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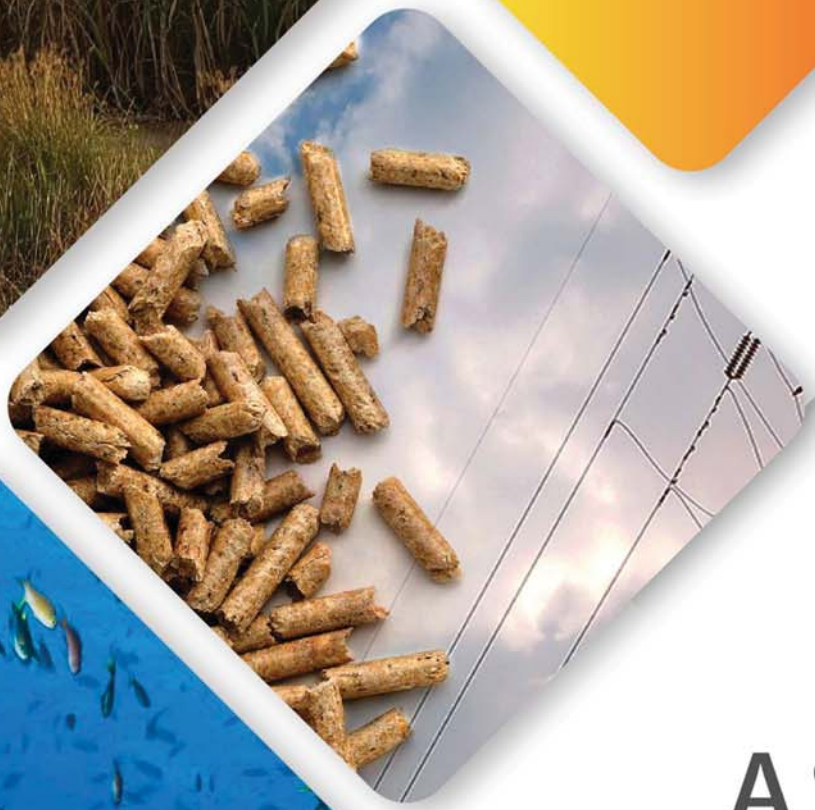

ASEAN BIO energy& economy Conference 2019

ASEAN Bioenergy and Bioeconomy Conference 2019:

Sustainable Bioresources for Green Energy and Economy

June 6th, 2019

BITEC, Bangkok, THAILAND

With ASEAN Sustainable Energy Week 2019

June 5th -8th, 2019

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Kasertsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI),

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PROCEEDINGS OF

ASEAN Bioenergy and Bioeconomy Conference 2019

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ASEAN Bioenergy and Bioeconomy Conference 2019: Sustainable Bioresources for Green Energy and Economy

June 6th, 2019

BITEC, Bangkok Thailand

With ASEAN Sustainable Energy Week 2019: June 5th -8th, 2019

The first ABB conference was organized in 2017 with focus on sustainable management and partnership. Since then, ABB conference promoted the exchange of knowledge of bioenergy and bioeconomy, providing opportunities to update database, status and situation in ASEAN countries. The conference has a strong participation of researchers, private sectors, business developers and policy makers and has been an important platform for a dialogue between academic and industrial scientists and technologists.

Objectives

1) To exchange and transfer useful information on biomass and bioenergy database, status and situation, new knowledge and technologies and innovation among ASEAN countries
2) To enhance sustainable biomass for energy production and utilization in ASEAN region to relieve environmental crisis and increase competitiveness of biomass for energy from ASEAN countries to the world market
3) To strengthen the biomass and bioenergy network within ASEAN countries

Conference Topics

Bioenergy

- Biomass
- Biofuel

Bioeconomy

- Biorefinery
- Biocomposite
- Biopharmaceutical

Total 200 Participants

Organizing Committee

- Kasetsart Agricultural and Agro-industrial Product Improvement Institute (KAPI), Kasetsart University, Thailand
- Asian Development College for Community Economy and Technology, Chiang Mai Rajabhat University, Mae Rim Campus, Thailand
- King Mongkut's University of Technology Thonburi (KMUTT), Thailand
- UBM Asia (Thailand) Co. Ltd, Thailand
- Peterson Projects & Solutions (Thailand) Company Limited

PROGRAM

ASEAN Bioenergy and Bioeconomy Conference 2019:
Sustainable Bioresources for Green Energy and Economy
June 6th, 2019
Bangkok International Trade and Exhibition Centre (BITEC),
Bangkok, Thailand

08.00-08.45	**Registration**	
08.45-09.00	**Opening ceremony** by **Assoc. Prof. Dr. Sornprach Thanisawanyangkura** *Vice President of Kasetsart University, Thailand*	1
09.00-09.30	**Keynote speaker 1** Overview of new Thailand energy planning, and its influence to ASEAN bioenergy **Dr. Twarath Sutabutr** *Inspector General, Ministry of Energy, Thailand*	3
09.30-10.15	**Keynote speaker 2** Bioenergy research trends under disruptive changes in electric power utility and transport mobility **Assoc. Prof. Dr. Supachart Chungpaibulpatana** *Coordinating Office for EGAT-TRF R&D Joint Funding Project,* *Sirindhorn International Institute of Technology,* *Thammasat University-Rangsit Campus, Thailand*	3
10.15-10.30	**Coffee break and *separate Sessions***	

SESSION 1 : BIOENERGY

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SESSION 1 : BIOENERGY (cont.)

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13.45-14.00	**Oral presentation 4** **O-EN4**: Symbiosis of Halophilic Anaerobic Bacteria *Halocella* sp. SP3-1 and *Halanaerobium praevalens* SP3-14: Role toward on cellulose degradation **Ms. Sobroney Heng** *School of Bioresources and Technology,* *King Mongkut's University of Technology Thonburi, Thailand*	74
14.00-14.45	**Coffee break and *Poster session***	
14.45-16.30	**Panel discussion** "Sustainable Biomass and Markets in Asia" **Moderator: Mrs. Piyathip Eawpanich** • **Dr.Gary Denes** *Senior Vice President, Asian Technology Service Pte Ltd.* • **Ms. Carol Rius** *CFO & Co-founder Husk Ventures* • **Mr. Wattanapong Thongsoi** *Chairman, the Thai Biomass Trade Association (TBTA)*	47

SESSION 2 : BIOECONOMY

ASEAN Bioenergy and Bioeconomy Conference 2019

SESSION 2 : BIOECONOMY (cont.)

ASEAN Bioenergy and Bioeconomy Conference 2019

POSTER

ASEAN Bioenergy and Bioeconomy Conference 2019

POSTER (cont.)

ASEAN Bioenergy and Bioeconomy Conference 2019

POSTER (cont.)

ASEAN Bioenergy and Bioeconomy Conference 2019

PREFACE

It gives me great pleasure to extend to you all a very warm welcome on behalf of Kasetsart University to “ASEAN Bioenergy and Bioeconomy Conference 2019”.

Allow me to extend a special welcome to the distinguished keynote speakers Dr. Twarath Sutabutr, Inspector General of Ministry of Energy, and Associate Prof. Dr. Supachart Chungpaibulpatana, Sirindhorn International Institute of Technology, Thammasat University, and to our honourable invited speaker from each institute. We would like to sincerely thank you for honouring our invitation in spite of your very busy and tight schedule.

We do believe that today’s symposium will open more opportunities for all researchers, scientists and technologists to share the knowledge, innovation and research methods, together with developing the Sustainable Biomass in ASEAN.

Sornprach Thanisawanyangkura
Vice President for Planning
Kasetsart University, Thailand

I would like to express my sincere appreciation to the organising committee led by Dr. Maliwan Haruthaithanasan, Director of Kasetsart Agricultural and Agro-Industrial Product Improvement Institute (KAPI), for taking the initiative to start this unique symposium. My gratitude is given also for those from companies, universities, and organizations, who co-operate and contribute to this important event.

In closing, let me finally wish you a successful symposium and fruitful discussion. I am delighted in declaring this conference open. I am confident that this symposium will achieve its objectives and hopefully will reward back to the society tremendously.

Keynotes

Keynote 1

Twarath Sutabutr

Ministry of Energy

Dr.Twarath Sutabutr is Inspector-General attached to the Ministry of Energy since October 2018. Dr. Twarath has experiences in a wide range of energy policy coordination and initiatives such as renewable energy, energy efficiency and international energy cooperations. His other current positions include Board of Directors of PTT Exploration and Production Public Company Limited (PTTEP) and (National Security and Dual – Use Technology Center.

Prior to this current position, he served as Director-General of Energy Policy and Planning Office (EPPO) from 2015 and Deputy Permanent Secretary from 2014 and Deputy Director-General of Department of Alternative Energy Development and Efficiency from 2009 to 2014. Moreover, when he served as Director of Policy and Strategy Coordination Office, he assumed parts of the work with National Economic and Social Development Board in energy infrastructure development planning, and involved in liberalization of electricity and natural gas sectors, which led to his strong relationship with major energy players in Thailand such as Energy Generating Authority of Thailand (EGAT) and PTT Public Company Limited, resulting in the successful development in such energy sectors.

Dr. Twarath holds Sc.D. in Civil and Environment Engineering from Massachusetts Institute of Technology (MIT), USA, Master of Geotechnical Engineering from Asian Institute of Technology (AIT) and Bachelor of Civil Engineering from Chulalongkorn University, Thailand.

Keynote 2

Supachart Chungpaibulpatana

Sirindhorn International Institute of Technology

Associate Prof. Dr. Supachart Chungpaibulpatana holds Doctor of Engineering and Master of Engineering in Energy Technology from Asian Institute of Technology, Thailand, and Bachelor of Science (Honors) in Mechanical Engineering, Prince of Songkhla University, Thailand. He also received Certificate in Lead Assessor of Quality Systems for the ISO 9000 Series from Registrar Accreditation Board (RAB), American Society for Quality Control (ASQC). Dr. Supachart is currently at Sirindhorn International Institute of Technology, Thammasat University-Rangsit Campus and Coordinating Office for EGAT-TRF R&D Joint Funding Project. He has extensive management experience as Head of School of Power, Energy and Environmental Technology, SIIT and School of Energy Technology, Industrial and Mechanical as well as many other department and institutions.

His background is in mechanical engineering and energy technology. His research activities and interests include energy equipment design, development and applications, as well as energy system planning and management. His core research topic is solar energy; both thermal and electrical applications. He is an expert in the design and development of low cost solar water heaters using local materials, solar-photovoltaic refrigerators for use in remote areas where electricity from the utility grid is not available, modeling of solar PV/thermal systems under various types of applications, development of standard methods for testing solar energy equipment, software packages for optimum sizing of solar energy systems.

Invited Presentation

BIOENERGY

Invited presentation 1
Mr.Tsuyoshi Teramae

Coal Business Department,
Idemitsu Kosan Company Limited, Japan
Tsuyoshi.teramae.7140@idemitsu.com

Mr.Tsuyoshi Teramae received his Bachelor degree from Faculty of Mining and Master degree from Graduate School of Engineering in Kyushu University. After that, he started his professional experience in Coal & Environment Research Laboratory, Idemitsu Kosan Co., Ltd. His interests include Utilization technology fly ash from coal fired boiler, Analysis technology of inorganic matter in coal by SEM/EDS, Study of coal combustion behavior by experimental method and numerical simulation, Study of ash behavior in coal combustion boiler such as slagging and fouling, Development of low temperature biomass gasification technology, Study of Particulate Matter generation during coal combustion. In 2016, he was promoted as a Technical Manager, in charge of the Environment & Biomass Section, Coal Business Department, for the project "Torrefied pellet production technology and its marketing in Japan".

UTILIZATION OF TORREFIED PELLET FOR CO-COMBUSTION WITH COAL IN JAPAN

Tsuyoshi Teramae, Masashi Machida, Naotsugu Otani and Sayaka Minami
Idemitsu Kosan Co.,Ltd., Japan
E-mail: tusyoshi.teramae.7140@idemitsu.com

ABSTRACT

The ideal compositions of power sources in FY2030 to achieve 3E+S (Energy Security, Economic Efficiency, Environment and Safety) based on basic policies of energy in Japan were announced in 2017. 'Environment' in the 3E+S includes execution of a reduction of large amount of CO_2 emission. 'Energy Mix' policy shows a portion of coal energy in power source is expected to become 26% in 2030 (about 33%, at present). Coal is still important energy source for Japan where there is no natural energy resources. Japanese government is developing high efficient power generation technology for coal power plant such as A-USC, IGFC. However, it would take a long time for the technologies to be realized. Co-combustion of biomass and coal contributes CO_2 emission reduction from coal-fired boiler in the short term.

Co-combustion of biomass and coal in large coal-fired power plant has advantages in comparison with in small-scale boiler used only biomass as a fuel. All cases of power plant using only biomass are; 1)Newly constructed power plant that have to be concerned about grid connection issue, 2)Adopting fluidized bed combustion system which is low efficient power generation compared with pulverized coal combustion system. Co-combustion in large coal-fired power plant also has an advantage to enable load adjustment for other renewable energy although biomass itself is renewable energy.

Many power companies with large-scale coal-fired power plants in Japan are using woody biomass (white pellet) at power stations. However, mixing ratio is very low, 1 - 3 wt.% because white pellet cannot be pulverized by mill. Utilization of torrefied pellet is effective way to increase the amount of biomass to be used, which leads to decrease CO_2 emission. Torrefaction is defined in ISO as "a mild pyrolysis process performed at temperatures between 200 - 300°C in inert atmosphere". By the thermally treating, grandability is improved. Thermal treatment also can bring an improvement of calorific value and hydrophobicity of woody pellet.

We are actively engaging in development of production technology of torrefied pellet with two companies in Thailand. We constructed demonstration-plant with a rotary kiln technology and are investigating an optimum production condition such as temperature and retention time.

Energy mix policy by FY2030 in Japan shows a portion of biomass energy in power source is expected to become 3.7 - 4.6% in 2030 (1.5%, at present). This is equivalent to 39 - 49 Twh (Terawatt hour) a year in power. Forest Agency said the amount of biomass generated in Japan is estimated to be equal to 13 Twh. Biomass produced overseas is required to meet the target of Energy mix policy. The most essential condition for expanding the amount of use of biomass is to reduce biomass procurement cost. Diversification of woody biomass feedstock should be studied to achieve large-volume provision of woody biomass with low cost.

Keywords : Torrefied pellet, Co-combustion, Biomass, Coal

Invited presentation 2
Assoc.Prof. Adisak Pattiya

Faculty of Engineering,
Mahasarakham University, Thailand
adisak.p@msu.ac.th, adisak_pattiya@yahoo.com

Associate Professor Dr.Adisak Pattiya holds Bachelor degree in Chemical Engineering from Thammasat University in 2001. After that, he graduated Master of Science in Advanced Chemical Engineering from Imperial College London in 2004 and Doctor of Philosophy in Chemical Engineering from Aston University, United Kingdom in 2008. He started his career as a lecturer at Faculty of Engineering, Mahasarakham University. Dr. Adisak has a lot of experience and produces more than 30 scientific publications in fast pyrolysis process, biomass conversion technologies, as well as energy production from agricultural residues. In 2015, he received the Innovation Award on Bio-oil Production Prototype from the National Research Council of Thailand (NRCT). He is also the reviewer for several international journals and the Secretariat and Chief Editor for the International Conference on Science, Technology and Innovation for Sustainable Well-Being (STISWB) in 2009 in Thailand and in 2010 in Vietnam.

PYROLYSIS TECHNOLOGIES FOR SOLID AND LIQUID BIOFUELS

Adisak Pattiya
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ABSTRACT

Pyrolysis is a thermochemical conversion process for transforming solid biomass into 3 forms of products including solid char, liquid biofuel and gas. These products have potential to be utilised not only as biofuel, but also as renewable biochemicals. Pyrolysis can be manipulated via process parameters such as temperature, heating rate and residence time in order to obtain desired products. With an aim to produce liquid biofuel, “fast pyrolysis” is designed to minimise the production of char and gas by providing high heating rate, optimum temperature, short vapour residence time and quick condensation of vapour. If the solid biofuel is targeted, “torrefaction” is considered a mild pyrolysis process for production of solid biofuel having properties equivalent to coal. Consequently, torrefied biomass can be called “biocoal”. The two key biomass pyrolysis technologies including fast pyrolysis and torrefaction will be presented to provide an overview of the technologies and the recent R&D progress.

Keywords : Pyrolysis, Biofuel

Invited presentation 3
Dr. Zhaoyuan Zhang

Guangxi Forestry Research Institute, P.R. China
Tsuyoshi.teramae.7140@idemitsu.com

Dr. Zhaoyuan Zhang, Senior Engineer, graduated from the Forest Tree Genetics and Breeding major of Chinese Academy of Forestry. Major in tree genetic improvement and efficient cultivation, such as Eucalyptus and Oil Tea, with some research experience in genetic diversity by molecular markers, genome-wide association study on some typical economic characters-oil and fat content with Oil Tea. Now is working at Guangxi Forestry Research Institute, Nanning Guangxi, China. By now, has presided or participated in 16 scientific research projects which also have won some provincial awards, such as Guangxi science and technology progress award, and Chinese Liangxi forestry science and technology award. Published 26 papers and 4 monographs co-edited with others.

THE INDUSTRIAL DEVELOPMENT OF *Aleurites montana* IN GUANGXI

Zhaoyuan Zhang[1], Riqing Zhang[2] , Wenhui Liang[1], Jinlin Ma[1,*], and Xing Zhang[1]
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[2]Central South University of Forestry and Technology, Changsha City Hunan PRC, 410000, P.R. China
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ABSTRACT

A.montana is an economic forest species with Chinese characteristics. It has a history of cultivation and utilization for more than one thousand years. The utilization is mainly Tung oil that is an important industrial oil. In recent years, due to the breakthrough in the deep processing and development of Tung oil, the demand is increasing day by day, which also brings new development opportunities for tung oil production. As one of the main producing areas of tung oil in China, Guangxi has a long history of cultivation and has made contributions to national construction. This paper mainly expounds the development of tung oil industry in Guangxi from the aspects of its industrial history, current situation of production, cultivation, processing and utilization, variety resources and scientific research, and analyses the existing problems in the development, and puts forward concrete countermeasures and suggestions for speeding up the development of tung oil industry.

Keywords : *A.montana*, Guangxi, Current situation, Development countermeasures

Aleurites montana, a deciduous tree of Euphorbiaceae, is native to China and has a history of cultivation and utilization for more than 1,000 years. It is a special economic forest species in China. It is also known as the four major woody oil tree species in China with *Camellia oleifera*, *Juglans regia* and *Sapium sebiferum* . It has the characteristics of fast growth, high yield per unit area, high oil content in seeds, wide adaptability, strong cultivation and wide economic use. Tung oil as the main product is an important industrial oil. It can be used to make paints and coatings. It can also synthesize many kinds of materials. It is a traditional export commodity in our country. It has a high reputation and influence in the world.

Guangxi is one of the main producing areas of *A. montana* in China. It has been an important economic tree species for farmers in mountainous areas in history and contributed to national construction. As a result of the rise of petrochemical industry, tung oil industry has undergone a considerable period of

downturn, sharp reduction in area and price decline. However, in recent years, due to the breakthrough in the deep processing and development of Tung oil, the price has risen year after year, the enthusiasm of farmers to grow *A.montana* has risen, and the planting efficiency has been continuously improved. How to rediscover the precious resources of *A.montana*, promote the rapid and large-scale development industry of *A.montana*, and play its important role in material industry, national defense industry, biomass energy and other aspects is a new issue faced by governments at all levels and forestry departments.

1. Utilization

Tung oil is the main product of *A.montana*. Tung oil is the oil extracted from Tung seeds. Its main component is a mixture of fatty acid triglycerides. Tung oil contains about 80% of the unique fatty acids in all plants on the earth - Tung acid, Tung acid has three conjugated double bonds, chemical properties of flexible, is a high-quality, multipurpose industrial raw materials. Tung oil is the best drying oil in vegetable oil in the world, and is the basic raw material for making the best quality paint and ink. It has many excellent characteristics such as fast drying, strong adhesion, light weight, luster, acid resistance, alkali resistance, high temperature resistance, frost cracking resistance, waterproof, anticorrosive, insulation, radiation resistance and penetration resistance. It is widely used in weapons, vehicles, ships, electronics, electrical appliances, buildings, decoration, machinery and fisheries. Environmental-friendly coatings with waterproof, anticorrosive and rust-proof properties. Tung oil is a high-quality raw material for biodiesel production. If *A.montana* is cultivated as a biomass energy tree species, it may become one of the most promising biomass energy tree species to alleviate energy shortage in China.

In recent years, the application of *A.montana* has shifted from the traditional field to the flourishing electronic industry, which has been widely used to produce impregnating materials for printed circuit boards as the heart of electronic products.

2. History and present situation of *A.montana* industry in Guangxi

2.1 Development History of A.montana in Guangxi

The 20-30's of the 20th century witnessed the rapid development of *A.montana* in Guangxi. In 1939, the total output of *A.montana* in Guangxi reached 19,500 tons, second only to Sichuan and Hunan, ranking third in the country, with a cultivated area of more than 266,000 hectares, which is the highest level in the history of *A.montana* production and cultivated area in Guangxi.

After the 1940s, due to the blockage of export, the output of tung oil and planting area decreased gradually. By the end of the 1940s, the output of tung oil in the whole region had risen to over 9700 tons, and the area of Tung forest had been reduced to over 160,000 ha. Basically, it is planted spontaneously by farmers, purchased by peddlers and hawkers. Private capitalists export tung oil after pressing and processing, or directly use it as waterproof paint.

In the early 1950s, with the recovery of the national economy and the implementation of the first Five-Year plan, tung oil production in Guangxi has been restored and developed to a certain extent. By 1957, the output of tung oil in the whole region had reached 10,050 tons, and the area of Tung forest had recovered to 2.55 million mu. But since 1958, because of various reasons, the production of tung oil has deteriorated. By 1976, the output of tung oil in the whole region was only 5,000 tons, and the area of Tung forest was more than 80,000 ha.

Since 1979, due to the implementation of a series of rural policies, tung oil production in Guangxi has been developed to a certain extent. Since 2007, as the price of tung oil has risen, the cultivation area of tung oil has risen, but the development is slow. According to the latest second-class survey data, the area of tung oil in Guangxi is 161,200 ha . The average yield of tung oil increased from 64-80 kg/ha in the past to 195 kg/ha in the whole area, and the average yield of tung oil in small area exceeded 495 kg/ha.

2.2 Area and Distribution of A.montana in Guangxi

According to incomplete statistics, the existing area of *A.montana* in the whole region is 161,200 ha, accounting for 24.4% of the existing tung oil forest area of 661,600ha in the whole country; the annual output of tung oil is about 20,000 tons, accounting for 29.6% of the total annual output of tung oil of 67.5 million tons in the whole country, ranking first in the country.

A.montana is distributed all over Guangxi, mainly in Baise and Hechi cities, of which Baise accounts for 65.5% of the total area and Hechi accounts for 29.8%. The traditional *A.montana* producing areas in

central and southeastern Guangxi are shifting to northwest Guangxi, mainly because the comparative benefits of *A.montana* in central and southeastern Guangxi are not as good as those of eucalyptus, citrus and sugarcane industries, while the benefits of *A.montana* planting in Western Guangxi are relatively high.

The reserved area of *A.montana* in Guangxi is relatively large and its distribution is relatively concentrated, mainly concentrated in seven counties, such as Tian'e, Leye, Longlin, Xilin, Nandan, Donglan, Jinzhongshan and Yachang, with a total area of 156573.1hm^2, accounting for 97% of the *A.montana* area in the whole region. This is conducive to the regionalization of the development of *A.montana* industry, improve the competitiveness of tung oil industry.

From the picture, we can see that the yield of *A.montana* seed reached the lowest level in 2003. To a great extent, the purchase price of Tung seed in 2003 was too low. The cost of collecting *A.montana* seed was lower than the income of selling, and the seed rotted on the mountain.

2.3 Variety Resources of T A.montana in Guangxi

There are abundant germplasm resources of *A.montana* in Guangxi (Table 4). It is mainly planted with *A. fordii*. Nandan centenary tung, Longsheng pagoda, Gongcheng are famous for the clones of annua Tung and Guiyong 27. Guiyong 27 and other four millennial Tung clones have passed the approval of the National Committee for the Examination and Approval of Fine Varieties, which can be vigorously popularized and applied within its suitable range. At present, Chongzuo Forestry Institute and Hechi Forestry Institute have preserved relatively complete experimental demonstration forests of families and clones. There are also a large number of selected excellent trees, which are good materials for breeding and further promotion and application, providing a rich variety of good gene types.

2.4 Scientific Research Status of A.montana in Guangxi

From 1935 to 1939, Lin Gang, a forestry scholar, conducted an experiment of *A.montana* grafting in Shatang, Liuzhou, Guangxi, and achieved good results. From 1938 to 1942, when Mr. Wu Qingquan, a forestry scientist, was appointed Technical Commissioner of Guangxi Extension and Breeding Station of the Central Ministry of Agriculture and Forestry in Liuzhou, Guangxi, he was engaged in the research of Tung varieties and the promotion of Tung cultivation techniques. He published the article "Study on Tung Varieties" and proposed that Tung varieties with high oil content should be selected and bred. The scientific research level of *A.montana* in Guangxi is in the forefront of the whole country. The selection of fine trees of tung tree in Guangxi has been carried out earlier and a series of scientific research achievements have been achieved. In 1986, it won the third prize of scientific and technological progress in Guangxi. In 1987, it won the third prize of national invention, the third prize of high-yielding fine clones and breeding and Cultivation Techniques of tung tree in China, such as Guiyong 27, Guiyong 1, Guiyong 2 and Guiyong 6. Resources Research was awarded the second prize of Science and Technology Progress of Ministry of Forestry in 1995 and 1996, the third prize of National Science and Technology Progress, the second prize of Guangxi Science and Technology Progress in 1989, the third prize of Guangxi Science and Technology Progress in 1989, and the third prize of Guangxi Science and Technology Progress in 1989. After years of efforts, 4 high-yielding clones of Millennium Tung have been bred, which is more than twice the yield of other Millennium Tung. Among them, Guiyong 27 achieved an average yield of nearly 1,500 kg/ha in 6 years, which greatly exceeded the requirement of 220 kg/ha put forward by the Ministry of Forestry. Guangxi Academy of Forestry has compiled Technical Regulations for High Yield Forest of *A.montana* in Guangxi, which was promulgated by Guangxi Quality and Technical Supervision Bureau. It has made normative requirements for *A.montana* cultivation in Guangxi and standardized cultivation in Guangxi.

2.5 Production Base of A.montana Varieties

Quality seed orchards and nurseries of collection: The construction of Tung seed orchards and nurseries of collection began in the 1980s. As early as 1985, Guangxi established a germplasm resource nursery and nursery of collection for *A.montana* in Guangxi. Thirty-six superior trees of 51 varieties and 225 superior trees of 5 groups of tung trees in three years and 225 superior trees of 3 groups of *A.montana* in Millennium were collected. The area of *A.montana* area was 2.0 ha and 1.9 ha respectively. The *A.montana* area was also divided into Hechi area, Baise area, Guilin area, Liuzhou area, Nanning area, Wuzhou area and other provinces. It provides material basis for the development of *A.montana* in Guangxi.

Maternal forest: After successive construction, the maternal forest of *A.montana* in Guangxi has reached 5000 mu, which can produce and supply 86000 kg/year seeds. In the middle of the period, seven varieties identified in the first batch have been established in the original place to purify and rejuvenate the maternal forest, with 100 mu of each variety. Although the current management of maternal forest is not in place, most of them have been gone out of cultivation, but after tending, rejuvenation and other work, the seed production capacity of maternalforest can be restored for 1-2 years.

2.6 Situation of A.montana Processing Industry in Guangxi

China is the largest producer and exporter of tung oil in the world. In recent years, the output of tung oil has generally stabilized at 130,000-180,000 tons, and the extraction of tung oil has reached 30,000-50,000 tons. It is exported to Europe and North America in large quantities every year, with a broad market prospect. The annual output of tung oil in Guangxi is about 40,000 tons (according to the data obtained from interviews with various tung oil processing factories in Guangxi), of which about 30,000 tons are crushed oil, the quality is well-known in the country, and about 10,000 tons are extracted oil. The quality standard of extracted tung oil products has not been unified, which restricts the promotion of *A.montana* to a certain extent. Guangxi Academy of Forestry has taken on the local standard of "extracting Tung oil" issued by the Forestry Department. The contract number "LYDB-450027" will make standardized rules to follow for improving the market of extracting tung oil in Guangxi, . Guangxi has many tung oil processing enterprises, of which Tian'e Tianquan Tung oil Co., Ltd. is the largest and best one. The company designs an annual production capacity of 50,000 tons, and produces 20,000 tons of tung oil in 2009. The company imports drying units from Wuhan. The oil yield of fresh seeds reaches 25%-28%, which is about 3 percentage points and is higher than traditional methods. The products are popular in the domestic market and seldom exported. They are mainly sold to Jiangsu, Zhejiang and Guangdong.

3. Problems in the Development of *A.montana* Industry in Guangxi

3.1 Serious loss of A.montana Germplasm Resources

Due to the influence of market instability in recent years and the impact of other industries, the state and local governments have suspended all their funding for research and production of *A.montana* from the early 1990s. A large number of researchers have turned to other economic tree species and research bases have also been used for other purposes. Even if a small number of bases have been retained, because of long years of barrenness, a large number of *A.montana* have died, and some of them are preliminary. Breeding of fine types and germplasm resources has disappeared, and seed orchard industry of *A.montana* is almost exhausted, which is not conducive to the breeding of fine varieties of *A.montana* and afforestation of fine varieties. At present, the management and protection of collection nurseries and scion nurseries in Guangxi are not in place. Some of the collection nurseries with poor growth have already been other species of this species. At present, *A.montana*germplasm resources are being re-collected, preserved and improved variety breeding, seed orchards and heading nurseries are also being restored.

3.2 Extensive management and low comparative benefit

Very few places adopt specifications to dig ridges and prepare land for afforestation, and the mixed planting of Tung crops is widely used. After three years of mixed planting, the soil fertility declines, that is to say, the exclusive farming of *A.montana* is abandoned. After entering the fruiting period, the people only cut down grass once a year before the *A.montana* fruit ripened to facilitate, never reclaimed or fertilized, and did not loosen the soil and fertilize. Under such extensive management conditions, the soil compaction affects the growth of root system, and it is difficult to restore the growth of tree potential after the results, resulting in premature senescence, low yield per unit area and low planting efficiency. In addition, the diseases and insect pests are serious. For example, Fusarium wilt is common in Tian'e County, which causes death every year.

4. Countermeasures and Suggestions for the Development Industry of *A.montana* in Guangxi

Based on the basic strategic considerations of the development of *Vernicia fordii* industry, it should be developed into a new energy industry, environmental protection industry and new material industry, which are all the industrial fields actively advocated and emphatically developed by the state. Therefore, the state and autonomous region must correctly guide, increase investment and scientific and technological innovation,

strengthen the development of new products, vigorously develop resources and bring into production. Business is bigger and stronger.

(1) To include industry in the development of characteristic industries in the autonomous region. It should be formally included in the development of key industries with strategic biomass energy, new materials and environmental protection, and priority should be given in terms of policies, funds and technology. We should further strengthen the leadership and technical guidance of the industry, do a good job in the management and protection of existing resources, and strictly prohibit any damage to resources. At the same time, we will further strengthen publicity and information services and expand the sales channels and market development of *A.montana*. Establish a subsidy system for the development of biomass energy forest and biodiesel enterprises to ensure *A.montana* as a strategic need for biomass energy development and sustainable development needs of the industry.

(2) Do a good job in the planning and layout of industry. According to the cultivation and distribution,the quantity of resources and the need of national construction for energy, new materials and environmental protection, the industrial layout and overall planning of *A.montana* should be done well, so as to develop new products to stimulate the development of resources, to cultivate and expand the industrial scale with resources, to fully tap the productive potential, to optimize the allocation of resources, to do a good job in the industrial layout and to increase the total amount of resources. In the south subtropical region and part of the middle subtropical region, we should focus on the development of *A.montana* to meet the national energy demand for biomass energy (biodiesel); in the middle subtropical region, we should focus on the development of *A.montana* to ensure the national demand for new materials, high-grade environmental protection special paints, and promote the healthy and orderly development of *A.montana* industry.

(3) Increase the breeding of fine Tung varieties. In view of the insufficiency of breeding ability and low proportion of fine Tung varieties in Guangxi at present, the basic work of breeding fine Tung varieties in Guangxi should be strengthened. Therefore, the research on breeding and introduction should be strengthened to breed varieties of *A.montana* with high yield, high quality and strong resistance as soon as possible.

(4) Strengthen the renewal and transformation of existing low-yielding forests. In view of the fact that the proportion of low-yielding tung trees is very high at present, first, the Tung forests with aging, decay and poor quality should be regenerated and transformed with good varieties to cultivate new high-yielding and good-quality Tung forests directly; second, for those low-yielding forests with better varieties, better site conditions and potential for increasing yield, measures such as medium tillage, weeding, tending and fertilization should be taken to improve the unit aspect of Tung forests. Accumulated output and operating benefit. Thirdly, we should synchronize the development of Camellia oleifera industry. We should guide the majority of oil-tea planters to promote the increase of *A.montana* area by interplanting, and solve the problems of shading and income in the early stage of oil. In the processing, we can combine the existing oil-tea pressing equipment to carry out the initial processing and reduce the duplicate investment.

(5) Fostering leading enterprises and well-known brands. We should integrate the existing *A.montana* processing enterprises scientifically and rationally, take the market as the guide, strengthen the allocation of resources, take appropriate preferential measures, support the existing leading enterprises in *A.montana*processing from the aspects of policy, capital, technology and forest land circulation, encourage them to cooperate with farmers in building raw material forests, strengthen technological innovation, widen the field and scope use, and develop intensive and high-end processing products. To increase the added value of products and promote the development of industry. At the same time, we should establish brand concept, strengthen quality management, create well-known brands, and create new well-known enterprises and brands.

(6) Innovating the system and mechanism for the development industry. Innovating the management mode of production. Leading enterprises should be guided and encouraged. According to the principles of government guidance, voluntary participation, free withdrawal and democratic management, professional cooperative

economic organizations and industry associations should be set up to actively explore such business models as "enterprise + base + peasant household", "company + Economic Cooperation Organization + peasant household", "branch + association + peasant household", and gradually establish and improve leading enterprises and economic cooperation. Organize and share risks and interests with farmers. Establish the mechanism of industry leading agriculture and promoting industry by agriculture. Accelerate the pace of marketization, industrialization, scale and standardization of industry development.

(7) Increase investment in scientific research and strengthen scientific and technological support. Increase investment in the development industry, and include cultivation, breeding and processing in relevant research projects such as national scientific and technological support. Making full use of the technical strength of domestic universities, scientific research institutes and professional institutions, building a capable and effective professional technical team, vigorously carrying out scientific research work, cultivating new varieties, expanding the area resources, and developing new products , to provide strong technical support for the development. Comprehensive utilization of existing fine varieties and High-yielding Cultivation techniques, strengthen tending management, increase yield per unit area and economic benefits, and increase farmers' enthusiasm for managing *A.montana.*

Invited Presentation

BIOECONOMY

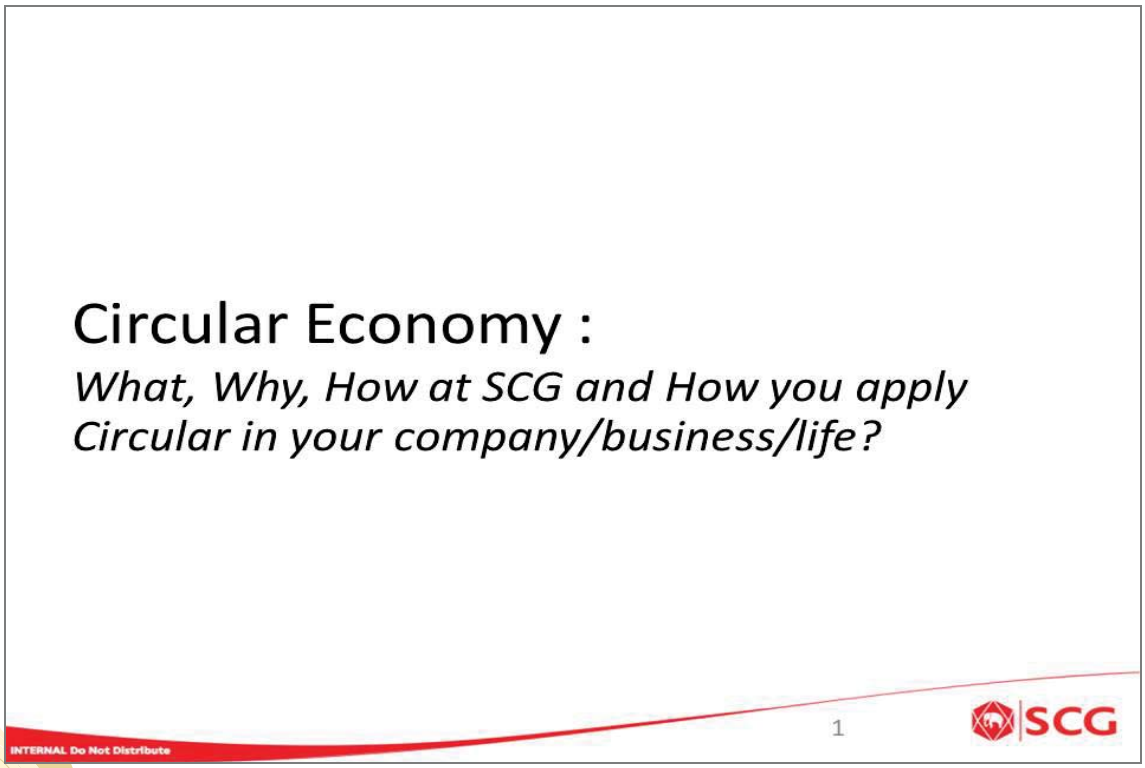
Invited presentation 1
Mr.Narongrit Pinyotrakool

Environment Manager
Sustainable Development Office,
SCG Cement Company Limited, Thailand
narongrp@scg.com

Mr.Narongrit Pinyotrakool holds Bachelor degree of Science (Chemistry) from Chulalongkorn University. He started his work as the Chemist-Quality Control at National Petrochemicals Co., Ltd. After that, he moved to Bangkok Synthetics Co., Ltd. as the Assistant Technical Manager for laboratory. Since 1996, Mr.Narongrit has joined Siam Cement Group (SCG) Public Company Ltd. During 1996-2008, he was the manager of laboratory at Rayong Olefins Co., Ltd, SCG Chemicals and the manager of SHE at Rayong Olefins Co., Ltd, SCG Chemicals during 2007-2009. In 2009, he became the Executive Consultant-Quality Assurance/Product Liability at Corporate Excellence and Sustainable Development, SCG. He is now the Environment Manager at Sustainable Development Office, SCG. He is also the member of Environment Excellence Committee, Water Management Committee and Waste Management Committee at SCG.

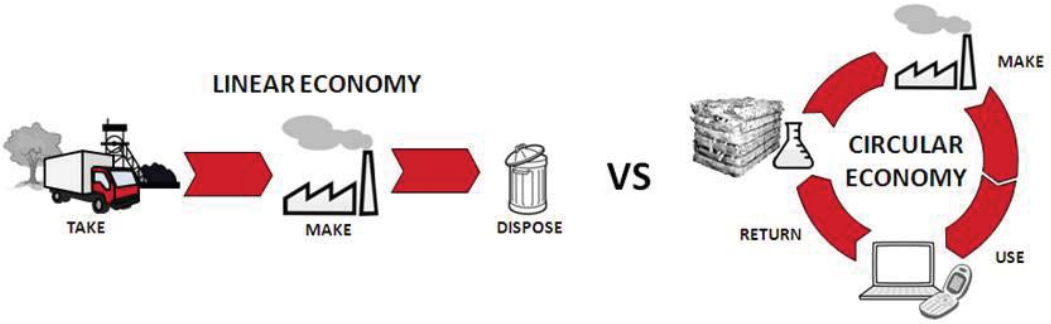
BIO ECONOMY ACTIVITIES IN SCG

Narongrit Pinyotrakool
Sustainable Development Office, SCG Cement Company Limited, Thailand
E-mail: narongrp@scg.com



What is Circular Economy?

From linear to circular



From **take-make-dispose** to **make-use-return**

Recommended Reading

wbcsd

World Business Council For Sustainable Development
https://www.wbcsd.org/

CEO Guide to the Circular Economy
https://www.wbcsd.org/Programs/Circular-Economy/Factor-10/Resources/CEO-Guide-to-the-Circular-Economy
https://www.wbcsd.org/contentwbc/download/3418/44718

ELLEN MACARTHUR FOUNDATION

Ellen MacArthur Foundation
https://www.ellenmacarthurfoundation.org/

Why we have to Change...in 2050 world population is expected to be 9.8 Billion, with this consumption rate we need the resource 4 times of what the world have and what will also happen...

What we have

What we need in 2050

+ Climate Change

Shortage of natural resources

World population has jumped dramatically from 7.6 billion in 2018 to 9.8 billion in 2050.

The global demand for resources is expected to reach 130 billion tons by 2050, which is more than 4 time of earth's total capacity

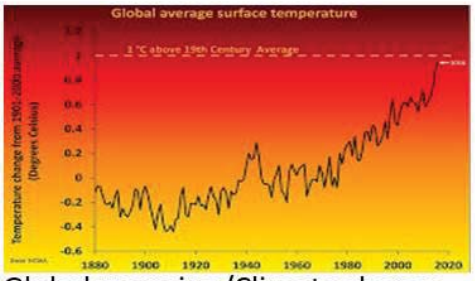
Source: CEO Guide to the Circular Economy, WBCSD

Why we have to Change...increasing in world population generates more emissions & problems and consumes more natural resources...

Overwhelming of garbage

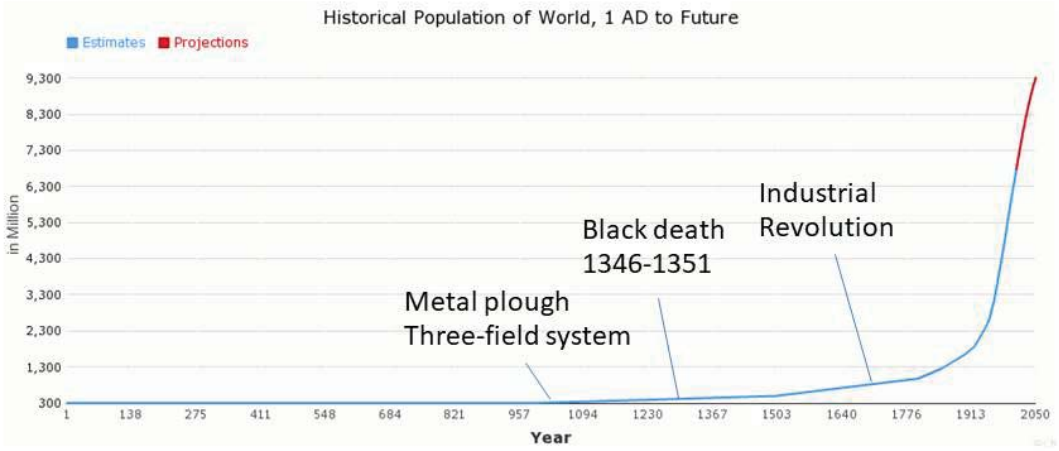
Plastic in the ocean

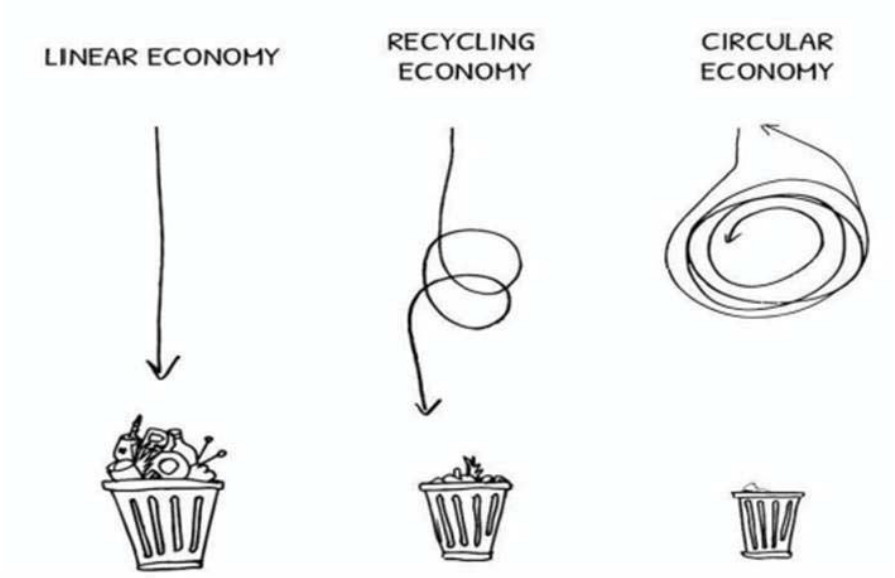
Resource scarcity

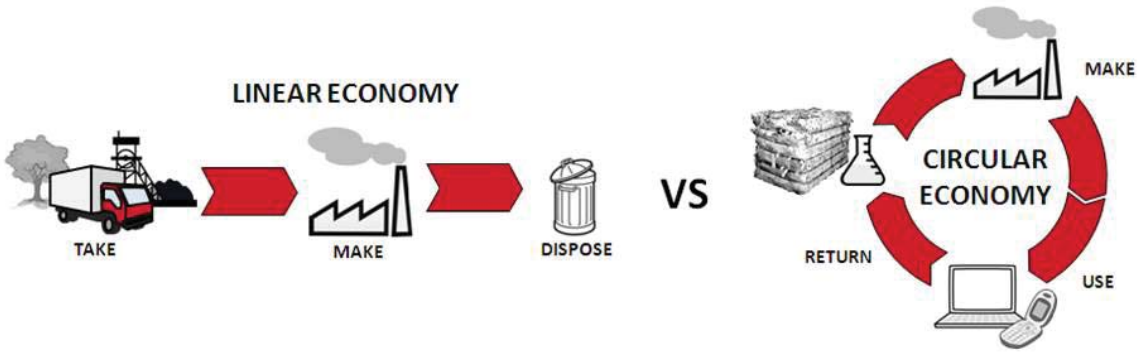


Global warming/Climate change

World Population







Circular Economy Activities Along The Value Cycle

BUY materials that are regenerative or recycled

COLLECT AND RECYCLE products and materials at the end of their current life to close the loop

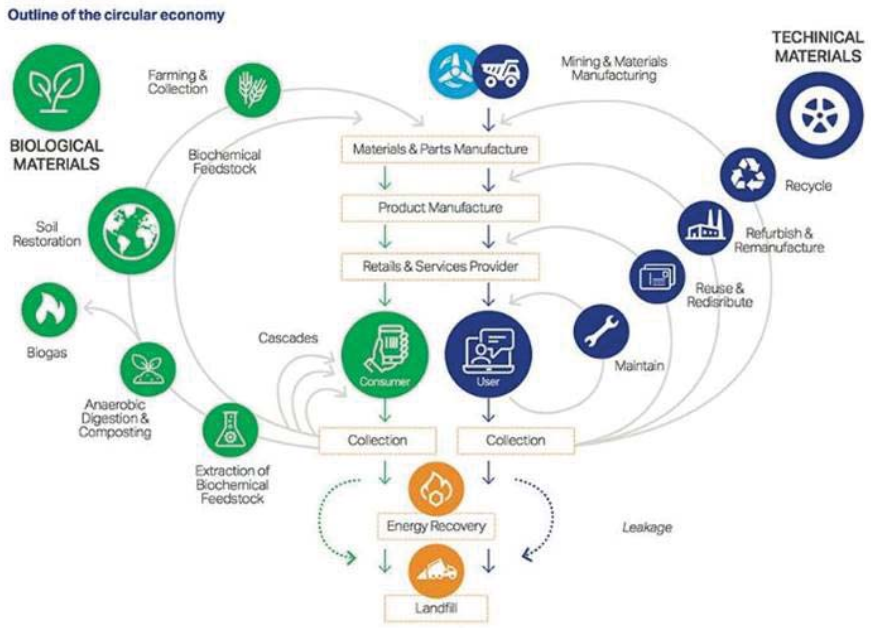
DESIGN products that are recyclable and reusable

MAKE products as waste-free and resource-efficient as possible

SELL access (rather than ownership), leasing or sharing products

USE products responsibly, not excessively, to extend their lifetime and to reduce pollution

Circular Economy-Butterfly Model



Circular Economy...the change of use of natural resource to maximize use and minimize taken of natural resource as much as possible

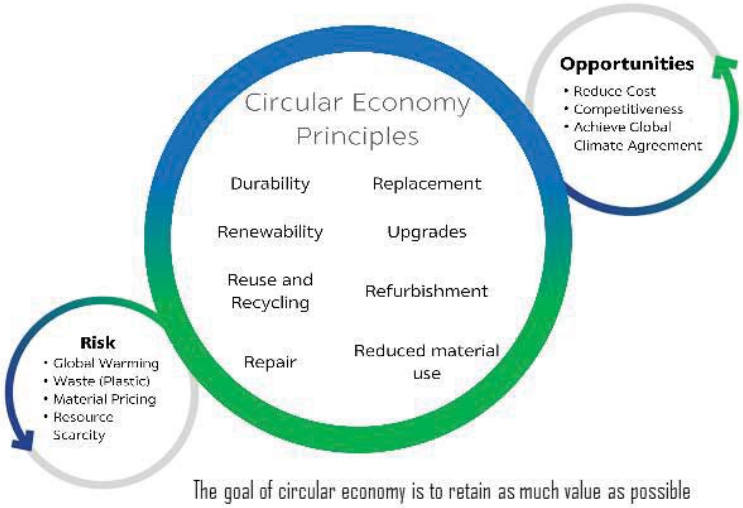
LINEAR ECONOMY

We take the natural resources, make the products, use them and dispose them without recycle

CIRCULAR ECONOMY

We take the natural resources, make the products, use them until end of life and return them to make the products again

8 Basic Principles and 5 Business Models that will help you implement the circular economy



The goal of circular economy is to retain as much value as possible from resources, products, parts and materials to create a system that allows for long lifetime, optimal reuse, refurbishment & remanufacturing and recycling

- PRODUCT LIFE-EXTENSION
- CIRCULAR SUPPLIES
- RESOURCE RECOVERY
- PRODUCTS AS A SERVICE
- SHARING PLATFORM

INTERNAL Do Not Distribute

12

SCG

Global Practices in Circular Economy

P&G stores PP from plastic packaging by removing contamination and color and use in board range of applications

Unilever uses recycle plastic from sachet waste, and the plastic is then used to create new sachet for Unilever products

Canon collect used parts and remanufactured new products from reused parts and recycled plastic

MICHELIN creates Fleet Tire Management solution in which customers pay a monthly fee for traveled kilometers (or miles) instead of tires

PHILIPS provides lighting as a service instead of selling light bulb

DSM innovates for fully recycled carpets

IKEA designs furniture that customer can create their own combination and offer the cover to change the look or replace cover when it is worn out or dirty

NETFLIX started as a disruption to the video rental industry, offering DVDs delivered to residences instead of requiring customers to visit brick-and-mortar stores.

INTERNAL Do Not Distribute

13

SCG

Call to Action!

Set Circular Vision

Choose Circular Model

Work with team

Start small and scale-up

Collaborate

Track progress

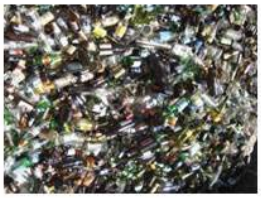
Materials that can be Recycle now a day as Usual

Scrap to Steel

Cans to Aluminum

PET to Fiber

Waste Paper to Paper



Glass Bottles to Glass

Plastic to ??

Electronic parts to ??

SCG takes a leading role in Circular Economy and successfully gain traction through SD symposium in 2018

- >1,000 participants + 55 CEOs from all sectors
- 4 cases of collaboration shared in the forum
- Gained awareness from public and private
- Stimulate collaboration among stakeholders

SCG SD Symposium 2018, Bangkok

SCG Circular Economy Business Cases: Circular Loops

Circular Packaging Paper

Circular Plastics

Circular Building Materials

CIRCULAR ECONOMY

3 strategies

REDUCED MATERIAL USE / DURABILITY

UPGRADE & REPLACEMENT

REUSE & RECYCLE

GREEN CARTON
Reduce **25%** raw material usage

SINGLE-USE PLASTIC
reduce from 36% to **20%** within 10 years

MARINE CEMENT
Last longer than ordinary cement

ACTIVE AIRFLOW
Reduce electricity consumption in habitat

POLYETHYLENE: SMX
Higher strength, use less materials, and recyclable

HYBRID CEMENT
Use less raw materials in production process

PAPER BAILING STATION

HEAT INSULATION
from used glass bottles

CIERRA

FUNCTIONAL MATERIALS
make more mono materials packaging

FISH HOME
from plastic waste

INTERNAL Do Not Distribute

Partnership Model for Enhancing Circular Economy

Greenovation Lube Packaging Circular Economy

Plastic wastes mix Asphalt, a Circular Economy for Road Construction

Paper Recycling Through Value Chain Collaboration

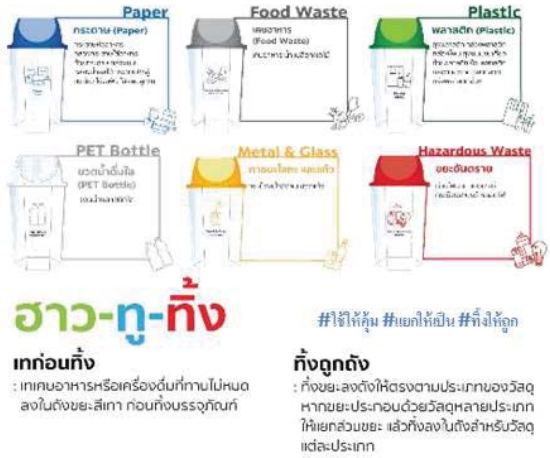
Glasswool Insulation Waste Recycling Collaboration

19

SCG

INTERNAL Do Not Distribute

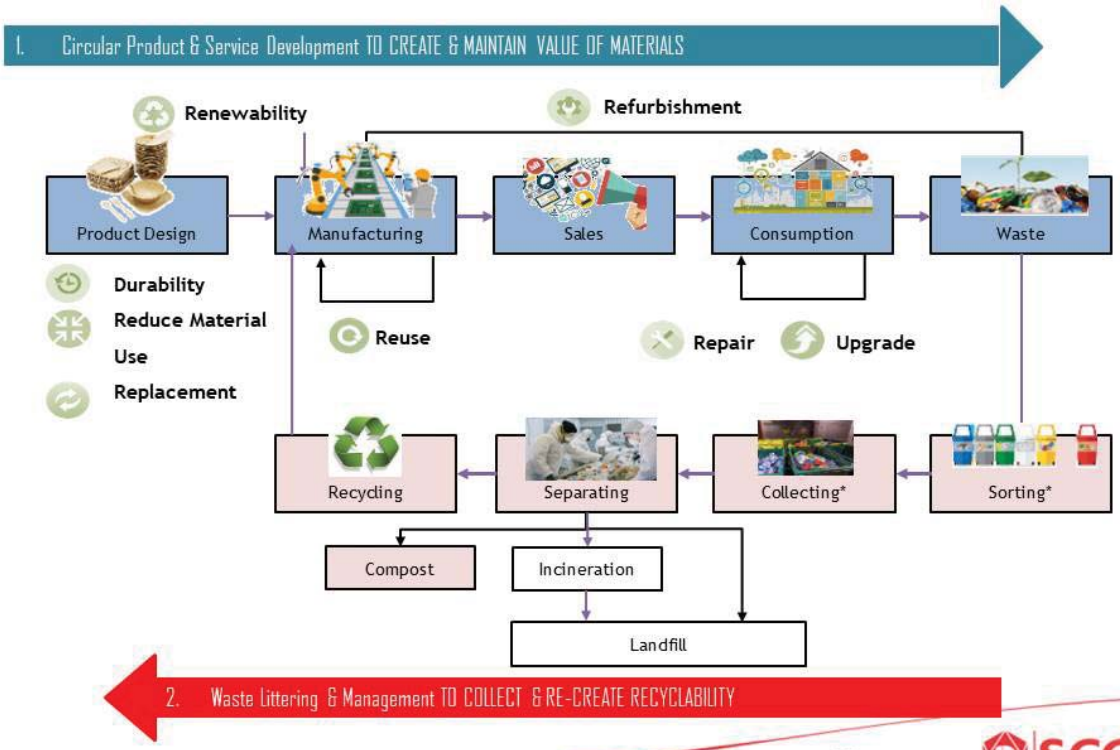
Create Model at Bangsue by Raising Employee Awareness



- **Replaced** all random number of trash bins with packs of trash bins at all locations in Bangsue **(160 packs)**
- **Kicked-off** in Leadership Forum: 25th October 2018
- **Launched** at Bangsue Complex: 29th October 2018

20

Circular Economy Business Model



21

Thailand's Waste Infographic...Is it now the time to minimize our waste problem? Good waste management and turn to benefit by Circular Economy

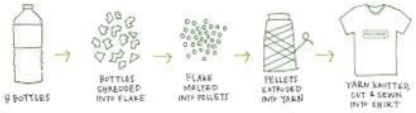
Plastic Wastes / M Tons
-Recycle 1 M Tons
-Not Recycle 6 M Tons

Suggested Solution for Municipal Waste to 100% Managed/Recycle minimized/zeroing Landfilled

- Food Waste → Soil, EM
- Recyclable Waste
  - Metal, Al, Glass, Paper, PET Bottle, Batteries → Recycled Materials
  - Plastic Bag, Bottle, Wrap, Tire → PCR / Upcycling / Plastic Road
- Non-Recyclable Waste → Contaminated Plastic & Paper → RDF-Wet / RDF-Dry / Landfilled → Waste to Energy
- Hazardous Waste
  - Electronic Waste, LED Lamp → Parts Recycled & Proper Treatment → Landfilled
  - Lighting Batteries

Upcycling/Down-cycling...in case of not recycle to its loop, materials are useful in other loops or turn it to energy

PET to Fiber



PET Bottle or used fishnet to shirts or sport shoes or carpets

Asphalt-plastic road

Waste to energy

Energy Recovery and Alternative Raw Materials from Waste

- Reduce material use
- Renewability/ Recycle

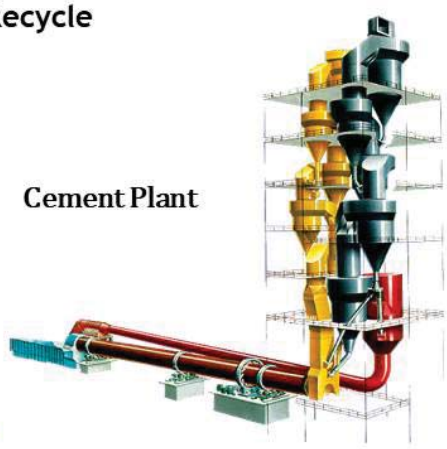
Alternative Raw Material (AR) from Waste

Sludge

Ash

Slag

Cement Plant



Alternative Fuel (AF) from Waste and Biomass

Industrial Waste

Biomass

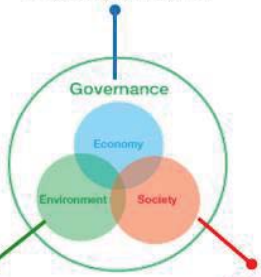
RDF

SCG balances value of the triple bottom lines in Sustainable Development framework reinforced by Circular Economy concept

SCG Sustainable Development Framework

Growing Economy

Create values not exclusively for profitability, but create mutual benefits for all stakeholders



Healthy Environment

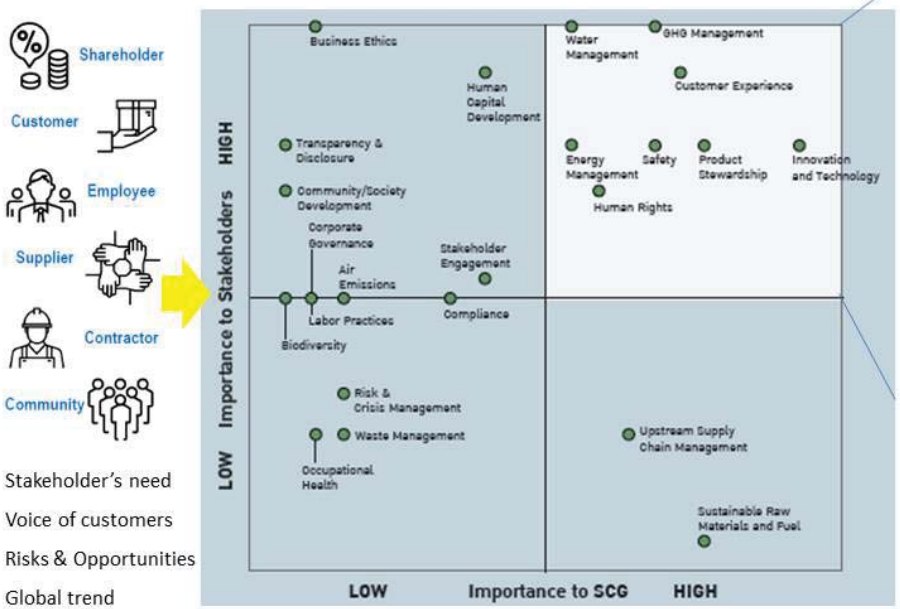
Commit to conservation of the environment and natural resources wisely & maintaining ecological balance

