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Research Article

Cloning, protein expression and biochemical characterization of *Carica papaya* esterase

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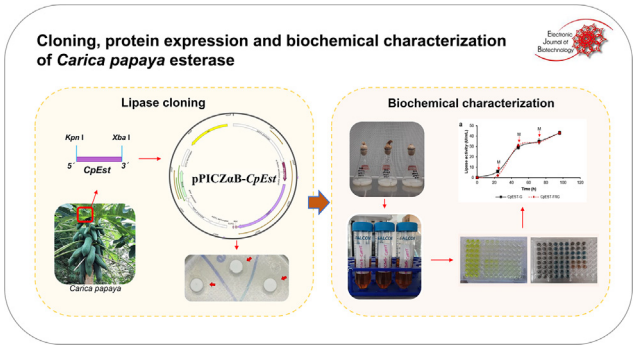
GRAPHICAL ABSTRACT



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ABSTRACT

Background: GDSL-like esterase/lipase proteins (GELPs) are enzymes that possess unique characteristics, they contain four invariable catalytic residues. Advances in the study of these proteins are interesting. The cloning and functional expression of a papaya esterase have not been reported. Therefore, in this work we evaluated the heterologous production of *Carica papaya* esterase CpEST in the yeast *Komogataella phaffii* (*Pichia pastoris*).

Results: The cloning and expression of the protein was performed under the P_{AOX1} promoter, and productions of up to 43 AU/mL were achieved using residual glycerol from biodiesel in the batch phase and methanol for the induction phase. Enzyme activity assays determined that CpEST has a high preference for short-chain substrates (*p*-NP C4 and *p*-NP C8), and optimal activity conditions were observed at 30°C and pH 10. The enzyme showed the highest stability to acetone, ethanol and *tert*-butanol solvents, retaining approximately 55% of its initial enzymatic activity after 1 h of exposure.

Conclusions: Cloning and functional expression of papaya CpEST esterase was achieved. During fermentation, the yeasts used as a carbon source residual glycerol from biodiesel production. Based on the results obtained from the characterization of the esterase, it was found that it has a high potential for use in the bioenergy and detergent industry.

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1. Introduction

Lipases (triacylglycerol ester hydrolases EC. 3.1.1.3) are ubiquitous enzymes found in different sources, such as microorganisms, animals, and plants [1,2,3]. These proteins catalyze the hydrolysis of fats and oils. However, in nonaqueous solvents, they are also able to catalyze synthesis, transesterification and interesterification reactions [4]. Due to their robustness, high specificity, and generation of higher quality products, lipases have become a tool with great potential, across various industries, including for use in the food and bioenergy industry in the production of aromatic esters, structured lipids, and biodiesel by esterification and transesterification, among others [5,6]. Previous studies have shown that the latex fraction of *Carica papaya* can be used as a biocatalyst for lipid modification and is therefore considered a source of plant lipases [5]. However, due to the matrix in which the enzymes are found, they are not commonly isolated and purified. One of the strategies to bypass this problem is heterologous expression in yeast cell factories, which are capable of secreting the enzymes of interest into the medium [7]. Under this system, the expression and recombinant production of lipase 1 from *C. papaya* (CpLIP1) was successfully achieved [8,9]. The product of this process, is considered highly functional; it was immobilized in Lewatit VPOC 1600 and successfully evaluated as a biocatalyst in the transesterification of crude oil of *Jatropha curcas*, demonstrating its efficiency in these processes [10]. On the other hand, the presence of an esterase with a GDSL esterase/lipase motif (UniProt ID: p86276) with a molecular weight of 38 kDa and 343 amino acid residues has been evidenced. This has great lipolytic activity due to its high preference for short-chain fatty acids [11]. GDSL-like esterase/lipase proteins (GELPs) have unique characteristics. They possess a conserved GDSL sequence motif at their N-terminus different from the conserved GxSxG motif of classical lipolytic enzymes. They are also called SGNH hydrolases because these four catalytic residues are conserved and invariant in blocks I, II, III and V [12,13,14]. These enzymes have been studied recently, and the function of the predicted GDSL gene in plants is still limited [15,16,17]. Recent reports indicate that the cloning of a strawberry GDSL lipase/esterase enzyme (*FvGELP1* gene) expressed in *Saccharomyces cerevisiae* allowed us to determine the role of the protein in the fruit ripening and hemicellulose degradation process [18]. The GDSL-type enzyme from *Ipomoea batata* was expressed in different expression systems, *Escherichia coli*, *S. cerevisiae* and *Komogataella phaffii*, and enzyme activity was detected only when extracts of the latter were used [19]. Therefore, studies on the heterologous expression of these plant enzymes are a fairly new. In addition, and considering the importance of esterases in different sectors, such as the food industry, where they have been used to synthesize more stable antioxidant molecules by esterifying vitamin A with a long-chain fatty acid chain, they show the relevance of the study of these enzymes [20,21].

