RGC Ref. No.:
UGC/FDS25/M04/15
(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

Working hand-in-hand: building engineered yeasts for semi-synthetic cephalosporins

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, 2016	N/A	N/A
Project Completion Date	December 31, 2017	June 30, 2018	November 17, 2017
Duration *(in month)*	24	30	November 17, 2017
Deadline for Submission of Completion Report	December 31, 2018	June 30, 2019	November 17, 2017

Part B: The Final Report

5. Project Objectives

5.1 Objectives as per original application

1. To create a collection of yeast strains that express scaffoldin, TvDAAO, PsGL-7-ACA acylase, and catalase;
2. To examine the catalytic activity and the phenotypic determinants of the enzyme-displayed yeast strains; and
3. To evaluate the optimal ratio of different yeast populations for one-spot biosynthesis of 7-ACA and other structurally related cephem nuclei.

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):
Cohesins of *Clostridium cellulolyticum* (Cc), *C. thermocellum* (Ct), and *Ruminococcus flavefaciens* (Rf) have been obtained by PCR, cloned into plasmid pCTCON2 in an in-frame C-terminal fusion to yeast Aga2, and transformed into *Saccharomyces cerevisiae* EBY100 using lithium acetate. The resulting transformants express the three cohesins from Cc, Ct, and Rf on cell surface to form a trifunctional modular scaffoldin.

Dockerins of Cc, Ct, and Rf have been obtained by PCR and cloned separately into plasmid pCEL15. Full length sequences of TvDAAO and PsGL-7-ACA acylase have been obtained by PCR and catalase gene has been obtained by total gene synthesis. The sequences of TvDAAO, PsGL-7-ACA acylase, and catalase have been cloned in an in-frame manner into plasmid pCEL15 containing the three different dockerins. These plasmids have been transformed into *S. cerevisiae* BY4742. The resulting transformants express and secrete dockerin-tagged TvDAAO, PsGL-7-ACA acylase, and catalase respectively.

Objective 2 (100% achieved):
Expression of the trifunctional modular scaffoldin on the surface of transformed *S. cerevisiae* EBY100 was examined using mouse anti-C-myc antibody and Alexa Fluor 488-conjugated anti-mouse antibody. Display of scaffoldin was evident as green fluorescence could be observed under fluorescent microscope.

Protein expression and secretion of dockerin-tagged TvDAAO, PsGL-7-ACA acylase, and catalase were examined from the supernatant of the respective transformed *S. cerevisiae* BY4742. TvDAAO activity was determined spectrophotometrically by measuring the formation of hydrogen peroxide; PsGL-7-ACA acylase activity was determined by HPLC; and catalase activity was determined by measuring the

decomposition of hydrogen peroxide. All enzymes were active to the respective substrates, suggesting that they all possess catalytic activity.

Objective 3 (100% achieved):
By virtue of the specific interactions of the cohesins and dockerins, spontaneous self-assembly of TvDAAO, PsGL-7-ACA acylase, and catalase to the trifunctional modular scaffoldin was achieved by incubating the trifunctional modular scaffoldin with the three enzymes to generate an array of yeast strains that incorporate the three enzymes at different positions. The catalytic activities of the different combinations of the assembly of modular enzymes were examined in an in-house thermostated reactor aerated with O_2 under agitation. Similar catalytic activities were detected in all combinations of the assembly of modular enzymes regardless the relative positions of TvDAAO, GL-7-ACA, and catalase within the trifunctional modular scaffoldin. The optimal yeast populations consisting of a TvDAAO:PsGL-7-ACA acylase:catalase ratio of 1:1:1 was evident for biosynthesis of 7-ACA.

The potentials of the assembly of modular enzymes to catalyze the biosynthesis of other structurally related cephem nuclei were also examined. The assembly of modular enzymes was able to bioconvert cephamycin C (CMC) to 7-MACA and deacetoxycephalosporin C (DAOC) to 7-ADCA.

