RGC Ref. No.:
UGC/FDS25/M02/16
(please insert ref. above)

RESEARCH GRANTS COUNCIL
COMPETITIVE RESEARCH FUNDING SCHEMES FOR
THE LOCAL SELF-FINANCING DEGREE SECTOR

FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report
(for completed projects only)

Submission Deadlines:
1. *Auditor's report with unspent balance, if any: within **six** months of the approved project completion date.*
2. *Completion report: within **12** months of the approved project completion date.*

Part A: The Project and Investigator(s)

1. Project Title

Made-to-order: engineering and optimization of "tunable" yeasts for phytate hydrolysis

2. Investigator(s) and Academic Department(s) / Unit(s) Involved

Research Team	**Name / Post**	**Unit / Department / Institution**
Principal Investigator	TSANG Wai-kei / Associate Professor	School of General Education and Languages / Technological and Higher Education Institute of Hong Kong
Co-Investigator(s)	FONG Wing-ping / Professor	School of Life Sciences / The Chinese University of Hong Kong
Others	N/A	N/A

3. Project Duration

	Original	**Revised**	**Date of RGC / Institution Approval** *(must be quoted)*
Project Start Date	January 1, 2017	N/A	N/A
Project Completion Date	December 31, 2018	June 30, 2019	November 27, 2018
Duration *(in month)*	24	30	November 27. 2018
Deadline for Submission of Completion Report	December 31, 2019	June 30, 2020	November 27, 2018

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. To create novel *Pichia* yeast strains that express and secret phytases for phytate hydrolysis;
2. To generate a Pi-responsive promoter library and examine the correlation of phytase production with external Pi concentrations; and
3. To evaluate the effect of activation of secretory pathway in *P. pastoris* for optimization of phytase production.

5.2 Revised objectives

Date of approval from the RGC: N/A

Reasons for the change: N/A

5.3 Realisation of the objectives
(Maximum 1 page; please state how and to what extent the project objectives have been achieved; give reasons for under-achievements and outline attempts to overcome problems, if any)

Objective 1 (100% achieved):
The nucleotide sequences of the two thermostable bacterial phytases (i.e. *Yersinia frederiksenii* phytase variant Ser51Thr, Yf, and *Bacillus* sp. MD2 phytase, MD2) were optimized for enhanced expression in *P. pastoris* using DNA2.0 software. The two "optimized" bacterial phytase genes were obtained by total gene synthesis and the nucleotide sequences were verified by automated DNA sequencing. Afterwards, the two "optimized" bacterial phytase genes were cloned into plasmid pPICZαA in which the P_{PHO89} promoter sequence (Genbank: EU938135) had been inserted to generate plasmid pPICZαA-P_{PHO89}-Yf and plasmid pPICZαA-P_{PHO89}-MD2.

These two plasmids were transformed into *P. pastoris* strain GS115 using electroporation and ten transformants were randomly selected for characterization. Phytase expression in the *Pichia* transformants was determined by measuring the amount of released Pi in culture supernatant using the ammonium molybdate method. Phytase activity was detected in all selected *Pichia* transformants.

Objective 2 (100% achieved):
The library was constructed using the P_{PHO89} promoter sequence as template. We employed error-prone PCR to generate a promoter library using P_{PHO89}-specific primers and plasmid pPICZαA-P_{PHO89}-yEGFP. Plasmid pPICZαA-P_{PHO89}-yEGFP was made by

replacing the Yf sequence in plasmid pPICZαA-P_{PHO89}-Yf with the open reading frame of yeast-enhanced green fluorescent protein (yEGFP). Upon completion of error-prone PCR, the PCR mixture was cloned into plasmid pPICZαA-P_{PHO89}-yEGFP to replace the resident P_{PHO89}, and transformed into *P. pastoris* strain GS115 using electroporation.

The responsiveness of the Pi-responsive promoter library to external Pi concentrations was examined by measuring the expression of yEGFP using fluorescence microplate reader at excitation wavelength of 488 nm and emission wavelength of 515 nm over a range of Pi concentrations at 0.11, 0.25, 0.5, 0.75, and 1 mg/mL. Out of some 5000 *Pichia* transformants we examined, the expression of yEGFP of 36 of them was found dependent on external Pi concentrations. Three mutant promoters were selected for further analysis as they exhibit an inverse concentration-dependent relationship with external Pi concentrations (i.e. the higher the external Pi concentration, the lower the fluorescence intensity).

