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Proteolytic Enzyme Activity of *Rhizopusoligosporus*Sp

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ABSTRACT
The objectives of this study was to obtain protease enzymes from two (2) isolates of RhizopusoligosporusSp, namely isolates of R. oligosporus WS and R. oligosporus RT. The short-term objectives of this study were to perform qualitative tests of protease enzyme activity, produce and optimize the production time of protease enzymes and to test the activity of protease enzymes.
Two isolates of R.oligosporus WS and R.oligosporus RT were first rejuvenated on Potato Dextro Broth (PDB) media. Characterization was done by observing the macroscopic and microscopic characteristics of the fungus. Macroscopic observations included colony color, color and density of mycelium. Microscopically the isolates were observed under a microscope to see the color and shape of the conidia, sporangiospore length, length and shape of the columella.Then the isolates were tested for their proteolytic activity on Skim Milk Agar (SMA) selective media and then produced on selective liquid media. This study used a randomized block design (RAK) with variations in the incubation time of protease enzyme production, namely 3, 5, 7, 9 and 11 days with Three (3) replications for each treatment with parameter 1). Qualitative test of protease enzyme activity, 2) production and optimization of protease enzyme production time, 3) test protease enzyme activity (U/ml), protein content and specific enzyme activity (U/ml).
The results showed that the two isolates R. oligosporus WS and R. oligosporus RT were identified macroscopically and microscopically as having brownish gray conidia with conidia lengths of 11.4 μm and 12.0 μm. Both isolates had the ability to hydrolyze protein with a hydrolysis index of 1.82 mm and 1.56 mm. The isolates of R. oligosporus WR and R. oligosporus RT produced the highest protease enzymes at an incubation time of 7th days which were 1.83 ± 0.014 U/mL and 1.51 ± 0.084. R. oligosporus WR and R. oligosporus RT isolates were able to produce the highest protease-specific enzymes at incubation time of day 7 which were 1.52 ± 0.03 U/mL and 1.20 ± 0.06 U/ml respectively
Keywords: *Activity, Enzyme, Proteolitic. R. oligosporus*

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I. INTRODUCTION

In the last decade, there has been a more rapid increase in the use of enzymes because they are efficient, selective, catalyze reactions without side products and are environmentally friendly. One source of proteases is microbes. Microbial proteases can be classified as serine proteases (EC.3.421), sulphydryl (EC. 3422), acid proteases (EC.3.423) and metalproteases (EC. 3424).

Protease is one of the most important industrial enzymes and has commercial value reaching 60% of total enzyme sales worldwide and is one of the mainstay products of thermophilic enzymes that are widely used in the food processing, detergent and pharmaceutical industries (Uyar*et al*., 2011). Enzymes used in industry in Indonesia are still imported. Whereas Indonesia is able to produce protease enzymes by looking for sources of microorganisms that produce these enzymes.

Proteolytic microbes are microbes that can hydrolyze proteins into smaller peptides or amino acid units. Protease is a proteolytic enzyme that catalyzes the breaking of peptide bonds in proteins. Proteases are physiologically needed for the life of organisms in plants, animals and microbes. Proteases not only play a role in cellular metabolic processes, but can also be applied in the industrial sector (Gupta *et al*., 2002; Rao *et al*., 1998). Protease is an important enzyme and has high economic value because of its wide application. Industries that use protease include the detergent, leather, textile, food, dairy processing, pharmaceutical, food, beer and waste industries (Moon and Parulekar, 1993; Ward, 1985).

Microbes are the most potential source of enzymes compared to plants and animals. The use of microbes is more profitable because they grow fast, can grow on cheap substrates, are easier to increase yields through regulation of growth conditions and genetic engineering. Various types of bacteria and molds were reported to be able to produce proteases (*Bacillus amylolique, B. licheniformis, B. subtilis, B. cereus, B. polymyxa, B. thermoproteolyticus, Mucorpucillus, M. miehei, Aspergillusoryzae, A. sojae*dan*A. phoenicis*), some

of which have been used on an industrial scale (Saono and Basuki, 1978). Species *R. oligosporus*Sp is one of the potential protease-producing microbes. *R. Oligosporus* is currently used in tempeh fermentation. According to Baehaki*et al*. (2011), several microorganisms are known to produce protease enzymes for commercial applications: *Bacillus, Lactobacillus, Mucor, Aspergillus* and *Rhizopus.* The importance of this enzyme is so important that it is necessary to look for enzymes from microbes with different habitats so that it is hoped that the enzymes produced have unique characters and meet industrial needs, both chemical, medical and agricultural and food industries.

