Session 3: Interactions between host physiology and process technology **Poster 2**

Human Prolactin (hPRL) and Growth Hormone (hGH) distinct behavior under bacteriophage lambda P_L promoter control

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Background

When producing recombinant protein in *E. coli* for therapeutic use, it is desirable not only to obtain substantial amounts of it, but also make sure that potential contaminants such as antibiotics or inducing agents (isopropyl-beta-D-thiogalactopyranoside, IPTG or nalidixic acid) will not taint the final product. To prevent this shortcoming we can use expression systems where the promoter is activated by temperature shift, which denatures the controlling repressor protein cIts, allowing promoter activity. While in our hands hGH was successfully expressed and secreted in *E. coli* periplasm with yields in general well above 1 μg/mL/A_{600}, after a temperature shift from 30°C to 42°C [1], attempts to express a related protein hormone (hPRL) with basis on the same protocol were not successful, providing 0.03 μg/mL/A_{600} at the most. Knowing that hPRL compared with hGH is a much more labile protein, we tried to obtain it from the same strain, but without the presence of the repressor protein and under optimized temperature conditions.

Results

Human growth hormone periplasmic secretion in a bacterial host that has also been transformed with the plasmid pRK-248cIts, which contains the thermosensitive transcription repressor (cIts) gene [2, 3] has been studied at different activation temperatures (Table 1). We can observe that the λP_L promoter is almost totally repressed up to 37°C, while at 42 °C its derepression permits a useful hGH periplasmic secretion that is acceptable according to previously established parameters [1]. In Table 1 we also can see the results obtained in a set of different experiments observing that without repressor still a quite high hGH secretion (1μg/mL/A_{600}) is obtained at 30 °C while an apparently higher secretion is obtained at 42°C. Under the same conditions, considering the described expression vector into which the hGH gene has been substituted by the hPRL gene, still with the presence of cIts, an approximately 130-180-fold lower secretion level for this hormone is observed at 42 °C. This led us to better study the behaviour of our hPRL-producing *E. coli* strains, either transformed or not with the plasmid pRK-248cIts. Considering not only that hPRL has been found a particularly labile protein but also that the bacterial periplasmic enviroment can even be more detrimental to protein stability, especially at high temperatures [4], it was decided to carry out a study on hPRL periplasmic secretion by "activating" at 30°, 35°, 37° and 42°C, with and without the presence of the repressor. The "activating" temperature of 37°C, and the bacterial strain lacking the cIts repressor, thus provided the highest hPRL secretion level, i.e. approximately 30-fold higher that those obtained with the equivalent strain containing the repressor gene. In Table 2 we can appreciate the statistical difference for prolactin yields obtained under different temperatures. As already observed for hGH (Table 1) the strain lacking the repressor gene is producing a significantly higher ($P < 0.001$) amount of hPRL, even at 42°C.

Since it has been reported that the lack of repressor could easily lead to plasmid loss [3], a study was carried out determining hPRL periplasmic yield in the strain lacking cIts after two growth periods corresponding to 10 and 50 *E. coli* generations, obtaining 0.64 ± 0.05 and 0.78 ± 0.03 μg hPRL/mL/A_{600} respectively. Also the presence or not of antibiotic (amp) did not influence the specific expression yield for at least 40 generations.

This same strain is being utilized for setting up a rapid and flexible feed batch fermentation in a laboratory bioreactor, obtaining up to now ~7 μg hPRL/mL with an optical density of 42.4 A_{600}.

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Table 1

temperature	phGH-DsbA-λP_L + pRK-248 clts ($\mu g/mL/A_{600} \pm SD$)	phGH-DsbA-λP_L ($\mu g/mL/A_{600} \pm SD$)
30 °C	0.01	1.0 ± 0.14 (n=2)
35 °C	0.02	-
37 °C	0,06	-
42 °C	1,31 ± 0.38 (n=4)	1.61 ± 0.11 (n=3)

hGH periplasmic secretion level activating at different temperature and utilizing hGH-secreting W3110 strains, with or without the repressor gene (clts).

