Department of Biology, Healthcare and Environment.
Microbiology Section. Faculty of Pharmacy and Food Sciences.
University of Barcelona.
PhD Program
Biotechnology

Characterization of active inclusion body

PhD candidate : Shirin Shoja Chaghervand
Tutor : Montserrat Busquet
Angeles Manresa

Outline

- Introduction
- Objectives
- Results
- Conclusion

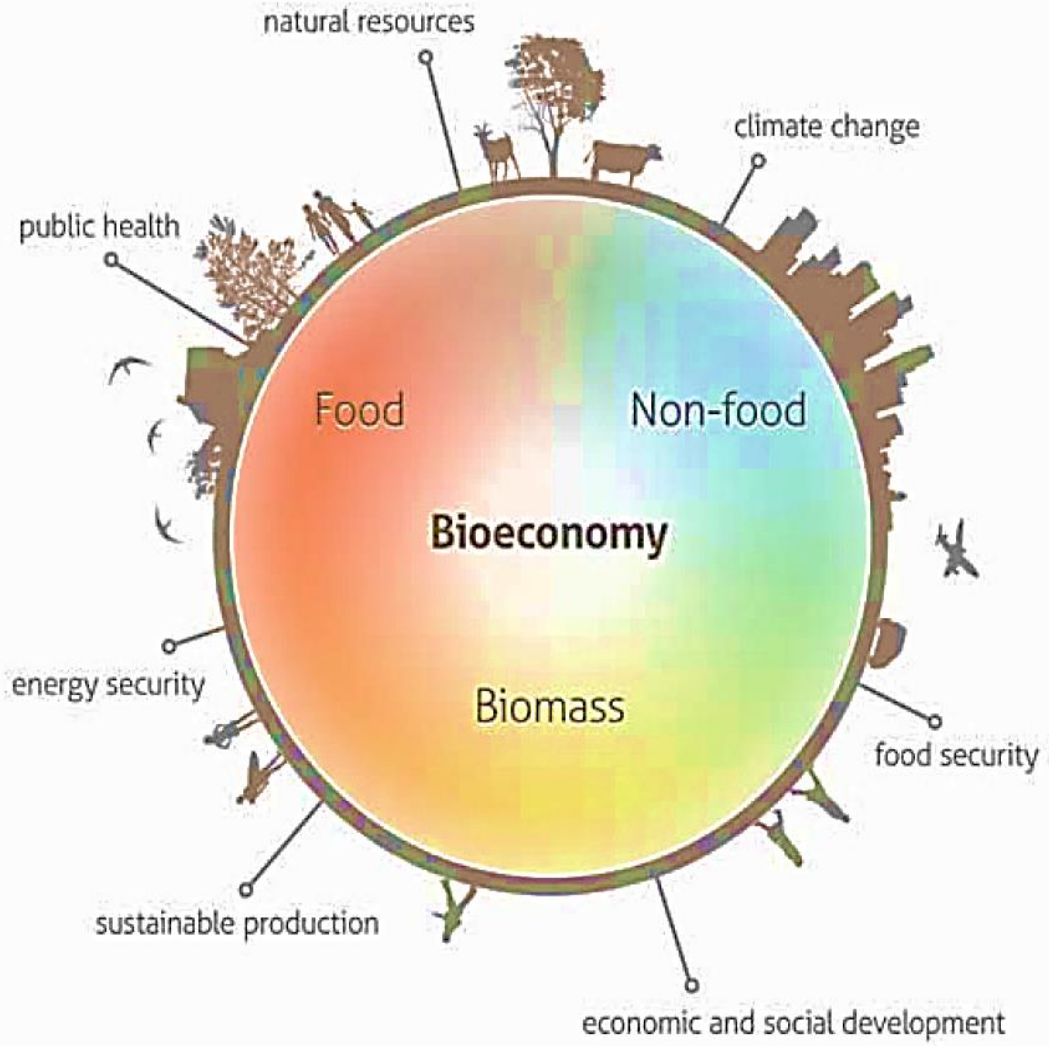
Introduction

Introduction

Bioeconomy

- Now we are in in the area of the Bioeconomy, which goes further than Biotechnology, it refers to the sustainable production and conversión of biomass into a range of food, health and industrial products.

- The bioeconomy will improve nutrition and health, create Smart bio-based products and biofuels, forestry and other ecosystems to adapt to climate change.



Introduction

Industrial Biotechnology

- Also known as White Biotechnology.
- White biotechnology contain the production of a variety of different chemical compounds using microorganism.

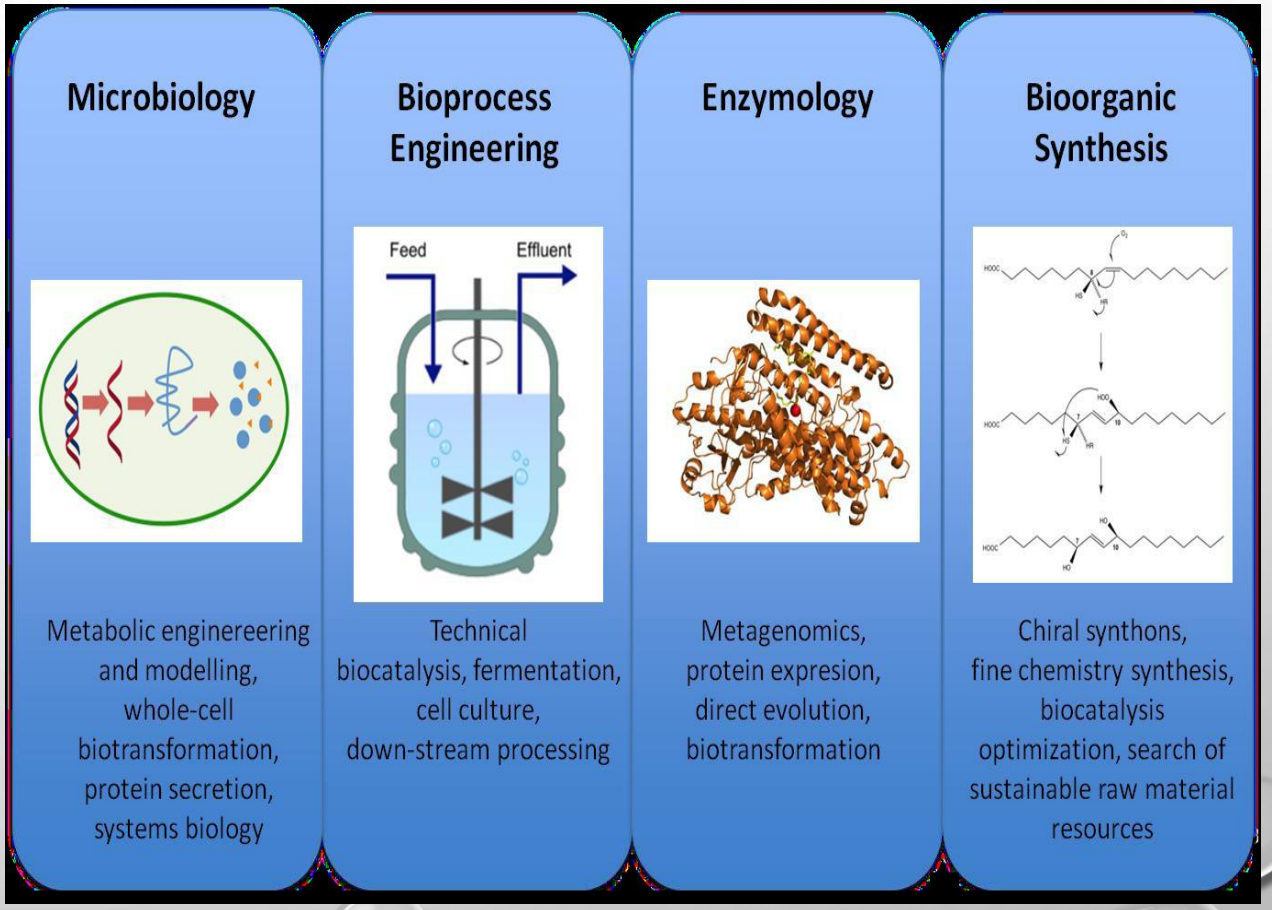
Introduction

- One of the molecular tools of White Biotechnology are enzymes.

- Enzymes act as biocatalysts in the chemical industry to:

1. Catalyse chemical reactions for which no suitable chemical catalysts are available.

2. Conduct Green chemistry by replacing chemical processes.



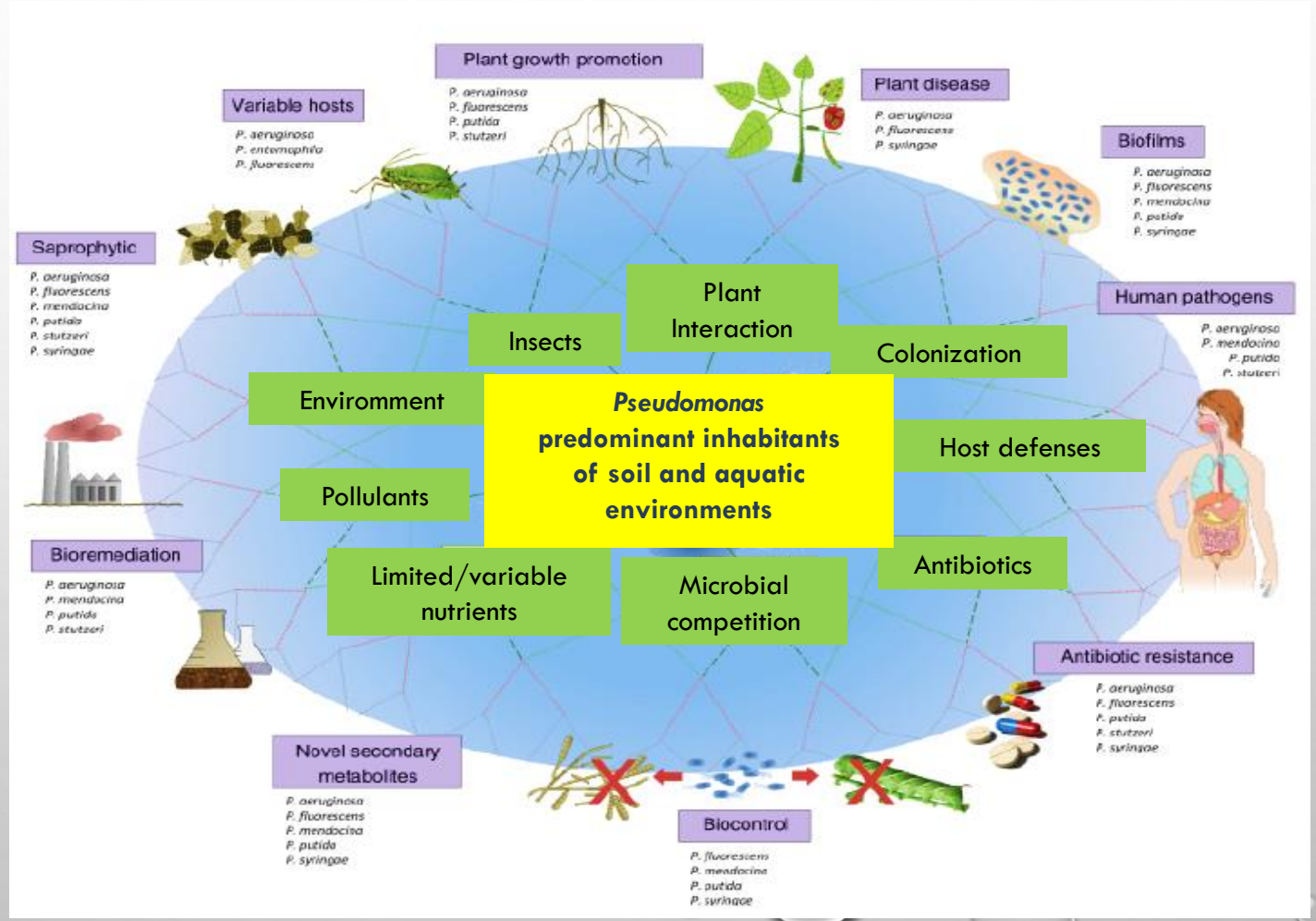
Drepper *et al.*, 2006

Introduction

- Microbial technology constitutes the core of Industrial Biotechnology

The genus *Pseudomonas*

Natural and poweful tool



FEMS Microbiol Rev 35:652-580 (2011)

