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October, 10th 2018

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"Innovative Bio-Production in Indonesia (ibioI):Integrated Bio-Refinery Strategy to Promote Biomass Utilization using Super-Microbes for Fuel and Chemicals Production"

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PUI Biorefinery Terpadu

Research Center for Biotechnology-LIPI, Science and Technology Research Partnership for Sustainable Development (SATREPS), The Japan International Cooperation Agency (JICA), Kobe University, The Japan Science and Technology Agency (JST), Innovative Bioproduction Indonesia, Ministry of Research, Technology and Higher Educationof the Republic of Indonesia (RISTEK DIKTI), and Center Excellence of Integrated Biorefinery (PUI Biorefineri Terpadu)

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Foreword

Innovative Bio-production Indonesia (iBioI) are pleased and proud to inform you the five International Symposium on Innovative Bio-Production (ISIBio2018), which held on October10th 2018 at IPB International Convention Center Bogor, Indonesia already succeeded. We received high appreciation from 220 attendants which werecome from science, education and industries communities.

As usually former ISIBio symposium, ISIBio2018 provide an excellent orientation of experience and premier forum related to technological advances and research result in integrated bio-refinery fields. We received 32 abstracts, 15 fullpapers, and20poster presentations. According to the accurate selecting and editing by our editorial team, in this 5th International Proceeding ISIBio 2018, we presented11 abstracts from invited speaker and 15 fullpapers from participants.

This International symposium will be held continuously and iBioI with full of honour will dedicate this proceeding as a contribution to biorefinery field in Indonesia specially and to support the international cooperation with others renowned scientists, professors and research professionals, young researchers, industrial delegates and student communities across the globe under a single roof, where they can discuss research, achievements and advancements in Bio-refinery and Bio-process engineering for the bright future. The 5th ISIBio2018 also designated final period of SATREPS Biorefinery Project which has good achivement in research and cooperation.

Finally, the opportunities of this continuously symposium will present us to be able to continue fulfilling our great intentions in biorefinery field for better future in science and technology.

Cibinong, November 2018

Dr. Ade Andriani
Chief Editor

Foreword from the Project Leader of Indonesian Side JST-JICA SATREPS Biorefinery - Innovative Bio-Production Indonesia (iBioI)

On Behalf of Innovative Bio-Production Indonesia (iBioI) members and the International Symposium on Integrated Biorefinery (ISIBio2018) committee, we are very pleased to hold the 5th ISIBio Symposium on the theme: “Innovative Bio-Production in Indonesia (Ibiol) :Integrated Bio-Refinery Strategy to Promote Biomass Utilization using Super-Microbes for Fuel and Chemicals Production”. This event is supported by JST-JICA SATREPS-Biorefinery Project with high contribution of Innovative Bio-Production Kobe (iBioK), Innovative Bio-Production Indonesia (iBioI), Indonesian Institute of Sciences (LIPI), and Kobe University (Japan). This year, we were also supported by Ministry of Research, Technology and Higher Educationof the Republic of Indonesia (RISTEKDIKTI) and Center of Excellent of Integrated Biorefinery (PUI Biorefinery Terpadu).

In order to achieve of the establishment of biorefinery concept in Indonesia, the objective of this symposium are to improve and to develope globalization of bio-refinery project for facing the world challenges renewable energy from scientists, research professionals, young researchers, industrial delegates and educational communities. Collaboration of related researchers from different expertise such as microbiology, biology molecular, protein engineering, biochemistry, agriculture, biochemical engineering, fermentation, machine engineering and so on will be created an integrated core-research on bio-process engineering and bio-refinery in Indonesia.Organizing committee is very grateful and honored to have 32 abstracts and 15 fullpapers from invited speaker and participants. Those are delivered from Research Center for Biotechnology-LIPI, Research Center for Biology-LIPI, Research Center for Chemistry-LIPI, Research Center for Biomaterial-LIPI, Research Fisheries of Freshwater and Extension Fisheries,Research & Development Centre for Social, Economy, Policy and Climate Change;University of Indonesia, Gajah Mada University, Bogor Agricultural University, PelitaHarapanUniversity, Research Center for Marine and Fisheries Product Processing and Biotechnology, PT. Smart tbk,Central for Pulp and Paper, Central Batik Indonesia, Ministry of Industry,PT Rajawali Nusantara Indonesia etc.

I would like to give my high appreciation to all institutions that supporting us and every kind person who have contributed and to all organizing committee of the 5th ISIbio 2018. Finally, I would like to wish you all the best toward the success of ISIBio 2018 as a continuously symposium in the coming years.

Cibinong, November 2018

Prof. Dr. Bambang Prasetya
Project Leader of Indonesian Side JST-JICA SATREPS Biorefinery

Invited Speakers

The Importance of Development of Biorefinery Technology to address the Sustainable Development Goals (SDGs)

Bambang Prasetya

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Abstract

The suitable and breakthrough-way of technology which very significant for facing to the nowadays global challenges has been studied and partial implemented in the last decade. Belong to this issues, some ecofriendly technology has been become intensively discussed in various field of research and development activities. In JST-JICA SATREPS Biorefinery Project there are many achievements have been published in several international publications. The activities range varies from development of suitable pretreatment process to development process by using potential microbe, and continuing development of products derived from developed process. By a seri of research activities from exploration and selection microbe, continued by development of engineered microbe and cloning based on yeast arming way have already some resulted super microbe which able to convert of biomass and it component become very prospective intermediate products. The mechanism of process and product partially are subjected to patent pending. A project downstream project also has been studied and assessed. A survey on mapping potential standardization to related of resulted research results will also important issues. Moreover, in this paper also discussed about the importance of research result of this project to achieve some important global challenges especially related to Sustainable Development Goals (SDGs).
.

Keywords: global challenges, biorefinery, engineered microbe, product and process, dowstream-commercialisation, patent and standardisation, SDGs

Overview and Perspectiveof Our Collaboration in Indonesia for Future

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Abstract

This work was supported in part by a Science and Technology Research Partnership for Sustainable Development (SATREPS) from collaborating project between JST and JICA, National biological research center (NBRC) of National institute of technology and evaluation (NITE) for providing strains, and a Special Coordination Fund for Promoting Science and Technology, Creation of Innovative Centers for Advanced Interdisciplinary Research Areas (Innovative Bioproduction Kobe) from the Ministry of Education, Culture, Sports and Technology (MEXT) Japan.

We believe they are many broad candidates available in nature, which can move beyond difficulties to out boundary so that the good platform for fermentation of lignocellulosic biomass could be built without too much genetic modification. However, it is not easy to achieve the candidates due to the unique adaptation possessed by many yeasts upon the environmental stress.

In this study, we have developed the simultaneous method for screening the candidate yeasts, which capable to grow and ferment lignocellulosic lysate into ethanol and fine chemicals such as lactate. By using culture collection in National Bio-resource Collection (NBRC) Japan, and Indonesian National Culture Collection (InaCC), the screening of the yeast strain, having a potential of growing and fermentation in the medium containing fermentation inhibitors, was conducted. Regarding the possibility of bio-resources in Asian for bio-refinery, we would like to discuss also in this presentation.

.

Keywords: Metallic iron, lignocellulose, hydrothermal liquefaction, catalytic cracking, upgrading, light olefins

Microbial Genetic Resources: from Collection to Industrialization

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Abstract

The collection of microbial genetic resources in Indonesia was first documented in 1800s by Dutch researcher in Bogor Botanical Garden. In 1934, the collection of microbial genetic resources in Bogor Botanical Garden were stops and was not continued. The activity was starting again in 1960s by Indonesian researchers. After the declaration of Convention of Biological Diversity (CBD) in 1992, the consideration of the richness of Indonesia as mega biodiversity country has been increased. The attempt for collection of the microbial genetic resources has been also increased. Collaboration researches with other countries had been developed on the scheme of CBD to collect, conserve, and preserve microbial genetic resources. Many microbial genetic resources were collected and Indonesian Culture Collection (InaCC) was developed in 2007. Although, attempts to improve the microbial genetic resources used in agriculture, food, health, and energy industries have had some success, but still no impact on industries. With our experiences in three SATREPS (Science and Technology Research Partnership for Sustainable Development) Projects in series, correlation between three those projects will be described from the collection of microbial genetic resources until the uses of the microbial products for industry. In the very near future, it is believed that with collaboration and connection, sustainable use of the microbial collection will give the great impact to human welfare.

Keywords: microbial genetic resources, SATREPS, collection, sustainable uses

The Establishment of Pretreatment Protocol for Lignocellulose Biomass from Oil Palm and Sugarcane Industry

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Abstract

Oil palm empty fruit bunch (OPEFB) and sugarcane bagasse (SB) are among potential feedstocks abundantly available in Indonesia for producing bioethanol. The conversion of the this biomass, which mainly contains cellulose, hemicellulose and lignin, into ethanolrequiresthreeprocessing steps, namely pretreatment, hydrolysis(saccharification) of cellulose and/or hemicelluloses to simple sugars, andfermentation of the sugars into ethanol.Since pretreatment of biomass is an important step in the conversion, an appropriate pretreatment protocol should be established. Pretreatment using acids is one of pretreatment methods that can effectively modify the chemical structures of lignocellulose. In this case, acids serve as catalysts to hydrolyze carbohydrates, especially hemicellulose, so that they loosen the lignin-hemicellulose barrier, which can facilitate the penetration of cellulase enzyme to cellulose matrices in the biomass. The degradation of biomass needs high temperature. Therefore, the pretreatment usually conducted at high temperature, either using conventional heating or other heating methods, for example heating in pressurized thermostirrer reactors and microwave heating. In this project we used organic acids, such as maleic acid and oxalic acid for pretreatment of OPEFB and SB in a conventional thermostirrer reactor and microwave digester. We also compared the results of those using sulfuric acid. The use of some surfactants combine with organic acid in the pretreatment was also evaluated.

Raw materials used in this project are OPEFB and SB, which were obtained from Sukabumi and PG Rajawali, Subang Indonesia, respectively. The materials were dried and ground into particle sizes 40-60 mesh, then they were kept in sealed plastic bags and stored in a container.Chemical compositions of the OPEFB and SB were determined.The pretreatment methods that were conducted and developed in this project at the laboratory scale were 1) Pretreatment of SB and OPEFB using hot water and sulphuric acid in an autoclave; 2) Pretreatment of SB and OPEFB using organic acids in thermostirrer reactors; 3) Pretreatment of sugarcane bagasse and OPEFB using combined of maleic acid and surfactant in thermostirrer reactor; 4) Pretreatment of OPEFB using organic acids in microwave digester.Following the pretreatment, the soluble fraction was separated from the insoluble fraction by filtration. The effectiveness of the pretreatment was evaluated by determining the sugars obtained in the soluble fraction and in the hydrolyzate after enzymatic saccharification of the pretreated biomass. Besides that, the amount of inhibitors in the soluble fraction was also determined.

Biomass pretreated with acids, eithersulphuric, maleic or oxalic acid, produced more sugars than those pretreated by liquid hot water. Sugars obtained from enzymatic saccharification of OPEFB were higher than those obtained from SB. The higher cellulose content in the OPEFB indicates the potential to produce more reducing sugars than SB, and this has been proved contributes to the higher sugars yield from OPEFB. The use of organic acids, especially maleic acid is potential for substitute the use of sulphuric acid in pretreatment, because the former produced comparable amount of sugars with less inhibitors than the latter. Pretreatment using acids mainly removed hemicellulose, and also removed small part of lignin.

The addition of surfactant (Tween 20) in maleic acid pretreatment could increase both sugar release in the soluble fraction and in the hydrolyzates after enzymatic saccharification of the pretreated insoluble fraction.

The addition of the surfactant during enzymatic hydrolysis also could increase sugar release from enzymatic saccharification. However, it is more advantageous if the surfactant is added in the pretreatment. This can reduce the use of enzyme in the saccharification.

Both heating methods, using thermostirrer reactors and microwave digester, combine with organic acids resulted in comparable sugars yields from the soluble fraction and from enzymatic hydrolysis of the pretreated samples. Even though microwave-assisted acid pretreatment took much shorter duration of heating than did pretreatment using thermostirrer reactors, the use of microwave for pretreatment of biomass in large scale still needs more elucidation and elaboration. It was probablynot as easy and practical as the use of pressurized or thermostirrer reactors. Therefore, at this moment, pretreatment using conventional heating in a pressurized reactor is still the method of choice.

The chemical composition, morphology and chemical structures of biomass were changed during the pretreatment. Experiment on the OPEFB pretreatment by microwave-assisted oxalic acid with various heating temperature shows that heating temperature affected OPEFB chemical components, structures, and morphology, confirmed by the results of chemical, SEM and FTIR analyses.

Keywords: biomass conversion, conventional heating, microwave heating, organic acids, sugar yield, surfactant

Development of Halomonas Cell Factory Producing γ-Aminobutyric Acid from Salty Waste Biomass

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Abstract

The moderate halophile, *Halomonas elongata*, can assimilate a variety of sugars and amino acids derived from biomass as a carbon and nitrogen sources. Thus, *H. elongata* could be a practical cell factory for bioproduction of fine chemicals including amino acid derivatives such as ectoine. In this study, we aim to develop a novel *Halomonas* cell factory, which can produce valuable fine chemicals other than ectoine. Using the salt-sensitive deletion mutant, *H. elongata* KA1 strain, lacking the ectoine biosynthesis operon (*ectABC*) in the genome, we successfully isolated one salt-sensitive suppressor mutant, which can over-produce glutamic acids (Glu) instead of ectoine in the cells. The Glu-over-producing mutant *H. elongata* strain designated as GOP was chosen as a host strain for further development of the γ-aminobutyric acid (GABA) producing *Halomonas* cell factory. The codon-optimized *HeGadBmut* gene encoding Glu decarboxylase with activity in broad pH range was designed and introduced into *H. elongata* GOP strain to generate a GABA producing recombinant *H. elongata* GABA strain. The *H. elongata* GABA strain could biosynthesize GABA as one of major compatible solutes instead of ectoine from simple carbon and nitrogen sources under high-salinity conditions. Thus, we proposed that *H. elongata* GABA strain could be used as a promising cell factory producing GABA from salty waste biomass.

Keywords: γ-aminobutyric acid (GABA), Glutamic acid, Halomonas elongata, salty waste biomass.

Production and evaluation of lignocellulose-degrading enzymes for saccharification of sugarcane bagasse

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Abstract

Indonesia is one of the most species-rich countries in the world, this biodiversity should also be reflected by microbial species diversity. Therefore especially Indonesian soils and marine should be a source for unknown industrial actinomycetes strains, for enzymes production. Therefore, screening of actinomycetes from Indonesia with is rich in rare actinomycetes is very important for establishment novel strains with industrial applications. As tropical country Indonesia rice in biomass resources which is abundant cellulose and hemicellulose carbohydrates from various plantation industrial, agriculture and forestry such as OP EPB and sugarcane baggase. The aimed of this research was to screened and optimization of sugarcane bagasse degradation with identified enzymes from actynomycetes, constructed of protein production system by actinomycetes, and study saccharification of sugarcane baggase for sugar production. About 500 and 50 isolates of soil and marine actinomycetes was screened. About 500 and 50 isolates of soil and marine actinomycetes was screened. At least two potential candidates for cellulase and xylanase enzyme production were chosen. The combination of this isolates was abble to degradation of pretreated sugarcane baggase biomass and produced fermentable sugar. In order to increased production of enzymatic activity, the genes from selected isolates was cloned and heterologous production using S. lividans as host cell, this method was significantly increased enzymes activity.

.

Keywords: actinomycetes, sugarcane baggase, cellulase, xylanase, heterologous production, saccharification and fermentable sugar

Utilization of Oleaginous Yeast *Lipomyces starkeyi* as A Microbial Platform for Production of Biochemical Building Blocks

Ario Betha Juanssilfero

Research Center for Biotechnology, Indonesian Institute of Sciences, LIPI

Abstract

Oleaginous microorganisms are able to convert carbon sources into storage lipid as intracellular lipid droplets yielding in more than 20% lipid per dry biomass. These lipids are also known as single cell oils (SCOs). Yeasts are recognized as suitable candidates for biotechnological experiments as they have many advantages over other microbial sources. Considering the depletion of crude oil, the controversial use of plant oils for biodiesel production and the overfishing of the oceans, SCO produced from oleaginous yeasts are considered as the suitable oil substitutes for crude, plant and fish oil. Among oleaginous yeasts, *Lipomyces starkeyi* has considerable potential as a viable SCO producer due to its ability to produce high amounts of oils from hemicellulose-derived sugars including xylose. In addition to sugars, unfortunately, inhibitory chemical compounds (ICCs) also are generated as byproducts during biomass pretreatment, and these interfere with microbial growth during the fermentation process. The potent ICCs released during pretreatment are furfural, 5-hydroxymethylfurfurral, syringaldehyde, and vanillin coupled with some cell-membrane permeative acids such as acetic, formic, and levulinic. The deleterious effect of ICC on cell is considered the most immediate technical barrier for lipid production from lignocellulose. Therefore, oleaginous yeasts using lignocellulose derived from sugar feedstock should not only be capable of accumulating high levels of intracellular lipid but should also be tolerant to various ICCs. *L. starkeyi* is a well-known strain and promising candidate to produce SCO. This strain has the capability to accumulate over 70% of its cell biomass as lipid under defined culture conditions, and can produce lipid on various carbon sources as well as other wastes. Several studies also have shown that *L. starkeyi* could produce a considerable amount of SCO in hemicellulose hydrolysate under the presence of inhibitory chemical compounds. As concerns of this finding, *L. starkeyi* can be considered a promising oleaginous yeast platform for industrial scale production of lipid.

Keywords: Lipomyces starkeyi, Single cell oils, lipid production, fatty acid methyl esters

Development of Platform Indonesia Yeast Strain Capable of Direct Fermentation of Raw Biomass to Ethanol and Lactic Acid

Ahmad Thontowi[1], Lutfi Nia Kholida[1], Urip Perwitasari[1], Filemon Jalu N Putra[1], Ario Betha Juanssilfero[1], Apridah Cameliawati Djohan[1], Atit Kanti[1], Senlie Oktaviani[1], Hans Wijaya[1], Awan Purnawan[1], Yopi[1], Bambang Prasetya[2], Prihardi Kahar[3], Chiaki Ogino[2], Akihiko Kondo[3,4]

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Abstract

Sustainable and economically feasible ways to produce ethanol and or fine chemicals are becoming increasingly relevant due to the limited supply of fossil fuels and the environmental consequences associated with their consumption. Microbial production of fuel compounds has gained a lot of attention and focus has mostly been on developing bio-processes involving non-food plant biomass feedstocks. Consolidated bioprocessing (CBP), which combines enzyme production, saccharification, and fermentation in a single step, has gained increased recognition as a potential bioethanol and lactic acid production system from biomasses. The key to CBP is the engineering of a microorganism that can efficiently hydrolyze lignocellulosic polysaccharides to fermentable sugars and efficiently ferment this mixed-sugar hydrolysate to produce ethanol and lactic. The promising strategy for effective breeding and advanced fermentation by applied developed microbes with the exploration of the potential indigenous yeast from Indonesia. We screened 800 yeast strains from Indonesia Culture Collection (InaCC) and Biotechnology Culture Collection (BTCC) as a platform yeast. Then this study provides insight observation based on the biochemical, gene expression, the metabolomic analysis of the natural robust yeast during the fermentation in the medium containing inhibitory chemical complexes (ICC) at different concentrations, and also modified of wild-type strain is available as a platform for high yield production of ethanol and lactic acid.

Keywords: biomass, cell factory, fermentation, bioetahnol, lactic acid

Sustainable Resource and Energy Supply for Yeast Cell Factories

Kiyotaka Hara

Abstract

Our aim of study is addition of value for food residues, such as residual non-food parts and byproducts from food production processes. Our approach is producing valuable products from food residues by microbial fermentation. Our main target product is fine chemical (e.g., supplement, functional food, food additives, feed supplement and functional fertilizers) that maintain and stimulate health of animal and crops. We are challenging to improve microbial cell factories to develop lower energy required, higher efficient and ecological friendly bioprocess converting ecologically negative resources to value-added fine chemicals.

1. Conversion of food residues to ingredients for microbial fermentation. In many cases, it is difficult to use food residues directly as ingredients for microbial fermentation. We developed methods to treat these resources for easy utilization by microorganisms.
2. Assessment and improvement of microbial cell factories. We studied to measure fundamental metabolic parameters of microorganisms owing to fermentation performance, especially energy and redox metabolism. We engineered these metabolisms to improve yeast to develop tough and efficient cell factories for production.
3. Improvement of fine chemical fermentation. We improved microbial fine chemical fermentation. For example, we improved yeast to enhance productivity of glutathione as a supplement and functional food for health of human, astaxanthin as feed supplement for health of fish.

.

Keywords: Biorefinery, Yeast, Energy metabolism, Fine chemical production

Synthesis and Composite of Poly Lactic Acid (PLA) Bio-based polymer

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Abstract

PLA synthesis was carried out using a commercial culture solution. As a result, it became possible to synthesize polylactic acid from lactic acid using the environmentally friendly chemical catalyst developed with bentonite clay as a support (Sn/Bt).The molecular weight of the synthesized polylactic acid polymer here can reach value till 140,000 g / mol.

The work then continue to carry out chemical modification and physical modification PLA. By PLA-PP-PPgMA blending biocomposite, it shown the addition of PP on PLA are significantly increase thermal degradation resistance. And the addition of PLA can improved biodegradation properties. Another result shown the blend of PLA and chitosan will decreased bacterial activity compared to neat PLA but increased water vapour permeability.

This Project team also has established a method for producing microfibrillated cellulose (MCF) from sorghum. In this method, the Project team succeeded in making MCF with high crystallinity by using alkali treatment. By blending MCF with polylactic acid, it was possible to obtain drastic improvement in physical properties (elongation, tensile strength, elasticity) of polylactic acid.

Keywords: poly lactic acid, MCF, composite, celluolose, degradation

Economic Assessment of Current Main Use of Oil Palm Empty Fruit Bunches (EFB) and Its Potential Use as Raw Material for Biofuel Production

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Abstract

Indonesia has the largest oil palm plantation and is the the largest CPO production in the world. In 2012 oil palm plantain area was 10.59 million Ha and in 2014 was approximately 10,85 million Ha (BPS, 2014). With an average of productivity level of 16.87 ton per Ha (PDSIP, 2014) equivalent to 183 million tons of Oil Palm FFB or 38.4 million ton EFB.This paper describes economic value of Oil Palm EFB in its current two types of main uses-- as mulch and compost organic fertilizer. This study describes detailed costs of dispersion, the cost of composting process so as to obtain compared to an increase in productivity level, and an estimate of economic value if the EFB converted into Bioethanol. Methodology uses in this study is analytical descriptive and case study. Data collection is conducted through field survey using questionnaire as a guide during the interview. Managers of three anonymous Oil Palm plantations and CPO Mills were interviewed. This study concludes that current main uses of oil palm EFB in Indonesia are as (a) organic materials in its primary form as mulch directly dispersing to the plantation and (b) organic fertilizer by composting them in the mill site then dispersing them to the plantation. On a 4-year-trial of using Oil Palm EFB as compost organic material and as mulch shows an increase of FFB productivity level of 1.1% and 1.07% per Ha respectively. This equivalent to an average increase of 187 g and 182 Kg per Ha, respectively. These uses do not reduce the use of inorganic chemical fertilizer. At a 40 ton/Hr operating capacity CPO mill it is found that the use of 10% of EFB as compost (current practice), 100% as compost, and 100% as mulch create a loss of approx. Rp. 5.3 billion 1.9 billion and Rp. 1.5 billion to the firm per year. While if the OP-EFB converting into bio-ethanol, it can generate Rp. 11.5 billion gross profit per year assuming wet to dry weight conversion of 60% and 70% of production cost. Therefore, theoretically the use of OP EFB as a raw material for bio-ethanol through enzymatic production process within the mill location can potentially provide a much better economic value to the firm and create a more environmentally friendly practice. However, the technology readiness level of this enzymatic production process technology has yet to be enhanced.
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Keywords: Oil Palm Empty Fruit Bunches, Biofuel, Bioethanol, Enzymatic Process

Concurrent Session

Production of Citric Acids by *Aspergillus niger* on Sorghum Bagasse

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Abstract

Citric acids demand increase yearly. The objective of study was to produce citric acid on sorghum bagasse using *Aspergillus niger* on solid state fermentation. Sorghum bagasse was obtained from Sorghum field trial site in Cibinong. The effect of particle size, initial pH, nitrogen sources on citric acid production was evaluated. The incubation time was determined before evaluation of the effect of nitrogen sources. The optimal particle size were 1.5 and 1.9 mm. Optimum fermentation time was 7 days, optimum pH was 5.0 and best nitrogen sources was ammonium sulphate. Maximum citric acid production was 14.5 g citric acid /100 g of dry solid. Addition of 1 % sucrose increased citric acid production. Sorghum bagasse is a good sources for citric acids production.

Keywords: *Aspergillus niger*, citric acid, sorghum bagasse.

Introduction

Citric acid ($C_6H_8O_7$, 2–hydroxy–1,2,3–propane tricarboxylic acid), a natural constituent and common metabolite of plants and microorganism. It is widely used organic acid. Citric acid is GRAS (generally recognized as safe) substances and is extensively used in food industry to adjust pH and improve flavor, account for 70% of its application. Citric acid also used in pharmaceuticals and cosmetics for acidification and metal ion chelation (Kamzolova et al. 2011, Najafpour 2015, Rossi et al. 2009). There is constant increase (3.5-4%) each year in its consumption, showing the need of finding new alternatives for its manufacture (Najafpour 2015).

Citric acid production system has been developed since 1917, the first microorganism used for the submerge fermentation was *Aspergillus niger* with sugar as the main carbon sources (Ali et al. 2002, Najafpour 2015). Significant improvement of citric acid yield was started in the 1950s when the glycolytic pathway and the tricarboxylic acid cycle (TCA) as biochemical basis of citric acid synthesis was proposed (Akram 2014, Najafpour 2015). Complexity of citric acid synthesis and its dependency on several complex nutritional conditions for effective fermentation requires intensive biochemical and production system engineering investigations (Den Haan et al. 2013). The most popular conventional citric acid production is the submerged culture using high-yielding mutant strains of *Aspergillus niger*, but this system still need further investigation on manufacturing process and effective microorganism for efficient fermentation to increase yield and subsequently minimize overall operating costs (Chaturvedi 2010).

The most common substrate for citric acid production is high sugar content substrate such glucose and sucrose which are quite expensive. To reduce production cost, a variety of media have been proposed such as molasses, several starchy materials, and agricultural byproduct (Dhillon et al. 2011). There are two groups of raw materials used for citric acid production: (i) substrate with a low ash content from which the cations could be removed by standard procedures (e.g. cane or beet sugar, dextrose syrups and crystallized dextrose); (ii) raw materials with a high ash content and high amounts of other non sugar substances (e.g. cane and beet molasses, crude unfiltered hydrolysates) (Nidhee & Raj 2012). We used sorghum bagasses for production of citric acid to reduce the feeding cost processing industry innoculated with *A. niger* in both SSF and submerged fermentation were proposed by Kieliszek (Kieliszek et al.

2017). Other works, used pineapple peel as a cheap medium to produce citric acid of 60.6 mg/L. Citric acids also produced using apple pomace solid waste, citrus waste, brewery spent grain, and sphagnum peat moss as main C-sources (Rossi et al. 2009).

Efficient citric acid producing microbes is one of key issue on achieveing cost effective citric acids production. *Aspergillus niger* has been used during the past 50 years as a commercial producer of citric acid. Due to complexicity of citric acid production process, selection of efficient citric acid producer may not offer the only solution for cost effective citric acid production. But understanding over all interlinked factors that influence fermentation processes, which include detail citric acid synthesis and its dependency on several nutritional conditions for cell growth and citric acid synthesis are critical to obtain high yield fermentation process (Ciriminna et al. 2017). Although conventional citric acid production by submerged culture of high-yielding mutant strains of *Aspergillus niger* has been optimized, but there is still interest in redesigning the traditional manufacturing process to increase yield and subsequently to minimize overall operating costs.

Sorghum contains fermentable sugars about 11.8 %, it is higher than energy cane i.e 9.8 %. The sweet sorghum bagasse contains 45% cellulose, 27% hemicellulose, and 21% lignin (Kusumah et al. 2016). Due to its high fermentable sugar composition, juice of sweet sorghum could be a good substrate for citric acid production (Rooney 2014).

Materials and Methods

Organism and culture condition

Aspergillus niger InaCC F539 was obtained from Indonesian Culture Collection (InaCC), was used in this study. Stock cultures were stored at -80 °C on glycerol stock. *Aspergillus niger* InaCC F539 was inoculated on PDA agar and cultured at 28 °C for 120 h. Spores were eluted with 20 mL 0.1% (v/v) Tween-80, of which approximately 15 mL was filtered through lens paper, transferred to a sterilized 50-mL centrifuge tube, and then separated by centrifugation (3000×g, 7 min). The supernatant was removed and the spores resuspended in 20 mL of 0.1% (v/v) Tween-80, and then centrifuged again.

Substrate

Sorghum bagasse was obtained from Field Trial of SATREPS Project (Project for Production of Biomass Energy and Material through Revegetation of Alang-alang (*Imperata cylindrica*) field. The particle size was fractionized into (1 cm <, 1-2 cm, 2-5 cm and 5-8 cm) by sieving it through appropriate mesh-size sieves. 15 g of each size was taken in triplicate in 500 ml flask with 50 ml of solutions containing (g/L) NH_4NO_3, 0.5; KH_2PO_4, 0.5; $MgSO_4.7H_2O$, 0.1; peptone, 7.0; $ZnSO_4.7H_2O$,0.001; ferrous ammonium sulphate, 0.001 and $CuSO_4$. $5H_2O$, 0.0006 were added. The flasks were autoclaved at 121°C for 30 minutes. After cooling 5 ml spore suspension of *Aspergillus niger* (6×10^8 spore per ml) were added and mixed in substrate. To study the effect of sucrose on citric acids fermentation 0.1 to 1.2 % sucrose was added to the substrate. The cultures were then incubated at 30°C for 9 days.

Mycelia growth

Mycelia growth was observe morphologically. One gram of substrate was taken and observed under stereo microscope, and the extent of mycelia growth was determined visually based on the extent of mycelia covering the substrate which expressed in percentage.

Determination of citric acid concentration

Citric acid concentration was determined with a Shimadzu Series high-performance liquid chromatography (HPLC) instrument equipped with a UV/Vis detector and Eclipse Plus C18 column (250 × 4.6 mm × 5 μm; Agilent Technologies, Santa Clara, CA, USA). Two grams of fermentation substrate was removed every 24 h under aseptic condition and added with distilled water, mixed, and centrifuged at 6000 rpm. The substrate was diluted 10-fold in the phosphate buffer (25 mM, pH 2.4), filtered through a 0.22-μm membrane, and injected into 2.0-mL autosampler vials. Citric acid was separated with a mobile phase composed of methanol and phosphate buffer (25 mM, pH 2.4) at a 1:9 ratio (v/v) with a flow rate of 1.0 mL/min at 30 °C. The injection volume was 20 μL and we performed three replicates of each trial.

Citric acid quantitation was performed at the wavelength of maximum absorbance for each analyses (λ = 210 nm; A210) obtained from UV spectrophotometer spectra determination. Citric acid was identified by comparing its retention time with that of the standard substance.

Statistical analyses

The data were analyzed using SPSS 18 (SPSS, Chicago, IL, USA). The statistical significance of differences was calculated using Tukey's *HSD, P<0.005*. The primary analyses were paired comparison of citric acids production and biomass at the various initial total reducing sugar concentration at 6 day fermentation time. Data are presented as mean ± SD. or medians if the data were not normally distributed.

Result and Discussion

Citric acid production by *Aspergillus niger* InaCC F539

On sorghum bagasse, *Aspergillus niger* InaCC F539 produced maximum 14.4± 2.4 g/100 g dry substrate extracellular citric acid at 8 days incubation (Figure 1). Less citric acids production was obtained at 4 days. The production of extracellular citric acid was much affected by incubation time (Figure 1). Citric acid production increased when initial total reducing sugar increased. There was significant increase of citric acid production rate when incubation time increased (Figure 1). Maximum citric acid production rate was 3.2 g/100 g substrate/day (Figure 1). Therefore it can be assumed that sorghum bagasse of *Sorghum bicolor* var1 is a good medium for citric acids production.

Biomass fungal growth

On sorghum bagasse, biomass growth was associated with incubation time (Table 1). Increased incubation time result in higher biomass production (Table 1). Maximum biomass production was after 7 days. Addition of sucrose increase rate of biomass growth (Table 1). Addition of 1 % (w/w) sucrose significantly increased mycelia growth. After 5 days incubation mycelia growth was already covering 100 % of media.

Effect of Particle size on citric acid production

Particle size affects citric acids production (Figure 2). The most optimum particle size for ctiric production was beteween 1-2 cm. Increasing particle size seems to reduce citric acids production Maximum citric acids production was 14.4 g/100 g dry substrate.

Effect of sucrose on citric acid production

Sucrose stimulates production of citric acids. In basal medium the production of citric acids was around 14.4 g/100 g dry substrate, but when sucrose was added the citric acids production increase to 17.5 g/100 g substrate. The rate of citric acids production also increases significantly when sucrose was added (Figure 3).

Table 1. Course growth of mycelia in medium bagasse without addition and with addition of sucrose

Substrate	Incubation Time (day)					
	4	5	6	7	8	9
Bagasse	50	70	90	100	100	100
Bagasse + 0.1 %	55	75	95	100	100	100
Bagasse + 0.2 %	58	78	95	100	100	100
Bagasse + 0.4 %	60	82	98	100	100	100
Bagasse + 0.8 %	62	88	100	100	100	100
Bagasse + 1 %	64	96	100	100	100	100
Bagasse + 1.2 %	65	100	100	100	100	100

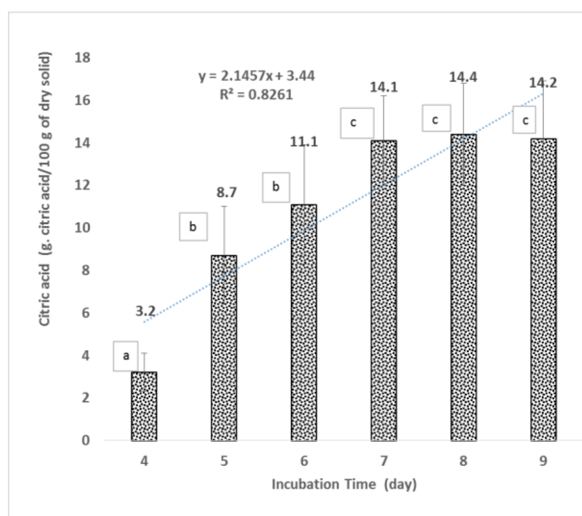

Fig. 1. Citric acid production by *Aspergillus niger* InaCC F539 at various 8 days. Bars with different letter are significantly different (Tukey's HSD, P <0.05)

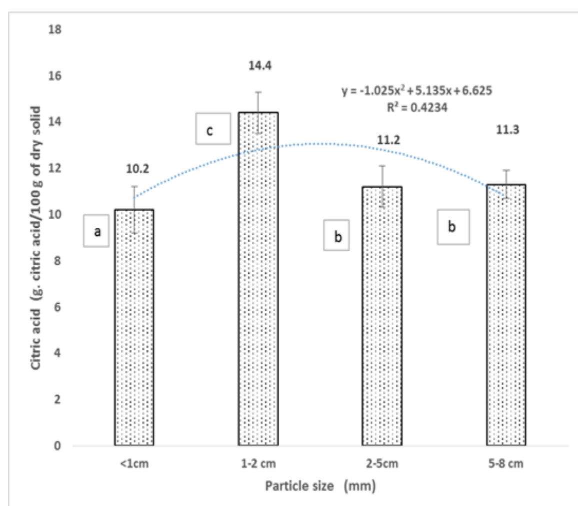

Fig. 2. Effect of particle size on Citric acid production. Bars with different letter are significantly different (Tukey's HSD, P <0.05)

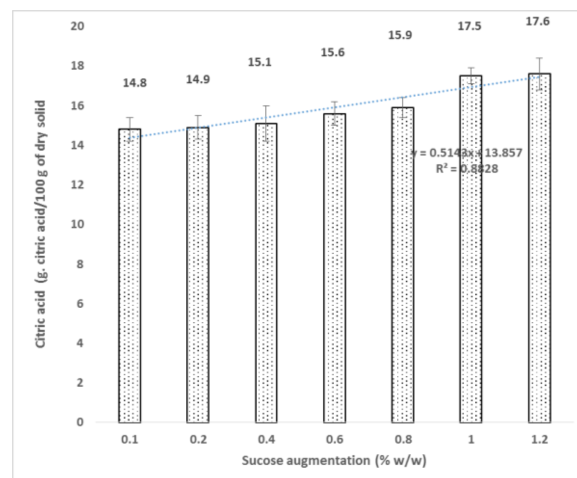

Fig. 3. Th effect of sucrose supplemnattion on citric acid production. The fermentation was 8 days at 30°C.

In sweet sorghum bagasse, average content of cellulose, hemicelluloses and lignin is 34-44%, 27-25%, and 18-20% respectively (Ballesteros et al. 2003, Kim & Day 2011, Sipos et al. 2009). The polysaccharide is hydrolyzed by hydrolytic enzyme produced by *Aspergillus niger* to produce fermentable sugar (Kulev & Sharova, n.d.). Those fermentable substrate are easily converted into citric acids (Geanta et al. 2013, Papanikolaou 2008). Sweet sorghum also contain of protein and lipid, which are required for new cell synthesis. Sweet sorghum also rich in micronutrient i.e Fe, Cu, K, Na, Fe, Mn which are needed for enzyme activities on citric acid synthesis and cell division (Ferreyra et al 2002, Sanchez-Marroquin et al. 1970). We found that sorghum baggasse produced about 14.44 g/100 g ddry substrate. This confirmed that sweet sorghum baggse is potential feeding materials for citric acids production. Sucrose was clearly stimulate citric acid and cell biomass production (Figure 3). The increase of citric acid production due to sucrose addition was from 10 to 35 %. Sucrose is commonly used as additional carbon source on solid state fermentation (Aranda et al. 2006).

The rate of citric acids production is affected by the fermentation time (Figure 2), and addition of sucrose increase citric acids production rate (Figure 6). Biomass production rate also increased when fermentation time increased. The efect of fermentation time on citric acids production is quite logical. Increased fermentation time

means increase contact between susbtrate and enzymes. Increased citris acids production due to increase fermentation time was also observed by Chaudhary & Raj on submerge type fermentation of citric production on cane molases Chaudhary & Raj (2012).

In addition to *Aspergillus niger*, there are numerous of microorganisms used for citric acids production, which includes *A. aculeatus*, *A. awamori*, *A. carbonarius*, *A. wentii*, *Penicillium janthinelum*. Recently several yeasts have been proposed i.e. *Saccharomycopsis lipolytica*, *Candida tropicalis*, *C. oleophila C. guilliermondii C. parapsilosis, C. citroformans,* and *Hansenula anamola.* Which suggest that there is an urgency to explore both diversity of citric acids producing microorganism and feeding substrate to get most cost effective citric acids production. Addition of other carbon sources such as sucrose increase citric acids production (Kola et al. 2017, Yu et al. 2017), which offer posibility to increase effectiveness of sweet sorghum baggase for citric acid production.

Molasses could be used as a carbohydrate source for the production of citric acid. Strain type, addition of whey, methanol and tricalcium phosphate had a significant impact on citric acid production by *Aspergillus niger* (Yu et al. 2017) which implies that augmentation of molasess other than sucrose might possible to increase citric acid production. The effectivness of solid state feremnation for citric acid production need further studies. Several studies have reported high enzymatic activity titers by solid-state fermentation (SSF) over SmF when high glucose concentrations were used. These observations have provoked a great controversy, because several authors considered that SSF was a process where the catabolic repression of inducible enzymes did not take place or was minimized.

Several hypotheses have been proposed to explain such differences in the enzyme activity titers, among which are the low water content, the nature of SSF, the diffusion of nutrients on solid matrix and the changes in the ratio between the substrate uptake rate and diffusivity substrate coefficient. The fatty acid composition of cell membrane from *Aspergillus niger* grown in SmF and SSF for pectinase production, concluding that those structural differences are directly (Barman et al. 2015) which implies both system infleunces cell physiology of fungi.

Conclusions

The results from the present work demonstrate that sweet sorghum is a good medium for citric acids production. Initial fermentable sugar concentration affect citric acids production, and maximum citric acid production on sweet sorghum reach 18.96 g/L at 75 g/L initial total reducing sugar concentration. Addition of 4% of methanol increases citric acid production by 65 %. The physiological mechanism by which methanol stimulate citric acid and biomass production need further verification.