Table 2

temperature	phPRL-DsbA-λP_L + pRK-248 clts ($\mu g/mL/A_{600} \pm SD$)	phPRL-DsbA-λP_L ($\mu g/mL/A_{600} \pm SD$)	Statistical significance [a]
30 °C	0.001 (n=1)	0.14 ± 0.02 (n=6)	-
35 °C	-	0.73 ± 0.07 (n=5)	P < 0.001
37 °C	0.03 (n=1)	0.92 ± 0.10 (n=6)	P < 0.01
39 °C	-	0.60 ± 0.12 (n=8)	P < 0.001
42 °C	0.02 (n=1)	0.19 ± 0.05 (n=4)	P < 0.001

hPRL periplasmic secretion level in *E. coli* W3110, at different temperatures utilizing a vector containing (phPRL-DsbA-clts -λP_L) and one not containing (phPRL-DsbA-λP_L) the repressor gene.

[a] Student´s T-test comparing each value to the previous one

Conclusion

A relatively high hPRL periplasmic secretion (up to 0.9 $\mu g/mL/A_{600}$), never reported before, has been obtained by constitutive expression of the unrepressed λP_L promoter, at 37 °C. The expression level is approximately 10-fold higher than that obtained in previous work [5] by using an IPTG-activated tac promoter. We can conclude that these data open the way to the utilization of *E. coli* instead of insect or mammalian expression systems for the production of an authentic and highly homogeneous hPRL.

Acknowledgements

Supported by FAPESP and CNPq.

References

[1]. CRJ Soares, FIC Gomide, EKU Ueda, P Bartolini: **Periplasmic expression of human growth hormone via plasmid vectors containing the λP_L promoter: use of HPLC for product quantifications.** *Protein Eng* 2003, **16**: 1131-1138

[2]. HU Bernard, DR Helinski: **Use of the phage promoter P_L to promote gene expression in hybrid plasmid cloning vehicles.** *Methods Enzymol* 1979, **68**: 482-492

[3]. R Crowl: **Expression of human interferon genes in E. coli with the lambda P_L promoter.** *Methods Enzymol* 1986, **119**: 376-383

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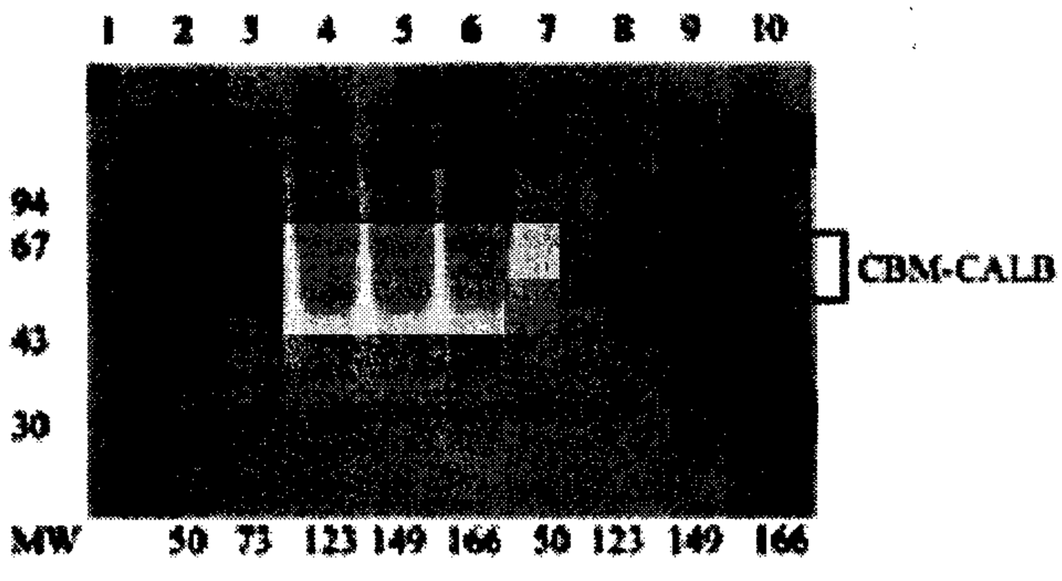
[5]. L Morganti, CRS Soares, R Affonso, PW Gout, P Bartolini: **Synthesis and characterization of recombinant authentic human prolactin secreted into the periplasmic space of *E. coli*.** *Biotechnology Appl* 1998, **27**: 63-70

