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

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Version justification: Versão 3 do artigo de revisão contemplando as melhorias sugeridas pelos revisores da revista científica Protein Expression and Purification. Basicamente, houve correções quanto às referências bibliográficas e avanços na seção sobre oportunidades adicionais para explorar a biossíntese de interferon solúvel pela bactéria E. coli.

Title

Strategies for manufacturing soluble human alphainterferons in *Escherichia coli*: expression, purification, and testing.

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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 capable of synthesizing enormous amounts of heterologous polypeptides, especially when no complex post-translational modifications are involved, such as the case of alphainterferons, which are small cytokines used against viral infections and tumors. A significant drawback of this bacterial system is that target molecules are commonly obtained as insoluble and inactive inclusion bodies in the cytoplasm, raising the need for laborious and expensive steps of solubilization and renaturation before the product can be purified. Here, we review past experiences and advances that have delivered IFN-α in its soluble and functional form, including optimization of culture conditions and induction, the use of engineered strains, fusion partners that enhance solubility, and translocation to the periplasm, among others. Also, we assessed downstream processing and analytical techniques that ensured the product's purity and quality. Finally, we identified some gaps that may represent future opportunities to improve soluble yields.

Keywords: recombinant, alphainterferon, bioprocess, soluble expression, purification.

1. Introduction

1.1 Interferon alpha

Interferons (IFNs) are pleiotropic cytokines, i.e., signaling molecules from the immune system that can act on several cell types, and are classically known for their antiviral, antiproliferative, and immunomodulatory activities [1,2], both autocrinally and paracrinally [3]. They can be secreted by several human cell types in response to damage and danger signals such as viruses, tumors, and other agents [4], mediating pluripotent effector functions from innate and adaptive immunities [5].

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 [6]. Recent classification [7] describes type I family with almost twenty members, encompassing 14 IFNα subtypes that were initially called "leucocyte IFNs" due to their cellular origin [8]. Type I also encompasses IFNβ, originally described as derived from fibroblasts; and the less understood interferons ε, κ, ω, and τ [9].

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 [10,11]. However, natural sources are scarce and lead to low yields [12], in addition to the risk of contamination by adventitious agents [13]. With the development of recombinant DNA technology, industrial manufacturing has mainly moved to biotechnological methods since the mid-80s [14].

F. Hoffman-La Roche and Schering-Plough licensed their mainstage biopharmaceuticals based on interferon alpha-2a (Roferon A®) and -2b (Intron A®), with either lysine or arginine in position 23, respectively. These molecules are allelic variants displaying 19.2 kDa and 165 aminoacids, and they are often considered equivalent, following the same Pharmacopeial Monograph [15]. 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 [16].

1.2 Some structural features

1.2.1 Post-translational modifications (PTM)

Alphainterferon-2 may be *O*-glycosylated in humans [17], so several groups have manufactured it in yeast [18] and mammalian cell lines [13], which are complex, slow-growing, and expensive eukaryotic expression hosts equipped with glycosylation machinery. It was even demonstrated that a bacterial host can glycosylate IFN-α2b in the presence of co-expressed glycosyltransferases [19]. But as happens with many small and simple heterologous proteins, alphainterferons have been produced primarily in engineered *Escherichia coli* (*E. coli*), thanks to its rapid growth and productivity, low costs, ease of manipulation, full understanding of genomic features [20], and viral safety [21]. This is possible because this cytokine is one of the rare cases where such post-translational modification (PTM) is not essential for the recombinant protein's activity and stability [22].

On the other hand, disulfide bonds are often the most critical PTM and are vital for the proper folding, solubility, stability, and functionality of proteins [23], and it is not different with interferon [11]. IFN-α2, for instance, displays two conserved bonds between cysteines 1-98 and 29-138, and the latter is essential for its potency [24], as seen in a study in which the molecule incubated with reducing agents lost all biological activity [8]. Such bonds are formed in compartmentalized organelles within eukaryotes, whereas bacteria as *E. coli* rely on the oxidative environment and specialized enzymes in the periplasmic space for the conversion of free sulfhydryls into covalent S-S bonds.

Other clinically relevant IFNs were not included in this review due to their structural differences. Used against multiple sclerosis, IFN-β1a (e.g., Rebif®, Avonex®) is a type I IFN that binds the same receptors targeted by alpha subtypes, but it is an N-glycosylated protein that needs the eukaryotic machinery to be synthesized [17,25], generally the mammalian line CHO [26]. IFN-γ is a non-glycosylated protein expressed in *E. coli*, but it is a type II IFN, non-covalent homodimer [27,28].

1.2.2 N-terminus heterogeneity

In vivo, the start codon AUG not only signals the beginning of translation, but also ensures an initial methionine (Met) before the first aminoacid from the native primary sequence. *E. coli*'s methionyl-aminopeptidase (MAP) is the enzyme responsible for excising such Met, and older studies showed that its action is directly favored when the neighboring aminoacid displays small side chain and radius of gyration [29,30], which is the case of Cys_1 in IFNα2.

On the other hand, there are reports about the inefficiency of MAP, leading to an N-terminal heterogeneity in alphainterferon. Several species have been described: Met-IFN, acetylated Met-IFN, native IFN, and acetylated IFN; variants are concerning because they could affect immunogenicity and stability profiles. Biological activity may also be influenced if the N-terminus is associated with receptor-binding and resistance to proteases [31].

Possible solutions include the co-expression of MAP in *E. coli* or the manufacturing in yeasts [32]. Sharma and cols. [33] reported the expression of IFNα2b with less initial Met simply by conducting the fermentation at low temperature (20ºC). Taken together, these data reinforce the importance of correctly characterizing the expression strain and its genetic features when aiming for batch-to-batch consistency. IFNα2's current pharmacopeial monograph [15] predicts peptide mapping as one of the identity tests, but it could be beneficial to add a method for its N-terminal sequencing.

