

The Second International Conference on Genetic Resources and Biotechnology

Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture

Bogor, Indonesia • 24–25 May 2021

Editors • I Made Tasma, Dwinita Winkan Utami, Ika Roostika,
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Preface: The Second International Conference on Genetic Resources and Biotechnology

The Second International Conference on Genetic Resources and Biotechnology, which is the continuation of the first event held in 2018, focuses on topics related to advances in biotechnology to create more opportunities for effective conservation and sustainable utilization of genetic resources for food and agriculture. This year conference's theme is Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture. The conference was organized by Indonesian Agency for Agricultural Research and Development (IAARD), Ministry of Agriculture, Indonesia, in collaboration with Indonesian Biotechnology Consortium and held on 24th-25th of May 2021 virtually due to the pandemic of COVID-19.

The conference aims to share and exchange current scientific information and technological developments on biotechnology and their applications for conservation and sustainable use of genetic, to encourage and promote quality, efficiency, and modernization of management and utilization of genetic resources, and to facilitate national and international collaboration among participants. There are five scopes discussed in this conference. They are effective management of conservation and sustainable use of genetic resources for food and agriculture, application of genomics and molecular markers for genetic resource conservation and crop adaptation to climate change, application of innovative crop improvement techniques for conservation and sustainable use of plant genetic resources for food and agriculture, plant cell and tissue culture for conservation and effective utilization of genetic resources, and the use of microbial genetic resources as biological control agents of agricultural pests and diseases, and for soil bioremediation.

Five speakers from the United States of America, Japan, India and Indonesia were invited to discuss about their expertise and knowledge on relevant subjects in the plenary sessions. This conference was attended by more than 100 participants including 75 presenters and 44 listeners worldwide. They came from diverse governmental, private, or academic institutions and also scientific communities. The presented materials have undergone peer review processes and only qualified papers were selected. Furthermore, all papers were subjected to double blind peer-review and expected to meet the scientific criteria of significance and academic excellence to be published in a conference proceedings indexed in a well-known, reputable service.

We would like to express our sincere gratitude to our speakers, presenters and all participants for their contributions in this conference. We would also like to express our appreciation for the generosity of our sponsors that support this conference: PT CropLife, PT ITS Science Indonesia, PT Fajar Mas Murni and PT Prima Instrument Analitika. Lastly, special thanks to all committee members for their exceptional work and contributions in the conference and publication.

Chair of Organizing Committee

Dr. Toto Hadiarto

Table of Contents

THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture



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- [20](#)
- [50](#)

- [100](#)
- [all](#)

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EFFECTIVE MANAGEMENT OF CONSERVATION AND SUSTAINABLE USE OF GENETIC RESOURCES FOR FOOD AND AGRICULTURE

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Harnessing plant genetic resources through biotechnology for food security in Indonesia

[Mastur](#), [Reflinur](#), [Nurul Hidayatun](#), [Sustiprijatno](#), [Fatimah](#), [Tri Puji Priyatno](#) and [Puji Lestari](#)
AIP Conference Proceedings **2462**, 020001 (2022); <https://doi.org/10.1063/5.0075671>

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DNA barcoding of *Vatica bantamensis*, a critically endangered tree endemic to Banten, Indonesia

[Muhammad Rifqi Hariri](#), [Iyan Robiansyah](#), [Dipta Sumeru Rinandio](#), [Dodo](#), [Desi Siti Sundari](#), [Cecep H. Sukmawan](#) and [Bayuntoro Ardi](#)
AIP Conference Proceedings **2462**, 020002 (2022); <https://doi.org/10.1063/5.0075529>

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Genetic parameters of agronomic traits in soybean (*Glycine max* [L.] Merrill) genotypes tolerant to drought

[Made J. Mejaya](#), [Suhartina](#), [Purwantoro](#), [Novita Nugrahaeni](#) and [Titik Sundari](#)
AIP Conference Proceedings **2462**, 020003 (2022); <https://doi.org/10.1063/5.0075159>

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Yield stability performance of soybean (*Glycine max* [L.] Merrill) lines tolerant to drought

[Suhartina](#), [Purwantoro](#), [Novita Nugrahaeni](#), [Abdullah Taufiq](#) and [Made Jana Mejaya](#)
AIP Conference Proceedings **2462**, 020004 (2022); <https://doi.org/10.1063/5.0075158>

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FreeJanuary 2022

Polymorphisms and associations of the *RACK1* genes with antibody response to Newcastle disease in KUB chickens

[Ifa Manzila](#), [Puji Lestari](#), [Tike Sartika](#), [Tri Puji Priyatno](#), [Risa Indriani](#), [Kristianto Nugroho](#) and [Rerenstradika Tizar Terryana](#)

AIP Conference Proceedings **2462**, 020005 (2022); <https://doi.org/10.1063/5.0075622>

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Rice grain quality evaluation of promising lines of rice under irrigation and for salinity tolerance

[Dody D. Handoko](#), [Nafisah](#), [Aris Hairmansis](#), [Trias Sitaresmi](#), [Heni Safitri](#), [Satoto](#), [Ali Imamuddin](#), [Cucu Gunarsih](#) and [Untung Susanto](#)

AIP Conference Proceedings **2462**, 020006 (2022); <https://doi.org/10.1063/5.0075956>

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Existing diversity profile for kernel characteristics of maize germplasm in IAARD-ICABIOGRAD gene bank

[Andari Risliawati](#), [Sobir](#), [Trikoesoemaningtyas](#), [Willy B. Suwarno](#) and [Puji Lestari](#)
AIP Conference Proceedings **2462**, 020007 (2022); <https://doi.org/10.1063/5.0075178>

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Characterization of Japansche citroen rootstock somaclones and *in vitro* selection for aluminium tolerance

[Deden Sukmadjaja](#), [Mia Kosmiatin](#) and [Tiwi Wati](#)
AIP Conference Proceedings **2462**, 020008 (2022); <https://doi.org/10.1063/5.0077888>

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FreeJanuary 2022

Resistance to brown planthoppers (*Nilaparvata lugens* Stål) in rice accessions originated from Sumatra Island, Indonesia

[Dodin Koswanudin](#), [Nurul Hidayatun](#) and [Muhamad Ace Suhendar](#)
AIP Conference Proceedings **2462**, 020009 (2022); <https://doi.org/10.1063/5.0075680>

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Morphological identification of underutilized local fruits in Kutai Barat Regency to support their conservation and sustainable use

[Fitri Handayani](#), [Nurbani](#) and [Asep Pebriandi](#)

AIP Conference Proceedings **2462**, 020010 (2022); <https://doi.org/10.1063/5.0075594>

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Genetic resources of adlay (*Coix lacryma-jobi* L.) in East Kalimantan as source of functional food

[Fitri Handayani](#), [Muhammad Amin](#) and [Muhammad Taufiq Ratule](#)

AIP Conference Proceedings **2462**, 020011 (2022); <https://doi.org/10.1063/5.0075593>

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-
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Screening of soybean genotypes resistance to rust disease (*Phakopsora pachyrhizi*)

[Sumartini](#) and [Kurnia Paramita Sari](#)

AIP Conference Proceedings **2462**, 020012 (2022); <https://doi.org/10.1063/5.0075674>

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Identification of soybean promising lines resistant to pod-sucking bug, *Riptortus linearis* (Fabricius)