Happy Society

Conduct business with ethics and concern for social responsibility, improve the quality of life where SCG operates

Defining Priorities - SCG Materiality

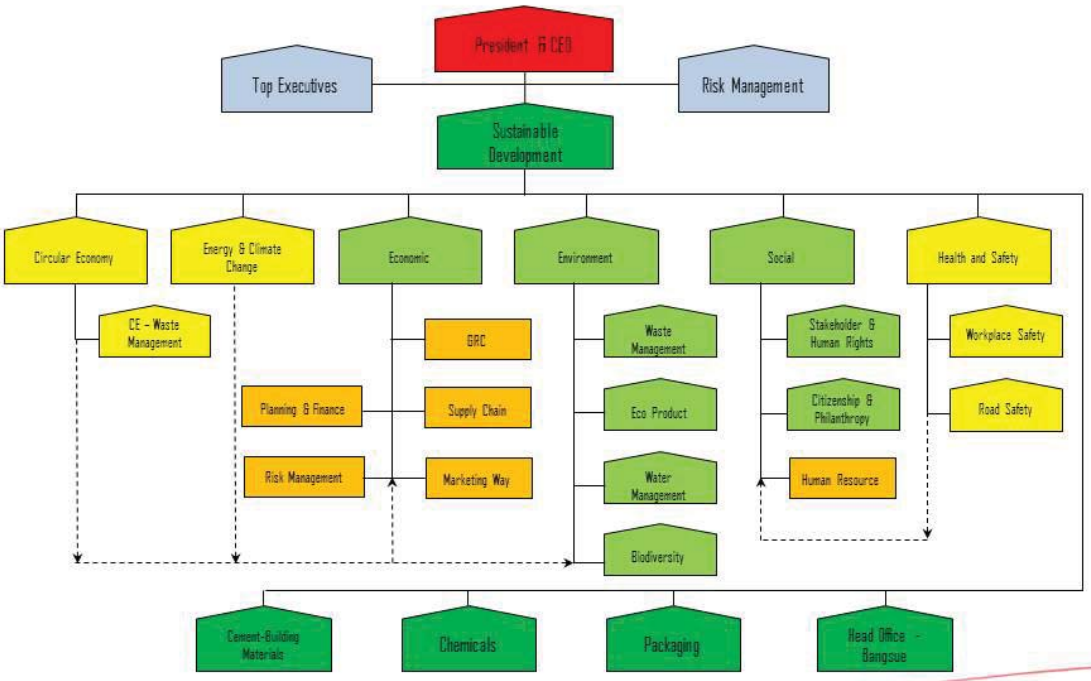
SCG Materiality Matrix 2018 - 2020



SCG Materiality

1. Customer Experience
2. Innovation & Technology
3. GHG Management
4. Product Stewardship
5. Safety
6. Water Management
7. Energy Management
8. Human Rights

Sustainability Management Structure



… and develops challenging Circular Economy goals for sustainable business executions

SCG's Sustainable Development Goals

Reduce 10%
GHG per production unit in 2020

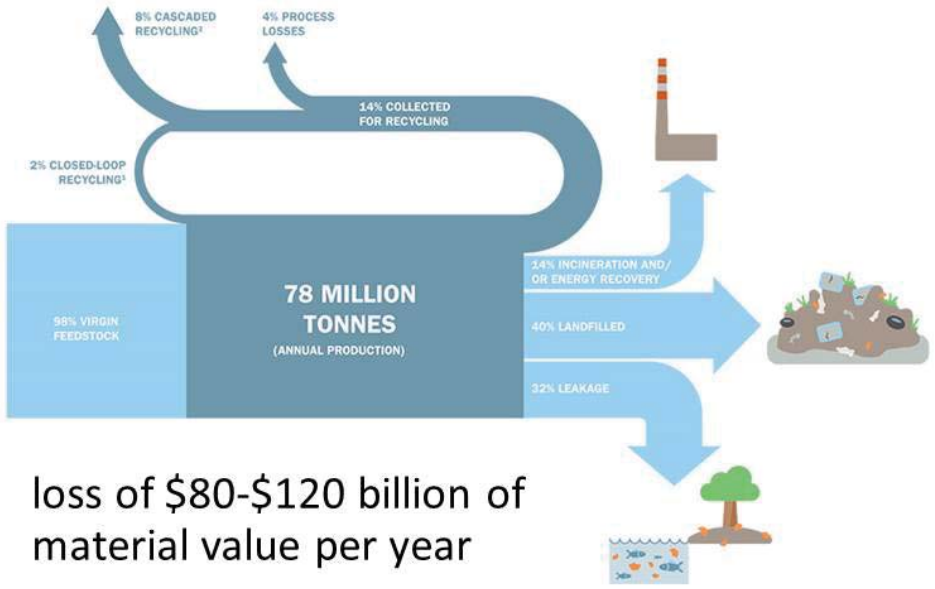
Zero waste to landfill
Reduce 20%
waste disposal per production unit in 2020

Reduce 20%
water withdrawal per production unit in 2020

Increase >1/3
sales of SCG eco value (products & services)

- **Lead collaboration** for end-to-end waste management
- **Reduce single-use applications** through innovations
- Launch **more recyclable or reusable** products

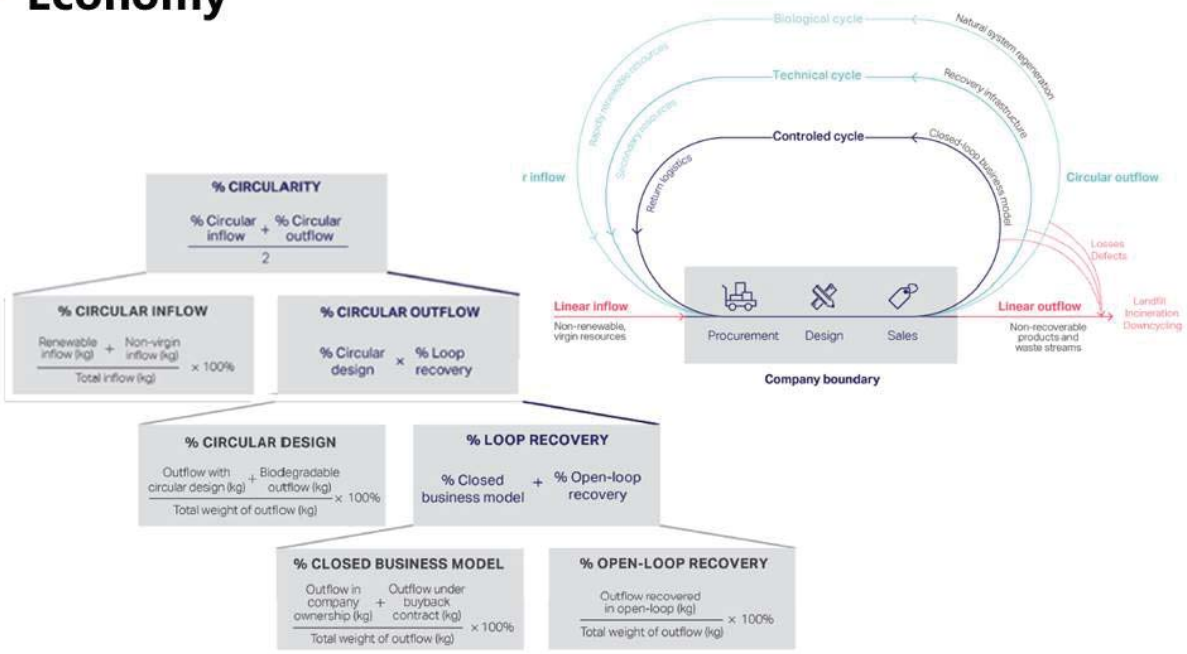
Circularity Metrics-Circular Flow Model



loss of $80-$120 billion of material value per year

https://www.packagingstrategies.com/articles/89932-addressing-the-plastic-recycling-issue

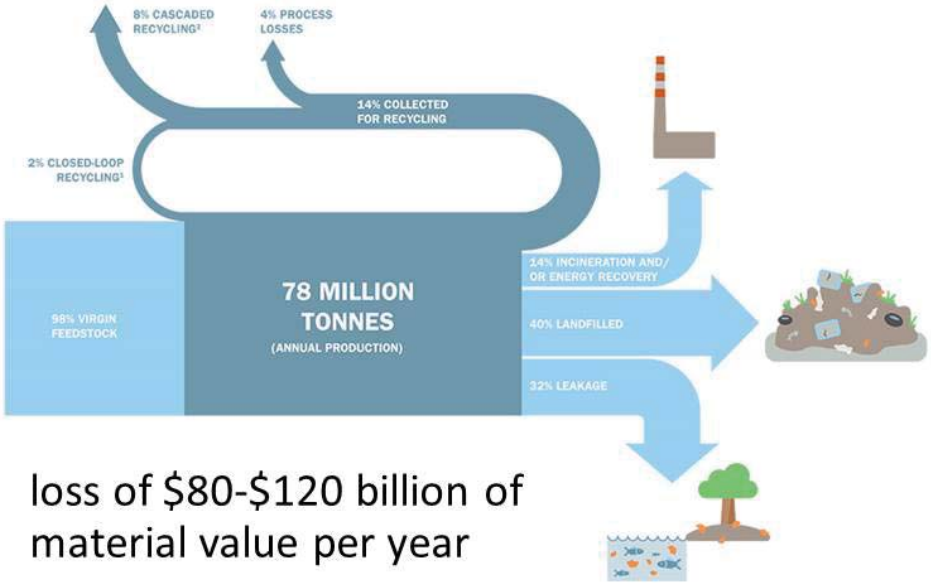
Circular Metrics-Measurement of Circular Economy



Now Factor10 Group/WBCSD is currently updating the guideline and will publish again.

Green Packaging
บรรจุภัณฑ์เพื่อสิ่งแวดล้อม

Circularity of Plastics



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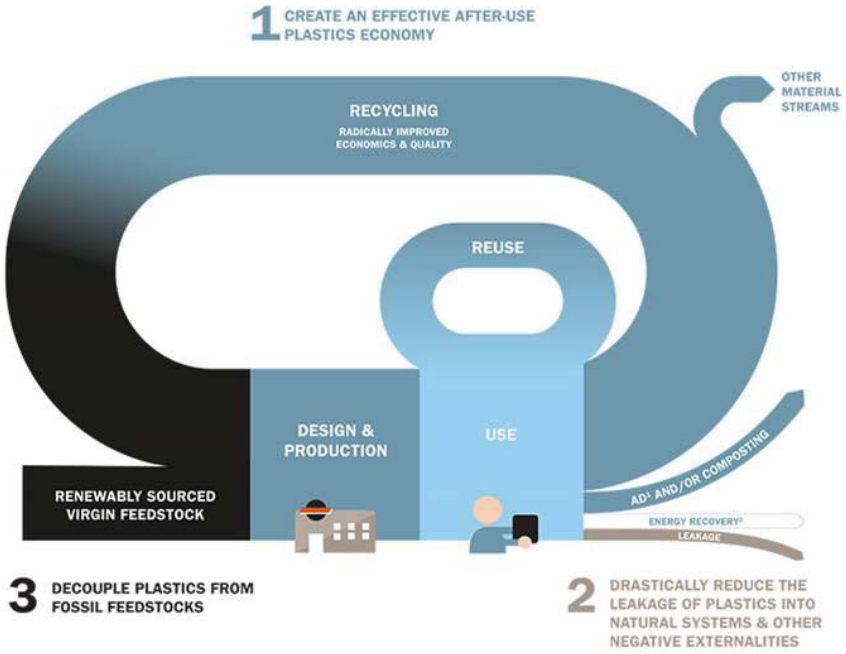
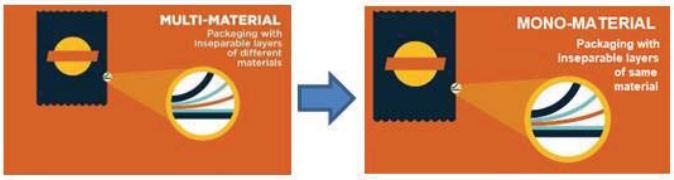
Circularity of Plastics



Circular Economy Strategic Direction to Reduce Plastic Wastes

CIERRA™

Functional Material developed for many application: such as:
Oxygen barrier in food packaging that replace Aluminum which made multilayer packaging non recyclable

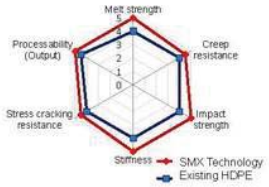


Non-Recyclable Recyclable

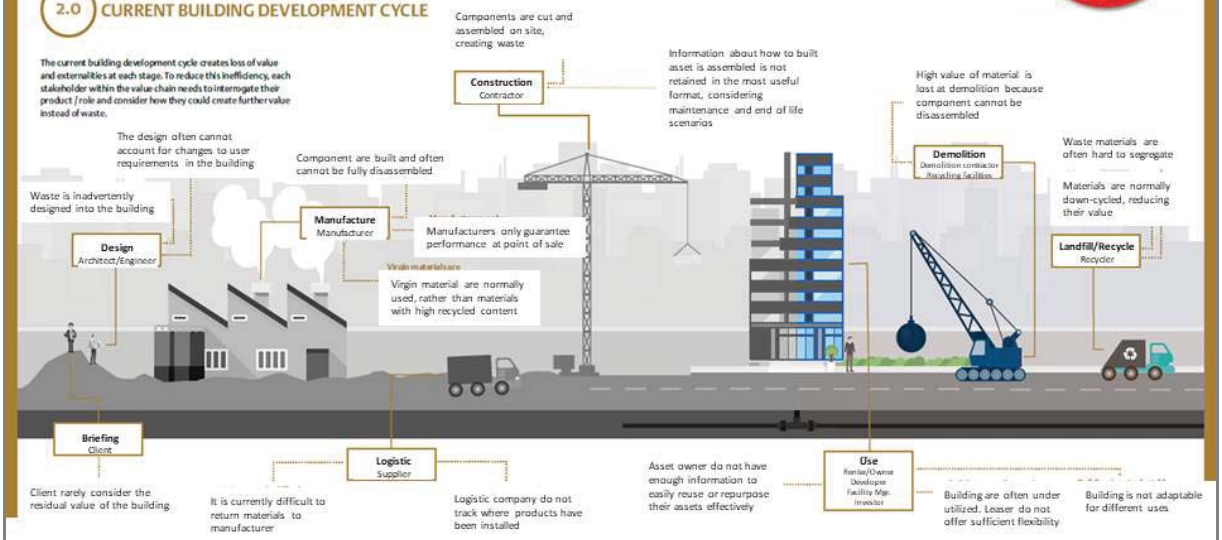
New PE Resin by SMX Technology

- Higher strength & wider film
- Lighter weight cap & closure
- More % recycle content to make recycle materials

Reduce Single use non circular plastics
Develop plastic product portfolio to reduce domestic sales in single use non-circular applications



Circular Economy for Construction Industry



- Design and product more durable products
- Make the product that reduce waste at construction site
- Using BIM-Building Information Modelling
- Recycling/Upcycling demolition waste

Success Factors help Circular Economy

Awareness

Knowledge

Mindset

Leadership

Role Model

Scale-up

Communications

Innovation

Product Design

Government Support

Law

Collaboration

Business Models

Infrastructure

Promotion vs. Ban

Consumer Mindset & Behavior

Invited presentation 2
Dr.Kunn Kangvansaichol

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PERSPECTIVE OF MICROALGAL BIOREFINERIES

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ABSTRACT

Microalgae typically contain high protein, low or moderate fat and carbohydrate but depending on species, condition and stage of development. For some species, when stressed with low nutrient, they start to accumulate higher lipid, while some accumulate carbohydrate.

Microalgae has multiple commercial applications. First, they are used as the live feed for zooplankton and some early stage creatures such as bi-valve, shrimp, crab, etc. in aquaculture industry. Although, there are some interesting development to replace live microalgae with freeze-dry or oven-dry or other encapsulated format, live or concentrate microalgae still perform the best. Second, we know that microalgae are being considered as super food. Therefore, they are sold as food supplement such as *Spirulina, Chlorella, Haematococcus*, etc. They can be in capsule or tablet. Many developments use microalgae as food ingredient such as that in bakery, drink and flavoring. Third, there are use of carotenoid which are Astaxanthin from *Haematococcus pluvialis*, Anthocyanin from Spirulina, and others. Fourth, microalgae are used in tertiary wastewater treatment but not widely use as it is slow and requires large area of land.

As we face multiple issues such as shortage of water, food and arable land, one obvious application is "food". MIcroalgae can be a good source of protein in current and future food application such as New Wave Food, Food from Spirulina, Food from Algae Protein etc. The term "Single cell" means you grow what you want. High protein, or high fat, or high carbohydrate is considered strength but if we could find the use of some specific protein for some applications, it will be more profitable. Carotenoid is also another strength but it needs

to make sure that no other competitive route can achieve the same level of concentration and economic.The dilemma of lower water and lower energy for microalgae cultivation is somewhat questionable. In theory, it is possible but in reality, it may take sometimes before we reach that target. Currently we can achieve the spirulina biomass at 3-5 USD/kg from China (feed grade) and some other places in the world, dry weight. Soy with 0.4 USD/kg with approx. 10% moisture or 0.45 USD/kg that is around 10x lower than spirulina biomass.

The creative schemes include algae secreting products, milking algae product and maintaining the cell concentration, extraction without harvesting (some targeted product should be carefully selected). This is applicable for those high volume, moderate to low value. For those high value applications, the use of photobioreactor and small-scale production should justify the cost but novel discovery of specific molecule is the key driver. One of the high-value usages is in health and medicine. That includes the use of microalgae as vehicle to transfer the drug and medicine to targeted area inside our body, microalgae with specific drug molecule to act as vaccine to animal esp. in fish and aquaculture, etc.

The future is bright, but it yet needs breakthrough in the field of microalgae production, and the use of modified strains in some specific areas.

Keywords : Microalgae, Biorefinery

Invited presentation 3

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UNDERLYING MOLECULAR MECHANISMS OF OIL AND STARCH ACCUMULATION IN MICROALGAE TO ENHANCE ITS BIOFUEL POTENTIAL

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ABSTRACT

Microalgae accumulate triacylglycerols (TAGs), a promising feedstock for biodiesel production, under unfavorable environmental conditions for their survival. However, the underlying molecular mechanism of TAG accumulation in microalgae remains unclear. In our previous study, we showed that the target of rapamycin (TOR), a highly conserved serine/threonine protein kinase that plays pivotal roles in nitrogen and other signaling pathways in eukaryotes, is a checkpoint kinase for TAG accumulation in microalgae. For example, TAG accumulation can be induced after treatment of rapamycin, a TOR specific inhibitor, even under normal growth conditions. However, TOR-inactivation also resulted in an inhibition of cell growth. The same phenomenon is also observed under TAG accumulation conditions, such as nitrogen depletion, high-light conditions, etc., indicating “TAG accumulation” and “cell growth” are generally in a trade-off relationship. Thus, the achievement of “TAG accumulation” and “normal growth” at the same time may significantly improve TAG productivity. In this article, we will discuss our recent study in which we succeeded in improving the TAG productivity of a unicellular red alga *Cyanidioschyzon merolae* by more than 56-fold using molecular genetic approaches. Furthermore, a fundamental molecular mechanism for starch accumulation in the alga and one example of a novel utilization of algal starch will be described.

Keywords : Glycerol-3-phosphate acyltransferase, Triacylglycerol, Target of rapamycin, Endoplasmic reticulum, Red alga, Starch

INTRODUCTION

The effects of global warming are becoming more serious every year due to the increment of greenhouse gases such as CO_2 generated by the large consumption of fossil fuels. At COP24, which aims to solve this problem, the implementation rules of the Paris Agreement were adopted, and it was confirmed that developed countries and developing countries would work within the same framework for preventing global warming after 2020 (Cozier, 2019). However, the current reduction targets indicated by each country are pointed out as insufficient to prevent the serious effects of global warming, and effective measures are still required.

In order to reduce global CO_2 emissions, it is effective to use renewable energies instead of fossil fuels. Although there are several types of renewable energy that can be used by us, it is important to combine them well to stably supply the necessary energies, since each has its own merits and demerits. Oil production using microalgae is one of the renewable energy production systems that is expected to contribute significantly to the reduction of CO_2 emissions since microalgae use CO_2 from the air as a carbon source and have features such as high biomass production per unit time and unit area (Chisti, 2007). Among the oils produced by algae, triacylglycerols (TAGs) can be used as a liquid fuel (jet fuel or diesel fuel) after its transesterification (Chisti, 2008). For this reason, an increase in TAG production by microalgae directly leads to higher biofuel production.

Despite the superior properties of microalgae, construction of large-scale commercial energy production systems using microalga has not been realized so far. One of the obstacles behind this is the lack of understanding of the molecular mechanisms involved in TAG production. In this paper, we introduce a novel mechanism that regulates TAG production in the model microalga *Cyanidioschyzon merolae*, and show how TAG productivity was drastically improved based on our finding. Further, we have mentioned an underlying molecular mechanism of starch accumulation in the alga and its novel utilization for valuable chemical production in a biorefinery manner.

MATERIALS AND METHODS

Strains and growth conditions

Cyanidioschyzon merolae F12, SF12, C12 and T1 strains were grown in a liquid MA2 medium (Imamura *et al*., 2013; 2017). The cells were grown at 40°C, under continuous light (50 $\mu molm^{-2}s^{-1}$). The air was supplemented with the bubbling of 2% CO_2 (Imamura *et al*., 2017). For rapamycin treatment, *C. merolae* cells (OD_{750} = 0.2~0.4) were treated with rapamycin at a final concentration of 1.0 μM. For control experiments, an equal amount of DMSO was added to the culture medium, as rapamycin was dissolved in the DMSO solvent. Nitrogen depletion experiments were performed as described in Imamura *et al*. (2009). For cultivation of T1 and SF12 strains, uracil and 5-fluoroorotic acid were added to MA2 medium at a final concentration of 0.5 mg/mL.

Detection of lipid droplets and determination of TAGs in C. merolae

Lipid droplets (LDs) were detected in the *C. merolae* cells as described in Imamura *et al*. (2015). Briefly, BODIPY (400 nM) was added to 1 ml of the culture and incubated in the dark for 5 min at room temperature. Next, cells were centrifuged at 1000 X *g* for 5 min at room temperature and the supernatant was removed. The stained cells were observed in the light microscope (Olympus BX51). TAGs content in cells were determined by gas chromatography (Imamura *et al*., 2015). Briefly, *C. merolae* cells (40-50 ml) were harvested by centrifugation and the resulting cells were immediately frozen in the liquid nitrogen and stored at -80ºC until use. Lipid was extracted from 10-20 mg of freeze dried cells according to Bligh and Dyer (1959). The extracted lipids were separated using one-dimensional thin layer chromatography using hexane:diethyl ether:acetic acid (40:10:1, v/v). Lipids were visualized with 0.01% (w/v) primulin in 80% acetone (v/v) under UV light (365nm). The TAGs were extracted from the plate, converted to fatty acid methyl ester and analyzed with gas chromatography (Shimadzu GC-2014) as described in Imamura *et al*. (2015).

Construction of CmGPAT1 and CmGPAT2 overexpression strains

The two GPAT genes [*CmGPAT1* (*CMA017C)* and *CmGPAT2* (*CMK217C*) in *C. merolae* database, http://merolae.biol.s.u-tokyo.ac.jp] were amplified by PCR using primer sets:
for *CmGPAT1*, A017_pSUGA_F (5′-CGTTCGTTGACCCCCATGGCAGCGACCACCGCC-3′) and A017_pSUGA_R (5′-GTCGACTCTAGACCCGAAGCGAGCCGTCTTGGG-3′); for *CmGPAT2*, K217_pSUGA_F

(5´-CGTTCGTTGACCCCCATGCTTTTTGTACGCAACTG-3´) and K217_pSUGA_R (5´-GTCGACTCTAGACCCGCGCCAAATGCGCCGGCA-3´). Wild-type *C. merolae* genomic DNA was used as a template for the PCR. The amplified fragments were cloned into the *Sma*I-digested pSUGA vector (Fujii *et al*., 2013) using an In-Fusion cloning kit to prepare pSUGA-A017 (for CmGPAT1ox) and pSUGA-K217 (for CmGPAT2ox) plasmid. The pSUGA-A017 and pSUGA-K217 plasmids were transformed to T1 strain as described previously (Imamura *et al*., 2013) to construct overexpression strains of CmGPAT1 (CmGPAT1ox) and CmGPAT2 (CmGPAT2ox). A control strain (GPc) was obtained by transforming an empty vector pSUGA to T1 strain.

RESULTS AND DISCUSSION

Microalgae generally accumulate TAGs under environments that are not suitable for their growth. Among these environmental conditions, research on TAG accumulation has been conducted mostly under the nitrogen depletion conditions (Scott *et al*., 2010). However, the underlying molecular mechanisms of how TAG accumulation is regulated in response to the nitrogen depletion conditions is not well understood, although the information is indispensable for improvement of TAG productivity.

We previously showed that TOR (target of rapamycin) kinase contributes to transcriptional regulation in response to nitrogen depletion (Imamura *et al*., 2015). TOR is a highly conserved serine/threonine protein kinase that receives and transmits various signals related to cell growth such as nutrition, and thus is thought to be a key regulator for cell growth (Wullschleger *et al*., 2006; Beck and Hall, 1999). Rapamycin is a specific inhibitor of TOR kinase and its application has contributed to elucidate the function of TOR (Beck and Hall, 1999).

We constructed *C. merolae* F12 and SF12 strains that are sensitive to rapamycin (Imamura *et al*., 2013; 2017) and are conducting research to elucidate the function of TOR in algae. One such outcome is the relationship between TOR and transcriptional regulation in response to nitrogen depletion. When rapamycin was added to the culture medium of F12 strain, transcript levels of the nitrogen depletion responsive genes were increased even under the nitrogen sufficient condition (Imamura *et al*., 2015). This demonstrates that TOR exerts regulation of the gene expression, and at the same time, TOR-inactivation by rapamycin mimics a nitrogen depletion condition.

Based on this result, we hypothesized that TAG accumulation could be induced by TOR-inactivation using rapamycin since TAG accumulation is generally observed under the nitrogen depletion conditions in microalgae as mentioned above. To examine this possibility, we measured TAG contents before and after rapamycin treatment. The results showed that LDs and TAGs accumulate after TOR-inactivation by rapamycin in *C. merolae* F12 strain (Figure 1). A similar phenomenon was also observed in the unicellular green alga *Chlamydomonas reinhardtii* (Imamura *et al*., 2016). Therefore, we concluded that TOR activity determines the ON/OFF switch for TAG accumulation, and the regulation is likely to be conserved among eukaryotic alga of divergent lineages (Imamura *et al*., 2015; 2016)

By using the ON/OFF switch of TAG accumulation by TOR, it is possible to increase TAG accumulation in algae at any time by inhibiting the activity of TOR. This method is very effective compared to the conventional method of nitrogen depletion in which the culture is shifted from a nitrogen-sufficient to nitrogen-deficient conditions that require a lot of effort and time. As mentioned above, TOR plays an important role in cell growth. Therefore, TOR-inactivation results in cell growth inhibition in addition to the TAG accumulation (Imamura *et al*., 2015). The similar relationship between TAG accumulation and cell growth inhibition is also observed under nitrogen depletion conditions (Scott *et al*., 2010). Thus, in general, there is a trade-off relationship between "TAG accumulation" and "cell growth", which is a large barrier for the improvement of TAG productivity.

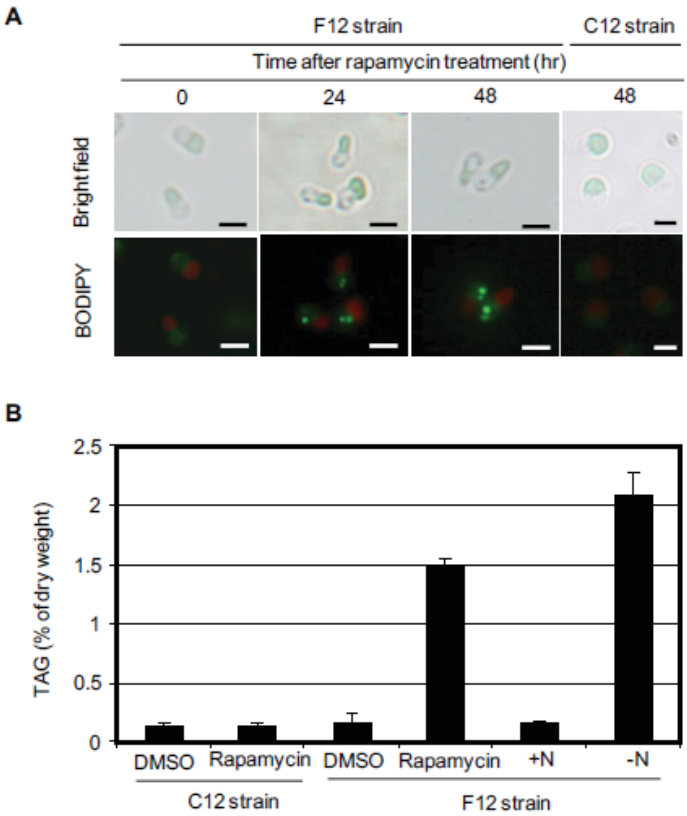

Figure 1. Accumulation of lipid droplets and TAG under TOR-inactivation conditions in *C. merolae* F12 strain. (**A**) BODIPY staining of cells after rapamycin treatment. Rapamycin sensitive F12 strain and its control strain, C12, were incubated with rapamycin for the indicated times. Bright field (top) and BODIPY staining (bottom) images are indicated. Each BODIPY staining image was merged with the relevant chlorophyll fluorescence image. Bar 2 μm. (**B**) Intracellular TAG content after rapamycin treatment and nitrogen depletion. TAG contents in F12 and C12 strains were quantified 48 h after DMSO (the solvent of rapamycin) or rapamycin treatment. TAG contents in the F12 strain were also quantified 48 h after nitrogen depletion (-N) or nitrogen-replete (+N) conditions. Values are averages of three independent experiments and represent the percentage of dry weight at indicated time points. Error bars indicate standard deviation (SD). Figure modified from Imamura *et al*. (2015).

Therefore, we next tried to overcome that problem by elucidating how TOR regulates the TAG accumulation after TOR-inactivation. For this purpose, we first investigated gene expression related to *de novo* fatty acid and TAG syntheses (Figure 2). When *C. merolae* F12 strain was cultured under TOR-inactivation or nitrogen depletion conditions, only transcripts of *CmGPAT1* and *CmGPAT2* were increased in both conditions (Imamura *et al*., 2015). CmGPAT1 and CmGPAT2 are genes encoding glycerol-3-phosphate acyltransferase (GPAT) that catalyzes the first step of *de novo* synthesis of TAGs/phospholipids in the endoplasmic reticulum (ER) or galactolipids in the chloroplast (Figure 2). To examine their intracellular localization and roles in TAG synthesis in the cell, we constructed Flag-fused CmGPAT1 and Flag-fused CmGPAT2 overexpression strains, named GP1 and GP2, respectively. Using the FLAG epitope tag as a marker, we investigated intracellular localization of CmGPAT1 and CmGPAT2 using immunostaining (Figure 3) and cell fractionation (Fukuda and Hirasawa *et al*., 2018). The results demonstrated that CmGPAT1 and CmGPAT2 localize in the ER, and implied that both proteins could be involved in TAG synthesis (Figure 2). If the ER-localized CmGPAT1 and CmGPAT2 play critical roles in TAG synthesis in *C. merolae,* overexpression of each GPAT may lead to accumulation of cytoplasmic LDs containing TAG. We, therefore, examined whether LD and TAG accumulation were induced in the GP1 and GP2 strains under normal growth conditions. In the GP1 strain, a drastic increase in the LD formation was observed (Figure 4A). Further, the TAG accumulation was 2.1% of dry cell weight, which was 19-fold higher compared to the control strain (GPc) (Figure 4B). This amount of TAGs is approximately the same as that of the control (GPc) cells exposed to nitrogen depletion conditions for 48-72 hours (Figure 1B). On the other hand, the amount of TAGs in the GP2 strain was equivalent to that of GPc. So far, the reason why the

same effect as GP1 is not observed in GP2 is unknown. The above results indicate that the reaction catalyzed by CmGPAT1 is the rate-limiting step of TAG biosynthesis in *C. merolae*.

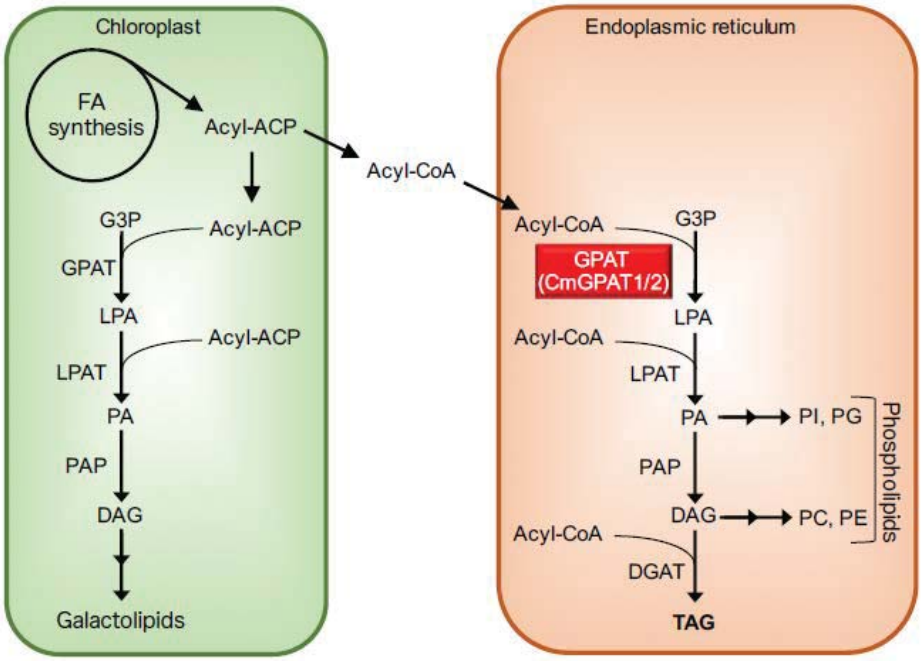

Figure 2. A schematic representation of synthesis pathways of galactolipids, phospholipids, and TAGs in *C. merolae*. The full name of each abbreviation in the figures is as follows: GPAT, glycerol-3-phosphate acyltransferase; LPAT, lysophosphatidic acid acyltransferase; PAP, phosphatidic acid phosphatase; DGAT, acyl-CoA:diacylglycerol acyltransferase; G3P, glycerol-3-phosphate; LPA, lysophosphatidic acid; PA, phosphatidic acid; DAG, diacylglycerol; TAG, triacylglycerol; PI, phosphatidylinositol; PG, phosphatidylglycerol; PC, phosphatidylcholine; PE, phosphatidylethanolamine.

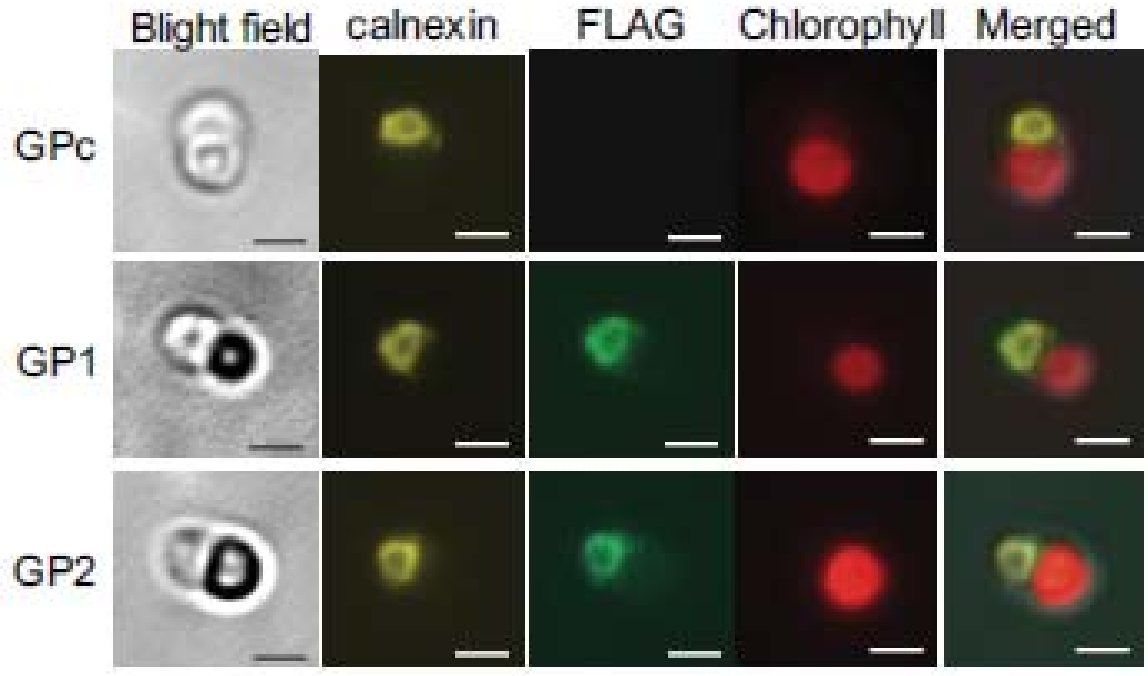

Figure 3. Intracellular localization of FLAG-fused CmGPAT1 and CmGPAT2. Fluorescence derived from calnexin (yellow signal, calnexin), FLAG-fused CmGPAT1 or CmGPAT2 (yellow-green signal, FLAG), intrinsic chlorophyll fluorescence (red signal, Chlorophyll), merged image of calnexin, FLAG, and Chlorophyll (Merged), and bright field image (Bright field) in indicated strains are shown. Bars correspond to 1 μm. Figure modified fromFukuda and Hirasawa *et al*. (2018).

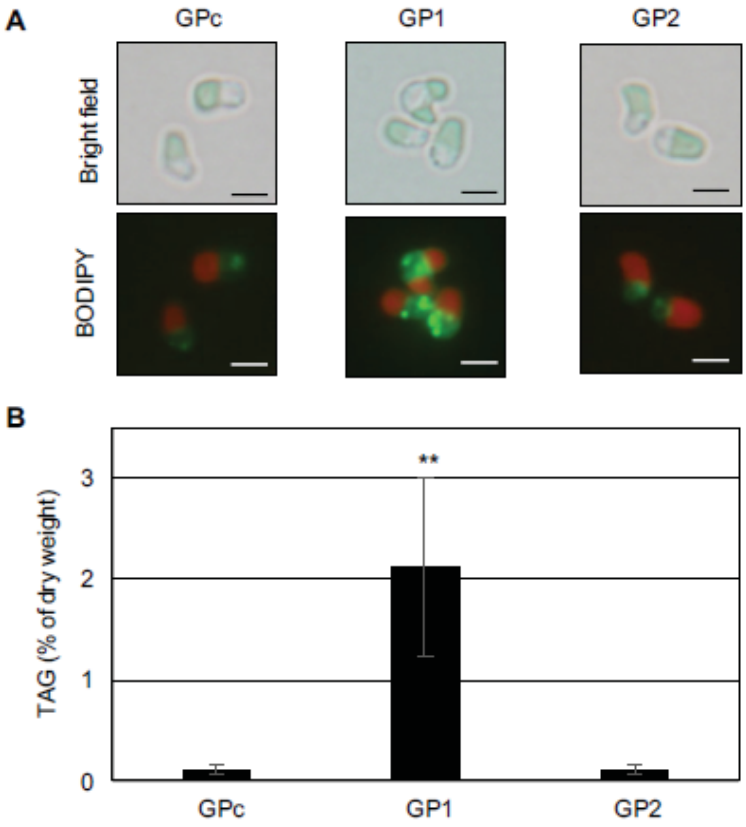

Figure 4. Accumulation of lipid droplets and TAGs by CmGPAT1 overexpression. (A) BODIPY staining of GPc, GP1, and GP2 cells. Each strain was grown under normal growth conditions until OD750 = 0.4–0.6, and was stained with BODIPY. Other details are as in Figure 1. (B) Intracellular TAG content in GPc,GP1, and GP2 cells. Values are averages of three independent experiments and represent percentages of dry weight. Error bars indicate standard deviation (SD). ** indicates a significant difference compared with GPc (Student's *t*-test, $P<0.01$). Figure modified from Fukuda and Hirasawa *et al*. (2018).

Next, we checked the growth of GP1 strain under the same conditions. Interestingly, the growth of GP1 strain was comparable to GPc, despite the high level of TAG accumulation in GP1 (Figure 5A). To calculate the TAG productivity of GP1 in three growth phases (mid-exponential, late-exponential and after-exponential phases), we quantified the TAG amounts during each phase (Figure 5B). The amount of TAGs was the highest during the late-exponential phase (about 4.6% of dry weight) and was slightly reduced during the after-exponential phase. The TAG productivity (mg/L/day) was drastically increased compared to that of GPc and increased with incubation time to 1.2 (29.9-fold increase compared with the control), 3.6 (24.7-fold), and 5.7 mg/L/Day (56.1-fold) during the mid-exponential, late-exponential and after-exponential phases, respectively (Figure 5C). These results clearly indicated that CmGPAT1 overexpression improves the TAG productivity without any growth inhibition. In other words, we succeeded in constructing the algal strain that is compatible with "TAG accumulation" and "cell growth".

The biosynthetic pathway of TAGs is partially shared with the synthesis pathway of phospholipids that constitute the cell membrane (Figure 2). Thus, the intensification of the reaction catalyzed by ER-localized GPAT should increase the accumulation of not only TAGs, but also phospholipids. However, the amount of phospholipids in the GP1 strain did not change compared to the GPc (Fukuda and Hirasawa *et al*., 2018). The reason behind this is unclear, but it suggests the existence of a mechanism that strictly maintains the appropriate amount of phospholipids in the cells.

Based on these results, Figure 6 shows a possible regulatory model of TAG biosynthesis in *C. merolae*. TOR activity is depending on the external environment and is inhibited under stress conditions, such as nitrogen depletion and rapamycin treatment. The TOR-inactivation results in upregulated expression of CmGPAT1, which catalyzes a rate-limiting step of TAG biosynthesis. Then, the increased CmGPAT1 triggers the TAGs accumulation (Imamura *et al*., 2015; Fukuda and Hirasawa *et al*., 2018).

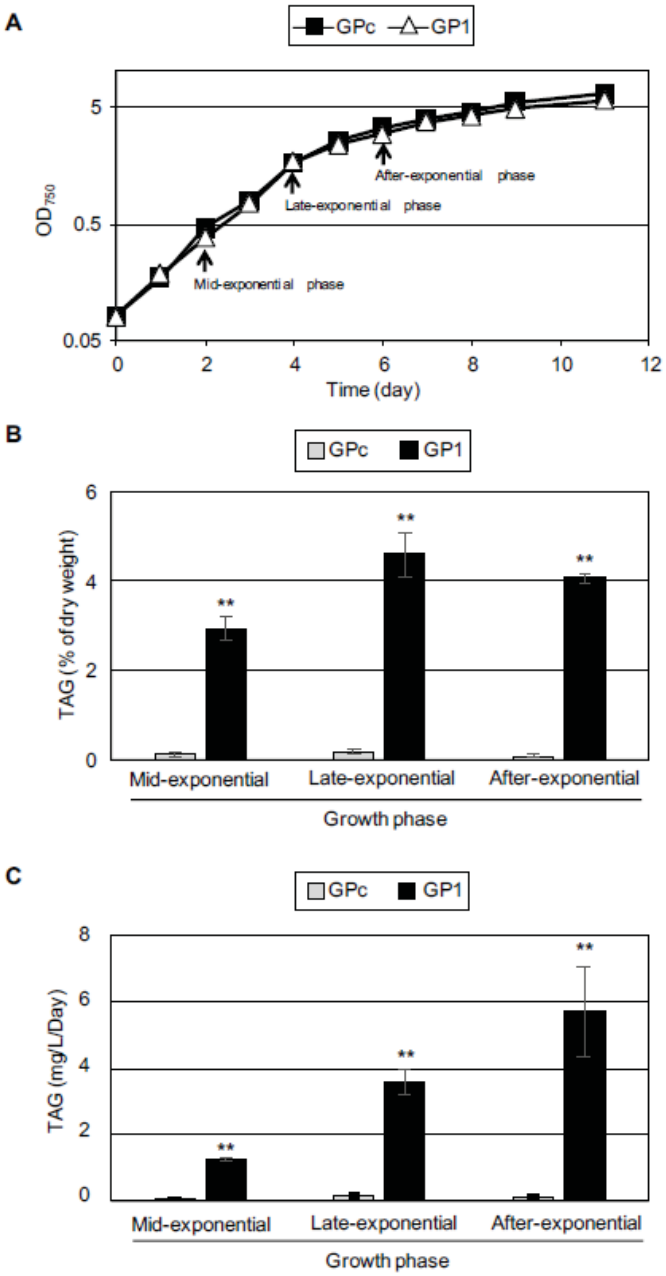

Figure 5. Improved TAG productivity by CmGPAT1 overexpression. (A) Growth of the GPc and GP1 strains. The growth of both strains under normal growth conditions was monitored by OD750. Arrows indicate the sampling times for the TAG measurements shown in panels B and C. (B and C) TAG productivity in the GP1 strain. The TAG contents in GPc and GP1 were measured at the mid-exponential, late-exponential, and after exponential phases, and are shown using two different indexes, percentage of dry weight (B) and mg/L/Day (C). Other details are same as in Figure 4. Figure modified from Fukuda and Hirasawa *et al*. (2018).

The TAG productivity observed in GP1 strain (1.2-5.7 mg/L/day) is higher than that observed under phosphorus depletion in *Nannochloropsis* (about 1 mg/L/day), which is considered to be one of the practical algal strains for oil production (Iwai *et al*., 2015). Therefore, the reaction catalyzed by CmGPAT1 can be considered as an excellent target for increasing TAG accumulation. In other microalgae, especially in unicellular green alga *Chlamydomonas*, several studies have indicated that the rate-limiting step of TAG biosynthesis is the final step in TAG biosynthesis that is catalyzed by diacylglycerol acyl-CoA acyltransferase (DGAT) (Du and Benning, 2016) (Figure 2). Since TOR and ER-localized GPAT are widely conserved proteins in eukaryotes, it is thought that the regulatory mechanism elucidated in this study might also be conserved in other algae. Understanding of the basic control mechanism of TAG biosynthesis by ER-localized GPAT and DGAT is a future subject to be solved, and the information will further improve TAG productivity.

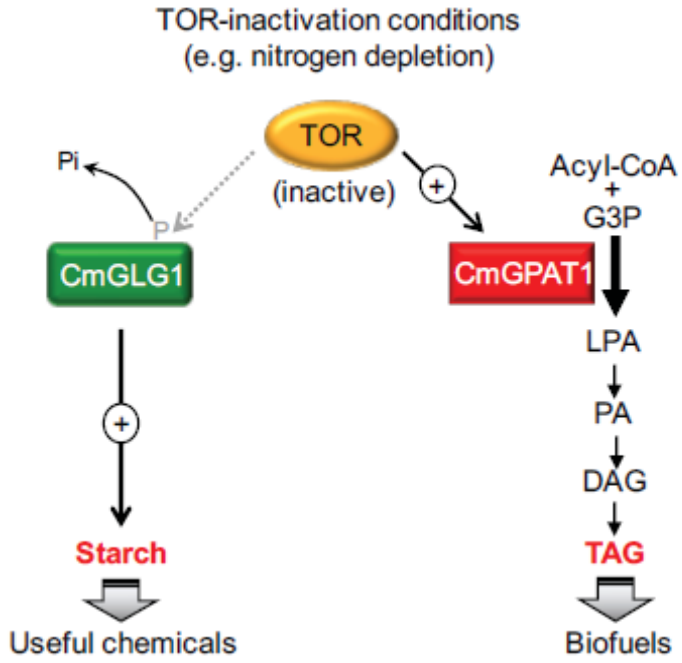

Figure 6. A possible model of TAG/starch accumulation by TOR-signaling in *C. merolae*. TOR-inactivation conditions, such as nitrogen depletion and rapamycin treatment, lead to increase in the expression of CmGPAT1 or dephosphorylation of CmGLG1, which results in the accumulation of TAG or starch. For details, please refer the text.

Starch, another carbon storage molecule, can be a potential target for the production of biofuels and valuable chemicals (Daroch *et al*., 2013; Yamaguchi *et al*., 2017). For example, we previously reported that starch from *C. merolae* cells or de-oiled biomass of *C. merolae* can be converted into the valuable chemicals like methyl lactate or methyl levulinate using $Sn(OTf)_2$ or $SnBr_4$ as a catalyst (Yamaguchi *et al*., 2017). Recently, we also revealed that the phosphorylation status of CmGLG1, a glycogenin protein, which is regulated by TOR-signaling pathway, controls starch accumulation in *C. merolae* (Pancha *et al*., 2019) (Figure 6). Based on the finding, we demonstrated that overexpression of CmGLG1 results in 4.7-fold higher starch in *C. merolae* compared to its control strain (Pancha *et al*., 2019). These findings raise the possibility that we can efficiently produce these chemicals using the CmGLG1 overexpression strain and increase TAGs and starch contents in one algal strain by overexpression of CmGPAT1 and CmGLG1. Further understanding the molecular mechanisms of biomass production in microalgae will improve the production of TAGs, starch, and other useful algal biomass.

CONCLUSION

- TOR is a conserved protein kinase among eukaryotes and plays a critical role in growth and energy metabolism.
- TOR is a key regulator of TAG and starch accumulation in microalgae and its inactivation by rapamycin can easily induce TAG and starch contents.
- TOR-inactivation leads to increased expression of ER-localized CmGPAT1, and the step catalyzed by CmGPAT1 is the rate-limiting step for TAG accumulation in *C. merolae.*
- TOR modulates starch accumulation in *C. merolae* by changing the phosphorylation sta.tus of CmGLG1.
- Microalgal starch is a good alternative to produce valuable chemicals.

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Panel Discussion

MODERATOR

Piyathip Eawpanich

Mrs. Piyathip Eawpanich is the ecologist for business development. Her background is in forestry and experiences on eco-efficiency and climate change policies development. Piyathip has become one of a few Thai experts in integration of ecology for sustainable business development. She is also a trainer on group moderation, result-based project development and monitoring, biodiversity, and the concept of TEEB: The Economic of Ecosystem and Biodiversity to formulate economic instruments for integration of ecosystem and biodiversity into landscape management and business operation.

From her active role in promoting Thai green and bio-based economy, she has been involved in the development of FSC's Thailand Standard, FSC's Regional Technical Advisory Group for small-holders, as well as in the piloting of Thailand Forest Management Standard (TSI 14061) for a group certification.

Gary Denes

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Dr. Gary Denes received his Ph.D. of Chemical Engineering in Sung Kyun Kwan University, Seoul, Korea. In 1982, he started his career as a Technical sales for formulations of fuel and lubricant in EXXON Abingdon R&D, UK and Singapore, and hold his last position as the Asia fuel and lube technical marketing Director. In 1994, he stepped up to the Vice President of Texaco International Asia Ltd. President and CEO, and achieved the highest growth in sales and profit. In 1997, as the President and CEO of Taxaco International Asia Ltd., apart from his success in managing fuel, lubricant and its additives production and sales for Asian market, he successfully launched renewable energy projects such as bioethanol in China. Since then, he involved in various renewable energy projects in the US and other countries. In 2013, he was a Senior consultant of Kentec Energy which generate the electricity from gasification of municipal waste in Washington State. Based on his professional, he had associated in Steering Committee for ASIA Society of Automotive Engineers, Technical Committee of Korean EPA G-7 for Future technology and Senior Technical advisor of Indonesian Government in Ministry of Energy and Mining. Now, he is the Senior Vice President of Asian Technology Service Pte Ltd. The business involves power production from solar and biomass as well as fuel trade and Vectorcide in Thailand.

Carol Rius

CFO & Co-founder Husk Ventures

Carol has over 10 years experience in international business, is founder of INQUVE and specialized in supporting impact oriented business. She has worked as a consultant for the Spanish Public Administration, visiting professor at UAB and ESCI-UPF and has delivered over 500 consultancy projects. Carol is also a coach and mentor for managers and social entrepreneurs, has an extensive network in India and South East Asia. Carol leads the investment and financial strategy of Husk Ventures.

Wattanapong Thongsoi

Chairman, Thai Biomass Trade Association (TBTA)

Wattanapong Thongsoi is Managing Director at Tipawat Corporation Limited, an energy forest plantation and biomass manufacturing company in Thailand. He is also Chairman of the Thai Biomass Trade Association (TBTA) founded by biomass manufacturers in Thailand. He was previously General Cargo Department Manager at SGS (Thailand). Wattanapong has 20 years of experience in engineering, trade inspection and manufacturing related to biomass. Wattanapong obtained his B.Eng in Instrumentation Engineering and MBA in Marketing.

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Mr. Parnthep Pourpongpan received his Bachelor degree in civil engineering from King Mongkut's University of Technology Thonburi and Master degree in business management from New York University, United States of America. He is now the Dean of Institute of Integrative Medicine and Anti-Aging, Rangsit University. Mr. Parnthep has experiences in medical business management, including the managing director of Bangkok Integrative Medicine Poly Clinic and the managing director of MW Wellness Clinic. He is also the writer of "Suriyan Ganja" (สุริยัน กัญชา), the book describes in details about the cannabis: what is it?, how it can be used as medicine?, cannabis and cancer?

Trin Rujiravanich

Founder, CannaFlower Medical Wellness

Mr. Trin Rujiravanich holds 2 Bachelor degrees from Chulalongkorn University, the first one is in Communication Arts in 1997 and the second one is in Law in 2007. He has experiences in a wide range of marketing, coordination and initiatives, especially in the Cannabis projects. He has made many field trips for cannabis study in Canada and Israel and also has experience in the international cannabis for more than 3 years. He has been also invited as the keynote speaker on cannabis for various conferences. He is currently positioned as founder of CannaFlower Medical Wellness.

Stephan Moreels

General Manager Control Union Cambodia

Mr. Stephan Moreels graduated in International Business Law and Maritime Law. He has now gained and developed deep knowledges in the certification and quality controls for commodities by working with Control Union since nearly 3 years. His background gave him a high sense of responsibility, especially when it comes to compliance and liability. As a General Manager in Cambodia, his role is to understand the needs of tomorrow from different industries and how it will match with the customers' requirements, along with applicable regulations.

Oral Presentation

BIOENERGY

O-EN1

EFFECTS OF PYROLYSIS TEMPERATURES ON LIQUID YIELD FROM OIL PALM BIOMASS USING AGITATED BED PYROLYSIS REACTOR

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ABSTRACT

Biomass is one of the promising sources of renewable energy due to its abundance and carbon neutral in nature. The conversion of biomass into liquid biofuels by using pyrolysis process is one of the challenging technologies. The objective of this study was to investigate the effects of pyrolysis temperatures on the product yields, including bio-oil, bio-char and pyrolysis gas by using agitated pyrolysis reactor. The biomass used in the study was oil palm trunk (OPT). The initial properties of OPT were considered from proximate analysis, ultimate analysis, thermogravimetric analysis (TGA), lignocellulose content and heating value. The experiments were performed at the pyrolysis temperature of 450-600 ^{o}C, while other parameters were fixed. The results from the determination of OPT properties showed that the OPT was the promising biomass from the oil palm tree replantation to be converted into bio-oil. The ratio of hydrogen and carbon for OPT was relatively appropriate to obtain a good yield of bio-oil. The higher heating value (HHV) of OPT was 16.11 MJ/kg. The results from thermogravimetric analysis (TGA) showed that most decomposition of OPT sample was decomposed under temperature ranging from 200 to 450 ^{o}C. The lignocellulosic analysis revealed that the OPT contained a relatively high amount of cellulose and hemicellulose i.e. 47.81 % and 23.19 % respectively. Pyrolysis of OPT under investigated conditions revealed that the highest liquid yield was obtained at 500 ^{o}C.

Keywords: Alternative energy; Bio-energy; Bio-oil; Oil palm biomass; Pyrolysis

INTRODUCTION

The world is facing problem of energy crisis because of the increase in population and excessive usage of fossil energy. The use of conventional fossil fuels such as coal, natural gas or petroleum products is decreasing because of the concern and awareness of people about the reduction and environmental impacts of fossil fuels, which result in the higher utilization of renewable and alternative energy resources. The sustainable policies adopted by the developing nations are to reduce the dependence on fossil-based fuels and to decrease harmful gas emissions in order to control the drastically changing climate. Biomass is considered as an attractive option because of its abundance and renewability of the feed. The utilization of biomass as energy is overall CO_2 neutral and short time carbon cycles. Thailand involves the biomass based energy due to the presence of a huge number of agricultural residues and wastes (approximately 120 million tons per year) [2]. The abundant biomass resources in Thailand are oil palm, rubberwood, rice husk, rice straw and sugarcane bagasse [2]. These biomasses can be used for renewable energy purposes in Thailand and even for exportation. For the oil palm biomass, it is available in the south of Thailand. The oil palm biomass can be obtained from harvesting, replantation and processing of oil palm fruits. The oil palm trunk, oil palm fronds and oil palm roots are an example of oil palm biomass which is obtained from harvesting and

replantation. For other oil palm biomass such as oil palm shell, oil palm fiber and empty fruit bunches, they are biomass obtained from the processing of oil palm fruits at oil palm factory.

There are many processes that can be applied to convert biomass into biofuels or bioenergy, such as mechanical, thermochemical and biological process. Pyrolysis is one of the thermochemical conversion processes that can be used to convert biomass into biofuels, including bio-oil, biochar and pyrolysis gas. In general, pyrolysis runs at temperature ranging from 200 to 900 °C under inert conditions or absence oxygen. Reaction pathways and end-product distribution depend on many factors such as biomass composition and properties, operating parameters, pyrolysis types and reactor configurations [3]. Pyrolysis temperature is one of the operating parameters that plays an important role in pyrolysis process, leading to its different product yields and qualities. This is due to the fact that thermal decomposition of each biomass occurs at a different temperature. At higher temperatures, the production of gaseous products increases; however, char is a dominant product at lower temperatures [4, 5].

Beside the pyrolysis temperature, biomass composition and properties also strongly affect the bio-oil yield and quality. The particle size, moisture content, volatile matter, elemental composition, chemical constituent and mineral composition are an important biomass property for pyrolysis processes. Particle size of biomass has strong effects on the heat and mass transfer during the pyrolysis process. Moisture and oxygen content in biomass affect the water content of bio-oil or liquid product. The volatile matter is the main part of biomass that can be converted into vapour both condensable and non-condensable vapours, which is resulting in the bio-oil or liquid yield. For the chemical composition, it was found that pyrolysis of cellulose or hemicellulose produces higher oil yield than that of lignin [5]. In addition, biomass also contains traces amounts of inorganic compounds metals (K, Na, P, Ca, and Mg) which appear in pyrolysis products both liquid and solid phases [6]. Composition of mineral matters in biomass is also an important parameter for the secondary pyrolysis reactions and influences the reactivity of pyrolysis char [6]. In fact, minerals might decrease the amount of liquid oils and tend to increase char and gas formation [5]. Thus, production and characterization of bio-oils produced from various biomass resources are essential to identify their chemical composition and properties.

As mentioned above, the objective of this study was to investigate the effects of pyrolysis temperatures on liquid yield of oil palm biomass using agitated pyrolysis reactor. The biomass used in this study was oil palm trunk (OPT). The initial properties of OPT were determined via proximate analysis, ultimate analysis, thermogravimetric analysis, lignocellulose content and mineral composition. The OPT was pyrolyzed at the temperature of 400-500 ^{o}C under specific other parameters.

MATERIALS AND METHODS

The OPT biomass used in this study was collected from Klong Thom District, Krabi Province, Thailand. The fresh OPT with bark was sawed to reduce the size (about 0.05 m x 0.05 m x 1.5 m). Then, the fresh sawed OPT was chopped by chopping machine (MCH-420, Machinery789, Thailand) to reduce the size again before drying. The sample was then dried by solar greenhouse for 3–4 days to reduce the moisture content below 10 % (w.b.). The dried sample was ground by the grinding machine which is equipped with 1 mm sieve size. The sample was then stored in plastic bags at room temperature for future use.

Proximate analysis

The proximate analysis is the determination of moisture content, volatile matter, fixed carbon content and ash content. These compositions were determined by Thermogravimetric analyzer (TGA) using the Macro TGA 701 (LECO, UK), according to the ASTM D7582 procedure.

