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Is *Pichia pastoris* a realistic platform for industrial production of recombinant human interferon gamma?

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ABSTRACT

Human interferon gamma (hIFNγ) is an important cytokine in the innate and adaptive immune system, produced commercially in *Escherichia coli*. Efficient expression of hIFNγ has been reported once for *Pichia pastoris* (Wang et al., 2014) – a proven heterologous expression system. This study investigated hIFNγ expression in *P. pastoris* replicating the previous study and expanding by using four different strains (X33: wild type; GS115: $HIS^{-}Mut^{+}$; KM71H: Arg^{+}, Mut^{-} and CBS7435: Mut^{S}) and three different vectors (pPICZαA, pPIC9 and pPpT4αS). In addition, the native sequence (NS) and two codon-optimised sequences (COS1 and COS2) for *P. pastoris* were used. Methanol induction yielded no expression/secretion of hIFNγ in X33, highest levels were recorded for CBS7435: Mut^{S} (~16 μg. L^{-1}). mRNA copy number calculations acquired from RT-qPCR for GS115-pPIC9-COS1 proved low abundance of mRNA. A 10-fold increase in expression of hIFNγ was achieved by lowering the minimal free energy of the mRNA and 100-fold by Mut^{S} phenotypes, substantially lower than reported by Wang et al. (2014). We conclude that commercial production of low cost, eukaryotic recombinant hIFNγ is not an economically viable in *P. pastoris*. Further research is required to unravel the cause of low expression in *P. pastoris* to achieve economic viability.

1. Introduction

Natural human interferon gamma (hIFNγ) is a glycoprotein comprised of 166 amino acids including a secretory signal sequence of 23 amino acids, encoded by a single gene on chromosome 12 [1,2]. hIFNγ is classed a cytokine with miscellaneous functions in the regulation of innate and adaptive immune system responses. It has been reported to be an immuno-modulatory clinically effective drug due to its pleiotropic effects against a wide range of diseases like cancers, hepatitis and tuberculosis [3].

To date, commercial production of recombinant hIFNγ is limited to expression in *E. coli*, which is branded as Actimmune® and approved by the US-Food & Drug Administration, (FDA) for the treatment of chronic granulomatous disease and severe malignant osteopetrosis [1,2]. This recombinant form of hIFNγ is an unglycosylated monomer composed of 143 amino acids, rendering it less protease-resistant, resulting in a shorter half-life in the bloodstream compared to the glycosylated form [1–3]. Other drawbacks associated with *E. coli* expression systems include: potential for endotoxin contamination and the formation of intracellular protein aggregates, termed inclusion bodies, requiring a complex purification and protein refolding process. This increases the final cost of the product [3].

To overcome these limitations, expression of recombinant hIFNγ was attempted in various hosts like *Saccharomyces cerevisiae* 20B-12 [4], insect cells lines *Spodoptera frugiperda*, *Spodoptera exigua*, and *Spodoptera litura*. [5], Chinese hamster ovary (CHO) [3,6], wild-type mice strain C57BL/6 [7], rat cell line 3Y1-B [8], monkey and human cells [9]; however; high costs of cultivation and purification, contamination, low yields, low biological activity and short half-life of the product also adversely impacted on the use of these expression systems [10].

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Abbreviations

AGRF	Australian Genome Research Facility
AOX	alcohol oxidase
BMGY	buffered glycerol complex medium
BMMY	buffered methanol complex medium
CHO	Chinese hamster ovary
COS1	codon-optimised sequence 1
COS2	codon-optimised sequence 2
GOI	gene of interest
hIFNγ	human interferon gamma
HPLC	high pressure liquid chromatography
MFE	minimum free energy
MGY	minimal glycerol medium
MM	minimal methanol medium
NS	native sequence
T_m	melting temperature
YNB	yeast nitrogen base

Another yeast-based expression system for recombinant hIFNγ is the methylotrophic yeast, *Pichia pastoris* (synonym. *Komagataella pastoris*), a proven successful heterologous expression system for the production of hundreds of recombinant proteins [11]. The *P. pastoris* expression systems offer distinct advantages such as easy manipulation, high cell densities, cultivation in low acidity reducing the chance of contamination, low cost of production, eukaryotic post-translational modification and secretion, including protein folding and glycosylation [11].

Commercially available *P. pastoris* strains are the auxotrophic strains GS115 (the *HIS4* mutant), KM71H (the *AOX1* and *ARG4* mutant), the reconstituted prototrophic strain X-33 and protease-deficient strains such as SMD1168. However, use of these strains for commercial applications is restricted by intellectual property [11]. In contrast, some strains of *P. pastoris* like CBS7435, are not protected by patent and, thus represent an alternative for production purposes [11].

The most commonly used promoter capable of driving recombinant protein expression in *P. pastoris*, is the strong alcohol oxidase (*AOX*) promoter which is only inducible with methanol [12]. Two *AOX* operons can be found in the *P. pastoris* chromosome: *AOX1* is responsible for the major *AOX* activity, and *AOX2*, which plays a minor role [12]. Recombinant gene techniques for transformation of *P. pastoris* can leave either or both *AOX* gene sets functional, only the *AOX2*, or neither. Thus, the resulting phenotypes are referred to as Mut^+ (methanol utilisation plus), Mut^S (methanol utilisation slow), or Mut^- (methanol utilisation minus), respectively. Expression efficiency for a recombinant protein in a particular recombinant is not predictable, and available information is at odds in this respect [12].

This study was based on the study by Wang et al. (2014) using native and *P. pastoris* codon-optimised sequences of hIFNγ and expanded the study using eight combinations of *P. pastoris* strains, vectors and sequences. Surprisingly, expression were orders of magnitudes lower than previously reported [10]. Based on our low expression/secretion results for all constructs and in agreement with very recently published results [13], we conclude that the *P. pastoris* expression/secretion system is at present not economically viable for commercial production of eukaryotic recombinant hIFNγ (Animation 1).

Supplementary video related to this article can be found at http://dx.doi.org/10.1016/j.biologicals.2016.09.015.

