CHEMICAL ENGINEERING TRANSACTIONS
VOL. 93, 2022
A publication of AIDIC
The Italian Association of Chemical Engineering
Online at www.cetjournal.it
Guest Editors: Marco Bravi, Alberto Brucato, Antonio Marzocchella

ISBN 978-88-95608-91-4; **ISSN** 2283-9216
DOI: 10.3303/CET2293029

Fed-batch Pre-industrial Production and Purification of a Consensus Tetratricopeptide Repeat (CTPR) Scaffold as Container for Fluorescent Proteins (FPs)

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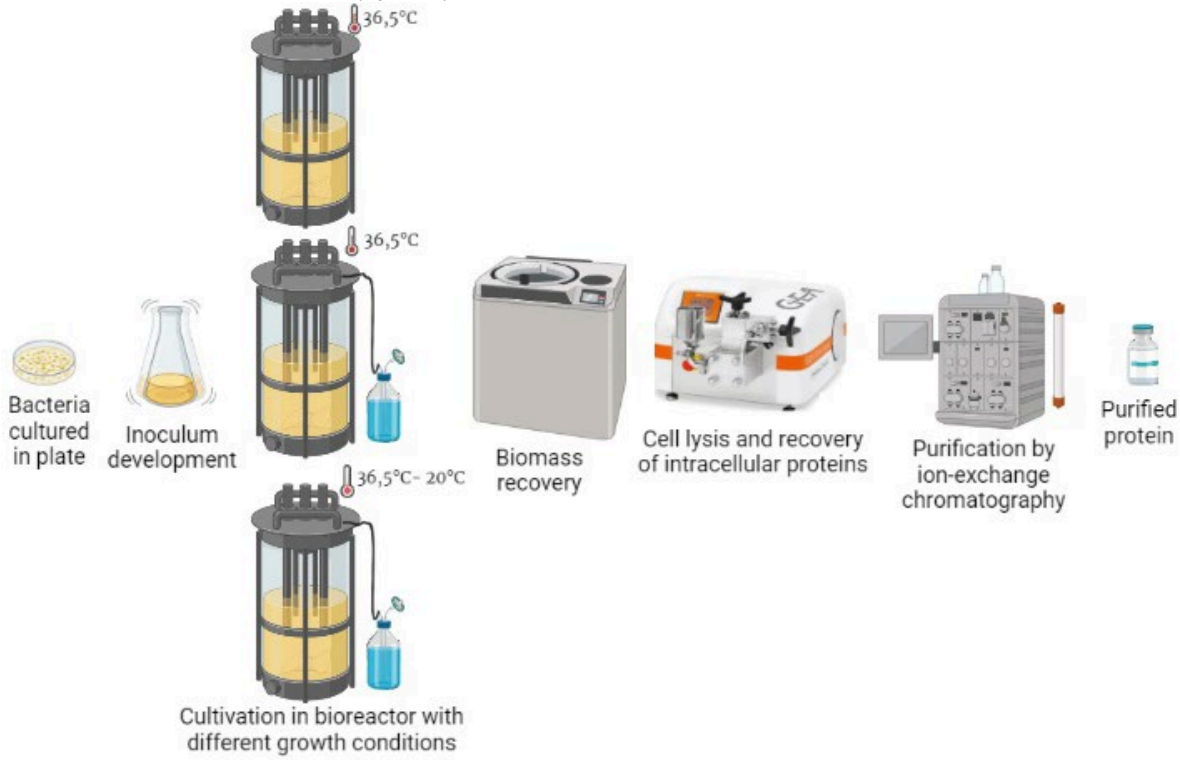
White light-emitting diodes (WLEDs) are big news in the field of lighting, however, current production processes are still very expensive or based on unsustainable inorganic metals such as inorganic phosphorus. The EU-funded ARTIBLED project aims to produce low-cost and high-efficiency Bio-hybrid light-emitting diodes (Bio-HLEDs). This can be achieved using artificially synthesized fluorescent proteins linked in biological scaffolds like the packaging to obtain LED for lighting applications containing a biogenic phosphor.
This study aims to optimize the protein scaffold CTPR10 production process to obtain a high number of scaffolds with a good purity level for Bio-HLEDs construction. Different fed-batch fermentation procedures were investigated and it was possible to produce more than two times of biomass and intracellular proteins compared to a conventional fed-batch strategy.
The improvement in production leads both to the reduction of costs related to the amount of IPTG used and the isolation of a consistent amount of CTPR10 through rapid and highly efficient purification techniques.
The realization of this project represents a significant milestone for Europe which will be at the forefront of innovation in the lighting sector.