[M. Muchlish Adie](#), [Titik Sundari](#), [Kurnia Paramita Sari](#) and [Ayda Krisnawati](#)
AIP Conference Proceedings **2462**, 020013 (2022); <https://doi.org/10.1063/5.0075343>

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Variation in pod shattering resistance among black soybean genotypes associated with agronomic traits

[Ayda Krisnawati](#), [Titik Sundari](#) and [M. Muchlish Adie](#)
AIP Conference Proceedings **2462**, 020014 (2022); <https://doi.org/10.1063/5.0075338>

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Preliminary characterization and identification of genetic integrity of velvet bean germplasm in IAARD-ICABIOGRAD gene bank

Nurwita Dewi, Andari Risliawati and Nurul Hidayatun

AIP Conference Proceedings **2462**, 020015 (2022); <https://doi.org/10.1063/5.0076355>

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Plant parasitic nematodes infesting three minor legumes (velvet bean, lablab bean, and jack bean)

Chaerani, Try Zulchi P. Hariyadi and Nurwita Dewi

AIP Conference Proceedings **2462**, 020016 (2022); <https://doi.org/10.1063/5.0075204>

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Proactive management approach of seed PGRFA conservation during the pandemic of coronavirus disease (COVID-19) in Indonesia

Nurul Hidayatun, Andari Risliawati, Nurwita Dewi, Lina Herlina and Dodin Koswanudin

AIP Conference Proceedings **2462**, 020017 (2022); <https://doi.org/10.1063/5.0075531>

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Evaluation of mung bean accessions in saline soil based on quantitative morphological characters

[Trustinah](#), [Ratri Tri Hapsari](#), [Rudi Iswanto](#) and [Rudy Soehendi](#)

AIP Conference Proceedings **2462**, 020018 (2022); <https://doi.org/10.1063/5.0075324>

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Screening and evaluation of 100 upland rice accessions for developing high-yielding upland rice varieties tolerant against acid soil

[Lina Herlina](#) and [Yusi N. Andarini](#)

AIP Conference Proceedings **2462**, 020019 (2022); <https://doi.org/10.1063/5.0075550>

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Morphological characters of sugarcane mutant (*Saccharum officinarum* L.) from *in vitro* selection for drought stress

Rr. Sri Hartati, Sri Suhesti and Nurya Yuniyati

AIP Conference Proceedings **2462**, 020020 (2022); <https://doi.org/10.1063/5.0075656>

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Identifying potential seedless citrus accessions through floral structure and pollen performance

Baiq Dina Mariana, Anis Andrini and Sri Andayani

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Secondary characters based selection of Indonesian kenaf (*Hibiscus cannabinus* L.) germplasm for developing superior varieties

Taufiq Hidayat R. S., Marjani, Nurindah, Muhammad Rasyidur Ridho, Cynthia Lestari Hertianti and Widya Fatriasari

AIP Conference Proceedings **2462**, 020022 (2022); <https://doi.org/10.1063/5.0075716>

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Genetic relationship of pigmented rice (*Oryza sativa* L.) collected from Eastern Indonesia based on morpho-agronomical traits and SSR markers

[Yusi Nurmalita Andarini](#), [Willy Bayuardi Suwarno](#), [Hajrial Aswidinnoor](#) and [Hakim Kurniawan](#)
AIP Conference Proceedings **2462**, 020023 (2022); <https://doi.org/10.1063/5.0075706>

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Rejuvenation and morphological characterization of local rice from the province of Yogyakarta

[Setyorini Widyayanti](#), [Sutarno](#), [Endang Wisnu Wiranti](#) and [Kristamtini](#)
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Characterization of plant architecture and yield trait of castor (*Ricinus communis* L.) germplasm suitable for mechanical harvesting

[Tantri Dyah Ayu Anggraeni](#) and [Rully Dyah Purwati](#)

AIP Conference Proceedings **2462**, 020025 (2022); <https://doi.org/10.1063/5.0075155>

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Characterization and interrelationships of the number of vessel bundles with yield components in various genotypes of soybean (*Glycine max* [L.] Merrill)

[Anna S. Karyawati](#) and [Dyah P. Fitrawantio](#)

AIP Conference Proceedings **2462**, 020026 (2022); <https://doi.org/10.1063/5.0075693>

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Tuber starch content of edible canna (*Canna indica* L.) from different geographical origins

[Surya Diantina](#), [Randy Sanjaya](#), [Kristina Dwi Atmini](#), [Ace Suhendar](#) and [Dodin Koswanudin](#)

AIP Conference Proceedings **2462**, 020027 (2022); <https://doi.org/10.1063/5.0075922>

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The diversity of morpho-agronomic characters and identification of early maturity cassava (*Manihot esculenta* Crantz.) germplasm

[Tinuk Sri Wahyuni](#), [Kartika Noerwijati](#) and [Made J. Mejaya](#)
AIP Conference Proceedings **2462**, 020028 (2022); <https://doi.org/10.1063/5.0075658>

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Radiosensitivity and phenotypic characterization of gamma ray-induced mutant population of four *Capsicum annum* L. cultivars grown in screen house

[Andri Fadillah Martin](#), [Dyah Retno Wulandari](#), [Tri Muji Ermayanti](#), [Betolini Widhi Hapsari](#), [Erwin Al Hafiih](#) and [Laela Sari](#)
AIP Conference Proceedings **2462**, 020029 (2022); <https://doi.org/10.1063/5.0075173>

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Morphological performances of mutant butterfly pea (*Clitoria ternatea* L.)

[Try Zulchi](#), [Ali Husni](#), [Dwinita Wikan Utami](#), [Reflinur](#), [Mia Kosmiatin](#), [Tarkus Suganda](#) and [Agung Karuniawan](#)

AIP Conference Proceedings **2462**, 020030 (2022); <https://doi.org/10.1063/5.0075592>

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Screening of beta carotene and its correlation with tuber flesh color in sweet potato

[Kristina Dwi Atmini](#), [Surya Diantina](#), [Muhamad Sabda](#) and [Dodin Koswanudin](#)

AIP Conference Proceedings **2462**, 020031 (2022); <https://doi.org/10.1063/5.0075618>

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Evaluation of morpho-agronomical characters and grain quality of red rice lines

[Heni Safitri](#) and [Puji Lestari](#)

AIP Conference Proceedings **2462**, 020032 (2022); <https://doi.org/10.1063/5.0078807>

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Growth variation and relationship of clove progenies of high-yielding mother trees collected from various regions in Indonesia

[Mariana Susilowati](#), [Sri Wahyuni](#), [Adi Setiadi](#) and [Nurliani Bermawie](#)

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Screening on bast fiber plants resistant to spiral stem borer, *Agrilus acutus* (Coleoptera: Buprestidae)

[Sujak](#), [Nurindah](#), [Dwi Adi Sunarto](#), [Marjani](#) and [Nurul Hidayah](#)

AIP Conference Proceedings **2462**, 020034 (2022); <https://doi.org/10.1063/5.0075691>

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Characteristic of indigenous *Leuconostoc mesenteroides* EN 17-11 protease and its stability during storage at cold and freezing temperatures

[Tatik Khusniati](#), [Ika](#), [Harry Noviard](#) and [Sulistiani](#)

AIP Conference Proceedings **2462**, 020035 (2022); <https://doi.org/10.1063/5.0076004>

