Babes-Bolyai University

Faculty of Chemistry and Chemical Engineering

Identification of new natural sources of hydrolytic enzymes with potential applications in selective biotransformation

-Summary-

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Doctoral Thesis

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Contents

Introduction

Biocatalysis plays an important role in numerous fields especially in modern chemistry. It aims to improve various reactions and to bring milder and environmentally friendly conditions.

Enzymes are biocatalysts of great importance. Produced and modified by natural forces in all kingdoms, they are underexplored and possibly, underestimated. In present there are many efforts put into finding the appropriate biocatalysts in several domains (Arpigny and Jaeger, 1999). The importance of these enzymes is materialized in numerous papers describing the purification and characterization of such novel sources.

In the past few years more and more enzymes from thermophilic microorganisms, with an emphasis on hydrolytic ones, are gaining much attention. There is a constant search of such novel sources, mostly microbial and their enzymes are characterized and screened for possible applications.

This thesis resumes to the search and description of a novel indigenous source of possible biocatalyst. The carboxyl-esterase from a newly thermophilic bacterium belonging to *Anoxybacillus sp*. was purified and characterized.

Chapter I.

The first chapter of the present thesis is a short literature review. It aimed the description of hydrolytic enzymes such as lipases and esterases, methods of verifying activity of such enzymes and also the most useful methods for purification. We also mention molecular cloning of carboxyl esterases. The last part of the first chapter is destined for thermostable microorganisms and their special enzymes.

Chapter 2. Isolation, identification and characterization of a bacterial strain, source of a thermostable hydrolase

This chapter describes the isolation and characterization of an indigenous strain of *A. flavithermus* T1. In addition, lipolytic activity was tested in relation with temperature, time, pH and inducers.

Results and discussions

The water samples were transported from each location in sterile thermostatted vessels and grown on LB-agar medium. From the 4 plates obtained, one colony was grown in LB liquid medium and lipolytic activity was measured against *p*-nitrophenol palmitate.

The sample taken from Tăşnad presented extracellular lipolytic activity and was chosen for further experiments. The morphological and biochemical characteristics of the isolated bacterial strain were assessed concomitantly with genetic determination of the bacterium.

The genetic analysis showed that we have isolated a thermophilic strain of *Anoxybacillus flavithermus* (maximum identity with *Anoxybacillus flavithermus* AE3), which is deposited at the National Collection of Agricultural and Industrial Microorganisms, Budapest, Hungary, under the accession number NCAIM B 02482.

The main characteristic of the species are listed in Table 1.

Table 1. Characterization test results for the isolated strain. + indicates a positive, - a negative, +/- a weak result.

Morphology		**Biochemistry**	
Cell shape	Rod	Hydrolysis of:	
Size (average)	length 4.0-5.0 µm, width >0.5 µm	Gelatine	+
Endospore	+	Starch	+

Position	Terminal	Urea	-
Shape	ellipsoidal/cylindrical	Production of catalase	-
Motility	+/-	Production/formation of:	
Colonies	circular, yellow, convex, Φ1-3 mm	H_2S	-
Gram staining	Variable	Indol compounds	-
Gram chemotype	Positive	Acetoin	-
Biochemistry		**Physiology**	
Growth on:		Anaerobic growth	+/-
NaCl 0.5-3%	+	pH range	5.5-8.5
5%	-	Temperature (°C)	37-70
Egg broth	+/-		
Blood-agar	-		
Meat broth	-		
Utilisation of:			
D-Glucose	+		
Manitol	-		
Inositol	-		
D-Sorbitol	-		
L- Rhamnose	-		
D- Sucrose	+		
D- Melibiose	+		
Amygdaline	-		
L- Arabinose	+		
Citrate	-		

The carboxyl-esterase production was monitored related with temperature, pH and time of growth of *Anoxybacillus flavithermus.* Maximum secretion was achieved at 60°C, pH 8 and after 16 h of cell growth. Also, the enzyme secretion was induced by supplementing the medium with various commercial oils such as: sunflower oil, palm oil and olive oil. The best inducer was sunflower oil.

Chapter 3. Purification of native carboxyl-esterase

The third chapter describes the partial purification of a native carboxyl-esterase from *A. flavithermus* T1 by classical methods such as ammonium sulphate precipitation, acetone precipitation and anion-exchange chromatography. In addition, a new procedure based on specific precipitation was

employed, previously described by Gorokhova *et al*. (2002) as a method of immobilization and activation of lipases.