1. Introduction

The WLEDs represent the future of lighting globally (Cho et al., 2017). This new technology will make it possible to reduce electricity consumption, reducing annual CO_2 emissions.
Although the use of WLEDs is advantageous both for the economy and the environment, attention must be paid to the filters applied to them, which are mainly composed of inorganic phosphorus to obtain a yellow light, cadmium and cesium to obtain other colours. However, the use of these rare metals is disadvantageous in economic and environmental sustainability terms; the research aims to find low-cost green alternatives.
In particular, the project ARTIBLED (Engineered ARTIficial proteins for Biological Light-Emitting Diodes), funded by the European community, aims to engineer fluorescent proteins in combination with biological scaffolds to obtain LED for lighting applications containing biogenic phosphorus.
The strategy employed by the ARTIBLED researchers is to design and synthesize stable protein scaffolds to which functionalized organic chromophores are linked, then select artificial fluorescent proteins with greater stability and emissivity to insert them into the coating of the filters for the colour conversion of Bio-HLEDs (Costa, 2021).Consensus Tetratrico Peptide Repeat (CTPR) proteins are small proteins made up of repeats (TPR units) of 34 amino acid residues that form a helix-turn-helix structural pattern (Blatch, Lässle, 1999).

Paper Received: 22 December 2021; Revised: 9 March 2022; Accepted: 15 May 2022
Please cite this article as: Cinà P., Salamone M., Rigogliuso S., Paternostro R., Liutkus M., Cortajarena A.L., Ghersi G., 2022, Fed-batch Pre-industrial Production and Purification of a Consensus Tetratricopeptide Repeat (CTPR) Scaffold as Container for Fluorescent Proteins (FPs), Chemical Engineering Transactions, 93, 169-174 DOI:10.3303/CET2293029

The main function of TPR motifs is to mediate protein-protein interactions (Kajander et al., 2007); the number of repeats is variable. Still, the smallest functional unit observed has 2 repeats (CTPR2), and 20 (CTPR20) is the largest. The binding domains are present in the concave face of the repeat and bind the target peptide with high affinity (D'Andrea, Regan, 2003).
CTPR proteins can form nanofibers and thin films that are mechanically robust and exhibit anisotropic properties tuned by the other molecules constituting the biomaterial scaffold. These characteristics make the CTPR10 protein (10 repeats per unit) ideal as a component of the protein scaffold of Bio-HLED filters (Sanchez-deAlcazar et al., 2019).
This study aims to optimize the protein scaffold CTPR10 production process through optimized fermentation procedures and use ion-exchange chromatography to obtain a high number of scaffolds with a good purity level for Bio-HLEDs construction (figure 1).



2. Methods

2.1 Bacterial strain and plasmid

The strain BL21-AI One Shot Chemically Component *Escherichia coli* Invitrogen (Genotype: F- ompT hsdSB (rB-mB-) gall dcm araB::T7RNAP tetA) was transformed by electroporation with a pET plasmid containing sequence encoding for CTPR10 protein. The strain holds a chromosomal insertion of a cassette containing the T7 RNA polymerase (T7 RNAP) gene in the araB locus, allowing T7 RNAP to be regulated by the araBAD promoter (Guzman et al., 1995).
The pET plasmid vector holds a lac operator (LacO) sequence just downstream of the T7 promoter that can be acted upon by the lac repressor (LacI) protein to block transcription of the T7 promoter. The addition of Isopropyl-β-D-thiogalactoside (IPTG) blocks the inhibitory action of LacI by removing the inhibition of the transcription of the target sequence (Sivashanmugam et al., 2009). The vector also contains a kanamycin resistance cassette to select of the transformed strain.

2.2 Cultivation medium and inoculum development

The culture medium chosen was terrific broth (tryptone 12g/L, yeast extract 24g/L, glycerol 5ml/L, KH_2PO_4 2.3g/L, K_2HPO_4 16.4g/L) supplemented with kanamycin [50µg/ml].
In fed-batch configuration, 300ml of ultrapure glycerol and 900ml of a solution containing 130g of tryptone and 130g of yeast extract was used as feeding.

The inoculum was developed as follows: a loopful of glycerol stock was streaked on an LB plate containing kanamycin [50µg/ml] and incubated at 37 °C overnight. A single isolated colony was transferred into 20ml of LB medium, incubated on a rotary shaker at 31°C and 180 rpm for 6 h. This pre-inoculum was transferred into the main inoculum (250ml LB with kanamycin [50µg/ml]) and incubated for 15 h at 37 °C, stirring at 220 rpm.