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Performance of introduced lines based on morphological markers for diversity enrichment of Indonesian chili pepper (*Capsicum annum* L.) varieties

[Rinda Kirana](#), [Catur Hermanto](#), [Reflinur](#) and [Derek W. Barchenger](#)

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[Chotimatul Azmi](#), [Imas Rita Saadah](#), [Nazly Aswani](#) and [Asih Kartasih Karjadi](#)

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Genetic diversity analysis of *Castanopsis argentea* using random amplified polymorphic DNA markers

Muhammad Imam Surya, Lily Ismaini, Decky Indrawan Junaedi, Aisyah Handayani, Taufikurrahman Nasution, Muhammad Efendi, Andes Hamuraby Rozak, Zaenal Mutaqien, Musyarofah Zuhri, Imawan Wahyu Hidayat, Fitri Kurniawati, Vandra Kurniawan, Dwindi Mariska Putri and Risha Amilia Pratiwi
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Current status of tidal swamp rice varieties and its improvement for Fe toxicity tolerance and biofortification

Muhamad Sabran, Dwinita Wikan Utami and Susilawati
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Agroforensic, a new emerging study using molecular marker technique

[Edy Listanto](#), [Ahmad Warsun](#), [Ahmad Dadang](#), [Eny Ida Riyanti](#), [Saptowo Jumali](#)

[Pardal](#), [Sustiprijatno](#) and [Mastur](#)

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Molecular diversity comparison in local rice accessions originated from Kalimantan and other islands of Indonesia

[Puji Lestari](#), [Rerenstradika Tizar Terryana](#), [Kristianto Nugroho](#), [Andari Risliawati](#), [Nurul](#)

[Hidayatun](#), [Priatna Sasmita](#), [Yudhi Sastro](#), [I. Gusti Komang Dana Arsana](#) and [Ikhwani](#)

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Genetic variation of Adan, a Krayan local rice mutant, using microsatellite markers

[Joko Prasetyono](#), [Tio Fadel Rafsanjani](#), [Tri Aminingsih](#), [Tasliah](#) and [Sugiono Moeljopawiro](#)

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[Ida Rosdianti](#), [Dani Satyawati](#), [Muhamad Yunus](#) and [Dwinita Wikan Utami](#)
AIP Conference Proceedings **2462**, 030005 (2022); <https://doi.org/10.1063/5.0075676>

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Field adaptation and molecular characterization of Code-*qTSN4* and Code-*qDTH8* rice lines at two different locations

[Tasliyah](#), [Kurniawan Rudi Trijatmiko](#) and [Joko Prasetyono](#)
AIP Conference Proceedings **2462**, 030006 (2022); <https://doi.org/10.1063/5.0075661>

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Hybrid purity assessment in F₁ hybrids segregating for phytophthora root rot resistance genes of chili pepper (*Capsicum annuum* L.)

[Fatimah](#), [Reflinur](#), [Joko Prasetyono](#), [Wartono](#), [Kristianto Nugroho](#), [Rinda Kirana](#), [Dani Satyawan](#), [Rerenstradika Tizar Terryana](#), [Aqwin Polosoro](#), [Puji Lestari](#) and [I. Made Tasma](#)
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Characterization of genomic variation on three Indonesian oil palm genotypes analyzed using next-generation sequencing HiSeq

[I. Made Tasma](#), [Habib Rijzaani](#), [Dani Satyawan](#), [Ida Rosdianti](#), [Edy Supriyanto](#) and [Razak Purba](#)
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Cytological and molecular identifications of seedless tangerine derived from endosperm culture

[Chaireni Martasari](#), [Mia Kosmiatin](#), [Ali Husni](#), [Kurniawan Budiarto](#) and [Innez Candri Gilang Purnama](#)
AIP Conference Proceedings **2462**, 030009 (2022); <https://doi.org/10.1063/5.0076395>

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Improvement of sex determination of salak plant using sequence characterized amplified regions

Reflinur, Ma'sumah, Namira Nur Arfa, Budi Setiadi Daryono and Azis Natawijaya
AIP Conference Proceedings **2462**, 030010 (2022); <https://doi.org/10.1063/5.0075698>

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-
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THE SECOND INTERNATIONAL CONFERENCE ON GENETIC RESOURCES AND BIOTECHNOLOGY: Harnessing Technology for Conservation and Sustainable Use of Genetic Resources for Food and Agriculture



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I Made Tasma, Dwinita Winkan Utami, Ika Roostika, Yadi Suryadi, Chaerani, Eny Ida Riyanti, Puji Lestari, Toto Hadiarto, Reflinur, Joko Prasetyono, Fatimah, Surya Diantina, Tru Puji Priyanto, Kusumawaty Kusumanegara, Wening Enggarini, Rerenstradika Tizar Terryana and Dani Satyawan

Volume number: 2462

Published: Jan 19, 2022

DISPLAY :

- [20](#)
- [50](#)

- [100](#)
- [all](#)

APPLICATION OF INNOVATIVE CROP IMPROVEMENT TECHNIQUES FOR CONSERVATION AND SUSTAINABLE USE OF PLANT GENETIC RESOURCES FOR FOOD AND AGRICULTURE

FreeJanuary 2022

Design and *in vitro* test of sgRNA for the CRISPR/Cas9 plasmid construct of the *SQS* gene of *Artemisia annua* L.

Sri Koerniati

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The efficacy of genetically modified (GM) corn Bt11 against *Ostrinia furnacalis* (Guenee) and *Helicoverpa armigera* (Hubner)

Bahagiawati and Diani Damayanti

AIP Conference Proceedings **2462**, 040002 (2022); <https://doi.org/10.1063/5.0075312>

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Construction and introduction of OsAER1::LeAlaAT cassette to improve the nitrogen use efficiency in rice cv. Mekongga

Atmitri Sisharmini, Aniversari Apriana, Intan Kamila, Aqwin Polosoro, Wening Enggarini, Tri Joko Santoso, Toto Hadiarto, Bahagiawati A. Husin and Kurniawan Rudi Trijatmiko

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FreeJanuary 2022

Environmental safety assessment of genetically engineered potato resistant to late blight caused by *Phytophthora infestans*

Alberta Dinar Ambarwati, Eny Ida Riyanti, Edy Listanto, Tri Joko Santoso, Toto Hadiarto and Kusmana

AIP Conference Proceedings **2462**, 040004 (2022); <https://doi.org/10.1063/5.0075612>

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Backcrossing of soybean lines containing aluminium tolerance gene into superior soybean variety, Biosoy

[Saptowo J. Pardal](#), [Amalia Prihaningsih](#), [Suharsono](#), [Ratna Utari](#) and [Riri Sundasari](#)
AIP Conference Proceedings **2462**, 040005 (2022); <https://doi.org/10.1063/5.0075187>

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Phenotypic and genetic stability evaluation of the targeted *GA20ox-2* gene mutation in CRISPR/Cas9 mutant rice derived from Mentong cultivar

[Aniversari Apriana](#), [Tri Joko Santoso](#), [Atmitri Sisharmini](#), [Reflinur](#), [A. Dinar Ambarwati](#), [Toto Hadiarto](#), [Sustiprijatno](#) and [Nuryati](#)
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FreeJanuary 2022

Transformation of *csp* gene into tobacco plant mediated by *Agrobacterium tumefaciens*

