Production of Wild-Type and Peptide Fusion Cutinases by Recombinant *Saccharomyces cerevisiae* MM01 Strains

Cecília R. C. Calado,[1] Maurice Mannesse,[2] Maarten Egmond,[2] Joaquim M. S. Cabral,[1] Luis P. Fonseca[1]

[1]Centro de Engenharia Biológica e Química, Instituto Superior Técnico, Av. Rovisco Pais, 1049-001 Lisboa, Portugal; telephone: 351-21-8419065; fax: 351-21-8419062; e-mail: lfonseca@alfa.ist.utl.pt
[2]Unilever Research Laboratory, Vlaardingen, 3133 AT Vlaardingen, The Netherlands

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Abstract: This study focused on the growth of *Saccharomyces cerevisiae* MM01 recombinant strains and the respective production of three extracellular heterologous cutinases: a wild-type cutinase and two cutinases in which the primary structure was fused with the peptides $(WP)_2$ and $(WP)_4$, respectively. Different cultivation and strategies were tested in a 2-L shake flask and a 5-L bioreactor, and the respective cell growth and cutinase production were analyzed and compared for the three yeast strains. The highest cutinase productions and productivities were obtained in the fed-batch culture, where wild-type cutinase was secreted up to a level of cutinase activity per dry cell weight (specific cell activity) of 4.1 Umg^{-1} with activity per protein broth (specific activity) of 266 Umg^{-1}, whereas cutinase-$(WP)_2$ was secreted with a specific cell activity of 2.1 Umg^{-1} with a specific activity of 200 Umg^{-1}, and cutinase-$(WP)_4$ with a specific cell activity of 0.7 Umg^{-1} with a specific activity of 15 Umg^{-1}. The results indicate that the fusion of hydrophobic peptides to cutinase that changes the physical properties of the fused protein limits cutinase secretion and subsequently leads to a lower plasmid stability and lower yeast cell growth. These effects were observed under different cultivation conditions (shake flask and bioreactor) and cultivation strategies (batch culture versus fed-batch culture). © 2002 Wiley Periodicals, Inc. *Biotechnol Bioeng* **78**: 692–698, 2002.

Keywords: cutinase; *Saccharomyces cerevisiae;* heterologous protein; secretion; hydrophobic peptides; fusion tags

INTRODUCTION

Cutinase from *Fusarium solani pisi* is a versatile enzyme showing several noteworthy properties for industrial applications (Carvalho et al., 1999). Cutinase has been cloned in *Saccharomyces cerevisiae* as this yeast has become an important host in industrial biotechnology (Barthel and Kula, 1993; Shi et al., 1993).

Unlike *Escherichia coli*, *S. cerevisiae* lacks detectable endotoxins, is a generally-regarded-as-safe organism for the production of food and pharmaceutical products, and it performs some post-translational and post-transcriptional modifications that are characteristics of eukaryotes (Romanos et al., 1992). Furthermore, because of a long history of commercial use, substantial information already exists on traditional yeast cultivations (Calado et al., 2001; Ejiofor et al., 1994, 1996). Yeast genetics are relatively well known and, from a bioprocessing point of view, their extracellular secretion capability of the yeast allied to a very small secretion of its own proteins to the medium is an attractive feature (Calado et al., submitted; Chisti and Moo-Young, 1994).

The secretion of a fully activated enzyme eliminates many of the processing steps required to isolate activated cytoplasmatic proteins. Unfolding in denaturing steps and refolding, which is frequently inefficient in vitro, are not required. The export of the product protein from the host cell also reduces the risk of protein degradation by intracellular proteases, allows glycosylation, and favors disulfide bond formation (Bitter et al., 1987; Das and Shultz, 1987; Novick et al., 1981; Smith et al., 1985). The extracellular secretion of a recombinant protein also simplifies downstream processing. This last capability is very important because the cost of the product recovery with importance for pharmaceutical/clinical research and industrial use becomes critical to the overall process economics, representing 50 to 90% of the total costs (Diamond and Hsu, 1992; Raghavarao et al., 1995).

