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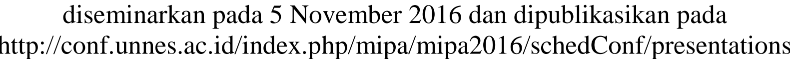

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Thermophile Metagenomic: A Resource For Novel Enzyme

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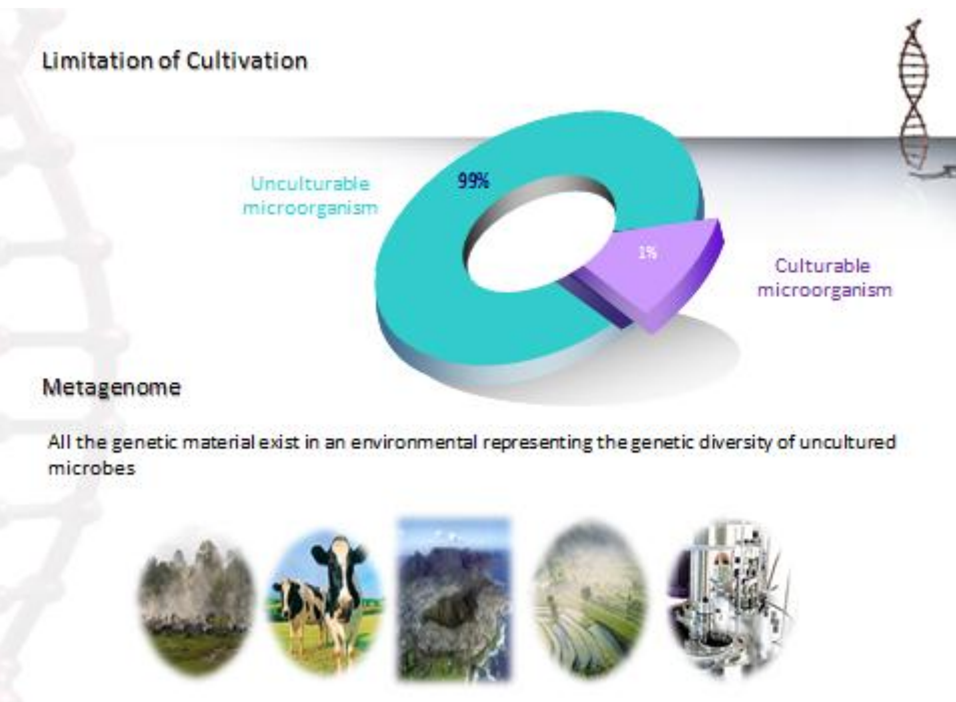
Abstract

Studies of geothermal ecosystems have revolutionized our understanding of global diversity and led to major technological advances in medicine, industry and biotechnology. Microbial life in extreme environments is attracting many scientists in order to discover new many thermostable enzymes. These microorganisms have unusual and desirable enzymes of particular interest for biotechnological and industrial processes. However, traditional method based on culture-dependent techniques produced only small fraction microorganisms from extreme environments cultured under standard laboratory condition. Therefore, other approaches including sequence based screenings and metagenomics have been successful in providing novel thermozymes. Functional metagenomics has the advantage of not requiring the cultivation of microorganisms or previous sequence information to known genes, thus representing a valuable approach for mining enzymes with new features. Recent reports have suggested that the establishment of industrially relevant enzyme collections from environmental genomes has become a routine procedure.

Keywords : Metagenomic, Thermophilic Microorganisms, enzymes

- Metagenomics is the culture-independent genomic analysis of microbial communities.
- The term is derived from the statistical concept of meta-analysis (the process of statistically combining separate analyses) and genomics (the comprehensive analysis of an organism's genetic material)

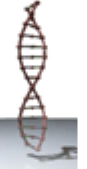
- the estimation that >99% of microorganisms in most environments are not readily culturable, and therefore not accessible for biotechnology or basic research.
- Metagenomics can be used to solve the challenge of studying prokaryotes in the environment that very little is known about their genomes, genes and encoded enzymatic activities



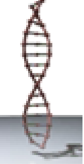
- Research institutes and companies established collections of microorganisms from a variety of ‘common’ environments (e.g. soil or seawater) and extreme environments (e.g. hot springs, Antarctic ice, etc.).

