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Title

"Strategies for the production of soluble human alphainterferons in Escherichia coli: expression, purification, and characterization."

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Abstract

Escherichia coli has been the favorite expression host for the last decades when it comes to simple recombinant proteins used as biopharmaceuticals. This organism is well-characterized and able to deliver enormous amounts of heterologous polypeptides, especially when no complex post-translational modifications are needed, such as the case of alphainterferons, which are small cytokines used against viral infections and tumors. One major disadvantage of this bacterial system is that target molecules are commonly synthesized as insoluble and inactive inclusion bodies in the cytoplasm, bringing up the need for laborious and expensive steps of solubilization and renaturation before the product may be purified. Here we review past experiences, advances, and gaps that may represent future opportunities to deliver interferon-α in its soluble and functional form, including optimization of culture conditions, use of engineered strains, and solubility fusion partners, among others.

Keywords: recombinant, alphainterferon, bioprocess, soluble expression.

1. Introduction

1.1 Interferon alpha

Interferons (IFNs) are pleiotropic cytokines, i.e., signalizing molecules from the immune system which may act on several cell types, and are classically known for their antiviral, antiproliferative, and immunomodulatory activities (González-Navajas et al., 2012; Mesev, 2019), both autocrinally and paracrinally (Zitvogel et al., 2015). They can be secreted by almost all human cell types in response to damage and danger signals such as viruses, tumors, and other agents (Parker et al., 2016), mediating pluripotent effector functions from innate and adaptive immunities (Vidal, 2020).

IFNs are currently divided into types I, II, or III based on: (a) their molecular structure, (b) stimuli for secretion, (c) specific binding to membrane receptors, and (d) signal transduction cascades (Castro et al., 2021). Recent classification (Renren et al., 2022) describes type I family with almost twenty members, encompassing 14 IFNα subtypes that were initially called “leucocyte IFNs” due to their cellular origin (Goeddel et al., 1981). Type I also encompasses IFNβ, originally described as derived from fibroblasts; and the less understood interferons ε, κ, ω, and τ (George et al., 2012).

Subtype alpha quickly conquered great therapeutic importance, and early commercial batches were directly extracted from virally-stimulated cells, such as the case of Alferon-N® (IFN alfa-n3), a pool of 14 natural alphainterferons derived from leucocytes that were induced by incomplete infection with the avian Sendai virus (Friedman-Kien, 1995; El-Baky and Redwan, 2015). However, natural sources are scarce and lead to low yields (Taylor, 2014), besides the risk of contamination by adventitious agents (Dumont et al., 2016). With the development of recombinant DNA technology, industrial manufacturing has mainly moved to biotechnological methods since the mid-80s (Borden, 2019).

F. Hoffman-La Roche and Schering-Plough licensed their mainstage biopharmaceuticals based on interferon alpha-2a (Roferon A®) and -2b (Intron A®), respectively, both displaying 19,2 kDa and 165 aminoacids; these molecules are allelic variants with lysine or arginine in position 23, and they are often considered equivalent, following one same Pharmacopeial Monograph (EDQM, 2023b). Next, second-generation alphainterferons in their peguilated form were licensed, exhibiting improved pharmacokinetic profiles; and several biosimilars were approved when patents expired. Type I IFNs have been used either alone or in combination with chemo- and radiotherapy (Aricò et al., 2019).

As with many small and simple heterologous proteins, alphainterferons have mostly been produced in engineered *Escherichia coli* (*E. coli*), thanks to its rapid growth and productivity, low costs, ease of manipulation, the full understanding of genomic features (Baeshen et al., 2015), and safety (Salunkhe et al., 2009; Shein et al., 2024). As a matter of fact, these cytokines may be originally *O*-glycosylated in humans (Adolf et al., 1991), but this is one of the few cases where such post-translational modification (PTM) is not essential for the recombinant protein's activity and stability (O'Flaherty et al., 2020). This feature avoids the need to use more complex and expensive expression systems, such as eucaryotic cells from yeasts or mammals (Dumont et al., 2016). On the other hand, another PTM, the formation of disulfide bridges, plays an important role in interferon's structure and solubility. IFN-α2, for instance, displays two conserved bonds between cysteines 1-98 and 29-138, and the latter is essential for the cytokine's potency (Neves et al., 2004), as the molecule incubated with reducing agents loses its biological activity (Goeddel et al., 1981).

Other clinically relevant IFNs were not included in this paper due to their structural differences. Used against multiple sclerosis, IFN-β1a (e.g. Rebif®, Avonex®) is a type I IFN, thus able to bind the very receptors used by alpha subtypes, but it is an *N*-glycosylated protein which needs to be synthesized by eukaryotic cells (Adolf et al., 1991; EDQM, 2023c), namely the mammalian line CHO (Zhu et al., 2017). IFN-γ is a non-glycosylated protein expressed in *E. coli*, but it is a type II IFN, non-covalent dimer composed of two identical monomers (EDQM, 2023d).

1.2 Classical route of expression

Current mainstream bioprocesses using *E. coli* rely on (a) transforming cells with plasmids regulated by strong promoters upstream of the gene of interest, (b) cultivating the microorganism at its optimum growth temperature (37ºC), and (c) using inducers to start the synthesis of target proteins (Salunkhe et al., 2009). One of the most common systems involves strains whose genome displays a sequence known as λDE3 lysogen, with the *lacUV5* operon regulating transcription of the RNA polymerase from bacteriophage T7 (T7 RNA pol). In regular conditions, transcription is repressed by the product of gene *LacI*, which prevents *E. coli*'s RNA polymerase from docking. However, lactose or its non-metabolizable synthetic analog, isopropyl β-d-1-thiogalactopyranoside (IPTG), may bind *lac* and de-repress the system, allowing the synthesis of T7 RNA pol. This enzyme is extremely active and binds to its specific promoter on an engineered expression vector (e.g. pET plasmids), leading to the biosynthesis of a given recombinant protein (Rosano et al., 2019).

