读书报告

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RESEARCH ARTICLE

Est10: A Novel Alkaline Esterase Isolated from Bovine Rumen Belonging to the New Family XV of Lipolytic Enzymes

María Cecilia Rodríguez[1], Inés Loaces[1], Vanesa Amarelle[1], Daniella Senatore[2], Andrés Iriarte[3], Elena Fabiano[1], Francisco Noya[1]*

1 Departamento de Bioquímica y Genómica Microbianas, Instituto de Investigaciones Biológicas "Clemente Estable", Montevideo, Uruguay, **2** Laboratorio de Ecología Microbiana, Instituto de Investigaciones Biológicas "Clemente Estable", Montevideo, Uruguay, **3** Departamento de Genómica, Instituto de Investigaciones Biológicas "Clemente Estable", Montevideo, Uruguay

* fnoya@iibce.edu.uy

01 Abstract

研究对象：Est10

One positive clone with lipolytic activity isolated from a metagenomic fosmid library from bovine rumen (牛瘤胃).

The 367 amino acids sequence harbors a signal peptide, the conserved secondary structure arrangement of alpha/beta hydrolases, and a GHSQG pentapeptide which is characteristic of esterases and lipases.

异源表达：

heterologously expressed in *Escherichia coli* as a His-tagged fusion protein

Esterase: showed maximum activity towards C4 aliphatic chains and undetectable activity towards C10 and longer chains

optimum pH is 9.0 optimum temperature is 40℃

02 Introduction

Lipolytic enzymes

carboxylesterases (EC 3.1.1.1)　　羧酸酯酶

triacylglycerol lipases (EC 3.1.1.3)　甘油三酯脂肪酶

用于洗涤剂、食品、药物、纸张、纺织品和精细化学品等的制造和加工；

α/β-水解酶超家族，含有催化三联体，通常由GXSXG五肽基序中的亲核丝氨酸与氢键合至组氨酸残基的酸性残基（天冬氨酸或谷氨酸）组成；

分为8个家族（I-VIII）；后增加家族（IX至XVI）；家族XV：DUF3089。

metagenomics

functional metagenomics turns the problem around by first identifying specific functions present in a microbial population and then isolating the genes responsible for them. To date, numerous novel biocatalysts from various microbial habitats, such as lipases, esterases, cellulases, proteases, amylases, lacasses, were identified by functional metagenomic approaches.

03 Materials and methods

Sample collection and processing

One hundred and fifty grams of fresh cow rumen digesta of a Holando bull (2 years old, 482 kg, pasture fed in southern Uruguay)

The liquid fraction of digesta was obtained by compressing whole digesta between two layers of cheesecloth.

The cells were harvested from this fraction by centrifugation at 10.000×g for 20 min at room temperature. The cells were suspended in 1 ml of PBS buffer pH 8.0.

Isolation of bacterial metagenomic DNA

Lq fraction:Percoll= 1:1v/v → centrifugation: 4℃ 14000g 20 min → pellet: suspended in lysis buffer → cells: disrupted by vortexing at maximum speed 30s (涡旋破碎) → mixture: incubated for 15 min at 70℃) → centrifugation: 4℃ 16000g 5 min → supernatant: 15 µl Proteinase K (0.2 mg/ml) incubated for 1 h at 37℃ →10% CTAB in 0.7M NaCl incubated for 10 min at 65℃

Isolation of bacterial metagenomic DNA

two consecutive extractions: equal volumes phenol , 4℃ 10400g 10 min → remove aqueous phase (去水相) → two consecutive extractions: chloroform → precipitated: 0.6 volumes isopropanol 0.1 volumes 3 Msodium acetate (pH 5.2) → 30 min on ice → centrifugation → washed on 70% ethanol →air dried → re-suspended in water (20 µl RNAse A 0.4 mg/ml) → resolved on a 0.8% agarose gel →20 kb → QIAEX II Gel Extraction Kit

Library construction and screening for lipolytic clones

CopyControl Fosmid Library Production kit with the pCC1FOS Vector (Epicentre)

MaxPlaxLambda Packaging Extracts (Epicentre)
packaging and infection of *E. coli* EPI300-T1R (Epicentre)

LB agar medium supplemented with 12.5 µg/ml chloramphenicol (LB-Cm) at 37°C for 16 h.

