Effect of Tween-80 on Stability and Secretion of Hydrophobic Tagged-cutinases

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Dedicated to the memory of Professor Dr. Valentin Koloini

To significantly enhance the downstream processing, two cutinase variants were constructed by genetic fusion small hydrophobic peptides $(WP)_2$ and $(WP)_4$, respectively. However, the fusion of these peptides impairs cutinase secretion by the host cell *Saccharomyces cerevisiae* and increases cutinase inactivation in the culture broth due to cutinase aggregation, resulting in cutinase activities per biomass of 56 % of cutinase-$(WP)_2$ and of 7 % of cutinase-$(WP)_4$, in relation to cutinase without the hydrophobic tags. It was observed that the addition of non-ionic surfactant Tween-80 into the culture broth could minimise the cutinase inactivation. The addition of Tween-80 also results in the enhancement of cutinase secretion by the yeast cell, leading to 1.25 and 2.51 fold-higher extracellular cutinase-$(WP)_2$ and cutinase-$(WP)_4$, respectively, in relation to cultivations performed in the absence of surfactant. Therefore, the addition of Tween-80 on the culture broth partly minimises the effect of fusion of the hydrophobic tags on the inactivation of the enzymatic activity and on the reduction of the protein secretion. By this way, the use of Tween-80 on the *S. cerevisiae* cultivation may contribute to the efficiency enhancement of the downstream processing of tagged cutinases.

Key words:
Cutinase, tagged-cutinase, Tween-80, secretion

Introduction

Cutinase (EC 3.1.1.3) is an extracellular protein, secreted by phytopathogenic fungi, that catalyses the hydrolysis of ester bonds in the cutin,[1,2] a polyester of n-C_{16} and n-C_{18} hydroxy fatty acids, that acts as a structural component of the cuticle of higher plants and is thought to play a key role in plant protection because it is a barrier against the entry of pathogens.[1] Cutinase belongs to a superfamily of proteins, with a common structural framework the α/β hydrolase fold.[3] All the lipases belongs to this superfamily, in which cutinase is the smallest known member.[4] However, cutinase differs from classical lipases as it does not exhibit interfacial activation, as it is active on soluble as well as on emulsified triglycerides, being potentially suitable for fat-stain removal applications.[5]

Due to the importance of the potential applications of cutinase and in order obtain an efficient and low-cost production system, the cutinase from *Fusarium solani pisi* has been cloned and expressed in recombinant *Saccharomyces cerevisiae* strains.[6,7] This expression system resulted from improve-ments in the development of *S. cerevisiae* as a cell factory,[8] where for heterologous expression of the cutinase cDNA, the mature fragment of the gene was cloned into a yeast expression vector derived from pMIRY2. In this plasmid, the cutinase gene is fused to the invertase signal sequence, and the expression is controlled by the GAL7 promoter. The yeast invertase signal sequence enables the secretion of the heterologous enzyme into the cultivation medium, leading to a relatively high initial degree of purity of the product that allowed the selectivity of the yeast product secretion facilitates to target product recovery.[9,10] Since for industrial production of food and detergent enzymes, such as cutinase,[11] the downstream processing costs should be as low as possible, the specific export of the target protein for the extracellular medium constitutes a very attractive production route.

To enhance the cutinase recovery process from the culture medium by an aqueous two-phase system, two hydrophobic variants of cutinase were genetically constructed within a European Union (EU) project.[12,13] These variants contained attached to the C-terminal of the enzyme the peptide tags rich in tryptophan and proline $(WP)_2$ and $(WP)_4$. However, the *S. cerevisiae* strains producing the

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cutinases with tags rich in tryptophan presented lower cutinase secretion efficiencies.[12]

The scope of this work is to improve the secretion by the recombinant *Saccharomyces cerevisiae* of the cutinases fused to the peptide tags (WP)$_2$ and (WP)$_4$ using non-ionic surfactants. The non-ionic surfactant, polyoxyethylene sorbitan monooleate (Tween-80), has been used to enhance the production and secretion of several enzymes, as α-amylase,[14] human granulocyte colony-stimulating factor by recombinant *S. cerevisiae*,[15,16] FGD1 (a F420-dependent glucose-6-phosphate dehydrogenase) by *Mycobacterium* smegmatis[17,18] and the recombinant endoglucanase by *Streptomyces lividans*.[19] To understand better the effect of Tween-80 on the cutinase activity, the effect of non-ionic surfactants (Tween-80 and Triton X-100) on cutinase activity was also evaluated.

