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Effects of carbon sources on extracellular lipase production and *lip*A transcription in *Acinetobacter calcoaceticus*

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The effects of lactic acid, oleic acid, gum arabic and their mutual interactions, on the production of extracellular lipase and the regulation of the expression of the lipase encoding gene (*lip*A) in *Acinetobacter calcoaceticus* were investigated. Formation of extracellular lipase was measured in culture supernatants of wild-type strain BD413 and expression of the *lip*A gene was monitored *in vivo* with a chromosomal fusion of *lip*A to *lac*Z. At the level of *lip*A expression only oleic acid had a significant effect; it lowered expression. Neither gum arabic nor lactic acid had any effect on *lip*A expression. On the other hand, the yield of extracellular lipase increased 2–5 times by the addition of gum arabic, possibly due to the release of cell surface-bound lipase. An interaction between oleic acid and lactic acid was also detected. *Journal of Industrial Microbiology & Biotechnology* (2000) **24,** 25–30.

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Introduction

Microbial lipases have been used extensively in the food and detergent industries. Recently, lipases have become of interest to the chemical and pharmaceutical industries because of their catalytic properties in organic solvents, and their ability to resolve racemic mixtures [8,11,17]. This has led to the cloning and characterisation of a large number of genes encoding bacterial lipases (for a review see [9]).

Many lipases have been studied regarding optimisation of their production [6,7,22,27] and the mechanisms of lipase production (including regulation of gene expression and the mechanisms of translation and secretion), are quite different among different bacterial species.

Acinetobacter calcoaceticus BD413 produces several lipolytic enzymes, among which is an extracellular lipase and at least four esterases [12,14,15]. The extracellular lipase is encoded by the *lip*A gene [14]. LipA is highly similar to lipases of several *Pseudomonas* species, and a *Pseudomonas*-type specific lipase chaperone (LipB) also plays a vital role in production of the extracellular lipase in *Acinetobacter*. The two corresponding genes (*lip*A and *lip*B) are organised in a single operon in both organisms [6,9,15].

The mechanism of formation and export of the lipase requires lipase translocation across the inner and outer membrane [13,15]. Several post-transcriptional steps contribute to the control over the rate of lipase production. In addition, the amount of active lipase in the extracellular medium is influenced by proteolytic degradation of mature Lip A [13].

As all components of a growth medium may affect lipase production including the post-transcriptional levels described above, the design of an optimal medium for lipase production is complicated.

Carbon sources such as fatty acids, triglycerides and sugars, or complex polysaccharides such as glycogen, hyaluronidate and alginate can stimulate or repress lipase production [28–30]. Here we report on the effect of three medium components, lactic acid, oleic acid and gum arabic, separately and in combination, on lipase production. Lipase production was measured at the level of *lip*A transcription in a strain harbouring a chromosomal *lip*A::*lac*Z fusion and of extracellular lipase activity in wild-type strain BD413.

Medium components to be analysed were selected as follows: (i) gum arabic, as an inert polysaccharide, was expected to stimulate lipase production [29,30]; (ii) oleic acid acts as a repressor of lipase production [13,22] and (iii) lactic acid is an excellent carbon source for *A. calcoaceticus*, providing high growth rates.

The experimental conditions were arranged in the form of a random fractional factorial design [3] and evaluated by surface response analysis [1,4,18,20,23,24,26]. Surface response plots were generated separately for *lip*A expression and production of extracellular lipase.

Significant effects of the individual nutrients, as well as their interactions, were analysed and linked to critical control levels in lipase production.

Materials and methods

Strains

A. calcoaceticus BD413 [10] was kindly provided by E Juni (University of Michigan). In recent publications this strain is also referred to as '*Acinetobacter* BD413' [5,16]. Strain AAC320-1 is derived from BD413; it carries a transcriptional fusion of the promoter region of *lip*A, encoding its extracellular lipase, with a promoterless *lac*Z, in its chromosome [15]. As a consequence, AAC320-1 does not produce lipase.

