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Characteristics and Selulotic Activities of Endophytic Fungi in Macroalgae (*Sargassum* sp., *Gracilaria* sp., *Gelidium* sp., and *Caulerpa* sp.) from Seagrass Habitat in Pari Island, Thousand Islands, Jakarta

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Abstract

Macroalgae is one source of energy feedstock for bioethanol that has the potential to be developed as a renewable energy. Bioethanol is a product of fermentation of carbohydrates that require molds to produce cellulose decomposer enzyme complex into simpler forms. The purpose of this study were to determine the diversity of endophytic fungi associated with living macroalgae in seagrass habitats Pari Island, Thousand Islands, as well as measuring the activity of the endophytic fungus selulotik. A total of 21 isolates of fungi were isolated from four types of macroalgae, such as *Sargassum* sp., *Caulerpa* sp, *Gracilaria* sp. and *Gelidium* sp. The fungus was isolated on medium CMC (*Carboxy Methyl Cellulose*), congo red staining, and for the calculation of enzyme activity using the Milles method. The highest selulotic index value obtained from isolates of *Aspergillus westerdijkial* from *Caulerpa* sp. (PCLAR 6).

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Cellulase activity for *Aspergillus versicolor* and *Aspergillus sydowii* optimum on day 2, while *Penicillium citrinum* and *Aspergillus westerdijkial* optimum on day 1. *Aspergillus versicolor* and *Aspergillus sydowii* selulotik showed optimum activity at pH 7 and temperature phosphate 40 ^{0}C.

Keywords: fungus; cellulose enzyme; endhophytic; macroalgae.

1. Introduction

Seagrass is a habitat that has a fairly high of biodiversity. It can be seen from the number of associated marine organisms in seagrass habitats. Seagrass habitats provide habitat and shelter for a number of marine organisms. One of marine organisms that live in seagrass habitats are macroalgae (seaweed).

Macroalgae including one marine biological resources that are mainly founded growing in Indonesian waters. Macroalgae has great potential to be developed, because it has an important role in terms of both ecological and economical. Currently, macroalgae can be used as a source of energy or raw materials of bioethanol feedstock. Bioethanol is one of the alternative renewable energy. Bioethanol has a higher energy efficiency compared to petroleum. In addition, ethanol also is more environmentally friendly. Bioethanol is a product that can be made from the fermentation of substrates containing carbohydrates (sugar, starch, or cellulose). Cellulose is a polysaccharide that abundant in the earth and can be converted into glucose by hydrolysis [1]. Cellulose is the main component of plant cell wall constituent and a number of the most abundant biopolymer in nature [2].

Naturally cellulose degraded by insects, earthworms, fungi and bacteria. Although fungus are commonly as cellulose decomposer. Fungi that is able to decipher cellulose derived from groups of Ascomycota, Basidiomycota, Zigomycota, and Deuteromycota [3]. Fungi degrade cellulose to monomer in produce extracellular enzymes, namely cellulase [4, 5, 2]. Cellulase enzymes capable to hydrolyze cellulose into simple sugars or glucose and can be used in bioethanol production. Production of bioethanol requires glucose as substrate of fermentation. Cheapest source of glucose are come from the breakdown of cellulose. Cellulose is available abundance in nature and potentially to be used as a feedstock for ethanol production. The hydrolysis of cellulose into monosaccharide, disaccharides and oligosaccharides by using chemical and biological process. Hydrolysis by chemical means using strong acids while biological hydrolyze by using pure enzyme or microorganisms producing cellulase enzyme [6].

During this hydrolysis process done to change the bioethanol feedstock into simple sugars done acid, but acid hydrolysis using has some shortcomings such costs are relatively expensive, which produced lower monosaccharide, a process that is carried out long enough , and acidic waste handlers are not easy [6]. In Indonesia, the use of cellulose and cellulase enzymes as one of the new renewable energy sources at the research stage. The potential is large enough to encourage the utilization of cellulose and cellulase enzymes as a source of renewable energy need to be developed further. The purpose of this study was to determine some of which type of mold that is of some macroalgae, determine the ability of the activity of the fungus-mold selulotic the qualitative and quantitative characteristics and determine the optimal enzyme cellulase to hydrolyze.

