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DESENVOLVIMENTO DE UM BIOPROCESSO LIVRE DE METANOL PARA A PRODUÇÃO CONSTITUTIVA DO PRECURSOR DE INSULINA HUMANA EM *KOMAGATAELLA PHAFFII* SOB CONTROLE DO PROMOTOR pPGK1

DEVELOPMENT OF A METHANOL-FREE BIOPROCESS FOR THE CONSTITUTIVE HUMAN INSULIN PRECURSOR PRODUCTION IN *KOMAGATAELLA PHAFFII* DRIVEN BY THE pPGK1 PROMOTER

DESARROLLO DE UN BIOPROCESO LIBRE DE METANOL PARA LA PRODUCCIÓN CONSTITUTIVA DEL PRECURSOR DE INSULINA HUMANA EN *KOMAGATAELLA PHAFFII* GUIADA POR EL PROMOTOR pPGK1

Michele Caldeira Magdalena Ribeiro
MSc, Postgraduate Program in Biotechnology from Federal University of Amazonas, Brazil.
E-mail: engbiomichele@gmail.com

Enedina Nogueira de Assunção
DSc, Federal University of Amazonas, Brazil.
E-mail: dinanog@ufam.edu.br

Edson Júnior do Carmo
DSc, Federal University of Amazonas, Brazil. E-mail: ejcarmo@ufam.edu.br

Livia Miyuki Takahashi
Graduate, Postgraduate Program in Biotechnology from Federal University of Amazonas, Brazil.
E-mail: livia.takahashi.lt@gmail.com

Flavia Cabral Netto Resende
MSc, Brazilian Agricultural Research Corporation, Brazil.
E-mail: flaviacabralnetto@gmail.com

Fernando Araripe Gonçalves Torres
DSc, University of Brasília, Brazil.
E-mail: ftorres@unb.br

Ninoska Isabel Bojorge Ramirez
DSc, Fluminense Federal University, Brazil.
E-mail: nbojorge@id.uff.br

Nei Pereira Junior
DSc, Federal University of Rio de Janeiro, Brazil.
E-mail: nei@eq.ufrj.br

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Spartaco Astolfi Filho
DSc, Federal University of Amazonas, Brazil. E-mail: spartaco.biotec@gmail.com

Resumo

O desenvolvimento de novas plataformas biotecnológicas de produção de insulina assume papel importante no cenário de saúde global devido ao aumento do diabetes mellitus, tornando-se ainda mais relevante quando associada a rotas sintéticas sustentáveis que viabilizem a segurança e a escalabilidade do sistema. Este estudo investiga a produção de um precursor de insulina humana (PI) em *Komagataella phaffii*, modificada geneticamente para expressar o gene correspondente sob o controle do promotor constitutivo pPGK1, utilizando diferentes estratégias de cultivo em um biorreator de tanque agitado (STR). A secreção do precursor de insulina para o sobrenadante da cultura foi confirmada por SDS-PAGE, ELISA indireto e MALDI-TOF/MS. Para viabilizar a produção secretória de PI, foi projetado e validado experimentalmente um processo específico em biorreator. Para o processamento *downstream*, utilizou-se um sistema de membranas de fibra oca para concentrar o produto de interesse. O desempenho do processo foi avaliado comparando-se as operações em batelada e em batelada alimentada, e os resultados demonstraram que a estratégia de batelada alimentada proporcionou um aumento de aproximadamente 4 vezes na produtividade de PI. No geral, o sistema proposto representa uma plataforma de prova de conceito viável para a produção constitutiva de precursores de insulina humana heteróloga, estabelecendo a base genética e de bioprocessos para futuros estudos de otimização e dimensionamento.
Palavras-chave: promotor constitutivo pPGK1; precursor de insulina; *Komagataella phaffii*.

Abstract

The development of novel biotechnological platforms for insulin production plays a major role in the global health landscape due to the rise in diabetes mellitus, becoming even more relevant when coupled with sustainable synthetic routes that ensure system safety and scalability. This study investigates the production of a human insulin precursor (IP) in *Komagataella phaffii*, engineered to express the corresponding gene under the control of the constitutive pPGK1 promoter, using different cultivation strategies in a stirred-tank reactor (STR). Secretion of the insulin precursor into the culture supernatant was confirmed by SDS-PAGE, indirect ELISA, and MALDI-TOF/MS. To enable secretory IP production, a dedicated bioreactor-based process was designed and experimentally validated. For downstream processing, a hollow fiber membrane system was used to concentrate the target product. Process performance was assessed by comparing batch and fed-batch operations, and the results showed that the fed-batch strategy yielded an approximately 4-fold increase in IP productivity. Overall, the proposed system represents a viable proof-of-concept platform for the constitutive production of heterologous human insulin precursors, establishing the genetic and bioprocess foundation for future optimization and scale-up studies.
Keywords: constitutive pPGK1 promoter; insulin precursor; *Komagataella phaffii*.

Resumen

El desarrollo de nuevas plataformas biotecnológicas para la producción de insulina desempeña un papel crucial en el escenario de la salud global debido al incremento de la diabetes mellitus, adquiriendo una relevancia aún mayor cuando se asocia a rutas sintéticas sostenibles que viabilicen la seguridad y la escalabilidad del sistema. Este estudio investiga la producción de un precursor de insulina humana (PI) en *Komagataella phaffii*, modificada genéticamente para expresar el gen correspondiente bajo el control del promotor constitutivo pPGK1, utilizando diferentes estrategias de cultivo en un biorreactor de tanque agitado (STR). La secreción del precursor de insulina hacia el sobrenadante del cultivo se confirmó mediante SDS-PAGE, ELISA indirecto y MALDI-TOF/MS. Para

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viabilizar la producción de tipo secretoria de PI, se diseñó y validó experimentalmente un proceso específico en biorreactor. Para el procesamiento *downstream*, se empleó un sistema de membranas de fibra hueca con el fin de concentrar el producto de interés. El rendimiento del proceso se evaluó comparando las operaciones en lote y en lote alimentado (fed-batch), y los resultados demostraron que la estrategia de lote alimentado proporcionó un incremento de aproximadamente cuatro veces en la productividad de PI. En general, el sistema propuesto representa una plataforma de prueba de concepto viable para la producción constitutiva de precursores de insulina humana heteróloga, estableciendo la base genética y de bioprocesos para futuros estudios de optimización y escalado.
Palabras clave: promotor constitutivo pPGK1; precursor de insulina; *Komagataella phaffii*.

