รายงานวิจัยฉบับสมบูรณ์

Development of oligopeptide biosynthesis in *Aspergillus oryzae* through non-ribosomal peptide synthetase (NRPS) system

โดย

ดร. ชนิกุล ชูตระกูร

ดร. กอบกุล เหล่าเท้ง

นางสาวสโรชา ปัญจนวพร

ดร. อติกร ปัญญา

ดร. สุกัญญา จีนเหนาะ

นางสาวจุฑามาศ อนันทยานนท์

เมษายน 2565

สัญญาเลขที่ RSA6280066

รายงานวิจัยฉบับสมบูรณ์

Development of oligopeptide biosynthesis in *Aspergillus oryzae* through non-ribosomal peptide synthetase (NRPS) system

โดย

ดร. ชนิกุล ชูตระกูร

ดร. กอบกุล เหล่าเท้ง

นางสาวสโรชา ปัญจนวพร

ดร. อติกร ปัญญา

ดร. สุกัญญา จีนเหนาะ

นางสาวจุฑามาศ อนันทยานนท์

ศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

สนับสนุนโดยสำนักงานกองทุนสนับสนุนการวิจัย
และศูนย์พันธุวิศวกรรมและเทคโนโลยีชีวภาพแห่งชาติ

(ความเห็นในรายงานนี้เป็นของผู้วิจัย สกว. และต้นสังกัดไม่จำเป็นต้องเห็นด้วยเสมอไป)

Acknowledgements

This work was supported by Thailand Science Research and Innovation, and National Center for Genetic Engineering and Biotechnology (RSA6280066).

Abstract

Project Code : RSA6280066

Project Title : Development of oligopeptide biosynthesis in *Aspergillus oryzae* through non-ribosomal peptide synthetase (NRPS) system

Investigator : Dr Chanikul Chutrakul, National Center for Genetic Engineering and Biotechnology

E-mail Address : chanikul@biotec.or.th

Project Period : 30 April 2019 – 29 April 2022

There has been an increasing demand of oligopeptides for pharmaceutical, food and feed applications, which lead to accelerating the exploration of new strategies for the peptide production. Non-ribosomal peptide synthetase (NRPS) system offers an alternative system for biological synthesis of small peptides. Through re-programming the regulatory control of targeted gene expression mediated by CRISPR-Cas9 system, the strain engineering of *A. oryzae* for production of non-ribosomal ACV tripeptide, a precursor of β-lactam antibiotic biosynthesis, was successfully achieved. The promoter exchange of the AoGpdA strain was efficient to drive the *acv* expression resulted in the ACV tripeptide production. In addition, several cultivation parameters strongly contributed to the production. It was found that the titre and content continually increased and reached the maximum level at the stationary phase, suggesting that the tripeptide product was growth-associated. A comparative study of transcriptomes of the ACV-producing and non-producing strains was implemented, suggested the up-regulated expression of the genes involved in basic nutrient and amino acid/peptide precursor with co-factor transport, and perhaps small molecule excretion, presumably supporting the biosynthesis of oligopeptide metabolite. Accordingly, the role of ammonium transporters (Amts) in *A. oryzae* was explored by genetic perturbation, showing that the Aoamt3 of *A. oryzae* displayed a functional role in ammonium transporting capacity required for cell growth. By overexpressing the *Aoamt3* gene, the production yield of mycelial mass was enhanced, and its glucose consumption rate was less than the wild type. Apart from the tripeptide (ACV) production, the strain improvement for heterologous cyclic dipeptide production in *A. oryzae* was performed, yielding the engineered strain with capability in the production of the bio-pigment indigoidine. The enhanced production titre was achieved by manipulating the cultivation conditions. Taken together, our study provided an insight into the production development of non-ribosomal oligopeptides in *A. oryzae* through synthetic biology and bioprocessing approaches that is applicable to produce other valuable peptides with industrial interest.

Keywords: *Aspergillus oryzae*, non-ribosomal peptide synthetase (NRPS), oligopeptide, CRISPR-Cas9 system, synthetic biology

Table of Contents

	Page
Acknowledgements	i
Abstract	ii
Table of Contents	iv
List of Tables	v
List of Illustrations	vi
List of abbreviations	vii
Introduction to the research problem and its significance	1
Literature review	2
Objectives	13
Materials and Methods	14
Results and Discussion	27
Conclusion and suggestion	54
References	55
Output	66

List of Tables

Table		Page
1	Oligopeptides and their functions	4
2	Production of oligopeptides	6
3	Effect of nitrogen sources on ACV tripeptide production of the AoGpdA strain	35
4	Effect of C:N ratio on the ACV tripeptide production by the AoGpdA strain	36
5	Effect of $MgSO_4$ concentration on the ACV tripeptide production of the AoGpdA strain	37
6	Biomass and residual glucose concentration of the OEAoamt3 and wild type strains grown in SM medium	47
7	Biomass, residual glucose concentration and indigoidine production of the AoInK strain grown in the InK production medium	53

List of Illustrations

Figure		Page
1	Schematic diagram of peptide synthesis by the ribosomal system and non-ribosomal system	10
2	Schematic diagram of Acv synthetase structure	12
3	The *acv* gene expression and cell biomass of *A. oryzae* at different cultivation times	28
4	PCR analysis of the exchanged promoter fragments in the engineered strains	29
5	Relative *acv* expression levels under the control of native and exchanged promoters in *A. oryzae* strains	31
6	ACV tripeptide analysis of the engineered strains	33
7	Profile of cell growth and ACV production of the AoGpdA-engineered strain	38
8	Volcano plot of differential expressed genes (DEGs) of the AoGpdA strain (ACV producer) and the recipient	41
9	Differential expressed genes (DEGs) up-regulated in the AoGpdA strain (ACV producer)	44
10	Fungal colonies of indigoidine-producing strain of *A. oryzae* overexpressing *AoinK* gene and the recipient	50

List of abbreviations

ΔAoamt	=	ammonium transporter disruptant strain
Aaa	=	L-α-aminoadipic acid
ABC	=	ATP-binding cassette transporter
acv	=	L-α-aminoadipyl-L-cysteinyl-L-valine synthetase gene
ACV	=	L-α-aminoadipic acid-L-cysteine-L-valine tripeptide
ACP	=	L-α-aminoadipic acid-L-cysteine-L-proline
A-domain	=	adenylation domain
amt	=	ammonium transporter gene
Amt	=	ammonium transporter
AoinK	=	codon-optimised indigoidine synthetase gene
AoInK	=	indigoidine-producing strain
AoPgpdA	=	gpdA promoter of *A. oryzae*
AoGpdA	=	ACV-producing strain
AoPtef1	=	tef1 promoter of *A. oryzae*
CD	=	Czapek Dox medium
C-domain	=	condensation domain
CRISPR-Cas9	=	Clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein 9 endonuclease (Cas9)
Cys	=	L-cysteine
DEG	=	differentially expressed gene
DMSO	=	dimethylsulfoxide
E-domain	=	epimerisation domain
EL	=	L-glutamine-L-leucine
ESI-MS	=	electrospray ionisation mass spectrometry
FPKM	=	fragments per kilobases of exon region per million mapped reads
Gln	=	L-glutamine
GpdA	=	glyceraldehyde-3-phosphate dehydrogenase
GRAS	=	Generally Recognised As Safe
HPLC	=	high-performance liquid chromatography

hts1	=	HC-toxin synthetase gene
inK	=	indigoidine synthetase gene
InK	=	indigoidine synthetase
LB	=	Luria–Bertani medium
LC-MS	=	liquid chromatography-mass spectrometry
Leu	=	L-leucine
LLE	=	liquid-liquid extraction
MEP	=	methylammonium permease
MFS	=	major facilitator superfamily transporter
$MgSO_4$	=	magnesium sulfate
$NaNO_3$	=	sodium nitrate
NH_4Cl	=	ammonium chloride
nrps	=	non-ribosomal peptide synthetase gene
Nrps	=	non-ribosomal peptide synthetase
NRPS	=	non-ribosomal peptide synthetase system
OEAoamt	=	ammonium transporter overexpressed strain
PCR	=	polymerase chain reaction
PEL	=	L-proline-L-glutamine-L-leucine
PL	=	L-proline-L-leucine
PMT	=	PEG-mediated method
4′-PPT	=	4′-phosphopantethine
PPTase	=	phosphopantetheinyl transferase
Pro	=	L-proline
PtPtoxA	=	toxA promoter from *Pyrenophora triticirepentis*
RP-HPLC	=	reverse-phase high-performance liquid chromatography
RT-PCR	=	reverse transcription PCR
RT-qPCR	=	real-time quantitative PCR
ScindC	=	indigoidine synthetase gene of *Steptomyces chromofuscus*
SD	=	yeast selective medium
simA	=	cyclosporine synthetase gene
SM	=	semi-synthetic medium
SPE	=	solid-phase extraction

SPPS	=	solid phase peptide synthesis
T-domain	=	thiolation domain
TE-domain	=	thioesterase domain
tex	=	*T. virens* peptaibol synthetase gene
TFA	=	trifluoroacetic acid
Val	=	L-valine
YPD	=	yeast complete medium

1. Introduction to the research problem and its significance

Oligopeptides are functional peptides consisting of 2 to 20 amino acid residues, which have versatile industrial applications ranging from animal feed additive to food supplement, nutraceuticals and pharmaceuticals. The demand of oligopeptides has continuously increased due to the population growth and socio-economic changes over the past decades (https://www.businesswire.com; https://globenewswire.com). In 2018, the US was the global peptide market. However, the Asia-Pacific region has forecasted as one of the fastest growing peptide market by 2024 (https://globenewswire.com; https://www.researchandmarket.com).

In the context of industrial production, oligopeptides available in the global market are mostly derived from chemical synthesis. However, an environmental concern on chemical waste generated from the production process has been arisen. Therefore, the biotechnological production of such oligopeptides is more preferable as an alternative approach. Oligopeptides can be biologically synthesised by the non-ribosomal peptide synthetase (NRPS) system, which naturally exists in certain microorganisms. The peptide-bond formation requires a mega-enzyme activity of Nrps, which is known as a chain of modules. Each module of the structural enzyme recognises a specific amino acid residue by adenylation (A-) domain, which is able to incorporate such amino residues into the growing peptide chain. Recognition of the A- domain is conducted by specific amino acid residues spanning in the substrate-binding pocket. Engineering of Nrps enzyme in certain microbes by genetic modification has shown a prospect in biosynthesis of tailor-made oligopeptides. Production of oligopeptides in a surrogate host may induce physical stress affecting cell functions, such as efficiency of peptide production. On the other hand, genetic manipulation of peptide biosynthesis in a native host where the system is available is considered to be a more practical approach.

Aspergillus oryzae is a filamentous fungus, which has been widely utilised for producing an array of bioproducts and thus is known to be a Generally Recognised As Safe (GRAS) microorganism. In addition to application in food products, *A. oryzae* has been employed as a workhorse for the synthesis of unnatural natural products, due to the existence of efficient NRPS system in this fungus. Therefore, this study aimed to rationally engineer *A. oryzae* BCC 7051 for biosynthesis of di- and tripeptides using the genome editing approach (CRISPR-Cas9 system), and available genomics information of this fungal strain. This would towards a biotechnological production platform of non-ribosomal oligopeptides through the fungal system for food and feed applications as well as other industrial sectors.

2. Literature review

2.1 *Aspergillus oryzae*: A fungal platform for the production of bio-products

A. oryzae is one of the filamentous fungi, which has been widely utilised for food production. For example, traditional Japanese food, including soy sauce, soybean paste, and sake are fermented products of *A. oryzae*. Moreover, it has been used for the production of C4 dicarboxylic acid, which is a chemical building block for food preservatives and polymerisation starter units (Ochsenreither *et al.*, 2014). Very recently, a genome characterisation of the selected strain of the *A. oryzae* BCC 7051 was conducted. This promising strain has an ability in utilising several carbon and nitrogen sources for cell growth, and thus the biomass production yield was higher than the reference strain, *A. oryzae* RIB40. Genomic information strongly confirmed that it is a non-aflatoxin strain as a result of a truncation in biosynthetic gene cluster (Thammarongtham *et al.*, 2018). This genome-based study provided an insight into the cellular metabolisms that could be exploited for the strain improvement for industrial applications through systematic approach.

2.2 CRISPR-Cas9 and its application for *A. oryzae*

Recently, an advanced genome editing, Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) and CRISPR-associated protein 9 endonuclease (Cas9) (CRISPR-Cas9) system, has been established for this fungus (Katayama *et al.*, 2015; Nakamura *et al.*, 2017). Using this emerging technology, our group has established the genome editing system for *A. oryzae* BCC 7051, generating *pyr*G auxotrophic strain via antibiotic marker-free disruption process. Moreover, we identified a novel U6 RNA polymerase III promoters of other *Aspergillus* strains, that efficiently functioned in *A. oryzae* BCC 7051, which are genetic tools for further strain development by multi-gene editing approach (Chutrakul *et al.*, 2019). Therefore, the availability of this robust host strain and genome editing tools permit the study of cell biology as well as the production of bioproducts by this fungal strain through synthetic biology approach.

2.3 Oligopeptides: Significance and functionality

Global peptide market has been dramatically expanded in the last decades. For instance, the market value of peptide therapeutics is at 16% increasing from 2015 to 2020 (https://globenewswire.com), whereas collagen peptide is expected to grow close to 8% from 2017 to 2021 (https://www.businesswire.com). Oligopeptides are composed of 2-20 amino residues linked by the amide bond (Hou *et al.*, 2017). These peptides exhibit specific physiological functions and properties beyond free amino acids. They have been used as nutrients, medical treatment and food/feed supplements for human and animals (Yagasaki and Hashimoto, 2008; Mills *et al.*, 2011; Santos *et al.*, 2012). As dietary supplement, some oligopeptides are widely utilised for enhancing growth performance of animals. Oligopeptides available in the markets with various functions are shown in Table 1.

Table 1 Oligopeptides and their functions

Application	Compound/composition	Activity	Producer/reference
Nutraceuticals and functional foods	L-glutamyl-L-threonine (Glu-Thr)	Enhancing umami taste of L-glutamate (saltiness-strengthening agent)	(Koike, 2012; Arai *et al.*, 2013; Shimono and Sugiyama, 2009)
	L-leucyl-L-serine (Leu-Ser)		
	Carnosine: β-alanyl-L-histidine (β-Ala-His)	Antioxidant	(Di Bernardini *et al.*, 2011)
	Aspartame: L-aspartyl-L-phenyl-O-methyl ester) (L-Asp-L-Phe-OMe)	Sweetener	(Özcengiz and Öğülür, 2015)
	Collagens: - L-glycyl-L-hydroxyproline (L-Gly-L-Hyp) - L-glycyl-L-proline-L-hydroxyproline (L-Gly-L-Pro-L-Hyp) - L-glycyl-L-proline-L-X* (L-Gly-L-Pro-L-X*) - L-glycyl-L-X*-L-hydroxyproline (L-Gly-L-X*-L-Hyp)	Strengthening bone, muscles, tendons promoting; healthy growth	(Li and Wu, 2018; https://www.genacol.ca/en/)
	L-glycyl-L-tyrosine (L-Gly-L-Tyr)	Ingredients of patient infusions	(Özcengiz & Öğülür, 2015)
	L-alanyl-L-glutamine (L-Ala-L-Gln)		
Medicine/therapeutic agents	L-histidyl-β-alanine (His-β-ala)	Induction of sedative and hypnotic effects	(Tsuneyoshi *et al.*, 2008)
	Angiotensin-I converting enzyme (ACE)-inhibitory peptides: - L-isoleusyl-Lprolyl-L-proline (L-Ile-L-Pro-L-Pro) - L-valyl-L-prolyl-L-proline (Val-Pro-Pro)	Anti-hypertensive effects	(Hou *et al.,* 2017)
Cosmetics	L-phenylalanyl-β-alanine (Phe-β-Ala)	Inhibition of hair-growth	(Tsuji & Moriwaki, 2001)
Animal feed	L-lysyl-L-glycine (Lys-Gly)	Supplemented diets on digestive tract in juvenile yellow perch and carp	(Ostaszewska *et al.*, 2010; Ostaszewska *et al.*, 2012)
	Opioid peptides	Animal health and stress alleviation as well as modulation of satiety	(Hou *et al.*, 2017)
	Cyclic dipeptides (diketopiperazines)	Feed additives	US patent number 20110295006A1

	L-arginyl-L-aspartate (Arg-Asp) and L-lysyl-A-aspartate (Lys-Asp)	Increase in amino acid absorption	Cysal company (http://www.cysal.de/technology)
Anti-microbial agent	Bacilysin: L-alanyl-L-anticapsin	Anti-bacteria and anti-fungi	(Özcengiz and Öğülür, 2015)

X* = any amino acid

2.4 Production of oligopeptides

The vast growing demand of oligopeptides in nutritional and medical products prompts manufacturers and suppliers to seek new strategies for the production. Chemical synthesis and chemical hydrolysis of proteins from animal by-products, marine organisms and plant sources have been used to generate small peptides. However, amino acids incorporated to peptide chain by such processes are always varied as a result of complicated reaction steps. Enzyme digestion has been employed to generate peptides with various lengths. The advantages and drawbacks of the various techniques used in peptide manufacturing are summarised in Table 2.

