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BIOTECHNOLOGY | ORIGINAL ARTICLE

Extracellular expression, purification and bioreactor production of tambaqui (*Colossoma macropomum*) growth hormone in the yeast *Komagataella phaffii* (formerly *Pichia pastoris*)

Elson Antonio SADALLA-PINTO[1,10,*], Edmar de ANDRADE[1,2,3], Edson Junior do CARMO[1,2], Júlio Nino de SOUZA-NETO[2], Larissa Matos BATISTA[3], Alexandra Regina BENTES-SOUSA[4], Luis André Morais MARIÚBA[1,5,6,7], Douglas de França PASSOS[8], Rodrigo Pimentel FERNANDES[8], Nei PEREIRA JUNIOR[8], Jorge Ivan Rebelo PORTO[9], Spartaco ASTOLFI-FILHO[1,2,3]

[1] Universidade Federal do Amazonas, Programa de Pós-Graduação em Biotecnologia, Manaus-AM, Brazil

[2] Universidade Federal do Amazonas, Centro de Apoio Multidisciplinar, Manaus, AM, Brazil

[3] Universidade Federal do Amazonas, Departamento de Genética, Instituto de Ciências Biológicas, Manaus, AM, Brazil

[4] Embrapa Amazônia Oriental, Núcleo de Apoio à Pesquisa e Transferência de Tecnologias do Baixo Amazonas, Santarém, PA, Brazil

[5] Fundação Oswaldo Cruz, Instituto Leônidas e Maria Deane, Manaus, AM, Brazil

[6] Fundação Oswaldo Cruz, Programa de Pós-Graduação em Biologia da Interação Patógeno-Hospedeiro, Manaus, AM, Brazil

[7] Universidade Federal do Amazonas, Programa de Pós-Graduação em Imunologia Básica e Aplicada, Instituto de Ciências Biológicas, Manaus, AM, Brazil

[8] Universidade Federal do Rio de Janeiro, Escola de Química, Laboratórios de Desenvolvimento de Bioprocessos e Bioprodutos, Cidade Universitária, Rio de Janeiro, RJ, Brazil

[9] Instituto Nacional de Pesquisa da Amazônia, Coordenação de Biodiversidade, Laboratório de Genética Animal, Manaus, AM, Brazil

[10] Instituto Federal de Educação, Ciência e Tecnologia do Amazonas, Manaus, AM, Brazil

*Corresponding author: elson.sadalla@ifam.edu.br; https://orcid.org/0000-0002-7474-5481

ABSTRACT

Due to its zootechnical importance, the gene encoding growth hormone (GH) from several fish species has been isolated and expressed in heterologous systems. In this study, we isolated the cDNA sequence of GH of tambaqui (*Colossoma macropomum*), a species native to the Amazon basin and considered promising for Brazilian fish farming, and expressed it in the yeast *Komagataella phaffii* GS115. Heterologous expression was regulated by the AOX1 promoter and recombinant tambaqui GH (rtGH) secreted to the culture supernatant via the alpha factor of *Saccharomyces cerevisiae*. The rtGH was successfully expressed in shaken flask and bioreactor systems and purified using affinity chromatography. In the bioreactor, the production reached 250 mg L^{-1}. This is the first report of the heterologous expression, purification and production of rtGH in a bioreactor. Our results contribute to making tambaqui a target species for future biotechnological innovations based on the significant levels of rtGH obtained in the bioreactor and the future zootechnical applications of this protein, which can be exploited in aquaculture.

KEYWORDS: somatotropic hormone; Amazonian fish; heterologous expression; shake flasks; bioreactor

Expressão extracelular, purificação e produção em biorreator do hormônio de crescimento de tambaqui (*Colossoma macropomum*) na levedura *Komagataella phaffii* (anteriormente *Pichia pastoris*)

RESUMO

Devido à sua importância zootécnica, o gene que codifica o hormônio de crescimento (GH) de diversas espécies de peixes tem sido isolado e expresso em sistemas heterólogos. Neste estudo, isolamos a sequência de cDNA do GH do tambaqui (*Colossoma macropomum*), espécie nativa da bacia amazônica e considerada promissora para a piscicultura brasileira, e a expressamos na levedura *Komagataella phaffii* GS115. A expressão heteróloga foi regulada pelo promotor AOX1 e GH de tambaqui recombinante (rtGH) foi secretado para o sobrenadante da cultura através do fator alfa de *Saccharomyces cerevisiae*. O rtGH foi expresso com sucesso em frascos agitados e sistemas de biorreatores e purificado por cromatografia de afinidade. No biorreator a produção atingiu 250 mg L^{-1}. Este é o primeiro relato da expressão heteróloga, purificação e produção de rtGH em biorreator. Nossos resultados contribuem para tornar o tambaqui uma espécie alvo para futuras inovações biotecnológicas baseadas nos níveis significativos de rtGH obtidos no biorreator e nas futuras aplicações zootécnicas desta proteína, que pode ser explorada na aquicultura.

PALAVRAS-CHAVE: hormônio somatotrópico; peixe amazônico; expressão heteróloga; frascos agitados; biorreator

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INTRODUCTION

Genetic studies involving the search for and cloning and expression of genes that encode proteins associated with important physiological processes and advantageous characteristics from the zootechnical point of view have made biotechnological research increasingly promising. Due to its importance in medicine, veterinary and agricultural sciences, the gene encoding growth hormone (GH) of several vertebrate species, including fish, has been widely expressed in heterologous systems (Tsai et al. 1997; Anathy et al. 2001; Acosta et al. 2007; Sekar et al. 2014) to produce a recombinant protein with preserved biological activity (Li et al. 2003; Funkenstein et al. 2005; Acosta et al. 2009).

