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DOI: 10.2225/vol16issue5-fulltext-12 *RESEARCH ARTICLE*

Production of heterologous cutinases by *E. coli* and improved enzyme formulation for application on plastic degradation

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Abstract

Background: The hydrolytic action of cutinases has been applied to the degradation of plastics. Polyethylene terephthalate (PET) have long half-life which constitutes a major problem for their treatment as urban solid residues. The aim of this work was to characterize and to improve stable the enzyme to optimize the process of degradation using enzymatic hydrolysis of PET by recombinant cutinases.
Results: The wild type form of cutinase from *Fusarium solani pisi* and its C-terminal fusion to cellulose binding domain N1 from *Cellulomonas fimi* were produced by genetically modified *Escherichia coli*. The maximum activity of cutinases produced in Lactose Broth in the presence of ampicillin and isopropyl β-D-1-thiogalactopyranoside (IPTG) was 1.4 IU/mL. Both cutinases had an optimum pH around 7.0 and they were stable between 30 and 50ºC during 90 min. The addition of glycerol, PEG-200 and $(NH_4)_2SO_4$ to the metabolic liquid, concentrated by ultra filtration, stabilized the activity during 60 days at 28ºC. The treatment of PET with cutinases during 48 hrs led to maxima weight loss of 0.90%.
Conclusions: Recombinant microbial cutinases may present advantages in the treatment of poly(ethylene terephthalate) PET through enzymatic treatments.

Keywords: cutinase, environmental application, poly(ethylene terephthalate).

INTRODUCTION

Cutinase (EC 3.1.1.74) also known as cutin hydrolases are versatile enzymes that demonstrate various useful properties applied to industrial products and processes. These biocatalysts have hydrolytic activity on various compounds, ranging from natural to synthetic esters, whether soluble or insoluble with long chain triglycerides such as triolein, tricaprylin and emulsified triglycerides (Egmond and De-Vlieg, 2000).

Biocatalysis by cutinases have been applied to the degradation of polyesters among other polymers and to the esterification or transesterification of small molecules. For example, cutinases from *Thermobifida fusca* (Thf42_Cut1) and *Thermobifida cellulolysitica* (Thc_Cut1 and Thc_Cut2) were cloned and the kinetic properties of poly(ethylene terephthalate) PET hydrolysis were characterized and compared with closely related cutinases so as to explain their different hydrolysis efficiencies. Similar behaviour between enzymes was determined by kinetic parameters in the presence of the

soluble substrate, in the hydrolysis products of PET and in PET hydrophilization (Herrero-Acero et al. 2011). The authors Herrero-Acero et al. (2013) compared these to Thc_Cut2, mutants carrying Arg29Asn and/or Ala30Val exchanges and observed considerable higher specific activity and higher kcat/KM values in soluble substrates. The results described their specific activities and kinetic parameters on soluble substrates and their ability to hydrolyze PET. This investigation described a PET model substrate bis(benzoyloxyethyl) terephthalate (3PET).

Recently, two novel class II HFBs (hydrophobins) from *Trichoderma* sp. were described and these cutinases can stimulate activity in PET. The authors suggest individual HFBs can display different properties, and could be used in the enzymatic hydrolysis of aromatic-aliphatic polyesters such as PET (Espino-Rammer et al. 2013).

The construction of chimeric cutinases by fusing the cutinase gene with genes coding carbohydrate-binding modules (CBM) resulted in higher enzyme concentrations on the surface of the cellulose-based solid substrates. Different gene constructs were obtained so that different CBMs fused independently to the C-terminus of cutinase and produced different substrate affinities. The type B CBM of the endoglucanase C from *Cellulomonas fimi* is capable of binding to amorphous cellulose and was selected based on substrate affinity. The results demonstrated the hydrolysis of the substrate and increased the reactivity and hydrofilicity of the cellulose acetate fibers with higher reactive dye uptake in fabrics treated when compared to the fibers treated with the wild type of cutinase (Matamá et al. 2010).

Enzyme characterization regarding optimum pH and optimum temperature as well as pH and thermal stabilities during the reaction time is very important for the application of bioproducts with catalytic activity. These parameters can be further improved by the addition of certain chemicals in the formulated product which could increase and/or stabilize the enzyme activity during storage and operating conditions which are the key factors for marketing the enzyme (Vermelho et al. 2008).

Plastic polymers have high versatility and applicability worldwide. Poly(ethylene terephthalate) PET is a thermoplastic that was mass-produced in the twentieth century (Mano et al. 2004; Canevarolo, 2006). PET is a thermoplastic polymer resin of the polyester family to use for food and other liquid containers. In Brazil, the market in PET commercial bottles produces 9 billion units/year and more than 50% are discarded in the environment and are not reused (http://www.abipet.org.br/index.html). The recycling rate of PET is a very interesting strategy for industrial organizations considering to takes into account social, economic and environmental aspects (McBean et al. 2005; Barboza et al. 2009; Singh et al. 2009). In addition, the environmental incentive, recycling of PET as an industry is getting its driving force from the increasing value and applications of virgin and modified PET (Awaja and Pavel, 2005). Those polymers can only react to produce partly crystalline structures called "semicrystalline" partly crystalline structures, usually (Strobl, 1997).

