

Production in Fed-Batch Reactor of *Bacillus subtilis* LipaseA Immobilized on its own Producer *Saccharomyces cerevisiae* Cells

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Lipases (EC 3.1.1.3, triacylglycerol hydrolases), a subclass of the esterases (EC 3.1.1.1, carboxyl ester hydrolases) are one of the most important groups of biocatalysts for biotechnological uses such as synthesis of biopolymers and biodiesel, production of enantiopure pharmaceuticals, detergent formulation or the production of flavour compounds. Lipases can be commercialized as free or immobilized form. Nowadays it is of great interest to obtain immobilized enzymes especially for industrial applications since immobilization confers stability giving the possibility to recover the biocatalyst after its use, simplifying downstream processing. In the present work, Lipase A from *Bacillus subtilis* has been expressed in different strains of the yeast *S. cerevisiae* as a fusion protein with the yeast cell-wall mannoprotein Pir4. The corresponding gene fusion was created by inserting all the coding sequence of the lipase A gene in the *Bgl*II restriction site of *PIR4* gene, leading to the expression of a fusion protein still containing the four cysteine residues responsible for the anchorage of Pir4 to the yeast cell wall.

Production of lipase as a naturally immobilized biocatalyst was carried out allowing yeast cells to proliferate in a fed-batch reactor under glucose limitation to promote a fully respiratory metabolism and consequently high biomass yield. The performance of the producer strains was evaluated in terms of cell density and enzyme productivity. A preliminary study of economic feasibility of a single fermentation run has been performed based on the experimental results obtained.

1. Introduction

Lipases are used in several industrial applications such as biodiesel production, pharmaceutical synthesis, detergent and food industry. One of the most important applications is in the baking industry, where lipases are used to modify the components of the flour and, as a result, the physical-chemical properties of the dough and/or the bread quality parameters (Poutanen et al., 1997).

In this work, Lipase A from *Bacillus subtilis* has been expressed in three different strains of the conventional yeast *Saccharomyces cerevisiae*: Y306 (10a12-13X28b4; Randez-Gil and Sanz, 1994), a polyploid derivative of an industrial baker's yeast strain bearing one auxotrophy, and CEN.PK113-5D and BY4741, both of them haploid laboratory strains bearing one and four auxotrophies respectively.

Lipase A from *B. subtilis* is one of the smallest lipases known (19 KDa), it shows good hydrolytic activity in medium-length substrate and is used for biotechnological applications including bread making or preparation of technological additives.

The expression strategy used (Mormoneo et al., 2008) permitted to produce Lipase A as a naturally immobilized enzyme on its own producer yeast cells. Immobilization permits multiple uses of enzymes and often enhances their thermal and chemical stability, thus leading to predictable decay rates. It also enhances opportunities for better control of both the process and product quality. In fact, the incremental costs of using an immobilized biocatalyst in a continuous process are more than 20 times lower than with a traditional one, arising primarily from the cost of the relatively large amount of non-reusable enzyme required by the latter process (Murty et al., 2002). In this work, the production of the naturally immobilized

lipase A was carried out in aerated fed-batch reactor and the results obtained by using the three *S. cerevisiae* strains were compared each other.

Based on the experimental results obtained, a preliminary study of economic feasibility of the production process has been performed with the *S. cerevisiae* Y306 strain, highlighting that the process developed would allow for a good profit margin.

2. Materials and methods

2.1 Strains

The strains (and their genotypes) used in this work are the following: *S. cerevisiae* CEN.PK113-5D (MATa ura3-52 HIS3 LEU2 TRP1 MAL2-8c SUC2), *S. cerevisiae* BY4741 (MATa,ura3Δ0, leu2Δ0, met15Δ0, his3Δ1) and *S. cerevisiae* Y306 (10a12-13X38b4), a polyploid strain auxotroph for tryptophan kindly provided by Dr. F. Ranz-Gil (IATA, CSIC, Valencia).