2. Material and methods

2.1. Strains, sequences, vectors and cloning

2.1.1. Strains

Four strains of *P. pastoris* with different characteristics were used; **X33:** wild-type strain containing two active *AOX* genes resulting in Mut^+ phenotype, **GS115:** A His^- mutant (mutation of *HIS4*), with the His^- Mut^+ phenotypes, **KM71H:** A mutant strain with *ARG4* (arginosuccinate lyase) and disruption of *AOX1*, creating a Mut^S Arg^+ phenotype, **CBS7435, Mut^S**: a knockout of the *AOX1* gene derived from the wild-type CBS7435 strain.

2.1.2. Sequences

In this study, two distinct codon-optimised sequences of *hIFNγ* were synthesised based on the codon preference of *P. pastoris* by Invitrogen™, GeneArt™ Strings, and one copy of the native sequence of *hIFNγ* "NCBI: NM_000610.2; UniProtKB: P01579" was used as a positive control (Fig. 1).

2.1.3. Vectors

The vectors used in this study for transformation of *P. pastoris* are shown in (Fig. 2). **pPIC9:** was provided by Invitrogen™ (catalogue no. K1710-01). This plasmid contains a methanol-inducible *AOX1* promoter, the α-mating secretion signal at the 5′ end of the gene of interest (GOI) and the *HIS4* gene for selection enabling the GS115 strain to biosynthesise histidine. The sequence of the GOI was inserted at *NotI* and *EcoRI* restriction sites. Then, the construct was linearized using *SalI* restriction endonuclease prior to transformation. **pPICZαA:** was provided by Invitrogen™ (catalogue no. K1710-01). This plasmid contains a methanol-inducible *AOX1* promoter, the α-mating secretion signal and a polyhistidine tag (HIS-tag) at 5′ and 3′ ends of the GOI, respectively. The Zeocin™ resistance gene (*Sh ble*) is placed in the plasmid which allows selection of successful transformants on Zeocin™ containing medium plates. The sequence of the GOI was inserted at *EcoRI* and *NotI* restriction sites. Then, 5 μg of the construct was linearized using *SacI* restriction endonuclease prior to transformation. **pPpT4αS:** was provided by Protein Expression Facility at The University of Queensland (Brisbane, Australia). This plasmid contains the methanol-inducible *AOX1* promoter and the α-mating secretion signal at the 5′ end of the GOI. The Zeocin™ resistance gene (*Sh ble*) was placed in the plasmid which allows selection of successful transformants on Zeocin™ containing medium plates [14] The native secretion signal was omitted from the sequence of the GOI followed by insertion at *SnaBI* and *NotI* restriction sites. Then, 5 μg of the construct was linearized using *SwaI* restriction endonuclease prior to transformation.

Gene sequences in vectors (pPICZαA and pPpT4αS) were verified by using ABI BigDye Terminator v3.1 sequencing, conducted by the Australian Genome Research Facility (AGRF). Data analysis was performed using the software Sequencer™ 4.7 (Gene Codes Corporation).

[1] In this study, several codon-optimised sequences were designed for *P. pastoris* based on codon preference. COS1 was selected according to similarity of GC% and T_m to the NS. Upon review, RNA truncation due polyadenylation (poly A) signals appeared possible. COS2 was designed by replacing putative poly A signals (bases 292–297, 331–338 and 457–466) and lowering the predicted minimum free energy (MFE) of the mRNA compared to COS1 (Section 2.8).

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NS      ATGAAATATACAAGTTATATCTTGGCTTTTCAGCTCTGCATCGTTTTGGGTTCTCTTGGC
COS1    ATGAAGTACACATCCTACATCTTGGCTTTCCAGTTGTGTATCGTTTTGGGTTCCTTGGGT
COS2    ATGAAATACACCTCTTATATACTTGCCTTCCAGTTATGCATTGTCTTGGGTAGTCTGGGG
        ***** ** **    ** **  * ** ** *** * ** ** ** ****** * **

NS      TGTTACTGCCAGGACCCATATGTAAAAGAAGCAGAAAACCTTAAGAAATATTTTAATGCA
COS1    TGTTACTGTCAGGACCCATACGTTAAGGAAGCTGAGAACTTGAAGAAGTACTTCAACGCT
COS2    TGTTATTGCCAAGATCCATATGTGAAAGAAGCCGAAAACCTGAAAAAGTACTTCAACGCC
        ***** ** ** ** ***** ** ** ***** ** *** * ** ** ** ** ** **

NS      GGTCATTCAGATGTAGCGGATAATGGAACTCTTTTCTTAGGCATTTTGAAGAATTGGAAA
COS1    GGTCACTCCGACGTTGCTGATAACGGTACTTTGTTCTTGGGTATCTTGAAGAACTGGAAA
COS2    GGACATAGTGATGTTGCAGACAACGGCACACTATTTCTTGGTATTCTAAAGAACTGGAAG
        ** **    ** ** ** ** ** ** **  * **  * ** **  * ***** *****

NS      GAGGAGAGTGACAGAAAAATAATGCAGAGCCAAATTGTCTCCTTTTACTTCAAACTTTTT
COS1    GAAGAGTCCGACAGAAAGATCATGCAGTCCCAGATCGTTTCATTCTACTTCAAGTTGTTC
COS2    GAGGAGTCTGACCGTAAAATTATGCAATCACAAATCGTTTCTTTTTACTTTAAACTATTC
        ** ***   *** * ** ** *****    ** ** ** ** ** ***** **  * **

NS      AAAAACTTTAAAGATGACCAGAGCATCCAAAAGAGTGTGGAGACCATCAAGGAAGACATG
COS1    AAGAACTTCAAGGACGACCAGTCCATCCAGAAGTCCGTTGAGACTATCAAAGAAGATATG
COS2    AAAAATTTTAAAGATGACCAATCAATTCAAAAGTCCGTTGAGACGATTAAAGAAGATATG
        ** ** ** ** ** *****    ** ** ***   ** ***** ** ** ***** ***