- However, the culturable microorganisms constituting these resources represent only a tiny fraction of the microbial diversity, which is limiting the spectrum of search for new enzymes (biocatalysts) for the bioprocess industry.

- 'Metagenome technology' tries to overcome this bottleneck by developing and using culture-independent approaches.
- The generation and analysis of (meta)genomic libraries is thus a powerful approach to harvest and archive environmental genetic resources

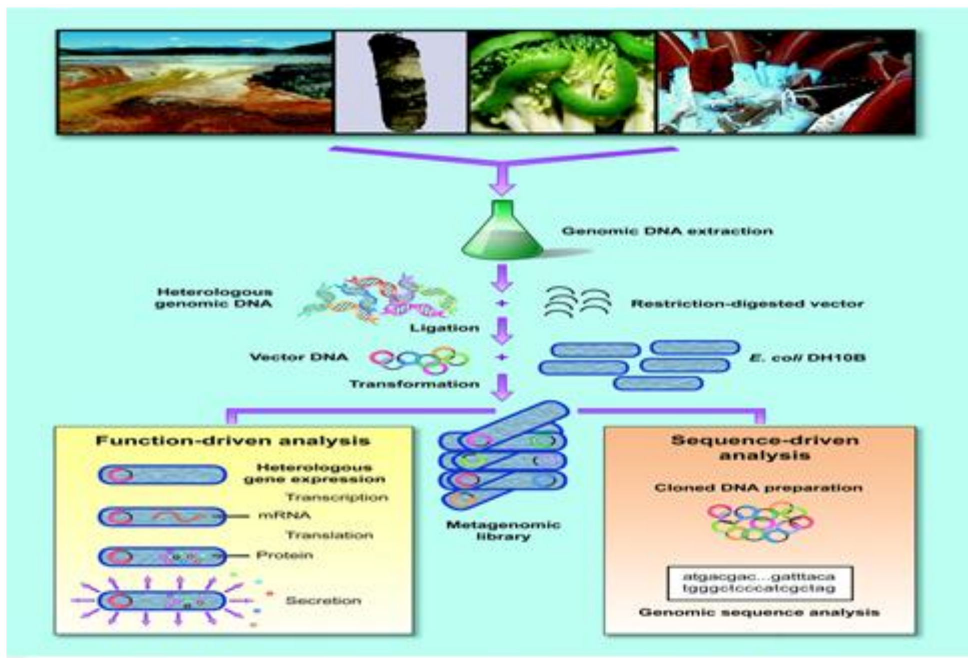


Metagenomics could be a powerful tool for making such discoveries: containing both culturable and unculturable organisms, have been demonstrated to be a largely untapped genetic reservoir for the discovery of new biomolecules, including novel biocatalysts



- Two types of analysis have been used to obtain information from metagenomic libraries :

