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The production and recovery analysis of a promising recombinant phytase from *Yersinia intermedia* expressed in *Escherichia coli* and its potential use in biotechnology

Ruth Maria Rocha RIBEIRO[1], Sandro Braga SOARES[2], Quemuel Quesley Nunes MENDES[2], Juan Pablo SOARES[2], Amanda Ferrari das NEVES[2], Nathália Zenaide Durães SOARES[2], Ludmila Ribeiro MENDES[2], Izabella Mendes LIMA[1], Ivan Pires de OLIVEIRA[2], Gustavo Leal TEIXEIRA[2], Bruna Mara Aparecida de Carvalho MESQUITA[1,2], Gabriela da Rocha Lemos MENDES[2], Igor Viana BRANDI[1,2], Mariana Sousa VIEIRA[3,5], Renato Ramos GODOI[3,5], Thaís Paiva Porto de SOUZA[3,5], Marina Quádrio Raposo Branco RODRIGUES[4,5], Daniel Gonçalves BONOTO[4,5], Alexsandro Sobreira GALDINO[3,5], William James Nogueira LIMA[1,2*]

Abstract

Recombinant phytases produced in *Escherichia coli* offer an alternative to inorganic phosphorus (P) use in monogastric diets, reducing production costs and minimizing soil and water pollution. The enhanced bioavailability of phytic P, minerals, proteins, and carbohydrates, along with the benefits of inositol, is highly valued by specialists when formulating feeds with optimal levels of enzymatic additives.

Keywords: fermentation; downstream process; animal nutrition; enzymes; cell disruption.

Practical Application: *Yersinia intermedia* recombinant phytase is a potential enzymatic additive for use in monogastric diets.

1 INTRODUCTION

Phytases are industrially relevant feed enzymes that catalyze the sequential hydrolysis of phosphate groups from phytate, the salt of phytic acid, which serves as the primary phosphorus (P) storage form in plant tissues, notably in cereal grains and oilseed meals (Herrmann et al., 2022; Jatuwong et al., 2020).

Among the microorganisms that produce them, metabolically engineered Escherichia coli has emerged as a cell factory because of many advantages including high growth rate, the availability of gene and genome engineering tools, established high cell density culture techniques, and various systems metabolic engineering (de Souza et al., 2018; Yang et al., 2020). Microbial phytases are the most competent, economically stable, potential bioinoculants, and environmentally friendly enzymes (de Souza et al., 2024; Rizwanuddin et al., 2023).

In this study, the gene that encodes the production of bacterial phytases from *Yersinia intermedia* was inserted into *Escherichia coli* BL21 (λDE3) with a C-terminal 6His tag, enabling the purification of the enzyme by immobilized metal affinity chromatography (IMAC). The recombinant phytase yPHY from *Y. intermedia* (National Institute of Industrial Property [INPI] BR1020140168796) is a 6-phytase, histidine acid phosphatase (HAP) (Lemuchi et al., 2013; Vieira et al., 2019).

Phytate P is not available to monogastric animals since they possess very low levels of phytase activity in their digestive tracts. So, it is excreted along with animal waste, and this is known to cause algal blooms and eutrophication in surface waters (Jatuwong et al., 2020). An alternative to supply the P demand in poultry, fish, and swine organisms is the addition of inorganic P in the feed formulation (de Souza et al., 2018).

The application of this resource in the feed industry competes with its use as a fertilizer for crop production. It is known that the sustainable management of P in livestock-dominated food systems requires reliance on animal feed concentrates (Rothwell et al., 2020). It is also widely recognized that phosphate rock reserves are finite and critical natural resources. The high demand for the inorganic fertilizer P, and the heavy reliance on

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[1]*Universidade Estadual de Montes Claros, Postgraduate Program in Biotechnology, Bioprocess Development Laboratory, Montes Claros, MG, Brazil.*
[2]*Universidade Federal de Minas Gerais, Institute of Agricultural Sciences, Montes Claros, MG, Brazil.*
[3]*Universidade Federal de São João Del-Rei, Laboratory of Microorganism Biotechnology, Divinópolis, MG, Brazil.*
[4]*Universidade Federal de São João Del-Rei, Department of Biotechnology, São João del-Rei, MG, Brazil.*
[5]*National Institute of Science and Technology in Industrial Biotechnology, Brasília, DF, Brazil.*
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imports, makes Brazilian agriculture particularly vulnerable to future P scarcity, or sudden fluctuations in the cost of P (Withers et al., 2018).

