วารสารวิทยาศาสตร์ประยุกต์
THE JOURNAL OF APPLIED SCIENCE

ISSN 1513-7805 [Print] ISSN 2586-9663 [Online] ปีที่ 17 ฉบับพิเศษ [2561] Vol.17, Special issue [2018]



Contents **Page**

1. Evaluation of *Bacillus amyloliquefaciens* for control of potato blackleg caused by *Pectobacterium carotovorum* 1
2. Transesterification reaction for palm based wax esters by immobilized lipase EQ3 isolated from wastewater of fish canning industry 9
3. Enhanced succinic acid production from L-arabinose by *Corynebacterium glutamicum* CS176-Δ*ldhA* disruptant 18
4. A preliminary study on differential gene expression in hemocyte of the mantis shrimp *Harpiosquilla raphidea* by next generation sequencing technique 27
5. Ethanol production from cellulosic cassava waste 37
6. Biohydrogen production from xylose by the newly isolated *Clostridium beijerinckii* PS-3 47
7. Transformation of artemisinin to deoxyartemisinin by *Aspergillus terricola* TISTR 3019 55
8. Anti-aging efficacy of Thai red rice callus cosmetic product 63
9. Simultaneous extraction of oil and protein from perilla seed by three-phase partitioning and their application in serum 73

วารสารวิทยาศาสตร์ประยุกต์
THE JOURNAL OF APPLIED SCIENCE
ISSN 1513-7805 ปีที่ 16 ฉบับพิเศษ [2560] Vol. 16, Special issue [2017]

วารสารวิทยาศาสตร์ประยุกต์ เป็นวารสารที่ตีพิมพ์และเผยแพร่บทความวิจัยและบทความทางวิชาการเพื่อเป็นสื่อกลางในการเผยแพร่ความรู้และวิทยาการด้านวิทยาศาสตร์และเทคโนโลยี และเพื่อเป็นแหล่งแลกเปลี่ยนเรียนรู้ระหว่างคณาจารย์ นักวิชาการและนักศึกษา ทั้งภายในและภายนอกมหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ บทความที่เสนอมาอาจเขียนเป็นภาษาไทยหรือภาษาอังกฤษก็ได้ วารสารจัดตีพิมพ์ปีละ 2 ฉบับ ผู้สนใจสามารถส่งต้นฉบับแบบออนไลน์ได้โดยตรงที่ http://www.journal.sci.kmutnb.ac.th โดยบทความดังกล่าวจะต้องไม่เคยตีพิมพ์เผยแพร่ในการประชุมวิชาการหรือวารสารวิชาการอื่นใดมาก่อน และเป็นข้อคิดเห็นของผู้ส่งบทความเท่านั้น

- **วัตถุประสงค์**

เพื่อเผยแพร่/พัฒนาผลงานจากการวิจัยและผลงานทางวิชาการที่เกี่ยวข้องทางวิทยาศาสตร์พื้นฐาน วิทยาศาสตร์ประยุกต์ และเทคโนโลยี ที่เกี่ยวข้องกับคณิตศาสตร์ สถิติ ฟิสิกส์อุตสาหกรรม อุปกรณ์การแพทย์ เทคโนโลยีสารสนเทศ เทคโนโลยีชีวภาพ อุตสาหกรรมเกษตร สิ่งแวดล้อม เคมีอุตสาหกรรม สถาปัตยกรรม และวิศวกรรมศาสตร์

- **กำหนดการเผยแพร่วารสาร** ปีละ 2 ฉบับ คือ ฉบับที่ 1 มกราคม-มิถุนายน ฉบับที่ 2 กรกฎาคม-ธันวาคม

"บทความทุกบทความจะต้องผ่านการพิจารณาจากผู้ทรงคุณวุฒิ อย่างน้อย 2 ท่าน ก่อนที่จะตอบรับการตีพิมพ์"

"เมื่อวารสารส่งบทความไปให้ผู้นิพนธ์แก้ไขแล้ว หากไม่มีการติดต่อกลับมาภายใน 4 สัปดาห์ ทางวารสาร ขอปฏิเสธการตีพิมพ์บทความนั้น"

- **การเผยแพร่วารสาร**

จัดส่งให้หน่วยงานห้องสมุด บริการวิชาการและหน่วยงานที่เกี่ยวข้องทั่วประเทศและสามารถค้นหาได้ในเว็บไซต์นี้

- **การส่งบทความ**

ส่งไฟล์บทความ .doc และ pdf
ที่ **http://www.journal.sci.kmutnb.ac.th**

- **สำนักงาน**

งานวิจัยและประกันคุณภาพการศึกษา
สำนักงานคณบดี คณะวิทยาศาสตร์ประยุกต์
มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
1518 ถ.ประชาราษฎร์ 1 บางซื่อ กรุงเทพฯ 10800

● ที่ปรึกษา

รศ.ดร.สุรพันธ์	ยิ้มมั่น	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
ดร.พีรพงษ์	พรวงศ์ทอง	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
ผศ.ดร.โดม	โล่ห์เพ็ชร์	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ

● บรรณาธิการ

รศ.ดร.สมโภชน์	น้อยจินดา	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ

● บรรณาธิการร่วม

รศ.ดร.กิตติโพธิปัทมะ		มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ

● ผู้ช่วยบรรณาธิการ

รศ.ดร.เสาวณิต	สุขภารังษี	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
รศ.ดร.วีระศักดิ์	อัศวางศ์อารยะ	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
ผศ.ดร.สุมนต์ทิพย์	คงตันจันทร์ฟัก	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
ผศ.ดร.วลัยพร	ปฤษณารุณ เอื้อใจ	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
ผศ.ดร.เบญจพร	ลิ้มธรรมาภรณ์	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
ผศ.ดร.อภิชาต	ศุรธณี	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
ดร.สุกัญญา	แพรสมบูรณ์	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ

● ผู้ทรงคุณวุฒิในกองบรรณาธิการ

ศ.ดร.ประนอม	จันทรโณทัย	มหาวิทยาลัยขอนแก่น
ศ.ดร.ยงค์วิมล	เลณบุรี	มหาวิทยาลัยมหิดล
ศ.ดร.สังวรณ์	กิจทวี	มหาวิทยาลัยมหิดล
Professor Dr. Jennifer Ayla	Jay	University of California Los Angeles, USA
Professor Dr.rer. nat. Carsten	Harms	University of Applied Sciences Bremerhaven, Germany
Professor Dr.Woo-Kul	Lee	Dankook University, South Korea
รศ.ดร.มนตรี	มาลีวงศ์	มหาวิทยาลัยเกษตรศาสตร์
รศ.ดร.ดุษฎี	อุตภาพ	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี
รศ.ดร.เฉลิมชัย	วงษ์อารี	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี
รศ.ดร.สอาด	ริยะจันทร์	มหาวิทยาลัยธรรมศาสตร์
รศ.ดร.สิทธิวัฒน์	เลิศศิริ	มหาวิทยาลัยมหิดล
รศ.ดร.มารินา	เกตุทัต-คาร์นส์	มหาวิทยาลัยเทคโนโลยีสุรนารี
รศ.ดร.วราฤทธิ์	พานิชกิจโกศล	มหาวิทยาลัยธรรมศาสตร์
Associate Professor Dr.Haruhiko Toyohara		Kyoto University, Japan
ผศ.ดร.กันต์พงษ์	วรรัตน์ปัญญา	สถาบันเทคโนโลยีพระจอมเกล้าเจ้าคุณทหารลาดกระบัง
ผศ.ดร.เสน่ห์	รุจิวรรณ	มหาวิทยาลัยวลัยลักษณ์
ผศ.ดร.ศุภกานต์	พิมลธเรศ	จุฬาลงกรณ์มหาวิทยาลัย
พ.อ.หญิง ผศ.ดร.ปนัดดา	หัตถโชติ	วิทยาลัยแพทยศาสตร์ พระมงกุฎเกล้า
ดร.ชีวารัตน์	ม่วงพัฒน์	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าธนบุรี
Dr.Elvin James	Moor	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ

● ฝ่ายประสานงานและจัดการ

นางภควรรณ	สีสวย	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
นายภิรมย์	พาบุ	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
นายปิยะวัฒน์	สุธา	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
นางสาววิลัยพร	ขันแก้ว	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ
นางสาวรมณ	ทองศรี	มหาวิทยาลัยเทคโนโลยีพระจอมเกล้าพระนครเหนือ

"บทความที่ลงพิมพ์ เป็นข้อคิดเห็นของผู้เขียนเท่านั้น
และจะต้องเป็นผู้รับผิดชอบต่อผลทางกฎหมายใดๆ ที่อาจจะเกิดขึ้นจากบทความนั้น"

The Journal of Applied Science
วารสารวิทยาศาสตร์ประยุกต์
ISSN 1513-7805 (Printed in Thailand)

Vol. 17 Special issue: 1-8 [2018]
doi: 10.14416/j.appsci.2018.02.S01
ISSN 2586-9663 (Online)

Research Article

Evaluation of *Bacillus amyloliquefaciens* for control of potato blackleg caused by *Pectobacterium carotovorum*

Athidtaya Kumvinit[1,2,3] and Angsana Akarapisan[1,2,3]*

[1]Division of Plant Pathology, Department of Entomology and Plant Pathology, Faculty of Agriculture, Chiang Mai University, Chiang Mai 50200, Thailand
[2]Center for Agricultural Biotechnology, Kasetsart University, Kamphaeng Saen Campus, Nakhon Pathom 73140, Thailand
[3]Center of Excellence Agricultural Biotechnology (AG-BIO/PERDO-CHE), Bangkok 10900, Thailand.
***E-mail**: Angsana.a@cmu.ac.th

Abstract

Potato is an important crop in Chiang Mai, Thailand. Blackleg disease is one of the constraints of potato production. The objective of this study was to evaluate the efficacy of antagonistic bacteria to control the causal pathogen. Diseased potato stems were collected in Chai Prakan District, Chiang Mai Province. The symptoms included black stem lesions, vascular discoloration, and foliar yellowing and wilting. The pathogen was identified as the bacterium *Pectobacterium carotovorum* CK3-2 by morphological characteristics and sequencing the 16S rDNA gene. *Bacillus amyloliquefaciens* isolates BB06, BB18, BB23 and NSB4 recovered from the rhizosphere of a number of different plants species, and identified by 16S rDNA sequence, were provided by the Bacteria Plant Disease Laboratory, Faculty of Agriculture, Chiang Mai University. These *B. amyloliquefaciens* isolates produced zones of inhibition of *P. carotovorum* CK3-2 of 6.80, 5.80, 6.60 and 8.20 mm, respectively. Co-inoculation experiments on potato tubers indicated that *B. amyloliquefaciens* BB06 was the most effective treatment in inhibiting invasion of wounds.

Keywords: Potato, *Pectobacterium carotovorum*, *Bacillus amyloliquefaciens*, biological control, blackleg, bacterial soft rot

Introduction

Potato (*Solanum tuberosum*) is considered the fourth most important crop after rice, maize and bean in Chiang Mai Province, Thailand. The cultivation area of potato is around among 2,213 ha with 41,223 tons of production. The period of planting is October to April (Dry season) (Chiang Mai Agriculture, 2015) Blackleg and soft rot diseases are caused by bacteria belonging to *Enterobecteriaceae* family and the genera *Pectobacterium* species (formerly classified as pectinolytic *Erwinia* spp.). *Pectobacterium* spp. cause disease in a wide range of plants, including potato, broccoli, carrot, tomato, cabbage and eggplant. These bacteria cause soft rot of tubers and blackleg of stems on field-grown potato (Czajkowski et al., 2011; Katarina et al., 2014; Sonia et al., 2015). The bacteria produce plant cell-wall degrading enzymes such as cellulase, pectinase, polygalacturonase and protease. The disease always occurs in the field as well as transportation and storage (Lee et al., 2014).

Vol. 17 Special issue: 1-8 [2018]
doi: 10.14416/j.appsci.2018.02.S01

A number of *Pectobacterium* species and subspecies cause soft rot and blackleg disease on potato, including *Pectobacterium atrosepticum*, *Pectobacterium wasabiae*, and *Pectobacterium carotovorum*, which has been divided into three subspecies including *Pectobacterium carotovorum* subsp. *carotovorum*, *Pectobacterium carotovorum* subsp. *odoriferum* (Gardan et al., 2003) and *Pectobacterium carotovorum* subsp. *brasiliensis* (Duarte et al., 2004). *Bacillus* spp., a plant growth promoting rhizobacterium (PGPR) has been associated with suppression of a number of pathogens, and produces endospores which enable it to resist environmental stresses (Jacobsen et al., 2004). The biological mechanisms behind pathogen inhibition by PGPR include habitat occupation, antibiosis, and induction of plant defense systems. The objective of this study was to evaluate the efficacy of *Bacillus amyloliquefaciens* against *Pectobacterium carotovorum*.

Material and methods

Bacterial Isolates

Diseased potato stems were collected in Chai Prakan District, Chiang Mai Province in 2016. The symptoms included black stem lesions, vascular discoloration, and foliar yellowing and wilting. Infected tissue at the leading edge of the infection was excised with a sterile knife, macerated in sterile water, and a loopful of the macerate was streaked on nutrient agar (NA). Pure cultures were cultivated on NA and Eosin methylene blue (EMB) agar for 48 h at room temperature. Isolates of antagonistic rhizosphere bacteria were provided by the Bacterial Plant Disease Laboratory, Faculty of Agriculture, Chiang Mai University and included BB06 (from chilli pepper), BB18 (from tomato), BB23 (from coffee) and NSB4 (from banana).

Identification of bacteria

Extraction of the DNA from Gram-negative and Gram-positive bacteria used a modification of the protocol of Cheng & Jiang (2006). Bacterial isolates were cultivated in Lubia-Bertani broth (LB) on a rotary shaker at 30ºC and 180 rpm for 18 h. About 2 ml of the cell suspension was centrifuged at 8,000g for 2 min. The pelleted cells were washed with 400 µl of STE Buffer (100 mM NaCl, 10 mM Tris/HCl, 1 mM EDTA, pH 8.0) twice and mixed by a vortex. Then the cells were centrifuged at 8,000g for 2 min. The pellets were resuspended in 200 µl of TE buffer (10 mM Tris/HCl, 1 mM EDTA, pH 8.0), and 100 µl Tris-saturated phenol (pH 8.0) was added and mixed by a vortex. The samples were centrifuged at 13,000g for 5 min. The supernatant containing the DNA (160 µl) was removed into a new sterile 1.5 ml tube. TE buffer (40 µl) was added and mixed with 100 µl of chloroform and centrifuged for 5 min at 13,000g. The supernatant (160 µl) was transferred to a clean 1.5 ml tube. TE buffer (40 µl) was added, mixed well and incubated at 37° C for 10 min. Chloroform (100 µl) was added and the sample was mixed. After centrifugation at 13,000g for 5 min, 150 µl of the supernatant was transferred to a clean 1.5 ml tube. Genomic DNA in TE buffer was visualized in 1% (w/v) agarose gels. DNA concentration was determined by gel electrophoresis.

PCR was performed using the primers 16SF (5'-AGAGTTTGATCCTGGCTCAG-3') and 16SR (5'-AAGGAGGTGATCCAGCCGCA-3') amplify a fragment of about 1500 bp (Liu et al., 2009). PCR reactions were performed in a total volume of 50 µl, containing genomic DNA, 10X PCR buffer, 20 µM of each of dNTP, 50 mM $MgCl_2$, 1 U of *Taq* DNA polymerase and 20 µM of each primer. The reaction mixtures were incubated in a Peltier-based Thermal Cycler A100/A200 (LongGene Document Version 1.4). Following an initial denaturation at 94°C for 5 min, the DNA templates were amplified for 35 cycle consisting of 30 s at 94°C, 30 s at 64°C,

Vol. 17 Special issue: 1-8 [2018]
doi: 10.14416/j.appsci.2018.02.S01

and 2 min at 72°C followed by a final extension at 72°C for 10 min. Amplification products were separated in 1% (w/v) agarose gels, and viewed by gel electrophoresis.

The PCR products were purified and directly sequenced by Fluorescent dye-terminator sequencing ABI Prism™ 3730xl DNA sequencer (Bio Basic Inc). The obtained sequences were edited and assembled using MAGA5 software and then compared with available sequences in the GenBank (http://www.ncbi.nlm.nih.gov/Genbank) using Basic local Alignment Search Tool (BLAST) for nucleotide alignments.

Inhibition assay

The pathogen (3 ml of 10^8 cfu/ml) was transferred to a 250-ml flask containing 97 ml of NA and poured into petri-dishes. After that, the antagonistic bacteria (10^8 cfu/ml) were evaluated by a disc diffusion assay. Paper discs (6 mm diameter) were plated on the agar, followed by incubation at 28°C for 3 d, and measurements of the inhibition zone have been done. The experimental was designed as a CRD with five replications.

Co-inoculation Experiment

Potato tubers were washed with a 1% sodium hypochlorite solution for 5 min and rinsed twice with sterile water. The tubers were dried and then were sprayed with 70% ethanol. The pathogen strain and antagonistic bacteria were diluted in sterile 0.8% NaCl to 10^8 cfu/ml. The tubers were inoculated with 10 µl of the bacterial pathogen suspension and each bacterial antagonist suspension, and incubated at room temperature. The experimental design was a CRD with 4 replications (Esmaeil et al., 2011).

Results and Discussion

The pathogen isolate CK3-2 of blackleg disease was isolated from a potato field in Chai Prakan District, Chiang Mai, Thailand. Typical symptoms of the disease were observed including black stem lesions, vascular discoloration, and foliar yellowing and wilting (Figure 1A). The isolate CK3-2 showed motile with peritrichous flagella (Figure 1B). The isolate produced white to creamy colonies on NA (Figure 1C) and emerald green colonies on EMB which developed after 2 d (Figure 1D). Therefore, the isolate was preliminarily identified as belonging to the genus *Pectobacterium* (Golkhandan et al., 2013). The phylogenetic tree was based on a 1500 bp alignment sequence of 16S rDNA of the isolate from the potato blackleg sample. The pathogen was identified as *Pectobacterium carotovorum* and clustered closely with the type strain of *P. carotovorum* DSM30168 (GenBank NR_041971.1) and *P. carotovorum* CFBF2046 (GenBank NR_118227.1). The database showed 99% sequence similarity with the type strain (Figure 2). Fujimoto et al. (2017) studied potato blackleg disease in Japan and identified the causal pathogen as *P. carotovorum* subsp. *brasiliense* by 16S rDNA sequences. Similar results were obtained by Duarte et al. (2004). All strains from blackleg and stem rot disease samples yielded a specific amplified (322 bp) in a PCR with primers Br1f and L1r for *P. carotovorum* subsp. *brasiliense.*

The antagonistic bacterial isolates BB06, BB18, BB23 and NSB4 were identified as *Bacillus amyloliquefaciens* by 16S rDNA sequences. They were grouped with *B. amyloliquefaciens* BCRC11601 (GenBank NR_116022.1), *B. amyloliquefaciens* MPA1034 (GenBank NR_117946.1), *B. amyloliquefaciens* NBRC15535 (GenBank NR_112685.1) and *B. amyloliquefaciens* (GenBank NR_118227.1) (Figure 3). The disc diffusion assay found that isolates NSB4 and BB06 produced equivalent zones of inhibition that were significantly greater than BB10. BB23 produced an intermediated zone of inhibition (Figure 4).

The Journal of Applied Science **Vol. 17 Special issue: 1-8 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S01

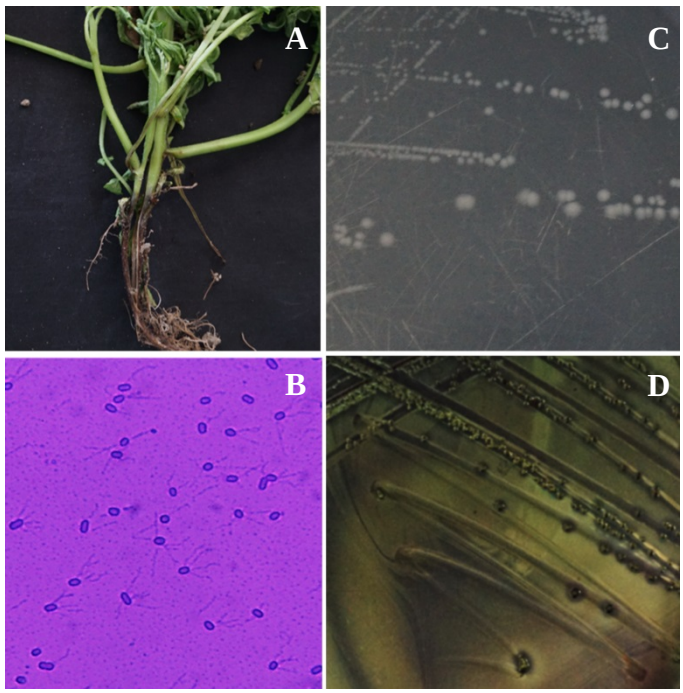


Figure 1. Blackleg symptoms and morphology of *Pectobacterium carotovorum* CK3-2: (A) blackleg symptom on potato stem, (B) peritrichous flagella, (C) the colony on NA, (D) the colony on EMB agar.

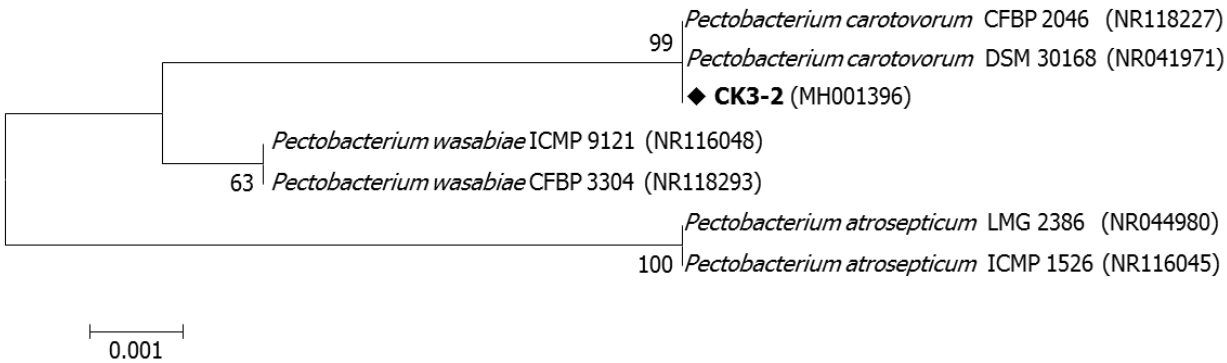


Figure 2. Phylogenetic tree based on comparative 16S rDNA sequence analysis of isolate CK3-2.

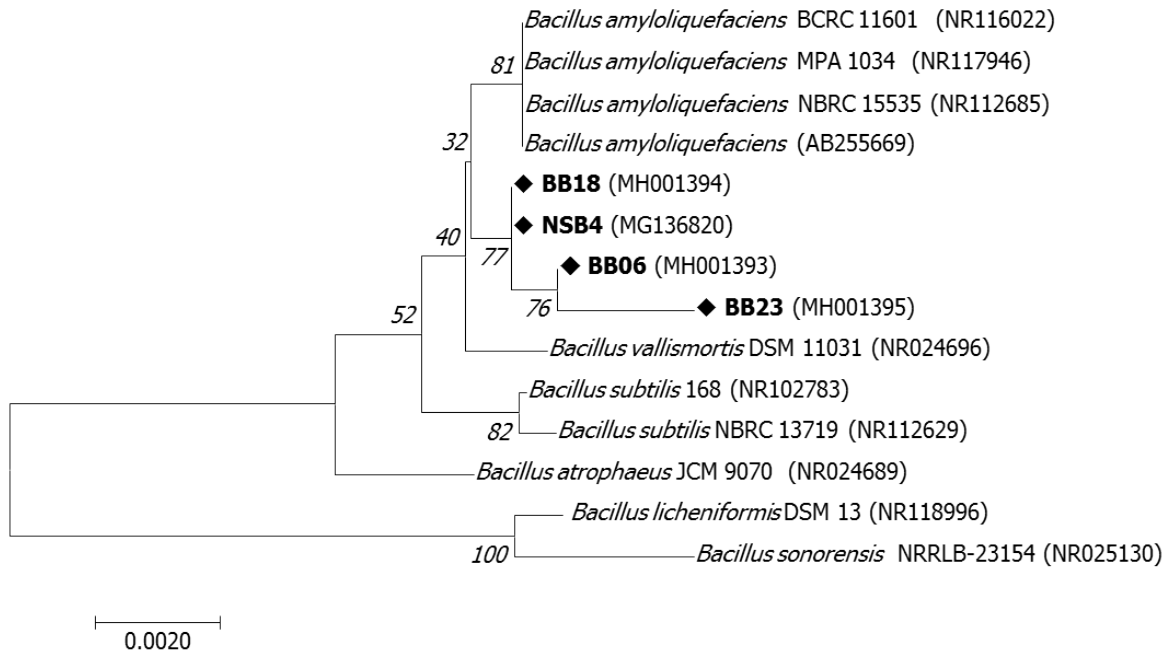


Figure 3. Phylogenetic tree based on comparative 16S rDNA sequence analysis of isolates BB06, BB18, BB23 and NSB4.

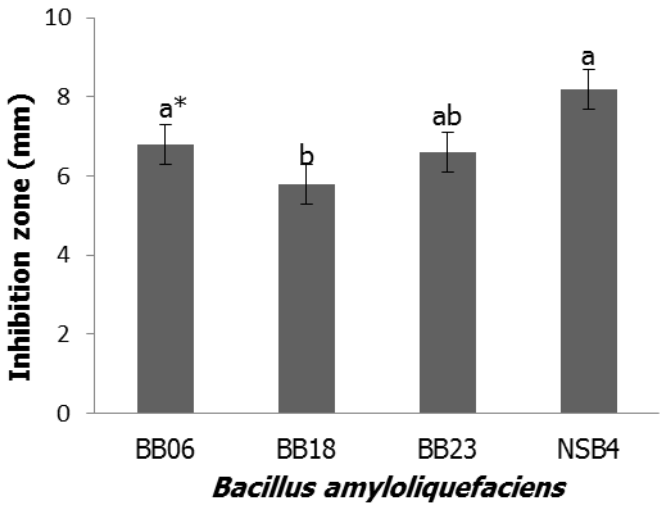


Figure 4. Inhibition zone of *Bacillus amyloliquefaciens* BB06, BB18, BB23 and NSB4 against *Pectobacterium carotovorum* CK3-2 by a disc diffusion assay at 28ºC after incubation for 3 d on NA. *The values within the table with different superscripts are significantly different by LSD (p=0.05).

The Journal of Applied Science **Vol. 17 Special issue: 1-8 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S01

The antagonism of the four isolates of *B. amyloliquefaciens* was tested in the co-inoculation experiment on potato tuber. The results showed that the direct inoculation of *P. carotovorum* CK3-2 gave tissue maceration after 72 h of incubation. As shown in Figure 5 and Table 1, the co-inoculation of the pathogen isolate CK3-2 and the antagonistic isolates BB06, BB18, and NSB4 led to significant reductions in tissue maceration area, measuring 1.73, 9.30, and 11.72 mm^2, respectively, when compared to the pathogen alone (23.56 mm^2). Co-inoculation with isolate BB23 did not significantly reduce the maceration area. *Bacillus amyloliquefaciens* BB06 was the most effective show in inhibiting invasion of wounds and reducing tissue maceration. Similar results were obtained by Esmaeil et al. (2011). The inoculation of *P. carotovorum* subsp. *carotovorum* EMPCC caused extensive tissue maceration in 48 h, while the ability of EMPCC to macerate potato tubers tissue was reduced when co-inoculated with by *Bacillus* sp. DMA133. Yin et al. (2010) reported that *B. amyloliquefaciens* showed biocontrol efficacy against *P. carotovorum* subsp. *carotovorum* on carrot. *Bacillus subtilis*, *Bacillus thuringiensis*, *Bacillus megaterium*, *Bacillus cereus*, and *Bacillus pumilus* showed good activity by zones of inhibition against *P. carotovorum* subsp. *carotovorum* (Issazadeh et al., 2012). Furthermore, Hoda & Kamal (2016) found that antagonistic isolate *Bacillus subtilis* Bs3 restricted the growth of *Pectobacterium atrosepticum*, a causal agent of blackleg disease of potato. Isolate BB06 will be tested for disease control efficacy under field conditions by mixing the tuber with a formulation of the bacterium before planting or by spraying.

Table 1. Effectiveness of antagonistic bacteria in reducing disease on potato 72 h after inoculation.

Treatment	**Diameter of wound (mm^2)***
Negative control (0.8% NaCl)	0.00^{c}**
Positive control (pathogen (CK3-2) only)	23.56^{a}
Co-inoculation of CK3-2 and BB06	1.73^{c}
Co-inoculation of CK3-2 and BB18	9.30^{b}
Co-inoculation of CK3-2 and BB23	19.00^{a}
Co-inoculation of CK3-2 and NSB4	11.72^{b}
LSD (p=0.05)	6.08
CV (%)	30.84

*The average was calculated using data from four replications.
**The values within the table with different superscripts are significantly different by LSD (p=0.05).

Conclusion

The causal agent of potato blackleg disease in Chai Prakan District, Chiang Mai Province was identified as the bacterium *Pectobacterium carotovorum* CK3-2 by morphological characteristics and sequencing of the 16S rDNA. The symptoms included black stem lesions, vascular discoloration, and foliar yellowing and wilting. The pathogen was tested with *Bacillus amyloliquefaciens* BB06, BB18, BB23 and NSB4 by a disc diffusion assay. The results indicated that, isolates NSB4 and BB06 showed the greatest inhibition zones of *P. carotovorum* CK3-2. The co-inoculation experiment on potato tubers found that *B. amyloliquefaciens* BB06 was the most effective in inhibiting the invasion of wounds by *P. carotovorum* CK3-2.



Figure 5. Attenuation of the maceration capacity of *Pectobacterium carotovorum* CK3-2 by *Bacillus amyloliquefaciens* BB06, BB18, BB23 and NSB4 at 72 h after incubation.

Acknowledgement

This research is partially supported by the Center of Excellence on Agricultural Biotechnology, Science and Technology Postgraduate Education and Research Development Office, Office of Higher Education Commission, Ministry of Education (AG-BIO/PERDO-CHE).

References

Chiang Mai Agriculture. (2015). *An economic fruit cultivation area information in Chiang Mai Province.* Retrieved July 21, 2017, from http://www.chiangmai.doae.go.th/ Stat_Plan.html.

Cheng, H. R. & Jiang, N. (2006). Extremely rapid extraction of DNA from bacteria and yeasts. *Biotechnology Letters, 28,* 55-59. http://dx.doi.org/10.1007/s10529-005-4688-z.

Czajkowski, R., Perombelon, M. C. M., van Veen, J. A. & vander Wolf, J. M. (2011). Control of blackleg and tuber soft rot of potato cause by *Pectobacterium* and *Dickeya* species; a review. *Plant Pathology, 60*, 999-1013. http://dx.doi.org/ 10.1111/aab.12166.

Duarte, V., DoBoer, S. H., Ward, L. J. & de Oliveira, A. M. R. (2004). Characterization of atypical *Erwinia carotovora* strains causing blackleg of potato in Brazil. *The Society for Applied Microbiology, 96*, 535-545. http://dx.doi.org/10.1111/j.1365-2672.2004.02173.x.

Esmaeil, M., Badraldin, E. S. T. & Vittorio, V. (2011). Virulence attenuation of *Pectobacterium carotovorum* using N-Acyl-homoserine Lactone degrading bacteria isolated from potato rhizosphere. *The Plant Pathology Journal*, *27(3)*, 242-248. http://dx.doi.org/10.5423/PPJ. 2011.27.3.242.

Fujimoto, T., Yasuoka, S., Aono, Y., Nakayama, T., Ohki, T., Sayama, M. & Maoka, T. (2017). First Report of Potato Blackleg Caused by *Pectobacterium carotovorum* subsp. *brasiliense* in Japan (disease notes). *The American Phytopathological Society*, *101(1)*, 241. http://dx.doi.org/ 10.1094/PDIS-06-16-0928-PDN.

Garden, L., Gouy, C. Christen, R. & Samsom, R. (2003). Elevation of *Pectobacterium atrosepticum* sp. nov., *Pectobacterium betavasculorum* sp. nov. and *Pectobacterium wasabiae* sp. nov. *International Journal of Systematic and Evolutionary Microbiology, 53,* 381-391. http://dx.doi.org/10.1099/ ijs.0.02423-0.

Golkhandan, E., Kamaruzaman, S., Sariah, M., Zainal Abidin, M. A. & Nasehi, A. (2013). Characterisation of *Pectobacterium carotovorum* causing soft rot on Kalanchoe gastonis-bonnierii in Malaysia. *Archives of Phytopathology and Plant Protection*, *46(15)*, 1809-1815. http://dx.doi.org/10.1080/ 03235408.2013.778452.

Hoda, H. E-H. & Kamal, A. M. A-E. (2016). Combination of different antagonistic bacteria to control of potato blackleg disease caused by *Pectobacterium atrosepticum* under greenhouse and field condition. *International Journal of Phytopathology, 5(1),* 53-43.

Issazadeh, K., Rad, S. K., Zarrabi, S. & Rahimibashar, M. R. (2012). Antagonism of Bacillus species against *Xanthomonas campestris* pv. *campestris* and *Pectobacterium carotovorum* subsp. *carotovorum*. *African Journal of Microbiology Research*, *6(7)*, 1615-1620. http://dx.doi.org/ 10.5897/AJMR12.075.

Jacobsen, B. J., Zidack, N. K. & Larson, B. J. (2004). The role of Bacillus-based biological control agents in integrated pest management systems. *Photophatology, 94,* 1272-1275. http://dx.doi.org/ 10.1094/PHYTO.2004.94.11.1272.

Katarina, G., Veljko, G., Nenad, D., Nenad, T., Svetlana, Z., Danijela, R. & Aleksa, O. (2014). *Pectobacterium carotovorum* subsp. *carotovorum* – the causal agent of broccoli soft rot in Serbia. *Pesticide Phytomed (Belgrade), 29(4)*, 249-255. http://dx.doi.org/10.2298/ PIF1404249G.

Lee, D. H., Kim, J. B., Lim, J. A., Han, S. W. & Heu, S. (2014). Genetic diversity of *Pectobacterium carotovorum* subsp. *brasiliensis* isolated in Korea. *Plant Pathology Journal, 30(2)*, 117-124. http://dx.doi.org/10.5423/PPJ.OA.12.2013.0117.