Introduction

Microorganism/Biotransformation

- *Pseudomonas aeruginosa*

Gram-negative bacteria

Bioremediation

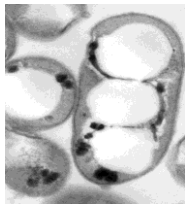
Oxylipin synthesis

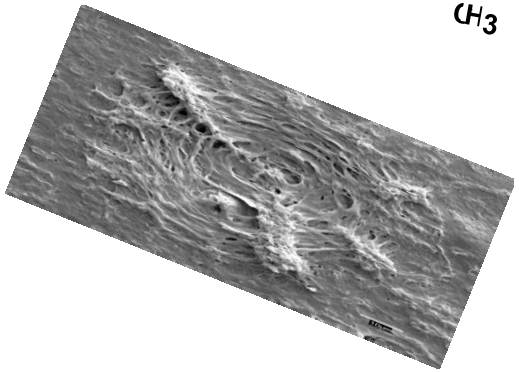
Introduction

Oxylipin

- Family of oxygenated fatty acids
- Emulsifying agent in food and cosmetics industries
- Biologically active antibacterial or antifungal substance
- Intermediates in the synthesis of fine chemicals and pharmaceuticals

Introduction





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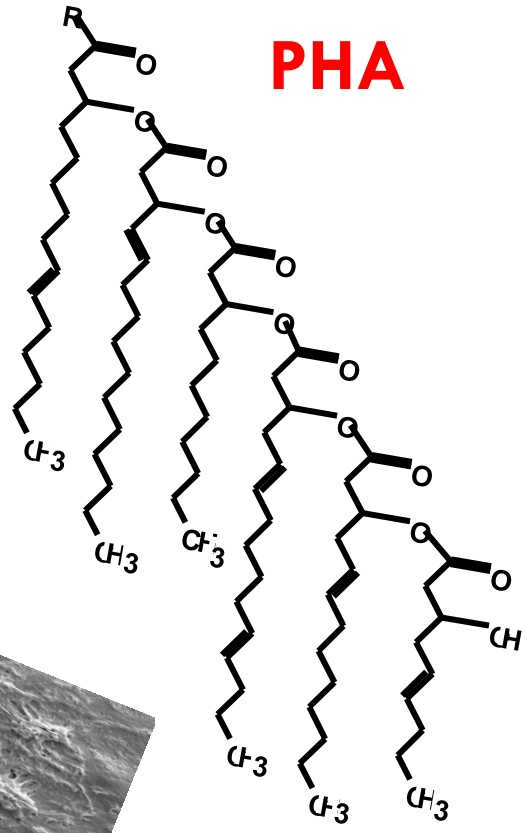
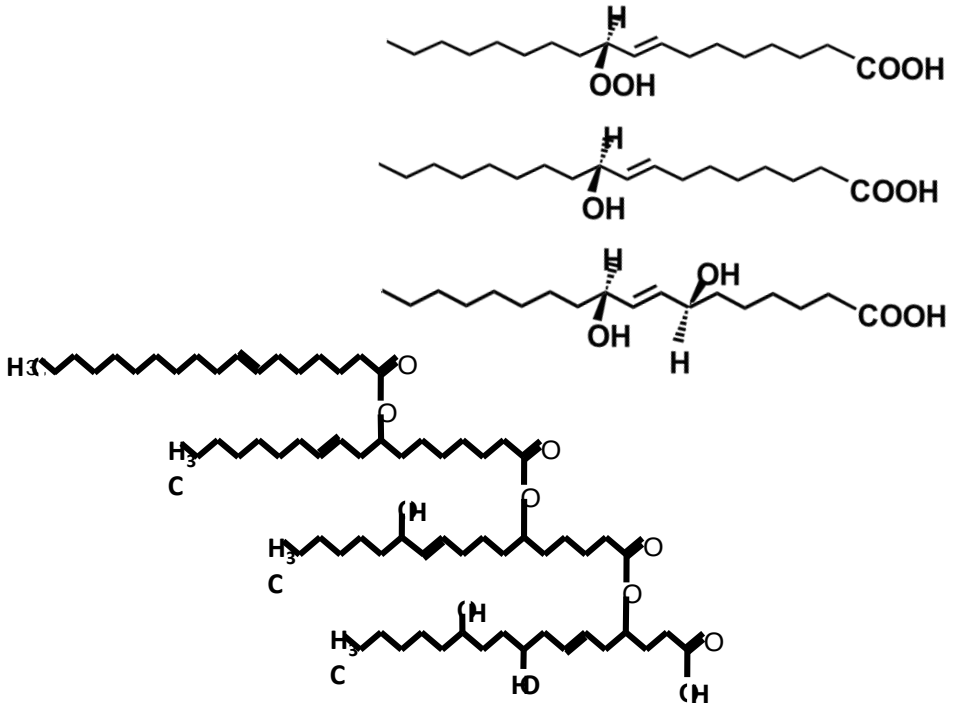


Fernandez *et al* Biochem Eng J 26: 159-167 (2005)
Bassas-Galià *et al* J Non-Crystal Solids 352:2259-2263
Rodriguez-Carmona *et al*. JAOCS 89:111-122 (2012)

Hydroxy-fatty acids and Estolides



Martin-Arjol, 2014; Estupiñan, 2015

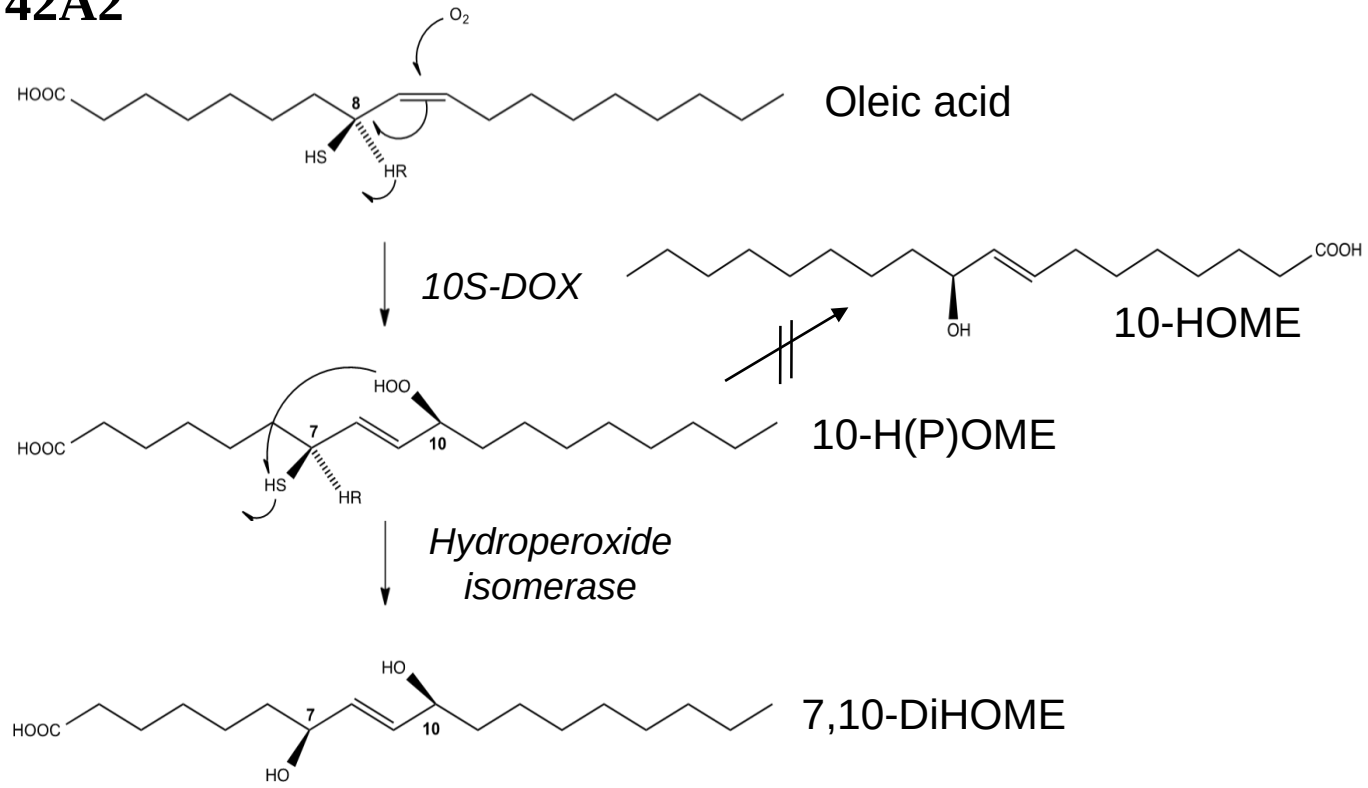
Introduction

Oxylipin synthesis in *Pseudomonas aeruginosa* 42A2

Oleate-diol synthase pathway:

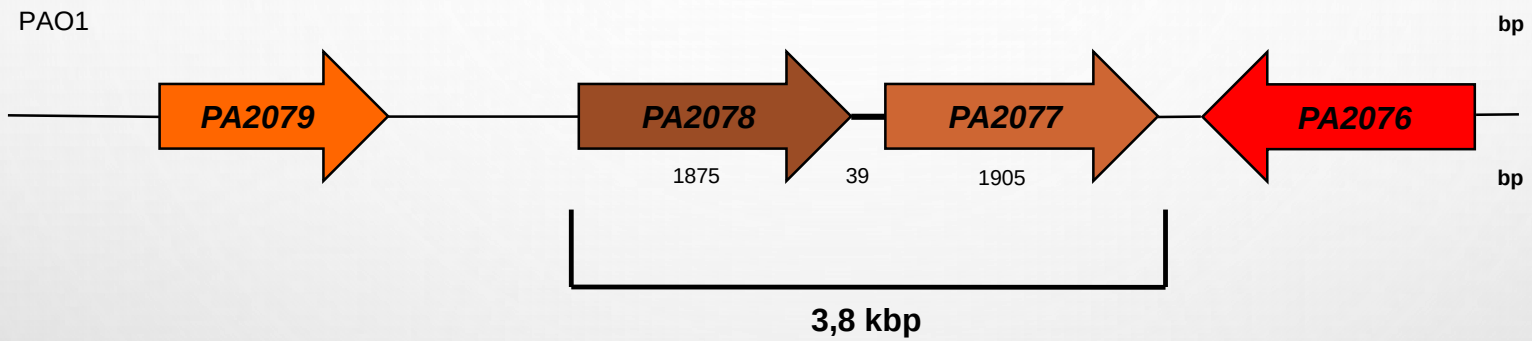
1. oleic acid, is initially converted into hydroperoxide 10-H(P)OME by a 10*S*-Dioxygenase (10-DOX) (PA2077).