In the last decade, the amount of plastic polymers in the composition of municipal solid waste has increased exponentially. In addition, strategies to cope with this involve structural modification and biodegradation and these are being developed as alternatives so as to reduce the accumulation of these polymers. The goal of this work was to improve stable recombinant cutinase production by *Escherichia coli* to be applied in the enzymatic degradation of PET.

MATERIALS AND METHODS

Microorganisms

Cultures of *E. coli* genetically modified, kindly provided by Prof. Artur Cavaco-Paulo, from the University of Minho - Portugal, were used for the production of the following cutinases:

• Wild type CUT from *Fusarium solani*;

• CUT-N1: cutinase from *F. solani* fused to the type B CBM N1 of endoglucanase C from *Cellulomonas fimi*.

These cultures were maintained in Lysogeny broth (LB) (1% tryptone, 0.5% yeast extract, 1% NaCl, pH 7.0) and 2% agar, supplemented with 100 µg/ml ampicillin.

Poly(ethylene terephthalate)

PET bottles were obtained from commercial use.

Production of cutinases

Cultures of *E. coli* CUT and *E. coli* CUT-N1 with 24 hrs of incubation were separately inoculated in Erlenmeyer flasks with LB-medium, supplemented with 100 µg/ml of ampicillin and incubated under orbital agitation at 150 rpm at 37ºC for 12 hrs. These cultures were used (0.1% v/v) to inoculate fresh LB-medium, supplemented with Ampicillin and maintained under the same conditions. The production of cutinases CUT and CUT-N1 was induced by the addition of isopropyl β-D-1-thiogalactopyranoside (IPTG) after the cultures reached an absorbance at 600 nm (A600) of 0.6. The culture media were centrifuged at the orbital speed of 5000 rpm for 20 min at 4ºC and the cell-free supernatants were used for measuring cutinase activity and determining pH. The cultures were grown in triplicate.

Determination of enzyme activity

The esterase activity was determined by spectrophotometry at 405 nm by the hydrolysis of p-nitrophenyl butyrate (p-NPB). An aliquot of the cell-free supernatant (0.07 mL) was added to 3.43 ml solution containing 1.12 mM p-NPB, 50 mM phosphate buffer pH 7.2, Triton X-100 0.2% v/v and tetrahydrofuran 0.43 M (Pio et al. 2008). The cutinases quantifications were determined using the p-nitrophenol extinction coefficient of 15.1 $mmol^{-1}$ x cm^{-1} at 405 nm. According to international parameters, one unit of enzyme (U) is defined as the amount of enzyme required to convert 1 µmol of p-NPB into p-nitrophenol (p-NP) per minute. All activity assays were performed in triplicate.

Ultrafiltration

The supernatant (cell-free) was subjected to ultrafiltration in order to concentrate the enzymes. A flat membrane filter (Pellicon XL - Millipore) with a 10 kDa cutoff was used under a pressure of 2.5 bar. The sample was separated into two: retentate (concentrate) and permeate. The process was conducted in the laboratory of Bioenzima Indústria e Comércio Ltda. After the concentration process, anti-microbial preservatives (0.25% sodium sorbate and 0.25% sodium benzoate) were added to the concentrated metabolic liquid.

Effects of pH and temperature on the stability of cutinases

The optimum pH for esterase activity in the concentrated and formulated enzyme solutions was determined in the presence of the substrate (p-NPB) in 0.1 M phosphate buffer (pH 8.6) and glycine-NaOH buffer (pH 9). Aliquots were removed for activity determination at intervals of 30, 60 and 90 min.

The concentrated and formulated enzyme extract of cutinases at optimum pH was incubated at different temperatures between 30 and 70ºC. Aliquots were withdrawn at 30, 60 and 90 min of incubation to determine thermal stability.

Assays of optima pH and investigation of temperature were performed in triplicate. Esterase activity was determined as previously described.

Application of engineered cutinases

The PET used in the treatment experiments was produced industrially and discarded in the environment.

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These synthetic polymers were cut into pieces smaller than 20 x 20 mm. The samples were subjected to a pretreatment solution of Tween-80 at 2% v/v, at 50ºC for 1 hr. Then, they were washed in distilled water for 1 hr and dried in an oven at 40ºC for 24 hrs.

The pretreated polymers were weighed and subjected to UV radiation for 1 hr; 0.01% of PET was added to a solution containing 0.1 M phosphate buffer pH 7.5 and 1% of concentrated and formulated cell-free culture medium with esterase activity. The samples were incubated in Erlenmeyer flasks and subjected to orbital shaking 90 rpm, at 37ºC for 48 hrs. After the treatment, the samples were washed in a solution of 2 g/L Na_2CO_3 for 2 hrs, in order to stop the enzymatic reaction. Then, the polymers were washed in 10 g/L Tween-80, at 25ºC for 1 hr and subsequently washed with distilled water (O'Neill and Cavaco-Paulo, 2004).

The treated polymers were dried in an oven (40ºC for 24 hrs) and weighed.

