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Comparative Expression Levels of Methylotrophic Yeast Strains of *Pichia Pastoris*

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Abstract. This investigation compared the expression levels of diverse phenotypes of *Pichia pastoris* GS115, X-33 and KM71H in various nutrient media, including BMMY, BMMH, MMY and MMH. The MMY nutrient media demonstrated a higher expression level of HBsAg protein compared to the other minimal media. Mut^+ was chosen as an acceptable phenotype because of its high growth density and relatively short expression period of recombinant HBsAg protein. Furthermore, the X-33 yeast strain showed a greater growth rate than the GS115 and KM71H yeast strains, leading to higher levels of expression of the HBsAg protein. In conclusion, BMMY and BMM media are considered to be suitable for HBsAg protein expression, since the expression levels of the protein in both media are nearly identical.

Keywords: GS115, X-33, KM71H, hepatitis B, *pichia pastoris*

Introduction

Since hepatitis B virus (HBV) infection is a major global health concern and an efficient vaccine production system is required, comparative expression levels of *Pichia pastoris* strains in nutrient medium have become an area of active research with respect to HBsAg protein production (Liu *et al*., 2009). *Pichia pastoris* holds several advantages over *Saccharomyces cerevisiae* as a cell factory such as thermotolerance, the ability to grow respiratively in very high cell densities and the ability to express high levels of heterologous proteins using constitutive promoters or its inducible methanol oxidoreductase promoter (Puxbaum *et al*., 2015; Ahmad *et al*., 2014; Mattanovich *et al*., 2012). The popular strains GS115 and X-33 are derivatives of CBS7435 and are components of a commercial protein-expression kit that is distributed by invitrogen/life technologies/ Thermo Fisher Scientific. GS115 was derived from CBS7435 by random nitrosoguanidine mutagenesis and carries the HIS4 mutation in addition to a variety of other point mutations (Shen *et al*., 2018; Love *et al*., 2016). X-33 is a subline of GS115 in which the HIS4 gene has been converted to the wild-type gene sequence by site-directed mutagenesis (Kurtzman, 2009; Blanchard *et al*., 2008).

This methylotrophic yeast is a highly useful eukaryotic host due to its ability to efficiently secrete proteins into the culture broth, the availability of sophisticated quality control and post-translational modification systems, high growth rates and the availability of advanced DNA engineering tools, including strong promoters, selectable markers and genome editing platforms (Tsuda and Nonaka, 2024; Unver and Dagci, 2024). The methylotrophic yeast *Pichia pastoris* has been established as a commercial host for production of heterologous proteins (Morvarid *et al*., 2012; Cereghino *et al*., 2000). The expression levels obtained in the eukaryotic pichia system are high and most of the recombinant proteins are soluble (Shao *et al*., 2003). *Pichia* can be grown to very high cell densities in a simple mineral medium, thus avoiding the endotoxin contamination problems of the bacterial expression systems and the viral contamination problems of animal-cell cultures. Moreover, *Pichia pastoris* can grow in the presence of methanol as carbon source when glucose is not present. The *Pichia pastoris* expression system uses the methanol-inducible alcohol oxidase (AOX1) promoter that drives the expression of the alcohol oxidase gene,

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which catalyzes the first step of methanol metabolism (Hartner and Glieder, 2006). The *Pichia pastoris* system is licensed to over 160 companies in the biotechnology, pharmaceutical, vaccine and food industries (Hu *et al*., 2011).

Expression and efficient folding of hepatitis B surface antigen (HBsAg) have been described for *Pichia pastoris* after integration of a single copy of the HBsAg gene under control of the AOX1 promoter (Sobirdjan *et al*., 2021; Sobirdjan *et al*., 2020; Rachman *et al*., 2011). Moreover, introduction of multiple copies of the recombinant HBsAg gene into *Pichia* strain GS115 has been found to be a good way to improve the protein expression (Vassileva *et al*., 2001).