GH is a polypeptide produced in the pituitary gland of all vertebrates (Moriyama 2016). In fish, its action includes the regulation of ions and lipids, osmotic balance, carbohydrate and protein metabolism, growth of soft and skeletal tissues, reproduction, immune functions, energy mobilization, gonadal development, appetite, social behavior and aging (Björnsson 1997; Bartke et al. 2021). Several studies have shown that the cDNA of the GH in fish encodes a mature polypeptide with a high degree of conservation, whith length ranging from 178-191 amino acid residues and a secretion signal peptide of between 17-22 residues (Agellon and Chen 1986; Akiko et al. 1988; Koren et al. 1989; Rentier-Delrue et al. 1989a,b; Funkenstein et al. 1991; Lemaire et al. 1994; Pendón et al. 1994; Venugopal et al. 2002; Moriyama 2016; Lima et al. 2023). As it has a function that is comparable to natural GH, which is produced in the pituitary gland, this polypeptide can be used in studies that focus on physiology (Li et al. 2003) and improvement of zootechnical performance in fish (Acosta et al., 2007), as growth manipulation is an important subject in aquatic biotechnology. The successful expression of gene encoding fish GH has been reported in prokaryotes [e.g., *Escherichia coli* Escherich, 1885 (Agellon and Chen 1986; Jeh et al. 1998), *Bacillus subtilis* Cohn, 1872 (Ng Alan et al. 2016) and cyanobacteria (Liu et al. 2008)] and in eukaryotes [e.g., *Saccharomyces cerevisiae* Pasteur, 1856 (Piyaviriyakul et al. 2002), algae (Chen et al. 2008), insect cells (Tsai et al. 1994) and transgenic fish such as the Atlantic salmon (*Salmo salar* Linnaeus, 1758) developed and marketed by the Canadian company AquAdvantage® (Du et al. 1992)].

The methylotrophic yeast *Komagataella phaffii* Phaff, 1956 (formerly *Pichia pastoris*) has been successfully used as a heterologous eukaryotic gene expression system due to that (a) it has a high frequency of transformation by heterologous DNA; (b) transformation occurs by means of integrative vectors by homologous complementation directly in the cellular genome, which leads to greater genetic stability of recombinant cells; (c) it has high levels of intra or extracellular protein expression under the control of strong and inducible promoters, such as the AOX1, the promoter of the alcohol oxidase I enzyme gene; (d) the genetic manipulation and fermentative techniques are well establishe; (e) detailed information on the structures of N-linked oligosaccharides on secreted proteins; (f) the presence of post-translational modifications that are compatible with those found in superior eukaryotes; and (g) it is generally recognized as safe (GRAS) (Cregg et al. 1993; Cereghino and Cregg 2000; Cereghino et al. 2002), which allows its use in the production of proteins for use in aquaculture.

The tambaqui (*Colossoma macropomum* Cuvier, 2018) is a fish native to the Amazon basin with great economic importance as one of the most appreciated and consumed fish in northern Brazilian cuisine (Araújo-Lima and Goulding 1998; Morais and O'Sullivan 2017). It has characteristics that make it appropriate for farming, as well as an ample consumer market, standing out as the most produced native species in fish farming in Brazil (IBGE 2022). For this reason, tambaqui has become the focus of extensive research efforts regarding feeding and reproductive management (Melo et al. 2001; Izel et al. 2013; Morais and O'Sullivan 2017). Studies include the evaluation of the effects of different nutritional constituents and probiotics combined with food management on physiological and zootechnical responses (Pereira Junior et al. 2013; Sandre et al. 2017; Sadalla-Pinto et al. 2021).

The present study aimed to isolate the cDNA sequence of the tambaqui growth hormone (tGH) from the pituitary gland and part of the brain of juvenile tambaqui and to use this sequence for extracellular expression in the *K. phaffii* phenotypes Mut+ and Muts and the subsequent purification of the recombinant protein secreted (recombinant tambaqui GH, rtGH) in the culture supernatant, as well as its production in a bioreactor system.

MATERIAL AND METHODS

Strains, vector and culture media

The strain *E. coli* DH10B, used for plasmid propagation, was obtained from New England BioLabs (NEB®). The yeast strain *K. phaffii* GS115 and the vector pPIC9 were obtained from Invitrogen®. Luria-Bertani medium (LB) was prepared according to the description in the molecular cloning manual (Sambrook and Russell 2001). Buffered glycerol-complex media (BMGY), buffered methanol-complex media (BMMY), minimal dextrose media (MD) and minimal methanol (MM) were prepared as described in the instruction manual of the Invitrogen® *Pichia* expression kit. The FM22 basal salts medium was prepared with the following components: KH_2PO_4 42.9 g L^{-1}, $(NH_4)SO_4$ 5 g L^{-1}, $CaSO_4.2H_2O$ 1.0 g L^{-1}, K_2SO_4 14.3 g L^{-1}, $MgSO_4.7H_2O$ 11.7 g L^{-1}, glycerol 80 g L^{-1} and PTM4 1 mL L^{-1}. The mixture was completed with distilled water and the pH adjusted to 5.0 with KOH. The PMT4 trace element solution was prepared with $CuSO_4.5H_2O$ 2.0 g L^{-1}, NaI 0.08 g L^{-1}, $MnSO_4.H_2O$ 3.0 g L^{-1}, $Na_2MoO_4.2H2O$ 0.2

g L^{-1}, H_3BO_3 0.02 g L^{-1}, $CaSO_4.2H_2O$ 0.5 g L^{-1}, $CoCl_2$ 0.5 g L^{-1}, $ZnCl_2$ 7 g L^{-1}, $FeSO_4.7H_2O$ 22 g L^{-1}, biotin 0.2 g L^{-1} and [H_2SO_4] 1 mL. The PMT4 solution was also added to methanol as a supplement (at a concentration of 4 mL $L^{-1)}$.

tGH cDNA acquisition from transcriptome

The tGH cDNA sequence was obtained from a cDNA library. Total RNA was extracted using the guanidine isothiocyanate method and using the TRIZOL kit (Invitrogen®) according to the manufacturer's recommendations. The poly(A)+ fraction (containing mRNAs) was isolated via centrifugal affinity chromatography in an oligo(dT)-cellulose column, using the FastTrack 2.0 kit (Invitrogen®). The cDNA library of the pituitary tissues and part of the brain of 12 juvenile tambaqui of approximately 15 cm length each (obtained from the fish farming station at the National Institute for Amazonian Research - INPA in its acronym in Portugues) was built following the protocol of the SuperScript® Plasmid System and using the Gateway® Technology for cDNA Synthesis and Cloning Kit. The vector used for cloning the fractionated cDNA fragments was pCMVSPORT6-SuperScript™ (Invitrogen®). To obtain the fragments corresponding to tGH, the following GHP-F primers were used: TCAGACAACCAGCGGCTCTTC and GHP-R: CAGGGTGCAGTTGGAATCCAG (Pinheiro et al. 2008). The PCR-amplified products were sequenced using the method of Sanger et al. (1977) and were then compared with GH sequences of fish species already described. The consensus of all the sequences obtained served as the basis for the chemical synthesis of the open reading frame (ORF) of the tGH.