Today, engineered phytases already contribute to efficient P mobilization in the feeding industry and might pave the way to a circular P-bioeconomy (Herrmann et al., 2022). Feed enzymes increase the flexibility and range of feedstuffs in feed formulations, helping reduce feed costs. Besides, the alteration of gut microbiota toward favorable bacterial species improves gut health and provides a protective effect on the animal's overall health (Glitsoe et al., 2015).

To support the advancement of purification studies aimed at developing products and processes within the national industry, this study sought to identify the factors influencing enzyme production and recovery. These insights will aid in optimizing the affinity chromatography step in IMAC at a semi-preparative scale.

2 METHODOLOGY

This study was developed in Bioprocess Laboratory of the Agricultural Sciences Research Center of the Universidade Federal de Minas Gerais (UFMG) Montes Claros.

2.1 Materials and equipment

The following materials and equipment were used in this study: BioFlo 3000 benchtop fermenter, New Brunswick Scientific Co., Inc. (NBS), Canada; culture medium (Mat. Suppl. 1–3) (KH_2PO_4, K_2HPO_4, $Na_2HPO_42H_2O$, $(NH_4)_2SO_4$, NH_4Cl, $MgSO_4.7H_2O$, citric acid, oligodynamic solution [OLD], yeast extract, and casein peptone), glucose, and lactose (15 g/L); EmulsiFlex-C5 high-pressure homogenizer, Avestin, Inc., Ottawa, ON, Canada; Thermo Scientific Sorvall ST 16R Centrifuge, Thermo Fisher Scientific Inc., Osterode am Harz, Germany; a refrigerated incubator with an orbital shaking benchtop shaker TH.6440 Touch Line, Thoth, Piracicaba, São Paulo, Brazil; Shimadzu model UV-1280 spectrophotometer, Kyoto, Japan; Alltion optical microscope, SN 1600226, Wuzhou, China; 30% acrylamide (AAm) mix (30 g AAm, 0.8 g N,N'-Methylenebisacrylamide [MBAAm], and water q.s.p. 100 mL); 1.5 M Tris (pH 8.8); 10% sodium dodecyl sulfate (SDS); 10% ammonium persulfate (APS); tetramethylethylenediamine (TEMED); 1.0 M Tris (pH 6.8); 2x Laemmli sample buffer, Bio-Rad Laboratories Inc., CA, USA; Amersham Low Molecular Weight Calibration Kit for SDS electrophoresis, GE Healthcare, Buckinghamshire, UK; bovine serum albumin (BSA), Saint Louis, USA; and Bradford reagent. Most reagents are from Sigma-Aldrich.

2.2 Cloning of the yPHY

The gene corresponding to the *Y. intermedia* phytase, hereafter named yPHY (GenBank accession no. DQ986462.1) (Huang et al., 2006), was synthesized by Epoch Biolabs (USA), with preferential codons for expression in *E. coli* BL21 (λDE3) pLysS cells, cloned in the expression vector pET21a. Six C-terminal histidines were added for further purification using an immobilized nickel affinity column.

The addition and optimization of codons for expression in *E. coli* BL21 (λDE3) resulted in a change in the characteristics of the modified phytase yPHY (INPI accession no. BR1020140168796) (Vieira et al., 2019) in relation to the wild-type APPA (GenBank accession no. ABI95370.1), whose host was *Komagataella phaffii*.

The APPA, classified as a 6-phytase (EC 3.1.3.26) and histidine acid phosphatase (HAP), has a molecular mass of 45 kDa, an optimal pH of 4.5, an optimal temperature of 55°C, and a specific activity at 37°C for sodium phytate of 3,960 U/mg (Huang et al., 2006). The yPHY has a molecular mass of 50 kDa, an optimal pH of 8.0, an optimal temperature of 40°C, and a specific activity at 37°C of 5,333 U/mL (without Cu^{2+} ions) and 7466 U/mL (with Cu^{2+} ions). It is thermostable between 37 and 40°C (Vieira et al., 2019).

2.3 Fermentation of E. coli *cells*

A culture of *E. coli* BL21 (λDE3)-yPHY was initially grown in three 500 mL Erlenmeyer flasks, each containing 100 mL of Jung seed medium, for approximately 24 h. The composition of the culture medium followed the methodology described in (Jung et al., 1988), adapted.

2.4 Recovery of phytase

From the cells obtained via fermentation in a BioFlo 3,000 bench-top bioreactor, the downstream steps of the process proceeded as follows: cell collection, cell disruption, and enzyme collection (Rao et al., 2008; Santos et al., 2012; Vieira et al, 2019). The sample of cells after mechanical lysis was observed via an optical microscope in order to evaluate their fragmentation.