1. Introduction

In industrial biotechnology, the yeast *Komagataella phaffii* (formerly *Pichia pastoris*) has demonstrated several advantages over other expression systems for bioprocess development. These include ease of genetic manipulation, the ability to achieve high cell densities with diverse carbon sources such as glucose and glycerol, the availability of well-established protocols for heterologous protein overexpression, and a fully sequenced genome (CEREGHINO and CREGG, 2000; KHLEBODAROVA *et al*., 2024). An additional advantage is its GRAS (Generally Recognized as Safe) status, granted by the U.S. FDA in 2006 (VOGL, HARTNER and GLIEDER, 2013; KHLEBODAROVA *et al*., 2024).

Typically, recombinant protein production in *K. phaffii* is driven by the inducible pAOX1 promoter, derived from the alcohol oxidase 1 gene. However, this expression system requires methanol as an inducer, raising significant safety and operational concerns due to methanol toxicity (CARNEIRO *et al*., 2022).

As an alternative, gene expression can be controlled by constitutive promoters such as pPGK1, which drives phosphoglycerate kinase, a key glycolytic enzyme. Constitutive promoters simplify bioprocess management in fermentation by eliminating the need for hazardous chemical inducers and enabling sustained transcription of the gene of interest under different carbon-source regimes (CANKORUR-CETINKAYA *et al*., 2018; BARROS *et al*., 2025; KASTBERG *et al*., 2025). Literature suggests that the pPGK1 promoter optimizes energy partitioning between biomass synthesis and heterologous protein secretion (MAURY *et al*., 2018). The glycolytic flux-regulated activity of pPGK1 favors the maintenance of cell viability in parallel with protein synthesis, avoiding the premature depletion of cellular

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resources (ARRUDA *et al*., 2016).

Among the biopharmaceuticals produced by *K. phaffii*, insulin is particularly noteworthy due to its essential role in diabetes mellitus therapy since the early 1980s (SEKARINGTYAS *et al*., 2021). Since then, multiple strategies have been proposed to enhance insulin production in yeast platforms, aiming at higher titers, improved productivity, and more robust and scalable operation. Recent studies reported insulin precursor production in *K. phaffii* spanning from 4.19 mg/L up to 2.267 g/L using methanol-inducible AOX-based systems (THONGYOO *et al*., 2019; NURDIANI *et al*., 2021).

Diabetes prevalence continues to rise worldwide, and projections from the International Diabetes Federation estimate that the global diabetic population may reach 853 million patients by 2050 (MAGLIANO and BOYKO, 2025). Despite the relevance of yeast-based insulin platforms, there remains a strong demand for safer and more scalable processes that avoid methanol induction while maintaining high productivity in controlled bioreactor operation (BERNAT-CAMPS *et al*., 2023).

In this context, the present study aims to produce the human insulin precursor (IP) in *K. phaffii* under the control of the constitutive pPGK1 promoter, replacing the typical methanol-dependent pAOX promoter regulation, using a mechanically agitated and instrumented stirred-tank reactor (STR). Batch and fed-batch strategies were systematically compared to evaluate their effects on biomass accumulation, secretion performance, and overall IP productivity. Additionally, a hollow fiber system was incorporated into the downstream processing stage to concentrate the target product, representing a crucial step toward an end-to-end process for industrial scalability (WANG *et al*., 2019). By integrating a constitutive expression system with optimized STR cultivation modes and a downstream concentration approach, this work provides a proof-of-concept framework for efficient and scalable processes for recombinant insulin precursor production.

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2. Literature review

2.1. Methanol-Free expression strategies for recombinant protein in *K. phaffii*

A variety of expression systems have been investigated for heterologous protein production in *K. phaffii.* Optimizing recombinant protein yields necessitates evaluating key variables, including the target protein's structural characteristics, choice of promoter and signal peptide efficiency (HAN *et al*., 2026). Recombinant insulin expression in *K. phaffii* typically involves cloning the gene encoding an insulin precursor (IP), which consists of the A and B chains linked by a connecting peptide (MUNIRAH *et al*., 2025).

Recombinant protein expression in *K. phaffii* conventionally relies on methanol-induced expression systems by AOX1 promoter (pAOX1) (HAN *et al*., 2026). Using the pAOX1, Nurdiani *et al*. (2021) established a two-phase heterologous expression system for an insulin precursor: an initial growth phase on glycerol followed by methanol induction. Optimizing five key process variables (inoculum density, methanol concentration, induction time-point, pH, and temperature) yielded a maximum titer of 2.267 g/L. Although significant yields have been achieved in heterologous expression by pAOX1, the dependence on methanol for induction introduces operational and safety constraints owing to its toxic and flammable nature (WANG *et al.*, 2017; BERNAT-CAMPS *et al*., 2023).

As an alternative to the pAOX1, gene expression can be driven by constitutive promoters which eliminate the need for inducers and enable efficient expression across various carbon sources (MAURY *et al*., 2018). Recent research has dedicated significant effort to expanding the *K. phaffii* expression system toolbox, facilitating the efficient production of complex proteins with distinct processing requirements (BERNAT-CAMPS *et al*., 2023). Although the glyceraldehyde-3-phosphate dehydrogenase promoter (pGAP) is the standard constitutive promoter, other candidates including pGCW14, pTEF1, pPGK1, and pYPT1 have emerged as viable alternatives (SONG *et al*., 2026).

The constitutive pPGK1 promoter regulates phosphoglycerate kinase, an essential

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glycolytic enzyme and emerges as an alternative to both pGAP and pAOX1. Moderate-activity promoters like pPGK1 facilitate pathway optimization while reducing metabolic burden. Additionally, heterologous promoters improve strain stability by preventing unwanted recombination upon integration (HAN *et al*., 2026). Literature suggests that pPGK1 optimizes energy allocation between biomass synthesis and heterologous protein secretion (MAURY *et al*., 2018; BERNAT-CAMPS *et al*., 2023). Regulated by glycolytic flux, pPGK1 activity sustains cell viability alongside protein synthesis, thereby preventing premature cellular resource depletion (ARRUDA *et al*., 2016).

2.2. Fed-Batch operation in Biopharmaceutical Bioprocesses

Biotechnology production commonly employs fed-batch operation, predominantly utilizing aerated stirred-tank reactors or bubble columns (LÜBBERT, 2026). Fed-batch has become highly sought after in commercial production because it yields higher product concentrations while retaining the operational simplicity of a batch process (ABIDI, JONES and GEROGIORGIS, 2026).