On the other hand, during the production of biodiesel, by products such as glycerol are also generated. If this byproduct, had high levels of purity, it could be used in various sectors. However, to date, it has not been possible to determine an efficient methodology, so glycerol becomes a waste pollutant [22,23,24]. Therefore, in applying the concept of a circular economy, the search for new strategies to take advantage of the materials available for proposes has led to the consideration of esterases as a viable alternative [25,26,27]. The circular economy method helps in the transition to reuse and proper management of waste generated during each stage of product development through the adoption of ethical and sustainable solutions [28,29].

In light of this, the present work focused on the cloning, expression and characterization of the CpEST esterase from *C. papaya* using the yeast *K. phaffii* as a cell factory using residual glycerol as a carbon source for growth and methanol for induction.

2. Materials and methods

2.1. Synthetic gene design

The protein sequence of CpEST was obtained on Universal Protein Resource Knowledgebase UniProt ID: p86276 [11]. The mature protein-coding sequence was optimized with codon usage for *K. phaffii* and flanked by restriction sites *Kpn* I and *Xba* I (New England Biolabs, NEB).

2.2. Construction and expression of pPICZαB-CpEst

The mature protein-encoding sequence without the signal peptide of CpEST was inserted between the *Kpn* I and *Xba* I sites of *K. phaffii* under regulation of P_{AOX1} by using the vector pPICAZαB (Invitrogen) to generate the pPICAZαB-*CpEst* plasmid arranged in frame with an N-terminal secretory signal of the *S. cerevisiae* α-factor. The plasmid was linearized with *Pme* I and transformed into *P. pastoris* X-33 electrocompetent cells. High resistance to Zeocin (100 mg/ml) was used as a selection marker for transformed cells, and subsequently, these colonies were reseeded in culture medium supplemented with 1% tributyrin. The colonies that formed the halo with the largest diameter around the microbial growth were selected for further study. The plasmid construction was confirmed by colony PCR and sequencing. The gene was amplified with two primers: α-factor_F (5′-TAC TATTGCCAGCATTGCTGC-3′) and AOX1_R (5′-GCAAATGGCATTCTGACATCC-3′). The PCR conditions were as follows: one cycle of denaturation at 95°C for 5 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 30 sec, and a final extension step at 72°C for 5 min. The PCR product was visualized in 1% agarose at 100 V for 40 min. All restriction enzymes and One*Taq* 2X Master mix with standard buffer for polymerase chain reaction (PCR) were purchased from New England Biolabs. Direct Load 1 kb DNA Ladder and Gen ruler DNA Ladder Mix were purchased from Sigma and Thermo Scientific, respectively. UltraPure agarose was purchased from Invitrogen.

2.3. Enzyme production

Recombinant protein was produced by heterologous expression in the cell factory *K. phaffii.* The selected transformed strains were incubated in 25 mL of YPG broth (10 g/L yeast extract, 20 g/L peptone, 20 g/L glycerol ACS reagent and residual biodiesel production) for 24 h. Then, 250 mL of BMM broth (100 mM potassium phosphate, pH 6.0, 1.34% YNB, 4×10^{-5}% biotin, 1% methanol) in 1000 mL flasks was inoculated with 25 mL of preculture and shaken at 250 rpm and 30°C. Protein production induction was

performed by adding 0.5% methanol every 24 h. After cultivation was carried out for four days, the culture broth was centrifuged at 11000 × *g* for 30 min at 4°C, and finally, the obtained supernatant was tested for lipase activity [7]. The residual glycerol was donated by the company Bioil de México (Guadalajara, Jal., México).

2.4. Characterization of CpEST

2.4.1. Total protein determination

Protein concentration was determined using Bradford's method with bovine serum albumin (BSA) as the standard [30]. Bradford Reagent was purchased from Sigma. All reagents for SDS–PAGE analysis were obtained from Bio-Rad. Measurements were performed in duplicate.