To study the efficiency of mechanical lysis in a homogenizer at an operating pressure between 3,447 and 103,425 kPa, a combination of two factors was performed: the number of passages, with three levels being 1, 3, and 5, and the cell feed concentration, with two levels being 100% (a concentrated lysate, 6.4 g dry cell weight [dcw]/L) and 50% (the sample diluted twice in the concentrated lysate, 3.2 g dcw/L). Table 1 presents the experimental design performed.

2.5 Protein quantification and sodium dodecyl sulfate-polyacrylamide gel electrophoresis

The Bradford methodology (Bradford, 1976) was used to quantify the total proteins in mg/mL, in triplicate, using BSA protein as standard (0.2–2.0 mg/mL), in the supernatant and

Table 1. Experimental design of cell disruption in Avestin Homogenizer, Emulsiflex-C5.

Feed concentration (%)	Number of passages
100	1
100	3
100	5
50	1
50	3
50	5

precipitate fractions of the lysed sample. The presence and concentration of the enzyme phytase were detected by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Simpson, 2006) using the ImageJ 1.53e program (approved by the National Institute of Health [NIH], USA).

2.6 Statistical analysis

A two-way analysis of variance (ANOVA) was conducted on a sample of 18 data points to assess the effects of feed concentration and the number of passages on total protein concentration in the supernatant and precipitate fractions of the lysed sample. Additionally, Tukey's test was applied to construct confidence intervals for all paired differences between the means of the three levels of the passage number factor, maintaining an overall error rate at a 5% significance level (Mat. Suppl. 4 and 5).

3 RESULTS AND DISCUSSION

3.1 Fermentation of E. coli *cells*

The batch cell growth curve (Figure 1) illustrates the growth, decline, and stationary phases of the *E. coli* BL21 (λDE3) culture prior to lactose induction.

Applying a linear regression in the growth phase that follows Equation 1, the first-order autocatalytic reaction, a specific growth rate of 0.0169 min^{-1} was obtained as shown in Equation 2:

$$\ln x = \ln x_0 + \mu t, \quad (1)$$

$$\ln x = -0.2665 + 0.0169t \quad (2)$$

Several phases of cell growth are observed in the batch culture. During the growth and decline phases of the batch culture,

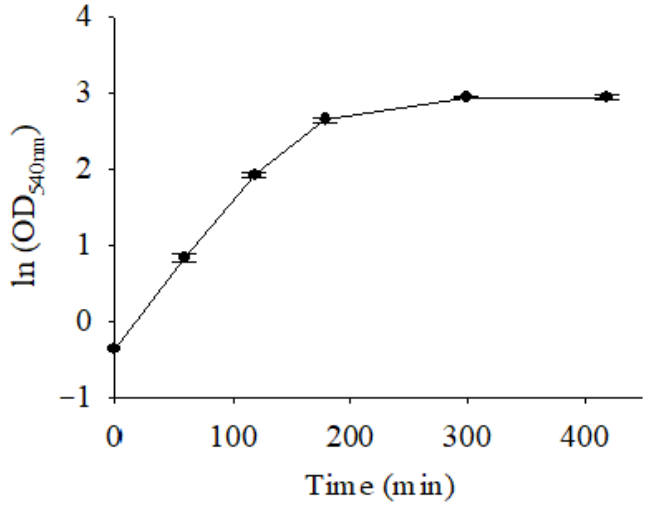

Figure 1. *E. coli* BL21 (λDE3) cell growth curve via optical density at 540 nm, with standard deviation during 7 induction hours.

the specific growth rate of cells is dependent on the concentration of nutrients in the medium. Often, a single substrate exerts a dominant influence on the rate of growth; this component is known as the growth-rate-limiting substrate or, more simply, the growth-limiting substrate. As nutrients in the culture medium become depleted or inhibitory products accumulate, the growth slows down and the cells enter the decline phase. After this transition period, the stationary phase is reached during which no further growth occurs (Doran, 1995).

From this mode of operation, it is clear that the initial operating conditions are highly important, such as the composition of the complex medium, which must be rigorously delimited. Investigating the effect of minimal and complex media on the global metabolic profiles of *E. coli* and *Saccharomyces cerevisiae* employing metabolomics (Kim & Kim, 2017) suggests that the cells prepare their metabolism for survival in the minimal media, whereas they are actively engaged in rapid growth in the complex media.