To enhance heterologous recovery and purification processes, genetic engineering can be used because of its ability to confer particulate properties to a protein, where a most common modification involves the fusion of peptide tails to the protein (Bandmann et al., 2000; Berggren et al., 2000; Carlsson et al., 1996; Costa et al., 2001; Eiteman et al., 1994; Hassinen et al., 1994; Köhler et al., 1991). In an effort to combine the advantages of cutinase secretion to the extracellular medium by recombinant *S. cerevisiae* and the advantages of low cost and scalability of product recovery from cultivation

Correspondence to: L. P. Fonseca

medium by aqueous two-phase system, two cutinase variants were constructed by genetic fusion small hydrophobic peptides $(WP)_2$ and $(WP)_4$ to cutinase. However, the hydrophobicity of cutinase can lead to impaired protein secretion in *S. cerevisiae* (Sagt et al., 1998). Indeed, many factors influence the secretion efficiency and productivity of heterologous products including host strain and protein characteristics, signal/leader sequence, promoter strength, expression vector, chaperon availability, and environmental factors such as bioreactor operational strategy and medium composition (Das and Shultz, 1987; Novick et al., 1981; Smith et al., 1985).

The aim of this study was to evaluate the effect of the fused $(WP)_2$ and $(WP)_4$ peptides on the yeast growth and cutinase production. Thus, the extracellular cutinase activity and cell growth of recombinant *S. cerevisiae* MM01 strains producing wild-type cutinase (wt-cutinase) and peptide fusion cutinases [cutinase-$(WP)_2$ and cutinase-$(WP)_4$], were compared at different cultivation conditions. To improve the heterologous cutinase production, a fed-batch culture strategy was developed because this type of cultivation by providing a more tight control over environmental variables, such as the state variable glucose, may improve the overall product yield compared with simple batch cultures (Ejiorfor et al., 1994; Lang et al., 1997). Finally, the effect of $(WP)_2$ and $(WP)_4$ peptides fusion to cutinase in relation to the wild-type strain using batch and fed-batch culture strategies was also investigated.

MATERIALS AND METHODS

Microorganism and Inoculum Preparation

The cutinase wild-type-, cutinase-$(WP)_2$-, and cutinase-$(WP)_4$-producing *S. cerevisiae* MM01 strains (Mata, leu2-3, *ura*3, *gal*1: URA3, *MAL*-8, *MAL*3, *SUC*3) contained the expression vectors pUR7320, pUR807, and pUR806, respectively. The synthetic cutinase gene (van Gemeren et al., 1995) was placed behind the invertase signal sequence under control of a *GAL*7 promoter. The plasmids were integrated on the chromosomal ribosomal DNA locus and the constructs contained a Leu2d gene (Erhart and Hollenberg, 1983) enabling selection on Leu-lacking plates.

Both pUR807 and pUR806 were derived from pUR7320 by direct polymerase chain reaction methods using primers containing the sequence enconding the peptide extensions $(WP)_2$ and $(WP)_4$, respectively. The *Hin*III/*Bg*III polymerase chain reaction fragments were cloned into the statement vector pUR7320 resulting in pUR807 [cutinase-$(WP)_2$] and pUR806 [cutinase-$(WP)_4$]. These strains were constructed and provided by Unilever Research Laboratory, Vlaardingen, The Netherlands.

The stock cultures (50% (v/v) were maintained in glycerol and selective medium (on Leu-lacking agar plates) at −80°C. The composition of the medium for inoculum preparation (selective medium) was: 20 $g \cdot L^{-1}$ D(+)-glucose anhydre (Merck, Darmstadt, Germany), 6.7 $g \cdot L^{-1}$ of yeast nitrogen base without amino acids (Difco Laboratories, Detroit, MI). The pre-cultivations were performed in shake flasks at 30°C and 200 rpm in an orbital shaker (Agitorb 160E, Aralab, Lisbon, Portugal) during 24 h.

Cultivation

Yeast cells cultured in the selective medium were transferred, at an inoculum volume of 10% (v/v) in relation to the final fermentation medium volume to a 2-L shake flask or to a 5-L bioreactor (Biostat MD, B. Brown Biotech Int., Germany) with working volumes of 1 L and 4 L, respectively. The cultivations in shake flasks were performed in an orbital shaker (Agitorb 160E, Aralab) at 30°C, 200 rpm, where the pH was manually controlled at pH 5.5 ± 0.5.

Cultivations performed in the bioreactor were maintained at pH 5.5 by automatic control through NaOH or HCl 2N addition, and at 30°C with a minimum of dissolved oxygen tension of 15% of air saturation, achieved by aeration at a constant air flow rate of 4 $L \cdot min^{-1}$ (41 vvm) and by adjusting automatically the agitation rate.

The batch cultivation medium consisted of 10 $g \cdot L^{-1}$ yeast extract (Difco), 10 $g \cdot L^{-1}$ peptone bacteriological (BDH), 20 $g \cdot L^{-1}$ glucose (Merck) and 25 $g \cdot L^{-1}$ of D(+)-galactose anhydrous (Sigma Chemical Co., St. Louis, MO).

