Bioteknologi
Biotechnological Studies

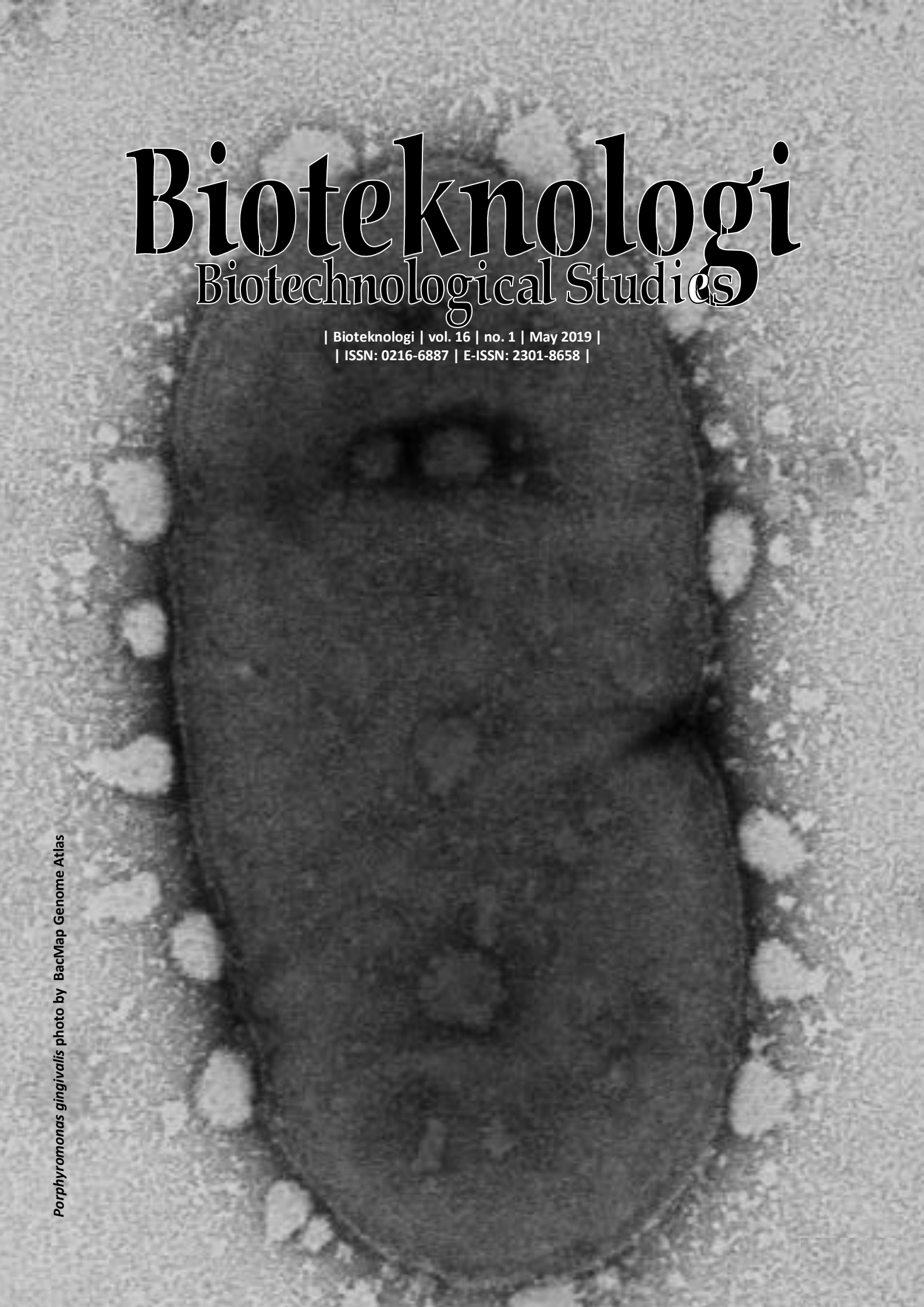
| Bioteknologi | vol. 16 | no. 1 | May 2019 | ISSN: 0216-6887 | E-ISSN: 2301-8658 |

Published semiannually **PRINTED IN INDONESIA**

9 772301 865312

Bioteknologi
Biotechnological Studies

| Bioteknologi | vol. 16 | no. 1 | May 2019 |
| ISSN: 0216-6887 | E-ISSN: 2301-8658 |



Porphyromonas gingivalis photo by BacMap Genome Atlas

Bioteknologi
Biotechnological Studies

| Bioteknologi | vol. 16 | no. 1 | May 2019|

ONLINE
http://smujo.id/bbs

p-ISSN
0216-6887

e-ISSN
2301-8658

PUBLISHER
Society for Indonesian Biodiversity

CO-PUBLISHER
Universitas Sebelas Maret, Surakarta, Indonesia

OFFICE ADDRESS
Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Sebelas Maret.
Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia. Tel./Fax.: +62-271-663375, email: bs@smujo.id, jbioteknologi@gmail.com

PERIOD OF ISSUANCE
May, November

EDITOR-IN-CHIEF
Suranto – Universitas Sebelas Maret, Surakarta, Indonesia

MANAGING EDITOR
Ari Pitoyo – Universitas Sebelas Maret, Surakarta, Indonesia

ASSOCIATE EDITOR
Ahmad Dwi Setyawan – Universitas Sebelas Maret, Surakarta, Indonesia

EDITORIAL BOARD
Artini Pangastuti – Universitas Sebelas Maret, Surakarta, Indonesia
Asish Kumar Parida – CSIR-Central Salt & Marine Chemicals Research Institute, Bhavnagar, India
Brijmohan Singh Bhau – CSIR-North-East Institute of Science & Technology (CSIR-NEIST), Jorhat, Assam, India
Ghulam Nabi – Institute of Hydrobiology, the Chinese Academy of Sciences, Wuhan, China
Heru Kuswantoro – Indonesian Legumes and Tuber Crops Research Institute (ILETRI), Malang, Indonesia
Indrastuti A. Rumanti – Indonesian Center for Rice Research (ICRR), Sukamandi, Subang, Indonesia
Mallappa Kumara Swamy – Universiti Putra Malaysia, Malaysia
Martin Mkandawire – Cape Breton University, Nova Scotia, Canada
Megga Ratnasari Pikoli – Universitas Islam Negeri (UIN) Syarif Hidayatullah Jakarta, Indonesia
Mohammed Arifullah – Universiti Malaysia Kelantan, Jeli, Malaysia
Saifeldin A. F. El-Nagerabi – College of Arts and Sciences, Nizwa University, Nizwa, Oman
Yosep Seran Mau – Universitas Nusa Cendana, Kupang, Indonesia

Society for Indonesian Biodiversity

Universitas Sebelas Maret Surakarta, Indonesia

Published by Smujo International for The Society for Indonesian Biodiversity and Sebelas Maret University, Surakarta, Indonesia

GUIDANCE FOR AUTHORS

Aims and Scope *Bioteknologi* or *Biotechnological Studies* encourages submission of manuscripts dealing with all aspects of biotechnology that emphasize issues germane to improve human life, includes cell biochemistry, enzymes and proteins, applied genetics and molecular biotechnology, genomics and proteomics, applied microbiology and cell physiology, environmental biotechnology, improvement of crop productivity, public health and waste management, as as well as product formation and manipulation of biological processes for use in the agricultural, pharmaceutical biotechnology and allied industries.

Article types The journal seeks original full-length research papers, reviews, and short communication. Manuscript of original research should be written in no more than 8,000 words (including tables and picture), or proportional with articles in this publication number. Review articles will be accommodated, while, short communication should be written at least 2,000 words, except for pre-study.

Submission The journal only accepts online submission through system or email to the editors at **jbiotechstud@gmail.com** (until the end of 2017). Submitted manuscripts should be the original works of the author(s). The manuscript must be accompanied by a cover letter containing the article title, the first name and last name of all the authors, a paragraph describing the claimed novelty of the findings versus current knowledge. Submission of a manuscript implies that the submitted work has not been published before (except as part of a thesis or report, or abstract); and is not being considered for publication elsewhere. When a manuscript written by a group, all authors should read and approve the final version of the submitted manuscript and its revision; and agree the submission of manuscripts for this journal. All authors should have made substantial contributions to the concept and design of the research, acquisition of the data and its analysis; drafting of the manuscript and correcting of the revision. All authors must be responsible for the quality, accuracy, and ethics of the work.

Acceptance The only articles written in English (U.S. English) are accepted for publication. Manuscripts will be reviewed by editors and invited reviewers (double blind review) according to their disciplines. Authors will generally be notified of acceptance, rejection, or need for revision within 1 to 2 months of receipt. The manuscript is rejected if the content does not in line with the journal scope, does not meet the standard quality, inappropriate format, complicated grammar, dishonesty (i.e. plagiarism, duplicate publications, fabrication of data, citations manipulation, etc.), or ignoring correspondence in three months. The primary criteria for publication are scientific quality and biodiversity significance. **Uncorrected proofs** will be sent to the corresponding author as *.doc* or *.rtf* files for checking and correcting of typographical errors. To avoid delay in publication, corrected proofs should be returned in 7 days. The accepted papers will be published online in a chronological order at any time, but printed in May and November.

Ethics Author(s) must obedient to the law and/or ethics in treating the object of research and pay attention to the legality of material sources and intellectual property rights.

Copyright If and when the manuscript is accepted for publication, the author(s) still hold the copyright and retain publishing rights without restrictions. Authors or others are allowed to multiply article as long as not for commercial purposes. For the new invention, authors are suggested to manage its patent before published.

Open access The journal is committed to free-open access that does not charge readers or their institutions for access. Readers are entitled to read, download, copy, distribute, print, search, or link to the full texts of articles, as long as not for commercial purposes. The license type is CC-BY-NC-SA.

A charge The journal is committed to free of charge for submission and publication of non-institutional funded research (waiver).

Reprints The sample journal reprint is only available by special request. Additional copies may be purchased when ordering by sending back the uncorrected proofs by email.

Manuscript preparation Manuscript is typed on A4 (210x297 mm^2) paper size, **in a single column**, single space, 10-point (10 pt) Times New Roman font. The margin text is 3 cm from the top, 2 cm from the bottom, and 1.8 cm from the left and right. Smaller lettering size can be applied in presenting table and figure (9 pt). Word processing program or additional software can be used, however, it must be PC compatible and Microsoft Word based (*.doc* or *.rtf*; **not *.docx***). **Scientific names** of species (incl. subspecies, variety, etc.) should be written in italic, except for italic sentence. Scientific name (genera, species, author), and cultivar or strain should be mentioned completely for the first time mentioning it in the body text, especially for taxonomic manuscripts. Name of genera can be shortened after first mentioning, except generating confusion. Name of the author can be eliminated after first mentioning. For example, *Rhizopus oryzae* L. UICC 524, hereinafter can be written as *R. oryzae* UICC 524. Using trivial name should be avoided, otherwise generating confusion. **Biochemical and chemical nomenclature** should follow the order of the IUPAC - IUB. For DNA sequence, it is better used Courier New font. Symbols of standard chemical and abbreviation of chemistry name can be applied for common and clear used, for example, completely written butilic hydroxyl toluene (BHT) to be BHT herein after. **Metric measurement** use IS denomination, usage other system should follow the value of equivalent with the denomination of IS first mentioning. Abbreviations set of, like g, mg, mL, etc. do not follow by dot. Minus index (m^{-2}, L^{-1}, h^{-1}) suggested to be used, except in things like "per-plant" or "per-plot". **Equation of mathematics** does not always can be written down in one column with text, in that case can be written separately. **Number** one to ten are expressed with words, except if it relates to measurement, while values above them written in number, except in early sentence. The fraction should be expressed in decimal. In the text, it should be used "%" rather than "percent". Avoid expressing ideas with complicated sentence and verbiage, and used efficient and effective sentence.

Title of the article should be written in compact, clear, and informative sentence, preferably not more than 20 words. Name of author(s) should be completely written. **Name and institution** address should also be completely written with street name and number (location), postal code, telephone number (O), facsimile number (O), and personal email address. For Indonesian universities, use local name. Manuscript written by a group, author for correspondence along with address is required. First page of the manuscript is used for writing above information.

Abstract should not be more than 200 words. **Keywords** is about five words, covering scientific and local name (if any), research theme, and special methods which used; and sorted from A to Z. All important **abbreviations** must be defined at their first mention. **Running title** is about five words. **Introduction** is about 400-600 words, covering the background and aims of the research. **Materials and Methods** should emphasize on the procedures and data analysis. **Results and Discussion** should be written as a series of connecting sentences, however, for manuscript with long discussion should be divided into subtitles. Thorough discussion represents the causal effect mainly explains for why and how the results of the research were taken place, and do not only re-express the mentioned results in the form of sentences. **Concluding** sentence should be given at the end of the discussion. **Acknowledgments** are expressed in a brief; all sources of institutional, private and corporate financial support for the work must be fully acknowledged, and any potential conflicts of interest are noted.

Figures and Tables of maximum of three pages should be clearly presented. Title of a picture is written down below the picture, while title of a table is written above the table. Colored figures can only be accepted if the information in the manuscript can lose without those images; chart is preferred to use black and white images. Author could consign any picture or photo for the front cover, although it does not print in the manuscript. All images property of others should be mentioned source. **There is no appendix**, all data or data analysis are incorporated into Results and Discussions. For broad data, it can be displayed on the website as a supplement.

References Author-year citations are required. In the text give the authors name followed by the year of publication and arrange from oldest to newest and from A to Z. In citing an article written by two authors, both of them should be mentioned, however, for three and more authors only the first author is mentioned followed by et al., for example: Saharjo and Nurhayati (2006) or (Boonkerd 2003a, b, c; Sugiyarto 2004; El-Bana and Nijs 2005; Balagadde et al. 2008; Webb et al. 2008). Extent citation as shown with word "*cit*" should be avoided. Reference to unpublished data and personal communication should not appear in the list but should be cited in the text only (e.g., Rifai MA 2007, pers. com. (personal communication); Setyawan AD 2007, unpublished data). In the reference list, the references should be listed in an alphabetical order (better, if only 20 for research papers). Names of journals should be abbreviated. Always use the standard abbreviation of a journal's name according to the **ISSN List of Title Word Abbreviations** (www.issn.org/2-22661-LTWA-online.php). The following examples are for guidance.

Journal:

Saharjo BH, Nurhayati AD. 2006. Domination and composition structure change at hemic peat natural regeneration following burning; a case study in Pelalawan, Riau Province. Biodiversitas 7: 154-158.

Book:

Rai MK, Carpinella C. 2006. Naturally Occurring Bioactive Compounds. Elsevier, Amsterdam.

Chapter in book:

Webb CO, Cannon CH, Davies SJ. 2008. Ecological organization, biogeography, and the phylogenetic structure of rainforest tree communities. In: Carson W, Schnitzer S (eds) Tropical Forest Community Ecology. Wiley-Blackwell, New York.

Abstract:

Assaeed AM. 2007. Seed production and dispersal of *Rhazya stricta***.** 50th Annual Symposium of the International Association for Vegetation Science, Swansea, UK, 23-27 July 2007.

Proceeding:

Alikodra HS. 2000. Biodiversity for development of local autonomous government. In: Setyawan AD, Sutarno (eds.) Toward Mount Lawu National Park; Proceeding of National Seminary and Workshop on Biodiversity Conservation to Protect and Save Germplasm in Java Island. Universitas Sebelas Maret, Surakarta, 17-20 July 2000. [Indonesian]

Thesis, Dissertation:

Sugiyarto. 2004. Soil Macro-invertebrates Diversity and Inter-Cropping Plants Productivity in Agroforestry System based on Sengon. [Dissertation]. Universitas Brawijaya, Malang. [Indonesian]

Information from internet:

Balagadde FK, Song H, Ozaki J, Collins CH, Barnet M, Arnold FH, Quake SR, You L. 2008. A synthetic *Escherichia coli* predator-prey ecosystem. Mol Syst Biol 4: 187. www.molecularsystemsbiology.com. DOI:10.1038/msb.2008.24

THIS PAGE INTENTIONALLY LEFT BLANK

BIOTEKNOLOGI
Volume 16, Number 1, May 2019
Pages: 1-4

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c160101

Short Communication: Evaluation of antimicrobial activity of hydroxyapatites from *Anadara granosa* and *Achatina fulica* against *Porphyromonas gingivalis*

SILVIA ANITASARI[1,2,♥], YUNIATI YUNIATI[2], SYLVIA AGUSTIN[1]

[1]Laboratory of Dental Material and Devices, Dentistry Program, Faculty of Medicine, Universitas Mulawarman. Jl. Krayan, Gunung Kelua, Samarinda 75123, East Kalimantan, Indonesia. Tel.: +62-541-748581, ♥email: carabelli74@yahoo.com

[2]Laboratory of Medical Microbiology, Faculty of Medicine, Universitas Mulawarman. Jl. Krayan, Gunung Kelua, Samarinda 75123, East Kalimantan, Indonesia

Manuscript received: 16 January 2019. Revision accepted: 9 May 2019.

Abstract. *Anitasari S, Yuniati Y, Agustin S. 2019. Short Communication: Evaluation of antimicrobial activity of* Anadara granosa *and* Achatina fulica *hydroxyapatites against* Porphyromonas gingivalis. *Bioteknologi 16: 1-4.* Hydroxyapatite bioceramic (HAp) plays a key role in reconstructive surgeries in both medicine and dentistry. However, its use is often saddled with the occurrence of post-surgery infection, thereby creating the need for antimicrobial therapy. The antibiotics used should support the body's defense mechanisms. Applying antimicrobials locally after surgery is promising, but the concentration must be lethal to pathogens and must be safe for normal human flora, as most oral antibiotic therapies are not effective. This study aimed to create HAp with good antimicrobial activity without causing harm to normal human flora. Antimicrobial activity of various combinations of hydroxyapatites from *A. granosa* and *A. fulica* against *P. gingivalis* were determined by the *Kirby-Bauer* method. The results showed that the inhibitory zone of HAp *A. granosa* (8.3±0) was similar to positive control Chlorhexidine (8.5± 0.1), but no activity was found in HAp of *A. fulica.* The HAp combination of *A. granosa* and *A. fulica*, at the ratio of 6: 4, had the smallest inhibitory zone (3.5±0 mm). It can be concluded that the differences in antibacterial activity of HAp from *A. granosa*'s and *A. fulica*'s might be influenced by concentration and mineral of HAp. Based on the results, it can be concluded that the inhibitory concentration was obtained at the ratio of 6: 4.

Keywords: Antibacterial, hydroxyapatite, *Porphyromonas gingivalis*

INTRODUCTION

In this new era of biomaterials, the ability for the human body to accept materials used for producing medical devices should be considered. This can include several factors, i.e., (i) the chemical and biological properties of the materials, (ii) biomaterials biocompatibility, and (iii) recipient's health condition (Nathanael et al. 2018).

In the field of dentistry, biocompatibility is the material capability to be compatible with dentition, oral mucosa, and the entire body systems without causing injury or damage to the cells and tissues. Biomaterials are used to replace a part of the body in dentistry, but there have been many cases of graft failure due to bacterial infections in the tooth area. Therefore, biomaterials must have antimicrobial activity so that they can reduce the failure of the grafting process (Pandey et al. 2016). Antibacterial agents are in high demand for sanitary materials.

Therefore, new materials with antibacterial activities are widely studied (Pandey et al. 2016; Nathanael et al. 2018). Antimicrobials influence the biocompatibility of the material. One of the biomaterials used in the dentistry field is hydroxyapatite. It is bioceramic with excellent biocompatibility, bioactivity, osteoinductive, and osteoconductive properties. This material is widely used to repair and reconstruct bone damage in the human skeleton. The process of filling the bone defect to repair was called augmentation or guided bone regeneration (Pandey et al. 2016). The hydroxyapatites from *Anadara granosa* and *Achatina fulica* are composed of calcium phosphate, both have antimicrobial activity against gram positive and negative bacteria, yet their combined mechanism of action has not been revealed (Berniyanti et al. 2007; Santana et al. 2012; Pandey et al. 2016). When two antimicrobial agents are combined, the effect of the combination may equal the sum of the antimicrobial effects of its components (addition), it may exceed this sum (synergism), or it may be smaller (antagonism) (Hafidh et al. 2011).

The study aims to determine whether the combination of hydroxyapatites from *A. granosa* and *A. fulica* has antimicrobial properties against *Prophyromonas gingivalis*.

MATERIALS AND METHODS

Materials

The sampling and collection of *Anadara granosa* and *Achatina fulica* was conducted in Samarinda, East Kalimantan, Indonesia; and identification was carried out in the Ecology and Animal Systematics Laboratory, Mulawarman University, Samarinda, East Kalimantan, Indonesia.

Calcination of *Anadara granosa* and *Achatina fulica*

The shells of *A. granosa* and *A. fulica* were cleaned and dried. The process was continued with calcination twice. The first calcination was done at the temperature of 1000°C for 12 hours to get calcium, and at the second calcination was done at the temperature of 1000°C for 24 hours (Anitasari et al. 2018).

Hydroxyapatite synthesis (HAp synthesis)

The HAp synthesis was carried as follows: 1 M Ca $(OH)_2$ solution was prepared by weighing 24.81 grams of CaO in a beaker glass and mixed with 600 mL of distilled water. The solution was stirred using a magnetizer at a speed of 150 rpm until homogeneous. After being homogenous, the solution was added with 300 mL of 1.8 M phosphoric acid, heated on a hot plate at 40°C, and stirred for 60 minutes at a speed of 300 rpm and then left for 24 hours. The product was filtered with Whatman membrane and dried at 100°C for 3 hours. Finally, the sintering process was carried out at 900°C for 5 hours (Anitasari et al. 2018).

Antimicrobial sensitivity test

The culture medium of Mueller-Hinton Agar was prepared as follows: 38 gram of Mueller Hinton Agar was suspended in one liter of distilled water and heated with frequent agitation. It was then boiled for one minute to completely dissolve the medium, followed by autoclaving at 121°C for 15 minutes. 20-25 mL of the freshly prepared and cooled MHA was poured into sterile Petri dishes. After solidification, 100 µL of *P. gingivalis* bacteria cultured in nutrient broth (with a concentration of 0.5 Mc Farland Standard) were swabbed evenly on each of the agar plates using a sterile cotton bud and clearly labeled. After that, holes were on the surface of agar were made with a punch hole. A different combination of HAp was put into the hole. Chlorhexidine used as a positive control and sterile saline as a negative control. Finally, Petri dishes were incubated at 37°C for 24 hours, and the clear zone of inhibition was observed after incubation (Sudarna et al. 2011).

Statistical analysis

The data was expressed as means ± SE, and the statistical analysis was performed according to SPSS software version 23.00.

RESULTS AND DISCUSSION

Table 1 shows that the diameter of the inhibitory zone of HAp from *A. granosa* was similar to positive control chlorhexidine. It indicated that the HAp of *A. granosa* had significant antimicrobial activity against *P. gingivalis*. *P. gingivalis* is gram-negative anaerobic bacterium as the main cause of Periodontitis. It utilizes cysteine protease, called gingipains. The gingipain family includes two related proteases, Arg-gingipain and Lys-gingipain. The Arg-gingipains include two members, namely Arg-gingipain A (*RgpA*) and Arg-gingipain B (*RgpB*), encoded by two closely related genes, *RgpA* and *RgpB*. The Lys-gingipain (*kgp*) is encoded by a single gene, *kgp*, which is the major virulence of the *P. gingivalis* (Guo et al. 2010; Tang et al. 2011; Jerala et al. 2012). The HAp of *A. granosa* may have neutralized the *kgp* gene due to its capability to inactivate the process of cationic antimicrobial peptides on their surface. Different conditions were observed in the HAp of *A. fulica*, as they could not kill or inhibit the growth of the bacteria (Viera et al. 2004; Dos Santos et al. 2006; Verma et al. 2016). The combination of HAp of *A. granosa* and *A. fulica* was antagonistic (Table 2). Increasing HAp from *A. fulica* cause in decreasing the inhibitory zone against *P. gingivalis*. The HAp of *A. fulica* hindered the effect of *A. granosa* and the circumstances that influenced their antagonism enabled us to understand their benefit better.

The shells of *A. granosa* and *A. fulica* contained glycosaminoglycan (GAGs). The common GaGs include glycosaminoglycans (heparin, keratan sulfate, heparan sulfate, and hyaluronic acid), galactosaminoglycan (chondroitin sulfate and dermatan sulfate) and acharan sulfate which can only be found in snails (*A. fulica*) (Verma et al. 2016).

The glycosaminoglycans can inhibit protease enzymes, such as gingipain, but their activity depends on the pH and temperature of hydroxyapatites because there are several sensors in the proteases that can be activated by pH and temperature. Hence, it is still unexplainable that the HAp of *A. granosa* inhibits the growth of *P. gingivalis*, but the other one from *A. fulica* did not inhibit the growth of *P. gingivalis* (Hocevar et al. 2018).

Table 1. Antimicrobial activity of HAp of *Anadara granosa* and HAp of *Achatina fulica* by Kirby-Bauer Method (n=3).

	HAp *A. granosa*	**HAp *A. fulica***	**Control +**	**Control-**
Inhibition zone in diameter (mm)	8.3±0	0	8.5±0.1	0

Table 2. Antimicrobial activity of various combination of hydroxyapatites (*Anadara granosa: Achatina fulica*) by Kirby-Bauer Method (n=6).