Alternatively, biosynthetic approaches relying on enzyme activities are employed for manufacturing the customised peptide production. The NRPS system is attractive for short peptide synthesis. The system offers a simple synthetic process, which targeted common and uncommon amino acids are incorporated into peptide chain product. In response to the growing markets of functional oligopeptides, the development of peptide biosynthetic process has become a domain of interest.

Table 2 Production of oligopeptides

Method	Reaction/ Enzyme	No. of amino acids	Advantages	Drawbacks	Reference
1.Chemical hydrolysis	Acid/alkali hydrolysis	2-7	- Cheap	- Destruction and loss of some amino acids -Chemical waste	(Hou *et al.*, 2017)
2. Chemical synthesis	Solid phase peptide synthesis (SPPS)	30-50	- Long chain with specific sequence -Common and unique peptides	- Cost - Chemical waste - By-product formation	https://www.thermofisher.com/th/en/home/
3.Enzymatic hydrolysis	-Pancreatin, trypsin, pepsin, carboxylpeptidases, aminopeptidases, papain, proteases	2 to > 20	- Mild hydrolysis condition - Specific and precise cleavage - Small amount used	-Expensive - Enzyme inhibitors concern	(Bah *et al.*, 2016; Hou *et al.*, 2017)
4. Biological synthesis	L-amino acid ligase (Lal): RizA, RizB and TabS	2-5	-Small gene (1.2 kb) - Branched-chain or uncommon amino acid incorporated	- Arg, Val, Leu, Ile or Met restricted at the N-terminus - Homo-peptide by-products	(Kino *et al.*, 2009; Kino *et al.*, 2010; Arai *et al.*, 2013)

	Ribosomal peptide synthesis (RPS)	2 (aspartame)-84 (bacteriocin)	Short and long chain available	- Proteinogenic amino acids incorporated - Amino acid modification in post translation process - Low productivity and separation difficulty in *E. coli*	(Doel *et al.*, 1980)
	NRPS	2-22	-*In vivo* synthesis - Non-proteinogenic amino acids incorporated - Amino acid modification coupling with growing peptide chain - Available system in bacteria and fungi	- Mega gene cluster (11 kb onwards - Co-factor (4'-phosphopantetheine) required	(Hashimoto, 2006)

2.5 Oligopeptide production by non-ribosomal synthesis

Non-ribosomal peptide synthetase (Nrps) structure

Apart from the ribosomal-dependent peptide biosynthesis (Figure 1A) (Hashimoto, 2006), NRPS is a mega-peptide synthetase that carries out the synthesis of short peptides bypassing the ribosomal system. The incorporation of amino acid monomers into the nascent peptide chain and catalysis of peptide bond are directed by its functional modules. The Nrps generally consists of the initiation, elongation and termination modules (Figure 1B) (Lautru and Challis, 2004). In each Nrps, the existence of multiple elongation modules is possible; therefore, the number of these elongation modules usually indicates the number of amino acid residues in the synthesised peptide chain. In addition to proteinogenic incorporation, Nrps can further add uncommon amino acid building blocks and cyclises the peptide, yielding a variety of peptide products with diverse functions.

A modular architecture of Nrps comprises multi-functional domains, displaying specific roles in recognising, activating, tailoring, condensing target substrates and finally releasing or cyclising the peptide product (Ackerley, 2016) (Figure 1B). Selectivity of amino acid substrates depends on the adenylation (A-) domain. The first amino acid is recognised and activated with ATP by this domain (step 1) similar to the activity of aminoacyl-tRNA synthetase (AATS) of the ribosomal system (Figure 1A). Activated amino acid forms a thioester bond onto a conserved 4’-phosphopantethine (4’-PPT) co-factor of a thiolation (T-) domain. Next, condensation (C) domain catalyses the amide bond formation between this new amino acid and the existing peptide chain (step 2), which attaches to the T-domain of the downstream module. The extended peptide now forms a thioester bond to the current T-domain for iterative elongation process. Finally, a thioesterase (TE-) domain hydrolyses and releases the peptide chain from the last T-domain (step

3). Moreover, a unique TE-domain enables the cyclisation of the peptide molecule. In addition to the core domains, tailoring domains with catalytic activities of epimerisation, methylation or heterocyclisation can be inserted into targeted modules. It should be noted that amino acid specificity of the A-domain is determined by 10 amino acids spanning through the domain acting as "codons" of NRPS (Stachelhaus *et al.*, 1999; Challis *et al.*, 2000; Weber and Marahiel, 2001). The NRPS is encoded by *nrps* gene, which is responsible for arranging modular structure as presented in the protein sequence.

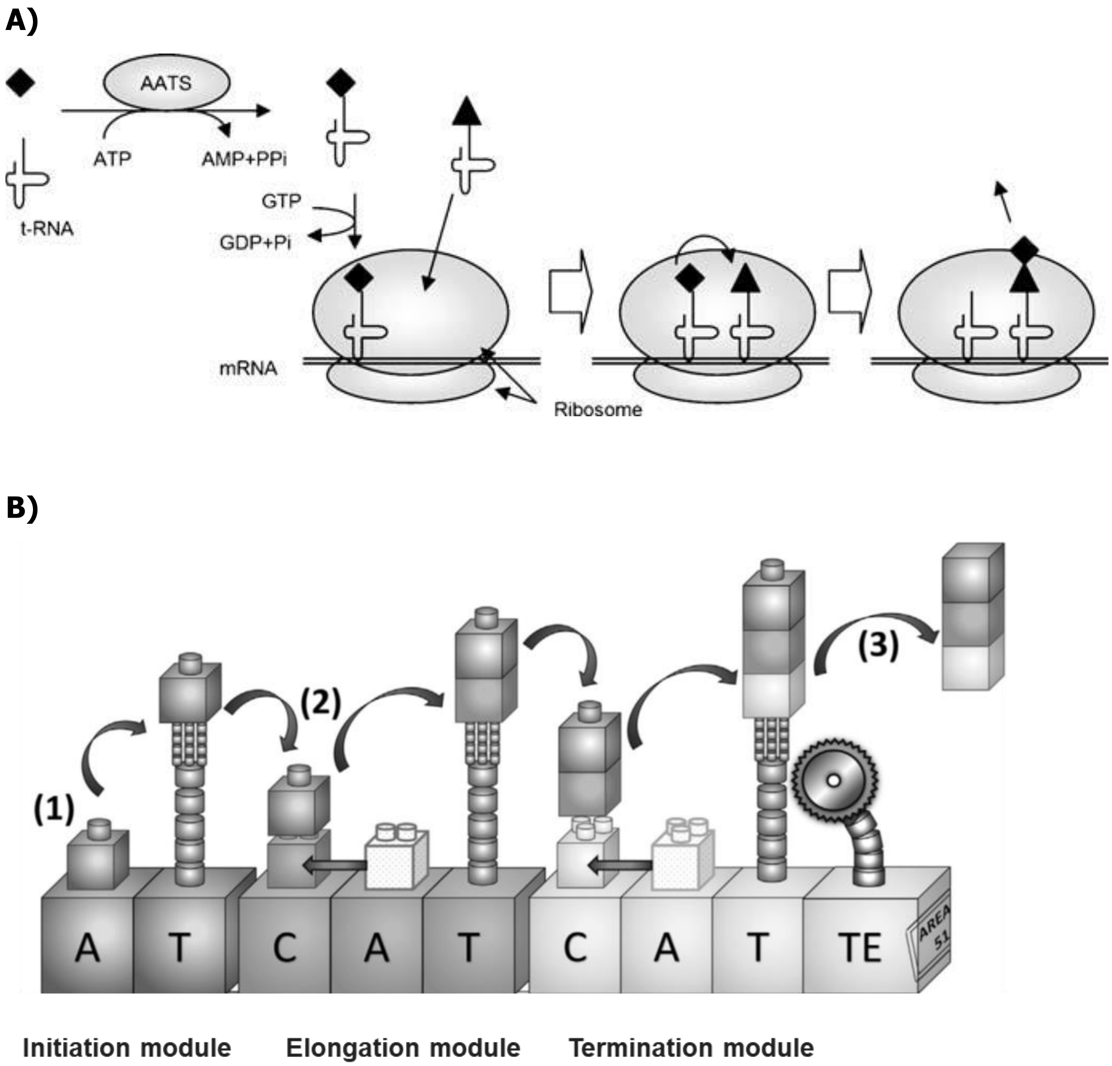


Figure 1 Schematic diagram of peptide synthesis by the ribosomal system and non-ribosomal system. A) Ribosomal peptide synthesis machineries consisting of mRNA, ribosome, tRNA and amino acids (closed diamonds and triangles). Aminoacyl-tRNA synthetase (AATS) recognises and catalyses the attachment of amino acid to tRNA during the growing of peptide chain. B) Organisation of the Nrps comprising of an initiation, elongation and termination modules. Additional elongation modules can be presented for incorporating more amino acid residues into the peptide chain. Adenylation (A), thiolation (T) and condensation (C) domains are core domains of a minimal module. Initiation module always lacks of the C-domain, whereas the termination module possess a thioesterase (TE) domain.

The approach that has been used for the production fo artificial peptide product is modular or domain shuffling of the *nrps* genes. Customised peptides have been successfully created in bacteria via interchanging of NRPS structures (Mootz *et al*., 2000; Finking and Marahiel, 2004; Nguyen *et al*., 2006; Beer *et al*., 2013; Calcott *et al*., 2014; Winn *et al*., 2014; Kries *et al*., 2015). So far, there has not been a report on the production of customised peptides using the NRPS system in *A. oryzae*. Therefore, the availability of *nrps* genes and gene editing tools, it is conceivable that the NRPS system in this fungus could be exploited for the production of oligopeptides with diverse applications.

Acv synthetase

Acv (L-α-aminoadipyl-L-cysteinyl-D-valine) synthetase is categorised as a Nrps, which shares mechanism in the peptide synthesis similar to other Nrps. The *acv* synthetase gene has been discovered in Ascomycetes, such as *Penicillum chrysogenum, Aspergillus nidulans* and *A. oryzae* (Tahlan et al, 2017). Modular structure of the enzyme is shown in Figure 2 (Keller *et al*., 2005). This enzyme recognises α-aminoadipic acid (Aaa), cysteine (Cys) and valine (Val) molecules in conjunction with the generation of D-Val by the function of epimerisation (E-) domain. Previously, it has been shown that overexpression of ACV synthetase under the control of the strong *tef1* promoter in *A. oryzae* could enhance the production of penicillin, which is an acv-derived product (Marui *et al*., 2010). Penicillin yield was increased in *A. nidulans* by 30% with the replacement of the heterologous *alcA* promoter (Kennedy and Turner, 1996). Therefore, engineering of the *acv* gene is feasible as a rational re-designing of other oligo-peptide synthesis.

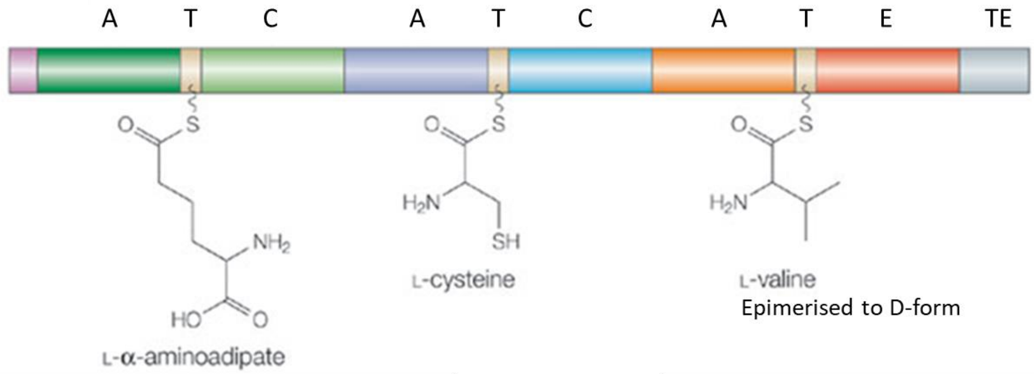


Figure 2 Schematic diagram of Acv synthetase structure. The enzyme structure consists of three modules. Module 1 contains A- and T-domains. Modules 2 and 3 contain three core domains: C-, A- and T-. The E- domain is incorporated into the last module for converting L- to D-form of valine. This last module also contains TE-domain for releasing the peptide molecule. The A-domain in each module is specific to different amino acid as indicated.

3. Objectives

Main objective: To rationally engineer *Aspergillus oryzae* for the biotechnological production of oligopeptides through the CRISPR-Cas9 mediated multi-gene editing technology

Specific objectives:

1. To reconstitute the *acv* gene and investigate its functional activity for dipeptide (Glu-Leu) and tripeptide (Pro-Glu-Leu) synthesis
2. To evaluate the phenotypes of engineered strains compared with the wild type
3. To study the role of nitrogen, C:N ratio and physical conditions in oligopeptide production of engineered strains
4. To analyse transcriptional response of engineered strain using different nitrogen sources

4. Materials and methods

4.1 Strains and cultivation

The wild type strains of *Aspergillus oryzae* BCC 7051 and *Trichoderma virens* TBRC 4129 were routinely grown on Czapek Dox (CD) and potato dextrose agar media, respectively at 30 °C for 3-5 days and subcultured every month. Spore inoculum of *A. oryzae* was prepared for submerged cultivation by cultivating the mycelial cells on sterile rice for 5 d. Spores were harvested by suspension in 0.05 % (v/v) Tween 80 solution, filtrated through miracloth, and subjected to centrifugation. *T. virens* TBRC4129 was grown in 10 ml PDB at 30 °C for overnight and subjected to genomic DNA extraction.

Escherichia coli DH5α (ThermoFisher Scientific, USA) (*supE44*, *ΔlacU169*, (*Φ80*, *lacZΔM15*), *hsdR17*, *recA1*, *endA1*, *gyrA96*, *thi1*, *relA1*) was used as a recipient for propagation of the constructed recombinant plasmids. *E. coli* transformant was grown in Luria–Bertani medium (LB) containing 100 mg/l of ampicillin at 37°C with shaking at 200 rpm.

Saccharomyces cerevisiae strain INVSCI (Invitrogen, USA) (*MAT*α, *his3-Δ1*, *leu2*, *trp1-289*, *ura3-52*, *MAT*, *his3-Δ1*, *leu2*, *trp1-289*, *ura3-52*) was used for plasmid construction. The yeast was grown in either a complete medium, YPD (1%, w/v yeast extract, 2%, w/v peptone and 2%, w/v glucose) or a selective medium, SD (0.67%, w/v yeast nitrogen base and 2%, w/v glucose) with supplementation of L-tryptophane, L-histidine-HCl and L-leucine at concentrations of 20, 20 and 30 mg/l, respectively. The yeast cultures were incubated at 30 °C with shaking at 200 rpm.

4.2 Reprogramming the regulatory control mode of *acv* expression by promoter exchange and construction of ACV-producing strain

4.2.1 Searching of L-α-aminoadipyl-L-cysteinyl-D-valine synthetase gene (*acv*) from *A. oryzae* genome

The *acvA* sequence of the penicillin producer of *Aspergillus nidulans* (GenBank accession number An_CAA38631.1) (MacCabe *et al.*, 1991) was employed as a query for searching against the available genome of *A. oryzae* BCC7051 (Thammarongtham *et al.*, 2018) through BLAST homology search of the NCBI database (http://www.ncbi.nlm.nih.gov/).

4.2.2 Construction of recombinant plasmids for promoter exchange

The pCAP-dPacv plasmid carrying the Cas9-sgRNA cassettes was constructed. Two sgRNA cassettes of the Cas9 plasmid targeting at the 5′- and 3′-ends of the *acv* promoter were designed from the genome sequence of *A. oryzae* BCC 7051, and their expressions were controlled by two PU6 promoters. These DNA fragments and the linearised pCAP plasmid were combined using yeast assembly technique (Pahirulzaman *et al.*, 2012), and the resulting plasmid was propagated in *E. coli*. DNA sequencing analysis was carried out to verify the assembly regions within the plasmid.

To construct the pPAotef1, pPAogpdA and pPPttoxA plasmids, the promoter fragment of *toxA* (*PtoxA*) sequence was amplified from the pCT74 plasmid (Lorang *et al.*, 2001) carrying the promoter sequence from *Pyrenophora tritici-repentis*, and the promoter fragments of *Aotef1* and *AogpdA* (*AoPtef1* and *AoPgpdA*, respectively) were amplified from the genomic DNA of *A. oryzae* BCC 7051 using Platinum™ Tag DNA polymerase Hi-Fidelity with overlapping primer sets. The homologous sequences were designed, wherein the 5′- and 3′-fragments were homologous to the 5′-UTR of the *acv* synthetase gene. The promoter fragment and two homologous fragments were assembled with the YEp356 plasmid backbone in the yeast cell, and the constructed plasmid was then propagated in *E. coli* cell. The plasmid analysis by enzyme restriction was carried out, and DNA sequences at the assembly points were verified to ensure the in-frame translation start site. Each restricted promoter fragment was co-transformed with the pCAP-dPacv plasmid in *A. oryzae.*

4.2.3 Fungal transformation

For fungal transformation, 2-5 μg of plasmid or DNA fragments were transformed into the protoplast cells by the PEG-mediated transformation method (Chutrakul *et al.*, 2016). Fungal transformants were grown on Czapek Dox medium and incubated at 30 °C for 5 - 14 days. Single spore isolation was carried out to obtain pure culture. The PyrG re-cycling step was performed by addition of 5-FOA, uridine and uracil into the medium. For investigating the mutation point derived from the homologous replacement event, the genomic DNAs extracted from the transformant mycelia were subjected to PCR using specific oligonucleotide primers and Platinum™ Tag DNA polymerase Hi- Fidelity. Amplicons were used for sequence analysis.

