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Denika Dellanerra, Aditya Risandi, Anggun Sunari, Dalia Sukmawati, Shabrina Nida Al Husna, and Hesham Ali El-Enshasy

Screening and Characterization of Amylolitic Mold Originated from Ghost Crab (*Ocypode* sp.) in Cidaon, Ujung Kulon National Park, Indonesia

Denika Dellanerra[1)], Aditya Risandi[1)], Anggun Sunari[1)], Dalia Sukmawati[1, 2, a)], Shabrina Nida Al Husna[3)] and Hesham Ali El-Enshasy[4, 5)]

[1]*Department of Biology, Faculty of Mathematics and Natural Sciences, Universitas Negeri Jakarta, Campus A, Jl. Rawamangun Muka East Java. Hasyim Ashari Building, 9th floor, Indonesia.*
[2]*Universitas Negeri Jakarta Culture Collection* (UNJCC), *Campus A, Jl. Rawamangun Muka East Java. Hasyim Ashari Building, 9th floor, Indonesia.*
[3]*Department of Microbiology, School of Life Sciences and Technology, Institut Teknologi Bandung, Indonesia*
[4]*Institute of Bioproduct Development, Universiti Teknologi Malaysia (UTM), Skudai, Johor Bahru, Malaysia.*
[5]*City of Scientific Research and Technology Applications, New Burg Al Arab, Alexandria, Egypt.*

[a)]Corresponding author: Dalia-Sukmawati@unj.ac.id

Abstract. Amylases have been used since centuries in textile, feed, food and paper industries. Of different biofactories applied in amylases production, molds have been widely used in industries based on their high capacity for production and excretion of the enzyme. Amylolytic molds can be isolated from different environments. This study aims to select and characterize amylolytic fungi from ghost crabs (*Ocypode* sp.), at Ujung Kulon National Park, Indonesia. Sampling was carried out by purposive random sampling method, followed by mold isolation by using the direct and washing method. The screening was carried out by the agar diffusion method, and the selected strain was fully characterized using microscopic and macroscopic studies. The data obtained were analyzed by one way ANOVA followed by DMRT test with a significant level of 5%. Based on the results, it can be seen that ghost crab (*Ocypode* sp.) is a good substrate for mold growth. There are 30 representative mold isolates has successfully isolated from crabs from 2 sampling sites. Almost 46% of mold isolates were found to have the potential ability to produce amylase enzymes. However, isolate UNJCC F111 has the highest IA value of 2.73 mm and based on observation, it is thought to belong to the *Penicillium* group. As many as 71.4% of the 14 amylolytic potential mold isolates were thought to belong to the genera *Aspergillus*. The isolated strains have high potential as biofactory of amylases production and could be applied for industrial production.

INTRODUCTION

Amylase enzyme needed in the industrial as a biocatalyst. Amylase enzyme production reaches 30% of the total industrial production of enzymes in the world [37]. Amylase enzyme finds a wide range of applications in pharma, food, feed, textile, paper and pulp industries [12-13]. In general, amylases are characterized by their ability to hydrolyze starch molecule to glucose monomers [5,14,29]. In addition, glycogen, which is composed of glucose polymer branching with a very large size can be hydrolyzed by amylase enzymes [7].

Amylase enzyme can be obtained from various organisms, one of which is microorganisms such as mold (Amylolytic molds). Microorganisms such as mold can produce amylase enzymes in high mass production since it has a higher reproduction rate than other microorganisms. Amylolytic molds can be isolated from the marine environment. Amylolytic molds were derived from the marine environment of the genera *Penicillium* and *Aspergillus* [34]. Amylolytic molds can be found in crabs due to the nutrient content of crabs such as carbohydrates (glycogen), protein, fat and mineral elements (Na, K, Ca, Mg, Fe, Zn, and Se) that can support the growth of amylolytic molds [26].

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Crab is a member of the Crustacea class which has an important role as an indicator of the waters because of its relatively constant habitat. According to Castro and Huber [27], there are around 68,000 types of Crustacean class that have been identified and around 150,000 species that have not been identified, including crabs. Ghost crabs (*Ocypode* sp.) Can be found in ecosystems around the waters such as on the beach and become benthos on sand beaches. Every crab has the ability to tolerate certain environmental conditions [33]. The presence of mangroves, seagrasses and coral reefs will also affect the existence of crabs [2,32]. Ujung Kulon National Park has marine habitats with various substrates that can represent ghost crab habitat [15].