Materials and methods

Microorganism and inoculum preparation

Three *Saccharomyces cerevisiae* MM01 (Mata, leu2-3, ura3, gal1: URA3, MAL-8, MAL3, SUC3) strains, expressing cutinase wild type (wt-), cutinase-(WP)$_2$ and cutinase-(WP)$_4$ contained the plasmids pUR7320-9, pUR807-1 and pUR806-6, respectively. The *S. cerevisiae* strains were constructed at Unilever (Vlaardingen, The Netherlands).

The strains were stored at –80 °C in cryogenic tubes containing selective medium and 50 % (v/v) glycerol (Merck, Germany). The selective medium consisted of 20 g L^{-1} D(+)-glucose anhydrous (Merck, Germany) and 6.7 g L^{-1} of yeast nitrogen base without amino acids (Difco, USA). The pre-cultures grown on selective medium were conducted in shake flasks at 30 °C and 200 rpm in an orbital shaker (Agitorb 160E, Aralab, Portugal) until a cell concentration between 1.1 and 1.8 g L^{-1} was achieved.

Cultivation

The pre-cultures were used for inoculation of the batch culture at 10 % (v/v). The batch production medium contained 20 g L^{-1} glucose (Merck, Germany), 10 g L^{-1} yeast extract (Difco, USA), 10 g L^{-1} peptone bacteriological (BDH, U.K.) and 2 g L^{-1} of D(+)-galactose anhydrous (Sigma, USA). The cultivations were performed in an orbital shaker (Agitorb 160E, Aralab, Portugal) at 30 °C, 200 rpm in 250 mL shake flasks with 100 mL working volume and each experiment was performed in triplicate.

The batch cultivations with surfactants were conducted as previously described, but adding polyoxyethylene-(20)-sorbitan monooleate (Tween-80) (Merck, Germany) to the shake-flask before medium sterilization. Tween-80 was added in order to achieve a final Tween-80 concentration on the culture medium between 0 and 2 % (w/v).

The cultivation was monitored for 72 hours by taking one sample of 5 mL per day. Samples were immediately analysed for biomass concentration, cutinase activity, protein concentration and secretion efficiency.

Biomass, protein, cutinase activity and enzyme secretion efficiency

Biomass concentration as dry cell weight and protein concentration were determined as described in Calado *et al.*[12]

The cutinase estereolytic activity was determined spectrophotometrically, following the hydrolysis of *p*-nitrophenylbutyrate (Sigma, USA) at 400 nm. 20 μL of sample were added to 980 μL of a 0.56 mmol L^{-1} *p*-nitrophenylbutyrate solution in 50 mmol L^{-1} potassium phosphate buffer pH 7 with 11.3 mmol L^{-1} sodium cholate (Sigma, USA) and 0.43 mol L^{-1} tetrahydrofuran (Merck, Germany). The reactions were monitored 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 one minute under the specified conditions. The extinction coefficient of *p*-nitrophenol was considered to be $1.84 \cdot 10^4$ M^{-1} cm^{-1}, as indicated by the supplier Sigma (USA).

Enzyme secretion efficiency 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 (Merck, Germany), during 5 min, with 1 min intervals in ice. The interplasmatic cutinase was analysed by gentled resuspended cellular pellets in phosphate buffer 50 mmol L^{-1} pH 6.0 containing 20 μL lyticase (10 U μL^{-1}, Sigma, USA). The digestion mixture was incubated for 8 h at 30 °C. After this digestion, a new centrifugation was performed and cutinase activity of the supernatant analysed.

The biomass concentration, protein and cutinase activity were determined in triplicate. Cutinase specific activity was defined as the ratio of the cutinase activity and the broth protein concentration. The purified three cutinase variants, with or without fused peptides, presented the same specific cutinase activity (unpublished data).

Effect of non-ionic surfactants on cutinase activity

Samples obtained from batch cultures were clarified by centrifugation, and 2 mL of the clarified sample was immediately diluted at 1/2 (v/v) in polyoxyethylene-(20)-sorbitan monooleate (Tween-80) (Merck, Germany) or polyethylene-glycol mono [4-(1,1,3,3-tetramethylbutyl)phenyl] ether (Triton X-100) (Merck, Germany) to achieve a final surfactant concentration of 0.2 % (w/v). Samples were incubated in a water batch at 22 °C.

Some cutinase samples were denatured by diluting the clarified broth at 1/2 (v/v) with a 6 mol L^{-1} guanidine hydrochloride (Merck, Germany) solution, in a 2 mL final volume. Cutinase activity was monitor in intervals of 15 min. The refolding of cutinase was conducted by dilution of 100 μL of the previous solution at 1/20 (v/v) in stock solutions of phosphate buffer (Merck, Germany) or Tween-80 or Triton X-100 in order to achieve final concentrations of 50 mmol L^{-1} phosphate buffer pH 7.0, 0.2 % (w/v) Tween-80 or 0.2 % (w/v) Triton X-100. Samples were constantly maintained in a water bath at 22 °C.