2. the bioconversion of the hydroperoxide into 7,10-DiHOME by an 7,10-diol synthase (7,10-DS) (PA2078).



Estupiñan, M. PhD, 2015; Martinez et al 2010

GENE IDENTIFICATION: FROM FUNCTION TO GENE

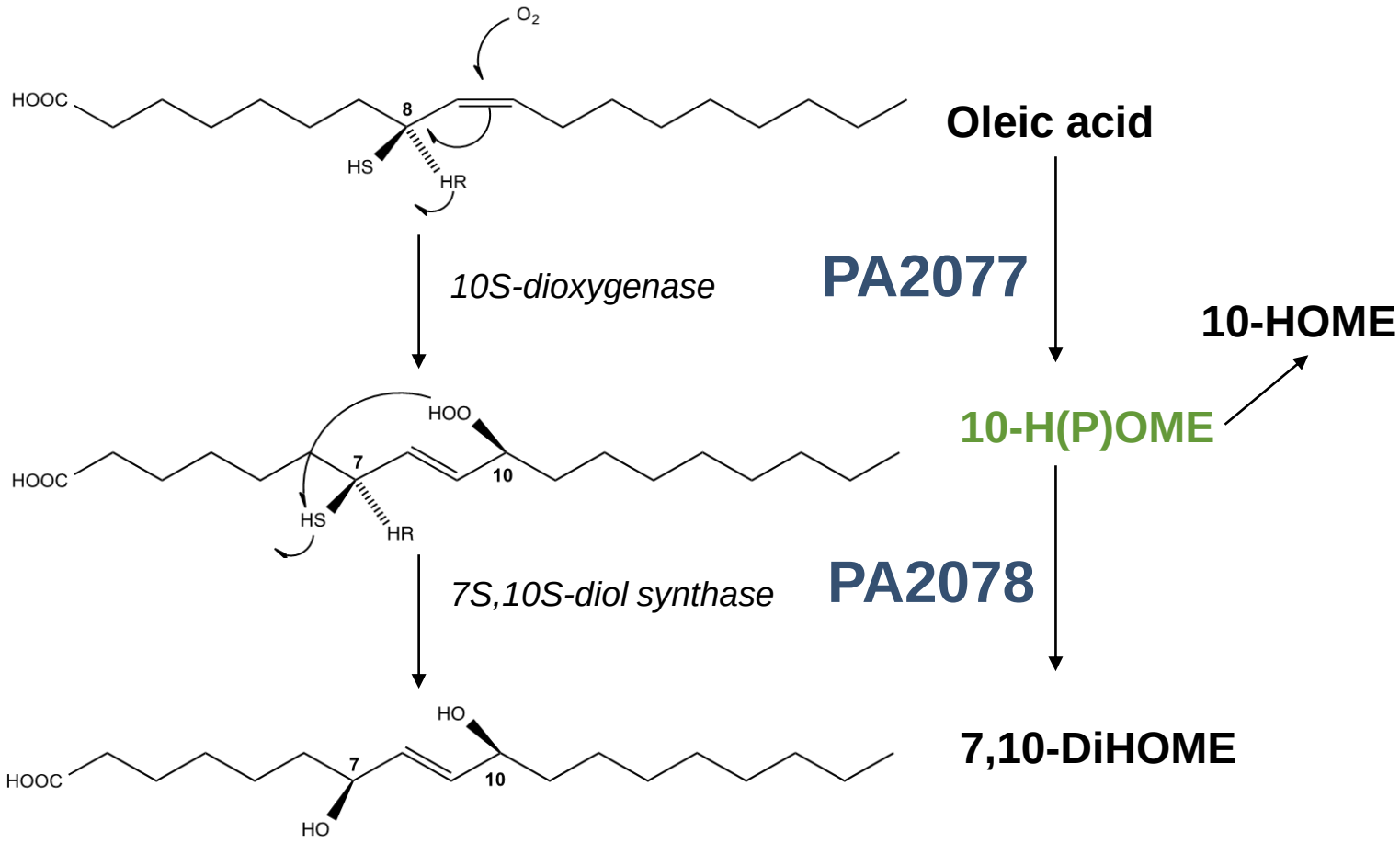


Genomic organization
of ORFs PA2077 and PA2078

Introduction

Final pathway..

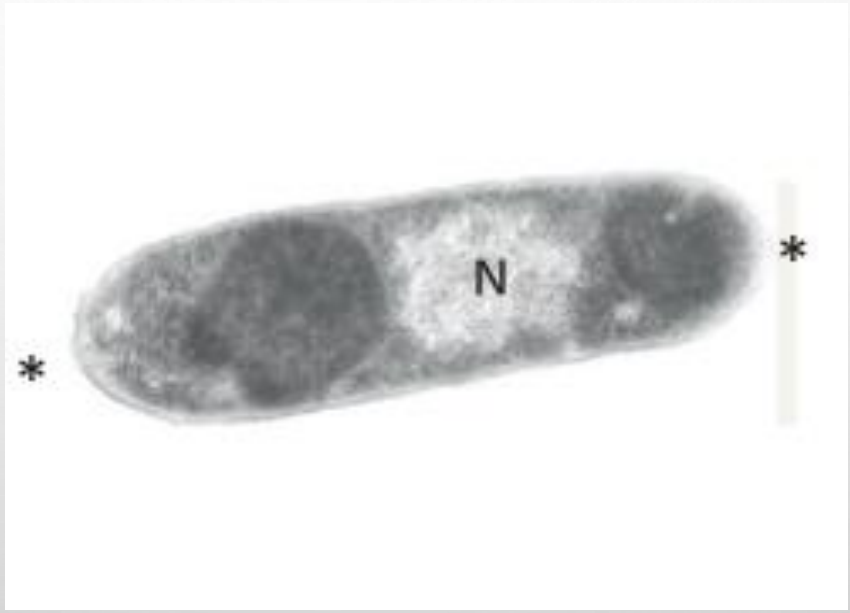
Oleate-Diol Synthase (DS) pathway



Introduction

Inclusion body

- Accumulation of aggregate protein that produced in *E. coli* during high level expression of heterologous proteins.



Rinas *et al.*, 2017

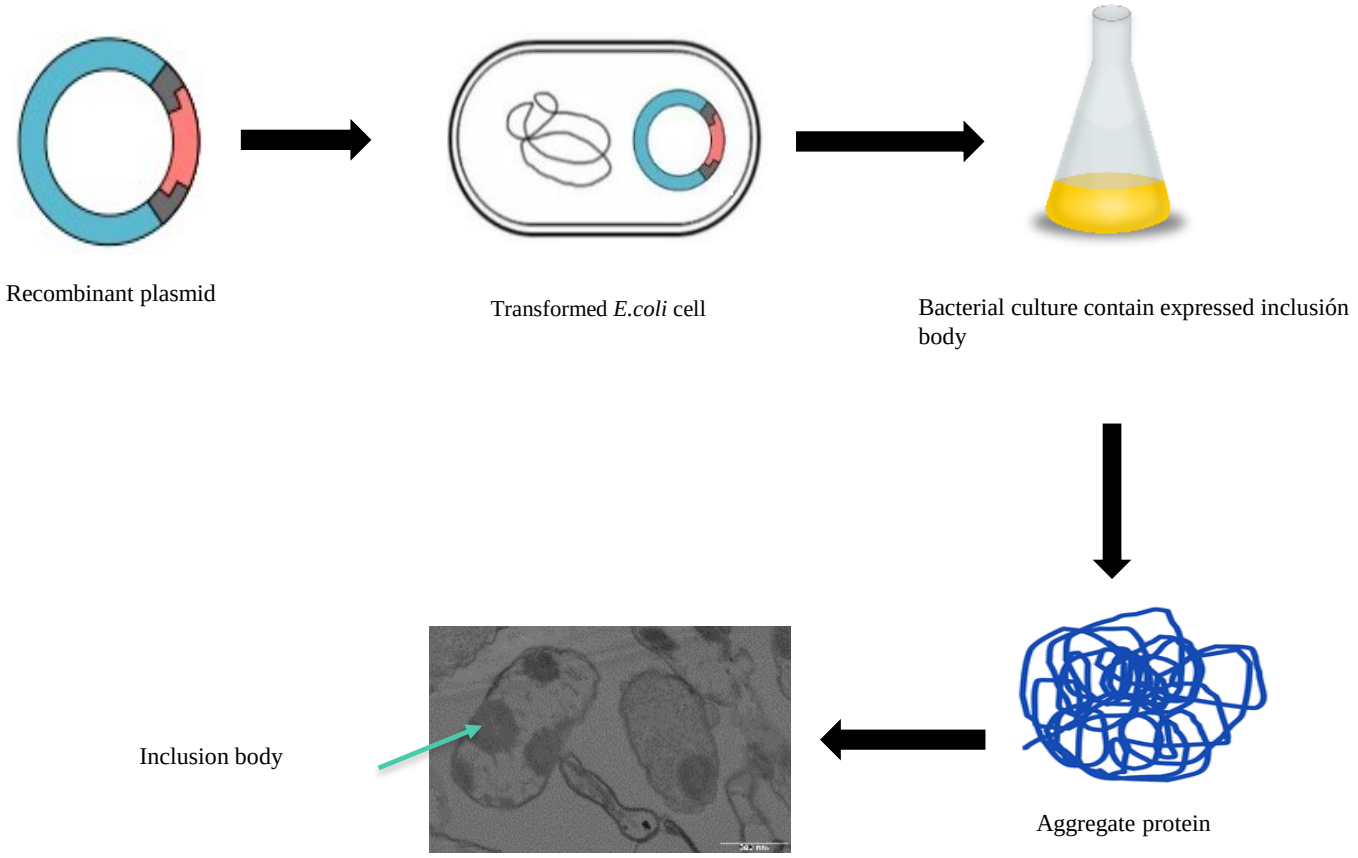
Introduction

- **Formation of inclusion body**
  - Heat shock stress
  - Strong inducer or strong promoter in vectors
  - Chaperons
  - Amino acidic sequence (hydrophobic)