A two-stage fed-batch cultivation was performed in a 5-L B. Braun bioreactor (Biostat MD). The first phase was a batch growth phase initiated by transfer of inoculum cells to a 2-L culture medium containing 20 $g \cdot L^{-1}$ yeast extract (Difco), 10 $g \cdot L^{-1}$ peptone bacteriological BDH, and 20 $g \cdot L^{-1}$ of D(+)-glucose (Merck). After 18 h of the batch growth phase, 100 g of D(+)-galactose (Sigma) in 400 mL of water was added for induction of cutinase expression.

After induction, an exponential feeding phase was started by addition of 2.1 L of a concentrated a mixture of 260 $g \cdot L^{-1}$ of glucose and 60 $g \cdot L^{-1}$ of yeast extract sterilized separately, considering a constant specific growth rate of $\mu = 0.14\ h^{-1}$, according to the following:

$$F = \frac{\mu V_o X_o}{C_s Y_{X/S}} \cdot \exp^{\mu \cdot t}$$

where F is the feed flow rate, μ the biomass specific growth rate, V_0 the bioreactor volume of the batch phase, X_0 the biomass concentration at the point where batch mode is changed to fed-batch mode, C_s the substrate (glucose) concentration in the feed, and $Y_{X/S}$ the biomass to substrate yield and t the cultivation time.

Assays

Cell concentration, cutinase activity, specific cutinase activity (cutinase activity relative to total protein in the broth), specific cell activity (cutinase activity relative to dry cell weight in the broth), and productivity (enzyme activity relative to the cultivation time and dry cell weight in the broth) were the parameters used to characterize the cultivation performance.

Cell Concentration as Dry Cell Weight per Volume of Culture Medium

Optical density was measured at 600 nm after appropriate dilution with 0.8% (w/v) NaCl. Dry cell weights (dcw) were obtained by filtrating the culture and subsequently drying the filter in a oven at 105°C until constant weight. Measurements of dcw were correlated with optical density. Subsequently optical density measurements were converted to dcw per volume of culture medium using the previous determined correlation factor.

Plasmid Stability

The plasmid-containing fraction of the population was determined by replica plating from non-selective medium to a selective medium (similar to selective medium but with 20 mg/L L-leucine; Merck).

Cell Viability

Cell viability represents the fraction of viable cells, determined after optical microscopic examination of cellular suspension stained with methylene blue as described by Lange et al. (1993).

Protein Determination and Cutinase Activity Assay

The protein concentration was determined by the method of Bradford (1976). The cutinase estereolytic activity was determined spectrophotometrically, following the hydrolysis of *p*-nitrophenylbutyrate at 400 nm. Twenty microliters of sample was added to 980 μL of a 0.56 m*M* p-nitrophenylbutyrate solution in 50 m*M* potassium phosphate, buffer pH 7, with 11.3 m*M* sodium cholate and 0.43 *M* tetrahydrofuran (reaction mixture). The reactions were followed for one minute against the blank solution. One unit of activity was defined as the amount of enzyme required to convert one mole of *p*-nitrophenylbutyrate in *p*-nitrophenol in 1 min under the specified conditions. The extinction coefficient of *p*-nitrophenol was considered to be 1.84×10^4 $M^{-1}cm^{-1}$, as indicated by the supplier Sigma. The three heterologous purified cutinases, with or without fused peptides, presented the same specific cutinase activity (unpublished data).

Enzyme Secretion Efficiency

It was estimated as the ratio between extracellular cutinase activity and total cutinase activity (extracellular and intracellular) after cell disruption. Cell disruption was performed by vigorous mixing with 500-μm glass spheres, at 50% (v/v) glass spheres and suspended yeast cells in 0.8% (w/v) NaCl, during 5 min, with 1-min intervals in ice. The high cell disruption efficiency obtained, of almost 100%, was measured after cellular optical microscopic examination.

RESULTS AND DISCUSSION

Effect of Cultivation Conditions

S. cerevisiae strains were grown in a 2-L shake flasks with 1 L working volume (Fig. 1). The growth profiles of the *S. cerevisiae* strains producing wt-cutinase and cutinase-$(WP)_2$, respectively, were very similar. However, the yeast-producing cutinase-$(WP)_4$ presented after 60 h slight lower biomass concentration (Fig. 1A). Both producing strains of cutinase with hydrophobic peptides, in relation to wt-cutinase producing strain, presented always lower cutinase activities (Fig. 1B), specific

Table I. Percentage of viable cells, percentage of cells containing plasmids, and secretion efficiency for the wt-cutinase, cutinase-$(WP)_2$, and cutinase-$(WP)_4$ producing strains, along the batch cultivation in 2-liter shake flasks.