	The ratio of Ag: Af										
	9: 1	**8: 2**	**7: 3**	**6: 4**	**5: 5**	**4: 6**	**3: 7**	**2: 8**	**1: 9**	**C+**	**C-**
Inhibition zone in diameter (mm)	7.8±0.2	5.1±0.	5±0	3.5±0.5	0	0	0	0	0	8.5±0.1	0

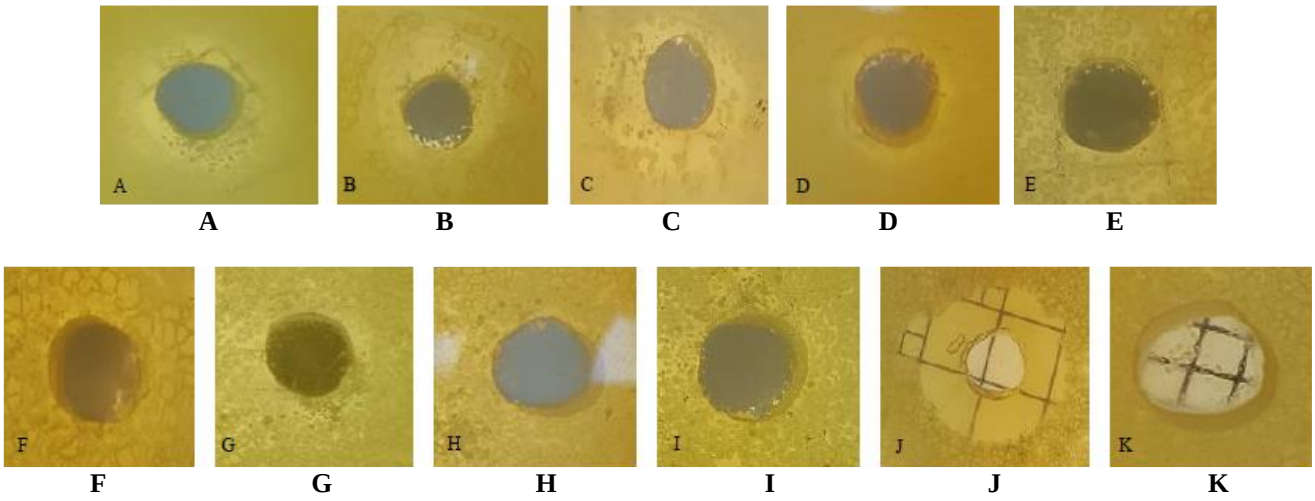


Figure 1. Photographs of inhibitory zone of various hydroxyapatite combination of *Anadara granosa* (Ag) and *Achatina fulica* (Af). A. Ag: Af (9: 1), B. Ag: Af (8: 2), C. Ag: Af (7: 3), D. Ag: Af (6: 4), E. Ag: Af (5: 5), F. Ag: Af (4: 6), G. Ag: Af (3: 7), H. Ag: Af (2: 8), I. Ag: Af (1: 9), J. C+, K. C-.

A combination of HAp of *A. granosa* with *A. fulica* showed antagonism due to the presence of acharan sulfate that could inhibit the GAGs on the *A. granosa* (Adhya et al. 2016; Chernikov et al. 2013; Shyamasree 2017; Toda et al. 1980; Tuane et al. 2004). Acharan sulfate, the new GAGs, was first isolated and characterized by Kim et al (1996). The inhibitory zones of various combinations of the HAp from *A. granosa* and *A. fulica* were presented in Figure 1. Antibacterial ability is very important in the field of dentistry when using them on the human body (Dos Santos et al. 2006; Kim et al. 2007).

The use of biomaterials in the field of dentistry is influenced by many factors, including antimicrobial characteristics and its toxicity to tissues. Hence, it cannot be proposed as a dental material if it is toxic to tissues. Therefore, employing the right ratio between HAp *A. granosa* and HAp *A. fulica* was important to prevent tissue toxicity due to the long-term use of the material. The best ratio between two hydroxyapatites was 6: 4, because it was the smallest concentration for inhibiting or killing bacteria (Leekha et al. 2011; Li et al. 2018; Yousef et al. 2018).

ACKNOWLEDGEMENTS

This research was supported funding of the Project Implementation Unit IsDB University of Mulawarman, Samarinda, Indonesia (No: 2248/UN17.11/PL/2018) and the authors declare no conflict of interest.

REFERENCES

Adhya M, Biswajit S. 2016. Gal/GalNAc Specific Multiple Lectins in Marine Bivalve *Anadara granosa*.Fish Shellfish Immunol 50: 242-246.

Anitasari A, Mu'ti A, Hutahaean YO. 2018. Effect on drying on the porosity of the hydroxyapatite and cellulose nata de coco composite as bone graft candidate. IOP Conf Ser Earth Environ Sci 144: 012043 DOI: 10.1088/1755-1315/144/1/012043.

Berniyanti T, Edy BW, Suwarno.2007. Biochemical Characterization of An Antibacterial Glycoprotein from *Achatina fulica ferussac* Snail Mucus Local Isolate and Their Implication on Bacterial Dental Infection. Indonesian J Biotechnol 12 (1): 943-951.

Chernikov OV, Molchanova IV, Chikalovets V, Kondrashina AS, Li W, Lukyanov PA. Lectins of marine hydrobionts. Biochemistry Moscow 78: 760-770. DOI: 10.1134/S0006297913070080.

Dos Santos AVF, Onofre GR, Oliviera DMP, Machado EA, Allodi S, Silva LCF. 2006. Heparan sulfate is the main sulfated glycosaminoglycan species in internal organs of the male cockroach, *Periplaneta americana*. Micron 37: 41-46.

Guo YH, Nguyen KA, Potempa J. 2000. Dichotomy of gingipains action as virulence factors: from clearing substrates with the precision of a surgeon's knife to meat chopper-like brutal degradation of proteins. Periodontol 54 (1): 15-44.

Hafidh RR, Abdulamir AS, Vern LS, Bakar FA, Abas F, Jahanshiri F, Sekawi Z. 2011. Inhibition of growth of highly resistant bacterial and fungal pathogens by a natural product. Open Microbiol 5: 96-106.

Hocevar K, Potempa J, Turk B. 2018. Host cell-surface proteins as substrates of gingipains, the main proteases of *Porphyromonas gingivalis*. Biol Chem 399 (12): 1353-1361.

Jerala NK. 2012. The role of cysteine proteinases and their inhibitors in the host-pathogen cross talk. Curr Protein Pept Sci 13: 767-775.

Kim HS, Lee YH, Lee YR, Sun AI, Lee JK, Kim YS, Sim JS, Choi HS, Lee CK. 2007. Activation of professional antigen presenting cells by acharan sulfate isolated from giant African Snail, *Achatina fulica*. Arch Pharm Res 30 (7): 866-870.

Leekha S, Terrel CL, Edson RS. 2011. General principle of antimicrobial therapy. Mayo Clin.Proc86 (2): 156-167.

Li X, Wang L, Fan Y, Feng QL, Cui FZ. 2012. Biocompatibility and toxicity of nanoparticles and nanotubes. J Nanomater 1: 1-19.

Nathanael AJ, Mangalaraj D, Hong SI. 2018. Biocompatibility and antimicrobial activity of hydroxyapatite/titania bio-nanocomposite. Proceeding of 18th International Conference on Composite Materials*:* 1-6.

Pandey E, Srivastava K, Gupta S, Srivastava S, Mishra N. 2016. Some biocompatible materials used in medical practise. Intl J Pharm Sci Res 7 (7): 2748-2755.

Santana WA, De Melo CM, Cardoso JC, Pereira-Filho RN, Rabelo AS, Reis FP, Cavalcanti RL. 2012. Assessment of antimicrobial activity

and healing potential of mucous secretion of *Achatina fulica*. Intl J Morphol 30 (2): 365-373.

Shyamasree B. 2017. Sialic Acid Binding Lectins (SABL) from mollusca, A review and in silico study of SABL from *Solen grandis* and *Limax flavus*. J Entomol Zool Stud 5 (6): 1563-1572.

Sudarna, Setiorina, Suprapto H. 2011. Efektivitas ekstrak tanaman maniran (*Phyllanthus niruri*) sebagai antibakteri *Edwariella farda seca* in vitro. Jurnal Ilmiah Perikanan dan Kelautan 3 (1): 103-108. [Indonesian]

Tang YQ, Potempa J, Pike RN, Wijeyewickrema LC. 2011. The lysine-specific gingipain of *Porphyromonas gingivalis*. In: Robinson MW, Dalton JP (eds). Cysteine Proteases of Pathogenic Organism. Landes Bioscience and Springer Science, USA.

Toda N, Doi A, Jimbo A, Matsumoto I, Seno N. 1980. Interaction of sulfated glycosaminoglycans with lectins. J Biol Chem 256 (11): 5345-5349.

Tuane CRG, Viera, Costa-Filho A, Norma C, Saldago, Alloa S, Valente AP, Nasciutoti LE, Silva LC. 2004. Acharan sulfate, the new glycosaminoglycan from *Achatina fulica* Bowdich 1820, structural, heterogeneity, metabolic labeling, and localization in the body, mucus, and the organic shell matrix. Eur J Biochem 271: 845-854.

Verma S, Dixit R, Pandey KC. 2016. Cysteine protease: Modes of activation and future prospects as pharmacological targets. J Front Pharmacol 7 (107): 1-12.

Viera TCRG, Costa-Filho A, Salgado NC, Allodi S, Valente AV, Nasciutti LE, Silva LCF. 2004. Acharan sulfate, the new glycosaminoglycan from *Achatina fulica* Bowdich 1822. Eur J Biochem 271: 845-854.

Yousef JM, Danial EN. 2012. In vitro antibacterial activity and minimum inhibitory concentration of zinc oxide and nano-particle zinc oxide against pathogenic strains. J Health Sci 2 (4): 38-42.

Zhao J, Liu XY, Kao C, Zhang E, Li Q, Zhang F, Linhardt RJ. 2016. Kinetic and structural studies of interaction between glycosaminoglycans and langerin. Biochemistry 55: 4552-4560.

BIOTEKNOLOGI
Volume 16, Number 1, May 2019
Pages: 5-10

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c160102

Identification and antimicrobial activity of lactic acid bacteria from the digestive tract of eels (*Monopterus albus*)

RIDWAN[1], ENDAH RETNANINGRUM[2,♥], MIFTAHUL ILMI[2], BUDI SETIADI DARYONO[3]

[1]Graduate Program in Biology, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia
[2]Microbiology Laboratory, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia. Tel./fax.: +62-274-580839, ♥email: endahr@ugm.ac.id
[3]Molecular Genetics and Breeding Laboratory, Faculty of Biology, Universitas Gadjah Mada. Jl. Teknika Selatan, Sekip Utara, Sleman 55281, Yogyakarta, Indonesia

Manuscript received: 4 January 2019. Revision accepted: 12 May 2019.

Abstract. *Ridwan, Retnaningrum E, Ilmi M, Daryono BS. 2019. Identification and antimicrobial activity of lactic acid bacteria from the digestive tract of eels (*Monopterus albus*). Bioteknologi 16: 5-10.* This study aims to identify the species of lactic acid bacteria from the digestive tract of eels (*Monopterus albus*) which have inhibitory activity against pathogenic bacteria. The eel used in this study was obtained from the eel cultivation site in Yogyakarta. The isolation results obtained thirteen isolates; the isolated bacteria were then phenotypically characterized to select lactic acid bacteria. The results of phenotypic characterization obtained seven isolates that were in accordance with the character of lactic acid bacteria. Seven lactic acid bacterial isolates were then screened to select the three best isolates in inhibiting the growth of pathogenic bacteria (*Aeromonas hydrophila, Staphylococcus aureus, and Vibrio harveyi*). The results showed that seven isolates of lactic acid bacteria had antimicrobial activity, which were able to inhibit the growth of pathogenic bacteria. Three of the best isolates were selected to be used for molecular identification and monitoring of antimicrobial activity. The results showed that the three selected isolates produced an antimicrobial extract which was able to inhibit the growth of pathogenic bacteria. Furthermore, the results of the molecular identification showed that the three selected isolates were *Lactococcus lactis* species.

Keywords: 16s rRNA gene, antimicrobial, eel, lactic acid bacteria, *Monopterus albus*

INTRODUCTION

Lactic acid bacteria have various important roles for host health; for example, stimulating the host's immune system (Gatesoupe 2008). In addition, lactic acid bacteria have antimicrobial activity due to the production of lactic acid, acetic acid, bacteriocin, bacteriocin-like compounds and bioactive molecules such as ethanol, formic acid, fatty acids, hydrogen peroxide, diacetyl, reuterin, reutericyclin (Awen et al. 2012). The antimicrobial activity of acid bacteria in inhibiting the growth of pathogenic bacteria attracted the attention of researchers to isolate these bacteria in various sources.

A source that can be used to isolate lactic acid bacteria is the digestive tract of fish. Previous researchers have used the digestive tract of fish to isolate lactic acid bacteria. Lactic acid bacteria isolated from the digestive tract of fish generally have antimicrobial activity, which is stable to high temperatures, pH and stable to organic solvents such as chloroform. Loh et al. (2017) reported that *Lactococcus lactis* subsp. lactis CF4MRS isolated from the digestive tract of fish can inhibit bacterial growth of *Escherichia coli*, *Edwardsiella tarda*, *Aeromonas hydrophila*, *Pseudomonas fluorescens*, *Pseudomonas aeruginosa*, *Klebsiella pneumonia* and *Serratia marcescens.*

One group of fish that can be used as a source for isolating lactic acid bacteria are eels (*Monopterus albus*). Eels are aquaculture animals that are very tolerant of low dissolved oxygen content and can live in temperature which are close to freezing (Roy 2013). The ability of eel (*Monopterus albus*) to live on low oxygen content and near-freezing temperatures is likely due to the presence of superior lactic acid bacteria compared to other aquaculture animals. So, further research regarding the antimicrobial characterization of lactic acid bacteria from the eel digestive tract (*Monopterus albus*) is needed. In addition, molecular identification is needed to find out the species of lactic acid bacteria which have the best antimicrobial activity.

MATERIALS AND METHODS

Procedures

Isolation of lactic acid bacteria

Isolation of lactic acid bacteria in this study used eel (*Monopterus albus*) obtained from the Pingit Market, Yogyakarta, Indonesia. Three eels were taken to the Microbiology Laboratory, Faculty of Biology, University Gadjah Mada, Yogyakarta. The eels were then differentiated and the intestines were taken and then mashed using mortar. After that, isolation was carried out using a dilution technique with the pour plate method. The sample was diluted using sterile distilled water, and then a 400 µL 10^{-2} to 10^{-6} diluent was put into a petri dish and then added to MRSA liquid. Petri dishes that had the sample added and MRSA media, were then incubated at room temperature for 2 x 24 hours. The growing bacterial

colonies were selected based on the formation of clear zones around the colonies for purification. The purification results were propagated on the sloping MRSA media and stored in the refrigerator as stock for further research (Bajpai et al. 2016).

Screening of inhibitory activity of lactic acid bacteria

The screening of lactic acid bacteria used the Well diffusion method. This involved making a 5 mm well in the NA media (agar nutrient) in a petri dish which had been previously tested by bacterial culture (Aeromonas hydrophila, Vibrio harveyi, Staphylococcus aureus) using the pour plate method. Next, 50 µL of lactic acid bacteria culture was added into the well. Then the well was incubated for 24-48 hours at room temperature. The clear zone formed after incubation was measured as the activity of inhibiting lactic acid bacteria (Edalatian et al. 2015).

Antimicrobial production

Lactic acid bacteria culture was grown in 20 mL MRSB media, then incubated in the shaker incubator for 24 hours at 37ºC. After 24 hours incubation, the culture was centrifuged at 10,000 rpm at 10ºC for 15 minutes. Then the supernatant was taken as a crude antimicrobial extract and stored in the refrigerator (Hyronimus et al. 1998, Bizani and Brandelli 2002).

Cell-Free Supernatant (CFS) test

Test of CFC's activity used the Well diffusion method. First this involved making a 5 mm well on NA media (agar nutrient) in a petri dish which had previously been added to test bacteria (*Aeromonas hydrophila, Vibrio harveyi, Staphylococcus aureus*) using the pour plate method. Followed by adding 50 µL of antimicrobial crude extract, which was filtered using a bacterial filter measuring 0.22 µL pore. Then the well was incubated for 24-48 hours at room temperature. The clear zone formed was measured and calculated using the formula:

$$Inhibition\ Score\ (IS) = \frac{\text{Ø clear zone of inhibition (mm)(mm)}}{\text{Ø size of well (mm)}}$$

Antimicrobial inhibitory activity of CFC (Cell-Free Supernatant) is said to be low ($1 < IS < 3$), moderate ($3 \leq IS < 5$), strong ($5 \leq IS < 7$), or very strong ($7 \leq IS < 9$) (Tremonte et al. 2017).

Molecular identification

Bacterial DNA extraction

DNA extraction was carried out by following the procedure contained in the Geneaid Presto™ Mini gDNA Bacteria Kit. This was to prepare bacterial culture on MRSB media for 48 hours. Bacterial culture of 1.5 mL was then transferred to the microcentrifugation tube. Then it was centrifuged for 1 minute at a speed of 14-16,000 x g and then the supernatant was removed. 200 µL gram (+) buffer was inserted into the centrifuge tube and lysozyme (4 mg/mL) was added. Then it was vortexed until lysozyme was completely dissolved. A gram (+) of buffer solution that has been added to lysozyme was put into the sample then the pellet was resuspended using a vortex or pipette. The samples were then incubated at 37 ° C for 20 minutes, and it was reversed several times during incubation. Next, 20 µL proteinase K was added and vortexed until homogeneous. It was incubated at 60ºC for 10 minutes, and it was reversed every 3 minutes during incubation. Next the sample was added with 200 µL GB buffer before being vortexed. Afterwards, it was incubated for 10 minutes at 70ºC. Next the sample was added with 200 µL ethanol absolute and then was vortexed for 10 seconds. It was then put into GD column and centrifuged at a speed of 14-16,000 g for 2 minutes. The previously used tube was then replaced with a new one. After that, 400 µL of W1 buffer was added and was centrifuged at a speed of 14-16,000 g for 30 seconds. Then 600 µL of W1 buffer was added and centrifuged at a speed of 14-16000 g for 30 seconds. Next the GD column was transferred into a sterile Eppendorf tube and added with 100 µL of pre-heated elution. Lastly, it was centrifuged at a speed of 14-16000 g for 3 minutes. The supernatant which was still in the Eppendorf cane was DNA extract (Geneaid Biotech Ltd., 2017).

Amplification of the 16S rRNA gene Lactic Acid Bacteria

The amplification reaction was carried out in a PCR tube. The amplification used TaKara Eq Taq as much as 0.25 µL, 10 x Ex Taq Buffer (containing Mg2 + 2.5 mM) as much as 5 µL, dNTP mixture (2.5 mM) as much as 4 µL, primer 27f (5 '-AGAGTTTAGTCCTGGCT CAG-3') and 1492r (5'-GGTTACCTTGTTA CGACTT-3 ') with 0.5 µL each, 1 µg of the genome extract and 10 µL of distilled water added. Amplification was carried out to obtain a single band. The amplification process was initiated with pre-denaturation at 96ºC for 4 minutes, followed by 30 cycles at 94ºC for 1 minute, and annealing at 51.5ºC for 1.30 minutes. After extension at 68ºC for 8 minutes, PCR was completed by elongation for 10 minutes at 68 °C. PCR products were then taken and stored at 4 ° C and examined using 1% agarose (Lawalata et al. 2011).

Agarose electrophoresis

1% agarose was heated to boiling with TAE buffer solvent (tris acetate). After it was cooled and poured it into the electrophoresis tray, it was left to be solidified. Then the comb was lifted. Furthermore, into the electrophoresis tool, 1x TAE buffer was poured. The tray containing the solid agarose was then placed on electrophoresis until it was submerged by a buffer. The loading dye was prepared on top of the parafilm then was mixed with 4 µL PCR products and put into agarose well using a micropipette. In addition, a 3 µL marker was prepared. The electrophoresis device was conducted at a voltage of 100 volts for 30 minutes. Agarose results from electrophoresis were immersed in an ethidium bromide dye solution for 10 minutes, then they were visualized in the transilluminator. The presence of DNA bands with a size of ± 1500 bp indicated that the 16S rRNA gene has been amplified, then the results were documented using a camera.

Data analysis

The results of 16S rRNA gene sequencing were analyzed on the website of www.ncbi.nlm.nih.gov. The BLAST program was used to determine the similarity of nucleotide sequences so that identification can be done based on the similarity of nucleotide sequences. Then Phylogeny Tree Construction was carried out to determine the level of closeness between species using the application of MEGAX (Kumar et al. 2018).

RESULTS AND DISCUSSION

Isolation of lactic acid bacteria

Thirteen isolates from this study were phenotypically characterized. Phenotypic characterization being carried out was colony morphology, cell morphology, catalase test and gram staining properties. The results of the phenotypic characteristics of the thirteen bacterial isolates are listed in Table 1.

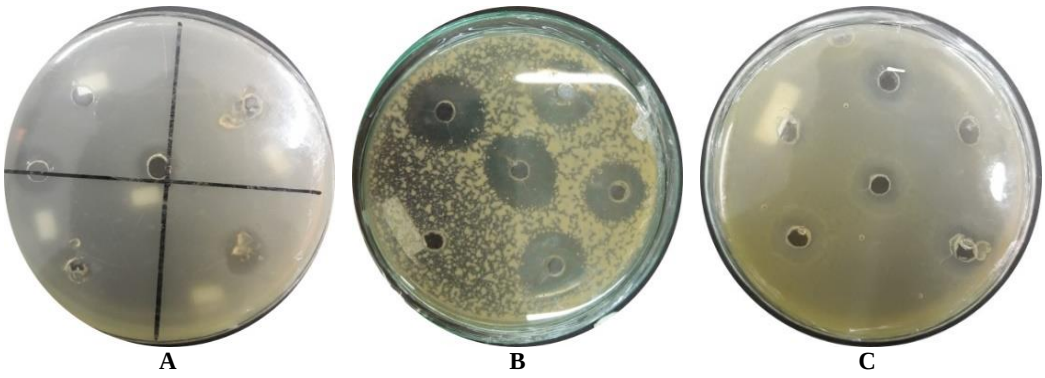
The results of phenotypic characterization listed in Table 1 show that there are seven bacterial isolates that are suitable for the characterization of lactic acid bacteria. These seven isolates were then screened to find three isolates that had the best antimicrobial activity in inhibiting the growth of test bacteria (*Aeromonas hydrophila*, *Staphylococcus aureus*, and *Vibrio harveyi*).

Screening of inhibitory activity of lactic acid bacteria

Screening the ability of lactic acid bacteria is carried out on seven bacterial isolates. This screening aims to select lactic acid bacteria which have the best activity in inhibiting the growth of pathogenic bacteria (*Aeromonas hydrophila, Staphylococcus aureus* and *Vibrio harveyi*). The inhibition ability is best seen based on the diameter of the clear zone formed around the well (Figure 1). The results of screening for inhibitory activity of lactic acid bacteria are listed in Table 2.

Table 1. Characteristics of lactic acid bacteria strain isolated from eels (*Monopterus albus*).

Character	Bacterial Isolates												
	B1A	**B1B**	**B1C**	**B1D**	**B1E**	**B1F**	**B1G**	**B1H**	**B1I**	**B1J**	**B1K**	**B1L**	**B1M**
Cell shape	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci	Cocci
Gram strain	+	+	+	+	+	+	+	-	-	+	-	-	-
Catalase	-	-	-	-	-	-	-	+	+	+	+	+	+
Color													
White	-	-	+	+	-	+	+	+	+	+	+	+	+
Cream	+	+	-	-	+	-	-	-	-	-	-	-	-
Size													
Small	+	+	+	+	-	+	-	+	+	-	-	+	+
Pintpoint	-	-	-	-	+	-	+	-	-	+	+	-	-
Elevation													
Convecs	+	-	+	+	+	+	+	+	+	+	+	+	+
Flat	-	+	-	-	-	-	-	-	-	-	-	-	-



A **B** **C**

Figure 1. Zone of inhibition of lactic acid bacteria at the screening stage i.e.: A. Inhibition zone of *Aeromonas hydrophila*, B. Inhibition zone of *Vibrio harveyi*, C. Zone of inhibition of *Staphylococcus aureus*.

Table 2. Screening results of seven isolates of lactic acid bacteria isolated from the eel digestive tract (*Monopterus albus*).

Strain code	Ø Well (mm)	Ø Mean inhibitory zone (mm)		
		Vibrio harveyi	*Aeromonas hydrophila*	*Staphylococcus aureus*
B1A	5	23.33 ± 1.15^{ab}	11.67 ± 0.58^{a}	16.67 ± 0.58^{a}
B1B	5	21.00 ± 1.00^{cd}	11.33 ± 1.15^{a}	14.67 ± 1.15^{ab}
B1C	5	21.00 ± 3.46^{cd}	12.33 ± 2.31^{a}	9.00 ± 1.73^{c}
B1D	5	25.33 ± 2.89^{a}	12.33 ± 2.52^{a}	13.00 ± 2.65^{b}
B1E	5	22.33 ± 3.21^{bc}	12.00 ± 3.00^{a}	11.33 ± 1.15^{bc}
B1F	5	21.00 ± 1.73^{cd}	11.33 ± 1.15^{a}	11.67 ± 2.08^{bc}
B1G	5	18.67 ± 0.58^{d}	11.67 ± 0.58^{a}	13.67 ± 4.04^{ab}
K-	5	-	-	-

Note: Ø = Diameter. Different letters in the same column show the level of real difference – Duncan test α <0.05 (mean ± std)

Table 2 shows that antimicrobial extract of the three isolates had inhibitory activity against the test bacteria (*Aeromonas hydrophila*, *Staphylococcus aureus* and *Vibrio harvei*). The inhibition activities of seven isolates of lactic acid bacteria were then analyzed statistically using SPSS 17.0 software. The results of statistical analysis showed that the inhibitory activity of lactic acid bacteria had a significant effect on inhibiting the growth of pathogenic bacteria. Ringo (2005) states that lactic acid bacteria isolated from aquatic animals can inhibit the growth of pathogenic bacteria such as *Aeromonas hydrophila* and *Vibrio harveyi*. Buntin et al. (2008) reported that lactic acid bacteria isolated from the digestive tract of fish were able to inhibit the growth of *Staphylococcus aureus* bacteria. Based on the results of the screening, three isolates were selected for further testing. Selected isolates in this study were B1A, B1D and B1E.

Cell-Free Supernatant (CFS) test

The CFC test was conducted to determine the activity of antimicrobial crude extract in inhibiting the growth of test bacteria. The antimicrobial crude extract was obtained from 24-hour-old culture of lactic acid bacteria. Antimicrobial extracts were filtered using a bacterial filter measuring 0.22 µL pore to separate cells from supernatants. The crude extract filtered was tested for its ability to inhibit the growth of test bacteria. The results of the antimicrobial crude extract activity test are listed in Table 3.