2.4.2. Lipase activity assay

Lipase activity using p-nitrophenyl butyrate as a substrate was determined by measuring the amount of *p*NP released after the addition of the enzyme. The standard assay was conducted at 30°C for 15 min in a final volume of 200 µL of a reaction mixture containing 100 mM carbonate buffer (pH 10.0), 1 mM *p*NP-butyrate dissolved in 2-methyl-2-butanol (Sigma Aldrich, USA) and 20 µL of enzyme extract diluted in carbonate buffer. The liberated *p*NP was quantified at 402 nm using a microplate spectrophotometer (Costar, USA). One unit (1 U) of lipase activity was defined as the amount of enzyme needed to liberate 1 µmol of *p*NP per minute under the conditions described above.

2.4.3. Substrate specificity

This parameter was evaluated by measuring the activity toward *p*-nitrophenyl acetate (*p*NP-C2), *p*-nitrophenyl octanoate (*p*NP-C8), *p*-nitrophenyl decanoate (*p*NP-C10), *p*-nitrophenyl dodecanoate (*p*NP-C12), *p*-nitrophenyl myristate (*p*NP-C14), and *p*-nitrophenyl palmitate (*p*NP-C16) under the same conditions used to measure activity by hydrolysis of *p*-nitrophenil butyrate (Sigma–Aldrich, USA).

2.4.4. Effect of temperature on lipase activity

The temperature effect on lipase activity was studied by carrying out the assays at different temperatures in the range of 25°C to 45°C and pH 10.0 using 100 mM Tris-HCl buffer.

2.4.5. Effect of pH and ionic strength on lipase activity

The pH effect was evaluated by measuring the activity using different buffers at 100 mM concentration and 30°C. The corresponding buffers were used as follows: acetate buffer (pH 3–6), Tris-HCl (pH 7–9), and carbonate buffer (pH 10–11). The ionic strength effect was evaluated by measuring the activity using different buffer concentrations (50, 100, 150, 200, 400 and 1000 mM) at 30°C.

2.4.6. Effect of solvent on lipase activity

The solvent effect on lipase activity was determined by measuring the residual activity after incubation of the enzyme in the presence of 20% v/v solvent and 100 mM Tris-HCl buffer pH 10.0 for one hour. After solvent treatment, the activity was measured under standard conditions. The solvents were purchased from Sigma–Aldrich and Merck. The statistical analysis of experimental data represents the mean SE of two independent experiments.

2.4.7. Statistical analysis

The statistical analysis of experimental data represents the mean of three independent experiments, and bars represent the SE. Calculations were performed using the statistical software R version 3.5.1[31].

3. Results

3.1. Expression of CpEST

After the transformation process in the *P. pastoris* yeast, 11 colonies were identified by forming a hydrolysis halo in agar plate medium supplemented with tributyrin (Fig. 1a). An amplicon of approximately 1237 bp demonstrate the presence of the CpEST gene within the genome of the selected yeast (Fig. 1b), for which the plasmid and enzyme construction was successfully transformed.

The extracellular secretion of the recombinant enzyme was carried out because the CpEST signal peptide was replaced by the α-factor secretion sequence of *S. cerevisiae*. Therefore, the biochemical characteristics were carried out using culture supernatant of the selected and transformed clones with the pPICAZαB-*CpEst* construct. A maximum enzyme activity of approximately 43 AU/mL was reached at 96 h of fermentation in shake flasks and after the addition of 0.5% methanol as an enzyme production inducer (Fig. 2a). The initial use of two types of glycerol as a carbon source, glycerol ACS reagent (G) and filtered residual glycerol (FRG), allowed a biomass of approximately 6 g/Lto be reached at 48 h. After that time and due to the beginning of the enzyme production induction phase, the yeasts experienced slow growth, as expected, since the added methanol is metabolized by the cell for the formation of products and in a smaller amount to produce biomass [32,33]. However, the growth kinetics of the yeasts in the media supplemented with different glycerol sources showed significant similarity (Fig. 2b). A previous study revealed that the utilization of residual glycerol on *Yarrowia lipolytica* yeast biomass production is possible, and moreover, no negative effects on cell morphology and extracellular lipase production were found [34]. Therefore, the use of residual glycerol from biodiesel can be exploited as a carbon source for obtaining high cell density in the production of lipolytic enzymes.