Design and synthesis of tGH ORF

The cDNA sequence of the tGH was adapted with the use of preferential codons from *K. phaffii* and the reduction of the high content of adenine-thymine (AT) in the native sequence. In this step, we used the software Blast (https://blast.ncbi.nlm.nih.gov/Blast.cgi), Oligocalc (http://biotools.nubic.northwestern.edu/OligoCalc.html), NEBcutter® V2.0 (https://nc3.neb.com/NEBcutter/), ExPASy (https://www.expasy.org/), ProtParam (https://protparam.net/index.html), NetNGlyc 1.0 Server (https://services.healthtech.dtu.dk/services/NetNGlyc-1.0/) and NetOGlyc 3.1 Server (https://www.hsls.pitt.edu/obrc/index.php?page=URL1043959855), which are available online. The adapted sequence was obtained via chemical synthesis and cloned into the pUC19 vector by GenOne Biotechnologies Ltd. (Rio de Janeiro, RJ, Brazil). Restriction sites for the EcoRI and NotI enzymes flanking the synthetic sequence were added. Flanking the START Codon, the sequence Kozak ACCATGG was added to confer greater efficiency at the start of the translation. Histidine tail codons were inserted in the 3' region for confirmation and purification of the expressed protein.

pPIC-tGH expression plasmid acquisition

The synthetic sequence of the tGH gene and the pPIC9 vector were digested with the enzymes EcoRI and NotI and were purified and linked by the enzyme T4-DNA ligase (NEB®). *Escherichia coli* DH10B cells were transformed with the recombinant plasmid pPIC-tGH, and the selected colonies were obtained via colony PCR to confirm the transformants containing the tGH gene sequence. For this, the primers 5'AOX1-*forward* 5´-GACTGGTTCCAATTGACAAGC-3´ and 3'AOX1-*reverse* 5´-GCAAATGGCATTCTGACATCC-3´ (Invitrogen®) were used. The colonies selected for PCR were resuspended in 5 μL of water. PCR conditions were as follows: 1 μL of cells, 1x *Taq* DNA polymerase enzyme buffer, 1.5 mM MgCl2, 0.3 mM dNTPs, 5 pmol of each primer, 1.5 U Taq DNA enzyme (NEB®), 2 minutes at 95°C, followed by 25 cycles of 94°C for 40 seconds, 62°C for 40 seconds, 72°C for 1 minute and a final extension at 72°C for 5 minutes. The resulting products were analyzed in 0.8% agarose gel stained with ethidium bromide 0.5 μg mL^{-1} and visualized under UV light.

Transformation of *K. phaffii* and phenotype determination

The pPIC-tGH recombinant plasmid was linearized with BglII and used to transform *K. phaffii* GS115 via electroporation (1900 V, Electroporator 2510 Eppendorf, 1.0 mm cuvette). The transformants were selected in MD medium and the colonies were visible after 2-3 days of incubation at 30 ºC. For the Mut+ and Muts phenotypic determination, the transformants were seeded in MM medium and identified according to their growth capacity in methanol as the sole carbon source, following the manufacturer's instructions (Invitrogen®).

tGH gene expression in shaken flasks

Komagataella phaffii GS115/pPIC-tGH transformants of both the Mut^+ and Mut^s phenotypes selected for tGH gene expression were inoculated in 25 mL of BMGY medium and incubated for 16-20 hours at 30 ºC under shaking at 250 rpm until the culture reached an optical density at 600 nm (OD_{600}) of between 2-6. The cultures were then centrifuged for 10 minutes at 4,000 g at 4 ºC and the pellets were inoculated in 100 mL of BMMY in 500 mL flasks and incubated for 96 hours at 30 ºC under shaking at 250 rpm. Absolute methanol was added to the culture every 24 hours to a final concentration of 0.5 % (v/v). Samples of the cultures were collected and preserved at -80 ºC for further analysis via SDS-PAGE and Western blotting.

Analysis of rtGH

Samples of supernatants from the expression were precipitated with 10% TCA and resuspended in 1x sample buffer for SDS-PAGE (200 mM Tris pH 6.8;

0.1% bromophenol blue (w/v); 4% SDS (v/v); 4% β-mercaptoethanol; 20% glycerol (v/v)). The samples were loaded into a mini gel SDS-PAGE system (BioRad), according to the methodology of Sambrook and Russell (2001). After the electrophoretic run, the proteins were stained with Coomassie brilliant blue R-250 overnight and transferred to a nitrocellulose membrane in a semi-dry transfer system (Trans-Blot®SD, Semi-Dry Transf Cell, Bio-Rad). For the development of the Western blot, the C-terminal anti-His-tag primary antibody was used at a dilution of 1:5000 (v/v) (Life Technologies, catalog: 46-0693) along with the WesternBreeze® Chromogenic Western Blot Immunodetection kit, following the manufacturer's instructions (Invitrogen®).

Purification of rtGH

After induction by methanol for 96 hours, samples of the supernatant of *K. phaffii* GS115/pPIC-tGH phenotype Mut+ were purified via affinity chromatography (Akta Purifier system, GE Healthcare), using the commercial column His Trap HP 5 mL (GE Healthcare), previously equilibrated with buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 10% glycerol (v/v)). The rtGH was eluted with buffer B (buffer A with 500 mM imidazole) in the linear gradient from 0 to 100% at a flow of 2 mL per minute. The fractions collected were analyzed via SDS-PAGE.

rtGH production in bioreactor

The cell mass of *K. phaffii* GS115/pPIC-tGH/Mut$^+$ grown in BMGU was inoculated in FM22 medium containing glycerol 80 g L^{-1} for biomass formation in a mechanically agitated and instrumented bioreactor (BIOSTAT B, B. Braun Biotech International), with real-time monitoring sensors of pH, temperature and dissolved oxygen (DO), coupled to a vessel with a capacity of 4 L and a working volume of 3 L. The pH of the medium was adjusted to 5.0 using 28% ammonium hydroxide solution, temperature 30 ºC, aeration 5-18 L min^{-1}, DO at over 20% saturation and shaking between 50 and 1,000 rpm. Antifoam (polypropylene oxide) was added to eliminate excess foam. The bioreactor operated in single batch mode until the glycerol was fully consumed (about 26 hours), followed by a period of starvation (60-90 min) for consumption of any glycerol residues and inhibitors of heterologous expression. After this period, induction of gene expression was initiated with 7.5 g L^{-1} of methanol containing PTM4, increasing to 15 g L^{-1} after the second pulse at intervals of approximately 3 hours. Throughout the fermentation, the supernatant samples were collected to quantify the rtGH. Cell biomass was quantified by means of a standard curve that indicated optical density and cell concentration (X) in dry weight, which, after linear adjustment, provided the following equation: X (g L^{-1}) = 0.27 • OD_{600}. Optical density was measured with a spectrophotometer (Shimadzu, UV 1601). The bioprocess lasted 80 hours. The determination of glycerol and methanol was performed by high performance liquid chromatography (HPLC) using a Shimadzu liquid chromatograph that was equipped with Hi-Plex H column (100 mm x 7.7 mm) and refractive index detector (RID). The temperature was maintained at 60°C, and a 5 mM sulfuric acid solution was used as the mobile phase. The volume of the injected sample was 0.5 mL.

