times during growth (Fig. 1). The profiles of the lipase specific activities

in glycerol and glucose media were quite different (Fig. 1). The peak specific activity achieved in glycerol was nearly twice that obtained in glucose, and this peak was achieved early during growth, after only about 20 to 30 h of incubation. At this point, the gross activity had reached about two thirds of its maximum value (Fig. 1). Most striking is the great difference between the lipase specific activities in the two media between 20 and 50 h of growth: in glycerol the specific activity is maximal throughout this period, in glucose it remains low or at baseline.

The efficiency of 30 mM glycerol as a substrate for lipase production was compared with that of olive oil, a carbon source frequently used in lipase production (Fig. 2). Two olive oil concentrations were examined: 1% (v/v), a value typical of those used in media for lipase production, and 0.153% (v/v), which provides approximately 30 mM glycerol equivalents of carbon to the culture. Extracellular protein accumulated at similar rates in all three media during the first 70 h of growth (data not shown), at which time the protein concentration in the high oil medium plateaued at 25 to 30 $\mu\text{g}/\text{mL}$. Protein continued to accumulate in the other two media, reaching concentrations between 35 and 45 $\mu\text{g}/\text{mL}$ by the end of the experiment.

Extracellular lipase accumulated in all three media (Fig. 2A). At the lower olive oil concentration, total lipase activity increased at a nearly constant rate throughout the first 100 h of incubation, reaching a peak of 12.1 U/mL. In the high oil medium lipase activity rose steadily to 4 U/mL during the first 40 h of growth, then stabilized and rose no higher during further incubation. In the glycerol medium, lipase levels rose to a peak value of 10.6 U/mL 60 h after inoculation