Currently, the hydrolysis process to produce bioethanol is performed by using acid, but has some disadvantages

such as costs are relatively expensive, produced lower monosaccharide, a longer process, and requires acidic waste handlers [6]. In Indonesia, the use of cellulose and cellulase enzymes as a source of renewable energy is still at the research stage. Because the potential is quite large, so that utilization of cellulose and cellulase enzymes as a source of renewable energy need to be developed further. The purpose of this study was to determine some kind of fungus isolated from several types of macroalgae, determine the ability of the fungus selulotic activity qualitatively and quantitatively and determine the characteristics of the optimal enzyme cellulase to hydrolyze.

2. Materials and Methods

Macroalgae was collected from seagrass habitat of Pari Island, Thousand Islands, Jakarta, Indonesiaa on March 2013 and transported to laboratory of Surfactant dan Bioenergy Research Center and laboratory of Marine Bioprospecting, Department of Marine Science and Technology, Bogor Agricultural University, Bogor, Indonesia. Isolation of fungi on macroalgae started by washing samples using sterile sea wate , then cut into pieces at size 1 cm x 1 cm. Furthermore, placed it on Petri dishes containing Chloramphenicol Potato Dextrose Agar (PDA). Then, mold incubated at room temperature for 3-7 days.

2.1 Purification and Identification of Fungus

Colonies of mold that grows during the isolation process, purified by colony propagation technique that is cut and transferred aseptically a half of mold mycelium into a new culture medium. Isolates of fungi that have been grown on PDA Chloramphenicol selected and transferred into a new petri dish containing Chloramphenicol PDA medium. Furthermore, colonies were incubated at room temperature for 3-7 days until sporulation. Colony that has pure and can grow well selected and replanted in a new petri dish as much as two replications. Furthermore, this pure mold isolates identified in SEAMEO BIOTROP Laboratory.

2.2 Cellulase Activity

Selulotic activity qualitatively was tested with Congo red staining method 0.1 % and isolate of mold spotted on agar medium CMC 1 %. Fungi were incubated for 5 days at room temperature, then tested the selulotic activity by congo red as much as 15 ml and allowed to stand for 30-60 minutes. Diameter of clear zone and colony diameter formed was measured on 3 replications. Cellulase activity can be calculated from cellulase index. Cellulase index is the ratio between diameter of the clear zone and a diameter of colony. The larger selulotic index, the greater enzymes produced by the fungus isolates. Selulotic activity index (IS) is obtained by using the following formula [7]:

$$\text{IS} = \frac{(\mathbf{dzb - dk})}{\mathbf{dk}}$$

where :

IS = Selulotic Index

dzb = clear zone diameter (mm)

dk = colony diameter (mm)

2.3 Determination of Optimum Time for Cellulase Enzyme Production

Determination of the optimum time of cellulase enzyme production began with the determination of the time of pouring the inoculum. This is conducted in order to know the exponential growth time of mold in inoculum to be used. The timing of inoculum was carried out by culturing 4 selected loops isolates in cellulase activity qualitatively in 50 mL PDL (Potato Dextrose Liquid). The cultures were incubated at room temperature in hot shaker with agitation speed of 150 rpm. Sampling was conducted every day for 5 days with range of 24-hour sampling period to measure the spore density. After that, mold growth curve was made to determine the best time for inoculum pouring of production medium. After inoculum pouring in media production was known, then it followed by determination of the optimum activity of cellulase enzymes. Total of 10 mL nutrient broth that contains cell cultures was inoculated in PDL media. The inoculum was poured into 1% CMC liquid medium as much as 90 mL. Samples were stored in water bath at temperature of 50°C. Then 3 mL of the filtrate was taken every day for crude extract. Crude extract was obtained by centrifuging the culture filtrate at speed of 4000 g for 15 min at room temperature. Then the supernatant solution was taken as 1 mL. Daily cellulase activity was determined by Miller [8] by mixing 1 mL of crude extract with 1 ml of liquid CMC. Then, the mixture was added 3 ml of DNS, strong shaking with vortex, and then incubated at temperature of 100°C for 25 minutes. After the solution was cold, the absorbance was measured at wavelength of 550 nm. The optimum production of cellulase enzymes is determined based on the highest activity in incubation period.

