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

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Title

Strategies for manufacuring 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 able to synthesize 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 synthesized 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 interferon-α in its soluble and functional form, including optimization of culture conditions and induction, use of engineered strains, fusion partners that enhance solubility, and translocation to the periplasm, among others. Also, we have assessed downstream processing and analytical techniques that assured 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, chromatography.

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 [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], besides 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 expressed it in complex, slow, and expensive eukaryotic systems such as yeast [18] and mammalian cell lines [13]. 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 mainly 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 [20], and safety [21,22]. This is possible because this cytokine is one of the few cases where such post-translational modification (PTM) is not essential for the recombinant protein's activity and stability [23,24].

On the other hand, disulfide bonds are often the most critical PTM and essential for the proper folding, solubility, stability, and functionality of proteins [25], as is the case with interferon's structure [26]. IFN-α2, for instance, displays two conserved bonds between cysteines 1-98 and 29-138, and the latter is essential for its potency [27], as seen in a study where the molecule incubated with reducing agents lost all biological activity [8]. *In vivo*, 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 S-S.

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, hence it can bind the same receptors targeted by alpha subtypes, but it is an N-glycosylated protein which needs to be synthesized by eukaryotic cells [17,28], generally the mammalian line CHO [29]. 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 [30,31].

1.2.2 N-terminus heterogeneity

In vivo, the start codon AUG not only signals the beginning of translation, but also assures 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 reported that its action is directly favored when the neighboring aminoacid displays smaller side chain and radius of gyration [32,33], which is the case of Cys_1 in IFNα2.

On the other hand, there are reports about MAP's inefficiency, 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 the molecule's immunogenicity and stability. Biological activity may also be affected if the N-terminus is linked to receptor-binding and resistance to proteases [34].

Possible solutions include the co-expression of MAP in *E. coli* or the manufacturing in yeasts [35]. Sharma et al 2016 [36] reported the expression of IFNα2b with less initial Met simply by conducting the fermentation at low

temperatures (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 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 [21,19, 37]. 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 *lac*I 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 [38]. 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 [39,40], as depicted in Figure 1(b), or by limitation of a nutrient [41]. Additionally, the final recombinant protein may either be located in the cytosol or be translocated to the periplasmic space.

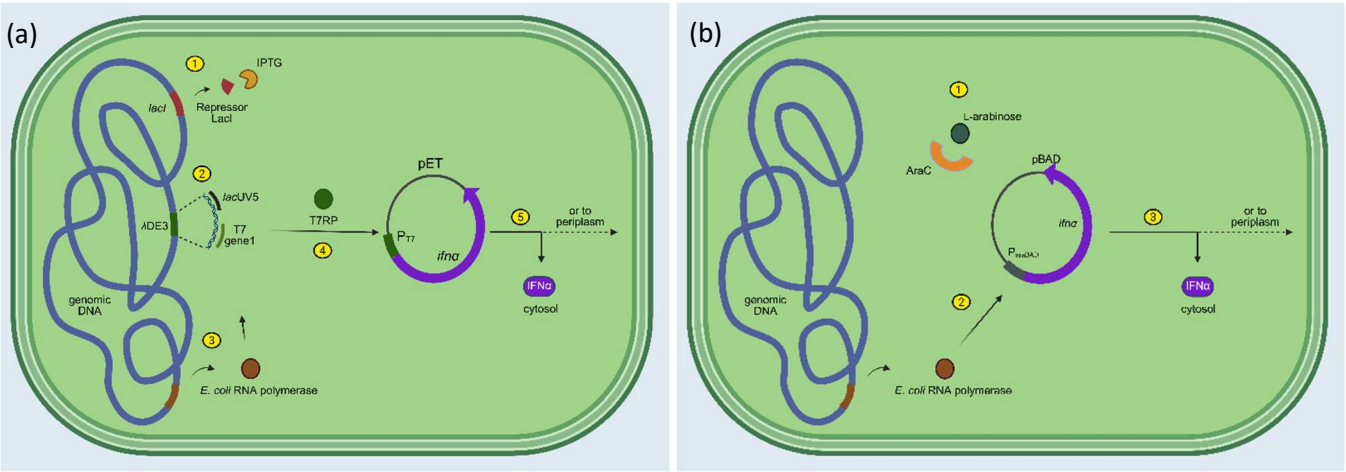

Figure 1: **Simplified two main pathways for the expression of alphainterferon in *E. coli* reviewed in this paper. (a)** IPTG binds repressor LacI (1), which stops blocking the promoter *lac*UV5 (2), thus allowing *E. coli* RNA polymerase (3) to read phage T7 gene 1. This leads to the synthesis of T7RP (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 present in the cytosol or exported to the periplasmic space, if fused to an appropriate signal peptide. 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 [42]. 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 precipitate as amorphous inclusion bodies (IBs) in the cytoplasm [6].

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 their original biological activity [43]. A lot can go wrong in these steps, and one may generate proteins incorrectly folded, which are difficult to remove during purification steps. Overall, renaturation of proteins is the most troublesome and expensive phase when dealing with *E. coli* bioprocesses [44]; up to 50% of the expressed polypeptide may be lost in such step [45].

This manuscript reviews strategies that influenced the soluble expression of human recombinant IFNα in *E. coli*, and thus provides a starting point for researchers attempting to optimize the conditions for expressing the same or a similar target protein using such host.

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 the upstream stage may do so by reducing the common overexpression that occurs at 37ºC [44]. 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 folding machinery; (b) inhibit hydrophobic interactions between apolar side chains in aminoacids, therefore minimizing protein aggregation; (c) partly eliminate heat-shock proteases; (d) increase the activity of *E. coli*'s chaperones [46,47], which are proteins that help others achieve their correct structure; and (e) convert the activity of some enzymes from proteolytic (at high temperatures) into chaperone-like [48].

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 [49]. 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.

In 2016, Sharma et al. [36] reported using the strain BL21 Gold, which is improved in transformation efficiency and shows less degradation of plasmid 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 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.

Zhang and colleagues [50] 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 deliver 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 regular concentration of 1 mM IPTG at a lower temperature (30ºC) for 6h.

Yan [51] produced the rather uncommon interferon alpha-4, which is highly expressed in peripheral blood mononuclear cells to combat viruses and tumors. 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 was conducted for 12-16h at 15-18ºC, resulting in part 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 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 exhibits optimized codons for translation by *E. coli* [52]. An autoinduction medium containing lactose was used, so that when cells consumed all the glucose, the former sugar was internalized and used as fuel, in addition to prompting *lac*UV5. 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 [53].

Lighter induction conditions also helped Fanhong and Tengjie [54]. Following an initial growth at 37 °C, cultures were treated with low concentrations of IPTG (e.g., 0.1–0.5 mM) for a few hours, which allowed the expression of IFN-α1b from DE3 strains; in contrast, most studies with the usual 1 mM IPTG dose [55] at high temperatures (37ºC) resulted in insoluble IFN. Similarly, Kuruganti et al. [56] 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.

Relying exclusively on the leaky expression of BL21(DE3) cells transformed with a non-specialized pET9a plasmid, Bretas et al. [57] 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 the early-midlog phase, and then the culture was incubated at 16ºC for 48h, 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 [58]. 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 [47]. 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 [25,59]. 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 [60] 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. Following induction with a high dose of inducer for 8h at 30ºC, Lin et al. [61,62] 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 [39,50,52,57], as avoiding codon bias can significantly affect protein folding and improve solubility [55]. 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 [63]. Another study showed IFN-α2 was unexpressed in a regular *E. coli* RV308 strain even when exported to the periplasm; the cytokine was only detectable after correcting the codon bias and/or expression in the strain BL21-CodonPlus(DE3)-RIPL [64].