The practical significance of the present study arises from the need for cheap and scalable production of HBsAg for the production of vaccine and diagnostic reagents, especially in developing countries where HBV prevalence is still high (Naully *et al*., 2020; Gurramkonda *et al*., 2009). Despite these advances, there remains a difficulty in optimizing HBsAg yield using different *Pichia pastoris* strains and nutrient media formulations. The main challenges are due to strain phenotypes (Mut^+ vs. Mut^S) variability in expression levels, gene copy number and induction protocols (Tam *et al*., 2014; Morvarid *et al*., 2012).

There is still a lack of information about comparative performance of various strains under different nutrient media for HBsAg production in *Pichia pastoris*. In this study, the expression of HBsAg in three *Pichia pastoris* strains GS115, X-33 and KM71H, cultivated in different minimal media, BMMY, BMMH, MMY, MMH had been investigated.

Materials and Methods

Strains and vector. *Escherichia coli* TOP 10 (Invitrogen, USA), *Pichia pastoris* strains GS115, X-33, KM71H (Invitrogen, USA) and the expression vector pPICZαA (Invitrogen, USA) containing the HBsAg gene were used in this study.

Media for cultuvation *Pichia pastoris* strains. MMH **(**Minimal Methanol Histidine) 1.34% YNB, $4x10^{-5}$ % biotin, 1% methanol, 0.004% histidine. BMMY (Buffered Methanol-complex Medium) 1% yeast extract, 2% peptone, 100 mM potassium phosphate, pH 6.0, 1.34% YNB 4×10^{-5}% biotin, 1% methanol. BMMH (Buffered Minimal Methanol Histidine) 100 mM potassium phosphate, pH 6.0, 1.34% YNB 4×10^{-5}% biotin, 1% methanol, 0.004% histidine. MMY (Minimal Methanol Complex Medium) 1.34% YNB, $4x10^{-5}$% biotin, 1% methanol, 1% yeast extract, 2% peptone)

Electroporation of pPICZαA-HBsAg into *Escherichia coli*. pPICZαA-HBsAg was transformed into *Escherichia coli*. After transformation, plate transformation was mixed onto low-salt LB plates (1% tryptone, 0.5% Yeast Extract, 0.5% NaCl) with 25 μg/mL Zeocin™ and Zeocin™ resistant colonies were selected. Zeocin TM-resistant transformants were inoculated into 1 mL LB medium with 25 μg/mL Zeocin™ and grown overnight at 37 °C with shaking. Plasmid DNA was isolated using miniprep for restriction analysis.

Electroporation of pPICZαA-HBsAg into *Pichia pastoris.* Electroporation was performed as described by Wu *et al*. (2004) with minor modifications. The yeast strains GS115, X-33 and KM71H were grown overnight in YPD medium (1% yeast extract, 2% peptone, 2% dextrose) at 30°C. Thirty microliters of yeast culture were transferred to 100 mL of YPD medium and incubated at 30°C until the cell density reached 1-2A (absorbance) at 600 nm (A_{600}). The cells were collected by centrifugation. The cells were pelleted from 100 mL and were suspended at room temperature for 30 min in 10 mL of 100 mM LiAc, 10 mM DTT, 0.6 M sorbitol and 10 mM Tris-HCl, pH 7.5. The cells were then pelleted, resuspended in 5 mL of ice-cold 1 M sorbitol, washed three times with 5 mL ice-cold 1 M sorbitol, pelleted and resuspended in 0.5 mL 1 M ice-cold sorbitol. The recombinant vector, pPICZαA-HBsAg, was linearised with SalI (Invitrogen, USA) restriction enzyme. The 80 mkl cells were mixed with 50 ng linearised pPICZαA-HBsAg in 2 μL of water, transferred to a 0.2 cm gap vial and incubated for 5 min on ice. The electroporating pulse was applied at 1.5 kV using a Electroporator 2510 (Eppendorf, Germany). The electroporated cells were immediately diluted in 1 mL of ice-cold 1 M sorbitol, incubated 1-2 h at 30 °C. The transformants were plated on yeast extract peptone dextrose medium (YPDS+Zeocin agar) (1% yeast extract, 2% peptone, 2% dextrose, 1 M sorbitol, 2% agar, 100 μg/mL Zeocin™). The plates were incubated at 30 °C for approximately 3-5 days until single colonies were observed.