4.2.4 Gene expression determination by real-time quantitative PCR (RT-qPCR)

The *A. oryzae* wild type culture was grown in SM medium (4%, w/v glucose, 0.5%, w/v yeast extract, 0 .02%, w/v NH_4Cl , 0.24%, w/v KH_2PO_4, 0.05%, w/v $MgSO_4 \cdot 7H_2O$, 0.01%, w/v $CaCl \cdot 2H_2O$, 0.0015%, w/v $FeCl_3 \cdot 7H_2O$, 0.001%, w/v $MnSO_4 \cdot H_2O$, and 0.008%, w/v $ZnSO_4 \cdot 7H_2O$) (Laoteng *et al.*, 2008) and subjected to total RNA extraction using PureLink™ RNA Mini kit (Invitrogen, CA). The reverse transcription of total RNA to cDNA was performed using the 1× RevertAid First Strand cDNA Synthesis Kit (Thermo Fisher Scientific) according to the manufacturer's instruction. RT-qPCR was carried out according to the previous method (Jeennor *et al.*, 2019). 18S rRNA gene was used for normalisation of the targeted transcript level. The experiments were independently analysed in triplicate.

4.2.5 Submerged cultivation of the engineered strain for ACV production

The effect of glucose on fungal growth and ACV production was carried out using the SM by varying glucose concentrations (4, 6 and 8%, w/v). Using the optimized glucose concentration (6%, w/v), the inorganic nitrogen (NH_4Cl and $NaNO_3$), organic nitrogen (urea and yeast extract), and mixed nitrogen sources (NH_4Cl plus yeast extract) were further investigated. In addition, the C:N ratio (10:1, 20:1 and 40:1; mol:mol) and $MgSO_4$ concentrations (0, 2, 5, 10 and 50 mM) were variables for the cultivation optimisation. For the experiments with different C:N ratios, the nitrogen molar concentrations were varied, in which the initial glucoses concentration (60 g/l) was kept constant.

4.2.6 Biomass determination and residual glucose concentration measurement

Mycelial samples were collected at different time points by filtration and washed with sterile water followed by baking in a hot-air oven until the weight was constant. The culture filtrate was subjected to residual glucose measurement by HPLC. The HPLC condition was set up at 60 °C for 30 min with a flow rate of 0.6 ml/min using Aminex® HPX-87H column (300 x 7.8 mm) and 18 mM sulfuric acid as a mobile phase. The residual glucose concentration was calculated using a calibration curve of glucose standard at the concentrations of 0.1 to 10.0 mg/ml.

4.2.7 Preparation of fungal crude extracts

Crude extract was prepared from the ground fungal mycelia and suspended in absolute methanol followed by incubation on ice for 0.5 h. The methanol phase was collected by centrifugation at 4 °C. A volume of 20 µl was subjected to reverse-phase high-performance liquid chromatography (RP-HPLC) or liquid chromatography-mass spectrometry (LC-MS).

4.2.8 Analysis of ACV tripeptide by RP-HPLC

The ACV trifluoroacetate salt (BACHEM, Switzerland) was used as a tri-peptide standard. The solution was prepared by resolving the standard in deionised water and diluted to the final concentration of 1.0 mg/ml. Serial dilution was carried out, and 5 μl solution was subjected to HPLC analysis using C-18 Acclaim™120 column (4.6 × 250 mm, C18, 5 μm ∅). The gradient system was set up using the solvent mixture A (0.05%TFA in deionised water) and mixture B (0.065%TFA in acetonitrile) as follows: 15 to 50% mixture B for 15 min, then 50 to 65 % mixture B for 2 min and finally 65 to 15% for 3 min with the flow rate of 1 ml/min. The eluents were monitored at 220 nm with running time of 20 min. A volume of 20 μl fungal extract was subjected to HPLC analysis, and the ACV concentration was calculated using the calibration curve of the ACV standard (0.0156-1.0000 mg/mL).

4.2.9 Analysis of ACV tripeptide by LC-MS

For LC-MS analysis, electrospray ionisation mass spectrometry (ESI-MS), a micrOTOF instrument (Bruker Daltonis®, Germany) and Agilent 1200 series HPLC (Agilent Technologies, USA) were used to analyse the oligo-peptides by on-line coupling of the instruments. Injection volume was 10-20 μl, and the HPLC condition was set up as a gradient system of solvent mixture A (0.05% formic acid in deionised water) and mixture B (0.05% formic acid in 5% acetonitrile) with the flow rate of 0.3 ml/min. Elution peaks were detected at the wavelengths between 196-600 nm. A micrOTOF mass spectrometer with positive mode was used to determine mass spectra at the set capillary of 4,500 V, dry gas flow rate of 4.0 l/min and the dry temperature of 180 °C. The mass spectra of the samples were determined in a range of 100-1000 Da.

4.3 RNA sequencing-based transcriptome analysis

4.3.1 RNA isolation and sequencing

The ACV production medium (60 g/l glucose and 2.55 g/l urea with the C:N ratio of 20:1, and 10 mM $MgSO_4$) was used for cultivation of the AoGpdA and recipient strains, which were ACV-producer and non-producer, respectively. Mycelia were collected from the 3-d cultures and washed with DEPC-treated water. Total RNA was isolated using PureLink™ RNA Mini kit (Invitrogen). The experiments were carried out in three independent experiments for each strain. Total RNA content was measured using BioPhotometer® D30 (Eppendorf AG, Germany). Extracted RNA was DNase-treated (RNase-free DNase I, Thermo) to remove the genomic DNA. The obtained total RNA was sent to stranded paired-end mRNA-seq sequencing using the Illumina Novaseq Platform of Novogene Co., Ltd (Singapore).

4.3.2 Transcriptome analysis

Based on the transcriptome data of *A. oryzae*, DESeq2 and EdgeR software provided by Novogene Co., Ltd. were used for analysing the differentially expressed genes (DEGs) by the value of fragments per kilobases of exon region per million mapped reads (FPKM). Using $\log_2$ fold change ≥ 2.0, *p*-values 0.001, and FPKM ≥ 100 as cut-off values, the significant DEGs were grouped based on their functions.

4.4 Studying the functional role of ammonium transporters in *A. oryzae*

4.4.1 Identification of ammonium transporter homologs of *A. oryzae*

To identify putative ammonium transporter genes, BLAST search against the available genome sequence of *A. oryzae* strain BCC 7051 was performed using NCBI database (http://www.ncbi.nlm.nih.gov/) and *amt* sequences of *Aspergillus flavus* as queries. cDNA cloning of these putative genes was carried out by r*everse transcription polymerase chain reaction* (*RT-*

PCR) using total RNA of *A. oryzae* BCC 7051 as a template. Four cDNA sequences were obtained, namely *Aoamt1*, *Aoamt2*, *Aoamt3* and *Aoamt4*.

4.4.2 Submerged cultivation of *A. oryzae* for the differential gene expression analysis and ammonium consumption

Spore suspension (10^6 spores) of the wild type of *A. oryzae* strain BCC 7051 was inoculated into 50 ml modified Czapek Dox medium (mCD) (10 g glucose, 5 g yeast extract, 1 g K_2HPO_4, 0.5 g $MgSO_4.7H_2O$, 0.5 g KCl, 15 mg $FeCl_3{\cdot}7H_2O$, 10 mg $MnSO_4{\cdot}H_2O$ and 7.5 mg $ZnSO_4{\cdot}7H_2O$ with initial pH adjusted to 4.5) as a basal medium. Fungal cultivation was carried out at 30 °C with shaking for 16 h. Mycelial cells were harvested, washed twice with sterile distilled water, and resuspended in 50 ml mCD without nitrogen (ammonium-free medium) at 30 °C with shaking for 4 h. Then fungal mycelia were transferred to the new ammonium-containing medium, which yeast extract was replaced with 1 or 20 mM NH_4Cl (Sigma-Aldrich) as a sole nitrogen source, and the cultivation was further carried out at 30 °C. Mycelial samples were then harvested at different cultivation times for studying the differential gene expression by RT-qPCR as described above. The culture broths were subjected to analyse the ammonium consumption (see below). All experiments were independently performed in triplicate.

4.4.3 Construction of Aoamt disrupted and overexpressed strains

To construct the disruption plasmids, pDAoamt2 and pDAoamt3, the specific 5′ and 3′ DNA fragments with homologous sequences of individual *Aoamt* genes were amplified using the genomic DNA of *A. oryzae* BCC 7051 as a template, Platinum™ Tag DNA polymerase Hi Fidelity (Invitrogen) and overlapping primer sets. DNA assembly of the homologous fragments and the linearized backbone plasmid BPO152 carrying *AopyrG* marker was performed in *S. cerevisiae*. Plasmids were extracted from the yeast transformants using Zymoprep™ Yeast plasmid Miniprep I (Zymo Research), and were then shuttled to *E. coli* cells. The constructed plasmids were confirmed by DNA sequencing. To generate the overexpression plasmids, pOEAoamt2 and pOEAomt3, the *toxA* promoter and the *nos3* terminator fragments were amplified from the plasmid pCT74 (Lorang *et al.*, 2001). The *Aoamt* cDNAs was amplified from the cloning plasmids by using overlapping primers. Then, DNA assembly of these fragments with the linearized backbone plasmid BPO152 was carried out in the yeast cells. The plasmid or DNA fragment (2 – 5 µg) was transformed into the protoplast cells by the PMT method as described above. Transformants were selected on CD medium after incubation at 30 °C for 3–7 d. Spore re-isolation was performed to obtain pure culture. To verify the disruptive and overexpressing strains, total RNA extracted from the mycelial cells of the transformants were subjected to the Super Script III One-Step RT-PCR with Platinum *Taq* DNA Polymerase (Invitrogen, CA) using specific oligonucleotide primers.

4.4.4 Radial growth and dry cell weight measurements

To investigate mycelial growth on solid agar, spore inoculum (5000 spores) of the recipient (*pyrG* prototroph) and the recombinant strains was individually dropped onto the mCD agar added with 1 or 20 mM NH_4Cl and incubated at 30°C for three days. Each experimental set was carried out in three technical replicates.

For dry cell weight measurement, mycelial samples grown in SM broth were collected at different cultivation times by filtration with gentle suction, then washed with sterile water and dried in hot-air oven until the constant weight was obtained. The biomass titre is represented as dry weight per litre (g/l).

4.4.5 Determination of ammonium concentration

Ammonium concentration was determined calorimetrically using the Phenol-hypochlorite method with some modifications from the published method (Watherburn, 1967). The filtrated broth (0.1 ml) of the culture grown in the medium containing ammonium was thoroughly mixed with 0.5 ml of phenol (5%, v/v) and nitroprusside (0.005%, w/v), and then 0.5 ml alkaline hydrochlorite solution (0.5%, w/v sodium hydroxide and 0.8%, v/v sodium hypochlorite) was added. The mixture was incubated at 37°C for 20 min and subjected to measure the calorimetrical absorbance at 625 nm. Ammonium chlorite (0.1-0.5 mM) was used as a standard. Concentrations were reported as millimoles of ammonium per milliliter (mM/ml).

4.5 Module reconstitution of *acv* gene in *A. oryzae*

4.5.1 Prediction of Nrps adenylation (A-) domain specificity

The query A-domains of Nrps mega-enzyme were predicted for substrate specificity by using the NRPSpredictor2 web server (Röttig *et al.*, 2011: http://nrps.informatik.uni-tuebingen.de/). The prediction of A-domain specificity was based on the configuration of amino residues spanning in the active site of an A-domain. *nrps* gene sequences of *hts1* (HC-toxin synthetase accession no. AAA33023), *simA* (cyclosporine synthetase accession no. Z28383), *tex1* and *tex2* (*T. virens* peptaibol synthetases accession no. AF4690454 and EHK23788, respectively) retrieved from the available database were submitted to the web server for the analysis of signature sequences of Pro, Glu and Leu.

4.5.2 Construction of recombinant strains for module reconstitution of *acv* gene

The Cas9-sgRNA plasmid, pCAP-dA1A3acv, was constructed. Two sgRNA cassettes of the pCAP-dA1A3acv plasmid targeting at the 5′- and 3′-ends of the *acv* gene were designed from the genome sequence of *A. oryzae* BCC 7051, and the expression was controlled by PU6 promoters.

Each DNA fragment was assembled with the linearised pCAP backbone plasmid in yeast cells, and the resulting plasmid was propagated in *E. coli* cell as aforementioned. Restriction enzyme analysis and DNA sequencing were carried out to verify the sequences of the constructed plasmids.

Construction of the plasmids for two module reconstitution (pGluLeu (EL), pProLeu (PL)), three module reconstitution (pProGluLeu (PEL), pAoProGluLeu (AoPEL), and pAoAaaCysPro (AoACP)) of *acv* gene was performed. The *glu*, *leu*, *pro* gene modules were amplified from the genome sequence of *T. virens*, while *Aoaaa, Aocys* and *Aopro* were amplified from *A. oryzae* genomic DNA*.* The 5′- and 3′-homologous sequences were designed to recombine the promoter of AogpdA and the 3′-end of the *acv* gene, respectively. Selected gene modules with the 5′- and 3′-homologous fragments were assembled with the YEp356 plasmid backbone in yeast cell, and individual plasmid was further propagated in *E. coli*.

The module engineering was carried out by co-transformation of two or three module reconstitution plasmid linearised by enzyme restriction, and the pCAP-dA1A3acv plasmid into *A. oryzae* protoplast cell*.* Recombinant clones were selected on CD medium without nutrient supplementation and re-isolated to acquire pure culture. Reconstitution of the *acv* gene with tailor-made module genes was verified by RT-PCR using the SuperScript® III One-Step RT-PCR system, and the DNA amplification was performed using Platinum® Taq DNA polymerase (InvitrogenTM, USA) with a primer set specific to leucine or proline A-domain as a target.

4.5.3 Fungal cultivation

A defined medium modified from the SM lacking yeast extract, which the C:N ratio was maintained, was prepared. In addition, the complete medium (20 g/l (w/v) glucose, 20 g/l (w/v) malt extract and 10 g/l (w/v) peptone) (http://www.fgsc.net/methods/anidmed.html) was used in this study. The culture was incubated with shaking at 250 rpm for 7 d.

4.5.4 Fungal extraction and analysis

Fresh mycelial cells were extracted with 100-200 ml methanol and left for 1-2 h followed by centrifugation. Clear supernate was evaporated and then suspended in methanol to obtain the final concentration of 10 mg/ml. To prepare crude extracts from the culture broth, liquid-liquid extraction (LLE) was performed. A volume of ethylacetate solution was added to the broth and vigorously shaken for two hours. Broth extracts were collected from the solvent phase and left to dryness before suspending in methanol as described for mycelial extracts.

Solid-phase extraction (SPE) was adopted for oligopeptide preparation from the fungal samples. In this study, the SPE method and resolving agents used for siderophore and anti-microbial peptide extraction were employed with some modifications (Munawar *et al.*, 2013; พึ่งแพง *et al.*, 2555). Fresh ground mycelia were suspended in phosphate buffer pH 7.5. 100 ml culture broth was also adjusted to the same pH value. Then, the pre-equilibrated amberlite XAD-7 resin (3 - 5 g) was used to trap putative peptide metabolites, which were finally removed by 40-100% methanol solution. Dry extracts were suspended in methanol and then subjected to LC-MS analysis as described above.

4.6 Heterologous expression of indigoidine synthetase gene (*inK*) in *A. oryzae*

Indigoidine (5,5'-diamino-4,4'-dihydroxy-3,3'-diazadiphenoguinone-(2,2'), InK) is a natural dark blue pigment consisting of two L-glutamine residues. It is produced by several groups of bacteria during the stationary phase. Biosynthesis of InK is done by indigoidine synthetase, a member of Nrps family. In this study, Ink-producing strain of *A. oryzae* was constructed and heterologous production of the pigment was studied.