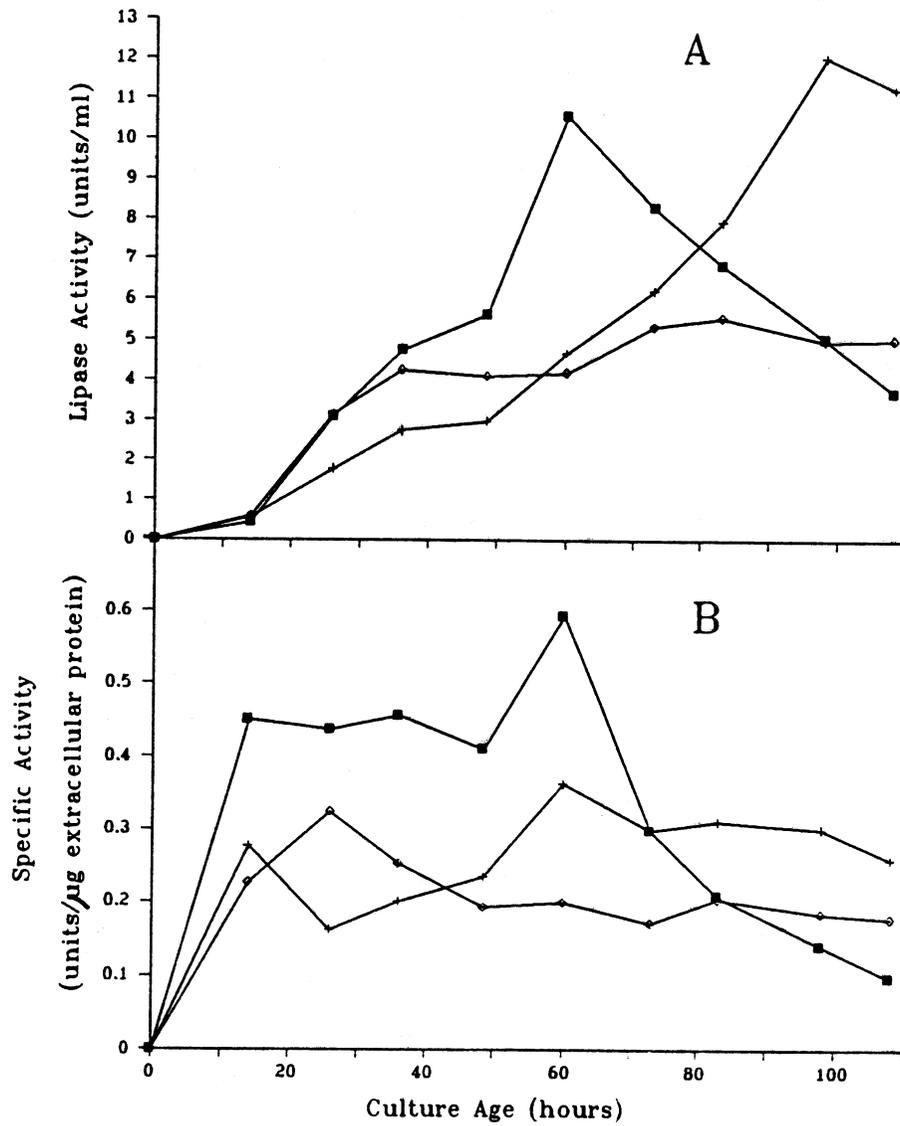


FIGURE 2

Time course of (A) net and (B) specific lipase activities during growth in media containing 30 mM glycerol (■), 0.153% olive oil (+) or 1% olive oil (◇). Data shown are the averages of duplicate determinations.

and then declined steadily. Throughout the first 80 h the net lipase levels in glycerol equaled or exceeded those in olive oil, at times by nearly 3 fold.

Lipase specific activities displayed similar profiles in the two olive oil media, reaching constant values within 18 h after inoculation (Fig. 2B). Lipase specific activity also rose to a rapid plateau in glycerol medium, remaining there until 60 h of growth, and declining thereafter. The greatest specific activity was obtained in glycerol, exceeding that in oil media by as much as 3 fold throughout the first 70 h of incubation (Fig. 2B). The data in Figure 2 demonstrate the superiority of glycerol over olive oil as a substrate for lipase production.

The pHs and the concentrations of glycerol and glucose were determined in samples taken at various times after inoculation. During the first 15 h of incubation there was little change in the concentration of glycerol in the media (Fig. 3). During this time little mycelial mass could be seen in the cultures. Subsequently, mycelial mass became clearly present and visibly increased over time. Glycerol levels fell rapidly between 20 and 40 h after inoculation. This period, especially between 15 and 30 h after inoculation, was also the time during which lipase levels rose rapidly in the cultures (Fig. 1). By 60 h after inoculation, glycerol was essentially depleted (Fig. 3). These data indicate that, in glycerol media, lipase production began soon after inoculation.

When glucose was the carbon source, it was very rapidly removed by metabolism, especially between 15 and 20 h after inoculation (Fig. 3). After 30 h of incubation no glucose could be detected (Fig. 3). Significant lipolytic activity did not appear until more than 40 hr after inoculation (Fig. 1), by which time the glucose had been depleted.

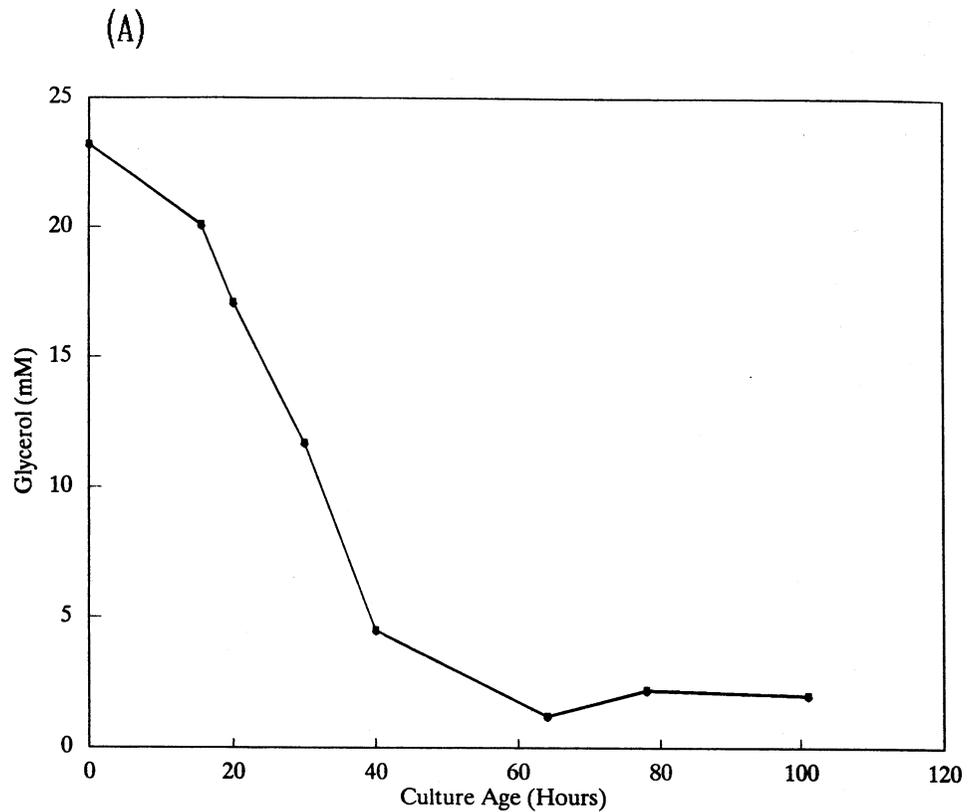


FIGURE 3

The concentrations of glycerol (\square) and glucose (\blacklozenge) in their respective media during growth of *R. delemar*. Data are the averages of three independent determinations.

