BIOTECHNOLOGY LETTERS
Volume 18 No.6 (June 1996) p.633-638
Received as revised 29th March.

EFFECT OF NITROGEN SOURCES ON THE REGULATION OF EXTRACELLULAR LIPASE PRODUCTION IN *Acinetobacter calcoaceticus* STRAINS

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SUMMARY

Extracellular lipase production and *lipA* expression were studied in *A.calcoaceticus* strains in relation to the nitrogen sources. Addition of casamino-acids or tryptone improved 2-3 times lipase yield as compared to ammonium, yeast extract or proteose-peptone. Further increase in lipase yield and enzyme stability were achieved when ammonium was also added. Differences in yields were not related to *lipA* transcription. Proteolytic activity was detected in culture broths simultaneous with lipase degradation. Post- transcriptional processes including enzyme protection, inactivation and secretion must be considered as important factors affecting lipase production.

INTRODUCTION

The development of technologies using lipases for the synthesis of novel compounds has extended the field for new applications of this enzyme to the food, detergent, chemical and pharmaceutical industries.
As a consequence of this, a steadily increasing number of bacterial lipase genes have been cloned and sequenced, and the corresponding proteins were purified and characterized (for a review, see Jaeger *et al.*, 1994).
Recently, the lipase of *Acinetobacter calcoaceticus* BD413 (Juni and Janik, 1969) has been cloned and characterized (Kok *et al*, 1995a). The enzyme showed structural and functional similarities to *Pseudomonas* lipases, including the requirement of a specific chaperone for translocation across the outer membrane (Kok *et al* ., 1995b).
Several environmental factors have been described to regulate the synthesis and release of lipases in many bacterial strains (Jaeger *et al.*, 1994; Marcin *et al.*, 1993), but this knowledge is still scarce for the *A. calcoaceticus* BD413 lipase.
In this paper, the effect of several nitrogen sources on *lipA* expression and lipase production is presented, as well as evidences on the simultaneous excretion of protease/s, which may also contribute to regulate lipase yields.

MATERIALS AND METHODS

STRAINS: 1- *Acinetobacter calcoaceticus BD413*, a mini-encapsulated mutant secreting lipase and esterase/s (Juni and Janik,1969). 2- *A. calcoaceticus* AAC320-1, a transcriptional lipA::lacZ fusion, generated by the insertion of a promoterless *lac Z* cassette under control of the lipA -promoter into the chromosome of *A.calcoaceticus BD413* (Kok, 1995). This strain has the phenotype lipA- lacZ+.

MEDIA AND CULTURE CONDITIONS: The bacterial strains were cultivated aerobically at 30°C in Erlenmeyer flasks containing 30 ml of medium. Base medium consisted of 0.81 mM $MgSO_4$, 68 μM $CaCl_2$, 11 mM KH_2PO_4, 95 mM Na_2HPO_4, 1.8 μM $FeSO_4$, and -per liter- 1 ml of a spore-solution containing 50 g EDTA, 2.2 g $ZnSO_4$. $7H_2O$, 5 g $FeSO_4.7H_2O$, 1.6 g $CuSO_4.5H_2O$, 5 g $MnCl_2.4H_2O$, 1.1 g $(NH4)_6Mo_7O_{24}$. $4H_2O$, 50 mg H_3BO_3, 10 mg KI , 50 mg $CoCl_2.6H_2O$. This solution was set at pH=6.75 and autoclaved at 120 °C for 20 min. After sterilization, a filter-sterilized glucose solution was added (0.22μm Millipore) up to 1% final concentration, as well as the different nitrogen sources. Nitrogen sources were prepared and autoclaved separately (120°C, 15 min) and added in the following concentrations: 37 mM NH_4Cl; 1% Tryptone (Oxoid); 1.65% Casamino-acids (DIFCO) +0.1mg/L Trp +0.1mg/L Cys; 1% Proteose-peptone (Difco); 1% Yeast extract (Merck). Ammonium, Tryptone and Casamino-acids media are further indicated as A, T and CA, respectively.

An early stationary phase culture of the strains, grown in the A medium, was used to inoculate the flasks. Initial Optical Density at 580 nm ($O.D._{580}$ nm) was around 0.1. Cultures were incubated on a rotatory shaker at 250 rpm, and 1 ml samples were collected and analyzed for lipase or β-galactosidase.