Liu, B., Qiao, L., Huang, L., Buchenauer, H., Han, Q., Kang, Z. & Gong, Y. (2009). Biological control of take-all in wheat by endophytic *Bacllus subtilis* E1R-j and potential mode of action. *Biological Control*, *49*, 277-285. https://doi.org/10.1016/j.biocontrol.2009.02.007.

Sonia, N. H., Graig, G. E., Rachel, K., Neil, M. P., Leighton, I, Ian, K. T. & Geny, S. S. (2015). Detection of the bacterial potato pathogens *Pectobacterium* and *Dickeya* spp. using conventional and Real-Time PCR. *Plant Pathology, 1302*, 1-16. http://dx.doi.org/ 10.1007/978-1-4939-2620-6_1.

Yin, X-T., Xu, L., Fan, S-S., Xu L-N., Li, D-C. & Liu, Z-Y. (2010). Isolation and characterization of an AHL lactonase gene from *Bacillus* amyloliquefaciens. *World Journal of Microbiology and Biotechnology, 26(8)* 3161-3167. http://dx.doi.org/ 10.1007/s11274-010-0308-8.

The Journal of Applied Science
วารสารวิทยาศาสตร์ประยุกต์
ISSN 1513-7805 (Printed in Thailand)

Vol. 17 Special issue: 9-17 [2018]
doi: 10.14416/j.appsci.2018.02.S02
ISSN 2586-9663 (Online)

Research Article

Transesterification reaction for palm based wax esters by immobilized lipase EQ3 isolated from wastewater of fish canning industry

Pakpimol Ungcharoenwiwat[1*] and Aran H-Kittikun[2]

[1]School of Science, Walailak University, Thasala, Nakhon Si Thammarat 80161, Thailand.
[2]Faculty of Agro-Industry, Prince of Songkla University, Hat Yai, Songkhla 90112, Thailand.
***E-mail**: pakpimol.ung@gmail.com

Abstract

Palm olein based wax esters were synthesized by tranesterification for increasing value of palm olein and applications as well as reducing chemical usage. The immobilized lipase EQ3 from *Burkholderia* sp. EQ3 which was isolated from wastewater of fish canning factory was used as biocatalyst in liquid wax esters synthesis reaction. The optimal reaction conditions for palm olein based wax esters synthesis were studied for immobilized lipase EQ3 dosages, temperatures, the molar ratios of palm olein to oleyl alcohol, substrate concentrations and organic solvents. All of these factors had an effect on efficiencies of enzymatic transesterification reaction. The optimal conditions were 10 U of immobilized lipase loading, temperature of 37°C, the molar ratio of palm olein to oleyl alcohol at 1:3 (mol/mol) and the substrate concentration of 0.33% (w/v) in isooctane, and 12 h of the reaction time producing the palm olein based wax esters reached the highest synthesis more than 80%.

Keywords: *Burkholderia* sp. EQ3, lipase EQ3, transesterification, wax esters

Introduction

Lipases are one of the most usage in industrial applications that are also growing rapidly (Schmid et al., 2001). The microbial lipases produce the great variety of catalytic activities, rapid growth of microorganisms on inexpensive media with independent seasonal fluctuations. *Burkholderia* lipases have been widely researched in terms of their commercial potential in a variety of biotechnological applications such as in food, dairy, detergent and pharmaceutical industries. Indeed, the *Burkholderia* genus is one of the most important lipase-producing microorganism (Mullen et al., 2007). Generally, the enzymatic reactions provide for an environmentally friendlier, more energy efficient and cost-effective techniques due to low-energy demanding operation and simpler downstream processing. However, using native enzyme as a biocatalyst presents some drawbacks, such as poor stability under operational conditions, difficulty of product recovery, and impossibility of multiple reuses in industrial process. The enzyme immobilization techniques have been improved their functionality, stabilized catalytic properties and reusability of enzyme, for example, the enzyme immobilized on carriers via covalent binding (Yemul & Imae, 2005; Yong et al., 2008), entrapment (Ge et al., 2009), or adsorption (Sörensen et al., 2010). Moreover, the immobilization enzyme provides

Vol. 17 Special issue: 9-17 [2018]
doi: 10.14416/j.appsci.2018.02.S02

for its repeated use as well as it is easy separation from the reaction medium (Minovska et al., 2005).

Wax esters can be extracted from animal and plant materials such as beeswax, jojoba oil and carnauba wax. They are widely used in plasticizer, lubricant, pharmaceutical and cosmetic industries (Gunawan et al., 2004). Wax esters composed of long chain fatty acids from fat or oil and fatty alcohol (Petersson et al., 2005). For wax esters synthesis, the chemical-catalyzed method has many disadvantages, such as corrosive acids required, hazards in handling corrosive chemicals, high energy consumption and degradation of synthesized esters (Knox & Cliffe, 1984; Yadav & Lathi, 2003). On the other hand, lipase-catalyzed process has been increasingly used for wax esters synthesis because of mild reaction condition and environmental friendly process. Gunawan et al. (Gunawan et al., 2004) synthesized palm-based wax esters from palm oil and oleyl alcohol by Lipozyme IM. Decagny et al. (Decagny et al., 1998) studied alcoholysis between triolein and stearyl alcohol to produce wax esters. Moreover, Radzi et al. (Radzi et al., 2005) studied large scale production of liquid wax ester from oleic acid and oleyl alcohol using Novozyme 435. From the above information, the immobilized lipases are widely used, and the immobilized forms make them achieving the stability for industrial applications.

The aims of this research were to investigate the liquid wax esters synthesis from palm olein and oleyl alcohol catalyzed by immobilized lipase EQ3 with optimization the transesterification reaction.

Materials and methods

Bacterial strain and immobilized lipase EQ3

Burkholderia sp. EQ3 producing an extracellular lipase was isolated from wastewater treatment system of the tuna canning factory in southern of Thailand on basal medium containing 1% fish oil at 37°C for 12 h (Ungcharoenwiwat & H-Kittikun, 2013). The crude lipase EQ3 from acetone precipitation was immobilized onto Accurel MP-100 as hydrophobic carrier obtained from Akzo Nobel Membrana (Obernburg, Germany). The immobilized lipase EQ3 was prepared with various enzyme activities by adsorbtion technique (Ungcharoenwiwat et al., 2016). The immobilized lipase EQ3 was stored at 4°C until use.

Chemicals

Palm olein was supplied from the local market (Hat Yai, Thailand). Oleyl alcohol was supplied by Sigma-Aldrich Corp. (St. Louis, Missouri, USA). All other chemicals used were of analytical grade.

Analyses

Lipase activity

The lipase activity was assayed in a two-phase system according to Lee and Rhee (Lee & Rhee, 1993) using 10% (w/v) palm olein dissolved in isooctane as a substrate. One unit of enzyme activity was defined as the enzyme necessary to release 1 µmol of palmitic acid per minute at the specified condition.

Wax esters analysis

Wax esters synthesis through transesterification reaction was analyzed on silica-coated quartz rods, chromarods S III (Silica gel powder coated) from Mitsubishi Chemical Medicine Corporation (Tokyo, Japan) using a thin layer chromatography-flame ionization detection

Vol. 17 Special issue: 9-17 [2018]
doi: 10.14416/j.appsci.2018.02.S02

system (TLC-FID) (IATROSCAN MK5, Iatron Laboratories Inc. (Tokyo, Japan)) (Ungcharoenwiwat & H-Kittikun, 2013).

The factors effecting on transesterification reaction for palm oil based wax esters synthesis by immobilized lipase EQ3

The reaction mixture consisted of palm olein (150 µmol, 142.8 mg), oleyl alcohol (450 µmol, 120.8 mg), immobilized lipase and hexane (1 mL) in screwed-cap tube. The reaction was incubated at 37°C and 150 rpm for 72 h. All experiments were assayed in duplicate. The samples were collected and diluted with chloroform (1:2 v/v). The percentage of wax esters synthesis for each reaction was determined using TLC-FID analysis. The factors following below were studied while the other conditions were fixed.

1. *Effect of enzyme concentration:* 2 U, 5 U and 10 U
2. *Effect of temperature:* 30°C, 37°C and 45°C
3. *Effect of substrate molar ratio:* 1:1, 1:2, 1:3 and 1:4 mol/mol (palm olein/oleyl alcohol)
4. *Effect of substrate concentration:* the optimum molar ratio of palm olein and oleyl alcohol (1:3 mol/mol, 1.0X). The substrate concentration levels at 0.33X, 0.66X and 1.0X were investigated.
5. *Effect of organic solvent:* toluene (log P=2.5), hexane (log P=3.5), heptane (log P=4.0) and isooctane (log P=4.5)

Results and discussion

Factors effecting on palm olein based wax esters synthesis by immobilized lipase EQ3

Amount of immobilized lipase EQ3

The amount of enzyme is one of the most important factors for wax esters synthesis. It can increase the reaction rate at appropriate amount of enzyme. For all cases studied, the reaction rate of palm olein based wax esters synthesis enhanced rapidly when increasing the immobilized enzyme dosage from 2 (56%) to 10 U (87%) (Figure 1). Moreover, the immobilized lipase EQ3 obtained the highest percentage of wax esters when using 10 U of enzyme with highest wax esters for 24 h (84.31%). The wax esters synthesis had been slightly increased after 24 to 72 h. This result was agreement with Gunawan and Suhendra (Gunawan & Suhendra, 2008), the production of palm kernel oil based wax esters was not increased beyond the time that might due to the mass-transfer limitations and the reaction rate of forward and reverse equilibrium. Thus, the immobilized lipase at 10 U was selected for wax esters synthesis in the subsequent experiments.

Temperature

The temperature effects on the substrate solubility as well as its influencing on the enzyme molecule and substrate solubility. The temperatures in reaction mixture were investigated in ranges at 30-45°C, and the reaction time was maintained at 24 h (Figure 2). The temperature had slightly affected on transesterification, and the palm olein based wax esters were found to be a maximum of 85.60% at 45°C for 24 h. In the previous work, the optimum temperature for wax esters synthesis of crude fish fat and cetyl alcohol using the free lipase EQ3 from acetone precipitation was at 37°C (Ungcharoenwiwat & H-Kittikun, 2013), and

Vol. 17 Special issue: 9-17 [2018]
doi: 10.14416/j.appsci.2018.02.S02

the immobilized form of lipase EQ3 on Accurel could improve the thermal stability of tranesterification.

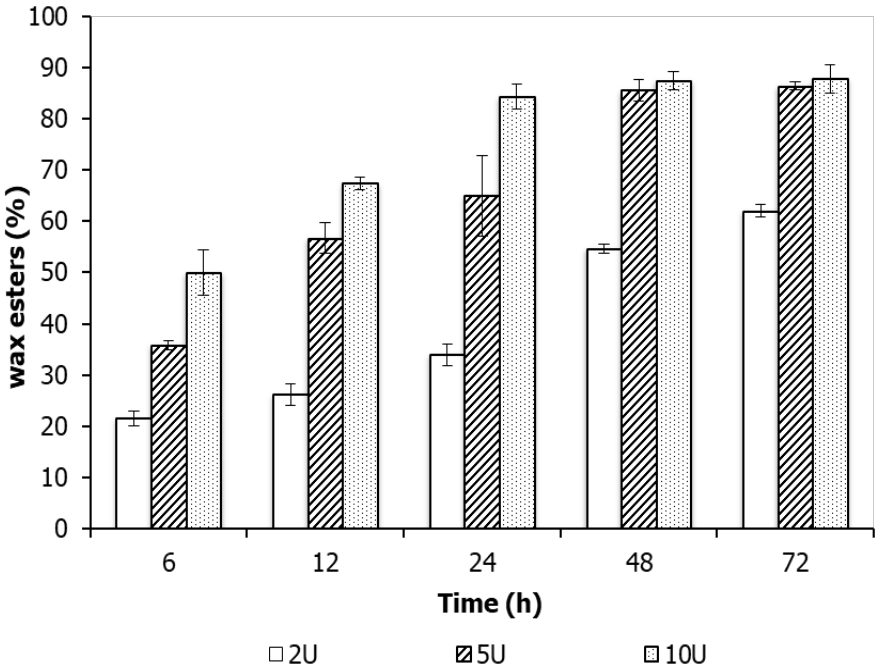


Figure 1. Effect of enzyme concentrations on palm olein based wax esters synthesis by immobilized lipase EQ3 (reaction mixture: substrate molar ratio 1:3, hexane 1 ml, at 37°C and 150 rpm).

Substrate molar ratio

The substrate molar ratio influences the product yield. The results given in Figure 3, show that the increasing the palm olein to oleyl alcohol molar ratio from 1:1 to 1:3 (mol/mol) led to increase the percentage of wax esters. The optimum molar ratio of palm olein and oleyl alcohol was 1:3 (mol/mol) with the highest wax esters of 80.27% for 24 h. Moreover, the rate of wax esters synthesis was reduced at 1:4 (mol/mol). Gunawan and Suhendra (Gunawan & Suhendra, 2008) also found the highest palm kernel oil alcoholysis at 1:3 (mmol of palm kernel oil/mmol of oleyl alcohol) with 86.4% wax esters. The decreasing of tranesterification reaction indicated that oleyl alcohol might inhibit the lipase activity. Due to the access alcohol could distort the essential water layer around the enzyme and then obstruct the oil diffusion and reduce the catalytic activity (Gunawan & Suhendra, 2008; Ungcharoenwiwat & H-Kittikun, 2013).

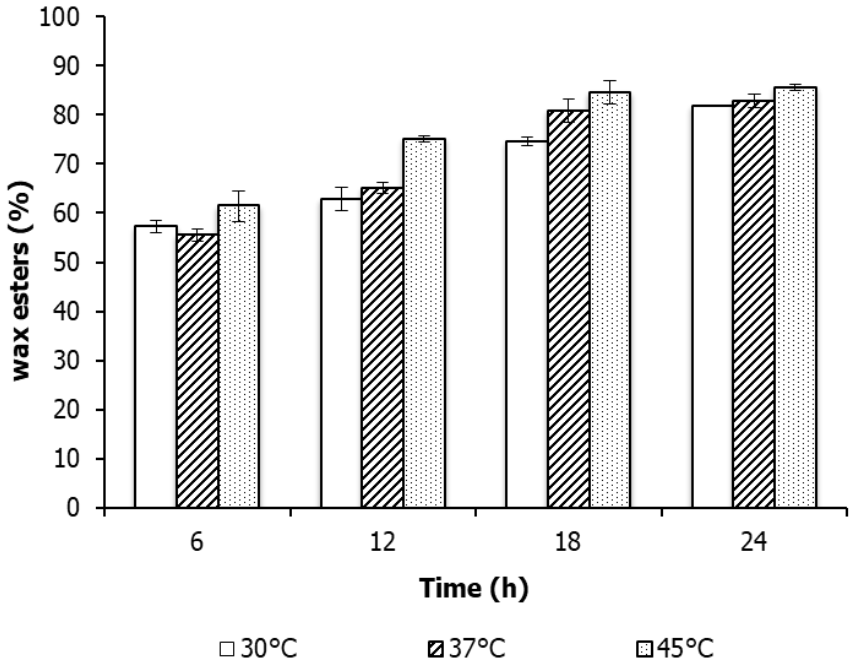


Figure 2. Effect of temperature on the synthesis of palm olein based wax esters by immobilized lipase EQ3 (reaction condition: enzyme 10 U, substrate molar ratio 1:3, hexane 1 ml and 150 rpm).

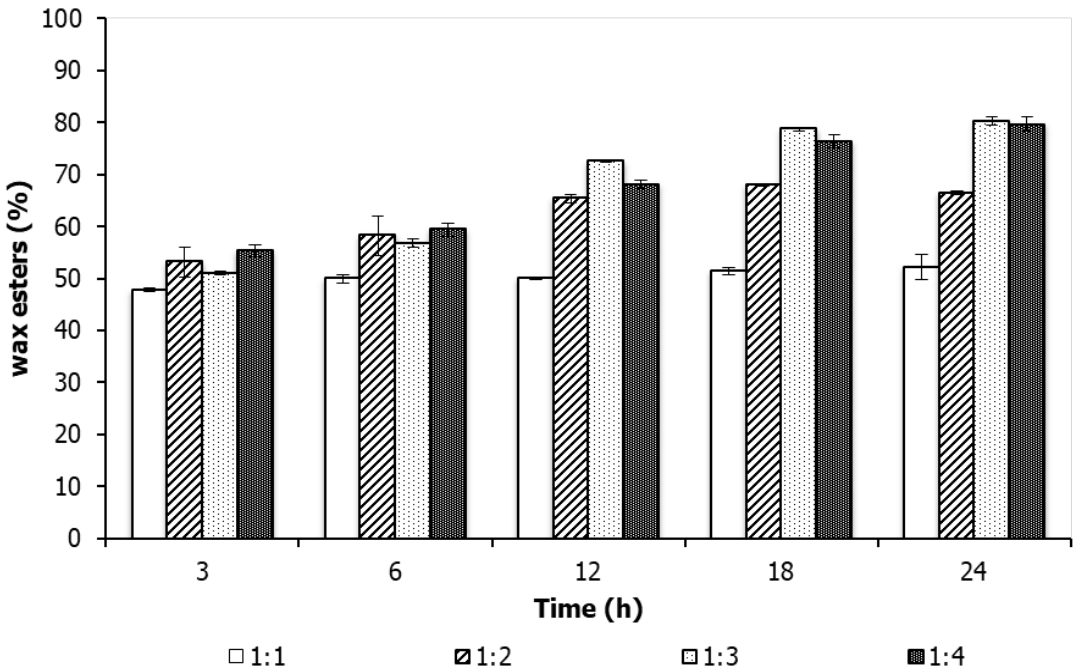


Figure 3. Effect of molar ratio of palm olein and oleyl alcohol on wax esters synthesis by immobilized lipase EQ3 (reaction condition: enzyme 10 U at 45°C, hexane 1 ml and 150 rpm).

The Journal of Applied Science **Vol. 17 Special issue: 9-17 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S02

Substrate concentration

The substrate concentrations seem to play an important role for increasing the synthesis yield of wax esters and the reaction rate. The result was shown in Figure 4. The three levels of substrate concentration were varied at 0.33X, 0.66X and 1.0X of the optimum molar ratio of substrate. The palm olein based wax esters synthesis by the immobilized lipase EQ3 was exhibited in minimum substrate concentration (0.33X) with the highest alcoholysis level. At 0.33X, the wax esters synthesis was rapidly increased to 87.59% for 12 h. Moreover, the wax esters synthesis was slowed down when increasing the substrate concentration. At high substrate concentration had negative effect on attachment of enzyme and substrate (Al-Zuhair, 2005).

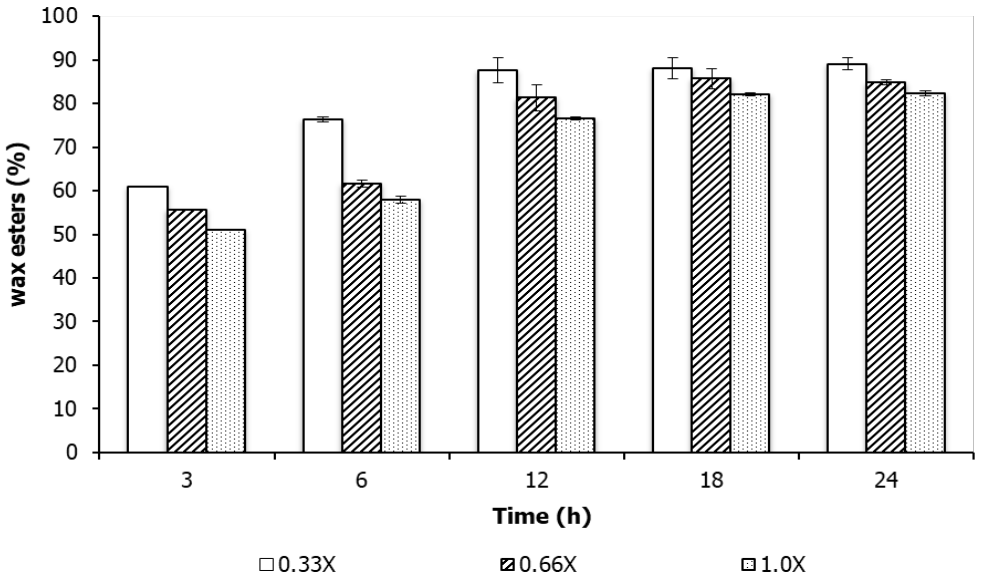


Figure 4. Effect of substrate concentration on palm olein based wax esters synthesis by immobilized lipase EQ3 (reaction condition: enzyme 10 U, substrate molar ratio 1:3, at 45°C, hexane 1 ml and 150 rpm).

Various organic solvents

The palm olein based wax esters synthesis by transesterification had been performed in various organic solvents with immobilized lipases. The synthesis with organic solvent system displayed more efficiency of enzymatic process. In this study, the polarity of organic solvents in terms log P value had an influence on alcoholysis reaction (Figure 5). The result showed that the immobilized lipase EQ3 exhibited high wax esters synthesis in non-polar organic solvents (log P ≥ 3.5). The wax esters synthesis was the highest of 89.05% at 12 h in isooctane. Moreover, the immobilized lipase EQ3 synthesized low amount of wax esters in higher polarity of organic solvent due to they could strip off the essential water layer around the enzyme molecule that preserved its active conformation (Gunawan & Suhendra, 2008).

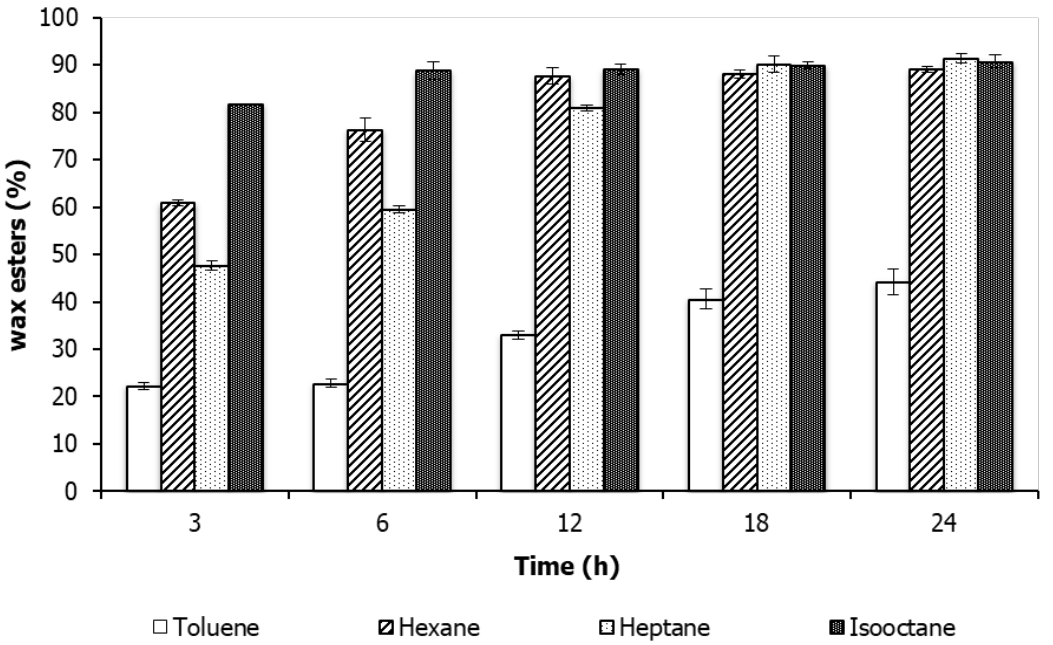


Figure 5. Effect of organic solvents on palm olein based wax esters synthesis by immobilized lipase EQ3 (reaction condition: enzyme 10 U, substrate molar ratio 1:3 (0.33X), at 45°C and 150 rpm).

Conclusion

In this study, the immobilized lipase EQ3 on Accurel MP-100 has enable to catalyst the wax esters synthesis reaction by tranesterification of palm olein and oleyl alcohol with reasonable yield. The optimum condition for wax esters synthesis was 10 U of immobilized lipase EQ3, 45°C and substrate molar ratio 1:3 (mol/mol) dissolved in isooctane with 89% at 12 h. In this research, the study in physicochemical properties of palm olein based wax esters is continued interestingly to know its application. The palm olein based wax esters was long chain liquid wax esters that could be potentially applied in cosmetics, pharmaceuticals or lubricants industries.

Acknowledgement

This research was supported by Enzyme Biotechnology Laboratory, Department of Industrial Biotechnology, Faculty of Agro-Industry, Prince of Songkla University.

References

Al-Zuhair, S. (2005). Production of biodiesel by lipase-catalyzed transesterification of vegetable oils: a kinetics study. *Biotechnology Progress*, *21*(5), 1442-1448. doi: 10.1021/bp050195k

Decagny, B., Jan, S., Vuillemard, J. C., Sarazin, C., Séguin, J. P., Gosselin, C., Barbotin, J. N. & Ergan, F. (1998). Synthesis of wax ester through triolein alcoholysis: Choice of the lipase and study of the mechanism. *Enzyme and Microbial Technology*, *22*(7), 578-582. doi: 10.1016/S0141-0229(97)00240-8

Desai, P. D., Dave, A. M. & Devi, S. (2004). Entrapment of lipase into K-carrageenan beads and its use in hydrolysis of olive oil in biphasic system. *Journal of Molecular Catalysis B: Enzymatic*, *31*(4-6), 143-150. doi: 10.1016/j.molcatb.2004.08.004

Gao, S., Wang, Y., Wang, W., Luo, G. & Dai, Y. (2010). Enhancing performance of lipase immobilized on methyl-modified silica aerogels at the adsorption and catalysis processes: Effect of cosolvents. *Journal of Molecular Catalysis B: Enzymatic*, *62*(3-4), 218-224. doi: 10.1016/j.molcatb.2009.10.010

Ge, J., Lu, D., Wang, J. & Liu, Z. (2009). Lipase nanogel catalyzed transesterification in anhydrous dimethyl sulfoxide. *Biomacromolecules*, *10*(6), 1612-1618. doi: 10.1021/ bm90 0205r

Gunawan, E. R., Basri, M., Rahman, M. B. A., Salleh, A. B. & Rahman, R. N. Z. A. (2004). Lipase-catalyzed synthesis of palm-based wax esters. *Journal of Oleo Science*, *53*(10), 471-477. doi: 10.5650/jos.53.471

Gunawan, E. R. & Suhendra, D. (2008). Synthesis of wax esters from palm kernel oil catalyzed by lipase. *Jurnal Matematika dan Sains*, *13*, 76-83.

Knox, T. & Cliffe, K. R. (1984). Synthesis of long-chain esters in a loop reactor system using a fungal cell bound enzyme. *Process Biochemistry*, *19*, 188-192.

Lee, S. Y. & Rhee, J. S. (1993). Production and partial purification of a lipase from *Pseudomonas putida* 3SK. *Enzyme and Microbial Technology*, *15*(7), 617-623. doi: 10.1016/0141-0229(93)90026-X

Li, Y., Gao, F., Wei, W., Qu, J.-B., Ma, G.-H. & Zhou, W.-Q. (2010). Pore size of macroporous polystyrene microspheres affects lipase immobilization. *Journal of Molecular Catalysis B: Enzymatic*, *66*(1-2), 182-189. doi: 10.1016/j.molcatb.2010.05.007

Minovska, V., Winkelhausen, E. & Kuzmanova, S. (2005). Lipase immobilized by different techniques on various support materials applied in oil hydrolysis. *Journal of the Serbian Chemical Society*, *70*(4), 609-624.

Mullen, T., Markey, K., Murphy, P., McClean, S. & Callaghan, M. (2007). Role of lipase in *Burkholderia cepacia* complex (Bcc) invasion of lung epithelial cells. *European Journal of Clinical Microbiology & Infectious Diseases*, *26*(12), 869-877. doi: 10.1007/s10096-007-0385-2

Petersson, A. E. V., Gustafsson, L. M., Nordblad, M., Börjesson, P., Mattiasson, B. & Adlercreutz, P. (2005). Wax esters produced by solvent-free energy-efficient enzymatic synthesis and their applicability as wood coatings. *Green Chemistry*, *7*(12), 837-843. doi: 10.1039/B510815B

Radzi, S. M., Basri, M., Salleh, A. B., Ariff, A., Mohammad, R., Rahman, M. B. A. & Rahman, R. N. Z. R. A. (2005). Large scale production of liquid wax ester by immobilized lipase. *Journal of Oleo Science*, *54*(4), 203-209. doi: 10.5650/jos.54.203

Schmid, A., Dordick, J. S., Hauer, B., Kiener, A., Wubbolts, M. & Witholt, B. (2001). Industrial biocatalysis today and tomorrow. *Nature*, *409*(6817), 258-268. doi: 10.1038/35051736

Sellami, M., Aissa, I., Frikha, F., Gargouri, Y. & Miled, N. (2011). Immobilized *Rhizopus oryzae* lipase catalyzed synthesis of palm stearin and cetyl alcohol wax esters: Optimization by response surface methodology. *BMC Biotechnology*, *11*, 68. doi: 10.1186/1472-6750-11-68

Sörensen, M. H., Ng, J. B. S., Bergström, L. & Alberius, P. C. A. (2010). Improved enzymatic activity of *Thermomyces lanuginosus* lipase immobilized in a hydrophobic particulate mesoporous carrier. *Journal of Colloid and Interface Science*, *343*(1), 359-365. doi: 10.1016/j.jcis.2009.11.014

Ungcharoenwiwat, P. & H-Kittikun, A. (2013). Synthesis of wax esters from crude fish fat by lipase of *Burkholderia* sp. EQ3 and commercial lipases. *Journal of the American Oil Chemists' Society*, *90*, 359-367. doi: 10.1007/s11746-012-2183-y

Ungcharoenwiwat, P., Canyuk, B. & H-Kittikun, A. (2016). Synthesis of jatropha oil based wax esters using an immobilized lipase from *Burkholderia* sp. EQ3 and Lipozyme RM IM. *Process Biochemistry, 51*(3), 392-398. doi: 10.1016/j.procbio.2015.12.019

Yadav, G. D. & Lathi, P. S. (2003). Kinetics and mechanism of synthesis of butyl isobutyrate over immobilised lipases. *Biochemical Engineering Journal*, *16*(3), 245-252. doi: 10.1016/S1369-703X(03)00026-3

Yemul, O. & Imae, T. (2005). Covalent-bonded immobilization of lipase on poly(phenylene sulfide) dendrimers and their hydrolysis ability. *Biomacromolecules*, *6*(5), 2809-2814. doi: 10.1021/bm050285d

Yong, Y., Bai, Y.-X., Li, Y.-F., Lin, L., Cui, Y.-J. & Xia, C.-G. (2008). Characterization of *Candida rugosa* lipase immobilized onto magnetic microspheres with hydrophilicity. *Process Biochemistry*, *43*(11), 1179-1185. doi: 10.1016/j.procbio.2008.05.019

The Journal of Applied Science
วารสารวิทยาศาสตร์ประยุกต์
ISSN 1513-7805 (Printed in Thailand)

Vol. 17 Special issue: 18-26 [2018]
doi: 10.14416/j.appsci.2018.02.S03
ISSN 2586-9663 (Online)

Research Article

Enhanced succinic acid production from L-arabinose by *Corynebacterium glutamicum* CS176-*ΔldhA* disruptant

Pinnita Fueangbangluang[1, 2] and Savitr Trakulnalaemsai[1, 2]*

[1]Department of Microbiology, Faculty of Science, Kasetsart University, Bangkok, Thailand, 10900
[2]Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University, Bangkok, Thailand
***E-mail**: rdisat@ku.ac.th

Abstract

Corynebacterium glutamicum CS176 (CS176) was previously isolated from chicken feces contaminated soil from Suwanvajokkasikit Farm at Kasetsart University. The CS176 strain is among few native strains of industrial bacteria which can utilize L-arabinose, a pentose sugar as fermentative substrate. Recently, many attempts have been made to engineer *C. glutamicum* for efficient succinic acid production. In this research, we aimed to improve succinic acid production of CS176 strain. To do this, the gene encoded lactate dehydrogenase enzyme (*ldhA*) of CS176 was disrupted by construction of plasmid pK19*mobsacB-ΔldhA* harboring deleted *ldhA* and cloning to CS176. Subsequently, the disruptant, CS176-*ΔldhA*-C12 (C12) was obtained and its succinic acid production was investigated. The production was carried out by two-stage fermentation in 16x125 mm screw-cap tube containing 15 ml basal salt (BT) medium without nitrogen source. The results indicated the succinic acid concentration in BT-arabinose of C12 disruptant reached 1.74 g/l with a productivity yield of 0.89 g g^{-1}dried cell within 2 h after 10.7 mM sodium bicarbonate was added. C12 disruptant enhanced succinic acid in arabinose with 1.62-fold as compared with the wild type. In addition, the succinic acid concentration in BT-mixed glucose and arabinose reached 1.31 g/l with a productivity yield of 0.63 g/g dried cell which was 2.52-fold higher than that of wild type. Thus, this study suggested that disruption of *ldhA* gene of CS176 strain can improve succinic acid production especially in arabinose. The C12 disruptant would be suitable for starting as succinic acid producer and further works required for successful fermentative production especially from pentose sugar derived from lignocellulosic biomass.

Keywords: arabinose-utilizing *C. glutamicum*, bio-based succinic acid production, gene disruption

Introduction

Succinic acid has been used as an important building block for deriving high-value chemicals including 1,4-butanediol, γ-butyrolactone, adipic acid and tetrahydrofuran as well as biodegradable polymer such as polybutylene succinate (PBS) and polybutylene succinate adipate (McKinlay et al., 2007; Zeikus et al., 1999). Because succinic acid synthesized by petroleum chemical process may release CO_2 into environment and make a high environmental cost, bio-based production a green technology, has been considered for succinic acid production.

The Journal of Applied Science **Vol. 17 Special issue: 18-26 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S03

Corynebacterium glutamicum, the facultative anaerobic non-pathogenic bacteria has been used for amino acids production industry (Kinoshita et al., 1985). Under O_2 deprivation condition, the cell growth was arrested but it could produce organic acids such as lactic acid, succinic acid and acetic acid from glucose-minimal medium passed by reductive TCA cycle pathway (Inui et al., 2004; Okino et al., 2005; 2008, Cheng et al., 2013; Wang et al., 2014).