1.3 Classical route of expression

Current mainstream bioprocesses using *E. coli* rely on (a) transforming fast-growing 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 [19,34]. One of the most common systems involves strains whose genome displays a sequence known as λDE3 lysogen, with the *lac*UV5 promoter regulating transcription of the RNA polymerase from bacteriophage T7 (T7RP). Under regular conditions, transcription is repressed by the product of *lac*I gene, 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 LacI and de-repress the system, allowing the synthesis of T7RP. This enzyme is extremely active and binds its specific promoter on an engineered expression vector (e.g., pET plasmids), leading to the biosynthesis of a given recombinant protein [35]. Figure 1(a) illustrates this approach.

There are also cases in which the target plasmid is directly de-repressed by adding the inducer L-arabinose [36,37], as depicted in Figure 1(b), or by limitation of a nutrient [38]. Additionally, the final recombinant protein may be located in the cytosol, or it may be exported to the periplasmic space or external medium, if fused to an appropriate signal peptide.

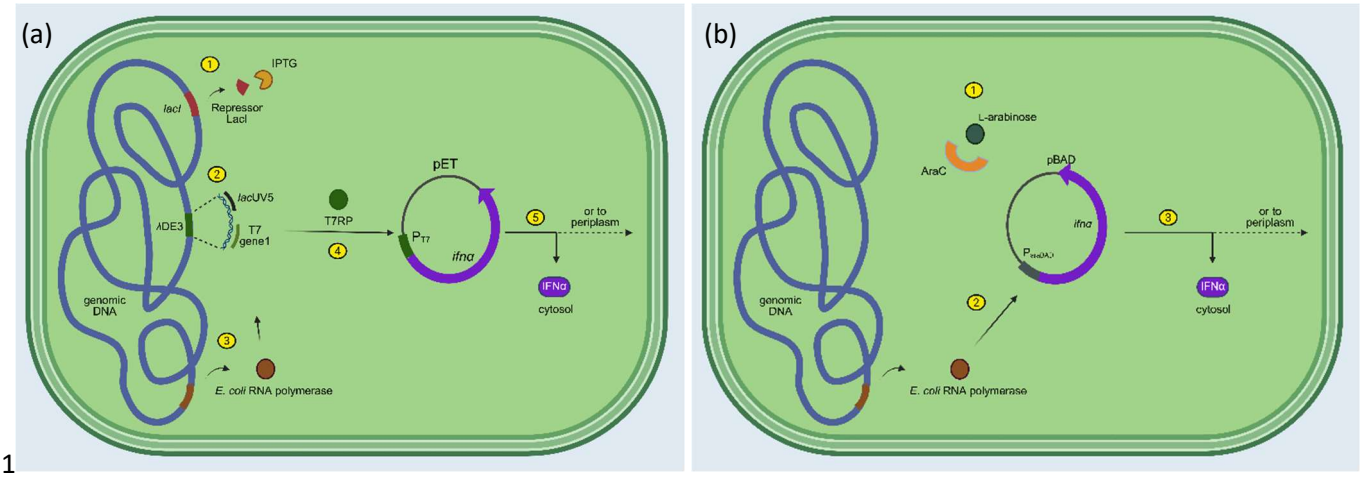

Figure 1: **Simplified two main pathways for the expression of alphainterferon in *E. coli* reviewed in this article. (a)** IPTG binds repressor LacI (1), which stops blocking the promoter *lac*UV5 (2), allowing *E. coli* RNA polymerase (3) to read phage T7 gene 1. This leads to the synthesis of T7RP (4), enzyme that binds the strong promoter P_{T7}, expressing the target gene in a pET plasmid (5). **(b)** the sugar L-arabinose binds the repressor AraC (1), allowing *E. coli*'s RNA polymerase (2) to transcribe from the weaker promoter P_{araBAD} within a pBAD vector (3). For both cases, the resulting IFN may be either present in the cytosol or translocated/exported. Created with BioRender.

Reflecting the phage's aim to parasitize its host machinery, viral T7RP overcomes the intrinsic *E. coli*'s RNA polymerase in competition for precursors [39]. 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 require an initial phase to increase biomass at this enterobacterium's 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, resulting in misfolded and denatured proteins that precipitate as amorphous inclusion bodies (IBs) in the cytoplasm [6].

Working with IBs is sometimes claimed to have a few 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 to their native molecular structure [40] so that they can exhibit biological activity. Many things can go wrong in these steps and one may generate proteins incorrectly folded, which are difficult to remove during purification steps. In general, protein renaturation is the major hurdle when dealing with *E. coli* bioprocesses [41]; up to 50% of the expressed polypeptide may be lost in this step [42].

This manuscript reviews strategies that favored the soluble expression of human IFNα in *E. coli*, providing a starting point for researchers attempting to manufacture the same or a similar protein using this host. Downstream processing (purification) and quality control tests are also explored.

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, fine-tuning of the upstream stage may do so by reducing the common overexpression that occurs at 37ºC [43]. Lower temperatures (16-25ºC or even 30ºC) are 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 folding machinery; (b) inhibit hydrophobic interactions between apolar side chains in aminoacids, minimizing protein aggregation; (c) partly eliminate heat-shock proteases; (d) increase the activity of *E. coli*'s chaperones [44], which are proteins that help others achieve their correct structure; and (e) convert the activity of some enzymes from proteolytic into chaperone-like [45].

An early report had already demonstrated the expression of ≥70% soluble IFN-α2 merely by reducing culture conditions 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 [46]. Table 1 summarizes all cases that succeeded in expressing soluble alphainterferons; yields are shown as reported by authors.

In 2016, one group [33] used the strain BL21(DE3) Gold, which is improved in transformation efficiency and shows less degradation of plasmidial DNA (https://www.agilent.com/cs/library/usermanuals/public/230130.pdf). Cells were transformed with a pET plasmid and followed a fed-batch scheme using two types of media, initially between 30-40ºC for cell duplication, then induced with 200 – 1000 μM IPTG at moderate temperature (20ºC).

Zhang and colleagues [47] described the production of IFNα2b mostly in its soluble form by using simple BL21(DE3) cells with a codon-optimized target sequence, i.e., an adapted genetic sequence comprising codons that are preferentially used by the host to select a certain aminoacid during the formation of a nascent polypeptide chain. Cell growth in LB was initially conducted at 37ºC, followed by induction with the common concentration of 1 mM IPTG at a lower temperature (30ºC) for 6 h.