Elemental composition analysis

The elemental compositions, including carbon (C), Hydrogen (H), Oxygen (O), Nitrogen (N) and Sulphur (S0 were determined by Thermo Scientific FLASH 2000 Organic Elemental Analyzer (Thermo Scientific, Italy). The C, H and N will be determined by following the EN15104 procedure and S content will be performed according to ASTM D4239 procedure.

Lignocellulosic analysis

The percentage of cellulose, hemicellulose and lignin was determined in terms of acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL) [6]. The major and minor elements in biomass will be determined using inductively coupled plasma optical emission spectrometry (ICP-OES) (Optima 3300, Perkin Elmer, USA), according to EN 15297 procedure.

Experimental procedure

The experimental procedure has been performed by the pyrolysis system shown in figure 1. The sample is added to the reactor chamber having a wall thickness of 10 mm. The sample is added to the chamber by using a hopper arrangement and continuous stirring has been performed by magnetic agitator place on the top of the reactor chamber. Two K-type thermocouples sensor is equipped at the top of the reactor chamber to measure the temperature of the biomass and connected to PID controlled system. The reactor power, pyrolyzed time, pyrolysis temperature can be controlled by adjusting the control system. The system is equipped with three-stage condensation process and most of the volatile vapors are converted into bio-oil.

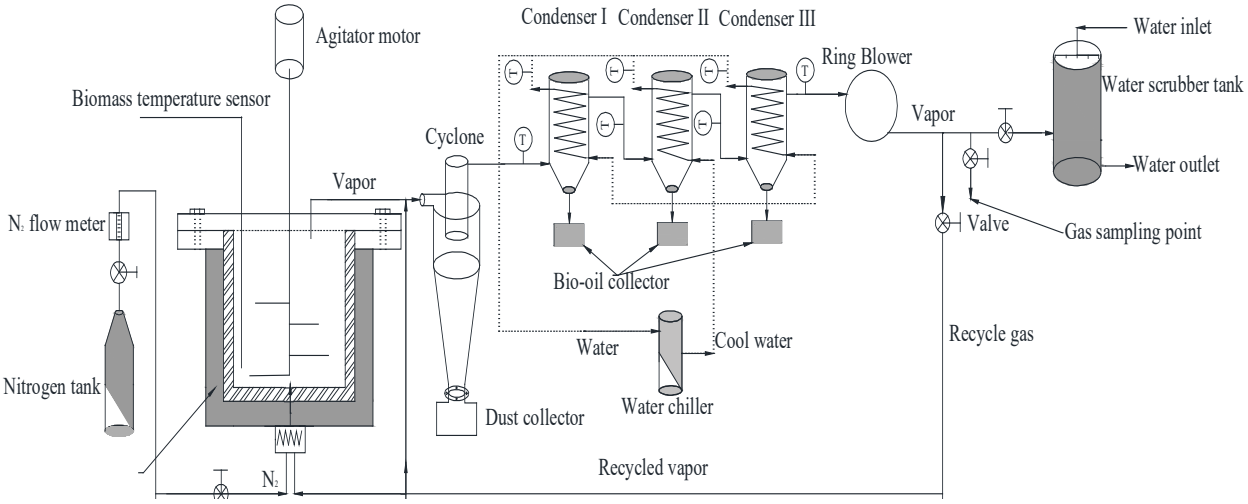

Figure 1. Schematic diagram of pyrolysis system

RESULTS AND DISCUSSION

Biomass characterization based on elemental composition

Table 1 shows the contents of alkali metals of the biomass feedstocks that are related to their ash contents. As studied by different researches, the metal contents of rice straw are much higher than those of other feedstocks. In fact, high metal content in the biomass might result in catalysing secondary reactions, leading to a decrease in its bio-oil yield and an increase in water and char formation [8]. In contrast, oil palm trunk possesses a low content of Na + K metals and this is one of the positive features of this feedstock. In general, biomass with higher holocellulose content is likely to give a high bio-oil yield, but high ash content brings a reverse direction [9].

The chemical composition and properties of Thailand oil palm biomass is quite comparable with those obtained from previous works [9-13]. This implies that the Thai biomass can be used as substituted resources for the biofuel industry.

Table 1 Comparison of typical contents of alkali metals in the feedstock based on dry basis [13-18,23]

	Oil palm trunk	Rice straw	Rice husk	Bagasse	Corn cob
Na + K, ppm	616	9980	1728	1510	3410
Mg + Ca, ppm	1679	5071	1215	1462	1056

Effect of biomass chemical composition on product yield

Bio-oil yield nearly the sum of cellulose and hemicellulose fractions in biomass. On the other hand, it is well known that lignin content is also important for fast pyrolysis leading to more liquid product [14]. Characterization results of the biomass are described in this section. It can be observed that oil palm

biomass samples possess a higher proportion of holocellulose (cellulose + hemicellulose), with over 70 wt.% than the others. As a result, higher bio-oil yields are expected on this feedstock. This agrees with Nowakowski's observation [15].

The bio-oil yield strongly depends on the type of biomass used as feedstock, i.e., biomass chemical composition. Research shows that the bio-oil yields from high contents of the total cellulose and hemicellulose feedstocks are significantly higher [14]. In fact, oil palm trunk has high contents of the total cellulose and hemicellulose (~ 72%) and low amounts of ash (~ 2%), whereas the other feedstocks have lower contents of the total cellulose and hemicellulose (~ 62%) and much higher contents of ash (> 10%) as shown in Table 2. Normally, water is formed mainly from either feedstock containing water or due to the cleavage of glucosidic bonds of cellulose and hemicelluloses.

Table 2 Comparison of lignocellulosic components in the feedstock based on dry basis [13-18,23]

Biomass	Lignocellulose contents (% wt, dry basis)		
	Cellulose	Hemicellulose	Lignin
Oil palm trunk	49.50	35.97	6.64
Palm Kernel shell	46.75	20.52	38.15
Rice husk	19.64	27.20	51.48
Rice straw	34.00	27.20	14.20
Bagasse	35.00	30.00	20.00
Corn Cob	45.00	35.00	15.00

Effect of volatile and fixed solids composition on product yield

Table 3 shows the comparison of volatile and fixed solid composition of the different feedstocks. Oil pal biomass has the highest amount of volatile matter (74.06 %) and very low amount of ash contents (3.42 %). Oil palm biomass shows promising composition in comparison with other biomass and hence considered suitable to produce bio-oil. Feedstock properties (e.g., ash content and alkali metal contents) and pyrolysis conditions (e.g., pyrolysis temperature, hot vapor residence time, and char particles) are also responsible for secondary cracking reactions, leading to further water formation [7, 16, 17]. Feedstock with higher amount of volatile matter will result in a higher amount of oil yield. It is generally accepted that a high-ash-content feedstock leads to higher water content in bio-oil. In fact, the higher water content in bio-oil results in higher pH value, lower viscosity, and heating value. This can be explained that water could not release energy, prevent the combustion process, and improve the viscosity of bio-oil [18].

Table 3 Comparison of volatile components in the feedstock based on dry basis [13-18,23]

Biomass	Proximate Analysis			
	M	FC	VM	ASH
Oil palm trunk	7.60	14.92	74.06	3.42
Palm Kernel shell	10.00	23.00	74.00	3.00
Rice husk	8.00	9.27	73.18	9.55
Rice straw	7.50	10.30	72.60	2.90
Bagasse	7.85	19.70	72.17	8.13
Corn Cob	2.52	24.44	70.78	2.26

Effect of reaction temperature on product yield

In previous studies [2, 19, 20], they found that bio-oil yield rises on increasing reaction temperature to reach a maximum at a certain temperature. In this study, the typical effect of reaction temperature on product yields from oil palm trunk pyrolysis is shown in Figure 2. It could be explained that temperature affects the competition between two major reaction pathways occurring in biomass thermal conversion. In fact, decomposition of biomass forms vapour products that can be condensed as primary pyrolytic products or have a secondary cracking to produce non-condensate gases. At low temperature, decomposition reactions will prevail, but the decomposition process of biomass may take place incompletely, leading to a low yield of bio-oil. As reaction temperature increases, these reactions become more intense, resulting in a

higher liquid yield. However, when the temperature is too high, cracking of the product vapor will dominate, leading to a lower liquid yield and a higher gas yield [5].

In addition, high temperature favours a further decomposition of char [21,22,24]. In this study, the highest bio-oil yields obtained from oil palm trunk is at 500 ^{o}C (42.32 %) followed by 450 ^{o}C (39.52 %) and 400 ^{o}C (38.81 %). This result revealed the relation between the reaction temperature and bio-oil yield.

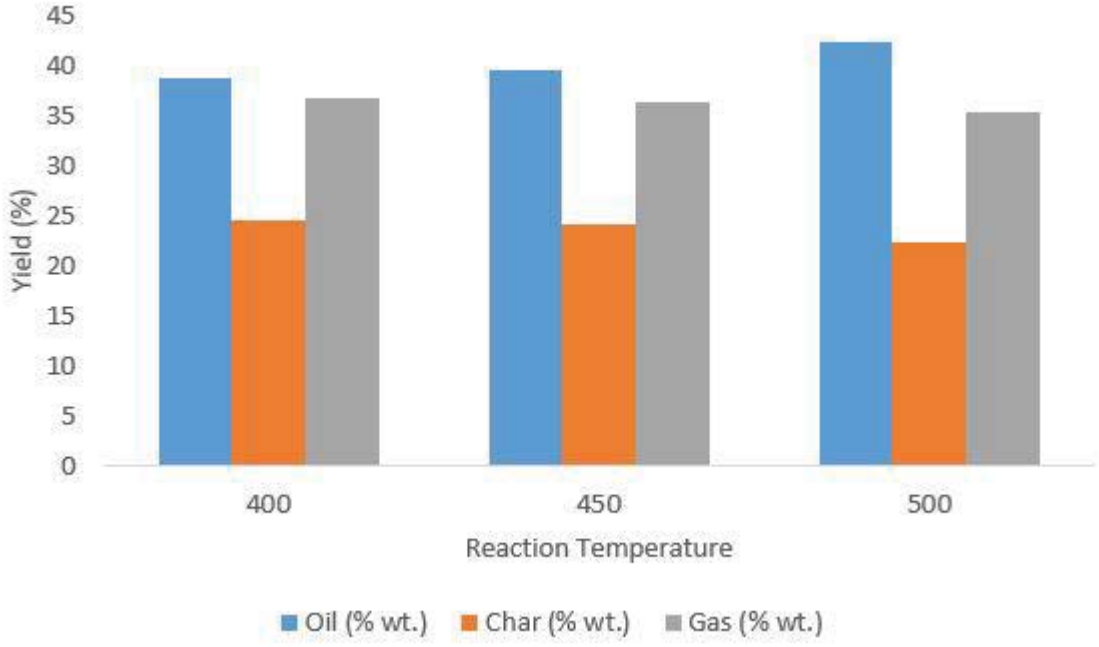

Figure 2. Product yield at different temperature of oil palm trunk

CONCLUSION

Oil palm trunk was investigated as feedstocks for bio-oil production in detail. The bio-oil yield was increased with the proportion of the main components (cellulose, hemicellulose, and lignin), whereas the high-ash content caused a loss in bio-oil yield. In addition, the effects of reaction conditions on bio-oil yield from oil palm trunk feedstock are more prominent. The lignocellulosic analysis revealed that the OPT contained a relatively high amount of cellulose and hemicellulose i.e. 47.81 % and 23.19 % respectively. Pyrolysis of OPT under investigated conditions revealed that the highest liquid yield was obtained at 500 ^{o}C. Hence, control of the pyrolysis process to achieve a high yield of bio-oil in case of oil palm trunk is quite strict compared to the other biomass. In summary, the finding might provide a strategy for bio-based producers to use various resources of biomass for bio-oil production and give a useful database for operational conditions as well as bio-oil yields and physical properties from different biomass feedstocks. Blending two or more feedstocks to ensure sustainable supplies of feedstock for biorefineries as well as to obtain bio-oil with its suitable composition and properties should be considered.

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O-EN2

POTENTIAL OF RUBBERWOOD BIOMASS FOR PRODUCING TORREFIED BIOMASS AND USING AS CO-FIRING FUEL VIA PHYSIOCHEMICAL PROPERTY

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ABSTRACT

Biofuels and bioenergy have gained significant interest in many countries due to the depletion and environmental impacts of fossil fuels. Thailand is an agro-industrial country which has a large volume of agricultural biomass. The objective of this study was to evaluate the potential of rubberwood biomass for producing torrefied biomass and using it as a co-firing fuel via its physicochemical properties. Rubber tree roots and branches (RTR and RTB) were used in this study. Proximate analysis, ultimate analysis, lignocellulose content, thermogravimetric analysis (TGA), higher heating value (HHV), and potential use as energy equivalent with fossil fuels were the physicochemical properties of biomass investigated in this work. The properties of RTR and RTB were also compared to those reported for other biomass materials in other studies. The results showed that RTR and RTB had a relatively high volatile matter and fixed carbon content comparable to other biomasses. The RTR and RTB had oxygen/carbon and hydrogen/carbon ratios close to other biomasses. The cellulose and hemicellulose of both RTR and RTB were higher than lignin. Most of the thermal decomposition of RTR and RTB clearly occurred at temperatures in a 250-400 °C range. The estimated HHV of RTR and RTB was in the range of 19.19-20.45 MJ/kg, depending on different correlations. The HHV of RTR and RTB was not significantly different from that of other biomasses. The potential use as energy equivalent with fossil fuels of RTR and RTB was 0.50 L, 0.67 kg, and 0.50 m^3 for crude oil, coal, and natural gas, respectively. These results indicated that the RTR and RTB had potential for producing torrefied biomass to be used as fuels in combustion or co-firing systems based on the mentioned physicochemical properties.

Keywords: Biofuels, Biomass, Co-firing, Rubberwood biomass, Torrefaction, Torrefied biomass

INTRODUCTION

The increase of the global population leads to higher energy consumption. In 2017, the global total primary energy consumption was approximately 13,511.20 million tons of oil equivalent, of which 80% was obtained from fossil fuels. It is estimated that the global energy demand will increase by approximately 28% from 2015 to 2040 (EIA, 2017; BP, 2018). Thailand is an energy-intensive country in the ASEAN region. Most of the energy used in Thailand is obtained from fossil fuels, including crude oil, coal, and natural gas. Reliance on fossil fuels or fossil energy, particularly in countries that need to import fossil fuels, not only affects their energy security and sustainability but also strongly influences their economic growth and the environments. It is well known that emissions of greenhouse gases (GHG) from the utilization of fossil fuels causes global warming and climate change (Chen *et al*., 2015; Vassilev *et al*., 2017; BP, 2018; Ribeiro *et al*., 2018). With these concerns, renewable and alternative energies have received increasing interest in many countries, including Thailand. Therefore, the government of Thailand has a policy to reduce the proportion of energy consumption from fossil fuels by increasing the utilization of renewable and alternative energy (RE

and AE), following the Alternative Energy Development Plan 2015 (AEDP2015). The RE and AE that have high potential in Thailand include solar, wind and biomass energy (Ministry of Energy of Thailand, 2015).

Thailand is an agro-industrial country which has great potential of biomass energy. The replanting, harvesting, and processing of crops and agricultural products provide many residual and waste biomass. Examples of biomasses that are available in Thailand are bagasse, rice husk, corn stalk, cassava roots, oil palm shell, and rubberwood (DEDE, 2012). Rubberwood is biomass mostly available in the south of Thailand. In 2018, the plantation area of rubber trees in the south accounted for 60% (approximately 1.36 million hectares) of total rubber plantation area in Thailand (Rubber Authority of Thailand, 2018). The biomass obtained from cutting down old rubber trees and processing of rubberwood includes trunk, branches, roots, bark, and sawdust. Some of which have been used as solid biofuel for boiler in factories and power plants. Besides the direct use of rubberwood biomass, it is also used as raw material for wood chip and wood pellet production (Office of Industrial Economics, 2013). The rubberwood biomass still remains, however, of great potential to produce biofuels in the future, which would help reduce reliance on fossil fuels of Thailand.

The conversion of biomass into biofuels or bioenergy can be performed by several processes or methods such as mechanical, biochemical, thermochemical, and combined processes. The criteria for selecting the biomass conversion processes depend on many factors such as biomass properties, the final form of fuels or energy, costs, storage, and transportation, as well as social and environmental impacts (UNEP, 2013; Adams *et al*.,2018). Thermochemical conversion processes are conventional processes widely used in many sectors for heat and power generation. Thermochemical conversion processes include torrefaction, pyrolysis, gasification, and combustion. These processes provide different forms of fuels or energies, and are operated at different conditions. Torrefaction is a process that consumes heat to convert biomass into solid biofuel or coal-like biomass (torrefied biomass). Torrefaction is operated at the temperature typically ranging between 200 and 300 °C in the absence or controlled oxygen conditions, with a heating rate below 50 °C/min, and a heating time of 30-120 min. The common reactions that occur during biomass torrefaction include devolatilization, depolymerization, and carbonization of lignocellulosic compositions (cellulose, hemicellulose, and lignin). The main product obtained from the torrefaction process is in the form of solid biofuel with brown to black color. This process also provides condensable (water, organics, and lipids) and non-condensable gases (CO_2, CO, and CH_4). Typically, after completing the torrefaction process, 70% of the initial mass is retained as a solid product which has 90% of its initial energy content (Prins *et al*., 2006; Tumuluru *et al*., 2011; Sukiran *et al*., 2017). Previous studies have shown that the quality and quantity of torrefied biomass depend on many factors such as types and properties of raw biomass, types of reactor, and operating conditions (Daya *et al*., 2014; Satpathy *et al*., 2014; Granados *et al*., 2017; Proskurina *et al*., 2017). Biomass properties strongly influence the quality and quantity of torrefied biomass. This is because each biomass has different physical and chemical properties such as size, density, moisture content, ash content, elemental composition, lignocellulose content, and thermal decomposition. Thus biomass properties not only affect the quality and quantity of torrefied biomass but also influence the reactor designs, the energy consumption of torrefaction process and production cost (Chen *et al*., 2013; Kopczyński *et al*., 2017; Ribeiro *et al*., 2018; Rokni *et al*., 2018).

The objective of this study was to evaluate the potential of rubberwood biomass for producing torrefied biomass to be used as co-firing fuel via its physicochemical properties. The physicochemical properties of rubberwood biomass (rubber tree roots and branches) were determined via proximate analysis, ultimate analysis, lignocellulose content, thermogravimetric analysis (TGA), higher heating value (HHV), and potential use of rubberwood biomass as energy equivalent with fossil fuels. The properties of rubberwood biomass were also compared to those of other biomass materials obtained by other studies.

MATERIALS AND METHODS

Rubberwood biomass collection and preparation

The biomass material used in this study was roots and branches of a RRIM 600 rubber tree (RTR and RTB). The rubber tree was harvested at Khlong Thom District, Krabi Province, Thailand and its age was approximately 28 years old. The tree was sawed to separate the trunk from the branches. The roots were removed from the ground and then manually cleaned to remove the soil. Fresh RTR and RTB were sawed and chopped for size reduction. The rubberwood chips were further chopped using a chopping machine (MCH-420, Machinery789, Thailand) to reduce their size smaller than 5 mm before drying in a solar

greenhouse. The prepared biomass was dried until it reached a moisture content lower than 10% (w.b.). The dried sample was further ground to 1 mm using a grinding machine (Bonny, 2HP, Thailand). The ground biomass was kept in a sealed plastic bag until the determination of its physical and chemical properties was performed.

Determination of the physical and chemical properties of rubberwood biomass

The proximate analysis that is the determination of the moisture content (MC), volatile matter (VM), fixed carbon (FC) and ash content (AC) was performed by a macro thermogravimetric analyzer (TGA 701, LECO, USA) based on the ASTM D7582-15 procedure. The result was reported as the mean value (%wt. dry basis).

The elemental composition of rubberwood biomass, including carbon (C), hydrogen (H), oxygen (O), nitrogen (N), and sulphur (S) was determined by an ultimate analysis using a CHNS/O Analyzer (FLASH 2000, ThermoScientific, Italy). The determination was performed following an in-house method. The results of determination were reported as %wt. (dry basis).

The lignocellulose content, including cellulose, hemicellulose, lignin and extractives were determined in terms of acid detergent fiber (ADF), neutral detergent fiber (NDF) and acid detergent lignin (ADL). The composition was determined by the modification method of Georing and Van Soest, 1970; Van Soest, 1991 and George W. Latimer, Jr. 2016. The results of determination were reported as %wt. (dry basis).

The thermal decomposition of the rubberwood biomass sample was observed via thermogravimetric analysis (TGA) using a thermogravimetric analyzer (TGA 8000, Perkin Elmer, USA) at temperatures ranging from 50 °C to 1000 °C with a heating rate of 10 °C/min.

The higher heating value (HHV) of the rubberwood biomass was estimated by proposed correlations as shown in equations (1) – (3) (Sheng and Azevedo, 2005). The calculations were performed based on the results from the proximate analysis, ultimate analysis, and lignocellulose analysis. Then, the HHV of biomass obtained from equation (2) was also used to calculate the potential use as energy equivalent with fossil fuels, including crude oil, coal, and natural gas (NG).

The estimation of HHV based on the proximate analysis result:

$$HHV_{(1)} = -3.0368+0.2218VM+0.2601FC \text{ (MJ/kg)} \quad (1)$$

The estimation of HHV based on the ultimate analysis result:

$$HHV_{(2)} = -1.3675+0.3137C+0.7009H+0.0318O \text{ (MJ/kg)} \quad (2)$$

The estimation of HHV based on the lignocellulose content:

$$HHV_{(3)} = 0.1739Ce+0.2663L+0.3219E \text{ (MJ/kg)} \quad (3)$$

where, FC, VM, C, H, O, N, Ce, L and E are fixed carbon, volatile matter, carbon, hydrogen, oxygen, nitrogen, cellulose, and hemicellulose content, lignin content, and extractives (wt.%, dry basis), respectively.

The properties of the biomass obtained from this study was also compared to those of other biomass materials which were obtained from a short literature review, including Peng *et al*., 2013; Toptas *et al*., 2015 and Wang *et al*., 2019.

RESULTS AND DISCUSSION

The results of proximate analysis

Figure 1 shows the proximate analysis results, including volatile matter (VM), fixed carbon content (FC) and ash content (AC), of RTR, RTB and other biomass materials which were obtained from other studies. In this study, the fresh RTR and RTB had an initial moisture content of 39.25 and 43.46 (wt.%, wet basis), respectively. After preparation and drying with solar energy, the final MC of samples was about 5 (wt.%); (the sample was dried to a very low final MC to protect the biomass from biological decomposition during storage). For solid fuels, the moisture content of fuels has a strong influence on the thermal efficiency of combustion or co-firing, particularly the useful heat. This is because biomasses with high MC require thermal energy to remove or evaporate the water present in biomass before combustion or burning, which results in heat loss or thermal energy loss. The MC of biomass also affects storage duration and

transportation costs. Biomass with high MC also presents some risks of biological decomposition during storage. Regarding the volatile matter, it was found that the VM of RTR and RTB was relatively high compared to Olive tree pruning, Pine bark, and Spruce. The VM is a combustible composition of biomass. It is a mixture of short and long chain hydrocarbons and aromatic hydrocarbons. Biomass with high VM indicates that it can easily be combustible and subsequently complete oxidation (Lu *et al*., 2012). Moreover, the fuel ratios (FC/VM) of selected biomass as shown in Figure 1 (b) were considered and it was found that biomass with not significantly different and had a near tendency to have higher heating value. When considering the ash content, it was found that RTR and RTB had low ash content compared to Olive tree pruning, Pine bark, and Spruce, which was in the same order of magnitude than other biomasses. For combustion or co-firing, the ash content significantly affects the design of combustion chambers, exhaust cleaning systems (cyclone, bag filter and ESP) and ash removal systems. Biomass with high ash content can also cause slagging and fouling problems due to the melting of ash at high-combustion temperature conditions. These problems lead to high maintenance costs and also loss of operating time (Wannapeera *et al*., 2011; Ramos-Carmona *et al*., 2018). Moreover, the remaining ashes after biomass burning need appropriate management or utilization. This is because ashes are considered as waste according to the regulation of the Pollution Control Department. This result revealed that RTR and RTB have a high potential to be used as solid biofuel for combustion, co-firing or to be used as raw biomass for producing torrefied biomass.

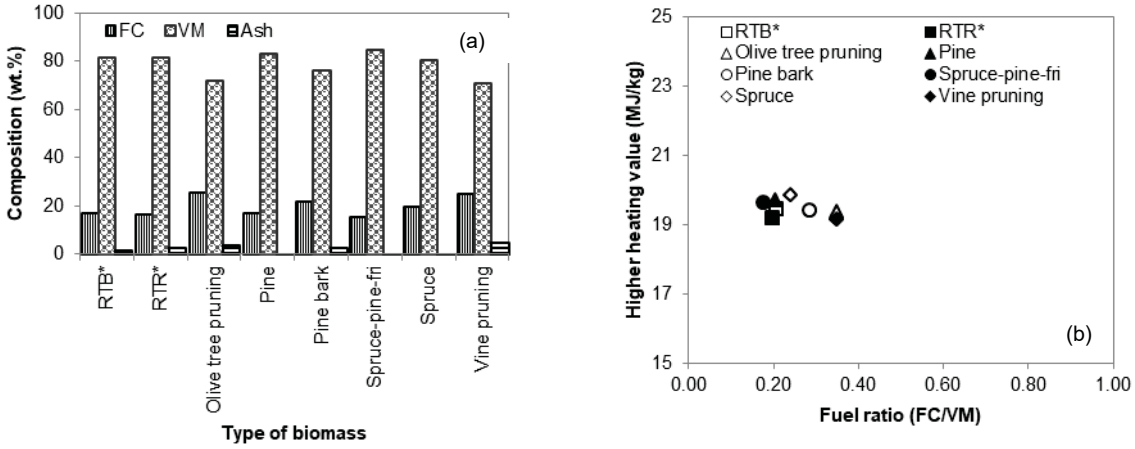

Figure 1 Proximate analysis result (a) and Relationship between fuel ratio and higher heating value (b) of biomass.

Note: *Sample of this study and other biomasses adapted from Peng *et al*., 2013; Toptas *et al*., 2015 and Wang *et al*., 2019, respectively. All values were presented as wt.% (dry basis).

The results of ultimate analysis

Figure 2 (a) and 2 (b) show the atomic ratio and the relationship between higher heating value and carbon content of biomasses. The ultimate analysis results were presented in atomic ratio or Van Kervelen diagram. It is seen that RTR and RTB had O/C and H/C ratios of 0.98 and 0.13, respectively. The ratios of O/C of RTR and RTB are similar to Spruce-pine-fri and H/C of RTR and RTB are similar to other biomasses such as Olive tree pruning, Spruce-pine-fri, and Vine pruning. Normally, general biomasses have high oxygen content, low carbon and hydrogen content which are weak properties for application as solid fuel, unlike coal. Previous works showed that upgrading biomass using torrefaction process improve the O/C and H/C ratios. Higher H/C and lower O/C ratios lead to higher fuel properties which are consistent with the fuel properties of peat, coal or anthracite (Phanphanich and Mani, 2011; Chen *et al*., 2015; Wilk *et al*., 2015; Ribiro *et al*., 2018). In combustion or co-firing processes, it is well known that the combustion of biomass with C, H and O provides flue gas containing CO_2 and H_2O. Although the oxygen contained in biomass helps enhance the air or oxygen supply in the combustion process, it also interrupts the heat released by hydrogen oxidation and further results in H_2O formation. For solid fuels, the carbon and hydrogen content relates to the content of VM and FC. For good quality biomass, it should contain high H and C while the oxygen content

should be low. On the other hand, higher N and S content in biomass has negative effects on the flue gas composition. This is because of the oxidation of nitrogen and sulphur with oxygen risk to form NO_X and SO_X during the combustion process. NO_X formation may occur during the combustion process and this depends on many factors such as nitrogen composition in fuels and air supply (Fuel NO_X), combustion temperature (Thermal NO_X) and reaction during combustion (Prompt NO_X). The sulphur content is generally low in biomass compared to coal. Combustion of biomass with sulphur, however, leads to the formation of SO_2 and SO_X. Both SO_2 and SO_X have negative impacts on human, combustion systems and lead to extra operating costs (Jenkins *et al*., 1998; Obernberger and Thek, 2004; Kopczyński *et al*., 2017).

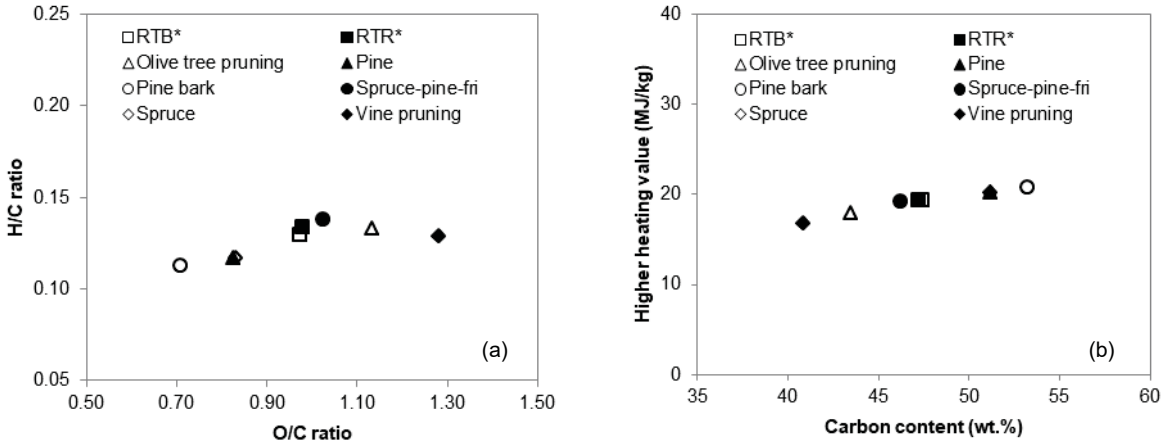

Figure 2 Van Kervelen diagram (a) and Relationship between carbon content and higher heating value (b) of biomass.

Note: *Sample of this study and other biomasses adapted from Peng *et al*., 2013; Toptas *et al*., 2015 and Wang *et al*., 2019, respectively. All values were presented as wt.% (dry basis).

Lignocellulose contents

Figure 3 (a) shows the lignocellulose contents of RTR, RTB and other biomasses. The results are shown in terms of cellulose/lignin and hemicellulose/lignin ratios. RTR and RTB had relatively high cellulose/lignin compared to other biomass materials such as Olive tree pruning, Pine bark, and Vine pruning. In addition, RTR and RTB provided high hemicellulose/lignin ratios as compared to other biomasses but are similar to Pine. These results also revealed that RTR and RTB had cellulose and hemicellulose contents higher than their lignin content. The results indicated that most of the biomass composition was decomposed vapor or gas phase at medium temperature (250-400°C) as shown in TGA curves. This is because, at that temperature range, the thermal decomposition of hemicellulose and cellulose is relatively complete. For the lignin, it is decomposed by heat at a wider range of temperature (i.e. 200-900 °C). Thus, combustion or co-firing of biomass at a temperature higher than 800 °C leads to complete thermal decomposition of lignin. In addition, the higher heating values that were estimated by the lignocellulose content using equation (3) revealed that the biomass with high lignin content has high HHV. Regarding the cellulose and hemicellulose content, they have a small effect on the HHV of biomass, although they are the main components in biomass. This is due to the different elemental structures of those compounds, particularly the hydrocarbon and oxygen content of lignin, cellulose and hemicellulose (Saidur *et al*., 2011; Daya *et al*., 2014).

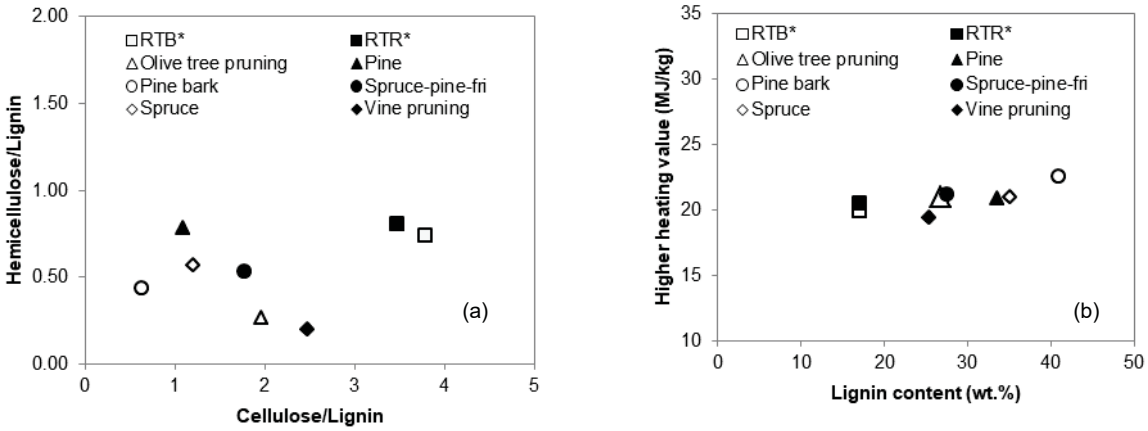

Figure 3 Cellulose, hemicellulose/Lignin ratios (a) and Relationship between lignin content and higher heating value (b) of biomass.

Note: *Sample of this study and other biomasses adapted from Peng *et al*., 2013; Toptas *et al*., 2015 and Wang *et al*., 2019, respectively. All values were presented as wt.% (dry basis).

Thermal decomposition via thermogravimetric analysis (TGA)

Figure 4 (a) and 4 (b) show the thermal decomposition of RTR and RTB at different temperatures. They revealed that the change of this biomass was classified into four periods. During the first period (50-100 °C), the mass of biomass slightly decreased due to evaporation of moisture. During the second period (100-240 °C), it is seen that the mass of biomass was constant because most of the moisture has evaporated and the heat supplied to biomass is used to increase its temperature. This period is normally called the “heating up period”. During the next period (240-400 °C), hemicellulose and cellulose were decomposed by heat. The last period (400-1000 °C) corresponds to the main decomposition of lignin. These results are consistent with previous studies which found that hemicellulose, cellulose and lignin were decomposed at temperature ranges of 200-400, 275-430 and 127-900 °C respectively (Yang *et al*., 2007; Daya *et al*., 2014). The results also showed that RTR and RTB had thermal decomposition close to the trend of mass loss due to torrefaction at 200-300 °C. It revealed that torrefaction of rubberwood biomass at this temperature range will provide higher solid yield than liquid and gas yield. The mentioned thermal decomposition of other biomasses via TGA can be seen from the literature review (Toptas *et al*., 2015).

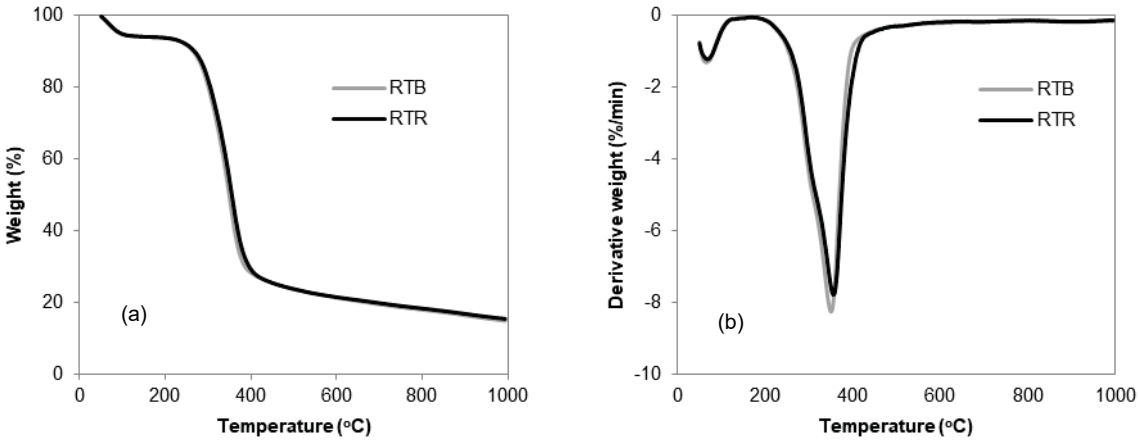

Figure 4 TGA (a) and DTA (b) profiles of samples at heating rate of 10 °C/min

Higher heating value (HHV) and potential use as energy equivalent with fossil fuels

Table 1 and Table 2 show the higher heating value (HHV) and potential use of biomass as energy equivalent with fossil fuels, respectively. In general, HHV is the total energy available in fuels which can be estimated or determined from the heat released during complete combustion. It is the combination of thermal energy from the heat of formation and heat from water vapor condensation. The results showed that the HHV of RTR and RTB was in the range 19.19-20.45 MJ/kg, depending on the different correlations previously mentioned. When the HHV of rubberwood biomass is compared to other biomasses from other studies, the results showed that the HHV of all biomass was in the range 16.81-22.56 MJ/kg. These results indicated that the HHV of rubberwood biomass is in the same range as those from other biomasses. The higher heating value depends on several factors such as moisture content, ash content, elemental composition and chemical component. In addition, the HHV of different biomass materials was compared with the HHV of fossil fuels, including crude oil, coal, and natural gas (NG), and the results were presented as potential use as energy equivalent with fossil fuels. The HHV of crude oil, coal (Bituminous), and NG was equal to 38.78 MJ/L, 28.87 MJ/kg, and 38.95 MJ/m^3, respectively (IEA, 2010a; IEA, 2010b). The results revealed that the energy equivalent with fossil fuels of rubberwood biomass and other biomasses were comparable to those of crude oil, coal, and natural gas as shown in Table 2. For rubberwood biomass, one kilogram of RTR and RTB could be used to replace approximately 0.50 L, 0.67 kg and 0.50 m^3 of crude oil, coal, and natural gas, respectively. Thus, the use of rubberwood biomass as biofuels or bioenergy via combustion or co-firing could help reduce fossil fuel dependence and reduce greenhouse gas emissions in Thailand.

Table 1 Higher heating value (HHV) of different biomass materials

Type of biomass	$HHV_{(1)}$ (MJ/kg)	$HHV_{(2)}$ (MJ/kg)	$HHV_{(3)}$ (MJ/kg)
RTB*	19.45	19.29	19.84
RTR*	19.19	19.34	20.45
Olive tree pruning[(a)]	19.37	17.89	21.00
Pine[(b)]	19.70	20.24	20.87
Pine bark[(b)]	19.39	20.73	22.56
Spruce-pine-fri[(c)]	19.63	19.12	21.11
Spruce[(b)]	19.86	20.25	20.97
Vine pruning[(a)]	19.14	16.81	19.40

Note: * means sample of this study and (a), (b) and (c) adapted from Toptas *et al*., 2015; Peng *et al*., 2013; and Wang *et al*., 2019, respectively. All values were presented as wt.%, (dry basis).

Table 2 Potential use of RTR, RTB and other biomass as energy equivalent with fossil fuels

Type of biomass	$HHV_{(2)}$ (MJ/kg)	Potential use as energy equivalent with fossil fuels		
		Crude oil (L)	Coal (kg)	Natural gas (m^3)
RTB*	19.29	0.50	0.67	0.50
RTR*	19.34	0.50	0.67	0.50
Olive tree pruning[(a)]	17.89	0.46	0.62	0.46
Pine[(b)]	20.24	0.52	0.70	0.52
Pine bark[(b)]	20.73	0.53	0.72	0.53
Spruce-pine-fri[(c)]	19.12	0.49	0.66	0.49
Spruce[(b)]	20.25	0.52	0.70	0.52
Vine pruning[(a)]	16.81	0.43	0.58	0.43

Note: * means sample of this study and (a), (b) and (c) adapted from Toptas *et al*., 2015; Peng *et al*., 2013 and Wang *et al*., 2019, respectively. All values were presented as wt.%, (dry basis).

CONCLUSION

The potential of rubberwood biomass (rubber tree roots and branche) for producing torrefied biomass and using it as co-firing fuel via its physiochemical properties was evaluated in this study. The physiochemical properties of biomass were determined via proximate analysis, ultimate analysis, lignocellulose content, thermogravimetric analysis (TGA), higher heating value (HHV), and potential use as energy equivalent with fossil fuels. The properties of RTR and RTB were also compared to those of other biomass materials from other studies. The results showed that RTR and RTB had a relatively high volatile matter and fixed carbon content but relatively low in ash content, compared to other biomasses. The Van Kervelen diagram showed that the RTR and RTB had O/C and H/C ratios close to other biomasses. The RTR and RTB had higher cellulose and hemicellulose than lignin. The main thermal decomposition of RTR and RTB clearly occurred at temperature 250-400 °C. The estimated HHV showed that the values obtained for RTR and RTB was in the range 19.19-20.45 MJ/kg, which were similar to other biomass materials. The potential use of RTR and RTB as energy equivalent with fossil fuels was 0.50 L, 0.67 kg and 0.50 m^3 for crude oil, coal, and natural gas, respectively. These results indicated that the RTR and RTB had potential for producing torrefied biomass and using it as fuels in combustion or co-firing systems.

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O-EN3

POTENTIAL EVALUATION OF LIGNOCELLULOSIC BIOMASS VIA PHYSICOCHEMICAL PROPERTIES FOR PRODUCING BIO-OIL BY USING PYROLYSIS PROCESSES

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ABSTRACT

Pyrolysis is one of the thermochemical conversion processes widely used for conversion of biomass into bio-oil and bio-char. The main advantages of bio-oil are easy storage and transport, high energy density compared to raw biomass, application for biofuels and high value-added products. However, the quantity and quality of bio-oil obtained from pyrolysis processes depend on many factors, such as pyrolysis types, reactor types, operating conditions and biomass properties. The objective of this study was therefore to determine the physicochemical properties of lignocellulosic biomasses, including oil palm trunk (OPT) and rubber wood sawdust (RWS) compare to other biomasses for evaluating the potential of bio-oil production. The properties of biomass samples were evaluated via proximate analysis, ultimate analysis, higher heating value, lignocellulose content and thermogravimetric analysis (TGA). The results of this study showed that volatile matter and fixed carbon of RWS was higher than OPT. The carbon and hydrogen content of OPT were lower than RWS, which was consistent with their heating value. The lignin and cellulose contents of OPT was relatively higher than those of RWS. The main thermal decomposition of OPT and RWS was occurred at the temperature of 250–400 °C, which is not different from other biomasses. The thermal decomposition trend of OPT and RWS was relatively similar due to they had similar lignocellulose content. Based on these results, OPT and RWS displayed a potential for using as raw biomass to produce bio-oil via pyrolysis processes.

Keyword: Bio-oil, Rubber wood sawdust, Oil palm trunk, Pyrolysis, physicochemical property of biomass

INTRODUCTION

The utilization of renewable and alternative energy (RE and AE) to replace or substitute fossil fuels or fossil energy is increasing worldwide. This is because fossil fuels are finite resources but RE and AE are renewable. The depletion in fossil fuels sources strongly influences to many countries, like Thailand, that need to import fossil fuels. Reliance on fossil fuels not only affects the energy security and sustainability but also influences the environmental and social aspects. Thus, the utilization of RE and AE based on their potential in each region or country is a challenge (Demirbas, 2001; Tanger *et al*., 2013; Yelmen and Tarık, 2016). This is due to the fact that the potential of RE and AE in each region or country is different, depending on many factors such as location, weather, as well as agricultural and industrial activity. Solar, wind, hydro, tidal, wave, geothermal and biomass energy are examples of RE and AE. These RE and AE can be applied to generate heat, power and both. Biomass is available in many countries across the world including Thailand (Abbasi and Abbasi, 2010; Bribian *et al.*, 2011; Fernando *et al.*, 2010). Biomass plays an important role in energy systems of Thailand because it is an agro-industrial country with high quantity of available. The harvesting, replanting and processing of sugar cane, rice, cassava, rubber tree and oil palm fruits

provide large amount of biomasses. Some of these biomasses have been already used as fuel for both heat and power generation. Most of rubber wood and oil palm biomass resources is available in the south of Thailand. However, rubber wood and oil palm biomass still remain potential for future use.

Biomass is defined as organic substances that may be obtained from plant, animal, and human. It also includes agricultural and forestry products, wastes and residue from the processing industry (Jagustyn *et al.*, 2013). Most of the biomass is presents in solid form which can be classified as woody and non-woody biomass, their main compositions are cellulose, hemicellulose and lignin (Collard F-X and Blin, 2014). This biomass is known as lignocellulosic biomass. Beside the lignocellulosic composition, biomass may contain protein, carbohydrate, fat, starch, and mineral. It has been reported that biomass energy accounted for about 14% of the world's total primary energy supply (International Energy Agency, 2015). For energy purposes, the conversion of biomass into biofuels or bio-energy can be performed by several processes, including mechanical, biological, thermochemical, and combined processes. The criteria for selecting biomass conversion into biofuels or bioenergy depend on many factors such as types and forms of biomass, biomass property, final energy or fuel forms, conversion cost, and environmental impacts. Thermochemical processes, including torrefaction, pyrolysis, gasification, and combustion, are available for converting biomass to biofuels or bioenergy (Lam *et al.*, 2016). Conversion of biomass into such products with mentioned the thermochemical processes provide different forms of biofuels such as solid, liquid and gas phases which may be easy to use, store, and transport.

Pyrolysis is one of alternative routes to overcome the limitation of fossil fuels in sustainable basis [Yan, Acharjee, Coronella and Vasquez, 2009]. Pyrolysis processes are a relatively new concept for transformation of biomass with heat in the absence of oxygen or air at elevated temperature higher than 400 °C into bio-oil, bio-char, and pyrolysis gas. (Bridgewater., 1999; Williams *et al.*, 2000; Zhang *et al.*, 2005; Abnisa *et al*., 2011; Cunha *et al.*, 2011; Jahirul *et al.*, 2012). Normally, there are two types of pyrolysis processes, fast pyrolysis and slow pyrolysis. These pyrolysis processes provide different product yields. The fast pyrolysis provides liquid or bio-oil yield higher that the slow pyrolysis. The bio-oil or liquid product obtained from pyrolysis process is a complex mixture of aromatics, carbohydrate and oxygenated which are generated from the fragmentation of cellulose, hemicellulose, and lignin (Oasmaa, 1999; Williams, 2000; Czernik, 2004; Goldemberg, 2008; Cunha, 2011). The bio-oil can be used as fuels for the boiler and engine (Kelkar *et al*., 2015; Yusoff, 2006). However, the quantity and quality of liquid products not only depend on the biomass property and compositions but also are influenced by pyrolysis reactor types and pyrolysis conditions. For biomass property and composition, previous studies revealed that both property and composition of biomass strongly influenced the quantity and quality of liquid products. This is because each biomass has different compositions. The potential of each biomass for applying pyrolysis processes can be evaluated by basic property and composition via physicochemical properties such as particle size distribution, proximate analysis, ultimate analysis, higher heating value, thermal decomposition, chemical composition, and elemental component.

The objective of this study was to evaluate the potential of biomass form oil palm trunk (OPT) and rubber wood sawdust (RWS) for producing bio-oil by using pyrolysis processes via physicochemical properties. The property of these biomasses was also compared to that of other biomasses obtained by other studies.

MATERIALS AND METHODS

Biomass preparation

The biomass used in this study was oil palm trunk (OPT) and rubber wood sawdust (RWS). The OPT was obtained from a 22 years old oil palm tree planted at Klong Thom District, Krabi Province, Thailand. The RWS was residue from a rubber wood processing factory located at Khlong Ngae, Sadao District, Songkhla Province, Thailand. The fresh oil palm trunk was sawed and chopped to reduce the size and then dried in a solar greenhouse for 3 days to reduce the moisture content to below 10 % (w.b.). The dried samples were ground by a grinding machine (model YPS-102, Bonny, Thailand), which was equipped with 1 mm sieve size. The ground sample was stored in plastic bag to protect moisture and for future use. The selected biomass samples were also compared to other biomass such as pinewood (PW), timothy grass (TG) and wheat straw (WS) (Nanda *et al.,* 2013).

Determination of biomass properties

The proximate analysis was performed by thermogravimetric analyzer (TGA) using Macro TGA (LECO, USA) following the procedure of ASTM D7582. The results were presented in terms of moisture content, volatile matter, fixed carbon content, and ash content (%wt. as received).

The ultimate analysis was used to determine the elemental composition of biomass, including carbon (C), hydrogen (H), nitrogen (N), oxygen (O), and sulphur (S). They were determined by CHNS/O Analyzer (FLASH 2000, Thermo Scientific, Italy), following the ASTM D4239 and EN15104 standard. The results were reported as dry basis by weight.

The higher heating value (HHV) of the biomass sample was measured by IKA C5000 bomb calorimeter (IKA, Germany) and the result was reported in the unit of MJ/kg.

The thermal decomposition of biomass was observed via thermogravimetric analysis (TGA) using thermogravimetric analyzer (TGA 7, Perkin Elmer, USA), according to ASTM E1131 procedure. The test was performed at temperature ranging from 50 °C to 1000 °C and at heating rate of 10 °C/min.

The lignocellulose content of biomass sample was determined by percentage of cellulose, hemicellulose and lignin. They were determined via acid detergent fiber (ADF), acid detergent lignin (ADL) and neutral detergent fiber (NDF). This determination was performed by modification method of Georing and Van Soest (1970), Van Soest (1991) and William (2000). The results of ADF, NDF, ADL and inorganic values were then used to calculate the percentage of cellulose, hemicellulose and lignin following the equations below (Omar *et al.,* 2011).

Cellulose (%) = ADF-ADL (1)

Hemicellulose (%) = NDF-ADF (2)

Lignin (%) = ADL (3)

RESULTS AND DISCUSSION

Proximate analysis results

Figure 1 shows results of the proximate analysis of oil palm trunk (OPT) and rubber wood sawdust (RWS) compared to that of pinewood (PW), timothy grass (TG) and wheat straw (WS) which was described in Nanda *et al*. (2013). The biomass samples in this study had the moisture content lower than 10% which is appropriate for thermochemical conversion processes. For pyrolysis, the moisture content of biomass samples should be low as much as possible. This is because pyrolysis of biomass samples with high moisture content provide bio-oil or liquid products with high water content. The water content in bio-oil is not good for heating value. The volatile matter of selected biomass was higher than 70%. The volatile matter is the main composition in biomass that can be converted into condensable and non-condensable vapors. These volatiles contain the structure of hydrogen, carbon monoxide, carbon dioxide, moisture, tars and light hydrocarbon. For pyrolysis processes, the biomass should have high volatile mater, particularly condensable volatile matter. Fixed carbon was lower than 20% (wt.). The OPT and RWS had fixed carbon lower than those of PW and WS. Fixed carbon of biomass is the composition that cannot be converted into vapors at elevated temperature. This composition may contain of some lignin. For ash content, it was found that the selected biomass had cash content lower than 5% (wt.) which is good for pyrolysis processes. This is because biomass with high ash content provides low bio-oil or liquid yield. Moreover, ash content also influences to the solid content in bio-oil because the ash in reaction chamber may carried out the condenser if the reactor is operated at high sufficient inert gas (Kalinovsky, 2009; Dhyani *et al.*, 2018). Thus, pyrolysis of biomass with high ash content needs to consider this problem.

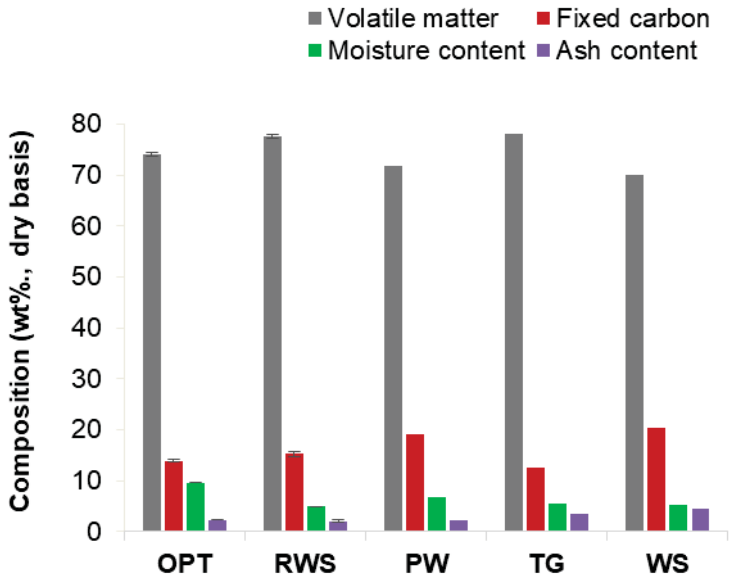

Figure 1 Proximate analysis result of biomass samples

Ultimate analysis results

Figure 2 shows the ultimate analysis results, including carbon (C), nitrogen (N), hydrogen (H), oxygen (O) and sulphur (S) of the studied biomass samples. Carbon and hydrogen content of biomass was higher than 40 and 5% (wt.), respectively. The pinewood (PW) contained highest carbon content. The hydrogen content of biomasses was not much different. The RWS had carbon content higher than the OPT. These results are consistent with the proximate analysis results. For pyrolysis processes, the biomass with high carbon, high hydrogen and low oxygen is favorable (Pimenidou and Dupont, 2012). This is because carbon and hydrogen are formed as useful aromatics liquid products. On the other hand, the oxygen will be bonded with hydrocarbon molecules which is a disadvantage of biomass for producing bio-oil. In addition, pyrolysis of biomass with high oxygen content is a risk to obtain the bio-oil or liquid products with high water content. This is due to the fact that the reaction water can be formed from the reaction between hydrogen and oxygen. For nitrogen and sulphur, they have a small bio-oil or liquid composition. These elements are transformed to be pyrolysis gas products. For the formation of NO_x and SO_x in pyrolysis gas products, it has very less possibility if the pyrolysis process is operated with absent oxygen or air at not too high temperature. The sulphur may also remain in bio-char and deposits at the reactor wall, or releases as salts or H_2S (Berndes *et al*., 2008). The sulphur content also influences the problem of corrosion (Jenkins *et al*., 1998; Obernberger and Thek, 2004).

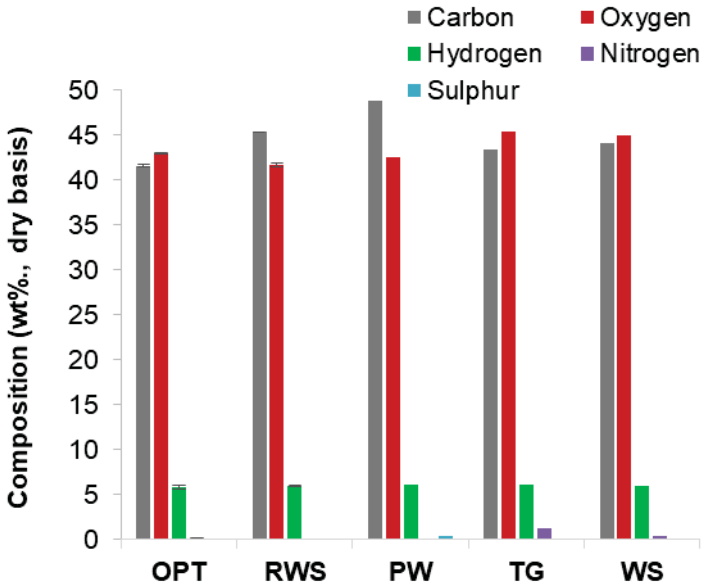

Figure 2. Ultimate analysis result of biomass samples

Higher heating value of biomass samples

The higher heating value (HHV) of biomass samples was in the range of 16.3-18.6 MJ/kg (Table 1). The pinewood (PW) had highest HHV which was better than that of rubber wood sawdust (RWS), wheat straw (WS), timothy grass (TG) and oil palm trunk (OPT). The HHV of RWS was higher than OPT, TG and WS. The biomass with high HHV provides the bio-oil or liquid product with high heating value. This is because the hydrocarbon composition in bio-oil is also high. The results of HHV were consistent with the results of proximate and ultimate analysis. In practice, the HHV of biomass or solid fuels can be estimated from proximate analysis results and ultimate analysis results.

Table 1 Higher heating value of biomass samples

Type of biomass sample	Higher heating value (MJ/kg)
OPT	16.3
RWS	17.7
PW	18.6
TG	16.3
WS	16.4

*HHV of PW, TG and WS was obtained from Nanda *et al*. (2013)

Lignocellulose content of selected biomass samples

Table 2 shows the lignocellulose content of oil palm trunk (OPT) and rubber wood sawdust (RWS), compared to pinewood (PW), timothy grass (TG), and wheat straw (WS). The results showed that chemical composition of biomass was mainly cellulose, which was in the range of 34.2-65.7 (%wt.). The hemicellulose and lignin content were in the range of 11-30.1 and 5.2-20.4 (%wt.), respectively. The OPT had highest cellulose content while PW had highest lignin. Generally, the main components of lignocellulosic biomass are cellulose, hemicellulose and lignin. The results of this study revealed that most cellulose and hemicellulose content was found in OPT and RWS samples. Previous studies found that cellulose and hemicelluloses contributed to the bio-oil production yield, while lignin yield provided larger proportion of solid char. Moreover, recent studies reported that the higher lignin content in biomass may increase the average molecular weight and viscosity of bio-oil but decrease the water concentration of bio-oil. The thermogravimetric analysis results also showed the thermal decomposition of the biomass samples within the temperature range of 250-400°C. This is also confirmed by the predictions of thermal decomposition of biomass samples (Mansaray and Ghaly, 1998; Luangkiattikhun *et al*., 2008; Chen and Kuo, 2010; Carrier *et al.*, 2011).

Table 2 Lignocellulose content of biomass samples

Type of biomass sample	Chemical composition (% wt., dry basis)			
	Cellulose	**Hemicellulose**	**Lignin**	**Extractives**
OPT	65.7	11.0	18.2	5.1
RWS	44.9	18.0	5.2	31.9
PW	38.8	23.6	20.4	17.2
TG	34.2	30.1	18.1	17.6
WS	39.1	24.1	16.3	20.5

PW, TG and WS was obtained from Nanda *et al.* (2013)

Thermogravimetric analysis (TGA) of biomass

Figure 3 shows the thermal decomposition of the biomass samples at different temperatures. There were three stages of decomposition of the samples due to heat. The first stage was at 50-240°C, during this stage the weight of the samples decreased in a steep manner. This is due to the weight decreasing from the evaporation of the moisture content and the light volatile matter. After the evaporation of the moisture and light volatile matter, the temperature of the sample also increased. The TGA curves revealed that the thermal decomposition of the biomass samples took place within the range of 200–400°C. Then, the thermal degradation of the samples was very slow, decreasing in the final state. The weight of the samples decreased in the second stage due to the decomposition of lignocellulose content and its transformation into

vapors. There are three principal classes of thermally degradable biopolymers in a lignocellulosic biomass, namely cellulose, hemicelluloses and lignin. The degradation path ways of these components have been investigated separately. Previous study found that the decomposition of hemicellulose, generally represented by xylan, mainly takes place within the temperature range of 250-350°C, followed by cellulose decomposition, which primarily occurs in the temperature range of 325-400°C with levoglucosan as the main pyrolysis product. Recent investigations have reported that lignin was the most stable component, decomposing in the higher temperature range of 300–900°C (Vaibhav Dhyani and Thallada Bhaskar, 2018)

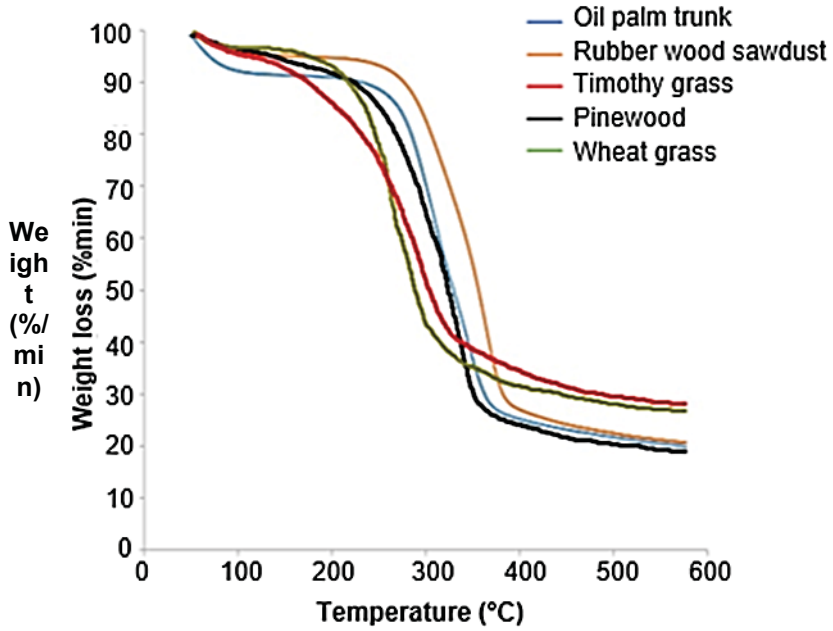

Figure 3. TGA curves biomass samples (PW, TG and WS was obtained from Nanda *et al.* (2013)

CONCLUSION

The physicochemical properties of lignocellulosic biomass, including oil palm trunk (OPT), rubber wood sawdust (RWS) and other biomasses were studied. The proximate analysis, ultimate analysis, higher heating value, thermogravimetric analysis (TGA), and lignocellulose content were used to evaluate biomass properties. The results indicated that the prepared biomass samples had low moisture content, relatively high volatile matter, and low ash content compared to other biomass form literatures. The biomass samples had high carbon and oxygen content, low hydrogen, and very less sulphur content. The higher heating value (HHV) of RWS was relatively high compared to other biomass samples. The main thermal decomposition of biomass samples took place within the range of 250-400 °C, depending on the lignocellulose content. These results revealed that RWS and OPT have potential for use as raw biomass to produce bio-oil via pyrolysis processes when considered from mentioned physiochemical properties.