C. glutamicum CS176 was previously isolated from chicken feces contaminated soil (Kitjakarn and Trakulnaleamsai, 2007). This is a few native strains which can utilize L-arabinose, pentose sugar as fermentative substrate. The aim of this study was to disrupt *ldhA* gene encoded lactate dehydrogenase enzyme of *C. glutamicum* CS176 to block lactic acid formation and characterize the capability of the Δ*ldhA* disruptant on succinic acid production using two-stage fermentation. The first stage was aerobic condition to increase the cell mass and the second stage was O_2 deprivation condition to produce the succinic acid. At the second stage, sodium bicarbonate was added to generate CO_2, the co-substrate of pyruvate carboxylase and phosphoenol-pyruvate carboxylase activities for improvement of the bio-based succinic acid production by *C. glutamicum.*

Materials and methods

Bacterial strains and plasmids

The bacterial strains and plasmids used in this work were shown in Table 1.

DNA Techniques

Genomic DNA was extracted from *C. glutamicum* CS176 followed by Sambrook et al. (2001) method. Plasmid DNA was isolated by using a GeneJET Plasmid Miniprep Kit (Thermo Scientific) according to the manufacturer's instructions. Restriction endonucleases were purchased from Takara. PCR (Perkin Elmer GeneAmp PCR System 9700) was performed using total volume of 25 µl with 100 ng of genomic DNA, 0.16 mM dNTPs and 0.75 units of USB® *Taq* DNA Polymerase in 10X PCR buffer (10X PCR buffer with provided 1.5 mM $MgCl_2$ in the final reaction volume, Affymetrix, Inc.) for 25 cycles at 95 °C for denaturation (30 sec), 56 °C for annealing (30 sec) and 72 °C for polymerization (1 min). T4 DNA ligase and 100 bp Plus DNA ladder were purchased from Thermo Scientific.

Construction of *ldhA* deletion mutant and plasmids

A portion of *ldhA* gene (1.1-kb) from genomic DNA of *C. glutamicum* CS176 was amplified with ldhF (5'-CTCTGTCGACATCAGGAAGTGGGATCGAAA-3') and ldhR (5'-CTCTGTCGAC TTCCATCCAACAGTTTCATT-3') primers (Inui et al, 2004). The 1.1-kb PCR product was ligated into pGEM-T easy vector, yielding pGEM-*ldhA* plasmid. The *ldhA* fragment was separated from pGEM-T easy vector by cutting with *Sal*I enzyme and inserted into the unique *Sal*I site of pUC18 vector, yielding pUC18-*ldhA*. The 3.8-kb pUC18-*ldhA* was digested with *EcoR*V and *Nae*I enzymes to remove 322 bp fragment and self-ligated yielding pUC18-*ΔldhA.* The 730 bp *ΔldhA* fragment was digested with *EcoR*I/*Hind*III and ligated into *EcoR*I/*Hind*III-digested pK19*mobsacB* plasmid yielding pK19*mobsacB*-*ΔldhA* and all of positive clones were confirmed by DNA sequencing method.

C. glutamicum CS176 was transformed by electroporation with plasmid pK19*mobsacB*-*ΔldhA* and selection for the first and second recombination events on brain heart infusion/ sorbitol (BHIS) plate containing kanamycin and S10 medium containing 10% sucrose, respectively (S10 medium per litre containing 10 g peptone, 10 g yeast extract, 5 g NaCl and

Vol. 17 Special issue: 18-26 [2018]
doi: 10.14416/j.appsci.2018.02.S03

100 g sucrose). Transformation of *E. coli* was performed by $CaCl_2$ procedure and selection on LB plate containing appropriate of antibiotic (Sambrook et al., 1989).

Table 1. Bacterial strains and plasmids used in the work

Strain or plasmid	Relevant characteristics	Source or reference
E. coli		
HIT-DH5α	*F-(80d lacZ M15)(lacZYA-argF)U169 hsdR17 (r- m+) recA1 endA1 relA1 deoR*	RBC Bioscience
Stellar (dam-/dcm-)	*F- ara Δ(lac-proAB)[Φ80d lacZΔM15] rpsL(str) thi Δ(mrr-hsdRMS-mcrBC) ΔmcrA dam dcm*	Clonetech
C. glutamicum		
CS176	Wild type, the arabinose utilizing strain	Kitjakarn & Trakulnaleamsai, (2007)
C12	*ldhA* disrupted mutant (*ΔldhA*)	This work
Plasmids		
pGEM-T easy	Ap^r; α-lac MCS, M13 ori	Promega
pGEM-T easy-*ldhA*	Ap^r; pGEM-T easy containing the *C. glutamicum* CS176 *ldhA* gene	This work
pUC18	Ap^r; α-lac MCS, M13 ori	Takara
pUC18-*ldhA*	Ap^r; pUC18 with 1.1 kb *Sal*I-*Sal*I fragment containing the *C. glutamicum* CS176 *ldhA* gene	This work
pUC18-**Δ***ldhA*	Ap^r; pUC18 with 730 bp *Sal*I-*Sal*I fragment containing *ΔldhA* gene	This work
pK19*mobsacB*	Km^r; plasmid for allelic exchange in *C. glutamicum*	Schäfer et al. (1994)
pK19*mobsacB*-**Δ***ldhA*	Km^r; with 730 bp *EcoR*I-*Hind*III fragment containing *ΔldhA* gene	This work

Vol. 17 Special issue: 18-26 [2018]
doi: 10.14416/j.appsci.2018.02.S03

Two-stage fermentation for succinic acid production

The CS176 and its disruptant were activated in 5 ml of A medium and incubated at room temperature with shaking at 350 rpm for 16-18 h (A medium per litre in dH_2O containing 2 g urea, 2 g yeast extract, 7 g casamino acid, 7 g $(NH_4)_2SO_4$, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O_2$ 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin and 0.2 mg thiamine).

Transferred 0.1 ml culture broth from A medium into 5 ml BTN medium (BTN medium per litre in dH_2O containing 10 g glucose, 3 g NH_4Cl, 2 g urea, 0.5 g KH_2PO_4, 0.5 g K_2HPO_4, 0.5 g $MgSO_4 \cdot 7H_2O$, 6 mg $FeSO_4 \cdot 7H_2O$, 4.2 mg $MnSO_4 \cdot H_2O$, 0.2 mg biotin and 0.2 mg thiamine). Cultivation was performed under aerobic stage at room temperature, 350 rpm for 16-18 h. Under oxygen deprivation stage, 11 ml of culture broth from aerobic phase was transferred to screw cap tube (size 16×125 mm) containing 4 ml BT medium (BTN without N source). Cultivation was performed at room temperature, 120 rpm and 10.7 mM sodium bicarbonate was added at 4 h after cultivation and further incubation in CO_2 condition for 0-10 h. The carbon sources were varied (per litre): 10 g arabinose, 10 g glucose, 10 g sucrose, 5 g glucose and 5 g arabinose, 5 g glucose and 5 g sucrose, 5 g arabinose and 5 g sucrose, 0.33 g each of glucose, arabinose and sucrose.

Analytical methods

Cell growth was measured by spectrophotometry at optical density 600 nm (OD_{600}) (UV/VIS Spectrophotometer U-3310, Hitachi, Japan) or Klett Summerson colorimeter. Samples were collected at 0-10 h after bicarbonate was added and centrifuged at 10,000 rpm, 4 °C for 5 min. Supernatants were analyzed for sugars and organic acids. The qualitative organic acids were detected by Thin-layer chromatography (TLC) on TLC Silica gel 60 F_{254} (Merck, Germany) (Kraiker and Burch, 1973). The quantitative organic acids were quantified by HPLC (Varian ProStar, USA) equipped with UV detector and C18 ChromSep HPLC Column SS (Varian, Inc., USA) operating at 210 nm at 25 °C using 1 mM H_2SO_4 and 8 mM Na_2SO_4 pH 2.8 as mobile phase at flow rate 1 ml/min. Sugars concentrations were determined by HPLC (brand and model) equipped with ELSD detector and Rezex RPM-Monosaccharide Pb^{2+} Column (Phenomenex, USA) operating at 60 °C using deionized water as mobile phase at flow rate 0.6 ml/min.

Results and discussion

Screening of CS176-*ΔldhA* disruptant

In general, *C. glutamicum* could produce L-lactic acid as a major product and also produced succinic acid under oxygen deprivation condition via reductive branch of TCA cycle pathway. Many attempts have been drawn to improve succinic acid production in *C. glutamicum* by using metabolic engineering. The native *ldhA* gene was disrupted to block L-lactic acid formation pathway and shifted to succinic acid formation pathway.

Construction of recombinant *ΔldhA* plasmid was confirmed by DNA sequencing analysis. The result showed that *ΔldhA* fragment consists of 727 bp and the nucleotide positions from 271-603 was removed as compared with the *ldhA* gene from CS176 wildtype (Figure 1).

The Journal of Applied Science **Vol. 17 Special issue: 18-26 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S03

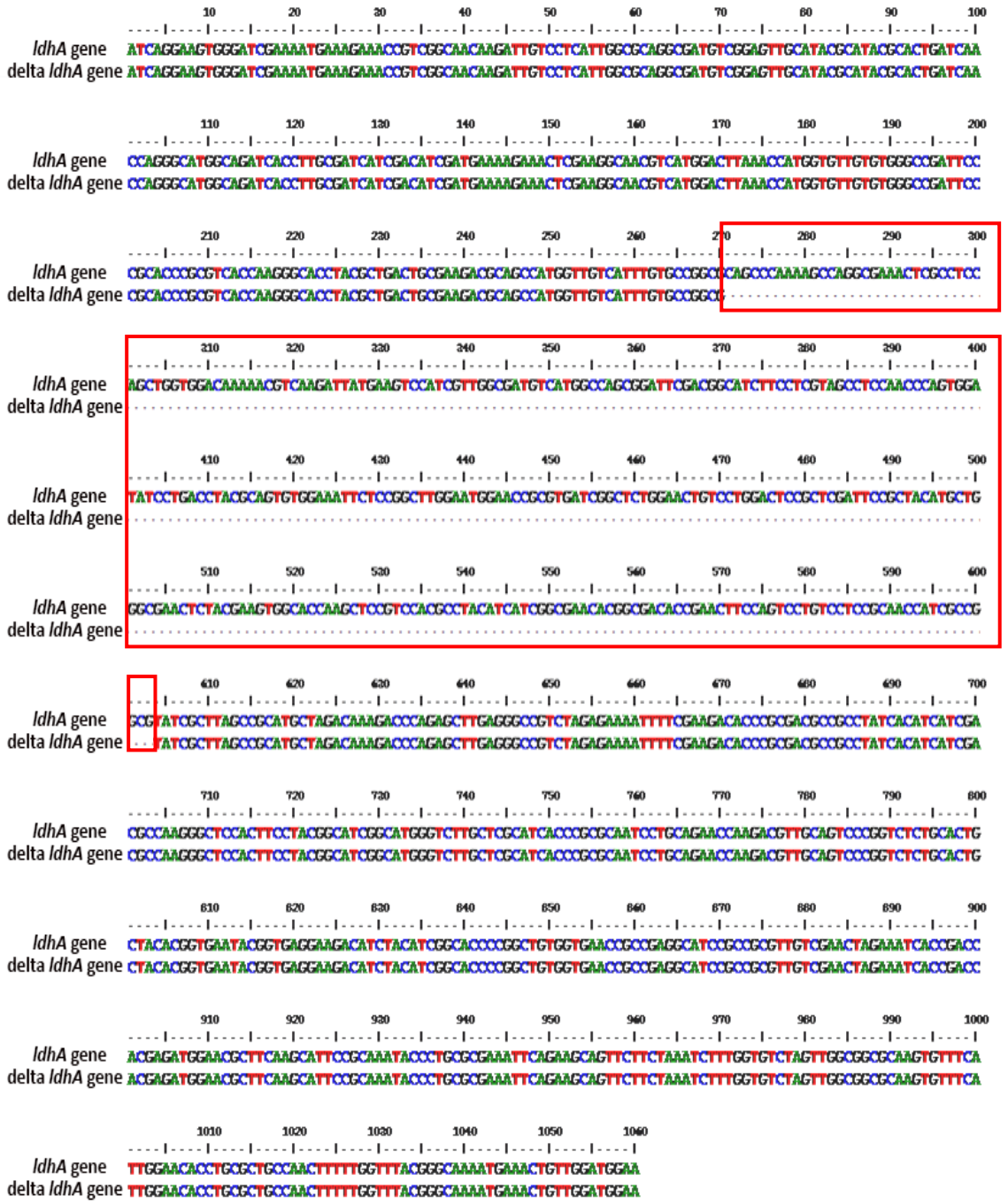


Figure 1. Comparison of nucleotide sequences between *ldhA* gene (top line) from *C. glutamicum* CS176 and *ΔldhA* fragment (bottom line) from recombinant pK19*mobsacB-ΔldhA* plasmid. Region in the red box was deleted.

Vol. 17 Special issue: 18-26 [2018]
doi: 10.14416/j.appsci.2018.02.S03

The *ldhA* disruption in *C. glutamicum* CS176 was constructed in two-step homologous recombination with pK19*mobsacB-ΔldhA* plasmid. The first step of recombination event, the clones harboring two bands of 1.1 kb (full length of *ldhA* gene from CS176, wild type) and 730 bp of *ΔldhA* gene were selected by kanamycin resistant trait on BHIS plate containing kanamycin (50 µg/ml). Then, the second recombination event was selected by sucrose resistant growth on S10 plate containing 10% sucrose. 10 out of 23 sucrose resistant colonies (clone C11-C20) which appeared on S10 plate were randomly picked up to screen positive disruptant by colony PCR. As a result, 9 clones possessed 750 bp PCR fragment indicating positive clones harboring deleted *ldhA* gene while only C17 was not detected due to no amplification (Figure 2). Then, 9 disruptants (except C17) were screened for succinic acid production ability on BTN-glucose plate containing 1% $MgCO_3$ under O_2 deprivation condition to observe clear zone after $MgCO_3$ hydrolized by organic acid. The clone named C12 showed the largest of clear zone on screening plate (data not shown) thus, C12 disruptant was selected for further characterization on succinic acid production.

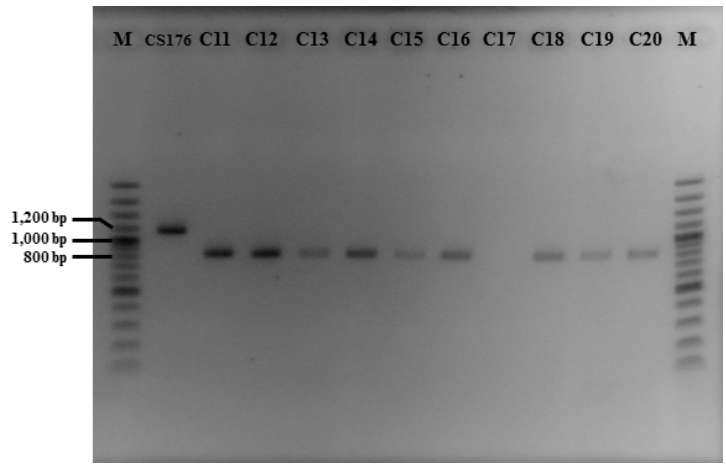


Figure 2. PCR product of *ldhA* gene from CS176, wild type and sucrose resistant clones on 1.2% agarose gel; M: DNA marker, 100 bp Plus DNA ladder.

Succinic acid production of CS176 wild type and C12 disruptant

Both of CS176 and selected C12 disruptant could grow and increase the number of cell mass in BTN medium containing glucose, arabinose or sucrose as sole and mixed carbon source(s) at high aeration condition and produced succinic acid under oxygen deprivation condition (as described in material and methods). However, high carbon dioxide has been shown to elevate succinic acid in *Anaerobiospirillum succiniciproducens* and *C. glutamicum* (Samuelov et. al. 1991, Okino et. al., 2005). Thus, the succinic acid production in various sugars of *C. glutamicum* CS176 and C12 disruptant were analyzed in the conventional fermentation with adding 10.7 mM bicarbonate as a maximum concentration in tested condition. The results are shown in Figure 3 and Table 2. The results revealed that C12

disruptant could produce succinic acid in BT medium containing either arabinose or mixed sugars better than that of CS176 wild type. The succinic acid production in BT-arabinose of C12 disruptant reached 1.74 g/l within 2 h after adding 10.7 mM sodium bicarbonate with a yield of 0.89 g/gCDW while CS176 reached the highest succinic acid concentration of 1.07 g/l at 8 h. The succinic acid yield of C12 disruptant was increased 1.62 folds with highest production rate of 0.45 g/gCDW/h. The result revealed that the consumption of arabinose to succinic acid in C12 was increased as compare to that of CS176. In term of mix carbon sources, succinic acid yield from C12 disruptant was higher 2-4.4 folds. The highest succinic acid concentration in BT medium containing glucose and sucrose from C12 disruptant was 3.31 g/l. Moreover, other organic acids as observed by shorter retention time peaks appeared on HPLC profile could be detected along with succinic acid analysis (profile not shown).

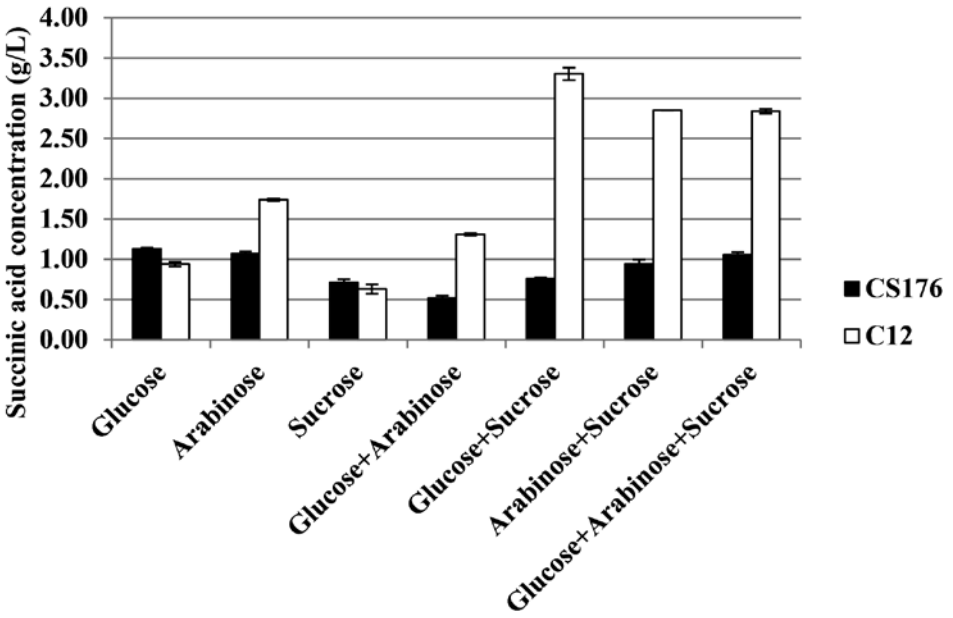


Figure 3. Succinic acid production under oxygen deprivation condition with addition of 10.7 mM bicarbonate by *C. glutamicum* CS176 wild type (black bar) and C12 disruptant (white bar) in medium with various sugars. Data were average of duplicate experiments.

In this study, the disruption of lactate dehydrogenase gene (Δ*ldhA*) of *C. glutamicum* CS176, an arabinose utilizing strain, by homologous recombination was successful. The disruptant, namely, *C. glutamicum-ΔldhA*-C12 was obtained. The result reveals C12 disruptant improved succinic acid production as compare to CS176 wild type under conventional two-stage fermentation supplemented with 10.7 mM bicarbonate. The maximum succinic acid production rate of 0.45 g/gCDW/h was achieved in BT-arabinose within two hours after adding bicarbonate. The succinic acid yield was enhanced 1.62 folds as compared to that of wild type. Thus, this study indicated that disruption of *ldhA* gene of CS176 strain can improve succinic acid production in arabinose. The C12 disruptant would be suitable strain for further

Vol. 17 Special issue: 18-26 [2018]
doi: 10.14416/j.appsci.2018.02.S03

improvement required for successful fermentative production of succinic acid especially from pentose sugars derived from lignocellulosic biomass.

Table 2. Summary of succinic acid production by *C. glutamicum* CS176 and C12 disruptant in various carbon sources in the presence of 10.7 mM bicarbonate

Strain	Carbon source(s)	Titer (g/l)	Time giving highest succinic acid after adding bicarbonate (h)	Productivity (g/l/h)	Succinic acid yield (g/gCDW)	Production rate (g/gCDW/h)
C. glutamicum CS176	Glucose	1.13±0.01	8	0.14	0.47	0.06
	Arabinose	1.07±0.03	8	0.13	0.54	0.07
	Sucrose	0.71±0.04	6	0.12	0.34	0.06
	Glucose and arabinose	0.52±0.03	4	0.13	0.24	0.06
	Glucose and sucrose	0.76±0.01	6	0.13	0.33	0.06
	Arabinose and sucrose	0.94±0.06	8	0.12	0.42	0.05
	Glucose, sucrose and arabinose	1.10±0.03	8	0.14	0.53	0.06
C. glutamicum-*ΔldhA*-C12 disruptant	Glucose	0.94±0.03	6	0.16	0.47	0.08
	Arabinose	1.74±0.01	2	0.87	0.89	0.45
	Sucrose	0.63±0.06	8	0.08	0.33	0.04
	Glucose and arabinose	1.31±0.01	6	0.22	0.63	0.11
	Glucose and sucrose	3.31±0.08	8	0.41	1.48	0.19
	Arabinose and sucrose	2.85±0.00	8	0.36	1.28	0.16
	Glucose, sucrose and arabinose	2.84±0.03	8	0.36	1.17	0.15

Acknowledgements

Plasmid pK19*mobsacB* was kindly provided by Associate Professor Dr. Toshiharu Yakushi, Department of Biological Chemistry, Faculty of Agriculture, Yamaguchi University, Japan. This work was financial supported by The Center for Advanced Studies in Tropical Natural Resources, NRU-KU, Kasetsart University and Graduate Thesis Grant from The National Research Council of Thailand (NRCT).

The Journal of Applied Science **Vol. 17 Special issue: 18-26 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S03

References

Cheng, K.-K., Wang, G.-Y., Zeng, J. & Zhang, J.-A. (2013). Improved succinate production by metabolic engineering. *BioMed Research International, 2013*, *Article ID 538790*, 12 pp. http://dx.doi.org/10.1155/2013/538790

Inui, M., Murakami, S.,Okino, S., Kawaguchi, H., Vertès, A. A. & Yukawa, H. (2004). Metabolic analysis of *Corynebacterium glutamicum* during lactate and succinate productions under oxygen deprivation conditions. *Journal of Molecular Microbiology and Biotechnology*, *7*, 182-196. https://doi.org/10.1159/000079827

Kitjakarn, S. & Trakulnaleamsai, S. (2007). *Isolation and characterization of the L- glutamic acid producing bacteria, Corynebacterium glutamicum thermotolerant strain isolated from Thailand* (M.S. thesis), Faculty of Science, Kasetsart University, Bangkok.

Kinoshita, S. (1985). Glutamic acid bacteria. In: A. L. Demain & N. A. Solomon (Eds.), *Biology of Industrial Microorganisms.* pp. 115-145. London: Benjimin Cummings.

Kraiker, H. P. & Burch, R. E. (1973). Thin layer chromatography of Krebs cycle acids. *Zeitschrift Fur Klinische Chemie Und Klinische Biochemie*, *11*, 393-397.

McKinlay, J. B., Vieille, C. & Zeikus, J. G. (2007). Prospects for bio-based succinate industry. *Applied Microbiology and Biotechnology, 76*, 727-740. doi: 10.1007/s00253-007-1057-y

Okino, S., Inui, M. & Yukawa, H. (2005). Production of organic acids by *Corynebacterium glutamicum* under oxygen deprivation. *Applied Microbiology and Biotechnology, 68*, 475-480. doi: 10.1007/s00253-005-1900-y

Okino, S., Noburyu, R., Suda, M., Jojima, T., Inui, M. & Yukawa, Y. (2008). An effient succinic acid production process in a metabolically engineered *Corynebacterium glutamicum* strain. *Applied Microbiology and Biotechnology, 81*, 459-464. doi: 10.1007/s00253-008-1668-y.

Sambrook, J., Fritsch, E. F. & Maniatis,T. (1989). *Molecular Cloning: A Laboratory Manual.* New York: Cold Spring Habor Laboratory Press.

Sambrook, J. & Russell, D. W. (2001). *Molecular Cloning: A Laboratory Manual* (2nd ed.). New York: Cold Spring Habor Laboratory Press.

Samuelov, N. S., Lamed, R., Lowe, S. & Zeikus, J. G. (1991). Influence of CO_2-HCO_3^- levels and pH on growth, succinate production and enzyme activities of *Anaerobiospirillum succiniciproducens*. *Applied and Environmental Microbiology, 57*, 3013-3019.

Schäfer, A., Tauch, A., Jäger, W., Kalinowski, J., Thierbach, G. & Pühler, A. (1994). Small mobilizable multi-purpose cloning vectors derived from the *Escherichia coli* plasmids pK18 and pK19: selection of defined deletions in the chromosome of *Corynebacterium glutamicum*. *Gene, 145*, 69-73.

Wang, C., Zhang, H., Cai, H., Zhou, Z., Chen, Y., Chen, Y. & Ouyang, P. (2014). Succinic acid production from corn cob hydrolysates by genetically engineered *Corynebacterium glutamicum*. *Applied Biochemistry and Biotechnology, 172*, 340-350. doi: 10.1007/s12010-013-0539-x

Zeikus, J. G., Jain, M. K. & Elankovan, P. (1999). Biotechnology of succinate production and markets for derived industrial products. *Applied Microbiology and Biotechnology, 51*, 545-552.

The Journal of Applied Science **Vol. 17 Special issue: 27-36 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S04
ISSN 1513-7805 (Printed in Thailand) ISSN 2586-9663 (Online)

Research Article

A preliminary study on differential gene expression in hemocyte of the mantis shrimp *Harpiosquilla raphidea* by next generation sequencing technique

Naiyasit Sodsee[1], Sarawut Wongphayak[2], Hidehiro Kondo[3], Ikuo Hirono[3] and Rachanimuk Hiransuchalert[1*]

[1]Faculty of Marine Technology, Burapha University, Chanthaburi Campus, Chanthaburi 22170, Thailand
[2]Vishuo Biomedical (Thailand) Ltd. 17th Floor Alma Link Building, Bangkok 10330 Thailand
[3]Laboratory of Genome Science, Tokyo University of Marine Science and Technology, Tokyo, Japan
***E-mail**: rachanimuk@buu.ac.th

Abstract

This study aims to determine gene expression profiling in hemocyte of *Harpiosquilla raphidea* by using bioinformatics technology and Next Generation Sequencing (NGS) technique. The *De novo* RNA-sequencing was performed using Illumina HiSeq2000. In total, 93,344 assembly consensus sequences were identified by using Trinity software. These 93,344 transcripts were estimated the expression level of each transcript in the hemocyte of *H. raphidea* (both infected by *Vibrio parahemolyticus* for 24 hours and normal condition) in terms of FPKM (Frackments Per Kilobase of target transcript length per Million reads mapped). The 219 differential expression of transcripts were classified into 4 clusters, i.e., down regulated expression levels (cluster 1/1 and 2/1) and up regulated expression levels (cluster 1/2 and 2/2). Clusters were further functional and annotation analyzed by using Blast2GO software. The important genes that relevant in the immune system of *H. raphidea* were found this study. These included histone H1-delta-like, Cyclooxygenases, 5-aminolevulinate erythroid-mitochondrial, electron transfer flavo-ubiquinone mitochondrial and CCR4-NOT transcription complex subunit 7isoform X1 gene. The basic information related to these immune genes in *H. raphidea* was investigated in this study and will be the guidance for further studies on the important process in the immune system of *H. raphidea* and for its commercial culture in the future.

Keywords: *Harpiosquilla raphidea*, NGS, RNA-seq, *de novo*, transcriptome assembly

Introduction

Recently, mantis shrimps are popular for food consumption especially in Asia. *Harpiosquilla raphidea* is one of the commercial mantis shrimp species. *H. raphidea* become more desirable food for customers (Tangkrock-olan & Ruksar, 2012), however, most of *H. raphidea* is not come from aquaculture. These affect natural populations of *H. raphidea* because of the over fishing and ore potential fishing gear were used.

The Journal of Applied Science **Vol. 17 Special issue: 27-36 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S04

Therefore, Department of Fisheries tries to culture mantis shrimps aiming to increase the natural population and commercially culture of mantis shrimp (Srimukda & Champasri, 1994). Unfortunately, mantis shrimps culture still unsuccessful cause of lacking in some biological knowledge, i.e., immune system and reproductive system.

Next Generation Sequencing (NGS) technology enables us to identify known as well as novel gene (Buermans & den Dunnen, 2014) and multiomics approaches including interactome sequencing (Ohashi et al., 2015). We have demonstrated the NGS technology for *de novo* assembly and annotation of genes without prior genome information, as well as its reliability and complementarily by alignment with sequence database. Moreover, it also can apply this technique for the study of gene expression level in transcription level under physical state or diseases. RNA-seq maybe covered part of mRNA in genome for gene expression analysis (Wilantho et al., 2012). NGS technology can investigate gene structure and gene function by complementary DNA (cDNA), cDNA synthesis from RNA that call genetic data is RNA-seq data, therefore the study of *H. raphidea* in reproductive and immune organ by bioinformatics tools to know novel transcriptome of *H. raphidea* for gene expression in importance organ. These will be the useful information for commercial culture and increase nature population of *H. raphidea* in the future.

Materials and methods

Samples

The wild *H. raphidea* were derived from Trad province, Thailand and separated by control (normal condition; N=2) and treatment (infection by 10^4 CFU of *Vibrio parahemoluticus* at 24 hours; N=2). The hemolymphs were collected from all samples and centrifuge at 3000g for 10 minutes. The hemocytes were kept to 10 mL of RNA later by incubated in 4 °c for 12 hours after that incubated in -80 °c until used.

Total RNA extraction

All hemocytes samples (approximately 0.5 g per each sample) were transferred to a microcentrifuge tube containing 500 µL of TRI REAGENT® and homogenized. Additional 500 µl of TRI REAGENT® were added. The homogenate were left for 5 minutes, before adding 0.2 ml of chloroform. The homogenate was vortexed for 15 seconds and left at room temperature for 2 – 15 minutes and centrifuged at 12000 g for 15 minutes at 4 °C. RNA was precipitated by an addition of 0.5 ml of isopropanol and washed with 1 ml of 75% ethanol. RNA was dissolved in DEPC-treated H_2O for immediately used. Alternatively, the RNA pellet was kept under absolute ethanol in a -80 °c freezer for long storage.

Library construction and sequencing

RNA-seq library preparation and sequencing was carried out by Professor Dr. Hidehiro Kondo and Professor Dr. Ikuo Hirono, Laboratory of Genome Science Tokyo University of Marine Science and Technology, Japan. Total RNA of each sample was quantified and qualified by Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA), NanoDrop (Thermo Fisher Scientific Inc.) and 1% agrose gel. 1 µg total RNA with RIN value above 7 was used for following library preparation.

Vol. 17 Special issue: 27-36 [2018]
doi: 10.14416/j.appsci.2018.02.S04

Next generation sequencing library preparations were constructed according to the manufacturer's protocol (NEBNext® Ultra™ RNA Library Prep Kit for Illumina®). The poly(A) mRNA isolation was performed using NEBNext Poly(A) mRNA Magnetic Isolation Module (NEB). The mRNA fragmentation and priming was performed using NEBNext First Strand Synthesis Reaction Buffer and NEBNext Random Primers. First strand cDNA was synthesized using ProtoScript II Reverse Transcriptase and the second-strand cDNA was synthesized using Second Strand Synthesis Enzyme Mix. The purified (by AxyPrep Mag PCR Clean-up (Axygen)) double-stranded cDNA was then treated with End Prep Enzyme Mix to repair both ends and add a dA-tailing in one reaction, followed by a T-A ligation to add adaptors to both ends. Size selection of Adaptor-ligated DNA was then performed using AxyPrep Mag PCR Clean-up (Axygen), and fragments of ~360 bp (with the approximate insert size of 300 bp) were recovered. Each sample was then amplified by PCR for 11 cycles using P5 and P7 primers, with both primers carrying sequences which can anneal with flow cell to perform bridge PCR and P7 primer carrying a six-base index allowing for multiplexing. The PCR products were cleaned up using AxyPrep Mag PCR Clean-up (Axygen), validated using an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA) , and quantified by Qubit 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA).

Then libraries with different indices were multiplexed and loaded on an Illumina HiSeq instrument according to manufacturer's instructions (Illumina, San Diego, CA, USA). Sequencing was carried out using a 2x150bp paired-end (PE) and 10 million reads per sample configuration; image analysis and base calling were conducted by the HiSeq Control Software (HCS) + RTA 2.7 (Illumina) on the HiSeq2000 instrument.

Gene differential expression

Illumina build RNA-seq data like a mass nucleotide reads that made a lot of same length reads. *De novo* transcriptome assembly made by include RNA-seq data in to Trinity software that contain 3 step first Inchworm for connect reads to contig that can reconstructs linear transcripts, Chrysalis builds de Bruijn transcript graphs of cluster minimally-overlapping Inchworm contigs into sets of connected components, and constructs complete de Bruijn graphs for each component, Butterfly reconstructs plausible full-length, linear transcripts by reconciling the individual de Bruijn graphs generated by Chrysalis with the original reads and paired-ends.

The expression levels (FPKM, Frackments Per Kilobase of target transcript length per Million reads mapped) were compared in each transcript by R studio software that generated gene expression profile, first expunge the transcripts that have FPKM value = 0 in treatment ro control, convert FPKM values to $\log_{10}$ format, estimate standard deviation of each transcript between treatment and control, expunge the transcripts that have standard deviation of each transcript between treatment and control less than 2, cluster transcripts depend on expression structure by gPlots package (function heatmap = 2).

Transcripts were cluster by R studio software not contain nucleotide in each transcript. Before gene annotation analysis transcripts were match with nucleotide sequences and saved with another transcript in same cluster in fasta format.

Vol. 17 Special issue: 27-36 [2018]
doi: 10.14416/j.appsci.2018.02.S04

Function and annotation of transcripts made in each cluster by blast2go software (default setting), follow by 1.) Blast*X* towards transcripts without of intron, 2.) Map transcripts with all kinds of protein database in blast2go, 3.) Gene ontology with GO database and 4.) Pathway identification from Kyoto Encyclopedia of Genes and Genomes Pathway database (KEGG).