As shown in Figure 1, there are also cases in which the target plasmid is de-repressed by an added inducer as L-arabinose (Mohammed et al., 2012), limitation of a nutrient (Voss et al., 1994), or it is directly read by *E. coli*'s own RNA polymerase. Additionally, the final recombinant protein may either be located in the cytosol or be translocated to the periplasmic space.

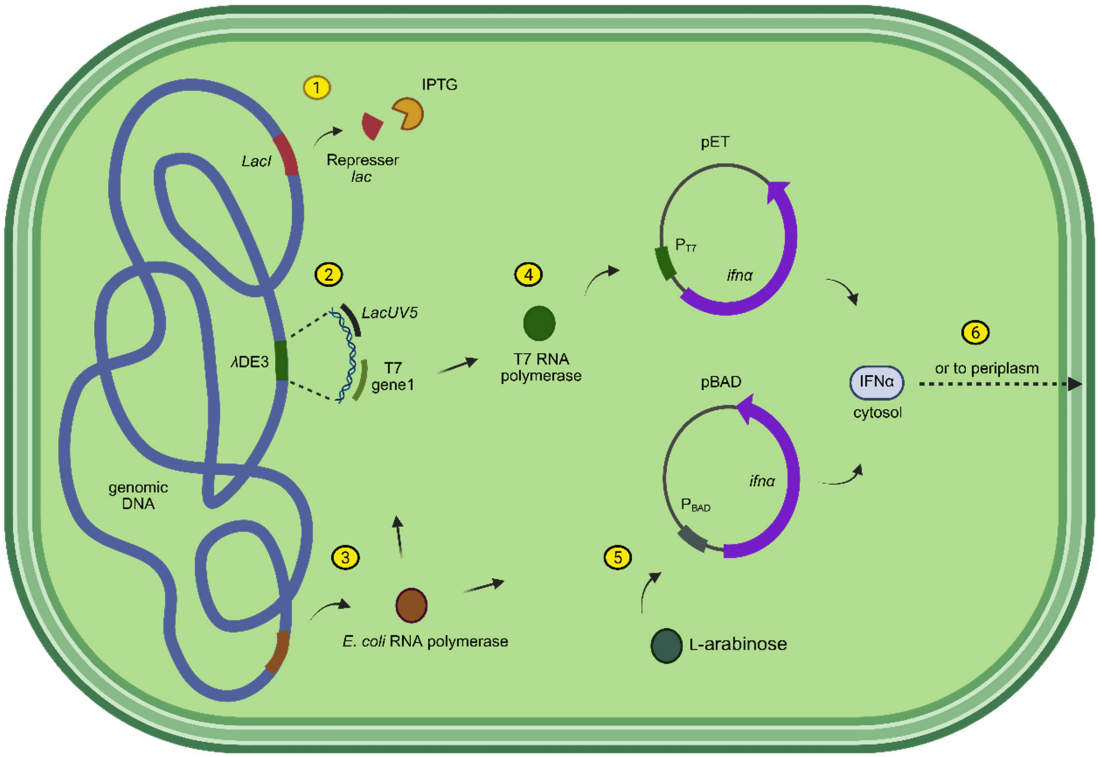

Figure 1: **Simplified two main pathways for the expression of alphainterferon in *E. coli* reviewed in this paper.** IPTG binds to *LacI*'s product (1), which stops blocking the strong promoter *lacUV5* (2), thus allowing *E. coli* RNA polymerase (3) to read phage T7's gene 1. This leads to the synthesis of T7 RNA pol, enzyme that binds to the also strong promoter P_{T7} (4), expressing the target gene in a plasmid. Alternatively, inducer L-arabinose de-represses the weaker promoter P_{BAD} (5), allowing *E. coli*'s RNA polymerase (3) to transcribe from a vector (e.g. pBAD plasmid). The resulting IFN may be present in the cytosol or exported to the periplasmic space (6), if fused to an appropriate signal peptide. Created with BioRender.

Reflecting the phage's aim to parasite its host machinery, viral T7 polymerase overcomes intrinsic *E. coli*'s RNA polymerase in the competition for precursors (Studier and Motaff, 1986). This causes a metabolic burden that prioritizes redirecting the host's resources toward the synthesis of a recombinant protein rather than cell duplication. Therefore, these processes generally first require a phase to increase biomass at the optimal doubling temperature, followed by an induction stage to produce the heterologous protein.

Activation of strong promoters such as P_{T7} results in excessive transcription and translation rates that exceed the cell's capacity to process the nascent polypeptide correctly, thus resulting in misfolded (denatured) proteins that deposit in the form of insoluble and inactive inclusion bodies (IBs) in the cytoplasm (Castro et al., 2021). Some examples of insoluble IFNα were assessed in the present manuscript to compare the bioprocesses (Tisminetzky and Baralle, 2003; Valente et al., 2006; Ahmed et al., 2015), including a Cuban biosimilar currently in the market (Beldarraín et al., 2001), a case of the uncommon IFNα5 (Algirdas, 2012), and an osmotically-induced BL21(SI) strain that expresses IFNα2b (Neves et al., 2004).

Working with IBs is sometimes claimed to have some advantages because the recombinant protein is partly pure and protected from bacterial proteases. The downside is the need to solubilize these structures with reducing and denaturing agents such as β-mercaptoethanol, DTT, or guanidine, and then renature proteins back to their native molecular structure so that they can exhibit the original biological activity (Wingfield, 2015). A lot can go wrong in these steps and one may generate proteins incorrectly folded, which are difficult to remove along purification steps. As a whole, renaturation of proteins is the most troublesome and expensive phase when dealing with *E. coli* bioprocesses (San Miguel et al., 2013); up to 50% of the expressed polypeptide may be lost in such step (Beldarraín et al., 2001).