One of the protease-producing molds, especially extracellular proteases, is mostly produced by the *Rhizopusoligosporus* Sp.Rhizopusspecies belonging to the order Mucorales, family Mucoraceae. The genus *Rhizopus* consists of several species, including *R. arrhizus, R. oligosporus, R. microsporus, R. shipperae, R. stolonifer* and *R. oryzae*. Tempe fermentation was first discovered using *R. oryzae* then *R. oligosporus* until now used in tempeh fermentation. *R. oligosporus* is a mold that is widely used in the manufacture of tempeh, widely found in nature because of its saprophytic nature (Weng and Chen,2011). This mold is known as a mold that is capable of producing lipase enzymes and protease enzymes to break down fat and protein media (Aunstrop, 1979).

R. oligosporus WS is a mold isolated from the hibiscus plant, namely from hibiscus leaves that grow in the Sanur area of Bali, while *R. oligosporus* RT is isolated from the tempe yeast which is used in the manufacture of unbranded tempeh in the Sesetan area. These two types of molds had the following characteristics: *R. oligosporus* WS had white mycelia, conidia gray-brown color, conidia length 11.4 um, *sporangiospore* length 160 um, and *columella*length 27.1 um, while *R. oligosporus* RT has the characteristics of having white mycelia, brownish gray conidia color, gobos conidia shape, conidia length 12 um, *sporangiospore* length 160 um, and *columella* length 27.1 um

The production of protease enzymes is influenced by the time factor of enzyme production. Proper production time will result in maximum enzyme activity. Suganthi*et al*. (2013) reported that *Bacillus licheniformis* species produced maximum enzyme activity at 24 hours of production. Enzyme production in this study used variations in production time between 3 to 11 days with 2 day intervals. The appropriate production time is expected to maximize the protease enzyme activity obtained. Therefore, the aim of this study was to obtain protease enzymes from two isolates of *R. oligosporus* WS and *R. oligosporus* RT and to test their activity at production times between 3 days and 11 days.

II. METHODE AND MATERIAL

Isolate rejuvenation

A total of one ozeneedle of *R. oligosporus* derived from pure isolate in oblique stock was taken and inoculated into a test tube containing Potato Dextro Agar (PDA) media, then incubated for 5 days at room temperature.

Characterization of *R. oligosporus.*

Characterization was done by observing the macroscopic and microscopic characteristics of the fungus. Macroscopic observations included colony color, color and density of mycelium. Microscopically, isolates were observed under a microscope to see the color and shape of the conidia, spongiospore length, length and shape of the columella.

Qualitative Test of Proteolytic Activity.

*R.oligosporus*sp isolate rejuvenated on Potato Dextro Broth (PDB) media for 24 hours at 37°C. Activity qualitative test protease enzymes are carried out byinoculated *R.oligosporus*sp isolate on Skim Milk Agar (SMA) media. SMA medium contains peptone (0.1%w/v), NaCl (0.5% w/v), agar (2.0% w/v) and skim milk (10% v/v) (Chu, 2006). A positive test is indicated by the presence of clear zone around bacterial colonies on high SMA media surface.

