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Metagenomic-based Approach for the Analysis of Yeast Diversity Associated with Amylase Production in Lai (*Durio kutejensis*)

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Abstract

This study reported the application of a next generation sequencing (NGS) analysis of yeast diversity in native Indonesian fruit, *Durio kutejensis*, collected from Borneo, Central Kalimantan. The analysis was designed to observe the microbial consortium associated with solid state fermentation (SSF) for amylase production. Together with the additional data from culture-dependent analysis, we observed the morphological features, molecular characteristics, and amylase concentration produced by each isolate. We performed Solid State Fermentation (SSF) for amylase production and the enzyme activity was then determined using UV-Vis spectrophotometer at 540 nm. Result obtained from metagenomic approach consist of 4 group that fungal species included in the Ascomycota identified as *Botryosphaeria dothidea* (1.35%), *Lasiodiplodia crassispora* (17.62%), *Aureobasidium pullulans* (55.02%), *Paraphoma chrysanthemicola* (11.38%), *Preussia funiculate* (1.90%), *Sporormiella intermedia* (0.82%), *Myrothecium gramineum* (1.35%), *Fusarium oxysporum* (6.24%), *Fusarium proliferatum* (3.25%) and *Phialemoniopsis curvata* (1.08%). The results of isolation using culturable medium in the form of YMA obtained 40 yeast isolates. A total of 40 representative isolates from durian fruit were screened, two positive amylase isolates based on clear zones formed were DU 4.2 (*Candida sorboxylosa*) and DU4.22 (*Cyberlindnera fabianii*) isolates with amylolytic index of DU 4.2 isolates at 0.24 and DU 4.22 at 0.72 with an incubation time of 48 h. The highest amylase enzyme activity was found in isolate DU 4.2 of 31.21 U / mL.

Keywords: Yeast diversity, *Durio kutejensis*, Metagenomic, Amylase production, Solid State Fermentation (SSF)

INTRODUCTION

Durian is a tropical fruit native to Southeast Asia, especially Indonesia, regarded as the "King of Fruits" due to its distinctive (large, covered by spines) shape and rich flavor. Scientifically, durian belongs to the group of Malvaceae, family Bombacaceae and genus Durio. Kostermans[1] and David[2] have recorded 27 species of durian, with the total of 19 species are found in Kalimantan, Indonesia and 11species in Malacca Peninsula, 7 species in Sumatra and 1 species in Myanmar. Among all, only 7 species are edible for human, which are *Durio zibethinus* (durian), *Durio kutejensis* (lai), *Durio oxleyanus* (kerantongan), *Durio dulcis* (lahong), *Durio graveolens* (labelak), *Durio grandiflorus* (durian monkey), and *Durio testinarium* (turtle durian)[3,4]. *Durio zibethinus* is the most widely cultivated by Indonesians because of its delicious taste and smooth texture. One of Indonesia's original fruits from East Kalimantan is Lai Durian (*Durio kutejensis*)[5] which has drier pulp, yellow-orange with distinctive aroma (not like durian) and less pungent[6]. However, information about Lai Durian is still limited.

Lai cultivars were reported to have less diverse sulfurs and esters as compared to the other durian, which were most probably the key reason for the different aroma characteristics[8]. Because of its distinctive flavor and aroma, lai becomes one of Indonesia's tropical fruit commodities which is potentially developed as raw materials for fermented food and beverages, and have large domestic market opportunities and exports, especially in pharmaceutical industry[10],. Besides aromatic compounds (Methyl acetate, Ethyl acetate, Methyl propionate, Ethyl propionate, n-Propyl propionate, Ethyl iso-butyrate, Ethyl butyrate, Methyl-methylbutyrate), ester compounds (Ethyl-2-methylbutanoate, Ethyl iso-butyrate, Ethyl butyrate, Methyl-methylbutyrate), ether compounds (Ethyl-2-methylbutanoate, volatile Ethyl acetate), and Sulphur compounds (Hydrogen sulphide, Methanethiol, Ethanethiol, Propanethiol)[7]. Lai consists of biomass containing hemicelluloses, and lignin which can be used as carbohydrate resources[5].

Lai Durian (*Durio kutejensis*) with its distinctive flavor and aroma becomes one of Indonesia's tropical fruit commodities which is potentially to be developed as raw materials for fermented food and drinks and have large domestic market opportunities and exports[8]. The main content of durian seeds is starch and protein[9]. Some researches on starch contained in durian seed have been carried out, including characterization[10], hydrolysis with acids, and its application to as raw materials of nuggets and even bioplastic material. Like all durian seeds,

Lai seeds also have the same source of starch, but the extraction of starch from its flesh and the measurement of functional properties have not been explored further.

The presence of chemical compounds in lai have been reported to be associated with endophytic microorganisms exist in the fruit. The physicochemical, microbial diversity and sensory profile of lai changes during post-harvest because the natural fermentation occurs. During fermentation, yeasts are responsible for material degradation from complex into simpler compounds. Yeasts like *Saccharomyces* spp. have been successfully isolated from durian fruit (*Durio zhibetinus*) and was used as starter inoculum for bioethanol production from cassava (*Manihot esculentao*[18]. Yeasts are single-celled microorganisms that can be found in various kind of fruits, such as *Aureubasidium pullulans* in grapes[12]; found in jackfruit[13] with amylase enzyme activity (0.88 U/ mL); *Pichia* sp. from apple (*Malus domestica*) was also found[14]. Yeasts has been found to produce various enzymes for many industrial applications[15-17]. Yeasts like *Saccharomyces* spp. have been successfully isolated from durian fruit (*Durio zhibetinus*) and used as starter inoculum for bioethanol production from cassava (*Manihot esculenta*)[18].