2. *Successful cases expressing human recombinant IFNα in its soluble form*

2.1 Adaptation of culture conditions

When strain and plasmid are not specifically planned to deliver soluble proteins, the fine-tuning of upstream stage may do the trick mainly by reducing the common overexpression that takes place at the high value of 37ºC (San-Miguel et al., 2013). Lower temperatures (16-25ºC or even 30ºC) were the first choice, especially during induction, since they can (a) slow down transcription and translation rates, which in turn reduces the burden on the whole protein folding machinery; (b) inhibit hydrophobic interactions between apolar side chains in aminoacids, thus minimizing protein aggregation; (c) partly eliminate heat-shock proteases; (d) increase the activity of *E. coli*'s chaperones (Sorensen and Mortensen, 2005; Rabhi-Essafi et al., 2007), which are proteins that help others achieve their correct folding; and (e) convert the activity of some enzymes from proteolytic (at high temperatures) into chaperone-like (Ryan & Henehan, 2013).

An early report had already demonstrated the expression of ≥ 70% soluble IFN-α2 merely by reducing culture setup from 37ºC to 28-30ºC. The study employed two strains transformed with three distinct plasmids; furthermore, interferon became insoluble when exposed to reducing reagents and conditions such as DTT, β-mercaptoethanol and even the lysate from *E. coli* cells grown at 37ºC (Schein and Noteborn, 1988). Table 1 summarizes all cases that succeeded in delivering soluble alphainterferons; yields are shown as reported by authors, and not all were expressed in terms of the mass of purified protein per original cultivation volume.

Zhang and colleagues (2008) described the production of IFNα2b mostly in its soluble form by using simple BL21(DE3) cells with the codon-optimized target sequence. Cell growth in LB was initially conducted at 37ºC, but followed by induction with the regular (and high) concentration of 1 mM IPTG at a lower temperature (30ºC) for 6h.

In 2016, Sharma et al. reported using the strain BL21 Gold, which is improved in transformation efficiency and shows less degradation of plasmid DNA[1]. Cells were transformed with a pET plasmid and underwent a fed-batch scheme with Luria HighVeg broth or yeast extract with supplements, initially between 30-40ºC for cell duplication, then induced with 0.2 – 1 M IPTG at 20ºC.

Yan (2006) produced the rather uncommon interferon alpha-4, which is highly expressed in peripheral blood mononuclear cells to combat viruses and tumors. The team chose a plain BL21(DE3) strain transformed with a plasmid responsive to T7 RNA pol. Reactivated cells were cultivated in a 500-mL bioreactor with LB initially at 32ºC, under a fed-batch regimen with the addition of different nutrients, then induction with IPTG took place for 12-16h at 15-18ºC, resulting in most of the protein in its soluble form.

Some groups worked with interferon consensus - IFNc or alfacon-1 (e.g. Infergen®) -, a 19,4 kDa synthetic molecule engineered with the 166 most prevalent aminoacids among 14 IFNα subtypes. The highest yield reported for an alpha-IFN was accomplished with this molecule, resulting from cultivation for one day at 25-30ºC with the strain BL21-CodonPlus DE3, which exhibits optimized codons for the translation by *E. coli* (El-Baky et al., 2015). An autoinduction medium containing lactose was used, so that when cells consumed all glucose, the former sugar was internalized and used as a fuel, besides prompting *lacUV5*. This approach exempted the need for constant monitoring of cell biomass (e.g. by measuring optical density at 600 nm) and adding IPTG.

Lighter induction conditions also helped, including lower concentrations of IPTG (e.g. 0.1 – 0.5 mM) during fewer hours. Fanhong & Tengjie (2003) cloned the gene for IFN-α1b into two strains displaying lysogen DE3, cultivated in LB firstly at 37ºC and afterwards induced with 0.2 mM IPTG, or alternatively galactose and lactose. In contrast, most studies with the common concentration of IPTG (1 mM) at high temperatures (37ºC) resulted in insoluble IFN.

[1] As per the supplier's manual available at https://www.agilent.com/cs/library/usermanuals/public/230130.pdf.

Table 1: expression of soluble alpha interferon.

Strategy	IFN subtype	*E. coli* strain[1] (and genomic $P_{promoter}$)	Plasmid[1] (and its $P_{promoter}$)	Culture (growth; induction)	Yield[2]	Reference
Customization of upstream step	α2(?)	HB101 and DS410	pP1T7α2 (P_{T7}); pNeo-cop-α2 (P_{colE1}) or pM215a (P_{Amp})	30ºC/20-24h or 44h (Neo-cop-α2)	73-85%	Schein and Noteborn, 1988
	alfacon-1	BL21-CodonPlus DE3 (P_{lacUV5})	pET101/D-TOPO (P_{T7})	lactose[3], 25-30ºC/24h	270 mg/L	El-Baky et al., 2015
	α2b	BL21(DE3) (P_{lacUV5})	pET43 (P_{T7})	37ºC/4-5h (OD 0.8); 1 mM IPTG 30ºC/6h	*n.i.*	Zhang et al., 2008
	α2b	BL21 Gold(DE3) (P_{lacUV5})	pET20b (P_{T7})	30-40ºC; 0.2-1 M IPTG/20ºC	*n.i.*	Sharma et al., 2016
	α4	BL21(DE3) (P_{lacUV5})	pTYB11 (P_{T7})	32ºC; IPTG 15-18ºC/12-16h	*n.i.*	Yan, 2006
	α1b	BL21(DE3) and JM109(DE3) (P_{lacUV5})	pEAM2 (P_{T7})	37ºC until OD 1.0; 0.2 mM IPTG/4h	*n.i.*	Fanhong & Tengjie, 2003
	α2a	BL21(DE3) (P_{lacUV5})	pET9a (P_{T7})	37ºC until early-mid log; 16ºC/48h (no inducers)	16 mg/L	Bretas et al., 2024
Specialized strain	α2a	BL21 (*gor- trxB+*) (P_{lacUV5})	pET26b(+) (P_{T7})	37ºC until mid-log (OD 0.5-0.8); 1 mM IPTG/3h	*n.i.*	Schilling & Diederich, 2012
	α2b	Rosetta-gami2 (DE3) (P_{lacUV5})	pET26a (P_{T7}), *pelB* signal peptide	37º/4h; 1 mM IPTG 30ºC/8h	40.7 - 74.64%	Lin et al. 2012, 2013 (also periplasmic expression)
Strength of promoter	α8	BL21-CodonPlus (DE3)-RIPL (P_{lacUV5})	pBAD-TOPO (P_{BAD})	37ºC until mid-log; 0.02% (w/v) arabinose, 37ºC/6h	100 mg/L	Mohammed et al., 2012
	α2a	MC1061	pBAD18 (P_{BAD})	30ºC/3h; 1% arabinose 30ºC/11h	$3,5 \times 10^9$ IU[4]/L	Chung & Jung, 2002
	α(?)	MC1061	pΔMa (P_{arab})	37ºC until early log; 2.7 mM arabinose/9-10h	~ 80% (~5% total cell protein)	Lim et al., 2000