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 [65]. 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 allows cells to grow and express foreign proteins simultaneously. Mohammed et al. [39] used a codon-optimized BL21 strain and delivered45.8% 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.

Table 1: Expression of soluble alphainterferon.

Strategy	IFN subtype	*E. coli* strain[1] (and genomic $P_{romoter}$)	Plasmid[1] (and $P_{romoter}$)	Culture (growth; induction)	Scale / Medium	Yield[2]	Reference
Customization of upstream step	α2b	BL21 Gold(DE3) (P_{lacUV5})	pET20b (P_{T7})	30-40ºC; 0.2 - 1 M IPTG 22ºC/25h	Complex	~1.5 g/L^{ly}	[36]
	α2	HB101 and DS410	pP1T7α2 (P_{T7}); pNeo-cop-α2 (P_{colE1}) or pM215a (P_{Amp})	30ºC/20-24h or 44h (Neo-cop-α2)	LB	73-85%^{ly}	[49]
	α2b	BL21(DE3) (P_{lacUV5})	pET43 (P_{T7})	37ºC up to OD 0.8; 1 mM IPTG 30ºC/6h	5 mL LB (test tubes)	≥ 45% soluble^{ly}	[50]
	α4	BL21(DE3) (P_{lacUV5})	pTYB11 (P_{T7})	32ºC; IPTG 15-18ºC/12-16h	2 - 5 L LB (bioreactor)	> 30% soluble^{ly}	[51]
	alfacon-1	BL21-CodonPlus(DE3) (P_{lacUV5})	pET101/D-TOPO (P_{T7})	lactose, 25-30ºC/24h	1 L, AutoInd (shake flask)	70% soluble^{ly} 270 mg/L^{dsp}	[52]
	α1b	BL21(DE3) and JM109(DE3) (P_{lacUV5})	pEAM2 (P_{T7})	37ºC up to OD 1; 0.2 mM IPTG/4h	800 mL LB (shake flask)	> 30% soluble^{ly}	[54]
				37ºC up to 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}	[56]
	α2a	BL21(DE3) (P_{lacUV5})	pET9a (P_{T7})	37ºC up to OD 0.4; 16ºC/48h (no inducers)	150 mL LB (shake flask)	≥ 63% soluble^{ly} 16 mg/L^{dsp}	[57]
Specialized strain	α2a	BL21 (*gor- trxB+*) (P_{lacUV5})	pET26b(+) (P_{T7})	37ºC up to OD 0.5-0.8; 1 mM IPTG/3h	15 mL, Complex (shake flask)	*n.i.*	[60]
	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}	[61,62]

Strength of promoter	α8	BL21-CodonPlus (DE3)-RIPL (P_{lacUV5})	pBAD-TOPO (P_{BAD})	37ºC up to OD 0.6 - 0.8; 0.02% (w/v) arabinose, 37ºC/6h	100 mL M9 (shake flask)	46% soluble^{ly} 100 mg/L^{dsp}	[39]
	α2a	MC1061	pBAD18 (P_{BAD})	30ºC/3h; 1% arabinose 30ºC/11h	20 L, Complex (bench bioreactor)	8 mg/L^{dsp}	[66]
	α (*n.i.*)	MC1061	pΔMa (P_{arab})	37ºC up to early log; 2.7 mM arabinose/9-10h	1 L TB + M9 (bioreactor, high cell density)	~ 80%^{ly} (~5% total cell protein)	[67]
Solubility tag	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}	[47]
	alfacon-1	BL21(DE3) (P_{lacUV5})	pET28a(+) (P_{T7}) + 6His-Fh8 tag	25-30ºC up to OD 0.6; 30ºC/6h	100 mL AutoInd	8 mg/L^{dsp}	[68]
	α2b	BL21(DE3) (P_{lacUV5})	pUC57-derived plasmid + MBP tag	37ºC up to OD 0.5; 1 mM IPTG 18ºC/12h	500 mL LB	80% soluble^{ly} 14.4 mg/L^{dsp}	[69]
	α2a	BL21(DE3) (P_{lacUV5})	pET28a (P_{T7}) + 6His-SUMO tag	37ºC up to OD 1.0; 0.2 mM IPTG/16ºC/16h	1 L LB	16 mg/L^{dsp}	[70]
	α2-Tα1	BL21(DE3) (P_{lacUV5})	Champion® pET (P_{T7}) 6His+ SUMO tag	37ºC up to OD 0.7; 0.5 mM IPTG 37ºC/4h	50 mL	80% soluble^{ly}	[71]
	alfacon-1	SHuffle T7 (P_{lacUV5})	Champion® pET (P_{T7}) 6His + SUMO tag	37ºC up to OD 5 - 7; 0.1 - 1 mM IPTG 37ºC/4h	500 mL TB	87% soluble^{ly} 50 mg/L^{dsp}	[72]
Coexpression of chaperone(s)	α2b	BL21(DE3) (P_{lacUV5})	pGZ10 (P_{T7}) 6His tag + Erv1p + hPDI	30ºC/24h or 40h autoinduction medium (rich or defined)	24 x 2 mL, AutoInd (deep well plates)	33,3 mg/L^{dsp}	[26]
	α2	Origami B (P_{lacUV5})	pET23 (P_{T7}) 6His or MBP tag + IFN pLysS (P_{aran}) + Erv1p + hPDI	30ºC up to 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	[41]
	α2a and α2b	BL21(DE3) (P_{lacUV5})	pET14b (P_{T7}) + *STII* signal peptide	IPTG 3h	LB	*n.i.*	[75]
	α1b	BL21(DE3) (P_{lacUV5})	pET22b(+) (P_{T7}) + *pelB* signal peptide	36-38ºC up to OD 1.0; 1 mM IPTG 20-25ºC/12-20h	ChemDef	*n.i.*	[76]
	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}	[77]
	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.*	[78]
	αA	Rosetta (DE3) (P_{lacUV5})	pET32-based (P_{T7}) + 6His + lichenase	37ºC up to OD 0.6; 0.5 mM IPTG 37ºC/16h	*n.i.*	38% soluble^{ly}	[79]
Secretion to extracellular medium	α2 mutated	EPEC *ΔsepD*	pIFN (P_{T7}) + *EspB* signal peptide	37ºC up to OD 0.7; 0.25 mM IPTG	LB + DMEM	*n.i.*	[80]

Legend: [1]promoters in genome or plasmid are shown within parentheses; [2] soluble IFN after lysis$^{(ly)}$ or downstream processing$^{(dsp)}$; *n.i.:* not informed; *OD:* optical density at 600 nm

Complex: media with undefined components (e.g., yeast extract); AutoInd: autoinduction media (e.g., with lactose for a strain with *lac* UV5 operon); ChemDef: chemically defined culture media.