Also, the controlled addition of the carbon source circumvents kinetic limitations associated with substrate inhibition and product feedback inhibition typically observed in batch cultures (PHILIP *et al*., 2018; BOLMANIS *et al*., 2023). Coetzee *et al* (2024) evaluated the production of a novel engineered β-fructofuranosidase under control of the constitutive promoter pGAP in *K. phaffii*, and the results indicate that the enzymes were successfully produced through a fed-batch strategy in stirred-tank reactor.

Stirred-tank bioreactors represent a robust method for the expression of heterologous proteins. Noseda *et al*. (2023) evaluated the production of the SARS-CoV-2 Spike protein using *K. phaffii* as a cell factory in a stirred-tank bioreactor (STR) across different cultivation strategies and demonstrating successful bioprocess scale-up.

2.3. Recovery steps of secreted precursors using Hollow Fiber membranes

Clarification is typically required post-fermentation in extracellular protein bioprocesses to isolate the product from fermentation broth. Traditionally, the clarification step was considered to play a limited role in modulating Critical Quality

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Attributes (CQAs) (YEOP *et al*., 2025). The CQAs represent a fundamental element of Quality by Design (QbD) in biopharmaceutical manufacturing (DEMMON *et al*., 2020).

Reid *et al*. (2024) evaluated alternative microfiltration modalities for the clarification of recombinant proteins, demonstrating that tangential flow filtration techniques (vibratory membrane filtration - VMF and hollow fiber – HF) offered high efficiency alongside straightforward optimization and operation.

Hollow fibers are thin-walled membrane structures capable of separating substances based on their size and chemical properties, making them highly suitable for filtration applications. Variations in membrane module design directly influence the efficiency and efficacy of target protein isolation (RATNANINGSIH *et al*., 2021; WOJCIECHOWSKI *et al*., 2024). Consequently, key membrane specifications determine whether the process achieves like broth clarification or protein concentration.

3. Methodology

3.1 Plasmids and genetic constructions

The human insulin precursor (IP) gene (212 bp) was synthesized by GenOne Biotech (Brazil), with codon optimization for expression in *K. phaffii* (GenBank). The gene was cloned into the pPGK1 yeast expression vector provided by the Molecular Biology Laboratory of the University of Brasília (ARRUDA *et al*., 2016). The IP coding sequence was inserted into the pPGK-AMY vector using the XhoI and NotI restriction sites, generating the recombinant plasmid pPGKA1 (Figure 1a). The primary sequence of the IP in the context of the secretion signal sequences present in pPGKA1 is shown in Figure 1b.

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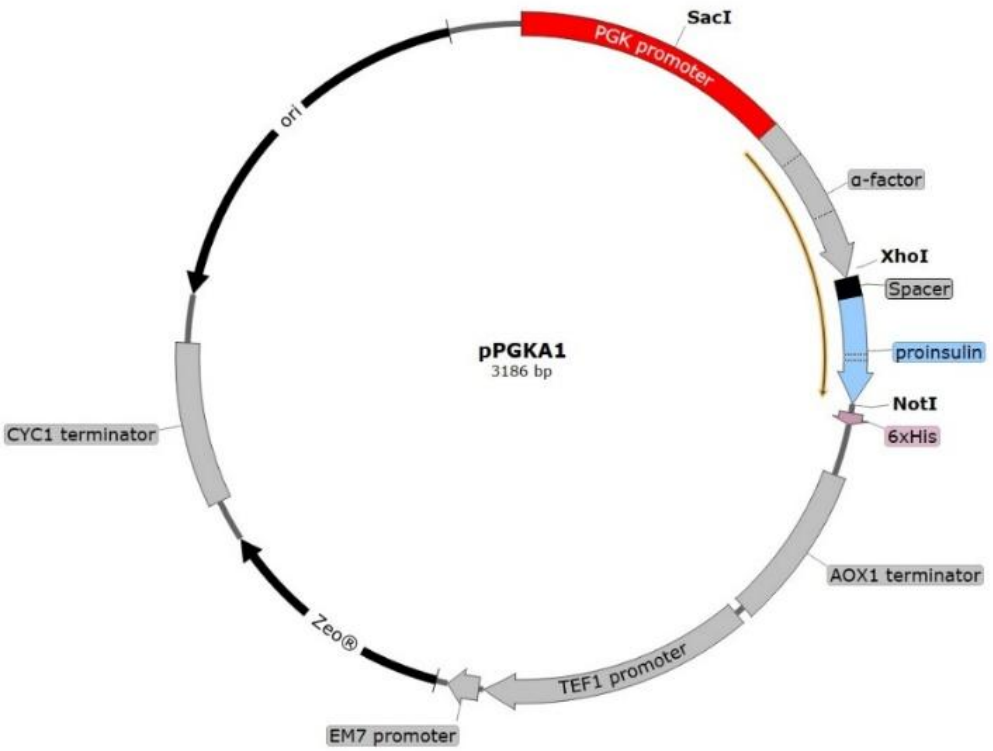
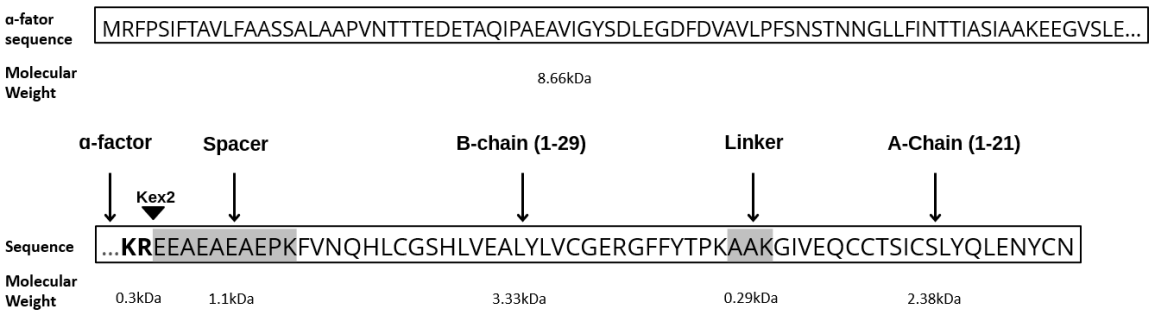
Figure 1 (a) Physical map of yeast integrative vector pPGKA1 (3186 bp). ori – replication origin of pUC; PGK promoter – promoter of the *K. phaffii* 3-phosphoglycerate kinase gene (pPGK1); α-factor – signal peptide from Saccharomyces cerevisiae; Spacer – spacer peptide; Proinsulin - single-chain polypeptide precursor to insulin, containing the B-chain (B29), C-chain – linker (AAK) and A-chain (A21), 6xHis – His Tag sequence; AOX1 terminator – AOX1 Terminator; TEF1 (translation elongation factor) and EM7 (T7 phage) promoters; ZeoR – gene that confers resistance to the antibiotic Zeocin for selection in bacteria and yeast cells; CYC1:cytochrome c terminator. Only selected restriction sites are shown. (b) Predicted Human Insulin Precursor (IP) sequence produced in yeast prior to protein pro-cessing. The black triangle denotes the Kex2 cleavage site.