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Media and culture conditions

The strains were cultivated aerobically at 30°C in a mineral medium containing 37 mM NH_4Cl, 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 1 ml (per L) of a solution containing 50 g EDTA, 2.2 g $ZnSO_4 \cdot H_2O$, 5 g $FeSO_4 \cdot 7H_2O$, 1.6 g $CuSO_4 \cdot 5H_2O$, 5 g $MnCl_2 \cdot 4H_2O$, 1.1 g $(NH_4)_6 Mo_7O_{24} \cdot 4H_2O$, 50 mg H_3BO_3, 10 mg KI and 50 mg $CoCl_2 \cdot 6H_2O$. Lactic acid (0–1%, v/v) and oleic acid (0–10 mM) were used as carbon sources. Where indicated, gum arabic was added (0.4%, w/v; see also Tables 1 and 2). The pH of all media was adjusted to 6.8. Media were autoclaved at 120°C for 20 min. Cultures were inoculated with an early stationary phase culture, pre-grown in the same medium, to an initial optical density at 580 nm ($OD_{580\,nm}$) of approximately 0.1 in 250-ml Erlenmeyer flasks, containing 30 ml of medium, on a rotatory shaker at 250 rpm for 12 h. Every 2 h duplicate 1-ml samples were collected and analysed.

Analytical methods

Biomass production was monitored by measuring $OD_{580\,nm}$. Cells from cultures containing oleic acid were washed twice by centrifugation at 12500 $\times$ *g* for 3 min and suspended in a buffer containing 50 mM Tris-HCl, 2 mM $MgCl_2$ and 2 mM $CaCl_2$ (pH 7.5) prior to $OD_{580\,nm}$ measurements.

Extracellular lipase was measured in culture supernatants of *A. calcoaceticus* BD413, as previously described [14], using *p*-nitrophenyl palmitate (*p*-NPP, Sigma, St Louis, MO, USA) as the substrate. One unit (U) enzyme activity equals the amount of enzyme forming 1 μmol *p*-nitrophenol per min. Lipase activity per litre of culture is divided by the $OD_{580\,nm}$ of the sample, to obtain the specific lipase yield (U L^{-1} $OD_{580\,nm}$). Maximum specific lipase yield, calculated for each experiment, was selected and pooled for the statistical tests (see Table 1).

Table 1 Experimental design and maximal lipase specific yields obtained

Carbon sources			Y_{Lip}[a] (U L^{-1} $OD_{580\,nm}$)
Lactic acid (%)	Oleic acid (mM)	Gum arabic (%)	
0	0.5	0	220 ± 10
0	10	0	0 ± 2
0.25	0	0	165 ± 6
0.25	0.20	0	118 ± 5
0.50	0	0	111 ± 6
0.50	1	0	138 ± 8
0.50	10	0	0 ± 3
1	0	0	79 ± 4
0	0.50	0.4	374 ± 15
0	10	0.4	0 ± 2
0.25	0	0.4	414 ± 18
0.25	0.20	0.4	217 ± 11
0.50	0	0.4	345 ± 13
0.50	1	0.4	243 ± 10
0.50	10	0.4	0 ± 3
1	0	0.4	203 ± 4
1	1	0.4	98 ± 4
1	10	0.4	62 ± 3

[a]Mean values are given of duplicate samples (plus and minus standard error). Y_{lip} is the maximum specific lipase yield.

Table 2 Experimental design and maximal β-galactosidase specific yields in *A. calcoaceticus* AAC 320-1

Carbon sources			$Y_{\beta\text{-Gal}}$[a]
Lactic acid (%)	Oleic acid (%)	Gum arabic (%)	
0.25	0	0	739
0.25	0.5	0	608
0.25	1	0	609
0.50	0	0	643 ± 32[c]
0.50	0.5	0	578
0.50	1	0	530
1	1	0	535
0	0.5	0.4	330
0.25	0.2	0.4	647 ± 33[c]
0.25	1	0.4	530
0.25	10	0.4	200
0.5	0	0.4	626 ± 26[c]
0.5	0.2	0.4	635
0.5	1	0.4	578 ± 28[c]
0.5	10	0.4	317
1	0	0.4	513 ± 24[b]
1	1	0.4	513
1	10	0.4	122

[a]$Y_{\beta\text{-Gal}}$ is the yield of β-galactosidase, expressed in Miller units. The standard deviation, as determined from duplicate assays of two [b] or three [c] separate cultures, has been indicated.