Estupiñan, M. PhD, 2015; Martinez et al 2010

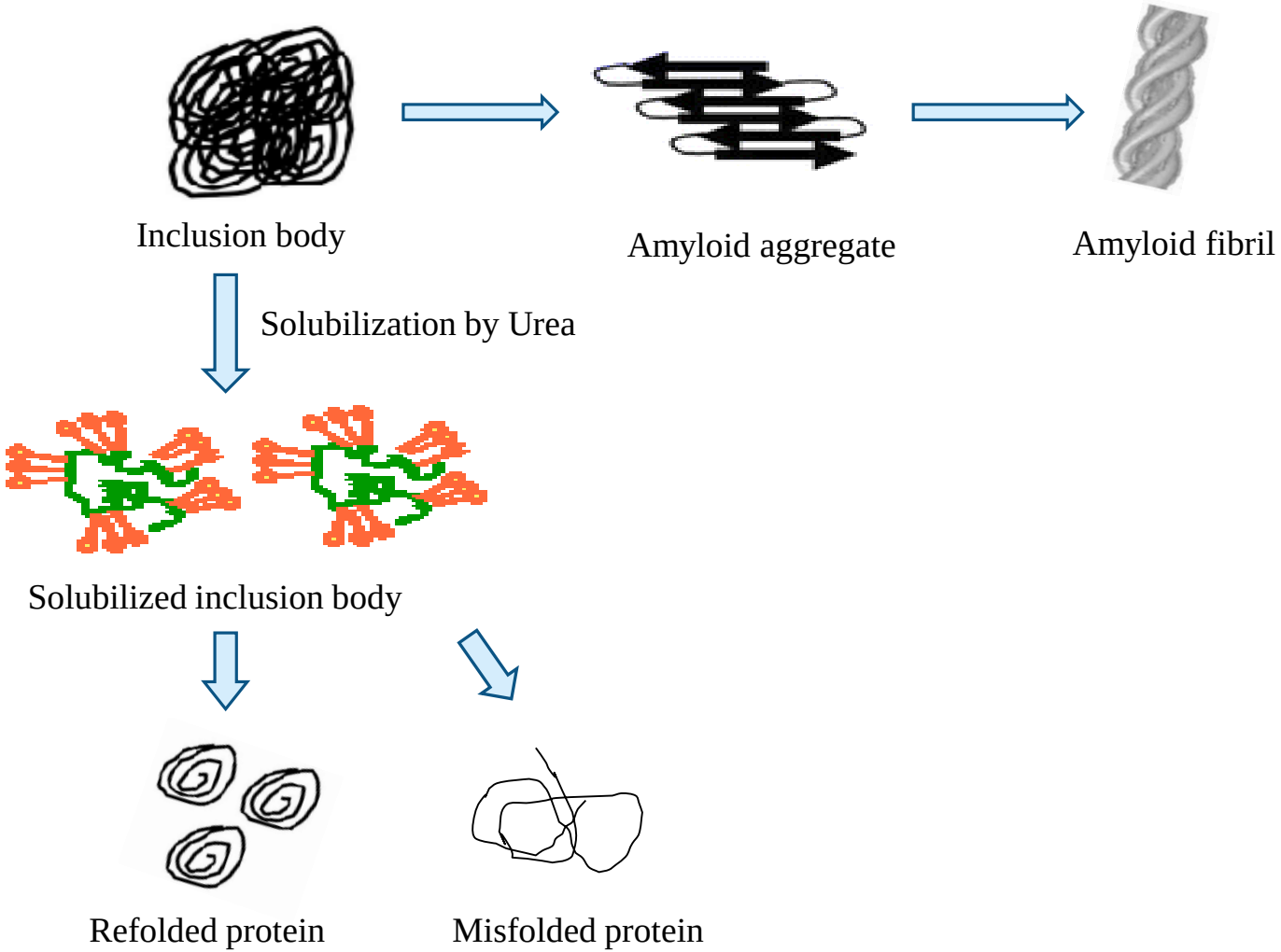
Introduction

- Expressed DH5α (pMMB-77) and BL21(pET 28 a-78) as IBs in *E.coli*.



Introduction

- **structure of IB and protein refolding**



Objetives

Objetives

Aim 1

- Produce recombinant protein in *E.coli*

Aim 2

- Study the structure of the aggregates formed protein

Aim 3

- Demonstrate activity of purified & refolded inclusion body

Results

Results

SDS-PAGE analysis of DH5α (pMMB-77) and BL21 (pET 28a-78) over expression in *E. coli*. . lane 1, crude IBs-77; lane 2 crude IBs-78; lane 3 refolded IBs-77, lane 4 refolded IBs-78

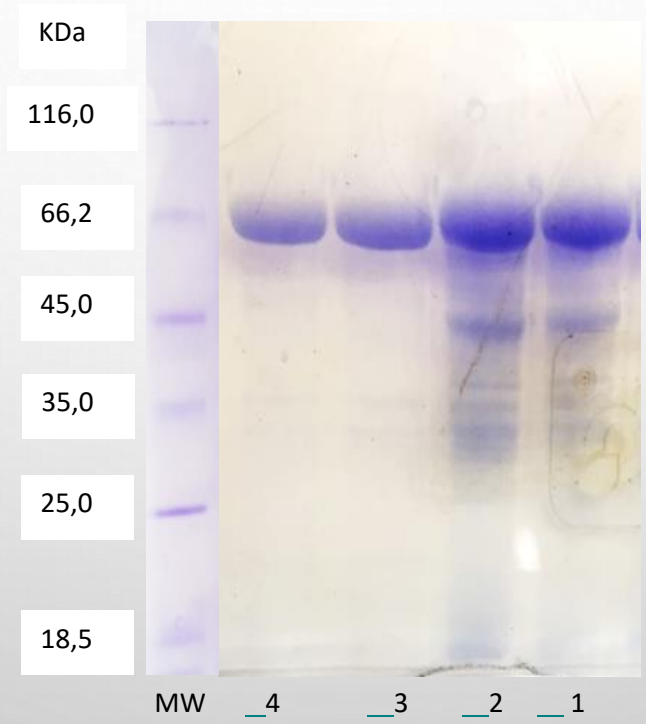


Fig .1

Results

Protein concentration obtained in the production of inclusion bodies

Protein (mg/mL)	**IBs-77**	**IBs-78**
Pure IBs	0.875	1.398
Soluble protein in supernatant	1.8	2.12
Fraction of IBs protein (%)	32.5	39.7
Refolded protein	0.333	1.335

Results

Enzymatic activities of the 10S-dioxygenase and 7,10 (S,S)-diolsynthase, inclusion bodies.

Functional protein	refolded protein/IBs (%)	Specific activity UI/mg			Recovered activity (%)
		Soluble protein	Pure IBs	Refolded protein	
10S- dioxygenase 10-DOX	37.7	0.8×10^{-3}	1.0	0.05	0.4
7,10 (S,S) diol synthase 7,10-DS	95.5	0.8×10^{-3}	2.2	nd*	nd

* no detected

Results

- **Morphology of inclusion body**

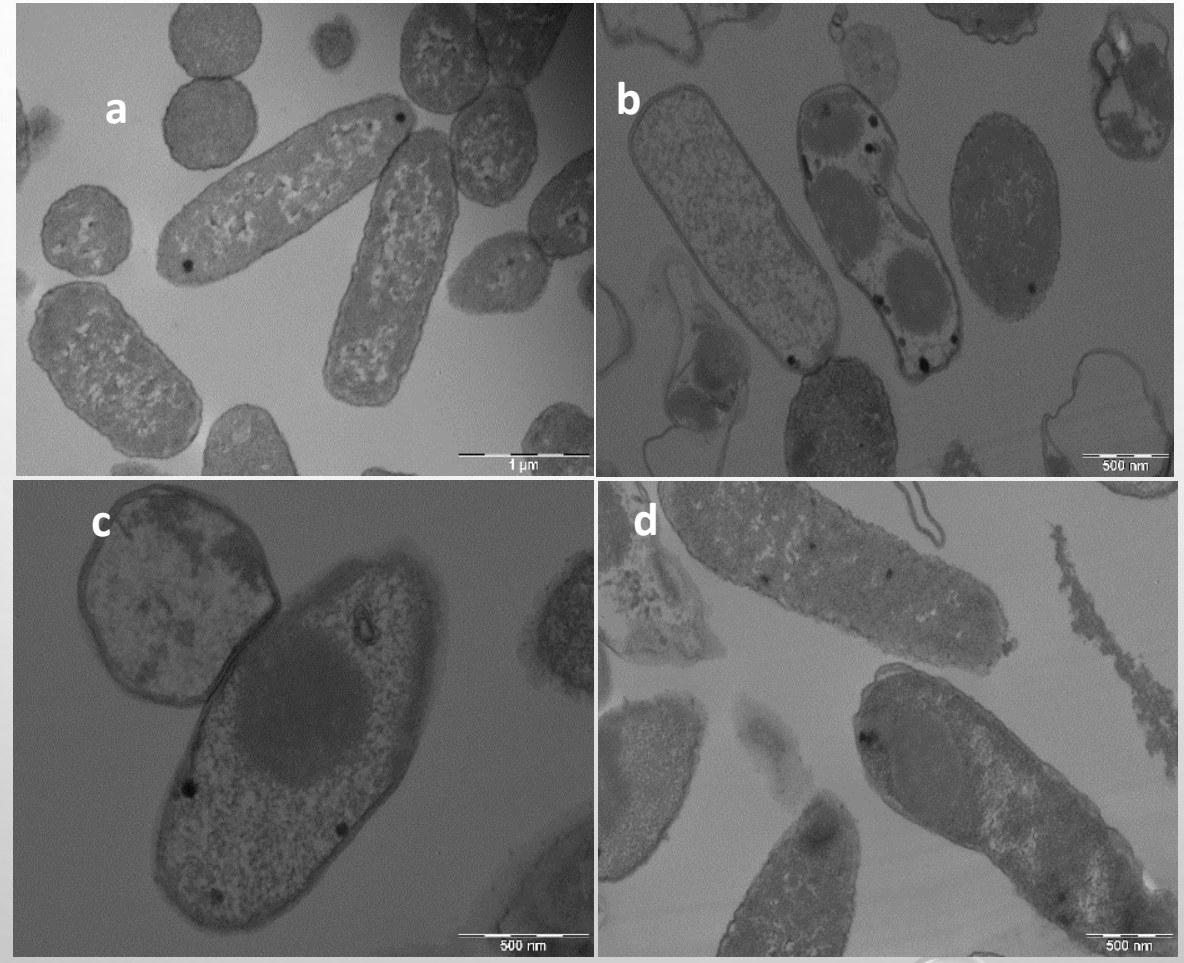
  - Transmission electron microscopy (TEM)
  - Fourier-transform infrared spectroscopy (FT-IR)
  - Congo red (CR)
  - Proteinase K (PK)
  - Thioflavin T flurescence (ThT)
  - Atomic Force Microscope (AFM)