The three mutant promoters were used to drive the expression of phytase upon addition of different concentrations of Pi. It was found that the levels of phytase activity were inversely concentration-dependent on Pi concentrations.

Objective 3 (100% achieved):
The *HAC1* gene sequence was obtained by total gene synthesis and the nucleotide sequence was verified by automated DNA sequencing. We have cloned the *HAC1* gene into plasmid pPICZαA-P_{PHO89} and generated plasmid pPICZαA-P_{PHO89}-Hac1p. Linearized plasmid pPICZαA-P_{PHO89}-Hac1p was transformed into phytase-containing *Pichia* transformants and the expression of phytase was evaluated by measuring the amount of Pi release using the ammonium molybdate method. No significant change in the expression of phytase was evident in the presence of the *HAC1* gene.

5.4 Summary of objectives addressed to date

Objectives *(as per 5.1/5.2 above)*	**Addressed** *(please tick)*	**Percentage Achieved** *(please estimate)*
1. To create novel *Pichia* yeast strains that express and secret phytases for phytate hydrolysis;	√	100%
2. To generate a Pi-responsive promoter library and examine the correlation of phytase production with external Pi concentrations; and	√	100%
3. To evaluate the effect of activation of secretory pathway in *P. pastoris* for optimization of phytase production	√	100%

6. Research Outcome

6.1 Major findings and research outcome
(Maximum 1 page; please make reference to Part C where necessary)

1. **Creation of novel *Pichia* yeast strains that express and secrete phytases**
   Two thermostable bacterial phytases were cloned (Fig. 1) and transformed into *P. pastoris* strain GS115. Expression of these two bacterial phytases in a heterologous host was enhanced by codon optimization. Upon examination of the culture supernatant of the randomly selected *Pichia* transformants, phytase activity was detected (YF: 0.23 U/mL; MD2: 0.14 U/mL), suggesting successful expression of these two bacterial phytases in *P. pastoris*.

2. **Generation of improved Pi-responsive promoters for "made-to-order" expression of phytases in *P. pastoris***
   A Pi-responsive promoter library was generated by three rounds of error-prone PCR. The strengths of the mutant promoters were evaluated at different external Pi concentrations (Fig. 2a). Out of 36 mutant promoters, three (i.e. P3, P4, P4-TT) were selected for further analysis (Fig. 2b). Among them, P4-TT was found to exhibit enhanced sensitivity to regulate the expression of phytase in response to external Pi concentrations (Fig. 3a). DNA sequence analysis revealed a deletion of two T nucleotides at positions -597 and -598 (Fig. 3b). There is a CACGTT motif at position -583, which is very close to the double T nucleotides deletion. Previous study reported a *PHO* regulation system in which the Pi signal could be conveyed via binding of Pho4p to the promoter binding sites UASp1 and UASp2. The P_{PHO89} promoter has the same consensus binding site, suggesting that the *P. pastoris*-derived *PHO89* gene is also under the control of the *PHO* regulatory system. Therefore, the P4-TT promoter might have a stronger strength and an improvement in transcription efficiency as the structure of the binding site UASp1 was altered.

3. **Coexpression of transcriptional factor Hac1p does not have positive effect on the expression of phytase in our engineered *Pichia* yeast strains**
   We hypothesized that coexpression of Hac1p could increase the yield of phytase. However, no significant change in the expression of phytase was evident in our engineered *Pichia* yeast strains as the measured phytase activities were similar to the controls (YF with Hac1p: 0.21 U/mL; MD2 with Hac1p: 0.13 U/mL).

4. **Potential applications of our engineered *Pichia* yeast strains in *in vitro* digestion**
   We also evaluated the effectiveness of our engineered *Pichia* yeast strains in a simulated *in vitro* digestion model that mimics the digestive conditions (temperature and pH) of monogastric animals. Using YF, a substantial increase in Pi release was evident in the first hour of digestion using hydrated wheat-based meal as substrate, and a total of ~ 1.4 g/L of Pi was detected at the end of the whole process (3.25 h) (Fig. 4a). Moreover, it was found that Pi release decreased with an increase in Pi concentration (Fig. 4b), suggesting that a lower Pi concentration environment stimulates the expression of phytase which digests the wheat-based meal more efficiently (i.e. made-to-order).