Results

The results showed that by ammonium sulphate precipitation the carboxyl-esterase lost a high amount of activity. Passages of ammonium sulphate or acetone precipitate through anion-exchange chromatography column yielded no or low activity recovery, but highly contaminated samples.

The partially purified enzyme (acetone precipitate) is defined by a high optimum temperature (fig. 1) and a high stability at elevated temperatures even than the optimum. Optimum pH for activity is 7.8 (fig. 2). The enzyme is fairly stable at pH's in the low alkaline range (7.4-9.0), retaining at least 70% of its activity after a 30 min incubation. Outside this range, it quickly looses activity.

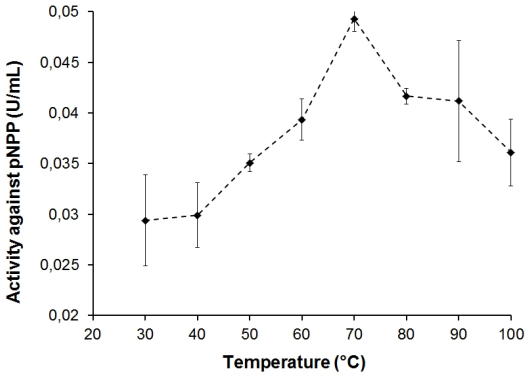

Figure 1. Temperature profile of carboxyl-esterase (acetone precipitate)

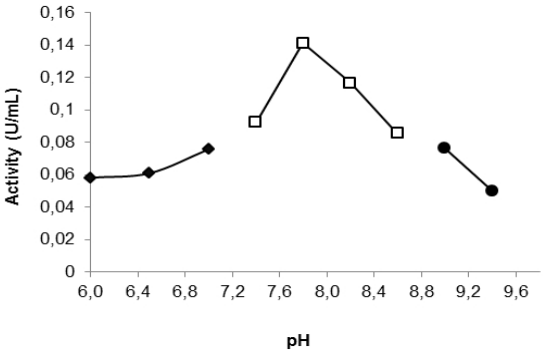

Figure 2. pH range curve for partially purified Est/Lip from *A. flavithermus*

Our enzyme in its partially purified state showed improved activity when incubated with 1mM Ba^{2+}, Mn^{2+} and Fe^{3+} 30 or 45 minutes at 60°C, but all the other cations (Ca^{2+}, Mg^{2+}, Co^{2+}, Cu^{2+}, Zn^{2+}, Hg^{2+}, Sn^{2+}) seemed to have no effect or an inhibitory effect on carboxyl-esterase activity.

Among several organic solvents used for carboxyl-esterase stability, acetone and isopropanol increased the enzyme activity after 15h incubation at 60°C. However, ethyl acetate, methanol, ethanol and acetonitrile displayed moderate to strong inhibitory effect on the carboxyl-esterase activity.

The enzyme showed no substantial inhibition or activation in the presence of 1 mM the chosen modulators. EDTA inhibited the Est/Lip only with 12%, and SDS activated it with more than 50%.

Assaying the Est/Lip for specificity we obtained the following activities, expressed in U/mL*10^3: *p*NP - acetate: 34,3; *p*NP – propionate: 35,5; *p*NP- butyrate: 9,0; *p*NP- methyl butyrate (chiral substrate): 10,0; *p*NP- palmitate: 35,9 and *p*NP- oleate: 39,4.

The method used for specific precipitation of carboxyl-esterase, in our case was proven to precipitate mainly S-layer glycoproteins, according to MS analyses.

Chapter 4. Cloning and characterization of a thermophilic Est/Lip from *Anoxybacillus flavithermus* T1

In the fourth chapter of the present thesis we describe the molecular cloning of the carboxyl-esterase gene found in *A. flavithermus* (Saw *et al*., 2008) and expression in *E.coli* BL21 DE3. The purification and detailed characterization of the recombinant *A. flavithermus* T1 Est/Lip is also presented.

Results

Based on the already sequenced genome of *A. flavithermus* we were able to create primers sequences on order to clone the Est/Lip gene. We used MyTaq for cloning, pET 20b (+) and *E.coli* XL1 Blue/*E.coli* BL21 DE3 for plasmid multiplication or carboxyl-esterase expression, respectively.