2.2 Gene fusion strategy and expression of Lipase A in *S. cerevisiae* strains

The gene fusion *PIR4/LipA* used consisted in the insertion of the coding sequence of *Bacillus subtilis lipA* gene (Dartois et al., 1992), minus the 5' region coding leader peptide, in the *BglII* site of *PIR4*. For this, a 542-bp fragment of *lipA* was amplified using oligonucleotides LBGL5 (TTCAGCAGATCTTAGCTGAACACAATCCAGTC) and LBGL3 (GGTATAAGATCTTATTCGTATTCTGGCCCC) and plasmid pBR322lipA as template. The oligonucleotides included the restriction sites for the enzyme *BglII* and had been designed so that the *lipA* fragment was inserted in-frame in *PIR4* in construction pIA1 (Andrés et al., 2003). The PCR fragment, amplified using Expand High Fidelity DNA Polymerase (Roche), was subcloned in the *HincII* site of pUC18; digested out with *BglII* and inserted in pIA1 previously digested with *BglII*. The correct orientation of the inserts was monitored by PCR performed directly on the colonies of transformants using specific oligonucleotides.

The resulting construction was transformed into the different strains and expression of lipase activity was confirmed on tributyrin agar plates (0.25 % peptone, 0.25 % casein, 0.3 % yeast extract, glucose 2 % w/v, 1 % v/v glycerol tributyrate) by determining the presence of haloes of degradation around the colonies.

2.3 Shake flask cultures

500 mL shake flasks were inoculated (0.2 initial Optical Density at 590 nm, OD₅₉₀) from pre-cultures prepared by using stock cultures kept at -80 °C in 12.5 % v/v glycerol. In both cases, flasks contained 100 mL of a defined mineral medium (Verduyn et al., 1992) with 5 % w/v initial α-D glucose pH 5.0, supplemented with 1% w/v hydrolyzed casein (BD Bacto™ Casamino Acids, Becton Dickinson & Co., Sparks, MD 21152 USA), and, when there was the need, also uracil (7.5 mL of a 0.2 % w/v stock solution) or tryptophan (0.8 mL of a 0.5 % w/v stock solution). Pre-cultures and cultures were incubated at 30 °C at 220 rpm (Stuart Scientific S150 Orbital Incubator) for 24 h.

2.4 Fed-batch cultures

Fed-batch cultures have been performed at 30 °C in a 2.0 L working volume of a stirred fermenter, Bioflo 110 (New Brunswick Scientific). The fermenter initially contained 1 L of the defined mineral medium above mentioned and was inoculated to give an initial OD₅₉₀ of 0.04. After a 15 h batch phase, which allowed glucose in the medium to be completely consumed, fed phase of 25 h, started by applying an exponentially increasing feed to allow the cells to proliferate at a constant specific growth rate (0.16 h⁻¹), lower than the critical one (Enfors, 2001). The feeding solution (0.9 L) contained glucose (50 % w/v), salts (KH₂PO₄ 15.70, KCl 5, MgSO₄·7H₂O 5.83, CaCl₂·2H₂O 1.20, NaCl 0.44, FeSO₄·7H₂O 0.25 g L⁻¹), trace elements (ZnSO₄·7H₂O 50, CoCl₂·6H₂O 2, CuSO₄·5H₂O 40, MnCl₂·4H₂O 50 mg L⁻¹), glutamic acid (1 g L⁻¹), vitamins (biotin 4, calcium pantothenate 40, nicotinamide 90, myo-inositol 50, thiamine HCl 100, pyridoxine HCl 20 mg L⁻¹), and hydrolyzed casein, the concentration of which was calculated according to Paciello et al. (2010), taking into account the value of biomass yield for the given amino acid under aerobic conditions (Pronk, 2002). Oxygen was supplied by air sparging (DOT 30 % air saturation). The culture pH was maintained at 5.0 by automatic addition of 2 N KOH during batch phase and 10 % v/v NH₄OH during exponential phase. The foam level in the fermenter was controlled by the automatic addition of the antifoam B (dil. 1:10) (Sigma Aldrich).