Grow: 37°C overnight LB-Cm
Store: 25% (v/v) glycerol -20°C

Library construction and screening for lipolytic clones

For lipolytic activity screening, clones were replica plated with a 48-pin array onto LB-Cm agar medium containing 1% (v/v) tributyrin (Sigma-Aldrich), 12.5 µg/ml chloramphenicol and 0.01% (w/v) L-arabinose to increase the fosmid copy number. Cells were grown at 30°C and periodically checked for enzymatic activity. Clones expressing lipolytic activities were identified by the formation of clear halos surrounding the colonies after 2 to 3 days.

In vitro transposon mutagenesis and DNA sequencing

In vitro transposon mutagenesis: EZ-Tn5 <KAN-2> Insertion Kit

LB-tributyrin (50 μg/mL kanamycin): wild-type and mutant fosmids were digested with BamHI and XhoI.

After comparing the fragments sizes between them, single insertion mutants were selected because only one of the fragments from the wild-type was split into two smaller fragments. Flanking DNA was sequenced by conventional Sanger method (Macrogen). ORFs were called using getORF from the EMBOSS suite.

Est10 cloning

Primers→ PCR → 1% (w/v) agarose gel → purified (1100bp) → digestion with NdeI and BamHI → ligated into expression vector pET14b → electroporated into *E.coli* DH5α cells → sequence

5'-AAAAACATATGATCATGAAAAAACAGAATTTCTTCG-3'
containing a NdeI site shown in bold
5'-ATTAGGATCCAATCAGTTCTCCATACGG-3'
containing a BamHI site shown in bold

Overexpression and purification of Est10

E.coli BL21 (DE3)-pET14b-Est10 → 1L 2X YT media → 1 mM IPTG (OD620=0.5-0.7) → 18 h at 20℃ with shaking → 4℃, 15min → 4℃, 1600g, 30 min → imidazole, Suspended → Sicated → 1 ml 50% Ni-NTA agarose resin, purify Est10 → imidazole, Suspended → dialyzed twice with 10% (v/v) glycerol → SDS-PAGE (测试纯度) → BCA (测试浓度)

- Determination of preferred chain length
- The effect of temperature on activity and thermostability
- Effect of pH, cations, chelating agents and detergents on activity
- Phylogenetic analysis
- Three dimensional modeling of Est10
- Nucleotide sequence accession number

04 Results

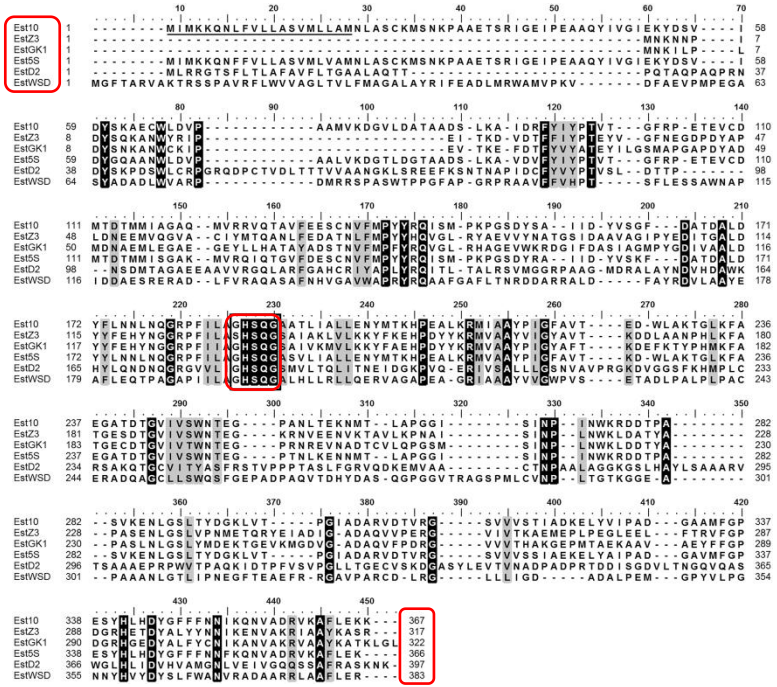
Metagenomic library construction and screening

To identify genes associated with lipolytic activity, a metagenomic library was generated using DNA isolated from the non-associated bacteria present in the Lq or liquid fraction of cow rumen. The library contained 27500 clones with average insert size of 42 kbp. The quality and size of inserts were verified by analyzing 40 randomly picked clones. The majority of analyzed clones contained inserts of approximately 35-45 kbp. Restriction analysis revealed a high level of diversity among the cloned DNA fragments.