Yan [48] worked with the rather uncommon interferon alpha-4. This patent dealt with a plain BL21(DE3) strain cultivated in a bench bioreactor with LB initially at 32ºC, under a fed-batch regimen with the addition of different nutrients, then induction with IPTG for 12-16 h at 15-18ºC, resulting in part of the protein in its soluble form.

Table 1: Strategies to express soluble alphainterferon.

Strategy	IFN subtype	*E. coli* strain[1] (and genomic $P_{promoter}$)	Plasmid[1] (and $P_{promoter}$)	Culture (growth; induction)	Scale / Medium	Yield[2]	Reference
Customization of upstream step	α2b	BL21 Gold(DE3) (P_{lacUV5})	pET20b (P_{T7})	30-40ºC; 200 – 1000 µM IPTG 20ºC/25h	*n.i.* / Complex	~1.5 g/L[ly]	[33]
	α2	HB101 and DS410	pP1T7α2 (P_{T7}); pNeo-cop-α2 (P_{colE1}); pM215a (P_{Amp})	30ºC/20-24h or 44h (Neo-cop-α2)	*n.i.* / LB	73-85%[ly]	[46]
	α2b	BL21(DE3) (P_{lacUV5})	pET43 (P_{T7})	37ºC until OD 0.8; 1 mM IPTG 30ºC/6h	5 mL / LB (test tubes)	≥45% soluble[ly]	[47]
	α4	BL21(DE3) (P_{lacUV5})	pTYB11 (P_{T7})	32ºC; IPTG 15-18ºC/12-16h	2 - 5 L / LB (bioreactor)	> 30% soluble[ly]	[48]
	alfacon-1	BL21-CodonPlus(DE3) (P_{lacUV5})	pET101/D-TOPO (P_{T7})	25-30ºC/24h	1 L / AutoInd (shake flask)	70% soluble[ly] 270 mg/L[dsp]	[49]
	α1b	BL21(DE3) and JM109(DE3) (P_{lacUV5})	pEAM2 (P_{T7})	37ºC until OD 1; 0.2 mM IPTG/4h	800 mL / LB (shake flask)	> 30% soluble[ly]	[51]
				37ºC until OD 10; 2 mM lactose/6h	150 L / LB (bioreactor, high cell density)		
	α1, α2a, α2b, α4, α5, α14	BL21(DE3) (P_{lacUV5})	pPAL7 (P_{T7})	37ºC until OD 0.6; 20ºC/20h	500 mL / AutoInd (shake flask)	6-40 mg/L[dsp]	[53]
	α2a	BL21(DE3) (P_{lacUV5})	pET9a (P_{T7})	37ºC until OD 0.3-0.6; 16ºC/48h (no inducers)	150 mL / LB (shake flask)	≥ 63% soluble[ly] 23.5 mg/L[dsp]	[54]
Specialized strain	α2a	BL21 (*gor- trxB+*) (P_{lacUV5})	pET26b(+) (P_{T7})	37ºC until OD 0.5-0.8; 1 mM IPTG/3h	15 mL / Complex (shake flask)	*n.i.*	[58]
	α2b	Rosetta-gami2 (DE3) (P_{lacUV5})	pET26a (P_{T7}) + *pelB* signal peptide	37º/4h; 1 mM IPTG 30ºC/8h	50 mL / TB (shake flask)	40.7 - 74.64%[dsp]	[59,60]

Strength of promoter	α8	BL21-CodonPlus (DE3)-RIPL (P_{lacUV5})	pBAD-TOPO (P_{BAD})	37ºC until OD 0.6-0.8; 0.02% (w/v) arabinose, 37ºC/6h	100 mL / M9 (shake flask)	~46% soluble[ly] 100 mg/L[dsp]	[36]
	α2a	MC1061	pBAD18 (P_{BAD})	30ºC/3h; 1% arabinose 30ºC/11h	20 L / Complex (bench bioreactor)	8 mg/L[dsp]	[67]
	α (*n.i.*)	MC1061	pΔMa (P_{arab})	37ºC until early log; 2.7 mM arabinose/9-10h	1 L / TB + M9 (bioreactor, high cell density)	~ 80%[ly] (~5% total cell protein)	[68]
Solubility tag	α2a	BL21(DE3) (P_{lacUV5})	pET28a (P_{T7}) + 6His-SUMO tag	37ºC until OD 1.0; 0.2 mM IPTG/16ºC/16h	1 L / LB	16 mg/L[dsp]	[61]
	alfacon-1	SHuffle T7 (P_{lacUV5})	Champion® pET (P_{T7}) + 6His + SUMO tag	37ºC until OD 5 - 7; 0.1 - 1 mM IPTG 30ºC/4h	500 mL / TB	87% soluble[ly] 50 mg/L[dsp]	[62]
	α2b	BL21(DE3) (P_{lacUV5})	pUC57-derived plasmid + MBP tag	37ºC until OD 0.5; 1 mM IPTG 18ºC/12h	500 mL / LB	80% soluble[ly] 14.4 mg/L[dsp]	[63]
	alfacon-1	BL21(DE3) (P_{lacUV5})	pET28a(+) (P_{T7}) + 6His-Fh8 tag	25-30ºC until OD 0.6; 30ºC/6h	100 mL / AutoInd	8 mg/L[dsp]	[64]
	α2b	Origami B (P_{lacUV5})	pGEX-Δ (P_{tac}) + GST tag	37º; 0.5 mM IPTG 25ºC/37ºC	100 mL / LB	≥ 80% soluble[ly] 100 mg/L[dsp]	[70]
	α2-Tα1	BL21(DE3) (P_{lacUV5})	Champion® pET (P_{T7}) + 6His + SUMO tag	37ºC until OD 0.5; 0.5 mM IPTG 37ºC/4h	50 mL / LB + TB	80% soluble[ly]	[71]
Coexpression of chaperone(s)	α2b	BL21(DE3) (P_{lacUV5})	pGZ10 (P_{T7}) 6His tag + Erv1p + hPDI	30ºC/24h or 40h	24 x 2 mL (deep well plates) / AutoInd (rich or defined)	33,3 mg/L[dsp]	[72]
	α2	Origami B (P_{lacUV5})	pET23 (P_{T7}) 6His or MBP tag + IFN-α2 pLysS (P_{arab}) + Erv1p + hPDI	30ºC until OD 0.4, pre-induction 0.5% (w/v) arab 30 min, IPTG 0.5 mM/4h.	25 mL / ChemDef (shake flasks)	16 mg/L[dsp]	[73]