2.3 Bioreactor cultivation

Batch and fed-batch culture experiments were carried out in a 10 L Bioflo celligen benchtop bioreactor (New Brunswick Scientific Co., USA) at pH of 7.12 and 36.5 °C. Sodium hydroxide and citric acid were used to control pH. Dissolved oxygen (DO) was monitored using a polarographic steam oxygen electrode (Mettler–Toledo International Inc. Switzerland) and reported as a percentage of air saturation. The DO maintained over 20% saturation by varying airflow and impeller speed.

2.4 Batch cultivation

Batch cultivation was performed at 36.5°C in 3L of TB by adding 20% (v/v) of pre-inoculum. Induction with arabinose (0.2% w/v) and IPTG [2mM fc] was performed at the beginning of the exponential phase, approximately 3h after inoculation, at an OD_{600} of 3.3. The induction was maintained from a minimum of 3h up to a time necessary to bring the culture to the start of the stationary phase.

2.5 Fed-batch cultivation

Two different fed-batch fermentation strategies were used: the first was performed canonically at 36.5 °C, the second was carried out at 36.5 °C until before the insertion of the inductors. After which, the temperature was brought to 20 °C. A lower growth temperature is reflected in slower growth kinetics; for this reason, other parameters such as the IPTG concentration and the induction time were changed in the experiment conducted from 36.5° C to 20°C. Each fermentation was interrupted at the beginning of the stationary phase in order not to lower the yield of CTPR10
The following table summarizes the different conditions between the two strategies used:

Table 1: Summary of the Different Conditions for the Two Fed-Batch Experiments

	IPTG final concentration	feeding solutions ratio	Time of induction
Strategy 36.5°C	2mM	Glycerol: 150ml/h Fed: 450ml/h	3h
Strategy 36.5°C-20°C	0,5mM	Glycerol: 23ml/h Fed: 70ml/h	19h

For both strategies, 5L of TB culture medium and 0,2%w/v of arabinose were used and the same pH and DO conditions were maintained (7.12 and over 20% respectively).

2.7 Analytical methods

Bacterial cell concentration was evaluated offline by optical density at a 600 nm spectrophotometer (BMG specktrostar). The fresh cell weight was obtained by centrifugation (8000xg) and elimination of the spent medium.
The biomass was lysed by the French press (Niro Soavi Panda). The intracellular protein concentration was measured using Bradford assay (Bradford, 1976). The protein samples obtained from each fermentation experiment were loaded onto an SDS PAGE 7.5 % gel (Sambrook et al.,1989) to assess the level of CTPR10 induction obtained. Densitometric analysis using the ImageJ software was carried out (Alonso Villela., 2020) to reveal the percentage of induction of CTPR10 on the total proteins produced.

2.8 CTPR10 purification

Intracellular protein was dialysed against Tris-HCl 20 mM pH 7.5, 0,025 mM NaCl, for 24 h at 4°C with repeated changes in the same buffer (after 8 and 16 h). After dialyses, proteins were fractionated using Anionic Exchange Chromatography (Q sepharose High Performance, GE Healthcare Life Sciences, Uppsala, Sweden 5 ml column). The fractionation was performed using the AKTA Start chromatography system (GE Healthcare Life Science, Uppsala, Sweden) as follows: 40 mg of total protein was loaded in a 5 ml column. After washing with Tris-HCl 25 mM, 0,025 NaCl pH 7.4, proteins were eluted in 60 fractions of 1ml for each with NaCl gradient (0,2–1 M). Then, 10 µl of the fractions of the major peak containing CTPR10 (from 43 to 47 fractions) were observed in SDS PAGE.

