Harnessing alkaline-pH regulatable promoters for efficient methanol-free expression of enzymes of industrial interest in *Komagataella phaffii*

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ABSTRACT

Background The yeast *Komagataella phaffii* has become a very popular host for heterologous protein expression, very often based on the use of the *AOX1* promoter, which becomes activated when cells are grown with methanol as a carbon source. However, the use of methanol in industrial settings is not devoid of problems, and therefore, the search for alternative expression methods has become a priority in the last few years.

Results We recently reported that moderate alkalinization of the medium triggers a fast and wide transcriptional response in *K. phaffii*. Here, we present the utilization of three alkaline pH-responsive promoters (*TSA1*, *HSP12* and *PHO89*) to drive the expression of a secreted phytase enzyme by simply shifting the pH of the medium to 8.0. These promoters offer a wide range of strengths, and the production of phytase could be modulated by adjusting the pH to specific values. The *TSA1* and *PHO89* promoters offered exquisite regulation, with virtually no enzyme production at acidic pH, and limitation of Pi in the medium further potentiated alkaline pH-driven phytase expression from the *PHO89* promoter. An evolved strain based on this promoter was able to produce twice as much phytase as the reference *AOX1*-based strain. Functional mapping of the *TSA1* and *HSP12* promoters suggests that both contain at least two alkaline pH-sensitive regulatory regions.

Conclusions Our work shows that the use of alkaline pH-regulatable promoters could be a useful alternative to methanol-based expression systems, offering advantages in terms of simplicity, safety and economy.

Keywords: heterologous protein expression; methanol-free bioprocesses; phytase production, alkaline stress, promoter mapping, *PHO89*; *HSP12*; *TSA1*; *Komagataella phaffii* (*Pichia pastoris*)

INTRODUCTION

Heterologous expression in diverse hosts is an important source for protein production with very diverse uses, from food and beverages to pharmaceuticals. Among the possible hosts, the yeast *Pichia pastoris* (renamed *Komagataella phaffii*) is widely used for heterologous protein production [1,2]. The popularity of this organism derives from several advantageous features, such as high cell density growth in defined media and the ability to perform eukaryotic posttranslational modifications and to release target products extracellularly. In addition, a wide range of genetic modification tools, including genome editing toolkits (CRISPR/Cas9 system), are available for this organism [3].

Expression in *K. phaffii* can be driven from both constitutive and inducible promoters [4]. Examples of the first class are the strong *GAP* and *TEF1* promoters and the somewhat weaker *PGK1*. By far, the most widely employed inducible promoter is that of the *AOX1* gene, which is responsible for most of the alcohol oxidase activity in the cell and allows this organism to grow on methanol as a carbon source. The *AOX1* promoter (p*AOX1*) is induced by methanol and is repressed by carbon sources such as glucose, ethanol, glycerol or acetate [5,6]. The acceptance of this promoter is based on its strength, which allows the production of some secreted recombinant proteins at >15 g/L yield and tight regulation. However, the need to grow on methanol represents a significant drawback because 1) methanol is a hazardous and explosive material that needs special handling, posing a problem in larger-scale industrial processes; 2) it is highly toxic and not suitable for producing certain edible and medical products; 3) its metabolism produces hydrogen peroxide as a byproduct, leading to oxidative stress, which may result in the degradation of recombinant proteins [7–9]; and 4) the use of methanol requires a higher oxygen supply and heat removal than other carbon sources [10,11], thus increasing production costs and making scaling-up difficult. The limitation associated with the use of the *AOX1* promoter has prompted the search for alternative, methanol-free expression systems in *K. phaffii*. Diverse novel regulated promoters have been proposed that do not require the use of methanol, such as p*THI11* (see [12] for references), but thus far, none of them has been able to replace the *AOX1* promoter in industrial settings.

Most yeasts, including *K. phaffii*, grow preferentially at acidic pH, and shifting the pH of the medium to moderate alkalinization (pH 7.8-8.2) results in an adaptive response that is mainly mediated by the rewiring of gene expression. Such a response has been characterized in detail in diverse species, such as *Saccharomyces cerevisiae*, *Candida albicans*, and *Aspergillus nidulans* [13–17]. We have recently reported short-term changes in the transcriptional landscape of *K. phaffii* in response to moderate alkalinization of the medium [18]. In this report, we identified several genes that were induced in cells shifted to

pH 8.0 at a level comparable to that of strong constitutive genes such as *TEF1* or *GAP1*. We then conceived the idea that such promoters could be an advantageous alternative to *AOX1*-based expression systems because they would allow the culture of cells on any desired nontoxic carbon source, thus eliminating safety and regulatory issues. Here, we present the use and characterization of three different alkali-regulatable promoters (p*PHO89*, p*HSP12* and p*TSA1*) for the expression of an *E. coli* derived phytase enzyme. This enzyme was selected because, among other interesting characteristics, it represents one of the largest enzyme segments in the feed industry [19]. We show here that these promoters offer a useful range of strength and, in some cases, could compete with p*AOX1* in terms of potency and fast induction.

MATERIALS AND METHODS

Yeast strains and growth conditions

K. phaffii X-33 and the new strains generated in this work were grown at 28 °C with continuous shaking (230 rpm/min) in YP medium (10 g/L yeast extract, 20 g/L peptone) supplemented with 20 g/L glycerol (YPGly) or glucose (YPD). YPD plates were supplemented with 200 µg/ml hygromycin B when selection of transformants was needed. The *AOX1*-based phytase-producing strain (appaT75, CGMCC 12056) was usually grown in YPGly. Low phosphate (low-Pi) medium was prepared by phosphate precipitation from YP as previously described [20]. The estimated Pi concentration in this medium was 0.7-0.8 mM.

Recombinant DNA techniques

The *Escherichia coli* strain DH5α was used as a plasmid host and grown at 37 °C in LB medium supplemented with hygromycin B (100 µg/ml) when needed for plasmid selection. *E. coli* transformation and standard recombinant DNA techniques were performed as described [21]. Transformation of yeast cells by electroporation was carried out as in [22] with a Gene Pulser electroporator (Bio-Rad).

Construction of gene reporters and phytase producer strains

The *TSA1*-GFP reporter used in this work was made as follows. The *TSA1* promoter was amplified by PCR from genomic DNA obtained from the X-33 strain using Q5 polymerase (New England Biolabs) and primers TSA1_BstBI_Fw and TSA1-GFP_Rv_KpnI. The amplified genomic region (-765/+13, from the initiating ATG codon) was digested with BstBI and KpnI and subsequently ligated into the same sites of plasmid pAHYB-GFP [23]. The *PHO89*-GFP reporter was described in [18].

For the generation of phytase constructs, we used a two-step PCR strategy. First, we amplified the α-factor secretion signal together with the Phytase ORF from the genome of the appaT75 strain. Second, we amplified the *PHO89* (-772/-1), *HSP12* (-749/-1) and *TSA1* (-765/-1) promoters from genomic DNA of the X-33 strain as described above. All these amplicons were designed so that they contained a 3'-region that hybridized with the first 24 nucleotides of the α-factor secretion signal. Finally, each promoter was fused to the phytase ORF by overlapping PCR, and the final product was digested with the restriction enzymes KpnI and NotI. The final products were then cloned at the same sites of the pAHYB-GFP plasmid, replacing the *GFP* reporter. This process was repeated to generate several versions of *HSP12*- and *TSA1*-Phytase constructs, with shortened or extended versions of the promoter region used. Constructs were then used to transform *E. coli* cells, and positive clones were selected from LB plates supplemented with hygromycin B. Positive clones were subjected to DNA