1. A function-driven analysis
2. A sequence-driven analysis

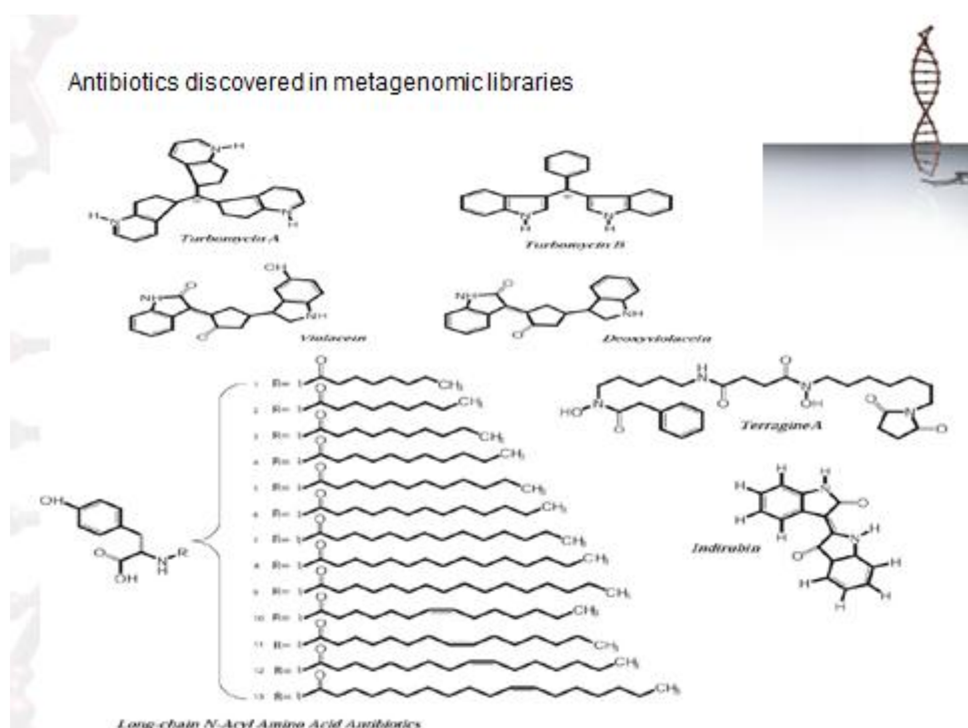


Schematic analysis to obtain information from metagenomic libraries

The function-driven analysis

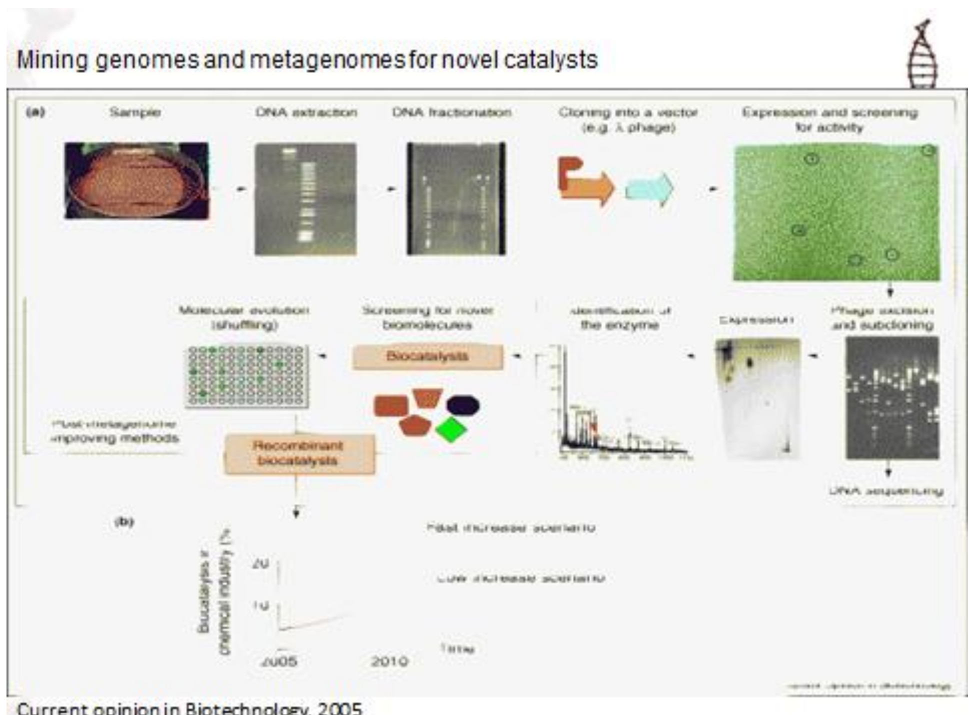
- in which metagenomic libraries are initially screened for an expressed trait

- Functional screens of metagenomic libraries have found both novel and previously described :
  - antibiotics (turbomycin A and B)
  - antibiotic resistance gene
  - Lipases
  - enzyme for 4-hydroxybutyrate
  - enzyme for biotin synthetic



The sequence-driven analysis

- Sequence-driven analysis relies on the use of conserved DNA sequences to design hybridization probes or PCR primers to screen metagenomic libraries for clones that contain sequences of interest