The presence of the enzyme in the cell culture extract was verified by SDS–PAGE. The estimated theoretical molecular weight for the CpEST enzyme is approximately 38 kDa [11]. However, previous reports noted that a difference of approximately 3 kDa was observed between the weight obtained by molecular prediction and that observed on SDS–PAGE [11]. This was also observed in the present work (Fig. 3) and is possibly because the protein was found to be glycosylated, since 4 N-glycosylation sites were previously predicted for this protein.

3.2. Substrate specificity

A protein colorimetric assay using *p*NP esters of different chain lengths was used to determine substrate specificity [35,36,37]. Although they are not natural substrates, activity on *p*NP esters could confirm the behavior of the enzyme as that of an esterase or "true" lipase. Recombinant CpEST exhibited the highest activity on *p*NP-C4 after this substrate and an exponential decrease when a fatty acid chain increase was observed. Finally, no activity was observed on *p*NP-C16 (Fig. 4). In contrast to plant esterases, lipolytic enzymes of the GDSL family of extremophilic microorganisms mostly have a preference for short-chain fatty acids such as *p*NP-C2 and *p*NP-C4 [38,39,40]. The evidence of enzymatic activity on the substrates tested is directly attributed to the recombinant CpEST protein secreted by the yeast, since the host microorganism does not secrete native extracellular lipases. As a result, this yeast continues to be a powerful platform for the efficient expression and secretion of enzymes or other proteins of industrial interest [33].

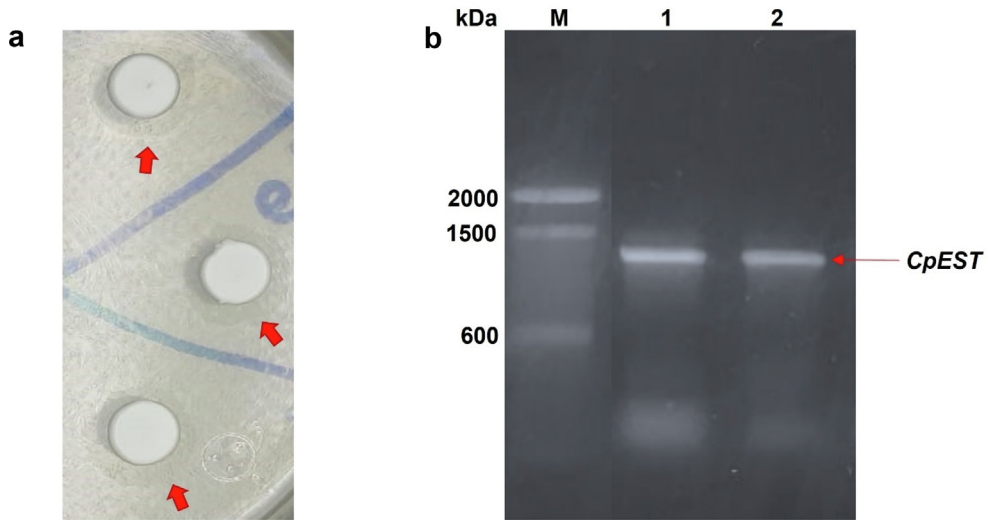

Fig. 1. Selection and identification of positive colonies expressing the *CpEST* gene. (a) Formation of hydrolysis halo in medium with tributyrin. The red arrows indicate the halo generated after 24 h of incubation at 30°C of the colonies that grew in medium supplemented with zeocin. **(b)** Electrophoresis in agarose 1%/100 V/40 min of colony PCR product. M: Marker 100 bp. 1 and 2: 1237 bp amplicon PCR product using transformation-positive colonies as a template.