NS      AATGTCAAGTTTTTCAATAGCAACAAAAAGAAACGAGATGACTTCGAAAAGCTGACTAAT
COS1    AACGTTAAGTTCTTCAACTCCAACAAGAAAAAGAGAGATGACTTCGAGAAGTTGACTAAC
COS2    AATGTCAAGTTTTTCAACTCAAACAAAAAGAAGAGGGACGACTTTGAAAAGTTAACTAAT
        ** ** ***** *****    ***** ** **  * ** ***** ** *** * *****

NS      TATTCGGTAACTGACTTGAATGTCCAACGCAAAGCAATACATGAACTCATCCAAGTGATG
COS1    TACTCCGTTACTGACTTGAACGTTCAGAGAAAGGCTATCCACGAGTTGATCCAGGTTATG
COS2    TATTCCGTAACCGATTTGAACGTTCAGAGGAAGGCAATCCATGAGTTGATACAGGTTATG
        ** ** ** ** ** ***** ** **  * ** ** ** ** **  * ** ** ** ***

NS      GCTGAACTGTCGCCAGCAGCTAAAACAGGGAAGCGAAAAAGGAGTCAGATGCTGTTTCGA
COS1    GCTGAATTGTCTCCAGCTGCTAAGACTGGTAAGAGAAAAAGATCCCAGATGTTGTTCAGA
COS2    GCAGAACTGTCCCCCGCTGCCAAAACAGGAAAGAGAAAAAGATCACAAATGCTTTTCAGG
        ** *** **** ** ** ** ** ** ** *** *******    ** *** * **  *

