Characterization of active inclusion body

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PhD Program
Biotechnology
Introduction
Introduction

Bioeconomy

• Now we are in the area of the Bioeconomy, which goes further than Biotechnology, it refers to the sustainable production and conversion 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.
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.
Introduction

- Microbial technology constitutes the core of Industrial Biotechnology

The genus *Pseudomonas*

Natural and powerful tool
Introduction

Microorganism/Biotransformation

- *Pseudomonas aeruginosa*
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

Hydroxy-fatty acids and Estolides

Bassas-Galià et al. J Non-Crystal Solids 352:2259-2263

Martin-Arjol, 2014; Estupiñan, 2015
Oxylipin synthesis in *Pseudomonas aeruginosa 42A2*

Oleate-diol synthase pathway:

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

2. the bioconversion of the hydroperoxide into 7,10-DiHOME by an 7,10-diol synthase (7,10-DS) (PA2078).
Genomic organization of ORFs PA2077 and PA2078
Introduction

Final pathway..

Oleate-Diol Synthase (DS) pathway

Oleic acid

10S-dioxygenase

PA2077

10-H(P)OME

7S,10S-diol synthase

PA2078

7,10-DiHOME
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)
• Expressed DH5α (pMMB-77) and BL21(pET 28 a-78) as IBs in *E.coli*.
• structure of IB and protein refolding
Objetives
Objectives

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

<table>
<thead>
<tr>
<th>Protein (mg/mL)</th>
<th>IBs-77</th>
<th>IBs-78</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure IBs</td>
<td>0.875</td>
<td>1.398</td>
</tr>
<tr>
<td>Soluble protein in supernatant</td>
<td>1.8</td>
<td>2.12</td>
</tr>
<tr>
<td>Fraction of IBs protein (%)</td>
<td>32.5</td>
<td>39.7</td>
</tr>
<tr>
<td>Refolded protein</td>
<td>0.333</td>
<td>1.335</td>
</tr>
</tbody>
</table>
Results

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

<table>
<thead>
<tr>
<th>Functional protein</th>
<th>refolded protein/IBs (%)</th>
<th>Specific activity UI/mg</th>
<th>Recovered activity (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Soluble protein</td>
<td>Pure IBs</td>
</tr>
<tr>
<td>10S- dioxygenase 10-DOX</td>
<td>37.7</td>
<td>0.8 x 10^{-3}</td>
<td>1.0</td>
</tr>
<tr>
<td>7,10 (S,S) diolsynthase 7,10-DS</td>
<td>95.5</td>
<td>0.8 x 10^{-3}</td>
<td>2.2</td>
</tr>
</tbody>
</table>

* 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 fluorescence (ThT)
  - Atomic Force Microscope (AFM)
Results

- TEM

Native cells of DH5α (pMMB-77), a

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

The Size range: 214.28 - 460.5 nm

Fig. 2
Results

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

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

The Size range: 294 - 529 nm

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](image1.png) ![IBs-78](image2.png)

Fig. 4
Results

- **FT-IR**

Row and deconvoluted spectra of IBs-77 and control cells

Amide I band 1600-1700 cm⁻¹

Amide II band 1500-1550 cm⁻¹

control cells

IBs-77

Fig. 5
Results

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

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

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

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

Digested IBs collapse up to 60-65%

Fig. 13
Amplitude AFM images of BL21 (pET 28a-78) IBs before and after PK digestion.

Digested IBs collapse up to 70-83%
Conclusion
Conclusion

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