Based on Table 3, the ability of antimicrobial extract from the three bacterial isolates is known now. The results showed that the antimicrobial extract of the three isolates had inhibitory activity against the test bacteria. The three isolates had an Inhibitor Score (IS) <3 or a weak category against *Aeromonas hydrophila* and *Vibrio harveyi*. Furthermore, isolates B1A and B1D have Inhibitor Score (IS) <3 or weak categories while isolates B1E have Inhibitor Score (IS) > 3 or moderate categories against *Staphylococcus aureus* bacteria.

Molecular identification

Molecular identification aims to determine the isolate species of lactic acid bacteria. Molecular identification used the 16S rRNA gene. Amplification of the 16S rRNA gene used 27F primers (5'-AGAGTTTA GTCCTGGCTCAG-3 ') and 1492R (5'-GGTT ACCTTGTTACGACTT-3'). The amplification results are listed in Figure 2.

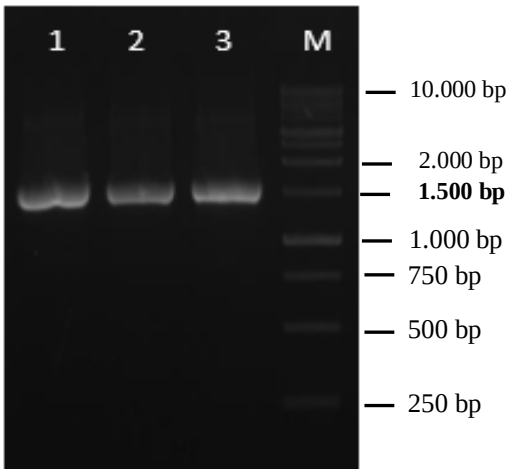


Figure 2. Results of amplification of the 16S rRNA gene.

Table 3. Test results for antimicrobial crude extract activity.

Test bacteria	Strain code	Ø Well (mm)	Ø Inhibition zona (mm)			Mean Ø Inhibition zona (mm)	*Inhibition Score* (IS)
			U1	U2	U3		
Vibrio harveyi	B1A	5	13	15	14	14.00 ± 1.00	2.80^{a}
	B1D	5	14	13	13	13.33 ± 0.58	2.67^{a}
	B1E	5	15	15	14	14.67 ± 0.58	2.93^{a}
	K-	5	-	-	-	-	-
Aeromonas hydrophila	B1A	5	13	14	14	13.67 ± 0.58	2.73^{a}
	B1D	5	14	15	14	14.33 ± 0.58	2.86^{a}
	B1E	5	14	15	15	14.67 ± 0.58	2.93^{a}
	K-	5	-	-	-	-	-
Staphylococcus aureus	B1A	5	15	15	14	14.67 ± 0.58	2.93^{a}
	B1D	5	15	14	15	14.33 ± 0.58	2.86^{a}
	B1E	5	14	16	16	15.78 ± 1.15	3.16^{b}
	K-	5	-	-	-	-	-

Note: Ø = diameter. ^{a} = weak, ^{b} = moderate, ^{c} = strong

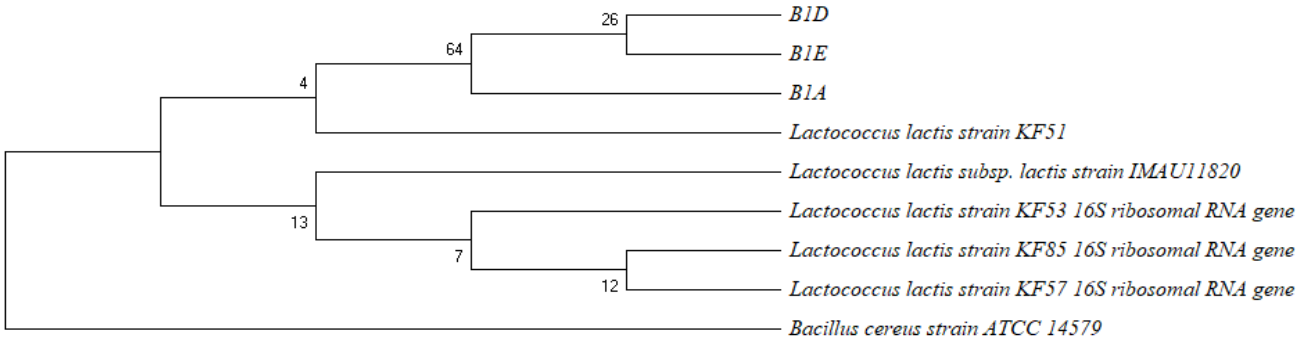


Figure 3. Tree of phylogeny isolates of lactic acid bacteria from eel (*Monopterus albus*) (bootstrap = 1000)

The results of 16S rRNA gene amplification were then sequenced using the same primer as the amplification process, 27F and 1492R primers. The sequencing results were then analyzed using BLAST on the site of www.ncbi.nlm.nih.gov with a database of ribosomal 16S rRNA genes. The BLAST analysis results showed that the three isolates had 99% similarity with *Lactococcus lactis* species. Then the phylogenetic tree construction was carried out to determine the evolutionary relationship between bacterial species from isolation and BLAST results. The phylogenetic tree was reconstructed using MEGAX software. The results of the phylogenetic tree reconstruction are shown in Figure 3.

The phylogeny tree in Figure 3 shows that isolates B1A, B1D, and B1E isolated from the eel digestive tract were in one cluster with *Lactococcus lactis*. The results showed that the isolates of lactic acid bacteria isolated from the eel digestive tract (*Monopterus albus*) are a species of *Lactococcus lactis.* Marrifield et al. (2014) reported that lactic acid bacteria include indigenous bacteria in the digestive tract of fish. Lactic acid bacteria that occupy the digestive tract of fish include *Lactococcus lactis*, *Streptococcus* spp., *Weissella cibaria*, and *Lactobacillus* spp. Kato et al. (2016) reported that Lactococcus spp. from the digestive tract of fish have antimicrobial activity and can inhibit the growth of *Staphylococcus aureus* (inhibitory zone 6 ± 0.17), *Escherichia coli* (inhibition zone 7 ± 0.10) and *Proteus* spp. (3.5 ± 0.10).

Discussion

Lactic acid bacteria have many important roles; for example, it can stimulate the host's immune system (Verschuere et al. 2000). It can also produce antimicrobial compounds that can inhibit the growth of harmful pathogenic bacteria (Muthukumar and Kandepan 2015). Previous studies by Balcazar et al. (2008) reported that lactic acid bacteria isolated from the digestive tract of fish were able to inhibit the growth of pathogenic bacteria such as *Aeromonas hydrophila, Aeromonas salmonicida, Yersinia ruckeri dan Vibrio anguillarum.*

This study shows that isolated lactic acid bacteria from the eel digestive tract could potentially inhibit the growth of pathogenic bacteria. Lactic acid bacteria that have the potential to inhibit the growth of pathogenic bacteria are then identified molecularly. The purpose of this study was to find lactic acid bacteria that can inhibit the growth of *Vibrio harveyi*, *Staphylococcus aureus*, and *Aeromonas hydrophila*. *Staphylococcus aureus* is one of the food pathogenic bacteria found throughout the world. Then *Vibrio harveyi* is one of the causes of gastroenteric disease and tissue invasion, especially in hosts who experience a decline in the immune system (Auerbach et al. 2016). *Aeromonas hydrophila* is one of the causes of infectious diseases in fish (Percival and Williams 2014). This study found that lactic acid bacteria were able to inhibit the growth of *Vibrio harveyi*, *Staphylococcus aureus*, and *Aeromonas hydrophila*. These lactic acid bacteria are included in the species *Lactococcus lactis*.

ACKNOWLEDGEMENTS

We thank all of those who have given criticism and suggestions for improving this manuscript. Thanks to the Microbiology Laboratory and the Falitma Laboratory, Faculty of Biology, Gadjah Mada University, Indonesia for being willing to facilitate research.

REFERENCES

Auerbach PS, Cushing TA, Harris NS. 2016. Auerbach's Wilderness Medicine, 7th ed. Elsevier, New York.

Aween M, Hassan Z, Belal M. 2012. Evaluation on antibacterial activity of *Lactobacillus acidophilus* strains isolated from honey. Amer J Appl Sci 9: 807-817.

Bajpai VK, Han JH, Nam GJ, Majumder R, Park C, Lim J, Paek WK, Rather IA, Park YH. 2016. Characterization and pharmacological potential of *Lactobacillus* sakei 1I1 isolated from freshwater fish *Zacco koreanus*. DARU J Pharamaceut Sci 24: 8. DOI: 10.1186/s40199-016-0147-8

Balcazar JL, Vendrell D, de Blas I, Girones O. 2008. Characterization of probiotic properties of lactic acid bacteria isolated from intestinal microbiota of fish. Aquaculture 278 (1-4): 188-191.

Bizani D, Brandelli A. 2002. Characterization of a bacteriocin produced by a newly isolated *Bacillus* sp. Atrain 8A. J Appl Microbiol 93: 512-519.

Buntin N, Chanthachum S, Hongpattarakere T. 2008. Screening of lactic acid bacteria from gastrointestinal tracts of marine fish for their potential use as probiotics. Songklanakarin J Sci Technol 30 (1): 141-148.

Edalatian MR, Habibi NMB, Mortazavi A. 2015. Identification of antimicrobial producing *Enterococci* isolated from Iranian raw milk cheeses using polyphasic approach. Appl Food Biotechnol 2(1): 19-24.

Gatesoupe FJ. 2008. Updating the importance of lactic acid bacteria in fish farming: natural occurrence and probiotic treatments. J Mol Microbiol Biotechnol 14 (1-3): 107-14.

Geneaid Biotech Ltd. 2017. Presto™ Mini gDNA Bacteria Kit, Geneaid Instruction Manual, For Research Use Only, Ver. 02.10.

Hyronimous BC, Marrec L, Urdaci MC. 1998. Coagulin, a bacteriocin-like inhibitory substance produced by *Bacillus coagulansI4*. J Appl Microbiol 35: 42-50.

Kato CD, Kahuma CE, Namulawa VT, Nasser Kasozi N. 2016. Antibacterial activity of *Lactobacillus* spp. and *Lactococcus* spp. isolated from various parts of pebbly fish, *Alestes baremoze*. British Microbiol Res J 17 (2): 1-7.

Kumar S, Stecher G, Li M, Knyaz C, Tamura K. 2018. MEGA X: Molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol 35 (6): 1547-1549.

Lawalata HJ, Sembiring L, Sembiring ES. 2011. Molecular identification of lactic acid bacteria producing antimicrobial agents from Bakasang, An Indonesian traditional fermented fish product. Indonesian J Biotechnol 16 (2): 93-99.

Loh JY, Lim YY, Ting ASY. 2017. Bacteriocin-like substances produced by *Lactococcus lactis* subsp. lactis CF4MRS isolated from fish intestine: Antimicrobial activities and inhibitory properties. Intl Food Res J 24 (1): 394-400.

Merrifield DL, Balcazar J, Daniels C, Zhou Z, Carnevali O, Sun Y, Hoseinifar SH, Ringo E. 2014. Indigenous lactic acid bacteria in fish and crustaceans. In: Aquaculture Nutrition: Gut Health, Probiotics and Prebiotics, 1st ed, Wiley-Blackwell, New York.

Muthukumar P, Kandeepan C. 2015. Isolation, identification, and characterization of probiotic organisms from intestine of freshwater fishes. Intl J Curr Microbiol Appl Sci 4 (3): 2319-7706.

Ringo E, Schillinger U, Holzapfel W. 2005. Antimicrobial activity of lactic acid bacteria isolated from aquatic animals and the use of lactic acid bacteria in aquaculture. In: Holzapfel WH, Naughton PJ. (eds.). Microbial Ecology in Growing Animals. Elsevier, New York.

Roy R. 2013. Budidaya Belut. PT Media Agro Pustaka, Jakarta. [Indonesian]

Tremonte P, Pannella G, Succi M, Tipaldi L, 1Sturchio M, Coppola R, Luongo D, Sorrentino E. 2017. Antimicrobial activity of Lactobacillus plantarum strains isolated from different environments: a preliminary study. Intl Food Res J 24 (2): 852-859.

Verschuere L, Rombaut G, Sorgeloos P, Verstraete W. 2000. Probiotic bacteria as biological control agents in aquaculture. Microbiol Mol Biol Rev 64 (4): 655-671.

BIOTEKNOLOGI
Volume 16, Number 1, May 2019
Pages: 11-20

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c160103

Molecular characterization of *Edwardsiella* species isolated from *Oreochromis niloticus* and *Clarias gariepinus* in Wakiso District, Uganda

MARY NANTONGO, ERNATUS M. MKUPASI♥, DENIS K. BYARUGABA
Sokoine University of Agriculture. PO Box 3021, Chuo Kikuu, Morogoro, Tanzania. Tel./fax.: + 255-23-2603511-4, ♥email: emkupasi@suanet.ac.tz

Manuscript received: 10 February 2019. Revision accepted: 15 May 2019.

Abstract. *Nantongo M, Mkupasi EM, Byarugaba DK. 2019. Molecular characterization of* Edwardsiella *species isolated from* Oreochromis niloticus *and* Clarias gariepinus *in Wakiso District, Uganda. Bioteknologi 16: 11-20. Edwardsiella tarda* (Ewing & McWhorter, 1965) is well-known as an opportunistic pathogenic bacterium causing Edwardsiellosis in the host body of cultured and wild fish. The disease is among the most important bacterial diseases causing severe economic losses in fish worldwide. Here, we determined the occurrence and performed characterization of *E. tarda* in cultured Nile tilapia and African catfish from selected 17 fish farms in Wakiso District, Uganda. Fish samples were collected in the duration between September 2016 and February 2017. Bacteriological examination of the internal organs (kidney, liver, spleen) was followed by clinical and post-mortem examination of the overall fish body. The bacterium was identified using conventional biochemical tests, API 20E kits, and sequencing of 16S rRNA. Phylogenetic analysis was done by the Neighbor-Joining method in MEGA 7.0.2 against the 16S rRNA gene sequences retrieved from the GenBank. The isolate was further screened for the presence of selected virulence genes by polymerase chain reaction (PCR). One isolate from *Oreochromis niloticus* (Linnaeus, 1758) was confirmed to be *E. tarda* by the 16S rRNA sequencing. The isolate gave 99.9% identity to other members of *E. tarda* compared to known 16S rRNA sequences in the GenBank database. In the phylogenetic analysis, the isolate did not cluster with any of the *E. tarda* isolates, indicating a distant relationship with the isolates whose sequences were included in this study. Six virulence genes that enhance bacterial survival and host pathogenesis were identified in the isolate, including; CitC, muk, gadB, katB, esaV and fima. This study confirmed one positive *E. tarda* isolates. Nevertheless, the isolate possessed several virulence genes indicating their potential to cause disease in fish. Because the bacterium is of public health importance, awareness should be created amongst fish farmers and stakeholders to take precautions to avoid disease outbreaks.

Keywords: *Clarias gariepinus*, *Edwardsiella* species, *Oreochromis niloticus*, Wakiso District

INTRODUCTION

The aquaculture industry is one of the fastest-growing food production sectors globally, with fish production accounting for 44.1% of 2014 (FAO 2016). In Uganda, fish is an important commodity and the source of animal protein for 34.5 million people. Still, the current consumption rate is only 5.7 kg per capita, well below the WHO recommended 12.5 kg per capita (Directorate of Fisheries Resources 2011). Thus, there is a growing need to increase fish production as the population grows rapidly by 3.4% annually (UNBOS 2014). The country needs to be more aggressive in increasing sustainable fish production to reach the goal of the agriculture sector of ensuring sustainable and market-oriented production, food security, and household incomes (NDPII 2015). Uganda's second National Development Plan has identified fish farming as one of the twelve agricultural enterprises on which the focus is to provide food security and contribute to export earnings (NDPII 2015).

Furthermore, the country will have to intensify aquaculture to ensure food security and maximize profits (Öztürk and Altınok 2014). However, fish kept in high densities are more likely to experience stress factors like poor water quality and physical damage, which predispose them to infections. Fish diseases are a major constraint to aquaculture, causing significant economic losses due to mortalities, reduced growth, and increased cost of production through disease management (Faruk et al. 2004). Bacteria are among the most encountered causes of diseases in cultured fish (Mohanty and Sahoo 2007). The *Edwardsiella* species is known to have a major impact on the economy, as it causes mass mortality in a considerable number of commercially important fish populations around the world (Park et al. 2012). The occurrence of *Edwardsiella* in fish has been reported in various countries, including Ethiopia (Kebede and Habtamu 2016); India (Das et al. 2014); Egypt (El-Seedy et al. 2015); Uganda (Walakira et al. 2014); Malaysia (Najiah and Lee 2006) and from the Mediterranean (Katharios et al. 2015).

Edwardsiella tarda (Ewing & McWhorter, 1965) is commonly found in water and healthy fish populations. However, the bacterium is opportunistic, so disease outbreaks usually occur when the environment is imbalanced, such as when the water quality is poor, the population is crowded, or the organic content is high (Park et al. 2012). Wyatt et al. (1979) reported *E. tarda* in African catfish and water bodies, increasing incidence with increasing organic pond content and water temperature. The *E. tarda* is a public health concern as it is known to be zoonotic; it causes disease in fish and other aquatic animals and causes gastroenteritis in humans (Park et al. 2012).

There is little information on the presence and prevalence of *Edwardsiella* species in Uganda, possibly

due to limited studies of fish diseases. Akoll and Mwanja (2012) attribute this to the lack of diagnostic tools and the high cost of identifying and characterizing such pathogens in subsistence aquaculture. Therefore, this study aimed to generate knowledge on *Edwardsiella* infections in farmed African catfish and Nile tilapia in different production systems in selected fish farms, which is useful in planning control measures.

The objectives of this study are: (i) to determine the presence of *Edwardsiella* species in cultured African catfish and Nile cichlids from selected farms in Uganda; (ii) to determine the phylogenetic relatedness of *Edwardsiella* isolates from farmed African catfish and Nile cichlids in Uganda; (iii) Determine the virulence genes present in *Edwardsiella* isolates recovered from Nile cichlids and African catfish in Uganda.

MATERIALS AND METHODS

Study area

This study was conducted in the Wakiso District of Central Uganda, which covers 1,906.7 km^2, as shown in Figure 1. It is located at 004'N North latitude, 32045'E East latitude, and 1,218 m above sea level. One of the main economic activities in the district is fishing on Lake Victoria and fish farming, both commercial and subsistence, in ponds, basins, and cages. The district was selected by FAO (2016) as one of the country's districts suitable for fish farming. The district has 15 sub-counties with a population of over 2 million people and an annual growth rate of 6.6% (UBOS 2014).

Study design and sampling strategy

The study used a cross-sectional research design to characterize *E. tarda* isolates from fish in the Wakiso District. Fish samples were collected and isolated, and bacteria were identified using biochemical assays, API 20E kits, and sequencing. Phylogenetic analysis was performed using MEGA 7.0 software, and isolates were screened for selected virulence genes by PCR.

Fish farms were specifically selected for sampling, with preference given to farms reporting fish diseases and depending on the farmer's willingness to provide samples. Simple random sampling was done to select healthy fish from the farming system, while diseased fish were deliberately selected from the farming system in case of disease.

Sample size

The size of the sample was determined using the formula by Naing et al. (2006);

$$n = \frac{Z^2P\ (1\text{-}P)}{d^2}$$

Where; "n" is the sample size, "Z" is the confidence interval (1.96), "P" is the disease prevalence of 9.67%, as previously reported by Kebede and Habtamu (2016) in Ethiopia and "L" is the expected error (0.05). Hence, sample size (number of fish) = 134. However, one hundred and eleven fish samples of Nile tilapia and African catfish were collected from 17 farms in the Wakiso District, Uganda.

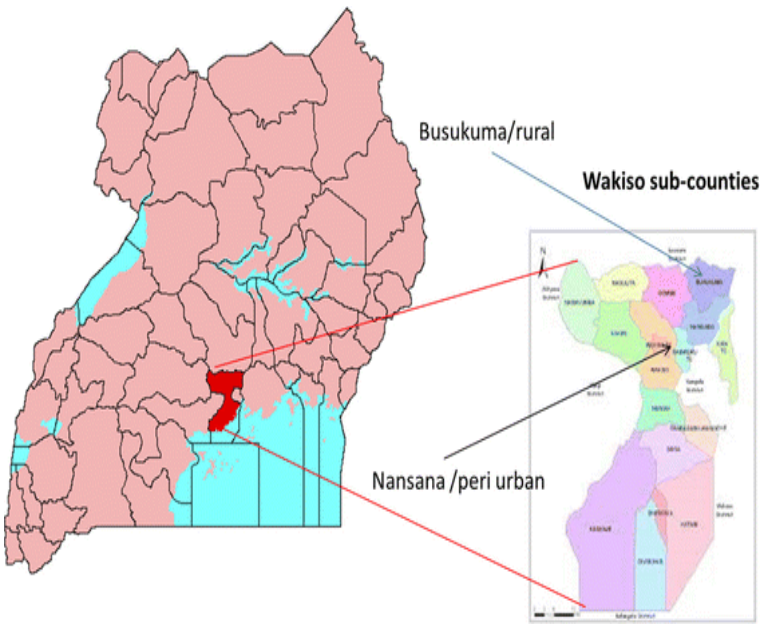


Figure 1. Map of Uganda showing the location of Wakiso District (source: Kalyesubula et al. 2017)

Sample and data collection

Samples were taken from 17 fish farms. A biographical data form was provided to fish farmers whose farms were visited to collect information on management practices and farm history. Five to ten fish were randomly sampled from the culture system (pond, basin, or cage) using a seine net, and if the system was a mixed culture, 10 fish were sampled, 5 of each variety. As many as 111 fish samples were obtained from the 17 farms, including apparently healthy, dying, and recently dead fish. Of the 111 fish samples collected, 81 were Nile tilapia, and 30 were African catfish, as more farms raised Nile tilapia than African catfish. Fish were collected from various culture systems, including in-ground ponds, concrete basins, and cages. Living and dying fish have been transported in plastic pools filled with water and with or without oxygen depending on the distance from the laboratory, while recently, dead fish, animal resources, and biological security at university maker.

Sample examination

There was 7 catfish fry crushed and inoculated directly onto xylose-lysine-deoxycholate (XLD) agar plates. The healthy and dying Nile tilapia was humanly sacrificed by joining together. At the same time, the African catfish was covered over the eyes with a cloth and hit on the head with a hard material. These plus dead fish were weighed, and their length was measured using a digital scale and ruler. The fish then underwent clinical and post-mortem examination to identify external and internal abnormalities. After examination, skin and gill swabs were taken and inoculated directly onto XLD agar and incubated at 370°C for 24 hours. The fish were then dissected using sterilized scissors and forceps by making a transverse incision in front of the anus towards the ventral part of the head. As far as the gill covers, another incision was made at the level of the anus running craniodorsally to the lateral line along the dorsal margin, from the abdominal cavity to the branchial arches, and a third cup connecting the ends of the two cups. The side panel was then removed to reveal the internal organs.

Organ examination was done by looking for any abnormalities, including location, size, color, and other signs of the disease. Next, the digestive tract, gonads, and visceral organs were removed by cutting the esophagus and disconnecting them from the kidneys. Next, samples of organs of interest (kidney, spleen, and liver) were taken using a surgical blade. Finally, organs were cultured separately from gills and skin swabs from the same fish; thus 208 plates were used for 104 fish samples, and the remaining 7 were for crushed African catfish for 215 plates.

Bacterial isolation

The samples were homogenized, seeded on XLD agar plates, and incubated for 24 hours at 37°C, after which the plates were examined for primary cultures. The examination of the plates was carried out by visual inspection for colonial morphology. The *E. tarda* forms small circular colonies 1 mm to 3 mm in diameter with black centers due to lysine decarboxylation (Buller 2004). A single colony well-differentiated from these black colonies was picked and subcultured onto XLD agar plates to produce a pure culture subjected to various biochemical tests.

Phenotypic identification of isolates

Identification of the bacterium was carried out using cultural and morphological characteristics. A series of conventional biochemical tests and the Analytical Profile Index (API 20E) system (BioMerieux, France) were performed according to the manufacturer's instructions. Conventional biochemical tests identifying The *E. tarda* included Gram stain, motility, indole production, cytochrome oxidase, H_2S production, methyl red, Voges-Proskauer, gelatin hydrolysis, urease, citrate, esculin, hydrolysis, and lactose utilization. The suspects from the conventional tests were re-identified using the API 20E kit, and their biochemical profiles were determined. These were preserved in cryovials containing BHI broth and glycerol and then stored in the freezer at -4°C until further testing.

Molecular characterization

Genotypic characterization

Preserved *E. tarda* isolates were recovered and inoculated onto nutrient agar slopes and transported to the Norwegian University of Life Sciences (NMBU) microbiology laboratory. The bacteria were subcultured and partially stored at -80°C as glycerol stock. The remainder was used to extract genomic DNA at Genlab, NMBU, where further molecular identification and characterization were performed.

DNA isolation

According to the manufacturer's instructions, genomic DNA extraction was performed using the QIAamp DNA Mini Kit (Qiagen). Briefly, the bacterium was added to 200 µL of lysis buffer with lysozyme and incubated at 37°C for 30 minutes. Then 20 µL of Proteinase K and 100 µL of buffer AL (lysis buffer) were added and incubated for 30 minutes at 56°C. DNA was precipitated by adding 200 µL of 100% ethanol to the sample and mixed by pulse vortexing for 15 seconds and then centrifuged briefly.

The mixture plus the precipitate were gently applied to the QIAamp Mini spin column in a 2 mL collection tube to bind the DNA to the column. The cap was closed and centrifuged at 6000 x g (8000 rpm) for 1 minute. 500 µL of buffer was added to the QIAamp column and centrifuged at 6000 x g (8000 rpm) for 1 minute. 500 µl of Buffer AW2 was added to the QIAamp column and centrifuged at full speed (20,000 x g; 14,000 rpm) for 3 minutes. The QIAamp column was placed in a new 2 mL collection tube and centrifuged at full speed for 1 minute. The QIAamp column was placed back into a new clean 1.5 mL microcentrifuge tube, and the collection tube containing the filtrate was discarded. 30 µL of Buffer AE was added to the column and incubated for 1 minute at room temperature and then centrifuged at 600 x g (8000 rpm) for 1 minute. The resulting DNA was stored at -200°C.

