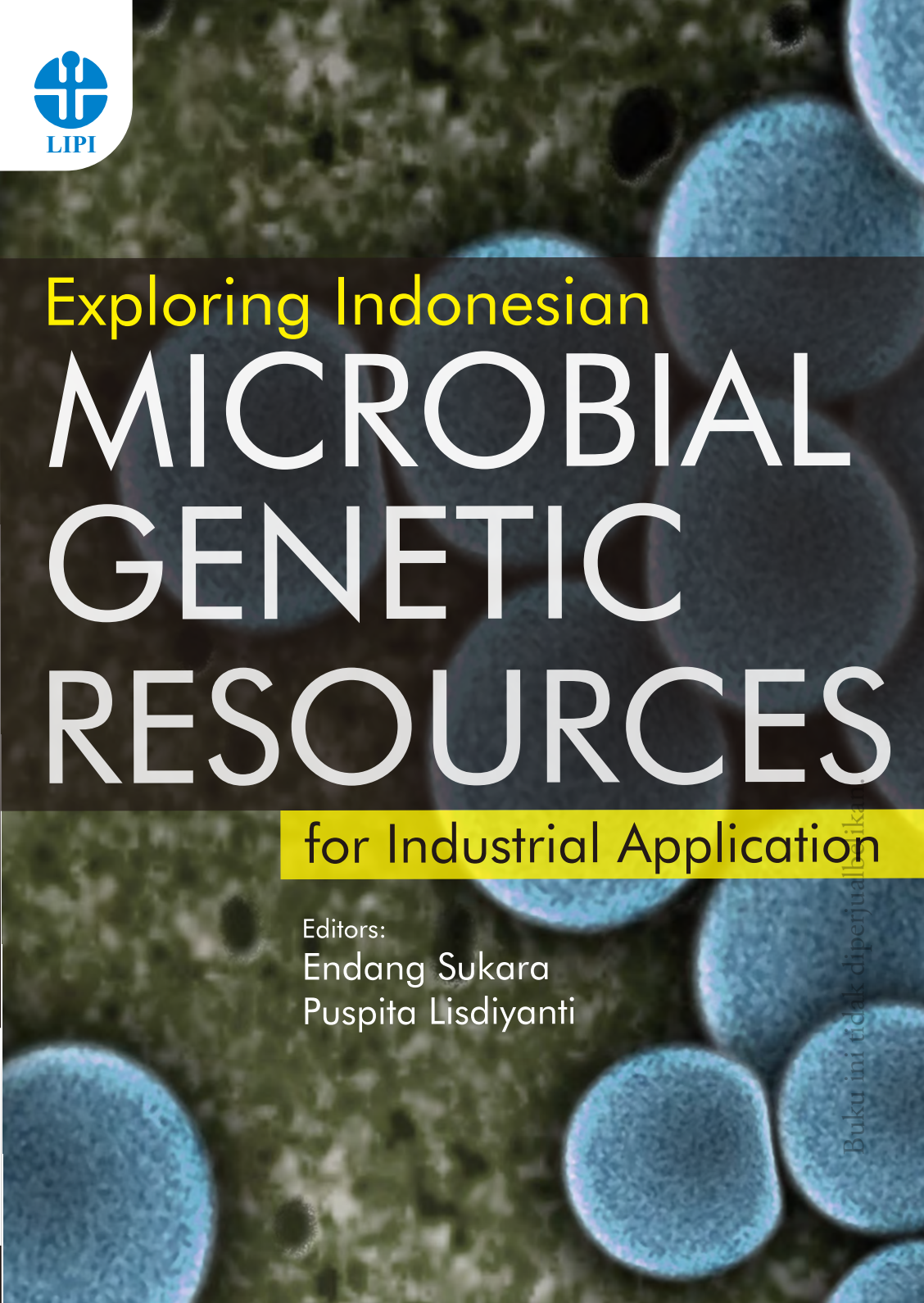
LIPI
Exploring Indonesian
MICROBIAL GENETIC RESOURCES
for Industrial Application
Editors:
Endang Sukara
Puspita Lisdiyanti
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LIPI Press

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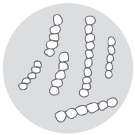

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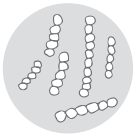


Editorial Note

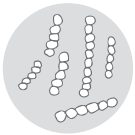
As a scientific publisher, LIPI Press holds on high responsibility to provide high-quality scientific publication. The provision of qualified publication is the epitome of our works to participate in enlightening society intelligence and awareness as stated in The 1945 Constitution of the Republic of Indonesia.

The edited book, *Exploring Indonesian Microbial Genetic Resources for Industrial Application* offers some important findings on the exploration and utilization on the microbial genetic resources in Indonesia. Through this book, some intensive exploration on microbial genetic resources in Indonesia, many new species and even new genus of important microbial taxa have continuously been discovered. This book serves to highlight the significant achievements, especially in the area of microbiology, carried out by the microbiologists in Indonesian Institute of Sciences (LIPI).

We surely hope this book could give new insights and information, especially on diversity of the microbial genetic resources in Indonesia and its potential utilization.

As a final note, we would like to deliver our heartfelt gratitude to everyone taking part in the process of this book.

LIPI Press



Foreword

I am very delighted and I really appreciate this bright initiative of LIPI's researchers to publish a book entitled *Exploring Indonesian Microbial Genetic Resources for Industrial Application*.

This publication certainly helps LIPI to highlight the work being conducted and sets of achievement reached by LIPI's microbiologists. I witness that there are number of significant achievements in the area of microbiology carried out at LIPI. The dedication of LIPI's microbiologists marks a new era for LIPI. The number of international scientific cooperation on exploration and utilization of Indonesian microbial genetic resources has been increasing steadily. Through this cooperation, LIPI has successfully upgraded human resources and research capability. The most important achievement is that through the intensive exploration on microbial genetic resources in Indonesia, many new species and even new genus of important microbial taxa have continuously been discovered. With this, LIPI has proven a significant contribution to the advancement of science in the field of microbiology at global level.

The number of microorganism collection continuously increases, some have been studied for its potential utilization, and some are still awaiting for further studies. Upon this achievement, collection of LIPI is not limited to those of plant specimens at Herbarium Bogoriense,

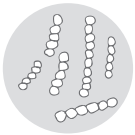
living plant species at Botanic Gardens, and the collection of animal specimens at Museum Zoologicum Bogoriense, but also include living microbial genetic resources. It is also important to note that through a hard work and high dedication of LIPI's microbiologists, we could now demonstrate the future value of microbial genetic resources in supporting sustainable development of the country. I do believe that our microbial genetic resources could provide solution to various problems faced by Indonesia and even other countries. We could demonstrate and offer the usefulness of our collection at the grass root level to support pro poor, pro growth, pro job, and pro green initiatives.

It is well-timed for LIPI to search strategic partners to convert research output to become outcome and provide impact for Indonesian sustainable economic development. At the same time, it is also opportunity for LIPI to build and establish instrument to conserve and sustain the use of microbial genetic resources. LIPI has storage facilities for the conservation of microbial genetic resources, which is called Indonesian Culture Collection (InaCC) which meets international standard as set out by the Budapest Treaty. With this facility, Indonesia now has a facility which can deposit microbial genetic resources, including patent materials and ready for broader collaboration.

Jakarta, 12 June 2016

Deputy Chairman for Life Sciences,
Indonesian Institute of Sciences (LIPI)

Prof. Dr. Ir. Enny Sudarmonowati



Preface

Indonesia is a maritime country sparkled by more than 17,000 islands. Each island is isolated one and another for hundred thousands years. Indonesia is also a volcanic archipelago that forms a ring of fire. Indonesia has diverse ecosystem types, ranging from mountains covered by snow in Papua to a deep sea in Wallace Weber imaginary line region. These facts are gifts for Indonesia's biodiversity as Indonesia becomes a truly mega biodiversity country. In fact, Indonesia is the second largest mega biodiversity country in the world after Brazil.

Nevertheless, biodiversity of microbes described by many scientists in Indonesia is limited compared to plant and animal biodiversity. The biodiversity of microbes in the country has yet to be explored extensively. Scientific work on Indonesian microbial resources has already been started in late 1800 by Dr. M. Treub at Bogor Garden. However, the information on Indonesian microbial genetic resources is still very limited. Treub himself was a botanist. He, for nearly 30 years between 1880 and 1909, collected and described many microorganisms such as fungi, microalgae, and bacteria. The work of Treub, however, came to a halt as no one followed on his footsteps. Only a little more than 50 years later, the study on microbes in Indonesia was continued in 1960s by Indonesian scientists. Yet, the progress was slow due to limited human resources and research infrastructure.

Only in the mid 1990s, the work on microbiology was growing. And just recently, during the last 15 years, exploration on Indonesia microbial resources has been accelerated by LIPI's scientists. Cooperation with Japanese scientists, under the auspice of Japan Society for the Promotion of Sciences (JSPS), Japan Bioindustry Association (JBA), and lately with National Bioresource Center of the National Institute for Technology Evaluation (NITE) Japan has significantly improved research facilities, human resources and number of collections. Many new species of microorganisms which have significant value to agriculture, food, feed, and bioenergy were collected.

It is obvious that Indonesia is rich in microbial resources and their value is significant, not only for the prosperity of Indonesian people but also for the future of humanity. On the study of fungi and actinomycetes, a large amount of new species and even new genus are found. The information on diversity of Indonesian microbial resources and its potency is continuously being accumulated in accelerated fashions. The collections are now safely maintained at the newly established Indonesian Culture Collection (InaCC). InaCC is now well recognized by the World Federation of Culture Collection (WFCC), and the management of collection is progressively being perfected. It is aimed to be part of microbial culture collection according to the Budapest Treaty. It is now timely for LIPI microbiologists to write review on progress made and to disseminate that scientific information widely to the public nationally and around the world. It is our hope that the awareness on the important of microbial genetic resources for the prosperity of the nation could become the reality.

Jakarta, 12 June 2016

Prof. Dr. Endang Sukara

PROLOGUE: Research Experiences on the Use of Indonesian Microbial Genetic Resources by Microbiologists at the Indonesian Institute of Sciences (LIPI)

✍ Endang Sukara and Puspita Lisdiyanti

Introduction

Exploration on Indonesian biological diversity was started on 16th century by Jacob Bontius and Rhumpius Expedition. Then, Reinwardt established Land's Plantentuin (Kebun Raya) in Bogor in 1817, and Melchior Treub established Foreigner Laboratory (later known as Treub Laboratory, Figure 1) in 1884 inside the Land's Plantentuin, later known as Bogor Botanical Garden. Bogor Botanical Garden was truly a working place for world class scientists at that time (Frodin, 1998).

The inventory and sustainable use of microbes in Indonesia was started at Treub Laboratory. The name of Treub Laboratory was to commemorate Dr. Melchior Treub (1851–1910), the botanist and the Director of the Bogor Botanical Garden for nearly 30 years

Figure 1. Treub Laboratory in 1930 (Harvey, 1930) (left) and in 2016 (right)

(1880–1909). During his period, work on microbiology was intensified. Many microbes such as mychorrizal fungi, fresh water microalgae and plant disease bacteria such as *Bacillus saccharis*, *Aspergillus* sp., and *Penicillium* sp., had been collected and studied. Collection and research on Indonesian microbes were carried out by Dr. Treub himself (Cittadino, 2002). Later, many scientists from all over the world (mostly from Europe) visited Treub Laboratory to carry out research on tropical rich biodiversity, including microbial diversity. Most of microbes studied were related to tropical estate crops development program. Treub Laboratory became a well known laboratory for foreign researchers. A mychorryza fungus was first reported by researchers who work in Cibodas Botanical Garden nearly 200 years ago (Dammerman, 1945).

In 1934, research work at Treub Laboratory of Bogor Botanical Garden was stopped and the collection of microbes was not continued. The research in this Laboratory was started again in 1959 by Indonesian researchers supported by the Government (Figure 2). The first president of Republic of Indonesia was really enthusiastic on basic science which he believed to be the foundation for his program known as "Pembangunan Semesta Berencana". The research on using microbes, in particular, was started in 1960.

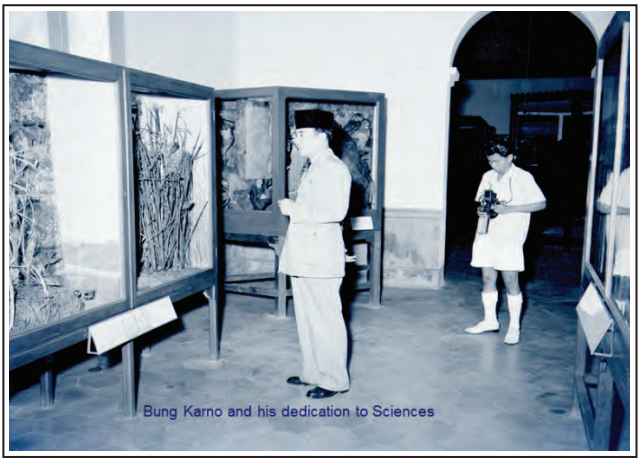


Figure 2. Ir. Soekarno, the First President of Republic of Indonesia, visited Bogor Botanical Garden (photo courtesy of Herbarium Bogoriense's collection).

The aim of this book is to highlight the microbiology works being done and achievement reached by LIPI's microbiologists on the use of Indonesian microbial genetic resources for human welfare. The works include development of biofertilizer, inoculums for fermented foods, silage for feed, functional food, enzymes, and active pharmaceutical ingredients for industries. The works have been started since 1970 and the detailed achievements of selected research experiences were described in nine chapters of this book. The works are mainly based on exploration, identification, and characterization of the potential uses of the microbes.

Microbial Research by Indonesian Microbiologists in LIPI from 1970 to 2000

Actually, Indonesian researchers in LIPI studying on Indonesian microbial genetic resources were started in 1960s, but the intensity of

research was very low due to the limited number of scientist and poor laboratorium infrastructures (Carlyle et al., 1961; Saono et al., 1974; Saono & Ganjar, 1972). Research topics were tailored or inclined to support government programs, mainly on the application of science to improve human welfare. The inventory of microbes, therefore, was dominated for uses in food and agriculture. In September 1973, microbiologist community in Indonesia succeeded in establishing the Indonesian Society for Microbiology. The main aim of this society is to maintain communication among microbiologists in Indonesia. It should be noted that microbiologists from LIPI are actively involved in this society. The number of the members has flourished from around 100 to more than 2,000 at present, and this society is very active in organizing both annual national and international seminar and publishing their works in some reputable journals. In LIPI, the number of microbiologists has increased significantly and the laboratory infrastructure has also improved, which eventually provide big contributions in advancing microbiology in Indonesia.

With a better funding, more microbiologists, better laboratories and facilities, more international research cooperation, and more collection of bacteria, yeast and fungi, microbial researches in LIPI have significantly developed since 1975. At the beginning, the focus was on microbes in fermented foods (Saono & Basuki, 1979). Sukara et al. (1992) described solid substrate fermentations as in the production of *tempe* and other similar products from *Rhizopus oligosporus*. Kanti (2000) continued the work of Saono & Basuki (1979) to identify yeasts in *ragi* and *tape*. Melliawati and Sukara (2015) succeeded in patent certified of paste inoculums for making nata de coco.

Although for centuries microbes have been used in Indonesia to prepare traditional fermented foods such as *tempe* (fermented soybean by *R. oligosporus*), *oncom* (fermented tofu waste by red mold), *tape* (fermented glutinous rice), *terasi* (fermented fish paste), *kecap* (sweet

soy sauce), and beverages likes *brem* (fermented rice), and *tuak* (fermented beverage from rice), there are no large scale industries in these microbial processes (Steinkraus, 1995). Until now, these fermented foods and beverages have been produced through home-based operations; the techniques and equipments was recycled from generation to generation. It is clearly understood that the production processes have rarely changed and much less improved. Modern technology has not entered into these traditional food production methods although they could become important industrial resources. Currently, only the production of inoculums for *tempe* is produced using modern techniques at commercial scale by LIPI.

Furthermore, the focus had been put on microbial resources for agriculture. Saono (1977) and Saono and Karsono (1979) initiated the development of legume innoculant and the works were reviewed by Ladha et al. (1988). Ozawa et al. (1999) selected the low pH and aluminium tolerance of *Bradyrhizobium* isolated for acid soils in Indonesia. Harmastini and Ariani (2010) developed the "Kedelai Plus" technology from the collection of rhizobial strains.

Microbial Research by Indonesian Microbiologists in LIPI from 2001 to 2015

The international collaborations to further explore microbial genetic resources in Indonesia is continuously growing. International cooperation between LIPI and overseas partners under a variety of cooperative schemes in operation between 1980s and 1990s such as between LIPI-EU (European Union), LIPI and Australia through Australia-ASEAN scheme, and LIPI-JSPS was established. The results of these collaborations were not only in increasing the capacity of microbiologists of LIPI, but also significantly increasing the number of important microbial taxa important for sustainable agriculture and fermentation

technology. The number of publications by LIPI's scientists in the field of microbiology was also improved. Two culture collections, namely LIPI Microbial Collection (LIPIMC) at Research Center for Biology and Biotechnology Culture Collection (BTCC) at Research Center for Biotechnology LIPI, were also developed rapidly in this period. These two Culture Collections were recognized by World Federation of Culture Collection (WFCC). With this competencies, LIPIMC and BTCC could support the *ex situ* conservation and maintenance of Indonesian microbes. These two culture collection facilities are recently merged to form Indonesian Culture Collection (InaCC) with better facilities and better management.

After the declaration of Convention of Biological Diversity (CBD) in 1992, the richness of biodiversity, including microbial diversity becomes obvious. More international cooperations to further explore, study, and search for future prospects steadily increase. The number of microbial cultures (stored at InaCC), in addition to the number of specimen of flora (stored at Herbarium Bogorienses and Botanical Gardens) and fauna (stored at Museum Zoologicum Bogoriense), is undergone acceleration. In this context, research collaborations with other countries need to be conducted in the future to further advance our capacity to harness benefit from this invaluable asset, microbial genetic resources. The schemes under the CBD and its related convention should be used as a means to conserve and sustainably use microbial genetic resources for the benefit and prosperity of Indonesian people.

In collaboration with Centre for Agriculture and Biosciences International (CABI) and International Mycology Institute (IMI), England under Darwin Initiative Project from April 1994 to March 1996, 6 fungal taxonomists and 22 staffs have been trained to master on isolation, identification, and preservation of microorganisms. The aim of this collaboration is to enhance the capacity of researchers at

LIPI. This project provided funding to facilitate collection, identification, and exploration of local microbial diversity. The activities were focused on the collection of fermented foods and 400 freshly isolated fungi have been added to the InaCC collection.

In collaboration with LIPI, Agency for Assessment and Application of Technology (*Badan Pengkajian dan Penerapan Teknologi* (BPPT)), and Japan Bioindustry Assosiation-New Energy and Industrial Technology Development Organization (JBA-NEDO) from April 1993 to March 1999, the diversity of economic important bacteria, acetic acid bacteria, and lactic acid bacteria in natural resources were studied and preserved at LIPI Culture Collections. Intensive studies through this cooperation revealed that Indonesia has high diversity of these two groups. It was a big achievement that gained worldwide recognition as we were able to identify two new genus in acetic acid bacteria groups, namely *Asaia* gen. nov and *Kozakia* gen. nov., as well as seven new species of the genus *Acetobacter* (Yamada et al., 2000; Lisdiyanti et al., 2000, 2001, 2002). This was the first contribution of LIPI's scientists to the global advancement in the field of microbiology. Regarding the lactic acid bacteria, a number of species have been identified and these species are of importance in traditional fermentation as well as in silage making. Around 100 isolates of acetic acid bacteria and 200 isolates of lactic acid bacteria (Widyastuti et al., 1999) were collected, well-identified, and added to the InaCC collection.

Through nine intensive meetings (about 2½ years), collaboration between LIPI and the National Institute for Technology Evaluation (NITE) Japan on "The Taxonomical and Ecological Study of Fungi and Actinomycetes in Indonesia" was established, run from April 2003 to March 2009. This model of scientific cooperation may be adopted by other research institutions in Indonesia and in other parts of the world as a mechanism to properly implement the protocol related to CBD, including the implementation of Cartagena and Nagoya Protocol.

Through this cooperation, characterization and identification of economically important fungi and Actinomycetes were successfully carried out. The researchers have also expanded further cooperation with the aim to improve their capacity in characterizing the Indonesian microbial resources for oil degrading microbes. Work on isolation of specific microbes from Indonesian marine environments was carried out between April 2006 and March 2009. During 6 years of activities, the number of economically important collections were significantly increased, man power capacity was improved, and the number of international publications and patent were also increased. From this collaboration, more than 6,500 of fungi, Actinomycetes and oil degrading microbes were collected, well identified, and well preserved (Widyastuti & Ando, 2009).

During the course of collaboration between LIPI and NITE, LIPI also developed mechanism on access to microbial genetic resources for research. Now, many Indonesian researchers from government institutions, universities, and private sectors including those from overseas could have access to our collection through strategic partnerships and specific material transfer agreement (MTA) to ensure the implementation of Nagoya Protocol in the term of Access and Benefit Sharing (ABS). Several research cooperation between LIPI and university in Japan have been conducted using the isolates obtained from collaboration research between LIPI and NITE. Many important results have been produced, e.g. through screening program (cooperation between LIPI and Hokkaido University, Japan) on 100 Actinomycetes, we have been able to identify a candidate to produce inulin fructotransferase enzymes which is very important in converting inulin to di-D-fructofuranose 1,2':2,3' dianhydride (DFA III). This enzyme can work perfectly well at 65°C and at a pH of 5.5. DFA III is a valuable compound as it can accelerate translocation calcium ion in the blood stream which means that it is a potential compound for

bone formation for curing osteoporosis (Pudjiraharti et al., 2011). This should be future business in pharmaceutical industry. The other research on using the collection was screening to find a better manannase, cellulase, xylanase (Yopi et al., 2006; Ratnakomala et al., 2015). Some isolates were also tested for the inhibition of ATPase activity of RNA helicase of Japanese encephalitis virus (JEV) or hepatitis virus, in order to find drug candidate to combat viral infections (Ratnakomala et al., 2011). Evaluation on diversity of fungal and actinomycetal strains by Chugai Pharmaceutical Company in Japan clearly shows that some fungi and Actinomycetes strains should be of important in developing compounds related to metabolic disorders and immunology.

Further progress is also important to note when LIPI, Forest Research Agency, Department of Forestry (FORDA), and Institut Teknologi Bandung (ITB) established research cooperation with University of California Davis (UC-Davis) from 2009–2011. The researchers were able to explore microbial diversity in Mekongga, South East Sulawesi for energy and health. Many microbes show a good ability in producing enzymes for converting lignocelluloses to glucose as an important feedstock in bioethanol industry in the near future. Some microbes could accumulate lipid as a candidate for the production of landless biodiesel. Some microbes were able to produce important metabolites to cure degenerative diseases and cancers.

Furthermore, from April 2011 to March 2016, LIPI with NITE Biological Resource Center (NBRC) were continuing the collaboration for "The Development of International Standardized of Microbial Resources Center in Indonesia". This collaboration is of great value to LIPI in materializing InaCC.

With those progresses, the accumulation of knowledge on Indonesian microbial genetic resources increases significantly. It is time for LIPI to share the knowledge to wider audiences, beyond scientific

community. For this reason, the researchers compile and review their work to highlight some of the important microbial groups which should be of great value for human welfare. This book illustrates the important of *Rhizobium* in supporting plant growth and improve soybean production, the diversity of acetic acid bacteria and its potential application in industry, bacterial cellulose, lactic acid bacteria and its value in food and feed industry, the diversity of actinomyces and its prospect, as well as potential value of yeast and fungi in food and industrial enzymes production.

Conclusion

Indonesia, truly, is an integral part in the global history of microbiology. Exploration on Indonesian biological diversity started in 16th century, and the inventory and sustainable use of Indonesian microbial resources was started at Treub Laboratory located in Bogor Botanical Garden under the leadership of Dr. Melchior Treub (1851–1910). Many scientists from all over the world (mostly from Europe) visited Treub Laboratory to carry out research on tropical rich biodiversity, including microbial diversity since then. It is true that the work on microbiology was decreasing and there was no more activity until 1934. Research in microbiology started again in 1960s by Indonesian scientist and was accelerated in 1980s. Many Indonesian scientist returned home from overseas in 1990s, more advanced laboratories and infrastructures were built, and more cooperations with international scientific community were flourished. Now LIPI has InaCC, a modern facilities to conserve living microbial genetic resources in addition to the Herbarium Bogoriense and Museum Zoologicum Bogoriense conserving flora and fauna speciments and depositing information on the value of biological resources to be utilized for the prosperity of the nation.

Acknowledgement

The authors would like to acknowledge and give their sincerest thanks to Dr. Susono Saono, senior and prominent microbiologist of LIPI, for his leadership and his dedication to build the capacity of microbiologists at LIPI. They are also grateful for his kindness to set up international cooperation through diverse strategic partnerships of LIPI with other research institutes in Indonesia, cooperation with other laboratorium overseas in ASEAN, developing cooperation within the framework of ASEAN-Australia, ASEAN-Japan, and ASEAN-EU.

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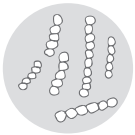


Rhizobium, and Its Potential to Support Plants Growth and Improve Soybean Production

✍ Harmastini Sukiman and Sylvia J. R. Lekatompessy

Introduction

The unique soil bacteria, rhizobia, are widely known as the symbiotic bacteria with special potential on fixing nitrogen taken from the air, thus providing more readily-used nitrogen for plant (Yutono, 1989; Saono, 1989; Widiastuti, 1989; Boonkerd & Singleton, 2002). Nitrogen is the most limiting factor for supplying nutrients for plants. Legumes plant has potential on accessing nitrogen from the air since they live symbiotically with Rhizobia (Simon et al., 2014). In this case the bacteria obtain the energy from the plant as a result of plant metabolism process and the plant will use available nitrogen from the result of nitrogen fixing process (Usman & Joko, 2001). Nitrogen is needed by the plants, especially for synthesis of enzymes, protein, chlorophyll, DNA and RNA, thus essential for food and feed production (Matiru & Dakora, 2004). The bacteria have been studied deeply since decades, and it has been confirmed that the bacteria could be developed further as biofertilizer.

Most of Rhizobia belong to the family Rhizobiaceae in the alpha-Proteobacteria, and consist of the genera of *Rhizobium, Mesorhizobium, Ensifer,* and *Bradyrhizobium* (Weir, 2016). Later, taxonomists give names for the genus *Rhizobium* based on the host plant in which the bacterium originally lives symbiotically with and also the character of the bacterium itself (Table 1).

Table 1. *Rhizobium* Classification Based on Host Plant, and Crossed Inoculation Groups

Name of host plant	***Rhizobium* (fast growing)**	**Another host plant (cross inoculation)**
Leguminosae	*Rhizobium leguminosarum*	*Pisum* sp., *Vicia* sp., *Lena culinaria*
Phaseolus	*Rhizobium phaseoli*	*Phaseolus vulgaris, Phaseolus coccineus*
Trifolii	*Rhizobium trifolii*	*Trifolium subterraneum, Trifolium* sp.
Mellilotus	*Rhizobium melilotti*	*Medicago sativa, Mellilotus* sp., *Trigonella* sp.
Name of host plant	***Rhizobium* (slow growing)**	**Another host plant (cross inoculation)**
Lupinus	*Rhizobium lupini*	*Lupinus* sp., *Ornithopus* sp.
Glycine	*Rhizobium japonicum*	*Glycine max*
	Rhizobium sp.	*Vigna* sp., *Macroptilium* sp., *etc.*

Source: Somasegaran & Hoben, 1985

Characterization of Rhizobia is very important for finding bacterial strains with effective rhizobium-legume association to maximize the agriculture production (Berada et al., 2012). Since 1975, Indonesian Institute of Sciences (*Lembaga Ilmu Pengetahuan Indonesia* (LIPI)) has conducted huge research activities on nitrogen fixing bacteria, especially the genus *Rhizobium* by starting with the collection of biodiversity of Rhizobia from many places in Indonesia and

many host plants. However, the focus of research was put on diversity of *Rhizobium* from soybean crops. The collections of bacterium have been maintained at Biotechnology Culture Collection (BTCC) although there was no formal culture collection division has been established at that time in LIPI. The numbers of collection have been recorded and noted tidily, while the pure isolates were maintained by using conventional method (Sukiman et al., 1995; Ozawa et al., 1995; Sukiman et al., 1996; Sukiman et al., 1998).

Screening activities were conducted to select the best isolates which have high potential on nitrogen fixing process, in addition to their compatibility with several host plants and adaptability to the extreme conditions of environment such as acid soil, soil salinity, nonfertile soil and other specific conditions which will influence the existancy of the bacterium in symbiosis living with plant (Sukiman et al., 1998; Ozawa et al., 1999). The best potential on nitrogen fixing process very much depended on the functional state of the legume plant and the optimum environmental conditions supporting the symbionts, since the stresses could affect the host plant and symbiotic Rhizobia (Graham, 1992; Hungria & Vargas, 2000).

Considering the potential of *Rhizobium*, many scientists in the world study the possibility of the bacterium to be used as fertilizer substitute. As indicated, the soil pollution caused by chemical fertilizer application spreads widely. The agriculture area becomes highly contaminated with chemicals which is harmful for human life. To solve and reduce the contamination, biofertilizer is very much recommended to be used instead of chemical fertilizer. *Rhizobium* has been reported as one of the bacteria which could fix nitrogen taken from the air. The bacterium is able to fix nitrogen ranging from approximately 100–300 kg per hectar in one crop season (Sutanto, 2002) or in average the nitrogen input of soil ranges from 0–60 kg per hectare per year (Reghuvaran et al., 2014). Moreover, there is also some amount

of nitrogen left in the soil which comes from the composting of rest nodules and green material of plant residue. Therefore, the use of *Rhizobium* to inoculate the crop seed could save the use of chemical fertilizer, especially nitrogen up to 60 percent. Sustainable environment is the main target from using bioproduct to fertilize the soil.

This paper aims to describe the importance of *Rhizobium* as potential microbe for supporting the growth of soybean and introducing the simple technology of *Rhizobium* inoculation into seeds which will help the farmer on the application of biofertilizer.

Mass Production of *Rhizobium* for Biofertilizer

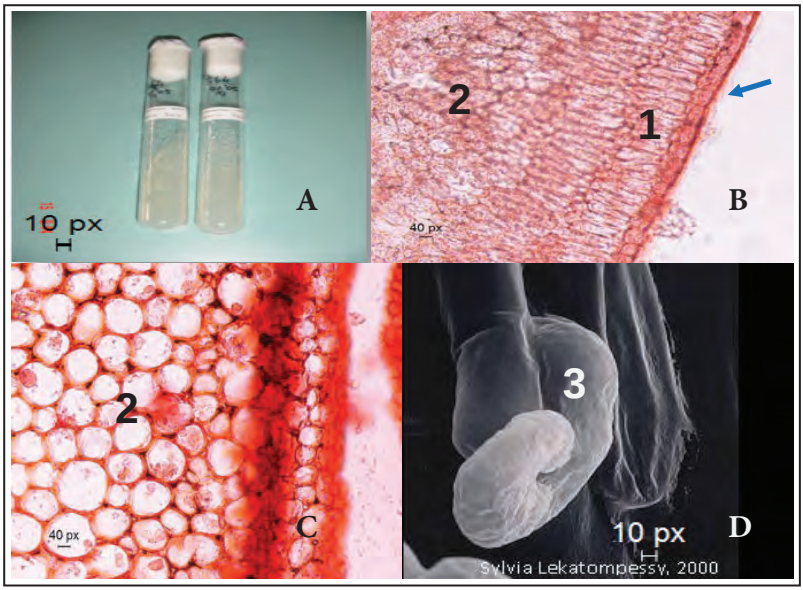
Mass production of bacterial cells of *Rhizobium* becomes the main important subject to be learned since normally the bacterium grows in the selective media such as Yeast Extract Mannitol Agar (Vincent, 1970). To fulfill the requirement of mass production of bacterial cell and keep it economically reasonable, effort has been done to obtain cheap but good quality media for growing the bacterium. Study on providing the best media for growing *Rhizobium* by using cheap material was conducted by our research group in 1996. Evaluation on the assessment of bacterial growth media was done by using cassava starch as main material for carbon sources. Cassava starch was fermented by selected fungus namely *Aspergillus awamori* which converts cassava starch into sugar. Double fermentation process, then, was applied to raw material and the sugar produced during fermentation process by the use of *Rhizobium* to grow. The results indicated that *Rhizobium* could be used for many different types of sugar like glycerol, sucrose, glucose, and fructose besides mannitol for their grow (Lekatompessy, 1996). Cheap material such as cassava starch could be the alternative media for growing the bacteria through the activity of fungus which help convert the starch to sugar.

Inoculum production of *Rhizobium* has been studied by several methods. *Rhizobium* cell could be incorporated into the carrier such as peat soil, vermiculate, or sand. The population of bacterium needed for root infection is 10^7–10^9 cell per ml. Inoculum which is prepared with carrier is actually simple and easy to apply, but sometimes the soybean farmers do not pay much attention on the survival of bacteria which have been coated the soybean seeds. Therefore, some of the cell might have died because of their susceptibily on the heat since usually farmers mix the bacteria under the sunshine. In addition, the farmers do not use suitable glue (sugar, arabic gum, etc.) to stick the bacteria on the surface of the seed. As a result, the numbers of cell are not suitable enough for the bacteria to infect the root tissue, so finally the effect of inoculation could not be sophisticated and performed.

