UNIVERSITÀ DEGLI STUDI
DELL'INSUBRIA

"Production and Engineering of Recombinant Proteins"

Coordinator: Prof. Elena Rosini

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Keywords: Recombinant proteins, protein engineering, E. coli, purification, biochemical characterization

Purpose: The facility focuses on the selection, development, and production of proteins in heterologous systems (recombinant proteins). Examples of applications include the production of: i) novel antigens; ii) biotechnological drugs for the immunotherapy of infectious diseases and cancer; iii) enzymes for industrial applications.

Location: Department of Biotechnology and Life Sciences, Via Dunant 3, Varese, Italy

Organization: The facility integrates a network of research groups with multidisciplinary expertise and advanced technological resources. This organization represents a reference point for research and the development of tailored biotechnological solutions in the field of recombinant protein production.

The facility is organized into three sub-units:

- production of proteins in eukaryotic CHO cells;
- production of proteins in microbial systems (bacteria and yeasts);
- design and implementation of protein engineering studies.

Connection with the CRIETT Technological Platforms and the University Scientific Platforms:

This facility operates in collaboration with:

- the Sustainability Platform, within the objectives of: 1) resource recovery and sustainability of industrial processes; 2) green chemistry; 3) valorization of biomass and production of natural substances; 4) sustainability and protection of natural resources.

- the Technologies for Energy, Health and Environment Platform, within the objective of developing technologies, methods, and approaches for biomedical investigation.

Sub-unit "Production of Recombinant Proteins in CHO Cells"

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Keywords: mammalian cells, bioreactors, heterologous expression, CHO, protein purification

This facility is dedicated to the production of recombinant proteins in Chinese hamster ovary (CHO) cells. The integration of advanced technologies and strict control of operating conditions allows the optimization of every production step, ensuring high efficiency, reproducibility, and quality standards suitable for the production of proteins for preclinical studies, biotechnological applications, and pharmaceutical purposes. The laboratory is equipped with a CO_2 incubator and a bioreactor (Cytiva, WAVE 25), designed for large-scale CHO cell growth, with precise and automated control of key parameters such as pH, temperature, oxygenation, and nutrient concentration. Additional equipment includes a biological safety cabinet for sterile manipulation, a centrifuge, a thermostatic water bath, a microscope, and an automated cell counter. The facility also includes an ultrafiltration system (Millipore) for protein concentration and purification, together with the instrumentation required for the biochemical characterization of the produced proteins.

Sub-unit "Production of Recombinant Proteins in Microbial Cells"

Heads: Dott.ssa Francesca Berini, Prof. Luciano Piubelli, Prof. ssa Elena Rosini, Prof.ssa Silvia Sacchi

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Keywords: recombinant proteins, *E. coli*, *S. cerevisiae*, *P. pastoris*, fermentation

The recombinant protein production facility in microbial cells supports the production of recombinant proteins in bacteria (Gram-positive and Gram-negative) and yeasts, using the host system best suited to the target protein. The flexibility of the platform lies in the possibility of scaling operational and production conditions from flask cultures to fermenters, thus facilitating transfer to industrial production. The facility is equipped with bioreactors featuring continuous monitoring and regulation of pH, temperature, liquid level, and oxygenation: three 3 L biofermenters (P100 Applikon) and one 7 L Livit Flex bioreactor (Getinge). Supporting equipment includes autoclaves, sterile laminar flow hoods, stereomicroscopes and optical microscopes, a plate incubator, temperature-controlled shakers, and a temperature-controlled room equipped with shakers. The sub-unit interfaces with the other facilities for purification and biochemical characterization of the produced proteins.

Sub-unit "Protein Engineering and Protein Purification"

Heads: Prof. Loredano Pollegioni, Prof. Gianluca Molla, Prof.ssa Elena Rosini,

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Keywords: protein engineering, rational design, directed evolution, high-throughput screening, biochemical characterization, molecular docking

Protein engineering, rational design, directed evolution, high-throughput screening, biochemical characterization, molecular docking. Protein engineering techniques based on rational design and directed evolution approaches are routinely employed for protein evolution. These techniques make it possible to improve enzyme activity, affinity for specific (including non-natural) substrates, stability, oligomerization state, and other functional properties. The facility employs computational approaches, including molecular modelling, molecular dynamics, and molecular docking, to optimize research timelines and improve the operational performance of engineered proteins. In addition, the facility integrates an automated high-throughput screening system (Opentrons), enabling large-scale enzymatic assays. The sub-unit interfaces with the "Production of Recombinant Proteins in Microbial Cells" facility for the production of engineered proteins and is equipped with the instrumentation required for the biochemical characterization of the produced proteins.