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Crude Oil Degradation by Fungal Co-culture System of *Pestalotiopsis* sp. NG007 and *Polyporus* sp. S133 under Saline-Alkaline Stress

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Abstract

Petroleum oil is currently the primary energy source in modern society. The demand for oil increases dramatically during the development of a social economy. However, the exploration, mining, storage, transportation, and usage of oil products and also various leakage accidents have frequently resulted in the gross contamination of environments. These processes generate huge volumes of unwanted oily sludge and cause serious problems for surrounding ecosystems, especially marine environments and coastal areas. The aims of the present study were to select a potential fungal co-culture for crude oil degradation in sea sand medium, to select a suitable pregrown medium for the selected fungal co-culture, to compare the ability of fungal co-culture in different solid media, and to investigate the effect of nutrient amendment treatment during degradation. In this study, Co-culture system of Pestalotiopsis sp. NG007 and Polyporus sp. S133 with ratio 25/75 (v/v) was found as the most suitable fungal co-culture among fungal co-culture studied on crude oil degradation at sea sand medium due to stimulation of ligninolytic and non-ligninolytic enzyme production during incubation. This study also demonstrated that combination of kapok as pre-grown medium, the periodic biostimulation of malt extract (10%) as a nutrient source, tween 80 (0.5%) as a surfactant, and mineral mixture of $MnSO_4$ and $CuSO_4$ (1 mM) as an enzyme inducer enhanced enzymatic activities in the co-culture, even after 120 d, by contributing to a new generation of mycelia during biodegradation. The condition was found to enhance enzymatic activities, leading to the greater degradation of TPH than control treatment.

Keywords: *fungal-coculture, crude oil, biodegradation, enzyme inducers*

Introduction

Crude oil is a major source of Polycyclic Aromatic Hydrocarbons (PAHs) contamination in the environment. PAHs removing method in the environment cannot be separated with the ability to degrade crude oil as a complex mixture. Since crude oil consists of different fractions with various chemical and physical properties, degradation of crude oil by using organism consortium has been reported more effective compared with using a single organism. The number of studies reporting more effective bioaugmentation using a consortium or a co-culture rather than a single microbe has greatly increased (Kotterman et al. 1998, Yanto et al. 2014). Li et al. (2016) reported that bacteria consortium from Bohay Bay (China) was effective to degrade marine crude oil (*Ochrobactrum, Brevundimonas, Brevundimonas, Bacillus*, and *Castellaniella*). Tao et al. (2017) also reported the effectivity of indigenous bacteria consortium and exogenous bacteria *Bacillus subtilis* on crude oil degradation. Most studies have been limited to the development of bacterial compared with other organisms.

On the other hand, indigenous bacteria consortium has been reported to have no effectivity on PAHs degradation on in situ treatment (Baker and Herson 1994, Boufadel et al. 2016). In the recent study, Yanto et al. (2014) reported that fungal co-culture system of Pestalotiopsis sp NG007 and Polyporus sp S133 could also enhance biodegradation of aromatic fraction (with its major components are PAHs) of three types crude oil in soil medium. The effectivity of this co-culture on aromatic fraction degradation might be related to the ability of *Polyporus* sp. (a kind of WRF) to produce the ligninolytic system. Study of fungal co-culture, which one or more of them

are WRF, for the degradation of crude oil under saline-alkaline stress such as crude oil contaminated sea sand need to be examined in more detail. The aims of the present study were to select a potential fungal co-culture for crude oil degradation in sea sand medium, to select a suitable pregrown medium for the selected fungal co-culture, to compare the ability of fungal co-culture in different solid media, and to investigate the effect of nutrient amendment treatment during degradation.

Materials and Methods

Chemicals

Three types of crude oil, namely heavy oil A (HOA), heavy oil C (HOC) and asphalt were obtained from Taiyo Petroleum Co. Ltd. (Japan). Solvents for analysis were purchased from Wako Co., Ltd. (Osaka, Japan). The fraction composition of three crude oil was shown in Figure 1.

Microorganisms

Five fungal strains were used in the present study: Trametes hirsuta D7, Bjerkandera adusta SM46, Polyporus sp S133) and the two fungal strains are Ascomycetes: Pestalotiopsis sp NG007 and Fusarium sp F092. All fungi are the fungal collection of Plant Chemistry Laboratory, Faculty of Agriculture, Ehime University.

Selection of a potential fungal co-culture for crude oil degradation in sea sand medium

Degradation experiments were performed in 400 ml-autoclavable plastic trays containing sea sand (30 g) mixed with glucose (10% w/w sea sand) and shitake no sato 15% (w/w sea sand). The medium was adjusted with 30% (w/w sea sand) distilled water. The sea sand was sterilized at 121°C for three h and allowed to cool. Each tray was added with 30 mg of crude oil (HOC type) which dissolved in 1 mL dichloromethane to a final concentration of 1000 mg/kg sea sand. The solvent was evaporated for four h in air laminar flow. Four milliliters of fungal preculture in malt extract medium pH 4.5 (7 days) was added to HOC-contaminated sea sand and incubated in the dark at 25°C for 15 and 30 d.

In the present study, Pestalotiopsis sp NG007, Fusarium sp F092, Trametes hirsuta D7, Bjerkandera adusta SM46, Polyporus sp S133 were used as inoculated fungi in several combination treatments. The system was conducted using several comparisons of a percentage each inoculated preculture (100-0; 75-25; 50-50; 25-75; 0-100).

Selection of a suitable pregrown lignocellulose medium for the selected fungal co-culture

Rice straw and kapok fibers were compared as a pregrown medium for the selected fungal co-culture. Three grams of each of these materials, 10% (w/w) glucose, 15% (w/w) shiitake no sato (a kind of sugar-based nutrient from mushrooms), and 60% (v/w) distilled water were added to an autoclavable plastic tray and then autoclaved at 121°C and 1 atm for 3 h. Total for 4 ml of the growing fungus selected co-culture in ME liquid medium were inoculated on the tray and incubated for approximately one month. Fungi were ready to be used in subsequent experiments when fully grown mycelia appeared on the tray. The growth of mycelia in the lignocellulosic material was monitored daily and confirmed by microscopic analysis. The material that showed the greatest growth, colonization of the fungus, ligninolytic enzyme production, and biodegradation rate of crude oil was selected for subsequent experiments.

Degradation of crude oil by the selected fungal co-culture in three different media

The experiments were performed in 400 ml-autoclavable plastic trays containing one of solid medium (sea sand, black mud, and agricultural soil) 30 g, and then mixed with glucose (10% w/w soil) and shitake nutrient 15% (w/w soil) was adjusted with 30% (w/w sea sand) distilled water. The medium was autoclaved at 121°C for three h and allowed to cool. Crude oil (HOC) with concentration 1000 ppm were transferred to the sea sand. The solvent allowed evaporating for four h. 7.5% dried weight pre-grown of fungal co-culture NG007 and S133 on kapok medium (1-month incubation) was added to contaminated soil, black mud, and sea sand. The medium was incubated in the dark at 25o C for 15 and 30 d. The Total petroleum hydrocarbons (TPHs) degradation rate and enzyme activities such as laccase (Lac), Mangan peroxidase (MnP), Lignin Peroxidase (LiP), 1,2-

Dioxygenase (1,2-D), and 2,3-Dioxygenase (2,3-D) were measured.

Effect of periodic biostimulation of nutrient and mineral inducer during crude oil degradation by the co-culture

Based on preliminary study, the fungal growth of co-culture tends to decrease after 60 d incubation. The nutrient addition and inducers on 15d, 30d, 60d, and 90d have been expected to stimulate the fungal growth as well as degrading related enzymes for longer incubation day. Malt extract solution as a fungal nutrient source and three types of inducers (CuSO4, MnSO4, and veratryl alcohol) as a stimulator for degrading related enzyme production were used in this study. A suitable concentration for each added solution was an investigation by optimization analysis. The detailed experimental design is described in Figure 2.

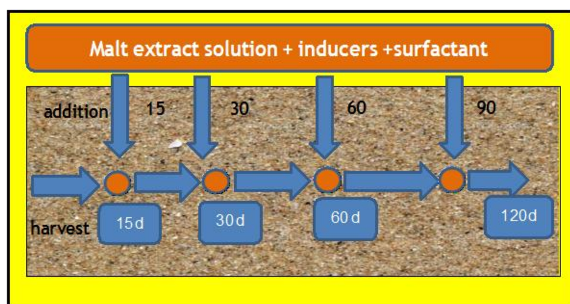

Fig. 2. Experimental design of periodic biostimulation using malt extract solution, mineral inducer and surfactant during crude oil degradation by the selected fungal co-culture

Degradation analysis of crude oil

The biodegradation of crude oil and fractions (aliphatic, aromatic, resin and asphaltene) were analyzed as described by Mishra et al. (2001) with slight modifications. The petroleum hydrocarbons remaining in the soil were extracted using n-hexane, dichloromethane (DCM), and chloroform, respectively with 100 mL of each solvent. The hydrocarbons were analyzed using the gravimetric method. n-Hexane was used to separate asphaltene from the asphalt. The precipitate was filtered off, placed in Whatman filter paper, and washed with n-hexane to separate n-hexane soluble fraction and asphaltene. The n-hexane-insoluble fraction which remained in the filter paper consisted of asphaltenes. The asphaltenes were washed out from the filter paper with DCM. The weight of asphaltene was analyzed by the gravimetric method. The n-hexane-soluble fractions were further separated to obtain aliphatic, aromatic and resin fractions by purification on a 6 grams silica gel (C-200) column eluted with 100 mL of n-hexane, 100 mL of toluene and 200 mL of chloroform : methanol (1:1 (v/v)), respectively. The aliphatic and aromatic biodegradation was analyzed by gas chromatography (GC-FID Shimadzu, 2014), with a TC-5 capillary column (30 m, id x 0.25mm x 0.25 mm) (Andriani & Tachibana 2016).

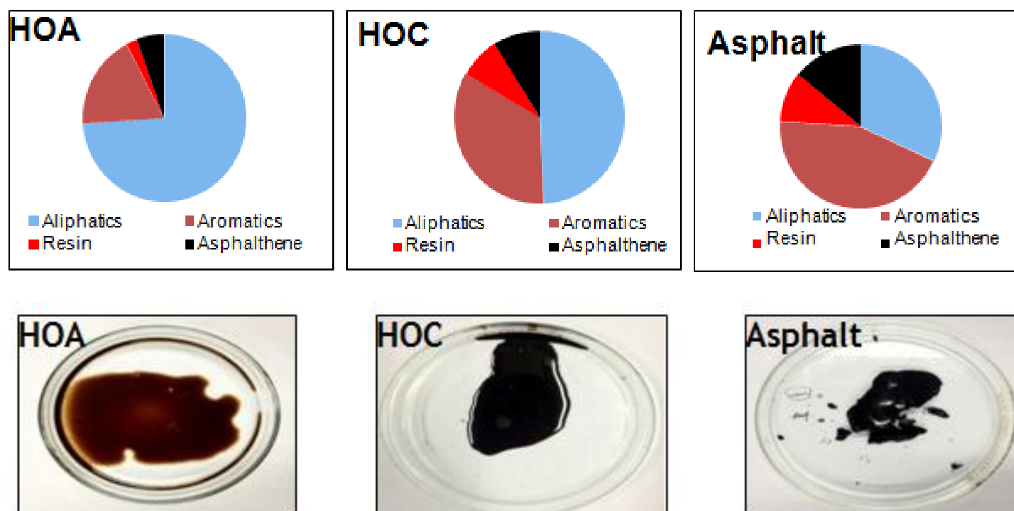

Fig. 1. Fraction composition (a) and the appearance forms of three crude oil types (b).

Result and Discussion

Selection of a potential fungal co-culture for crude oil degradation in sea sand medium

In the present study, the degrading ability of fungal co-culture between NG007 and other basidiomycetes and filamentous fungus was first examined on HOC contaminated sea sand medium. All the co-culture treatment showed a fungal growth and degrading ability with various degradation rates. On the other hand, a single culture of basidiomycetes in contaminated sea sand showed no a fungal growth and significant degrading related enzymes production. A double inhibition of crude oil as a contaminant compound and high salinity of sea sand medium may be associated with the phenomenon. A Basidiomycota, including a white rot fungus, was reported to have lower competitive characteristics compared with other fungal groups such as ascomycetes and Deuteromycetes for application in wastewater because most often they demand specific growth conditions (Saparrat & Hammer 2006). However, basidiomycetes, especially WRF can mineralize many aromatic compounds into non-toxic products such as carbon dioxide and water (McGenity et al. 2012). Therefore, a combination of filamentous fungi and basidiomycetes, as a synergistic co-culture system may be a suitable way for accelerating the biodegradation of pollutants (Yanto & Tachibana 2014).

The biodegradation of HOC and enzyme activities produced by the co-culture of NG007 with strains D7, F092, SM46, and S133, are shown in Figure 6.4. Trametes hirsuta D7 exhibited the very low activity of 1,2-dioxygenase (20 U/L) and did not produce ligninolytic enzymes when incubated for 15 d in HOC contaminated sea sand. Ligninolytic enzymes were inhibited in the treatment. Pure D7 degraded 3.19 and 4.25 % of HOC in 15 and 30 d, respectively (Figure 6.4a). In the NG007/D7 co-culture with combination ratio 75/25, the enzyme activity of ligninolytic enzymes was higher than pure D7 and NG007. The amount of HOC degraded (63% for 30 d) was also higher than that by pure D7 and NG007. Fungal growth analysis of D7 in HOC contaminated sea sand showed no hyphal growth detected. However, two kinds of fungal hyphae were grown in the NG007/D7 co-culture. The phenomenon indicated the synergistic effect of the NG007/D7 co-culture with ratio 75/25 (Figure 3a,b).

In the NG007/F092 co-culture, only 1,2-dioxygenase and low activity of MnP were detected in 15 d and 30 d. LiP and laccase were not detected in the treatment. In 30 d, 1,2- dioxygenase was higher more 2-fold than pure F092. The biodegradation rate of HOC in 15d of the co-culture was lower than pure F092 but increased in 30 d. NG007/F092 is a synergistic filamentous fungal co-culture which is effective for dioxygenase production, but it showed no potency for ligninolytic enzyme production (Figure 3c, d).

Another basidiomycete from the previous study, SM46, was shown to be capable of ligninolytic enzymes production under saline-alkaline stress (Andriani et al. 2016). However, both pure SM46 and NG007/SM46 co-culture in the HOC contaminated sea sand showed no enhancement on ligninolytic enzyme activity of Laccase, MnP, and LiP. Also, no ligninolytic activities were detected in any of the compositions after the 30 d incubation, resulting in a lower level of degradation rate than other fungal co-culture treatments (Figure 3e, f).

The NG007/S133 co-culture revealed as the most potential co-culture among fungal co-culture tested in this study for HOC degradation and degrading related enzymes production. The biodegradation rate of the NG007/S133 co-culture with ratio 25/75 in 15 d and 30 d were 48.5 and 70.6%, respectively. Ligninolytic enzymes were enhanced in the treatment. Laccase, MnP, and LiP were detected with high activity: 2066 U/L, 597 U/L, and 275 U/L, respectively in 30 d.

Based on fraction degradation analysis (Table 1), the co-culture of NG007/S133 with ratio 25/75 could degrade all HOC fractions. The aromatic fraction was the highest degraded fraction during incubation. The degradation of the aromatic fraction reached 86% in 30 d. The rate was higher than the aliphatic fraction degradation rate with 82% removal in 30 d. But, in sea sand medium, fungal hyphal did not appear in mono-culture S133 100%. 007 and S133.

The synergistic effect of fungal co-culturing has potential as a mechanism for accelerating the biodegradation of HOC by

providing highly degradative enzymes. Screening among four potential fungal co-culture on HOC degradation revealed that NG007/S133 as the most synergistic co-culture for HOC degradation under saline-alkaline stress. The co-culture could produce both high dioxygenase and ligninolytic enzyme activity. In the present study, use of a white rot fungus for crude oil contaminated sea sand could not be applied as a single culture. Fungal mycelia did not appear in all a single culture of D7, SM46, and S133. High salinity and pH at sea sand were not a suitable condition for white rot fungi studied for growing well. However, co-culturing with NG007 could enhance the growth rate of WRF and ligninolytic system of S133. Two different fungal mycelia were found in the co-culture treatment indicating the ability of S133 to grow in the presence of NG007. Microbial interactions are important for metabolism as they can improve or reduce metabolic efficiency. The synergistic effect among microbes involved is the key to early success in a microbial consortium (Varjani et al. 2015). For further investigation, this co-culture of NG007/S133 with ratio 25/75 will be used as crude oil degrader in the study.

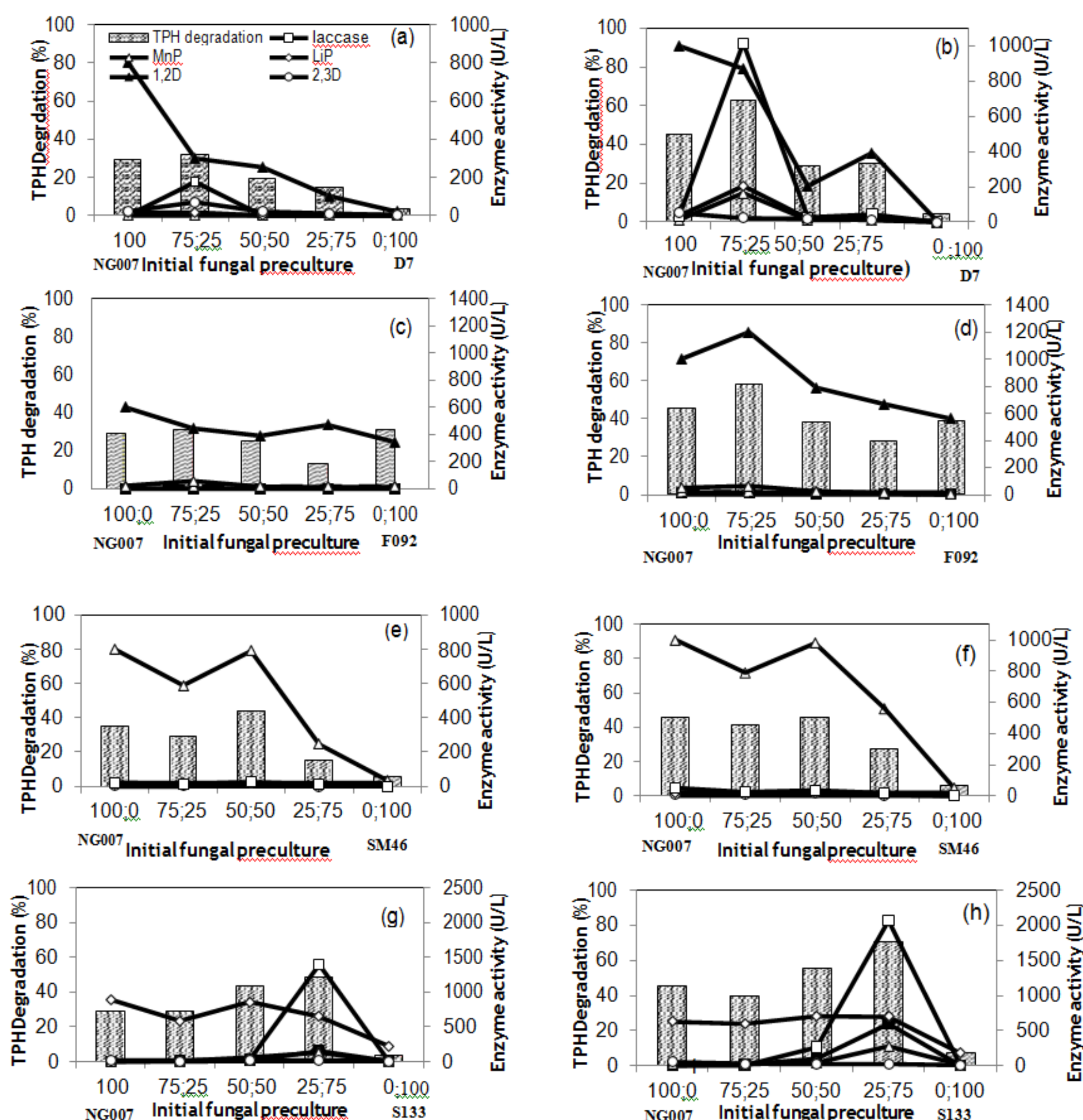

Fig. 3. Biodegradation of HOC and enzyme activities detected in the co-cultures of Pestalotiopsis sp. NG007 with T. hirsuta D7 in 15 d (a), 30 d (b); Fusarium sp F092 in 15 d (c), 30 d (d); B. adusta SM46 in 15 d (e), 30 d (f); Polyporus sp. S133 in 15 d (g), 30 d (h) in the sea sand

Table 1. Biodegradation of asphalt fractions by the co-culture of NG007 with strains D7, SM46, F092, and S133

Fungal strain combination	Incubation time (d)	Composition (%)	Biodegradation fractions (%)			
			Aliphatics	Aromatics	NSO	Asphaltene
NG007	15	100	43.35	19.69	14.04	30.10
	30	100	69.40	35.74	17.49	33.55
D7	15	100	6.25	1.25	0.29	3.23
	30	100	7.60	2.54	0.30	4.10
SM46	15	100	7.04	2.30	1.90	0.00
	30	100	9.52	3.60	4.13	0.00
F092	15	100	42.40	25.00	24.40	21.94
	30	100	62.97	24.50	30.90	25.60
S133	15	100	5.10	3.60	5.00	0.00
	30	100	7.65	10.72	6.53	0.00
NG007/D7	15	75/25	48.16	21.97	14.33	34.19
		50/50	34.88	10.43	10.01	12.4
		25/75	29.15	1.46	9.7	15.6
	30	75/25	61.42	89.66	24.07	32.70
		50/50	41.18	27.49	11.10	15.50
		25/75	29.61	43.81	9.00	15.81
NG007/SM46	15	75/25	35.11	31.24	17.47	20.60
		50/50	62.05	44.12	19.73	15.80
		25/75	14.07	19.07	18.67	0.00
	30	75/25	43.34	55.52	17.01	19.06
		50/50	65.52	58.35	21.32	14.14
		25/75	49.33	44.50	18.67	0.00
NG007/F092	15	75/25	52.64	39.01	10.80	10.32
		50/50	34.83	21.06	28.00	2.58
		25/75	15.18	20.70	0.00	0.00
	30	75/25	79.32	77.73	15.20	12.40
		50/50	49.56	44.12	20.00	5.00
		25/75	20.18	23.00	3.50	4.60
NG007/S133	15	75/25	37.32	22.99	15.80	22.00
		50/50	60.70	40.52	28.00	12.58
		25/75	67.42	45.00	33.00	14.00
	30	75/25	63.60	29.34	23.46	21.32
		50/50	74.08	57.09	31.40	17.06
		25/75	82.41	86.15	37.07	25.13

Selection of a solid support medium for the selected fungal co-culture

Kapok fiber (KF) and rice straw are lignocellulose biomasses that potential as a solid support medium for fungi, especially for a WRF. The biomasses can provide a natural substrate for fungi and enhance specific bioactive substances such as degrading related enzymes during incubation. In the previous study, use of rice straw as a pregrown medium for B. adusta SM46 could significantly enhance the ligninolytic enzymes even under saline-alkaline stress condition (Andriani & Tachibana 2016). KF also has been reported to have the ability as a natural sorbent in petroleum hydrocarbon biodegradation by Pestalotiopsis sp. Addition of 7.5% KF-associated NG007 to crude oil-contaminated soil increased 25.6% degradation compare with no addition of kapok (Yanto & Tachibana 2016). Therefore, in this study, we investigated the role of KF and RS in the degrading related enzyme production and TPH degradation by fungal co-culture NG007-S133. Based on the results (Figure 4), the use of KF as a single solid support medium (100%) resulted in higher degrading related enzymes and TPH that than the use of rice straw and the combination. The use of KF enhanced 1,2-Dioxygenase 3-fold (3072 U/L) compared to control treatment (1006 U/L). Laccase also was enhanced 25% in 15 d. Also, TPH degradation rate also increased by more than 8% in 15 d. KF has been reported to

improve oil absorbed and microbial interaction during incubation (Yanto & Tachibana 2016). Oil absorbed in KF can be more easily attacked by the fungal co-culture so the degradation rate of oil can be enhanced. The very low surface energy and extreme hydrophobicity of KF made the oils highly penetrable into the lumen and inter-fiber pores, resulting in minimal differences in their absorbencies by KF. The hydrophobic–oleophilic characteristics of the KF could be attributed to its waxy surface, while its large lumen contributed to its excellent oil absorbency and retention capacity (Lim & Huang 2007).

Degradation of crude oil by the selected fungal co-culture in three different media

Type of soil affects the crude oil removal by microbes. Soil parameters such as moisture content, pH, mineral content, oxygen circulation in the medium have become important factors in the crude oil degradation process (Haghollahi et al. 2016). In this study, the biodegradation of crude oil in three different types of contaminated soils was compared to investigate the most suitable medium for fungal co-culture NG007-S133 to apply. Sea sand, agricultural soil, and black mud with different molecular properties were used as crude oil contaminated medium for co-culture application (Figure 5).

Among three media, the co-culture in sea sand and agricultural soil resulted in high degradation of TPH (more than 86 and 90%, respectively). High laccase was detected in both media (4108 and 1578 U/L in 30 d, respectively). 1,2-dioxygenase revealed as the predominant enzyme produced by the co-culture (10062 and 13208 U/L in 30 d, respectively). However, TPH degradation in black mud reached only 45% after 30 d and also the co-culture produced lower enzyme activity compared to other two media (Figure 6). Subtle black mud characteristics were likely to cause low oxygen circulation that affects the low fungal growth of the co-culture.

Based fungal growth observation (Figure 7), the co-culture could grow most rapid and well in the sea sand medium. This is interesting finding since sea sand is a saline-alkaline medium (Table 2) and not many microorganisms could grow well in the medium. The fungal co-culture could grow well and show the degrading ability of TPH under saline-alkaline stress indicating the potency to apply in a wide range location such as coastal areas.

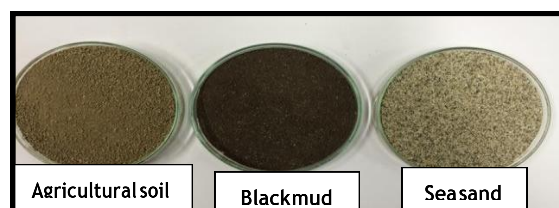

Fig. 5. Three different types of soil used in the crude oil degradation by co-culture NG007/S133

Table 2. pH of three soil media

No	Sample	pH
1	Soil	5.73 ± 0.01
2	Black Mud	6.63 ± 0.05
3	Sea Sand	8.05 ± 0.02

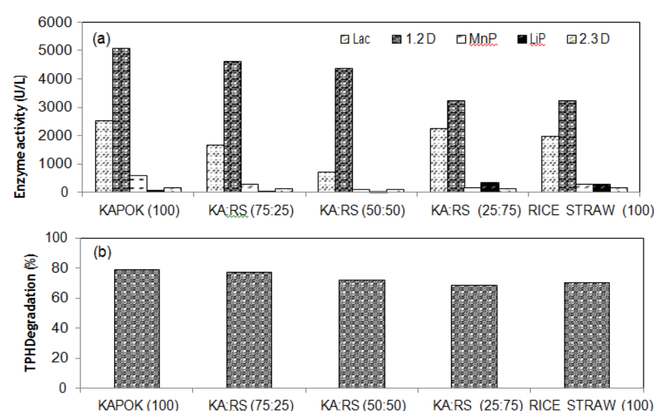

Fig. 4. Comparison of enzyme activity (a) and TPH degradation (b) in the different composition solid support medium

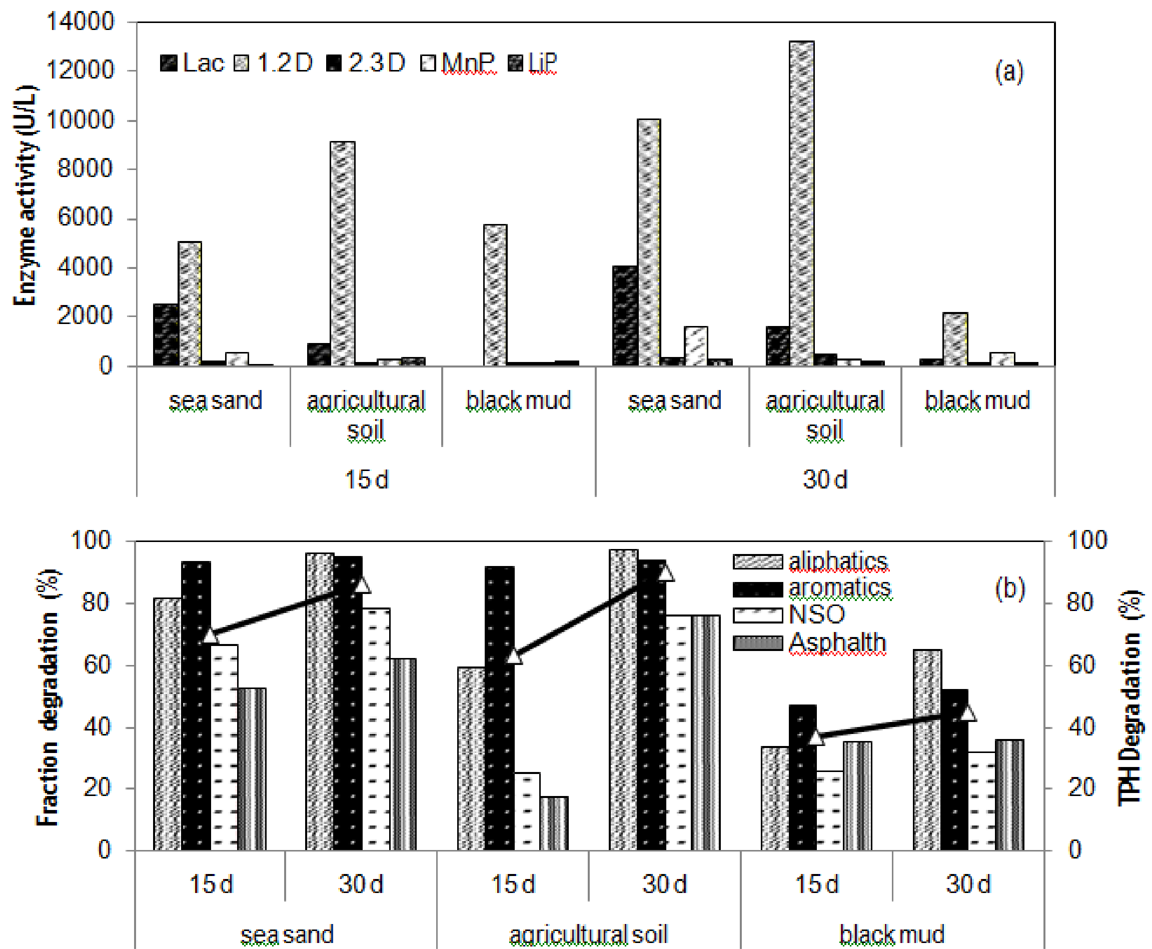

Fig. 6. Enzyme activity (a) and TPH degradation (b) in three different types medium by co-culture NG007/S133

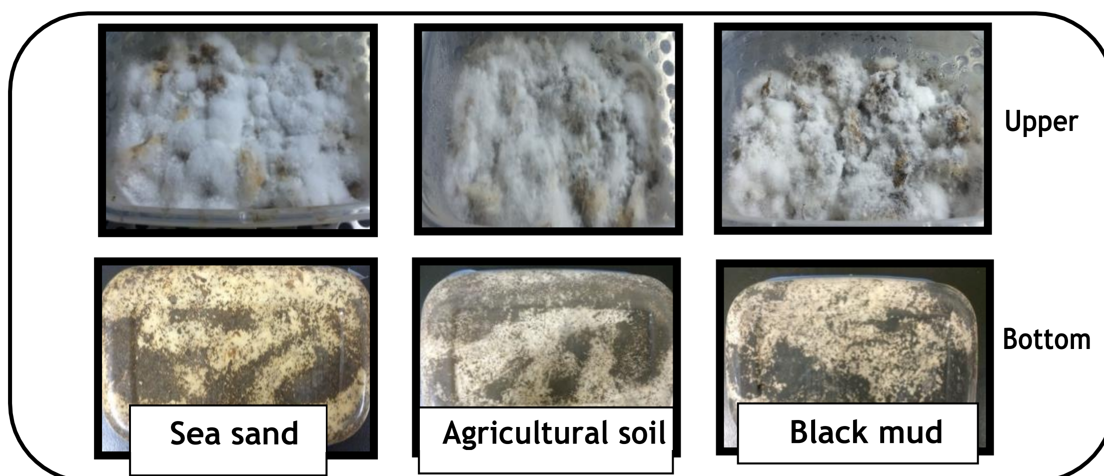

Fig. 7. Fungal growth in three different media by co-culture NG007/S133

Effect of periodic biostimulation of nutrient and mineral inducer during crude oil degradation by the co-culture

Based on a preliminary study of crude oil degradation at sea sand medium, the fungal growth and also degrading related enzyme production of fungal co-culture tend to decline after incubation over 30 incubation days. In the present study, strategies to maintain enzymatic oxidation during the extended bioremediation of oily sea sand were examined using periodic biostimulation using fungal nutrient, surfactant, and enzyme inducer. Biostimulation refers to stimulation of microbes using a nutrient or other substances needed by microbes for growing and producing their bioactive (Baker & Herson, 1994, Yanto & Tachibana 2017). In the present study, we selected the most suitable inducer, surfactant, and nutrient concentration for the fungal co-culture. Asphalt as the most difficult crude oil to degrade by the co-culture was used as the model compound in the optimization.

As shown in Figure 8, of the three inducers and their mixture added to the crude oil contaminated sea sand (CuSO4 as the laccase inducer, MnSO4 as the MnP inducer, and veratryl alcohol as the LiP inducer), the mixture of MnSO4 and CuSO4 was identified as the most suitable ligninolytic enzyme inducer for the fungal co-culture under saline-alkaline stress conditions. Manganese is an essential part of the catalytic cycle of MnP. MnSO4 has two functions in fungal cultures: a ligninolytic enzyme inducer and redox mediator to enhance degrading of pollutants (Hofrichter 2002). CuSO4 has been recognized as laccase inducer, however, in the recent study, CuSO4 has been reported to alter the activities of several lignocellulolytic enzymes such as MnP (Mishra et al. 2017). In the present study, the mixture of MnSO4 and CuSO4 could enhance not only ligninolytic enzymes (especially for laccase and MnP) but also 1,2-dioxygenase activity. Laccase and 1,2-dioxygenase increased 2-fold and 3-fold, respectively in 30 d. TPH degradation rate also increased more than 21% in 30 d.

For a selection of suitable surfactant for co-culture, Tween 80 (T-80) revealed capable of enhancing both the ligninolytic enzymes and dioxygenase enzymes activity. As shown in Figure 9 (a) enhancement by T-40 was effective not only for low concentration but also a high concentration of crude oil (15000 and 30000 ppm). Degradation of asphalt for 1000, 15000, and 30000 ppm were 80.0, 42.5, and 52.6%, respectively in 30 d. Surfactants may have a beneficial effect on the biodegradation of asphalt by increasing the diffusivity of biodegradable components and/or enhancing the bioavailability of asphalt via the surfactant/asphalt interaction. Surfactants enhance the bioavailability of pollutants by stabilizing the substrates in micelles, by solubilizing the pollutants in the aqueous phase, or by facilitating the transport of pollutants from the solid phase to the aqueous phase (Yanto & Tachibana 2014).

The extended bioremediation causes reduction of nutrient content required by the fungus. Malt extract (ME) is generally known as the most common media for growth and sporulation of fungi (Ajdari et al. 2011). In the present study, nutrient addition (ME solution) at several incubation days (15, 30, 60, and 90 d) could enhance TPH degradation significantly compared to no nutrient addition treatment (Figure 9). At concentration ME 10%, degradation rate of asphalt enhanced 6, 11, and 13%, respectively for 1000, 15000, and 30000 ppm in 30 d. ME solution could supply nutrient sources, such as carbon and nitrogen, for fungal co-culture system pregrown on kapok to grow well even after long incubation days. The good fungal growth consequently might stimulate the degrading related enzyme system.

Periodic biostimulation by a combination of surfactant, malt extract, and mineral inducer treatment of the fungal co-culture improved the biodegradation rates of asphalt, HOA, and HOC during extended bioremediation compared to the control and only malt extract addition treatment. As shown in Figure 10, the fungal co-culture of NG007/S133 treatment enhanced biodegradation rates by 31% and 39 % for asphalt at a concentration of 15000 and 30000 ppm, respectively. Degradation rates of HOC increased more than 19% at both concentrations of 15000 and 30000 ppm. Degradation rates of HOA were the highest results among three crude oil used in the experiment with degradation rates more than 95% and 93%, respectively at concentration 15000 and 30000 ppm, respectively. Low concentration of crude oil (in HOC and HOA

treatment) was almost completely degraded by the fungal co-culture. These results indicated that the crude oil degradation by the fungal co-culture was strongly influenced by the crude oil type. HOA, a light crude oil, could be more easily degraded than heavier crude oil types such as HOC and asphalt

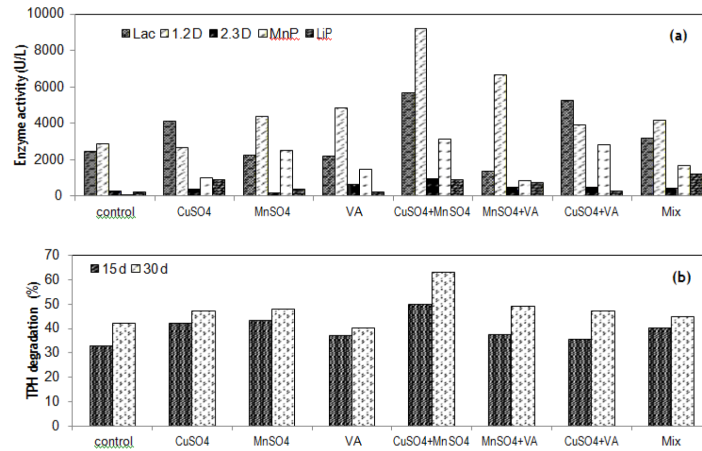

Fig. 8. Effect of enzyme inducer on degrading related enzymes production (a) and TPH degradation by the fungal co-culture (b)

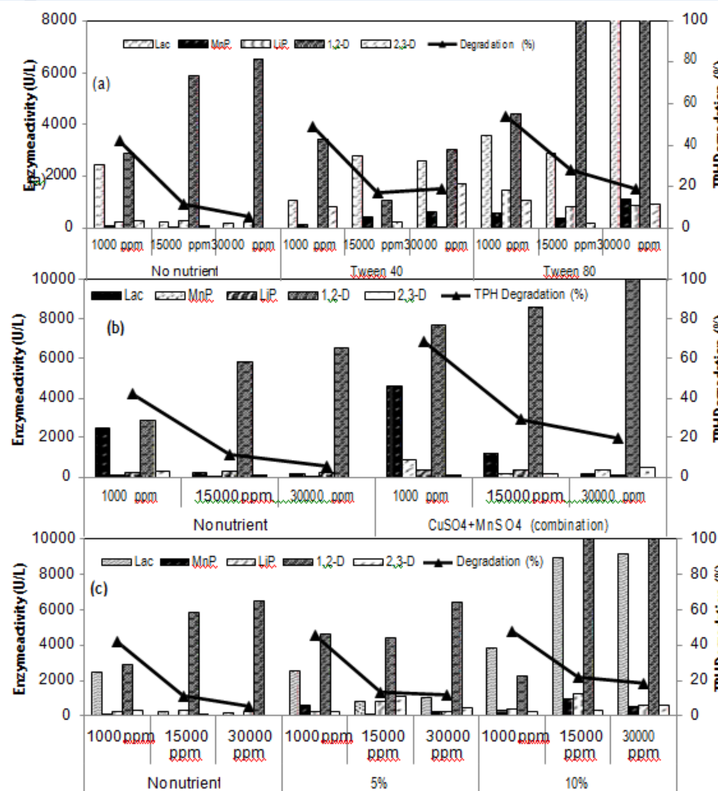

Fig. 9. Effect of surfactant (a), enzyme inducer (b), malt extract concentration (c) on degrading related enzymes production and TPH degradation by the fungal co-culture

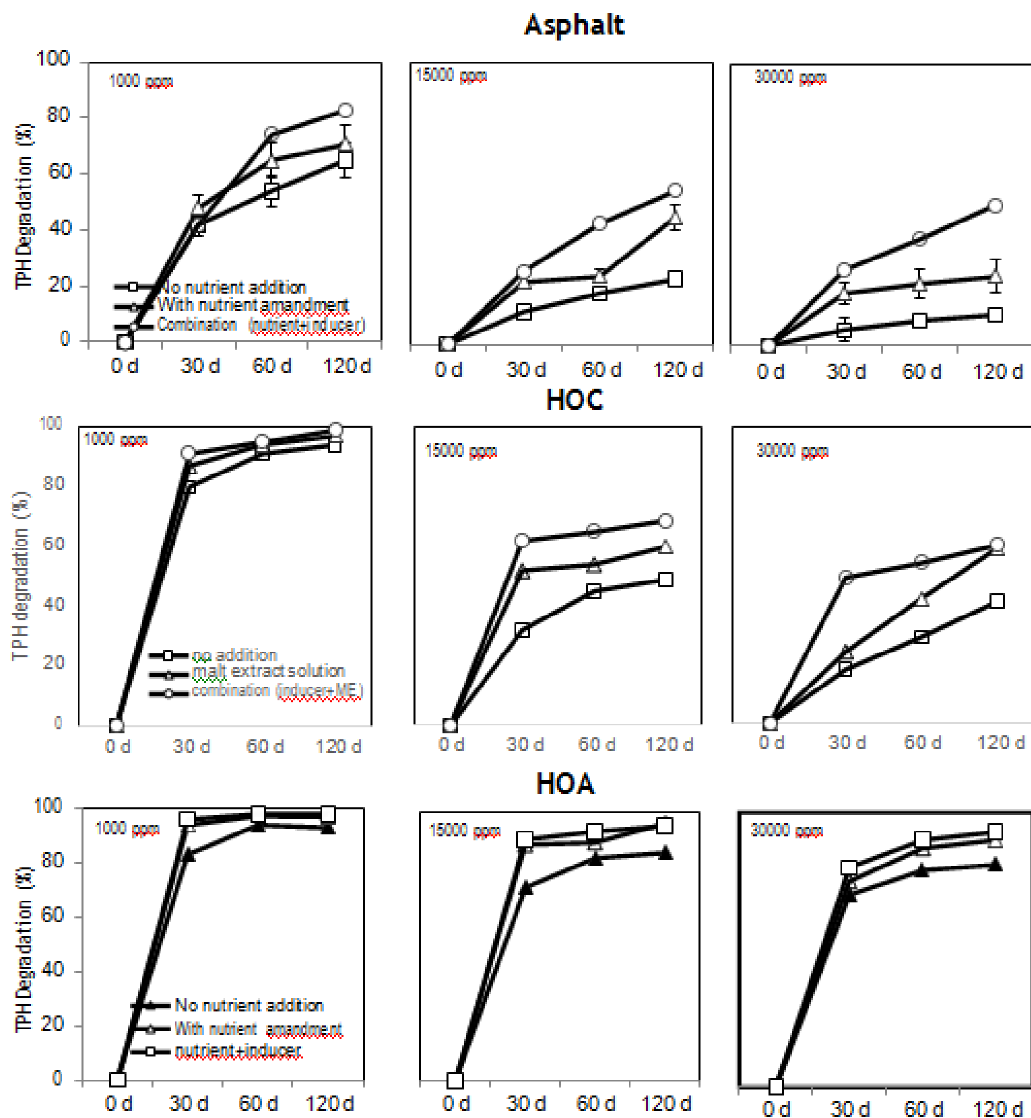

Fig. 10. Degradation of asphalt, HOC, and HOA with combination nutrient amendment at three concentrations 1000 ppm; 15000 ppm; and 30000 ppm

The enzymatic activities of the fungal co-culture NG007/S133 with or without periodic biostimulation in crude oil biodegradation are shown in Table 3 for asphalt, Table 4 for HOC, and Table 5 for HOA. In all treatments (with periodic biostimulation and control), activities generally decreased after 30 d depending on the type of enzyme system. In asphalt, the co-culture produced maximum activities for 1,2-dioxygenase and laccase (194.7 and 94.9 U/g, respectively) at a concentration of 30000 ppm in 30 d incubation. These activities significantly decreased after that until 60 d. Activities of only approximately 12.1 and 0.47 U/g were detected at the end of the experiment (120 d).