Metagenomics using next-generation sequencing is a technology that enables massive and parallel DNA sequencing. Called high throughput-sequencing, which means that in one time, this technology can run millions of DNA sequences from one sample of the same environment in a relatively short time. By using metagenomics, data can be gathered to develop a profile of yeast diversity and taxonomy[19] In this study, we use metagenomics-based approach to obtain information of yeast diversity profile in post-harvested lai which might be associated with the metabolite secondary production, in this case is amylase enzyme[20]. Amylase enzymes can be obtained during fermentation done by yeast[21,22] α-amylase enzyme is one of the enzymes used in the enzymatic hydrolysis process to convert starch to glucose. This enzyme cuts the α-1,4-glucoside bond specifically at a certain point to form dextrin[11].

In this study, we also support the data with the rational reason for production optimization of amylase enzyme in Solid State Fermentation (SSF) by the culture-dependent[24]. This method has been widely used for optimal cultivation in low water activity condition[24,25]. We expect that this study will provide information about yeast diversity of lai and recommendation for alternative source of amylase enzyme for industrial needs. We hope that the result will be reliable and reproducible as guidance for experiments further.

MATERIALS AND METHODS

Yeast isolation and total genomic extraction of Lai Durian (*Durio kutejensis*). A fresh-harvested lai fruit was collected from Desa Bukit Sawit, Central Kalimantan, Indonesia, and lai with specific criteria of good ripening, no deformed husks and normal odor and texture of pulp, were used in this study. Lai with the code of DU4 are chosen to be performed for yeast diversity analysis of whole pulp content using metagenomics approach. 0.5 g of Durian pulp was collected aseptically and was put into a conical tube. Total genomic DNA from samples were extracted using CTAB/SDS method. DNA concentration and purity was monitored on 1% agarose gel. The extracted DNA was then diluted to reach the concentration of 1 ng/μL using sterile water. The rRNA/ITS genes of distinct regions. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). The PCR Products confirmation was done by mixing it with 1×loading buffer (contained SYB green) and was run for electrophoresis on 2% agarose gel for detection. The PCR products was followed by purification with Gene JETTM Gel Extraction Kit (Thermo Scientific). The reads were compared with the reference database (Silva database, https://www.arb-silva.de/) using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html) to remove the chimera sequences [The clean reads were finally obtained and the species annotation for each representative sequence was compared to the Silva Database (https://www.arb-silva.de/) based on Mothur algorithm to annotate taxonomic information. Phylogenetic relationship construction to obtain yeast diversity of different OTUs and the pre-dominant species in the sample was conducted using multiple sequence alignment by the MUSCLE software (Version 3.8.31 http://www.drive5.com/muscle/).

A fresh Lai fruit was collected from Desa Bukit Sawit, Central Kalimantan, Indonesia, and Lai durian with the code DU4 are chosen to perform yeast diversity analysis of whole pulp content using metagenomics approach. Durian with specific criteria such as good ripening, no deformed husks and normal odor and texture of pulp were used in this study. 0.5 g of Durian pulp was collected aseptically and was put into a conical tube. Total genomic DNA from samples were extracted using CTAB/SDS method. DNA concentration and purity was monitored on 1% agarose gel. According to the concentration, DNA was diluted to 1 ng/µL using sterile water. Amplicon Generation 16S rRNA/18S rRNA/ITS genes of distinct regions (16S V4/16S V3/16S V3-V4/16S V4-V5, 18S V4/18S V9, ITS1/ITS2, Arc V4) were amplified used specific primer (e.g. 16S V4: 515F-806R, 18S V4: 528F-706R, 18S V9: 1380F-1510R, et. al) with the barcode. All PCR reactions were carried out with Phusion® High-Fidelity PCR Master Mix (New England Biolabs). PCR Products Mixing and Purification Mix same volume of 1×loading buffer (contained SYB green) with PCR products and operate electrophoresis on 2% agarose gel for detection. PCR products was mixed in equidensity ratios. Then, mixture PCR products was purified with GeneJETTM Gel Extraction Kit (Thermo Scientific). The reads were compared with the reference database (Silva database, https://www.arb-silva.de/) [26] [2] using UCHIME algorithm (UCHIME Algorithm, http://www.drive5.com/usearch/manual/uchime_algo.html)[27,3] to detect chimera sequences, and then the chimera sequences were removed[28,4]. Then the Clean Reads finally obtained. Species annotation for each representative sequence, the Silva Database (https://www.arb-silva.de/)[26,2] was used based on Mothur algorithm to annotate taxonomic information. Phylogenetic relationship Construction In order to study phylogenetic relationship of different OTUs, and the difference of the dominant species in different samples (groups), multiple sequence alignment were conducted using the MUSCLE software (Version 3.8.31 http://www.drive5.com/muscle/).

Yeast isolation from *Durio kutejensis* using culture-dependent analysis

Lai with the code DU4 were collected to perform yeast isolation using culture-dependent analysis. Yeast isolation was done by inserting pieces of durian flesh into Erlenmeyer flask containing 45 mL of MEB medium and homogenized for 24 h. 0.1 mL of durian suspension and was diluted by 9.9 mL of sterile distilled water (10^{-2} dilution), followed by serial dilution until 10^{-6}. 0.1 mL of 10^{-6} dilution was put into petri dish containing YMA pH 4 by spread technique. The pH was adjusted by adding 0.5 mL of 35% HCL to 300 ml of Yeast Malt Agar (YMA) media. The sample solution was flattened using the Drygalski spatula and incubated for 24 h at room temperature. The growing colonies were then purified to obtain pure culture on the YMA pH 4 medium with the quadrant streak method, which was incubated for 24-48h at room temperature; and carried out 2-3 times transfer until a single colony was obtained. The growing colonies of the yeast isolates were observed macroscopically after 48h as described by morphological characters such as color, texture, profile and margin were noted refereed to Sukmawati et al.[29].