NS      GGTCGAAGAGCATCCCAGTAA
COS1    GGTAGAAGAGCTTCCCAGTAA
COS2    GGTAGACGTGCTTCTCAATAA
        *** ** * ** ** ** ***

POI     MQDPYVKEAENLKKYFNAGHSDVADNGTLFLGILKNWKEESDRKIMQSQIVSFYFKLFKN
        FKDDQSIQKSVETIKEDMNVKFFNSNKKKRDDFEKLTNYSVTDLNVQRKAIHELIQVMAE
        LSPAAKTGKRKRSQMLFRGRRASQ
```

Fig. 1. DNA sequences of *hIFNγ*; **NS**: Native sequence, **COS1**: Codon-optimised sequence 1, **COS2**[1]: Codon-optimised sequence 2. **POI**: Protein of interest *i.e.* amino acid sequence of hIFNγ. **(*)**: Presence of this symbol shows the similarity in the bases. The first 23 amino acid sequence (eq. 69 bp nucleotides) is the native secretion signal at the N-terminal of the amino acid sequence.

2.2. Transformation into Pichia pastoris

2.2.1. Order of transformation

In order to generate the construct; each sequence was flanked with suitable restriction enzymes listed in (Fig. 2) and inserted into the vectors between the same restriction sites. Prior to transformation, each construct was linearized using suitable restriction enzymes for the vector (Fig. 2). The combinational order for generating each transformant is listed in (Table 1).

2.2.2. Electroporation of Pichia pastoris

Each plasmid pPIC9-COS1 & pPIC9-COS2 was linearized with suitable corresponding restriction enzymes (Fig. 2) and then transformed into the *P. pastoris* GS115 strain by electroporation (Electroporator 2510™, Eppendorf) following the protocols for electro-competent cell production and electroporation (Invitrogen™).

Each plasmid pPICZα-NS, pPICZα-COS2, pPpT4αS-NS, pPpT4αS-COS2, were linearized with suitable corresponding restriction enzymes (Fig. 2) and then transformed into either X33, KM71H or CBS7435 strains of *P. pastoris* by electroporation (Electroporator Thermo Hybaid CelljecT Pro®, ADP-400) following the protocols for electro-competent cell production and electroporation [15].

2.2.3. Screening for transformants

pPIC9-COS1 & pPIC9-COS2-transformed GS115 (Table 1) were screened for HIS^+ phenotype on Minimal Dextrose (MD) (1.34% Yeast Nitrogen Base (YNB), 2% dextrose) agar plates in 30 °C, as successful transformants should have regained histidine auxotrophy. With the intention of determining the methanol utilisation (Mut) phenotype of the strain, colonies with HIS^+ phenotype were re-plated on Minimal Methanol (MM) (1.34% YNB, 0.5% methanol) as the sole carbon source, the methanol utilisation plus (Mut^+) phenotype was chosen by the ability to grow on both media agar plates after 24 h while methanol utilisation slow (Mut^S) cells grow normally on MD, but their growth on MM was negligible.

The remainder of the transformants which were obtained from other strains of *P. pastoris* i.e. X33, KM71H and CBS7435 (Table 1) were selected by plating onto selective medium (1% Yeast 2% Peptone 1% Dextrose plus Zeocin™ 100 μg mL^{-1}) after 5 days' incubation at 30 °C.

2.2.4. Confirmation of integration into gDNA by PCR

In order to determine, whether *hIFNγ* was integrated into the *P. pastoris* genome, colony PCR was conducted for X33, KM71H and CBS7435 transformants, while genomic DNA was extracted from GS115 transformants using the Wizard® Genomic DNA Purification Kit, (Promega) for PCR. The integration of *hIFNγ* into the genome of *P. pastoris* was confirmed by PCR using primers listed in Table 2. Genomic DNA of untransformed *P. pastoris* strains was used as a negative control. PCR amplification was run according to the standard protocol for *P. pastoris* (Invitrogen™).

Successful integration of *hIFNγ* into *P. pastoris* genome was demonstrated by detecting the expected ~500–800 bp fragment size using agarose (1.5%) gel electrophoresis.

2.3. Expression of hIFNγ

2.3.1. Standard expression in P. pastoris in buffered medium

Transformant cells (GS115-pPIC9-COS1 and GS115-pPIC9-COS2) were cultivated in 25 mL buffered Minimal Glycerol (BMGY) medium (1.34% YNB, 1% glycerol, 100 mM potassium phosphate, pH 6.0) in a 250 mL baffled flask and incubated for 48 h at 28 °C on a shaking plate at 200 rpm until reaching an $OD_{600} \geq 2$ (log-phase growth) (EnSpire® Multimode Plate Reader, PerkinElmer). Next, the cells were harvested by centrifugation for 5 min at 3000 *g* at room temperature. Then, cell pellets were resuspended in 50 mL PBS buffer (0.1 M Phosphate Buffer Saline, pH 7.4) to wash off residual glycerol. Finally, cell pellets were resuspended in 50 mL buffered methanol-complex (BMMY) medium (2% peptone, 1% yeast extract, 1.34% YNB, 1% methanol, 100 mM potassium phosphate, pH 6.0) to a starting $OD_{600} = 1$ in a 250 mL baffled flask. Methanol was added to a final concentration of 1% (v/v) every 24 h to induce expression of hIFNγ. The culture supernatant was obtained after 72 h of cultivation by centrifugation at 1500 *g* for 5 min to analyse the expression of hIFNγ, untransformed *P. pastoris* GS115 cell culture was used as negative control [16].

2.3.2. High throughput expression in P. pastoris in buffered medium

Small-scale expression screens were performed in 24 deep-well plates for the transformant strains of X33, KM71H and CBS7435. It was shown that oxygenation for the deep well plates used was comparable to levels achieved in baffled flasks [17]. Following inoculation of 5 mL BMGY media with a single colony, cultures were grown at 30 °C with shaking at 250 rpm for 48 h. Then, the BMGY media was removed by centrifugation at 1000 *g* for 10 min and each cell pellet was washed with 4 mL of PBS twice. After the second wash, the cell pellets were resuspended in 5 mL of BMMY medium to induce expression. Methanol was added to 1% (v/v) every 24 h until harvest. Samples were harvested 96-h post-induction (hpi) for