The same results were also observed in the biodegradation of HOC and HOA which indicated decreases in enzymatic activities in the co-culture after the maximum enzyme activities in 30 d. Biostimulation with a combination of malt extract, surfactant, and mineral inducer could significantly enhance the enzyme activities, especially in 60 and 120 d incubation. In asphalt, the treatment enhanced laccase more than 6-fold, 5-fold, and 8-fold at concentration 1000, 15000, and 30000 ppm, respectively in 60 d. 1,2-dioxygenase, as the enzyme that has a role in the aliphatic degradation of crude oil, also increased 4-fold, 8-fold, and 3-fold at

concentration 1000, 15000, and 30000 ppm in the end of incubation day (120 d).

Extended bioremediation will lead to a reduction of nutrient needed by the co-culture to grow and produce their metabolic system such as degrading related enzymes (Hadibarata & Kristanti 2012). Decreases in the activity of each enzyme after the optimum point may occur in a specific manner due to the different biochemical properties of each enzyme. However, it is reasonable to speculate that reductions in growth nutrient levels in the medium (sea sand) may be the main reason for the decreases observed in the enzymatic activities of co-cultures during extended biodegradation. This study demonstrated that the periodic biostimulation of malt extract as a nutrient source, tween 80 as a surfactant, and a mineral mixture of MnSO4 and CuSO4 as enzyme inducers enhanced enzymatic activities in the co-culture, even after 120 d, by contributing to a new generation of mycelia during biodegradation. Also, nutrient biostimulation may maintain enzymatic activities, leading to the greater degradation of TPH than that without nutrient biostimulation treatment.

Table 3. Enzyme activity degradation of asphalt with combination nutrient amendment at three concentrations

Concentration (ppm)	Incubation day	Treatment	Enzyme activity (U/gram)				
			Lac	1.2D	LiP	MnP	2.3 D
	30 d	No nutrient addition	14.75	17.31	1.81	0.53	1.4
		With nutrient	22.85	13.5	1.31	1.89	2.46
		Combination	14.7	9.72	0.73	4.03	2.98
	60 d	No nutrient addition	1.02	16.65	0.49	0.47	3.5
1000 ppm		With nutrient	4.05	19.54	0.08	0.99	2.54
		Combination	6.81	21.89	0.007	1.24	3.01
	120 d	No nutrient addition	0	0.68	0	0	0.14
		With nutrient	0	1.44	0	0.21	0.34
		Combination	0.02	2.53	0.06	0.46	0.25
	30 d	No nutrient addition	1.32	35.1	0.49	0.25	1.76
		With nutrient	53.87	128.07	1.8	5.74	7.64
		Combination	70.21	177.6	1.22	7.17	5.32
	60 d	No nutrient addition	2.04	22.28	0	0.9	12.16
15000 ppm		With nutrient	10.56	79.48	0.28	2.61	11.27
		Combination	11.9	80.98	0.67	2.89	10.45
	120 d	No nutrient addition	0	1.18	0	0	0.22
		With nutrient	0	7.59	0	0.53	0.63
		Combination	0.15	8.9	0.08	0.72	1.24
	30 d	No nutrient addition	1.32	35.1	0.49	0.25	1.76
		With nutrient	53.87	128.07	1.8	5.74	7.64
		Combination	94.88	194.78	2.71	5.74	5.79
	60 d	No nutrient addition	3.91	35.44	0	0.72	2.65
30000 ppm		With nutrient	22.74	112.79	1.52	1.4	4.07
		Combination	24.51	120.9	2.31	1.54	2.3
	120 d	No nutrient addition	0	4.07	0	0	0.07
		With nutrient	0	9.2	0.54	0.27	0.38
		Combination	0.47	12.71	0.43	0.98	0.34

Table 4. Enzyme activity degradation of HOC with combination nutrient amendment at three concentrations

Concentration (ppm)	Incubation day	Treatment	Enzyme activity (U/gram)				
			Lac	1,2-D	LiP	MnP	2.3 D
	30 d	No nutrient addition	24.65	60.37	1.39	9.53	1.86
		With nutrient	42.61	73.94	1.50	8.04	2.29
		Combination	57.18	53.73	10.31	10.73	7.69
	60 d	No nutrient addition	5.50	7.39	0.11	1.67	0.20
1000 ppm		With nutrient	20.61	41.83	1.09	6.95	0.49
		Combination	26.15	43.13	5.41	8.11	3.52
	120 d	No nutrient addition	0.00	0.67	0.00	0.14	0.00
		With nutrient	0.94	4.76	0.05	1.00	0.36
		Combination	3.15	7.41	0.79	0.42	0.58
	30 d	No nutrient addition	126.34	112.39	2.22	18.54	2.61
		With nutrient	151.50	85.65	2.52	21.30	2.22
		Combination	207.87	176.85	6.14	22.41	12.77
	60 d	No nutrient addition	14.15	15.26	0.20	2.20	0.32
15000 ppm		With nutrient	51.65	28.29	0.90	8.23	5.40
		Combination	61.54	52.41	1.42	8.21	8.72
	120 d	No nutrient addition	0.00	1.34	0.00	0.28	0.10
		With nutrient	33.40	11.76	1.15	2.22	0.64
		Combination	45.68	8.30	2.15	1.94	0.36
	30 d	No nutrient addition	192.24	170.13	3.77	32.72	6.30
		With nutrient	275.34	192.00	3.36	36.48	7.20
		Combination	283.35	241.65	28.47	27.59	12.77
	60 d	No nutrient addition	53.15	34.06	0.39	2.83	0.59
30000 ppm		With nutrient	154.02	147.27	1.28	13.09	5.11
		Combination	179.15	159.59	5.62	8.21	9.81
	120 d	No nutrient addition	0.14	1.90	0.00	0.31	0.08
		With nutrient	41.44	27.27	2.35	8.74	0.97
		Combination	44.26	26.80	17.03	13.61	1.31

Conclusion

The co-culture of NG007/S133 with ratio 25/75 was selected as the most suitable fungal co-culture among four fungal combinations studied for crude oil degradation at sea sand medium. This study also demonstrated that the periodic biostimulation of malt extract (10%) as a nutrient source, tween 80 (0.5%) as a surfactant, and mineral mixture of $MnSO_4$ and $CuSO_4$ (1 mM) as an enzyme inducer enhanced enzymatic activities in the co-culture, even after 120 d, by contributing to a new generation of mycelia during biodegradation. The nutrient biostimulation was found to enhance enzymatic activities, leading to the greater degradation of TPH than control treatment (without nutrient biostimulation).

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Table 5. Enzyme activity degradation of HOA with combination nutrient amendment at three concentrations

Concentration (ppm)	Incubation day	Treatment	Enzyme activity (U/gram)				
			Lac	1.2D	LiP	MnP	2.3 D
	30 d	No nutrient addition	20.72	29.44	2.45	2.20	0.06
		With nutrient	27.65	17.28	1.31	1.89	2.46
		Combination	35.09	32.67	2.39	2.76	1.90
	60 d	No nutrient addition	1.51	8.84	0.69	0.67	0.00
1000 ppm		With nutrient	4.55	13.55	3.10	1.47	0.05
		Combination	6.57	15.62	2.45	2.08	0.12
	120 d	No nutrient addition	0.74	0.58	0.00	0.17	0.02
		With nutrient	2.24	0.89	0.08	0.38	0.00
		Combination	3.78	1.93	1.65	1.37	0.04
	30 d	No nutrient addition	32.80	51.98	3.09	2.77	1.27
		With nutrient	41.14	58.80	1.80	5.74	7.64
		Combination	48.33	62.89	4.67	6.08	6.49
	60 d	No nutrient addition	2.43	20.54	0.81	1.57	0.40
15000 ppm		With nutrient	7.78	50.25	4.06	2.83	2.90
		Combination	9.56	58.79	3.88	4.96	3.24
	120 d	No nutrient addition	3.28	1.28	0.35	0.25	0.19
		With nutrient	10.49	3.13	1.75	0.45	1.39
		Combination	7.64	10.98	2.67	2.85	2.44
	30 d	No nutrient addition	67.25	95.33	2.71	12.02	2.16
		With nutrient	119.26	100.73	3.60	3.29	3.58
		Combination	132.88	105.84	6.87	6.43	4.21
	60 d	No nutrient addition	17.90	23.48	1.35	2.13	0.75
30000 ppm		With nutrient	35.39	74.80	4.12	4.01	5.26
		Combination	46.51	80.43	3.86	4.65	6.44
	120 d	No nutrient addition	7.25	8.08	0.46	1.12	0.17
		With nutrient	14.34	25.75	1.39	2.10	1.17
		Combination	18.96	56.42	1.20	2.89	4.29

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Effect of the Exposure of North- and South-Pole of Static Magnet on Biomass and Lipid Productivity of Lipid-Producing Microalgae, *Choricystis* sp. LBB13-AL045

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Abstract

The effect of the exposure of both pole of a static magnet on biomass and lipid productivity of lipid-producing microalgae, *Choricystis* sp., was studied. The exposure of static magnetic field onto microalgae culture was conducted for 24h during its cultivation. The results showed that the microalgae culture exposed by the south-pole magnetic field gave the best enhancement on growth (biomass productivity) and lipid productivity. The application of the south-pole magnetic field gave the best result in enhancing microalgae's biomass productivity and lipid productivity (81,12 ± 0,56 mg biomass. L^{-1}. day^{-1} and 39,17 ± 0,56 mg lipid. L^{-1}. day^{-1}, respectively) compared to the north-pole magnetic field (48,47 ± 1,53 mg biomass. L^{-1}. day^{-1} and 30,90 ± 1,02 mg lipid. L^{-1}. day^{-1}) and the control treatment (39,31 ± 0,42 mg biomass. L^{-1}. day^{-1} and 20,79 ± 0,77 mg lipid. L^{-1}. day^{-1}). The enhancement of the biomass and lipid productivity by the south-pole magnetic field exposure could reach up to more than two times compared to the control treatment. On the other hand, the exposure of the north-pole magnetic field showed different role in the cultivation of lipid-producing microalgae, *Choricystis* sp. LBB13-AL045. It (63,76 ± 0,09%) gave significant increase in the microalgal lipid content over the control (52,92 ± 2,51%) and the south-pole magnetic field (48,29 ± 1,02%).

Keywords: Magnetic field, north-pole, south-pole, microalgae, biomass productivity, lipid productivity, Choricystis.

Introduction

Microalgae have attracted considerable attention due to their high potency to be used as biofuel feedstock, especially biodiesel (Chisti 2007). Microalgal biomass was known as great source of lipids and other metabolites, such as carbohydrates, proteins, antioxidants and pigments (Chen & Vaidyanathan 2013). The utilization of microalgal biomass as biodiesel feedstock in commercial scale required high amount of biomass production which could be achieved by optimizing the growth process (Liu et al. 2017). Various techniques had been applied with the intention to increase microalgal biomass and lipid productivity (Abu-ghosh et al. 2018, Ramanna et al. 2017). One such promising technique was the exposure of static magnetic field to microalgal culture (Deamici et al. 2016a).

The potential link between magnetic field and the effect caused on living microorganism, including microalgae, is due to the fact that it causes oxidative stress, i.e., the magnetic field can alter the energy levels and orientation of the spin of electrons, thus increasing activity, concentration and time life of free radicals (Repacholi & Greenebaum 1999, Sahebjamei et al. 2007). The effect of the magnetic field exposure on the metabolism of microalgae has been studied, with an optimization of biotechnological processes, such as wastewater treatment (Tu et al. 2015), protein production (Yang et al. 2011), biomass production (Deamici et al. 2016a, Luna et al. 2011, Small et al. 2012) and pigments concentrations (Deamici et al. 2016b)

However, among the authors who conducting the research associated to microalgal growth and magnetic field didn't mention which pole that effectively enhanced microalgal growth. On the other hand, Davis and Rawls stated that north- and south-pole of a magnet giving opposed influence for microorganism growth (Davis & Rawls 1974). Therefore, the aiming of this study was at

observing the effect of the exposure of north- and south-pole of a static magnet in enhancing the productivity of biomass and lipid of lipid-producing microalgae, *Choricystis* sp. LBB13-AL045.

Materials and Methods

Microalgae and cultivation condition

Choricystis sp. LIPI-LBB13-AL045 was part of the microalgal collection of Bioenergy and Bioprocess Laboratory, Research Center for Biotechnology (LIPI), and was isolated from Bengkulu province, Indonesia. The microalgal culture was maintained and grown in a medium containing (mM) 5.95 $NaHCO_3$; 1.65 $NaNO_3$; 0.47 $(NH_4)_2SO_4$; 0.12 $MgSO_4.7H_2O$; 0.074 KH_2PO_4; 0.029 K_2HPO_4; 0.008 $C_6H_5FeO_7$; and 0.01 $C_6H_8O_7$. Cells were grown in a 600-mL bottle containing 500 mL of the microalgal culture with aeration and under continuous illumination with the light intensity of 20000 lux. White LED flood light (50 Watt) was used as a light source and its light intensity was measured at the outer-surface of the bottle (on the middle of the culture's height) by using light meter.

Application of north- and south-pole of magnet in microalgal culture

Neodymium magnets N50 with the dimension of 30 x 20 x 10 mm (length x width x thickness) were used in this study. The field magnitude of the magnet on its center-surface was 4082 Gauss (based on the calculation of online rectangle-magnetic calculator addressed at https://www.dextermag.com/field-calculations-for-rectangle/). The pole of the magnet was determined by using compass. In the microalgal cultivation, the magnet was placed inside the microalgal culture, on the bottom of the reactor bottle, during cultivation with the north- or south-pole intentionally facing upwards.

Growth measurement

Microalgae's growth performance during cultivation in the presence of magnetic field was measured by counting the cells number under 400x magnification of the light microscope using Neubauerhaemocytometer. A growth curve of microalgae under each treatment was generated by plotting the cell number against time (days).

Microalgal Growth Property

The microalgal strains of *Choricystis* sp. LBB13-AL045 were cultivated in aerated bottle and cultured at room temperature under continuous illumination with the light intensity of 20000 Lux for 9 days. The specific growth rate of each strain was calculated based on the equation below(Nascimento et al., 2013):

$$\mu = \ln (N_y/N_x)/ (t_y - t_x)$$

N_x : the cell number at the start (t_x) of the logarithmic growth phase
N_y :the cell number at the end (t_y) of the logarithmic growth phase

Whereas the cell doubling time (D) were computed from the growth rate (μ) by employing this equation:

$$D = \frac{\ln 2}{\mu}$$

Biomass productivity (P_{dwt}) was determined as the dry biomass produced during the logarithmic growth phase. While lipid productivity (L_p) was calculated according to the equation (Nascimento et al. 2013):

$$L_p = P_{dwt} \times L_c$$

L_p : Lipid productivity (mg/L/day)
P_{dwt} : Biomass productivity (mg/L/day)
L_c: Lipid content (%)

Determination of Lipid Content

Lipid content reported as percentage of the total biomass (in % dry weight). Lipid extraction was conducted by adapting the modified method from Ryckebosch et al. (2012). Chloroform : methanol = 1: 1 were used as mix-solvent in the microalgal lipid extraction. 6 mL of mix-solvent was added to 100 mg microalgae biomass and the tube was vortex mixed for 30 s. 2 mL of mix-solvent and water were then added and the tube was

vortex mixed again and subsequently centrifuged at 2000 rpm for 10 min. The aqueous layer was removed and the solvent layer was transferred into the clear tube. The remaining solid were re-extracted with 4 mL mix-solvent. The re-extraction of lipid was repeated until the remaining-microalgae biomass turned to be colorless. The solvent was removed by letting it evaporated in the open air and the lipid content was determined gravimetrically. The extraction was performed in triplicate. The resulting percentage of extracted lipids is the sum of two extractions performed in series.

Result and Discussion

Growth Curve

In Figure 1, the microalgal growth performance in 9 day-cultivation under the magnetic field and control treatment could be compared. The exposure of south-pole magnetic field showed the best performance over the control and north-pole magnetic field treatment. Compared to control treatment, south-pole magnetic field exposure could obviously enhance *Choricystis*' growth better. Both condition showed that the microalgae could reach the initial stationary phase in 7 days, while north-pole magnetic field exposure could shorten the time for the algae to reach the initial stationary phase (6 days). This means that the exposure of north-pole magnetic field to microalgae culture could increase its biomass and lipid productivity compared to the treatment with no magnetic field (as shown in Table 1 and 2). In large scale microalgal biomass production, such benefit derived from the north-pole magnetic field exposure could significantly reduce the cost usage (Kadir et al. 2018).

Both treatments of magnetic field exposure showed predominance over the control treatment with each different characteristic. However, south-pole magnetic field undoubtedly showed best performance in enhancing the microalgal growth (cell number) over the other treatments. According to Davis and Rawls (1974), the exposure of south-pole magnetic field to the plant seed could increase the carbon dioxide intake. The same mechanism may also be applied to microalgae during its cultivation; further research would surely be necessitated. The enhancement of *Choricystis* sp. cell number under 0.4082 T (4082 Gauss) of south-pole magnetic field resulted higher cellular densities in the shorter duration of time compared to control. This result was coherent with the study conducted by Luna et al. (2011), they reported that the magnetic field of 0,03 T could enhance higher cellular densities of *Chlorella vulgaris* in shorter culture time.

Enhancement of Microalgal Growth

The cell growth profile of *Choricystis*sp LIPI-LBB13-AL045 under the influence of north- and south-pole magnetic field compared to control treatment are shown in Table 1. The growth parameter of magnetic field treatment shows better result over the control treatment in term of microalgal growth performance. According to Small et al. (2012), the mechanism of magnetic field in increasing microalgal growth was associated to the increased of oxidative stress and free radical in microalgal cell metabolism. Another mechanism was suggested by Li et al. (2007), they stated that enhanced growth rates may be associated with the increased uptake of nutrients due to permeability of the membrane. Thus, the high rate of nitrogen and phosphorus consumption would take place and cause the rapid growth of microalgae. The growth enhancement of various species of microalgae by the magnetic field exposure have been reported, such as in *Chlorella vulgaris* (Luna et al. 2011, Yang et al. 2011), *Chlorella kessleri*(Small et al. 2012), *Chlorella fusca* (Deamici et al. 2016a), *Spirulina*sp(Deamici et al. 2016b) and *Scenedesmus obliquus*(Tu et al. 2015). This study which used another species of microalgae (*Choricystis* sp.) gives the coherent results with the previous studies and confirms that magnetic field is suitable method to increase microalgal growth.

Of three treatments in this study, the exposure of south-pole magnetic field gave the highest value of specific growth rate (0.468 day^{-1}), followed by north-pole magnetic field (0.401 day^{-1})and control treatment (0.381 day^{-1}), respectively. According to Davis & Rawls (1974), the effect of south-pole energies from the magnet for the plant seed was increasing carbon dioxide intake and also fertilizer acceptance, the same mechanism could also be

considered occurred in microalgae when exposed to south-pole magnetic field.

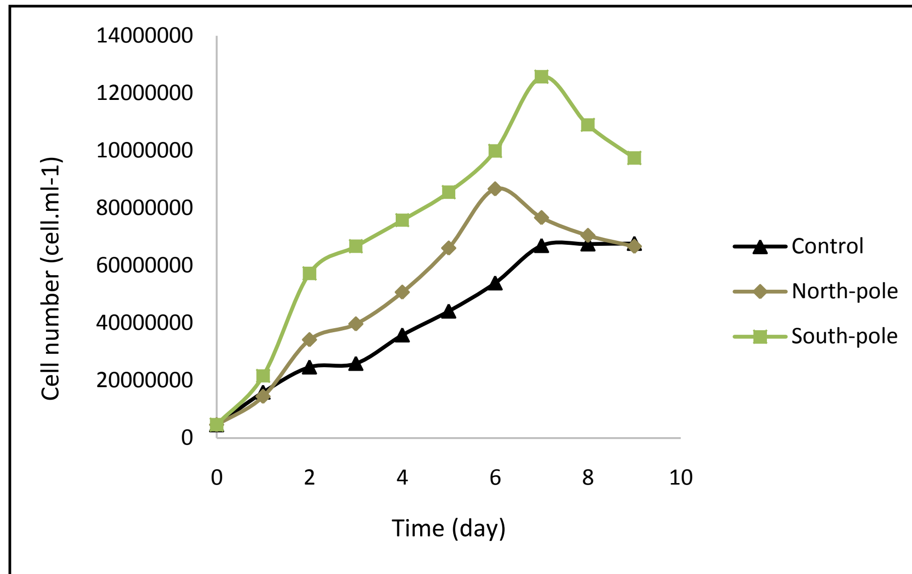

Fig. 1. The growth curve of Choricystis sp. LIPI-LBB13-AL045 under the treatment of north- and south-pole magnetic field influence and control treatment for 9 days of cultivation.

Table 1. Growth profile of *Choricystis* sp. LIPI-LBB13-AL045 microalgae strain cultivated under the influence of north- and south-pole magnetic field.

Magnetic Treatment	Specific growth rate (day^{-1})	Doubling time (day)	Biomass productivity (mg L^{-1} day^{-1})
Control	0.381	1.82	39.31 ± 0.42
North-pole	0.401	1.73	48,47 ± 1.53
South-pole	0.468	1.48	81.12 ± 0.56

Since all metabolic reactions (including in microalgae) are based on the difference of electrical charges and ions of the system (Teng 2005), then all magnetic forces, either south- or north-pole magnetic force, can cause changes in metabolization in the microalgal cells (Santos et al. 2017). In this study, it particularly seems that the physiological and biochemical constitution in *Choricystis* sp. cells is more susceptible to the action of south-pole magnetic field rather than to north-pole magnetic field. This high susceptibility to south-pole magnetic field would deliver higher microalgal biomass productivity compared to control and north-pole magnetic field treatments, as shown in Table 1. The result of this study was consistent with the result of the research conducted by Small et al. (2012), Bauer et al. (2017) and Luna et al. (2011) in term of increasing microalgal biomass production.

Table 2. Lipid content and lipid productivity of *Choricystis* sp. LIPI-LBB13-AL045 microalgae strain cultivated under the influence of north- and south-pole magnetic field.

Magnetic Treatment	Lipid content (%)	Lipid productivity (mg L^{-1} day^{-1})
Control	52.92 ± 2.51	20.79 ± 0.77
North-pole	63.76 ± 0.09	30.90 ± 1.02
South-pole	48.29 ± 1.02	39.17 ± 0.56

Enhancement of Lipid Content and Lipid Productivity

Table 2 shows comparison of lipid content and lipid productivity of *Choricystis* sp. LIPI-LBB13-AL045 between culture treated with the magnetic field and control. Overall, in regards to lipid production, microalgal culture treated by magnetic field exposure shows better result compared to the culture with no magnetic field exposure. The enhancement of lipid content up to 21.2% compared to the control treatment could be seen in the microalgae cultivated under the exposure of north-pole magnetic field. Bauer et al. (2017) was also reported that lipid content of *Chlorella kessleri* was increased 13.7% under magnetic field exposure compared to

controlculture. The growth and lipid content of *Choricystis* sp. LIPI-LBB13-AL045 culture treated under north-pole magnetic field exposure was higher compared to the control treatment, but, in regards to microalgal growth performance, the influence of south-pole magnetic field was giving the best result among others. Those results indicate that the exposure of north-pole magnetic field on microalgal culture is suitable for increasing microalgal lipid content per dry-weight basis, while the exposure of south-pole magnetic field was suitable for enhancing microalgal growth (biomass production). Such benefits could be applied for further microalgal cultivation in order to maximize microalgal growth by applying south-pole magnetic field during logarithmic phase and to maximize microalgal lipid content by turning the magnetic field into the north-pole one when microalgae initiate to enter its stationary growth phase. Thus, maximum lipid productivity, as a key characteristic for biodiesel production from microalgae (Griffiths & Harrison 2009), could be achieved by means of magnetic field exposure. Moreover, magnet itself is a very economical tool due to its low price that would possibly make the large scale microalgal biomass production become economically feasible (Bauer et al. 2017). However, further research in regards to magnetic field application in large scale photobioreactor would be necessitated.

Conclusions

The application of magnetic field in the cultivation of lipid-producing microalgae, *Choricystis* sp. LIPI-LBB13-AL045, is proven to be able to increase microalgal biomass and lipid productivity regardless of which pole is applied. The application of north- and south-pole magnetic field gave distinguished results for microalgal growth and lipid production as well. The growth of microalgae was significantly improved under the exposure of south-pole magnetic field, while the application of north-pole magnetic field to *Choricystis* culture could increase its lipid content. However, such benefits would eventually affect the microalgal lipid productivity in significant and positive way.

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Development of Gypsum Particleboard (GPB) made from Natural and PlantationRed MerantiWood

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Abstract

Gypsum board is the panel products that consist of gypsum, and a paper surfacing on the face, back and long edges This product is water and fire resistance, termite resistance, and can be used for some purposes. Gypsum board were developed from natural and plantation of red meranti (*Shorealeprosula*) obtained from PT Sari BumiKusuma. The purpose of this study was to characterize the physical and mechanical properties of gypsum particleboard by effects of red meranti wood particles-gypsum ratios.The log was processed into particle using ring flaker. Before gypsum board manufacture, hydration temperature was observed to know the compatibility of gypsum-wood particles. The ratio between wood particles to gypsum used varied to 1:2.3, 1:4, and 1:9 based on weight for natural and plantation red meranti. Water was used about 60%-100% of gypsum weight. Borax ($Na_2B_4O_7$) at 1% of gypsum weight was added. Firstly, borax was dissolved in water, then wood particles and gypsum were mixed until evenly in the borax solution. The mixtures were hand-matt formed and cold pressed with a pressure of 10 MPa for 30 minutes. The size of the board was 25x25x1.2 cm with a targeted density of 1.2 g/cm^3. The boards were kept for 21 days before tested. Physical and mechanical tests were conducted according to JIS A5417. Results showed that the density of boards was decreased and the moisture content, thickness swelling and water absorption were increased by increasing amount of wood particles. In the other hand the mechanical properties also decreased by adding wood particles. The physical and mechanical properties of gypsum board from red meranti natural wood particles showed a little higher than from plantation ones.

Keywords : Gypsum board, red meranti, natural, plantation.

Introduction

Red meranti (*Shorealeprosula*) is one of the fastest growing species of Dipterocarp in South East Asia rainforestregions such as Thailand, Malaysia,and Indonesia (Appanah & Weinland 1993). This species can grow well in a location that has good drainage, usually hilly areas with a high up to 700 m above sea level. This species generally found in areas with climate types A and B, the lands latosol, red-yellow and yellow podzolic. *S. leprosula*wood has a density of 300-865 kg/m^3 at 15% moisture content (Lemmens & Soerianegara 1994). The wood is primarily used for veneer and plywood, besides it can also be used for residential buildings and can also be used as a shipping timber, packing crates, coffins, and musical instruments (Martawijaya et al. 1981).

Gypsum board is the panel products that consist of gypsum, and a paper surfacing on the face, back,and long edges. It has been primarily used for residential construction, such as wall and roof sheathing/ceilings.

As a good building construction material, gypsum board has a number of outstanding advantages such as non-formaldehyde emission, high fire-retardant property, sound isolation, durability, ease of installation, economy, and versatility.

On the other hand, some researcher (Deng and Furuno 2002, Bekhta and Dobrowolska 2006, Espinoza-Herrera & Cloutler 2011) were used wood particles as strengthing materials on the gypsum board manufacturing (Gypsum particle board, GPB). The wood particles were obtained from waste materials generated by lumber and wood panel manufacturers. The use of these particles promotes the reclamation

of wood waste that would otherwise be sent to landfills.

In order to utilize the potential wood particles as by-product from wood industries, the wood particles of natural and plantation red meranti were used in the manufacturing of GPB.The aim of this study was to determine the chemical composition of red meranti natural and plantation woods, to investigate the compatibility of a mixture between gypsum, red merantiwood particles and borax (as retarder), and to characterize the physical and mechanical properties of gypsum particleboard by effects of red meranti wood particles-gypsum ratios.

Materials and Methods

The natural red meranti and 11-year-old plantation red meranti (*Shorealeprosula*) obtained from PT Sari BumiKusumawas used to observe hydration test and manufacture GPB. Gypsum based on Indonesian standard (SNI) was used as a binder, and borax ($Na_2B_4O_7$) at 1% of gypsum weight was used as a retardant.

Chemical content analysis

The chemical contents of natural and plantation red merantiwere determined by using the standard of TAPPI 222 om-88 for lignin, TAPPI T17m-55 for cellulose, TAPPI 204 om-88 for solubility in alcohol benzene, and method from Browning (1967) to measure the content of holocellulose, cellulose, and hemicellulose.

Hydration Test

The hydration test method used was the same as described by Hermawan (2001). The hydration temperature was measured in an insulated box. The gypsum/wood ratio of 6.9: 1.0 and powder size of 20 pass/30 on the mesh. Borax was added to each mixture in the range of 0% - 2.0% based on gypsum weight. The mass water/gypsum ratios were 0.5. A thermocouple wire was inserted approximately at the center core of gypsum paste and connected to Graphtec midi LOGGER GL220. All the experiments were conducted at room temperature.

To calculate the inhibitory index (I) of the species it was used inthe following equation (Hofstrand et al. 1984) :

$$I = 100[((t_2 - t'_2)/t'_2)*((T'_2 - T_2)/T'_2)*((S'_2 - S_2)/S'_2)]$$

where

t_2 = time to reach a maximum temperature of the inhibited gypsum (wood-gypsum-water mixture)

t'_2 = time to reach a maximum temperature of the uninhibited gypsum (gypsum-water mixture

T_2 = Maximum temperature of the inhibited gypsum

T'_2= Maximum temperature of the uninhibited gypsum

Gypsum manufacture

In this study, the ratio between wood particles to gypsum used varied to 1:2.3, 1:4, and 1:9 based on weight for natural and plantation red meranti. Water was used about 60-100% of gypsum weight. Borax at 1.0% of gypsum weight was added for all ratio. Firstly, borax was dissolved in water, then wood particles and gypsum were mixed until evenly in the borax solution. The mixtures were hand-matt formed and cold pressed with a pressure of 10 MPa for 30 minutes. The size of the board was 25x25x1.2 cm with a targeted density of 1.2 g/cm^3. The boards were kept for 21 days before tested. Physical and mechanical tests such as density, thickness swelling, modulus of elasticity (MOE), modulus of rupture (MOR), and internal bond (IB) were conducted according to BISON standard for Gypsum Board with Reinforcement by Wood Flake (Hubner 1985).

Result and Discussion

Chemical content analysis

The value of the chemical compound of natural and plantation red meranti are presented in Table 1.

Table 1. The chemical content of red meranti

Chemical Components (%)	Red Meranti Natural Wood		Red Meranti Plantation Wood	
	Average	**SD**	**Average**	**SD**
Moisture Contents	7.17	*0.26*	10.56	*0.22*
Alcohol-Benzene Extrative	1.74	*0.18*	2.17	*0.52*
Cold Water Extractive	3.87	*0.62*	5.07	*0.68*
MC after Free Extractive	1.38	*0.20*	2.85	*0.33*
Lignin	37.28	*0.38*	39.62	*0.59*
Holocellulose	55.73	*0.79*	50.29	*0.64*
Alfa-celulose	33.23	*1.13*	29.51	*0.79*

The chemical content of natural red meranti is slightly different with plantation one. Natural red merantihasa lower amount of moisture content, alcohol benzene extractive, cold water extractive, and lignin, but have a larger amount of holocellulose and alfa-cellulose than plantation red meranti. The difference in the chemical content of these two kinds of wood is not significantly different, so it can be expected that even the physical and mechanical properties of GPB will not significantly different.

Hydration Test

The value of the inhibitory index is presented in Table 2.

Table 2. Inhibitory index of natural and plantation red meranti bonded gypsum particleboard

Mixtures	Inhibitory index (%)	
	Natural red meranti	Plantation red meranti
Gypsum/water without particles (as a control)	0.000	
Gypsum/water/particles	2.251	-0.091
Gypsum /water/particles/1.0% borax	4.088	2.677
Gypsum/ water/particles/1.5% borax	37.897	18.448
Gypsum /water/particles/2.0% borax	33.873	57.336

The mixture of natural and plantation red meranti without borax and with 1.0% borax as retarder were classified as "low inhibition" (I<10) (Okino et al. 2004).The mixture of natural and plantation red merantiwith 1.5% borax addition and the mixture of natural red merantiwith 2.0% borax addition were classified as "moderate inhibition" (10<I<50), while the mixture of plantation red meranti with 2.0% borax addition were classified as "high inhibition" (I>50).

Based on these data, the addition of borax can inhibit the hardening reaction of the mixture of gypsum and wood particles. The greater the borax content added, the greater the inhibitory index of the mixture. This is in accordance with the purpose of using borax as a retarder so that the gypsum mixture and particles are not too fast to harden. This can provide additional time in making GPB. The addition of 1% borax can be used for gypsum particleboard manufacture, because its low inhibition.

Physical and mechanical properties of GPB

The physical properties of gypsum particleboard (GPB) are presented in Table 3.
All of the GPB have the lower density (0.69-0.98 g/cm^3) compared to the gypsum board (1.11 g/cm^3) and the BISON standard (1.2%). This is related to the wood density (0.6-0.7 g/cm^3) that lower than gypsumdensity (>2.2 g/cm^3).

The values of moisture content (MC) of GPB ranged from 15.4% to 16.6%. It is shown that all of MC's values of GBP were higher than gypsum board (15.2%), and also were ahigher than the BISON Standard (6-12%). These phenomena happen because wood particles in GPB can absorb much water.

Table 3. Physical properties of Gypsum Particleboard

GPB	Density (g/cm^3)	MC (%)	TS (%)	WA (%)
Gypsum/water	1.11	15.2	1.06	27.12
Gypsum /water/Natural red meranti /1.0% borax with wood /gypsum ratio of 1:2.3	0.75	15.9	4.48	48.49

Gypsum/ water/ Natural red meranti /1.0% borax with wood /gypsum ratio of 1:4	0.98	15.4	2.56	29.74
Gypsum/ water/ Natural red meranti /1.0% borax with wood /gypsum ratio of 1:9	0.95	16.6	1.88	34.10
Gypsum /water/Plantation red meranti /1.0% borax with wood /gypsum ratio of 1:2.3	0.69	15.7	3.53	47.81
Gypsum/ water/ Plantation red meranti /1.0% borax with wood /gypsum ratio of 1:4	0.86	15.9	1.21	31.66
Gypsum/ water/ Plantation red meranti /1.0% borax with wood /gypsum ratio of 1:9	0.94	16.2	1.08	35.99

The values of thickness swelling (TS) of GPB ranged from 1.08% to 4.48%. All of TS values of GPB were higher than gypsum board without wood particle (1.06%), but some of GPB were met the BISON standard (2.5%). Similar with TS, the values of water absorption (WA) of GPB (29.74%-48.49%) also higher than gypsum board without wood particle (27.12%). These phenomena happen because the effect of wood particle addition that hasthe ability to absorb water.

The mechanical properties of GPB are presented in Table 4.The values of the modulus of rupture (MOR) ranged from 1.08 to 2.80 MPa. Compared to the MOR value of a gypsum board without wood particles (3.55 MPa) and BISON standard (8.3-8.8 MPa), these values are still smaller. This occurs because of the limited ability of gypsum to cover the addition of wood particles, and also the lower compatibility between gypsum and red meranti wood.

Similar with the MOR, the MOE values of GPB (133.9-2098.2MPa) were lower than the gypsum board without wood particles, but higher than BISON standard (44.1-49.0 MPa). It means that the wood particles addition can increase the elasticity of GPB.The internal bond (IB) values of GPB (0.217-2.33 kgf/cm^2) were also lower than the gypsum board without wood particle (2.16 MPa) except for GPB with natural red merantiwith wood particle/gypsum ratio of 1:9 (2.33 kgf/cm^2). All of the GPB were lower than BISON standard (3.9 kgf/cm^2)

Table 4. Mechanical properties of Gypsum Particleboard

GPB	MOR (MPa)	MOE (MPa)	IB (kgf/cm^2)	SW (N)
Gypsum/water	3.55	3418.6	2.16	92
Gypsum /water/Natural red meranti /1.0% borax with wood /gypsum ratio of 1:2.3	1.83	225.4	0.424	105
Gypsum/ water/ Natural red meranti /1.0% borax with wood /gypsum ratio of 1:4	2.80	589.4	0.995	183
Gypsum/ water/ Natural red meranti /1.0% borax with wood /gypsum ratio of 1:9	2.33	1865.5	2.330	134
Gypsum /water/Plantation red meranti /1.0% borax with wood /gypsum ratio of 1:2.3	1.08	133.9	0.217	33
Gypsum/ water/ Plantation red meranti /1.0% borax with wood /gypsum ratio of 1:4	1.77	551.1	0.707	130
Gypsum/ water/ Plantation red meranti /1.0% borax with wood /gypsum ratio of 1:9	2.22	2098.2	1.310	141

Different with others mechanical properties, the SW value of GPB (105-183 N) is greater than that of a gypsum board (92 N), except for GPB with plantation red merantiwith wood particle/gypsum ratio of 1:2.3. This shows that the particles in GPB have a better impact to be able to withstand screws

Conclusions

The density of boards was decreased and the moisture content, thickness swelling and water absorption were increased by increasing amount of wood particles. In the other hand the mechanical properties also decreased by adding wood particles. Based on the results, it can be concluded that the physical and

mechanical properties of red meranti natural wood particles showed a little higher than plantation ones.