The study aimed to select amylolytic molds from the ghost crab substrate in Ujung Kulon National Park. Characterization of morphology amylolytic molds were also carried out as a data of identification.

EXPERIMENTAL DETAILS

Place of Study

The research was conducted in September - November 2018, sampling was conducted in Cidaon, Ujung Kulon National Park, Banten, Indonesia. Screening potential of producing amylase enzymes and characterization was carried out at the Microbiology Laboratory on the 9th floor of the Hasyim Ashari Building, Universitas Negeri Jakarta, Jakarta, Indonesia.

Growth and Selective Media

The media used in this study contained growth media for isolation and selective media for screening potential of producing amylase enzymes. The growth media for isolation used Potato Dextrose Agar (PDA) media (39 g/L). Selective media used Starch Agar (SA) (Starch 1%, KH_2PO_4 0.5%, Agar 2%). Each medium made was added with chloramphenicol 200 mg/L then sterilized using an autoclave at 121 ºC for 20 min, and the final pH was made into 7 [23].

Samples and Sampling Location

The samples were used is ghost crabs (*Ocypode* sp.), which was taken from Cidaon, Ujung Kulon National Park, Banten (Fig. 1). The technique of collecting data using a method Purposive Random. Locations and stations which were used are the West Coast of Cidaon and to be divided to 2 stations. Each station is repeated into 2 sampling points, with 2 repetitions at each sampling point.

Mold Isolation

Isolation of molds from ghost crab was carried out based on the method of Romanenko *et al.* [22] with some modifications. Total of 8 crab samples was obtained from 2 different locations. The crabs obtained are rinsed using sterile seawater, then coarsely crushed crabs use a mortar and sterile pestle to make small pieces. Mold isolation from samples was carried out using washing and direct methods [9]. In the washing method, the sample was diluted by taking 1 mL of suspension added to 9 mL of sterile seawater and then homogenization. The sample results of dilution 100 and 10^{-1} were cultured using the spread plate method by adding 0.1 mL on PDA plate. In direct method the crab pieces on the conical tube are placed directly into the PDA plates after that, Inoculated samples were incubated for 48 h at 27 °C.

Screening of Amylolytic Mold Based on Amilolytic Index

Screening of amylolytic mold based on the amylolytic index [4]. Amylolytic mold selection was carried out by inoculated fungi in SA selective media using the agar diffusion method with making wells, by inoculated 20 µL in wells in each quadrant. Making wells using sterile straws was carried out in the SA media which had been divided into four quadrants. The mold suspension was made by adding 5 mL of sterile distilled water to the working culture of sporulated mold isolates and then the homogenization suspension using vortex. The medium containing the suspension of mold cells was then incubated at 27 ºC for 72 h. Positive mold parameters produced an amylase enzyme

characterized by the presence of a clear zone around the colonies in the test medium (SA) after adding iodine solution to the media. Determination of the amylolytic index value using the following formula [31]:

$$\text{Index of Amylolytic} = \frac{\text{The diameter of the Clear Zone} - \text{Diameter of the Colony}}{\text{Diameter of the Colony}} \quad (1)$$

Characterization of Molds

Characterization of molds was carried out based on macroscopic and microscopic observations according to the method of Afzal *et al.* [16]. The macroscopic characteristics observed included; colony surface, texture, zoning, colour opposite the colonies, growing zone, radial furrow and exudate drops. Whereas, for microscopic characteristics include; the form of conidia, forms of vesicles, and bulkhead in hyphae. Mold isolates to be observed were first grown on PDA (Potato Dextrose Agar) medium and then incubated for 7 days [3].

Data Collection and Analysis Techniques

The data obtained in this study is the amylolytic index of each isolate analyzed using SPSS 16.0 for Windows software with one-way ANOVA (One way ANOVA). If the results show significance, then proceed with Duncan multiple range test (DMRT) with a significant level of 5% to determine the presence of significant differences in the amylolytic index formed from each mold isolate. While the characteristic data of amylolytic potential isolates were analyzed descriptively.