Results

Gene expression profile

In total, 93,344 transcript sequences were identified by *De novo* transcriptome assembly, after removing disreputable data left 28,323 sequences, after compare standard deviation in $\log_{10}$ format between normal and 24 hours infection of *Vibrio parahemolyticus* get 214 sequences that have standard deviation more than 2. Gene expression profile displayed in heatmap that contain two main clusters and each cluster contain two sub clusters. The difference of the main cluster 1 and 2 are that cluster 1 contained a low differentially level between normal and *Vibrio parahemolyticus*. Whereas, cluster 2 contained a high differentially level between normal and *Vibrio parahemolyticus* infection. By each sub cluster, they contained down regulated cluster (/1) and up regulated cluster (/2), respectively (Figure 1).

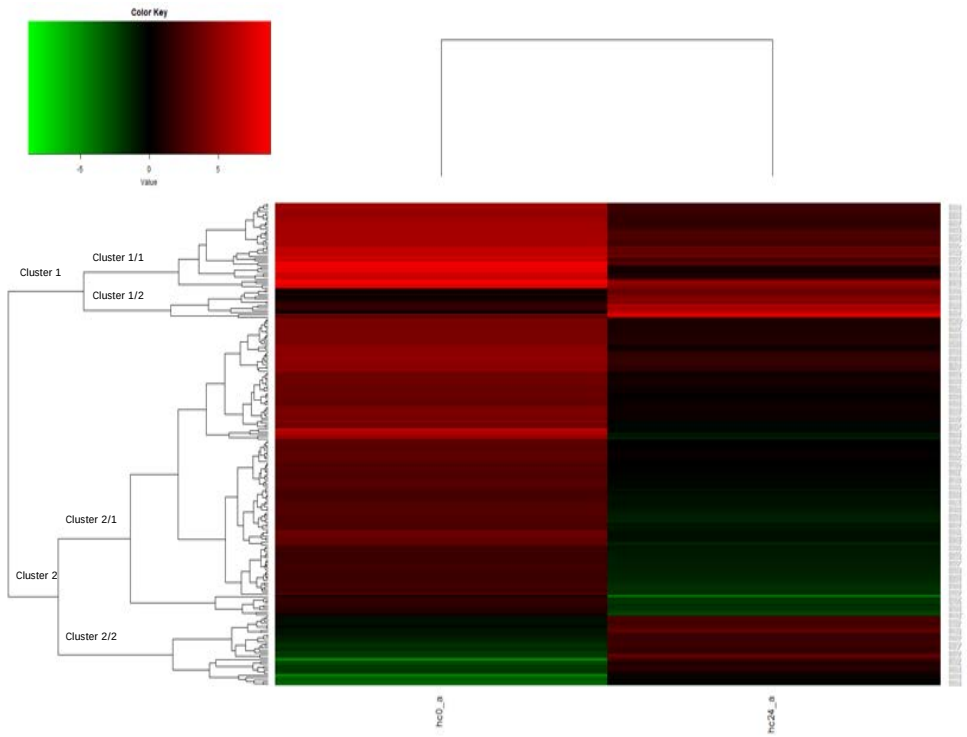


Figure 1. Gene expression profile of *H. raphidea* hemocyte between normal condition (hc0_a) and 24 hours infection of *Vibrio parahemolyticus* (hc24_a).

The Journal of Applied Science **Vol. 17 Special issue: 27-36 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S04

Functional annotation of genes

All of 219 transcripts were divided to two main clusters, one main cluster contains two subcluster that up regulated and down regulated expression. After blast to GenBank using Blast*X*, 68 transcripts were identified as known transcript. Moreover, each cluster was specified the gene functional and annotation in biological process, molecular function and cellular component. Gene function and annotation was specified by gene ontology format from GO database. Down regulated expression while infection of *Vibrio paprhemolyticus* condition classified in cluster 1/1 and cluster 2/1. Whereas, up regulated expression while infection of *Vibrio paprhemolyticus* condition classified in cluster 1/2 and cluster 2/2 (Table 3).

Table 1. Gene function of down regulated expression transcripts in clusters 1/1 and 2/1.

description	biological process (level 2)	molecular function (level 2)	cellular component (level 2)
cluster 1/1			
1. histone H1-delta-like	cellular process, cellular component	binding	macromolecular complex, organelle, cell part, cell, organelle part
2. histone H1-delta-like	cellular process, cellular component	binding	macromolecular complex, organelle, cell part, cell, organelle part
3. venom toxin	regulation of biological process, biological regulation, growth, cellular process, cellular component, single-organism process	binding	macromolecular complex, extracellular region part, extracellular region
4. 40S ribosomal S30	cellular process, cellular component, metabolic process (translation)	binding, structural molecule activity	macromolecular complex, organelle, cell part, cell
5. histone H1-delta-like	cellular process, cellular component	binding	macromolecular complex, organelle, cell part, cell, organelle part
cluster 2/1			
1. crustacean hyperglycemic hormone	cellular process, single-organism process, regulation of biological process, biological regulation, signaling, response to stimulus	binding	extracellular region
2. 5-aminolevulinate erythroid-mitochondrial	metabolic process, cellular process, single-organism process	binding, catalytic activity	membrane-enclosed lumen, cell part, cell, organelle part, organelle
3. venom toxin	growth, cellular component, cellular process, single-organism process, regulation of biological process, biological regulation	binding	mcromolecular complex, extracellular region part, extracellular region
4. bumetanide-sensitive sodium-(potassium)-chloride cotransporter isoform X1	localization	-	membrane
5. tyrosine decarboxylase	metabolic process, cellular process, single-organism process	binding, catalytic activity	-
6. 5-aminolevulinate erythroid-mitochondrial	metabolic process, cellular process, single-organism process	binding, catalytic activity	membrane-enclosed lumen, cell part, cell, organelle part, organelle
7. low-density lipo receptor	cellular process, single-organism process, regulation of biological process, biological regulation, signaling, response to stimulus	binding	-
8. 70 kDa heat shock	-	binding	-
9. crustin-like peptide type	-	molecular function regulator	extracellular region
10. Crustin antimicrobial peptide	-	molecular function regulator	extracellular region
11. cryptochrome-1-like isoform X2	-	catalytic activity	-
12. programmed cell death 5	-	binding	-
13. Ovalbumin-related Y	-	-	extracellular region part, extracellular region

The Journal of Applied Science **Vol. 17 Special issue: 27-36 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S04

Table 2. Gene function of up regulated expression transcripts in clusters 1/2 and 2/2.

description	biological process (level 2)	molecular function (level 2)	cellular component (level 2)
cluster 1 / 2			
1. ccaat enhancer-binding	regulation of biological process, biological regulation, metabolic process, cellular process	nucleic acid binding, binding	macromolecular complex, cell part, cell
2. cyclooxygenase	detoxification, multicellular organismal process, single-organism process, biological reguration, metabolic process, cellular process response to stimulus	antioxidant activity, binding, catalytic activity	membrane part, membrane
3. phosphoenolpyruvate carboxykinase	single-organism process, metabolic process	binding, catalytic activity	-
4. MAP kinase-interacting serine threonine- kinase 1 isoform X1	single-organism process, metabolic process, cellular process	binding, catalytic activity	-
5. elongation factor 1-alpha	growth, locomotion, multicellular organismal process, single-organism process, developmental process, regulation of biological process, biological regulation, metabolic process, cellular process, reproduction	binding, catalytic activity	macromolecular complex, cell part, cell, organelle
6. Phosphoenolpyruvate carboxykinase	single-organism process	binding, catalytic activity	-
7. prophenoloxidase	single-organism process, metabolic process	binding, catalytic activity	-
8. serum amyloid A -like	-	-	extracellular region
cluster 2 / 2			
1. CCR4-NOT transcription complex subunit 7 isoform X1	biological regulation, metabolic process, cellular process, regulation of biological process	catalytic activity, binding	-
2. 39S ribosomal mitochondrial-like	metabolic process, cellular process, cellular component	structural molecule activity	cell part, cell, macromolecular complex, organelle
3. transforming growth factor-beta receptor-associated 1-like	biological regulation, localization, cellular process, single-organism process, regulation of biological process	binding	cell part, cell
4. hypothetical protein Y032_0655g1201	biological regulation, metabolic process, cellular process, regulation of biological process	binding	-
5. 5-3 exoribonuclease 2 homolog	biological regulation, metabolic process, cellular process, regulation of biological process	catalytic activity, binding	cell part, cell, organelle
6. electron transfer flavo - ubiquinone mitochondrial	metabolic process, cellular process, single-organism process, response to stimulus	catalytic activity, electron carrier activity, binding	membrane, organelle part, cell part, cell, organelle, membrane part
7. PREDICTED: uncharacterized protein KIAA2013-like	-	-	membrane, membrane part
8. serine ase inhibitor	-	-	extracellular region part, extracellular region

The Journal of Applied Science **Vol. 17 Special issue: 27-36 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S04

Table 3. The summary of transcripts by step after functional annotation of genes using blast2go software.

Clusters	Total (transcripts)	Blast*X* (transcripts)	Unknown / *De novo* (transcripts)	Annotated (transcripts)	Pathway (transcripts)
Cluster 1/1	18	9	4	5	-
Cluster 1/2	34	9	1	8	2
Cluster 2/1	135	34	21	13	2
Cluster 2/2	32	16	8	8	-
Total	219	68	34	34	4

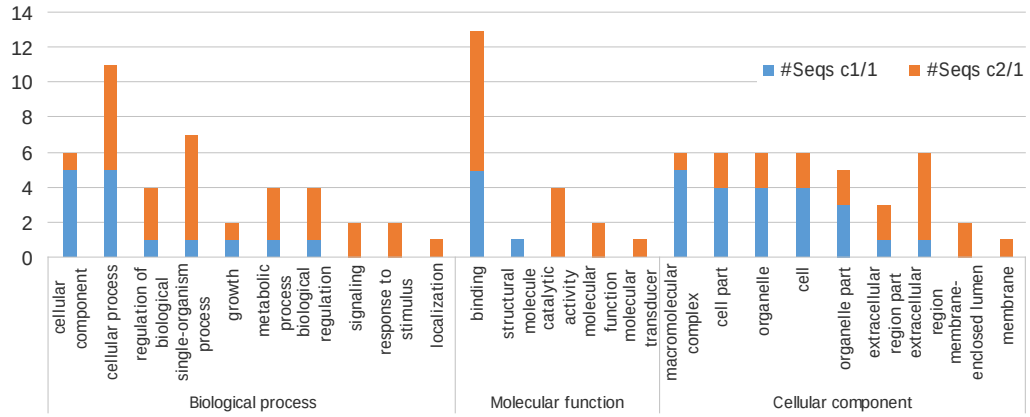


Figure 2. Gene ontology and annotation result (level 2) of Cluster 1/1 and 2/1 showing the number of down regulated expression transcripts while infection of *Vibrio paprhemolyticus* at 24 hours.

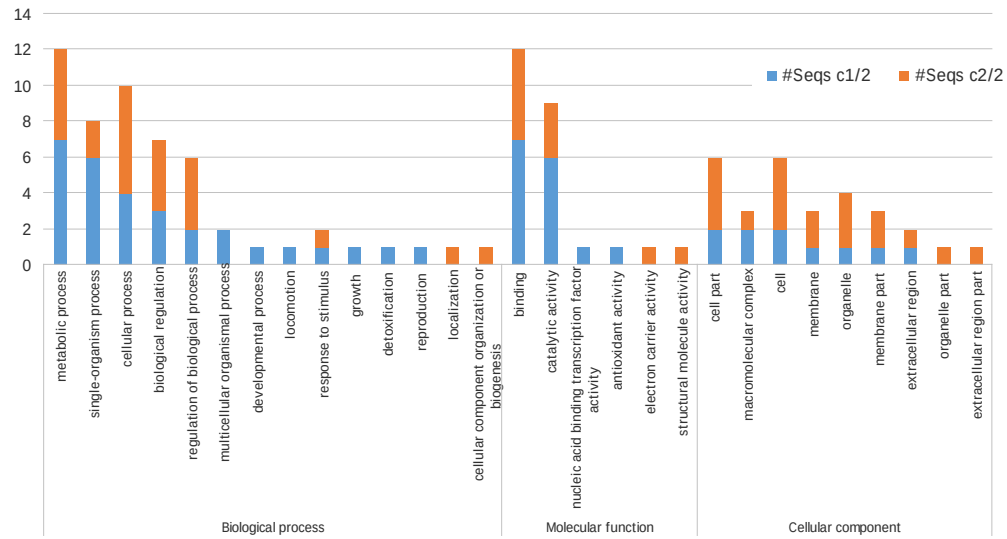


Figure 3. Gene ontology and annotation result (level 2) of Cluster 1/2 and 2/2 showing the number of up regulated expression transcripts while infection of *Vibrio paprhemolyticus* at 24 hours.

The Journal of Applied Science **Vol. 17 Special issue: 27-36 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S04

Pathway analysis

Six pathways were identified from cluster 1/2 (4 pathways) and cluster 2/1 (2 pathways). Cluster 1/2 contained Arachidonic acid metabolism, Phenylpropanoid biosynthesis, Thiamine metabolism, Purine metabolism. While Cluster 2/1 contained Glycine, serine and threonine metabolism and Porphyrin and chlorophyll metabolism (table 4).

Table 4. The metabolism pathway identified from the up regulation transcripts (cluster 1/2) and the down regulation transcripts (cluster 2/1).

Seq	Pathway	Pathway ID	#Enzs in Pathway	Enzyme
Cluster 1/2				
TR33858|c0_g1_i1	Arachidonic acid metabolism, Phenylpropanoid biosynthesis	map00590, map00940	1, 1	ec:1.14.99.1 - synthase, ec:1.11.1.7 - lactoperoxidase
TR4161|c0_g1_i1	Thiamine metabolism, Purine metabolism	map00730, map00230	1, 1	ec:3.6.1.15 - phosphatase, ec:3.6.1.15 - phosphatase
Cluster 2/1				
TR31322|c0_g1_i1	Glycine, serine and threonine metabolism, Porphyrin and chlorophyll metabolism	map00260, map00860	1, 1	ec:2.3.1.37 - synthase, ec:2.3.1.37 - synthase
TR31322|c0_g2_i1	Glycine, serine and threonine metabolism, Porphyrin and chlorophyll metabolism	map00260, map00860	1, 1	ec:2.3.1.37 - synthase, ec:2.3.1.37 - synthase

Discussion

Nucleotide data analysis in hemocyte of *H. raphidea* by Next Generation Sequencing (NGS) technology and bioinformatic tools study in different condition between normal *H. raphidea* and 24 hours infected *Vibrio parahemolyticus* that reconstruct transcripts of *H. raphidea* without referent genome to know more biological data of *H. raphidea* that importance for commercial culture of *H. raphidea* in future. This study found importance gene that concerned in immune system of *H. raphidea*.

Histone H1-delta-like transcript was found in cluster 1/1 down regulated while infection of *Vibrio paprhemolyticus* condition Transcripts (TR8251|c0_g3_i1, TR51934|c0_g1_i1 and TR30957|c0_g1_i1). Histone is the protein in chromatin structure that contains core histones (H2A, H2B, H3, H4) and linker histones (H1, H5). Linker histone H1 link DNA between neucleosome and compact chromatin (Hergeth & Schneider, 2015). Accordingly, Nam et al. (2012) studied on antimicrobial function of histone H1-like protein in olive flounder (*Paralichthys olivaceus*), fish lived in microbe-rich environment and exposed to high concentration of pathogenic microbes. To protect the pathogenic microbes, most of fish can protect them from antimicrobial peptides (AMPs). AMPs are key mediator of innate immunity particularly from primary

Vol. 17 Special issue: 27-36 [2018]
doi: 10.14416/j.appsci.2018.02.S04

barriers such as the skin, mucus, gills, and intestinal mucus. These include pardaxin, pleurocidin, piscidin, misgurin, bass hepcidin, myxinidin. All of AMPs are peptides. In addition, some small protein has antimicrobial property such as histone-likeproteins (HLPs). That can specify by N- or C- terminus (fish histone). From Nam et al. (2012) study by compare between antimicrobial proteins and histone H1 that represent antimicrobial property, positive gram contain *Bacillus subtilis, Staphylococcus aureus, Streptococcus iniae*, negative gram contain *Aeromonas hydrophila*, *Escherichia coli* D31, *Vibrio parahemolyticus*, *Candida albicans*. cDNA cloning and tissue distribution of hiatone H1 from olive founder expressed depend on development stage so anyway without histone H1 in other immunity organ such as kiddy and spleen and represent histone H1 importance play in innate immunity and reproduction stage.

Cyclooxygenase transcript was found in cluster 1/2 up regulated while infection of *Vibrio paprhemolyticus* condition. Varvas et al. (2009) studied on cyclooxygenase pathway of prostaglandin synthesis in arthropods. The cloning of cyclooxygenase (COX) gene from *Gammarus* spp. and *Caprella* spp., amphipod organisms, represent COX protein from amphipods role are N-glycosylate and same thin position in endoplasmic reticulum and nuclear envelope of vertebrate organism. COX activate arachidonic acid to prostaglandin are lipid mediator in vertebrate organism and play in reproductive system, immune system and ion transport of arthropods including crustacean *Daphnia* (Heckmann et al., 2008).

Conclusion

The basic information related to these immune genes in *H. raphidea* was investigated in this study. These transcripts had never been reported and studied. A better understanding of their biochemistry and genetic control in *H. raphidea* will help to improve our understanding of their information for commercial culture and increase nature population of these organisms in the future.

Gene expression value file and nucleotide sequences file are separated. After clustering gene expression profiles in each cluster was merge with nucleotide sequences by developed GUI software for this thing. Those develop by Mr. Suphachip boonta, a bachelor degree student in major information technology, faculty of science and arts, Burapha University in visual studio platform. Mention in searching principle between transcript names in each cluster and transcript names in nucleotide sequences file (fasta files) and export files in fasta format. That working well than searching by “VLOOKUP” function in Microsoft excel because of too more nucleotide sequences (93,344 row).

Acknowledgements

A research grant was supported by National Research Council of Thailand (research ID 10/2560). A travel grant was supported by Faculty of Marine Technology, Burapha University, Chanthaburi Campus.

References

Buermans, H. P. & den Dunnen, J. T. (2014). Next generation sequencing technology: advances and applications. *Biochimica et Biophysica Acta, 1842*, 1932–1941. doi: 10.1016/j.bbadis.2014.06.015

Collart, M. A. & Panasenko, O. O. (2012). The Ccr4-Not complex. *Gene*, 492, 42-53. doi: 10.1016/j.gene.2011.09.033

Heckmann, L. H., Sibly, R. M., Timmermans, M. & Callaghan, A. (2008). Outlining eicosanoid biosynthesis in the crustacean *Daphnia*. *Frontiers in Zoology*, *5*(11), 1-9. doi: 10.1186/1742-9994-5-11

Hergeth, S. P. & Schneider, R. (2015). The H1 linker histones: multifunctional proteins beyond the nucleosomal core particle. *EMBO Reports*, *16*(11), 1439-1453. doi: 10.15252/embr.201540749

Hunter, G. A. & Ferreira, G. C. (2011). Molecular enzymology of 5-Aminolevulinate synthase, the gatekeeper of heme biosynthesis. *Biochimical et Biophysica Acta*, *1814*, 1467-1473. doi: 10.1016/j.bbapap.2010.12.015

Nam, B. H., Seo, J. K., Go, H. J., Lee, M. J., Kim, Y. O., Kim, D. G., et al. (2012). Purification and characterization of an antimicrobial histone H1 like protein and its gene from the testes of olive flounder, *Paralichthys olivaceus*. *Fish and Shellfish Immunology*, *33*, 92-98. doi: 10.1016/j.fsi.2012.04.006

Ohashi, H., Hasegawa, M., Wakimoto, K. & Miyamoto-Sato, E. (2015). Next-generation sequencing technologies for multiomics approaches including interactome sequencing. *BioMed Research International*, *Article ID 104209*, 9 pp. http://dx.doi.org/ 10.1155/2015/104209

Srimukda, B. & Champasri, S. (1994). *Preliminary study on breeding and nursing of mantis shrimp, Harpiosquilla raphidea (Fabricius, 1798).* pp. 453-459. In The 32nd Kasetsart University Annual Conference: Animal Science, Veterinary Science and Fisheries, 3-5 February 1994, Kasetsart University, Bangkok. (In Thai)

Tangkrock-olan, N. & Ruksar, P. (2012). Effect of salinity on blood osmolality of mantis shrimp, *Miyakea nepa* (Latreille, 1828). *Burapha Science Journal*, *17*(1), 50-58. (In Thai)

Varvas, K., Kurg, R., Hansen, K., Järving, R., Järving, L., Valmsen, K., et al. (2009). Direct evidence of the cyclooxygenase pathway of prostaglandin synthesis in arthropods: Genetic and biochemical characterization of two crustacean cyclooxygenase. *Insect Biochemistry and Molecular biology*, *39,* 851-860. doi: 10.1016/j.ibmb.2009.10.002

Watmough, N. J. & Frerman, F. E. (2010). The electron transfer flavoprotein: Ubiquinone oxidoreductases. *Biochimical et Biophysica Acta*, *1797*, 1910-1916. https://doi.org/10.1016/j.bbabio.2010.10.007

Waiho, K., Fazhan, H., Shahreza, M. S., Moh, J. H. Z., Noorbaiduri, S., Wong, L. L., et al. (2017). Transcriptomy analysis and differential gene expression on the testis of organ mud crab, *Scylla olivacea*, during sexual maturation. *PLOS ONE*, *12*(1), e0171095. doi: 10.1371/journal.pone.0171095

Wilantho, A., Praditsup, O., Charoenchim, W., Kulawonganunchai, S., Assawamakin, A. & Tongsima, S. (2012). Next generation sequencing (NGS) technologies and their applications in omics-research. *Thai Journal of Genetics*, *5*(2), 104-129. (In Thai)

The Journal of Applied Science **Vol. 17 Special issue: 37-46 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S05
ISSN 1513-7805 (Printed in Thailand) ISSN 2586-9663 (Online)

Research Article

Ethanol production from cellulosic cassava waste

Phakamas Rawaengsungnoen[1], Saijai Leklai[1] and Sukon Tantipaibulvut[1]*

[1]Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, 10140
***E-mail**: sukon.tan@mail.kmutt.ac.th

Abstract

Cassava cellulosic waste obtained from starch processing was utilized in this work for bio-ethanol production. The 10% (w/v) of cassava waste was pretreated with either water or 1% (v/v) acetic acid and steam treated by autoclaving at 121 °C under pressure of 15 pound/inch^2 for 15 min. After neutralization of the acid-autoclaved pretreated waste with 2% (w/v) $NaHCO_3$, the mixture was hydrolyzed further by using 3% or 6% (w/w) cellulase for sugar production. It appeared that the optimum hydrolysis condition was 1% (v/v) acetic acid and 6% (w/w) enzyme (shaking at 50 °C, 150 rpm for 96 h), which gave the maximum sugar yield of 199.82 ± 0.27 mg/g dried cellulosic waste. A total of 515.39 ± 0.48 mg ethanol/g dried cellulosic waste was obtained from 9% (w/v) of *Saccharomyces cerevisiae* in 7-days fermentation at room temperature. This indicates that cassava wastes actually could be used as substrate for biochemical production, such as fuels, organic acids and etc. In addition, the pretreatment method using dilute acetic acid and steam is environmentally friendly and not complex.

Keywords: acetic acid, cassava waste, cellulase, fermentation

Introduction

Cassava is considered one of the most important economic crops in Thailand. Nearly all the harvested roots are processed into dry chips and pellets for export as animal feed, as well as into starch, both for domestic use and export (Ratanawaraha et al., 2000). Waste material from cassava-starch processing is about 1.1% of cassava tubers that enters the factory. This is approximately 2 million tons per year. If this waste is not properly managed, it may cause foul smell and unattractive sight (Sackey & Bani, 2007; Deeprasert et al., 2012). The technologies currently existing for treatment of this waste include land filling of solid waste, use as animal feed, utilization as a media for mushroom cultivation, or use to produce compost. Major components in this waste are cellulose and starch. If this material can be degraded and hydrolysed to sugar, it can be used as raw material to produce many value-added components; for example, lactic acid, ethanol, biogas and so on (Ubalua, 2007; Sackey & Bani, 2007).

A total of 32.4% alcohol (2.7 g ethanol/15 g cellulosic waste) was obtained from cassava cellulosic waste when the waste was first hydrolysed by 0.1% α-amylase, followed by hydrolysed using 0.5 M HCl. Finally, amyloglucosidase was used to saccharify the hydrolysed starch into reducing sugar (Elemike et al., 2015). Thongchul et al. (2015) reported that the maximum starch recovery (88%) and the highest glucose production (98 g/L) were obtained when hydrolyzing cassava pulp at 121°C for 15 min with 1.0 M HCl (1 g dry pulp to 9 mL HCl

Vol. 17 Special issue: 37-46 [2018]
doi: 10.14416/j.appsci.2018.02.S05

solution). Recently, a soaking assisted thermal pretreatment was introduced for optimum fermentable sugar release from cassava peels waste. Maximum reducing sugar of 89.80 ± 2.87 g/L corresponding to a fermentable sugar yield of 0.93 ± 0.03 g/g cassava peels was achieved when 9.65% w/v of milled cassava peels were soaked in 3.68 v/v hydrochloric acid concentration at 69.62 °C for 2.57 h followed by autoclave thermal treatment (121 °C) for 5 min (Aruwajoye et al., 2017).

However, these works used strong acid in the pretreatment of the waste. Therefore, the aim of this report was to study the utilization of cassava cellulosic waste as raw material for ethanol production using weak acid (acetic acid) and cellulase in the pretreatment method. The factors that were investigated included the pretreatment condition, the concentration of cellulase, the hydrolysis time and the concentration of yeast used in the fermentation.

Materials and methods

Raw material

The cassava cellulosic waste used in this study was provided by Thai Wah Starch Public Company Limited, Nakhon Ratchasima Province. The waste was collected directly from the plant, milled into fine particles and dried at 100 °C for 24 h. Then, it was kept in moisture-proof bag at room temperature.

Proximate analysis

Some biochemical parameters such as crude fibre content, protein content and total carbon of the cellulosic cassava waste was determined. The protein content was determined by Kjeldahl method using the conversion factor of 6.25 (AOAC, 2012). The fibre content was also determined by using AOAC, 2012 method. The sample is allowed to boil with 1.25% dilute H_2SO_4, washed with water, further boiled with 1.25% dilute sodium hydroxide and the remaining residue after digestion was taken as crude fibre. This fibre content was roughly estimated as cellulose content. Total carbon content was estimated based on the volatile solids (VS) content. % Carbon equal to (% VS) / 1.8 (Adams et al., 1951).

Microorganism

Saccharomyces cerevisiae used in this study was commercial instant dry bakers yeast, Fermipan Red.

Pretreatment

The wastes were pretreated by either water or 1% (v/v) acetic acid to increase the accessibility of cellulose, at solid to liquid ratio of 1 : 10 (w/v). The condition used was either incubation at room termperature for 24 h or steam-treatment by autoclaving at 121°C and 15 pound/inch^2 pressure, with reaction time of 10, 15 and 20 min. Reaction mixture was allowed to cool to room temperature and 1 mL of hydrolysate was pipetted out to estimate the concentration of total reducing sugar (TRS) using dinitrosalicylic (DNS) acid method (Miller, 1959). Glucose was used as a standard in this assay. The best pretreatment that gave the highest reducing sugar was used further for enzyme hydrolysis.

Enzyme hydrolysis

The effects of enzyme concentration and hydrolytic conditions were evaluated by adjusted the pH of the acid-autoclaved pretreated waste to 5.5 using 2% (w/v) $NaHCO_3$ solution. The neutralized waste was then subjected to enzymatic hydrolysis using commercial

Vol. 17 Special issue: 37-46 [2018]
doi: 10.14416/j.appsci.2018.02.S05

cellulase solution Cellic® CTec2 (Novozymes, Denmark). The wastes were hydrolyzed with 3% or 6% of Cellic® CTec2 (W enzyme/W waste) at 3 different condition for 48 h : stand at room temperature; shaking at 30°C, 150 rpm; and shaking at 50°C, 150 rpm. The samples were harvested at 6-h interval to assay for the concentration of TRS using dinitrosalicylic (DNS) acid method (Miller, 1959).

Ethanol fermentation

After enzymatic treatment for 48 h, the waste hydrolysate was separated into 2 groups, one was sterilized by carried out in an autoclave at 15 pound/inch^2 pressure, 121°C for 15 min, the other was not autoclaved. Then, they was inoculated with 3 different concentrations of yeast; 3%, 6% and 9% (w/v). Each experiment was carried out in duplicate. In addition, the extension of enzymatic treatment to 72 and 96 h, without sterilization of the waste hydrolysate before yeast inoculation, was also carried out. The samples were harvested each day to assay for the concentration of TRS using dinitrosalicylic (DNS) acid method (Miller, 1959), and ethanol using gas chromatograph with a 10% Carbowax 20 M support (6 ft. x 1/8 in), at oven temperature of 150°C and flame ionization detector (FID) at 200 °C. Nitrogen with a flowrate of 30 mL/min was used as carrier gas.

Results and discussion

Effect of pretreatment condition

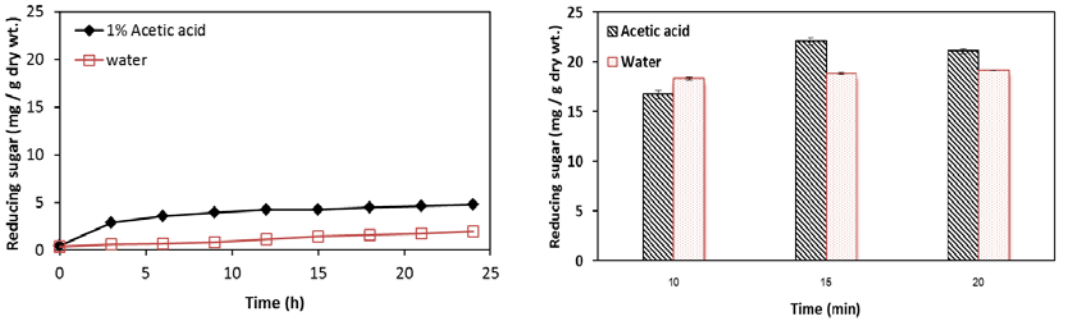
Cassava cellulosic waste contained (wt. %): 2.33 protein, 13.30 cellulose and 58.04 total carbon. The composition of cellulose is in agreement with the previously reported values, 14.35% and 15.25% w/w dry basis (Yoonan, 2003; Deeprasert et al., 2012).

Most acid hydrolysis pretreatment for cellulosic material used concentrated strong acids such as H_2SO_4 and HCl. Although they are powerful agents for cellulose hydrolysis, concentrated strong acids are toxic, corrosive, hazardous, and thus require reactors that are resistant to corrosion, which makes the pretreatment process very expensive. In addition, the concentrated strong acid must be recovered after hydrolysis to make the process economically feasible (Kumar et al., 2009).

Dilute-acid hydrolysis has been successfully developed for pretreatment of lignocellulosic materials. High temperature in the dilute-acid treatment is favorable for cellulose hydrolysis (Kumar et al., 2009). Furthermore, it was reported that the presence of weak acids can improve glucose utilization, ethanol production and tolerance to hydroxymethylfurfural and furfural in *S. cerevisiae* (Greetham et al., 2016). Therefore, acetic acid was used in this study to pretreat cassava waste compared to the use of water either at room temperature or in the autoclave.

According to Figure 1, the condition that gave the highest yield of reducing sugar was the pretreatment of cassava waste with 1% (v/v) acetic acid in autoclave at 121 °C for 15 min (22.08 ± 0.35 mg/g dry wt.) The value was not significantly different from that of the pretreatment with 1% (v/v) acetic acid at 121 °C for 20 min (21.08 ± 0.24 mg/g dry wt.). This condition was better than the condition that used only water in autoclave and only acetic acid at room temperature for 24 h (19.17 ± 0.00 and 4.76 ± 0.04 mg/g dry wt., respectively). This is due to the entrance of steam into the biomass expanding the walls of fibers leading to partial hydrolysis and increasing the accessibility of enzymes for cellulose. After this, the pressure is reduced to atmospheric condition. During this pretreatment, the hydrolysis of cellulose into glucose monomers is carried out by the acetic acid (Kumar & Sharma, 2017). Water can act as an acid at high temperatures. Addition of acid [typically 0.3-3% (w/w)] in steam explosion can

decrease time and temperature, effectively improve hydrolysis and decrease the production of inhibitory compounds. Steam provides an effective vehicle to rapidly heat cellulose to the target temperature without excessive dilution of the resulting sugars (Kumar et al., 2009). The pH of acid-autoclaved pretreated waste was adjusted to 5.5 by 2% (w/v) $NaHCO_3$ solution before using further.



(a) Room temperature (b) In autoclave (121°C, 15 lb/inch^2)

Figure 1. Effect of acetic acid concentration (0% and 1% (v/v)), incubation condition and time on the sugars yield of cassava waste.

Effect of enzyme loading

To increase the efficiency of the acid pretreatment, enzymatic hydrolysis was performed using the neutralized sample obtained after acid autoclaving. The different enzyme concentration (3% and 6% (w/w) Cellic® CTec2) and incubation temperature (room temperature, shaking at 30°C and 50°C) was evaluated. The highest yield of glucose (138.61 ± 0.37 mg/g dry wt.) was obtained from the enzymatic hydrolysis using 6% w enzyme/w of cassava waste, shaking at 50°C for 48 h, after acid autoclaving (Figure 2). On the other hand, 110-115 mg glucose/g dry wt. of cassava waste was obtained when 3% (w/w) cellulase was used at either 30°C or 50°C for 48 h. Increasing enzyme concentration gave higher sugar yield. This was similar to that reported by Bayitse et al. (2015). Enzymatic hydrolysis of cassava peels with 2.5% (v/w) cellulases (NS22186) at 4 h released 0.9 g/l of glucose which rose to 1.2 g/l in 48 h. When the enzyme loading was increased to 10% cellulase, glucose concentration was doubled from 1.02 g/l at 4 h to 2.17 g/l at 48 h of hydrolysis which increased marginally to 2.6 g/l at 120 h. The amount of fermentable sugar obtained increased as the enzyme load increased while cellulose load decreased (Azhar et al., 2017). Deeprasert et al. (2012) can produce 65.80 g/l of reducing sugar from cassava solid waste (100 g/L), using 3 steps of enzymatic hydrolysis, Cellic® CTec2 for 6 h, α-Amylase (Novozyme) for 2 h and Glucoamylase (Novozyme) for 12 h. However, the presence of high sugar concentration in the fermentation medium may lead to substrate inhibition and results in the inhibition of cell growth and ethanol production (Azhar et al., 2017).