Figure 1 (abstract P24)



SDS-PAGE analysis of samples withdrawn from the bioreactor in two *P. pastoris* mTLFB cultivation processes. 20 L of the culture supernatant was loaded in each lane. The two strong upper bands, in the lanes 2 to 6, represent protein of interest, which appears as two diffuse bands due to glycosylation. Lanes 7 to 10 correspond to samples from reference cultivation of the same *P. pastoris* strain, transformed with linear vector lacking DNA coding for the protein of interest (thus, the contaminating proteins).

technique, a modified cultivation technique was developed. The modification included the reduction of the medium salt concentration, which was then kept constant by regulating the medium conductivity at low value (about 8 mS cm^{-1}) by salt feeding. Before loading, the low conductivity culture broth was diluted only to reduce viscosity, caused by high cell density. The concept was applied to a one-step recovery and purification procedure for a fusion protein composed of a cellulose binding module (CBM) from *Neocallimastix patriciarum* cellulase 6A fused to lipase B from *Candida antarctica* (CALB).

Results: The modified cultivation technique resulted in lower cell death and consequently lower concentration of proteases and other contaminating proteins in the culture broth (see Figure 1). Flow cytometry analysis showed 1% dead (propidium stained) cells compared to 3.5% in the reference process. During the whole process of cultivation and recovery, no proteolysis was detected and in the end of the cultivation the product constituted 87% of the total supernatant protein. The lipase activity in the culture supernatant increased at an almost constant rate up to a value corresponding to 2.2 g L^{-1} of CBM-CALB. In the EBA process no cell-adsorbent interaction was detected but the cell density had to be reduced by a two-times dilution to keep a proper bed expansion. At flow velocity of 400 cm h^{-1}, the breakthrough capacity was 12.4 g L^{-1}, the product yield 98%, the concentration factor 3.6 times, the purity about 90%, and the productivity 2.1 g L^{-1} h^{-1}.

Conclusion: Our achievements in the modified cultivation stage that increased the quality of the feedstock for the separation stages were: higher target protein to total protein concentration ratio in the culture broth, low protease activities, and lower salt concentration and conductivity. The low salt and conductivity should also make the feedstock more suitable for loading on ion exchanger EBA media. These achievements made the expanded bed adsorption technique more efficient for initial recovery of CBM-CALB in particular but should also make the EBA technique more attractive for the recovery of other recombinant proteins from *P. pastoris* system.

Acknowledgements
This work is part of the BiMac Enzyme Factory programme and financed by the Södra Skogsägarnas Stiftelse for Forskning, Utveckling och Utbildning.

P25
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Microbial Cell Factories 2006, **5(Suppl 1)**:P25

Background: When producing recombinant protein in *E. coli* for therapeutic use, it is desirable not only to obtain substantial amounts of it, but also make sure that potential contaminants such as antibiotics or inducing agents (isopropyl-beta-D-thiogalactopyranoside, IPTG or nalidixic acid) will not taint the final product. To prevent this shortcoming we can use expression systems where the promoter is activated by temperature shift, which denatures the controlling repressor protein clts, allowing promoter activity. While in our hands hGH was successfully expressed and secreted in *E. coli* periplasm with yields in general well above 1 $\mu g/mL/A_{600}$, after a temperature shift from 30°C to 42°C [1], attempts to express a related protein hormone (hPRL) with basis on the same protocol were not successful, providing 0.03 $\mu g/mL/A_{600}$ at the most. Knowing that hPRL compared with hGH is a much more labile protein, we tried to obtain it from the same strain, but without the presence of the repressor protein and under optimized temperature conditions.

