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Xanthomonas campestris as a host for the production of recombinant *Pseudomonas aeruginosa* lipase

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Recombinant plasmid pBP13, which expresses the alkaline lipase from *Pseudomonas aeruginosa* IGB83 under the *tac* promoter was transferred to *Xanthomonas campestris* pv *campestris* IBT148. Different fermentation conditions were tested for lipase productivity by strain IBT148 carrying plasmid pBP13, and a fermentation process was established in an instrumented bioreactor, where lipase production was increased more than 12-fold with respect to the initial culture conditions in shake flasks. Xanthan gum stabilized the activity of the alkaline lipase.

Keywords: lipase; recombinant *Xanthomonas*; fed-batch; bioreactor

Introduction

Lipases are esterases that hydrolyse water-insoluble esters such as long-chain triglycerides. Lipases also catalyze the formation of esters (esterification) and the exchange of ester bonds (transesterification), when present in non-aqueous media [2,14]. Lipases display a high degree of specificity and enantioselectivity for esterification and transesterification reactions, thus their potential uses in industry are very wide [2,15]. These potential industrial applications have been an important driving-force for lipase research, and in particular for the study of bacterial lipases [15]. *Pseudomonas* lipases display special biochemical characteristics not common among the lipases produced by other microorganisms, such as thermo-resistance and activity at alkaline pHs. Recently, several *Pseudomonas* genes encoding lipases have been cloned and sequenced, and the regulation of their expression is beginning to be understood [13,15,31].

We have characterized the lipase produced by *Pseudomonas aeruginosa* IGB83 [22]. This strain was isolated after screening natural populations for lipolytic bacteria native to the Mexican tropical rain forest. The apparent molecular weight of this enzyme (58 kDa), is similar to that of *P. fluorescens* lipases, but substantially different from those of other *P. aeruginosa* strains. The optimum conditions for the strain IGB83 lipase activity are 55°C and pH 10; it has a half life at 55°C and pH 8.5 of 13 min, and has an isoelectric point of 8. The lipase from strain IGB83 is completely inactivated by freezing. This behavior suggests that hydrophobic interactions are important for the structure of the enzyme. Inactivation upon freezing has not been reported for other lipases.

Lipases produced by Gram-negative bacteria, including pseudomonads, are secreted to the culture medium as an aggregate with lipopolysaccharide [31]. Secretion of the *P. aeruginosa* lipase has been reported to depend on the '*xcp* pathway', which is a general route for the translocation of extracellular proteins from the periplasmic space through the outer membrane of Gram-negative bacteria [24,34]. *Xanthomonas campestris* pv *campestris* has been reported to present the *xcp* pathway, and the genes coding for the proteins which participate in this route are homologous to the *P. aeruginosa* genes [6,8]. The similarity between *P. aeruginosa* and *X. campestris* is not restricted to their mechanism for protein secretion since they are closely related organisms, and their phylogenetic distance is similar to the distance between different *Pseudomonas* species belonging to RNA group I [20]. The phylogenetic relationship between *P. aeruginosa* and *X. campestris* was the rationale behind the use of *X. campestris* pv *campestris* as a host to express the alkaline lipase from *P. aeruginosa* IGB83 that we report here. The IGB83 lipase cannot be expressed in the native bacteria for its production at an industrial scale since *P. aeruginosa* is an opportunistic human pathogen, and strain IGB83 in particular, has a very high virulence (unpublished results).

X. campestris pv *campestris* produces the exopolysaccharide xanthan gum. This biopolymer has several commercial applications due to its high viscosity and pseudoplastic behavior under a wide range of pHs, temperatures and salt concentrations [11]. Polysaccharides increase protein stability [29]. Xanthan gum is a very good viscosifying and emulsifying agent [11], and the activity of *P. aeruginosa* lipase increases in the presence of polysaccharides [30,35]. We evaluate here the effect of xanthan gum on the stability of the alkaline lipase from *P. aeruginosa* IGB83.

We used an expression plasmid containing the *tac* promoter since it is active in a broad spectrum of Gram-negative bacteria [1], and has been used specifically for the expression of *P. aeruginosa* genes [21]. Another advantage of the *tac* expression system is its simple induction and the high ratio between repression and derepression.