DNA purification

DNA was purified using a QIAquick® gel extraction kit (cat. 28704 and 28706) according to the manufacturer's instructions as follows; Absolute ethanol was added to the PE buffer and mixed thoroughly. A conventional tabletop microcentrifuge performed all centrifugation steps at 17,900 x g (13,000 rpm). The DNA fragment was excised using a clean, sharp scalpel from the agarose gel. Next, the gel slice was weighed into a colorless tube, and 3 volumes of QG buffer were added to 1 volume of gel (100 mg of gel-100 µL). The sample was incubated at 50°C for 10 minutes after the gel slice had completely dissolved, while the tube was vortexed every 2 minutes to help dissolve the gel. Finally, a volume of isopropanol gel was added to the sample and mixed. Safety glasses were used to protect the eyes from UV rays.

DNA binding was achieved by applying the sample to the QIAquick column and centrifuging for 1 minute until all samples passed through the column. The flow-through was discarded, and the QIAquick column was placed back into the same tube. Next, a 500 µL QG buffer was added to the QIAquick column and centrifuged for 1 minute. The continuous flow was again discarded, and the QIAquick column returned in the same tube. Then 750 µL of Buffer PE was added to the QIAquick column and centrifuged for 1 minute for washing. The continuous flow was again discarded, and the QIAquick column returned in the same tube. The column was left to stand for 5 minutes after adding the PE buffer. The QIAquick column was then centrifuged for 1 minute in the 2 mL collection tube provided to remove residual wash buffer. The QIAquick column was then placed in a clean 1.5 mL microcentrifuge tube. To elute the DNA, 30 µL of Buffer EB (10 mM Tris. Cl, pH 8.5) was added to the center of the QIAquick membrane, and the column was allowed to stand for 1 minute and then centrifuged for 1 minute.

Amplification of the 16S rRNA genes

The 16S rRNA gene was amplified by Polymerase Chain Reaction (PCR) using the 16S universal bacteria primers 27F (5' -AGAGTTTGATCCTGGCTCAG-3'), and 1492R (5'-GGTTACCTTGTTACGACTT-3') with the expected amplicon size of 1465bp (Lane 1991) ordered from Invitrogen, Thermo Fisher Scientific (Waltham, MA USA), PCR machine used in this research was iCycler from Bio-Rad. Each PCR reaction was performed in a final volume of 25 µL containing 2.5 µL of 10x reaction buffer (50 mM KCl, 75 mM Tris-HCl (pH 9.0), 2 mM $MgCl_2$, 20 mM $(NH_4)_2SO_4$), 0.5 µL 10 mM deoxyribonucleotide mix, 0.2 µL of *Taq* DNA polymerase, 1 µL 10 mM of each forward and reverse primer, 2 µL of DNA template and 16.8 µL of sterile ultrapure water. PCR conditions included initial denaturation at 94°C for 3 minutes, followed by 30 cycles of amplification as follows; Denaturation at 940°C for 30 seconds, annealing at 560°C for 30 seconds, and elongation at 720°C for 2 minutes. This was followed by a final extension step at 72°C for 5 minutes and left at 40°C until collected for further analysis. The PCR products were analyzed on 1% agarose gel (Ultrapure Agarose from Invitrogen, Thermos Fisher Scientific) using PowerPac 300 (Bio-Rad) at 100 volts for 60 minutes. The amplified DNA on the gel was visualized using Invitrogen's Safe Imager™, and bands of interest were excised using a scalpel blade for gel purification. The ChemiDoc ™ XRS (Bio-Rad) molecular image was used to view and capture gel photos. DNA concentration was measured using the NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Inc).

DNA sequencing

The PCR products were sequenced on ABI genetic analyzer with 16S 27F and 16S 1492R universal bacteria primers by Sanger sequencing techniques at GATC Biotech, Germany.

Phylogenetic analysis

The 16S rRNA gene sequence was edited using Bioedit, and Basic Local Alignment Search Tool (BLAST) searches performed in GenBank, specifically the National Center for Biotech Information (NCBI). Related sequences were obtained, and several sequence alignments were performed using the ClustalW algorithm. The gene sequences used in the phylogenetic analysis are shown in Table 3. These sequences were used to construct a phylogenetic tree.

Detection of selected virulence genes in the *Edwardsiella tarda* isolate

To characterize the virulence attribute of the isolate of *E. tarda*, seven virulence genes were screened in this study. These included: *gadB* (glutamate decarboxylase; resists host phagocyte killing activity), *muk* (putative killing factor), *citC*, *esaV*, *fimA* (allows bacteria to attach to host for invasion), *esrB* (helps penetration by encoding a regulatory protein for the type III secretion system (T3SS) and *katB* (provides the bacterium with resistance to the host's phagocytic activity) The genes were amplified by PCR using the primers in Table 1. The PCR reaction was performed in a final volume of 25 µl containing 0.25 µl of 10x reaction buffer (5 mM KCl, 7.5 mM Tris-HCl (pH 9.0), 0.2 mM MgCl2, (NH4)2SO4 2 mM), 0.05 µL of 1 mM deoxyribonucleotide mixture, 0.02 µL of Taq DNA polymerase, 0.1 µL of 1 mM sense and antisense primer, 0.2 µL of template DNA and 1.68 µL of sterile ultrapure water. The PCR program used was as follows: initial denaturation at 94°C for 3 minutes, followed by 32 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for citC, muk, esrB; 580C for catB; 570°C for gadB and 600°C fimA for 1 minute, extension at 720°C for 1 minute and final extension at 720°C for 10 minutes and left at 40°C until collected for analysis further.

PCR products were run on 1% agarose gel by PowerPac 300 (BioRad) at 100Volts for 60 minutes. The amplified products on the gel were observed and captured using ChemiDoc™XRS Molecular imager (Bio-Rad).

Data analysis

Data was saved, summarized, and analyzed using Microsoft Excel and Windows 10. Quantitative data (weight and height) were averaged. The sequences were edited in the MEGA 7.0 software and aligned using the ClustalW algorithm. Finally, a phylogenetic tree was constructed in MEGA 7.0 (Kumar et al. 2016) using the Neighbor-Joining method (Saitou and Nei 1987).

Table 1. Primers used in the amplification of the virulence genes

Target gene	Sequence 5' → 3'	Product size (bp)	Source
gadB (F)	5′- ATTTGGATTCCCGCTTTGGT-3′	583	Wang et al. 2012
gadB (R)	5′- GCACGACGCCGATGGTGTTC-3′		
muk(F)	5′- TTGCTGGCTATCGCTACCCT-3′	357	Wang et al. 2012
muk (R)	5′- TTGCTGGCTATCGCTACCCT-3′		
citC(F)	5′- TTTCCGTTTGTGAATCAGGTC-3′	591	Wang et al. 2012
citC (R)	5′- AATGTTTCGGCATAGCGTTG-3′		
fimA(F)	5′- CTGTGAGTGGTCAGGCAAGC-3′	441	Wang et al. 2012
fimA(R)	5′- TAACCGTGTTGGCGTAAGAGC-3′		
esrB(F)	5′-TCGTTGAAGATCATGCCTTGC-3′	311	Wang et al. 2012
esrB (R)	5′-TGCTGCGGGCTTTGCTT-3′		
katB(F)	5′-CTTAGCCATCAGCCCTTCC-3'	1417	Wang et al. 2012
katB (R)	5′-GCGAGTGCCGTAGTCCTT-3'		
esaV (F)	5'-GGTCAATAGCTGGCTACACAA-3'	955	Li et al. 2010
esaV (R)	5'-GCGCCTCAGCGAGTATGCGAT-3'		

RESULTS AND DISCUSSION

General description and farm history

Seventeen fish farms were visited in the Wakiso District, Uganda, where 111 healthy, dying, and recently dead fish were sampled and examined clinically and post-mortem. The whole Nile tilapia was 81 (72.9%), with an average body weight of 113.1 g and an average length of 17.42 cm. The total number of African catfish was 30 (27%), with an average body weight of 154.34 g and an average length of 25.1 cm. Seven of the 30 African catfish were juveniles, so their weight and length were not measured. Fish were collected from three rearing systems, namely in-ground ponds (10), basins (1), and cages (4). Two farms had both ponds and reservoirs. 82.3% of all farms practiced monoculture, Raising either Nile tilapia or African catfish, while 17.6% had mixed crops where both species were raised in the same system and were in soil ponds systems.

All farms with ponds were located in remote low-lying areas or agricultural areas and their water sources were streams or wells. Cages were located on the lake and river shores, while the farm with basins was only located in a residential area with its water source being tap and rainwater. All farms reported feeding the commercial fish feed twice or once a day, depending on feed availability, time, and the response of the fish. All farms with bottom ponds used organic fertilizers to boost primary production. Most farms used chicken manure (75%) for fertilization, while others used cow manure (25%).

Two farms (11.7%) reported having a history of disease during dry and wet seasons. Others reported observing signs of disease, including loss of appetite, low growth, proptosis, dropsy, abnormal swimming, parasites, and mortality. The farmers also admitted to using antibiotics, formalin, potassium permanganate and salt to treat sick fish. Farms that reported a history of the disease also reported overfeeding and poor water quality

Clinical signs and a post-mortem examination

The infected Nile tilapia showed ascites and abdominal petechial bleeding of the fish examined. In contrast, the African catfish showed abnormalities, including petechial bleeding on the skin and fins, lordosis, pallor of the liver, and ulcers on the abdominal and opercula regions (Figure 2).

Isolation of *Edwardsiella tarda*

Fifty-one plates out of 215 plates had produced clear colonies with black centers surrounded by reddened media due to the decarboxylation of lysine on xylose-lysine deoxycholate (XLD) medium after 36 hours at 370°C (Figure 3), and these have been subjected to conventional treatment biochemical tests submitted.

Phenotypic identification of *Edwardsiella tarda* isolates

Presumptive identification of *E. tarda* by conventional biochemical assays yielded eight (7.2%) *E. tarda* in four (3.6%) tilapia and four (3.6%) African catfish. All isolates were Gram-negative, and microscopic examination of the wet media showed mobile rods 2 µm in length and 1 µm in diameter. All isolates were negative for cytochrome oxidase, citrate, aesculin hydrolysis, gelatin hydrolysis, urease, and lactose fermentation. All isolates produced H2S and tested positive for indole production. There were variations in the MRVP test as only two isolates tested positive for MR and negative for VP (Table 2).

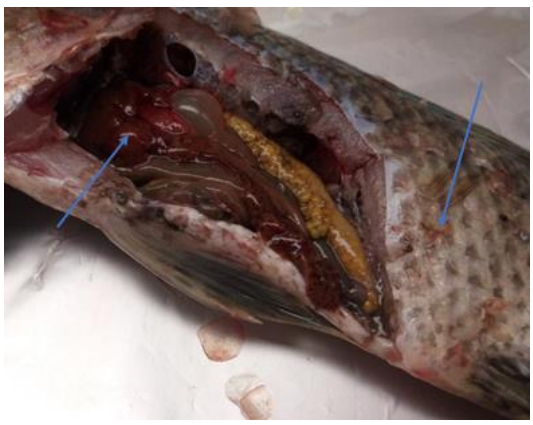


Figure 2. Hemorrhages and accumulation of ascitic fluid in Nile tilapia (arrows)

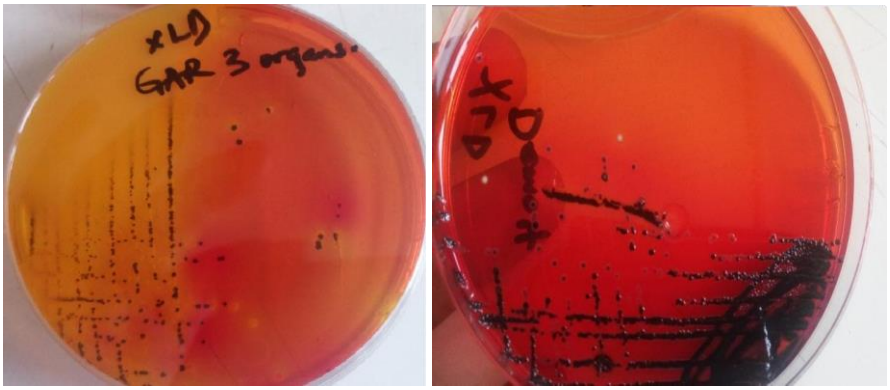


Figure 3. Clear colonies with black centers on XLD agar plates

Table 2. Biochemical properties of the eight *Edwardsiella tarda* isolates

Test	Isolates							
	***O.n*1**	***O.n*2**	***O.n*3**	***O.n* 4**	***C.g*1**	***C.g 2***	***C.g* 3**	***C.g*4**
Gram stain	-	-	-	-	-	-	-	-
Oxidase	-	-	-	-	-	-	-	-
Indole production	+	+	+	+	+	+	+	+
Citrate	-	-	-	-	-	-	-	-
Motility	+	+	+	+	+	+	+	+
Methyl Red	+	-	-	-	+	-	-	-
Voges-Proskauer	-	+	+	+	-	+	+	+
Esculin hydrolysis	-	-	-	-	-	-	-	-
Gelatin hydrolysis	-	-	-	-	-	-	-	-
Urease	-	-	-	-	-	-	-	-
Lactose utilization	-	-	-	-	-	-	-	-
H_2S production	+	+	+	+	+	+	+	+

Note: + means a positive result, - means a negative result.

Identification of the isolates using API 20E kit

One isolate was re-identified as 99.4% *E. tarda* using Analytical Profile Index (API) 20E kit (Code No.4744000) at 37°C. In the biochemical tests, the seven isolates identified as *E. tarda* were identified by the API 20E kit as Plesiomonas shigelloides, a fish pathogen belonging to the family Enterobacteriaceae. Plesiomonashigelloides has a worldwide distribution, and human outbreaks have been reported in Cameroon from cold fish. It has many host plants, including aquatic environments, aquatic animals, mammals, and humans, that cause gastroenteritis (Janda et al. 2016).

Sequence analysis of the 16S rRNA gene for identification of *Edwardsiella tarda*

16S rRNA sequencing has been widely used to understand bacterial evolution and phylogeny and is considered an essential tool in bacterial systematics and identifying new species. Therefore, in this study, the bacterial isolate identified as *E. tarda* using the API 20E kit was identified and confirmed to be *E. tarda* from amplification of the 16S rRNA gene resulting in a PCR product with the expected size of 1465 bp (Figure 4).

Phylogenetic analysis

The *E. tarda* isolates with known 16S rRNA sequences in the GenBank database using the BLAST program showed that the isolate had a 99.9% identity compared to that of other E. late. The phylogenetic relationship of *E. tarda* was probed using the 16S rRNA gene with other strains of *E. tarda* downloaded from NCBI for phylogenetic analysis, including a type *E. tarda* strain ATCC 15947, *Escherichia coli*, *Salmonella enterica* and *Pleisomonas shigelloides* strains. Their taxonomic status, strain collection numbers, and GenBank accession numbers are listed in Table 3. A phylogenetic tree can be seen in Figure 5.

Next to the branches is shown the percentage of replicate trees in which the corresponding taxa are grouped together in the bootstrap test (500 replicates) (Felsenstein 1985).

Detection of virulence genes in the *Edwardsiella tarda* strain

The *E. tarda* strain was screened for 7 virulence genes, including; gadB, muk, citC, fimA, ESRB, katB, and esaV, by Polymerase chain reaction (PCR) technique. Six of the seven virulence genes screened for were present in *E. tarda* isolated in this study, including; CitC, muk, gadB, katB, esaV and fimA.

Table 3. Isolates whose sequences were used in phylogenetic analysis

Name of culture	Accession number
E. tarda strain T1	KX388234.1
E. tarda strain C6	FJ607400.1
E. tarda strain UMT-WD-ON	FJ600537.1
*E. tarda*strainVMCU06	KU860461.1
E. tarda strain C7-5m	HQ663902.1
E. tarda strain ATCC 15947	JX866952.1
E. tarda strain29-907R	KX828266.1
P. shigelloides ATCC 14029	M59159.1
E. tarda strainSY-ED14	KX388234
P. shigelloides ATCC14029T	X74688.1
*E. tarda*strain59-907R	KX828321.1
Salmonella enterica subsp *enterica* ATCC 13076	LSHA01000019.1
E. coli ATCC 11775	NZ_JMST01000035.1

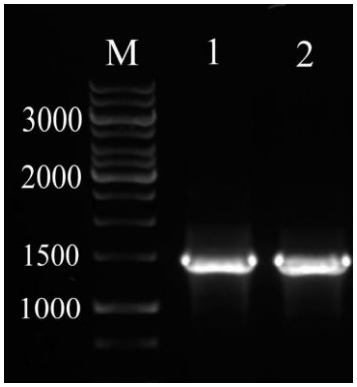


Figure 4. 16S rRNA gene PCR products on a 1% agarose gel. Lane M: Molecular marker, lanes1 and 2: *Edwardsiella tarda*

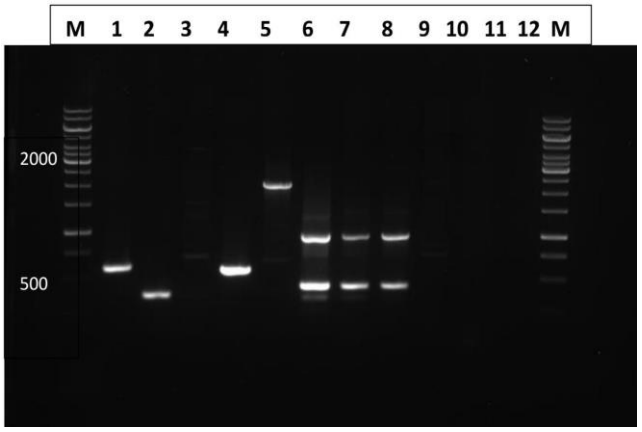


Figure 6. Agarose Gel electrophoresis of PCR of virulence genes for the isolated *Edwardsiella tarda*. Note: lane 1 is citC, lane 2 ismuk, lane 3 isesrB(-ve), lane 4 isgadB, lane 5 is katB,lanes 6, 7 and 8 arefimA and esaV, Lanes 9 to 12 are not considered, and lane M is DNA marker.

Discussion

The present study determined the presence of *Edwardsiella* species at 7.2% by conventional bacteriology and molecular technique. It confirmed *E. tarda* at 0.9% in one of the fish samples from farms in the Wakiso District of Uganda. The *E. tarda* is a significant pathogenic bacterium that is said to cause Edwardsiellosis in more than 20 fresh and marine fish species, both farmed and wild (Bullock and Herman 1985; Mohanty and Sahoo 2007). It reduces the marketability of diseased fish and causes massive mortality in different age groups, leading to severe economic losses in fish farms worldwide (Faruk et al. 2004; Abraham et al. 2015).

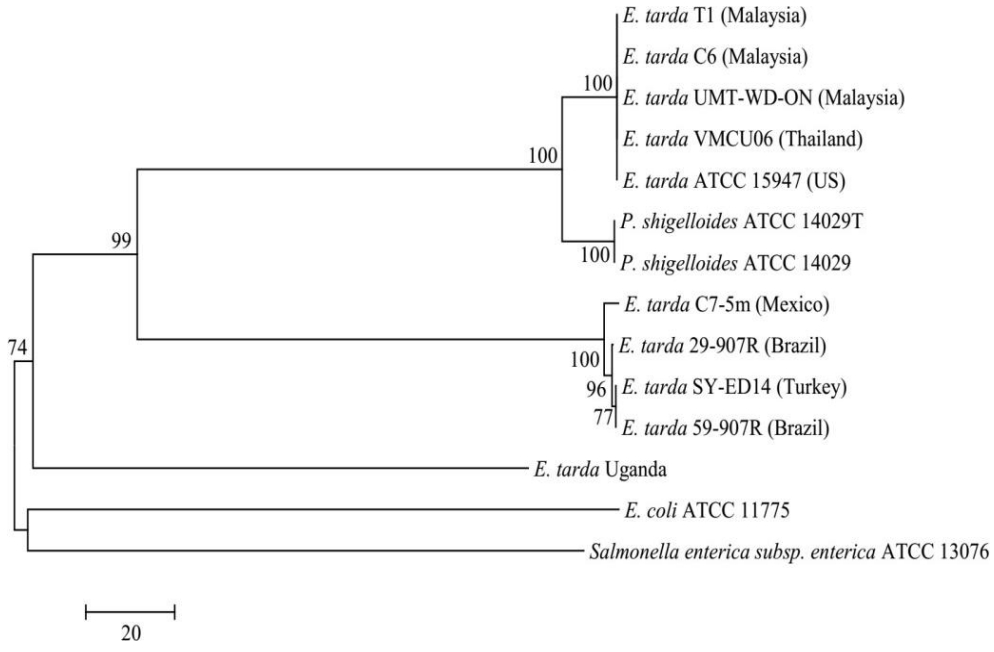


Figure 5. Phylogenetic relationship of *Edwardsiella tarda* from Uganda with other strains

In this study, *Oreochromis niloticus* (Linnaeus, 1758), which produced the *E. tarda* isolates, showed signs of ascites and petechial abdominal hemorrhages. These presentations were similar to those reported for *E. tarda* infection (El-Refaey 2013; El-Seedy et al. 2015). However, Edwardsellosis symptoms and macroscopic lesions are unreliable in arriving at a conclusive diagnosis because several bacterial infections also share similar signs and symptoms (Mohanty and Sahoo 2007). Additionally, diseased fish, with or without clinical signs of disease, are equally important as they can transmit pathogens that pose a risk of disease transmission to other species, including humans. Thereby, detection of these pathogens is crucial for their effective prevention and control (Castro et al. 2014)

The successful isolation and correct identification of a given pathogen depend on the standardized bacteriological culture methods. In the present study, the bacterium was grown on XLD agar and, due to lysine decarboxylation, produced clear colonies with black centers surrounded by reddened backgrounds (Figure 3) (Wyatt et al. 1979; Najiah and Lee 2006; Wei and Musa 2008). All isolates in this study were gram-negative, and microscopic examination of wet slides revealed motile rods 2 µm long and 1 µm in diameter. These results were similar to those of other researchers who isolated *E. tarda* from cultured freshwater cichlids, African catfish, Chinook salmon, and sea bream (Amandi et al. 1982; El-Refaey 2013; Griffin et al. 2013; Abram et al. 2015; Katharios et al. 2015; Emanet al. 2016)

All isolates showed negative results for cytochrome oxidase, citrate, esculin hydrolysis, gelatin hydrolysis, urease, and lactose fermentation. All isolates have been tested positively for H_2S and indole production (Table 2). These results are consistent with those of other studies (Wyatt et al. 1979; Amandi et al. 1982; Joh et al. 2010; El-Refaey 2013; Abraham et al. 2015; Eman et al. 2016). There were variations in MRVP tests as only 2 isolates tested positive for MR and negative for VP. These results did not agree with the literature, which could mean these new strains of E. late. However, variations in phenotypic traits are attributed to the presence and absence of plasmids that control the metabolic traits of the phenotypic traits of the isolates (Acharya et al. 2007).

Biochemical identification using conventional bacteriological techniques failed to distinguish between *E. tarda* and Plesiomonas shigelloides. When the API 20E kit was used on the eight isolates identified as *E. tarda* by biochemical tests, only one isolate was found to be *E. tarda* at 99.4%. Indeed, it tested positive for citrate but was citrate negative in conventional biochemical tests, so it would have been 99.9% *E. tarda*. According to Buller (2004), reaction differences between the API system and conventional biochemical tests are particularly reported for decarboxylase, citrate, urea, indole and Voges-Proskeur tests. El-Seedy et al. (2015) also reported that 22.2% of their *E. tarda* tested positive for citrate utilization and concluded that these isolates were typical strains of *E. tarda*.

More isolates have been confirmed as *E. tarda* by conventional biochemical tests than by molecular techniques, possibly due to the high degree of phenotypic diversity within bacterial species, which limits the accuracy of biochemical identification, especially when only a few isolates are tested, which requires more work for the investigation is needed on the phenotypic diversity of *E. tarda* (Griffin et al. 2013).

Phylogenetic analysis separated strains of *E. tarda* into two groups by excluding *E. tarda* isolated in this study from any group. One group clustered with *Pleisomonasshigelloides* with high bootstrap support (100%). The *P. shigelloides* is deeply rooted in the Enterobacteriaceae family and aligned closer to *E. tarda*. Therefore, this may explain the misidentification in biochemical tests as *E. tarda* in this study (Janda et al. 2016). The second group shares greater similarity with *E. tarda* of this study with 74% bootstrap support than the first group. These results are consistent with other comparative phylogenomic studies comparing two distinct genetic groups of E. spät (Griffin et al. 2013). This finding, therefore, suggests that *E. tarda* isolated from this study is genetically distant from the GenBank included in this study

The ability of a bacterium to cause disease depends on the expression of virulence factors that allow the bacteria to enter the host, produce pathological effects, and evade the host's defenses. The ability of a microorganism to invade the host is the most important aspect of its pathogenicity (Roberts 2012). Identifying the virulence genes needed to establish infection in hosts helps to understand the mechanisms of pathogenesis in bacteria, aiding in developing vaccines and the formulation of therapies to protect fish from infection. The *E. tarda* is believed to be multifactorial, and several authors have reported several potential virulence factors involved in its pathogenesis (Mohanty and Sahoo 2007; Mendez et al. 2012). Several potential pathogenic properties are thought to contribute to the infection process of *E. tarda*, including ability to invade epithelial cells, secretion of degradative enzymes, adhesions, type III and IV secretion systems, production of toxins to spread infection, siderophores, catalase, ability to survive and replicate in phagocytes (Mohanty and Sahoo 2007; Park et al. 2012; Xu and Zhang 2014; Eman et al. 2016). Pathogenic bacteria are thought to have virulence genes absent in non-pathogenic bacteria. While virulence can be present in both pathogenic and non-pathogenic bacteria, they are functional only in pathogenic ones.