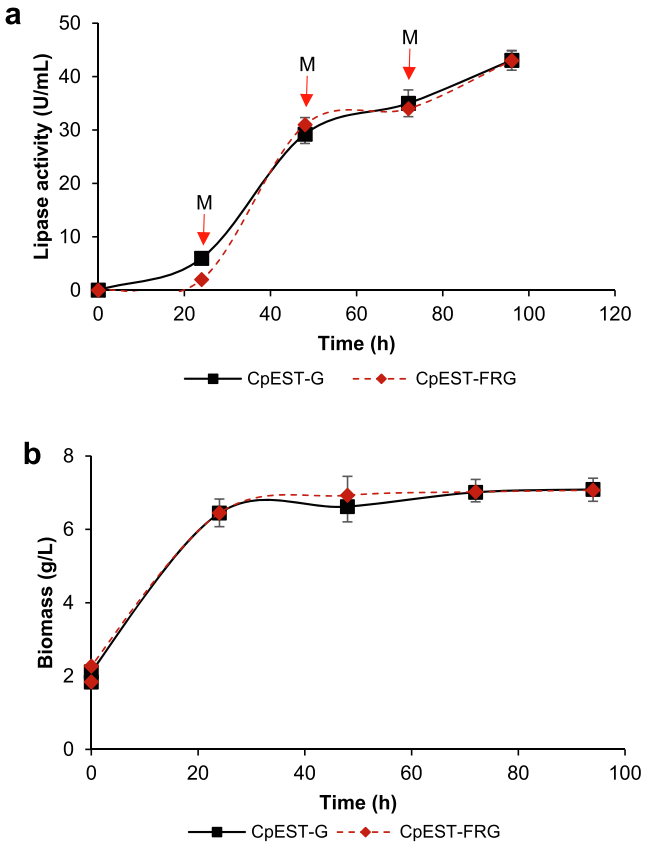

Fig. 2. Enzymatic production of recombinant CpEST and cell growth. **(a)** Lipase activity using extracts from production media using reactive and residual glycerol. **(b)** Biomass. Data represent the mean ± SD of two independent experiments. G = glycerol, ACS reagent; FRG = filtered residual glycerol. The red arrows indicate the addition of methanol (M) to induce enzyme production. The error bars represent the standard deviation.

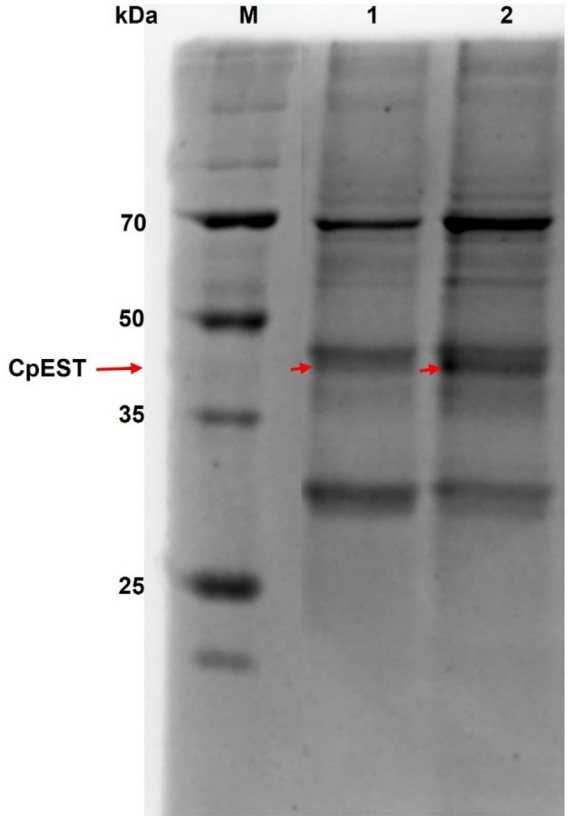

Fig. 3. SDS–PAGE analysis of the recombinant CpEST from *C. papaya*. Lane M, molecular size marker; lanes 1 and 2 extract from cell-free culture media. The arrow points to the CpEST protein in the study.

3.3. Effects of pH, ionic strength, and temperature on lipase activity

The pH and temperature of recombinant CpEST were determined using *p*NP-C4 as a substrate. It was observed that for this enzyme, there was a marked tendency toward alkalinity since activity was detected from a pH range of 5.0 to 10.0 (Fig. 5a). Since the highest specific activity of the enzyme was reached at pH 10.0 in a reaction medium incubated at 30°C, evaluation of different buffer concentrations was performed at this pH (Fig. 5b). The presence of high or low concentrations of salts in the medium can impede enzymatic activity since enzymes require an adequate