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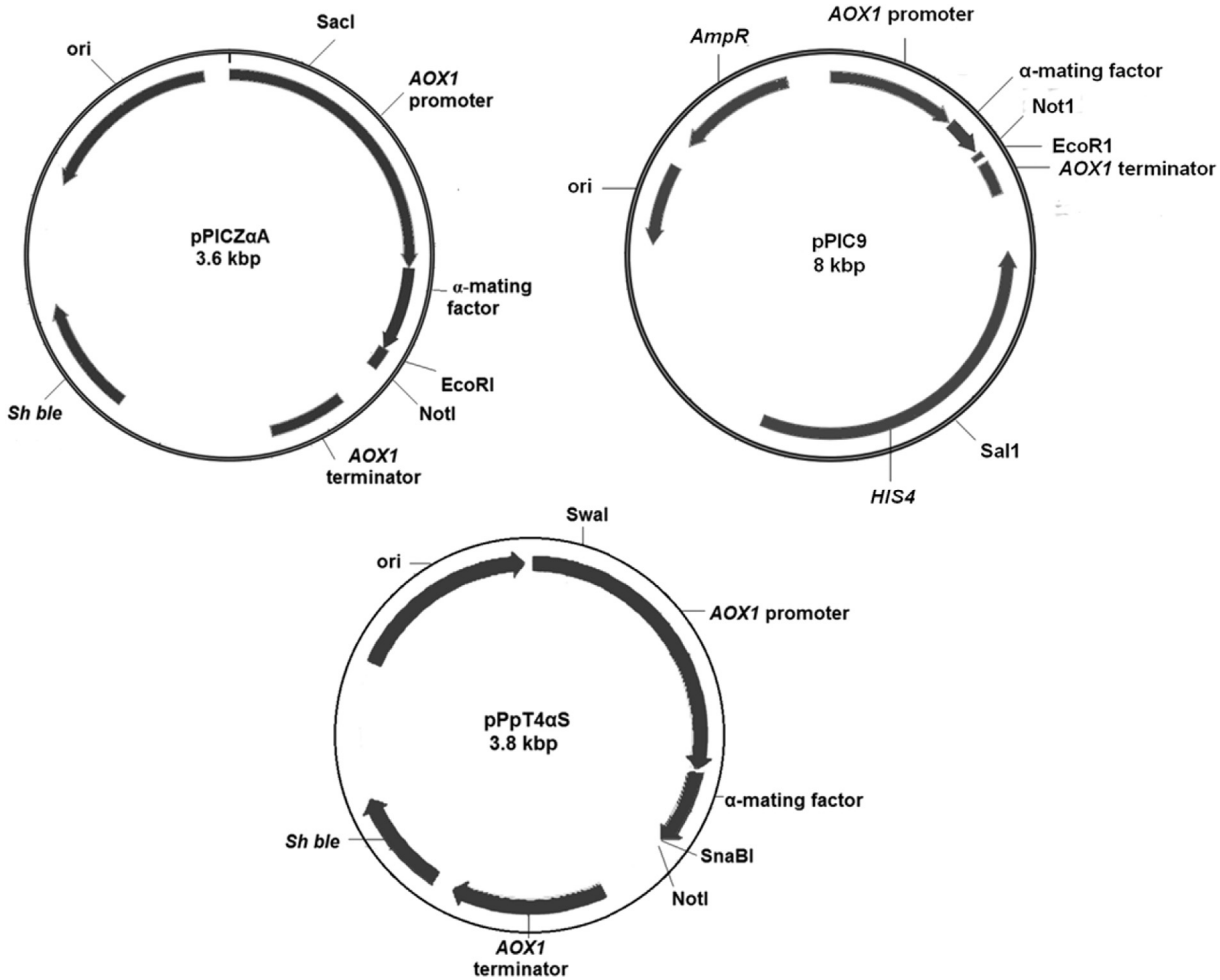

Fig. 2. Generic plasmid vector maps of pPIC9, pPICZαA, pPpT4αS. **Ori**: the origin of replication, for more information consult the text. **6His-tag**: polyhistidine tag. ***Sh ble***: The Zeocin™ resistance gene ***AOX***: alcohol oxidase gene.

analysis. Untransformed strains of X33 (Mut^+), CBS7435 (Mut^S) and KM71H (Mut^S) were used as negative controls and a positive control (P-Protein) was cultured along with the test clones.

2.3.3. Expression in P. pastoris in unbuffered medium

There are some recombinant proteins susceptible to proteases after secretion into the culture medium. In this case, it is usually possible to use unbuffered media such as Minimal Glycerol Medium (MGY) (1.34% YNB and 1% glycerol) and Minimal Methanol (MM) (1.34% YNB and 1% methanol) to inactivate secreted proteases, as the pH drops to 3 or below during cultivation, inactivating many proteases, while *P. pastoris* is tolerant to the acidic condition [18] Expression was performed in unbuffered MGY/MM instead of BMGY/BMMY following the same protocol as detailed in 2.3.1.

2.4. Cell lysis for protein extraction

A modified version of the standard cell lysis technique for *P. pastoris* was used (Invitrogen™) following this procedure; 1 mL of culture (as detailed in 2.3.1) was centrifuged for 5 min at 3000 *g* to remove the supernatant then washed once in Breaking Buffer (50 mM sodium phosphate, pH 7.4, 1 mM EDTA, 5% glycerol plus 1 dissolved tablet of Sigma FAST™ Protease Inhibitor Cocktail Tablet #S8830 in 100 mL of distilled water) by resuspension and

Table 1
Combinational order of expression systems, strains, vectors and sequences which have been used for cloning and transformation.

Expression system	Strain	Vector	Sequence of GOI	Transformant	Phenotype
Pichia pastoris	GS115	pPIC9	COS1	GS115-pPIC9-COS1	His^+Mut^+
			COS2	GS115-pPIC9-COS2	
	X33	pPICZαA	NS	X33-pPICZαA-NS	Mut^+
			COS2	X33-pPICZαA-COS2	
		pPpT4αS	COS2	X33-pPpT4αs-COS2	Mut^+
	KM71H	pPICZαA	NS	KM71H-pPICZαA-NS	Mut^S
			COS2	KM71H-pPICZαA-COS2	
	CBS7435	pPpT4αS	COS2	CBS7435-pPpT4αs-COS2	Mut^S

NS: Natural sequence, **COS1:** Codon-optimised sequence 1, **COS2:** Codon-optimised sequence 2.

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Table 2
Primer sequences for each vector and their hybridising points on the target DNA.

Transformant	Primer sequence		Hybridising point
GS115-pPIC9-COS1/COS2	Forward	5′TACTATTGCCAGCATTGCTGC3′	5′ end of the α-factor region
	Reverse	5′GCAAATGGCATTCTGACATCC3′	3′ end of the *AOX1* TT
X33-pPICZαA-NS	Forward	5′GAGAAAAGAGAGGCTGAAGCTCAGGACCCATATGTAAAAGAAGC3′	5′ end of the GOI
	Reverse	5′GTTCTAGAAAGCTGGCGGCCTTAATGATGATGGTGGTGATGCTGGGATGCTCTTCGACCT3′	3′ end of the GOI
X33-pPICZαA-COS2	Forward	5′GAGAAAAGAGAGGCTGAAGCTCAAGATCCATATGTCAAAGAAGC3′	5′ end of the GOI
	Reverse	5′TTCTAGAAAGCTGGCGGCCTTAATGATGATGGTGGTGATGCTGAGAAGCTCTTCTACCTC3′	3′ end of the GOI
X33-pPpT4αS-COS2	Forward	5′GACTGGTTCCAATTGACAAGC3′	3′ end of the *AOX* promoter
	Reverse	5′GGCATTCTGACATCCTCTTGATTACTGAGAAGCTCTTCTACCTC3′	3′ end of the GOI
KM71H-pPICZαA-NS	Forward	5′GACTGGTTCCAATTGACAAGC3′	3′ end of the *AOX* promoter
	Reverse	5′GTTCTAGAAAGCTGGCGGCCTTAATGATGATGGTGGTGATGCTGGGATGCTCTTCGACCT3′	3′ end of the GOI
KM71H-pPICZαA-COS2	Forward	5′GAGAAAAGAGAGGCTGAAGCTCAAGATCCATATGTCAAAGAAGC3′	5′ end of the GOI
	Reverse	5′TTCTAGAAAGCTGGCGGCCTTAATGATGATGGTGGTGATGCTGAGAAGCTCTTCTACCTC3′	3′ end of the GOI
CB7435-pPpT4αS-COS2	Forward	5′GAAGAGAGAGGCCGAAGCTCATCACCACCATCATCATCAAGATCCATATGTCAAAGAAGC3′	5′ end of the GOI
	Reverse	5′CCCAAACCCCTACCACAA3′	3′ *AOX1* TT

GOI: gene of interest (*i.e. hIFNγ*), ***AOX1* TT:** *AOX* transcription terminator.

centrifugation at 3000 *g* at 4 °C for 5 min. Cells were resuspended in 1 mL of Breaking Buffer and an equal volume of acid-washed glass beads was added, the mixture was vortexed for 30 s, then incubated on ice for 30 s, which was repeated 7 times. The sample was centrifuged at 4 °C for 5 min at 12,000 *g* and hIFNγ was quantified by ELISA of the cleared supernatants. Cell lysates of untransformed *P. pastoris* GS115 were used as negative controls.

2.5. SDS-PAGE and western blotting

Sample supernatants were loaded on 4–12% Bis-Tris SDS-PAGE under denatured and reduced conditions (using NuPAGE® LDS sample buffer, catalogue no. NP0007). Novex® Sharp Pre-Stained Protein Standard (Catalogue no: LC5800, ThermoFishers™) was used as molecular weight ladder ranging 3.5–260 kDa. Gels were blotted to PVDF membrane and probed with the polyhistidine-HRP conjugated antibody (Miltenyi Biotec, catalogue no. 130-092-785, Lot# 5141126111) at a dilution of 1: 6000. The analysis was performed using a Bio-Rad Chemi-Doc™ XRS + imaging system and the molecular weight was calculated using ProtParam.

2.6. ELISA

Recombinant hIFNγ protein levels were quantified by indirect ELISA. A standard curve 0, 1.25, 2.5, 5, 10, 20 μg L^{-1} ($R^2 = 0.9904$ and $y = 13.981x - 1.7371$) was prepared by serial dilution of the recombinant hIFNγ (Abcam catalogue no. ab51240) [16].

(18) primers for priming cDNA synthesis; using the poly-A tail found at the 3′ end of the eukaryotic mRNAs that ensures the 3′ end of mRNAs is represented, and random hexamer primers which randomly cover all regions of the RNA to create a cDNA pool. The synthesised cDNA was divided into equal triplicates for treatment with the QuantiTect SYBR® Green PCR Kit (Qiagen catalogue no. 204141) for the two-step reverse transcription-PCR (RT-qPCR; determining copy number of mRNA transcript) using primers forward 5′ACTTCAACGCTGGTCACTC 3′ and reverse 5′ CGGACTTCTGGATGGACTG 3′ to amplify 168 bp amplicon close to the 5′ end of the COS1 *hIFNγ* sequence [16].