Cellulase activity is expressed in International Units U / mL. One unit is the amount of enzyme needed to break 1 mol of cellulose into reducing sugars per minute in test conditions. Glucose levels resulting from cellulose hydrolysis by cellulase enzymes was based on absorbance at wavelength of 575 nm.

$$Abs = (As-Ab)$$

where :

Abs = Absorbance

As = Sample Absorbance

Ab = Blank Absorbance

Absorbance values were then entered into equation obtained from standard curve of glucose. The result of equation is amount of obtained glucose. Figure 1 is the equation of standard curve of glucose and glucose levels.

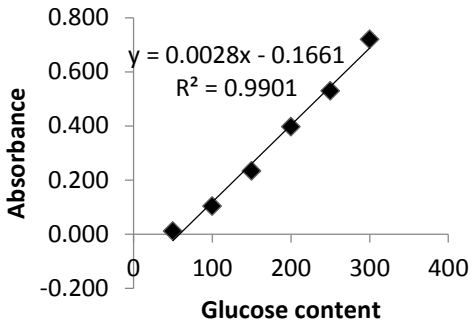

Figure 1: Standard curve of glucose

The equation of glucose level is :

$$y = 0.002x – 0,166$$

Where y = Sample absorbance

x = Glucose level

Glucose level that obtained can be entered into the equation of cellulase enzyme activity. Cellulase activity was calculated by the following modified formula [9].

$$\text{Sellulose enzyme activity (U/mL)} = \frac{glucose\ content\ X\ 1000}{vxtxBM}$$

where :

v = enzyme volume (1 mL)

t = incubation time (30 min)

BM = glucose molecule weight (180 Dalton)

2.4. Crude Enzyme Production

Production of cellulase enzymes is based on procedures and incubation time that have been known for the highest cellulase activity in which cellulase activity curves were generated. Production growth was incubated at 50°C in hot shaker with agitation speed of 150 rpm, and then the enzyme cellulase was harvested during the peak production period which has been obtained previously. Cell culture in production media containing

extracelluler cellulase enzyme production was centrifuged at speed of 10,000 x g for 15 minutes to separate the enzyme solution. The supernatant from centrifugation then was stored at temperature of 10°C as crude enzyme extract.

2.5. Characterization of Cellulase Enzymes

2.5.1. Optimum pH

pH Effect on enzyme activity was tested by adding 0.2 mL of enzyme reacted with 1.8 mL of the substrate. The substrate was prepared by mixing CMC into the buffer with various pH levels such as 3,4,5,6,7,8 and 9. Each enzyme was incubated at 30°C for 30 minutes. Cellulase enzyme activity was measured according to the test procedures before.

2.5.2. Optimum Temperature

Effect of temperature on enzyme activity was carried out by reacting 0.2 mL to 1.8 mL of enzyme substrate in which the substrate was prepared by mixing 1.8 g of CMC in optimum buffer pH. Enzymes that have been mixed with substrate and then incubated at temperature level between 30-90°C with range 10°C for 30 minutes of incubation time. Cellulase enzyme activity was measured according to the test procedures before.

3. Results and Discussions

3.1. Mold Endophytic Species of macroalgae

The results of isolation and identification of some endophytic mol from macroalgae *Gracilaria* sp., *Sargassum* sp., *Gelidium* sp. and *Caulerpa* sp. were 21 isolates. The Nast produced mold is from the genus *Aspergillus*, and there is one strain from genus *Penicillium*. (Table 1).