121 sequencing of the inserts to verify the absence of unwanted mutations and subsequently
122 linearized by PmeI digestion and introduced into X-33 cells by electroporation to promote
123 integration into the *AOX1* promoter locus. Positive X-33 transformants were selected on YPD
124 plates containing 200 µg/ml hygromycin B. The correct insertion of the constructs was
125 confirmed by PCR. The oligonucleotides used in this work are documented in Additional File
126 1.
127
128 Posttransformational vector amplification (PTVA)
129 The PTVA strategy was used to increase the copy number of the *PHO89*-Phytase construct.
130 To this end, we grew X-33 *PHO89*-Phytase cells with increasing concentrations of hygromycin
131 B (from 200 to 4000 µg/ml) as described in [23]. However, instead of using agar plates, we
132 adapted the procedure shown in [24], and PTVA was carried out in liquid cultures, increasing
133 the concentration of hygromycin B in the medium every 48 hours.
134
135 GFP reporter assays
136 For the induction of GFP expression by alkaline stress, cells were grown overnight on YPGly
137 in duplicate, and then cultures were diluted in 20 mL of fresh medium at OD_{600} = 0.5 in baffled
138 flasks (250 ml). Growth was resumed for 16 hours, with the cultures reaching an OD_{600} of
139 approximately 20-25. At this point, cultures were centrifuged for 5 min at 1000 xg, and
140 supernatants were discarded. One set of cultures was then resuspended in the same volume
141 of YPGly supplemented with 50 mM TAPS adjusted to pH 8.0 to induce the expression of GFP.
142 In parallel, the second set was grown in YPGly 50 mM TAPS at pH 5.5 as a noninduced control.
143 Immediately after resuspending the cells, 1 mL samples were taken (t = 0 h) and fixed with
144 formaldehyde as described in [18]. Subsequent samples were taken and fixed at 4, 8, 24, 32
145 and 48 hours after induction. During cell growth, 1% glycerol (final concentration) was added
146 to the cultures at 8, 24 and 32 hours. In addition, after 24 h of growth, nutrients other than the
147 carbon source were refreshed by the incorporation of 2 mL of 2x concentrated YP medium.
148 The pH of the alkali-stressed cultures was measured after every sampling using a Crison
149 GLP21 pH meter and readjusted to 8.0 (when indicated) by the addition of the appropriate
150 volume of a concentrated KOH solution. The fluorescence of the samples was analyzed in a
151 CytoFLEX (Beckman Coulter) flow cytometer using the FIT-C filter (525/40).
152
153 Phytase expression induction and sample collection
154 For the induction of phytase expression by alkaline stress, X33 cells carrying *PHO89*-, *HSP12*-
155 or *TSA1*-based phytase constructs were grown overnight on YPGly or YPD as above, and then
156 cultures were diluted in 25 mL of fresh medium at OD_{600} = 0.5 in baffled flasks (250 ml). Growth
157 was resumed for 16 hours, with the cultures reaching an OD_{600} of approximately 20-30. At this

point, cultures were centrifuged (5 min, 1000 xg) and resuspended in the same volume of YPD or YPGly supplemented with 50 mM TAPS adjusted to pH 8.0 (unless otherwise indicated) or in noninducing media (see above) as a control.

For *AOX1* promoter-controlled phytase expression, the appaT75 strain was grown overnight on YPGly medium, and then cultures were diluted in 25 mL of fresh YPGly medium at OD_{600} = 0.5. Cultures were grown and processed as above, except that they were resuspended in fresh YP supplemented with 1% methanol. One ml aliquots were taken and centrifuged (3 min, 13000 xg) immediately after cell resuspension in fresh media (t = 0 h). The supernatants were stored at -20 °C for further analysis. This process was repeated at 8, 24, 48, 72, 96 and 144 hours for most assays. The pH of the alkali-stressed cultures was measured and readjusted to 8.0 (or the indicated pH) as above after each sampling. At these same time points, the carbon source, either glucose, glycerol or methanol (depending on the culture), was newly added to a 1% final concentration. Every 24 h, 2 mL of 2x concentrated YP media was also added to the cultures to refresh the nutrients.

For those experiments involving strains designed for functional mapping of the *HSP12* and *TSA1* promoter regions, multiple colonies from each transformation were grown overnight on YPGly until saturation. From this point, cultures were diluted in 5 mL of YPGly 50 mM TAPS adjusted to pH 8.0 (plus an aliquot in noninducing media) at OD_{600} = 0.2, and growth resumed. During these tests, neither nutrients nor carbon sources were refreshed, and the pH of the induced cultures was not readjusted to 8.0. For *TSA1*- and *HSP12*-based constructs, cells were grown for 48 and 72 hours, respectively. Then, 1 mL aliquots were centrifuged (3 min, 13000 xg), and supernatants were analyzed for phytase activity.

Phytase activity assay

Determination of phytase activity was performed essentially as described in the ISO 30024:2009 protocol (https://www.iso.org/standard/45787.html) but scaling down the final volume to 0.4 ml. Briefly, the appropriate dilutions of the fermentation medium were incubated for 30 min at 37 °C in the presence of 5 mM sodium phytate (Sigma–Aldrich, #68388, inorganic phosphorus <0.1%). The released phosphate was determined by reaction with a mixture of ammonium heptamolybdate and ammonium vanadate reagents plus nitric acid and measuring the A_{405} in a Multiskan Ascent plate reader (Thermo). Phytase activity (U/ml) was calculated using the slope of a previously generated standard curve. One unit is defined as the amount of enzyme that releases 1 µmol of inorganic phosphate from phytate per minute at 37 °C.

Other techniques

For SDS-polyacrylamide gel electrophoresis analysis, a volume of the phytase-containing supernatants was mixed with 4x SDS–PAGE loading buffer (200 mM Tris–HCl pH 6.8, 8% sodium dodecyl sulfate (SDS), 0.4% bromophenol blue, 40% glycerol and 20% β-mercaptoethanol) so that the final concentration was 1x. Subsequently, the mixture was heated at 95 °C for 5 min, and 5 μL of the mixture (equivalent to 3.75 μL of medium supernatant) was resolved by SDS–PAGE at a concentration of 10% acrylamide/bis-acrylamide (37.5:1 ratio). Gels were stained using a Coomassie Blue solution (10% acetic acid, 25% isopropanol and 0.25% Coomassie Brilliant Blue R250, AMRESCO).

Identification of putative transcription factor-binding sites in the promoters of *HSP12* and *TSA1* was performed with the matrix-scan tool from the (RSAT) server [25] using the JASPAR core nonredundant fungi collection [26] and a *p* value threshold of 10^{-4}.

Statistical significance was calculated by the unpaired Student's t test.

207 **RESULTS**

208 Long-term expression of GFP from the *PHO89* and *TSA1* promoters in response to
209 alkalinization.

210 The gene *PHO89* (PAS_chr2-1_0235) encodes a high-affinity Na+/Pi plasma-
211 membrane transporter needed to adapt to situations of phosphate scarcity. Our previous
212 work with the PHO89-GFP reporter [18] was restricted to rather short time-course
213 experiments (4-6 h after induction by alkalinization). We considered it necessary to test
214 whether the strong induction observed could be maintained or even increased during
215 prolonged cultures. To this end, we initiated the cultures as described in the Materials and
216 Methods and established two sets of alkali-induced cultures. The first one was induced at
217 time zero, and growth was continued without further modification of the pH (reaching a value
218 of 6.2 at the end of the experiment). In contrast, in the second set, pH was monitored at
219 different times, and pH 8.0 was restored by the addition of potassium hydroxide (Figure 1A).
220 As can be observed, GFP production in the first set increased steadily until 24 h after
221 induction and then decreased gradually. In contrast, maintenance of alkaline stimulation
222 resulted in a continuous increase in the GFP content, reaching a difference of approximately
223 1.8-fold at the end of the experiment when compared with cells for which stimulation was not
224 sustained over time. We then tested the response of the *TSA1*-based reporter restoring pH
225 8.0 as for *PHO89-GFP*. The *K. phaffii* gene *TSA1* (PAS_chr2-2_0220) encodes a thioredoxin
226 peroxidase known to be involved in oxidative stress responses. As shown in Figure 1B,
227 substantial GFP production was already detected 4 h after induction, and GFP levels
228 increased throughout the entire experiment, reaching values approximately 25% higher than
229 those detected for the *PHO89* promoter. Thus, recursive maintenance of alkaline pH in the
230 medium greatly improved GFP production driven by the tested alkaline pH-sensitive
231 promoters.

232

233 Expression of phytase from the *TSA1* promoter

234 The observation that maintenance of the stimulus was beneficial for protein
235 production and that the ratio of detected GFP in induced *vs* noninduced cells with each
236 promoter was very favorable (over 80-fold increase) prompted us to test the ability of these
237 promoters, plus an additional alkaline-pH sensitive promoter (*HSP12*), to drive efficient
238 expression of an industrially relevant enzyme, such as phytase. In our previous work
239 analyzing the short-term response upon alkalinization, we found that *TSA1* mRNA levels
240 could match those of the powerful constitutive *GAP* promoter, particularly in cells grown on
241 glucose [18]. Therefore, we constructed strains expressing, under the control of the *TSA1*
242 promoter, a phytase form that was engineered to be secreted into the medium and exposed