Materials and methods

Bacterial strains and plasmids are shown in Table 1. *X. campestris* strain IBT148 was routinely grown on NYGB

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Table 1 Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference
Bacteria:		
E. coli		
HB101	*leu, pro, recA, hsdR, hsdM, rpsL*	[3]
P. aeruginosa		
PAO2003	*argH32, str-39, rec-2*	[28]
IGB83	wild type strain, highly lipolytic	[22]
X. campestris pv *campestris*		
IBT148	Rif^r derivative of strain NRRL B1459	[18]
Plasmids:		
pMMB22	RSF1010 derivative containing the *tac* promoter, Ap^r, Str^r	[1]
pJB3JI	RP4 derivative, Tra$^+$, Ap^r, Tc^r	[4]
pBP1	pMMB22 derivative containing the *P. aeruginosa* IGB83 *lip* gene, Lip$^+$, Ap^r	This work
pBP13	pBP1 cointegrate with pJB3JI, Lip$^+$, Tra$^+$, Ap^r, Tc^r	This work

medium [5], lacking glycerol. The two culture media used in the fermentations are: AL1 medium which contains (g L^{-1}): NH_4Cl 4.12, $(NH_4)_3PO_4$ 0.6978, KH_2PO_4 3.06, citric acid 1.3, $MgSO_4 \cdot 5H_2O$ 0.23, $FeCl_3 \cdot 6H_2O$ 0.0014, ZnO 0.0072, $CaCO_3$ 0.0024, boric acid 0.0028, and sucrose 20, pH 7.5; and medium AL2 which contains (g L^{-1}): NH_4Cl 4.12, KH_2PO_4 5.0, citric acid 2.3, Na_2SO_4 0.114, $MgCl_2 \cdot 6H_2O$ 0.163, $CaCl_2$ 0.012, boric acid 0.006, Na_2CO_3 0.5, $FeCl_3 \cdot 6H_2O$ 0.0014, ZnO 0.007, sucrose 20 and pH 7.5. AL1 and AL2 media were derived from media described previously for xanthan gum production [9,23], but have decreased C/N ratio (by increasing N) to minimize xanthan gum production. *P. aeruginosa* and *E. coli* strains were cultured on LB medium [19]. *X. campestris* IBT148 and *E. coli* HB101 were grown on M9 minimal medium [27]; in the case of *E. coli* HB101 leucine and proline were added. *P. aeruginosa* strains were grown on FAM minimal medium [22], using 1% olive oil as carbon source. Xanthan gum production was determined by its dry weight and culture viscosity was determined as described previously [25]. Biomass concentration was monitored by optical density at 600 nm, and was converted to cell dry weight using a calibration curve done with *X. campestris* pv *campestris* IBT148(pBP13). Sucrose was determined as described previously [9].

The antibiotic concentrations used (μg ml^{-1}) were: carbenicillin 800, rifampicin 50, streptomycin 200 and tetracycline 30. Carbenicillin was used instead of ampicillin to select for the presence of plasmid pMMB22 and derivatives, since different *Pseudomonas* strains have high basal levels of resistance to the latter antibiotic. 1-Isopropyl-β-D-1-thiogalactopyranoside (IPTG) was used at a concentration of 2 mM as reported previously to be optimum for *Pseudomonas putida* [1], unless otherwise stated.

Matings were done by plating a 1 : 1 mixture of the donor and recipient strains on agar plates of NYGB medium without glycerol, and incubating the mixture overnight at 30°C; the mixture was serially diluted and plated onto selective medium.

Fermentation conditions

Batch fermentations were carried out in a 2-L tank bioreactor with a working volume of 1 L. This tank was fitted with two Rushton turbines. Temperature was kept at 29°C, pH was controlled between 7.3 and 7.7, and dissolved oxygen tension (DOT) was maintained at 10% with respect to air saturation. The fermentations were conducted at 200 or 500 rpm (depending on the oxygen demand). DOT was kept constant by an automatic control operated via a Macintosh computer which manipulated the oxygen partial pressure, using pure oxygen and pure nitrogen. Fed-batch fermentations were carried out under the same conditions as the batch cultures, and 20 ml of a 10× salt stock solution of medium AL1, lacking citric acid and $CaCO_3$, was intermittently supplemented after 12 h of incubation.