Macroalgae endophytic mold are molds that come from inside of macroalgae. The results shows that the identification of *Aspergillus* is widely available in endophytic macroalgae. In addition, *Penicilium* sp. was identified as isolate from *Gracilaria* sp. There are three types of unidentified isolates because of the unknown morphology. All isolates were grown in 1% agar CMC and formed a clear zone after given congo red.

3.2. Qualitative Cellulase Activity of Some Isolates

Among 21 isolates, four isolates were selected based on the highest selulotic index of each type of macroalgae. Four isolates were PGRAR1, PSAR3, PGEL9 and PCLAR6. Selulotic index of those four isolates are 2.06 (PGRAR1 / *Penicillium citrinum*), 3.34 (PSAR3 / *Aspergillus versicolor*), 3.22 (PGEL9 / *Apergillus sydowii*) and 4.33 (PCLAR6 / *Aspergillus westerdijkial*). The four isolates were selected for qualitative testing. All isolates that tested qualitatively has the ability to degrade 1% CMC substrate. It can be seen from clear zone that formed after the mold colony was colored by congo red as the effect of strong interaction with polysaccharides contained binding of ß-1, 4-glycosidic and ß-1, 3-glycosidic [10]. Formed clear zone around mold colony

indicates that the cellulose in the zone has already broken down into simpler unit and showed the presence of extracellular cellulase enzyme activity. The wide clear zone produced depends on CMC concentration and gelatin that used. The more the CMC and agar given, it will cause shrunk media pores so that the cellulase enzymes secreted will be more difficult to pass through and cause inhibition of cellulose degradation [11]. Zverlova *et al*. [12] states that the clear zone diameter is generally larger than colony diameter, since the cellulase enzymes secreted into the environment or media around the colonies is to degrade cellulose.

Table 1: Mold species generated from several macroalgae

No	Mold	Isolate code
1	*Penicilium citrinum*	PGRAR 1
2	*Aspergillus niveus*	PGRAR 2
3	*Aspergillus versicolor*	PSAR 1
4	*Aspergillus sydowii*	PSAR 2
5	*Aspergillus versicolor*	PSAR 3
6	*Aspergillus candidus*	PGEL 1
7	*Aspergillus sydowii*	PGEL 2
8	*Aspergillus sydowii*	PGEL 3
9	*Aspergillus sydowii*	PGEL 4
10	*Aspergillus sydowii*	PGEL 5
11	*Tidak teridentifikasi*	PGEL 6
12	*Tidak teridentifikasi*	PGEL 7
13	*Aspergilus sydowii*	PGEL 8
14	*Aspergilus sydowii*	PGEL 9
16	*Aspergillus sydowii*	PGEL 10
18	*Aspergillus sydowii*	PCLAR 1
19	*Aspergillus tereus*	PCLAR 2
20	*Penicilium citrinum*	PCLAR 3
21	*Aspergillus sydowii*	PCLAR 4
22	*Aspergillus sydowii*	PCLAR 5
23	*Aspergillus westerdijkial*	PCLAR 6

3.3. Optimum Time of Cellulase Enzyme Production

Mold growth was based on the spore density that measured daily for 5 days (Figure 1). Fungal isolates from *Sargassum* sp. (PSAR3/*Aspergillus versicolor*) was increased in density of mold or experienced exponential phase on 2nd and 3rd with total of 70 x 10^4 cells/mL. On day 4 and 5, A. *versicolor* fungal growth experienced declining phase. Fungal isolates from *Gracilaria* sp. (PGRAR1 / *Penicillium citrinum*) growth began to increase

at day 2 (30 x 10^4 cells / mL), and then at day 3 and 5, it decreased. Fungal isolates from *Caulerpa* sp. (PCLAR6 / *Aspergillus westerdjikal*) was increased (exponential phase) at day 2 to 3, with the highest cell number of 72.5 x 10^4 cells/mL. Fungal isolates from *Gelidium* sp. (PGEL9 / *Apergillus sydowii*) began to increase at day 2 to 4, with the highest cell number of 88 x 10^4 cells / mL. *Aspergillus sydowii* (PGEL9) isolated from macroalgae *Gelidium* sp. has the highest spore density than any other types (Figure 2).