For the detection of proteolytic activity, culture supernatants were filtrated (0.22μm Millipore filter),concentrated by lyophilization, reconstituted in water and further de-salted using Sephadex PD10 micro-columns. The eluates were again liophylized and reconstituted in buffer 10mM Tris-ClH + 2mM $CaCl_2$ + 2mM $MgCl_2$ (pH=7.5). The concentration factor was 50x

ANALYTICAL METHODS

Cell growth was monitored turbidometrically at 580 nm (OD_{580}).

Extracellular lipase was measured in culture supernatants of the strain *A. calcoaceticus* BD413, using p-nitrophenyl palmitate (p-NPP, Sigma Chem Co) as the substrate (Kok et al., 1993). One unit (U) of enzyme activity is defined as the amount of enzyme forming 1μmol p-nitrophenol per min. Volumetric lipase production was calculated per litre culture (U/L) and divided by the Optical Density of the culture sample, in order to obtain the specific lipase activity (U/L.OD).

Production of β-galactosidase activity was measured in the strain *A. calcoaceticus* AAC 320-1. This was done essentially according to Miller, 1972, using o-nitrophenyl β-D-galactopyranoside (oNPG) as the substrate. Cells were harvested from the culture medium, centrifuged and washed twice in Buffer 1 and resuspended in a known volume of the same buffer, upon which the $O.D._{580}$ nm value was recorded and the samples were frozen until further use. Prior to the β-galactosidase assay, cells were thawed, diluted appropriately to reach an O.D. under 3.0 (corresponding to 0.5 g/L biomass), pelleted via centrifugation and resuspended in **Buffer Z** containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.001 % SDS and 50 mM β-mercaptoethanol.

Permeabilization of cells was routinely performed in 1 ml samples in Z-buffer, by adding 100 μl ethanol and 20 μl toluene, and incubating the samples at 37° C for 20 min. 50 μl of permeabilized cell suspension was added to 2 ml of pre-warmed (37°C) Z-buffer (without β-mercaptoethanol) containing 0.4 mg/ml oNPG. Further incubation followed at 37°C. Reactions were stopped by addition of 0.85 ml 1 M Na2CO3. After 10 min, the absorbance was measured at 420 nm and 580 nm. Specific β-galactosidase activity in the samples was calculated according to Miller and is given in Miller Units (M.U). All reactions were analyzed in duplicate samples from two or three independent cultures.

SDS- gelatin-polyacrylamide gel electrophoresis was done according to Heussen and Dowdle, 1980. Gels were incubated during 15 hours at 30^0C in Buffer 250 mM Tris-ClH pH=6.8 prior to staining. Absence of cross-contamination with intracellular enzymes was checked by assaying isocitrate dehydrogenase activity (Sigma Diagnostic kit)

STATISTICAL ANALYSIS: Lipase yields were analyzed by ANOVA and F test (significance level (α) = 0.1)

RESULTS AND DISCUSSION

Extracellular lipase production by *A. calcoaceticus* strains has been characterized as a late-growth-phase event: in complex medium containing Nutrient Broth (NB,GIBCO) as sole carbon and nitrogen source, enzyme activity reached a maximum at the moment of transition to the stationary phase, and then decreased sharply to the levels observed during exponential growth (Kok *et al.*, 1993).

This time-dependent expression, and further decay, has been the subject of these studies. More precisely, the influence of components of culture media, preferently nitrogen sources, in this process.

Switching from NB to a mineral base medium containing glucose as sole carbon source and several mineral or complex nitrogen sources (see composition in M&M) did not change the growth-phase pattern, although the amounts of biomass and lipase produced varied significantly. Due to the differences in growth, specific lipase yields were calculated (in U/L.OD) and plotted, as shown in fig. 1.

In relation to lipase yields, nitrogen sources could be divided in two groups: one, yielding a low response, consisted of ammonium (A), yeast extract and proteose-peptone,. The other group, producing high lipase yields, consisted of casamino-acids (CA) and tryptone (T). Differences between both groups were significant while differences between T and CA were not (α = 0.1). From this experiment we concluded that, in principle, feeding with amino acids and relatively small peptides (as present in tryptone) favoured lipase production, in contrast to mineral nitrogen (ammonium) or large peptides and proteins, as present in proteose-peptone and yeast extract.

Moreover, addition of Ammonium to CA or T further increased lipase yields by a factor of two, as it is shown in Table 1. Also maintenance of lipase in culture broths was improved, as shown during prolonged incubation (fig.2).