Results and discussion

Effect of non-ionic surfactants on enzyme activity

The effect of Tween-80 on the activity of wild-type cutinase (wt-cutinase) and tagged cutinases (cutinase-(WP)$_2$ and cutinase-(WP)$_4$,) was evaluated by incubating culture samples, previously clarified by centrifugation, with 0.2 % (w/v) Tween-80 during 72 h. During this incubation period, the activity of the three cutinase variants increased, achieving a maximum of activity during the first 48 h of incubation. Furthermore, as the length of the hydrophobic tag increases the higher activity increase was observed. The maximum activity for the three cutinase variants was achieved during the first 48 h, being attained 1.3, 1.5 and 1.7-fold higher activities of wt-, –(WP)$_2$ and cutinase-(WP)$_4$, respectively in relation to the initial activity (Fig. 1).

Since, the formation of aggregates is one of the processes leading to cutinase thermal inactivation,[20] it could be hypothesised that, as the size of the fused-tag increases, global cutinase hydrophobicity also increases leading to protein aggregation and consequently to inactivation. Tween-80 by interacting with the hydrophobic amino acids residues of the peptide tag will reduce protein aggregation tendency. This hypothesis is corroborated by Bae *et al.*[15,16] observations, that the addition of Tween-80
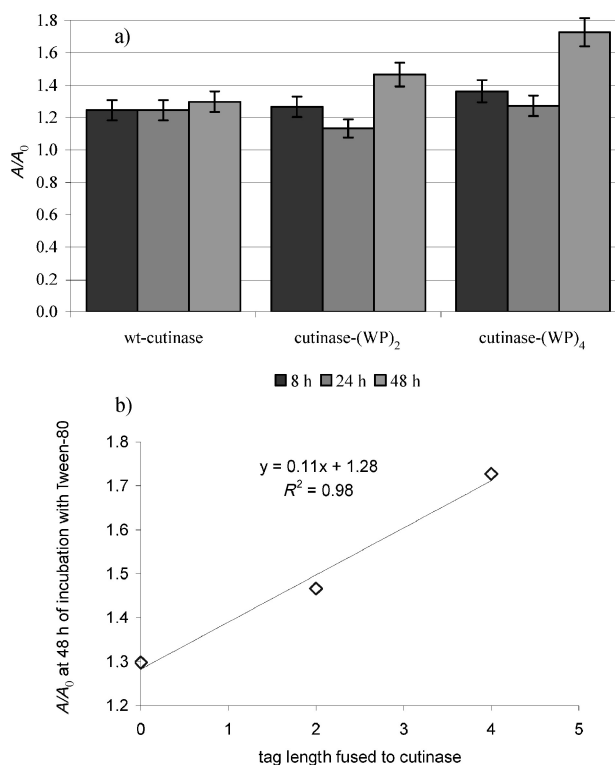

F i g . 1 – *Effect of 0.2 % (w/v) Tween-80 on cutinase activity (A) of clarified broth samples in relation to initial activity (A$_0$) along the incubation period. The initial cutinase activity (A$_0$) for the –wt, –(WP)$_2$ and cutinase-(WP)$_4$ was 36, 20 and 2.6 U mL^{-1}, respectively. b) Relationship between the increase of cutinase activity observed after 48 h of incubation with Tween-80 and the length of the fused tag.*

to cultures of *S. cerevisiae* also prevents the formation of multimers of heterologous human granulocyte colony-stimulating factor, stabilising, by this way, the product formation.

The fact that the highest increases of cutinase activity were observed only after 48 h of incubation could be due to the strong interaction between the more hydrophobic cutinases. To speed the solubilisation of cutinase multimers, the cutinase was denatured by incubation with a strong denaturant agent, followed by its refolding by dilution in buffer or on a non-ionic surfactant as Triton-100 and Tween-80. The three cutinase variants were denatured by diluting the clarified culture broth at 50 % (v/v) with a solution of 6 mol L^{-1} guanidine hydrochloride (GdnCl), as for globular proteins, the following series of the denaturing capacity holds: GdnCl > urea > sodium dodecyl sulfate > high temperature > extremes of pH > high hydrostatic pressure.[21] After 1 h of incubation in the denaturing solution, the cutinase activity in all the samples was inferior to 5 % of the initial activity (data not shown).