Formulation of the concentrated liquid metabolic

The 2^3 factorial design with four center point replicates was used to optimize the enzyme stability by adding the following compounds: glycerol, polyethylene glycol (PEG-200) and ammonium sulphate ($(NH_4)_2SO_4$) as the factors (Table 1). Aliquots were removed at 0, 30 and 60 days of storage at room temperature (28ºC) to determine esterase activity.

Table 1. Factors and levels of full factorial design investigated in the stabilization of cutinases.

	Full factorial design		
Factors (%)	Levels		
	-	0	+
Glycerol	0	2.5	5
PEG-200	5	7.5	10
$(NH_4)_2SO_4$	5	7.5	10

RESULTS AND DISCUSSION

Microbial growth curve

Figure 1 illustrates the growth curves of both cultures of *E. coli* CUT and *E. coli* CUT-N1, grown in LB-medium at 37ºC. At the initial time of the cultivation the pH was 7.0 which value increased in the exponential phase of the cell growth to pH 7.2-7.3 that remained until the end of the experiment.

The lag phase of microbial growth was during the first two hours of cultivation. The exponential phase of *E. coli* CUT and *E. coli* CUT-N1 occurred between the second and sixth hours. The maximum specific growth rates (μ_{max}) estimated by the maximum gradient in the exponential growth phase were 1.3 h^{-1} and 1.2 h^{-1} for *E. coli* CUT and *E. coli* CUT-N1, respectively. The highest biomass production was determined in the maximum stationary phase, reaching 1.3 g/L after 24 hrs for the two cultures of *E. coli* that, under the working conditions, showed a similar behaviour.

The literature presents kinetic data of microbial *E. coli* genetically modified. Vaz et al. (2009) evaluated the kinetic parameters of transformed *E. coli* clones for the expression of antigens of *Leishmania chagasi*. The growth in the culture medium containing tryptone, yeast extract, sodium chloride at pH 7 led to μ_{max} values ranging between 0.11 to 0.22 h^{-1}. These authors also observed that the exponential growth phase finished within 6 of culture and the maximum biomass obtained was equal to 3.14 g/L after 24 hrs of cultivation. Cell productivity reached 0.13 g/L x hr which was greater than the value 0.05 g/L x hr calculated for *E. coli* CUT and *E. coli* CUT-N1 in our study.

The Crabtree effect may explain the low biomass production obtained in the cultures of *E. coli* CUT and *E. coli* CUT-N1. Cell growth can be inhibited by the production of heterologous proteins by the genetically modified cultures. Another aspect to be considered is the growth inhibition of these cultures because of the presence of IPTG itself. The addition of inducers is an important variable in the production of proteins, especially on a large scale, since IPTG is costly and it can be toxic to cells (Einsfeldt et al. 2011). Pan et al. (2008) confirmed that IPTG used to induce expression of cloned genes is a toxic compound that in high concentrations drastically reduces cell growth in some cultures of recombinant *E. coli*.

Effects of the concentration of IPTG in the production of cutinases

Table 2 illustrates the influence of IPTG concentration on the production of cutinases by *E. coli*. IPTG was added at the start of the exponential phase after 3 hrs (absorbance at 600 nm = 0.4-0.6). The maximum activity concentration of both cutinases was obtained after 24 hrs of cultivation. The cutinase activity produced by *E. coli* cultures showed different behaviours regarding the effect of this inductor. The cutinase activity by *E. coli* CUT-N1 was strongly dependent on the IPTG concentration; CUT-N1 production was inhibited by increasing the IPTG concentration. The maximum cutinase activity was 1.4 U/ml by *E. coli* CUT-N1 in the presence of 0.2 mM of IPTG after 24 hrs of cultivation. *E. coli* CUT expressed the maximum of 0.7 U/ml of cutinases at the concentration of 0.2 mM of IPTG. This value was not significantly changed by doubling the IPTG concentration.

Table 2. Activity of cutinases in the supernatant cell-free of *E. coli* CUT and *E. coli* CUT-N1 under submerged culture in the presence of different concentrations of IPTG.

E. coli	**IPTG**	**Activity of cutinases (U/mL)**					
	(mM)	3 hrs	4 hrs	5 hrs	18 hrs	20 hrs	24 hrs
E. coli CUT	0.2	0.02	0.08	0.1	0.5	0.6	0.7
E. coli CUT–N1	0.2	0.06	0.30	0.4	0.8	1.2	1.4
E. coli CUT	0.4	0.01	0.06	0.1	0.4	0.5	0.6
E. coli CUT-N1	0.4	0.04	0.30	0.4	0.7	0.8	0.9

Therefore, in this study, the concentration of 0.2 mM of IPTG was chosen for the production of both cutinases CUT and CUT-N1.

The decrease of the esterase activity may be partially due to the toxic effect of IPTG at the concentration of 0.4 mM. On the other hand, the induction of protein expression in the presence of IPTG induces stress to cells, which can lose the plasmid that contains the recombinant gene of interest (Sorensen and Mortensen, 2005). Kosinski and Bailey (1991) observed the temporary inhibition of protein during the exponential growth phase after the addition of IPTG to a culture of *E. coli*. Furthermore, high levels of recombinant protein expression in the presence of IPTG can also induce the expression of several proteases the enzymatic activity of which influences the activity of the heterologous enzyme (Han and Lee, 2006). Parekh and Patel (2012) investigated the IPTG concentrations: 0.1, 0.5 and 1 mM during 2, 4 and 6 hrs of induction of the protein Fcε-Bik by *E. coli*. The level of expression of this protein was determined by electrophoresis in polyacrylamide gel. The reduction of the expression of the recombinant protein in the presence of IPTG at 1 mM was observed when compared to other concentrations of the inducer.