Screening of Potential Amylase-Producing Yeast Isolates

Qualitative assay for amylolytic activity was done by diffusion method [30. A total of 28 UNJCC yeast isolates from Lai durian fruit were spread by streak method to the YMA (Yeast Malt Agar) medium and was followed by incubation at 30°C for 72 h. Each isolate was then inoculated into YPSA (Yeast Peptone Starch Agar) medium with 1% starch addition and making wells. The wells were made using sterile straws on YPSA media which has been divided into 8 quadrants with 2 replications in each quadrant. One pick of each yeast isolates was collected and homogenized with 0.9 mL of sterile distilled water in Eppendorf tube. About 20µL of the yeast suspension was inoculated into YPSA media and was incubated at 30°C for 72 h. Amylase activity can be seen by the presence of clear zone around the colony on YPSA media after the addition of Lugol drops. The clear zone was then measured using analytic calipers. Amylolytic index was calculated[31].

Inoculum and Medium Preparation for Solid State Fermentation (SSF)

The positive yeast isolates (DU4.2 and DU4.22) were inoculated into YMA slant agar medium by 15 streak methods, followed by incubation at room temperature for 48 h. The two isolates were then picked and put into 5 mL of distilled water, followed by homogenization using

vortex. A total of 10% (v/w) of yeast suspension (0.5 mL in 5 grams) was put into a sterile fermentation medium. Fermentation medium composition was made in Erlenmeyer with 70% humidity as follows: 5 gram of wheat husk; 3.5 mL of nutrients (1% starch, 5 g peptone, 2 g yeast extract, 0.1 g CaCl2, 0.5 g KH2PO4 and 200 mg chloramphenicol) were dissolved in 1000 ml Mc'Ilvaine buffer (pH 3 - 5)[24]. The media is then homogenized using rod stirrer.

Optimization of Amylase Enzyme Extraction

Amylase enzyme extraction was carried out by solid state fermentation (SSF). Fermentation was run in incubator for 24h, 48h, 72h, and 96h at room temperature (28°C). Production of crude amylase enzyme extracts was carried out by adding 50 ml of sterile distilled water and agitation at 150 rpm rotator shaker for an hour (Oliveira et al. 2015). The medium was filtered using muslin cloth and Whattman paper and the crude enzyme extract were then put into the Falcon tube. The extract was centrifuged for 10 minutes at 3000xg and 4°C. The results obtained are in the form of filtrate (crude enzyme extract) and its sediment. The filtrate was expected to be α-amylase crude extract and collected for enzyme activity test based on Yalcin and Corbaci (2014) method. 0.5 ml of enzyme crude extract were placed in test tube and added with 0.5 ml of 1% starch solution containing Mcllavaine pH 4 buffer. The reaction was run in water bath with temperature of 60°C for 10 minutes. 1 ml of 3,5-dinitrosalicylate (DNS) reagent was then added to the tube. The enzymatic reaction was stopped by heating at 100°C for 5 minutes. The addition of 8 ml of sterile distilled water was carried out into the sample using pipette

Quantitative Amylolytic Activity Assay of Yeast Isolates

Quantitative amylolytic activity assay was performed based on absorbance using OD (Optical Density) spectrophotometer at wavelength of 540 nm. Amylase enzyme activity of each sample was tested three times based on[33] method. Calculation of enzyme activity uses the formula[34]:

Molecular Identification of Isolate DU 4.2 and DU 4.22

Among 28 yeast isolates, isolate DU4.2 and isolate DU4.22 showed amylolytic activity and therefore were collected for molecular identification. The DNA yeast was extracted using the Genetic Plant Gneaid kit. DNA was amplified based on D1/D2 rDNA region using the following primers: forward- ITS5 (5'-GGAAGTAAAAGTCGTAACAAGG-3') and reverse-ITS4 (5'TCCTCCGCTTATTGATATGC-3'). PCR cycling conditions for yeasts consisted of an initial denaturation step at 95°C for 2 min; 33 cycles of denaturation at 95°C for 15 sec, annealing at 58°C for 30 sec and extension at 68°C for 2 min; and a final extension at 68◦C for 10 min. The PCR product was purified with first base service. The sequences were aligned and compared with the NCBI database by the Internet using Basic Local Alignment Search Tool. Phylogenetic trees were made using MEGA 5 software with the Neighbor Joining (NJ) method. Phylogenetic trees are used to determine kinship relationships between species sampled with various other species. Molecular phylogenetic combines molecular biology techniques with statistics to reconstruct phylogenetic relationships.

RESULT AND DISCUSSION

Yeast Diversity Information of Lai Durian Using Metagenomic Approach In this study, yeast diversity information was obtained based on ITS4 specific gene which is known to be conserve region of fungal groups. Next-Generation Sequencing (NGS) technology makes it possible to sequence millions of DNA fragments in one analysis at a relatively low cost compared to Sanger's sequencing technology. Metagenome analysis was performed to see the diversity of culturable and unculturable microorganisms[35]. The sample of Lai durian with the code of DU4 was used for metagenomics analysis. This sample was chosen that is in good condition, without any damage and disease seen. The result of showed that only group of Ascomycota is present in the fruit with two genera consisting of four clade classes based on OTU analysis, which are Dothideomycetes, Eurotiomycetes, Leotiomycetes, and Sordariomycetes (Fig. 1). The class of Sordariomycetes dominated the yeast community, consisting of *Botryosphaeria, Lasiodiplodia, Aureobasidium, Paraphoma, Preussia, Sporormiella*. Lim T.K has reported some lesser known mycoflora of durian in Malaysia which are black mildew Ascomycetes species, *Meliola durionis*, and 7 sooty mould species:

3 from the Deuteromycetes and 4 from the Ascomycetes. *Polychaeton* sp., *Leptoxyphium* sp. and *Tripospermum* sp. belong to the former class while *Scorias spongiosa*, *Phragmocapnias betle*, *Trichomerium grandisporum*, and *Trichopeltheca asiatica* to the latter[36].