These results were presented in the "9th International Congress on Biocatalysis" held in Hamburg, Germany in August, 2018; and published in a peer-reviewed journal "Enzyme and Microbial Technology" (Impact Factor: 3.553) in 2020. Financial support from RGC was acknowledged. (Note: P4-TT is shown as $P_{PHO89}\Delta TT$ in the journal article.)

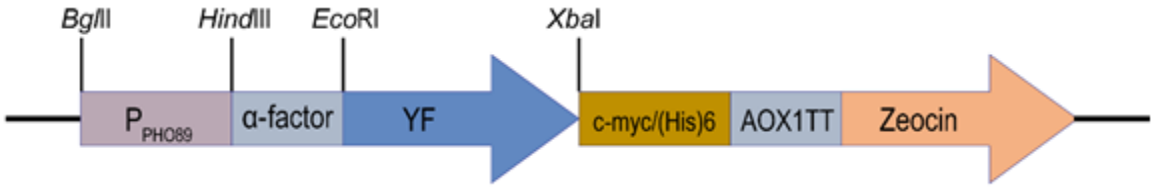


Fig. 1 Schematic diagram of the plasmid construct for the expression of phytase YF in *P. pastoris*. P_{PHO89}: Pi-responsive promoter; α-factor: N-terminal α-factor secretion signal; YF: phytase gene of *Yersinia frederiksenii*; c-myc/(His)6: c-myc/(His)6 antibody targeting sites; AOX1TT: AOX1 transcription termination region; Zeocin: zeocin resistance gene.

Note: In plasmid construct for the expression of phytase MD2 in *P. pastoris*, YF is replaced by MD2.