All fermentations were inoculated with 100 ml of a culture of *X. campestris* IBT148(pBP13) grown overnight on NYGB medium without glycerol, and supplemented with rifampicin and carbenicillin. The fermentations presented here were done twice and the results were reproducible with a variability of ±5%.

Lipase assay

Lipase was quantified by titrating the butyric acid released from tributyrin at pH 8.5 and 55°C with 50 mM NaOH. Lipase activity was calculated from the rate of alkali consumption, corrected for the rate given by a boiled sample and expressed as lipase units (U: 1 unit is defined as the release of 1 μmol of titrable fatty acid min^{-1}). This method has a variability of approximately ±200 U.

A qualitative method to measure lipolytic activity on plates was developed which consisted of a 33-mM tributyrin emulsion in 50 mM Tris-HCl buffer pH 8.5 supplemented with the pH indicator *m*-cresol purple and solidified with 1.5% agarose. When these plates were incubated at 37°C, lipolytic activity was visualized by halo formation and from the change of color of the medium from purple to yellow.

Nucleic acid procedures

DNA isolation and cloning procedures were carried out as described by Sambrook *et al* [27]. Plasmid visualization was done as described by Rosenberg *et al* [26].

Isolation of clones coding for the alkaline lipase

A genomic library of *P. aeruginosa* IGB83 was made in plasmid pMMB22 [1] digested with *Eco*RI endonuclease. *P. aeruginosa* IGB83 total cellular DNA was partially digested with *Eco*RI* and ligated. This ligation mixture was transformed into *E. coli* HB101. Cells containing this genomic library were grown on plates of M9 medium with 2% (vol/vol) of glycerol as carbon source, proline and leucine, and supplemented with 33 mM tributyrin and 1 mM IPTG. Clones which formed a halo after 2 weeks at room temperature were considered as putative lipase clones. One of these, containing plasmid pBP1, was used in this study.

Plasmid pBP1 was transferred to *P. aeruginosa* PAO2003 using plasmid pJB3JI for its mobilization, a cointegrate was formed between these two plasmids which is called plasmid pBP13 and which was further characterized in this work.

Results

Construction of plasmid pBP13 and transfer to X. campestris IBT148

Plasmid pBP13 is a cointegrate between the broad host range plasmid pJB3JI and the recombinant plasmid pBP1 which expresses the alkaline lipase under the *tac* promoter of the vector pMMB22 (Figure 1). In the *P. aeruginosa* and *X. campestris* genetic backgrounds pBP13 plasmid was maintained without dissociating into its components, since in gel electrophoresis the band with the expected size of plasmid pBP1 is not apparent (Figure 1).

P. aeruginosa strain PAO2003(pBP13) has a lipase activity of 1100 ± 200 U ml^{-1} when induced with IPTG, but has no activity if not induced (Table 2). Strain PAO2003 did not present lipolytic activity, under our experimental conditions (Table 2).

Plasmid pBP13 was transferred by conjugation to *X. campestris* either from *E. coli* HB101 or from *P. aeruginosa* PAO2003. Carbenicillin-resistant clones were selected and those which present lipolytic activity in the presence of IPTG were selected. As shown in Table 2, strain IBT148(pBP13) exhibited a lipase activity as high as that of *P. aeruginosa* PAO2003(pBP13) under the same culture conditions.

Stability of plasmid pBP13 and conditions for inoculum production

In order to determine the stability of plasmid pBP13 in *P. aeruginosa* PAO2003, this strain was grown for approximately 25 generations on LB medium without selective pressure for the plasmid. All the colonies retained the plasmid carbenicillin resistance after the subculturing. When lipolytic activity was measured through halo formation of the colonies on FAM plus olive oil, only 2% of the bacteria lost lipase activity.