Ultrafiltration of metabolic liquid

The (cell-free) supernatants with cutinolytic activity from the two genetically modified *E. coli* were concentrated twice by ultrafiltration (Table 3). The retention of the activity of cutinases on the concentrated bioproduct reached 93% when compared with the sample controls.

The cutinases determined in the concentrates showed approximately double the activity of the control samples. Few enzyme molecules crossed through the membrane; the activities in permeates reach only 0.10 and 0.15 U/ml for *E. coli* CUT and *E. coli* CUT-N1, respectively. The average molecular mass of cutinases (24 kDa) and the pores of the membrane (10 kDa) are responsible for these results (http://www.uniprot.org/uniprot/P00590). The substances cross through the pores by tangential force in the ultrafiltration process and, consequently, molecules larger than the pore size can also cross the membrane.

There was no loss of cutinolytic activity in the process of ultrafiltration. Under working conditions, the membrane has not retained any essential substance for the enzyme activity; neither did the addition of anti-microbial preservatives influence the enzyme activity.

Table 3. Enzymatic activity in the concentrated metabolic liquid.

Microorganism	Cutinolytic activity (U/mL)		Retention of activity (%)
	Control	Concentrate	
E. coli CUT	0.7	1.3	93
E. coli CUT-N1	1.4	2.6	93

pH optimum and pH stability of cutinases

Figures 2 and Figure 3 show the optimum pH and stability at different pHs of the concentrated and formulated supernatant with esterase activity during 30, 60 and 90 min at 28ºC. Cutinases expressed by *E. coli* CUT and *E. coli* CUT-N1 showed the same behaviour as a function of pH. The maximum activity was determined at pH 7.0 (optimum pH) and the maximum stability was determined during 90 min of incubation at the optimum pH. The pH stability of cutinases produced by *E. coli* CUT and *E. coli* CUT-N1 decreased from pH 7.0 to pH 9.0. The esterase activity at pH 9.0 reached only 20% of the maximum value in the initial time and was not determined during the incubation. The enzyme activity at pH 6.0 decreased during the incubation and reached 80-90 % of the maximum value during 90 min.

In the tested range of pH (4 to 10), the optimum pH 7.0 for cutinases from *Fusarium oxysporum* was also found by Pio et al. (2008). Moreover, cutinases produced by *F. solani pisi* and *T. fusca*, after isolation and purification, showed optimum pH 8.0 for the hydrolysis of triolein and pNPB. These enzymes were produced in specific media under orbital agitation of 200 rpm at 50ºC (Chen et al. 2008). Speranza et al. (2011) characterized cutinases produced by *F. oxysporum* and also determined maximal activity at pH 8.0 in different solid culture media in the presence of wheat bran, soybean rind and rice bran; and at pH 9.0 in the presence of *Jatropha curcas* seed cake. Although the maximum activities took place in slightly alkaline medium, the pH stability of these cutinases was higher at pH 6.0.

The optimum pH of three cutinases constructed by fusion with different CBM and expressed in *E. coli* was the same value as the native enzyme optimum pH (pH 8.0). The pH stability was highest for the three chimeric enzymes after incubation for 24 hrs at 37ºC under optimum pH (Zhang et al. 2010).

Thermal stability of cutinases

Figure 4 shows the activities of CUT-N1 at optimum pH and after incubation at temperatures of 30 to 70ºC during 30, 60 and 90 min. CUT showed the same behaviour. The activities were retained after incubation at 30, 40 and 50ºC, during 90 min. The retention of activity at 60ºC was only 38% and non-enzymatic activity was determined after incubation at 70ºC, during 90 min.

The literature shows several references of optimum temperature of cutinases equal to/above 50ºC. Zhang et al. (2010) determined optimal temperature of 50ºC for recombinant cutinases and observed the half-life of 53 hrs after incubation at optimum temperature. Ronkvist et al. (2009) characterized cutinases produced by different microorganisms and determined thermostability at 50ºC for cutinases

produced by *Pseudomonas mendocina* and *F. solani*, while cutinases from *Humilica insolens* showed thermostability at 70-80ºC.

Moreover, mesophilic cutinases are also produced by microorganisms. Speranza et al. (2011) compared characteristics of cutinases produced by *F. oxysporum* in different solid media. The optimum temperature and the thermal stability ranged between 30 and 37ºC depending on the substrate. Chen et al. (2008) characterized cutinases produced by *F. solani pisi* and *T. fusca* and determined optimum temperatures of 30 and 40ºC, respectively. Cutinases from *T. fusca* exhibited higher thermal stability; the residual activity was higher than 80% after 160 hrs, at 40ºC.