The dominant yeast present in durian fruit and its ability to produce volatile sulfur compounds (VSCs) has been found[37]. Durian is rich in carbohydrates, proteins, and fats relative to other fruits, allowing yeasts and molds to grow and develop. *Botryosphaeria dothidea* is a pathogenic yeast found in postharvest durian which is also found in fruits such as olives[38]. These yeast also produce volatile compounds such as fatty acids, esters, sterols and fatty acid methyl esters were some types of compounds recovered. Furthermore, the antioxidant capacity was measured, with the most promising result as 38.4±3.1 µmol TEAC/µg extract[39]. *Lasiodiplodia crassispora*, the species belongs to the family group of Botryosphaeriaceae, was also found, which can cause diseases in fruits such as fruit rot, leaf spots, dieback, cankers and root rot of Angiosperms and Gymnosperms worldwide[40].

As shown in Fig. 2, 10 species belong to the group of Ascomycota were identified as *Botryosphaeria dothidea* (0.008%,1.35%), *Lasiodiplodia crassispora* (0.106%,17.62%); *Aureobasidium pullulans* (0.331%,55.02%), *Paraphoma chrysanthemicola* (0.068%,11.38%), *Preussia funiculate* (0.011%,1.90%), *Sporormiella intermedia* (0.005%,0.82%), *Myrothecium gramineum* (0.008%,1.35%), *Fusarium oxysporum* (0.037%,6.24%), *Fusarium proliferatum* (0.019%,3.25%), and *Phialemoniopsis curvata* (0.006%,1.08%). From the result obtained, it can be seen that *Aureobasidium pullulans* accounted for most Ascomycota phylum present in Lai durian. *Aureobasidium pullulans* is a ubiquitous black, yeast-like fungus that can be found in different environments (e.g. soil, water, air and limestone). The discovery of some yeasts and molds in this study provides new information regarding the role and function of these microorganisms on durian fruit. The dominance of yeast *A. pullulans* benefits both fruit and human health. *Aureobasidium pullulans* plays an important role in the process of the food industry and the biocontrol of pathogenic molds in post-harvest fruits. Yeast like fungi *A.*

Fig. 1. Yeast diversity information of Lai durian (*Durio kutejensis*) collected from Desa Bukit Sawit, Central Kalimantan, Indonesia, using metagenomic approach based on ITS4 gene. Only Ascomycota was present and there are four large phylum exist with the domination of Sordariomycetes (blue).

pullulans has ability to produce amylase enzymes[41]; producing α-amylase[42] acting as biocontrol agent controlling *Fusarium* sp.[43]; found in grapes[44]. The biocontrol activity of *Aureobasidium pullulans* has been reported in some researches; *A. pullulans* multiplied rapidly and controlled decay caused by either *B. cinerea* or *P. expansum*, reducing the incidence of gray and blue mold of apple by 89 and 67%, respectively, compared to the water-treated control[45]. *Aureobasidium pullulans* strain Ach1-1 was also successfully isolated for its biocontrol effectiveness against *Penicillium expansum*, the causal agent of blue mold on harvested apples[46]. Yeast species such as *Saccharomyces cerevisae, Saccharomyces blourdeous, Zygosaccharomyces fermentatii, Candida sorboxylosa, Zygosaccharomyce bisporus, Saccharomyces blourdeous,* can be found in many local fruit in Indonesia[47].

Culture-dependent Analysis of Yeasts Isolated from Lai Durian

The results showed that from 3 collected Lai durian, 28 yeast isolates were successfully isolated (with code DU4) (Table 1). Based on morphological observation, all yeast isolated from Lai durian have white colony with mucoid and butyrous texture. The mucoid texture of the yeast was due to the presence of polysaccharide substances contained in the extracellular component of yeast[48]. Some yeast is covered by extracellular components in the form of slimy polysaccharides and heteropolysaccharides[49]. Research by Nyanga et al. reported that yeasts obtained from Masau (*Ziziphus mauritiana*) ripe have white to creamy colonies[50]. Research by Lentz et al. obtained all yeast isolates from Pindo palm fruit (*Butia capitata*), loquat (*E. japonica*), blackberry (*Rubus* sp.), and hackberry (*Celtis* sp.) have white to cream colonies[51]. Yeast can produce various kinds of pigments such as carotenoids and melanin but white yeast isolates indicate that yeast does not produce pigments. Carotenoid pigments are indicated in red to orange while black indicates melanin pigment[52]. Research Sukmawati et al. reported that yeast isolates obtained from the surface of Saeh leaves (*Broussonetia papyrifera*)