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Thermal Characterizations and Color Changes of Natural Pulp Fibers as Reinforcing Agent in Polymer Composites

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Abstract

Manufacturing of thermoplastic-based biocomposites require a thermally stable of reinforcing agents, especially when natural fibers was incorporated into polymer. Here, we studied thermal behaviors of three types of natural fibers, namely kenaf (Kena), pineapple leaf fiber (Pine) and coconut fiber (Coco). Those fibers were isolated from fiber-bundle into single-fiber by soda, soda, and kraft pulping process, respectively. The thermal behaviors of pulp fibers were analyzed using thermogravimetry analysis (TGA) which the energy of activation (Ea) of pulp fibers degradation was analyzed using the Kissinger method, while the color changes of pulp fibers during pulping process and biocomposite manufacturing were observed under Colorimeter in CIELab color coordinates. Results showed that kenaf pulp fiber was thermally stable, suggested the highest potential for biocomposites manufacturing with activation energy (Ea) ~233.83 kJ/mol. The color changes can be visually help to analyze the degradation of natural fiber during manufacturing process quickly. These findings provide information for material selections during composite production in order to understand the decomposition of natural filler which further could be applied in biocomposites such as for automotive interior applications.

Keywords : Pulp, kenaf, the Kissinger, biocomposite, thermal behavior, color change

Introduction

There are several issues which most reasearcher concern and must be accounted when designing new advance composite materials. There are global warming issue, fossil fuel depletion, eco friendly technology and renewable materials (Holbery & Houston 2006, John et al. 2008). To the two later issues, incorporating natural fiber into composite material has gaining attention and resulted in huge revolution. It was reported in Reinforced plastics Magazine (2003) that by 2010 the weight of the cars will be decreased from 1,400 kg to 1,150 kg by substitution of 10% steel components and increase the use of 19% polymer matrix composite. The percentage is increase to date.

Introducing natural fibres into polymer composites has several drawbacks due to incompatibility with the hydrophobic polymer matrix, the tendency to form aggregates during processing, poor resistance to moisture and low thermal stability, which are greatly reduced the potential of natural fibers to be used as reinforcing agents in polymers (Saheb and Jog 1999; Subyakto et al. 2011 and; 2013).

In our previous study on characterization of pulp fibers i.e. their chemical compositions and morphology, it was showed that the choice of perfect fiber as reinforcing agent will lead to better interface interaction with polymer matrix (Masruchin & Subyakto 2012). We also assessed the crystal structure of pulps fiber which showed that kenaf pulp fiber has higher degree of crystallinity, suggested high modulus reinforcing agents (Masruchin 2011). Further, in order to comprehend the analysis of material selection of those three kinds of pulp fibers for composite materials applications, here, series of thermal analyses were conducted by studying the kinetic degradation, calculated the energy activation and studied the color changes of pulp fibers after processing and during manufacturing of biocomposites.

From this study, it could be taken industrial point of view which requires high speed analysis of product quality. We reported a result of assessment of color changes by using Colorimeter methods to address the initial

degradation of natural fiber composites. Thus techniques are non-destructive, easy and quick. Meanwhile, qualitative and quantitavie thermal degradation of natural fiber as reinforcing agent in polymer composite were also reported.

Materials and Methods

In this study, three kinds of fibers were used. Coconut and pineapple fibers were collected from local farmer industry in Sukabumi and Subang regency, respectively. Kenaf bast fiber was provided by PT. Abadi Barindo Autotech (ABA) from Pasuruan. Those three kinds of fibers normally can be used directly as reinforcing agent in polymer composite, however, different fiber lengths and morphology will affect and produce unisotropic polymer composite. Therefore, fiber modification will lead to homogen properties of fiber and it's composite. For that kind of purposes, all fibers were processed into pulp fibers to obtain single fiber dimension. The brightnes color of Whatman™ (What) filter paper number 5, Cat No 1005047 was used for color reference. Commercial bacterial cellulose (BC) as pure cellulose sources was bought from local producer which usually it uses for food comsuption (*Nata de-coco*) also used for reference.

Pulping process

In order to obtain higher yield of pulping process, the fibers were treated at different optimum pulping condition due to different chemical raw material compositions. For example, due to higher lignin content (John et al. 2008), coconut fibre was pulped using Kraft pulping method while kenaf and pineapple fiber were enough using soda treatment. In brief, Kraft pupling was processed as follows; active alkali and sulfidity was kept at 18% and 30%. Pulping was conducted at 165 ^{o}C for 4 h in total. On the other hand, soda process was applied for kenaf and pineapple with active alkali level 17% at 170 ^{o}C for 3 h and 10% at 160 ^{o}C for 3.5 h, respectively. All pulping process was kept at solid to liquid ratio at 1 to 4. Pulp fibers were fibrillated using a disc refiner for 8 cycles. Pulps were dried at 75 ^{o}C for three days and kept at sealed plastic for further characterizations. Pulp fibers were marked as kena for kenaf, pine for pineapple and coco for coconut fiber.

ATR-FTIR spectroscopy

Perkin Elmer FTIR Spectrometer Spectrum Two was used. ATR (Attenuated Total Reflectance) is an infra red technique commonly used to probe surface properties of materials rather than their bulk properties. The dried pulps were pressed on diamond plate surface at 80% pressure scale. Observation was recorded in the absorption range 600 – 800 cm^{-1} with accumulation of 25 scans at 4 cm^{-1} resolution. Those range was chosen to identified the crystal structure of the pulps which then was compared to bacterial cellulose —as pure cellulose sources (purity 98% cellulose) which mainly consist of triclinic (Iα) allomorph) —Sassi et al. 2000.

Thermogravimetry analysis (TGA)

Thermogravimetry (TG) and derivative thermogravimetry (DTG) analyses were carried out using a thermogravimetry instrument (PerkinElmer Thermogravimetry). About 5-6 mg of the dried powder pulp sample was heated from 25 to 600 °C at four different heating rates of 5, 10, 20 and 35 °C/min under atmosphere condition. The activation energy (Ea) of thermal fiber degradation was calculated using the Kissinger method.

In the Kissinger method (Kissinger 1957), $\ln(\beta/Tmax^2)$ is plotted against 1/Tmax from different series of heating rates. Tmax was the temperature maximum obtained from derivative thermogravimetry (DTG) peak of the pulp sample. The equation is as follow (Eq. 1):

$$ln\left(\frac{\beta}{Tmax^2}\right) = ln\frac{AR}{Ea} - \frac{Ea}{RTmax}$$

where β is the heating rate ($^{o}C.s^{-1}$) (Cho et al. 1998), Tmax is maximum temperature degradation, A is the pre-exponential factor (s^{-1}), R is the gas constant (8.3145 J mol^{-1} K^{-1}) and Ea is the apparent activation energy of the process (kJ mol^{-1}) (Fabiyi et al. 2009; Alwani et al. 2014).

Color changes measurement

Color changes of pulp fibers and their composites after pulping process and

composite manufacturing were observed using Colorimeter Konica Minolta CR-100 in CIELab coordinates (L* for lightness; a* for redness; b* for yellowness). Total color change was calculated using Eq.2 as follow:

$$E = \sqrt{L^2 + a^2 + b^2}$$

while the the relative total color changes ΔE, ΔL, Δa, Δb were obtained automatically from the equipment by measured the initial and final values of surface color. An increase in L means the sample is lightened.

Biocomposite manufacturing

Polymer composite of polypropylene (PP) reinforced with natural fiber was manufactured using industrial injection molding at PT. Inter Aneka Lestari Kimia, Tangerang. Prior to injection molded, the blend of PP and pulp fibers was mixed in compounding machine with addition of compatibilizer (MAPP, maleic acid grafted polypropylene) at 7.5% of total weight. The natural fiber loading was varied from 30, 40 and 50% of total weight. Before loaded to the injection molding feeder, the compound was crushed and sieved to provide a homogenous particle size. Injection temperature was 180 °C with about 12 seconds injection leadtime. The images of the injected samples are presented in the next section.

Results and Discussions

Most of thermoplastic polymer composites are processed at high temperatures, around 170-190 °C, for example: mixing process between polymer and natural fiber, extrusion process for pelletizing steps and finally, injection moulding for specimen purpose or product manufacturing purpose. In addition, natural fiber processing such as pulping process, chemical modification also require higher temperature processing. Those overwhelming exposure of temperature into natural fiber need to be assased in order to understand its effect on properties of polymer composite. This study observed the behavior of three kinds of natural pulp fibers under accelerate heating condition. Here we observed the ATR-FTIR spectra of pulp for underlining the crystal allomorph of the pulps, after that, the thermal stability of different pulp was measured using TG analysis and the energy activation was calculated. Finally, the color changes were also investigated.

ATR-FTIR spectroscopy

Beside cellulose, pulp fibers used in this study still consist of lignin, hemicellulose and extractive (Masruchin and Subyakto 2012). Those chemical components have different response to thermal exposure. The crystal allomorph of cellulose can be detected by ATR-FTIR spectra analysis. Native cellulose is a composite of two distinct crystalline structures, namely Iα (triclinic) and Iβ (monoclinic), whose fractions vary depending on the origin of the cellulose (Gumuskaya et al. 2006). The two absorbance peaks presence at 710 cm^{-1} and 750-760 cm^{-1} observed by FTIR are characteristics of monoclinic and triclinic allomorph, respectively (Sassi et al. 2000). Figure 1 shows the trasmittance of kenaf, pineapple and coconut pulps at wavenumber between 600 and 800 cm^{-1}. All of pulps show high intensity at 710 cm^{-1} and no absorbtion at around 760 cm^{-1} while bacterial cellulose, as a reference, shows peak at around 750 cm^{-1}. Those suggested that all pulp fibers consist of monoclinic (Iβ) type cellulose crystal structure. Monoclinic crystal is thermodynamically stable than triclinic structure, since it is not only dense, but also tends to be final product in the heat annealing of cellulose which is irreversible process (Sassi et al. 2000, Wan et al. 2017). Generally speaking, triclinic structure is more degradable than monoclinic structure. Therefore, all three pulps are suitable for composite reinforcing agents.

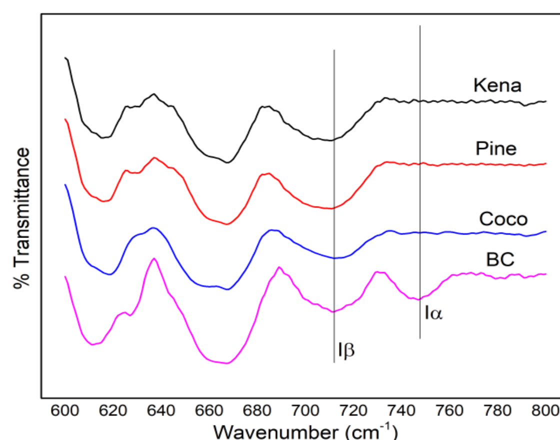

Fig. 1. ATR-FTIR pulps fiber

Thermogravimetry (TG) analysis

Figure 2 shows the TG analysis of different pulp fibers. Figure 2a depicts the percentage weight loss of pulp fiber over accelerate temperature in the presence of oxygen. It shows that the decrease of weight loss was in the order of kenaf<pineapple<coconut, suggested that kenaf has higher thermal stability. Figure 2b elaborates the derivative weight loss over temperature. From the results it shows that all pulps have a single major degradation peak, while natural fibres usually present two major degradation peaks regarding to non cellulosic and cellulose degradation peaks. This result might be caused by the present of oxygen in TGA analysis used in this study. Therefore, the degradation was more accelerated. In case of heating rate 10 ^{o}C/min the maximum degradation temperature degradation for kenaf, pineapple and coconut pulps fibers are 372.3, 376.3 and 368 ^{o}C, respectively. Pineapple pulp has a high degradation temperature degradation, probably due to it has higher cellulose content which reported in our previous publication (Masruchin and Subyakto 2012).

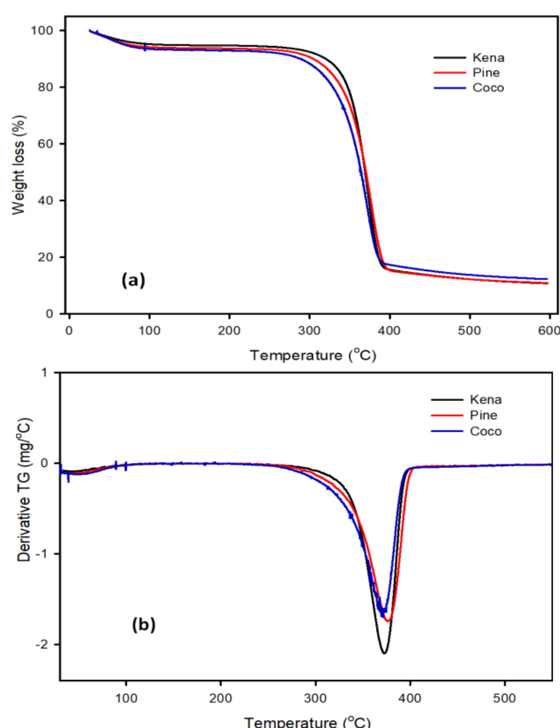

Fig. 2. (a) TGA and (b) DTG of three kinds of pulp fibers at heating rate 10 oC.min-1

The degradation temperature of natural fiber is expected to be related to the heat stability of its constituents, chemical compositions (Shebani et al. 2008). Kim et al. (2006) confirmed that the hemicelluloses degrade between 180 and 350 ^{o}C, the lignin degrades between 250 and 500 ^{o}C while the degradation of cellulose takes place between 275 and 350 ^{o}C. Therefore, a lower thermal stability of natural fiber can be attributed to high lignin and hemicelluloses content, while the high thermal stability is due to a higher cellulose content. However, at higher temperature, lignin appears to be more heat resistant than hemicelluloses and cellulose due to its low degradation rate (Tserki et al. 2005), which confirmed that the decomposition of lignin occurs in a wider temperature range (250 to 500 ^{o}C) and resulted in higher char residue as can be seen in Figure 2b.

From TG analysis with varying the heating rate, it can be obtained the maximum temperature (Tmax) for each natural fiber and calculated to obtain the activation energy (Ea) as fitted in Equation 1 and plotted to obtain the sloped of the equation (Figure 3).

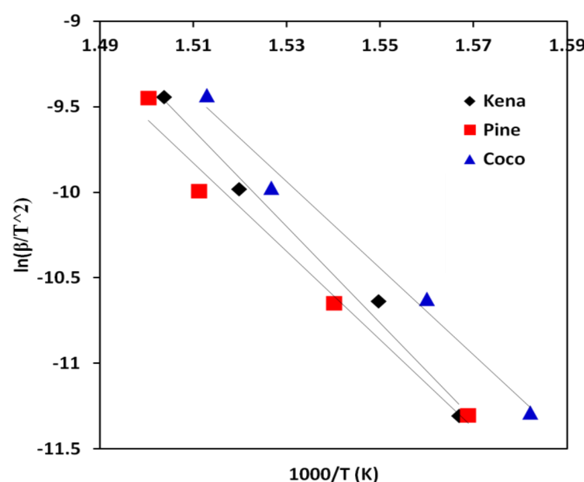

Fig. 3. Linear plots of ln(β/T2) versus 1000/T for various pulps in the Kissinger methods at different heating rates from 5 to 35 oC.min-1

The result of the calculation is presented in Table 1. The higher the activation energy, the more thermally stable the material. Alwani et al. (2014) reported that the Ea value of coconut fiber with the Kissinger method was around 200 kJ. mol^{-1}, which is quite similar with the result from this study, although in here coconut fiber was converted into single

fibers (pulp) instead of bundle of coconut fibers.

In the study from Bouafif et al. (2009), they had summarized the activation energy of natural fibres constituent as follows; Ea of lignin was between 54 and 80 kJ.mol^{-1}, Ea of hemicelluloses was between 63 and 109 kJ.mol^{-1} while Ea of cellulose component was between 151 and 251 kJ.mol^{-1}. Therefore, the Ea resulted in this study majority belongs to cellulose component, which was also supported the single degradation peak as presented in Figure 2b.

Table 1. Activation energy calculated by the Kissinger method

Pulp fibers	Ea (kJ.mol^{-1})	R^2
Kena	233.83	0.9879
Pine	215.11	0.9801
Coco	210.73	0.9867

Color changes

Natural fibre degradation during processing may adversely affect the mechanical properties of composite for some reasons. First, it changes the fiber structure, which adversely affects the mechanical properties of the fiber. Second, some volatile degradation products usually create microvoids across the interface of fibre that act as critical flaws and lead to extensive debonding and failure of the material under service. While the color changes might result in aesthetic judgement and visually inspection for the degradation of the natural fibers,the degradation is usually followed by the strong odors from the component.

Table 2. Color parameter of fiber and its composite

Pulp fiber				
	L*	a*	b*	E
What	99.0	0.1	6	99.182
Kena	81.2	3.8	13.7	82.435
Pine	81.7	3.2	12.4	82.698
Coco	56.5	8.9	20.3	60.692
Composite at 30% pulp fiber				
PP	62.8	-1.4	-1.0	62.824
Kena	41.1	3.4	6.2	41.704
Pine	35.1	4.4	8.0	36.268
Coco	33.1	1.3	2.3	33.205

Here, we reported the color changesof natural pulp fibers during pulping process and its biocomposite manufacturing. Figure 4 shows the color different of pulp fibers from different sources compared to the What (Whatman™) filter paper. The quantification of different L*, a* and b* are presented in Table 2. E value was calculated using the equation 2. Pulp fibers from kenaf and pineapple show almost similar lightness and total E, while coconut pulp shows the lowest scale. However, when those pulps were incorporated into polymer composite and subjected into thermal exposure during manufacturing processing, the total E decreased (Table 2). Figure 5 depicts the color changes of pure polypropylene and its composite for different pulp fibers at 30% fiber loading. The total E value was decreased by a half of the initial value. The color for PP reinforced coconut pulp fibers became dark-brown. Those color changes indicated that degradation probably occur during composite processing

Table 3 summarizes the relative lightness and total color changes from the initial pulp fiber to PP-composite and from pure PP to PP-composite. It shows that pineapple has higher ΔE. The higher color changes alarms that the degradation might be occurred in the pineapple composite processing. That also supported with the ΔE from pure PP to its composite which show that kenaf composite has low ΔE. The ΔE different between pineapple and coconut pulp fibers are insignificant. Those aforementioned phenomena were reported in quite low fiber contents (30%). Therefore, in case of pineapple pulp composite, the amount of fiber loading was increased up to 50%. The result of color changes is shown in Figure 6 and Table 3.

Table 3. Color changes from different items

Pulps fiber to composite (30%)				
	ΔL	Δa	Δb	ΔE
Kena	-41.1	-0.1	-7.0	41.7
Pine	-49.2	1.6	-4.4	49.4
Coco	-30	-7.4	-18.6	36.1
Pure PP to composite (30%)				
Kena	-20.8	5.4	7.9	22.9
Pine	-28.3	6.0	8.9	30.3
Coco	-31.8	3.4	3.6	32.3

Pure PP to composite at different pulp fiber loading				
Pine30%	-28.7	7.4	10.4	31.4
Pine40%	-24.8	7.7	11.3	28.3
Pine50%	-25	7.9	11.4	28.6

Surprisingly, the results were not as expected, since at higher pineapple fiber loadings, the ΔE at 40% and 50% were almost the same and the values are lower than 30% pulp fiber loading. As can be seen by naked eyes in Figure 6, the surface of composite at 40% and 50% was covered by slightly thin white layer. Those phenomena was more aggressively also happened for coconut pulp fibers composite at 50% fiber loading (picture was not shown). This pattern influences the measurement of ΔE using our equipment. Therefore, more samples and varying the leadtime of injection molding need to be assessed to validate the color changes in conjugation to the degree of fiber degradation.

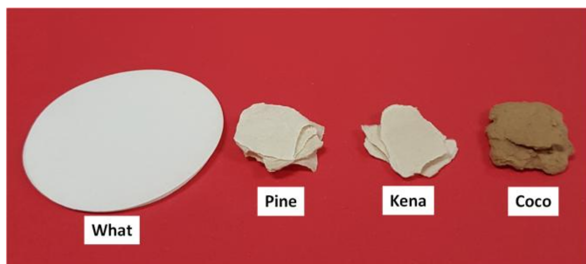

Fig. 4. Images of different pulp fibers and reference using WhatmanTM

Fig. 5. Images of color changes of biocomposites from their initial pulp color.

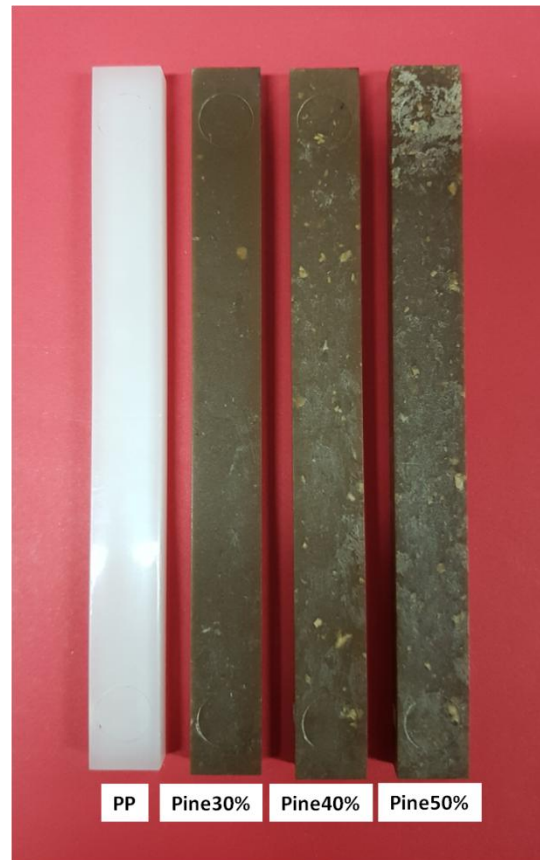

Fig. 6. Images of pure polypropylene and its biocomposite at different loadings of pineapple pulp fiber

Conclusions

Here, we reported the thermal analysis study of three kinds of pulp fibers, kenaf, and pineapple and coconut fiber by the Kissinger method. The activation energy for degradation peak showed value around 200 °C.

Kenaf pulp fiber was thermally more stable compared to pineapple and coconut fiber as proof by TG analysis, energy activation and color changes measurement. Colorimeter analysis is very helpful to asses the degradation of natural fiber in composite quickly.

Acknowledgment

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Potential Utilization of Nano Carbon Wrapping Paper From Bamboo for Packaging of Brownies Cake

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Abstract

Wood is a lignocellulosic material commonly used as raw material for making wrapping paper. Wood production is getting lower due to the decreasing number of forest land. So, it is necessary to search the other lignocellulosic materials that can be used as raw material for making wrapping paper and its modifications so that it can be applied to certain products. In this research, the paper made of the modified two types of bamboo (Tali and Ampel) with nanocarbon and tested on brownies cake. Bamboo is semi-chemically prepared to produce pulp and added 20% nanocarbon particles. The pulp + nanocarbon were formed with a sheet size of 60 gram / m2 and then the physical properties tested. The application of this wrapping papper has been done for the brownies cake and then the freshness and the nutrition of brownies cake was analyzed. Microbe contamination analyzed tend to decrease from 8,9 x 103 colonies/gram in fresh brownies to 1,1 x 103 colonies/gram in brownies wrapped with wrapping paper-nanocarbon Tali. The results showed that in general, the utilization of ampel- wrapping paper with nanocarbon modification can maintain the nutrition of the brownies cake.

Keywords: paper wrap, nanocarbon, bamboo

Introduction

Woods as one of the many kinds of ligno-cellulosic fiber stuffs are prevalently still used as raw material for the production of paper, further utilized for various purposes, such as writing, copying, printing, drawing, and wrapping/packaging. Wood production is getting lower due to the decreasing number of forest land (Ramdhonee & Jeetah 2017). Meanwhile, concurrently Indonesia's paper production and export increased as much as 9.76% in 2017 (Rini 2017), including wrapping/packaging paper, so that alternative raw materials are needed to meet the needs for the manufacture of wrapping paper. Several researches associated with wrapping paper manufacture have been conducted using non-wood sources, like banana fibers and cotton (Ramdhonee & Jeetah 2017). Other fiber sources, highly potential to be advisably used for wrapping paper are bamboos, as they are abundantly available and remarkably growing fast. Wrapping papers typify as a specific kind of paper which are numerously consumed; and as much 40% of those papers presents as paperboard. Consequently, the potency and utilization of wrapping papers might become high.

Among the benefits of wrapping papers is to facilitate the distribution and maintain the quality of the wrapped products. Particularly, the use of wrapping papers is intended to get the food more durable and to protect them from contaminations by organisms which can bring about food deterioration or spoilage (Rachmani et al. 2015), cheaper and easier to degrade (Rudra et.al. 2013). The wrapping paper can be modified to become more functional when given a certain additive and it is called active paper, expectedly are able to maintain and even enhance the service life of the wrapped products. Accordingly, new technology in wrapping paper manufacture can become a one step; and provide an answer to the community and industry necessities (Dobrucka & Cierpiszewski 2014). Several filler additives incorporated in the wrapping paper for food stuffs are among others chitosan, MgO, ZnO, SiO2 (Swaroop & Shukla 2018). One of the additives indicatively able for its use in wrapping papers

is nanocarbon. Nanocarbon technology has been indicated capable of detecting the presence of poisons, rotting processes, and deteriorating organisms in food; and even those papers can serve as smart and intelligent packaging materials (Tully et.al. 2006, Wang & Irudayaraj 2008). Nano carbon has a large pore; and therefore is expectedly able to serve as adsorbent agent to adsorb particular matters, such as water, oxygen, and other unwanted items. The presence of water in food stuffs can accelerate the food spoiling and lower their quality of the food.

In this research, the wrapping paper already manufactured from two species of bamboos separately (tali and ampel) and incorporating nano active-carbon additives, were experimentally tried for the wrapping of brownies cakes. The aim of this research is? to look into the effect of the using of those papers for the freshness and nutrition of the wrapped brownies cakes.

Materials and Method

Materials and Equipment

The materials used in this research were the papers (with 60 gram/m2), from two bamboo species separately (tali and ampel) and incorporated nano active-carbon as additive. The material which was experimentally to be used for the wrapping was brownies cakes. Nano-active-carbon was obtained from the activation process on sawdust charcoal. The equipment as employed consisted of retort for the charcoal activation; electrically heated digester for pulping; handsheet former; and laboratory instruments for examining cake freshness, nutrition, and hygienic value.

Research Method

The bamboo-manufactured papers either with or without nano active-carbon additives were at first placed in the conditioned room under fixed and controlled temperature (25-28°C [t]) and humidity (75-77%[Rh]) for 24 hours. In the wrapping, the top side of papers (indicatively less hygroscopic) was in direct contact with the cake, while the wire side (indicatively more hygroscopic) not in direct contact (Smook 2002).

Afterwards, all those bamboo papers in which the cakes were wrapped, then together with the unwrapped cakes, were placed again in the same conditioning room (25-28°C t and 75-77% Rh); and let there for 14 days. Thereafter, brownies cakes with or without wrapping; and the fresh cakes (Table 1) were analyzed for their freshness, nutrition contents, and microbe contaminations.

Table 1. Sample code data

No	Sampel	Kode sampel
1.	Wrapping paper-control Tali	WP-CT
2.	Wrapping paper-nanocarbon Tali	WP-NCT
3.	Wrapping paper-control Ampel	WP-CA
4.	Wrapping paper-nanocarbon Ampel	WP-NCA
5.	Brownies without wrapping paper	BW-WP
6.	Fresh Brwonies	FB

Analysis on Cake Freshness, Nutrition, and Hygiene

The freshness, nutrition, and hygiene analysis on the wrapped was performed by examining peroxide number, moisture content, ash content, carbohydrate content, vitamin B1, vitamin B2, folic acid, and microbe contamination.

Results and Discussion

The wrapping of food stuffs is regarded as one of the effective attempts to protect food from organism contaminations; and to keep them from the changes in their chemical, physical, and biology properties during the storage as well as transportation and distribution (Khaneghah, Hashemi, & Limbo 2018). One of the chemical changes that occur is the change of food, especially oxidized fats form peroxide so that free radicals will be formed and ultimately endanger human health because it can damage DNA, cell death or cause cancer (Rohmawati et.al. 2017). Peroxide numbers can also be pointed out as an indication to know the rancidity of food (Hasibuan, 2014).

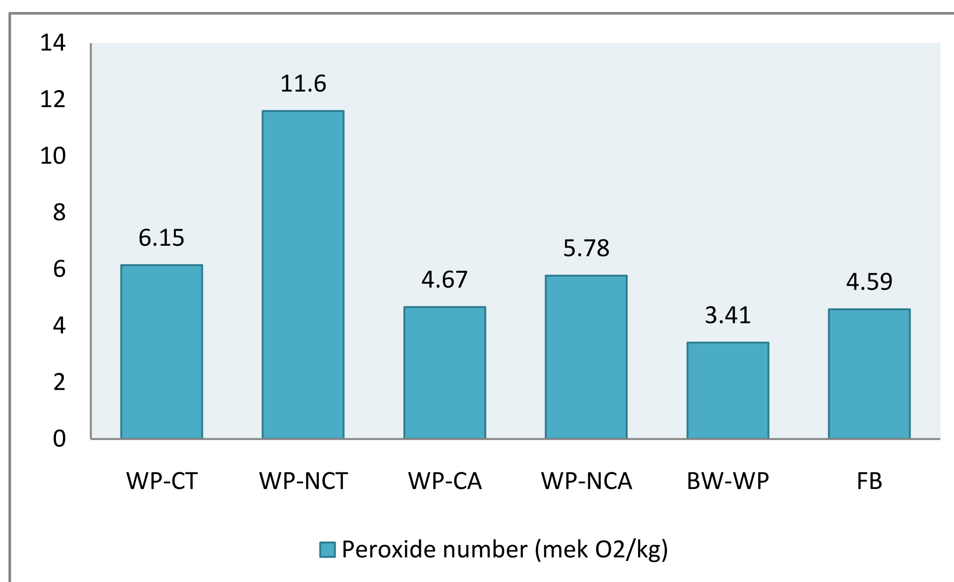

Fig. 1. Analysis of peroxide numbers from brownies cake.

Figure 1 reveals that the peroxide numbers values as examined on each of the six items were notably different from each other. The highest value was at brownies cake wrapped in tali bamboo paper incorporated with nano active-carbon additive, whereas the lowest value at the unwrapped cakes) and at the fresh cakes. Fresh brownies cakes could still undergo changes, but seemingly not so large, because their condition was still fresh, thereby not affecting much of their peroxide values. In the wrapped cake, the peroxide number formed tends to increase compared without wrapping paper even if it is stored for 14 days, presumably due to the influence of open air, degraded again into lower molecular weight compounds and more volatile, so peroxide numbers are not as high as in the wrapped cakes. The wrapping on the cake, allegedly leads to the presence of trapped oxygen so that the oxidation process tends to be easier and the peroxide is easily to formed. Peroxide numbers on brownies cake without activated nanocarbon are lower than the addition of activated nanocarbon. It is possible that activated nanocarbon can reduce the hygroscopic nature of the paper so that the wrapping paper without the addition of activated nanocarbon is more hygroscopic and more capable of absorbing oxygen and reducing the potential for peroxide formation.

Table 2. Proximate analysis of brownies cake samples

No	Sample	Proximate analysis (%)				
		Water content	Ash content	Protein	Fat	Carbohydrat e
1.	WP-CT	24,2	1,23	6,22	22,2	46,2
2.	WP-NCT	22,4	1,24	6,34	20,3	49,7
3.	WP-CA	23,6	1,25	6,39	23,5	45,3
4.	WP-NCA	24,6	1,21	6,22	20,6	47,4
5.	BW-WP	23,3	1,23	6,22	27,3	42,0
6.	FB	32,0	1,18	5,64	20,8	40,4

In Table 2, it reveals that the moisture content in fresh brownies cakes was higher than the content in all five other specified cakes. This could be explained due to the long storage time, the desorption of water molecules from the cake might occur more easily. High moisture content could affect the cakes negatively, as this would trigger degradation as well as changes in their chemical components; and enhance the potency of microorganism growth. Consequently, the wrapping of cakes in this

regard was better to maintain the cake freshness. For ash, protein, fat and carbohydrate levels, each of these levels tends to increase after the treatment of wrapping compared to the content of fresh brownies cake and brownies without wrapping. The ash content of a material indicates the mineral content in the material (Kaderi 2015). Ash content contains certain minerals needed in the body such as for physiological processes, such as enzyme work aids, as well as organ formation (Arifin 2008).

Protein content which was lower in the unwrapped cakes could be indicatively due to more organism contaminations, thereby inducing more degradation of protein compound. The fat content of the brownies cake for 14 days has the highest value, and otherwise the fat content of wrapping paper using nanocarbon both on bamboo (Tali and Ampel) paper is low, this is possible due to the adsorption, dehydration and fat volatilization of some fat when cake wrapped in nanocarbon paper. Then, this happens also on carbohydrate levels, its value increases when compared with fresh brownis cake or brownies cake without wrapping paper. This is possible because of the dehydration or volatilization of non-carbohydrate content in the paper especially when incorporated with nanocarbon, so that the carbohydrate levels are increasing. Protein content, fats and carbohydrates balanced are needed for the body in order to maintain heart resistance and body activities (Pertiwi & Murbawani 2012).

Table 3. Analysis on nutrition in brownies cakes

No	Items / Treatment codes	Nutrition levels			
		Vitamin B1 (mg/100 gram)	Vitamin B2 (mg/100 gram)	Folic acid (mg/kg)	Microbe contaminations (colonies/gram)
1.	WP-CT	0,36	< 0,025	1,43	$4,4 \times 10^3$
2.	WP-NCT	0,35	< 0,025	1,71	$1,1 \times 10^3$
3.	WP-CA	0,35	< 0,025	2,99	$3,4 \times 10^5$
4.	WP-NCA	0,34	< 0,025	2,30	$6,7 \times 10^4$
5.	BW-WP	<0,25	< 0,025	0,25	$5,7 \times 10^3$
6.	FB	0,40	< 0,025	< 0,25	$8,9 \times 10^3$

Analysis on the nutrition in brownies cakes was performed by examining vitamin B1, vitamin B2, folic acid levels; and the microbe contaminations. Vitamin B1 on fresh brownies cake has the highest levels compared with other treatments; this is because in the fresh cake, vitamin content is maintained while the cake without wrapping provides the lowest levels, so the packaging process is considered enough to maintain vitamin B1 levels, either by adding nanocarbon or without nanocarbon. For vitamin-B2 level, all the levels were below 0.025 mg/100 gram. Accordingly, so far the effect of different specifications in brownies cakes, with respect to the wrapping, being with or without nanocarbon, and fresh condition on that level could not be assessed or evaluated.

Folic acid level in the cakes after being wrapped in the bamboo papers, either with or without active carbon additive tended to be higher, compared to the level in unwrapped cakes and fresh cakes (Table 3). Meanwhile, the situation was on the contrary for the level of microbe contaminations, particularly the contamination in the cakes wrapped in tali bamboo paper with nano active-carbon additive. This is expectedly due to the adsorption or dehydration of other compounds than folic acid so that the value of folic acis tends to increases. Folic acid is the preferred compound in food because it can help the formation of the brain, especially in the fetus so that the risk of premature birth, low birth weight and abnormalities can be minimized (Murti et.al. 2017).

The value of microbial contamination in food for the human body is desired as low as possible, because it is associated with the level of hygienic food. Foods that are less hygienic can interfere with human health and cause illness. The process of wrapping the cake with paper, especially from the Tali bamboo, with the addition of activated carbon is allegedly able to suppress microbial contamination quite well, because nanokarbon has an important role in controlling microbial growth (Gintu et.al. 2018), so the wrapping process can keep

the cake better without being wrapped. Results of this research were almost similar to the results by Shankar & Rhim (2018), who modified the inside surface of wrapping paper by layering it with the extracts of Bali's orange seeds. Those extracts contained antimicrobe compounds, thereby bringing out the specific wrapping papers which were able to inhibit microbe contaminations in particular food stuffs.

Conclusion and Suggestions

The results showed that bamboo can potentially become the raw material of fiber to make paper wrap, as well as the application of wrapping paper from bamboo along with modification with activated carbon is known to keep the nutrients from food, especially lowering the level of microbial contamination on brownies cake.

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Implementation of Cleaner Production in Batik Industry at Banyumas Regency

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Abstract

Batik is an Indonesian cultural heritage that continues to develop so that in every region in Indonesia has an unique batik motifs that represent the identity of the area. One of the unique batik is well known as Batik Banyumasan. The growth of Small Middle Industry (SMIs) batik in Banyumas regency develops every year to preserve and enliven the golden era of banyumasan motif. However, the increase of batik SMIs in Banyumas Regency is followed by increasing of environmental pollution problems. This is mainly due to the lack of optimal use of raw materials, water and energy, so that the waste disposal will burden the environment. Centre of Handycrafts and Batik, Yogyakarta introduced one of tools of environmental management effort that is cleaner production principle. Cleaner production is a concept of environmental improvement that is done in an integrated and systematic by all parties concerned towards the achievement of environmental, economic and social balance. The selected areas for implementation of cleaner production at SMIs batik in Banyumas Regency are BASOKA Batik and Batik Pringmas. The hope is applying this cleaner production concept, not only will be able to change the pattern of raw material consumption and production process in batik industry of Banyumas batik SMIs, but also get production efficiency and can reduce production cost. Further, it can minimize the potential of environmental pollution and public health.

Keywords: Batik SMIs, Cleaner Production, Environmental Pollution

Introduction

The UNESCO have awarded batik as a cultural heritage of the Indonesian people and led by an increase in the quantity of batik industry and growth of new batik industry centers. Batik industry sector has a strategic role in development, especially to grow the level of employment and contribution in driving national economic growth. Along with the increase in the batik industry, environmental problems are also increasing. These problems are mainly due to the production process often resulting in waste of raw materials, water and energy, and also waste disposal that burden the environment. Today, the application of environmental management tools leads to environmental improvements carried out in an integrated and systematic manner. This concept was conducted by all interested parties towards achieving a balance of environmental, economic, and social aspects. Therefore, it is very necessary to form environmental management tools that can be applied to Batik SMIs. considering that, it is a challenge to implement a form of comprehensive management efforts in small and medium industries.

Every year, the growth of SMIs in Banyumas Regency increase especially for batik industries, although some centers of batik industries such as 'Batik Mruyung' has closed, but new batik centers have emerged, namely, the Sokaraja Original Batik Community (BASOKA) and Batik Pringmas. This fact shows the return of the golden era of of Banyumasan batik in Banyumas Regency. The BASOKA consists of approximately 20 (twenty) Batik SMIs, while Pringmas batik center has 120 members of batik craftsmen who preserve the banyumasan batik motif.

On average, 1(one) company has 15 employees. So, in every year, an average of one company uses 10,950 kg of batik wax; 182,880 meters of cloth; 1600 liters of H2O; 2000 kg kostik; and in the production process requires 15,000 liters of clean water and 100

liters of kerosene. Whereas, the production process for the last five years left 12.5% used wax out of the failed product of 9,144 meters, 200 liters of H2O2 liquid, 24 kg of Kostik, and 80% of the wastewater produced from clean water (Setyowati 2007). This condition certainly has negative impact on the environment and batik SMIs itself if management does not carried out in an integrated manner. The fact shows that efforts to manage the environment and save factors process such as raw, additional materials, clean water and energy are still faced with constraints of lack of practical knowledge regarding environmental management and limited quality of human resources.

Along with the development of batik centers in Banyumas Regency, Centre of Craft and Batik, Yogyakarta implements cleaner production as an effort to manage the environment for increase savings on the use of raw materials, water and energy in SMIs batik. In this activity, the Sokaraja Original Batik (BASOKA) and the Batik Pringmas, Papringan were chosen as partners in the implementation of cleaner production activities in 2017. Through the implementation of sustainable cleaner production, it is expected that the use of raw materials and others can be reduced. This reduction will reduce the cost of production, so that SMIs batik in Banyumas can compete with other batik industries. The other most important thing is to reduce the impact of environmental and health pollution on workers and the community due to production process in the batik industry.

The term cleaner production began to be introduced by the United Nation Environment Program (UNEP) in May 1989 and formally submitted in September 1989 at a seminar on The Promotion of Cleaner Production in Canterbury. Indonesia agreed to adopt the definition conveyed by UNEP (2003), namely, net production is a preventive and integrated environmental management strategy applied continuously to production process, products and services.

Therefore, this strategy needs to be applied continuously to the production process and life cycle product with the aim of reducing risks to humans and the environment (Nastiti 2009).

Meanwhile, the United Nation Industrial Organization (UNIDO 2002) added that cleaner production is an environmental management strategy that is directed towards prevention and integrated so that it can be applied to the entire production cycle.

The two definitions above have the same goal, namely to increase productivity by providing a better level of efficiency. The eficiency involves the use of raw materials, energy, water and encourage better environmental performance through reducing waste sources and emissions an impact product for the environment from the product life cycle with eco-friendly design and cost-effective.