Translocation to periplasm	α2c	W3110 (K12-based)	pDH13 (P_{phoA}) + *STII* signal peptide	37ºC	7 L / AutoInd (bioreactor)	14% or 190 µg IFN/g biomass	[38]
	α2a and α2b	BL21(DE3) (P_{lacUV5})	pET14b (P_{T7}) + *STII* signal peptide	IPTG 3h	*n.i.* / LB	*n.i.*	[76]
	α1b	BL21(DE3) (P_{lacUV5})	pET22b(+) (P_{T7}) + *pelB* signal peptide	36-38ºC until OD 1.0; 1 mM IPTG 20-25ºC/12-20h	*n.i.* / ChemDef	*n.i.*	[77]
	α2b	Rosetta-gami 2(DE3) (P_{lacUV5})	pET26a (P_{T7}) + *pelB* signal peptide	37ºC/4h; 1 mM IPTG 30ºC/8h	50 mL / TB	329.2 µg/L^{ly}	[78]
	α2b	BL21(DE3) (P_{lacUV5})	pET23-based + *TorA* signal peptide + Erv1p + hPDI + 6His	37ºC up to OD 0.5; 1 mM IPTG/ 20-30ºC/3h	50 mL / LB	*n.i.*	[79]
	αA	Rosetta (DE3) (P_{lacUV5})	pET32-based (P_{T7}) + 6His + lichenase	37ºC until OD 0.6; 0.5 mM IPTG 37ºC/16h	*n.i.* / LB	38% soluble^{ly}	[80]
Secretion to extracellular medium	mutated α2	EPEC *ΔsepD*	pIFN (P_{T7}) + *EspB* signal peptide	37ºC until OD 0.7; 0.25 mM IPTG	*n.i.* / LB + DMEM	*n.i.*	[81]

Legend: Different approaches to manufacture soluble alphainterferons in *E. coli*. [1]promoters in genome and plasmid are shown within parentheses; [2] yield of soluble IFN after lysis$^{(ly)}$ or after downstream processing$^{(dsp)}$; *n.i.*: not informed; OD: optical density at 600 nm; Complex: media, other than LB/TB, with undefined components (e.g., yeast extract); AutoInd: autoinduction media (e.g., with lactose for a strain harboring *lac* UV5 operon); ChemDef: chemically defined culture media.

Some groups expressed interferon consensus - IFNc or alfacon-1 (e.g., Infergen®) -, a 19.4 kDa synthetic molecule engineered with the 166 most prevalent aminoacids among IFNα subtypes. The highest yield reported for an alpha-IFN was accomplished with this molecule, following cultivation for one day at 25-30ºC employing a DE3 strain which was, additionally, codon-optimized for translation by *E. coli* [49]. An autoinduction medium containing lactose was used, so when cells consumed all glucose, the former sugar was internalized, then it induced *lac*UV5 and also worked as fuel for cell metabolism. This kind of medium exempts the need for constant monitoring of cell biomass (e.g., by measuring optical density at 600 nm) and adding IPTG [50].

Fanhong and Tengjie [51] were also helped by mild induction conditions, i.e., lower values for inducer concentration, step duration, and temperature. Following initial growth at 37°C, cultures were treated with 0.1 – 0.5 mM IPTG for just a few hours, resulting in the soluble expression of IFN-α1b from DE3 strains; in contrast, most studies with the usual 1 mM IPTG dose [52] at high temperatures (37ºC) resulted in insoluble IFN. Similarly, Kuruganti et al. [53] expressed six soluble and bioactive IFN-α subtypes using a codon-optimized BL21(DE3) strain by simply employing autoinduction medium at 20°C for approximately a day. Since a considerable number of successful cases employed autoinduction media, it is reasonable to speculate that these solutions may resemble the use of low inducer concentrations, as lactose may enter cells gradually as glucose is depleted.

Relying exclusively on the optimization of leaky expression using BL21(DE3) cells transformed with a non-specialized pET9a plasmid, Bretas et al. [54] recently expressed up to 23.5 mg/L of purified and bioactive IFN-α2a, largely in its soluble form and with native disulfide bonds. As the cytokine was constitutively produced from the very beginning in LB, initial growth was briefly conducted at 37ºC until the early-midlog phase, and then the culture was incubated at 16ºC for 48 h, without IPTG.

2.2 Customized strains

Some researchers relied on more specialized cells derived from *E. coli* B strains, which are deficient in the major proteases *ompT* and *lon*, able to degrade recombinant proteins [55]. Origami B was engineered with the genotype *trxB-/gor-*, so it is deficient in two main *E. coli* reductases, namely thioredoxin reductase and glutathione oxido-reductase [56]. 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 indicates in its very name the relation to -SH groups. These cells were derived from Origami B, but additionally synthesize the disulfide bond isomerase DsbC (also a chaperone) without its original signal peptide [57]. 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 [58] developed a *gor- trxB+* BL21 strain altered to resemble (DE3) and transformed it 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. Following induction with a high dose of IPTG for 8 h at 30ºC, Lin et al. [59,60] expressed soluble IFN-α2b using a pET26a plasmid, which displays the *pelB* signal sequence for translocation to the periplasm.

The use of codon-optimized strains was a smart choice in many published cases [36,47,49,54,61,62,63,64], as avoiding codon bias can significantly improve protein folding and solubility [52]. A recent comparison between gene design tools highlighted the importance of preventing rare codons to achieve high expression rates outside the gene's native context; it also reinforced that *E. coli*'s genome is CG-rich, so optimized sequences must have a high (51-64%) percentage of guanine and cytosine [65]. Another study showed IFN-α2 was expressed in a regular *E. coli* RV308 strain only after correcting the codon bias or when the host was replaced by strain BL21-CodonPlus(DE3)-RIPL [66].