The Est/Lip was purified from the periplasmic space by a two-step procedure involving membrane-destabilization and Ni-affinity chromatography (fig. 3).

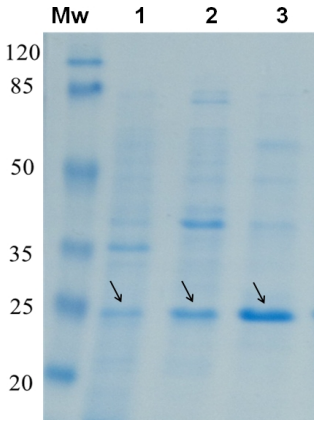

Figure 3. Denaturing SDS-PAGE gel showing purification steps of the cloned Est/Lip. MW: molecular weight markers; lane 1: crude extract of *E.coli* BL 21 DE3 cells, 5 h after IPTG induction; lane 2: periplasmic fraction; lane 3: purified Est/Lip after Ni^{2+}-affinity chromatography

Analysis of the modeled tridimensional structure of *A. flavithermus* T1 Est/Lip showed that the central β-sheet is composed of seven strands, with the short N-terminal β-strand antiparallel to the rest, while the lid, apparently a helical region distinct from the core domain, consists of two α-helices and one 3_{10}-helix (fig. 4).

The protein has the alpha-helices and beta sheets specific structure. By comparison with the carboxyl-esterase Est 30 from the *Geobacillus stearothermophilus* (Liu *et al*., 2004; Montoro-Garcia *et al*., 2009) we proposed that the catalytic amino acids are Ser 93, Asp 192 and His 222.

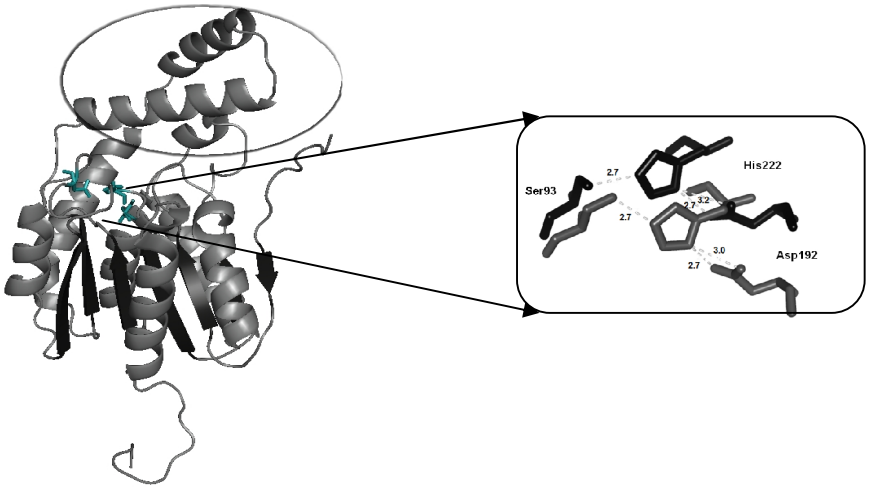

Figure 4. Cartoon diagram of the tridimensional structure of the Est/Lip. The lid is marked the top of the structure. The blue stick models represent the catalytic amino acids, detailed in the right square: *A. flavithermus* T1 Est/Lip (in grey) and of *G. stearothermophilus* Est30 (in black), respectively.

The features of the purified enzyme differ to a certain extent from the partially purified enzyme. The recombinant purified Est/Lip has an optimum temperature of 60°C, when activity was measured against *p*NPA and 65°C when *p*NPP was used as a substrate. At 60°C the Est/Lip had a half-life of about 6h. The pH optimum is rather a range of pH values: from 6.5 to 8.

All metal ions tested inhibited Est/Lip activity. Metals of groups I and II had a limited effect, except for magnesium, which depressed activity by about 70%. Of the transitional metals, Co^{2+} exerted the strongest inhibitory effect, inactivating the enzyme completely. Est/Lip retained a residual activity close to 10% in the presence of Fe^{3+}, Cu^{2+} and, surprisingly, Hg^{2+}.