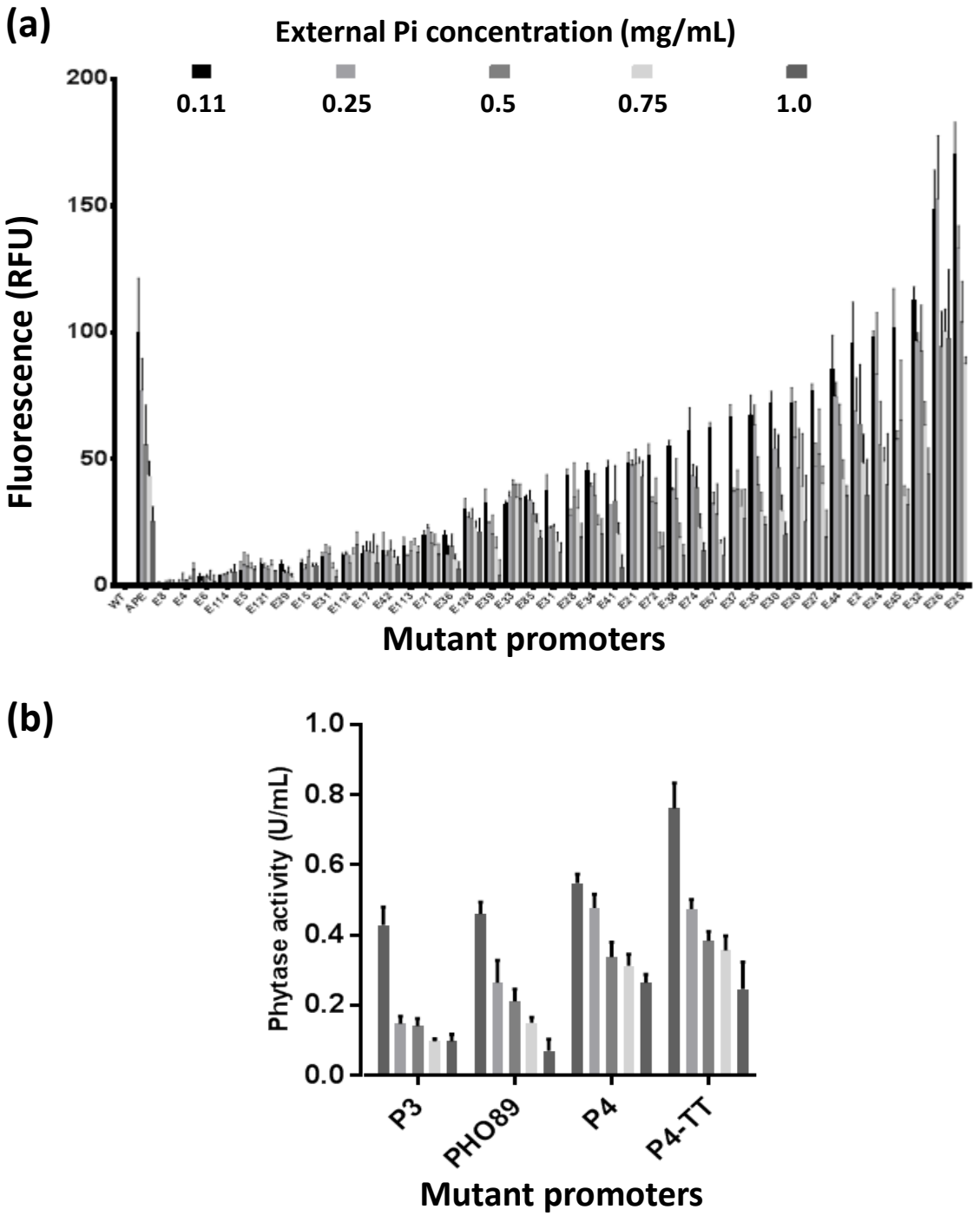
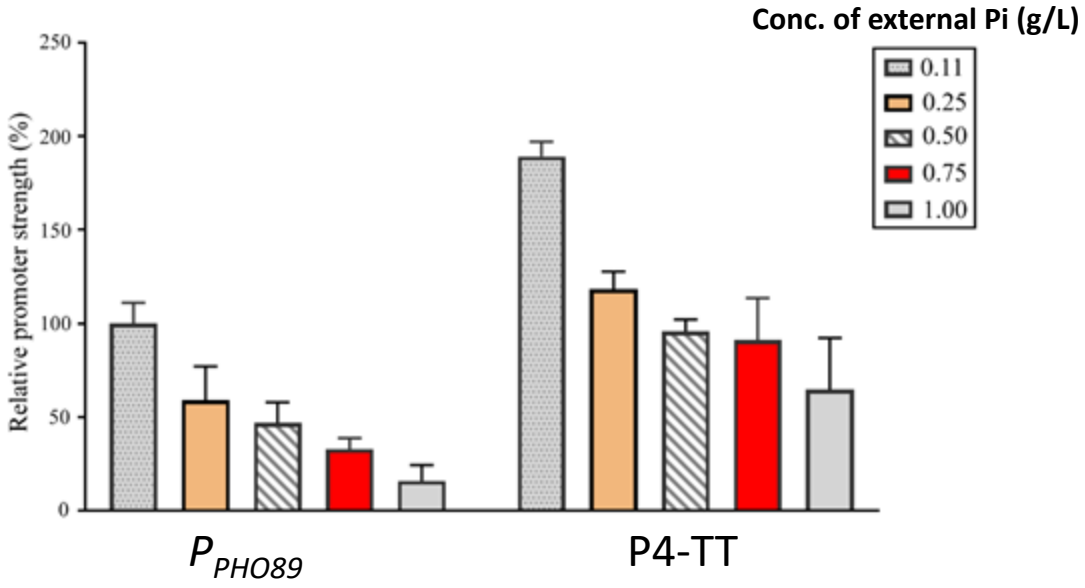


Fig. 2 Evaluation of the correlation of external Pi concentrations to Pi-responsive promoter library. (a) Expression of yEGFP was dependent on external Pi concentrations. **(b)** Three mutant promoters (P3, P4, P4-TT) were cloned into plasmid to drive phytase expression. Phytase activity was responsive to external Pi concentrations in an inverse proportion manner. Wild type P_{PHO89} promoter (PHO89) was included for comparison.