In the present study, six of the seven screened virulence genes were present in *E. tarda* isolate, including; citC, muk, gadB, katB, esaV and fimA (Figure 6). Two of these genes (muk and gadB) were also identified by Eman et al. (2016) and are considered specific for *E. tarda*. According to the literature, the genes detected in this study are considered to be mainly virulent genes because they give the bacteria resistance to the host's phagocytic activity (gadB and katB), allow cell adhesion (fimA), which promotes intracellular survival and replication of the bacterium in the host allows and muk is a putative killing factor (Mohanty and Sahoo 2007; Katharios et al. 2015). It has also been suggested that the presence of the fimA gene

indicates the ability of *E. tarda* to bind to specific receptors in fish and thus define the site of entry and colonization (Wang et al. 2009)

It is known that the virulence genes detected are present only in virulent strains and are therefore considered biomarkers in the diagnosis of pathogenic *E. tarda*. Furthermore, these genes determine how pathogenic bacteria interact with the host to cause systemic infections and vaccine development. In addition, they are used to develop new therapies and shared antigens (Mendez et al. 2012). Although the current study did not assess the virulence of the isolate based on in vivo experiments, these virulence genes coupled with clinical signs of disease provide evidence for the presence of Edwardsialosis in fish in which the isolate was found.

The *E. tarda* is a zoonotic bacterium affecting not only fish but also invertebrates, amphibians, reptiles, birds, and mammals, including humans (Bullock and Herman 1985; Park et al. 2012). The *E. tarda* bacteremia (ETB) has been described as a serious food- and water-borne infection that results in high mortality rates, especially in patients with serious underlying conditions (Hirai et al. 2015). In addition, the pathogen was reported by Litton and Rogers (2016) in a fisherman who was pierced by a hook. As no biosecurity measures were adopted in all farms visited in this study, this poses a threat to human health, contributing to the introduction of pathogens from fish to humans and creating potential contamination between humans and animals (Takyi et al. 2012).

In conclusion, this study established the presence of *E. tarda* in *O. niloticus* in the district of Wakiso, Uganda. The isolate has been successfully characterized, which is of the utmost importance in diagnosing the pathogen. Isolation of *E. tarda* from farmed fish intended for human consumption poses a threat to the fishing industry because it causes heavy economic losses and to the public sector because it is zoonotic. The present study results revealed that the *E. tarda* isolate had six virulence genes that play an important role in E. late pathogenicity. This information will facilitate effective control strategies and the development of vaccines to prevent Edwardsialosis in fish.

REFERENCES

Abraham TJ, Mallick PK, Adikesavalu H, Banerjee S. 2015. Pathology of *Edwardsiella tarda* infection in African catfish, *Clarias gariepinus* (Burchell 1822), Fingerlings. Arch Pol Fish 23: 141-148. DOI: 10.1515/aopf-2015-0016.

Acharya M, Maiti NK, Mohanty S, Mishra P, Samanta M. 2007. Genotyping of *Edwardsiella tarda* isolated from freshwater fish culture system. Comp Immunol Microbiol Infect Dis 30: 33-40. DOI: 10.1016/j.cimid.2006.10.003.

Akoll P, Mwanja WW. 2012. Fish health status, research and management in East Africa: Past and present. Afr J Aquat Sci 3 (2): 117-129. DOI: 10.2989/16085914.2012.694628.

Amandi A, Hiu SF, Rohovec JS, Fryer JL. 1982. Isolation and characterization of *Edwardsiella tarda* from fall chinook salmon (*Oncorhynchus tshawytscha*). Appl Environ Microbiol 43 (6): 1380-1384. DOI: 10.1128/aem.43.6.1380-1384.1982.

Buller NB. 2004. Bacteria and Fungi from Fish and Other Aquatic Animals: A Practical Identification Manual. 2nd edition. CABI, US. DOI: 10.1079/9780851997384.0000.

Bullock GL, Herman RL. 1985. *Edwardsiella* Infections of Fishes. US Fish and Wildlife Publications, Lincoln.

Castro N, Toranzo AE, Magariños B. 2014. A multiplex PCR for the simultaneous detection of *Tenacibaculum maritimum*and *Edwardsiella tarda* in aquaculture. Intl J Microbiol 17: 111- 117. DOI: 10.2436/20.1501.01.213.

Das BK, Sahu I, Kumari S, Sadique M, Nayak KK. 2014. Phenotyping and whole cell protein profiling of *Edwardsiella tarda* strains isolated from infected freshwater fishes. Intl J Curr Microbiol Appl Sci 3 (1): 235-247.

Directorate of Fisheries Resources. 2011. Annual Report 2010/2011. Ministry of Agriculture, Animal Industry and Fisheries, Government of Uganda.

El-Refaey AME. 2013. Studies on major bacterial diseases affecting fish; tilapia *Oreochromis niloticus*, catfish *Clarias gariepinus* and mullets in Port Said, Egypt with special references to its pathological alterations. Researcher 5 (2): 5-14.

El-Seedy FR, Radwan IA, Abd El-Galil MA, Sayed HH. 2015. Phenotypic and genotypic characterization of *Edwardsiella tarda* isolated from *Oreochromis niloticus* and *Clarias gariepinus* at Sohag Governorate. Am J Sci 11 (11): 68-75.

Eman MM, Omar AAE, Abdo WS. 2016. Insight into the virulence-related genes of *Edwardsiella tarda* isolated from cultured freshwater fish in Egypt. Vet World J 6 (3): 101-109. DOI: 10.5455/wvj.20160874.

FAO. 2016. State of World Fisheries and Aquaculture 2016. Contributing to Food Security and Nutrition for All. Rome.

Faruk MAR, Sarker MMR, Alam J, Kabir MB. 2004. Economic loss from fish diseases on rural freshwater aquaculture of Bangladesh. Pak J Biol Sci 7 (12): 2086-2091. DOI: 10.3923/pjbs.2004.2086.2091.

Felsenstein J. 1985. Confidence limits on phylogenies: An approach using the bootstrap. Evolution 39: 783-791. DOI: 10.2307/2408678.

Griffin MJ, Quiniou SM, Cody T, Tabuchi M, Ware C, Cipriano RC, Mauel MJ, Soto E. 2013. Comparative analysis of *Edwardsiella* isolates from fish in the Eastern United States identifies two distinct genetic taxa amongst organisms phenotypically classified as *E. tarda*. Vet Microbiol 165: 358-372. DOI: 10.1016/j.vetmic.2013.03.027.

Hirai Y, Asahata-Tago S, Ainoda Y, Fujita T, Kikuchi K. 2015. *Edwardsiella tarda* bacteremia. A rare but fatal water and foodborne infection: Review of the literature and clinical cases from a single centre. Can J Infect Dis Med Microbiol 26 (6): 313-318. DOI: 10.1155/2015/702615.

Janda JM, Abbott SL, McIver CJ. 2016. *Plesiomonas shigelloides* revisited. Clin Microbiol Rev 29: 349-374. DOI: 10.1128/CMR.00103-15.

Joh S, Kim M, Kwon H, Ahn E, Jang H, Kwon J. 2010. Characterization of *Edwardsiella tarda* isolated from farm-cultured eels, *Anguilla japonica*, in the Republic of Korea. J Vet Med Sci 73 (1): 7-11. DOI: 10.1292/jvms.10-0252.

Kalyesubula R, Nankabirwa JI, Ssinabulya I, Siddharthan, T, Kayima J, Nakibuuka J, Salata JA, Mondo C, Kamya,MR, Hricik D. 2017. Kidney disease in Uganda: A community based study. BMC Nephrol 18: 116-125. DOI: 10.1186/s12882-017-0521-x.

Katharios P, Kokkari C, Dourala N, Smyrli M. 2015. First report of Edwardsiellosis in cage-cultured sharpsnout sea bream, *Diplodus Puntazzo* from the Mediterranean. BMC Vet Res 11: 155-161. DOI: 10.1186/s12917-015-0482-x.

Kebede B, Habtamu T. 2016. Screening for the presence and prevalence of *Edwardsiella tarda* infection in fish harvested from Lakes Zeway and Langano, Southern Oromia, Ethiopia. Fish Aquac J 7: 184-190. DOI: 10.1080/23311932.2017.1312757.

Kumar S, Stecher G, Tamura K. 2016. MEGA7: Molecular evolutionary genetics analysis version 7.0 for bigger datasets. Mol Biol Evol 33: 1870-1874. DOI: 10.1093/molbev/msw054.

Lane DJ. 1991. 16S/23S rRNA sequencing. In: Stackebrandt E, Goodfellow M (eds). Nucleic Acid Techniques in Bacterial Systematics. John Wiley and Sons, New Jersey, US.

Li GY, Li J, Xiao P, Guo YH, Mo ZL. 2010. Detection of type III secretion gene as an indicator for pathogenic *Edwardsiella tarda*. Lett Appl Microbiol 52: 213-219. DOI: 10.1111/j.1472-765X.2010.02984.x.

Litton KM, Rogers BA. 2016. *Edwardsiella tarda* endocarditis confirmed by Indium-111 white blood cell scan: An unusual pathogen and diagnostic modality. Case Rep Infect Dis 2016: 1082160. DOI: 10.1155/2016/108216.

Méndez J, Reimundo P, Pérez-Pascual D, Navais R, Gómez E, Cascales D, Guijarro JA. 2012. An overview of virulence-associated factors of Gram-negative fish pathogenic bacteria. Health Environ Aquac 5: 133-156. DOI: 10.5772/29837.

Mohanty BR, Sahoo PK. 2007. Edwardsiellosis in fish: A brief review. J Biosci 32: 1331-1344. DOI: 10.1007/s12038-007-0143-8.

Naing L, Winn T, Rusli BN. 2006. Practical issues in calculating the sample size for prevalence studies. Arch Orofac Sci 1: 9- 14.

Najiah M, Lee SW. 2006. Phenotypic characterization and numerical analysis of *Edwardsiella tarda* in wild asian swamp eel, *Monopterus albus* in Tereengganu. J Sustain Sci Manag 1 (1): 85-91.

National Development Plan (NDPII). 2015. Second National Development PLAN (NDPII) 2015/16-2019/20. Uganda Govt, Kampala.

Öztürk RÇ, Altınok İ. 2014. Bacterial and viral fish diseases in Turkey. J Fish Aquat Sci 14:275-297. DOI: 10.4194/1303-2712-v14_1_30.

Park SB, Aoki T, Jung TS. 2012. Pathogenesis of and strategies for preventing *Edwardsiella tarda* infection in fish. Vet Res 43: 67-78. DOI: 10.1186/1297-9716-43-67.

Roberts RJ. 2012. Fish Pathology. 4th Edition. John Wiley and Sons, Limited, United Kingdom.

Saitou N, Nei M. 1987. The neighbor-joining method: A new method for reconstructing phylogenetic trees. Mol Biol Evol 4: 406-425. DOI: 10.1093/oxfordjournals.molbev.a040454.

Takyi RF, Nunoo KE, Ziddah P, Oddoye J. 2012. Occurrence of bacterial infection in two commonly cultured fish species on two fish farms in Southern Ghana. World J Biol Res 5 (2): 81-92.

UBOS. 2014. The National Population and Housing Cencus 2014. Kampala, Uganda.

UNBOS. 2014. Uganda National Bureau of Statistics Statistical Abstract. [http://www.ubos.org/onlinefiles/uploads/ubos/statistical_abstracts/Statis tical_Abstract_2014.pdf] site visited on 20th February 2016.

Walakira J, Akoll P, Engole M, Sserwadda M, Nkambo M, Namulawa V, Kityo G, Musimbi F, Abaho I, Kasigwa H, Mbabazi D, Kahwa D, Naigaga I, Birungi D, Rutaisire J, Majalija S. 2014. Common fish diseases and parasites affecting wild and farmed Tilapia and Catfish in Central and Western Uganda. Uganda J Agric Sci 15 (2): 113-125.

Wang K, Liu E, Song S, Wang X, Zhu Y, Ye J, Zhang H. 2012. Characterization of *Edwardsiella tarda* rpoN: Roles in family regulation, growth, stress adaptation and virulence toward fish. Arch Microbiol 194 (6): 493-504. DOI: 10.1007/s00203-011-0786-6.

Wang Q, Yang M, Xiao J, Wu H, Wang X, Lv Y, Xu L, Zheng H, Wang S, Zhao G, Qin Q, Zhang Y. 2009. Genome sequence of the versatile fish pathogen *Edwardsiella tarda* provides insights into its adaptation to broad host ranges and intracellular niches. PLoS One 4 (10): e7646. DOI: 10.1371/journal.pone.0007646.

Wei LS, Musa N. 2008. Phenotyping, genotyping and whole cell protein profiling of *Edwardsiella tarda* isolated from cultured and natural habitat freshwater fish. Am-Eur J Agric Environ Sci 3 (5): 681-691.

Wyatt LE, Nickelson R, Vanderzant C. 1979. *Edwardsiella tarda* in freshwater catfish and their environment. Appl Environ Microbiol 38 (4): 710-714. DOI: 10.1128/aem.38.4.710-714.1979.

Xu T, Zhang X. 2014. *Edwardsiella tarda*: An intriguing problem in aquaculture. Aquaculture 431: 129-135. DOI: 10.1016/j.aquaculture.2013.12.001.

BIOTEKNOLOGI
Volume 16, Number 1, May 2019
Pages: 21-30

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c160104

Bagasse pretreatment by some wood-rotting fungi in ethanol production

DAWIT TEWOLDE, AMARE GESSESSE♥
Department of Microbial, Cellular and Molecular Biology, Addis Ababa University. PO Box 1176, NBH1, 4killo King George VI St, Addis Ababa, Ethiopia. Tel.: +251-111239706, ♥email: amare.gessesse@aau.edu.et

Manuscript received: 6 April 2019. Revision accepted: 21 May 2019.

Abstract. *Tewolde D, Gessesse A. 2019. Bagasse pretreatment by some wood-rotting fungi in ethanol production. Bioteknologi 16: 21-30.* Biomass from lignocellulosic materials is a sustainable feedstock for ethanol production. During ethanol production, pretreatment modifies the lignin barrier to make cellulose more accessible to enzymatic hydrolysis. The biological pretreatment involves selecting rot fungi that preferentially degrade lignin while retaining a minimum amount of polysaccharides. Despite not being well developed, the enzymatic nature of the reaction is an advantage over other pretreatment methods. These fungi produce ligninolytic enzymes, predominantly lignin peroxidase (LiP), manganese peroxidase (MnP) and laccase (Lac) in different combinations. Biological pretreatments have shown weight loss of lignin and improved yields of ethanol. However, only a few well-characterized white rots have been explored. This study evaluated 15 wood rotting fungi isolated from Ethiopia for pretreatment during 15 days of solid state fermentation using bagasse as a lignocellulosic substrate. The production of ligninolytic enzymes by *Fomitiporia aethiopica*, *F. pseudopunctata*, *Fomitopsis carnea* and *Vanderbylia vicina* were reported for the first time in this study. The white rots, *F. aethiopica*, *Perenniporia tephropora*, *Inonotus* sp. and *Pleurotus sajor-caju*, were highly selective based on maximum and minimum productivity of ligninolytic and polysaccharide degrading enzymes, respectively. The pretreatment by the white rots caused ligninolysis and better cellulose digestibility was obtained with higher lignin loss. Among the selective degraders, *P. tephropora* caused the highest lignin loss (7.71%) and cellulose digestibility (29.44%) after enzyme hydrolysis of the pretreated bagasse. This digestibility showed an improvement of 38.74 % in comparison with untreated bagasse. In addition to high MnP productivity (55.87 U/g), *P. tephropora* also produced high titers of Lac (79.65 U/g) in contrast to the other selective degraders that might have attributed to better lignin loss. The ethanol yield from the fermentation of cellulase enzyme hydrolyzed *P. tephropora* pretreated bagasse was 1.87 g/L, which improved by 27.21 % compared with untreated bagasse (1.47 g/L). Therefore, *P. tephropora* pretreatment enhances ethanol production from bagasse through partial degradation of lignin, which improves the accessibility of cellulose to enzyme hydrolysis.

Keywords: Bagasse, ethanol, ligninolytic enzymes, pretreatment, rot fungi

INTRODUCTION

Ethanol has been used as a biofuel to provide an alternative energy source and thus reduce oil consumption. Simple sugars and starchy biomass serve as raw materials for ethanol production. However, these substrates are in limited quantity and constitute animal feed and human food (Sun and Cheng 2002). Therefore, the use of lignocellulosic biomass as a raw material is attracting growing interest. It is abundant, cheap, and the ethanol production process has very low net CO_2 emissions compared to other feedstocks (Tomas-Pejo et al. 2008). However, process technology for producing ethanol from lignocellulosic biomass is complicated, energy-intensive and under development. The biggest challenge lies in processing the raw material due to the structural arrangement of the fiber components. Cellulose, the main sugar reservoir, is surrounded by a wall of lignin which becomes a barrier to enzymatic hydrolysis. Therefore, biomass must be pretreated in the production process to remove lignin, increase porosity and decrease cellulose crystallinity (Sanchez and Cardona 2008).

There are several pretreatment systems, which are divided into physical, physicochemical, chemical and biological. Biological pretreatment involves mainly selective lignin-degrading fungi capable of removing lignin with minimal loss of polysaccharides (Itoh et al. 2003). Selective lignin degraders preferentially degrade lignin and hemicelluloses and degrade cellulose only in the later stages (Hakala 2007). Biological pretreatment is performed under mild reaction conditions with few side reactions. As a result, the system has very low chemical consumption, lower energy consumption and less susceptibility to pressure and corrosion than other pretreatments (Lee 1997; Samsuri et al. 2008).

Selective degradation is characteristic of some species of white rot fungi. These fungi degrade lignin mainly through the action of extracellular oxidative enzymes, lignin peroxidase, manganese peroxidase and laccase (Hakala 2007). Different fungi produce these enzymes in different combinations (Tuor et al. 1995). The degradation mechanism is the oxidation of lignin to form cationic radicals which initiate a non-enzymatic chain reaction and ultimately mineralize the polymer (Hakala 2007). Biological pretreatment studies of various lignocellulosic biomasses have produced a loss of lignin with a low degradation of polysaccharides with consequent improvement in ethanol production (Itoh et al. 2003; Munoz et al. 2007; Samsuri et al. 2008; Bak et al. 2009). Although these studies show promising results, only a few well-characterized white rot species have been evaluated for pretreatment. Therefore, there is a need to assess the

potential of different putrefactive fungi seeking a better lignin degradation system than bare cellulose without significant self-consumption.

Sugar cane bagasse can be used as a lignocellulosic biomass substrate for ethanol production. Bagasse is the residue of fiber that remains after the mechanical extraction of the sap from the cane stalks. It has a high cellulosic composition, 30-43% (Kadam 2000), making it suitable for ethanol production (Buaban et al. 2010). Sugar mills burn bagasse as fuel to generate steam and continue to produce a 15-25% surplus. This product can be further increased if steam generators are improved to reduce consumption, facilitated by solar energy, and if bagasse is replaced by waste piping (Kadam 2000). In the Ethiopian context, with the development of hydroelectric projects, the electricity supply could be surplus soon. Thus, given the increased demand for liquid fuel, the sugar industry has the opportunity to replace bagasse fuel with electrical energy. With an oversupply of bagasse and the integration of appropriate ethanol processing technology with existing molasses at ethanol fermenters in the sugar industry, ethanol production from bagasse could be possible. However, as with all other lignocellulosic biomasses, the accessibility of polysaccharides in bagasse biomass is limited. Therefore, different pretreatment systems should be studied to develop a process for converting bagasse into ethanol.

MATERIALS AND METHODS

Most experiments were conducted in the Microbial Biotechnology Laboratory, Unit of the Biotechnology Program, Addis Ababa University, Ethiopia.

Culture preparation

The 15 rot fungi in Table 1 were generously provided by Dr. Adane Bitew (Department of Medical Laboratory Technology, School of Medicine, Addis Ababa University, Ethiopia), as slant or plate cultures. These rot fungi were subcultured on 41 g/L potato dextrose agar (PDA) plates and were incubated for 5-7 days at 27°C.

Solid state fermentation

Production of ligninolytic enzyme and pretreatment by the rot fungi were studied during SSF of bagasse substrate. The SSF conditions were prepared as follows.

Substrate preparation

Sugar cane bagasse residues from mechanical crushing of the cane stalks were purchased from a local cane juice shop. The bagasse was dried in the sun, cut into smaller pieces with a cleaver, ground with a blender and passed through a 1 mm sieve (to remove smaller particles). The residual sucrose on the fiber was removed by immersing it in water (approximately 1: 100 w/v) for 1 hour in two turns with regular agitation. This bagasse preparation is dried in the sun and used as a lignocellulose substrate.

Seed culture preparation

With additional carbon and nitrogen supplements, a mineral salt solution was prepared from Basal III medium and trace elements (10: 1 v/v) (Tien and Kirk 1988; Kumar et al. 2006). Basal III medium was prepared from KH_2PO_4, 20 g/L; $MgSO_4$, 5 g/L and $CaCl_2$, 1 g/L. The trace element solution consisted of (g/L) $MgSO_4$, 3; $MnSO_4$, 0.5; NaCl, 1; $FeSO_4.7H_2O$, 0.1; $CoCl_2$, 0.1; $ZnSO_4.7H_2O$, 0.1; $CuSO_4$, 0.1; AlK (SO_4) $2.12H_2O$, 0.01; H_3BO_3, 0.01 and $Na_2MoO_4.2H_2O$, 0.01 dissolved in a nitrilotriacetate solution, 1.5 g/L adjusted to pH 6.5 with 3% KOH. Glucose (1 g/L) and peptone (10 g/L) were added as a supplement of carbon and nitrogen, respectively. The bagasse preparation (5 g) in a 500 mL Erlenmeyer flask was autoclaved at 121°C for 30 minutes. After cooling, 15 mL of the mineral salt solution with the supplements was membrane filtered (0.2 µm) on the substrate.

Each flask was inoculated with four agar cube blocks of approximately 6 x 5 mm^2 (Samsuri et al. 2008) cut from the peripheral sides of the mycelium on the PDA cultures. The SSF culture was incubated stationary at 27°C for 15 days. The growth characteristics of each fungal mushroom on PDA and SSF media were compared by visual observation of the mycelium.

Enzyme extraction and assay

The SSF substrate was immersed in 50 mL of sodium acetate buffer (25 mM, pH 4.5) on a rotary shaker (120 rpm) at room temperature for 90 minutes. The liquid homogenate was filtered through cotton gauze. The extract was centrifuged at 3000 rpm for 5 minutes to remove solids and kept in aliquots at -20°C.

The crude extract was used directly as an enzyme in the test tubes. The concentration between enzyme and substrate was maintained at 1:10 (v/v). The absorbance of each tube was measured on a 1 mL 1 cm (wide) cuvette using a Jenway 6300 visible spectrophotometer (Jenway Ltd., England). The productivity of the enzyme (U/g) was determined using equation 1, where U (U/mL) is the unit expressing the activity per mL of enzyme used, and (mL) is the volume of the enzyme extract collected from the SSF substrate and s (g) is the weight of the SSF substrate. All enzymatic reactions were performed in triplicate according to the respective protocols.

$$\text{Enzyme productivity} = U \times e / s \qquad [1]$$

Manganese peroxidase assay

MnP activity was determined according to Kuwahara et al. (1984) based on the oxidation of phenol red, characterized by a color change from red to pink-purple, under alkaline conditions. The reaction mixture consisted of phenol red (0.01%), sodium lactate (25 mM), $MnSO_4$ (100 µM) and egg albumin (0.1%) in 20 mM sodium succinate buffer (pH 4 ,5). To start the reaction, H_2O_2 (100 µM, final concentration) was added, and the tubes were incubated at 30°C for 5 minutes. The reaction was terminated with 2N NaOH (40 µL/mL) and the absorbance was measured at 610 nm. One unit (U) was defined as the amount of enzyme required to increase by 0.1 optical density (OD) units per minute.

Laccase assay

Lac activity was detected based on the oxidation of guaiacol (C_6H_4 (OH) (OCH_3)), causing the colorless substrate to turn brown (Coll et al. 1993). The reaction mixture was 1 mM guaiacol in 50 mM sodium acetate buffer (pH 4.5). The tubes were incubated at 37°C for 1 hour and the absorbance was measured at 465 nm. According to Arora and Sandhu (1985), a unit is defined as the amount of enzyme required to increase by 1 OD unit per hour.

Lignin peroxidase assay

The LiP test based on the Azure B bleaching method, according to Archibald (1992) using an absorption spectrophotometer, failed in the present study. The readings of the reagent blank containing the dye were unstable. Vares et al. (1995) have encountered similar problems with the protocol. Therefore, a qualitative test based on blue B discoloration was performed on agar plates by inoculating the rotting fungi on solid media according to Zhao et al. (1996). The medium composition was designed to simulate the SSF condition to predict the actual LiP productivity in the bagasse substrate. SSF simulation medium contained peptone, 10 g/L; glucose, 10 g/L; Azure B, 0.1 g/L and agar, 15 g/L dissolved in a mineral salt solution and the cultures were incubated at 27°C for 10 days. However, MnP could discolor Azure B under certain conditions and produce distortions (Archibald 1992). Thus, the LiP activity of putrefactive fungi showing dye discoloration on SSF simulation medium was confirmed on Mn-deficient agar plates (confirmation medium) supplemented with veratryl alcohol (Zhao et al. 1996; Levin et al 2004). The medium was composed of a mineral salt solution (without $MnSO_4$); glucose, 10 g/L; NH_4NO_3, 26 mM; aspartic acid, 15 mM; thiamine, 1.68 mg/L; sodium succinate, 0.1 M; veratrilic alcohol, 2 mM; agar, 15 g/L and blue B, 0.02 g/L. Cultures were incubated at 27°C for 10 days. All detections of discoloration were confirmed by visual observation of a cubic section removed from areas very close to the growth of the mycelium.