Standard curves for qPCR were prepared with purified DNA amplicons of 699 bp of which 501 bp belong to the hIFNγ gene (Section 2.2.4). Dilution series of DNA amplicons according to copy number (n/per total volume of reaction) were used to prepare standard curve with 0, 1.33×10^2, 1.33×10^3, 1.33×10^4 and 1.33×10^5 copy number ($R^2 = 0.998$, Overall efficiency = 101% and $y = 10^9 .e^{-0.699x}$).

Each 50 μL reactions contained 25 μL (2×) QuantiTec SYBR® GreenPCR Master Mix, 10 μM forward and reverse primers with final concentrations of 0.3 μM (5 μL each), 6 μL sample (~2 μg cDNA quantified by NanoDrop®, ND-1000 Spectrophotometer) and 9 μL RNase-free water. qPCR reactions were run on a Peltier Thermal Cycler-200 (BioRad) as per [16] Total RNA of untransformed *P. pastoris* GS115 was used as a negative control.

Approximate copy number of hIFNγ mRNA transcripts per cell were calculated as per Equation (1) (Eq. (1));

$$\text{Approx. hIFN}\gamma\text{ mRNA transcript}\left(\frac{\text{Copy number}}{\text{Cell number per batch of reaction}}\right) \approx \text{Determined copy number of transcripts per volume of reaction} \Big/ 16.66 \times 10^3 \quad (1)$$

2.7. Detection and determination of mRNA copy number by RT-qPCR

PureLink® RNA Mini Kit (Life Technologies™ catalogue no. 12183018A) was used for extraction of total RNA equivalent to ~16.66×10^3 amount of yeast cells (GS115-pPIC9-COS1), followed by reverse transcription to cDNA using the Tetro cDNA Synthesis Kit (BIOLINE catalogue no. BIO-65050). This kit applies both Oligo dT

2.8. Prediction of mRNA secondary structure

RNA secondary structure regulates expression of many gene transcripts, and plays a substantial role in regulating transcription, splicing RNA, editing and transcript degradation and translation. RNA secondary structure is formed through hydrogen-bonding between pairs of complementary nucleotides in a transcript. In order to study the functional role of a transcript, it

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suffices to know its RNA secondary structure [19]. All RNA folding tools calculate the minimum free energy (MFE) by adding up individual energy contributions from base pair stacking, bulges, hairpins, internal loops and multi-branch loops. The RNAfold server was selected for analysis, because it uses both the Wuchty algorithm and the McCaskill algorithm, which offers a wide variety of functions, having the benefit of computing all possible secondary structures within a narrow free-energy range [20,21]. This approach leads to the creation of one most likely structure. In contrast, some software servers predict secondary structures by calculating thermodynamics, *e.g.* UNAfold and RNA Structure use the Zuker algorithms to calculate the MFE and systematically sample structures within a percentage range of free-energy to create a set of diverse sub-optimal structures without providing a preference for the most likely outcome. Thus in this study, in order to predict the secondary structure of each hIFNγ mRNAs, minimum free energy (MFE) structures and base pair probabilities from single RNA sequences, RNAfold WebServer (Vienna RNA Websuite) was used [20].

3. Results

3.1. Confirmation of integration into P. pastoris

PCR products were amplified at the expected size for ~50 clones for all constructs using gene-specific forward and reverse primers. Amplification resulted in bands of the correct sizes of 0.5–1 kbp which was confirmed by sequence analysis at the AGRF demonstrating successful integration of the *hIFNγ* gene into the yeast genome.

3.2. SDS-PAGE & western blotting

Secretion of hIFNγ in *P. pastoris* cultures was not detected using SDS-PAGE and anti-His Western blotting analysis. In contrast, expression of secreted positive control (P-Protein) was observed.

3.3. ELISA

Low expression of secreted hIFNγ was detected in some transformant cells of *P. pastoris* (Table 3), with highest levels found in CB7435-pPpT4αS-COS2 and lowest in GS115-pPIC9-COS1, indicating that codon-optimisation of the sequence had no impact on expression yields. This was confirmed by non-detectable yields in the X33 strains transformed with the NS and COS2 sequences of hIFNγ, respectively. As the latter results were also independent of vector used, this also indicates that expression of hIFNγ was strain-dependent and affected by their resulting phenotype rather than the choice of vector and gene codon optimisation.

ELISA analysis of cell lysates of *P. pastoris* GS115-pPIC9-COS2 transformants demonstrated that hIFNγ was successfully secreted to medium (Table 3). GS115-pPIC9-COS2 transformant cells cultivated in unbuffered media yielded similar amounts of secreted hIFNγ to those using buffered medium (Table 3) suggesting that extracellular proteolytic degradation of the product was not the cause of low yields. Despite the low expression, expression was enhanced 10-fold in using the codon-optimised sequence COS2 which had a lower MFE (GS115-pPIC9-COS2 in comparison to GS115-pPIC9-COS1 (Table 3) (Section 3.4.1).

3.4. RNA analysis

3.4.1. mRNA secondary structure

Although the secondary structures of mRNAs appeared to be different in bi-dimensional models, both codon-optimised sequences possessed similar predicted levels of the MFE compared to the natural sequence of *hIFNγ*. Thus, a similar level of RNA stability would be expected (Fig. 3).

3.4.2. Detection of hIFNγ mRNA

