Increasing the production of insulin glargine in *Pichia pastoris* through medium modification

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Abstract. Insulin is the primary drug for managing diabetes mellitus, especially for individuals with type 1 diabetes mellitus. Producing insulin glargine, a long-acting insulin analogue, using *Pichia pastoris* is a notable advancement in biopharmaceutical manufacturing. This research aims to increase the yield of insulin glargine in *Pichia pastoris*. The effects of these mediums and vitamins on cell growth and insulin glargine expression levels were evaluated. The findings revealed that the addition of vitamins to the minimal medium (MM) and ½ basal salt medium (BSM) increased insulin glargine production. This study highlights the critical role of vitamins in maximizing the efficiency of insulin glargine production in *Pichia pastoris*. The addition of vitamins to MM and ½ BSM mediums enhances the production of insulin glargine.

1 Introduction

The global increase in diabetes necessitates the development of insulin therapeutics, particularly insulin glargine, which is essential for maintaining glycaemic control in diabetic patients. Insulin glargine is a the first commercially available long-acting insulin analogue and widely used for the management of diabetes mellitus [1]. Insulin glargine is a modification of human insulin with the addition of two arginine molecules to the carboxyl terminal of the B peptide chain and the replacement of aspartic acid with glycine at the 21st amino acid position on the A peptide [1]–[3]. Its long duration of action and stable pharmacokinetic profile make it the main reason insulin glargine is widely used for the treatment of diabetes mellitus. As the demand for these therapeutics increases, the need for efficient and cost-effective production methods also increases.

Recombinant DNA technology has revolutionized recombinant protein. Two major pathways for large-scale production of insulin and its analogues are *Escherichia coli* and

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Saccharomyces cerevisiae. *E. coli* produces inclusion bodies that need solubilisation and oxidative refolding, whereas *Saccharomyces cerevisiae* secretes insulin immediately into the culture supernatant [4], [5]. *Pichia pastoris* leading the way due to their high protein yield, either intracellularly or extracellularly, and post-translational modifications [6]–[15]. *Pichia pastoris*, a methylotrophic yeast, is the platform of choice for large-scale insulin glargine production due to its high cell density [6], [8], [11], [12], [14]–[19], strong protein expression capability and ease of genetic manipulation [6], [7], [20]. However, maximizing the yield of recombinant insulin glargine remains essential for efficient and cost-effective diabetes therapy. This study explores the potential of medium modification as a promising strategy to improve insulin glargine production in *Pichia pastoris*.

Medium composition, including specific nutrients and supplements, significantly impacts protein expression levels and functionality. Researchers investigated the impact of pH [14], [21]–[24], temperature [14], [21]–[23], and methanol concentration [11], [21] on Production of recombinant protein using *Pichia pastoris*. The nitrogen [13], [25], [26] and carbon source [13], [25]–[29] significantly impacted heterologous protein production.

This study focuses on the modification of MM and ½ BSM medium by incorporating vitamins to investigate their impact on the production of insulin glargine in recombinant *Pichia pastoris*. By systematically analyzing the effects of these modifications, we aimed to identify optimal culture conditions that improve insulin glargine yield and activity.

2 Materials and methods

2.1 Preparation for glargine production

Recombinant *Pichia pastoris* from stock culture in -80 ºC was grown on YPD agar medium (1% yeast extract, 2% peptone, 2% dextrose, and 2% agar) containing 100 µg/mL zeocin and incubated for 72 hours at 30'C. Single colony was selected and regenerated on YPD medium containing 100 µg/mL zeocin and incubated for 48 hours at 30 ºC. Single colonies were selected and regenerated on YPD medium containing zeocin 100 µg/mL and incubated for 48 hours at 30 ºC. Selected recombinant *Pichia pastoris* were inoculated into 10 ml of BMGY medium (1% yeast extract, 2% peptone, 1% glycerol, 1.34% YNB, 100 mM potassium phosphate at pH 6.0, and 4x biotin) containing 100 µg/mL zeocin and incubated in a 200 rpm shaker incubator at 30 ºC for 24 hours.