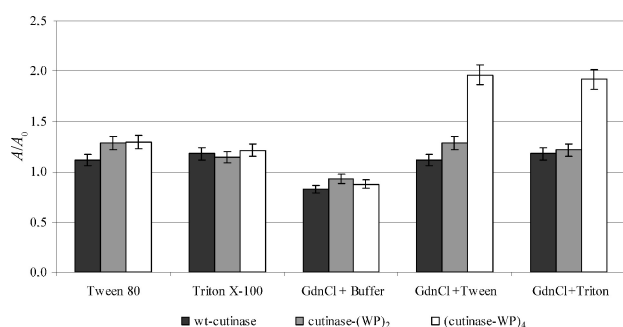The cutinase refolding was performed by dilution at 1/20 (v/v) with stock solutions of phosphate

F i g . 2 – *Effect of 1 h incubation of clarified broth samples with 0.2 % (w/v) Tween-80 and with 0.2 % (w/v) Triton X-100 on cutinase activity (A) in relation to the initial activity (A_0). Some clarified broth samples were submitted to a denaturation solution of 3 mol L^{-1} GdnCl. The subsequent refolding was performed by 1/20 (v/v) dilution in order to achieve a final solution of 50 mmol L^{-1} phosphate buffer pH 7.0, or 0.2 % (w/v) Tween-80 or 0.2 % (w/v) Triton X-100. The cutinase activities (A) were analysed after 1 h of the dilution step and compared in relation to samples that were not denatured (A_0).*

F i g . 3 – *Effect of 1 h incubation of intracellular cutinase activity (A), present in extracts of disrupted yeast cells, with 0.2 % (w/v) Tween-80 and with 0.2 % (w/v) Triton X-100 in relation to the initial activity observed before the addition of the non-ionic surfactant (A_0). The intracellular cutinase, present in extracts of disrupted cells, was submitted to a denaturation solution of 3 mol L^{-1} GdnCl, and subsequently refolded by 1/20 (v/v) dilution in order to achieve a final solution with 50 mmol L^{-1} phosphate buffer pH 7.0 or 0.2 % (w/v) Tween-80 or 0.2 % (w/v) Triton X-100. The cutinase activities (A) were analysed after 1 h of the dilution step and compared in relation to samples that were not denatured (A_0).*

buffer or with Tween-80 or with Triton X-100, in order to achieve a final solution concentration of 50 mmol L^{-1} phosphate buffer pH 7.0, or 0.2 % (w/v) Tween-80 or 0.2 % (w/v) Triton X-100 (Fig. 2). The cutinase activity was monitored for a period of 24 h. After dilution, the maximum activity was immediately achieved after 1 h. The samples that were not submitted to the denaturing agent, and were incubated with Tween-80 or Triton X-100 exhibited after 1 h activity increases between 1.1 and 1.3-fold in relation to the initial activites. The refolding by dilution in phosphate buffer resulted in cutinase recoveries between 82 and 93 %.

The cutinase refolding with Tween-80 or with Triton X-100 presented increases of cutinase activity between 1.1 and 1.2-fold for wt-cutinase, between 1.2 and 1.3-fold for cutinase-$(WP)_2$ and between 1.9 and 2.0-fold for cutinase-$(WP)_4$, respectively, in relation to the activity analysed before the denaturation step. All these observations corroborate the hypothesis that as the length of the hydrophobic tag $(WP)_n$ fused to cutinase increases, cutinase tends to strongly interact, resulting in cutinase aggregation and inactivation. To avoid this aggregation and consequent inactivation, Tween-80 can be added to the clarified culture broth. To speed the desaggregation of cutinase, a strong denaturating agent could be used followed by refolding by dilution in Tween-80.

The previous experiments were also conducted with intracellular cutinase, present in extracts of disrupted yeast cells (Fig. 3). It was observed that the incubation with the non-ionic surfactants also resulted in activity increases, where the more hydrophobic cutinases presented the highest increases in activity, corroborating the hypothesis that the in-
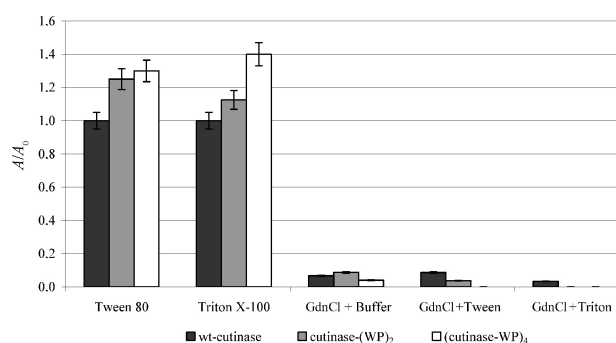
creased length of the fused tag favours the hydrophobic interactions between cutinases leading more easily to their aggregation and consequent inactivation.