Metagenomic library construction and screening

Fosmid clones encoding esterase activity were identified by their halo-forming ability on agar plates containing tributyrin. A total of 3 clones were identified in these plates. None of them showed similar activities in tricaprylin or triolein plates, suggesting that the encoded enzymes are not lipases.

Identification of lipolytic genes



GXSXG
五肽基序

	序列同一性
Est10	
EstZ3	41%
EstGK1	39%
Est5S	92%
EstD2	29%
EstWSD	29%

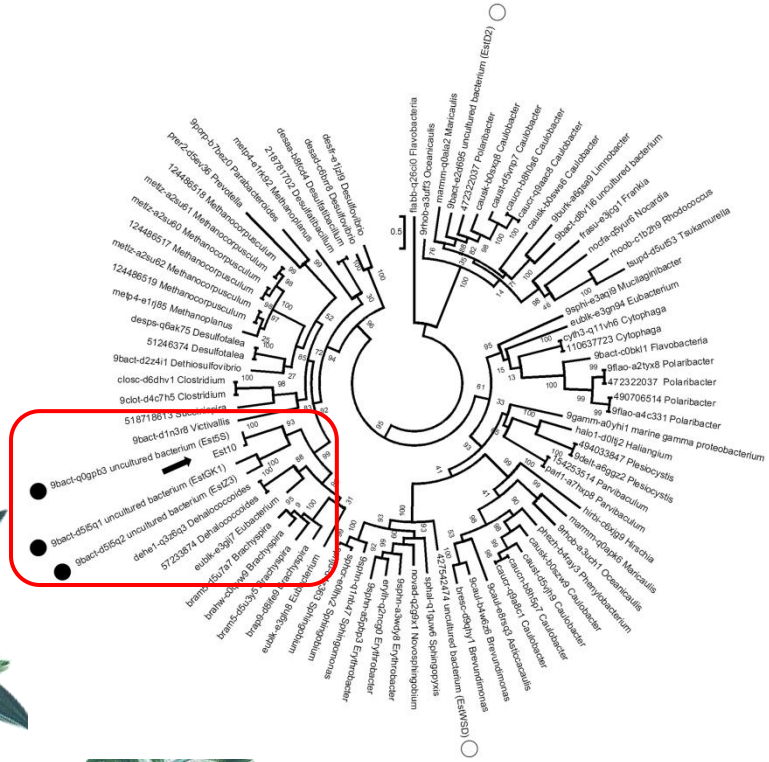
Est5S
an uncultured bacteria in cow rumen

EstZ3 EstGK1
metagenomic library of sheep rumen

EstD2 EstWSD
soil metagenomes

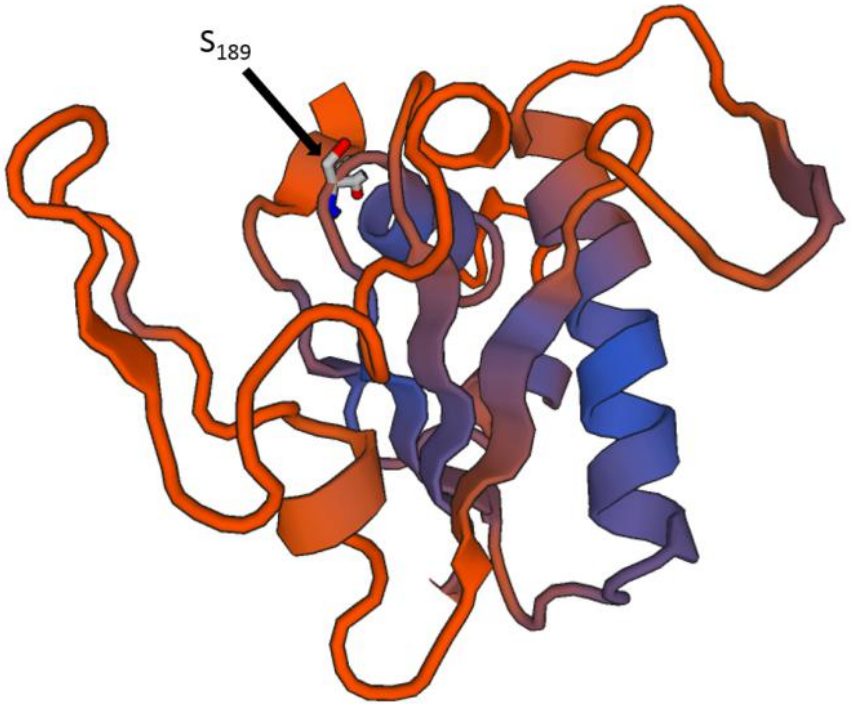
Interestingly, all of them come from unidentified bacteria.