Solution has been made by developing new technology, that is by inserting bacteria cell directly into the cell tissue of soybean seed. By this technique, bacterial cell could be translocated to the seed tissue especially to the palisade tissues where there is enough space to accomodate bacterial cell. For the insertion of bacterial cell into the cell tissue of soybean seed, LIPI has designed simple equipment to inject bacterial cell into the the tissue of seed. Injection is performed by applying air pressure generated by initial vacuuming condition of the chamber containing the mixure

Source of photograph: Sukiman, 2010

Figure 1. Equipment for the Insertion of Rhizobial Cell into Soybean Seed that Is Designed by Endang Sukara and Fabricated by Research Center for Calibration and Metrology of Indonesian Institute of Sciences (LIPI)



Note: (⟶) the surface of the seed coat, epidermis (1) palisade tissue, (2) cotyledon, and (3) pleomorphic *Rhizobium* cell

Source of photograph: Lekatompessy, 2000 & 2013.

Figure 2. A) Selected Strain of *Rhizobium* in Slant Agar Media; B) a longitudinal slice of soybean seed inserted by *Rhizobium* cell; C) a longitudinal slice of soybean seed as control; and D) *Rhizobium* cell in pleomorphic stage

of seed and bacterial cell. This equipment is suitable for inserting bacterial cell into soybean seed as it is illustrated in Figures 1 and 2.

Application of "Soybean Plus"

With the vacuum seed technology, the researchers have been able to provide farmers with the seed containing bacterial biomass, which later being promoted as *Kedelai Plus* or "Soybean Plus". Now, the farmers can easily and directly feed the soybean seed into their field

and wait for a better harvest. This technology has succesfully been socialized and applied to the farmer in many locations in Indonesia, such as Sukabumi in West Java, Yogyakarta, Grobogan in Central Java, Malang in East Java, and Jambi and Lampung in Sumatra. Numbers of demonstration plot have been conducted to confirm the technology and the result showed that the production of soybean could increase from 50–100%. Successful application of "Soybean Plus" in several locations in Indonesia are listed in Tables 2 and 3. In average, the

Table 2. Multilocation Tests of "Soybean Plus" in Numbers of Location in Indonesia during Rainy Season 2010–2011

Locations	Type of site	Seed production without LIPI technology (ton/Ha)	Seed production of "Soybean Plus" (ton/Ha)
Ciomas, West Java	Dry Land	0.8	1.7
Cihideung, West Java	Rice Field	0.7	1.7
Cikampak, West Java	Dry Land, Farm	0.7	1.8
Taman Sari, West Java	Marginal Land	1.3	1.4
Bodogol, West Java	Dry Land, Farm	0.8	1.0
Cicurug, West Java	Dry Land, Farm	1	1.7
Cililin, West Java	Dry Land, Farm	0.8	1.6–2.0
BBI, Central Java	Research Station Farm	1.3	1.5
Bantul, Imogiri, Yogyakarta	Irrigated rice field	1.0	2.2
Bandung, Gunung Kidul, Yogyakarta	Dry Land, Farm	0.8	1.0
Bleberan, Gunung Kidul, Yogyakarta	Dry Land, Farm	1.2–1.6	2.0
Grobogan, Central Java	Irrigated Rice Field	3.0	3.2
Musirawas, Sumatra	Dry Land	2	3.6
Palembang, South Sumatra	Dry Land	1	1.5
Bedali, Lawang, East Java	Dry Land	1	1.2

Source: Sukiman, 2012 (unpublished data)

Table 3. Multiadaptation Test of "Soybean Plus" during 2011 Dry season in Several Locations in Indonesia

Locations	Seed Production without technology (ton/ha)	Seed Production of "Soybean Plus" (ton/ha)
Cihideung, West Java	0.80	1.75–2.0
Cikampak, West Java	0.80	1.70–2.0
Cicurug, West Java	0.70	1.70–2.0
Ciomas, West Java	0.70	1.28–1.45
Taman Sari, West Java	0.70	1.37–1.74
Grobogan, Central Java	2.00	2.50–3.00
Bantul, Central Java	1.12	1.30–1.50
Bandung, West Java	1.47	1.57–2.00

Source: Sukiman, 2012 (unpublished data)

productivity of soybean increases twice when the soybean seeds are inoculated with *Rhizobium*. This experience confirms that the bacterium have a special potential to be developed further as biofertilizer (Sukiman et al., 1998)

In regard to the viability of rhizobial cells inside the seed tissue, research has been done to confirm the condition of bacteria during the storage period. It has been confirmed that the bacteria will stay in dormant stage when it is inserted to the tissue of the seeds. Soybean seed can normally be storaged for more than 3 months. After three months of storaging, the seed germination will decrease followed by the vigor of seed. However, the bacteria inside the seed are still very active. Phycological character and the effectiveness of the bacteria are still very high.

The potential of *Rhizobium* as biofertilizer have been confirmed again by multilocation tests under the coordinator of National Innovation Committee (*Komite Inovasi Nasional* (KIN)). The multilocation tests include other bioproducts produced by other institutions, namely Agriculture Department, Bogor Agriculture University, and Agency

for Assessment and Application of Technology (BPPT). The aim of these activities is to show the effectiveness of bioproduct on supporting the growth of crop or plant and hopefully could increase the seed productivity. The multilocation tests have especially been done at the marginal soil since the extensification of agriculture land will be focused outside Java, because soil outside Java is usually unfertile and very acid (Figures 4–6).

Recently, LIPI has launced the acid resistant bacteria to be used on its biofertilizer. Research about the bacteria has been done since many years ago. It was reported by Ozawa et al. (1999) that the selected Rhizobia are acid resistant strain. They could live at pH 4.0 and effective on running the nitrogen fixing process.

Genetic molecular study of the Rhizobia has been done in several ways especially concerned with the isolation and identification of megapalasmid and DNA sequence of bacteria (Sukiman et al., 1996; Nuswantara et al., 1996; and Nuswantara et al., 1997). Phylogeny of bacterial symbiont has been studied to understand the classification of the bacteria (Sukiman et al., 1997). It is showed that selected bacteria that have specific potential belong to the genus *Bradyrhizobium* sp. Identification of the bacteria has been done using 16S rRNA gene analysis and the result showed that selected bacteria with a specific potential belong to the *Bradyrhizobiun* sp. *Bradyrhizobium* sp. is a slow growing rhizobia, and it normally reaches the optimal grow after 4–5 days of incubation time. Other genetic studies have been conducted by many scientists and they mostly focus on understanding the timing of excretion of plant root and microbes excudates during plant microbes interaction. It is understood that the colonization of the bacteria in the surface of the root of plants very much depends on the root excudate produced by the plant root, so the timing of when the root exudates are produced by the plant becomes crucial for the success of colonization. Besides, the functions of genes in nodulation on certain host are

also very important to study since the gene function will confirm the competitiveness among the microbes surrounding the root system. Basically, biological phenomenon involving many regulated processes in both bacteria and host plant is very important to understand.

Rhizobium could be combined with other beneficial microbes, such as Soil Fungus (*Endo-mycorrhizae*), Plant Growth Promoting Bacteria (*Azospirillum, Spirillum, Bacillus, Azotobacter,* etc.), Phosphate Solubilizing Bacteria (*Pseudomonas* sp.) and packed as consortia inoculum. Bioproducts promoted as Byonic LIPI have been recently lauched to the farmers. Byonic products for promoting the growth of plants consist of consortia microbes, namely Biorhizin, BioVam, Bioazos and Biocompost of LIPI. Those bioproducts will support the

Source of photograph: Sukiman, 2013

Figure 4. The Application of Technology in the Production of "Soybean Plus" (*Kedelai Plus*) at a Bench Scale with Capacity of 50 kg per Batch

Source of photograph: Sukiman, 2012

Figure 5. Cultivation of Soybean Using "Soybean Plus" Seed at Experimental Site Showing a Good and Healthy Root Nodules

"Soybean Plus" to complete other nutritions needed by the plants. Recent field test results inform that "Soybean Plus" applied by other bioproduct shows increase in production twice when it is compared with single inoculation (Ariani & Harmastini, 2010; Harmastini & Ariani, 2010).

Conclusion

It can be confirmed that nitrogen fixing bacterium, *Rhizobium,* could be used as biofertilizer for crops plant especially soybean. Application of *Rhizobium* could increase the soybean yield twice from normal

production. In addition to that, application of *Rhizobium* into the field soil could repair the condition of soil since almost all of Indonesian soil is destroyed because of high accumulation of chemical residues which are poisonous for the plant. Besides, application of *Rhizobium* could maintain the soil structure, and soil becomes loose.

Insertion of *Rhizobium* cell into the seed tissue through the vacuum technology could make the application of nitrogen fixing bacteria more effective and easier. Biofertilizer based on potential microbes could be applied easier without any risk of loosing the bacteria, and even more the technology provides security of the purity of bacterial cell. Development of equipment should be done in regard to adjust the technical matter of seed inoculation.

Source of photograph: Sukiman, 2012

Figure 6. Cultivation of Edamame Using Soybean Seed which Initially Inserted by Rhizobial Cell Using Technique Developed by Indonesian Institute of Sciences (LIPI) at West Java

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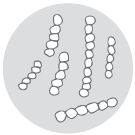


Taxonomy, Diversity, and Industrial Application of Acetic Acid Bacteria

✍ Puspita Lisdiyanti

Introduction

Acetic acid bacteria comprise a group of a Gram-negative, aerobic and rod shaped bacteria, which incompletely oxidize alcohols, leading to the accumulation of organic acids as end-products. Using ethanol as a substrate, these bacteria produce acetic acid, from which their common name is derived. They also exhibit a relatively high tolerance to acidic conditions (pH < 5). This acid tolerance is commonly essential for an organism producing large amounts of acid. Acetic acid bacteria belong to the family of Acetobacteraceae and are ubiquitous in various environments (Kersters et al., 2006).

According to Asai (1968), acetic acid bacteria were first found and described in 1822 by Persoon, who isolated them from the surface of wine and beer, and considered them as a member of the animal kingdom at that time. In 1879, Pasteur recognized that the cause of acetic acid fermentation during wine production was a microorganism. Nevertheless, he was unable to identify the microorganisms

as bacteria. In 1894, Hansen found that the cause of the acetic acid fermentation of wine or beer was acetic acid bacteria. According to Asai (1968), Beijerinck (1898), Hoyer (1899), Henneberg (1898), and Rothenbach (1898) classified these bacteria on the basis of morphology appearance.

From the view point of systematics, a number of changes have occurred in the number of both genera and species of the family Acetobacteraceae. Until June 2016, 22 valid genera in the family Acetobacteraceae are recognized, namely *Acetobacter* (with 39 species), *Acidicaldus* (with 1 species), *Acidiphilium* (with 6 species), *Acidisoma* (with 2 species), *Acidisphaera* (with 1 species), *Acidocella* (with 3 species), *Acidomonas* (with 1 species), *Ameyamaea* (with 1 species), *Asaia* (with 7 species), *Belnapia* (with 2 species), *Bombella* (with 1 species), *Craurococcus* (with 1 species), *Crenalkalicoccus* (with 1 species), *Endobacter* (with 1 species), *Gluconacetobacter* (with 18 species), *Gluconobacter* (with 11 species), *Granulibacter* (with 1 species), *Humitalea* (with 1 species), *Komagataeibacter* (with 1 species), *Kozakia* (with 1 species), *Muricoccus* (with 1 species), *Neoasaia* (with 1 species), *Neokomagataea* (with 1 species), *Nguyenibacter* (with 1 species), *Paracraurococcus* (with 1 species), *Parasaccharibacter* (with 1 species), *Rhodopila* (with 1 species), *Rhodovastum* (with 1 species), *Rhodovarius* (with 1 species), *Roseococcus* (with 2 species), *Roseomonas* (with 15 species), *Rubritepida* (with 1 species), *Saccharibacter* (with 1 species), *Stella* (with 2 species), *Swaminathania* (with 1 species), *Swingsia* (with 1 species), *Tanticharoenia* (with 1 species), *Teichococcus* (with 1 species) and *Zavarzinia* (with 1 species) (Malimas et al., 2013; Thi et al., 2013; Cole et al., 2014; Corby-Harris et al. 2014; Parte, 2014; Li et al., 2015; Ming et al., 2016). All genera share the following characteristics: Gram-negative to Gram-variable, aerobic, rod-shaped bacteria; do not form endospores; a respiratory type of metabolism; in glucose-containing media, their pH drop to 4.5.

As mentioned before, until 2000's the majority of strains of acetic acid bacteria currently preserved and available from culture collections were isolated from Europe and Japan, countries in temperate regions. In collaboration between Indonesian Institute of Sciences (*Lembaga Ilmu Pengetahuan Indonesia* (LIPI)) with Agency for Assessment and Application of Technology (*Badan Pengkajian dan Penerapan Teknologi* (BPPT)), and Japan Bioindustry Assosiation-New Energy and Industrial Technology Development Organization (JBA-NEDO) from April 1993–March 1999, the researchers had studied a large number of acetic acid bacteria isolated from traditional fermented foods, fruits and flowers collected from Indonesia. Investigation of new niches led to the introduction of two new genera, *Asaia* (Yamada et al., 2000) and *Kozakia* (Lisdiyanti et al., 2002). In addition, several new species and new combinations of the genus *Acetobacter* have been described. Furthermore, identification of collection of acetic acid bacteria from Thailand and the Philippines is also conducted. It is no doubt that the isolation of this bacteria from new niches has changed the taxonomical position, elucidated the diversity, and given the broad prospect of industrial application of acetic acid bacteria.

In this chapter, the research on the taxonomical study especially to genus *Acetobacter*, *Asaia,* and *Kozakia* that have been first found in Indonesia are described. Diversity of these three genera in various environments and their industrial applications or further prospect is also described.

Taxonomical Study of the Genus *Acetobacter*

The history of the genus *Acetobacter* until 1968 was described in detailed by Asai (1968). According to Asai (1968), taxonomically strains belonging to the genus *Acetobacter* had been given a number of generic names: "*Ulvina*" Kützing 1834, "*Mycoderma*" Thompson 1852,

"*Termobacterium*" Zeidler 1896, "*Acetobacterium*" Ludwig 1898 and "*Acetimonas*" Orla-Jensen 1909. In the 8th edition of Bergey's Manual of Determinative Bacteriology (De Ley & Frateur, 1974), it is written that actually Beijerinck did not formally propose the generic name *Acetobacter* because after 1898 the name *Acetobacter* had been used without further explanation. However, in any case morally, Beijerinck is to be considered as the authors of the genus *Acetobacter*.

After that, the bacterial taxonomy has been rearranged in 1980 (Skerman et al., 1980). More than 50 specific epithets of *Acetobacter* have been published before the rearrangement of bacterial species names in 1980, but after rearrangement, 3 species were recognized as valid name: (i) *Acetobacter aceti*, which contains 4 subspecies, *A. aceti* subsp. *aceti*, *A. aceti* subsp. *orleanensis*, *A. aceti* subsp. *xylinum*, and *A. aceti* subsp. *liquefaciens*; (ii) *Acetobacter pasteurianus*, which contains 5 subspecies, *A. pasteurianus* subsp. *pasteurianus*, *A. pasteurianus* subsp. *lovanensis*, *A. pasteurianus* subsp. *estunensis*, *A. pasteurianus* subsp. *ascendens*, and *A. pasteurianus* subsp. *paradoxus*; (iii) *Acetobacter peroxydans*. These taxonomy positions were cited in Bergey's Manual of Determinative Bacteriology 8th edition (De Ley & Frateur, 1974; Buchanan & Gibbons, 1974).

Furthermore, Gosselé et al. (1983), on the basis of numerical analysis using 177 phenotypic features and protein profiles of 98 acetic acid bacterial strains, rejected the subspecies concept in the previous classification and delineated 4 species in the genus *Acetobacter*: (i) *A. aceti* (Pasteur 1864) Beijerinck 1898; (ii) *Acetobacter hansenii* De Ley 1983; (iii) *Acetobacter liquefaciens* (Asai 1935) De Ley 1983; and (iv) *A. pasteurianus* (Hansen 1879) Beijerinck 1916. Three of these species (*A. aceti*, *A. hansenii* and *A. liquefaciens*) showed a fair internal homogeneity, but the species *A. pasteurianus* was a heterogeneous species. Its heterogeneity was reflected by its broad mol% G+C of DNA ranging from 52.8–62.5 mol%, and by whole protein profiles. These

conclusive results were cited in the Bergey's Manual of Systematic Bacteriology (De Ley et al., 1984).

Yamada (1983) revived *Acetobacter xylinum* as an independent species and a separate entity from *A. pasteurianus* and *A. aceti* on the basis of ubiquinone systems. Entani et al. (1985) succeeded in isolating acetic acid bacteria from vinegar fermentors using AE medium and proposed a new species "*Acetobacter polyoxogenes*". Uhlig et al. (1986) isolated acidophilic facultative methylotrophic strains with typical features of acetic acid bacteria and proposed the species *Acetobacter methanolicus*. Gillis et al. (1989) proposed a new N_2-fixing acetic acid bacteria, *Acetobacter diazotrophicus*. Sievers et al. (1992) proposed *Acetobacter europaeus* isolated from vinegar fermentors in Europe. However, it is worth noting that "*A. polyoxogenes*" strains isolated from fermentors in Japan may have been the same strains as those isolated in Europe. However, due to problems in propagation and preservation, "*A. polyoxogenes*" strains are not available from Japan Collection of Microorganisms (JCM), hence cannot be confirmed (Sievers et al., 1992).

From the description of history above, it is noted that a lot of new species of the genus *Acetobacter* have been described. However, it is no doubt that the elevation of the genus *Gluconacetobacter* and the revival of the genus *Acidomonas* by Yamada et al. (1997) significantly decreased the number of the species in the genus *Acetobacter* into 2 species only, namely *A. aceti* and *A. pasteurianus*. These 2 species could be easily differentiated from other species of other genera in the family Acetobacteraceae by their ability to oxidize acetate and lactate to CO_2 and H_2O, and their having Q-9 as the major ubiquinone.

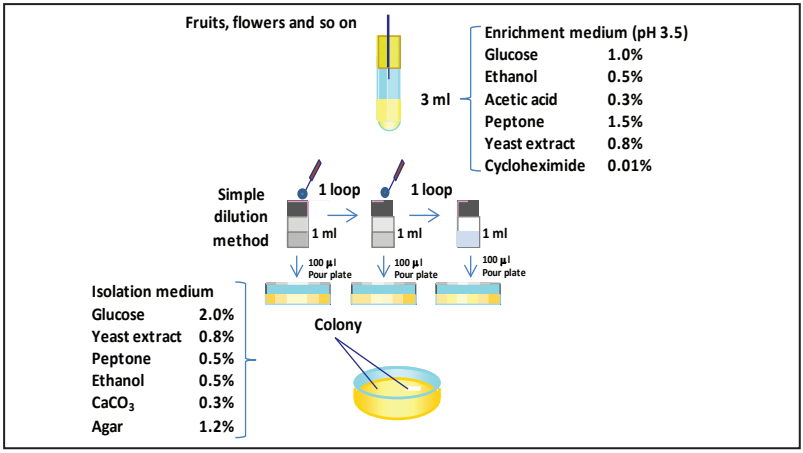
Sokollek et al. (1998) proposed two new species in the genus *Acetobacter*, namely, *Acetobacter pomorum* and *Acetobacter oboediens*, which were isolated from fermented vinegar using AE medium. Boesch et al. (1998) proposed the species *Acetobacter intermedius* for

an isolates from vinegar fermentors using AE medium. Yamada (2000) transferred *A. oboediens* and *A. intermedius* to the genus *Gluconacetobacter* because these two species had Q-10 as the major ubiquinone and were clustered in the genus *Gluconacetobacter* on the basis of 16S rRNA gene sequences. This increased the number of *Acetobacter* species to 3 species: *A. aceti*, *A. pasteurianus*, and *A. pomorum*. Therefore, after this time, the oxidation test and determination of ubiquinone type to differentiate the genus *Acetobacter*, *Gluconacetobacter*, and *Acidomonas* were recognized as test to be done.

Lisdiyanti et al. (2000, 2001) contributed a big effort for establishing the systematic concept of the genus *Acetobacter* using strains isolated from Indonesian sources together with strains obtained from Culture Collections by polyphasic taxonomy, determination of DNA-DNA hybridization and 16S rRNA gene analysis. As a result, five new species and three new combinations were established in the genus *Acetobacter*. Using enrichment medium in combination with simple isolation method, 45 isolates belong to the genus *Acetobacter* were found in first course study of acetic acid bacteria in Indonesia (Yamada et al., 1999) (Figure 1). All the isolates shared the same biochemical characteristics, and it is very difficult to differentiate them by phenotypic characterization. Therefore, to elucidate their characteristics, 31 *Acetobacter* strains obtained from culture collections were investigated for their phenotypic characteristics, ubiquinone systems, DNA base compositions and levels of DNA-DNA hybridization compared with 45 isolates from Indonesian sources. Lisdiyanti et al. (2000) described that several strains previously assigned to the species of *A. aceti*, *A. pasteurianus* and *A. peroxydans* were scattered over the different species and stated that it is evident that the value of DNA-DNA hybridization between strains comprising a new species should be determined for the establishment of the species, whereas current bacterial species without data of DNA-DNA hybridization should be

reexamined for the stability of bacterial nomenclature. Therefore, 9 species in the genus *Acetobacter*, namely, *A. aceti*, *A. pasteurianus*, *A. pomorum*, *A. peroxydans*, *A. orleanensis*, *A. lovaniensis*, *A. estunensis*, *A. indonesiensis* and *A. tropicalis* were described. By studying another 46 strains of acetic acid bacteria newly isolated from flowers, fruits, and fermented foods collected in Indonesia, Lisdiyanti et al. (2001) described another 3 new species of the genus *Acetobacter*, namely, *Acetobacter syzygii*, *Acetobacter cibinongensis*, and *Acetobacter orientalis*.

In the same time, this work was also conducted by co-workers from Belgian Coordinated Collections of Microorganisms/Laboratory of Microbiology, Department of Biochemistry and Microbiology, Faculty of Sciences of Ghent University (BCCM/LMG) Bacteria Collection, Belgium. Cleenwerck et al. (2002) examined 34 *Acetobacter* strains in their Culture Collection to a polyphasic study that included DNA-DNA hybridization, DNA base ratio determinations, 16S rRNA gene sequence analysis and phenotypic characterization. This work confirmed the study of Lisdiyanti et al. (2000, 2001) that the genus



Source: Lisdiyanti, 2001

Figure 1. Isolation Method of the Genus *Acetobacter*

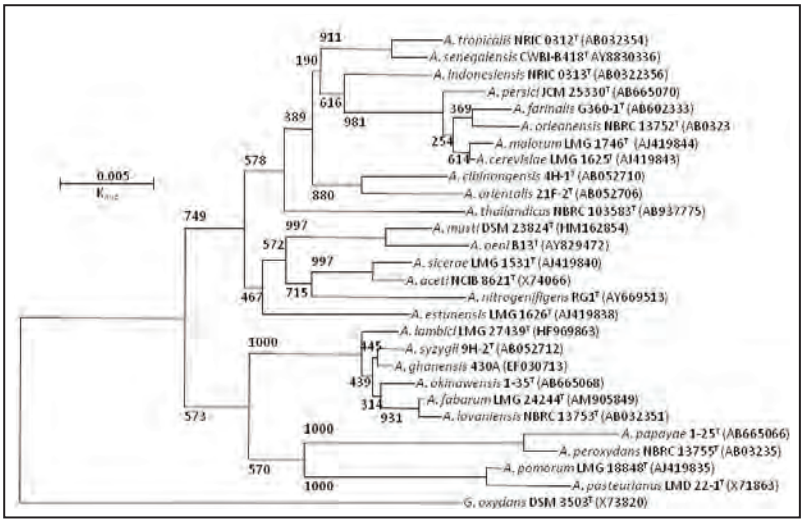


Figure 2. Phylogenetic Tree of the Genus *Acetobacter* Based on 16S rRNA Gene Sequence Analysis. A., *Acetobacter*; G., *Gluconobacter*; T, Type strain (Constructed by Lisdiyanti in 2016).

Acetobacter consists of 12 species plus 2 more species, namely *Acetobacter cerevisiae* and *Acetobacter malorum*.

At that time, the number of species in the genus *Acetobacter* remains increasing due to the new sources used in isolation process. Silva et al. (2006) described *Acetobacter oeni* isolated from spoiled red wine; Dutta and Gachhui (2006) described *Acetobacter nitrogenifigens,* a nitrogen fixing *Acetobacter* isolated from kombucha tea; Cleenwerck et al. (2007) described *Acetobacter ghanensis* from cocoa fermentation; Ndoye et al. (2007) described *Acetobacter senegalensis,* a thermotolerant acetic acid bacterium isolated from mango fruit; Cleenwerck et al. (2008) described *Acetobacter fabarum* from cocoa bean; Tanasupawat et al. (2011) described *Acetobacter farinalis* from fermented foods, Iino et al. (2012) described *A. okinawensis*, *A. papayae* and *A. persici* from fruits; Spitaels et al. (2014) described *A. lambici* from fermenting

lambic beer; Li et al. (2014) described *A. sicerae*; Ferrer et al. (2016) described *A. musti* from grape must; Pitiwittayakul et al. (2015, 2016) described *A. thailandicus*, and *A. surathanensis* from fruits. Therefore, currently 28 species are recognized (Figure 2).

Taxonomical Study of the Genus *Asaia*

As mentioned by Yamada et al. (2000), during the second course of taxonomic study of acetic acid bacteria from Indonesian sources, eight interesting bacterial strains were isolated from flowers of the orchid tree (*Bauhinia purpurea*) and of plumbago (*Plumbago auriculata*), and from fermented glutinous rice. The isolates had unusual characteristics compared with those of known acetic acid bacteria and showed no or a scanty production of acetic acid from ethanol and a complete inhibition of growth by 0.35% acetic acid. Nevertheless, the strains grew on medium adjusted to pH 3.0 with hydrochloric acid, and oxidized acetate and lactate to carbon dioxide and water. The isolates were obtained using D-sorbitol as substrate in enrichment medium, and this enrichment medium was differed from those of previous studies (Yamada et al., 1999), in that there was no acetic acid supplementation. Actually, the first target of the second course of isolation was to obtain *Gluconobacter* strains that are important for industrial application. On the basis of 16S rRNA gene sequences, the 8 isolates showed a phylogenetic location in the acetic acid bacteria lineage, but distant from the genera *Acetobacter*, *Gluconobacter*, *Acidomonas* and *Gluconacetobacter*. Furthermore, on the basis of 16S rRNA gene sequences, these isolates are located in the family *Acetobacteraceae* in α-*Proteobacteria*. On the basis of these characteristics, the isolates could be included in the category of acetic acid bacteria. Therefore, the genus *Asaia* was described.

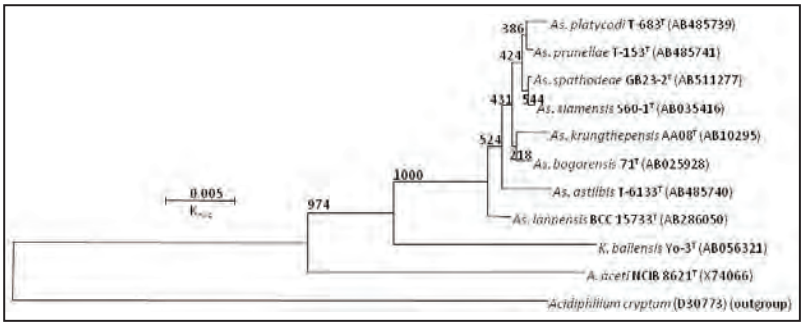


Figure 3. Phylogenetic Tree of the Genus *Asaia* Based on 16S rRNA Gene Sequence Analysis. A., *Asaia*; ^{T}, Type strain (Constructed by Lisdiyanti in 2016).

Asaia strains had unusual characteristics compared with those of known acetic acid bacteria because of none or limited production of acetic acid from ethanol and complete inhibition of their growth by 0.35% acetic acid. However, the strains grew on a medium adjusted to pH 3.5 with hydrochloric acid, and weakly oxidized acetate and lactate to CO_2 and H_2O (Yamada et al., 2000; Katsura et al., 2001). This genus was also found in the insect gut (Crotti et al., 2010).

Currently, this genus comprises of 8 species, namely, *Asaia bogorensis* (Yamada et al., 2000), *Asaia siamensis* (Katsura et al., 2001), *Asaia krungthepensis* (Yukphan et al., 2004), *Asaia lannensis* corrig (Malimas et al., 2008,), *Asaia spathodeae* (Kommanee et al., 2010), *Asaia astilbis* (Suzuki et al., 2010), *Asaia platycodi* (Suzuki et al., 2010), and *Asaia prunellae* (Suzuki et al., 2010) (Figure 3).

Taxonomical Study of the Genus *Kozakia*

In the first course of isolation of acetic acid bacteria from Indonesian sources, four isolates that were tentatively identified as *Gluconacetobacter* species by Yamada et al. (1999) were interesting because they

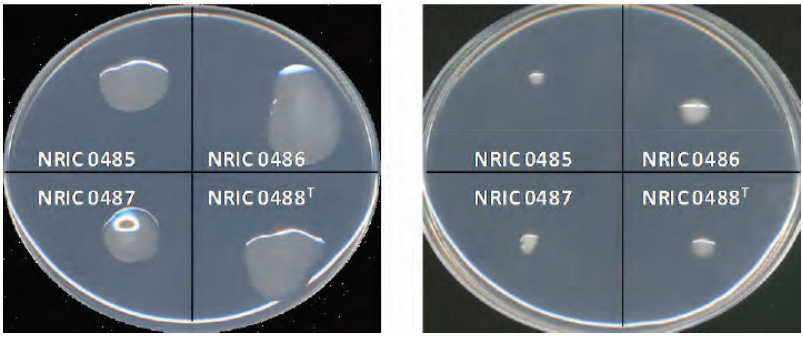
Table 1. Sources of *Kozakia baliensis* Isolates

Name of strain	Source	Place of isolation
Yo-3^T = NRIC 0488^T	palm brown sugar	Bali
Ri-1 = NRIC 0485	*ragi* (starter)	Yogyakarta
Wa-5 = NRIC 0486	*ragi* (starter)	Yogyakarta
Wa-2 = NRIC 0487	*ragi* (starter)	Bali

NRIC: Nodai Research Institute Culture Collection

Source: Lisdiyanti et al., 2000

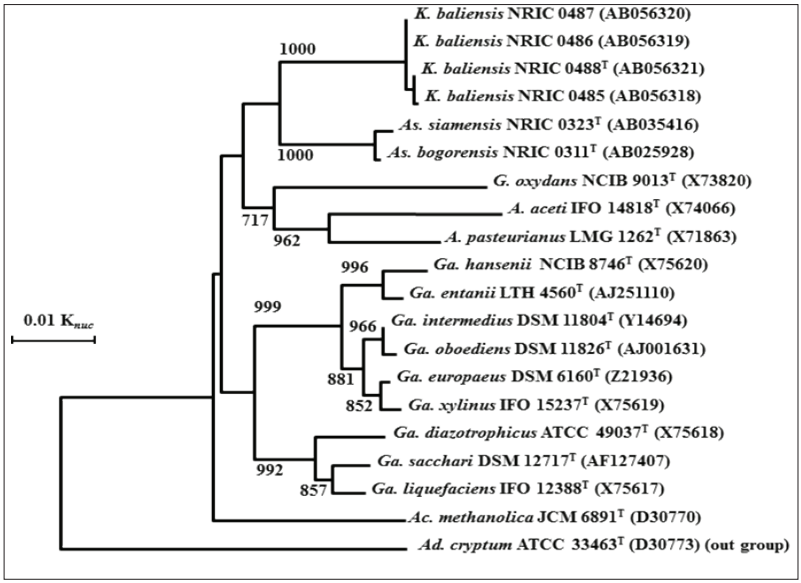
oxidized acetate and lactate to carbon dioxide and water and had Q-10 as the major quinone, but their taxonomic allocation was not within the genus *Gluconacetobacter*. Therefore, Lisdiyanti et al. (2002) conducted the detailed characterization of these four strains and proposed *Kozakia baliensis* as a new cluster of acetic acid bacterium on the basis of 16S rRNA gene sequences. Using enrichment medium as described by Yamada et al. (1999) and Lisdiyanti et al. (2000), palm brown sugar and starter for making *tape* (*ragi*) collected in Bali and Yogyakarta, Indonesia, were used as samples for isolation of this genus (Table 1). The isolates were clearly different from the genera *Asaia*,



Sucrose medium Glucose medium

Source: Lisdiyanti, 2001

Figure 4. Levan-like Mucous Substances Polysaccharides by *Kozakia baliensis* on Sucrose as a Sole of Carbon Source (left) Compared on Glucose as a Sole of Carbon Source (right).



Source: Lisdiyanti et al., 2000

Figure 5. Phylogenetic Tree of the Genus *Kozakia* Based on 16S rRNA Gene Sequence Analysis. A., *Acetobacter*; As., *Asaia*; Ac., *Acidophilus*; Ad., *Acidomonas;* G., *Gluconobacter*; Ga., *Gluconacetobacter*; ^{T}, Type strain.

Gluconacetobacter, *Gluconobacter*, *Acidomonas*, and *Acetobacter* in the production of acid from raffinose. Interestingly, four isolates produced a large amount of levan-like mucous substance(s) from sucrose and fructose. The production of mucous substance(s) was a possible marker for differentiating the isolates from other genera (Figure 4). Until now, this genus has only consisted of a single species, *Kozakia baliensis* (Figure 5).

Diversity of Acetic Acid Bacteria

The habitat of acetic acid bacteria is in association with sugar-rich fruits and flowers, but most frequently in alcoholic beverages, such as

wine or cider, their presence may be a nuisance since they can cause spoilage and off-flavors. Alternatively, they may be found in vinegar fermentors where various forms of alcoholic mash are converted in vinegar (Kersters et al., 2006). These habitats are relatively well-known because of considerable economic profits and losses of these bacteria in the food industry. In nature, these bacteria are particularly adapted well to alcoholic or sugary habitats, such as beer, wine, vinegar, flowers, fruits, beehives, teafungus, palm wine and so on.