Shake flask culture conditions. The shake flask experiments were carried out in 500 mL baffled flasks

(50 mL working volume). Minimum glycerol medium MGY (1.34% yeast nitrogen base, 2% glycerol, 0.0004% biotin) was used. The expression of the recombinant protein was allowed to grow in flasks at 30 °C at a shaking rate of 200 rpm for 24 h until A_{600}=2-6. The cells were harvested and resuspended in a 50 mL minimum methanol medium (BMMY, MMY, BMMH, MMH) and induced by adding 99.9% methanol to a final concentration of 0.5-1.0% for 96-120 h.

High-pressure cell disruption. Cell disruption was performed using a high-pressure homogeniser (APV Systems SPX 2000 bar Laboratory Homogenizer, USA). Cells were harvested and accumulated from the culture by centrifugation (4,500×g, 10 min, 4 °C), followed by two washes with distilled H_2O. The cell culture was disrupted by 4 passes at 600 bar.

Enzyme-linked immune-sorbent assay (ELISA). HBsAg detection was performed using ELISA according to the manufacturer's instructions (ELISA kit specific for HBsAg detection; Diagnostic Systems (Russia). First, 20 µL of the sample dilution solution was added to 96 well plates. Cell extracts were centrifuged (4,500×g, 10 min, 4 °C) and 100 µL was added. The mixture was then incubated for 60 min at 37 °C. After incubation, 50 µL of the conjugate solution was added to each well. The well containing the mixture was washed five times with 350 µL of washing buffer (phosphate-buffered saline (PBS), Tween-20) at 30 s intervals. The plate was then dried and 50 µL of 3,3',5,5'-tetramethyl-benzidine (TMB) substrate was added to each well before incubation for 30 min at 37 °C. The reaction was stopped by adding 50 µL stop reagent to each well. The results were observed at a reading wavelength of 450 nm and reference wavelength of 650 nm using an ELISA reader (Microplate Reader, Rayto, China).

Results and Discussion

Transformation of recombinant plasmid pPICZαA-HBsAg in *Pichia pastoris*. An expression vector, pPICZαA-HBsAg, was linearised with the SacI restriction enzyme and successfully transformed into *Pichia pastoris* GS115, X-33 and KM71H strains *via* electroporation. All transformants were selected on YPDS plates containing 100 mkg/mL Zeocin™ reagent. The transformation of the target HBsAg gene incorporated into linearised plasmid DNA was mainly occurred within the 5' AOX1 region of the *Pichia pastoris* strains X-33 and GS115 and Mut+ transformants were generated. In contrast, the KM71H strain only produced Mut- transformants. This difference is due to the replacement of the chromosomal AOX1 locus in KM71H with the *Saccharomyces cerevisiae* gene ARG4; thus, all of the transformants from KM71H exhibit the Mut- phenotype (Morvarid *et al*., 2012). (Fig. 1)

Yeast cells from a single colony of *Pichia pastoris* GS115, X-33 and KM71H strains were grown in MMY medium. The cell culture was pelleted and genomic DNA was extracted using 1% SDS and 100 mM LiOAc (Oybek *et al*., 2023). The presence of HBsAg (681 bp) inserts in transformants was verified by direct PCR amplification (Fig. 2). The overall length of the DNA product was 938 bp using α-factor F (5'-TACTATTGCCAGCATTGCTGC -3') and AOX1 R (5'-GCAAATGGCAT TCTGACATCC -3') primers.