243 these cells to pH 8.0 using either glycerol or glucose as a carbon source. As shown in Figure
244 2A (for glycerol) and Supp. Fig. 1 (for glucose), phytase activity could be detected 8 h after
245 alkalinization for all three clones selected. However, in contrast with what could be expected
246 from the short-term transcriptomic data, the amount of secreted activity was higher
247 (approximately 1.5-fold) for cells grown on glycerol. In any case, the activity produced from
248 the *TSA1* promoter was clearly lower (approximately 25%) than that obtained from the
249 reference *AOX1* strain growing on methanol. We then investigated the sensitivity of the
250 promoter to different levels of stress. To this end, we carried out cultures as in Figure 2A, but
251 using a range of alkalinization from slight (pH 7.4) to moderate (pH 8.2). As shown in Figure
252 2B, even the lower pH was able to trigger a noticeable production of phytase, albeit the
253 response was somewhat delayed (compare activities at t=8 h). The responses at pH 7.6 and
254 7.8 were rather similar and somewhat higher than at pH 7.4, and they were further increased
255 at the highest pH values tested. These results suggest that modulation of the stress signal
256 may be used as a tool for the regulation of this promoter.
257 Remarkably, the production of phytase was virtually null in cells grown at acidic pH,
258 suggesting that alkalinization led to positive inputs to the *TSA1* promoter or/and released it
259 from repressive activities. To gain further information, we prepared constructs expressing
260 phytase from different versions of the promoter progressively shortened from the 5' end
261 (Figure 3A). As shown in Figure 3B, removal of a region spanning from -765 to -459 resulted
262 in a strong decrease in the response to alkaline pH, which was reduced approximately 4-fold,
263 and this behavior was not modified by further deletions of the promoter (to note that the
264 experimental conditions of this experiment are very different from those employed for Figure
265 2A, leading to a lower phytase production). This result suggested that the functional region
266 relevant for the alkaline pH of the *TSA1* promoter could be restricted to a region of nearly
267 300 nt spanning from positions -765 to -459. To further refine the analysis, we created four
268 additional deletions (a to d) covering this 300-bp region and generated the corresponding
269 strains. As shown in Figure 3C, the response to alkaline pH for all four strains was reduced to
270 roughly one-half, and at the same time, the phytase activity released by unstimulated
271 cultures became noticeable, suggesting some derepression of the promoter. As a result, the
272 induced/noninduced ratio, which was approximately 200-fold for TSA1-1, decreased to 2- to
273 2.5-fold for these four newly constructed strains. Interestingly, additional removal of the
274 region spanning from -527 to -458 (yielding TSA1-2) not only further decreased the amount
275 of secreted phytase activity in alkali pH-stimulated cells but also abolished the production of
276 phytase in unstimulated cultures. These results suggest that the upstream region of the
277 *TSA1* promoter might contain two independent regulatory components sensitive to
278 alkalinization.
279

280 Alkaline induction yields high levels of p*HSP12*-driven phytase activity.

281 *HSP12* (PAS_chr4_0627) encodes a small heat shock protein that is known to
282 respond to diverse stresses. Figure 4A shows the time course of phytase production for three
283 independent clones expressing phytase from a version of the *HSP12* promoter starting at
284 position -749 relative to the initiating Met (denoted by a red inverted triangle in Figure 4B).
285 Alkalinization resulted in the rapid release of phytase activity, which reached a maximum at
286 the end of the experiment. It is worth noting that, in this case and for some time-points (48
287 and 72 h), phytase accumulation in the medium was similar to that of the commercial appT75
288 strain growing on YP plus methanol. At the end of the experiment, the accumulation of
289 phytase from the *HSP12* promoter was approximately 65% of that obtained from the AOX1-
290 based strain. However, we observed that the uninduced cells also produced detectable
291 phytase activity (see discussion). Consequently, the induced/noninduced ratio was relatively
292 low (3- to 4-fold, depending on the sampling time).

293 We wanted to identify possible regions in the *HSP12* promoter that could be sensitive
294 to alkalinization. To this end, we prepared five different versions of the promoter, starting from
295 position -1000 (that is, extending approximately 250 nucleotides upstream in comparison
296 with the promoter present in the strain described above). As shown in Figure 4B, the largest
297 region (H1) provided an induced/noninduced activity ratio of 2.7, quite close to that observed
298 for the *HSP12*-749 version (note that culture conditions differ from those of Figure 4A).
299 Deletion of the upstream 400 nucleotides (H2) resulted in a noticeable decrease in the
300 induced/noninduced activity ratio, albeit it was not considered statistically significant (*p* value
301 0.08). Further reduction to positions -460 or -330 (H3 and H4) yielded a similar (and
302 significant) value (≈ 2.0). Finally, when only the upstream 160 nt were included (H5), we
303 observed a further decrease in the activity ratio (1.5). Notably, this last version still provided
304 robust levels of noninduced activity. From these results, it can be hypothesized that at least
305 two independent regions sensitive to alkalinization exist in the *HSP12* promoter.

306

307 Effective expression of phytase from the *PHO89* promoter

308 We prepared a construct in which a promoter region of the same size used for GFP
309 expression was hooked to the phytase ORF and integrated into the yeast genome. As shown
310 in Figure 5A, for three different clones, alkalinization allowed the accumulation of phytase
311 activity to values similar to those attained with the *HSP12* promoter, reaching levels of
312 approximately 50% in comparison with the reference *AOX1* strain. However, in this case, the
313 activity in the control cultures was virtually undetectable, yielding an induced/noninduced
314 activity ratio of > 1000-fold. Previous work using the GFP reporter suggested that the *PHO89*
315 promoter could be stimulated effectively at pH values lower than 8.0 [18]. Therefore, we
316 carried out a dose-dependence study using one of the clones characterized above (clone

P89-J) and tested the production of phytase in a range of pH values from 7.4 to 8.2. As observed from Figure 5B, even the lowest pH tested was able to substantially induce the expression of phytase. Production was increased in cells cultured at pH 7.6 (≈ 1.8-fold at 72 h), although a further rise in the intensity of the stimulus was not reflected in higher enzyme production.

The exquisite response of the *PHO89* promoter to alkaline pH prompted us to attempt to improve phytase production by means of the posttransformational vector amplification approach (see Material and Methods). We started with clone P89-J, and at the end of the process, we obtained dozens of clones able to proliferate in the presence of 4000 µg/ml hygromycin (20-fold higher than that used for initial clone selection). After screening for phytase production, we selected three evolved strains (J5, J6 and J7) for additional time-course experiments. As presented in Figure 6A and B, all clones exhibited higher production than the parental P89-J strain (from 1.6 to 3-fold) at all times tested. The improved production of the evolved strain could also be assessed by SDS–PAGE analysis of the production media (Supp. Fig. 2), which also shows that phytase is virtually the only protein detected in the supernatant of the cultures. Importantly, the amount of phytase produced was consistently higher for the pH-regulated strains than for the *AOX1*-based reference strain, with a maximum increase ranging from 2 to 2.5-fold after 72 h of culture (Figure 6B). This difference could be confirmed by SDS–PAGE analysis. Figure 6C compares the production of phytase from the reference *AOX1*-based strain and the evolved P89-J6. The amount of protein correlated well with the measured activity. Scanning of the gels and integration of the phytase signals in comparison with known amounts of BSA suggests that the maximal production phytase in the *AOX1*-based strain was 0.59 mg/ml, whereas in the evolved J-6 strains it was 1.32 mg/ml, indicating a specific activity of~50000 U/mg of protein.

<u>Phosphate limitation improves alkaline pH-triggered expression from the *PHO89* promoter.</u>

PHO89 encodes a putative Na+/Pi high affinity transporter and as expected, a previous report demonstrated that the *PHO89* promoter positively responds to limitation of Pi availability [27]. We wanted to test if a moderate limitation in Pi could reinforce the response of the *PHO89* promoter to alkalinization. To this end, inorganic phosphate was removed from YP medium by precipitation to decrease its level to about 5-fold (0.7-0.8 mM). The time-course experiment shown in Figure 7 demonstrates, using clone P89-J, that partial limitation of Pi in the medium strongly potentiates (nearly 3-fold) the production of phytase, likely by increasing transcription from the promoter. This result is interesting because it illustrates the possibility of modulating alkaline pH response, in a promoter-specific fashion, through judicious manipulation of the culture medium.

DISCUSSION

We present here three promoters that are plausible, methanol-free alternatives for expression in *K. phaffii* of proteins of industrial interest and that offer a wide range of responses. These promoters were first tested for GFP production and then employed to produce a secreted form of a modified *E. coli* phytase. The choice of phytase as a test enzyme was based on its industrial importance: it is widely used as an additive to monogastric animal feed to improve inorganic phosphate absorption and reduce environmental pollution [28] and its commercial impact since its market value was estimated at USD 517.36 million in 2022 and is growing steadily [29].