4.6.1 Construction of expression plasmid and generating the indigoidine-producing strain (AoInK)

The indigoidine-overexpressing plasmid, pAoInk-PyrG, was constructed. The 4134-bp indigoidine synthetase gene (*Aoin*K) with intronless was retrieved from *Streptomyces chromofuscus*, and the codon-optimised gene for heterologous expression in *A. oryzae* was designed based on the OptimumGene™ algorithm by Genscript (Piscataway, USA), and then was amplified from the pUC57-AoIndC plasmid (GeneScript, USA) by PCR using Platinum™ Tag DNA polymerase Hi Fidelity and overlapping primer sets. The DNA fragments, including a constitutive promoter, a terminator and the *pyrG* marker, were also amplified by PCR. Using 20-bp overlapping primer sets, all four DNA fragments and *Eco*RI-linearized backbone plasmid (pPNGB) (Punya *et al.*, 2013) were co-transformed to *S. cerevisiae* cells using the PEG/lithium acetate method (Invitrogen, USA). After *in vivo* recombination in the yeast cells, the yeast transformant carrying the pAoinK-pyrG plasmid was grown in uracil-lacking medium and subjected to the extraction of circular plasmids using Zymoprep™ Yeast Plasmid Miniprep (Zymo Research, USA). Purified plasmid was then transformed into *E. coli* for its propagation. Restriction enzyme analysis and DNA sequencing were carried out to verify the accuracy of the constructed plasmid. Plasmid transformation into the *pyrG*-deficient strain *A. oryzae* was done by PEG-mediated method (PMT) as described above. After cultivation on Czapek Dox (CD) medium without supplementation at 30 °C for 10-14 days, the transformants were selected based on dark blue colonies because of the pigment production. Spore re-isolation was performed to obtain pure culture of Ao-InK strain.

4.6.2 Submerged cultivation of AoInK strain

SM was used as a basal medium for fungal cultivation. Initial pH conditions (4.5, 5.5, 6.5 and 7.5) were variables. The culture temperatures (25, 30, and 35°C) were further investigated using the optimal initial pH condition. With the optimal temperature and initial pH condition, L-glutamine concentrations (2, 5, and 10 mM) and $MgSO_4$ concentrations (2, 5, 10, and 50 mM) were also investigated to optimise the indigoidine production. Polyethylene glycol (PEG 4000), Tergitol NP-40, Triton X-100 and Tween 80 (Sigma-Aldrich, USA) at different concentrations (1, 3 and 5%, w/v) were used to investigate the extracellular indigoidine production. Fungal cultures were incubated at 25°C for 5 days with shaking at 200 rpm. The fungal cultivation in the presence of selected surfactant at optimal condition was performed to examine the growth kinetic and InK production yield. Determination of biomass and residual glucose concentration was performed as described above.

4.6.3 Indigoidine extraction and quantification

Fresh fungal mycelia were ground with liquid nitrogen, and then subjected to extract indigoidine by adding 1 ml of dimethylsulfoxide (DMSO) with sonication as previously described (Wehrs *et al.*, 2019). For extracellular pigment extraction, the culture broth was diluted with 100% (v/v) DMSO solution. The prepared samples were subjected to analyse indigoidine using a spectrophotometer (Jasco V-730BIO) with a wavelength of 612 nm. Indigoidine standard (ViabLife, PRC) was used to prepare a calibration curve for calculating the pigment concentration of the crude extract.

5. Results and discussion

5.1 Promoter exchange of the cryptic non-ribosomal peptide synthetase gene for oligopeptide production in *A. oryzae*

5.1.1 Identification, characterisation and expression analysis of *acv* gene in *A. oryzae*

Blast analysis using the *acvA* of *A. nidulans* as a query revealed the presence of a single copy of 11.22-kb *acv* sequence with intronless in the putative penicillin biosynthesis gene cluster of *A. oryzae* BCC 7051 (accession number Ao_OOO08980.1). Its deduced amino acid sequence contained 3741 residues rendering the unique modular characteristics of Nrps. In each module, the conserved domain with substrate binding pocket sequence, which plays role in recognition of α-Aminoadipic acid (Aad), cysteine (Cys) and valine (Val) for incorporation into the newly synthesised peptide product, was identified. The amino acid sequence of this putative Acv sequence shared 67 – 99% identity to that of *A. nidulans* (An_CAA38631.1), *A. oryzae* strain RIB40 and *P. chrysogenum* PCBAB, which are known as the industrial penicillin producer.

RT-qPCR analysis revealed that the expression level of *acv* gene of the fungus did not much alter during the cultivation time (18-60 h) (Figure 3). However, the highest gene expression level was observed at 60 h of the cultivation (stationary phase), which 1.6-fold increase of expression level was relatively detected when compared to the basal expression of the early logarithemic cultures (18-24 h). This result was rather common for the biosynthesis of secondary metabolites which occurred during the stationary or non-growth phase (Calvo *et al.*, 2002; Gerke and Braus, 2014).

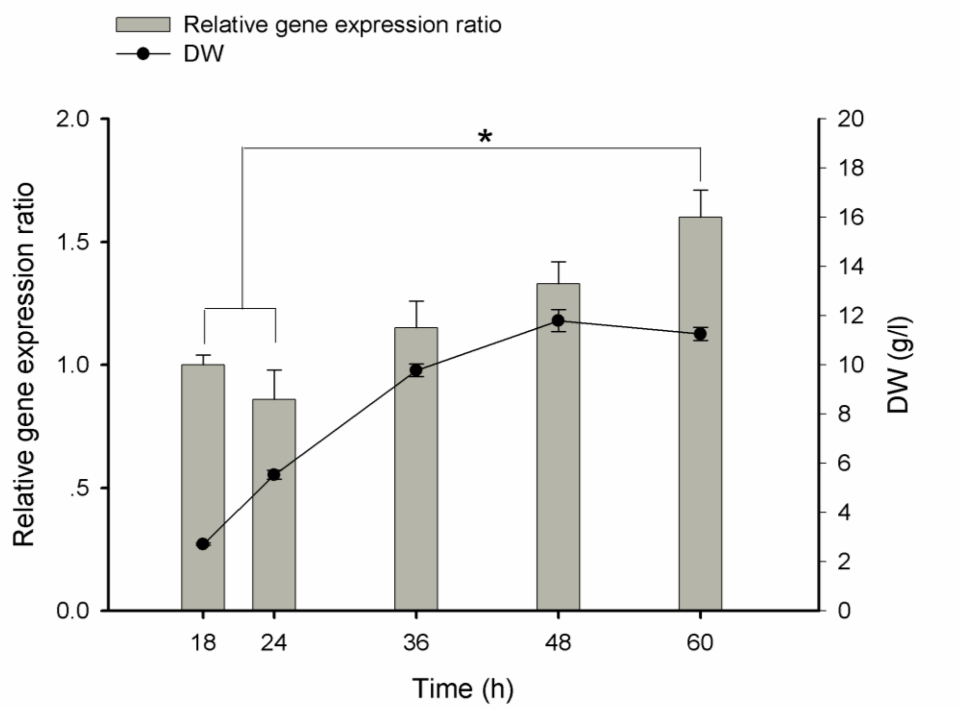


Figure 3 The *acv* gene expression and cell biomass of *A. oryzae* at different cultivation times. Mycelial samples were harvested at different time points for determination of cell growth. RT-qPCR analysis of *acv* expression in *A. oryzae* was carried out, showing relative expression level at different cultivation times. The expression of 18-h culture (early log phase) was used as a control. LSD post hoc test was performed in SPSS indicating the significant difference (p value < 0.05) in expression levels marked by an asterisk.

5.1.2 Control of *acv* gene expression in *A. oryzae*

The control of *Acv* gene expression can be manipulated by a strong promoter. Reprogramming *acv* expression in *A. oryzae* was implemented by replacement of a native promoter with different constitutively strong promoters through the CRISPR-Cas9 technique. The constitutively strong promoters used in this study were *AoPgpdA, AoTtef1* and *PtPtoxA*. For conducting promoter exchange, a set of plasmids were constructed including the pCAP-dPacv for removing the native *acv* promoter, and pAoPgpdA, pAoPtef1 and pPtPtoxA for promoter replacement. These plasmids were co-transformed into the *pyrG*-deficient strain (recipient), generating the respective AoGpdA-, AoTef1-, and PtToxA-engineered strains. By PCR and DNA sequence analyses, the 5′-UTR of the *acv* gene in individual engineered strains was successfully replaced with the constitutive promoter (Figure 4).

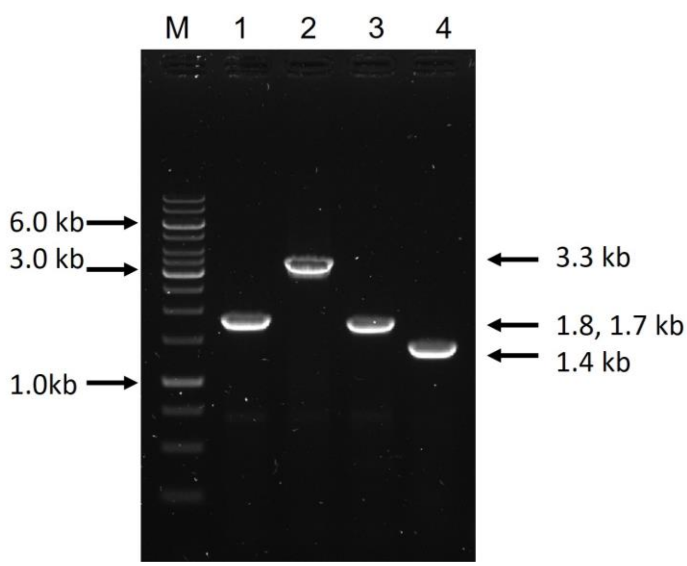


Figure 4 PCR analysis of the exchanged promoter fragments in the engineered strains. Lane 1 indicates the amplified DNA fragments of 1.8-kb of the recipient strain (native promoter). Lanes 2, 3 and 4 indicate the 3.3-, 1.7- and 1.4-kb DNA fragments amplified from the engineered strains harboring *AoPgpdA, AoPtef1*, and *PtPtoxA* promoters, respectively. M is Thermo Scientific GeneRuler 1-kb DNA Ladder.

The efficiency of *acv* transcription under the control of exchanged promoters was evaluated by RT-qPCR. In relation to the 18S rRNA transcript, the *acv* expression levels of the engineered strains carrying those promoters grown for 24 h were determined in comparison with that of the recipient strain (Figure 5). It was found that the relative expression level of the recipient strain was extremely low similar to that of the wild type. The highest transcript level of *acv* was detected in the AoGpdA culture followed by the AoTef1 culture. Notably, the expression level of the PtToxA culture was still low, but it was slightly higher than the expression in the wild type and recipient strain (native promoter). This result indicated the preference in its own promoters for driving the targeted gene in the host cell. The *AoPgpdA* is a promising strong promoter which would be used for overexpressing the targeted gene in addition to the gene involved in secondary metabolite production for addressing the gene function or strain improvement.

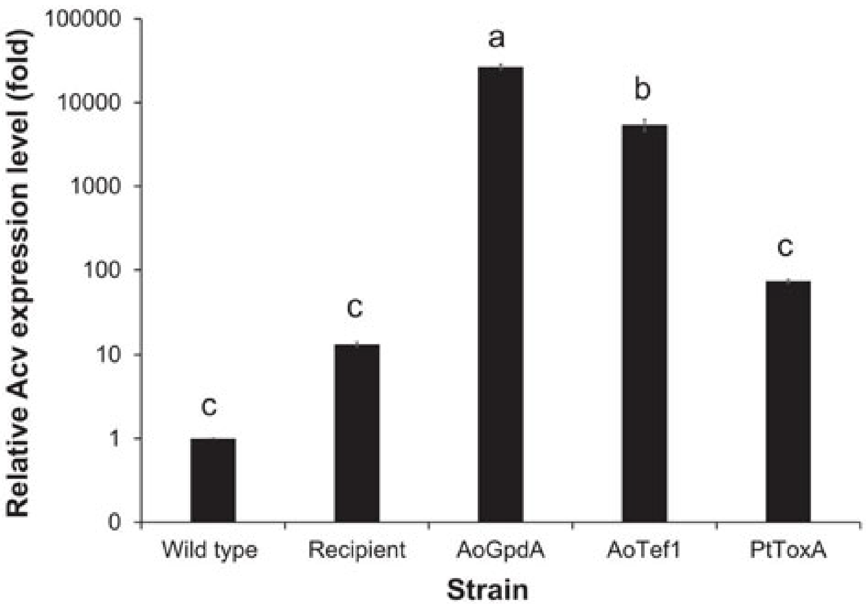
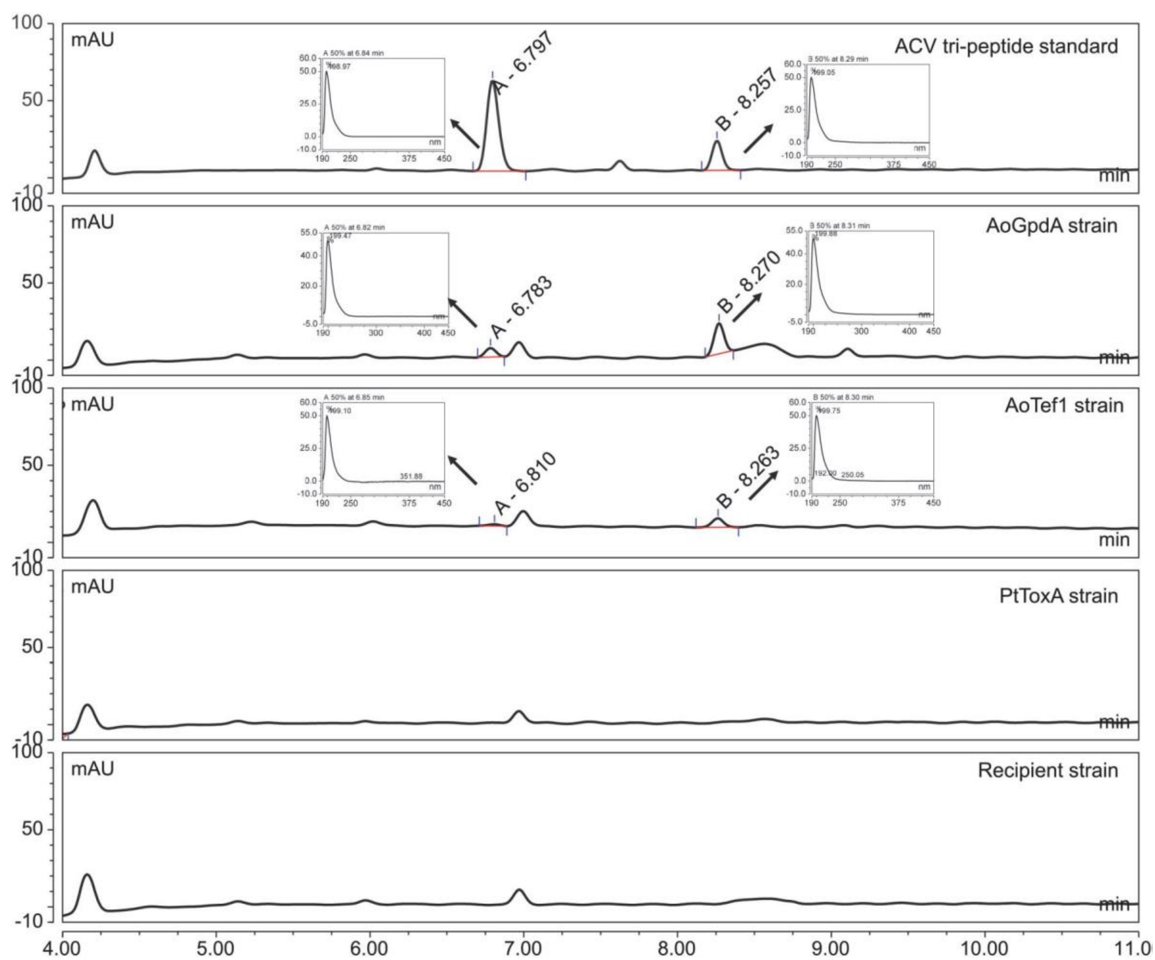


Figure 5 Relative *acv* expression levels under the control of native and exchanged promoters in *A. oryzae* strains. The wild type, and recombinant strains carrying the *AoPgpdA, AoPtef1*, and *PtPtoxA* promoters were subjected to RT-qPCR analysis. The relative expression level of the wild type was adjusted to 1. Letters (a and b) above the bars indicate a statistical significance of the *acv* transcript levels among different strains, which were analysed by Duncan's multiple range test (MRT) (p value < 0.05). The experiments were carried out in triplicates.

5.1.3 ACV tripeptide production in the engineered strains of *A. oryzae*

Functional activity of Acv mega-synthetase enzyme in the biosynthesis of ACV tripeptide of the engineered strains was analysed. RP-HPLC analysis showed that the methanol crude extracts of the AoGpdA and AoTef1 cultures contained two UV-visible spectral peaks (peaks A and B) with retention times of 6.78–6.81 and 8.25–8.27 min, respectively (Figure 6A). They were identified as ACV tripeptide molecules when compared with the peptide standard. However, both of them were undetectable in the PtToxA and recipient extracts. Noticeably, penicillin G was not observed in the extracts of all strains tested. To verify the molecular mass of the putative ACV tripeptides generated, the crude extract obtained from the AoGpdA strain was further subjected to LC–MS analysis. Two chromatographic peaks (peaks A and B) identified in the extract of the AoGpdA strain contained molecular mass ions at *m/z* 364.20 and 727.41 (peak A), whereas the ions at *m/z* 363.20 and 725.39 of peak B were detected (Figure 6B). Molecular masses derived from the engineered strain were reliable to the respective standard. These results indicated that the engineered strains of AoGpdA and AoTef1 were able to synthesise ACV tripeptide molecules in contrast to the recipient and PtToxA strains.