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 a collection of yeast strains that express scaffoldin, TvDAAO, PsGL-7-ACA acylase, and catalase;	√	100%
2. To examine the catalytic activity and the phenotypic determinants of the enzyme-displayed yeast strains;	√	100%
3. To evaluate the optimal ratio of different yeast populations for one-spot biosynthesis of 7-ACA and other structurally related cephem nuclei	√	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 an array of yeast strains that express scaffoldin, TvDAAO, PsGL-7-ACA acylase and catalase**
   Cohesins of Cc, Ct, and Rf have been cloned and transformed into *S. cerevisiae* EBY100 to create yeast strains for expression. The trifunctional modular scaffoldin is displayed on the cell surface of *S. cerevisiae* and confirmed by fluorescence microscopy (Fig. 1). Dockerins of Cc, Ct, and Rf have been cloned in an in-frame manner with TvDAAO, GL-7-ACA acylase, and catalase to create different forms of dockerin-enzyme pairs.

2. **Production of dockerin-tagged TvDAAO, PsGL-7-ACA and catalase**
   Protein expression and secretion of dockerin-tagged TvDAAO, GL-7-ACA acylase, and catalase were evident from the supernatant of cell lysates (Fig. 2). The catalytic activities of the dockerin-tagged enzymes were determined spectrophotometrically using chromogenic substrates or HPLC (Table 1), suggesting that addition of a dockerin module does not result in significant conformational changes which may lead to loss in catalytic activity.

3. **Evaluation of the optimal ratio of yeast populations for one-pot biosynthesis of 7-ACA and other structurally related cephem nuclei**
   The different dockerin-tagged enzymes were incubated with the trifunctional modular scaffoldin to generate an array of yeast strains that incorporate the three enzymes at different positions (Fig. 3a). Similar catalytic activities were detected in all combinations of the assembly of modular enzymes regardless the relative positions of TvDAAO, PsGL-7-ACA, and catalase within the trifunctional modular scaffoldin (Fig. 3b), suggesting that the positioning of the three enzymes is not a critical consideration for one-pot production of 7-ACA. The optimal yeast populations consisting of a TvDAAO:PsGL-7-ACA acylase:catalase ratio of 1:1:1 was concluded for biosynthesis of 7-ACA (Fig. 3b).

   The potentials of the assembly of modular enzymes to catalyze the biosynthesis of other structurally related cephem nuclei were also examined. The assembly of modular enzymes was able to bioconvert CMC to 7-MACA and DAOC to 7-ADCA (Fig. 3c).

The above-mentioned major findings and research outcome were presented in the following two conferences:

1. “THEi 2018 Research Symposium” held in Hong Kong on May 17, 2018.
2. “International Conference on Applied Education, Technology and Innovation” held in Hong Kong on April 16-18, 2019.

Financial support from RGC was acknowledged.

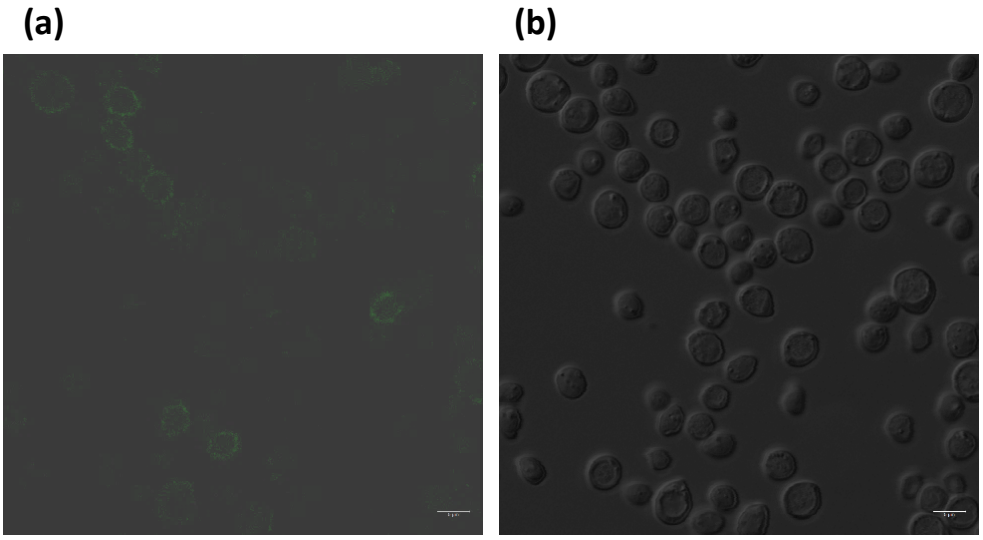

Fig. 1 Confirmation of displayed scaffoldin by confocal microscopy. (a) Trifunctional modular scaffoldin on cell surface was detected by mouse anti-C-myc antibody and Alexa Fluor 488-conjugated anti-mouse antibody. **(b)** Bright field.