Quantification of rtGH

The amount of rtGH secreted into the supernatant was determined using the immunodetection method and the 96-well Cell Biolabs His-tag Protein ELISA kit, as recommended by the manufacturer. The microplate reading was at 450 nm and the rtGH concentration was quantified by means of a standard curve generated with the C-terminal His-tag Rhotekin RBD protein (10 kDa) of a known concentration, which was provided by the kit.

RESULTS

Acquisition of tGH cDNA, comparison with other species and translational alignment

From the PCR A fragments of approximately 536 bp was obtained from the transcriptome of the pituitary and brain tissue. The sequencing of the fragment revealed it to be the cDNA of mature tambaqui GH. The comparative analysis of the predicted amino acid sequence had 100% identity with the tGH sequence deposited by Souza et al. (2016) (GenBank accession # ANG09167.1), as well as high similarity with GH sequences from other teleost species (Table 1). Translational analysis of the tGH sequence revealed a polypeptide of 186 amino acid residues including the five conserved cysteine residues responsible for the formation of the disulfide bridges that are essential for maintaining the structural integrity and biological activity of the molecule (Chang et al. 1992) and two N-glycosylation sites at positions 125 and 177 (Figure 1). The predicted molecular mass for non-glycosylated rtGH was 21.5 kDa, including the histidine tail.

pPIC-tGH acquisition and expression in *K. phaffii*

The cloning of the synthetic sequence of the tGH gene in the pPIC9 vector (Figure 2a) and the purification of the pPIC9 vector, the insertion of the tGH gene (Figure 2b), and the transformation of the PCR of ten colonies with the pPIC-tGH recombinant plasmid (Figure 2c) were successfully performed.

The rtGH (~23 kDa) was secreted to the extracellular medium in both the phenotypes Mut+ and Muts, starting at 24 hours until 96 hours (Figure 3). Western blotting analysis confirmed the presence of rtGH in the supernatant of cultures of *K. phaffii* GS115/pPIC-tGH of both the phenotypes Mut+ and Muts, in the induction intervals from 24 to 96 hours (Figure 4).

Table 1. Acquisition of the cDNA of mature GH from tambaqui. Comparative analysis (BlastX) of the predicted amino acid sequence of the cDNA of tambaqui GH obtained in this study (see Figure 1) with GH amino acid sequences deposited in GenBank.

Species	Max score	Total score	Query cover (%)	E value	Percent identity (%)	Accession length	Accession number
Colossoma macropomum	371	371	99	3E-129	100	200	ANG09167.1
Colossoma macropomum	371	371	99	3E-129	100	200	XP_036420034.1
Piaractus mesopotamicus	365	365	99	3E-127	98.9	178	ABA46816.1
Pygocentrus nattereri	365	365	99	4E-127	98.3	200	XP_017572862.1
Electrophorus electricus	357	357	99	2E-123	93.8	200	XP_026882530.1
Astyanax mexicanus	328	328	99	4E-112	89.4	201	XP_022522456.1
Myxocyprinus asiaticus	341	341	99	2E-117	88.8	210	ABC61680.1
Triplophysa rosa	341	341	99	3E-117	88.8	210	KAI7803802.1
Ictiobus bubalus	341	341	99	3E-117	88.8	210	AAR24538.1
Myxocyprinus asiaticus	341	341	99	5E-117	88.8	231	XP_051571425.1
Xyrauchen texanus	340	340	99	7E-117	88.8	208	XP_051987672.1
Schistura balteata	340	340	99	7E-117	88.3	199	ACJ26019.1
Misgurnus anguillicaudatus	340	340	99	1E-116	88.3	210	AAQ19263.1
Misgurnus anguillicaudatus	340	340	99	1E-116	88.3	210	ABC61681.1
Paramisgurnus dabryanus	338	338	99	3E-116	88.3	210	ABC61679.1

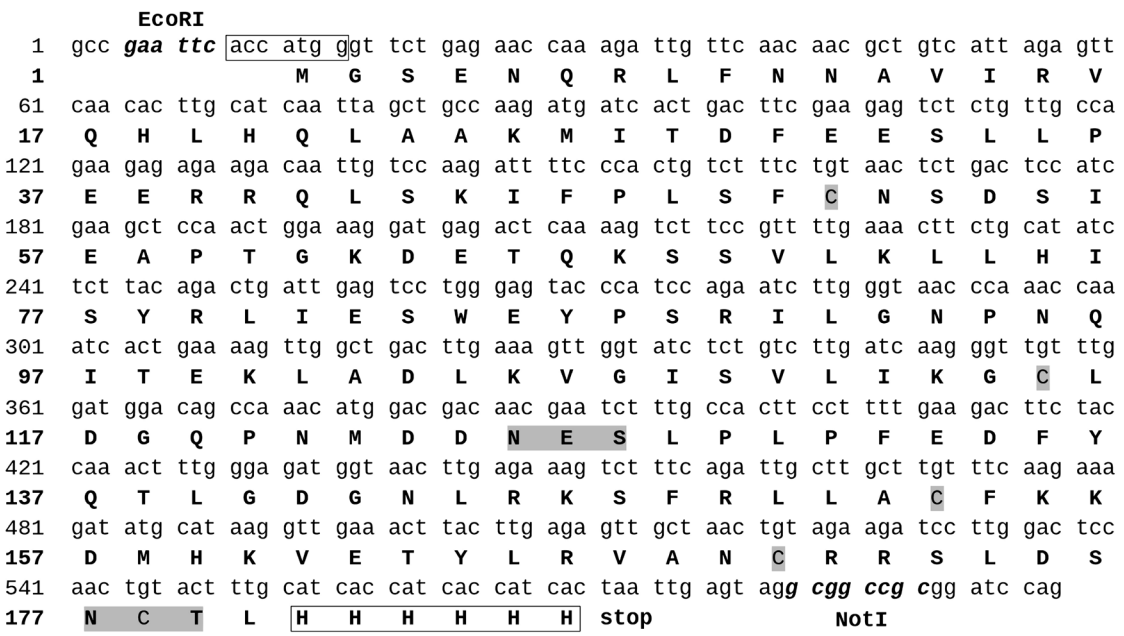

Figure 1. Predicted amino acid sequence of tambaqui GH. Translational alignment of tambaqui cDNA adapted for expression in *Komagataella phaffii*. Gray highlight indicates the existence of two signal sites for N-glycosylation, five cysteine residues and the restriction sites in 5'-EcoRI and 3'-NotI. Also highlighted are the 5'- Kozak sequence and the histidine tail in the C-terminal region.