(a)



(b)



3.2 Extraction of pPGKA1 from *E. coli*

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Plasmid pPGKA1 was introduced into *Escherichia coli* DH5α by electroporation, and transformants were selected for Zeocin resistance. Bacterial colonies were plated onto low-salt Luria–Bertani (LB) agar (0.5% yeast extract, 1.0% peptone, 1.0% NaCl, and 1.5% agar) supplemented with Zeocin (100 µg/mL; Invitrogen®) and incubated at 37 °C. Selected colonies were grown in liquid low-salt LB medium supplemented with Zeocin (100 µg/mL), and plasmid pPGKA1 was isolated using the Fast-n-Easy Plasmid Mini-Prep Kit (Cellco), according to the manufacturer's instructions.

3.3 Transformation of *K. phaffii* and selection of IP-producing clones

Amplified pPGKA1 was linearized with SacI and used to transform *K. phaffii* GS115 (His4) by electroporation (Eppendorf Electroporator 2510; 1,500 V; 1.0 mm cuvette) to contribute its integration into the yeast genome via homologous recombination (DALY and HEARN, 2005). Transformants were selected on YPD agar (1.0% yeast extract, 2.0% peptone, 2.0% dextrose, and 2.0% agar) supplemented with Zeocin (100 µg/mL). Colonies were visible after 72 h of incubation at 30 °C (ZHU *et al*., 2018).

3.4 Bioreactor cultivation and sampling

Inocula of the *K. phaffii* strain PGKA1 were prepared in shake flasks containing 100 mL of YPD medium (20 g/L glucose, 10 g/L yeast extract, 20 g/L peptone) supplemented with 100 µg/mL Zeocin. Cultures were incubated at 30 °C and 180 rpm for 24 h until late exponential phase. The culture was not washed prior to inoculation into the bioreactor. The exponential-phase seed culture was inoculated into YPD medium in a BioFlo 115 bioreactor (New Brunswick) to achieve an initial biomass concentration of 0.1 g/L. The bioreactor was equipped with two Rushton-type impellers and a sparger, with integrated sensors for real-time monitoring of temperature, pH, and dissolved oxygen (DO). The pH was adjusted manually using 1 M hydrochloric acid and 1 M sodium hydroxide solutions. Two cultivation strategies were systematically evaluated: (1) Batch mode: Initial YPD medium (20 g/L glucose) without further additions; (2) Fed-Batch mode: Initial YPD medium followed by pulse additions of concentrated glucose solution. Operating conditions were maintained at 30 °C and pH 6.0, with agitation at 180 rpm and an aeration rate of 2.0 vvm. Fed-

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batch cultivation was initiated with a working volume of 1 L, followed by intermittent additions of 100 mL of glucose solution (200 g/L) at t = 12, 24, 36, and 48 h. The choice of low agitation (180 rpm) and high aeration (2.0 vvm) was based on preliminary experiments (data not shown). Samples were collected periodically for biomass quantification via cell dry weight (CDW), glucose concentration, and IP secretion analysis. The culture broth was centrifuged at 2,200 × g for 10 min at 4 °C, and the supernatant was stored at -80 °C for subsequent analysis of IP concentration and residual glucose levels.

3.5 Concentration of IP using hollow fiber membranes

After fermentation, the culture broth was centrifuged at 2,200 x g for 40 min at 4 °C to remove cells and residual debris. The resulting supernatant was subjected to a two-step membrane-based process combining microfiltration and ultrafiltration to clarify and concentrate the human insulin precursor (IP). Filtration was performed using a peristaltic pump (Watson Marlow 323).

In the microfiltration the supernatant was passed through a hollow fiber cartridge with a 0.1 µm pore size membrane (surface area: 420 cm²) to remove remaining particulate matter and obtain a clarified solution (GE Healthcare Bio-Sciences Corp.), with a transmembrane pressure of 0.34 bar (5 psig).

In the ultrafiltration the clarified supernatant was subsequently concentrated using a hollow fiber cartridge with a molecular weight cut-off (MWCO) of 3 kDa and a surface area of 1400 cm² (GE Healthcare Bio-Sciences Corp.), and a transmembrane pressure of 0.7 bar (10 psig).

3.6 Cell density and glucose measurement

Cell density was monitored by measuring the optical density (OD) at 600 nm. Biomass concentration was estimated as cell dry weight (CDW). Briefly, 1 mL samples were centrifuged at 10,000 × g for 5 min, and the resulting pellets were dried at 75 °C until constant weight.

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The supernatants obtained from this centrifugation step were used for glucose analysis to monitor carbon source consumption throughout fermentation. Glucose concentration was determined using a glucose oxidase (GOD) assay kit (InVitro Diagnostica), according to the manufacturer's instructions.

3.7 Human insulin precursor analysis: SDS-PAGE and indirect ELISA

Supernatant samples were precipitated using analytical-grade acetone (25:75 v/v; supernatant: acetone). The presence of the insulin precursor (IP) in the culture supernatant was confirmed by SDS-PAGE (15% resolving gel and 5% stacking gel), run at 200 V and 75 mA. Gels were stained with Coomassie Brilliant Blue.

The concentration of secreted IP in the supernatant was quantified by enzyme-linked immunosorbent assay (ELISA). Indirect ELISA was performed using an anti-insulin/proinsulin antibody (Invitrogen). For plate coating, 50 µL of concentrated supernatant diluted in carbonate buffer (pH 9.6) was added per well and incubated for 1 h at 37 °C. Subsequently, ELISA plates (E.I.A./R.I.A. Plate High Binding, Corning) were blocked with 3% (w/v) BSA in PBS (phosphate-buffered saline) at room temperature (28 °C). After washing, wells were incubated with an anti-insulin/proinsulin antibody (1:1,000) for 60 min.

After a final wash, anti-mouse IgG antibody conjugated to peroxidase (1:1,000 dilution) was added and incubated for 60 min. The reaction was developed using an ultrasensitive TMB solution (3,3′,5,5′-tetramethylbenzidine) and stopped with 2 M sulfuric acid. Absorbance was measured at 450 nm using a microplate reader (Hidex Chameleon Plate Reader).