(a)



(b)

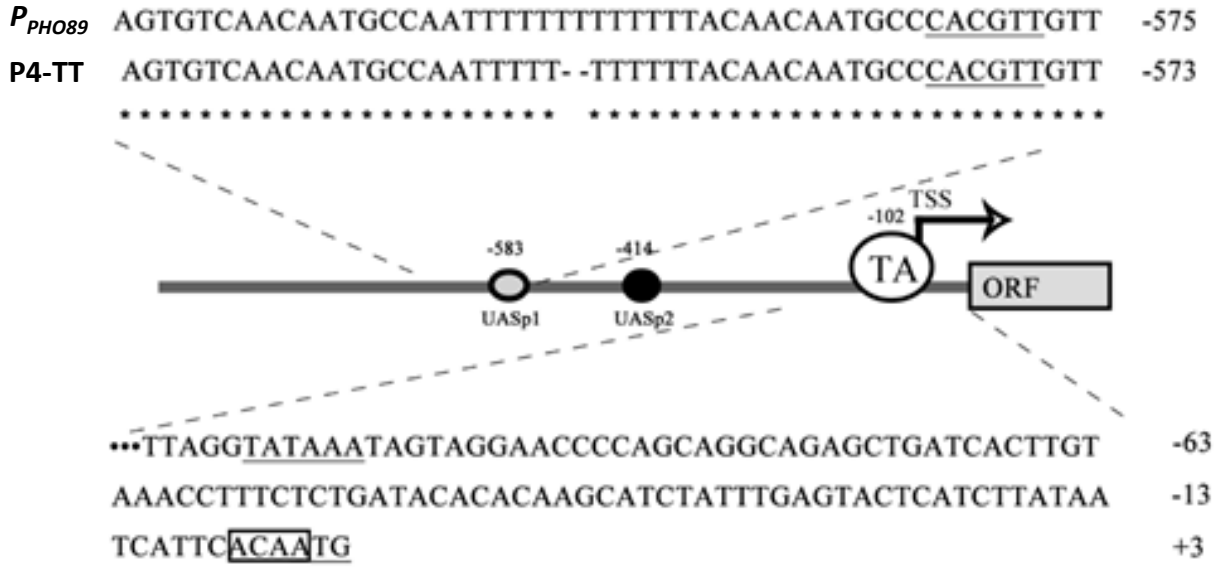


Fig. 3 Evaluation of P_{PHO89} mutant promoters. **(a)** Comparison of the strengths of wild type P_{PHO89} and P4-TT promoters at different Pi concentrations. **(b)** Sequence analysis of wild type P_{PHO89} and P4-TT promoters reveals a TT-deletion. UASp1/UASp2: Pho4p binding sites (CACGTT); TA: TATA box (TATAAA); Kozak sequence at position -1: boxed; TSS: transcription start site; ORF: open reading frame.