In *X. campestris* IBT148(pBP13) a similar stability was found and 100% of the colonies tested retained carbenicillin resistance even after seven subcultures (approximately 50 generations) without selective pressure. However, in contrast with the results obtained with PAO2003(pBP13), the stability of lipase production was very low (Figure 2). The lipolytic activity of four IBT148(pBP13) independent transconjugants (two obtained from the cross with HB101(pBP13) and two using strain PAO2003(pBP13) as donor in mating) was completely lost after approximately 50 generations on NYGB medium supplemented with 0.2% glucose and 2 mM IPTG. Even after six subcultures (approximately 40 generations), none of the 30 colonies tested derived from one of the clones mentioned above presented a halo of lipolytic activity.

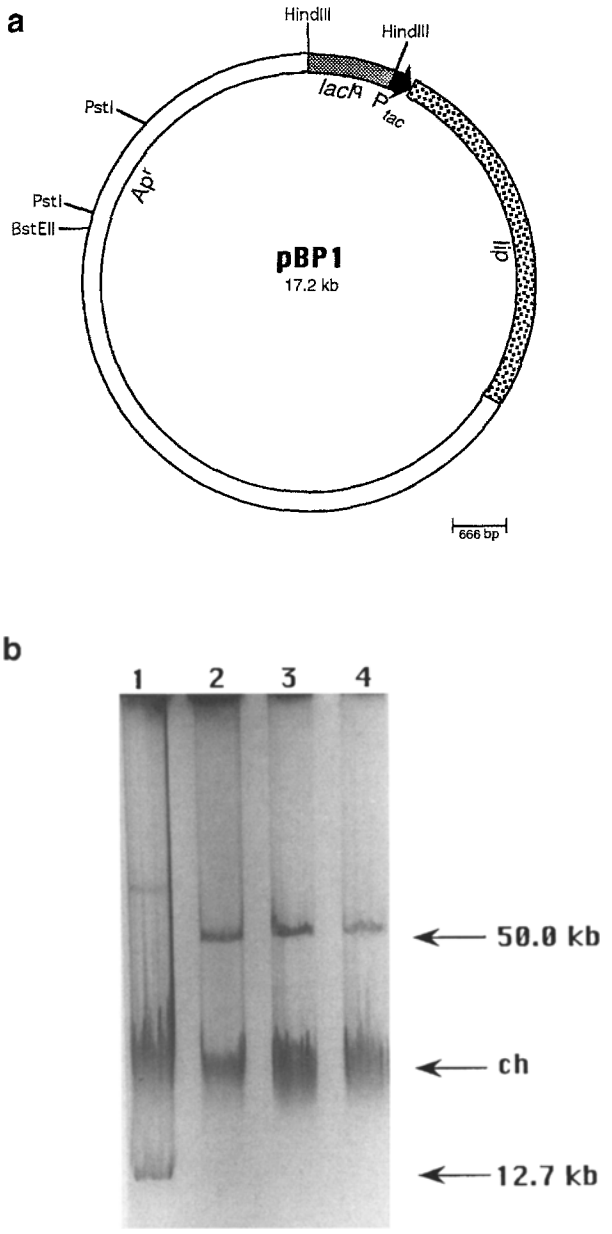

Figure 1 (a) Schematic representation of plasmid pBP1. (b) Plasmid electrophoretic profile, lanes correspond to: (1) *E. coli* HB101(pMMB22), (2) *E. coli* HB101(pJB3JI), (3) *P. aeruginosa* PAO2003(pBP13), and (4) *X. campestris* IBT148(pBP13). The size in kilobases (kb) of plasmids pMMB22 and pJB3JI and the migration of the chromosome (ch) are shown.

Table 2 Extracellular lipolytic activity of different strains grown on minimal medium[a]

Strain	IPTG	Carbon source	Lipase activity (U ml^{-1})
P. aeruginosa			
IGB83	−	olive oil	2850 ± 200
IGB83	− or +	glucose	ND
PAO2003	−	olive oil	300 ± 180
PAO2003	− or +	glucose	ND
PAO2003(pBP13)	−	glucose	ND
PAO2003(pBP13)	+	glucose	1100 ± 180
X. campestris			
IBT148	− or +	glucose	ND
IBT148(pBP13)	−	glucose	ND
IBT148(pBP13)	+	glucose	1500 ± 210

[a]Lipolytic activity was measured in the cell-free supernatant after 24 h of growth in the case of *P. aeruginosa* strains, and in the case of *X. campestris* strains activity was measured after 48 h of growth.
ND = not detectable.

