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Poster Presentation

Limitations using GFP as a protein expression reporter in *Pichia pastoris*

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Background

The development of fluorimetric sensors during the last decade and the advantages of fluorimetry as a non invasive, highly specific and sensitive technique have favoured the utilization of this signal not only in biology but also in bioprocesses. In this context, the *Aequoria victoria* green fluorescent protein (GFP) has appeared as a popular reporter protein to study both prokaryotic and eukaryotic systems. It does not demand any cofactors to fluoresce, is a small molecule and requires no fixation techniques. Among different applications, GFP has been used as a reporter in gene delivery, as a tracer in subcellular trafficking and as a fusion partner to monitor protein location. For bioprocess development, GFP has been used as a protein fusion partner to monitor and optimise recombinant protein production [1]. However, production of soluble, secreted GFP or protein-GFP fusions in *P. pastoris* has proved to be a difficult task. Also, decrease of the target protein production levels when fused to GFP is case dependent.

In this work, GFP (S65T) has been fused to a *Rhizopus oryzae* lipase (ROL) produced in *P. pastoris* to study its applicability in process monitoring.

Results

In the present work, wild type X-33 *P. pastoris* strain was used for the extracellular expression of ROL fused to GFP (S65T), either at its N- or C- terminal end. The nitrogen source regulated formaldehyde dehydrogenase promoter, PFLD, was utilized to drive recombinant protein expression and, the *Saccharomyces cerevisiae* α-factor signal peptide was selected for secretion. Both constructions were tested for extracellular expression. Only when GFP was fused to the ROL N- terminal, extracellular lipolytic activity was detected. Therefore, this construction was selected for further expression studies.

A batch culture using sorbitol and methylamine as carbon and nitrogen sources, respectively, was carried out in order to test the expression levels and growth performance of the strain expressing the GFP-ROL fusion protein in bioreactor controlled conditions.

Notably, the culture producing the GFP-ROL fusion protein achieved a lower extracellular expression level compared to that obtained with the ROL expressing strain under the same culture conditions. Western Blot analysis of culture supernatants confirmed that the fusion protein was properly processed and secreted when using the α-factor signal peptide.

Confocal microscopy images of the cells showed two GFP fluorescence distribution patterns. In some of them, GFP was found in the periphery of the cells, while other cells showed GFP located in large compartments such as vacuoles. Similar results have been reported by Lenassi *et al.* when expressing GFP intracellularly. A possible explanation for this fact could be that a bottleneck might exist in the fusion protein folding and secretion pathway, resulting in protein intracellular accumulation.

Flow cytometry analyses confirmed this hypothesis. GFP intracellular fluorescence from batch samples was meas-

ured and normalized by cell size. Consistently with the former results, the normalized intracellular fluorescence increased with the culture time.

Although lipolytic activity was detected in culture supernatants with the GFP-ROL expressing strain, measured extracellular GFP fluorescence levels were essentially identical to those obtained with the control strain, i.e. expressing only the fungal lipase.

The GFP variant used in this work (S65T), has an excitation and emission spectra close to that of riboflavin, which is a biogenic fluorophore of *P. pastoris*. Consequently, when measuring fluorescence from culture supernatants riboflavin signal overlaps GFP emission signal and the fusion protein can not be optically detected by means of GFP fluorescence analysis.

Interestingly, no riboflavin intracellular fluorescence could be measured spectrophotometrically. Moreover, intracellular GFP could be detected both by spectrofluorometry and by flow cytometry.

To assess that the GFP fusion protein was fluorescent after secretion, riboflavin was removed from culture supernatant by ultrafiltration. Thereafter, GFP fluorescence could be detected at its emission maximum at 510 nm, as expected for this mutant.

Conclusion

In this study, GFP was used as a fusion partner to monitor protein expression and secretion, as well as subcellular localization. As GFP fusions are being increasingly used for this kind of study, the results from our work reveal that some considerations must be taken into account when using this strategy in *P. pastoris*:

Firstly, the expression levels of the GFP fusion protein should be compared to those with the strain expressing the not fused protein. This goal is especially critical when attempting to use GFP fusions for bioprocess development. In this sense, both the fusion construction and the secretion signal play an important role. In our work, results point out a possible bottleneck in the secretion process.

Secondly, care must be taken when selecting the optimal GFP mutant. In our experiments, no riboflavin fluorescence was observed intracellularly. Therefore GFP mutants with an excitation and emission spectra close to those for riboflavin might be useful to monitor intracellular events. Nevertheless, if GFP fusions are desired to monitor protein secretion or extracellular/total fluorescence for bioprocess monitoring and optimization, GFP mutants with spectral characteristics close to those of riboflavin should be avoided. Red shifted mutants or those with excitation wavelengths around 395 nm, such as GFPuv, might be a better option to avoid possible interferences [3]. Care should also be taken when using mutants with excitation wavelengths around 360 nm, where NADH and other media components are also excited [4].

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