The isolation of acetic acid bacteria from beer, wine, and vinegar has been carried out by pioneers of microbiology, such as Pasteur, Hansen, Henneberg, and Beijerinck. Brown, Hoyer, and Janke have also attempted to isolate acetic acid bacteria from these habitat. Asai (1968) described that Takahashi in 1908, Miyazi in 1924, Yasui, and Tanaka in 1937 isolated acetic acid bacteria from sake (Japanese rice wine). The major beer-spoiling strains were found as *Ga. xylinus*, *A. pasteurianus* and *G. oxydans*. Fruits, as sources of sugar and ethanol, are excellent niches for acetic acid bacteria. Acetic acid bacteria have been found in apples, apricots, almonds, beets, bananas, custard apples, figs, grapes, mandarins, mangoes, oranges, pears, peaches, pineapples, plums, persimmons, strawberries, and tomatoes. The presence of acetic acid bacteria in fruits can cause problems during the manufacture of cider and wine (Kersters et al., 2006).

Acetic acid bacteria can cause bacterial rot in pears and apples, which is characterized by different shades of browning and tissue degradation. Research in microbe-insect symbiosis has shown that acetic acid bacteria establish symbiotic relationships with several insects of the orders Diptera, Hymenoptera, Hemiptera, and Homoptera, which all are relying on sugar-based diets, such as nectars, fruit sugars or phloem sap (Crotti et al., 2010). From Indonesian sources, the distribution of the genus *Acetobacter* is shown in Table 3.

Table 3. The Distribution of *Acetobacter* Strains in Indonesian Sources

Species	Flower	Fruit	Fermented Food	Other
A. pasteurianus (9)	-	-	Palm vinegar, pickle, *tape* cassava, water of *nata*, mash of fermented rice (*ciu*)	-
A. orleanensis (11)	Unidentified flower (1)	Guava, sapodilla, star fruit (6)	*Nata de coco*, mash of fermented rice (*ciu*) (4)	-
A. lovaniensis (27)	Unknown flower (2)	Coconut, star fruit, marquise, mangosteen, mango, sapodilla, java grape (16)	*Nata de coco*, *moromi soya*, palm wine, pickle (7)	Palm seed, coconut juice
A. indonesiensis (20)	Unknown flower (4)	Banana, papaya, zirzak, star fruit, mango, durian, coconut (14)	Palm wine (1)	Coconut juice (1)
A. tropicalis (9)	-	Lime, orange, guava, coconut (8)	Palm wine (1)	-
A. syzygii (4)	Unknown flower (2)	Malay rose apple, star fruit (2)	-	-
A. cibinongensis (2)	-	Montana (1)	Curd of tofu (1)	-
A. orientalis (9)	Canna flower (1)	Star fruit, coconut (5)	Curd of tofu, *tempe* (3)	-

A., *Acetobacter*; ( ), number of isolated data from Lisdiyanti et al. (2000 and 2001)

Application of Acetic Acid Bacteria

A number of industrial applications of acetic acid bacteria have also been determined, including production of gluconic acid, an antioxidant in the food industry; production of L-sorbose, an important intermediate in the chemical synthesis of vitamin C; production of

dihydroxyacetone, a major ingredient of suntanning agents; production of cellulose, which is broadly used in the film industry.

Cultures of acetic acid bacteria are used for commercial production of vinegar. Vinegar is used as a flavoring ingredient in salad and other foods, and because of its acidity, it is also used as pickling. Vinegar can be also produced from a mixture of pure alcohol and water, in which case it is called distilled vinegar, the term distilled referring to the alcohol from which the product is made rather than the vinegar itself. Aerobic acetic acid bacteria are an interesting group of bacteria; they are different from most other aerobes in that they do not oxidize their energy sources completely to CO_2 and water. Therefore, when provided with ethyl alcohol as an electron donor, they oxidize it only to acetic acid, which are accumulated in medium. Acetic acid bacteria are quite acid-tolerant and are not killed by the acidity resulting from their activity. They have a high oxygen demand during growth. The main problem in vinegar production is to ensure sufficient aeration of the medium. The active ingredient in vinegar is acetic acid produced by acetic acid bacteria that oxidize alcohol-containing fruit juice. Adequate aeration is the most important consideration in ensuring a successful vinegar manufacturing process. Acetate produced by acetic acid bacteria and homoacetogens are also used (in the form of the calcium salt) as a road deicer.

In addition to ethanol, acetic acid bacteria carry out an incomplete oxidation of organic compounds such as higher alcohols and sugars. For instance, glucose is oxidized only to gluconic acid, galactose to galactonic acid, and arabinose to arabonic acid. This ability of under oxidizing sugars is exploited in the manufacture of ascorbic acid (vitamin C). Ascorbic acid can be formed from sorbose, but sorbose is difficult to synthesize chemically. However, it can be conveniently obtained through the use of acetic acid bacteria, which oxidize sorbitol (a readily-made sugar alcohol) only to sorbose, a process called

bioconversion. The use of acetic acid bacteria makes the manufacture of ascorbic acid economically feasible.

Another interesting property of some acetic acid bacteria is their ability to synthesize cellulose. The cellulose formed by these bacteria is not significantly different from that of plant cellulose, but instead of being a part of the cell wall, bacterial cellulose is formed as a matrix outside the wall and the bacteria become embedded in the tangled mass of cellulose micro fibrils. When cellulose-producing species of acetic acid bacteria grow in an unshaken vessel, they form a surface pellicle of cellulose in which the bacteria develop. Since these bacteria are obligate aerobes, the ability to form such a pellicle may be a means by which the organisms are assured of remaining at the surface of the liquid where oxygen is readily available. Although acetic acid can be easily synthesized chemically from alcohol, the microbial product, vinegar, is a distinctive material, particularly the flavor being due in part to other substances present in the starting material. For this reason, the fermentation process has not been replaced by a chemical process.

Conclusion

During the study of acetic acid bacteria isolated from new sources obtained from several places in Indonesia, new genera and new species were found. In the genus *Acetobacter*, seven new species were described (*A. orleanensis*, *A. lovaniensis*, *A. indonesiensis*, *A. tropicalis*, *A. syzygii*, *A. cibinongensis*, and *A. orientalis*); in the genus *Asaia*, two species were described (*As. bogoriensis* and *As. siamensis*), and a new genus and a new species, *Kozakia baliensis*, were described. Acetic acid bacteria were widely distributed in sources of Indonesia. It is also concluded that systematic study was important to elucidate the microbial diversity.

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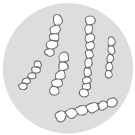


Bacterial Cellulose

Ruth Melliawati

Introduction

Indonesia is a rich biodiversity country with diverse tropical flora and fauna, including microbes. A large number of microbes have been isolated and many of them have potential value for food, feed, health, and bioenergy. Among them is *Gluconacetobacter.* This genus, which is characterized by its ability to convert ethanol to acetic acid in the present of oxygen, belongs to acetic acid bacteria. *Gluconacetobacter* can easily be distinguished in the laboratory by its ability to grow on medium containing 7% ethanol with enough calcium carbonate and turning the medium to partially opaque. When *Gluconacetobacter* colonies producing enough acetic acid from the ethanol, the calcium carbonate around the colonies is dissolved, which is indicated by the formation of distinct clear zone.

Several genera of bacteria such as *Acetobacter, Achromobacter, Aerobacter, Agrobacterium, Alcaligenes, Azotobacter, Gluconacetobacter, Pseudomonas, Rhizobium*, and *Sarcina*, were reported to have the ability to synthesize cellulose from simple sugar (Deinema et al., 1971). However, only *Gluconacetobacter* could produce cellulose for industries. The most extensively studied member of the

Gluconacetobacter species is *Gluconacetobacter xylinus*, formerly known as *Acetobacter xylinum*. Although the name of the genus and species scientifically change, the name of *Acetobacter* is still widely used until today. This bacterium extrudes glucan chains from cell biomass into the growth medium. Aggregate of glucan chains is formed and converted into micro fibrils, which bundled to make microbial cellulose ribbons. Various kinds of sugars can be used as substrate for the production of cellulose. The production of cellulose occurs mostly at the interface between liquid and air. *G. xylinus* is a Gram negative, aerobic bacterium and most widely known for its ability to synthesize cellulose (Schramm & Hestrin, 1954). *G. xylinus* is a non-pathogenic mesophile bacterium that was first identified by Brown (1886), and the biosynthesis of cellulose was described by Haigler and Weimer (1991). In nature, *G. xylinus* is found in soil and is commonly found on rotting fallen fruits (O'Neills Skinner & Cannon, 2000). Bacterial cellulose is interesting for commercial application because it is known to have higher purity, excellent crystalline property, good water absorption, better tensile strength, ultra fiber networks with diameter of 20–100 nm, low degree of polymerization, and stronger biological adaptability (Wan et al., 2006).

The aim of this chapter is to highlight the importance of bacterial cellulose in industry and to summarize research work carried out at Research Center for Biotechnology of the Indonesian Institute of Sciences (LIPI).

Applications of bacterial cellulose

Food application

According to Africa (1949), the utilization of microbial cellulose, a white gelatin like substances, was found in the Philippines, which dates back to the year of discovery of *nata* in 1861. *Nata* is derived

from the Latin word, *natare*, meaning "to float". The gelatinous gel was later attributed to *G. xylinus*. This white gelatin-like substance was also found when *G. xylinus* grown on substrate composed of pineapple juice and peel, and the product was called *nata de pina* (Sanchez, 1990). Later on, whole-year production of the bacterial gel was made possible through the use of coconut water and coconut milk which were readily available as raw materials. The product was then named *nata de coco.* When *nata* was cut into cubes, de-acidified and sugar was added to make it a delicious dessert widely appreciated by many people around the world (Sanchez, 1990).

Okiyama et al. (1992) showed that when alginate was added during processing the gelatinous gel by *G. xylinus*, the texture was comparable to grape making and it was applicable for salads, low calorie desserts, and other food items. When water was added during processing, the gelatinous gel formed a paste-like material which swelled and dispersed homogenously (Okiyama et al., 1992). Furthermore, the paste-like cellulose material in food formulations was achieved in chocolate drink, pasty condiment like seasoned clam extract, ice cream, *tofu* (soybean curd), *kamaboko* (boiled fish paste), and low–calorie products such as hamburger patty, sausage, and Japanese red bean jam (Okiyama et al., 1992). Okiyama et al. (1993) reported that bacterial cellulose could replace one third of the beef and beef extract required during hamburger formulation and producing a product with 25% decreased energy value without affecting tenderness and juiciness of the product.

Nata bacterium can utilize fruit syrup to grow, reproduce, and secrete micro fibrils *in situ* and acquire the nature flavor and pigment of the fruit. The type of cellulose can be produced in range of shapes and texture, such as films, multi-shaped, pulps, filaments, spheres, particles, and whiskers suited for many different applications in food industries (Sukara & Melliawati, 2014). Bacterial cellulose can

be used as prominent ingredient in low-calorie food products and can be used as an important ingredient in the production of low-cholesterol products. Another application is in an active food packaging industry where it can increase or confirm the shelf life or safety of products. Antimicrobial agents, oxygen and ethylene scavengers, moisture removers and taint removers may also be integrated with biocellulose based active food packaging systems (Shi et al., 2013).

Medical applications

The application of bacterial cellulose has also inspired many researches in the field of medicine. White and Brown (1989), Brown (1992) and Yamanaka (1989) reported that high crystallinity, purity (free of lignin and other biogenic products), high water absorption capacity and mechanical strength in wet state were the features that merited its new applications. The formation of two dimensional analogues of spherulites approximately more than 1 cm diameter in pellicle of bacterial cellulose produced by static culture of *G. xylinus* was reported by Colvin (1965). The spherulite was a unit of spherical crystalline body made up of radiating crystal fibers and, therefore, was found to improve the mechanical properties of the microbial cellulose. The formation of spherulites was recognized as a characteristic mode of crystallization of many synthetic polymers (Colvin, 1965). In the medical field, bacterial cellulose produced in static liquid culture *G. xylinus* may be applied for treatment as temporary skin substitute to treat skin wound and second and third degree burns, as a surgical wipe, treatment pad, bandages or tissue/organs draping (Ring et al., 1986; Fontana et al., 1990; Fontana et al., 1991). Furthermore, Geyer et al. (1994) developed a process in the preparation of hallow-shaped bacterial cellulose. This cylindrical biomaterial is useful as a substitute for medical materials such a blood vessel, lymphatic vessel, ureter, and trachea.

Gabriel et al. (2013) reported that gamma irradiation treatment could modify the bacterial cellulose surface properties and enhancing its potential for biomedical applications. Physical modification of bacterial cellulose is believed to be one of the future alternative routes for transdermal drug delivery. The fabrication of a nanofibrillar patch by using bacterial cellulose and its application as a new wound-healing platform for traumatic tympanic membrane (TM) perforation was demonstrated by Kim et al. (2013). TM perforation is a very common clinical problem worldwide and presents as conductive hearing loss and chronic perforations.

Nimeskern et al. (2013) reported that bacterial nanocellulose is recognized as a novel non-degradable biocompatible material that promotes chondrocyte adhesion and proliferation. Bacterial nanocellulose, therefore, can be used for ear cartilage and biofabrication to produce patient-specific bacterial nanocellulose-based ear cartilage for ear cartilage replacement.

Other Industrial applications

Bacterial cellulose has remarkably high modules of elasticity, high internal loss, low density, and high sound propagation velocity (Yamanaka, 1989). The young modulus of a sheet prepared by squeezing and drying alkaline treated gelatinous gel of bacterial cellulose is about 30 Gpa (Nishi et al., 1990). Some of the products produced from bacterial cellulose sheet can replace the traditional material cone paper as diaphragms for electroacoustic transducers such as loudspeakers and headsets. Bacterial cellulose is an interesting, renewable, and biodegradable material with extended commercial application. The loudspeakers with biocellulose membranes have been found to exhibit acoustic response in a wider frequency range and of higher effectivity in comparison to that produced of a woody tissue. The mechanical properties of biocellulose are strongly connected with

electroacoustic parameters of the loudspeakers, due to the mechanical compliance of the membrane which is inversely proportional to young's modulus of the membrane material. Bacterial cellulose sheet has been found to have better physical characteristics required for these products. Microbial cellulose product sold as Cellulon, is produced in agitated fermentor (Kent et al., 1991). Cellulon may be used as binder for ceramic powders and minerals, thickeners for paint, ink, adhesives, and even as paper coating (Cannon & Anderson, 1991). This material has advantages for its good adhesion, good fluid (water and electrolyte) retention, and low cost. Several studies reported that bacterial cellulose was seen to have potential as a new material for the production of high quality paper (Johnson & Winslow, 1990), paint additives (Cannon & Anderson, 1991), and ultra filtration membrane (Takai et al., 1991).

Another useful trait of bacterial cellulose sheet is that it is found to have better physical characteristics required for the manufacturing of electroacoustic transducers products (Sukara & Melliawati, 2014). Gutierrez et al. (2013) reported that bacterial cellulose was used as template to design hybrid inorganic-organic composites. It combined the excellent properties of bacterial cellulose with optical, magnetic, electrical, and chemical properties of inorganic nanoparticles. Fabricated hybrid inorganic/organic nanopapers modified with vanadium and titanium oxide nanoparticles had potential application as sensitive displays, biosensors, and others optical devices (Gutierrez et al., 2013). Other potential application of bacterial base carbon nanofiber aerogel may include 3D electrode materials for lithium-ion batteries and super-capacatiors, catalyst supports, and advanced sensors.

Bacterial Cellulose Research in the Research Center for Biotechnology-LIPI

Research Center for Biotechnology-LIPI intensively collects diverse bacteria responsible for the production of cellulose from several places in Indonesia. Selection and screening of bacteria producing cellulose has been done intensively. Two isolates, namely *Acetobacter* sp. RMG-2 and *Acetobacter* sp. EMN-1, were selected for the study. Both bacteria has potential value to be used as inoculums for the production of biocellulose and *nata de coco*. To increase the performance of the selected bacteria, the reseach to study the effect of sterilizations and types of coconut water for the production of biocellulose by *Acetobacter* sp. EMN-1 was carried out. The pure culture of *Acetobecter* sp. EMN-1 and commercial inoculums obtained from Cianjur were compared during the course of the study. Three methods of sterilization *i.e.* boiling the substrate for 5 minutes at 100°C (1), sterilization of the substrate at 121°C for 15 minutes (2), and 30 minutes (3) using two different types of coconut water were investigated. The results showed that the production of bacterial cellulose on unfresh and boiled substrates inoculated by pure culture *Acetobacter* sp. EMN-1 was higher than the production of bacterial cellulose using commercial inoculums. The yield using the pure *Acetobacter* sp. EMN-1 reached up to 472 g/L (wet weight) and 24 g/L (dry weight), while the yield of commercial inoculum only reached 411 g/L (wet weight) and 23.4 g/L (dry weight). The length of incubation for both production was 10 days at room temperature. The total amount of bacterial cellulose increased with an increasing of inoculums concentration. This best condition was used further for the production of bacterial cellulose in larger capacity (scale up). The average wet weight of bacterial cellulose increased up to 778.5 g/L (wet weight) (Melliawati et al., 1998)

Research was also conducted to see the effect of mix extract fruit and coconut water on the production of bacterial cellulose using

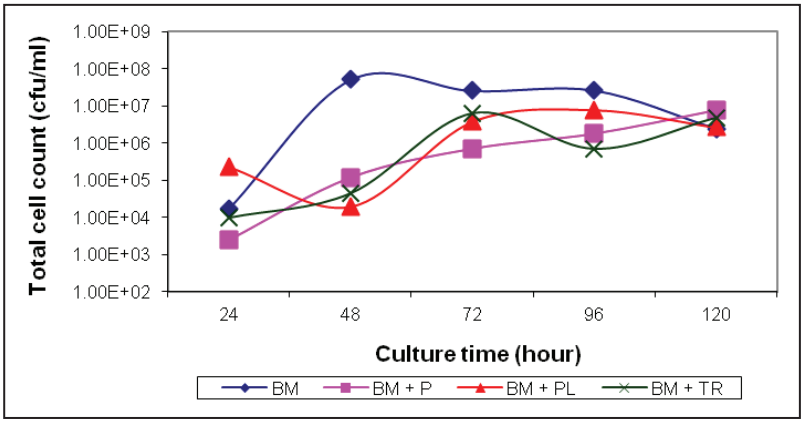
Acetobacter sp. EMN-1. Six different media, namely HS (2% glucose, 0.5% Bacto peptone, 0.5% yeast extract, 0.27% Na_2HPO_4, 0.12% acetic acid glacial, 0.04% $MgSO_4.7\ H_2O$ in sterile water), HB-K (10% glucose, 0.22% yeast extract, 0.5% K_2HPO_4, 0.06% $(NH_4)_2.7H_2O$, 0.04% $MgSO_4.7\ H_2O$ in coconut water), HB-T (10% glucose, 0.22% yeast extract, 0.5% K_2HPO_4, 0.06% $(NH_4)_2.7H_2O$, 0.04% $MgSO_4.7\ H_2O$ in tomatoes extract and coconut water), HB-N (10% glucose, 0.22% yeast extract, 0.5% K_2HPO_4, 0.06% $(NH_4)_2.7H_2O$, 0.04% $MgSO_4.7\ H_2O$ in pineapple extract and coconut water), TPSS (10% glucose, 0.5% Bacto peptone, 0.30% yeast extract, 0.02% $MgSO_4.7\ H_2O$, 0.01% K_2HPO_4, 0.01% NaCl in tomatoes extract and sterile water), and NPSS (10% glucose, 0.5% Bacto peptone, 0.30% yeast extract, 0.02% $MgSO_4.7\ H_2OH$, 0.01% K_2HPO_4, 0.01% NaCl in pineapple extract and sterile water), were used in this study. Results showed that HB-K media was the best for cellulose production. The highest bacterial cellulose produced was 0.16 g/mL using *Acetobecter* sp. EMN-1 after 24 hours incubation. The weight of cellulose produced on the HB-K medium was 239.6 g/L wet weight or 34.2 g dry weight, while the cellulose rendement was 47% and thickness of cellulose at 5 days of incubation was 0.47 cm. At the end of fermentation, the pH of the media always decreased. The extract of tomatoes and pineapple could be used as alternative mixtures for this purpose (Melliawati et al., 1999).

Environmental factors such as growth temperature, pH medium, aeration, and agitation affected the growth of microbes. *Acetobacter* sp. EMN-1 and RMG-2 were used to study the effect of pH medium (pH 4.0-7.0) on the cell growth and cellulose production. The media used in this study were HB-K and GAA (0.3% glucose, 0.5% acetic acid, 0.5% $(NH_4)_2\ SO_4$ in coconut water). The results indicated that both media composition (HB-K and GAA) and pH medium had an effect on the growth and the production of cellulose of *Acetobacter* sp. EMN-1 and RMG-2. The best cell growth of *Acetobacter* sp. EMN-1 was

on the medium with the pH range between 6.5 and 7.0. Meanwhile, the highest cell population (1.19×10^{11} CFU/mL) was obtained in the medium with the pH 6.5. The heaviest cellulose yield was 293.8 g/L (wet weight) or 15.68 gram (dry weight) and the thickest of cellulose produced was about 2 cm on the medium with pH 7.0. On the other hand, *Acetobacter* sp. RMG-2 showed the best growth condition at pH range between 5.5 and 6.5 for 5 days incubation. The highest cell population was 2.54×10^{10} CFU/mL obtained at pH 5.5. The heaviest and thickness of cellulose yield were 229.6 g/L (wet weight) or 29.3 g (dry weight) and 1.5 cm thick. In general, the pH medium on the end of fermentation process (7 days incubation) decreased both for the *Acetobacter* sp. EMN-1 and RMG-2. The number of the cells on HB-K and GAA media reached up to 1.12×10^{11} CFU/mL and 2.89×10^{10} CFU/mL respectively after 7 days fermentation. The experiment was carried out also in an Airlift fermentor with a 2 L working volume. The results showed that the number of the cells reached up to 2.61×10^{10} CFU/mL after 6 days incubation (Melliawati et al., 2000).

Two methods of shaking, namely rotary and reciprocal shaking were also studied. There were different effects on cell growth and biomass yield. Using a rotary shaking at 170 rpm and 72 hours fermentation, the total number of cell was 6.05×10^{8} CFU/mL with cell biomass of 114 g/L (wet weight) or 1.7 g dry weight. Meanwhile, using a reciprocal shaking at 120 rpm, the total of 1.05×10^{8} CFU/mL with a biomass yield of 136 g/L (wet weight) or 20 g dry weight. The use of yeast extract, peptone, polypeptone, or tryptone as a nitrogen source could accelerate the growth rate of the culture and reduced the fermentation time to only 48 hours. Effect of nitrogen sources on cell growth of *Acetobacter* sp. EMN-1 for 120 hours is shown in Figure 1. Scaling up was carried out using a 2 L Air Lift Fermenter but the maximum cell population reached was only 5.3×10^{7} CFU/mL after 72 hours. The total amount of biomass produced was 65.2

g/L (wet weight) or 13/5 g dry weight. Preliminary study using a 10 L Stirred Tank Fermenter was carried out and the result showed that the amount of cell was lower than of obtained in both of rotary agitated flask and air lift fermenter at the same incubation time (Melliawati et al., 2001).



Source: Melliawati et al., 2001

Figure 1. Effect of Nitrogen Sources on Cell Growth of *Acetobacter* sp. EMN-1. BM., Basal Medium; P., Peptone; PL., Polypeptone; TR., Tryptone.

Carbon and nitrogen are two compounds required for growth of microorganisms. Total of 14 different carbon sources, including sucrose, glucose, mannitol, serbose, ethanol, glycerol, sitric acid, arabinose, maltose, fructose, starch, galactose, lactose, and manose, and 4 different nitrogen sources such as yeast extract, peptone, polypeptone and tryptone, were subject to study. *Acetobacter* sp. RMG-2 grew on all carbon sources tested. Glucose and sucrose, however, were found to be the best carbon source for the growth of bacterium with cells number was 1.39×10^{11} CFU/mL and 9.50×10^{10} CFU/mL, respectively, after 144 hours incubation. The cells number of *Acetobacter* sp. RMG-2

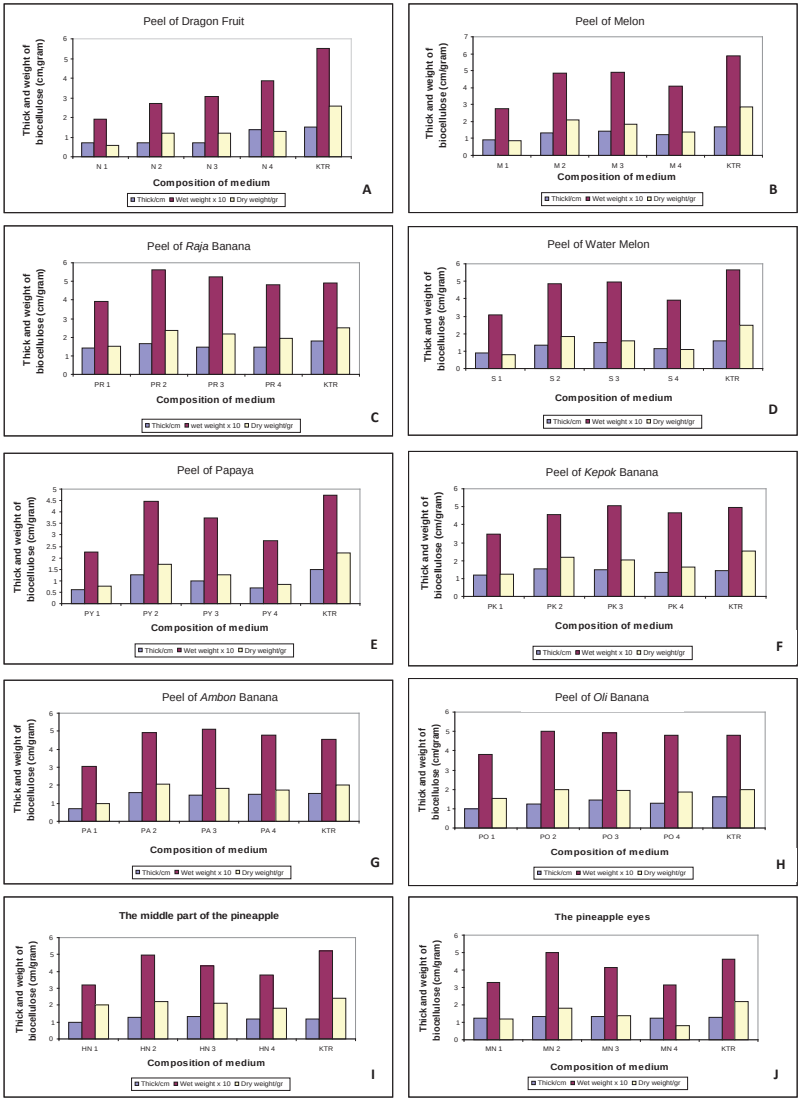
on basal medium containing 10% glucose, 0.25% yeast extract, 0.6 % K_2HPO_4 , 0.06% $(NH_4)_2$ SO_4 , 0.02% $MgSO_4$ $7H_2O$ with the addition of peptone, was 1.44×10^8 CFU/mL after 48 hours. The addition of tryptone did not support the cell growth, thus resulting in a low number of cells and longer fermentation time (Melliawati et al., 2003).

Another issue on *nata* production is the demand for better inoculums with high purity and easy handling. The existing inoculums type is in cell suspension culture and placed in a glass bottle. It is bulky, thus causing any means of transporting them to be difficult. To maintain the purity and to ease transport, the inoculums in the form of pasta have been developed. Various carriers have been introduced to maintain the viable bacterial cell. For this purpose, four carrier material, namely Carboxy Methyl Cellulose (CMC), Agar, Sagu Starch and Bacterial Cellulose Pulp were introduced. The texture of carrier gel, population of cells that can be maintained, and the capacity in producing bacterial cellulose were studied using *Acetobacter* sp. RMG-2 and *A. xylinum*. The results showed that all gel/pasta inoculums could be used as an inoculums replacing liquid inoculums. Both CMC and Bacterial Cellulose Pulp had good texture and could be used to met the standard quality of inoculums. Cells population of *A. xylinum* was 1.28×10^9 CFU/mL (in CMC), 1.6×10^6 CFU/mL (in Bacterial Cellulose Pulp) after 15 weeks. Using these to inoculums gel, the weight of bacterial bellulose was 500 g/L and 740 g/L (wet weight) respectively, while the cells population of *Acetobacter* sp. RMG-2 on CMC and biocellulose pap carrier was 1.79×10^8 CFU/mL and 7.75×10^7 CFU/mL producing bacterial cellulose of 630 and 775 g/L respectively. It was concluded that CMC and Bacterial Cellulose Pulp were able to keep bacteria without losing their capability to produce bacterial cellulose (Melliawati, 2008)

Inoculums became one of the most important factors in *nata* production process and also a determinant of product quality. The

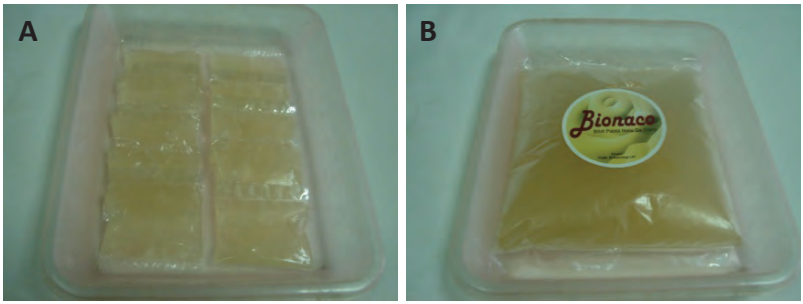
research was conducted to obtain the most suitable packaging of inoculums for better storage and efficiency. Straws and plastic bags were evaluated. Inoculums were stored for 20 weeks at 4°C. The number of cells, wet weight, dry weight and thickness of bacterial cellulose were evaluated. Inoculums gel with CMC as carrier which were packed in straw was better than that on plastic bag. The cells population of *Acetobacter* sp. RMG-2 was 4.53×10^8 cells/mL, while in the plastic bag only 3.5×10^7 cells/mL after 20 weeks (five months) of storage. The production of bacterial cellulose using 1 to 15 ml inoculum in 1,250 mL of medium was varied. With 1 ml of inoculum, the average of 725 g/L wet weight with a thickness of 0.6 cm of bacterial cellulose was obtained. It was found that 0.1% inoculums with CMC as a carrier materials stored for 8 week can still be used for the production of bacterial cellulose with the average of 314.5 g/L (wet weight) or 16 g dry weight and thickness of 0.8 cm during the seven days incubation (Melliawati & Nuryati, 2011).

Indonesia is rich agricultural country, plentiful with vegetables and fruits. Waste from vegetables and fruits is abundantly available. Utilizing both waste organic materials for the production of bacterial cellulose was carried out. Fruit peel of pineapple, watermelon, *ambon* banana, *raja* banana, *oli* banana, *kepok* banana, papaya, and dragon fruit were untapped. Fruit peel (*i.e.* inner part of fruit peel) was blended with the addition of enough water and then filtered (extract). The extract was used as a medium. It was inoculated with *Acetobacter* sp. RMG-2. The composition of media was a mixture of fruit peel extract and coconut water with a ratio of 100:0; 75:25; 50:50; 25:75; and 0:100 for control (Figure 2). Observations were conducted on the thickness of bacterial cellulose, its wet weight and dry weight after 7, 9 and 11 days incubation period. The results showed that 10 kinds of fruit peel were suitable for the production of bacterial cellulose, but the weight and thickness of bacterial cellulose was varied. For peel of



Source: Melliawati & Nuryati, 2012

Figure 2. Bacterial Cellulose Graph of 10 kinds of Peel Fruit, Thick, Heavy Wet and Dry Weight of Bacterial Cellulose Performed in Jars Containing 100 ml Medium for 11 Days of Incubation (A–C) and 9 Days of Incubation (D–J)



Source of photographs: Melliawati in 2015

Figure 3. A Paste of *Acetobacter sp*. RMG-2 Inoculum. A) Paste in Sachet Contents of 15 mL; B) Paste in Plastic Contents of 500 g

raja banana, ambon banana, *oli* banana, *kepok* banana, watermelon and pineapple, the bacterial cellulose had more weight and was thicker compared to the peel of dragon fruit, papaya, and melon. Extracts of *raja* banana yielded the highest bacterial cellulose after coconut water (as control), with thickness, wet weight, and dry weight of 1.4 cm, 392 g/L, and 15 g respectively after 11 days incubation. Coconut milk medium remained superior with 1.8 cm, 489 g/L, and 2.5 g. Medium containing a mixture of banana peel extract and coconut water (75:25) gave promising results with 1.6 cm thick of bacterial cellulose, wet weight of 491.1 g/L, and 2.05 g dry weight, and the incubation only 9 days. This result showed that this process may be useful for handling of waste to produce bacterial cellulose (Melliawati & Nuryati, 2012). Figure 3 shows earlier stage of inoculums in the form of a paste of *Acetobacter* sp. RMG-2 in sachet and plastic.

The inoculums in gel/paste form could boost the production of bacterial cellulose. It is time to market this inoculums type and establish more small and medium-sized bacterial cellulose industry in areas where substrate is abundantly available. Gel/paste inoculums of *Acetobacter* sp. RMG-2 could be transported to outside Java or remote areas to provide opportunity to establish bacterial cellulose industry.

Gel/paste inoculums is now available. The technology was registered for a patent in 2001 (P00200101012) and was granted in 2015 (IDP 000038337). It is therefore ready for licencing through the Research Center for Biotechnology, Indonesian Institute of Sciences (LIPI).