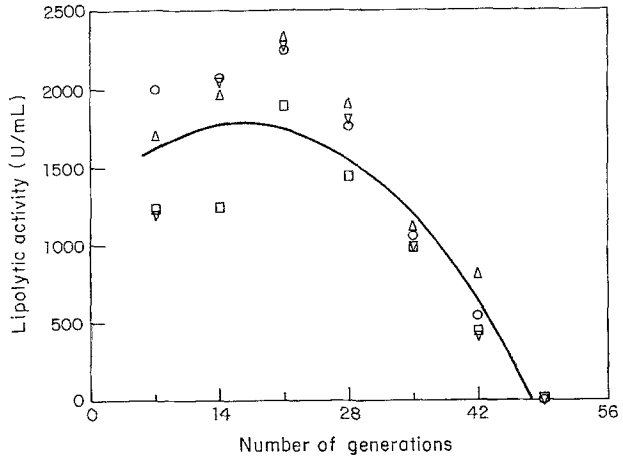

Figure 2 Instability of lipase production by *X. campestris* IBT148(pBP13). Different symbols represent the lipolytic activity exhibited by different subclones of this strain during subculturing.

In order to circumvent the instability of lipase production by strain IBT148(pBP13), the inoculum for all the fermentations presented in this work was done with recently isolated transconjugants which had lipolytic activity, and in no case were bacteria used that had been subcultured more than three times (approximately 20 generations).

Influence of medium composition on lipase production

Lipase productivity of strain IBT148(pBP13) was higher on AL1 medium, than on AL2 medium (data not shown). The effect of other variables was analyzed using AL1 medium, since it gave the best results. When IPTG concentration was increased to 5 mM, a decrease in both growth rate and final biomass concentration of the strain IBT148(pBP13) was found (data not shown), but similar lipase production was attained (fermentations 1 and 2, Table 3).

Lipase production on fed-batch cultures

In order to increase the biomass concentration of the fermentations, 20 ml of a 10× salt stock solution of medium, lacking citric acid and $CaCO_3$, was added every 2 h after 12 h of culture. This scheme of salt addition was established since neither sucrose availability, which was high at the end of these fermentations, nor dissolved oxygen, which was maintained at 10%, were limiting for the growth of strain IBT148(pBP13) (data not shown). This fed-batch fermentation resulted in a marked increase in biomass concentration and sucrose consumption (Figure 3). Under this fermentation condition, IPTG was added after 38 h of culture and lipase production was followed for 12 additional hours. We found a much higher lipase production with this scheme of induction under fed-batch conditions since we obtained 13200 U ml^{-1} (fermentation 3), compared with the 3400 U ml^{-1} obtained on fermentations 1 and 2 (Table 3).

In order to increase lipase productivity, by increasing biomass yield and time of lipase production, a fed-batch fermentation similar to that described above was done, but at 60 h of growth sucrose was supplemented, and at 46 h of cultivation, 2 mM IPTG was added. This fermentation resulted in a very high lipase activity and productivity, reaching 19800 U ml^{-1} of culture (Figure 3 and Table 3).

Influence of xanthan gum concentration on lipase thermo-stability

We determined the lipolytic activity remaining after 20 min at 55°C of a *P. aeruginosa* IGB83 supernatant medium in the presence of different xanthan gum concentrations (Figure 4). Xanthan gum stabilized lipase activity. A similar experiment was carried out with the alkaline lipase produced by strain IBT148(pBP13), where the time of total inactivation at 55°C of the lipase was measured as a function of the xanthan gum concentration. The increased lipase stability in the presence of increasing xanthan gum concentrations was also apparent (Table 4).

Discussion

We report the construction of a recombinant plasmid which expresses the alkaline lipase from *P. aeruginosa* IGB83 under the control of the *tac* promoter. The recombinant lipase is efficiently produced and secreted by *X. campestris*.