The data on lipase levels presented above was based upon measurement of enzymatic activity. One potential explanation for the differences between the activities in glucose and glycerol is that the pHs of the cultures differ, due to the metabolism of the two different carbon sources, and that the stability of the lipase is pH dependent and differs between the cultures as a result of the pH differences. To test this theory, the pH stability of the lipase was determined (Fig. 4).

(B)

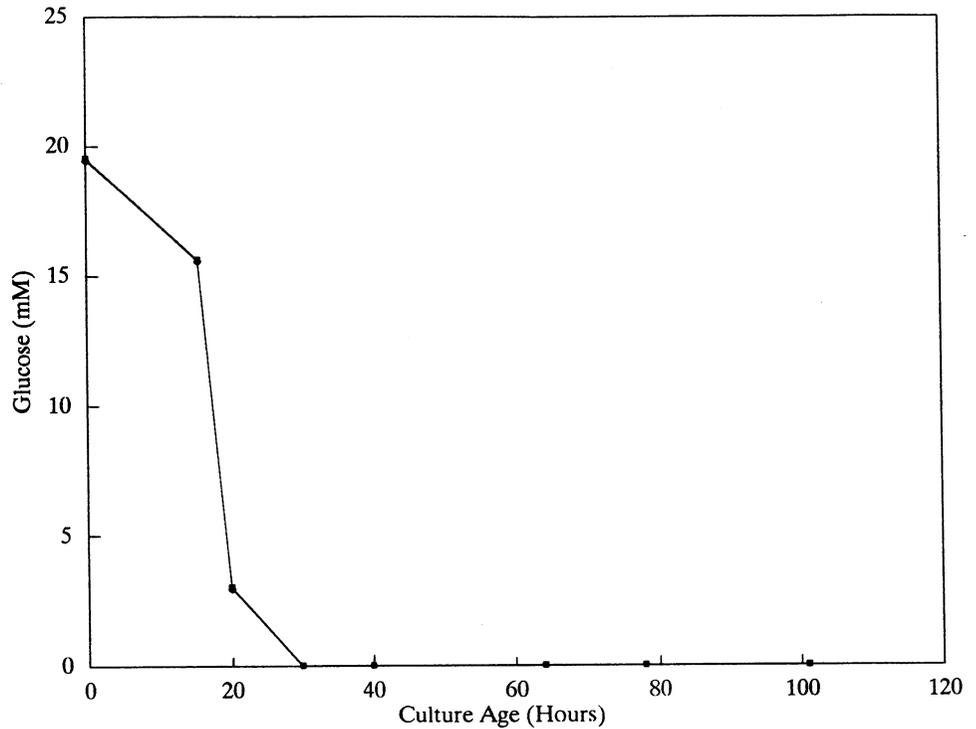


FIGURE 3 Continued

The enzyme was relatively stable. Seventy per cent or more of the activity was retained after exposure to pHs as low as 4 (Fig. 4). Even after incubation at pH 3 the enzyme retained 30% of its activity (Fig. 4).

Figure 5 presents the pHs of *R. delemar* cultures at various times during growth on glycerol or glucose. In glycerol media the pH rose steadily from an initial value of 6 to a plateau of about 8, which was attained about 80 hours after inoculation (Fig. 5). Thus, throughout the incubation, the pH of the glycerol culture was within the range wherein lipase is stable.