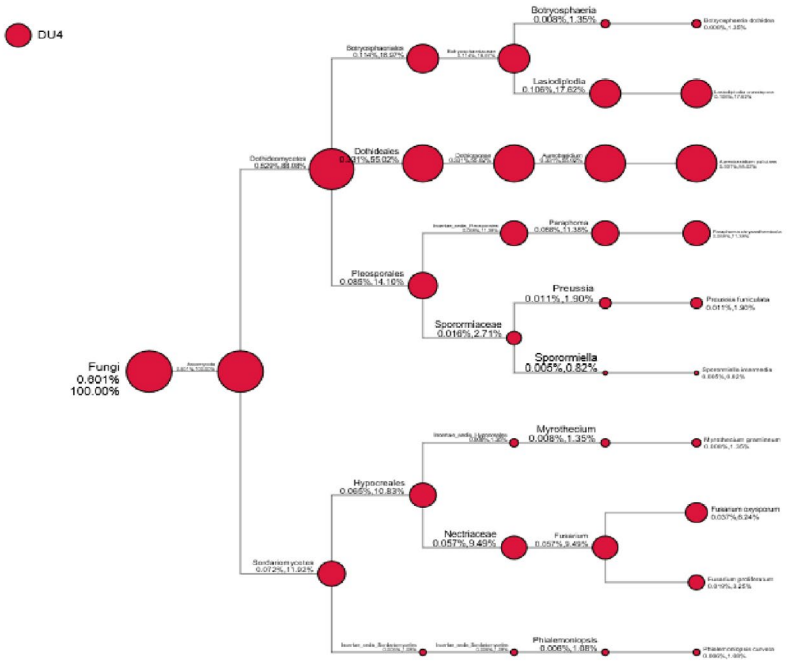

Fig. 2. Taxa diversity information of Lai durian (*Durio kutejensis*) collected from Desa Bukit Sawit, Central Kalimantan, Indonesia, using metagenomic approach based on ITS4 gene. 10 yeast species are present in the community.

were dominated by pigmented yeasts[29]. This might be caused by the leaf surface which experienced environmental stress. Leaf surfaces can be directly exposed to UV rays, air, and rain under varying conditions[53].

Yeast can be found from a variety of substrates and has different roles either in amylase-producing or cellulose-producing isolates[54,30,15,17], as well as controlling pathogenic fungi[55]. Research by Wulandari et al. has successfully isolated yeast from jackfruit and obtained 191 yeast isolates[13]. Amorim et al. obtained 132 yeast isolates from pineapple[56]. The population and yeast community on fruit is influenced by several factors such as: fruit type, geographical location, and fruit growth phase[57]. Yeast is rarely found in the fruit development phase and in immature fruit, but the number of yeast will increase when the fruit is ripe[58]. This is because ripe fruit contains more simple carbohydrates compared to unripe fruit,

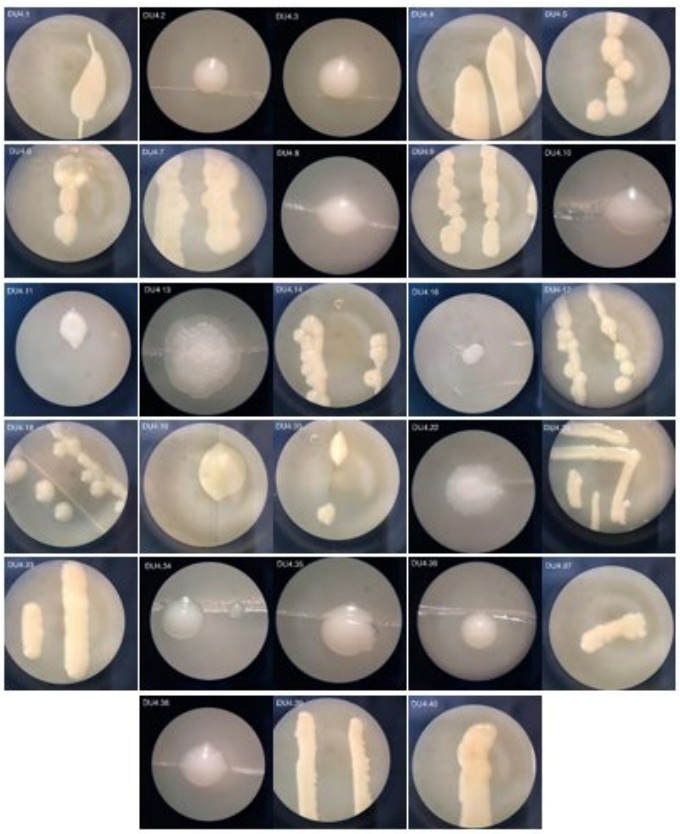

Fig. 3. Morphological characteristic of yeast isolated from Lai durian with code DU4, incubated on YMA medium at 28°C for 24 h.

Table 1. Yeasts isolated from Lai durian DU4 on YMA medium, incubated at 28°C for 48h. 40 isolates were obtained with various morphological characteristics

Sample code	Number of isolates	Colony color	Colony texture			
		White	Smooth	Rough	Butyrous	Mucoid
DU4	28	28	26	2	1	27
Total	28	28 (100%)	26 (92,85%)	2 (7,14%)	1 (3,57%)	27 (96,42%)

so the presence of yeast in ripe fruit is much more. The yeast isolates obtained were then purified to obtain single colony and were observed macroscopically (Fig. 3).

Screening Results of Yeast Isolates Producing Amylase Enzyme

Screening results showed that there are 2 isolates (DU4.2 and DU4.22) showing the presence of clear zones around the well after Lugol drops, indicating that the two isolates are having amylolytic activity (Fig. 4). Furthermore, the isolates with positive amylolytic activity were tested using digital calipers and the amylolytic index values were calculated[59]. The amylolytic index of isolate DU4.2 and isolate DU4.22 are 0,24±0,57 and 0,72±0,39, respectively.