Two groups worked with *E. coli* strain MC1061, which is also devoid of the T7 system. Chung & Jung [66] employed the vector pBAD18 carrying the sequence for IFNα2a and reported good soluble yields at 25ºC and 30ºC. Lim et al. [67] 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 induced with 2.7 mM L-arabinose for 9 – 10h; conversely, IBs were formed under high inducer concentrations (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 bioaffinity chromatography. The best approach seems to be their expression on the N-terminal portion so they can assist from the very beginning of protein synthesis; the disadvantage is that these tags generally deliver low protein yields [68]. Common examples include thioredoxin, glutathione-*S*-transferase (GST), maltose-binding protein (MBP), small ubiquitin-related modifier (SUMO, derived from yeast), and N-utilizing substance A (NusA) [47].

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. Therefore, 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 yields the best outcomes. Rabhi-Essafi and cols. [47] 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. [69] 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 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 achieved.

In 2021, Grabarz and colleagues [68] 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 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 with medium plus 0.1 mM IPTG and a DsbC (chaperone) tag.

Bis and her team [70] published a successful case using a BL21(DE3) strain transformed with a pET plasmid. They expressed IFNα2a fused to a polyHistidine (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 16h under a much lower temperature (16ºC); the yield was 16 mg/L of purified interferon. The same genetic construct was able to express ~80% soluble IFNα2 fused to Tα1, a small immunoadjuvant peptide [71]. The induction step was stimulated by 0.5 mM IPTG at 37ºC for 4h.

Peciak et al. [72] 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 6His tag. Induction with 0.1 – 1 mM IPTG for 4h 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 bioaffinity chromatography (IMAC), but their large hydrophobic side chains might enhance the insolubility of a target protein [58].

2.5 Co-expression of chaperones

A cytoplasmic disulfide formation system was tested in small plates, employing a single plasmid coding for IFNα2a, the thiol oxidase Erv1p, and human protein disulfide isomerase (hPDI), using a codon-optimized strain. When compared to SHuffle, a high yield was obtained with autoinduction medium (either rich or chemically

defined), most likely because the SHuffle just supplies the isomerase DsbC in a non-reducing environment, but lacks an oxidase to turn SH into S-S [26].

Previously, another group 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]. Both groups 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, wherelocal 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 [41] 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 [17]. 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 [75]; strains were cultivated in LB and then induced with IPTG for 3h. In a separate study [77], low temperature cultivations at 20-25ºC additionally helped the production of soluble periplasmic IFN-alpha1b, working with BL21(DE3) transformed with a pET plasmid and induced with 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, besides the gene for IFN-α2b. Tan et al. [77] bet on a statistical analysis to study medium optimization, in which 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 formulated with low glucose and high concentrations of yeast extract and peptone. The other case was previously described in section 2.2 [61,62].

In a different approach, Alanen et al. [78] chose the periplasmic route 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 into the extracellular medium.

Tyurin and cols. [79] inserted IFN-αA (166 aminoacids) into a domain of the uncommon 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, and induction was performed with 0.5 mM IPTG for 16h. 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 [80] 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* (ETEC). 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 more related to the genetic design of the expression system (strain + plasmid) and induction parameters.

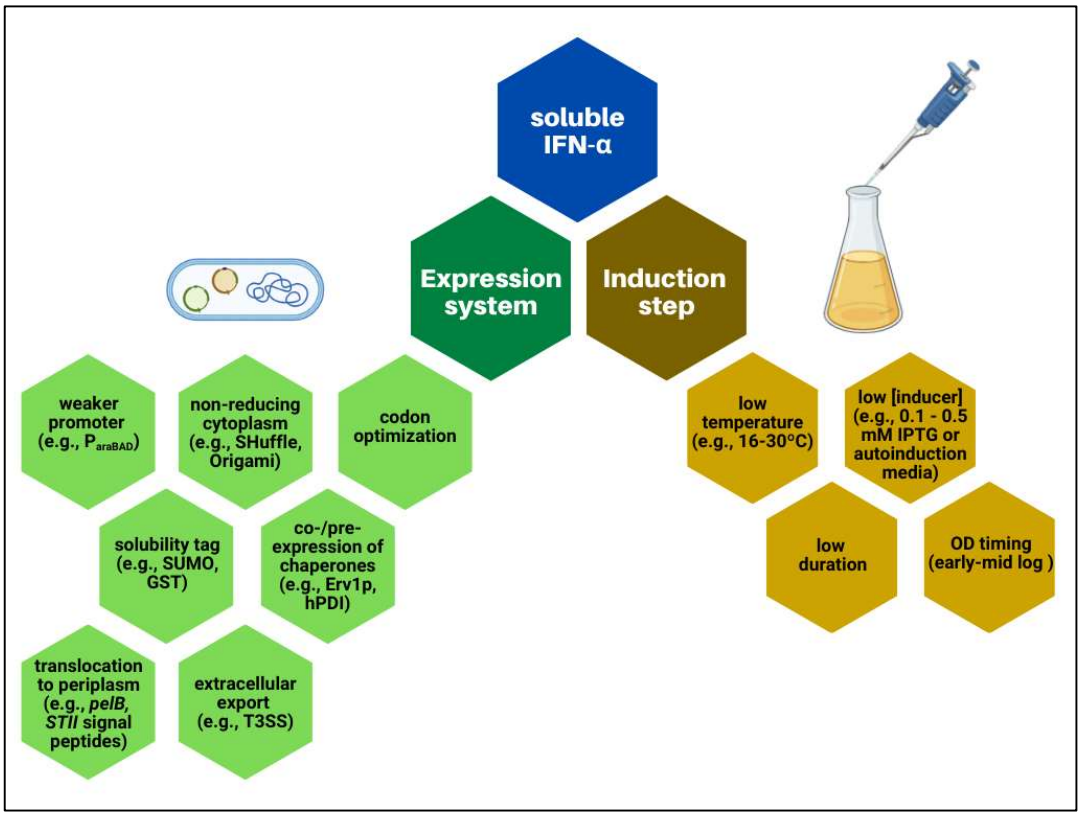

Figure 2: **Schematic diagram comprising the main strategies applied to improve soluble expression of alphainterferon in *E. coli.*** The genetic features of strain and plasmid can favor the formation of disulfide bonds and correct folding. Upstream conditions, specifically during induction, are the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

3. Downstream processing

3.1 Primary recovery: harvest and lysis

As the target product is mainly intracellular (only one group exported it to the medium), teams harvested cells by centrifugation and a couple of them used microfiltration to remove debris [36,72] after lysis.

Most protocols lysed bacterial pellets by lab-scale sonication [39, 47,50,52,60,69,71], alternatively aided by lysozyme [67,72], but the use of this protease also means an additional process-related impurity to be removed. Mechanical methods encompassed homogenizers [36,66]. These procedures provoke total cell breakage that releases more contaminants such as gram-negative's lipopolysaccharides (LPS), also named endotoxins [74], as well as host cell nucleic acids and non-target proteins.

Following cell lysis, just Sharma et al. [36] treated the product with polyethyleneimine (PEI) or protamine sulphate to remove nucleic acids. However, as noted by Gundinger and Spadiut [81], flocculation may be beneficial in removing cells, debris, DNA, endotoxins, and colloidal proteins. Periplasmic IFN might be more easily purified since fewer bacterial proteins are freed in the absence of a full cell breakage [76] 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 [61,62,75,77], osmotic shock with lysozyme [78] or acidic treatment [41].

Some groups reported cell lysis by freeze-thaw cycle(s), either by itself [57] or aided by lysozyme [26,49]. 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 [82]. 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 [83]. 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 poly(His) tails and solubility enhancers that also acted as affinity tags [26,47,68,69,70,71,78]. 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 [69,68]. In the absence of fusion partners, older studies reported affinity columns displaying specific anti-IFNα antibodies covalently linked to the resin [17]. Lastly, Kwon et al. [75] reported blue Sepharose as the capture affinity resin.