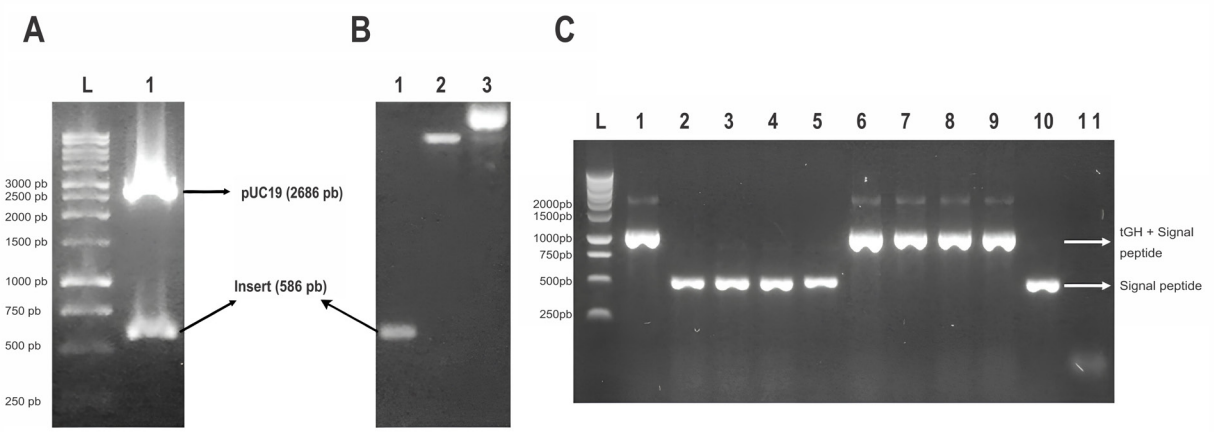

Figure 2. Acquisition of the pPIC-tGH vector for tambaqui GH expression in *Komagataella phaffii*. Electrophoretic profiles in 0.8% agarose gel. **A** – double digestion of the pUC-tGH vector and pPIC9 with the enzymes EcoRI and NotI, L: 1KB molecular mass marker (Promega), well 1: digested pUC-tGH plasmid; **B** – purification of pPIC9 vector and tGH gene insert, well 1: purified tGH gene insert; well 2: linearized and purified pPIC9 vector, well 3: non-linearized pPIC9 vector; **C** – PCR of *Escherichia coli* DH10B colony transformed with the product of linkage between synthetic tGH gene sequence and pPIC9 vector, L: 1 kb molecular mass marker (Promega), wells 1 and 6-9: *E. coli* DH10B/pPIC-tGH transformant, wells 2-5 and 10: *E. coli* DH10B/pPIC9 transformant, well 11: negative PCR control. The bands of wells 1 and 6-9 have 1078 bp (corresponding to the 586 bp tGH gene insert + 492 bp pPIC9 vector signal peptide coding region).

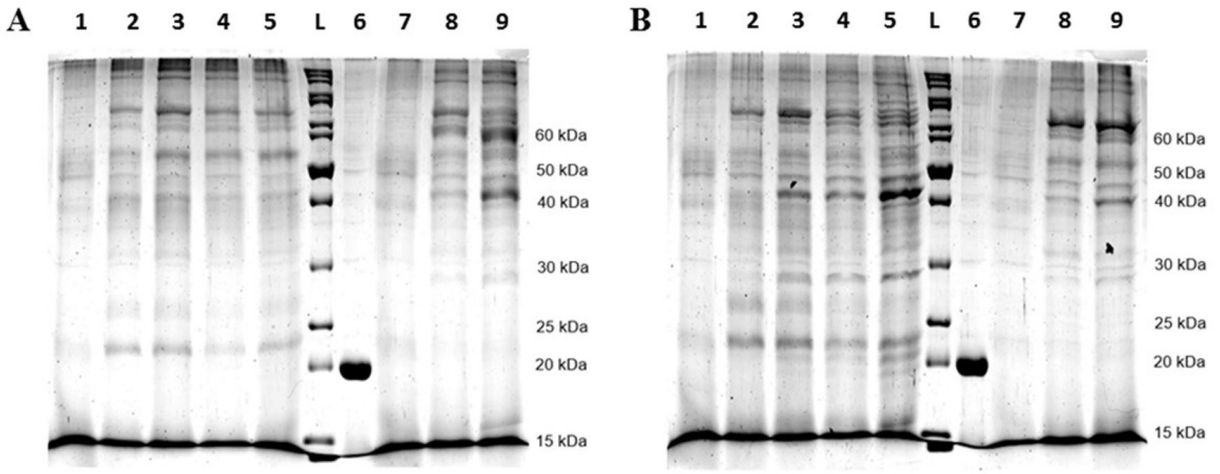

Figure 3. Expression of rtGH in *Komagataella phaffii*. Electrophoretic profiles of SDS-PAGE 15% gel proteins of rtGH expression. **A** – *K. phaffii* GS115/pPIC-tGH Mut+, wells 1-5: methanol induction times of 0 h, 24 h, 48 h, 72 h and 96 h, L: Unstained Protein Ladder PageRuler marker (Fermentas), well 6: human GH isoform 20 kDa (kindly provided by Cristália-Produtos Químicos Farmacêuticos, São Paulo, Brazil), wells 7-9: times 0 h, 48 h and 96 h of the C- (GS115/pPIC9/Mut+, negative control); **B** – *K. phaffii* GS115/pPIC-tGH Muts, wells 1-5: methanol induction times of 0 h, 24 h, 48 h, 72 h and 96 h, L: Unstained Protein Ladder PageRuler marker (Fermentas), well 6: 20 kDa human GH isoform, wells 7-9: times 0 h, 48 h and 96 h of the C- (GS115/pPIC9/Mut+, negative control).