The enzyme retained a high amount of activity (over 60%) in the presence of 10% methanol and 10% DMSO, although increasing concentrations of methanol inactivated it. DMSO was especially compatible with Est/Lip, allowing significant levels of activity at a concentration as high as 30%. Acetonitrile also appeared to be relatively well tolerated leaving more 10% activity at 20% concentration.

SDS in concentrations up to 100 μM and of Triton X-100 in concentrations of 0.01-0.5% exerted a slight inhibitory effect upon Est/Lip activity in unincubated samples. However, the presence of a low concentration of both detergents (1 μM SDS, respectively 0.01-0.05% Triton X-100), rendered the enzyme significantly more thermostable and concurrently facilitated a temperature-induced activation effect. At 1 mM, β-ME did not affect the activity and slightly enhanced the thermostability of the enzyme. When associated with prolonged incubation at 60°C, 100 mM β-ME led to complete inactivation of the enzyme.

We tested again the Est/Lip for specificity and the results showed a high difference from the partially purified enzyme: *p*NP - acetate: 61.3±1.5; *p*NP – propionate: 84.1±3.4; *p*NP- butyrate: 167.1±2.7; *p*NP- methyl butyrate (chiral substrate): 149.1±32.6; *p*NP-caprate: 73.6±2.9; *p*NP-palmitate: 1.4±0.3, *p*NP- oleate: 3.2±0.4, triolein: 14.3. The activities are expressed in U/mg.

Kinetic parameters were calculated for the purified enzyme and the following table summarizes the results.

Table 2. Kinetic parameters for *A. flavithermus* T1 Est/Lip, for *p*NPA and *p*NPP. Data were fitted with a Hill equation, in OriginPro 6.1

Substrate	vmax (μmol min-1)	K_M (μM)	k_{cat} (min-1)	k_{cat}/K_M (μM-1 min-1)	N
pNPA	82.89 ± 1.56	34.14 ± 1.37	7.34	0.21	1.96±0.16
pNPP	1.44 ± 0.08	23.28 ± 2.32	0.12	0.0052	1.85±0.32

Chapter 5. Optimizing protein expression and purification

Molecular cloning and expression of heterologous proteins has become in recent years a widespread technique. Improved yield, ease of purification, cost-effectiveness made this method into a convenient way for obtaining high purity target proteins. Heterologous protein expression induced in host organisms can be a burden to the cell (Han and Lee, 2006). The lactose derivative IPTG (Isopropyl β-D-1-thiogalactopyranoside), frequently used for induction, is also toxic to the cell (Farmer *et al*., 2002; Yan *et al*., 2004).

In this chapter we describe optimization of the expression process, aiming to identify a set of experimental conditions which would allow optimal expression and recovery of the recombinant Est/Lip. The investigated parameters referred to the nature of the inducer, to the concentration of IPTG, and to temperature and time of induction. Additionally, several methods for membrane destabilization were tested.

Results

The results show that a high expression yield can be attained if induction is carried out at 37°C, using 5 mM IPTG for induction and allowing 8h after induction. However, almost similar results were obtained when induction was carried out at 30°C, with a much lower IPTG concentration (0.4 mM) and after only 1 hour of induction. Considering the aspects of both cost and time-effectiveness, the latter might be a satisfactory compromise and the best practical option.

Cell growth was better if 10 mM lactose, rather than 0.1 mM IPTG, was added to the expression-host culture. As proven by both activity assays and SDS-PAGE, expression of the active

Est/Lip was also higher with lactose than with IPTG. Consistently with previous results, the best yield was attained at 8 hours after induction.