Phylogenetic analysis of Est10

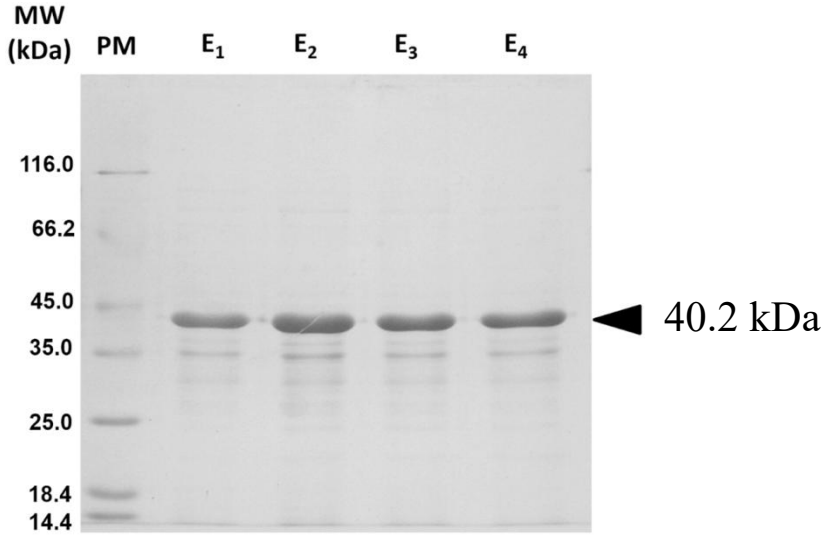


Est10 clustered with esterases from family XV, such as EstD2 and EstWSD.

Phylogenetic analysis of Est10

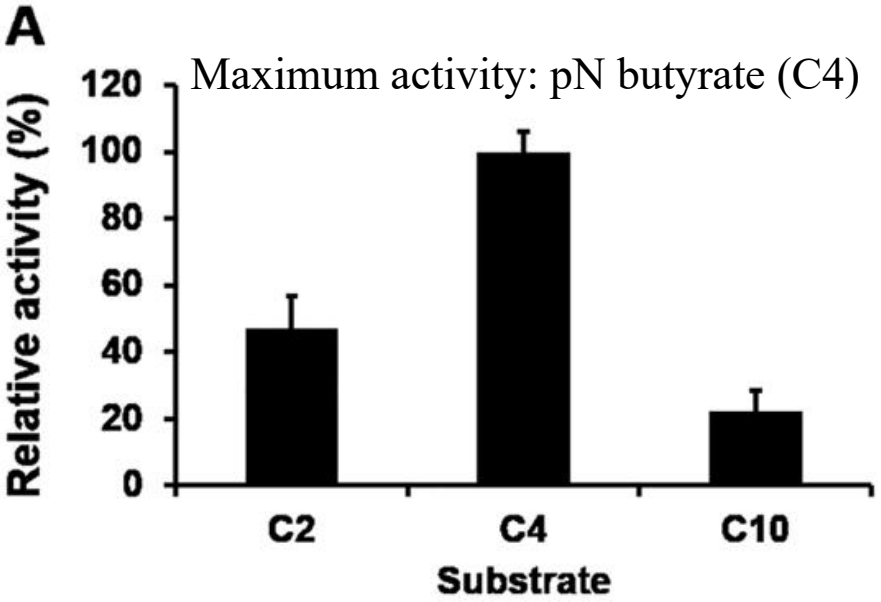


Determination of substrate specificity, effect of pH, temperature and thermostability on Est10 activity



Est10 was expressed as a His-tagged fusion protein and purified by affinity chromatography using a Ni^{2+} NTA resin.