2.3 Strength of the promoter

As depicted in Figure 1(b), 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. P_{BAD} is weaker

than T7, *lac*, and others (*tac*, *trp*, P_L, P_R), so transcription rates and the metabolic burden are lower, which may even allow cells to grow and express foreign proteins simultaneously [56]. Mohammed et al. [36] used a codon-optimized BL21 strain and delivered 45.8% of interferon-α8 in its soluble form, even at the high temperature of 37ºC, considering an induction for 6 h with 0.02 w/v of the sugar.

Two groups worked with *E. coli* strain MC1061, which is devoid of the T7 system. Chung & Jung [67] employed the vector pBAD18 carrying the sequence for IFNα2a and reported good soluble yields at 25ºC and 30ºC. Lim et al. [68] conducted both pre-culture and main fermentation at 37ºC, and still at such high temperature, the bioactive cytokine was mostly expressed in its soluble form when induced with 2.7 mM L-arabinose for 9 – 10 h; conversely, IBs were formed under high inducer dosage (10.8 mM) and longer induction hours.

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 part of extremely soluble polypeptides, and some may even act as chaperones or aid in affinity chromatography. The best approach seems to be their expression on the N-terminal portion so they can assist nascent proteins from the very beginning. Common examples include thioredoxin, glutathione-*S*-transferase (GST), maltose-binding protein (MBP), small ubiquitin-related modifier (SUMO), and N-utilizing substance A (NusA) [69].

Expressing human IFN fused to the abovementioned tags has clearly improved its solubility, but this strategy results in polypeptides with altered primary structures. Therefore, if the goal is to use the molecule as a biopharmaceutical without possible immunogenicity issues, fusion proteins must undergo an additional proteolytic step, as well as the removal of proteases, cleaved tags, residual unprocessed species, and chaperones.

There is no general rule on which tag yields the best outcomes for all proteins. Rabhi-Essafi and cols. [70] 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, reporting an expressive yield of 100 mg of purified protein per liter of medium. On the other hand, Vu et al. [63] 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 80% solubility of IFN-α2b in strain BL21(DE3). The host was first grown in LB at 37ºC for 5 h and then induced with 1 mM IPTG for 12 h at 18ºC. After purification, a final yield of 14.4 mg/L was achieved.

In 2021, Grabarz and colleagues [64] 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 worked with BL21(DE3) transformed with pET displaying a polyHistidine (6xHis) tag, 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 using medium with 0.1 mM IPTG and a DsbC (chaperone) tag.

Bis and her team [61] published a successful case using a BL21(DE3) strain transformed with a pET plasmid. They expressed IFNα2a fused to a 6xHis + SUMO tag on the N-terminal site; following initial cultivation at 37ºC, the culture was induced with a fifth of the usual IPTG dose and kept in a shaker for another 16 h under a much lower temperature (16ºC); the yield was 16 mg/L of purified interferon. The same genetic construct was used by another group who expressed ~80% soluble IFNα2 fused to Tα1, a small immunoadjuvant peptide [71]. The induction phase was performed with 0.5 mM IPTG at 37ºC for 4 h.

Peciak et al. [62] 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 4 h showed better results at 30ºC than at 16ºC, and a final yield of 50 mg/L.

Polyhistidine tags alone are suitable for purification with immobilized metal affinity chromatography, but their large hydrophobic side chains might enhance the insolubility of a target protein [55].

2.5 Co-expression of chaperones

A cytoplasmic disulfide formation system was tested in small plates, employing a single plasmid coding for IFNα2b, the thiol oxidase Erv1p, and human protein disulfide isomerase (hPDI), in a codon-optimized strain. When compared to SHuffle, a high yield was obtained with autoinduction medium (either rich or chemically defined), most likely because SHuffle just supplies the isomerase DsbC in a non-reducing environment, but lacks an oxidase to turn SH into S-S [72].

Previously, the same group had reported success when pre-expressing Erv1p and DsbC from a separate plasmid induced by arabinose, first creating a favorable scenario for the following expression of IFNα2 from an IPTG-induced vector [73]. The team argued that supplying the disulfide formation system (sulfhydryl oxidase + isomerase) led to better results than just impairing *E. coli*'s reductive pathways.

2.6 Export to the periplasmic space

A few researchers engineered alphainterferon to be translocated via the *Sec* pathway, in which unfolded proteins reach the periplasm, where local enzymes and the oxidative environment allow the formation of disulfide bonds and correct folding [74]; nonetheless, this pathway still delivers poor yields. Voss and colleagues [38] reported the use of strain W3110 with the sequence for IFNα2c, which is a minor allelic form that displays arginine in positions 23 and 34 [75]. The vector displayed the heat-stable enterotoxin II (*STII*) as a fusion protein, which served 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 [76]; strains were cultivated in LB and then induced with IPTG for 3 h. In a separate study [77], moderate-temperature cultivations at 20-25ºC additionally helped the production of soluble periplasmic IFN-alpha1b, working with BL21(DE3) transformed with a pET plasmid and using 1 mM IPTG.

Two groups combined the enhanced strain Rosetta-gammi 2 (DE3) with the plasmid pET26a, coding for the peptide *pelB* for periplasmic translocation of IFN-α2b. Tan et al. [78] bet on a statistical analysis to study medium optimization, in which baffled shake flasks underwent cultivation at 37ºC for 4 h and then induction at 30ºC for 8 h with 1 mM IPTG, delivering soluble interferon in the periplasmic space, with best results when the medium was formulated with low glucose and high concentrations of yeast extract and peptone. The other case was previously described in section 2.2 [59,60].

In a different approach, Alanen et al. [79] chose the periplasmic route just to facilitate the purification step rather than relying on its oxidative environment. The team targeted human IFN-α2b via the *Tat* secretory pathway, which recognizes correctly folded proteins. They co-expressed the cytoplasmic disulfide bond-forming duo Erv1p + hPDI, resulting in the cytokine's native form, which was then exported by an appropriate signal peptide. Despite the success, recombinant proteins still have limited storage space in the periplasm compared to expression in the cytosol or even secretion to the extracellular medium.