[Sustiprijatno](#), [Seagames Waluyo](#) and [Suharsono](#)

AIP Conference Proceedings **2462**, 040007 (2022); <https://doi.org/10.1063/5.0075571>

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PLANT CELL AND TISSUE CULTURE FOR CONSERVATION AND EFFECTIVE UTILIZATION OF GENETIC RESOURCES

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The application of gamma ray irradiation to increase triterpenoid compounds in embryogenic calli of *Centella asiatica* L. Urban

[Ika Roostika](#), [Suci Rahayu](#) and [Nurliani Bermawie](#)

AIP Conference Proceedings **2462**, 050001 (2022); <https://doi.org/10.1063/5.0076402>

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The effect of FeSO₄ concentration on the callus growth of two chili (*Capsicum annum* L.) varieties

[Rossa Yunita](#), [Endang Gati Lestari](#), [Iswari S. Dewi](#), [Mastur](#) and [Bambang Sapta Purwoko](#)

AIP Conference Proceedings **2462**, 050002 (2022); <https://doi.org/10.1063/5.0075223>

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Evaluation of ratooning ability in several sweet sorghum (*Sorghum bicolor* [L.] Moench) mutant lines

[Endang Gati Lestari](#), [Iswari Saraswati Dewi](#), [Rossa Yunita](#) and [Amin Nur](#)

AIP Conference Proceedings **2462**, 050003 (2022); <https://doi.org/10.1063/5.0075542>

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Response of gamma ray irradiation derived-cultures of three sugarcane varieties to drought stress induced by polyethylene glycol

[Ragapadmi Purnamaningsih](#) and [Suci Rahayu](#)

AIP Conference Proceedings **2462**, 050004 (2022); <https://doi.org/10.1063/5.0075185>

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Sucrose and putrescine increased callus induction in tomato anther culture

Iswari Saraswati Dewi, Imam Nur Kholis, Bambang Sapta Purwoko and Ratna Ningsih
AIP Conference Proceedings **2462**, 050005 (2022); <https://doi.org/10.1063/5.0075666>

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Field evaluation of elephant grass mutant lines (*Pennisetum purpureum* Schumach.) in highlands

Ali Husni, Muhammad Rifay, Mia Kosmiatin and Vyta W. Hanifah
AIP Conference Proceedings **2462**, 050006 (2022); <https://doi.org/10.1063/5.0076418>

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FreeJanuary 2022

Increasing drought tolerance of sugarcane through gamma ray irradiation and *in vitro* selection

Sri Suhesti, Syafaruddin, I. Ketut Ardana, Endang Hadipoentyanti and Rr. Sri Hartati
AIP Conference Proceedings **2462**, 050007 (2022); <https://doi.org/10.1063/5.0076155>

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Cells density affects cell production of *Citrus limonia* in flask and air-lift bioreactor cultures and limonin farming

Dita Agisimanto, Farida Yulianti and Hidayatul Arisah

AIP Conference Proceedings **2462**, 050008 (2022); <https://doi.org/10.1063/5.0075651>

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THE USE OF MICROBIAL GENETIC RESOURCES AS BIOLOGICAL CONTROL AGENTS OF AGRICULTURAL PESTS AND DISEASES, AND FOR SOIL BIOREMEDIATION

FreeJanuary 2022

In Silico functional prediction of CAS2, a protein specifically expressed in appressorium and required for pathogenicity of *Colletotrichum gloeosporioides*

Tri Puji Priyatno, Farah Diba Abu Bakar, Rohaiza Ahmad Redzuan, Abdul Munir Abdul Murad and Ifa Manzila

AIP Conference Proceedings **2462**, 060001 (2022); <https://doi.org/10.1063/5.0075625>

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Biofertilizer increases nutrient use efficiency (NUE) of nitrogen, phosphorus, and potassium at leaves level of *Artemisia annua* L.

Wiguna Rahman, Arthur A. Lelono, Erwin Al Hafiih and Tri Muji Ermayanti

AIP Conference Proceedings **2462**, 060002 (2022); <https://doi.org/10.1063/5.0075503>

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Effect of nitrogen fixation and phosphate solubilizing bacteria on growth and yield of lowland rice in different soil type

Ikhwani, Higa Afza, Siti Yuriyah and Waluyo

AIP Conference Proceedings **2462**, 060003 (2022); <https://doi.org/10.1063/5.0077914>

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The effect of coating application using chitosan enzymatic depolymerization on anthracnose disease suppression in mango (*Mangifera indica* L.) cv. ‘Arumanis’

[Yadi Suryadi, Dwi Ningsih Susilowati, I. Made Samudra, Alina Akhdiya and Karsinah](#)
AIP Conference Proceedings **2462**, 060005 (2022); <https://doi.org/10.1063/5.0075183>

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Understanding yeast tolerance as cell factory for bioethanol production from lignocellulosic biomass

[Eny Ida Riyanti and Edy Listanto](#)
AIP Conference Proceedings **2462**, 060006 (2022); <https://doi.org/10.1063/5.0075157>

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Isolation and pathogenicity test of fusarium basal rot and purple blotch fungal pathogens from shallot and *Allium* spp

Chaerani, Ragapadmi Purnamaningsih and Suci Rahayu

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Morphological characters and efficacy of thirteen entomopathogenic fungi of *Aschersonia aleyrodis* Webber isolates on whitefly (*Bemisia tabaci* Gennadius)

Yusmani Prayogo, Marida Santi Yudha Ika Bayu, Sri Wahyuni Indiati and Made Jana Mejaya

AIP Conference Proceedings **2462**, 060008 (2022); <https://doi.org/10.1063/5.0076067>

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Physicochemical characteristics of yoghurt from various beans and cereals

Heny Herawati, Diana Nur Afifah, Eni Kusumaningtyas, Sri Usmiati, Agus S. Soemantri, Miskiyah, Elmi Kamsiati and Muchamad Bachtiar

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The potential use of zeolite and exopolysaccharide bacteria for reduction of degradation and carbon emission on oil palm plantation in tropical peatland

[Laksmita P. Santi](#), [Haryo T. Prakoso](#) and [Donny N. Kalbuadi](#)
AIP Conference Proceedings **2462**, 060010 (2022); <https://doi.org/10.1063/5.0075506>

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Application of phosphate solubilizing microbes to promote the effectiveness of rock phosphate on cacao seedling growth in acid soil

[Kurnia Dewi Sasmita](#), [Iswandi Anas](#), [Syaiful Anwar](#), [Sudirman Yahya](#) and [Gunawan Djajakirana](#)
AIP Conference Proceedings **2462**, 060011 (2022); <https://doi.org/10.1063/5.0075843>