Determination of substrate specificity on Est10 activity

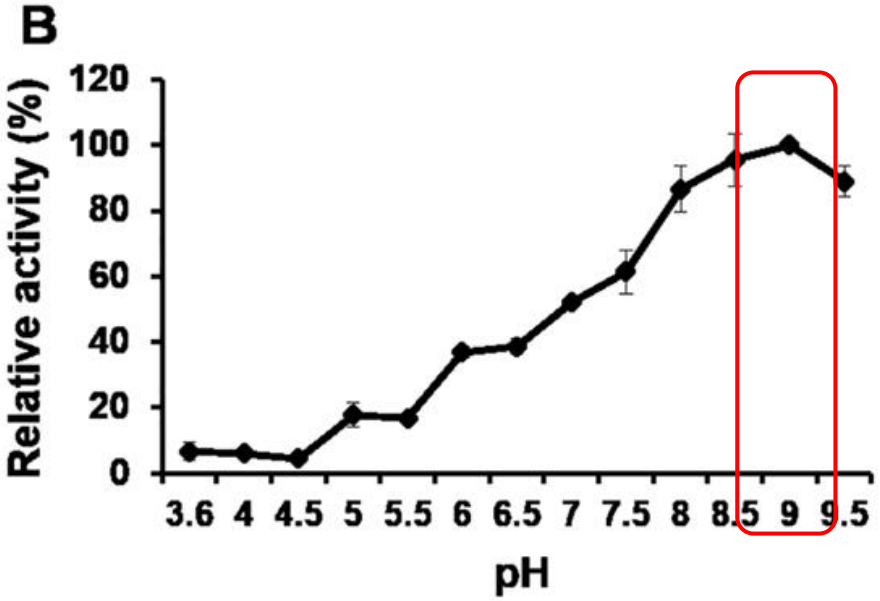


Maximum activity: pN butyrate (C4)

Against activity:
pNP dodecanoate (C12)
pNP myristate (C14)
pNP palmitate (C16)

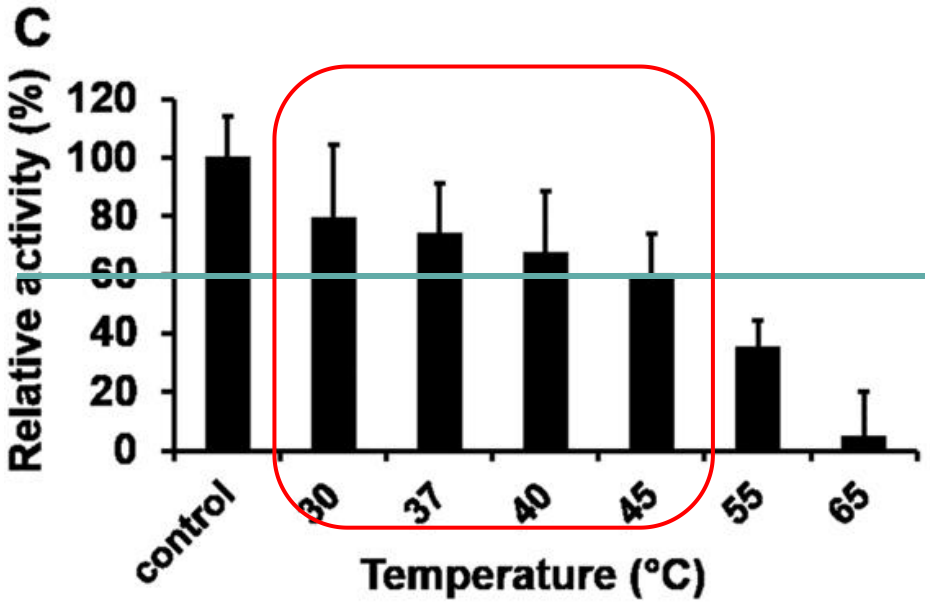
Substrate specificity of the purified enzyme was initially assayed using fatty acids esters of p-nitrophenol.(对硝基苯酚)

Effect of pH on Est10 activity



The activity of Est10 was tested under buffered conditions over the pH range 3.6 to 9.5, using pNP butyrate (C4) as substrate, at 40°C.