In order to investigate the effect of the concentration of acetic acid in the pretreatment before enzyme hydrolysis, the acetic acid concentration was increased to 3% and 5% (v/v) before hydrolysed by 6% (w/w) Cellic® CTec2. The result showed that the sugar yield obtained from 3% and 5% acetic acid pretreatment was not significantly different from that of 1% acetic acid (Figure 3). Esteghlalian et al. (1997) found that high temperature in the dilute acid treatment is favourable for cellulose hydrolysis. Dilute sulfuric acid (<2% w/w) pretreatment can achieve high reaction rate and makes the cellulose more susceptible to enzymatic

digestion. Horst et al. (2011) reported that acetic acid showed to be potential used for lignocellulosic hydrolysis. The hydrolysis efficiency levels of acetic acid against several types of wood chips was mantained between 21.47% and 32.62%, and the ethanol production rates stayed between 6.32 L/100 kg and 9.63 L/100 kg of biomass. Zhao et al. (2014) found that pretreatment of corn stover with 0.25% acetic acid at 191 °C for 7.74 min was the most optimal condition under which the production of acids in acidogenic fermentation can reach the highest level. Schneider et al. (2016) mentioned that acetic acid and formic acid are rather mild acid yielding low total reducing sugar levels compared to the sulfuric acid, when it was used to convert lignocellulosic barley straw to valuable sugars.

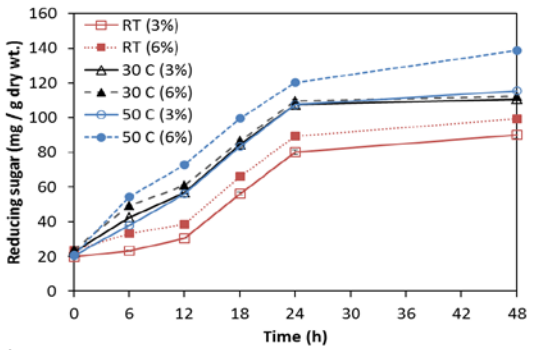


Figure 2. Effect of Cellic® CTec2 concentration (3% and 6% W enzyme/W cassava) and hydrolysis condition on the sugar yield of cassava waste pretreated with 1% (v/v) acetic acid in the autoclave for 15 min. The cellulolysis condition was stand at room temperature (RT), shaking at 30 °C (30 C) and 50 °C (50 C) for 48 h.

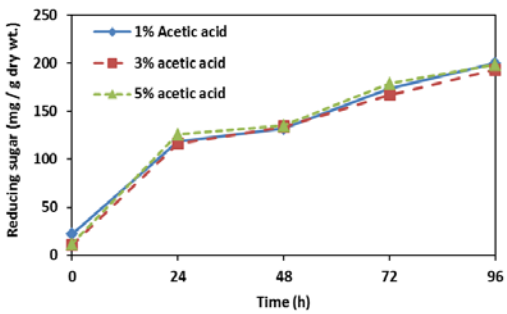


Figure 3. Effect of acetic acid concentration (1%, 3% and 5% (v/v)) in the autoclave for 15 min before enzyme hydrolysis on the sugar yield of cassava waste.

Furthermore, the longer the hydrolysis time was, the higher the sugar yield obtained. The sugar yield of 96-h hydrolysis of the cassava waste pretreated with 1%, 3% or 5% (v/v) acetic acid before enzyme hydrolysis were 199.82±0.27, 193.20±1.20 and 198.61±1.85 mg glucose/g dry wt; compared to 136.37±2.76, 133.97±2.41 and 134.97±2.33 mg glucose/g dry wt of 48-h hydrolysis, respectively (Figure 3). Therefore, the hydrolysis time may be more important than the acid concentration used. This was similar to the result of Bayitse et al. (2015), as mentioned above.

Vol. 17 Special issue: 37-46 [2018]
doi: 10.14416/j.appsci.2018.02.S05

Ethanol fermentation

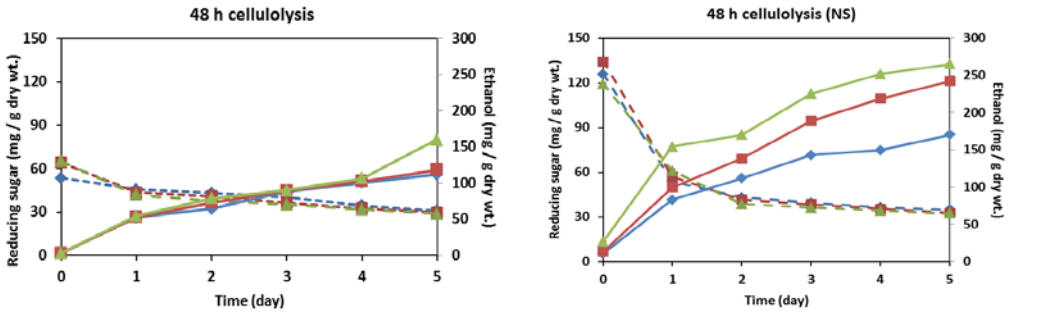
The acid-autoclaved pretreatment in this study did not need complex process of detoxification. After neutralization of the acid-autoclaved pretreated waste with $NaHCO_3$, the mixture can be hydrolysed further by Cellic® CTec2 and fermented by instant dry bakers yeast (*S. cerevisiae*) for ethanol production. The ethanol yield was investigated between sterilization and non-sterilization of the hydrolysate before yeast inoculation and different concentration of yeast. After acid-autoclaved pretreatment, enzymatic hydrolysis is used to convert the residual cellulose into monomeric sugars. The sugar is then fermented to ethanol by yeast. When enzymatic hydrolysis and fermentation are performed sequentially, it is referred to as separate hydrolysis and fermentation (SHF). However, the two process steps can be performed simultaneously, i.e. simultaneous saccharification and fermentation (SSF) (Öhgren et al., 2007).

In this study, the fermentation of non-sterile hydrolysate is similar to SSF with the delayed time of yeast incoculation and the sterile hydrolysate is similar to SHF owing to the denaturation of enzyme by autoclave. As can be seen from Figure 4, the non-sterile hydrolysate gave more yield of ethanol than the sterilized one (264.96 $\pm$ 0.51 and 159.96 $\pm$ 0.74 mg/g dry wt, respectively). During SSF, the faster saccharification rates result because the glucose product is immediately removed, considerably diminishing its inhibitory effect on the cellulase system (Abe & Takagi, 1991). Furthermore, small amount of fermentable sugar continuously gradually released by the enzyme, as can be seen from the stable low amount of sugar after 2-3 days of fermentation in Figure 4 & 5.

Rattanachomsri et al. (2009) found that the enzyme saccharification reaction can be performed simultaneously with the ethanol fermentation process using a thermotolerant yeast *Candida tropicalis* BCC7755. This process produced 14.3 g/l ethanol from 4% (w/v) cassava pulp after 30 h of fermentation. The productivity rate of 0.48 g/l/h is equivalent to 93.7% of the theoretical yield based on total starch and cellulose, or 85.4% based on total fermentable sugars. Öhgren et al. (2007) has compared the yield of ethanol from steam-pretreated corn stover by two different process of fermentation, SSF and SHF, using 8% water-insoluble solids. They reported that SSF gave a 13% higher overall ethanol yield than SHF (72.4% versus 59.1% of the theoretical). In addition, Apiwatanapiwat et al. (2013) also found that the ethanol concentration produced from cassava pulp using the SSF process was higher than that using the SHF process. Combination of the two process steps also results in a lower capital cost, the reduction of the requirements for many reactors and the fact that the ethanol concentration is higher during SSF than SHF reduces the risk of contamination (Adekunle et al., 2016). Zhang et al. (2013) found that the simultaneous co-saccharification of cassava starch/cellulose and ethanol fermentation process provided a cost effective option of cassava cellulose utilization for ethanol production. However, the temperature optima for the yeast and the enzymes used differ, which means that the conditions used in SSF cannot be optimal for both the enzymes and the yeast (Öhgren et al., 2007).

Furthermore, increase the hydrolysis time and the size of yeast inoculum provided more ethanol, as shown in Figure 4 and 5. Fermentable sugars should be as high as possible in the bioethanol fermentation system to reduce the cost (de Albuquerque Wanderley et al, 2013). The increase in sugar concentration up to a certain level caused fermentation rate to increase. The initial sugar concentration also has been considered as an important factor in ethanol production. High ethanol productivity and yield in batch fermentation can be obtained by using higher initial sugar concentration. Fermentation time also affect the growth of microorganisms. Shorter fermentation time causes inefficient fermentation due to inadequate growth of microorganisms. On the other hand, longer fermentation time gives toxic effect on microbial

growth especially in batch mode due to the high concentration of ethanol in the fermented broth (Zabed et al., 2014).



(a) Sterilization before yeast inoculation (b) non-sterilization before yeast inoculation

Figure 4. Effect of substrate sterilization and yeast concentration (3%, 6% and 9% (w/v)) on ethanol production, using 1% (v/v) acetic acid pretreated cassava waste with 6% (w/w) Cellic® CTec2 at 50°C for 48 h as substrate, fermented for 5 days at room temperature. (dash line for sugar, solid line for ethanol; ♦ = 3% yeast, ■ = 6% yeast, ▲= 9% yeast)

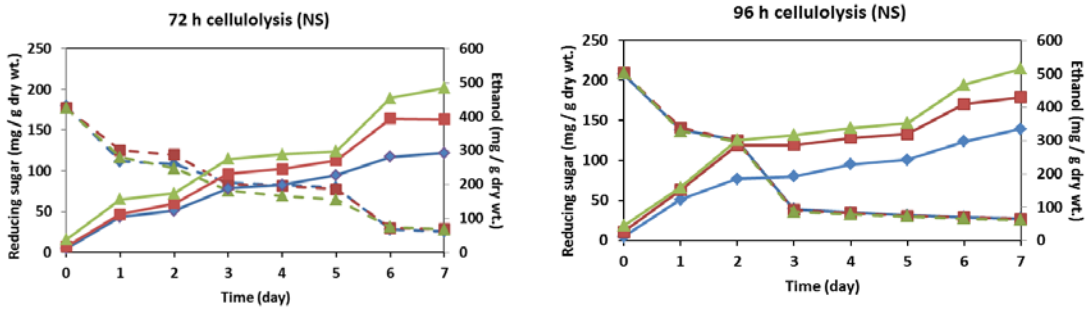


Figure 5. Effect of cellolytic time of cassava waste (72 h and 96 h) on ethanol production, using various concentration of yeast (3%, 6% and 9% (w/v)) fermented for 7 days at room temperature without substrate sterilization before yeast inculation. (dash line for sugar, solid line for ethanol; ♦ = 3% yeast, ■ = 6% yeast, ▲= 9% yeast)

Inoculum concentration may give significant effects on the ethanol production. Duhan et al., (2013) found that when the inoculum size was increased from 5 to 10%, ethanol production was also increased but above 10%, rate of alcohol production decreased after 48 h of incubation. The production of ethanol was increased with the increase in cell numbers from 1×10^4 to 1×10^7 cells per ml but there was no significant ethanol production found between 10^7 and 10^8 cells per ml. This is because the increase in cell concentration within certain range reduces fermentation time as the cells grow rapidly and directly consumes sugars into ethanol (Zabed et al., 2014). Breisha (2010) reported that increasing the yeast inoculum volume from 3% to 6% showed positive effects on fermentation from 25% sucrose and reduced the fermentation time from 72 h (3%) to 48 h (6%). The fermentation time shorten along with the raise in inoculum size which was due to the fast cell growth within the reactor. Most of the substrate was immediately converted to ethanol. The common inoculum size employed in bioethanol production is 5% and 10% (Azhar et al., 2017).

Vol. 17 Special issue: 37-46 [2018]
doi: 10.14416/j.appsci.2018.02.S05

Conclusion

This study shows that cassava cellulosic waste obtained from starch processing can be utilized for the production of bioethanol. The actions of acetic acid pretreatment and commercial cellulase have a remarkable capacity to degrade this waste. Pretreatment of the waste with 1% (v/v) acetic acid in the autoclave, followed by hydrolysis with cellulase at conditions of pH 5.5, temperature 50 °C, time 96 h produces more reducing sugar. The yield of 515.39 ± 0.48 mg ethanol/g dry wt was obtained from 9% (w/v) of *Saccharomyces cerevisiae* in 7-days fermentation at room temperature. Further study will be the application of this pretreatment method with other types of waste to see how well it works.

Acknowledgement

The authors would like to thank the Department of Microbiology, Faculty of Science, KMUTT for their support of the research facilities.

References

Abe, S. & Takagi, M. (1991). Simultaneous saccharification and fermentation of cellulose to lactic acid. *Biotechnology and Bioengineering, 37*(1), 93-96. doi: 10.1002/bit.260370113

Adams, R. C., MacLean, F. S., Dixon, J. K., Bennett, F. M., Martin, G. I. & Lough, R. C. (1951). The utilization of organic wastes in N.Z.: Second interim report of the inter-departmental committee. *New Zealand Engineering* (November 15, 1951), 396-424.

Adekunle, A., Orsat, V. & Raghavan, V. (2016). Lignocellulosic bioethanol: A review and design conceptualization study of production from cassava peels. *Renewable and Sustainable Energy Reviews, 64*, 518-530. http://dx.doi.org/10.1016/j.rser.2016.06.064

AOAC. (2012). Official Methods of Analysis of AOAC international (19th ed.). Gaithersburg, Maryland: AOAC International.

Apiwatanapiwat, W., Rugthaworn, P., Vaithanomsat, P., Thanapase, W., Kosugi, A., Arai, T. et al. (2013). Ethanol production at high temperature from cassava pulp by a newly isolated *Kluyveromyces marxianus* strain, TISTR 5925. *AIMS Energy*, *1*(1), 3-16. doi: 10.3934/energy.2013.1.3

Aruwajoye, G. S., Faloye, F. D. & Kana, E. G. (2017). Soaking assisted thermal pretreatment of cassava peels wastes for fermentable sugar production: Process modelling and optimization. *Energy Conversion and Management*, *150*, 558-566. https://doi.org/10.1016/j.enconman.2017.08.046

Azhar, S. H. M., Abdulla, R., Jambo, S. A., Marbawi, H., Gansau, J. A., Faik, A. A. M. et al. (2017). Yeasts in sustainable bioethanol production: A review. *Biochemistry and Biophysics Reports, 10*, 52-61. http://dx.doi.org/10.1016/j.bbrep.2017.03.003

Bayitse, R., Hou, X., Bjerre, A.-B. & Saalia, F. K. (2015). Optimisation of enzymatic hydrolysis of cassava peel to produce fermentable sugars. *AMB Express, 5*, 60. doi: 10.1186/s13568-015-0146-z.

Breisha, G. Z. (2010). Production of 16% ethanol from 35% sucrose. *Biomass and Bioenergy, 34* (8), 1243-1249. https://doi.org/10.1016/j.biombioe.2010.03.017

de Albuquerque Wanderley, M. C., Martín, C., de Moraes Rocha, G. J. & Gouveia, E. R. (2013). Increase in ethanol production from sugarcane bagasse based on combined pretreatments and fed-batch enzymatic hydrolysis. *Bioresource Technology, 128*, 448-453. http://dx.doi.org/10.1016/j.biortech.2012.10.131

Deeprasert, S., Boonmunma, S., Boottongmol, W., Chinchoedwong B. & Loha, V. (2012). Utilization of cassava soild waste for sugar production. *Thaksin University Journal, 15* (3, Special), 39-46. (in Thai)

Duhan, J. S., Kumar A. & Tanwar, S. K. (2013). Bioethanol production from starchy part of tuberous plant (potato) using *Saccharomyces cerevisiae* MTCC-170. *African Journal of Microbiology Research, 7* (46), 5253-5260, doi: 10.5897/AJMR2013.6122

Elemike, E. E., Oseghale, O. C. & Okoye, A. C. (2015). Utilization of cellulosic cassava waste for bio-ethanol production. *Journal of Environmental Chemical Engineering, 3*, 2797-2800. http://dx.doi.org/10.1016/j.jece.2015.10.021

Esteghlalian, A., Hashimoto, A. G., Fenske, J. J. & Penner, M. H. (1997). Modeling and optimization of the dilute-sulfuric-acid pretreatment of corn stover, poplar and switchgrass. *Bioresource Technology, 59*(2-3), 129-136. https://doi.org/10.1016/S0960-8524(97)81606-9

Greetham, D., Hart, A. J. & Tucker, G. A. (2016). Presence of low concentrations of acetic acid improves yeast tolerance to hydroxymethylfurfural (HMF) and furfural. *Biomass and Bioenergy, 85*, 53-60. https://doi.org/10.1016/j.biombioe.2015.11.026

Kumar, A. K. & Sharma, S. (2017). Recent updates on different methods of pretreatment of lignocellulosic feedstocks: a review. *Bioresources and Bioprocessing*, *4*, 7. doi: 10.1186/s40643-017-0137-9

Kumar, P., Barrett, D. M., Delwiche, M. J. & Stroeve, P. (2009). Methods for pretreatment of lignocellulosic biomass for efficient hydrolysis and biofuel production. *Industrial Engineering Chemistry Research*, *48*(8), 3713-3729. doi: 10.1021/ie801542g

Horst, D. J., Behainne, J. J. R. & Petter, R. R. (2011). *Analysis of hydrolysis yields by using different acids for bioethanol production from Brazilian woods*. XVII International Conference on Industrial Engineering and Operations Management. Belo Horizonte, Brazil, 4-7 October 2011. Retrieved Sep 13, 2017, from http://www.abepro.org.br/biblioteca/enegep2011_TI_ST_143_902_17745.pdf

Miller, G. L. (1959). Use of dinitrosalicylic acid for determining reducing sugars. *Analytical Chemistry, 31*, 426-428. doi: 10.1021/ac60147a030

Öhgren, K., Bura, R., Lesnicki, G., Saddler, J. & Zacchi, G. (2007). A comparison between simultaneous saccharification and fermentation and separate hydrolysis and fermentation using steam-pretreated corn stover. *Process Biochemistry 42*, 834-839. doi: 10.1016/j.procbio.2007.02.003

Ratanawaraha, C., Senanarong N. & Suriyapan, P. (2000). *Status of cassava in Thailand: Implications for future research and development*. A review of cassava in Asia with country case studies on Thailand and Viet Nam. Proceedings of the validation forum on the global cassava development strategy, Rome, 26-28 April 2000. Retreived Oct 2, 2017, from http://www.fao.org/docrep/009/y1177e/Y1177E04.htm#ch3.2.4

Rattanachomsri, U., Tanapongpipat, S., Eurwilaichitr, L. & Champreda, V. (2009). Simultaneous non-thermal saccharification of cassava pulp by multi-enzyme activity and ethanol fermentation by *Candida tropicalis*. *Journal of Bioscience and Bioengineering, 107*(5), 488-493. doi: 10.1016/j.jbiosc.2008.12.024.

Sackey, I. S. & Bani, R. J. (2007). Survey of waste management practices in cassava processing to gari in selected districts of Ghana. *Journal of Food, Agriculture & Environment, 5*(2), 325-328.

Schneider, L., Dong, Y., Haverinen, J., Jaakkola, M. & Lassi, U. (2016). Efficiency of acetic acid and formic acid as a catalyst in catalytical and mechanocatalytical pretreatment of barley straw. *Biomass and Bioenergy, 91*, 134-142. https://doi.org/10.1016/j.biombioe.2016.05.015

Thongchul, N., Navankasattusas, S. & Yang, S.-T. (2008). *From raw cassava pulp to L-lactic acid via fermentation by Rhizopus oryzae.* 2008 AIChE Annual Meeting: Food, Pharmaceutical & Bioengineering Division, 16-21 November 2008. Retrieved Sep 13, 2017, from https://www.researchgate.net/publication/267319378

Ubalua, A. O. (2007). Cassava wastes: treatment options and value addition alternatives. *African Journal of Biotechnology, 6* (18), 2065-2073. doi: 10.5897/AJB2007.000-2319

Yoonan, K. (2003). *Study of the optimum condition for sugars production from cassava peel and pulp* (M.Sc. thesis) Department of Biochemical Technology, School of Bioresource Technology, King Mongkut's University of Technology Thonburi, Bangkok. (in Thai)

Zabed, H., Faruq, G., Sahu, J. N., Azirun, M. S., Hashim, R. & Boyce, A. N. (2014). Bioethanol production from fermentable sugar juice. *The Scientific World Journal*, *2014*, *Article ID 957102*, 11 pp. http://dx.doi.org/10.1155/2014/957102

Zhang, J., Fang, Z., Deng, H., Zhang, X. & Bao, J. (2013). Cost analysis of cassava cellulose utilization scenarios for ethanol production on flowsheet simulation platform. *Bioresource Technology, 134,* 298-306. https://doi.org/10.1016/j.biortech.2013.02.041

Zhao, X., Wang, L., Lu, X. & Zhang, S. (2014). Pretreatment of corn stover with diluted acetic acid for enhancement of acidogenic fermentation. *Bioresource Technology, 158,* 12-18. https://doi.org/10.1016/j.biortech.2014.01.122

The Journal of Applied Science
วารสารวิทยาศาสตร์ประยุกต์
ISSN 1513-7805 (Printed in Thailand)

Vol. 17 Special issue: 47-54 [2018]
doi: 10.14416/j.appsci.2018.02.S06
ISSN 2586-9663 (Online)

Research Article

Biohydrogen production from xylose by the newly isolated *Clostridium beijerinckii* PS-3

Pongsak Noparat [1,2]*

[1]Program in Environmental Science, Faculty of Science and Technology, Suratthani Rajabhat University, Suratthani 84100, Thailand
[2]Renewable Energy and Environment Research for Local Community Unit (REERCU), Suratthani Rajabhat University, Suratthani 84100, Thailand
***E-mail**: pongsak@sru.ac.th

Abstract

The optimal condition for hydrogen production using *Clostridium beijerinckii* PS-3 was at an initial pH of 6.3–6.7 and cultivation at room temperature (30±2ºC). Among various types of substrate tested, the hydrogen yield of 1.73 mol/mol-xylose was about 1.2, 1.6 and 5.6 fold higher than those of glucose, starch and cellulose, respectively. From the xylose concentrations (5-30 g/L) tested, the optimum value was 10 g/L, giving the cumulative hydrogen production and hydrogen yield of 3254 ml/L and 2.18 mol/mol-xylose, respectively. Soluble metabolites from xylose were composed mostly of butyric acid (39.0-48.7mM), acetic acid (35.0-39.9 mM), ethanol (30.2-35.3 mM) and propionic acid (4.9-6.5 mM).

Keywords: hydrogen production, xylose, *Clostridium beijerinckii*

Introduction

Hydrogen is an alternative energy source that is cost-effective, environment-friendly and renewable. It has high specific energy content per unit mass (a high energy yield of 122 kJ/g), which is 2.75 times greater than hydrocarbon fuels (Chong et al., 2009a). Among various hydrogen production processes, the biological method is known to be less energy intensive as it can be carried out at ambient temperature and pressure (Lavarack et al., 2002) and at high temperature (Prasertsan et al., 2009).

Renewable biomass is the best non-petroleum based resource that is generated from various agro-industries as waste materials (Chong et al., 2009b). Felled oil palm trunk (OPT) (25 years old) provides an abundant biomass in oil palm plantations, especially in Southern Thailand. The composition of the OPT used was 31.28-42.85% cellulose, 19.73-25.56% hemicellulose, 10.74-18.47% lignin, 1.63-2.25% protein, 1.60-1.83% fat, 1.12-1.35% ash and with trace amount of minerals (0.01-0.40%) (Noparat et al., 2011). Glucose is easily biodegraded during hydrogen production by fermentation, giving the values of 1.2-2.52 mol/mol-glucose (Ishikawa et al., 2006; Xing et al., 2008; Chin et al., 2003). Conversely, fermentative hydrogen production from xylose is generally inefficient due to the lack of efficient hydrogen-producing bacteria for this substrate. Therefore, it is necessary to find a certain bacterial strain that prefers xylose to glucose in hydrogen production. Besides the bacterial strain, fermentative hydrogen production is influenced by many factors such as pH, temperature and substrate (Zhu and Yang, 2004; Lin et al., 2008; Lo et al., 2008).

Vol. 17 Special issue: 47-54 [2018]
doi: 10.14416/j.appsci.2018.02.S06

A number of studies have examined the potential of using hydrogen-producing bacteria, mainly in the genus *Clostridium*, from various sources such as anaerobic sludge digesters (Chen et al, 2005) and compost piles (van Ginkel et al, 2001). *Clostridium* sp. are able to degrade both simple sugars such as glucose and xylose and more complex substrates such as rice winery wastewater (Yu et al., 2002), sugarcane bagasse hydrolysate (Fangkum and Reungsang, 2011), palm oil mill effluent (O-Thong et al., 2007) and oil palm sap (Noparat et al., 2011). In the present study, the potential of newly isolated *Clostridium beijerinckii* PS-3 for hydrogen-producing from xylose was investigated.

Materials and methods

Microorganism and growth medium

C. beijerinckii PS-3, newly isolated from oil palm sap (Noparat et al., 2011), was grown in the synthetic medium (Chimtong et al., 2009) at 30±2°C under anaerobic condition for 18 h and kept at 4°C as stock culture for 1 month. Prior to cultivation, *C. beijerinckii* PS-3 was activated by transferring 1 mL of the stock culture at a cell concentration of OD_{660} = 0.5 into 5 mL of fresh synthetic medium. Then, a serum bottle was flushed with nitrogen to create anaerobic condition and incubated at 30±2°C for 18 h. The synthetic medium contains 10 g/L xylose, 1.5 g/L KH_2PO_4, 2.9 g/L K_2HPO_4, 2.1 g/L urea, 4.5 g/L yeast extract, 0.05 g/L $MgCl_2.6H_2O$, 0.0075 g/L $CaCl_2{\cdot}2H_2O$ and 0.015 g/L $FeSO_4{\cdot}7H_2O$ (Chimtong et al., 2009).

Optimization for Production of Hydrogen

Using batch fermentation, 5 mL of bacterial suspension at an exponential phase (OD_{660}=0.5) was transferred into 45 mL synthetic medium (Chimtong et al., 2009) supplemented with xylose as a carbon source to a final concentration of 10 g/L. To determine the effects of the operating parameters on hydrogen production, the xylose concentration, pH value and temperature were varied. The initial pH was set in the range of 5.9-7.3 in serum bottles using 2 N HCl and 2 N NaOH. The incubation temperature varied from 25 to 45°C. The xylose varied from 5 g/L to 30 g/L. All the experiments were done in triplicate. Culture broth from the serum bottles was taken for determining the pH, substrate utilization and fermentation end products. The volumetric H_2 production (ml/L) was calculated from the total gas production and the concentration of H_2 in the headspace. The hydrogen yield was calculated as mol/mol-xylose.

Analytical methods

The evolved biogas was collected in the headspace of a serum bottle. The total volume at each time interval was measured at room temperature by syringe. Hydrogen in the biogas was measured by gas meter (MX2100 OLDHAM, MULTIGAS Type MX2100) (O-Thong et al., 2008). The culture broth was centrifuged at 10,000×g for 10 min. Fermentation end products (volatile fatty acids (VFA) and ethanol) in the supernatant were determined by gas chromatography (GC HP 6890 series) equipped with a flame ionization detector (FID) and Innowax column (dimensions 30 m × 320 um × 0.25 um). The GC-FID conditions were set according to Miyazaki et al. (2008). The concentrations of xylose were determined using high performance liquid chromatography (HPLC) (Agilent 1200 series), equipped with Aminex HPX-87H ion exclusion column. The HPLC was operated with a refractive index detector under conditions of 0.005 M H_2SO_4 as effluent for 0.6 mL/min at 65°C (Delgenes et al., 1996). Data showed the average of three replicated assessments.

The Journal of Applied Science **Vol. 17 Special issue: 47-54 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S06

Results and discussion

Hydrogen production with various xylose concentrations

When xylose was used as the carbon source, the initial xylose concentration (5, 10, 15, 20, 25 and 30 g/L) had an influence on hydrogen production in the synthetic medium. The optimum xylose concentration for hydrogen production by *C. beijerinckii* PS-3 at 30ºC and pH 7 was found to be 10 g/L (Figure 1.), where the cumulative hydrogen production and hydrogen yield were 2675 ml/L and 1.79 mol/mol-xylose , respectively (Table 1). A significant decrease in the hydrogen yield was obtained when the xylose concentration was raised from 10 g/L to 15 g/L, beyond which the yields were not significantly different (1.33-1.37 mol/mol-xylose) at 15 to 25 g/L xylose. These results suggested that higher substrate concentrations may induce a shock load (van Ginkel et al., 2001). In addition, the metabolism for hydrogen production was related to that of volatile fatty acid production. The lower hydrogen yield at a higher substrate concentration suggested that the carbon flux was more directed to the production of reduced by-products such as organic acids and alcohol (van Ginkel et al., 2001). In view of the cumulative hydrogen production and hydrogen yield, 10 g/L xylose was selected for the following experiments.

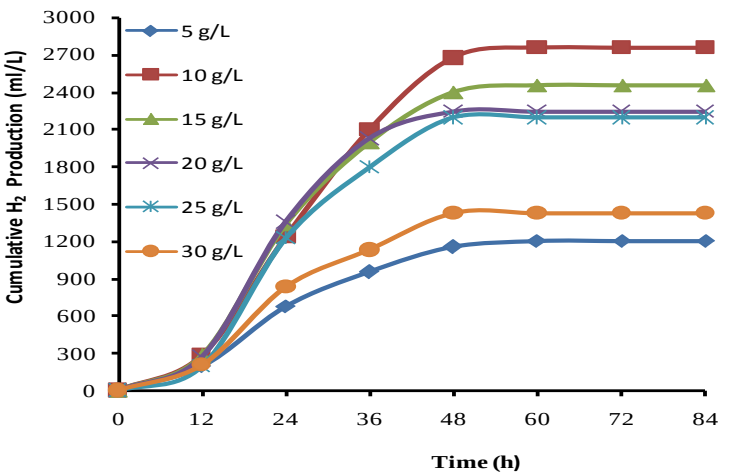


Figure 1. Effect of xylose concentrations on cumulative hydrogen production by *Clostridium berjerinckii* PS-3 in synthetic medium under anaerobic condition at 30ºC.

The Journal of Applied Science **Vol. 17 Special issue: 47-54 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S06

Table 1. Comparison of hydrogen production and soluble metabolites occurred during cultivation of *Clostridium berjerinckii* PS-3 in synthetic medium under different conditions

Conditions	H_2 (ml/L)	H_2 yield (mol/mol -xylose)	H_2 rate (ml/L/h)	Ethanol (mM)	Acetic acid (mM)	Butyric acid (mM)	Propionic acid (mM)
A: Xylose concentration (g/L)							
5	1150	1.54	24.0	22.8	35.2	28.6	6.5
10	2675	1.79	55.7	30.5	39.8	45.6	9.8
15	2399	1.34	50.0	26.5	34.7	39.2	8.1
20	2245	1.37	46.8	24.3	35.4	45.5	7.9
25	2191	1.33	45.6	24.3	35.4	45.5	7.9
30	1427	0.96	29.7	18.8	21.2	25.7	5.4
B: pH value							
5.9	2548	1.71	53.1	29.8	30.2	32.4	6.1
6.3	3120	2.09	65.0	33.6	34.9	44.5	9.4
6.7	2978	2.00	62.0	32.5	34.8	39.0	7.9
7.1	2365	1.59	49.3	22.5	33.7	42.8	8.2
7.3	2267	1.52	47.2	23.3	33.5	41.3	8.7
C: Temperature (^oC)							
25	2468	1.65	51.4	31.8	36.2	42.4	8.4
30	3254	2.18	67.8	35.3	39.9	48.7	9.5
37	2650	1.78	55.2	32.2	34.8	44.5	9.2
45	2345	1.57	48.9	24.5	33.7	32.6	7.9

Effect of initial pH

In order to determine the optimum pH of the medium, the cultivation of *C. beijerinckii* PS-3 at the initial pH of 4.7, 5.3, 5.9, 6.3, 6.7, 7.1, 7.3 and 8.1 with fixed xylose concentration of 10 g/L was investigated. The pH values were adjusted with 0.10 M phosphate buffer. As shown in Figure 2. The optimum pH range was 6.3-6.7 where the cumulative hydrogen production and hydrogen yield were not significantly different (2978-3120 ml/L and 2.00-2.09 mol/mol-xylose, respectively) (Table 1).

It should be emphasized that no hydrogen production was observed at pH 4.7 as the high acidity (low pH) had destroyed the cell's ability to maintain internal pH. This finding was consistent with most previous studies, in which the optimum pH value for hydrogen-producing system occurred in the pH range of 5.0-7.0 (Li and Fang, 2007). The results clearly demonstrated that the initial pH affects the hydrogen production considerably. The effect of pH may be on the inhibition of the hydrogenase activity that resulted from a higher pool of reduced ferredoxin in the cells which is reoxidized via NAD(P)H-ferredoxin oxidoreductase (Dabrock et al., 1992). In addition, this might be attributed to the carbon repression encountered during complex anaerobic metabolism (Venkata et al., 2007).

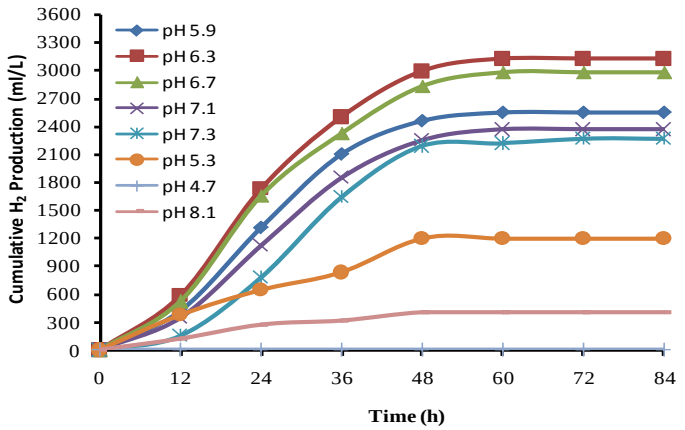


Figure 2. Effect of initial pH on cumulative hydrogen production by *Clostridium berjerinckii* PS-3 in synthetic medium under anaerobic condition at 30°C.