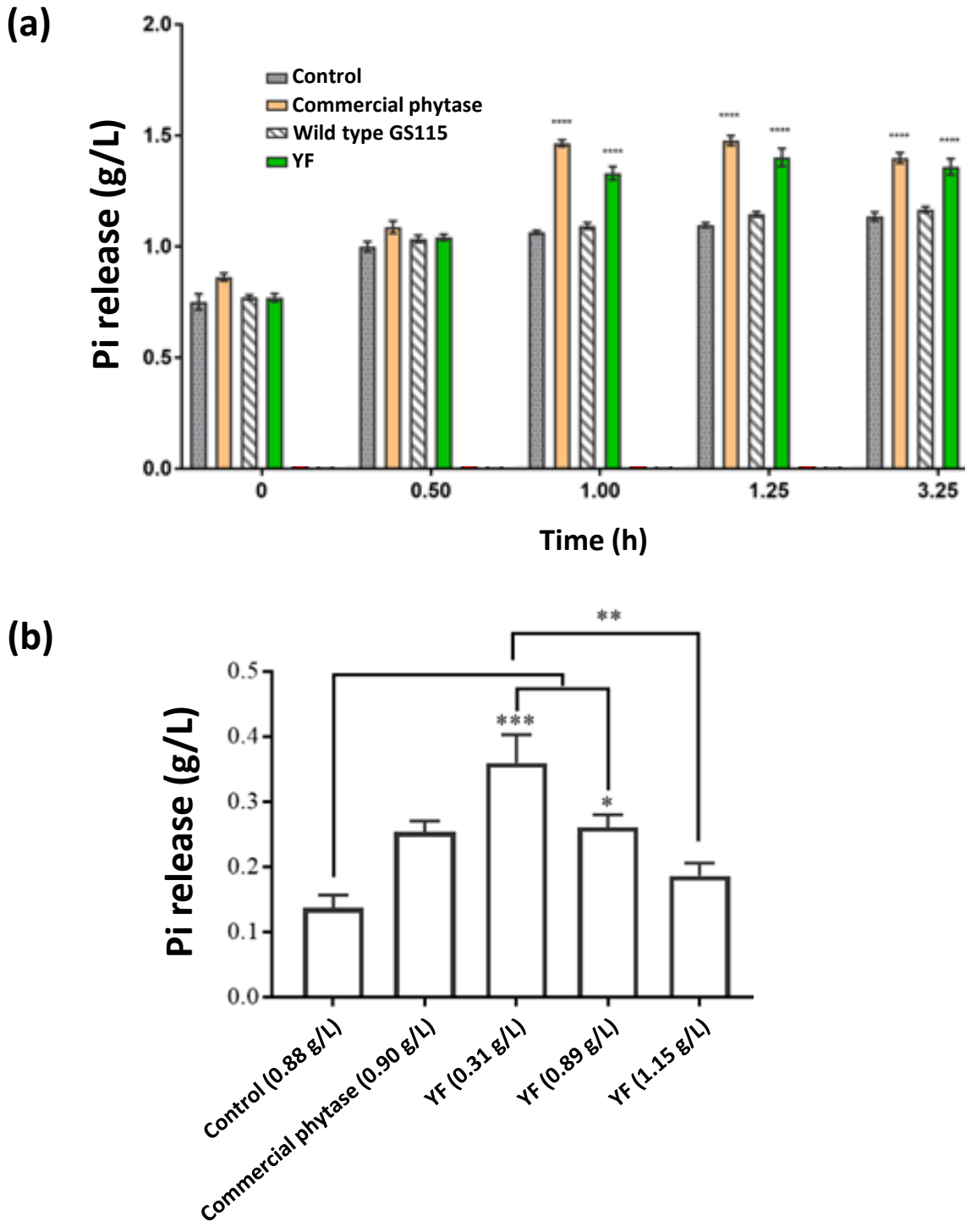


Fig. 4 Simulated *in vitro* digestion model. **(a)** Digestion of hydrated wheat-based meal with engineered *Pichia* yeast strains. **** $p < 0.0001$. **(b)** Pi release in the presence of different initial Pi concentrations (g/L, shown in parenthesis) in the environment. The data represent mean ± SEM of three independent experiments, each performed in duplicate. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

6.2 Potential for further development of the research and the proposed course of action *(Maximum half a page)*

1. Exploring potential applications as whole-cell biocatalyst in animals

The engineered *Pichia* yeast strains could be employed as probiotics to improve nutritional value associated with Pi release in monogastric animals. Further feasibility studies are warranted by adding the engineered *Pichia* yeast strains in feed pellet in experimental animal models. The effectiveness could be determined by measuring changes in growth rate, body gain, intestinal microbiome, and other biochemical parameters.

2. Determination of Pi concentrations in environment

The Pi-responsive promoter could be used to determine Pi concentrations in ambient environment by virtue of its high sensitivity. The levels of Pi could be readily examined by measuring the intensity of green fluorescence using pPICZαA-P4-TT-yEGFP. In addition, the promoter library could be further improved by more rounds of error-prone PCR and screening to generate more sensitive mutant promoter(s).

7. Layman's Summary

(Describe in layman's language the nature, significance and value of the research project, in no more than 200 words)

Phytate is the major storage form of phosphorus in plants. It is present in cereals and raw materials of vegetable origin used in animal and human diets. However, simple-stomached animals, including humans, swine, and poultry, have little phytase activity in their guts, and therefore cannot digest phytate. Thus, almost all dietary phytate is discharged into the environment, causing phosphorus pollution. Phytate is also considered as an "antinutrient" because it forms insoluble and stable complexes with metal ions, thus reducing dietary absorption of essential minerals. It is a dire need to develop sustainable approaches for environmentally-friendly utilization of this valuable and abundant natural resource.