The dry weight at the end of the batch was 6.4 g/L. This value is in line with expectations. The dcw of *E. coli* in batch cultures is typically 5–10 g dcw/L, while fed-batch cultures can achieve concentrations of over 50 g dcw/L. Some high cell-density culture (HCDC) techniques can even reach concentrations of over 100 g dcw/L (Yee & Blanch, 1992).

Using the BioBLU 3f Single-Use Vessel, the maximum biomass concentration of *E. coli* ATCC® 25922GFP™ gained in batch operation modes in complex and chemically defined media was 3.9 g dcw/L and 27.3 g dcw/L, respectively. Ease of operation and long downtime between runs are the common features of both batch operation modes. The complex medium showed limited biomass and yield, while the chemically defined medium showed good biomass accumulation and high biomass yield on glucose (Yang & Sha, 2019).

3.2 Recovery of yPHY

Figure 2 shows *E. coli* BL21 (λDE3) cells before induction with lactose (A), the presence of dense protein aggregates, inclusion bodies (IBs), in the cytoplasm of *E. coli* BL21 (λDE3) after induction with lactose (B), as well as the cell lysate after passing through a high-pressure homogenizer (C). From the lysis, the soluble and insoluble proteins available along with cellular debris were evaluated.

Research has shown that mechanical lysis using a high-pressure homogenizer is advantageous over other techniques for the isolation of active nanoparticles, designated as non-classical IBs (ncIBs).

Olbrich (1989) *apud* Wong et al. (1997) addressed this by centrifuging the homogenate at 2,000 × *g* for 26 min and taking the IB sample as the concentrate and the cell debris sample as the supernatant. As the measured size distribution of the cell debris is affected by the presence of IBs in the overlapping range, and vice versa, a mixture of particles occurs in the two centrifuged fractions due to the size heterogeneity of the cell debris and IBs.

Peternel and Komel (2010), in their study on *E. coli* cell disruption, compared enzymatic lysis, high-pressure

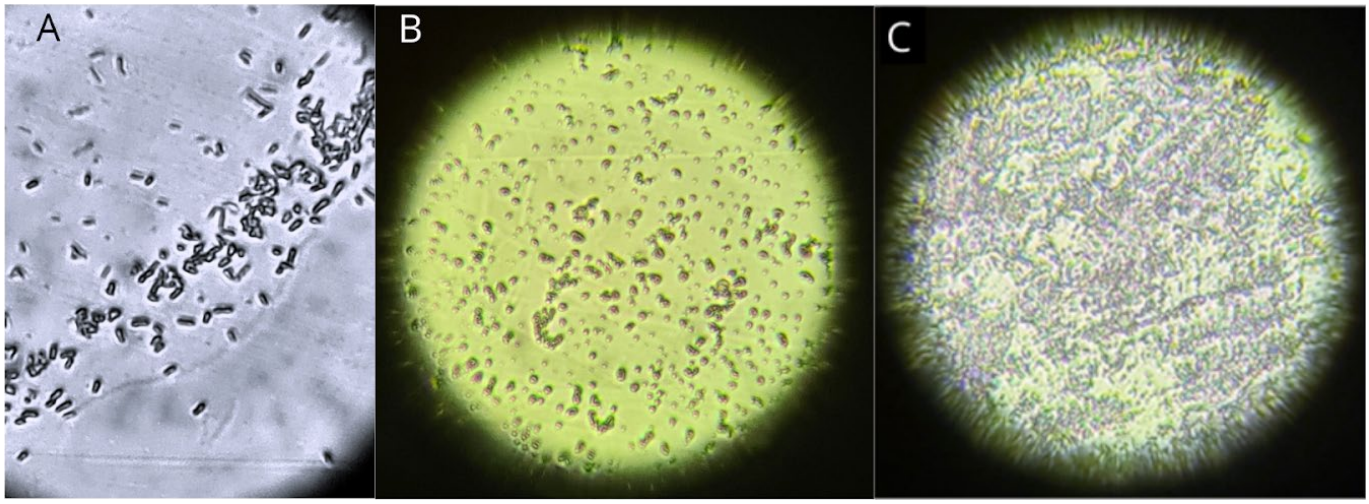

Figure 2. Images obtained via optical microscopy (Alltion, SN 1600226) with 1,000× amplification. (A) Broth fermented with *E. coli* BL21 (λDE3) cells before induction with lactose. (B) Broth fermented with *E. coli* BL21 (λDE3) cells showing the presence of phytase in IBs. (C) The lysed broth of *E. coli* BL21 (λDE3) cells, one pass, showing their fragmentation.

homogenization, and sonication. They concluded that the application of lysozymes in enzymatic lysis introduces an additional impurity into the engineered nanoparticles. In sonication, the acoustic output power compromises the biological activity of the proteins. High-pressure homogenization proves to be the most suitable method, as it achieves high purity and yield, with a minimal impact on the protein biological activity, despite causing surface disruptions (breaks) to the IBs.