Effect of temperature

Temperature is often a critical factor in the performance of a fermentative process. The hydrogen production by *C. beijerinckii* PS-3 at temperatures ranging from 25°C to 45°C are given in Figure 3. and Table 1. The maximum cumulative hydrogen production (3254 ml/L) and the hydrogen yield (2.18 mol/mol-xylose) were obtained at 30°C. The hydrogen yields dropped by 18% and 28% compared to those at 37°C (1.78 mol/mol-xylose) and 45°C (1.57 mol/mol-xylose), respectively. These results demonstrated that *C. beijerinckii* PS-3 can grow and produce hydrogen both at 30°C and 45°C, hence it could be thermoterolant. At high temperatures, the denaturation rate of the enzymes in the fermentative hydrogen production process increased (Chittibabu et al., 2006), which could lead to the inactivation of hydrogenase and a decrease in the hydrogen yield.

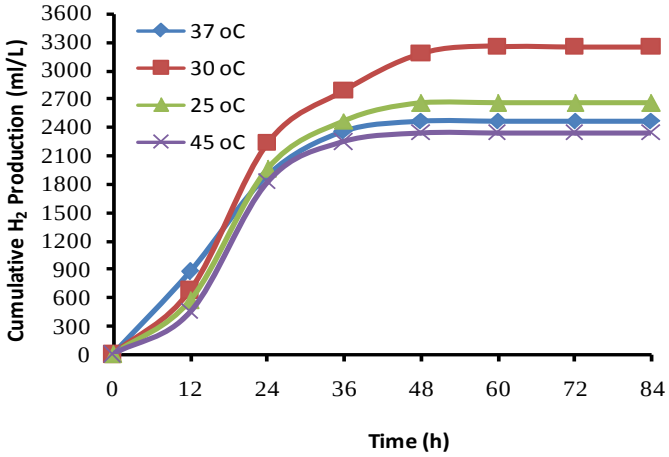


Figure 3. Effect of temperature on cumulative hydrogen production by *Clostridium berjerinckii* PS-3 in synthetic medium under anaerobic condition at 30°C.

The Journal of Applied Science **Vol. 17 Special issue: 47-54 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S06

The hydrogen production capability of *C. beijerinckii* PS-3 was compared with those of other fermentative hydrogen-producing microorganisms (Table 2). Due to the drastic differences in conditions, substrate and other process conditions, it was difficult to compare the hydrogen yields. Nevertheless, the characteristic values of hydrogen production by *C. beijerinckii* PS-3 were 1.1-4.1 fold higher than most other hydrogen-producing strains including mixed cultures. The maximum hydrogen yield of this strain using xylose was 15% lower than that obtained from *C. beijerinckii* Fanp3 using glucose (Pan et al., 2008).

Table 2. Comparison of *Clostridium berjerinckii* PS-3 with various fermentation hydrogen-producing microorganisms.

Microorganisms	Xylose concentration (g/L)	Maximum H_2 yield (mol/mol-sugar)	References
Enterobacter sp. CN1	16.15	2.00	Datar et al. (2007)
Clostridium sp. HR-1	12	1.63	Long et al. (2010)
Enterobacter cloacae IIT-BT 08	10	0.95	Xu et al. (2010)
Clostridium tyrobutyridum ATCC 25755	10	0.77	Lo et al. (2008)
Clostridium beijerinckii PS-3	10	2.18	This study

Conclusion

Optimization for hydrogen production from *Clostridium beijerinckii* PS-3 revealed that the optimal initial pH was 6.3–6.7 at 30±2°C and 10 g/L xylose. Under this condition, the maximum cumulative hydrogen production of 3254 ml/L and the maximum hydrogen yield of 2.18 mol/mol-xylose were achieved. Therefore, *C. beijerinckii* PS-3 is a potential candidate for fermentative hydrogen production from xylose and ability application from xylose in the lignocellulosic hydrolysate.

Acknowledgements

This work was financially supported by a scholarship from the Office of the Higher Education Commission. The experiments were conducted in Environmental Science laboratory at Suratthani Rajabhat University.

References

Chen, W. M., Tseng, Z. J., Lee, K. S. & Chang, J.S. (2005). Fermentative hydrogen production with *Clostridium butyricum* CGS5 isolated from anaerobic sewage sludge. *International Journal of Hydrogen Energy*, *30*, 1063-1070. https://doi.org/10.1016/j.ijhydene. 2004. 09.008

Chimtong, S., Soontorngun, N., Tachaapaikoon, C., Pason, P., Kyu, K. L. & Ratanakhanokchai, K. (2009). Sugar production from agricultural residues by Xylanolytic-Cellulolytic enzyme from *Thermoanaerobacterium* sp. strain NOI-19. *Agricultural Scicene Journal*, *40*, 373-376.

Chin, H. L., Chen, Z. S. & Chou, C. P. (2003). Fed-batch operation using *Clostridium acetobutylicum* suspension culture as biocatalyst for enhancing hydrogen production. *Biotechnology Progress*, *19*, 383-388. https://doi:10.1021/bp0200604

Chittibabu, G., Nath, K. & Das, D. (2006). Feasibility studies on the fermentative hydrogen production by recombinant *Escherichia coli* BL21. *Process Biochemistry*, *41*, 682-688. https://10.1016/j.procbio.2005.08.020

Chong, M. L., Sabaratnam, V., Shirai, Y. & Hassan, M.A. (2009a). Biohydrogen production from biomass and industrial wastes by dark fermentation. *International Journal of Hydrogen Energy, 34*, 3277-3287. https://doi.org/10.1016/j.ijhydene.2009.02.010

Chong, M. L., Rahman, N. A. A., Rahim, R. A., Aziz, S. A., Shirai, Y. & Hassan, M. A. (2009b). Optimization of biohydrogen production by *Clostridium butyricum* EB6 from palm oil mill effluent using response surface methodology. *International Journal of Hydrogen Energy*, *34*, 7475-7482. https://doi.org/10.1016/j.ijhydene.2009.05.088

Dabrock, B., Bahl, H. & Gottschalk, G. (1992). Parameters affecting solvent production by *Clostridium pasteurianum*. *Applied and Environmental Microbiology*, *58*, 1233-1239.

Datar, R., Huang, J., Maness, P., Mohagheghi, A., Czernik, S. & Chormet, E. (2007). Hydrogen production from the fermentation of corn stover biomass pretreated with a steam-explosion process. *International Journal of Hydrogen Energy*, *32*, 932-939. https://doi.org/10.1016/j.ijhydene.2006.09.027

Delgenes, J. P., Moletta, R. & Navarro, J. M. (1996). Effects of lignocellulose degradation products on ethanol fermentations of glucose and xylose by *Saccharomyces cerevisiae*, *Zymomonas mobilis*, *Pichia stipitis*, and *Candida shehae*. *Enzyme and Microbial Technology*, *9*, 220-225.

Fangkum, A. & Reungsang, A. (2011). Biohydrogen production from sugarcane bagasse hydrolysate by elephant dung: Effects of initial pH and substrate concentration. *International Journal of Hydrogen Energy*, *36*, 8687-8696. https://doi.org/10.1016/j.ijhydene.2010.05.119

Ishikawa, M., Yamamura, S., Takamura, Y., Sode, K., Tamiya, E. & Tomiyama, M. (2006). Development of a compact high-density microbial hydrogen reactor for portable bio-fuel cell system. *International Journal of Hydrogen Energy*, *31*, 1484-1489. https://doi.org/10.1016/j.ijhydene.2006.06.014

Lavarack, B. P., Griffin, G. J. & Rodman, D. (2002). The acid hydrolysis of sugarcane bagasse hemicellulose to produce xylose, arabinose, glucose and other products. *Biomass and Bioenergy*, *23*, 367-380. https://doi.org/10.1016/S0961-9534(02)00066-1

Li, C. L. & Fang, H. H. P. (2007). Fermentative hydrogen production from wastewater and solid wastes by mixed cultures. *Critical Reviews in Environmental Science and Technology*, *37*, 1-39. https://doi.org/10.1080/10643380600729071

Lin, C.-Y., Wu, C.-C. & Hung, C.-H. (2008). Temperature effects on fermentative hydrogen production from xylose using mixed anaerobic cultures. *International Journal of Hydrogen Energy*, *33*, 43-50. https://doi.org/10.1016/j.ijhydene.2007.09.001

Long, C., Cui, J., Liu, Z., Liu, Y., Long, M. & Hu, Z. (2010). Statistical optimization of fermentative hydrogen production from xylose by newly isolated *Enterobacter* sp. CN1. *International Journal of Hydrogen Energy*, *35*, 6657-6664. https://doi:10.1016/j.ijhydene.2010.04.094

Miyazaki, K., Irbis, C., Takada, J. & Matsuura, A. (2008). An ability of isolated strains to efficiently cooperate in ethanolic fermentation of agricultural plant refuse under initially aerobic thermophilic conditions: Oxygen deletion process appended to consolidated bioprocessing (CBP). *Bioresource Technology*, *99*, 1768-1775. https://doi.org/10.1016/j.biortech.2007.03.045

Noparat, P., Prasertsan, P. & O-Thong, S. (2011). Isolation and characterization of high hydrogen-producing strain *Clostridium beijerinckii* PS-3 from fermented oil palm sap. *International Journal of Hydrogen Energy*, *36*, 14086-14092. https://doi.org/10.1016/j.ijhydene.2011.04.143

O-Thong, S., Prasertsan, P., Intrasungkha, N., Dhamwichukorn, S. & Birkeland, N.-K. (2007). Improvement of biohydrogen production and treatment efficiency on palm oil mill effluent with nutrient supplementation at thermophilic condition using an anaerobic sequencing batch reactor. *Enzyme and Microbial Technology*, *41*, 583-590. https://doi.org/10.1016/j.enzmictec.2007.05.002

O-Thong, S., Prasertsan, P., Karakashev, D. & Angelidaki, I. (2008). Thermophilic fermentative hydrogen production by the newly isolated *Thermoanaerobacterium thermosaccharolyticum* PSU-2. *International Journal of Hydrogen Energy*, *33*, 1204-1214. https://doi.org/10.1016/j.ijhydene.2007.12.015

Pan, C. M., Fan, Y. T., Zhao, P. & Hou, H. W. (2008). Fermentative hydrogen production by the newly isolated *Clostridium beijerinckii* Fanp3. *International Journal of Hydrogen Energy*, *33*, 5383-5391. https://doi.org/10.1016/j.ijhydene.2008.05.037

Prasertsan, P., O-Thong, S. & Birkeland, N.-K. (2009). Optimization and microbial community analysis for production of biohydrogen from palm oil mill effluent by thermophilic fermentative process. *International Journal of Hydrogen Energy*, *34*, 7448-7459. https://doi.org/10.1016/j.ijhydene.2009.04.075

van Ginkel, S., Sung, S. & Lay, J.-J. (2001). Biohydrogen production as a function of pH and substrate concentration. *Environmental Science & Technology*, *35*, 4726-4730. doi: 10.1021/es001979r

Venkata, M. S., Vijaya, B. Y., Murali, K. P., Chandresekhara, R. N., Lalit, B. V., Sarma, P. N. et al. (2007). Biohydrogen production from chemical wastewater as substrate by selectively enriched anaerobic mixed consortia: influence of fermentation pH and substrate composition. *International Journal of Hydrogen Energy*, *32*, 2286-2295. https://doi.org/10.1016/j.ijhydene.2007.03.015

Xing, D. F., Ren, N. Q, Wang, A. J., Li, Q. B., Feng, Y. J. & Ma, F. (2008). Continuous hydrogen production of auto-aggregative *Ethanoligenens harbinense* YUAN-3 under non-sterile condition. *International Journal of Hydrogen Energy*, *33*, 1489-1495. https://doi.org/10.1016/j.ijhydene.2007.09.038

Xu, J. F., Ren, N. O., Wang, A. J., Qiu, J., Zhao, Q. L., Feng, Y. J. et al. (2010). Cell growth and hydrogen production on the mixture of xylose and glucose using a novel strain of *Clostridium* sp. HR-1 isolated from cow dung compost. *International Journal of Hydrogen Energy*, *35*, 13467-13474. https://doi.org/10.1016/j.ijhydene.2009.11.129

Yu, H., Zhu, Z., Hu, W. & Zhang, H. (2002). Hydrogen production from rice winery wastewater in an upflow anaerobic reactor by using mixed anaerobic cultures. *International Journal of Hydrogen Energy*, *27*, 1359-65. https://doi.org/10.1016/ S0360-3199(02)00073-3

Zhu, Y. & Yang, S. T. (2004). Effect of pH on metabolic pathway shift in fermentation of xylose by *Clostridium tyrobutyricum*. *Journal of Biotechnology*, *110*, 143-157. doi: 10.1016/j.jbiotec.2004.02.006

The Journal of Applied Science **Vol. 17 Special issue: 55-62 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S07
ISSN 1513-7805 (Printed in Thailand) ISSN 2586-9663 (Online)

Research Article

Transformation of artemisinin to deoxyartemisinin by *Aspergillus terricola* TISTR 3019

Wannipa Pipatsamut[1], Sirirat Chancharunee[1], Nisa Chawapun[2] and Angkana Wipatanawin[1,3]*

[1] Bio-organic Synthesis and Molecular Biochemistry, Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, 50200, Thailand
[2] Department of Radiology, Faculty of Medicine, Chiang Mai University, Chiang Mai, 50200, Thailand
[3] Faculty of Agro-Industry, King Mongkut's Institute of Technology Ladkrabang, Bangkok 10520, Thailand
***E-mail**: angkana.wi@kmitl.ac.th

Abstract

Artemisinin, a sesquiterpene lactone endoperoxide compound, was transformed using *Aspergillus terricola* TISTR 3019. The transformed product was isolated from the culture broth and identified as deoxyartemisinin (yield 21%). Investigation of the biological activities of deoxyartemisinin was carried out *in vitro* for both antimicrobial and cytotoxic activities. Deoxyartemisinin showed more effective than artemisinin in inhibitory of cell growth against human mouse melanoma B16F10 (ATCC-CRL-6475) cell line. However, the transformed product derived from *A. terricola* TISTR 3019 did not exhibit antimicrobial activities against *Salmonella typhimurium* TISTR 292, *Escherichia coli* TISTR 780, *Candida albicans* BCC 5390 and *Aspergillus niger* TISTR 3254.

Keywords: artemisinin, transformation, *Aspergillus terricola*, deoxyartemisinin, cytotoxicity, antimicrobial activity

Introduction

Artemisinin and its derivatives are well-known as antimalarial agents that are effective against multidrug-resistant *Plasmodium falciparum* (Meshnick, 2002). Artemisinin is a sesquiterpene lactone endoperoxide compound occurring in the herb *Artemisinin annua.* It has low solubility in both water and oil, leading to certain limitations in its clinical application (Meshnick et al., 1996). Microbial transformation has become the focus of attempts to modify structures either to enhance their biological activities or to develop new bioactive substances. This transformation has the ability to overcome the inaccessibility through chemical reaction, requires mild conditions, and produces less pollution (Loughlin, 2000). Additionally, this approach can also assist in predicting how drugs are metabolized in mammal species (Asha & Vidyavathi, 2009). Derivatives of artemisinin have been developed successfully via the microbial transformation approach in various studies. A number of these derivatives show considerable potential, particularly those transformed using fungi. For example, *Mucor polymorphosporus, Cunninghamella elegans* ATCC 9245*, Umbelopsis ramanniana,* and *Aspergillus niger* VKM F-1119 are able to transform artemisinin into 9β-hydroxyartemisinin which cannot be prepared

The Journal of Applied Science **Vol. 17 Special issue: 55-62 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S07

through a chemical approach (Parshikov et al., 2006; Parshikov et al., 2005; Parshikov et al., 2004; Zhan et al., 2002; Zhan et al., 2017). The hydroxygenated compounds, 9β-hydroxyartemisinin and 10β-9α-hydroxyartemisinin, demonstrate better anti-malarial activity and water solubility when compared with artemisinin (Avery et al., 1999; Parshikov, et al., 2005; Zhan, et al., 2017). Goswami et al. (2010) also revealed that *Penicillium simplissimum* transforms artemisinin into 3β-acetoxyartemisinin. This artemisinin derivative shows particularly inhibitory tendency toward colon HCT-15, lung A549, and neuroblastoma IMR-32 cell lines. Several fungi, such as *Penicillium chrysogenum* ATCC 9480, *M. polymorphosporus*, *Aspergillus flavus, A. niger* and *Rhizopus stolonifer*, show the ability to deoxygenate artemisinin (Parshikov, et al., 2005; Gaur et al., 2014; Lee et al., 1989; Srivastava et al., 2009). The deoxygenated artemisinin was found to exhibit higher antibacterial activity against *Staphylococcus aureus*, *Staphylococcus epidermidis,* and *Streptococcus mutans* compared to artemisinin (Srivastava, et al., 2009).

In this study, the transformation of artemisinin into its derivatives using *Aspergillus terricola* was assessed*. A. terricola* is a typical soil filamentous fungus, which can produce a wide range of enzymes such as inulase, diastase, invertase, maltase, alcoholoxydase, lipase, protease, amidase and xylanase (Michelin et al., 2011; Scales, 1914). *A. terricola* is a bioconverter in the transformation of bicyclo[3.2.0]hept-2-en-6-one to (−)-(1*S*, 5*R*)-lactone which is used for the synthesis of prostaglandins (Borges et al., 2009), and it is also capable of degrading endosulfan from contaminated soil (Hussain et al., 2007). *A. terricola* was selected for this study on account of the characteristics described. The first stage of the study involved the artemisinin transformation and was followed by *in vitro* tests for cytotoxicity and antimicrobial activities in order to evaluate the effect of modified-artemisinin on biological activities.

Materials and methods

Chemicals and reagents

Chemicals and reagents used for biotransformation process were methanol (Merck, Germany), ethyl acetate (Fisher Scientific, England), dichloromethane (Fisher Scientific, England), hexane (Carlo Erba, Italy). For *in vitro* cytotoxicity and antimicrobial assays involved the use of RPMI 1640 medium, fetal bovine serum (FBS), glutamine, penicillin, streptomycin, trypsin-EDTA (Invitrogen, England), 4)-3,-5dimethylthiazol--2yl)-2,-5 diphenyltetrazolium bromide or MTT, dimethylsulfoxide (Sigma-Aldrich, USA), agar, nutrient agar or NA, Mueller Hinton agar or MHA (Criterion, USA), Luria-Bertani or LB, Sabouraud dextrose broth or SDB (Scharlau, Spain), potato dextrose broth or PDB (Difco, France), ampicillin and amphotericin B (Applichem, Germany).

Microorganism methods, maintenance, and preservation

Aspergillus terricola TISTR 3019, *Candida albicans* BCC 5390 and *Aspergillus niger* TISTR 3254 were maintained on potato dextrose agar (PDA) slants, while a slant of LB was

Vol. 17 Special issue: 55-62 [2018]
doi: 10.14416/j.appsci.2018.02.S07

used for *Escherichia coli* TISTR 780 and a NA slant was used for *Salmonella typhimurium* TISTR 292.

Cell lines and cultivation

The mouse melanoma B16F10 and normal mouse fibroblast L929 cell lines were obtained from the American Type Culture Collection (ATCC). All cell lines were cultured at 37°C in an atmosphere of 5% carbon dioxide (Sanyo, Japan) in RPMI 1640 containing 10% (v/v) FBS and 1% penicillin (100 IU/mL) and streptomycin (100 mg/mL).

Biotransformation technique

The spore suspension of *A. terricola* TISTR 3019 was inoculated to SDB at final concentration 2×10^6 spore/mL and incubated at 37°C for 48 h on the 160 rpm rotary shaker (N-Biotek, Taiwan). The artemisinin at concentration 25 mg/mL was then introduced to the culture flask to achieve a final concentration of 0.5 mg/mL. The culture was grown for a further period of 96 h at 37°C on the rotary shaker. In the experiments, a transformed medium without the fungal culture was also prepared to use as the control. In the meantime, the substrate control was prepared under the same experimental conditions without adding artemisinin.

Isolation and identification of the transformed product

The cultured medium and mycelia were separated by filtration. The filtrate was extracted with ethyl acetate and evaporated *in vacuo* (Buchi, Switzerland). The residue was purified using column chromatography eluted with increasing proportions of methanol, which is, 1, 3 and 5% (v/v), respectively, in dichloromethane. Fractions containing the transformed product were collected and detected on TLC plates (Merck, Germany) using ethyl acetate/hexane (1:1 v/v) as the mobile phase. The TLC plate was developed with artemisinin reagent at a high temperature. The purified transformed product was further characterized by Mel-Temp melting point apparatus, IR, ESI-MS, ^{1}H-NMR, and ^{13}C-NMR. Deoxyartemisinin (yield = 21%), mp 110-111 °C, IR (KBr): 2941, 1748, 1385, 1140, 1016 cm^{-1}; ESI-MS *m/z*: 267.1617 $[M]^+$, calcd. for $C_{15}H_{23}O_4$: 267.3407; ^{1}H-NMR (400 MHz, $CDCl_3$) δ_H: 0.96 (3H, *J* = 5.6 Hz, H-14), 1.03 (1H, m, H-8a), 1.15 (1H, m, H-9a), 1.22 (3H, d, *J* = 7.2 Hz, H-13), 1.27 (1H, m, H-2a), 1.29 (1H, m, H-10), 1.30 (1H, m, H-1), 1.55 (3H, s, H-15), 1.65 (1H, m, H-3a), 1.77 (1H, m, H-3b), 1.78 (1H, m, H-9b), 1.92 (1H, m, H-2b), 1.95 (1H, m, H-8b), 2.03 (1H, m, H-7), 3.20 (2H, dq, H-11), and 5.71 (1H, s, H-5); ^{13}C-NMR (100 MHz, $CDCl_3$) δ_C: 12.8(C-13), 18.8(C-14), 22.3(C-2), 23.7(C-8), 24.2(C-15), 33.0(C-11), 33.7(C-9), 34.2(C-3), 35.6(C-10), 42.7(C-7), 44.9(C-1), 82.6(C-6), 99.9(C-5), 109.4(C-4), 172.1(C-12).

In vitro cytotoxicity assay

The effect of deoxyartemisinin on the growth of mouse melanoma B16F10 and normal mouse fibroblast L929 cell lines were determined by using MTT assay (Mosmann, 1983). The process required the seeding of cell lines at a 10,000 cell/well in 96-well plates (Corning, USA) followed by culturing in a 5% carbon dioxide atmosphere for 48 h at 37°C before washing in

Vol. 17 Special issue: 55-62 [2018]
doi: 10.14416/j.appsci.2018.02.S07

phosphate buffer saline (PBS) at pH 7.4. The RPMI 1640 which contained concentrations of 0.0001 to 2.5 mg/mL artemisinin or deoxyartemisinin was introduced and incubated for a further 48 h, while 20 μL of MTT solution (100 μg/mL in PBS) was introduced to the individual well for incubation extending an additional 4 h. The formazan product was solubilized by the addition of 100 μL of dimethylsulfoxide to the medium containing sample. The reduction in the amount of formazan product by the mitochondrial dehydrogenase was determined by measuring in a microplate reader (Biotek, Taiwan) at absorbance at 550 nm. The cytotoxic dose (CD_{50}) of the transformed product was calculated and then compared with the value obtained from the untreated sample. All experiments were performed in triplicate and repeated two times in an independent experiment.

Antimicrobial assays

Agar disc methods were used to determine the antimicrobial activity of the transformed product (Bizani & Brandelli, 2002). The purified transformed product 18 mg was dissolved in 3 mL of 5% (v/v) dimethylsulfoxide, diluted at the final concentration of 1, 2 and 3 mg/mL, respectively. The diluted transformed product was applied on discs on MHA plates previously inoculated with a swab of *S. typhimurium* TISTR 292, *E. coli* TISTR 780 and *C. albicans* BCC 5390 which corresponded to 0.5 McFarland turbidity standard solution or a spot of *A. niger* TISTR 3254. All plates were incubated at 37°C for 24 h. The clear zones of inhibition were observed and the diameter of the clear zones were measured and compared with that of ampicillin and amphotericin-B.

Statistical analysis

Data values of cytotoxicity were given as mean ± standard deviation (SD). Statistical differences were analyzed by one-way analysis of variance (ANOVA) carried out using SPSS 11.5 for windows (SPSS Inc, Chicago, USA). Scheffe and Tamhane tests were used as a post-hoc method to determine differences among the groups of LD_{50} or IC_{50}. Differences were considered statistically significant when $p < 0.05$.

Results and discussion

The isolation and identification of transformed product in the culture broth of *A. terricola* TISTR 3019

A. terricola TISTR 3019 was used in the production of artemisinin derivatives. The resulting transformed product was then extracted and purified from the culture broth for determining the chemical structure. The ESI-MS, ^{1}H-, and ^{13}C-NMR spectra suggesting that an oxygen atom had been removed from the endoperoxide position of artemisinin molecule. Further examination through the FT-IR spectrum found the important absorptions band of the transformed product were 2941 (C-H), 1748 (C=O), 1385 (C-H), 1140 and 1017 (C-O-C) cm^{-1}, which revealed the existence of lactone carbonyl group or C=O at υ_{max} 1748 cm^{-1} and absence of the C-O-O-C at υ_{max} 1117 cm^{-1}, which was presented in artemisinin. The resulting non-endoperoxide derivative had a melting point within the range from 110-111 °C. On the whole,

The Journal of Applied Science **Vol. 17 Special issue: 55-62 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S07

the transformed product was identified as deoxyartemisinin (Figure 1) which was reported to be the transformed product of artemisinin by *Nocardia coralline* ATCC 19070, *P. chrysogenum* ATCC 9480, *M. polymorphosporus*, *A. niger, A. flavus*, and *R. stolonifer* (Gaur, et al., 2014; Lee, et al., 1989; Parshikov, et al., 2005; Srivastava, et al., 2009). The yield of purified transformed product was 21% that was higher than deoxyartemisinin production by *N. coralline* ATCC 19070, *P. chrysogenum* ATCC 9480, *M. polymorphosporus*, *A. niger* and *R. Stolonifer* (Gaur, et al., 2014; Lee, et al., 1989; Parshikov, et al., 2005).

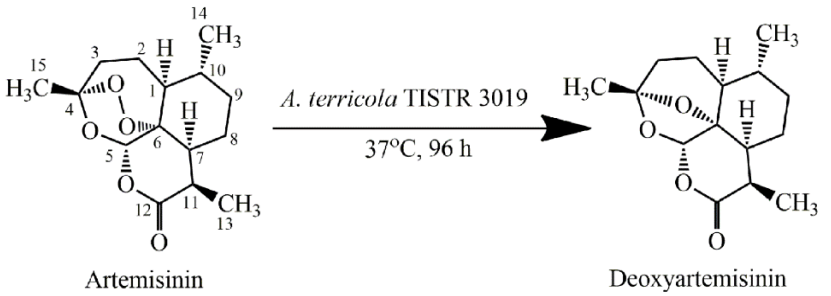


Figure 1. The biotransformation of artemisinin to deoxyartemisinin by *A. terricola* TISTR 3019.

In vitro cytotoxicity

To date, cytotoxicity for artemisinin and deoxyartemisinin towards mouse melanoma B16F10 and normal mouse fibroblast cell lines remain undetermined. Accordingly, the cytotoxicity of artemisinin and the deoxygenated artemisinin against mouse melanoma B16F10 and normal mouse fibroblast cell lines was evaluated by determining the reduction in mitochondrial dehydrogenase of the treated cells. An *in vitro* cytotoxicity study, deoxyartemisinin showed the cell growth inhibition at the CD_{50} value of 1.621 ± 0.095 mg/mL for normal mouse fibroblast L929 and 0.585 ± 0.058 mg/mL for mouse melanoma B10F16 cells (Table 1). Notably, deoxyartemisinin was shown to be 1.5 times less cytotoxic towards normal mouse fibroblast L929 cell lines than artemisinin, but considerably more effective in inhibiting the growth of mouse melanoma B16F10 cells (Table 1). These findings suggest that the endoperoxide bridge might not be essential for cytotoxicity in mouse melanoma B16F10 cell lines. This confirms the work of Beekman, who found that the presence of the endoperoxide in artemisinin and its derivatives have a minimal influence upon cytotoxicity in either the CFU-GM progenitor or EAT cells (Beekman et al., 1998). However, the cytotoxic mechanisms of artemisinin and deoxyartemisinin have not yet been precisely determined.

Antimicrobial assays

The capacity of deoxyartemisinin to inhibit Gram-positive bacteria, *S. aureus*, *S. epidermidis* and *S. mutans*, represented an improvement over artemisinin (Srivastava, et al., 2009). However, there remains a gap in the literature regarding the inhibitory effects of deoxyartemisinin against Gram-negative bacteria. It was therefore of interest to assess the

The Journal of Applied Science **Vol. 17 Special issue: 55-62 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S07

antimicrobial activity of deoxyartemisinin towards *S. typhimurium* TISTR 292 and *E. coli* TISTR 780 which is pathogenic Gram-negative bacteria. The results from this research demonstrated that deoxyartemisinin could not inhibit of *S. typhimurium* TISTR 292, *E. coli* TISTR 780, *C. albicans* BCC 5390 and *A. niger* TISTR 3254 for all concentrations up to 3 mg/mL (Figure 2).

Table 1. The *in vitro* cytotoxicity of deoxyartemisinin, artemisinin and doxorubicin against normal mouse fibroblast L929 and mouse melanoma B16F10 cell lines

Compounds	CD_{50} (mg/mL)*	
	L929	B16F10
Artemisinin	1.108±0.130[a]	0.871±0.070[a]
Deoxyartemisinin	1.621±0.095[b]	0.585±0.058[b]
Doxorubicin	0.002±0.000[c]	0.001±0.000[c]

*Data are representative of mean±SD of triplicate experiments.

[a-c] Means within a column with different superscript are considered significantly different ($p < 0.05$).

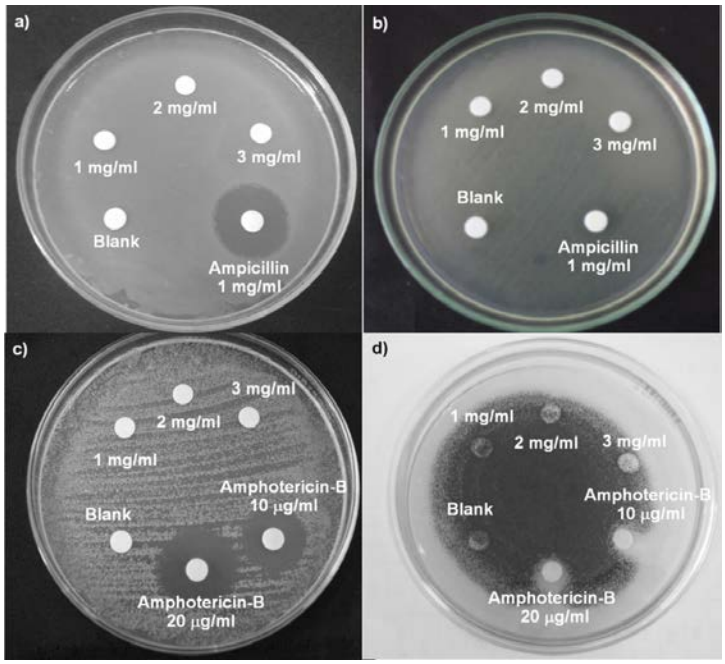


Figure 2. Antimicrobial activity of deoxyartemisinin determined by agar disc diffusion assay against, *E.coli* TISTR 780 (a), *S. typhimurium* TISTR 292 (b), *C. albicans* BCC 5390 (c), and *A. niger* TISTR3254 (d).

Vol. 17 Special issue: 55-62 [2018]
doi: 10.14416/j.appsci.2018.02.S07

Conclusion

The findings demonstrate that *A. terricola* TISTR 3109 was able to produce derivatives of artemisinin. The transformed product extracted from the culture broth was identified as deoxyartemisinin. The *in vitro* biological activity of deoxyartemisinin in terms of cytotoxicity activity indicated that deoxyartemisinin was significantly effective than artemisinin in B16F10 cell line. It was also found that the deoxygenation of artemisinin served to alter the cytotoxic capabilities of artemisinin towards the normal mouse fibroblast L929 and mouse melanoma B10F16 cell lines. Furthermore, the assessment of deoxyartemisinin on antimicrobial activity did not exhibit antimicrobial activities against *S. typhimurium* TISTR 292, *E. coli* TISTR 780, *C. albicans* BCC 5390 and *A. niger* TISTR 3254. In conclusion, the findings of this study have provided insights into a number of areas regarding the production and biological applications of deoxyartemisinin which the authors sincerely hope others will find to be of use and interest.

Acknowledgement

This work was supported by grants from the Chiang Mai University (Young Scientist Program) and Graduate School, Chiang Mai University, Thailand.

References

Asha, S., & Vidyavathi, M. (2009). *Cunninghamella*--a microbial model for drug metabolism studies--a review. *Biotechnology Advances*, *27*(1), 16-29. doi: 10.1016/j.biotechadv.2008.07.005S0734-9750(08)00087-6 [pii]

Avery, M. A., Alvim-Gaston, M., & Woolfrey, J. R. (1999). Synthesis and structure-activity relationships of peroxidic antimalarials based on artemisinin. *Advances in Medicinal Chemistry*, *4*, 125-217. https://doi.org/10.1016/S1067-5698(99)80005-4

Beekman, A. C., Wierenga, P. K., Woerdenbag, H. J., Van Uden, W., Pras, N., Konings, A. W., et al. (1998). Artemisinin-derived sesquiterpene lactones as potential antitumour compounds: cytotoxic action against bone marrow and tumour cells. *Planta Medica*, *64*(7), 615-619. doi: 10.1055/s-2006-957533

Bizani, D., & Brandelli, A. (2002). Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. Strain 8 A. *Journal of Applied Microbiology*, *93*(3), 512-519. doi: 10.1046/j.1365-2672.2002.01720.x

Borges, K. B., Borges, W. d. S., Durán-Patrón, R., Pupo, M. T., Bonato, P. S., & Collado, I. G. (2009). Stereoselective biotransformations using fungi as biocatalysts. *Tetrahedron: Asymmetry*, *20*(4), 385-397. https://doi.org/10.1016/j.tetasy.2009.02.009

Gaur, R., Darokar, M. P., Ajayakumar, P. V., Shukla, R. S., & Bhakuni, R. S. (2014). *In vitro* antimalarial studies of novel artemisinin biotransformed products and its derivatives. *Phytochemistry*, *107*(Supplement C), 135-140. https://doi.org/10.1016/j.phytochem.2014.08.004

Goswami, A., Saikia, P. P., Barua, N. C., Bordoloi, M., Yadav, A., Bora, T. C., et al. (2010). Bio-transformation of artemisinin using soil microbe: Direct C-acetoxylation of artemisinin at C-9 by *Penicillium simplissimum*. *Bioorganic & Medicinal Chemistry Letters*, *20*(1), 359-361. https://doi.org/10.1016/j.bmcl.2009.10.097

Hussain, S., Arshad, M., Saleem, M., & Zahir, Z. A. (2007). Screening of soil fungi for in vitro degradation of endosulfan. *World Journal of Microbiology & Biotechnology*, *23*(7), 939-945. doi: 10.1007/s11274-006-9317-z

Lee, I. S., elSohly, H. N., Croom, E. M., & Hufford, C. D. (1989). Microbial metabolism studies of the antimalarial sesquiterpene artemisinin. *Journal of Natural Products*, *52*(2), 337-341.