Two of these promoters, *HSP12* and *PHO89*, provided expression levels close to or even higher (in the evolved strains) than those of an *AOX1*-based strain, while *TSA1* produced phytase at lower levels (approximately 25%). We show that both *PHO89* and *TSA1* offer exquisite regulation, with undetectable phytase production when cells grow at acidic pH. This might be an invaluable tool when proteins to be expressed could be detrimental to the cell. It is worth noting that in the case of *TSA1* (and to a lesser extent in *PHO89)*, it was possible to somewhat modulate the level of phytase expression by adjusting the medium to the appropriate pH (Figures 2B and 5B). This sensitivity to the inducing stimulus provides a way to control the time course of product accumulation. The *PHO89* promoter was proposed earlier as a possible alternative for protein expression, first for the expression of a lipase [27] and later to produce bacterial and fungal phytases [30]. However, in both cases, regulation of the promoter was achieved by using conditions of phosphate starvation, which negatively affect growth. In contrast, we did not observe detrimental effects on growth (measured as OD_{600}) in cultures readjusted at pH 8.0 at any of the sampling times (not shown). Comparison of our results and those provided in reference [30] indicates that, even considering that the enzyme assays are not identical, our production of phytase activity exceeds that obtained by phosphate limitation by nearly two orders of magnitude. In addition, we observe that moderate limitation of Pi in the medium clearly potentiates alkaline pH-driven phytase production without detectable impact on cell growth. In the case of *HSP12*, phytase is also expressed (albeit at lower levels) at acidic pH. This is likely because, in our experimental setting, cells might suffer nutrient limitation for some periods, and it is known that the *HSP12* promoter is induced by glucose starvation in *S. cerevisiae* [31] and is highly and transiently induced upon carbon source limitation in *K. phaffii* [32].

The signaling pathways leading to the regulation of gene expression have been explored in detail in some fungi, such as *S. cerevisiae*, *C. albicans*, *A. nidulans*, and *C. neoformans*. These works have revealed the involvement of several protein kinases [33–39]

389 and transcription factors, such as the conserved PacC/Rim101 [38,40–42], Crz1/CrzA, related
390 to calcium signaling [41,43–47], Pho4, involved in response to phosphate starvation [44,48],
391 Yap1, connected with oxidative stress response [43,49], and likely Mac1 and Aft1, implicated
392 in adaptation to iron/copper scarcity [50,51]. In contrast, virtually nothing is known for *K.*
393 *phaffii*, except our recent report that alkali-dependent expression from the *K. phaffii PHO89*
394 promoter was largely dependent on two canonical CDREs, thus strongly suggesting that this
395 response is mediated by the calcineurin/Crz1 pathway [18], as was found earlier for diverse
396 fungi [41,52,53]. In this regard, the response of *PHO89* to alkalinization would be
397 mechanistically independent from that of phosphate limitation, which is likely mediated by
398 putative Pho4-binding sites located at positions -583 and -414 of the *PHO89* promoter [27].
399 This notion is reinforced by our observation that the combination of slight limitation of Pi in
400 the medium, unable by itself to trigger phytase production, and alkalinization strongly
401 potentiates the amount of enzyme recovered from the medium. Such potentiation suggests
402 that the *PHO89* promoter is integrating two independent signaling pathways, and highlights
403 the importance of elucidating the signals elicited by alkalinization and the molecular events
404 occurring downstream.
405 We also show here a functional mapping of *TSA1* and provide evidence that elements
406 present in two regions, -765/-698 and -527/-458, are important for the alkaline pH response.
407 It is worth noting that none of the stress-responsive transcription factor binding sequences
408 (Rox1, Yap1/6, Aft2) that we could predict to be involved in the alkaline response are located
409 within these regions. The upstream one includes a putative Arr1 binding element (*p* value
410 $1.7E^{-5}$) at position -723/-716. Arr1 (also known as Yap8) is a transcriptional activator needed
411 for the transcription of genes involved in resistance to arsenic compounds [54]. However, as
412 far as we know, Arr1 has not been previously connected to the response to alkalinization.
413 Our data also show that some responses persist when the promoter is trimmed up to position
414 -113. We identified a Met31 binding site (*p* value < $3.6E^{-6}$). Met31 is involved in activating
415 genes needed for the incorporation of inorganic sulfate and the synthesis of sulfur-containing
416 amino acids. Remarkably, we recently reported that in *K. phaffii,* alkalinization is
417 characterized by a fast induction of genes involved in sulfate assimilation and Met and Cys
418 biosynthesis [18]. This would support a possible role of Met31 in the alkaline response of the
419 *TSA1* promoter.
420 The study of the *HSP12* promoter was carried out by generating five different
421 fragments, from position -1000 to -160. We did not trim the promoter further because a
422 recent report showed that a region starting at nt -97 had no activity at all [32]. Our data
423 suggest that this promoter contains two regions sensitive to alkalinization. The first one would
424 lie between nt -1000 and -460 (or possibly -600). This region contains an Adr1-binding

425 sequence ($p<1.5E^{-5}$), which could be recognized by Mxr1, a proposed homolog in *K. phaffii*
426 for yeast Adr1 [55,56]. On the other hand, our results discard the relevance of Rap1, a
427 multifunctional and conserved transcription factor related to oxidative stress conditions in
428 humans and yeast [57,58] that has been identified as important for the expression of catalase
429 (*CAT1*) in *K. phaffii* [59] or Aft1, which in *K. phaffii* has been related to carbohydrate and not
430 to iron metabolism [60]. In contrast, the region between -330 and -160 appears relevant for
431 alkaline induction. This segment contains a putative site for the Com2 transcription factor (*p*
432 value $3.5E^{-6}$). This factor has been found in *S. cerevisiae* to be necessary for the expression
433 of genes involved in sulfur assimilation (also observed in *K. phaffii* upon alkalinization, see
434 above) when cells are exposed to sulfur dioxide [61]. It must be noted, however, that the
435 sequence recognized by this factor includes a canonical stress-responsive element (STRE,
436 5'-CCCCT-3'), which is recognized by Msn2/Msn4, and that there is recent evidence for the
437 involvement of these factors in the regulation of *HSP12* in *S. cerevisiae* [62]. In any case, it
438 would be most desirable to investigate the specific signaling pathways governing the
439 transcriptional response to alkalinization in *K. phaffii*.
440 The use of alkaline pH-regulatable promoters would allow, in principle, the utilization
441 of any carbon source for cultivation of yeast cells. This does not imply that the carbon source
442 would be irrelevant since it might affect the potency of the response, as we show here for the
443 case of the *TSA1* promoter. It is evident that the specific growth conditions for optimal
444 production must be set for each specific promoter and produced protein. In any case, these
445 promoters avoid the use of methanol and hence circumvent the associated problems, such
446 as flammability, toxicity (either by ingestion, inhalation, or absorption through the skin), as
447 well as the requirement for high oxygen supply and concomitant heat production. In contrast,
448 in the expression systems presented here, induction of the desired protein can be achieved
449 without any modification of the medium, simply by the addition of the appropriate amounts of
450 KOH, possibly one of the cheapest chemicals. Potassium hydroxide would be preferable to
451 sodium hydroxide to avoid the accumulation of sodium cations, which are more toxic than
452 potassium [63], in the culture medium. In addition, pH is a very easy parameter to follow in a
453 bioreactor. On-line monitoring can be coupled to the addition of KOH to counteract possible
454 acidification caused by fermentation, thus maintaining the appropriate pH profile for the
455 desired period. Finally, while alkaline-pH regulatable promoters could be used, in principle, to
456 express any protein (preliminary work in our laboratory suggests that it is also suitable for the
457 production of the CRL1 lipase from *Candida rugosa*), the induction conditions would be
458 particularly suitable for secretion of proteins that are unstable at the acidic pH found in
459 standard yeast fermentations, such as methionine adenosyltransferase [64] or the fungal
460 Fsl2 lipase [65].

ADDITIONAL MATERIAL

Additional file 1 (xlsx) Oligonucleotides used in this work.

Additional file 2 (pdf). Supplemental figures 1 (alkali pH-regulated expression of phytase from the *TSA1* promoter in cells growing on glucose) and 2 (SDS–PAGE analysis of the medium recovered from cultures of the parental P89-J and the evolved P89-J-6 strains).

DECLARATIONS

Ethics approval and consent to participate: Not applicable.

Consent for publication: Not applicable.

Availability of data and materials: Any data not presented in the paper are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: MA designed and constructed plasmids and yeast strains and carried out yeast cultures and treatments and protein gels. AC contributed to the design and preparation of DNA constructs, yeast strains and cultures for induction experiments and sampling. JA performed the phytase assays, supervised the work and drafted the manuscript. All the authors have read and approved the final manuscript.