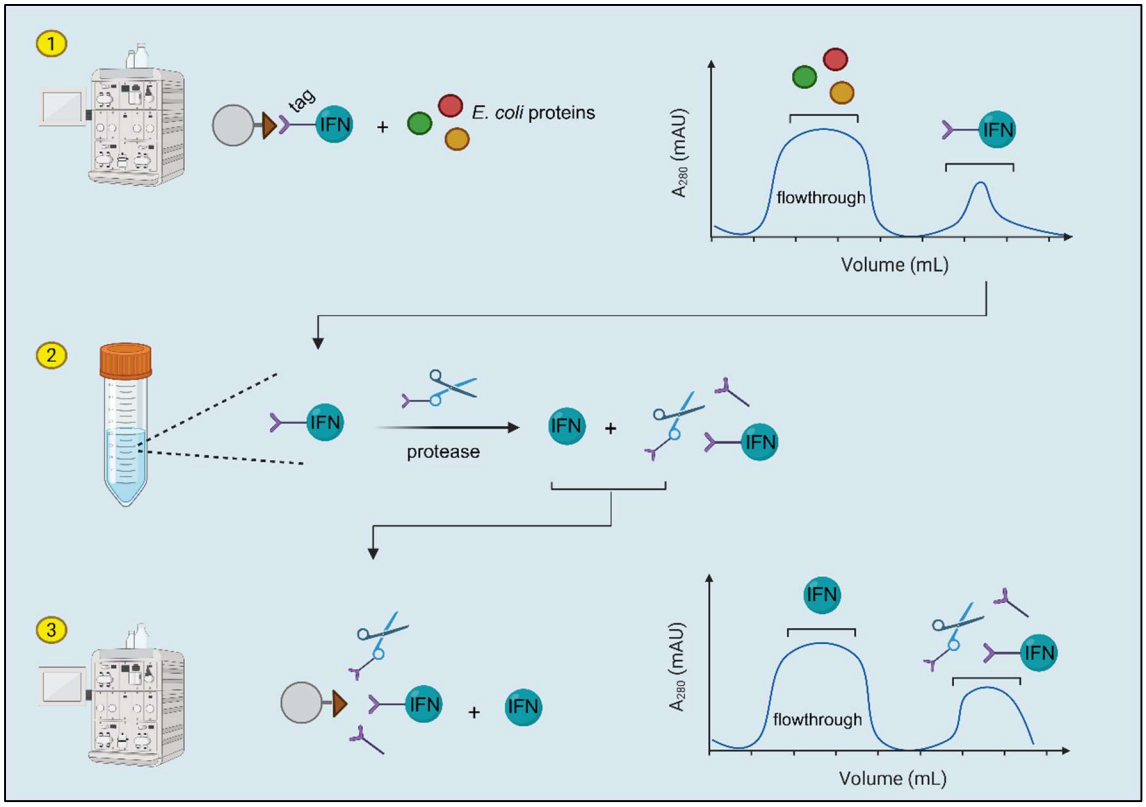

Figure 3: Purification of alphainterferon by affinity chromatography. (1) IFN is expressed with a protease recognition site and a bioaffinity tail (e.g., 6xHis or SUMO) that binds a ligand (brown arrow) on a chromatographic resin (gray sphere) while *E. coli* contaminating proteins are cleared away in 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 target 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, alternatively with detergents (Triton) to destabilize membranes, besides protease inhibitors, even when working with B strains. The pH was within the range 7 – 8, most likely to avoid the generalized precipitation described for *E. coli* proteins in acidic solutions [43,82]. 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 [39,52] or part of a sequence composed of two [50,51,69], three [54,68,75], or even four chromatographic steps [36,41,66].

Cationic exchange using functional groups carboxymethyl and sulphopropyl was performed with pH ≤ 5.3 and the target molecule positively charged [41,54,66,75], thus allowing removal of anionic contaminants along

368 flowthrough, such as LPS and residual DNA. Eluting with shallower gradient slopes allowed the separation of
369 IFNα aggregates and proteins displaying distinct positive charge intensities [57].

370 For both anionic and cationic columns, it is expected that neutral species are carried out during washing steps,
371 such as proteins in their i.p. and non-charged molecules like some lipids. Small species (IPTG, protease inhibitors)
372 are also likely to be cleared along some processes with membranes, such as dialysis [70,75], diafiltration [54,69],
373 or ultrafiltration. These steps were performed in some studies in order to exchange buffers or to concentrate
374 alphainterferon [36,50,51,72].

375 Size exclusion chromatography (SEC) was also used. Rather than a purification strategy *per se*, this technique
376 was mainly chosen as a final polishing step to desalt, exchange buffer, and/or to remove fusion tags, proteases
377 and accessory proteins [47,50,54], and could even be replaced by ultrafiltration in one article [66].

378 Another relevant approach included hydrophobic interaction chromatography, basically with phenyl sepharose
379 columns [41,51,66,76]. One group [45] chose a preparative reverse-phase high-performance liquid
380 chromatography (RP-HPLC) as the second (among four) chromatographic steps to purify their IB-derived IFN
381 alpha-2b. Indeed, although it is water-soluble, IFNα2 exhibits a relatively high hydrophobic character due to many
382 apolar aminoacid lateral groups exposed; such feature was increased by using buffers with pH values near its i.p.
383 and/or with the addition of ammonium sulphate. Finally, one group adsorbed IFN to a silica column as the first
384 downstream step [41].

385 Minor strategies to purify IFN-α2b from the periplasm included differential partitioning in aqueous two-phase
386 systems [61,62]. After osmotic shock, the team employed the water-soluble polymer PEG and potassium
387 phosphate, achieving a purification factor (Pf) of 26.3 e yield of 40.7%. Next, the group tested a system composed
388 of alcohol and salt, reaching Pf = 16.24 with yield = 74.64%. The molecule was analyzed by SDS-PAGE, but
389 there was no mention of its biological activity or the correct tertiary structure.

390 Two missed purification techniques were ceramic hydroxyapatite chromatography and membrane
391 chromatography. The former may interact with proteins through multimodal interactions such as ion exchange
392 (both anionic and cationic), metal affinity, and hydrogen bonding; the latter may display functionalized groups to
393 adsorb impurities [29]. Moreover, researchers neither explored stepwise elution schemes (only linear gradients),
394 which may be faster in an industrial context; nor the strategy of varying the buffer's pH along elution to modify
395 IFN's charge.

396

397 ***4. Testing***

398 SDS-PAGE was the most common method to monitor IFNα in soluble vs. insoluble fractions and throughout
399 downstream processing. Interferon's identity was inferred by comparison to analytical standards, molecular
400 weight standards, and, in some cases, confirmed by western blotting [75] and ELISA.

401 When it comes to the impurity profile of the final product, groups reported at least 95% pure IFN according to
402 electrophoretic methods; one also employed RP- and SEC-HPLC analyses [68]. Only two teams dosed residual
403 endotoxins [66,69], and another measured host cell DNA [57]. These are important impurities that must follow
404 regulatory limits if the molecule is intended to become a licensed biopharmaceutical [15].