Tyurin and cols. [80] inserted IFN-αA (166 aminoacids) into a domain of the lichenase from *Clostridium thermocellum*, a solubility enhancer, using a pET32-based plasmid with a translocation signal peptide in a Rosetta (DE3) line. All upstream step was conducted at 37ºC, then induction was performed with 0.5 mM IPTG for 16 h. Following purification, 38% of the fusion protein was found soluble in the periplasmic space, while 11% was soluble in the cytoplasm.

2.7 Secretion to the extracellular medium

Rostovsky and his group [81] fused a mutated form of IFN-α2 to a signal peptide that enabled export to the cultivation medium via the type III secretion system, using an enteropathogenic strain of *E. coli* (EPEC). Further assays demonstrated interferon's biological activity. Nonetheless, the study lacks data on the manufacturing scale and the yield of recombinant protein.

A critical analysis of all reviewed articles and patents with IFN-α in *Escherichia coli* allows us to organize the strategies as depicted in Figure 2. The cytokine's solubility is related to the genetic design of the expression system (strain + plasmid) and induction parameters.

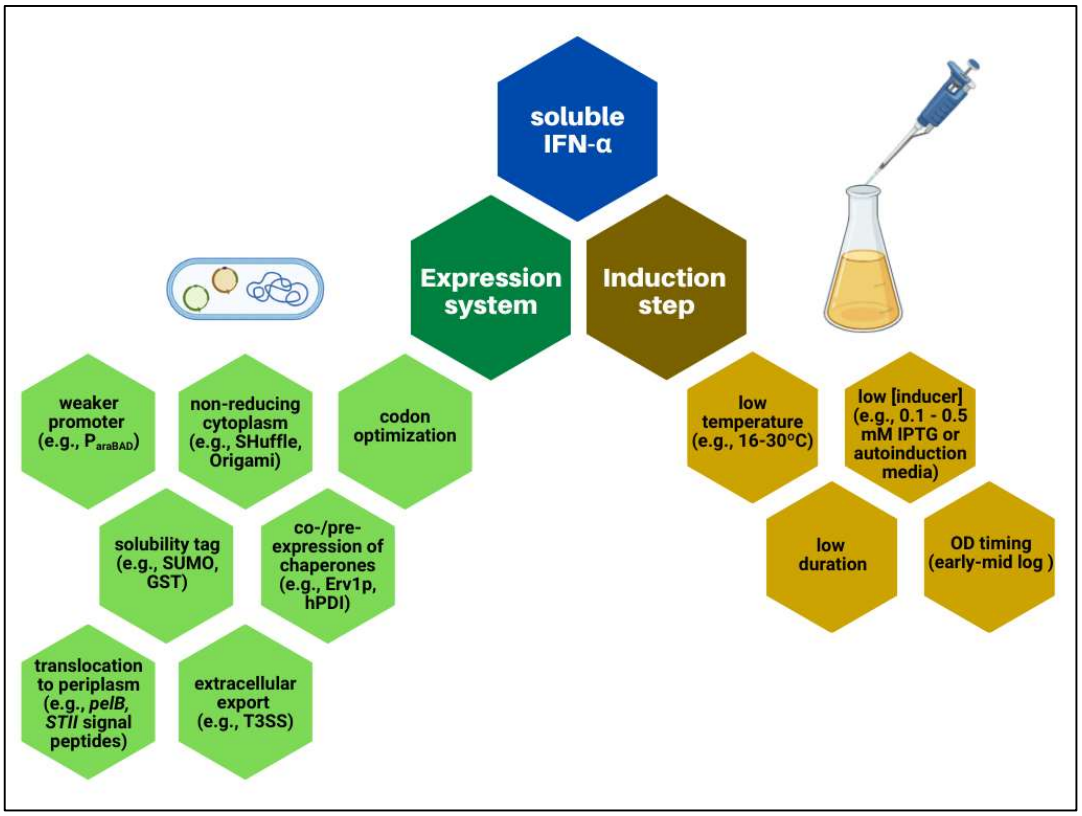

Figure 2: **Schematic diagram comprising the main strategies to improve soluble expression of alphainterferon in *E. coli.*** Genetic features of the strain and plasmid can favor the formation of disulfide bonds and correct folding. Upstream conditions, specifically during induction, are the easiest way to customize variables to favor soluble expression.

3. Downstream processing

3.1 Primary recovery: harvest and lysis

As the target product is mainly intracellular (only [81] exported IFN to the medium), teams harvested cells by centrifugation and a couple of them used microfiltration to remove debris before and/or after lysis [33,54,62].

Most protocols lysed bacterial pellets by lab-scale sonication [36,47,49,58,63,70,71], alternatively aided by lysozyme [62,68], but the use of this enzyme also means an additional process-related impurity to be removed. Mechanical methods encompassed homogenizers [33,38,67,80]. These procedures provoke total cell breakage that releases more contaminants such as gram-negative's lipopolysaccharides (LPS or endotoxins) [74], as well as host cell nucleic acids and non-target proteins. When cells were disrupted in acidic solutions, almost all IFN was lost in the insoluble fraction [54]; this phenomenon was also detected for other recombinant proteins that co-precipitated or got trapped in the generalized precipitation of *E. coli* proteins at low pH [82].

Following cell lysis, two teams [33,38] treated the product with polyethylenimine or protamine sulphate to remove nucleic acids. As discussed by Gundinger and Spadiut [82], this flocculation may be beneficial for removing cells, debris, DNA, endotoxins, and colloidal proteins.

Periplasmic IFN might be more easily purified since fewer bacterial contaminants are released in the absence of a full cell breakage [83] and because gentler extraction techniques may be used. 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 [59,60,76,78], osmotic shock with lysozyme [79] or acidic treatment [38].

Other groups reported cell lysis by freeze-thaw cycle(s), either by itself [54] or aided by lysozyme [46,72]. 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 [84]. 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 [85]. Unfortunately, scaling up this thermal technique remains challenging for the industry.