Production of β-galactosidase [21] was measured in the *lip*A::*lac*Z fusion strain *A. calcoaceticus* AAC320-1, using *o*-nitrophenyl β-D-galactopyranoside (*o*-NPG, Sigma) as the substrate. Cells were harvested by centrifugation, washed twice, and suspended in a known volume of the same buffer. After measurement of the $OD_{580\,nm}$, these samples were frozen until further use. Prior to the β-galactosidase assay, cells were thawed, diluted appropriately to reach an $OD_{580\,nm}$ of less than 3, and suspended in the same volume of Z-buffer (containing 60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.001% sodium dodecylsulphate and 50 mM β-mercaptoethanol).

Cells were permeabilized in 1-ml samples with 100 μl ethanol and 20 μl toluene and incubated at 37°C for 20 min. The level of β-galactosidase expression was calculated in Miller Units [21]. Maximum specific β-galactosidase yield ($Y_{\beta\text{-gal}}$), calculated for each experiment, was selected and recorded for the statistical tests (see the Results section). All growth conditions were analysed by taking duplicate samples from one, two or three independent cultures. Values displayed are mean values.

Experimental design, statistics and calculation methods

Tables 1 and 2 show the set of experimental conditions tested in a randomised fractional factorial design for the two strains. The kinetics of extracellular lipase formation in strain BD413, and β-galactosidase (under control of the *lip*A promoter) in strain AAC320-1, were significantly different. Presumably this is a consequence of the fact that LipA is secreted and β-galactosidase is retained in the cyto-

plasm. Therefore, measurements at a single point after inoculation do not reflect the maximal amount of each of the enzymes formed. In addition, cell yields vary with the amount of carbon source available. For these reasons, the criteria of maximal specific lipase yield (Y_{lip}) and maximal specific β-galactosidase yield ($Y_{\beta\text{-gal}}$) were adopted. Enzyme specific yields were calculated as the ratio between enzyme activity and cell density ($OD_{580\ nm}$), for each sample. As indicated, maximal Y_{lip} or $Y_{\beta\text{-gal}}$ values, calculated for each experiment, were pooled and used for the statistical tests.

These yields were analysed by ANOVA [3] in order to select significant main effects and interactions between the different carbon sources (95% confidence limits) and fitted to the general linear model, using statistical software (Statistix 4.1 Analytical Software). Results were presented using surface response methodology [2,20,23].

Results

The aim of this study was to analyse the effect of various concentrations of oleic acid and lactic acid, in the presence and absence of the polysaccharide gum arabic, on the multistep process of extracellular lipase production in *A. calcoaceticus*. Formation of active extracellular lipase (LipA) was studied in batch cultures of the wild-type strain BD413, while expression of the lipase encoding *lip*A gene was monitored by formation of β-galactosidase in the BD413-derived strain AAC320-1 (*lip*A::*lac*Z).

The experimental conditions were arranged as two independent fractional factorial designs, one for each strain, displayed in Tables 1 and 2. From hourly measurements, maximal specific enzyme yields were calculated both for lipase and β-galactosidase, and the data were analysed separately, using various statistical criteria (see below).

Oleic acid has a significant effect on lipA transcription

Main effects and possible interactions between oleic acid, lactic acid and gum arabic on expression of the lipase-encoding *lip*A gene in cultures of strain AAC320-1 were calculated from the β-galactosidase data, using the ANOVA procedure. The results indicated that only oleic acid significantly affected this process (ie *lip*A expression) ($P < 0.0001$); lactic acid had a minor effect ($P = 0.0616$), gum arabic did not have significant effects, nor were interactions between these medium components significant.