Conclusion

The application of bacterial cellulose as environmental friendly materials is now flourishing. It is now possible to use bacterial cellulose as raw materials in food and biomedical industries. Bacterial cellulose may be used for the production of synthetic blood vessel, urether, and trachea. This leads to open new opportunities for Indonesian scientists to further explore microbial genetic resources of Indonesian origin and search for better candidate for the production of better microbial cellulose and other microbial polymer for the future of industrial development in the country. Continuous exploration, isolation, and screening for better bacterial cellulose and microbial polymer producing microbes is in high urgency for future food, health, medicine, and advance materials industry in Indonesia.

Acknowledgements

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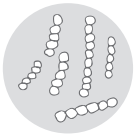


Lactic Acid Bacteria from Fermented Food and Feed

✍ Yantyati Widyastuti

Introduction

Lactic acid bacteria are specifically characterized by their positive reaction to Gram staining and negative reaction to catalase test. Cell morphology of lactic acid bacteria varies from straight or curved long to short rod and cocci in several configurations, including pair, chain, and tetrad. The lactic acid bacteria belong to family *Lactobacteriaceae* with several genera, in which *Lactobacillus* represents as the largest genus that contains over 100 species (Canchaya et al., 2006). The revision of grouping in the genus *Lactobacillus* representing phylogenetic structure has been changing from time to time, with the latest comprising *Lactobacillus acidophilus* group, *Lactobacillus casei* group, *Lactobacillus plantarum* group, *Lactobacillus reuteri* group, *Lactobacillus buchneri* group, and *Lactobacillus salivarius* group (Giraffa et al., 2010). In this family, several other genera including *Streptococcus, Pediococcus, Lactococcus, Leuconostoc, Bifidobacterium, Carnobacterium, Enterococcus,* and *Sporolactobacillus* (Wood & Holzapfel, 1995) are recognized.

Lactic acid bacteria are generally recognized as safe (GRAS) microorganisms and well known for their important role in improving the life of human and animal. Lactic acid bacteria have been widely applied in foods and feeds fermentation all over the world in both traditional and modern fermentation industries. Fermentation is simply defined as conversion of substrate to other new products by the presence of lactic acid bacteria as converting agent. As the result, the fermented product has longer shelf life than its raw material, which is originally perishable. In fermentation, through their ability to ferment sugars they produce lactic acid as the main catabolic product, together with other organic acids according to the type of fermentation, homofermentation or heterofermentation. Lactic acid has such variety of roles either to improve the flavor, texture and nutrition or as preservative of the products. Preservative functions to control putrefactive microorganisms and pathogens, improving the safety of food. There are 2 different substrates for lactic fermentation, i.e. dairy and non-dairy. In dairy fermentation, such as cheese and yoghurt, lactic acid contributes to improve fermented product with fresh mild acid taste. Meanwhile, improvement of the texture of fermented products is caused by the secretion of a polysaccharide polymer called exopolysaccharide (EPS). Ruas-Madiedo et al., (2002) and Welman & Maddox (2003) reviewed the perspectives, challenges, and functionality of EPS from lactic acid bacteria. So far, EPS production is low and inefficient; therefore, effort to enhance the production of EPS from lactic acid bacteria may be carried out through selection of superior strains and optimization of fermentation conditions and engineering of EPS synthesis metabolically. Properties of lactic acid bacteria that are relevant to quality improvement of fermented products include their proteolytic activity (Khalid and Marth, 1990; Griffiths & Tellez, 2013) and bacteriocins production (Yang et al., 2012; Zacharof & Lovitt, 2012).

In addition, the live cultures of lactic acid bacteria are also very popular for probiotics use, with certain terms and conditions. Lactobacilli are declared as model of gut bacteria, offering exciting research prospects to microbiologist (Tannock, 2004). Several species under the genus of *Lactobacillus* have been used for probiotics commercially with documented health benefits (Giraffa et al., 2010), showing that they have potent capability as probiotics. Due to the importance of lactic acid bacteria in our life, isolation, collection, and characterization of lactic acid bacteria are widely carried out, especially in mega biodiversity country like Indonesia.

The objective of this chapter is to describe the work on collection of lactic acid bacteria in Indonesia during the last 20 years carried out mainly at the Research Center for BiotechnologyIndonesian Institute of Sciences (LIPI). Collected isolates of lactic acid bacteria were preserved and some potential isolates were used in several studies on food and feed fermentation as well as probiotics.

Application of Lactic Acid Bacteria

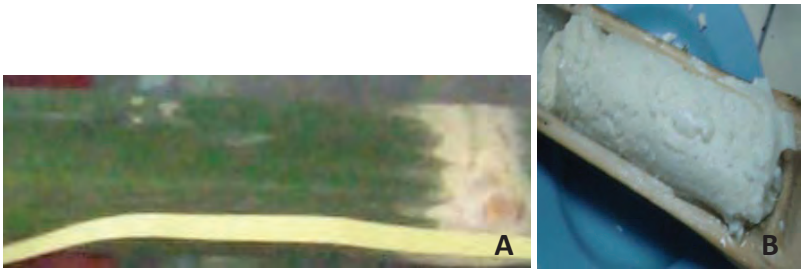
Most of us are familiar with lactic acid bacteria, because food fermentation using lactic acid bacteria has been practiced since ancient times. Traditional food fermentation was carried out mostly in Asia and recipes were handed over from one generation to another within a family, without knowing and considering the real role of lactic acid bacteria. On the other hand, modern industries have also been developed that make lactic acid bacteria become popular commercially and widely used in new fermented food products. Recently, people are aware that fermented foods give impact on health and demand of fermented foods has been increasing. Nuraida (2015) reported the presence of several lactic acid bacteria from Indonesian fermented

foods, which had health promoting effect through their probiotics property.

Food fermentation

Lactic acid bacteria are most likely present in various fermented food. Fermented food can be categorized from the materials used into dairy and non-dairy fermented food. In most parts of Europe, lactic acid bacteria are widely used in producing dairy fermented food, especially cheese. There are various famous cheese products made using certain potential lactic acid bacteria, since they give significant roles in improving the flavor of cheese products (Broome, 2007). In most parts of Asia, lactic acid bacteria are used in traditional fermented food, both dairy and non-dairy fermented food, but more in non-dairy substrate. During fermentation, lactic acid bacteria also provide a selective environment favoring other fermentative microorganisms to produce desirable flavors in various fermented food (Rhee et al., 2011), increase shelf life of fruits and vegetables, enhance several beneficial properties such as nutritive value and reduce toxicity as well as potential source of probiotic strains of lactic acid bacteria (Swain et al., 2014).

Fermented food in Asia have been practiced and produced for thousands of years and modified in various ways depending on the availability of raw materials. However, there is also growing interest to develop better products using modern technology and available facilities. China has claimed that they are the first country to utilize lactic acid bacteria for food fermentation. Representatives of traditional fermented foods using lactic acid bacteria in China include *koumiss*, *suan-tsai*, stinky tofu, and sausage (Liu et al., 2011). In Indonesia, there are traditional dairy fermented products, called '*dadih*' or '*dadiah*', in Sumatra, and '*dangke*', in Sulawesi islands. The main material of those products is buffalo milk, but recently *dangke* is also prepared using cow milk. *Dadih* is produced from artisanal buffalo milk that is



Source of photographs: Widyastuti, 2012

Figure 1. *Dadih*. A: *Dadih* in the Bamboo; B: Fresh *Dadih* Inside Bamboo.

directly put in one 'room' of bamboo after collecting the milk and kept for several days. During incubation, lactic acid bacteria are growing spontaneously. The curd in the bamboo is called *dadih* (Figure 1), that usually served and consumed uncooked. On the other hand, *dangke* is prepared in a different way from dadih. The curd formation of dangke is developed by adding few drops of papain enzyme that are squeezed from fresh papaya leaves. Procedure of *dangke* preparation according to the local people of South Sulawesi is shown in Figure 2. *Dangke* has harder texture compared to that of *dadih* and is served as fried *dangke. Lactobacillus casei* D11 isolated from dadih was reported to produce milk clotting enzyme that significantly influence the production of curd in mozzarella cheese (Rohmatussolihat et al., 2015).

Feed fermentation

So far, silage is the only product of forage preservation through fermentation that keeps the forage fresh for animal feed. The nature of fermentation of silage using lactic acid bacteria is suitable for all weather conditions including the tropics. Lactic acid content in silage acts as preservatives that prevent microbial spoilage; consequently, silage will become sour forage, but is still palatable. Silage can be stored for years as long as we can keep the condition free from oxygen.

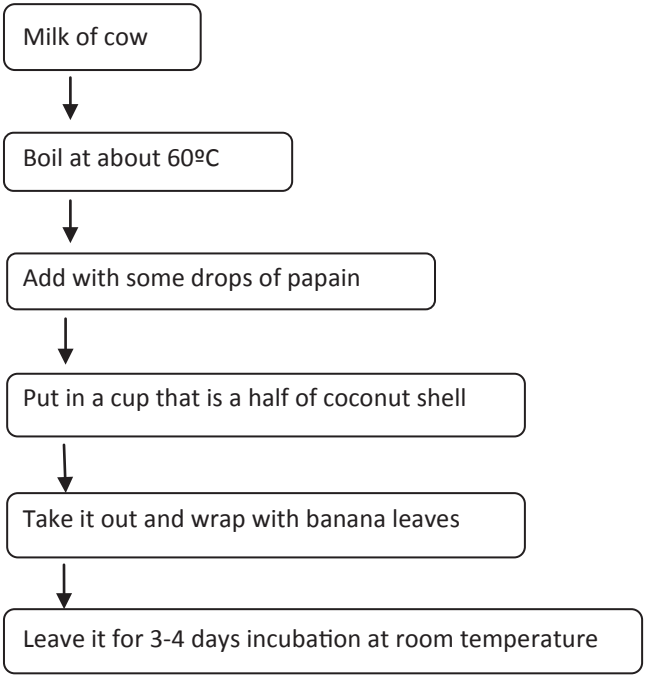


Figure 2. General Steps of *Dangke* Production

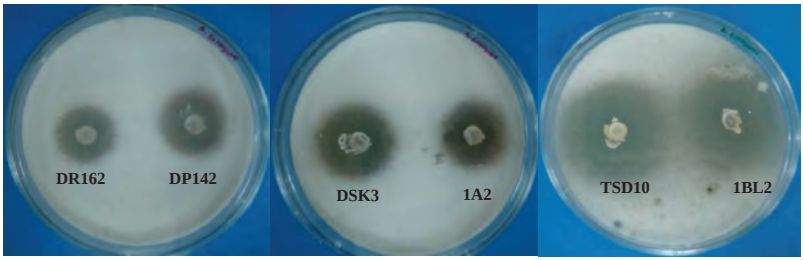
Silage inoculants are selected lactic acid bacteria which show properties suitable to be used in making silage. Research on screening and development of silage inoculants has been conducted for more than 15 years in the Research Center for Biotechnology-LIPI. First screening was done using lactic acid bacteria isolates from a variety of samples, including fermented foods, fruits, and flowers. They were screened through their acid production. The more the lactic acid produced, the higher their rank in the list of silage inoculants candidates (Yatno, 1999). Lactic acid bacteria isolates from *dadih* and *dangke* from several locations in Sumatera and South Sulawesi were also screened (Novianti, 2000). *Lactobacillus plantarum* 1A-2, which was originally isolated from fermented cassava, and *L. plantarum* 1 BL-2,

Source of photographs: Widyastuti, 2007

Figure 3. Silage Making Processes. A) Silage Inoculants *L. plantarum* 1A-2; B) Mixing Grass with Inoculants; C) Packaging of Grass in Plastic Bag as Silo.

from strawberry, were selected as the best isolates for silage inoculants. They were then used for several silage making using mainly elephant grass (*Pennisetum purpureum)* as the raw material. *L. plantarum* 1A-2 was used as inoculants for elephant grass silage (Ridwan et al., 2005) and vegetable wastes (Astuti et al., 2013) with several level of rice bran as addtitive, while *L. plantarum* 1 BL-2 was used in several different concentrations (Ratnakomala et al., 2006). Considering that multi cultures of lactic acid bacteria for silage inoculants are better than single culture, co-cultures of *L. plantarum* 1A-2 with other potential lactic acid bacteria cultures are being studied and hopefully will come out with better quality of silage inoculants in the future. *L. plantarum* 1A-2 as silage inoculants has been distributed and used in some locations when we disseminated the silage making technology to the farmers (Figure 3).

In general, silage making using these inoculants has been successful, which means that, so far, we have produced good quality of silage as preserved grass for animal feed. We observed high number of lactic acid bacteria and very low number of fungi as contaminant. Among contaminant fungi, *Aspergillus fumigatus* was one of the predominant fungi contaminating moldy silages (Cole et al., 1977). Six strains of



Source: Fauziah, 2012

Figure 4. Antifungal Activity of 6 Strains of *L. plantarum* Against *A. fumigatus*.

L. plantarum from our collection were studied for their antifungal against *A. fumigatus* and they produced clear zone of 22–30 mm using double layer agar method (Fauziah, 2012) as shown in Figure 4.

Collection of Lactic Acid Bacteria

Lactic acid bacteria are distributed widely and can be found in a variety of habitats, such as plants and animals, including its digestive tracts and milk. The presence of lactic acid bacteria in fermented products is interesting since they may originally present in the raw materials. Based on the potential role of lactic acid bacteria, collection of new lactic acid bacteria is important.

Collection of indigenous strains of lactic acid bacteria has been carried out in Indonesia through two bilateral collaborative projects between Indonesia and Japan. They are project on Conservation and Sustainable Use of Tropical Bioresources and project on Development of Internationally Standardized Microbial Resources Center as a Core of Biological Resources Center to Promote Life Science Research and Biotechnology. The first project (1993–1999) was organized by New Energy and Industrial Development Organization (NEDO) and Japan Bioindustry Association (JBA) from the Japan side and Agency for

the Assessment and Application Technology (BPPT), LIPI and universities as Indonesian side. The second project was funded by Japan Science and Technology (JST) and Japan International Cooperation Agency (JICA) and LIPI. The 5-year project (2011–2016) focuses on the development of a Biological Resources Center including the management of holdings as well as collection and handling of new taxa of several groups of microorganims.

From the first project, samples collection was from plants including flowers and fruits and several fermented foods, collected from Bogor and its vicinity. Around 200 isolates of lactic acid bacteria were collected throughout the project. By employing conventional identification methods, at that time, the collected isolates from tropical fruits, either fresh or ripened, were identified as *Lactobacillus plantarum, Lactobacillus* sp., *Enterococcus* sp. and *Streptococcus* sp. (Widyastuti et al., 1999). Later on, identification method based on 16S rRNA gene analysis was applied to the isolates. The isolates were then deposited in a culture collection in Indonesia, InaCC, which was newly established in 2016.

In the second project, there were two activities on lactic acid bacteria isolation based on samples used. The first activity focused on collection of lactic acid bacteria from traditional fermented foods samples, whereas the other focused on samples from digestive tract of chicken, cattle, and silages. More than 200 isolates were collected; among others *Lactobacillus* sp., *Enterococcus* sp., *Weisella* sp., *Lactococcus* sp. and *Pediococcus* sp. They were collected from traditional fermented foods such as *tape* with several variant, *tempe*, and *oncom*. The second activity aimed to develop animal probiotics, for chicken and cattle, derived from lactic acid bacteria from the collected isolates. The activity was initiated with isolation of lactic acid bacteria from the representative habitats followed by screening for probiotics purpose and identification of the isolates. Lactic acid bacteria were

isolated from digestive tract including crop, caecum, and gizzard of the chicken and the rumen of cattle. New taxa of lactic acid bacteria was highly expected to be found from this project that could be deposited in the Microbial Culture Collection or Microbial Resources Center. There are three potential *Lactobacillus salivarius,* each from crop, cecum, and gizzard of local *Cemani* chicken reported to have characteristics of probiotics. They showed high inhibitory activity against *E. coli* and *Salmonella enteritidis,* high cell surface hydrophobicity and inter-isolate coaggregation ability of lactic acid bacteria, high survival at low pH, high phytase, and protease activity (Jannah et al., 2014). Isolates from the rumen were dominated by *Streptococcus bovis* whereas from silage were *L. plantarum*.

Diversity of Lactic Acid Bacteria

Lactic acid bacteria from *Dadih* and *Dangke*

Lactic acid bacteria isolated from *dadih* were collected from three different places i.e., West Sumatera, Palembang, and Riau (Table 1). The dominant species are *L. plantarum* and *L. lactis.* The presence of *E. faecium* and *E. faecalis* in *dadih* was possibly related to the condition that *dadih* fermentation was not occurred in a hygienic condition, due to the simple facilities available during production of *dadih* in Riau. Meanwhile, *dangke* was dominated by species of *Pediococcus acidilactici.*

Lactic acid bacteria from silage

The species of *L. plantarum* is among the most dominant species in plants as its original habitat. It is a homofermenter lactic acid bacteria producing only lactic acid from its metabolism. This characteristic is suitable for silage fermentation since high concentration of lactic acid from inoculants is required, although recently heterofermenter

Table 1. Number of Lactic Acid Bacteria Isolated from *Dadih* Collected from West Sumatra, Palembang, and Riau (Widyastuti, unpublished data)

Lactic acid bacteria species	West Sumatra	Palembang	Riau
L. plantarum	1(3)	2(4)	6(23)
Lactococcus lactis	1(3)		9(23)
Pediococcus pentosaceus		1(4)	
Lactobacillus paracasei			1(23)
Lactobacillus sp.		1(4)	
Enterococcus faecium	1(3)		3(23)
Enterococcus faecalis			2(32)
Enterococcus thailandicus			1(32)
Enterococcus sp.			1(32)

Note: number in the bracket represents total isolates from each location

lactic acid bacteria are also used in the mix cultures to abate oxygen during fermentation. The role of inoculants is to grow well in the substrate, dominate among population of bacteria, and produce lactic acid abundantly.

Lactic acid bacteria from silage prepared without inoculants collected from Yogyakarta and Cibinong in 2011 were dominated by *L. plantarum* followed by *L. casei* (Table 2). From corn silage in 2012, were also dominated by *L.plantarum* followed by *L. fermentum*, and *L. brevis*.

Table 2. Lactic Acid Bacteria from Grass and Corn Silages Produced in Yogyakarta and Cibinong (Widyastuti, unpublished data)

Species	Yogyakarta		Cibinong	
	Grass silage	Corn silage	Grass silage	Corn silage
L. plantarum	6(6)	3(4)	10(15)	2(4)
L. casei		1(4)	5(15)	-
L. farciminis				1(4)
L. crustorum				1(4)

Note: number in the bracket represents total isolates from each location and sample

Grass and corn silages were dominated by *L. plantarum*. Pang et al. (2011) reported that *L. plantarum* was dominant on sorghum and paddy rice silages, while *L. pseudomesenteroides, L. paraplantarum* and also *L. plantarum* were dominant species in alfalfa silage.

Conclusion

Lactic acid bacteria represent a group of useful bacteria with some new information for further study. For the purpose of research that will end with application of lactic acid bacteria for a certain product, it is necessary to isolate and collect new isolates of lactic acid bacteria from various sources. Characterization of new taxa of lactic acid bacteria undoubtedly provide us with new information and prospects for its application. The author emphasized here the importance of a representative culture collection to deposit the existence of important lactic acid bacteria, in particular. Establishment of a representative National Culture Collection in Indonesia comes to its time.

Acknowledgement

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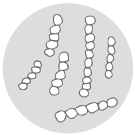


Future Challenges of Yeast and the Associated Microbes in *Ragi* and *Tape*

Atit Kanti

Introduction

Common perception for yeast is associated with bread and beer. The english word 'yeast' is based on words meaning "foam" and "to rise", produced during fermentation processes. Yeast is popular as fermentative ascomycetous fungi similar to *Saccharomyces cerevisiae* (Kurztman et al., 2011). Discovery of yeast belongs to basidiomycetes extending the definition and perception on the nature of yeast. Taxonomists generally define yeast as covering unicellular fungi that asexually reproduce by budding or fission. This definition may not fit to cover yeasts and those dimorphic filamentous fungi that produce abundant yeast-like growth. Yeast are those fungi whose growth is predominantly asexual as a result of budding or fission, and which do not form their sexual states within or upon a fruiting body. Phylogenetic analyses are able to distinct ascomycetous yeast reproduced by budding or fission and those belonged to *Euascomycetes* (*Pezizomycotina*). One exception is the genus *Eremascus*, which has unenclosed asci,

but budding cells are not formed. Molecular analyses are also able to differentiate basidiomycetous yeasts, which are often phylogenetically separated from the mushrooms and other taxa that form complex fruiting bodies. Ascomycetes or basidiomycetes yeasts are indicated by asexual reproduction via budding or fission, and their sexual states are not enclosed in fruiting bodies.

This paper discusses future challenges of yeast in *ragi* and *tape,* covers the role of yeast and associated microbes in fermentation process. Special focus is on the evaluation of yeast species diversity and physiological relevance of microbial group in *ragi*.

Yeast in Fermented Foods

Yeast is beneficial microbes intensively exploited for wide range of important industrial product covering food, medical, and agricultural sectors. Yeasts act on biotransformation processes in traditional fermentation such as *tape*, beers, cider, wines, sake, distilled spirits, bakery products, cheese, sausages, and other fermented foods. The advent of biotechnology is pioneering exploitation of yeast in fuel ethanol, single cell protein (SCP), feeds and fodder, industrial enzymes, and small molecular weight metabolites production. Progresses in modification of yeast genomic, gene expression of *Komagataella* (*Pichia*) *pastoris*, *S. cerevisiae*, and *Ogataea* (*Hansenula*) *polymorpha,* create industrial organisms for the heterologous production of enzymes and proteins, including protein pharmaceuticals. Archaeologists found evidences that alcoholic fermented beverages produced by yeast was consumed in Neolithic times (8,500–4,000 BC) in China, Iran, and Egypt, and other areas of the world (Cavalieri et al., 2003; McGovern, 2003). Babylonian stone tablets more than 6,000 years old illustrated beer and wine recipes (Ulber & Soyez, 2004; Legras et al., 2007). Many other fermented foods are also originated in Neolithic times, such as

African kafir and sorghum beer, pulque in Mexico, leavened breads in various regions and several fermented food and beverages from soy, rice, and vegetables (Beuchat, 1995; Boekhout & Robert, 2003; Steinkraus, 2004; Aidoo et al., 2006; Chilton et al., 2015).

Great variabilities of traditional fermented foods are found in the world, especially in Asian countries, covering wide varieties of ancient and modern food, prepared from raw or heated raw materials. To obtain unique aroma and taste, the process is mediated by microbes naturally occurred or inoculated. Several classifications had been used to categorize the wide spectrum of fermented food including the diversification of microbes, different food groups, and types of fermentation involved (Yokotsuka & Goto, 1955; Dirar, 1994; Steinkraus, 1996). Fermented foods are generally produced from plant or animal-based raw materials in combination with fungi or bacteria, which are either present in the natural environment, or intentionally added by human to obtain the desirable end products. According to Nout (2003), fermented food are typically unique and varied, according to regions due to the different of climate, social patterns, consumption practices and most importantly the availability of raw materials.

Rice wine is one of traditional fermented food that is very popular as alcoholic beverages in Asian countries in which yeasts play role in the fermentation process. '*Tape ketan*' is made from glutinous rice (black or white glutinous) rice (Figures 1.A). The rice should be throughly washed to remove the aleurone to get *tape* with sweet flavor. The taste and flavor of *tape* is greatly influenced by microflora of *ragi*, of which amylolytic and fermentative yeast play major role. *Tape ketan* is mostly made in Java and Bali, and now *tape ketan* has been made in various region thoughout Indonesia, albeit it is still mostly made by people with Javanese and Balinese root. The local tradition will conserve *tape* and its microflora as valuable genetic resources. Other than rice, cassava tuber (*Manihot utilisima*) plantations are

commonly encountered in tropical regions, supporting nearly half a billion people to meet their acceptable daily intake of calories and as a source of income. After African region with 48% of total production, Asian region contributes nearly 32% of the world cassava. Several very popular fermented food in Asian countries are made of fermented cassava tuber (Figure 1. B). Microflora in *ragi* (inoculants) especially yeasts liquefies cassava to physically soft and juicy (Figures 1.C).

A B C

Source of photographs: Kanti, 2016

Figure 1. Traditional Indonesian Fermented Food and Starter for Fermentation Process. A: *Tape* Glutinous Rice; B: *Tape* Cassava; C: *Ragi Tape*.

Alcoholic flavor and acidic taste are unique for certain product, depending on raw material, active yeasts microflora and environment at which *tape* cassava is produced. Unfortunately, *tape* cassava is perishable product, and therefore should be consumed immediately (within 3 to 4 days) after the optimum stage of fermentation. *Tape* cassava with special aroma, flavor, and unique taste are commonly produced homemade, or by small scale traditional manufacture.

Tape glutinous rice and *tape* cassava are consumed as a snack and commonly sold in traditional market, department stores, and commonly used as unique giveaway after visiting any *tape* producing region (Ganjar, 2003).Traditionally, *tape* is served during traditional ceremonies to celebrate birth of babies, marriage, and religious activities. In some eastern state of Peninsular Malaysia, *tape* from cassava is

very popular and used to prepare sweet delicacies. They can be consumed as such or used as an ingredient in homecooking and baking. There are many recipes with *tape* as main substrate. The *tape* cassava is sometimes baked as a cake (cheese *tape* cake) or cooked in coconut milk with palm sugar as a delicious snack.

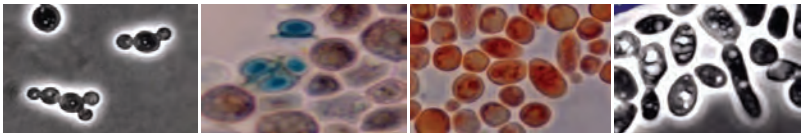
Other important alcoholic beverages that yeasts playing its role are local wines derived from the inflorescences of palm such as coconut, talipot palm and *nipah* (enau, giwang). The groups of palm are commonly found in Indo-China and Sri Lanka. Fresh *nipah* palm (*Nypa fruticans*) sap and neera (sap obtained from by tapping the unopened spadix of the coconut palm are popular beverages in the region. For Muslim consumers, palm juice (fresh saps) are consumed within 2 days after tapping as it is highly susceptible to spontaneous fermentation to produce alcohols and acetic acids. Fermented palm saps can also be used to produce alcohol, vinegar or alcoholic beverage such as palm wine. The fermented beverage is called "*panam culloo*" in Sri Lanka, "*tuba*", "*soom*" in the Philippines, "*nuoudua*" in Vietnam, "*arak*" in Indonesia, and "*tuak*" (tuack) or toddy in Indonesia, Malaysia, India, and Bangladesh (Lee & Fujio, 1999). Palm wine is obtained by the natural fermentation of palm sap and collected through the tapping of unopened inflorescence. Palm wine has mild alcoholic flavor, sweet in taste, vigorous effervescence, and milky white in color as it contained suspension of numerous bacteria and yeast. Palm wine from coconut flower juice is most popular among Southeast Asian regions. A community survey on the non-Muslim Balinese village in Indonesia showed there were approximately 40% excessive consumption of locally produced palm wine in 1990 (FAO & WHO, 1996).

The *ragi tape* is composed of several functional microbial groups mediating the bioconversion of raw or cooked material producing unique physical, chemical, and aesthetic fermentation product. The active microbial group of *ragi* is a major determinant influencing the quality and hygiene of desirable product.

Yeast and Associated Microbes in *Ragi* and *Tape*

To explore species diversity and to verify active microbial group of *ragi*, the Indonesian Institute of Sciences has been conducting research on Indonesian fermented food since the 1970s. The focus of the work is investigating the microflora in *ragi* to see consistency of microflora and to verify their taxonomy affiliation. The intensive work was started with the Institute of Mycological Collection (IMI), focused on introducing microbiological preservation technique to enable safely preserved the isolated culture from natural resources in 1994. Then, our work was continued with Dr. Hiroshi Kuriyama and Dr. Takema Fukatsu of Advanced Industrial Science and Technology (AIST), Japan in 1995, focusing on physiological characterization of microflora in *ragi*. We further expanded the microflora study, focused on taxonomy studies under supervision of Prof. Dr. Kazuo Komagata of Tokyo University of Agriculture, Japan (1998–2000). We were introducing chemotaxonomy analyses to verify species richness, physiological characters, and metabolites properties of yeast in traditional *ragi* and *tape*. From the last study, the important microflora in *ragi* and *tape* was identified, *Candida* sp.*, Candida guillermondii, Candida parasilopsis, Candida pelliculosa, Candida rugosa, Pichia anomala, S. cerevisiae*, Basidiomycetous yeast, *Saccharomycopsis* sp., *Saccharomycopsis fibuliger*, *Debaryomyces hansenii* (Table 1) (Kanti, 2000). This was the first report of yeast found in *ragi* and *tape* which showed diversity of *Candida* group. Several species of *Candida* were also found in other Indonesian fermented food such as red *oncom* and black *oncom* (Kanti, 2000). Our study showed great diversity of yeast in *ragi* and *tape*. Other species commonly found in *ragi* and *tape* as reported by previous study was *S. cerevisiae* and *Sm. fibuliger*. The presence of *P. anomala* was also reported by Dwidjoseputro and Wolf (1970), Ko (1972) and Kreger van Rij (1984). Meanwhile, *D. hansenii*

was merely reported in this study. Figure 2 shows the morphological characteristics of yeast used in Kanti (2000).



A B C D

Source: Kanti, 2000

Figure 2. Morphological Character of Yeast Isolated from *Ragi Tape*. A) Budding Process of *Candida*; B) Ascospore of *Pichia anomala* and Hat Shape Ascospore; C) Vegetative Stage of *Saccharomyces cerevisiae*; D) Vegetative Cell of Basidiomycetous yeast. = 5 µm

Table 1. Yeast and Associated Microbes in Indonesian Traditional Fermented Foods

Species commonly found in Indonesian traditional fermented food	**Major quinone**	**Sources**	**This study**	**Other studies**
Candida sp.		*ragi*, *tape* cassava	√	Hadisepoetro et al., 1979
Candida guillermondii	CoQ: 7	*ragi*, red *oncom*, *tape* cassava	√	
Candida parasilopsis		*tape* cassava, red *oncom*, black *oncom*	√	
Candida mesenterica	CoQ: 9	Black oncom	√	
Candida pelliculosa		red *oncom*, black *oncom*, *ragi*	√	Ardana & Fleet, 1989
Candida tropicalis	CoQ: 9	red *oncom*	√	
Candida reukaufii		black *oncom*	√	

Species commonly found in Indonesian traditional fermented food	Major quinone	Sources	This study	Other studies
Candida solani	CoQ: 7	black *oncom*, red *oncom*	√	
Candida glabrata		*ragi*		Hajar et al., 2011
Candida rugosa		*ragi tape*	√	
Pichia anomala		*ragi tape*, *tape* glutinous rice, *tape* cassava	√	Kreger van Rij, 1984. Dwidjoseputro & Wolf, 1970; Ko, 1972; Kuriyama et al., 1997
Saccharomyces cerevisiae	CoQ: 6	*ragi*, *tape* glutinous rice	√	Hajar et al., 2012; Ardhana & Fleet, 1989
Basidiomyceteus yeast		*ragi*	√	
Saccharomycopsis sp.		*ragi tape*	√	
Saccharomycopsis fibuliger	CoQ: 8	*ragi*	√	Saono *et al*., 1974; Steinkraus, 1983; Kuriyama et al., 1997
Trichosporon pullulans				
Amylomyces rouxii		*tape* glutinous rice		Saono et al., 1974; Dwidjosepoetro & Wolf, 1970; Ko, 1972; Ellis et al., 1976; Steinkraus, 1983; Ardhana & Fleet, 1989
Rhizopus sp.				Saono et al., 1974
Mucor sp.				Saono et al., 1974
Aspergillus sp.				Saono et al., 1982

Species commonly found in Indonesian traditional fermented food	Major quinone	Sources	This study	Other studies
Saccharomycopsis malanga				Hesseltine et al., 1976; Hesseltine et al., 1988
Debaryomyces hansenii	Q-8	*tape* glutinous rice	√	
Streptococcus sp.		*ragi*		Suprianto et al., 1989
Rhizopus sp.		*ragi*		Suprianto et al., 1989
Bacillus sp.		*tape* glutinous rice		Ardhana and Fleet, 1989.
Acetobacter sp.		*tape* glutinous rice		Ardhana and Fleet, 1989.

Source: Kanti, 2000

Note: C: *Candida*

Identification of Yeast in *Ragi* and *Tape* Using Current Methods

In *tape* production, yeasts play an essential role in biochemical changes, especially in starch hydrolysis. These changes result in the production of maltose and glucose, which renders a sweet taste to the product. Moreover, yeast is a ferment part of the sugar to alcohol and some are to organic acid (Flores et al., 2015). With this information, the study of yeast is essential to give a better understanding on Indonesian fermented foods.

Yeasts are defined as unicellular fungi reproducing by budding or fission. The knowledge of yeast taxonomy has been improved greatly and rapidly in recent years. Despite this advancement in the systematic

of yeast, detailed work of yeasts found in Indonesian fermented food are rather limited. Therefore, there is still a great task that has to be undertaken to identify yeast found in *ragi*. To deepen the authors' knowledge on yeast diversity in *ragi tape*, they collected *ragi* from several *tape* producing area in Java (Table 2), including 11 strains isolated from new *ragi* samples and 31 strains obtained from *ragi* sample that were isolated in 1974 and preserved in Indonesian Culture Collection (InaCC). The focus of the study was evaluating species richness of old *ragi* and new *ragi* type. The data of microflora in old *ragi* was obtained from the previous work conducted by Saono et al. (1974), and the new *ragi* type was collected from prominent area for *tape* production.