Wong et al. (1997) introduced a method for assessing *E. coli* cell debris size post-homogenization, utilizing cumulative sedimentation analysis under centrifugal force combined with SDS-PAGE to analyze membrane-associated proteins. Additionally, the spectrophotometric quantification of peptidoglycans (van Hee et al., 2004) was employed. The median debris size was found to decrease substantially from approximately 0.5 µm to 0.3 µm as the number of homogenization passages increased from 2 to 10. Recombinant cells containing protein IBs exhibited the smallest debris size post-homogenization in comparison with uninduced host cells.

3.3 Bradford and SDS-PAGE assays

Total protein quantification was performed using the Bradford method, as shown in Figure 3, in order to verify the performance of mechanical lysis in a high-pressure homogenizer. A two-way ANOVA was performed to examine the effect of feed concentration and the number of passages on total protein concentration in the supernatant and precipitate fractions of the lysed sample. In both fractions, the interaction between the two factors was not significant at the 5% significance level.

The null hypothesis was rejected, which states that the contrasts of the treatment means are null, for the individual factor feed concentration, referring to the supernatant fraction, and the two factors, number of passages and feed concentration, referring to the precipitate fraction, when $p \leq 0.05$ and $F_{calc} > F_{crit}$ according to Snedecor's F-distribution at 5% probability.

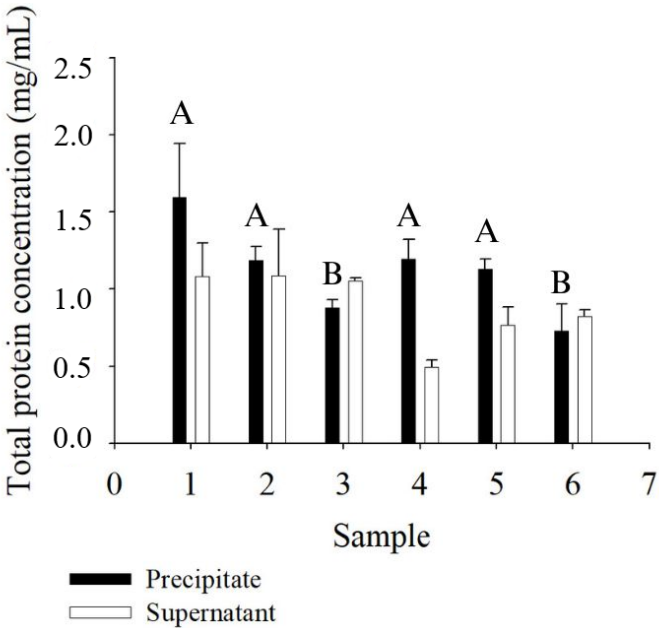

Figure 3. Total protein concentration (mg/mL) in the supernatant and precipitate samples, with standard deviation. 1, 2, and 3, 100% concentrated lysates from passages 1, 3, and 5, respectively; 4, 5, and 6, lysate 2-fold dilution (50%) of the concentrated lysate from passages 1, 3, and 5, respectively. Different letters (A and B) indicate the statistically significant difference among the precipitate samples at the same concentration ($p < 0.05$).

On analyzing the number of passages factor in the precipitate fraction, by Tukey's test, there was a significant difference between the averages of the group of five passages (B), in relation to the other groups (A), which was considered equal.

From Figure 4, the phytase was identified after expression at the end of fermentation, after cell collection, and after lysis. The densitometry of the bands, taking the low-molecular-weight

marker as a reference, generated the phytase profile, as shown in Figure 4B. It was noted that after cell lysis, clearer bands were revealed in wells 3–5, whose molecular weights were between 20.1 and 30 kDa, as well as in wells 6–8, with lower intensity. They are probably membrane-associated proteins.

In Figure 4, phytase was detected following an expression at the end of fermentation, cell collection, and cell lysis. The densitometric analysis of the bands, using the low-molecular-weight marker as a reference, produced the phytase profile, as illustrated in Figure 4B. After cell lysis, distinct bands were observed in wells 3–5, with molecular weights between 20.1 and 30 kDa, as well as in wells 6–8, though with lower intensity. These bands are likely indicative of membrane-associated proteins.