A standard calibration curve was prepared using known concentrations of commercial insulin (Humulin® Regular, 3.47 mg/mL) to quantify IP levels in the samples.

3.8 MALDI-TOF/MS analysis

MALDI-MS analysis was performed using an UltrafleXtreme MALDI-TOF/TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Final fermentation products

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concentrated via hollow fiber were lyophilized in a freeze-dryer (LABCONCO) and separated by preparative reverse-phase C18 chromatography (Strata C18-T) to remove residual salts and sugars from the sample. A 1 mL volume of the sample was loaded onto the column, which was subsequently washed with 10 column volumes (10 CV) of water. The column-bound sample was then eluted with 1 mL of 95% acetonitrile and collected in a microcentrifuge tube. The resulting fractions were frozen in liquid nitrogen and subsequently dried using a SpeedVac system (LABCONCO).

For MALDI analysis, samples were resuspended in water and mixed with α-cyano-4-hydroxycinnamic acid (α-CHCA) matrix at a 3:1 (sample: matrix) ratio. An aliquot of 0.8 µL of the mixture was spotted onto the MALDI target plate. The matrix solution consisted of 5 mg/mL α-CHCA in 50% acetonitrile (ACN) and 0.3% trifluoroacetic acid (TFA). Mass calibration was performed using the Peptide Calibration Standard (Bruker Daltonics). Mass spectra was acquired using FlexControl software (Bruker Daltonics) in the protein mass range and processed with FlexAnalysis software (Bruker Daltonics). According to the manufacturer's specifications, the instrument achieves a mass accuracy within an error of 10 ppm, which corresponds to one significant digit after the nominal mass (decimal point).

4. Results and Discussion

4.1 IP expression in transformant *K. phaffii* PGKA1

This study successfully established a constitutive expression platform to produce a human insulin precursor (IP) in *Komagataella phaffii*, using the pPGK1 promoter and the *S. cerevisiae* α-factor signal peptide to enable extracellular secretion. Based on literature reports indicating enhanced cleavage efficiency (Kjeldsen, 2000), a spacer peptide (EEAEAEAEPK) was introduced between the secretion signal and the IP coding sequence to promote Kex2 endoprotease processing.

The genotypic stability of the recombinant lineage is a critical determinant of long-term culture (DALY and HEARN, 2005). In this study, the pPGKA1 plasmid was linearized using SacI to facilitate its integration into the yeast genome via homologous

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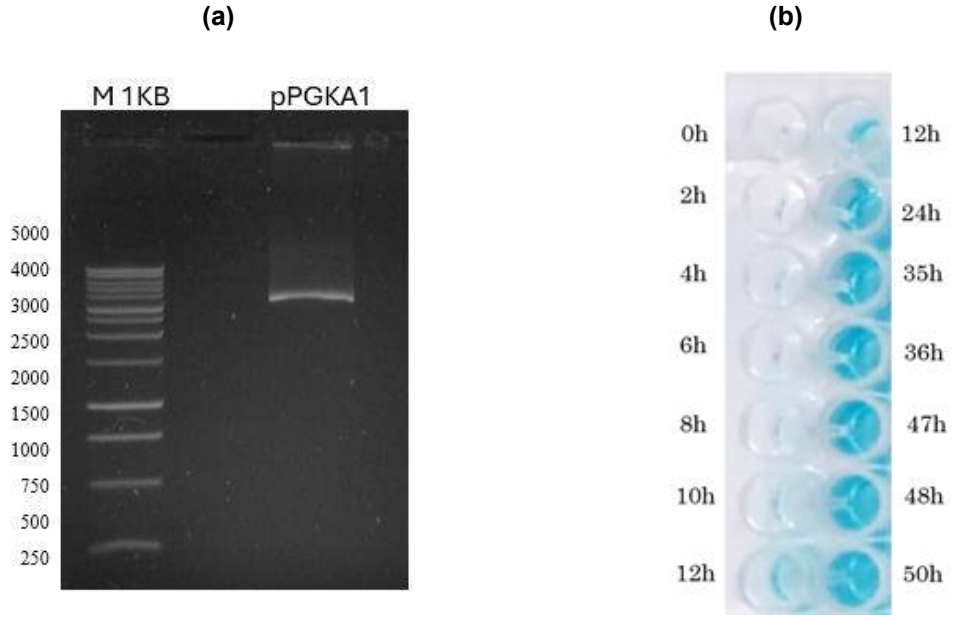
recombination (Figure 2a). The IP-producing transformant (*K. phaffii* PGKA1) was confirmed by ELISA (Figure 2b).

Figure 2 (a) Electrophoretic profile on 0.8% agarose gel of the recombinant plasmid pPGKA1. 1 – 1 kb marker, 2 – pPGKA1 plasmid digested with SacI (linearized with around 3000 bp). (b) Confirmation of IP expression by *K. phaffii* recombinants transformed with pPGKA1 (*K. phaffii* PGKA1) over time in the ELISA test.



4.2 Production of IP in STR bioreactor

Production of the insulin precursor (IP) by *K. phaffii* PGKA1 was evaluated in a stirred-tank reactor (STR) under batch and fed-batch strategies (Figure 3). In fed-batch mode, the glucose concentration was selected based on preliminary findings (data not shown), and samples taken immediately following addition reflect the accumulated medium concentration.

In relation to substrate kinetics, the initial glucose concentration of 1.89% (w/v) in the batch cultivation was completely depleted within 24 hours. In contrast, the fed-batch process started with 2.66% (w/v) glucose, maintaining an accumulated substrate level approximately 2.20%-2.74% (w/v) during intermittent feeding.