Table 2. Isolate and *Ragi* Sources (Kanti, 2000)

Strain no.	Source	No. of strains
Bg/3,Bo/1.2,Bo/2.2, Bo/1.1	*Ragi tape*, Bogor	4
CII/5.1, CI/3.2, CI/4.2 CII/3.1, CII/4.1	*Ragi tape*, Cianjur	5
Cm/1	*Ragi tape*, Cimande	1
Yg/1	*Ragi tape*, Yogyakarta	1
315,354-1,354-2,381	*Ragi tape*, Bandung	4
366,376	*Ragi tape*, Bogor	2
364-1, 364-2, 360,361	*Ragi tape*, Cianjur	4
313	*Ragi tape*, Garut	1
383,384	*Ragi tape*, Karawang	2
367,368	*Tape* cassava, Bandung	2
321-1,321-2,337	*Tape* cassava, Bogor	3
340,358	*Tape* cassava, Cianjur	2
371	*Tape* cassava, Karawang	1
311,323	*Tape* cassava, Sukabumi	2
369,370	*Tape* cassava, Bandung	2
386	Red *oncom*, Bandung	1
307-1,307-2,308-1,308-2	No data	4
	Total	42

The yeast isolations were based on conventional approach using serial dilution, and taxonomy study was done through following chemotaxonomy analyses as recommended by Kurtzman et al. (2011). In total, 42 strains were used in this study. Yeasts grouping were done through conventional morphological, physiological, and chemotaxonomy analyses. The microflora of *ragi* are divided into: ascomycetous, basidiomyceteous, and imperfect yeast based on the G+C content and DNA-DNA relatedness between Ascomyceteous and imperfect yeast, the microflora of *ragi* are divided into three clusters.

The G+C content and DNA-DNA relatedness value between Ascomycetous and imperfect yeasts were isolated from new *ragi* samples was in the range of 70–100% DNA relatedness value. Three strains showed high homology value with the type strain of *P. anomala*; 2 strains with *Sm. fibuliger* type strain; and 1 strain with *C. parapsilosis*. Three strains could not be identified until species level. The G+C content percentage confirmed the identity of the isolates.

One strain previously classified as *C. parapsilosis* and 9 strains previously classified as *C. pelliculosa* were identified as *P. anomala* owing to their high DNA relatedness with the type strain of this species. The G+C contents supported this identity. This results agreed with past report made by Kurtzman et al. (2011) citing *C. pelliculosa* as an anamorph stage of *P. anomala*. The previous observation which seems to be ascospore was not observed since the spore observation technique was not yet well-developed. However, one strain formerly identified as *C. pelliculosa* did not hybridize with the previous mention of 10 strains and formed a separate homology group. Further study was obviously needed to identify this strain up to certain species level.

The G+C content and DNA-DNA relatedness between imperfect yeast, however, showed different pattern which was the G+C content and DNA-DNA relatedness between ascomyceteous yeast. Two strains of imperfect yeasts were identified as *C. guilliermondii*. Two strains

formerly identified as *Candida humicola* and one strain formerly identified as *Candida intermedia*, respectively were identified as *Candida parapsilosis*. Finally, two strains of *Candida brumptii* and *Candida catenulata* were identified as *C. catenulata*. Again these identifications were supported by G+C content data.

It is interesting to note that the eight strains had been previously classified different from one distinct homology species group, based on the DNA relatedness data. This group did not hybridize with any type strain from species of yeasts used in this study. This homology group remained to be unidentified. This group requires further identification with 18S ribosomal DNA sequence. It is important to note that most of the previous and current identifications were not tally. This can be explained by the fact that previous identification method relied on a classification system that used only traditional phenotypic data as main bases. Chemotaxonomic data and molecular data were not considered, hence the high possibility of error in identification. It is considered that identification made in this study provided reliable information on the identity of yeasts found in *ragi*.

Comparison of the different yeasts isolated from old and new *ragi* observed that two species namely *C. parapsilosis* and *P. anomala* were present in both *ragi* samples, suggesting that the two yeasts were standard *ragi* yeasts. The other species shown were found either in the old or in the new *ragi* (Table 3). This showed that yeasts found in *ragi* were quite diverse, and it also indicated the lack of standard method for *ragi* production. *Sm. fibuliger* was found in newly produced *ragi*, and it was reported to play important role in the fermentation process particularly in the hydrolysis of the starch into glucose and maltose. However, *Sm. fibuliger* was not found in old *ragi* sample. The reason was not fully understood. It might be that they were not isolated, or that they might have died during subculturing or during preservation or they were not deposited in our culture collection. The diversity

Table 3. Occurance of Yeasts in *Ragi* and *Tape* (Kanti, 2000)

Source	Identified as								
	C. glabrata	*C. guillermondii*	*C. parapsilosis*	*C. rugosa*	*C. utilis*	*P. anomala*	*Sm. fibuliger*	*Basidiomycetous*	*Candida sp.*
Ragi tape, Bandung	■■	■							■
Ragi tape, Bogor				◆◆		■	◆◆		◆
Ragi tape, Cianjur			◆			■■◆◆		◆	■
Ragi tape, Cimande								◆	
Ragi tape, Garut		■							
Ragi tape, Karawang									■■
Ragi tape, Yogyakarta						◆			
Tape cassava, Bandung						■■			
Tape cassava, Bogor			■■						
Tape cassava, Cianjur									■■
Tape cassava, Karawang						■			
Tape cassava, Sukabumi			■		■				
Tape cassava, Bandung						■■			
Red *oncom,* Bandung									
	■	Old ragi	◆	New ragi					

implying that there was no standard method for *ragi* production. It was therefore, opined that the standardization of *ragi* production process was necessary to produce standard fermented foods in Indonesia.

Morphology of Vegetative Cells, Assimilation, and Fermentation Pattern of Yeasts in Traditional Fermented Food

Candida guilliermondii

This yeast species (teleomorph *Meyerozyma* (Pichia) *guilliermondii*) was isolated from *ragi* and other environmental sources. Morphologically, pseudohyphal formation varied in abundance from strain to strain. True hyphae, however, were not produced by this species. The blastoconidia of *C. guilliermondii* were found in short chains or clusters.

This species followed these physiological properties: Fermentation of Glucose +, Sucrose +. Assimilation of Glucose +, Inulin +/w, Sucrose +, Raffinose +, Trehalose +Maltose +, Melezitose +, Soluble starch -, Cellobiose +, Salicin +, D-Xylose +, Ethanol +, Glycerol +

Candida parapsilosis

This yeast was commonly found in *ragi tape* and the physiological role of this species in traditional food could be fermentation of a reducing sugar end product of saccharification. This species had the following physiological characters:

Fermentation of glucose (+). Assimilation of Glucose +, Sucrose ++, Trehalose +, Maltose ++, Melezitose ++, Soluble starch - , Salicin ++, L-Sorbose +, L-Rhamnose +, D-Xylose +

Candida mesenterica

This species was not common in *ragi,* but some species were found in 'oncom'. This species were generally different from yeast of *ragi*, having weak ability to ferment glucose. The physiological characters of this species were: Fermentation of Glucose -/s, Galactose -, Sucrose -, Maltose -. Lactose -. Assimilation of Glucose +, Sucrose +, Trehalose +, Maltose +, Methyl-D-glucoside +, Soluble starch –,

Cellobiose +, Salicin +, L-Sorbose +, Ethanol +, Glycerol +, Erythritol +, Succinate +.

Candida pelliculosa

This species had the ability to ferment Glucose, Galactose, and Sucrose.

Candida tropicalis

This species was not commonly found in *ragi*, but this strain had wide physiological properties: Fermentation of Glucose +, Galactose +, Maltose +. Assimilation of Glucose +, Ethanol +, Galactose + , Ribitol v, Lactose – , Trehalose +, D-Mannitol +, Maltose +, D-Glucitol +, Soluble starch + , Succinate +, D-Xylose +, Hexadecane +.

Candida solani

This yeast were commonly found in *ragi tape,* and their role could be for fermentating of reducing sugar. The physiological character of this species were:

Fermentation of Glucose + Assimilation of Glucose +, Sucrose +, Trehalose +, Maltose +, Melezitose +, Soluble starch -, D-Xylose +.

Candida etchellsii

This species was osmophilic, being isolated from fermenting cucumber brine, lemon concentrate, and *shoyu* fermentation. According to Yokotsuka & Goto (1955), *Torulopsis etchellsii* and *Torulopsis versatilis* contributed to the flavor in *shoyu*. It fermented only glucose and maltose and did not form pseudomycelium. It assimilated nitrate, glucose, and maltose but not sucrose, lactose, cellobiose, or trehalose. Characteristically for the genus, it did not produce ascospores and had multipolar budding.

Saccharomycopsis fibuligera

Sm. fibuligera was found in *ragi* and also commonly found in starchy substrates containing amylum. This species had the ability to ferment glucose. This species was grow in the various kind of carbon sources Glucose, Sucrose, Soluble starch, Cellobiose, Salicin, Ethanol, and Glycerol. *Sm. fibuliger* was well-known amylolytic yeast and commonly found in *ragi*. Some strains were arepectolytic, and they were able to grow and actively co-hydrolyze pectin in the presence of *Candida utilis* which required different incubation condition. Amylases produced by *Sm. fibuliger* had been studied with the intent of identifying the kinetic mechanism and product distribution, and modelling the production of D-glucose during starch hydrolysis (Chen & Liu, 2014).

The original isolate of this species was made from "chalky bread". Strains had been isolated from Chinese yeast, *ragi*, and *tape*. It assimilated sucrose and also maltose, cellobiose and soluble starch. Ascospores were hat-shaped with two to four spores per ascus. Often no conjugation of cells was observed. Characteristic of the genus was the production of long oval to cylindrical vegetative cells with pseudomycelium. Although the yeasts appeared to be the important ones in oriental food fermentation, there might be others which were misidentified or not yet reported.

Saccharomycopsis malanga

This was an important food yeast of the Orient, having been isolated repeatedly from *ragi* and Chinese yeast. It was represented by a number of isolates in the ARS Culture Collection at the Northern Regional Research Center. Originally described as *Hansenula malanga* in isolates from *ragi*, it was characterized by the extracellular production of 3-d-hydroxy-palmitic acid, which was not encountered in *Sm. fibuligera*. It contained two hat-shaped ascospores and produced asci

that often rupture at maturity. Fermentation of glucose was slow and weak, as it was also on maltose. It was separated from a second species of *Saccharomycopsis*, *Sm. fibuligera*, because it did not assimilate sucrose.

Saccharomyces cerevisiae

Most individuals tend to think of *S. cerevisiae* as being solely monomorphic. However, it did form pseudohyphae and chains of budding yeasts under the appropriate conditions. For example, nitrogen-poor media induced pseudohyphal growth in *S. cerevisiae* (Gagiano et al., 2002). This alternate growth form of *S. cerevisiae* was the result of a well-developed nutritional sensing mechanism that impacts cellular morphogenic programs (Gimeno et al., 1992). Like other species of *Saccharomyces*, vegetative reproduction was solely made by multilateral budding with no true mycelium. Ascospores were spherical and numbered one to four per ascus. Cellobiose and salicin were not utilized; soluble starch was not fermented, but raffinose, sucrose, maltose and galactose.

This species was commonly found in *ragi*, and very popular in biotechnology industry. Their growth tolerance to alcohol was one of most the unique physiological characteristics of this strain. Fermentation: Glucose +, Sucrose +, Maltose +, Lactose -, Raffinose +, Assimilation: Glucose +, Sucrose +, Raffinose +, Trehalose +, Maltose +, Soluble starch -, Ethanol +.

Sm. cerevisiae, the common species, had been reported from various fermented food. This species occurrence was reported in *papadam* (a condiment) and *janjabi waries* (fermented leguminous grain paste), both are in India (Kuriyama et al., 1997; Jespersen, 2003; Kurztman et al., 2011).

Functional Microflora in *Ragi* and *Tape*

Yeasts

Candida krusei, Candida kefyr, and *Candida glabrata* played important roles on flavour formation, alcohols and esters, and stimulation of lactic acid bacteria, thus increasing lysine availability. *C. rugosa*, *Issatchenkia tropicalis* were responsible for starch modification and flavour generation. *D. hansenii* was flavour generator and stimulated lactic acid bacteria (LAB), while *Endomycopsis fibuligera* was amylolytic starters, alcohol, and flavour formation. *Hansenula* sp. was responsible for alcohol and flavour formation (Steensels et al., 2014).

P. anomala was responsible for flavour formation and stimulated growth of LAB. *S. cerevisiae* was responsible for alcohol and ester formation and interaction with LAB. *Trichosporon pullulans* was responsible for flavor generation and stimulated growth of LAB.

Filamentous Fungi

Amylomyces rouxiiragi were amylolytic starters glucose released from starch by amyloglucosidase. *Aspergillus* sp. had amylolytic, proteolytic activity. *Mucor circinelloides*, *Mucor rouxii*, *Mucor indicusragi* were responsible for enzymic transformation and flavour production. *Rhizopus microsporus*, *Rhizopus oligosporus*, *Rhizopus oryzae koji* were amylolytic starters, texture formation, enzymic transformations, and vitamins producer.

Bacteria

Acetic acid bacteria contribute to flavour by formation of acetate esters. *Bacillus* sp. produced enzymes degrading starch, proteins, lipids, and phosphates. *Enterococcus faecalis* was amylolytic starters and contributed to flavour by mixed fermentation *Lactobacillus brevis, Lactobacillus curvatus, Lactobacillus buchneri* to produce acid, flavour formation, and texture improvement. *Lactobacillus plantarum*

produced acids and some strains degraded starch. *Leuconostoc mesenteroides* produced gas (leavening) and flavour. *Pediococcus acidilactici* and *Pediococcus pentosaceus* were amylolytic starters and produced acid, while *Pediococcus dextrinicus* produced acid.

Conclusion

Fermented food is now a part of the culture of modern society and posseses a natural and healthy image. In the case of traditional rice wine, ethanol as a major product of alcoholic fermentation has been implicated with human intoxication. The alcoholic brews is a part of tradition for local people in several occasion and amount of alcohol consumption are difficult to be estimated. The production processes of this traditional wine are unique for every area and representing traditional forms of alcoholic food products, lacking of sufficient hygienic precautions. Besides, knowledge about the significance of the presence of the metabolite and their chemical interaction is still insufficient. Furthermore, little scientific literature is found to date relating to health relevant constituents and contaminants in these alcoholic beverages and foods. Enormous varieties of traditional food are found easily in the market place and in the street vendors. Indonesia is considered one of the most richest nations in fermented foods. The authors have observed inconsistency of microorganisms in *ragi* and *tape* production. For example, *C. guillermondii* and *P. anomala* are consistently discovered in *ragi tape*, whereas *C. parasilopsis* and *S. cerevisiae* are commonly observed in *tape* implying that environment at which *tape* is produced influencing the dominance of microbes in *tape*. The technology of traditional fermentation is simple and without sufficient hygienic precautions, hence natural contamination is always possible. Casava's *tape* technology which is perishable in nature should need better attention on its shelf life and quality control.

Hygienic *tape* production protocol should be disseminated and quality of inoculants *ragi* has to focus on quality and hygiene improvements of traditional fermented food. The InaCC can play as a mediator in supplying well-identified and physiologically maintained microbes for helping local people and small scale enterprises. The InaCC serves industries exploiting full potential of microbial collections.

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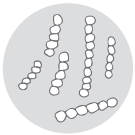


Oligosaccharides, Its Related Microbes and Enzymes

✍ Yopi

Introduction

Oligosaccharides are known as main component of functional food. They have potential value as prebiotic, which is important as a specific carbon and energy source for bifidobacteri (Gänzle & Follador, 2012). The term glycoscience and glycotechnology are becoming popular to improve the value of oligosaccharide's function in food industry, beverage, and pharmacy. The application of enzymes is a key element in the production of oligosaccharides. Oligosaccharides industries are actually the unification process of enzymes invention and advancement of production tehnique. The production of oligosaccharides begins to start when several potential enzymes with specific function are discovered. Through rapid development on enzyme technology, the opportunity to produce new oligosaccharides from starchy rich materials and other carbohydrates is widely opened. Mostly, the enzymes are involved in the process of hydrolysis and transglycosilation (Nakanuki, 1998; 2003). Study on the development of new oligosaccharides from several types of biomass material has been expanded rapidly.

New type of oligosaccharides, such as arabinogalacto-oligosaccharide (Van Laere et al., 2000), agar-oligosaccharide (Wang et al., 2004), and mannan-oligosaccharide (Kurakake et al., 2006) may be produced by enzimatic processes.

Indonesia is rich in microbial diversity. The opportunity to find potential indigenous microbes for the production of enzymes to be used in the production of new types of oligosaccharides is widely opened. It is our objective to illustrate our work on the exploration of enzymes and its application in the production of oligosaccharides from diverse organic biomass abundantly available in Indonesia.

Oligosaccharides

Oligosaccharides are oligomers composed of 3–10 simple sugars. Oligosaccharides can be found naturally in bananas, honey, milk, and vegetables such as garlic, onions, artichokes, etc. Oligosaccharides steadily become an important commodity because the demand of oligosaccharides in the food industry is increasing, along with high assessment for the functional side of saccharide in relation to its health benefits for living beings as bioactive. Basically, saccharide (sugar) has a primary function as a source of energy for living things and a secondary function associated with flavors and physical properties. In the wake of the development of oligosaccharides in the early 1970s, researches on the function of saccharides, such as a tertiary effect of improving the microflora in the colon, the effects of high mineral absorption, increased immunity and anti-allergic, were done. In addition to that, the application of the production of some types of oligosaccharides was also performed. In the production of these oligosaccharides, all of them could not be separated from the use of the enzymes. Basically, the oligosaccharides industry is the combination of the invention of several enzymes that are crucial in the

production of oligosaccharides and technique production. Oligosaccharides are still being developed, including those that are still in the research study. The number has increased steadily, but only 25 types that have been produced and used in the food industry. Almost all oligosaccharides production used hydrolase and trans-glycosylase enzyme. Some oligosaccharides were produced directly by extraction methods, such as oligosaccharides for soybean (Okada & Nakakuki, 1992; Nakakuki, 1993).

The utilization of enzymes dominates various types of oligosaccharides production. The important focus of the enzyme utilization is its high specification of reaction that can effectively produce the required oligosaccharides types. With a good catalyst, the reaction does not require special equipment such as when using a chemical reaction method. In addition to the safety for consumer, oligosaccharide production using enzyme can be performed more easy and efficient. The oligosaccharides production utilize three type of reactions: hydrolysis reactions, transfer, and condensation (Crout & Vic, 1998). There are also purification and filtration methods, degradation using acid as in the production of oligosaccharides from soybean (Kim et al., 2003).

The major material source for the production of oligosaccharides is carbohydrates. Several types of carbohydrates are available in Indonesia, such as starch carbohydrate and non-starch carbohydrates like cellulose, hemicellulose, agar, chitin, and others. These types of carbohydrates can be degraded by microbes producing specific enzyme that can hydrolyze carbohydrate into simple sugars that will be used as a source of energy. Non-starch carbohydrate sources can be used as targets for non-digestible oligosaccharides production (Mussato & Mancilha, 2007). Horticulture industry, plantation, and Indonesian marine field will be able to produce various types of non-starch biomass, but their uses are still limited. Some of the specific carbohydrates that are found in tropical environment are biomass from oil industries,

minor tuber, marine biomass such as agar, alginate and carrageen. The uses of biomass are still few in number so it is necessary to develop the derivative products from that biomass; one method is by the production of oligosaccharides in biological method, which focuses on the use of microbial enzymes for hydrolyzing the biomass. Local microbial biodiversity is very diverse so it will be difficult to get the potential microbial degrading non-starch biomass.

Sugar Metabolism System

Basically, every bacteria, molds and yeasts has specific sugar metabolism system. Among the types of microbes, bacteria groups have still maintained and have the metabolic system for various types of sugar. Besides monosaccharide, the bacteria have the ability to insert a disaccharide or oligosaccharide into cells. For example, *Escherichia coli* has a maltose system to insert the maltose into the cell. In the maltose system, the two regions in *E. coli* genome contained 9 proteins/enzymes that were involved in the process of maltose insertion into the cell, then it changed into maltoheptaose (Boos & Shuman, 1998). The *E. coli* also had a trehalose system and other sugar metabolic system (Boos et al., 1990). Unlike *E. coli*, Gram-positive bacteria were reported to have cyclodextrin system consisting of 10 types of proteins such as *Klebsiella oxytoca* (Fieldler et al., 1997). In addition to proteins that acted to bind and/or transport proteins, there were two main enzymes; cyclodextrinase for hydrolyze cyclodextrin and cyclodextrin glycosyltransferase that produces cyclodextrin from other simple sugars. Cyclodexrin metabolism system in *K. oxytoca* was spesific only for inserting the cylodextrin (Pajatsch et al., 1998). The similar system reportedly contained in *Thermoactinomyctes vulgaris* R-47, which had a maltose-cylodextrin system that involving a single gene operon consisting of protein binder and transporter, enzyme

amylase, and glucoamylase. In this system, a binding protein had wider binding activity, such as for maltose, maltotriose, maltotetraose, and cyclodetrin. With this system, the *T. vulgaris* had high competitive value if the environment only had maltose and cyclodextrin (Yopi et al., 2002). There was other sugar metabolism reported in *Bacillus* sp. A2-5a that the products of the gene clusters were engaged in the metabolism for cyclodextrins (Ohdan et al., 2000). In *K. oxytoca* and *Bacillus* sp. A2-5a, genes of cyclodextrin glucanotransferases had been found in the flanking regions of the genes of clyclodextrin binding protein, but the *T. vulgaris* seemed to have cyclodextrin-utlization system, although it was unclear that *T. vulgaris* had a cyclodextrin glucanotransferase gene.

Sugar metabolism system was more widely reported from some types of bacteria that had metabolic system as a specialized tool to defend themselves in an environment full of dynamics. All the microbes could use simple sugars such as glucose, but not all bacteria had sugar metabolism system for a complex sugar, such as trehalose, maltose, cylodextrin, and oligosaccharides (Boos & Shuman, 1998). Given these diverse systems, the bacteria became more competitive and were able to survive. In addition to the presence of enzymes to turn the monosaccharides into oligosaccharides, they also gave a distinct advantage to the bacteria, because the enzymes could function in the processes of oligosaccharides production and hydrolysis that were usually specifically presented in bacteria, adhered to the sugar metabolic system.

The focus on sugar metabolism system becomes an important issue when it is used for developing the enzyme product and oligosaccharides. An example is the use of oligosaccharides as a prebiotic compound, that is a special food for microflora probiotic bacteria in stomach or probiotics contained in commercial yogurt products. Oligosaccharides products are specifically metabolized by the probiotic

bacteria, whereas the *E. coli* and other microbes could not use those oligosaccharides. In another case, a group of enzymes that can be used for the production of oligosaccharides are in the bacterial metabolism system, e.g. cyclodextrin glycosyltranferase, endo-amylase and others. In the last two decades, many discoveries have been reported dealing with functional enzyme for saccharide production, including a phenomenal issue for industrial scale such as the development of trehalose from starch carbohydrate material by using the two types of enzymes, namely malto-oligosyltrehalose synthase and malto-oligosyltrehalose trehalohydrolase (Michio et al., 2004). Hayashibara, which is a big company in Japan, has managed to find microbes that can produce up to 2 type of enzymes related to trehalose production from starch. By using these 2 enzymes, trehalose can be produced from starch product with a yield of about 80%. With a more efficient production system, trehalose becomes an affordably-priced material for foods, cosmetics, and pharmaceutical industries' applications.

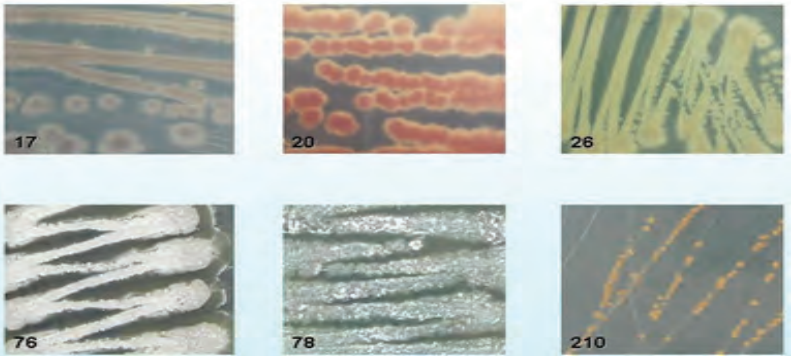
Exploration of the New Oligosaccharide

The prospects of new enzyme for the development of oligosaccharide type are new challenges. The Indonesian biodiversity of carbohydrate source materials and microbial biodiversity are amazing, as sources of wealth. It will be able to support the process of finding the enzyme-producing microbes that can be used for the production of new type oligosaccharides that have yet to be developed.

Introduction of microbial diversity is the first step to obtain the enzyme that can be used in oligosaccharides production. The most important in the development of these oligosaccharides is by making the oligosaccharide production system effective. It would be difficult to apply if the process requires a high production cost. There are two ways to develop a new oligosaccharide production using enzymes.

The first way is the development of methods to modify the terms of existing enzyme reactions. In this process, the development is conducted by changing the type of material, the material composition, the concentration of the material, the enzyme characters, and the reaction conditions. These processes will give higher the probability of the development of new oligosaccharides. The second way is to find new types of natural enzyme. In this case, the common practice is firstly by isolating the desired microbes. Industrial-scale production needs enzymes in large quantities. Due to that, the next step is how to increase the production of enzymes by microbes. By using microbes, breeding strain microbes to increase the enzyme production could be done in a short time; examples are a new fermentation process for large-scale production of human milk oligosaccharides by metabolically engineered bacteria (Priem et al., 2002) and a report related to strategies for oligosaccharides production such as sialylated oligosaccharides using metabolically engineered bacteria (Endo & Koizumi, 2000).

In the focus of the utilization of microbial biodiversity and its application for the production of oligosaccharides, there are some studies that have been conducted by researchers from Indonesian Institute of Sciences (LIPI). One of them is the screening for mannanase producing microbes for mannan biomass hydrolysis and production of manno-oligosaccharides. Mannan is a source of biomass besides cellulose and xylan, which are still not widely used and specifically available in our country. The main source of heteromannan biomasses in Indonesia are palm kernel cake and coconut. In addition, there is a tuber named Porang which contain high glucomannan. Porang plant (*Amorphophallus muelleri* blume), a typical local tuber that grows wild in the woods and similar with konjac root (*Amorphophallus konjac*), is popular in China and Japan (Chua et al., 2010). Glucomannan is a dietary fiber that plays an important role in human health. This



Source of photographs: Collection of Laboratorium Biocatalyst & Fermentation Research Center for Biotechnology LIPI in 2006.

Figure 1. Profile of 6 potential isolates for cellulase and hemicellulase production screened from 488 isolates belong to biotechnology culture collection (BTCC).

dietary fiber is the main polysaccharide that could be hydrolyzed into oligosaccharides enzymatically. The porang tubers contained glucomannan up to 70% of the dry weight, while palm kernel cake contained galactomannan which is quite high at around 30% (Masashi et al., 2008). Hydrolysis of mannan carbohydrate could be performed more efficiently by using the endo-β mannanase and produce oligosaccharides with a variety of sizes. Screening of mannanase activity from approximately 488 local microbial collections of Biotechnology Culture Collection (BTCC) LIPI obtained at least 6 isolates that have high activity in mannan substrate degradation, 4 isolates of *Streptomyces* sp., *Kitasatospora* sp. and *Streptacidiphilus lutealbus* (Yopi et al., 2006) (Figure 1 & Table 1). These isolates had an interesting character because the isolations were from many areas in Indonesia and they had unique characters that could metabolize different types of saccharides such as glucose, mannose, raffinose and others. These potential strain microbes could also live in medium containing biomass

Table 1. Information of 6 Potential Isolates of BTCC

No. Isolate	Code Isolate	Isolation place	Source	Microbial identification
17	ID05-A0260	Enrekang	under bamboo, near flow	*Streptomyces cyaneus*
20	ID05-A0291	Enrekang	under Cotton tree	*Streptomyces purpurascens*
26	ID05-A0323	Enrekang	Coffea sp.	*Streptomyces alboniger*
76	ID04-0555	Cibinong	near dried pond, *Penisetum* sp. Area	*Kitasatospora* sp.
78	ID04-0571	Cibinong	Star fruits area	*Streptomyces tumescens*
210	ID05-A0975	Enrekang	Cacao	*Streptacidiphilus lutealbus*

Source: Widyastuti & Ando, 2009.

of raw material such as palm kernel cake, coconut cake, and husk. This experiment showed that there were several types of enzyme that could be produced at once and also demonstrated that the potential microbes could be resistant to inhibitors that were exerted from the biomass and the enzyme production in its stable condition (Table 2).

There were 3 strain of microbes from those 6 potential strains known to have the ability to produce 3 types of enzyme: mannanase, cellulase, and xylanase which were suitable for the cellulose and hemicellulose biomass degradation processes. Strain No. 76 (*Kitasatospora* sp.) which had the highest mannanase enzyme activity than other isolates were selected for fermentation process using raw biomass. The initiation of fermentation used the substrate concentration of 2.5% palm kernel cake (PKC) and the highest enzyme activity reached at 24 hour by using stirrer fermentor. The oligosaccharide was formed

Table 2. Analysis of the 6 Potential Isolates BTCC for Degrading Commercial and Raw Biomasses. Number with plus (+) shows the activity of mannanase, xylanase and cellulase.

No. Isolate	Mannan	Xylan	Cellulose	Palm kernel cake	Copra cake	Hay
17	++	+++	+++	++++	++++	++++
20	++	+++	+++	+++	+++	+++
26	++	-	-	-	-	-
76	++++	++++	++++	+++++	+++++	+++++
78	++	-	-	-	-	-
210	++	-	-	++	-	-

Source: Unpublished data of Laboratorium Biocatalyst & Fermentation Research Center for Biotechnology LIPI, 2006.

in 2 L scale fermentation along with the high activity of enzymes produced by the fermentation.

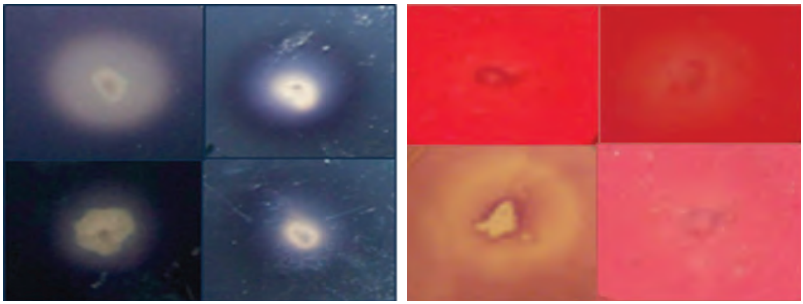
The optimation of the fermentation condition gave results that temperature and pH did not affect the production of oligosaccharides during the fermentation process. The best concentration of PKC fermentation in oligosaccharide production process was 2.5% with 200 rpm agitation. The first fermentation product for 24 hours of incubation was monosaccharide and after 28 hours of fermentation of the oligosaccharide products such as disaccharides and trisaccharides were formed. Optimum production of oligosaccharides occurred at 44 hours and after that oligosaccharides were degraded on a regular basis. The results showed that the production of oligosaccharides from PKC could be done even if the percentage of production was not high (Yopi et al., 2007; Yopi et al., 2010)

Utilization of local Indonesian tuber for the production of oligosaccharides that could serve as a component of functional foods was expected to increase the economic value of the local tubers. By

using the mannanase enzyme which was produced by strain *Kitasatospora*, the result of analysis and reaction time between mannanase and mannan substrate of porang tuber powder for 24 hours showed that oligosaccharides began to form immediately at first and increases over the reaction time. Reaction time of mannan from porang tuber and manannase for 8 hours gave the best oligosaccharide concentration mixture. The HPLC result analysis showed that samples from the mananase enzyme reactions with mannan from porang tuber could produce oligosaccharides such as disaccharides, trisaccharides, and pentasaccharides. (Yopi et al., 2009).

Observing the potential of oligosaccharides as a prebiotic compound indicated that the oligosaccharide mixture results from mannan porang tuber hydrolysis could stimulate the growth of *Lactobacillus* sp. culture specific AA0014 and *Lactobacillus* sp. FU0811 with the highest specific growth rate at 0.13 hour/L. In simulation model test of *in vitro* gastrointestinal tract of six adult healthy donor feces, it was known that the population of bacteria lactobacilli and bifidobacteria tended to be higher when getting a carbon source oligosaccharide mixture from mannan porang tuber hydrolysis product, respectively by 10.9% and 3.3% of the total microflora than the other carbon sources. Total short-chain fatty acid was produced in cultures with carbon sources oligosaccharide mixture (79.57 mmol/L) with the dominance of lactic acid and acetic acid. The results of this study indicated that the enzymatic hydrolysis products from mannan porang tuber compounds had potential as a prebiotic compounds with stimulatory activity on the gut bacteria that had a positive impact on the health of its host (Dinoto et al., 2013).

The development of oligosaccharides depends on the availability of materials to be used and the indigenous microbes that can produce a variety of needed enzymes. Microbes from the marine environment becomes an attractive alternative because the exploration of marine



A. Amylolytic microbes B. Mannolytic microbes

Source: Djohan et al., 2010

Figure 2. Analysis of the Marine Microbes Potency for Polysaccharide-hydrolyzing Enzyme Production. A) Four isolates of marine bacteria that have ability to produce amylase, B) Four isolates of marine bacteria that have ability to produce mannanase.

microbes are still rare and there are a lot of carbohydrate sources in the marine. Indonesia is a country having tropic marine conditions with plenty of tropical indigenous marine biodiversity microbes. Researches focusing on identification potential marine microbes and further utilization of enzimatic benefit for degrading polysaccharides compound have been conducted by researcher of LIPI. The qualitative analysis showed that there were potential microbes which could produce several types of enzyme, 24 microbes producing cellulase, 28 microbes producing mannanase, 36 microbes producing agarase and 28 microbes producing amylase (Djohan et al., 2010) (Figure 2). Collection of marine microbes is an interesting object, especially if the enzyme that is produced has the appropriate characteristics for the oligosaccharides production. Carbohydrate source from marine are plenty such as agar, carrageenan, and alginate obtained from the extraction of seaweed. Agar carbohydrate consists of galacturonate that bonds and forms polymers which can be hydrolyzed by the agarase enzyme to produce agarose, agarobiose, and agarotriose. Oligosaccharide

derivated products from agar carbohydrate have been reported to have a specific function and can be applied in industry as an anti inflammation for cosmetic as well as a prebiotic for healthy food industry.