Analysis of RT-qPCR result showed that approximately 2–3 copy number of hIFNγ mRNA were found per cell (Table 4), which is considered as low abundance RNA.

4. Discussion

Codon preference between the recombinant gene and expression host has been established to be one bottleneck for protein translation in heterologous expression systems, hindering the translation of the recombinant gene transcript [22]. To overcome this potential problem, codon optimisation was used to adapt the foreign recombinant gene for successful and efficient heterologous expression in yeast systems [23]. Studies investigating the correlation between codon usage patterns and expression level for *hIFNγ* proved that codon bias exists in CHO cells [3,22]. In contrast, our results did not show a significant impact on the expression of codon-optimised (COS1 & COS2) and non-codon-optimised, native (NS) sequences (Table 3). The potential for premature RNA truncation due to poly A signals in COS1 was refuted by proving that the poly A tail was in the correct location of the hIFNγ mRNA using reverse transcription with the Oligo dT (18) primer and a primer pair binding close to the 5′ end. This would suggest that RNA instability was not the cause of low expression. Moreover, analysis of the predicted secondary structures of mRNAs revealed more similar MFE levels of two sequences (NS, COS1) and slightly lower MFE level for the COS2 (Fig. 3) which could be the reason for enhancing the expression of the COS2 by 10 folds, this result is in conformity with previous reports indicating that manipulation of secondary structure of mRNA can effectively improve translation and heterologous expression of recombinant proteins in yeast [24].

Table 3
Maximal yield of secreted hIFNγ expressed in *P. pastoris* after 72 hpi **(Mean ± SD, n = 3)**.

Transformant (Strain-vector-sequence)		Phenotype	Material analysed	Max. yield (μg L^{-1})
Pichia pastoris	GS115-pPIC9-COS1	His^+Mut^+	Supernatant/buffered	0.17 ± 0.02
	GS115-pPIC9-COS2	His^+Mut^+	Supernatant/buffered	1.8 ± 0.06
			Cell lysate/buffered	0.4 ± 0.2
			Supernatant/unbuffered	1 ± 0.2
	X33-pPICZαA-NS	Mut^+	Supernatant/buffered	n.d.
	X33-pPICZαA-COS2	Mut^+	Supernatant/buffered	n.d.
	X33-pPpT4αS-COS2	Mut^+	Supernatant/buffered	n.d
	KM71H-pPICZαA-NS	Mut^S	Supernatant/buffered	12 ± 0.02
	KM71H-pPICZαA-COS2	Mut^S	Supernatant/buffered	9 ± 0.5
	CB7435-pPpT4αS-COS2	Mut^S	Supernatant/buffered	16 ± 3

n.d.: not detected.

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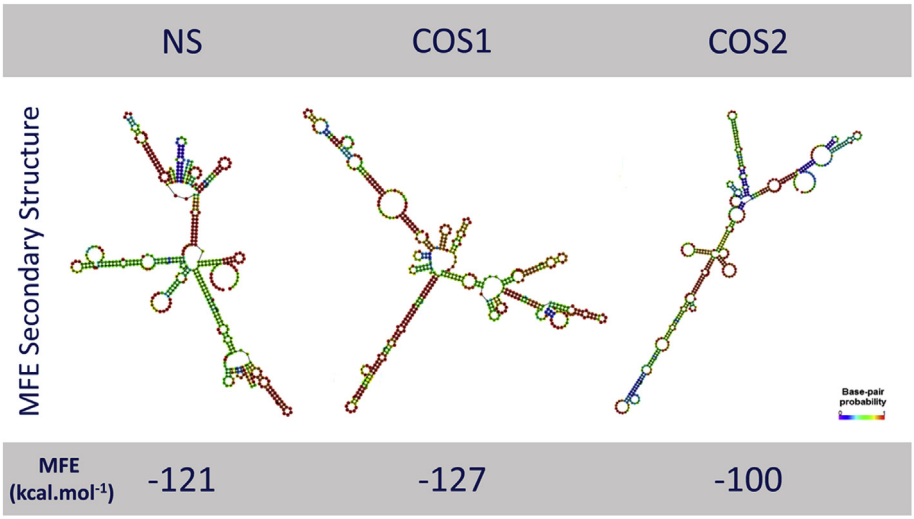

Fig. 3. Bi-dimensional modelling of mRNA secondary structure predicted based on the MFE. **NS**: Native sequence, **COS1**: Codon-optimised sequence 1, **COS2**: Codon-optimised sequence 2.

Table 4
hIFNγ cDNA (= mRNA) copy number of GS115-pPIC9-COS1 *P. pastoris* transformants **(Mean ± SD, n = 3)**.

C(t)	14.4 ± 0.3
Initial copy number of *hIFNγ* cDNA per volume reaction	$43 \times 10^3 \pm 7 \times 10^3$
Approx. hIFNγ mRNA copy number per cell	2.6 ± 0.4

All transformants showed low to no expression of hIFNγ, however, an improvement of 100-fold was, achieved with transformants of the Mut^S phenotype (Table 3). Several hypotheses can be formulated to explain this outcome.

1. The large difference in yields between Mut^S and Mut$^+$ phenotypes could indicate that lack of the *AOX2* gene in the Mut^S phenotype would result in higher yields. This raises the question whether the activity of *AOX2* interferes with transcription of *hIFNγ*. This hypothesis can be tested by using constitutive promoters in *P. pastoris e.g.* GAP promoter.

2. Undesired proteolysis of heterologous proteins expressed in *P. pastoris* and/or inefficient secretion could have caused lower product yields of the recombinant protein [11]. Proteolysis may occur in two ways; firstly, intracellularly, during vesicular transport of recombinant protein by secretory pathway-resident proteases and secondly extracellularly, by proteases being secreted or released into the culture medium [11]. For example, the yield of ovine interferon τ decreased due to proteolysis and eventual degradation of the recombinant protein [25]. In contrast, our results render the low expression of hIFNγ due to extracellular proteolytic activity and/or incomplete secretion unlikely. Furthermore, as the α-mating factor secretion signal was incorporated into the design, neither addition nor deletion of the native secretion signal altered expression levels, as expected, and presence or absence of the His tag also had no impact.

3. A distinct possibility for the observed low expression in our study could be the low abundance of mRNA (estimated 2–3 copy numbers of hIFNγ mRNA transcripts). To explain this, it needs to be noticed that a typical average cell contains ~10–30 pg total RNA composed of ~360,000 mRNA molecules. Low abundance mRNA species may have a copy number as low as 5–15 molecules per cell [26] This could be explained by assuming that either the *AOX* promoter was not activated to initiate transcription of hIFNγ mRNA or induction of *AOX2* adversely impacted on transcription of hIFNγ mRNA.

4. Intracellular degradation of the recombinant protein may be an additional cause for the low yields, which is supported by the apparent mismatch between anti-His Western blot detection and ELISA results which could have resulted in the removal of the His-tag prior to secretion. To examine this hypothesis, the use of protease-deficient strains of *P. pastoris* in future studies could be useful.

5. Protein mis-folding in the endoplasmic reticulum (ER) has been reported as one of the possible reasons for low production rates of proteins in yeast [13].