Microorganisms are indispensable in production and biopharmaceutical industries. Scientists have been engineering these tiny "workhorses" for industrial manufacture of fine chemicals at low cost. Here, we have created novel *Pichia* yeast strains that secrete heat stable bacterial phytases for direct hydrolysis of phytate. In particular, phytase production is designed in a "made-to-order" manner in response to different levels of inorganic phosphate (Pi). Completion of this research project represents a proof-of-concept approach to create novel *Pichia* yeast strains with potential applications for food processing, animal waste treatment, and other biotechnological processes using Pi-controlled circuitry.

Part C: Research Output

8. Peer-Reviewed Journal Publication(s) Arising Directly From This Research Project
(Please attach a copy of the publication and/or the letter of acceptance if not yet submitted in the previous progress report(s). All listed publications must acknowledge RGC's funding support by quoting the specific grant reference.)

The Latest Status of Publications				**Author(s)** *(denote the corresponding author with an asterisk*)*	**Title and Journal / Book** *(with the volume, pages and other necessary publishing details specified)*	**Submitted to RGC** *(indicate the year ending of the relevant progress report)*	**Attached to this Report** *(Yes or No)*	**Acknowledged the Support of RGC** *(Yes or No)*	**Accessible from the Institutional Repository** *(Yes or No)*
Year of Publication	**Year of Acceptance** *(For paper accepted but not yet published)*	**Under Review**	**Under Preparation** *(optional)*						
2020	N/A	N/A	N/A	XIE, Zhenming , FONG Wing-ping, TSANG Wai-kei*	Title: Engineering and optimization of phosphate-responsive phytase expression in *Pichia pastoris* yeast for phytate hydrolysis Journal: Enzyme and Microbial Technology, 137, 109533	N/A	Yes	Yes	Yes

9. Recognized International Conference(s) In Which Paper(s) Related To This Research Project Was / Were Delivered
(Please attach a copy of each conference abstract)

Month / Year / Place	**Title**	**Conference Name**	**Submitted to RGC** *(indicate the year ending of the relevant progress report)*	**Attached to this Report** *(Yes or No)*	**Acknowledged the Support of RGC** *(Yes or No)*	**Accessible from the Institutional Repository** *(Yes or No)*
August / 2018 / Hamburg, Germany	High level expression of secretory phytases in *Pichia pastoris*	9th International Congress on Biocatalysis	2018	Yes	Yes	Yes

10. Whether Research Experience And New Knowledge Has Been Transferred / Has Contributed To Teaching And Learning
(Please elaborate)

The research experience and results of the development of engineered *Pichia* yeast strains for secretory production of phytase in a Pi-dependent manner have provided a successful proof-of-concept approach for environmentally-friendly utilization of phytate. The underlying principles and new knowledge of this innovative idea from the present research project have been incorporated into the two teaching areas (i.e. Genetic engineering and

microbial biotechnology; Harnessing the power of microorganisms to human activities) of a General Education Elective Module (Title: Journey to the Exotic World of Microorganisms) in which the PI is the Module Convenor. The concepts are delivered to students and an accompanied experiment has been designed for students to evaluate the efficiency of the engineered *Pichia* yeast strains for direct phytate hydrolysis under different conditions.

11. Student(s) Trained

(Please attach a copy of the title page of the thesis)

Name	Degree Registered for	Date of Registration	Date of Thesis Submission / Graduation
N/A	N/A	N/A	N/A

12. Other Impact

(e.g. award of patents or prizes, collaboration with other research institutions, technology transfer, teaching enhancement, etc.)

This research project is a collaborative work between Technological and Higher Education Institute of Hong Kong and The Chinese University of Hong Kong.

13. Statistics on Research Outputs

	Peer-reviewed Journal Publications	Conference Papers	Scholarly Books, Monographs and Chapters	Patents Awarded	Other Research Outputs (please specify)	
					Type	No.
No. of outputs arising directly from this research project	1	1	0	0	N/A	0

14. Public Access Of Completion Report

(Please specify the information, if any, that cannot be provided for public access and give the reasons.)

Information that Cannot Be Provided for Public Access	**Reasons**
N/A	N/A