These results were confirmed by linear regression analysis: oleic and lactic acid were the variables selected (fitted) in the model, while gum arabic was discarded. The surface response plot displayed in Figure 1 shows a (linear) negative effect of oleic acid on *lip*A expression within the entire range of concentrations tested (0–10 mM oleic acid).

The effect of lactic acid is less clear. Although the fitting procedure includes a significant quadratic term, the relevance of this term may be questioned on the basis of the ANOVA results (mentioned above) and the smoothness of the β-galactosidase surface (reflected in the small differences between minimum and maximum values).

Data for β-galactosidase (*lip*A expression) fitted best to an equation that lacks significant x/y interaction terms (ie the xy, xy^2 and yx^2 terms were discarded), based upon the ANOVA test and a regression analysis of the data. Therefore, Figure 1 shows a smooth quadratic surface, with an acceptable fit.

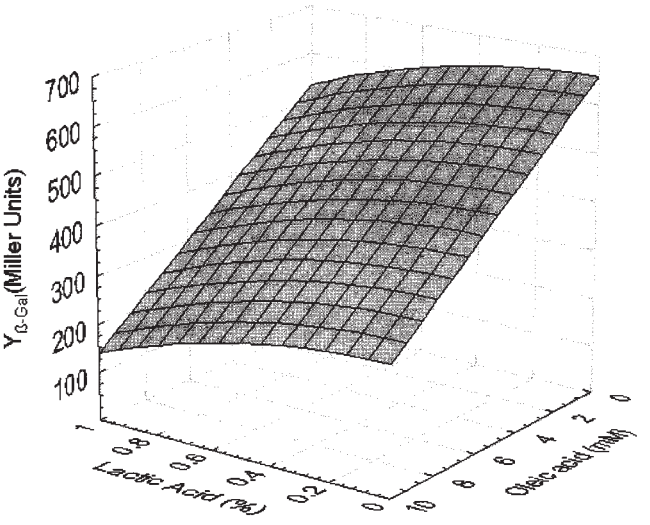

Figure 1 Effect of oleic and lactic acid on β-galactosidase yields. Data were fitted to the following model: $z = 655.64 - 39.19\ y - 123.8\ x^2$; $r^2 = 0.91$ where x is the lactic acid concentration, y is the oleic acid concentration and z is the β-galactosidase yield. Gum arabic was discarded from the model ($P > 0.1$).

From these results we conclude that there is no significant interactive effect between oleic and lactic acid regarding lipase production, as measured at the level of the initiation of *lip*A transcription.

Gum arabic stimulates lipase production

Similar to the analysis of *lip*A expression described above, the maximal lipase yields obtained in cultures of *A. calcoaceticus* BD413 were analysed by the ANOVA procedure.

The results obtained indicated that gum arabic had a significant effect on the extracellular lipase yield ($P = 0.008$), as did oleic acid ($P = 0.003$), while lactic acid did not have significant effects.

Total lipase production was approximately 2 to 5-fold higher in media containing gum arabic than in media without this polysaccharide (Table 1). Due to these large differences in extracellular lipase yield, both sets of data (with and without gum arabic) were analysed separately, as shown in the corresponding surface response plots (Figure 2a,b). Separate analysis of lipase yields obtained in media with and without gum arabic increased the sensitivity of the test, thus including lactic acid as a significant variable.

Oleic acid has a (linear) negative effect on lipase production (Figure 2), with a strong descent upon an increase of the fatty acid concentration. In fact, cultures of *A. calcoaceticus* BD413, grown with an initial oleic acid concentration of 10 mM or higher, lacked detectable extracellular lipase activity, even in the presence of gum arabic.