Plasmid pBP13 was stably replicated both in *P. aeruginosa* PAO2003 and in *X. campestris* IBT148, but the lipolytic activity which it encodes is very unstable in the *X.*

Table 3 Comparison of lipase production under different culture conditions

Fermentation number	Type of culture	Growth phase[a] induction (IPTG)	Lipase activity[b]	
			(U ml^{-1})	(U g cells^{-1}) × 10^{6}[c]
1	batch	start (2 mM)	3400	4.5
2	batch	start (5 mM)	3300	8.4
3	fed-batch	log (2 mM)	13200	10.0
4	fed-batch	late log (2 mM)	19800	12.0

[a]The growth phase was determined from the growth curve and corresponds to a different time, depending on the specific growth rate during each fermentation.
[b]The maximum lipase activity attained in each fermentation is reported.
[c]Dried cell weight.

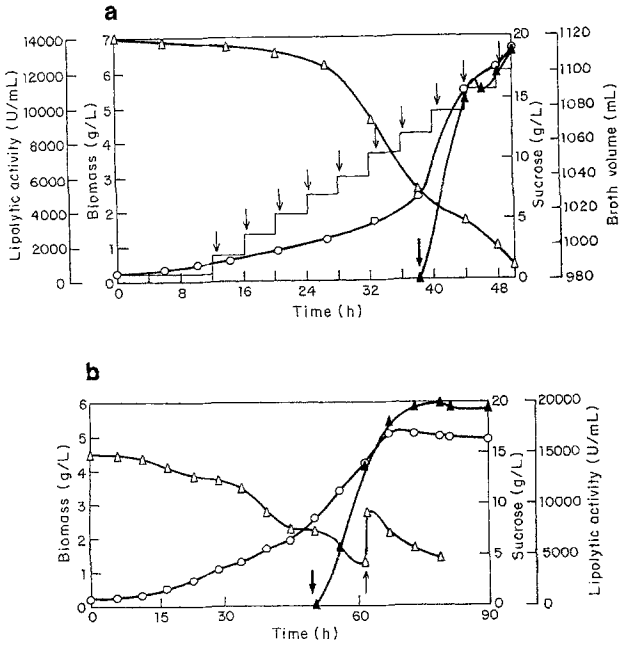

Figure 3 Lipase production on a fed-batch culture on medium AL1. (a) Fermentation number 3. Arrows indicate the addition of 20 ml salt stock to the culture. The addition of 2 mM IPTG is indicated (bold arrow). (b) Fermentation number 4, conditions the same as in (a) but IPTG was added at 46 h (bold arrow), and at 60 h 50 ml of a 10% solution of sucrose was added (arrow). Symbols correspond to: biomass (○), sucrose (△) and lipolytic activity (▲).

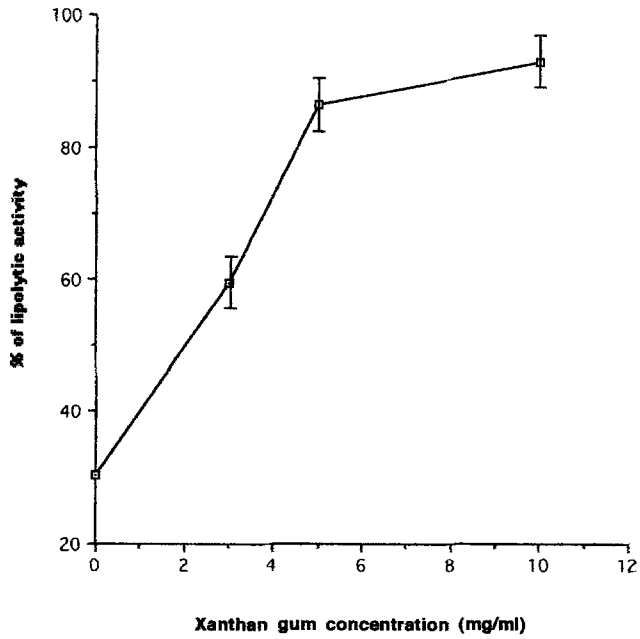

Figure 4 Effect of xanthan gum concentration on the stability of the alkaline lipase produced by *P. aeruginosa* IGB83. The percentage of lipolytic activity remaining after 20 min of incubation at 55°C is shown, 100% = 2120 ± 150 U ml^{-1} (lipase activity prior to incubation).