Application of CUT and CUT-N1 in the degradation of plastics

Table 4 presents the weight difference after the treatment of PET with cell-free culture media produced by *E. coli* CUT and *E. coli* CUT-N1 with cutinase activity (1.3 and 2.6 U/mL, respectively). The higher mass loss during the treatment of PET was verified in the presence of CUT-N1. The decrease of the weight was approximately one percent of this plastic mass after incubation at 37ºC during 48 hrs.

Table 4. Treatment of PET by cutinases in phosphate buffer pH 7.2 during 48 hrs.

Samples	Initial weight (mg)	Final weight (mg)	Weight loss (%)
Control	167.4 ± 0.04	167.4 ± 0.04	0
E. coli CUT	183.0 ± 0.15	182.9 ± 0.15	0.05
E. coli CUT-N1	167.2 ± 0.22	165.7 ± 2.77	0.90

Vertommen et al. (2005) observed a surface modification of PET by the catalytic activity of commercial lipases and cutinases. After 120 hrs of incubation, the degradation of this plastic was observed, beyond the determination of co-polymers of PET, presents in the aqueous phase.

Ronkvist et al. (2009) compared the catalytic activity of cutinases produced by *H. insolens, F. solani* and *P. mendocina* in the presence of PET films as substrates. After 96 hrs of incubation, the catalytic activities of cutinases produced by *F. solani* and *P. mendocina* resulted in a weight loss of 5% of this plastic at 40 and 50ºC, respectively. However, the hydrolysis of cutinases produced by *H. insolens* at 70ºC, resulted in a weight loss of 97 ± 3% after 96 hrs. The authors determined a loss of the thickness film with low-crystallinity of 30 µm/day. Moreover, the analysis of the aqueous products of the three treatments showed the presence of terephthalic acid and ethylene glycol.

Herrero-Acero et al. (2011) evaluated cutinases produced by *T. cellulosilytica* DSM44535 (Thc_Cut1 and Thc_Cut2) and *T. fusca* DSM44342 (Thf42_Cut1) in the PET hydrolysis. The cutinase Thc_Cut1 hydrolyzed this substrate and released large amounts of terephthalic acid, among other compounds. Different hydrolytic properties were determined although the cutinases Thc_Cut1 and Thc_Cut2 showed a high degree of homology. The modeling of these enzymes revealed that electrostatic and hydrophobic differences in the surface properties of the active site could be responsible for these characteristics. These authors observed an increase in the PET hydrophilicity due to a decrease in the contact angle with the water.

Ribitsch et al. (2012) characterized the hydrolysis of PET from cutinases produced by *Thermobifida alba* (Tha_Cut1). The authors determined the decrease in the water contact angle of 87.7º to 45.0º at the surface of PET hydrolyzed, leading to an improvement in the polymer hydrophilicity, a fundamental characteristic in the treatment of plastic degradation.

The literature shows the versatility of cutinases used in the treatment of other polymers. Wang et al. (2010) showed results of cutinase applications in enzymatic processes of pretreating the tissue. The authors showed that cutinases act as modifier agents of the polymer surface, by facilitating the absorption of other substances in different treatments.

Formulation of concentrated metabolic liquid with cutinolytic activity

Table 5 shows the activities of cutinases produced by *E. coli* CUT-N1 in the concentrated metabolic liquid and formulated with glycerol, PEG-200 and $(NH_4)_2SO_4$. The activity of the control sample was 2.6 U/ml in the absence of the chemical additives whereas the formulated bioproduct showed cutinolytic activities of 1.4 to 2.6 IU/mL. The activity of cutinases was independent of the concentrations of PEG-200 (5 and 10%) and glycerol (0 and 5%) while the concentration of the salt influenced the enzyme activity the maximum value of which was determined in the presence of 10% of $(NH_4)_2SO_4$ (assays 5 and 8) at initial time.

Table 5. Decoded matrix of the factorial design, cutinolytic activity at initial time and retention of activity of the bioproduct produced by *E. coli* CUT-N1.

Assay	Glycerol (%)	PEG-200 (%)	$(NH_4)_2SO_4$ (%)	Activity (U/mL)	Retention of activity (%)	
					30 days	60 days
1	0	5	5	2.2	100	50
2	5	5	5	1.8	100	100
3	0	10	5	1.8	100	100
4	5	10	5	1.8	100	100
5	0	5	10	2.6	70	75
6	5	5	10	2.4	90	70
7	0	10	10	1.4	100	80
8	5	10	10	2.6	70	70
9	2.5	7.5	7.5	2.2	100	100
10	2.5	7.5	7.5	2.2	100	100
11	2.5	7.5	7.5	2.2	100	100
12	2.5	7.5	7.5	2.2	100	100

At low concentrations, salts can stabilize proteins by non-specific electrostatic interactions, depending only on the ionic strength of the medium. At high concentrations, salts exert specific effects on protein, resulting in the precipitation of these compounds. Salts act on the increase of the interfacial tension between the protein surface and the solvent, changing the solubility (Hamada et al. 2009).