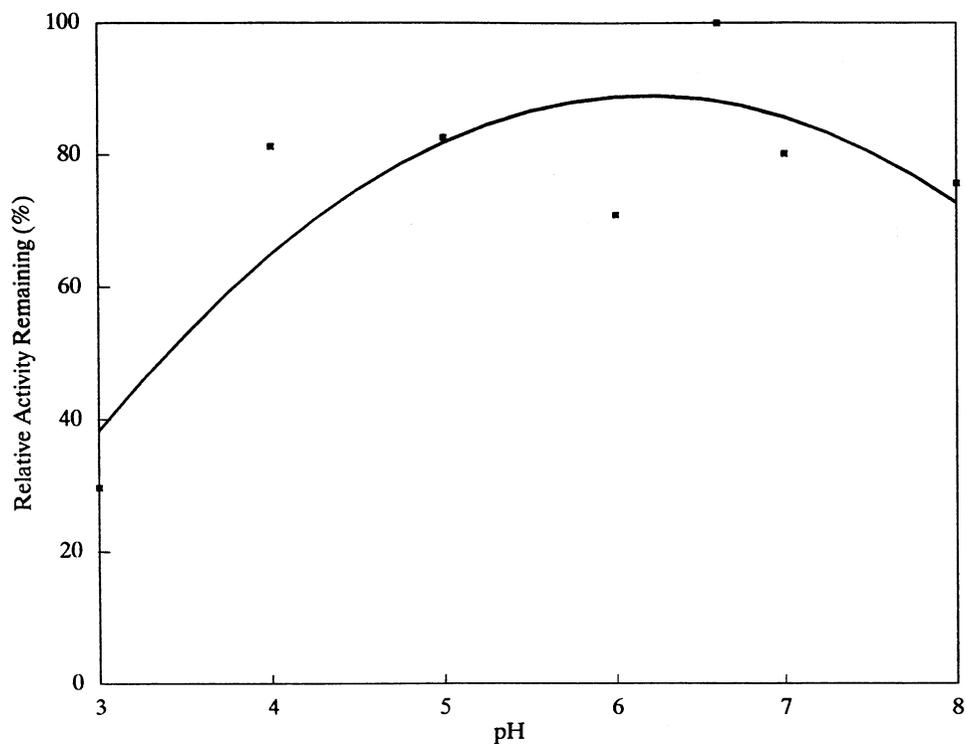


FIGURE 4

Lipolytic activity retained by unpurified *R. delemar* lipase following one hour incubation at the indicated pH values, re-adjustment to their original pH (6.6), and overnight incubation at room temperature. Data are expressed relative to a sample of enzyme whose pH was not adjusted, and are the averages of duplicate determinations.

The pH of glucose media fell during the first 20 h of growth, from an initial value of 6 to a final pH slightly below 4 (Fig. 5). The pH then rose steadily, reattaining a value of 6 at about 55 h (Fig. 5). The trough in the pH curve corresponds roughly to the point of depletion of glucose (Fig. 3). The subsequent increase in pH probably corresponds to a metabolic

shift by the culture, most likely to metabolism of the amino acids in the media and the release of their nitrogen as ammonium ions. Although cultures growing on glucose became acidic, the pH stayed within the region where lipase retains substantial activity (Fig. 4). Therefore, it is unlikely that the absence of lipolytic activity in glucose during the first 40 h of incubation (Fig. 1) is due to the synthesis, and pH-mediated inactivation, of amounts of lipase comparable to those synthesized in glycerol.

Immunoblotting techniques were employed to determine the kinetics of synthesis of the lipase polypeptide during growth in glycerol and glucose. The lipase consists of a single polypeptide with a molecular mass of 30,300 (30.3 kDal) (Haas et al. 1992). In immunoblots of glycerol-grown cultures, a cross-reacting protein with the electrophoretic mobility of lipase was present at least as early as 15 h after inoculation (Fig. 6A). Over the next 6 h the intensity of this band increased greatly, indicating vigorous synthesis. The band remained prominent for the duration of incubation, and was still intense after 100 h of incubation (Fig. 6A). The time of appearance and changes in the intensity of this band correspond to the kinetics of appearance of lipolytic activity in glycerol cultures (Fig. 1). Thus, during growth on glycerol, the synthesis of active lipase began soon after inoculation, with the appearance of the lipase protein paralleling the appearance of lipolytic activity. The enzyme was present and active throughout incubation.

A protein of slightly larger molecular mass, 34.5 kDal, which also cross-reacted with lipase antibodies was present in cultures grown in glycerol (Figure 6A). This protein predominated in young cultures (15 h) and was gradually reduced

(A)