RESULT AND DISCUSSION

Samples and Sampling Locations

The number of crab samples obtained was 8 individuals from 2 different locations. The crab used as a sample is a ghost crab from the genus *Ocypode*. Based on the characteristics of the crab samples obtained had an average size of 3 cm, with morphology the brownish carapace and on the upper surface of the crab body, there were black spots (Fig. 1). Genus *Ocypode* has the characteristics of a brownish carapace, in the form of a box without anterolateral teeth, its eyes soaring up and long above the cornea [30].

FIGURE 1. Ghost crab sample (*Ocypode* sp.)

The sampling locations for crabs at 4 points obtained environmental parameters with a pH range of around 7 - 8 and a temperature range of 27 ºC – 28 ºC (Table 1). The environmental parameters were measured and used as a

reference for the incubation temperature and pH of the medium. The optimum pH and temperature of mold growth are in accordance with the pH and temperature of the mold source environment isolated [28]. Each mold has a character that is influenced by several environmental factors [6]. Based on the measurement of environmental parameter values, it was found that the optimal conditions for the growth of mold from crabs were at 27 ºC and pH 7.

TABLE 1. Environmental Parameter Values for Crab Sampling Location.

Station	Location	Coordinate Point	Temperature (^{O}C)	pH
1	1	-6,7602485,105,2639405	27	7
(South Pier)	2	-6,7597484,105,2646698	27	7
2	1	- 6,7596592,105,2652853	28	7
(North Pier)	2	- 6,7576268,105,2660722	27	7

Mold Isolation

Based on the isolation results from the crab samples from 4 locations, there are 46 isolates from the crab origin obtained by washing and direct method (Table 2). Total of 46 mold isolates was taken 30 representative mold isolates (UNJCC F108, UNJCC F109, UNJCC F110, UNJCC F111, UNJCC F112, UNJCC F113, UNJCC F114 UNJCC F115, UNJCC F116, UNJCC F117, UNJCC F118, UNJCC F119, UNJCC F120, UNJCC F121). The presence of mold isolates obtained from crabs proves that crabs are a good substrate for the growth of microorganisms such as mold. The nutrient and mineral content of crabs can be used by molds as a source of nutrients and support microbial growth [7].

TABLE 2. Results of isolation molds

Location	Amount of Mold Isolate
1	10
2	14
3	11
4	11

Amylolytic Mold Selection Based on Amylolytic Index Value

This test was conducted on 30 isolates from isolation. Determination of the potential amylolytic index of mold isolates was carried out using well diffusion method [4]. Based on results of selection amylolytic potential mold isolates from 30 mold isolates, there were 14 potential amylolytic mold isolates (UNJCC F108, UNJCC F109, UNJCC F110, UNJCC F111, UNJCC F112, UNJCC F113, UNJCC F114, UNJCC F115, F116, UNJCC F117, UNJCC F118, UNJCC F119, UNJCC F120 and UNJCC F121). With the percentage of potential amylolytic molds reaching 46.67%. Amylolytic potential mold isolates are characterized by the presence of clear zones around the colonies. This proves that in crabs there are amylolytic molds that play a role in the ecosystem as a component of glycogen decomposers in crabs.