After denaturation of the intracellular cutinases, it was not possible to recover the cutinase activity by dilution in buffer or in the non-ionic surfactants, suggesting that, during the unfolding step, the cutinase structure tends to be more susceptible to proteolysis by vacuolar proteases released during cellular disruption (Fig. 3). Indeed, cutinase is a very stable enzyme to proteolysis. Even after 30 min incubation with trypsin, chymotrypsin, elastase, proteinase K, V8 protease or clostripain no loss in enzymatic activity was detected.[22] Only after drastic conformational changes it was possible to increase the cutinase susceptibility to proteolysis. Particularly anionic detergents such as sodium dodecyl sulphate may have a drastic effect on structural integrity, because hydrophobic patches at the surface together with positively charged amino acid residues may lead to binding of SDS molecules followed by unfolding of the protein structure.[22,23]

Samples from the extracellular medium submitted to denaturation followed by renaturing conditions did not present decreases in cutinase activity (Fig. 2) probably due to a negligible secretion of extracellular proteases by the yeast host.

Effect of non-ionic surfactant Tween-80 on cutinase secretion

In batch cultures performed without the addition of Tween-80 it was observed that as the length of the fused tag to cutinase increased the secretion

efficiency of the heterologous enzymes significantly decreased. *S. cerevisiae* expressing wt-cutinase, cutinase-(WP)$_2$ and cutinase-(WP)$_4$ presented secretion efficiencies of 72 %, 65 % and 32 %, respectively. The retention of cutinase in the yeast host resulted in a cellular metabolic stress and consequently in lowers biomass concentrations (Fig. 4), as previously been observed in Calado *et al.*[12] For example, the yeast strain producing cutinase-(WP)$_4$ presented 88 % of the biomass concentration, 27 % of cutinase produced per biomass and consequently 11 % of the extracellular cutinase activity in relation to the culture producing wt-cutinase.
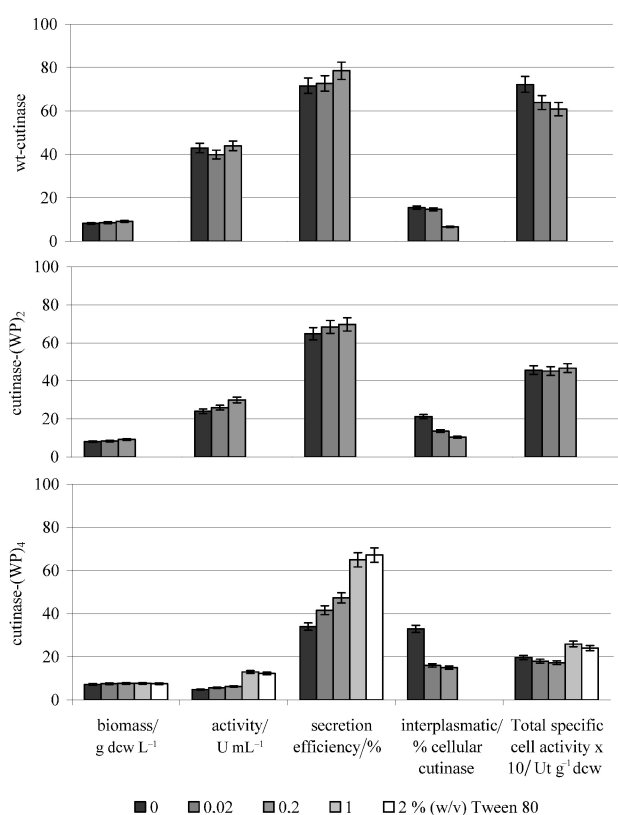

F i g . 4 – *Effect of adding Tween-80 to batch cultures of S. cerevisiae strains. The total specific cell activity was determined as the total produced cutinase (intra- and extracellular) per biomass concentration. Data refers to the end of 72 h of cultivation. The extracellular cutinase activity obtained in the batch cultures without the surfactant for the –wt, –(WP)$_2$ and cutinase-(WP)$_4$ was 36, 20 and 2.6 U mL$^{–1}$, respectively.*

To evaluate the effect of adding Tween-80 on yeast cultures, several batch cultivations were conducted using different concentrations of Tween-80. To account the effect of Tween-80 on cutinase activity, all the clarified culture samples were diluted at 1/10 (v/v) with a 2 % (w/v) Tween-80 solution, and subsequently incubated at 22 °C for 6 hours before cutinase activity analysis.

The addition of Tween-80 to yeast cultures producing wt-cutinase, resulted in decreases of the interplasmatic cutinase (from 15 % to 7), slight increases of cutinase secretion (from 72 % for 81 %) and slight increases of dry cell weigh (from 8.3 to 9.2 g L$^{–1}$). However, a slight decrease in produced cutinase per biomass was registered, and consequently the addition of Tween-80 did not result with an increase in the extracellular activity of wt-cutinase (Fig. 4). In the case of the strain producing cutinase-(WP)$_2$ it was observed that, as the surfactant concentration increased in the culture medium, the extracellular cutinase activity slightly increased due to the enhanced secretion efficiency (from 65 % to 70 %), while the total cutinase produced per biomass was maintained (Fig. 4).

