# Key stress factors and parameters for batch production optimisation of silk-elastin-like proteins in *E. coli*

Tony Collins, João Azevedo-Silva, André da Costa, Raul Machado, Margarida Casal

Centre of Molecular and Environmental Biology (CBMA), Department of Biology, University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal.

## Introduction

- Silk-elastin-like proteins (SELPs) are a family of biopolymers based on the highly repetitive amino acid sequence blocks of the naturally occurring fibrous proteins silk and elastin..
- In contrast to conventional synthetic polymers, the composition, sequence and length of these biopolymers can be strictly controlled, leading to monodispersed, precisely defined polymers which can be biosynthesised in an ecologically friendly manner, are biodegradable and biocompatible.
- SELPs combining the physicochemical and biological properties of the high tensile strength silk with highly resilient elastin allow for the fabrication of diverse materials with a high potential for use in the pharmaceutical, regenerative medicine and materials fields, yet their development for use is restrained by their typically low production levels.
- A series of novel SELPs composed of multiple blocks of the silkworm silk consensus sequence GAGAGS in various combinations with a mutated variant (VPAVG) of the natural mammalian elastin repetitive sequence block VPGVG have recently been prepared.
- The genes encoding the newly designed SELPs have been synthesised and the novel recombinant polymers expressed in *E. coli* by use of the pET25b-*E. coli* BL21(DE3) expression system.

## Aims

- To optimise the shake flask production levels of the novel SELP $(S_5E_9)_{10}$ by means of a comprehensive empirical approach examining all process variables (see 1 to 4 below).
- To obtain a better understanding of the factors limiting further improved recombinant protein production of shake flask cultivations with the expression system used (see 5 to 7 below).

## 1. Culture Medium

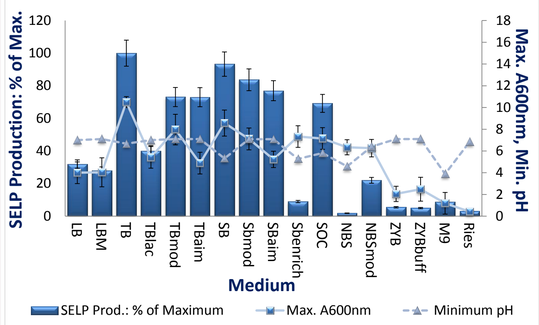


**Fig. 1: Media Optimisation**. SELP production, expressed as a percentage of the maximum, biomass production (OD600nm) and minimum pH measured, as a function of the culture medium used. SELP production levels evaluated by SDS-PAGE.

- Highest biomass and SELP production were observed with rich buffered media such as terrific broth (TB) and super broth (SB).
- Variation of the sugar supplement in TB, modification of its ingredient concentrations, or use of additives such as ammonium, various amino acids, magnesium or NaCl did not improve production levels further.

## 2. Temperature

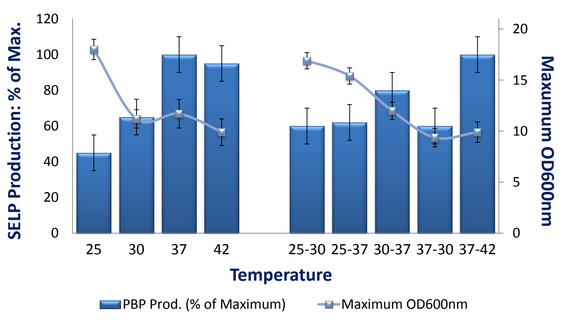


**Fig. 2: Effect of temperature on biomass and SELP production.** The right hand side of the curve (25-30, 25-37, 30-37, 37-30, 37-42) represent the temperature shift experiments: the initial temperatures used and the temperatures used after induction.

- 37 – 42ºC allowed for highest production levels
- Highest biomass production was observed at 25ºC but SELP production was low, possibly due to repression of the T7-based expression system at this temperature.
- Upshifts in temperature on induction did not lead to significantly higher production levels.