Protease Production (Charles *et al*, 2008;Fatoni*et al*, 2008)

Fungal isolates were grown for 5 days on slanted medium containing Dextro Agar (PDA). A total of 0.5 mL of spore suspension (10^7 spores/mL) of mold isolate was inoculated into Potato Dextro Agar (PDA) medium, 50 mL containing (g/mL). L): Sucorse (30), KCl (0.5), MgSO4 (0.5), FeSO4 (0.01), K2HPO4 (1.0), NaNO3 (2.0), Casein (10) (pH = 7). Phosphate buffer was added to the medium. The cultures were then incubated at room temperature on a Rotary Shaker at a speed of 120 rpm, and were taken at incubation times of 3,5,7,9 and 11 days. The enzyme that has been produced is then filtered with Whatman paper no 1 to get the supernatant which is a crude enzyme, then centrifuged at 10,000 rpm for 20 minutes at 4^oC.

Protease enzyme activity test

The crude extract of the isolated enzyme was then determined for its proteolytic activity based on Cupp and Enyard (2008). A total of 1 mL of crude extract of the enzyme was added to 0.65% casein substrate (0.65 g of casein in 100 mL of 0.05 M K-phosphate buffer pH 7.5). The reaction mixture was incubated at 37°C for 10

minutes. Termination of the reaction was carried out by adding 5 mL of 110 mM TCA reagent and incubating again at 37°C for 30 minutes. A total of 2 mL of filtrate was separated by centrifugation at 10000 rpm for 10 minutes. A total of 5 mL of Na2CO3 and 1 mL of folinciocalteaureagent were added to the filtrate and incubated at 37°C for 30 minutes. The absorbance of the mixture was measured using a spectrophotometer at a wavelength of 660 nm. One unit of enzyme activity was defined as the amount of enzyme required to release 1µmol of tyrosine on a casein substrate per minute.

Determination of protein content

Determination of protein content by Lowry Method (Plummer, 1979). A total of 0.1 mL of crude enzyme extract solution (pH 7) added 9.8 mL of Na2CO3 and 0.1 mL of K-Na-tatrate and 0.1 mL of CuSO4 were shaken slowly. Then it was rapidly incubated with 1 mL of Folin-Ciocalteau and incubated at room temperature for 30 minutes. The absorbance of the solution was then measured at the maximum wavelength of BSA using a UVVis spectrophotometer. The protein content was determined by linear regression against the BSA standard curve using the following formula: Protein (mg protein/mL) = y – cm

Description:

Y = Sample Absorbance

c = Slope on the standard curve BSA

m = Intercept on BSA standard curve

Determination of specific enzyme activity

The specific activity of the enzyme was obtained by determining the unit of enzyme activity divided by the protein content of the enzyme. Thus the formula used is:(units/mg protein) = Units of activity (Units)/Protein content (mg)

III. RESULTS AND DISCUSSION

Confirmation Test of *R. oligosporus* WR and *R. oligosporus*RT isolates

The results of macroscopic and microscopic observations showed that two (2) isolates of *R. oligosporus* WR and R. oligosporus RT were confirmed as isolates used in this study to test their ability to produce proteolytic enzymes. Macroscopically, the two isolates can be seen in Figure 1.

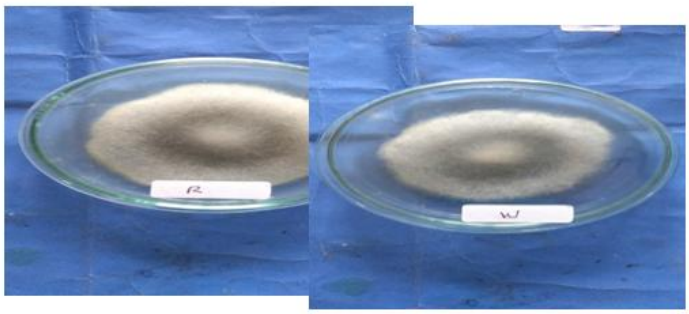

Figure 1. Isolates of *R. oligosporus* WR and *R. oligosporus* RT

The results of observations on the density of mycelia and conidia of the two isolates of *R. oligosporus*WR and *R. oligosporus* RT can be seen in Table 1

Table 1. Mycelium density of *R. oligosporus* WR and *R. oligosporus* RT

Code Isolate of*R. oligosporus*	Origin of Isolate	Color of Conidia	Observation in slant PDA	
			Mycelia	Conidia
WR	Leave of Herbicus	Brownish gray	+++	+++
RT	Mold of Tempeh from Bali	Brownish gray	+++	+++

Description : + = Congested , ++ = a bit dence, +++ = Very dence

Figure 1 and Table 1 show that macroscopically *R. oligosporus* has a brownish-gray characteristic, this shows that the two isolates *R. oligosporus* WR and *R. oligosporus* RT have the same characteristics as the characteristics of *R. oligosporus* according to the results of Pitt and Hocking's research. (1997) and Samson et al. (1995). The results of this study also showed the growth of the two isolates on Potato Dextro Agar slanted media with very dense mycelia and conidia.