Current opinion in Biotechnology, 2005

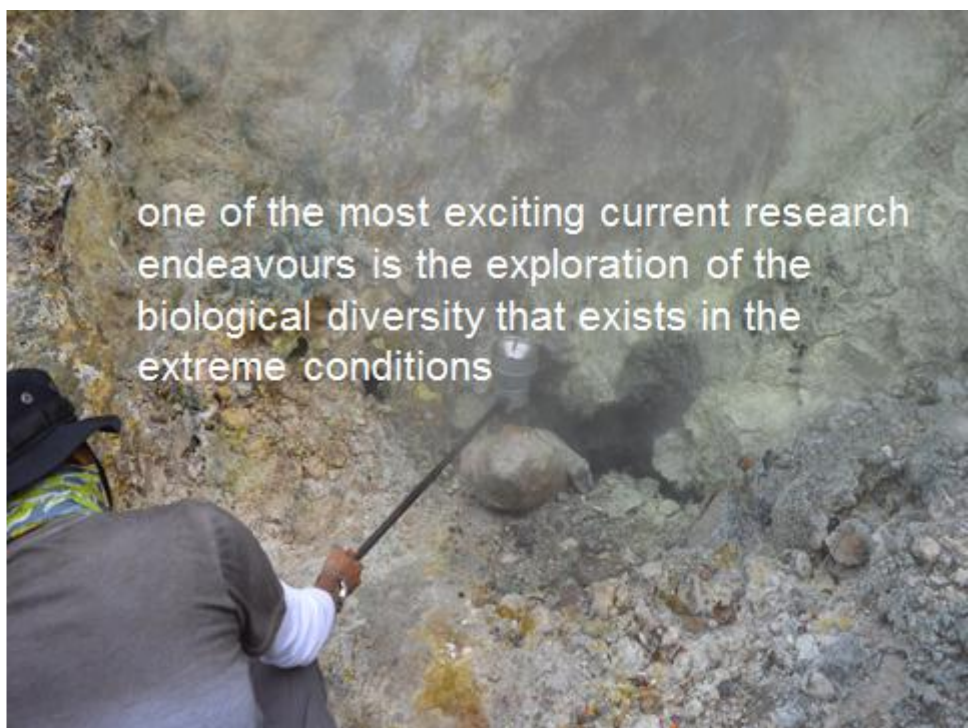
Table 1

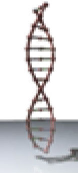
Recently identified metagenome-derived functional genes and enzymes with a high potential for industrial and/or pharmaceutical applications.

Functional genes/enzymes identified	References
Lipases/esterases	[9*,12*,18*,21*,25]
Polysaccharide-modifying enzymes	
α-Amylases	[9*,12*,21*,34]
α-1,4 Glucan branching enzymes	[9*,21*]
β-Agarases	[9*]
Chitinases	[35]
Cellulases	[8]
Oxidoreductases/dehydrogenases	
Alcohol oxidoreductases	[10,36]
Di-keto-D-gluconic acid reductases	[31]
4-Hydroxybutyrate dehydrogenases	[11]
Other enzymes	
Proteases	[37]
Nitrilases	[38]
Vitamin biosynthesis	
Biotin biosythesis	[7]
Production of bulk chemicals	
1,3-Propanediol biosynthesis	[39]
Antibiotics and drug biosynthesis	
Turbomycin A,B	[40]
Polyketide synthases	[16*,41]
Violacein	[42*]
Long-chain *N*-acyltyrosines	[43,44]
Indirubin	[45]
Terragines	[17]

Functionality of these metagenome-derived genes and enzymes has been demonstrated in biochemical assays and/or by genetic complementation. The original host organisms are all unknown and cultivation in the laboratory as a pure culture was not demonstrated.

Whatever approach is adopted to search the metagenome, these new microbial genetic resources will provide a wealth of potential new biotechnological applications for biotech enterprises specialising in biocatalysis, bioremediation, and natural products for the pharmaceutical and agricultural industry

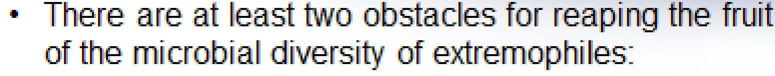




- Extreme environments on Earth are colonized by microorganisms called extremophiles, which can thrive under diverse harsh conditions

- extremophilic organisms are possibly the least well understood because our ability to study and understand their metabolic potential has been hampered by our inability to isolate pure cultures.

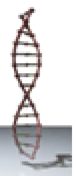
- The extremophiles not only survive, but thrive in the harshest environments on earth previously thought to prohibit all forms of life
- Environments with extreme serve as natural reservoirs for more robust biocatalysts that in the future could become indispensable for industrial applications.