Solubility tag	α2b	Origami B (P_{lacUV5})	pGEX-Δ (P_{tac}) + GST tag	37º; 0.5 mM IPTG 25ºC/37ºC	≥80% soluble	Rabhi-Essafi et al., 2007
	α2b	BL21(DE3) (P_{lacUV5})	pUC57-derived plasmid + MBP tag	37ºC until OD 0.5; 1 mM IPTG 18ºC/12h	14.4 mg/L	Vu at al., 2016
	α2a	BL21(DE3) (P_{lacUV5})	pET28a (P_{T7}) + 6His-SUMO tag	37ºC until OD 1.0; 0.2 mM IPTG/16ºC/16h	16 mg/L	Bis et al., 2014
	alfacon-1	Shuffle T7 (P_{lacUV5})	Champion® pET (P_{T7}) + 6His-SUMO tag	37ºC/3h (OD 0.5-0.7); 0.1-1 mM IPTG/30ºC/4h	50 mg/L	Peciak et al., 2014
	alfacon-1	BL21(DE3) (P_{lacUV5})	pET28a(+) (P_{T7}) + 6His-Fh8 tag	25-30ºC until OD 0.6; 1 mM IPTG or lactose[3] 30ºC/6h	8 mg/L	Grabarz et al., 2021
Translocation to periplasm	α2c	W3110 (derived from K12)	pDH13 (P_{phoA}) + *STII* signal peptide	37ºC; induction[3] by low [PO_4^{-2}]	14% or 0.19 mg IFN/g biomass	Voss et al., 1994
	α2a and α2b	BL21(DE3) (P_{lacUV5})	pET14b (P_{T7}) + *STII* signal peptide	IPTG 3h	*n.i.*	Kwon et al., 2004
	α1b	BL21(DE3) (P_{lacUV5})	pET22b(+) (P_{T7})	36-38ºC until OD 1.0; 1 mM IPTG 20-25ºC/12-20h	*n.i.*	Xinrong et al., 2022
	α2b	Rosetta-gami 2(DE3) (P_{lacUV5})	pET26a (P_{T7}) + *pelB* signal peptide	37ºC/4h; 1 mM IPTG 30ºC/4h	329.2 μg/L	Tan et al., 2009

Legend: [1] promoters in genome or plasmid are shown within parenthesis; [2] soluble IFN after purification; [3] autoinduction medium; [4] International Units (measure of biological activity; (?) no information on IFN subtype; *n.i.* : not informed; *OD* : optical density at 600 nm.

Relying exclusively on the leaky expression of BL21(DE3) cells transformed with a non-specialized pET plasmid, Bretas et al. (2024) recently expressed up to 16 mg/L of purified and bioactive IFN-α2a, largely in its soluble form. As the cytokine was constitutively produced from the very beginning in LB, initial growth was briefly conducted at 37ºC until early-mid log phase and then the culture was incubated at 16ºC for 48h, without IPTG.

2.2 Customized strains

Most researchers relied on more specialized cells derived from *E. coli* B strains (rather than K12), which are deficient in the major proteases *ompT* and *lon*, able to degrade recombinant proteins (Hayat et al., 2018). Origami B (FÅ113) was engineered with the genotype *trxB-*/*gor-*, meaning it is deficient in two main *E. coli* reductases, namely thioredoxin reductase and glutathione oxido-reductase (Rabhi-Essafi et al., 2007). This feature results in an oxidative cytoplasmic environment that helps reduced sulfhydryl (-SH) groups become S-S disulfide bridges and stabilize polypeptides.

SHuffle B strain (SMG96) indicates in its very name the relation to -SH groups. As Lobstein and her team (2012) characterized, these cells were derived from Origami B, but additionally synthesize the disulfide bond isomerase DsbC (also a chaperone) without its original signal peptide. This enzyme acts in the cytoplasm, shuffling S-S linkages that might be generated with mismatched disulfides by the *trxB-/gor-* system alone; such target proteins could be mis-oxidized and inactive. Schilling & Diederich (2012) developed a *gor- trxB+* BL21 strain altered to resemble (DE3) and transformed with a pET vector that expressed soluble interferons -α2a, -beta, and –gamma, even at high values for temperature and IPTG.

Rosetta-gami2 (DE3) was derived from an *E. coli* K12 background and combines features from Rosetta - the expression of $_{t}$RNA for seven rare codons – and Origami, i.e. the formation of disulfide bonds. After induction with a high dose of inducer for 8h at 30ºC, Lin et al. (2012, 2013) expressed soluble IFN-α2b using a pET26a plasmid, which displays the *pelB* signal sequence for translocation to the periplasm.

Codon optimization was a smart choice in many published cases (Zhang et al., 2008; Mohammed et al., 2012; Bis et al., 2014; Peciak et al., 2014; El-Baky et al., 2015; Vu et al., 2016; Grabarz et al., 2021; Bretas et al., 2024). It is no wonder that IFNα translated according to *E. coli*'s preferential codons would result in more stable $_{m}$RNA and final protein, ultimately with a positive impact on its solubility.

2.3 Strength of the promoter

As depicted in the bottom part of Figure 1, a couple of groups expressed IFNα from pBAD plasmids controlled by the promoter P_{araB} (or P_{BAD}), which is positively regulated by the non-toxic and inexpensive sugar L-arabinose (Terol et al., 2021). P_{BAD} is weaker than T7, *lac* (negatively regulated by *lacI*), and others (*tac*, *trp*, P_L, P_R), so transcription rates and the metabolic burden are lower, which allows cells to grow and express foreign proteins simultaneously, contrasting with classical systems with strong promoters. Mohammed et al. (2012) used a simple codon-optimized BL21 strain (without the T7 system) and delivered an expressive amount (45.8%) of interferon-α8 in its soluble form, even at the high temperature of 37ºC, considering an induction for 6h with 0.02 w/v of the sugar.