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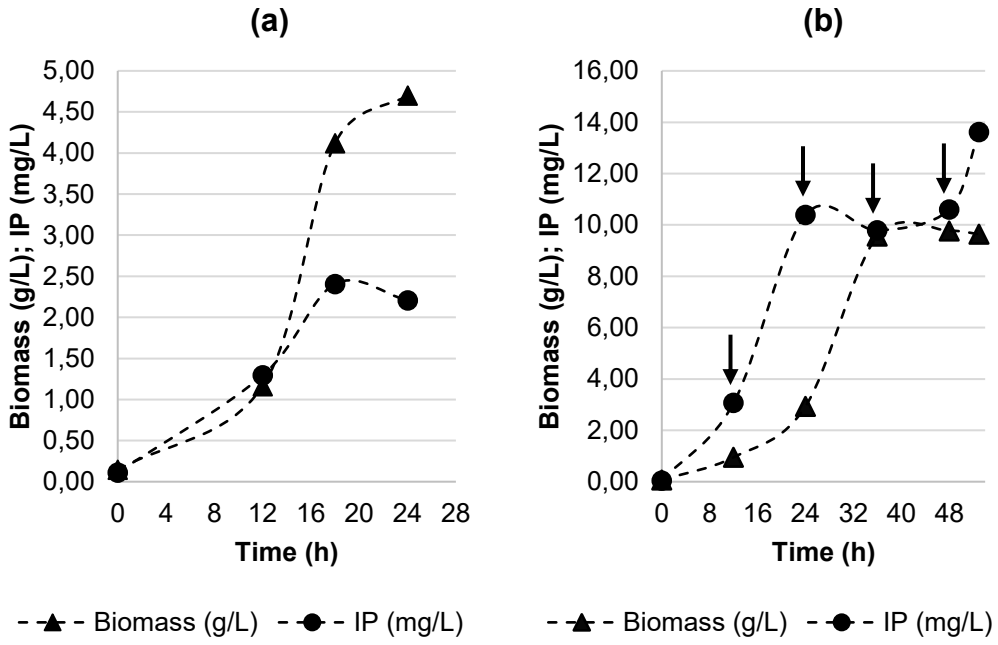
Figure 3. Kinects profile of *K. phaffii* PGKA1 in batch (a) and fed-batch (b) fermentations. (YPD medium; pH 6.0; 30 °C; 180 rpm; 2 vvm). In the fed-batch graphic, arrows indicate the intermittent glucose pulses applied to maintain a concentration near 2.6%. The experiments were carried out in a single replicate per experimental condition (n = 1).



IP concentrations were determined by ELISA using a monoclonal anti-insulin/proinsulin antibody, with quantification based on a calibration curve prepared from commercial insulin (Humulin®, 100 IU/mL; ranging from 0.07 mg/L to 34.70 mg/L). The ELISA analysis were carried out in a single replicate per experimental condition (n = 1). Humulin®, a recombinant human polypeptide hormone structurally identical to native insulin, was used as an immunoreactive insulin standard to generate the calibration curves for the ELISA assays. The mouse anti-insulin/proinsulin monoclonal antibody (D3E7 (5B6/6)) cross-reacts with both human insulin and proinsulin, an affinity constant of 6.3 x 10^8 M^{-1} (Invitrogen). IP concentrations were calculated using a four-point linear regression standard curve forced through the origin (Equation 1). The coefficient of determination (R^2) was consistently greater than 0.95:

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Equation 1: $[IP] = 35.531 \times OD_{450nm}$

Table 1 contains the heterologous production parameters of insulin precursor by *K. phaffii* PGKA1 in batch and fed-batch fermentation. The product-to-biomass yield ($Y_{P/X}$) is the mass of product per mass of biomass (Equation 2) and the volumetric productivity of the insulin precursor (Q_P) represents the mass of product generated per unit working volume per unit time (Equation 3). The calculations accounted for the working volume, which remained constant during batch operation but varied over time in fed-batch mode due to intermittent substrate feeding.

Equation 2: $Y_{P/X} = \left(\frac{P_f \times V_f - P_i \times V_i}{X_f \times V_f - X_i \times V_i}\right)$, where P is the insulin precursor concentration, X is the cell concentration, and V is the culture volume, with subscripts *i* and *f* denoting the initial and final states, respectively.

Equation 3: $Q_P = \left(\frac{P_f \times V_f - P_i \times V_i}{\Delta t}\right)$, where P is the insulin precursor concentration and V is the culture volume, with subscripts *i* and *f* denoting the initial and final states over the time interval (Δt).

The volumetric productivity of the insulin precursor increased from 0.087 in batch to 0.359 mg/L·h in fed-batch, representing a ~4-fold improvement. Importantly, the product-to-biomass yield ($Y_{P/X}$) increased from 0.460 to 1.417, suggesting that the intermittent feeding not only contributed with higher insulin precursor productivity but also enhanced the efficiency of IP synthesis and secretion relative to growth (Table 1). These results support the potential of the fed-batch strategy for further optimization under constitutive expression driven by the PGK1 promoter.

Table 1. Heterologous production parameters of insulin precursor by *K. phaffii* PGKA1 in STR (YPD medium; pH 6.0; 30 °C; 180 rpm; 2 vvm).

Kinetic Parameter	Unit	Batch	Fed-Batch
Maximum specific growth rate (μ_{max})	h^{-1}	0.128	0.094
Product/Biomass Yield ($Y_{P/X}$)	mg/g	0.460	1.417

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Product Volumetric Productivity (Q_P)	mg/L.h	0.087	0.359
Cultivation Time	h	24	53

A notable finding was that the maximum specific growth rate (μ_{max}) in fed-batch mode was lower than in batch mode (0.094 h^{-1} and 0.128 h^{-1}, respectively). Although expression driven by a constitutive promoter is expected to increase with higher growth rates, this study demonstrated that a lower growth rate provided a higher product volumetric productivity (Q_P). A similar trend was reported by Herrera-Estala *et al*. (2022) during the expression of the enzyme β-propeller phytase (BPP) under the control of the constitutive GAP promoter in *K. phaffii*, where the specific growth rate was inversely proportional to the volumetric productivity of the heterologous protein.

The distinct glucose consumption dynamics observed in the two processes could affect overall yield and productivity. In batch mode, the culture relied on an initial glucose load, which was rapidly depleted, likely limiting sustained expression. In contrast, the fed-batch maintained moderate glucose levels through intermittent feeding. This approach could conceivably attenuate metabolic stress, which is hypothesized to reduce catabolite repression and potentially limit byproduct accumulation. The increase in yield $Y_{P/X}$ suggests that the intermittent feeding not only increased cell density but also optimized the cell factory for IP secretion.

The influence of glucose availability on heterologous protein expression is consistent with regulation under the pPGK1 promoter, which is associated with glycolytic flux (ALMEIDA *et al*., 2005). Moreover, intermittent feeding may have redirected cellular metabolism toward secretory protein synthesis rather than solely biomass formation. This interpretation aligns with prior reports on the metabolic burden imposed by heterologous expression and the benefits of controlled growth regimes for secretion efficiency (BAUMANN *et al*., 2011; NOCON *et al*., 2014).