The results of the study on the characteristics of the two isolates of *R. oligosporus* WR and *R. oligosporus* RT microscopically can be seen in Table 2. Table 2. Microscopic characteristics of *R. oligosporus* WR and *R. oligosporus* RT

Table 2. Microscopic characteristics of *R. oligosporus* WR and *R. oligosporus* RT

Characteristics	*R. oligosporus* WR	*R. oligosporus* RT	*R. oligosporus* (Samson *et al.*, (2010).
Color of Mycelia	White	White	White*
Color of Conidia	Brownish gray	Brownish gray	Brownish gray*
Conidia shaoe	Globose-Elipsoidal (Oval)	Globose	Globose-ellipsoidal (Oval)
Length of conidia	11.4 µm	12 µm	7-24 µm
Length of sporangiospore	160 µm	170.1 µm	150-400 µm
Length of sporangium	90 µm	92 µm	80-120 µm
Texture of sporangiospore	Smooth	Smooth	Smooth**
Length Columella	27.1	27.1	25 -27 µm *
Columela Shape	Globose-sub globose	Globose-sub globose	sub globose- Globose
Chlamidospore	Single-Short Chain	Single-Short chain	Abundant, single (short chain) **

Qualitative Enzyme Activity

Proteins contained in SMA selective media act as inducers for protease enzymes. The resulting clear zone is the result of hydrolysis of protein substrates contained in SMA media by protease enzymes produced by isolates of *R. oligosporus* sp. SMA media contained peptone and skim milk as the main carbon sources for the metabolic needs of *R. oligosporus* sp. Proteolytic activity was indicated by the formation of a clear circle around the mold colony. The two isolates *R. oligosporus* WS and *R. oligosporus* RT had the ability to hydrolyze protein with a hydrolysis index of 1.82 mm and 1.56 mm respectively.

Protease Enzyme Activity

The results showed that the two isolates *R.oligosporus* WR and *R. oligosporus* RT were able to produce protease enzymes during the incubation period. Table 3shows the activity of the protease enzyme during the incubation period.

Table 3. Protease Enzyme Activities

Isolate	**Time of Incubation (Days)**				
	3	5	7	9	11
***R. oligosporus* WR**	1.48 ± 0.028	1.80 ± 0.056	1.83 ± 0.014	1.76 ± 0.014	1.65 ± 0.014
***R. oligosporus* RT**	1.09 ± 0.042	1.29 ± 0.028	1.51 ± 0.084	1.43 ± 0.014	1.28 ± 0.014

In Table 3, it can be seen that the longer the incubation time until the 7thday there was an increase in protease enzyme activity, after that the protease enzyme activity decreased on the 9th and 11th days. Cells were able to synthesize proteases as a reaction to inducers in the medium (Said, and Likadja, 2012; Suhartono Suhartono and Wiwit Artika, 2017). Protease is an inducible enzyme because its synthesis requires an inducer, namely casein (Falcb, 1991). The presence of casein in the medium causes cells to synthesize proteases. The production of protease enzymes was influenced by *R. oligosporus* WR and *R. oligosporus* RT by the time factor of enzyme production. The appropriate production time will result in maximum enzyme activity. The breakdown of casein substrates into amino acids is needed as a building block for proteins. According to Kamelia*et al*. (2005) the existence of an induction and repression mechanism allows cells to regulate the content and activity of enzymes according to cell needs and nutritional conditions. The induction mechanism makes cells more economical in utilizing nutrients from their environment.