Since the strain producing cutinase-(WP)$_4$ was the one that presented the lowest secretion efficiency, higher quantities of Tween-80 were tested in relation to the other two yeast strains (Fig. 4). The increase of the surfactant concentration up to 0.2 % resulted in a significant enhancement of secretion efficiency (from 34 % to 65 %), a slight decrease in total cutinase produced per biomass, and a final significant increase in extracellular cutinase activity. The highest value of cutinase-(WP)$_4$ per biomass was observed with 1 % Tween-80, that allied to a high secretion efficiency, resulting in the highest extracellular cutinase-(WP)$_4$ activity of 13 U mL$^{–1}$, corresponding to a 2.7-fold higher cutinase activity in relation to the cultivation performed without Tween-80 addition.

The specific cutinase activity represents the quantity of cutinase present in the extracellular medium in relation to whole proteins presented, that includes secreted proteins from the yeast cell and proteins from the yeast extract used as N-source. Therefore, the specific activity represents the purification degree of cutinase in whole broth, where a high specific activity in the culture broth could simplify the downstream processing. It was observed that, in general, the specific cutinase activity increases along the cultivation, reflecting that cutinase represents the major protein secreted by the yeast cells. Indeed *S. cerevisiae* usually directs only a small number of proteins for transport.[24] As the production of cutinase along the cultivation time increases, the proportion of cutinase over whole proteins (from the culture medium (yeast extract) and other secreted proteins) also increases (Fig. 5a). Indeed, there exists a direct relationship between the extracellular cutinase activity measured along the cultivation period and the corresponding cutinase specific activity (Fig. 5b). Since the production of extracellular tagged-cutinases decrease proportionally with the length of the fused tag, also a proportional lower cutinase specific ac-
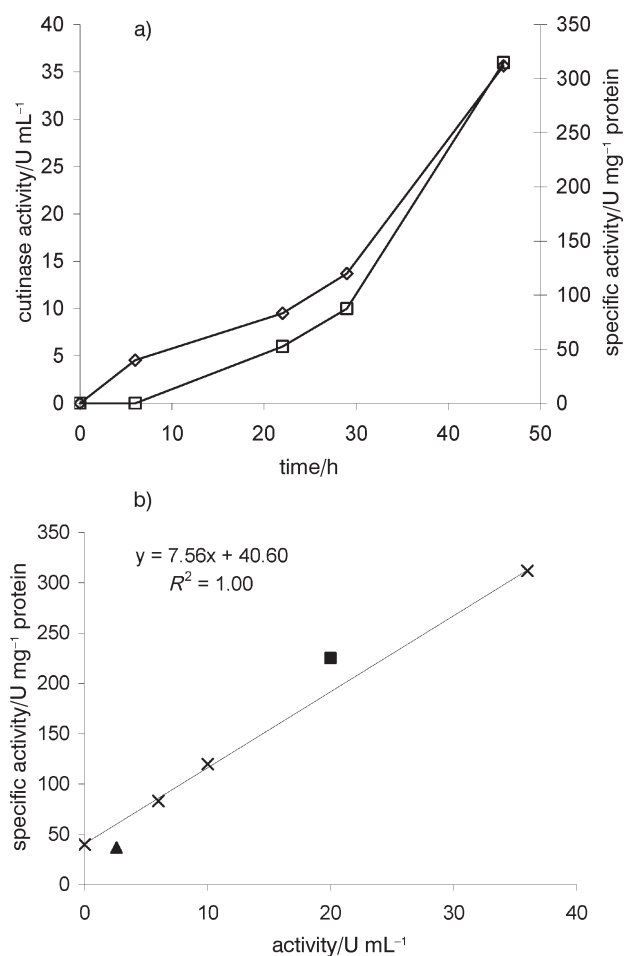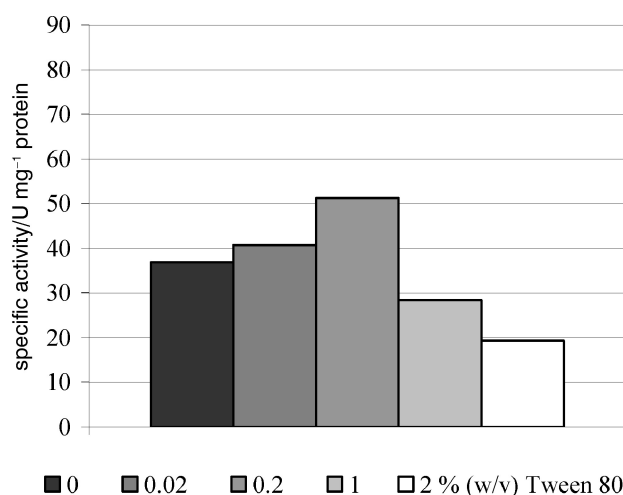
F i g . 6 – *Effect of adding Tween-80 on the cutinase specific activity achieved at the end of 72 h of cultivation of the yeast producing cutinase-(WP)₄*