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O-EN4

SYMBIOSIS OF HALOPHILIC ANAEROBIC BACTERIA *Halocella sp.* SP3-1 AND *Halanaerobium praevalens* SP3-14: ROLE TOWARD ON CELLULOSE DEGRADATION

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ABSTRACT

Microbial communities in natural ecology play an important role in the evolutionary of biomass degradation. Recently, the combination of microorganisms in nature known as in term of the microbiome was detected using next-generation sequencing. However, the microbiome can be underlined by the number of species and also can be only predicted some functions of species. Therefore, the symbiotic relationship of beneficial microorganism is essential to elucidate for more understand the role of them. In this study, a symbiotic of cellulose degrading ability between the extremely halophilic anaerobic bacteria was isolated from saltern pond. It comprised of two strains in the stable co-culture. Both of them were successfully separated using different carbon sources and designed as strain SP3-1 and SP3-14. Base on 16S rRNA gene sequence analysis, the strain SP3-1 was most closely related to *Halocella cellulolytica* DSM 7362 (92.77%), whereas the strain SP3-14 showed the nearest with *Halanaerobium praevalens* DSM 2228 (99.6%). The isolated strain SP3-1 produced cellulase, xylanase, amylase, without β-glucosidase, whereas the strain SP3-14 produced only extracellular β-glucosidase. The result strongly indicated why both strains are living together in the natural environment. Furthermore, the crude enzymes of both strains were characterized. Cellulase produced from the strain SP3-1 revealed optimum pH, temperature, and NaCl concentration at 5.0, 60^{o}C and 15% (w/v), respectively. In the case of β-glucosidase from the strain, SP3-14 can active, even NaCl concentration up to 20 % (w/v). This symbiosis could be used for degrading cellulose under high salt concentrations processes could be used this symbiosis.

Keywords: Cellulase, β -glucosidase, *Halocella cellulosilytica, Halanaerobium praevalens,* Symbiosis

INTRODUCTION

Halophiles are the microorganisms that can tolerate to high salt concentration. They can be found among all kingdoms of life such as bacteria, eukarya, and archaea. It has mainly been isolated from saltern crystalized ponds, the Dead Sea, solar lakes, and hypersaline lakes (Oren, 2002).

Cellulose, the most abundant and renewable natural biopolymer, is a linear polymer of D-glucose linked by β-1, 4- glycosidic linkages. Cellulose can be potentially subjected to bioconversion into a variety of value-added products. It is formed by a rigid crystalline structure with some amorphous regions. The biodegradation of cellulose requires the combined activities of several cellulolytic enzymes. The cellulose-degrading enzymes are divided into three major groups: endoglucanases (EC 3.2.1.4), cellobiohydrolases (EC 3.2.1.91), and β- glucosidase (EC 3.2.1.21) (Sadhu *et al*., 2013).

Many microorganisms, such as fungi and other aerobic and anaerobic bacteria that produce cellulosic enzymes, have been reported and characterized. Presently, many efforts at finding effective enzymatic systems from various cellulolytic microorganisms are still kept going because it will be impacted by the economic feasibility of the conversion process. The biodegradation of plant cell wall polysaccharides through the use of microbial co-culture or complex communities have been proposed as a highly efficient approach for biotechnological application because it avoids the problems of feedback regulation and metabolite repression posed by using only one strain (Wongwilaiwalin *et al*., 2018). Symbiotic interaction between cellulolytic and non-cellulolytic microorganisms in cellulose degradation have been reported

(Valášková *et al.*, 2009). However, a few reports showed the symbiosis of cellulose-degrading bacteria that can grow under anaerobic and high salt concentration, which produces primarily extracellular of cellulolytic, xylanolytic and amylolytic-enzymes *(*Simankova *et al*., 1993).

Recently, cellulose-degradation bacteria in the high salt concentration were isolated from the salt evaporation ponds (Heng *et al*., 2019). In fascinate studying about their symbiotic behavior was proficient of degradation of cellulose and produces the cellulolytic enzymes was observed. A previous study, an anaerobic, halophilic, and cellulolytic bacterium, *Halocella* sp. strain SP3-1 and *Ha. praevalens* strain SP3-14, have been successfully isolated from a consortium of bacteria. In this study, an enzymatic system was elucidated in which a symbiotic relationship was produced when co-culturing on cellulose.

MATERIALS AND METHODS

Microorganisms and Medium

Halocella sp. strain SP3-1 and *Ha. praevalens* strain SP3-14 were isolated from soil at salt evaporation in Samut Sakhon province, Thailand (Heng *et al.*, 2019). The fresh cultures were maintained by routinely transferring 1% (v/v) inoculum into new basal medium (BM) containing (per liter) 1.5 g of K_2HPO_4, 2.9 g of KH_2PO_4, 2.1 g of urea, 4.5 g of yeast extraction, 0.001 g of resazurin, 0.5 g of cysteine hydrochloride, and 0.2 mL of mineral solution. The mineral salt solution contained (per liter) 250 g of $MgCl_2.6H_2O$, 37.5 g of $CaCl_2.2H_2O$, and 0.3 g of $FeSO_4.6H_2O$. The pH of the media was adjusted to 7.0 and autoclaved at 121^{o}C for 15 min. The media were prepared under anaerobic conditions, as previously described (Chimtong *et al*., 2014).

Screening and Isolation

Halocella sp. strain SP3-1 and *Ha. praevalens* strain SP3-14 were isolated using PASC (phosphoric acid swollen cellulose) as the sole carbon source. The cultures were incubated at 37^{o}C under anaerobic conditions. The screening step was carried out several times to obtain the target candidates. The samples were showing that the most effective PASC degradation was enriched. The isolation was carried out on BM agar medium by using the roll-tube technique (Hungate, 1969) under sticky anaerobic conditions and incubated at 37^{o}C. Two kinds of colonies characteristics appeared on agar rolling tube. However, a single colony was picked by using a single colony technique with a needle and inoculated into fresh BM contained PASC as the sole carbon source. One of the strains (namely SP3-1) could grow well on PASC as the sole carbon source, and it was repeated several times on agar rolling tube until there was single pattern appearance. In case of strain, SP3-14 could not grow on PASC agar rolling tube, but it was growing well on cellobiose as the sole carbon source was culture several times until we get a single pattern appearance. The pure isolates, SP3-1 and SP3-14, were kept in appropriate culture broths containing PASC and cellobiose as the sole carbon sources, respectively.

16S rRNA Gene Analysis

Genomic DNA was prepared using Phenol-Chloroform-Isoamyl Alcohol (PCI) DNA Extraction method. 16S rRNA gene analysis was performed as described previously (Chimtong et al., 2014). The 16S rRNA gene was amplified by PCR using the following primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was performed with standard conditions according to the manufacturer's instructions. PCR products were purified using the QIAquick PCR purification kit (Qiagen, Germany). The determined sequence was compared with references available in the GenBank databases using the BLAST program.

Preparation of Enzyme from Strains SP3-1 and SP3-14

Strains SP3-1 and SP3-14 were growth in BM medium consisted of 1% (w/v) PASC and cellobiose as the sole carbon source, respectively. The culture was incubated at 37^{o}C until the late exponential growth phase (3 days) and harvested by centrifugation (8,000 rpm for 15 min) at 4^{o}C. Subsequently, the supernatant was used as the crude enzyme. Ammonium sulfate was added to the supernatant to 80% (w/v) saturation. Then, it was left overnight for the precipitate and was recovered by centrifuged (8000 rpm) for 20 min at 4^{o}C. The precipitate was then dissolved in 50 mM of phosphate buffer (pH 7.0) and dialyzed in the same buffer for 48 hrs. Dialysis was perform using tubing cellulose membrane with a molecular weight cut-off of 6000-8000. The dialyzed fraction was used as the crude enzyme.

Protein Determination and Enzyme Assays

All experiments were conducted in triplicate. Protein concentration was measured using the Lowry method (Lowry, 1951). A standard curve was generated using solutions of 1 µg/mL bovine serum albumin. Enzyme activities were determined using 50 µl of enzyme mixed with 50 µl of the substrate in 50mM phosphate buffer (pH 7.0) and incubated at 60^{o}C for 10 min. One percent (w/v) PASC were used as the substrates. The release of reducing sugars was quantified by spectrometric determination of reducing sugars by 3, 5-dinitrosalicylic acid (DNS) method (Miller, 1959), and used glucose as standard. The absorbance of protein and release sugars were estimated visually and determining by the OD_{660nm} and OD_{520nm} in the spectrophotometer (MULTISKAN; Labsystems), respectively. One unit of activity was defined as the amount of enzyme that releases 1µmol of glucose equivalents from substrate per minutes. The β-glucosidase activity was assayed by the amount of *p*-nitrophenol liberated from PNPG (Araujo *et al*., 1993). The β-glucosidase activity was expressed as µmols of *p*-nitrophenol released per minute per milliliter of enzyme solution.

RESULTS AND DISCUSSION

Symbiotic microbial associations play an essential role to degrade biomass in the environment. In this study, a halophile microbial capable of degrading the cellulose was screened from an extremely salty evaporation environment. After several times of enrichment and isolation on Hungate rolling tube, it was found that the capable candidate still contained two kinds of colonies of anaerobic bacteria based on colony morphology, named as strain SP3-1 and strain SP3-14. After attempts to purify each strain by single colony isolation technique (Hungate rolling tube technique), they were purified using different carbon sources. It was found that both strains could be separated as described in the materials and methods section.

The isolated strains SP3-1 and SP3-14 showed the highest similarities with *H. cellulolytica* DSM 7362 (92.77%) and *Ha. praevalens* DSM 2228 (99.6%), respectively, when they were identified by 16S rRNA gene analysis. According to the Ad Hoc Committee on Reconciliation of Approaches to Bacterial System, the 16S rRNA gene sequence identity of 97 and 95% have been used as cut-offs to classify bacteria isolates as novel taxa at the genus and species levels, respectively, when compared with their phylogenetically closest neighbours with validly published names (Stackebrandt *et al.*, 2002). Therefore, they were designated *Halocella* sp. for strain SP3-1, whereas strain SP3-14 designed as *Ha. praevalens* strain SP3-14. Moreover, both strains growth together during isolation. However, they were successfully isolated using different carbon sources. Hence, the symbiotic behavior of these strains in cellulose cultivation was studied and monitored in terms of an enzymatic symbiosis.

The enzyme production and cell growth were investigated by cultivating *Halocella* sp. strain SP3-1 and *Ha. praevalens* strain SP3-14 individually and together using cellulose as the sole carbon source. Enzyme activities were determined from culture supernatant after the culture reached the stationary growth phase (Table 1). The results showed that the strain SP3-1 produced cellulase, xylanase, and amylase as the main enzymes, but did not produce β-glucosidase. While strain SP3-14 produced the extracellular β-glucosidase. These result indicated that both strains contained different enzyme production. The SP3-1 strain produced main chain cleaving enzymes, whereas SP3-14 produced the β-glucosidase, which is the short and side-chain cleaving enzymes. Therefore, both strains are the symbiotic relationships to the actively promoting of cellulose degradation by mixed microbial culture under the high salt concentration.

Table 1. Enzymatic activities of the crude enzyme from *Halocella* sp. strain SP3-1 and *Ha. praevalens* strain SP3-14.

Enzymes	Specific Activity (U/mg)	
	Halocella sp. strain SP3-1	*Ha. praevalens* strain SP3-14
Amylase	53.9 ± 0.001	ND
Cellulase	79.3 ± 0.019	ND
Xylanase	63.7 ± 0.001	ND
β-glucosidase	ND	89.02±0.014

ND: Not determined

Therefore, *Halocella* sp. strain SP3-1 produced the extracellular cellulase and *Ha. praevalens* strain SP3-14 produced the extracellular β–glucosidase. The effects of pH, temperature, and NaCl on the enzyme activities produced from both strains were examined. The pH profiles of cellulase and β–glucosidase were studied using pH in the range of 3.0 to 9.0. The results demonstrated that cellulase and β–glucosidase was activated across a broad pH range between pH 4.0 to 7.0, but decreased drastically at pH 3.0 or 9.0 (Figure 1). The optimum pH of cellulase and β–glucosidase from strains SP3-1 and SP3-14 were found to be 5.0 and 4.0, respectively, which indicated the acid-tolerant enzymes. The temperature profiles of the cellulase and β–glucosidase of strains SP3-1 and SP3-14 were performed between 25 and 80°C at pH 5.0. Both of those enzymes had an optimum temperature of 60°C (Figure 2). However, β–glucosidase of the strain SP3-14 still active at 70°C with nearly the same level of 60°C. The cellulase of *Halocella* sp. strain SP3-1 and β–glucosidase of *Ha. praevalens* strain SP3-14 exhibited appreciable in the present of NaCl between 0 to 25% (w/v) NaCl at pH 5.0 and temperature 60°C (Figure 3). The optimum NaCl for an activity of cellulase and β–glucosidase was found at 15% and 10% (w/v), respectively. The activity was reduced to 46.6% of the original activity at 20% (w/v) NaCl, showing the halotolerance as the same observed in other extracellular enzymes from halophiles, such as *Halomonas meridian* (Coronado *et al.*, 2000) and *Chromohalobacter* sp. TVSP 101 (Prakash *et al*., 2009). Previous study has been reported that cellobiose is not only found to be accumulated in the cellulosic hydrolysate when hydrolyzed with endocellulases but also inhibited these enzymes (Martins *et al*., 2008, Andric' *et al*., 2010). Hence, adding β-glucosidase of *Ha. praevalens* SP3-14 could improve degradation ability, which reduced production inhibition. Altogether the results, the symbiosis of halophilic anaerobic bacteria *Halocella* sp. SP3-1 and *Ha. praevalens* SP3-14 have the role toward on cellulose degradation process. Moreover, with these characteristics, the obtained cellulase and β–glucosidase could be used in many interesting applications in biotechnological processes which are carried out under harsh conditions, such as high temperature, pH, and salinity.

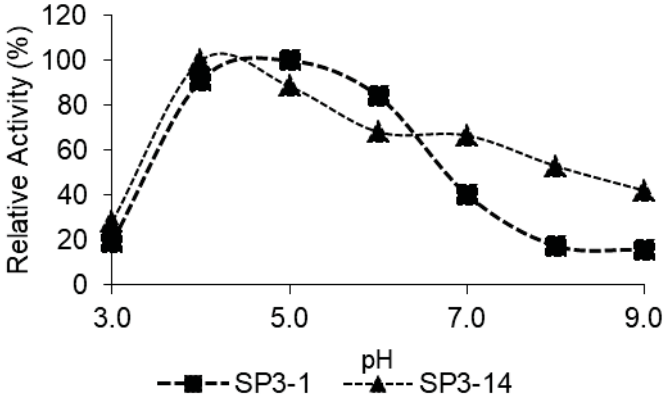

Figure 1. The activity of cellulase from *Halocella* sp. strain SP3-1 and β-glucosidase from *Ha. praevalens* strain SP3-14 under various pH at 60°C.

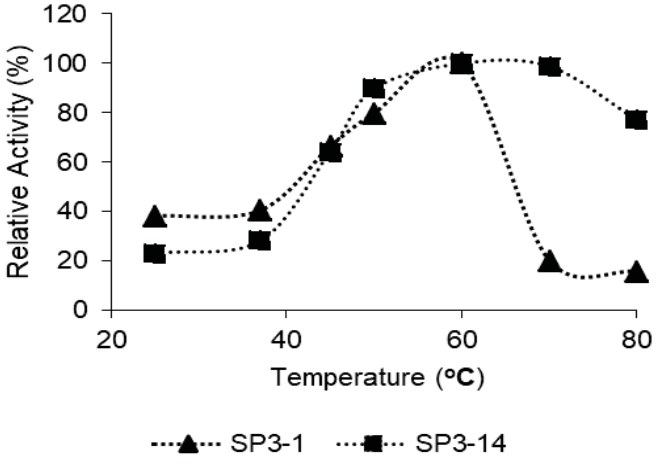

Figure 2. The activity of cellulase from *Halocella* sp. strain SP3-1 and β-glucosidase from *Ha. praevalens* strain SP3-14 under various temperatures at pH 5.0 and 4.0, respectively.

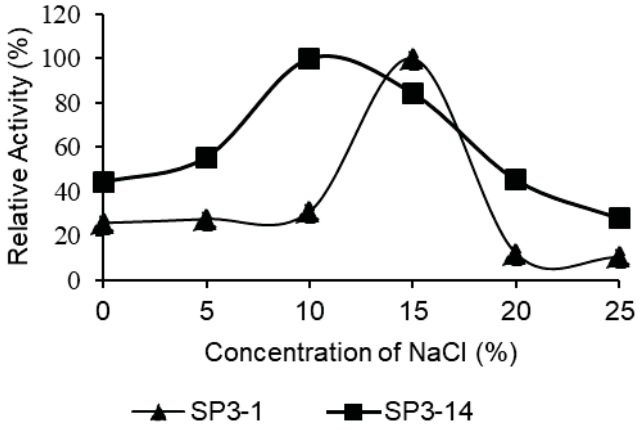

Figure 3. The activity of cellulase from *Halocella* sp. strain SP3-1 and β-glucosidase from *Ha. praevalens* strain SP3-14 under various NaCl concentrations at 60°C, and pH 5.0 and 4.0, respectively.

CONCLUSION

The biomass-degrading bacteria with the ability to competently degraded cellulose in the high salt concentration were enriched. On the other hand, two halophilic bacteria strains, SP3-1 and SP3-14, were successfully isolated from the salt evaporation ponds by using different carbon sources. Moreover, this is the first report of co-culturing of *Halocella* sp. strain SP3-1 and *Ha. praevalens* strain SP3-14 on cellulose in terms of an enzymatic symbiosis. Therefore, it can be concluded that the symbiosis between strains SP3-1 and SP3-14 are co-produced the extracellular cellulase and extracellular β–glucosidase, respectively. The symbiotic mechanism between strains SP3-1 and SP3-14 was studied by comparing the enzymatic system. It was successful in separating microorganism to hydrolysis cellulose under the harsh condition as high salt concentration.

ACKNOWLEDGMENTS

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Oral Presentation
BIOECONOMY

O-EC1

BIODECOLOURIZATION OF TEXTILE SYNTHETIC AZO DYE (METHYLENE BLUE) BY *Bacillus subtilis* STRAIN JSG1

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ABSTRACT

Degradation of a synthetic azo dye is particularly essential challenges before releasing of textile dye effluents into the natural resource. One of them, the methylene blue is a primary synthetic azo dye used in the textile industry, which produced effluents that extremely cause the highest source of water pollution and health problems. Over the years, several of physical-chemical decolonization techniques have been reported, but the textile industries accepted few due to the high cost and non-ecofriendly procedure. Therefore, biodecolourization is considered an alternative method for textile dye removal with environmental-friendly. In this study, the newly azo dye degrading bacterium was screened and isolated from agricultural residue composts, and soils in Suphanburi province of Thailand. A methylene blue-degrading bacterium, designed as strain JSG1 showed the highest dye removal ability. Based on 16S rRNA gene analysis, the isolated strain was identified belonging to *Bacillus subtilis* with 99.35% similarity. The optimum temperature and pH of growth were 37^{o}C and pH 7.0, respectively. The absorbance of methylene blue removal was measured by spectrophotometrically ($OD_{\lambda max}$= 592nm). The strain JSG1 could decolorize more than 93% of methylene blue (20 mgL^{-1}, w/v) within 72 hours under the optimal condition. The results support that *B. subtilis* strain JSG1 decolorized the azo textile dye efficiently, and could reduce the environmental impact of industrial wastewater containing this dye.
Keywords: *Bacillus subtilis*, Biodecolourization, Methylene blue, Synthetic azo dye, Textile industry

INTRODUCTION

Industrialization and urbanization are importantly attributed to the pollution topics because a large amount of industrial effluents has been released directly into the natural resource that has augmented wastewater troubles (Rajan, 2015). The textile industry is one of the main zones which produced an enormous amount of industrial effluents annually causing the highest source of water pollution which harmful, as well as mutagenic and cancer disease (Gomaa, 2016). Synthetic azo dyes are commonly used inside the leather, printing, cosmetic, pharmaceutical, food industries and increasing used in the textile industries because of their easiness and cost-effectiveness in synthesis, firmness, excellent strength to light, temperature, detergent and microbial attack, and multiplicity of colour compared with natural dyes (Rodríguez, 2009).

Approximately, there are 10,000 different synthetic dyes are used in industrial, representing an annual consumption of around 7×10^5 tonnes worldwide including methylene blue (MB) (Ulson *et al*., 2007). Approximately 10-15% of synthetic dyes are lost during different processes of the textile industry (Khehra *et al.*, 2005). The effluents with high levels of Biochemical Oxygen Demand (BOD) and Chemical Oxygen Demand (COD) values are highly toxic to the ecosystem (Jayan *et al*., 2016). Synthetic azo dyes are characterized by the presence of one or more azo bonds (R_1–N=N–R_2) linkage aromatic ring compounds (Popli *et al.*, 2015).

According to the previous reports, different microorganisms, belonging to different groups of bacteria, fungi, algae, and yeast, are involved in biodegradation of different synthetic azo dyes by their metabolic pathways (Jafari *et al.*, 2014). Reducing synthetic azo dye in wastewater instigated by the textile industry is hugely considered as environmentally friendly approach (Min *et al.*, 2015). Therefore, the

treatment of textile effluents becomes necessary for wastewater management. The aim of this work is to show the positive impact of *B. subtilis* strain JSG1, isolated from the agricultural residue composts in Suphanburi province, on its degradability of methylene blue in textile effluents.

MATERIALS AND METHODS

Synthetic Dye, Culture Medium, Screening and Isolation of Methylene Blue Decolourizing Bacteria

The synthetic dye, MB ($C_{16}H_{18}ClN_3S.H_2O$) was purchased from Sigma-Aldrich. All chemicals used were analytical grade. Nutrient Broth (NB) contained peptone 5.0 g, NaCl 5.0 g, meat extract 1.5 g, and yeast extract 1.5 g per liter. Methylene blue-degrading bacteria were isolated from agricultural residue composts in Suphanburi province of Thailand. In brief, each sample was cultured in 5ml of NB medium (pH 7.0) that supplemented with MB initial concentration (20mg.L^{-1}) and incubated at 37^{o}C by 120 rpm for 24 hrs. The final methylene blue-degrading enrichment culture was diluted serially in 0.8% of NaCl and spread on NB agar plates added with dye. The positive candidate showed a clear halo zone on the plate. The purity of MB-degrading single colonies was picked up and streaked on MB agar plate. Then, MB-degrading pure colonies were cultured into 250 ml Erlenmeyer flask containing 50ml of NB with MB for 72 hrs. The highest dye removal ability of isolate was selected in this study.

16S rDNA Partial Gene Sequencing and Phylogenetic Analysis

Genomic DNA of the isolate was prepared using Phenol-Chloroform-Isoamyl Alcohol (PCI) DNA extraction technique. The 16S rRNA gene was amplified by PCR using the following primers: 8F (5'-AGAGTTTGATCCTGGCTCAG-3') and 1492R (5'-GGTTACCTTGTTACGACTT-3'). PCR was performed with standard conditions according to the manufacturer's instructions. The determined sequence was compared with online references available in the GenBank database by the BLAST program. Phylogenetic analysis was constructed using MEGA10 program.

Decolourization Analysis

Culture broths in each day of MB decolourization were centrifuged at 13,000 rpm for 15 min. The supernatant was further absorbance determined. The absorbance of methylene blue removal was measured by UV-VIS spectrophotometer at its wavelength (λ_{max}= 592nm). All decolourization experiments were performed in terms of the percentage (%) of decolourization.

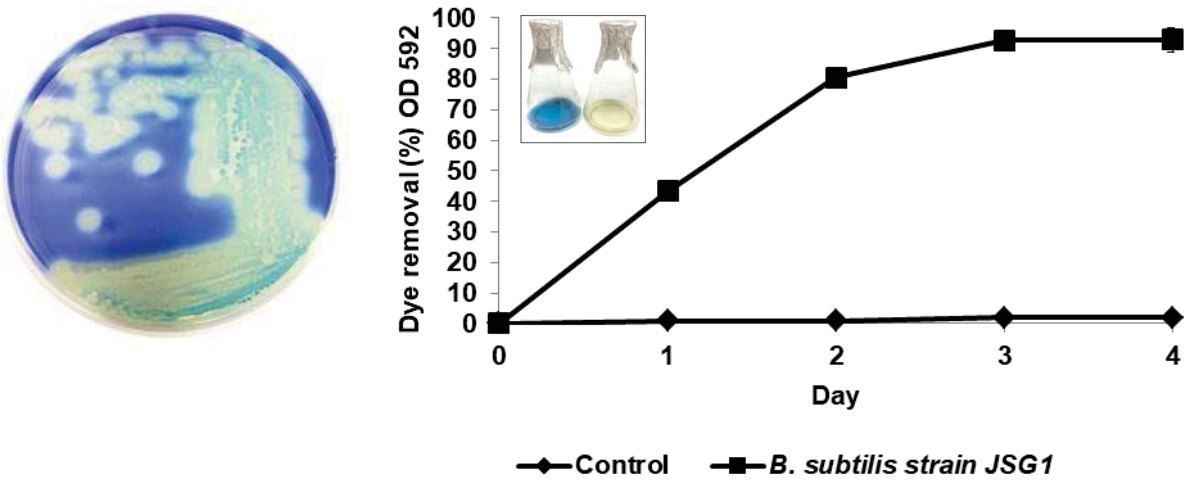
Effects of Temperature, pH, and Culture Conditions on MB Decolourization

The results of temperature, pH, and incubation condition on MB degradation were analyzed. Three different temperatures as 30, 37, and 45^{o}C were studied at an initial pH 7.0 by 120 rpm for 72 hrs. The effect of pH on MB decolourization was done in the range between 4.0 to 9.0, which incubated at 37^{o}C by 120 rpm for 5 days. In case of shaking (200 rpm) and static conditions on the MB decolourization were observed at 37^{o}C, pH 7.0 for 72 hrs.

RESULTS AND DISCUSSION

Isolation of MB-degrading Bacterium

The MB-degrading bacterium was successfully isolated from the composts. The selected strain namely JSG1 was shown MB removal on the agar plate (Fig.1A). Afterward, the strain JSG1 was determined to dye removal by culturing in 50ml of NB broth medium with MB to confirm dye removal ability. The result indicated that the MB-degrading bacterium could decolorize more than 93% of MB within 72 hrs (Fig.1B). Many microorganisms have been reported to decolorize MB previously such as *Acinetobacter baumannii, Corynebacterium* sp., *Cytophaga columnaris, Escherichia coli, Pseudomonas fluorescence,* and *Pseudomonas iuteola.* Interestingly, the isolated strain JSG1 can remove nearly complete whereas the previous report revealed the consortium of *E. coli* and *P. luteola* decolorized MB approximately 37% and increased decolourization rate up to 58% of dye removal after 95 hrs (Ghanem *et al*., 2011). The result implied that the isolated strain JSG1 was more attractive for MB decolourization in wastewater treatment.



(A) **(B)**

Figure 1. MB degrading bacterium on MB+NB agar plate (A). Dye removal of *B. subtilis* strain JSG1 toward MB within 96 hrs (B). Each data represents as mean ± SD (n=3)

Identification and Phylogenetic Analysis of MB-degrading Bacterium

The 16S rRNA gene of the strain JSG1 was sequenced and constructed a phylogenetic tree. The partial sequence of the 16S rRNA gene was 1,411 bp. After BLAST with GenBank databases, the result revealed that the strain JSG1 showed the highest similarity with *B. subtilis* strain X-c at 99.35% and followed by *B. toyonensis* strain VITSN1505 in 99.21%. Therefore, the strain JSG1 was classified as *B. subtilis* strain JSG1. The Neighbour-Joining phylogenetic tree indicated that strain JSG1 belonged to *Bacillus* genera (Fig.2).

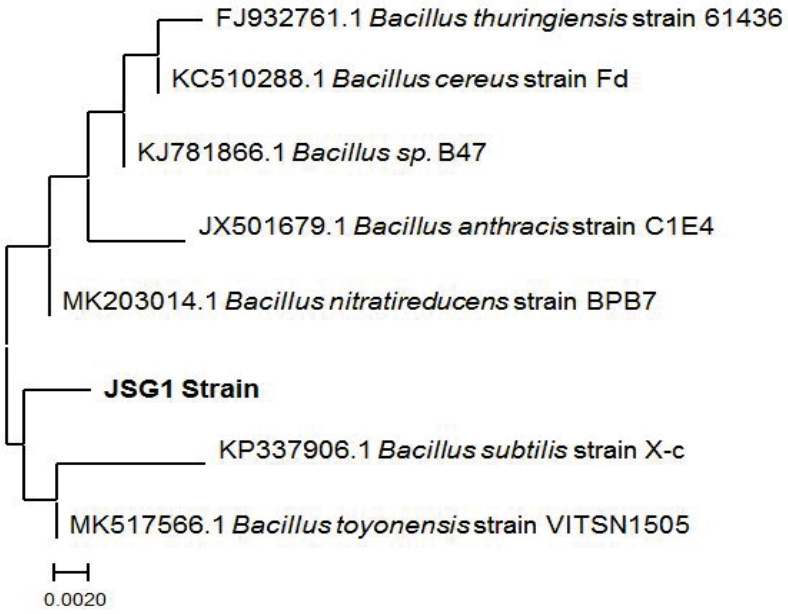


Figure 2. Phylogenetic relationship based on the 16S rRNA gene sequences of strain JSG1 and other related strains.

Effect of Temperature on the Decolourization Efficiency

The results of the temperature of growth on the MB decolourization were assessed three different temperatures at 30, 37, and 45°C at pH 7.0 under shaking condition by monitoring for 96 hrs. As a result, the optimum temperature for MB decolourization by *B. subtilis* strains JSG1 was found more than 93% in 37°C (Fig.3). Moreover, this strain can remove MB around 67 and 24% at 30 and 45°C, respectively. The strain JSG1 can be applied in the wastewater system that is mostly operating under the average environmental temperature.

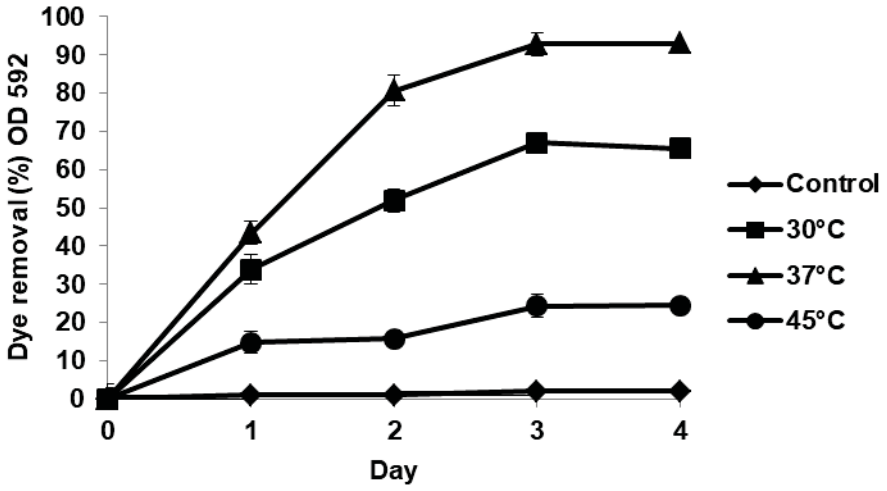


Figure 3. Effect of temperature on decolourization of MB by *B. subtilis* strain JSG1 at pH 7.0 under shaking (120 rpm) condition. Each data represents as mean ± SD (n=3)

Effect of pH on the Decolourization Efficiency

The impact of the pH to the decolourization of MB by *B. subtilis* strain JSG1 was analysed in the 5th day at 37°C by shaking cultivation. It found that this strain still high efficiency in removing MB at various pH 5.0-7.0 (Fig. 4), with the highest MB removal at pH 7.0. Moreover, it can remove 32 and 27% at pH 8.0 and 9.0, respectively. The results indicated that the strain JSG1 could be applied under the wide range of pH. However, it could not be used in acidic condition (pH 4.0). In contract with *Aspergillus wentii*, it can decolorize MB nearly 85.04% from aqueous solution in pH 10.0 (Acemio *et al*., 2010).

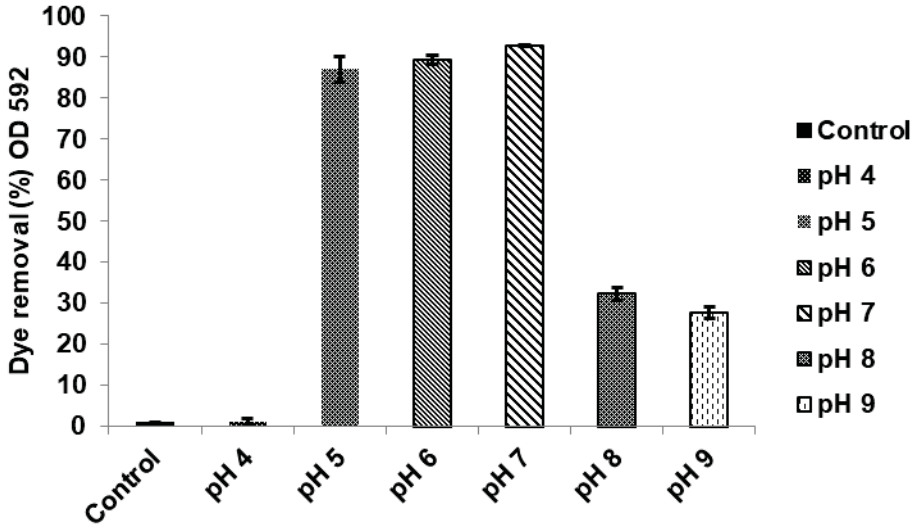


Figure 4. Effect of pH on decolourization of MB by B. subtilis strain JSG1 at 37oC under shaking (120 rpm) condition in the 5th day. Each data represents as mean ± SD (n=3)

Effect of Incubation Conditions on the Decolourization Efficiency

The result of shaking and static conditions on the dye decolourization was studied. The experiment was carried out by measuring MB decolourization assay under static and shaking (200 rpm) at 37°C, pH 7.0 for 72 hrs. Decolourization of MB was removed in 93% under shaking condition and only 40% at static condition (Fig.5). The result showed that shaking was better than static condition because of the isolated strain JSG1 is an aerobic bacterium, thereby oxygen is necessary for growth (Somerville and Proctor, 2013).

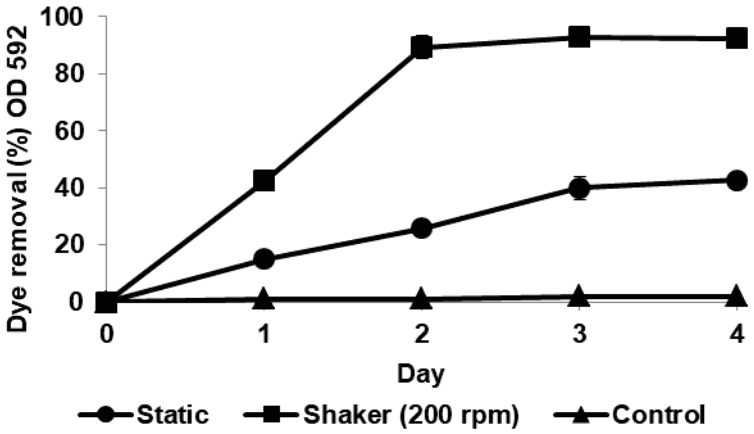


Figure 5. Effect of incubation conditions on decolourization of MB by *B. subtilis* strain JSG1 at 37°C and pH 7.0. Each data represents as mean ± SD (n=3)

CONCLUSION

Although MB dye exhibited a great group of chemical compounds, however, it could have negative impacts on environmental pollution. The MB-degrading bacterium was screened and isolated from agricultural residue composts in this study. The isolated strain JSG1 was identified as *B. subtilis* by 16S rRNA gene analysis. The physical-biochemical parameters such as pH, temperature, and culture incubation conditions were determined for the MB decolourization. The optimized condition for MB decolourization was at pH 7.0, 37°C with shaking state. It near entirely removed MB ca. 93%. Furthermore, the enzymatic, involving in MB decolourization of the strain JSG1, will be studied in the future.

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INTERGRATED ECONOMIC ANALYSIS OF AGRICULTURAL WASTE-BIOGAS TO INVESTMENT OF BIO-METHANOL INDUSTRY

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ABSTRACT

Bio-methanol is methanol produced from bio resources. In this case, it was obtained from biogas containing methane (CH_4) and carbon dioxide (CO_2). Biogas was first removed H_2S, and then it was concentrated by removing CO_2 until obtaining 85% and 95% methane composition for substituting CBG (Compressed Biogas) and LNG (Liquefied Natural Gas) respectively. CO_2 separated from the process was returned to mix with H_2S removed biogas until obtaining mixed gas ratio CH_4/CO_2 (30/70). The mixed gas was run in steam reforming reactor with temperature 700 °C and atmospheric pressure under modified Ni/Al_2O_3 catalyst then obtaining syngas as a product. The syngas was pressurized and fed into the methanol synthesis reactor with 200 °C and 40 to 50 bar under $Cu/ZnO/Al_2O_3$ catalyst yielding approximately 98% bio-methanol purity. The bio-methanol can be refined to 99.5% purity for replacing commercial methanol in various applications: solvent, biodiesel, and additives. All experimental data received from previous work which used for this calculation and it was showed that the optimal process was under 50% off gases recycle, 3 THB/kg biogas price, 12 THB/kg of methanol price and available production capacity over 14 to 20 metric tons/day (MT/day) and was fitted to most biogas producer obtaining feasible methanol price of 12,000 baht per ton. The economic parameters were internal rate of return (IRR) 15%, return on investment (ROI) 6.8%, and payback period (PB) 5.4 years. This feasible result was applied for linking a triangle of social connection (Agricultural waste supplier-Biogas maker-Bio-methanol producer). In Thailand, it was evaluated that Bio-methanol can substitute to imported commercial methanol which was around 0.88 MT in 2018 and forecasted 1.70 MT in 2036. The calculation showed that only 10% of agricultural residues from equal of corn, cassava, and palm was 4.95 MT. This number can generate 5,210 million cubic meters (Mm^3) of biogas, transform to 2.74 MT of bio-methanol, and compensate electricity 1,543 MW. The three stakeholders have valued 2,478 MTHB, 15,662 MTHB, and 33,078 MTHB for Organic waste supplier, Biogas maker, and Bio-methanol producer. Hence, it can save up to 20,000 MTHB for 1.7 MT of importing commercial methanol in 2036. This cash cycle is distributed to 18.75%, 37.50%, and 62.50% for the agricultural sector, biogas plant, and bio-methanol industry respectively. In conclusion, bio-methanol from biogas obtained from agricultural residues is feasible and supports basic industry, and not only creates the linkage of upstream, midstream, and downstream sectors but also strengthens all stakeholders.

Keywords: Bio-methanol, Biogas, Agricultural waste, Economic Analysis

INTRODUCTION

Awareness of energy usage with value and responsibility in conjunction with environmental conservation, biofuels are therefore being used to replace fossil fuel. Biogas is one of the biofuels and it can be manufactured from organic and agricultural waste such as manure, food, fruit, tapioca starch, and sugarcane residue. Biogas comprises mainly methane and carbon dioxide. Because methane is hydrocarbon gas, it is used as an alternative fuel for heat and electricity generation. Biogas has been produced for a long time and has been used in the industry. However, the factors that affect the productivity of the biogas industry are two aspects: low economic return rate and high carbon dioxide emission to the environment. To minimize the problems and subsidize the biogas maker, CO_2 utilization has been studied by many researchers. Attractive research is transforming biogas to bio-methanol. Commercial methanol (produced from fossil sources such as coal or natural gas) can be replaced by bio-methanol (produced from

syngas obtained from reforming of biogas). Methanol is an important chemical and it can either be used directly or further transformed to produce a wide range of chemicals using in diverse sectors such as solvent, biodiesel, biofuels and additives (Richardson and Paripatyadar, 1990).

Direct using, methanol is used as a solvent and a raw material by reacting with vegetable oil to produce biodiesel (Mayra and Leiviska, 2008). Indirect consuming, it is transformed to formaldehyde, MTBE/TAME (blending component for gasoline), dimethyl ether (DME), MTO (methanol to olefin), and MTP (methanol to paraffin), etc. Global methanol demand is rising an average of 5% to 6% each year by 63.96 MMT (2014), 92.30 MMT (2016), and forecasted 107 MMT (2023) (HIS Markit, 2017). In case of Thailand, methanol was absolutely imported and was approximately consumed 10% of the World methanol usage from 0.66 MMT (2014), 0.78 MMT (2016), 0.88 MMT (2018) and predicted 1.7 MMT (2036) (Hazardous Substance Report, 2018). A large amount of methanol will be required because biodiesel is projected for 20% blending with commercial diesel in 2036 (AEDP plan, 2015). As a result, the production of bio-methanol is interested not only solving CO_2 problem form using biogas as electricity but also alleviating the amount of methanol importing from outside country. In addition, it will be an alternative route for the biogas industry and a promising way for bio-methanol and further transformed to biofuels and biochemicals.

Bio-methanol, methanol derived from biogas, is synthesized by two main steps: reforming of methane and methanol synthesis. The first methane can be transformed to syngas composing carbonmonoxide and hydrogen by two reactions: Dry reforming reaction (1) is methane and carbondioxide reacting and obtaining syngas and steam reforming reaction (2) methane and water converting to syngas. However, water gas shift reaction (3) can be as a side reaction. The latter carbonmonoxide or carbondioxide are used to produce methanol. The reaction has occurred via two reactions: hydrogenation on carbon monoxide (4) and hydrogenation on carbon dioxide (5) (Bussche *et al.*,1996) ; (Skrzypek *et al*.,1991) ; (Snoeck *et al.*,1997).

Methane Reforming

$CH_4 + CO_2$	↔	$2CO + 2H_2$	ΔH = +274.7 kJ/mol	(1)
$CH_4 + H_2O$	↔	$CO + 3H_2$	ΔH = +206.0 kJ/mol	(2)
$CO + H_2O$	↔	$CO_2 + 2H_2$	ΔH = -41.12 kJ/mol	(3)

Methanol Synthesis

$CO + 2H_2$	↔	CH_3OH	ΔH = -90.55 kJ/mol	(4)
$CO_2 + 3H_2$	↔	$CH_3OH + H_2O$	ΔH = -49.43 kJ/mol	(5)

In most novel industry, the process feasibility and economic analysis are essential factors to boost the research study to commercialize scale. Process simulation is one of the alternative tools which it is useful through predict commercial process and to evaluate economic feasibility before the process is invested. It comprises of two steps: (1) Obtaining input data from laboratory or pilot scale comparing with basic theory. (2) Calculating those variable parameters or condition affecting to process for prediction and comparison with the alternative process and design process.

The purpose of this article is to make technical feasibility of a BTM (Biogas to Methanol) process and meanwhile explores the opportunities of upstream (agricultural sector), midstream (Biogas maker) and downstream process (Bio-methanol) relating to electric producer from biogas.

MATERIALS AND METHODS

Raw materials

1. Biogas and mixed gas contained a ratio of CH_4 and CO_2 55/45 vol% and 30/70 vol% respectively received from Thai Special Gas Co., Ltd.
2. CO_2 (95%) obtained from LNG process of CRYOTECH Co., Ltd.

Process reaction modeling

Data obtained from 1 L/D experimental pilot scale. The process of bio-methanol production consists of two main reactions, including methane reforming reaction and methanol synthesis reaction. Two main reactors were made from 304 stainless steel with the inside diameter of 16 cm and belonging dimension of 30 cm. The flow of mixed gas was controlled by mass-flow controller length and data of the experiment as shown in Table 1. (Jitrwung *et al*., 2019)

Table 1 Data of 1 L/D bio-methanol obtained from an experimental pilot scale

MeOH	Bio-methanol capacity: 1 L/day			
	Biogas To Syngas (BTS)		Syngas to methanol (STM)	
Feed Flow rate	1.18	L/min	1.7	L/min
catalyst density	1	kg/L	1.49	kg/L
	0.685	kg	1.02	kg
Volume of bed	0.685	L	0.685	L
WHSV	1.72		1.67	

The simulation work of bio-methanol process from biogas was developed by using laboratory operating condition. Non-random two-liquid model (NRTL-RK) was used for model illustration. Catalytic reactions in fixed-bed reactors were simulated in the one-dimensional heterogeneous model. Temperature and concentration gradients are accounted in an axial direction (Tamnitra *et al.*, 2018).

Process Flow Diagram of the H_2S removed Biogas to Bio-methanol process

Process flow Diagram of the H_2S removed biogas to the bio-methanol process shown in Figure 1. Biogas is preheated in a heat exchanger (HX-1), mixed with water in a mixer (MX-2), and then heated up in furnace to raise high temperature to meet up the desired temperature in the first reactor (R-1). After the reaction is going on in the reactor, the fluid product is come out and cooled down in a heat exchanger (HX-2) then steam is condensed and trapped in the vessel (V-1). The fluid is separated into two phases. Liquid phase is water returning by the pump (P-1), mixing with steam in the mixer (MX-1). Vapour phase or syngas is compressed by a compressor (CP-1) to reach required pressure then sent to an absorption unit (AB-1) for removing the water before passing the syngas to the second reactor. The methanol reaction is occurred in the reactor (R-2) under operating temperature (170-200 ^{o}C) and pressure (40 to 50 bar). The methanol in the gas phase combining with residue gas is cooled down to 40 ^{o}C by cooling water in a heat exchanger (HX-3). Methanol is condensed and firstly trapped in the vessel (V-2), and it depressurized to atmospheric pressure by a relief valve (P-2) then sending to vessel (V-3) for second trapped and finally obtaining methanol. The residue gas from V-2 and V-3 is mixed in the mixer (MX-4) and sent to the splitter (SP-1). The residue gas is partially purged (50%) and returned (50%) to the mixer (MX-3) for returning to the methanol synthesis.

Process economic evaluation (IRR, NPV, ROI, PB)

The feasible assumption of studying bio-methanol was shown in Table 2. Basic economic calculation tools such as Net Present Value (NPV), Internal Rate of Return method (IRR), Return on Investment (ROI) and Payback Period (PB) as shown in the equation (6)-(8) (Benguerba *et al*., 2015) ; (Richardson *et al.*,1990)

$$NPV = \sum_{t=1}^{t} \frac{C_t}{(1+r)^t} - C_o \quad (6)$$

NPV = net present value
C_0 = initial investment
C_t = net cash flow over time
r = discount rate
t = duration

$$I - \left(\sum_{t=1}^{t} \frac{C_t}{(1+r)^t} - C_o\right) = 0 \quad (7)$$

I = first investment payment
C_t = net cash flow over time
n = amount of interest / number of years
r = discount rate
t = duration

Payback period = initial investment / net cash per year after tax (8)

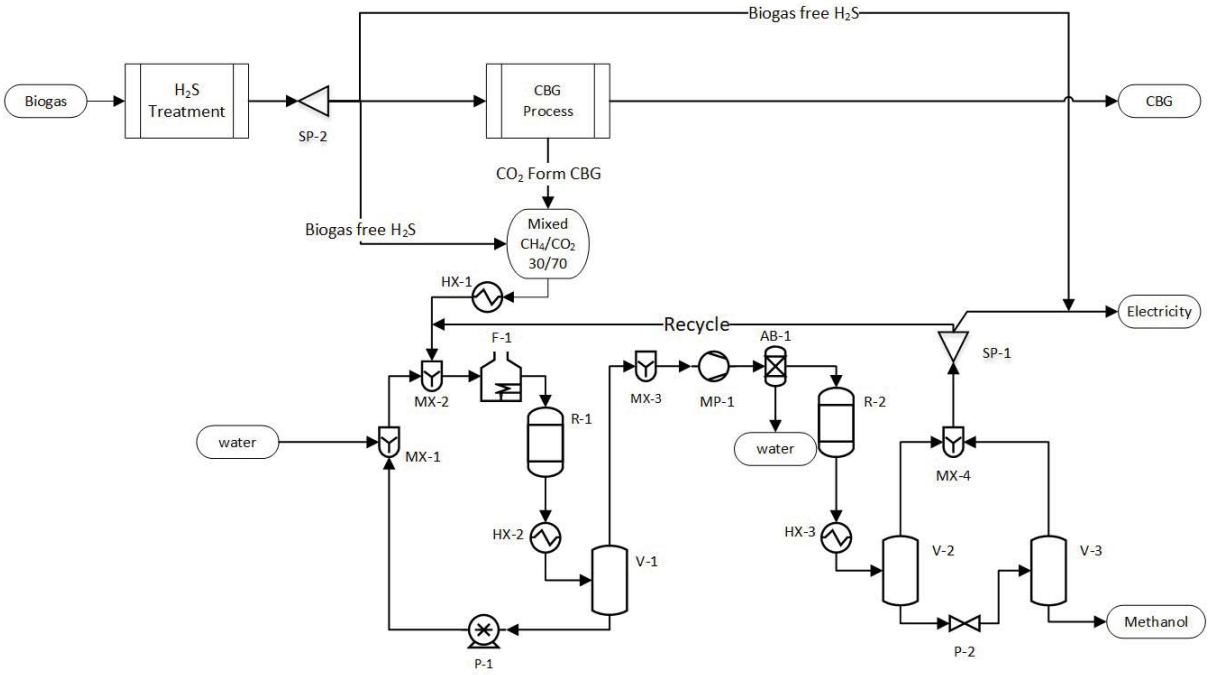

Figure 1. Process flow diagram of the H_2S removed biogas to bio-methanol process

Table 2 Assumption of feasibility study of Bio-methanol

ASSUMPTION	AMOUNT	UNIT
Simulation Assumption		
Production day per year	330	Days
Selling day per year	330	Days
Methanol rate	10,000	L/D
Price Methanol	12.00	THB/kg
Price Biogas (CH_4/CO_2=55:45)	3.00	THB/m^3
Cost Index	0.39	
Economic Assumption		
Depreciation	10	Years
Demand growth rate	5%	%
Incremental fixed costs	1,000,000	THB
SG&A growth rate	1%	%
WACC (or interest rate)	7%	%
Tax Rate	20%	%
Cash conversion cycle (CCC)	50	Days
Capital Expenditures (CAPEX)	66.78	MTHB

RESULTS AND DISCUSSION

Off gas recycle versus electricity production and CO_2 consumption

The bio-methanol process gave off gases containing syngas composition which can be returned to the process to compensate the raw material using. The result showed that increasing percent of gas recycle from 0 % to 100 % increased economic feasibility by NPV (13.992 MTHB to 60.279 MTHB), IRR (10.6% to 20.9%), ROI (2.7% to 13.5%) and reduced PB (6.40 yr to 4.43 yr) as shown in Table 3. This result reflected that returning off gases to use for raw material brought about increasing the positive economic feasibility. However, when considering the percent recycle to electricity production and CO_2 consumption, it is found that increasing the recycle gas decreased electrical production and CO_2 consumption as shown in Table 4.

The optimal off gases recycle was around 50 % because the process generated enough electricity to supply the bio-methanol itself and consumed optimum CO_2 relating to the optimum IRR around 15%.

Table 3 Sensitivity analysis of recycle off gases

SENSITIVITY ANALYSIS				
CASE STUDY	NPV	IRR	ROI	PB
No Recycle	13,992 k	10.6%	2.7%	6.40 y
25% Recycle	17,273 k	11.4%e	3.4%	6.21 y
50% Recycle	32,977 k	15.0%	6.8%	5.42 y
75% Recycle	48,877 k	18.5%	10.5%	4.80 y
100%Recycle	60,279 k	20.9%	13.5%	4.43 y

Table 4 Effect of recycle off gases to electricity production and CO_2 consumption

CASE STUDY	Electrical		CO_2 consumption	
	kW	THB/day	m^3/day	m^3/kg
No Recycle	597	39,963	9,719	1.23
25% Recycle	285	19,069	9,021	1.14
50% Recycle	0	0	7,175	0.91
75% Recycle	0	0	5,415	0.68
100% Recycle	0	0	4,140	0.52

Sensitivity analysis of production capacity (10,000 kg/day to 20,000 kg/day) versus methanol price and biogas price

When using the optimum 50% recycle off gases, the increasing process capacity from 10 to 20 MT/day versus to varying methanol price from 10 to 15 THB/kg showed in Table 5. The IRR study was resulted in positive economic feasibility starting from 12 THB/kg of methanol in all production capacity. When the methanol price was lower than 12 THB/kg, the IRR feasible showed negative. In the case of the methanol price is 11 THB/kg, the feasible is originated positive after increasing 14 MT/day methanol production capacity. When the methanol price is 10 THB/kg, the feasible is all negative in all production capacities.

The lowest possible methanol price was 12 THB/kg that used to evaluate the production capacity related to biogas price as shown in Table 6. The biogas price was equal to or lower than 3 THB/m^3 yielded the positive IRR. It is started to negative IRR when the biogas price started from 3.50 THB/m^3 and production capacity 10 MT/day. The negative IRR showed in all cases of methanol production capacity from10 to 18 MT/day.

Table 5. IRR of the effect of methanol production capacity versus methanol price

	IRR					
MeOH	10 THB/kg	11 THB/kg	12 THB/kg	13 THB/ kg	14 THB/ kg	15 THB/kg
10 MT/Day	-14.0%	-3.5%	3.9%	9.8%	15.0%	19.8%
12 MT/Day	-11.2%	-0.8%	6.7%	12.8%	18.3%	23.3%
14 MT/Day	-8.9%	1.4%	9.0%	15.4%	21.1%	26.4%
16 MT/Day	-7.1%	3.3%	11.1%	17.7%	23.6%	29.1%
18 MT/Day	-5.5%	5.0%	12.9%	19.8%	25.9%	31.7%
20 MT/Day	-4.2%	6.5%	14.6%	21.6%	28.0%	34.0%

Table 6. IRR of the effect of methanol production capacity versus biogas price

	IRR			
MeOH/BG	2.50 THB/m^3	3.00 THB/m^3	3.50 THB/m^3	4.00 THB/m^3
10 MT/Day	8.5%	3.9%	-1.6%	-8.4%
12 MT/Day	11.5%	6.7%	1.1%	-5.7%
14 MT/Day	14.1%	9.0%	3.3%	-3.5%
16 MT/Day	16.3%	11.1%	5.2%	-1.7%
18 MT/Day	18.3%	12.9%	6.9%	-0.2%
20 MT/Day	20.2%	14.6%	8.4%	1.2%

Sensitivity analysis of production capacity (10,000 kg/day to 20,000 kg/day) versus incremental fixed cost

The relationship of incremental fixed cost with methanol prices effected to IRR showed in Table 7. The biogas cost was reasonable at 3.00 THB/m^3, and the methanol price was fixed at 12 THB/kg. The incremental fixed cost was 4 to 5 MTHB and the methanol capacity was 10 MT/day, the result yielded the negative IRR. Other situations showed positive IRR.

Table 7. IRR of the effect of methanol production capacity versus Incremental fixed cost

	IRR				
MeOH/BG	1 MTHB	2 MTHB	3 MTHB	4 MTHB	5 MTHB
10 MT/Day	3.9%	2.2%	0.5%	-1.3%	-3.2%
12 MT/Day	6.7%	5.2%	3.8%	2.2%	0.7%
14 MT/Day	9.0%	7.8%	6.5%	5.1%	3.8%
16 MT/Day	11.1%	9.9%	8.8%	7.6%	6.4%
18 MT/Day	12.9%	11.9%	10.8%	9.7%	8.6%
20 MT/Day	14.6%	13.6%	12.6%	11.6%	10.6%

Triangle bio-economical bio-methanol values

Bio-methanol produced from biogas will create chain value from upstream to downstream as shown in Table 8. The upstream is the agricultural sector which leaves organic residues such as corn, cassava, and palm. These three plants generate approximately 60% of the products. Thereby it takes 10% or 4.95 MMT/year to make the biogas (Food and Agriculture Organization of the United Nations. 2019.), the cash is circulated 2,478 MTHB/year to this point. This vast amount of these agricultural residues can supply to 5,210 Mm^3 of biogas and can evaluate around 15,662 MTHB/year (Assessment of the biogas potential from agricultural waste in Northern Thailand. 2017). The biogas is transformed to 2.74 MT/year of bio-methanol and its value is around 33,078 MTHB/year. This process is not only creating the value chain for agriculture, biogas and bio-methanol but also help to reduce electrical power 1,543 MW or 5,401 MTHB/year. The triangle combining of these sectors for social connection of bio-methanol can be drawn in Figure 2. The only bio-methanol value is around 14,938 MTHB/year.

Profit of bio-methanol = 33,078 MTHB/yr - 15,662 MTHB/yr - 2478 MTHB/yr
= 14,938 MTHB/yr

Table 8 Social income of secondary generation of bio-methanol

Waste Materials	Agricultural Sector	Biogas Maker	MeOH Producer	Electricity Producer
	Million THB/year	Million THB/Year	Million THB/Year	Million THB/Year
Prices	500 Baht/Ton	3.0 Baht/M^3	12,000 Baht/Ton	3.50 Baht/KW
Corn (10.15%)	0.50 MMT/yr* 251 MTHB/yr	503 MM^3/yr 1,511 MTHB/yr	0.26 MT/yr 3,192 MTHB/yr	521 MTHB/yr
Cassava (61.42%)	3.04 MMT/yr* 1,522 MTHB/yr	3195 MM^3/yr 9,586 MTHB	1.68 MT/yr 20,246 MTHB/yr	3,306 MTHB/yr
Palm (28.43%)	1.41 MMT/yr* 705 MTHB/yr	1,512 MM^3/yr 4,536 MTHB/yr	0.80 MT/yr 9,640 MTHB/yr	1,574 MTHB/yr
Total	4.95 MMT/yr 2,478 MTHB/yr	5,210 MM^3 15,662 MTHB/yr	2.74 MT/yr 33,078 MTHB/yr	5,401 MTHB/yr

*(10% residue)

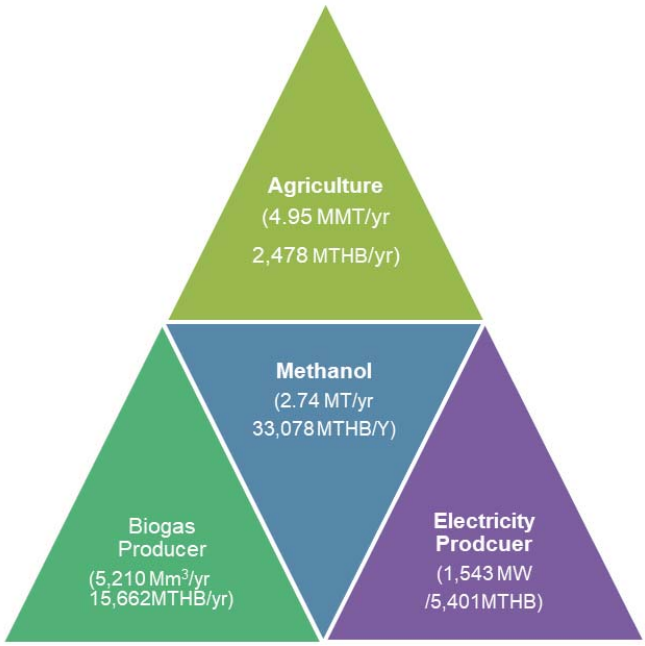

Figure 2 Social connection of bio-methanol

CONCLUSION

Bio-methanol is produced from biogas mixed with extra carbon dioxide. The optimal process was under 50% off gases recycle, 3 THB/kg biogas price, 12 THB/kg of methanol price and production capacity over 14 MT/day to 20 MT/day. The incremental fixed cost can be accepted from 1 to 3 MTHB and yielded positive IRR 6.5% to 14.6%. The integration value of bio residues (corn, cassava, and palm etc.) linked to biogas and bio-methanol provided 2,478, 15,662, and 33,078 MTHB/year respectively. The electricity is compensated around 5,401 MTHB/year.