TABLE 1: EFFECT OF NITROGEN SOURCES ON EXTRACELLULAR LIPASE YIELDS

	NITROGEN SOURCE	SPECIFIC LIPASE YIELD (U/L.OD)	INCREASE IN YIELD (%)*
SOLE NITROGEN SOURCE	ammonium	27 ± 1	100
	proteose-peptone	28 ± 2	104
	yeast extract	25 ± 2	90
	casamino-acids (CA)	53 ± 7	196
	tryptone (T)	78 ± 9	289
COMBINED NITROGEN SOURCE	ammonium+ CA	94± 10	348
	ammonium+CA+dipeptide	113 ± 6	418
	ammonium+CA+pentapeptide	120 ± 9	444
	ammonium+ T	164 ± 20	607

* calculated as the ratio between specific lipase yield in each medium relative to lipase specific yield in ammonium medium, and expressed as percentage.

In order to test whether the large increase in lipase yields (ca. 6 times) in medium containing A+T in relation to sole ammonium could be due to increase

transcription of the *lipA* gene (encoding the extracellular lipase of *A.calcoaceticus BD413),* the transcriptional fusion AAC 320-1 was used. *A. calcoaceticus* AAC320-1 was cultured in medium containing A or A+T, and β-galactosidase activity was recorded during the time course of the experiment.
As shown in fig.3, no significant differences in β-galactosidase patterns were observed between both culture conditions, indicating that the higher lipase yields obtained with the simultaneous addition of both nitrogen sources could not be attributed to transcriptional activation of the *lipA* gene.
Another important observation during this experiment was that, although lipase activity always fell (more or less sharply) during stationary phase, β-galactosidase activity remained constant, at maximum level, far beyond reaching stationary phase, indicating that the promoter has not been inmediately switched-off during this transition.
As displayed in Table 1, differences in lipase yield between A+ CA and A+T were significant (α=0.1). Again, no significative differences in β-galactosidase activities were measured between cells corresponding to both culture conditions (data not shown) indicating that the effect was not due to increased transcriptional activity.
Peptides may partially contribute to improve lipase yields, as shown by addition of two different peptides (in size and composition) to A+CA medium (Table1).
On the other hand, differences in free or peptidic amino-acids consumption during the time course of the experiments between both media (A+CA and A+T) were not observed, as calculated by amino-acid analysis of free and hydrolized samples (data not shown).
All toghether, these results suggest that post-transcriptional processes, including modification in secretion rate and/or enzyme inactivation/ protection, could be important factors regulating lipase production (see below).
Inactivation of lipase in culture broth was further investigated in concentrated supernatants, corresponding to 8, 10, 12, 15 and 17 hours culture, using copolymerized acrylamide-gelatin gel electrophoresis. As shown in fig.4, a time-dependent proteolytic activity could be detected, reaching its maximum at 17 hours culture and accompanying lipase inactivation.
Simultaneous production of lipase and protease have been reported in *Serratia* and *Pseudomonas* strains (Henriette *et al.*, 1993; Guillou *et al.*, 1995) but not described previously in *Acinetobacter,* except for some evidences suggesting proteolytic inactivation, i.e:
- increased stability of lipase in culture broths after the addition of the protease inhibitor PMSF (Bompensieri *et al.*, 1996)
- lipase degradation products detected in stationary phase supernatants using specific lipase antibodies (Kok *et al.*, 1993).
To our knowledge, there have been only one periplasmic (insulin)-peptidase from *A. calcoaceticus* which was isolated and purified (Fricke and Aurich, 1992).
Provided that a proteolytic activity has been demostrated, and nitrogen sources are important factors regulating this activity (Nigam *et al.*, 1981; McKellar, 1982; Rowe and Gilmour, 1983; Fairbairn and Law, 1986) the effect of the nitrogen sources assayed can be explained as follows:
- Large peptides and proteins stimulate early protease expression (and lipase degradation), while amino-acids, either alone or in addition to small peptides, repress protease activity (and therefore protect lipase).
- The addition of ammonium to CA or T further delays proteolytic expression.

