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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.

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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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