3.2 Purification: column and membrane-based operations

Bioaffinity chromatography was the obvious choice to purify IFN fused to 6xHis tails and solubility enhancers that also acted as affinity tags [61,62,63,64,70,71,72,79]. 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 [61,62,63,64]. A couple of studies reported affinity columns displaying antibodies covalently linked to the resin [17,38]. Lastly, Kwon et al. [76] reported blue Sepharose as the capture affinity resin.

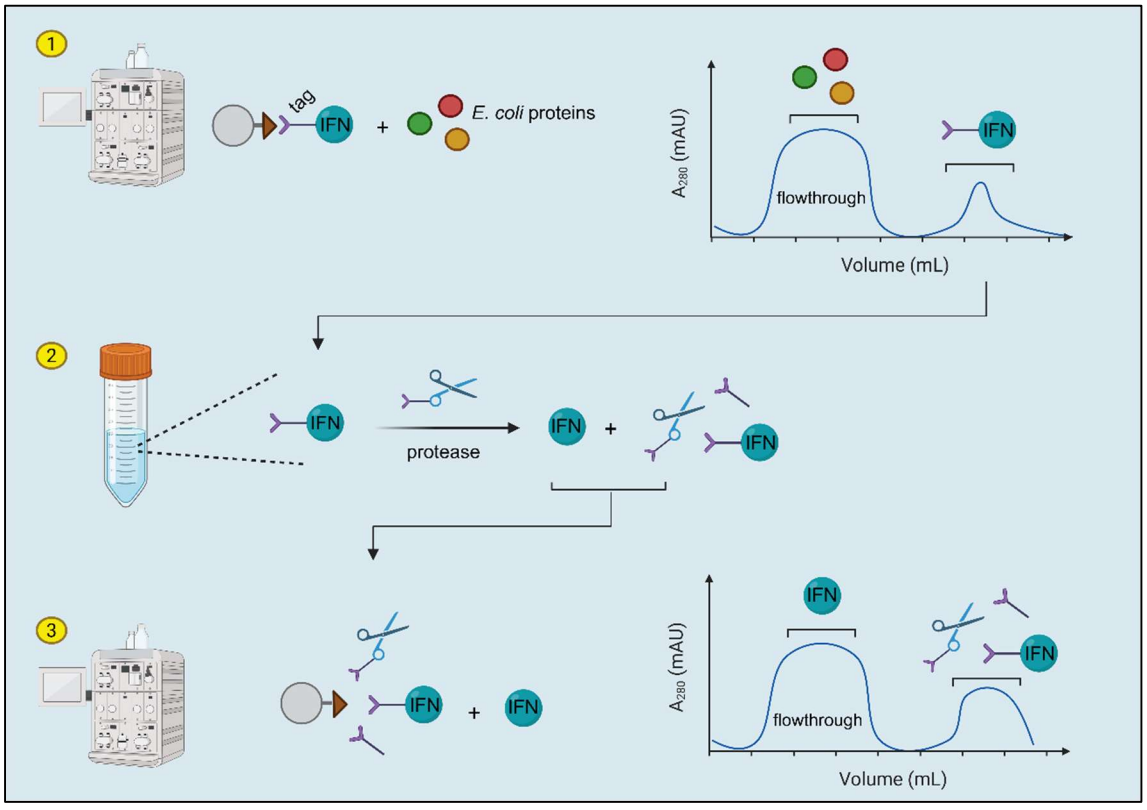

Figure 3: Purification of alphainterferon by affinity chromatography. (1) IFN is connected through a protease recognition site to an affinity tail (e.g., 6xHis or SUMO) that binds a ligand (brown arrow) on a chromatographic resin (gray sphere) while *E. coli* contaminating proteins are cleared away in the flowthrough. (2) Next, in a separate reaction, a protease cleaves the fusion tag, but some residual [IFN+tag] remains unprocessed. The protease itself is fused to the same tag, but lacks the hydrolysis sequence. (3) Finally, the pool goes through a second affinity chromatography, but now only tagged species are retained while free IFN is collected as the flowthrough. Created with BioRender.

Almost all studies used lysis buffers based on phosphates and/or Tris with pH 7 – 8, alternatively with detergents (Triton) to destabilize membranes, besides protease inhibitors, even when working with B strains. IFNα2's isoelectric point 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-affinity purification technique. Resins based on DEAE and Q-Sepharose were either the only ones used [36,49] or part of a sequence composed of two [47,48,54,63], three [51,64,76], or even four chromatographic steps [33,38,67].

Cationic exchange using functional groups carboxymethyl and sulphopropyl was performed with pH ≤ 5.3 and the target molecule positively charged [33,38,51,67,76], thus allowing removal of anionic contaminants along flowthrough, such as LPS and residual DNA. Eluting with shallower gradient slopes helped isolate IFNα2 monomers from aggregates and contaminating proteins displaying distinct positive charge intensities [54].

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 molecules (IPTG, protease inhibitors) are also likely to be cleared in steps with membranes, such as dialysis [38,54,61,64,67,71,76,80] and ultrafiltration [33,47,48,62,67,80]. These operations were performed in reviewed studies, respectively, to exchange buffers and concentrate alphainterferon. Diafiltration was not cited.

Size exclusion chromatography (SEC) was also employed [33,47,51,70], but mostly as a final polishing stage to desalt and exchange buffers. Only a few examples applied it as a main purification step *per se* to remove fusion tags, proteases, and accessory proteins, and it could even be replaced by ultrafiltration in one study [67].

Another relevant approach included hydrophobic interaction chromatography with phenyl/butyl Sepharose or silica columns [33,38,48,67,77]. One group [42] 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 IFNα2 is water-soluble, it 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 by the addition of ammonium sulphate.

Minor strategies included purifying periplasmic IFN-α2b by differential partitioning in aqueous two-phase systems [59,60]. After osmotic shock, the team employed the water-soluble polymer PEG and potassium phosphate, achieving a purification factor (Pf) of 26.3 and yield of 40.7%. Next, they tested a system composed of alcohol and salt, reaching Pf = 16.24 and yield = 74.64%. The molecule was analyzed by SDS-PAGE, but there was no mention of its biological activity or the correct tertiary structure.