Two groups worked with *E. coli* strain MC1061, which is also devoid of T7 system. Chung & Jung (2002) worked with the vector pBAD18 carrying the sequence for IFNα2a and reported good soluble yields at 25ºC and 30ºC. Lim et al. (2000) conducted both pre-culture and main fermentation at 37ºC, and even at such high temperature, the bioactive cytokine was mostly expressed in its soluble form when the group tested low L-arabinose concentrations (2.7 mM) and induction times (9 – 10h); conversely, IBs were formed only under much higher inducer concentrations (10.8 mM) and long induction hours. These findings suggest that lower temperatures are less important for weaker promoters, and confirm Terol et al.'s (2021) point that P_{BAD} may synthesize most of the target protein at the soluble fraction without expressive leaky expression.

2.4 Fusion tags to enhance solubility

Solubility tags may be engineered into plasmids to be expressed as fusion partners to heterologous proteins. Such sequences code for full or partial polypeptides extremely soluble, and some of them may even act as chaperones or aid in bioaffinity chromatography. The best approach seems to be their expression on the N-terminal portion so they can help from the very beginning of protein synthesis; the disadvantage is that these tags generally deliver low protein yields (Grabarz et al., 2021). Common examples include thioredoxin, glutathione-*S*-transferase (GST) for processes with glutathione-based resins, maltose-binding protein (MBP), SUMO (small ubiquitin-related modifier, a protein derived from yeast), and N-utilizing substance A, also called NusA (Rabhi-Essafi et al., 2007).

Expressing human IFN fused to the abovementioned tags has clearly improved its solubility. Nonetheless, this strategy results in an altered primary structure for the polypeptide sequence, so, if the goal is to use the molecule as a biopharmaceutical, fusion proteins must undergo an additional proteolytic step, as well as the removal of proteases, cleaved tags, residual unprocessed species, and accessory proteins like chaperones.

There is no general rule about which tag provides the best outcomes. Rabhi-Essafi and cols. expressed ≥ 80% soluble interferon-α2b fused to GST using a plasmid displaying a *tac* promoter in an Origami B strain. Initial cultivation at 37ºC was followed by induction best tuned with 0.5 mM IPTG at either 25ºC or 37ºC. On the other hand, Vu et al. (2016) had their worst result with the same tag (GST) when testing several fusion partners to enhance solubility. Their best outcome was achieved with MBP, delivering 45% expression and 80% solubility IFN-α2b in strain BL21(DE3). The host was firstly grown in LB at 37ºC for 5h and then induced with 1 mM IPTG for 12h at 18ºC. After purification, a final yield of 14.4 mg per liter of expression medium was reached.

Bis and her team (2014) published a successful case using BL21(DE3) cells transformed with a pET plasmid. They expressed IFNα2a fused to a polyHistidine (6xHis) + SUMO tag on the N-terminal site; after initial growth at 37ºC, the culture was induced with a fifth of the usual IPTG dose and kept in a shaker for another 16h under a much cooler temperature (16ºC). The yield was 16 mg of purified interferon per liter of expression medium.

Peciak et al. (2014) employed Shuffle T7 (a K12-based strain) transformed with a Champion® pET plasmid to produce soluble IFN-con1. The expression vector comprised the SUMO sequence and a 6xHis tag. Induction with 0.1 – 1 mM IPTG for 4h showed best results at 30ºC than at 16ºC, and a final yield of 50 mg/L.

In 2021, Grabarz and colleagues succeeded in expressing soluble IFN-con1 linked to the fusion partner Fh8, which is a 7.6 kDa antigen from the parasite *Fasciola hepatica*; this tag may also increase stability and help downstream processing with hydrophobic resins. The group chose strain BL21(DE3) transformed with a pET vectot displaying a 6xHis tail, initially grown in LB and then in an autoinduction medium containing lactose, at 30ºC. The downstream processing yielded 8 mg/L of functional protein. Secondary results were obtained with medium plus 0.1 mM IPTG, and a DsbC tag. The use of a polyHis tag by itself resulted in insoluble IFNc, as well as with media with 0.1 mM IPTG or higher cultivation temperature.

Polyhistidine tags alone may be very useful for purification with immobilized metal affinity chromatography (IMAC), but their large hydrophobic side chains might enhance the insolubility of the target protein (Hyat et al., 2018). One such example was described when Neves et al. (2004) expressed a mutated IFNα in which cysteines 1 and 98 were replaced by serines, thus eliminating the disulfide bond between these aminoacids. The gene was cloned into a pAE plasmid, linked to an N-terminal 6xHis tail and expressed in BL21 (SI), which is an *E. coli* strain whose T7 RNA polymerase is controlled by the osmotically-induced *proU* promoter. After induction with 300 mM NaCl for 12h, interferon was found insoluble in IBs.

2.5 Export to the periplasmic space

A few researchers engineered proteins to be translocated to the periplasm, where local enzymes and the oxidative environment allow the formation of disulfide bonds (Niazi and Magoola, 2023). Voss and colleagues (1994) reported the use of *E. coli* strain W3110 harboring a plasmid pDH13 with the sequence for IFNα2c, which is a minor allelic form that displays arginine in positions 23 and 34 (Adolf et al., 1991). The vector

displayed the heat-stable enterotoxin II (*STII*) as a fusion protein that worked as the signal-peptide responsible for translocation. All cultivation was conducted at 37ºC as a fed-batch operation and resulted in soluble IFN.

Another team also fused *STII* to IFNα2a and IFNα2b sequences, using *E. coli* BL21(DE3) transformed with a plasmid from the pET family (Kwon et al., 2004); strains were cultivated in LB and then induced with IPTG for 3h. Cooler cultivation conditions of 20-25ºC additionally helped the production of soluble periplasmic IFN-alpha1b by Xinrong et al. (2022), who worked with BL21(DE3) transformed with a pET plasmid and induced with 1 mM IPTG.

