Isolation and screening of amylolytic yeast from *Paphiopedilum* sp., originating from Bedugul Botanical Garden, Bali, Indonesia

Isolation and screening of amylolytic yeast from *Paphiopedilum* sp., originating from Bedugul Botanical Garden, Bali, Indonesia

A Risandi[1], R Fuady[1], D Sukmawati[1,2,*], S N A Husna[3], M Nurjayadi[4], H E Enshasy[5,6] and R Ridawati[7]

[1] Biology Department, 9th Floor Hasyim Ashari Building, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Jakarta, Indonesia
[2] Universitas Negeri Jakarta Culture Collection, 9th Floor Hasyim Ashari Building, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Jakarta, Indonesia
[3] Department of Microbiology, School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia
[4] Chemistry Department, 6th Floor Hasyim Ashari Building, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Jakarta, Indonesia
[5] Institute of Bioproduct Development (IBD), Universiti Teknologi Malaysia (UTM), 81130 UTM, Skudai, Malaysia
[6] Department of Bioprocess and Polymer Engineering, School of Chemical and Energy Engineering, Faculty of Engineering, Universiti Teknologi Malaysia, 81310, Skudai, Johor, Malaysia
[7] Food and Nutrition Department, Faculty of Engineering, Universitas Negeri Jakarta, Jakarta, Indonesia

*dalia-sukmawati@unj.ac.id

Abstract. Amylase (E.C.3.2.1.1) are the enzyme that works as catalyst in the hydrolysis of starch into simple monomers. Amylase enzymes are widely used in various industrial fields such as textile, food, paper and other industries. Compared to other organisms, yeasts can produce enzymes more effectively and safer for the environment. Amylolytic yeast can be isolated from flower substrates as it contains sugar for the very limited condition of yeast growth. This study aims to isolate, select and characterize amylolytic yeasts on the substrate of *Paphiopedilum* sp. from Bedugul Botanical Garden, Bali. Yeast isolation was carried out with direct and washing method, followed by screening conducted on YPSA medium with diffusion agar method. Results showed that 19 yeast isolates were obtained with characteristics of 73.9% white-mucoid, 21.05% cream-mucoid and 5.26% light flesh-mucoid. Screening results showed that 10 isolates which coded by P1, P4, P6, P10, P11, P12, P13, P14, P15 and P19, were able to produce amylase enzyme. The potential yeast isolates in yielding amylase with P12 isolate codes had amylolytic index 0,45 mm. from this research, it can be found the symbiosis between yeasts and plants are happening in certain ways.

1. Introduction

Amylase (E.C.3.2.1.1) is an enzyme that has widely used in bioindustry application since it can hydrolyze starch polymers into glucose monomer units [1]. About 30% of the world's enzyme production industry is amylase enzymes [2]. Amylase enzyme works to degrade starch polymers by breaking down α-1,4-glycosidic bonds [1]. The α-amylase enzyme are used for various industrial needs such as bakery, paper industry, detergent, food and pharmaceutical industries [3].

Amylase enzymes can be obtained from various organisms such as yeast. Yeast is a unicellular fungus and is widespread in various environments, from aquatic, terrestrial to atmospheric environment [4]. Yeasts are able to associate with plants without causing damage or disease [5]. Mutual relationship with plants and the presence of antifungal compound can result a function as biocontrol agents for pathogenic fungi [6]. Although yeast is not the main degradator of organic matter, interaction with other organisms is important for the microhabitat of a substrate.

As it occupies a wide range of ecological niche, yeast can be found in various substrates such as the fruit [7], leaves [8] and flower. Orchid flowers (*Paphiopedilum* sp.), for example, is a good habitat of yeasts since it contains limited nutrients for yeast to grow. Besides, it can live in a tropical climate where there is not too much water available, being exposed to direct exposure to sunlight and shelves of the trees. This flower is generally an ornamental plant because of its special shape and color. The potential cellulolytic yeasts were found from the rhizosphere of *Pecteilis susannae* flower [9]. *Candida* sp. and *Saccharomyces* sp. were obtained from the flower epidermis. However, research on yeast from *Paphiopedilum* sp. has still not done yet. Therefore, in this study, the isolation and selection of amylolytic yeast from *Paphiopedilum* sp will be conducted.