Carboxymethyl cellulase and xylanase assay

The extracellular enzymatic activities of carboxymethylcellulose (CMCase) and xylanase were determined by the DNA method (dinitro salicylic acid, $C_7H_4N_2O_7$), according to Bernfeld (1955). The assay is based on reducing 3,5-DNA (yellow) to 3-amino-5-nitrosalicylic acid (reddish brown) by the respective reducing sugars released from the polysaccharides. DNA reagent was prepared from DNA, 10 g/L; C6H6O, 2g/L; Na2SO3, 0.5 g/L and NaOH, 5 g/L. Substrates contained carboxymethylcellulose (0.5%) and birch xylan (1%) in 50 mM citrate phosphate buffer (pH 5.0) for CMCase and xylanase assays, respectively. The tubes were incubated at 40°C for 15 minutes. The reaction was terminated by adding the DNS reagent to the reaction solution (2: 1 v/v), followed by boiling in a water bath for 5 minutes. The absorbance of each reaction tube was measured at 540 nm after cooling. The activities of CMCase and xylanase were expressed in international units where 1 U is the amount of enzyme required to release 1 µmol of glucose (0.18 mg/mL) (Ghose 1987) and xylose (0.15 mg/mL). respectively per minute. The standard curves were made with known glucose and xylose concentrations (appendices 1A and 1C, respectively).

Screening for selective rot fungi

Highly selective rot fungi were examined, showing maximum and minimum productivity of ligninolytic and polysaccharide degradation enzymes, respectively. The aggregate metric index analysis was used as the screening methodology, similar to the multimetric approach of Barbour et al. (1999) for the environmental data analysis. Productivity of enzymes was used as a measure. Each metric was classified into 4 levels of productivity: high, moderate, low and marginal. The range (score range) between tiers was determined by dividing the range (the difference between maximum and minimum productivity) by 4 (the number of tiers). Unitless scores were then assigned to these levels. Score values range from a maximum of 4 for high to a minimum of 1 for marginal productivity for ligninolytic enzymes. The scoring criterion was reversed for CMCase and xylanase; thus, the score values range from a maximum of 4 for marginal to a minimum of 1 for high productivity. The level of each mushroom was identified based on the metric value (U/g) and scores were assigned accordingly. The aggregate index for each mushroom was determined by summing the scores of all metrics. These indices have been divided into 4 levels of selectivity: high, moderate, low and poor. The interval between levels was determined as described for the metrics and each mushroom was ranked based on its index value.

Minimal xylanase activity was set as a selection criterion in the present study, although residual xylan was not used as a substrate for saccharification and fermentation. This was done to consider its potential as a major component of the bagasse.

Screening for effective ligninolysis

Lignin compositions and cellulose digestibility of bagasse, pretreated by the highly selective rot fungi during the SSF, were compared with untreated bagasse to screen for effective ligninolysis.

Lignin degradation

According to Munoz et al. (2007) by hydrolysis of polysaccharides with concentrated acid. Acid-soluble lignin was also analyzed by UV absorbance (Ferraz et al. 2000; Sluiter et al. 2008) of acid-hydrolyzed filtrate. Before hydrolysis, residual non-structural materials or extractable (since some of their composition was removed during soaking) were removed to facilitate acid penetration. Mineralized lignin components, water-soluble inorganic materials, non-structural sugars and nitrogenous materials were removed by autoclaving in an aqueous suspension at neutral pH to avoid hydrolysis hemicelluloses. According to Sluiter et al. (2005) by Soxhlet extraction with ethanol.

First, the fungal biomass on the pretreated bagasse was removed by soaking twice in 2 L of distilled water for 30 minutes with continuous agitation. The floating biomass of

low-density fungi was decanted and the bagasse was recovered (Kumar et al. 2006). Next, the bagasse was suspended in water (1: 100 w/v), adjusted to pH 7-7.5, and sterilized in an autoclave at 121°C for 15 minutes. The liquid was discarded, and the bagasse was washed with 200 mL of boiling water. Other extractants were removed with ethanol (96%) in a Soxhlet extractor (Glassco Laboratory Equipment Ltd., UK) set to reflux for 9 hours. After extraction, the fiber was washed with 80 mL of fresh ethanol (96%) and air dried.

The extract-free bagasse (0.1 g) was treated with 1.5 mL of H_2SO_4 (72%) in a test tube. The tube was shaken vigorously on a vortex and continuously every 10 minutes for 2 hours. The resulting acid hydrolyzate was diluted in 56 mL of distilled water to bring the concentration to 1.88%. The hydrolyzate was autoclaved at 1210°C for 1 hour and filtered through a 0.45 µm calibrated Whatman glass fiber filter. The residue on the filter (the acid-insoluble lignin) was washed with 100 mL of water and dried in an oven at 105°C to constant weight. The filtrate collected from acid hydrolysis was used to determine acid-soluble lignin. The absorbance of the filtrate was analyzed at 205 nm using a Lambda 19 UV-Vis spectrophotometer (Perkin Elmer Inc., Germany). The acid-soluble lignin concentration (%) was determined using Equation 2, where A is the absorbance, V (L) is the filtrate volume, and ε (105 L/g cm) is the absorbance of soluble lignin, l (~1 cm) is the trough path length, and w1 (g) is the initial weight of bagasse without extract.

$$\text{Acid soluble lignin} = A \text{ x } V\text{x } 100/\text{s x } l \text{ x w1} \qquad [2]$$

The lignin composition of the extract free bagasse was the sum of acid-insoluble and acid-soluble lignin. Therefore, lignin weight losses caused by the selected rot fungi pretreatments were determined from the difference in composition with the lignin content of the untreated extract-free bagasse.

Cellulose digestibility

Cellulose digestibility was defined as the percentage of the theoretical maximum of glucose obtained from the hydrolysis of cellulose (Bak et al. 2009). The glucose released from enzyme hydrolysis and the cellulose composition of untreated bagasse was determined as follows.

Enzyme hydrolysis

The bagasse substrate for enzymatic hydrolysis was prepared by removing the fungal biomass and water-soluble mineralized lignin (to avoid enzyme inhibition), without ethanol extraction. Cellulase enzyme (Endoglucanase, EC 3.2.1.4) extracted from Aspergillus niger (chemical group Sigma, USA) was used to hydrolyze cellulose. The enzyme (60 U/g cellulose) was dissolved in 50 mM citrate-phosphate buffer (pH 5.0) and centrifuged at 3000 rpm for 5 minutes to precipitate the particulate. The bagasse (1 g) was hydrolyzed by the solid-state enzyme (1:10) and sterilized on a filter (0.2 µm) at 37°C for 5 days. Glucose was extracted by immersing the hydrolyzate in 10 mL of water at room temperature on a rotary shaker (120 rpm) for 20 minutes. The amount of glucose was estimated using the standard curve. A small change has been made to the composition of the DNS reagent, with the addition of sodium and potassium tartrate, 200 g/L to increase the stability of the reduced DNS (Miller 1959).

Cellulose composition

The water-soluble and ethanol extracts were removed from untreated bagasse according to Sluiter et al. (2005) by Soxhlet extraction, refluxed for 18 and 9 hours, respectively. Then, the bagasse was air dried and the composition of the remaining extracts was estimated.

The free extractive residue (1 g) was delignified according to Ferraz et al. (2003) in a solution of 0.1 mL of acetic acid and 0.6 g of sodium chlorite in 32 mL of aqueous solution. The reaction was carried out in a water bath at 80°C for 1 hour. Equal amounts of sodium chlorite and acetic acid were added every hour over 4 rounds. The mixture was filtered through a porous glass filter No. 4 and washed with cold water and acetone. The filtered residue was extracted with 95% ethanol in the Soxhlet extractor for 4 hours. Finally, the residue was washed with cold water and dried in an oven at 105 ° C to constant weight.

The oven-dried holocellulose residue (0.5 g) was treated with 2.5 mL NaOH (17.5%) at 20°C. NaOH (5 mL) was added 3 times at 5-minute intervals. The mixture was allowed to stand for 30 minutes, then diluted with 8.25 mL of distilled water and left to stand again at 20°C for 1 hour. The fiber was filtered through a #4 fritted glass crucible and soaked in 50 mL of NaOH (8.3%) for 2 minutes. The crucible was soaked in 3.25 mL of acetic acid (10%) for 3 minutes and washed with 300 mL of water. The residual cellulose was dried in an oven at 105°C to constant weight (Rowell et al. 2005). The cellulose composition C (%) was calculated using equations 3, 4 and 5, where c '(%) is the cellulose composition in the extractive free holocellulose, w2 (g) is the weight of the extractive free holocellulose residue, w3 is (g) the weight of cellulose in the extractive free holocellulose, H (%) is the holocellulose composition in the bagasse, L (g) is the extractive free lignin composition per gram of bagasse and E (g) is the composition of the remaining extracts per gram of bagasse. The digestibility of cellulose (%) was estimated using equation 6 where g (g) is the amount of glucose per gram of substrate.

$$c' = \frac{\text{w3}}{\text{w2}} \text{ x } 100 \qquad (3)$$

$$H = (1- L)\,(1-E) \text{ x } 100 \qquad (4)$$

$$C = c' \text{ x } H \text{ x } 100 \qquad (5)$$

$$\text{Cellulose digestibility} = \frac{g}{c} \text{ x } 100 \qquad (6)$$

Ethanol fermentation and GC analysis

The substrates for the ethanol fermentation were cellulase hydrolyzate from a pretreatment, causing the highest lignin loss and cellulose digestibility; Untreated bagasse cellulose hydrolyzate and standard glucose. Saccharomyces cerevisiae strain TAYI 4-2 was obtained from Amare Gesese (Ph.D.). The yeast stem cells for the inoculum were cultured aerobically in 50 mL of broth consisting of (g/L) glucose, 10; yeast extract, 1; KH_2PO_4, 0.1; $MgSO_4.7H_2O$, 0.1 and $(NH_4)2SO_4$, 0.1 (Samsuri et al. 2008). The fermentation broth was prepared from 5 mL of substrate and additional nutrients (g/L): yeast extract, 1; NaH_2PO_4, 1.2; $MgSO_4.7H_2O$, 0.025 and NH_4HPO_4, 0.5. The pH of the broth was adjusted to 5.5 with NaOH (Vaithanomsat et al. 2011). Finally, a 10% (v/v) yeast inoculum was added and anaerobic fermentation of 5 mL broth was carried out in 7 mL tubes for 72 hours at 30°C.

Samples of fermented broth (1 mL) were centrifuged at 10,000 rpm for 5 minutes to precipitate cells and other particles. The supernatant was diluted 10:1 with isopropanol (1 g/L, internal standard). Gas chromatography (GC) was performed on a DANI GC 1000 (DANI Instruments Ltd., Italy) with a flame ionization detector (FID) under optimized operating conditions. An Alltech Econo-Cap ECTM-5 capillary (30 m long, 0.32 mm diameter) coated with 95% methylpolysiloxane (stationary phase) was used as the column. The nitrogen (carrier gas) and hydrogen (FID fuel) flow rates were set at 5 and 0.65 bar, respectively. The GC was conditioned splitless and the inlet, oven and detector temperatures were set to 210, 155 and 250°C, respectively. The starting oven temperature was 50°C, 1 minute hold time at a heating rate of 30°C per minute. After a running time of 4.5 minutes, the peak areas (mV/s) of ethanol and isopropanol were recorded. The data was used to determine the ethanol concentration using the standard curve equation, developed by the same method using a known ethanol concentration. Fermentation broth samples were also analyzed for unfermented glucose composition by the DNA.

RESULTS AND DISCUSSION

Growth characteristics of the rot fungi

The intense growth of 8 rotting fungi was observed on SSF media (Table 1). Only 3 rot had poor growth, as seen on the handheld. Additionally, 11 rotting mushrooms changed the faded yellow color of the bagasse to red or light red.

Production of ligninolytic and polysaccharide degrading enzymes

The productivity of the MnP and Lac enzymes is shown in Figures 1A and 1B, respectively. These enzymes were also distributed in 11 putrefying fungi. However, in terms of productivity, MnP was produced by many putrefying fungi at relatively high titers compared to the Lac (Appendixes 7 and 8). *Laetiporus sulphureus* and *Schizophyllum commune* showed no activity, while *Polyporus* sp. and *Inonotus* sp. produced neither MnP nor Lac. The activities of the MnP and Lac enzymes of *Perenniporia* sp. and *Pleurotus sajor-caju* were also negligible. The highest productivity of MnP and Lac was recorded in *Fomitiporia aethiopica* (55.87 ± 0.37 U/g) and *Perenniporia tephropora* (79.65 ± 1.78 U/g), respectively. The *P. tephropora* also showed high MnP productivity (51.73 ± 1.12 U/g). Marginal productions of MnP and Lac were obtained from *Vanderbylia vicina* (5.56 ± 0.26) and *Lentinus edodes* (2.37 ± 1.11 U/g), respectively. CMCase and xylanase activities were detected from all putrefying fungi (Figures 1C and 1D, respectively). The *P. sajor-caju* had the lowest CMCase productivity (0.3 ± 0.02 U/g), while *Tyromyces* sp. produced the enzyme at the highest titers (8.53 ± 0.19 U/g). The *F. aethiopica* was the least producer of xylanase (2.25 ± 0.14 U/g), while *L. edodes* recorded the highest productivity (19.52 ± 1.12 U/g).

LiP was produced from 7 rotting fungi exhibiting varying intensities of blue B discoloration (Table 2). The discoloration was initially observed in 8 decaying mushrooms grown on SSF simulation media. Among them, Phellinus sp. did not discolor the blue B on the mounting support, excluding the possibility of LiP production. The complete discoloration was observed only in *Polyporus* sp. on both media, while other rot fungi could only fade or partially fade the dye.

Table 1. Growth characteristics of the rot fungi based on visual observation on PDA and SSFmedia

Species	Growth on		Bagasse color
	PDA	SSF	
Fomitiporia pseudopunctata	++++[c]	++++	R[f]
Fomitiporia aethiopica	++[a]	++++	R
Fomitopsis carnea	++++	++++	-[d]
Inonotus sp.	+++[b]	++++	R
Perenniporia tephropora	++	++++	R
Pleurotus sajor-caju	++++	++++	R
Polyporus sp.	++++	++++	-
Vanderbylia vicina	++	++++	R
Lentinus edodes	++++	+++	LiR[e]
Phellinus sp.	++++	+++	R
Schizophyllum commune	++++	+++	
Microporous sp.	+++	+++	LiR
Perenniporia sp.	++	++	LiR
Tyromyces sp.	++	++	LiR
Laetiporus sulphureus	++	++	-

Note: [a]poor, [b]medium, [c]intense, [d]no colour change observed, [e]light red, [f]red

Table 2. The intensity of azure B decolorization on SSF simulating and confirmation media

Species	SSF simulating media	Confirmation media
Polyporus sp.	++++[e]	++++
Fomitiporia aethiopica	+++	+++
Inonotus sp.	+++	+++
Tyromyces sp.	++[c]	+++[d]
Pleurotus sajor-caju	+[b]	+
Microporous sp.	+	+++
Vanderbylia vicina	+	+
Phellinus sp.	++	-[a]

Note: [a]no decolorization, [b]mild fade, [c]fade, [d]partial decolorization, [e]complete decolorization

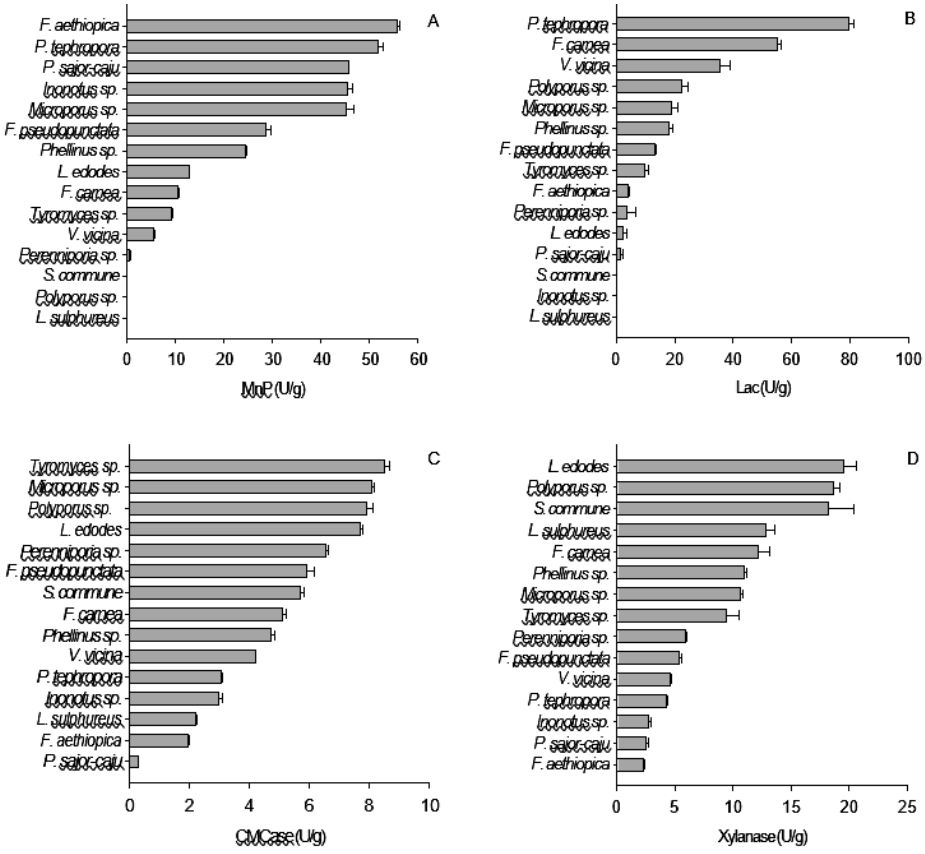


Figure 1. Productivity of ligninolytic and polysaccharide degrading enzymes by the rot fungi. A: MnP, B: Lac, C: CMCase and D: Xylanase

Categorization by ligninolytic enzyme production profile

Based on the production of ligninolytic enzymes, 13 rot fungi were categorized into 5 groups: LiP, MnP and Lac; LiP and MnP; LiP and Lac; MnP and Lac and only Lac producers (Table 3). There were 5 rot fungi grouped under MnP and Lac producers and 4 were LiP, MnP and Lac producers. At least two of the ligninolytic enzymes were simultaneously produced by 12 rot fungi.

Screening for selective rot fungi

The *F. aethiopica*, *P. tephropora*, *Inonotus* sp. and *P. sajor-caju* were highly selective among the rot fungi (Table 4). These fungi belonged to the white rot group. They showed high MnP, marginal xylanase and either low or marginal CMC productivity.

Screening selective rot fungi for effective ligninolysis

Lignin degradation analysis

The lignin composition of the untreated extract-free bagasse was determined to be 25.93% (acid-insoluble, 24% and acid-soluble, 1.93%). The residual lignin compositions in the 4 pretreated bagasse were determined and weight losses of lignin were observed in 3 pretreatments (Table 5). The major loss of lignin (7.71%) was due to pretreatment with *P. tephropora*. Degradation of *P. tephropora* was impossible to determine *P. sajor-caju* as the lignin composition after pretreatment (32.62%) was higher than that obtained in the untreated bagasse.

Cellulose digestibility

The untreated washed bagasse comprised 8.58% residual extractives, 67.72% holocellulose and 42.42% cellulose. All pretreatments increased glucose yields from enzymatic hydrolysis and thus cellulose digestibility (Table 6). Pretreatment with *P. tephropora* resulted in the maximum: glucose yield (7.345 g/L), cellulose digestibility (29.44%), and bagasse saccharification (12.49%, w / w). Compared to untreated bagasse, digestibility was improved by 38.74%. Pretreatment with *P. sajor-caju* had the lowest improvement in digestibility (1.7%). Therefore, *P. tephropora* was chosen as the selective degrader, displaying the most effective ligninolysis among the rot fungi based on lignin loss and cellulose digestibility results.

Ethanol yield

The ethanol yield from the fermentation of the enzyme hydrolysate of *P. tephropora* pretreated bagasse was 1.87 g/L (Table 7). This showed an improvement by 27.21% compared with the untreated bagasse (1.47 g/L). The *P. tephropora* pretreatment hydrolysate had a relatively higher content of unfermented glucose than the untreated bagasse. However, the glucose conversion efficiency (50.87%) was lower than the untreated bagasse (55.56%). The highest conversion efficiency (83.53%) was obtained from fermentation of the standard glucose under the same conditions.

Table 3. Categorization of the rot fungi by ligninolytic enzyme production

Group	Enzymes	
I	LiP, MnP and Lac	*Fomitiporia aethiopica, Microporus* sp., *Tyromyces* sp., *Vanderbylia vicina*
II	LiP and MnP	*Inonotus* sp., *Pleurotus sajor-caju*
III	LiP and Lac	*Polyporus* sp.
IV	MnP and Lac	*Fomitiporia pseudopunctata, Fomitopsis carnea, Lentinus edodes, Perenniporia tephropora, Phellinus* sp.
V	Lac only	*Perenniporia* sp.

Table 4. Comparison based on aggregate metrics index analysis

Species	Scores of metrics					Aggregate index	Relative selectivity
	MnP	Lac	LiP	CMC	Xylanase		
Fomitiporia aethiopica	4	1	3	4	4	16	High
Perenniporia tephropora	4	4	1	3	4	16	High
Inonotus sp.	4	1	3	3	4	15	High
Pleurotus sajor-caju	4	1	1	4	4	14	High
Fomitiporia pseudopunctata	3	1	1	2	4	11	Moderate
Vanderbylia vicina	1	2	1	3	4	11	Moderate
Microporous sp.	4	1	1	1	3	10	Low
Fomitopsis carnea	1	3	1	2	2	9	Low
Laetiporus sulphureus	1	1	1	4	2	9	Low
Polyporus sp.	1	2	4	1	1	9	Low
Perenniporia sp.	1	1	1	1	4	8	Low
Phellinus sp.	2	1	1	2	2	8	Low
Tyromyces sp.	1	1	2	1	3	8	Low
Schizophyllum commune	1	1	1	2	1	6	Poor
Lentinus edodes	1	1	1	1	1	5	Poor

Table 5. Residual lignin compositions and weight losses of bagasse after pretreatments

	Acid insoluble lignin (%)	Acid soluble lignin (%)	Total lignin (%)	Lignin loss (%)
Perenniporia tephropora	21.90	2.03	23.93	7.71
Fomitiporia aethiopica	22.45	1.74	24.19	6.71
Inonotus sp.	23.50	1.92	25.42	1.97
Pleurotus sajor-caju	30.80	1.82	32.62	ND[a]

Note: [a]not determined

Table 6. Comparison of glucose yield and cellulose digestibility among pretreatments andwith untreated bagasse

	Glucose yield		Cellulose digestibility	Improvement in digestibility
	g/L	g/g of substrate	(%)	(%)
Untreated	52.950	0.0900	21.22	
Perenniporia tephropora	73.450	0.1249	29.44	38.74
Fomitiporia aethiopica	71.700	0.1219	28.73	35.39
Inonotus sp.	65.250	0.1109	26.15	23.23
Pleurotus sajor-caju	53.850	0.0915	21.58	1.70

Table 7. The *S. cerevisiae* fermentation profile of pretreated and untreated bagasse enzymehydrolysates and standard glucose

Fermentation substrate	Ethanol yield (g/L)[a]	Glucose conversion efficiency (%)	Unfermented glucose (g/L)
Perenniporia tephropora pretreated bagasse hydrolysate	1.87	50.87	2.36
Untreated bagasse hydrolysate	1.47	55.56	1.82
Standard glucose	4.18	83.53	0.97

Discussion

Pretreatment during SSF can be attributed to favorable conditions for the growth of fungi and the production of ligninolytic enzymes. Factors that may have contributed include the similarity of the solid state to the natural environment (Lee 1997), the stimulation of secondary metabolism by the peptone (Kimura et al. 1990; Kaal et al. 1995; Martinez et al. 1996) and a small entity, glucose supplement (Lee 1997) to promote the production of ligninolytic enzymes. Production of MnP and Lac enzymes was more common than LiP in the present study. MnP and Lac producers are common in white rot (Hatakka 2001). MnP is produced by most white rot (Kaal et al. 1995) and is the most studied enzyme from natural substrate degradation (Vares et al. 1995).

Conversely, LiP is less common in white rot (Hakala 2007) and several factors in SSF conditions may have affected production. LiP activity can be regulated by Mn^{2+} in SSF media. Mn reduces the accumulation of veratril alcohol (Hamman et al. 1999), an important redox mediator of the LiP-catalyzed reaction (Hakala 2007). Bonnarme and Jeffries (1990) reported a 2.5-fold increase in LiP activity in an Mn-free medium. Poor LiP productivity makes detecting enzymatic activity difficult (Kaal et al. 1995) and may have influenced the low intensity of the blue B discoloration in the present study.

The production of all predominant ligninolytic enzymes by *F. aethiopica* is the first report of this activity by this redescribed taxa species. The ability to produce high titers of MnP arouses interest to characterize further and study the potential application of the enzyme. The other elevated MnP producer, *P. tephropora*, has previously been studied for its MnP activity (Ralph et al. 1996); however, the enzyme has not been well characterized. MnP activity has also been studied previously in *P. sajor-caju* (Boyle et al. 1992; Tuor et al. 1995; Martinez et al. 1996), *Inonotus* sp. (Palma et al. 2011) and *Microporus* sp. (Song 1997). However, for *Microporus* sp. the role of MnP is not related to the degradation of lignin.

The high productivity of the Lac enzyme by *P. tephropora* has been reported by Ralph et al. (1996). Although Lac's productivity was moderate and low due to *Fomitopsis carnea* and nearby *V. vicina*, this is the earliest report of ligninolytic enzyme activity. Several studies have identified LiP activities from several species of the genus *Polyporus*. LiP activity was detected by the white rot species *P. tulipiferae* (Rothschild et al. 2002), *P. platensis*, *P. brumalis*, *P. pinsitus* and *P. varius* (Tuor et al. 1995) and the brown rot species, *P. ostreiformis* (Dey et al. 1991). Although the productivity was low or marginal, *Microporus* sp., *Tyromyces* sp. and *V. vicina* showed LiP activity which has not been reported before. *Tyromyces* sp. will be added to the few LiP-producing brown rot fungi (Dey et al. 1991; Mtui and Masalu 2008). LiP activities have already been reported by *P. sajor-caju* (Tuor et al. 1995) and *Inonotus* sp. (Risna and Suhirman 2002).