Tan et al. (2009) bet on a statistical analysis to study medium optimization. The expression system was composed of a Rosetta-gami 2(DE3) strain transformed with a pET26a plasmid containing the sequence for IFN-α2b and the signal peptide *pelB*. Baffled shake flasks underwent cultivation at 37ºC for 4h and then induction at 30ºC for 8h with 1 mM IPTG, delivering soluble interferon in the periplasmic space, with best results when the medium was compounded with low glucose content and high concentrations of yeast extract and peptone.

3. Downstream processing

3.1 Primary recovery: harvest and lysis

As the target product is intracellular (cytosolic or periplasmic), all groups harvested cells by centrifugation and a couple of them used microfiltration to remove debris (Peciak et al., 2014; Sharma et al., 2016) after lysis. Following solubilization of IBs + renaturation, downstream processing was not different from what is expected for other biopharmaceuticals. Figure 2 illustrates all the steps in the publications reviewed.

Most teams lysed bacterial pellets by lab-scale sonication (Lim et al., 2000; Rabhi-Essafi et al., 2007; Zhang et al., 2008; Mohammed et al., 2012; Schilling & Diederich, 2012; Peciak et al., 2014; El-Baky et al., 2015; Vu et al., 2016), alternatively aided by lysozyme (Lim et al., 2000; Peciak et al., 2014); the use of this protease also means an additional process-related impurity to be removed. Mechanical methods encompassed homogenizers (Chung and Jung, 2002; Sharma et al., 2016) and French press (Neves et al., 2004). These procedures provoke total cell breakage that releases more contaminants such as gram-negative's lipopolysaccharides (LPS), also named endotoxins (Niazi and Magoola, 2023), as well as host cell nucleic acids and proteins.

Periplasmic IFN might be more easily purified since fewer bacterial proteins are freed in the absence of the cell's full breakage (Xinrong et al., 2022), and because gentler extraction techniques may be used; nonetheless, this strategy still delivers poor yields due to the limited processing capacity in the translocation pathway. Among the cases assessed, IFNα was recovered simply by disarranging *E. coli*'s outer membrane with an osmotic shock, e.g. with a 20% sucrose solution (Kwon et al., 2004; Tan et al., 2009; Lin et al., 2012 and 2013) or acidic treatment (Voss et al., 1994).

Only two groups reported cell lysis by repeated freeze-thaw cycles, either by itself (Bretas et al., 2024) or aided by lysozyme (Schein and Noteborn, 1988). As another gentle extraction, this method is claimed to allow small and soluble recombinant proteins, highly expressed intracellularly, to exit partly pure through transient pores caused by ice crystals (Johnson and Hecht, 1994). Fast freezing coupled with slow thawing might help the formation of large crystals that damage cell envelopes, since the exact opposite is recommended in cryopreservation protocols that aim to preserve cellular viability (Freshney, 2010). Unfortunately, scaling up this thermal technique remains challenging for the industry.

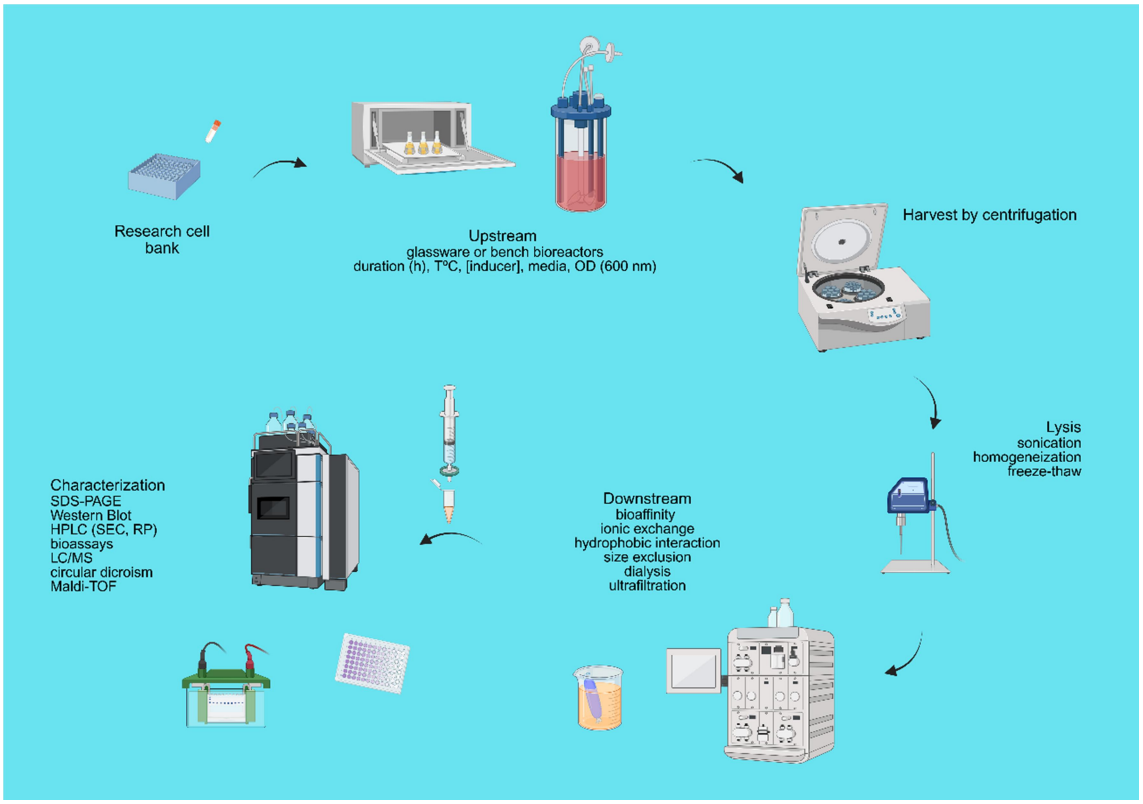

Figure 2: **Schematic steps in bioprocesses reviewed.** For each batch, a vial of the cell bank was thawed and grown in increasing scales using erlenmeyers/shake flasks or small bioreactors. After induction and expression, cells were collected; IFN was extracted, purified and tested via different physicochemical, biochemical and biological assays. Created with BioRender.