Results

- **TEM**

The Size range: 214,28 - 460,5 nm

Native cells of DH5α (pMMB-77), a



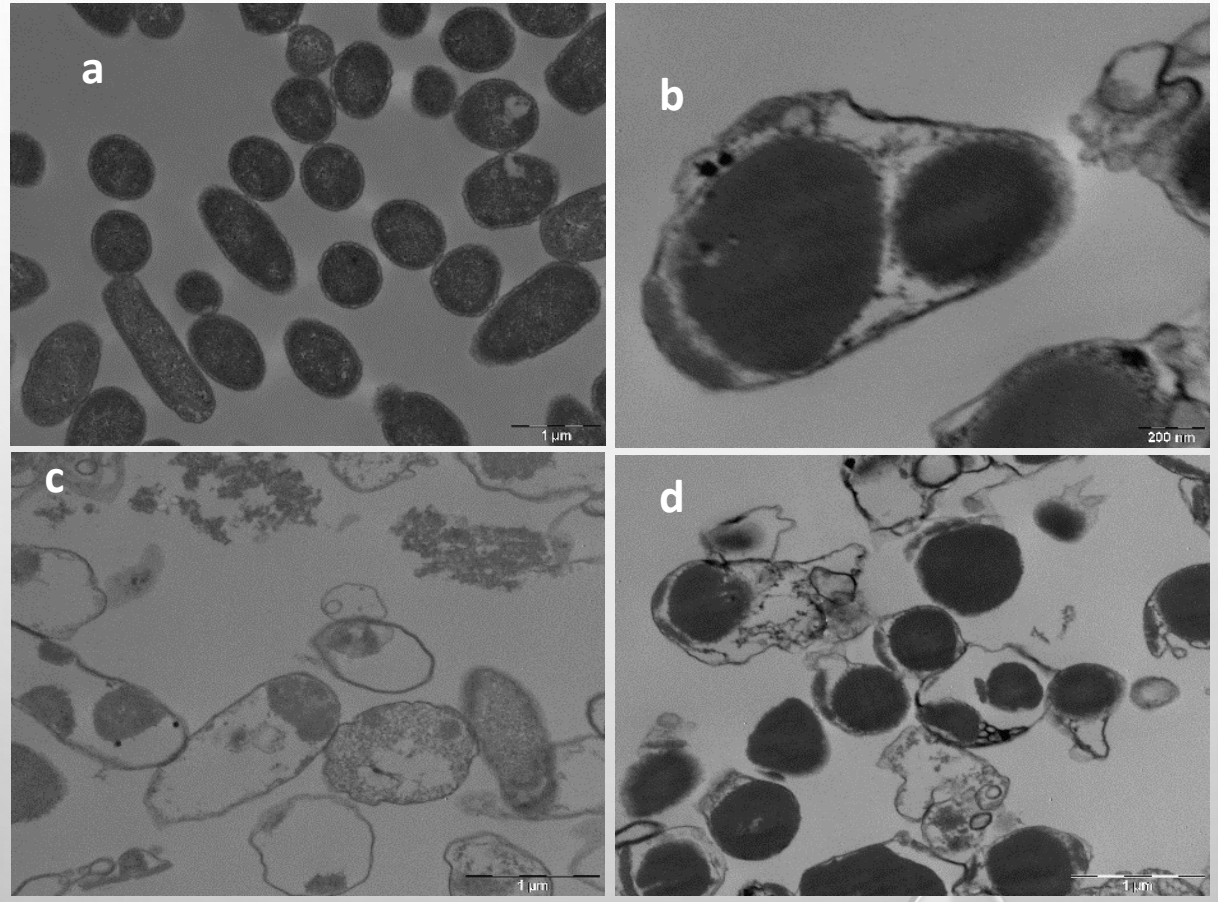
Induced cells of DH5α (pMMB-77), (b-d)

Fig .2

Results

The Size range: 294 - 529 nm

Native cells of BL21(pET 28a-78), a



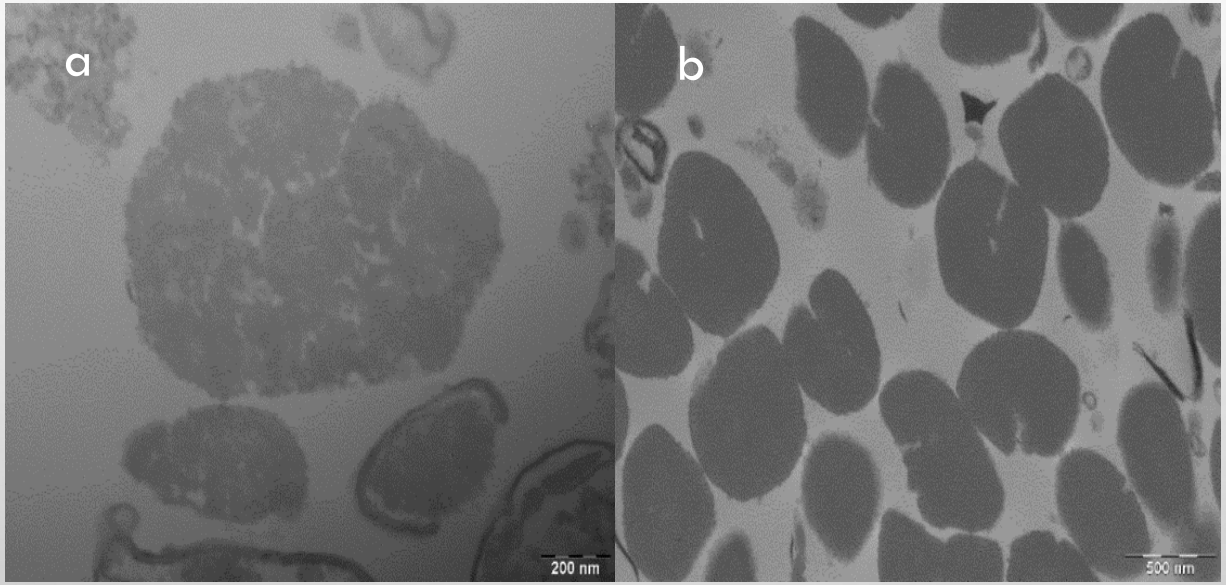
Induced cells of BL21(pET 28a-78) cells (b-d)

Fig .3

Results

- Transmission Electron Microscopy of purified inclusion bodies after fresh staining with uranyl acetate 2%. (a) IBs-77, (b) IBs-78.