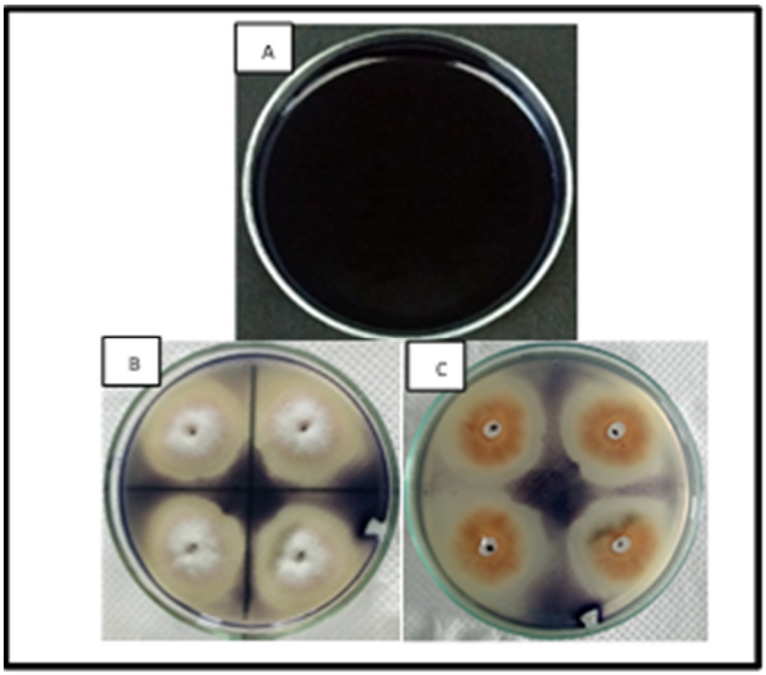

FIGURE 2. The ability of mold isolates based on the amylolytic index with the well method. (A) Negative control, (B) Clear surface zone of amylolytic positive colonies (surface view); and (C) Clear zones of amylolytic colonies (reverse view). Data were taken after incubation for 72 h at 27 ºC using SA medium)

The agar diffusion method is based on the ability of fungal isolates to hydrolyze compounds in the medium after iodine reagents are pressed and produce clear zones around the test wells [11]. The clear zone formed around the colonies shows that starch in the SA medium is hydrolyzed by the amylase enzyme [24]. The starch on the media after iodine reagent is cut will form a solid blue complex due to the presence of helical amylose and iodine in forming I_3^- which fills the helical nucleus [39] whereas the formation of clear zones is not caused by starch not being hydrolyzed by the amylase enzyme so that the star reacts with iodine and forms a deep blue colour [19].

The use of diffusion method so that by making wells in amylolytic mold selection can get good mold growth. The mold suspension which was inoculated into the well obtained the growth of mold colonies only around the well and did not spread which ease the measurement of the clear zone. The agar diffusion method by making wells is a method based on the ability of molds to hydrolyze compounds in the medium after iodine reagents are pressed and produce a clear zone around the test well [11].

Based on IA measurements, we found mold isolates with varying IA values. IA values with a range of 0 - 0.5 mm are produced by 8 mold isolates (UNJCC F108, UNJCC F112, UNJCC F113, UNJCC F114, UNJCC F116, UNJCC F118, UNJCC F120, UNJCC F121), the range of values IA 0.51 - 1 mm is produced by 4 mold isolates, UNJCC F109, UNJCC F110, UNJCC F117, UNJCC F119, and ranges of IA values 1 mm are produced by 2 mold isolates UNJCC F111 and UNJCC F114. The UNJCC F111 mold isolate has the highest IA value of 2.73 mm. While UNJCC F112 isolates had the lowest IA value of 0.21 mm (Table 3).

Amylolytic index values were processed using one-way ANOVA to see the difference between the isolates used. The results of the one-way ANOVA analysis (Table 3) show that there are differences in the amylolytic index for each mold isolate, with a value of α (0.05)>Sig. 0.00. This shows that all mold isolates have a significantly different amylolytic index value. Because there were significant differences in the amylolytic index of all the isolates tested, the Duncan Multiple Range Test (DMRT) test was used to classify molds based on their amylolytic index. Duncan Multiple Range Test (DMRT) test shows that isolates UNJCC F108, UNJCC F112, UNJCC F113, UNJCC F115, UNJCC F116, UNJCC F117, UNJCC F118, UNJCC F120 and UNJCC F121 have amylolytic index values that are not significantly different from the range 0.20-0.50 mm. Isolates UNJCC F110, UNJCC F111 and UNJCC F119 have amylolytic index values that are not significantly different from 0.5 - 0.8 mm. Isolates of UNJCC F109 F110, F114 , and UNJCC F119 have amylolytic index values that are not significantly different from the range 0.70 - 1.11 mm. Whereas UNJCC F111 has an amylolytic index value that is significantly different from the value of 2.73 mm against 13 other isolates at a confidence level of 5% (Table 3).