A)



B)

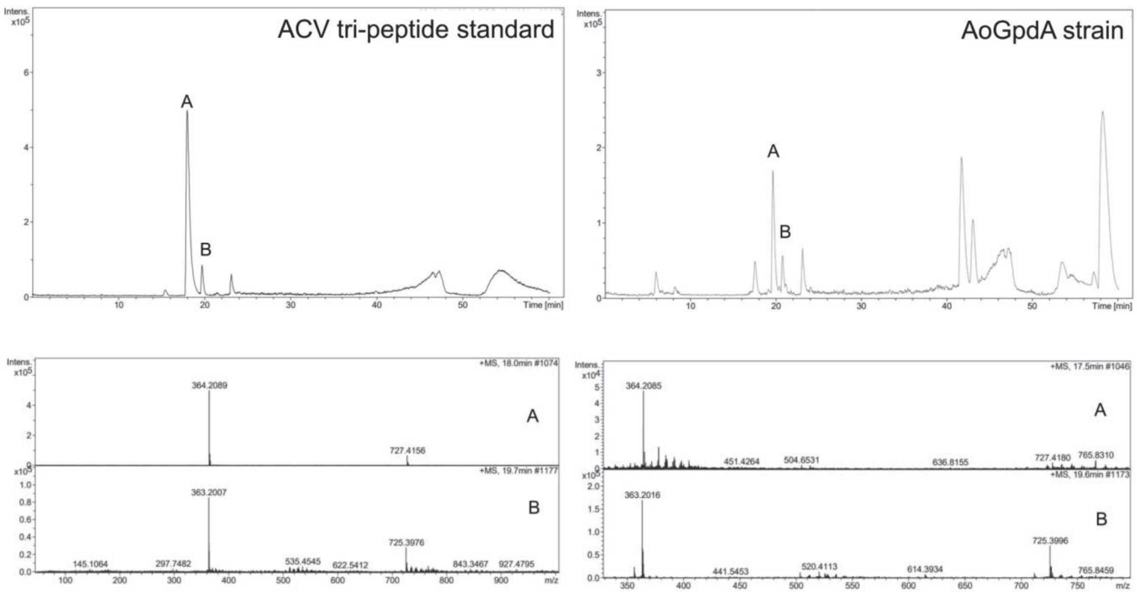


Figure 6 ACV tripeptide analysis of the engineered strains. RP-HPLC analysis of the ACV tripeptide standard, AoGpdA, AoTef1, PtToxA, and recipient (control) extracts (A). Arrows indicate chromatograms of ACV peptides with different retention times (peaks A and B). Molecular mass determination of ACV molecules of the AoGpdA strain (right) compared with its authentic standard (left) by LC-MS (B). Mass ions of peaks A and B are shown.

5.1.4 Effects of cultivation conditions on ACV tripeptide production in the AoGpdA strain

As a result of AoGpdA-regulated expression of *acv*, the ACV tripeptide production in the AoGpdA strain was detected at the logarithmic phase (24-h) when cultivated in a semi-synthetic medium with 4% glucose. The total ACV titre increased with the increase of cultivation time, and the maximal titre (36.94±5.07 mg/ml) was observed in the 72-h culture, which glucose was completely consumed. This result clearly showed that *AoPgpdA* was the strong promoter for transcriptional control of *acv* gene. With respect to the nature of such promoter in driving glyceraldehyde-3-phosphate dehydrogenase gene in the glycolysis pathway (https://en.wikipedia.org/wiki/), it could function in a similar cultivation condition in the presence of glucose. The effect of glucose concentration was investigated in the AoGpdA strain by cultivation in the medium broths with different concentrations of glucose (4, 6 and 8% (w/v) glucose). Clearly, the biomass titre increased reliable on the increasing of glucose concentrations (20, 25 and 32 g/l in 4, 6 and 8% glucose conditions, respectively). However, the ACV titres were highest in the cultures using 6 and 8% glucose concentrations, which were approximately 40 – 45 mg/l.

Apart from carbon source, nitrogen substrate has been reported to contribute to the cell growth and development as well as the formation of secondary metabolites, including NRPs (Rabha and Jha, 2017). In this study, the effects of nitrogen source and concentration on the ACV production were studied in the AoGpdA strain grown in SM with 6% (w/v) glucose concentration for 4 d, which was a chosen condition for investigating cell growth and ACV production. Using 6% glucose, the biomass titres of all cultures were comparable except for the culture using NH_4Cl as a nitrogen source. The very low biomass titre found in the NH_4Cl-grown culture was caused by extreme acidic conditions (pH ≤ 1.0) generated during cell growth that significantly affected the ACV production. It was found that $NaNO_3$, urea and the mixed nitrogen sources (NH_4Cl and yeast extract) were suitable for both biomass and ACV production, yielding an ACV titre of approximately 40 mg/l, whereas yeast extract was the poor nitrogen source for ACV production (Table 3). These three nitrogen sources were chosen for further studied.

Table 3 Effect of nitrogen sources on ACV tripeptide production of the AoGpdA strain

Nitrogen source	Biomass (g/l)	ACV titre (mg/l)
Mixed nitrogen	24.25±1.08[a]	42.23±1.78[a]
Yeast extract	25.51±2.73[a]	20.86±4.17[b]
Urea	24.97±0.98[a]	39.23±6.59[a]
$NaNO_3$	25.22±0.80[a]	43.84±2.77[a]
NH_4Cl	8.34±0.13[b]	1.62±0.52[c]

Superscript letters ([a], [b] and [c]) indicate significant differences ($p < 0.05$) in biomass or ACV titres between the cultures using various nitrogen sources. The experiments were carried out in triplicates.

The effect of nitrogen concentrations on ACV production in the AoGpdA strain was investigated. All nitrogen concentrations had no negative attribute on cell growth (Table 4). Cultivation in $NaNO_3$-containing medium showed that there was no significant difference in the ACV titres when using different C:N ratios, at which approximately 40 mg/l was obtained. Low C:N ratios (10:1 and 20:1) enhanced tripeptide production when using urea or mixed nitrogen sources for fungal cultivations (Table 4), in which the ACV titre was over 60 mg/l, and the highest ACV titre was found in the urea-grown cultures, approximately 70 mg/l. These results revealed a significance of urea and its concentration on enhancing the ACV production by the AoGpdA strain, which might be explained by the nitrogen regulatory mechanism for controlling the secondary metabolism in fungi including the NRP group (Miethke and Marahiel, 2007; Tudzynski, 2014). The urea was a preferable nitrogen substrate of this fungus, which provided ammonia groups to amino acid residues, subsequently served as building blocks for NRP-based biosynthesis.

Table 4 Effect of C:N ratio on the ACV tripeptide production by the AoGpdA strain

C:N ratio (mol:mol)	Nitrogen source	Biomass (g/l)	ACV titre (mg/l)
10:1	Mixed nitrogen	29.18±1.27[a]	65.54±7.53[ab]
	Urea	23.02±0.49[d]	70.71±1.08[a]
	$NaNO_3$	20.63±1.20[e]	37.61±1.75[c]
20:1	Mixed nitrogen	27.63±0.32[b]	62.06±3.43[b]
	Urea	25.48±0.43[c]	70.53±1.04[a]
	$NaNO_3$	21.79±0.81[de]	42.27±3.38[c]
40:1	Mixed nitrogen	27.73±0.45[b]	36.73±2.25[c]
	Urea	24.97±0.98[c]	39.23±6.59[c]
	$NaNO_3$	25.22±0.80[c]	43.84±2.77[c]

Superscript letters ([a], [b], [c], [d] and [e]) indicate significant differences ($p < 0.05$) in biomass or ACV titres between the cultures using various nitrogen molar concentrations. The experiments were carried out in triplicates.

Posttranslational modification is a crucial step for synthesising functional Acv synthetase in the active *holo*-form. The posttranslational process is catalysed by a specific phosphopantetheinyl transferase (PPTase). The enzyme is become activated by the divalent cations of magnesium (Mg^{2+}) as co-factors (Lambalot *et al.*, 1996; Martín *et al.*, 2010; Muller *et al.*, 2012). Therefore, various $MgSO_4$ concentrations were investigated for ACV production by the engineered strain. It was found that $MgSO_4$ was crucial for fungal growth, which was indicated by a very low biomass titre when the divalent cation was absent (Table 5). Certain amounts of $MgSO_4$ could enhance the ACV tripeptide production by the engineered strain cultivated in the medium consisting of glucose and urea with the C:N ratio of 20:1. An increase in $MgSO_4$

concentration up to 10 mM had a strongly positive effect on the ACV production. However, the ACV titres were not different between the cultures containing 10 and 50 mM $MgSO_4$.

Table 5 Effect of $MgSO_4$ concentration on the ACV tripeptide production of the AoGpdA strain

$MgSO_4$ (mM)	Biomass (g/l)	ACV titre (mg/l)
0	1.78 ± 0.13^{c}	0.00 ± 0.00^{c}
2	25.48 ± 0.43^{b}	70.53 ± 1.04^{b}
5	25.3 ± 0.58^{b}	72.84 ± 8.19^{b}
10	24.91 ± 0.84^{b}	99.50 ± 20.04^{a}
50	28.2 ± 0.40^{a}	107.46 ± 9.64^{a}

Superscript letters (^{a}, ^{b} and ^{c}) indicate significant differences ($p < 0.05$) in biomass and ACV titres between the cultures using various $MgSO_4$ concentrations. The experiments were carried out in triplicates.

The ACV titres at different cultivation time of the AoGpdA strain in the ACV production medium (60 g/l glucose, 2.55 g/l urea, 10 mM $MgSO_4$) is shown in Figure 7. Apparently, the ACV titres and contents increased with increasing biomass titres (Figures A and B), and the maximal ACV titre (137.39±22.31 mg/l) was observed at the 4th-day of cultivation, wherein the engineered and the recipient strains entered to the stationary phase. At this time point, the ACV content in dry biomass also reached the maximal level. After glucose exhaustion, both the biomass titre and ACV content markedly decreased, which coincided with the ACV titre. These findings suggested that ACV was a growth-associated metabolite as a result of transcriptional control of gene expression. A sharp decrease in ACV production after glucose exhaustion presumably indicated that ACV did not display a secondary metabolite trait, which is commonly produced and highly accumulated in the cell during the late stationary phase or nutrient-limited conditions (Calvo *et al.*, 2002; Nielsen *et al.*, 2019).

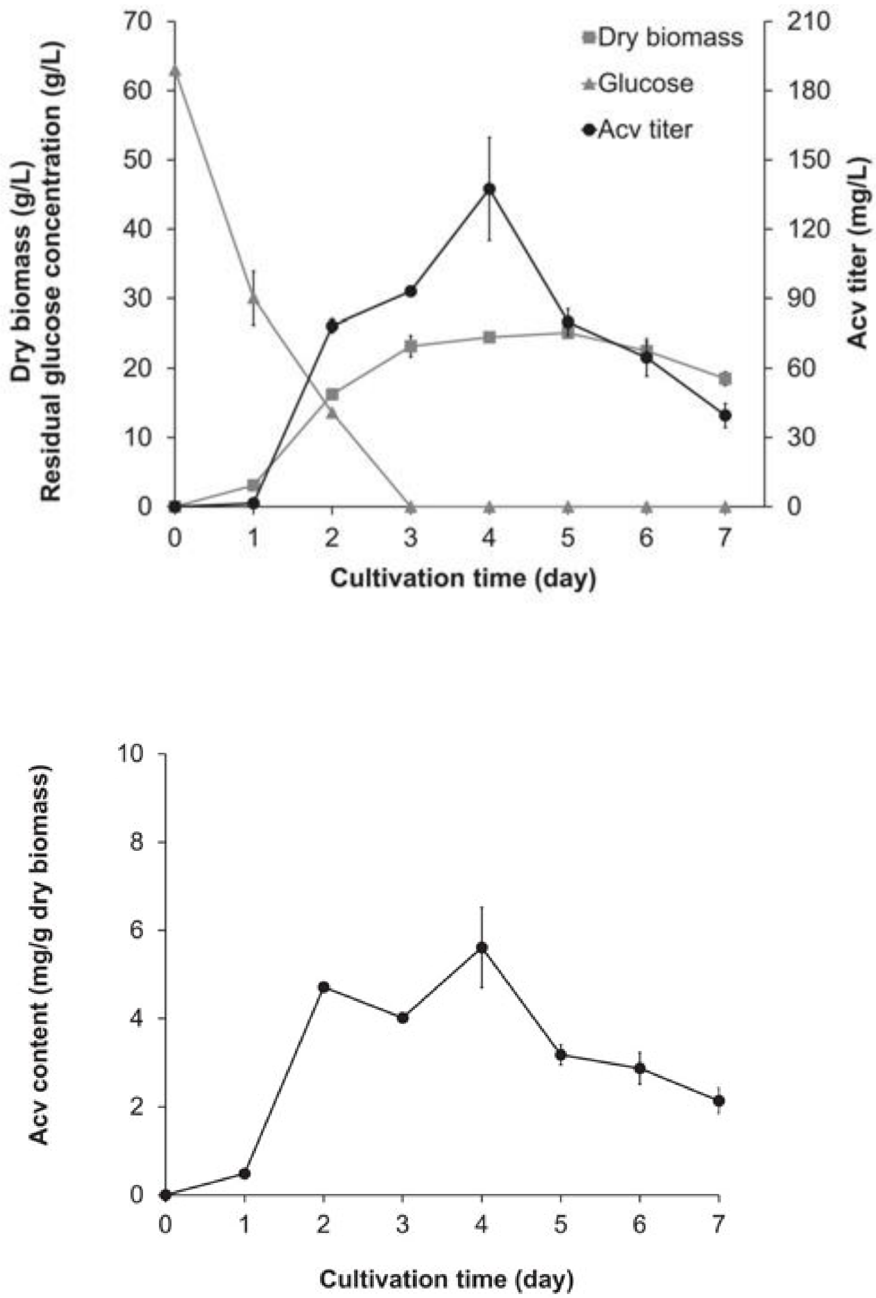


Figure 7 Profile of cell growth and ACV production of the AoGpdA-engineered strain. The cultures were grown at 30 °C in the ACV production medium. (A) Dry biomass, residual glucose and ACV titres from the AoGpdA strain. (B) ACV content in dry biomass of the AoGpdA strain.

5.2 RNA-sequencing based transcriptome analysis of the AoGpdA strain (ACV-producer) in response to ACV production

Reprogramming of transcriptional regulation of the *acv* gene might lead to alterations in expression of genes involved in cellular metabolism, particularly in the penicillin biosynthesis. To explore this, the transcriptome analysis of the AoGpdA strain (ACV producer) was carried out in comparison with the recipient strain. A set of differentially expressed genes (DEGs) between ACV producer and recipient were identified based on $\log_2$ fold change ≥ 0 with $p \leq 0.05$. The volcano plot showed that 12712 genes were separated into three groups of DEGs, in which 3,361 genes were up-regulated, and 3340 genes were down-regulated in the ACV-producing strain. In addition, 6011 genes were not significantly different from the recipient (Figure 8). Based on FPKM ≥ 100 with $\log_2$ fold change ≥ 2.0 and $p \leq 0.001$, the result showed that 192 of DEGs were up-regulated in the ACV-producing strain. Among these, 17 DEGs were found involved in transportation, which were 5 putative genes encoded for sugar and carbon transporters, 3 putative genes for oligopeptide and amino acid transporters, 1 putative gene for magnesium ion (Mg^{2+}) transporter, and 8 putative genes for major facilitator superfamily (MFS) and *ATP-binding cassette (ABC) transporters* (Figures 9A - D). The high expression of sugar and carbon, peptide, amino acid and magnesium transporters might contribute to the supply of precursors and the enzyme co-factor for the biosynthesis of ACV tripeptide. One of the well-known features of MFS and ABC membrane transporters is the export of small molecules to the surrounding environment; therefore, they might have a role for excretion of such oligopeptide or other secondary metabolite molecules.

Apart from the carbon substrates, ammonium is also a crucial nutrient required for fungal growth and oligopeptide biosynthesis. Ammonium can be taken up into the cell via ammonium transporters. Based on the transcriptome data, different transcript levels of ammonium transporter genes (*Aoamts*) were investigated. It was found that *Aoamt2, Aoamt3 and Aoamt4* were DEGs up-regulated in the ACV producer, whereas *Aoamt1* was expressed equally in both ACV producer and the recipient according to the FPKM values (Figure 9E). This result might suggest the different roles of ammonium transporters encoded by these genes in *A. oryzae*.

Focusing on the penicillin biosynthesis, the over-expression of the *acv* gene led to the increase of transcriptional levels of isopenicillin-N synthetase and isopenicillin-N epimerase genes in the ACV producer strain compared with those of the recipient as shown by FPKM values in Figure 9F. However, a slight increase of expression level of the isopenicillin-N N-acyltransferase gene in both ACV producer and recipient might explain the undetectable penicillin metabolite. Taken together, the transcriptomic results suggested that the ACV-producing strain required basic nutrient and amino acid/peptide precursors, and also the magnesium as a co-factor for biosynthesis of oligopeptide metabolite and possibly molecule secretion by the function of protein transports.