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649

650

651 **LEGENDS TO FIGURES**

652 **Figure 1.-** Long-term GFP expression from the *PHO89* and *TSA1* promoters in response to
653 alkalinization. A) A strain with an integrated *PHO89*-GFP reporter was grown as indicated in
654 the Materials and Methods. At time 0, the cells were resuspended in medium buffered at pH

655 5.5 (noninduced, NI) or pH 8.0 (induced). The pH of a set of induced cultures was readjusted
656 (R) to pH 8.0 at specific times (denoted by the red arrow), whereas the pH of a parallel set
657 (Not R) was not modified. Samples were fixed, and fluorescence was determined by flow
658 cytometry. Data are presented as the mean ± SEM from 4 to 8 independent cultures. B) A
659 *TSA1*-GFP reporter strain was cultured as in panel A. In this case, the pH of the induced
660 cultures was readjusted (R) to pH 8.0 after 8, 24 and 32 h of culture. Fluorescence data are
661 the mean ± SEM from 4 independent cultures.
662
663 **Figure 2.-** Alkali-induced production of phytase from the *TSA1* promoter. A) Three
664 independent clones containing an integrated *TSA1*-phytase construct were grown on YP with
665 glycerol as the carbon source and processed as described in the Materials and Methods.
666 Cells were shifted to pH 8.0 at time 0 (or maintained at pH 5.5), and cultures were subjected
667 to periodic restoration of the pH and carbon source. Samples were taken at the indicated
668 times, and phytase activity was determined after appropriate dilution with water. The activity
669 produced by an *AOX1*-phytase strain grown on YP medium is included for comparison. Data
670 are presented as the mean ± SEM from 3 to 5 independent cultures for each clone. B) The
671 TSA1-1 clone was processed as in panel A except that cells were shifted at the indicated pH
672 values. Data are the mean ± SEM from 3 independent cultures.

673 **Figure 3.** Mapping the pH-dependent activation of the *TSA1* promoter. A new ser of yeast
674 strains that contained the indicated versions of the *TSA1* promoter driving the expression of
675 phytase were created (panel A). The location of predicted transcription factor-binding sites
676 with a *p* value $<2x10^{-5}$ is denoted at the bottom of the cartoon. Cells were grown on YPGly
677 and tested for phytase activity after 48 h of induction (no restoration of pH or nutrients) as
678 indicated in Material and Methods (B and C). Data are presented as the mean ± SEM from 4
679 (noninduced) or 8 (induced) independent experiments.
680
681 **Figure 4.-** Expression of phytase from the *HSP12* promoter and its functional mapping. A)
682 Three independent clones containing an integrated *HSP12*-phytase construct were
683 processed as in Figure 2A, and phytase activity was determined in the medium supernatant
684 at different times after induction. Data are the mean ± SEM from 2 or 3 independent cultures
685 for each clone. B) Constructs containing diverse versions of the *HSP12* promoter fused to
686 the phytase ORF were integrated into the genome. The inverted red triangle denotes the
687 position of the 5' end of the construct assayed in panel A (denoted as HSP12 in the bar
688 graph). Predicted transcription factor-binding sites (with a *p* value $<2x10^{-5}$) are mapped at the
689 bottom of the cartoon. Phytase activity was determined after 72 h of induction for all strains,

690 and the induced/noninduced ratio was calculated. Data are presented as the mean ± SEM
691 from 8 independent cultures for H1 to H5 and 4 cultures for HSP12. *, $p < 0.05$, ***, $p < 0.001$.
692
693 **Figure 5.-** Tightly regulated, alkali-induced expression of phytase from the *PHO89* promoter.
694 A) Three independent strains, P89-A, P89-H, and P89-J), containing an integrated *PHO89*-
695 phytase construct, were treated as in Figure 2A, and the produced phytase activity was
696 determined. Data are the mean ± SEM from 3 or 4 independent cultures for each clone. B)
697 Strain P89-J was induced by resuspending cells in medium buffered at different pH values
698 (from 7.4 to 8.2), which was readjusted (as appropriate) during the experiment. Data are the
699 mean ± SEM from 2 or 3 independent cultures.
700
701 **Figure 6.-** An evolved *PHO89*-phytase strain surpasses the production levels of the *AOX1*-
702 phytase strain. A) Three evolved *PHO89*-phytase strains (J5, J6 and J7), derived from P89-J,
703 were tested for phytase production as shown in Figure 2A. Data are presented as the mean
704 ± SEM from 3 to 5 independent cultures for each strain. B) The production level of parental
705 P89-J and the three evolved strains was compared for each time point with that of the *AOX1*-
706 based strain, taken as the unit. *, $p < 0.05$; **, $p < 0.01$; *** $p < 0.005$. C) SDS–PAGE analysis
707 of the medium recovered from cultures of the *AOX1*-based and the evolved P89-J6 strain.
708 Samples corresponding to 3.75 µl of medium were run on 10% polyacrylamide gels and
709 stained with Coomassie Blue. Specific quantities (1 and 3 µg) of bovine serum albumin
710 (BSA) were included in the gel for comparison of the amounts of expressed proteins. M
711 denotes molecular mass standards.

712 **Figure 7.-** Increased phytase production from the *PHO89* promoter by combination of high
713 pH and phosphate limitation. Three colonies from clone P89-J (a to c) were grown and tested
714 for phytase production as in Fig. 2A. Low Pi medium was YPD medium partially depleted for
715 Pi (0.7-0.8 mM). Data are presented as the mean ± SEM from 3 independent experiments for
716 each culture.

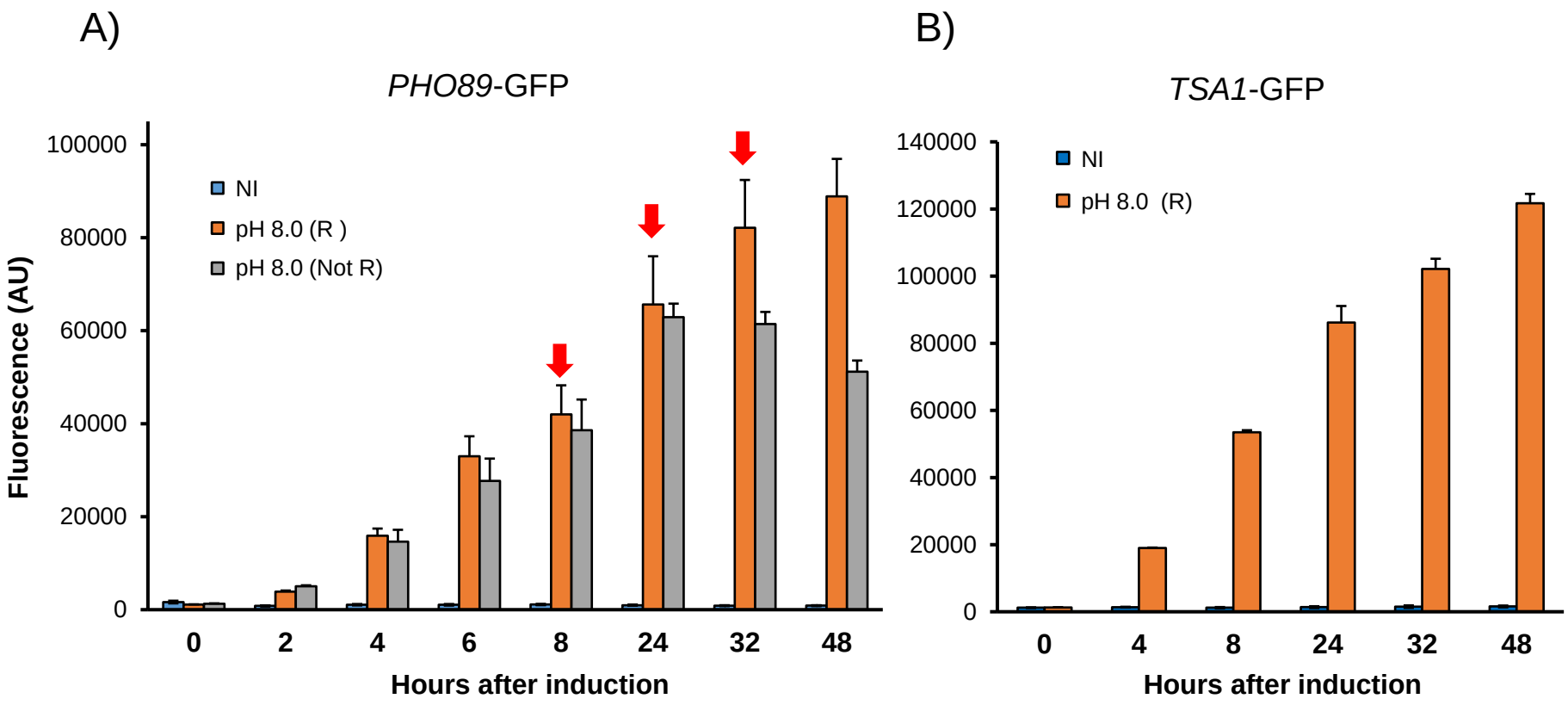
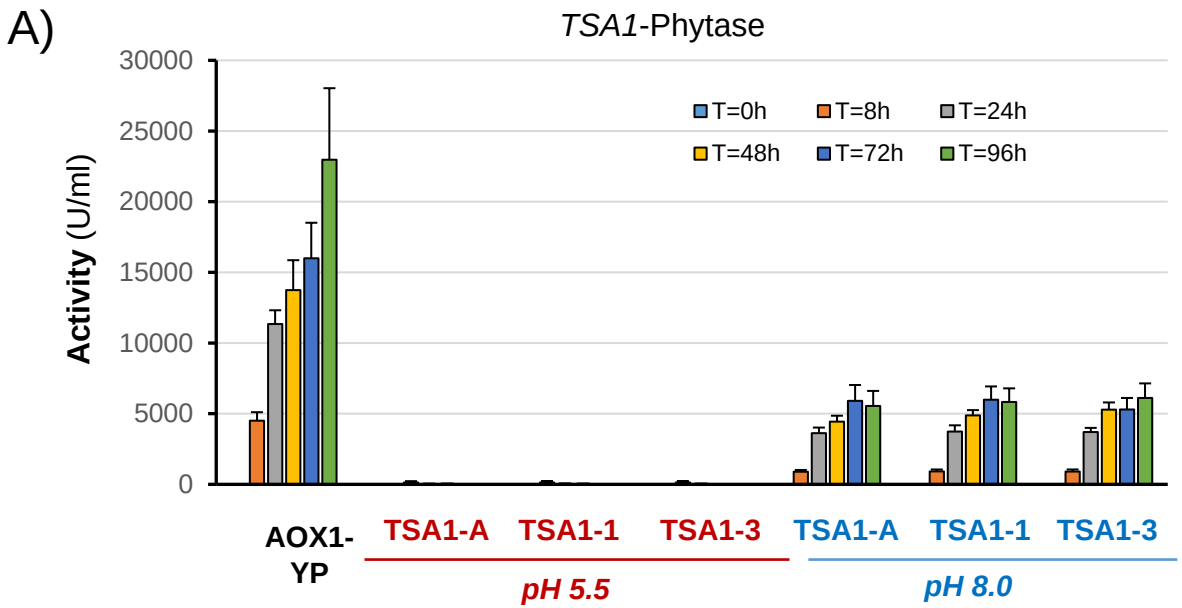