	Time (h)	wt-Cutinase	Cutinase-$(WP)_2$	Cutinase-$(WP)_4$
Cell viability (%)	24	99	99	97
	48	99	98	96
	60	97	97	96
Plasmid stability (%)	24	98	96	80
	48	98	77	76
	60	88	77	63
Secretion efficiency (%)	24	62	–[a]	10
	48	70	62	32
	60	72	65	34

[a]Not determined

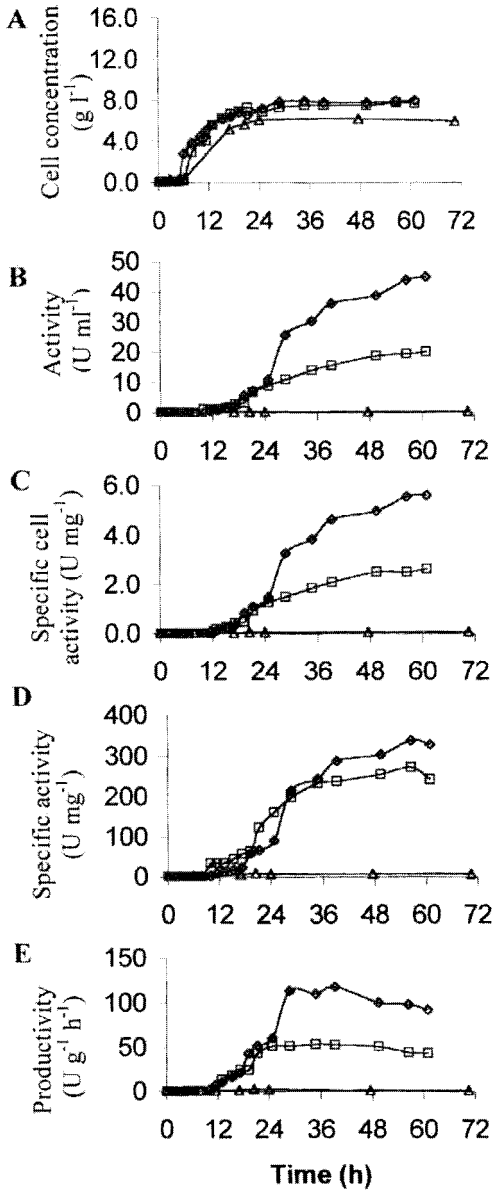

Figure 1. Heterologous cutinase production and cell growth of *S. cerevisiae* by batch cultivation in a 2-L shake flask at 30°C, 200 rpm. wt-Cutinase (◇), cutinase-$(WP)_2$ (□), cutinase-$(WP)_4$ (△).

cell activities (Fig. 1C), specific activities (Fig. 1D), and productivities (Fig. 1E). Thus, the strains producing wt-cutinase, cutinase-$(WP)_2$ and cutinase-$(WP)_4$ presented at 48 h of fermentation cutinase specific cell activities of 5.0, 2.5, and 0.1 U mg^{-1} dcw, respectively.

The extracellular activity decreases in cultures of hydrophobic cutinase production strains resulted most probably from the cellular retention of the more hydrophobic cutinase variants and from a decrease cutinase gene expression (Table I). Indeed, along the cultivation, the three strains that displayed a similar and high cell viability presented, however, different plasmid stabilities (Table I). Where, the wt-cutinase producer presented always high plasmid stability, whereas the cutinase-$(WP)_4$-producing strain presented the lowest plasmid stability.

The three strains also present different secretion efficiencies, where the wt-cutinase-producing strain exhibited the highest cutinase secretion efficiency, whereas cutinase-$(WP)_4$ presented the lowest cutinase secretion efficiency. This last observation is in agreement with Sagt et al.'s (1998) experiments concerning a large series of *Fusarium solani pisi* cutinase mutants expressed in *S. cerevisiae* SU50 that were constructed to improve the interaction with lipid substrates. These authors observed that the hydrophobic mutant CY028 was secreted at a very low level when compared to cutinase wild type, the differences in cutinase secretion due to a retention of the more hydrophobic cutinase mutant in the endoplasmatic reticulum, probably as a result of an interaction with the immunoglobulin heavy-chain binding protein.