Mold growth at optimum phase of each fungus was used as the best time determination for pouring the inoculum to production medium. This was conducted so the mold isolate will have the optimum spore amount, then at harvest time, media production of cellulase enzymes can produce more rugged enzyme amount. Isolates mold of *Sargassum* sp. / *Aspergillus versicolor* which is optimum at day 2, *Gracilaria* sp. / *Penicillium citrinum* at day 1, *Caulerpa* sp. / *Aspergillus westerdijikal* at day 2 and *Gelidium* sp. / *Aspergillus sydowii* on day 3.

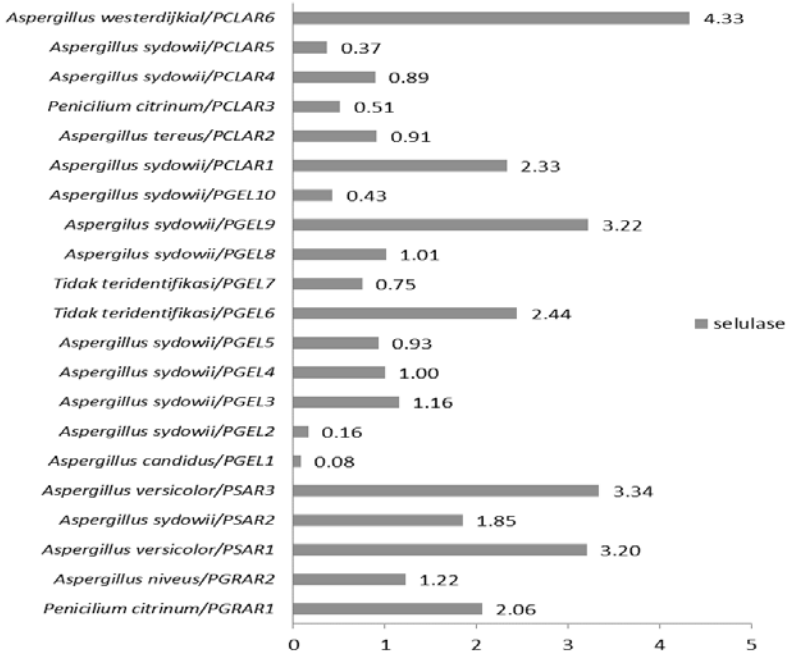

Figure 1: Index Value of Selulotic Mold

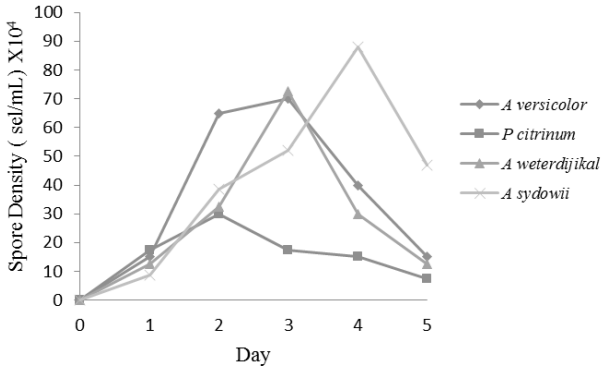

Figure 2: Growth curves of isolated mold

3.4. Cellulase Enzyme Activity

The enzyme activity of cellulase (CMC-ase) of four selected fungi isolates have varied values (Figure 3). *Caulerpa* sp (PCLAR6) with the type of *Aspergillus westerdjikal* has the highest enzyme activity at day 2 with value of 22,860x10^{-4} U/ml, the isolates from *Gracillaria* sp. (PGRAR1) with fungus *Penicillium citrinum* has the highest enzyme activity value at day 1 with value of 17,489x10^{-4} U/ml, the isolates from *Gelidium* sp. (PGEL9) with *Aspergillus sydowii* has the highest value of enzyme activity at day 3 with 24,554x10^{-4} U/mL and isolates of *Sargassum* sp. (PSAR3) with *Aspergillus versicolor* has the highest value of enzyme activity at day 3 with 20,575x10^{-4} U/mL. Determination of of cellulase enzymes production optimum time is conducted by determining the amount of cellulase enzyme activity with 1 day interval for 5 days. The optimum time of each mold was also used to harvest the fungus spores because it is the effective state in terms of time and number of spores. Isolates mold of *Sargassum* sp./ *Aspergillus versicolor* is optimum at day 2, *Gracilaria* sp. / *Penicillium citrinum* is at day 1, *Caulerpa* sp. / *Aspergillus westerdijikal* is at day 1 and *Gelidium* sp / *Aspergillus sydowii* is at day 2. Fardiaz [13] explains that enzyme production in bioprocess requires several factors, such as types of microbes, growth curve and the optimum conditions to increase enzyme activity.