Recent Researchers Related to Oligosaccharides in Indonesia

Research on oligosaccharide was conducted by researchers from LIPI and other related institutions from 2011–2015 to explore the potential oligosaccharide producing microbes and their abilities to produce several enzymes. There are several reports regarding production and characterization biocatalyst such as cellulase and hemicellulase from *Aspergillus ustus* (Thontowi et al., 2013), mannanase from *Nonomurae* sp. (Ratnakomala et al., 2014), xylanase from *Bacillus substilis* (Gading et al., 2015), and xylanase from *Panebibacillus* sp. (Kurrataa'yun et al., 2015). Cellulase & hemicellulase (xylanase, mannanase) reported in the papers had potential to be used for hydrolyzing various lignocellulose biomasses such as baggase, rice straw, empty fruit bunch of palm tree and others to produce valuable bio-products. Further study including the consortia analysis of those enzymes, construction of the recombinant enzyme, upscale production system of each enzymes needs to be conducted to promote and disseminate the product of enzyme into appropriate industries.

There are reports regarding oligosaccharides production from carbohdyrate strach such as maltooligosaccharides production from black potato starch (Rahmani et al., 2013) and cassava cultivar Kuning (Rahmani et al., 2015), and some reports related to oligosaccharides production from non-starch carbohydrate such us cello-oligosaccharides from cellulosic biomass (Yopi et al., 2013), manno-oligosaccharides from porang using mannanase from *Streptomyces violascens* (Safitri et al., 2014), manno-oligosaccharides from copra meal

(Ariandi et al., 2015), manno-oligosaccharides from *Amorphophallus* sp. (Sasongko et al., 2015), xylo-oligosaccharides production from xylan concorbs (Salupi et al., 2015), and xylan tabacco stalk (Kholis et al., 2015). Compared to starch derived oligosaccharides that are already available in the market, oligosaccharides produced from non-starch carbohydrate are still on the developing process. Those previous researches above indicated the potency of indigenous microbes and its enzymes from hydrolyzing the cellulose-based biomass to produce nutraceutical compound such us manno-oligosaccharides, xylo-oligosaccharides and others. Biotechnology approaches are needed for research improvement to get the stable microbe and its enzyme production for oligosaccharides production.

Conclusion

Consumption of oligosaccharide product as a component of functional foods is increasing in Indonesia as much as the increasing awareness of a healthier life. These oligosaccharides can be produced considering that the availability of specific basic material is widely available in the country as well as the collection of local microbial biodiversity which has high potential in producing the various enzymes. In this case, the challenges have to be solved as soon as possible so the microbial utilization of biological resources and the development of derivative products from hydrolysis process of carbohydrates can produce new type of oligosaccharide products. Therefore, there is an obvious need to focus in research and product development for the desired targets.

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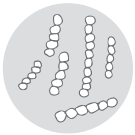

Industrial Potential of *Rhizopus oligosporus*

✍ Endang Sukara

Introduction

According to Alexopoulos et al. (1996), *Rhizopus* belongs to the Kingdom of Fungi. This kingdom comprises to only four phyla: Chytridiomycota, Zygomycota, Ascomycota, and Basidiomycota. *Rhizopus* belongs to the phylum Zygomycota, Class Zygomycetes, and ordo Mucorales. In nature, the member of Zygomycota is active decomposers of organic materials. They grow very fast and the results of their organic degradation are important components which become substrates for yeasts and bacteria (Gandjar, 2005).

Rhizopus species grow as filamentous, branching hyphae that generally lack cross-walls (i.e., they are coenocytic). They reproduce by forming asexual and sexual spores. In asexual reproduction, sporangiospores are produced inside a spherical structure, the sporangium. Sporangia are supported by a large apophysate columella on a long stalk of the sporangiophore. Sporangiophores arise among distinctive root-like rhizoids. In sexual reproduction, a dark zygospore is produced at the point where two compatible mycelia fuse. Upon

germination, a zygospore produces colonies that are genetically different from either parent.

Rhizopus is easily found in nature in both subtropical and tropical climates. *Rhizopus* can be isolated from soil, garden compost, fresh plant parts, wood pulp, decaying garbage, rotting fruits and vegetables, nuts, legume seeds, wheat, rice, water, sea, rumen and air (Boedijn, 1958; Inue et al., 1965; Scholer & Mueller, 1971; Gandjar, 1977; 2005; Liou et al., 1990; Samson et al., 1995; Alexopoulos et al., 1996; Surbakti, 1999).

There are 10 species of *Rhizopus*. Two species namely *Rhizopus oryzae* and *Rhizopus oligosporus* have been long known in industry. In Indonesia, these two species in particular, *R. oligosporus* are widely use as starter for the production of *tempe*, a traditional fermented soy bean, as the main source of protein for many people in the country. The use of this starter in *tempe* production has actually been practiced for centuries and it is still being practiced today. *R. oligosporus* is used for solid state fermentation process. The use of *Rhizopus* in liquid fermentation is not common until Sukara & Doelle (1986; 1987; 1988a; 1988b) and Sukara (1988) did series of experiments to grow this fungus in liquid fermentation. It is proved that *Rhizopus* was not only important as starter culture in *tempe* production using solid state fermentation, but could also be used for production of myco-protein and for the production of enymes in liquid fermentation. Many among the species, are good enzyme producers: protease, α-amylase, lipases, chitinase, pectinase, phytases and also producers of organic acids, bioactive compounds and biotransformers (Crueger & Crueger, 1984; Azeke et al., 2011). Kobayashi et al. (1992) found that *R. oligosporus* produced antibiotic which was active against *Bacillus subtilis, Staphylococcus aureus*, and *Streptococcus cremoris,* and Roubos-van den Hil et al. (2010) found that soya bean *tempe* extracts showed antibacterial activity against *Bacillus cereus*.

This chapter is aimed to illustrate *R. oligosporus* in the history of Indonesia, the development of *tempe* industry, and the potential use of *R. oligosporus* in future industry.

Rhizopus oligosporus in Indonesian History

R. oligosporus is very specific to Indonesia. It has been undergone domestication process by Indonesian unintentionally for more than a century. It is obvious that this species can not be separated from the Indonesians daily life. *De facto*, rarely do Indonesians have scientific knowledge on these fungi. However, this species is used commonly by *tempe* maker in the form of inoculum or starter in small scale *tempe* industry in many part of Indonesia, especially in Java. Most of *tempe* makers have never actually seen the true image of *R. oligosporus* under the microscope. The use of *R. oligosorus* in the form of inoculums for making *tempe* and many other traditional fermented foods is transmitted from generation to generation. The knowledge is transferred without any understanding on microbiology and biochemistry of the fungi (Sukara, 2005).

Tempe may be new to the west. *Tempe* is a soy food that has been eaten in Asia for hundreds of years. *Tempe* is made from cooked fermented soybeans and formed into a patty like shaped. *Tempe* has a unique taste and is mildly flavorful on its own. Unlike tofu, *tempe* is not only high in protein and calcium, but also it contain beneficial isoflavones. *Tempe* has a textured and nutty flavor. *Tempe* is a low-fat and high-protein food. With this nature, many vegetarians choose to include it in their diet on a regular basis. 100 gram of *tempe* provide around 200 calories and 18.2 grams of protein, and sufficient calcium and iron. *Tempe* is a naturally cholesterol-free food. It is reasonable if tempe becomes the interest of researchers from other countries. Researcher also carries out works on *tempe* and more work on *R.*

oligosporus. Currently, the use of *R. oligosporus* in making *tempe* has been implemented by many countries outside Indonesia. Several patents have been issued. Nowadays, *tempe* is commercially produced in Malaysia. *Tempe* is also produced by at least 53 small industries in U.S. *Tempe* is even the main diet of vegetarians in U.S, which reaches the number of 15,000,000 people (Sukara, 2005).

Development of *Tempe* Industry

Since centuries ago, many Indonesians without any knowledge of microbiology and chemistry have successfully developed a fermentation technique to process soy beans into a product identical to meat (meat analogue) which is called *tempe*. Nevertheless, scientific contribution in the *tempe* industry is very poor which results in the stagnation of *tempe* industry in Indonesia, even though the industry has been around since a very long time ago (Sukara, 2005).

In the early 60s, *tempe* was introduced to U.S by an Indonesian scientist. At the beginning, production of *tempe* for the U.S market also used traditional methods. Nevertheless, scientists succeeded to improve *tempe* production techniques. Steinkraus et al. (1965) developed *tempe* production process in a pilot scale. The peeling process of the soy bean skins and other pre-production methods done before the soy beans were fermented was developed through many sequences. There were also several findings on certain optimum conditions in the preparation phase before the fermentation started, including the importance of lactic acid addition (1%) into sterile water, which was to be used to soak the soy beans before the fermentation process was conducted. Nowadays scientists have also been able to brilliantly eliminate fermentation failures. Martinelli and Hesseltine (1964) developed a new method in *tempe* incubation by controlling aeration which enables *R. oligosporus* to have access towards enough

oxygen in the *tempe* production process. To improve *R. oligosporus* growth and guarantee success in *tempe* production, a stainless steel incubator was developed as shown in Figures 1 and 2. This incubator could also be used as a *tempe* pasteurization device by increasing its temperature to 80°C. After pasteurized, *tempe* was then to be chopped and packed and finally stored in the freezer. This method was very useful to produce more hygienic *tempe* and also to increase storage time (Sukara, 2005).

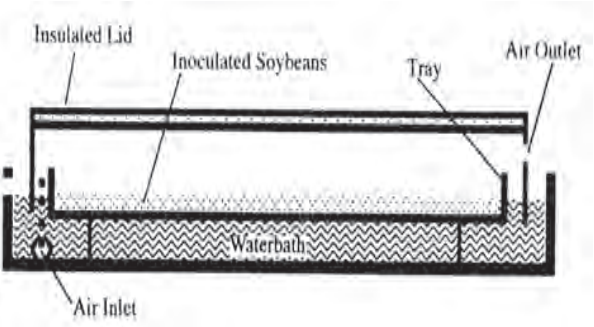

Figure 1. Diagram of a *Tempe* Incubator with Temperature and Humidity Control (Martinelli & Hesseltine, 1964).

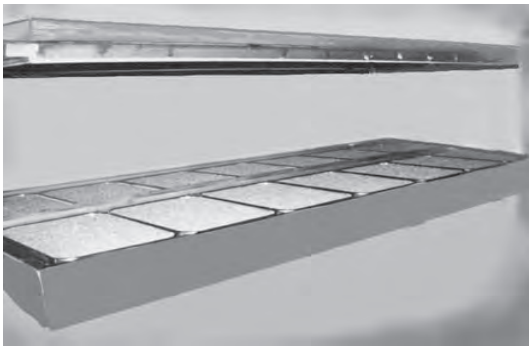

Figure 2. An Incubator with 14 Trays to Pasteurize and Produce *Tempe*. The tray cover is slightly raised and filled in with substrate, each tray can produce up to 2 kg of *tempe* every 22 hours (Martinelli & Hesseltine, 1964).

In the fermentation process, besides improvement of the soy bean structure, a part of protein and fat is also hydrolyzed. This process leads to the enrichment of amino acids and fatty acids. Not just that, the fermentation process also contributes towards the reduction of stachyose, a tetrasaccharide compound which can not be digested by humans. Riboflavin also increases for almost twofold. Niacin increases to sevenfold. Vitamin B_{12}, which usually does not exist in vegetarian diets, is also synthesized by *R. oligosporus*—bacteria symbioses. The vitamin B_{12} producing bacterium is identified as *Klebsiella pneumoniae* (a non pathogenic bacterium). Therefore, a high protein of *tempe* (approximately 40%) does not only supply essential protein needs, but also vitamin B_{12} needs for the human body. Sukara et al., (1988b) conducted studies on flavor transformation during the course of *tempe* fermentation. The research required to be continued to find the right flavor composition for various kinds of needs. By this way, raw material composition, method, and fermentation time could be distinguished to get the proper aroma for the market demand.

Sudarmadji (2005) reported that *Rhizopus* had an antioxidant property. *Rhizopus* had a tendency to prevent rancidity in polyunsaturated oils (up to 1.5 years). *Rhizopus* in *tempe* fermentation would increase the soluble solids, soluble nitrogen, soluble crude fibers, nitrogen free extract, and ammonia. *Rhizopus* had strong protease activities with its ability to increase soluble protein. Free amino acids, peptides, medium size proteins increased and became soluble in water. *Rhizopus* increased protein efficiency ratio of soybean *tempe*. It increased riboflavin, niacin and vitamin B6. *Rhizopus* had strong lipase activities which released free fatty acids from soybean lipids and reduced the triglycerides content of *tempe*. At the same time, there was an increase in antioxidant activities which was provided by its low peroxidase level in *tempe* and an antioxidant trihydroxy isoflavone was detected. When *tempe* was consumed, it reduced cholesterol level.

There are at least five vegetarian communities in U.S, including the Farm, Summertown, and Tennessee which have used *tempe* as their source of protein to replace meat. In California, Nebraska, and Canada (Toronto), there are at least 6 commercial *tempe* factories. The acceptance of Indonesia's food technology in the U.S and Canada, has enabled the opportunity for other developing countries (besides Indonesia) to develop this industry, which leads towards food diversification programs and improvement of nutrition in diet for poor people all over the world.

Tempe has been so quickly adopted as Americans' diet for vegetarians, and now it is easily found in health-food stores and even large supermarkets east and west of America. *Tempe*-burgers are also provided in the West Coast and *tempe* is anticipated as daily diet food for Americans. It is also possible that *tempe* may become a daily diet menu for people all around the world.

Rhizopus oligosporus in Industry

Since 1984, there have been certain efforts to form and find the possibilities of utilization of *R. oligosporus* in other industry besides *tempe*. This fungus has a very complex and inclusive enzyimatic system. It has branching hyphae, but lacks cross-walls. This fungus is acoenocytic fungi. Physically, this fungus is very fragile. If the fungus mycelium is damaged or raptured, its cytoplasm constituent may leak and could lead to the fungal death. Therefore, it is not a surprise if this fungus is used widely only in solid substrate fermentations as in the production of *tempe* and other similar products (Sukara, 2005). Meanwhile, we have also learned from the Indonesian people's traditional knowledge that this fungus does not need too much oxygen for optimal growth. This fungus has micro-aerophylic traits. It can grow white immense mycelium covering the soy beans in the *tempe* production process.

In the process, soy beans are wrapped in banana leaves or plastic without hindering the growth of the fungi. In fact, extensive oxygen is disadvantageous for *tempe* production because it triggers the induction of *R. oligosporus* sporulation, which makes the *tempe* become grayish black. This phenomenon is caused by the emergence of spore (Sukara, 2005). Using these unique characteristics, Sukara and Doelle (1986) successfully grew this fungi in submerged fermentation and proved that *R. oligosporus* could convert cassava starch into single cell protein (*R. oligosporus* biomass). Sukara and Doelle (1986, 1987) developed a special bioreactor (bubble column fermenter) which was very suitable to support *R. oligosporus* growth and its enzymatic activity. The fermentation process was perfected further by Sukara and Doelle (1989a, 1989b, 1990), and by Sukara and his Japanese partners at International Center for Biotechnology, Osaka University (Sukara et al., 1989, 1992). Using bubble column fermenter, a special medium mixture and a soft aeration process, which was also used to facilitate agitation, could increase the productivity and efficiency of the fermentation process to produce single cell protein and glucoamylase enzyme directly from cassava starch as the main source of carbon and energy (Sukara et al., 1988a & 1992).

By using the submerged fermentation system, the growth behavior of *R. oligosporus* was also studied (Sukara, 1988; Sukara & Doelle, 1988a; 1989b). This method also allowed the optimization of single cell protein production. (Sukara & Doelle, 1988b). Sukara and Doelle (1989a) introduced the fermentation process to produce single cell protein and glucoamylase together in a single process. After that, Sukara and Doelle (1990) introduced the potency of using *R. oligosporus* and *Zymomonas mobilis* to produce single cell protein, glucoamylase enzyme and ethanol all at once from cassava starch. Along with a Japanese researcher, Sukara (1988) found that an enzyme produced by *R. oligosporus* was able to degrade raw starch (without

gelatinization prior to enzymatic degradation). This process was very ideal for further development because the enzymatic process could be done at a high starch concentration (extensive viscosity factor/starch gelling property is avoided).

Starzyńska-Janiszewska et al. (2012) studied the effect of simultaneous mixed-culture fermentation of grass pea seeds on selected parameters of products as compared to traditional *tempe*. The inoculum contained different ratios of *R. oligosporus* and *Aspergillus oryzae* spores. The simultaneous fermentation of grass pea seeds with inoculum consisting of 1.2×10^6 *R. oligosporus* and 0.6×10^6 *A. oryzae* spores (per 60 g of seeds) resulted in improved quality product, as compared with traditionally made *tempe* (obtained after inoculation with 1.2×10^6 *R. oligosporus* spores), at the same fermentation time. This product had radical scavenging ability, 70% higher than the one obtained with pure *R. oligosporus* culture and contained 2.23 g/kg dm of soluble phenols. The thiamin and riboflavin levels were more than threefold (340 μg/g dm) and twofold higher (50.50 μg/g dm) than in traditionally made *tempe*, respectively. The product had 65% *in vitro* bioavailability of proteins and 33% in vitro bioavailability of sugars. It also contained 40% less 3-N-oxalyl-L-2, 3-diaminopropionic acid (0.074 g/kg dm), as compared to traditional *tempe*.

Another research group from the National Research Institute of Brewing, Tokyo-Japan, accomplished to purify and characterize a type of antibiotic produced by *R. oligosporus* IFO 8631 (Kobayashi et al., 1992). They purified the antibiotic from the liquid fermentation process of *R. oligosporus* IFO 8631 using CM-Cellulofine chromatography and HPLC. The type of antibiotic produced by *R. oligosporus* didn't show a wide spectrum. Nevertheless, the antibiotic produced by *R. oligosporus* IFO 8631 was very active towards the *Bacillus* class, especially *Bacillus subtillis* (*B. natto*) in a very low concentration (less than 1 ppm). The antibiotic produced by *R. oligosporus* IFO 8631 also

showed activity towards Gram-positive bacteria including *Staphylococcus aureus* and *Streptococcus cremoris*. The type of antibiotic from *R. oligosporus* IFO 8631 were simple proteins with a molecule weight of 5,500 Daltons. The amino acid component consisted of abundant cystine. This characteristic showed that the antibiotic from *R. oligosporus* IFO 8631 was an antibiotic that was stable towards pH and high heat. The activity of the antibiotic was still working at least 50% after it was treated with boiling water for 1 hour. Meanwhile, Hassan et al. (2014) evaluate the hypocholesterolemic effects of soybean and sweet lupine *tempe* fermented with *R. oligosporus* in hypercholesterolemic rats and indicated that the product could significantly improved the levels of lipid profiles, activity of liver enzymes, concentrations of uric acid and urea nitrogen, and induced a better protective effect in the hepatocytes. It was also proved that the product could exhibit hypocholesterolemic, anti-atherogenic, and hepatoprotective effects. Incorporating it into the diet might help in treating and preventing hypercholesterolemia.

Phytase is an enzyme which has the ability to degrade phytate, a chemical compound that is very hard to degrade inside the bird digestion system. Pytatic acid is always found inside chicken food which consists of cerealia and seeds as its main ingredient. By using the phytase enzyme, phytatic acid can be degraded, while phosphor and calcium attached to it can be loosen from and ready to be used as food. If the phytase enzyme is not used, the phosphor disposed from in manure will pollute the environment which results to eutrophication. Research at Iowa State University in 2001 has proven that the application of phytase in feed for pig was able to increase phosphor availability in corn-soy bean based feed with a number ranged from 15 to 45%. Therefore, addition of phosphor into feed formulation is not needed anymore. The use of phytase can also increase mineral absorption and amino acid digestion. The positive side of phytase enzyme

used as feed additive also reduces phosphor accumulation in manure that can result in reducing phosphor pollution to the environment. This particular enzyme may be produced by *R. oligosporus* (Sukara, 2005; Azeke et al., 2011). The production of phytase by *R. oligosporus* was optimized using solid state fermentation on rice bran by Suresh & Radha (2016). They could improved phytase production by fourfold due to chemical mutagenesis and media composition.

Rozan et al. (1966) demonstrated the process of detoxication of rapeseed meal by *R. oligosporus* sp-T3: The first step in the production of rapeseed protein concentrate from rapeseed meal for food was through *R. oligosporus* sp-T3 fermentation. Fermentation of rapeseed meal for 40 hours could degrade about 84% of rapeseed carbohydrate, break down 30% of lignin and other polyphenolic compounds which could not be degraded by non-ruminant animals. Around 47% of glucosinolates compounds are responsible for goiter syndrome were also degraded. Fermentation process using *R. oligosporus* could increase the nutritive value of rapeseed meal. *R. oligosporus* was able to detoxicate rapeseed meal.

Guava is known as fruit which is rich in antioxidant and ascorbic acid. However, commercial value of guava waste from guava processing industry is very limited. Correia et al. (2004) conducted an experiment to improve fonolic compound of guava waste by *R. oligosporus.* Solid waste of guava was mixed with soybean flour as a source of nitrogen before fermented. β-Glucosidase activity and antioxidant presented in crude extract and resulted from fermentation process were evaluated. In his experiment, Correia and his co-worker prepared the medium by mixing 90% of waste and 10% of soybean flour. They also did the same for another medium by mixing 50% of waste and 50% of soybean flour. Both media were then fermented by using *R. oligosporus*. Fermentation process could increase dissolved fenolic compounds to 12.9% and to 98.6% when first and second medium

were used respectively. For those two media used, they found out that the antioxidant activity increased at the first stage of fermentation, but then decreased when the fermentation time extended. Mobilization of fenolic antioxidant during the course of fermentation associated with the activity of β-Glucosidase might correlate with the mobilization of polymeric form of fenolic compounds. Bioconversion of guava waste with the addition of soybean flour was a new strategy to improve the content of antioxidant and should open a new opportunity to develop the commercial value of guava waste. *R. oligosporus* could also improve total phenolic content, antioxidant activity, and hypoglycemic functional of chikpea, *Cicer ariatinum* L. (Sanchez-Magaña et al., 2014) and fermented coastal sand dun wild legume, *Canavalia* sp., to produce high bioactive principles and antioxidant (Niveditha & Sridhar, 2014). When *R. oligosporus* was grown on buckwheat, *Fagopyrum esculentum* Mbench, the amount of water soluble vitamin (thiamine, pyridoxine, L-ascorbic acid) and tocopherol content increased and it might be used as *tempe*-like functional buckwheat-based foods with reduced antinutritional factor (Malgorzata et al., 2015). Norliza and Ibrahim (2005) published their work on the production of benzaldehyde by *R. oligosporus* USM R1 in solid fermentation system of soy bean meal and rice husk. This must be a new era to use *R. oligosporus* to provide natural compounds as flavor replacing synthetic aromas. Benzaldehyde is the second most important flavoring compound after vanillin (Welsh et al., 1989). Natural benzaldehyde can be found as a glycoside (amyugdalin) in the pits of almonds and cherries and is extracted using chemical methods which some time lead to the formation of toxic byproducts such as hydrocyanic acid. Benzaldehyde can also be synthesized chemically via toluene chlorination which is unfriendly to the environment (Norliza & Ibrahim, 2005). The production of benzaldehyde through fermentation surely is one of good alternatives. Solid state fermentation using *R. oligosporus* may than be

considered to be an economical approach for the production of fungal metabolites including in particular the production of benzaldehyde.

R. oligosporus was also used in biosynthesis of gamma amino butyric acid (Qureshi & Anwar, 2011). For this purposes, *R. oligosporus* was grown on rice medium using solid state fermentation process. Gamma amino butiryc acid (GABA) was depressive neurotransmitters in the sympathetic nervous system (Ohkuma et al., 1997; Nishikawa, 1996). GABA also had a physiological function to depress the elevation of systolic blood pressures (Vemulapalli & Barletta, 1984) and induced hypotensive effect, diuretic and treatment of epilepsy, and also had tranquilizer effects (Jakobs et al., 1993; Cohen et al., 2002; Komatsuzaki et al., 2005).

Nowadays, there is a high interest to utilize fungi from *Rhizopus* genus/group in the process to develop industries. Other species such as *Rhizopus delamar*, is now used to decaffeinate coffee (Tagliari et al., 2003) and the process to absorb heavy metal (Tsekovaa et al., 2002).

Conclusion

R. oligosporus has very complex enzymatic systems and hence it can grow well in a large variety of substrates. This mould is also very unique and have been used in *tempe* making process for centuries. *Tempe* is now becoming a choice food, especially for groups of vegetarian in many parts of the world including in the U.S. *R. oligosporus* based food products are accepted by many people all around the world as a healthy food. Its use is not limited to solid fermentation as we learn in the production of *tempe* and other traditional fermented foods, this mould can also be grown in liquid fermentation. The researcher's experience pointed out that in liquid fermentation, the separation of fungal biomass from fermentation broth was easy. Now, it is the right moment to manipulate *R. oligosporus* through

modern biology. Indonesia should lead this new initiative to develop *Rhizopus* based industry.

It is obvious from current literatures that the use of fungi from *Rhizopus* group in many kinds of industries is dramatically increasing.

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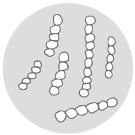


Amylases of *Aspergillus awamori* KT-11 and Its Industrial Uses

✍ Trisanti Anindyawati

Introduction

The application of enzymes as biocatalyst in Indonesia have been increasing steadily in many industries. However, all the enzymes used for application have to be imported from advanced industrial countries such as Denmark, America, Japan, England, Germany, etc. A suitable organisms for a specific use in the production of industrial enzymes should posses a number of useful attributes. These includes an easy and rapid growth under a certain conditions for microbes or with comparatively cheap and simple nutrients without certain inducers. A high yield of enzymes should be obtained by manipulating a particular method to produce a starch hydrolyzing enzyme, and also by using an easy procedure to isolate a selected microbe in a certain medium (Wiseman, 1985).

In regard to Indonesia, there are two main points which should have a great potential value for developing the production of enzymes. Firstly, Indonesia as a tropical country produces a large amount of starch such as cassava, sago, taro, etc, which could be used as a

substrate for enzymatic processes. Secondly, Indonesia is one of the richest countries for its germ plasm including microbes. Based of those facts, it is reasonable enough to develop the process of enzymes production from various natural resources, both in terms of exploiting starch sources and specific microbes that produce the available enzymes for industrial use commercially.

The industrial enzymes are generally obtained from a specific fermentation process by utilizing certain raw materials. In this process, the microbes are grown, maintained, and controlled at certain conditions in order to obtain enzyme economically. Enzymes produced by microbial cells are more useful than those derived from plants or animals. About half of industrial enzymes are produced by filamentous fungi (Lubertozzi & Keasling, 2009). The industrial production of enzymes started when Dr. Takamine began the production of digestive enzymes preparation by wheat bran koji culture of *Aspergillus oryzae* in 1894 (Komaki, 1988).

Amylolytic enzyme is one of the most important enzymes industrially, generally known as the enzyme which catalyzes the hydrolysis of starch and glycogen to produce glucose, maltose, and maltodextrin (Hollo et al., 1982; Yamamoto, 1988). It is classified into α-amylase (EC. 3.2.1.1), β-amylase (EC. 3.2.1.2), glucoamylase (EC. 3.2.1.3), α-glucosidase (EC. 3.1.2.20), and debranching amylase (EC. 3.2.1.41) as well as enzyme that has transferring activity such as cyclomaltodextrin glucanotransferase (EC. 2.4.1.19). Those enzymes are produced by microbes, plants or animals. One of the popular microbes for amylolytic production is *Aspergillus* sp. (Ueda, 1956). The most popular enzymes in industries are starch hydrolyzing enzymes including enzyme isomerizing of glucose to fructose, particularly α-amylase, glucoamylase and glucose isomerase (EC. 5.3.1.5).

There are several microbes especially fungi which have been found to produce raw starch digesting amylase, such as one of *Aspergillus*

awamori glucoamylase being active on raw starch absorption and digestion (Ueda et al., 1974), *A. oryzae* glucoamylase I being highly active in raw corn starch digestion (Miah & Ueda, 1977), and *Rhizopus niveus* having an activity to degrade raw corn starch stronger than *Aspergillus* sp. (Yamamoto, 1995).

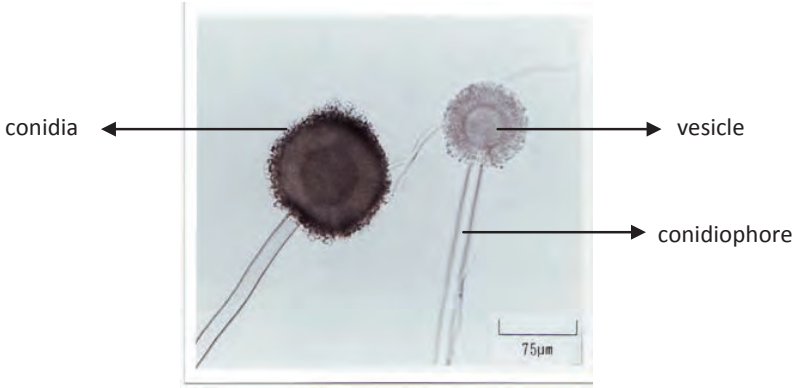
Generally, the enzymatic production of glucose from starch is performed by the joint action of α-amylase and glucoamylase, sometimes with debranching enzyme and gelatinization of starch by heat. This saccharification of starch consumes much energy. Therefore, a potent raw starch digesting enzyme is expected for the production of glucose in order to reduce energy. Enzymatic digestion of raw starch to produce glucose depend on the action of enzyme, the concentration of starch and some conditions for the enzymatic reaction, type of starches, and degrees of starch susceptibility to enzyme (Kerr, 1950; Noda et al., 1993). The mechanically damaged starch granules treated by using sonicator and stirring with magnetic stirrer could make the enzyme easier in digesting the starch granules. This phenomenon might be due to mechanical force on the surface structure of starch granule. Physical method such as sonication can be used as a supplementary way to simplify the process and increase the yield (Isono et al., 1994).

The aim of this chapter is to review the local strain, *A. awamori* KT-11 that is potential for producing amylase.

A Novel Type of Amylolytic Enzymes from *A. awamori* KT-11

A local strain of black mold isolated from the air in Bogor, Indonesia was identified as *A. awamori* KT-11 by Japan Research Laboratories, Osaka (Figure 1). It was found to have a potent mold for producing amylolytic enzymes which were quite different in term of the

enzymatic, protein natures, and substrate specificity from those of other black molds, especially *Aspergillus* sp. The strain secreted three kinds of amylolytic enzymes with potent activity, two forms of α-glucosidases, three forms of α-amylases, and two forms of glucoamylases.



Source of photographs: Private collection taken by Japan Research Laboratories, Osaka

Figure 1. Fruiting Body of *Aspergillus awamori* KT-11

α-Glucosidases

The two α-glucosidases (α-G I and α-G II) had molecular weights of 108 and 106 kDa respectively as detected by FPLC. They were composed of two hetero subunits with molecular weights estimated by SDS-PAGE were 62 and 31 kDa for α-G I and 59 and 31 kDa for α-G II. The action pattern of both enzymes was almost similar to hydrolyze on maltotetraose, isopanose, maltotriose, maltotriitol, panose, maltopentaose, maltose, maltohexaose, isomaltose, and maltoheptaose in order of the initial reaction velocity and acted very weak on nigerose, maltitol, and amylose to produce only glucose. However, they didn't act on trehalose and sucrose. The best conditions for production of panose from maltose by transfer action of α-G I were at pH 6.7 and 27°C on 60% (w/v) of substrate concentration. Both α-glucosidases,

from *A. awamori* KT-11 were suggested to be novel enzymes since they were different from the enzymes of other black mold, especially *A. awamori* and *A. niger* (Anindyawati et al., 1998a). The products from maltose were isomaltose, panose, and other maltooligosaccharides with higher molecular weight than panose, and the products of transfer action from leucrose by the mixture of α-G I and α-G II were α-1.6 glucosyl leucrose and α-1.6 isomaltosyl leucrose (Anindyawati et al., 1995). The comparison of some *Aspergillus* sp. α-glucosidases is shown on Table 1.