In the formulation to stabilize CUT-N1 produced by *E. coli*, the maximum activities (2.4-2.6 U/mL) determined at zero time showed low storage stability. On the other hand, the cutinolytic activity of 2.2 U/ml determined on center point assays 9, 10, 11 and 12, showed 100% of retention of the activity in the presence of 2.5% v/v glycerol, 7.5% v/v PEG-200 and 7.5% of $(NH_4)_2SO_4$, during 60 days of storage at 28ºC. Therefore, concentrations of these chemical additives used on the center point are proposed to stabilize the CUT-N1.

Figure 5 illustrates the Pareto chart that shows the effects by the factors: glycerol, PEG-200 and $(NH_4)_2SO_4$ on the response variable: esterase activity of the bioproduct produced by *E. coli* CUT-N1 at the initial time.

Considering the effects of the factors statistically significant, the increase of the concentration of 5 to 10% of $(NH_4)_2SO_4$ in the activity of cutinases showed positive effect and led to higher catalytic action of the enzyme produced by *E. coli* CUT-N1. Talekar et al. (2012) stabilized alpha-amylase from *Bacillus amyloliquefaciens* immobilized using $(NH_4)_2SO_4$. As the concentration of this salt increased the enzyme activity also increased. The maximum activity was at 70% of $(NH_4)_2SO_4$. The authors concluded that the precipitation reduces the contact surface with the solvent and increases the stability of proteins.

PEG is one of the most versatile water soluble polymers for refolding recombinant proteins and for stabilizing proteins by chemical modification (Hamada et al. 2009). In this study, the Pareto chart (Figure 5) shows the positive effect of the interaction between glycerol and PEG-200 in the activity of CUT-N1.

The interpretation of the factorial design by Pareto chart in Figure 6, using the retention of the esterase activity during 30 days of storage as response, shows that the only significant variable (factor) was the $(NH_4)_2SO_4$ concentration. The increase of 5 to 10% of this salt negatively affected the stability of both cutinases, *i.e*., at higher concentrations of the salt, the enzyme stabilitydecreased. The negative interaction between glycerol and PEG-200 in the stability of cutinases during 30 days of storage indicated that the increase of the concentrations of these two variables, at working conditions, decreased the activity of cutinases.

The effects of the factors on the activity during 60 days of storage at room temperature were not statistically significant.

All the tested samples of CUT - formulated with glycerol, PEG-200 and $(NH_4)_2SO_4$ - showed lower enzyme activity than the control sample (non-formulated). The retention of the activity decreased in 83% of assays during 60 days of storage at room temperature.

CONCLUDING REMARKS

• Maximum productions of recombinant cutinases CUT and CUT-N1 is induced by 0.2 mM IPTG.

• Ultrafiltration membrane of 10 kDa is effective to concentrate cutinases in cell-free metabolic liquid.

• Cutinases CUT and CUT-N1 produced by cultures of *E. coli* genetically modified have optimum pH 7.0 and thermal stability at 30-50ºC.

• Enzymatic treatment of PET by cutinases during 48 hrs decreases the mass of this plastic.

• 2.5% v/v glycerol, 7.5% $(NH_4)_2(SO_4)$, and 7.5% v/v PEG-200 added at the metabolic liquid stabilize recombinant cutinases during 60 days of storage at 28ºC.

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Figures

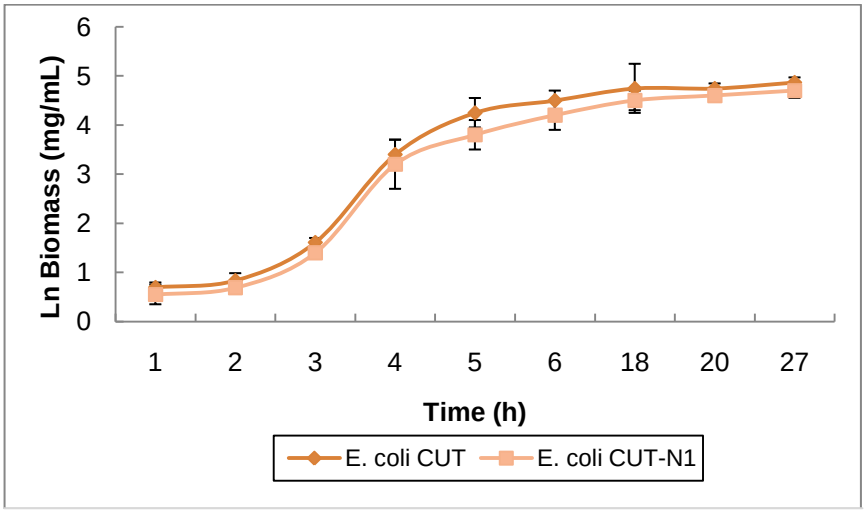

Fig. 1 Cell growth curve of cultures of *E. coli* CUT and *E. coli* CUT-N1 in LB medium supplemented with 100 µg x mL^{-1} Ampicillin.