2.5 Determination of cell density

Cellular density was determined by optical density (OD₅₉₀) and dry weight. The calibration curve relating OD₅₉₀ values to biomass density provided a correlation factor of 1.85, 2.1, and 2.45 OD₅₉₀ per mg mL⁻¹ for *S. cerevisiae* Y306, CEN.PK, and BY4741 strains.

2.6 Determination of lipolytic activity

Lipolytic activity was determined by a colorimetric assay according to Prim et al. (2000; 2001) in which the release of *para*-nitrophenol (*p*NP) from *p*NP-butyrate was measured at OD₄₀₅. One international unit of activity (U) was defined as the amount of enzyme necessary to release 1 μ mol of Pnp/h under the assay conditions described.

3. Results and discussion

3.1 Lipase activity

For each strain examined, as expected, most lipolytic activity resulted, to be associated to the microbial cells, catalytic activity in the supernatants being quite negligible (Table 1). Even after cell-breakage with Ballottini beads (\varnothing 425 ÷ 600 μ m) no increase in enzymatic activity was observed in the supernatant (data not shown), accordingly, cytoplasmic localization of lipolytic activity was excluded.

S. cerevisiae strains employed in this work, exhibited different levels of lipase activity (Table 1). The best performer strain resulted to be BY4741 which displayed the highest values of both volumetric and specific lipolytic activity (Table 1).

Table 1: Lipolytic activity of transformed *S. cerevisiae* strains growing in shake flask culture

Transformed strain	Cell density [g d.w. L ⁻¹]	Volumetric Activity [U L ⁻¹]		Specific Activity [U g ⁻¹]	
		Supernatant	cells	Supernatant	cells
CEN.PK	3.43	17.0 ± 0.8	431 ± 20	5.0 ± 0.2	125 ± 6
BY4741	3.39	26 ± 1.3	590 ± 25	7.7 ± 0.4	174 ± 8
Y306	6.06	25 ± 1.2	88 ± 5	4.1 ± 0.2	15.0 ± 0.8

3.2 Fed-batch growth of untransformed and transformed *S. cerevisiae* strains

Fed-batch runs (Figure 1A and 1B) were set up with untransformed and transformed cells of the three *S. cerevisiae* strains, to look for any metabolic burden due to the peculiar lipase expression system used and to evaluate the naturally immobilized lipase production. The untransformed strains (Figure 1A), except for BY4741, a weak strain bearing four auxotrophies (Paciello et al., 2009; Landi et al., 2011), displayed a behavior very near to the ideal one whereas the transformed strains (Figure 1B) arrested their growth relatively early, achieving a cell density significantly lower than expected. This behavior suggests that lipase A expression during fed-batch propagation represents a strong metabolic burden.

