IITB-Monash Research Academy

Indian Institute of Technology Bombay | MONASH University

An Indian-Australian research partnership

Production of Recombinant Proteins in *Bacillus Subtilis*

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Research Academy theme/s

List only the research academy theme/s that is relevant to the project

1. **Biotechnology and stem cell research** ✓

Project aims

1. Feasibility studies of *Bacillus subtilis* as an expression host for the production of therapeutically important proteins through high cell density fermentations.
2. Studies on intein mediated purification of therapeutically important proteins expressed in *Bacillus subtilis*.
3. Case studies with a few proteins of research/commercial relevance including malaria proteins that are likely vaccine candidates.

Motivation

1. **Advantages of *Bacillus subtilis* over *E. coli* system**

   Proteins expressed in *E. coli* tend to form inclusion bodies; the yield after refolding of the protein is low. In the *Bacillus* system, proteins can be secreted directly into the growth media. This facilitates better recovery and cheaper downstream processing.

2. **Advantages of *Bacillus subtilis* over fungal expression systems**

   Process times for *subtilis* cultures are smaller than for a fungal system. Handling is relatively straightforward, given that it has been extensively studied for the production of industrial enzymes.

3. **Native and heterologous protein production in Bacillus subtilis.**

   Bacillus subtilis has the ability to produce enzymes in the order of grams per liter. Recent studies showed its ability to produce functionally active recombinant proteins like IL-3, staphylokinase etc., secreted

directly into the growth media. Therefore this is one of the most promising hosts for heterologous protein expression.

4. **Intein Mediated Protein Purification**

   A rapid and economical purification of recombinant proteins is a major challenge in biotechnology. A typical recovery process involves a number of chromatographic steps. Each step has to be separately standardized for recovery of a particular protein. This is time consuming and costly. For large scale production of proteins of industrial or therapeutic importance, downstream processing costs can account for up to 80% of total production costs. The affinity tag fusion technology is a frequently used alternative. A major problem with the use of such tags is in the subsequent use of proteases for tag removal. This necessitates additional chromatography steps. The use of inteins as a fusion technology simplifies protein recovery. Addition of DTT or change in pH induces self-cleavage of inteins and thereby separates the recombinant protein from the intein tag.

5. **Industrial importance of the project.**

   Ease of handling, higher yields of Bacillus subtilis and simplifying purification by using self splicing inteins would make a difference over the existing methods for heterologous protein production.

Background:

1. *Bacillus subtilis* as an Expression Host

There are substantial research efforts ongoing for the development of a novel heterologous gene expression system with superior growth and protein production characteristics. Production of heterologous proteins at high levels by bacteria is commonly achieved using *Escherichia coli* as the host. However, the *E. coli* expression system still has disadvantages. For example, it is a pathogenic bacterium and heterologous proteins must be endotoxins free. It secretes protein into the peri plasm and often into inclusion bodies. *Bacillus subtilis* is an alternative host for expression of heterologous secretory proteins. Advantages of *B. subtilis* as an expression host are:

1. Huge capacity for secreting proteins directly into the growth medium, which greatly facilitates their downstream processing.
2. Widely used for the production of industrial enzymes, so cultivation techniques are already standardized.
3. Genetically highly amenable host organism from which a large variety of genetic tools have been developed. Several staphylococcal plasmids (pUB110 and pE194) can function in *B. subtilis*, and these plasmids form the basis for the development of various cloning and expression vectors.
4. *B. subtilis* is non-pathogenic, and free of endotoxins, and has “generally recognized as safe” (GRAS) status.

Disadvantages:

1. Plasmid instability and
2. Low level of heterologous protein production, which limit its application potential.

2. INTEINS

An **intein** is a segment of a protein that is able to excise itself and rejoin the remaining portions (the exteins) with a peptide bond. The first intein was discovered in 1987. Since then, inteins have been found in all three domains of life (eukaryotes, bacteria, and archaea). There are more than approx. 200 inteins identified to date; their sizes range from 100-800 aa.