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O-EC3

SEGMENTATION OF GREEN CONSUMERS: IMPLICATION FOR BIOPLASTIC PACKAGING

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ABSTRACT

This research aimed to study segmentation of green consumers based on attitude toward environmental awareness and lifestyle related to the use of bioplastic packaging. Questionnaires were collected from 400 respondents and were analyzed using a two-step cluster analysis. Findings showed that easy to use style, price, and safety were the main factors influencing decision on the use of packaging, while environmental friendly factor was ranked fifth, giving that consumers were aware of environmental impact of packaging choice. Most respondents preferred thermoplastic starch (TPS) tray comparing to regular plastic tray. However, respondents were willing to pay additional price less than 5 THB for bioplastic packaging per piece. Results from the cluster analysis indicated that green consumers were segmented into four clusters viz. young no green group (33.0%), young go green group (28.8%), green concerned group (24.5%) and powerful green group (13.8%). Each group was different in additional price for bioplastic packaging, demographics, attitude toward environment, and green lifestyle. Overall, the young go green group and powerful green group, which together accounted for 42.6%, had a high potential for using bioplastic packaging. This study revealed the characteristics of different green consumer groups leading to develop green marketing strategies differently and consumers need to be informed and encouraged on environmental awareness.

Keywords: Bioplastic packaging, Customer segmentation, Two-step cluster analysis, Attitude, Lifestyle

INTRODUCTION

Consumption of plastic packaging has increased by 3-5% annually (Economic Intelligence Center, 2018). The changes of consumer lifestyle toward urbanization and convenience led to more demand on plastic usage. In addition, the growth of food service businesses (i.e. restaurants, café and bakery shops) and delivery/takeaway businesses caused more single-use plastic packaging for food and beverage products (Jinkarn, 2015). In 2017, the market value of delivery/takeaway business in Thailand was 26,000 million baht (BLT Bangkok, 2018). With a large scale consumption of plastic, improper disposal of household and industrial in Thailand has caused poor waste management and plastic pollution.

Waste management is one of the critical problems in Thailand. In 2018, there were 27.8 million tons of solid waste, but only 10.88 million tons were treated appropriately and 9.58 million tons were reusable (Pollution Control Department, 2019). A total of 7.36 million tons (27% of total of solid waste) was mismanaged waste, of which 2 million tons were plastics wastes such as plastic bottles, plastic bags and plastic trays. As a result, Thailand became the 6th worst contributor of plastic marine debris in the world with 1.03 million tons of plastics wastes (Komchadluek, 2018). Moreover, 70% of the plastic marine debris are durable and non-degradable which endanger marine species and cause ocean pollution. As a response to this problem, the use of bioplastics is an alternative way for solving environmental pollution (Kaewpirom, 2014).

In 2018, the amount of global bioplastic production was about 2.11 million tons, of which up to 65% of the total production were developed for the packaging market (European Bioplastics, 2018a). The advancement and innovation of the bioplastic industry in new materials have continuously improved the properties of bioplastics close to those of petroleum based plastics. Thermoplastic starch (TPS), includinges starch blends made of thermo-plastically modified starch and other biodegradable polymers, is a popular bioplastic type for producing packaging. The TPS packaging accounts for 18.2% of the total bioplastic packaging (European Bioplastics, 2018b; Ministry of Industry, 2018). In Thailand, the development of TPS packaging has been a focus of domestic and international investors since the main sources of starch come

from rice and cassava, which are the economic crops of Thailand. Furthermore, the growth of environmental awareness have driven the demand for green products and reduced the use of plastics.

Despite the increasing of environmental concerns, some consumers may or not accept the use of bioplastic due to various factors. Bioplastic being a new innovation for consumers and applications of bioplastic are limited in a niche market. Moreover, the cost of bioplastics is higher when compared to conventional plastics. The purpose of this research was to classify green consumers based on attitude toward environmental awareness and lifestyle. Bioplastic packaging of bakery products was used in this study because of selective applications of bioplastic sties. The outcomes can provide useful information for food packaging businesses and marketers to have a better understanding of green consumers and bio-economy markets.

MATERIALS AND METHODS

A questionnaire was developed in consultation with experts, and pre-tested with 30 respondents to obtain the final version. Face-to-face interview was conducted to collect information for the questionnaire during May to August 2018. The interviewees were consumers who had purchased bakery products in the past week. In this study, the applications use of bioplastic with TPS properties (i.e. poor thermal property and tensile strength) are limited and suit to a single packaging, so the bakery market was selected. In total, 400 respondents were interviewed.

The collected data was summarized to explain the demographic and consumption behavior of the respondents with descriptive statistics. The part of consumer attitude toward environmental awareness and lifestyle was applied under 5-point Likert scales (1 = strongly disagree and 5 = strongly agree). The part of knowledge about bioplastic was asked by using true or false questions. Two-step cluster analysis, identifies groupings by running pre-clustering first and then hierarchical methods, was used to classify green consumer groups related to the use of bioplastic packaging.

RESULTS AND DISCUSSION

Demographic data

Results of demographic data are presented in Table 1. Female respondents accounted for 67.75%. The majority of respondents (69.75%) was 21-38 years of age. For education, 61% of the respondents had a bachelor degree and 31.5% completed a master degree or higher. Most of the respondents were public employee (33%) and student (29.5%). Twenty nine per cent of the respondents had monthly income less than 10,000 THB while another 29.5% had monthly income 10,001-20,000 THB. Only 7.5% of the respondents earned more than 50,000 THB per month.

Consumption behavior

The consumer behavior on bakery products is shown in Table 2. Over 60% of the respondents bought bakery products from bakery shops, and 43.71% of the respondents used both service at the shop and take home. Most respondents bought bakery products 1-2 days/week and spent 51-100 THB/time. On the factors affecting decision on the use of packaging, easy to use (60%) is the most important factor followed by price (57.43%), safety (49.43%), design (42%) and environmental friendly (40%), giving that consumers have positive attitude toward environment. Most respondents were willing to pay less than 5 THB extra for bioplastic packaging. When respondents were asked to choose three types of tray packaging for bakery products, most preferred TPS tray (70.75%) to paper tray (17.75%) and plastic tray (11.50%) respectively.

Table 1 Demographic of respondent (n=400)

Personal Characteristic	Specification	Percentage (%)
Gender	Male	32.25
	Female	67.75
Age	< 21 years	10.00
	21-38 years	69.75
	39-53 years	15.50
	>53 years	4.75
Education	Below bachelor degree	7.50
	Bachelor degree	61.00
	Master degree or more	31.50
Occupation	Student	29.50
	Business owner	6.00
	Private employee	10.00
	Public employee	33.00
	Other	21.50
Income	< 10,000 THB	29.00
	10,001-20,000 THB	29.25
	20,001-30,000 THB	13.00
	30,001-40,000 THB	14.25
	40,001-50,000 THB	7.00
	> 50,000 THB	7.50

Table 2 Consumer behavior on bakery product.

Behavior	Specification	Percentage (%)
Type of bakery shop (can choose more than one)	Bakery and drink shop	48.00
	Coffee shop	56.00
	Bakery shop	61.71
	Restaurant	50.29
	Don't buy bakery	14.29
Type of service	At shop	19.14
	Take home	37.14
	At shop and take home	43.71
Buying frequency	Everyday	1.71
	5-6 days/week	3.14
	3-4 days/week	22.86
	1-2 days/week	70.00
	Less than 1 day/week	2.29
Purchasing costs per time	Less than 50 THB	13.14
	51-150 THB	62.29
	151-300 THB	20.86
	More than 300 THB	3.71
Additional price for bioplastic packaging of bakery product	Do not willing to pay	13.00
	Less than 5 THB	57.00
	6-10 THB	13.50
	11-15 THB	14.00
	More than 15 THB	2.50
Factors for decision on the use of packaging for bakery product (Top 5)	Easy to use	61.71
	Price	57.43
	Safety for food	49.43
	Packaging design	42.00
	Environmental friendly	40.00
Packaging type for bakery product	TPS tray (Thermoform technique)	70.75
	Paper tray	17.75
	Plastic tray	11.50

Attitude toward environmental awareness and lifestyle

There were 10 statements related to four attitudes toward environmental awarenesses (S1-S4) and six lifestyles (S5-S10) as shown in Table 3. The reliability test of questionnaire by Cronbach's alpha was 0.755, indicating that the data was reliable. Almost half of the respondents (40.5%) agreed to choose bioplastics for single-use. Almost 40% of the respondents strongly agreed and one third of the respondents agreed that sorting of disposal waste was a social responsibility. Over 70% of the respondents always kept up with news about technology and innovation. Almost 50% of the respondents preferred to purchase products from the stores that used environmental friendly packaging, giving that consumers would have a positive image of business that supports environmental issues.

Table 3 Attitude toward environmental awareness and lifestyle (n=400)

Statement	Strongly disagree	Disagree	Neutral	Agree	Strongly agree
S1. The use of packaging types has impact on the environment.	1.3	6.0	40.0	36.0	16.8
S2. You will choose bioplastics for single-use products.	0.5	6.0	40.5	40.5	12.5
S3. You do not take any part on reducing global warming.	11.3	38.8	37.5	10.5	2.0
S4. Sorting of disposal waste is responsibility of everyone.	0.0	2.0	27.5	34.5	36.0
S5. You will not receive plastic bags from the stores, if it is not necessary.	1.8	11.0	32.0	37.5	17.8
S6. You always like to try new products, technologies and innovations about foods.	2.5	9.8	43.3	31.3	13.3
S7. You always keep up with news about technology and innovation.	1.0	13.3	45.5	32.3	8.0
S8. You prefer to buy products from stores with environmental friendly packaging.	1.3	10.8	45.5	33.0	9.5
S9. You concern about the impact of innovative products that contact to food (i.e. bioplastic packaging).	0.3	2.3	35.3	48.0	14.3
S10. You prefer to join in environmental activities (i.e. the use of recycle packaging or cloth bags)	3.3	17.3	42.8	27.0	9.8

Knowledge about bioplastics

Findings of knowledge about bioplastics are illustrated in Figure 1. With the total of 5 questions, respondents who answered correctly for 4-5 choices represented as high level, those answering correctly 2-3 choices represented as moderate level, and those answering correctly only 1 choice or zero represented as low level. Most respondents (60%) had moderate level of knowledge about bioplastics, while about 34.5% of respondents had high level of knowledge about bioplastics.

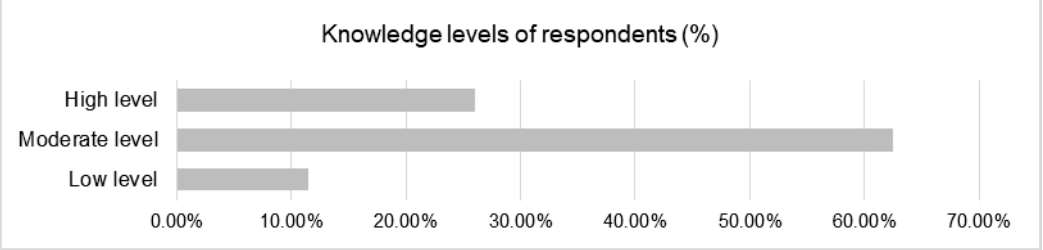

Figure 1 Knowledge levels of respondents (%)

Cluster analysis

In this part, two-step cluster analysis was used to segment green consumers regarding the use of bioplastic packaging. The selection of variables was based on high mean scores and high potential for clustering. The eleven variables was used to classify green consumers into four clusters as shown in Table 4. Five types of factors were captured in the analysis, which included two demographic variables, one variable of purchasing behavior, one variable of knowledge about bioplastics, two variables of attitude toward environment, and five variables of lifestyle.

Table 4 Cluster analysis of green consumers

Description	Cluster			
	1	2	3	4
	Young no green	Young go green	Green concerned	Powerful green
Additional price for bioplastic packaging	≤ 5 THB	≤ 5 THB	≤ 5 THB	11-15 THB
Lifestyle (S8)	Quite negative (2.77)	Strongly positive (4.12)	Quite positive (3.34)	Quite positive (3.44)
Attitude (S2)	Quite positive (3.06)	Strongly positive (4.24)	Quite positive (3.44)	Moderately positive (3.73)
Lifestyle (S9)	Quite positive (3.22)	Strongly positive (4.27)	Moderately positive (3.80)	Moderately positive (3.76)
Education	Bachelor degree	Bachelor degree	Bachelor degree and above	Bachelor degree
Lifestyle (S7)	Quite negative (2.80)	Strongly positive (3.81)	Moderately positive (3.50)	Quite positive (3.29)
Lifestyle (S10)	Quite negative (2.66)	Strongly positive (3.81)	Moderately positive (3.32)	Quite positive (3.22)
Attitude (S1)	Quite positive (3.11)	Strongly positive (4.13)	Moderately positive (3.66)	Moderately positive (3.62)
Lifestyle (S6)	Quite positive (3.13)	Strongly positive (3.89)	Moderately positive (3.49)	Quite positive (3.09)
Age*	Gen Z	Gen Z and gen Y	Gen Y and gen X	Gen Y
Knowledge about bioplastic	Moderate level	Moderate level	High level	Low level
Percentage (%)	33.0	28.8	24.5	13.8

*Generational definitions (Gray *et al*., 2016)

Results indicated that the demographic factor (age and education levels) affected the use of bioplastic packaging, which is supported by the study of eco-products by Kantaputra (2011). However, demographic and knowledge factors did not play an important role in study on the characteristics of green consumers. The outcome is supported by the study of Bureerat (2009) indicating that consumers having knowledge and understood the facts of environment were more likely to pay a high premium on green products. The main factors influenced segmenting green consumers were lifestyle and additional price. Similarly, lifestyle factors had a positive relationship with the acceptance for environmental friendly products. (Impitak, 1997)

Cluster 1: Young no green group.

The largest group of respondents (33.0%) had the least concern about the environmental impacts. They were the youngest group with the lowest mean score of age (Gen Z) and held a bachelor degree. Respondents of this group had moderate level of knowledge about bioplastic, but were less concerned about the impact of packaging types on the environment. They were quite negative for green lifestyle such as participating in environmental support activities and buying products from stores that use environmentally friendly packaging.

Cluster 2: Young go green group.

The second largest of respondents (28.8%) belonged to this group, which had the highest concern about environment with moderate additional price for bioplastic packaging. Respondents in this group had higher mean score of age than cluster 1 (Gen Z and gen Y), completed a bachelor degree and had moderate knowledge level about bioplastic. They were strongly positive for green lifestyles, but had moderate willingness to pay extra for bioplastic packaging. The young go green group was a new generation who had a positive attitude towards environment, followed-up technology and innovation news, as well as was willing to support green activities and stores using environmentally friendly packaging. This group was considered a major group for targeting green marketing strategies such as the use of environmentally friendly packaging or cloth bags, and environmental activities.

Cluster 3: Green concerned group.

This group, accounted for 24.5% of total respondents, had moderate concern about environment, but was sensitive to paying additional price for bioplastic packaging. Respondents of this group had the highest mean score of age (Gen Y and gen X), had the highest education level, and had high knowledge level about bioplastic. In this group, respondents were quite positive for promotion of the stores with environmental friendly activities and preferred to use bioplastic for single-use products.

Cluster4: Powerful green group.

The smallest group of respondents (13.8%) has high concern about environment and was willing to pay extra for bioplastic packaging at the highest additional price. Respondents in this group were middle-age (Gen Y) and held a bachelor degree. This group had the lowest knowledge about bioplastic, but had high purchasing power to support green products (i.e. bioplastics for single-use products) and environmental activities. This group was considered as a minor group for targeting green marketing strategies.

CONCLUSION

In this study, consumers are heterogeneous in attitude toward environment and lifestyle to the use of bioplastic packaging. Top five factors affecting decision on the use of packaging for bakery products were easy to use style, price, safety, design, and environmental friendly property. Four clusters of green consumers consisted of young no green group, young go green group, green concerned group, and powerful green group. Only 13.8% in the powerful green group had high willingness to pay additional price for bioplastic packaging, giving that additional price was an important factor for decision on the use of green products. For the major group, young go green group (28.8%) was a new generation who had positive attitude toward environment and accepted new technology easily. The green concerned group (24.5%) was an interesting group for marketing campaign with promotions and discounts. For the last group, young no green group (33%) needs to be encouraged with information about the environmental impacts when introduces the green products (Suankramdee and Sukkumnoed, 2015), as well as communicates with new activities and creative ideas to attract the young generation.

According to the findings, the food packaging businesses and marketing managers should consider the difference of additional prices for green products, demographics, purchasing behaviors, and lifestyles among segments when designing the marketing strategies.

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O-EC4

RECOMBINANT TILAPIA LAKE VIRUS (TiLV) PROTEIN FROM BACULOVIRUS EXPRESSION INSECT CELL SYSTEM FOR VACCINE PRODUCTION

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ABSTRACT

Since the discovery in 2014, tilapia lake virus (TiLV) has been reported on three continents (Asia, Africa and South America) and therefore posed the global threat to the Tilapia as an important food source. However there is no vaccine available at present to prevent the spreading of this newly emerged virus. Thus, as a division of cooperative team, this study aims to develop a TiLV vaccine using a recombinant protein of Tilapia lake virus segment 6 generated by the baculovirus expression system in an insect cell. Our results showed that the recombinant TiLV-S6 protein at approximately 48 kDa was detected from infected cell lysates by Western blot analysis using anti-TiLV antibody. Optimal multiplicity of infection (MOI) for the recombinant TiLV-S6 baculovirus to infect $2x10^6$ cells/ml was at MOI 1 giving maximum recombinant TiLV-S6 protein on 72 h post-infection.

Keywords: Tilapia lake virus (TiLV), Recombinant TiLV-S6 protein, TiLV-S6 vaccine, Baculovirus expression insect cell system

INTRODUCTION

Tilapia (*Oreochromis* sp.) is the third most important fish in aquaculture after carp and salmon, with estimated global production increase from 4.5 million metric tons to 7.3 million metric tons by 2030 (Bacharach *et al.*, 2016) and create a current value of over $7.5 billion U.S. dollars (USD. China, Philippines, Taiwan, Indonesia and Thailand are the leading suppliers, and these countries altogether produced about 76% of the total aquaculture production of tilapia worldwide (Bacharach *et al*., 2016). Since 2009, tilapia aquaculture has been threatened by mass mortality in Israel (Bacharach *et al.*, 2016), from 316 metric tons of wild catch in 2005 to a low of 8 metric tons in 2009, at the same time, massive losses of farmed tilapia with mortality level of above 80% were recorded (Eyngor *et al.* ,2014). In 2014, extensive damages of wild and farmed tilapia have been reported in different countries including Israel, Ecuador, Colombia, Egypt and Thailand (Eyngor *et al*., 2014; Bacharach *et al.*, 2016; Fathi *et al.*, 2017; Kembou Tsofack *et al.*, 2017; Surachetpong *et al.*, 2017). A novel, orthomyxo-like virus, tilapia lake virus (TiLV) was identified as a causative agent of these outbreaks (Eyngor *et al.*, 2014; Bacharach *et al*., 2016; Surachetpong *et al.*, 2017). Based on its morphology of round to oval enveloped and filamentous/tubular viral particles, the TiLV virus was classified as an Orthomyxo-like virus with negative-sense, single-stranded RNA genomes with 10,323 kb total length (Bacharach *et al*., 2016; Del-Pozo *et al.*, 2017). Unlike other orthomyxo-viruses such as influenza that are made up of eight segments, the TiLV virus is made of 10 RNA segments (Palese and Schulman, 1976). All segments have conserved, complementary sequences at 5′ and 3′ termini, which are characteristic of orthomyxo influenza virus. Interestingly, only segment one of the viruses shares weak homology to the PB1 subunit of the influenza C virus. Other genomic segments of TiLV do not resemble any sequences of other organisms in the database. International Committee of Virus Taxonomy (ICVT) classified TiLV as a single new species known as Tilapia tilapinevirus in the new genus Tilapinevirus (Bacharach *et al.*, 2016; Fathi *et al*., 2017; Kembou Tsofack *et al.*, 2017; Surachetpong *et al.*, 2017). The clinical signs and pathology of infected fish include poor body conditions, abnormal swimming, severe anemia, skin erosion and congestion, ocular degeneration, scale protrusion, and abdominal swelling. The most common

microscopic lesions associated with TiLV infections include SHT (syncytial hepatitis of tilapia) and encephalitis lesions.

Unlike other viral diseases of tilapia, TiLV appears to be widely spread and may be present in many countries where it is not yet recognized. To limit the negative impact and to prevent further spread of the virus, combined approaches are required. Control of TiLV will be improved by biosecurity efforts, rapid response to emerging diseases, development of surveillance diagnostic tools and most importantly, the use of vaccines to reduce the impact of this emerging viral disease. However, there is no vaccine currently available to protect the Tilapia from this virus. It was proposed that recombinant TiLV structural proteins would be a good choice for vaccine development. Since the TiLV natural host is fish, baculovirus expression insect cells system was chosen for the production of TiLV structural proteins. In this study, TiLV segment 6 gene (TiLV-S6) was expressed and produced in insect cells.

MATERIALS AND METHODS

Construction of recombinant plasmid pFastBac-S6

A 954 bp segment 6 cDNA of TiLV (TiLV-S6) was a gift from Center of Excellence for Shrimp Molecular Biology and Biotechnology (CENTEX Shrimp), Faculty of Science, Mahidol University, Thailand. It was PCR amplified using specific primers; TiLV-S6 F (CGC GGC CGC TAT ATG CAT TTT TAT CTA C) and TiLV-S6 R (GGA CTC GAG TCA CAT GTA TTT ATT G) and sub-cloned into a baculovirus, pFastBac HTb, transfer vector. The recombinant pFastBac-S6 DNA was obtained and subjected to DNA sequencing.

Generation of recombinant baculovirus expressing TiLV *segment 6 gene*

Recombinant TiLV-S6 baculovirus was generated by the following instruction in Bac-to-Bac Invitrogen instruction (Bac-to-Bac® Baculovirus Expression System). Briefly, the recombinant pFastBac-S6 was transformed into MAX Efficiency® DH10Bac™ *E. coli* competent cells that contain baculovirus genome bacmid. The white colony containing recombinant bacmid with TiLV segment 6 gene inserted into the *LacZ* was selected, cultured and the bacmid genomic DNA was purified. Insertion of TiLV-*S6* into the bacmid genome was confirmed by PCR analysis method using primers specific to *Lacz* (M13 forward and M13 reverse) and primers specific to TiLV-S6. Sf9 insect cells were then transfected with the recombinant TiLV-S6 bacmid in the presence of Cellfectin® II (Invitrogen, USA) to produce recombinant baculovirus. The transfected Sf9 cells were cultured for 4 days when the obvious signs of viral infection appeared, the first passage of recombinant TiLV-S6 baculovirus, namely rBV-S6 (P1) was obtained from the culture medium. The rBV-S6 P1 viral stock was amplified in a 15 ml suspension culture at 2×10^6 cells/ml, incubated at 27℃ for 72 hours to collect the supernatant to obtain the rBV-S6 P2 viral stock. The virus titer was determined by End-Point dilution method (Reed and Muench, 1938).

Detection of TiLV-S6 *gene expression by RT-PCR*

RNA samples were isolated from Sf9 cells infected with recombinant TiLV-S6 baculovirus, using a combination of the TRIzol (GIBCO, Life Technologies) and RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) techniques as protocol recommended. Briefly, 1ml of Trizol was added to the 5-10×10^6 Sf9 cell pellet, pipetting and allow the sample to stand for 5 minutes at room temperature. A 0.2 ml of chloroform was added per 1ml of Trizol used and vigorously shake for 15 seconds before left for 5 minutes at room temperature. The resulting mixture was centrifuged at 12,000g for 15 min at 4℃. The colorless upper aqueous phase was transferred to a new clean tube followed by isopropanol precipitation and 75% ethanol washing of the RNA pellet. Dried pellet was suspended in RNase-free water. TiLV-S6 cDNA synthesis was carried out using the RevertAid First Strand cDNA Synthesis Kit that has an integrated step for the removal of contaminated genomic DNA. The negative control sample was prepared without adding transcriptase for the first strand cDNA synthesis. The PCR reaction was performed in a 25 μl reaction containing 10x PCR buffer (Thermo Fisher Scientific, USA), 50 mM of $MgCl_2$, 10 mM of dNTP mix, 10 μM of each TiLV-S6 specific primer, 0.25 μl of 5 U/μl Taq DNA polymerase and 100 ng of cDNA. The PCR reaction was carried out in a T100 thermal cycler (Bio-Rad, USA) with cycle conditions of initial denaturation at 94°C for 3 minutes, 35 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 30 s and extension at 72°C for 45 s, with a final extension at 72°C for 45 s.

Detection of recombinant TiLV-S6 *protein in infected insect cells by Western blot analysis*

Recombinant TiLV-S6 baculovirus-infected cell pellet was suspended in 100 μl PBS and mix with 5x SDS/PAGE loading buffer and subjected to Western blot analysis using either anti-6x His-Tag antibody or anti-TiLV antibody from TiLV infected fish serum as primary antibody and Goat anti-mouse IgG conjugated with Anti-tilapia IgM as a secondary antibody, the color was developed using 3, 3', 5, 5'-Tetramethylbenzidine (TMB).

Optimization of the recombinant TiLV-S6 *protein production in Sf9 cell culture*

Sf9 cells at 2×10^6 cells/ml in log phase with viability higher than 95% were infected with rBV-S6 recombinant baculovirus. Varieties of MOI 0.01, 0.1, 1 and 10 were used for infection. The recombinant TiLV-S6 protein production from the same numbers of infected cells was determined by Western blot analysis as previously described. Recombinant TiLV-S6 protein produced was estimated by band intensities on the membrane.

RESULTS AND DISCUSSION

Construction of recombinant pFast Bac-S6 transfer vector

A full length (954 bp) of TiLV-S6 gene was amplified by PCR using pGEM-S6 as a template. Agarose gel electrophoresis showed a specific band at around 947 bp (Fig. 1a) which indicated that the gene of interest TiLV-S6 was successfully amplified. The TiLV-S6 PCR amplified was sub-cloned into the pFastBac HTb transfer plasmid at the *Not* I and *Xho* I cloning sites. Thus, the *Not* I and *Xho* I digested recombinant pFast Bac-S6 plasmid was detected as two specific bands appeared at 4,856 bp and 954 bp (Fig. 1b) in accordance with the expected sizes of pFastBac HTb and TiLV-S6. This result and DNA sequence analysis of the recombinant pFast Bac-S6 confirmed that TiLV-S6 gene was correctly inserted and the recombinant plasmid was successfully constructed.

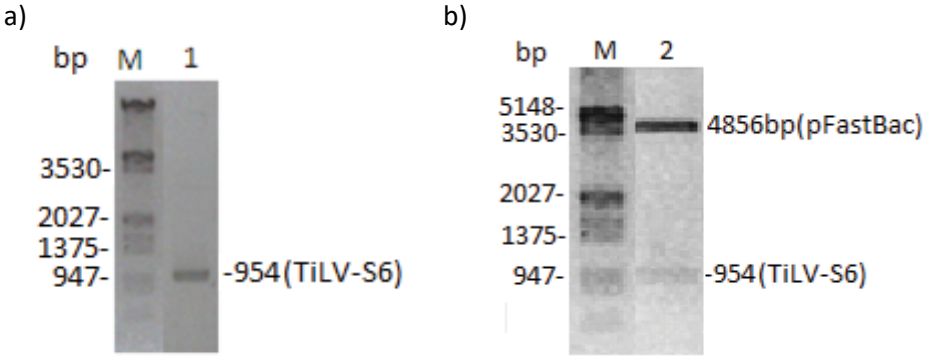

Figure 1. PCR product of TiLV-S6 (a) and restriction enzyme analysis of recombinant plasmid (b)
M : marker λ DNA digested with *EcoR*I and *Hind*III
Lane 1: TiLV-S6 PCR
Lane 2: recombinant plasmid pFastBac-S6 digested with *Not*I and *Xho*I

PCR Analysis of Recombinant Bacmid DNA

The size of bacmid DNA is >I35 kb. It is difficult to verify the insertion of the gene of interest by restriction enzyme digestion. Therefore, the polymerase chain reaction (PCR) with the M13 F and M13 R primers to confirm the insert location since M13 *LacZ* specific primers directed at sequences on either side of the mini-attTn7 site within the *LacZ*-complementation region of the bacmid and TiLV-S6 gene specific-primers for the specific TiLV-S6 gene insertion. It was demonstrated in Fig. 2 that a PCR product at 3,530 bp (lane 1) represents an insertion of a DNA fragment at 945 bp in *LacZ* and a PCR product between 3,530 bp and 2,027 bp (lane 2) confirmed the presence of the TiLV-S6 gene which proved that the transposition is successful and the recombinant rBV-S6 bacmid was generated.

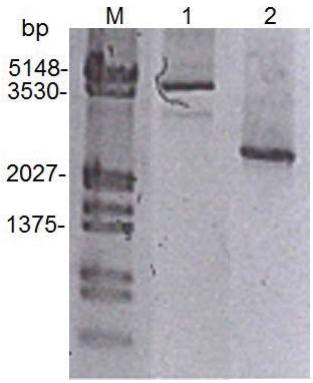

Figure. 2. PCR analysis of recombinant TiLV-S6 Bacmid
M：marker λ DNA digested with *EcoR*I and *Hind*III
Lane 1: PCR products using *LacZ* specific primers M13 F and M13 R
Lane 2: PCR products using *LacZ* specific primers M13 F and TiLV-S6 R

Detection of TiLV-S6 *gene expression by RT-PCR*

Insect cell line Sf9 was infected with high titer of recombinant TiLV-S6 baculovirus and incubated at 27℃, both supernatant and cell pellet were collected after 72 hours. Expression of the TiLV-S6 could be confirmed by the presence of mRNA transcribed by the infected insect cells. Thus, total RNA was extracted from the infected insect cells and mRNA was converted to cDNA. As it showed that TiLV-S6 specific PCR detected a specific band at 954 bp while none was shown in the negative group (Fig. 3). This result proved that TiLV-S6 gene was successfully expressed and hence recombinant TiLV-S6 protein production could be expected.

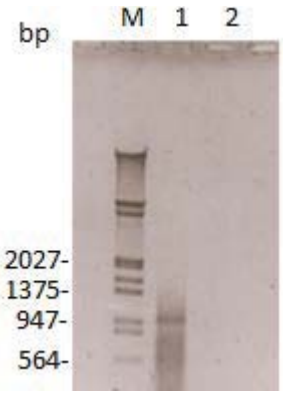

Figure 3. Detection of TiLV-S6 gene expression by RT-PCR
M：marker λ DNA digested with *EcoR*I and *Hind*III；
Lane 1: RT-PCR using template from reaction with the reverse transcriptase
Lane 2: Negative control, RT-PCR using the template from reaction without enzyme

Detection of recombinant TiLV-S6 *protein expression by Western blot analysis*

Western blot analysis showed a band specific to anti-His antibody and anti-TiLV appeared only in the protein extracts of infected cell pellet, located at around 48 kDa (Fig. 4), while no such band appeared in culture supernatant, normal Sf9 cell pellets and normal Sf9 cell culture supernatant, which indicated that TiLV-S6 gene was expressed inside the Sf9 insect cells.

a) b)

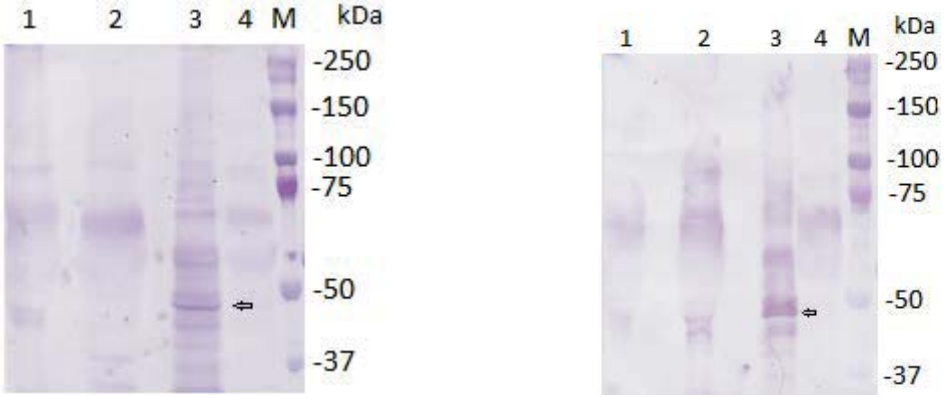

Figure 4. Recombinant TiLV-S6 protein detected by Western blot using anti-TiLV antibody (a) and Anti-His antibody (b)

M: protein marker
Lane 1: Culture supernatant from normal Sf9 cell culture
Lane 2: Culture supernatant obtained from Sf9 infected by recombinant TiLV-S6 baculovirus
Lane 3: Cell pellet obtained from Sf9 infected by recombinant TiLV-S6 baculovirus
Lane 4: Cell pellet from normal Sf9 cell culture

Optimization of recombinant TiLV-S6 *protein production in insect cells*

Sf9 cells were cultured in a 6-well plate to a suitable concentration of 2×10^6 cells/well and infected with recombinant baculovirus with TiLV-S6 gene (rBV-S6). A variety of MOI, i.e. 0.01, 0.1, 1 and 10 used for determination of an optimal MOI used for the production of recombinant TiLV-S6 protein production in insect cells. The infected cells were collected after 24 h intervals with Sf9 uninfected cells served as a negative control. The intracellular recombinant TiLV-S6 protein was detected by Western blot analysis. The results showed that the highest amount of recombinant TiLV-S6 protein was detected from the MOI 1 infected cell pellets (Fig. 5 lane 2). The MOI 1 was then used to determine the optimum time of harvest. It was found that the highest amount of recombinant TiLV-S6 protein was detected from infected cell pellets after 72 h (Fig.6 lane 3). Thus, the optimal MOI for recombinant TiLV-S6 baculovirus to infect $2x10^6$ cells/ml is MOI 1 and the time of harvest of recombinant TiLV-S6 protein is 72h post infection.

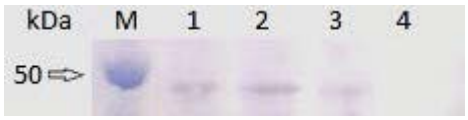

Figure 5. Western blot using anti-TiLV antibody for detection of recombinant TiLV protein produced from 2×10^6 cells/ml Sf9 cells after 96 hours infection with recombinant TiLV baculovirus at MOI 10 (Lane 1), MOI 1 (Lane 2), MOI 0.1 (Lane 3) and MOI 0.01 (Lane 4). M: 50 kDa protein marker.

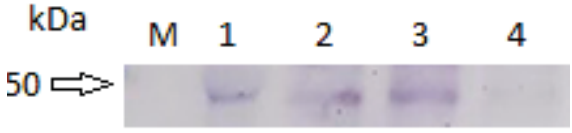

Figure 6. Western blot using anti-TiLV antibody for detection of recombinant TiLV protein produced from 2×10^6 cells/ml Sf9 cells infected with recombinant TiLV baculovirus at MOI 1 after 24h (Lane 1), 48 h (Lane 2), 72 h (Lane 3) and 96 h (Lane 4). M: a 50 kDa protein marker.

CONCLUSION

Among several heterologous protein expression systems such as bacteria, yeasts, plants and mammalian, the baculovirus expression vector system is suited for producing eukaryotic target protein in many applications including the production of pharmaceuticals, pesticides, vaccines and more. Baculovirus is used as a gene expression vector to express target gene in an insect cell host (Murphy *et al.,* 2004). Baculovirus does not replicate in eukaryotic cells besides their insect cell hosts: therefore, insect cell expression in the laboratory does not require particular safety measures (Murphy and Piwnica-Worms, 1994a, b; Murphy *et al.,* 2004). Large proteins with several hundred kilodalton molecular weight can be produced by the baculovirus expression insect cell system, and the proteins are, in most cases, functionally active with post-translational modification. Besides, insect cell cultures are easily grown with basic facilities (Weber *et al.*, 2002). Since its discovery in 2014, tilapia lake virus (TiLV) has emerged as a newly emerging viral pathogen that poses a potential threat to the global tilapia industry. As current knowledge on this virus is still limited, several important knowledge gaps that remain to be filled. Here, we report the production and optimization of Tilapia lake virus (TiLV) recombinant TiLV-S6 protein using the baculovirus expression vector system. This work provides an initial molecular characterization of TiLV-S6 gene as its DNA sequence had no similarity with the published sequences. As a newly emerging pathogen for tilapia culture worldwide, our findings have provided the raw data and basis for the further study on the development of a vaccine to the Tilapia lake virus in order to limit the negative impact and to prevent further spread of the virus.

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Poster

EN-P001

FUEL PROPERTIES OF SOME NATIVE TREE SPECIES FOR BIOMASS ENERGY IN THAILAND

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ABSTRACT

Thailand is a tropical country and rich in plant species diversity. At present, most of the forest plantations in Thailand are established using exotic species. Many native species are expected to have potential as biomass energy but information on wood yield, coppicing ability and fuel properties is lacking. This study determined some fuel properties in seven native species (*Albizia lebbeck*, *Albizia procera*, *Broussonrtia papyrifera*, *Cassia siamea*, *Combretum quadrangulare*, *Melia azedarach* and *Trema orientalis*). All species were analyzed for wood density, higher heating value (HHV), and components of the proximate and ultimate analysis. Results showed highly significant differences (*P<0.001*) in all but one trait (*P<.01* for hydrogen content) among the species. The HHV was in the range 4285-4653 kcal/kg (17.9-19.5 MJ/kg) which was comparable to some commonly used bioenergy species. Three species (*T. orientalis*, *M. azedarach* and *B. papyrifera*) had high HHV, high volatile matter and low ash content, suggesting their suitability for biomass energy. The results indicated the potential of native plant species in Thailand for use as bioenergy.

Keywords: Native species, Fuel properties, Biomass energy

INTRODUCTION

In order to strengthen Thailand's long-term energy security and global economic competitiveness, the country has committed to develop its alternative energy capabilities. This policy emerged at the national level as the Alternative Energy and Development Plan (AEDP) with a 10-year initiative (2012- 2021) to better diversify and build a more sustainable energy sector. With this plan, Thailand has set the target of increasing alternative energy consumption from 9,025 kilotons of oil equivalent (kloe) in 2014 to 24,638 ktoe in 2021 (AEDP, 2015). Energy sourced from biomass is one of the main players in the AEDP initiative to ramp up renewable energy production. The end goal of the AEDP is to achieve 25% reliance on total national energy generation from alternative or renewable sources by the year 2021 and to set the country's socioeconomic development on a path towards a low-carbon usage base, away from fossil fuels.

Thailand is a tropical country and rich in native plant species diversity. According to Flora of Thailand and research from Forest Herbarium, Department of Forestry, there are more than 10,000 native species of 1763 genera in 245 families in Thailand. However, less than 100 species are used for plantation establishment and ornamental planting. The majority of the species are not used in agriculture, forestry or other industries (RECOFTC, 1993) and therefore information about their potential uses is scarce. Fast-growing tree forest plantation is a potential source of bio-energy. At present, most of the forest plantations in Thailand are established using exotic species such as *Eucalyptus sp*., *Acacia sp*. and *Leucaena sp.* Some of the exotic species, especially *Eucalyptus* sp. still face resistance from people because of environmental concerns. Many native species are expected to have potential as biomass energy but information on wood yield, coppicing ability and fuel properties is lacking.

In the study, fuel properties of selected native species were determined with a view to develop plantations for bio-energy production and to increase the choice of species for plantation development.

MATERIALS AND METHODS

Wood samples were collected from seven native species known to have good adaptability over a range of soil and climate conditions. These were Cassod tree (*Cassia siamea*), White Siris (*Albizia procera*), Siris tree (*Albizia lebbeck*), Charcoal tree (*Trema orientalis*), Bead tree (*Melia azedarach*), Paper mulberry

(*Broussonetia papyrifera*) and Sakae Naa (*Combretum quadrangulare*). The sampled trees were located in the central region of Thailand and had a diameter at breast height of 7 – 10 cm. The wood samples of each species were divided into two parts for physical and chemical property tests. The samples of block size 16 cm^3 (25 mm x 25 mm x 25 mm) were used for density test, while those ground to powder smaller than 1 mm were used for chemical property test.

For density test, the samples were conditioned at 105 ± 2 ºC and 65 ± 5 % relative humidity unless constant mass was obtained. The blocks were measured for volume in dry condition using the mercury displacement method (Chauhan and Walker 2011). These blocks were then oven dried at 105 ± 2 °C until they achieved constant mass. Basic density (B_D) was determined using equation $B_D = W_{OD}/ V_g$, where W_{OD} is the oven-dry biomass and V_g is the green volume of biomass.

For other property test, the wood samples were ground to pass through a 0.25 mm sieve. Moisture content was determined by drying the samples at 105 ± 2 ºC until they reached a constant weight (ISO 18134: 2015). The samples were then burnt at 900 ± 10 ºC with inert condition for 7 min (ISO 18123: 2015) to determine volatile matter content. Ash content was determined after burning in a muffle furnace at 550 ± 10 ºC to constant weight (ISO 18122: 2015) using an automated proximate analyzer (LECO TGA-701). Fixed carbon content was estimated by subtracting the sum of moisture content, volatile content and ash content from 100%.

Higher heating value (HHV) was determined in accordance with the ISO 18125: 2017 standard. The samples were combusted in the IKA C 5000 automated adiabatic calorimeter, which was calibrated with a benzoic acid standard (heat of combustion 6,315 kcal/kg). The ground sample (approximately 0.5 g) was compacted into a tablet and burnt in an oxygen bomb calorimeter to determine the calorific value. The elemental parameters (carbon, hydrogen, nitrogen and sulfur) were determined using an automated CHN analyzer (LECO- CHN-628). Percent oxygen was calculated by subtracting the sum of percent C, H, N, S, and ash from 100%. Na and K elements were determined in accordance with the ISO 16967: 2015 standard. The samples were digested at resistance heating 220 °C, digestion with HNO_3 (65%), HF (40%), H_3BO_3 (4%), according to ISO 16967: 2015, and detected with flame atomic absorption spectrometry (FAAS). In order to minimize experimental and instrumental errors, experiments were repeated three times (i.e. they were treated as three replicates) and mean values were calculated.

Analysis if variance was performed using GenStat v18 (VPN International, Hemel Hemstead, UK). Least significant difference (L.S.D.) ($p<0.05$) was used to compare the differences among pairs of means.

RESULTS AND DISCUSSION

Highly significant differences ($p<0.001$) were observed in all but one trait ($p<0.05$ for hydrogen) among the species. Basic wood density of the seven native species varied from 0.29 g/cm^3 to 0.78 g/cm^3 with an overall mean of 0.53 g/cm^3 (Table 1).The highest density was recorded for *C. quadrangulare* and the lowest density was recorded for *T. orientalis*. The physical structure cell wall and lumen (Prabir, 2010) affect the density. In general, biomass having higher density is preferred as fuel due to its high energy content per unit volume (Kumar and Chandrashekar, 2014). However, other fuel property characteristics should also be taken into account.

Proximate analysis of volatile matter, fixed carbon and ash content varied greatly among species (Table 1). *T. orientalis* had the highest volatile matter (81.75 % as dry) and lowest in ash content (0.79% as dry) but moderate amount of fixed carbon (17.46%). *B. papyrifera* and *A. lebbeck* had high volatile matter (81.37 and 81.49%) and low ash content (1.72 and 1.6%), but both had the lowest fixed carbon (16.9%). In the case of *M. azedarach*, this species had the highest fixed carbon (18.27%), was relatively low ash content (1.85%) and its volatile matter (79.88%) was not statistically different from that of *T. orientalis*. *C. quadrangulare* which recorded the highest wood density had the highest ash content of 6.47% and lowest volatile matter of 75.5%. Volatile matter and fixed carbon content have a positive effect on burning properties. Volatile is a combustible composition of biomass, and the biomass with high volatile can easily be combustible and subsequently complete oxidation (Demibas, 2012). In contrast, ash content of bio-fuel should be as low as possible in wood. High ash will have a negative impact on the combustion process (HytÖnen and Nurmi, 2014). Low ash content in biomass will also benefit the end users in terms of energy content and time for emptying ash (Uemura et al., 2010).

The ultimate analysis is very important in order to determine the theoretical air-fuel ratio in thermo-conversion processes and also to gain knowledge of the pollution potential. This study showed that carbon

(C), hydrogen (H), nitrogen (N), sulfur (S) and chloride (Cl) differed significantly among the species. *T. orientalis* had the highest C (45.59% as dry) but lowest N (0.083 % as dry) and S (0.005% as dry). *C. quadrangulare* had the highest Cl (0.2035 as dry) but lowest C (42.707% as dry) and H (5.877% as dry). The main element for heat source was carbon and hydrogen while nitrogen, sulfur, and chloride had a negative effect on the calorific value. In general, low carbon and hydrogen content reflexes weak fuel properties of the biomass (Kumar *et al.*, 2010), Furthermore, sulfur and nitrogen compounds are transformed into acidifying substances. When these substances reach the ground, they cause acidification of the soil and water, resulting in acid soil (Sardans *et al*., 2015). This phenomenon is an important cause of damage to the aquatic environment and may severely impair the life of plant and animal species.

Heating value is one of the most important fuel properties which explains the higher energy content and determines the efficiency of biomass fuels. Higher Heating Value (HHV) of the native species in this study varied from 4,285 kcal/kg to 4,653 kcal/kg (17.9-19.5 MJ/kg). The highest HHV was obtained for *T. orientalis* and the lowest for *C. quadrangulare*. These HHV values are comparable to that shown by other tree species commonly planted for production of biomass energy, such as eucalypts 17.6-19.6 MJ/kg, casuarinas 19.1-19.3 MJ/kg and acacias 19.2-19.8 MJ/kg (Ritesh *et al.*, 2011, Telmo *et al*., 2010). Carbon and volatile content have a positive effect on the HHV property, the higher the carbon and volatile content the higher the HHV. The volatile is a liquid phase in wood (not including water).

In this study, it was found that species with low wood density species, such as *T. orientalis* and *M azedarach* had the highest HHV among all the species tested. A plausible explanation is that if the density is high the wood will have low porous and less empty space for volatile matter. On the contrary low density wood is more porous with more space for volatile to trap the heat.

Table 1 Solid fuel characteristics of some native species in Thailand (wood with bark).

Species	Density (g·cm^{-3})	HHV (cal/g)	Proximate analysis (% as dry)			Element analysis (% as dry)				
			Volatile	Ash	Fix C	C	H	N	S	Cl
A. *lebbeck*	0.76	4,488	81.49	1.60	16.90	45.02	6.17	0.09	0.012	0.022
A. *procera*	0.39	4,496	79.76	2.78	17.36	43.82	6.17	0.37	0.018	0.120
B. *papyrifera*	0.32	4,585	81.37	1.72	16.91	45.25	6.11	0.11	0.008	0.052
C. *siamea*	0.68	4,566	79.23	3.51	17.26	43.35	6.28	0.27	0.012	0.130
C. *quadrangulare*	0.78	4,285	75.50	6.47	18.04	42.71	5.87	0.30	0.017	0.203
M. *azedarach*	0.48	4,650	79.88	1.85	18.27	44.44	6.09	0.12	0.017	0.051
T. *orientalis*	0.29	4,653	81.75	0.79	17.46	45.59	6.22	0.08	0.005	0.032
Overall mean	0.53	4,532	79.85	2.67	17.46	44.31	6.13	0.19	0.013	0.087
F probability	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.01	<0.001	<0.001	<0.001
L.S.D. (P<0.05)	0.25	34	0.55	0.17	0.53	0.83	0.19	0.06	0.004	0.021

CONCLUSION

We believe this study was the first to investigate the fuel properties of native plant species of Thailand. Results showed the potential of several species that can be developed for bio-energy production. Three species i.e. *T. orientalis*, *M. azedarach* and *B. papyrifera* had high HHV, high volatile matter and low ash content, which are important traits of bioenergy species, However, this research was only preliminary. More studies should be conducted with a systematic sampling of materials from a wider range of distribution in Thailand.

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EN-P002

RUBBER WOOD PROPERTIES TESTING FOR BIOMASS ENERGY USING NEAR INFRARED SPECTROSCOPY

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ABSTRACT

When the rubber tree is 20-25 years old, the latex yield is not economically worthwhile. The rubber tree will be cut down. The rubber wood cut down is processed into dried rubber wood and will be used in downstream industries. The remaining are roots of trees and wood or branches that are not utilized, including rubber waste from wood processing plants and wood production plants, including wood slabs, sawdust, wood (flawed) and shavings. At present, there are a lot of demands for biomass fuel in biomass power plants. The 1 MW power plant will use about 10,000 tons of biomass/year. Based on the assessment of the availability of raw materials, the number of rubber trees in the southern region is large enough for the 1,000 MW biomass power plant to be distributed almost throughout the year. Many parts of rubber trees can be used as fuel. The study of timber that provides quality wood for biomass fuel is therefore necessary. Traditional rubber wood quality analysis (conventional method) is complicated, takes time and high cost. Therefore, this research aimed to develop a quality analysis of the biomass rubber with near-infrared technique in order to create new alternative testing. In this study, near-infrared (NIR) spectroscopy was employed to determine hemicellulose, cellulose, N, C, H and moisture contents in rubber wood as biomass for energy utilization. The 120 rubber samples from 10 varieties were selected from three parts of trunk, limb, and root with each aged twenty years. All samples were ground and measured in the NIR region of 1100 – 2500 nm. Partial least squares regression (PLSR) models for the quantitative determination of hemicellulose, cellulose, N, C, H and moisture contents in rubber wood samples were built. PLSR models were developed from the whole region of NIR spectra and the analyzed contents were determined by the reference method. The PLSR model predictive performance of moisture was very good, hemicellulose and N were good, C and H were moderate, and cellulose was fair, respectively. This study showed that NIR spectroscopy could be used to predict the necessary properties of para wood for biomass.
Keywords: Rubber wood, Near-infrared spectroscopy, Wood properties, Biomass, Energy

INTRODUCTION

Rubber wood is a type of economic wood that is important to Thailand. There are 25-30% of the remaining rubber wood that has not been utilized, which can be processed into biomass energy. The production and quality of rubber wood are important in determining and selecting rubber species for the purpose of biomass energy. The conversion of waste rubber wood to energy is influenced by its physical characteristics and chemical composition. Therefore, the accurate compositional analysis of waste rubber woods is necessary because they may have different properties and chemical composition, which will affect the energy properties when burning, for example, minerals in wood, carbon, hydrogen, oxygen, nitrogen, and sulfur, have features directly related to the heating value. Those biomass materials with high carbon and hydrogen contents will have a high heat value as well (Lewandowski and Kicherer, 1997).

However, the chemical method for analysis biomass material is complicate and time consuming. Therefore, an alternative testing has to be focused on user-friendly and rapid method. Near-infrared (NIR) spectroscopy has these benefits and has been investigated to determine its potential to predict qualitative and quantitative attributes in the biomass analysis. Matt *et al.* (1996) employed the NIR technique to analyze

components in biomass raw materials by creating various predictive equations including ethanol extractives, ash, lignin, uronic acids, arabinose, xylose, mannose, galactose, glucose, C, H, N and O from wood biomass feedstocks. Xu *et al.* (2012) reported by comparison of NIR technique and chemical methods for the determination of lignocellulosic biomass properties. Nicole *et al.* (2008) applied NIR method for classification biomass samples including red oak, yellow poplar tree, walnut family tree, switch grass, corn cob, and bagasse.

Therefore, the opportunity to bring an alternative analysis method by mean of NIR for chemical analysis of rubber wood as biomass energy is likely to have a lot of potentials. The objective of this study was to determine the potential of NIR spectroscopy in conjunction with chemometrics to predict the hemicellulose, cellulose, N, C, H and moisture contents in waste rubber wood as biomass for energy utilization.

MATERIALS AND METHODS

Samples

The rubber wood samples were selected from clone trial aged twenty years at Rubber Research Center, Buriram province, Thailand. The experimental design was randomized complete block (RCB) including four replicated treatments of ten varieties of rubber wood, namely BPM24, PB235, PB260, PB310, PR255, RRIC110, PR305, RRIT226, GT1, and RRIM 600. Three rubber trees of each variety were randomly selected. Then, they were cut at a height of 30 cm from the ground. The trunks, limbs, and roots were separated from the cut rubber tree. The fresh woods were weighted and dried before grinding to a particle size of 60 mesh. A total of 120 ground rubber wood were used in the experiment.

Spectra Measurement

NIR spectrometer (SpectraStar™ 2500; Unity Scientific; USA) was used to acquire reflectance spectra (log 1/R) of the rubber wood samples. Samples were filled and scanned in a standard cup for powder sample (Fig. 1). The spectral acquisition and instrument were controlled using the Info star software version 3.10.0 (Unity Scientific; USA). NIR spectra were recorded in a wavelength range of 680-2500 nm. Each sample was scanned in triplicate and the averaged spectrum with an average resolution every 2 nm, was computed before further model calculations.

Figure 1. A sample cup uses in the NIR measurement.

Standard methods to determine chemical composition of rubber wood samples

All chemical analysis results are shown in Table 1. Hemicellulose and cellulose contents were determined by acid chlorite method (Browning, 1967 and Rowell, 2005). Moisture content was determined according to National Renewable Energy Laboratory (Sluiter *et. al.*, 2008). An organic elemental analyzer was employed to investigate carbon, nitrogen and hydrogen contents (LECO, 2003).

Data analysis

The data analysis was performed with Unscrambler (Ver. 9.8: CAMO AS, Trondheim, Norway). The NIR spectra were pretreated with second derivative (2D), multiplicative scatter correction (MSC), and

standard normal variate (SNV) before development of the calibration models, and then do the comparisons. A calibration model was calculated by partial least squares regression (PLSR) method, and validated by the full cross validation method in Visible and a shorter-NIR wavelength range (680-1246 nm) or Visible and a longer-NIR wavelength range (680-2500 nm).

RESULTS AND DISCUSSION

The contents of each chemical component were depicted in Table 1. Hemicellulose, cellulose, carbon, and moisture values showed the standard deviation higher than one. Because the rubber samples were collected from different varieties that reflected the influence of genetics to their chemical properties. The quality of wood for bioenergy production depends on the chemical and mechanical properties. Cellulose and hemicellulose were generally found in the cell walls of rubber wood. The capability of biomass process depends on their contents in rubber wood, therefore, high cellulose and hemicellulose values affect the great amount of bioenergy production (McKendry, 2002). Carbon and hydrogen, are the wood minerals. Their amounts are directly related to the heating value of biomass. (Lewandowski and Kicherer, 1997). Rubber wood samples with high carbon and hydrogen values will also have a high heat value. Adler *et al.* (2006) reported that the amount of nitrogen must not be too large in biomass. Nitrogen can be interacted to oxygen as nitrous oxide that is a kind of greenhouse gas affecting the global warming. Therefore, nitrogen content in biomass should not exceed 0.600% (Obernberger *et al*., 2006). Table 1 shows that the rubber wood samples contained nitrogen in the range of 0.159 to 0.749% with the average value of 0.303% that falls within the suggested value. Moisture content of dried rubber wood samples is very important for changing energy by burning process. This process requires that the value of moisture content in biomass must not exceed 30-50%. If the biomass has higher humidity, the heat value will decrease and also affect the combustion efficiency. Biomass should be in a dry condition or with minimal moisture. Our dried rubber wood samples remained less of moisture value in the range of 1.970-6.900% (Table 1).

Table 1 The statistical summary of chemical contents in rubber wood samples.

Component (%)	Range	Mean	Standard deviation
Hemicellulose	12.337-24.141	17.809	3.070
Cellulose	31.217-62.145	46.355	4.576
N	0.159-0.749	0.303	0.098
C	43.300-49.700	45.390	1.238
H	6.040-7.210	6.647	0.237
Moisture	1.970-6.900	4.096	1.108

The original and pretreated NIR spectra of rubber wood samples are shown in Fig 2. The summary of statistic results is shown in Table 2. From the results, the calibration model using second derivative pretreated NIR spectra in region of 680-1246 nm yielded the suitable model for prediction hemicellulose content. This PLS model provided the small error of validation (root mean square error of cross-validation, RMSECV) of 1.86% with a reasonable factor number of 8 giving the coefficient of determination (R^2) of 0.835. By the obtained statistic values, the calibration model of hemicellulose can be clarified as a good predictive performance. The band at 1194 nm related with second overtone of C-H stretching. The band at 1372 nm related with C-H vibration of methyl group. The bands at 2274 and 2336 nm corresponded to O-H/C-O in glucose molecule and C-H stretching/CH_2 deformation combination in polysaccharide structure, respectively (Workman and Weyer, 2007). They were assigned and shared by cellulose and hemicellulose information, which demonstrated that parts of the structures were similar among them. We found that the PLS calibration for hemicellulose employed the short NIR region of 680-1246 nm that covered the most information of methyl group. As for PLS model for cellulose prediction, the best model used of second derivative NIR spectra in the longer region of 680-2500 nm. However, the obtained statistic result (R^2 = 0.596) given the level of fair predictive performance (Williams, 2007).

Nitrogen, carbon, and hydrogen, they all shared NIR information with the cellulose and hemicellulose that are composed of these elements. The calibration model for prediction of nitrogen value built by SNV pre-treated NIR spectra in the longer region of 680-2500 nm including the band of N-H first overtone at 1472 nm. The model predictive performance was good with the statistic results of R^2 of 0.847 at 9 factor number,

in which it yielded the less RMSECV of 0.046%. The calibration models for determine of carbon and hydrogen content were developed by using original NIR spectra in the range of 680-2500 nm, and MSC pretreated NIR spectra in the range of 680-1246 nm, respectively. They showed the same predictive performance of moderate level ($0.66 \leq R^2 \leq 0.81$).

The O-H stretching bands due to water bands clearly showed at 1426 and 1920 nm (Fig. 2). So, the calibration model for determination of moisture content was calculated by the second derivative pretreated NIR spectra covering the water bands (680-2500 nm). The statistic results were R^2 for calibration of 0.839, RMSEC of 0.445%, and the lowest RMSECV of 0.488% at an optimal factor number of 4. Note that the level of predictive performance for the PLS calibration model was justified according to the value of coefficient of determination (Williams, 2007). The distributions of the NIR predicted value versus measured value by those reference methods for hemicellulose (a), cellulose (b), nitrogen (c), carbon (d), hydrogen (e), and moisture (f) are shown in Fig. 3. The accurate predictive performance of PLS calibration model can be seen from the distributions that the high accuracy will present the highest slope of regression line close to one (R^2=1), and the dots representing of predicted values placed at the closest to the regression line.

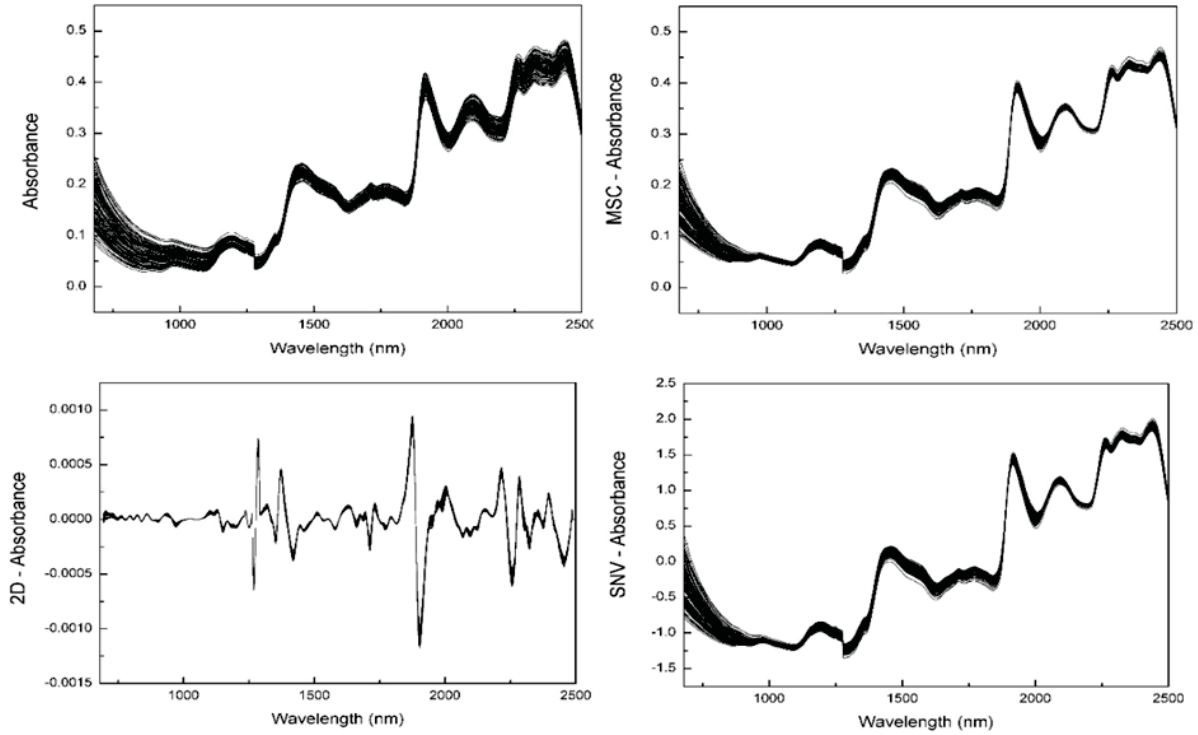

Figure 2. Original and pretreated NIR spectra of rubber wood samples in the region of 680 – 2500 nm.