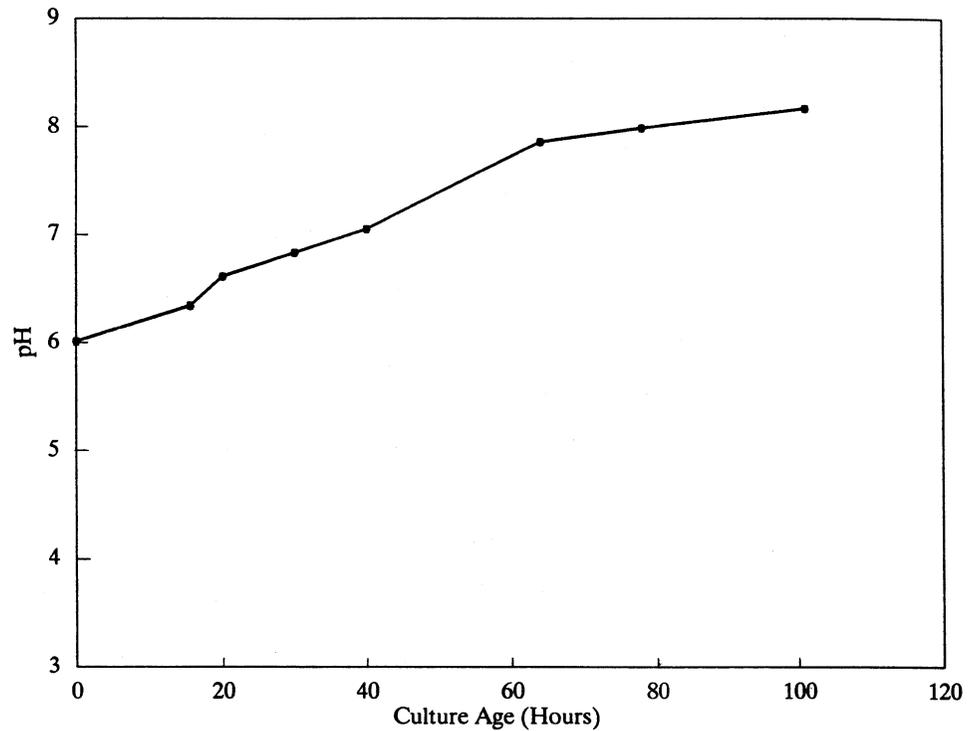


FIGURE 5

The pHs of *R. delemar* cultures as a function of time during growth on either glycerol (\square) or glucose (\blacklozenge) as the primary carbon source. Data are the averages of three independent determinations.

in amount as incubation proceeded. The absence of detectable lipolytic activity 15 h after inoculation (Fig. 1) indicates that this protein is not an active lipase. Preliminary studies in this laboratory suggest that it may be a lipase precursor which is converted over time to the 30.3 kDal 'mature' lipase.

On immunoblots of *R. delemar* cultures growing on glucose, a cross reacting protein band with the mobility of mature lipase