4. Testing

SDS-PAGE was the most common method to monitor IFNα in soluble vs. insoluble fractions and throughout downstream processing. Interferon's identity, concentration, and purity were inferred by comparison to standards (analytical or other proteins). Immunological methods were used in several cases - western blotting [38,54,70,71,76,80] and ELISA [36,70].

When it comes to the impurity profile of the final product, groups reported at least 95% pure IFN according to electrophoretic methods; RP- and SEC-HPLC analyses were also performed [38,54,62,64]. Few teams measured residual endotoxins [33,36,63,67] and host cell DNA [33,54]; these are important impurities that must follow regulatory limits if the molecule is intended to become a licensed biopharmaceutical [15].

All but three publications [59,60,78] reported bioassays to demonstrate *in vitro* activity of alphainterferon, therefore implying that its 3D structure was correct [86,87]. Antiviral assays based on the literature [15,88] were the first choice, followed by antiproliferative tests using tumoral lines [36,49,61,71] and reporter gene assays [63,70]. Results were displayed 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.

Few research teams performed further analyses to confirm purified IFN's structural features [38,61]. Methods included circular dicroism to certify the predominant α-helical secondary structure of interferons, NMR to verify the tertiary arrangement, analytical centrifugation to assess the aggregation state, LC coupled with mass spectrometry (MS) for tryptic mapping, and MALDI-TOF [62] to check the precise molecular weight. Peptide

mapping coupled to MS was employed by two teams [38,54] to prove the presence and native position of disulfides after protease digestion. Data on purification and analytical testing are summarized in Table 2.

Table 2: Downstream processing and testing.

Purification technique / Quality control		Description	References
Purification technique	Microfiltration	Removal of debris	[33,54,62]
	Ultrafiltration	Concentration of target protein	[33,47,48,62,67,80]
	Dialysis	Buffer exchange	[38,54,61,64,67,71,76,80]
	Bioaffinity	Separation by binding to ligand	[17,38,61,62,63,64,70,71,72,76,79]
	Anion exchange	Separation by net superficial charge	[33,36,38,47,48,49,51,54,63,64,67,76]
	Cation exchange		[33,38,51,54,67,76]
	Size exclusion	Desalting, buffer exchange, separation by hydrodynamic raius	[33,47,51,67,70]
	Hydrophobic interaction	Separation by interaction with apolar residues	[33,38,42,48,67,77]
	Aqueous two-phase system	Partitioning between immiscible solutions	[59,60]
Qualty control			
	SDS-PAGE	Identity, purity, concentration	Nearly all
	HPLC	Identity, purity, concentration	[38,54,62,64]
	Bioassays	Functionality, potency	Nearly all
	Immunoassays	Identity, purity, concentration	[36,38,54,70,71,76,80]
	Circular dicrosim	α-helix pattern	[38,61]
	Mass spectrometry	Identity, disulfide pattern	[38,54,61,62]

Legend: Main methods used to purify and test alphainterferons.

5. Further opportunities

Some strategies from other articles may be applied to IFNα in future studies, either on their own or in combination with techniques reviewed herein. When designing the expression vector, replication origins (*ori*) such as p15A deliver low plasmid copy numbers that reduce metabolic burden and may result in higher protein yields. In addition, if such *ori* is associated with weaker promoters (P_{BAD}), higher amounts of soluble proteins may be achieved [89].

Although reported for other soluble heterologous proteins, *Arctic Express (DE3)* [35] was not chosen as the host in any of the reviewed studies. Considering the strain's ability to express cold-adapted chaperones that show high refolding activity at up to 4ºC [35] and the low metabolic burden at this temperature, these cells are a promising candidate to express aggregation-prone proteins [52]. Furthermore, the co- or pre-expression of other chaperones could also be tested, such as Ero1α and QSOX oxidases, PDIs from different species, GroEL/ES, DnaK-DnaJ-GrpE, and trigger factor (TF). Finally, exportation to the extracellular medium via the type II secretion system has not been explored with alphainterferons thus far.

In the upstream stage, supplementation of culture media or lysis buffers with chemical chaperones and cofactors may improve solubility and bioactivity by protecting proteins and increasing the amount of osmolytes and chaperones. Examples include sugars, polyols, glycerol, small thiols, DMSO, and aminoacids, as suggested by some general reviews on recombinant proteins [35,44,52,55] and difficult-to-express targets [90].

Concerning purification techniques, no study with IFNα has tried ceramic hydroxyapatite chromatography and membrane chromatography yet. 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 [26]. Moreover, researchers neither explored stepwise elution schemes (only linear gradients), which may be more efficient in an industrial context; nor the strategy of buffers with pH gradients to modify IFN's charge along elution. Nonetheless, downstream steps were more related to the final purity and yield than to the cytokine's solubility.

6. Conclusions and Future Trends

Based on the reviewed cases, we conclude that IFN-α is not a difficult-to-express protein *per se* in the absence of codon bias; however, it is challenging to be expressed in its soluble form under common cultivation practices, i.e., 37°C using strong promoters with ≥ 1 mM IPTG. Its solubility is directly related to the genetic features of the expression system (cell line + plasmid) and conditions of the induction step. Rather than isolated interventions, many groups succeeded by adopting multiple effective strategies simultaneously, such as choosing a codon-optimized strain with an oxidizing cytoplasm, engineered to co-express a solubility fusion tag or a chaperone, inducing at early-midlog phase under mild conditions, and disrupting cells in neutral or alkaline buffers. Among the cases assessed, exportation to the periplasm and extracellular medium showed the least promising yields.

Despite all cases analyzed, biotech industries currently still manufacture alphainterferons as inclusion bodies, and some argue that the large amounts achieved might compensate for the time and costs involved in processing these denatured proteins. However, the theoretical advantage of IBs being protected from bacterial proteases may be easily compensated by using inhibitors and Δ*lon*/*ompT* strains, as well as purifying proteins fast and under refrigeration. Some unexplored strategies might still improve manufacturing; probably, the execution of well-planned pharmacoeconomic studies would reveal, for each specific bioprocess, whether it is more profitable to express the target IFN in its soluble or insoluble form.

Conflict of interest

The authors declare no conflict of interest.

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Data availability statement

All data are available in this manuscript text.

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Authors' contribution statement (CRediT)

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

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