Table 2 The statistical results of PLS models for hemicellulose, cellulose, N, C, H, and moisture in rubber wood developed from original and pretreated NIR spectral data.

PLS model	Range (nm)	Pretreatment spectra	F	Calibration		Full-Cross Validation	
				R^2	RMSEC (%)	R^2	RMSECV (%)
Hemicellulose	680-1246*	None	15	0.912	0.901	0.674	1.748
		2D*	8	0.835	1.231	0.630	1.860
		MSC	16	0.920	0.857	0.605	1.923
		SNV	14	0.905	0.935	0.646	1.821
	680-2500	None	27	0.973	0.500	0.612	1.915
		2D	15	0.919	0.866	0.479	2.219
		MSC	26	0.979	0.446	0.649	1.821
		SNV	15	0.821	1.289	0.507	2.158
Cellulose	680-1246	None	9	0.612	2.701	0.466	3.198
		2D	4	0.549	2.912	0.435	3.289
		MSC	9	0.613	2.698	0.459	3.218
		SNV	9	0.612	2.701	0.457	3.225
	680-2500*	None	3	0.473	2.938	0.438	3.059
		2D*	4	0.596	2.573	0.503	2.878
		MSC	7	0.592	2.583	0.488	2.921
		SNV	7	0.612	2.521	0.494	2.903
N	680-1246	None	1	0.291	0.075	0.276	0.077
		2D	5	0.582	0.058	0.394	0.070
		MSC	6	0.434	0.067	0.335	0.073
		SNV	6	0.433	0.067	0.332	0.074
	680-2500*	None	12	0.879	0.034	0.803	0.044
		2D	8	0.884	0.033	0.755	0.049
		MSC	10	0.866	0.036	0.789	0.045
		SNV*	9	0.847	0.038	0.785	0.046
C	680-1246	None	9	0.652	0.692	0.516	0.823
		2D	4	0.612	0.730	0.508	0.829
		MSC	9	0.652	0.692	0.517	0.822
		SNV	9	0.649	0.695	0.517	0.822
	680-2500*	None*	8	0.664	0.680	0.583	0.764
		2D	3	0.605	0.737	0.553	0.790
		MSC	5	0.598	0.743	0.539	0.803
		SNV	5	0.613	0.729	0.554	0.790
H	680-1246*	None	3	0.660	0.134	0.660	0.135
		2D	1	0.677	0.131	0.670	0.134
		MSC*	5	0.747	0.116	0.705	0.126
		SNV	5	0.745	0.116	0.701	0.127
	680-2500	None	1	0.614	0.143	0.606	0.146
		2D	4	0.721	0.122	0.652	0.137
		MSC	5	0.699	0.126	0.650	0.137
		SNV	5	0.706	0.125	0.658	0.136
Moisture	680-1246	None	9	0.830	0.456	0.766	0.540
		2D	4	0.826	0.462	0.788	0.515
		MSC	7	0.807	0.487	0.741	0.569
		SNV	8	0.827	0.461	0.765	0.542
	680-2500*	None	6	0.815	0.477	0.785	0.519
		2D*	4	0.839	0.445	0.806	0.488
		MSC	4	0.806	0.488	0.784	0.519
		SNV	4	0.808	0.486	0.785	0.518

F: The number of factors; R^2: the coefficient of determination; RMSEC: root mean square error of calibration; RMSECV: Root mean square error of cross-validation; *The selected PLS calibration model

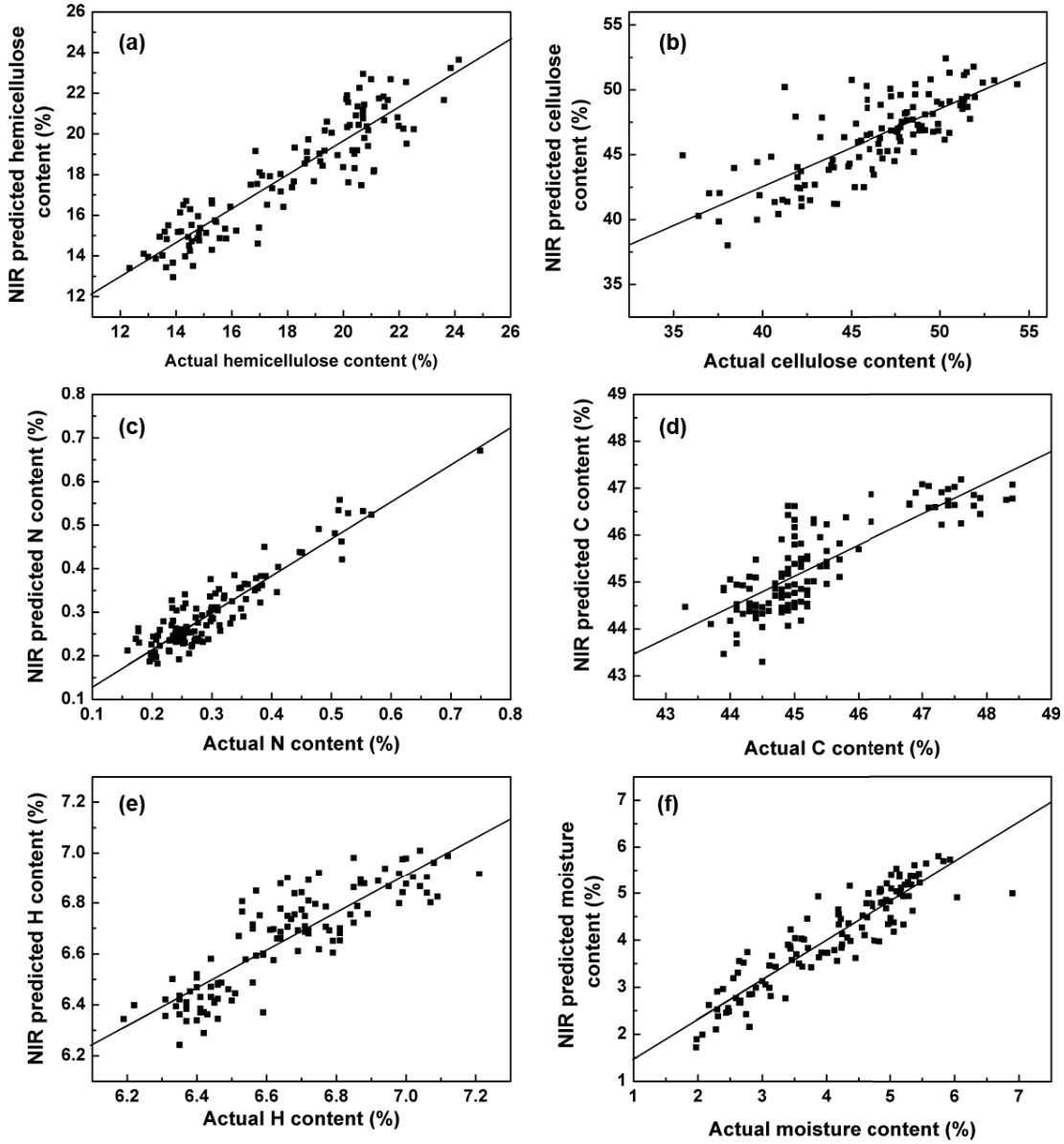

Figure 3. Correlation plot between actual (x axis) and NIR predicted (y axis) contents of hemicellulose (a), cellulose (b), nitrogen (c), carbon (d), hydrogen (e), and moisture (f) in rubber wood.

CONCLUSION

From all the results, we found that the NIR spectroscopic method can be used to quantitative determination of hemicellulose, nitrogen and moisture in the rubber wood with good accuracy, and of cellulose, carbon and hydrogen with moderate accuracy. The NIR spectra significantly showed bands of important chemical structure, which is the fundamental information of biomass material. These calibration models may be possible for estimating the contents of chemical components in rubber wood sample for bioenergy production process.

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EN-P003

POTENTIAL ABILITY OF BIOMASS AND LIPID PRODUCTION IN GREEN MICROALGAE *CHALMYDOMONAS REINHARDII* ZEAXANTHIN DEFICIENT AND ACCUMULATING STRAIN

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ABSTRACT

Microalgal biomass and lipid production have a high potential for alternative biofuel production. However, the processes of microalgal biomass and lipid production are very costly. Thus, increasing of biomass, biofuel, and natural product production becomes a way to reduce cost. Characterization of various microalgal species and strains have been challenged to discover the high biomass and lipid production strains. In this study, biomass and lipid production in *Chalmydomonas reinhardii* CC3682 and CC3683 which zeaxanthin deficient and accumulating strain respectively, were evaluated. In response to nitrogen starvation, biomass yield of CC3683 was increased up to 1.8 fold in comparison to wild type strain. In contrast to CC3683, biomass yield of CC3682 was not changed. The raising up of total lipid content (g/cell) was only observed in CC3682. According to total lipid content, triglyceride content of CC3682 was increased up to 1.13 fold in comparison to wild type. This finding revealed that *Chalmydomonas reinhardii* CC3682 and CC3683 have the potential for biomass and lipid production.
Keywords: *Chalmydomonas reinhardii*, Lipid, Triglyceride

INTRODUCTION

Current increasing of the population is the major problem in the world. This situation leads to the depletion of food and energy as petroleum, coal and natural gas. Thus, searching for new sources of fuel have been considered. Biomass, biodiesel and bioethanol which obtained from natural sources are interest (Widjaja *et al*., 2009). Among the natural sources of biodiesel, microalgae have many advantages in comparison with the other sources. For example, their cultivation is easy and inexpensive. In addition, they have high productivity in term of yield per area. Finally, biomass and biofuel production from microalgae reduce the competition between food and energy. (Chisti, 2007). Several microalgae have been reported to apply in biodiesel production. The common algae (*Chlorella*, *Dunaliella*, and *Tetraselmis*) can accumulate the oil content in the range of 20-50% and reach high productivity (Mata *et al*., 2010). Although microalgal biofuel production has many advantages, but this process still has limitations. Firstly, large scale production is very costly (in term of cost per yield). Secondly, lipid extraction and purification is energy intensive. Finally, monitoring of microbial community contamination is difficult (Tanvi *et al.*, 2016). To overcome these limitations, three characteristics of microalgae for biofuel production have been proposed including high lipid content production, high growth rate, and high biomass production. To achieve these characteristics, several lipid induction strategies have been challenged. Many researchers have been considered the methods for increasing of lipid content in microalgae, such as screening of high lipid production species and strain, optimization of stresses and culture conditions, and genetic engineering (Guiheneuf *et al.,* 2016).

Beyond the production of biofuel, microalgae can produce biomass that contains various valuable substances especially carotenoid pigments. Zeaxanthin which one of oxygenated carotenoids or xanthophylls is more attractive. Zeaxanthin can play an important role in non-photochemical quenching by quenching the singlet excited state of chlorophyll (Baroli, *et al*., 2003) According to the photoprotection properties, zeaxanthin was recommended for the health benefit and prevention the chronic diseases such as age-related macular degeneration (AMD), atherosclerosis, retinal neural damage, and ultraviolet radiation (Bernstein, *et al.,* 2016 and Nwachukwu, *et al.,* 2016) Many zeaxanthin-accumulating microalgae including *Chalmydomonas reinhardii and Dunaliella spp.* were established and characterized in terms of its physiology and adaptation to stress conditions (Niyogi K, *et al.*, 1997 and Thaipratum, *et al*. 2009). Since then, there

have been no evidence regarding its biomass and lipid productivity, or further development or optimization of biomass production. In this study, we evaluated the potential abilities of *Chalmydomonas reinhardii zeaxanthin deficient and accumulating strain* for biomass and lipid production. Growth, biomass productivity, total lipid content, and triglyceride content were compared.

MATERIALS AND METHODS

Microalgal growth condition

Chlamydomonas reinhardtii strains (wild type: CC-124, zeaxanthin deficient: CC-3682 and zeaxanthin accumulating: CC-3683) were obtained from the Chlamydomonas Genetic Center (Duke University, Durham, NC). Microalgal cells were pre-cultivated in Tris-acetate phosphate (TAP) medium under constant light intensity at 50 µmol photons m^{-2} s^{-1} at 25 °C. To treat cell by nitrogen starvation, miroalgal cells were grown in TAP medium for 96 h, harvested, washed twice in TAP-N medium, and transferred to fresh TAP (control), TAP-N (TAG induction). These cultivations were grown under constant light intensity 50 µmol photons m^{-2} s^{-1} at 25 °C.

Growth and biomass determination

Growth of cells from all culture conditions was measured daily by optical density at 680 nm for 4 days. Cell number of these microalgae were determined by hemocytometer in the final day of microalgal growth. Microalgal biomass cell was measured in term of dry weight. PVDF membrane filter was dried at 60 ºC in overnight and pre-weighted before use. Ten milliliter of microalgal culture was filtered through the filter and then dried in the oven at 60 ºC for 3 days. The filtered were weighted. Biomass concentration was calculated as follow; *Biomass concentration (g/ml) = (Final weight filter – initial weight filter) / volume*

Total lipid and triglyceride content (TAG) measurement

Microalgal cells were collected and extracted by three milliliters of chloroform and methanol (1:2 v/v) according to modified from Bligh and Dyer (Bligh & Dyer, 1959). The supernatant from lipid extractions were transferred to pre-weighted tube. Then these supernatants were evaporated by air-flow for 3 days in a chemical hood. The amount of total lipid was measured gravimetrically and calculated as follow; *Lipid content (g/cell) = (Final weight test tube - initial weight filter) / cell number*. TAG contents were analyzed by thin layer chromatography (TLC). Chloroform: methanol (1:2) was performed to dissolve the crude lipid in to the final volume of 200 µl per 1.75×10^8 cells. Soy bean oil was used as a standard TAG. The lipid dissolved and standard were spotted on TLC plate (Silica gel 60 F254, Merck). The mobile phase composes of hexane: diethyl ether: acetic acid (70:30:1). Visualization of lipid band can be done by iodine vapor standing for 30 minutes. The bands were quantified by an ImageJ program. Nile-red fluorescent dye at the final concentration of 0.5 µg ml^{-1} was performed to stain microalgal cells for 30 minutes in the dark. Then, cell and compartments were fixed by 4% paraformaldehyde (v/v) for 30 minutes. Microalgal cells were washed by phosphate buffer saline solution twice. The fluorescent signal was observed by confocal Laser Scanning Microscope (FV10i-DOC) using 430 and 690 nm of excitation and emission wavelength, respectively.

Pigment analysis

Microalgae cells were centrifuged at 3,000 g. TritonX-100 at 0.7% (v/v) was added in cell pellet to lyse cell and incubate overnight. Then the cell lysates were dissolved in acetone (final volume 80% acetone). The cell debris was removed by centrifugation at 14,000 g for 15 minutes. All of microalgae pigments were detected by high performance liquid chromatography (HPLC 717 water) combine with photodiode array detector (PDA). The absorbance spectrum was collected between 300-700 nm and victualed at 450 nm. C18 reverse phase column (Hypersil™ ODS-2 25 cm x 4.6 nm 5 µ particle size) was performed to separate these pigments. HPLC running condition and pigment identity were described previously (Yokthongwattana, *et al.,* 2005).

RESULTS AND DISCUSSION

Pigment accumulation

To confirm the phenotype of all microalgal strains, the pigment of wild type, zeaxanthin deficient, and zeaxanthin accumulating strain were evaluated. Chromatographs were shown in Fig 1. These results represented that zeaxanthin peak was clearly observed in zeaxanthin accumulating strain, while it was

slightly observed in wild type strain. In contrast to wild type and zeaxanthin accumulating strain, zeaxanthin peak was not observed in zeaxanthin deficient strain.

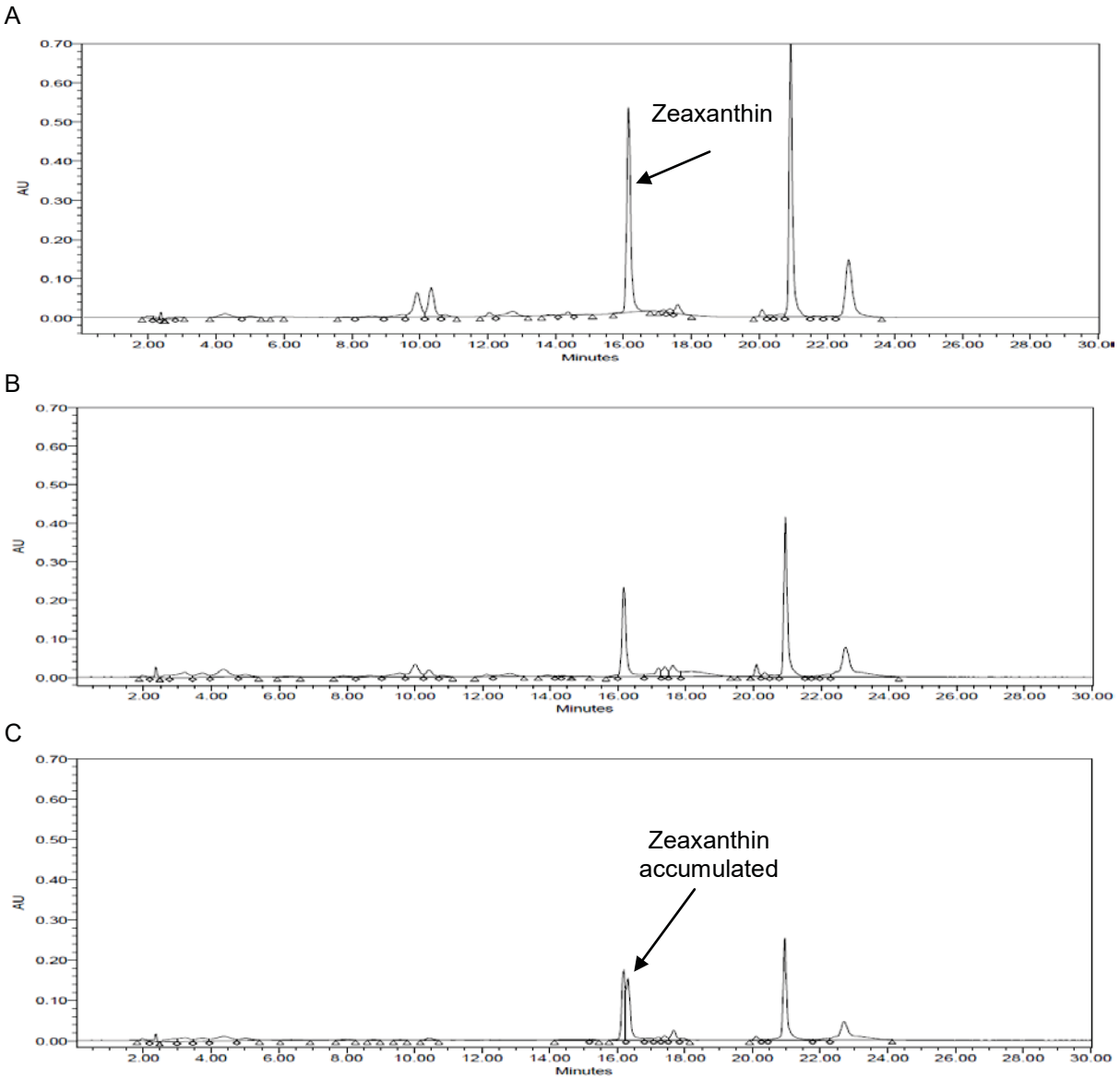


Figure 1. Pigment chromatogram of *C. reinhardtii* from HPLC analysis couple with C18 reverse phase column. The zeaxanthin peaks were labeled in the chromatogram. A) CC124 (wild type), B) CC3682 (zeaxanthin deficient), C) CC3683 (zeaxanthin accumulating)

Growth characteristic, final cell and biomass concentration

Growth performance of microalgae is the crucial factors for microalgal biomass and lipid production. Therefore, growth characteristic of wild type, zeaxanthin deficient, and zeaxanthin accumulating were investigated by optical density at OD680 for 8 days under normal light intensity at 50 µmol photons m^{-2} s^{-1}. Growth characteristic showed that all microalgal strains entered a log phase and stationary phase with no significant difference between them (Fig 2). It suggested that these microalgal strains did not cause significant impairment of growth in this condition. The final cell concentration and dry cell weight of microalgal cells were determined after nitrogen starvation treatment. The result showed that the final cell concentration of both zeaxanthin deficient and zeaxanthin accumulating were decreased in comparison to wild type strain. Many evidences have been earlier reported that the microalgal zeaxanthin deficient and accumulating strains impair growth and photosynthesis performance because the lacking of non-photochemical quenching mechanism to dissipate energy (Niyogi, *et al.*,1997 and Ait Ali, *et al.* 2008). Thus, the lacking of protection mechanism possibly affected the growth of microalgal strains under nitrogen

starvation. In contrast to final cell concentration, the dry cell weight of zeaxanthin deficient and accumulating strain were increased in comparison to wild type strain under all growth conditions (Fig 3). According to previous studies in *Arabidopsis* (Kromdijk, *et al.*,2016) and *Chalmydomonas* (Berteotti, *et al.*, 2016). Photosynthetic organisms have the ability to quenching states to dissipate the energy absorbed in excess through heat. In that state, photosynthetic biomass production is reduced. Thus, the limitation of non-photochemical quenching in zeaxanthin deficient and accumulating strain possibly increased biomass productivity. However, this study did not evaluate the non-photochemical quenching of both microalgal strains. Therefore, analysis of non-photochemical quenching and photosynthesis performance will be investigated further.

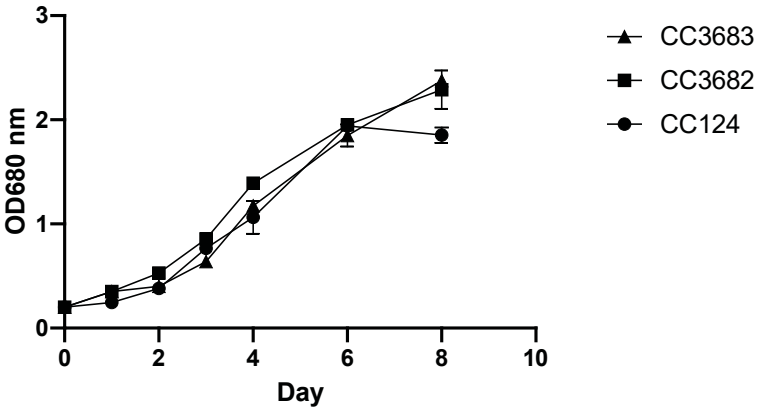
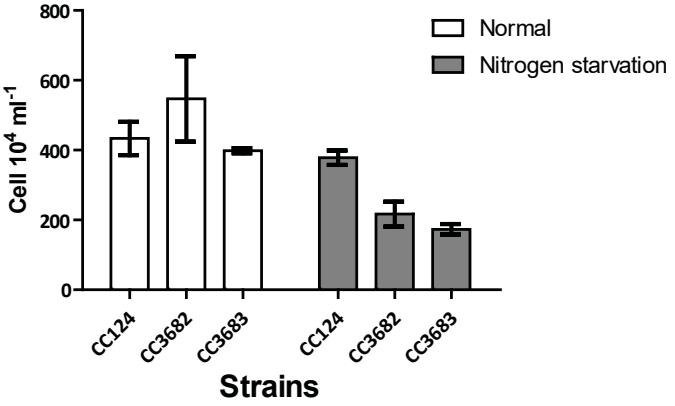


Figure 2. Growth characteristic of microalgae cells. All *C. reinhardtii* strains were grown in TAP medium for 8 days. The optical density at OD680 was detected at each day of microalgae growth. Each data represents as mean ±SD (n = 3).

A



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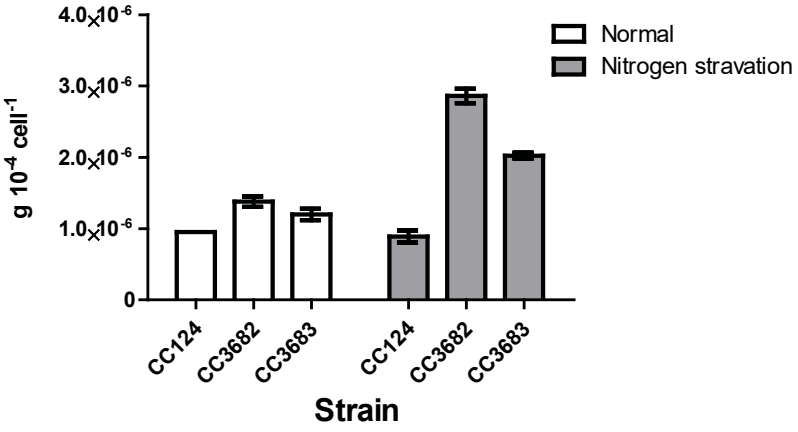

Figure 3 Final cell concentration and biomass concentration under normal and nitrogen starvation condition. A) Final cell concentration B) biomass concentration. *C. reinhardtii* CC124 (wild type), CC3682 (zeaxanthin deficient), and CC3683 (Zeaxanthin accumulating) were grown in TAP medium for 96 h and transferred to TAP medium (normal) and TAP-N medium (nitrogen starvation). Each data represents as mean ±SD (n = 3).

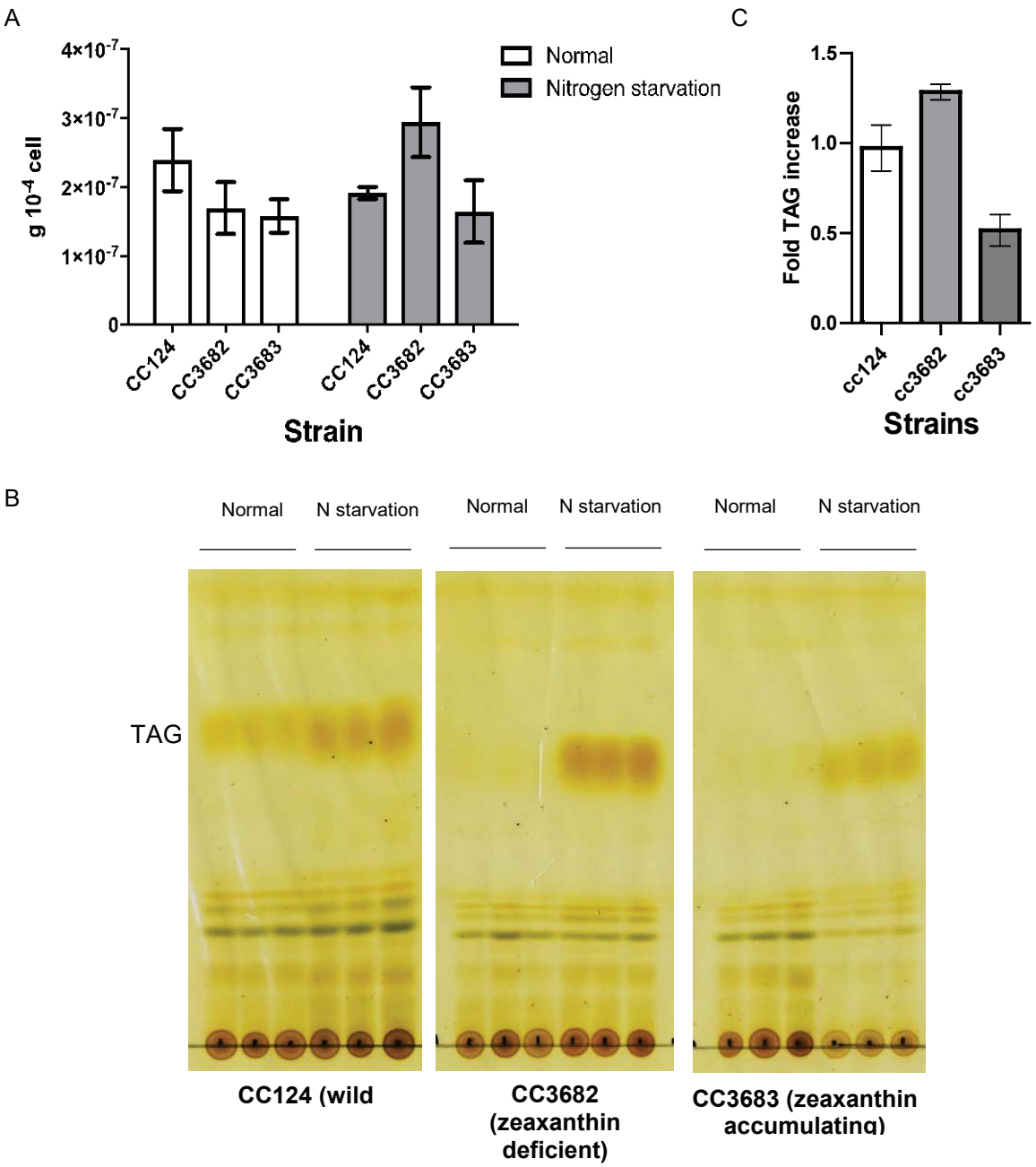

Figure 4 Total lipid and triglyceride content under normal and nitrogen starvation condition. *C. reinhardtii* CC124 (wild type), CC3682 (zeaxanthin deficient), and CC3683 (Zeaxanthin accumulating) were grown in TAP medium for 96 h and transferred to TAP medium (normal) and TAP-N medium (nitrogen starvation). A) Total lipid extract from these microalgae. B) Thin layer chromatography showing TAG from microalgal cells. C) Intensity of TAG content quantified from the TLC chromatogram. Each condition represents as mean ±SD (n = 3).

Total lipid and triglyceride content

The accumulation of total lipid and triglyceride level of all strains were determined. The microalgal cells were cultivated under normal and nitrogen starvation condition. Under nitrogen starvation, the total lipid content (g cell^{-1}) of wild type and zeaxanthin accumulating strain were lower than that when compared with the normal condition (Fig 4A). However, the level of lipid content (g cell^{-1}) was increased in zeaxanthin deficient strain. To analysis further, the total lipid extracts from the equal amount of cell number were separated by TLC plate. In general, TAG of total lipid extract was constituent increased in microalgae species under nitrogen starvation condition (Minhas, *et al.*,2016). The TAG contents of all microalgae were

increased under this condition (Fig 4B-C). However, zeaxanthin deficient strain accumulated the highest amount of TAG. Then, the lipid production of all microalgae were confirmed by Nile red staining. Nile red (9-diethylamino-5H-benzo[a]phenoxazine-5-one) is the commonly dyed for the lipophilic stain of intracellular TAG in microalgae (Rumin, *et al.*, 2015). Like the TAG content, the lipid droplet abundance in zeaxanthin deficient strain was higher than that of wild type and zeaxanthin accumulating strain (Fig 5). The increasing of triglyceride content in zeaxanthin deficient strain was related to the increase of dry cell weight as described previously. However, we did not claim that this phenotype originates from the lacking of non-photochemical quenching. Such this phenotype might be part of the background genetics of insertional mutagenesis to construct zeaxanthin deficient strain (Niyogi, *et al.*,1997). This hypothesis and unexplainable result regarding the triglyceride contents of this strain needs further characterization.

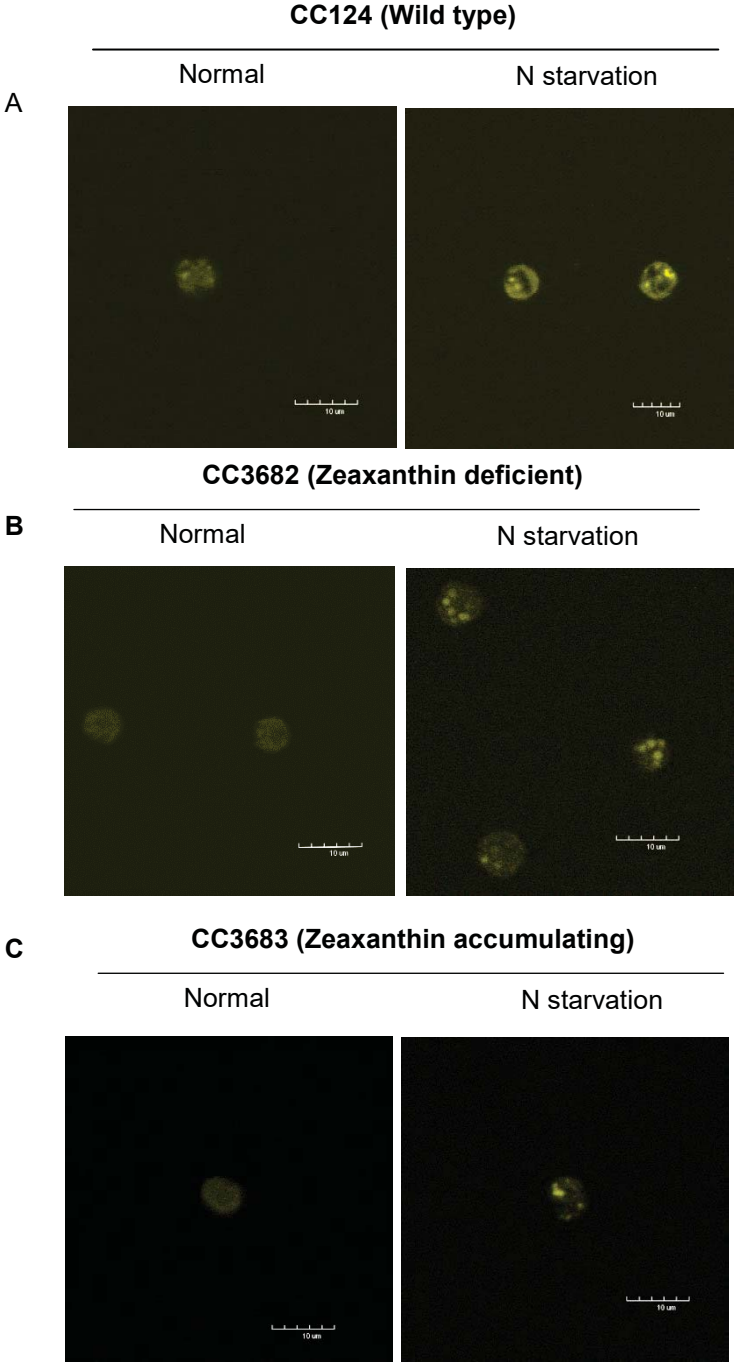

Figure 5 Nile red fluorescent signal from A) *C. reinhardtii* CC124 (wild type), B) CC3682 (zeaxanthin deficient), and C) CC3683 (Zeaxanthin accumulating) under normal and nitrogen starvation condition. The fluorescent signal was observed by Confocal Laser Scanning Microscope (FV10i-DOC) using 430 and 690 nm of excitation and emission wavelength, respectively.

CONCLUSION

Screening and characterize microalgal strains are the way to get the good candidate strains for biomass and biofuel production. The microalgal *Chlamydomonas reinhardtii* zeaxanthin deficient and accumulation strains were found to efficiently produce high biomass (cell dry weight) under normal and nitrogen starvation. In addition, the highest total lipid and triglyceride content were obtained by zeaxanthin deficient strain under nitrogen starvation. Our results demonstrated that zeaxanthin deficient strain is a noticeable and promising microalgae for biomass and lipid production.

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EN-P004

SCREENING OF ANAEROBIC BACTERIA FOR OIL-PLAM EMPTY FRUIT BUNCH DEGRADATION

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ABSTRACT

Oil-palm empty fruit bunch (EFB) is a lignocellulosic waste produced in the palm oil industry and an attractive lignocellulosic material for production of value-added products. EFB mainly consists of cellulose, hemicellulose and lignin, forming a complex structure which is difficult to hydrolyze, and requires the synergistic action of many enzymes. Converting EFB to fermentable sugars and biogas is prove a more appropriate technology than treating EFB as a waste. This study aimed to isolate thermophilic anaerobic bacteria that showed high performance EFB degrading ability and utilization. Soil samples were collected from various sources in Thailand, and inoculated directly into basal medium (BM) pH 7.0 containing 1% (w/v) EFB as the carbon source at 60°C under anaerobic conditions. The results showed that KB9 possessed greater EFB degrading ability (45.49%) into 1.17 times than *C. thermocellum* ATCC27405 (38.85 %). Composition analysis reveal that cellulose and xylan in EFB was degraded more than 61.94% and 57.87%, respectively, by KB9 after 5 days incubation. The crude enzyme pattern of KB9 on EFB was indicated 18 visual protein bands (19-215 kDa) on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) after CBB staining. Zymogram analysis revealed 14 xylanases, 8 cellulases and 9 mannanases production by KB9, leading effective EFB degradation. Furthermore, the isolated KB9 promoted high methane yield of 40.51 mol CH_4/gEFB. Thus, the isolated KB9 would indicate the high biodegradable potential in utilizing EFB and might apply to EFB bio-refinery industry.
Keywords: Anaerobic bacteria, Degradation, Lignocellulosic waste, Oil palm empty fruit bunch

INTRODUCTION

Oil Palm (*Elaeis guineensis*) is one of the most versatile crops in the tropical world. Oil palm mill plant also generates a large amount of solids waste such as empty fruit bunches (EFB) (23%), mesocrap fiber (12%) and shell (5%) for every ton of fresh fruit bunches (O-Thong *et al*., 2012). One of the most challenging problems is the management of EFB (Nieves *et al.*, 2011). EFB constitutes nearly 50 % of these solid wastes, and composed of cellulose, hemicellulose, lignin and other extractive materials. Mostly, EFB has great potential to be processed as bioenergy, fertilizer and other products with higher economic value. Because of the high content of lignin up to 21 %, EFB carries similar hardness as wood (Kumar *et al.*, 2009). However, a high moisture content of 60-70% and high content of organic matter has potential to be used for biogas production (Kim *et al*., 2013) and bio-refinery industry.

Degradation of lignocellulosic biomass, with high lignin concentration, requires a pretreatment process that can be performed through a physical, chemical, mechanical, as well as biological method using microbes or enzymes (Atlas and Bartha, 1986). The purpose of pretreatment is to remove or break down lignin structure and increase the digestibility of cellulose fraction. Natural decomposition process of lignocellulosic biomass, instead of physical and chemical process, is performed by a biological pretreatment. Anaerobic bacteria can degrade cellulosic biomass and produce ethanol, hydrogen and organic acids as products. Therefore, many researches aimed in obtaining new microorganisms producing cellulolytic and xylanolytic enzymes with greater degradation efficiency. Bacteria, which has high growth rate as compared to fungi has good potential to be used in sugar and biogas production (Ariffin *et al.*, 2008). Especially,

thermostable cellulase can increase the rate of reaction, decrease amount of enzyme needed, longer half-life and decrease the possibility of microbial contamination (Ibrahim and El-diwany, 2007). The aim of this study was to isolate thermophilic anaerobic bacteria that showed high performance on EFB degrading ability and biogas production.

MATERIALS AND METHODS

Microorganisms and Medium

The strain KB9 was isolated from soil sample in Krabi province, Thailand. The fresh cultures were maintained by routinely transferring 1% (v/v) inoculum into new basal medium (BM) containing (per liter) 0.45 g of K_2HPO_4, 0.45 g of KH_2PO_4, 0.9 g of NaCl, 0.9 g of $(NH)_4SO_4$, 5 g of Yeast extract powder, 0.12 g of $CaCl_2.2H_2O$, 0.18 g of $MgSO_4.7H_2O$, 0.001 g of resazurin, 4 g of Na_2CO_3 and 0.5 g of cysteine hydrochloride. The pH of the media was adjusted to 7.0, purge with CO_2 and autoclaved at 121°C for 15 min. The media were prepared under anaerobic conditions, as previously described (Tachaapaikoon *et al.*, 2012).

Screening and Isolation

The strain KB9 was isolated using oil palm empty fruit bunch (EFB) as the sole carbon source. The cultures were incubated at 60°C under anaerobic conditions. The screening step was carried out several times to obtain the target candidates. The samples showing the most effective EFB degradation was enriched. The isolation was carried out on BM agar medium by using the roll-tube technique (Hungate, 1969) under sticky anaerobic conditions and incubated at 60°C. Many kinds of colonies characteristics appeared on agar rolling tube. However, a single colony was picked up by using a single colony technique with a needle and inoculated into fresh BM contained cellulose powder as the sole carbon source and it was repeated several times on agar rolling tube until there was single pattern appearance. The pure isolates were kept in appropriate culture broths containing EFB as the sole carbon sources. Degradation of EFB was determined using equation 1.

$$\text{Degradation of EFB (\%)} = 100 - \left[\frac{WR \times 100}{WI}\right] \quad \text{--------- (1)}$$

Where: W_I = Initial dry weight of EFB in basal medium (g)

W_R = Remaining dry weight of EFB after cultures were incubated at 60°C until the late exponential growth phase (5 days).

Preparation of Enzyme from Isolated Strains

The isolated strains were grown in BM medium consisted of 1% (w/v) EFB as the sole carbon source. The culture was incubated at 60°C until the late exponential growth phase (5 days) and harvested by centrifugation (8,000 rpm for 15 min) at 4°C. Subsequently, the supernatant was used as the crude enzyme. Vivaspin™ 20 was used to concentrate crude enzyme with a molecular weight cut-off (MWCO) of 10,000 kDa.

Gel Electrophoresis Analysis and Zymograms

All samples contained 100 µg of protein. SDS–PAGE was performed on a 10% polyacrylamide gel by the method of Laemmli (Laemmli, 1970). After electrophoresis, the proteins were stained with Coomassie brilliant blue R-250 (CBB). The molecular weight standards were used as a high-molecular-weight calibration kit (Bio-Rad). Xylanase, CMCase and Mannanase zymograms were prepared from polyacrylamide gels containing 0.1% (w/v) soluble xylan, 0.1% (w/v) CMC and 0.1% (w/v) locust bean gum as described previously. Zymogram gels were regenerated by 1% triton-X for 2 times and incubated with 50 mM sodium phosphate buffer pH 7.0 at 50°C for 5 min. Then stained with 0.1% (w/v) Congo red solution and de-stained by 1M NaCl.

Chemical Composition Analysis and Structural Characterization

The polysaccharide and lignin contents were determined according to National Renewable Energy Laboratory (NREL) protocol (Sluiter *et al*., 2008).

Analytical Biogas Yields Procedures

The amount of biogas produced was recorded daily using the water displacement method. The contents of hydrogen, carbon dioxide and methane in the biogas were analyzed by a gas chromatograph (Shimadzu GC-2014) with a thermal conductivity detector. The column (4 m x 3 mm) used for biogas quantification was filled with a 5 Å molecular sieve using argon as a carrier gas. The column temperature was 40°C. The injection temperature was 110°C and the detector temperature was 120°C. One milliliter of the gas phase from the culture was injected directly into the gas chromatography column with a 10 ml syringe. The volume of the gas phase of the culture was measured at the same time. Yields of biogas were indicated as moles of H_2, CO_2 and CH_4 produced per grams of oil palm empty fruit bunch as the carbon source.

RESULTS AND DISCUSSION

Screening of Anaerobic Bacteria for EFB Degradation

Thermophilic anaerobic bacteria that showed high performance EFB degrading ability was isolated and their biogas yields were measured. More than 100 soil samples were collected from Krabi province, Thailand and placed in first-screen hungate tubes (40 ml) containing 10 ml of BM medium and 10 gl^{-1} of EFB as a carbon source. The tubes were incubated at 60°C under anaerobic conditions. The cultures were transferred to fresh medium more than three times to ensure that the EFB degrading microorganisms were predominant compared with *C. thermocellum* ATCC27405. Using agar roll tube containing cellulose powder, single colonies were isolated after one-day' growth at 60°C. A colony that could degrade cellulose was selected and tube was re-rolls more than three times to obtain a pure culture. The isolated, namely KB9, KB17 and KB30, could grow well on cellulose powder as the sole carbon source, and were effective EFB degradation shown in Figure 1. KB9 possessed greater EFB degrading ability (45.49%) into 1.17 times than *C. thermocellum* ATCC27405 (38.85 %), while degrading ability of KB17 and KB30 were 28.52 and 32.10 %degradation, respectively. Therefore, KB9 is an interesting option for further study because of its high efficiency of EFB degradation.

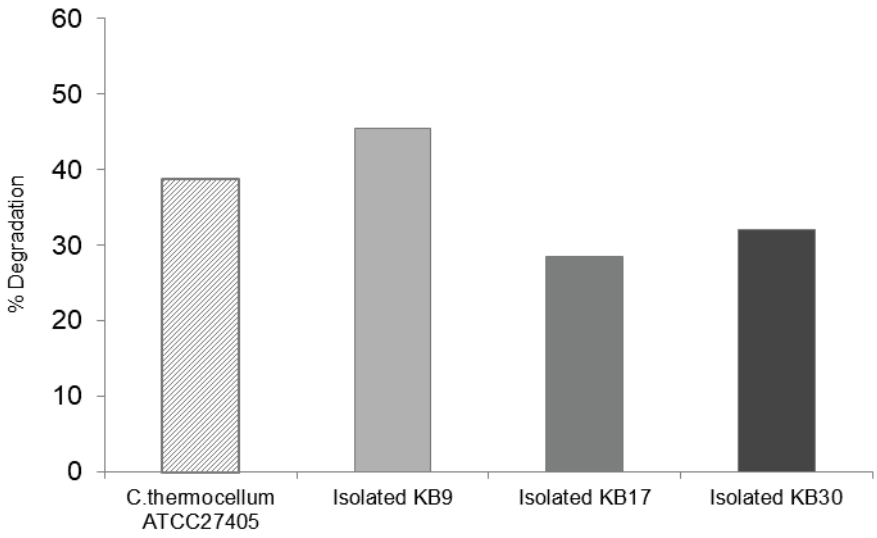

Figure 1. Degradation of EFB from isolated strains and *C. thermocellum* ATCC27405.

Biogas Production

The total biogas production was determined by direct measurements at the late exponential growth phase (5 days) and the results were shown in Table 1. When KB9 was cultured, the biogas production showed only methane at 40.51 mol/g EFB.

Table 1. The degradation, biogas yields from isolated strains and *C. thermocellum* ATCC27405 and chemical composition of EFB after incubated at 60°C for 5 day.

Strains	Degradation (%)	Biogas yields (mol/g EFB)			Chemical composition in remaining residue (%)		
		H_2	CH_4	CO_2	Cellulose	Hemicellulose	Lignin
Raw EFB (control)	N/D	N/D	N/D	N/D	46.27	32.38	21.03
C. thermocellum ATCC27405	38.85	2.54	0	53.15	32.69	33.40	28.53
Isolated KB9	45.49	0	40.51	62.61	32.31	25.02	30.51

*N/D = not determined

Chemical Composition Analysis

The composition of cellulose, hemicellulose and lignin content of the EFB after cultures were incubated at 60°C until the late exponential growth phase (5 days). The composition of EFB after cultures by isolated KB9 for 5 day consisted of 32.31% cellulose, 25.02 %hemicellulose and 30.51 %lignin (Table 1). The cellulose content was decreased towards the end of culture process by isolated KB9 and *C.thermocellum* ATCC27405 with 61.94% and 56.78% of reduction, respectively, KB9 showed effective degradation of cellulose more than *C.thermocellum* ATCC27405. At the same time, hemicellulose content decreased by KB9 more than *C.thermocellum* ATCC27405 with decrement of 57.87% and 36.93% of reduction, respectively. The lignin content was reduced slightly towards the end of culture process by isolated KB9 and *C. thermocellum* ATCC27405 with 20.92% and 17.07% of reduction, respectively.

SDS-PAGE and Zymograms Analysis of Crude Protein Components

The protein compositions of the crude enzyme KB9 was analyzed by SDS-PAGE analysis and zymogram, using CMC for cellulase activity, soluble xylan from birch wood for xylanase activity and locust bean gum for mannanase activity. When subjected to SDS-PAGE, the crude enzyme KB9 showed at least 18 proteins with molecular masses in the range of 215 to 19 kDa (Figure 2A). Zymogram analysis revealed 14 proteins band showed xylanase activity (Figure 2B), 8 proteins band showed cellulase activity (Figure 2C) and 9 proteins band showed mannanase activity (Figure 2D). Seven high-molecular-mass proteins, of 215, 180, 104, 85, 80, 72 and 69 kDa showed tri-functional enzyme of xylanase, cellulase and mannanase activities. Cellulose and hemicellulose degrading enzyme increased EFB degradation and utilization.

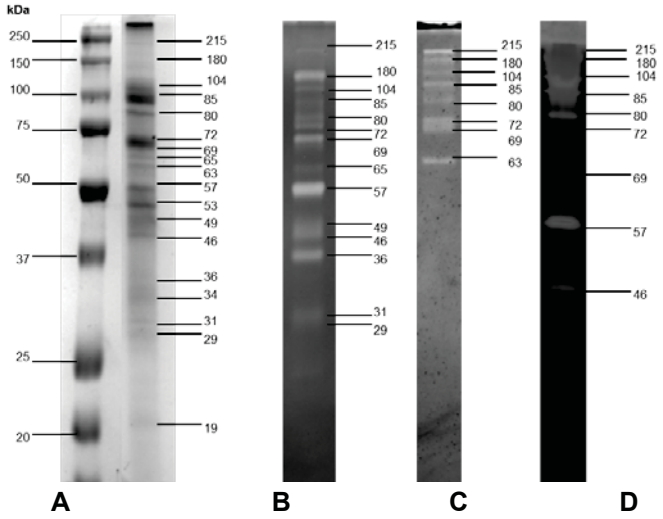

Figure 2. SDS-PAGE (A) and zymogram analysis of xylanase activity (B), cellulase activity (C) and mannanase activity (D) of crude enzyme from Isolated KB9. All samples contained 100 µg of protein. Molecular masses, in kilodaltons, are indicated.

CONCLUSION

The biomass degrading thermophilic anaerobic bacteria namely KB9 with the ability to competently degrade EFB were enriched. KB9 was successfully isolated from soil sample in Krabi province, Thailand by using EFB as the sole carbon source. Interestingly, the isolated KB9 containing of many cellulase, xylanase and mannanase enzymes that had high biodegradable potential to utilizing EFB. Especially, isolated KB9 showed effective degradation of cellulose and hemicellulose more than *C.thermocellum* ATCC27405, and it promoted high methane yield of 40.51 mol CH_4/gEFB.

ACKNOWLEDGMENTS

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EC-P001

THE EFFECT OF EVAPORATION TEMPERATURE ON THE CONTENTS OF KEY COFFEE AROMA COMPOUNDS IN COFFEE BREW

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ABSTRACT

Coffee aroma is one of the significant factors that indicates the quality and freshness of the coffee . This study evaluated the volatile compounds of coffee aroma using a rotary evaporator to recover the aroma compounds by headspace solid phase micro-extraction (HS-SPME). Gas chromatograph-mass spectroscopy (GC-MS) was used for analytical purposes. The temperature of evaporation was investigated at 120° C, 125°C and 130°C with constant pressure at 1013 mbar .The interested volatile compounds were classified into chemical groups which are ketones, pyridines, pyrazines, furans, pyrroles, and phenols .The study has found that rising temperature of evaporation led to a reduction of the concentrations of most of the volatile compounds .However, three odorants, i.e., 2-Furanmethanol, 2-Ethyl-5-methylpyrazine and Furfurly acetate, were found to increase with the higher temperature which, as a consequence, has elevated a burnt note in the coffee brew. This research aims to improve the aroma extraction process of instant coffee production.

Keywords: Coffee aroma, Coffee brew odor, Odor analysis coffee brew, Volatile compounds

INTRODUCTION

Aroma consists of more than a hundred of volatile compounds. Most of which present at low concentrations, i.e. at ppm or ppb. The main constituents are classified into chemical groups which are hydrocarbons, alcohols, aldehydes, ketones, acids, anhydrides, esters, lactones, phenols, furans, pyrans, thiophenes, pyrroles, oxazoles, thiazoles, pyridines, pyrazines, and sulfur compounds. (Ivon Flament., 2002). From all of a hundred of volatile compounds, there are only some significant ones called key odorants.

Aroma volatile components in the brew of Arabica and Robusta coffees were diacetyl, 2,3-pentandione, furaneol, sotolon, abhexon, 4-ethylguaiacol, 4-vinylguaiacol, 3-mercapto-3-methylbutylformate, and (E)-β-damascenone. They were analysed by gas chromatography/ olfactometry (GC/O). The results showed significantly higher concentration of 4-ethylguaiacol in the Robusta coffee compared to the Arabica coffee (Blank *et al.,* 1991). The study has revealed potent odorants that are responsible for characteristic notes in the odor profile of brews prepared from roasted Arabica and Robusta coffees. Calculation of odor activity values (OAVs; ratio of concentration to odor threshold) indicated 2-furfurylthiol, 3-mercapto-3-methylbutyl formate, methanethiol, β-damascenone, methylpropanal, and 3-methylbutanal as the most potent odorants (Peter and Werner., 1996). The volatile profiles of two different coffee brews, Turkish coffee and French press coffee, were analyzed by gas chromatography-mass spectrometry (GC-MS) technique. 2,3-butanedione, guaiacol and furfury acetate were the most potent odorants of both coffee brews (Asghar and Serkan., 2015)

The aim of this work was to investigate the effects of evaporation temperature, 120°C, 125°C, and 130°C, on the levels of key odorants in coffee brew and recovered in the condensate to choose the optimal temperature. Moreover, this study cosidered the behavior of key aroma compounds on evaporation temperature relate to odor type of odorant which might be relevant to the good coffee aroma.

MATERIALS AND METHODS

Preparation of coffee aroma

The coffee brew samples, 100% Robusta coffee, were supplied by a local company in Thailand. 500 mL of the coffee brew was distilled in a rotary evaporator (Buchi model Rotavapor® R-300 equipped with a heating bath model B-300 Base and a 1000 mL round-bottom flask). The temperature of evaporation was

investigated at 120°C, 125°C, and 130°C with a rotation speed of 40 rpm and constant pressure at 1013 mbar.

Headspace solid phase micro-extraction (HS-SPME) Sampling

2 mL of sample were directly placed in a 20 mL vial. It was exposed to the volatile compounds with a SPME (DVB/CAR/PDMS) 50/30 µm fiber (Sigma-Aldrich Co.) for 30 minutes at 60°C in the headspace. After exposing, the fiber was followed by a desorption step into the injection port of the GC-MS.

GC-MS Analysis and Identification of the volatile compounds

Gas-chromatography coupled with mass detection (5979, Agilent) and He as the carrier gas was used. A 2 mL headspace sample was injected into a Free Fatty Acid (FFAP) capillary column (25 m x 0.32 mm x 0.3 µm film thickness; Agilent Technologies). The oven temperature was programmed at an initial temperature of 40°C for 5 minutes, followed by an increase of 3°C/minute to 150°C and increase of 6°C/minute to 250°C. The volatile compounds identification was primarily based on the NIST library v.2.0a (2002), followed by mass spectra interpretation.

RESULTS AND DISCUSSION

Volatile components of the headspace of coffee brew

The gas chromatograms is shown in Figures 1. Forty-eight volatile compounds were identified by headspace analysis from coffee brew are shown in Table 1. From a total of 48 volatile compound, 23 odorant volatile compound were classified by their chemical groups which are 1 ketone, 1 pyridine, 7 pyrazines, 5 furans, 4 pyrroles, and 5 phenols (Table 2).

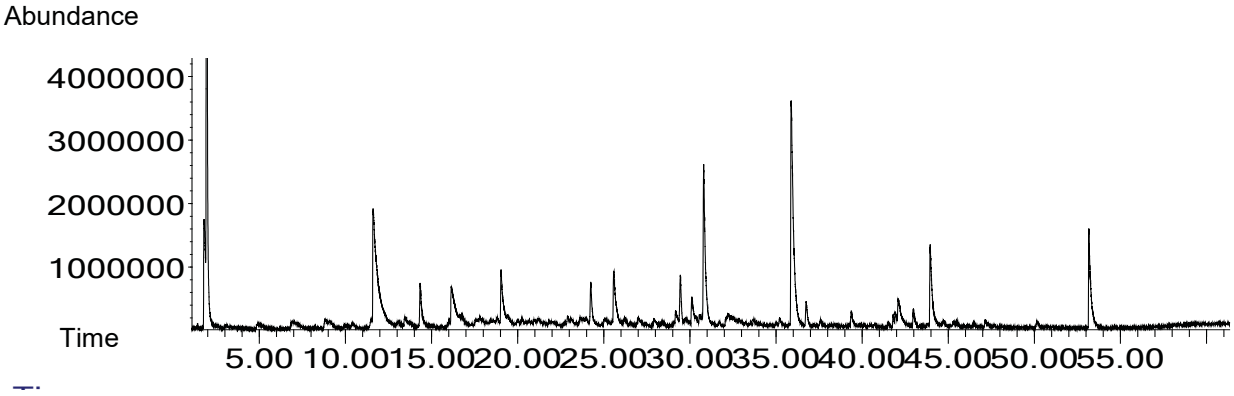

Figure 1. Typical total ion chromatograms of dynamic headspace Volatiles from the coffee brew.

Table 1. Volatile compounds found in the headspace of coffee brew.

No.	Retention Time	Compounds	Peak area
1	1.983	5-Methyl-2-hexanone	542,415,995
2	2.388	2-Methyl-5-nonanone	1,546,931
3	2.571	2-Heptanone	1,880,321
4	3.126	Pyridine	2,784,856
5	4.927	Methylpyrazine	15,316,493
6	7.018	Ethylpyrazine	22,439,400
7	8.824	2-Ethyl-6-methylpyrazine	9,905,034
8	9.091	2-Ethyl-5-methylpyrazine	12,982,322
9	9.984	2-Ethyl-3-methylpyrazine	3,703,274
10	10.449	2-Ethyl-3,5-dimethylpyrazine	4,053,150
11	11.535	2-Ethyl-3,6-dimethylpyrazine	8,024,061
12	11.639	Furfural	235,857,215
13	13.092	3,3-Dimethyl-3,3a,4,7-tetrahydropyrazolo[1,5-a]pyridine	7,376,509
14	13.482	2-Acetylfuran	8,999,472
15	14.361	2-Furylmethyl acetate	36,347,032

16	16.039	1-(2-Furyl)-1-propanone	3,301,223
17	16.179	5-Methyl-2-furancarboxaldehyde	42,344,937
18	16.781	2-(2-Furylmethyl)furan	17,931,621
19	17.823	2-Formyl-1-methylpyrrole	18,836,662
20	19.067	2-Furanmethanol	40,597,347
21	19.42	5-Methyl-2-furfurylfuran	2,866,308
22	22.92	2-Pentynoic acid ethyl ester	4,322,271
23	24.263	1-(2-Furylmethyl)-1H-pyrrole	33,918,706
24	25.622	Guaiacol	40,564,180
25	26.249	Pilocarpine	5,834,385
26	29.193	1-(1H-Pyrrol-2-yl)ethanone	7,457,790
27	29.465	Difurfuryl ether	23,248,557
28	29.749	4-Pentylcyclohexanone	5,729,061
29	30.139	Phenol	16,198,791
30	30.559	1H-Pyrrole-2-carbaldehyde	4,114,774
31	30.824	4-Ethyl guaiacol	103,903,907
32	35.252	4-Ethylphenol	3,694,314
33	35.904	4-Vinylguaiacol	213,937,862
34	36.758	α-Furfuryliden-α-furylmethylamine	13,063,400
35	37.577	β-Elemene	3,847,616
36	39.387	3,5-Di-tert-butylphenol	10,590,886
37	41.514	4,4a,5,6-Tetrahydro-2(3H)-naphthalenone	3,964,484
38	41.804	1H-Indole	7,595,236
39	41.896	6-Methyl-3-pyridinol	18,388,491
40	42.062	3-Hydroxypyridine	48,231,799
41	42.984	3-Methyl-1H-indole	12,767,934
42	43.937	5-Hydroxymethylfurfural	106,604,378
43	45.281	Vanillin	2,659,919
44	45.482	2-Ethyl-4-[3-(2-ethyl-1,3,2-dioxaborolan-4-yl)propyl]-1,3,2-dioxaborolane	2,581,289
45	46.469	(3E)-4-(1-Acetyl-2,2-dimethylcyclopentyl)-3-buten-2-one	3,537,419
46	47.094	[(E)-2-Chloroethenyl](ethoxy)dimethylsilane	4,330,358
47	50.103	n-Hexadecanoic acid	6,121,980
48	53.131	Caffeine	125,364,004

Table 2. Key odorants of volatile compounds identified in coffee brew.

