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

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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 degrader 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 susanae 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°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°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°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]:

\[
IA = \frac{E - \alpha \cdot c}{E - \alpha \cdot c - d - \beta \cdot c}
\]
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 28°C. 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](image1.png)

**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](image2.png)

**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; 28-30°C; 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 α = 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.

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