TABLE 3. Amylolytic Index value (AI) origin of crab mold isolate in medium SA at 27 ºC for 72 h.

Code Isolate	AI Value (mm) (Mean±SE)
(UNJCC F108)	0.41^{a}±0.03
(UNJCC F109)	0.90^{c}±0.14
(UNJCC F110)	0.81^{bc}±0.13
(UNJCC F111)	2.73^{d}±1.90
(UNJCC F112)	0.21^{a}±0.08
(UNJCC F113)	0.26^{a}±0.20
(UNJCC F114)	1.11^{c}±0.12
(UNJCC F115)	0.25^{a}±0.09
(UNJCC F116)	0.44^{a}±0.02
(UNJCC F117)	0.51^{ab}±0.07
(UNJCC F118)	0.41^{a}±0.06
(UNJCC F119)	0.79^{bc}±0.05
(UNJCC F120)	0.42^{a}±0.03
(UNJCC F121)	0.22^{a}±0.03

The difference in amylolytic index obtained is because each mold has a different ability to produce amylase enzymes. Type and Characteristics affect the ability of the mold to produce amylase enzymes [8]. It can also be influenced by environmental factors. Environmental factors that can influence the production of amylase enzymes are temperature, pH, specific substrate and agitation intensity [36].

Characterization of Amylolytic Potential Molds

Characterization of amylolytic potential mold was carried out by observing the characteristics of mold isolates. Characteristic macroscopically and microscopically of mold was observed using PDA media incubated for 7 days at 28 ºC [3]. Based on microscopic observations, 71.42% of mold isolates had uniseriate and conidia radiate vesicles, uniseriate-branched molds of 7.14% and biseriate-branched molds of 7.14%. Mold with hyphae which had septa were 21.42 % and molds in the hyphae do not have septa (asepta) as much as 78.57% (Table 4).

TABLE 4. Microscopic characteristics of amylolytic mold isolates after 7 days incubation at 27 ºC on PDA medium.

Code of Isolate	Vesicles	Conidia	Hyphae
(UNJCC F108)	Uniseriate	Radiate	Aseptate
(UNJCC F109)	Uniseriate	Radiate	Aseptate
(UNJCC F110)	Uniseriate	Branched	Aseptate
(UNJCC F111)	Biseriate	Branched	Aseptate
(UNJCC F112)	-	-	Septate
(UNJCC F113)	-	-	Septate
(UNJCC F114)	Uniseriate	Radiate	Septate
(UNJCC F115)	Uniseriate	Radiate	Aseptate
(UNJCC F116)	Uniseriate	Radiate	Aseptate
(UNJCC F117)	Uniseriate	Radiate	Aseptate
(UNJCC F118)	Uniseriate	Radiate	Aseptate
(UNJCC F119)	Uniseriate	Radiate	Aseptate
(UNJCC F120)	Uniseriate	Radiate	Aseptate
(UNJCC F121)	Uniseriate	Radiate	Aseptate

Based on the macroscopic observations of 14 mold isolates, 35.71% of brown ocher-colored mold isolates were fluffy textured, 14.28% were white fluffy textured, 7.14% for cream-fluffy color, texture, ivory-powdey, blue green-rocky, warm grey-fluffy, cream-velvet, olive green-fluffy, gold-fluffy. The texture of mold isolates is dominated by fluffy texture, which is 78.57%. A total of 14 mold isolates had a characteristic of zonation of 64.29%, there was a growing zone of 92.86%, there was an exudate drop of 71.43% and a radial furrow of 62.29% (Table 5).

The results obtained clearly show that 71.4% of isolates belong to the genus *Aspergillus* because they have a surface colour of brown ocher, there are zonation, growing zones, exudate drop and radial furrow. Based on microscopic characteristics of the *Aspergillus* genus have radiate shape conidia (Fig. 3F). This is in accordance with

characteristics of the genus *Aspergillus* has the surface colour dark brown to black, zonation, exudate drop, growing zone, radial furrow and microscopically seen from conidia shaped radiate [10]. The UNJCC F111 mold isolate which has the highest AI value is thought to belong to the genus *Penicillium* because it has a blue-green colour, without obvious zonation, and exudate drop and radial furrow. Microscopic characteristics of conidia in the form of branched (Fig. 3C). Characteristics of genus *Penicillium* has white to blue-green and has a branched conidial head [17].