The ligninolytic enzyme production profiles of *F. aethiopica*, *F. pseudopunctata*, *F. carnea*, and *V. vicina* have not been previously reported. Adults of *Inonotus* sp., *Phellinus* sp., *Perenniporia* sp., and *P. sajorcaju* and the absence of any *L. sulphureus* and *S. commune* activity were not entirely consistent with previous studies (Table 1). This could be due to variations in the species or strains of fungi responsible for the rotting and fermentation conditions in the present study. In the case of *L. sulphureus* and *S. commune*, enzymatic activities have been reported by Mtui and Masalu (2008) and Asgher et al. (2008) in liquid cultures, according to Tien and Kirk (1984, 1988). These media also contained a limited source of inorganic N and veratryl alcohol as a lignin substrate. The effect of media on the production of ligninolytic enzymes was examined by Kimura et al. (1990). They achieved high LiP productivity by white rot P. chrysosporium only in crops consisting of limited inorganic nitrogen; while on the contrary *Bjerkandra* sp. produces high titers in the presence of nitrogen-rich organic supplements.

The red color formed on the bagasse substrate during growth may indicate lignin degradation during SSF. Fackler et al. (2007) observed red discoloration of wood, during short-term treatment, due to the release of free phenoxy radicals and resulting quinoid structures due to lignin degradation. In this study, this evidence was seen throughout highly selective white rot.

The productivity of the ligninolytic and polysaccharide degrading enzymes was inversely related to the highly selective degraders. As white rot, these fungi could follow a particular degradation pattern with a higher preference for lignin degradation (Hakala 2007). The SSF conditions may have further influenced this physiology in this study. In addition to stimulating secondary metabolism by dietary supplements, glucose can suppress the production of polysaccharide-degrading enzymes (Levonen-Munoz and Bone 1985). This white rot did not use the polysaccharides despite a better accessibility probably after modification of the lignin. Therefore, the supplements made lignin more likely to be metabolized as a carbon and energy source. This metabolic pathway was suitable for the pretreatment system.

The better degradation of lignin by *P. tephropora* can be attributed to the high productivity of Lac. White rot had no LiP activity, and MnP productivity was comparable to other selective lignin degraders. Arora et al. (2002) noted that white rot with high Lac productivity showed better lignin degradation and that combining one of the ligninolytic enzymes with Lac led to greater lignin loss.

The lignin loss by *P. tephropora* (7.71%) was comparable with *Coriolus versicolor* (6-7%) and *P. chrysosporium* (9%) pretreatments of hardwood lignin (Kashino et al. 1993). In otherstudies, lignin losses of up to 15.7% (Samsuri et al. 2008), 21% (Bak et al. 2009) and 21.7% (Itoh et al. 2003) have been reported. The relatively lower lignin degradation in the present study could be due to incomplete pretreatment from the short SSF period (15 days). This was evident in the red color formation of the bagasse substrate, a characteristic of short-term pretreatment (Fackler et al. 2007). Other factors might include culture conditions and or simply the natural lignin degradation capability of *P. tephropora*.

The lignin composition of bagasse after pretreatment with *P. sajor-caju* was higher than that of untreated

bagasse. This indicated a loss of weight of cellulose and hemicelluloses by degradation, which was compensated by the accumulation of unmodified lignin. However, white rot had only marginal CMCase and xylanase productivity. It is therefore suspected that white rot may have cell-bound CMCase and/or xylanase activity that has not been detected. In a study by Valaskova and Valerian (2006), 66% of the activities of P. ostreatus cellulose, xylan and mannan degrading enzymes were cell-bound. Although weight loss could not be determined, lignin degradation by *P. sajor-caju* was still evident due to the red coloring of the bagasse substrate and high MnP productivity.

A pretreatment that resulted in higher lignin loss showed better cellulose digestibility. Samsuri et al. (2008) and Bak et al. (2009) noted that as lignin weight loss increased, glucose yield was increased by enzymatic hydrolysis. Therefore, the modification of the lignin barrier increased the accessibility of the cellulose for enzymatic hydrolysis.

In previous studies, ethanol yield has been improved while sustaining 2.94% (Samsuri et al. 2008) and 5% (Itoh et al. 2003) cellulose weight losses from pretreatments. The present study also observed improvements in glucose yield, despite CMCase activities. The highest improvement in digestibility was observed from *P. tephropora* pretreatment while having low CMCase productivity, which was higher than only marginal productivity by the other highly selective degraders. Thus, the impact of lignin modification has overshadowed the effect of cellulose degradation by the enzyme.

The saccharification of bagasse pretreated with *P. tephropora* (12.49%) was superior to that of beech wood (9.5%) pretreated with P. chrysosporium (Sawada et al. 1995). The digestibility of cellulose (29.44%) was also higher than that of P. chrysosporium from corn stalks (0.3-12%) and was comparable to Cyathus stercoreus pretreatment (8.3-35.7%) (Keller et al. 2003). In other studies, the digestibility of cellulose reached around 44% (Samsuri et al. 2008) and even 64.9% (Bak et al. 2009). However, these results were obtained from white rot pretreatments which caused greater lignin losses.

Pretreatment with *P. sajor-caju* showed a slight improvement in the digestibility of cellulose (1.7%), despite the impossibility of determining the loss of lignin. The relatively low improvement indicates some form of modification of the lignin. It also implies that the suggested cell-bound polysaccharide-degrading enzyme activity might be dominated by xylanase. However, this suggestion needs to be confirmed by further analysis.

The increase in glucose yield due to *P. tephropora* pretreatment consequently enhanced ethanol production compared with the untreated bagasse. However, the glucose conversion efficiency was slightly reduced due to the pretreatment. As a general rule, the fermentation was highly affected by factors in the biomass saccharification process, as evidenced by the large difference in conversion efficiency between fermentations of standard glucose.

In conclusion, there is a greater prevalence of MnP and Lac enzymes among the studied rot fungi, while LiP is less frequent and exhibits low activity. The white rots, *F. aethiopica*, *P. sajor-caju*, *Inonotus* sp. and *P. tephropora* are relatively highly selective lignin degraders. CMCase and xylanase activity are low, but high quantities of ligninolytic enzymes are produced. The pretreatment of lignin with these selective degraders results in lignin weight losses, and lignin degradation results in an increase in cellulose accessibility. Minimal polysaccharide consumption under these pretreatment conditions does not highly affect the glucose yield (*P. sajor-caju* pretreatment is an exception). The *P. tephropora* is the most effective lignin degrader among these selective white rots. Better ligninolysis is attributed to the high productivity of Lac enzyme in combination with MnP. The pretreatment of bagasse with *P. tephropora* partially degrades lignin, thereby increasing cellulose accessibility to enzyme hydrolysis.

REFERENCES

Archibald FS. 1992. A new assay for lignin-type peroxidases employing the dye azure B. Appl Environ Microbiol 58 (9): 3110-3116. DOI: 10.1128/aem.58.9.3110-3116.1992.

Arora DS, Sandhu DK. 1985. Laccase production by a white rot fungus *Daedalea flavida*. Enzyme Microb Technol 7: 405-408. DOI: 10.1016/0141-0229(85)90131-0.

Arora DS, Chander M, Gill PK. 2002. Involvement of lignin peroxidase, manganese peroxidase and laccase in degradation and selective ligninolysis of wheat straw. Intl Biodeterior Biodegrad 50: 115-120. DOI: 10.1016/S0964-8305(02)00064-1.

Asgher M, Kausar S, Bhatti HN, Shah SAH, Ali M. 2008. Optimization of medium for decolourization of solar golden yellow R direct textile dye by *Schizophyllum commune* IBL- 06. Intl Biodeterior Biodegrad 61: 189-193. DOI: 10.1016/j.ibiod.2007.07.009.

Bak JS, Ko JK, Choi I, Park Y, Seo J, Kim KH. 2009. Fungal pretreatment of lignocelluloses by *Phanerochaete chrysosporium* to produce ethanol from rice straw. Biotechnol Bioeng 104 (3): 471-482. DOI: 10.1002/bit.22423.

Barbour MT, Gerritsen J, Synder BD, Stribling JB. 1999. Rapid Bioassessment Protocols for Use in Streams and Wadeable Rivers: Periphyton, Benthic Macroinvertebrates and Fish. 2nd ed. U.S. Environmental Protection Agency, Washington DC.

Bernfeld P. 1955. Amylases, alpha and beta. Method Enzymol 1: 149-158. DOI: 10.1016/0076-6879(55)01021-5.

Bonnarme P, Jeffries TW. 1990. Mn (II) regulation of lignin peroxidase and manganese dependent peroxidases from lignin degrading white rot fungi. Appl Environ Microbiol 56 (1): 210-217. DOI: 10.1128/aem.56.1.210-217.1990.

Boyle CD, Kropp BR, Reid ID. 1992. Solubilization and mineralization of lignin by white rot fungi. Appl Environ Microbiol 58 (10): 3217-3224. DOI: 10.1128/aem.58.10.3217-3224.1992.

Buaban B, Inoue H, Yano S, Tanapongpipat S, Ruanglek V, Champreda V, Pichyangkura R, Rengpipat S, Eurwilaichitr L. 2010. Bioethanol production from ball milled bagasse using an on-site produced fungal enzyme cocktail and xylose fermenting *Pichia stipitis*. J Biosci Bioeng 110 (1): 18-25. DOI: 10.1016/j.jbiosc.2009.12.003.

Coll PM, Fernandez JM, Villanueva JR, Santamaria R, Perez P. 1993. Purification and characterization of a phenoloxidase (Laccase) from the lignin degrading basidiomycete PM1 (CECT 2971). Appl Environ Microbiol 59 (8): 2607-2613. DOI: 10.1128/aem.59.8.2607-2613.1993.

Dey S, Maiti TK, Bhattacharyya BC. 1991. Lignin peroxidase production by a brown rot fungus, *Polyporus ostreiformis*. J Ferment Bioeng 72: 402-404. DOI: 10.1016/0922-338X(91)90096-Y.

Fackler K, Gradinger C, Schmutzer M, Tavzes C, Burgert I, Schwanninger M, Hinterstoisser B, Watanabe T, Messner K. 2007. Biotechnological wood modification with selective white rot fungi and its molecular mechanisms. Food Technol Biotechnol 45 (3): 269-276.

Ferraz A, Baeza J, Rodriguez J, Freer J. 2000. Estimating the chemical composition of biodegraded pine and eucalyptus wood by DRIFT spectroscopy and multivariate analysis. Bioresour Technol 74: 201-212. DOI: 10.1016/S0960-8524(00)00024-9.

Ferraz A, Cordova A, Machuca A. 2003. Wood biodegradation and enzyme production by *Ceriporiopsis subvermispora* during solid state fermentation of *Eucalyptus grandis*. Enzyme Microb Technol 32: 59-65. DOI: 10.1016/S0141-0229(02)00267-3.

Ghose TK. 1987. Measurements of cellulase activities. Pure Appl Chem 59 (2): 257-268. DOI: 10.1351/pac198759020257.

Hakala TK. 2007. Characterisation of the Lignin Modifying Enzymes of the Selective White Rot Fungus *Physisporinus rivulosus*. [Ph.D. dissertation]. University of Helsinki, Helsinki.

Hamman OB, Rubia T, Martinez J. 1999. The effect of manganese on the production of phanerochaete flavido-alba ligninolytic peroxidases in nitrogen limited cultures. FEMS Microbiol Lett 177: 137-142. DOI: 10.1111/j.1574-6968.1999.tb13724.x.

Hatakka A. 2001. Biodegradation of lignin. In: Hofrichter M, Steinbuchel A (eds.). Biopolymers. Wiley-VCH, Weinheim. DOI: 10.1002/3527600035.bpol1005.

Itoh H, Wada M, Honda Y, Kuwahara M, Watanabe T. 2003. Bioorganosolve pretreatment for simultaneous saccharification and fermentation of beech wood by ethanolysis and white rot fungi. J Biotechnol 103: 273-280. DOI: 10.1016/S0168-1656(03)00123-8.

Kaal EEJ, Field JA, Joyce TW. 1995. Increasing ligninolytic enzyme activities in several white rot basidiomycetes by nitrogen sufficient media. Bioresour Technol 53: 133-139. DOI: 10.1016/0960-8524(95)00066-N.

Kadam KL. 2000. Environmental Life Cycle Implications of Using Bagasse Derived Ethanol as a Gasoline Oxygenate in Mumbai (Bombay): Technical Report. National Renewable Energy Laboratory, Colorado. DOI: 10.2172/772426.

Kashino Y, Nishida T, Takahara Y, Fujita K, Kondo R, Sakai K. 1993. Biomechanical pulping using white rot fungus IZU-154. TAPPI J 76 (12): 167-171.

Keller FA, Hamilton JE, Nguyen QA. 2003. Microbial pretreatment of biomass: Potential for reducing severity of thermochemical biomass pretreatment. Appl Biochem Biotech 105: 27-41. DOI: 10.1007/978-1-4612-0057-4_3.

Kimura Y, Asada Y, Kuwahara M. 1990. Screening of basidiomycetes for lignin peroxidase genes using a DNA probe. Appl Environ Microbiol 32: 436-442. DOI: 10.1007/BF00903779.

Kumar AG, Sekaran G, Krishnamoorthy S. 2006. Solid state fermentation of *Achras zapota* lignocellulose by *Phanerochaete chrysosporium*. Bioresour Technol 97: 1521-1528. DOI: 10.1016/j.biortech.2005.06.015.

Kuwahara M, Glenn JK, Morgan MA, Gold MH. 1984. Separation and characterization of two extracellular H_2O_2- dependent oxidases from ligninolytic cultures of Phanerochaete chrysosporium. FEBS Lett 169 (2): 247-250. DOI: 10.1016/0014-5793(84)80327-0.

Lee J. 1997. Biological conversion of lignocellulosic biomass to ethanol. J Biotechnol 56: 1-24. DOI: 10.1016/S0168-1656(97)00073-4.

Levin L, Papinutti L, Forchiassin F. 2004. Evaluation of Argentinean white rot fungi for their ability to produce lignin modifying enzymes and decolourize industrial dyes. Bioresour Technol 94: 169-176. DOI: 10.1016/j.biortech.2003.12.002.

Levonen-Munoz E, Bone DH. 1985. Effect of different gas environments on bench scale solid state fermentation of oat straw by white rot fungi. Biotechnol Bioeng 27: 382-387. DOI: 10.1002/bit.260270324.

Martinez MJ, Ruiz-Duenas J, Guillen F, Martinez AT. 1996. Purification and catalytic properties of two manganese peroxidase isoenzymes from *Pleurotus eryngii*. Eur J Biochem 237: 424-432. DOI: 10.1111/j.1432-1033.1996.0424k.x.

Miller GL. 1959. Use of dinitrosalicylic acid reagent for determination of reducing sugar. Anal Chem 31 (3) :426-428. DOI: 10.1021/ac60147a030.

Mtui G, Masalu R. 2008. Extracellular enzymes from brown rot fungus *Laetiporus sulphureus* isolated from mangrove forests of coastal Tanzania. Sci Res Essays 3 (4): 154-161.

Munoz C, Mendonca R, Baeza J, Berlin A, Saddler J. 2007. Bioethanol production from bioorganosolve pulps of *Pinus radiata* and *Acacia dealbata*. J Chem Technol Biotechnol: Intl Res Process Environ Clean Technol 82 (8): 767-774. DOI: 10.1002/jctb.1737.

Palma C, Contreras E, Urra J, Martinez MJ. 2011. Eco-friendly technologies based on banana peel use for the decolourization of the dyeing process waste water. Waste Biomass Valorization 2 (1): 77-86. DOI: 10.1007/s12649-010-9052-4.

Ralph JP, Graham LA, Catcheside DEA. 1996. Extracellular oxidases and the transformation of solubilised low rank coal by wood-rot fungi. Appl Microbiol Biotechnol 46: 226-232. DOI: 10.1007/s002530050809.

Risna RA, Suhirman. 2002. Ligninolytic enzyme production by Polyporaceae from Lombok, Indonesia. Fungal Divers 9: 123-134.

Rothschild N, Novotny C, Sasek V, Dosoretz CG. 2002. Ligninolytic enzymes of the fungus Irpex lacteus (*Polyporus tulipiferae*): isolation and characterization of ligninperoxidase. Enzyme Microb Technol 31: 627-633. DOI: 10.1016/S0141-0229(02)00171-0.

Rowell RM, Pettersen R, Han JS, Rowell J, Tshabalala MA. 2005. Cell wall chemistry. In: Rowell RM (eds). Handbook of Wood Chemistry and Wood Composites, CRC Press, Florida. DOI: 10.1201/9780203492437.

Samsuri M, Gozan M, Hermansyah H, Prasetya B, Nasikin M, Watanabe T. 2008. Ethanol production from bagasse with combination of cellulase-cellubiase in simultaneous saccharification and fermentation using white rot fungi pretreatment. J Chem Nat Resour Eng 3: 20-32.

Sanchez OJ, Cardona CA. 2008. Trends in biotechnological production of fuel ethanol from different feedstocks. Bioresour Technol 99: 5270-5295. DOI: 10.1016/j.biortech.2007.11.013.

Sawada T, Nakamura Y, Kobayashi F, Kuwahara M, Watanabe T. 1995. Effects of fungal pretreatment and steam explosion pretreatment on enzymatic saccharification of plant biomass. Biotechnol Bioeng 48: 719-724. DOI: 10.1002/bit.260480621.

Sluiter A, Hames B, Ruiz R, Scarlata C, Sluiter J, Templeton D, Crocker D. 2008. Determination of Structural Carbohydrates and Lignin in Biomass: Laboratory Analytical Procedures (LAP). National Renewable Energy Laboratory, Colorado.

Sluiter A, Ruiz R, Scarlata C, Sluiter J, Templeton D. 2005. Determination of Extractives from Biomass: Laboratory Analytical Procedures (LAP). National Renewable Energy Laboratory, Colorado.

Song H. 1997. Biodegradation of aromatic hydrocarbons by several white rot fungi. J Microbiol 35 (1): 66-71.

Sun Y, Cheng J. 2002. Hydrolysis of lignocellulosic materials for ethanol production: A review. Bioresour Technol 83: 1-11. DOI: 10.1016/S0960-8524(01)00212-7.

Tien M, Kirk TK. 1984. Lignin-degrading enzyme from *Phanerochaete chrysosporium*: Purification, characterisation, and catalytic properties of a unique H_2O_2 requiring oxygenase. Proc Natl Acad Sci 81: 2280-2284. DOI: 10.1073/pnas.81.8.2280.

Tien M, Kirk TK. 1988. Lignin peroxidase of *Phanerochaete chrysosporium*. In: Wood WA, Kellogg ST (eds.). Methods in Enzymology: Biomass, Part B, Lignin, Pectin and Chitin, Academic Press, Inc, San Diego. DOI: 10.1016/0076-6879(88)61025-1.

Tomas-Pejo E, Olivia JM, Ballesteros M. 2008. Realistic approach for full scale bioethanol production from lignocelluloses: A review. J Sci Ind Res India 67: 874- 884.

Tuor U, Winterhalter K, Fiechter A. 1995. Enzymes of white rot fungi involved in lignin degradation and ecological determinants of wood decay. J Biotechnol 41: 1-17. DOI: 10.1016/0168-1656(95)00042-O.

Vaithanomsat P, Apiwatanapiwat W, Chumchuent N, Kongtud W, Sundhrarajun S. 2011. The potential of coconut husk utilization for bioethanol production. Kasetsart J Nat Sci 45: 159-164.

Valaskova V, Baldrian P. 2006. Estimation of bound and free fractions of lignocellulose degrading enzymes of wood rotting fungi *Pleurotus ostreatus*, *Trametes versicolor* and *Piptoporus betulinus*. Res Microbiol 157: 119-124. DOI: 10.1016/j.resmic.2005.06.004.

Vares T, Kalsi M, Hatakka A. 1995. Lignin peroxidase and other ligninolytic enzymes produced by *Phlebia radiata* during solid state fermentation of wheat straw. Appl Environ Microbiol 61 (10): 3515-3520. DOI: 10.1128/aem.61.10.3515-3520.1995.

Zhao J, Koker HT, Janse BJH. 1996. Comparative studies of lignin peroxidases and manganese dependent peroxidases produced by selected white rot fungi in solid media. FEMS Microbiol Lett 145: 393-399. DOI: 10.1111/j.1574-6968.1996.tb08606.x.

BIOTEKNOLOGI
Volume 16, Number 1, May 2019
Pages: 31-36

ISSN: 0216-6887
E-ISSN: 2301-8658
DOI: 10.13057/biofar/c160105

Virulence and genetic diversity of *Fusarium oxysporum* f.sp. *cepae* as the cause of root rot in garlic

M. RIFQI CHOIRUDDIN, ZAINAL D. FATAWI, HADIWIYONO♥
Agronomy Program, Faculty of Agriculture, Universitas Sebelas Maret. Jl. Ir. Sutami 36A Surakarta 57126, Central Java, Indonesia.
Tel./Fax.: +62-271-637457, ♥email: hadiwiyono@staff.uns.ac.id

Manuscript received: 6 April 2019. Revision accepted: 21 May 2019.

Abstract. *Choiruddin MR, Fatawi ZD, Hadiwiyono. 2019. Virulence and genetic diversity of* Fusarium oxysporum *f. sp.* cepae *as the cause of root rot in garlic. Bioteknologi 16: 31-36.* Garlic (*Allium sativum* L.) is a well-known name in the community. The development of garlic in an area intensively and continuously has a positive impact on increasing farmers' income but also has a negative impact with a significant increase in garlic disease attacks. Root rot caused by *Fusarium oxysporum f.* sp *cepae* is one of the factors causing garlic yield loss, both in the field and during storage. Recently, this disease has also become endemic in the garlic production center in Tawangmangu, Indonesia. This study aims to obtain information about the virulence and genetic diversity of *Fusarium oxysporum* f. sp. *cepae* from endemic and non-endemic areas of garlic based on RAPD analysis. This research was conducted from August to December 2009 in Indonesia. Based on the virulence test, there is no significant difference in the damage caused by various isolates between endemic and non-endemic areas. There is no difference in the pattern of DNA fragments in endemic and non-endemic areas, but 3 groups of DNA strains can be obtained from these results. Still, the grouping is unrelated to the virulence and origin of endemic or non-endemic isolates. The grouping between lines does not include differences between endemic and non-endemic areas. In line 1, all isolates are isolates from endemic areas; in line 2, there are isolates from endemic areas (FCp2 isolates) and non-endemic (FCp7 isolates); while strain 3 is also a mixture of isolates from endemic areas (FCp3 isolates) and non-endemic areas. (FCp5, FCp6, and FCp8) isolates. It proves that there is genetic diversity in *F. oxysporum* f. sp. *cepae* isolated from Tawangmangu. However, the genetic diversity of these pathogens is not related to the disease status of the area of origin of the isolates.

Keywords: *Fusarium oxysporum* f.sp. *cepae*, garlic, genetic, root rot, virulence

INTRODUCTION

Garlic (*Allium sativum* L.) is a well-known name in the community. This tuber vegetable is one of the main kitchen spices. Garlic with the surname *Allium* is a descendant of the wild onion *Allium longicurpis* Regel, which grows in Central Asia with a subtropical climate (Wibowo 2003). High public demand for garlic has caused many farmers to plant this vegetable. However, domestic garlic production has not been able to cover this demand, so garlic imports are still an option. According to *Dinas Pertanian Daerah Istimewa Yogyakarta* (2008), Indonesian garlic imports amounted to 295 thousand tons with a value of not less than US$ 103 million. Increasing domestic awareness to reduce dependence on imported garlic has encouraged the development of garlic in Indonesia. This increase in production can also support the price of garlic in the market.

In general, garlic is only suitable for cultivation in the highlands, although some lowland tolerant varieties are now found. Tawangmangu, Indonesia, is one of the central areas for garlic production. The development of garlic in an area intensively and continuously has a positive impact on increasing farmers' income but also has a negative impact with a significant increase in garlic disease attacks. In garlic cultivation, the disease becomes an important obstacle. Root rot caused by *Fusarium oxysporum f.* sp *cepae* is one of the factors causing garlic yield loss since 1973, both in the field and during storage (Widodo et al. 2008).

Recently, this disease has also become endemic in the garlic production center in Tawangmangu. More than 92% of the area's garlic planting area has been infected with *F. oxysporum* sp. f. *cepae* (Hadiwiyono et al. 2009). Based only on experience, the current farming practice can lead to changes in the genetic character of pathogens that can trigger an explosion of pathogenic attacks. Therefore, there is a need for research on various aspects of the ecology and epidemiology of the disease, including its genetic character and its relationship to the pathogen's virulence.

The phenotype testing and observation showed that the explosion of *F. oxysporum* sp. f. *cepae* in some areas is caused by changes in the genetic virulence character of the pathogen. For example, the continuous cultivation of garlic mixed with shallots and garlic and the intensive use of agrochemicals are thought to be the cause of the explosion of base rot disease in Tawangmangu (Fatawi et al. 2003). In addition, continuous garlic cultivation can also cause changes in the phenotype of *F. oxysporum* sp. f. *cepae*. However, phenotype characterization has a weakness; namely, the phenotype is strongly influenced by the environment, so the characters that appear are often pseudonymous. Therefore, it causes the information obtained to be often biased. Therefore, to know more about changes in phenotype due to changes in genotype, it is necessary to carry out the genetic characterization of the Fusarium pathogen, for example, by DNA fingerprinting.

The molecular characterization of pathogens through DNA fingerprinting is more reliable because it is directly based on the source of genetic character information, namely DNA. One of the DNA fingerprinting methods that have been proven to be highly discriminatory and high reproducibility but inexpensive is random amplified polymorphism DNA (RAPD) analysis (Ruiz et al. 2000). This PCR-RAPD method was used for genetic characterization of *F. oxysporum* f. sp. *cepae* to detect genetic changes.