405 All but three publications [61,62,77] reported bioassays to demonstrate *in vitro* activity of alphainterferon,
406 therefore implying that its 3D structure was correct [24,84]. Antiviral assays [15,85] were the first choice,
407 followed by three antiproliferative tests using tumoral lines [39,52,70]. Results were reported as the effective dose
408 impacting half the cells (EC_{50}) or as international units (IU/mg or IU/mL) whenever the laboratory possessed an
409 official standard with known potency.

410 Few research teams performed further structural analyses to confirm the purified IFN's secondary and tertiary
411 features. Methods included circular dicroism to confirm the predominant α-helical structure of interferons, nuclear
412 magnetic resonance, analytical centrifugation [70], LC coupled with mass spectrometry (MS), and MALDI-TOF
413 [72] to check the molecular weight. Peptide mapping coupled to MS [41] was also employed following an
414 approach to confirm the presence and position of disulfides after protease digestion under non-reducing conditions
415 [86]. 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	[36,72]
	Ultrafiltration	Concentration of target protein	[54,69]
	Dialysis	Exchange buffers	[70,76]
	Bioaffinity	Separation by binding to ligand	[26,47,68,69,70,71,78]
	Anion exchange	Separation by net superficial charge	[36,41,39,50,51,52,54,66,68,69,76]
	Cation exchange		[41,54,57,66,76]
	Gel filtration	Separation by size	[47,50,54,66]
	Hydrophobic interaction	Separation by interaction with apolar resin	[41,45,51,66,77]
Qualty control			
	SDS-PAGE	Identity, purity	Nearly all
	HPLC	Identity, purity	[68]
	Bioassays	Functionality	Nearly all
	Circular dicrosim	α-helix pattern	[70]
	Mass spectrometry	Identity, disulfide pattern	[41,72,87]

Legend: references are cited in the order they appear in the text.

5. Unexplored opportunities

Some overlooked strategies may be adopted in future studies, either on their own or in combination with techniques already described. In the upstream stage, supplementation of culture media or lysis buffers with chemical chaperones and cofactors may increase 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 [38,46,55, 58] and difficult-to-express targets [87].

Although reported for other soluble heterologous proteins, *Arctic Express (DE3)* [89] was not chosen as the host in any of the studies reviewed. Considering the strain's ability to express cold-adapted chaperones that show high refolding activity at up to 4ºC [38] and the low metabolic burden at such reduced temperature, these cells are a promising candidate to express aggregation-prone proteins [55]. 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 thus far.

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 standard cultivation practices, i.e., 37°C and ≥ 1 mM IPTG with a strong promoter. Its solubility is directly related to the genetic features of the expression system (cell line + plasmid) and the specificities of the induction step. Rather than isolated interventions, many groups succeeded in 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, and inducing it at early-midlog phase under lighter conditions of temperature and inducer dose. 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 mutated strains, as well as purifying proteins fast and under refrigeration. There are some unexplored strategies that 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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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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References

[1] González-Navajas, J.M., Lee, J., David, M., Raz, E., 2012. Immunomodulatory functions of type I interferons. Nat. Rev. Immunol. 12(2), 125-135. https://doi.org/10.1038/nri3133.

[2] Mesev, E.V., Ledesma, R.A., Ploss, A., 2019. Decoding type I and III interferon signaling during viral infection. Nat. Microbiol. 4(6), 914–924. https://doi.org/10.1038/s41564-019-0421-x.

[3] Zitvogel, L., Galluzzi, L., Keep, O., Smyth, M.J., Kroemer, G., 2015. Type I interferons in cancer immunity. Nat. Rev. Immunol. 15(7), 405–414. https://doi.org/10.1038/nri3845.

[4] Parker, B.S., Rautela, J., Hertzog, P.J., 2016. Antitumour actions of interferons: implications for cancer therapy. Nat. Rev. Cancer. 16(3), 131–144. https://doi.org/10.1038/nrc.2016.14.

[5] Vidal, P., 2020. Interferon α in cancer immunoediting: from elimination to escape. Scand. J. Immunol. 91(5), e12863. https://doi.org/10.1111/sji.12863.

[6] Castro, L.S., Lobo, G.S., Pereira, P., Freire, M.G., Neves, M.C., Pedro, A.Q., 2021. Interferon-based biopharmaceuticals: overview on the production, purification, and formulation. Vaccines. 9(4), 328-379. https://doi.org/10.3390/vaccines9040328.

[7] Renren, Y., Zhu, B., Chen, D., 2022. Type I interferon-mediated tumor immunity and its role in immunotherapy. Cell. Mol. Life Sci. 70(3), 191. https://doi.org/10.1007/s00018-022-04219-z.

[8] Goeddel, D.V., Leung, D.W., Dull, T.J., Gross, M., Lawn, R.M., McCandliss, R., Seeburg, P.H., Ullrich, A., Yelverton, E., Gray, P.W., 1981. The structure of eight distinct cloned human leukocyte interferon cDNAs. Nature. 290(5801), 20–26. https://doi.org/:10.1038/290020a0.

[9] George, P.M., Badiger, R., Alazawi, W., Foster, G.R., Mitchell, J.A, 2012. Pharmacology and therapeutic potential of interferons. Pharmacol. Ther. 135(1), 44-53..

[10] Friedman-Kien, A., 1995. Management of condylomata acuminata with Alferon N injection, interferon alfa-n3 (human leukocyte derived). Am. J. Obstet. Gynecol. 172(4, 2), 1359-1368. https://doi.org/10.1016/0002-9378(95)90404-2.

[11] El-Baky, N.A., Redwan, E.M., 2015. Therapeutic alpha-interferons protein: structure, production, and biosimilar. Prep. Biochem. Biotechnol. 45(2), 109-127. https://doi.org/10.1080/10826068.2014.907175.

[12] Taylor, M.W., 2014. Interferons, in: Viruses and man: a history of interactions. Springer, Cham. https://doi.org/10.1007/978-3-319-07758-1.

[13] Dumont, J., Euwart, D., Mei, B., Estes, S., Kshirsagar, R., 2016. Human cell lines for biopharmaceutical manufacturing: history, status, and future perspectives. Crit. Rev. Biotechnol. 36(6), 1110-1122. https://doi.org/10.3109/07388551.2015.1084266.

[14] Borden, E.C., 2019. Interferons α and β in cancer: therapeutic opportunities from new insights. Nat. Ver. Drug. Discov. 18(3), 219–234, 2019. https://doi.org/10.1038/s41573-018-0011-2.

[15] European Directorate for the Quality of Medicines & Healthcare (EDQM), 2023a. Interferon alpha-2 concentrated solution, in: European Pharmacopeia. 11.0, 3101-3104.

[16] Aricò, E., Castiello, L., Capone, I., Gabriele, L., Belardello, F, 2019. Type I interferons and cancer: an evolving story demanding novel clinical applications. Cancers. 11(12), 1943..

[17] Adolf, G.R.; Kalsner, I.; Ahorn, H.; Maurer-Fogy, I.; Cantell, K., 1991. Natural human interferon-alpha2 is *O*-glycosylated. Biochem. J. 276(2), 511-518. https://doi.org/10.1042/bj2760511.

[18] Chronopoulou, S., Tsochantaridis, I., Tokamani, M., Kokkinopliti, K.D., Tsomakidis, P., Giannakakis, A., Galanis, A., Pappa, A., Sandaltzopoulos, R., 2023. Expression and purification of human interferon alpha 2a (IFNα2a) in the methylotrophic yeast *Pichia pastoris*. Protein Expr Purif. 211, 106339. https://doi.org/10.1016/j.pep.2023.106339.