Purification of rtGH

The rtGH was purified directly from the culture supernatant of *K. phaffii* GS115 pPIC-tGH Mut$^+$ induced with methanol for 72 hours. Elution occurred in the gradient of approximately 20% imidazole (100 mM) (Figure 5). The existence of three purified bands may be related to the existence of rtGH isoforms.

rtGH production in bioreactor

The fermentation for the production of rtGH was successfully conducted in a total time of 80 hours. The specific growth rate in glycerol was 0.216 h^{-1}. The dry cell mass achieved after total glycerol consumption was 31.5 g L^{-1} in 28 hours. During the induction phase, the specific growth rate in methanol was reduced to 0.0172 h^{-1}. The dry cell mass achieved in methanol was 62.8 g L^{-1} in 52 hours of induction. The rtGH was detected in the culture medium from the first 6 hours of induction and its concentration continued with a linear growth until the end of fermentation, reaching a peak production of 250.7 mg L^{-1} in 80 hours (Figure 6a). Overall, there was low pH variation due to the addition of ammonium hydroxide throughout the culture, yet there was a marked variation in the saturation level of DO, from zero to peaks of ≅ 95% (Figure 6b).

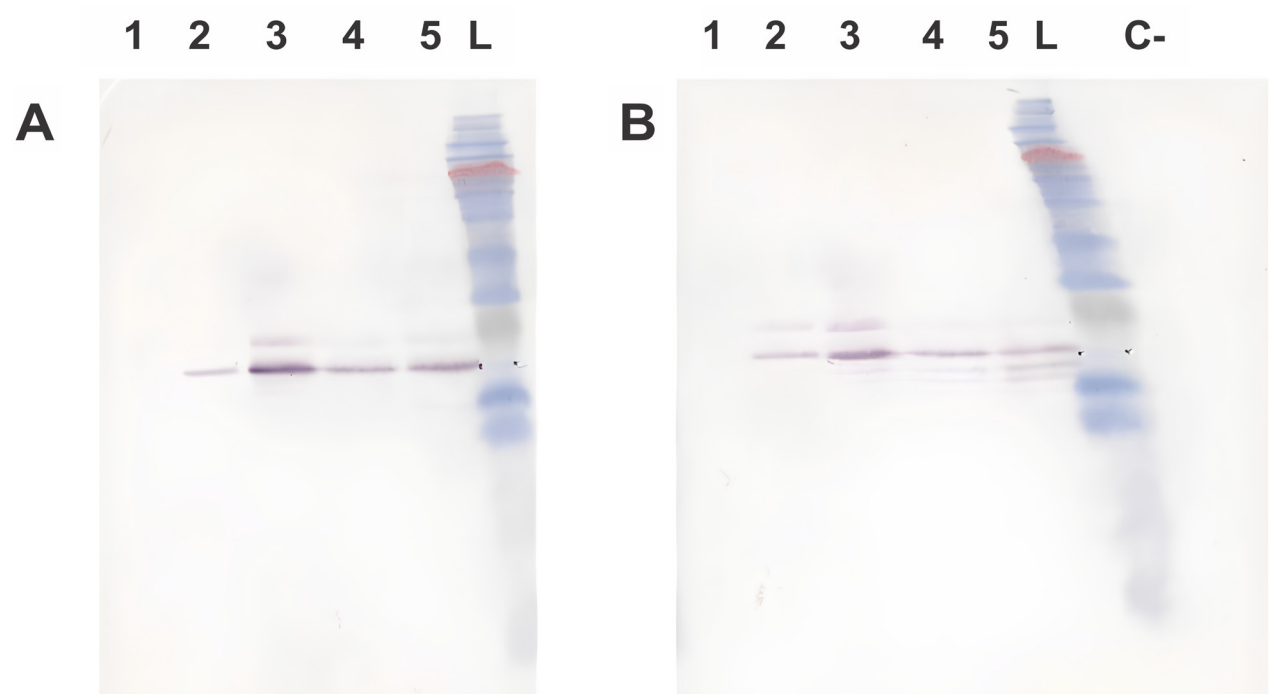

Figure 4. Western blotting analysis of rtGH expression. **A** – *Komagataella phaffii* GS115/pPIC-tGH Mut+, wells 1-5: methanol induction times of 0 h, 24 h, 48 h, 72 h and 96 h, L: molecular mass marker ColorPlus Prestained Protein Ladder**,** broad range of 10-230 kDa (New England BioLabs); **B** – *K. phaffii* GS115/pPIC-tGH Muts, wells 1-5: methanol induction times of 0 h, 24 h, 48 h, 72 h and 96 h, L: molecular mass marker ColorPlus Pretained Protein Ladder, broad range of 10-230 kDa (New England BioLabs), C-: negative control (GS115/pPIC9/Mut+) with 72 h induction.