This study aims to obtain information about the virulence and genetic diversity of *F. oxysporum* f. sp. *cepae* from endemic and non-endemic areas of garlic based on RAPD analysis.

MATERIALS AND METHODS

Research time and place

This research was conducted from August to December 2009 in Indonesia at the Laboratory of Plant Pests and Diseases, Universitas Sebelas Maret, Surakarta, and the Laboratory of Agricultural Biotechnology, Universitas Gadjah Mada, Yogyakarta.

Materials and Tools

The materials used in this study were plants infected with the fungus *Fusarium oxysporum f.* sp. *cepae* garlic, distilled water, lactic acid 25%, furelox 5%, sublimate 0.1%, alcohol 90%, CTAB 2% Tris-HCl 50 mM, Chloroform Isoamyl Alcohol (CIAA), EDTA 100 mM pH 8, NaCl 1.4 M, Mercapto Ethanol 1%, quartz sand, TBE (Tris-Boric-EDTA) 0.5X, Ethidium bromide 0.1%, Mega Mix Royal (MMR), Miliq water, Potato Dextrose Broth (PDB) medium and Potato Dextrose Agar (PDA) medium.

The tools used in this study were a digital camera, cooling flask, microscope, refrigerator, Laminar Air Flow (LAF), autoclave, inoculation needle, ose needle, 250 mL, and 100 mL Erlenmeyer tubes, sterile petridish, denatured alcohol lamp, razor blade, tweezers, beaker, label paper, filter paper, cotton, 1.5 mL Eppendorf tube, electrophoresis bath, oven, PCR tube, and ThermoCycler PCR machine (BioRad).

Surveys and collections of *F. oxysporum* f. sp. *cepae*

The survey was conducted to obtain endemic and non-endemic garlic plantations for root rot disease. The criteria for endemic areas were crops that were attacked by pathogens with more than 30%, while non-endemic areas have a disease intensity of less than 1%. Each of these areas was isolated from *F. oxysporum* f. sp. *cepae*. The survey was conducted by looking for plants that were attacked or had symptoms of root rot attack with the characteristics of the plants drying out, and there was white and rotten mycelium when removed from the tubers. Plants that experience attack symptoms were brought for isolation and collection of pathogens.

The *F. oxysporum* f. sp. *cepae* isolated from plants showing symptoms of infection by cutting necrotic tissue. The tissue pieces were placed on acidified PDA medium by adding 2 mL of 25% lactic acid L-1 and incubated for 7 days (Kim et al. 2001). A total of 32 isolates were collected for identification to obtain isolates with special characteristics. A total of 32 isolates were grouped based on the origin, and isolates were selected for use in the virulence test.

Identification of colonies of *F. oxysporum* f. sp. *cepae* of each isolate was carried out under a microscope and identified as *F. oxysporum* f. sp. *cepae* transferred to the PDA. Pure isolate from *F. oxysporum* f. sp. *cepae* stored in sterile liquid paraffin solution and stored at room temperature as a collection and can be used for virulence testing and genetic diversity analysis. The isolates obtained were then grouped based on the similarity of colony structure, color, and colony growth.

Virulence test

This virulence test used garlic seeds. The test was carried out by 2 methods as follows:

(i) Inoculation of garlic seeds on colonies of *F. oxysporum* f. sp. *cepae* on PDA medium. The garlic seeds to be inoculated were washed with 70% alcohol and rinsed with sterile distilled water, and then the seeds were dried and wiped with a tissue. Garlic seeds were cut on one side with a sterile ose needle. Afterward, the seeds were placed on the colonies of *F. oxysporum* f. sp. *cepae* with the injured side in direct contact with the pathogenic colony. The test was arranged according to a Completely Randomized Design (CRD) with 4 replications. Observations were made 2 weeks after inoculation by observing the intensity of root rot disease in garlic bulbs by measuring the percentage of tuber tissue with rot symptoms and colonized by pathogens. (ii) Inoculation of garlic seeds on soil infested with *F. oxysporum* f.sp. *cepae*. Garlic seeds were planted on soil infested with *F. oxysporum* f. sp. *cepae*. There are 2 varieties of seeds, namely the Chinese garlic variety and the Tawangmangu local variety. The soil was placed in a 10 x 20 cm tray with a soil depth of 5 cm, then watered until the soil was wet. The seeds were cleaned from the dry outer layer of the tubers, washed with 70% alcohol, rinsed with sterile distilled water, then planted in the soil in trays. Half of the tuber was immersed in the soil infested with the pathogen, especially the base of the tuber, to accelerate growth. Treatment was done by watering every morning to maintain soil moisture. The test was arranged in a completely randomized design with 3 replications. Observations were made 2 weeks after inoculation. Observations were made by observing the intensity of disease in the seeds planted by dismantling the planted tubers and then measuring the percentage of tuber tissue showing signs of rot. The percentage of damage was calculated by looking directly at the amount of damage that occurred in the seeds, and the results were converted into a percentage to make it easier to see the virulence. The amount of damage was calculated from the number of parts of garlic rotted or colonized by *F. oxysporum* f. sp. *cepae* and expressed in percent between 0-100%.

Analysis of the results of the virulence test observations using analysis of variance based on the F test at levels of 5% and 1%, if there is a significant difference, then proceed with the DMRT test.

Genetic diversity test of *F. oxysporum* f. sp. *cepae*

Breeding of F. oxysporum *f. sp.* cepae *for DNA extraction*

Selected pathogen isolates tested for virulence were cultured in a PDB medium. DNA extraction was initiated by preparing the culture of *F. oxysporum* f. sp. *cepae* from the existing PDA medium. Preparation for making a PDB medium is to sterilize 50 mL of PDB medium in a 100 mL Erlenmeyer tube for 15 minutes at 121°C. Next, the available cultures of *F. oxysporum* f. sp. *cepae* were put into the PDB medium, which had been cold and incubated in a rotary shaker for 5 days. The fungus that had grown was separated from the PDB medium by centrifugation and then washed with sterile distilled water until no medium was carried away. Next, the fungus suspension that had been cleaned was filtered with filter paper to separate the fungus and liquid. Finally, the fungus was ready for the DNA extraction process with CTAB.

The next process was the extraction of DNA from *F. oxysporum* f. sp. *cepae* with CTAB, starting with weighing as much as 0.5 grams of fungus mycelium. Next, the mycelium fungus was ground in a mortar with the addition of 2% CTAB 300 L and a little quartz sand to make it smooth quickly. Next, the finely ground mycelium was transferred to an Eppendorf tube to be heated in a water bath of 65°C for 30 minutes and then shaken for 10 minutes. Next, the Eppendorf tube was centrifuged for 5 minutes at a speed of 5000 rotations, the supernatant (top clear liquid) was transferred to another Eppendorf tube, and the pellet was left in the tube.

The precipitate or pellet in the Eppendorf tube was added with CIAA until the tube was full and shaken until homogeneous, then centrifuged at 12000 rotations for 10 minutes, and the supernatant contained in the tube was transferred to another Eppendorf tube while the pellet was left in an Eppendorf tube. The pellets were then added with absolute ethanol/alcohol until the tube was full and stored in a refrigerator at -20°C for 1-3 hours.

The alcohol (supernatant) was discarded, and the pellet was diluted again with 70% alcohol until the tube was full. The Eppendorf tube was centrifuged at 12000 rotations for 10 minutes. The alcohol (supernatant) was discarded, and the DNA pellet was dried in LAF for ± 2 hours. The DNA pellet was dissolved in a suspension of Miliq water and stored in a refrigerator at -20°C. Extraction results were seen by electrophoresis on 0.8% gel with ethidium bromide dye to ensure the presence of DNA in the pellet.

PCR-RAPD

The DNA amplification fingerprinting (DAF) system was carried out according to Bentley and Bassam (1996) with minor modifications, including amplification reactions by PCR, thermal cycle conditions, and electrophoresis to be carried out. The primary sequence used in the RAPD analysis was 5"-GATGAGCC-3" (Bentley et al. 1998).

The PCR kit used was Mega Mix Royal (MMR). In one PCR process, 5 µL of MMR and other mixtures, namely 5 µL Primer, 2 µL template, and 8 µL Miliq water were needed. PCR-RAPD was performed using a Thermal cycler (BioRad) machine. The extracted DNA would be directly carried out by the PCR process in a PCR machine with an initiation program of denaturation at a temperature of 94°C for 5 minutes for 1 time, denaturation at a temperature of 94°C for 30 seconds for 30 times, annealing (separation) at a temperature of 35°C for 30 seconds for 30 times, extensions at 72°C for 30 seconds for 30 times, final extensions at 72°C for 5 minutes for 30 times.

The PCR program process ended with equilibration at a temperature of 20°C until the process ended on its own. After all processes end, the DNA from the PCR-RAPD would be directly electrophoresed or stored at 4°C until electrophoresis was carried out.

Electrophoresis for visualizing PCR-RAPD results

Electrophoresis began with the manufacture of an electrophoretic gel. Preparation began with weighing 0.8% agarose as much as 0.325 g (for 40 mL TBE gel molds with 17 gel wells) and heated in the oven for a maximum of 3 minutes. After it was not too hot, 40 µL of 0.1% ethidium bromide was added, and the agarose was shaken until homogeneous and then poured into a mold fitted with a perforation comb. After more than 20 minutes or hardened, the comb was removed. Next, the gel with the gel mold base was transferred to an electrophoresis bath to be used in the electrophoresis process.

The tub was filled with 0.5X TBE until the gel was immersed in the solution. Then, each gel well was filled with 5-20 µL of PCR result DNA, and the final well was filled with 10 µL of marker (DNA marker). Finally, the electrophoresis machine was closed, and the power supply was turned on at 100 volts for 35 minutes. After the electrophoresis process was complete, the DNA fragment pattern was seen, and the characteristics of the DNA fragment similarity pattern were tested using the NTYSIS program.

Analysis of PCR-RAPD results

The similarity analysis of DNA fragment patterns was analyzed with the NTYSIS software. In addition, analysis of gel electrophoresis data was carried out using FreeTree software (Hampl et al. 2001) with the Unweighted Pair Group Method with Arithmetic mean (UPGMA) method to determine the similarity of patterns between isolates and grouping and relationships between isolates in the form of a dendrogram. The similarity pattern was between 0.0-1 (0-100%) based on the numbers that appear in the NTYSIS program and the UPGMA method.

RESULTS AND DISCUSSION

***Fusarium* survey results and collection**

The survey results showed that endemic garlic plantations were from the Gondosuli area, while non-endemic garlic plantations were from the Blumbang area. The results of the isolation of *F. oxysporum* f. sp. *cepae* from the two areas produced 32 isolates. A number of these isolates were grouped into 8 groups based on growth structure and colony color with extracellular. Each group was represented by 1 isolate to test for virulence and genetic diversity. Those 8 isolates consisted of 4 from endemic areas and 4 from non-endemic areas (Table 1).

Table 1. Isolates of *F. oxysporum* f. sp. *cepae* used for virulence and DNA characteristics testing

Isolates code	Origin	Description
FCp1	Gondosuli	Endemic
FCp2	Gondosuli	Endemic
FCp3	Gondosuli	Endemic
FCp4	Gondosuli	Endemic
FCp5	Blumbang	Non-endemic
FCp6	Blumbang	Non-endemic
FCp7	Blumbang	Non-endemic
FCp8	Blumbang	Non-endemic

Virulence test results

Inoculation of garlic seeds on colonies of F. oxysporum *f. sp.* cepae *on PDA medium*

The inoculation of garlic seeds on colonies of *F. oxysporum* f. sp. *cepae* (Figure 1) showed that the intensity of disease attack between isolates on garlic seeds was less varied. All existing isolates caused disease intensity or were virulent, while for controls, there were no damage symptoms. In addition, all isolates caused a high average disease intensity, which was more than 75% (Figure 2).

Based on the intensity of disease in the PRC varieties, it can be concluded that the virulence between isolates of *F. oxysporum* f. sp. *cepae* varies. However, if it is associated with the area of origin, the grouping is not related to the disease status of the area of origin. Not all isolates from endemic areas showed high virulence; vice versa, not all isolates from non-endemic areas showed low virulence. For example, FCp1 isolates from endemic areas were not virulent, with a disease intensity of 0.00%. On the other hand, FCp5 isolates from non-endemic areas showed the highest virulence by showing the highest disease intensity, 100%.

Different results were shown by inoculation of garlic seeds on soil infested with *F. oxysporum* f. sp. *cepae* with local varieties of garlic Tawangmangu which results are less varied. All pathogen isolates can cause disease intensity.

Inoculation of garlic seeds on soil infested with F. oxysporum *f. sp.* cepae *with local varieties of garlic Tawangmangu*

Local varieties of garlic Tawangmangu is one type of onion widely cultivated in Tawangmangu. Still, this variety is only a local superior, so it is not widely known and cultivated in other areas. The testing of the Tawangmangu Local variety was also carried out in the same way as the PRC variety. It was used as a comparison to find out how far the virulence caused by the various isolates was. Results of inoculation of garlic seeds on soil infested with *F. oxysporum* f. sp. *cepae* with local varieties of garlic Tawangmangu showed less varied results. The highest disease intensity was caused by FCp4 isolates, which was 73.33% from endemic areas, but isolates from non-endemic areas, namely FCp5 and FCp7 isolates, also caused quite serious damage, reaching more than 60% (Figure 3).

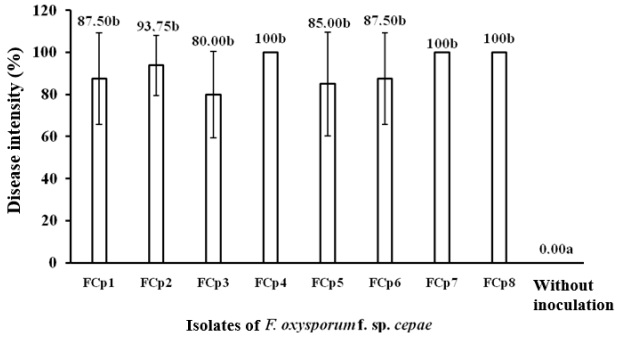


Figure 1. The average disease intensity of garlic seeds over a colony of 8 isolates of *F. oxysporum* f. sp. *cepae* on a PDA medium in a petri dish. Note: Numbers followed by the same letter show no significant difference based on DMRT 5% based on data transformed to Arc sin X

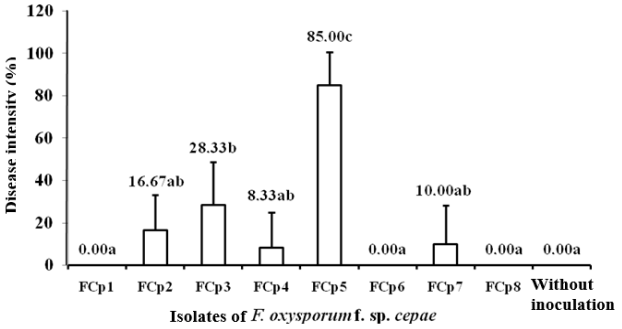


Figure 2. The average percentage of disease intensity in garlic seeds of PRC varieties grown on soil infested with *F. oxysporum* f. sp. *cepae.* Note: Numbers followed by the same letter show no significant difference based on DMRT 5% based on data transformed to Arc sin X

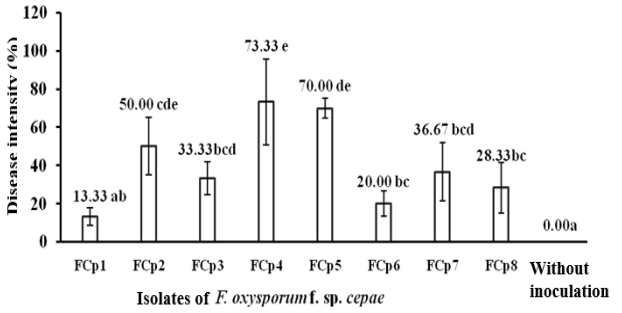


Figure 3. The average percentage of disease intensity in local varieties of garlic seeds Tawangmangu grown on soil infested with *F. oxysporum* f. sp. *Cepae.* Note: Numbers followed by the same letter show no significant difference based on DMRT 5% based on data transformed to Arc sin X

This result was different from inoculation using the PRC variety. It is thought to be caused by all the isolates that have long been associated with the Tawangmangu local variety so that the fungus is adaptive as a pathogen in that variety. The higher attack of *F. oxysporum* f sp *cepae* on the Tawangmangu local variety compared to the PRC variety was different from the results of field observations

which showed the opposite, namely, the Tawangmangu local variety was more resistant (Hadiwiyono 2004). This difference in results is thought to be caused by different environmental conditions; namely, the virulence test was carried out in the lowlands, 110 m above sea level (asl), while the Tawangmangu field was above 600 m asl. Environmental differences, especially temperature due to altitude differences, can affect the ability of pathogen infection and plant resistance. In addition, plants that grow outside their ecological area can cause crop stress. Environmental stress in plants can predispose them to pathogen infection by weak parasites such as *F. oxysporum* f. sp. *cepae* (Agrios 2005).

Genetic diversity test of *F. oxysporum* f. sp. *cepae*

The results of the visualization of DNA fragments through PCR-RAPD are shown in Figure 4. The length of the fragments ranges from 80 to 200 base pairs (bp). The dendrogram (Figure 5) shows that the coefficient of genetic similarity between the sample isolates is between 0.25-1.00 (25%-100%). At the coefficient value of 25%, it was divided into 2 groups of strains, namely group 1 (FCp3, FCp5, FCp6, FCp8) and group 2 (FCp1, FCp4, FCp2, and FCp7), so that the overall isolate group was divided into 3 isolate strains, namely strain 1 (FCp1 and FCp4), strain 2 (FCp2 and FCp7), and strain 3 (FCp3, FCp5, FCp6, and FCp8). Of the three strains, only strain 1 (FCp1 and FCp4) and strain 2 (FCp2 and FCp7) had the closest relationship of 51%, while the highest relationship of 100% was found in each strain. The furthest kinship between strains 1, 2, and 3 was only 25%.

Analysis of the level of genetic diversity of 8 isolates based on RAPD analysis had a coefficient of genetic similarity of 25% or genetic variation of 75%. It proves that there has been a genetic change in *F. oxysporum* f. sp. *cepae* with high genetic variation due to the continuous cultivation of garlic. These results are also in line with studies on isolates of *F. oxysporum* f. sp. *cubense* with RAPD-PCR, which shows very high genetic diversity with a similarity level of 0.25-0.95 (25%-95%) and can be grouped into 4 groups (Anonymous 2008).

Based on the virulence test, there is no significant difference in the damage caused by various isolates between endemic and non-endemic areas. These results are also seen in the DNA pattern of PCR-RAPD. There is no difference in the pattern of DNA fragments in endemic and non-endemic areas, but from these results, 3 groups of DNA strains can be obtained (Figure 5). The grouping between lines does not include differences between endemic and non-endemic areas. In line 1, all isolates are isolates from endemic areas; in line 2, there are isolates from endemic areas (FCp2 isolates) and non-endemic (FCp7 isolates); while strain 3 is also a mixture of isolates from endemic areas (FCp3 isolates) and non-endemic areas. (FCp5, FCp6, and FCp8) isolates. It proves that there is genetic diversity in *F. oxysporum* f. sp. *cepae* isolated from Tawangmangu. However, the genetic diversity of these pathogens is not related to the disease status of the area of origin of the isolates.

The results of this study indicate that the virulence of the pathogen is not the only factor that causes endemic disease or the low intensity of base rot disease in certain fields. According to Hadiwiyono and Widono (2008) and Hadiwoyono et al. (2008; 2009), the root rot of garlic in Tawangmangu is determined by the physical, chemical, and biological characteristics of the soil. The high content of organic matter and N and low P and K causes an increase in the intensity of garlic root rot. In addition, non-endemic soils have a higher microbial population (fungi, bacteria, and actinomycetes) than endemic soils.

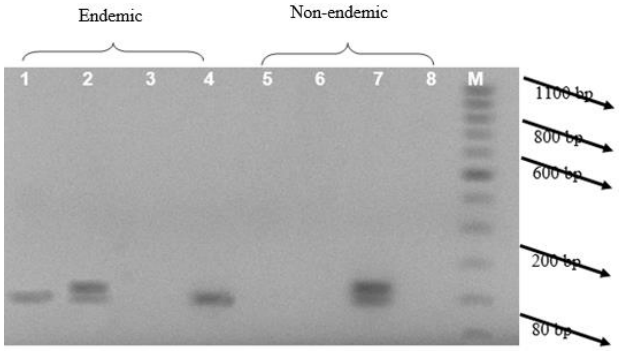


Figure 4. The pattern of DNA fragments of *F. oxysporum* f. sp. *cepae* via PCR-RAPD. Note: 1: FCp1 isolate, 2: FCp2 isolate, 3: FCp3 isolate, 4: FCp4 isolate, 5: FCp5 isolate, 6: FCp6 isolate, 7: FCp7 isolate, 8: FCp8 isolate, and M: Marker

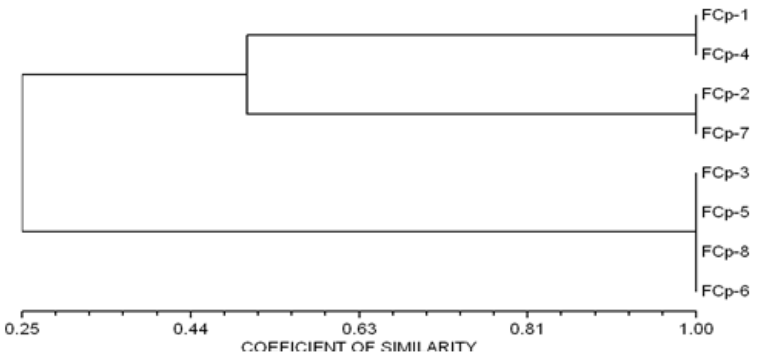


Figure 5. Dendrogram-UPGMA based on the pattern of DNA fragments from PCR-RAPD results in 8 isolates of *F. oxysporum* f. sp. *cepae* (Coefficient of similarity)

In conclusion, the PCR-RAPD analysis of *F. oxysporum* f. sp. *cepae* could be grouped into 3 strains. Still, the grouping is unrelated to the virulence and origin of endemic or non-endemic isolates.

REFERENCES

Agrios GN. 2005. Plant Pathology. 4th Ed. Academic Press, San Diego, California.

Anonim. 2008. Variasi genetik isolat-isolat *Fusarium oxysporum* f. sp. *cubense*. Badan Litbang Pertanian. www. hortikultura.litbang.deptan.go.id. Diakses: 10 Juni 2010 (*Abstr*.). [Indonesian]

Bentley S, Bassam BJ. 1996. A rabust DNA aplification fingerprinting system applied to analysis of genetic variation within *Fusarium oxysporum* f.sp. *cubense*. J Phytopathol 144: 207-213. DOI: 10.1111/j.1439-0434.1996.tb01516.x.

Bentley S, Pegg KG, Moore NY, Davis RD, Buddenhagen IW. 1998. Genetic variation among vegetative compatibility gruops of *Fusarium oxysporum* f. sp. *cubense* analyzed by DNA finger printing. J Phythopathol 88: 1283-1293. DOI: 10.1094/PHYTO.1998.88.12.1283.

Dinas Pertanian Provinsi Daerah Istimewa Yogyakarta. 2008. Bawang Putih Dataran Rendah. Bidang Produksi Tanaman Pangan dan Hortikultura Seksi Sayuran dan Aneka Tanaman, Dinas Pertanian Provinsi Daerah Istimewa Yogyakarta. [Indonesian]

Fatawi ZD, Gutomo HS, Hadiwiyono. 2003. Studi Lini Dasar Terjadinya Epidemi Penyakit Busuk Pangkal Bawang Putih di Tawangmangu. Laporan Hasil Penelitian Sumber Dana DUE-Like TA.2003. Fakultas Pertanian, Universitas Sebelas Maret, Surakarta. [Indonesian]

Hadiwiyono H, Widono S, Fatawi ZD, Novianti N. 2008. Analisis hubungan keharaan N, P, dan K tanah terhadap intensitas penyakit busuk pangkal (*Fusarium oxysporum* f. sp. *cepae*) pada bawang putih. Agrosains 10 (1): 21-24. [Indonesian]

Hadiwiyono H, Widono. 2008. Hubungan faktor lingkungan tanah terhadap intensitas busuk pangkal bawang putih di Tawangmangu. Agrin 12 (1): 15-22. [Indonesian]

Hadiwiyono H, Wuspada RD, Widono S, Poromarto SH, Fatawi ZD. 2009. Kesupresifan tanah terhadap busuk pangkal (*Fusarium oxysporum* f.sp.*cepae*) bawang putih di Tawangmangu, Karanganyar. Sains Tanah 6 (1):1-6. [Indonesian]

Hadiwiyono H. 2004. Penyakit-Penyakit Tanaman Hortikultura di Indonesia. Universitas Gadjah Mada Press, Yogyakarta. [Indonesian]

Hampl V, Pavlicek A, Flegr J. 2001. Construction and bootstrap analysis of DNA fingerprinting-based phylogenetic trees with the freeware program FreeTree: Application to trichomonad parasites. Intl J Syst Evol Microbiol 51: 731-735. DOI: 10.1094/PHYTO.1998.88.12.1283.

Kim JT, Park IH, Hahm YH, Yu SH. 2001. Crown and root rot of greenhouse tomato caused by *Fusarium oxysporum* f.sp. *radicis-lycopersic*i in Korea. Plant Pathol J 17 (5): 290-294.

Ruiz RA, Vacek DC, Parker PE, Wendel LE, Schaffner U, Sobhihan R, Richard RD. 2000. Using randomly amplified polymorphic DNA polymerase chain reaction (RAPD-PCR) to match natural enemies to their host plant. In: Proceedings of the X International Symposium on Biological Control of Weeds.

Wibowo S. 2003. Budidaya Bawang. Penebar Swadaya, Jakarta. [Indonesian]

Widodo, Kondo N, Kobayashi K, Ogoshi A. 2008. Vegetative compatibility groups within *Fusarium oxysporum* f. sp. *cepae* in Hokkaido-Japan. J Mikrobiol Indones 2 (1): 39-43. DOI: 10.5454/mi.2.1.8.