2. Material and methods

2.1. Sample collection and yeasts isolation

Two stalks of orchid flower (*Paphiopedilum* sp.) were collected from Bedugul Botanical Garden, Bali, Indonesia. The isolation method used in yeast isolation is direct method and washing method [10]. The flower samples used were cut and weighed as much as 1 gram, and were then put into eppendorf tubes containing 20 mL sterile distilled water. The sample was homogenized by placing it in the rotator shaker at a speed of 150 rpm for 20 minutes. 1 ml of homogenized sample were then put into a test tube which filled with 9 ml sterile distilled water (10^{-1} dilution), the solution was homogenized using vortex for 1 minute, then the same protocol was done for a 10^{-3} and 10^{-5} dilutions. 0.1 ml of sample in each dilution was then inoculated into PDA medium in duplicate with the spread plate method. The samples were flattened using Drygalski spatula and incubated for 48 hours at 28^{o}C [11]. The growth isolates were picked using toothpicks and colony libraries were made using PDA medium. Purification for each isolate were done and were inoculated into PDA Slants and followed by incubation at room temperature 28-30^{o}C for 48 hours.

2.2. Screening and selection of amylolytic yeasts

To screen and obtain potential yeast with amylolytic activity, starch hydrolysis test was performed. Wells are made using sterile plastic straws in each quadrant. The 72-hour-incubated yeast suspension is inoculated as much as 20 uL using a micropipette into the well on the agar starch medium which is divided into 4 quadrants with two repetitions. Isolates were incubated at room temperature 28-30^{o}C for 72 hours. Selection was done by the presence of clear zones after introducing iodine reagents on starch agar media. The magnitude of the amylolytic index value of each isolate was calculated by the clear zone diameter and colony diameter using analytic calipers.

The amylolytic index is calculated based on [12]:

$$IA = \frac{D \quad o\ c \quad z \quad -d \quad o\ c}{D \quad o\ c}$$

2.3. Morphological yeasts identification

Identification of yeast morphology is done by observing yeast morphology macroscopically [13]. Observation of yeast morphology was carried out by growing yeast isolates on Yeast Malt Agar (YMA) media, incubated for 48 hours at 28ºC. The macroscopic characteristic of yeast observed including colour, texture, and the edge of the colony.

3. Results and discussion

19 yeast isolates were obtained from orchid flower samples (Figure 1). It showed that several group of yeasts can live in the plant tissues and symbiosis between flower and plants can be found [14]. The presence of yeast in the orchid flower indicated that yeast has a mutual relationship with plants in terms of nutrients sharing. Yeasts can receive nutrients and place while plants get protection from pathogenic attacks because yeast produces bioactive secondary metabolites [15].

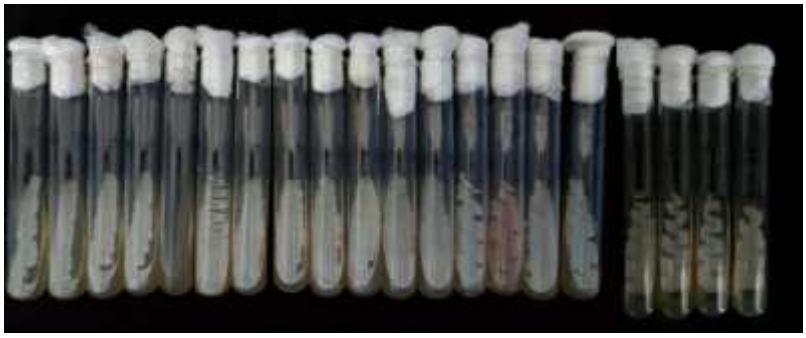

Figure 1. Yeast isolates inoculated from orchid flower samples with code isolates P1 - P20 from left to right (Media PDA; temperature 28-30 ° C for 48 hours).

Amylolytic yeast selection was carried out on 19 yeast isolates. 10 of 19 isolates obtained were able to produce amylase enzymes by the presence of clear zones (Figure 2) while other 9 isolates were not capable of producing clear zones.

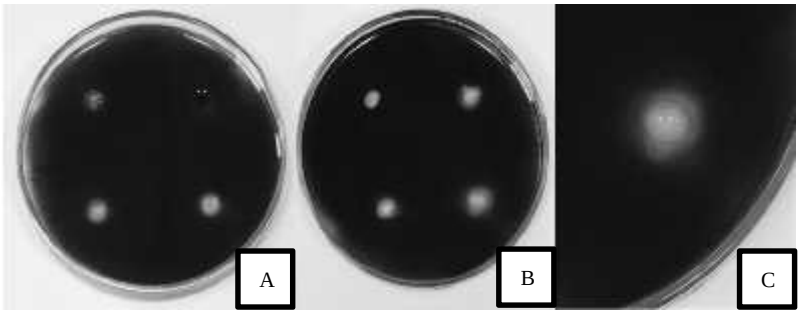

Figure 2. Results of selection of potential yeast isolates producing amylase enzyme (A) Isolate P1 (B) Isolate P12 (C) Clear zone formed. (Medium Starch Agar; 28 - 30ºC; 72 hours).