Compounds	Molecular Weight	Odor Type	Odor Threshold	Boiling Point[1]	Key odorants selection
Ketone					
2-Heptanone	114.19	cheesy	140 ppb or 0.14 ppm	149.00 to 150.00 °C	yes
Pyridine					
Pyridine	79.10	fishy	0.23-1.9 ppm	115.00 to 116.00 °C	yes
Pyrazine					
2-Ethyl-3,5-dimethylpyrazine	136.20	nutty	0.00004 ppm	180.00 to 182.00 °C	No
2-Ethyl-3,6-dimethylpyrazine	136.20	nutty	0.0086 ppm	180.00 to 182.00 °C	yes
2-Ethyl-5-methylpyrazine	122.17	coffee	0.1 ppm	79.00 to 80.00 °C[2]	yes
2-Ethyl-3-methylpyrazine	122.17	nutty	0.13 ppm	169.00 to 171.00 °C	No
2-Ethyl-6-methylpyrazine	122.17	potato	20 ppm	170.00 to 171.00 °C	yes
Ethylpyrazine	108.14	nutty	21 ppm	152.00 to 153.00 °C	No
Methylpyrazine	94.12	nutty	105 ppm	136.00 to 137.00 °C	No
Furan					
Furfuryl acetate	140.14	fruity	0.1 ppm	175.00 to 177.00 °C	yes
Furfural	96.09	bready	3 ppm	161.00 to 162.00 °C	No
2-Furanmethanol	98.10	bready/burnt	5 ppm	169.00 to 171.00 °C	yes
Difurfuryl ether	178.19	coffee	10 ppm	101.00 °C[3]	yes
2-Acetylfuran	110.11	balsamic	80 ppm	173.00 to 175.00 °C	No
Pyrrole					
1H-Indole	117.15	Powerful floral notes	0.3 - 2.0 ppm	253.00 to 254.00 °C	yes
1-(2-Furylmethyl)-1H-pyrrole	147.18	vegetable	5 ppm	77.00 to 79.00 °C[4]	yes
1-(1H-Pyrrol-2-yl)ethanone	109.13	musty	170 ppm	220.00 °C	No
1H-Pyrrole-2-carbaldehyde	95.10	musty		216.00 to 218.00 °C	No
Phenol					
2-Methoxy phenol ; Guaiacol	124.14	phenolic	2.5 - 3.0 ppb	205.00 to 206.00 °C	yes
4-vinylGuaiacol	150.18	woody	3 - 5 ppb or 0.005 ppm	224.00 °C	yes
4-ethylGuaiacol	152.19	spicy/smoky	50 ppb or 0.050 ppm	235.00 to 236.00 °C 82.00 to 85.00 °C[5]	yes
4-ethylphenol	122.17	smoky	600 ppb or 0.6 ppm	218.00 to 219.00 °C	No
Phenol	94.11	phenolic	5.9 ppm	181.00 to 182.00 °C	No

[1]Boiling Point at 760.00 mm Hg
[2]Boiling Point at 50.00 mm Hg
[3]Boiling Point at 2.00 mm Hg
[4]Boiling Point at 1.00 mm Hg
[5]Boiling Point at 1.30 mm Hg

Key odorants selection of volatile compounds in coffee brew

The first step was to select representative odorant of chemical group. The next step was that odor threshold, the lowest concentration of a certain odor compound that is perceivable by the human sense of smell, was arranged in order from least to greatest. Odor type of volatile compounds was a third thing to consider. The final was to present 13 volatile compounds as key odorants of coffee aroma related to chemical group, odor thresholds, and odor type. 2-heptanone and pyridine are in an odorants a representative of ketone group and pyridine group respectively. The representative odorants of pyrazine group are 2-ethyl-3,6-dimethylpyrazine, 2-ethyl-5-methylpyrazine, and 2-ethyl-6-methylpyrazine. Three odorants; furfuryl acetate, 2-furanmethanol, and difurfuryl ether are representatives of furan group. 1H-Indole and 1-(2-Furylmethyl)-1H-pyrrole are representatives of pyrrole group. Three representative odorants of phenol group are guaiacol, 4-vinylguaiacol, and 4-ethylguaiacol. There are many type of odor of key volatile compounds, including cheesy, fishy, nutty, coffee, potato, fruity, bready/burnt, floral, vegetable, phenolic, woody, and spicy/ smoky. However, 2 volatile compounds of key odorants that related to coffee flavors are 2-ethyl-5-methylpyrazine and difurfuryl ether.

Effect of evaporation temperature on the coffee aroma

The percentage of the key odorants was remained and recovered in the condensate of coffee brew shown in Figures 2, Figures 3 and Figures 4. The highest percentage of recovered key odorants in the condensate of feed at the evaporation temperature of 120°C was 64.97% of 2-ethyl-6-methylpyrazine, 62.28% of 2-ethyl-3,6-dimethylpyrazine, and 58.67% of difurfuryl ether (Figures 2). The highest percentage of recovered key odorants in the condensate of feed at the evaporation temperature of 125°C was 53.69% of 2-ethyl-6-methylpyrazine, 50.28% of 2-ethyl-3,6-dimethylpyrazine, and 46.08% of 4-ethylguaiacol (Figures 3). The highest percentage of recovered key odorants in the condensate of feed at the evaporation temperature of 130°C was 59.33% of difurfuryl ether, 54.58% of 2-ethyl-6-methylpyrazine, and 54.47% of 2-ethyl-3,6-dimethylpyrazine (Figures 4). The highest percentage of remained of coffee brew were 2-furanmethanol.

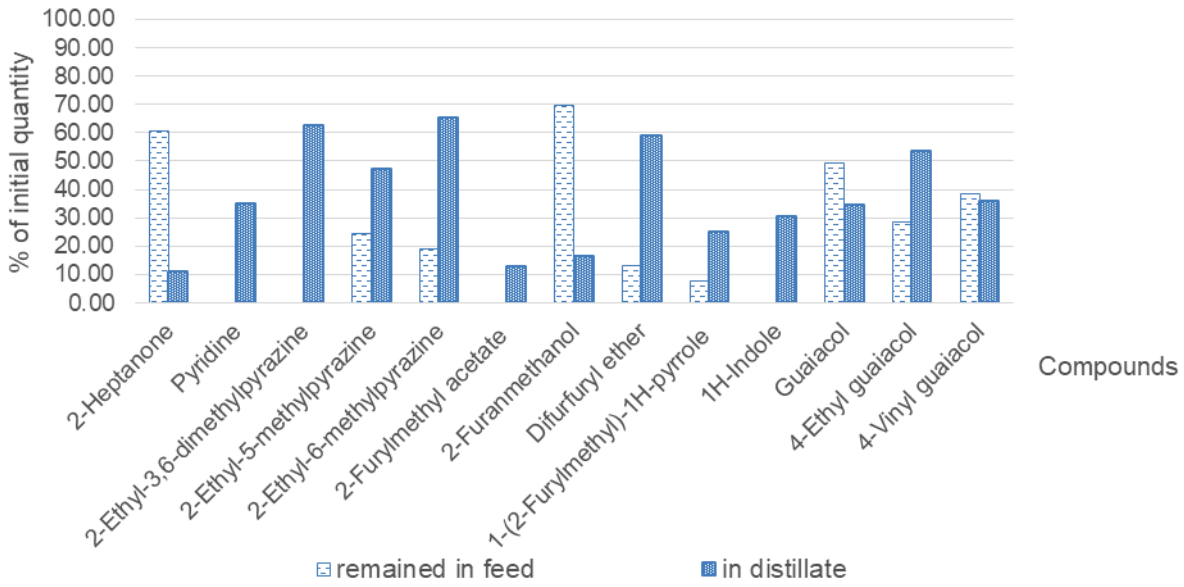

Figure 2. Percent of aroma compounds remained in the feed and recovered in the distillate when the temperature of evaporation was 120°C.

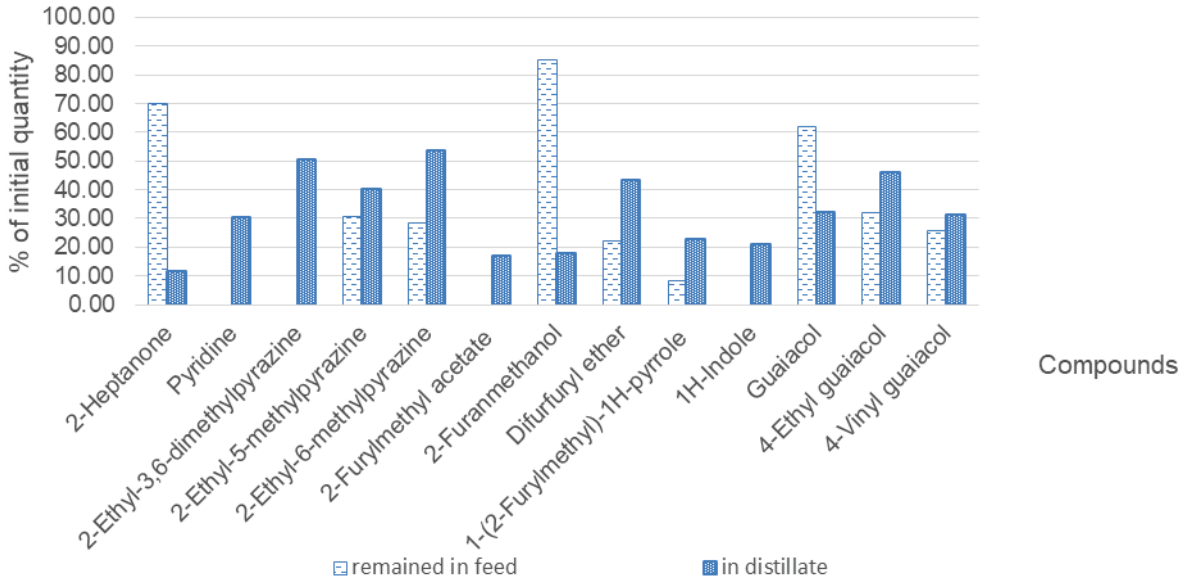

Figure 3. Percent of aroma compounds remained in the feed and recovered in the distillate when the temperature of evaporation was 125°C.

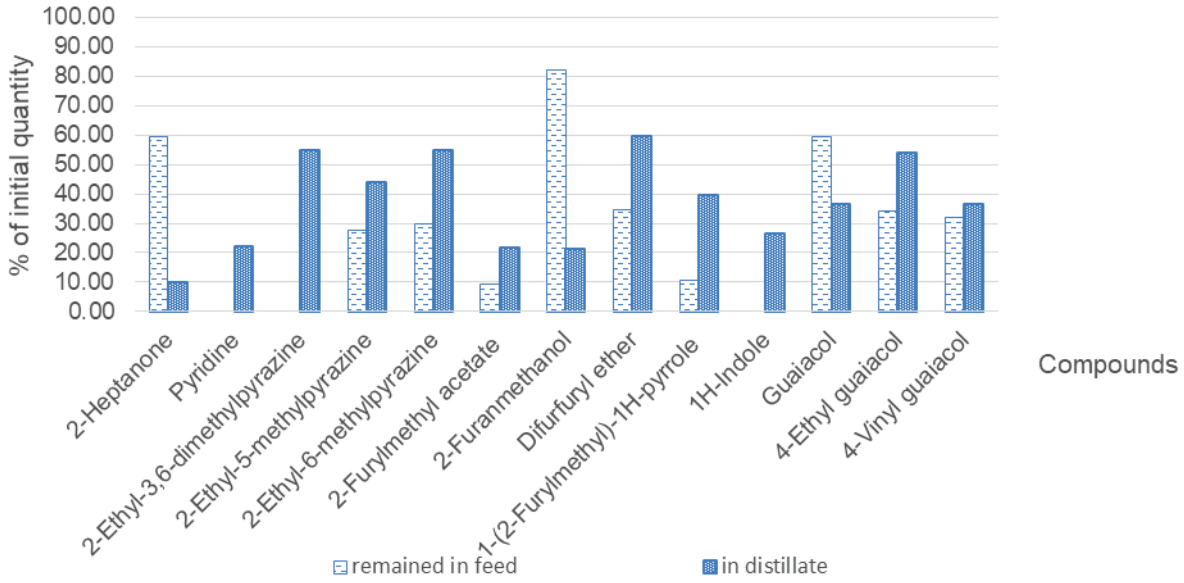

Figure 4. Percent of aroma compounds remained in the feed and recovered in the distillate when the temperature of evaporation was 130°C.

CONCLUSION

The highest percentage recovered in the condensate of coffee brew was two pyrazines; 2-ethyl-6-methylpyrazine (64.97%) and 2-ethyl-3,6-dimethylpyrazine (62.28%), and one funran, difurfuryl ether (59.33%). For the optimal temperature of evaporation was 120°C because there are obtain the most of coffee odor quantities by 2-ethyl-5-methylpyrazine and difurfuryl ether. However, when the temperature increased related to a burnt note in the coffee brew.

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EC-P002

ANTI-MICROBIAL ACTIVITY OF ENDOPHYTIC ACTINOMYCETE CRUDE EXTRACTS ISOLATED FROM THAI MEDICINAL PLANTS

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ABSTRACT:

Actinomycete bacteria, especially genus *Streptomyces*, are such a great antibiotic resource. The objective of this study is to isolate endophytic actinomycetes from two native medicinal plants such as *Cuminum cyminum* and *Andrographis paniculata* and to test antimicrobial activity using their crude extracts against 7 bacterial pathogens (*Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Candida albicans* ATCC10231, *Pseudomonas aeruginosa* ATCC278533, *Micrococcus luteus* ATCC25923, Methicillin resistance *Staphylococcus aureus* (MRSA)). After surface sterilization and isolation, we found 52 and 37 isolates of endophytic actinomycetes from *Cuminum cyminum* and *Andrographis paniculata*, respectively. All endophytic actinomycetes were grouping into 10 groups by using primary screening of their antimicrobial activities and morphological analysis. A representative in each group (CMR-50, CMR-13, CMR-30, CMR-17, CMR-42, CMR-45, CMR-62, CMR-55, APR-1 and APR-11) was tested antimicrobial activity by using agar disc diffusion. The result showed that 125µg/mL and 500µg/mL of CMR-17 crude extract was able to inhibit *C. albicans* and *B. subtilis*, respectively. Furthermore, 125, 500 and 1000 µg/mL of APR-1 crude extracts were able to inhibit *C. albicans*, *M. luteus*, MRSA and *S. aureus*. The result of both 16S rRNA analyses showed that CMR-17 and APR-1 had 99.93% and 99.65% similarity to *Streptomyces misionensis* and *Streptomyces bacillaris*, respectively. From this preliminary study, we found that the typically endophytic actinomycetes isolated from *Cuminum cyminum* and *Andrographis paniculata* were able to inhibit gram-positive bacteria and most of those endophytic actinomycetes belong to genus *Streptomyces*. We further plan to do more endophytic actinomycetes screening to find a new species that can be a promising antibiotic producer.

Keywords: Endophytic Actinomycetes, *Cuminum cyminum*, *Andrographis paniculata*, Agar disc diffusion, Antimicrobial activity

INTRODUCTION

Actinomycetes are gram positive bacteria and well-known as antibiotic producers, especially, genus *Streptomyces* (Zin *et al.*, 2007). They have been typically found in soil and rhizosphere as a decomposer (saprophytic bacteria) and the plant growth promoter including pathogenic fungi inhibitor, respectively (Crawford *et al*., 1993 and Cao *et al.*, 2004). However, some actinomycetes are able to associate and colonize in plant internal tissue called "endophytic actinomycetes". In recent years, endophytic actinomycetes with bioactive potential have revolutionized in natural product research (Ezra *et al.*, 2004, Zin *et al.*, 2007, Ryan *et al*., 2008, Li *et al*., 2008). Thailand is an agricultural country having a variety of native medicinal plants.

Here we used Thai medicinal plants, *Cuminum cyminum* and *Andrographis paniculata*, to seek a novel endophytic actinomycete species or some species to be a promising antibiotic producer. We found most of the endophytic actinomycetes belong to genus *Streptomyces* by morphological analysis. From 16S rRNA analysis, we found two actinomycete representatives were *Streptomyces misionensis* and *Streptomyces bacillaris* and they were able to inhibit *C. albicans*, *B. subtilis*, *C. albicans*, *M. luteus*, MRSA and *S. aureus*.

MATERIALS AND METHODS

Plant Samples Collection

Cuminum cyminum and *Andrographis paniculata* were collected from their field plots in Kampangpetch province, Thailand. The samples were stored in the plastic bags, which were transferred to the laboratory within the same day.

Isolation of Endophytic Actinomycetes

Endophytic actinomycetes were isolated by modified protocol of Cao *et al*. (2004), plant samples were cut into small pieces (1 x 1 cm) and separated into 3 parts (root, stem and leaf). Plant samples in each part were done separately. Surfaced-sterilized plant samples were soaked in 0.1% Tween-20 for 30 sec, washed in sterilized water 3 times, soaked in 95% EtOH and followed by 1% sodium hypochlorite for 7 min for leaf and stem and 13 min for root. The samples finally were washed in sterilized water 3 times and they were ground with 0.4 mL of sterilized water. An aliquot of 0.1 mL suspension was inoculated on starch casein agar adding 0.2 mL of Nystasin and incubated in the room temperature for 14 days. Endophytic actinomycetes were isolated on ISP medium No.2 agar (ISP2) until they were purified.

Primary Antimicrobial Activity and Morphological Screening

Isolated actinomycetes were primary screening according to their secreting metabolite and morphological criteria on ISP2 (Goodfellow and Cross 1984). The isolated actinomycete was cultured for 14 days, then 7 bacterial pathogens, *Bacillus subtilis* ATCC 6633, *Escherichia coli* ATCC25922, *Staphylococcus aureus* ATCC25923, *Candida albicans* ATCC10231, *Pseudomonas aeruginosa* ATCC278533, *Micrococcus luteus* ATCC25923, Methicillin resistance *Staphylococcus aureus* (MRSA) were inoculated as shown in Fig. 1. The antimicrobial activity was recorded in the next day. The morphological criteria including characteristics of colonies on the plate, morphology of substrate and aerial mycelium, the morphology of spores and pigment produced was characterized by using 40X long working distance objective lens and compared with the NBS-ISCC color system. The strains were grouped based on these primary results and representative ones were used for the next experiment.

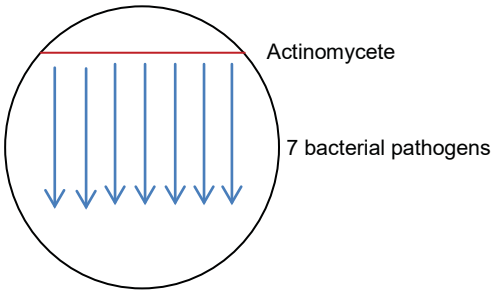

Figure 1. The primary screening technique. Actinomycete was grown for 14 days (red line), then was tested the microbial activity against with 7 bacterial pathogens for one day.

Antimicrobial Activity from Crude Extracts by Agar-disc Diffusion Method

The actinomycete was extracted followed by Taechowisan et al. (2005)'s protocol. Briefly describe, actinomycetes were cultured in 200 mL of ISP2 medium and shaken under 30 °C at 200 rpm for 14 days. Next, they were filtered by Whatman filter paper No.1 and crude extracts were performed by using Ethyl acetate (1:1) for 5 min, repeat this extraction step for 3 times. The crude extract was finally evaporated by the rotary evaporator and stored as a powder in -20°C until ready to use.

To test the antimicrobial activity by using agar-disc diffusion, the 7 bacterial pathogens were freshly inoculated on Mueller-Hinton agar (MHA) at 37 °C for overnight and then mixed with 0.85% NaCl to adjust their concentrations into McFarland standard No.0.5. Each pathogen suspension was aseptically spread onto MHA and the plates were dried under a laminar for 5 min. Next, discs loaded with extracts were placed onto pathogen plates and incubated at 37 °C for overnight. The inhibition zones were measured to determine antimicrobial activity in the next day.

Genotypic Identification

Genomic DNA (gDNA) was prepared by using HiPurA^{TM} Bacteria Genomic DNA Purification Kit (MB505), HiMedia, India. We next sent all genomic DNA samples out to U2Bio (Thailand) Co., Ltd to amplify and sequence 16S rRNA by using a forward primer (9F) 5'-GAGTTTGATCCTGGCTCA-3' and reverse primer (1541R) 5'-AAGGAGGTGATCCAGCC-3'. Finally the 16S rRNA sequences are blasted in https://www.ezbiocloud.net/ to identify the actinomycete species.

RESULTS AND DISCUSSION

Isolation of Endophytic Actinomycete

To isolate endophytic actinomycetes from *Andrographis paniculata* (AP) *and Cuminum cyminum* (CM), we did surface sterilization to eliminate exophytic actinomycete contamination and then screened endophytic actinomycetes on ISP2 agar. We found a total number of endophytic actinomycetes 52 and 37 isolates from AP and CM, respectively (Table. 1). Most isolates were found in root tissues of both plants. This finding was not surprised since actinomycetes were typically found in soil and rhizosphere (Crawford *et al*., 1993 and Cao *et al.*, 2004).

Table 1 Total number of endophytic actinomycetes isolated from *A. paniculata and C. cyminum*.

Plant sample	Plant part	No. of Isolates
Cuminum cyminum (CM)	Root (CMR)	43
	Stem (CMS)	4
	Leaf(CML)	5
	Total	52
Andrographis paniculata (AP)	Root (APR)	28
	Stem (APS)	9
	leaf (APL)	-
	Total	37

Primary Antimicrobial Activity Screening and Morphological Analysis

To discard non-antimicrobial-activity actinomycetes and group the similar active strains, we performed primary antimicrobial screening and analyzed their morphological criteria. From the experiment, we found only 42 out of 89 actinomycetes had antimicrobial activity and they were able to inhibit *B. subtilis* and *C. albicans* (Table 2). Based on their morphology criteria (data not shown), we were grouping all 42 strains into 10 groups. However, this finding was just the preliminary result, and the antimicrobial activity occurred from some metabolite secreted by actinomycete cells. We were interested in testing the antimicrobial activity from their crude extracts.

Table 2 The primary antimicrobial activity screening.

Group	Samples	*P. aeruginosa*	*B. subtilis*	*S. aureus*	*E. coli*	*M. luteus*	MRSA	*C. albicans*
1	CMR-34, 46, 50*	-	+	-	-	-	+	+
2	CMR-13*, 64, 75	-	+	-	+	+	-	-
3	CMR-2, 30*	+	+	-	-	-	-	-
4	CMR-9, 17*, 65 CML-3	-	+	+	-	-	-	+
5	CMR-42*, 54, 56	-	+	-	-	-	-	+
6	CMR-38, 39, 45*, 46, 50 CML-2	-	+	-	-	-	+	+
7	CMR-62*, 69, 70	-	-	-	-	-	-	+
8	CMR-55*, 74, 86	-	-	-	+	-	-	-
9	APR-1*, 2, 3 , 4, 5, 6, 7, 8, 9, 13, 20			+		+		+
10	APR-11*, APL-2,3							+

* = representative in each group, CMR = *Cuminum cyminum* Root, CML= *Cuminum cyminum* Leaf, APR = *Andrographis paniculata* Root, APL = *Andrographis paniculata* Leaf

Antimicrobial Activity Analysis from the Crude Extracts by Agar-disc Diffusion Method

We next set out the experiment to get the actinomycete crude extracts to test their antimicrobial activity by using agar-disc diffusion. From 10 representative strains, we found only two crude extracts from CMP-17 and APR-1 were able to inhibit some pathogens. To illustrate, 1,000 µg/mL of CMR-17 crude extract could inhibit *B. subtilis* and *C. albicans* as shown the inhibition zone at 11 and 28 mm, respectively (Table 3). While 1,000 µg/mL of APR-1 crude extract could inhibit *S. aureus*, *M. luteus*, MRSA and *C. albicans* as shown the inhibition zone at 33, 17, 19.4 and 9 mm, respectively (Table 3).

The results suggested that these endophytic actinomycetes were able to inhibit gram positive but were unable to inhibit gram negative bacteria. This finding might be explained that the crude extracts had the efficiency to inhibit a substrate for peptidoglycan synthesis and decrease transpeptidation activity since gram positive bacteria did contain thicker peptidoglycan than gram negative bacteria consists of Lipopolysaccharide in their cell wall. Therefore, the gram positive bacteria pathogens seemed to have highly sensitive to the crude extracts (Boudjella *et al*., 2006).

Table 3 Antimicrobial activity from crude extract by using agar-disc diffusion.

Group	Samples	Inhibition zone (mm)						
		P. aeruginosa	*B. subtilis*	*S. aureus*	*E. coli*	*M. luteus*	MRSA	*C. albicans*
1	CMR-50	-	-	-	-	-	-	-
2	CMR-13	-	-	-	-	-	-	-
3	CMR-30	-	-	-	-	-	-	-
4	CMR-17	-	11	-	-	-	-	28
5	CMR-42	-	-	-	-	-	-	-
6	CMR-45	-	-	-	-	-	-	-
7	CMR-62	-	-	-	-	-	-	-
8	CMR-55	-	-	-	-	-	-	-
9	APR-1	-	-	33	-	17	19.4	9
10	APR-11	-	-	-	-	-	-	-

Genotypic Analysis

We next identified what species of CMR-17 and APR-1 is. We extracted genomic DNA of CMR-17 and APR-1, sequence and blasted 16S rRNAs by using E2biocloud database. We found that 16S rRNA of CMR-17 and APR-1 was closely related to *Streptomyces misionensis* and *S. bacillaris* with 99.93% and 99.65% similarity, respectively. This finding showed that genus *Streptomyces* was commonly found in these two medicinal plants, corresponding to several researches reported that 80% antibiotic producers were from genus *Streptomyces* from endophytic actinomycetes (Rahman 2000, Lakshmanan *et al.*, 2011, Qiu *et al*., 2015).

CONCLUSION

In this study, we aim to find a novel species or some endophytic actinomycete species to be used as a good antibiotic resource. We did endophytic actinomycete isolation from two native medicinal plants in Thailand. Here we found *Streptomyces misionensis* isolated from *Andrographis paniculata* was able to inhibit *B. subtilis* and *C. albicans*, efficiently. While *S. bacillaris* isolated from *Cuminum cyminum* was able to inhibit *S. aureus*, *M. luteus*, MRSA and *C. albicans*. The finding suggested that two endophytic actinomycetes were able to mostly inhibit gram positive bacteria. However, we also would like to seek a new endophytic species with a great antimicrobial potential. Thus, we further planned to do more endophytic actinomycete screening.

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EC-P003

IN VITRO ANTIOXIDANT, α-GLUCOSIDASE, α-AMYLASE INHIBITORY ACTIVITIES AND MAIN CHEMICAL CONSTITUENTS OF DENDROBIUM EXTRACT

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ABSTRACT

This research aimed to determine antioxidant, anti-glucosidase and anti-amylase activities of ethanolic extracts from the stem of 3 dendrobium orchids including *Dendrobium hercoglossum*, *Dendrobium huoshanense*, *Dendrobium chrysotoxum*, and to analyze their main chemical compound by HPLC. The extracts were tested for their free radical scavenging activity using the DPPH, ABTS, Nitric oxide, Superoxide anion**,** and FRAP assays. The extract of the *D. hercoglossum* showed the highest scavenging activity, which had the lowest IC_{50} DPPH (0.121±0.002 mg/mL), IC_{50} ABTS (2.631±0.018 mg/mL) IC_{50} Nitric Oxide (0.607+0.023 mg/mL) and the highest FRAP value (870.181±4.378 µmol/g extract), respectively. While *D. chrysotoxum* extract showed a great ability to scavenge superoxide anion radical (IC_{50} 0.100±0.002 mg/mL), in which it was much stronger than those of vitamin C. The *in vitro* study of anti-diabetic activity by alpha-amylase and alpha- glucosidase inhibitory assays showed that *D. hercoglossum* and *D. chrysotoxum* had the most potent inhibitory activity with IC_{50} value of 11.423±0.135 and 16.827±0.242 mg/mL, respectively. The extract of *D. chrysotoxum* had the greatest inhibitory effect on α-amylase, followed by *D. hercoglossum*, and *D. huoshanense* with the IC_{50} values of 2.663±0.737 mg/mL, 3.050±0.072 mg/mL, and 7.653±0.325 mg/mL, respectively. The active chemical compositions of the orchid extracts were determined by HPLC. It was found that the main compositions of the ethaolic extracts of *D. hercoglossum* were dendrobine followed by moscatilin: 74.035±0.715 and 14.372±0.169 mg/g extract, respectively. The major compounds of *D. chrysotoxum* were dendrobine, scoparone and moscatilin as 63.055±1.164, 25.683±0.212 and 12.746±0.891 mg/g extract, respectively. While scoparone and moscatilin were the main constituents of *D. huoshanense* extract with the content of 9.334±0.242 and 2.032±0.334 mg/g extract, respectively. The results indicated that the extract of *D. hercoglossum and D. chrysotoxum* orchid had high potential to be used in the future as an active pharmaceutical ingredient for anti-diabetic therapy.

Keywords: Dendrobium orchids, Antioxidation, α-Glucosidase, α-Amylase, *D. hercoglossum*, *D. chrysotoxum*

INTRODUCTION

Dendrobium orchids have long been used as herbal medicines in Asia. Various species of Dendrobium are used as anti-inflammatory, anti-oxidant, anti-cancer, anti-mutagenic, anti-platelet, anti-diabetic, anti-allergic and immunomodulatory agent etc. (Gutiérrez, 2010)**.** This activity is due to various constituents presented in these orchids. *Dendrobium chrysotoxum, D. hercoglossum*, *D*. *huoshanense* are commonly used for species of medicinal Dendrobium in many countries. The purpose of this work was to evaluate antioxidant, anti-glucosidase and anti-amylase activities and to identify the chemical constituents of ethanolic extracts from the stem of these orchids.

MATERIALS AND METHODS

Plant material preparation

All samples of orchid stems were cleaned and cut into small pieces. Samples were dried in hot air oven at 45 °C and for 48 hours. The samples were then ground to a fine powder in a mechanical blender and stored in plastic bag at 4°C and ready for further analysis.

Extraction method

Dried powder samples (10 g) were extracted with ethanol under 1:20 solid–liquid ratio at 70 °C for 60 minutes. The extract was filtered through Whatman filter paper No. 1 before removing the solvent using rotary evaporator. The dried extracts were weighed and stored at 4 °C in storage vials for further analysis.

In vitro free radical scavenging activity

In this study, five methods of free radical scavenging activity were evaluated for 3 dendrobium orchid extracts. Vitamin C was used for reference of free radical scavenging activity.

DPPH radical scavenging activity

The scavenging effect of the extracts was assessed according to the DPPH assay described by Zhu *et al*. (2006) with some modifications. IC_{50} which is an inhibitory concentration of sample required to reduce 50% of the DPPH radical was determined**.**

ABTS radical scavenging activity

The scavenging effect of the extracts was assessed according to the ABTS assay described by Re *et al*. (1999) with some modifications. IC_{50} which is an inhibitory concentration of sample required to reduce 50% of the ABTS radical was determined.

Superoxide anion radical ($^{\bullet}O2^{-}$) scavenging activity

This assay was based on the capacity of the extract to inhibit the photochemical reduction of nitro blue tetrazolium (NBT) as described by Kumar *et al.* (2008) with slight modifications. IC_{50} which is an inhibitory concentration of sample required to reduce 50% of the superoxide anion radical was determined**.**

Nitric oxide scavenging activity

Nitric oxide radical generated from sodium nitroprusside (SNP) was measured according to the method of Pooja *et al*. (2010). IC_{50} which is an inhibitory concentration of sample required to reduce 50% of the nitric oxide radical was determined.

Ferric reducing/antioxidant power (FRAP) assay

FRAP assay was based on the reduction of Fe^{3+}–TPTZ to a blue-coloured Fe^{2+}–TPTZ. The FRAP assay was adapted from Kubola and Siriamornpun (2008). The antioxidant potential of sample was determined from a standard curve plotted using the $FeSO_4$. $7H_2O$ linear regression equation to calculate the FRAP values of the sample with expressed as µmol of Fe (II)/g extract**.**

Alpha-amylase Inhibitory Assay

Alpha-amylase inhibitory assay of extracts was carried out according to method of Rege and Chowdhary (2014) with slight modification. The result is expressed as percentage inhibition. Acarbose was used for reference of alpha- amylase inhibitory assay.

Alpha-Glucosidase Inhibitory Assay

Alpha -glucosidase activity of extracts was carried out according to method of Kazeem *et al.* (2013 ; Feng *et al*., 2011) with some modification. The result is expressed as percentage inhibition.
Acarbose was used for reference of alpha-glucosidase inhibitory assay.

Quantification of main compounds in Dendrobium orchid stems extract.

RP-HPLC with UV detection was employed for the quantification of Scoparone, Moscatilin and Dendrobine compounds using the method described by Xu *et al*. (2010) with some modification.

RESULTS AND DISCUSSION

The results showed that the extracts of *Dendrobium hercoglossum*, *D. huoshanense*, *D. chrysotoxum* exhibited high free radical scavenging effect (Table 1). The extract of the *D. hercoglossum* showed the highest scavenging activity. These results had a strong correlate with those of the bioactive compound content. It was observed that the greater the amount of bioactive compounds in the extract, the

stronger antioxidant capacity was obtained. This result supported by the works of Zhang *et al*. (2013) and Kowitdamrong *et al*. (2013) which reported that scoparone and moscatilin exhibited antioxidative effect.

Table 1. Free radical scavenging activities of the extract of Dendrobium orchid

Sample	Free Radical-Scavenging Activities IC_{50} (mg/mL)				FRAP value (μmol(Fe(II)/g extract)
	DPPH assay	ABTS assay	Nitric Oxide assay	Super oxide assay	
Dendrobium chrysotoxum	0.159±0.004^{c}	3.104±0.076^{c}	1.273±0.116^{c}	0.100±0.002^{a}	799.109±7.7208^{b}
Dendrobium hercoglossum	0.121±0.002^{b}	2.631±0.018^{b}	0.607±0.023^{b}	0.192±0.008^{b}	870.181±4.3779^{b}
Dendrobium huoshanense	0.486±0.001^{d}	5.426±0.214^{d}	3.019±0.206^{d}	0.578±0.007^{c}	309.626±2.911^{c}
Vitamin C	0.004±0.000^{a}	0.230±0.004^{a}	0.348±0.008^{a}	0.187±0.008^{b}	11029.908±116.458^{a}

Mean values followed by different superscript in a column are significantly different ($p<0.05$)

Alpha-amylase and alpha-glucosidase inhibitory assay

The result of α-amylase and α-glucosidase inhibition showed that *D. chrysotoxum* possessed the highest inhibitory activity against α-amylase (IC_{50} 2.663±0.737 mg/ml) while *D. hercoglossum* extract exhibited the highest potential to inhibit α-glucosidase with IC_{50} value of 11.423±0.135 mg/ml. (Table 2)

Table 2. α-Glucosidase and α-amylase inhibitory activities of the extracts of Dendrobium orchids

Sample	α-Amylase IC_{50} (mg/mL)	α-Glucosidase IC_{50} (mg/mL)
D. chrysotoxum	2.663±0.737^{b}	16.827±0.242^{b}
D. hercoglossum	3.0500±0.072^{c}	11.423±0.135^{a}
D. huoshanense	7.6533±0.325^{d}	41.8187±0.148^{d}
Acarbose	0.3090±0.009^{a}	33.048±0.882^{c}

Mean values followed by different superscript in a column are significantly different ($p<0.05$)

The active chemical compositions of the orchid extracts were determined by HPLC. (Table 3) It was found that the main compositions of the ethanolic extracts of *D. hercoglossum* were dendrobine followed by moscatilin: 74.035±0.715 and 14.372±0.169 mg/g extract, respectively. The major compounds of *D. chrysotoxum* were dendrobine, scoparone and moscatilin as 63.055±1.164, 25.683±0.212 and 12.746±0.891 mg/g extract, respectively. While scoparone and moscatilin were the main constituents of *D. Huoshanense* extract with the content of 9.334±0.242 and 2.032±0.334 mg/g extract, respectively.

Table 3. Main chemical composition in Dendrobium orchid extracts

Sample	Scoparone (mg/g crude extract)	Moscatilin (mg/g crude extract)	Dendrobine (mg/g crude extract)
D. chrysotoxum	25.683±0.212^{a}	12.746±0.891^{a}	63.055±1.164^{a}
D. hercoglossum	0.000±0.000^{c}	14.372±0.169^{a}	74.035±0.715^{a}
D. huoshanense	9.334±0.242^{b}	2.032±0.334^{b}	0.000±0.000^{b}

Mean values followed by different superscript in a column are significantly different ($p<0.05$)

CONCLUSION

The results indicated that the extract of *D. hercoglossum and D. chrysotoxum* orchid had high potential to be used in the future as an active pharmaceutical ingredient for anti-diabetic therapy.

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EC-P004

EFFICIENCY OF ACTINOMYCETES AS A BIOLOGICAL FACTORY TO SYNTHESIZE SILVER NANOPARTICLES

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ABSTRACT

Silver nanoparticles (AgNPs) have been widely used in biomedical fields because of their intrinsic therapeutic properties. Microorganisms are important for nanofactories that hold immense potential as ecofriendly tools for physiochemical synthesis. AgNPs production by the green chemistry approach was investigated using an isolated actinomycetes strain. Two hundred ninety three isolates of actinomycetes isolated from soil samples in 3 different locations; Chanthaburi, Rayong and Lop Buri provinces in Thailand were tested for the ability of AgNPs biosynthesis. Among 293 isolates of actinomycetes, only 144 isolates could synthesize AgNPs, which were 49 % of all tested isolates. Three isolates; HH120-12, IS 50-16 and IS 120-3 showed high tendency to synthesize AgNPs. The morphology and structure of synthesized silver nanoparticles and some critical factors such as strains of actinomycete, carbon source, inoculum size and incubation time, $AgNO_3$ concentration and the time taken for formation particles on AgNPs synthesis were carried out. The high AgNPs synthesis was achieved by inoculation 20 % of seed culture of IS 50-16 on ISP-2 medium. This IS-2 medium contained dextrose as carbon source and incubated for 48 h at room temperature in a rotary shaker at 200 rpm. Silver nanoparticles in the range of 80.2 – 146.0 nm was achieved by incubation of seed media supernatant obtained after separating the cells with 2 mM $AgNO_3$ for 24 h. Identification by 16S rRNA gene sequencing of IS 50-16 found that 99.36 % of the species most closely related to the pelvic isolate was *Streptomyces cyslabdanicus*.

Keywords: Biosynthesis, Green synthesis, Silver nanoparticles, Actinomycetes, Antimicrobial activity

INTRODUCTION

Nanotechnology is the synthesis of particles with at least one dimension in the range of 1–100 nm.(Dos Santos et al., 2014). Among metal nanoparticles, silver nanoparticle (AgNPs) has wide application in industry and medicine due to its antibacterial, antifungal, larvicidial and anti-parasitic characters. Because of their wide applications beneficial to humans (Wei *et a*l., 2015). Nanoparticles are traditionally synthesized broadly by physical and chemical procedures. The chemical methods are the most popular but the use of toxic chemicals during synthesis produces toxic by-products (Song and Kim, 2009). The physical methods require large amount of energy to maintain high pressure and temperature required for the reaction (Rajasekharreddy *et al*., 2010). Thus the chemical and physical methods have their own limitations; these are considered expensive and unsuitable for sustainable ecosystem (Kumar and Yadav, 2009). The synthesis of silver nanomaterials using biological entities is gaining momentum as; biological methods are providing, nontoxic and environmentally acceptable “green chemistry” procedures. Hence, this study was aimed to screen actinomycete strains for the selection of strains having high potential for the synthesis of AgNPs. The study extent is to optimize the biosynthetic conditions for AgNPs production.

MATERIALS AND METHODS

Collection of soil samples

Soil samples were randomly collected using a sterile 50 mL conical centrifuge tube from 3 different locations; Chanthaburi, Rayong and Lop Buri provinces in Thailand. Among 10 soil samples were kept on ice while being transported back to the laboratory. The soil samples were spread on humic acid–vitamin agar (supplemented with cyclohexamide [25 µg/mL] and nalidixic acid [10 µg/mL] as antifungal and antibacterial compounds, respectively) (Hayakawa and Nonomura, 1987) after serial dilution upon reaching laboratory. Actinomycete strains were picked from colonies present on humic acid–vitamin agar after

incubation at room temperature (30°C) for 7-14 days. The strain was then purified and maintained on yeast extract-malt extract agar (ISP 2 medium) and kept at 4 °C for further studies.

Screening of actinobacteria for AgNPs biosynthesis

Actinomycetes were isolated from soil samples and were tested for the ability of silver nanoparticles (AgNPs) biosynthesis. The isolated cultures were inoculated into Nitrate broth (5 g/L Peptone, 3 g/L Meat extract, 1 g/L KNO_3, pH 7.5) and incubated in the orbital shaker (200 rpm) at room temperature for 7 days. After the incubation period, the broth was centrifuged at 10,000 rpm for 15 min. Aqueous solution of 1 mM silver nitrate (50 ml,) was mixed with actinomycete supernatants (10 ml) and the pH was adjusted to 8.5. The mixture was incubated in a rotary shaker at 37 °C and 200 rpm in the dark for 4 days. All the reactions were analyzed by UV−visible spectroscopic (Shimadzu-UV 1700) absorption by scanning the samples between 200 and 800 nm range. In each reaction vessel colour change was observed to yellowish brown in the silver nitrate solution incubated with actinomycetes supernatant (Selvakumar *et al*., 2012).

Optimization of parameters the biosynthesis of AgNPs

To scale up the biosynthesis of AgNPs, It require to optimize the critical factors during synthesis. Some critical factors such as strains of actinomycete, carbon source, inoculum size and incubation time, $AgNO_3$ concentration and the time taken for formation particles on AgNPs synthesis were carried out. All the experiments were performed in triplicate.

RESULTS AND DISCUSSION

Among 293 isolates of actinomycetes, only 144 isolates could synthesize AgNPs, which were 49 % of all tested strains. Three isolates; HH120-12, IS 50-16 and IS 120-3 showed high tendency to synthesize AgNPs. The high AgNPs synthesis was achieved by inoculation 20 % of seed culture of IS 50-16 on ISP-2 medium. This IS-2 medium contained dextrose as carbon source and incubated for 48 h at room temperature in a rotary shaker at 200 rpm. Silver nanoparticles in the range of 80.2 – 146.0 nm were achieved by incubation of seed media supernatant obtained after separating the cells with 2 M $AgNO_3$ for 24 h as shown in Figure 1 (a-d). Identification by 16S rRNA gene sequencing of IS 50-16 was found that 99.36 % of the species was most closely related to the pelvic isolate of *Streptomyces cyslabdanicus* as shown in Figure 2 (a-c).

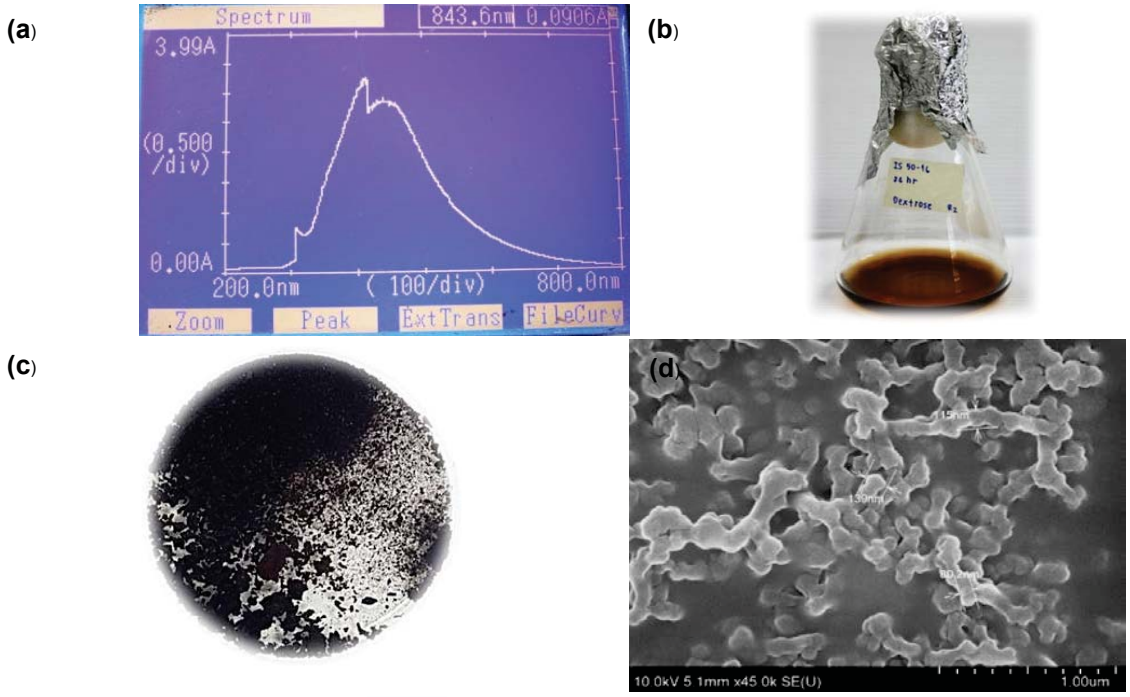

Figure 1. AgNPs biosynthesis from *Streptomyces cyslabdanicus* IS 50-16. (a) UV–visible spectra of AgNPs synthesized by *S. cyslabdanicus* IS 50-16. (b) ErlenMeyer flask with *S. cyslabdanicus* IS 50-16 cell-free supernatant and after exposure to $AgNO_3$ solution (2 mM) for 48 h of incubation. (reddish-brown color). (c) AgNPs freeze dried powder. (d) SEM analysis of produced AgNPs.

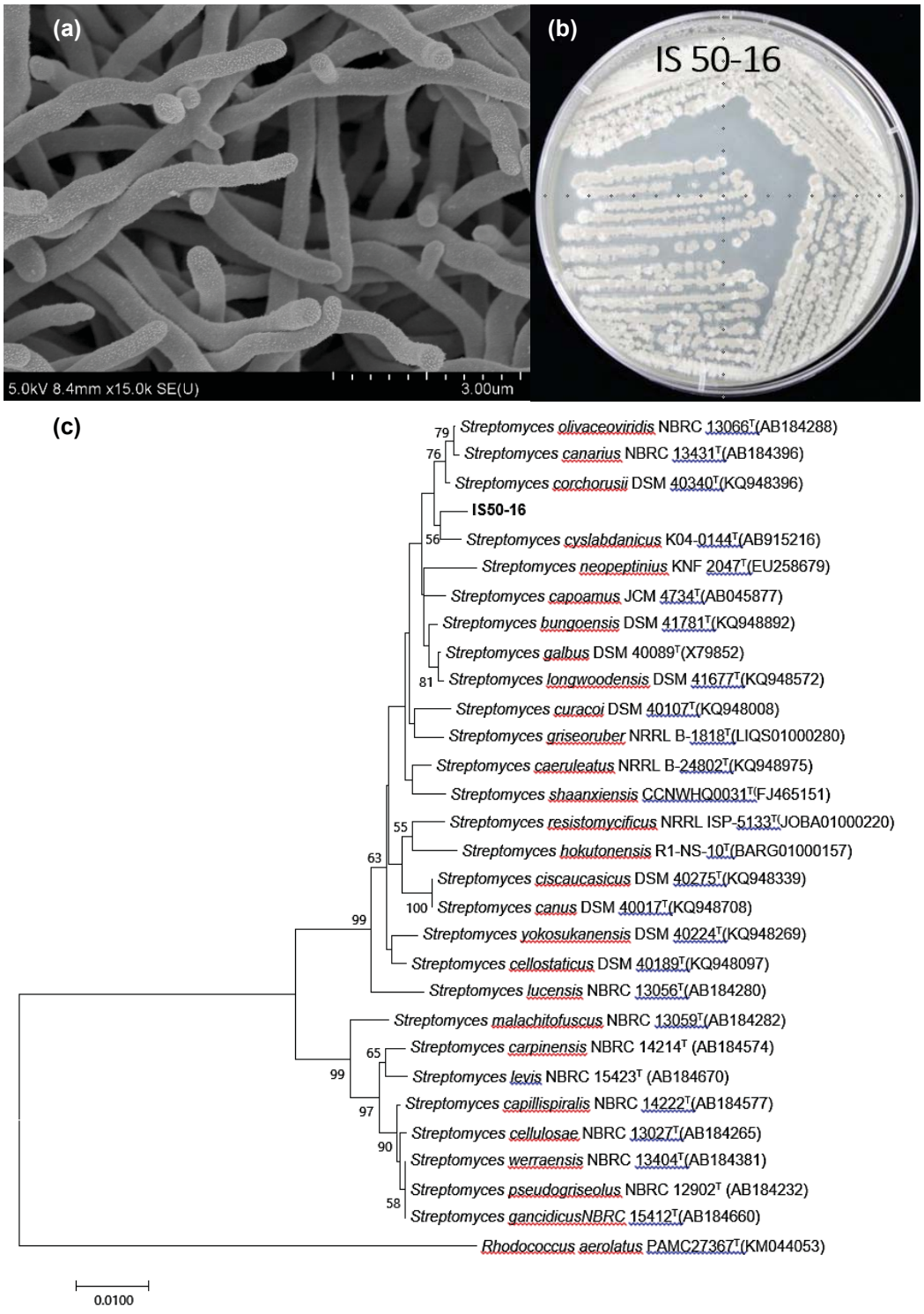

Figure 2 Morphological characterization and taxonomic features of the soil isolate identified as *Streptomyces cyslabdanicus* (a) Scanning electron image showing the presence of hyphae and spores. (b) The growth of *S. cyslabdanicus* IS 50-16 on ISP-2 medium after incubation for 7 days. (c) Phylogenic tree of *S. cyslabdanicus* IS 50-16.

CONCLUSION

A crucial need in the field of nanotechnology is the development of eco-friendly and reliable process for nanoparticles synthesis. It is thus concluded from this study that the *Streptomyces cyslabdanicus* has been used efficiently for AgNPs synthesis. Some critical factors such as strains of actinomycete, carbon source, inoculum size and incubation time, $AgNO_3$ concentration and incubation time for treatment had a great impact on this bioprocess. The present route could be easily scaled up for the industrial applications to increase the yield of the AgNps extensively, which would establish its commercial applicability. The biosynthesis of AgNps derived from microbial origin has lower toxicity and excellent biomedical application.

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EC-P005

STREPTOMYCETES SAMSUNGENSIS RB-4 : PROMISING SOURCE OF ANTIFUNGAL SUBSTANCE FOR RICE SHEATH BLIGHT FUNGUS, *RHIZOCTONIA SOLANI*

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ABSTRACT

Searching for new sources of antifungal substances from nature is becoming increasingly important especially in organic agriculture. In Thailand, *Rhizoctonia solani* is a very common soil borne pathogen with a great number of host plants such as chili, rice, corn, watermelon, soybeans and peanuts which experienced the most damage in every years. Actinomycetes could be represented as a promising alternative source for fungal control in those economic plants. A total number of 293 actinomycetes isolates was used in a screening assay for antifungal activity and *Streptomycetes samsungensis* RB-4 showed the highest antifungal activity against *R. solani* isolated from paddy field by agar well diffusion method. This strain has been studied for their taxonomic evaluation by a polyphasic taxonomic approach. The result revealed that the whole-cell hydrolysates of the isolate were rich in the LL-A_2pm (diaminopimelic acid). The 16S rDNA gene sequence analysis which is a standard method in bacterial taxonomy and identification, revealed that the strains were related to *S. samsunensis* with 99.92% similarity. In this paper, this strain can represent as a promising alternative source for antifungal control in economic plants.

Keywords: Streptomycetes, Rhizoctonia solani, Economic plants

INTRODUCTION

In Thailand, blast and sheath blight disease has been focused as the responsible for major loss in economics plants (Kanjanamaneesathian, 2009). Especially in rice planting, fully half of Thailand's cultivated land is devoted to rice. The fungi cause sheath blight disease of those crop mainly *Rhizoctonia solani*, considered a soil-borne pathogen which distribute in wide agricultural zone (Pohanka, 2006) *R. solani* can cause serious plant losses by attacking leaf sheath . The most found symptom of the disease is called "sheath blight", the infected leaf sheath shows the lesions with narrow blackish or dark brown margins. (Sivalingam, 2006). This disease shows a serious damage on economic crops and the alternative substance from natural to control this pathogen has been focused.

Actinomycetes are well-known as antibiotics producer and used by many scientific works as a source of antibiotics and secondary metabolites for the treatment of various pathogen in human animals and plants. Especially in genus *Streptomyces*, the outstanding group with the ability to produce bioactive compound or secondary metabolites, such as antifungal, antiviral, antitumoral, anti-hypertensive, immunosuppressant, and especially secondary metabolites that have antifungal properties. (Procópio et al, 2012). *Streptomyces* plays a great role in ecosystem by decomposing complex mixtures of polymers in dead organism such as plants, animals and microbial especially in fungal, with the powerful effect to degrading in various kinds of polymers, such as chitin, keratin, and lignocelluloses in fungal cell wall (Goodfellow and Williams 1983, McCarthy and Williams 1992, Stach and Bull 2005)

This research aims to investigation the potential strain RB-4 against economics plant fungal, *R. solani.* Optimization of production mediums is required to maximize the secondary metabolite yield and employ a polyphasic taxonomic approach which includes: morphological observations, 16S rDNA sequences and chemotaxonomic analysis for identification the effective strain.

MATERIALS AND METHODS

Microorganisms and culture conditions

The phytopathogenic fungus *R. solani* isolated from paddy field was obtained from Plant Protection Research and Development office. The procured fungus was grown on potato dextrose agar (PDA) plates

and incubated at 30°C for 4-7days. Stock culture of *R. solani* was maintained on PDA slants and stored at 4°C. Actinomycete strain RB-4 isolated from soil sample in Thailand was maintained on ISP2 agar medium and kept at 4°C as stock culture.

Morphological characteristics

Strain RB-4 was transferred to Yeast extract-malt extract agar (ISP 2; Shirling and Gottlieb, 1985) and oatmeal agar (ISP 3; Shirling and Gottlieb, 1985) plates for 14 days, compared aerial spore mass color and substrate mycelium color with standard color using NBS/IBCC color chart and examined morphological characteristics by SEM (JEOL JSM 5600 LV).

Chemotaxonomic analysis

The presence of isomer of diaminopimelic acid (A_2pm) in whole cell hydrolysates of strain RB-4 was determined according to the methods described by Becker *et al.* (1964) and Hasegawa *et al.* (1983). Strain RB-4 biomass were obtained from a culture grown on ISP 2 medium.

16S rDNA gene amplification and analysis

Genomic DNA from strain RB-4 was extracted using standard method (Kieser *et al.*, 1992). The 16S rDNA was amplified by PCR with the universal bacterial primer 27f (5'AGAGTTTGATCMTGGCTCAG 3') and 1525r (5'AAGGAGGTGWTCCARCC 3'). Amplification was performed using GeneAmp PCR System 9700, according to the following profile: initial denaturation at 95 °C for 3 min followed by 30 cycles of 95 °C for 1 min, 55 °C for 1 min and 72 °C for 1 min; these cycles were followed by a final extension at 72 °C for 5 min. The PCR products were purified and sequenced using universal primers. The resultant 16S rDNA gene sequences were aligned against corresponding sequences of the type strains of actinomycete species, retrieved from the EMBL / GenBank database, using the CLUSTAL_X and PHYDIT programs. An unrooted phylogenetic tree was inferred using the neighbour-joining algorithms. The tree topology was evaluated by bootstrap analyses of the neighbour-joining data based on 1000 resamplings. All analyses were carried out using TREECON software.

Medium selection for antifungal metabolites

Strain RB-4 was grown in the glucose yeast extract (GYE) medium containing glucose, 20 g/L; yeast extract, 5 g/L; $CaCl.2H_2O$, 0.2 g/L; and distilled water, 1000 mL at pH 7.0 and temperature of 30 °C. Three full loops from 7 days old of strain RB-4 was transferred to 100 ml of ISP 2 broth for use as an inoculum.

At first, 3% of inoculum of strain RB-4 was used to inoculate each 250 mL fermentation flask containing 100 mL of six media:

- Starch-Casein (SC) media containing soluble starch, 10 g/L; casein, 0.3 g/L; KNO_3, 2 g/L; NaCl 2 g/L; $MgSO_4.7H_2O$, 0.05 g/L; $CaCO_3$, 0.02 g/L; $FeSO_4.7H_2O$, 0.01 g/L; K_2HPO_4, 2 g/L; and distilled water, 1000mL.
- Yeast extract-Malt extract agar (ISP 2 formula 1) containing yeast extract, 4 g/L; malt extract, 10 g/L; dextrose, 4 g/L, and distilled water, 1000mL.
- Yeast extract-Malt extract agar (ISP 2 formula 2) containing malt extract, 3 g/L; yeast extract, 3 g/L; peptone, 5 g/L; glucose, 10 g/L, and distilled water, 1000mL.
- Basal medium 1 containing rice bran, 10 g/L; K_2HPO_4, 1 g/L; KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 5 g/L; $MgSO_4.7H_2O$, 0.1 g/L; NaCl, 1 g/L; and distilled water, 1000mL.
- Basal medium 2 containing rice bran, 2 g/L; grist, 5 g/L; K_2HPO_4, 1 g/L; KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 5 g/L; $MgSO_4.7H_2O$, 0.1 g/L; NaCl, 1 g/L; and distilled water, 1000mL.
- Basal medium 3 contains rice bran, 10 g/L; K_2HPO_4, 1 g/L; KH_2PO_4, 2 g/L; $(NH_4)_2SO_4$, 5 g/L; $MgSO_4.7H_2O$, 0.1 g/L; NaCl, 1 g/L; and distilled water, 1000mL.

All flasks were incubated at 30 °C with shaking at 150 rpm for 7 days. Cell-free supernatants were collected by centrifugation (10,000×g) and follow by filtration (Whatman® cellulose filter paper; ø 0.45 µm). The culture filtrates were tested antifungal activity against *R. solani* by the modified agar diffusion method (Cappuccino and Sherman, 2004). The Petri dishes were incubated under 30 °C for 7 days. The diameters of inhibition growth zones were measured.

RESULTS AND DISCUSSION

Morphological characteristics and chemotaxonomy analysis

Strain RB-4 was identified using a polyphasic approach. Phylogenetic tree (Figure 1) based on 16s rDNA gene sequences, consisting of 1,418 nucleotides, showed that strain RB-4 was phylogenetically closely related to *Streptomycetes samsungensis* species. The 16S rDNA gene sequences of strain RB-4 shared 99.87% similarity with *S. samsungensis* species. In addition, the *S. samsungensis* M1463^T, the type strain which reported in 2011 by Sazak et al., recovered from the rhizosphere of *Robinia pseudoacacia*.

The phylogenetic assignment of strain RB-4 was supported by the results of LL-A_2pm diaminopimelic acid in cell wall amino acid. Strain RB-4 grew well on ISP 2 and ISP 3 agars. The substrate mycelium was orange yellowish and greyish yellow on ISP 2 and ISP 3, respectively. The aerial mycelium on both media was grey and diffusible pigments are not produced in these agar. Spiral chains of unsegmented, helix-spores with rugose-surface were found in this strain (Figure 2). This morphological characteristics were similar to *Streptomyces malaysiensis*, which showing spiral chains of rugose ornamented spores after grown on ISP 3 at 28 °C for 12 days (Sazak *et al*., 2011).

Chemotaxonomic analysis of whole cell hydrolysates revealed strain RB-4 presence of the *LL*-A_2pm isomer in cell wall, assigned to streptomycete group. Based on polyphasic taxonomy, strain RB-4 was identified as *Streptomyces samsunensis* RB-4.