The three strains grown in a 5-L Biostat bioreactor presented differences in yeast cell growth (Fig. 2A), extracellular cutinase activity (Fig. 2B), cutinase specific cell activity (Fig. 2C), cutinase specific activity (Fig. 2D) and productivity (Fig. 2E). The strains producing wt-cutinase, cutinase-$(WP)_2$, and cutinase-$(WP)_4$ presented at 48 h of bioreactor culture cutinase specific cell activities of 2.4, 0.7, and 0.1 U mg^{-1} dcw, respectively.

By comparing the batch cultivations performed in shake flask and in the bioreactor, it is observed that the environment influenced both the production of the cloned protein and the growth of *S. cerevisiae*. At 48 h of cultivation, in a controlled environment, with a oxygenation at a minimum of 15% dissolved oxygen, in relation to the 2-L shake flask cultivations, the wt-cutinase and cutinase-$(WP)_2$ strains presented higher biomass concentrations, but lower cutinase activities and specific activities. With the cutinase-$(WP)_4$ strain, it was observed higher biomass concentration in the bioreactor, but in opposition with the other two strains higher cutinase activity and higher specific activity were obtained.

These differences could result in part from different levels of oxygenation between the 2-L shake flask and the 5-L bioreactor. The higher dissolved oxygen concentrations obtained in the 5-L bioreactor can minimize the fermentation of sugars to ethanol and consequently maximizing biomass yield due to the higher ATP yield from respiratory metabolism. However, higher dissolved oxygen concentrations can minimize cutinase production. Indeed, in a previous work Calado et al. (submitted) performed with a recombinant *S. cerevisiae* SU50 strain containing the cutinase expression vector pUR7320, the same expression system used for production of wt-cutinase in *S. cerevisiae* MM01 strain, it was also observed that low dissolved oxygen concentrations is more suitable for cutinase production.

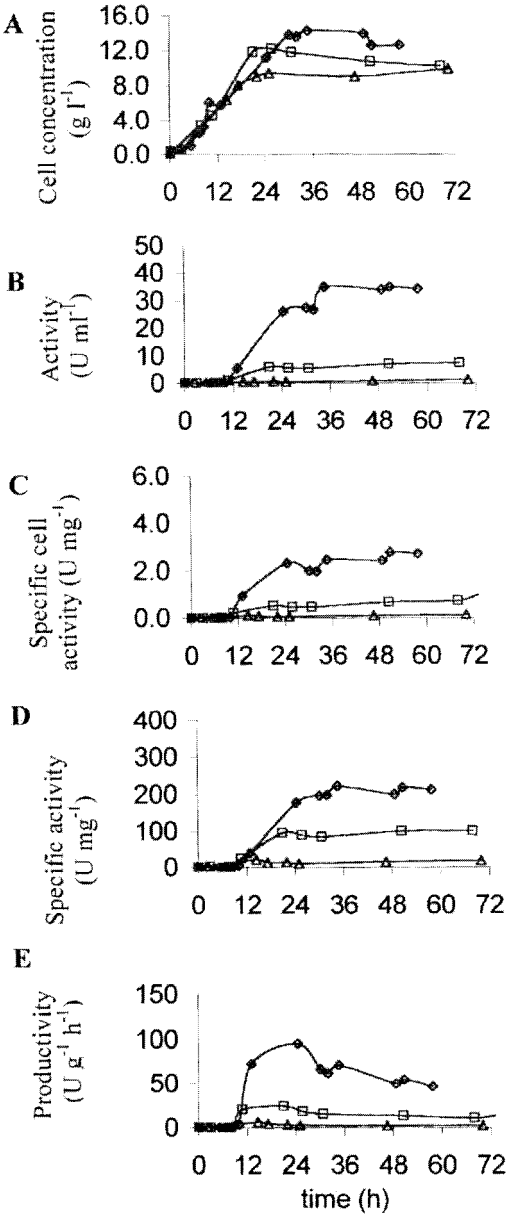

Figure 2. Cutinase production and cell growth of *S. cerevisiae* by batch cultivation a 5-L bioreactor. The conditions were maintained at pH 5.5, 30°C with a minimum 15% dissolved oxygen (controlled by agitation rate) and 1.1 vvm air. wt-cutinase (◇), cutinase-$(WP)_2$ (□), cutinase-$(WP)_4$ (△).

In summary, the increase of hydrophobic length of the peptide $(WP)_n$ fused to wt-cutinase, from n = 2 and n = 4, increased the retention of heterologous cutinase on host yeast cells and decreased the plasmid stability. The different growth profiles and plasmid stabilities observed between the several yeast strains are most probably due to the increased metabolic stress, which resulted from this impaired secretion of the more hydrophobic cutinases. As major consequences of the increased hydrophobic peptide length were the decrease on cell concentration, cutinase specific cell activity, cutinase specific activity and productivity.