2.2 Glargine production

Recombinant *Pichia pastoris* cultures in BMGY medium were centrifuged at 5,000 rpm for 5 min at 4'C. The pellet ($OD_{600} \pm 1$) was resuspended in 10 mL ½ BSM medium (13.35 mL 85% phosphoric acid, 0.465 g calcium sulfate, 9.1 g potassium sulfate, 2.065 g potassium hydroxide, 7.45 g magnesium sulfate, 20 g g glycerol, 13.2 g ammonium sulfate) containing per Liter 4.35ml PTM 1 (per Liter contains: 6 g copper sulfate-5H2O, 0.08 g sodium iodide, 3 g manganese sulfate-H2O, 0.3 g sodium molybdate-2H2O, 0.02 g boric acid, 0.5 g cobalt chloride, 20 g zinc chloride, 65 g iron sulfate-7H2 O, 0.2 g biotin, 5 mL sulfuric acid) as additional minerals in ½ BSM medium and 10 mL MM medium (containing: 1.34% YNB, 2% methanol, 4x biotin) with or without 0.25% vitamin (per Liter contains: 0.8 g calcium pantothenate, 8 g myo-inositol, 0.8 g thiamine dichloride, 0.8 g pyridoxine hydrochloride, 0.2 g nicotinic acid, 0.8 g D(+)-biotin, and 4 g K2HPO4). Then incubated in a 200-rpm shaker incubator for 96 hours at 30'C and induced with 2% methanol every 24 hours. After 96 h of incubation, cultures were harvested, centrifuged (1500 - 3000 g, 5 min, room temperature),

OD_{600} was measured, and the glargine in the supernatant was characterized by SDS-PAGE gel electrophoresis.

3 Results

Recombinant *Pichia pastoris* grew on YPD agar containing zeocin due to its zeocin resistance gene (Fig. 1).

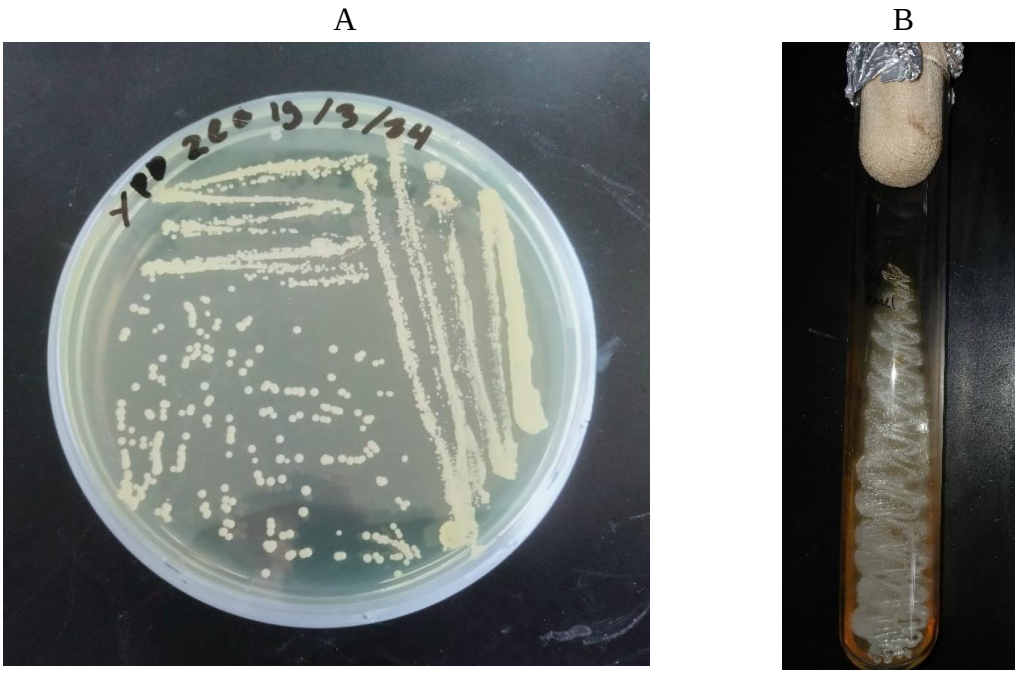

Fig. 1. Recombinant *Pichia pastoris* grew on YPD agar containing Zeocin, A. Regeneration of recombinant *Pichia pastoris* from culture stock, B. Regeneration of recombinant *Pichia pastoris* from selected single colony