The surface response plot of lipase yield, obtained with media that did not contain gum arabic, is shown in Figure 2a. Oleic acid ($P = 0.003$) and lactic acid ($P = 0.05$) were included in the model. The corresponding plot of lipase

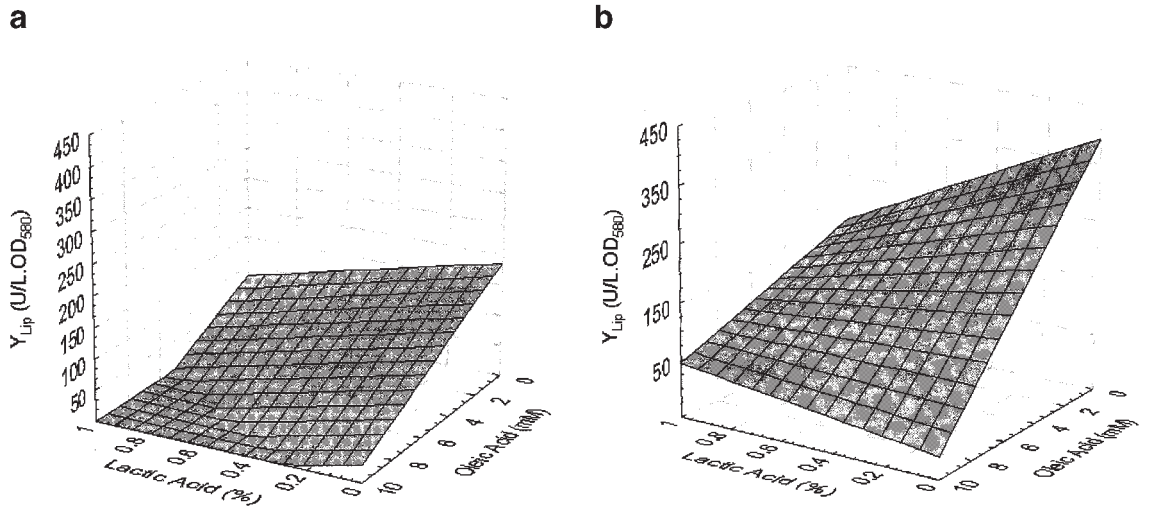

Figure 2 Effect of oleic and lactic acid on lipase yields in cultures with and without gum arabic. (a) Without gum arabic. Data were fitted to the following model: $z = 185.92 - 15.82\ y - 104.40\ x$; $r^2 = 0.86$; $P = 0.007$. (b) With gum arabic. Data were fitted to the following model: $z = 394.18 - 40.53\ y - 225.84\ x + 28.32$ xy; $r^2 = 0.87$; $P = 0.005$. In both equations x is the lactic acid, concentration y is the oleic acid concentration and z is the lipase yield.

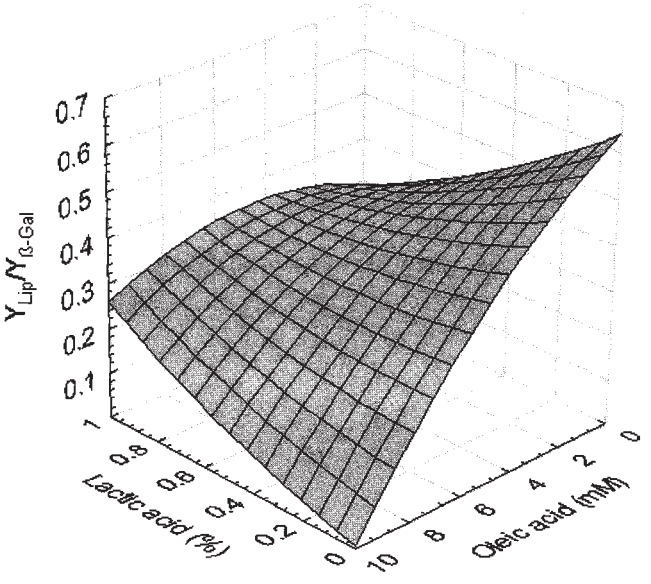

Figure 3 Ratio between the calculated lipase yields (data from Figure 2b) and β-galactosidase yields (data from Figure 1) in relation to the oleic and lactic acid concentrations.

yield of cells grown in media containing gum arabic is shown in Figure 2b. As under the former culture conditions, oleic and lactic acid were also the variables fitted in this model ($P = 0.002$ and 0.03, respectively). The *xy* interaction term between both carbon sources (lactic and oleic acid) markedly increased the goodness-of-fit of the model; its effect is incorporated into the corresponding surface response plot (Figure 2b).