3. Results

3.1 Comparison of growth and production performances

To optimize the cost-production ratio of CTPR10, three different fermentation strategies were investigated: the first in batch configuration and the other two in fed-batch (Figure 2).
The growth curves relating to the three different strategies used are shown below:

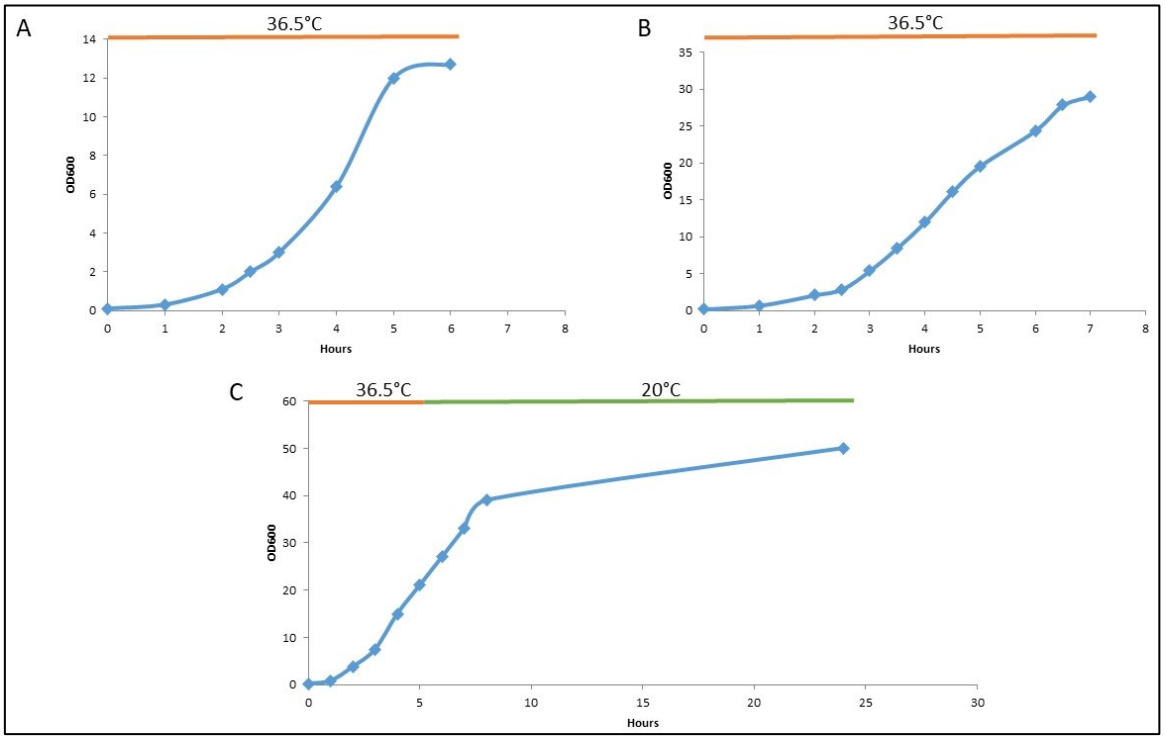


Figure 2: Comparison of the three different growth curves: batch 36.5°C (A); fed-batch 36.5°C (B); fed-batch 36.5°C- 20°C (C); each fermentation was interrupted at the beginning of the stationary phase in order not to lower the yield of CTPR10

The results obtained reveal how the batch strategy has a dramatically lower yield, in biomass production, compared to the fed-batch method. Although this limiting result is already well known, batch fermentation made it possible to set fundamental parameters, such as pH and DO, that allowed the strategy to switch to fed-batch fermentation, limiting costs.
Surprisingly, the two fed-batch strategies also showed markedly different results. In fact, by lowering the temperature to 20 °C and extending the induction time from 3 to 19 hours, it was possible to produce more than double the biomass and total intracellular proteins, compared to a fed-batch fermentation conducted conventionally at a constant temperature. This was possible for two reasons: IPTG has a cytotoxic effect and by decreasing the concentration it impacts less negatively on cell growth; the lower administration of inducer is totally compensated by a much longer induction period which is made possible by the lower temperature which determines a slower growth kinetics.
The following table summarizes the performances obtained with the three strategies used:

Table 2: Performance Summary

	Batch 36.5°C	Fed-batch 36.5°C	Fed-batch 36.5-20°C
Fresh weight	54g	230g	484g
Intracellular protein	4g	17.8g	45g

Another parameter that has been evaluated is the percentage of induction of CTPR10 on the total of intracellular proteins produced. The results obtained from the densitometric analysis conducted on polyacrylamide gel show that this value mainly remained constant in the three experiments carried out:; about 20% of the total (Figure 3).