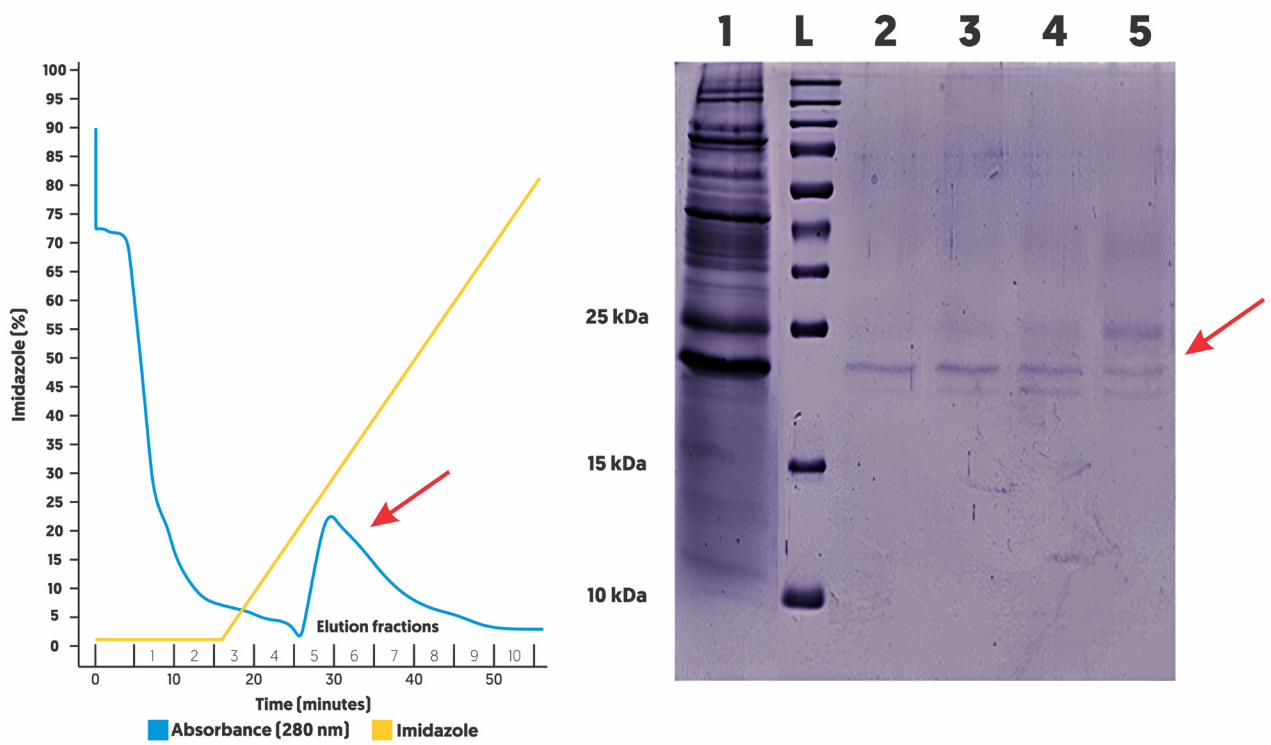

Figure 5. Purification of rtGH expressed by *Komagataella phaffii*. **A** – rtGH purification chromatogram obtained from the supernatant of the induced culture of *K. phaffii* GS115/pPIC-tGH/Mut+. The red arrow indicates the peak elution of rtGH with 20% imidazole (100 mM); **B** – electrophoretic profile of proteins on SDS-PAGE 15% gel of the rtGH elution fractions from the chromatographic column. Well 1: crude extract of the culture supernatant, L: protein molecular mass marker (Thermo Scientific Spectra Multicolor Broad Range Protein Ladder), wells 2, 3, 4 and 5: elution fractions 8, 7, 6 and 5, respectively.

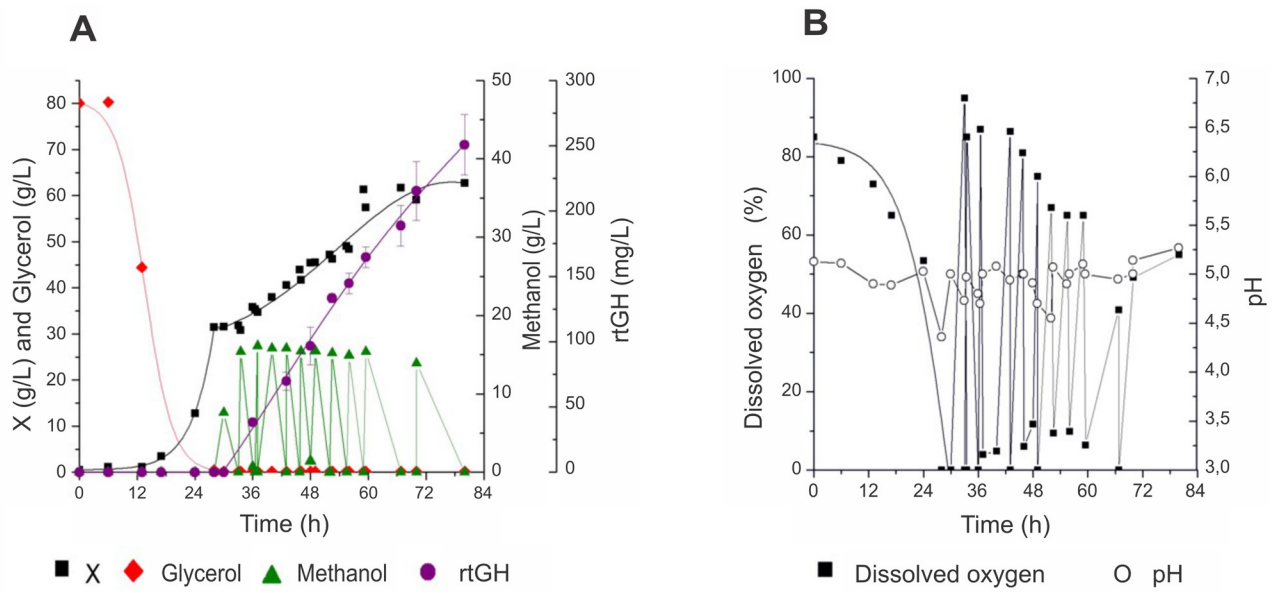

Figure 6. Kinetic profile of rtGH production by *Komagataella phaffii* in FM22 culture medium. The system was operated in a batch fed by pulses in a mechanically agitated bioreactor. **A** – variation of cell concentration (X), glycerol, methanol and rtGH; **B** – variation of dissolved oxygen and pH.

DISCUSSION

The cDNA sequence of tambaqui GH was successfully obtained from the pituitary and part of the brain of juvenile tambaqui. This sequence was adapted for extracellular expression in the yeast *K. phaffii* GS115. During the adaptation of the sequence, the AT content was reduced from over 65% to 58% and the preferential codons of *K. phaffii* were used. These were decisive steps for the success of heterologous expression, since in the attempts at expression of the native cDNA we were unsucessful (data not shown). The high AT content (> 65%) of the tGH cDNA sequence is consistent with reports in the literature that sequences with an AT content above 60% are associated with low levels of expression or no gene expression in the *K. phaffii* system due to premature termination of transcription of the RNA in formation (Boettner et al. 2007).

The 100% identity between the gene sequence described in here and the sequence deposited in GenBank (Souza et al. 2016) demonstrated the reliability of our results. This high similarity is related to that the GH coding region tends to be highly conserved among different vertebrate groups, therefore the GH gene has been used to evaluate evolutionary relationships among fish at different taxonomic levels (Meier et al. 2006; Bart et al. 2010; Kamenskaya and Brykov 2020). In our analysis of the amino acid sequence, tGH showed high amino acid similarity with the GH of other teleost fish species, such as Cypriniformes, Siluriformes and Characiformes.

SDS-PAGE and Western blotting analyses confirmed the presence of rtGH in the culture supernatant starting from the first 24 h until 96 h of induction. The rtGH was well secreted into the extracellular medium due to the signal peptide alpha factor of *S. cerevisiae* present in the vector pPIC9. The efficient secretion in the *K. phaffii* system represents an important advantage in the heterologous production of GHs of fish by facilitating its detection and subsequent purification (Cregg et al. 1993; Cereghino and Cregg 2000; Cereghino et al. 2002). Due to the GRAS status of *K. phaffii*, the rtGH expressed by it can be used in aquaculture. Several studies have already demonstrated that the use of yeast in fish feed increases digestibility (El-Bab et al. 2022), feed efficiency and immunological resistance (Li and Gatlin 2004; El-Bab et al. 2022), feed conversion and productive performance (Ozório et al. 2012; El-Bab et al. 2022), in addition to serving as a source of vitamins (Zhang et al. 2019).