The presence of amylase enzymes is indicated by the presence of a clear zone around the yeast isolate colony. Clear zone formed around the yeast colony shows that the isolate is able to hydrolyze starch. Starch will form a deep blue complex with iodine reagents. The iodine-starch reaction is caused by the presence of the helical amylose and iodine in forming I_3^- which place the helical core. Active hydrolysis of starch by amylase enzyme will cause the starch-iodine complex to decompose to form a clear zone. The absence of a clear zone around the colony indicates a reaction between the iodine reagent and the starch that is not hydrolyzed in the YPSA medium[60]. The ability or power to produce amylase enzymes in a microbe is characterized by the formation of clear zones in a medium containing starch[61].

Morphological and Molecular Identification Result of Isolate DU4.2 and Isolate DU4.22

Identification of potential yeast probiotics was carried out by macroscopic and microscopic observations of isolate DU4.2 and isolate DU4.22 at 48 h. Macroscopic observations showed that both isolate DU4.2 and isolate DU4.22 showed white colony, mucoid texture, smooth colony surfaces, mounting colony profiles, and without hyphae (Fig. 5). Isolate DU4.2 has flat colony edge, cylindrical cell shape, and multilateral germination, while isolate DU4.22 has undulate colony edge, circle cell shape and germination at every colony edges. Phylogenetic trees are constructed to see the kinship of yeast species that have potential as probiotic agents. The results of the mapping of D1/D2 rDNA yeast isolates were aligned using the MUSCLE (Multiple Sequence Comparison by Log-Expectation) method[62]. The phylogenetic tree was constructed using the Neighbor Joining method with a bootstrap value of 1,000 replications[63]. Molecular identification of isolate DU4.2 and isolate DU4.22 was performed based on ITS DNA region. Results showed that isolate DU4.2 has the closest homology to *Cyberlindnera fabianii* NRRL 1871 with 100% homology, while isolate DU4.22 showed sequence homology of 99.16% to *Candida sorboxylosa* ADR2-2 (Table 2).

Cyberlindnera fabianii is an ascomycetous 'uncommon' yeast which was previously named *Candida fabianii*[64]. The yeast of *C. fabianii* has a morphology in the form of a colony of creamy white cells, smooth surface, flat edges, round to oval shaped cells, and has budding[65]. This is consistent with macroscopic and microscopic observations

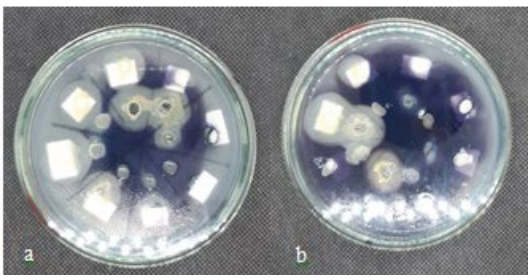

Fig. 4. The presence of clear zone appeared after Lugol drops by isolates DU4.2 (a) and isolates DU4.22 (b) incubated on YPSA medium at 30°C for 72 h.

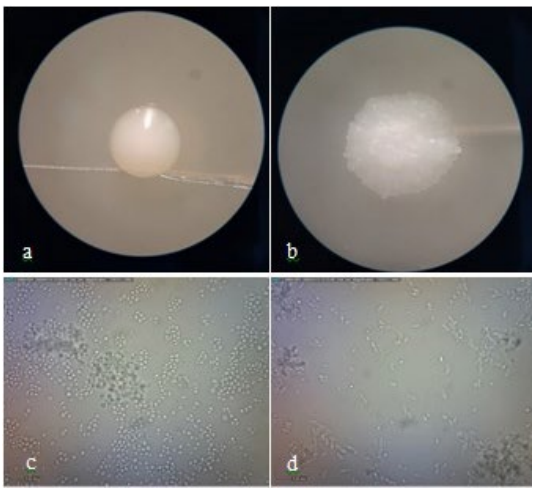

Fig. 5. Macroscopic observation of isolate DU4.2 (a) and isolate DU4.22 (b), and microscopic observation of isolate DU4.2 (c) and isolate DU4.22 (d)

of *C. fabianii* isolates which have macroscopic and microscopic characteristics similar to those of the yeast *C. fabianii.* Macroscopic observations showed that white *C. sorboxylosa* yeast isolate, mucoid textured, smooth colony surface, undulate colony edge and mountainous colony profile (Table 3). Microscopic observations showed that the isolate of yeast *C. sorboxylosa* has a round cell shape and buds at every place on the cell surface. The yeast of *C. sorboxylosa* has a morphology in the form of white cell colony, smooth surface, raised profile, and medium size[66]. This is consistent with macroscopic and microscopic observations of *C. sorboxylosa* isolates which have characteristics such as *C. sorboxylosa.*

The phylogenetic tree showed that the isolate DU4.2 was in monophyletic clade with *Cyberlindnera fabianii* NRRL 1871 with bootstrap value of 82. Isolate DU4.22 was in a monophyletic clade with *Candida sorboxylosa* ADR2-2 with bootstrap value of 100 (Fig. 5). Bootstrap value in phylogenic trees shows the tree topology formed.