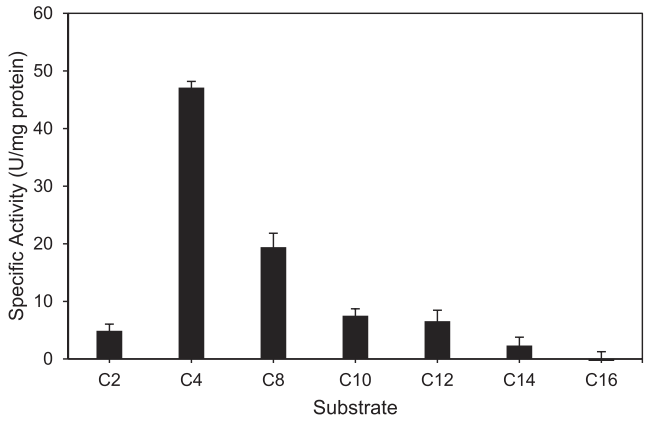

Fig. 4. Substrate specificity of recombinant CpEST. C2, *p*NP-C2; C4, *p*NP-C4; C8, *p*NP-C8; C10, *p*NP-C10; C12, *p*NP-C12; C14, *p*NP-C14; C16, *p*NP-16.

ionic strength to maintain their charge and structure. The results related to the ionic strength revealed the maximum value of the specific activity that was obtained when the buffer used was 100 mM, indicating a tendency to decrease as the buffer concentration was higher (Fig. 5b). Regarding temperature, the optimum was reached at 30°C with an important loss of specific activity for the highest temperatures (Fig. 5c).

3.4. Effects of organic solvents on lipase activity

The tests to determine the effect of different solvents on the catalytic activity of recombinant CpEST showed that it maintains over 50% of its activity in acetone, ethanol and tert-butanol, while in isooctane it loses almost 70% biocatalytic capacity (Fig. 6).

4. Discussion

The presence of active esterases produced by microorganisms was identified by an agar plate assay. When tributyrin, is sometimes emulsified in different culture media and poured into a petri dish, lipolysis or stereolysis can be observed via the formation of a halo around the producer colony by changes in the appearance of the substrate [41]. The tributyrin hydrolysis zones observed around the halo-shaped colony in Fig. 1a are an indicator that there is secretion of the CpEST enzyme by different colonies of yeast under study, as no extracellular native lipase/esterase production from the host cell has been reported [32].

During the production of biodiesel, in addition to biofuel, residual glycerol is obtained; stoichiometrically, the reaction requires 3 mol of methanol and 1 mol of triglycerides to produce 3 mol of biodiesel and 1 mol of glycerol [42,43]. In most cases, since residual glycerol is of low purity, few application alternatives have been identified [23]. Although some advances have been made in the purification of this residue, the options explored have not achieved the desired degree of purity [24]. Therefore, methodologies are still being sought to use this waste and minimize environmental impacts. In this sense, the use of residual glycerol as a substrate for the production of lipases in the batch phase to generate biomass as quickly as possible with a high $Y_{X/S}$ can be a viable alternative, since, as seen in Fig. 2, the production of the recombinant enzyme CpEST had the same production kinetics and biomass as the lipase medium culture in which reagent grade glycerol was used. Thus, possible contaminants present in crude glycerol do not affect the growth kinetics [44].

Because lipase production is associated with a methanol induction system (*pAOX1*) and not with cell growth, as occurs with production systems where constitutive vectors (*pGAP*) are used, biomass growth has a lower specific growth rate once the production inducer is added, according to the change in carbon source from glycerol to methanol, while the lipase activity titers increase, as shown in Fig. 2 [45,46].