Protein content of *R. oligosporus* WR and *R. oligosporus* RT

The results showed that the two isolates *R.oligosporus* WR and *R. oligosporus* RT during incubation there was a decrease in protein content. Table 4 shows the protein levels of the two isolates during the incubation period.

Table 4. Protein content during incubation (mg/mL)

Isolate	**Time of Incubation (Days)**				
	3	5	7	9	11
R. oligosporus WR	1.63 ± 0.042	1.48 ± 0.056	1.21 ± 0.098	1.16 ± 0.014	1.09 ± 0.014
R. oligosporus RT	1.39 ± 0.014	1.25 ± 0.028	1.26 ± 0.098	1.12 ± 0.056	1.12 ± 0.014

In Table 4 it can be seen that two isolates *R.oligosporus* WR and *R. oligosporus* RT produce the protein ranging 1.63 ± 0.042 - 1.09 ± 0.014mg/mLand 1.39 ± 0.014- 1.12 ± 0.014mg/mL during incubation period. During the incubation time from 3 days to 11 days there was a decrease in the protein content of the two isolates*R.oligosporus* WR and *R. oligosporus* RT. But the two isolates showed differences in the amount of protein produced. *R. oligosporus* WR produced higher protein than *R. oligosporus* RT. This is due to differences in the source of the isolates and the stress from which the isolates originate (Duniaji*et al*., 2019)

Enzyme is a protein, so a high amount of enzyme can be seen from the high protein content. According to Rahayu (2014) based on its function, proteins are divided into functional proteins and structural proteins.

Functional proteins are examples of digestive enzymes, while structural proteins are used for growth such as cell formation.

In Figure 3. It is seen that there is a tendency for enzyme protein levels to decrease during incubation. This is probably because *R. oligosporus* WR and *R. oligosporus* RT are in the logarithmic phase. In the logarithmic phase, *R. oligosporus* WR and R. oligosporus RT required a lot of protein for mycelia growth.

Specific Enzyme Activities

The results showed that during the incubation period there was an increase in specific enzymes up to day 7 of the two isolates *R. olgosporus* WR and *R. oligospotus* RT and on day 9 to day 11 the specific enzyme activity of the two isolates *R. olgosporus* WR and *R. oligospotrus*RT. decrease. Table 3 shows the specific enzyme activity during incubation.

Table 4. Protease-Specific Enzyme Activities

Isolate	**Time of Incubation (Days)**				
	3	5	7	9	11
***R. oligosporus* WR**	0.090± ± 0.04	1.21 ± 0.02	1.51 ± 0.07	1.52 ± 0.03	1.51 ± 0.02
***R. oligosporus* RT**	0.784 ± 0.02	1.03 ± 0.04	1.19 ± 0.03	1.20 ± 0.06	1.14 ± 0.06

Specific enzyme activity indicates the purity of an enzyme. The higher the specific activity of the enzyme, the higher the level of purity of the enzyme. This is due to the loss of non-enzyme proteins at several stages of separation that are passed in the purification of enzymes (Suganthi*et al*., 2013). Specific activity also indicates that the protein produced by microbes into the growth medium is the desired target protein

IV. CONCLUSIONS AND SUGGESTIONS

The results showed that the two isolates *R. oligosporus* WS and *R. oligosporus* RT were identified macroscopically and microscopically as having brownish gray conidia with conidia lengths of 11.4 µm and 12.0 µm. Both isolates had the ability to hydrolyze protein with a hydrolysis index of 1.82 mm and 1.56 mm. The isolates of *R. oligosporus* WR and *R. oligosporus* RT produced the highest protease enzymes at an incubation time of 7^{th} days which were 1.83 ± 0.014 U/mL and 1.51 ± 0.084. R. oligosporus WR and *R. oligosporus* RT isolates were able to produce the highest protease-specific enzymes at incubation time of day 7 which were 1.52 ± 0.03 U/mL and 1.20 ± 0.06 U/ml respectively.

It is recommended that the use of *R. oligosporus* to produce protease enzymes requires an incubation period of 7^{th} days

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