Before concept of cleaner production begin to be developed, initially, environmental management was based on a Carrying Capacity Approach due to limited natural carrying capacity to neutralize ever increasing of pollution. The efforts to overcome pollution problem have changed waste processing approach that is formed (End Of Pipe Treatment). The concept of cleaner production is hierarchy principle of 5 (five) R insisted of Rethink, Reduce, Reuse, Recycled, and Recovery that must be applied directly in pipe recycle pipe so that the resolution of environmental problems is emphasized on pollution sources not at the end of the process as in the end-of-pipe treatment technology, including the efficient use of natural resources which also means the depletion of waste produced, pollution, and risk reduction for human health and safety. This concept does not always require expensive activities or sophisticated technology but often results in potential savings so as to increase competitiveness in the market. What is needed is a change in attitude, responsible environmental management and assessment of technology choices.

Several concepts in the cleaner production strategy introduced by Center of Craft and Batik in the Banyumas Batik SMIs are as follows (Bohnet 2010):

a. Reducing or minimizing the use of raw materials, water and energy and avoiding the use of toxic and hazardous raw materials and reducing the formation of waste at the source, thus preventing or reducing the occurrence of pollution and environmental damage problems as well as the risk to humans.

b. Changes in production and consumption patterns apply both to the process and products produced, so it must be well understood the product life cycle analysis.
c. This cleaner production effort cannot be successfully implemented without any change in mindset, attitudes and behavior of all relevant parties from the government, the community and the industrial world. In addition, it is also necessary to apply management patterns among industry and government by considering environmental aspects.
d. Applying familiar environmental technology, management and standard operating procedures in accordance with the requirements set. These activities do not always require high investment costs, even if they occur often the time needed for the return on investment capital is relatively short.
e. The implementation of this cleaner production program is more directed to self regulation that is negotiated approach. So the the implementation of cleaner production program does not only rely on government regulations, but is based more on awareness of behavioral change.

Material and Methods

Sampling Location

Sampling location were 25 (twenty five) batik SMIs incorporated in the BASOKA and 25 (twenty five) workers batik which is part of the Batik Pringmas, Papringan at Banyumas Regency.

Methods

1. The socialization of the concept of cleaner production in two centers of batik in Banyumas Regency, namely 25 (twenty five) batik SMIs incorporated in the BASOKA and 25 (twenty five) workers batik which is part of the Batik Pringmas, Papringan. At Banyumas regency.
2. Distributed and filling out questionnaires, interviews and observations about the production process and habits carried out by the cough batik industries in the two SMIs.
3. The direct implementation at the work site towards improvements in several matters relating to the application of cleaner production.
4. Analyzed the questionnaire data and data from direct observation.

Result and Discussion

Result

In the concept of cleaner production, known understanding of Non Product Output (NPO) is the first step in conducting an analysis before applying the concept of cleaner production itself. NPO is defined as all material, energy and water used in the production process but not contained in the final product. NPO forms can be identified in the batik industry, among others, are less quality raw materials in the form of less quality dye, reject products because they are not in accordance with the ordering specifications, reprocessing, solid waste in the form of wax, liquid waste (amount of contaminants, whole water that is not contained in the final product), energy (not contained in end products, such as steam, electricity, oil, diesel, etc.), emissions (including noise and odor), losses due to lack of maintenance and losses due to health and environmental problems, the total cost of NPO is the sum of NPO costs from inputs, NPO costs from the production process, and NPO costs from output. In general, the total cost of NPO ranges from 10-30% of the total production costs. Therefore, it is important for NPO batik to identify the NPO sources in the production, detail of process, in order to reduce production costs and increase productivity.

Through filling out questionnaires distributed to both two batik industrial centers in Banyumas, BASOKA and Batik Pringmas to obtain data on pattern of consumption of raw materials and the daily production process. From these data, it is known that the problems faced by batik SMIs incorporated in BASOKA among others, the lack of identification and calculation of detailed sources of wasteful resources. This is because one of the resource components such as water for the production process uses water from the Serayu River. Whereas wood as a source of heat energy in the wax removing process is

available at low prices from the surrounding wood collectors. Although the recycled process of used waxs with the used wax collection process has been carried out, it is only limited to resale to used wax collectors at a price of approximately 30 percent of the fresh or new wax price on the market. The used wax recycling process to be reused in the production process has not been carried out so that because there is no value in production cost savings from used wax use so NPO is mainly late at wax which is dissolved in waste water. The absence of used wax trap is called Koen. The management of chemicals has not been done optimally by reusing used dyestuffs so that the cost of consumption of coloring agents cannot be reduced and increasing the amount of liquid waste used in the coloring process. Environmental burden is very large due to the unavailability of Waste Water Treatment Plant (WWTP) for proper waste treatment.

Batik Pringmas in Papringan. Banyumas is an area that is famous for having many batik workers known as 'pengobeg' who get batik jobs from batik merchants. In 2010 Bank Indonesia (BI) representatives of Purwokerto fostered and conducted mentoring to unite the collectors into a Joint Business Group which they called Batik Pringmas. Until now Batik Pringmas has a gallery to showcase its batik products and often hold exhibitions in several cities in Indonesia and open batik training for batik enthusiasts around it.

In the implementation of cleaner production at Batik Pringmas has made several efforts but it has not been done optimally.

These efforts include processing used wax, replacing the energy of the stove from kerosene with an electric stove. Unlike the BASOKA, this group carried out the dyeing or coloring process in one place, namely in the workshop room. This was done because of the availability of WWTP facilities for Bank Indonesia assistance to treat batik waste. However, it is necessary to optimize the utilization of staining residual waste to reuse dyes so that in addition to reducing the volume of waste can also reduce production costs. Batik Pringmas has also begun to try to use natural colors to replace the use of synthetic dyes.

Discussion

Clean Batik Initiative (2010) explains that the steps of a simple cleaner production concept for batik SMIs to be applied include:

1) Substitution of Raw Materials and Auxiliary Materials

a. Replacing raw materials containing hazardous ingredients with ingredients that do not or less contain hazardous and toxic substances. The use of natural dyes from plant extracts such as tarum (Indigofera sp), Secang (Caesalpinia sappan), Tegeran (Maclura cochinchinensis), Mengkudu (or Morinda citrifolia) began to be developed to replace or substitute the position of synthetic dyes which allegedly contained several types of azo compounds which are carcinogenic. Even the Loba plant (Symplocos sp) was also investigated to contain Allum (Al) and Ferum metals which can be used as color reinforcing material in the mordanting process and fixation process.

b. Replacing solvents and cleaning materials containing hazardous ingredients such as the use of tapioca flour in the process of agreement on the fabric will be safer than the addition of sodium silica (water glass) in the wax removing process. Both ways are to facilitate the process of wax removing and batik wax.

2) Improving the Good House Keeping)

a. Reducing the loss of raw materials, products and energy as a result of leaks, and spills. Providing and using droplets, spills and leaks.

b. Placing equipment properly to avoid spills and contamination.

c. Inventory of equipment used in production including machine tools and machine maintenance equipment, as well as equipment for fire extinguishers. Make work instructions (Work Instructions) to facilitate understanding of the execution of the work make a regular maintenance schedule for the equipment so that the equipment is maintained and can be operated optimally.

d. Revents mixing of waste streams from different sources.

3) Product Modification

Re-formulate the product design to reduce the negative impact on the environment after the product is used. For example, equipment modification is carried out in a dyeing process that uses an inaccurate dipping tub, which is 2.0 X1.6 to dye a 1.35 X 1.6 meter cloth. By shortening or reducing the size of the tub, it is adjusted to the size of the dyed fabric. waste of about 50% of the costs due to immersion. Some best practices that can be implemented such as:

a. Eliminating excessive and unnecessary packaging
b. Increase product life time
c. The product so that can be recycled

4) Process Modification

a. Replace damaged equipment and layout improvements to optimize material flow and product efficiency.
b. Improve process conditions such as flow velocity, temperature, pressure and time.
c. Storage, to improve the quality of the final product and reduce the formation of waste.

It is expected that with the application of simple things like the above will be able to change the pattern of consumption of raw materials and production processes in the batik industry so that the batik industry gets production efficiency and can reduce production costs. The concept of the cleaner production principal above is translated into good practical things that aim to help implement it in the production process. Some of the best practices of cleaner production that can be implemented in batik industry are as follows (Center of Craft and Batik, 2017):

Workshop

The things that need to be considered in the workshop are:

a. Making layout (layout) of the production space to facilitate understanding of the production flow;
b. Making division of job responsibilities to facilitate employee coordination, in the event of product or process failure will facilitate the search for the cause;
c. Make a schedule for the division of tasks for employees;
d. The division of tasks and responsibilities is useful for you to facilitate coordination of employees and if a product / process failure occurs, it will facilitate the search for the cause.

Fabric Storage and Handling

Fabric storage

The raw material for batik in the form of mori or white cloth has a width of about 105 cm. After processing, the fabric usually shrinks between 5-8% both in the direction of the length (warp direction) and in the direction of the width (direction of feed), so to make 1 meter of batik a mori with a length of about 105 cm is needed.

Steps in handling fabrics are:

1. Record the incoming fabric
2. Monitor the quality of fabric by providing an inspection table
3. Record the return of the fabric if there is an inappropriate stock.
4. Label the fabric according to the type of fabric and the size of the fabric.
5. Fabric placement is separated between fabric material and product.
6. Record the product produced
7. Handling of fabric warehouse:
8. The room is kept from leaking, and keeps it clean to avoid damage to the fabric.
9. Providing adequate ventilation to maintain the moisture of the fabric.
10. Complete the warehouse with a smoking ban warning.
11. There is periodic cleaning of the room.
12. If there is no fabric warehouse, store the cloth in a separate place from storing chemicals.

Chemical Storage and Handling

Steps in handling chemicals:

1. Record the amount and price of incoming chemicals
2. MSDS (Material Safety Data Sheet) for each chemical
3. Purchase of chemicals tailored to the needs to avoid expiration and the occurrence of stock hospitalization.
4. Strived to use chemicals from certain suppliers (which are of high quality) so as to reduce the occurrence of failed products.
5. Put the chemicals into a closed jar / container

6. Label each chemical
7. Chemical substances placed on the floor should be given a pallet (buffer) to avoid damage Record chemicals that are expired / damaged
8. Record the consumption of chemicals
9. Apply the principle of FIFO (First In First Out), which is the chemical used is the first to enter.
10. The balance used needs to be repeated every certain period.
11. Employees need to use Personal Protective Equipment (PPE) when handling chemicals such as masks and gloves.
12. Employees need to be careful in avoiding spills and chemical leaks

It is necessary to socialize and direct how to handle chemicals to the employees involved.

Water and Wastewater

1. Matters that need to be considered in handling water and wastewater:
2. Monitor and record water and wastewater consumption
3. Water consumption and the amount of waste water can be recorded from the number indicated in the flow meter installed.
4. Analyzing the quality of water and wastewater periodically into the laboratory
5. Containing waste water is a particular container so it is not scattered
6. Installing a flow meter in each water pump used for the production process
7. Install the flow meter in the waste water outlet pipe
8. Turn on the water pump when the tub (washing, soaking, etc.) is full of water
9. Reducing the use of water for washing tools
10. Elevate the front side of the sink to reduce water spills around the production room
11. Check regularly and repair if there is a leakage of pipes, tools and water lines
12. Giving a filter to the ditch, so that solid waste does not enter into
13. Pay attention to caution in using water to reduce spills
14. Rearrange the drainage system (also need to pay attention to the slope of the floor) on the work floor to avoid standing water
15. Response to cleaning when there is a pool of water.
16. Installing posters saving water
17. Installing warnings not to throw garbage in the toilet
18. Selling certain wastes (paper, glass, plastic, aluminum, steel, etc.) to scavengers.

Energy

The things that need to be considered in handling energy are:

1. Monitor and record electricity and fuel consumption for the production process
2. Turn on a lamp that is not needed
3. Using energy-saving lamps
4. Reducing electricity consumption by using glass tiles
5. Make or increase ventilation in the production room
6. Perform periodic maintenance of electrical equipment.
7. Respond if the electricity is cut off
8. Separating the electricity meter production space with households
9. Firing walls and ceilings in bright colors
10. Installing electricity saving posters
11. Using electric stoves for batik compared to wood or kerosene

Occupational Health and Safety Protection

The things that you need to pay attention to as an effort to protect occupational health and safety are:

1. Ensure that every container, tool and rack is safe and does not topple
2. Ensure that every room and stairs are safe even when it rains here are periodic checks and maintenance of electrical equipment and cables
3. Install safety signs for each tool
4. Make WI (Work Instruction) for each tool
5. Get rid of unnecessary tools / objects from the production room
6. Cleaning the bathroom and toilet regularly
7. Installing warnings against smoking and eating at work
8. Provides adequate air circulation / air ventilation in the production room (windows, doors, vents and chimneys)
9. Install a soundproof wall on a loud sounding device or machine

10. Installing an anti-vibration system on a device or machine that vibrates violently
11. Provide first aid box
12. Providing training in first aid use for employees
13. Make WI for handling accidents
14. Providing telephone numbers for the nearest doctor, hospital, ambulance and fire department to anticipate the accident
15. Provides fire extinguisher in a room that has the potential to cause a fire, check the expiration date.
16. Providing training to employees on how to use fire extinguishers.
17. If there is an empty room, it can be used for employee break rooms or changing rooms for work clothes or employee parking.
18. Providing, maintaining and storing Personal Protective Equipment (PPE): gloves, rubber boots, masks, ear plugs, ear muffs in a safe place.
19. There are instructions to always use PPE while working
20. Regular replacement of PPE

Conclusions

The application of cleaner production is a series of work procedures applied at each stage of batik production. The work procedure is expected to result in changes in mindset and patterns of resource consumption, raw materials and handling of waste so as to reduce environmental pollution. It is expected that batik SMIs in Banyumas Regency especially BASOKA and Batik Pringmas can apply the principle of cleaner production continuously so as not only to obtain production efficiency and reduce production costs, but also can minimize the potential for environmental pollution and public health.

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Impact Ionic Liquid [Emim]OAc to Cellulose Hydrolysis Activities of Indigenous Microorganisms (IMO's) on Cassava Peels Substrate

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Abstract

An ionic liquid (IL), *1-ethyl-3-methylimidazolium acetate* ([Emim]OAc), was used as pretreatment catalyst follow by hydrolysis of cellulose on cassava peels using local selected indigenous microorganisms (IMO's). Under 25% (w/v) loading mass of substrate and liquid fermentation condition, cellulolytic of cellulase activities and growth of IMO's were investigated under a series of IL concentration. Optimal temperature and pH were 37°C and 5.5 respectively. Enzyme activity was determined by analyzing the hydrolysis of carboxymethylcellulose (CMC) at 37°C using the dinitrosalicylic acid. In general, 0.1 M of IL did not inhibit cell growth significantly. In addition, the hydrolytic activities of obtained-cellulases were most active in presence of 0.1 M of IL, while 0.5 M of IL pushed down the activity into an half and 1.0M extremely was losing the activity. The optimum experimental conditions for hydrolysis of pretreated cassava peels using 0.1 M of [Emim]OAc was established at 37 °C, for 72 h when yield of glucose rose up to 64.49%.

Keywords: ionic liquid, indigenous microorganisms, cellulose hydrolysis, cassava peels

Introduction

Lampung Province is the largest producer of cassava in Indonesia with an average contribution of cassava production in the period 2012-2016 of 33.93% of the total national contribution of 91.21%. Meanwhile, the trend of cassava production productivity in Indonesia during 1980-2016 rising around 2.64% a year. This condition has a potency to result the surplus of cassava production in 2015 rose to 1,027 million tons. However, the utilization of cassava in the industrial sector remains the cassava peels (26.98%) and onggok (15.87%) as solid waste (Departemen Pertanian and Pangan, 2016). The waste have economic value because cassava peels contains a high starch and cellulose (Cui et al. 2014).

Being substitute starch from first generation to second generation ethanol substrate, cellulose is the most common important natural polymer as a source of carbon in ethanol fermentation. Cellulose is being major component of polymers consists in lignocellulose beside hemicellulose and lignin (Isikgor and Becer 2015). Utilization of lignocellulosic feedstock in ethanol production has promising more advantage for reduction CO_2 emission than using starch substrate (Marquardt et al. 2010). The abundance of cellulose is valuable as a renewable source of energy. Cellulose degradation and its subsequent utilizations has importance for global carbon sources significantly. This reason has considered cellulose hydrolysis as the subject of intense research and industrial interest (Moreira 2005). Many research objective have purpose to obtaining new microorganisms cellulase producing which have higher specific activities and greater efficiency (Maki, et al. 2010).

Numerous microorganisms which are able to produce cellulase have been isolated and identified. In particular, many studies have focus on fungi because of the abundance and simple procedure to extract, and some of the fungal cellulases have already produced as commercial enzyme (Gusakov and Sinitsyn 2012). On the other hand, various bacteria which produce cellulases are more intensive studied in recently years. Bacterial cellulase properties such as their fast growth, expression of multi-enzyme complexes, and resistance to extreme environments were considered the studies (Liang et al. 2014; Maki, et al. 2010; Rawway, et al. 2018; Shanmugapriya, et al.

2012; Verma, et al. 2012; Waeonukul et al. 2009).

The first step to utilizing cellulose biomass is deconstruction the compact structure of polymers, namely pretreatment process. The pretreatment has primary aim to diminish the crystalline structure of cellulose for efficient hydrolysis the cellulose chemically or biochemically by enhancing enzyme accessibility to the cellulose during hydrolysis step. In addition, the pretreatment accommodates the solubilization major components of biomass (Menon and Rao 2012) and it has impact on hydrolysis to provide a high sugars concentration (Mosier et al. 2005).

The discovery of ionic liquids (ILs), the organic salts which have a melting point below 100 °C, as pretreatment agents are promising technological invention. The ability of ILs to reduce the degree of cellulose crystallization by breaking hydrogen bonds intra- and inter-molecules of cellulose is a paradigm that provides convenience in hydrolysis steps (Wu et al. 2011; Remsing et al. 2008). It has been discovered that 1-ethylpyridiniumchloride ([C2pyr]Cl) can dissolve cellulose. Then, it has been examined that a range of ILs, typically with 1,3-dialkylimidazolium cations, are also effective cellulose solvents (Swatloski et al. 2002; H. Zhang et al. 2005). Clear viscous mixture solutions are resulted, showing the polymer solutions in general and solutions of cellulose in particular (Gericke et al. 2009).

Antagonistically, the synergic of ILs for the fermentation process is faced by ILs toxicity to microbial fermentation. Some imidazolium ILs that have great pretreatment abilities such as Emim Acetat ([Emim] OAc) and 1-butyl-3-methylimidazolium chloride ([C_4C_1im] Cl) are toxic while some ILs are biocompatible (Ouellet et al. 2011; Ganske and Bornscheuer 2006; X. Zhang et al. 2016).

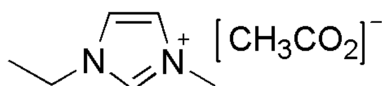

Fig.1. Chemical structure of [Emim] OAc

The aim of this study are to observe growth of indigenous bacteria isolates and their cellulase activities in presence of [Emim]OAc. The chemical structure of [Emim] OAc is shown in Figure 1.

Materials and Methods

Pretreatment of Cassava Peels.

The cassava peels was collected then rinse in tap water for overnight to reduce HCN content. After rinsing period, the cassava peels were collected then, and were dried at 65 °C. Dried cassava peels then were grinded and meshed using 250 µm mesh sieve. The obtained cassava peels powder (5.0 g) then were immersed in a series of ILs weight and were stirred gently at 50 °C for 24 h (Cui et al. 2014).

Isolation and Screening of Bacterial.

The cassava peels were sliced and were immersed in sterile nutrient broth (NB; Difco) then it was incubated at 37 °C for 24 h. After incubation period, a dilution series of cultivated bacteria from cassava peels on NB were done in 0.85% physiological solution and were poured on to nutrient agar (NA; Difco). The cultivated NA then were incubated at 37 °C for 48 h. Bacterial colonies that appeared on NA medium were picked using needle and grown onto enriched NA containing 1% CMC (Sigma). The isolate that resulted clear zone after 0.1% congo red (Merck) pored followed by 1% NaCl (Merk) was selected as cellulase isolate (Yin 2010).

Growth Assay

The assay medium contains NB medium enriched by 25% of cassava peel powder in a series of IL's concentrations (0.00; 0.01; 0.05; 0.10; 0.50; 1.00 M) was prepared. Pre-culture of isolated bacteria was prepared by inoculation 1 colony of isolate into 5.0 mL NB aseptically and was incubated in a shaker incubator at 160 rpm, 37ºC, for 18-24 hours. The density of pre-cultured cells was measured after 18-24 hours by using a spectrophotometer, and the absorbance at 600 nm then was defined as OD_{600}. The appropriate volume of pre-cultured isolate was taken to set up initial OD_{600} at 0.1 in the 2.0 mL assay medium and was transferred into micro-tube and then was centrifuged at 15,000rpm, 4ºC, for 5 minutes. The filtrate was then discharged, and the cells were re-

suspended using the assay medium. Suspended of inoculum cells was inoculated into assay medium, then was incubated in a shaker incubator at 160 rpm, 37ºC, for 24 hours. The measurement of OD_{600} was done at 6, 12, and 24 hours regularly. Relative OD_{600} 24h then was calculated using the following equation:

$$ROD_{600}\ 24h = \frac{OD_{600}\text{ of sample the IL at 24 hours}}{OD_{600}\text{ of 0.00 M at 24 hours}}$$

(Kuroda et al. 2017)

Enzyme Production.

Appropriate NB were added into pretreated cassava peels mixture aseptically to obtain the series concentration of ILs. The loading mass of cassava peels in the solution mixture was 20 g/L. The bacterial isolates were pre-culture overnight in NB medium at 37 °C and 160 rpm. Pre-cultured isolates then collected into centrifuge tube and appropriate volume of inoculum were then used to give initial OD 1 in the series concentration of ILs in the cassava peels solution mixture. The mixtures then were incubated at 37 °C and 160 rpm for 96 h (Bayitse et al. 2015).

Enzyme Assay

Enzyme production during cultivation was assayed at 24 h intervals up to 4 days. The culture were centrifuged at 10.000 rpm for 15 min at 4 °C. The supernatants were collected as crude enzyme for enzyme assay. Cellulase activities was determined using the 3,5-dinitrosalicyclic acid (DNS). The buffer used for dissolving or resuspending substrate (CMC) was 200 mM sodium citrate buffer (pH 5.5). The reaction system was prepared as follows 250 μL of crude enzyme mixed 250 μL of 2% (w/v) of CMC. The mixtures were incubated at 50◦C for 30 min. Then, the reactions were stopped by adding 1mL of DNS reagent. All the mixtures were heated in boiling water for 5 min for color development. Subsequently, 500 μL of each sample was transfered to cuvet and the absorbance at 550 nm were determined using *uv-vis* spectrophotometer (Adney and Baker 2008).

Glucose yields were calculated using following equation:

$$\text{Glucose Yield (\%)} = \frac{W_{glucose}\ (g)}{W_{potential\ glucose}\ (g)} \times 100\%$$

where $W_{glucose}$ is weight of obtained glucose, and $W_{potential\ glucose}$ is weight of cellulose (g) × 180/(180 − 18) (Tsai and Meyer 2014).

One unit of cellulase activity was defined as the amount of enzyme that could hydrolyze CMC and release 1 μmol of glucose within 1 min of reaction (Rawway et al. 2007).

Result and Discussion

A total of 45 cellulose-degrading aerobic bacterial strains were isolated from local cassava peels in Bandar Lampung, Indonesia, which were cultured in agar medium containing pretreated cassava peels powder as the sole carbon source. Out of these strains, 3 isolates showed hydrolyzing zones on agar plates containing CMC-Na after Congo-red staining (Figure 2). The isolates, SCWb-3, SCWb-13 and SCWb-17, were cultivated to produce cellulase in presence of [Emim]OAc. The crude cellulases of each isolate have activities at 22.25 U/mL, 23.97 U/mL, and 23.59 u/mL, respectively. These crude enzymes were produced in medium containing cellulose from cassava peels. The production system allowed 5 g of cassava peels powder loading into 200 mL of medium solution. It means the loading mass in this study was 25 g/L.

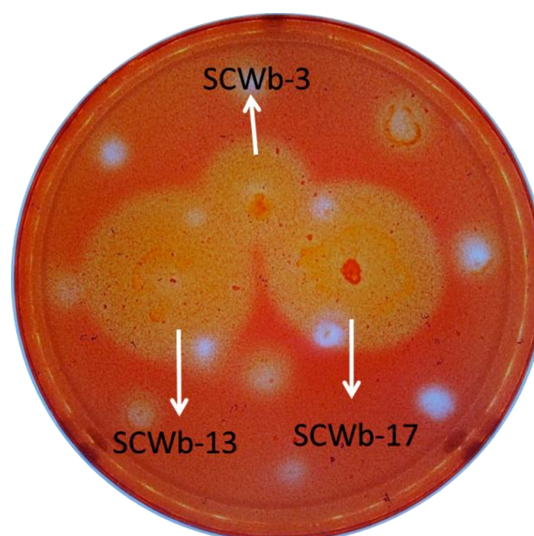

Fig.2. Hydrolyzing zones produced by bacterial isolates on agar plate containing CMC after Congo-red staining. The cellulase positive were given by SCWb-3, SCWb-13

and SCWb-17 and were indicated by clear zone appearance.

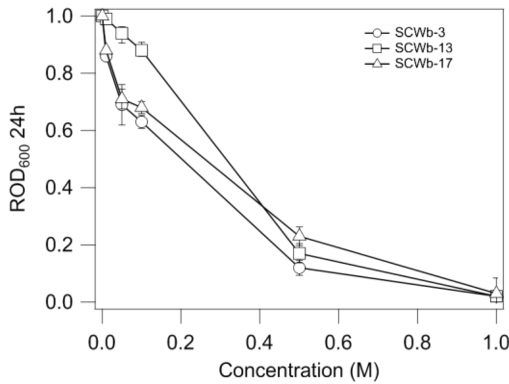

Fig.3. ROD_{600} 24h of isolate SCWb-3, SCWb-13 and SCWb-17 that describe growth of isolat in medium consist of [Emim] OAc

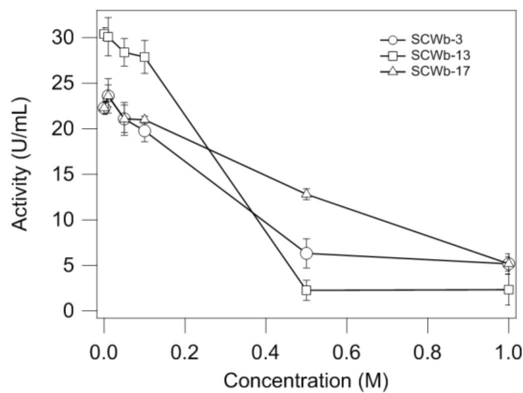

Fig.4. Cellulase activities of isolate SCWb-3, SCWb-13 and SCWb-17 in the presence of [Emim]OAc

To determine whether [Emim]OAc was the cause of growth inhibition and cellulase activity, the isolates were grown in NB medium cultures were supplemented with 0-1.0 M of [C2mim]OAc. Fig-3 expresses the growth of isolate compare with control when they cultivate in medium. It shows that the presence of [Emim]OAc below 0.5 M inhibite the grow of isolate less than 50%, since the concentration above 0.5 M the inhibition effects are increasing dramatically. Effect of ILs on cellulase activity during cultivation of isolated presents in Fig-4. It appears that the trend between the growth of isolate and enzyme activites are almost similar. Firstly, [Emim]OAc pushed down the cellulase activities for all of isolate cellulases, starting in the presence of 0.01 M of [Emim]OAc the isolate cellulases activities were placed under the control (un-presence of [Emim]OAc). However, cellulase activitiy of SCWb-13 was higher than others. When the concentration of [Emim]OAc increasing to 0.5 M, the cellulase activities decrease almost an half of initial isolate cellulase activity. It seems that [Emim]OAc presence was the main cause of inhibition.

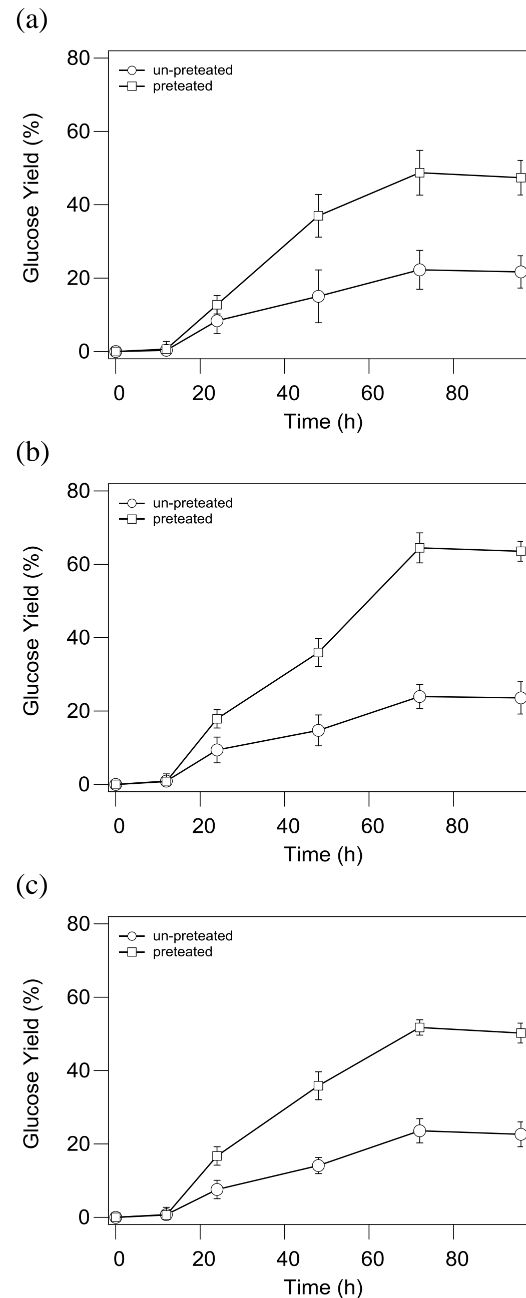

Fig.5. Effect of pretreatment [Emim]OAc on cassava peels to the yield of glucose resulted from cellulase activities of isolate SCWb-3 (Fig-5a), SCWb-13 (Fig-5b) and SCWb-17

(Fig-5c).

Ouellet et al. (2011) were observed that cation Emim+ has the primary source of inhibition than anion OAc- when ionic liquid [Emim]OAc interact with fermentated microbial. Since the pH in this study were detected between 5.07-6.57 was not a factor explaining the inhibition observed.

When we evaluated the amount of producing glucose that expressed as yield of glucose in this study, using 0.1 M of [Emim]OAc as pretreatment agent of cassava peels, it was appeared that the pretreatment gave positive impact into hydrolysis of substrate significantly. It was seen clearly in Fig-5 that after 24 h cultivated there were differences between pretreated cassava peels and un-pretreated one in glucose yield. It is known that [Emim]OAc is one of effective ILs using in pretreatment. It work to break down the crystallinity of cellulose via destruct hydrogen bonding intra- and inter-molecular of cellulose structure. In nature, cellulose is microcrystalline and its native cellulose I structure is recalcitrant to enzymatic hydrolysis. In the cellulose I lattice form, cellulose chains align in a parallel position fashion via hydrogen bonding and van der Waals forces to form a high compact and ordered micro-fibril highly, which consists of crystalline and amorphous regions. The others form of cellulose detected from recent progress involving experimental studies and computer simulations have shown that other forms of cellulose such as amorphous cellulose, cellulose II, and cellulose III are less recalcitrant (Cheng et al. 2012).

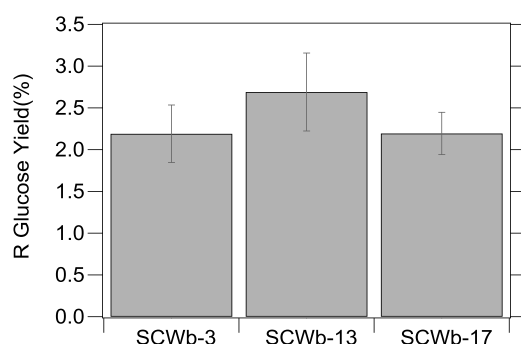

Fig.6. The glucose yield relative given by hydrolysis cassava peels during 72h cultivation using isolate SCWb-3, SCwb-13 and SCWb-17.

We consider that the glucose yield of un-pretreated substrate was lower than pretreated ones. To find out how high the difference in glucose yield, in particular calculation of relative glucose yield made from the data in Figure 5. The assumed was made based on comparation the glucose yield during the 72h hydrolysis process between preteated and un-pretreated substrate. The calculation resulted that after 72h cultivation the pretreated substrate hydrolyzed almost more two times effective than un-pretreated substrate (Figure 6). Moreover, the SCWb-13 isolate has the highest point which it showed 2.5 times more effective to hydrolysis pretreated cassava peels compare to control.

Conclusions

Three isolate of indigenous bacteria showed hydrolysis of cellulose ability. All of isolates gave the cellulase activity in presence of [Emim]OAc up to 0.1 M while 0.5 M of IL decreasing the activity into an half. Pretreated cassava peels using 0.1 M of [Emim] OAc was effective to increase the yield of glucose relative more than two times compare by un-pretreated ones.

Acknowledgment

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Characteristic of Poly (Lactic Acid) - Betung Bamboo Acetylated Pulp Composites

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Abstract

Betung bamboo is one of potential non-wood lignocellulosic fibers source. Kraft pulping and bleaching using hydrogen peroxide was conducted to remove lignin from Betung bamboo fibers. Subsequently, bleached pulp of Betung bamboo was acetylated with acetic anhydride. Prior to acetylation, Betung bamboo pulp was activated with acetic acid glacial by ratio of pulp and acetic acid, 1:20 (w/v) for an hour. Afterward, acetic anhydride and sulfuric acid were added by ratio of pulp, acetic acid dan sulfuric acid, 1:10:0.05 (w/v/v), for 1, 3, 5 hours. The composites were prepared using acetylated pulp of Betung bamboo and poly (lactic acid) (PLA) via a solvent casting process. Surface topography of the composites was examined using scanning electron microscopy. The presence of the chemical functional groups of Betung bamboo pulp and acetylated pulp of Betung bamboo were confirmed via FTIR analysis. Tensile test was conducted on composites containing 20 wt% acetylated-bleached-pulp of Betung bamboo showed the highest tensile strength (18.84 N/mm^2), compared to other composites in this study.

Keywords: poly lactic acid, betung bamboo pulp, acetylation, composites.

Introduction

Environmentally sustainable material processing has been a consideration when developing new plastic materials, due to the increasing scarcity of fossil resources. Poly (lactic acid) or PLA is a biodegradable aliphatic polyester that can be produced by fermentation of carbohydrate from renewable agricultural plants such as corn (Ajioka et al. 1995) or cassava (Yuwono & Kokugan 2008). Moreover, PLA has high mechanical properties and good processability, so that PLA is one of the most promising biodegradable polymers for plastic application (Pantani & Sorrentino 2013). However, PLA has some drawbacks such as brittle, low impact strength and low resistance in thermal deformation (Shukor & Hassan 2014). To overcome those problems, some researchers have been investigated to utilize natural fibers in form of microfibrillated cellulose (Nagakaito et al. 2009) or cellulose nanofibers (Kowalczyk et al. 2011) in PLA matrice.

Bamboo is a potential source of natural fibers to develop sustainable composite because it grows rapidly and its fibers have superior mechanical properties (Scurlocka et al. 2000). The development of bamboo-plastics composites has grown remarkably over the past decade. Nevertheless, the addition of bamboo fibers into plastic matrix still encounter some difficulties especially fibers dispersion in plastic matrix (Okubo et al. 2009). The incompatibility between bamboo fibers and plastics was due to the poor interfacial adhesion. Natural fibers have hydroxyl groups on the surface, whereas, plastic is a nonpolar matrix, leading to an inefficient stress transfer under load (Arrakhiz et al. 2013). Research on improvement of the interface adhesion between natural fibers and plastic has conducted through chemical treatment of natural fibers.

Various surface treatments with alkali using sodium hydroxide (Islam et al. 2010) or silane coupling agent pretreating (Lu et al., 2014) on natural fiber have been reported. Kenaf fibers treated with NaOH increased PLA composite flexural strength, flexural modulus and impact strength by 34%, 69% and 50%, respectively (Huda et al. 2008). The alkali treatment successful remove lignin, hemicelluloses and other non-cellulose

components from fibers surfaces which prevent interfacial adhesion between natural fibers and PLA. While silane coupling-agent molecules created tiny thin film on the surface of bamboo cellulosic fibers represented linkage between PLA and bamboo fibers. The epoxy end groups on silane which had grafted onto bamboo cellulose molecules could react with the matrix (PLA) and formed chemical bonds, which significantly improved the adhesive force between the interfaces of the components (Lu et al. 2014). Other researcher said that acetylation results better interfacial bonding and strength enhancement than silane and alkali treatment (Abdul 2014).

In this study, PLA composites with untreated and bleached-acetylated pulp of Betung bamboo were prepared and examined to evaluate the effect of bamboo pulp bleaching and acetylation on the composite characteristics.

Materials and Methods

Materials

Betung bamboo was obtained from Bogor Botanical Garden. Chemical for pulping and bleaching were sodium hydroxide (NaOH), sodium sulfide (Na_2S), hydrogen peroxide (H_2O_2). Chemical for acetylation were acetic acid as activating agent, acetic anhydride as acetylated agent, and sulfuric acid as catalyst. Those chemicals were on pure analysis grade. Whereas, ethanol 96% and acetone used for solvent exchange process, were on technical grade. Chemical for degree of substitution analysis were sodium hydroxide and hydrochloric acid on pure analysis grade. Poly lactic acid (PLA) semicrystalline, which trade name NatureWorks™ 3001D was supplied by Cargill Dow LLC (Minnesota, USA). Glycerol triacetate (Merck) was used as plasticizer of PLA.

Pulping and Bleaching

Kraft pulping was carried out at 30% sulphidity and 19% effective alkali. Thus, Na_2S solution, NaOH solution and water with total of 2000 ml were added to digester after 250g Betung bamboo fibers (dry basis). Pulping was conducted in 2 hours, at 170°C. After cooling of the digester, the pulp was collected and washed with fresh water several times until neutralized.

For the bleaching process, as much of 5 grams bamboo pulp (dry basis) and 190 ml distilled water were added into 250 ml erlenmeyer, then put in waterbath and heated at 75°C. The solution of 30% hydrogen peroxide was used as bleaching agent. Every 60 minutes, 2 ml of 30% hydrogen hydroxide was added into erlenmeyer, for 5 times. After bleaching process, bleached pulp was collected and washed with distilled water then stored in cold storage in condition of 80% moisture content. Unbleached pulp of bamboo was coded A, and bleached pulp of bamboo was coded B.

Acetylation

Five grams bamboo pulp (unbleached and bleached) was activated in 100 ml glacial acetic acid for 1 h in room temperature. Acetylation process was conducted with 50 ml of acetic anhydride and 0.25 ml of sulfuric acid within determined reaction time at 30°C of reaction temperature. Reaction times were varied in 1, 3, and 5 h. Then resulted acetylated cellulose was washed with distilled water, ethanol 96%, and acetone. Codes for samples were 1, 3, and 5 represent as reaction time.

Degree of substitution analysis

Prior to analysis, acetylated cellulose was oven dried at 40°C within 2 h. Degree of substitusion (DS) were analyzed by saponification method. As much as 100 mg of acetylated cellulose was immersed in 10 ml of ethanol 75% and then heated at 60°C within 30 min. Afterward, as much as 12 mL of sodium hydroxide 0.5 N was added then further heated at 60°C for 15 min. Samples were kept at room temperature for 72 h. Titration was performed using hydrochloric acid 0.5 N and then back titration using sodium hydroxide 0.5 N conducted after 24 h conditioning.

$$\text{Acetyl content (\%)} = \frac{[(D-C)+(A-B)]N \times 4{,}305}{W}$$

$$\text{DS (\%)} = \frac{3{,}86 \times acetyl\ content\ (\%)}{102{,}4 - acetyl\ content\ (\%)}$$

A is sodium hydroxide volume of samples titration, B is sodium hydroxide volume of blank titration, C is hydrochloric acid volume of samples titration, D is hydrochloric acid volume of blank titration, N is determined as 0.5, and W is samples weight.

Fourier Transform Infra Red (FTIR) analysis

Universal Attenuated Total Reflectance (UATR) was used to analyze specific chemical functional groups of untreated and treated pulp of bamboo. As much as 5 mg of samples was placed on diamond reflectors. Scanning was conducted in proper position. The spectrum of wavelength was determined in 400 – 4000 cm^{-1} and scanning repetition was 5 times.

Composite preparation

Eight grams of PLA were stirred in 50 ml dichlorometane until dissolved, then 0.8 ml glycerol triacetate (GTA) was added. On the other side, untreated or treated pulp of bamboo (1.6 g) was suspended gradually in 30 ml PLA and stirred in a beaker for 10 min at speed of 700 rpm. Afterward the suspended bamboo pulp was poured into dissolved PLA+GTA and stirred for 10 min. The bamboo pulp-PLA mixture was transfered into trays to evaporate dichlorometane at room temperature for 12h followed by oven drying at 60°C for 24h, to obtain a composite sheet.