Discussion

Nutrients often have multiple effects on cellular processes, depending on the concentration at which they are used. They may act as repressor, inducer, activator or inhibitor of an enzyme, catalysing a specific step in a metabolic pathway, leading to the formation of a valuable product. Multiple nutrients may even show synergistic effects and gradual nutrient consumption during growth in batch culture may further complicate matters by continuously altering the rate of formation of the desired product. Therefore, variation of the concentration of a single nutrient is often inadequate for optimisation of a specific production process [2,25]. One aspect that is often neglected is the fact that specific growth yields are not constant through the entire range of substrate concentrations of a particular carbon source, thus affecting product yields. For physiological optimisation of a production process, it is therefore advisable to vary the concentration of multiple substrates, such that the relative influence of each nutrient can be determined accurately. This type of analysis, potentially highly labour-intensive and time consuming, calls for a fractional factorial arrangement of experimental conditions, coupled to the type of surface response analysis used in this paper [1,2,4,18,20,23–26].

However, the application of surface response analysis to microbial physiology at different cellular levels (transcription, translation and post-translational processes) has not been reported so far. Here, we show that surface response analyses can aid in unravelling the complicated cellular performance in the production process of the extracellular lipase in *Acinetobacter* BD413.

For example, the analysis of β-galactosidase data in the transcriptional fusion strain AAC320-1, in response to the various carbon sources assayed, indicated that gum arabic does not affect *lip*A transcription. Since the fitted equations for lipase production showed an effect of gum arabic, the latter must have acted at a post-transcriptional level. This confirms previous suggestions that polysaccharides influence lipase production at the level of secretion, and/or mechanical liberation of accumulated lipase from the outer surface of the cell [29,30].

Diauxic growth effects have never been observed during

growth of *Acinetobacter* in media containing lactic and oleic acid, within the range of concentrations tested (data not shown). If biomass attained in culture broth is an important key to lipase production, as shown in *Candida* [22], then the criterion of maximal specific enzyme yield is adequate for comparisons.

Lipase expression/production ratio and the control of lipase formation

We have used the response surfaces of Figure 1 (*lip*A expression; derived from maximal β-galactosidase yields) and of Figure 2b (lipase production; derived from maximal yields of extracellular lipase) to calculate the ratio of lipase production over *lip*A expression, in relation to medium composition. This ratio, for the concentration range of oleic and lactic acid tested, is shown in Figure 3.

The significance of this figure is best illustrated by analysing the effect of changes in medium composition on the balance between lipase production and expression. If both processes are tightly coupled, then the ratio of the yields of lipase over β-galactosidase would be constant in the whole range of carbon source concentrations tested. This was not the case, however, in particular towards the more extreme conditions represented in this figure. The almost constant ratio observed at the higher lactic acid concentrations is lost at the lower concentrations, where changes in medium composition seem to specifically affect either *lip*A expression or lipase release from the cell. For instance, a significant decrease in the production/expression ratio is found towards the higher oleic acid concentrations, presumably due to the suppression of transcription (Figure 1). The opposite is observed towards the lower oleic and lactic acid concentrations.

Probably this type of dependence on oleic acid concentrations is the main reason why fed-batch and continuous cultures, by limiting the fatty acid or carbon supply, have been successful strategies for maximal lipase production [19,27]. Our analysis suggests that both *lip*A-transcription and lipase-excretion across the outer membrane are optimised under these conditions.

Summarising, the combined approach of using genetic methods (by generating a strain containing a specific transcriptional fusion construct [13]), as well as surface response analysis, allowed the identification of key regulatory points for *lip*A transcription, lipase production and their possible correlation. In addition to initiation of transcription, the secretion and post-secretion level play an important role in this process.

As different optimum conditions could be defined for *lip*A transcription and lipase production, it may be feasible to study these aspects separately in future experiments, aimed at the further optimisation of lipase production.

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