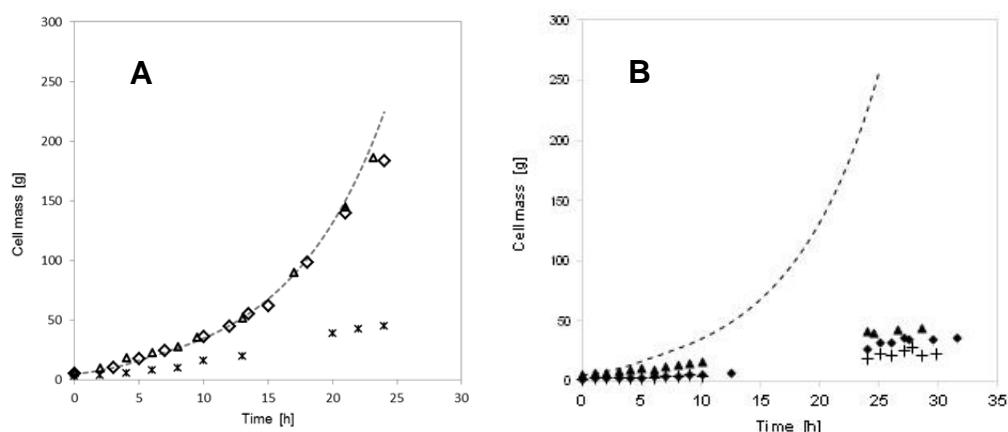


Figure 1: Growth in aerated fed-batch of the untransformed *S. cerevisiae* CEN.PK113-5D (empty rhombus), Y306 (empty triangle) and BY4741 (star) at the same μ value of 0.16 h⁻¹ (A). Growth in fed-batch reactor of the transformed strains *S. cerevisiae* CEN.PK113-5D (full rhombus), Y306 (full triangle) and BY4741 (plus) at μ value of 0.16 h⁻¹ (B). Dotted line represents the theoretical biomass obtainable with the feeding profile

Moreover, the cells of the transformed strains appeared significantly lengthened if compared to those of the untransformed strains. Optical microscopic observations of the phenomenon are reported in Figure 2 for untransformed (on the left) and transformed CEN.PK 113-5D (on the right) cells. The morphological change may be ascribed to the deleterious effect of lipase localization. The treatment of yeast cells with 10 mM DDT (dithiothreitol) did not release any lipase activity in the supernatant (data not shown), so that it was conceivable that the fusion protein Pir4-LipA could interact with yeast cell-wall through components different from cystein residues. This interaction could be responsible for both inhibition of budding process and yeast growth so as to reduce cell density in the fed-batch reactor (Figure 1B).

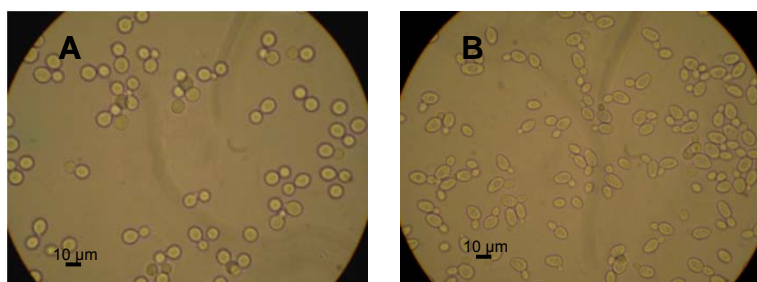


Figure 2: Cell morphology (o.m. 100x) of *S. cerevisiae* CEN.PK 113-5D strain before (A) and after (B) transformation during fed-batch proliferation

The evaluation of the naturally immobilized lipase A production in fed-batch reactor was made comparing enzymatic activities (either volumetric or specific) exhibited by the three selected strains after 10 and 25 h of feeding (Figures 3A and B). *S. cerevisiae* strains produced different amounts of lipase in terms of specific activity during fed-batch runs (Figure 3A), with BY4741 being the best producer. This activity decreased significantly after 10 h and dropped at the end of the feeding (25 h) (Figure 3A). Considering that the maximum volumetric activity achieved by each strain (Figures 3B) at the end of feeding was slightly higher than that obtained after 10 h (due to growth inhibition), it would be possible to interrupt the fermentation after 10 h of feeding with a considerable saving of the carbon source (glucose).