Mechanical Testing

Composite tensile properties were examined based on ASTM D-638 standard. Four specimens of composites were analyzed using a Shimadzu 50kN universal testing machine at a cross-head speed of 5 mm/min and a gauge length of 25 mm.

DSC analysis

Differential Scanning Calorymetry (DSC) (DSC4000 Perkin Elmer) was used to analyze thermal characterization of composites. Scanning method was performed as follow: 5 mg of samples was placed on calorymetry coloumn then heated from 20°C up to 200°C with heating rate of 10°C/min.

The thermal properties such as melting temperature (Tm), and enthalpy of melting (ΔHm) were determined from the heating scan and the melt crystallization temperature (Tc) and enthalpy of melt crystallization (ΔHc) was determined from the cooling scan of the samples. The percentage crystallinity of each sample was calculated by following equation:

$$\% \text{ crystallinity} = \Delta Hm / (\Delta Hm^\circ * w) * 100$$

In this equation, the heat of melting (ΔHm) is in terms of J/g. The term ΔHm° is a reference value and represents the heat of melting if the polymer were 100% crystalline. The theoritical melting enthalpy of 100% crystalline PLA was taken to be ΔHm° = 93 J/g and w is the weight fraction of PLA in the sample (Suryanegara et al. 2010).

Morphology analysis

The unbleached or bleached pulp bamboo and PLA composites were analyzed by scanning electron microscope/energy dispersive X-Ray analysis (SEM/EDX) JEOL JSM-6510LA, operated at 15 kV. Samples were coated with gold using a vacuum sputter-coater to improve conductivity of the samples and thus SEM images quality.

Results and Discussion

Bamboo pulp degree of substitution (DS)

Degree of substitution represents the ability of acetylation agent to substitute hydroxyl groups in cellulose with acetyl groups. Analysis of acetyl content and degree of substitution were based on Ernest-Saunders et al. (2014) works. Acetyl content was determined by titration of cellulose acetate with NaOH. The ethanol was used in the next step as swelling agent to optimize the reaction.

The degree of substitution and acetyl content of bamboo bleached pulp were higher than that of bamboo unbleached pulp, accordance to the increasing of reaction time. Popescu et al. (2012) reported that after 5 h acetylation reaction, the degree of substitution of unbleached pulp was lower than that of bleached pulp, but higher when acetylated for 24 h. Unbleached pulp has more hydroxyl groups than that of bleached pulp, because of the higher hemicellulose and lignin content. Furthermore, unbleached pulp structure was

less ordered, with the result that it was more susceptible to attack by reagent (acetylation).

Ifuku et al. (2007) declared that DS of 0.2-0.6 were not change the cellulose crystallinity significantly. In this study DS of unbleached and bleached pulp were 0.51-1.27 (Table 1), so cellulose crystallinity of those pulps could be changed, as explained in composite thermal properties section.

Table 1. Bamboo pulp degree of substitution

Samples	Degree of substitution (%)	Acetyl content (%)
Unbleached pulp of bamboo		
A1 (acetylated for 1 h)	0.51	10.05
A2 (acetylated for 3 h)	0.68	12.92
A3 (acetylated for 5 h)	0.62	11.48
Bleached pulp of bamboo		
B1 (acetylated for 1 h)	0.74	15.07
B2 (acetylated for 3 h)	1.05	19.37
B3 (acetylated for 5 h)	1.27	22.24

Untreated and treated pulp of bamboo chemical functional groups

Fourier transform infrared spectroscopy (FT-IR) is a well-known powerful analytical tool to detect functional groups by measuring fundamental molecular vibrations. The characteristic bands for unbleached and bleached pulp of bamboo and those associated to acetylated samples were identified and were assigned according to literature data (He et.al. 2009, Hu et al. 2011, Jebrane et.al. 2011).

On FTIR spectra of unmodified pulp of bamboo, there were peaks at 3331 cm^{-1} (O3 H3· · · O5 intramolecular in cellulose), 1029 cm^{-1} (C-O stretching ring in cellulose and hemicellulose), 896 cm^{-1} (β-glucosidic linkage between the sugar units in hemicelluloses and celluloses). While, on acetylated pulp of bamboo (Figure 1 and Figure 2), there were peaks at 2895 cm^{-1} (C-H symmetric vibration), 1723 cm^{-1} (C=O streching), 1368 cm^{-1} (CH_3 in-plane bending), 1243 cm^{-1} (C-O stretching in acetyl group). Furthermore, the intensity of C-O stretching increased at 1209 cm^{-1}, which was assigned to cellulose, hemicellulose and lignin. And the low intensity at 896 cm^{-1}, shows α-glucosidic linkage between the sugar units in hemicelluloses and celluloses.

The same acetylation effect was observed in both types of unbleached and bleached pulps. However, differences in signal intensities were stronger for bleached pulp than of for unbleached one. This means that the acetylation was more pronounced in the case of the bleached pulp sample.

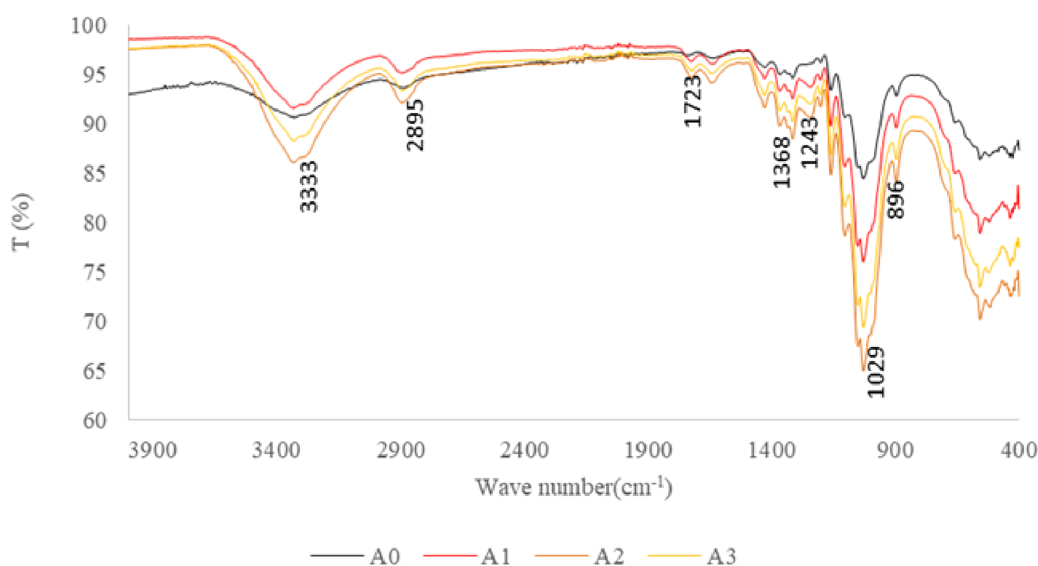

Fig. 1. FTIR Spectrogram of unbleached pulp of bamboo, acetylated in various time

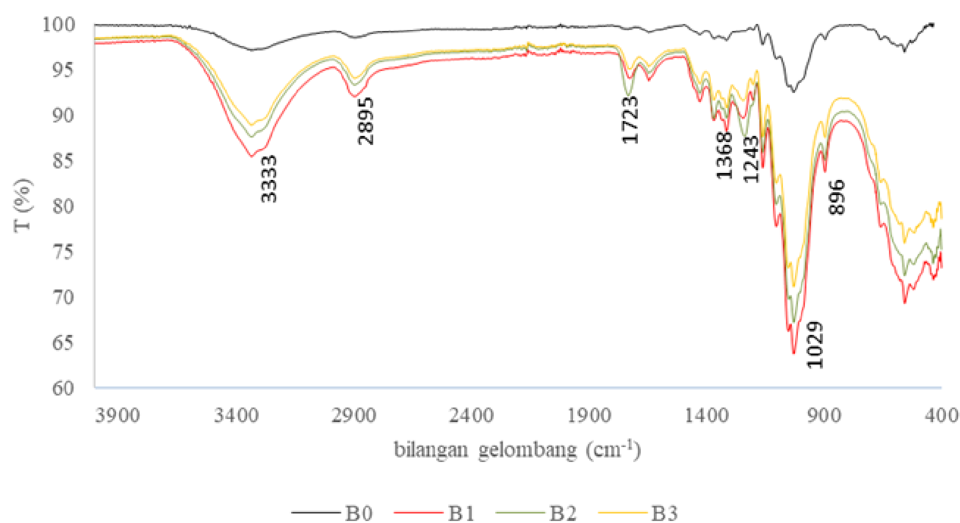

Fig. 2. FTIR spectrogram of bleached pulp of bamboo, acetylated in various time

Composite mechanical properties

Based on the result of pulp DS analysis, the composites were prepared using unbleached and bleached pulp of bamboo which were acetylated for 5 h. Figure 3 shows that mechanical properties of composite with PLA-bleached acetylated pulp of bamboo (18.84 N/mm^2) were higher than that of PLA-unbleached acetylated pulp of bamboo (17.07 N/mm^2). Bleached pulp could increase composite mechanical properties, because bleaching agent opened cellulose crystalline structure, so that cellulose was susceptible to be attacked by acetylation agent.

Hydrogen peroxide bleaching has proved to be a highly efficient and competitive bleaching chemical in term of delignification efficiency, low cost and reducing ecological impact (Walsh 1991, Abrantes et al. 2007). When hydrogen peroxide was used in kraft hardwood pulp bleaching, some wood components including lignin were dissolved out during the bleaching and resulted in the collapse of fiber lumens. Those conditions increased the contacting area between fibers and strengthened fiber-to-fiber bonding, then resulted in increasing of fibers mechanical properties. The xylan was also dissolved and subjected to loss of acetyl groups, then began to redeposit on fiber surface, which also can be expected to effect the strength properties of fibers (Li et al. 2011).

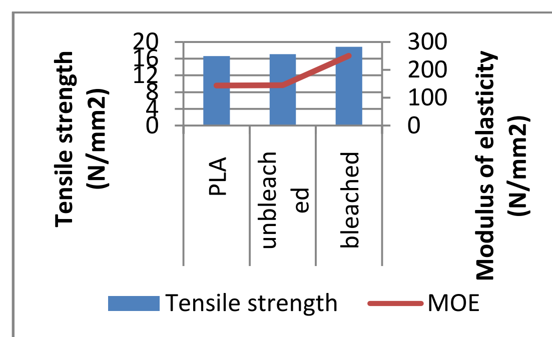

Fig. 3. Mechanical properties of PLA-unbleached- 5 h acetylated pulp of bamboo and PLA-bleached- 5 h acetylated pulp of bamboo.

In this study composite of PLA-bleached-acetylated pulp of bamboo shows higher tensile strength and modulus of elasticity than composite of PLA-unbleached-acetylated pulp of bamboo. Bleaching of bamboo pulp was expected to remove impurities. The use of hydrogen peroxide (H_2O_2) as an oxidizing bleaching agent causes discoloration of fiber. Perhydroxyl ions (HOO−) attack the light absorbing chromophoric groups of lignin and cellulose (carbonyl and conjugated carbonyl groups and quinones) (Rayung et al., 2014). The removal of pulp impurities, the collapse of fiber lumens and xylan redeposit on fiber surface were the reason of the increasing of PLA-bleached-acetylated pulp of bamboo composite's mechanical properties.

Acetylation has been shown to improve the dispersion of microfibrillated cellulose (MFC) in a PLA matrix. During the acetylation reaction, the primary hydroxyl groups in cellulose are esterified by the introduction of acetyl groups, consequently enhanced the cellulose dispersion in PLA matrix, due to the compatibility improvement (Bulota et al. 2012). The better dispersion of cellulose fiber in PLA matrix and improved fiber-matrix interaction lead to an enhancement in the mechanical properties of composites.

Composite thermal properties

Differential scanning calorymetry analysis was conducted to evaluate PLA thermal properties changes due to introduction of unmodified bamboo pulp and bleached-acetylated bamboo pulp. The melting point of PLA-unbleached-acetylated bamboo fibers (Figure 4) or PLA-bleached-acetylated bamboo fibers (Figure 5) were lower than that of PLA-unmodified bamboo fibers composite melting point. This condition will give positive effect on processing PLA composite due to the less energy needed and cost efficiency.

In PLA-unbleached-acetylated bamboo pulp composites (A1, A2, A3), the crystallinity was higher than that of PLA-untreated bamboo pulp (Table 2). Unbleached bamboo fibers still contain hemicellulose and lignin, beside cellulose. Therefore, more hydroxyl groups were replaced by acetyl groups after acetylation, and resulted in higher crystallinity. The introduction of unbleached bamboo pulp which was acetylated for 5 h resulted higher crystallinity in PLA composite, compare to unbleached bamboo pulp which were acetylated for 1 h or 3 h.

Composite of PLA-bleached 3 h acetyled bamboo pulp shows the highest crystallinity among others composites in this study.

Table 2. Thermal properties and crystallization degree of PLA-Bamboo pulp

Samples	Heating		Cooling		% X
	Tm (°C)	ΔHm (J/g)	Tc (°C)	ΔHc (J/g)	
Unbleached bamboo pulp					
A0 (PLA-untreated pulp)	163.54	35.1959	-	-	47.31
A1 (PLA-1 h acetylated pulp)	159.13	29.0598	95.23	-14.5126	58.57
A2 (PLA-3 h acetylated pulp)	158.13	32.3476	92.99	-12.2585	59.95
A3 (PLA-5 h acetylated pulp)	159.57	37.1398	93.04	-11.4130	65.26
Bleached bamboo pulp					
B0 (PLA-bleached-unacetylated pulp)	163.95	31.8532	96.49	-20.9848	71.02
B1 (PLA-bleached -1 h acetylated pulp)	154.40	25.6068	89.71	-10.9088	49.08
B2 (PLA-bleached -3 h acetylated pulp)	157.22	32.6135	94.08	-23.6089	75.57
B3 (PLA-bleached -5 h acetylated pulp)	158.95	28.8843	92.50	-17.4085	62.22

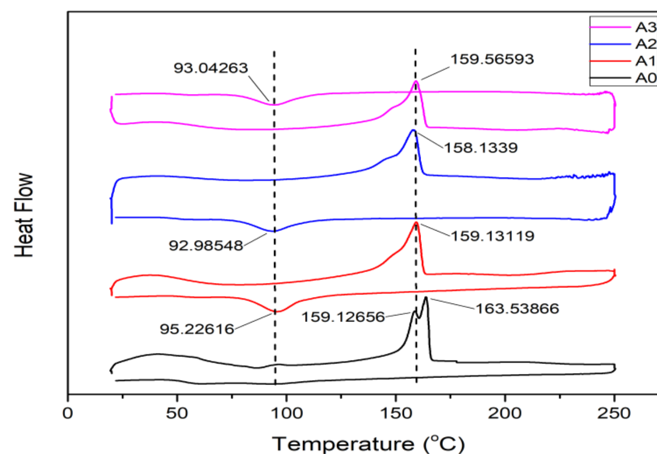

Fig. 4. DSC Spectrogram of unbleached bamboo pulp, acetylated in various time

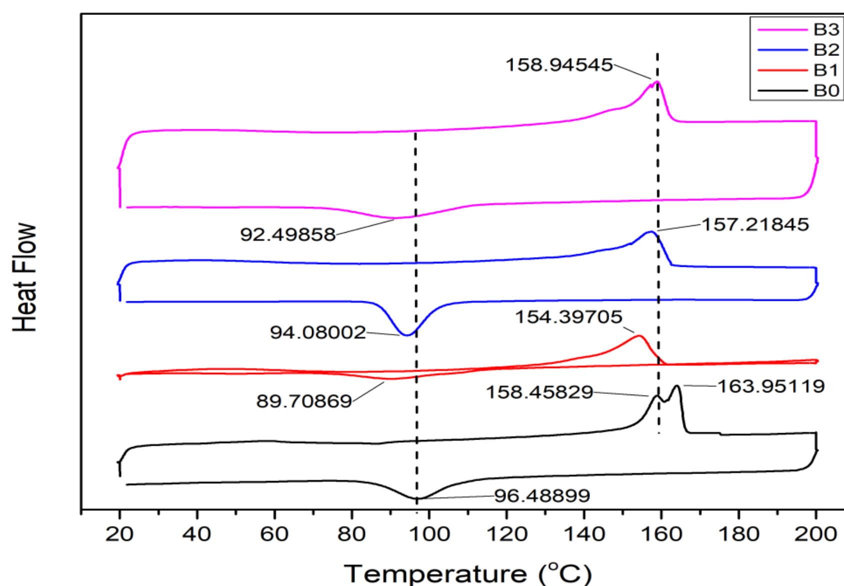

Fig. 5. DSC Spectrogram of bleached bamboo pulp, acetylated in various time

Composite morphology

Figure 7 shows SEM micrograph of PLA-untreated pulp of bamboo composite. The diameter of bamboo fibers was varied widely and not embedded in PLA matrix. Bamboo fibers were located at some spots and not well dispersed in PLA matrix. While, Figure 8 shows that bamboo fibers were well dispersed in PLA matrix, resulted higher mechanical properties. Bleaching process effected bamboo fibers in term of removing impurities, dissolving lignin and redepositioning of xylan (hemicellulose). Furthermore, acetylation replaced hydrophilic hydroxyl groups of bamboo fibers with more hydrophobic acetyl groups and increased the compatibility between bamboo fibers and PLA matrix.

Bulota et al. (2007) reported that the acetylation of cellulose hydroxyl groups proved to be an effective way of decreasing polarity and, in turn, improving compatibility with PLA, the non-polar matrix. The better dispersion of reinforcing agent and improved fiber-matrix interaction leads to an enhancement in the mechanical properties of composites.

Fig. 6. SEM of unbleached pulp of bamboo-PLA composite (500x magnification)

Acetylation on fiber surface increased fiber fibrillation from fiber bundle to become smaller diameter fibers. Thus, it initiates a better dispersion of fiber in matrix (Albano et al. 2003).

Zhang et al. (2012) stated that modified bamboo fibers caused a better adhesion with PLA compare to unmodified bamboo fibers. Agglomeration was observed in PLA composite with unmodified bamboo fibers.

Fig. 7. SEM of bleached-acetylated pulp of bamboo-PLA composite (500x magnification)

Conclusions

Unbleached pulp of Betung bamboo structure which still contain hemicellulose and lignin was less ordered, with the result that it was more susceptible to be acetylated. FTIR signal intensities were stronger for bleached pulp of Betung bamboo than for unbleached one. This means that the acetylation was more pronounced in the case of the bleached pulp of Betung bamboo.

Tensile test was conducted on composites containing 20 wt% bleached-5 h acetylated pulp of Betung bamboo showed the highest tensile strength (18.84 N/mm^2), compared to other composites in this study. Composite of PLA-bleached 3 h acetyled pulp of Betung bamboo shows the highest crystallinity among others composites in this study and lower melting point (157.22 °C).

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Evaluation of Lipid Content and Fatty Acids Profile from 5 Marine Microalgae

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Abstract

Each microalga has different lipid content and fatty acid profiles. This study aims to analyze lipid content and fatty acid profiles of microalgae. The five microalgae used are isolated from marine in Indonesia. The microalgae were cultured in AF-6 media. Lipids of microalgae were extracted using the Bligh & Dyer method and fatty acid profiles were analyzed using GCMS. The result showed that the highest lipid content is at the stationary phase which is 59, 57, 49.58 and 45 % respectively for microalgae FS001, FS002, FS003, FS005, and FS010. There are six fatty acids that dominant in five microalgae observed are palmitic acid, linoleic acid, linolenic acid, stearic acid, arachidic acid, and oleic acid. Fatty acids profile showed that oleic acid is a dominant fatty acid in FS 002, FS 005, and FS 010 microalgae with areas percent of 53.64, 45.17, and 50.83 % sequentially. Linoleic acid is a dominant fatty acid in FS 003 microalga with area percent of 43.64. Linoleic acid (omega 6) and oleic acid (omega 9) can be applied to food, supplement, pharmaceutical industries etc.

Keywords : microalgae, lipid, fatty acids, marine

Introduction

Microalgae are single cell microscopic organisms and can be found in fresh and marine water (Scott *et al*. 2010). Microalgae can be easily cultivated both on a small scale in the laboratory and on a large scale in the field. Microalgae contain three main components, namely carbohydrate, protein, and lipid (Hadiyanto &Azim 2012).

Microalgae have varying lipid content of 1-90 % in accordance with conditions of growth (Hu *et al*. 2008). Microalgae can be used in various fields such as pharmaceuticals, food preservatives, supplements, cosmetics etc. In this study, we analyzed lipid content and fatty acids profile of 5 microalgae, so that their potential is known and can be applied. These miroalgae were isolated from the sea in Indonesia.

Lipid is one of the main components highly related to the quality traits of processed meat products including nutritional value and flavor development. The content and composition of fatty acids are important indicators for evaluating the nutritional value of foods. Lipid degradation caused by lipolysis and oxidation in meat products during processing has an important impact on the final product quality (Gilles, 2009).

Table 1. Lipid content of microalgae

Microalgae	Lipid contet (% dry weight)
Botryococcus braunii	25-75
Chlorella sp.	28-32
Crypthecodinium cohnii	20
Cylindrotheca sp	16-37
Dunaliella primolecta	23
Isochrysis sp.	25-33
Monallanthus salina	20
Nannochloris sp.	20-35
Nannochloropsis sp.	31-68
Neochloris oleoabundans	35-54
Nitzschia sp.	45-47
Phaeodactylum tricornutum	20-30
Schizochytrium sp	50-77
Tetraselmis sueica	15-23

Source : (Alam *et al.* 2015)

Materials and Methods

The research materials consist of 5 microalgae namely FS 001, 002, 003, 005, and 010. Microalgae were cultivated in 630 mL AF6 media. Microalga preculture of 70 mL (cell density equal to 1) added to AF6 media. Microalgae culture were aerated and given light as an energy source. Microalgae were cultivated until stationary phase.

Lipid was analyzed using the Blight and Dyer (1959) method. Microalgae culture was taken 40 mL and centrifuged at a speed of 6000 rpm for 10 minutes. The supernatant was removed, while pellet was used for analysis of lipid content. The solvents were used to analyze lipid content is a methanol: chloroform: water with ratio of 1: 1: 1. The pellets were mixed with 3 mL of methanol and shaken for 1 hour at 28 ^{o}C, and then they were added with 3 mL of chloroform and shaken for 1 hour at 28 ^{o}C. The samples were added with 3 mL of water and shaken for 15 minutes at 28 ^{o}C.

Samples were centrifuged at speed 2000 rpm for 5 minutes, then incubated overnight. Samples separated become three layers. The top layer is methanol and water, the middle layer is microalgae biomass, and the lower layer is chloroform and lipid. The top layer was discarded, the middle layer was dried using an oven at 60 °C, and the bottom layer was evaporated to remove chloroform so that the lipid remains.

Lipids are used for transesterification. 0.1 g lipid was put into a flask and 4 mL of 2 % NaOH in methanol was added than refluxed at 80 ºC for 20 minutes. Sample was added with 4 mL BF3 in methanol and refluxed at 80 ºC for 20 minutes. Sample was added with 4 mL saturated NaCl and homogenized, then added 4 mL n-hexane and homogenized again for 10 minutes then refluxed again at 80 ºC for 20 minutes. Sample was put into separating funnel and settle until two layers are formed, namely glycerol layer at the bottom and biodiesel at the top. Biodiesel was used for analyzing fatty acid methyl ester (FAME) profile. Fatty acid methyl ester compound was characterized using Shimadzu QP 2010 GCMS. Gas Chromatograhy Mass Spectrometry instrument operation system are shown in table 2 below.

Table 2. GCMS instrument operation system

GC *Condition*	
Coloumn	C18 DB5-MS
Column Oven	Agilent
Temperature	60 ºC
Column Flow	1.00 mL/min
Injection Temperature	230 ºC
Injection Mode	Split
Presure	57.4 kPa
Carrier gas	Helium
Total Flow	104.0 mL/min
Oven temperature program	10 ºC/min to 230 ºC.
MS *Condition*	
Ion Source Temp.	250 ºC
Interface Temperature	270 ºC
Scan Speed	1111

Result and Discussion

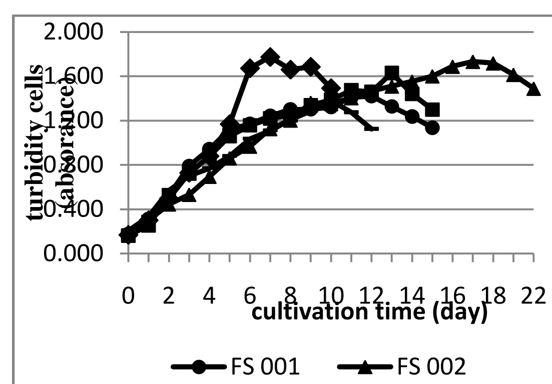

Fig.1. Growth curve of microalgae

Figure 1 shows the growth curves of the FS001, FS002, FS003, FS005, and FS010 microalgae. Microalgae growth was observed up to stationary phase. The five microalgae reached stationary initial phase at different cultivation times. The stationary initial phases of microalgae FS001, FS002, FS003, FS005, and FS010 are respectively 12th, 18th, 11th, 14th, and 8th days. Each type of microalgae has different growth.

Cultivation is a way to grow microalgae with certain controlled environmental conditions. Microalgae cultivation is influenced by external factors (environment) which include chemical, physical and biological factors. These environmental factors affect the growth rate and metabolism of microalgae. Some of parameters that affect the growth of microalgae are salinity, pH, nutrients, and light intensity (Lannan 2011).

Salinity affects the growth of microalgae. Almost all types of microalgae can grow optimally in salinity slightly below the original habitat. The most optimum salinity range for microalgae growth is 25-35 ‰ (Sylvester et al. 2002). The degree of acidity (pH) affects metabolism and growth of microalgae cultures because it alters the balance of inorganic carbon, nutrient availability, and affects cell physiology.

Nutrients are very important for microalga growth. Nutrients are divided into macronutrients and micronutrients. Macronutrients consist of C, H, N, P, K, S, Mg, and Ca. Micronutrients consist of Fe, Cu, Mn, Zn, Co, Mo, Bo, Vn, and Si (Dallaire et al. 2006).

Light is an energy source in photosynthesis which is useful for the formation of organic carbon compounds. In dark conditions, microalgae do not synthesize biomass. In this condition, microalgae do respiration, so that the culture becomes saturated with carbonate compounds that are not utilized by microalgae. This causes a reduction in the process of transferring CO_2 gas into culture media (Khuluq 2013).

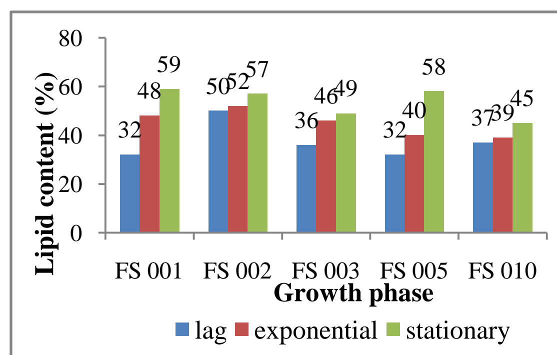

Fig 2. Lipid content of microalgae at lag, exponential, and stationary phase

Lipid content was analyzed at the phase of lag, exponential and stationary. Figure 2 shows the lipid content of the five microalgae increasing from the lag, exponential, and stationary phases. The highest lipid content is at the stationary phase which is 59, 57, 49.58 and 45 % respectively for microalgae FS001, FS002, FS003, FS005, and FS010.

According to Hartati *et al.* (2011), lipid content of *Nannochloropsis oculata* at the stationary phase was higher of 67.7% compared with at the exponential phase of 35.7%. This is because microalgae lack nutrients (stress) at the stationary phase. Stressful conditions in microalgae occur due to several things such as nutritional deficiencies, high or low temperatures, salinity, pH, light, and UV radiation (Sharma et al. 2012).

When microalgae lack nutrients for growth, microalgae will survive by accumulating the lipids they contain. Lipid is a source of energy stored during photosynthesis. Microalgae use it for growth, food reserves and survive from environmental stress (Khoo et al. 2011).

Microalgae under optimal growth conditions synthesize fatty acids primarily lead to the synthesis of membrane lipids. In stressful condition, microalgae change lipid biosynthesis pathway towards the formation and accumulation of neutral lipids in the form of TAG. The role of the TAG is as a form of carbon and energy storage. ncreased total lipid content in microalgae during stressful conditions, namely increased levels of neutral lipids, especially TAG. This is due to the enlargement of lipid metabolism from the synthesis of membrane lipid to the synthesis of neutral lipid or storage lipid (Hu, et al. 2008).

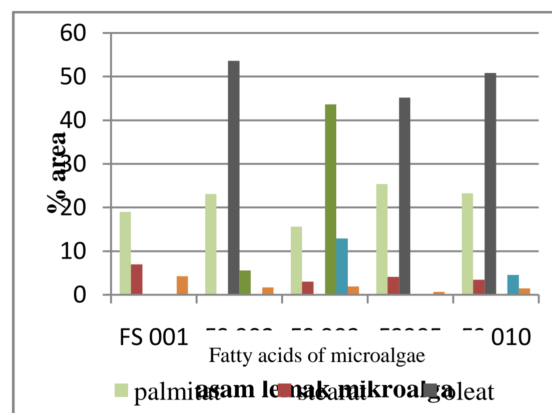

Fig 3. Profile of fatty acids

Fatty Acid Methyl Ester (FAME) profiles showed that there are 6 types of fatty acids namely palmitic acid (C16:0), steaeic acid (C18:0), arachidic acid (C20:0), oleic acid (C18:1), linoleic acid (C18: 2), and linolenic acid (C18:3). Palmitic acid, stearic acid, and arachidic acid are saturated fatty acids (SFA). Oleic acid is a monounsaturated fatty acid (MUFA). Likewise, linoleic acid and linolenic acid are polyunsaturated fatty acids (PUFA).

Oleic acid is a dominant fatty acid in FS 002, FS 005, and FS 010 microalgae with

areas percent of 53.64, 45.17, and 50.83 sequentially. Linoleic acid is a dominant fatty acid in FS 003 microalga with area percent of 43.64 (Figure 3).

Information about fatty acids profile is needed for industrial applications. Linoleic acid (omega 6) and oleic acid (omega 9) can be applied to food, supplement, pharmaceutical industries etc.

Linoleic acid (omega 6) is a member of the simplest PUFA. It called essential fatty acid because the body cannot synthesize it. PUFA regulate a wide variety of biological functions, depending on the location of the last double bond, which range from blood pressure and blood clotting to the correct development and functioning of the brain and nervous system (Wall et al. 2010).

Oleic acid (omega 9) reduces the inflammation associated with saturated fatty acid-induced inflammation in human aortic endothelial cells (Harvey et al. 2010). Further, the incorporation of milk enriched with oleic acid into the diet has resulted in reductions in total cholesterol, LDL-cholesterol, and triglyceride levels. The effects of which were observed among healthy individuals, those with increased risk for cardiovascular disease (Lopez-Huertas 2010). Oleic acid improves oxidative stability and cold flow properties on biodiesel.

Conclusions

The five microalgae studied had the highest lipid content in the stationary phase. Oleic acid is a dominant fatty acid in FS 002, FS 005, and FS 010. Linoleic acid is a dominant fatty acid in FS 003 microalga.

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Mannanase Production from *Kitasatospora sp*. using Batch Fermentation Method

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Abstract

Mannanase is a hemicellulose-degrading enzyme which is capable to hydrolyze mannan-rich biomass to mannooligosaccharides. *Kitasatospora* sp., filamentous Actinomycetes, has ability to produce this enzyme. In our previous work, the enzyme was obtained in a flask-shaker level. This study scaled-up the process to a 2 L vessel continuous stirred bioreactor using batch fermentation method. Fermentation was conducted at 28°C, the aeration and motor rotation were set at 2 vvm and 400 rpm, respectively. Bacterial growth was analyzed through its dry cell weight and enzyme activity was tested by DNS method using locust bean gum as substrate. Results indicated that enzyme obtained from this method exhibited highest enzymatic activity was 52.34 $\pm$ 4.91 U/mL at 90 h of production time.

Keywords: Mannnanase, Batch fermentation, Kitasatospora sp.

Introduction

Lignocellulose from agricultural waste is a potential renewable resource for several industries, such as biofuel and pharmaceutical, on account of its abundant presence in nature. It mainly consists of cellulose, hemicellulose, lignin and pectin (O'Neil & York 2003). One of hemicellulose constituents, mannans, structural polysaccharides found in softwood, are now taking scientists' attentions due to its prospects in future biorefinery applications (Soni & Kango 2013).

Generally, mannans are used in form of their hydrolysis products. For instance, mannooligosaccharides (MOS), degradation product of mannans, are known to contribute to human health and confer prebiotic benefits. Complete biodegradation of mannans involves mannan-degrading enzymes: β-mannosidase (1,4-β-D-mannopyranoside hydrolase), β-mannanase (1,4-β-D-mannan manno-hydrolase), β-glucosidase (1,4-β-D-glycoside glucohydrolase), α-galactosidase (1,4-α-D-galactoside galactohydrolase) and acetyl esterase. Of those mentioned enzymes, β-mannanase plays a major role in forming of mannooligo-saccharides (Soni & Kango 2013).

Microorganisms have been acclaimed as the richest source for enzyme production (Kirk et al. 2002). In addition, microbial enzymes are able to be produced in a large quantity and possible to be genetically modified (Anbu et al. 2015). Various species of yeast and bacteria are able to produce mannanase, and some of them are applicable for production at a commercial level (Soni & Kango 2013). Furthermore, the exploration of enzyme source from Actinomycetes becomes a 'hotspot' by reason of their diversity and metabolic versatility (Priyadharsini & Dhanasekaran, 2015). Also, several Actinomycetes showed mannolytic activity, including *Streptomycetes scabies* CECT 3340 (Montiel et al. 1999), *Kitasatospora* sp. (Rahmani et al. 2017) and *Nonomuraea* sp. (Ratnakomala et al. 2015).

Kitasatospora sp. ID04-0555, one of Actinomycetes strains previously studied, was able to produce endo-β-mannanase in a shake-flask scale. This enzyme showed high degrading activity towards various mannan polymers, viz. locust bean gum, ivory nut, konjac glucomannan and porang potato (Rahmani et al. 2017). However, the production of enzyme at higher scale had not been done; hence, in this study, the process was conducted in a 2 L batch fermentor. This

result would reveal the possibility for industrial use of this method.

Materials and Methods

Materials

Kitasatospora sp. (BTCC B-806, Gen-Bank database under the accession number KY576672) was obtained from Biotechnology Culture Collection, Indonesian Institute of Sciences (LIPI). Locus bean gum (LBG) was purchased from Sigma-Aldrich, USA. Malt extract, yeast extract and peptone were purchased from Becton, Dickinson and Company (BD), French. Other chemicals were purchased from Merck, Germany.

Fermentation

Kitasatospora sp. was refreshed (4 days) from glycerol stock solution and pre-cultured in medium which consisted of 1.0% malt extract, 0.4% yeast extract and 0.4% glucose. Incubation was conducted in a shaker at 28°C and 190 rpm for 3 days (pre-culture). Production culture was composed of 1.25% locus bean gum, 0.075% peptone, 0.05% yeast extract, 0.14% $(NH_4)_2SO_4$, 0.2% KH_2PO_4, 0.03% $MgSO_4{\cdot}7H_2O$, 0.039625% $CaCl_2$, 0.03% $CO(NH_2)_2$, 0.00036625% $CoCl_2$, 0.0005% $FeSO_4{\cdot}7H_2O$, 0.0976125% $MnSO_4{\cdot}7H_2O$, 0.00014% $ZnSO_4{\cdot}7H_2O$ and pH medium 8. The pre-culture was, then, inoculated into this medium (1:9, v/v) in New Brunswick BioFlo/CelliGen 115 Fermenter 2 L (Eppendorf, USA). Bioreactor was set at T = 28°C, aeration = 0.2 vvm and motor rotation = 400 rpm. During process, sampling was carried out through the injection tube with a syringe. Sample was, centrifuged for 40 min. at 8000 rpm and 4°C. After centrifugation, supernatant was collected and stored in a refrigerator.

Determination of cell mass

Enzyme (1 mL) was placed into dry Eppendorf tubes and centrifuged. The supernatant was discarded while pellets were dried in oven at 80°C for 24 h. The cell mass was calculated from its dry weight.

Evaluation of enzymatic activity

Mannanase enzymatic activity was measured by DNS method established by Miller (Miller 1959). Measurements were taken for sample and control. For sample, at first, 50 µL of diluted enzyme was mixed and vortexed together with 250 µL of LBG substrate (1%, w/v), 100 µL of distilled water, and 100 µL of phosphate buffer pH 6 (50 mM). The mixture was incubated for 15 minutes at 60°C. After that, 500 µL of DNS solution was added, vortexed, and incubated for 15 minutes at 100°C. The mixture was cooled in ice for 10 minutes and its absorbance was measured by spectrometer at λ = 540 nm. For control, the same procedure was conducted, but instead of adding the diluted enzyme, 50 µL of phosphate buffer pH 6 (50 mM) was added.

$$\textbf{Enzymatic activity (U/mL)} = \frac{\boldsymbol{c \cdot DF \cdot 10^3}}{\boldsymbol{t \cdot M}}$$

c : concentration of mannose (ppm)
DF : dilution factor
t : reaction time (15 min.)
M : molecular weight of mannose (~ 180.16)

Result and Discussion

In present study, the enzyme production was carried out using batch fermentation method. In this type of fermentation, most of materials required are filled onto bioreactor in the beginning of process and not removed until the end of the process (Cinar et al. 2003). The process was operated twice in a New Brunswick BioFlo/CelliGen 115 Fermenter 2L (Figure 1).

Fig. 1. Fermentation in New Brunswick

BioFlo/CelliGen 115 Fermenter 2L (T = 28°C, aeration = 0.2 vvm, motor rotation = 400 rpm)

The operation of first batch was aimed to ascertain the ability of stain to produce β-mannanase enzyme in fermentor scale. An inoculum (100 mL), which had already incubated for 3 days, was aseptically loaded onto fermentor containing 1 L of sterile medium. Medium composition and parameter of process used in accordance to method explained. The fermentation was conducted at 28°C and pH 8 owing to the fact that the strain exhibited high enzymatic activity at this condition (in a shaker flask method) (Rahmani et al. 2017). As a first trial, the aeration and motor rotation was set at 0.2 vvm and 400 rpm, respectively.

To examine its activity, the product was sampled after 72 h of fermentation process. The production was terminated by separating the enzyme and cell. The cell weight was 2.533 ± 0.5 g. Supernatant, on the other hand, consisted of enzyme and its activity was evaluated by DNS method. The activity of enzyme produced was 16.36 ± 3.63 U/mL (Tabel 1).

Table 1. Mannanase activity of enzyme produced (batch I)

	Absorbance		*c*	Enzyme
	Sample	Control	(ppm)	activity (U/mL)
1	0.404	0.227	54.25	20.09
2	0.342	0.259	34.67	12.84
3	0.345	0.219	43.63	16.16
				16.36 ± 3.63*

* Average ± STD

More detail observation was studied on the second batch. All parameters, such as composition of medium, temperature, aeration and motor rotation, were similar to previous batch. However, sample was collected at various times to figure out the change of enzyme activity and dry cell weight over time. Product was sampled at 0, 18, 24, 42, 48, 66, 72 and 90 h for dry cell analysis and at 0, 18, 48, 72 and 90 h for enzymatic activity analysis.

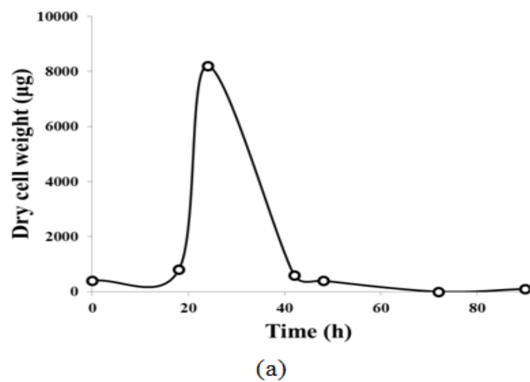
Table 2. Dry cell weight (batch II)

Time (h)	Dry cell weight (μg)
0	400
18	800
24	8,200
42	600
48	400
72	0
90	100

Table 3. Mannanase activity of enzyme produced (batch II)

Time	Absorbance		*c*	Enzyme
(h)	Sample	Control	(ppm)	activity (U/mL)
18	0.064	0.053	134.86	49.95
48	0.066	0.051	136.68	50.62
72	0.073	0.037	146.23	54.15
90	0.114	0.044	161.68	59.88
				52.34 ± 4.91*

* Average ± STD



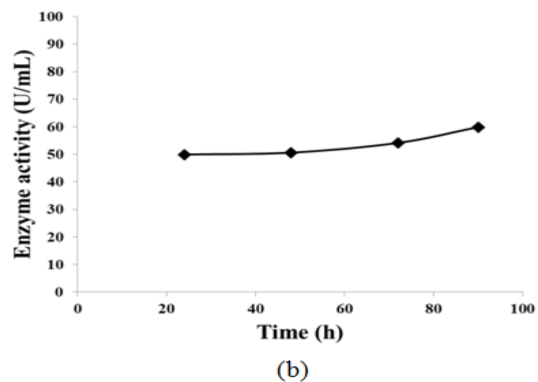


Fig. 2. The correlation of fermentation time and dry cell weight (a), or enzyme activity (b)

Bacterial growth during fermentation was studied through the change of dry cell weight within the process. Quantitative results were presented in Table 2. According to the data, dry cell weight significantly rose from 800 µg (at 18 h) to 8,200 µg (at 24 h), indicating the process was in a logarithmic phase within this period. However, downward trend started after it as the dry cell weight dropped to 600 µg (at 42 h) and no significant change occurred afterward, indicating the start of death phase. Within this period, the nutrient was no longer sufficient and the rate of cell death was higher than the rate of cell growth. This trend is clearly shown in Figure 2(a).