3.2 Purification: column and membrane-based operations

Bioaffinity chromatography was the obvious choice to purify IFN fused to poly(His) tails and solubility enhancers that also acted as affinity tags (Neves et al., 2004; Rabhi-Essafi et al., 2007; Bis et al., 2014; Vu et al., 2016; Grabarz et al., 2021). As depicted in Figure 3, commonly the whole fusion protein was first bound to the ligand matrix and then eluted; secondly, the fusion partner was excised by a protease that also harbored the affinity tag, allowing the target protein to be collected from the flowthrough of a second affinity column while tagged species were retained (Peciak et al., 2014; Grabarz et al., 2021). In the absence of fusion partners, older studies reported affinity columns displaying specific anti-IFNα antibodies covalently linked to the resin (Adolf et al., 1991). Lastly, Kwon et al. (2004) reported blue Sepharose as the capture affinity resin.

Almost all studies used lysis buffers based on phosphates and/or Tris, alternatively with detergents (Triton) to destabilize membranes, besides protease inhibitors, even when working with B strains. The pH was within the range 7 – 8, probably to avoid the generalized precipitation described for *E. coli* proteins in acidic solutions (Wingfield, 2015; Gundinger and Spadiut, 2020). IFNα2's isoelectric point (i.p.) is 5.9, so it exhibits a net negative charge in this environment, which helps explain the choice of anionic exchange as the number one non-bioaffinity purification technique. Resins based on DEAE and Q-Sepharose were either the only ones used (Mohammed et al., 2012; El-Baky et al., 2015) or part of a sequence composed of two (Kwan, 2004; Yan, 2006; Zhang et al., 2008; Vu et al., 2016), three (Fanhong & Tengjie, 2003; Kwon et al., 2004; Grabarz et al., 2021), or even four chromatographic steps (Voss et al., 1994; Chung and Jung, 2002; Sharma et al., 2016).

Cationic exchange using functional groups carboxymethyl and sulphopropyl was performed with pH ≤ 5.3 and the target molecule positively charged (Voss et al., 1994; Tisminetzky and Baralle, 2002; Chung and Jung, 2002; Fanhong & Tengjie, 2003; Kwon et al., 2004), thus allowing removal of anionic contaminants along flowthrough, such as LPS and residual DNA. Eluting with slower gradients allowed the separation of IFNα aggregates and proteins displaying distinct positive charge intensities (Bretas et al., 2024).

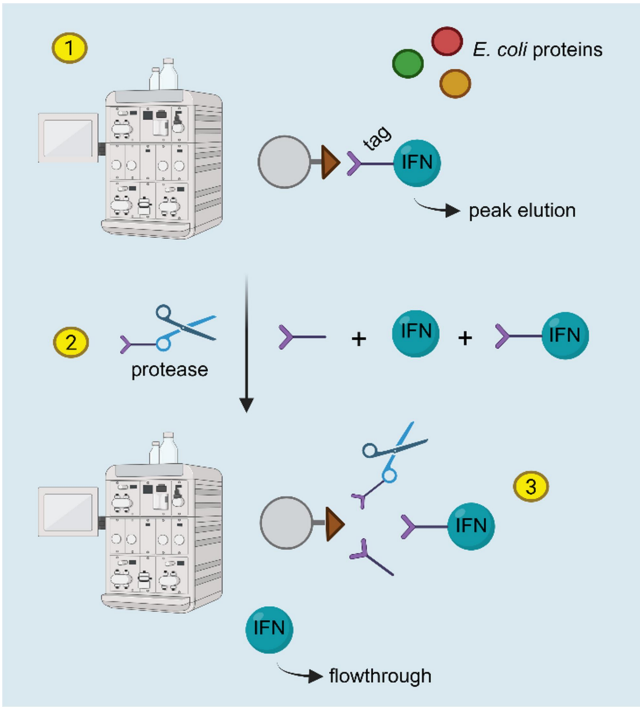

Figure 3: Purification of alphainterferon by affinity chromatography. (1) IFN is expressed with a protease recognition site and a bioaffinity tail (e.g. 6xHis tag) that binds a ligand (brown arrow) on a chromatographic resin (gray sphere) while *E. coli* contaminating proteins are cleared away in flowthrough. (2) Next, a protease cleaves the fusion protein, but some residual IFN-tag remains unprocessed. The protease itself is fused to the same tag, but lacks the proteolysis sequence. (3) Finally, the pool goes through the affinity column a second time, but now only tagged species are retained while free IFN is collected in the flowthrough fraction. Created with BioRender.

For both anionic and cationic columns, it is expected that neutral species are carried out during washing steps, such as proteins in their i.p and non-charged molecules like some lipids. Small species (IPTG, protease inhibitors) are also likely to be cleared along some processes with membranes, such as dialysis (Kwon et al., 2004; Bis et al., 2014), diafiltration (Fanhong & Tengjie, 2003; Vu et al., 2016), or ultrafiltration. These steps were performed in some cases in order to exchange buffers or to concentrate the target protein (Yan, 2006; Zhang et al., 2008; Peciak et al., 2014; Sharma et al., 2016).

Size exclusion chromatography (SEC) was largely used too. Rather than a purification strategy *per se*, this technique was mainly chosen as a final polishing to desalt, exchange buffer, and/or to remove fusion tags, proteases and accessory proteins (Fanhong and Tengjie, 2003; Rabhi-Essafi et al,. 2007; Zhang et al., 2008), and could even be replaced by ultrafiltration in one article (Chung and Jung, 2002).