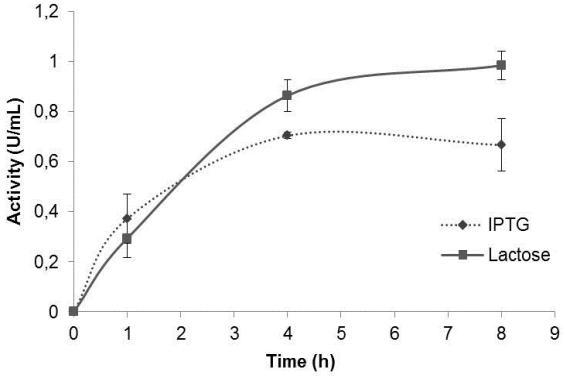

Figure 5. Est/Lip activity after induction with 0.1 mM IPTG or 10 mM lactose

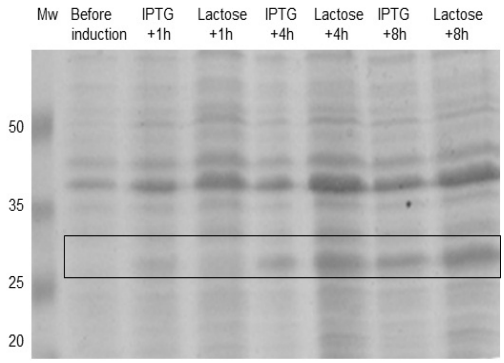

Figure 6. Denaturing SDS-PAGE: time course of Est/Lip expression after induction with 0.1 mM IPTG or 10 mM lactose

Membrane destabilization

The highest activity of the Est/Lip expressed in the periplasmic fraction was recovered with the freeze-thaw treatment, followed closely by the use of the standard Tris-sucrose-EDTA-$MgSO_4$ buffer. The use of DMSO and chloroform as membrane destabilizing agents yielded a lower, but still satisfactory recovery (Table 3).

Table 3. Recovery of Est/Lip activity from the periplasmic fraction following various membrane destabilization methods

Method	Concentration	Activity (U/mL) ± st.dev
Osmotic shock/EDTA/$MgSO_4$	1 mM/5 mM	1.36 ± 0.26
DMSO	1%	1.19 ± 0.01
Chloroform	1%	1.00 ± 0.12
Freeze-thaw (5 cycles)	-	1.47 ± 0.06

Chapter 6. Applications of recombinant Est/Lip from *A.flavithermus* T1 in biotransformations reactions

Among *Anoxybacillus* sp.there are several enzymes already described as being important in biotransformations.

The *Anoxybacillus* genus was only recently described. Ibrahim and Ahmed (2007) purified a cellulase of biotechnological interest from *Anoxybacillus flavithermus.* An esterase which acts a polyhydroxyalkanoates (PHA) depolymerase was reported to be secreted by *A. gonensis* G2 (Çolak *et*

al., 2005). A thermostable lipase from *A. kamchatkensis* has been described more recently (Olusesan *et al*., 2009), which retained over 50% of its activity after 30 minutes at 80°C. The partially purified carboxyl-esterase from *A. gonensis* A4 (Faiz *et al*., 2007) and the recombinant Est/Lip from *Anoxybacillus* sp.PDF were not used so far in biotransformations.

Results

The Est/Lip showed high enantioselectivity towards several chosen substrates. The results show that our enzyme can be used in mild hydrolysis reactions. Although the results are weaker than we expected, the enzyme thermostability can be exploited in directed evolution. The goal is to obtain a more biocatalytically active enzyme (Reetz, 2004).

General conclusions

In conclusion, the new strain of *Anoxybacillus flavithermus* we have successfully isolated is a local source of a thermostable hydrolytic enzyme, showing good esterolytic/lipolytic activity.

The crude enzymatic extract (acetone precipitate) with high optimum temperature for activity, high thermostability, good tolerance to organic solvents and low substrate specificity can be used in biotechnological applications. The purification of the native enzyme encountered difficulties possible due to aggregation tendency, which lead to a low recovery of enzyme and its activity.

Cloning process of the esterase/lipase was successfully achieved in mesophilic *E.coli* as a heterologous host, although the enzyme belongs to a thermophilic microorganism. The recombinant pure Est/Lip shows a good thermostability with a $t_{1/2}$ of 5 hours at 60°C and pH 8. It tolerates, although in small concentrations, organic solvents such as acetonitrile and DMSO up to 30%. Substrates specificity analysis revealed a preference towards *p*NP esterified with butyric acid (C_4), a specific substrate for esterases.

Optimizing the expression of the recombinant Est/Lip we found the following optimum parameters: growth at 30°C, 1 hour induction with 0.4 mM IPTG. Induction with lactose led to better cellular growth and higher protein expression. Purification of the Est/Lip from the periplasmic space was best achieved by osmotic shock and freeze-thaw repetead cycles.

Biocatalytic reactions performed with the pure recombinant Est/Lip indicated a high selectivity at moderate high temperatures (45-50°C) even with bulky substrates.

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