Meanwhile, the enzyme activity fluctuated, ranging from 49.95 U/mL (at 0 h) to 59.88 U/mL (90 h) (Table 3). The highest activity occurred at the end of process because the enzyme was accumulated during fermentation process. Figure of this trend is illustrated in Figure 2(b).

In the other hand, Dhawan and co-workers (2015) investigated the effect of inexpensive agroresidues (apple pomace, orange peel, potato peel, copra meal, oat bran, and wheat bran) on β-mannanase production by using *Paenibacillus thiaminollyticus*. According to their results, the β-mannanase activities were found as 50, 55, 80, 372, 527, and 590 U ml–1, respectively. Soni and co-workers (2015) optimized the production of mannanase from palm kernel cake (PKC) as substrate by using Aspergillus terrus FBCC 1369 via RSM, and the effect of particle size of substrate, pH, moisture content, and carbon and nitrogen supplements was studied. Optimized medium consisted of 5 g PKC of 0.5 mm particle size, LBG 1% (w/v), and urea 1% (w/v) as carbon and nitrogen supplementation with 12.8 ml moisture content, with a pH of pH 8.0, which resulted in a maximum yield of 417 U gds–1 β-mannanase. El-sahrouny and co-workers (2015) studied the optimization of the fermentation medium components using Placket-Burman design, and glucose and inoculum size were found as the most important factors enhancing the production of enzyme. Using optimized medium in the fermentation process, enzyme activity of 42.2 U ml–1 was achieved.

However, the effect of temperature, aeration and rotor rotation on the enzymatic activity and microbial growth should be further studied. Other parameters, such as pH and DO, also need to be monitored. The activity assay of purified enzyme is also essential to do so that the results could be compared to previous experiment. Overall, this study showed the prospective results and capability of enzyme production in fermentor-scale level.

Conclusions

Mannanase enzyme from *Kitasatospora* sp. could be produced using batch fermentor method. Under mentioned conditions, fermentation reached logarithmic phase at 24 h and the dry cell weight at the time was 0.0082 g. Mannanase activity of obtained enzyme was 52.34 ± 4.91 U/mL.

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The Ultrastructure and Enzyme Susceptibility of Oil Palm Empty Fruit Bunch Pretreated by Oxalic Acid under Microwave Irradiation

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Abstract

Pretreatment of lignocellulosic biomass is one of determining steps in the conversion of the biomass to ethanol. There are some changes of the ultrastructure in the biomass during pretreatment which might have correlation with the enzymatic saccharification of the pretreated biomass. The aim of this research was to investigate ultrastructure changes in oil palm empty fruit bunch (OPEFB) after pretreatment using oxalic acid under microwave irradiation. OPEFB particles (40-60 mesh) were pretreated using 1% oxalic acid at 180 °C for 5, 10 and 15 minutes in microwave oven. After separating the liquid fraction by filtration, the solid fraction was freeze dried for 24 hours, then was analyzed for its morphology using scanning electron microscope (SEM), its crystallinity using X-ray diffraction (XRD), and its functional groups using Fourrier transform infrared spectroscopy (FTIR). Enzymatic saccharification was performed at 50 °C for 120 hours. SEM analysis shows the more severe fiber disruption in the OPEFB pretreated with longer duration of heating. The band absorbance of functional group around 1732 cm^{-1} representing unconjugated carbonyl linkage between lignin and xylan was lost in the pretreated OPEFB. Crystallinity indexes of the pretreated OPEFB were higher than the untreated one. The highest glucose yield was 36.6% per initial biomass or equal to almost 80% of cellulose conversion. However, there was not clear correlation between the biomass crystallinity after pretreatment and enzyme susceptibility of the biomass, which was shown by the glucose yield obtained from the biomass.

Keywords: Crystallinity, glucose yield, fiber morphology, microwave pretretament, oxalic acid

Introduction

Indonesia is the highest producer of crude palm oil (CPO) in the world. In 2016 Indonesia produced 84 million tons of oil palm fresh fruit bunch (OPFFB) and resulted in 20 million tons of CPO (Statistics Indonesia 2016). According to Wong et al. (2000), the processing of OPFFB to CPO generated 21% of oil palm empty fruit bunch (OPEFB) having 70% moisture content. This means that in 2016 there was 17.64 million tons of wet OPEFB or 5.29 million tons of dry OPEFB generated from the manufacture of the CPO in Indonesia. Previous studies show that OPEFB contains significant amount of cellulose, ranging from 22.5 up to 56% (Nomanbhay et al. 2013, Ying et al. 2014, Kamcharoen et al. 2014, Akhtar et al. 2015, Medina et al. 2016, Palamae et al. 2017, Burhani et al. 2017, Rosli et al. 2017, Fatriasari et al. 2018). Therefore, OPEFB is considered as a potential biomass for producing glucose which could be further processed to obtain different kinds of products, such as ethanol, butanol, and lactic acid.

In order to hydrolyze the cellulose in the OPEFB, some pretreatment processes are needed. The aim of the pretreatment is basically to reduce the recalcitrance of the lignocellulose biomass, thus it better facilitates the cellulase enzyme for penetrating the fiber matrices to hydrolyze the cellulose. This could be reached for example by reduction of particle size of the biomass to increase its surface area, by heating of the biomass, and by using some chemicals to disrupt the fibers and to cleave some linkages in the lignin-carbohydrate complexes of the biomass. Among pretreatment methods of biomass, microwave pretreatment is one of potential methods, due to its short duration of heating. Microwave irradiation could be combined with

chemicals, such as acid, either organics or inorganics, so that the pretreatment was more effective. The use of sulfuric acid, which is an inorganic acid, in the pretreatment has been widely recognized and reported (Foston & Ragauskas 2010, Wang et al. 2011, Rocha et al. 2014, Zhang et al. 2016, Risanto et al. 2018). The use of organic acids, such as oxalic acid and maleic acid, have been reported as well (Kootstra et al. 2009, Gong et al. 2010, Barisik et al. 2016, Sari et al. 2016, Fatriasari et al. 2018). Comparison between dilute mineral and organic acid pretreatment has also been reported by Kootstra et al. (2009) and Zhang et al. (2013). The use of acids during pretreatment mostly removed hemicelluloses, and only has a little effect on the removal of lignin (Solihat et al. 2017, Fatriasari et al. 2018).

The changes of ultrastructure of OPEFB during sequential pretreatment using sulfuric acid and continued with sodium hydroxyde was reported by Burhani et al. (2017). Microwave-oxalic acid pretreatment at different temperatures have been reported in our previous report (Solihat et al. 2017). The study shows that temperature during microwave-assisted oxalic acid pretreatment affects the morphology of OPEFB. It also affects the chemical compositions of the OPEFB, and thus, the intensity of some functional groups related to the main chemical components of the biomass. Unfortunately, the effects of duration of heating and the correlation between fiber disruption and the enzyme susceptibility of the biomass have not yet revealed. Therefore, in this study the effects of duration of heating on the ultrastructure of OPEFB was observed, and its effects on the enzyme susceptibility was also discussed.

Materials and Methods

Materials

The OPEFB used was the same as that used in our previous report (Risanto et al. 2018). It was obtained from Sukabumi and it contains 8.44±0.04% of moisture, 2.91±0.03% of ash, 2.69±0.11% of extractives, 23.62±0.08% of acid insoluble lignin, 41.23±0.77% of cellulose, and 26.55±0.57% of hemicellulose.

Pretreatment of OPEFB

The pretreatment of OPEFB was the same as that in our previous study (Solihat et al. 2017). Briefly, 3 g of OPEFB (oven-dry weight) was added with 1% oxalic acid in a microwave tube with S/L (solid to liquid) ratio of 1:10, and degassed at -20 bar vacuum condition for 5 min before pretreatment process. The pretreatment in the microwave was maintained under 50% magnetic stirring condition at 180 °C for 5, 10, and 15 min in a digestion microwave (Milestone START D), not including approximately 12 min pre-heating time to targeted temperature and 10 min cooling time. After irradiation the tubes were cooled in an ice bath for 30 min. The insoluble fraction or pulp was obtained by filtering the slurry through filter paper by vacuum filtration (GAST DOA-P504-BN, USA) and washed with distilled water until neutral, indicated by blue litmus paper. The weight and moisture content of the insoluble fraction or pulp were measured to determine the pulp recovery, then, the pulp was put in a sealed plastic bag, and stored in a freezer.

Functional group analysis

As much as 2-5 mg of sample was mixed homogenously with about 0.05 g of KBr and put in a sample holder in the FTIR (FTIR Prestige 21, Shimadzu). The analysis was conducted at wave number of 4000-400 cm^{-1}. A total of 40 scans were accumulated in transmission mode with a resolution of 4 cm^{-1}.

Analysis using X-Ray Diffractometer (XRD) and determination of biomass crystallinity

The pretreated biomass samples were dried in a freeze drier (Alpha 1-2 LDplus, Martin Christ Gefriertrocknungsanlagen GmbH, Germany) for 8-9 h. The dry samples were analyzed using XRD (Rigaku Smartlab instrument) with copper (Cu) as radiant source. Analysis was performed at an energy of 40.0 kV, electric current of 30.0 mA, scanning speed of 2.0º/min., step size of 0.02º and scanning angle of 10─40º. Crystallinity index (CrI) was defined as the percentage ratio of crystalline cellulose, and calculated using a software (PANalytical X-pert Pro Diffractometer).

Morphological analysis

The morphological analysis of the untreated and the pretreated OPEFB were conducted using a Hitachi SU-3500 scanning electron microscope (SEM). The samples were put on a specimen stub (10 mm in diameter) and their surfaces were coated with a thin layer (approximately 400-500 Å thick) of gold using coating unit Ion Coater iB2 to make them electrically conductive.

Enzymatic hydrolysis of pretreated OPEFB and determination of glucose yield

The solid fractions were hydrolyzed using cellulase (Meicellase from *Trichoderma viride*, Meiji Seika, enzyme activity 200 FPU/g). The enzyme solution was prepared by dissolving 1 g of enzyme in 100 mL of water, to make enzyme concentration of 1%. The enzymatic hydrolysis was conducted in a 200 mL bottle, each containing 1 g (oven-dry weight) of OPEFB pulp, 10 mL of 0.05 M citrate buffer, pH 4.8, 1 mL of 2 % sodium azide, and excess cellulase enzyme (120 FPU/g substrate). Eventually, 0.1 M citrate buffer was added to get total final weight of 100 g. After this step, the bottles were tightly closed, and the samples were incubated at 50 °C for 120 h in an incubator shaker (Wisecube, WIS-30R) at 150 rpm.

The glucose release after enzymatic hydrolysis was analysed using Glucose CII test kit (Wako Junyaku, Co., Osaka) and quantified by a microplate reader (Paradigm P2 Spectramax) at a wavelength of 505 nm. After obtaining glucose concentration in the hydrolysate, the glucose yield per initial biomass were determined by Eq. 1. In this calculation we assumed that the total final weight of the enzymatic hydrolysis solution (100 g) was equal to 100 mL.

$$\text{Glucose Yield (\%)} = \frac{\text{GC} \times 100}{\text{PW}} \times \left(\frac{\text{PR}}{100}\right) \quad (1)$$

Where GC = glucose concentration (in g/L)
100 = the total final weight (volume) of enzymatic hydrolysis solution
PW = oven dry weight of pulp sample (g)
PR = pulp recovery (%)

Results and Discussion

Pulp recovery after pretreatment

The pulp recovery after microwave-oxalic acid pretreatment of OPEFB at 180 °C was ranging from 60.88 up to 65.17%, with the highest from pretreatment for 5 min. These were corresponded with the weight loss of the biomass which was ranging from 34.83 up to 39.12% with the lowest from pretreatment for 5 min. At longer duration of heating there was more chemical components broke down into smaller molecules and dissolved in the acid solution. Among the three major components in the lignocellulose, hemicellulose was the easiest component to be degraded by thermo chemical treatment, especially in acid solution (Barisik et al. 2016, Ying et al. 2014, Kootstra et al. 2009). Ying et al. (2014) reported that the hemicellulose content of OPEFB was decreased from 44% to 5% after pretreatment using 2% H_2SO_4 at 120 °C for 60 min, while the lignin and cellulose content did not change significantly. The loss of hemicellulose in OPEFB during pretreatment was confirmed by the loss of band at 1732 cm^{-1} in the FTIR spectra (Fig 1) of all pretreated samples. Therefore, the results of this study confirmed that the major part of OPEFB that was degraded was hemicellulose. The initial content of hemicellulose in the biomass was 26%, while the weight losses of the biomass were higher than 26%. This was probably due to the loss of some small parts of cellulose and lignin which were degraded and dissolved in the acid. The pulp recovery of OPEFB obtained in this study was slightly lower than that reported by Fatriasari et al. (2018) who obtained 66.97% pulp recovery after microwave-maleic acid pretreatment of OPEFB at 180 °C for 2.5 min with 1% maleic acid.

Table 1. OPEFB pulp recovery after microwave assisted oxalic acid pretreatment

Pretreatment	Pulp recovery (%)	Weight loss (%)
180 °C, 5 min.	65.17 ± 0.01	34.83 ± 0.01
180 °C, 10 min.	60.88 ± 1.57	39.12 ± 1.57
180 °C, 15 min	63.48 ± 3.51	36.53 ± 3.51

Functional groups in OPEFB

Major peaks obtained during the analysis of OPEFB using FTIR are listed in Tabel 2. Peaks around 3300 cm^{-1} were related to hydrogen bonds in cellulose, while those around 2905 cm^{-1} were due to symmetric stretching vibration CH in cellulose. There were some peaks missing in the treated biomass, i.e. at 2866 cm^{-1} for asymmetric CH_2 valence vibration and at 1732 cm^{-1} for unconjugated C=O in xylan

Table 2. Assignments of IR bands of untreated and microwave-assisted oxalic acid pretreated OPEFB at 180 °C

Absorption bands wavenumber (cm^{-1})				Assignments
Un-treated	5 min.	10 min.	15 min.	
3333	3381	3323	3304	Stretching vibration intramolecular hydrogen bonds in cellulose
2903	2905	2905	2905	Symmetric stretching vibration CH in cellulose
2866	-	-	-	Asymmetric CH_2 valence vibration
1732	-	-	-	Unconjugated C=O in xylan
-	-	1663	1690	C=O stretch in conjugated p-substituted aryl ketones in lignin
1599	1607	1599	1599	C=C and C=O stretching of aromatic lignin
-	1441	1452	1439	C=C stretching of the aromatic ring in lignin
-	1315	1327	1362	C-H vibration in cellulose C-O vibration in syringyl derivates in lignin
1248	1244	1225	1227	Guaiacyl ring and C=O stretch in lignin and xylan
-	-	1153	1148	C-O-C stretching of β–(1,4) glycosidic bond between sugars
1088	-	-	-	C-H stretching of sugars
1020	1036	1047	-	C-O/C-H stretching of sugars in cellulose
781	-	781	763	C-H_2 vibration in cellulose Iα in cellulose
679	648	635	-	C-O out of plane bending in cellulose

These were probably related to the loss of some chemical compounds, such as hemicellulose from the biomass caused by pretreatment. On the other hand, there were also some peaks that were only appear in the pretreated samples, such as around 1663-1990, 1439-1452, and 1315-1362 cm^{-1}, which mostly related to lignin. According to Auxefans et al. (2017), the bands at 1300-1600 cm^{-1} were for aromatic skeletal vibrations of lignin. The condensation or crosslinking of lignin during pretreatment could be attributed to the appearance of the peaks at the above wavenumbers. The peaks in the range of 890-1200 cm^{-1} show carbohydrate abundance in the biomass. Peak around 1160 cm^{-1} was for the C-O-C stretching of β–(1,4) glycosidic bond between sugars, while that around 1100 cm^{-1} was for C-H stretching of sugars, and that around 1034 cm^{-1} was for C-O/C-H stretching of sugars in cellulose (Auxefans et al. 2017).

XRD pattern and crystallinity of OPEFB

Crystallinity of cellulose in the biomass is one of factors that could affect enzyme susceptibility of the biomass. Fig 1 shows the XRD patterns of untreated and pretreated OPEFB, while Table 3 shows their crystallinity indexes (CI). The CI of untreated OPEFB was rather low (13.9%) compared to the CI of OPEFB reported by other researchers. Rosli et al. (2017) reported that the CI of native of OPEFB was 40%, while that of the OPEFB powder (<250 µm) was 37%. Nor et al. (2016) and Dahnum et al. (2017) reported similar results, which were 38.27 and 38.33%, respectively. The difference could be due to the size of OPEFB particles as well as the instrument and method or software that were used to analyze and calculate the CI. The CIs of pretreated OPEFB were higher (19-21%) than the untreated one (Table 3). The similar results were reported by Burhani et al. (2017) for the OPEFB pretreated by dilute H_2SO_4, NaOH and combined of dilute H_2SO_4 and NaOH as well as by Singh et al. (2015) for dilute acid pretreated corn stover. This was caused by the loss of amorphous part of the OPEFB during acid pretreatment. The amorphous part of the biomass, especially hemicelluloses, was degraded more easily than cellulose, which had more compact and crystalline structure. The detachment of hemicellulose from cellulose chains increased the formation of hydrogen bonding between cellulose chains, because there was a lack of competition from hemicellulose for hydrogen bonds, which turn out in increasing the crystalline form in the cellulose chains (Pingali et al. 2016). The CI increased until 10 min pretreatment, but after that it was slightly decreased when the pretreatment was prolonged until 15 min. The longer duration of heating probably caused some degradation in the crystalline part of the cellulose in OPEFB. These data were correlated with the result of glucose yield in Table 4.

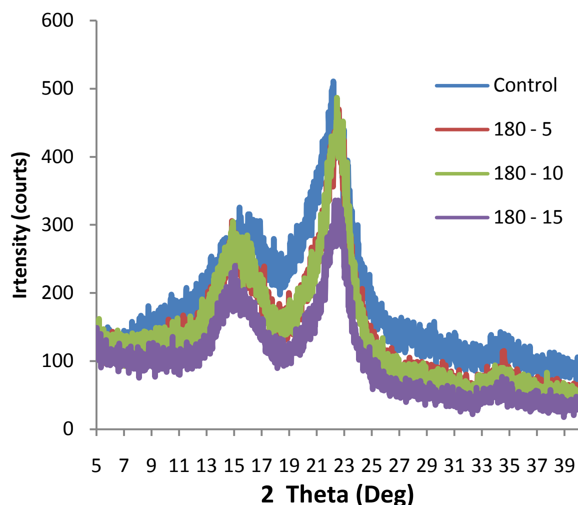


Fig 1. XRD patterns of untreated and microwave oxalic acid pretreated OPEFB at 180 °C for 5, 10, and 15 min.

Table 3. Crystallinity index of untreated and microwave oxalic acid pretreated OPEFB

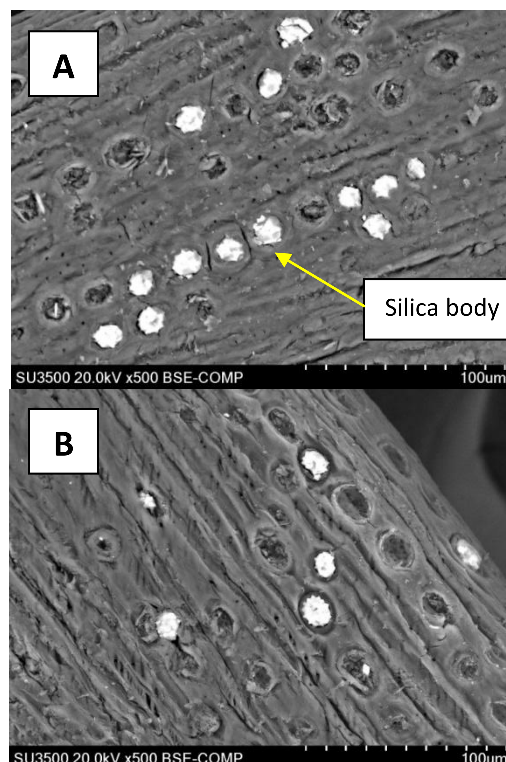
Pretreatment	Crystallinity Index (%)
Untreated (Control)	13.96
180 °C, 5 min	19.34
180 °C, 10 min	21.27
180 °C, 15 min	19.60

Morphology of OPEFB

The results of morphology analysis using SEM (Fig 2) show a stepwise degradation of OPEFB fiber due to longer heat treatment. In the untreated sample it can be seen that the structure of the fiber was still firm with so many silica bodies fill in the circular craters on the surface of the fiber. The appearance of silica bodies on the surface of OPEFB was also reported by other researchers, such as Law et al. (2007), Shamsudin et al. (2012), Nor et al. (2016), Solihat et al. (2017), and Burhani et al. (2017). In the sample experiencing heat treatment for 5 min there was a little fiber degradation and the silica bodies were still attached on the circular craters. The increase of duration of heating until 10 min caused a more pronounce of fiber degradation and removed more silica bodies from the craters, and surface of the fiber looked more loosened and disrupted. The bottom part of the craters is perforated (Law et al. 2007). Thus, when the silica bodies were removed, the cellulase enzyme could penetrate the fiber through the perforated craters. The most degraded fiber was found in the sample exposed to the longest heat treatment in the experiment (15 min). There was almost no silica body and more extensive of fiber disruption with perforated craters on the fiber surface. Nevertheless, the results of enzymatic saccharification (Table 4) of 10 min and 15 min pretreatment were almost the same.

Glucose yield after enzymatic saccharification

Table 3 shows that glucose yields from enzymatic saccharification of pretreated OPEFB were about three to four times higher than that of untreated OPEFB. This shows that microwave oxalic acid pretreatment removed some recalcitrance in the OPEFB, which increases the enzyme susceptibility of the biomass and better facilitates the conversion of cellulose to glucose. The glucose yield per initial biomass and the cellulose conversion were increased from 31.35% (313.5 mg glucose / g dry initial biomass) to 36.60% (366 mg glucose / g dry initial biomass) when the duration of heating increased from 5 to 10 min. However, they were did not change a lot when the duration of heating was further increased until 15 min. The glucose yield based on the weight of pretreated biomass or pulp (Table 2) shows that the glucose yield from pretreated OPEFB could reach five to six time higher than that form untreated OPEFB, with the highest 60.20% or 602 mg glucose / g dry treated biomass.



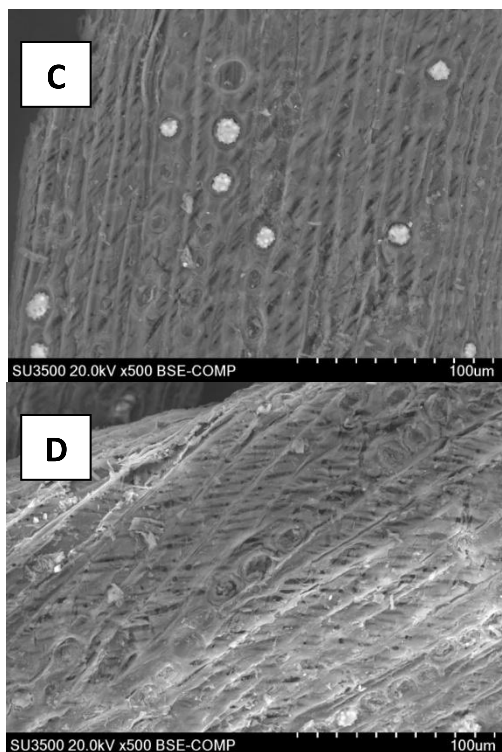


Fig 2 SEM micrographs of untreated OPEFB (A) and m.icrowave oxalic acid pretreated OPEFB at 180 °C for 5 (B), 10 (C), and 15 (D) min.

The pretreatment at this condition could convert as much as 80% of cellulose in the OPEFB. The glucose yield obtained in this study was slightly lower than was reported by Palamae et al. (2017), who could obtain 629.8 g/g dry pretreated OPEFB after enzymatic hydrolysis of two step pretreatment of the biomass using peracetic acid (9 h) and alkaline peroxide (12 h) at 20-35 °C. Another two step pretreatment using 4% H_2SO_4 (121 °C, 60 min) and 40% NaOH (121 °C, 15 min) resulted in 708 mg glucose / g pretreated biomass (Kim and Kim, 2013). Even though those pretreatments resulted in higher glucose yield, the two-step pretreatment takes longer time and probably consumes more energy than the microwave pretreatment conducted in this study. Hassan et al. (2013) could only obtain 282 mg glucose / g EFB after enzymatic saccharification of steam and acetic acid pretreatment of OPEFB, which was lower than that obtain in this study. The results of this study was comparable with those of Ying et al. (2014) who obtained glucose yields of 34.9 and 34.2% per initial biomass after water pretreatment (170 °C, 60 min) and sulfuric acid pretreatment (2%, 120 °C, 60 min), respectively.

Table 4. Glucose yield after enzymatic saccharification of OPEFB pretreated with oxalic acid under microwave irradiation

Pre-treatment	Glucose yield per pulp (%)	Glucose yield per initial biomass (%)	Cellulose conversion (%)
Untreated	9.99 ± 0.74	9.99 ± 0.74	21.81 ± 1.61
180 °C, 5 min.	48.10 ± 8.87	31.35 ± 5.78	68.43 ± 6.12
180 °C, 10 min.	60.20 ± 6.08	36.60 ± 2.75	79.90 ± 6.01
180 °C, 15 min	57.64 ± 6.15	36.48 ± 1.88	79.63 ± 4.09

Conclusions

Microwave-assisted oxalic acid pretreatment at 180 °C has changed the ultrastructure of OPEFB. The pretreatment has loosen the fiber structure, removed some amorphous part from the biomass, and improve enzymatic digestibility of the biomass. The highest glucose yield and cellulose conversion was obtained after enzymatic saccharification of the fiber pretreated for 10 min.

Acknowledgement

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Formulation of Palm Oil Based Surfactant for Fungicide Emulsifiable Concentrate

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Abstract

Natural materials are widely developed to replace petroleum-based materials because of their advantages. Natural oils and its derivatives are biodegradable, renewable and relatively safe for environtment and human. In Indonesia natural oil like crude palm oil has high productivity and it can be utilized for many applications such as surfactant. Surfactant contributes in lowering interfacial tension between oil and has been used for many industries. Fungicide formulation in emulsifiable concentrate (EC) use surfactant to make the active agent spontaneously emulsified in water. This active agent is commonly in oil phase which is hardly dissolves in water. It is obvious that surfactant play important role in emulsion stability. The most efficient emulsion formula optimization is using HLB system. In this study, combination of palm oil based surfactant was used to stabilize emulsion system contained essential oils for fungicide EC formulation. The best result was achieved at surfactant HLB value 10 with surfactant composition MES 20%, PDO 10% and PMO 70%, oil composition neem oil 33%, eugenol oil 33%, citronella oil 33% in oil/surfactant ratio 60/40 based on emulsions stability test for 6 hours. Furthermore, this study can be developed by investigating its efficacy and residue in environment.

Keywords: palm oil, surfactant, fungicide, emulsion, stability.

Introduction

Natural materials become interesting study to replace petroleum-based materials because of their advantages. Natural oils and its derivatives are biodegradable, renewable and relatively safe for environtment and human (Salimon et.al. 2012). Indonesia has abundant palm oil resources. In 2015 total crude palm oil (CPO) production was 31.3 million tons and it is estimated increase in 2020 for about 40 million tons (Permadi et.al. 2017). There is a huge opportunity to utilize this resource, for example as surfactant. Surfactant contribute in lowering interfacial tension between oil and water phase (Saxena et al. 2017).

The development of palm-oil based surfactant is widely investigated because of its many applications such as for enhance oil recovery (EOR) in oil well petroleum industry, pharmaceutical industry, nanotechnology and fungicide formulation in agricultural industry (Sonawane et.al. 2015, Muhpidah et al. 2017; Feng et al. 2016). Palm oil based surfactant used in this study was methyl ester sulfonate (MES), polyethyleneglycol monooleate (PMO) and polyethyleneglycol dioleate (PDO). Fungicide with emulsifiable concentrate (EC) formulation use surfactant to make active agent spontaneously diluted with water and practically applied (Allawzi et.al. 2016). Fungicide EC formulation should have good stability during its application in field, therefore surfactant combination become important to maintain its performance (Meliana et al. 2016). Essential oil naturally has anti fungal and anti microbe effect such as thyme, peppermint, lemongrass, citronella, eugenol and also neem (Sellamuthu et.al. 2013, Wang et al. 2010). A study by Ali et al. reported that nanoemulsion with citronella and neem oil mixture showed antifungal activity against phytopathogenic fungi (Ali et al. 2017). Eugenol oil nanoemulsion has antifungal activity against Fusarium wilt (Abd-Elsalam & Khokhlov 2015). But, these oils hardly dissolved in water which made surfactant has important role in stabilizing the oil in emulsion droplet (Hu et.al. 2016). In this study, the essential used for fungicide active agent was neem oil, citronella oil and eugenol oil.

The approach of formula optimization of surfactant combination can be done by varying wide types of surfactant with different HLB value. The most efficient way to save time in surfactant selection is using hydrophile-lipophile balance (HLB) system. HLB system provide number of ingredients which will be emulsified thus the blend of surfactant can be decided based on the same HLB number (Inc 1980). The HLB of the oils and surfactants are shown in Table 1.

Table 1. HLB Value of Oils and Surfactans

Substance	HLB
Neem Oil	9
Eugenol Oil	6
Citronella Oil	12
MES	0.5
PDO	8
PMO	13

This study aimed to optimize fungicide EC formulation based on HLB value system with essential oil neem, eugenol and citronella as fungicide active agent and palm oil derivative MES, PMO and PDO as surfactant. Emulsion stability was investigated by observing creaming formed in emulsion system for 6 hours aging every 10 minutes.

Materials and Methods

The materials used in this study were neem oil, eugenol oil, citronella oil as oil phase, methyl ester sulfonate (MES) as anionic surfactant HLB value 0.5, polyethylene glycol monooleat (PMO) HLB value 13, polyethylene glycol dioleat (PDO) HLB value 8, from Sigma Aldrich as nonionic surfactant, ethanol for analysis Merck and distilled water.

The variation of surfactant was based on (Hypo-Lypo Balance) HLB value and oil/surfactant ratio. The variation in this study is shown in Table 2.

Neem oil, eugenol oil, citronella oil were mixed altogether with surfactant for 2 hours with mixing speed 1000 rpm. This mixture was added by ethanol with ratio 1:3. The formulation was spontaneously emulsified by mixing it with water by ratio 1:10.

Emulsion stability was investigated by observing creaming height for 6 hours every 10 minutes.

Table 2. Variation of Fungicide Formulation based on Surfactant HLB Value and Oil/Surfactant Ratio.

Formula	Oil Fraction	Surfactant Fraction	Oil			Surfactant			HLB Surfactant
			Neem	Eugenol	Citronella	MES	PDO	PMO	
A1	60	40	33%	33%	33%	10%	35%	55%	10
A2	70	30							
A3	80	20							
A4	90	10							
B1	60	40	33%	33%	33%	10%	15%	75%	11
B2	70	30							
B3	80	20							
B4	90	10							
C1	60	40	33%	33%	33%	20%	30%	50%	9
C2	70	30							
C3	80	20							
C4	90	10							
D1	60	40	33%	33%	33%	20%	10%	70%	10
D2	70	30							
D3	80	20							
D4	90	10							

Result and Discussion

Oil has HLB value to know the required HLB values for O/W emulsion. Similar with surfactant which is also assigned an HLB numerical value. The combination two or more oil or surfactant can be calculated with equation below:

(Oil/Surfactant Percentage 1 X HLB) + (Oil/Surfactant Percentage 2 X HLB) + ... + (Oil/Surfactant Percentage n X HLB)

The HLB value combination neem oil, eugenol oil and citronella oil is 9 according to respective equation. The preliminary test based on approximate HLB range is plus or minus one from HLB value (Inc 1980). It was decided the surfactant HLB value at 9, 10 and 11 as described in Table 2.

The oil phase stability also observed to investigate the compatibility and solubility between oil and surfactant. The result of oil phase stability is shown in Table 3. Formula A3, A4, B2 and C4 were separated in minutes 360. Surfactant selection and combination should has theoretical miscibility of their lipophilic part with the oil formulated in emulsion system (Holtzscherer and Candau 1988). Solubility between two or more compounds can be predicted using Hansen Solubility Parameters (HSPs) to evaluate the ability of compound interaction (Peña-gil, Toro-vazquez, and Rogers 2016). Neem oil contain 42% oleic acid (Djibril et al. 2015), eugenol oil contain 33% linolenic acid (Al-jasass and Al-jasser 2012), citronella oil main substituent is citronellal which is C10 hydrocarbon chain (Silva et al. 2011). All surfactants mainly contain oleic acid. The Hansen Solubility Parameter of the oils and surfactant is shown in Table 4. Solubility of components were determined by matching solubility parameters of the components incorporated in the formulation with its medium (Junior et al. 2016). Neem and eugenol oil had similar fatty acid composition and solubility parameters with surfactant used, unlike citronella oil which had slight difference solubility parameters. This reason caused separation in oil phase. The difference of surfactant composition on each formulation also affected the compatibility between oil and surfactant. It was also seen that the separation occurred mostly in high oil/surfactant ratio.

Table 3. Stability of Fungicide Oil Phase for Emulsifiable Concentrate

Formula	Observation	Remarks	Separation Height (cm)
A1	Stable	-	-
A2	Stable	-	-
A3	Separated	Separated at minute 360	0.1
A4	Separated	Separated at minute 360	0.2
B1	Stable	-	-
B2	Separated	Separated at minute 360	0.1
B3	Stable	-	-
B4	Stable	-	-
C1	Stable	-	-
C2	Stable	-	-
C3	Stable	-	-
C4	Separated	Separated at minute 360	0.2
D1	Stable	-	-
D2	Stable	-	-
D3	Stable	-	-
D4	Stable	-	-

Table 4. Hildebrand Solubility Parameters and Hansen Solubility Parameters of Oils and Surfactants

Compound	Main Fatty Acid Composition	carbon number	δ_d	δ_p	δ_h	δ_t
Citronella Oil	Stearic Acid	10:0	16.2	4.2	8.3	18.7
Neem Oil	Oleic Acid	18:1	16.5	3.1	5.7	17.7
Clove Oil	Linolenic Acid	18:2	16.5	3.1	6.2	17.7

MES	Oleic Acid	18:1	16.5	3.1	5.7	17.7
PMO	Oleic Acid	18:1	16.5	3.1	5.7	17.7
PDO	Oleic Acid	18:1	16.5	3.1	5.7	17.7

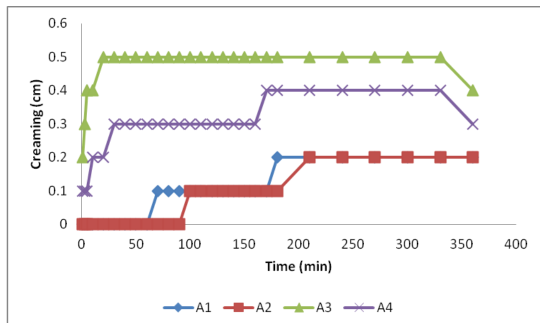


Fig. 1. Creaming of Formulation A for 6 Hours Aging.

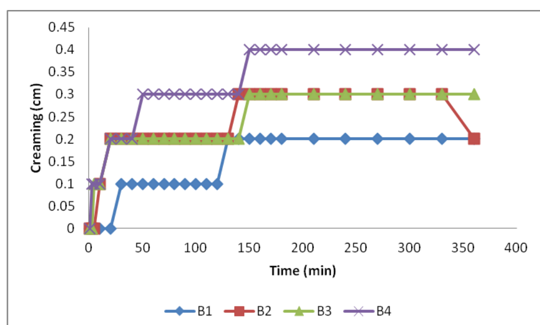


Fig. 2. Creaming of Formulation B for 6 Hours Aging.

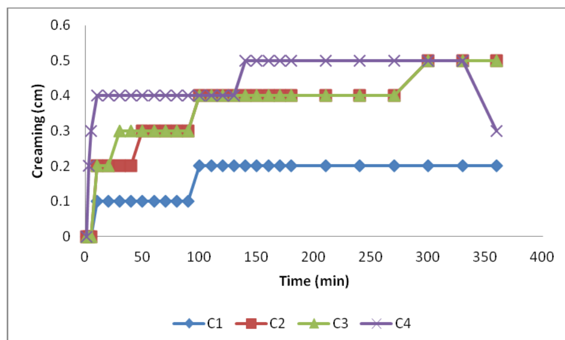


Fig. 3. Creaming of Formulation C for 6 Hours Aging.

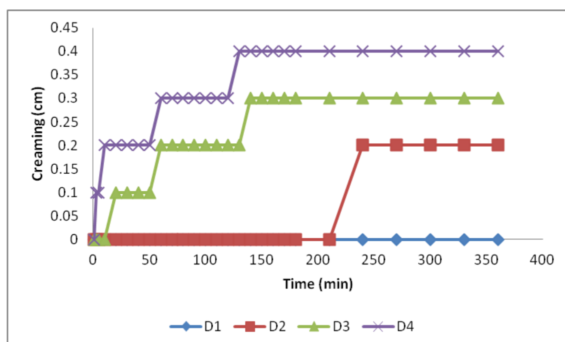


Fig. 4. Creaming of Formulation D for 6 Hours Aging.

Stability test of formula A1-A4 is shown in Figure 1. Creaming occurred in formula A1, A2, A3, A4 at minutes 70, 100, 1, 1 respectively.

Stability test of formula B1-B2 is shown in Figure 2. Creaming was formed in formula B1, B2, B3, B4 at minutes 30, 10, 5, 3, respectively.

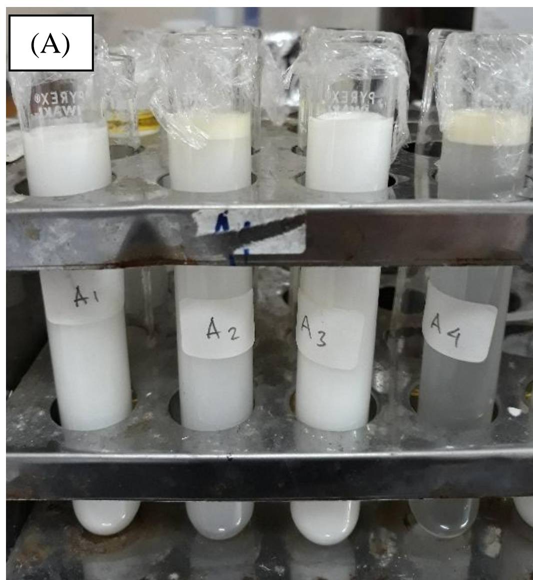
Creaming formation of formula C1-C4 is shown in figure 3. It occurred in formula C1, C2, C3, C4 at minutes 10, 10, 10, 3, respectively.

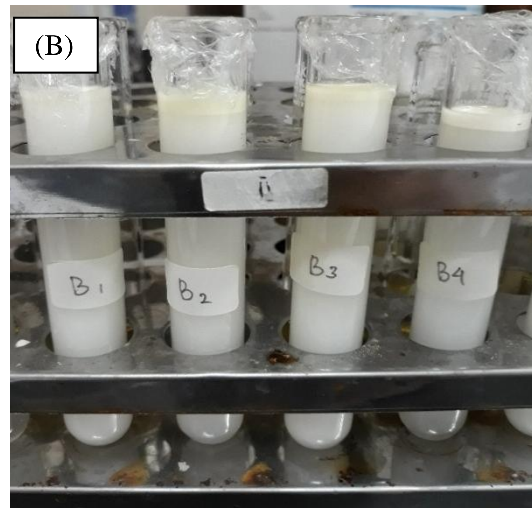
The emulsion physical appearance of Formula A-D is shown in Figure 5.