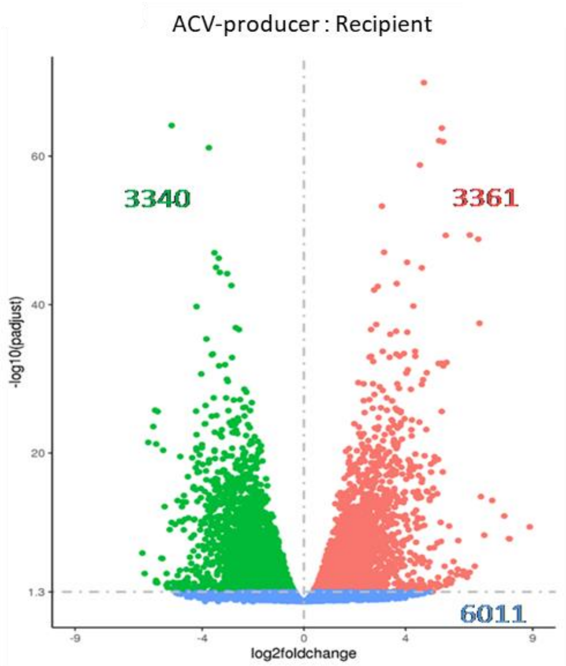
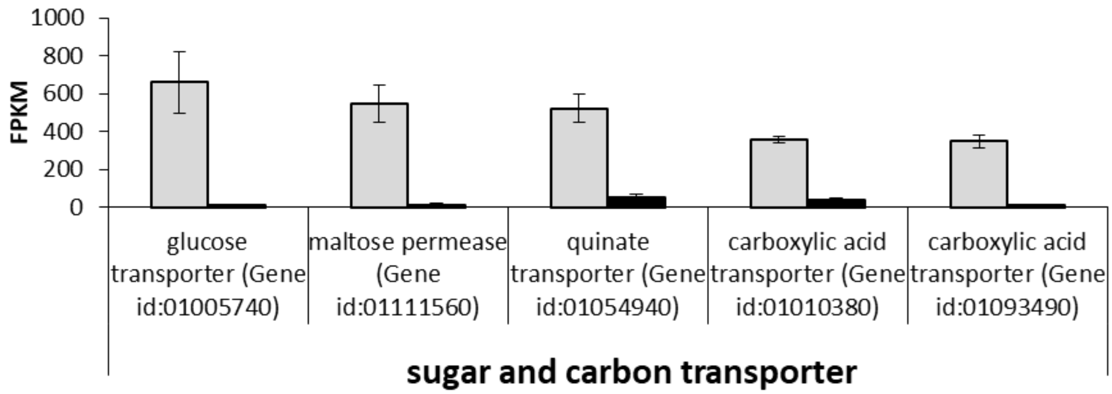
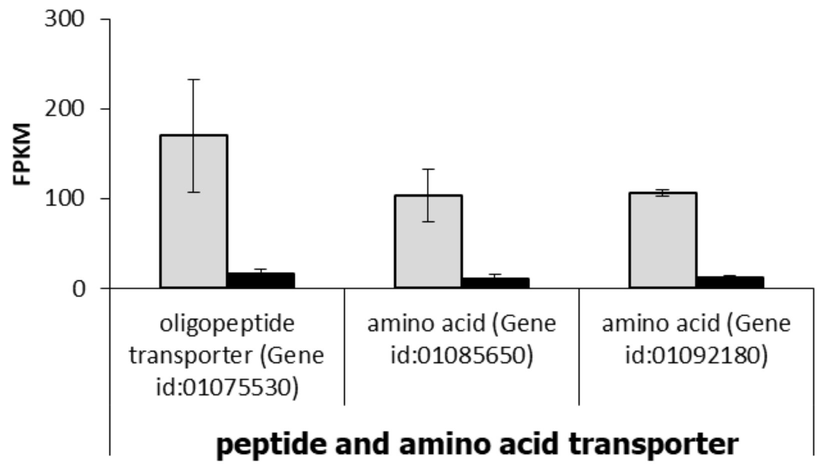


Figure 8 Volcano plot of differential expressed genes (DEGs) of the AoGpdA strain (ACV producer) and the recipient. Fungal cells were cultivated in the optimal ACV production medium for 3 d. The $\log_2$ fold change ≥ 0.0 with *p* value ≤ 0.05 was set as a cut-off. Significant up- and down-regulated genes obtained from the producer strain are shown by red and green dots, respectively. Genes with no significant difference in the expression levels are shown by blue dots.

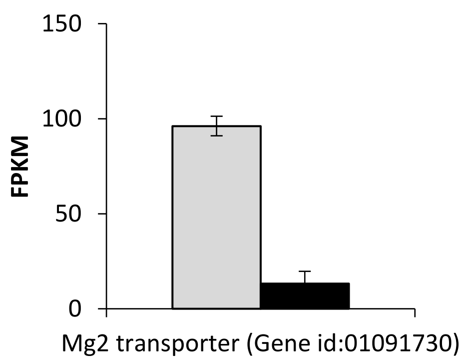
A)


1000
800
600
400
200
0
FPKM
glucose transporter (Gene id:01005740)
maltose permease (Gene id:01111560)
quinate transporter (Gene id:01054940)
carboxylic acid transporter (Gene id:01010380)
carboxylic acid transporter (Gene id:01093490)
sugar and carbon transporter

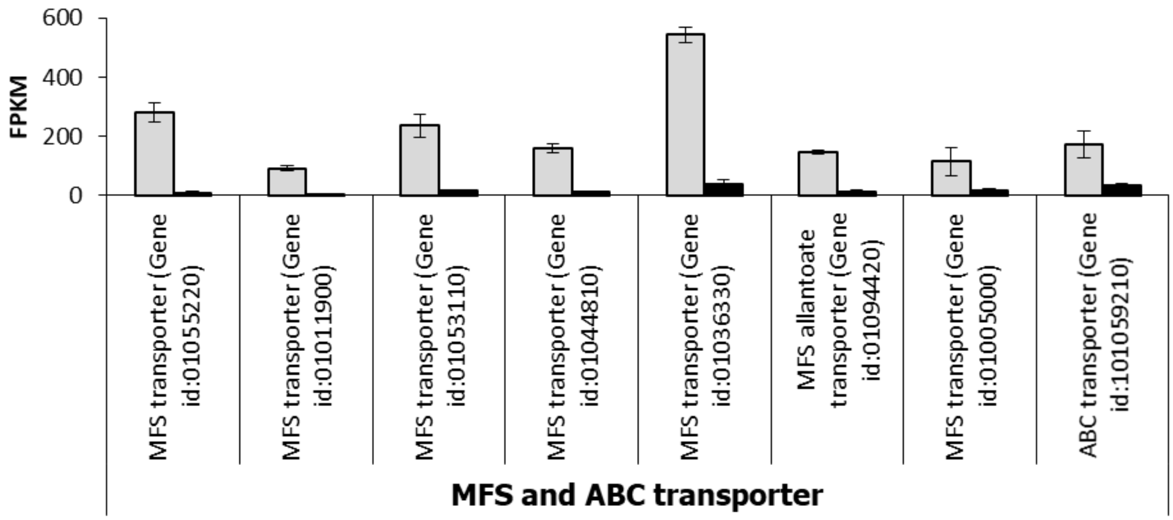
B)


300
200
100
0
FPKM
oligopeptide transporter (Gene id:01075530)
amino acid (Gene id:01085650)
amino acid (Gene id:01092180)
peptide and amino acid transporter

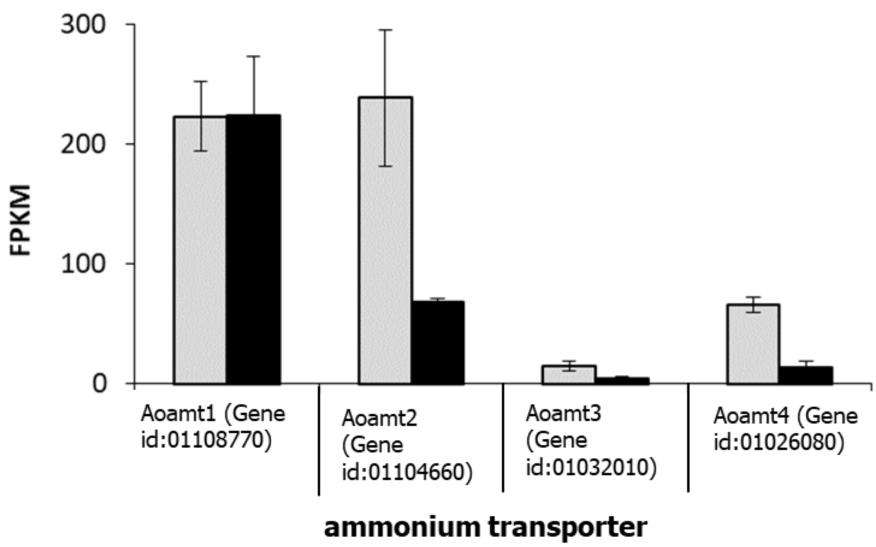
C)


150
100
50
0
FPKM
Mg2 transporter (Gene id:01091730)

D)


600
400
200
0
FPKM
MFS transporter (Gene id:01055220)
MFS transporter (Gene id:01011900)
MFS transporter (Gene id:01053110)
MFS transporter (Gene id:01044810)
MFS transporter (Gene id:01036330)
MFS allantoate transporter (Gene id:01094420)
MFS transporter (Gene id:01005000)
ABC transporter (Gene id:101059210)
MFS and ABC transporter

E)


300
200
100
0
FPKM
Aoamt1 (Gene id:01108770)
Aoamt2 (Gene id:01104660)
Aoamt3 (Gene id:01032010)
Aoamt4 (Gene id:01026080)
ammonium transporter

F)

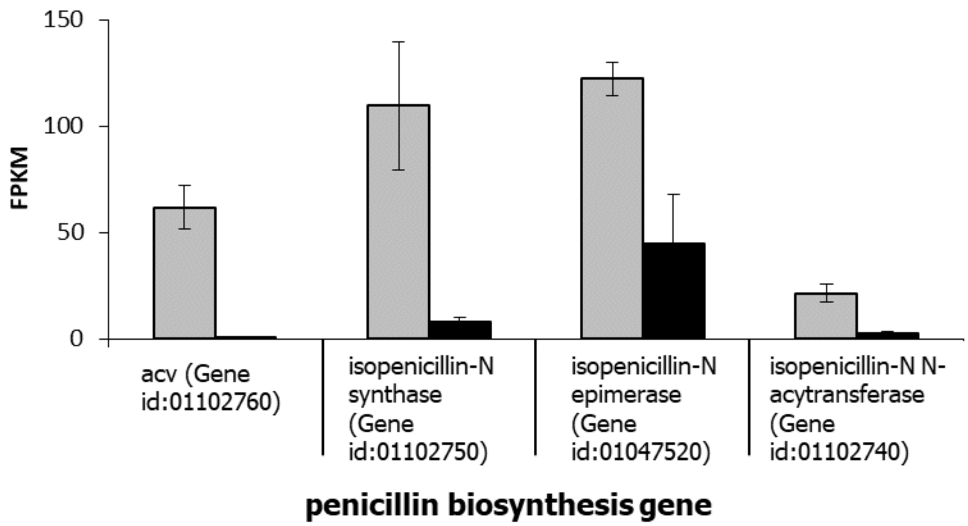


Figure 9 Differential expressed genes (DEGs) up-regulated in the AoGpdA strain (ACV producer). Sugar and carbon substrate transporter genes (A), peptide and amino acid transporter genes (B), magnesium co-factor transporter gene (C), MFS and ABC transporter genes (D), ammonium transporter genes (E) and penicillin biosynthesis genes (F). Gray and black bars represent the gene transcripts of the ACV producer and recipient, respectively.

5.3 Exploring functional role of ammonium transporters in *A. oryzae*

5.3.1 Identification and characterisation of ammonium transporter sequences in *A. oryzae*

Nitrogen is a crucial nutrient for all living organisms that is required for cell growth and development, and biosynthesis of several biomolecules. Ammonium is a simple inorganic nitrogen source preferred for filamentous fungi (ter Schure *et al.*, 2000; Jiang *et al.*, 2019). It can be taken up directly by the fungal cells via ammonium transporters (Amts), which are structural membrane proteins membered in the multigenic ammonium transporter/methylammonium permease (AMT/MEP) family (Marini *et al.*, 1994; Ninnemann *et al.*, 1994; Javelle *et al.*, 2003). In this study, characterisation of ammonium transporter genes in *A. oryzae* BCC 7051, and their functional role in ammonium uptake in relevant to cell growth and biomass production were explored. By genome analysis, four putative *Aoamt* homolog sequences of *A. oryzae*, *Aoamt1, Aoamt2, Aoamt3* and *Aoamt4,* were identified. Based on the analysis of amino acid sequence similarity, the gene-encoding proteins were verified to be members of the AMT/MEP family by the existence of the ammonium transporter family domain (PF00909). The deduced amino acid sequences of AoAmt1 – AoAmt4 shared 70 – 89% identities to the ammonium transporters of *Aspergillus nidulans* (Monahan *et al.*, 2002a; Monahan *et al.*, 2002b; Monahan *et al.*, 2006). The computational prediction demonstrated that AoAmt1, AoAmt2 and AoAmt3 contained 11 transmembrane helices with an $N_{(out)}$ - $C_{(in)}$ topology based on the TMHMM (Krogh *et al.*, 2001) in agreement with the conserved characteristic of other fungal ammonium transporters (Taichert *et al.*, 2008; Ellerbeck *et al.*, 2013; Neuhäuser, 2020). However, the AoAmt4 was distinctive, which contained 9 transmembrane helices with an $N_{(out)}$ - $C_{(in)}$ topology.

5.3.2 Differential expression of ammonium transporter genes in *A. oryzae*

The transcriptional response of *Aoamt* genes to the presence of ammonium in the cultivation conditions was investigated in relevant to the ammonium uptake of the fungal cells. When the active cells grown in the logarithmic phase were transferred to 1 mM NH_4Cl-containing medium for 1 h, ammonium concentration in the culture broth declined to undetectable level. Differential expression of the ammonium-contained culture clearly showed that *Aoamt* expression patterns were attributed by ammonium. The relative expression analysis demonstrated that *Aoamt2* and *Aoamt3* were ammonium-induced genes. Expression level of *Aoamt2* gene was detectable and significantly increased when ammonium was limited, and gradually declined in the ammonium starvation condition. However, the transcript level of *Aoamt3* was relatively maintained through the starvation condition. These results were not coincided with the previous findings for *A. nidulans*, where the *mepA* (*Aoamt2*-like) expression continually increased when encountered the ammonium starvation, and the expression of *mepB* (*Aoamt3*-like) was only detected in the complete nitrogen starvation condition (Monahan *et al.*, 2006). The transcript levels of *Aoamt1* and *Aoamt4* in the cultures grown in ammonium-available and limited conditions were not significantly different. However, the transcript level of *Aoamt4* declined in the nitrogen depleted condition. These resulted demonstrated the differential expression responses of *Aoamt* genes of *A. oryzae* to the ammonium sufficiency, limitation, and starvation conditions.

5.3.3 The role of ammonium transporters in cell growth and biomass production of *A. oryzae*

To study the role of AoAmt proteins in ammonium uptake reflected to cell growth, the genetic perturbation of *Aoamt2* or *Aoamt3* was performed by gene disruption and overexpression. Mycelial growth performance of these recombinant strains on solid agar medium adding with 1 mM NH_4Cl was evaluated in comparison to the recipient strain. It was noted that radial growths of both recombinant strains were lower than that of the recipient. Disruption of the *Aoamt2* did not affect the mycelial growth of the *Aoamt2* disruptant strain (ΔAoamt2), whereas the disruption of *Aoamt3* (Δ*Aoamt3*) showed a defective growth on the agar medium. The growth phenotype of the Δ*Aoamt3* strain differed from the previous report described in *A. nidulans* where the deletion of *mepB* (*Aoamt3*-like) gene showed negative attribute on cell growth even though a wide range of ammonium concentrations were tested (Monahan *et al.*, 2006). On the other hand, the constitutive overexpression of the *Aoamt3* gene (OEAoamt3 strain) showed the growth recovery of the ΔAoamt3 strain. Taken together, these results suggested that the Aoamt3 plays a functional role in ammonium transporting capacity required for cell growth of *A. oryzae.*

Furthermore, the functional characterisation of *Aoamt3* was implemented by gene overexpression. The result showed that when a mixture of nitrogen sources (ammonium and yeast extract) was used for submerged cultivation, the fungal growth and biomass titre of the OEAoamt3 strain were sustained, which were higher than that of the wild type, especially observed in the stationary phase (Table 6). Apparently, the maximum growth was observed in the 4-d culture. Moreover, the period of complete glucose consumption of the OEAoamt3 strain was prolonged as compared to that of the wild type. Growth parameters determined at 2-d cultivation indicated that the glucose consumption rate of the overexpressed strain was reduced by more than 20% while the biomass yield increased for more than 25% as compared to the wild type. These results indicated that the AoAmt3 was an attributive transporter for ammonium uptake involved in the cell growth and biomass production. Overexpression of *Aoamt3*; therefore, increased the ammonium transporting capacity for sustaining cell growth and biomass production with less glucose consumption.