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Abstract. Yeast is one of important microorganisms in the biofuel industry in recent years. Identification and characterization of yeast are important for determining the quality and purity of yeast strains. This study intended to characterize yeast isolated from persimmon, special woods from Indonesia, and commercial yeast as a comparison. Macroscopic and microscopic morphological observation was performed to preliminarily identify the species of yeast isolates, supported with molecular characterization based on ITS fragment. The physiological aspect of yeast was assessed on their sensitivity to ethanol and a comparative experiment with glucose and sucrose substrate. A total of seven isolates were obtained. All yeast isolates had milky white color and slimy consistency, except for YK1.3 and YBK1, which had a watery consistency. Several isolates have oval and round shapes with blue color. All isolates were successfully sequenced based on the internal transcribed spacer (ITS) 1 and ITS4 regions. Homology analysis of the sequences against the GenBank database using BLASTN showed that seven isolates have homology with the *Debaryomyces hansenii*, *Wickerhamomyces anomalus*, *Clavispora lusitaniae*, and *Metschnikowia agaves* species. Physiologically, YRt2.1, YK1.3, YK2.2, and YK3.2 belonging to *D. hansenii*, *C. lusitaniae*, *M. agaves*, and *W. anomalus*, respectively, had better growth on glucose substrate incubated for 24 h. In contrast, YRt1.2, YB3.1, and YBK1 belonging to *D. hansenii*, *W. anomalus*, and *D. hansenii*, respectively, grew well on sucrose rather than glucose substrate. All isolates with glucose substrate seemed to be more tolerant to ethanol. Moreover, isolates YRT1.1 produced the highest ethanol concentration level until 39.28 ppm compared to the other isolates. This study clearly illustrates that identification based morphological and physiological characters as well as ITS fragment could be useful taxonomic tools for rapid identification at the species level of unknown isolated strains and constitutes a basis for initial yeast strain selection for future application in the production of ethanol.

INTRODUCTION

Indonesia is a tropical country with abundant diversity of flora, fauna, and microorganisms [1]. Yeast, one of microorganisms defined as ascomycetes or basidiomycetes fungi that persist as single cells for at least some of their life cycle, do not form fruiting bodies, have a fairly wide ecological range and can be found in high organic environments [2]. Kurtzman *et al.* reported that isolation and identification of yeast diversity in the world have only been carried out in 1% of the 89 yeast genera registered on yeast monographs [3]. Research on yeast is mostly carried out by exploring various ecosystems, but many yeast in nature are still not yet known [4].

Yeast is an important microorganism for the life of other living things. These microorganisms can be used as starter agents for fermentation of feed factory waste [5, 6], alcoholic beverages [3, 7], bioethanol [8], and as biocontrol agents [9, 10]. Generally, yeasts are important microflora of many food products due to their ability to

grow on a substrate rich in proteins, sugars, and organic acids [18]. This considerable usage for microorganism makes Indonesia still imports dry yeast products. Therefore, it is necessary to isolate and identify yeast isolates found in nature to support these needs.

Yeast can be isolated from the surface of fruits and leaves [11]. Besides, yeast is also an epiphytic microbe in plants [12]. Indonesia has been known to have diverse fruits and woods across the country island for useful microorganism's source. Some of forest trees producing woods which could be used for local liquor are well grown in the main islands like Kalimantan, Sumatra, and Sulawesi. Some of the most common yeast genera found in tropical fruit are the genus *Candida*, *Hanseniaspora*, *Wickerhamomyces*, and *Rhodotorula* [13]. The most common yeast genera found in plants are *Cryptococcus*, *Rhodotorula*, *Hanseniaspora*, and *Metschnikowia* [14].

Yeast identification needs to be done to determine the genus or species of yeast, considering there are still many types and species of unknown yeast. Identification and characterization of yeast isolated species have traditionally been based on morphological and, in particular, physiological characteristics regarding their certain abilities [15, 16]. However, molecular techniques have been increasingly used recently for identifying yeast, especially when dealing with species with indistinguishable morphological or physiological characteristics. The application of the molecular approach has resulted in more studies on the classification, identification, and ecology of yeast species [17]. Molecular identification techniques employing sequencing the internal transcribed spacer (ITS) regions and partial D1/D2 large-subunit domains of the 26S ribosomal RNA (rRNA) have become the most frequent and convenient method for the identification of yeasts isolates [18]. This study aims to identify and characterize yeast isolated from persimmon, three Indonesian woods, and commercial yeast as a comparison based on molecular, morphology, and physiology.

MATERIALS AND METHODS

Isolation of yeast was conducted from persimmon (*Diospyros kaki*), special woods from Indonesia (Kalimantan wood (*Eusideroxylon zwageri*), "Raru" wood (*Cotilelobium melanoxydon*) from Sumatra and Sulawesi, and commercial yeast "NKL Harum Manis" as a comparison. Briefly, each sample was diluted 10-fold in sterile saline (0.85% NaCl) and incubated at ambient temperature for 90 min. Serial dilutions of each sample were performed, and 1 ml aliquots of appropriate dilutions were inoculated by the pour plate method on sterile yeast peptone dextrose (YPD) agar plates (containing 5 g/l yeast extract, 10 g/l peptone, 10 g/l glucose, and 15 g/l agar) and incubated at room temperature for 72–96 h [3]. Subsequently, colonies showing different morphologies were selected and inoculated thrice on YPD agar plates. The pure cultures were preserved at $8\pm 2^{\circ}\text{C}$ until further use.

The morphology of rejuvenated yeast (3-day old isolates) was examined macroscopically and microscopically. The morphology observed on the yeast viable cells was color, surface, shape, and consistency. Furthermore, microscopic observations were made by taking a loop of yeast isolate mixed with a little water on the glass slide. After that, it is levelled and covered with glass. The smears were observed under a 1,000 \times magnification in a light microscope.

Physiological observation was performed on the substrate-used efficiency and ethanol sensitivity for growth and reducing sugar. A single spot excised (3 mm in diameter) was placed on solid YPD agar media on a Petri dish and allowed a room temperature for 24 h for growth observation. The colony forming units (CFU) were measured by naked eye for all isolates. Reducing sugar was determined by preparing the liquid culture of each isolate. Single spot excised (3 mm in diameter) was inoculated in preculture medium containing 10 ml liquid YPD and incubated in a shaker for 24 h. The substrates used were glucose and sucrose as the source of carbon. For this treatment, 10% of glucose or sucrose (w/v) was transferred into 100 ml liquid YPD as cultivation media in Erlenmeyer flask. Two treatments (addition or absence of 6% ethanol) were applied to this solution. All the fermented yeast of each isolates in the preculture was transferred into cultivation media and subsequently were shaken for the next three days at room temperature. At the end of incubation, the fermented culture was centrifuged at room temperature at 3,500 $\times g$ for 10 min to remove the debris from the supernatant. The supernatant (0.5 ml) was added with 0.5 ml DNS (Dinitrosalicylic acid), placed in a test tube, then vortexed. Furthermore, the mixture was incubated in the boiling water at 100 $^{\circ}\text{C}$ for 5 min. After cooling down, it was added by 5 ml ddH₂O and homogenized by using a vortex, then measured using spectrophotometry UV-Vis at 540 nm. Calculating glucose levels was done by substituting the absorbance of the solution into the regression equation for the calibration curve of the standard glucose solution [19].

Preparation of preculture and cultivation of each isolate for ethanol content estimation was similar to that for reducing sugar measurement. Assessment of the ethanol produced was performed on the supernatant of each isolates

using gas chromatography (GC). Injection volume for the GC assay was done at a rate of 0.8 ml/min within the capillary column (HP-Innowax; length 60 m, diameter 0.25 mm, and film thickness 0.25 μ m) with helium (He) as the carrier gas. The GC system was attached with a flame ionization detector (FID; 250°C), while the injection port temperature was controlled at 200°C. The ethanol content was calculated by comparing the retention time of the sample to the ethanol standard. The standard curve was made by using pure ethanol with methanol as the solvent [20].