A protein with a molecular weight of around 45 kDa was found in all samples, indicating the presence of recombinant phytases produced after the induction of gene expression by lactose.

By the phytase concentration profile, a pattern was observed among the first five samples, whose initial cell concentration was 6.4 g dcw/L. The phytase concentration, taking ovalbumin as a reference, ranged from 0.31 to 0.79 g/L. The reduction in the phytase content in the last three samples was due to the reduction in the cell concentration to 3.2 g dcw/L.

The cell lysates from each mechanical lysis condition studied were separated into two fractions by centrifugation. The precipitate (Figure 5A) and supernatant (Figure 5B) fractions were submitted to SDS-PAGE to identify and quantify the proteins.

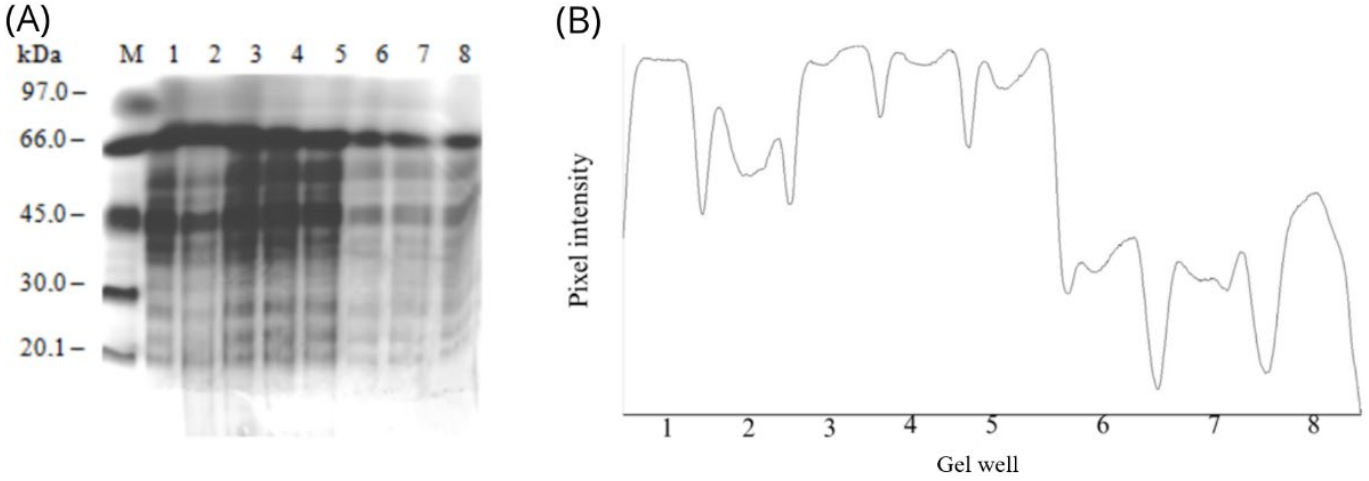

Figure 4. (A) Electrophoresis gel (12%), run at 100-150 V. M, low-molecular-weight standard; 1, sample of cells collected by centrifugation from sample 2; 2, sample of cells from the end of fermentation in a reactor; 3, 4, and 5, 100% concentrated lysate at passages 1, 3, and 5 through the homogenizer, respectively; 6, 7, and 8, lysate 2-fold dilution (50%) of the concentrated lysate at passages 1, 3, and 5 through the homogenizer, respectively. (B) The phytase concentration profile of gel wells 1-8 measuring the pixel intensity of peaks at 45 kDa.