[19] Du, T., Buenbrazo, N., Kell, L., Rahmani, S., Sim, L., Withers, S.G., DeFrees, S., Wakarchuk, W., 2018. A Bacterial Expression Platform for Production of Therapeutic Proteins Containing Human-like O-Linked Glycans. Cell Chem Biol. 26(2), 203-212. https://doi.org/10.1016/j.chembiol.2018.10.017.

[20] Baeshen, M.N., Al-Hejin, A.M., Bora, R.S., Ahmed, M.M.M., Ramadan, H.A.I., Saini, K.S., Baeshen, N.A., Redwan, E.M, 2015. Production of biopharmaceuticals in E. coli: current scenario and future perspectives. J. Microbiol. Biotechnol. 25(7), 953–962. https://doi.org/10.4014/jmb.1412.12079.

[21] Salunkhe, S., Prasad, B., Sabnis-Prasad, K., Apte-Deshpande, A., Padmanabhan, S., 2009. Expression and purification of SAK-fused human interferon alpha in *Escherichia coli*. J. Microb. Biochem. Technol. 1(1), 5–10. http://dx.doi.org/10.4172/1948-5948.1000002.

[22] Shein, A.M.S., Hongsing, P., Khatib, A. et al., 2024. Phage therapy could be key to conquering persistent bacterial lung infections in children. npj Antimicrob. Resist. 2, 31. https://doi.org/10.1038/s44259-024-00045-4.

[23] O'Flaherty, R., Bergin, A., Flampouri, E., Mota, L.M., Obaidi, I., Quigley, A., Xie, Y., Butler, M., 2020. Mammalian cell culture for production of recombinant proteins: a review of the critical steps in their biomanufacturing. Biotechnol. Adv. 43, 107552. https://doi.org/10.1016/j.biotechadv.2020.107552.

[24] Zhao, J., Yu, H.Y., Zhao, Y., Li, F.H., Zhou, W., Xia, B.B., He, Z.Y., Chen, J., Jiang, G.T., Wang, M.L, 2019. Soluble expression, rapid purification, biological identification of chicken interferon-alpha using a thioredoxin fusion system in *E. coli* and its antiviral effects to H9N2 avian influenza virus. Prep. Biochem. Biotechnol. 49(2), 192–201. https://doi.org/10.1080/10826068.2019.1566150.

[25] Lobstein, J., Emrich, C.A., Jeans, C., Faulkner, M., Riggs, P., Berkmen, M., 2012. SHuffle, a novel Escherichia coli protein expression strain capable of correctly folding disulfide bonded proteins in its cytoplasm. Microb. Cell Fact. 11(56). https://doi.org/10.1186/1475-2859-11-56.

[26] Castillo-Corujo, A., Uchida, Y., Saaranen, M.J., Ruddock, L.W., 2024. Escherichia coli Cytoplasmic Expression of Disulfide-Bonded Proteins: Side-by-Side Comparison between Two Competing Strategies. J. Microbiol. Biotechnol. 34(5), 1126-1134. https://doi.org/10.4014/jmb.2311.11025.

[27] Neves, F.O., Ho, P.L., Raw, I., Pereira, C.A., Moreira, C., Nascimento, A.L., 2004. Overexpression of a synthetic gene encoding human alpha interferon in Escherichia coli. Protein Expr Purif. 35(2), 353-359. https://doi.org/10.1016/j.pep.2004.02.005.

[28] EDQM, 2023b. Interferon beta-1a concentrated solution, in: European Pharmacopeia. 11.0, 3104-3106.

[29] Zhu, M.M., Mollet, M., Hubert, R.S., Kyung, Y.S., Zhang, G.G. Industrial Production of Therapeutic Proteins: Cell Lines, Cell Culture, and Purification. In: Kent, J., Bommaraju, T., Barnicki, S. (eds). Handbook of Industrial Chemistry and Biotechnology. Springer, Cham. 2017. https://doi.org/10.1007/978-3-319-52287-6_29.

[30] Capobianchi, M.R., Uleri, E., Cagloti, C., Dolei, A., 2015. Type I IFN family members: similarity, differences and interaction. Cytokine Growth Factor Rev. 26, 2, 103-111. https://doi.org/10.1016/j.cytogfr.2014.10.011.

[31] EDQM, 2023c. Interferon gamma-1b concentrated solution, in: European Pharmacopeia. 11.0, 3107-3110.

[32] Hirel, P.H., Schmitter, M.J., Dessen, P., Fayat, G., Blanquet, S., 1989. Extent of N-terminal methionine excision from Escherichia coli proteins is governed by the side-chain length of the penultimate amino acid. Proc Natl Acad Sci U S A. 86(21), 8247-8251. https://doi.org/10.1073/pnas.86.21.8247.

[33] Dalbøge, H., Bayne, S., Pedersen, J., 1990. In vivo processing of N-terminal methionine in E. coli. FEBS Lett. 266(1-2), 1-3. https://doi-org.ez27.periodicos.capes.gov.br/10.1016/0014-5793(90)90001-b.

[34] Ahsan, F., Gardner, Q.A., Rashid, N., Towers, G.J., Akhtar, M., 2018. Preventing the N-terminal processing of human interferon α-2b and its chimeric derivatives expressed in Escherichia coli. *Bioorganic chemistry*, 76, 294–302. https://doi.org/10.1016/j.bioorg.2017.11.016.

[35] Yu, L., Tao, L., Zhao, Y., Li, Y., Pei, D., Rao, C., 2020. Analysis of Molecular Heterogeneity in Therapeutic IFNα2b from Different Manufacturers by LC/Q-TOF. Molecules. 31, 25(17), 3965-. doi: 10.3390/molecules25173965. https://doi.org/10.3390/molecules25173965.

[36] Sharma, S.K., Kumar, S.B., Parab, R., Kankonkar, M., D'Souza, C., Bachate S., Sharma R.K., 2016. An improved process for the preparation of pharmacopoeial grade interferon alpha 2b. Patent WO2016079598A1. https://worldwide.espacenet.com/patent/search/family/056013352/publication/WO2016079598A1?q=WO2016079598A1.

[37] Gileadi, O., Recombinant Protein Expression in E. coli : A Historical Perspective, in: Burgess-Beown, N.A. (Ed.) Heterologous gene expression in E. coli, Methods and Protocols. Humana Press, Oxford, 2017. Doi: 10.1007/978-1-4939-6887-9_13.

[38] Rosano, G.L., Morales, E.S., Ceccarelli, E.A, 2019. New tools for recombinant protein production in Escherichia coli: a 5-year update. Protein Sci. 28(8), 1412–1422. https://doi.org/10.1002/pro.3668.

[39] Mohammed, Y., El-Baky, N.A., Redwan, N.A., Redwan, E.M., 2012. Expression of human interferon-α8 synthetic gene under P_{BAD} promoter. Biochemistry (Mosc). 77(10), 1210–1219. https://doi.org/10.1134/s0006297912100136.

[40] Ahmad, I., Nawaz, N., Darwesh, N.M., Ur, Rahman S., Mustafa, M.Z., Khan, S.B., Patching, S.G., 2018. Overcoming challenges for amplified expression of recombinant proteins using *Escherichia coli*. Protein Expr. Purif. 144, 12-18. https://doi.org/10.1016/j.pep.2017.11.005.