A)
PHO89-GFP
Fluorescence (AU)
100000
80000
60000
40000
20000
0
NI
pH 8.0 (R)
pH 8.0 (Not R)
0
2
4
6
8
24
32
48
Hours after induction
B)
TSA1-GFP
140000
120000
100000
80000
60000
40000
20000
0
NI
pH 8.0 (R)
0
4
8
24
32
48
Hours after induction

Figure 1


A)
TSA1-Phytase
Activity (U/ml)
30000
25000
20000
15000
10000
5000
0
T=0h
T=8h
T=24h
T=48h
T=72h
T=96h
AOX1-YP
TSA1-A
TSA1-1
TSA1-3
TSA1-A
TSA1-1
TSA1-3
pH 5.5
pH 8.0

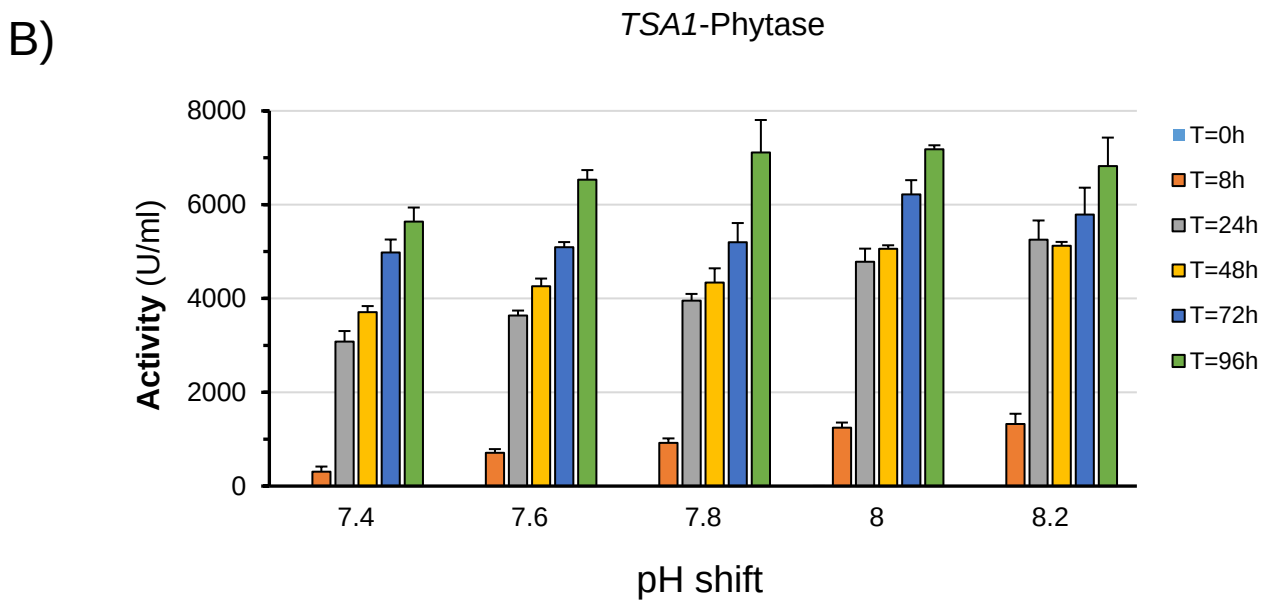

B)
TSA1-Phytase
Activity (U/ml)
8000
6000
4000
2000
0
7.4
7.6
7.8
8
8.2
pH shift
T=0h
T=8h
T=24h
T=48h
T=72h
T=96h

Figure 2

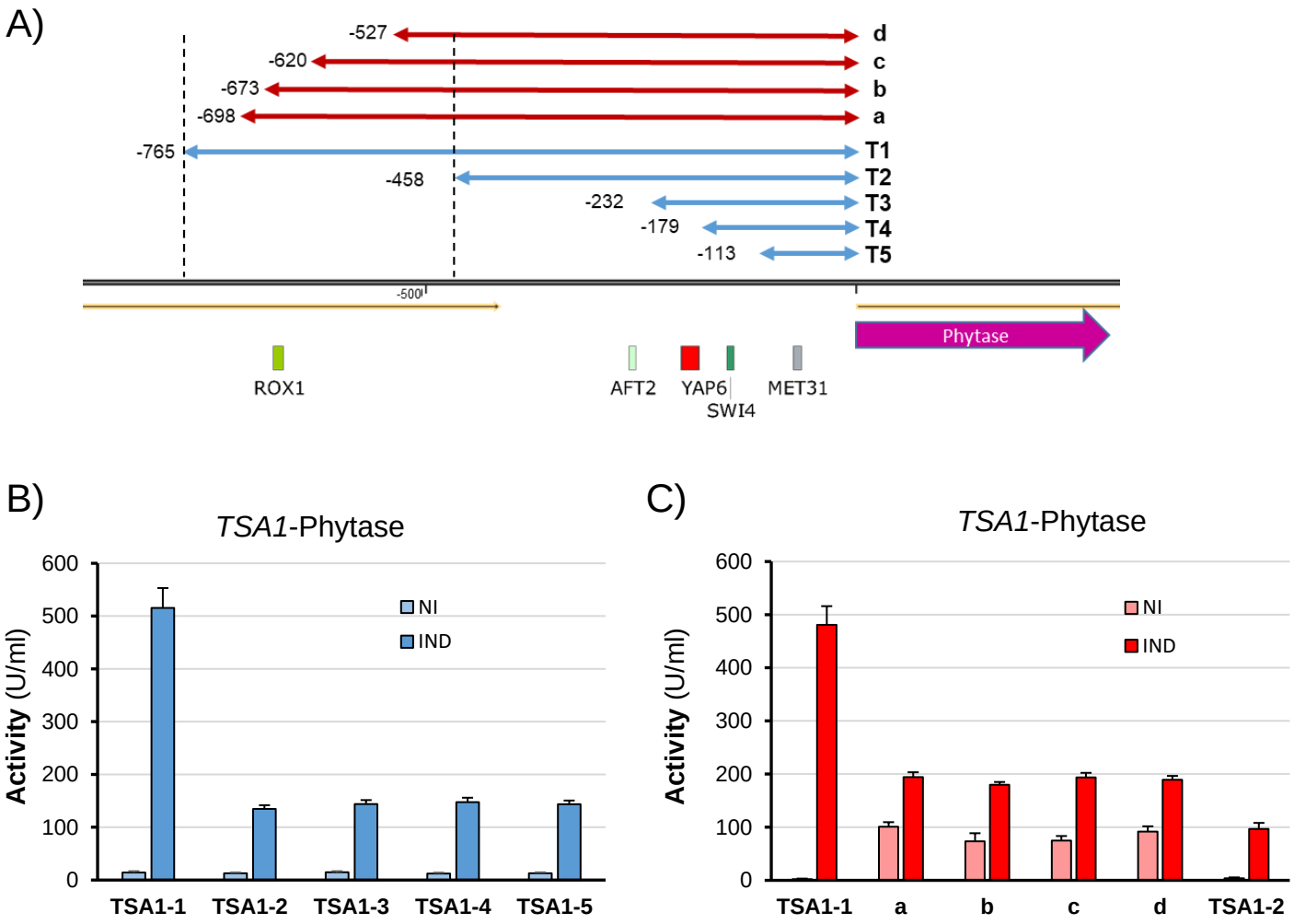

A)
-527
-620
-673
-698
-765
-458
-232
-179
-113
d
c
b
a
T1
T2
T3
T4
T5
-500
Phytase
ROX1
AFT2
YAP6
SWI4
MET31
B)
TSA1-Phytase
Activity (U/ml)
600
500
400
300
200
100
0
NI
IND
TSA1-1
TSA1-2
TSA1-3
TSA1-4
TSA1-5
C)
TSA1-Phytase
Activity (U/ml)
600
500
400
300
200
100
0
NI
IND
TSA1-1
a
b
c
d
TSA1-2