In this study, amylolytic molds originated from crabs is domination with genera *Aspergillus* and *Penicillium* were found. This is in accordance with the research of Safitri and Samingan [8], that the amylolytic potential mold is dominated by the genus *Aspergillus* and *Penicillium*.

TABLE 5. Macroscopic characteristics morphology of the amylolytic mold (Incubation 7 days at 27 ºC on PDA media).

Code Isolate	Colour		Texture	Zonation	Growing zone	Exudate drop	Radial furrow
	Surface	Reverse					
(UNJCC F108)	White	Ivory	Fluffy	-	v	-	v
(UNJCC F109)	Cream	Ivory	Fluffy	v	v	v	v
(UNJCC F110)	Ivory	Cream	Powdery	-	v	-	-
(UNJCC F111)	Blue green	Cream	Rocky	-	v	v	v
(UNJCC F112)	Warm Grey III	Sepia light	Fluffy	v	v	v	v
(UNJCC F113)	White	Light orange	Fluffy	-	-	v	-
(UNJCC F114)	Cream	Lemon	Velvety	v	v	-	v
(UNJCC F115)	Brown ochre	Cinnamon	Fluffy	v	v	v	v
(UNJCC F116)	Brown ochre	Cinnamon	Fluffy	-	v	v	-
(UNJCC F117)	Olive green	Bistre	Fluffy	v	v	v	v
(UNJCC F118)	Brown ochre	Cinnamon	Fluffy	v	v	v	v
(UNJCC F119)	Brown ochre	Cinnamon	Fluffy	v	v	v	-
(UNJCC F120)	Gold	Sanguine	Fluffy	v	v	-	v
(UNJCC F121)	Brown ochre	Cinnamon	Fluffy	v	v	v	-

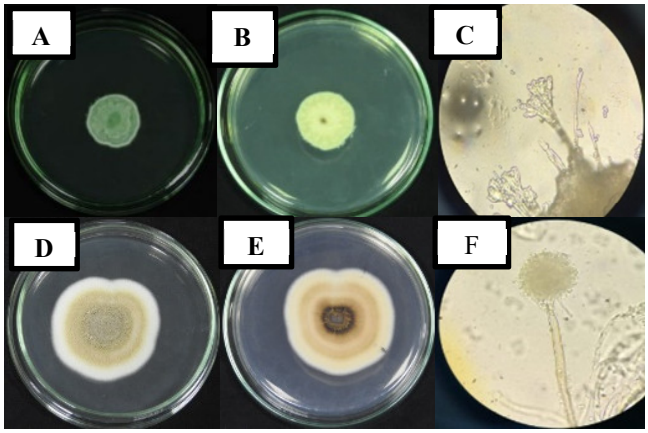

FIGURE 3. Morphological characteristics of amylolytic fungi from crabs. (A) top side UNJCC F111 colony; (B) reverse side the mold colonies of UNJCC F111 isolates; (C) Conidia of mold UNJCC F111 isolates (M = 1000×); (D) top side UNJCC F119 colony; (E) reverse side the mold colonies of isolates UNJCC F119; (F) The mold conidia of isolates UNJCC F119 (M = 1000× were incubated for 7 days at 28 ± 2 °C on PDA medium).

SUMMARY

Crab is a good substrate for mold growth. Based on the results of the isolation of mold from the crabs at 2 sampling locations, 30 representative mold isolates were obtained. Amylolytic mold selection in 30 mold isolates found that 46.7% of mold isolates had the potential to produce amylase enzymes. UNJCC F111 isolate has the highest IA value of 2.73 mm and is thought to belong to the *Penicillium*. As many as 71.4% of the 14 amylolytic potential mold isolates were thought to belong to the *Aspergillus*. Therefore, this study clearly demonstrates that the amylolytic molds isolated from crabs mainly belong to *Aspergillus* and *Penicillium*. However, both genera are recognized as GRAS (Generally

Regarded as Safe). Therefore, there is a high potential for using these isolated as potential biofactory for amylases production in the industry.

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