Publications:

Recombinant Protein Production in CHO Cells:

Rosini E, Pollegioni L. Optimized rapid production of recombinant secreted proteins in CHO cells grown in suspension: The case of RBD. *Biotechnol. Appl. Biochem.* 2022 Oct 3. doi: 10.1002/bab.2409.

Rosini E, Volpi NA, Ziffels B, Grimaldi A, Sacchi S, Neri D, Pollegioni L. An antibody-based enzymatic therapy for cancer treatment: The selective localization of D-amino acid oxidase to EDA fibronectin. *Nanomedicine.* 2021 Aug;36:102424. doi: 10.1016/j.nano.2021.102424. Epub 2021 Jun 24.

Rosini E, De Vito A, Ponti F, Scaldaferri D, Pollegioni L, Acquati F. Expression and purification of the human tumor suppressor protein RNASET2 in CHO-S cells. *Protein Expr Purif.* 2020, 174: 105675. doi: 10.1016/j.pep.2020.105675.

Recombinant Protein Production in Bacterial Cells:

Bernardini R, Tengattini S, Li Z, Piubelli L, Bavaro T, Modolea AB, Mattei M, Conti P, Marini S, Zhang Y, Pollegioni L, Temporini C, Terreni M. Effect of glycosylation on the affinity of the MTB protein Ag85B for specific antibodies: towards the design of a dual-acting vaccine against tuberculosis. *Biol Direct.* 2024,19(1):11. doi: 10.1186/s13062-024-00454-5.

Riva D, Orlando M, Rabattoni V, Pollegioni L. On the quaternary structure of human D-3-phosphoglycerate dehydrogenase. *Protein Sci.* 2024, 33(8):e5089. doi: 10.1002/pro.5089.

Bisaccia M, Binda E, Rosini E, Caruso G, Dell'Acqua O, Azzaro M, Laganà P, Tedeschi G, Maffioli EM, Pollegioni L, Marinelli F. A novel promising laccase from the psychrotolerant and halotolerant Antarctic marine *Halomonas* sp. M68 strain. *Front Microbiol.* 2023 Feb 10;14:1078382. doi: 10.3389/fmicb.2023.1078382.

Murtas G, Zerbini E, Rabattoni V, Motta Z, Caldinelli L, Orlando M, Marchesani F, Campanini B, Sacchi S, Pollegioni L. Biochemical and cellular studies of three human 3-phosphoglycerate dehydrogenase variants responsible for pathological reduced L-serine levels. *Biofactors.* 2023 Aug 31. doi: 10.1002/biof.2002.

Murtas G, Marcone GL, Peracchi A, Zangelmi E, Pollegioni, L. Biochemical and biophysical characterization of recombinant human 3-phosphoglycerate dehydrogenase. *Int. J. Mol. Sci.* 2021, 22, 4231. https://doi.org/10.3390/ijms22084231.

La Vitola P, Balducci C, Baroni M, Artioli L, Santamaria G, Castiglioni M, Cerovic M, Colombo L, Caldinelli L, Pollegioni L, Forloni G. Peripheral inflammation exacerbates α-synuclein toxicity and neuropathology in Parkinson's models. *Neuropathol Appl Neurobiol.* 2020. Feb;47(1):43-60. doi: 10.1111/nan.12644.

Vignali E, Gigli M, Cailotto S, Pollegioni L, Rosini E, Crestini C. The laccase-Lig multienzymatic multistep system in lignin valorization. *Chem. Sus. Chem.* 2022 Aug 2:e202201147. doi: 10.1002/cssc.202201147.

Engineered Protein Production:

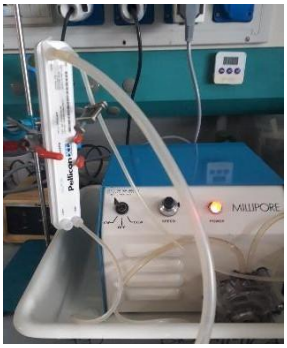
Pollegioni L, Campanini B, Good JM, Motta Z, Murtas G, Buoli Comani V, Pavlidou DC, Mercier N, Mittaz-Crettol L, Sacchi S, Marchesani F. L-serine deficiency: on the properties of the Asn133Ser variant of human phosphoserine phosphatase. *Sci Rep.* 2024, doi: 10.1038/s41598-024-63164-y.