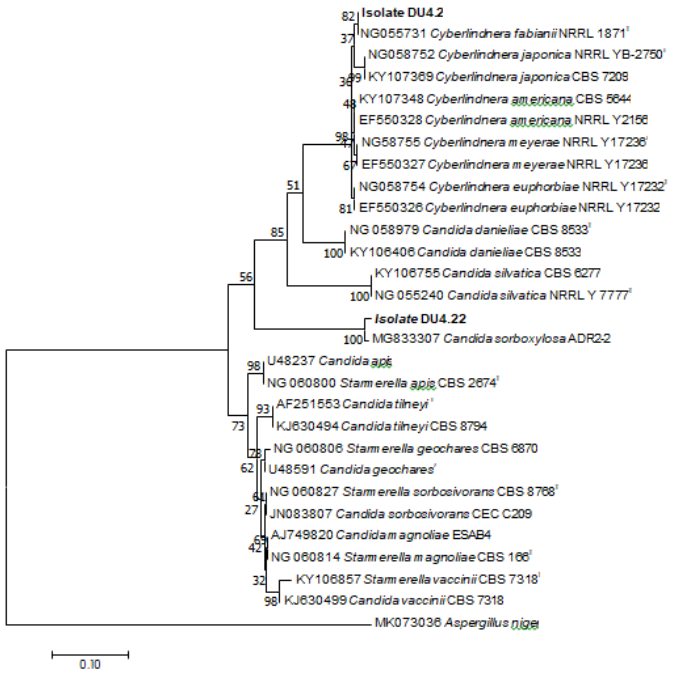

Fig. 6. Phylogenetic tree of isolate DU4.2 and isolate DU4.22, constructed using MEGA 5 software with the Joining Neighbor (NJ) method (1000x Bootstrap). *Aspergillus niger* was used as outgroup of the tree and the sequence collection were taken from NCBI.

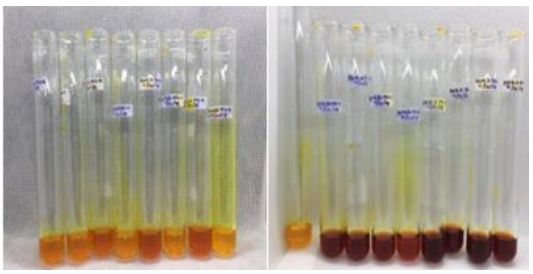

Fig. 7. Filtrate with the addition of 1% starch extract and DNS reagents before heating (left) and filtrate after heating (right).

Bootstrap value of 70-100% is a value that has a high level of confidence in the phylogenic tree produced[67].

Cyberlindnera fabianii NRRL 1871, reviously *Hansenula fabianii, Pichia fabianii*, and *Lindnera fabianii*), has been proposed to be novel gene after multigene sequence analysis[68]. *Candida sorboxylosa* ADR2-2 has been diversely colonized palm wines, among which some were related to a specific type of wine and the majority of them have the ability to digest starch, sugar, protein or lipid[69].

PCR-restriction fragment length polymorphism of ITS-5.8S rDNA combined to 26S rRNA gene and/or the partial ACT1 gene sequencing were applied for yeast characterization, and their enzymatic profiles assessed by using API ZYM kits. Thirteen genera and 23 species were identified, with the highest diversity (14 species) in raffia wine. Saccharomyces cerevisiae was dominant and common to all palm wines. Some potentially pathogenic yeasts were also isolated. The majority of tested strains displayed high amylo-peptidase, phosphatase, β-glucosidase and α-glucosidase activities and esterase activity.

Amylase Enzyme Activity of *Cyberlindnera fabianii* NRRL 1871 and *Candida sorboxylosa* ADR2-2 in *Solid State Fermentation* (SSF)

The fermentation medium with the SSF method is used in this study because it is one of the factors that influence the activity of amylase enzyme. Research conducted by Bhatti et al. obtained the optimum humidity to produce amylase enzyme at 70% humidity[70]. High humidity in the medium will cause the gas phase to be reduced and gas exchange to be hampered, causing the substrate to be anaerobic, while too low humidity will lead to poor microbial growth and decreased levels of nutrient acquisition[71]. After DNS reagent (3,5-dinitrosalicylate) application, the results of enzyme extract in solid state fermentation turned red as shown in Fig. 7. The principle of the DNS method with starch substrate is to reduce 3.5 dinitrosalicylate to 3-amino-5-nitrosalicylate resulting in a change from yellow to brownish red[32]. These reagents are commonly used to measure reducing sugars produced by microbes because of their high level of accuracy

Table 2. BLAST results of isolate DU4.2 and isolate DU4.22 based on D1/D2 rDNA region

Isolate code	NCBI result	Max Score	Query Cover	E-value	Accession	Identity	Gaps (%)
DU4.2	*Cyberlindnera fabianii* NRRL 1871	1113	96%	0,0	NG_055731.1	100%	0/579 (0%)
DU4.22	*Candida sorboxylosa* ADR2-2	675	93%	0,0	MG833307.1	99,16%	0/357 (0%)

Table 3. Average absorbance value of of *Cyberlindnera fabianii* NRRL 1871 and *Candida sorboxylosa* ADR2-2 at 540 nm wavelength

Isolate code	Repetition	Incubation time (h)			
		48	72	96	120
Cyberlindnera fabianii NRRL 1871	1	1.05	1.04	1.26	0.96
	2	1.18	1.08	0.87	0.93
	3	1.25	1.02	1.09	0.84
	4	1.15	1.19	0.92	0.54
Cyberlindnera fabianii NRRL 1871	1	0.79	0.63	0.56	0.89
	2	0.53	0.55	0.58	0.77
	3	0.73	0.66	0.45	0.82
	4	0.58	0.66	0.45	0.49

Table 4. Amylase activity value of *Cyberlindnera fabianii* NRRL 1871 and *Candida sorboxylosa* ADR2-2 at pH 4 in Solid State Fermentation method

Isolate code	Incubation time (h)	Amylase Activity (U/mL) (Mean±SE)
Cyberlindnera fabianii	24	$25.32^{b} \pm 1.84$
	48	$31.21^{c} \pm 1.20$
	72	$29.81^{bc} \pm 1.11$
	96	$27.32^{bc} \pm 2.52$
Candida sorboxylosa	24	$14.08^{a} \pm 1.45$
	48	$17.06^{a} \pm 1.72$
	72	$16.07^{a} \pm 0.73$
	96	$12.73^{a} \pm 0.97$

so that they can be applied to even small amounts of sugar. In an alkaline atmosphere reducing sugars will reduce 3,5-dinitrosalicylic acid (DNS) to form compounds that can be measured for absorption by a spectrophotometer at a wavelength of 540-550 nm[72].