Table 1. Comparison of *A. awamori* KT-11 α-Glucosidases with Others

Properties	*A. awamori* KT-11		*A. awamori*[1]			*A. niger*[2]	*A. niger* GRM[3]
	α-G I	α-G II	I	II	III		
Optimum pH	5.3	5.3	5.0	5.0	5.0	4.3	4.5
Mol. weight (kDa)	6.2 3.1	5.9 3.1	12.5	14.0	13.0	12.5	4.2 9.0
FPLC (kDa)	10.8	10.6					
Isoelectric point	4.0	4.5	5.6	5.6	5.6		

[1]Yamasaki et al. (1977)
[2]Kita et al., (1991)
[3]Kimura et al., (1997)

α-Amylases

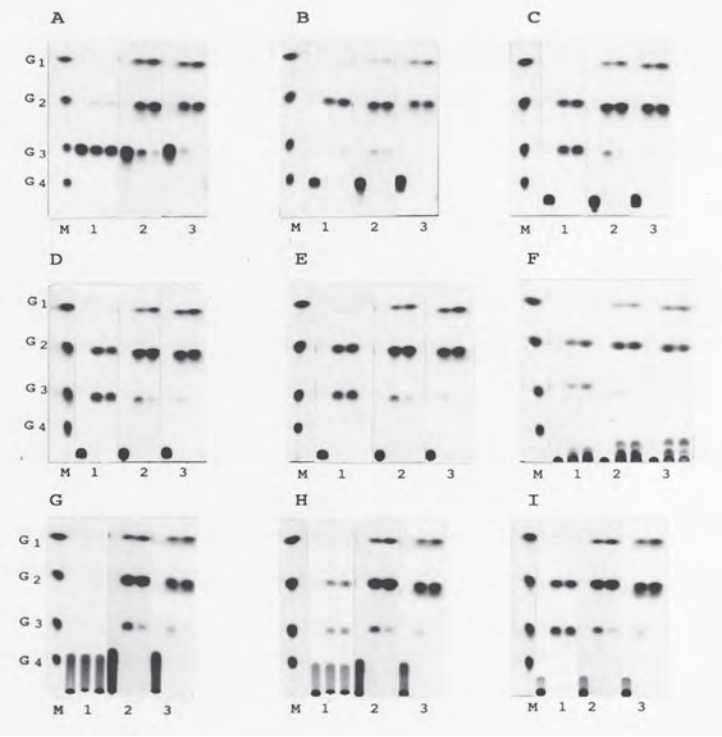
Three forms of soluble starch degrading enzymes (Amyl I, Amyl II, and Amyl III) were isolated by a complicated purification procedure from an extract of wheat bran koji culture of *A. awamori* KT-11. All of the enzymes were regarded as α-amylases from the anomeric form of their reaction products by ^{1}H-NMR analysis and the action mode on starch.

The three α-amylases had molecular weight of 49, 63, and 97 kDa by SDS-PAGE, respectively. Amyl I hydrolyzed maltotetraose, -pentaose, -hexaose, -heptaose, β-, and γ-cyclodextrin to produce maltose and maltotriose as a major products but not maltose, isomaltose, maltotriose, isomaltotriose, α-cyclodextrin and raw corn starch. On the other hand, both Amyl II and Amyl III hydrolyzed maltotriose as well as maltooligosaccharides described above and α-, β-, γ-cyclodextrin, and even raw corn starch as well as gelatinized corn starch to produce maltose as a major product and glucose and maltotriose as a minor products, but they didn't hydrolyze maltose, isomaltose and isomaltotriose (Anindyawati et al., 1998b). As shown in Figure 2, Amyl I hydrolyzed waxy maize starch to produce mainly maltose, maltotriose and some maltooligosaccharides with a higher degree of polymerization, while Amyl II and Amyl III produced maltose and some maltooligosaccharides of higher molecular weight than maltotetraose and small amount of glucose.

The physicochemical and enzymatic properties of each enzyme were found to be different in molecular weight and also some diffrerences were observed in their K_m values. Other properties such as optimal pH, temperature, and pH and thermal stabilities of Amyl II and Amyl III were almost similar, but these values were quite different from those of Amyl I. The carbohydrate contents of Amyl I, Amyl II and Amyl III were found to be 10.4, 6.3 and 11.3%, respectively (Anindyawati et al., 1998b). The effect of metal ions on the activity of the enzymes were examined. Among the metal ion tested, $HgCl_2$, $CuSO_4$ and $ZnCl_2$ strongly inhibited all of the enzymes. $CaCl_2$ inhibited both Amyl II and Amyl III to a certain degree, but not Amyl I. However, $FeSO_4$ was observed to double the activation of all enzymes.

The limit hydrolysis of soluble starch with three forms of enzymes were 33% for Amyl I, 35% for Amyl II, and 38% for Amyl III, and

the reaction products had α-anomeric formd by NMR analysis and blue color reaction with I_2 dissapeared completely at about 18% of hydrolysis of the starch for all enzymes (Anindyawati et al., 1998b).



Source: Anindyawati et al., 1998b

Figure 2. TLC Analysis of Hydrolyzates from Maltooligosaccharides and Glucans by α- Amylases. M, marker; G_1, glucose; G_2, maltose; G_3, maltotriose; G_4, maltotetraose. Incubation period (hour) were 0, 24 and 48 from left to right for each Amyl I, Amyl II and Amyl III. A: maltoriose; B: maltoetraose; C: maltopentaose; D: maltohexaose; E: maltoheptaose; F: waxy maize starch; G, H, I: α, β, γ-Cyclodextrin.

Table 2. Comparison of *Aspergillus awamori* KT-11 α-Amylases with others

Properties	*A. awamori* KT-11[1] Amyl			*A. awamori*[2]	*A. awamori* var. *Kawachi*[3]		*A. niger*[4]		*A. oryzae*[5]
	I	II	III		I	II	Acid stable	Un stable	
Optimum pH	4.0	5.5	5.5	4.9	4.5	5.0			
Molecular weight (kDa)	4.9	6.3	9.7	5.4	10.4	6.6	5.8	6.1	5.2
Isoelectric point	3.5	3.2	3.3	4.2	4.3	4.2	3.4	3.7	3.7
Specificities									
α-cyclodextrin	-	G_2, G_1	G_2, G_1						G_2, G_3
β-cyclodextrin	G_2, G_3	G_2, G_1	G_2, G_1						G_2, G_3
γ-cyclodextrin	G_2, G_3	G_2, G_1	G_2, G_1						G_2, G_3
Soluble starch	G_2, G_3	G_2, G_1	G_2, G_1		G_1,G_2,G_3	G_1,G_2,G_3			
Raw starch	-	G_2, G_1	G_2, G_1	*	*	*	*	*	*

*Not reported
[1]Anindyawati et al. (1998b)
[2]Bhella & Altosaar (1984)
[3]Mikami et al. (1987)
[4]Arai et al. (1968)
[5]Suetsugu et al. (1974)

Comparison of *A. awamori* KT-11 α-amylases with other *A. awamori*, *A. niger* as well as *A. oryzae* on both properties and specificities was shoen on Table 2. Each of three kinds of α-amylases was different in protein natures from α-amylases of other strains of black mold, especially *A. awamori.* Meanwhile, some certain distinctive differences between Amyl I and both Amyl II and Amyl III were observed on the action modes on maltooligosaccharides and glucans, though the action modes of Amyl II and Amyl III were quite similar to each other. Both Amyl II and Amyl III were found to be almost similar not only in its enzymatic properties (Table 2), but also in action patterns of hydrolyzing maltotriose besides the maltooligosaccharides, α, β, γ-CD as well as raw corn starch to produce maltose and small amount of glucose. *A. awamori* KT-11 was first to be shown to produce two types of α-amylases, one (Amyl II and Amyl III) attacking raw corn starch and another (Amyl I) having no ability to do this. Matsubara et al. (2004) reported that the action mode of Amyl III on starch granules was different from glucoamylase judging from the observation of granules under SEM before and after enzyme reaction, and also from the reaction product.

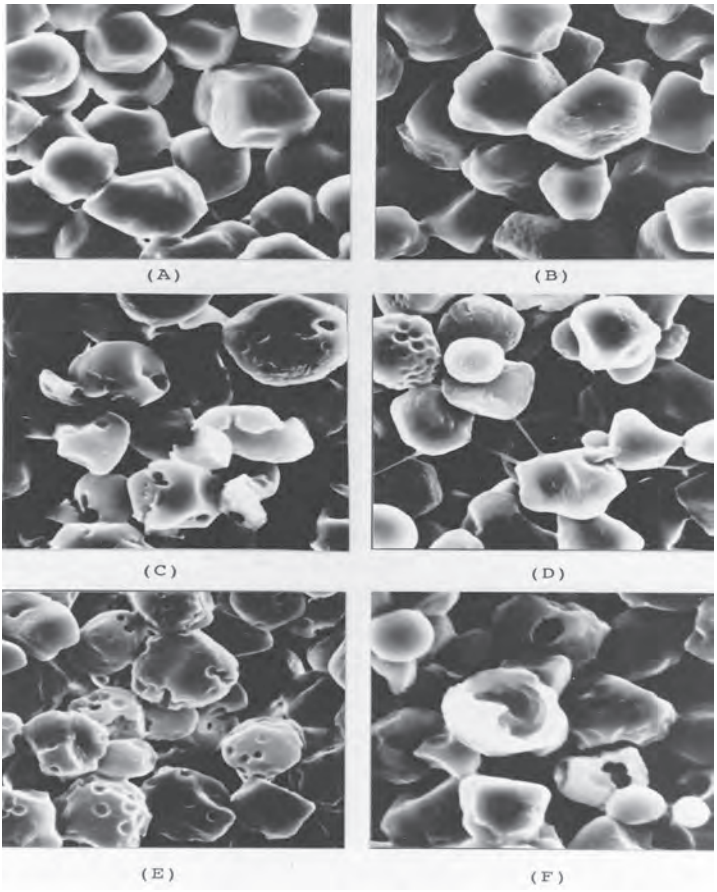
Table 3. Digestion of Some Raw Starches* by Amylolytic Enzymes Preparation

Enzymes	Digestion of starch, %**			
	Corn	Cassava	Sago	Potato
Crude enzymes	95	41	36.0	9.3
α-Amylases (Amyl III)	10	6	5.8	2.5
Glucoamylases (GA I + GA II)	19	16	6.7	2.6
α-Amylase (Amyl III) and Glucoamylases (GA I + GA II)	44	21	12.1	4.0

Source: Anindyawati, 2003

*With sonication treatment before enzyme digestion

**The values were expressed from acid hydrolysis of each starch as 100%



Source: Anindyawati, 2003.

Figure 3. Scanning electron micrograph of corn starch by amylolytic enzymes. A: corn starch; B: starch after sonication treated; C: after added with crude enzymes; D: after added with α-amylase (Amyl III); E: after added with the mixture of glucoamylase (GA I and GA II); F: after added with combination of α-amylase and the mixture of glucoamylases.

Table 4. Some Enzymatic and Physicochemical Properties of Amylases from *A. awamori* KT-11

Properties	α-Glucosidases		α-Amylases			Glucoamylases	
	α-G1	α-GII	Amyl I	Amyl II	Amyl III	GA I	GA II
Molecular weight SDS-PAGE (kDa)	6.2 3.1	5.9 3.1	4.9	6.3	9.7	15.0	15.8
FPLC (kDa)	10.8	10.6	4.6	5.9	12.0	13.5	12.5
Isoelectric point	4.0	4.4	3.5	3.2	3.3		
Carbohydrate content (%)	9.8	8.4	10.4	6.3	11.3	19.44	15.38
Optimum pH	5.2	5.2	4.0	5.5	5.5	5.0 4.58*)	5.0 4.5*)
pH stability	5–7	5–7	4.5–8.5	3.5–7.5	4.0–7.0	4.0–8.0	4.0–8.0
Thermal stability (°C)	50	50	50	70	70		
K_m for Maltotriitol (mM)	1.82	2.5					
Soluble starch (%)	10	6.7	0.45	0.33	0.14		
Amylopectin (%)			0.167	0.154	0.069		
B-limit dextrin (%)			0.143	0.25	0.167		
Anomeric forms of Product (^{1}H-NMR)	α-form	α-form	α-form	α-form	α-form	β-form	β-form

*)raw starch
Source: Anindyawati et al., 1998b

Glucoamylases

The two glucoamylases, GA I and GA II, with molecular weights of 152 and 158 kDa by SDS-PAGE, respectively were isolated from the extract of *A. awamori* KT-11. Both of them had the ability to hydrolyze raw starch. Corn starch with ultrasonication treatment was hydrolyzed much easier than that without treatment. It could reach up to 95% conversion to glucose by crude enzymes. Hydrolysis of corn starch by α-amylase and glucoamylase reached 10 and 19%, respectively. However, combination of both enzymes reached 44% conversion to glucose. In this case, addition of both enzymes could be more effective to hydrolyze all starches tested (Table 3). GA II was able to hydrolyze

some raw starch stronger than GA I. Mixture of GA I and GA II resulted in much less ability of digestion toward raw starch than those of crude enzymes.

The morphological of corn starch granules after 48 h digestion by enzymes was investigated by SEM, resulting that the crude enzymes attack the granules strongly to make it crumble. Meanwhile, α-amylase showed a very weak attack to the granules, but the joint action of both enzymes attacked the granules stronger as shown in Figure 3. It was also shown that there was difference in attacking mode on starch granule. The reason might be due to the preparation method and some unknown factors to aid the digestion of raw starch. When Amyl III was used together with glucoamylase for degradation of corn starch, the decomposition activity increased remarkably because of the synergetic effect of both enzymes.

The summary of properties of each amylolytic enzyme is shown in Table 4. The physicochemical and enzymatic properties of each enzyme were found to be different in molecular weight and also some differences on their K_m values. Other properties such as optimal pH, temperature, pH and thermal stability of the enzymes were also different.

Application of Amylases to Industry

Amylases which approximately represent 25% of the enzyme market have potential application in several industries (Sidhu et al., 1997). Amylase can be produced by fermentation process by using techno-economic feasibility study. The fermentation technology is through kinetic modeling of microbial growth, parameters control, scale-up, and commercialization. Some of amylases have been applied in industry; meanwhile industrial amylase should be developed in broader area with more economic efficiency (Sheoran & Dhankhar, 2016).

The efficiency of amylases in various sectors may be achieved by chemical modification of the enzyme or by protein engineering. In biotechnology, amylase can be applied in biopharmaceutical industries (Das et al., 2011) as well as in food and starch based industries (Saranraj & Stella, 2013). Amylase from fungal source, especially *Aspergillus* sp., has gained more attention because amylase extracted from fungal source are more acceptable and classified as generally regarded as safe (GRAS) (Dar et al., 2014).

Van der Maarel et al. (2002) highlighted that starch was a major storage product of many economically important crops such as wheat, rice, maize, tapioca, and potato. It is obvious that, a large-scale starch processing industry has emerged in the last century, including here in Indonesia. In the past decades, we have witnessed that there was a shift from the acid hydrolysis of starch to the use of starch-converting enzymes in the production of maltodextrin, modified starches, or glucose and fructose syrups. Currently, according to Van der Maarel et al. (2002), starch hydrolyzing enzymes comprises about 30% of the world's enzyme production.

Amylase family is used intensively in industrial production of glucose and fructose, bakery and anti-staling, cyclodextrin/cycloamylose formation and miscellaneous applications including warp sizing of textile, clarification of haze formed in beer or fruit juices, pretreatment of animal feed (to improve digestibility), and laundry and dish-washing detergents. Besides the use in starch hydrolysis, starch-converting enzymes are also used in a number of other industrial applications, such as laundry and porcelain detergents or as anti-staling agents in baking (Van der Maarel et al. 2002). Furthermore, de Souza & de Oliveria e Magalhaes (2010) illustrated the importance of amylase in fuel alcohol production, starch conversion, detergent, food, textile, and paper industries. One of fungal amylases, *A. fumigatus* NTCC1222 has potential application in textile industry, which has highest amylase

activity under solid state fermentation (Singh et al., 2014). While Aiyer (2005) highlighted the application of amylase in liquefaction process, manufacturing maltose, high fructose syrups, manufacturing mixture of oligosaccharides, maltotetraose syrup, production of anomalously linked oligosaccharides mixture (Alo mixture), manufacturing of high molecular weight branched dextrins, direct fermentation of starch to ethanol, treatment of starch processing waste water and many other applications.

The crude amylase of *A. awamori* KT-11 was evaluated for the ability in converting five different non food varieties of cassava as feedstock for bioethanol production. Evaluation was carried out on pure starch and fresh cassava tubers. Enzyme reaction was at 55–60°C for 72 hours. The best conversion efficiency reached 38.60% w/v of sugar when pure starch of Sapikuru was used. This process was scaled up using fermentor by using materials of cassava flour produced reducing sugar of 66.86 gr/L for 48 hours of enzymatic reaction (Melliawati et al., 2010). The optimum condition of crude amylase for making bioethanol feedstock using wheat bran as main component was at 60°C, pH 5.5 and 4% substrate concentration for 48 hours (Melliawati et al., 2012). The addition of additives was known to be able to maintain both stability and activity of the enzyme. Among three stabilizers used during storage of crude amylase, Tween 80 was the best when stored at room temperature for 7 days. For commercialization purpose of crude amylase of *A. awamori* KT-11, the techno-economic calculation showed that Internal Rate of Return (IRR) was 27.48% with Payback Period (PBP) for 3.64 years (Anindyawati & Melliawati, 2012).

Melikoglu et al. (2015) used packed bed bioreactor for the production of glucoamylase and protease by *A. awamori* using waste bread in solid state fermentation. Using appropriate aeration, the production of both enzymes were much higher compared to that of produced in Petri dishes at static conditions. Meanwhile, de Castro

et al. (2015) demonstrated that fixed-bed solid-state fermentation bioreactor with forced aeration could be used as an effective procedure for the production of hydrolases enzymes by *A. awamori*. This system claimed to be the solution in overcoming scale-up limitations of conventional try-type solid-state fermentation bioreactors. Using this system, *A. awamori* could produce six enzyme groups namely exoamylases, endoamylase, proteases, xylanases, cellulase and isoamylase. A fixed-bed bioreactors with forced aeration presented a promising alternative in terms of instrumented bioreactors for solid-state fermentation processes.

Kumar et al. (2015) illustrated that *A. awamori* could also produce tannase capable of hydrolyzing tannin to glucose and gallic acid. This opened the possibility of using *A. awamori* not only in food, brewing and beverage industry, but also in pharmaceutical industry. Only recently, Anuradha et al. (2016) used this species to clarify mango juice. This was an indication that this fungus could also produce pectolytic enzymes, polygalacturonase, to degrade pectin.

Saleh et al. (2014) highlighted that *A. awamori* had a potential role for probiotics as natural growth promoters in boiler nutrition and might provide an effective alternative to antibiotics in the production of boilers. In 2015, de Oliveira and his co-worker tried to evaluate the performance of beef cattle bulls in feed lots and fed on diets containing enzymatic complex derived from *A. awamori* (de Oliveira et al. 2015).

Only recently, Singh & Kaur (2016) identified *A. awamori*, an endophytic fungus, isolated from *Acacia nilotica* which had the ability to produce α-amylase and glucosidase inhibitor. This should open the opportunity to explore *A. awamori* strains further to produce important other molecules for the development of antidiabetic agents.

Conclusion

A strain of *A. awamori* KT-11 was found to be a potent fungus not only for the production of new amylolytic enzymes, which were quite different type in enzymatic and protein natures and even substrate specificity from those of other black fungi, especially *Aspergillus* sp., but also important for the production of many other hydrolytic enzymes. *A. awamori* could also be used as prebiotics and enzyme inhibitor as ingredient in formulating antidiabetics. The enzymes and diverse products derived from *A. awamori* were considered to be novel types of products to boost food, beverages, and pharmaceutical industry in the future.

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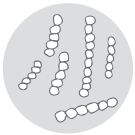


Collection of Indonesian Actinomycetes and Its Uses

✍ Shanti Ratnakomala, Puspita Lisdiyanti, and Yantyati Widyastuti

Introduction

Actinomycetes are group of prokaryotic organisms in current taxonomy. This group belongs to the Domain Bacteria, Phylum Actinobacteria, Class Actinobacteria, Subclass Actinobacteridae, and Order Actinomycetales. Currently, the Order Actinomycetales includes 14 suborders (Gao & Gupta, 2012). All members of this order are characterized in part by high G+C content (>55 mol %) in their DNA (Stackebrandt et al., 1997). They are filamentous bacteria which produce two kinds of branching mycelium, aerial, and substrate mycelium. The aerial mycelium is important as the part of the organism that produces spores. For this reason they have been considered as fungi, as it is reflected in their previous name; 'actino' means 'ray' and 'mykes' means 'mushroom/fungus', so Actinomycetes are called ray fungi.

Actinomycetes, also considered as actinobacteria, are the most widely distributed group of microbes in nature and are also well-known as saprophytic soil inhabitants (Takizawa et al., 1993). Some

of Actinomycetes produce a volatile compound called geosmin which literally translates to 'earth smell' (Gust et al., 2003). This organic compound is responsible as a contributor to the strong odor that occurs in the air when rain falls after a dry spell of weather. In natural habitats, the genus *Streptomyces* is common and, usually is a major component of the total Actinomycetes population that contains more than 400 species. This genus, together with genera *Kitasatospora* and *Streptacidiphilus,* is member of Streptomyces group; meanwhile, some Actinomycetes genera such as *Actinoplanes*, *Amycolatopsis*, *Catenuloplanes*, *Dactylosporangium*, *Kineospora*, *Microbispora*, *Micromonospora*, and *Nonomuraea* are grouped as rare Actinomycetes (Hayakawa, 2008).

Review on Actinomycetes research in Indonesia was presented comprehensively by Karossi et al. (1988), focusing on the screening and differentiation of Actinomycetes, biosynthesis of secondary metabolites and genetics in Actinomycetes as reported by workers from Indonesia until 1988. After that, the isolation and identification of Actinomycetes were conducted comprehensively as a part of collaboration research between Indonesian Government Research Center (GRC) and National Institute of Technology and Evaluation (NITE), Japan. The members of Indonesian GRC were Indonesian Institute of Sciences (LIPI) as representative, Bogor Agricultural University, University of Indonesia, Soil Research Center, Ministry of Agriculture, and Agency for Assessment and Application of Technology (BPPT). The title of collaboration research was "Taxonomic and Ecological Study of Fungi and Actinomycetes from Indonesian Sources" and called as LIPI-NITE Project. Further, LIPI with NITE Biological Research Center (NBRC), Japan continued the collaboration project entitled "Development of International Standardized of Microbial Resources Center in Indonesia" under the Science and Technology Research Partnership for Sustainable Development (InaCC SATREPS) Project, which was a research program structured as a collaboration

of the Japan Science and Technology Agency (JST) and the Japan International Cooperation Agency (JICA).

In this paper, the collection of Actinomycetes from the projects, description of new species of Actinomycetes found in Indonesia, and the prospect uses of Actinomycetes collection from Indonesia for health purposes are presented.

Collection of Actinomycetes from LIPI-NITE Project and InaCC SATREPS Project

Actinomycetes collection from LIPI-NITE Project

During the implementation of LIPI-NITE project from April 2003 to March 2009, 373 sample sources including 232 soil and 141 plant leaf-litter samples were used in this study as shown in Table 1 (Widyastuti & Ando, 2009). Collection of samples was carried out in 13 locations in Indonesia, mainly in 9 Botanical Gardens, including Eka Karya in Bedugul, Bali; Cibodas in West Java; Purwodadi in East Java; Bukit Sari in Jambi; Enrekang in South Sulawesi; Sungai Wain in East Kalimantan; Baturraden in Central Java; Pucak in South Sulawesi; and Liwa in Lampung, and 4 in other locations *i.e.* Kutai National Park, Cibinong Science Center (CSC) in the Campus of LIPI, and 2 fields LIPI station in Lombok and Kupang (Figure 1).

From that number of samples, more than 5,000 Actinomycetes were isolated, and finally, 3,193 Actinomycetes were selected for further study and became a project collection of Actinomycetes. In this project, isolation of particularly rare genus of Actinomycetes was carried out based on techniques and pretreatments that enable the researchers to collect certain Actinomycetes of interest. It was also the way to obtain desirable Actinomycetes by eliminating contaminants. Isolation methods that included a variety of selective isolation media supplemented with certain antibiotics and various enrichment and

Table 1. Number of Samples Used During Exploration of Actinomycetes in Indonesia Under LIPI-NITE Project (Widyastuti & Ando, 2009)

Year	Sampling site	Number of sample		Total
		Soil	Plant litter	
2003	1. Eka Karya	18	20	**38**
	2. Cibodas	24	13	**37**
2004	3. Bukit Sari	21	21	**42**
	4. Purwodadi	20	10	30
	5. CSC	10	4	**14**
2005	6. Kupang	20	20	**40**
	7. Enrekang	24	25	**49**
	5. CSC	6	3	9
2006	8. Kutai National Park	30	0	**30**
	9. Lombok	14	0	**14**
2007	10. Sungai Wain	20	10	**30**
	11. Baturraden	10	5	15
2008	12. Pucak	10	5	**15**
	13. Liwa	5	5	**10**
Total		232	141	373

pretreatment were also employed. Six isolation methods, *i.e.* 1) Dry Heat (DH) (Nonomura & Ohara, 1969); 2) SDS-yeast extract (SDS-YE) (Hayakawa & Nonomura, 1989); 3) phenol; and 4) RC + vancomycin as modification of Hayakawa & Nonomura's was supplemented with Vancomycin antibiotic (Hayakawa et al., 2000); 5) Rehydration and Centrifugation (RC) (Hayakawa et al., 2000; Otoguro et al., 2001); and 6). Oil Separation System (OSS) as modification of Ishigami's water-hexane distribution method use of olive-oil instead of hexane (Ishigami et al., 2004). HV agar medium (Hayakawa & Nonomura, 1987, 1989) supplemented with 50 µg/ml cycloheximide, 20 mg/ml nalidixic acid, and 20 mg/L kabisidin to minimize the growth of fungi and undesirable bacteria was used as isolation medium.

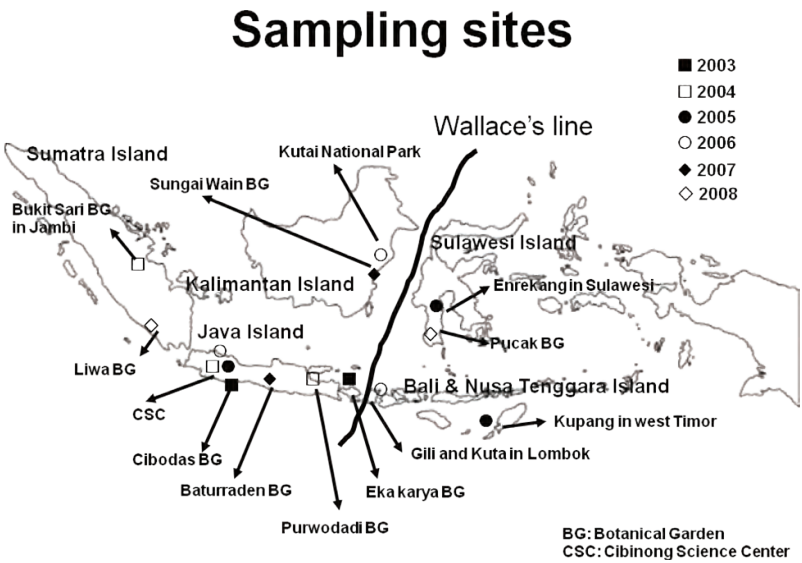


Figure 1. Location for Collection of Soil and Plant Litter Samples in Indonesia (Widyastuti & Ando, 2009)

All selected isolates were purified and then identified based on 16S rRNA gene analysis and phylogenetic analysis according to Lisdiyanti et al. (2010). The identification was based on homology search by BLAST compared with registered strains in data base on GeneBank/EMBL/DDBJ. Some of the interesting isolates were used for further taxonomical study. Molecular identification based on 16S rRNA gene revealed that 12 suborder, 19 families, and 64 genera of Actinomycetes were found from the sequence data as shown in Table 2 (Widyastuti & Ando, 2009). Many genera and species found in this study were never reported in Indonesia. The predominant genera of Actinomycetes were *Streptomyces*, *Actinoplanes*, and *Micromonospora*. Several new species were described, including *Streptomyces baliensis* sp. nov. (Otoguro et al., 2009), *Actinokineospora baliensis* sp. nov, *Actinokineospora cibodasensis* sp. nov, *Actinokineospora cianjurensis*

sp. nov. (Lisdiyanti et al., 2010); *Actinophytocolla timorensis* sp. nov, *Actinophytocolla corralina* sp. nov. (Otoguro et al., 2011); and *Dietzia timorensis* sp. nov. (Yamamura et al., 2010).

Based on data of BLAST homology search using 16S rRNA gene sequence data as queries, 919 strains showed homology more than 99% with strains in GenBank/DDBJ/EMBL data base, 1067 strains showed the homology value from 98% to 96%, and 104 strains showed homology less than 96%. This study revealed that 20% of isolates were predicted to be new taxa (Widyastuti & Ando, 2009).

Table 2. Actinomycetes from the LIPI-NITE Project (Widyastuti & Ando, 2009)

Suborder		Family		Genus		∑ of isolates
1	*Catenulisporineae*	1	*Catenulisporaceae*	1	*Catenulispora*	2
2	*Corynebacterineae*	2	*Corynebacteriaceae*	2	*Corynebacterium*	1
		3	*Dietziaceae*	3	*Dietzia*	1
		4	*Gordoniaceae*	4	*Gordonia*	4
		5	*Mycobacteriaceae*	5	*Mycobacterium*	18
		6	*Nocardiaceae*	6	*Nocardia*	78
				7	*Rhodococcus*	3
		7	*Tsukamurellaceae*	8	*Tsukamurella*	1
3	*Frankineae*	8	*Cryptosporangiaceae*	9	*Cryptosporangium*	36
		9	*Geodermatophylaceae*	10	*Geodermatophilus*	1
				11	*New genus*	3
4	*Glycomycineae*	10	*Glycomycetaceae*	12	*Glycomyces*	1
5	*Kineosporiineae*	11	*Kineosporiaceae*	13	*Kineococcus*	2
				14	*Kineosporia*	90
6	*Micrococcineae*	12	*Brevibacteriaceae*	15	*Brevibacterium*	2
		13	*Cellulomonadaceae*	16	*Cellulomonas*	2
				17	*Oerskovia*	1
		14	*Dermacoccaceae*	18	*Dermacoccus*	3
		15	*Dermatophilaceae*	19	*Dermatophilus*	1
		16	*Intrasporangiaceae*	20	*Lapilicoccus*	1
		17	*Microbacteriaceae*	21	*Agrococcus*	1
				22	*Agromyces*	1
		18	*Micrococcaceae*	23	*Kocuria*	1
				24	*Micrococcus*	1
		19	*Promicromonosporaceae*	25	*Isoptericola*	3

	Suborder		Family		Genus	∑ of isolates
				26	*Promicromonospora*	9
7	*Micromonosporineae*	20	*Micromonosporaceae*	27	*Actinoplanes*	471
				28	*Catellatospora*	3
				29	*Catenuloplanes*	47
				30	*Couchioplanes*	2
				31	*Dactylosporangium*	35
				32	*Hamadaea*	1
				33	*Krasilnikovia*	11
				34	*Micromonospora*	135
				35	*New genus*	1
				36	*Polymorphospora*	2
				37	*Pseudosporangium*	3
				38	*Verrucosispora*	3
				39	*Virgosporangium*	2
8	*Propionibacterineae*	21	*Nocardioidaceae*	40	*Aeromicrobium*	2
				41	*Kribbella*	28
				42	*Nocardioides*	11
9	*Pseudonocardineae*	22	*Actinosynnemataceae*	43	*Actinokineospora*	11
				44	*Actinosynnema*	4
				45	*Lentzea*	2
				46	*Saccharothrix*	6
		23	*Pseudonocardiaceae*	47	*Actinophytocola*	2
				48	*Amycolatopsis*	11
				49	*Pseudonocardia*	21
				50	*Saccharopolyspora*	12
				51	*Saccharomonospora*	1
10	*Streptosporangineae*	24	*Nocardiopsaceae*	52	*Nocardiopsis*	6
		25	*Streptosporangiaceae*	53	*Acrocarpospora*	2
				54	*Herbidospora*	1
				55	*Microbispora*	6
				56	*Microtetraspora*	2
				57	*Nonomuraea*	118
				58	*Planobispora*	1
				59	*Planotetraspora*	32
				60	*Sphaerosporangium*	9
				61	*Streptosporangium*	13
		26	*Thermomonosporaceae*	62	*Actinoallomurus*	1
				63	*Actinocoralia*	1
				64	*Actinomadura*	24

	Suborder		Family		Genus	∑ of isolates
11	*Streptomycineae*	27	*Streptomycetaceae*	65	*Kitasatospora*	1886
				66	*Streptacidiphilus*	
				67	*Streptomyces*	
12	*Solirubrobacterales*	28	*Patulibacteraceae*	68	*Patulibacter*	1
	TOTAL					**3193**

Actinomycetes collection from InaCC SATREPS Project

InaCC SATREPS Project was implemented from April 2011 to March 2016. Research Center for Biology, LIPI was the executing agency of this international competitive project. Through this project, Japanese experts from NITE, the University of Tokyo, as well as RIKEN, Japan, shared their knowledge and technologies in managing microbial bio-resources. The researchers under the project collected microbes that were potentially useful for agriculture and livestock production, determined their characteristics, and created a database that contributes to the preservation and sustainable use of microbial resources. The main outcome of these activities was to establish the largest depository center for microbes in Southeast Asia.

Actinomycetes group in InaCC SATREPS Project focused on isolation, identification, and preservation of filamentous Actinomycetes and marine Actinomycetes. Collection of samples was carried out in 7 locations in Indonesia i.e. Cibodas Botanical Garden, West Java; Gunung Pancar and Ciseeng Hot Spring, West Java; Satonda Island and Mount Tambora, West Nusa Tenggara; Rambut Island, Pari Island and Pramuka Island, DKI Jakarta; and Bali Island. Eighty-four samples consisted of 75 soil and sediment samples from mangrove areas and hot springs, and 9 plant leaf-litter samples were used in the study as shown in Table 3. Six isolation methods *i.e.* 1) SDS-YE method (Hayakawa & Nonomura, 1989); 2) Modified SDS-YE method incubated at 45°C; 3) RC method (Hayakawa et al., 2000; Otoguro et al., 2001); 4) Modified RC method incubated at 45°C; 5) Direct plated

method; and 6) Serial dilution method. HV agar medium (Hayakawa & Nonomura, 1987, 1989) supplemented with 50 µg/ml cycloheximide, 20 mg/ml nalidixic acid, and 20 mg/L kabisidin was used as isolation medium for the method No. 1 to 5, and NBRC medium No. 802 was used as isolation medium for method No. 6.

From this project, 102 isolates of filamentous Actinomycetes and 215 isolates of marine Actinomycetes were selected and studied. Molecular identification based on 16S rRNA gene revealed that 9 suborder, 18 families, and 30 genera of studied Actinomycetes were found from the sequence data (Table 4).