IBs-77



IBs-78

Fig .4

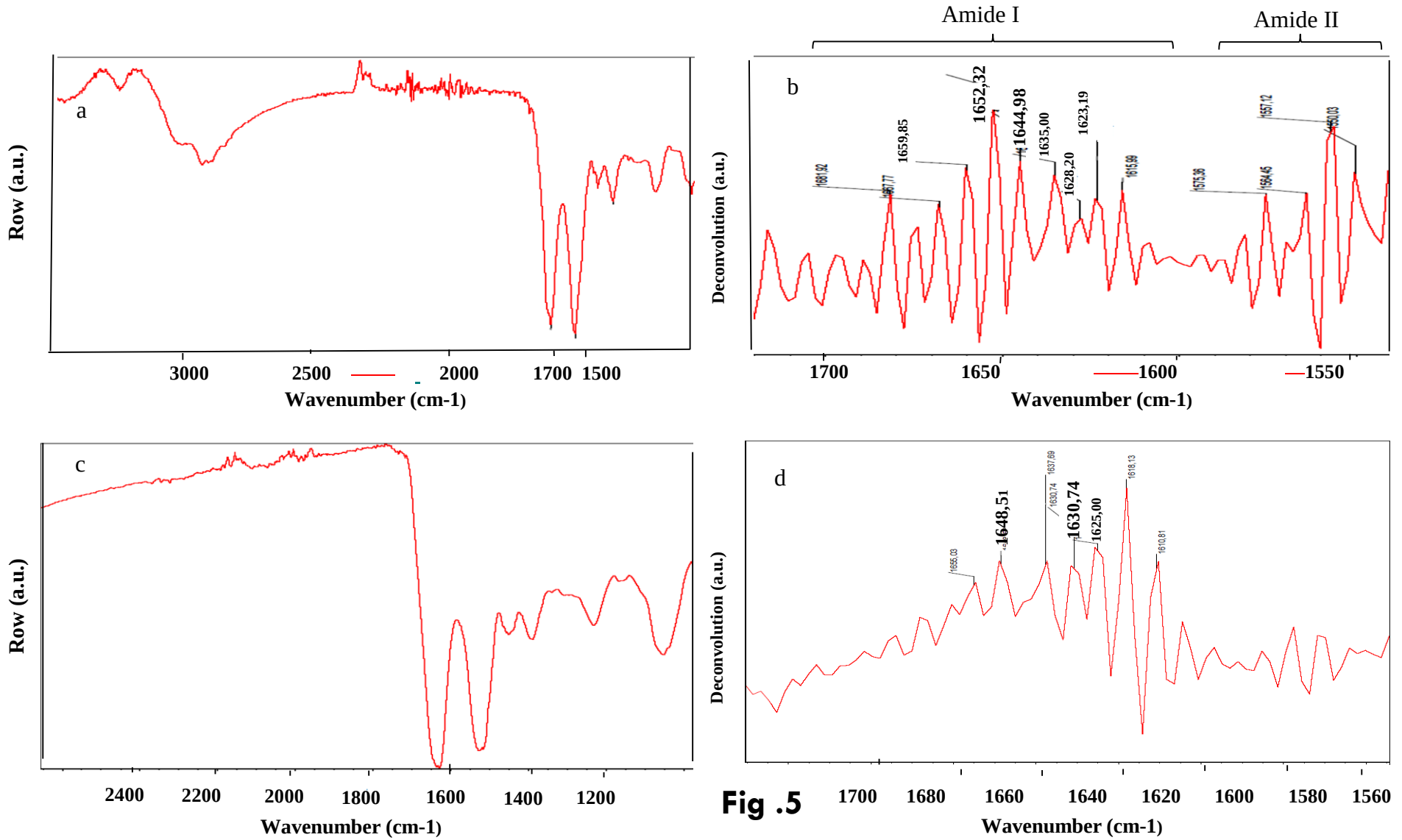
Results

- **FT-IR**

Row and deconvoluted spectra of IBs-77 and control cells

Amid I band
1600-1700cm-1

Amid II band
1500-1550cm-1



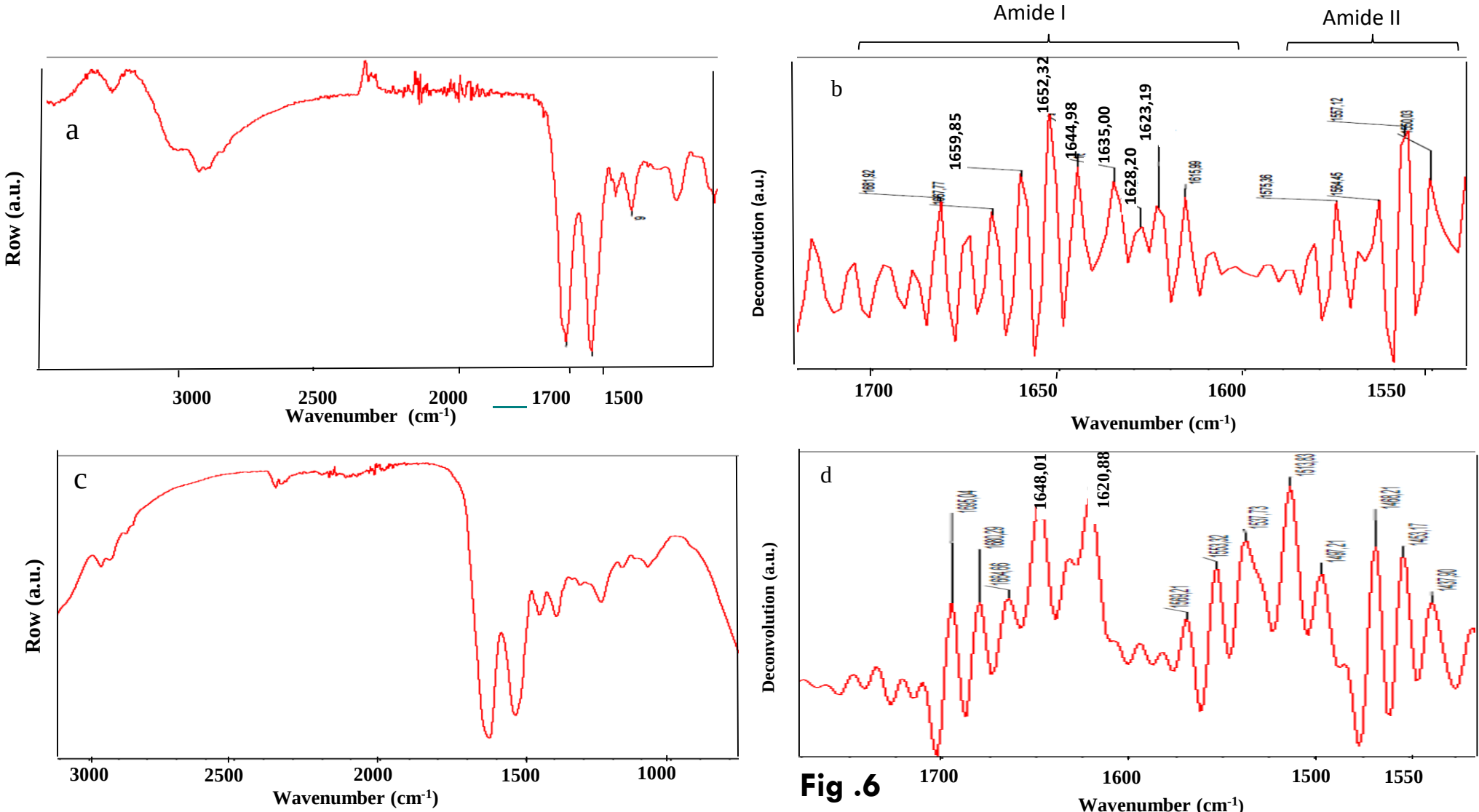
control cells

IBs-77

Fig .5

Results

Row and deconvoluted spectra of pure IBs-78 and control cells.



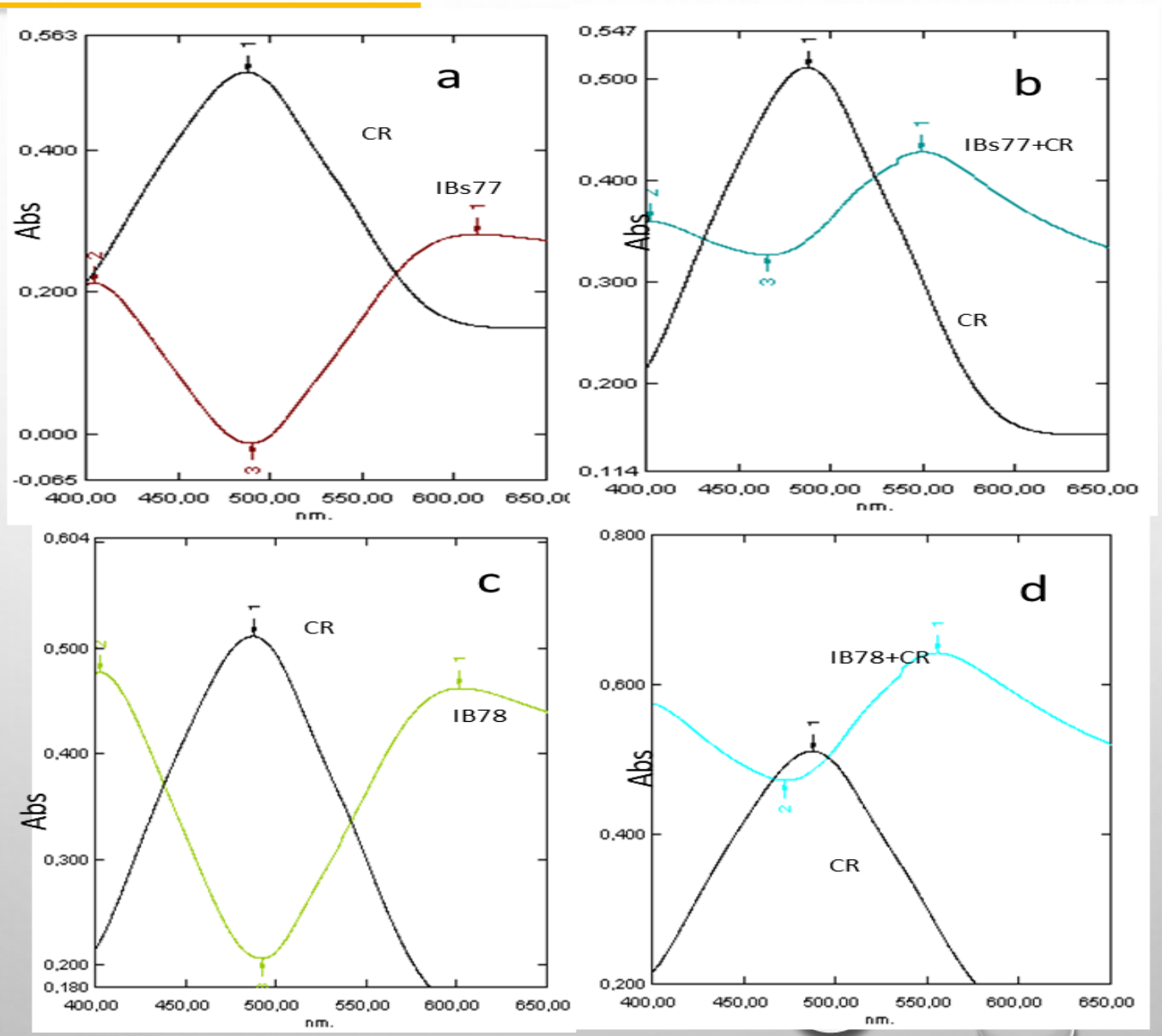
Control cells

IBs-78

Fig .6

Results

CR (Congo Red)



Control absorbance

Absorbance of the complex CR bund to IBs.

Fig .7

Results

Digestion with Proteinase K

Proteinase K digestion of DH5α (pMMB-77) and BL21(pET 28a-78) IBs. 1: molecular marker; 2-5 Coomassie blue marker; 6 control IBs protein; 7-10 digested protein IBs.