The results of testing the activity of the amylase enzyme required calibration to determine glucose levels by making a glucose standard curve. Glucose solution was chosen as the solution for making standard curves because glucose includes reducing sugars produced from hydrolysis of the substrate by the amylase enzyme. The results of the standard curve analysis have a linear equation y = 0.3889x + 0.0619 with a correlation value (R2) of 0.9772. The equation obtained is used to determine the concentration of glucose in the test sample amylase enzyme activity. Correlation value (R2) which is close to number 1 shows the correlation between absorbance value and glucose concentration[73]. Table 3 showed the average absorbances of *Cyberlindnera fabianii* NRRL 1871 and *Candida sorboxylosa* ADR2-2 at wavelength 540 nm.

Table 4 showed that based on the measurement of amylase enzyme activity, *Cyberlindnera fabianii* NRRL 1871 has higher value (31.21 U/mL; 48 h incubation time) than *Candida sorboxylosa* ADR2-2 (12.73 U/mL; 96 h incubation time). This result showed that there is an effect of the incubation time of solid state fermentation with pH 4 on the activity of the amylase enzyme produced by both isolates.

Both isolates produced the highest activity at 48 hours of incubation. Enzyme activity or enzyme work has U/ml units. One unit (U) of amylase enzyme activity is defined as the amount of enzyme that produces 1 µmol reducing sugar (glucose) per minute under conditions of enzyme activity testing (Lehninger, 2008). Martin et al. (1983) stated that enzyme activity is strongly influenced by the incubation time. The incubation time is the time required by the enzyme to interact with the substrate, if the enzyme is saturated with the substrate, the enzyme will not work optimally. Darwis (1995) also states that at the beginning of fermentation the enzyme activity is still very low. Enzyme activity will increase with increasing fermentation time. This follows the growth pattern of microorganisms that experience several growth phases, namely the adaptation phase, the exponential phase, the stationary phase, and the death phase. Amylase enzymes are produced by yeast in the adaptation phase and reach optimum during the final exponential phase (Sjofjan and Ardyati 2011). Amylase enzyme activity will decrease after the yeast reaches the final exponential phase because the starch in the fermentation medium starts to run out so the enzyme cannot be produced. Yeast plays a role in the process of maturation and control of pathogenic molds on durian fruit. Amylase activity increased at 48 hours incubation.

Research on the yeast *Candida sorboxylosa* and *Cyberlindnera fabianii* as amylase enzyme producers is still limited. *Candida sorboxylosa* isolated from local fruits of Ethiopia can ferment the type of fructose sugar present in bread dough[47]. Sugar breakdown that occurs will release carbon dioxide in the bread dough. This shows that *Candida sorboxylosa* has an enzyme that is responsible for fermenting most of the sugar in the bread dough. While the yeast *Cyberlindnera fabianii* has been known to have acetate ester hydrolase enzyme activity and can produce ethanol[78,79].

CONCLUSION

Metagenomic analysis result showed that yeast isolated from Lai durian (*Durio kutejensis*) collected from Bukit Sawit Village in Central Kalimantan, Indonesia was associated with Ascomycota phylum, consisting of 10 species including *Botryosphaeria dothidea* (0.008%, 1.35%), *Lasiodiplodia crassispora* (0.106%, 17.62%); *Aureobasidium pullulans* (0.331%, 55.02%), Paraphoma chrysanthemicola (0.068%, 11.38%), *Preussia funiculate* (0.011%, 1.90%), *Sporormiella intermedia* (0.005%, 0.82%), *Myrothecium gramineum* (0.008%, 1.35%), *Fusarium oxysporum* (0.037%, 6.24%), *Fusarium proliferatum* (0.019%, 3.25%), and *Phialemoniopsis curvata* (0.006%, 1.08%). Culture-dependent analysis showed that among 40 yeasts isolated from 3 Lai durian samples, isolate with the code DU4.2 and DU4.22 exhibited the clear zones with amylolytic activity value at 0.24 and 0.72. Molecular identification resulted that these two isolates have the closest homology to *Cyberlindnera fabianii* NRRL 1871 and *Candida sorboxylosa* ADR2-2, respectively.

Isolate DU4.2 *Cyberlindnera fabianii* NRRL 1871 has higher amylase activity value (31.21 U/mL) with an incubation time of 48h at pH 4 than *Candida sorboxylosa* ADR2-2 (12.73 U/mL) with 96 h incubation time. This research was a study describing yeast information that could be found from various native fruits of Indonesia, especially Lai durian. Yeast isolated from Lai durian are able to produce amylase enzyme which can be potentially developed in industrial application.

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CONFLICT OF INTEREST

The authors declares that there is no conflict of interest.

AUTHORS' CONTRIBUTION

DS, HEE conceived and designed the experiments; SN and ZN performed the experiments; DS, RW, DJD, analyzed the data; DS, SNA, HEE, DJD wrote the paper.

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None.

DATA AVAILABILITY

All datasets generated or analyzed during this study are included in the manuscript and/or the Supplementary Files.

ETHICS STATEMENT

Not applicable.

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