Figure 3

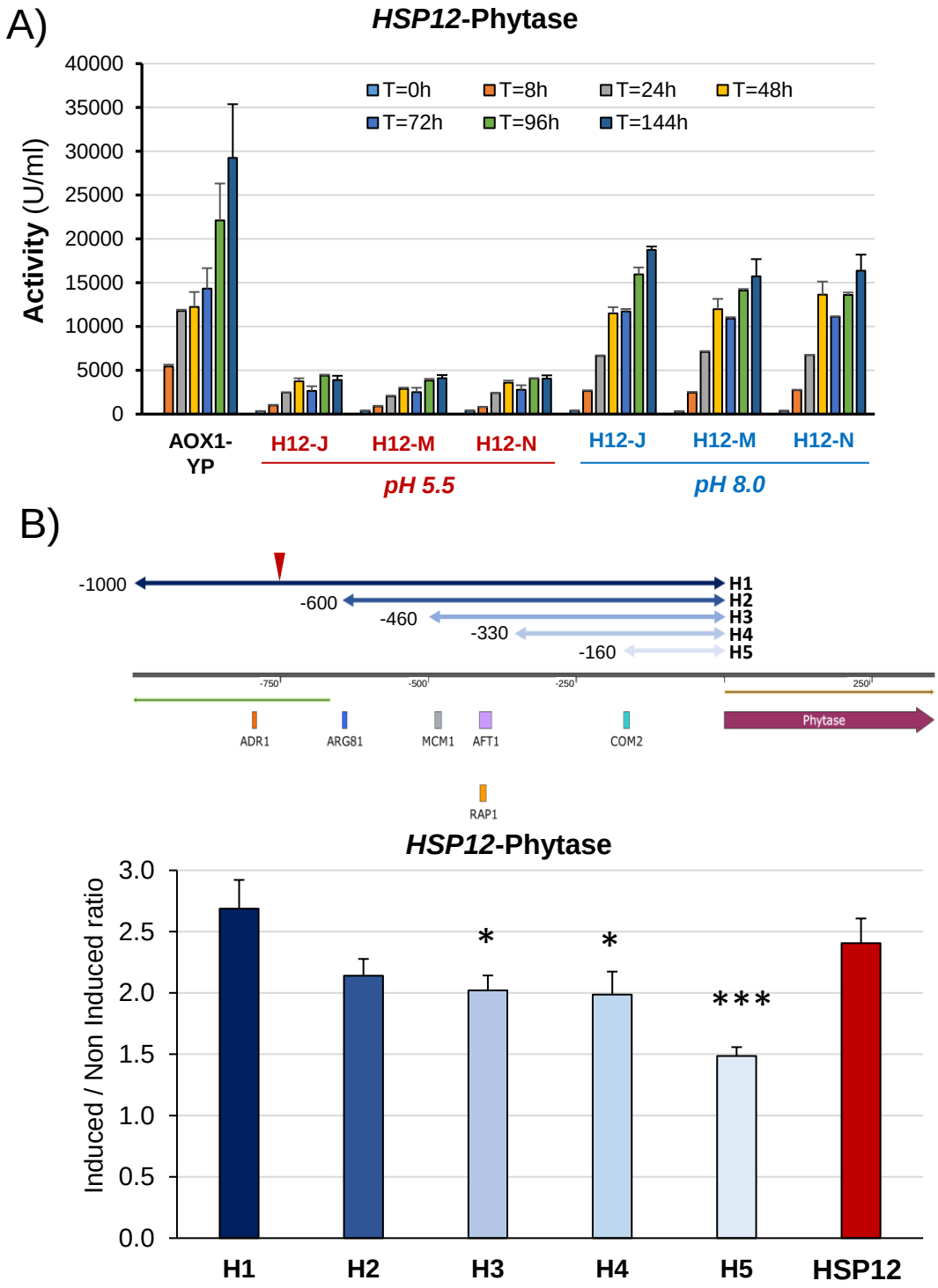
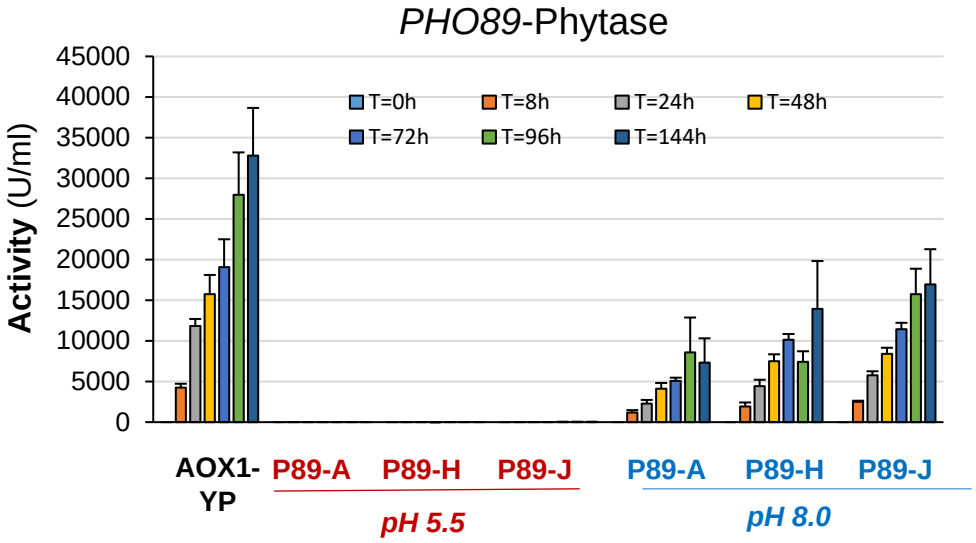

A)
HSP12-Phytase
Activity (U/ml)
40000
35000
30000
25000
20000
15000
10000
5000
0
T=0h
T=8h
T=24h
T=48h
T=72h
T=96h
T=144h
AOX1-YP
H12-J
H12-M
H12-N
H12-J
H12-M
H12-N
pH 5.5
pH 8.0
B)
-1000
-600
-460
-330
-160
H1
H2
H3
H4
H5
-750
-500
-250
250
ADR1
ARG81
MCM1
AFT1
COM2
Phytase
RAP1
HSP12-Phytase
Induced / Non Induced ratio
3.0
2.5
2.0
1.5
1.0
0.5
0.0
*
*
***
H1
H2
H3
H4
H5
HSP12

Figure 4

A)


PHO89-Phytase
Activity (U/ml)
45000
40000
35000
30000
25000
20000
15000
10000
5000
0
T=0h
T=8h
T=24h
T=48h
T=72h
T=96h
T=144h
AOX1-YP
P89-A
P89-H
P89-J
P89-A
P89-H
P89-J
pH 5.5
pH 8.0

B)