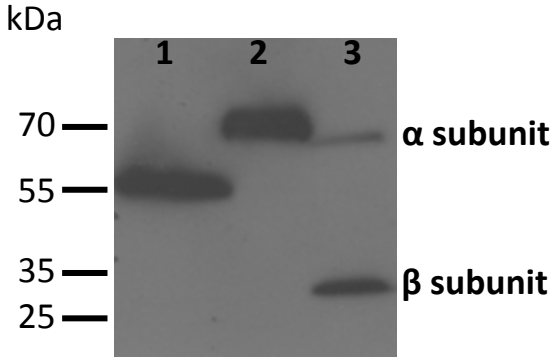

Fig. 2 Confirmation of protein expression and secretion by SDS-PAGE analysis. Lane 1: dockerin-tagged TvDAAO; lane 2: dockerin-tagged catalase; lane 3: dockerin-tagged PsGL-7-ACA acylase (α and β subunits are shown).

	Substrate		
Enzyme	**CPC**	**GL-7-ACA**	**H_2O_2**
TvDAAO	**29.3 ± 1.9**	**nd**	**nd**
GL-7-ACA acylase	**nd**	**25.9 ± 2.6**	**nd**
Catalase	**nd**	**nd**	**15.8 ± 1.3**

Table 1 Catalytic activities (U/mg) of the dockerin-tagged enzymes (mean ± SD; n = 3). nd: not determined

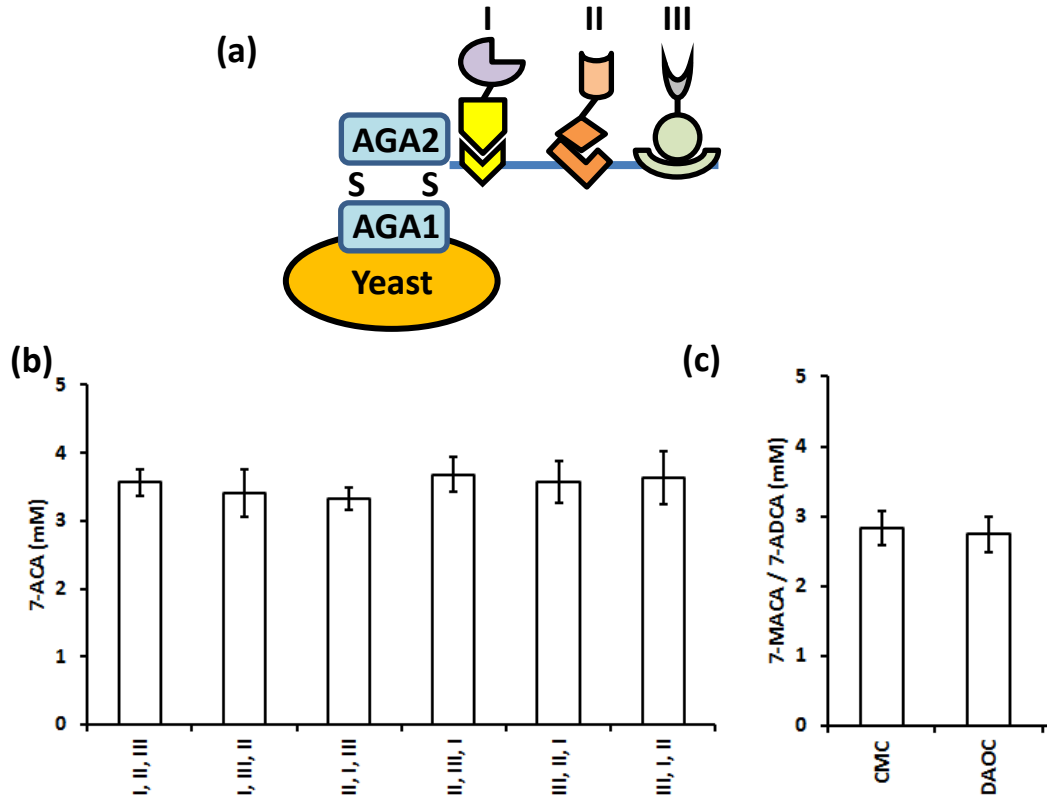

Fig. 3 Analyses of the one-pot biosynthesis of 7-ACA and other cephem nuclei. (a) Simplified schematic representation of the assembly of modular enzymes. The three positions (I, II, III) are shown. **(b)** Production of 7-ACA by the different combinations of the assembly. **(c)** Production of 7-MACA and 7-ADCA from CMC and DAOC respectively by the assembly.