## 3. Available Oxygen

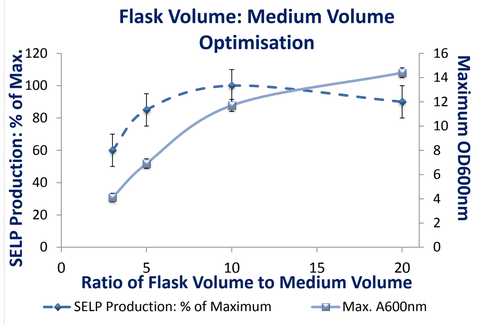


**Fig. 3 - Effect of varying the flask volume to culture medium volume ratio on biomass and SELP production.**

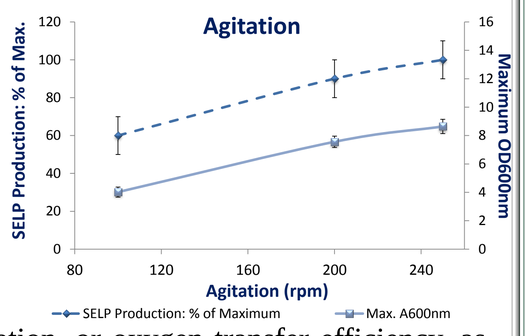


**Fig. 4: - Effect of varying the agitation rate on biomass and SELP production..**

- Increasing aeration, or oxygen transfer efficiency, as indicated by increasing flask vol. to liquid vol. ratio and increasing agitation, correlated with improved biomass and SELP levels up to a limit.
- At higher aeration rates toxic byproduct production (e.g. acetate) due to the higher growth rates observed probably interfere with production.

## 4. Induction

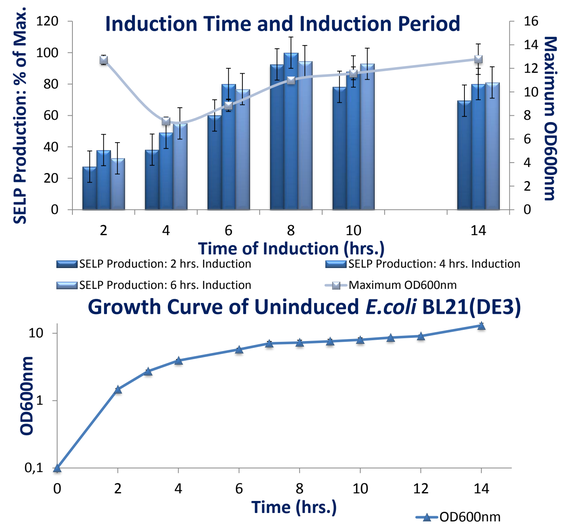


**Fig. 5 - Effect of induction time and induction period on biomass and SELP production.**
SELP and biomass production as a function of IPTG induction time (2-14 hrs. incubation) and induction period (2, 4 and 6 hrs.) (top). Growth curve of uninduced *E.coli* BL21(DE3) for comparison of the induction time with the stage of growth (bottom).

- In contrast to most studies which induce during the mid-log phase, we observed maximum production with induction at the end of the declining exponential phase.
- At least four hours induction is recommended for maximum production.

## 5. Acetate Production

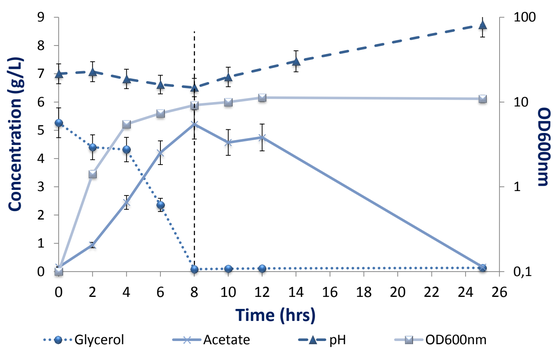


**Fig. 6: Monitoring of glycerol (HPLC), acetate (HPLC) and biomass levels (OD600nm) as well as pH as a function of incubation time using the optimised process conditions for SELP production.** The vertical line at 8 hours marks the point of induction with 0.5mM IPTG.