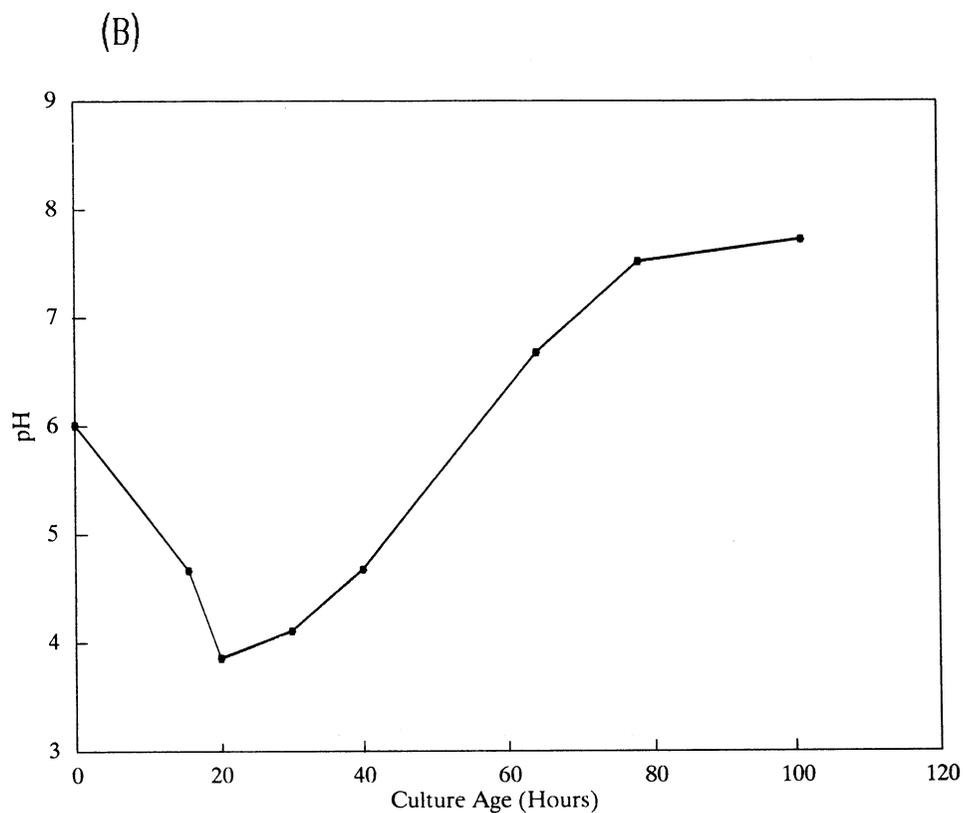


FIGURE 5 Continued

did not appear in significant amounts until at least 40 h after inoculation (Fig. 6B). Its appearance corresponded to the time when substantial amounts of lipolytic activity were detected in these glycerol cultures (Fig. 1). Thus, during growth in glucose, the appearance of activity parallels the appearance of the lipase polypeptide. This indicates that the low lipase levels in glucose grown cultures during the first 40 to 50 h of growth are the result of a failure to synthesize mature lipase, and are not due to inactivation of preexisting enzyme by the acidic pH or other conditions within the culture. Whether the

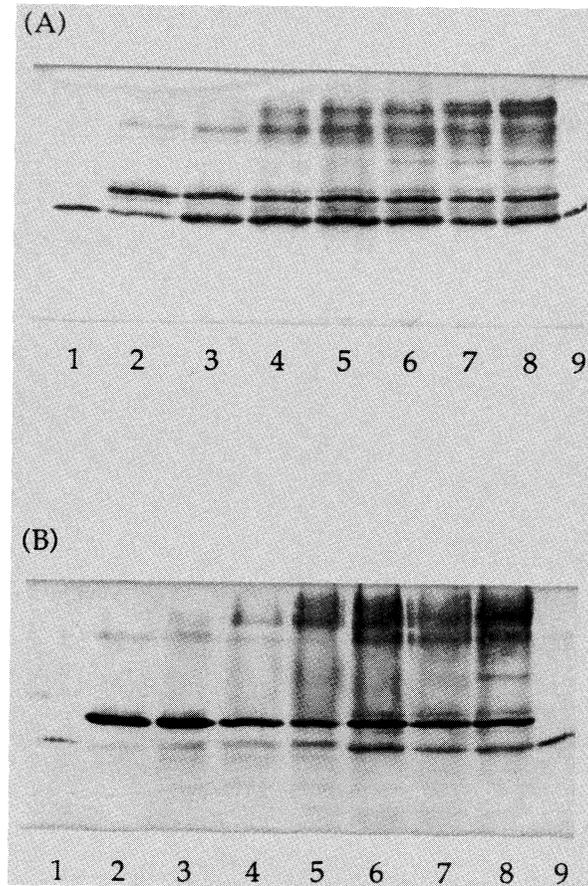


FIGURE 6

Immunologic characterization of the production of lipase polypeptide during growth of R. delemar on (A) glycerol or (B) glucose media. Four μg of cell-free growth media were subjected to electrophoresis and immunoblotting as described in Materials and Methods. Proteins with molecular masses between 14 and approximately 100 kDal can be detected using the techniques employed here. Lanes 1 and 9: 66 nanograms of pure R. delemar lipase. The times of sampling of the cultures were: lane 2: 15.5 h; lane 3: 20 h; lane 4: 30 h; lane 5: 40 h; lane 6: 64 h; lane 7: 78 h; lane 8: 101 h.

low levels of mature lipase are due to a failure to process the 34.5 kDal cross-reacting species or result from an inactivity of the lipase gene itself remains to be determined.

DISCUSSION

The production of a lipase benefits an organism only when the substrates, triglycerides, are available for metabolism. It is reasonable, therefore, that lipase synthesis would be coordinated with the availability of fats or oils. Indeed, numerous investigations have shown that lipids stimulate lipase production in a variety of fungi (Yoshida et al. 1968, Akhtar 1979, Sviridenko et al. 1979, Borgström and Brockman 1984, Espinosa et al. 1990, Del Rio 1990). It is possible that the lipids themselves are not the inducing molecule, due to their low water solubilities. A more soluble substance derived from them may be the actual inducer of high level lipase production. Glycerol is a highly soluble terminal product of glyceride hydrolysis. This investigation was undertaken to determine its capacity to stimulate lipase production.

Under the conditions employed here, glycerol is superior to other carbon sources tested as a substrate for the production of lipase by Rhizopus delemar. In terms of both total and specific lipase activities, it fosters levels generally greater than those obtained using either glucose or olive oil as carbon source. In addition, the maximum lipase level is achieved much earlier during growth on glycerol than when other carbon sources are employed. Another useful aspect of lipase production in glycerol is that enzyme activity remains stable at its peak value for a considerable period of time. Thus the recovery of maximum enzyme activity is not vitally dependent upon the time of harvest.