Identification of yeast isolates was done based on ITS regions. Genomic DNA of yeast was extracted from isolated colonies using Whatman FTA filter matrix technology [21]. Morphologically identified yeast were sequenced at the ITS5.8S rRNA gene domain using the universal primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') [16]. Amplification of ITS regions was performed using T1 Thermocycler (Biometra, Germany). The PCR reaction was performed as follows: i) pre-denaturation at 95°C for 5 min, ii) 35 cycles of denaturation at 95°C for 30 sec, iii) annealing step at 55°C for 35 sec, iv) extension at 72°C for 30 sec, and v) final extension at 72°C for 7 min. The PCR products were also subjected to agarose gel electrophoresis using 1.2% (w/v) agarose gel in 1 \times Tris Acetate-EDTA (TAE) buffer, stained with ethidium bromide (EtBr), and visualized using gel documentation system. The PCR products were also sequenced both in forward and reverse direction at 1st Base Laboratory in Malaysia. Using the Basic Locus Search Alignment Tool (BLASTN) program, the sequences were compared to database available in the GenBank of National Center for Biotechnology Information (NCBI) website (<http://www.ncbi.nlm.nih.gov/blast/>).

RESULTS AND DISCUSSIONS

Isolation of yeast species from various collected local fruit and woods was investigated with commercial yeast as a comparison. Seven yeast isolates were obtained and the observation showed that all of them had milky white color and slimy consistency except for YK1.3 and YBK1, which had a watery consistency (Table 1). Several isolates have oval and round shapes with blue paint. All yeast did not have a flagellum, and only YRt1.1 and YRt1.2 had hyphae (Fig. 1). Due to different morphological characters, it is most probable that the isolates belong to different species or strain. The isolates were then identified molecularly.

TABLE 1. Morphological identification of obtained yeast colonies.

Isolate ID	Sample sources	Color	Surface	Shape	Consistency
YRt2.1	Commercial yeast	Milky white	Smooth	Convex	Slimy
YRt1.2	Commercial yeast	Milky white	Smooth	Convex	Slimy
YK1.3	Kalimantan's wood	Milky white	Smooth	Convex	Watery
YK2.2	Sumatra's wood	Milky white	Smooth	Convex	Slimy
YK3.1	Maros wood	Milky white	Smooth	Convex	Slimy
YK3.2	Maros wood	Milky white	Rough	Convex	Slimy
YBK1	Persimmon	Milky white	Smooth	Convex	Watery

Physiological analysis on the isolates was performed by examining the ability of the isolates to grow on glucose and sucrose as a substrate of growth media. A variety of techniques can be used to measure the growth of yeast biomass on growth media, including turbidity, total cells, and living cells [22]. Yeast requires a source of carbon, nitrogen, mineral, and vitamin to spring up [23]. Generally, yeast growth medium contained glucose, malt extract, yeast extract, and bacto-peptone. Glucose is known to be very effective and most popular carbon source by the cells because it supports fast growth as it is a single molecule and easy consumption first. It is the initial substrate and the precursor of the main pathways [24]. However, using glucose as a substrate medium requires a high cost. Previous research reported that glucose at a very high concentration could easily be assimilated into the cells, causing accumulation and congestion, leading to the Crabtree effect that inhibits cell growth. Therefore, sucrose could be used as an alternative to glucose. Sucrose which belongs to the disaccharide, is another sugar composed of fructose and glucose [25]. The present study results showed that the YK3.1 isolated from Maros wood, YRt1.2 from commercial yeast, and YBK1 from persimmon had better growth on sucrose media (Fig. 2). Meanwhile, the others isolates had better growth on glucose substrate. Generally, the growth rate of different yeast cells is different in various media. For example, previous studies showed that yeast isolates belonging to *Debaryomyces*,

Wickerhamomyces, and *Metschnikowia* grew better in media containing glucose substrate that belongs to the monosaccharide group [20]. In contrast, the other previous studies reported that *Saccharomyces* grew better in sucrose than glucose [26, 27].

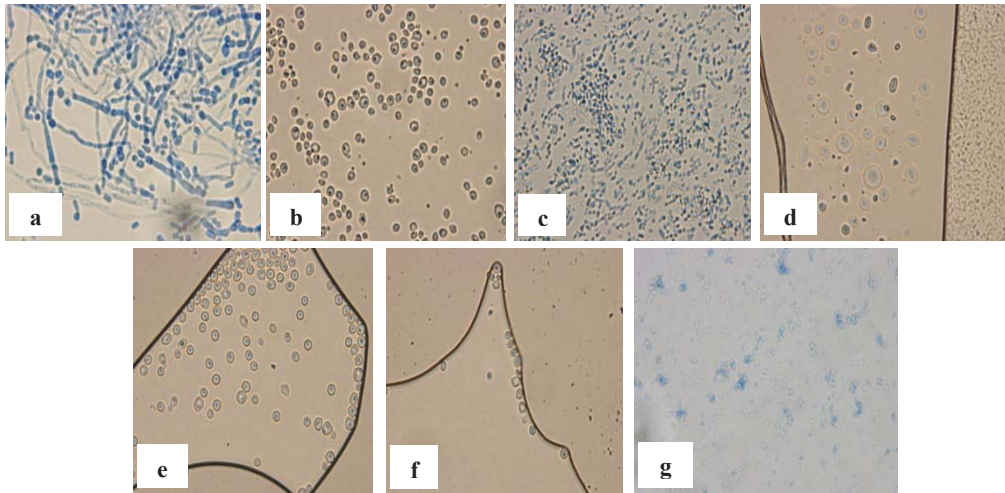


FIGURE 1. Microscopic observation of yeast cells from different resources as observed at 1,000× magnification. (a) YRt1.2. (b) YRt2.1. (c) YK1.3. (d) YK2.2. (e) YK3.1. (f) YK3.2. (g) YBK1.

Ethanol treatment was also observed in this study for the initial screening of yeast growth. Ethanol is known as a growth inhibitor of microorganisms. It has been reported that ethanol could damage mitochondrial DNA in yeast cells [28] and lead to the inactivation of some enzymes, such as hexokinase and dehydrogenase. Ethanol tolerance on yeast has yet to be clearly defined, although it has been reported to be reproducible under defined conditions and appears to be under complex genetic control [29]. Nevertheless, some strains of the yeast *S. cerevisiae* showed tolerance and could adapt to ethanol.

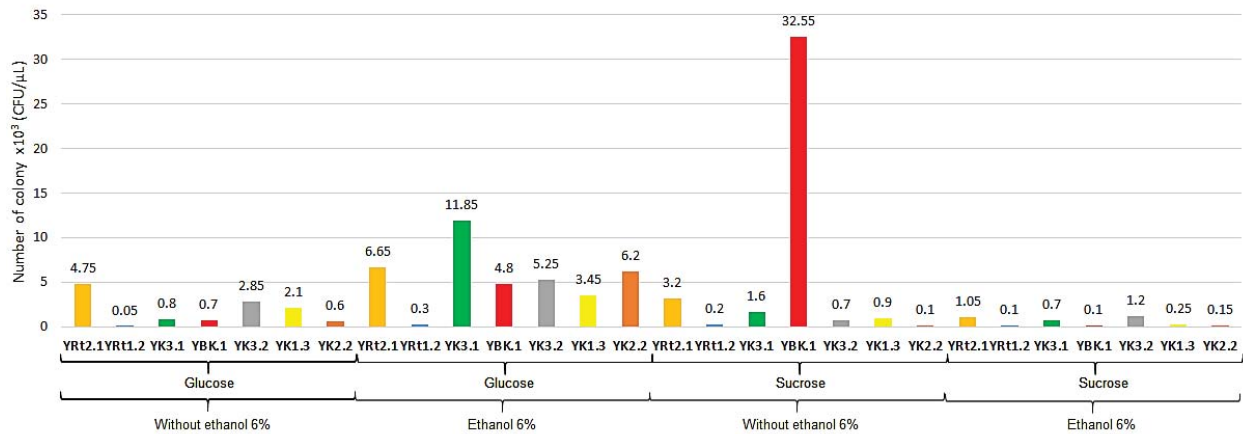


FIGURE 2. Growth of yeast colonies on glucose and sucrose substrates incubated for 24 h, with or without 6% (v/v) ethanol.