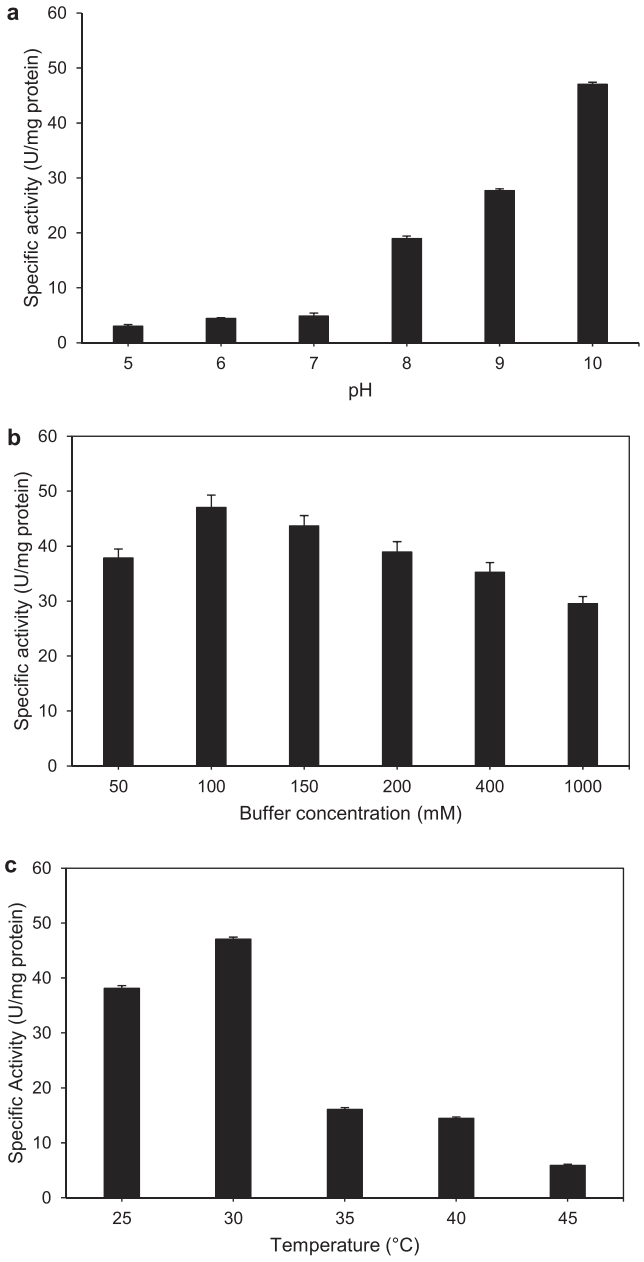

Fig. 5. Effects of ionic strength, pH and temperature on the specific activity of recombinant CpEST. (a) Determination of the optimal pH. **(b)** Evaluation of the effect of ionic strength. **(c)** Determination of the optimal temperature.

During enzyme characterization, *p*NP substrate assays demonstrated that the highest specific activity of recombinant CpEST (Fig. 3) exhibits a high preference for short-chain substrates (*p*NP-C4), as previously reported for CpEST esterase partially purified from the latex of *C. papaya* [11]. This affinity for short-chain substrates (*p*NP-C4) was also observed with esterases from *Geobacillus thermodenitrificans* T2 [40,47,48]. Other mainly thermophilic microbial esterases, such as *Fervidobacterium nodosum* and *Bacillus* sp. K91, have been identified for their high affinity for short-chain substrates, such as *p*NP-C2 [38,39]. The

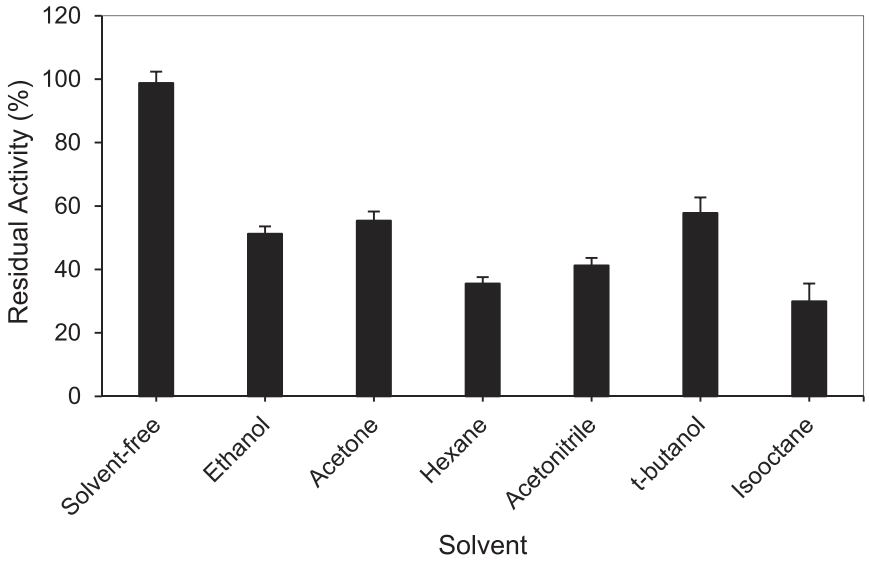

Fig. 6. Solvent stability of CpEST. Activity was measured by hydrolysis of pNP-C4 after 1 h of incubation.