Results: Human growth hormone periplasmic secretion in a bacterial host that has also been transformed with the plasmid pRK-248clts, which contains the thermosensitive transcription repressor (clts) gene [2, 3] has been studied at different activation temperatures (Table 1). We can observe that the λP_L promoter is almost totally repressed up to 37°C, while at 42°C its derepression permits a useful hGH periplasmic secretion that is acceptable according to previously established parameters [1]. In Table 1 we also can see the results obtained in a set of different experiments observing that without repressor still a quite high hGH secretion (1 $\mu g/mL/A_{600}$) is obtained at 30°C while an apparently higher secretion is obtained at 42°C. Under the same conditions, considering the described expression vector into which the hGH gene has been substituted by the hPRL gene, still with the presence of clts, an approximately 130–180-fold lower secretion level for this hormone is observed at 42°C. This led us to better study the behaviour of our hPRL-producing *E. coli* strains, either transformed or not with the plasmid pRK-248clts. Considering not only that hPRL has been found a particularly labile protein but also that the bacterial periplasmic enviroment can even be more detrimental to protein stability, especially at high temperatures [4], it was decided to carry out a study on hPRL periplasmic secretion by "activating" at 30°, 35°, 37° and 42° C, with and without the presence of the repressor. The "activating" temperature of 37°C, and the bacterial strain lacking the clts repressor, thus provided the highest hPRL secretion level, i.e. approximately 30-fold higher that those obtained with the equivalent strain containing the repressor gene. In Table 2 we can appreciate the statistical difference for prolactin yields obtained under different temperatures. As already observed for hGH (Table 1) the strain lacking the repressor gene is producing a significantly higher ($P < 0.001$) amount of hPRL, even at 42°C. Since it has been reported that the lack of repressor could easily lead to plasmid loss [3], a study was carried out determining hPRL

Table 1 (abstract P25) hGH periplasmic secretion level activating at different temperature and utilizing hGH-secreting W3110 strains, with or without the repressor gene (clts).

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42°C	1,31 ± 0.38 (n = 4)	1.61 ± 0.11 (n = 3)

Table 2 (abstract P25) hPRL periplasmic secretion level in *E. coli* W3110, at different temperatures utilizing a vector containing (phPRL-DsbA-clts -P_L) and one not containing (phPRL-DsbA-P_L) the repressor gene.

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References

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P26

Two-compartment bioreactor as a scale-down model to study the effect of glucose overflow and anaerobiosis on large-scale recombinant protein production processes

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Background: In high-cell density fermentations the host cells are often subjects of transient changes in microenvironment around them. This is true especially in large-scale bioreactors. The changes can be for example substrate gradient, differences in oxygen availability and pH variations. Our aim is to obtain more information about physiological changes of *E.coli* W3110 and its recombinant variants in such conditions for better understanding of the bottlenecks in recombinant protein production processes.

To mimic the conditions in large-scale fermentations, we have set-up a two-compartment bioreactor [1], in which cells are circulated between a regular stirred tank reactor (STR) and a plug-flow reactor (PFR) using a peristaltic pump. The glucose is fed into the bottom of the plug-flow reactor with the aim of maintaining the glucose limitation in the STR part.

The advantage of the model is that the conditions of the zones where the changes occur can be measured. We take samples from 4 positions A, B, C and D (See Fig 1) of the PFR and additionally from 1 sample position of the STR. The normal process parameters such as pH, DOT and temperature are measured from the STR and additionally we have placed DOT and pH sensors in two positions of the PFR (S1 and S2).

Results: From the initial fermentation experiments in the STR-PFR reactor we have seen that the model is a good simulator for conditions in large-scale fermentors. When the STR part only was monitored, no signs from anaerobic conditions or pH variations were observed. However, in the PFR part glucose was measured and the highest value