Effect of Cultivation Strategy (Batch Culture vs. Fed-Batch Culture)

To achieve high yield, high volumetric productivity and high product concentration, fed-batch cultivation is commonly used to attain high-density recombinant cell cultivation. The exponential feed was chosen as this strategy is common used in fed-batch cultures of recombinant microorganism for limiting the concentration of glucose while maximizing productivity (D'Anjou and Daugulis, 1997; Wangsa-Wirawan et al., 1997). A constant specific growth rate of $\mu = 0.14\ h^{-1}$ was chosen as this value represents an intermediate value between the used values by the authors previously refereed.

Plasmid instability is a major concern in recombinant yeast cultivations (Zabriskie and Arcuri, 1986). In the fed-batch cultivation in order to maximize plasmid stability and because the cutinase gene was cloned behind *GAL7* promoter, a two-stage culture was used, comprising one yeast growth phase followed by a cutinase production phase. In the batch growth phase, cutinase production is repressed due to the fact that yeast cells grow in a complex medium containing glucose as a carbon source and in the absence of galactose. In this phase, due to the initial high glucose concentrations, cells will use the fermentative pathway to growth on glucose, resulting in a buildup of ethanol, consequently a diauxic exponential growth on glucose, and in ethanol as sequential carbon sources will occur (data not shown). Thus, the feed phase was started only after the ethanol consumption. Just before the beginning of the exponential feed phase, the cloned cutinase production is induced by galactose addition and by maintaining low glucose concentrations.

By using a two-phase cultivation, higher cell density cultivations were obtained (Fig. 2A; Fig. 3A): The strains producing wt-cutinase, cutinase-$(WP)_2$ and cutinase-$(WP)_4$ presented at 48 h of fermentation in the batch culture performed in the bioreactor biomass concentrations of 13.9, 10.7, and 9.0 g dcw $\cdot$ L^{-1}, respectively, and cutinase yields on glucose of 1.7, 0.4, and 0.05 U $\cdot$ g^{-1} glucose, respectively, whereas, by fed-batch cultivation these strains presented biomass concentrations between 34 and 38 g $\cdot$ L^{-1} and cutinase yields of 2.4, 1.3, and 0.03 U $\cdot$ g^{-1} glucose, respectively.

Thus, by using this fed-batch strategy, in relation to the batch cultivation, it was possible to obtain higher cell density and cutinase activities. Furthermore, for the strains producing wt-cutinase and cutinase-$(WP)_2$ it was also possible to increase the specific cell activity, the specific activity, productivity and the cutinase yields on the carbon source.

By comparing the fed-batch cultivation of the three strains, it is also observed that the increased hydrophobic length of the peptide $(WP)_n$, from n = 2 to n = 4, led to a decreases of cutinase extracellular activity. The wt-cutinase, cutinase-$(WP)_2$ and cutinase-$(WP)_4$