The results from this study depicted in Fig. 2 showed that all yeast isolates with glucose-containing media could grow well and resist 6% (v/v) ethanol. However, their growth was inhibited and cannot resist ethanol when the glucose was replaced with sucrose. Glucose may trigger beneficial effects on cells, including stimulation of cell proliferation, mobilization of storage compounds such as glycogen and trehalose, and decreased resistance to cell stress. In contrast, negative impacts due to lack of glucose in the process can lead to several problems such as

decreased or blocked fermentations, instability of cellular viability, and low ethanol production, where the break of sugars, sucrose into simple sugars (glucose) occurs by an intracellular enzyme known as invertase located in cell wall [30]. Without ethanol 6% (v/v) treatment, YBK1 producing the highest colonies until 32.55×10^3 CFU/ μ l on sucrose substrate.

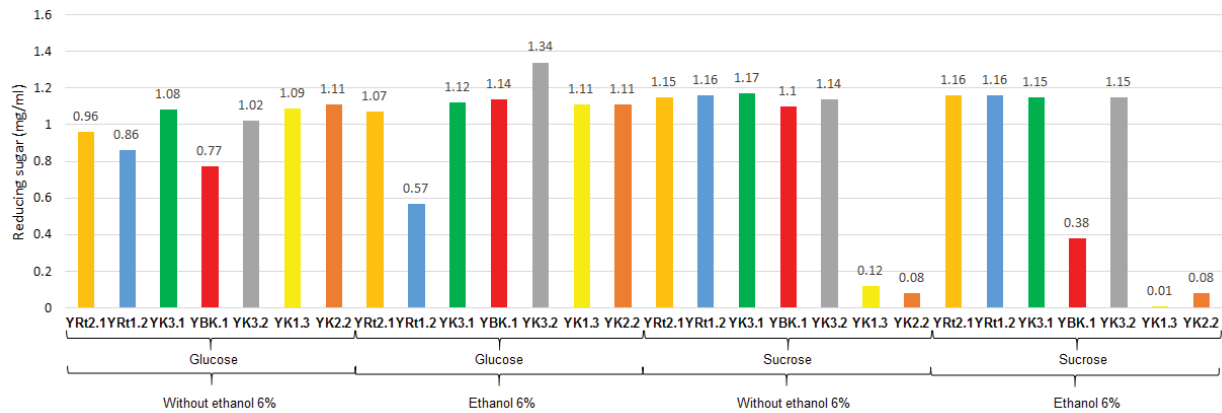


FIGURE 3. Reducing sugar (glucose and sucrose) on cultivation of yeast isolates, with or without 6% (v/v) ethanol.

Yeast is known to utilize the sugar contained in the fermentation medium for cell growth and ethanol production. These cells will break down sugar into ethanol. The overhaul is carried out through the process of glycolysis with the help of enzymes produced during fermentation [31]. Fig. 3 shows the histogram of reducing sugars of each isolate grown on glucose or sucrose substrates with or without ethanol 6%. It can be seen that the levels of reducing sugar were quite varied. The greater the reducing sugar content in the substrate, the more ineffective the substrate in the growing media used will be [32].

Based on the present results, the addition of ethanol is not necessarily effective in reducing the levels of reducing sugar. For example, isolate YK3.2 on glucose substrate produced the highest reducing sugar content of 1.02 mg/ml without 6% ethanol, lower than the addition of 6% ethanol, which was 1.34 mg/ml. On the other hand, isolate YRt1.2 on glucose substrate produced a reducing sugar content of 0.86 mg/ml without ethanol, higher than the addition of 6% ethanol, which was 0.57 mg/ml. The YBK1 isolate would be more efficient if grown on a glucose substrate without 6% ethanol, but with 6% ethanol if using a sucrose substrate. The more reducing sugars are used by yeast, the higher the concentration of ethanol produced, and conversely, the less reducing sugar used, the lower the concentration of ethanol produced. This is consistent with other studies where the more reducing sugars that can be utilized by yeast, the higher the ethanol concentration produced [33].

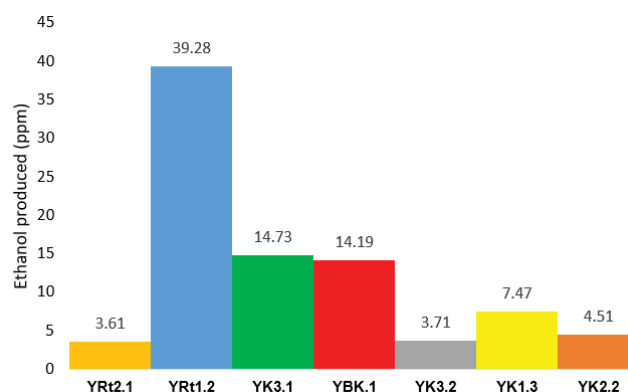


FIGURE 4. Ethanol production of yeast isolates.

Isolate YRT1.2 produced the highest ethanol concentration level of 39.28 ppm compared to the other isolates (Fig. 4). Clearly, the production of ethanol by yeast is a key technology in ethanol fermentation. However, ethanol is toxic to yeast cells. It can cause misfolding of proteins, increased membrane fluidity, changes in mRNA export from the nucleus, and activation of various stress signaling pathways, including the protein kinase A pathway. Ultimately, these may lead to cell death. In contrast, during most of the bioethanol fermentation, yeast cells do not die, either because the ethanol concentration does not reach a high enough level or because the fermentation period is not long enough to cause cell death. More tolerant yeast strains are important for highly efficient ethanol production because alcohol concentration only continues to increase during fermentation to levels that can be harmful or lethal to cells. In addition, yeasts that are tolerant of ethanolic stress may have physiological differences such as intracellular accumulation of ergosterol, trehalose, and proline [34], which helps them to survive. Several researchers reported that industrial yeast such as *S. cerevisiae* has proven to be efficient and rapid in fermenting hexose sugar (C6) into ethanol [35, 36].

Yeast is a eukaryotic organism with one unit of the rRNA region consisting of coding and non-coding regions. The rRNA region sequences often used for yeast identification are the D1/D2 domain of large-subunit (LSU) ribosomal DNA (rDNA) and ITS [37]. ITS region is one of the most used markers in the phylogenetic study of most yeast. The region has been reported to be used to analyses phylogenetic relationships at species and interspecies to understand systematic and fungal community [38]. This region consists of the highly conserved region as well as ITS1 and ITS4. The region contains multiple tandem repeats of ribosomal RNA mostly in the haploid genome which made it very fruitful in species identification, therefore has been proposed as a standard molecular marker in DNA barcoding of fungal species.