F i g . 5 – *a) Cutinase activity (◇) and cutinase specific (□) activity observed along the cultivation time conducted without surfactant of the yeast producing wild-type cutinase. b) Relationship between cutinase activity and cutinase specific activity observed along 72 h of the cultivation time of the yeast producing wild-type cutinase (×). The cutinase specific activity obtained at the cultivation end of cutinase-(WP)₂ (■) and cutinase-(WP)₄ (▲) are also represented.*

sources in relation to the total cutinase. Addition of Tween-80 in quantities higher than 0.2 % resulted in reduced cutinase specific activities, probably due to the increased secretion of proteins other than cutinase (Fig. 6).

Protein transport and secretion in *S. cerevisiae* involves a complex series of events associated with membrane-bound sub-cellular components. Synthesised proteins pass through the endoplasmatic reticulum and the Golgi apparatus and are packaged into secretory vesicles, which fuse with the plasma membrane to release the fully processed protein. Certain yeast proteins accumulate in the periplasm space, while others pass through the cell wall into the culture medium. One possibility explaining the effect of the non-ionic surfactant, is that Tween-80 is an artificial source of oleic acid, which after hydrolysis, is incorporated directly into the yeast plasma membrane and that, by virtue its amphiphilic properties, might then interact physico-chemically with the membrane,[15] that will potentially result in the passage of cutinase from the periplasmatic space to the extracellular medium. The aggregation of the more hydrophobic cutinase with the fused tags probably occurs in majority in the endoplasmatic reticulum.[7,8] Therefore, it could be deduced that the physical-chemical interaction between surfactant and the plasmalemma that will induce the passage of cutinase from the interplasmatic space to the extracellular medium, may also affect a driving force related to the passage of the hydrophobic cutinases from the endoplasmatic reticulum to the cell membrane. Indeed, as the Tween-80 concentration increases the percentage of cutinase on the periplasmatic space in relation to the total cellular cutinase decrease and the secretion efficiency increases.

tivity is observed in relation to the observed with wt-cutinase (Fig. 5b). The cultures producing wt-, –(WP)₂ and cutinase-(WP)₄ presented at the culture end cutinase specific activities of 312, 280 and 47 U mg⁻¹ protein, respectively. It is expectable, that so different cutinase specific activities will influence the downstream process.

The addition of Tween-80 in cultures producing wt- and cutinase-(WP)₂ did not significantly affect the cutinase specific activity (data not shown). However, in yeast cultures producing the most hydrophobic cutinase, cutinase-(WP)₄, the addition of Tween-80 up to 0.2 % resulted in slight increases of the specific activity, probably as the result of increase of cutinase secretion. Since the recombinant *S. cerevisiae* directs only a small number of proteins for transport,[24] this specific activity increase resulted probably from the decreased contribution of the medium proteins furnished by the nitrogen

In spite of the positive effect of the surfactant on the increase of extracellular cutinase-(WP)$_4$, the highest activity value achieved (of 13 U mL^{-1}) still represents 30 % of the wt-cutinase. Indeed, the addition of 1 % (w/v) Tween-80 to a yeast culture producing cutinase-(WP)$_4$ resulted in 36 % of total produced cutinase per biomass, 90 % of secretion efficiency, 93 % of yeast biomass and 8 % of cutinase specific activity in relation to the yeast culture producing wt-cutinase and conducted without Tween-80.

Conclusions

The cutinase modification by fusion small hydrophobic tags (as –(WP)$_2$ and –(WP)$_4$) may significantly simplify the downstream processing. However, the inclusion of these hydrophobic tags also results both in protein inactivation and reduced secretion by the host cell. Furthermore, these effects are exacerbated as the length of the hydrophobic tag increases. The addition of Tween-80 at concentrations of 0.2 % (w/v) on the culture broth of *S. cererisiae* producing wild-type cutinase and cutinase-(WP)$_2$ simultaneously: i) inhibit the cutinase aggregation by hydrophobic interactions, minimizing its inactivation along the culture process, and ii) will increase the cutinase secretion. In the case of cutinase-(WP)$_4$, it is necessary to use Tween-80 concentrations as high as 1 % (w/v) in the culture broth to maximise cutinase secretion by the yeast host cells, resulting in 2.7 fold-higher extracellular activities in relation to the culture performed without the surfactant. However, even with a high concentration of the surfactant, it was possible to achieve only 30 % of the extracellular activity of cutinase-(WP)$_4$ in relation to wt-cutinase. In order to evaluate if the use of tagged-cutinases will result in an overall economic production system (that includes the cultivation step and the downstream process), it is necessary to consider the effect of the hydrophobic tags on the reduction of the secretion efficiency, that partly may be overcome by using Tween-80 during the cultivation step.