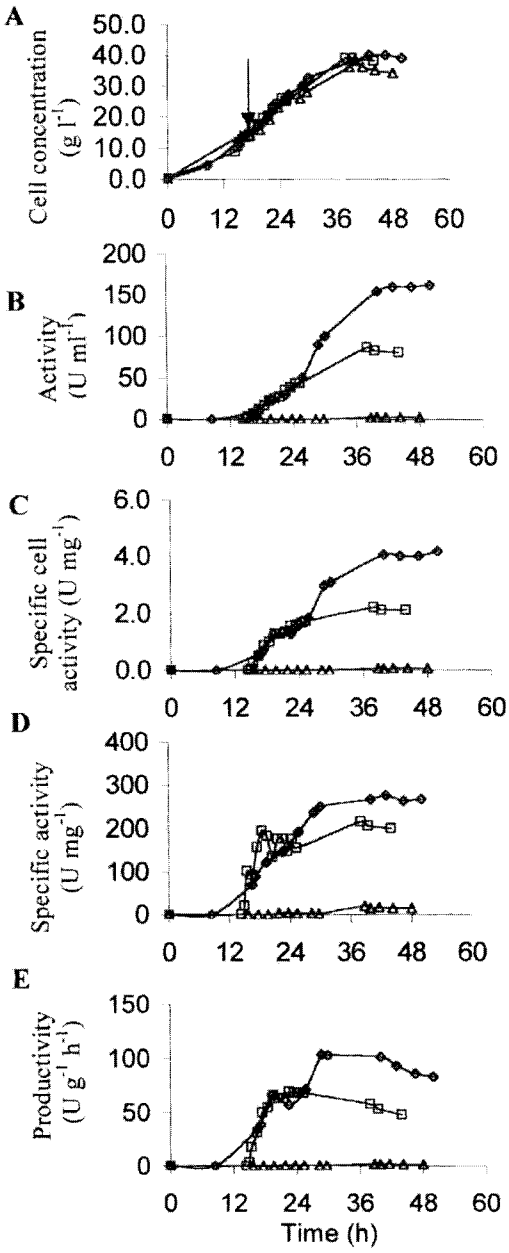

Figure 3. Cutinase production and cell growth of *S. cerevisiae* by fed-batch cultivation in a 5-L bioreactor. The conditions were maintained at pH 5.5, 30°C with a minimum 15% dissolved oxygen (controlled by agitation rate) and 1.1 vvm air. wt-Cutinase (◇), cutinase-$(WP)_2$ (□), cutinase-$(WP)_4$ (△). The arrow represents the induction of cutinase gene expression by galactose addition.

producing strains presented final extracellular activities of 162, 88, and 2 $U \cdot mL^{-1}$, specific cell activities of 4.1, 2.1, and 0.7 $U \cdot mg^{-1}$ dcw, specific activities of 266, 200, and 15 $U \cdot mg^{-1}$ and productivities of 84, 48, and 1 $U \cdot g^{-1}h^{-1}$, respectively.

It is interesting to observe that the effect of the fed-batch strategy on cultivation main characteristics depends on the *S. cerevisiae* strain. By using the previously described fed-batch strategy the cutinase-$(WP)_2$ producing strain presented the highest increases of the cultivation performance. The cutinase-$(WP)_4$ producing strain, in relation to the other two yeast strains presented the lowest increase in cutinase activity, presenting even lower values of specific activity, productivity and cutinase yields than obtained by batch cultivation.

Thus, by using this fed-batch strategy the negative effects of fusing the hydrophobic $(WP)_2$ peptide to cutinase on cultivation main characteristics were minimized, when comparing to the batch cultivation. The differences between wt-cutinase and cutinase-$(WP)_4$ strains in batch and fed-batch strategies continue to be observed.

In summary, it is possible to conclude that by using a high density recombinant cell fed-batch cultivation it was possible to achieve a higher cutinase productions than that in a conventional batch cultivation. As previous observed in batch cultivation, the fed-batch studies also showed the negative influence of fusion small hydrophobic $(WP)_n$ peptides on cutinase production. Furthermore, the effect of increased hydrophobic length of the peptide $(WP)_n$ fused to cutinase on cultivation main characteristics depends on the cultivation strategy. This effect was minimized by using a two-phase cultivation, a growth and a producing phase, where an exponential feed technique was used in the production phase.

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References

Bandmann N, Collet E, Leijen J, Uhlén M, Veide A, Nygren P-Å 2000. Genetic engineering of the *Fusarium solani pisi* lipase cutinase for enhanced partitioning in PEG-phosphate aqueous two-phase systems. J Biotechnol 79:161–173.

Barthel T, Kula M-R. 1993. Rapid purification of DesPro(2)-Val15-Leu17-Aprotinin from the culture broth of a recombinant *S. cerevisiae*. Biotecnhol Bioeng 42:1331–1336.

Berggren K, Tjerneld F, Veide A. 2000. Peptide fusions tags with tryptophan and charged residues for control of protein partitioning in PEG-potassium phosphate aqueous two-phase systems. Bioseparation 9:68–80.

Bitter GA, Egan KM, Koski RA, Jones MO, Elliott SG, Giffin JC. 1987. Expression and secretion vectors for yeast. Methods Enzymol 153:516–544.

Bradford M. 1976. A rapid and sensitive method for the quantification of micrograms quantities of protein utilising the principle of protein-dye binding. Anal Biochem 72:248.

Calado CRC, Hamilton GE, Cabral JMS, Fonseca LP, Lyddiatt A. 2001. Direct product sequestration of a recombinant cutinase from batch fermentations of *Saccharomyces cerevisiae*. Bioseparation, 10:87–97.

Calado CRC, Taipa MA, Cabral JMS, Fonseca LP. Optimisation of culture conditions and characterisation of cutinase produced by recombinant S*accharomyces cerevisiae*. Submitted.

Carlsson M, Berggren K, Linse P, Veide A, Tjerneld F. 1996. Effects of fused tryptophan rich peptides to a recombinant protein A domain on the partitioning in polyethylene glycol-dextran and Ucon-dextran systems. J Chromatogr A 756:107–117.

Carvalho CML, Aires-Barros MR, Cabral JMS. 1999. Cutinase: from molecular level to bioprocess development. Biotecnol Bioeng 66:18–34.