Molecular identification of all seven isolates based on homology analysis of the sequences against the GenBank database using BLASTN, resulted in identification of the isolates in four different species, namely *Debaryomyces hansenii*, *Wickerhamomyces anomalus*, *Clavispora lusitaniae*, and *Metschnikowia agaves* (Table 2). The species *D. hansenii* represents three identified isolates from commercial yeast (YRt2.1, YRt1.2) and persimmon (YBK1). *D. hansenii*, which has an oval and elliptical shape, the possibility of forming a pseudo mycelium or not forming a true mycelium is an osmotolerant microorganism. It can be found in water, cheese, meat, grapes, fruit, and soil habitats [39]. It is reported that osmotolerant property in yeast provides advantages for biotechnological applications in food industry sector to reduce production costs [40].

Sequencing results indicated that YK3.1 and YK3.2 isolates had high homology with *W. anomalus* isolate F2 and *W. anomalus* isolate F4, respectively (Table 2). *W. anomalus* is known to grow well in various carbon sources, resistant to low pH and high osmotic pressure, useful for wine aroma and biocontrol agents [41]. YK1.3 was similar with *C. lusitaniae* strain h76c, which is a pathogen type to humans, while YK2.2 had a high homology with *M. agaves* strain 94-267.2, which can be utilized in the alcohol fermentation process [42].

TABLE 2. Molecular identification of obtained yeast strains by DNA sequencing.

Isolate ID	Sample sources	Molecular identification	Query cover (%)	Sequence identity (%)	GenBank accession ID
YRt2.1	Commercial yeast	<i>Debaryomyces hansenii</i> strain SCSGAF0208	100	99	C859432.1
YRt1.2	Commercial yeast	<i>D. hansenii</i> strain SCSGAF0208	100	99	HE799661.1
YK1.3	Kalimantan's wood	<i>Clavispora lusitaniae</i> strain h76c	100	99	KP674780.1
YK2.2	Sumatra's wood	<i>Metschnikowia agaves</i> strain 94-267.2	99	92	KC859432.1
YK3.1	Maros wood	<i>Wickerhamomyces anomalus</i> isolate F2	100	100	KM603601.1
YK3.2	Maros wood	<i>W. anomalus</i> isolate F4	100	99	KM603609.1
YBK1	Persimmon	<i>D. hansenii</i> strain SCSGAF0208	100	99	JN851059.1

To corroborate the obtained data, phylogenetic analysis was performed, and a phylogenetic tree was constructed (Fig. 5). The phylogenetic analysis involved seven yeast isolates from this study against five reference isolates (*D. occidentalis*, *C. lusitaniae* CBS 6936, *M. agaves* 18S rRNA gene (partial), *W. anomalus* strain BCRC, dan *C.*

lusitaniae strain MTCC) used as an outgoing group. Isolate YK3.1 is suspected of having a close relationship with *W. anomalus* strain BCRC 23112 (Fig. 5) originating from the outgroup sequence compared to *W. anomalus* isolate F2 (Table 2). YK2.2 isolate was in one branch with *M. agaves* strains 94-267.2. This genetic relationship is in good agreement with the aligned sequences in the database to relate the two isolates closely. Furthermore, YRT1.2 and YRT2.1 isolates were in one subclade, and their relationship was closer to *W. anomalus* isolate F4 compared to *D. hansenii* strain SCSGAF0208. YBK1 and YK3.1 isolates showed the same results on the alignment results on the database and phylogenetic tree. Meanwhile, the YK3.2 isolate was closer to *W. anomalus* isolate F2 than *W. anomalus* isolate F4 due to sequence alignment in the database.

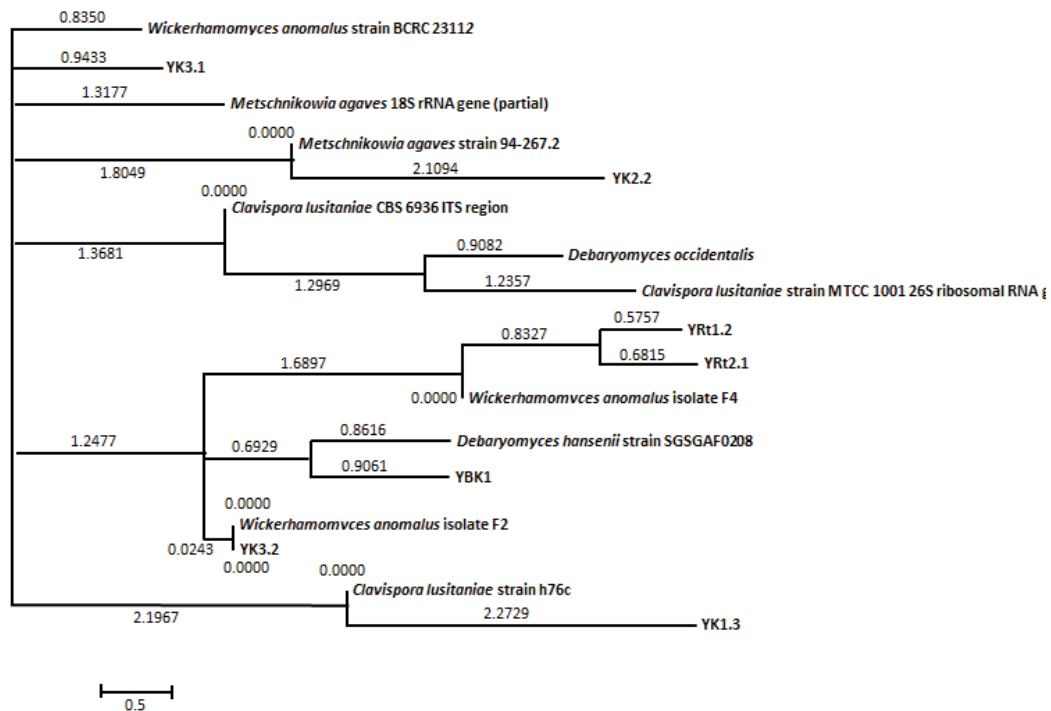


FIGURE 5. Phylogenetic trees of yeast isolates based on ITS sequences compared with NCBI data and sequences outside the group.

CONCLUSION

This study reported the occurrence of yeast species isolated from persimmon, special woods from Indonesia (Kalimantan, Sumatra, and Maros), and commercial yeast as a comparison. Isolates YRT2.1, YK1.3, YK2.2, and YK3.2 had better growth on glucose substrate. In contrast, YRT1.2, YB3.1, and YBK1 grew well on sucrose rather than glucose substrate. This study's lowest reducing sugar content was 0.01 mg/ml in YBK1 isolate on sucrose substrate with 6% ethanol. YRT1.1 produced the highest ethanol compared to the other isolates, suggesting its potential application for producing ethanol after further assessment. All seven yeast isolates were successfully identified to belong to four genera, they are *D. hansenii* (three isolates), *C. lusitaniae* (one isolate), *M. agave* (one isolate), and *W. anomalus* (two isolates). The morphological and physiological identification results was highly consistent with molecular identification ones. Molecular methods showed higher effectiveness in identifying yeast isolates in this study.

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