Another relevant approach included hydrophobic interaction chromatography, basically with phenyl sepharose columns (Voss et al., 1994; Chung and Jung, 2002; Yan, 2006; Xinrong et al., 2022). One team (Beldarraín et al., 2001) chose a preparative reverse-phase high performance liquid chromatography (RP-HPLC) as the second (among four) chromatographic steps to purify their IB-derived IFN alpha-2b. Indeed, although it is water-soluble, IFNα2 exhibits a relatively high hydrophobic character due to many apolar aminoacid lateral groups exposed; such feature was increased by using buffers with pH values near its i.p. and/or with the addition of ammonium sulphate. Finally, one group adsorbed IFN to a silica column as the first downstream step (Voss et al., 1994).

Minor strategies to purify IFN-α2b from the periplasm included differential partitioning in aqueous two-phase systems (Lin et al., 2012 and 2013). After osmotic shock, the team employed the water-soluble polymer PEG and potassium phosphate, achieving a purification factor (Pf) of 26.3 e yield of 40.7%. Next, the group tested a system composed of alcohol and salt, reaching Pf = 16.24 with yield = 74.64%. The molecule was quantified and analyzed by SDS-PAGE, but there was no mention of its biological activity or the correct tertiary structure.

Characterization

SDS-PAGE was the most common method to monitor IFNα in soluble vs. insoluble fractions and along downstream processing. Interferon's identity was inferred by comparison to analytical standards, molecular weight standards, and, in some cases, confirmed by western blotting (Kwon et al., 2004) and even ELISA. The cited blotting could also help identify possible IFN aggregates among the whole pool of host cell proteins.

When it comes to the impurity profile of the final product, groups reported at least 95% pure IFN according to electrophoretic methods; one also employed RP- and SEC-HPLC analyses (Grabarz et al., 2021). Only two teams dosed residual endotoxins (Chung and Jung, 2002; Vu et al., 2016), and another measured host cell DNA (Bretas et al., 2024). These are important impurities that must comply to regulatory limits if the molecule is intended to become a licensed biopharmaceutical (EDQM, 2023b).

All but two publications (Lin et al., 2012 and 2013) reported bioassays to demonstrate *in vitro* activity of alphainterferon, thus implying that its 3D structure was correct (Meager, 2002). Antiviral assays (EDQM, 2023a and b) were the first choice, followed by three antiproliferative tests using tumoral lines (Mohammed et al., 2012; Bis et al., 2014; El-Baky et al., 2015). Results were reported as the effective dose impacting half the cells (EC_{50}) or as international units (IU/mg or IU/mL) whenever the laboratory possessed an official standard with known potency.

A few research teams performed further structural analyses to confirm the purified protein's secondary and tertiary features. Methods included circular dicroism, peptide mapping coupled to mass spectrometry (MS) (Voss et al., 1994), LC/MS, nuclear magnetic resonance, analytical centrifugation (Bis et al., 2014) and MALDI-TOF (Peciak et al., 2014).

4. Unexplored opportunities

Some overlooked strategies might be adopted in future studies, either by themselves or combined with techniques already described. In the upstream stage, supplementation of culture media with chemical chaperones and cofactors may increase solubility, as suggested by previous general reviews on recombinant proteins (Sorensen and Mortensen, 2005; Hayat et al., 2018; Rosano et al., 2019).

Operation mode-wise, all examples reviewed were either simple batches in glassware (erlenmeyers and flasks up to 500 mL medium) or operated in batch and fed-batch modes in small bioreactors. No publication adopted perfusion (continuous) cultures, which are more common for slow-growing animal cells (Zhu et al., 2017).

Although reported for other soluble heterologous proteins, *Arctic Express (DE3)* was not chosen as the host in any of the studies reviewed, despite the strain's ability to express cold-adapted chaperones that show high refolding activity at up to 4ºC (Rosano et al., 2019). Moreover, no group co-expressed chaperones (Baeshen et al., 2015) in the same or additional plasmids (except for SHuffle, whose DsbC gene is inserted in the chromosome).

Following cell lysis, just Sharma et al. (2016) treated the product with polyethyleneimine (PEI) or protamine sulphate to remove nucleic acids. But as pointed out by Gundinger and Spadiut (2020), flocculation may prove helpful in removing cells, cell debris, DNA, endotoxins and colloidal proteins.

Finally, two missed purification techniques were ceramic hydroxyapatite chromatography and membrane chromatography. The former may interact with proteins through multimodal interactions such as ion exchange (both anionic and cationic), metal affinity and hydrogen bonding; the latter may display functionalized groups to adsorb impurities (Zhu et al., 2017). Moreover, groups neither explored stepwise elution schemes (only linear gradients), nor the strategy of varying the buffer's pH along elution to modify IFN's charge.

5. Conclusions and Future Trends

Successful biosynthesis of soluble and functional IFNα was closely related to the (1) selection of appropriate host strains with helpful genetic features; (2) design of optimized expression plasmids; and (3) adjusts in culture conditions. For non-specialized expression systems, the induction phase was critical and improved by a shorter duration, cooler temperatures, and the right timing (early - mid log phase) to add inducers at submaximal concentrations. The selection of poor media and weaker promoters helped as well to reduce the overexpression that leads to protein precipitation. On the other hand, customized systems delivered the soluble cytokine even at higher metabolic rates caused by more intense culture scenarios.

Despite all cases reviewed, biotech industries still manufacture alphainterferons as insoluble inclusion bodies currently, and some argue that the large amounts achieved might compensate for the time and costs involving the processing of these denatured proteins. However, the theoretical advantage of IBs being protected from bacterial proteases may be easily compensated by using inhibitors and *ompT*/*lon*-deficient B strains, as well as purifying proteins fast and under refrigeration. There are some untested strategies that might still improve bioprocesses; probably, the execution of well-planned and straightforward pharmacoeconomic studies would reveal, for each specific bioprocess, which approach is the best.

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Authors' contribution statement (CRediT)

Rodrigo M Bretas: conceptualization, methodology, investigation, formal analysis, visualization, and writing – original draft. **Sophie Y Leclerq**: conceptualization, supervision, and writing – review and editing. **Armando SC Jr.**: conceptualization, supervision, and writing – review and editing. **Luciana MS Lopes**: conceptualization, supervision, and writing – review and editing.

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