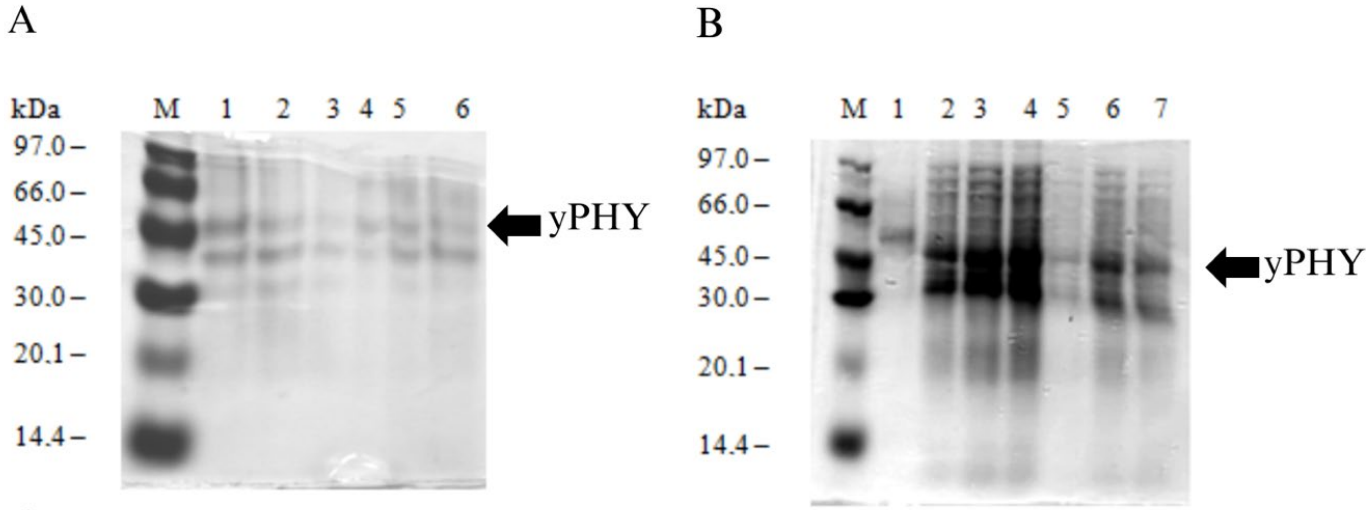

Figure 5. Electrophoresis gel (12%), run at 100-150 V. (A) The precipitate fraction from the lysed cell sample. M, low-molecular-weight standard; 1, 2, and 3, 100% concentrated lysate at passages 1, 3, and 5 through the homogenizer, respectively; 4, 5, and 6, lysate 2-fold dilution (50%) of the concentrated lysate at passages 1, 3, and 5 through the homogenizer, respectively. (B) The supernatant fraction from the lysed cell sample. M, low-molecular-weight standard; 1, phosmor TT phytase 10% (w/v); 2, 3, and 4, concentrated lysate at passages 1, 3, and 5 through the homogenizer, respectively; 5, 6, and 7, lysate 2-fold dilution (50%) of the concentrated lysate at passages 1, 3, and 5 through the homogenizer, respectively.

Figures 5A and 5B shows the presence of phytase from *Y. intermedia*, between 50 and 47 kDa. From the electrophoretic profile, it was observed that the phytase content in supernatant samples was higher than that in the precipitate samples (Figure 6). This suggests that phytase was expressed in greater quantity in its biologically active form.

The soluble fraction of phytase was predominant in the supernatant, due to its low density. The insoluble fraction, IBs, tended to be deposited predominantly in the precipitate. The precipitated fraction, despite the reduced phytase content, presented greater purity in samples 1 and 2 than the supernatant fraction. This indicates that the IB sample with greater purity brings cost reduction in the process by the elimination of purification steps.

At an operating pressure of 103.0 MPa, higher phytase content was achieved in the supernatant fraction in five passages. According to the homogenizer manual, one pass through the homogenizer at 103.0–117.0 MPa has been shown to rupture almost all of the cells in a batch of *E. coli*.

For the 100% supernatant and precipitate samples, cell fragmentation led to an increase and decrease proportional to the number of passages, respectively. While for the 50% precipitate samples, there was an increase proportional to the number of passages, for those with 50% supernatant samples, there was no pattern. This highlights the influence of only the feed concentration factor on the phytase concentration obtained after lysis for the supernatant. For the supernatant fraction, the optimal phytase concentration was in sample 3, with the highest phytase concentration obtained in the 100% lysed concentrated sample. For the precipitate fraction, the optimal phytase concentration was in sample 6, with five passages in 50% of concentrated samples.