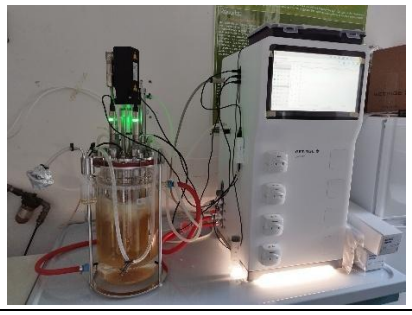
Pirillo V, Orlando M, Battaglia C, Pollegioni L, Molla G. Efficient polyethylene terephthalate degradation at moderate temperature: a protein engineering study of LC-cutinase highlights the key role of residue 243. *FEBS J.* 2023 Jan 24. doi: 10.1111/febs.16736.

Pirillo V, Orlando M, Tessaro D, Pollegioni L, Molla G. An efficient protein evolution workflow for the improvement of bacterial PET hydrolyzing enzymes. *Int. J. Mol. Sci.* 2022, 23(1), 264. doi: 10.3390/ijms23010264.

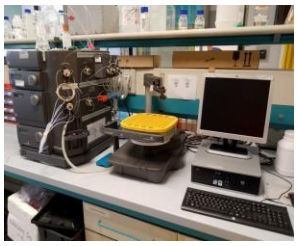
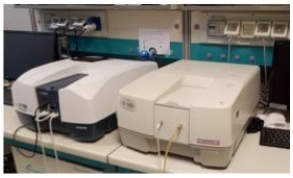
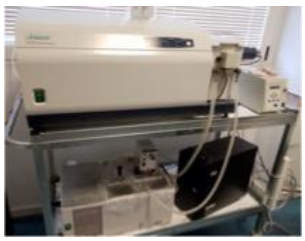
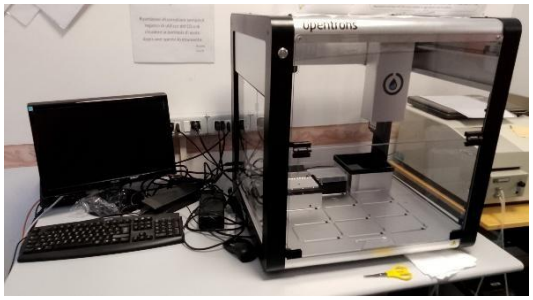
Melis R, Rosini E, Pirillo V, Pollegioni L, Molla G. *In vitro* evolution of an L-amino acid deaminase active on L-1-naphthylalanine. *Catal. Sci. Technol.* 2018, 8: 5359-5367. doi: 10.1039/c8cy01380b.

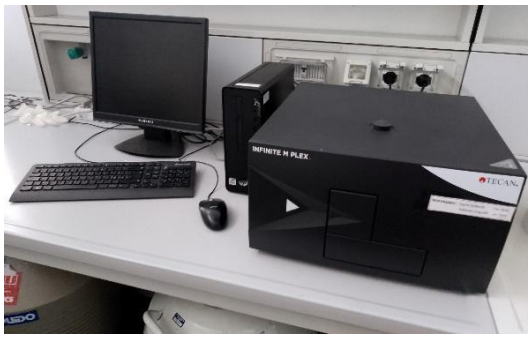
Rosini E, Pollegioni L. PEG-DAAO conjugate: a promising tool for cancer therapy optimized by protein engineering. *Nanomedicine.* 2020, 24:102122. doi: 10.1016/j.nano.2019.102122

• A) Facility for Recombinant Protein Production in CHO Cells	
	**The bioreactor (Cytiva, ReadyToProcess WAVE25)** provides a controlled environment for the efficient production of recombinant proteins by precisely regulating pH, oxygenation, temperature, and nutrient supply. Automation and real-time monitoring ensure high reproducibility and optimal yield.
	**Ultrafiltration systems (Millipore)** are used for the concentration and filtration of culture broths and for protein purification, enabling the removal of impurities, salts, and other small molecules. Thanks to selectively permeable membranes, these systems also allow buffer exchange while preserving protein integrity and biological activity.

• B) Facility for Recombinant Protein Production in Microbial Cells	
	**The 3 L biofermenters (P100 Applikon)**, with a maximum working volume of 2 L for each fermenter, are ideal for cell culture and small-scale biomolecule production. Equipped with electrodes for continuous monitoring of crucial parameters such as pH, temperature, foam level, and dissolved oxygen concentration (dO_2), these fermenters provide precise, real-time control of culture conditions, enabling the optimization of cell growth and protein expression in controlled environments.
	**The 7 L Livit Flex bioreactor (Getinge)**, with a maximum working volume of 5.5 L, is designed to provide advanced and precise control of fermentation processes. Thanks to the integrated SCADA software, it enables real-time monitoring and management of key parameters (pH, temperature, dissolved oxygen, nutrients), ensuring optimal growth conditions. This bioreactor is ideal for research, development, and small-scale production applications, guaranteeing high reproducibility and accurate process control.