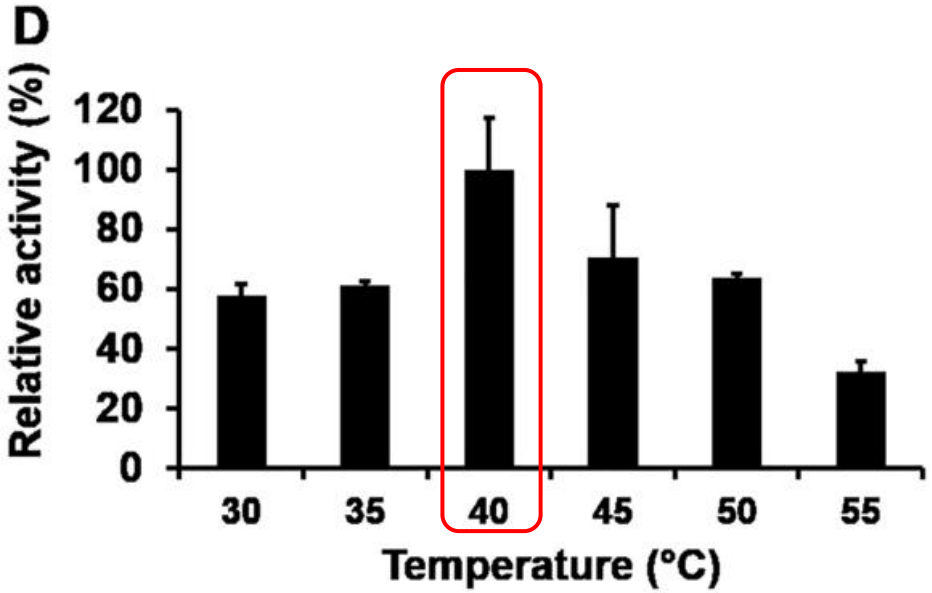
Effect of thermostability on Est10 activity



45°C孵育30min,保持60%以上活性。

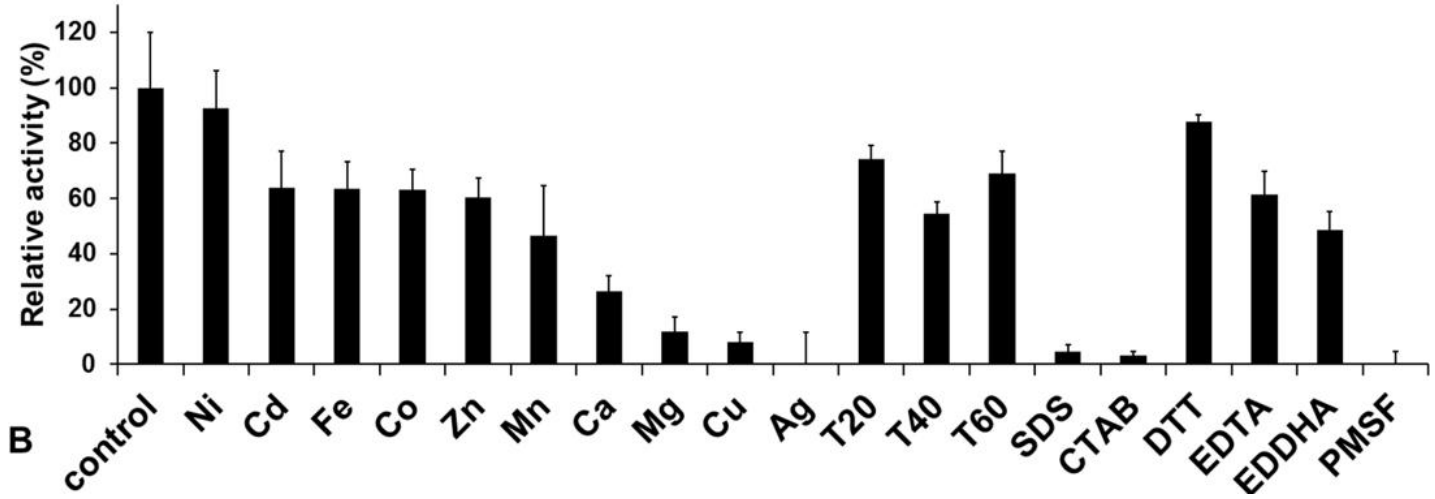
In order to test its thermostability the enzyme was pre-incubated at various temperatures between 30°C and 65°C for 30 min and its residual activity was assayed.