recombinant CpESt, although at a lower percentage in addition to hydrolyzing short-chain substrates, also hydrolyzes medium- and long-chain substrates such as *p*NP-C10 and *p*NP-C14, respectively. This result differs from what was previously obtained for the partially purified CpEST protein, which, as expected, showed a greater preference only for the natural substrate tributyrin but not for trioctanoin (C8) [11]. *G. thermocatenulatus* esterases/lipases have the same tendency as recombinant CpEST to hydrolyze medium- and long-chain *p*NPs, and unlike the study performed with partially purified CpEST, the triactanoin substrate is less soluble than the paranitrophenol substrates, which promotes lower affinity for the natural substrate. This is reflected in less activity when using this substrate [44].

On the other hand, it is known that the enzymatic activity of an enzyme is influenced by various factors present in the reaction medium, such as substrate concentration, pH and temperature [49,50]. In the case of the CpEST under study, it was determined that it is an enzyme that remains active in a pH range of 8 to 10, having its maximum activities at the most alkaline pH. At acidic pH, the activity observed is residual. From a neutral pH with a tendency toward acidity, the enzymatic activity of the enzyme decreases by up to 90%. Esterases and lipases with optimal activities at pH 9.0 and 9.5 have already been reported [40,49,51]. The concentration of salts in the reaction medium is crucial for the optimal reaction rate of the enzyme. In the case of CpEST, the effect of the ionic strength on the specific activity at 100 mM allowed the maximum value to be reached, with a decrease in value at higher levels of salt concentration. This is because a high concentration of salts in the medium affects the ionic strength of the protein, affecting its stability to maintain its charges and structure [52]. Active enzymes at highly alkaline pH values have great potential as biocatalysts in bioenergy and industrial applications for the manufacture of detergents. In this sector, most of the enzymes are used for the removal of greasy residues in clothes [53]. On the other hand, temperature can have significant effects on the catalytic activity of an enzyme because it can affect its stability or irreversibly denature it [54]. In the case of the CpEST protein, the reaction temperature range is from 25 to 45°C, with 30°C being optimal, so its use during catalytic processes can occur almost at room temperature [55]. High temperatures and temperatures higher than those evaluated could have negative effects on the enzyme activity caused by partial or total denaturation of the protein [56]. In addition to temperature, it is important to consider that the correct choice or the absence of solvents can affect the performance of the enzyme, since the physicochemical properties of the solvent used can control the molecular interactions between the enzyme, the substrates, and the reaction medium. Certain highly polar solvents are known to affect some enzymes. The CpEST enzyme presented less stability to the isooctane solvent since it retained 30% of its activity (Fig. 6). In solvents such as tert-butanol and acetone, the enzyme retained between 58% and 55% of its initial activity. This pattern was very similar to that observed for lipases secreted by *Pseudomonas aeruginosa* and *Pseudomonas fluorescens* P21 [57]. In fact, reports point to the lipases of the *Pseudomonas* genus as the most frequently used in biotechnological applications due to their high potential in the organic synthesis of different chemical products [58]. The retention behavior of 50% in ethanol by recombinant CpEST was also documented for the *Geobacillus* sp HBB-4 esterase, which also maintained 50% of its activity in the presence of methanol [59]. This relatively high residual activity with ethanol seems to predict an excellent performance in enzymatic biodiesel reactions when ethanol and, to a greater extent, methanol are causes of enzymatic deactivation [60,61].

5. Conclusions

In this work, we report the successful recombinant production of the enzyme CpEST from papaya, which was secreted into the culture medium and showed high functionality in hydrolysis reactions. This enzyme represents a new alternative esterase with high potential for use in the bioenergy industry and other sectors. The enzyme is robust, and neither the production nor the catalytic capacity was affected during enzyme production using residual substrates. Therefore, there is an added advantage of its use as a biocatalyst.

Author contributions

- Study conception and design: G Sandoval, F Valero
- Data collection: AL Reyes
- Analysis and interpretation of results: AL Reyes, F Valero, G Sandoval
- Draft manuscript preparation: AL Reyes

– Revision of the results and approved the final version of the manuscript: AL Reyes, F Valero, G Sandoval

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Conflict of interest

The authors declare no conflicts of interest.

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