The *P. pastoris* expression system has only recently been investigated for the commercial production of hIFNγ, reporting secretion of around 300 mg L^{-1} with a specific activity of 1×10^7–1.4×10^7 IU mg^{-1}, using the pPICZα vector and the alcohol oxidase (*AOX1*) promoter [10]. Our yields of hIFNγ were much lower and these outcomes were not improved using additional strains, vectors, codon-optimised sequences. In contrast to Wang, Ren [10], protein quantification was carried out in this study via ELISA instead of using the Bradford assay and purity-determination by HPLC. As we did not use the same restriction sites, it could be possible that KEX2 and/or STE13 cleavage was not efficient, leading to ineffective secretion, because the processing of the signal sequence of human interferon gamma occurs in two steps, firstly cleavage by KEX2 then STE13. Whilst we cannot exclude incomplete processing and secretion, our antibody detects the secreted protein, which would indicate that the antibody does not target the secretion signal sequence. In addition, we checked the cell lysate of GS115-pPIC9-COS2 and found that the protein was efficiently secreted with little remaining. We would therefore expect the same outcome for pPICZ, as we used the same cloning sites EcoRI/NotI. This, to our point of view, would suggest that the observed low yields are the result of more than just inefficient secretion, i.e. low mRNA copy numbers remain a highly likely reason.

Furthermore, the molecular weight of the unglycosylated recombinant hIFNγ expressed in *E. coli* is 17.6 kDa; however, generally, the molecular weight of proteins increases due to glycosylation [27] Therefore, the expected molecular weight of hIFNγ expressed in *P. pastoris* would be higher. The Western blot result, however, shown in Ref. [10] identified a 15 kDa band as hIFNγ which is theoretically impossible unless the target protein was truncated and unglycosylated, which highlights the possibility of misidentification in the small-scale experiments. In contrast, the authors verified the nature of the secreted protein by N-terminal

sequencing following HPLC purification, suggesting that obtained yields might be achievable.

Comparison of the translated amino acid sequence shown in Ref. [10] (derived from the published optimised DNA sequence) to the native amino acid sequence of hIFNγ revealed defects in three positions, *i.e.* there is a deletion of serine ^{43} and an addition of serine ^{51} and replacement of leucine ^{50} by phenylalanine ^{50} in the native polypeptide in the recombinant protein. While these differences may not impact on the determination of yields, it could alter the three-dimensional structure of the protein with potential impacts on biological activity.

Taken together, irrespective of sequence, vector and expression strain used, this study showed that the yield of hIFNγ expressed in *P. pastoris* is too low for industrial-scale production. Similarly, a very recent study by Prabhu et al. (2016) also did not achieve high expression yields of hIFNγ (<2.5 mg. L^{-1}), using a *P. pastoris* codon-optimised hIFNγ sequence, the same strain of *P. pastoris* (GS115) and vector (pPICZα) under the control of *AOX1* promoter, or a multiple copy number integration approach (pPIC9K, a multiple copy integrating vector) and process fermenter parameter optimisation, *viz*. agitation rate, inoculum size, methanol concentration, pH and temperature [13].

Although glycosylation of the protein by *P. pastoris* should lead to a longer half-life of the recombinant protein and higher biological activity, it needs to be considered that recombinant proteins can on occasions become over-glycosylated and content of high mannose glycans could cause immunogenicity in patients, which are two disadvantages of this expression system. Therefore, we recommend future studies to focus on improvement of expression of hIFNγ in mammalian systems, as glycosylation patterns should be more similar to those found in human cells [3].

Among mammalian expression systems; CHO was the focal point of studies from the 1990s onward, achieving laboratory-scale secretion of 15 mg L^{-1} of hIFNγ [28] and studies included overexpression, optimisation of cultivation, scale-up production and purification [3,6,29,30]. An order of magnitude greater expression yields of hIFNγ (570 mg mL^{-1}) was achieved in mammary glands of transgenic mice *in vivo*, which is comparable to productivity in *E. coli* [7,31]. Studies in mammalian expression systems are ongoing to improve productivities further and to lower the cost of production, which is essential to make mammalian expression systems at the industrial scale competitive with the currently used *E. coli* expression system.

5. Conclusions

Around 50 transformant colonies of *P. pastoris* were screened for expression of hIFNγ with yields ranging from not detectable to low. This was most likely the result of the low abundance of mRNA transcript and/or inefficient secretion. We, therefore, conclude that industrial production of hIFNγ in *P. pastoris* is economically unrealistic, unless transcription/translation can be significantly increased. It is therefore recommended that commercial production focuses on other eukaryotic expression systems *e.g.* CHO, mammary gland expression in transgenic mice or even human embryonic kidney 293 (HEK293) cells. In addition, research has to focus on unravelling the cause of low expression of hIFNγ in *P. pastoris* to overcome low yield hurdles to make the system competitive economically.

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