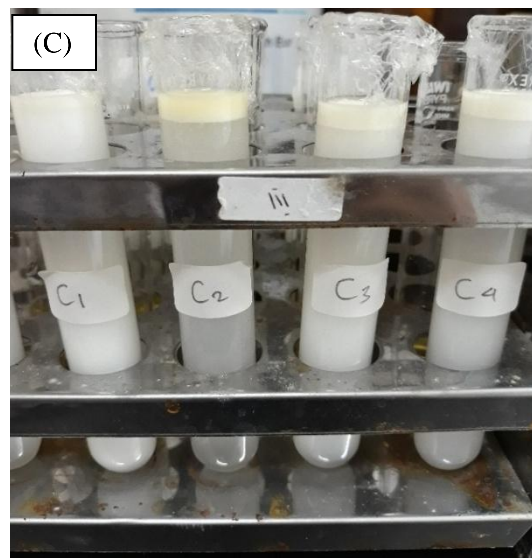
Stability test of formula D1-D4 is shown in figure 4. Creaming was formed in formula D2, D3, D4 at minutes 240, 20, 3, respectively. It was seen that destabilization occurred faster as oil/surfactant ratio increase. Formulation with higher surfactant content were easily self-emulsified (Agubata et al. 2014). This characteristic is very important since the application of the fungicide based on spontaneous emulsification system for practical use. It was also proved by the optimum condition in formula D1 where the surfactant amount was the highest among formula variation. This creaming occurred because the emulsion separate into two emulsion, the cream was richer in the disperse phase than the other where the droplets density was lower than the solvent density and this phenomenon leads to further destabilization called coalescene (Science 2011). It could be concluded that combination of surfactant unable to form stable droplets in higher oil ratio. Creaming induced by surfactant micelles yet formed a polydisperse droplet size (Bibette, Roux, and Pouligny 1992). It is known that narrow size distribution enhance emulsion stability compared than polydispersed emulsion (Meliana & Septiyanti 2016). A study by Matsaridou use oil/surfactant ratio in range 6:4 and 7:3 for formation of self-emulsifying microemulsion because this range covered the region of emulsification and produce smaller droplets size (Matsaridou et al. 2012).

It is commonly know that HLB affect stability of emulsion. HLB range of 10-15 is proposed for stable emulsion and finer droplet diameter (Matsaridou et al. 2012). It was proved by the optimum formula D1 where the HLB value of surfactant was 10. The surfactant HLB also near with HLB of oil combination. HLB correlated with critical micelle concentration of surfactant where the micelles start to form (Prakash 2010). The surfactant with HLB value 10 produce more stable emulsion despite of higer value than HLB value of the oil, this was because surfactant with higher HLB value than recommended range resulted in more stable emulsion (Mohamed et al. 2017).

Similar result also reported by Ali et all where stable nanoemulsion system contained neem oil and citronella oil for fungicide had been achieved at room temperature by centrifugation method (Ali et al. 2017). Abd-Elsalam reported a stable eugenol oil nanoemulsion for fungicide using Tween 20 as surfactant up to 1 month storage at room temperature (Abd-Elsalam and Khokhlov 2015).







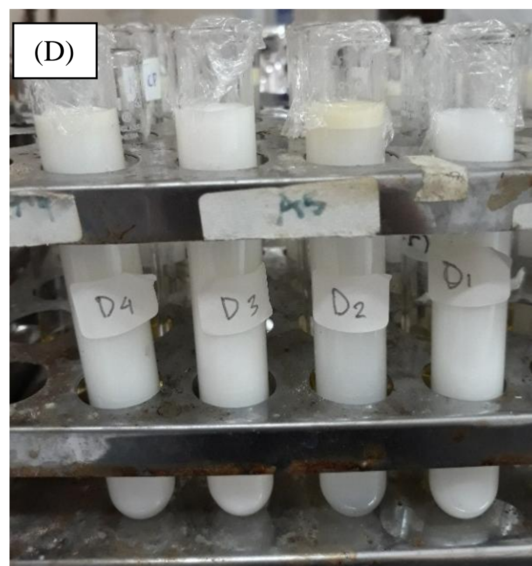


Fig 5. Emulsion Physical Appearance of (A) Formula A, (B) Formula B, (C) Formula C, (D) Formula D.

Conclusions

The best fungicide formulation is formula D1 with surfactant HLB value 10 with composition MES 20%, PDO 10% and PMO 70%, oil composition neem oil 33%, eugenol oil 33%, citronella oil 33% in oil/surfactant ratio 60/40 based on emulsion stability test for 6 hours. Furthermore, this study can be developed by investigating its efficacy and residue in environment.

Acknowlegment

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Screening of Yeast from InaCC (Indonesian Culture Collection) for Lactic acid Producing Yeast Candidate

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Abstract

Lactic acid production by employing yeast is interesting because of their tolerance to low pH and some inhibitors. Naturally, yeast can not produce lactic acid by themselves, but they can be genetically engineered to do this order. The purpose of this research was to screen about 96 InaCC isolates for its tolerance to low pH inhibitors, and could not produce ethanol to become a candidate for lactic acid producing yeast as a host cell. The 96 InaCC isolates were grown at various pH of YPD medium, pH 4.8, 3.0, 2.5, 2.0, inhibitors containing-medium (YS), and both low pH and inhibitors containingmedium.The parameters of the research were cell growth, glucose consumption, and ethanol production. The analysis was performed by using spectrophotometer UV-VIS and HPLC. The screening was yielded a candidate which had the capability to grow at low pH and inhibitors containing-medium, Pichia kudriavzevii.

Keywords:Yeast, lactic acid, screening

Introduction

Lactic acid ($C_3H_6O_3$) is aproduct occured in the fermentation of C6 sugar, resulted by utilizing NaDH from glycolysis process. It has widely applied in some industries, such as food, cosmetic, medical, and biodegradable plastic (Doran-Enderson et.al. 2008). Biodegradable plastic with lactic acid as a monomer is called PLA (Poly Lactic Acid), a renewable bio-plastic as an alternative to petroleum-based plastic (Tamakawa et. al. 2012). Lactic acid has two optical isomers, L-(+)-Lactic acid and D-(-)-lactic acid. Production of lactic acid by microbial fermentation using renewable source is developed because it results pure isomer of lactic acid (Tang et. al. 2013).

The microbes that commonly used for lactic acid production is lactic acid bacteria (LAB), from genus *Lactobacillus,* also *E. coli* and *Corynebacterium glutamicum* (Okano et. al. 2010, Klotz et. al, 2016). Unfortunatelly, using bacteria to produce lactic acid have some lacks, that areuntolerant to low pH and remaining high amount of gypsum (Sauer et. al 2010), the gypsum is used to control pH in medium during fermentation (Singh et. al, 2006).

Yeast is the eukaryotic unicellular organism which relativelly tolerance to low pH (Tamakawa et. al. 2012). Naturally, yeast could not produce lactic acid in their metabolism. Therefore, many studies interest to engineer them until they have the ability to produce lactic acid. the screening was selecting the yeast with low pH tolerant (Liu *et. al*, 2005). Yeast engineered by introducing the lactate dehydrogenase (LDH) gene into their cell, this gene leads the pyruvic acidto be converted to lactic acid (Liu et. al. 2005, Ikushima et. al. 2009). Some studies use ethanol producing yeast, such as *Saccharomyces cerevisiae*and *Candida utilis*. As the host cell for LDH gene, to enhance the production of lactic acid, they also deleting or decreasing the expression of pyruvate decarboxylase (PDC) gene to prevent the conversion of pyruvate to acetaldehyde and furthermore to ethanol (Adachi et. al. 1998, Ishida et. al. 2006, Ikushima et. al. 2009). The

study to employ yeast to produce lactic acid is still conducted until today. It is necessary to screen the potential one to enrich the research about lactic acid producing yeast.

Indonesian Culture Collection (InaCC) has many collections of yeasts from various sources that have not been utilized yet. The aim ofthis research were to screen and select the yeast that has potentiality to be lactic acid producing yeast by screening in low pH and inhibitor containing-medium.

Materials and methods

Microorganism

Yeasts are the collection of InaCC, Indonesian Culture Collection, Research Center for Biology, Indonesian Institute of Sciences. The 96 yeast isolates were various species and genus of yeasts and taken from several places. The medium were YPD and 0.2YS. YPD: yeast extract (Difco,USA) 10 g/L, peptone (Difco, USA) 20 g/L, glucose (NacalaiTesque, Japan) 20 g/L, xylose (NacalaiTesque, Japan) 20 g/L. 0.2YS: yeast nitrogen base w/o amino acids (Difco, USA) 6.7 g/L, glucose 50 g/L, xylose 50 g/L, and inhibitors: acetic acid (Merck, Germany) 15 mM, formic acid (Sigma-Aldrich, USA) 7.5 mM, furfural (NacalaiTesque, Japan) 15 mM, 5-HMF (NacalaiTesque, Japan) 2.5 mM, levulinic acid (NacalaiTesque, Japan) 1.25mM. also YPX medium, yeast extract (Difco,USA) 10 g/L, peptone (Difco, USA) 20 g/L, xylose (NacalaiTesque, Japan) 20 g/L.

Growing in pH 3.0 YPD and 0.2YS medium

Each single colony of 96 yeast from YPD agar plate were inoculated into 1 mL YPD liquidmedium of preculture in 96 deepwell plate (Eppendorf, Germany). The preculture was incubated in the deepwell maximizer from TaitecBioshaker M BR-0224UP (Japan) at 30°C, 1400 rpm, for 18 hours.

After 18 hours of theincubation period, as much of 100µL preculture was added to 900µL main culture contained pH 3.0 YPD medium in deepwell plate. The main culture was incubated in deepwell maximizer at 30°C, 1400 rpm, for 24 hours. In the same way, 0.1 mL preculture was added to 0.9 mL pH 4.8 0.2 YS medium in deep well plate. The culture was incubated in the deep well maximizer at 30°C, 1400 rpm, for 24 hours.

The cultures were cultivated after 24 hours fermentation. The cells from the culture were transferred to microtube for analysis purposes. The cultivated cultures were checked for optical density, glucose consumption, and ethanol production.The yeasts which could grow in that medium were selected to go through the next step screening.

Growing in pH 2.5 and pH 2.0 YPD medium

The selected yeasts from the previous step were grown at pH 2.5 and pH 2.0 YPD medium. A single colony was inoculated into1 mL YPD liquid medium as preculture, then incubated in deep-well maximizer at 30°C, 1400 rpm, for 18 hours. From each culture, 0.1 mL was transferred into 0.9 mL pH 2.5 YPD medium in the deep well plate. Then were incubated in deep well maximizer at 30°C, 1400 rpm, for 24 hours. The same way was applied for growing the yeast into pH 2 YPD medium.

After 24 hours, the cultures were cultivated and transferred into 1.5 mL microtube for further analysis. The cultivated cultures were checked for optical density, glucose consumption, and ethanol production. The survived yeasts were selected to perform next step of screening.

Growing in 12 mL pH 3.0 and pH 2.5 0.2YS medium

The selected yeasts from the previous step were grown at 12 mL pH 3.0 0.2YS medium in 100 mL erlenmeyer flask. Each single colony was inoculated into 12 mL YPD liquid medium in 100 mL flask scale, then incubated in a shaker incubator (BIOShaker BR-43FL, TAITEC, Japan) at 30°C, 1400 rpm, for 18 hours. From each preculture, as much of 1.2 mL was inoculated into 11.8 mL pH 3.0 0.2YS medium in flask scale 100 mL, then were incubated in shaker incubator BIOShaker BR-43FL, TAITEC (Japan) at 30°C, 1400 rpm, for 24 hours. Samples were collected periodically at 0, 3, 6, 9, and 24 hour of incubation period, and checked for optical density and glucose consumption.

The survived yeasts were checked for its capability to grow at pH 2.5 0.2YS medium in

100 mL erlenmeyer flask with the similar condition to previous fermentation method. After 24 hours incubation period, the cultures were cultivated and collected for further analysis. They were checked for the optical density and glucose consumption.

Xylose assimilation

The candidate was checked for xylose assimilation ability by inoculating the yeast at xylose containing medium, YPX. The similar step to the previous method was applied for this step.

Analysis

The optical density of the main culture after 24 hours incubation period was measured the absorbance at wavelength 600nm of spectrophotometer UV-Vis from Shimadzu mini-1240 (Japan). The optical density was obtained by multiplying the value of absorbance of with K factor. The optical density is related to the cell growth. The cell growth is the difference of optical density in each time during incubation period.

The quantity analysis of glucose consumption and ethanol production were performed using HPLC (HighPerformance Liquid Chromatography) from Shimadzu (Japan). The column was coregel 87H, with the conditions as follows, flow rate 0.6 mL/min, oven temperature 80°C, elution time 40 minutes, and eluent 5 mM H_2SO_4.

Result and Discussion

Initially, the yeasts of InaCC were checked for the capability to survive in pH 3 of rich medium YPD and pH 4.8 of 0.2 YS medium. To become lactic acid-producing yeast, the candidate must have the capability to survive in low pH, because they will produce lactic acid which the pH is around 3.0. Whereas, the optimum pH range for yeast growth is vary from pH 4 to 6, depending on temperature, oxygen presence, and yeast strain (Narendranath et. al. 2005). According to the figure 1, about 83 InaCC yeast strains were able to survive in pH 3 with the optical density at 600nm was more than 20. The highest one was isolate InaCC Y95, *Pichia silvicola* with optical density as much of 100.4.

YS is the stressed medium. This medium consists of the chemical compounds that estimated exist in the pretreated biomass, such as acetic acid, furfural, 5-HMF, and formic acid. These chemical compounds were organic acid that derived from the breakdown of lignin, cellulose, and hemicellulose. Organic acid inhibit the growth of yeast by affecting the enzymes and cytoplasm of yeast cell (Heer & Sauer 2008) (Iwaki et. al. 2012). This is related to the target of the screening, to get the potential host yeast that has the capability to produce lactic acid from the pretreated biomass.

In this study, we used 20% YS medium in an optimum pH (4.8) as initially condition of the screening. The survived yeasts were about 74 yeasts with optical density in 600nm was more than 20as seen on figure 2. While, isolate with the highest optical density was isolate InaCC Y58, *Rhodosporidium toruloides*as much of 71.

Glucose consumption and ethanol production during fermentation at YPD pH 3 and 0.2 YS medium were checked using HPLC. The graphic (Figure 2) showed the amount of remain glucose and the formed ethanol after 24 hours fermentation. as much of 30 isolates have no remain glucose from the fermentation in both YPD pH 3 and 0.2 YS medium. It showed that the yeast could consume the 50 g/L glucose completely after 24 hours. While, as much of 5 yeast isolates could consume the glucose completely in pH 3 YPD medium, but could not survive nor consume any glucose in pH 4.8 0.2YS medium. Some yeasts could produce ethanol in both YPD pH 3 and 0.2 YS pH 4.8 medium, but the amount of the produced ethanol in 0.2 YS pH 4.8 medium was higher than in YPD pH 3 medium (Figure 3). For the survived yeasts, this condition may be caused by the support of rich medium condition and the presence of inhibitor was not affect the yeast activity, while pH was normal for yeast, so it would not affect the growth.

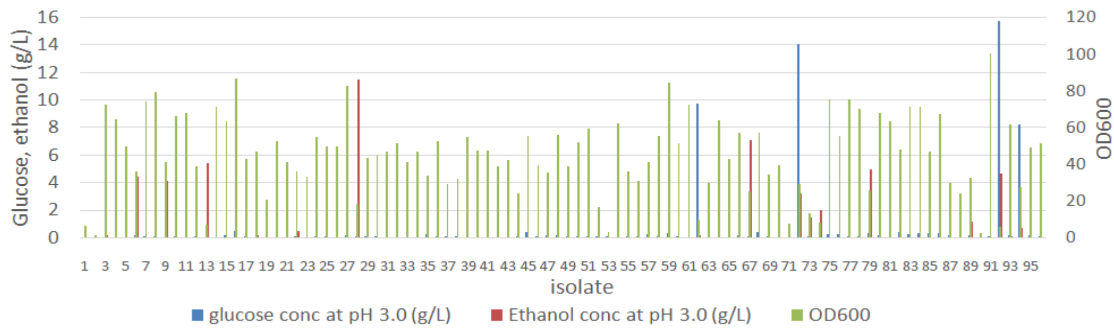

Fig. 1. Optical density (OD600), glucose remaining, and ethanol produced in pH 3.0 YPD medium after 24 hours fermentation

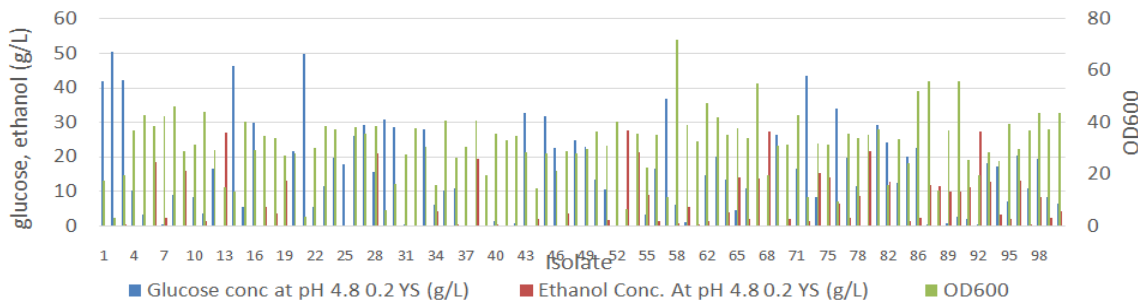

Fig. 2. Optical density (OD600), glucose remaining, and ethanol produced in pH 4.8 0.2YS medium after 24 hours fermentation

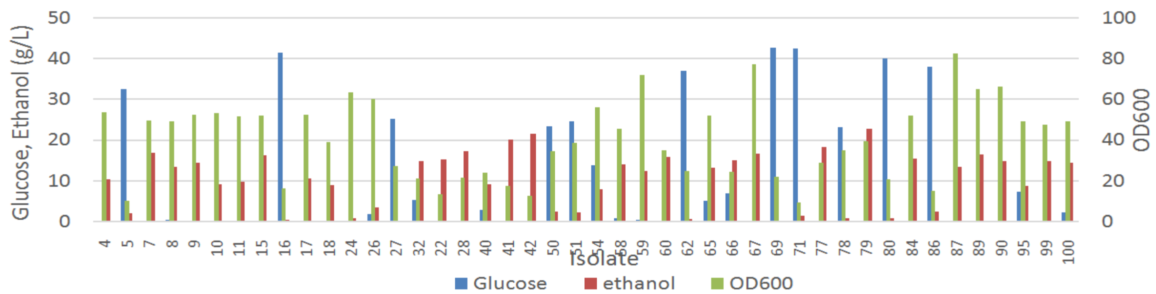

Fig. 3. Optical density (OD600), glucose remaining, and ethanol produced in pH 2.5 YPD medium after 24 hours fermentation

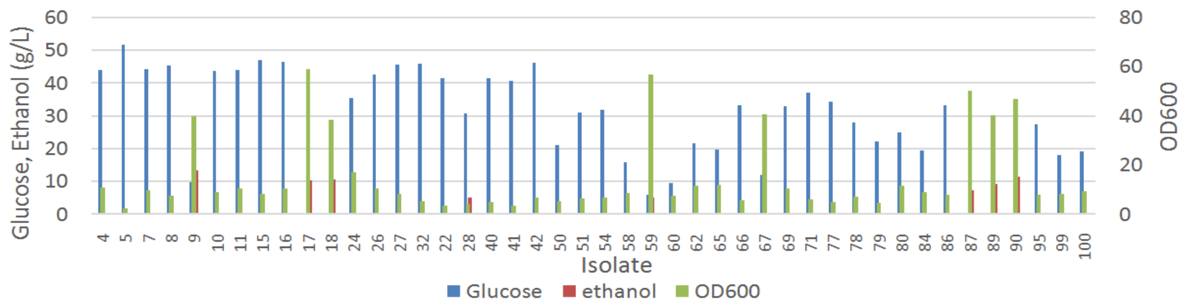

Fig. 4. Optical density (OD600), glucose remaining, and ethanol produced in pH 2 YPD medium after 24 hours fermentation

From these two condition of fermentation, as much of 44 isolates which could grow in both medium in a highest rate were continued to the next step of screening. The selected yeast isolates were grown at pH pH 2.5 and pH 2.0 YPD medium to check the capability of yeasts to survive in a lower pH with rich medium. This result indicate that pH can affect the growth of yeast. When the pH of medium is ajusted deviate to the optimum range, the enzyme of yeast may not work normally or will be deactivated, so it will not be able to grow (Narendranath et. al. 2005)

As much of 44 survived yeasts were grown in pH 2.5 and pH 2.0 YPD medium. After 24 hours of fermentation, almost all 44 yeasts could grow in pH 2.5 medium, with 22 isolates resulted optical density over 20 (Figure 3), but only 8 isolates could survive in pH 2.0 medium with optical density over 20, and consume 50 g/L glucose completely in 24 hours (Figure 4). These 8 survived isolates were Y9, Y59, Y89, and Y90 known as *Candida carpophila*, Y17 and Y18 known as *Pichia kudriavzevii*, Y67 and Y87 known as *Meyerozyma carribica*.

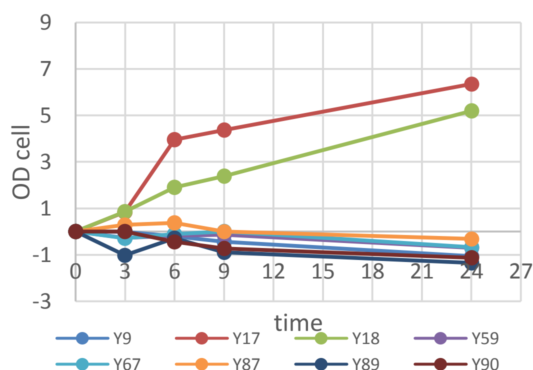

Fig. 5. Optical density (OD600) in pH 3.0 0.2YS medium after 24 hours fermentation(flask scale)

Further, these 8 isolates were checked for their capability to survive in medium contained both stressed and low pH medium, 0.2 YS medium pH 3.0. The fermentation were undergone for 24 hours in 100 ml flask with 12 mL working volume. Based on the data of optical density, 6 isolates could not grow at all, optical density was under 0 or minus, but another 2 isolates grew slowly, the optical density was only 6.36 for isolate Y17 and 5.2 for isolate Y18 after 24 hours, while another isolates were not growing in this media condition (Figure 5).

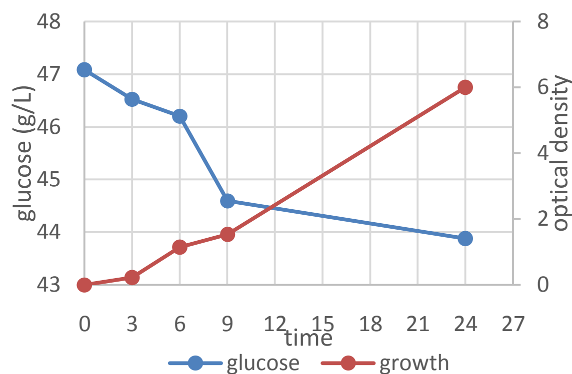

Fig. 6. Optical density (OD600) in pH 2.5 0.2YS medium after 24 hours fermentation(flask scale)

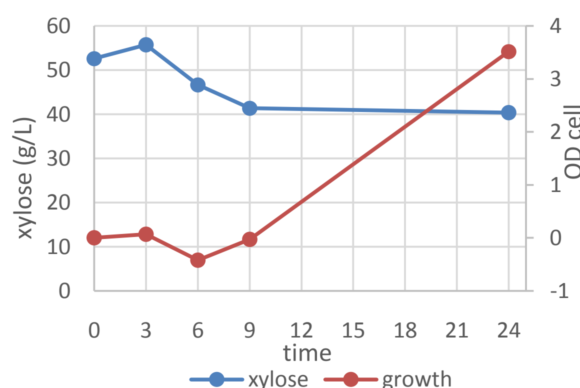

Fig. 7. Optical density (OD600) in xylose containing medium after 24 hours fermentation (flask scale)

The survived isolate Y17 was grew in pH 2.5 0.2 YS medium to checked the growth in higher stressed medium. Figure 6 showed that the yeast was able to grow although not as high as in normal medium. The optical density was quite similar to it when grew in pH 3.0 0.2 YS medium as much of 6. The glucose consumption also went slowly because the stressed medium affect the rate of metabolism of yeast. The isolate Y17 was *Pichia kudriavzevii*. Previous study reported that *P. kudriavzevii* tolerant to high salt (Na2SO4) and acid (H2SO4 at pH 2.0) with heat 43ºC, so this yeast also thermotolerant (Isono et. al. 2012). Yuangsaard (2013) also has studied for thermotolerant *P. kudriavzevii* from pork sausage to produce ethanol with optimal temperature for fermentation was at 40ºC.

P. kudriavzevii also checked for its capability for xylose assimilation, as this hemicellulose is a part of lignocellulosic biomass. Figure 7 showed the growth of the yeast in xylose containing medium, after 24

hours fermentation, xylose was only consumed in a little amount, about 10 g/L, and the growth was not significant, the optical density was under 4. Gallardo et al. (2011) has that *Issatchenkia orientalis* (former name of *P. kudriavzevii*) was negative assimilation of xylose based on the analysis using the API ID32C kit and the key of Middelhoven assay. By this reason, Toivari et al. (2013) was engineering this yeast to express D-xylose dehydrogenase to produce D-xylonate in a high yield.

Park et al. (2018) has engineered *P. kudriavzevii* strain to become lactic acid producing yeast by introducing D-lactate dehydrogenase gene (D-LDH) to replace pyruvate decarboxylase 1 (PDC1) gene. The engineered strain was succesfuly produce D-lactic acid up to 154 g/L. By this result, it is the promised way to employ the potential yeast *P. kudriavzevii* as lactic acid producing yeastusing the proper method, such as knock out PDC1 gene and other genes that lead to ethanol production and replace it with LDH gene from another microorganism.

This research was selecting the best candidate as host for lactic acid producing yeast. From 96 yeast isolates of InaCC, *Pichia kudriavzevii* is potential for this aim.

Conclusions

The 96 yeast isolates from Indonesian Culture Collection(InaCC)had been conducted the screening for low pH and stressed medium tolerant. The isolate InaCC Y17Pichia kudriavzevii has been selected as the potential candidate for lactic acid producing yeast which has the characters: low pH tolerant, stressed medium tolerant, minor xylose assimilation, and robust.

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Screening of Succinic Acid producing Lactic Acid Bacteria from Indonesia Culture Collection

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Abstract

A total 321 of *Lactobacillus* strain from *Indonesia Culture Collection* was screened for the ability to produce succinic acid in MRS medium. The detection of succinic acid was carried out by flourescent ring procedure and high performance liquid chromatography (HPLC). Lactobacillus strain were fermentated in MRS medium for 48h in incubator shaker 37°C, 150 rpm. The supernatants obtained from fermentated media were analyzed for the presence of succinic acid. Positive isolate which produce succinic acid was showed green flourence colour in reaction on flourescent ring procedure. 22 isolates represent type species of LAB which has positive result to produce succinic acic qualitatively was analyzed by HPLC. The highest concentration succinic acid was obtained by *Leuconostoc mesenteroides* with succinic acid concentration was 11,14 g/L. *Leuconostoc mesenteroides* may also produced acetic acid (2,8 g/L) and formic acid (8,7 g/L), but did not produced lactic acid. Other strains of LAB such as *Enterococcus durans, Lactobacillus* sp., *Enterococcus faecium, Lactobacillus plantarum, Lactobacillus brevis, Lactobacillus casei* did not produced succinic acid from citrat medium, but produced high lactic acid and acetic acid. Further investigation is needed to analyzed biochemical pathways for produced succinic acid from *Leuconostoc mesenteroides.*

Keywords : organic acids, indigenous bacteria, fermented food, bioprocess

Introduction

Product development from the application of Biorefenery technology is currently being a concern in the industry. The application of this technology is one of the alternative solutions to support the reduction of oil resource problems and environmental pollution. Nowadays more chemical compounds are produced by chemical processes, so the development of the production of chemical compounds biologicallly becomes important (Ragauskas et al. 2006). Among these chemical compounds, succinic acid is an attractive target because using this succinic acid can produce a variety of derivative products with economic value. Various derivative products produced from succinic acids include 1,4-butanediol, tetrahydrofuran, c-butyrolactone, and other chemical products that have a broad market. These products are widely used as solvents, industrial polymers and others. (Zeikus et al. 1998, Beuprez et al. 2010).

The market for succinic acid production in Asia is increasing quite high every year. Pacific Asia including Indonesia occupies the third world market for succinic acid. This increase in the world market spurs the development of efficient and low-cost succinic acid production. For the production of succinic acid efficiently there are two main key things, first is the microbial fermentation medium with the lowest cost and second is the isolate microbe that is able to use various sources of sugar from various biomass with high yield and productivity.

Several publications reported the development of fermentative succinic acid production by utilizing agricultural or food waste as a substrate, including using wheat waste (Du et al. 2008), corn (Li et al. 2009) as well as bread industry food waste (Lee et al. 2012). Some microorganisms that have been known to be capable of producing succinic acid include *Actinobacillus succinogenes* (Xi et al. 2012), *Anaerobbiospirilium succiniciproducens* (Lee et al. 2008), *Mannheimia succiniciproducens* (Lee et al. 2006), *Escherichia coli* (Liu et al. 2011), *Corynebacterium glutamicum* (Inui et al.

2004), and *Lactobacillus* strains (Kaneuchi et al. 1988).

Lactic acid bacteria (LAB) is a group of gram positive bacteria that can produce lactic acid. This bacterium found mostly in fermented foods and partly in the rumen of mammals. Succinic acid production using LAB was carried out by Kaneuchi et al. (1988) and Dudley & Steele (2005). The diversity of LAB collections obtained from Indonesian fermented products is very diverse. And there has been no research report regarding the microbial potential of LAB from Indonesia which can produce succinic acid.

Song & Lee (2006) states that the formation of acetic, formic, and lactic acid products is the main problem that must be overcome because it affects the yield and production of succinic acid. Thus the metabolic characteristics and fermentation of each microorganism will affect the production of succinic acid produced. Based on these informations, studies of microbial metabolism in succinic acid production are very interesting. In this paper, we tried to report the results of microbial selection from a collection of LAB collections belong to *Indonesia Culture Collection* (InaCC) that could potentially produce succinic acid.

Materials and Methods

Isolates of *Lactobacillus* strain (total 321 stains) were obtained from InaCC, LIPI, Cibinong, Bogor. Culture medium including for maintenance, fermentation and bacterial testing used MRS medium with a composition of 10 g peptone, 10 g beef extract, 5 g yeast extract, 20 g glucose, 1 mL Tween 80, 2 g ammonium citrate, 5 g sodium acetate, 0.1 g MgSO4 , 2 g dipotassium phosphate and 0.05 g MnSO4.

Preculture was prepared by adding one ose of bacterial isolate to the media containing MRS broth and incubated in the shaker incubator for 24 hours at 37°C, 150 rpm. After 24 hours, the preculture of bacterial isolates were measured OD at 600 nm and transferred to the main culture media by 1% from the volume of MRS broth media, then incubated in the shaker incubator for 48 hours at 37°C, 150 rpm. Supernatant from fermented media was separated from the bacterial cell by centrifuge at 10,000 rpm, 4°C for 10 minutes. Separate supernatants were used for the analysis using kits and HPLC.

Qualitative analysis was carried out to determine the ability of bacteria to produce succinic acid. The analyzed indicator is flourence color which characterizes the succinate content observed in the UV lamp detector. 200 µL of supernatant isolates were added 5 drops of sulfuric acid (p) then reacted with 300 µl of resorcinol 1%. After vortexing, store in an oven at 130 ° C for 1 hour, and cool in ice for 10 minutes. Add 1 mL of 10% ammonia and mix it. The flourence color formed can be checked using the UV lamp detector. As a positive control pure succinic acid is used.

Quantitative succinic acid content was analyzed using HPLC. The column used was Coregel 87H, with eluent of 1 mM sulfuric acid, flow rate of 0.6 mL / min, oven temperature of 80 ° C, the standard used was citric acid, succinic acid, formic acid, acetic acid, and lactic acid in concentration of 50, 100, 250, 500, and 750 ppm.

Analysis of succinic content was performed with Megazyme Succinic kit. Stages of quantitative tests with the Megazyme Succinic kit was based on the procedure stated in the kit instructions using the microplate assay method. The reagent kit consists of 6 solutions, the first solution is buffer + sodium azide, the second solution is NADH + stabilizer, the third solution is ATP + PEP and CoA, the fourth solution is pyruvat kinase + L-lactat dehydrogenase, the fifth solution Succinyl -CoA synthetase, and the sixth solution of Succinic acid. The first procedure was to react 210 µL samples, blanks and standards with 20 µL solution 1, 2 and 3 then add 2 µL solution 4 then mix and read the absorption at wavelength 340 nm after 3 min reaction then add 2 µL solution 5 then measure the absorption again at the wavelength of 340 nm after 6 min of the reaction.

Result and Discussion

This study aims to obtain a strain LAB in the InaCC collection which has the potential to produce succinic acid. Several studies have been carried out using strains of BAL to produce succinic acid using MRS media and several precursors (Kaneuchi et al. 1988, Dudley and Steele 2004). The results of the

study concluded that the use of citrat as a precursor capable of producing 70% succinic acid was higher than other precursors such as fumaric acid, malic acid, ketoglutaric acid, pyruvic acid, and lactic acid in various strains of LAB. Therefore, in this study MRS media which contain ammonium citrate (2 g/L) were used as a medium to select BAL strains that have the potential to produce succinic acid.

Total 321 strains of LAB from the InaCC collection were grown on MRS media to 37°C. The selection process were carried out qualitatively and quantitatively by using the supernatant produced by isolates on the MRS broth medium. Succinic acid is an extrasellular product produced by BAL. The supernatant is used for the analysis of the presence of succinate (Agarwal et al. 2005).

The qualitative test of the presence of succinic acid in supernatant samples was checked by the flourence ring test method using resorcinol reagents. This method has been carried out by Agarwel et al. (2005) and Thakker et al. (2006) to identify the presence of succinic acid effectively and efficiently. Resorcinol is a group of phenol compounds, succinic acid which is a group of carboxylic acids reacted with resorcinol under high temperature conditions with the addition of concentrated sulfuric acid, will produce a yellowish green florencent color (Jaism & Muhammad 2012). The chemical reaction that occurs can be seen in Figure 1 which shows an indication of the presence of succinic acid in the supernatant.

Fig. 1. Qualitative analysis of resorcionol test in BAL isolates compared with positive controls (Ks) and negative controls (K)

The results of the Flourencent ring analysis on LAB isolates were compared with negative controls (MRS) without inoculation and succinic acid) and positive controls (MRS without inoculation plus succinic acid). Figure 2. shows the results of color observations formed in several samples of LAB isolates. Qualitative values are given in the highest range of 3 plus (+) and lowest 1 plus (+). The recapitulation of the resorcinol test results on 321 isolates was obtained by 149 positive isolates to produce succinic acid. The 149 positive isolates consisted of 22 strains with the highest number of strains of Lactobacillus plantarum (Table 1).

Table 1. Grouping of strains of LAB isolates that are positive for succinic acid production based on strain name

No	Nama Strain	Jumlah			Total positive	Total sample
		+	++	+++		
1	Lactobacillus sp.	14	9	6	29	56
2	Lactobacillus brevis	1	0	0	1	3
3	Lactobacillus casei	0	1	0	1	3
4	Lactobacillus fermentum	1	0	1	2	2
5	Lactobacillus paracasei	1	0	0	1	2
6	Lactobacillus pentosus	0	0	1	1	5
7	Lactobacillus plantarum	21	21	8	50	100
8	Enterococcus durans	2	1	1	4	7
9	Enterococcus faecalis	1	4	1	6	15
10	Enterococcus faecium	1	5	0	6	21
11	Enterococcus gilvus	0	1	0	1	1
12	Enterococcus hirae	1	0	0	1	7
13	Enterococcus sp.	4	4	1	9	20
14	Bifidobacterium sp.	0	1	0	1	1
15	Lactococcus lactis	4	2	0	6	11
16	Lactococcus sp.	2	0	0	2	3
17	Leuconostoc mesenteroides	3	1	1	5	11
18	Leuconostoc sp.	0	2	1	3	7
19	Pediococcus pentosaceus	3	1	1	5	8
20	Staphylococcus hominis	0	1	0	1	2
21	Pediococcus sp.	2	2	1	5	10
22	Weissella sp.	2	5	2	9	18
	Total				149	

From the results of the grouping of positive LAB isolates in succinic acid production, quantitative analysis was then carried out using HPLC. A total of 22 positive isolates were subsequently cultured and the

supernatant obtained was analyzed by HPLC. The results of the analysis of succinic acid content from 22 supernatant samples with HPLC showed that the highest yield was produced by LAB isolates namely *Leuconostoc mesenteroides* (Table 2). This type of strain of lactic acid bacteria is a type of gram positive bacteria that is semi anaerobic and has high temperature resistance. Qualitatively, this isolate has a high bacterial activity which is indicated by its absorption value at a 600 nm wavelength in the UV-VIS spectrophotometer of 2.709. Quantitative levels of succinic acid based on HPLC analysis results were obtained at 11.14 g / L. compared with other LAB isolates, the characteristics of LAB isolates *L. mesenteroides* were able to produce high levels of succinic acid in the low lactic acid content produced. The citric acid content produced is also quite high.

Table 2. Analysis of the content of succinic acid produced by 22 LAB isolates selected using HPLC

No	Strain name	Concentration (g/L)				
		Succinic acid	Citric acid	Lactic acid	Acetic acid	Formic acid
1	*Lactococcus* sp.	1.761	21.073	0.375	2.842	1.462
2	*Lactobacillus brevis*	0.000	15.379	32.660	2.683	2.177
3	*Lactobacillus casei*	0.000	8.914	25.339	1.754	4.229
4	*Lactobacillus fermentum*	0.625	16.704	27.250	3.009	3.313
5	*Lactobacillus paracasei*	0.903	14.202	30.996	3.863	5.581
6	*Lactobacillus pentosus*	0.191	13.046	46.552	3.171	8.414
7	*Lactobacillus plantarum*	0.374	14.981	46.567	3.504	3.514
8	*Enterococcus durans*	0.000	12.168	18.676	1.011	0.000
9	*Enterococcus faecalis*	0.000	7.119	17.192	1.214	1.812
10	*Enterococcus faecium*	1.849	14.855	9.185	3.597	0.295
11	*Enterococcus gilvus*	0.325	17.663	34.073	2.668	4.337
12	*Enterococcus hirae*	0.345	10.043	33.947	0.777	4.395
13	*Enterococcus sp.*	1.341	14.629	20.139	4.376	4.384
14	*Bifidobacterium sp.*	1.071	14.534	38.080	2.667	4.075
15	*Lactococcus lactis*	1.180	20.831	0.000	2.492	0.000
16	*Lactococcus* sp.	0.000	0.749	0.000	0.000	0.000
17	*Leuconostoc mesenteroides*	11.147	18.973	0.000	2.831	8.708
18	*Leuconostoc sp.*	2.501	19.432	26.321	3.607	7.979
19	*Pediococcus pentosaceus*	0.997	15.024	28.450	5.243	3.584
20	*Staphylococcus hominis*	5.647	17.747	10.691	4.610	4.916
21	*Pediococcus* sp.	0.436	5.192	9.215	0.893	0.000
22	*Weissella sp.*	2.872	17.456	26.367	3.657	4.206
	Control Negative (K)	0.000	18.446	0.000	2.491	0.000
	Control Positive (Ks)	6.479	17.829	32.432	4.172	3.124

Several studies were conducted to determine the metabolic pathway of LAB strains in producing succinic acid. It is known, that certain types of LAB strains have different characteristics of metabolic pathways. Some precursors added to growth media affect the production of succinic acid produced. From the results of HPLC analysis, in addition to succinic acid, other organic acids were identified including citric acid, lactic acid, formic acid, and acetic acid. The organic acid content produced by several LAB strains is able to describe metabolic pathways with semi-anaerobic environmental conditions. Dudley and Steele (2005) in their study mapped the metabolic pathways of several strains of LAB. Isolate *L. mesenteroides* does not produce lactic acid, but can produce succinic acid 11.14 g/L, citrat acid 18.97 g/L, acetic acid 2.83 g/L and formic acid 8.79 g/L. The strain is known to have the ability to tolerate acidic conditions (Mcdonald et al. 1990). These results showed that *L. mesenteroides* isolates were able to produce succinic acid in semi anaerobic conditions. The metabolic pathways used by *L. mesenteroides* are predicted through the citric pathway. Because of the high content of citric acid, it is used as a precursor for producing succinic acid. However, further investigation is needed to explain the succinic acid production system in this *L. mesenteroides* isolate

Conclusion

A total of 321 isolates of the collection of lactic acid bacteria from InaCC were selected in their potential to produce succinic acid with MRS media. Qualitative analysis of resorcinol and quantitative results with HPLC showed that *L. mesenteroides* isolates were able to produce the highest succinic acid of 11.14 g/L compared to other LAB isolates. The metabolic pathway of the *L. mesenteroides* strain is predicted to use the metabolic jaur citric acid in producing succinic acid. Further investigation is needed to explain the pathway of succinic acid metabolism from these isolates. With the information on metabolic products, it is hoped that optimistic production of succinic acid using *L. mesenteroides* isolates can be developed

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