Value of cellulase enzyme activity indicates the amount of CMC degradation rate to be glucose. Inoculum culture that inoculated into CMC media was derived from previous growth curve. After reaching maximum day in each isolate then their production of cellulase enzyme activity will be decreased. This may be caused by glucose presence in given amount of time as the final product of cellulose breakdown that can be the allosteric inhibitor of enzyme. In addition, the diminishing substrate availability influences in reducing cellulase enzymes production.

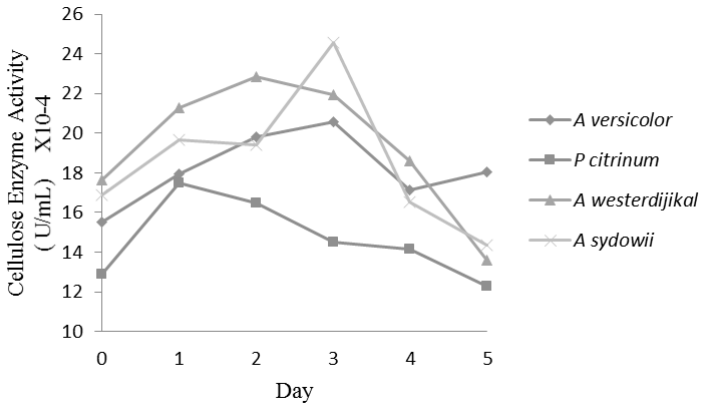

Figure 3: Curves of Cellulase Enzyme Activity

3.5. Characterictics of Cellulose Enzyme

3.5.1 Optimum pH

Activity of cellulase enzymes from *Sargassum* sp. fungi isolates (PSAR3/*Aspergillus versicolor*) at the highest optimum pH was obtained in phosphate buffer pH of 7 with values of cellulase activity 13,092x10^{-4} U/mL

(Figure 4) while from *Gelidium* sp. mold isolates (PGEL9/*Aspergillus sydowii*), the optimum pH was obtained at 7 with cellulase activity of 13,236x10^{-4} U/mL (Figure 4). The characteristics of enzymes measured in this study is the pH effect on selulotic activity of cellulase enzymes with pH range from 3 to 9. pH is one of the parameter that influence the enzyme activity [14]. Changes in pH affect the enzyme activity if conditions of low or high pH can also cause denaturation and enzyme activity will drop. The decreased enzyme activity due to pH changes is influenced by the presence of the media substrate enzyme ion. Reserved energy that began to run out and reduced nutrient can cause the decreased enzyme activity. Nevertheless, the graph (Figure 4) did not show this, caused by the estimated existence of multienzyme, which is the presence of several different types of enzyme in media production [15]. Both isolates showed maximum yield at phosphate pH 7, this shows that this number is the optimum pH for cellulase enzymes production in liquid CMC media. According to Bailey and Ollis [16], in cellulase enzyme, glucose consumption at pH 7 was more than any other pH.