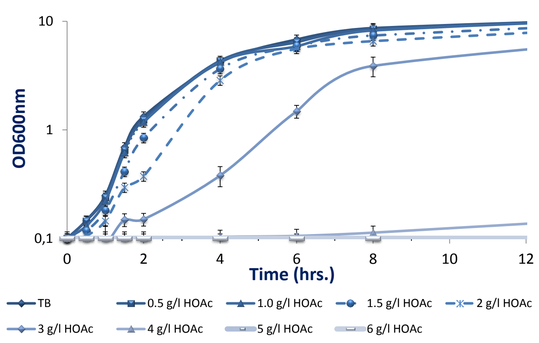


**Fig. 7 - Effect of acetic acid , added at 0 hours, on growth of *E. coli* BL21 DE3(+)/pET25b/SELP3 under the optimised shake flask conditions of the present study.**

- Glycerol levels rapidly decrease during the process with a concomitant accumulation of acetate to approximately 5 g/l and a decrease of pH.
- At low glycerol concentrations the cells switch to a utilisation of the accumulated acetate with an accompanying pH increase. This accumulated acetate provides the carbon source during recombinant protein production.
- As little as 1 g/L acetate has a bactericidal effect on the host under the conditions used.

## 6. Selection Agent Concentration

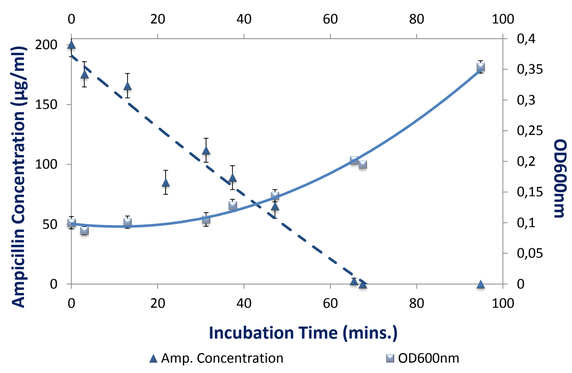


**Fig. 8: Ampicillin concentration (measured by the Kirby Bauer assay) and biomass levels as a function of incubation time**

- The ampicillin concentration rapidly decreases during the first hour of cultivation and is already depleted at a culture OD600nm of approximately 0.2.
- β-lactamase encoded on the pET expression vector used leads to the observed degradation of the selection agent.
- Productions without the use of a selection agent have allowed for similar SELP production levels to those with ampicillin.

## 7. Plasmid Stability

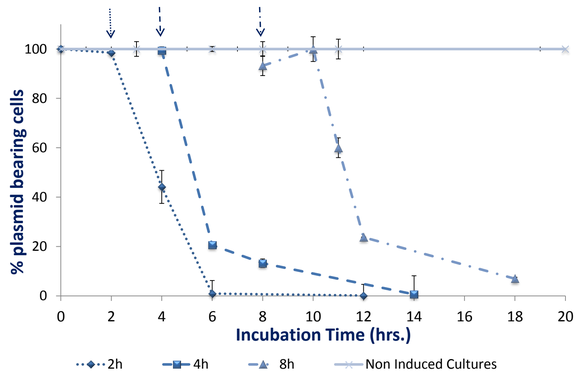


**Fig. 8: Plasmid stability, with and without induction, at various time points during the cultivation.**
Cultures induced at 2, 4 and 8 hours (as indicated by the arrow) as well as non-induced cultures are compared.

- Induction with IPTG leads to rapid loss of plasmid.
- Plasmid stability remains at maximum for the duration of cultivation in the absence of induction.
- Induction at the later stages of the growth curve leads to slower loss of plasmid.

## Conclusions

- **Maximum SELP production in shake flasks was obtained with terrific broth at 37ºC, pH7.0 with a liquid vol.:vessel vol. ratio of 1:10 and an agitation speed of 200 rpm.**
- **Maximum induction is obtained at the end of the declining exponential phase with an induction period of at least 4 hours. This allows for high cell density on induction and reduces the rate of plasmid loss after induction.**
- **Production levels of approximately 500 mg/L were obtained after purification.**
- **Rapid degradation of the selection agent used, plasmid instability on induction and high acetate byproduct formation are the principal factors preventing further improved production levels with the expression system used.**
- **We are presently investing the fed-batch approach for overcoming some of these restraints and to further increase production levels of the novel SELP.**

This work was financed by the European Commission via the 7th Framework Programme Project EcoPlast (FP7-NMP-2009-SME-3, collaborative project number 246176).