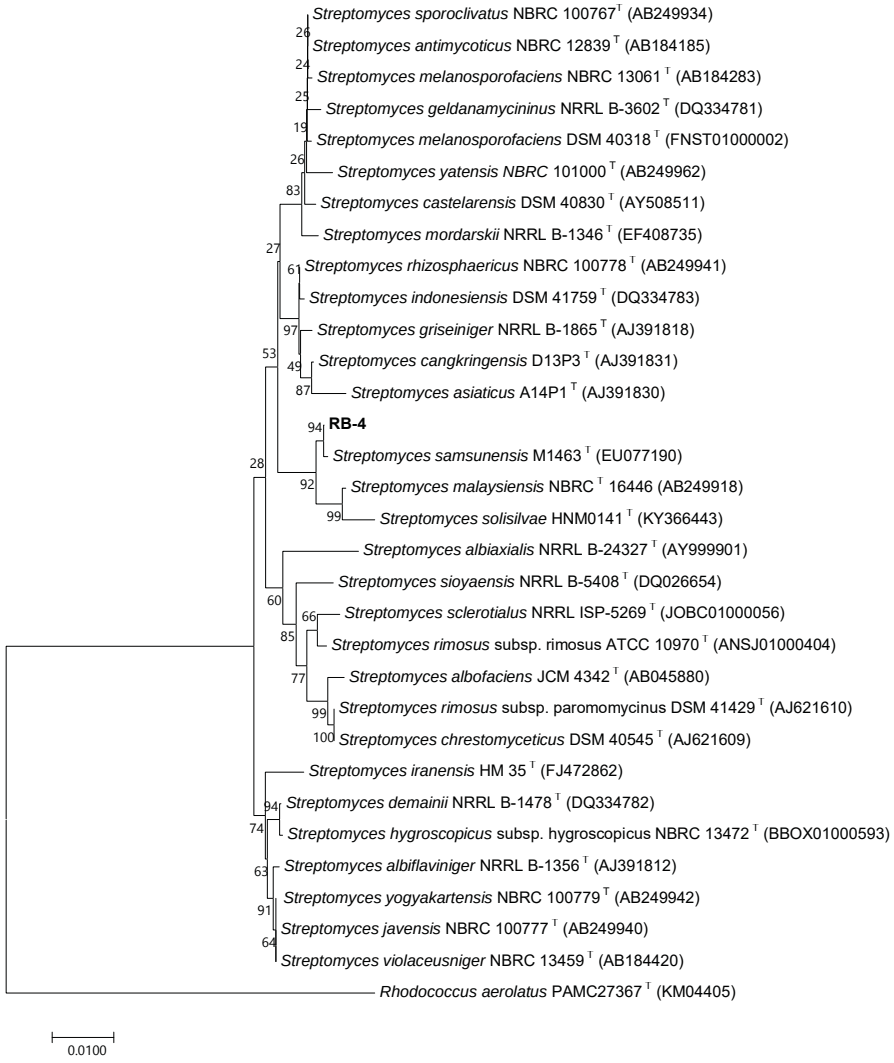

Figure 1. Neighbour-joining tree based on 16S rDNA sequences showing the relationship between isolate RB-4, and representatives of the Streptomycetes. *Rhodococcus aerolatus* PAMC27367^T (KM044053) is used as an outgroup. The numbers on the branches indicate the percentage bootstrap values of 1000 replicates (only values >50% are indicated). Bar, 0.01 substitutions per nucleotide position

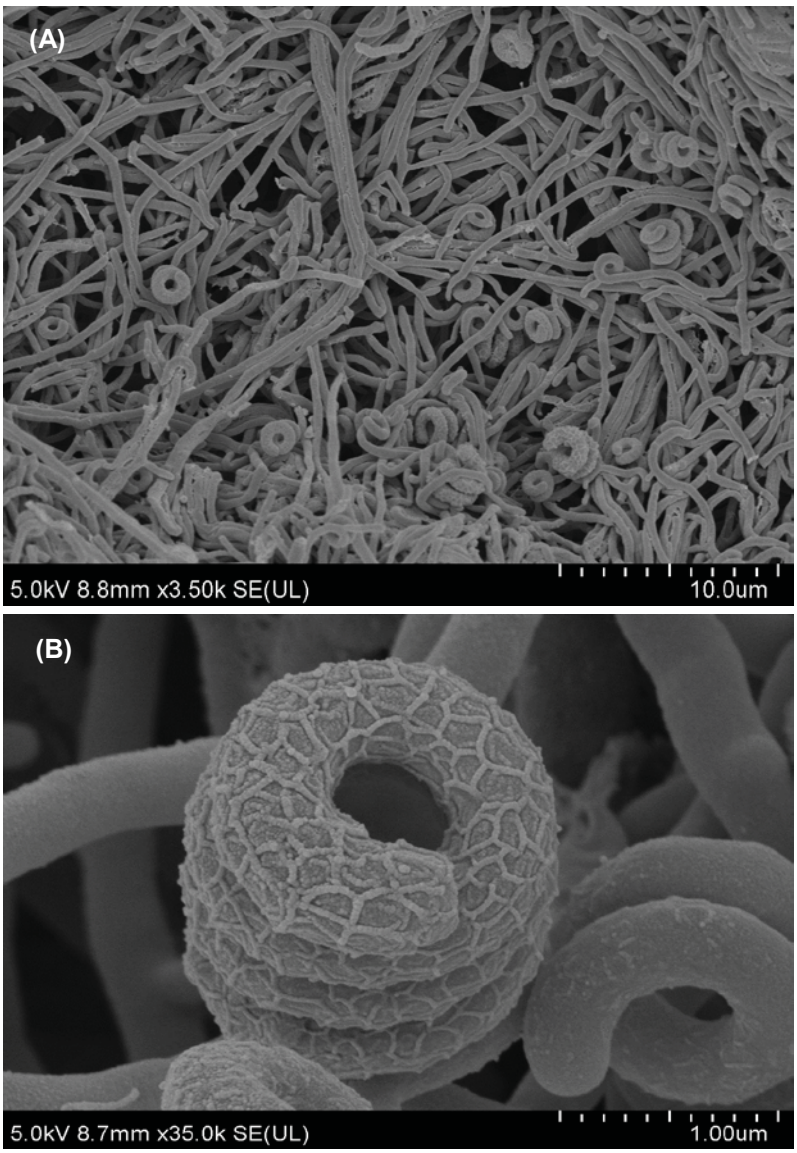

Figure 2. Scanning electron micrographs of strain RB-4 grown on yeast extract-malt extract agar (ISP 2) for 14 days at 30 °C. Magnification: X3,500 (A) and X35,000 (B).

Production of antifungal metabolites

A total of 6 media were used to tested antifungal metabolites of *R. solani*. The ability of antifungal metabolite production of *S. samsunensis* RB-4 on each medium was shown in Figure 3. It was found that the maximum of inhibition zone diameter (10.25 mm) was exhibited by *S. amsunensis* RB-4 grown in ISP 2 formula 1 as shown in Figure 3. Whereas, broth of basal medium 1 and 3 have not shown antifungal activity. This result was consistent with the research by Goudjal et al., (2014) who reported antifungal activity of the *Streptomyces* genus against phytopathogenic fungus *R. solani* damping-off. The six isolates had the greatest pathogen inhibitory capacities *in vitro* antifungal activity. The biocontrol potential on *R. solani* in both sterilized and non-sterilized soils showed that 4 actinomycete strains can reduce the disease incident by over 75%. Similarly, a potential actinomycete for the control of *R. solani* was reported by Mary et al., (2012). It was found that isolate I_9 showed good antagonistic activity against phytopathogen by production of toxic volatile and non-volatile metabolites and hydrolytic enzymes which cause hyphal lysis in *R. solani*.

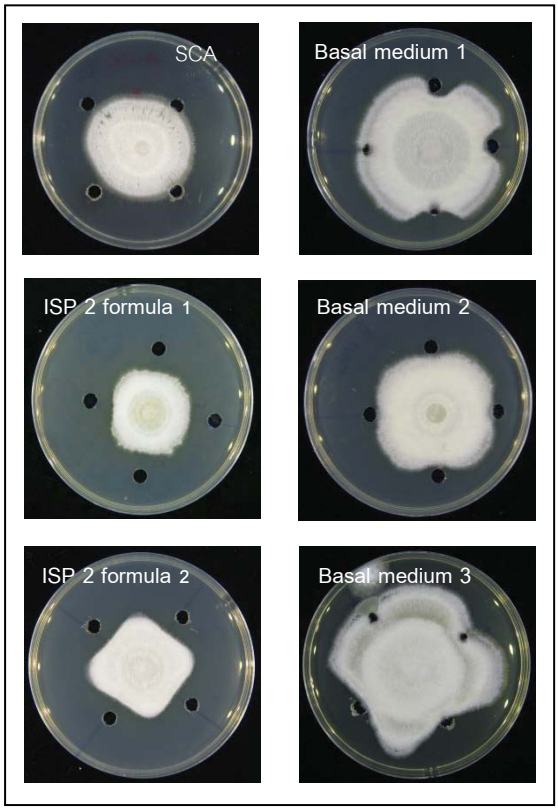

Figure 3. Antifungal metabolites efficiency of strain RB-4 grown in six media against *Rhizoctonia solani.*

CONCLUSION

The morphological of *S. samsunensis* RB-4 showed a rugose spiral chains with cylindrical spores. This strain can be related to of the *S. samsunensis* through a polyphasic approach. The *S. samsunensis* RB-4 showed a significant inhibition to *R. solani* and implies that this strain has effective antagonistic activities against *R. solani* a soil-borne fungal pathogen. The antifungal metabolites of this strain could be useful for agricultural especially in paddy cultivation in Thailand. In the next phase, we suggested that this result can formulate to produce the fungal bio control substance and apply for rice planting in paddy field.

ACKNOWLEDGMENTS

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EC-P006

EFFECT OF THAI RICE VARIETY AND CONDITION ON PRODUCTION OF KOJIC ACID BY *ASPERGILLUS ORYZAE* FOR APPLICATION OF COSMETICS

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ABSTRACT

Kojic acid is an organic acid, present as white crystal and easily dissolved in water, ethanol, methanol, acetone and ethyl acetate. It has been widely applied in cosmetics and pharmaceutical industries, due to its ability to inhibit tyrosinase activity, resulting in reduction of melanin production which caused spots in the skin. This study implicated kojic acid production from different Thai rice varieties by *Aspergillus oryzae* via a solid state fermentation. The optimal rice variety, initial moisture, spore suspension and incubation time were investigated. The suitable rice for kojic acid production was sticky rice namely Luem-Pua glutinous rice which has dark-purple pericarp and contains high nutrients and antioxidant activity. The appropriate initial moisture for kojic acid production from Luem-Pua glutinous rice was at 75% while spore suspension concentration 10^5 spores per ml promoted the highest kojic acid production. The highest kojic acid content was detected after 20 days of cultivation at 30°C. The kojic acid production was improved around 1.6 times under optimum condition. The results from this study demonstrate that Luem-Pua glutinous rice can be used as the substrate for kojic acid production by *Aspergillus oryzae*.
Keywords: Thai rice varieties, Kojic acid, Fermentation, Cosmetic, *Aspergillus oryzae*

INTRODUCTION

Kojic acid is a secondary metabolite that mostly is produced from *Aspergillus* sp. (Rodrigues *et al.*, 2011). It has been wildly applied in food, pharmaceutical, medical, chemical and especially in cosmetic industries (Devi *et al.*, 2014) due to its ability to inhibit tyrosinase activity which is a key enzyme for melanin formation (Saghaie *et al*., 2013). Kojic acid can be produced from many kinds of carbon sources such as glucose, pineapple residues, starch, and molasses (Zohri *et al.*, 2018: Nurashikin, Rusley and Husaini, 2013: Devi et al., 2014). Rice is another promising carbon source for kojic acid production, as well as red and black colored rice grains contain not only starch but also antioxidant properties and total phenolic compounds. Thus, kojic acid produced from this rice might contain both anti- tyrosinase and antioxidant activities. Usually, kojic acid is produced by a solid state process, where the carbon source, initial moisture, spore suspension concentration, incubation time and temperature are considered parameters.

This present work aims to investigate the optimization of rice variety, initial moisture, spore suspension concentration and incubation time for solid state fermentation of kojic acid production.

MATERIALS AND METHODS

Substrates and microorganism

White rice, Sung yod rice, Luem-Pua glutinous rice and Riceberry were purchased from the market. Samples were dried by oven at 60 °C for 24 h, milled and kept in a dry place before use. *Aspergillus oryzae* was used in this study for kojic acid production.

Effect of Thai rice variety on kojic acid production

White rice, Sung yod rice, Luem-Pua glutinous rice and Riceberry were used as substrates. Five grams of each substrate was placed in the flask. The moisture content was adjusted to 80% by addition of 4 ml distilled water into substrate. The medium was sterilized at 121°C for 15 min. Spore suspension of *A. oryzae* at 10^7 spores per ml was added into medium. The cultures were incubated on the incubator rotary shaker at 250 rpm at 30°C for 12 days. The extraction of kojic acid was done by introducing 50 ml of distilled water to the sampled solid cultures. The slurry suspension was mixed and centrifuged at 4,000 rpm for 20

minutes at 4°C. The supernatant containing kojic acid was filtered through a 0.45 µm filter for subsequent kojic acid assays. The most optimum substrate obtained was applied for subsequent experiments.

The optimum condition for kojic acid production

The optimum Thai rice variety from above section was used as substrate. The kojic acid fermentation process was performed following the above section. Selected parameters; initial moisture content, spore concentration and fermentation period; were varied to obtain highest kojic acid concentration. The initial moisture content on kojic acid production was varied from 60 to 85%. The final concentration of spore's suspension was carried out by adding 5 ml of 10^5, 10^6, 10^7 and 10^8 spores per ml into medium. The fermentation period was varied by collecting samples after 2, 4, 6, 8, 10, 12, 14, 18 and 20 days of fermentation.

Analysis of kojic acid

Analysis of kojic acid was modified from Bentley (1975) using a colorimetry method. One milliliter of diluted sample was mixed with 1 ml of ferric chloride ($FeCl_3$) solution. $FeCl_3$ solution was prepared by dissolving 1 g of $FeCl_3.6H_2O$ in 100 ml of 0.1 N HCl. The absorbance of the reaction mixture was measured using spectrophotometer at a wavelength at 500 nm. The kojic acid concentration was calculated by comparing to the standard curve.

RESULTS AND DISCUSSION

Effect of Thai rice variety on kojic acid production

The four different kinds of rice were used to get the optimal substrate for maximum kojic acid production viz. white rice, Sung yod rice, Luem-Pua glutinous rice and riceberry. Luem-Pua glutinous rice showed the highest kojic acid yield 0.23 g/g rice, followed by riceberry (0.17 g/g rice), white rice (0.14 g/g rice) and Sung yod rice (0.06 g/g rice), respectively, as shown in Figure 1. Thus, Luem-Pua glutinous rice was selected and used for the next experiment.

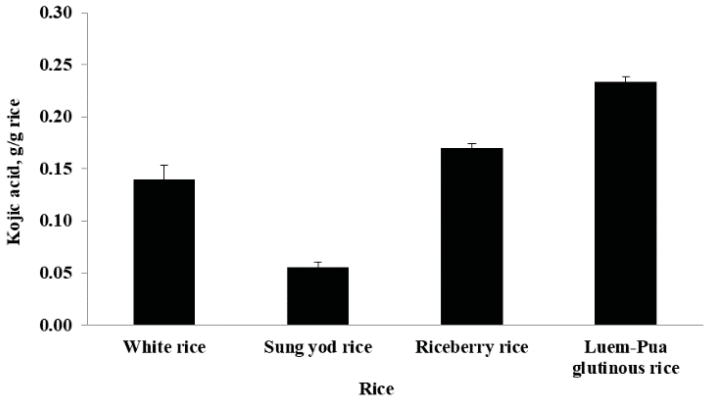

Figure 1**.** Effect of Thai rice variety on kojic acid production by *Aspergillus oryzae*

The optimum condition for kojic acid production

Effect of initial moisture content

The optimization of initial moisture content on kojic acid production was evaluated by varying initial moisture of substrate, Luem-Pua glutinous rice, from 60 to 85%. The initial moisture contents were adjusted by considering the weight of substrate with 4 ml distilled water, and the result is shown in Figure 2. Initial moisture content of 75% clearly enhanced the maximum production of kojic acid (0.32 g/g rice). Further increase in moisture content has a negative effect on kojic acid fermentation. The optimum initial moisture content attained at 75% was applied into the next experiment (Figure 2).

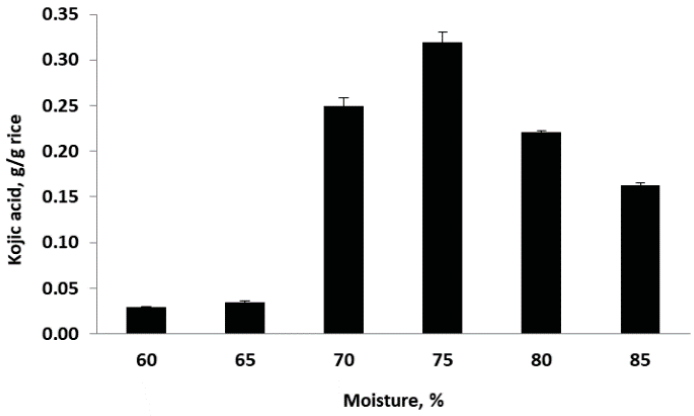

Figure 2. Effect of initial moisture content on kojic acid production by *Aspergillus oryzae*

Effect of spore suspension

Optimization of spore suspension concentration was conducted by varying the spore concentration at 10^5, 10^6, 10^7 and 10^8 spores per ml. Figure 3 shows the effect of spore suspension concentration on kojic acid production, in which the concentration at 10^5 spores per ml gave maximum kojic acid content (0.35 g/g rice). The increasing of spore suspension higher than 10^5 did not increase kojic acid production. This result was consistent with that from Kumar and Jayalakshmi (2016) who reported that the high number of viable cells caused the overgrowth of mycelium and this mycelium utilized the substrate for cell growth rather than utilized the substrate for kojic acid production. The optimum spore suspension concentration attained was applied for subsequent experiments.

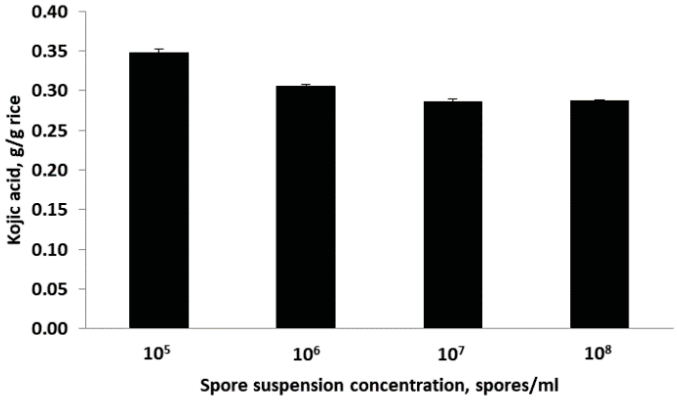

Figure 3. Effect of spore suspension on kojic acid production by *Aspergillus oryzae*

Effect of fermentation period

The effect of fermentation period on kojic acid production was conducted by collecting the samples at different time intervals from day 2 to 20. Figure 4 shows that the kojic acid was increased when the incubation time was increased and the optimum incubation time was at 20 days (0.36 g/g rice). The highest kojic acid production under optimum condition was higher than those produced from sucrose by *A. oryzae* (Chaves et al., 2012) and pineapple peel by *A. flavus* (Nurashikin *et al.,* 2013) at 0.31 g/g and 0.26 g/g, respectively.

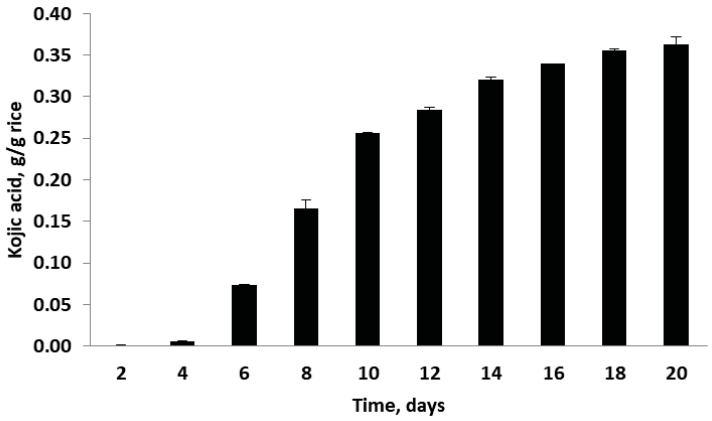

Figure 4. Effect of fermentation period on kojic acid production by *Aspergillus oryzae*

CONCLUSION

In conclusion, Luem-Pua glutinous rice showed promising potential to be utilized as an alternative substrate for kojic acid production by *A. oryzae* via solid state fermentation. The optimum condition for kojic production at 75% initial moisture content, 10^5 spores per ml, incubated at 30°C for 20 days could enhance the highest kojic acid production. The kojic acid production was improved around 1.6 times under optimum condition.

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EC-P007

DETERMINATION OF LYSINE IN THE FERMENTATION PROCESS OF PINEAPPLE STEM BY VISIBLE-NEAR INFRARED SPECTROSCOPY

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ABSTRACT

The pineapple (*Ananus comosus*) stems remain waste in the field after harvest. Their major composition is starch, which can be used as a raw material for amino acid production. In our process, the bacteria, namely *Corynebacterium glutamicum*, was used for the lysine fermentation. Three kinds of nitrogen as $(NH_4)_2SO_4$, NH_4Cl and urea were applied to the fermentation medium. The fermentation was carried out on incubator shaker at 30 °C and 180 rpm. The samples were collected at 0, 24, 48 and 72 hours. Visible-Near infrared (Vis-NIR) spectroscopy has been used in a variety of commodity, such as agricultural products, food and pharmaceuticals. Mostly, it was applied for raw material quality control. In this research, we focused on its potential for quantitative analysis of the amount of lysine obtained from the fermentation process. The thirty sample solutions were used to generate the calibration model and were validated by full cross validation model. The spectra of fermented solutions were pretreated with smoothing method and their selected region of 680-1260 nm provided optimal calibration model. The root mean square of standard error of cross validation (RMSECV) and the correlation of coefficient (R) of validation set are 0.1067 mg/mL and 0.8858, respectively. The results indicate that the implementation of Vis-NIR in the lysine fermentation process provides the capability to accurately monitor the lysine content, which can probably be applied to the continuous batch process in the further experiment.

Keyword: Lysine, Near infrared spectroscopy, Fermentation

INTRODUCTION

The pineapple (*Ananus comosus*) is one of Thai economic tropical fruit exported to various countries such as USA, Singapore and Japan. The products are also available in fresh cut, freeze dried fruit and canned pineapple. The pineapple stem is the waste of harvesting crop from the field; however, it contains a lot of valuable components which are very profitable. The extraction can produce bromelain enzyme, which is a very useful ingredient for pharmaceutical and cosmetic industries (Novaes *et al*., 2016). From our previous research, after bromelain extraction, the pulp of waste still contains a high percentage of the starch content. Therefore, it has high potential as a raw material for amino acid producing. It means that the waste material can be further changed to the valuable component.

As we have already known, amino acid is a subunit of proteins and peptides which are significantly substantial for human life. Recently, the demand of amino acid is great increasing for application in various industries such as food, cosmetics and pharmaceuticals. The high potential of utilization as substantial additives, nutrients, rejuvenators and drugs was captured the big business marketing. Several methods can be used for production, for example, the extraction, fermentation with micro-organism, enzyme catalysis and synthesis method. Among four production methods, the fermentation and enzyme catalysis are dramatic growth because of their economic and ecological advantages (Leuchtenberger *et al*., 2005). The fermentation is simple but powerful process for producing amino acids, such as the production of L-threonine, L-tryptophan and L-phenylalanine by *E.coli* (Ikeda 2003; Ding *et al*., 2016) and the production of L-glutamate and L-lysine by *C. glutamicum* (Wendisch *et al*., 2016). This research will focus on L-lysine. L-lysine is one of essential amino acids that have benefit for support the production of enzyme, antibodies and hormones. Together with promotes normal growth and development by increasing collagen formation. L-lysine is the third most frequently produced amino acid in a large industrial scale. Of that manufactured commercially, the largest amount (80%) is produced by fermentation and 20% by chemical synthesis (Coello *et al*., 2000). *Corynebacterium glutamicum* is wildly used for lysine production. Various recombinant and

genetic strain were also developed for high amino productivity (Tryfona *et al*., 2005; Koffas and Stephanopoulos, 2005).

The conventional analysis of amino acid particularly mentions in the chromatography analysis, such as reversed-phase high performance liquid chromatography (RPHPLC) or gas-chromatography (GC) combine with various detection system as mass spectrometry, ultraviolet/visible, fluorescence and electrochemical (Peris-Vicente et al., 2006; Poinsot *et al.,* 2006; Simpson *et al*., 1995; Woo, 2003). The pre- and post-column derivatizations were frequently used by reacting with ninhydrin, phenyl isothiocyanate and o-phthaldialdehyde (Cohen et al., 1987; Cronin *et al*., 1979; Friedman, 2004). The conventional method is time consuming and the sample preparation is complicate.

Near infrared (NIR) spectroscopy is the prominently technical analysis which using the wavelength in the region of 780-2500 nm. This analytical method is often used for direct measurement in the agricultural industry, especially in food and feed industries. Various food components were analyzed as moisture, protein and starch. Amino acid is one of the key factors which has potential to be analyzed by near infrared spectroscopy. Several reports indicted detection successfully in grain and seed. For example, the predicted equations of 12 kinds of amino acids in quinoa provided the correlation coefficient higher than 0.70 and the ratio of standard error of prediction to standard deviation (RPD) is in the range of 1.8-5.2 (Escuredo *et al.*, 2014). The detection of protein and amino acid in peanut which rambling from various provinces in China was reported. The correlation equation for protein prediction is very high as 0.99 and the amino acid prediction is about 0.8, respectively (Wang *et al.*, 2013). Furthermore, the detection of amino acid in rice shows a high correlation of determination in the range of 84.8-97.5%, however, the accuracy of cysteine, methionine and histidine is not adequate (Wu *et al*., 2002).

This research is focused on the development of the lysine detected method in the fermented process of pineapple stem by visible-near infrared spectroscopy. Time dependent of fermentation were collected. The calibration models were developed by considering the optimization of wavelength region and were validated by full cross validation method.

MATERIALS AND METHODS

Sample preparation

The waste pineapple stems were collected from the pineapple co-operated in Ratchaburi province, Thailand. Subsequently, they were cut and dry at 50 °C in oven for reducing the moisture content.

Investigate the optimum condition for production the lysine content from the waste of pineapple stem

Corynebacterium glutamicum bacteria was used for the lysine fermentation experiment. The optimum conditions of fermentation process to convert sugar to lysine were investigated as time and the culture medium. Seed culture was prepared in medium that contained glucose, peptone water, yeast extract, sodium chloride (NaCl), magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), manganese(II) sulfate monohydrate ($MnSO_4 \cdot H_2O$), dipotassium hydrogenphosphate (K_2HPO_4), potassium dihydrogen phosphate (KH_2PO_4) and biotin. *C. glutamicum* was incubated at 30ºC for 24 hrs in this medium before transferring into fermented medium. The fermented medium contained the pineapple stem hydrolysate, magnesium sulfate heptahydrate ($MgSO_4 \cdot 7H_2O$), manganese (II) sulfate monohydrate ($MnSO_4 \cdot H_2O$), dipotassium hydrogen phosphate (K_2HPO_4), calcium carbonate ($CaCO_3$), ammonium sulfate $(NH_4)_2SO_4$, Urea, biotin and L-leucine.

The suitable nitrogen source was determined. The ammonium sulfate was replaced with equimolar concentration of various nitrogen sources, namely ammonium sulfate ($(NH_4)_2SO_4$,), ammonium chloride (NH_4Cl), and urea while the carbon source was kept constant.

Lysine measurement

Lysine analysis was determined by using acid ninhydrin method of Chinard (1952). Add 1 mL of glacial acetic acid to 1 mL of the supernatant in the test tube and then add 1 mL of a reagent solution which contains an acid mixture of 0.4 mL of 6M orthophosphoric acid, 0.6 mL of glacial acid and 25 mg of ninhydrin per milliliter of the acid mixture. All sample solutions were capped and truly mixing with vortex mixture before heating at 100 °C in the oil bath for 1 hour. After complete the time, the reacted solution was cooled to room temperature. Then, 2 mL of glacial acetic acid was added to each test tube to present the final volume of 5

mL. The solution was measured against the blank at 515 nm with UV-Vis spectrometer (Genesys 105 UV-VIS, Thermo Scientific, USA).

Near infrared spectroscopic acquisition

Total of forty fermented samples were directly scanned using near infrared spectrometer (SpectraStar 500, Unity Scientific, USA) in the reflectance mode. The spectra were collected in the region of 680-2500 nm with 1 nm wavelength interval. The original spectra were pretreated with smoothing and Savizky-Golay derivative calculation methods as first derivative and second derivative before calibration development. The calibration model was performed using partial least squares regression method (PLSR) and was validated by full cross validation method by Unscrambler V9.8 (CAMO AS, Oslo, Norway).

RESULTS AND DISCUSSION

Near infrared absorption spectra of the fermented solutions present two dominant peaks at 1440 and 1920 nm with dominating by water absorption as shown in Figure 1. The second derivative spectra show two splitting peaks at 1410 and 1450 nm and peak at 1890 nm which normally exhibit phenomenal of water bands. The calibration models were developed with the spectra in the region of 680-1260 nm as visible and short wavelength NIR regions and 1300-2500 nm for long wavelength NIR region and the combination regions of 680-1260 nm and 1300-2500 nm. The optimum calibration model was evaluated by the basis of statistic result as the correlation coefficient (R) and the root mean square error of calibration (RMSEC) and the root mean square error of validation (RMSECV).

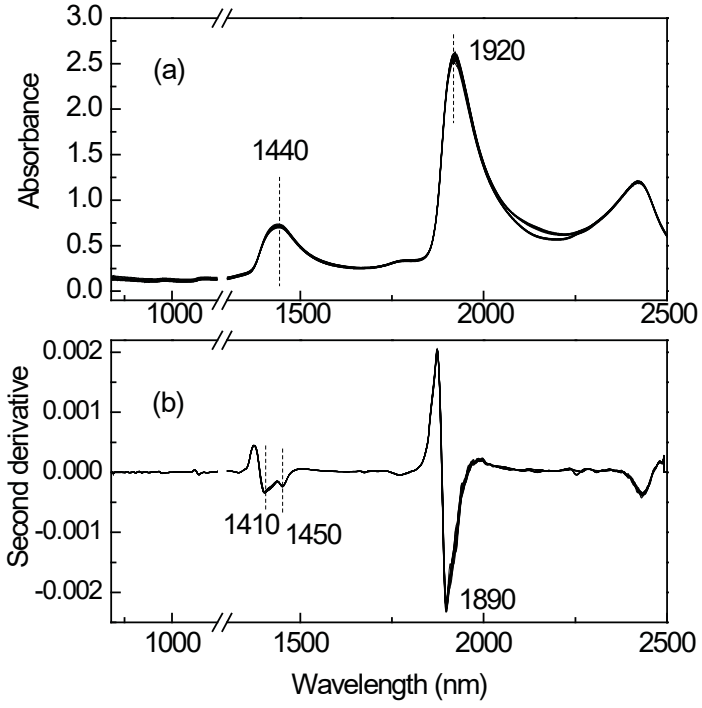

Figure 1. Absorbance (a) and second derivative (b) spectra of the fermented solutions.

Lysine contents which produced from the fermentation process comparing with three kinds of nitrogen source in the basis culture were comparing as $(NH_4)_2SO_4$, NH_4Cl and urea. The results indicate that the condition of fermented process obtained from $(NH_4)_2SO_4$ exhibit the highest lysine contents and the optimal time is 48 hours while the fermented solution with obtained from urea present the lowest value and the value reduces with increasing time, as shown in Figure 2. The statistic value of lysine content show in Table 1. The lysine amounts of 40 samples from all three-nitrogen source were used to calculate as the reference data in the model development.

Table 1. The lysine values from the standard method analysis

Type of Nitrogen source	Min (mg/mL)	Max (mg/mL)	Average (mg/mL)	Standard deviation
$(NH_4)_2SO_4$	0.478	1.667	0.893	0.393
NH_4Cl	0.515	0.719	0.619	0.072
Urea	0.151	0.151	0.251	0.082

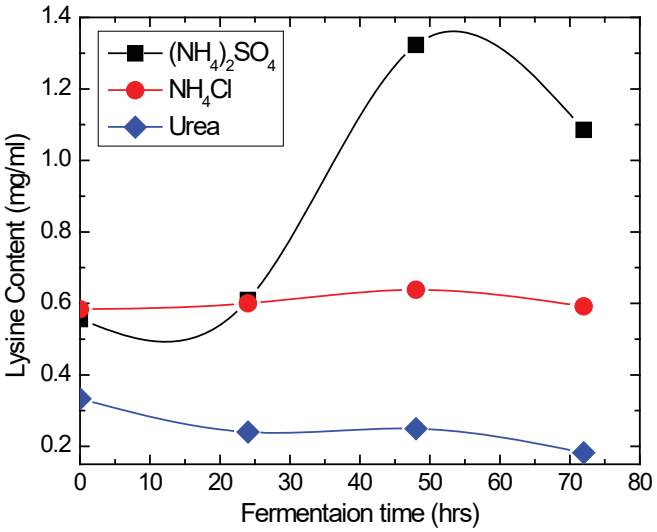

Figure 2. Amount of lysine contents were produced from the fermentation process with three kinds of nitrogen source.

The statistic results of the optimal model in each wavelength region show in Table 2. The spectra of all fermented solutions which were pre-treated with first derivative before developing the calibration model provided the lowest value of RMSECV than those which were pre-treated with smoothing, second derivative and combination method. Furthermore, the calibration obtained from the spectral data in the region of 680-1260 nm provided the lowest value of RMSECV than those of other regions. The optimal calibration model presents the highest correlation coefficient and lowest RMSECV of validation set as 0.8858and 0.1067 mg/mL. The results indicate that both regions of visible and near infrared spectroscopy are important for lysine detection.

Table 2. Calibration results of model developed for predicting the lysine content.

Region (nm)	Pre-treatment	Factor	Calibration		Validation	
			R	RMSEC (mg/mL)	R	RMSECV (mg/mL)
680-1260	First derivative	4	0.9419	0.0767	0.8858	0.1067
1300-2500	First derivative	4	0.8827	0.1073	0.8099	0.1347
680-1200 +1300-2500	First derivative	4	0.8828	0.1073	0.8100	0.1346

CONCLUSION

Vis-NIR spectroscopy in the short wavelength region, especially in the region of 680-1260 nm, shows the promising potential for analysing the amount of lysine content of fermentation process. The calibration model indicates high correlation between the spectral data and the lysine content obtained from the reference method. Therefore, the Vis-NIR spectroscopic method shows a feasibility to be used as rapid quantitative analysis for fermentation process.

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EC-P008

PERFORMANCE OF RICE STRAW PAPER COATED WITH LONGAN PEEL EXTRACT FOR ANTIMICROBIAL PAPER PACKAGING

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ABSTRACT

The aim of this study is to apply longan peel extract for coating rice straw paper in order to develop functional paper packaging for antibacterial activities. Longan peels were extracted by using maceration technique with 75% ethanol as a solvent. The minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of longan peel extract were evaluated against 4 bacterial strains of food borne pathogens by using broth microdilution method for gram-positive bacteria (*Staphylococcus aureus* TISTR 746 and *Bacillus cereus* TISTR 1395) and gram-negative bacteria (*Escherichia coli* TISTR 117 and *Salmonella typhimurium* TISTR 1469). The results indicated that the MICs and MBCs ranged in 25.6-51.2 mg/ml and 51.2-102.4 mg/ml, respectively. Rice straw papers were coated with longan peel extract at various concentrations i.e., 10, 15 and 20 % (w/v). The coated rice straw paper showed reduced brightness, while tensile strength increased when loading 20% of longan peel extract. However, the longan peel extract provided non-significant affecting on water drop penetration. In addition, rice straw paper coated with longan peel extract at 20% (w/v) inhibited growth of all tested bacteria. These findings demonstrated the potential of rice straw paper coated with longan peel extract as antibacterial paper for food packaging application.
Keywords: Rice straw paper, Longan peel extract, Antibacterial activity, Food borne pathogen, Paper packaging

INTRODUCTION

Paper packaging has been attracted to be replacing synthetic plastic based packaging, but it has some restricted in their performance such as lacking of antimicrobial properties. In addition, fruit processing industry that cause large amount of fruit peels waste has stimulated research on utilization of agricultural wastes. Longan fruit is one of a large agricultural fruit peels of Thailand contained high levels of polyphenolic compounds which can provide antibacterial activity. Therefore, the objectives of this study were focused on (i) extraction of longan peel extract and evaluate their antibacterial activity, (ii) utilization of logan peel extract for coating on rice straw paper and the effect of longan peel extract on physical, mechanical and antibacterial properties of coated paper was investigated.

MATERIALS AND METHODS

Longan peels (*Dimocarpus longan* Lour.) were extracted by using maceration technique with 75% ethanol as a solvent. The obtained crude extract was concentrated by rotary evaporator and then freeze dried into powder. The minimal inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) of longan peel extract were evaluated against 4 bacterial strains of food borne pathogens by using broth microdilution method (CLSI, 2012a). To prepare coating solution, longan peel extracts at 10, 15 and 20 % (w/v) were added into 8% (w/v) hydrophobic starch solution and then coated on rice straw paper by using rod coating technique. Brightness of papers was measured using a colorimeter (Hunter Lab Miniscan EZ 4500L, USA). Mechanical and water absorption properties of papers were analyzed according to standard methods of The Technical Association of the Pulp and Paper Industry (TAPPI) including basis weight (TAPPI T410 om-08), tensile strength (TAPPI T404 om-92), and water absorption (TAPPI T441). Moreover, antimicrobial activity of coated paper were evaluated by agar disc diffusion method (CLSI, 2012b). All

experimental values were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range test was used to determine the significant difference between treatments at $p < 0.05$ using the SPSS software.

RESULTS AND DISCUSSION

Antibacterial efficacy of the longan peel extract against 4 strains of food borne pathogenic bacteria was evaluated by using broth microdilution method and the results showed in Table 1. This results indicated that the MICs and MBCs of the longan peel extract ranged in 25.6-51.2 mg/ml and 51.2-102.4 mg/ml, respectively. This antibacterial mechanisms of longan peel extract might be attributed with its phenolic compounds.

Table 1 The minimal inhibitory and minimal bactericidal concentrations of longan peels extract against 4 strains of food borne pathogenic bacteria.

Bacteria test	MICs (mg/ml)	MBCs (mg/ml)
S. aureus	25.6	51.2
B. cereus	51.2	102.4
E. coli	51.2	102.4
S. typhimurium	25.6	51.2

Physical and mechanical properties of coated rice straw paper at various contents are shown in Table 2. Increasing of longan peel extract concentrations have no effect on basis weight of rice straw paper. Tensile index of rice straw paper increased after coated with 20% longan peel extract. While brightness of paper decreased after coating with higher concentration extracts due to the color of the extract. However, the coating of longan peel extract provided non-significant affecting on water absorption of coated papers.

Table 2 Properties of rice straw paper coated with longan peel extract at various contents.

Properties roperties	Longan peel extract (% w/v)			
	0	10	15	20
Basis weight (g/m^2)^{ns}	126.19 ± 5.93	118.20 ± 9.44	117.03 ± 20.95	118.95±13.32
Brightness (%)	32.76 ± 1.64^{a}	22.74 ± 1.39^{b}	20.76 ± 0.85^{b}	15.99 ± 0.54^{c}
Tensile index (N.m/g)	64.2 ± 4.33^{a}	32.7 ± 5.67^{c}	32.6 ± 3.15^{c}	52.9 ± 5.08^{b}
Water absorption (%)^{ns}	225.9 ± 30.52	235.55 ± 55.71	217.97 ± 35.32	216.79 ± 23.11

Different letters (a, b, c) in the same row means that the results are significantly different at $p < 0.05$ by Duncan's multiple-range test.
Ns in the same row means that the results are not significantly different at $p \geq 0.05$ by Duncan's multiple-range test.

Antibacterial activity of coated paper were investigated by agar disc diffusion method and the presence of inhibition zone was recorded as show in Table 3. It resulted that rice straw paper coated with 20% (w/v) of longan peel extract could be inhibited all of tested bacterial strains with the highest efficiency. Thus, the extract at 20% (w/v) is appropriated for using as antimicrobial agent on food packing paper.

Table 3 Antimicrobial activity of rice straw paper coated with longan peel extract at various contents which expressed as diameter of inhibition zone according to agar disc diffusion method.

Longan peel extract (% w/v)	Diameter of inhibition zone (mm)			
	S. aureus	*B. cereus* ^{ns}	*E. coli* ^{ns}	*S. typhimurium*
10	18.00±0.50^{b}	17.67±1.61	15.83±1.53	15.33±0.76^{ab}
15	18.50±1.83^{ab}	18.33±1.04	15.83±0.58	14.00±1.00^{b}
20	22.17±2.93^{a}	19.00±0.87	17.33±2.25	17.33±1.26^{a}

Different letters (a, b) in each row means that the results are significantly different at $p < 0.05$ by Duncan's multiple-range test.

CONCLUSION

Rice straw paper coated with longan peel extracts showed antibacterial activity with including enhancement in tensile strength of rice straw paper. Hence the coated paper can be used in food packaging for inhibit the growth of food pathogenic bacteria.

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EC-P009

PHYSICAL AND MECHANICAL PROPERTIES OF PINEAPPLE FIBER PAPER COATED WITH POLYHYDROXYBUTYRATE

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ABSTRACT

Polyhydroxybutyrate (PHB) is one of common biodegradable plastics, synthesized and utilized by various kinds of microorganisms as the carbon and energy sources under the nutrient limitation. The aim of this work is to apply PHB for coating pineapple fiber paper in order to improve their physical and mechanical properties of pineapple fiber paper for food packaging. Pineapple fiber papers were coated with PHB using the dip-coating method with chloroform used as a solvent. The effects of PHB contents at 5, 7.5 and 10 % (w/v) on properties of coated pineapple fiber paper were investigated. Physical appearance and color properties of pineapple fiber paper were improved by PHB coating. The morphology of the papers coated with PHB were illustrated by scanning electron microscopy (SEM) and showed that the 5% (w/v) PHB coated paper was less smooth, thinner and contained less bubbles than that coated with 10% (w/v) PHB. As the increasing of PHB contents, increasing in percentage of weight and folding index of coated paper were significantly increased while percentage of elongation, bursting index and tearing index tended to decrease. However, tensile index of the coated paper with varied PHB contents showed non-significant difference. In particular, water drop penetration on surface of coated paper was significantly improved when increasing of PHB content. Pineapple fiber paper coated with 7.5% (w/v) of PHB exhibited the good physical and mechanical properties and also has excellent water protection. These studies demonstrated that using PHB coating could be improved physical and mechanical properties of pineapple fiber paper and applied as potential paper for food packaging.
Keywords: Polyhydroxybutyrate (PHB), Pineapple fiber paper, Coating, Food packaging

INTRODUCTION

Polyhydroxybutyrate (PHB) is one of the most extensive semicrystalline bio-polyester and has mechanical properties closely to low-density polyethylene. PHB can be coated on paper *via* dip-coating method to provide water and oil resistance, and still be biodegradability for paper better than wax coating. In this study, pineapple fiber extracted from pineapple leaf which is agricultural wastes in field after fruit harvesting is used to produce handmade paper for packaging. Thus, this work aims to study the effect of PHB concentration on physical and mechanical properties of pineapple fiber paper.

MATERIALS AND METHODS

Pineapple fiber papers were coated with commercial PHB (Good Fellow, UK and Mw. = 550k g/mol) using the dip-coating technique which followed by the method of Cyras *et al.* (2007) and Obeso et al. (2013) with some modifications. PHB solutions were prepared at varied concentrations of 0, 5, 7.5 and 10 % (w/v) using chloroform as a solvent. Paper samples were immersed in PHB solutions at 60 °C for 15 min. Finally the paper samples were placed in a glass surface and allowed to dry at room temperature.

The morphology of coated papers were observed using a field emission scanning electron microscope (FE-SEM) (Hitachi SU8020) compared to uncoated paper. Whiteness index of papers were measured using a colorimeter (Hunter Lab Miniscan EZ 4500L, USA). Mechanical and water drop penetration properties of papers were analyzed according to standard methods of The Technical Association

of the Pulp and Paper Industry (TAPPI) including folding endurance (TAPPI T511 om-94), tensile index and elongation (TAPPI T404 om-92), tearing index (TAPPI T414 om-98), bursting index (TAPPI T403 om-97) and water drop penetration time (TAPPI 831 om-93). All experimental values were subjected to one-way analysis of variance (ANOVA) and Duncan's multiple range test was used to determine the significant difference between treatments at $p < 0.05$ using the SPSS software.

RESULTS AND DISCUSSION

The morphology of pineapple fiber paper coated with PHB at different concentrations was observed by scanning electron microscopy (SEM) as shown in Fig. 1. SEM images showed that the PHB coated paper have high surface smoothness than non-coated paper (A1). The 5% (w/v) PHB coated paper (B1) was less smooth, thinner and contained less bubbles than that coated with 10% (w/v) PHB (C1). Moreover, fractured surface images presented the thin layer of PHB coating on the surface area of paper contrasted to the entanglements of fibers in uncoated paper appearance.

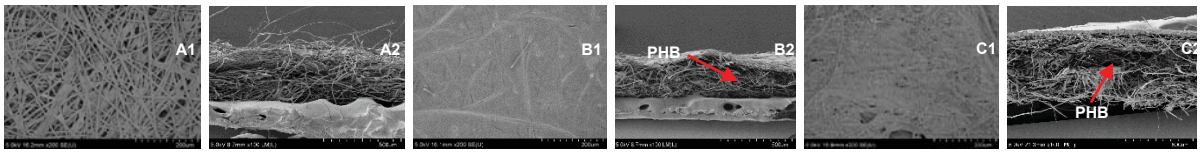

Figure 1. SEM images of surfaces (at 200x magnification) and fractured surfaces (at 100x magnification) of uncoated paper (A1-2) and paper coated with PHB at 5% (B1-2) and 10 %(w/v) (C1-2) concentration.

Physical and mechanical properties of PHB coated paper at various concentrations are showed in Table 1. Increasing of PHB contents affected to increase basis weight, percentage of increasing in weight, whiteness index and water drop penetration time of coated papers were increased. For mechanical properties of the papers, folding index of coated paper were–significantly increased while percentage of elongation, tearing index and bursting index tended to decrease. However, tensile index of the coated paper with varied PHB contents showed non-significant difference. This might be attributed to amount of PHB embedded between pineapple fibers resulting in different mechanical properties.

Table 1 Properties of pineapple fiber paper coated with polyhydroxybutarate (PHB).

Properties	Polyhydroxybutarate concentrations, % (w/v)			
	0	5	7.5	10
Basis weight (g)	99.64 ± 12.73^{b}	114.35 ± 16.56^{b}	150.09 ± 31.88^{a}	171.30 ± 30.02^{a}
Increasing weight (%)	0.00 ± 0.00^{c}	60.81 ± 16.04^{b}	64.69 ± 16.17^{b}	98.78 ± 12.13^{a}
Whiteness index	27.39 ± 0.60^{b}	41.98 ± 13.40^{a}	44.06 ± 2.38^{a}	45.13 ± 1.59^{a}
Folding endurance	1021 ± 965.02^{b}	1837 ± 718.59^{a}	1901 ± 879.13^{a}	1988 ± 712.29^{a}
Tensile index (N.m/g)	35.18 ± 12.56^{a}	28.13 ± 5.61^{a}	29.38 ± 5.97^{a}	28.39 ± 4.91^{a}
Elongation (%)	2.58 ± 0.46^{a}	1.86 ± 0.49^{bc}	2.06 ± 0.88^{ab}	1.46 ± 0.25^{c}
Tearing index (mN.m^2/g)	37.14 ± 7.84^{a}	21.85 ± 5.26^{b}	19.30 ± 4.07^{bc}	15.31 ± 4.44^{c}
Bursting index (kPa.m^2/g)	3.15 ± 0.28^{a}	2.80 ± 0.36^{a}	2.12 ± 0.46^{b}	2.26 ± 0.60^{b}
Water drop penetration time (min)	0	1.98 ± 0.36^{c}	34.71 ± 2.37^{b}	51.23 ± 5.23^{a}

Different letters (a, b, c) in the same row mean that the results are significantly different at $p < 0.05$ by Duncan's multiple-range test.

CONCLUSION

This study revealed that surface roughness and brightness of pineapple fiber paper can be improved by coating with PHB. The water surface hydrophobicity and folding endurance of pineapple fiber paper were highly improved at 7.5% (w/v) of PHB and its mechanical properties were still comparable with the uncoated paper. This pineapple fiber paper could be further developed for a promising paper packaging for food products.

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EC-P010

DEVELOPMENT OF GELLING AGENT FROM CASSAVA STARCH MODIFIED BY SIMULTANEOUS CARBOXYMETHYLATION AND CROSSLINKING FOR APPLICATION IN HERBAL GEL PRODUCTS

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ABSTRACT

Simultaneous modification of cassava starch by carboxymethylation and crosslinking process was developed in this work. The effect of degree of carboxymethyl substitution (DS) (low and high DS; DS = 0.4 and DS = 0.7, respectively) as well as crosslinking agent types (dichoroacetic acid and sodium trimetaphosphate) and their concentrations (0.025-0.150 mole/ mole AGU) on physico-chemical and gelling properties of crosslinked carboxymethyl cassava starch was investigated to develop gelling agent with comparative viscosity and stability to Carbopol® Ultrez 10, commonly used in cosmeceutical products. The results indicated that crosslinked carboxymethyl cassava starch with suitable gelling properties (HDS-DCA050) can be prepared by carboxymethylation reaction to obtain high DS (0.7) and crosslinking reaction with 0.05 mole dicholoacetic acid/mole AGU under the same condition (temperature and time of 40^{o}C and 3 h) . HDS-DCA050 gel at 1.5% w/w in water and 50% ethanol possessed comparable viscosity, clarity and pH with Carbopol® Ultrez 10 gel at 0.5% w/w. Its stability under various storage conditions in 50% ethanol solution was relatively higher than in water. HDS-DCA050, therefore, has high potential for further application as gelling agent in herbal gel products.
Key words: Cassava starch, Carboxymethyl starch, Crosslinking, Gelling agent

INTRODUCTION

Gelling agent, an essential ingredient in cosmeceutical products, is hydrocolloids capable of high water absorption and solubility providing semisolid gel with elasticity. Good gelling agents can swell in solvent, do not separate and possess proper rheological properties when incorporated into formulation (Kittipongpatana et al., 2009). Commercial gelling agent commonly used in cosmeceutical applications are synthetic polymers (e.g. Carbopol®) and natural polymers mainly polysaccharides and their derivatives including cellulose derivatives (hydroxypropyl methylcellulose (HPMC), methylcellulose (MC) and carboxymethyl cellulose (CMC)) which are usually imported and highly cost. With more consumer concerns on safety and environment, new gelling agents from biopolymers including carrageenan, xanthan gum, chitosan as well as starch with high potential have been developed.

Carboxymethyl starch with high viscosity is widely used as thickening agent in food and printing as well as pharmaceutical and cosmetic industries. Although carboxymethyl starch is cold water soluble providing highly viscous and stable paste, many gel formulations containing 30-50% alcohol, especially those with herbal extracts, required gelling agents with high alcohol tolerance. Crosslinking can be applied to further improve viscosity and stability of carboxymethyl starch gel. This work, therefore, aimed to simultaneously modify cassava starch by carboxymethylation and crosslinking to efficiently improve its gelling properties.

MATERIALS AND METHODS

Cassava starch was modified by carboxymethylation and crosslinking reactions according to modified method of Sangseethong et al. (2018) and Kittipongpatana and Kittipongpatana (2015), respectively. Cassava starch (150 g) was dispersed in 90% isopropanol and stirred at 40 ^{o}C. Sodium hydroxide solution (1 mole/mole AGU) was added and stirred. Sodium monochloroactate (0.5 and 1.0

mole/mole AGU) followed by crosslinking agent, sodium trimetaphosphate (STMP) or dichloroacetic acid (DCA), at varied concentrations (0.025-0.150 mole/mole AGU) were added and stirred for 3 h. The reaction was stopped by adjusting pH to neutral by hydrochloric acid. The modified starch was filtered and washed with 85% ethanol and dried at 40°C. The degree of substitution (DS) was determined by titration method as described (Sangseethong *et al*., 2018)

Viscosity of starch pastes and gels was analyzed by Physica MCR 300 Rheometer (Physica Messtechnik GmbH, Ostfildern, Germany) using parallel plate fixture at 25 °C and shear rate of 1-100 s^{-1}. Starch gel clarity before and after storage at various conditions was determined by measuring light transmittance (%T) at 650 nm using a spectrophotometer. The stability of starch gels in water and 50% ethanol was evaluated by analyzing their viscosity, paste clarity and pH before and after storage at various conditions.

RESULTS AND DISCUSSION

Crosslinked carboxymethyl starch with low (LDS; DS =0.4) and high (HDS; DS = 0.7) degree of carboxymethyl substitutions were obtained. DCA crosslinked carboxymethyl starches were soluble in cold water providing clear viscous paste and their viscosity increased with increasing DCA concentrations. Carboxymethyl starches crosslinked with STMP, on the other hand, were not soluble as their swelling was restricted by crosslinks. This result indicated that DCA was more effective as crosslinking agent than STMP. The gels from LDS crosslinked with 0.125 mole DCA/mole AGU (LDS-DCA125) and HDS crosslinked with 0.05 mole DCA/mole AGU (HDS-DCA050) at 1% (w/w) exhibited the highest viscosity when compared with those from carboxymethyl starches with similar DS. LDS-DCA125 and HDS-DCA050 gels at 3% and 1.5% (w/w) provided comparable viscosity with Carbopol® Ultrez 10 gel at 0.5% (w/w) as shown in Fig. 1 and Fig. 2, respectively. HDS-DCA050 was, therefore, further evaluated for its gelling properties and stability due to its lower concentration required and less amount of crosslinking applied.

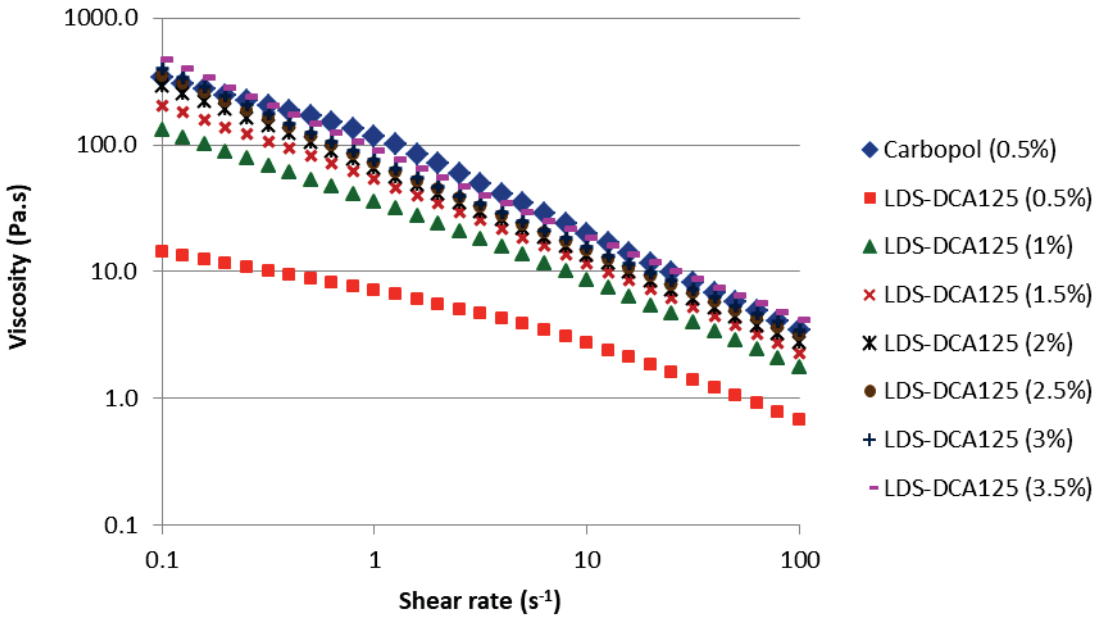

Figure 1. Viscosity of aqueous gels from low DS carboxymethyl cassava starch crosslinked with 0.125 mole DCA/mole AGU (LDS-DCA125) at 0.5-3.5% (w/w) compared to Carbopol® Ultrez 10 gel at 0.5% (w/w).

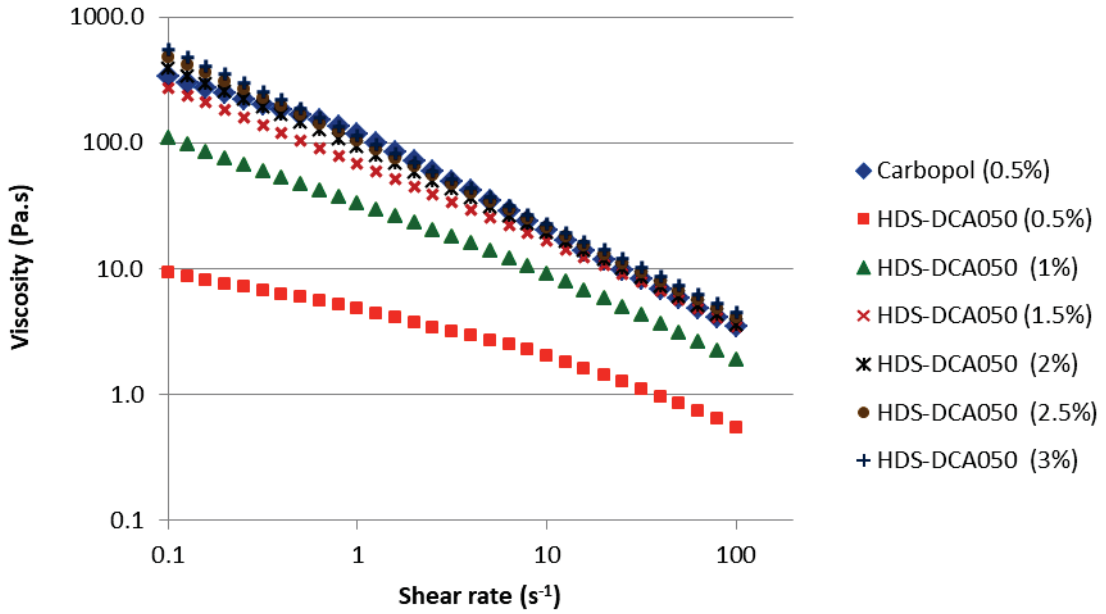

Figure 2. Viscosity of aqueous gels from high DS carboxymethyl cassava starch crosslinked with 0.05 mole DCA/mole AGU (HDS-DCA050) at 0.5-3% (w/w) compared to Carbopol® Ultrez 10 gel at 0.5% (w/w).

HDS-DCA050 gel at 1.5% (w/w) in water and 50% ethanol possessed comparative viscosity and pH with slightly less clarity than Carbopol® Ultrez 10 gel at 0.5% (w/w) (Fig. 3 and Table 1). After subjecting to 6 heating cooling cycles and storage at room temperature for 1 month, the viscosity of HDS-DCA050 gel in water significantly decreased. The stability of HDS-DCA050 gel in 50% ethanol was higher as determined by less decrease in viscosity.

Solvent/ Gelling agent	HDS-DCA050 (1.5%)	Carbopol® Ultrez 10 (0.5%)
water		
50% ethanol		

Figure 3. Appearance of gels from high DS carboxymethyl cassava starch crosslinked with 0.05 mole DSC/mole AGU (HDS-DCA050) (1.5% w/w) in water and 50% ethanol compared to Carbopol® Ultrez 10 gel (0.5% w/w).

Table 1 Viscosity, clarity and pH of HDS-DCA050 gels (1.5% w/w) in water and 50% ethanol after preparation and various storage conditions compared to Carbopol® Ultrez 10 gel (0.5% w/w).

Gelling agent	Solvent	Conditions	Viscosity (Pa.s)	Clarity (%T)	pH
HDS-DCA050 (1.5%)	water	Freshly prepared	3.44 ± 0.17^{a}	92.18 ± 0.11^{c}	6.27 ± 0.02^{c}
		6 heating cooling cycles	2.00 ± 0.06^{c}	95.47 ± 0.11^{a}	6.31 ± 0.01^{b}
		4 °C, 1 month	3.44 ± 0.04^{a}	91.71 ± 0.38^{d}	6.30 ± 0.00^{b}
		Room temperature, 1 month	2.44 ± 0.06^{b}	94.31 ± 0.05^{b}	6.33 ± 0.01^{a}
	50% ethanol	Freshly prepared	2.67 ± 0.01^{a}	95.23 ± 0.18^{d}	6.77 ± 0.01^{d}
		6 heating cooling cycles	2.14 ± 0.01^{b}	97.25 ± 0.08^{a}	6.81 ± 0.01^{c}
		4 °C, 1 month	2.78 ± 0.05^{a}	94.50 ± 0.09^{c}	6.84 ± 0.01^{b}
		Room temperature, 1 month	2.18 ± 0.06^{b}	96.49 ± 0.47^{b}	6.86 ± 0.00^{a}
Carbopol® Ultrez 10 (0.5%)	water	Freshly prepared	3.64 ± 0.08^{a}	98.33 ± 0.01^{a}	6.44 ± 0.03^{ns}
		6 heating cooling cycles	3.32 ± 0.10^{b}	98.53 ± 0.03^{a}	6.26 ± 0.08^{ns}
		4 °C, 1 month	3.90 ± 0.08^{a}	97.66 ± 0.31^{b}	6.33 ± 0.15^{ns}
		Room temperature, 1 month	3.32 ± 0.11^{b}	98.44 ± 0.19^{a}	6.25 ± 0.14^{ns}
	50% ethanol	Freshly prepared	2.30 ± 0.03^{ns}	99.70 ± 0.10^{ab}	7.08 ± 0.02^{b}
		6 heating cooling cycles	2.30 ± 0.09^{ns}	99.84 ± 0.05^{a}	7.10 ± 0.01^{ab}
		4 °C, 1 month	2.28 ± 0.02^{ns}	99.16 ± 0.05^{c}	7.12 ± 0.01^{a}
		Room temperature, 1 month	2.23 ± 0.09^{ns}	99.58 ± 0.13^{b}	7.10 ± 0.03^{ac}

Values of each gelling agent and condition in the same column with different superscripts are significantly different ($p\leq0.05$) by Duncan's multiple-range test.
ns = not significant ($p>0.05$).

CONCLUSION

Cassava starch with increased viscosity and improved stability can be prepared by carboxymethylation and crosslinking with dichloroacetic acid under the same condition. High DS carboxymethyl starch crosslinked with 0.05 mole DCA/mole AGU (HDS-DCA050) gel at 1.5% (w/w) in water and 50% ethanol showed comparable viscosity and stability with commercial gelling agent and has high potential for further use as gelling agent in herbal products.

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