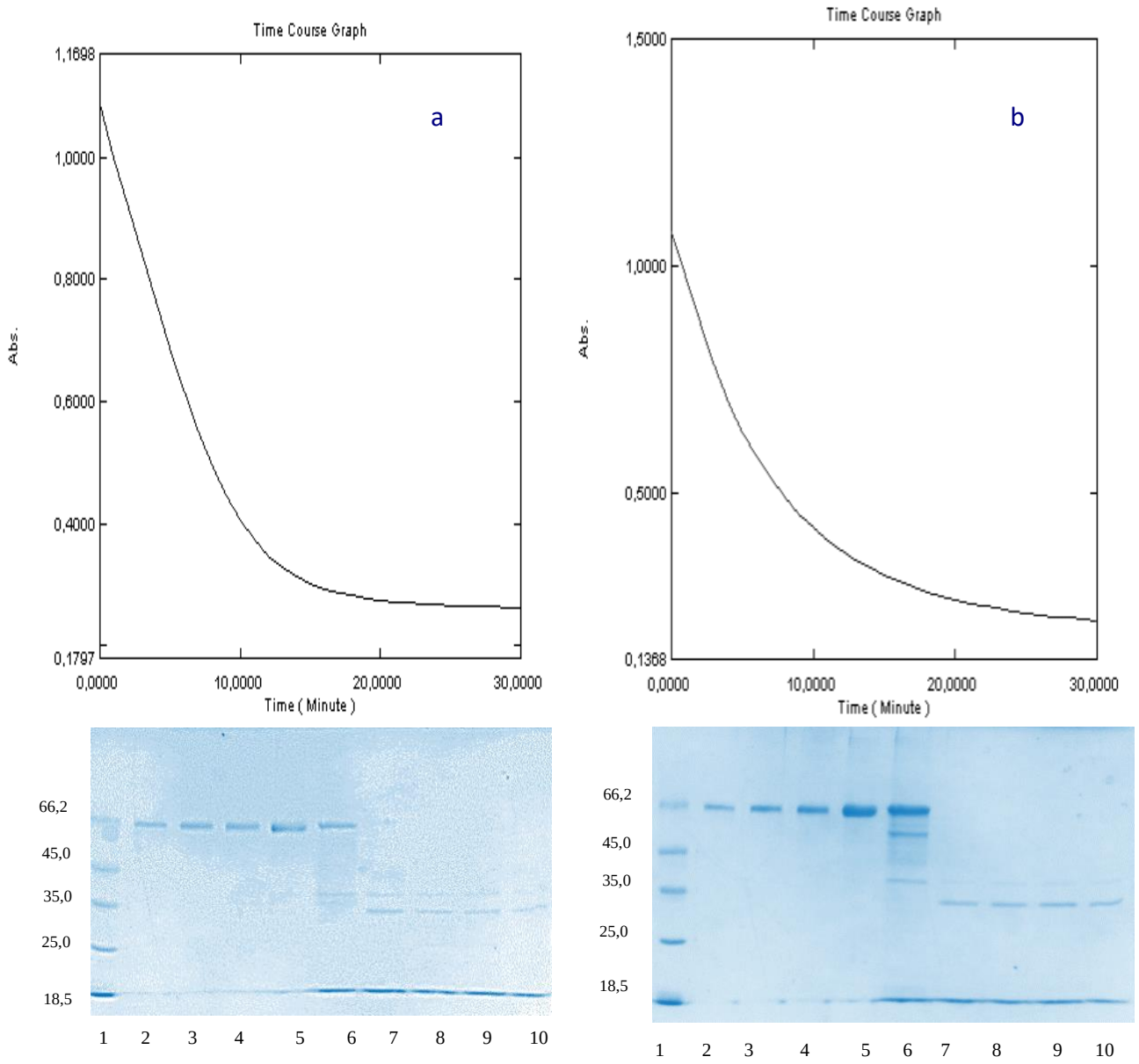


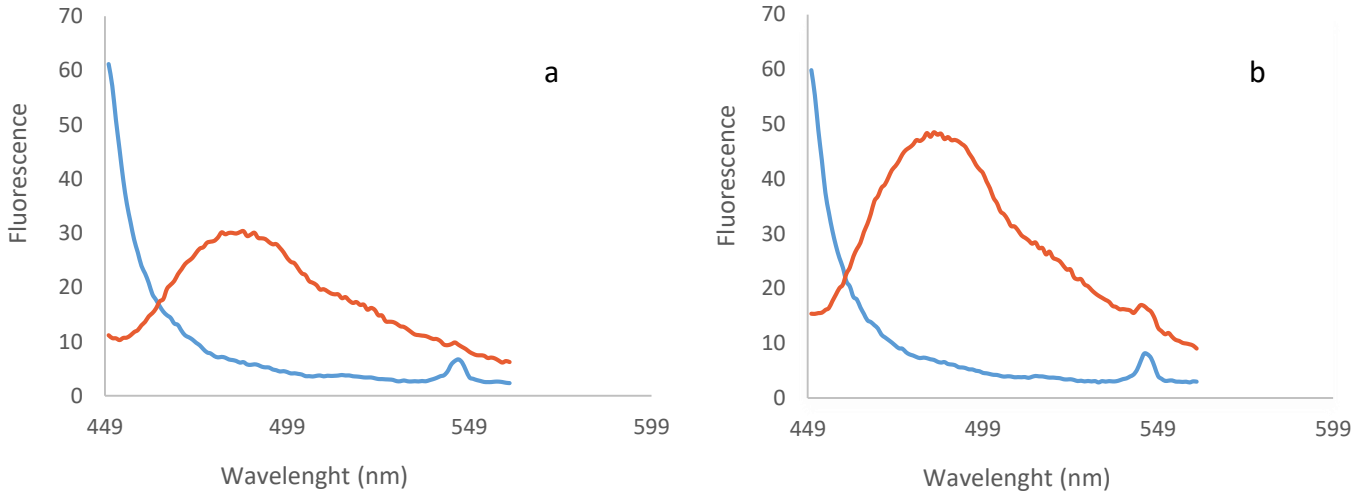
Fig .8

Results

Thioflavin binding florescence

Fluorescence emission spectra of IBs-77 (a) control(blue line) digested protein without Th-T; Fluorescence emission spectra of IBs-78(b) control (blue line) digested protein without Th-T

IBs 77



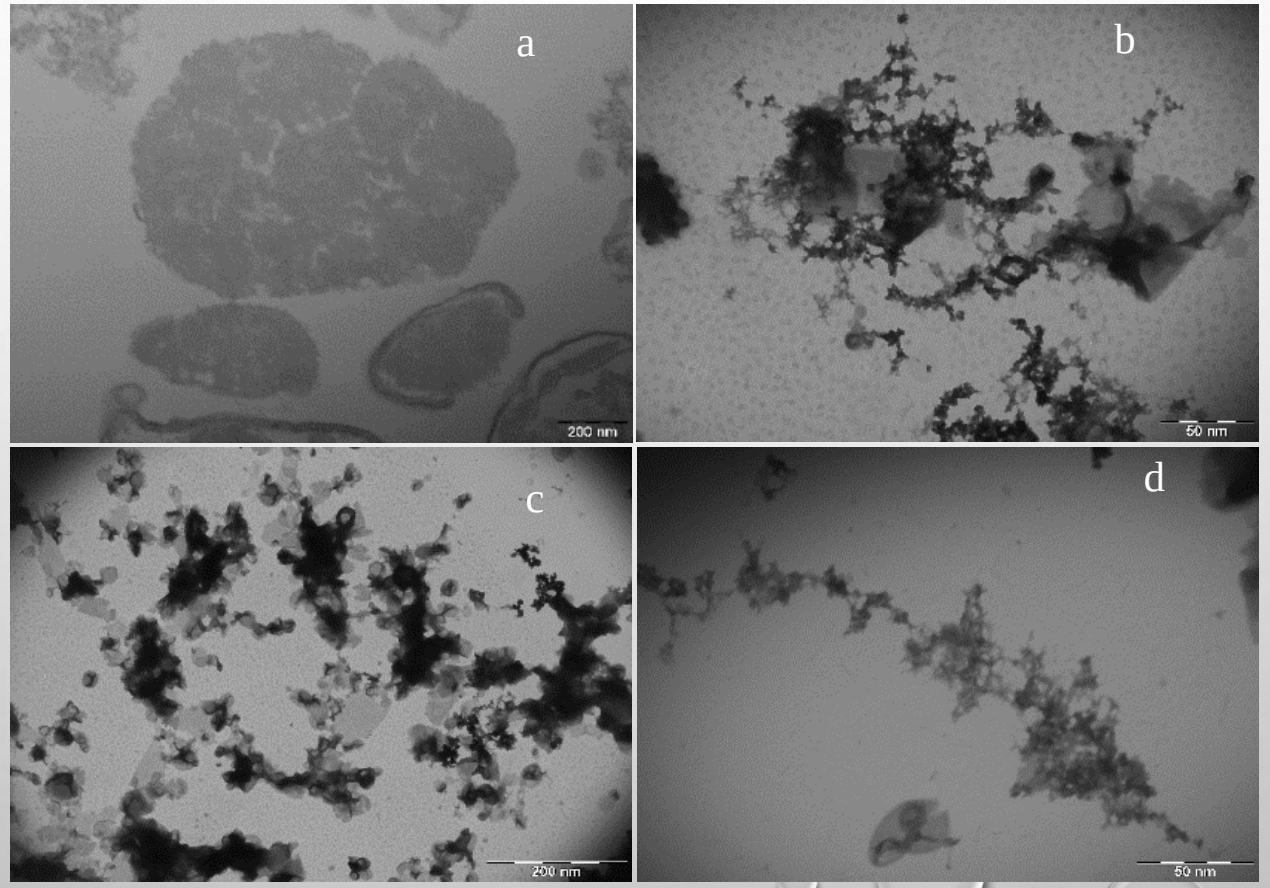
IBs 78

Fig .9

Results

Micrographs of IBs-77 aggregates. Purified IBs-77 before digestion (a); IBs-77 after digestion dark and amorphous material (c) amyloid fibrils (d) purified IBS-77 before digestion

IBs-77 before digestion



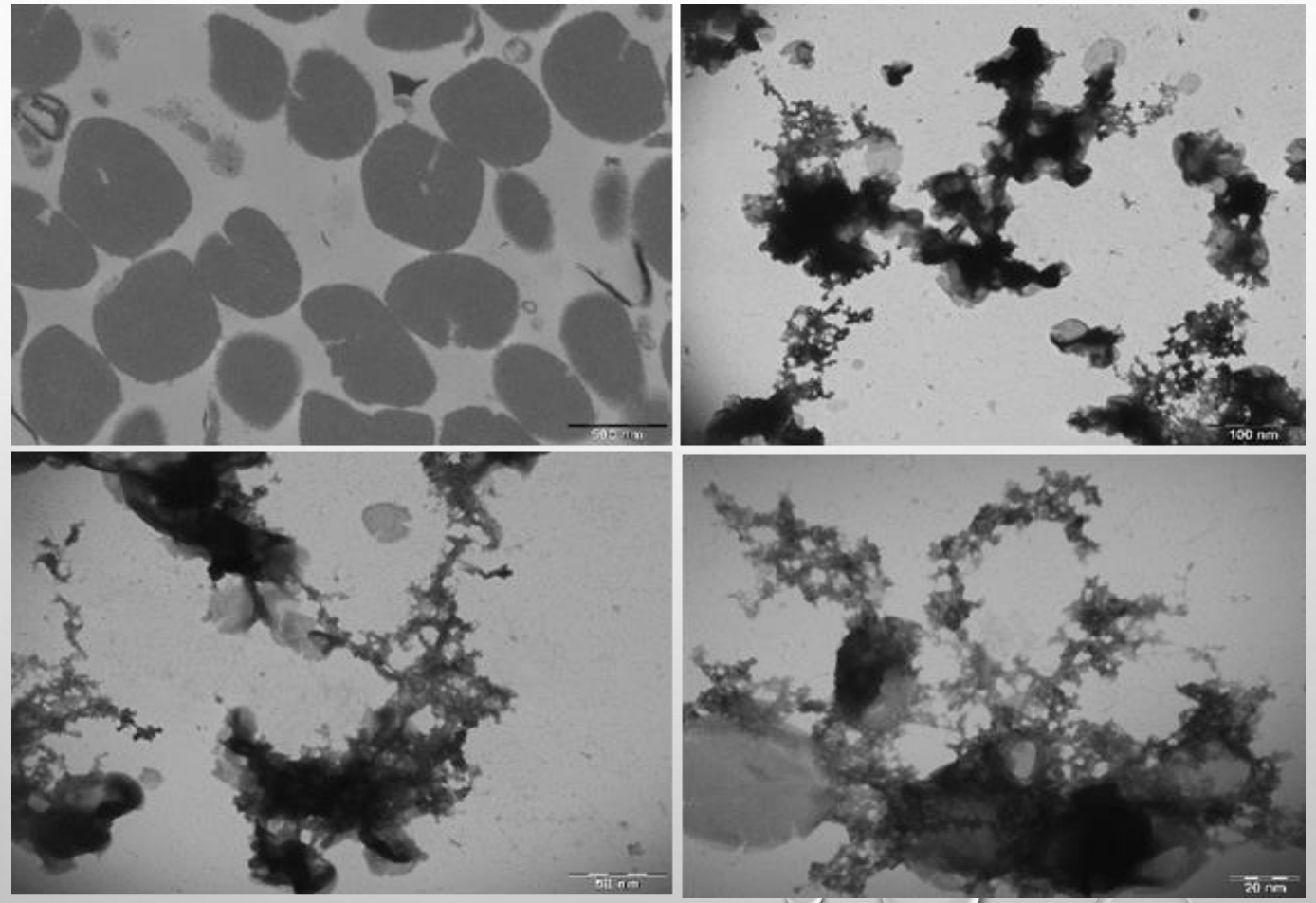
IBs-77 after digestion

Fig .10

Results

TEM micrographs of IBs-78 aggregates. Purified IBs-78 before digestion (a); IBs-78 after digestion