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

1. **Building multifunctional modular scaffoldin**
   Recent advances in surface display technology can accommodate more cohesin-dockerin pairs on microbial cell surface, as many as eight cellulolytic enzymes can be displayed for concerted catalysis to degrade plant matter (Stern *et al*., 2016). The trifunctional modular scaffoldin in the present research project could be enhanced by incorporating more sites for cohesion-dockerin pairs. For instance, addition of one extra site to include cephalosporin C deacetylase could convert the yeast strains to further hydrolyze 7-ACA to diacetyl-7-ACA, a biosynthetic precursor of 3-vinyl substituted cephalosporins.

   Stern J, Moras S, Lamed R, Bayer E (2016) Adaptor scaffoldins: an original strategy for extended designer cellulosomes, inspired by nature. mBio 7,e00083-16.

2. **Exploring potential applications in other multi-enzyme reactions**
   Another research project currently being carried out by the PI is the biodegradation of environmental phytate using microbial phytases. The trifunctional modular scaffoldin could be employed to accommodate phytases of different pH optima and / or substrate specificities to generate whole cell biocatalysts for concerted biodegradation of phytate with potential applications in food processing industry and animal waste treatment.

7. Layman's Summary

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

Semi-synthetic cephalosporins, considering as "magic bullets" to fight against a variety of microbial infections, represent the biggest annual sale in the global market of β-lactam antibiotics. The worldwide cephalosporin market was estimated at US$78,000 million in 2016 and it would go up by 14% by 2023. Most marketed cephalosporins are semi-synthetic, and the methodologies involved are environmentally-damaging because toxic chemicals are used. Recent advocates of environmental sustainability favor a new paradigm to develop alternative "greener" production of antibiotics.

Microbial biotechnology helps solve environmental and sustainable resources problems using single-celled living systems and enzymes to produce valuable fine chemicals. Here, we have generated an array of yeast strains that have been engineered to specifically express the three crucial enzymes (i.e. D-amino acid oxidase, glutaryl-7-aminocephalosporanic acid acylase, and catalase) for one-pot production of 7-aminocephalosporanic acid, a cephem nucleus for the production of nearly two-thirds of semi-synthetic cephalosporins. In particular, the enzymes are displayed on the cell surface of baker's yeast to eliminate the tedious and cumbersome purification steps. Completion of this research project represents a proof-of-concept approach to create novel yeast strains with potential industrial applications for antibiotics production. They may serve as platforms to further develop other yeast-based models to replace environmental-damaging chemical reactions.

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)*						
N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A	N/A

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)*
May / 2018 / Hong Kong	Construction of a functional assembly of modular enzymes for direct biosynthesis of cephalosporins	THEi 2018 Research Symposium	2019	Yes	Yes	Yes
April / 2019 / Hong Kong	A green journey to the making of magic bullets	International Conference on Applied Education, Technology and Innovation	2019	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 an array of yeast populations that express scaffoldin, TvDAAO, PsGL-7-ACA acylase, and catalase have provided a successful proof-of-concept approach for simplified production of major cephem nuclei such as 7-ACA. The underlying principles and new knowledge from the present research project have been

incorporated into one of the teaching areas (i.e. Harnessing the power of microorganisms to human activities) of a General Education Elective (GEE) Module (Title: Journey to the Exotic World of Microorganisms) in which the PI is the Module Convenor. An accompanied experiment has been designed for students to examine the production of cephem nuclei using the different yeast populations generated from the present research project.

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. 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

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

FACULTY DEVELOPMENT SCHEME (FDS)

Completion Report - Attachment
(for completed projects only)

RGC Ref. No.:	UGC/FDS25/M04/15
Principal Investigator:	TSANG Wai-kei
Project Title:	Working hand-in-hand: building engineered yeasts for semi-synthetic cephalosporins

Statistics on Research Outputs

	Peer-reviewed Journal Publications	Conference Papers	Scholarly Books, Monographs and Chapters	Patents Awarded	Other Research Outputs (Please specify)
No. of outputs arising directly from this research project [or conference]	0	2	0	0	0