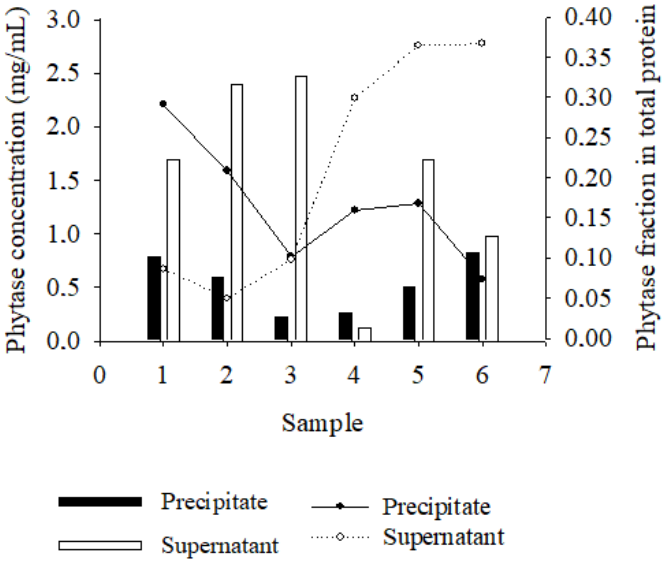

Figure 6. Phytase concentration (mg/mL) and phytase fraction (0-1.0) in supernatant and precipitate samples obtained by ImageJ from electrophoresis gels. 1, 2, and 3, 100% concentrated lysate from passages 1, 3, and 5, respectively; 4, 5, and 6, lysate 2-fold dilution (50%) of the concentrated lysate from passages 1, 3, and 5 passages, respectively.

3.4 Potential of phytase as an enzyme additive and the advantages of producing active IBs

With maximum recoveries of 37% and 29% of phytase in the supernatant and precipitate fractions of the cell lysate, *E. coli* demonstrated great potential for protein expression at a high cell density. Previous studies by the research group at the Universidade Federal de São João Del-Rei (UFSJ) on the enzyme yPHY (INPI accession number BR1020140168796) (Vieira et al., 2019) indicate the potential of the enzyme as an enzyme additive (de Souza et al., 2018).

The thermostability between 37 and 40°C is adequate to sustain phytase activity at the body temperature of chickens (41–42°C) (Nezhad et al., 2020). Protease resistance to trypsin (pH 7.5) and pepsin (pH 2.0), at a protease/phytase ratio of 0.02, retained 55% activity at 37°C over 2 h, with activity levels of 5,333 and 7,466 U/mL in the absence and presence of Cu^{2+} ions, respectively. When assessing the protease resistance of *Aspergillus niger* phytase (Natuphos, BASF) at pH 2.0, 37°C for 1 h at 2,000 U, 40–50% of activity was retained (Greiner & Konietzny, 2012).

In order to reach the quality standards provided by the Normative Instruction 13/2004 approved by Ministry of Agriculture and Livestock (Brasil, 2004), according to Law 6,198 of 1974, as well as the U.S. Food and Drug Administration (FDA) which regulates enzyme preparations in the USA (FDA, 2010) and the European Commission which implements an integrated Food Safety Policy in the European Union (EU) (European Commission, 2002), the purification of recombinant proteins, a downstream step after microbial fermentation, is essential.

The results obtained from the characterization of the yPHY phytase regarding the enzymatic activity after purification on the Ni-NTA affinity resin demonstrate the potential of the histidine tag added to the original sequence of the wild-type APPA enzyme. After elution with an elution buffer containing 500 mM imidazole, the protein recovery was 53.6%, with a specific activity of 83.33 U yPHY/mg, 8.45 times greater than the specific activity of the crude lysed extract (Vieira et al., 2019).

The purification of the soluble fraction of the lysed extract demonstrated that the production of the enzyme in its active form is possible (Vieira et al., 2019). Furthermore, the insoluble fraction has shown to be a promising alternative for the production of active enzymes with high purity.

The formation of physiologically active IBs is possible under the conditions of a non-regulated pH during growth, cultivation at 37°C, and expression at 15°C. The temperature shift benefits the optimal yield of active IBs. Furthermore, a high concentration of an inducer, usually isopropyl β-D-1 thiogalactopyranoside (IPTG), increases the overall production of active IBs. Recently, a novel strategy for creating IBs has been introduced through the fusion of target proteins with aggregation-supporting short peptide tags or domains. These pull-down domains ensure that the protein is purposefully folded into the form of IBs (Belkova et al., 2022; Castellanos-Mendoza et al., 2014; Lamm et al., 2020).

The purification of these protein aggregates is facilitated by their high density, enabling straightforward isolation from

host cells through centrifugation. This characteristic represents a primary advantage of IBs. However, it is necessary to keep in mind that the isolation process should neither disrupt IBs' structure nor denature proteins trapped inside IBs through cell lysis methods (Belkova et al., 2022). Thus, the production of ncIBs from bacterial cells is a promising strategy since it withstands high mechanical lysis pressures and achieves high purity with simple purification steps. The highly cross-linked spherical agarose, 6% matrix, is capable of retaining, by the IMAC, the protein of interest from an unclarified lysate sample without pre--centrifugation and filtration, according to the General Electric Company manual, HisTrap FF crude.

4 CONCLUSION

The application of an enzymatic additive in monogastric feed formulations reduces costs and environmental pollution associated with inorganic P.

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