Post-fermentation of fermentation, the culture broth supernatant obtained from the centrifugation step was subjected to a two-step membrane process combining

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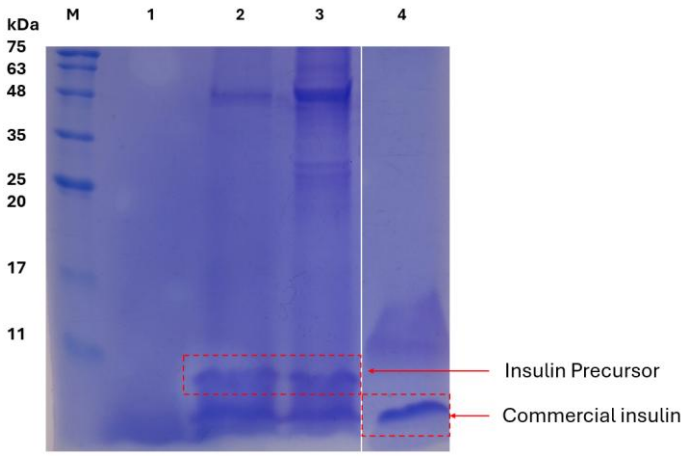
microfiltration and ultrafiltration to clarify and concentrate the working volume 10-fold. Also, the ELISA analysis of the retentate and permeate fractions confirmed that the insulin precursor was retained during ultrafiltration (data not shown).

4.3 Characterization of secreted IP by SDS-PAGE and MS analysis

SDS-PAGE analysis indicated that the insulin precursor (IP) secreted into the culture medium presented an apparent molecular weight of approximately 7 kDa. In contrast, commercial insulin showed a band at 5.7 kDa (Figure 4a). To further characterize the recombinant product, the supernatant was desalted by semi-preparative liquid chromatography and then freeze-dried. MALDI-TOF/MS was then used to analyze the resulting lyophilized material.

Figure 4 (a) SDS-PAGE analysis of heterologous protein expression. Lane M, prestained protein molecular weight marker; Lane 1, protein extracted from start of fermentation (0h) without expression of IP; Lane 2, protein extracted from exponential growing phase (24h); Lane 3, protein extracted from stationary growing phase (53h); Lane 4, commercial insulin 70N/30R. b) MALDI-TOF/MS spectra: Average mass of the major ions $[M1+H]^+$ = 7,054.14 Da, $[M2+H]^+$ = 7,138.94 Da, $[M3+H]^+$ = 7,217.89 Da of the protein content obtained from the extracellular product from *K. phaffii* PGKA1 fermentation.

(a)



(b)

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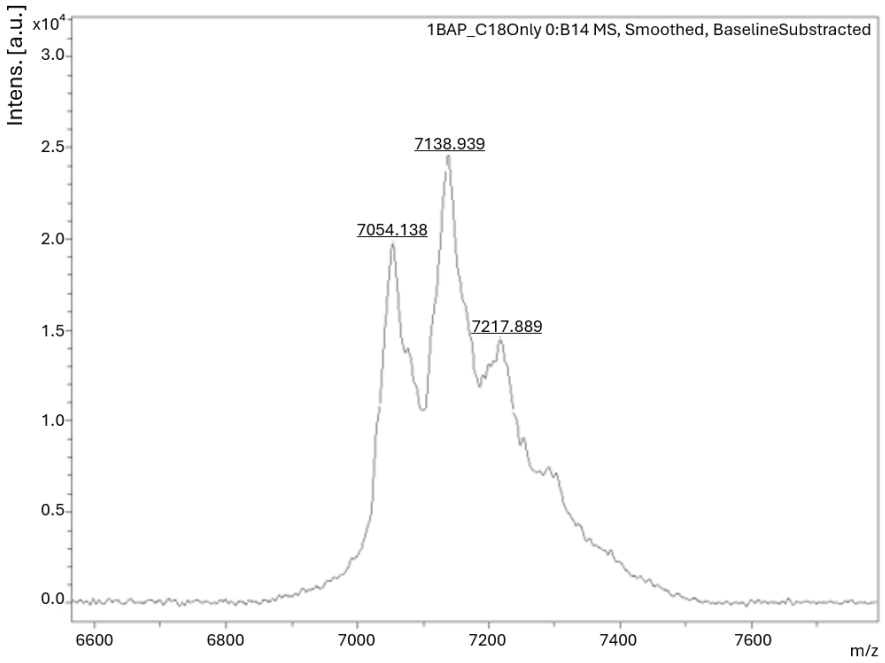

The mass spectrometry results (Table 2) corroborated the SDS-PAGE observations. As shown in the MS spectra (Figure 4b), the fed-batch sample exhibited a prominent signal consistent with the expected structure of the insulin precursor, comprising the spacer sequence followed by the B, C, and A insulin chains, with a molecular mass of approximately 7,049 Da, and indicates potential cleavage at the Kex2 site. Notably, this value is in close agreement with the molecular mass reported by Gurramkonda *et al* (2010) for the same construction (7,041 Da). In addition to the main peak, two signals near the expected mass of the insulin precursor were detected, suggesting the presence of related bioproducts with molecular weights close to that of the target peptide. Consequently, complementary approaches such as protein sequencing will be required to elucidate their exact identities and potential alternative processing events.

Table 2. Insulin precursor produced in *K. phaffii* PGKA1 fermentation detected by Mass Spectrometry.

Sequence	**Theoretical average m/z**	**Observed m/z**
EEAEAEAEPKFVNQHLCGSHLVEALYLVCGER GFFYTPKAAKGIVEQCCTSICSLYQLENYCN	7,049 Da	7,054 Da

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From an industrial perspective, the use of a constitutive promoter under methanol-free conditions offers a safer platform with potential for future scale-up development compared to conventional pAOX1-based expression systems. The pPGK1 promoter enables continuous expression without hazardous inducers, offering a significant advancement in the safety and operational benefits for biopharmaceutical manufacturing platforms. Moreover, intermittent feeding in fed-batch fermentation represents a practical strategy due to its simplicity and ease of implementation and control (BHARGAVA *et al*., 2003). The combination of a GRAS-status host, cultivation in a chemically defined medium (YPD), and a controllable feeding regime underscores the strength of this strategy as a proof-of-concept.

Further improvements in process performance may be achieved through promoter engineering to enhance transcriptional strength, screening of alternative secretion signal peptides to increase secretion efficiency, and optimization of fermentation variables under bioreactor control. In addition, feedback-regulated feeding strategies based on real-time monitoring of nutrients and oxygen availability may contribute to stabilizing productivity. Finally, multi-omics approaches (e.g., transcriptomics and proteomics) could provide deeper insights into potential cellular bottlenecks, supporting rational metabolic engineering strategies to enhance yield and reduce variability.