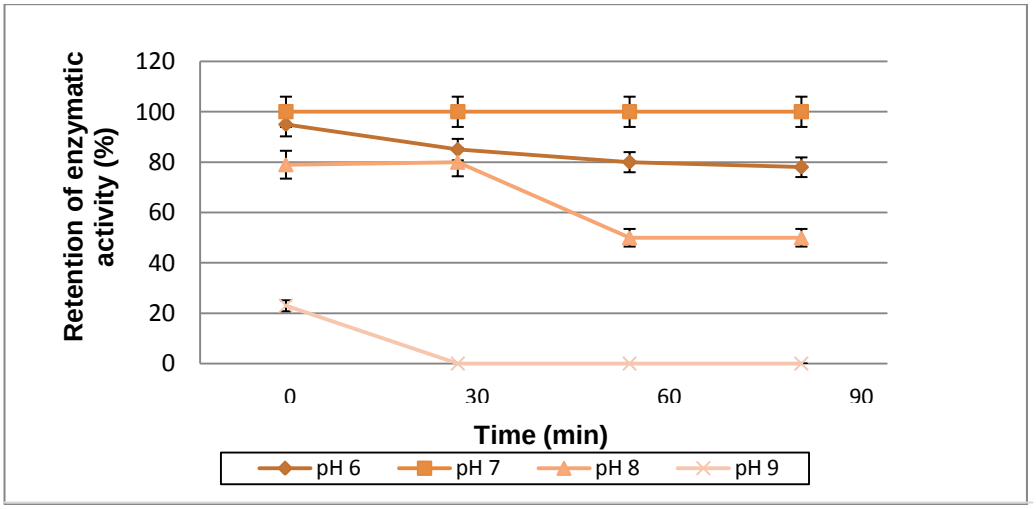

Fig. 2 Retention of enzymatic activity of the concentrated and formulated metabolic liquid from *E. coli* CUT at pH 6 to 9.

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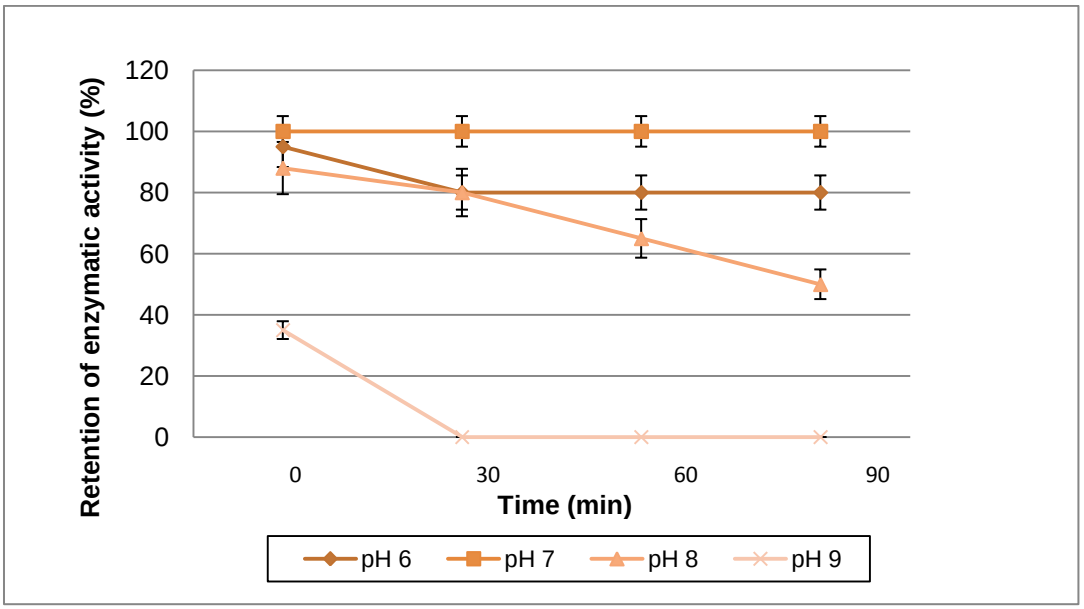

Fig. 3 Retention of enzymatic activity of the concentrated and formulated metabolic liquid from *E. coli* CUT-N1 at pH 6 to 9.