• C) Purification and Characterization of Recombinant Proteins

 	**The ÄKTA Purifier** and **ÄKTA Explorer FPLC** systems provide high efficiency and reproducibility in recombinant protein purification. Thanks to the precise control of critical parameters such as pH, salinity, and elution gradients, together with advanced automation, they ensure high sample purity and yield. Their modularity makes them suitable for different chromatographic purification strategies, including ion exchange, affinity, and size-exclusion chromatography.
 	**U V/Vis spectrophotometers (Jasco)** enable the quantitative analysis of biomolecules through absorbance measurements at specific wavelengths, ensuring accurate determination of sample concentration and purity.
	**The spectropolarimeter (Jasco)** enables the analysis of protein secondary structure by circular dichroism, providing essential information on protein conformation and stability. Thanks to its high sensitivity and precision, it is ideal for evaluating structural changes caused by amino acid substitutions, ligand interactions, temperature or pH variations, and denaturation processes.
	**The spectrofluorometer (Jasco)** enables the analysis of protein conformation, stability, and interactions with small molecules through fluorescence emission measurements. It is used to study structural variations, ligand binding, and the effects of pH and temperature changes. In addition, it is widely employed in fluorescence assays for enzymatic kinetics, affinity studies, and conformational dynamics analyses.
	**HPLC and GPC systems (Jasco)** allow the separation and analysis of proteins, peptides, and metabolites. HPLC provides high resolution and sensitivity for determining sample purity and composition, whereas GPC enables separation according to molecular size, allowing the characterization of molecular weight and protein aggregation state. These techniques are fundamental for stability studies, quality control, and biomolecular interaction analyses.
	**The automated liquid-handling and plate reader system (Opentrons OT-2)** combines automated liquid handling with multiwell plate reading, enabling high-throughput screening for enzymatic and inhibition assays. Thanks to its high precision in reagent dispensing and its ability to simultaneously analyze multiple samples, it optimizes workflow efficiency, reduces the risk of errors, and improves the reproducibility of results.

	**The 96-well plate absorbance, fluorescence, and luminescence reader (Tecan Infinite 200PRO)** is ideal for the quantification of metabolites and reaction products in biochemical and biological assays. Thanks to its capability to measure absorbance, fluorescence, and luminescence, it supports a wide range of applications, including enzymatic assays and binding studies. Compatibility with 96-well plates allows the simultaneous analysis of numerous samples, making it ideal for high-throughput screening and large-scale studies.