The predominant genus of Actinomycetes was *Streptomyces*, *Isoptericola*, and *Microbacterium*. Several new species were described, including *Tropicihabitans flavus* gen. nov., sp. nov. (Hamada et al., 2015a), *Serinibacter tropicus* sp. nov. (Hamada et al., 2015b), *Actinoplanes tropicalis* sp. nov., *Actinoplanes cibodasensis* sp nov., and *Actinoplanes bogoriensis* sp. nov. (Nurkanto et al., 2015, 2016a), and *Cryptosporangium cibodasense* sp. nov. (Nurkanto et al., 2016b).

Table 3. Number of Samples Used During Exploration of Actinomycetes in Indonesia under InaCC SATREPS Project

Year	Sampling site	Number of sample		Total
		Soil/ Sediment	Plant litter/ Coral/Sea weed	
2011	1. Cibodas	15	5	**20**
	2. Gunung Pancar Hot spring	4	-	**4**
	3. Ciseeng Hot spring	3	-	**3**
	4. Satonda Island	4	-	**4**
	5. Mount Tambora	5	-	**5**
2013	6. Kepulauan Seribu (Pari Island, Rambut Island, and Pramuka Island)	24	4	**28**
2014	7. Bali Island	20	-	**20**
Total		75	9	84

Source: Internal report InaCC Satreps Project

Table 4. Actinomycetes from the InaCC SATREPS Project

	Suborder		Family		Genus	∑ of isolates
1	*Corynebacterineae*	1	*Corynebacteriaceae*	1	*Corynebacterium*	6
		2	*Dietziaceae*	2	*Dietzia*	2
		3	*Nocardiaceae*	3	*Nocardia*	2
				4	*Rhodococcus*	8
2	*Propionibacterineae*	4	*Propionibacteriaceae*	5	*Microlunatus*	1
3	*Kineosporiineae*	5	*Kineosporiaceae*	6	*Kineosporia*	1
4	*Micrococcineae*	6	*Brevibacteriaceae*	7	*Brevibacterium*	5
		7	*Cellulomonadaceae*	8	*Demequina*	12
		8	*Dermabacteraceae*	9	*Brachybacterium*	10
		9	*Microbacteriaceae*	10	*Leucobacter*	1
				11	*Microbacterium*	22
		10	*Micrococcaceae*	12	*Kocuria*	17
				13	*Arthrobacter*	6
				14	*Citricoccus*	1
				15	*Micrococcus*	1
				16	*Zhihengliuella*	1
		11	*Promicromonosporaceae*	17	*Isoptericola*	92
				18	*Cellulosimicrobium*	3
5	*Micromonosporineae*	12	*Micromonosporaceae*	19	*Dactylosporangium*	1
				20	*Micromonospora*	3
6	*Propionibacterineae*	13	*Nocardioidaceae*	21	*Nocardioides*	1
7	*Pseudonocardineae*	14	*Actinosynnemataceae*	22	*Saccharothrix*	1
		15	*Pseudonocardiaceae*	23	*Amycolatopsis*	1
				24	*Saccharopolyspora*	1
8	*Streptosporangineae*	16	*Streptosporangiaceae*	25	*Microbispora*	1
				26	*Nonomuraea*	1
		17	*Thermomonosporaceae*	27	*Actinomadura*	2
9	*Streptomycineae*	18	*Streptomycetaceae*	28	*Kitasatospora*	3
				29	*Streptacidiphilus*	1
				30	*Streptomyces*	106
	TOTAL					313

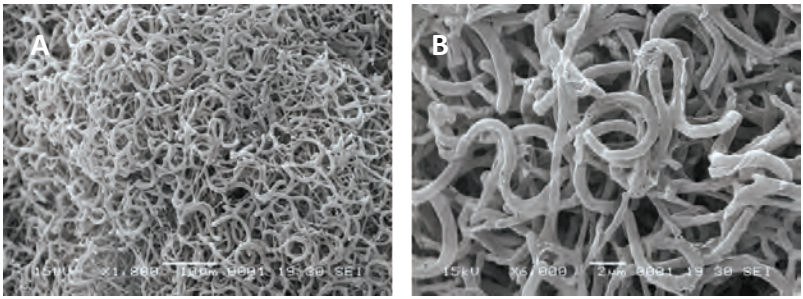
Source: Internal Report InaCC SATREPS Project

Description of New Species of Actinomycetes from Indonesia

The Genus *Streptomyces*

Sembiring et al. (2001) isolated large number of putatively novel streptomycetes from environmental samples collected from in and around the root system of the tropical angiosperm, *Paraserianthes falcataria*. Representative isolates were assigned to 37 multi-membered and 107 single membered color groups based on their ability to form pigments on oatmeal and peptone yeast extract iron agar. The largest taxon, color group 3, encompassed 94 isolates which had morphological properties typical of members of the *Streptomyces violaceusniger* clade. Twelve representatives of this taxon chosen on the basis of Curie-point pyrolysis mass spectrometric data were compared with representatives of the validly described species which constitute the *S. violaceusniger* clade. Six out of the twelve representative strains were readily distinguished from one another and from the marker strains using a combination of genotypic and phenotypic properties. These organisms were consequently considered to merit species status as *Streptomyces asiaticus* sp. nov., *Streptomyces cangkringensis* sp. nov., *Streptomyces indonesiensis* sp. nov., *Streptomyces javensis* sp. nov., *Streptomyces rhizosphaerius* sp. nov., and *Streptomyces yogyakartensis* sp. nov.

Otoguro et al. (2009) described the taxonomic positions of Actinomycetes strains ID03-0915^T and ID03-0825 isolated from soil of Bali Island, Indonesia using a polyphasic taxonomic approach which named *Streptomyces baliensis* (ba.li.en'sis. N.L. masc. adj. baliensis pertaining to the island of Bali, Indonesia, where the first strains were isolated). Phylogenetic analyses performed using almost-complete 16SrRNA gene sequences demonstrated that the strains were closely related to *Streptomyces glauciniger* and *Streptomyces lilacinus*. However, DNA–DNA hybridization and phenotypic characteristics revealed that the strains differed from known *Streptomyces* species, and



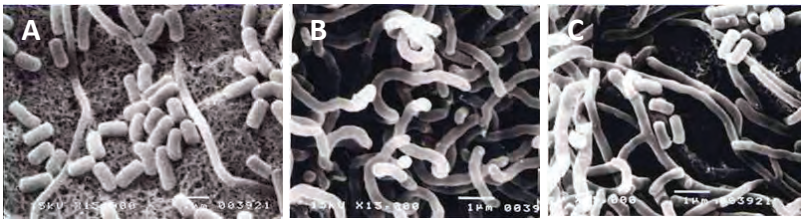
Source of photographs: Widyastuti & Ando, 2009

Figure 3. *Streptomyces baliensis* (A) x1800 Magnification and (B) x6000 Magnification

should be classified within a novel species of the genus *Streptomyces*, which the name *Streptomyces baliensis* sp. nov. was described. Photograph of the strains taken by Scanning Electron Microscope (SEM) was shown in Figure 3.

The genus *Actinokineospora*

Lisdiyanti et al. (2010) described six Actinomycetes strains belonged to the genus *Actinokineospora* that were found from soil and leaf litter sample in Eka Karya Botanical Garden, Bali, and Cibodas Botanical Garden, West Java, in 2003. These strains were isolated by RC method for the selective isolation of motile rare Actinomycetes group. From the genotypic, chemotaxonomic, and phenotypic data, the six Indonesian strains represented three novel species in the genus *Actinokineospora*. The names *Actinokineospora baliensis* sp. nov., *Actinokineospora cibodasensis* sp. nov., and *Actinokineospora cianjurensis* sp. nov. were described. Photograph of the new taxa taken by SEM is shown in Figure 4.

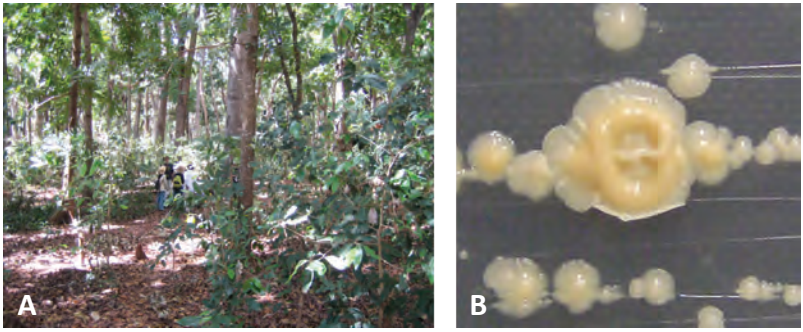


Source of photographs: Widyastuti & Ando, 2009

Figure 4. Photograph of Cell Morphology Taken by SEM of (A) *Actinokineospora cibodasensis*, (B) *Actinokineospora baliensis*, and (C) *Actinokineospora cianjurensis*.

The Genus *Dietzia*

Yamamura et al. (2010) described a rare Actinomycetes *Dietzia timorensis* pertaining to West Timor, Indonesia, from where the organism was first isolated (Figure 5). This strain was isolated using the SDS-yeast extract pre-treatment method from soil under mahogany (*Swietenia mahogani*) trees. The chemotaxonomic characteristics of strain ID05-A0528^T were consistent with its assignment to the genus *Dietzia*.



Source of photographs: Widyastuti & Ando, 2009

Figure 5. (A) Source of New Species of *D. timorensis,* Soil Under *Sweitenia mahogany* and (B) Colony Appearance of *D. timorensis* (ID05-A0528^T)

The Genus *Actinophytocola*

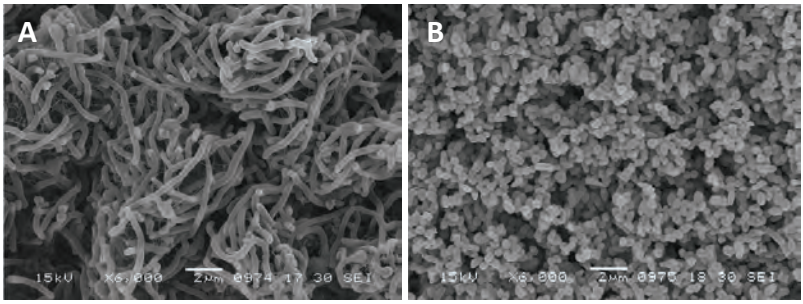
Otoguro et al. (2011) described two Actinomycetes strains, ID05-A0653^T and ID06-A0464^T isolated from soil of West Timor and Lombok Island, respectively. 16S rRNA gene sequence analysis clearly demonstrated that the strains belonged to the family *Pseudonocardiaceae* and were closely related to the genus *Actinophytocola*. These two new taxa were proposed as *Actinophytocola timorensis* (ti.mo.ren'sis. N.L. fem. adj. timorensis pertaining to West Timor, Indonesia, from where the type strain was isolated) and *Actinophytocola corallina* (co. ral.li'na. L. fem. adj. corallina coral red, because the organism produces coral-colored soluble pigment).

The Genus *Tropicihabitans*

From the rhizosphere of a mangrove and sea sediment sample collected from the foreshore of Rambut Island, DKI Jakarta, Indonesia, Hamada et al. (2015a) described two novel Gram positive Actinomycetes, designated PS-14-16^T and RS-7-1, respectively. These strains were distinguishable from the member of the genus *Sediminihabitans* and the other genera within the family *Cellulomonadaceae* based on chemotaxonomic characteristics and phylogenetic relationship, and represented a novel genus and species of the family *Cellulomonadaceae*, with the name *Tropicihabitans flavus* gen. nov., sp. nov. Photograph of the strain taken by SEM was shown in Figure 6.

The Genus *Serinibacter*

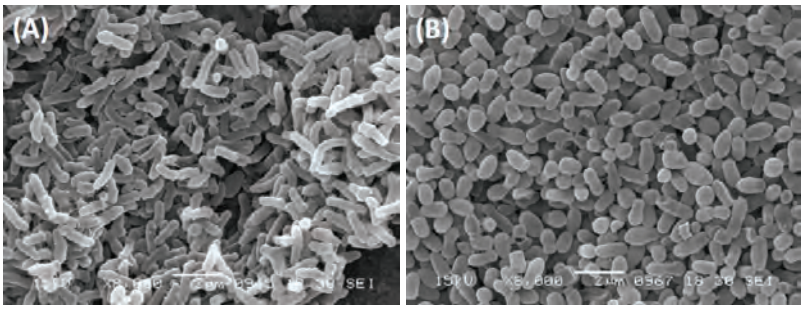
Discovery of novel microbes from natural environment is important because it helps to understand the microbial diversity of a given habitat and may also result in isolation of bacteria with possible industrial or other applications. In recent study on the isolation and diversity of Actinomycetes in seashore environments, Hamada et al. (2015b) isolated a novel Actinomycetes from a sediment sample that were



Source of photographs: Hamada et al., 2015a

Figure 6. Scanning Electron Micrographs of *Tropicihabitans flavus* sp. nov. grown on NBRC Medium 802 for (A) 1 day and (B) 4 days at 28°C; bars, 2 µm.

collected from the rhizosphere of a mangrove (*Rhizopora mucronata*) growing on the seashore of Pramuka Island, DKI Jakarta, Indonesia. A novel species of the genus *Serinibacter*, for which the name *Serinibacter tropicus* sp. nov. was proposed (Figure 7).



Source of photographs: Hamada et al., 2015b

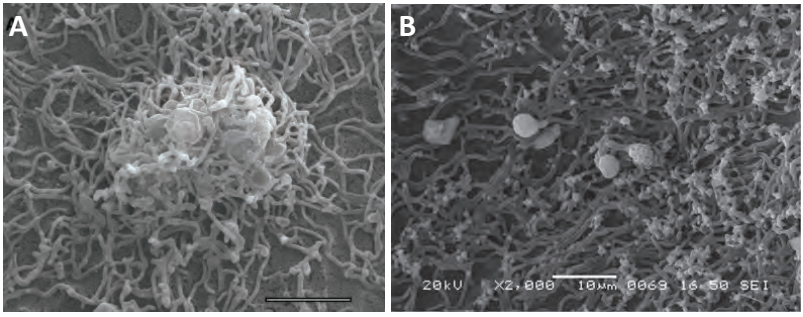
Figure 7. Scanning Electron Micrographs of Cells of *Serinibacter tropicus* sp. nov grown on NBRC Medium 802 for (A) 1 and (B) 7 days at 28 °C. Bars, 2 µm.

The Genus *Cryptosporangium*

Nurkanto et al. (2015b) described a novel actinomycete strain, designated LIPI11-2-Ac046^T isolated from a leaf litter sample obtained from Cibodas Botanical Garden, West Java, Indonesia, using the rehydration and centrifugation method. The results of DNA–DNA hybridization as well as physiological and biochemical analyses distinguished strain LIPI11-2-Ac046^T from known members of the genus *Cryptosporangium*. On the basis of these data, it was proposed that strain LIPI11-2-Ac046^T represents a novel species of the genus *Cryptosporangium*, with the name *Cryptosporangium cibodasense* sp. nov., pertaining to Cibodas Botanical Garden, West Java, Indonesia, where the organism was originally isolated (Figure 8A).

The Genus *Actinoplanes*

Nurkanto et al. (2015, 2016a) described three novel Actinomycetes strains, designated LIPI11-2-Ac034^T, LIPI11-2-Ac042^T, and LIPI11-2-Ac043^T that were isolated from leaf litter sample collected from Cibodas Botanical Garden, West Java, Indonesia (Figure 8B).



Source of photographs: Nurkanto et al., 2015; 2016

Figure 8. Scanning Electron Micrograph of strain (A). *Cryptosporangium cibodasense* sp. nov. (B). *Actinoplanes bogoriensis* sp. nov., Cultivated on Humic Acid-vitamin (HV) Agar for 2 weeks at 28°C. Bar, 10 µm.

Phylogenetic analysis based on 16S rRNA gene sequences suggested that the three isolates belonged to the genus *Actinoplanes*. The genus was one of the Actinomycetes that was well-known as producer for several bioactive compounds, and because of its value for drug discovery, studies on the bioprospecting and taxonomy of new species and genus of Actinomycetes were important.

It has been proven that Indonesia is a mega biodiversity country and rich in microbial diversity. At the time of writing, 13 new taxa of Actinomycetes from eight families were found in Indonesia and validly described as shown in Table 5. A huge number of new species and even new genus were still waiting to be described.

Screening for Bioactive Compounds of Actinomycetes

Actinomycetes have been proven to be a rich source of important natural products especially antibiotics. Thus far, approximately 10,000 antibiotics have been found and almost half of them are produced by *Streptomyces* originated in the soil (Lazzarini *et al.,* 2000). Recently, the discovery rate of new compounds from existing genera obtained from common soil has decreased, thus it is critical that novel Actinomycetes from unexplored habitats such as marine, hot spring be pursued as sources of novel antibiotics and others bioactive compounds.

Ratnakomala et al. (2011) screened 853 Actinomycetes isolated from soil and leaf litter samples from Kupang NTT and Enrekang, South Sulawesi, for its inhibition activity to RNA helicase of Japanese Encephalitis Virus (JEV). Those isolates were then tested for inhibition of ATPase activity of RNA helicase from JEV, in order to identify a drug candidate for the treatment of JEV infection. Results revealed that 14 isolates had relatively high inhibition activity on JEV ATPase activity of the JEV RNA-helicase, which ranged from 40.0–50.0%

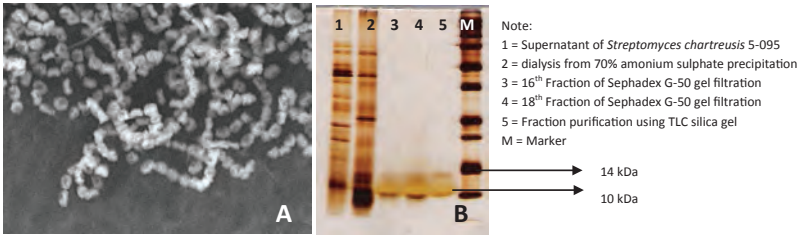
Table 5. Novel Taxa Discovered During Exploration of Actinomycetes in Indonesia

Family	Genus and Species	Isolation methods	Similarity with the closest species	G+C content of DNA	References
Streptomycetaceae	*Streptomyces baliensis* sp. nov	SDS/YE	*S. glauciniger* (98%), *S. lilacinus* (97.6%) and *S. abikoensis* (97.7%).	71.1 mol%.	Otoguro *et al.* (2009)
	Streptomyces asiaticus sp. nov. *Streptomyces cangkringensis* sp. nov. *Streptomyces indonesiensis* sp. nov. *Streptomyces javensis* sp. nov *Streptomyces rhizosphaerius* sp. nov *Streptomyces yogyakartensis* sp. nov.				Sembiring *et al.* (2001)
Actinosynnemataceae	*Actinokineospora baliensis* sp. nov	RC	*Actinokineospora diospyrosa* (99.4%)	71.4 mol%.	Lisdiyanti *et al.* (2010)
	Actinokineospora cibodasensis sp. nov	RC	*Actinokineospora auranticolor* (98.2%)	71.3 mol%	Lisdiyanti *et al.* (2010)
	Actinokineospora cianjurensis sp. nov.	RC	*Actinokineospora terrae* (99.4%)	70.2 mol%.	Lisdiyanti *et al.* (2010)
Corynebacteriaceae	*Dietzia timorensis* sp nov.	SDS/YE	*Dietzia cinnamea* (97.2%)	65.5 mol%	Yamamura *et al.* (2010)
Pseudonocardiaceae	*Actinophytocola timorensis* sp. nov	SDS/YE	*A. oryzae* GMKU367 ^{T} (98.1%)	69.7 mol%.	Otoguro *et al.* (2011)
	Actinophytocola corallina sp. nov	SDS/YE	*A. oryzae* GMKU367 ^{T} (98.2)	71.2 mol%.	Otoguro *et al.* (2011)
Cellulomonadaceae	*Tropicihabitans flavus* gen. nov., sp. nov	Serial dilution	*Sediminihabitans luteus* (97.94 %)	68.4–68.5 mol%	Hamada *et al.* (2015a)
Beutenbergiaceae	*Serinibacter tropicus* sp.nov.	Serial dilution	*Serinibacter salmoneus* Kis4-28^{T} (99.6%)	72.8 mol%	Hamada *et al.* (2015b)

Family	Genus and Species	Isolation methods	Similarity with the closest species	G+C content of DNA	References
Cryptosporangiaceae	*Cryptosporangium cibodasense* sp nov	RC	*C. minutisporangium* NBRC15962^T (98.9%)	72.7 mol%.	Nurkanto *et al.* (2016b)
Actinoplanaceae	*Actinoplanes tropicalis* sp. nov	RC	*A. ferrugineus* NBRC15555^T(98.2%)	71.5 mol%	Nurkanto *et al.* (2015)
	Actinoplanes cibodasensis sp. nov.	RC	*A. durhamensis* JCM 7625^T (97.7 %)	70.7 mol%.	Nurkanto *et al.* (2015)
	Actinoplanes bogoriensis sp. nov.	RC	*Actinoplanes abujensis* A4029^T (99.3%)	71 mol%.	Nurkanto *et al.* (2016a)

inhibition. The highest inhibition activity was identified in *Actinoplanes philippinensis* 5-849 and *Streptomyces chartreusis* 5-095, with the inhibition-activity of 49.9% and 49.2%, respectively. *Streptomyces chartreusis* 5-095 (Figure 9A) produced the inhibitor of JEV RNA helicase, an essential enzyme for virus replication. The study was objected to purify an extracellular protein inhibitor of JEV RNA helicase, particularly on its ATPase activity from the supernatant of *Streptomyces chartreusis* 5-095 culture. The protein inhibitor was purified by several steps of purification, and the specific activity after purification was 36.48 U/mg. Gradient polyacrilamide gel electrophoresis analysis showed that the relative molecular mass of the proteins were estimated to be 10,000 Da and 14,000 (Figure 9B) (Elfita *et al*., 2009; Ratnakomala *et al*., 2009).

Hairany et al. (2008) also screened the Hepatitis C virus (HCV) RNA helicase inhibitor, from Indonesian indigenous Actinomycetes, which might be used as a candidate for development of anti-HCV drug. The inhibitor protein was performed by screened ATPase inhibitor of HCV RNA helicase, due to the simple and easy use of the assay compared to RNA helicase and RNA binding assays. Of 2,000 isolates of Actinomycetes, some isolates produced the inhibitor of ATPase of the HCV RNA helicase. Seventeen isolates showed a relatively high



Source: Ratnakomala et al., 2009

Figure 9. (A) Microscope Appearance of *Streptomyces chartreusis* 5-095 and (B) SDS PAGE Profile from Protein Inhibitor ATPase

inhibition activity (40–75%) against ATPase activity of HCV RNA helicase. The highest inhibition activity was showed by *Streptomyces castaneus* ID06-313, *Streptomyces higroscopicus* ID06-469, and *Streptomyces* sp. ID05-271 with the inhibition activity of 75%, 57%, and 49%, respectively. These isolates were then used for purification of the HCV RNA helicase inhibitor.

Pudjiraharti et al. (2011, 2014) screened 100 isolates of Actinomycetes for inulin fructotransferase producing isolates. All isolates were inoculated in 10 mL of inulase induction medium and was incubated at 27 °C with shaking at 140 rpm for 5 days. As a result, an isolate that excretes inulin fructotransferase to the culture supernatant was able to produce di-D-fructofuranose 1,2':2,3' dianhydride (DFA III) from inulin, with the greatest rate of enzyme activity at 65 °C and at a pH of 5.5. Through chemotaxonomic and 16S rRNA gene analysis, this strain was identified into the genus *Nonomuraea* in the family *Streptosporangiaceae*. This is the first report of an inulin fructotransferase producer in this family.

Ratnakomala et al. (2015) studied mannanase producing Actinomycetes. The strain ID06-379 displayed significant mannanase activity. The strain ID06-379 was analyzed for its mannanase activity by determining the rate of enzyme production when cultured in the presence of palm kernel cake (PKC) as a substrate. The highest mannanase activity from ID06-0379 was 4.40 U/mL at 5% PKC concentration at 5 days incubation. Chemotaxonomic and phenotypic characterization of mannanolytic actinomycete was done, and based on phylogenetic analysis of its 16S rRNA gene sequence, it revealed that strain ID06-379 was closely related to species of *Nonomuraea jabiensis* A 4036^{T} with 99% nucleotide similarity in the family *Streptosporangiaceae*. A novel endo-β-1, 4-mannanases gene was successfully isolated and structurally analyzed from soil Actinomycetes *N. jabiensis* ID06-379. Alignment from several partial fragments sequences of mannanases

gene *N. jabiensis* ID06-379 gives 2,100 base pairs long. This segment contains a full length of β-mannanases gene of *N. jabiensis* ID06-379. The deduced sequence of β-mannanases gene *N. jabiensis* ID06-379 consisting of 1,338 bp encodes a 446-amino acid polypeptide. Crude enzyme of wild type *N. jabiensis* ID06-379 when grown in an optimized medium reached the highest enzyme activity of 2.3 U mL^{-1}, while the crude of the recombinant enzyme was obtained by 97.83 U mL^{-1}. The activity increased as much as 43 times in crude recombinant enzyme compared with the wild-type (Ratnakomala, 2015).

Conclusion

Actinomycetes represent important organism for industrial application. There are many interesting isolates of Actinomycetes from Indonesia. Only some of them have been isolated, identified, and characterized. Preservation of the isolates is important and highly needed to maintain the existence of the isolates for future uses. This time, Indonesia has been established a new ISO standardized culture collection InaCC, as a national reference collection and *ex-situ* conservation for sustainable utilization of microbial resource.

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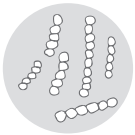

EPILOGUE: Microbiology for Better Future Life

✍ Puspita Lisdiyanti and Endang Sukara

Introduction

In the past, there were several microbes isolated from Indonesian environments used for researches, conducted by both Indonesian and foreign researchers. Some examples, Derx (1950) conducted further research on *Beijerinckia*, and then were continued by Ruinen (1956) in the 'phyllosphere' as also mentioned by Theunissen (1996). De Vries and Derx (1953) studied on the occurrence of *Mycoplana rubra* and its identity with *Protaminobacter ruber*. Saono and Basuki (1969) studied the amylolytic, lipolytic, and proteolytic activities of yeasts and mycelial molds from ragi and some Indonesian traditional fermented foods. Saono and Gandjar (1972) also studied the hydrocarbon utilizing soil yeasts from oil fields in Cepu region (Central Java), Indonesia. Saono et al. (1974) studied the microflora of *ragi* and some other traditional fermented foods of Indonesia that was continued by Dr. Atit Kanti and reported in a chapter in this book. Swings et al. (1976) reported that they used *Zymomonas* strains isolated from fermenting and fresh sap of *Arenga* for numerical analysis of electrophoretic protein patterns of

the genus (Figure 1). Brotonegoro and Abdulkadir (1978) described the decomposition of *Azolla pinnata* in moist and flooded soil. Becking (1979) conducted a topic research on nitrogen fixation by *Rubus ellipticus* found in Cibodas Botanical Garden. However, most of the microbial genetic resources were not preserved in Indonesian culture collections. Therefore, as described in this book, it is time to collect and deposit useful microbial genetic resources in Indonesia.

From this book, it is revealed that research on number of microbes, which have been isolated from various sources in Indonesia, has been continued. The process of identification, characterization, and preservation in culture collections has been conducted. The microbes that are useful for human welfare i.e. *Rhizobium* for biofertilizer; lactic acid bacteria for making silage; lactic acid bacteria, acetic acid bacteria, yeasts, and *Rhizopus* for starter culture of fermented foods; acetic acid bacteria for food and biomaterial production; *Aspergillus awamori* for enzyme production; and Actinomycetes for enzyme production and as source of pharmaceutical compounds are also described. This book highlights some achievements of small number of scientists in LIPI who are willing to share their experiences with the audience interested on the sustainable use of Indonesian microbial genetic resources. In the near future, when the output from the on-going research is accumulated, it is possible for LIPI to publish another book which should be important for us to know the value of the intangible asset, microbial genetic resources deposited at InaCC, Cibinong Science Center LIPI.

In this chapter, the authors described the need of private companies to involve in the research and prospect and challenges of microbial genetic resources for human welfare in this global and competitive era.

Table 1. *Origin of the additional Zymomonas strains used*

	Strain designation		
	Z. mobilis strain 3 Delft	*Z. mobilis* strain 6 Delft	*Z. mobilis* strain B70
Isolated from	Fermenting sap of *Arenga*	Fresh sap of *Arenga* (tuak)	Infected British ale
Place of isolation	Treub Laboratory, Bogor, Java, Indonesia	University, Bandung, Java, Indonesia	Licensed premises, England
Date of isolation	August 1949	1949	
Isolated by	Dr H. G. Derx	Professor C. O. Schaeffer	Dadds, Martin & Carr, 1973

Source: Swings et al., 1976

Figure 1. The Example of Using of Microbes from Indonesia

Involvement of Private Companies

It is an irony, that the interest of Indonesian private to explore new commercial product from Indonesian microbial genetic resources is still low. This fact is also mentioned by Saono (2005). The production of inoculums for tempe production is the only example done by industry for commercial purposes, but the other products are still very rare. This is only being initiated by internal LIPI scientists who form cooperation.

It is the time for the Government of Indonesia to set up a new initiative to provide appropriate condition e.g. tax incentive to the private sectors for their involvement in utilizing microbial genetic resources in industry. At the same time, funding for continuous fundamental research by research institute like LIPI has to be allocated. Allocation should also be dedicated to improve research facilities and the man-power development.

LIPI, as a scientific authority on biodiversity in Indonesia, should stay committed to continue the inventory of microbial genetic resources in Indonesia which is important to human welfare. In this near future, attempts should be conducted through strategic partnership with private sectors. It is believed that with such collaboration, the

Indonesian microbial genetic resources could be used for the prosperity of Indonesian nation and for great impact to human welfare.

Prospect and Challenges

As the second largest country in the world, in terms of tropical rain forest areas after Brazil in South America, Indonesia consist of many forest types and is also very rich in biological diversity. Microbe is one of biological diversities that is not explored yet. Riswan and Yamada (2006) predicted that the high pressure on tropical forest ecosystems in the form of natural and man-made disturbances, i.e. deforestation and other practices has caused the high rate of biodiversity losses on an unprecedented scale. They suggested curtailing this problem, doing more research, and acting actively to save our biodiversity, which includes ecosystem, species and genetic diversity as well as microbes and microbial genetic resources. Culture collections play a role in underpinning microbiology, supplying the genetic resources for study, innovation, and discovery (Smith, 2012). Indonesia has at least 18 culture collections registered at World Federation of Culture Collection (WFCC) for *ex situ* conservation of microbes, repositories for strains subject to publication, and patent deposits from researches. Therefore, researches on exploring of Indonesian microbes should be continued so that it can be useful for human welfare including food, feed, health and bioenergy.

Cultivating the microbes still poses many difficulties. Technical difficulties in cultivating novel microorganisms need modern approaches. This could help us to overcome these limitations. These are new topics that we need to improve our knowledge in. Genomic and proteomic approaches are a must. Prakash et al. (2013) described the role of microbial resource centers in the omics era. Networking to make global catalogue of microorganisms was also initiated by

World Data Center for Microorganisms (WDCM) (Wu et al., 2013). LIPIMC, which eventually merged with BTCC to form InaCC, is one of the members of this network that should have greater chances to participate. Using this feature, the authors can have comprehensive database and information retrieval, analysis, and visualization system for microbial resources.

However, the issue of the Nagoya Protocol (NP) on Access and Benefit Sharing (ABS) which is already been ratified by National Act No, 11/2013 poses challenges for biological collections (Watanabe, 2015). Even as researchers, they should have a better understanding of this issue. Another issue that is also important is about the concept of microbial patenting and requisite guidelines of the "Budapest Treaty" for establishment of an International Depositary Authority that is useful for application of microbes in industries.

Conclusion

Indonesia is rich in microbial genetic resources. Work on Indonesian microbial resources has been started long before Indonesia's independence. There is no significant work after independence. Research on Indonesian microbes has been started again in the late 1960s, but the progress is very slow. Human resources in this field are very limited and laboratory facilities are poor. It is fortunate that in the last 10–15 years, research on Indonesian microbial genetic resources has been accelerated by the establishment of laboratories infrastructure of LIPI at Cibinong Science Center Campus, Cibinong, Indonesia. Many Ph. D. researchers graduated in Microbiology gradually return home from overseas. With the hard work of LIPI researchers, the number of microbial collection collected from diverse Indonesian ecosystem rapidly increases, including those important for the advancement of sciences and future industrial development. New species and new

genus of microbe continuously identified. The establishment of InaCC mark the new era for microbiologist in Indonesia. The authors do believe that InaCC and its collection should be able to contribute to the advancement of sciences in the field of microbiology and for the development of industries e.g. agriculture (food and feed), health (chemical compounds important in pharmaceutical and drug industries), energy (oleochemicals and hydrogen based energy), environment (composting, oil splitting microbes), and other purposes benefited to human welfare. The journey has now begun.

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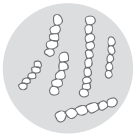

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Exploring Indonesian

MICROBIAL GENETIC RESOURCES

for Industrial Application

Indonesia is a mega biodiversity country consisting of various types of animals and plants, including genetic microbial resources. However, the exploration on microbes has not been yet extensively explored. This book highlights some important findings and achievements carried out by the microbiologists in LIPI on the sustainable use of Indonesian microbial genetic resources.

Through this book, some successful processes of identification, characterization, and preservation in culture collections of Indonesian microbial genetic resources have been showed vividly. Some of potential microbes useful for human welfare are also described in this book, including their utilization for food, feed, health, and bioenergy.

It is expected that this book can be a useful reference for those who are interested in the importance of microbial genetic resources for the prosperity of the nation as it revealed some significant findings on microbes, which have been isolated from various sources in Indonesia.

LIPI

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