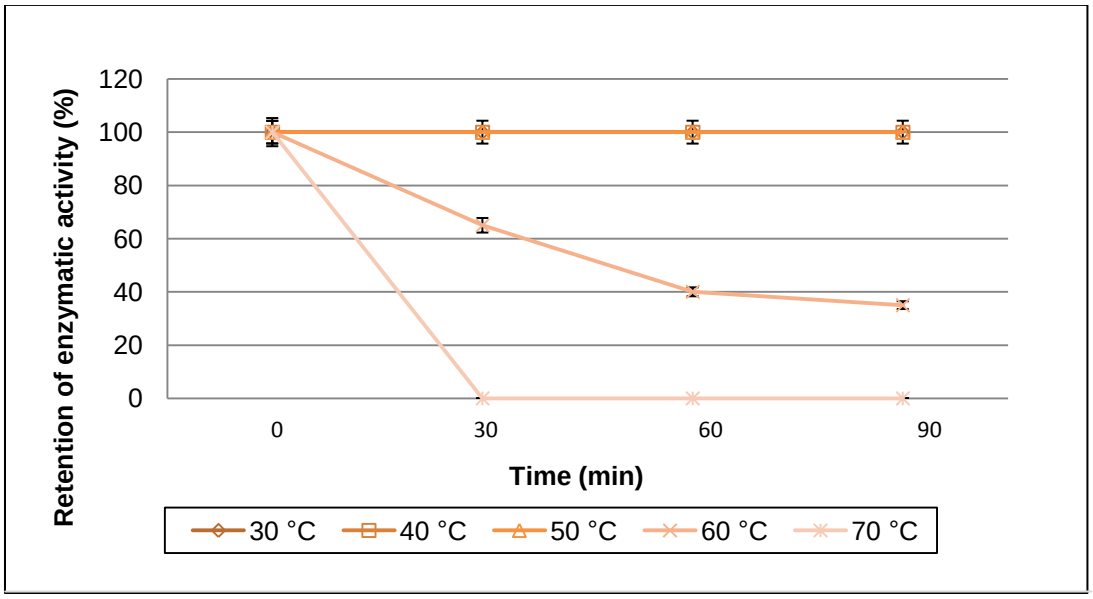

Fig. 4 Thermal stability of the metabolic liquid of *E. coli* CUT-N1 at optimum pH.

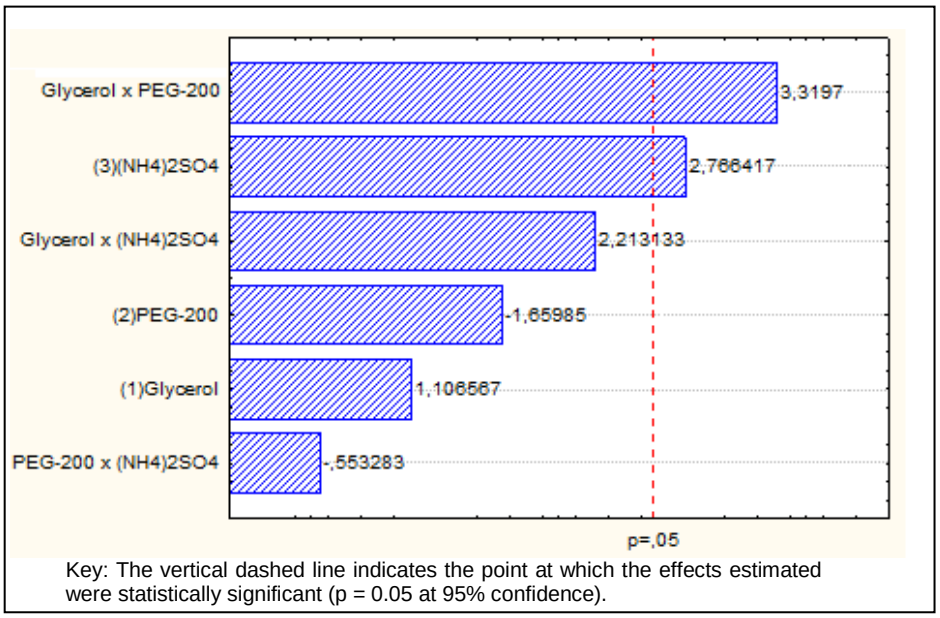

Fig. 5 Pareto chart of standardized effects for (1) glycerol, (2) PEG-200 and (3) $(NH_4)_2SO_4$ using activity of cutinases CUT-N1 as the response variable at initial time.

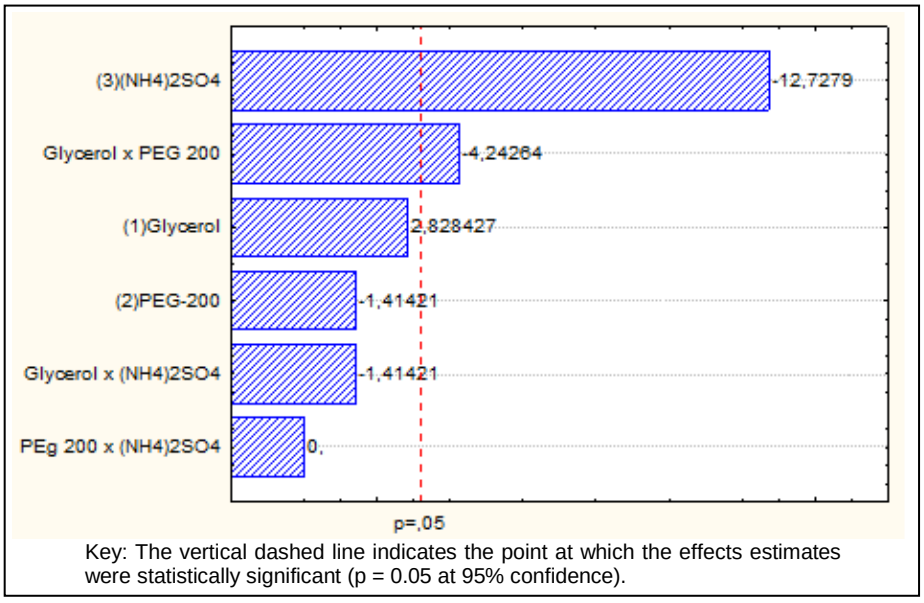

Fig. 6 Pareto chart of standardized effects for (1) glycerol, (2) PEG-200 and (3) $(NH_4)_2SO_4$ using activity of cutinases CUT-N1 as the response variable during 30 days of storage at room temperature.

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