4.4 Current limitations and perspectives for process optimization

This study establishes a proof-of-concept for methanol-free insulin precursor production in *K. phaffii* using constitutive expression. However, several limitations must be acknowledged. First, the biomass concentration and product titer (9.6 g/L biomass; 13.62 mg/L insulin precursor) achieved in this work are substantially lower than those reported for optimized fed-batch processes in *K. phaffii* pAOX-based systems, 59 g/L biomass and approximately 3 gram IP per liter of culture broth (GURRAMKONDA *et al*., 2010). This discrepancy is likely attributable to the intermittent feeding strategy employed, which creates cycles of glucose availability and depletion incompatible with the continuous metabolic flux required by glycolytic promoters. Second, the agitation speed (180 rpm) may have contributed to

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suboptimal oxygen transfer. Future work should implement continuous, feedback-controlled feeding strategies (exponential feeding, DO-stat, or pH-stat) and optimize aeration parameters to approach industrial-scale performance. Third, the downstream processing, while demonstrating the feasibility of hollow fiber membrane concentration, was not optimized for yield or purity. Additionally, biopharmaceutical manufacturing will require subsequent downstream purification processes to meet pharmaceutical purity standards, enzymatic conversion of the insulin precursor into mature human insulin, and functional bioactivity evaluation, all of which will be addressed in future work. Despite these limitations, the successful demonstration of constitutive expression, secretion, and recovery of insulin precursor in a methanol-free system represents a significant step toward safety and operational concerns in biopharmaceutical manufacturing platforms.

5. Conclusion

This study demonstrates a proof-of-concept for human insulin precursor production in *Komagataella phaffii* using a methanol-free constitutive expression system driven by the pPGK1 promoter. The engineered strain *K. phaffii* PGKA1 secreted IP into the extracellular medium, and downstream concentration using hollow fiber membranes enabled the product recovery. The use of the *S. cerevisiae* α-factor signal peptide, combined with a tailored spacer region, may have contributed to the precursor processing and secretion, as confirmed by SDS-PAGE and mass spectrometry analyses.

Overall, the proposed methanol-free, constitutive *K. phaffii* platform offers a safer, more operationally straightforward process with potential for regulatory compliance and future scale-up development.

Among the cultivation strategies evaluated, fed-batch operation clearly outperformed batch cultivation. Intermittent feeding supported controlled substrate availability and sustained IP production, resulting in improved process performance and the highest product-to-biomass yield. In contrast to batch fermentation, where glucose was rapidly depleted, fed-batch operation maintained adequate carbon

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availability throughout cultivation. These findings emphasize the importance of dynamic nutrient control in enhancing recombinant protein production, particularly under constitutive expression systems. Maintaining moderate, non-inhibitory glucose concentrations likely contributed to sustained metabolic activity and reduced physiological stress, supporting more consistent IP synthesis and secretion.

Although the precursor titer remains substantially below values reported for optimized high-cell-density methanol-induced systems, the results support the potential of constitutive, methanol-free *K. phaffii* platforms as safer and operationally simpler alternatives for biopharmaceutical production, with promising applicability to industrial manufacturing of insulin precursors. Additional studies addressing process reproducibility, quantitative analytical validation, product heterogeneity, biological activity, defined-media cultivation and scale-up parameters will be required before the platform can be considered for pharmaceutical manufacturing.

From a bioprocess engineering standpoint, the strategy adopted herein offers technical and regulatory advantages, including reduced risks associated with methanol handling and simplified process control. Furthermore, *K. phaffii* has an established history of industrial use, including FDA-reviewed GRAS notices for specific food ingredients produced using defined strains.

Future development of this system may benefit from deeper investigation of cellular responses through omics-based analyses, which could reveal limiting steps and guide rational metabolic or process interventions to further enhance robustness, productivity, and scalability.

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8. Supplementary Material

Figure 1S. The IP gene and polypeptide sequence of the recombinant *K. phaffii* PGKA1 construction. The downward arrows indicate the terminal ends of the IP gene and polypeptide. Amino acid residue numbers for the A and B chains are prefixed with 'A' and 'B', respectively, while the tripeptide linking the B and A chains is prefixed with 'C'. Black triangles denote trypsin cleavage sites. Nucleotides comprising the 5' and 3' restriction sites (XhoI and NotI) are shown in italics.

XhoI					Spacer sequence								
				↓									▼
Leu	Glu	Lys	Arg	Glu	Glu	Ala	Glu	Ala	Glu	Ala	Glu	Pro	Lys
CTC	*GAG*	AAG	AGA	GAA	GAA	GCT	GAA	GCT	GAA	GCT	GAA	CCA	AAG
B1	**B2**	**B3**	**B4**	**B5**	**B6**	**B7**	**B8**	**B9**	**B10**	**B11**	**B12**	**B13**	**B14**
Phe	Val	Asn	Gln	His	Leu	Cys	Gly	Ser	His	Leu	Val	Glu	Ala
TTT	GTT	AAC	CAA	CAT	TTG	TGT	GGT	TCT	CAT	TTG	GTT	GAA	GCT
B15	**B16**	**B17**	**B18**	**B19**	**B20**	**B21**	**B22**	**B23**	**B24**	**B25**	**B26**	**B27**	**B28**
Leu	Tyr	Leu	Val	Cys	Gly	Glu	Arg	Gly	Phe	Phe	Tyr	Thr	Pro
TTG	TAC	TTG	GTT	TGT	GGT	GAA	AGA	GGT	TTC	TTC	TAC	ACT	CCA
▼			▼										
B29	**C1**	**C2**	**C3**	**A1**	**A2**	**A3**	**A4**	**A5**	**A6**	**A7**	**A8**	**A9**	**A10**
Lys	Ala	Ala	Lys	Gly	Ile	Val	Glu	Gln	Cys	Cys	Thr	Ser	Ile
AAG	GCT	GCT	AAG	GGT	ATT	GTT	GAA	CAA	TGT	TGT	ACT	TCT	ATT
											↓		
A11	**A12**	**A13**	**A14**	**A15**	**A16**	**A17**	**A18**	**A19**	**A20**	**A21**	**END**	NotI	
Cys	Ser	Leu	Tyr	Gln	Leu	Glu	Asn	Tyr	Cys	Asn			
TGT	TCT	TTG	TAC	CAA	TTG	GAA	AAC	TAC	TGT	AAC	TAA	*GCGGCCGC*	

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