- There are at least two obstacles for reaping the fruit of the microbial diversity of extremophiles:

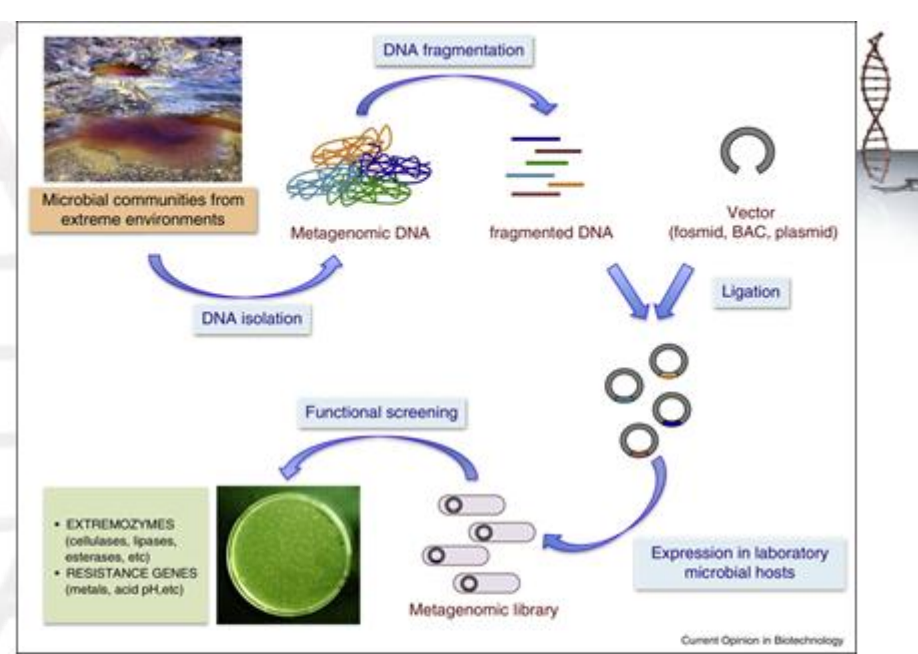
1. in spite of the recent progress in development of new culturing techniques most extremophiles cannot be cultured using traditional culturing technologies;
2. the problem of the very low biomass densities often occurs under the conditions hostile for life, which often do not yield enough DNA and reduces the effectiveness of cloning

- extremophiles have biomolecules called extremozymes that are catalytically active under extreme conditions and could therefore be used as biocatalysts.
- This will not only lead to the discovery of yet unknown enzymatic activities and reveal novel molecular structures and biochemistries, but will provide an understanding of the mechanistic basis of life under the most hostile conditions on Earth.



- Extremozymes have developed molecular mechanisms of adaptation to extremephysico-chemical conditions
- As they are active and/or stable under extreme conditions (i.e. thermophilic, psychrophilic, acidophilic, alkalophilic, halophilic and others) they have a great practical importance for industrial applications

- Activity based discovery in extreme environments of novel enzymes that are not related to any previously known proteins with the same function, or that exhibit unique structural features, marks the importance of these environments for enzyme mining through a metagenomic approach.



Schematic diagram describing the steps for finding extremozymes

Table 1

Metagenomic studies of extreme temperature habitats. Selected cultivation-independent studies are based on large-scale sequence datasets analysis, derived from DNA that has not been subjected to targeted amplification (PCR) and selective sequence analysis (e.g. 16S rRNA gene analysis).ᵃ