The data presented here are consistent with the repression of lipase production by glucose. In additional experiments we have observed that the stimulation of lipase production by glycerol is eliminated when glucose is also present in the growth medium. This observation, and the other data presented here, are consistent with catabolite repression of lipase synthesis by glucose. This phenomenon has been suggested previously as a result of investigations of fungal lipase synthesis (Baillargeon et al. 1989, Chopra and Chander 1983, Nakashima et al. 1988). To our knowledge, the data presented here are the first to monitor not only lipase activities but also the presence of the lipase polypeptide during growth.

While this work was underway, Del Rio et al. (1990) reported that lipase production by Candida rugosa was inhibited during growth on glycerol. Yoshida et al. (1968) previously reported that although triglycerides and fatty acids increased net lipase synthesis, the addition of 22 mM glycerol to a complex media had no effect on the production of this enzyme by Torulopsis ernobii. Baillargeon et al. (1989) found that in one of three strains of Geotrichum candidum examined, glycerol stimulated lipase production 1.6 fold over that obtained during growth on soybean oil. In the other two strains, lipase levels were reduced in glycerol media. In studies employing a different isolate of R. delemar and experimental conditions substantially different than those used here, Espinosa et al. (1990) found lipase levels in glycerol-grown cultures to equal those obtained on glucose. These reports, in conjunction with the data presented here, indicate that the effects of glycerol on lipase synthesis are dependent upon methodology and the organism under examination. Nonetheless, in at least some

situations, the use of glycerol provides an efficient means of producing lipase in a simple, homogenous medium, and provides insight into the mechanisms regulating fungal gene expression.

ACKNOWLEDGEMENT

Karen Kolaetis, Deborah Woolf and Bonnie Murray provided excellent technical support.

LITERATURE CITED

- Alford, J. A. and Smith, J. L. 1965. Production of microbial lipases for the study of triglyceride structure. *J. Am. Oil Chem. Soc.* 42: 1038-1040.
- Akhtar, M. W. 1979. Lipase induction in fungi. *Pak. J. Biochem.* 12: 115-124.
- Akhtar, M. W., Patterson, J. D. E. and Blain, J. A. 1974. Influence of olive oil on extracellular and cell-bound lipase production by fungi. *Pak. J. Biochem.* 7: 81-87.
- Baillargeon, M. W., Bistline, R. G., Jr. and Sonnet, P. E. 1989. Evaluation of strains of Geotrichum candidum for lipase production and fatty acid specificity. *Appl. Microbiol. Biotechnol.* 30: 92-96.
- Borgström, B. and Brockman, H. L. 1984. *Lipases*. Elsevier, New York, NY.
- Brocknerhoff, H. and Jensen, R. 1974. *Lipolytic Enzymes*. Academic Press, New York, NY.
- Chopra, A. K. and Chander, H. 1983. Factors affecting lipase production in Syncephalastrum racemosum. *J. Appl. Bacteriol.* 54: 163-169.
- Del Rio, J. L., Serra, P., Valero, F., Poch, M. and Sola, C.

1990. Reaction scheme of lipase production by Candida rugosa growing on olive oil. *Biotechnol. Lett.* 12: 835-838.
- Dordick, J. S. 1991. Principles and applications of nonaqueous enzymology. In: *Applied Biocatalysis*. Blanch, H. W. and Clark, D. S. (eds.), Marcel Dekker, Inc., New York, NY, Vol. 1, pgs. 1-51.
- Espinosa, E., Sanchez, S. and Farres, A. 1990. *Biotechnol. Lett.* 12: 209-214.
- Haas, M.J., Cichowicz, D. J. and Bailey, D. G. 1992. Purification and characterization of an extracellular lipase from the fungus Rhizopus delemar. *Lipids*. In press.
- Haraldsson G. G. and Höskuldsson P. A. 1989. The preparation of triglycerides highly enriched with ω -3 polyunsaturated fatty acids via lipase catalyzed interesterification. *Tetrahedron Lett.* 30: 1671-1674.
- Harwood, J. 1989. The versatility of lipases for industrial uses. *Trends Biochem. Sci.* 14: 125-126.
- Hayes, D. G. and Gulari, E. 1992. Formation of polyol-fatty acid esters by lipases in reverse micellar media. *Biotechnol. Bioeng.* 40: 110-118.
- Hills, M. J., Kiewitt, I. and Mukherjee, K. D. 1990. Enzymatic fractionation of fatty acids: enrichment of γ -linolenic acid and docosahexaenoic acid by selective esterification catalyzed by lipases. *J. Am. Oil Chem. Soc.* 67: 561-564.
- Iwai, M. and Y. Tsujisaka, 1974. The purification and the properties of three kinds of lipases from Rhizopus delemar. *Agr. Biol. Chem.* 38:1241-1247.
- Junge, W. 1984. Lipases. In: *Methods of Enzymatic Analysis*, Bergmeyer, J. and Graßl, M. (Eds.) Verlag Chemie, Deerfield Beach, FL, Vol. IV, pp. 15-25.