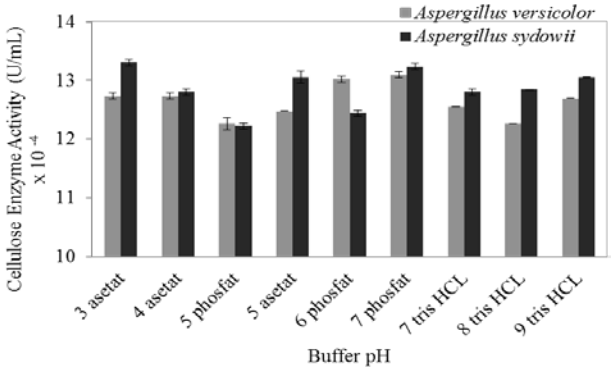

Figure 5: Effect of pH on Activity Cellulase Enzyme

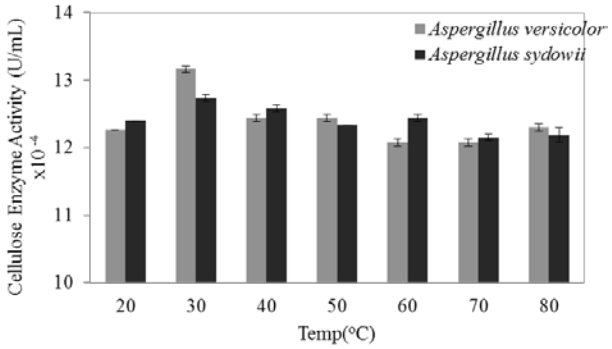

Figure 6: Effect of Temperature on Activity Cellulase Enzyme

3.5.2 Optimum Temperature

Each enzyme generally has maximum activity at certain temperature, increasing the temperature will increase the enzyme activity, but after the optimum temperature is reached there will occur protein denaturation that

causes the decreasing enzyme activity [17]. The highest activity of cellulase enzymes produced by both *Sargassum* sp. isolates (PSAR3/*Aspergillus versicolor*) and *Gelidium* sp. (PGEL9/*Aspergillus sydowii*) were obtained at 40°C with enzyme activity values respectively 12,528x10^{-4}U/mL and 12,367x10^{-4} U/mL (Figure 6). Temperature is one of the environmental factors that influence the development of cellulase enzymes activity. The cellulase enzyme activity becomes lower with the temperature increased after 30°C and reaches optimum temperature at 40°C. The effect of higher temperature can inhibit the activity of cellulase enzymes via protein denaturation process. From the results, it was found that the suitable optimum temperature for enzymes production is at 40°C. The increase in temperature will increase the reaction rate of cellulase enzyme activity at certain range [18].

4. Conclusions

21 endophytic fungi isolated from macroalgae *Gracilaria* sp., *Sargassum* sp., *Gelidium* sp. and *Caulerpa* sp. of Pari Island, Seribu Islands, has species such as *Aspergillus sydowii, Aspergillus niveus, Aspergillus versicolor, Aspergillus Candidus, Aspergillus tereus* and *Penicilium citrinum.* The highest four isolates from selulotic qualitative test such as *Aspergillus versicolor* isolate from *Sargassum* sp. (PSAR3), *Aspergillus sydowii* isolate from *Gelidium* sp. (PGEL9), A. *westerdijikal* from *Caulerpa* sp. isolates (PCLAR6) and *Penicilium citrinum* from *Gracilaria* sp. isolate (PGRAR1) have selulotic index value of 3.34, 3.22, 4.33 and 2.06 respectively. In quantitative test, *Aspergillus versicolor* (PSAR3) and *Aspergillus sydowii* (PGEL9) was optimum on day 2, while *Aspergillus westerdijikal* (PCLAR6) and *Penicillium citrinum* (PGRAR1) was optimum on day 1 and *Gelidium* sp. / *Aspergillus sydowii* was on day 2. Isolates of *Aspergillus versicolor* and *Aspergillus sydowii* was optimum at phospate pH at 7 and 40°C.

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