Loughlin, W. A. (2000). Biotransformations in organic synthesis. *Bioresource Technology*, *74*(1), 49-62. https://doi.org/10.1016/S0960-8524(99)00145-5

Meshnick, S. R. (2002). Artemisinin: mechanisms of action, resistance and toxicity. *International Journal for Parasitology*, *32*(13), 1655-1660. https://doi.org/10.1016/S0020-7519(02)00194-7

Meshnick, S. R., Taylor, T. E., & Kamchonwongpaisan, S. (1996). Artemisinin and the antimalarial endoperoxides: from herbal remedy to targeted chemotherapy. *Microbiological Reviews*, *60*(2), 301-315.

Michelin, M., Polizeli Mde, L., Silva, D. P., Ruzene, D. S., Vicente, A. A., Jorge, J. A., et al. (2011). Production of xylanolytic enzymes by *Aspergillus terricola* in stirred tank and airlift tower loop bioreactors. *Journal of Industrial Microbiology and Biotechnology*, *38*(12), 1979-1984. doi: 10.1007/s10295-011-0987-7

Mosmann, T. (1983). Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. *Journal of Immunological Methods*, *65*(1-2), 55-63. https://doi.org/10.1016/0022-1759(83)90303-4

Parshikov, I. A., Miriyala, B., Muraleedharan, K. M., Avery, M. A., & Williamson, J. S. (2006). Microbial transformation of artemisinin to 5-hydroxyartemisinin by *Eurotium amstelodami* and *Aspergillus niger*. *Journal of Industrial Microbiology and Biotechnology*, *33*(5), 349-352. doi: 10.1007/s10295-005-0071-2

Parshikov, I. A., Miriyala, B., Muraleedharan, K. M., Illendula, A., Avery, M. A., & Williamson, J. S. (2005). Biocatalysis of the antimalarial artemisinin by *Mucor ramannianus*. strains. *Pharmaceutical Biology*, *43*(7), 579-582. doi: 10.1080/13880200500301597

Parshikov, I. A., Muraleedharan, K. M., Avery, M. A., & Williamson, J. S. (2004). Transformation of artemisinin by *Cunninghamella elegans*. *Applied Microbiology and Biotechnology*, *64*(6), 782-786 doi: 10.1007/s00253-003-1524-z

Scales, F. M. (1914). The enzymes of *Aspergillus terricola*. *Journal of Biological Chemistry*, *19*(4), 459-472.

Srivastava, S., Luqman, S., Faima, A., Darokar, M. P., Negi, A. S., Kumar, J. K., et al. (2009). Biotransformation of artemisinin mediated through fungal strains for obtaining derivatives with novel activities. *Scientia Pharmaceutica*, *77*(1), 87-96. http://dx.doi.org/ 10.3797/scipharm.0803-15

Zhan, J. X., Zhang, Y. X., Guo, H. Z., Han, J., Ning, L. L., & Guo, D. A. (2002). Microbial metabolism of artemisinin by *Mucor polymorphosporus* and *Aspergillus niger*. *Journal of Natural Products*, *65*(11), 1693-1695. doi: np020113r

Zhan, Y., Wu, Y., Xu, F., Bai, Y., Guan, Y., Williamson, J. S., et al. (2017). A novel dihydroxylated derivative of artemisinin from microbial transformation. *Fitoterapia*, *120*, 93-97. https://doi.org/10.1016/j.fitote.2017.05.015

The Journal of Applied Science **Vol. 17 Special issue: 63-72 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S08
ISSN 1513-7805 (Printed in Thailand) ISSN 2586-9663 (Online)

Research Article

Anti-aging efficacy of Thai red rice callus cosmetic product

Wannisa Vichit[1], Nisakorn Saewan[1*] and Thanon Prinyarux[1]

[1] School of Cosmetic Science, Mae Fah Luang University, Chiang Rai, 57100
***E-mail**: nisakorn@mfu.ac.th

Abstract

Plant cell culture is an innovative technology to produce bioactive substances and anti-aging agents for cosmetic products. Red rice (*Oryza sativa*) is a special rice cultivar in Thailand that has high procyanidin content in seed coats. In this study, the red rice callus were cultured from rice seeds and extracted with ethanol. The extract contained 0.42 mg GAE/ml for phenolic compounds and 0.21 mg Glu/ml for amino acids. The anti-aging properties of the induced rice callus extract in solution form was evaluated on 28 volunteers, aged 30 – 55 years. The volunteers were separated into 3 groups; placebo (n = 8), 5 % of the induced stem cell extract cream (n = 10), and 5 % of the commercial red rice stem cell extract cream (n = 10). The tested creams were applied on facial skin twice a day for 12 weeks. Skin color, hydration and elasticity were assessed with Mexameter, Corneometer, and Cutometer, respectively. The results showed that anti-aging efficacies of the group treated red rice stem cell extract cream were greater than those of the commercial cream group, which were skin whitening (38.57%, 29.85%), skin moisture (74.62%, 33.48%) and skin elasticity (88.41%, 64.97%), respectively, however, the placebo group remained no change of skin in any parameters. In sensory evaluation, most of volunteers satisfied with product acceptability; appearance, texture, color, odor, moisture, spreadibility, absorption, smoothness, non-greasiness (> 85 %) and also the reduction of visible aging sign (> 90%). The results demonstrate that Thai red rice stem cell extract can be used as an effective anti-aging ingredient for cosmetic products functionalized in skin lightening, hydration and elasticity.

Keywords: anti-aging, cosmetics, red rice, rice stem cell

Introduction

Nowadays, the increased interest in fighting the aging skin has led to increase in the amount of worldwide cosmetic products which focus on anti-aging active ingredients such as antioxidants, vitamins, and bioactive compounds which scavenge free radicals from skin cells, restore skin elasticity and protect skin wrinkle.

Plant is a source of active compounds. However, the use of plants materials has certain limitation due to seasonal harvesting, plant growth rate, microorganism contamination, and pollution. Plant cell culture technology may help to overcome these essential problems in manufacturing cosmetic products (Schürch et al., 2008). Plant stem cells or callus contain a complex matrix of ingredients like salts, acids, phenols, sugars, lipids, proteins and other undefined fractions of compounds which may contribute to a beneficial protecting effect of skin stem cells (Schürch et al., 2008). Cosmetics industry interest the use of stem cells from plant as a source of materials for replenishing the aging body (Draelos, 2012).

The Journal of Applied Science **Vol. 17 Special issue: 63-72 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S08

Rice (*Oryza sativa*) consists of many phytochemicals such as γ-oryzanol, vitamin E homologues, and phenolic acids which produce beneficial health properties (Finocchiaro et al., 2007; Yawadio et al., 2007). Red rice is a special rice cultivar in Thailand that contain high procyanidin in the seed coated (Oki et al., 2002). Procyanidins, known as condensed tannins, are polymers of flavan-3-ol monomer units (catechin and epicatechin diastereoisomers) (Gu et al., 2004). Procyanidins have been reported to be high antioxidant, matrix metalloproteinases inhibition (Grimm et al., 2004), tyrosinase inhibition (Momtaz et al., 2008) and effective in protecting against cardiovascular diseases and cancer (Santos-Buelga & Scalbert, 2000).

The anti-aging efficacies of many plant stem cell such as apple, grape, raspberry, tomato have been previously reported. However, it has not been reported the anti-aging potential of Thai red rice stem cell. Therefore, the aim of the present study was to examine the effects of topical application of Thai red rice stem cell on 28 volunteers (aged 30 – 55 years) in comparison to commercial red rice stem cell based on skin color, hydration and elasticity.

Materials and methods

Materials

Red rice was cultivated in Chiang Rai, Thailand. Ethanol, folin-ciocalteu reagent, gallic acid, ninhydrin and sodium carbonate were purchased from Sigma-Aldrich Co., USA. 1-Naphthaleneacetic acid (NAA), 2,4-Dichlorophenoxyacetic acid (2,4-D), 6-Benzylaminopurine (BAP), gallant gum and Murashige and Skoog (MS) medium were purchased from PhytoTechnology Laboratories, USA. Commercially available rice stem cell extract was purchased from Water Doctor Company Limited, which extracted from red rice (*Oryza sativa*) callus. The claim of the extract is to provide anti-aging effect.

Preparation of red rice callus extract

The sterilized red rice seed was inoculated on agar solidified basal MS medium supplemented with 3 % sucrose, 0.2 % gellant gum, 2 mg/l of 2,4-D, 1 mg/l of NAA, and 1 mg/l of BAP. The pH of medium was adjusted to 5.8. The red rice was incubated in the controlled temperature conditions (25±2°C). The callus formation was observed and collected after incubation for 21 days. The harvested callus (1 g) was extracted with (10 ml) ethanol under sonicator for two hours. The extract was filtered with Whatman® no.1 filter paper and stored at 4°C until analysis.

Total phenolic content

Total phenolic content was analyzed using the Folin-Ciocalteu assay (Vichit & Saewan, 2015). Briefly, the extract (20 µl) was mixed with 50 µl of deionized water, 20 µl of Folin-Ciocalteu reagent and 125 µl of 7 % sodium carbonate. The mixture was incubated for 60 minutes at room temperature. The absorbance was measured at 750 nm using a microplate reader. The result was determined using gallic acid standard solution and expressed as gallic acid equivalents (mg GAE/ml extract).

Total amino acid content

Total amino acids content was determined by using the Ninhydrin assay (Friedman, 2004). The 200 µl of extract was mixed with 1 % ninhydrin solution and incubated at 80°C for 10 minutes. Absorbance was measured at 570 nm and then calculated using glutamic acid standard solution. The result was expressed as glutamic acid equivalents (mg Glu/ml extract).

The Journal of Applied Science **Vol. 17 Special issue: 63-72 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S08

Preparation of testing cream

Oil-in-water (O/W) emulsion cream (placebo) was formulated. Briefly, aqueous and oily phase were heated to 75°C. After heating, aqueous phase was then added to the oily phase with continuous stirring until complete homogenization by using homogenizer (IKA Werke, Germany). Finally, emulsion was cooled to room temperature. The testing cream was prepared by incorporation of 5 % of Thai red rice stem cell extract (F1) or commercial rice stem cell extract (F2) which was the maximum recommended dose for the commercial rice stem cell extract.

Skin irritation test

The protocol was approved by the Ethics Committee of Mae Fah Luang University (REH-60031). Each volunteer signed a written informed consent before participating in the clinical study which explained the type of study, the procedures to be followed, the general nature of the materials being tested, and any known or anticipated adverse reactions that might result from participation.

Twenty-eight healthy volunteers (aged 30 – 55 years) were subjected to the occlusive single patch test. Finn chambers (8 mm) were used for observation of skin irritation for a period of 24 h on the volar forearms of volunteers. Skin irritation severity was graded over arrange of 0 – 4. Data collected were used to calculate mean irritation index (M.I.I). A M.I.I value < 0.2 was classified as non-irritation.

Clinical anti-aging effects

Volunteers were randomized to one of three treatment groups. Group 1 received placebo cream. Group 2 received cream containing 5 % of Thai red rice stem cell extract. Group 3 received cream containing 5 % of commercially rice stem cell extract. Volunteers were instructed to apply 0.5 mg of the tested cream on facial skin twice daily, in the morning and before bedtime. Modification of the facial skin-care habits and concomitant use of other skin care products were not permitted during the study.

All skin measurements were conducted under controlled temperature (20 ± 5°C) and humidity (50 ± 10%) conditions. Volunteers washed their face with an assigned facial cleanser and allowed to acclimate to the room conditions for at least 30 min prior to measurements. Melanin content was determined with Mexameter. Skin moisture was measured by Corneometer. Skin elasticity was evaluated the net elasticity of the skin without viscous deformation by using Cutometer. The values of each volunteer were recorded and calculated as the percentage of change by following equation:

$$\% \text{ Change} = [(X_t - X_0)/X_0] \times 100 \quad (1)$$

Where X_0 is the initial value which measured before application (week 0) and X_t is the value that measured during application (Fox et al., 2014).

Sensory study

The products were sensory evaluated by all volunteers at the end of experiment. Each volunteer was asked to answer the questionnaire about the cream texture and self-assesment for anti-aging results which divided into 5 scores, as follows: (1) dislike extremely, (2) dislike, (3) neither like nor dislike, (4) like, and (5) like extremely (Herndon et al., 2014). Then, the

The Journal of Applied Science **Vol. 17 Special issue: 63-72 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S08

total number of volunteer who favorable responsed to the product properties (score 4 – 5) was calculated as preference percentage of each treatment group by using following equation:

Preference (%) = (Number of preferable response volunteer/ Total volunteer) x 100 (2)

Statistical analysis

All measurements were performed in triplicate. The obtained data were statistically analyzed using the SPSS program version 11.5 for Window (SPSS Inc, Chicago, IL, USA) and the differences were considered significant when $p<0.05$. For clinical trial, paired sample t-test was used to determine the skin improvement during sample application compared with initial condition (week 0) and * indicated significant differences ($p<0.05$).

Results and discussion

Preparation of red rice callus extract

In this study, auxins (2,4-D and NAA) and cytokinins (BAP) were used to induce red rice callus on agar solidified MS medium supplementing with sugar and gellant gum. The induced red rice callus appeared as yellow compact texture (Figure 1). The growth percentage was calculated based on the weight of rice seed and showed that stem cells were developed in range of 385 %.

Phenolic compounds and amino acids are two main groups of anti-aging agents in cosmetic products (Ganceviciene et al., 2012). Phenolic compounds are antioxidants which eliminate the causes of skin aging and skin damage, including wounds and burns. Moreover, phenolic compounds also help prevent or attenuate the progression of certain skin disorders such as wrinkles and acne (Dzialo et al., 2016). Amino acids are cell regulators which have the ability to stimulate collagen synthesis and activate dermal metabolism through topical application (Lupo & Cole, 2007). In addition, amino acids have the ability to stimulate collagen synthesis and restore dermal collagen impaired by UV irradiation (Lupo & Cole, 2007; Murakami et al., 2012).

In this study, the total phenolic and amino acid contents in the induced callus and the commercial extracts were determined by using the Folin-Ciocalteu and Ninhydrin assays (Friedman, 2004), respectively. The induced stem cell extract contained total phenolic content with 0.42 mg GAE/ml and total amino acid content with 0.21 mg Glu/ml, while, the total phenolic and amino acid of the commercial extract were 0.37 mg GAE/ml and 0.22 mg Glu/ml, respectively.



Figure 1. Appearance of red rice callus and ethanol extract.

Vol. 17 Special issue: 63-72 [2018]
doi: 10.14416/j.appsci.2018.02.S08

Skin irritation test

Skin irritation was evaluated by using single application closed patch test. The result showed that all volunteer were no any irritation or side effect. Then, the M.I.I value was calculated and showed that M.I.I value of Thai red rice stem cell and commercially rice stem cell was 0 and 0.1, respectively. Thus, the products were classified as non-irritant.

Clinical anti-aging effects

The clinical anti-aging effects of red rice stem cell cream were evaluated on facial skin of 28 volunteers compared with placebo and commercial rice stem cell cream for 12 weeks. The evaluation of wrinkles in the eye area by naked eye observation and photograph showed that placebo cream were no any change, while, the creams contained the induced stem cell (F1) and commercial extract (F2) showed reduction of fine wrinkles (Figure 2). The anti-aging efficacy was assessed for melanin content, skin moisture and elasticity. Then, the percentage change of all volunteers was calculated and statistical analyze was determined by comparing with week 0.

Skin moisture relate with stratum corneum water content and has an important role in skin function such as cell proliferation, skin hydration, and anti-inflammation process. The product which can improve moisture of skin can prevent skin dryness and alterations resulting from aging. The skin moisture was determined by using Corneometer. According to Figure 3, the percentage change of skin moisture of the group applied formula F2 was significant increased in week 2 and continuously increased to 33.48 % at week 12. The group treated formula F1 had caused an increase in skin moisture in all volunteers since first week of application (32.23 %) and enhanced skin moisture to 74.62 % at the end of application. The skin moisture effect may be due to the constituent of amino acid which are the main component of the natural moisturizing factor (NMF), helping maintain hydration in corneocyte and stratum corneum (Verdier-Sévrain & Bonté, 2007).

Melanin is a skin pigment which effect to the appearance of skin color. Therefore, determination of melanin content can imply the darker and lighter skin. The melanin content was determined by using Mexameter. The result showed that melanin content of volunteers who applied placebo were no any change values, while, volunteers who applied formulas F1 and F2 were continuously decreased. In overall, at week 12, the percentage change of melanin content was calculated and showed that no statistically significant difference ($p < 0.05$) observed in placebo group. The change of melanin content was significantly decreased by 38.57 and 29.85 % for F1 and F2, respectively (Figure 4).

The loss of skin elasticity is a main problem of aging, thus the active ingredient which can improve skin elasticity are interest to use as anti-aging active. The skin elasticity was determined by using Cutometer. The skin elasticity of each volunteer showed a little change values in placebo group, while, formulas F1 and F2 were increased skin elasticity evidently. After 12 weeks of application, formula F1 enhanced skin elasticity to 88.41 % which was significantly more than formula F2 (64.97 %) (Figure 5).

The induced stem cell extract showed higher anti-aging efficacy than the commercial exract may be due to it contain higher phenolic compounds which inhibit tyrosinase (the key enzyme responsible for melanin production) synthesis, dopachrome formation and melanogenesis (Chang, 2009; Couteau & Coiffard, 2016; Kim & Uyama, 2005). Moreover, phenolic compounds demonstrate the ability to promote the enzymatic inhibition of specific proteinases such as collagenase and elastase which can be useful to prevent loss of skin elasticity and thus skin sagging (Melzig et al., 2001; Silva et al., 2017; Thring et al., 2009).

The Journal of Applied Science **Vol. 17 Special issue: 63-72 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S08

In overall, the induced stem cell and the commercial stem cell extracts were found to be an effective ingredient for use as anti-aging ingredient in cosmetic formulation due to its ability to reduce the melanin content and enhance of skin moisture and elasticity.



Figure 2. Photographs of skin appearance at week 0 and week 12.

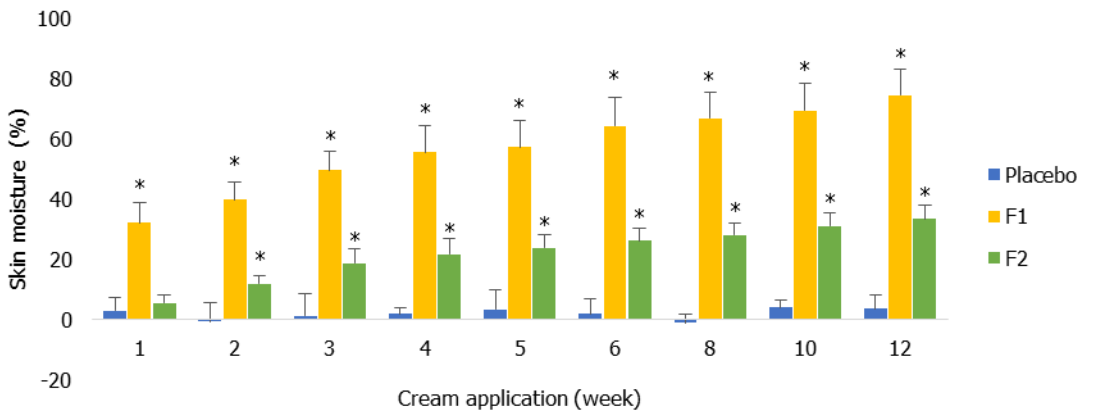


Figure 3. The change of skin moisture during experiment. * indicate significant differences ($p < 0.05$) between the skin property during experiment and baseline (week 0).

Vol. 17 Special issue: 63-72 [2018]
doi: 10.14416/j.appsci.2018.02.S08

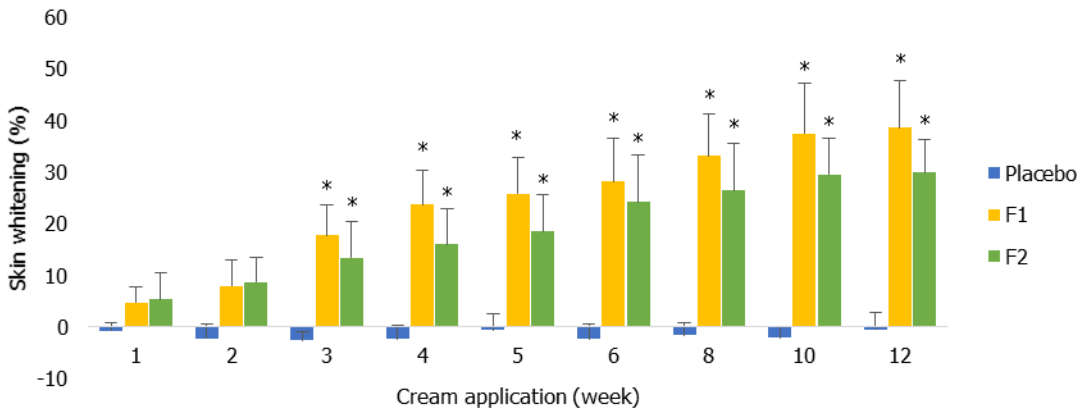


Figure 4. The change of skin whitening during experiment. * indicate significant differences ($p < 0.05$) between the skin property during experiment and baseline (week 0).

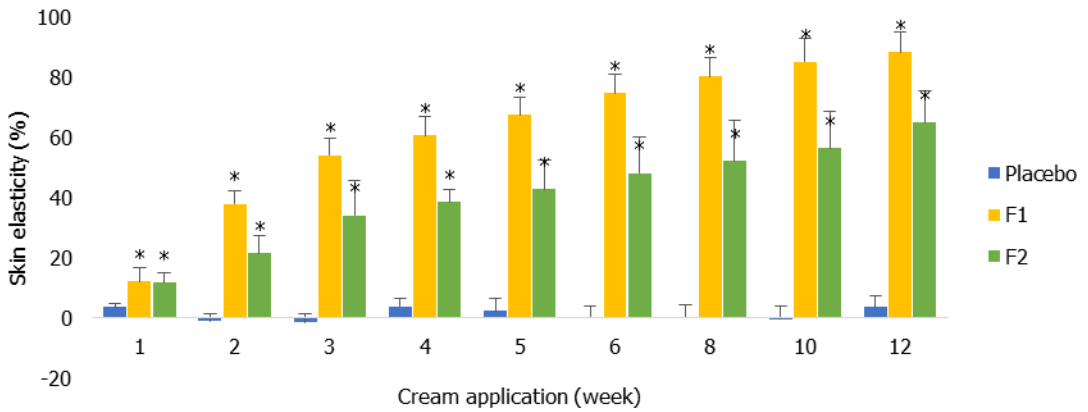


Figure 5. The change of skin elasticity during experiment. * indicate significant differences ($p < 0.05$) between the skin property during experiment and baseline (week 0).

Sensory study

The satisfaction of using placebo, Thai rice stem cell and commercial extracts creams were evaluated. The result showed that all volunteer groups prefer appearance of their product at similar level (90 %) in texture, skin absorption, smoothness, non-greasiness, and overall. The color, odor, and moisture of all products were little difference (Figure 6a). The efficacy preference of product was evaluated after application for 12 weeks. Volunteers who applied rice stem cell cream were more preferred the anti-aging efficacy of the product in skin whitening, moisture, smoothness, elasticity, firmness, wrinkle and dark spot reduction than the group obtained the commercial extract cream (Figure 6b).

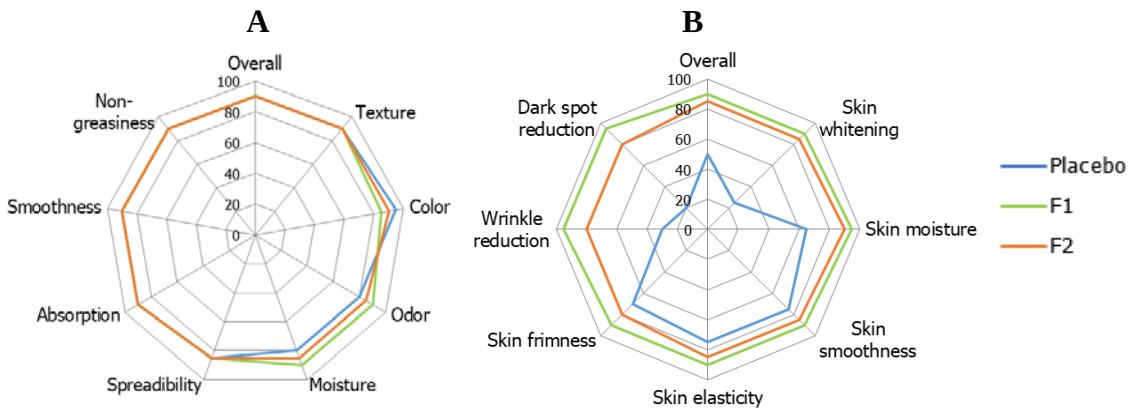


Figure 6. Sensory study of products with percentage of subject who extreamly like and like (A) texture and (B) efficacy.

Conclusion

The data from this study show that topical application of the product containing 5% Thai red rice stem cell extract offers anti-aging benefits. Statistical improvements were indicated in its ability to reduce the melanin content and enhance of skin moisture and elasticity of aging skin against baseline. Thus, red rice stem cell extract is an effective ingredient for use as anti-aging ingredient in cosmetic formulation.

Acknowledgements

The authors would like to acknowledge research funding from Agriculture research development agency (Public Organization). The authors express thanks to Mae Fah Luang University for providing scientific equipment and facilities for this work.

References

Chang, T. S. (2009). An updated review of tyrosinase inhibitors. *International Journal of Molecular Sciences*, *10*, 2440-2475. doi: 10.3390/ijms10062440

Couteau, C. & Coiffard, L. (2016). Overview of skin whitening agents: drugs and cosmetic products. *Cosmetics*, *3*(3), 27. doi: 10.3390/cosmetics3030027

Draelos, Z. D. (2012). Plant stem cells and skin care. *Cosmetic Dermatology*, *25*(9), 395-396.

Dzialo, M., Mierziak, J., Korzun, U., Preisner, M., Szopa, J. & Kulma, A. (2016). The potential of plant phenolics in prevention and therapy of skin disorders. *International Journal of Molecular Sciences*, *17*(2), 1-41. doi:10.3390/ijms17020160

Finocchiaro, F., Ferrari, B., Gianinetti, A., Dall'asta, C., Galaverna, G., Scazzina, F. et al. (2007). Characterization of antioxidant compounds of red and white rice and changes in total antioxidant capacity during processing. *Molecular Nutrition & Food Research, 51*(8), 1006-1019. doi: 10.1002/mnfr.200700011

Fox, L. T., du Plessis, J., Gerber, M., van Zyl, S., Boneschans, B. & Hamman, J. H. (2014). In vivo skin hydration and anti-erythema effects of *Aloe vera*, *Aloe ferox* and *Aloe marlothii* gel materials after single and multiple applications. *Pharmacognosy Magazine, 10*(2), S392–S403. doi: 10.4103/0973-1296.133291

Friedman, M. (2004). Applications of the ninhydrin reaction for analysis of amino acids, peptides, and proteins to agricultural and biomedical sciences. *Journal of Agricultural and Food Chemistry, 52*(3), 385-406. doi: 10.1021/jf030490p

Ganceviciene, R., Liakou, A. I., Theodoridis, A., Makrantonaki, E. & Zouboulis, C. C. (2012). Skin anti-aging strategies. *Dermato-Endocrinology, 4*(3), 308-319. doi: 10.4161/derm.22804.

Grimm, T., Schäfer, A. & Högger, P. (2004). Antioxidant activity and inhibition of matrix metalloproteinases by metabolites of maritime pine bark extract (pycnogenol). *Free Radical Biology and Medicine, 36*(6), 811-822. doi: 10.1016/j.freeradbiomed.2003.12.017

Gu, L., Kelm, M. A., Hammerstone, J. F., Beecher, G., Holden, J., Haytowitz, D. et al. (2004). Concentrations of proanthocyanidins in common foods and estimations of normal consumption. *The Journal of Nutrition, 134*(3), 613-617.

Herndon, J. H., Makino, E. T., Stephens, T. J. & Mehta, R. C. (2014). Hydroquinone-free skin brightener system for the treatment of moderate-to-severe facial hyperpigmentation. *The Journal of Clinical and Aesthetic Dermatology. 7*(5), 27-31.

Kim, Y. J. & Uyama, H. (2005). Tyrosinase inhibitors from natural and synthetic sources: structure, inhibition mechanism and perspective for the future. *Cellular and Molecular Life Sciences, 62*(15), 1707-1723. doi: 10.1007/s00018-005-5054-y

Lupo, M. P. & Cole, A. L. (2007). Cosmeceutical peptides. *Dermatologic Therapy, 20*(5), 343-349. doi: 10.1111/j.1529-8019.2007.00148.x

Melzig, M. F., Löser, B. & Ciesielski, S. (2001). Inhibition of neutrophil elastase activity by phenolic compounds from plants. *Die Pharmazie, 56*(12), 967-970.

Momtaz, S., Mapunya, B. M., Houghton, P. J., Edgerly, C., Hussein, A., Naidoo, S. et al. (2008). Tyrosinase inhibition by extracts and constituents of Sideroxylon inerme L. stem bark, used in South Africa for skin lightening. *Journal of Ethnopharmacology, 119*(3), 507-512. doi: 10.1016/j.jep.2008.06.006

Murakami, H., Shimbo, K., Inoue, Y., Takino, Y. & Kobayashi, H. (2012). Importance of amino acid composition to improve skin collagen protein synthesis rates in UV-irradiated mice. *Amino acids, 42*, 2481-2489. doi: 10.1007/s00726-011-1059-z

Oki, T., Masuda, M., Kobayashi, M., Nishiba, Y., Furuta, S., Suda, I. et al. (2002). Polymeric procyanidins as radical-scavenging components in red-hulled rice. *Journal of Agricultural and Food Chemistry, 50*(26), 7524-7529. doi: 10.1021/jf025841z

Santos-Buelga, C. & Scalbert, A. (2000). Proanthocyanidins and tannin-like compounds – nature, occurrence, dietary intake and effects on nutrition and health. *Journal of the Science of Food and Agriculture, 80*(7), 1094-1117. doi: 10.1002/(SICI)1097-0010(20000515)80:7<1094::AID-JSFA569>3.0.CO;2-1

Schürch, C., Blum, P. & Zülli, F. (2008). Potential of plant cells in culture for cosmetic application. *Phytochemistry Reviews, 7*(3), 599-605. doi: 10.1007/s11101-007-9082-0

Silva, S. A. M., Michniak-Kohn, B. & Leonardi, G. R. (2017). An overview about oxidation in clinical practice of skin aging. *Anais Brasileiros de Dermatologia, 92*(3), 367-374. doi:10.1590/abd1806-4841.20175481.

Thring, T. S. A., Hili, P. & Naughton, D. P. (2009). Anti-collagenase, anti-elastase and anti-oxidant activities of extracts from 21 plants. *BMC Complementary and Alternative Medicine, 9*, 27. doi: 10.1186/1472-6882-9-27.

Verdier-Sévrain, S. & Bonté, F. (2007). Skin hydration: a review on its molecular mechanisms. *Journal of Cosmetic Dermatology, 6*(2), 75-82. doi: 10.1111/j.1473-2165.2007.00300.x

Vichit, W. & Saewan, N. (2015). Antioxidant activities and cytotoxicity of Thai pigmented rice. *International Journal of Pharmacy and Pharmaceutical Sciences, 7*(7), 329-334.

Yawadio, R., Tanimori, S. & Morita, N. (2007). Identification of phenolic compounds isolated from pigmented rices and their aldose reductase inhibitory activities. *Food Chemistry, 101*(4), 1616-1625. doi:10.1016/j.foodchem.2006.04.016

The Journal of Applied Science
วารสารวิทยาศาสตร์ประยุกต์
ISSN 1513-7805 (Printed in Thailand)

Vol. 17 Special issue: 73-84 [2018]
doi: 10.14416/j.appsci.2018.02.S09
ISSN 2586-9663 (Online)

Research Article

Simultaneous extraction of oil and protein from perilla seed by three-phase partitioning and their application in serum

Punyawatt Pintathong[1,2], Phanuphong Chaiwut[1,2*], Natthawut Thitipramote[1,2], Nont Thitilertdecha[1,2], Witayapan Nantitanont[1,2], Sarita Sangthong[1,2] and Ninnapat Tiensri[1]

[1]School of Cosmetic Sciences, Mae FahLuang University, Chiang Rai, Thailand, 57100
[2]Green Cosmetic Technology Research Group, Mae FahLuang University, Chiang Rai, Thailand, 57100
***E-mail**: phanuphong@mfu.ac.th

Abstract

Perilla seeds contain high amount of oil and protein. This study was aimed to simultaneously extract oil and protein from perilla seed by using three-phase partitioning method (TPP). The extraction conditions were optimized by varying the ratio of crude extract to *t*-butanol of 1:2, 1:1 and 2:1 (v/v) and ammonium sulfate concentration of 20, 30 and 40% (w/v). The crude extract to *t*-butanol at 1:2 and 30% (w/v) ammonium sulfate resulted in the highest recovery of perilla oil (35.13±2.67%) and protein (13.18±0.65%). This provided 14 folds of time saving when compared to conventional method of oil and protein extraction separately. The obtained perilla protein and oil extracts were included in the facial serum formulation. Stability and satisfaction of the serum formulation were tested compared to its base formula. The perilla facial serum showed its good stability after accelerated conditions of centrifugation and heating-cooling for 4 cycles. The phase separation and pH changing was not observed. The viscosity and the color were slightly affected by the accelerated condition. The satisfaction test including moisturizing sensory of the serum was evaluated by 20 volunteers. The product was satisfied with average score 5.47 of the maximum 6 level. This study emphasized the valuable perilla seed protein and oil could be extracted by single step of TPP and feasibly included into moisturizing facial serum product.