Table 6 Biomass and residual glucose concentration of the OEAoamt3 and wild type strains grown in SM medium

Incubation time (day)	Biomass (g/l)		Residual glucose concentration (g/l)	
	OEAoamt3 strain	Wild type strain	OEAoamt3 strain	Wild type strain
0	0.00±0.00^{g}	0.00±0.00^{g}	41.88±1.24^{a}	41.88±1.24^{a}
1	8.02±0.03^{f}	11.44±0.68^{e}	32.00±1.41^{b}	18.73±1.24^{c}
2	16.91±0.34^{c}	16.77±0.39^{c}	11.03±1.08^{d}	0.01±0.00^{e}
3	19.58±0.62^{b}	19.19±0.01^{b}	3.34±0.68^{e}	0.00±0.00^{e}
4	20.59±0.33^{a}	17.35±0.50^{c}	0.13±0.07^{e}	0.00±0.00^{e}
5	18.80±0.65^{b}	14.94±0.40^{d}	0.00±0.00^{e}	0.00±0.00^{e}

Superscript letters indicate statistically different significance of biomass and residual glucose concentration between the OEAoamt3 and wild type strains across time points analysed by Duncan's Multiple Range Test (MRT) (p-value ≤ 0.05).

5.4 Reconstitution of *acv* gene by module engineering

5.4.1 Computational prediction of A-domain substrate specificity of Nrps

It was of interest to reconstitute the *acv* gene by manipulating particular modules for tailor-made oligopeptide biosynthesis. The Nrps mega-enzyme structure presents a modular architecture comprising of multi-functional domains (Ackerley, 2016). One of these, the Nrps A-domain appears to be the primary determinant of substrate selectivity, which is programmed by "signature sequence". It contains 10 amino acids spanning in the substrate-binding pocket. Accordingly, the structure-function relationship of A-domain substrate specificities has been characterised and elucidated by biochemical approach (Balibar and Walsh, 2006; Xu *et al.*, 2010; Bills *et al.*, 2014; Zhang *et al.*, 2018) and crystalisation (Conti *et al.*, 1997; Stachelhaus *et al.*, 1999; Challis *et al.*, 2000; Weber and Marahiel, 2001). Seeking the signature sequence and predicting the amino acid substrate were required to facilitate the exploitation of the Nrps for tailor-made oligopeptide biosynthesis. In this study, specificities of A-domains for Glu, Leu and Pro were predicted, and replacement of *acv* gene with the tailor-made oligopeptide module genes, which were designed from a basis of the A-domain substrate specificity, was carried out. The specificities of Nrps A-domains for Glu, Leu and Pro were computationally predicted from genomic DNA sequences of several fungi using the "NRPSpredictor2" web server. As a result of the prediction score and precision, three putative Nrps A-domains of Tex1 of *T. virens* specific for Glu, Leu and Pro substrate recognition were selected.

5.4.2 Module engineering of *acv* gene for tailor-made oligopeptide biosynthesis

To synthesise the tailor-made oligopeptides in *A. oryzae*, a series of module-reconstituted strains consisted of GluLeu, ProLeu, ProGluLeu and AoProGluLeu were constructed by substituting the structural *acv* gene with di- or tri-module genes driven by the potential constitutive *AoPgpdA* promoter using CRISPR-Cas9 technology based on the module fusion concept (Bozhüyük *et al.*, 2019). In addition, a substitution of the valine module gene of *acv* with the proline module gene was done to generate the ACP strain. After fungal transformation and selection, the gene expression in these tailor-made oligopeptide strains was verified by RT-PCR, which the last A-domain gene sequence specific to the leucine or proline residue was amplified. Verification of the structure-function relationship of the module-reconstituted strains for oligopeptide synthesis was carried out by cultivating thoese recombinant strains in the SM medium for 3 d. Using the methanol extraction method and LC-MS analysis; however, mass profiles of di- or tripeptide could not be detected in the module reconstituted strains. Attempts were done by optimising the cultivation media and extraction methods as described in Materials and Methods. Of these conditions, none of the expected peptides was observed even though the expressions were clearly observed in such strains. Probably, optimisation of cultivation condition might be required for investigating the oligopeptide production by these recombinant strains.

5.5 Potential of *Aspergillus oryzae* as a platform cell for production of non-ribosomal peptide pigment, indigoidine

5.5.1 Heterologous expression of indigoidine synthetase gene in *A. oryzae*

Our previous work has proved that *A. oryzae* is a potent organism platform for producing the non-ribosomal tripeptide (ACV) using its own NRPS system. Further, this study also explored the exploitation of this fungus as a chassis cell for production of the glutamine-derived cyclic dipeptide, indigoidine with dark blue colour through the available NRPS system. The 4134-bp codon-optimised sequence (*AoinK*) designed from indigoidine synthetase of *S. chromofuscus* (*ScindC*) was cloned into the expression plasmid, pAoInK-PyrG, bearing a constitutive strong promoter. After gene transformation, dark blue colonies as a distinguished phenotype were clearly visualised on the selective medium without nutrient supplementation. These colonies were re-isolated to obtain pure culture (Figures 10A and 10B, respectively). This result indicated the functional activity of the AoInK multi-functional synthetase encoded by *AoinK* gene of *A. oryzae.* DMSO extract of mycelial cells of the AoInK strain exhibited a spectral peak with visible-light absorption at 612 nm. The pigment production was growth-associated, and the highest accumulation (287 mg/l indigoidine) was obtained in the 5-day culture, which glucose was exhausted.

A) **B)**

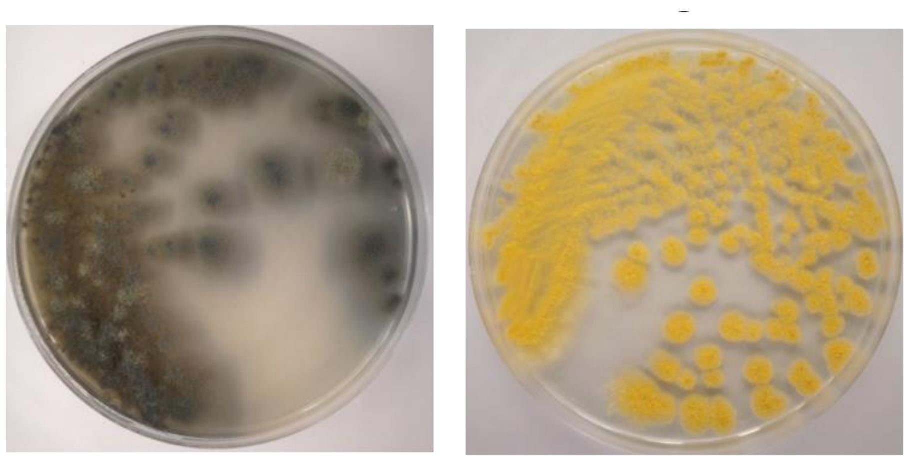


Figure 10 Fungal colonies of indigoidine-producing strain of *A. oryzae* overexpressing *AoinK* gene and the recipient*.* AoInK strain (A) and the recipient (B) of *A. oryzae.* Cultures were grown in SM agar at 30 °C for 10 d.

5.5.2 Indigoidine production of AoInK-producing strain at different cultivation conditions

In submerged fermentation, indigoidine was detected in the AoInK-producing strain when grown in the SM medium at 24-h cultivation afterwards. Different initial pH values for fungal cultivations were adjusted to 4.5, 5.5, 6.5 and 7.5. An optimal pH condition for total InK production by the AoInK strain was 6.5–7.5, which the pigment titres of 360–400 mg/l were obtained in the 5-d cultures, whereas pH 5.5 condition was optimal for biomass production. At initial pH 6.5, the InK titre was improved when grown at 25 °C, which more than 2-fold increase (850 mg/l indigoidine), was found as compared with the product titre at 30 °C (400 mg/l indigoidine). It seems that the Ink titre decreased when culture temperature was elevated as the finding of about 2-fold decrease of InK titre in the culture grown at 35 °C. The effect of glutamine, a precursor of indigoidine, on the pigment production of the engineered strain was investigated by growing the AoInK strain in the SM with initial pH 6.5 at 25 °C. The pigment product was enhanced up to 1000–1200 mg/l when supplemented with optimal concentrations of the amino acid (2- and 5-mM glutamine). It is noted that the high glutamine concentration (10 mM) negatively attributed the InK titre. Moreover, the addition of 2 mM magnesium (Mg^{2+}) was optimal for promoting the pigment production of the 2 mM glutamine-supplemented culture, whereas there was not much difference in biomass titres among the cultures with various $MgSO_4$ concentrations. Obviously, a substantial amount of indigoidine was localised inside the cells. To address such limitation, the effect of nonionic surfactants, polyethylene glycol (PEG), Tergitol, Triton-X100 and Tween 80, on the extracellular production of indigoidine was studied. It was found that the extracellular Ink titres of all AoInK cultures were significantly enhanced by addition of the nonionic surfactants. Of them, Triton X-100 and Tween 80 solutions could promote the extracellular InK titres over other surfactants. The secreted InK amounts were also dependent on the concentration of nonionic surfactants. Even though the Triton X-100 could promote pigment secretion, the supplementation over 1% (v/v) amount showed an adverse effect on total InK titres, which could be a result of low biomass. On the other hand, significant increases of extracellular pigment and cell biomass were achieved by addition of 1-3% Tween 80 solution as compared with the non-treated culture, giving the total InK titre of about 1400 mg/l. This result could be explained that the enhanced secretion might be a result of the amphipathic characteristic of Tween 80 involved in cell membrane integrity and permeability (Pardo, 1996; El-Batal and Al Tamie, 2016). In addition, the surfactant could be an alternative carbon source for fungal growth (Castro *et al.*, 2018; Li *et al.*, 2018).

5.5.3 Profiling of InK production and mycelial growth of the AoInK strain

The InK production during cell growth of the AoInK strain in the InK production medium (SM supplemented with 2 mM glutamine and 1% Tween 80 with initial pH 6.5) was investigated. The InK titre increased with increase of biomass titre along the cultivation time (Table 7). The maximal total InK titre was observed at the end of growth phase (5^{th}-day of cultivation). After glucose exhausted, the total pigment (intracellular and extracellular indigoidine) titre gradually declined suggesting the growth-associated trait of the pigment production. These results confirmed the strain improvement for heterologous production of non-ribosomal peptide pigment in *A. oryzae* and increase of product titer was done by cultivation conditions.

Table 7 Biomass, residual glucose concentration and indigoidine production of the AoInK strain grown in the InK production medium

Incubation time (day)	Biomass (g/l)	Residual glucose concentration (g/l)	Total indigoidine titre (mg/l)
0	0.00 ± 0.00^{f}	40.04 ± 0.20^{a}	0.00 ± 0.00^{e}
1	1.11 ± 0.09^{e}	36.85 ± 0.14^{b}	76.85 ± 0.22^{e}
2	8.67 ± 0.45^{d}	22.87 ± 0.45^{c}	507.93 ± 44.13^{d}
3	13.12 ± 0.77^{c}	17.65 ± 0.03^{d}	858.09 ± 3.20^{c}
4	15.61 ± 0.68^{a}	9.86 ± 0.65^{e}	1213.27 ± 121.30^{b}
5	15.61 ± 0.14^{a}	3.87 ± 0.05^{f}	1409.22 ± 95.33^{a}
6	15.51 ± 0.05^{a}	0.03 ± 0.05^{g}	1246.11 ± 55.95^{ab}
7	14.44 ± 0.37^{b}	0.00 ± 0.00^{g}	1075.55 ± 143.78^{b}

Superscript letters indicate statistically different significance of biomass, residual glucose concentration and indigoidine production across time points analysed by Duncan's Multiple Range Test (MRT) (p-value ≤ 0.05).

6. Conclusion and suggestion

The fungal platform technology was established for biosynthesis of non-ribosomal oligopeptides in *A. oryzae* based on synthetic biology approach and regulatory mechanism underlying the ammonium transport capacity of the particular ammonium transporter. This study could guide the potent approaches for rational improvement of fungal strains to produce bioactive non-ribosomal oligopeptides or other bio-products through synthetic biology. In addition, the bioprocess development of the engineered strains is required for optimising the production yield and cost that will lead to realistic applications in industrial sectors.

7. References

Ackerley, D.F. 2016. Cracking the Nonribosomal Code. *Cell Chemical Biology* 23, 535-537.

Arai, T., Arimura, Y., Ishikura, S., and Kino, K. 2013. L-Amino acid ligase from Pseudomonas syringae producing tabtoxin can be used for enzymatic synthesis of various functional peptides. *Applied and environmental microbiology* 79, 5023-5029.

Bah, C.S., Carne, A., McConnell, M.A., Mros, S., and Bekhit, A.D. 2016. Production of bioactive peptide hydrolysates from deer, sheep, pig and cattle red blood cell fractions using plant and fungal protease preparations. *Food Chemistry* 202, 458-466.

Balibar, C.J., and Walsh, C.T. 2006. GliP, a multimodular nonribosomal peptide synthetase in *Aspergillus fumigatus*, makes the diketopiperazine scaffold of gliotoxin. *Biochemistry* 45, 15029-15038.

Beer, R., Christiansen, T., Herbst, K., Ignatiadis, N., Kats, I., Kurzawa, N., Meichsner, J., Rabe, S., Riedel, A., Sachs, J., Schessner, J., Schmidt, F., Walch, P., Adlung, L., Genreith, K., Georgi, F., Heinemann, T., Meyer, H., Niopek, D., Di Ventura, B., and Eils, R. 2013. BBF RFC 100: Standard for Synthesis of Customized Peptides by Non-Ribosomal Peptide Synthetases. RFC100, pp. 1-16.

Bills, G., Li, Y., Chen, L., Yue, Q., Niuab, X.-M., and An, Z. 2014. New insights into the echinocandins and other fungal non-ribosomal peptides and peptaibiotics. *Natural Product Reports* 31, 1348-1375.

Bozhüyük, K.A.J., Linck, A., Tietze, A., Wesche, F., Nowak, S., Fleischhacker, F., and Bode, H.B. 2019. Modification and *de novo* design of non-ribosomal peptide synthetases using specific assembly points within condensation domains. *Nature Chemistry* 11, 653–661.

Calcott, M.J., Owen, J., Lamont, I.L., and Ackerley, D.F. 2014. Biosynthesis of novel pyoverdines by domain substitution in a nonribosomal peptide synthetase of *Pseudomonas aeruginosa*. *Applied and Environmental Microbiology* 80, 5723-5731.

Calvo, A.M., Wilson, R.A., Bok, J.W., and Keller, N.P. 2002. Relationship between secondary metabolism and fungal development. *Microbiology and Molecular Biology Reviews*, 447–459.

Castro, F.F., Pinheiro, A.B.P., Gerhardt, E.C.M., Oliveira, M.A.S., and Barbosa-Tessmann, I.P. 2018. Production, purification, and characterization of a novel serine-esterase from *Aspergillus westerdijkiae*. *J Basic Microbiol.* 58, 131-143.

Challis, G.L., Ravel, J., and Townsend, C.A. 2000. Predictive, structure-based model of amino acid recognition by nonribosomal peptide synthetase adenylation domains. *Chemistry & Biology* 7, 211-224.

Chutrakul, C., Jeennor, S., Panchanawaporn, S., Cheawchanlertfa, P., Suttiwattanakul, S., Veerana, M., and Laoteng, K. 2016. Metabolic engineering of long chain-polyunsaturated fatty acidbiosynthetic pathway in oleaginous fungus for dihomo-gammalinolenic acid production. *Journal of Biotechnology* 218, 85-93.

Chutrakul, C., Panchanawaporn, S., Jeennor, S., Anantayanon, J., Vorapreeda, T., Vichai, V., and Laoteng, K. 2019. Functional characterization of novel U6 RNA polymerase III promoters: Their implication for CRISPR-Cas9-mediated gene editing in *Aspergillus oryzae*. *Current Microbiology* 76, 1443–1451.

Conti, E., Stachelhaus, T., Marahiel, M.A., and Brick, P. 1997. Structural basis for the activation of phenylalanine in the non-ribosomal biosynthesis of gramicidin S. *EMBO Journal* 16, 4174–4183.

Di Bernardini, R., Harnedya, P., Bolton, D., Kerry, J., O'Neill, E., Mullen, A.M., and Hayes, M. 2011. Antioxidant and antimicrobial peptidic hydrolysates from muscle protein sources and by-products. *Food Chemistry* 124, 1296-1307.

Doel, M.T., Eaton, M., Cook, E.A., Lewis, H., Patel, T., and Carey, N.H. 1980. The expression in *E. coli* of synthetic repeating polymeric genes coding for poly(l-aspartyl-l-phenylalanine). *Nucleic Acids Research* 8, 4575-4592.

El-Batal, A.I., and Al Tamie, M.S.S. 2016. Optimization of melanin production by *Aspergillus oryzae* and incorporation into silver nanoparticles. *Der Pharmacia Lettre* 8, 315-333.

Ellerbeck, M., Schüßler, A., Brucker, D., Dafinger, C., Loos, F., and Brachmann, A. 2013. Characterization of three ammonium transporters of the Glomeromycotan fungus Geosiphon pyriformis. *Eukaryotic Cell* 12, 1554-1562.