The difference between the predicted molecular mass (21.5 kDa) and the equivalent band obtained in electrophoretic migration (~23 kDa) suggests the hypothesis of the occurrence of glycosylation in the recombinant protein, given the existence of two glycosylation sites in the molecule. The bands surrounding rtGH, observed both in Western blotting and purification, corroborate this hypothesis. In *K. phaffii*, approximately 0.5 to 1% of translated proteins are glycoproteins (Bretthauer and Castellino 1999; Daly and Hearn 2005). Glycosylation can increase the resistance of the protein against proteolytic degradation (Pratap et al. 2000), assist in correcting folding and passage through the secretory pathway, and also increase the stability of the mature protein (Daly and Hearn 2005). The majority of heterologous N- or O-glycosylated proteins expressed in *K. phaffii* preserved the bioactivity found in the native non-glycosylated protein (Li et al. 2007). The occurrence of glycosylation has also been

reported for human GH produced in *K. phaffii*, which has preserved the pharmacological effect and prolonged the half-life in the circulatory system of rats by a factor of 24 compared to the natural non-glycosylated variant (Flintegaard et al. 2010). These results demonstrate a positive aspect of glycosylation and make the expression of tGH in yeast even more promising for use in fish farming.

Our results showed that chromatography is a viable method for obtaining purified rtGH, which can be used in future biological activity assays, since the presence of the His-tag tail does not alter the biological activity of recombinant GHs (Doozandeh-Juibari et al. 2019). Recombinant growth hormones (rGH), which are isolated from host cell pellets, have been tested for their physiological effects and efficiency in the zootechnical performance of fish (Tsai et al. 1997; Jeh et al. 1998; Li et al. 2003; Funkenstein et al. 2005). However, purification of rGH is not economically viable for use in commercial cultivation (Liu et al. 2008). The extracellular production of rtGH in *K. phaffii* facilitates the use of this hormone in fish farming without the need for purification. The only study on the physiological effects of exogenous GHs on tambaqui used the commercial bovine GH (bGH) Lactotropin@ and demonstrated a significant weight gain in the first 15 days of fish treatment (Paz and Val 2018). However, the use of commercial bGH is impracticable for fish farming, both due to its high cost and that it is a hormone from another vertebrate class. With our study, it will be possible to carry out new studies using rtGH to evaluate the physiological and zootechnical performance of tambaqui subjected to the recombinant hormone with a focus on fish farming.

The good results obtaind for the heterologous production of rtGH in the bioreactor have room for improvement. The optimization of the production levels of heterologous proteins in *K. phaffii* depend on genetic factors, such as the native cDNA sequence, preferred codons and number of copies of the expression cassette integrated into the yeast genome, and physical, chemical and physicochemical parameters of the system, such as temperature, pH, inducer concentration and oxygen (Cregg et al. 1993; Brabander et al. 2023). In our system, the preferred codon parameters, temperature, pH and methanol concentration were maintained in the ranges considered optimal, which reflected in the significant production of rtGH. However, the specific growth rates, both in glycerol (0.216 h^{-1}) and methanol (0.0172 h^{-1}), were lower than the values recorded in the literature for the *K. phaffii* system (0.24 h^{-1} and 0.14 h^{-1}, respectively; Stratton et al. 1998), indicating the need to adjust the system, especially in relation to the oxygen supply. The abrupt variations of dissolved oxygen were due to the high cell density that avidly consumed oxygen. When the methanol was depleted, the oxygen concentration increased to near saturation, but, as soon as the bioreactor was fed with methanol pulses, oxygen consumption was restored. Dissolved oxygen frequently reached critical levels, below 5% saturation, especially after the first 28 hours, when the cell concentration exceeded 30 g L^{-1} (see Figure 6b). Peaks in dissolved oxygen concentration corresponded to intervals of depleted methanol in the medium, and drops in oxygen level corresponded to intervals of pulses of methanol in the medium as an inducer of AOX promoter activity, resulting in rtGH production and cell growth. In the metabolism of methanol, the increase in cell concentration demands a greater amount of oxygen, and meeting this demand results in greater heterologous production (Curvers et al. 2001). In our fermentation process, oxygen from filtered air was used. A possible alternative for maintaining the oxygen level above 20% saturation would be the supply of concentrated oxygen to the system at times of increased cellular metabolism. The level of 250.7 mg L^{-1} of rtGH production obtained in the fermentation is satisfactory in comparison to levels of rGH produced in bioreactor reported in the literature. For example, the pioneering study on recombinant human growth hormone (rhGH) in *K. phaffii* obtained in a bioreactor reported a production of 49 mg L^{-1} (Ecamilla-Treviño et al. 2000). Subsequent studies obtained 150 mg L^{-1} of rhGH in 24 h of induction (Çalik et al. 2010) and the highest level of rhGH in 24 h reported to date of 500 mg L^{-1} (Apte-Deshpande et al. 2009), twice as much as the level of rtGH obtained in our study. The latter authors used optimized conditions of continuous methanol feeding, dissolved oxygen maintained at 30% and supplementation with Tween 20 surfactant. Azadi et al. (2018) used an optimized strategy of mixed methanol/sorbitol feeding and supplementation with ascorbic acid in 30 h of induction to obtain an exceptional rhGH production of 1.4 g L^{-1}.

Our findings show a strong potential for tambaqui to be used as a target species for rGH production in biorector and its use in biotechnological innovations and zootechnical applications of this protein in aquaculture.

CONCLUSIONS

We demonstrated, for the first time, that gene expression and production of rGH derived from tambaqui can be successfully performed in *K. phaffii* yeast, either in shaken flasks or in a bioreactor, with efficient secretion into the culture supernatant and purification of the recombinant protein by affinity chromatography. Despite the great diversity of the Amazonian ichthyofauna, this is also the first time that the cDNA of the GH of a native Amazonian fish is expressed in a heterologous system and produced in a bioreactor, which opens the way for future work involving other Amazonian species that are important for fish farming in the region. The success of the heterologous expression of the tGH gene and the considerable level of bioreactor production of the rtGH shows great potential for future genetic, physiological and biotechnological research aimed at improving the productivity of tambaqui in fish farming.

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