From the study, it resulted that P12 isolate has the highest IA value with 0.45 mm compared to other isolates while P1 isolate has the lowest IA value 0.18 mm. Amylolytic index values were calculated using one-way ANOVA to see the difference between the isolates used. The results of one-way ANOVA analysis (Table 1.) show that there are differences in the amylolytic index of each yeast isolate with a value of α (0.05)> Sig. 0.00. This shows that all yeast isolates have a significantly different amylolytic index value.

Table 1. Amylolytic index value of yeast isolates producing amylase enzyme.

Isolate codes	Amylolytic index (mm) (Mean±SE)
P1	0.18 ± 0.01^{a}
P4	0.37 ± 0.02^{bc}
P6	0.27 ± 0.03^{ab}
P10	0.32 ± 0.02^{bc}
P11	0.41 ± 0.09^{cd}
P12	0.45 ± 0.01^{d}
P13	0.29 ± 0.01^{ab}
P14	0.27 ± 0.04^{ab}
P15	0.25 ± 0.04^{ab}
P19	0.40 ± 0.06^{cd}

Note: Numbers followed by the same letters are not significantly different at $\alpha = 0.05$ (Duncan test).

The formation of a clear zone around the yeast suspension shows the ability of these isolates to produce amylase enzymes as they can hydrolyse starch into a simpler compound. The reaction of starch with iodine reagent will make the colour of the media become solid blue. The ability of yeasts to produce amylase enzymes was characterized by the formation of clear zones in starch-containing media [16]. Starch hydrolysis by amylase enzyme will result hydrolysed starch that it does not form blue around the yeast suspension and form a clear zone. The absence of a clear zone around the colonies indicates a reaction between iodine reagents and non-hydrolysed starches in the Starch Agar medium [17]. The yeasts that positively produces the amylase enzyme were observed macroscopically by observing the colour, texture and edge of the yeast colonies (Table 1.)

Table 2. The macroscopic characteristics of yeast isolates with ability to produce amylase enzymes.

Isolate codes	Colony color	Colony texture	Colony edge
P1	*White*	*Mucoid*	Flattened
P4	*White*	*Mucoid*	Flattened
P6	*White*	*Mucoid*	Flattened
P10	*White*	*Mucoid*	Flattened
P11	*White*	*Mucoid*	Flattened
P12	*White*	*Mucoid*	Flattened
P13	*White*	*Mucoid*	Flattened
P14	*Light Flesh*	*Mucoid*	Flattened
P15	*White*	*Mucoid*	Flattened
P19	*Cream*	*Mucoid*	Flattened

From macroscopic data, amylolytic yeast isolates were obtained with 73.9% white-mucoid, 21.05% cream-mucoid and 5.26% light flesh-mucoid. The dominant white colour in yeast from flowers shows that yeast does not have colour pigmentation, different from the research of [18] yeast found on leaf surfaces, dominated by pigmented yeast. Yeast has a mucoid texture due to the presence of polysaccharide substances contained in yeast extracellular components. Based on [19], some yeasts are covered by extracellular components in the form of slimy polysaccharides and heteropolysaccharides. Further research should be conducted to determine the cause of differences in yeasts group found. Environmental factors such as the nutrient contents of the flower, temperatures and weather could contribute to the variety of yeasts found in certain plants.

4. Conclusion

19 yeast isolates were found from *Paphiopedilum* sp. and of 10 yeast isolates (P1, P4, P6, P10, P11, P12, P13, P14, P15, P19) showed the potential ability to produce amylase enzymes. P12 isolate has the

highest IA value of 0.45 mm, while yeast isolate P1 has the lowest IA value of 0.18 mm. Microscopic identification resulted that yeasts isolates were 73.9% white-mucoid, 21.05% cream- mucoid and 5.26% light flesh-mucoid. Information got from this research can be initial step in finding novel function of certain yeasts as biological agents against pathogenic fungi which will benefit farmers to increase agriculture products.

Acknowledgements
This research was funded by Dikti Grand Research as Dalia Sukmawati a pilot project, this is research in 2019-2020 with the title "**Aplikasi Omics Pada Produk Fermentasi Asli Indonesia: Upaya Standarisasi dan Pengembangan Industri Pangan Indonesia Untuk Bersaing Di Kancah Internasional**". We express deep gratitude and appreciation to the Department Biology Universitas Negeri Jakarta Research Grant supported this research. We thank the Lab. Microbiology and Universitas Negeri Jakarta Culture Collection (UNJCC) for the use of the facilities.