As can be seen in Fig. 2, the results obtained by the complete PCR analysis clearly demonstrated the existence of the HBsAg gene for all strains of *Pichia pastoris*. Consequently, it can be concluded that they successfully achieved the intricate process of cloning the HBsAg gene into each individual strain of *Pichia pastoris* without any complications.

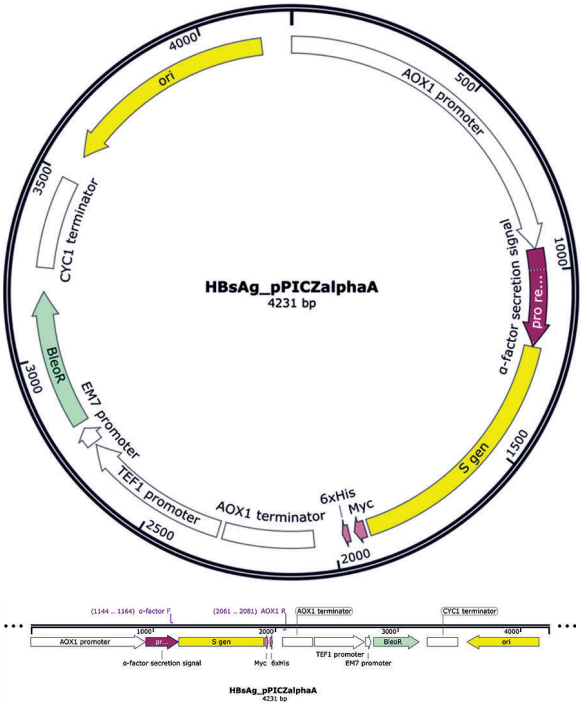

Fig 1. Construction of pPICZαA with HBsAg gene insertion.

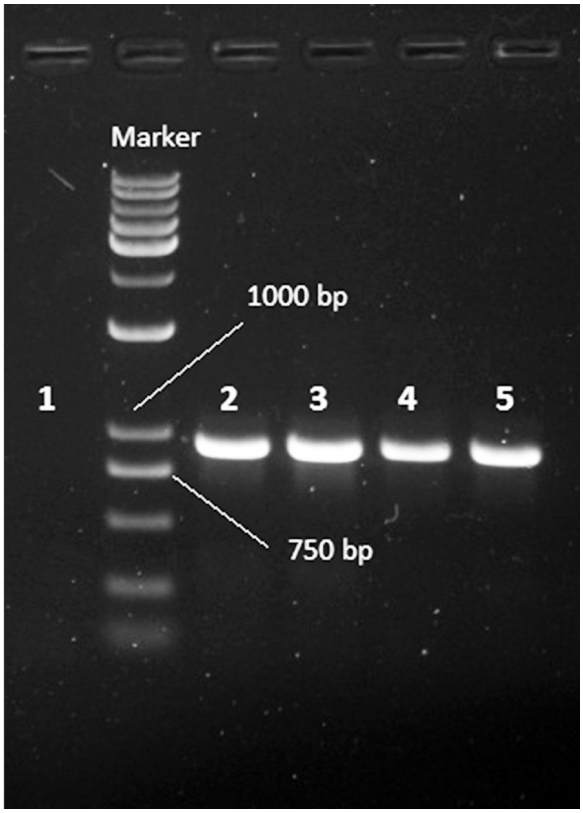

Fig. 2. Gel electrophoresis of PCR amplification of the HBsAg gene in 1% agarose gel.