[41] Voss, T., Falkner, E., Ahorn, H., Krystek, E., Maurer-Fogy, I., Bodo, G., Hauptmann, R, 1994. Periplasmic expression of human interferon-α2c in *Escherichia coli* results in a correctly folded molecule. Biochem. J. 298(3), 719–725. https://doi.org/10.1042/bj2980719.

[42] Studier, F.W., Moffatt, B.A., 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol. 189(1), 113-130. https://doi.org/10.1016/0022-2836(86)90385-2.

[43] Wingfield, P.T., 2014. Preparation of soluble proteins from Escherichia coli. Curr. Protoc. Protein Sci. 78, 6.2.1-6.2.22. https://doi.org/10.1002/0471140864.ps0602s78.

[44] San-Miguel, T., Pérez-Bermúdez, P., Gavidia, I., 2013. Production of soluble eukaryotic recombinant proteins in *E. coli* is favoured in early log-phase cultures induced at low temperature. Springer Plus. 2(1), 89. https://doi.org/10.1186/2193-1801-2-89.

[45] Beldarraín, A., Cruz, Y., Cruz, O., Navarro, M., Gil, M., 2001. Purification and conformational properties of a human interferon alpha2b produced in *Escherichia coli*. Biotechnol. Appl. Biochem. 33(3), 173–182. https://doi.org/10.1042/BA20010001.

[46] Sorensen, H.P., Mortensen, K.K., 2005. Soluble expression of recombinant proteins in the cytoplasm of *Escherichia coli*. Microb. Cell Fact. 4(1). https://doi.org/10.1186/1475-2859-4-1.

[47] Rabhi-Essafi, I., Sadok, A., Khalaf, N., Fathallah, D.M., 2007. A strategy for high-level expression of soluble and functional human interferon alpha as a GST-fusion protein in E. coli. Protein Eng. Des. Sel. 20(5), 201–209. https://doi.org/10.1093/protein/gzm012.

[48] Ryan, B.J., Henehan, G.T, 2013. Overview of approaches to preventing and avoiding proteolysis during expression and purification of proteins. Curr. Protoc. Protein Sci. unit 5.25. https://doi.org/10.1002/0471140864.ps0525s71.

[49] Schein, C.H., Noteborn, M.H.M., 1988. Formation of soluble recombinant proteins in *Escherichia coli* is favored by lower growth temperatures. Nat. Biotechnol. 6(3), 291–294. http://dx.doi.org/10.1038/nbt0388-291.

[50] Zhang, C., Zhou, D., Ji, C., Beijing Kawin Technology Share, 2008. Gene optimization and high-efficiency expression of recombinant human interferon a2b. Patent CN101508994A. China. https://worldwide.espacenet.com/patent/search/family/041001527/publication/CN101508994A?q=CN101508994A.

[51] Yan, C.S., QI Ao Pharmaceutical Group Co., 2006. Recombinant human interferon alpha 4 coded cDNA sequence, production and use thereof. Patent CN1746306A. China. https://worldwide.espacenet.com/patent/search/family/036166019/publication/CN1746306A?q=CN1746306A.

[52] El-Baky, N.A., Linjawi, M.H., Redwan, E.M., 2015. Auto-induction expression of human consensus interferon-alpha in *Escherichia coli*. BMC Biotechnol. 15(14). https://doi.org/10.1186/s12896-015-0128-x.

[53] Xu, J., Banerjee, A., Pan, S.H., Li, Z.J, 2012. Galactose can be an inducer for production of therapeutic proteins by auto-induction using *E. coli* BL21 strains. Protein Expr Purif. 83(1), 30-36. https://doi.org/10.1016/j.pep.2012.02.014.

[54] Fanhong, X., Tengjie, W., Shanghai Bio Products Inst., 2003. Method for preparing interferon. Patent CN1451748A. China. https://worldwide.espacenet.com/patent/search/family/029222793/publication/CN1451748A?q=CN1451748A.

[55] Incir, I., Kaplan, O., 2024. *Escherichia coli* as a versatile cell factory: advances and challenges in recombinant protein production. Protein Express. Purif. 219, 106463. https://doi.org/10.1016/j.pep.2024.106463.

[56] Kuruganti, S., Accavitti-Loper, M.A., Walter, M.R., 2014 Production and characterization of thirteen human type-I interferon-α subtypes. Protein Expr Purif. 103, 75-83. https://doi.org/10.1016/j.pep.2014.08.010.

[57] Bretas, R.M., Silva, M.V.A., Leclercq, S.Y., Lopes, L.M.S., Junior, A.S.C., 2025. Fine-tuning of leaky expression delivers soluble and functional recombinant alphainterferon in the cytoplasm of *Escherichia coli*. SCIELO PREPRINTS. https://doi.org/10.1590/SciELOPreprints.11736.

[58] Hayat, S.M.G., Farahani, N., Golichenari, B., Sahebkar, A., 2018. Recombinant protein expression in *Escherichia coli* (*E.coli*): what we need to know. Curr. Pharm. Des. 24(6), 718-725. https://doi.org/10.2174/1381612824666180131121940.

[59] Beygmoradi, A., Homaei, A., Hemmati, R., Fernandes, P, 2023. Recombinant protein expression: Challenges in production and folding related matters. Int. J. Biol. Macromol. 233, 123407. https://doi.org/10.1016/j.ijbiomac.2023.123407.

[60] Schilling, R., Diederich, B., Richter Helm Bio Tec GmbH & Co KG, 2012. Recombinant expression of soluble interferon. Patent WO2012160027A1, Germany. https://worldwide.espacenet.com/patent/search/family/046149442/publication/WO2012160027A1?q=WO2012160027A1.

[61] Lin, Y.K., Ooi, C.W., Ramanan, R.N., Ariff, A., Ling, T.C., 2012. Recovery of human interferon alpha-2b from recombinant *Escherichia coli* by aqueous two-phase system. Separation Science and Technology. 47, 1023–1030. https://doi.org/10.1080/01496395.2011.644018.

[62] Lin, Y.K., Ooi, C.W., Tan, J.S., Show, P.L., Ariff, A., Ling, T.C., 2013. Recovery of human interferon alpha-2b from recombinant *Escherichia coli* using alcohol/salt-based aqueous two-phase systems. Separation and Purification Technology. 120, 362–366. https://doi.org/10.1016/j.seppur.2013.09.038

[63] Demissie, E.A., Park, S., Moon, J.H., Lee, D., 2025. Comparative Analysis of Codon Optimization Tools: Advancing toward a Multi-Criteria Framework for Synthetic Gene Design. J. Microbiol. Biotechnol. 35, 1-11. https://doi.org/10.4014/jmb.2411.11066.

[64] Sletta, H., Tøndervik, A., Hakvåg, S., Aune, T.E., Nedal, A., Aune, R., Evensen, G., Valla, S., Ellingsen, T.E., Brautaset, T., 2007. The presence of N-terminal secretion signal sequences leads to strong stimulation of the total expression levels of three tested medically important proteins during high-cell-density cultivations of Escherichia coli. Appl Environ Microbiol. 73(3), 906-912. https://doi.org/10.1128/aem.01804-06.

[65] Terol, G.L., Gallego-Jara, J., Martínez, R.A.S., Vivancos, A.M., Díaz, M.C., Puente, T.D, 2021. Impact of the expression system on recombinant protein production in *Escherichia coli* BL21. Front. Microbiol. 12, 682001. https://doi.org/10.3389/fmicb.2021.682001.