Chisti Y, Moo-Young M. 1994. Separation techniques in industrial bioprocessing. I Chem E Symp Ser 137:135–146.

Costa MJL, Cunha MT, Cabral JMS, Aires-Barros MR. 2000. Scale-up of recombinant cutinase recovery by whole broth extraction with PEG-phosphate aqueous two-phase. Bioseparation, 9:231–238.

D'Anjou MC, Daugulis AJ. 1997. A model-based feeding strategy for fed-batch fermentation of recombinant *Pichia Pastoris*. Biotechnol Technol 11:865–868.

Das RC, Shultz JL. 1987. Secretion of heterologous proteins from *Saccharomyces cerevisiae*. Biotechnol Prog 3:43–48.

Diamond D, Hsu JT. 1992. Aqueous two-phase system for biomolecule separation. In: Fiechter A, editor. Advances in biochemical engineering/biotechnology, vol 47. Berlin: Springer-Verlag. p 88–135.

Eiteman MAA, Hassinen C, Veide A. 1994. A mathematical model to predict the partitioning of peptides and peptide-modified proteins in aqueous two-phase systems. Biotechnol Prog 10:513–519.

Ejiofor AO, Posten CH, Solomons BO, Deckwer WD. 1994. A robust fed-batch feeding strategy for optimal parameter estimation for baker's yeast production. Bioprocess Eng 11:135–144.

Ejiofor AO, Chisti Y, Moo-Young M. 1996. Fed-batch production of baker's yeast using millet (*Penniselum typhoides*) flour hydrolysates as the carbon source. J Ind Microbiol 16:102–109.

Erhart E, Hollenberg CP. 1983. The presence of defective Leu 2 gene on μ DNA recombinant plasmids of saccharomyces cerevisiae is responsible for curring and high copy number. J. Bacteriol 156: 625–635.

Hassinen C, Köhler K, Veide A. 1994. Polyethylene glycol-potassium phosphate aqueous two-phase systems. Insertion of short peptide units into a protein and its effects on partitioning. J Chromatogr A 668:121–128.

Köhler K, Ljungqvist C, Kondo A, Veide A, Nilsson B. 1991. Engineering proteins to enhance their partition coefficients in aqueous two-phase systems. Bio/Technology 9:642–646.

Lang C, Göllnitz C, Popovic M, Stahl U. 1997. Optimisation of fungal polygalacturonase synthesis by *Saccharomyces cerevisiae* in fed-batch culture. Chem Eng J 65:219–226.

Lange H, Bavouzet JM, Taillandier P, Delorme C. 1993. Systematic error and comparison of four methods for assecing the viability of *Saccharomyces cerevisiae* suspension. Biotechnol Tech 7: 223–228.

Novick P, Ferro S, Scheckman R. 1981. Order events in the yeast secretory pathway. Cell 25:461–469.

Raghavarao KSMS, Rastogi NK, Gowthaman MK, Karanth NG. 1995. Aqueous two-phase extraction for downstream processing of enzymes/proteins. Adv Appl Microbiol 4:97–171.

Romanos MA, Scorer CA, Clare JJ. 1992. Foreign gene expression in yeast: a review. Yeast 185:423–488.

Sagt CMJ, Müller WH, Boonstra J, Verkleij AJ, Verrips CT. 1998. Impaired secretion of a hydrophobic cutinase by *Saccharomyces cerevisiae* correlates with an increased association with immunoglobulin heavy-chain binding protein (BIP). Appl Environ Microbiol 64:316–324.

Shi Y, Ryu DDY, Yuan WK. 1993. Effects of oxygen and ethanol on recombinant yeast cultivation for hepatites B virus surface antigen production: modelling and simulations studies. Biotechnol Bioeng 41:55–66.

Smith RA, Duncan, MJ, Moir DT. 1985. Heterologous protein secretion from yeast. Science 229:1219–1224.

van Gemeren IA, Musters W, van den Hondel CAMJJ, Verrips CT. 1995. Construction and heterologous expression of a synthetic copy of the cutinase cDNA from *Fusarium solani pisi*. J Biotechnol 40:155–162.

Wangsa-Wirawan ND, Lee YS, Falconer RJ, Mansell CJ, O'Neill BK, Middelberg APJ. 1997. Novel fed-batch strategy for the production of insulin-like growth factor 1 (IGF-1). Biotechnol Lett 19:1079–1082.

Zabriskie DW, Arcuri EJ. 1986. Factors influencing productivity of fermentations employing recombinant microorganisms. Enzyme Microb Technol 8:706–717.