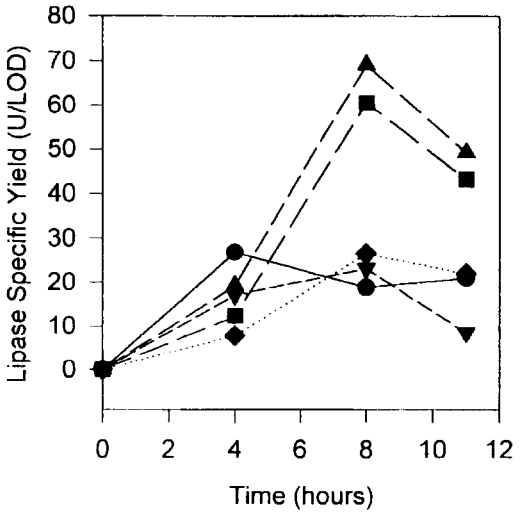

Fig. 1: lipase specific yields in medium containing different nitrogen sources: ● ammonium; ■ casamino-acids; ▲ tryptone; ▼ proteose; ◆ yeast extract

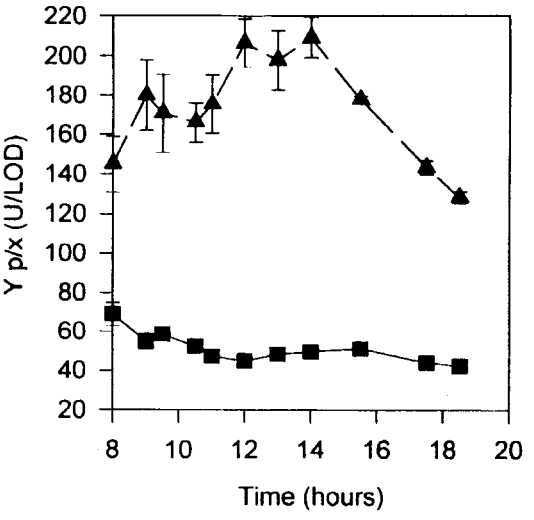

Fig. 2: Lipase specific yield in tryptone ■ and tryptone + ammonium ▲ containing medium up to 20 hours culture.

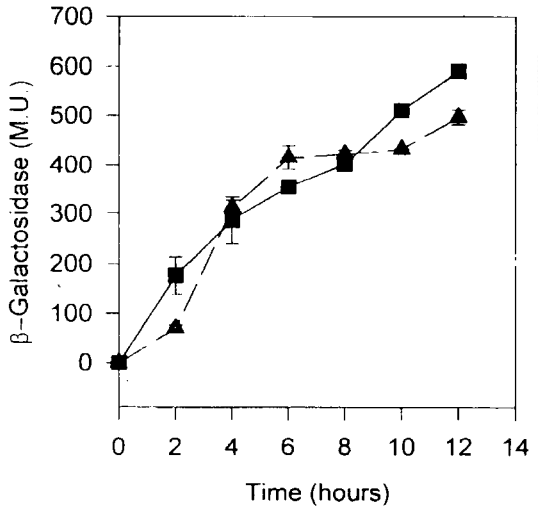

Fig. 3. β-Galactosidase activity in medium containing different nitrogen sources. Symbols: ■ ammonium; ▲ ammonium + tryptone 1%.

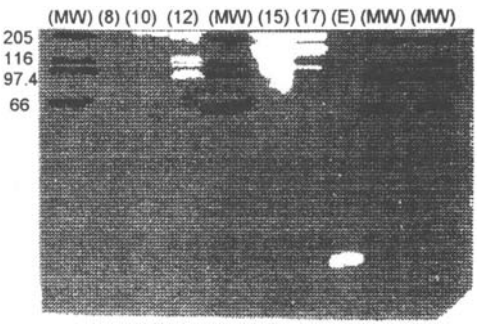

Fig. 4: Detection of proteolytic activity. Supernatants corresponding to 8, 10, 12 15 and 17 hours culture are indicated. (MW), molecular weight markers; Elastase, (E) was used as protease positive control.

In addition, protection of microbial lipase inactivation by some amino-acids and proteins is very likely (Naka and Nakamura, 1992), indicating that selected nitrogen sources may themselves contribute to increase the stability of lipase in culture broths.
More recently, the importance of the lipase secretion process in extracellular lipase production has been recognized, by the identification of specific lipase chaperones, essential for enzyme traslocation, both in *Pseudomonas* and *Acinetobacter* strains (Itzumi *et al.*, 1991; Kok *et al.*, 1995b), although it is not yet known how these proteins are regulated.
In conclusion, our results demonstrate that nitrogen sources, by regulating some post-transcriptional processes, in this case proteolysis, enzyme protection and inactivation, strongly influence extracellular lipase production in *A. calcoaceticus* strains.

Aknowledgments: We are very grateful to Daniel Guebel for helpful comments. This study was financially supported by a grant from the EC.

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