[66] Chung, K.D., Jung, J., 2002. Purification of recombinant human alpha-2a interferon without using monoclonal antibodies. J. Microbiol. Biotechnol. 12(6), 916-920.

[67] Lim, H.K., Jung, K.H., Park, D.H., Chung, S.I., 2000. Production characteristics of interferon-alpha using an L-arabinose promoter system in a high-cell-density culture. Appl. Microbiol. Biotechnol. 53(2), 201–208. https://doi.org/10.1007/s002530050009.

[68] Grabarz, F., Lopes, A.P.Y., Barbosa, F.F., Barazzone, G.C., Santos, J.C., Botosso, V.F., Jorge, S.A.C., Nascimento, A.L.T.O., Astray, R.M., Gonçalves, V.M., 2021. Strategies for the production of soluble interferon-alpha consensus and potential application in arboviruses and SARS-CoV-2. Life. 11(6), 460. https://doi.org/10.3390/life11060460.

[69] Vu, T.T.T., Jeong, B., Krupa, M., Kwon, U., Song, J-A., Do, B.H., Nguyen, M.T., Seo, T., Nguyen, A.N., Joo, C.H., Choe, H., 2016. Soluble prokaryotic expression and purification of human interferon alpha-2b using a maltose-binding protein tag. J. Mol. Microbiol. Biotechnol. 26(6), 359–368. https://doi.org/10.1159/000446962.

[70] Bis, R.L., Stauffer, T.M., Singh, S.M., Lavoie, T.B., Mallela, K.M.G., 2014. High yield soluble bacterial expression and streamlined purification of recombinant human interferon α-2a. Protein expression and purification, 99, 138–146. https://doi.org/10.1016/j.pep.2014.04.010.

[71] Aslam, M.S., Gull, I., Abbas, Z., Athar, M.A., 2018. Soluble Expression of IFNα2-Tα1 Fusion Protein in *Escherichia coli* by N-terminal SUMO Fusion and its Anti-Proliferative Activity. Pakistan J. Zool. 50, 1413-1419. http://dx.doi.org/10.17582/journal.pjz/2018.50.4.1413.1419.

[72] Peciak, K., Tommasi, R., Choi, J.W., Brocchini, S., Laurine, E., 2014. Expression of soluble and active interferon consensus in SUMO fusion expression system in E. coli. Protein Expr Purif. 99, 18-26. https://doi.org/10.1016/j.pep.2014.03.009.

[73] Nguyen, V.D., Hatahet, F., Salo, K.E., Enlund, E., Zhang, C., Ruddock, L.W., 2011. Pre-expression of a sulfhydryl oxidase significantly increases the yields of eukaryotic disulfide bond containing proteins expressed in the cytoplasm of *E.coli*. Microb. Cell Fact. 10, 1. https://doi.org/10.1186/1475-2859-10-1.

[74] Niazi, S.K., Magoola, M., 2023. Advances in *Escherichi coli*-based therapeutic protein expression: mammalian conversion, continuous manufacturing, and cell-free production. Biologics 3, 380–341. https://doi.org/10.3390/biologics3040021.

[75] Kwon, S.C., Jung, S.Y., Choi, K.D., Kim, C.S., Bae, S.M., Lee, G.S., 2004. Expression and secretion vector for human interferon alpha and process for producing human interferon alpha by employing same. Patent US2004151695A1. United States. https://worldwide.espacenet.com/patent/search/family/019639684/publication/US2004151695A1?q=US2004151695A1.

[76] Xinrong, Z., Chengwei, Y., Suofu, Q., 2022. Human interferon alpha 1b and preparation method thereof. Patent CN113930468A, China. https://worldwide.espacenet.com/patent/search/family/079286460/publication/CN113930468A?q=CN113930468A.

[77] Tan, J.S., Ramanan, R.N., Azaman, S.N.A., Ling, T.C., Shuhaimi, M., Ariff, A.B., 2009. Enhanced interferon-alpha2b production in periplasmic space of *Escherichia coli* through medium optimization using

response surface method. The Open Biotechnology Journal 3, 117-124. http://dx.doi.org/10.2174/1874070700903010117.

[78] Alanen, H.I., Walker, K.L., Suberbie, M.L.V., Matos, C.F.R.O., Bönisch, S., Freedman, R.B., Keshavarz-Moore, E., Ruddock, L.W., Robinson, C., 2015. Efficient export of human growth hormone, interferon α2b and antibody fragments to the periplasm by the Escherichia coli Tat pathway in the absence of prior disulfide bond formation, Biochimica et Biophysica Acta (BBA) - Molecular Cell Research. 1853(3), 756-763..

[79] Tyurin, A.A., Kabardaeva, K.V., Mustafaev, O.N., Pavlenko, O.S., Sadovskaya, N.S., Fadeev, V.S., Zvonova, E.A., Goldenkova-Pavlova, I.V., 2018. Expression of Soluble Active Interferon αA in Escherichia coli Periplasm by Fusion with Thermostable Lichenase Using the Domain Insertion Approach. Biochemistry (Mosc). 83(3), 259-269. https://doi.org/10.1134/s0006297918030069.

[80] Rostovsky, I., Wieler, U., Kuzmina, A., Taube, R., Sal-Man, N., 2024. Secretion of functional interferon by the type 3 secretion system of enteropathogenic *Escherichia coli*. Microb. Cell. Fact. 23, 163. https://doi.org/10.1186/s12934-024-02397-y.

[81] Gundinger, T., Spadiut, O., 2020. pH conditioning is a crucial step in primary recovery - a case study for a recombinant Fab from *E. coli*. Protein Express. Purif. 165, 105504. https://doi.org/10.1016/j.pep.2019.105504.

[82] Johnson, B.H., Hecht, M.H., 1994. Recombinant proteins can be isolated from *E. coli* cells by repeated cycles of freezing and thawing. Biotechnology (N Y). 12(13), 1357-1360. https://doi.org/10.1038/nbt1294-1357.

[83] Freshney, R.I., 2010. Cryopreservation, in: Culture of Animal Cells: a manual of basic technique and specialized applications, 6th ed. Wiley & Sons, Inc., New Jersey.

[84] Meager, A., 2002. Biological assays for interferons. J. Immunol. Methods. 261(1-2), 21-36. https://doi.org/10.1016/s0022-1759(01)00570-1.

[85] EDQM, 2023d. Assay of interferons, in: European Pharmacopeia. 11.0, 777-778.

[86] Lakbub, J.C., Shipman, J.T., Desaire, H., 2018. Recent mass spectrometry-based techniques and considerations for disulfide bond characterization in proteins. Anal. Bioanal. Chem. 410(10), 2467-2484. https://doi.org/10.1007/s00216-017-0772-1.

[87] Jiang, R., Yuan, S., Zhou, Y., Wei, Y., Li, F., Wang, M., Chen, B., Yu, H., 2024. Strategies to overcome the challenges of low or no expression of heterologous proteins in *Escherichia coli*. Biotehcnol. Adv. 75, 108417. https://doi.org/10.1016/j.biotechadv.2024.108417.

802 [88] Belval, L., Marquette, A., Mestre, P., Piron, M.C., Demangeat, G., Merdinoglu, D., Chich, J.F., 2015. A fast
803 and simple method to eliminate Cpn60 from functional recombinant proteins produced by *E. coli* Arctic Express.
804 Protein Expr Purif. 109, 29-34. http://dx.doi.org/10.1016/j.pep.2015.01.009.

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