Habitat	Sample type	Geographical location	Method	Reference	Comments
High temperature	Subsurface microbial mat sample	Hishikari gold mine (Japan)	Sequence screening of fosmids	Nunoura et al. [63]	Microbial mat within geothermal water stream, 320 m below the land surface. Temperatures at sampling sites: 50-69 °C
	Hot spring microbial mat samples	Mushroom Spring and Octopus Spring, YNP[b] (USA)	Sanger end-seq. of metagenomic library clones	Bhaya [41]	Samples collected from top green layer (~1 mm) of microbial mats. Temperature at sampling points ~60 °C and ~65 °C
	Fracture water from subsurface gold mine	South Africa	Sanger seq. and 454 pyroseq.	Chivian et al. [30]	~5600 L filtered fracture water, sampled from 2.8 km depth. Temperature at sampling sites: ~60 °C, pH 9.3
	Coat mineral surface biofilms of carbonate chimneys	Lost City Hydrothermal Field (Mid-Atlantic Ridge)	Sanger Shotgun seq.	Brazelton and Baross [37]	Temperature range at sampling point: 40–90 °C, pH 9–11
	Microbial mat and/or solid phase sample of hot springs	5 geothermal springs, YNP[b] (USA)	Sanger paired-end Shotgun seq.	Inskeep et al. [24]	Temperature at sampling sites: 65-80 °C, pH range 2.5–7.8
	Deep-sea sulfide hydrothermal vent chimney	Mothra Field, Juan de Fuca Ridge (Canada)	454 pyroseq. of fosmids	Xie et al. [38]	Outer layer of active/venting (~316 °C) black-smoker chimney. 16S rRNA gene seq of metagenomic fosmid library generated (no amplification)
	Hydrothermal fluids	Mariana Trough	454 pyroseq.	Nakai et al. [28*]	Sampling site at 2850 m water depth, fluid temperature ~106 °C. Cellular materials passed through 0.2 μm filter and collected by 0.1 μm filter. Isolated DNA randomly amplified using MDA WGA
	Subsurface oil reservoir sample	Norwegian Sea, NCS (Norway)	454 pyroseq.	Kotlar et al. [25*]	Pressurized sample (oil/water/gas). *In situ* temperature: 86 °C, *in situ* pressure: 240 bar
	Subsurface microbial mat sample	Hishikari gold mine (Japan)	454 pyroseq. of fosmids	Nunoura et al. [26]	Microbial mat within geothermal water stream, 320 m below the land surface. Temperature of sampling sites are 50-69 °C
	Hot spring microbial mat core samples	Mushroom Spring YNP[b] (USA)	454 pyroseq. and SOLiD seq. of cDNA	Liu et al. [23**]	High temperature (61-64 °C) alkaline habitat. Samples collected at different time-points (different sunlight levels). Metatranscriptomic analysis. Isolation of total RNA, cDNA synthesis and establishment of rRNA, gene and protein databases
	Hot spring microbial mat samples	Mushroom Spring and Octopus Spring, YNP[b] (USA)	Sanger end-seq. of metagenomic library clones	Klatt et al. [36]	High temp. alkaline habitat. Average temperatures for sampling sites: ~60 °C and ~65 °C
	Hot spring biofilm samples	5 sampling sites within the 'Bison Pool' (Rosette Geyser), Sentinel Meadows in the Lower Basin of YNP[b] (USA)	Sanger Shotgun seq.	Dick et al. [64]	5 sampling points with temperatures ~57 to ~88 °C
	Hot spring microbial mat samples	Octopus Spring, Mushroom Spring, YNP[b] (USA)	Sanger seq. of plasmids and BAC-vectors	Nelson et al. [36*]	Samples collected from top green layer (~1 mm) of microbial mats. Temperature at sampling sites: 60 °C and 65 °C. BAC clones generated by Bhaya et al. [65]

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New Thermophilic and Thermostable Esterase with Sequence Similarity to the Hormone-Sensitive Lipase Family, Cloned from a Metagenomic Library

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A gene coding for a thermostable esterase was isolated by functional screening of *Escherichia coli* cells that had been transformed with fosmid environmental DNA libraries constructed with metagenomes from thermal environmental samples. The gene conferring esterase activity on *E. coli* grown on tributyrin agar was composed of 936 bp, corresponding to 311 amino acid residues with a molecular mass of 34 kDa. The enzyme showed significant amino acid similarity (64%) to the enzyme from a hyperthermophilic archaeon, *Pyrobaculum calidifontis*. An amino acid sequence comparison with other esterases and lipases revealed that the enzyme should be classified as a new member of the hormone-sensitive lipase family. The recombinant esterase that was overexpressed and purified from *E. coli* was active above 30°C up to 95°C and had a high thermal stability. It displayed a high degree of activity in a pH range of 5.5 to 7.5, with an optimal pH of approximately 6.0. The best substrate for the enzyme among the *p*-nitrophenyl esters (C_4 to C_{16}) examined was *p*-nitrophenyl caproate (C_6), and no lipolytic activity was observed with esters containing an acyl chain length of longer than 10 carbon atoms, indicating that the enzyme is an esterase and not a lipase.