Intein-mediated protein splicing occurs after mRNA has been translated into a protein. This precursor protein contains three segments - an N-extein followed by the intein followed by a C-extein. Protein splicing involves the removal of an internal protein sequence from a precursor molecule and the ligation of the two flanking sequences to produce a mature protein product, in a post-translational event analogous to the removal of an intron from mRNA. The protein splicing mechanism is determined by the polypeptide sequence and does not require other accessory molecules.

Inteins have been used in the production of industrially important enzymes like lipase, human pituitary adenylate cyclase, Cre recombinase etc. They have also been used to produce therapeutically important proteins like GMCSF, HGH, HEGF etc. with single step recoveries being reported. In all these cases, the expression system is *E. coli*. Inteins have also been engineered for particular applications such as protein synthesis, and the selective labeling of protein segments (useful for NMR studies of large proteins).

Choice of Proteins to Be Expressed

Infectious diseases remain important causes of morbidity and mortality in the developing world. One of the most cost effective approaches to the control of infections is the development and deployment of protective vaccines. Vaccines may be formulated as attenuated living organisms or as various forms of subunit vaccines in which one or several pathogen molecules are presented in association with an adjuvant. For a number of parasitic diseases in particular, there is no attenuated vaccine available either because of technical or safety issues. For example, the blood stages of malaria caused by Plasmodium falciparum which is a major killer of children cannot be delivered as an attenuated vaccine because the parasite grows inside human red blood cells. There would be a danger that immunization with an attenuated parasite may raise an autoimmune response against the host red blood cell. Accordingly, this vaccine must be developed as a recombinant protein subunit vaccine. It is essential to keep the cost of the vaccine low and the yield of the protein high so that it may be widely deployed. This project will examine the use of Bacillus as a means of large scale production of malaria proteins.

Approach and Methodology:

1. Suitability of Intein mediated protein purification in *Bacillus subtilis*.
    - Selection of suitable intein and protein combination.
    - Vector construction and transformation
    - Protocol development for intein splicing
    - Protein Assays to confirm activity
    - Comparing the process with traditional (without intein mediated) purification method.
2. Achieving high productivity by means of high cell density fermentations via
    - Parameter optimization (ex: pH, DO, Temp etc.)
    - Media optimization
    - Feeding strategy (i.e. pH stat, DO stat, exponential.) optimization
3. Scale up studies
    - Studying process variations at 20 L scale
    - Optimization at 20 L scale.

Experimental Plan

Malaria proteins will be tested for their capacity to be expressed in Bacillus system

Proteins to be tested include current highly regarded candidate antigens for asexual stages:-

MSP1-$_{19}$

MSP4

MSP5

AMA-1

These will be stringent tests of the Bacillus system because they contain a number of cysteines that form conformational epitopes important for induction of protection.

Production of these proteins will assessed by:-

- Estimation of yield and integrity of product
- Physico-chemical parameters such as migration on reverse phase HPLC, Size exclusion and anion-exchange to determine heterogeneity of product
- Antigenicity and evidence of formation of conformational epitopes using a series of monoclonal and polyclonal antisera.
- Immunogenicity

Proteins from a number of different species of malaria could be tested. For example, if we produce the proteins from murine malaria, then we could test the capacity to protect mice from lethal challenge and this could be compared to what is elicited by the same protein produced in yeast and in *E. coli* in the case of MSP1-$_{19}$. Forms of the protein in *P. vivax* could also be produced and assessed.

Work To Be Done At IIT Bombay

1) Develop a peroxide inducible *B. subtilis* expression system
2) Test the feasibility of intein-mediated protein recovery in *B. subtilis*
3) Standardize and scale up *B. subtilis* cultivation in bioreactors.

Work To Be Done At Monash University

1) Construction of plasmids encoding various antigens using different codon biases
2) Assessment of antigenicity and immunogenicity of produced proteins
3) Challenge model in mice if we elect to produce some mouse malaria proteins