Expression of HBsAg protein in shake flask. To determine the most suitable media for the expression of HBsAg in *Pichia pastoris* expressed the recombinant HBsAg protein in different minimal media. Two different phenotypic classes of recombinant strains can be generated Mut^+ and Mut^S. Mut^S refers to the "Methanol utilization slow" phenotype caused by the loss of alcohol oxidase activity encoded by the AOX2 gene. A strain with a Mut^S phenotype has a mutant AOX1 locus but is wild type for AOX2. This results in a slow growth phenotype on methanol medium. Both X-33 and GS115 are Mut^+ and KM71H is Mut^S. Transformation of X-33 or GS115 with plasmid DNA linearized in the 5′ AOX1 region will yield Mut^+ transformants, while KM71H will yield only Mut^S transformants. For expression, recombinant proteins need BMGY/BMMY (buffered complex glycerol or methanol medium), BMG(H)/ BMM(H) (buffered minimal glycerol or methanol medium), or MGY/ MM(H) (minimal glycerol or minimal methanol medium) in a shake flask system. BMG(H), BMM(H), BMGY and BMMY are usually used for the expression of secreted proteins, particularly if pH is important for the activity of your protein (EasySelect™ Pichia Expression Kit).

The expression of recombinant *Pichia pastoris* strains GS115, X-33 and KM71H containing the HBsAg gene was studied through two-phase cultivation in a minimal medium. During the initial phase, cells underwent exponential growth for a duration of 24 h in MGY media. Cells were harvested in sterile centrifuge bottles by centrifugation at 4500 x g for 5 min. at room temperature. In the second phase, the supernatant was discarded and the cells were resuspended in various minimal media: BMMY, BMMH, MMY and MMH. Induction occurred under the pAOX1 promoter with 1% methanol and under the pAOX2 promoter with 0.5% methanol, administered every 24 h (Table 1). The induction time was established by administering 99.9% methanol to Mut^+ (GS115 and X-33 pPICZαA-HBsAg) for 96 h, while Muts (KM71H pPICZαA-HBsAg) were induced for 112 h. Fig. 3 illustrates the cultivation process.

Samples were taken to determine HBsAg levels using ELISA assays (Table 1).

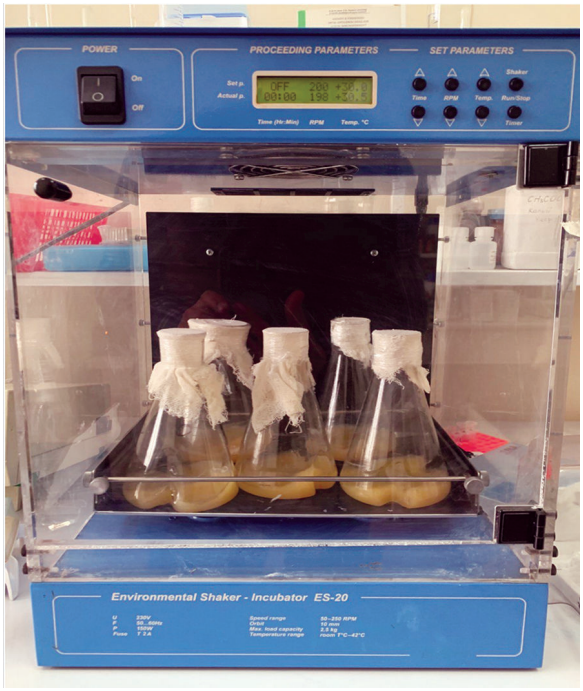

Fig. 3. Expression of HBsAg protein in shake flasks.

Table 1. ELISA results of yeast strains grown in different nutrient media

	Expression time after induction	Media	Optical density (OD_{600})	Result of ELISA
Mut^+ GS115 pPICZαA-HBsAg P_{AOX1}	96 h	BMMY	81	± 8 mg/L
		BMMH	60	± 1.2 mg/L
		MMY	83	± 8.5 mg/L
		MMH	20	± 0.75 mg/L
Mut^+ X-33 pPICZαA-HBsAg P_{AOX1}	96 h	BMMY	85	± 8.2 mg/L
		BMM	62	± 1.4 mg/L
		MMY	86	± 8.8 mg/L
		MM	25	± 0.8 mg/L
Mut^s KM71H pPICZαA-HBsAg P_{AOX2}	112 h	BMMY	70	± 8.5 mg/L
		BMMH	52	± 1.5 mg/L
		MMY	73	± 8.9 mg/L
		MMH	17	± 0.8 mg/L

The cell culture was disrupted using a homogeniser with four passes at a pressure of 600 bars. Following the disruption, an enzyme-linked immunosorbent assay (ELISA) was performed to evaluate the presence of the recombinant protein.