Finking, R., and Marahiel, M.A. 2004. Biosynthesis of Non-Ribosomal Peptides. *Annual Reviews Mibrobiology* 58, 453-488.

Gerke, J., and Braus, G.H. 2014. Manipulation of fungal development as source of novel secondary metabolites for biotechnology. *Applied Microbiology and Biotechnology* 98, 8443–8455.

Hashimoto, S. 2006. Occurrence, Biosynthesis, and Biotechnological Production of Dipeptides. In Wendisch, V.F. (ed.), *Amino acid biosynthesis-Pathways, Regulation and Metabolic Engineering*. Springer, Leipzig, Germany, Vol. 5, pp. 327-348.

Hou, Y., Wu, Z., Dai, Z., Wang, G., and G, W. 2017. Protein hydrolysates in animal nutrition: Industrial production, bioactive peptides, and functional significance. *Journal of Animal Science and Biotechnology* 8, 24.

Javelle, A., Morel, A., Rodríguez-Pastrana, B.-A., Botton, B., André, B., Marini, A.M., Brun, A., and Chalot, M. 2003. Molecular characterization, function and regulation of ammonium transporters (Amt) and ammoniummetabolizing enzymes (GS, NADP-GDH) in the ectomycorrhizal fungus Hebeloma cylindrosporum. *Molecular Microbiology* 47, 411–430.

Jeennor, S., Anantayanon, J., Panchanawaporn, S., Khoomrung, S., Chutrakul, C., and Laoteng, K. 2019. Reengineering lipid biosynthetic pathway of *Aspergillus oryzae* for enhanced production of gamma-linolenic acid and dihomo-gamma-linolenic acid. *Gene* 706, 106-114.

Jiang, J., Zhao, J., Duan, W., Tian, S., Wang, X., Zhuang, H., Fu, J., and Kang, Z. 2019. TaAMT2;3a, a wheat AMT2-type ammonium transporter, facilitates the infection of stripe rust fungus on wheat. *BMC Plant Biology* 19, 239.

Katayama, T., Tanaka, Y., Okabe, T., Nakamura, H., Fujii, W., Kitamoto, K., and Maruyama, J. 2015. Development of a genome editing technique using the CRISPR/Cas9 system in the industrial filamentous fungus *Aspergillus oryzae*. *Biotechnol Lett* 38, 637-642.

Keller, N.P., Turner, G., and Bennett, J.W. 2005. Fungal secondary metabolism — from biochemistry to genomics. *Nature Reviews Microbiology* 3, 937-947.

Kennedy, J., and Turner, G. 1996. Delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase is a rate limiting enzyme for penicillin production in *Aspergillus nidulans*. *Molecular and General Genetics* 253, 189-197.

Kino, K., Arai, T., and Tateiwa, D. 2010. A Novel L-Amino Acid Ligase from *Bacillus subtilis* NBRC3134 Catalyzed Oligopeptide Synthesis. *Bioscience, Biotechnology, and Biochemistry* 74, 129-134.

Kino, K., Kotanaka, Y., Arai, T., and Yagasaki, M. 2009. A novel L-amino acid ligase from *Bacillus subtilis* NBRC3134, a microorganism producing peptide-antibiotic rhizocticin. *Bioscience, Biotechnology, and Biochemistry* 73, 901–907.

Koike, M. 2012. Japan, Vol. JP 2012-165740.

Kries, H., Niquille, D.L., and Hilvert, D. 2015. A subdomain swap strategy for reengineering nonribosomal peptides. *Chemistry & Biology* 22, 640-648.

Krogh, A., Larsson, B., von Heijne, G., and Sonnhammer, E.L. 2001. Predicting transmembrane protein topology with a hidden Markov model: application to complete genomes. *J Mol Biol* 305, 567-580.

Lambalot, R.H., Gehring, A.M., Fluge, R.S., Zuber, P., LaCelle, M., Marahiel, M.A., Reid, R., Khosia, C., and Walsh, C.T. 1996. A new enzyme superfamily - the phosphopantetheinyl transferases. *Chemistry&Biology* 3, 923-936.

Laoteng, K., Jitsue, S., Dandusitapunth, Y., and Cheevadhanarak, S. 2008. Ethanol-induced changes in expression profiles of cell growth, fatty acid and desaturase genes of *Mucor rouxii*. *Fungal Genetics and Biology* 45, 61-67.

Lautru, S., and Challis, G.L. 2004. Substrate recognition by nonribosomal peptide synthetase multi-enzymes. *Microbiology* 150, 1629-1636.

Li, P., and Wu, G. 2018. Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth. *Amino Acids* 50, 29-38.

Li, Q., Lei, Y., Hu, G., Lei, Y., and Dan, D. 2018. Effects of Tween 80 on the liquid fermentation of *Lentinus edodes*. *Food Sci. Biotechnol.* 27, 1103–1109.

Lorang, J.M., Tuori, R.P., Martinez, J.P., Sawyer, T.L., Redman, R.S., Rollins, J.A., Wolpert, T.J., Johnson, K.B., Rodriguez, R.J., Dickman, M.B., and Ciuffetti, L.M.

2001. Green fluorescent protein is lighting up fungal biology. *Applied and Environmetal Microbiology* 67, 1987-1994.

MacCabe, A.P., van Liemptll, H., Palissall, H., Unkles, S.E., Riach, M.B.R., Pfeifer, E., von Dohren, H., and Kinghorn, J.R. 1991. Delta-(L-alpha-aminoadipyl)-L-cysteinyl-D-valine synthetase from *Aspergillus nidulans*: Molecular characterization of the *acvA* gene encoding the first enzyme of the penicillin biosynthetic pathway. *Journal of Biological Chemistry* 266, 12646-12654.

Marini, A.M., Vissers, S., Urrestarazu, A., and André, B. 1994. Cloning and expression of the MEP1 gene encoding an ammonium transporter in *Saccharomyces cerevisiae*. *EMBO J* 13, 3456–3463.

Martín, J.F., Ullán, R.V., and García-Estrada, C. 2010. Regulation and compartmentalization of beta-lactam biosynthesis. *Microbial Biotechnology* 3, 285-299.

Marui, J., Ohashi-Kunihiro, S., Ando, T., Nishimura, M., Koike, H., and Machida, M. 2010. Penicillin biosynthesis in *Aspergillus oryzae* and its overproduction by genetic engineering. *Journal of Bioscience and Bioengineering* 110, 8-11.

Miethke, M., and Marahiel, M.A. 2007. Siderophore-based iron acquisition and pathogen control. *Microbiology and Molecular Biology Reviews* 71, 413-451.

Mills, S., Stanton, C., Hill, C., and Ross, R.P. 2011. New developments and applications of bacteriocins and peptides in foods. *Annual Review of Food Science and Technology* 2, 299-329.

Monahan, B.J., Askin, M.C., Hynes, M.J., and Davis, M.A. 2006. Differential expression of *Aspergillus nidulans* ammonium permease genes is regulated by GATA transcription factor AreA. *Eukaryotic Cell* 5, 226–237.

Monahan, B.J., Fraser, J.A., Hynes, M.J., and Davis, M.A. 2002a. Isolation and characterization of two ammonium permease genes, meaA and mepA, from *Aspergillus nidulans*. *Eukaryotic Cell* 1, 85–94.

Monahan, B.J., Unkles, S.E., I, T.T., Kinghorn, J.R., Hynes, M.J., and Davis, M.A. 2002b. Mutation and functional analysis of the *Aspergillus nidulans* ammonium permease MeaA and evidence for interaction with itself and MepA. *Fungal Genetics and Biology* 36, 35-46.

Mootz, H.D., Schwarzer, D., and Marahiel, M.A. 2000. Construction of hybrid peptide synthetases by module and domain fusions. *Proceedings of the National Academy of Sciences of the United States of America* 97, 5848-5853.

Muller, M., Auslander, S., Auslander, D., Kemmer, C., and Fussenegger, M. 2012. A novel reporter system for bacterial and mammalian cells based on the non-ribosomal peptide indigoidine. *Metabolic Engineering* 14, 325-335.

Munawar, A., Marshall, J.W., and al., e. 2013. Isolation and characterisation of a ferrirhodin synthetase gene from the sugarcane pathogen *Fusarium sacchari*. *ChemBioChem* 14, 388-394.

Nakamura, H., Katayama, T., Okabe, T., Iwashita, K., Fujii, W., Kitamoto, K., and Maruyama, J. 2017. Highly efficient gene targeting in *Aspergillus oryzae* industrial strains under *ligD* mutation introduced by genome editing: Strain-specific differences in the effects of deleting EcdR, the negative regulator of sclerotia formation. *J. Gen. Appl. Microbiol.* 63, 172-178.

Neuhäuser, B. 2020. Distinct transport mechanism in *Candida albicans* methylammonium permeases. *Mycol Progress* 19, 1143-1149.

Nguyen, K.T., Ritz, D., Gu, J.Q., Alexander, D., Chu, M., Miao, V., Brian, P., and Baltz, R.H. 2006. Combinatorial biosynthesis of novel antibiotics related to daptomycin.

Proceedings of the National Academy of Sciences of the United States of America 103, 17462-17467.

Nielsen, J.C., Prigent, S., Grijseels, S., Workman, M., Ji, B., and Nielsen, J. 2019. Comparative transcriptome analysis shows conserved metabolic regulation during production of secondary metabolites in filamentous fungi. *mSystems* 4, e00012-00019.

Ninnemann, O., Jauniaux, J.C., and Frommer, W.B. 1994. Identification of a high affinity ammonium transporter from plants. *EMBO J* 13, 3464–3471.

Ochsenreither, K., Fischer, C., Neumann, A., and Syldatk, C. 2014. Process characterization and influence of alternative carbon sources and carbon-to-nitrogen ratio on organic acid production by *Aspergillus oryzae* DSM1863. *Applied Microbiology and Biotechnology* 98, 5449-5460.

Ostaszewska, T., Dabrowski, K., Kamaszewski, M., Grochowski, P., Verri, T., Rzepkowska, M., and Wolnicki, J. 2010. The effect of plant protein-based diet supplemented with dipeptide or free amino acids on digestive tract morphology and PepT1 and PepT2 expressions in common carp (*Cyprinus carpio* L.). *Comparative Biochemistry and Physiology, Part A* 157, 158-169.

Ostaszewska, T., Dabrowski, K., Kamaszewski, M., Kwasek, K., Grodzik, M., and Bierla, J. 2012. The effect of dipeptide, Lys-Gly, supplemented diets on digestive tract histology in juvenile yellow perch (Perca flavescens). *Aquaculture Nutrition* 19, 100-109.

Özcengiz, G., and Öğülür, İ. 2015. Biochemistry, genetics and regulation of bacilysin biosynthesis and its significance more than an antibiotic. 32, 612-619.

Pahirulzaman, K.A., Williams, K., and Lazarus, C.M. 2012. A toolkit for heterologous expression of metabolic pathways in *Aspergillus oryzae*. *Methods Enzymol.* 517, 241-260.

Pardo, A.G. 1996. Effect of surfactants on cellulase production by *Nectria catalinensis*. *Curr. Microbiol.* 33, 275-278.

Punya, J., Tachaleat, A., Wattanachaisaereekul, S., Haritakun, R., Boonlarppradab, C., and Cheevadhanarak, S. 2013. Functional expression of a foreign gene in *Aspergillus oryzae* producing new pyrone compounds. *Fungal. Genet. Biol.* 50, 55-62.

Rabha, J., and Jha, D.K. 2017. Metabolic diversity of *Penicillium*. In Gupta, V.K., and Rodriguez-Couto, S. (eds.), *New and future development in microbial biotechnology and bioengineering: Penicillium system properties and applications*. Elsevier, pp. 217-234.

Röttig, M., Medema, M.H., Blin, K., Weber, T., Rausch, C., and Kohlbacher, O. 2011. NRPSpredictor2: A web server for predicting NRPS adenylation domain specificity. . *Nucleic Acids Research* 39, W362–W367.

Santos, S., Torcato, I., and Castanho, M.A. 2012. Biomedical applications of dipeptides and tripeptides. *Biopolymers* 98, 288-293.

Shimono, M., and Sugiyama, K. 2009. Japanese patent WO2009/113563

Stachelhaus, T., Mootz, H.D., and Marahiel, M.A. 1999. The specificity-conferring code of adenylation domains in nonribosomal peptide synthetases. *Chemistry & Biology* 6, 493-505.

Taichert, S., Rutherford, J.C., Wottawa, M., Heitman, J., and Tudzynski, B. 2008. Impact of ammonium permeases MepA, MepB, and MepC on nitrogen-regulated secondary metabolism in *Fusarium fujikuroi*. *Eukaryotic Cell* 7, 187-201.

ter Schure, E.G., van Riel, N.A., and Verrips, C.T. 2000. The role of ammonia metabolism in nitrogen catabolite repression in *Saccharomyces cerevisiae*. *FEMS Microbiol Rev* 24, 67–83.

Thammarongtham, C., Nookaew, I., Vorapreeda, T., Srisuk, T., Land, M.L., Jeennor, S., and Laoteng, K. 2018. Genome characterization of oleaginous Aspergillus oryzae BCC7051: A potential fungal-based platform for lipid production. *Current Microbiology* 75, 57-70.

Tudzynski, B. 2014. Nitrogen regulation of fungal secondary metabolism in fungi. *Frontier in Microbiology* 5, 656.

Weber, T., and Marahiel, M.A. 2001. Exploring the domain structure of modular nonribosomal peptide synthetases. *Structure.* 10, R3-9.

Wehrs, M., Gladden, J.M., Liu, Y., Platz, L., Prahl, J.-P., Moon, J., Papa, G., Sundstrom, E., Geiselman, G.M., Tanjore, D., Keasling, J.D., Pray, T.R., Simmons, B.A., and Mukhopadhyay, A. 2019. Sustainable bioproduction of the blue pigment indigoidine: Expanding the range of heterologous products in *R. toruloides* to include non-ribosomal peptides. *Green Chem.* 21, 3394–3406.

Winn, M., Fyans, J.K., Zhuo, Y., and Micklefield, J. 2014. Recent advances in engineering nonribosomal peptide assembly lines. *Natural Product Reports* 33, 317-347.

Xu, W., Cai, X., Jung, M.E., and Tang, Y. 2010. Analysis of intact and dissected fungal polyketide synthase-nonribosomal peptide synthetase *in vitro* and in *Saccharomyces cerevisiae*. *Journal of the American Chemical Society* 132, 13604-13607.

Yagasaki, M., and Hashimoto, S. 2008. Synthesis and application of dipeptides; current status and perspectives. *Applied Microbiology and Biotechnology* 81, 13-22.

Zhang, F., Wang, Y., Jiang, Q., Chen, Q., Karthik, L., Zhao, Y.L., and Li, Z. 2018. Substrate selection of adenylation domains for nonribosomal peptide synthetase (NRPS) in bacillamide C biosynthesis by marine *Bacillus atrophaeus* C89. *Journal of Industrial Microbiology & Biotechnology* 45, 335-344.

พึ่งแพง, ส., ลิ้มสุวรรณ, ช., และ ภูรีวิโรจน์กุล, ว. 2555. การวิเคราะห์โปรตีนของสารต้านจุลินทรีย์จากเชื้อ *Bacillus subtilis* สายพันธุ์ B-1. วารสารวิจัยเทคโนโลยีการประมง 6, 84-93.

8. Output

8.1 ผลงานตีพิมพ์ในวารสารวิชาการนานาชาติ

Chutrakul, C., Panchanawaporn, S., Jeennor, S., Anantayanon, J. and Laoteng, K. (2022). Promoter exchange of the cryptic nonribosomal peptide synthetase gene for oligopeptide production in *Aspergillus oryzae*. Journal of Microbiology. 60: 47-56. (Q1, IF 2.845)

Panchanawaporn, S., **Chutrakul, C.**, Jeennor, S., Anantayanon, J. and Laoteng, K. (2022). Potential of *Aspergillus oryzae* as a promising production platform for nonribosomal peptide pigment, indigoidine, with antioxidant activity. ส่งผลงานตีพิมพ์ในวารสาร PLOS ONE (Q1, IF 3.240) อยู่ในระหว่างดำเนินการแก้ไขตามคำแนะนำของผู้เชี่ยวชาญ (major revision)

Chutrakul, C., Panchanawaporn, S., Vorapreeda, T., Jeennor, S., Anantayanon, J. and Laoteng, K. (2022). Exploring functional role of ammonium transporters of *Aspergillus oryzae* in nitrogen metabolism and challenging to cell biomass production. อยู่ในขั้นตอนการเตรียมยื่นต้นฉบับ โดยมีแผนจะส่งผลงานตีพิมพ์ในวารสาร International Journal of Molecular Sciences (Q1, IF 5.923)

8.2 การจดอนุสิทธิบัตรภายในประเทศ

ชนิกุล ชูตระกูร, สโรชา ปัญจนวพร, นกุล รัตนพันธ์, กอบกุล เหล่าเท้ง (2564) อนุสิทธิบัตรเรื่องราเส้นใยดัดแปลงพันธุกรรมสำหรับผลิตสารชีวรงควัตถุและกรรมวิธีการผลิตสารชีวรงควัตถุดังกล่าว วันที่ยื่นคำขอ 24 กันยายน 2564