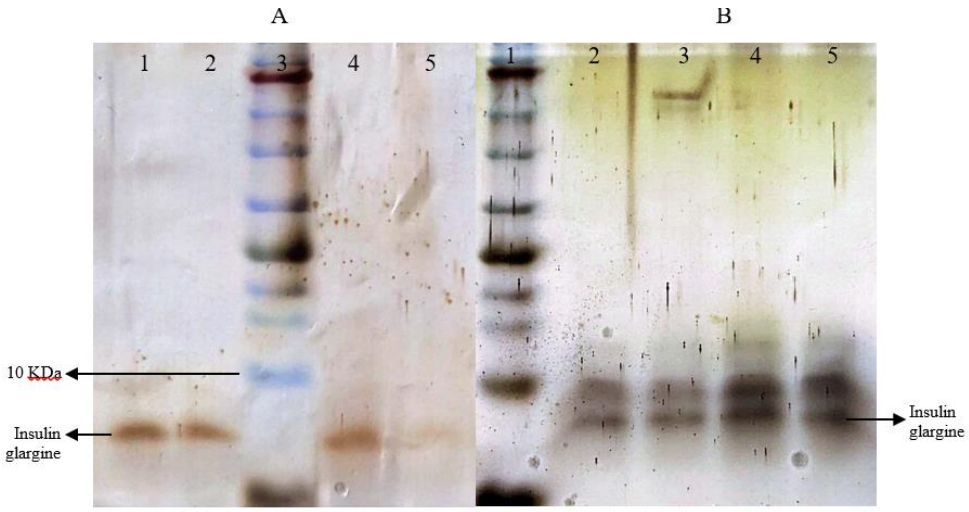

Fig. 2. Electropherogram of insulin glargine in SDS-PAGE gel. A. 1-2 insulin glargine from culture supernatant of MM medium with vitamins, 3. Protein ladder (Vivantis PR0624), 4-5 insulin glargine from culture supernatant of MM medium without vitamins; B. 1. Ladder protein, 3-4 insulin glargine from culture supernatant of ½ BSM medium without vitamins, 3. Ladder protein, 4-5 insulin glargine from culture supernatant of ½ BSM medium with vitamins

Recombinant *Pichia pastoris* was able to produce insulin glargine both on MM medium and ½ BSM medium extracellularly. The addition of vitamins in MM medium and ½ BSM medium increased insulin glargine production (Fig. 2).

4 Discussion

Recombinant *Pichia pastoris* can grow in YPD medium containing zeocin (Figure 1) because recombinant *Pichia pastoris* has the pPICZAα-g plasmid (Figure 3). This plasmid has a zeocin resistance gene with synthetic glargine. Recombinant *Pichia pastoris* containing the zeocin resistance gene produces proteins that efficiently inactivate zeocin. This protein binds to zeocin, preventing damage to the recombinant DNA of *Pichia pastoris* [30]–[32].

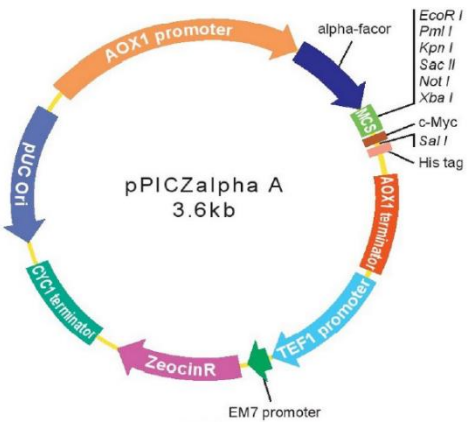

Fig. 3. The pPICZAα-g plasmid with zeocin resistance

Fermentation medium [13], [25]–[29] influence the amount of recombinant protein production in *Pichia pastoris*. The fermentation medium provides essential nutrients for cell growth and protein synthesis, and its composition can significantly affect the yield and quality of the target protein. Based on Pareto factors that affect the production of human interferon gamma in *Pichia pastoris* include: KH_2PO_4, amino acids, vitamins, triton-100, $MgSO_4$, EDTA, and trance elements [13]. The metabolic burden of protein production can be reduced by supplying amino acids, vitamins and fatty acids for cell metabolism [33]. The addition of vitamins and cofactors improves metabolism, leading to improved protein production [34]. The addition of vitamins and cofactors are also required when high levels of methanol are used [35]. The results of characterization by SDS-PAGE electrophoresis showed that the supernatant of MM and ½ BSM medium contained insulin glargine. And the addition of vitamins to MM and ½ BSM media increased the amount of insulin glargine (Figure 2). An interesting thing happened in the supernatant of ½ BSM where there were 2 bands on the results of SDS-PAGE electrophoresis (Figure 2).

5 Conclusion

The recombinant *Pichia pastoris* produced insulin glargine extracellularly and the vitamins supplementation increasing cell growth and insulin glargine production.

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