IBs-78 before digestion



IBs-78 after digestion

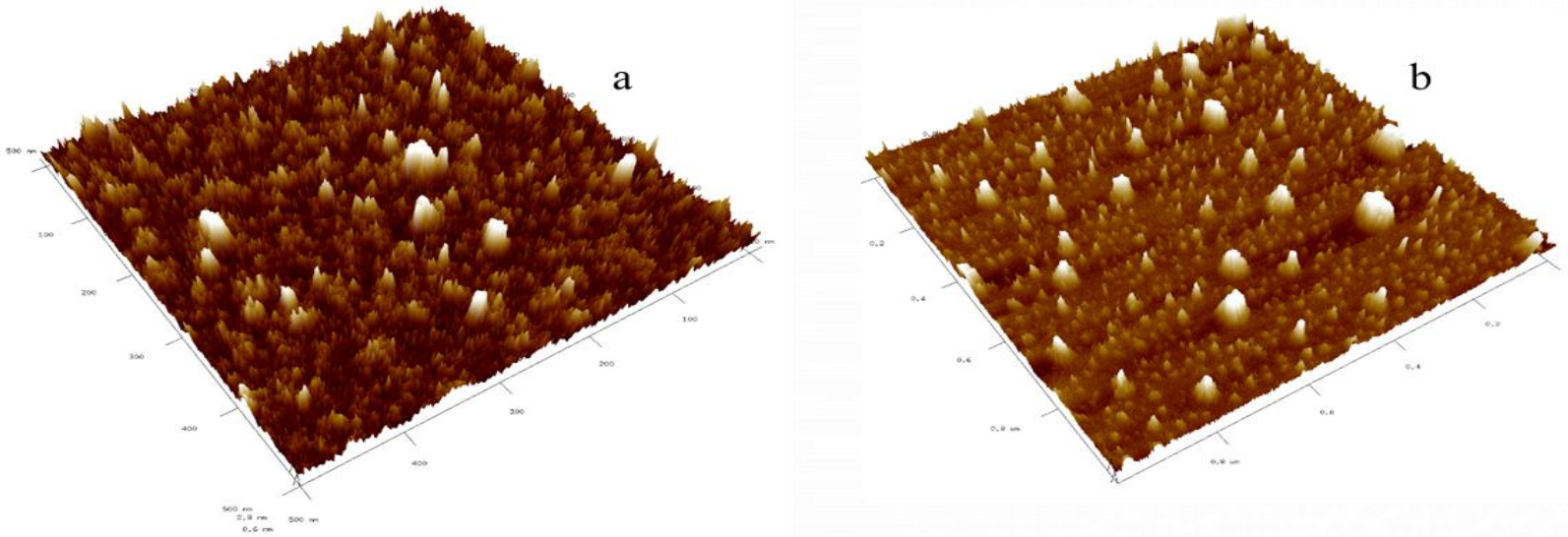
Fig .11

Results

AFM imaging

AFM 3-D overview of inclusion bodies produced by (a) DH5α (pMMB-77),the scan size is 5000nm

IBs 77



IBs 78

Fig .12

Results

Digested IBs colapse up to 60-65%

Amplitude AFM images of DH5α (pMMB-77) IBs before and after PK digestion.

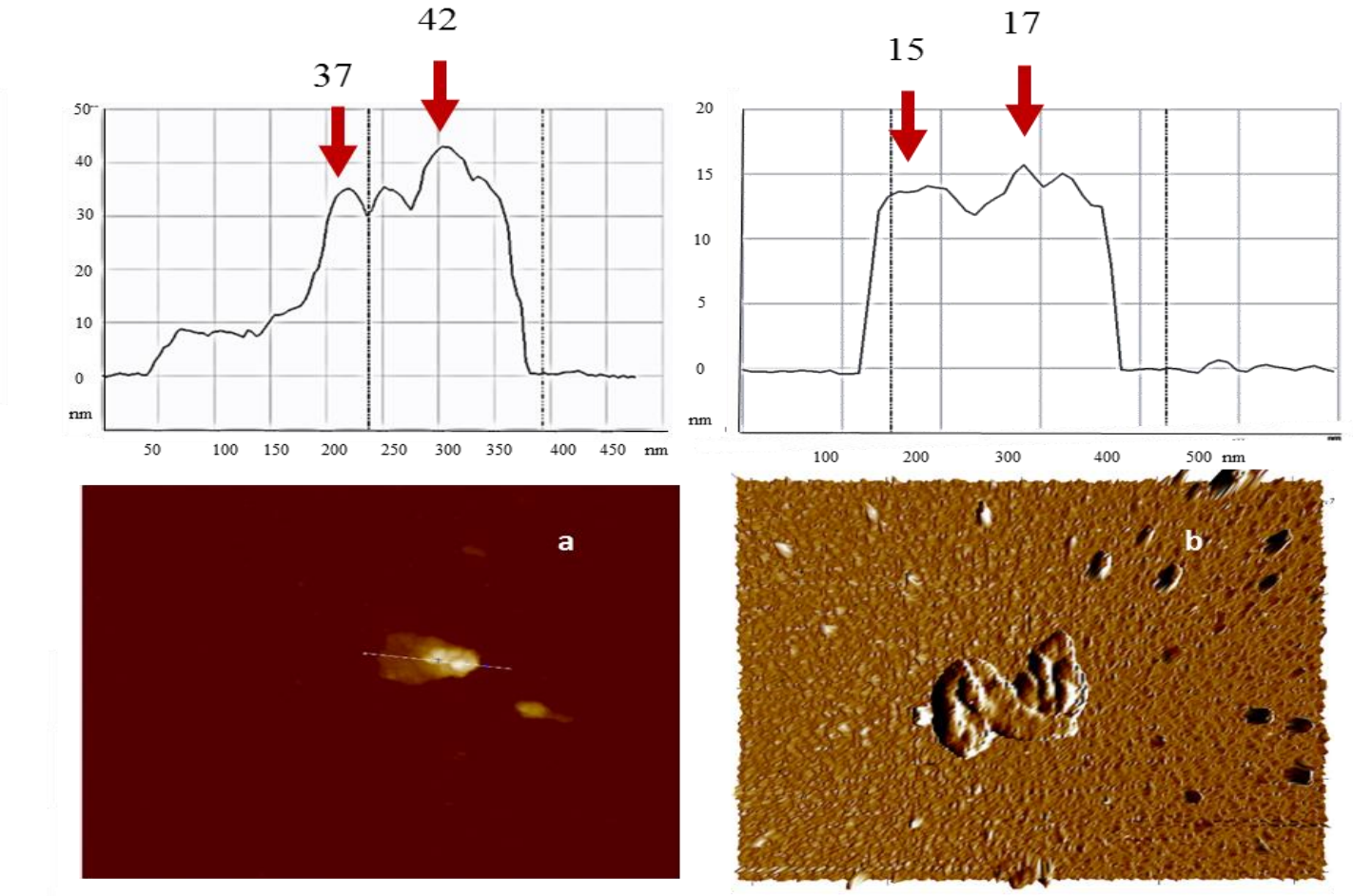


Fig .13

Results & Discution

Amplitude AFM images of BL21 (pET 28a-78) IBs before and after PK digestion.

Digested IBs colapse up to 70-83%

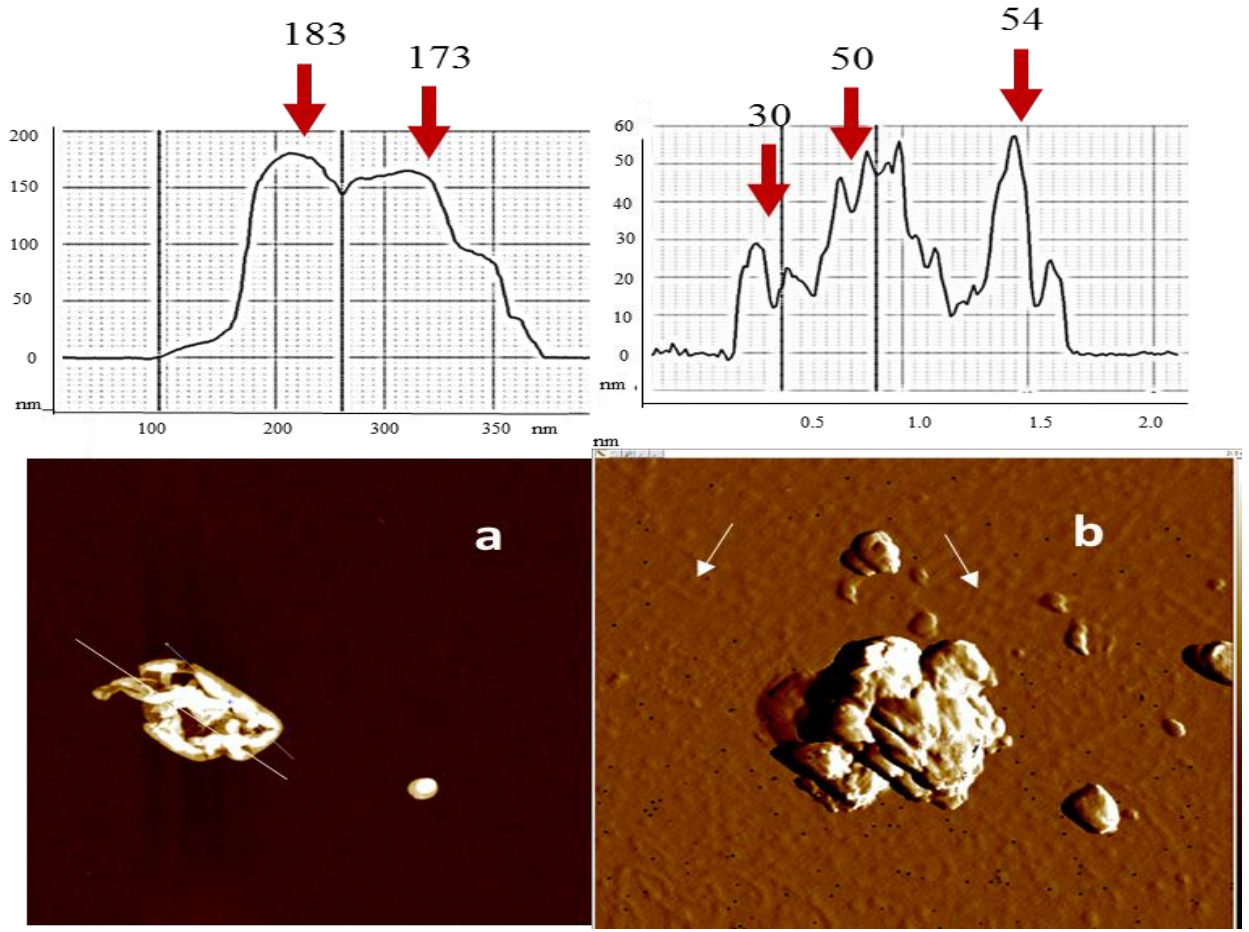


Fig .14

Conclusion

Conclusion

- Show acitivity of inclusion body and refolded protein
- Demontrated amyloid structure present in studied IBs
- Inclusion bodies are nanoparticles for biotransformation unsaturated fatty acid

GRACIAS