References

1. *Purdy, R. E., Kolattukudy, P. E.,* Biochemistry **14** (1975) 2824.
2. *Lin, T. S., Kolattukudy, P. E.,* Eur. J. Biochem. **106** (1980) 341.
3. *Ollis, D. L., Cheah, E., Cygler, M., Dijkstra, B., Frolow, F., Franken, S. M., Harel, M., Remington, S. J., Silman, I. M., Shrag, J., Sussman, J. L., Verschueren, K. H. G., Goldman, A.,* Protein. Eng. **5** (1992) 197.
4. *Martinez, C., De Geus, C. P., Lauwereys, M., Matthyssens, G., Cambillau, C.,* Nature **356** (1992) 615.
5. *Egmond, M. R., Van der Hijden, H. T. M., Musters, W., Peters, H., Verrips, C. T.,* J. De Vlieg, World Patent WO 94 (1994) 14964.
6. *van Gemeren, I. A., Musters, W., van den Hondel, C. A. M. J. J., Verrips, C. T.,* J. Biotechnol. **40** (1995) 155.
7. *Sagt, C. M. J., Müller, W. H., Boonstra, J., Verkleij, A. J., Verrips, C. T.,* Applied Envir. Microbiol. **64** (1998) 316.
8. *Verrips, T., Duboc, P., Visser, C., Sagt, C.,* Enzyme Microb. Technol. **26** (2000) 812.
9. *Calado, C. R. C., Hamilton, H. E., Cabral, J. M. S., Fonseca, L. P., Lyddiatt, A.,* Bioseparation **10** (2001) 87.
10. *Calado, C. R. C., Cabral, J. M. S., Fonseca, L. P.,* J. Chem. Technol. Biotechnol. **77** (2002) 1231.
11. *Carvalho, C. M. L., Aires-Barros, M. R., Cabral, J. M. S.,* Biotechnol. Bioeng. **66** (1999) 18.
12. *Calado, C. R. C., Mannesse, M., Egmond, M., Cabral, J. M. S., Fonseca, L. P.,* Biotechnol. Bioeng. **78** (2002) 692.
13. *Cunha, M. T., Costa, M. J. L., Calado, C. R. C., Fonseca, L. P., Aires-Barros, M. R., Cabral, J. M. S.,* J. Biotechnol. **100** (2003) 55.
14. *Goes, A. P., Sheppard, J. D.,* J. Chem. Technol. Biotechnol. **74** (1999) 709.
15. *Bae, C. S., Yang, D. S., Jang, K. R., Seong, B. L., Lee, J.,* Biotechnol. Bioeng. **57** (1998) 600.
16. *Bae, C. S., Yang, D. S., Lee, J., Park, Y.-H.,* Appl. Microbiol. Biotechnol. **52** (1999) 338.
17. *Goldstone, R. M., Moreland, N. J., Bashiri, G., Baker, E. N., Lott, J. S.,* Protein Exp. Purification **57** (2008) 81.
18. *Kim, E., Shin, D.-H., Irwin, D. C., Wilson, D. B.,* Biotechnol. Bioeng. **60** (1998) 70.
19. *Bashiri, G., Squire, C. J., Baker, E. N., Moreland, N. J.,* Protein Exp. Purification **54** (2007) 38.
20. *Lauwereys, M., De Geus, P., De Meutter, J., Stanssens, P., Mathyssens, G., Alberghina, G. L., Schmid, R. S., Verger, S.,* Cloning, expression and characterisation of cutinase, a fungal enzyme. In: lipases, structure, mechanism and genetic engineering, 16:243-251, SBF Monographes Braunschweig, Germany, 1990.
21. *Jaenicke, R., Rudolph, R.,* Folding proteins. In: Protein structure: a practical approach. *Creighton, T. E.,* (Ed.), IRL Press, UK, 1989, pp 101-223.
22. *Kolattukudy, P. E.,* Cutinases from fungi and pollen. In Lipases, *Borgström and Brockman H.,* (Eds.), Elsevier, Amsterdam, 1984, 471-504.
23. *Pocalyko, D., Tallman, M.,* Enzyme Microbial Technol. **22** (1998) 647.
24. *Calado, C. R. C., Taipa, M. A., Cabral, J. M. S., Fonseca, L. P.,* Enzyme Microbial Technol. **31** (2002) 161.