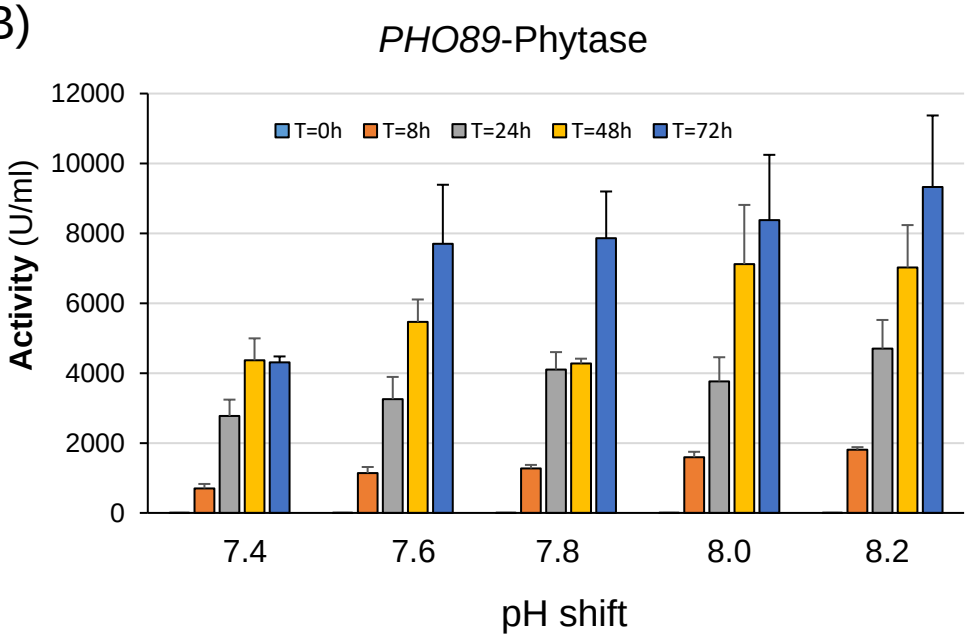

PHO89-Phytase
Activity (U/ml)
12000
10000
8000
6000
4000
2000
0
T=0h
T=8h
T=24h
T=48h
T=72h
7.4
7.6
7.8
8.0
8.2
pH shift

Figure 5

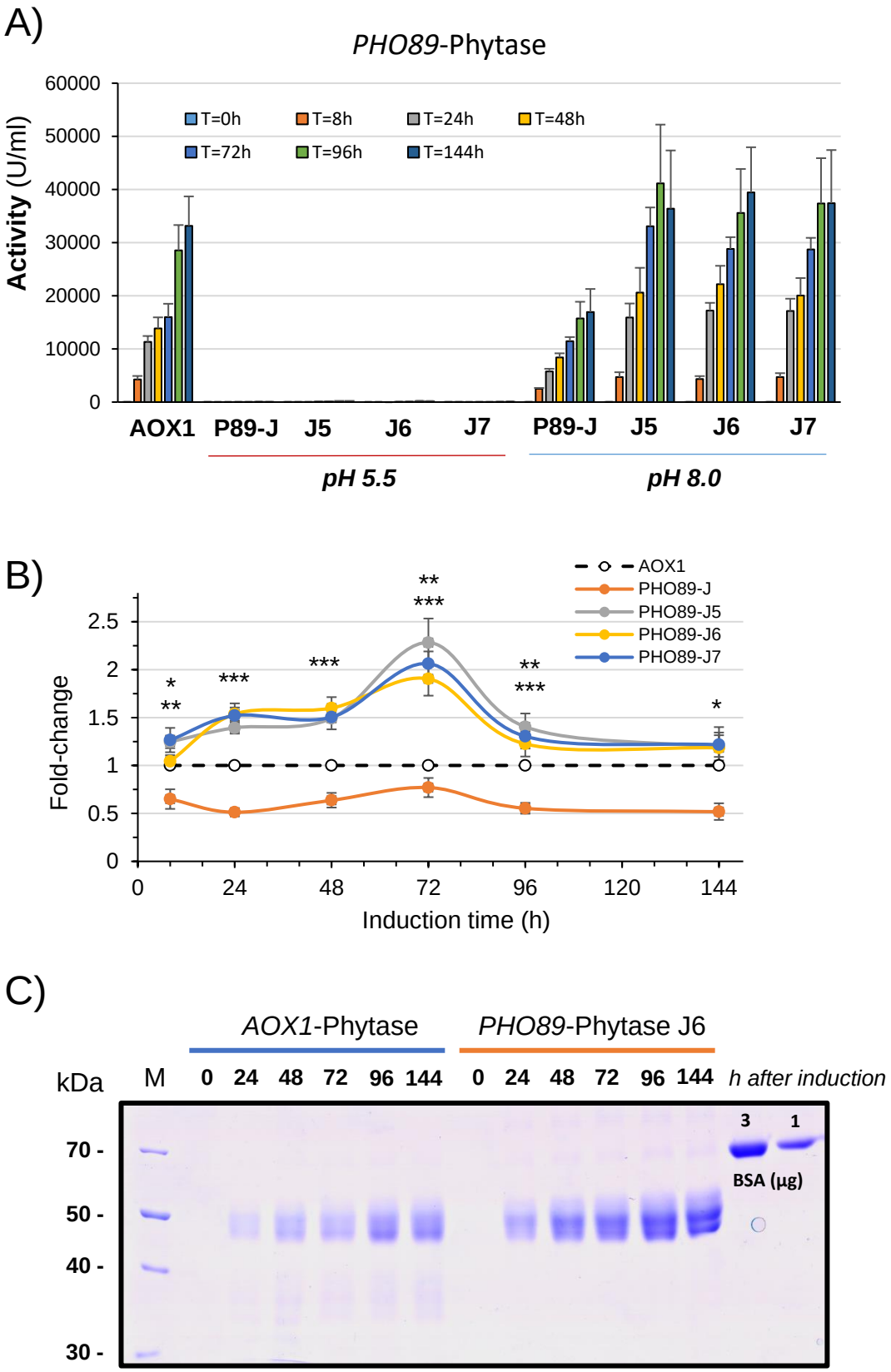

A)
PHO89-Phytase
Activity (U/ml)
60000
50000
40000
30000
20000
10000
0
T=0h
T=8h
T=24h
T=48h
T=72h
T=96h
T=144h
AOX1
P89-J
J5
J6
J7
P89-J
J5
J6
J7
pH 5.5
pH 8.0
B)
AOX1
PHO89-J
PHO89-J5
PHO89-J6
PHO89-J7
Fold-change
2.5
2
1.5
1
0.5
0
0
24
48
72
96
120
144
Induction time (h)
C)
AOX1-Phytase
PHO89-Phytase J6
kDa
M
0 24 48 72 96 144
0 24 48 72 96 144
h after induction
70 -
50 -
40 -
30 -
3
1
BSA (µg)

Figure 6

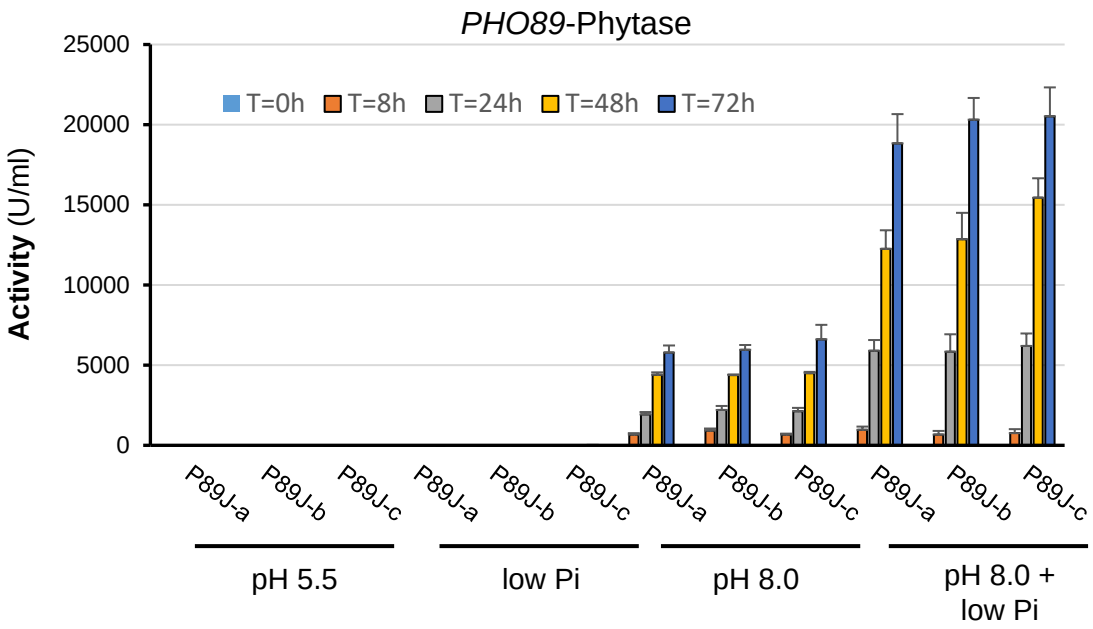

PHO89-Phytase
T=0h
T=8h
T=24h
T=48h
T=72h
Activity (U/ml)
25000
20000
15000
10000
5000
0
P89J-a
P89J-b
P89J-c
P89J-a
P89J-b
P89J-c
P89J-a
P89J-b
P89J-c
P89J-a
P89J-b
P89J-c
pH 5.5
low Pi
pH 8.0
pH 8.0 +
low Pi

Figure 7