Differing from strain X-33, which has an intact HIS4 gene, the host strains GS115 and KM71H have mutations at the HIS4 locus that prevent endogenous synthesis of histidine. Accordingly, histidine supplementation is appropriate for GS115 and KM71H cultures.

A commercial kit, Hepatitis-B Vaccine (rDNA) 20 mcg/ml from the Serum Institute of India, was used as a comparative control. The table illustrates that the expression level in all yeast strains was highest in the MMY nutrient medium. While most studies favour the BMMY medium, our research demonstrates that the MMY culture medium yields a superior expression of the HBsAg protein. Consequently, pH does not appear to be critical for optimising HBsAg protein expression. The MMH nutrient medium yielded the poorest results.

Heterologous expression in *Pichia pastoris* may take place either within the cell or through secretion. Secretion necessitates a signal sequence on the expressed protein to direct it to the secretory pathway. Various secretion signal sequences have been employed with varying degrees of success, including the native secretion signal found in certain heterologous proteins (Bardiya, 2006). This study utilized the pPICZαA plasmid, which includes an α-factor secretion signal. The findings indicated that the HBsAg protein was not released into the culture medium, even with the application of the α-factor secretion signal. The investigations indicate that the HBsAg protein may encounter challenges in recognition and transport *via* the secretory pathway in *Pichia pastoris*.

The Mut^+ phenotype facilitates increased protein production by utilizing methanol more efficiently, leading to improved growth rates and enhanced yields of recombinant proteins (Mastropietro *et al*., 2021). The Mut^+ phenotype demonstrates enhanced protein expression capabilities. Recent studies indicate that co-expressing essential enzymes from the methanol utilization pathway can further improve productivity in *Pichia pastoris* strains. The overexpression of dihydroxy-acetone synthase has been shown to significantly enhance substrate conversion efficiency, resulting in a 2- to 3-fold increase in methanol utilization; however, this improvement is associated with a decrease in volumetric productivity (Krainer *et al*., 2012). The study revealed that the Mut^S phenotype exhibited greater HBsAg expression compared to the Mut^+ phenotype, which contradicts the initial expectation that the Mut^+ phenotype would demonstrate higher expression levels. This finding indicates that the Mut^S phenotype may be a more appropriate option for the expression of HBsAg in *Pichia pastoris*.

Conclusion

This study compared the expression levels of different phenotypes of *Pichia pastoris* GS115, X-33 and KM71H in different nutrient media BMMY, BMMH, MMY and

MMH. The MMY nutrient medium showed a higher level of HBsAg protein expression than the other minimum media. Mut^+ was selected as the preferred phenotype because it resulted in a higher cell density and favored a relatively shortened cycle time to express the recombinant HBsAg protein. Moreover, it was noted that the X-33 strain had a higher growth rate than the GS115 and the KM71H strains and the expression of its HBsAg protein was also higher. In general, it can be concluded that the BMMY and BMM media are appropriate media for HBsAg protein expression since the expression profiles of produced proteins are similar. These results provide evidence that the optimization of the nutrient medium and the proper choice of yeast strains can significantly increase recombinant protein production, which is key to vaccine development and other biotechnological uses. By enhancing yields and lowering production costs, such optimization makes the process more efficient and accessible for large-scale pharmaceutical production.

Conflict of Interest. The authors declare that they have no conflict of interest.

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