List of available E. coli expression strains

STRAIN	*DESCRIPTION*	*RESISTANCE*	*APPLICATION*
BL21(DE3)	Standard strain for protein expression using T7 RNA polymerase	Ø	General protein expression
BL21(DE3) LOBSTR	Engineered to reduce background protein expression through the deletion of the Lon and OmpT proteases	Ø	Expression of proteins prone to degradation
BL21(DE3)pLysS	Contains a plasmid carrying the gene encoding T7 lysozyme, which inhibits the basal activity of T7 RNA polymerase	Chloramphenicol	Expression of toxic proteins
BL21(DE3)RIL	Contains a plasmid encoding specific tRNAs for rare codons (Arg, Ile, Leu)	Chloramphenicol	Expression of eukaryotic proteins containing rare codons
BL21(DE3)Star	Mutated RNase E for increased mRNA stability	Ø	Increased mRNA stability and protein expression
BL21CodonPlus(DE3)RIL	Engineered to contain additional copies of tRNAs for rare codons (Arg, Ile, Leu)	Chloramphenicol	Expression of eukaryotic proteins containing rare codons
BL21CodonPlusRIL	Similar to BL21CodonPlus(DE3)RIL, but lacking the DE3 lysogen	Chloramphenicol	Optimized expression of proteins containing rare codons
BL21pLysE	Contains the pLysE plasmid encoding T7 lysozyme to reduce basal expression	Chloramphenicol	Suitable for the expression of toxic proteins
C41(DE3) pRIL	Derived from BL21(DE3) Walker strains, characterized by low levels of T7 RNA polymerase and optimized for the expression of difficult proteins (e.g., membrane proteins)	Chloramphenicol	Expression of toxic or membrane proteins
Lemo21(DE3)	Derived from BL21 and optimized for the expression of difficult proteins; contains the pLEMO plasmid, which enables tight control of T/RNA polymerase levels	Chloramphenicol	Controlled expression of complex proteins
Origami (DE3) pLysS	Mutations in the trxB and gor genes to improve disulfide bond formation in the cytoplasm	Tetracycline, Chloramphenicol	Expression of proteins containing disulfide bonds
Origami 2(DE3)	Similar to Origami (DE3) but with enhanced mutations in the *trxB* and *gor* genes	Kanamycin, Tetracycline	Improved folding of disulfide bond-containing proteins expressed in the cytoplasm
Origami B (DE3)	Mutations in the *trxB* and *gor* genes, similar to Origami strains	Kanamycin	Suitable for the expression of proteins containing disulfide bonds
Origami B(DE3)pLysS	Combination of Origami B (DE3) with pLysS for tighter control of protein expression	Kanamycin, Chloramphenicol	Suitable for the expression of proteins containing disulfide bonds
Rosetta 2(DE3)pLysS	Enhanced tRNA genes for rare codons, with pLysS for tighter expression control	Kanamycin, Chloramphenicol	Expression of eukaryotic proteins containing rare codons
Rosetta-gami (DE3) pLysS	Combines rare codon tRNAs with mutations promoting disulfide bond formation	Kanamycin, Chloramphenicol, Tetracycline	Expression of complex proteins containing rare codons and disulfide bonds
Rosetta-gami B(DE3)pLysS	Combines the characteristics of Rosetta strains with the properties of Origami strains for disulfide bond formation	Kanamycin, Chloramphenicol	Expression of eukaryotic proteins containing rare codons and disulfide bonds

List of streptomycetes available as protein expression platforms

STRAIN	*DESCRIPTION*	*RESISTANCE*	*APPLICATION*
***Streptomyces albus* J1074**	Strains characterized by high secretion capacity	Ø	Recommended for the production of microbial proteins, especially for proteins that are difficult to express in soluble form in *E. coli*
Streptomyces coelicolor A3(2)		Ø	
***Streptomyces ghanensis* ATCC 14672**		Ø	
***Streptomyces lividans* TK24**		Ø	
***Streptomyces venezuelae* ATCC 10595**		Ø	

List of available expression plasmids

VECTOR	*RESISTANCE*	*PROMOTER*	*FUSION TAGS*	*PROTEASE CLEAVAGE SITE*
pBR328	Ampicillin	lac	Ø	Ø
pCold I	Ampicillin	cspA (cold shock)	His-tag	Factor Xa
pET11a	Ampicillin	T7	Ø	Ø
pET20b+	Ampicillin	T7	His-tag (C-terminal)	Ø
pET21+	Ampicillin	T7	His-tag (C-terminal)	Ø
pET21b con inserto in Nde/Xho	Ampicillin	T7	His-tag (C-terminal)	Ø
pET22b	Ampicillin	T7	His-tag (C-terminal)	Ø
pET24b+	Kanamycin	T7	His-tag (C-terminal)	Ø
pET26b+	Kanamycin	T7	His-tag (N-terminal), pelB signal sequence	Ø
pET28a con inserto in NdeI/XhoI	Kanamycin	T7	His-tag (N-terminal)	thrombin, enterokinase
pET29b con inserto in NdeI/Xho	Kanamycin	T7	His-tag (C-terminal)	thrombin, enterokinase
pET32b	Ampicillin	T7	His-tag (N-terminal), Trx-tag	thrombin, enterokinase
pET39b+	Kanamycin	T7	DsbA (N-terminal), His-tag (C-terminal)	Ø
pET9c	Kanamycin	T7	Ø	Ø
pET-DUET	Ampicillin	T7	Ø	Ø
pGex-6p-2	Ampicillin	tac	GST	PreScission
pG-Tf2	Chloramphenicol	GroESL	His-tag	Ø
pKJE7	Chloramphenicol, Kanamycin	arabinose (araBAD promoter)	Ø	Ø
pKK233-3	Ampicillin	tac	Ø	Ø
pMM1525	Ampicillin	trc	Ø	Ø
pPT7	Ampicillin	T7	Ø	Ø
pRSET A	Ampicillin	T7	His-tag (N-terminal), Xpress epitope	enterokinase
pT7	Ampicillin	T7	Ø	Ø
pT7-deltaBam-Hind	Ampicillin	T7	Ø	Ø
pTrc99A	Ampicillin	trc	Ø	Ø