Table 4 Effect of xanthan gum concentration on the stability of the recombinant alkaline lipase produced by strain IBT148(pBP13)

Xanthan gum (g L^{-1})	Time of lipase inactivation at 55°C (min)[a]
0	7.7
1	10.0
3	14.2
5	16.3

[a]The time of inactivation was considered as the last time when NaOH was added and pH remained unaltered for 2 additional minutes.

campestris IBT148 genetic background, and stable in *P. aeruginosa* PAO2003. The difference in stability might be due to the fact that strain PAO2003 is a *recA* mutant [28], while strain IBT148 is proficient in homologous genetic recombination [18]. We have recently isolated a *recA* mutant derived from *X. campestris* IBT148 [17], and we are currently evaluating the stability of the lipolytic activity encoded by plasmid pBP13 in it.

Under the conditions studied, the recombinant lipase was more efficiently expressed by strain IBT148(pBP13) if the *tac* promoter was induced at the late log phase of growth, this result is in accordance with the production of heterologous proteins by other recombinant bacteria such as *E. coli* [36,37]. The IPTG concentration used in this work was established on the basis of the results reported by Bagdasarian *et al* [1]. For the expression in *Pseudomonas putida* of genes cloned in plasmid pMMB22, they routinely used a concentration of 5 mM IPTG, but their reported level of gene expression was the same with a concentration of 2 mM IPTG. In *P. aeruginosa* PAO2003 and *X. campestris* IBT148 we found a good level of lipase expression with IPTG at a concentration of 1 mM and 2 mM, respectively. At 5 mM, IPTG decreased the growth rate of *X. campestris*.

The effect on lipase expression of lower IPTG concentrations was not determined.

Lipase activity reached a maximum when strain IBT148(pBP13) was grown on medium AL2 and then it declined (data not shown). This decline in lipase activity suggests that the enzyme is inactivated after its secretion. The presence of proteases in *X. campestris* has been reported [7], and the different stability of the lipase on medium AL1 and AL2 might be due to differences in protease expression on these culture media. An important advantage of AL1 medium is that it is suitable for the production of lipase and other enzymes, since being chemically defined, it does not show any problems in downstream operations.

Xanthan gum stabilizes the *P. aeruginosa* IGB83 alkaline lipase. This stabilizing effect was apparent whether the lipase was produced by strain IGB83 or, as an heterologous protein, by *X. campestris*. In this respect *X. campestris* presents advantages to be used as host for the expression of heterologous proteins: it is able to secrete at least some proteins which are secreted by Gram-negative bacteria and can produce xanthan gum which acts as a protein-stabilizing agent. Our results indicate that *X. campestris* has potential as a host for the production of recombinant extracellular proteins [32].

The stability of the alkaline lipase under the assay conditions was much higher when produced by *P. aeruginosa* IGB83 than when expressed by *X. campestris* IBT148(pBP13) (Figure 4 and Table 4). We have previously reported that the lipase half life at 55°C when produced by strain IGB83 is 13 min [22], and we report here that when produced by *X. campestris*, after less than 8 min at the same temperature, lipase was completely inactivated (Table 4). *Pseudomonas* lipases have been reported to form very tight aggregates with lipopolysaccharides, and it has also been reported that the enzyme is much more labile when this polymer is removed [15,31]. The different stability of the alkaline lipase when produced by these two different bacteria might be related to different interaction of the enzyme with the diverse lipopolysaccharides which they produced. Another possibility is that the different stability is a reflection of differences in the processing of the enzyme during the secretion process.

The manipulation of the culture conditions of recombinant bacteria can have a very important effect on the yields of recombinant proteins [10,12,37]. In the case of the lipases from *Pseudomonas*, the optimization of the fermentation conditions has been reported only for lipase production by the native *Pseudomonas* strain [16,33]. This is the first report on the manipulation of culture conditions for increased production of a recombinant *Pseudomonas* lipase: a high increase of lipase productivity was achieved by manipulation of the fermentation conditions of the recombinant strain IBT148(pBP13).

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