Keywords: perilla seed, protein, oil, serum, three-phase partitioning (TPP)

Introduction

Perilla frutescence (perilla) is an aromatic herb originated in some Asian countries including Thailand, Korea, India and China and now throughout world-wide. The perilla stalks, leaves and seeds have been repoted as a rich source of omega-3 polyunsaturated fatty acids (PUFAs), specifically alpha-linolenic acid (ALA). It also contains omega-6 and omega-9 fatty acids which are the essential for health (Mohammad & Atul, 2010). The seeds of perilla are a good source of oleic acid, stearic acid and palmitic acid (Chang et al., 2009; Peiretti et al., 2011). Earlier studies have shown that perilla seed is a potential source of fat (51.7%) and protein (17.0%) (Longvah & Deosthale, 1991). The lipids consisted of 91.2-93.9% neutral lipids, 3.9-5.8 % glycolipids and 2.0-3.0 % phospholipids (Shin & Kim, 1994). The contents of tocopherol were 734 mg/kg in perillain which γ-tocopherolis dominating isomer contributing 94.3% in perilla to the total amount of tocopherols (Scapin et al., 2017). The leaves and seeds

The Journal of Applied Science **Vol. 17 Special issue: 73-84 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S09

of perilla have been found to have many other components such as beta-sitosterol and ampesterol (Honda et al., 1986), flavonoids such as apigenin, luteolin, chrysoeriol, quercetin, catechin, and phenolic acids such as caffeic acid and rosmarinic acid (Lee et al., 2013; Mohammad & Atul, 2010). Perilla possesses various bioactivities, such as antioxidant (Jung et al., 2011), anti- inflammatory (Ueda et al., 2002), anti-allergic (Makino et al., 2013; Takano et al., 2004) and anticancer (Narisawa et al., 1994). In the North of Thailand, the perilla seeds are widely consumed as flavoring and nutritional sources. Perilla oil is widely used as dressing or cooking (Shin & Kim, 1994). Volatile oils of the plant are also used in aromatherapy and perfume (Povilaitytee & Venskutonis, 2000). Moreover, the perilla extract from the leaves, seeds and oils has also been used in nutritional and cosmetic formulations (Mohammad & Atul, 2010; Terranova et al., 2006). However, the perilla protein which is mostly readily digestible (Oita et al., 2008) has not been documented for industrial application.

Several methods have been reported for the extraction and purification of protein, such as alcohol and salt precipitation, ion exchange chromatography, membrane separation (Azarkan et al., 2003), and aqueous two-phase extraction (Tan et al., 2015). Although these methods are effective, there are many disadvantages such as high cost and time demands, or difficult to scale-up (Tan et al., 2015). Various methods for extracting the oil from the seeds have been reported and conventional method of solvent extraction is the most widely used technique, due to its high efficiency in oil recovery. However, the major disadvantage in using solvent extraction technique is its high energy input and toxicity from large amount of solvent used (Shivani et al., 2011).

An effective technique for the separation and purification of enzymes, proteins and edible oils called three–phase partitioning (TPP) has been introduced (Chaiwut et al., 2010; Rawdkuen et al., 2010; Gagaoua et al., 2014). This method uses ammonium sulfate to achieve a saturation and precipitation of protein and *t*-butanol was added to make three distinct phases and the purified biomolecules was found in the interphase (Chaiwut et al., 2010). It is simple, less time consuming, and maintains the nature of the protein (Rachana & Lyju, 2014). Purification of aloe polysaccharide (APS) and protein resulting in highly effective APS extract (Tan et al., 2015) was an example of using the TPP for single step extraction of bioactive compounds. Extraction of protein and oil from the plant source by using one step method has not been reported. Therefore, the aims of the study were to simultaneously extract the protein and oil from perilla seed by using the TPP method and develop facial serum containing perilla protein and oil.

Materials and methods

Plant sample and chemical reagent

Dry perilla seeds were collected from Hangdong district, Chiang Mai province, Thailand during January to Febuary 2016. The seeds were air dried for 3 days. Tert-butanol (2-methyl-2-propanol) and ammonium sulfate ($(NH_4)_2SO_4$) were obtained from Merck (Darmstadt, Germany). Coomassie Brilliant Blue G-250 was purchased from Sigma Chemical (St. Louis, MO, USA) and 95% ethanol was supplied from Quality Reagent Chemical. Bovine serum albumin (BSA) was obtained from Fluka (Buchs, Switzerland). Alpha tocopheryl acetate (Vitamin E acetate) was supplied from Eisal Co. Cosmetic raw materials were purchased from suppliers: Godrej Industries, Chemico Inter Corparation, Qingdao Fuso Refining & Processing, Innospec, Nam Siang Trading, and Lubrizol.

The Journal of Applied Science **Vol. 17 Special issue: 73-84 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S09

Preparation of crude extract from perilla seeds

The seeds of perilla were dried in a tray-dryer at 55°C until the weight was constant. The seeds were ground in a blender and then homogenized with water at a ratio of 1:2 w/v. Then the homogenate was filtered through a Whatman filter paper No.1. The filtrate was centrifuged at 5000 *g* at 4°C for 20 min. The obtained supernatant was referred to the "crude extract" and stored at -20°C until used.

Extraction of perilla protein and oil by three-phase partitioning (TPP)

The TPP composed of crude extract, *t*-butanol and $(NH_4)_2SO_4$. The mixture was partitioned by orbital shaking at 150 rpm for 10 min and centrifuged at 5000×*g* at 4°C for 20 min. The top phase (*t*-butanol) were collected and evaporated by rotary evaporater for oil determination and the interphase was collected for protein quantification. The partitioning compositions of the sample per *t*-butanol ratio and the concentration of $(NH_4)_2SO_4$ were varied for studying their effect on simultaneous extraction of protein and oil from perilla seed. The ratio of crude extract to *t*-butanol was varied as 1:2, 1:1 and 2:1 (v/v) with a constant 30% w/v $(NH_4)_2SO_4$ and the extraction was carried out as mention above. The ratio of crude extract to *t*-butanol which provided the highest recovery was chosen for study of the effect of $(NH_4)_2SO_4$ concentration on protein and oil partitioning. The $(NH_4)_2SO_4$ concentration was varied at 20, 30 and 40% (w/v).

Determination of protein and oil

The concentration of protein in the interphase was measured by Bradford method (Bradford, 1976) using bovine serum albumin (BSA) as protein standard. Protein recovery (%) was calculated from protein amount of interphase compared to initial protein amount in crude extract. Top phase from the TPP was subjected to remove *t*-butanol by using rotary evaporator. Oil recovery (%) was calculated from oil weight of the top phase compared to initial weight of perilla seeds.

Analysis of fatty acid compositions

Fatty acid compositions of perilla oil were analyzed by using GC-MS. The fatty acid methyl esters (FAMEs) of perrila seed oil was prepared by saponification of 3 grams oil with 0.9 M H_2SO_4 in methanol (3 mL) and 1 mL of toluene (Siriamornpun et al., 2006). The mixture was refluxed for 2 hours at 75-80°C and the solvent was removed by rotary evaporator at 40°C. Recovered FAMEs sample was adjusted to pH 6. The GC-MS analysis was carried out as the system composed of 0.25mm×30mm×0.25µm capillary column (Agilent 190915-43 HP-5MS) at 250°C using helium as a carrier gas with 1.0 mL/min. The injector and detector temperature were set at 220°C. The FAMEs was detected at 20-250 Da and identified by NIST08 database.

Development of serum containing perilla protein and oil

Oil/water facial moisturizing serum was developed by using ingredient listed on Table 1. The ingredients of the oil phase (phase A) were mixed at room temperature. In the same time, the aqueous part was mixed and then the oil phase was added to the water phase under mechanical stirring. The emulsion was continuously stirred and the pH was adjusted by using citric acid solution of part C. The proper texture of base formula was obtained after alteration amounts the ingredients. Stability of the base serum was checked by using centrifugation and heating-cooling accerelation test for 4 cycles. The formula providing the highest stability (pH, color and viscosity) and organoleptical characteristics (aspect, color and odor) was selected for

The Journal of Applied Science **Vol. 17 Special issue: 73-84 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S09

the base serum. In case of the serum containing oil and perilla protein, the perilla oil was added to replace jojoba oil and perilla protein solution was part D as described in the Table 1.

Stability test

The base serum and the serum containing perilla oil and protein (1.00 g) were centrifuged at 6000 rpm for 20 min. None of phase separation was observed. Then the base serum and the products were tested under accelerated condition of heating and cooling of 50°C and 4°C for 4 cycles. Before and after accerelation test, pH, viscosity and color of the products were measured. The pH of the serums was measured by using pH meter (NeoMet/pH-200L) at room temperature. The viscosity was measured by using the viscometer (Bookfield/RVD-II+P), 10 rpm and spindle no.4 under room temperature. Color measurement of base serum and sample were performed by using Chroma meter (Konica Minolta) through L*, a*, b* color scale of CIE. The L* value represents the brightness from black (0) to white (+100), a* value represents from green (-60) to red (+60) while b* value represents from blue (-60) to yellow (+60). The measurements were performed in three replicates.

Table 1. Formulas of base serum and serum containing perilla protein and oil

Part	Ingredients	Base Serum	Serum sample
A	Tocopheryl Acetate	0.10	0.10
	Caprylic/Capric Triglyceride	0.50	0.50
	Jojoba Oil	2.00	-
	Perilla Oil	-	2.00
	Isopropyl Palmitate	1.00	1.00
	Isopropyl Isostearate	1.00	1.00
	Dimethicone	1.00	1.00
	C12-15 Alkyl Benzoate	1.00	1.00
	PEG-40 Hydrogenated Castor Oil	0.20	0.20
B	DI Water	qs to 100	qs to 100
	Methyl Gluceth-20 and Glycerin	1.50	1.50
	Sodium Acrylates/Beheneth-25 Methacrylate Crosspolymer (and) Hydrogenated Polydecene (and) Lauryl Glucoside	1.50	1.50
	Hydroxyethyl Acrylate/Sodium Acryloyldimethyl Taurate Copolymer (and) Squalane (and) Polysorbate 60	2.50	2.50
C	DI Water	2.00	2.00
	Citric acid	0.15	0.15
D	Perilla Protein	-	1.00
	DMDM Hydantoin	1.00	1.00

The Journal of Applied Science **Vol. 17 Special issue: 73-84 [2018]**
วารสารวิทยาศาสตร์ประยุกต์ doi: 10.14416/j.appsci.2018.02.S09

Sensory evaluation

Sensory satisfaction of the finished products was evaluated by 20 volunteers (10 males and 10 females). The different skin feel attributes were selected to properly discriminate the serum on distinct stages of evaluation (Lee et al., 2005). These attributes were spreadability, moisturizing, absorbability, smell, feeling after application and texture and appearance. A 0-6 scale was used where 0 was the less and 6 was the most sensory satisfaction.

Statistical analysis

All experiments were done in triplicate and all results are presented as mean±SD. The statistical analyses of collected datafor protein and oil recovery were performed using SPSS version 19.0 (IBM). One-way analysis of variance (ANOVA) and multiple comparisons by Tukey's were performed to analyze the difference among data. Statistical differences were considered to be significant at $P<0.05$.

Results and discussion

Effect of crude extract to *t*-butanol ratio on protein partitioning

Effect of the crude extract to *t*-butanol ratio on protein partitioning and oil extraction is revealed in Figure 1. The highest protein recovery (13.09%) and oil recovery (36.63%) were obtained from the interphase and top phase of the TPP system at 1:2 crude extract:*t*-butanol. The high amount of *t*-butanol makes a flocculate and increase buoyancy and lead protein favor to be extracted in the interphase (Tan et al., 2015). The increasing of *t*-butanol providing a higher oil yield was agreed with the previous study of TPP system (Duttaet et al., 2015). From this result, the ratio of crude extract to *t*-butanol at 1:2 was selected to study the effect of ammonium sulfate concentration on protein partitioning.

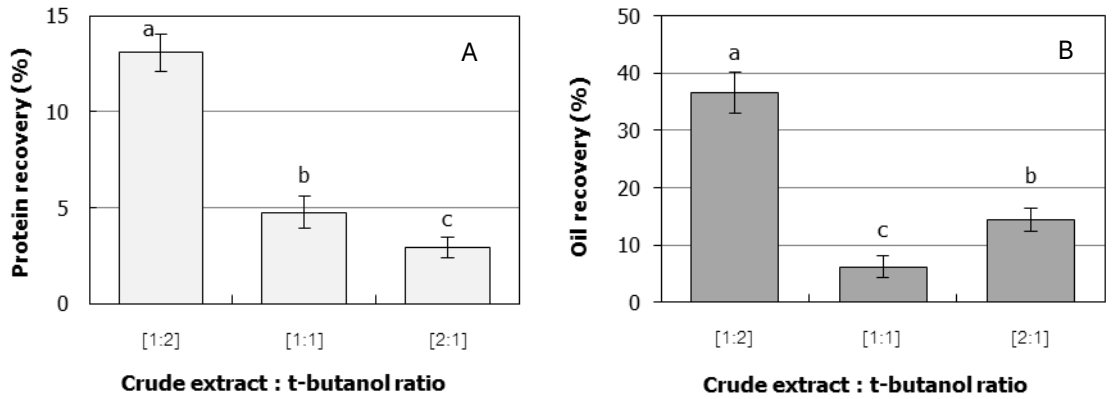


Figure 1. Effect of crude extract to *t*-butanol ratio on protein recovery (A) in the interphase and oil recovery in the top phase (B) of TPP

Effect of ammonium sulfate concentration on protein partitioning

The study on effect of ammonium sulfate concentration was explored by using the ratio of crude extract to *t*-butanol at 1:2 with varying the concentration of $(NH_4)_2SO_4$ at 20, 30 and 40% (w/v). As shown in Figure 2A, the salt concentration of 30% provided the highest recovery of protein (13.18±0.48 %). However, it was not significantly different from that of the 40%

$(NH_4)_2SO_4$ which exhibited 13.06±0.65 % recovery. Increasing the concentration of $(NH_4)_2SO_4$ increased protein amount in the interphase of TPP (Chaiwut et al., 2010). The 40% $(NH_4)_2SO_4$ giving the maximum protein in the interphase was revealed (Gagaoua et al., 2014). However, it has also been reported that the 30% (w/v) $(NH_4)_2SO_4$ was the best concentration for applying to TPP effectively (Rawdkuen et al., 2010). From the Figure 2B, the highest oil recovery (36.50±3.48 %) was obtained from top phase at 40% $(NH_4)_2SO_4$. This value was not significantly different from 35.13±2.67 recovery of the 30% salt. This might be attributed to the highest polarity in the aqueous bottom phase due to high amount of salt rendering the oil to be moved to the top phase.

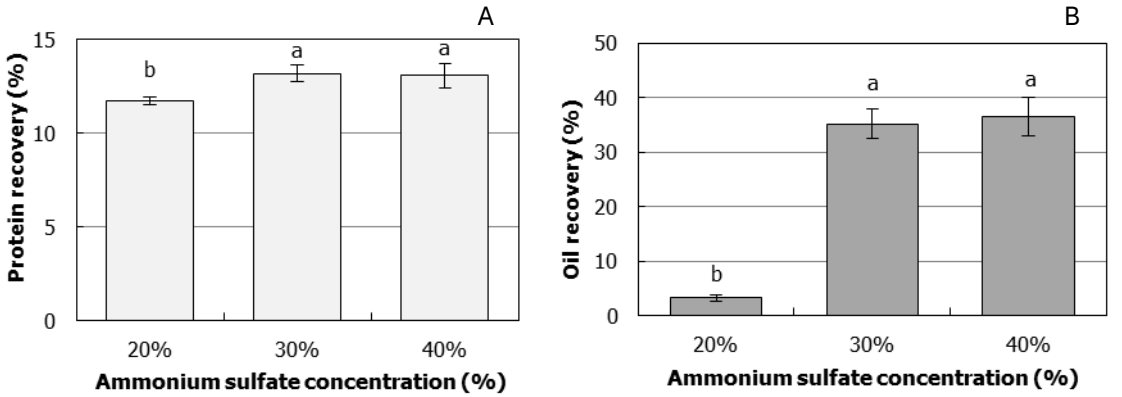


Figure 2. Effect of ammonium sulfate concentration on protein recovery in the interphase (A) and oil recovery in the top phase (B) of TPP

GC-MS analysis of fatty acid compositions in perilla oil

As shown in Table 2, two polyunsaturated fatty acids (PUFA) were identified in the perilla oil. The main PUFA was linolenic acid (18:3) of 70.63% followed by linoleic acid (18:2). Saturated fatty acids accounted for 16.51% was belonged to palmitic acid, stearic acid and arachidic acid. High amount of linoleic and linolenic acid were the significant fatty acids in term of quantity and quality of perilla oil (Siriamornpun et al., 2006). The linolenic acid (ALA) content is predominantly about 55-60% (Siriamornpun et al., 2006). It is noticed that the ALA in this study was higher than all previous reports. Ultrasonic-assisted aqueous enzymatic extraction using response surface provided 31.34% oil yield containing ALA as major component of 64.05% (Li et al., 2017). Similarly, soxhlet extraction using hexane as solvent and cold pressing extraction exhibiting 35.20% and 28.60%, respectively of perilla oil yields gave lower content of ALA at 62.26% and 60.11%, respectively (Li et al., 2017). Supercritical fluid extraction of perilla oil using carbondioxide and LPG could extract the linolenic acid as a majority fatty acid of 58.00% and 57.24%, respectively (Scapin et al., 2017).

Stability and satisfaction test of serum containing perilla protein and oil

The facial moisturizing serum was prepared by cold process and mixed using homogenizer. After centrifugation at 6000 rpm for 20 min, phase separations were not observed in both the base and serum containing perilla protein and oil. Then the serums were subjected to acceleration test by heating-cooling cycle. As shown in Table 3, the pH values of

Vol. 17 Special issue: 73-84 [2018]
doi: 10.14416/j.appsci.2018.02.S09

the base before and after heating and cooling test were 5.50 and 5.61, respectively, while the perilla serum exhibited 5.54 and 5.60, respectively. The results of pH values slightly changed indicating their high stability against acceleration test. Determination of the pH of a formulation intended for cutaneous application is extremely important, since it must be compatible with the pH of the application site. The serum in this study was designed to have pH between 5.50-5.60. The natural pH of the skin comes from the secretions of sweat and sebaceous glands, and lactic acid production, which leads to the formation of a protective film over the entire skin surface, designated hydrolipidic film. The skin normally has an average pH of 5.5, although this may vary slightly depending on the area of the body (Tichota et al., 2014).

Table 2. Fatty acid composition of perilla oil extracted by the TPP

Fatty acids	**% Content** (w/w)
Saturated fatty acids	
Palmitic acid (16:0)	10.23 ± 0.56
Stearic acid (18:0)	6.07 ± 0.12
Arachidic acid (20:0)	0.21 ± 0.003
Unsaturated fatty acids	
Linoleic acid (18:2)	11.17 ± 0.14
Linolenic acid (18:3)	70.63 ± 2.49
Other volatile components	1.69 ± 0.06

Table 3. Values of pH, viscosity, and color of serums before and after heating-cooling cycles

Formula	**Unit**	**Base serum**		**Perilla serum**	
		Before	**After**	**Before**	**After**
pH		5.50±0.16	5.61±0.07	5.54±0.21	5.60±0.11
Viscosity	**cP**	13,553±233	13,493±141	12,713±95	12,540±103
Torque	**%**	67.77±2.16	62.47±1.14	63.57±3.86	57.70±1.95
Color	**L***	64.70±0.13	64.42±0.06	67.77±0.20	66.87±0.43
	a *	-0.52±0.01	-0.68±0.02	-0.59±0.04	-0.70±0.04
	b*	-2.22±0.06	-2.14±0.04	-1.60±0.30	-1.59±0.24
	ΔE		0.33±0.07		0.91±0.25

The viscosities of the base serum before and after heating and cooling test were quite similar (Table 3). This implied the products contained enough stabilizing ingredients and the addition of perilla protein and oil was compatible with other ingredients. Amount of consistency agent was important to keep the viscosity of the formulation constant during the stress conditions applied (Miner, 1993). Presence of nonionic surfactant PEG–40 hydrogenated castor oil in the formula may cause a small reduction in viscosity (Miller & Loffler, 2006).

Vol. 17 Special issue: 73-84 [2018]
doi: 10.14416/j.appsci.2018.02.S09

The results of color measurement of the base serum and sample before and after heating-cooling test were aligned in Table 3. The L* means the amount of reflected light, and can range from 0 (black) to 100% (white); the a* and b* represent, respectively, the colors from green to red or blue to yellow, and the values range from –60 (close to green or blue) to +60 (close to red or yellow). Through the values of L*, a* and b*, the overall color change (ΔE) can be calculated. Minor changes of color in both base and serum were detected after acceleration test to give less ΔE of 0.33 and 0.91, respectively. This color changes were not visible to the naked eye. Thus, it was presumable that there were no changes in the color of the serum containing perilla protein and oil after heating-cooling cylcle. Color stability of the serum might suggest the absence of lipid degradation from oxidation reactions, and revealed lipid stability during thermal process and storage. Oxidative instability is a concern mainly associated with lipids consisting of unsaturated fatty acids (Jannin et al., 2008). Lipid oxidation reactions generate colored compounds and thus color change in the formulation can give information about lipid stability (Tichota et al., 2014; Gray, 1978).

The consumer satisfaction to a finish product received the mean value of very good score. Oils and proteins are generally considered as skin moisturizing agents. The oils provide occlusive barrier which prevents water loss from skin layer, on the other hand, the protein absorb water from atmosphere delivering skin moisturization. As shown in Figure 3, the perilla serum exhibited higher sensory satisfaction score than those of the base serum in all attribute tests. Moisturizing and sense of smell attributes of the perila serum possessed the highest satisfaction among the 20 volunteers. From the maximum scale of 6, they obtained similar mean score of 5.70±0.46, while the base serum showed lower satisfying scores of 4.80±0.46 and 4.30±0.85 for moisturizing and sense of smell, respectively. Spreadability property of perilla serum also showed higher gratifying score of 5.65±0.48 when compared to 5.20±0.61 of the base formula. The texture and feeling after application were also good satisfied which scored 5.45±0.50 and 5.35±0.74, respectively. The absorbability was less scored at 4.95±0.68 which might be from watery feeling of the product. The serum in this study was designed to have water breaking property during application which might need time for a while to absorb on to skin surface. However, feeling after application was satisfied by the volunteers and obtained high score as described above.

Conclusion

Protein and oil from perilla seed could be simultaneously extracted by using TPP. The TPP system consisted of the ratio of crude extract to *t*-butanol 1: 2 and 30% (w/v) $(NH_4)_2SO_4$ giving the highest protein recovery (13.18±3.48 %) and oil recovery (35.13±2.67 %). The oil and protein from perilla seed could be incorporated in moisturizing serum formulation providing high stability in pH, viscosity and color. The product also showed good satisfaction from 20 volunteers, especially spreadability, smell, moisturizing, feeling after application and texture properties.

Acknowledgement

Authors are grateful to Mae Fah Luang University for facility support.

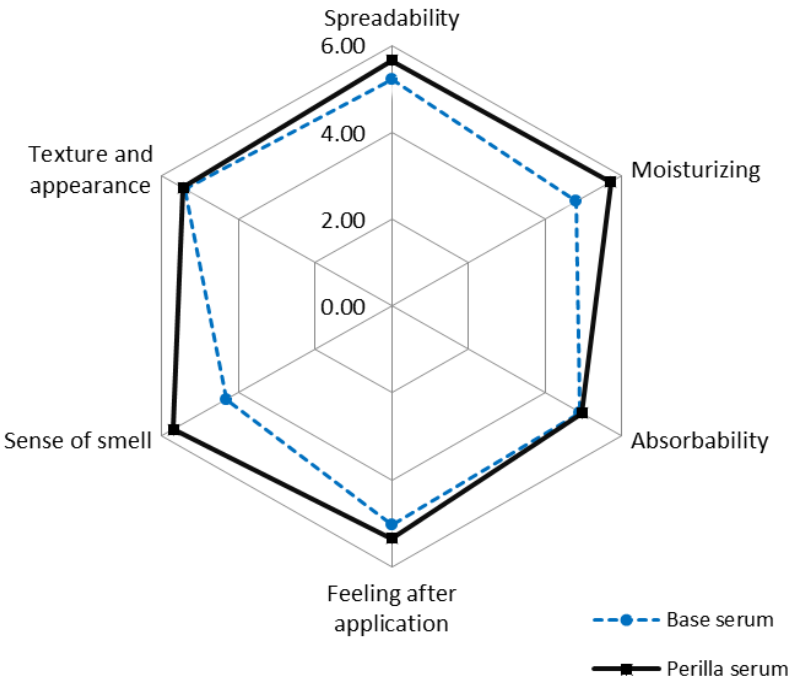


Figure 3. Satisfaction evaluation by 20 volunteers for perilla serum (—■—) compared to base formulation (---●---)

References

Azarkan, M., Moussaoui, A. E., van Wuytswinkel, D., Dehon, G. & Looze, Y. (2003). Fractionation and purification of the enzymes stored in the latex of *Carica papaya*. *Journal of Chromatography B*, *790*(1-2), 229-238. https://doi.org/10.1016/S1570-0232(03)00084-9

Bradford, M. M. (1976). A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry*, *72*(1-2), 248-254. https://doi.org/10.1016/0003-2697(76)90527-3

Chaiwut, P., Pintathong, P. & Rawdkuen, S. (2010). Extraction and three-phase partitioning behavior of proteases from papaya peels. *Journal of Process biochemistry*, *45*(7), 1172-1175. https://doi.org/10.1016/j.procbio.2010.03.019

Chang, H. H., Chen, C. S. & Lin, J. Y. (2009). Dietary perilla oil lowers serum lipids and ovalbumin-specific IgG1, but increase total IgE levels ovalbumin-challenged mice. *Food and Chemical Toxicology*, *47*(4), 848-854. https://doi.org/10.1016/j.fct.2009.01.017

Miller, D. & Löffler, M. (2006). Rheological effects with a hydrophobically modified polymer. *Colloids and Surfaces A: Physicochemical and Engineering Aspects, 288*(1-3), 165-169. https://doi.org/10.1016/j.colsurfa.2006.04.022

Dutta, R., Sarkar, U. & Mukherjee, A. (2015). Process optimization for the extraction of oil from *Crotalaria juncea* using three phase partitioning. *Industrial Crops and Products, 71*, 89-96. https://doi.org/10.1016/j.indcrop.2015.03.024

Gagaoua, M., Boucherba, N., Darenfed A. B., Ziane, F., Rabah S. N., Hafid, K. & Boudechicha, H. R. (2014). Three-phase partitioning as an efficient method for the purification and recovery of ficin from Mediterranean fig (*Ficus carica* L.) latex. *Separation and Purification Technology*, *132*, 461-467. https://doi.org/10.1016/j.seppur.2014.05.050

Gray, J. I. (1978). Measurement of lipid oxidation: a review. *Journal of American Oil Chemists' Society*, *55*(6), 539-546. https://doi.org/10.1007/BF02668066

Honda, G., Koezuka, Y., Kamisako, W. & Tabata, M. (1986). Isolation of sedative principles from *Perilla frutescens*. *Chemical and Pharmaceutical Bulletin*, *34*(4), 1672-1677. https://doi.org/10.1248/cpb.34.1672

Jannin, V., Musakhanian, J., & Marchaud, D. (2008). Approaches for the development of solid and semi-solid lipid-based formulations. *Advanced Drug Delivery Reviews*, *60*(6), 734-746. https://doi.org/10.1016/j.addr.2007.09.006

Jung, M., Chung, H., Choi, J., Jung, M. J., Chung, H. Y. & Choi J. S. (2011). Antioxidant activity of roasted defatted perilla seed. *Natural Product Science, 7*(3), 72-75. http://210.101.116.28 /W_files/kiss2/05003398_pv.pdf

Lee, I. S., Yong, H. M., Kim, J. W., Maeng, Y. J., Lee, C. W., Kang, Y. S., Rang, M. J. & Kim, H. Y. (2005). Terminology development and panel training for sensory evaluation of skin care products including aqua cream. *Journal of Sensory Studies*, *20*(5), 421-433. https://doi.org/10.1111/j.1745-459X.2005.00037.x

Lee, J. H., Park, K. H., Lee, M. H., Kim, H. T., Seo, W. D. & Kim, J. Y. (2013). Identification, characterization, and quantification of phenolic compounds in the antioxidant activity-containing fraction from the seeds of Korean perilla (*Perilla frutescens*) cultivars. *Food Chemistry*, *136*(2), 843-852. https://doi.org/10.1016/j.foodchem.2012.08.057

Li, H., Zhang, Z., He, D., Xia, Y., Liu, Q. & Li, X. (2017). Ultrasonic-assisted aqueous enzymatic extraction of oil from perilla seeds and determination of its physicochemical properties, fatty acid composition and antioxidant activity. *Food Science and Technology, Campinas*, *37*(Suppl. 1), 71-77. http://dx.doi.org/10.1590/1678-457x.29116

Longvah, T. & Deosthale, Y. G. (1991). Chemical and nutritional studies on Hanshi (*Perilla frutescens*) a traditional oil seed from Northeast India. *Journal of the American Oil Chemists Society, 68*(10), 781-784. https://doi.org/10.1007/BF02662172

Makino, T., Furata, Y., Wakushima, H., Fujii, H., Saito, K. & Kano, Y. (2013). Anti-allergic effect of *Perilla frutescens* and its active constituents. *Phytotherapy Research, 17*(3), 240-243. https://doi.org/10.1002/ptr.1115

Miner, P. E., (1993). Emulsion rheology: creams and lotions. In: D. Laba (Ed.), *Rheological Properties of Cosmetic and Toiletries* pp. 313-370. New York: Marcel Dekker.

Mohammad, A. & Atul, K. (2010). Nutritional and functional characterizations of *Perilla Frutescens* seed oil and evaluation of its effect on gastrointestinal motility. *Malaysian Journal of Pharmaceutical Sciences*, *8*(1), 1-12.

Narisawa, T., Fukaura, Y., Yazawa, K. & Ishikawa, C. (1994). Colon cancer prevention with a small amount of dietary perilla oil high in alpha linolenic acid in an animal model. *Cancer*, *73*(8), 2069-2075. https://doi.org/10.1002/1097-0142(19940415)73:8<2069::AID-CNCR2820730810>3.0.CO;2-1

Oita, S., Kimura, T., Shibuya, Y., Nihei, N. & Tanahashi, K. (2008). Extraction and digestibility of *Perilla frutescens* seed proteins. *Japan International Reseearch Center for Agricultural Sciences*, *42*(3), 211-214. https://doi.org/10.6090/jarq.42.211

Peiretti, P. G., Gasco, L., Brugiapaglia, A. & Gai, F. (2011). Effects of perilla (*Perilla frutescens* L.) seeds and supplementation on performance, carcass characteristics, meat quality and fatty acid composition of rabbits. *Livestock Science*, *138*(1-3), 118-124. https://doi.org/10.1016/j.livsci.2010.12.007

Povilaitytee, V. & Venskutonis, P. R. (2000). Antioxidative activity of purple peril (*Perilla frutescens* L.), moldavian dragonhead (*Dracocephalum moldavica* L.), and roman chamomile (*Anthemisnobilis*L.) extracts in rapeseed oil. *Journal of the American Oil Chemists Society*, *77*(9), 951-956. https://doi.org/10.1007/s11746-000-0150-1

Rachana C. R. & Lyju, J. V. (2014). Three Phase Partitioning—A Novel Protein Purification Method. *International Journal of ChemTech Research*, *6*(7), 3467-3472.

Rawdkuen, S., Chaiwut, P., Pintathong, P. & Benjakul, S. (2010). Three-phase partitioning of protease from *Calotropis procera* latex. *Biochemical Engineering Journal*, *50*(3), 145-149. https://doi.org/10.1016/j.bej.2010.04.007

Scapin, G., Abaide, E. R., Martins, R. F., Vendruscolo, R. G., Mazutti, M. A., Wagner, R. & Rosa, C. S. (2017). Quality of perilla oil (*Perilla frutescens*) extracted with compressed CO_2 and LPG. *The Journal of Supercritical Fluids*, *130*, 176-182. https://doi.org/10.1016/j.supflu.2017.08.007

Shin, H. S. & Kim, S. W. (1994). Lipid composition of perilla seed. *Journal of the American Oil Chemists' Society*, *71*(6), 619-622. https://doi.org/10.1007/BF02540589

Shivani, P., Khushbu, P., Faldu, N., Thakkar, V. & Shubramanian, R. B. (2011). Extraction and analysis of *Jatropha curcas* L. seed oil. *African Journal of Biotechnology*, *10*(79), 18210-18213. https://doi.org/10.5897/AJB11.776

Siriamornpun, S., Li, D., Yang, L., Suttajit, S. & Suttajit, M. (2006). Variation of lipid and fatty acid compositions in Thai perilla seeds grown at different locations. *Songkhlanakarin Journal of Science and Technology*, *28*(Suppl. 1), 17-21.

Takano, H., Osakabe, N., Sanbongi, C., Yanagisawa, R., Inoue, K., Yasuda, A., Natsume, M., Baba, S., Ichiishi, E. & Yoshikawa, T. (2004). Extract of *Perilla frutescens* enriched for rosmarinic acid, a polyphenolic phytochemical, inhibits seasonal allergic rhinoconjunctivitis in humans. *Experimental Biology and Medicine*, *229*(3), 247-254. https://doi.org/10.1177/153537020422900305

Tan, Z. J., Wang, C. Y., Yi, Y. J., Wang, H. Y., Tan, S. Y. & Li, F. F. (2015). Three phase partitioning for simultaneous purification of aloe polysaccharide and protein using a single-step extraction. *Process Biochemistry*, *50*(3), 482-486. https://doi.org/10.1016/j.procbio.2015.01.004

Terranova, F., Berardesca, E. & Maibach, H. (2006). Cellulite: nature and aetiopathogenesis. *International Journal of Cosmetic Science*, *28*(3), 157-167. https://doi.org/10.1111/j.1467-2494.2006.00316.x

Tichota, D. M., Silva, A. C., Lobo, A. M. S. & Amarai, M. H. (2014). Design, characterization, and clinical evaluation of argan oil nanostructured lipid carriers to improve skin hydration. *International Journal of Nanomedicine*, *9*(1), 3855-3864. https://doi.org/10.2147/IJN.S64008

Ueda, H., Yamazaki, C. & Yamazaki, M. (2002). Luteolin as an anti-inflammatory and anti-allergic constituent of *Perilla frutescens. Biological and Pharmaceutical Bulletin*, *25*(9), 1197-1202. https://doi.org/10.1248/bpb.25.1197