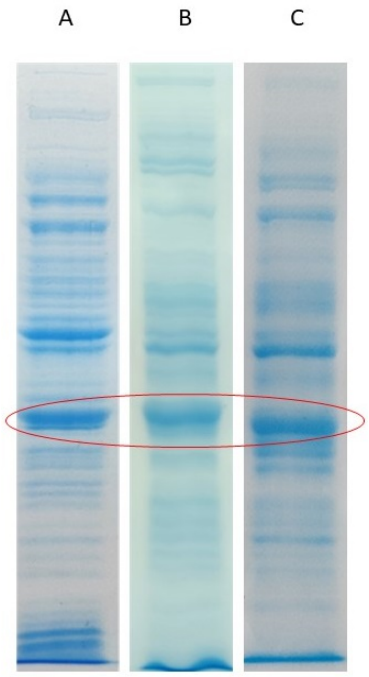


Figure 3: SDS-page of total intracellular proteins: batch 36.5°C (A); fed-batch 36.5°C (B); fed-batch 36.5°C-20°C (C); CTPR10 circled in red (35KDa).

Despite the four-fold reduction in the amount of IPTG administered in the 36.5°C-20°C fed-batch configuration, the induction percentage remains the same due to a longer induction period. Considering the lower amount of IPTG used and the increase in biomass obtained, a reduction in production costs of approximately 52% was calculated.

3.2 CTPR10 purification

To optimize the purification process, CTPR10 was separated from the total intracellular proteins using anionic Exchange Chromatography (Q-Sepharose). The CTPR10 was eluted in the last peak at the end of the NaCl gradient (0.8-0.9 M NaCl), as described in the experimental section. As shown in Figure 4, the fraction containing the CTPR10 were loaded to SDS PAGE.

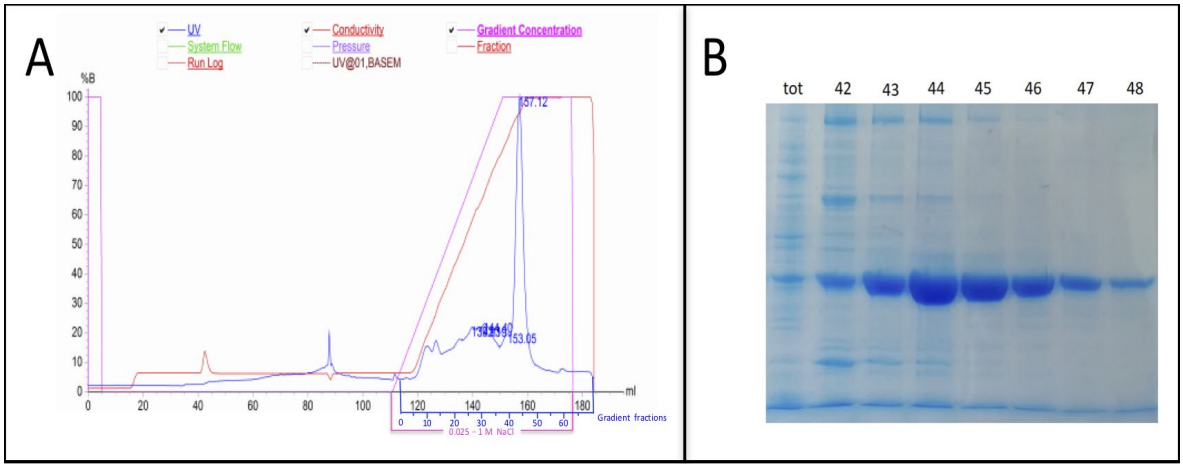


Figure 4: A) Q-Sepharose CTPR10 partial purification in a NaCl gradient 0.025 – 1.0 M. B) SDS-page of total intracellular proteins (tot) and fraction corresponding to the peak containing the CTPR10 (fractions 42-48)

The analysis of the peak (fractions 42-48) showed an enrichment of CTPR10 of 11.5 times compared to the unfractionated extract and its representation for about 21.76% of the total proteins.

4. Conclusions

In this work, different strategies of CTPR10 production were investigated. The fed-batch approach with temperature reduction from 36.5°C to 20°C after the administration of inducing agents showed a higher yield, in terms of biomass and intracellular proteins, more than two times compared to a conventional fed-batch strategy. The improvement in production was achieved by reducing costs (approximately 52%) due to a fourfold reduction in the amount of IPTG used and about double biomass and CTPR10 expression. Furthermore, due to the ion exchange chromatography method, it was possible to isolate CTPR10 from the total proteins produced. Further purification procedures are in place to ensure greater enrichment of the proteins of interest.
These findings could be functional to pre-industrial protocols development for this and other proteins; moreover, the synthesis condiction indicates the possibility to improve the process in direction of the best biomass production, such as in IPTG molecules induction/expression.

Acknowledgements

The project leading to these results has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 863170.

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