- Kilara, A. 1985. Enzyme-modified lipid food ingredients. *Process Biochem.* 20: 35-45.
- Kimura, Y., Tanaka, A., Sonomoto, K., Nihira, T. and Fukui, S. 1983. Application of immobilized lipase to hydrolysis of triacylglyceride. *Eur. J. Appl. Microbiol. Biotechnol.* 17: 107-112.
- King, R. C., (Ed.). 1974. *Handbook of Genetics*. Plenum Press, New York, NY. Vol. 1., p. 432.
- Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature.* 227: 680-685.
- Langrand, G., Triantaphylides, C. and Baratti, J. 1988. Lipase catalyzed formation of flavor esters. *Biotechnol. Lett.* 10: 549-554.
- Macrae, A.R. 1983. Lipase catalyzed interesterification of oils and fats. *J. Am. Oil Chem. Soc.* 60: 291-294.
- Maniatis, T., Fritsch, E. and Sambrook, J. 1982. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY. p. 440.
- Nakashima, T., Fukuda, H., Kyotani, S. and Morikawa, H. 1988. Culture conditions for intracellular lipase production by Rhizopus chinensis and its immobilization within biomass support particles. *J. Ferment. Technol.* 66: 441-448.
- Nicotra, R., Riva, S., Secundo, F. and Zucchelli, L. 1989. An interesting example of complementary regio selective acylation of secondary hydroxyl groups by different lipases. *Tetrahedron Lett.* 30: 1703-1704.
- Ota, Y., Miyairi, S. and Yamada, K. 1968. Sterol requirement for the lipase production by Candida cylindracea. *Agr. Biol. Chem.* 32: 1476-1478.

- Ota, Y., Suzuki, M. and Yamada, K. 1968. Lipids and related substances inducing the lipase production by Candida paralipolytica. Agr. Biol. Chem. 32: 390-391.
- Ratledge, C. 1984. Biotechnology as applied to the oils and fats industry. Fette Seifen Anstrichm. 86: 379-389.
- Smith, J. L. and Alford, J. A. 1966. Inhibition of microbial lipases by fatty acids. Appl. Microbiol. 14: 699-705.
- Sviridenko, Y. Y., Lobyreva, L. B., Marchenkova, A. I., Ruban, E. L. and Umanskii, M. S. 1979. Influence of the composition of the medium on the biosynthesis and properties of exolipases of microorganisms. Prikl. Biochim. Mikrobiol. 14: 677-682.
- Voderwulbecke, T., Kieslich, L. and Erdmann, H. 1992. Comparison of lipases by different assays. Enzyme Microb. Technol. 14: 631-639.
- Westergaard, M. and Mitchell, H. K. 1947. Neurospora V. A synthetic medium favoring sexual reproduction. Am. J. Bot. 34: 573-577.
- Winston, S., Fuller, A. S. and Hurrell, F. G. R. 1987. Western blotting. In: Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. and Struhl, K. (Eds.). Current Protocols in Molecular Biology. John Wiley, New York, NY. Vol. 2. pp. 10.8.1-10.8.6.
- Yadwad, V. B., Ward, O. P. and Noronha, L. C. 1991. Application of lipase to concentrate the docosahexaenoic acid (DHA) fraction of fish oil. Biotechnol. Bioengr. 38: 956-959.
- Yokozeiki, K., Yamanaka, S., Takinami, K., Hirose, Y., Tanaka, A., Sonomoto, K. and Fukui, S. 1982. Application of immobilized lipase to regio-specific interesterification of triglyceride in organic solvent. Eur. J. Appl. Microbiol. Biotechnol. 14: 1-5.

- Yoshida, F., Motai, H. and Ichishima, E. 1968. Effect of lipid materials on the production of lipase by Torulopsis ernobii. Appl. Microbiol. 16: 845-847.
- Zaks, A. and Klivanov, A. M. 1985. Enzyme-catalyzed processes in organic solvents. Proc. Natl. Acad. Sci. USA 82: 3192-3196.