References
[1] Aiyer P V 2005 Amylases and their applications *Afr. J. Biotechnol*. **4** (13) 1525–1529
[2] Sivaramakrishnan S, Gangadharan D, Nampoothiri K M, Soccol C R and Pandey A 2006 a-Amylases from microbial sources–an overview on recent developments *Food Technol Biotechnol* **44** (2) 173-184
[3] Gupta R, Gigras P, Mohapatra H, Goswami V K and Chauhan B 2003 Microbial α-amylases: a biotechnological prospective *Process. Biochem*. **38** 1599–1616
[4] Hong S G, Lee K H and Bae K S 2002 Diversity of yeasts associated with natural environments in Korea *The Journal of Microbiology* **40** (1) 55-62
[5] Abdel-Motaal F F, El-Zayat S A, Kosaka Y, El-Sayed M A, Nassar M S M and Ito S I 2009 Four novel ustilaginomycetous anamorphic yeast species isolated as endophytes from the medicinal plant Hyoscyamus muticus *Asian Journal of Plant Sciences* **8** (8) 526
[6] El-Tarabily K A and Sivasithamparam K 2006 Potential of yeasts as biocontrol agents of soil-borne fungal plant pathogens and as plant growth promoters *Mycoscience* **47** (1) 25-35
[7] Wulandari T P, Sukmawati D and Kurniati T H 2017 Isolasi Dan Seleksi Khamir Amilolitik Asal Buah Nangka (Artocarpus heterophyllus Lam.) *Bioma* **13** (1) 37-42
[8] Sukmawati D 2016 Antagonism Mechanism of Fungal Contamination Animal Feed using Phylloplane Yeasts Isolated from the Bintaro Plant (*Cerbera manghas*) Bekasi in Java Indonesia *Int. J. Curr. Microbiol. App. Sci* **5** (5) 63-74
[9] Diana L and Lasmini T 2016 Isolasi dan Identifiksi Khamir Selulolitik Dari Tanah Rizosfer Anggrek Puser Bumi (*Pecteilis susannae* L.) di Hutan Wonosadi Gunung Kidul DIY *Jurnal Biogenesis* **4** (1) 21-28
[10] Sjamsuridzal W, Oetari A, Kanti A, Saraswati R and Nakashima C 2009 "Section yeasts and yeastlike fungi. In: Taxonomic and Ecological Studies of Fungi and Actinomycetes," in Indonesia Volume II Widyastuti Y and Ando K (eds.) *Joint Research Project between Indonesian Institute of Sciences (LIPI) of the Republic of Indonesia and National Institute of Technology and Evaluation (NITE) of Japan* (Cibinong: LIPI Press) pp 468-540
[11] Chanchaichaovivat A, Ruenwongsa P and Panijpan B 2007 Screening and identification of yeast strains from fruits and vegetables: Potential for biological control of postharvest chilli anthracnose (Colletotrichum capsici) *Biological Control* **42** (3) 326-335
[12] Goldbeck R, Andrade C C P, Pereira G A G and Maugeri Filho F 2012 Screening and identification of cellulase producing yeast-like microorganisms from Brazilian biomes *African Journal of Biotechnology* **11** (53) 11595-11603
[13] Kurtzman C P and Fell J W 2006 "Yeasts systematic and phylogeny Implications of molecular identification methods for studies in ecology," in Rosa C A and Peter G *Biodiversity and ecophysiology of yeasts* (Germany: Springer)

[14] Rodriguez R J, White Jr J F, Arnold A E and Redman R S 2009 Fungal endophytes, diversity and functional roles *New Phytologist* **182** 314–330
[15] Higginbotham S J, Arnold A E, Iban A, Spadafora C, Coley P D and Kursar T A 2013 Bioactivity of fungal endophytes as a function of endophyte taxonomy and the taxonomy and distribution of their host plants *PLoS One* **8** 1–11
[16] Tan Gana N H T, Mendoza B C and Monsalud R G 2014 Isolation, screening, and characterization of yeasts with amyloytic, lipolytic, and proteolytic activities from the surface of Philippine bananas (Musa spp.) *Philipp. J. Sci*. **143** 81–87
[17] Cappuccino J G and Sherman N 2002 *Microbiology: A laboratory manual, 9th ed.* (California: Addison-Wilsey)
[18] Lindow S E and Brandl M T 2003 Microbiology of the phyllosphere *Appl. Environ. Microbiol.* **69** (4) 1875-1883
[19] Fardiaz S 1992 *Mikrobiologi Pangan 1* (Jakarta: PT Gramedia Pustaka Utama)