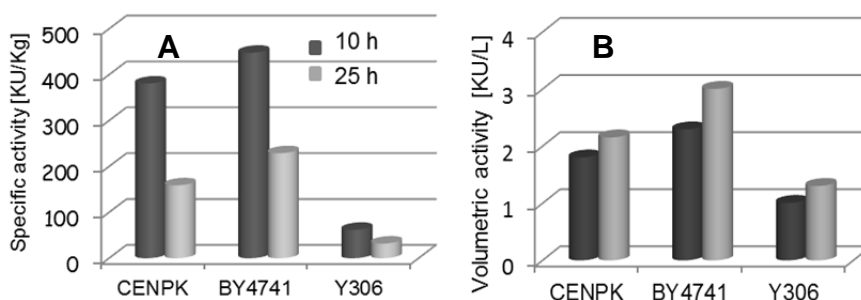


Figure 3: Specific (A) and volumetric (B) activity of the three *S. cerevisiae* lipase producer strains. The results refer to samples collected from the fermenter after 10 and 25 h of feeding

3.3 Evaluating the economic feasibility of lipase A production in fed batch reactor

The economic evaluation was carried out on an industrial scale using the experimental data obtained with *S. cerevisiae* Y306 strain which exhibited the lowest performance (Figure 3), so representing the less favourable case.

This study consisted on the evaluation of bioprocess costs, in terms of raw material and operating plant costs for a single fermentation cycle. The plant cost was not taken into consideration and has been considered as already amortized.

The fermenter chosen had a working volume of 25 m³ and the operative conditions were those employed in the fed batch runs described above, with batch and fed-batch phases lasting 15 and 10 h respectively. The volumetric lipolytic activity at the end of fermentation run was calculated to be 1 kU L⁻¹ of broth culture. Considering that the economic value of lipase A on the market is 4.6 US\$ (USD) per kU (Castilho et al., 2000) and considering the sum of the partial costs including the operating labor costs (Table 2), it's

possible to obtain the total cost of fermentation (53,450 US\$) and it's easy to verify that the unitary product cost (2.14 US\$/kU) is lower than the economic value of Lipase A on the market. Therefore, the industrial scale production of this enzyme, using this particular bioprocess, could be made profitable.

Table 2: Raw material and operating costs of an industrial scale fermenter producing heterologous lipase with *S. cerevisiae* Y306

Raw Materials	Unitary cost (US\$/t)	Quantity (t)	Total cost (US\$)
Glucose	26,140	1.284	33,564
Tryptone soya	72,700	0.228	16,576
(NH ₄) ₂ SO ₄	260	0.114	30
KH ₂ PO ₄	1,200	0.094	113
MgSO ₄ · 7 H ₂ O	130	0.021	3
Antifoam	2,500	0.045	113
KOH	1,350	0.045	61
NH ₄ OH	407	0.095	39
Utilities			
Steam	8 US\$/m ³	50 m ³	400
Cooling water	1.2 US\$/m ³	585 m ³	702
Electric energy	0.187 US\$/KWh	4,512 KWh	844
Operating labor			1,000
Total			53,450

4. Conclusions

Three *S. cerevisiae* strains able to produce a heterologous naturally immobilized lipase have been created by transformation of the strains with plasmids containing the *lipA* gene from *B. subtilis* fused with and under the promoter control of the *PIR4* cell-wall protein coding gene. Lipase immobilization has been pursued to facilitate downstream processes, that is, the separation of the catalyst from the broth-culture which, in this case, may take place simply by centrifugation, without forgetting the greater manageability, the increased storage and operative stability as well as the presence of the enzyme in a microorganism considered as GRAS by FDA. This allows, among other potential uses, the use of immobilized lipase in baking industry to render bread with a higher loaf volume and a more uniform crumb structure. At the moment it has not been yet elucidated how the fusion protein is so strongly associated to the yeast cell. The inabilities to release the enzyme by treatment with reducing agents suggest other forms of attachment, alternative or additional to disulphide bridges. Giving an answer to this question would be of great importance to understand the complete immobilization process.

Finally, of the three *S. cerevisiae* strains assayed in our study, BY4741 revealed to be the best performer in terms of both volumetric and specific activity. Notwithstanding this, the study of the economic feasibility of the bioprocess was carried out with the worst performer, Y306, to allow for any possible non-controlled factor and to make sure of the cost-effectiveness of the production process.

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