Effect of temperature on Est10 activity



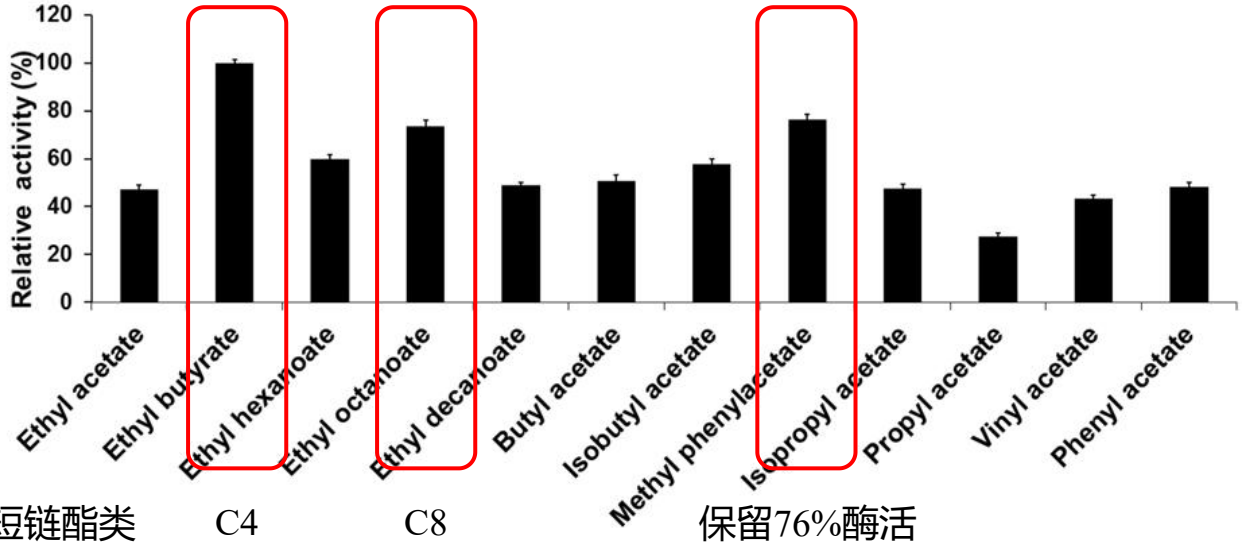
The effect of the reaction temperature on the activity of Est10 was determined between 30℃ and 55℃, using pNP butyrate as substrate.

Effects of cations, detergents, chelating agents and additives on Est10 activity



$NiCl_2$; Tween20, Tween40, Tween60 (非离子型洗涤剂) ; DTT (还原剂) : 无显著影响
$Cd(CH_3CO_2)_2$; $FeCl_3$, $CoCl_2$, $ZnSO_4$, $MnCl_2$; EDTA, EDDHA(螯合剂) : 部分抑制
$CaCl_2$, $MgCl_2$, Cu_2SO_4, $AgNO_3$; SDS, CTAB(离子型去垢剂) : 严重抑制
PMSF(丝氨酸水解酶抑制剂) : 完全抑制

Substrate specificity using an ester library



优选较短链酯类　　C4　　C8　　保留76%酶活

乙酸乙酯；丁酸乙酯；已酸乙酯；辛酸乙酯；癸酸乙酯；乙酸丁酯；乙酸异丁酯；苯乙酸甲酯；乙酸异丙酯；乙酸丙酯；乙酸乙烯酯；乙酸苯酯

Determination of kinetic parameters

hydrolysis of pNP acetate (C2), pNP butyrate (C4) and pNP decanoate (C10)

Substrate	Specific activity(U/mg of protein)[a]	K_M(mM)[a]	k_{cat} (s^{-1})[a]	k_{cat}/K_M(s^{-1} mM^{-1})[a]
C2	0.31 (0.04)	0.3 (0.1)	0.22 (0.03)	0.8 (0.4)
C4	4.4 (0.2)	0.16 (0.02)	3.1 (0.1)	19 (3)
C10	1.06 (0.06)	0.35 (0.06)	0.72 (0.04)	2.1 (0.5)

[a]Standard errors are indicated in parentheses.

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non-linear least squares implemented on the R package

Est10 elicited maximal specificity constant (kcat/KM) with pNP butyrate (C4).

05 Discussion

Est10活性对二价阳离子非常敏感。研究报导在其他酯酶中观察到在Mg^{2+}，Cu^{2+}和Ca^{2+}存在下的强抑制作用。

关于其在表面活性剂存在下的稳定性，Est10保留了它在非离子型去污剂中的大部分活性，而在离子型去污剂存中被灭活。

Est10和Est5S具相似底物特异性和最适温度。但Est10具有比Est5S更高的碱性最适pH。Est10最适pH值为9.0，该酶在pH8和9.5之间保持超过85%的活性。

Est10的最佳温度约为40°C，嗜温酯酶，高温下不耐热。Est10优选的作用温度在30和40°C之间，这恰好是瘤胃液的温度。

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